INVESTIGATION OF PIGMENTATION AND IMMUNE-RELATED CELL SURFACE MOLECULES IN CULTURED NORMAL AND VITILIGO MELANOCYTES.

SUSAN JENNIFER HEDLEY

THESIS SUBMITTED FOR THE DEGREE OF Ph.D.

UNIVERSITY OF SHEFFIELD
SECTION OF MEDICINE, DIVISION OF CLINICAL SCIENCES
NORTHERN GENERAL HOSPITAL

OCTOBER 1998

INVESTIGATION OF PIGMENTATION AND IMMUNE-RELATED CELL SURFACE MOLECULES IN CULTURED NORMAL AND VITILIGO MELANOCYTES.

Vitiligo is an acquired idiopathic hypomelanotic disorder which has an unknown aetiology and ultimately affects melanocytes, primarily of the skin. Many patients with vitiligo suffer from psychological distress and therefore it is important to determine why melanocytes are destroyed. The aim of this study was to learn more about the basic cellular physiology of normal and vitiligo melanocytes.

Morphology, proliferation and pigmentation in normal melanocytes were highly influenced by media additives, bovine pituitary extract (BPE), cholera toxin (CT), foetal calf serum (FCS) and phorbol 12-myristate 13 acetate (PMA). In the absence of these factors, extracellular matrix (ECM) proteins increased survival, proliferation and pigmentation of normal melanocytes, while ultraviolet irradiation (UVR) increased dendricity and pigmentation in the presence of these factors. Normal and vitiligo melanocytes were morphologically very similar and long-term cultured vitiligo melanocytes had proliferation which was not significantly different to that of normal melanocytes.

Constitutive and cytokine-stimulated expression of intercellular adhesion molecule-1 (ICAM-1), major histocompatibility complex (MHC) class I and II were not significantly different between normal and vitiligo melanocytes *in vitro*. The latter suggested that vitiligo melanocytes do not have an inherent defect in regulation of these adhesion molecules which could lead to their premature interaction with the immune system.

α-melanocyte stimulating hormone (α-MSH) has a controversial pigmentary role in man. In normal melanocytes, only under a limited number of media conditions did melanocytes exhibit increased melanogenesis to α-MSH. However α-MSH was found to be particularly effective at opposing the actions of TNF-α-stimulated ICAM-1 expression in melanocytes. This finding supported an immunomodulatory role for α-MSH in normal melanocytes.

Finally a surgical treatment using cultured epithelial autografts (CEA) was used to treat 3 patients with symmetrical (generalised) vitiligo, with mixed results. One patient showed good repigmentation, while the response was less promising in the other two patients.

ACKNOWLEDGMENTS.

I am very grateful to my supervisors, Dr Sheila Mac Neil and Dr David J Gawkrodger, for their advice and support during the course of my Ph.D. and for their comments on and proof-reading of my thesis.

I appreciate and thank enormously, the vitiligo patients who kindly donated their time and skin biopsies for experimental and therapeutic purposes. I would like to thank Mr Chris Caddy, for his surgical skills, and Dr David Gawkrodger (again) for their involvement in the grafting study (Chapter 5). I am also grateful to the Department of Plastic Surgery who provided skin samples for the culture of the normal melanocytes.

Several people have helped me in the laboratory throughout the four years. I would like to thank Ms Rebecca Dawson and Ms Susan Boyce for their help with the CEA culture and in addition Ms Louise Gould and Ms Jennifer Phillips who cultured the irradiated 3T3 cells. I am grateful to Rebecca who prepared the dermis, fibroblasts and keratinocytes in the epidermal/dermal reconstruct model (Chapter 3) and Mr Chris Layton who carried out all the histology and immunostaining work on these composites. I would like to thank Mr Russell Metcalfe for his involvement and advice in the flow cytometry studies (Chapter 4) and Mr Mark Wagner for preparing the rat tail collagen and his helpful advice in laboratory matters.

I am grateful to Dr Kausick Chakrabarty who allowed me to present some of his data including a photograph, in Chapter 3, and also to Dr David Ralston and Ms Jennifer Phillips for allowing me to add comments from their work to my thesis in Chapters 3 and 6 respectively.

I would like to thank Ms Sarah McGinley and Ms Kathryn Watson, for allowing me to use their computers and printer for printing the final version of my thesis. I am also grateful to Mr Robert Salthouse and Ms Annemarie Reed for taking the photographs of the epidermal/dermal composites and Medical Illustration, Northern General Hospital for printing all the photographs presented in this thesis.

I would like to thank my friends Ann-Margaret, Tim, Samantha and Gerard for keeping me sane, particularly during the writing up phase, and thank Mark Wagner and Sarah McGinley for their friendship at work.

I wish to dedicate this thesis to my mother, for her ever present support.

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ABBREVIATIONS.

ACTH	Adrenocorticotropic hormone
ADF	Adult T cell leukemia-derived factor
bFGF	Basic fibroblast growth factor
BPE	Bovine pituitary extract
cAMP	Adenosine 3':5'-cyclic monophosphate
CEA	Cultured epithelial autografts
CMV	Cytomegalovirus
CRE	cAMP response element
CT	Cholera toxin
DAF	Decay acceleration factor
DAG	1,2-Diacylglycerol
dbcAMP	Dibuytryl cAMP
DED	De-epidermised dermis
DHI	5,6-Dihydroxylindole
DHICA	Dihydroxyindole carboxylic acid
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethylsulphoxide
Dopa	3,4-Dihydroxyphenylalanine
ECM	Extracellular matrix
EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagle's modified essential medium
FBS	Foetal bovine serum
FCM	Fibroblast-conditioned medium
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward angle light scatter
cGMP	Guanosine 3':5'-cyclic monophosphate

GM-CSF	Granulocyte/macrophage-colony stimulating factor
GS	Goat serum
H and E	Haematoxylin and Eosin
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
dH_2O	Distilled water
HRP	Horseradish peroxidase
HSA	Human serum albumin
IBMX	3-Isobutyl-1-methylxanthine
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IR	Immunoreactive
i3T3	Irradiated 3T3 Swiss mouse fibroblasts
KCM	Keratinocyte conditioned medium
LCM	Lymphocyte conditioned medium
LFA-3	Leukocyte function antigen-3
LT-C_4/ D_4	Leukotriene C_4 or D_4
MC1R	Melanocortin 1 receptor
MCP	Membrane cofactor protein
MBEH	Monobenzyl ether of hydroquinone
MHC	Major histocompatability complex
MOP	Methoxypsoralen
MSH	Melanocyte-stimulating hormone
MTT	[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide
NKCA	Natural killer cell activity
NCS	Newborn calf serum
NDP-MSH	Nle^4DPhe^7-alpha-melanocyte-stimulating hormone
NFκB	Nuclear factor kappa B
NGF	Nerve growth factor

NO	Nitric oxide
OAG	1-Oleoyl-2-acetyl-glycerol
O.D.	Optical density
P1 (2,3,4)	Passage 1 (2,3,4)
PBS	Phosphate buffered saline
PE	R. phycoerythrin
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
POMC	Pro-opiomelanocortin
PUVA	Psoralen plus ultraviolet A radiation
RER	Rough endoplasmic reticulum
rpm	Revolutions per minute
SCF	Stem cell factor
SEM	Standard error of the mean
SSC	Side angle light scatter
TBS	Tris(hydroxymethyl)methylamine buffered saline
TNF-α	Tumour necrosis factor-alpha
TRP	Tyrosinase-related protein
Tween	Polyoxyethylenesorbitan monolaurate
UV	Ultraviolet
UVR	Ultraviolet irradiation
VKH	Vogt-Koyanagi-Harada syndrome
v/v	Volume to volume

CONTENTS.

CHAPTER 2 METHODS.

CHAPTER 3 INVESTIGATION OF THE INFLUENCE OF MEDIA, α-MELANOCYTE STIMULATING HORMONE, EXTRACELLULAR, MATRIX PROTEINS, ULTRAVIOLET IRRADIATION ANDCYTOKINES ON NORMAL AND VITILIGO HUMAN MELANOCYTE MORPHOLOGY, PROLIFERATION AND MELANOGENIC ACTIVITY.

CHAPTER 4 MODULATION OF IMMUNE-RELATED CELL SURFACE MOLECULES BY CYTOKINES AND α-MSH.

LIST OF FIGURES.

CHAPTER 4 MODULATION OF IMMUNE-RELATED CELL SURFACE MOLECULES BY CYTOKINES AND α-MSH.

CHAPTER 5 TREATMENT OF VITILIGO WITH CULTURED EPITHELIAL AUTOGRAFTS.

LIST OF TABLES.

CHAPTER 4 MODULATION OF IMMUNE-RELATED CELL SURFACE MOLECULES BY CYTOKINES AND α-MSH.

CHAPTER 5 TREATMENT OF VITILIGO WITH CULTURED EPITHELIAL AUTOGRAFTS.

CHAPTER 1

INTRODUCTION.

1.1 VITILIGO.

Vitiligo is described as an acquired idiopathic hypomelanotic disorder which indiscriminately affects persons of any race and any age and results in the clinical manifestation of characteristic milky white macules of the skin. The aetiology of the disorder remains enigmatic but several theories have been proposed, i.e. neuronal, self-destructive and immunological. The consensus prevalence of the disorder is 1% of the population (Lerner, 1971), although from a recent review (Nordlund, 1997), the author has suggested the prevalence is approximately 0.5% of the world population. This is in agreement with two large population studies from Denmark (Howitz *et al*, 1977) and India (Das *et al*, 1985a) where the prevalence of vitiligo was 0.4% and 0.46% respectively. The prevalence of familial cases of vitiligo have been reported to vary from 6.25% to 38% (Ortonne *et al*, 1983). Although not a life threatening disease, depigmentation can cause low-self esteem and psychological problems in many patients (see Porter *et al*, 1986, 1987 & 1990; Salzer & Schallreuter, 1995; Kent & Al' Abadie, 1996) and for this reason, amongst many, it is important to study the disease.

Melanocytes are derived from the neural crest (Rawles, 1947; Weston, 1970; Cramer, 1991), migrate during embyrogenesis and populate the epidermis at approximately 6-7 weeks estimated gestational age (Holbrook *et al*, 1989; Le Poole *et al*, 1996a), hair follicles, the mucous membranes (Laidlaw & Cahn, 1932; Zak & Lawson, 1974), the eye, the inner ear (Savin, 1965; Miyamoto & Fitzpatrick, 1957), the leptomeninges (Goldeieger, 1984) and various internal organs. Epidermal melanocytes are the primary target in vitiligo. Many investigators report that some patients additionally suffer from ophthalmologic and/or auditory complications (Albert *et al*, 1983; Wagoner *et al*, 1983; Cowan *et al*, 1986; Halder *et al*, 1987; Tosti *et al*, 1987). However, two additional studies suggest that an increased incidence of auditory disturbances does not occur in vitiligo patients (Orecchia *et al*, 1989; Escalante-Ugalde *et al*, 1991). Patients with Vogt-Koyanagi-Harada syndrome (VKH), a multisystem disease, often develop vitiligo-like hypomelanotic macules (Barnes, 1988). All the tissues involved in VKH contain melanocytes, suggesting that vitiligo, like VKH, could be a disease with a systemic component (Nordlund *et al*, 1980).

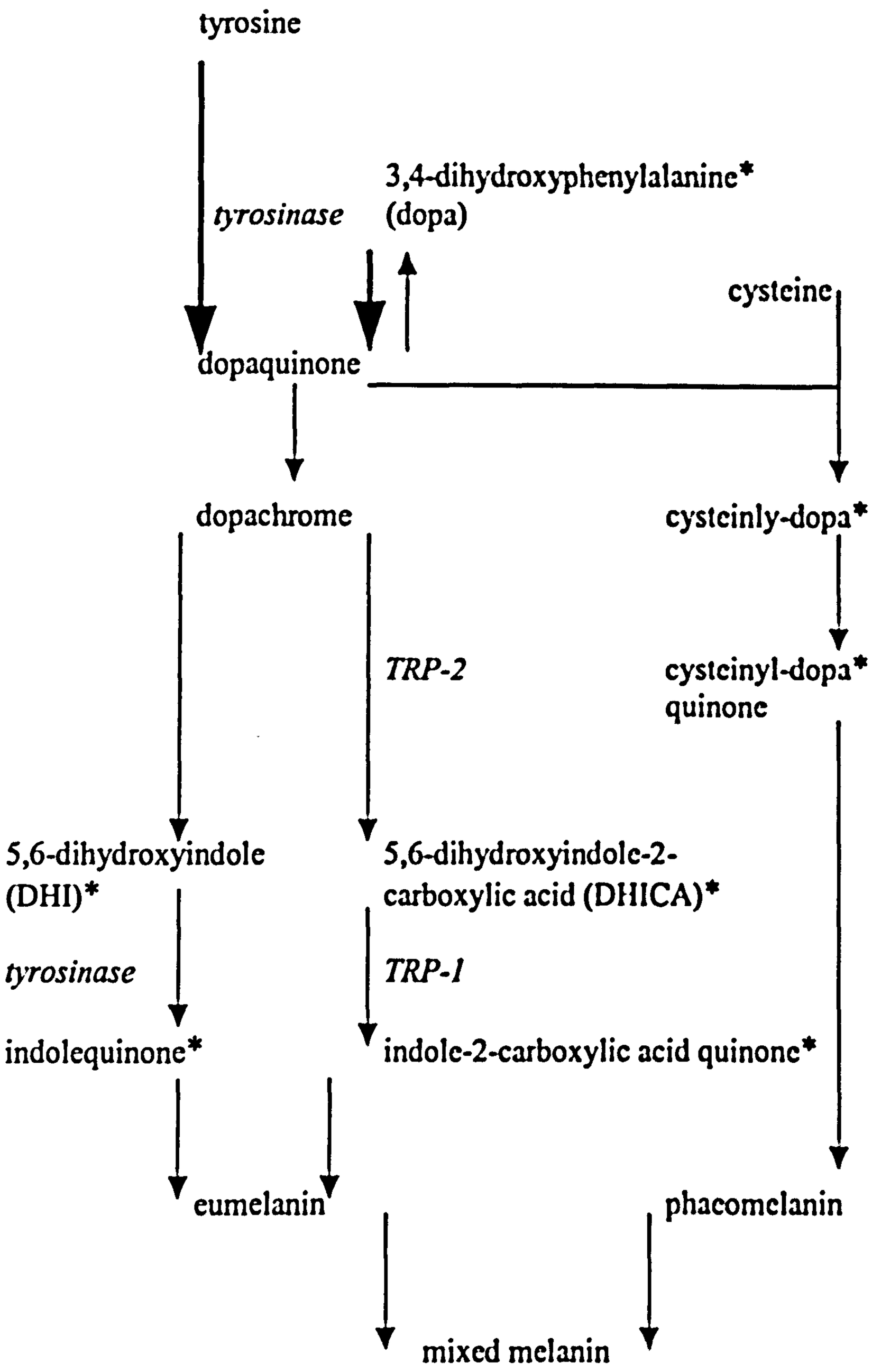

Figure 1.1 A schematic overview of melanogenesis. The pathway illustrated is based on Figure 2 in Riley (1998). Eumelanin and phaeomelanin are synthesised following the conversion of tyrosine to dopaquinone. Tyrosinase catalyses this important regulatory step in melanogenesis. The subsequent steps in eumelanogenesis, which can occur spontaneously, involve tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2 although the precise role of TRP-1 remains uncertain. Sulphydryl compounds such as cysteine (or glutathione) are required for phaeomelanogenesis. The melanin polymers not only include eumelanin and phaeomelanin but appear to incorporate several of the different intermediates of melanogenesis (intermediates incorporated are indicated by *)

1.1.1 Melanogenesis.

The main function of melanocytes is to synthesise the melanin pigments in specialised organelles termed melanosomes. The melanosomes are then transferred to keratinocytes by endocytosis of the dendrite ends (Wolff *et al*, 1974). As keratinocytes differentiate they migrate outwards and melanins are then able to form a protective barrier against the harmful rays of UV. At birth, epidermal melanocytes already transfer melanosomes to the keratinocytes (Glimcher *et al*, 1973). Melanins comprise the brown-black eumelanins and the reddish-yellow phaeomelanins. Eumelanins and phaeomelanins have distinct properties but only eumelanins appear to be photoprotective. While both forms have been detected in the epidermis of humans (Thody *et al*, 1991), dark skinned individuals form predominantly eumelanin, while light skinned individuals such as the red-haired Celts have a larger proportion of phaeomelanin (Prota, 1980).

The pigmentation pathway **(Figure 1.1)** is complex and undefined in many segments. The initial steps in the synthesis of melanin were initially described by Raper in the 1920s and then modified by Mason (1948). The pathway splits into the production of eumelanins or phaeomelanins at the point of dopaquinone. Phaeomelanogenesis relies on the presence of sulphydryl containing compounds such as cysteine or glutathione for the formation of the cysteinyl-dopa intermediates (Prota, 1980), while subsequent reactions from dopaquinone to eumelanin can progress slowly without enzymes (Prota, 1988). A family of related proteins, tyrosinase and tyrosinase-related protein-1 and -2 (TRP-1 and TRP-2) are now known to play a role in eumelanogenesis (described in **Section 1.1.1a)**.

Melanosomes in which melanogenesis proceeds (Seiji *et al*, 1961), are formed from the smooth endoplasmic reticulum and progress through four distinct stages as they mature (Quevedo, 1987). During maturation, the melanogenic enzymes are incorporated. Melanosomes containing eumelanin become elliptical in shape while phaeomelanosomes remain spherical. Melanosomes are required to enclose the pigmentary pathway as it is a dangerous process; many of the intermediates are highly reactive and exhibit significant cytotoxicity against melanocytes (e.g. Wick *et al*, 1977; Fujita *et al*, 1980; Pawelek *et al*, 1980; Urabe *et al*, 1994). Additionally melanin can

produce and absorb free radicals and active oxygen species (Korytowski *et al*, 1987). The dangerous nature of melanogenesis was highlighted in transfection studies where fibroblasts with only the full pigmentary machinery were capable of surviving the production of melanins (Bouchard *et al*, 1989).

1.1.1a The tyrosinase protein-related family.

Tyrosinase, a copper binding, highly glycosolated 70kDa enzyme, catalyses the main regulatory step in melanogenesis, the conversion of L-tyrosine to dopaquinone (Körner & Pawelek, 1982; Garcia-Carmone *et al*, 1982; Hearing & Jiménez, 1987). It additionally catalyses the oxidation of 5,6-dihydroxylindole (DHI) to indole 5,6 quinone (Körner & Pawelek, 1982; Hearing & Jiménez, 1987) in eumelanogenesis (see **Figure 1.1**). The level of tyrosinase activity has been proposed as a factor in deciding whether predominantly eumelanins or phaeomelanins are synthesised (Burchill *et al*, 1986 & 1989). Glutathione, which suppresses the translocation of tyrosinase from the Golgi complex to the melanosome may also contribute to which melanin pathway is followed (Imokawa, 1989). The human form of tyrosinase has been cloned and is located on the long arm of chromsome 11 which maps to the mouse *albino* locus on chromosome 7 (Kwon *et al*, 1987). The human tyrosinase gene, which is about 70kb in length, contains five exons (Shibahara *et al*, 1988) and tyrosinase negative oculocutaneous albinism (OCA-1) has been associated with numerous mutations in the gene (reviewed in Spritz & Hearing, 1994).

TRP-1, the melanosomal protein gp75, maps to the mouse *brown* locus on chromosome 4 (Jackson, 1988; Bennett *et al*, 1990; Bell *et al*, 1995) and is located on human chromosome 9 (Abbott *et al*, 1991; Murty *et al*, 1992). The human form has been cloned (Cohen *et al*, 1990; Vijayasarahdi *et al*, 1990). The catalytic activity of TRP-1 has been described as tyrosinase-like (Jiménez *et al*, 1991; Jiménez-Cervantes *et al*, 1993; Zhao *et al*, 1994b), a melanosomal specific catalase (Halaban *et al*, 1990) or dopachrome tautomerase (Winder *et al*, 1993). It was ascribed a dihydroxyindole carboxylic acid (DHICA) oxidase activity (Jiménez-Cervantes *et al*, 1994; Kobayashi *et al*, 1994) but this has recently been disputed for human TRP-1 (Boissy *et al*, 1998). In human pigment cells TRP-1 expression has been shown to correlate with

eumelanogenesis (del Marmol *et al*, 1993). TRP-1 has been reported to associate with stage I and II melanosomes while tyrosinase is associated with stage III and IV melanosomes (Orlow *et al*, 1993). TRP-1 may therefore have a role in melanosome formation as indicated by the predominantly spherical phaeomelanosomes of mice with mutations at the *brown* locus (Hearing & Jiménez, 1989). Winder *et al.* (1994 & 1995) have suggested that tyrosinase and other melanosomal enzyme activites may be stabilised by TRP-1. Mutations in the TRP-1 gene may lead to tyrosinase-positive albinism (OCA-3) (Boissy *et al*, 1996) or rufous OCA (Manga *et al*, 1997). A recent study analysing the TRP-1 gene from 100 Caucasian individuals with varying hair colour and two individuals with hypopigmented 9p- syndrome showed no polymorphisms and the authors suggested that the gene was not an important factor in normal human pigmentation differences (Box *et al*, 1998). The precise role of TRP-1 still remains unclear.

TRP-2, an iron and zinc binding glycoprotein (Solano *et al*, 1996; Furumura *et al*, 1998) has dopachrome tautomerase activity (Hearing & Tsukamoto, 1991; Tsukamoto *et al*, 1992; Yokoyama *et al*, 1994) and converts dopachrome to 5,6-DHICA through tautomerisation (Aroca *et al*, 1990). It may play a regulatory role in determining the proportion of DHI (black units) to DHICA (brown units) integrated into the eumelanin polymer (Urabe *et al*, 1993). In mice, the TRP-2 protein maps to the *slaty* locus on chromosome 14 (Jackson *et al*, 1992), while in humans the gene is located in chromosome 13 (Sturm *et al*, 1994). During development of the foetus, expression of TRP-2 begins prior to the expression of tyrosinase and TRP-1 (Steel *et al*, 1992). The difference in catalytic activities of the three melanogenic enzymes may be due to the differential binding of divalent metal cations (Furumura *et al*, 1998). In addition to the metal ions already mentioned, manganese, nickel and cobalt can influence melanogenic reactions (Palumbo *et al*, 1988), while pigmented melanocytes have high levels divalent cations (Jara *et al*, 1990) which are located in the melanosome (Shibata *et al*, 1993).

The three genes have been cloned and their genomic organisation described (Sturm *et al*, 1995). Regulatory elements in the promoter region of the tyrosinase gene, such as the M and E boxes were recently reviewed by Ferguson & Kidson (1997). Winder *et al.* (1994) suggested that the melanogenic enzymes existed in a protein matrix associated

complex and their expression was under the control of the tyrosinase promoter. Several studies indicate that the expression of the three tyrosinase-related proteins are not necessarily co-ordinated (e.g. Abdel-Malek *et al*, 1994b; Abdel-Malek *et al*, 1995; Kobayashi *et al*, 1995; Mengeaud & Ortonne, 1996; Maeda *et al*, 1997; Eberle *et al*, 1998; Kippenberger *et al*, 1998b). The adenosine 3':5'-cyclic monophosphate (cAMP)/protein kinase A (PKA) pathway, already known to have a pivotal role in the regulation of melanogenesis (e.g. Halaban *et al*, 1983; Hearing & Tsukamoto, 1991; Abdel-Malek *et al*, 1992; Englaro *et al*, 1995) has recently been shown to increase the promoter activites of tyrosinase through the E box promoter element (Bertolotto *et al*, 1996) and TRP-1 and TRP-2 through the M box promoter element (Bertolotto *et al*, 1998). A cAMP response element (CRE)-like motif was also involved in the increased TRP-2 promoter activity (Bertolotto *et al*, 1998). Microphthalmia, a basic helix-loop-helix transcription factor, transcribed from the *mi* gene in mouse (Hodgkinson *et al*, 1993; Hughes *et al*, 1994) and the human homolog, microphthalmia-associated transcription factor (Tachibana *et al*, 1994) activate the tyrosinase (Bentley *et al*, 1994) and TRP-1 promoters (Yavuzer *et al*, 1995; Yasumoto *et al*, 1997). The TRP-2 promoter is activated by microphthalmia (Bertolotto *et al*, 1998) but not by the human homolog (Yasumoto *et al*, 1997). In accordance with the presence of a CRE in the microphthalmia promoter (Fuse *et al*, 1996), Bertolotto *et al*. (1998) found that expression and binding of microphthalmia to the TRP-1 and TRP-2 M boxes was also increased by cAMP. Ultraviolet radiation (UVR), the major modulator of pigmentation, may act through a variety of different mechanisms. In addition to the PKA pathway and UVR, other hormonal and paracrine elements exist to regulate melanogenesis (discussed in **Chapter 3**).

1.1.1b Additional melanosomal proteins.

In addition to the tyrosinase family of proteins, three other melanosomal proteins have been discovered, which may play a role in melanogenesis. Pmel-17, which maps to the mouse *silver* locus on chromosome 10 and is located on chromosome 12 in humans (Kwon *et al*, 1991), may act distal to tyrosinase as a stablin in melanogenesis (Chakraborty *et al*, 1992). Mutations of the *silver* locus result in the graying of hair and a loss of melanocytes which may be related to the build up of toxic intermediates during

melanogenesis (Johnson & Jackson, 1992). Lysosome-associated membrane protein-1 may provide protection for the melanosomal membrane (Thody, 1995), or have a role in melanosomal development as it has been associated mainly with stage I and II melanosomes (Zhou *et al*, 1993). The p protein which maps the mouse *pink-eyed dilution* locus on chromosome 7 (Gardner *et al*, 1992; Rinchik *et al*, 1993) may be a melanosomal transport protein for tyrosine. Mutations in the human P gene located on chromosome 15 (Ramsay *et al*, 1992) lead to type II oculocutaneous albinism (Rinchik *et al*, 1993; Lee *et al*, 1994).

1.2 HISTOLOGICAL APPEARANCE OF NORMAL AND VITILIGO SKIN.

1.2.1 Histological appearance of normal skin.

In normal skin, melanocytes lie adjacent to the basal lamina, interspaced between the basal keratinocytes. They project dendrites through the keratinocyte layers and interact with keratinocytes in a tightly regulated fashion. One melanocyte contacts approximately 36 keratinocytes to form the so-called epidermal melanin unit (Fitzpatrick *et al*, 1967). The importance of keratinocytes has been illustrated in numerous *in vitro* studies (De Luca *et al*, 1998a & b; Valyi-Nagy *et al*, 1990; Haake & Scott, 1991; Scott & Haake, 1991; Donatien *et al*, 1993; Valyi-Nagy *et al*, 1993; Nakazawa *et al*, 1995a; Tenchini *et al*, 1995; Kippenberger *et al*, 1996; Nakazawa *et al*, 1997; Kippenberger *et al*, 1998a; Nakazawa *et al*, 1998). A precursor population of melanocytes have been detected in the skin by the use of c-Kit staining (Grichnik *et al*, 1996). c-Kit is expressed by melanocytes as they migrate from the neural crest (Wehrle-Haller & Weston, 1995). Melanocytes additionally contact Langerhans' cells and the epidermal melanin unit has been more recently labelled the keratinocyte-Langerhans' cells-melanocyte complex (Pelc & Nordlund, 1993). The interaction of normal melanocytes and the peripheral nervous system has been unclear for many years and only one study has indicated innervation of cutaneous melanocytes in normal skin by confocal microscopic analysis and electron microscopy (Hara *et al*, 1996). The connection between the nerve and melanocyte was through a synapse-like structure.

Active melanocytes populate the hair bulb matrix and infundibulium, while amelanotic melanocytes occupy the outer root sheath (Staricco, 1959; Cui *et al*, 1991; Horikawa *et al*, 1996a). Two distinct populations of melanocytes, amelanotic and pigmented, can be isolated from the hair follicle, and these in turn are distinct from epidermal melanocytes as seen by immunophenotyping and morphology (Tobin & Bystryn, 1996). The amelanotic melanocytes derived from the hair follicles were able to proliferate and were serially passaged, whereas the pigmented melanocytes derived from the hair follicles would not proliferate (Tobin & Bystryn, 1996).

1.2.2 Histological appearance and pathophysiology of vitiligo lesions.

1.2.2a The presence or absence of active/inactive melanocytes.

Within the depigmented area of vitiligo it is generally accepted that there is an absence of melanin granules and melanocytes. A detailed immunohistochemical study using a wide range of antibodies that react with pigmentary-related epitopes and non-pigmentary related epitopes (Le Poole *et al*, 1993c), presented evidence for the absence of melanocytes in the depigmented lesions. c-Kit staining also revealed the absence of melanocytes in vitiligo skin (Dippel *et al*, 1995). In perilesional skin, c-kit expression was reduced in comparison to control skin (Norris *et al*, 1996). Several light and electron microscopy studies and histochemical studies which rely on the detection of active melanin synthesis and the appearance of melanosomes, have verified this lack of melanocytes and melanin granules (Jarrett & Szabo, 1956; Hu *et al*, 1959; Birbeck *et al*, 1961; Zelickson & Mottaz, 1968; Bleehen, 1979; Ortonne *et al*, 1979; Ortonne *et al*, 1980; Moellmann et al, 1982; Bhawan & Bhutani, 1983; Cui *et al*, 1991; Hann *et al*, 1992b; Galadari *et al*, 1993; Ahn *et al*, 1994; Fargnoli & Bolognia, 1995). Early patches of vitiligo may demonstrate the continuing presence of a few dopa-positive melanocytes (Dourmishev *et al*, 1982; Abdel-Naser *et al*, 1994; Dourmishev & Pekov, 1993; Galadari *et al*, 1993; Horn & Abanmi, 1997). In perilesional regions, abnormal melanocytes which are fragmented, grossly enlarged or show vacuolization are noted (Morohashi *et al*, 1977; Iyenger & Misra, 1988; Kao & Yu, 1990; Hann *et al*,1992b; Galadari *et al*, 1993; Le Poole *et al*, 1993c; Abdel-Naser *et al*, 1994). Kao & Yu (1992) and Pitts & Grimes (1991) have additionally reported the presence of dopa-positive

melanocytes in the epidermis of lesions in some patients and related this to the ability of patients to respond to psoralen plus ultraviolet A radiation (PUVA) treatment. Occasionally some patients suffer from hyperpigmented areas (Seghal, 1974; Fargnoli & Bolognia, 1995; Schwarz & Janniger, 1997) and in these regions melanocytes are enlarged with elongated dendritic processes containing melanin (Fargnoli & Bolognia, 1995; Schwarz & Janniger, 1997).

Recently, though, light and electron microscopy suggest that melanocytes and/or granules of melanin are still present in vitiligo lesions of up to 20 or more years duration (Tobin & Schallreuter, 1997; Shoulder *et al*, 1998) although the immunohistochemical data (Shoulder *et al*, 1998) were in agreement with Le Poole *et al*. (1993c). In an earlier report, tyrosinase activity was detected in vitiligo lesions of short-term and long-term duration using a sensitive fluorimetric method (Husain *et al*, 1982). Bartosik *et al*. (1998) state that melanosomal complexes were present in the early stable lesions of 1-3 years, although melanocytes were not detected. Melanocyte cultures have occasionally been initiated from the depigmented lesions (Le Poole *et al*, 1993c; Shoulder *et al*, 1998).

1.2.2b Keratinocyte abnormalities.

Keratinocyte damage such as vacuolisation has been reported in perilesional regions of vitiligo (Breathnach *et al*, 1966; Ishii & Hamada, 1981; Moellmann *et al*, 1982; Bhawan & Bhutani, 1983; Hann *et al*, 1992b; Kao & Yu, 1992; Galadari *et al*, 1993; Ahn *et al*, 1994; Yagi *et al*, 1997). Vacuolar degeneration of keratinocytes was more prominent in actively spreading vitiligo when compared to marginal skin of stable vitiligo lesions. The presence of spongiosis and/or dyskeratosis was only seen in the lesions of active vitiligo (Hann *et al*, 1992b; Kao & Yu, 1992; Ahn *et al*, 1994). Yagi *et al*. (1997) also reported dyskeratosis in inflammatory borders of vitiligo lesions. Extracellular granular material that may be derived from the cytoplasm of altered keratinocytes was found in the adjacent normal-appearing and perilesional skin (Moellmann *et al*, 1982). At the margin of vitiligo lesions, disturbed expression of epidermal cytokeratins was detected by different specific antibodies (Abdel Naser *et al*, 1991).

1.2.2c Langerhans' cells.

The density of Langerhans' cells in vitiligo lesions has been reported variously as decreased, normal or increased (Brown *et al*, 1967; Riley, 1967; Mishima & Miller-Milinska, 1967; Zelickson & Mottaz, 1968; Bhawan & Bhutani, 1983; Claudy & Rouchouse, 1984; Kano *et al*, 1984; Westerhof *et al*, 1986; Hatchome *et al*, 1987; Moncada *et al*, 1987), and this controversy continues. Two recent studies demonstrated that Langerhans' cells were decreased in number in elevated inflammatory regions (Yagi *et al*, 1997) or increased (Horn &, Abanmi, 1997). In a definitive study, patients with active nonsegmental vitiligo had a marked decrease in CD1a-positive and ATPase-positive epidermal dendritic population, but there was a repopulation in patients with stable nonsegmental vitiligo (Kao & Yu, 1990). Patients with repigmenting lesions in response to PUVA or fluocinonide cream also showed a decreased in epidermal Langerhans' cells, suggesting a role for these cells in the disease (Kao & Yu, 1990).

Contact sensitivity, the induction of which requires the participation of Langerhans' cells, is diminished in lesional vitiligo skin (Uehara *et al*, 1984; Nordlund *et al*, 1985; Hatchome *et al*, 1987; Singh *et al*, 1987; Srinvas *et al*, 1987). Langerhans' cells from vitiligo lesions had a decreased capacity to induce proliferation of allogeneic peripheral blood lymphocytes suggesting functional impairment of the Langerhans' cells in vitiliginous skin (Hatchome *et al*, 1987). Gilhar *et al*. (1993) also reported decreased effectivity of Langerhans' cells in vitiligo lesions. Degenerative changes in Langerhans' cells have been reported (Kao & Yu, 1990; Marganian *et al*, 1990) while the Birbeck granules in Langerhans' cells showed altered morphology (Bartosik *et al*, 1998).

1.2.2d Merkel cells.

Focal gaps were seen in the basal layer of vitiligo lesions and these were shown to be due to the absence of Merkel cells (Bose, 1994c; Bose & Ortonne, 1994). Merkel cells have been associated with nerve terminals (Lacour *et al*, 1991) and it has been hypothesised that the reported autonomic dysfunction in lesional skin (Chanco-Turner

& Lerner, 1965; Dutta, 1965; Dutta & Dermat, 1972; Dutta & Mandal, 1982; Merello *et al*, 1993) may be due to disturbances in Merkel cells (Nordlund & Majumdar, 1997).

1.2.2e The presence of focal mononuclear infiltrates and the expression of immune-related cell surface molecules.

Focal mononuclear infiltrates containing lymphocytes are present in the perilesional epidermis and dermis of progressing lesions and inflammatory vitiligo (Michaëlsson, 1968; Ishii & Hamada, 1981; Moellmann *et al*, 1982; Bhawan & Bhutani, 1983; Gokhale & Meta, 1983; Hann *et al*, 1992b; Al Badri *et al*, 1993b; Ahn *et al*, 1994; Abdel-Naser *et al*, 1994; Le Poole *et al*, 1996b; Horn & Abanmi, 1997; Yagi *et al*, 1997). In trichrome vitiligo (described further in **Section 1.3.2b**), the inflammatory cell infiltrate was more prominent in the regions of intermediate hue than in the vitiliginous and unaffected skin (Hann *et al*, 1997). Fargnolia & Bolognia, (1995) found sparse infiltrates and melanophages in sections from both hypo- and hyperpigmented regions of a patient described as having pentachrome vitiligo (described in **Section 1.3.2b**). CD4+ T cells were significantly increased in the margins of depigmented lesions (Al Badri *et al*, 1993b) and in the raised region of inflammatory vitiligo (Horn & Abanmi, 1997; Yagi *et al*, 1997). CD4+ T cells were significantly increased in active lesions in comparison to stable lesion (Ahn *et al*, 1994). CD8+ T cells have been reported to be present (Ahn *et al*, 1994; Horn & Abanmi, 1997; Yagi *et al*, 1997) or significantly increased (Al Badri *et al*, 1993b; Le Poole *et al*, 1996b) in the perilesional regions. Many of the T cells were activated as cells expressed major histocompatibility complex (MHC) class II (Al Badri *et al*, 1993b) and the interferon gamma receptor (Al Badri *et al*, 1993b; Abdel-Naser *et al*, 1994) or interleukin (IL)-2 receptor expression (Gross *et al*, 1987; Al Badri *et al*, 1993b; Abdel-Naser *et al*, 1994; Le Poole *et al*, 1996b), of CD45RO memory subset (Al Badri *et al*, 1993b; Yagi *et al*, 1997) or expressed cutaneous lymphocyte associated antigen (HECA-452+) typical of skin-homing T cells (Al Badri *et al*, 1993b; Le Poole *et al*, 1996b). Macrophages were present in perilesional/lesional regions (Abdel-Naser *et al*, 1994; Le Poole *et al*, 1996b). Melanophage deposition has also been noted in the dermis (Hann *et al*, 1992b). B cells were present in a patient with VKH, with associated vitiligo (Okada *et al*, 1996) and in a patient developing vitiligo (Horn & Abanmi, 1997) but absent in several patients with

inflammatory vitiligo (Le Poole *et al*, 1996b). The presence of T cells and macrophages correlated with the disappearance of melanocytes in inflammatory vitiligo (Le Poole *et al*. 1996b). The infiltrates were not extensive in number but were associated with the remaining melanocytes (Le Poole *et al*, 1996b). The number of Factor XIIIa-positive dermal dendrocytes was reduced in the skin of patients with various forms of vitiligo (Aronson *et al*, 1990). Immunoglobulin (Ig) G was present within the skin in approximately 50% of vitiligo lesions (Uda *et al*, 1984) and C3 deposits have been demonstrated in the basement membrane of vitiliginous skin (Uda *et al*, 1984). However, this finding has not been confirmed by other workers.

Intercellular adhesion molecule-1 (ICAM-1) and MHC class II expression has been reported to be abnormally high in perilesional melanocytes (Al Badri *et al*, 1993a) but there was no correlation between the number of melanocytes displaying cell surface molecules and the number of T cells at the margin. ICAM-1 was expressed in the perilesional epidermis of all active but no stable lesions while MHC class II of the human leukocyte antigen (HLA)-DR locus was expressed in perilesional epidermis of all active and some stable lesions (Ahn *et al*, 1994). Keratinocytes (basal and suprabasal) and melanocytes expressed MHC class II in perilesional epidermis of inflammatory vitiligo but no ICAM-1 expression was noted (Le Poole *et al*, 1996b). In an independent study of inflammatory vitiligo, keratinocytes expressed both HLA-DR and ICAM-1 in the elevated regions, but not in normal or lesional skin (Yagi *et al*, 1997). Within the depigmented lesions ICAM-1 was actually downregulated when interferon-gamma (IFN-γ) was added (Nordlund & Majumder, 1997) although Gilhar *et al*. (1993) reported that there was no difference between vitiliginous skin and normal pigmented skin with regards to ICAM-1 and HLA-DR expression when sites were treated with IFN-γ.

1.2.2f Basement membrane aberrations.

In areas of disturbed keratinization, basement membrane antigen nicein and type IV collagen were missing (Abdel Naser *et al*, 1991). An alteration of tenascin levels, in which the tenascin content was increased from apparently normal skin into the depigmented lesion, has been reported (O'Donnel *et al*, 1991; Le Poole *et al*, 1997).

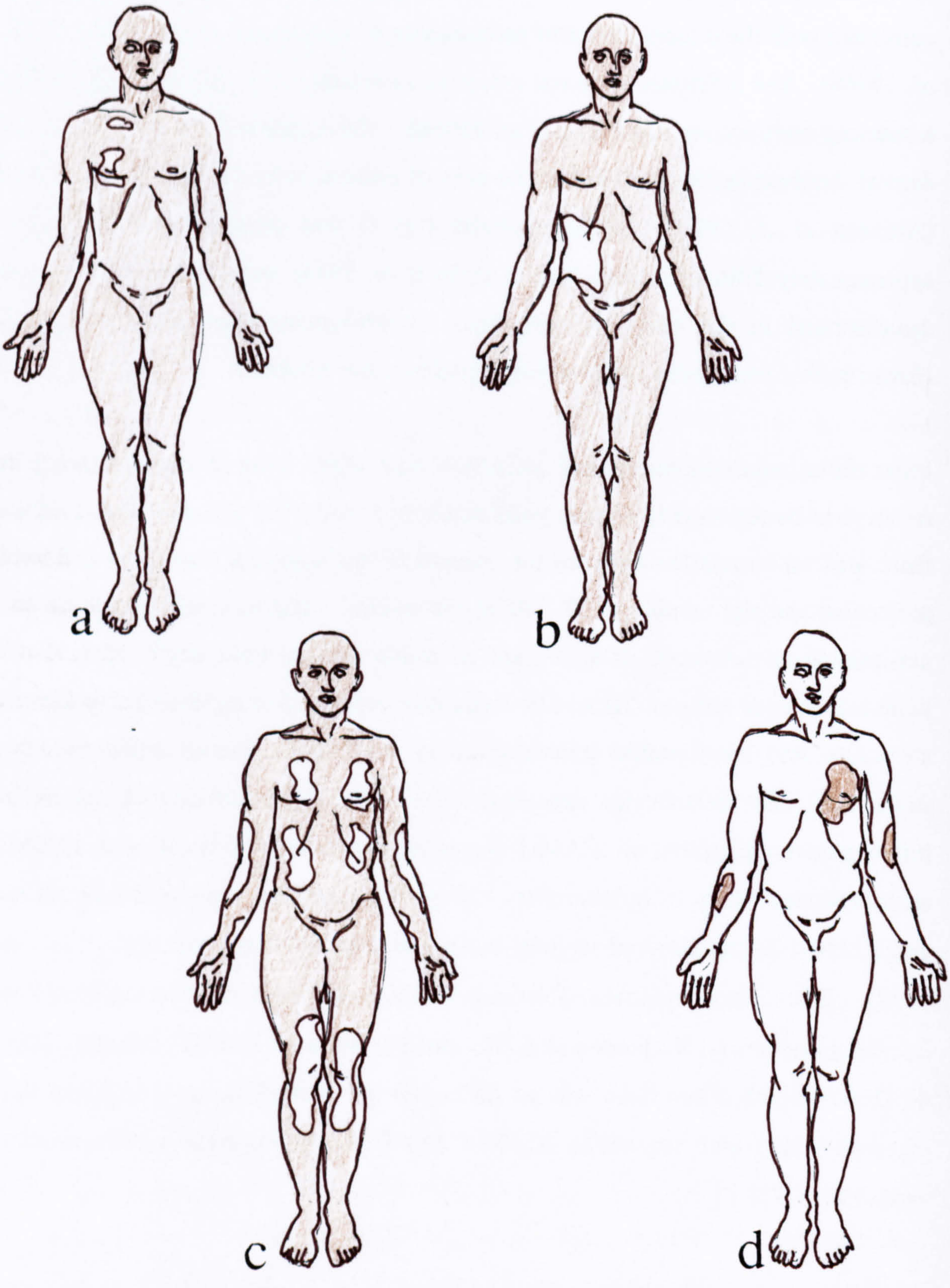

Figure 1.2 Distributional patterns of vitiligo lesions. The classification of vitiligo is described from the distribution of the lesions in a patient. The body maps illustrate four forms of the disease, focal (**a**), segmental (**b**), vulgaris (**c**) and universal (**d**). Idiopathic vitiligo can also be described as type A (nonsegmetal e.g. focal, acrofacial, generalised (vulgaris)) or type B (segmental) (Koga, 1977).

1.2.2g Association with the nervous system.

Early studies have shown dystrophic changes among nerve trunks and terminals in lesions (Chan-Gen, 1959) and degenerative changes in the dermal nerves of vitiligo lesions (Mori, 1964; Nozaki, 1976). Direct contact between dermal nerve endings and melanocytes in vitiligo have been shown (Breathnach *et al*, 1966; Morohashi *et al*, 1977; McBurney, 1979), while structural alterations such as the swelling of axons have been noted (Breathnach *et al*, 1966). Degeneration of nerves occurred in the segmental vitiligo lesions corresponding in location to the area of distribution of local nerve and regeneration of nerves was noted in repigmenting regions in reponse to therapy (Breathnach *et al*, 1966). Regenerative and degenerative changes in dermal nerves in lesional and perilesional vitiligo skin were noted (Al'Abadie *et al*, 1995). Basement membrane thickness increased in Schwann cells and other subtle ultrastructural evidence of axonal damage were seen (Al'Abadie *et al*, 1995). Liu *et al*. (1996) also found increased number of nerve growth factor immunoreactive (IR) nerve fibres and calcitonin gene-related peptide IR nerve fibres in the lesions in 18 patients compared to the control and uninvolved skin. Nerve growth factor (NGF) receptor IR basal keratinocytes were also reduced in lesions. Hypomelanotic macules seen in patients with leprosy also show decreased numbers of epidermal melanocytes and degenerative changes of superficical nerve fibers (Job *et al*, 1972).

1.3 CLINICAL FEATURES OF VITILIGO

The distribution of lesions (schematic examples illustrated in **Figure 1.2**) led to the classification of vitiligo as being focal, segmental, vulgaris or universal (Lerner, 1971) with this being modified so that patients were described as having localised (focal, segmental and mucosal), generalised (acrofacial, vulgaris, mixed) or universal vitiligo (Ortonne *et al*, 1983). Generalised vitiligo is the most common form of vitiligo and this is also seen in paedriatric patients (Halder *et al*, 1987; Janniger, 1993). Childhood vitiligo has been described as a "distinct subset of vitiligo" (Jaisankar *et al*, 1992; Halder *et al*, 1997). In all age groups, idiopathic vitiligo can be classified as type A (nonsegmental) or type B (segmental) (Koga, 1977). This was based on the reaction of

lesions to injections of physostigmine (a modulator of sympathetic nerves that induces sweat formation) (Koga, 1977). Koga (1977) suggested that the two types of vitiligo were associated with different aetiologies; nonsegmental vitiligo with immunological mechanisms, and segmental vitiligo with the dysfunction of the sympathetic nerves. Lesions in segmental vitiligo are generally confined to a particular dermatome, and do not cross the midline. Nonsegmental vitiligo can be further classified as familial or nonfamilial (Ando *et al*, 1993).

Additionally, some patients present lesional forms of idiopathic vitiligo such as trichrome, bilateral segmental and inflammatory vitiligo. Occupational leukoderma is difficult to distinguish from idiopathic vitiligo.

1.3.1 Comparison between segmental and nonsegmental vitiligo.

The prevalence of segmental vitiligo within populations of vitiligo patients varies from 5% to 27.9% (Koga, 1977; El Mofty & El Mofty, 1980; Park *et al*, 1981; Koga & Tango, 1988; Park *et al*, 1988; Bhatia *et al*, 1992; Janniger, 1993; Song *et al*, 1994a; Hann & Lee, 1996; Halder, 1997) while the prevalence of segmental vitiligo in childhood vitiligo has been reported from 19%-28% (Halder *et al*, 1987; Janniger, 1993; Halder, 1997). Segmental vitiligo generally affects persons of a younger age whereas nonsegmental vitiligo affects patients of all ages (Halder *et al*, 1987; Koga & Tango, 1988; Jaisankar *et al*, 1992; Janniger, 1993; Gwiezdzinski *et al*, 1994; Song *et al*, 1994a; Barona *et al*, 1995; Hann & Lee, 1996). For example, Koga & Tango (1988) reported that 82% of patients had onset of segmental vitiligo before the age of 30, while the nonsegmental group showed onset of disease at any age and in a recent Korean study (Hann & Lee, 1996), 87% of patients with segmental vitiligo had onset of their disease before the age of 30, while 41.3 % had onset before the age of 10 with the mean age of disease onset at 15.6 years.

In 208 Korean patients with segmental vitiligo (the largest study so far conducted on segmental vitiligo) the order of dermatomal involvement was trigeminal (52.1%), thoracic (22.8%), cervical (17.4%), lumbar (6.4%) and sacral (1.4%) (Hann & Lee, 1996). The progression of segmental vitiligo is generally rapid and usually ceases after

1-2 years (Koga & Tango, 1988; Song *et al*, 1994a) although Hann & Lee (1996) reported that the mean duration of disease activity was 4.8 years. Once the activity ceases, segmental vitiligo generally is a stable form of the disease and these patients therefore make good candidates for melanocyte transplantation (see **Section 1.5.2**). On very rare occasions though, patients initially diagnosed with segmental vitiligo later developed a nonsegmental form of vitiligo (Koga & Tango, 1988; Song *et al*, 1994a). The progression of lesions in nonsegmental vitiligo alternates between remission and activity (Koga & Tango, 1988). Nonsegmental forms of vitiligo generally exhibit symmetrical patterning in early as well as advanced lesions (Goudie *et al*, 1983). A recent study on patients with nonsegmental vitiligo has shown that when the initial sites involved the posterior trunk, hands or feet, there was a greater progression to other body sites (Chun & Hann, 1997). The pattern was usually contiguous to the initial site, except when the hands were the initial site. In this case the face usually become the next site for depigmentation (i.e. the development of acrofacial vitiligo). Chun & Hann (1997) were surprised that there appeared to be some form of progression pattern in many of the patients as nonsegmental has been associated with autoimmune disease (Koga & Tango, 1988), and there would be an expected generalised haphazard progression.

Nonsegmental vitiligo has a higher association with autoimmune diseases than segmental vitiligo (Koga & Tango, 1988; Song *et al*, 1994a; Barona *et al*, 1995). Autoimmune disease was not associated with any forms of childhood vitiligo (Halder *et al*, 1987; Janniger, 1993; Schallreuter *et al*, 1994d) although first and second degree relations had significantly higher association than those of adult patients (Halder *et al*, 1987; Halder, 1997). Strangely, children with vitiligo did have a significant incidence of positive organ-specific and nonorgan-specific autoantibodies (e.g. antinuclear, antithyroid, antiparietal) (Halder *et al*, 1987; Halder, 1997). Hann & Lee (1996) reported that only 3.4% of segmental vitiligo patients had association with autoimmune diseases or halo naevus and an earlier independent study stated that segmental vitiligo was not associated with autotimmune diseases (El Mofty & El Mofty, 1980). Koga & Tango (1988) described nonsegmental vitiligo as exclusively associated with halo naevus and the Koebner phenomenon in the study. However, in more recent studies, 5.3% of segmental patients (Hann & Lee, 1996) and 44.4% Columbian patients with

unilateral vitiligo (Barona *et al*, 1995) developed Koebnerisation but there was no significant difference between the two forms in terms of halo naevus. The high number of patients with unilateral vitiligo to develop Koebnerisation could be due to the inclusion of focal forms of vitiligo in addition to segmental forms in the Columbian study. The Koebner phenomenon, where the depigmentation develops at the site of trauma e.g. donor sites for grafting, occurs in many patients (Halder *et al*, 1987; Hatchome *et al*, 1990). Gauthier (1995) described a large number of vitiligo vulgaris lesions which were attributable to friction i.e. minor trauma caused by everyday washing for example and hence the Koebner phenomenon. In an early study, 68% of patients already with vitiligo developed further depigmentation following scarification due to the Koebner phenomenon (Gopinathan, 1965). Poliosis (white hairs) in the eyebrows, eyelashes and scalp hair is more common in segmental vitiligo than nonsegmental vitiligo, with 48.6% of patients with segmental vitiligo developing poliosis (Hann & Lee, 1996). An interesting finding in one study was an association of bilateral vitiligo with lighter skin types, I-III (Barona *et al*, 1995) and that 64.7% of all vitiligo patients in this study had one of these skin types.

1.3.2 Rare forms of vitiligo.

1.3.2a Bilateral segmental vitiligo.

Occasionally depigmented lesions affect the same or different dermatomes on both sides of the body and this is described as bilateral segmental vitiligo. Hann *et al.* (1997) described five cases of this rare segmental form of the disease. It is much harder to distinguish from nonsegmental forms of vitiligo although the clincal course appears to follow a similar one to unilateral segmental vitiligo, where stability is obtained after the cessation of rapid depigmentation (Hann *et al*, 1997).

1.3.2b Trichrome vitiligo.

Trichrome vitiligo involves macules where the loss of pigmentation is incomplete. An intermediate hue of varying width is seen between the normal and completely depigmented skin (Fitzpatrick, 1964; Bolognia & Pawelek, 1988; Hann *et al*, 1997).

One study has reported that the three areas were definitive, lacked gradual changes of colour and had stability (Dupre & Christol, 1978) but the general consensus is that trichrome vitiligo is a transitional active process, with the intermediate hues eventually depigmenting in a centrifugal manner (Pinkus, 1959; Fitzpatrick, 1964; Hann *et al*, 1997). Trichrome vitiligo occurred mainly in the trunk in active vitiligo vulgaris (Hann *et al*, 1997). In a Korean study (Hann *et al*, 1997), the authors noted a dark brown border surrounding the periphery of the intermediate hue and termed these lesions quadrichrome vitiligo while Fargnoli & Bolognia (1995) have described a patient with pentachrome vitiligo in which five colour shades of white, tan (the usual shade seen in trichrome vitiligo), medium brown (the unaffected skin), dark brown and black were present. As seen in the patient with pentachrome vitiligo, hyperpigmented borders may occur instead of borders of intermediate hue (Seghal, 1974; Bolognia & Pawelek, 1988; Schwarz & Janniger, 1997) with up to 5.5% patients demonstrating this phenomenon (Seghal, 1974).

1.3.2c Inflammatory vitiligo.

Inflammatory vitiligo is a rare form of vitiligo, described in only a few patients, where an erythematous, raised border occurs at the edge of the actively spreading lesion (Grab & Wise, 1948; Buckley & Lobitz, 1953; Michaëlsson, 1968; Ishii & Hamada, 1981; Le Poole *et al*, 1996b; Horn & Abanmi, 1997; Yagi *et al*, 1997).

1.3.2d Chemically induced/occupational vitiligo.

Chemically induced (occupational) vitiligo is less common than idiopathic vitiligo although the two can show very similar clinical pattern of disease and outcome. The leukoderma may be confined to the region exposed to the chemical i.e. the hands and forearms, but in some patients, distal sites become affected. This is suggestive of systemic exposure through percutanoeus adsorption, inhalation or ingestion; for example some patients suffering from p-tertiary butylphenol induced leukoderma also have abnormal liver function tests (Malten *et al*, 1971).

Compounds such as catechols, phenols, hydroquinone and monobenzone are known to induce cutaneous hypopigmentation and depigmentation; for example, p-tertiary butylphenol caused occupational vitiligo in workers who manufactured p-tertiary butylphenol formaldehyde resin, in shoe manufacturers and people who worked with cleaning fluids (Malten *et al*, 1971) while a similar agent, p-tertiary butylcatechol, which is used in oils as an antioxidant, also caused occupational vitiligo (Gellin *et al*, 1970). In tissue cultured melanocytes, incubation with tertiary butylcatechol produced beading of granules in melanocyte dendrites and irregular contours (Mansur *et al*, 1978). Inhibition of melanogenesis by alteration of tyrosinase activity, abnormal melanization and increased degradation of melanosomes was also shown (Jimbow *et al*, 1974). Some of these compounds are now specifically used for the depigmentation of certain vitiligo patients (see **Section 1.5.1g**).

Some compounds cause temporary depigmentation resembling vitiligo or unusual lesional colouring. Chloroquine which is used as an anti-malarial therapy was reported to have induced vitiligo-like depigmentation in 5 dark skinned individuals (Bentisi-Enchill, 1980; Weelden *et al*, 1982; Gonggrup & Kalla, 1992; Selvaag, 1997). Repigmentation gradually occurred once the chloroquine was stopped. Selvaag (1997) noted that the depigmentation followed sun exposure and hypothesised that a phototoxic reaction had occurred involving the chloroquine to induce damage to the melanocytes. Blue vitiligo refers to the colouration produced by zidovudine deposition in the vitiligo lesion (Ivker *et al*, 1994).

1.4 AETIOLOGY THEORIES OF VITILIGO.

The cause of melanocyte destruction is unknown. It has been suggested, due to the wide clinical presentations of the disease, that vitiligo is more than one disease (Ortonne & Bose, 1993; Morelli, 1995). Neuronal, autoimmune and self-destructive are three separate hypotheses, but Le Poole *et al.* (1993a) first reported the likelihood of interactions of various factors which converged to induce vitiligo. The most recent convergence theory described the pathogenesis of vitiligo as psycho-neuro-endocrin-immunological (Hautmann & Panconesi, 1997). A genetic component is also commonly discussed.

1.4.1 Genetic.

The familial aggregation of vitiligo (Ortonne *et al*, 1983) has indicated a genetic involvement for the pathogenesis of the disease. Butterworth & Strean (1962) suggested an autosomal dominant pattern of inheritance with modulating factors affecting the penetration and expression. While vitiligo tended to segregate with kinship (Hafez *et al*, 1982; Das *et al*, 1985a; Salamon, 1989; Majumder *et al*, 1993), a typical Mendelian pattern could not be seen. Several groups have suggested that the pattern of inheritance was polygenic/multifactorial in nature (Mehta *et al*, 1973; Hafez *et al*, 1982; Mohan *et al*, 1982; Ramaiah *et al*, 1988; Bhatia *et al*, 1992). Das *et al.* (1985b) associated vitiligo with 2 polymorphic genetic marker loci i.e. vitiligo was linked with genes on several different chromosomes. The pattern of inheritance for vitiligo vulgaris has been described as that of recessive alleles at 4 unlinked diallelic loci (Majumdar *et al*, 1988; Majumder *et al*, 1993; Nath *et al*, 1994).

The study of the MHC genes has revealed polymorphisms associated with different ethnic groups (Retornaz *et al*, 1976; Kachru *et al*, 1978; Metzker *et al*, 1980; Foley *et al*, 1983; Tiwari & Terasaki, 1985 ; Dunston & Halder, 1990; Finco *et al*, 1991; Poloy *et al*, 1991; Lorini *et al*, 1992; Orrechia *et al*, 1992; Venneker *et al*, 1992; Ando *et al*, 1993; Schallreuter *et al*, 1993a; Venneker *et al*, 1993; Al-Fouzan *et al*, 1995; Venataram *et al*, 1995; Buc *et al*, 1996; Ghaderi *et al*, 1996; Parish-Gause *et al*, 1997; Buc *et al*, 1998) (see **Table 1.1** for population examples). Some interesting results have been found; familial cases of vitiligo were associated with HLA-B46, whereas non-familial cases were associated with HLA-A31 and -Cw4, in a Japanese population (Ando *et al*, 1993). HLA-Bw4 segregated with patients whose parents were consanguinous in an Omani Arabian popoulation (Venkataram *et al*, 1993). Finco *et al.* (1991) described specific supratypes which were associated with different age groups; HLA-BfS, C4A3, C4B1, DR5(w11), DQw3 with paediatric patients, HLA-BfS, C4A3, C4B1, DR7, DQw2 were found with adult patients (further described in **Table 1.1**) and HLA-DR4 with black Americans of a young age (Dunston & Halder, 1990). In adult Germans with vitiligo, HLA-Bw60 was significantly increased (Schallreuter *et al*, 1993a). Venkataram *et al.* (1993) have shown that HLA-DR7 was significantly increased in

Table 1.1 Major histocompatibility complex molecules in ethnic groups of vitiligo patients.

Reference	ethnic origin	patient number[a]	MHC class I	MHC class II	MHC class III
Retornaz *et al*, 1976	Caucasian (French)	90 (341)	B13 ↑[b]		
Kachru *et al*, 1978	Black Americans	48 (107)	A1, A2, Aw31 ↑ A10 ↓		
Metzker *et al*, 1980[c]	Jewish Moroccans Yemenites	18 (462) 16 (462)	B13 ↑ B35 ↑ (B13 absent)[d]		
Foley *et al*, 1983	White Americans	48 (979)		DR4 ↑	
Dunston & Halder, 1990	Black Americans	24 (143)		DR4 ↑[e]	
Finco *et al*, 1991[f]	Caucasian Northern Italian	87 (388)	A30, B27, Cw6 ↑ B13 ↑ (ns)	DR7 ↑ DR1, DR3↓	C4AQ0↓, C4A3↑
Poloy *et al*, 1991	Caucasian Hungarians	57 (88/160)		DR1, (DR3)[g] ↑	C4AQ0↓, C4A4↑
Ando *et al*, 1993[h]	Japanese	131	A31, Cw4↑ Bw46 ↑		
Schallreuter *et al*, 1993a[i]	Caucasian	102 (400)	A2, Bw60 ↑	DRw12 ↑	
Venneker *et al*, 1993[j]	Dutch	48 (703)	Cw7 ↑	DR6 ↑ DR3 ↓	
Al Fouzan *et al*, 1995	Kuwaiti	40 (40) 15 (40)	B21[k], Cw6 ↑ A19↓	 DR53 ↑, DR52 ↓	
Venkataram *et al*, 1995[l]	Omani Arabians	50 (92/55)	Bw6 ↑	DR7 ↑	
Buc *et al*, 1996	Slovak	67 (760/152)	A2, Dw7[m] ↑		

Kovac *et al.* (1998) recently published a similar table (Table II) examining the linkage with MHC class molecules in vitiligo patients and patients with VKH. Results shown are significant unless otherwise noted for the number of patients listed.
The major histocompatibility molecules are divided in to three classes - class I and class II which encode histoglobulins at the A, B, C, DR, DQ, DP loci, while class III produce complement factors such as C4 and the Bf, properdin factor B. They are highly polymorphic.

[a] The patient number is shown along with control number from normal subjects in brackets.

[b] A significant increase in B13 was only seen in patients with antithyroid antibodies.

Table 1.1:

[c] Significant results were seen only in Jewish Moroccan (11/18) and Yemenites (9/16) whose age of onset for vitiligo was before the age of 20.

[d] B13 was absent in both the patient and control Yemenites.

[e] Dqw3 was also significantly increased and authors reported this due to a linkage disequilibrium with DR4. DR4 was associated with an early onset of disease (<20 years), and DR4/DQw3 segregated with a positive family history. Another finding was that DRw6 associated with late onset of disease and a negative family history.

[f] The results are shown irrespective of age of onset, while two separate supratypes were seen according to age. The paediatric form (<12 years) was represented as HLA-BfS, C4A3, C4B1, DR5(w11), DQw3 while the adult form (>16 years) was represented as HLA-BfS, C4A3, C4B1, DR7, DQw2.

[g] DR3 was only significant in patients with other autoimmune disorders (35 of the patients). Two further Northern Italian studies showed that HLA-A3 and HLA-A30, Cw6 were increased (Lorini *et al*, 1992; Orecchia *et al*, 1992; respectively), while HLA-DQw3 (Orecchia *et al*, 1992) and C4AQ0 (Lorini *et al*, 1992; Orecchia *et al*, 1992) were decreased. Gm (heavy chain of the immunoglobulin constant regions) allotypes - 3, 23, 5, 10, 11, 13, 14 - were significantly increased in association with A3 when patients had no autoantibodies (Lorini *et al*, 1993).

[h] A31 and Cw4 were found in patients with non-familial vitiligo (29 patients) while Bw46 was found in patients with familial vitiligo (102 patients; familial vitiligo was described as patients having members of 3 generations with vitiligo).

[i] A2 was increased in 65/102 patients, DRw12 in 9/76 patients, while Bw60 was only increased in 47 patients who were older than 21 years. Tiwari & Terasaki, (1985) reported that A2 and Bw60 were increased at similar frequenices in European Caucasians but these findings were not seen in the Italian Caucasians (Finco *et al.* 1991; Lorini *et al.* 1992; Orecchia *et al.* 1992).

[j] In an earlier report by this group, DR4 was significantly increased, but this had not been corrected for the number of antigens examined (Venneker *et al*, 1992)

[k] Southern Iranian patients associated with HLA-B21 in addition to A11, A23 and Cw4 (Ghaderi *et al*, 1996).

[l] DR7 was significantly increased in patients with acrofacial vitiligo compared to patients with focal disease. In addition HLA-Bw4 segregated with patients with a positive family history.

[m] HLA-Dw7 is an epitope of the α and β chains of HLA-DQ antigens (DQA1*0201 and DQB1*0202 respectively) (WHO, 1997). Therefore in an extension of this study, Buc *et al.* (1998) found that DRB1*0701, DQB1*0201 and DPB1*1601 alleles were significantly increased in vitiligo patients. Parish-Gause *et al.* (1997) have also reported HLA-DRB1 associations with vitiligo.

patients with acrofacial vitiligo compared to patients with focal disease, but no other studies have seen increased prevalence of HLA polymorphisms with clinical vitiligo types. The C4B gene, which is involved in complement production, may play a role (Venneker *et al*, 1992). However, with the wide variety of results, it is still difficult to draw any conclusions on the importance of the polymorphisms detailed by the different groups.

1.4.1a Intrinsic defect in the melanocyte.

An intrinsic defect in the melanocyte might indicate a genetic deposition to the disease. Melanocytes from unaffected regions of vitiligo patients skin show abnormalities in the rough endoplasmic reticulum (RER) (Boissy *et al*, 1991; Im *et al*, 1994) and these persist through the culture life of the patient cells (Boissy *et al*, 1991). However, this defect did not appear to be cytotoxic in human melanocytes under *in vitro* conditions (Boissy *et al*, 1991) and Im *et al*. (1994) showed that the vitiligo melanocytes had comparable rates of DNA synthesis and melanogenesis in response to UVB as did control melanocytes. Ortonne & Bose (1993) suggested that *in vivo* this abnormality may lead to an immune reaction by induction of cytoplasmic and/or cell surface modifications.

Vitiligo melanocytes cultured from the uninvolved and perilesional skin demonstrated growth defects (Puri *et al*, 1987) which could partially be corrected by the presence of extracts of fetal lung fibroblasts (Mojamdar *et al*, 1987), or fibroblast-derived growth factor (Puri *et al*, 1989). Ramaiah *et al*. (1989) proposed that a decreased concentration of melanocyte growth factor(s) could have a role in the pathogenesis of vitiligo. Another study, by an independent group (Jee *et al*, 1993) described conditions under which melanocytes derived from patients with segmental but not nonsegmental vitiligo could proliferate and be passaged. Catalase was shown to be vital in the initial isolation of vitiligo melanocytes, and their early culture, but not necessary for their later culture (Medrano & Nordlund, 1990). A growth defect may leave the vitiligo melanocytes unable to recover or replenish the skin after a precipating factor which damages the melanocyte.

In a recent review, it was suggested that other genes such as the b-cl2 gene, which determines premature melanocyte death (Plettenberg *et al*, 1995; Cosulich & Clark, 1996) be studied for signs of defects between vitiligo and normal melanoytes (Castenet & Ortonne, 1997).

1.4.2 Neuronal.

Several clinical observations have led to this theory including the common symmetrical distribution or the true dermatomal pattern of segmental vitiligo (Lerner, 1959; Koga, 1977), the occasional case reports of sparing by vitiligo of denervated skin (Lerner, 1959), requirement for innervation for skin transplants to repigment area (Spencer & Tolmach, 1952), association with decreased sweating within lesions of segmental vitiligo (Koga, 1977; Nordlund & Lerner, 1982), an association with viral encephalitis (Nellhaus, 1970) and vitiligo also developed in areas supplied by nerves damaged in a brachial plexus injury (Costea, 1961). Patients with inflammatory disorders which affect the peripheral nervous system such as leprosy may suffer from hypomelanotic macules (Job *et al*, 1972). Also, patients with neurodysplasias such as neurofibromatosis and tuberous sclerosis often present hyper- and hypo-pigmented lesions (Koplon & Shapiro, 1968; Zvulunov & Esterly, 1995). Melanocytes have been described as less differentiated neural cells which in the perilesional regions of vitiligo are capbable of neural differentiation (Iyengar & Misra, 1988). Melanocytes in the marginal zone of the lesions can produce noradrenaline as well as pigment (Iyengar & Misra, 1987).

An association with psychological stress (Nordlund & Lerner, 1982) has led to investigations in levels of acetylcholine, catecholamines, or related enzymes, with conflicting results (Lerner, 1959; Bamshad & Lerner, 1964; Dunerva, 1973). In more recent studies, Iyengar (1989) reported that acetylcholinesterase activity was reduced in vitiliginous skin as compared to normal skin. Levels of catecholamines were significantly increased in patients with actively progressing vitiligo (Morrone *et al*, 1992) but these were urinary levels, and release from cutaneous free nerve endings was not examined. These results were not confirmed by plasma levels of catecholamines in patients with acrofacial or generalised vitiligo (Orecchia *et al*, 1994) although, Orecchia

et al. (1994) did note a trend towards an increase in the levels of catecholamines in patients with a shorter duration of the disease. The "radical hypothesis" was put forward by Morrone *et al.* (1992) as a result of their findings and is described further in **Section 1.4.4.** Schallreuter *et al.* (1994d) found noreadrenalin levels in both the epidermis and plasma to be increased, due to defective catecholamine biosynthesis, but this is further discussed in **Section 1.4.4** as the findings may relate to keratinocyte catecholamine synthesis (Schallreuter *et al*, 1992) and not a neuronal abnormality. A role for catecholamines in the development of vitiligo was discussed in Bose (1994b), who described a patient with segmental vitiligo and naevus anemicus. Naevus anemicus has been associated with disturbances in catecholamines (Greaves *et al*, 1970). Additionally, met-enkephalin and beta-endorphin oscillations were no longer circadian in vitiligo patients (Mozzanica *et al*, 1992) and plasma met-enkephalin levels were generally higher in patients with active vitiligo. Although met-enkephalin is associated with the stress response (Heijnen *et al*, 1987; Khansari *et al*, 1990), Mozzanica *et al.* (1992) did not assess if patients experienced stress associated with their condition.

1.4.2a Alterations in nerves and neuronal related peptides in vitiligo skin.

Several studies have shown alterations in nerve structures and contact between melanocytes in vitiligo (Chan-Gen, 1959; Mori, 1964; Breathnach *et al*, 1966; Nozaki, 1976; Morohashi *et al*, 1977; McBurney, 1979; Gokhale & Mehta, 1983; Al'Abadie *et al*, 1995; Liu *et al*, 1996) as described further in **Section 1.2.2g.** Hypomelanotic macules seen in leprosy sufferers, also show decreased numbers of epidermal melanocytes and degenerative changes of superficial nerve fibres (Job *et al*, 1972). Al'Badie *et al.* (1994) reported increased neuropeptide Y IR in 5 out of 10 patients with nonsegmental symmetrical type vitiligo in marginal areas and in 3 of these patients with active disease, neuropeptide Y IR was increased in lesional skin. A minimal increase in vasoactive intestinal polypeptide and no difference in substance P or calcitonin gene-related peptide was seen (Al'Badie *et al*, 1994). Liu *et al.* (1996) reported changes in the number of NGF IR and calcitonin gene-related peptides-IR nerve fibres, but no change in the neuronal marker protein PGP9.5 and in S100 IR. While abnormalities in neuropeptides have been reported in vitiligo, only recently has a study examined the connection of the nervous system with melanocytes in normal skin (Hara *et al*, 1996)

and the authors suggested calcitonin gene-related peptide secretion may influence melanocyte behaviour. NGF and neurotrophin-3 have been reported to influence various aspects of normal melanocyte function (Peacocke *et al*, 1988; Yaar *et al*, 1991; Yada *et al*, 1994) and Lui *et al*. (1996) suggested a role for NGF in the pathogenesis of vitiligo. While patients do not complain about sensory abnormalities in the depigmented skin, autonomic dysfunction has been noted before (Chanco-Turner & Lerner, 1965; Dutta, 1965; Dutta & Dermat, 1972; Dutta & Mandal, 1982; Merello *et al*, 1993). This may be related to the absence of Merkel cells (as described in **Section 1.2.2e)** which are thought to interact with nerve endings.

1.4.2b The melatonin receptor hypothesis.

Hyperactivation of the melatonin receptor initiating a dysregulation of melanogenesis through a cascade of intracellular pathways was proposed as a precipating factor in the development of vitiligo (Slominski *et al*, 1989). This could lead to the build up of toxic metabolites or alteration in the immune cell surface related molecules. Iyenger (1994b) reported that melanocytes in the marginal regions of vitiligo lesions were receptive to incubation with melatonin and serotonin during a pulse of UV irradiation. The authors suggested that melanocytes had receptors for these neuromodulators. Dendricity was decreased in the presence of melatonin and could modulate a depigmentation effect (Iyengar, 1994b).

1.4.3 Autoimmune.

Several lines of evidence exist for vitiligo being an autoimmune disease such as autoantibodies against melanocytic cells, or mononuclear infiltrates, as discussed in this section. However, one of the major questions yet to be answered, is whether vitiligo is due to a primary dysfunction in the immune system or does it occur in response to some extrinsic factor such as a viral infection, or is there an intrinsic defect in the melanocyte.

1.4.3a Nonspecific immune markers.

Vitiligo has also been associated with other autoimmune diseases, such as autoimmune multiple endocrine glandular insufficiency (McGregor *et al*, 1972); thyroid disorders (Cunliffe *et al*, 1968; Hegedus *et al*, 1994; Schallreuter *et al*, 1994b), autoimmune hypoparathroidism (Fields *et al*, 1971), diabetes mellitus (Dawber, 1968; Gould *et al*, 1985), pernicious anaemia (Allison & Curtis, 1955), alopecia areata (Demis & Weiner, 1963; Fields *et al*, 1971), lupus erythematosus (Forestier *et al*, 1981), autoimmune atrophic gastritis (Zauli *et al*, 1986) and Addison's disease (Dunlop, 1963; Mulligan & Sowers, 1985). Autoantibodies associated with organ-specific autoimmune disorders have also been detected in patients (Cunliffe *et al*, 1968; Brastoff *et al*, 1969; Woolfson *et al*, 1975; Grimes *et al*, 1983a; Betterle *et al*, 1985; Halder *et al*, 1987; Lorini *et al*, 1992). In a recent study, Korean patients had an increased incidence of antinuclear (12.4%), antimicrosomal (7.1%) and antismooth muscle antibodies (25.7%) (Hann *et al*, 1993a). The appearance of these antismooth muscle antibodies related to vitiligo patients with early onset of disease, a long duration of disease and a positive family background. First-degree relatives, in the absence of clinical symptoms have also been shown to be positive for a variety of autoantibodies (Masala *et al*, 1982; Mandry *et al*, 1996). Vojdani & Grimes (1996) report that a significant number of patients with vitiligo have antibenzene ring antibodies in their sera. Exposure to chemicals (environmental, industrial or domestic) containing a benzene ring may thus set in motion an immunopathological injury in these patients leading to depigmentation (Vojdani & Grimes, 1996), although the effects of these chemicals can lead to depigmentation via other methods (as described in **Sections 1.3.2d**). It has still to be demonstrated whether anti-benzene ring antibodies are a marker for occupational leukoderma.

1.4.3b Autoimmunity due to autoantibodies.

Langhof *et al.* (1965) first reported antimelanin antibodies. Anti-melanocytic antibodies were then described by Hertz *et al*, 1977 and Howanitz *et al.* 1981 in patients with a complex syndrome associating hypoparathyroidism, Addison's disease, chronic muco-cutaneous candidiasis, and vitiligo but these antibodies were related to the chronic

muco-cutaneous candidiasis. Since then, strong evidence using various techniques shows that patient sera contain various types of anti-melanocytic antibodies, (Naughton *et al*, 1983a & b; Bystryn & Naughton, 1985; Bystryn & Pfeffer, 1988; Galbraith *et al*, 1988; Cui *et al*, 1992; Fishman *et al*, 1993; Yu *et al*, 1993; Merimsky *et al*, 1994; Cui *et al*, 1995; Hann & Kim, 1995; Park *et al*, 1996). The characterisation of the anti-melanocytic antibodies has shown that they select antigens of approximate molecular weights of 35, 40-45 (Vit40), 75 (Vit75), 90 (Vit 90) or 150kD (Cui *et al*, 1992; Cui *et al*, 1995). The 35 and 90kD antigens were found exclusively on pigment cells (Cui *et al*, 1992; Cui *et al*, 1995) while the others have reacted with a number of different cell types. In Korean patients, anti-melanocytic antibodies have been shown to select an antigen of approximately 65kD (Park *et al*, 1996). It has been suggested that melanocytes are much more sensitive to toxic or immune-mediated injury than other cutaneous cell types (Norris *et al*, 1988) and therefore the melanocyte would be more susceptible to these antigens common to many cell types. The Vit75 antigen is not thought to be related to tyrosinase, TRP-1 or TRP-2, which have similar molecular weights (Cui *et al*, 1995) but anti-tyrosinase antibodies (Song *et al*, 1994b; Baharav *et al*, 1996; Merimsky *et al*, 1996a, b & c; Kemp *et al*, 1997a & b) and anti-TRP-2 antibodies (Kemp *et al*, 1997b) have recently been reported in patient sera. The incidence of such antibodies in vitiligo patients can be quite low (Kemp *et al*, 1997a & b). The anti-melanocytic antibodies have been shown to belong to the IgG class (Harning *et al*, 1991) and subclasses IgG_{1-3} (Xia *et al*, 1991). Moreover, IgA levels have been reported to correlate best with vitiligo activity rather than IgG (Aronson *et al*, 1987 & 1989).

Patient sera have also been shown to induce immune damage to cultured human melanocytes via antibody-mediated complement activation and/or antibody-dependent cellular cytotoxicity (Norris *et al*, 1988; Park *et al*, 1991; Cui *et al*, 1993; Yu *et al*, 1993; Abdel-Naser *et al*, 1994) and disease activity correlates well with the titre of anti-melanocytic antibodies (Naughton *et al*, 1986; Harning *et al*, 1991). Additionally, an *in vivo* study using human skin grafts on mice showed that the IgG fraction of serum from patients with vitiligo caused melanocyte death (Gilhar *et al*, 1995). Decay acceleration factor (DAF) and membrane cofactor protein (MCP), which are involved in the protection against antibody mediated complement activation (Morgan & Meri, 1994;

Asghar, 1995; Liszewski *et al*, 1996), were expressed on normal melanocytes *in vitro* (Venneker *et al*, 1998). DAF contributed more than MCP in protecting melanocytes against complement attack. These findings together with the discovery that the expression of DAF and MCP were decreased in the skin of vitiligo (Venneker *et al*, 1994; Asghar, 1995) suggest an autoimmune role in the destruction of melanocytes.

Hair follicle melanocytes in culture expressed some antigens associated with alopecia areata but not with vitiligo while the reverse was true for epidermal melanocytes in culture (Tobin & Bystryn, 1996). These authors suggest this was a possible reason why hair follicle melanocytes are often spared in vitiligo.

Anti-pigment cell antibodies have been found in patients with VKH, which correspond to those found in vitiligo patient sera (Patel *et al*, 1992). The presence of anti-melanocytic (Cui & Bystryn, 1995) and anti-tyrosinase antibodies (Merimsky *et al*, 1996a, b & c; Fishman *et al*, 1997) have also been noted in malignant melanoma patients. It is known that patients with melanoma who develop hypopigmentation have an improved prognosis (Barnes & Nordlund, 1989). Patients treated with interleukin IL-2 therapy for melanoma often developed vitiligo-like depigmentation (Scheibenberger *et al*, 1994; Ho, 1995; Wolkenstein *et al*, 1995; Rosenberg *et al*, 1996). While anti-melanocyte antibodies present during melanoma and vitiligo bind to similar antigens shared by normal and malignant pigment cells (Cui & Bystryn, 1995), Berd *et al*. (1996) have warned against linking vitiligo and melanoma. Merimsky and co-workers have also suggested that different mechanisms may exist between the appearance of lesions in vitiligo and in patients with melanoma-associated hypopigmentation (Merimsky *et al*, 1994 & 1996b).

1.4.3c T cell mediated immunity.

Evidence exists for T cell mediated disease, in the form of a more prominent infiltrate of T cells in the perilesional epidermis in lesions (Abdel-Naser *et al*, 1994; Ahn *et al*, 1994; Le Poole *et al*, 1996b; Hann *et al*, 1997; Horn & Abanmi, 1997; Yagi *et al*, 1997) (as previously described in **Section 1.2.2e**). The upregulation of ICAM-1 and/or HLA molecules in the epidermis have been reported in the perilesional regions (Ahn *et al*,

1994; Le Poole *et al*, 1996b; Yagi *et al*, 1997) and specific increased expression of these molecules has been shown for melanocytes in perilesional biopsies of vitiligo (Al Badri *et al*, 1993a) (as previously described in **Section 1.2.2e**) and in ocular melanocytes of patients with VKH (Sakamoto *et al*, 1991). Infiltration of T cells and macrophages parallels the loss of pigment and the disappearance of melanocytes in inflammatory vitiligo (Le Poole *et al*, 1996b).

Peripheral T cells and subpopulations have been examined in many groups (Soubiran *et al*, 1985; Grimes *et al*, 1986; Halder *et al*, 1986; Ghoneum *et al*, 1987; Mozzanica *et al*, 1989; D'Amelio *et al*, 1990; Baumer *et al*, 1990; Mozzanica *et al*, 1990; Abdel-Naser *et al*, 1992; Mozzanica *et al*, 1990; Hann *et al*, 1993c, Al Fouzan *et al*, 1995; Durham *et al*, 1995) with conflicting results (as summarised in **Table 1.2**). Only two studies demonstrated that levels of CD3, CD4, CD8 and natural killer cells were normal (Baumer *et al*, 1990; Abdel-Naser *et al*, 1992). However, Baumer *et al.* (1990) found elevated numbers of peripheral monocytes (OKM5+) while Abdel-Naser *et al.* (1992) found a significant decrease of the CD4+/CD54RA+ subset (suppressor inducer lymphocytes) and a significant increase in activated peripheral T cells (expressing HLA-DR+). This has also been shown in other autoimmune disease such as insulin-dependent diabetes mellitus (Al-Kasseb & Raziuddu, 1990), rheumatoid arthritis (Morimoto *et al*, 1988) and systemic lupus erythematosus (Morimoto *et al*, 1987). The overall conclusion is that circulating T cell subpopulations are rarely normal.

Natural killer cell activity (NKCA) was examined (Ghoneum *et al*, 1987; Mozzanica *et al*, 1990 & 1992; Durham-Pierre *et al*, 1995) but only Durham-Pierre *et al.* (1995) compared NKCA against normal and malignant melanocytes from vitiligo patients and normal subjects (as detailed in **Table 1.2**). Enhanced activity of T cells has been suggested due to the finding of increased release of leukocyte migration inhibition factor (Zaman *et al*, 1992). Elevated serum levels of soluble IL-2 receptor were seen in patients with active nonsegmental vitiligo (Honda *et al*, 1997) but not in patients with segmental or non-progressive nonsegmental vitiligo. Elevated serum levels of soluble IL-2 receptor have been observed in autoimmune diseases (Wolf & Brelsford, 1988; Keystone *et al*, 1988) and relate to T cell activation (e.g. Tokano, 1989). In a recent study, peripheral mononuclear cells from patients with active vitiligo had several

Table 1.2 Peripheral blood cell abnormalities in vitiligo.

Reference	Number of patients[a]	Total T cells (CD3+)	Helper T cells (CD4+)	Suppressor T cells (CD8+)	CD4/CD8 ratio	Natural killer cells	B cells
Soubiran *et al*, 1985	32	normal	increased	increased	increased	ND[b]	decreased
Halder *et al*, 1986	25	decreased	decreased	decreased	decreased	increased[c]	ND
Grimes *et al*, 1986	20	normal	decreased	normal	decreased	ND	ND
Ghoneum *et al*, 1987	18	ND	ND	ND	ND	decreased function[d]	ND
Mozzanica *et al*, 1989	10	ND	ND	ND	ND	increased function[e]	ND
Baumer *et al*, 1990	25	normal	normal	normal	normal	normal[f]	ND
D'Amelio *et al*, 1990[g]	22	normal	increased	normal	increased	ND	ND
Mozzanica *et al*, 1990	12	ND	decreased	decreased	ND	ND	ND
Abdel-Naser *et al*, 1992[h]	16	normal	normal	normal	normal	normal[f]	ND
Hann *et al*, 1993c[i]	50	normal	decreased	normal	reversed	normal[f]	normal[j]
Al-Fouzan *et al*, 1995	40	normal[k]	increased	normal	ND	ND	ND
Durham-Pierre *et al*, 1995	21	ND	ND	ND	ND	normal function[l]	ND

Table 1.2: The design of the table is based on and expanded from table 1 in Abdel-Naser *et al.* (1992).
[a] Patient groups generally consisted of subjects from Causcasian or various ethnic backgrounds; Hann *et al.* (1993c) and Al-Fouzan *et al.* (1995) studied vitiligo patients from Korean and Kuwaiti ethnic groups respectively. Patients with a nonsegmental form of vitiligo e.g. generalised and localised, were generally studied.
[b] ND - not determined.
[c] Increased number of Leu7+ cells. The Leu 7 antibody also has affinity for some T and B cells; activity of natural killer cells was not discussed.
[d] Decreased natural killer cell activity (NKCA) against Molt-4 cells, a T lymphoid cell line derived from patients with acute lymphatic leukemia, but no change in activity against K562 cells, a myelogenous leukemia cell line.
[e] Increased NKCA against K562 cells. This was also reported in Mozzanica *et al.* (1992). Patients and normal subjects however had similar circadian levels of natural killer cells.
[f] Normal numbers of natural killer cells found; activity not determined.
[g] Similar results were obtained in 23 first degree relatives of the vitiligo patients.
[h] There was a significant decrease in the CD4+/CD54RA+ subset (suppressor inducer lymphocytes) of lymphocytes in vitiligo patients although CD4 and CD8 positive lymphocyte levels were the same between normal and vitiligo subjects.
[i] The results irrespective of the type and duration of vitiligo are shown. Patients with generalised but not localised vitiligo had a significantly lower number of CD4+ and increased number of CD8+ lymphocytes, while patients with localised vitiligo had significantly lower number of CD8+ lymphocytes than patients with generalised vitilgo.
[j] The results irrespective of the type and duration of vitiligo are shown. However patients with recent onset, i.e. duration of less than one year at the time of study had significantly higher numbers of B cells than control subjects or patients with a long duration of disease.
[k] Total T cells were measured using a CD2 antibody.
[l] NKCA was measured against K562 cells, normal and malignant melanocytes. The percent cytotoxicity was lower against normal and malignant melanocytes than K562 cells. Lymphokine-activated killer cell activity was also normal against normal melanocytes.

alterations in cytokine release (Yu *et al*, 1997). The authors suggested that increased IL-6, which can enhance ICAM-1 expression in normal melanocytes (Kirnbauer *et al*, 1992; Krasagakis *et al*, 1995), may induce lymphocyte attachment while decreased levels of granulocyte/macrophage-colony stimulating factor (GM-CSF), a growth factor for melanoytes (Imokawa *et al*, 1996b), may check melanocyte proliferation and retard recovery from an immunological attack.

Another theory proposed was that T lymphocytes have a primary dysfunction which induces them to form clones which are benign cutaneous lymphoma that home to corresponding anatomical sites on the two sides of the body. This could account for the symmetrical distributions of lesions (Goudie *et al*, 1990; Goudie *et al*, 1991).

1.4.3d Infection leading to an immune response.

An immune response, triggered by an infection has been proposed as a factor leading to the development of vitiligo in some patients (Le Poole *et al*, 1993a). For example hypopigmentation resembling vitiligo can occur in certain infectious disease e.g. leprosy (Shegan, 1971; Job *et al*, 1971; Olumide *et al*, 1990; Pavithran, 1991) (although as noted in **Section 1.4.2** leprosy also affects the nervous system). Schwann cells which are the preferential immunological targets in leprosy, are from the same bipotent intermediate progenitor cell (Stocker *et al*, 1991) as melanocytes, hence melanocytes may also be a target. Melanocytes are also capable of phagocytosis (Le Poole *et al*, 1993d) and of processing and presenting antigen including the *Mycobacterium leprae* heat shock protein 65 to T cell clones from leprosy patients (Le Poole *et al*, 1993b).

Patients with AIDS (Duvic *et al*, 1987; Tojo *et al*, 1991; Ivker *et al*, 1994) have developed vitiligo and recently cytomegalovirus (CMV) DNA was found in skin biopsies taken from 38% of patients with vitiligo (Grimes *et al*, 1996). It was present in the depigmented and uninvolved skin of some patients, being more prominent in biopsy specimens of progressive vitiligo of relatively brief duration and was absent in all of the control samples. Hence viral infections may trigger vitiligo in some patients.

1.4.4 Keratinocytes and free radical breakdown.

Many investigators report that keratinocytes show morphological abnormalities (as discussed in **Section 1.2.2b)** but the significance of these findings were rarely discussed. Moellmann *et al.* (1982) suggested that the destruction of keratinocytes may be due to toxic intermediates of melanogenesis but the presence of anti-keratinocyte antibodies (Yu *et al*, 1993) which are not effective suggest that keratinocyte damage may be secondary. Keratinocytes produce a whole range of growth factors and cytokines which influence melanocytes (discussed further in **Chapter 3**), and therefore it could be conceivable that a defect in the keratinocyte production of growth factors, results in a disturbance of the keratinocyte-melanocyte relationship. Liu *et al.* (1995) found elevated levels of α-melanocyte stimulating hormone (α-MSH) in the epidermis which may due to overproduction by epidermal cells (discussed further in **Chapters 3 & 4**). Additionally, the overproduction of tenascin, an extracellular matrix (ECM) component, could be important (O'Donnell, 1991; Le Poole *et al*, 1997). Vitiligo melanocytes did not exhibit defective integrin expression, but tenascin interfered with their attachment capacity (Le Poole *et al*, 1997). A preliminary study has suggested that there is a defect in vitiligo keratinocytes, as exhibited in a co-culture system with melanocytes (Le Poole *et al*, 1991), but this study has yet to be repeated. In an epidermal/dermal reconstitute model, which provides a more natural environment, mix and match studies with normal and vitiligo keratinocytes and melanocytes showed no significant differences (Bessou *et al*, 1997a), and the authors suggested that an extrinsic factor is required for the initiation of vitiligo.

1.4.4a Free radical breakdown.

Following the findings of elevated catecholamines in the urine of patients with active vitiligo, the "radical hypothesis" was proposed (Morrone *et al*, 1992). It was suggested that elevated catecholamine release from autonomic nerve endings in the microenvironment of the melanocyte would lead to the destruction of melanocytes via direct cytotoxic actions or by an indirect build-up of oxidative stress within the epidermis. As skin and mucosae possess alpha receptors, activation by abnormal catecholamine discharge could produce excessive vasoconstriction leading to epidermal

and dermal hypoxia-ischaemia which would cause hyperproduction of reactive oxidative species due to additional insults during reoxygenation (Morrone *et al*, 1992). In support of this theory was the recent finding that the epidermis of patients with active vitiligo had an imbalance of the intracellular redox status and a significant depletion of enzymatic and non-enzymatic antioxidants (Passi *et al*, 1998). The profile of reduced levels of $CoQ_{10}H_2$, vitamin E, glutathione reductase activity and catalase activity and the increase of oxidised glutathione seen in the vitiligo epidermis is an indication of increased oxidative stress (Davidson *et al*, 1994; Han *et al*, 1996). Previously the group had shown that the antioxidant status of the blood of patients with active vitiligo was not significantly different to control subjects, suggesting that effects were specific to the skin (Picardo *et al*, 1994). Normal melanocytes have lower levels of enzymatic and non-enzymatic antioxidants than keratinocytes and fibroblasts (Yohn *et al*, 1991) and cultured vitiligo melanocytes have altered levels of antioxidant defences (Maresca *et al*, 1997) rendering them very susceptible to epidermal alterations in oxidative stress. These changes in the epidermal oxidative stress levels could have direct cytotoxic effects or alter the antigenic profile of the melanocytes and additionally affect keratinocytes and Langerhans' cells.

Low levels of catalase activity (Schallreuter *et al*, 1991), defective tetrahydrobiopterin recycling and catecholamine synthesis and high levels of monoamine oxidase A in the epidermis of patients with vitiligo lead to the accumulation of toxic levels of hydrogen peroxide (Schallreuter *et al*, 1994a, c & d; Schallreuter *et al*, 1996b). The defective tetrahydrobiopterin synthesis can lead to the build-up of 6-biopterin which is toxic to melanocytes (Schallreuter *et al*, 1994a). Further evidence supporting this theory comes from the observation that phenylalanine accumulation occurred in the involved epidermis of patients (Schallreuter *et al*, 1998). Phenylalanine hydroxylase activity is decreased in the defective tetrahydrobiopterin system (Schallreuter *et al*, 1994c). Additionally catechol-*O*-methyl transferase, which methylates catecholamines to inactive them, was increased in lesional vitiligo skin (Le Poole *et al*, 1994a) and this was probably due to the increases in catecholamine levels. Keratinocytes are capable of catecholamine synthesis (Schallreuter *et al*, 1992) and this is significantly higher in the epidermis of vitiligo. A role for β2 adrenoreceptors and catecholamines in the pathophysiology of vitiligo is further substantiated by the abnormally high expression of

β-adrenoreceptors of differentiated keratinocytes in involved vitiligo skin (Schallreuter *et al*, 1993b). Melanocytes also express α-1-adrenoreceptors in response to noradrenaline (Schallreuter *et al*, 1996a).

Thioredoxin reductase is decreased in vitiliginous skin (Schallreuter *et al*, 1986; 1987; Schallreuter & Pittelkow, 1988; Schallreuter & Wood, 1989). Vitiligo keratinocytes cultured *in vitro* (Schallreuter & Pittelkow, 1988) and melanocytes (Schallreuter *et al*, 1996a) have defective calcium uptakes. Calcium plays a regulatory role in the free radical reduction capacity of thioredoxin reductase. Decreased uptake of calcium by keratinocytes and melanocytes would lead to a high concentrations of calcium which in turn would inhibit thioredoxin reductase. A build up in free radicals due to this defect may lead to keratinocyte and melanocyte damage (Ortonne & Bose, 1993). Thioredoxin reductase also plays a role in the tetrahydrobiopterin system (Schallreuter *et al*, 1994a).

1.4.4b Self destruction.

Both the tetrahydrobiopterin system (Schallreuter *et al*, 1994c) and thioredoxin reductase (Schallreuter & Wood, 1989) have been proposed to have a controlling role in melanogenesis through the conversion of phenylalanine to tyrosine and hence it has been proposed that defects in these systems in the epidermis of vitiligo patients could lead a route of self destruction through defective melanogenesis. This links in with the self-destructive theory in which melanocytes are destroyed by toxic products made by themselves. Melanogenesis is a beneficial yet a biochemically dangerous pathway which occurs within the melanocyte (as described in **Section 1.1.1**). In the pigmentary pathway, if the vitiligo melanocyte becomes defective in its ability to inactivate certain enzymes, or overproduces certain enzymes, or is unable to utilize intermediate products which are toxic to the melanocyte, then self destruction could occur (Pawelek *et al*, 1980; Moellman *et al*, 1982). Lerner (1971) hypothesised that a defect in the natural protective mechanism that removes toxic melanin precursors from the melanocytes leads to their destruction. Another theory is that melanin is unable to function as a scavenger of toxic free radicals. These build up and kill the cell (Nordlund & Lerner, 1982). As previously mentioned in **Section 1.1.1** the importance of the melanosome is

demonstrated by transfection studies in fibroblasts (Bouchard *et al*, 1989) where only the fibroblasts with the full machinery survived melanogenesis.

1.5 TREATMENT OF VITILIGO.

Vitiligo is not a life threatening disease, but as previously mentioned in **Section 1.1** can lead to psychological problems in many patients (e.g. Porter *et al*, 1986, 1987 & 1990; Salzer & Schallreuter, 1995; Kent & Al'Abadie, 1996). Accordingly, to correct lack of pigmentation for some patients is a very desirable objective. However, the treatment of vitiligo for dermatologists is a challenge as the aetiology of the disease is unclear. The reassurance that the disease is not fatal is enough for some patients while other patients prefer cosmetic camouflage, the choice of camouflage products now being extensive. Professional advice about the mixing and method of application of camouflage is important. The use of sunscreens is vital in lesional areas when the patient is exposed to the sun. Medical and surgical therapies are another option. The aim of these treatments is to achieve a uniform colour of the skin, resembling the patients' unaffected skin colour.

1.5.1 Medical therapies.

1.5.1a Topical and systemic psoralen with UVA.

Psoralens are furocoumarin compounds which are photosensitisers. They were first described for the treatment of various leukodermas as early as 1400BC (El Moftly, 1968). The effectiveness of 8-methoxypsoralen (-MOP) for the treatment of vitiligo was described by El Moftly (1948) and several early studies reported the efficacy of psoralens and UVR (Kanof *et al*, 1955; Lerner *et al*, 1959; Fulton *et al*, 1969; Parrish *et al*, 1976). Psoralens are optimal in combination with UVA irradiation (320-400nm wavelength) (Parrish *et al*, 1976) and form one of the major treatments for vitiligo. It is also an important treatment for many other skin diseases such as psoriasis (reviewed by Honig *et al*, 1994).

Repigmentation induced by PUVA treatment appears to act through several different mechanisms. Stimulation of melanocyte hypertrophy and migration from the hair follicle has been shown in PUVA treated patients (Ortonne *et al*, 1979; Fukuda *et al*, 1979; Ortonne *et al*, 1980; Cui *et al*, 1991). Arrunategui *et al*. (1994) from grafting studies proposed that a melanocyte reservoir exists in the lower third of the hair follicle while Cui *et al*. (1991) demonstrated that the amelanotic melanocytes of the outer root sheath were activated by PUVA. Staricco & Miller-Milinska (1962) reported the activation of the amelanotic melanocytes only with UVR. The importance of hair follicle melanocytes is emphasised by glabrous skin (distal ends of fingers, skin over knuckles, genitalia, lips) which, lacking in hair, is unresponsive to PUVA treatment. Leukotrichia (white hair) is also a poor prognostic feature for the repigmentation of vitiligo by medical methods (Seghal, 1974). The skin has been determined to have a reservoir of immature melanocytes as illustrated by c-Kit staining (Grichnik *et al*, 1996). Therefore, PUVA could also stimulate epidermal melanocytes in the perilesional region of vitiligo lesions to move into the lesions. Patients with the presence of residual melanocytes, as assessed by split DOPA technique, have a significantly greater mean of repigmentation than patients who demonstrated no follicular melanocytes prior to the PUVA treatment (Pitts & Grimes, 1991). *In vitro* melanocyte and fibroblast cell proliferation was stimulated by the serum of patients treated with oral PUVA (Abdel-Naser, 1997). It was suggested PUVA stimulated the release of growth factors from epidermal cells into the serum (Abdel-Naser, 1997). In a separate study, direct insult with UVA alone or in combination with psoralen actually inhibited *in vitro* melanocyte growth (Kao & Yu, 1992) but tyrosinase activity was increased. PUVA using 5-MOP also upregulated tyrosinase activity and melanin content in cultured normal human melanocytes, by modulating the regulatory pigmentary enzymes, tyrosinase, TRP-1 and TRP-2 (Mengeaud & Ortonne, 1996). The pattern of enzyme expression suggested that PUVA stimulated the metabolic pathway of the dark eumelanin (high ultraviolet (UV)-protective properties) (Mengeaud & Ortonne, 1996). It has been proposed that psoralen-fatty acid adducts, formed by UV exposure in melanocytes incubated with 8-MOP, activated melanogenesis through a protein kinase C (PKC) pathway (Anthony *et al*, 1997) while another theory suggested the stimulation of melanogenesis by psoralens was directed through binding to specific membrane receptors on the melanocytes (Laskin, 1985).

Morelli *et al.* (1993) hypothesised that PUVA therapy may enhance type-IV collagen secretion or alter the profile of melanocyte integrin receptor and thus change the melanocyte microenvironment. As discussed in an editorial (Fitzpatrick, 1997), PUVA may induce the release of cytokines and inflammatory mediators such as leukotriene (LT)-C_4 and transforming growth factor-α which stimulate melanocyte migration (Morelli *et al*, 1992). Earlier studies have also suggested that PUVA may be creating an environment favourable to the melanocyte (Grimes *et al*, 1988; Ashworth *et al*, 1989; Aubin *et al*, 1991) by recorrecting some of the humoral and cell-mediated immunological abnormalities reported in many patients. Additional studies have shown PUVA to induce immune system alterations (Gupta & Anderson, 1987), suppress delayed hypersensitivity and contact allergy, and alter distribution and function of T cells (Morison *et al*, 1981; Strauss *et al*, 1981; Morison, 1984), to stimulate selective cytotoxicity of mononuclear cells and inhibit degranulation of mast cells (Morison *et al*, 1981; Toda *et al*, 1986). Patients who responded to PUVA treatment appeared to have a decrease in the titre of melanocytes antibodies (Harning *et al*, 1991). A further *in vitro* study indicated that UVA/PUVA depleted expression of the epidermal growth factor (EGF) receptor and decreased associated melanocyte surface antigens (Kao & Yu, 1992) which could be a way for the melanocyte to escape from immune surveillance.

Topical and systemic PUVA treatments are the standard methods for delivery of psoralens as summarised in **Table 1.3**. Developments in PUVA therapy include psoralen baths plus UVA as an alternative to oral PUVA (Lüftl *et al*, 1997) (described in **Table 1.3**). Until recently, this treatment has only been used for other dermatological problems such as psorasis particularly in Scandinavian countries (Lüftl *et al*, 1997) with only one report of successful treatment of a 9 year old Causcasian girl with active vitiligo by this method (Mai *et al*, 1998). Vitamin E (Koshevenko, 1989) or minoxidil (Srinivas *et al*, 1990) have been used in conjunction with PUVA. Vitamin E was essentially used as an antioxidant to reduce skin erythema but it appeared to allow the build up of single UV doses. Repigmentation was quicker and there was enhanced melanocyte stimulation in the vitiligo lesion. Vitamin E has also been reported to increase the number of patients able to respond to weak to moderate topical corticosteroids or PUVA (Mandel *et al*, 1997). Recently it has been shown that topical

Table 1.3 Photochemotherapies for vitiligo.

Treatment	Patient suitability	Dose of chemical	Time chemical is taken prior to UV exposure	Initial UV dose[a]	UV dose increment[a]	Number of treatments per week
Topical psoralen	limited involvement (i.e. less than 20%); children older than 5 with localised lesions[b]	0.01% to 0.1% 8-MOP[c]	15-30 minutes	0.12 -0.25J/cm^2 UVA	0.12 -0.25J/cm^2 UVA[d]	1-3 times but not on consecutive days
Systemic psoralen	extensive vitiligo; recalcitrant to topical psoralens	0.2 to 0.4mg/kg 8-MOP	60-90 minutes	1-2J/cm^2 UVA	up to 1J/cm^2 UVA[g]	2-3 times but not on 2 consecutive days
	children older than 12[e]	0.6mg/kg TMP[f]	2 hours	0.25 - 0.5 J/cm^2 UVA		
Psoralen bath[h]	see footnotes[i]	8-MOP (0.5-5mg/l) or trioxsalen (0.2-0.5mg/l) in 150 litres of bath water for 15-20 minutes	no more than 30 minutes prior	<30% of minimal phototoxic dose	NS[j]	NS
Heliotherapy	suitable for patients unable to attend clinics for standard PUVA treatments	oral 0.3mg/kg trisoralen	2-4 hours	15 minutes sunlight	5 minutes per treatments	2-3 times but not on 2 consecutive days
Khellin	adults with up to 70% body invovlement; children >12years[k]	oral, 50-100mg	2 half hours	5 to 15J/cm^2 UVA	NS	NS
L-Phenylalanine	see Khellin[l]	oral, 50mg/kg	30-60 minutes	2 to 12J/cm^2 UVA	NS	NS
UVB irradiation[m]	see footnotes[n]	NA	NA	0.075J/cm^2 UVB	20% increments[d]	2 times but not on consecutive days
Balneophototherapy[o]	see footnotes[p]	salt-water bath[q]	for 20 mins	2.05J/cm^2 UVA 0.2J/cm^2 UVB[r]	NS	NS

Table 1.3: The data in the table is based on the guidelines in Drake *et al.* (1996) unless stated.
[a] Dose of ultraviolet irradiation adjusted to skin type.
[b] Children as young as 2 have been treated (Grimes *et al*, 1982; Halder *et al*, 1987).
[c] 8-methoxypsoralen (8-MOP).
[d] Increased until "mild erythema achieved".
[e] Children as young as 6 have been successfully treated (Grimes, 1993).
[f] Trimethylpsoralen (TMP).
[g] Increased until "asymptomatic erythema achieved".
[h] Data taken from Lüftl *et al.* (1997).
[i] This method has been used extensively for the treatment of psorasis (Lüftl *et al*, 1997) but there has been only one report of successful treatment of vitiligo (Mai *et al*, 1998).
[j] NS- not stated.
[k] Patient suitablility for khellin + UVA and phenylalanine + UVA taken from Antoniou & Katsambas (1992).
[l] Phenylalanine can be used for children younger than 12 years (Schulpis *et al*, 1989).
[m] Data taken from Westerhof & Nieuweboer-Krobotova, (1997).
[n] Patients treated by this method had active, extensive, generalised vitiligo (Westerhof & Nieuweboer-Krobotova, 1997).
[o] Data taken from Gambichler *et al.* (1997).
[p] The four patients treated had nonsegmental vitiligo further described as generalised vitiligo vulgaris.
[q] The salt-water bath contained 5-8% Tomesa salt and was comparable to the natural salt water of the Dead Sea.
[r] The patients were treated over a 11-48 month period with additional doses of upto 9.92J/cm^2 UVA and 1.22 J/cm^2 UVB received.

or oral adminstration of an extract of polypodium leucostomos stopped psoralen-induced phototoxic reactions (Gonzalez *et al*, 1997) and that this may be useful in combination with PUVA for treatment of vitiligo. Ultrapulse carbon dioxide laser induced two patients to respond to oral PUVA therapy (Knoell *et al*, 1997).

Recent studies showed that topical PUVA induced a mean repigmentation of 58% (178 patients studied) whilst oral PUVA induced a mean repigmentation of 63% (348 patients studied) (Grimes, 1997). In earlier studies, approximately 61% of patients responded with 25% or more repigmentation (Wildfang *et al*, 1992; Grimes, 1993) while a British study reported that only 10% of those treated had a good repigmentation response without any subsequent relapse (Anstey & Hawk, 1994). Children appear to have enhanced responses to PUVA treatment (Grimes, 1993; Grimes, 1997; Halder, 1997). Children treated with topical psoralens had a mean repigmentation of 78% in comparison to the adult population, who only had 58% mean repigmentation (Grimes, 1997) while the response rate in a separate study indicated 58% of children responded in comparison to 36% of adults (Halder, 1997). With oral PUVA treatment, 71% of children responded compared to 55% of adult (Halder, 1997). Paediatric patients with nonsegmental vitiligo had 70-90% repigmentation after 6 months of PUVA treatment while 3 children with segmental vitiligo had no repigmentation (Miklaszewska *et al*, 1993). Poor repigmentation has been noted in patients with segmental vitiligo (Koga, 1977; Hann & Lee, 1996) while in another study, the mean PUVA induced repigmentation was not significantly different between segmental and nonsegmental forms (Grimes, 1997). Three patients with bilateral segmental vitiligo were responsive to PUVA treatment (Hann *et al*, 1997). 5-MOP has been used in a Korean study and shown to have a similar potency with regard to results with 8-MOP but is associated with fewer side effects (Hann *et al*, 1991). Similarly, UVB (311nm) has been reported to be effective as topical PUVA treatment, with fewer side effects (Westerhof & Nieuweboer-Krobotova, 1997; discussed in **Section 1.5.1e)** but in one study a PUVA-induced tan was noted to be a more effective filter than one induced by UVB (Gschnait *et al*, 1978). Patients with a dark complexion most often obtained a maximal response to PUVA (Grimes *et al*, 1983b; Grimes, 1993). African-Americans and Hispanics achieved tolerance to a greater cumulative UVA dose than Caucasians (Grimes, 1993). The face and neck respond best to PUVA treatment (Grimes *et al*, 1982), followed by

the trunk and upper and lower extremities. The poorest response are generally seen on the dorsal regions of the hands; the fingers and glabrous skin will not respond. Generally, the genitalia are not treated due an increased susceptibility to cancer (Stern & Lange, 1988; Stern, 1990).

Disadvantages of topical PUVA include severe blistering reactions and perilesional hyperpigmentation (Grimes, 1993). It is vital that the level of lesional phototoxicity is not too great (Arora & Willis, 1976). While erythema is good for repigmentation, blistering induces no enhancement and often causes koebnerization (Grimes, 1993). The major advantages topical PUVA has over systemic PUVA are the lower cumulative UVA dose and lack of systemic and ocular toxicity (Grimes, 1993) while the major advantages of systemic PUVA over topical PUVA include the effectiveness in limiting active lesional progression, lower frequency of blistering reactions and superiority for the induction of repigmentation in persons with skin types I and II (Grimes, 1993). The side effects of oral PUVA include headaches, nausea, vomiting, pruritus, diffuse hyperpigmentation, photoageing and hypertrichosis (Gupta & Anderson, 1987). Long-term PUVA therapy has been associated with an increased susceptibility for skin cancers (Marx *et al*, 1983; Tanew *et al*, 1986; Henseler *et al*, 1987; Stern & Lange, 1988; Torinuki & Tagami, 1988; Abdullah & Keczkes, 1989; Forman *et al*, 1989; Bruynzeel *et al*, 1991; Chuang *et al*, 1992; Lever & Farr, 1994; Maier *et al*, 1996). However, skin cancers were not documented in patients with vitiligo (Wilfgang *et al*, 1992; Halder *et al*, 1995) until two recent studies described patients with squamous cell carinomas (Buckley *et al*, 1996; Takeda *et al*, 1998). Formation of cataracts in PUVA treated psoriasis patients was low or minimal (Tegner, 1983; Bourkes *et al*, 1985) but patients must wear UVA sunglasses following treatment to protect against cataract formation in the eyes (Stern *et al*, 1985). Grimes (1997) observed no cataracts or ocular abnormalities in patients treated with systemic PUVA. Interestingly, some patients with psoriasis and mycosis fungoides have developed vitiligo after PUVA treatment (Todes-Taylor *et al*, 1983). Finally PUVA treatment is very time consuming with over 100 sessions generally needed for the maximal response. A recent report illustrates this point, where 42% of patients that had received over 100 treatments had more than 75% repigmentation (Alkhawajah, 1997) but only 27% of all patients examined in the study received this many treatments. Approximately 25% of patients require maintenance, as

on cessation of therapy, follicular repigmentation generally fails (Kenny, 1971). Plott & Wagner (1990) also have experience of patients losing pigmentation within less than a year following the cessation of treatment.

1.5.1b Heliotherapy.

This is an additional psoralen treatment which combines oral trisoralen with sun treatment (see **Table 1.3**). It is suitable for patients who find it difficult to attend clinics for standard PUVA treatments. While oral trisoralen is less phototoxic than 8-MOP it is also less effective due to poor absorption from the gastrointestinal tract (Grimes, 1993). Another option includes using an extremely dilute form of 8-MOP applied topically (Grimes, 1997). Using this topical method, 125 patients had a mean repigmentation of 71% (Grimes, 1997) with blistering and mild perilesional hyperpigmentation significantly reduced amongst patients.

1.5.1c Khellin.

Khellin, a furanochrome derived from the seeds of the plant *Ammi visnagia*, resembles psoralens in the chemical structure and activity (Morliere *et al*, 1988) but it is less phototoxic (Abeysekera *et al*, 1983) and erythema does not occur (Abdel-Fattah *et al*, 1982; Ortel *et al*, 1988). Unlike psoralens, DNA is unaffected by khellin (Trabalzini *et al*, 1990; Riccio *et al*, 1992), although patients often suffer from elevated liver enzymes (Duschet *et al*, 1988; Ortel *et al*, 1988) and therefore, khellin could be hepatotoxic. The provocation of porphyria cutanea tarda has also been shown in response to khellin treatment (Jansen *et al*, 1995).

Early reports of the use of khellin were favourable; 50% (Ortel *et al*, 1988) to 76.6% (Abdel-Fattah *et al*, 1982) of patients repigmented to varying degrees when treated with oral khellin with UVA or sunlight respectively (a treatment strategy for oral khellin plus UVA is shown in **Table 1.3**). Ortel *et al*. (1988) also found promising results with topical khellin treatments. However, Procaccini *et al*. (1995) found that topical application of khellin did not improve a patient's response to UVA irradiation. In a separate study, topical khellin was no more effective than natural sunlight alone

(Orrechia & Perfetti, 1992) but Orrechia and co-workers found that an alteration in the gel formulation of khellin improved uptake into the skin and this in combination with UVA irradiation signifcantly increased pigmentation in lesions compared to lesions treated with just the gel and UVA (Orrechia *et al*, 1998). The best results from this study were seen in younger patients with a short duration of disease.

1.5.1d Phenylalanine.

The treatment of vitiligo with oral phenylalanine has been shown to be effective in several studies (Cormone *et al*, 1985; Kuiters *et al*, 1986; Westerhof *et al*, 1986; Antonoiu *et al*, 1989; Siddiqui *et al*, 1994) and found to be promising in a small study on children in Greece (Schulpis *et al*, 1989) (a treatment strategy is shown in **Table 1.3**). Cormone *et al.* (1985) reported 70% of patients had good to dramatic repigmentation of their lesions when treated with oral phenylalanine plus UVA. The same group showed that 26% of the patients had dense follicular repigmentation while 68% of the patients had sparse follicular repigmentation (Westerhof *et al*, 1986). Gradual repigmentation continued during 6-8 months following initiation of treatment. The follicular repigmentation pattern was seen in 81% of patients treated with oral phenylalanine and sunlight (Kruiters *et al*, 1986). Patients were treated with either topical or oral phenylalanine with UVA with good repigmentation in 82% and 90% of patients treated respectively (Antonoiu *et al*, 1989). The authors noted that arms, legs, eyelids and knees were the best areas to repigment. Various grades of repigmentation of up to 60% and 77% were seen in two trials of patients treated with oral phenylalanine and UVA (Siddiqui *et al*, 1994). They suggested that phenylalanine alone might at least prevent vitiligo from further expansion. In a retrospective study of phenylalanine plus UVA treatment, it was seen that 44% of patients had permanent repigmentation and that 52% of patients were satisfied with the treatment (Greiner *et al*, 1994). Two recent studies have suggested that phenylalanine plus UVA is not effective for the repigmentation of vitiligo (Rosenbach *et al*, 1993; Alkhawajah, 1996).

It is not obvious how this method works. Peak plasma levels of phenylalanine varied with dose (Kruiters *et al*, 1986) and time but there was no noticeable correlation between the peak levels and the level of pigmentation achieved (Antoniou *et al*, 1989;

Siddiqui *et al*, 1994). It has been suggested that the immunity of the patients is altered through changes in the Langerhans' cells (Westerhof *et al*, 1986) or through the inhibition of antibody production (Ryan & Carver, 1963) in response to phenlyalanine. Lesional vitiligo skin however becomes less photosensitve and normal skin becomes tanned (Grimes, 1993). No severe side effects were noted in any of the studies mentioned and there was no phototoxicity. It has been suggested that patients of all ages can be treated (Schulpis *et al*, 1989). Vitiligo involving less than 25% of the body surface, onset of vitiligo before the age of 21, a generalised and symmetrical distribution, and therapy of long duration were positive features for good repigmentation (Greiner *et al*, 1994). There are several contraindications to this treatment; phenylketonuria, impaired liver and kidney function, malignant skin disease, pregnancy, breast-feeding, history of arsenic exposure, prior radiotherapy and autoimmune disorder are all conditions where it is not prescribed (Grimes, 1993).

1.5.1e Additional photochemotherapies.

PUVA remains the main photochemotherapy for vitiligo but in addition to khellin plus UVA and L-phenylalanine plus UVA, photochemotherapies such as balneophototherapy (Gambichler *et al*, 1997), thalassotherapy (Dourmishev, 1991) and UVB irradiation (Köster & Wiskemann, 1990; Westerhof & Nieuweboer-Krobotova, 1997) are now being explored as possible alternatives.

Balneophototherapy combines a salt-water bath with subsequent UV irradiation (as detailed in **Table 1.3**). Four patients treated over a period of 11-48 months achieved 60-80% repigmentation (Gambichler *et al*, 1997) and this therapy lacks unwanted systemic side effects.

In a recent study, UVB treatment was compared with PUVA (Westerhof & Nieuweboer-Krobotova, 1997). Both treatments were effective but UVB had significant advantages over PUVA; a smaller cumulative UVB dose, less phototoxic effects, no photoallergic reactions, no hyperkeratosis, and a shorter treatment time as there was no need to administer drugs. After a year of treatment, 63% of patients had greater than 75% repigmentation. As with other treatments, the face showed best repigmentation,

- followed by the trunk with the distal regions of the extremities exhibiting minimal repigmentation.

1.5.1f Topical, systemic and intralesional steroids.

Topical steroids are often the first line of treatment with vitiligo. Many studies report on the effects of steroids (Kandil, 1970; Koopmans-van Dorp *et al*, 1973; Kandil, 1974; Bleehen, 1976; Clayton, 1977; Koga, 1977; Kumari, 1984; Geraldez & Gutierrez, 1987; Liu *et al*, 1990; Khalid *et al*, 1995). Betamethasone 17-valerate and clobetasol propionate are the main steroids used in these studies. Repigmentation of between 90-100% in 43% (Koopmans-van Dorp *et al*, 1973) to 92% of patients (Geraldez & Gutierrez, 1987) has been reported. The response rate in children has been reported at 45% compared to 29% in adults (Halder, 1997). Bleehen (1976) reported that only 1 out of 10 patients treated with clobetasol propionate responded and in a separate study only 9% of patients achieved good repigmentation (Clayton, 1977). The face and the extremities responded best to the treatment (Koopmans-van Dorp *et al*, 1973; Kumari, 1984; Geraldez & Gutierrez, 1987) with maximal results in dark complexioned individuals (Kumari, 1984, Geraldez & Gutierrez, 1987). Geraldez & Gutierrez (1987) also noted that "younger lesions" responded better. Koga (1977) showed that 5 patients with segmental vitiligo did not respond to topical corticosteroid treatment but 82.2% of 90 patients with generalized vitiligo responded with different levels of repigmentation. Stimulation of the melanocytes in the hair follicles has been shown (Bleehen, 1976). Although repigmented areas contained less melanocytes, dendrites were more prominent, dopa activity more intense while melanosomes were of normal shape and size (Bleehen, 1976). Side effects include capillary problems, epidermal atrophy and striae (Drake *et al*, 1996).

Imamura & Tagami (1976) found that only 35% of patients with generalised vitiligo who were treated with oral steroids had greater than 75% repigmentation. Patients with localised vitiligo appeared to be even less responsive. Two studies have investigated the use of oral mini-pulse therapy with betamethasone (Pasricha & Khaitan, 1993) and dexamethasone (Kanwar *et al*, 1995). Betamethasone halted the progression of disease in 89% of patients treated (Pasricha & Khaitan, 1993) while over a longer period 6

patients saw an increase of repigmentation between 76% and 100% and 13 patients saw an increase in repigmentation of between 26% and 75%. Several of the patients complained of side effects including headaches, acne, herpes zoster or glaucoma. The second study was less promising; only 44% of the patients saw a halt in the disease progression and some repigmentation (Kanwar *et al*, 1995). A Korean study showed that 29 of 37 patients with active progressive vitiligo had various degrees of repigmentation and/or cessation of disease progress in response to oral steriods (Hann *et al*, 1993b). Additionally, normal human melanocyte cytotoxicity by patient sera was reduced in patients treated with oral steroids.

Intralesional treatment with triamcinolone acetonide induced good to excellent repigmentation in 58% of patches treated in 26 patients, while 29% of patches showed satisfactory repigmentation (Kandil, 1970). Cutanous atrophy was evident in these patients. Vasistha & Singh (1979) could not recommend the use of intralesional steroid treatment after there was no significant difference in steroid versus water treated grouped. Several side effects were noted. In a recent study, 5 patients with symmetrical vitiligo who were treated with intralesional steroids showed no repigmentation after 4 months (Mandel *et al*, 1997).

A sex steroid/thyroid hormone mixture known as "Metharmon-F" was successfully used in 4 patients with generalised vitiligo (Muto *et al*, 1995). The study reported that melanocyte numbers were increased in the repigmented skin and that keratinocytes contained an increased number of melanin granules. *In vitro*, oestrogens have been reported to increase tyrosinase activity of normal melanocytes (Ranson *et al*, 1988b; Jee *et al*, 1994; McLeod *et al*, 1994). In a large study conducted in India, 560 patients were treated with a mixture of dapsone, vitamin B6 and topical steroid (hydrocortisone butyrate or clobetasol propionate) with promising results (Salafia, 1995). These patients also had dietary restrictions.

1.5.1g Depigmentation.

Depigmentation is a final option for patients with extensive vitiligo (i.e. greater than 50%-80% coverage), who are recalcitrant to conventional treatments such as PUVA,

who do not wish to undergo other repigmentation therapies and understand that this method is permanent and leads to problems (i.e. permanent photosensitivity).

Patients are generally treated with monobenzyl ether of hydroquinone (MBEH). MBEH was discovered as a depigmentation agent due to the occurrence of occupational leukoderma in a tannery (Oliver *et al*, 1939; Oliver *et al*, 1940). Hydroquinone alone induces depigmentation, but is less effective and not necessarily permanent (Spencer, 1965). Other depigmentation agents such as azaleic acid and isopropylcatechol have been considered for use in vitiligo (Verallo-Rowell *et al*, 1989). Patients require several sessions and depigmentation is generally carried out over small areas in succession. This treatment is also extensive and can takes several months to a year before completion. MBEH is less effective in patients with skin types I and II (Canizares *et al*, 1958). Mosher *et al*. (1977) treated 18 patients with 40-90% depigmentation. Only 44.4% of the patients treated achieved complete depigmentation with 16.7% showing no changes.

The side effects can include dermatitis (although this is generally eased with a topical corticosteroid treatment), pruritus and xerosis (Mosher *et al*, 1977). Distal depigmentation is often a problem (Catona & Lanzer, 1987) while conjunctival melanosis and corneal pigmentation can also occur (Hedges *et al*, 1983) but these do not affect visual acuity. Occasionally spontaneous repigmentation occurs, but this is rare (Oakley, 1996). In general, permanent, irreversible destruction of melanocytes occurs when patients are treated with MBEH with the histological changes similar to those seen in the already affected vitiligo lesions (Mosher *et al*, 1977). Jimbow *et al*. (1974) reported that hydroquinone decreased the number of melanised melansomes, altered melanosomal configuration and caused melanocyte organelle lysis while topical preparations with MBEH have been reported to not bleach pigment but significantly inhibit melanin synthesis (Engasser & Maibach, 1981; Passi & Nazzaro-Porro, 1981). MBEH is also shown to suppress lymphoproliferative responses (Grimes, 1993) and might increase specific subtypes of Langerhans' cells (Rheins *et al*, 1987).

The use of Q-switched ruby lasers has recently been explored as a depigmentation treatment (Thissen & Westerhof, 1997). Eight patients with minimal pigmentation

remaining on the arms and face were treated, with patients previously exhibiting the Koebner phenomenon, depigmenting in response to the laser treatment. Depigmentation was retained after 9 months and no severe side effects were reported. Low energy lasers have been used with mixed results in attempting conversely to repigment lesions of vitiligo (Renfro, 1992; Lanigan, 1996; see **Table 1.4**).

1.5.1h Additional medical therapies.

A variety of other techniques have been used for the treatment of vitiligo with mixed results (as summarised in **Table 1.4**). A recent treatment involved the application of topical pseudocatalase cream plus total-body suberythema UVB and topical calcium preparation with 90% of patients responding and complete repigmentation of the face and dorsa of the hands reported (Schallreuter *et al*, 1995; see **Table 1.4**). This methodology is based on findings of low levels of catalase (Schallreuter *et al*, 1991) and defective catecholamine metabolism in vitiligo epidermis (Schallreuter *et al*, 1994a&c). However a recent report on a double blind study of this treatment from this group was less promising (personal communication, Stiefel UK Ltd). A pool of antioxidants (described in **Table 1.4**) (Passi *et al*, 1995) have also been reported to halt disease progression but the results have not yet been reported in full. A nutritional appproach has been attempted using folic acid with vitamins C and B_{12}, following the discovery that folic acids were lower in the serum of several patients with vitiligo (Montes *et al*, 1990). Eight of the 15 patients treated in this study showed some improvement, while Juhlin & Olsson conducted a promising study in which 58/100 patients showed variable levels of repigmentation in response to folic acid, vitamin B_{12} and short sun exposure (see **Table 1.4**).

Immune modulators such as anapsos (Mohammed, 1989), cyclosporine (Gupta *et al*, 1990), isoprinosine (Grimes, 1993; Mandel *et al*, 1997) and levamisole (Pasricha & Khera, 1994) have had beneficial effects in some but not all patients (see **Table 1.4**). They are thought to ameliorate many of the observed baseline humoral and cell-mediated immune defects seen in patients (Grimes, 1993).

Table 1.4 Experimental therapies for vitiligo.

Reference	Treatment	Response
Schallreuter *et al*, 1995[a]	Pseudocatalase cream + total body suberythema UVB + topical calcium preparation[b]	Complete repigmentation of the face and dorsum of hands in 90% of 33 patients treated.
Passi *et al*, 1995[a]	Pool of antioxidants, vitamin E acetate, methioinine, selenio-methoinine, ubiquinone[c]	disease progression halted in 112 patients after one cycle, with recent lesions repigmenting after 2-3 cycles.
Montes *et al*, 1992[d]	oral folic acid + vitamin C and B_{12}	8/15 patients had steady and significant repigmentation
Juhlin & Olsson, 1997[d]	folic acid + B_{12} + sunlight[e]	64% of 100 patients disease halted, 6 patients total repigmentation, 52 patients some repigmentation
Cao *et al*, 1986[f]	melagenina + infrared radiation	84% of 732 patients responded with good repigmentation
Mal'tsev *et al*, 1995[f]	melagenina	62/366 patients showed 71-100% repigmentation in lesions, while 83% patients responded to varying degrees[g]
da Silva & Fontes Jr, 1997[f]	"Vitilikla's MC5"[h] + sunlight	37-48 months of treatment, 72% of 789 patients showed some repigmentation
Mohammed, 1989[i]	anapsos for 6 months	100% repigmentation in all patients
Gupta *et al*, 1990[i]	cyclosporine for 3 months	2/6 patients with perifollicular repigmentation
Grimes, 1993[i]	isoprinosine for 1 month	6/12 patients with variable repigmentation
Mandel *et al*, 1997[i]	isoprinosine for 4 month	no response in four patients
Pasricha & Khera, 1994[i]	levamisole ± 0.1% topical fluocinoline or 0.5% clobetasol propionate[j]	disease halted in 34/36 patients who had active vitiligo; variable degree of repigmentation in 54/64 patients treated[k]
Renfro, 1992[l]	Q-switched Ruby laser	no response in 7 patients
Lanigan, 1996[l]	Q-switched Nd/Yag laser	no permanent response in 7 patients
Gokhale & Gokhale, 1976[m]	ACTH for 6 months	81% of patients had repigmentation
Hernadez-Perez, 1979[m]	ACTH for 15 weeks	6/20 patients had repigmentation, but rapid depigmentation occurred following cessation of treatments

Table 1.4

[a] Antioxidant treatment of vitiligo.

[b] Patients were treated twice daily with the pseudocatalase/calcium chloride cream, and then twice weekly with UVB (1 hour after the application of the cream). There was a mean duration of 15.3 months for treatment.

[c] Patients with active phase vitiligo were treated with alternating cycles of the various antioxidants over a 2 month period.

[d] Folic acid treatment of vitiligo.

[e] The minimal treatment time was 3-6 months. If patients were repigmenting, treatment was continued as long as necessary.

[f] Natural treatment of vitiligo with placental extract known as melagenina or herbs.

[g] Paedriatric patients, aged 4-15 years, were treated with a Cuban source of melagenina.

[h] Vitilikla's MC5 consisted of 4 herbs from the Brazilian flora; *Echinodorus grandiflorus*, *Anchietea salutaris*, *Boerhaavia hirsuta*, *Baccharis trimera*.

[i] Immune modulator treatment of vitiligo.

[j] Patients were treated on 2 consecutive days each week over a period of 4-48 months.

[k] Variable repigmentation was seen in 9/14 patients treated with levamisole alone, 33/38 patients treated with levamisole and fluocinoline and all 12 patients treated with levamisole and clobetasol propionate.

[l] Laser treatment for repigmentation of vitiligo. Thissen & Westerhof (1997) use laser treatment for depigmentation as discussed in **Section 1.5.1f.**

[m] ACTH treatment of vitiligo.

Melagenina, a hydroalcoholic extract from the human placenta, was initially shown to be effective in a Cuban (Cao *et al*, 1986) and Ukrainian study (Malt'sev *et al*, 1995) (as summarised in **Table 1.4**) but Nordlund & Halder (1990) found that double-blind placebo studies carried out in Venezuela, Mexico and India did not confirm these reports. Pal *et al.* (1995) examined the chemical composition of hydroalcoholic human placental extract and suggested that the glycosphingolipids and endothelin content of the extract may play a role in skin pigmentation but Nordlund & Halder (1990) found that melagenina did not affect the growth rate of melanocytes or tyrosinase activity in cultured human or murine melanocytes. Traditional Chinese medication has been shown to work possibly through immuno-regulatory effects but the mixture and concentration of medicinal herbs in these treatments is unknown (Shao, 1995). A recent *in vitro* study showed that an aqueous extract of *Angelica sinensis*, a herb commonly used in the treatment of vitiligo in traditional Chinese medicine had no effects on mouse melanocyte proliferation (Raman *et al*, 1996). A product called "Vitilikla's-MCS" consisting of 4 herbs from Brazilian flora was used to treat 789 patients in an open study (da Silva & Fontes Jr, 1997) with promising results (**Table 1.4**). The majority of patients achieved some degree of repigmentation, with only 4.7% of patients showing any new lesions. Patients also received sun baths several times weekly, and the authors stated that double blind studies were now needed.

α-MSH and adrenocorticotrophic hormone (ACTH) have both been examined as possible treatments for vitiligo. Intradermal injections of α-MSH had no effect on vitiliginous skin or sites of Koeberisation following a cervical sympathectomy in a vitiligo patient (Lerner *et al*, 1966) yet enhanced normal skin pigmentation. $Nle^{4}DPhe^{7}$-alpha-melanocyte stimulating hormone (NDP-MSH), a potent analogue of α-MSH (Hadley *et al*, 1985) has been shown to induce pigmentation in normal skin (Levine *et al*, 1991) and earlier studies by Lerner and co workers showed that α-MSH, β-MSH and ACTH could enhance skin pigmentation (Lerner & McGuire, 1961 & 1964). However the enhanced pigmentation was generally at sun exposed sites. Treatment of vitiligo with ACTH had mixed results as described in **Table 1.4** (Gokhale & Gokhale, 1976; Hernandez-Perez, 1979). α-MSH was described as an ubiquitous cytokine by Nordlund (1991) and has many biological effects which have not yet been fully explored in humans.

1.5.2 Surgical therapies.

Medical methods are not successful in all patients, therefore to correct the lack of depigmentation a new source of melanocytes is required. Haxthausen (1947) and Spencer & Tolmachi (1952) were amongst the first to report successful repigmentation with surgical methods; one woman out of 3 had some repigmentation following treatment with Thiersch grafts (Hasxthausen, 1947) while good repigmentation was obtained in an African-American woman, using full-thickness grafts (Spencer & Tolmachi, 1952). Since then, many different surgical techniques have been described for the replacement of absent melanocytes in vitiligo lesions.

1.5.2a Blister technique.

The epidermis from suction blisters has been successfully used to repigment suction blistered (Mutalik, 1993; Suga *et al*, 1996), liquid nitrogen-treated (Suvanprakorn *et al*, 1985; Hatchome *et al*, 1990; Lee *et al*, 1991; Skouge *et al*, 1992; Hann *et al*, 1995; Skouge *et al*, 1995), PUVA blistered (Koga, 1988; Hatchome *et al*, 1990), dermabraded (Matsumura *et al*, 1993; Zachariae *et al*, 1993) or laser ablated (Yang & Kye, 1998) vitiligo macules (as summarised in **Table 1.5**). PUVA treatment was used after the grafts had healed to help pigment spread and coalesce in the lesions in some of the studies (Skouge *et al*, 1992; Mutalik, 1993; Hann *et al*, 1995; Yang & Kye, 1998). In one study, the donor site was treated with PUVA to induce a repigmented epidermal sheet prior to grafting (Suga *et al*, 1996). Lee & Jang (1998) recently described pretreatment of donor sites with PUVA significantly increased the number of melanocytes which improved the clinical results. Hann *et al*. (1995) saw that areas of leukotrichia also repigmented as noted in a previous study by this group (Hann *et al*, 1992a).

In some patients with generalised vitiligo, the initial repigmentation depigmented (Koga, 1988; Hatchome *et al*, 1990; Lee *et al*, 1991; summarised in the footnotes of **Table 1.5**). Hatchome *et al*. (1990) related this to donor site depigmentation and suggested that for patients with generalised vitiligo Koebnerisation was a possibility.

Table 1.5 Treatment of vitiligo with suction blister induced epidermal sheets.

Reference	Patients		Preparation of		Use of PUVA therapy[b]	Patient/lesional response
	Ethnic origin	Vitiligo type[a]	Donor site	Recipient site		
Suvanprakorn *et al*, 1985	Indian	5 A, 20 G, 5 S	suction blister	liquid nitrogen	No	28/30 patients responded with partial or complete repigmentation
Koga, 1988	Japenese	14 NS, 31 S	suction blister	PUVA induced blister	No	25/31 patients with segmental vitiligo had good repigmentation within 6 months, 8/14 patients with nonsegmental vitiligo repigmented[c]
Hatchome *et al*,1990	Japanese	11 G, 2 L, 5 S	suction blister	liquid nitrogen or PUVA induced	No	all patients except 4 with generalised vitiligo repigmented[d]
Lee *et al*, 1991	Korean	7 G, 7 L, 8 S	suction blister	liquid nitrogen	No	17/22 patients repigmented[e]
Skouge *et al*,1992	15 patients[f]		suction blister	liquid nitrogen	Yes	90% of grafts survived with repigmentation occuring in the majority of lesions treated
Matsumura *et al*,1993	Japanese	9 NS, 22 S	suction blister	dermabrasion[g]	No	88% of 88 lesions successfully repigmented
Mutalik, 1993	Indian	50 L[h]	suction blister	suction blister	Yes[i]	48/50 patients showed repigmentation within 3-4 months
Zachariae *et al*, 1993	Caucasian (2) Sri Lankan (1)[j]		suction blister	dermabrasion	No	all patients showed some degree of repigmentation
Hann *et al*,1995	Korean	30 F, 25 G, 45 S	suction blister	liquid nitrogen	Yes	89% of lesions showed >90% than repigmentation within 2 months
Suga *et al*, 1996	Japanese	10 S, 10 L, 8 G	suction blister[k]	suction blister	No	20% or less repigmentation seen in patients with generalised vitiligo, 60-100% repigmentation seen in patients with localised or segmental vitiligo
Yang & Kye, 1998	Korean	19	suction blister	laser ablation	Yes	16/19 repigmentation after 1 month, extending after 3 months

Table 1.5:

[a] The different clinical types of vitiligo are abbreviated as A - acrofacial, F - focal, G - generalised, L - localised (or focal), NS - nonsegmental (which includes generalised and localised) or S - segmental.

[b] PUVA treatment was given to patients once the graft sites had healed, to help pigment spread and coalesce.

[c] Four of the patients with segmental vitiligo and one patient with non-segmental vitiligo had not reached 6 months post operation but were showing initial signs of repigmentation. Three patients with non-segmental vitiligo had transient repigmentation.

[d] Two of the patients with generalised vitiligo were lost during follow-up. Four patients with generalised vitilgo has transient repigmentation.

[e] Two of the patients with localised vitiligo were lost during follow-up. Three patients with generalised vitiligo had transient repigmentation.

[f] The patients' ethnic origin and the type of vitiligo the patients were suffering from were not mentioned.

[g] The lesion was scrubbed with sterilized sandpaper.

[h] Patients with segmental vitiligo were included in this description of localised vitiligo, but the number was not mentioned.

[i] Only eleven of the patients in this study were treated with PUVA or PUVA-SOL (PUVA treatment in the sun) to help repigmentation.

[j] The type of vitiligo was not mentioned for any of the three patients.

[k] The donor sites which were used for suction blisters were previously treated with PUVA.

Repigmentation in patients with segmental vitiligo was maintained although a few patients failed to respond to the treatment (Koga, 1988; Lee *et al*, 1991; Matsumura *et al*, 1993). For example, Matsumura *et al.* (1993) noted that 91% of segmental lesions successfully repigmented while only 79% of non-segmental lesions successfully repigmented. Matsumura *et al.* (1993) also associated a good lesional response in patients that were anti-nuclear antibody negative. In contrast to these reports, Hann *et al.* (1995) noted that 4 patients with segmental vitiligo had recurrence and/or adjacent new lesions, while only 1 patient with generalised vitiligo developed donor site depigmentation but maintained recipient site repigmentation. Hann *et al.* (1995) concluded that the treatment success rate seen in the study was due to lesion site not type of vitiligo. Facial regions were good recipient sites (Lee *et al*, 1991; Hann *et al*, 1995; Suga *et al*, 1996) although the forehead could be a problem (Suvanprakorn *et al*, 1985; Yang & Kye, 1998). Lesions on the abdomen, back and legs achieved over 60% repigmentation in one study (Suga *et al*, 1996) while poor results were observed in lesions on the neck, axillae, overlying bony prominences and dorsum of the hands (Suvanprakorn *et al*, 1985; Koga, 1988; Hann *et al*, 1995; Yang & Kye, 1998).

Apart from the possibility of Koebnerisation and recipient site depigmentation, transient hyperpigmentation in the recipient and/or donor sites during this initial few months was the only other problem generally mentioned (Koga, 1988; Skouge *et al*, 1992; Zachariae *et al*, 1993; Hann *et al*, 1995; Suga *et al*, 1996; Yang & Kye, 1998). However Hann *et al.* (1995) reported that hypertrophic scarring occurred in 8 patients but by 6 months only 3 of the patients still had scarring. The authors suggested that this could have been caused if excessive liquid nitrogen was used in preparing the recipient area. Overall, this surgical method provides good results in both patients with segmental and non-segmental vitiligo without severe complications.

1.5.2b Epidermal/dermal grafts.

The application of epidermal/dermal grafts appears limited since Behl (1964) used thin Thiersch grafts to treat vitiligo lesions. Seventy per cent of the 107 patients treated had excellent results which was similar to the more recent report of successful Thiersch grafting of 85% (Bose, 1994a). A complication rate of 19% included depigmented

Table 1.6 The treatment of vitiligo with thin epidermal/dermal grafts.

Reference	Ethnic group	Vitiligo[a]	Graft	Patient Response
Agrawal & Agrawal, 1995	Indian	18 F, 3 S	split-thickness skin graft	90-100% repigmentation achieved in the 32 lesions grafted.
Kahn & Cohen, 1995[b]	Caucasian	5[c]	0.005 inch epidermal graft	98-100% repigmentation in all patients with good to excellent colour match
Olsson & Juhlin, 1997	2 Indian, 2 Arabian, 2 black African, 13 Caucasian	1 G, 1 S, 17 V	0.1mm epidermal graft	36/48 lesions treated obtained 70-100% repigmentation while 6/48 lesions obtained 10-50% repigmentation; all other lesions failed to respond

[a] The type of vitiligo is described as focal - F, generalised - G, segmental - S or vulgaris - V.
[b] Based on a method used for the treatment of depigmentation in burns (Kahn *et al*, 1991).
[c] The type of vitiligo was not stated, but all patients had stable forms

fringe at recipient site, infection, graft failure and donor site scarring with keloid formation the worst sort of scar seen (Behl, 1964). Free hand tangential excision of the area of vitiligo was a possible cause for some of the complications. Despite the complications, the author reported that permanent repigmentation was achieved.

Three studies summarised in **Table 1.6** (Agrawal & Agrawal, 1995; Kahn & Cohen, 1995; Olsson & Juhlin, 1997) have further examined the use of thin split thickness skin grafts and thin epidermal grafts. Agrawal & Agrawal (1995) and Kahn & Cohen (1995) reported repigmentation of between 90-100% in patients with stable vitiligo, generally of the focal and segmental forms, while Olsson & Juhlin (1997) reported variable repigmentation in a patients with the vulgaris form of vitiligo. Kahn & Cohen (1998) recently described a modification to their method in which the grafts were partially meshed and expanded. Successful treatment of 15/17 lesions in 12 patients was reported (Kahn & Cohen, 1998). No additional therapies were used although Olsson & Juhlin (1997) permitted their patients to expose the grafted regions to sunlight for a short period each day. A satisfactory colour match was obtained within 9-12 months. Kahn & Cohen (1995) reported that the hands, fingers, wrist, neck and forehead were the most successful while Olsson & Juhlin (1997) reported that the face and arms were the most successful. Hyperpigmentation again was noted and scarring was absent. Milia were a common problem for the patients but these either diasappeared of their own accord or were treated surgically. Agrawal & Agrawal (1995) also noted overhanging margin due to a thicker graft and irregular graft surface as complications.

1.5.2c Mini-grafting.

Mini-grafting involves the transfer of small punch biopsies of up to 2mm in diameter from the donor site to recipient site and has been successfully used in the treatment of stable segmental and focal forms of the disease (Falabella, 1983 & 1988). Mini-grafting can be a tool to determine which patients with stable vitiligo will pigment in response to various grafting methods (Boersma *et al*, 1995, Falabella *et al*, 1995a) or to complete pigmentation of lesions where the initial treatment did not cover the whole site (Falabella *et al*, 1995b). Pigment spread of four to six mini-grafts implanted within lesions was assessed at 3 months; a test was considered positive if the pigment spread

beyond 1mm and up to 2 to 3mm from the border of the grafts (Falabella *et al*, 1995a). Ninety-five percent of patients with focal or segmental vitiligo had a positive test in comparison to 48% of patients with other forms (bilateral) of vitiligo (Falabella *et al*, 1995a).

Boersma *et al.* (1995) found that 24 of 59 patients with vitiligo vulgaris had a positive mini-grafting result but they showed no clinical differences to the non-responders. This study highlighted the difficulty in selecting patients with more generalised/bilateral forms of the disease for grafting. Tweny-three of the 24 patients with a positive mini-grafting test were then grafted. Repigmentation of 80-99% was achieved in 14 lesions, 50-80% repigmentation in 10 lesions and zero to 50% in 12 lesions three months following grafting but the best results were seen by 9-12 months. Patients received UVA exposures soon after the grafted region had healed and no cobblestone appearance was evident (Boersma *et al*, 1995) although this has previously been a problem (Falabella, 1988).

1.5.2d Non-cultured and cultured autologous cells.

The use of autologous cultured cells for burns patients and patients who suffer from ulcers of the legs is well documented (e.g. O' Connor *et al*, 1981; Hefton *et al*, 1983; Gallico *et al*, 1984; Cuono *et al*, 1986; Madden *et al*, 1986; Faure *et al*, 1987; Leigh *et al*, 1987; De Luca *et al*, 1989; Phillips *et al*, 1989; Mol *et al*, 1991). The first report of successful treatment of a leukoderma, piebaldism, with cultured autologous melanocytes was by Lerner *et al.* (1987). Several groups have explored the possible uses of autologous cultured cells in the treatment of vitiligo (Falabella *et al*, 1989 & 1992; Brysk *et al*, 1989; Plott *et al*, 1989; Jha *et al*, 1993; Olsson & Juhlin, 1993; Zachariae *et al*, 1993; Löntz *et al*, 1994; Olsson *et al* 1994; Olsson & Juhlin, 1995; Kumagai & Uchikoshi, 1997; Andreassi *et al*, 1998; Arenberger & Matoušková, 1998; Kaufmann *et al*, 1998). The techniques differ in that cells were either grown and applied as co-cultures of epidermal cells (Falabella *et al*, 1989 & 1992; Brysk *et al*, 1989; Plott *et al*, 1989; Kumagai & Uchikoshi, 1997; Andreassi *et al*, 1998; Arenberger & Matoušková, 1998), or grown as co-cultures and then applied as suspensions (Jha *et al*, 1993) or grown as pure melanocyte cultures (Olsson & Juhlin, 1993; Zachariae *et al*, 1993; Löntz

et al, 1994; Olsson *et al*, 1994; Olsson & Juhlin, 1995; Kaufmann *et al*, 1998). These techniques are still evolving, but could be the way forward for the treatment of large areas of vitiligo. They offer the possibility of expanding cell number and also of storing cells eliminating the need for subsequent biopsies (Olsson *et al*, 1994). Non-cultured cells have also been successfully used to treat lesions (Gauthier *et al*, 1992; Olsson & Juhlin, 1998). These techniques are discussed further in **Chapter 5**.

1.5.2e Additional surgical therapies.

Single hair grafting has been explored in 4 patients with generalised vitiligo and 17 patients with localised or segmental vitiligo (Na *et al*, 1998). Perifollicular repigmentation was seen within 2-8 weeks and pigment had spread up to 10mm over a 12 month period. Na *et al.* (1998) recommended this treatment for small areas such as eyebrows. Arruntaegui *et al.* (1994) also reported on transplantation studies with the hair and discovered that a melanocyte reservoir exists in the lower third of the hair bulb.

Micropigmentation involves the injection of pigment (iron oxide pigment) into the dermis. While it is difficult to obtain a good colour match and the colour often fades, this methodology is good for lips and fingertips where other methodologies do not suffice (Halder *et al*, 1989).

Topical 5-fluorouracil was applied twice daily to a dermabraded lesion for 7-10 days in 28 patients (Tsuji & Hamada, 1983). Sixty four percent of the patients showed complete or almost complete repigmentation. Five patients showed partial repigmentation and 5 patients did not respond. Szekeres & Marta (1985) treated two patients with segmental vitiligo without success and 3 patients with symmetrical vitiligo with some success using this methodology. Two of the patients with symmetrical vitiligo had repigmentation persisting at 12 months while one patient had initial repigmentation followed by depigmentation. Dermabrasion is needed as patients treated with just fluorouracil in dimethyl sulphoxide (DMSO), to try and enhance skin absorption, did not respond (Monk, 1985) and patients treated with fluorouracil applied to skin that had been epidermal stripped with cellophane tape did not respond (Mandel *et al*, 1997). This technique results in complete but often darker repigmentation. There

is a long recovery time and there is also the possibility of infection, scarring and aggravation of vitiligo. Dermabrasion alone was reported to stimulate melanocyte migration from the hair follicle into the epidermis (Staricco, 1961) in normal skin.

1.6 AIMS OF THE STUDY

The overall aim was to learn more about the physiology of normal and vitiligo melanocytes. The approach taken had the following specific aims:

(1) To investigate the influence of (a) media composition, (b) ECM proteins, (c) UVR and (d) α-MSH on normal melanocyte morphology, proliferation and pigmentation.

(2) To compare morphological, proliferative and pigmentary responses of normal and vitiligo melanocytes to some of the conditions determined in (1).

(3) To examine pigmentary behaviour of normal melanocytes when added to a 3-dimensional epidermal/dermal reconstruct.

(4) To compare the constitutive and cytokine stimulated expression of several immune-related cell surface molecules in normal and vitiligo melanocytes.

(5) α-MSH has many biological activities and following the poor melanogenic respone to α-MSH it was decided to investigate a protective/anti-inflammatory role in melanocytes by examining the influence of α-MSH on cytokine-stimulated ICAM-1 expression in normal melanocytes.

(6) To explore the use of cultured autologous epithelial cells in the treatment of vitiligo lesions.

CHAPTER 2

METHODS.

Table 2.1 Composition of melanocyte maintenance media.

	Final Concentration	A	B[a]	C
Base Medium		MCDB153	MCDB151	MCDB153
Insulin	10μg/ml	+	+	+
Transferrin	10μg/ml	+	+	+
Hydrocortisone	2.8μg/ml	+	+	+
L-glutamine	2mM	+	+	+
α-tocopherol	1μg/ml	+	+	-
Foetal calf serum	2% v/v	+	+	+
Bovine pituitary extract	50μg/ml/25μg/ml (M1/M3)	+	+	+
Phorbol 12-myristate 13-acetate	10nM	+	+	+
Cholera toxin	100ng/ml	+	-	-
Basic fibroblast growth factor	0.6ng/ml	-	+	+
Heparin	unknown[a]	-	+	-
Penicillin/streptomycin	100IU/ml:100μg/ml	+	+	+
Nystatin	10U/ml	+	+	+

Melanocytes were maintained in one of the above three media until they were used in experiments. The presence of a supplement is indicated by +, while the absence is indicated by -.

[a] B is the commercially available medium from Sigma and concentrations of supplements were not available.

2.1 CULTURE OF NORMAL ADULT HUMAN CUTANEOUS MELANOCYTES[a].

2.1.1 Isolation of normal adult human cutaneous melanocytes.

Samples of normal skin were supplied by the Department of Plastic Surgery, Northern General Hospital, from patients undergoing abdominoplasties and breast reductions (consent for use of surgically removed tissue for research purposes is included in the Northern General Hospital Research Trust Surgical Consent Form). The subcutaneous fat was removed before the sample arrived at the laboratory. The skin which had been transported in saline solution was cut into small pieces. During the initial 2-3 months of the project, the pieces of skin were incubated overnight in dispase (2mg/ml) at 4^0C. The epidermis and dermis were separated with fine forceps and the epidermis then incubated in Difco trypsin (0.25%) at 37^0C for 10-15 minutes to disaggregate the epidermis. The trypsin was inactivated with basal MCDB153 + 10% foetal calf serum (FCS) and cells pelleted by centrifugation (Sorvall RT 6000D DuPont) at 200g for 5 minutes. The cells were resuspended in MCDB153 medium supplemented with chelated FCS[b], insulin, transferrin, hydrocortisone, α-tocopherol, bovine pituitary extract (BPE), cholera toxin (CT) and phorbol 12-myristate 13 acetate (PMA) referred to as maintenance medium A (see **Table 2.1** for details), plated into T25 tissue culture flasks at 2-4 x 10^6 epidermal cells/flask and incubated at 37^0C with 5% carbon dioxide (CO_2).

However the yield of epidermal cells and hence melanocytes could be quite low from this method. Accordingly a methodology used by the laboratory staff culturing keratinocytes was employed instead. This method involved incubating the pieces of skin overnight in 0.1% Difco trypsin, at 4^0C before separating the epidermis and dermis. The lower surface of the epidermis and the upper surface of the dermis were scraped gently using a scalpel blade to release cells. The epidermal cells were pooled in chelated FCS and pelleted by centrifugation as before. The epidermal cells were resuspended in one of the three maintenance media A, B or C (see **Table 2.1** for details) and seeded into T25 flasks and incubated as before. With this method, the yield of epidermal cells was much higher.

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[a] Details of the suppliers can be found on pages 187-188.
[b] The FCS was chelated with iminodiacetic acid (Chelex 100) for 24 hours at 4^0C to remove calcium.

2.1.2 Maintenance of normal adult human cutaneous melanocytes.

Three maintenance media have been used throughout this project. Initially, maintenance medium A was used as this was the medium in use for the culture of ocular melanocytes in the laboratory. The change to a basic fibroblast growth factor (bFGF) containing medium, came about after initial investigations with α-MSH were proving fruitless (as described in **Section 3.4.7**). Abdel-Malek *et al.* (1995) were suggesting that CT could be detrimental and block melanocyte responses to other cAMP modulators. They were using a bFGF containing medium and the decision to switch was made. A commercially available melanocyte medium was used to begin with (referred to as maintenance medium B) but the concentrations of supplements in this medium were unavailable, and therefore an in-house version of the medium used by Abdel Malek *et al.* (1995), referred to as maintenance medium C, was used eventually.

Every 3-4 days the spent medium was removed and cells received fresh medium. Geneticin (100μg/ml) was generally added to the cultures for 3-4 days at the second change of medium to prevent contamination from fibroblasts. Cells were passaged when near confluency, by removing spent medium and incubating for 2-5 minutes with trypsin/ethylenediaminetetracetic acid (EDTA) (0.5%/0.2%) at 37^0C. Ten percent FCS in MCDB153 medium was used to neutralise the effect of the trypsin/EDTA and cells pelleted as previously described. At passage, cell cultures were split in the ratio 1 to 3 through to 1 in 5 depending on the numbers required. Melanocytes were used as primaries, passage (P)1 or P4 cells. Purity of melanocyte cultures was assessed by dopa staining (as described in **Section 2.8.1**) at various passages.

2.1.3 Experimental conditions and experimental media for adult human cutaneous melanocytes (normal and vitiligo).

The experiments performed on pure cultured melanocytes were designed to investigate the following topics, (1) ECM protein effects on pigmentation, (2) UV effects on pigmentation, (3) media and α-MSH effects on pigmentation, (4) cytokine effects on immune-related cell surface molecule expression (e.g. ICAM-1, leukocyte function antigen-3 (LFA-3), CD40, MHC class I and class II) and (5) α-MSH/ACTH/3-isobutyl-

Table 2.2 Composition of experimental media.

	MEDIA CONSTITUENTS[a]				
	FCS 2%	BPE 50µg/ml	PMA 10nM	CT 100ng/ml	bFGF 0.6ng/ml
1[b]	+	+	+	+	-
2[c]	+	+	+	-	+
3	+	+	+	-	-
4	+	+	-	+	-
5	+	+	-	-	+
6	+	-	+	+	-
7	+	-	+	-	+
8	-	+	+	+	-
9	-	+	+	-	+
10	+	+	-	-	-
11	+	-	-	+	-
12	+	-	-	-	+
13	+	-	+	-	-
14	-	+	+	-	-
15	-	+	-	+	-
16	-	+	-	-	+
17	-	-	+	+	-
18	-	-	+	-	+
19	+	-	-	-	-
20	-	+	-	-	-
21	-	-	+	-	-
22	-	-	-	+	-
23	-	-	-	-	+
24	-	-	-	-	-
25[d]	+	+	+	-	+

Melanocytes were cultured in the above media during a 5 day experimental period. All media (excluding medium 25 - see footnote [d]) were based on MCDB153 medium supplemented with insulin, transferrin, hydrocortisone, L-glutamine and antibiotics. In addition, the presence of FCS, BPE, PMA, CT, or bFGF is indicated as + and the absence as -.

[a] FCS, foetal calf serum; BPE, bovine pituitary extract; PMA, phorbol 12-myristate 13 acetate; CT, cholera toxin; bFGF, basic fibroblast growth factor.

[b] Medium 1 closely resembles maintenance medium A (**Table 2.1**)

[c] Medium 2 closely resembles maintenance medium C (**Table 2.1**)

[d] Medium 25 is the commercially available medium and is also the maintenance medium referred to as B, **Table 2.1**.

1-methylxanthine (IBMX) effects on cytokine-stimulated ICAM-1 expression. All the experiments followed similar protoccols except for the UV irradiation experiments (as described in **Section 2.6**).

Melanocytes were seeded into appropriate tissue culture vessels, generally multiwell plates (96, 24 or 12 well) or T25 flasks (plating densities indicated throughout **Chapters 3-4**) in one of the three maintenance culture media. The culture vessels were either tissue culture plastic alone, or coated with individual or cell-derived ECM proteins. 24 hours following seeding (day 1), experimental medium replaced the normal culture medium. A full list of the experimental media used are shown in **Table 2.2**. The experimental media had the basic composition of MCDB153 supplemented with insulin, transferrin, hydrocortisone, L-glutamine and antibiotics except for medium 25, the commercially available medium. The most commonly used media were 1, 10 and 24. They were employed in the ECM experiments and UV experiments (**Chapter 3**) and cell-surface molecule expression experiments (**Chapter 4**). α-MSH pigmentation studies utilized all the media (**Chapter 3**), while α-MSH/ICAM-1 experiments initially used media 1, 10 and 24, but then employed medium 23 (**Chapter 4**).

Melanocytes were cultured for a further 5 days, receiving media changes on day 3 and day 5. 24 hours following the final feed, parameter analysis would take place. This protocol was decided from two timecourse experiments run over 14 and 15 day periods examining the effect of media and ECM proteins on melanocyte dopa oxidase (tyrosinase) activity at various timepoints. Clear morphological differences induced by various media could be seen at days 5 and 6, and good levels of dopa oxidase activity could be detected (as described in **Section 3.4.4**).

In the α-MSH studies, 10^{-10} or 10^{-8}M α-MSH was added to appropriate cells at each media change. In some of these studies 10^{-3}M IBMX was added at each of the media changes (only 5 media were used for this part of the study). Cell number, dopa oxidase activity and/or melanin were the parameters analysed at the end of the experimental period.

In the immune-related cell-surface molecule expression studies, cytokines were added for the final 24 hours (i.e. day 5, the final feed) at 100U/ml. 100U/ml was determined

from dose response curves to be the most useful concentration at which to compare the cytokines IFN-γ and tumor necrosis factor-α (TNF-α) (as described in **Section 4.2.1**). Other cytokines and lymphocyte conditioned media were used in a few of these studies (see **Chapter 4** for details). A cell based-enzyme linked immunosorbent assay (ELISA) method was used to analyse ICAM-1 expression (described in **Section 2.9**) while flow cytometry (described in **Section 2.10**) was used to analyse the expression of ICAM-1, MHC class I and class II, LFA-3 and CD40.

In the α-MSH/ICAM-1 experiments, α-MSH, ACTH, or IBMX were added to appropriate cells at each media change or just on day 5. TNF-α or IFN-γ, was added at the same time as the pro-opiomelanocortin (POMC) peptides/IBMX on day 5 and 24 hours later ICAM-1 expression was determined by an ELISA method and related to cell number (as described in **Sections 4.3.1-4.3.5**) or in the case of the IFN-γ experiments by flow cytometry (as described in **Section 4.3.6**).

2.2 CULTURE OF MELANOCYTES FROM UNAFFECTED REGIONS OF SKIN OF VITILIGO PATIENTS.

2.2.1 Cultured epithelial autografts (CEA) containing vitiligo melanocytes.

Skin biopsies (generally one per patient), approximately a 2 x 1cm elliptical shave, were taken from unaffected areas of skin of vitiligo patients, by Dr DJ Gawkrodger (Department of Dermatology, Royal Hallamshire Hospital, Sheffield). Dr DJ Gawkrodger selected patients for these studies. All patients were informed of the use of the biopsies and signed consent forms. The donor site was generally the arm (see **Table 2.3a** for further details), and the specimen was transferred to the laboratory in a phosphate buffered saline solution containing antibiotics. Epidermal cells were isolated as described in **Section 2.1.1** for normal melanocytes. A viable cell count using trypan blue was conducted on the resuspended pellet and preferably 0.7 x 10^6 cells/T25 flask were plated onto irradiated 3T3 (i3T3) cells (culture of these Swiss mouse fibroblast cells is described in **Section 2.3.1**) in Green's medium (see **Table 2.4** for composition)and cells incubated at 37^0C in 5% CO_2. The i3T3 cells had been plated out the previous day. Every 3 days spent medium was removed and cells recieved fresh medium. Cells were passaged after approximately 10 days. The spent medium was

Table 2.3a Details of the vitiligo skin biopsy donors and the initial cultures.

	Age	Sex	Biopsy site	Vtiligo type	Disease activity[a]	Intial plating surface	Initial medium used[b]	Time in culture for the following experiments (days)[c]		
								UV	ICAM	MHC
VM436	39	female	arm	symmetrical	yes	plastic	A	ND[d]	ND	ND
VM694	43	female	arm	symmetrical	yes	plastic	A	ND	ND	ND
VM695	55	female	buttock	symmetrical	yes	plastic	A	ND	ND	ND
VM1	70	female	arm	symmetrical	no	collagen I	Green's	82	82	
VM2	77	male	arm	symmetrical[e]	no	plastic/i3T3[f]	Green's		115	106
VM3	65	female	arm	symmetrical	yes	plastics	B	109		
VM4	34	female	arm	symmetrical	no	i3T3/collagen I[g]	Green's	81	77	79
VM5	66	see VM3 (same patient)				plastic	Green's		92	94

Table 2.3b Details of the normal skin sample donors and the initial cultures.

	Age	Sex	Elective surgery	Paired culture	Initial plating surface	Initial medium used	Time in culture for the following experiments (days)		
							UV	ICAM	MHC
M577	26	female	breast reduction	VM3	plastic	A	77		
M693	21	female	breast reduction	VM694/5	plastic	A	ND	ND	ND
M748	55	female	breast reduction	VM3	plastic	B	77		
M756	28	female	abdominoplast	VM1	plastic	B	50	50	
M767	33	female	breast reduction	VM2	plastic	B		50	
M813	29	female	breast reduction	VM2	plastic	C			119
M833	50	female	breast reduction	VM5	plastic	C		91	93
M843	31	female	breast reduction	VM4	plastic	C		47	49
M852	30	female	abdominoplast	VM4	plastic	C	43		

Table 2.3:
[a] disease activity at time of biopsy donation.
[b] the initial medium used refers to melanocyte maintenance medium A, B or C as detailed in **Table 2.1** and Green's medium for keratinocyte growth as detailed in **Table 2.4.**
[c] the numbers refer to the length of time in culture prior to experiments in examining the pigmentation of melanocytes in response to UVB (UV), ICAM-1 expression and expression of MHC class I and class II molecules.
[d] ND - not done.
[e] all the patients had symmetrical type vitiligo although patient VM2, exhibited some segmental type patterning.
[f] the cells from this biopsy were split into two cultures (1) plated initially onto plastic and used in the ICAM-1 experiment (paired with M767) and (2) plated initially onto i3T3 cells and used in the MHC class I and class II experiment (paired with M813).
[g] the cells from this biopsy were split into two cultures (1) plated initially onto i3T3 cells and used in the UV experiments (paired with M852) and (2) plated initially onto collagen I and used on the ICAM-1 and MHC class I and class II experiments (paired with M843).

Table 2.4 Green's Medium

Supplements	Final Concentration
Basal medium	DMEM:Ham's F12 (3:1v/v)
Foetal calf serum[a]	10%
Hydrocortisone	2.5µg.ml
Epidermal growth factor	10ng/ml
Cholera toxin	10^{-10}M
Adenine	1.8×10^{-4}M
Insulin[b]	5µg/ml
Transferrin	5µg/ml
Triiodo-L-thyronine	2×10^{-7}M
L-glutamine	2mM
Penicillin/streptomycin	100IU/ml:100µg/ml
Fungizone	0.313µg/ml

[a] Foetal calf serum is from a New Zealand source when used in the patient studies.
[b] In the skin composite experiements, insulin is from a bovine source, but in the patient work for CEA culture, the insulin is a human recombinant form.

removed, and monolayers washed in phosphate buffered saline (PBS), followed by a few minutes with EDTA to remove any i3T3 cells. A second PBS wash was followed by a 10-15 minute incubation with trypsin/EDTA to remove the keratinocytes (and the small population of melanocytes). Complete Green's medium (containing serum) was used to halt the action of the trypsin/EDTA and the cells were pelleted at 200g for 5 minutes in a MSE Mistral 1000 centrifuge. A viable cell count was performed on the resuspended pellet before cells were plated onto i3T3 cells in 90mm^2 petri dishes or frozen at this stage. Two of the patients who were grafted received secondary CEA sheets, while another patient who was grafted received tertiary CEA sheets.

When cells were frozen down, they were suspended at between 0.5 to 2 x 10^6 cells/ml in freezing down medium, consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 25% FCS and 10% DMSO. The cryovials were placed into the minus 80^{0}C freezer overnight in an isopropanol box. The vials were then transferred to the gaseous phase of the liquid nitrogen store for 4 hours, before being submerged into the liquid nitrogen. Cryovials were rapidly thawed, by incubating the vials in a water bath at 37^{0}C for a few minutes. The cells were then washed in medium, pelleted and counted before plating out as before.

2.2.2 Preparation of the CEA sheet for grafting.

A few days before the CEA sheets were due to be grafted, the medium was changed to a Green's medium deficient in CT and FCS. On the day of grafting, the spent medium was removed and sheets washed in PBS. Dispase (2mg/ml) was added for a maximum of 40 minutes, but after 15 minutes and then after every 5 minutes, the edges of the sheets were checked to see if they were starting to lift off. Once they were lifting, the dispase was removed and sheets briefly washed in EDTA and then PBS. Green's medium (deficient in CT and FCS) was added. A sterile scraper was used to gently loosen the edges all around the petri and a Tegapore backing dressing was added. The edges of the sheet were fastened to the backing dressing using liga clips. The sheets were gently peeled from the petri and the CEA sheet/backing dressing were placed in a large square petri and covered with medium. They were stored in the incubator at 37^{0}C for up to 1 to 2 hours until they were taken to theatre.

2.2.3 Procedure for grafting and after treatment of the vitiligo patients.

The surgeon, Mr C Caddy (Department of Plastic Surgery, Northern General Hospital) selected the region to be grafted in all three patients on the day of grafting. The area was cleaned and a local anaesthetic of 0.5% lignocane/1:200000 adrenaline injected into the region. Once the area became numb, it was dermabraded down to an even bleeding layer (illustrations of this are shown in **Chapter 5**). The CEA sheets were applied and the area dressed with yelonet followed by gauze. This was held secured by a crepe bandage in the case of patients VM1 and VM4, and by Effix in the case of patient VM5 (individual details if different or additional are discussed in **Chapter 5**). The dressings were changed generally a week after grafting and the backing of the CEA removed between 1 and 2 weeks, depending on the healing of the patient. Patient follow-up was initially with the surgeon, Mr C Caddy, until the area had healed and then the patients were referred back to the dermatologist, Dr DJ Gawkrodger. Patients were advised in this study not to expose the regions to sunlight while healing.

2.2.4 Culture of pure vitiligo melanocytes.

Epidermal cell suspensions were obtained as previously described in **Section 2.1.1** and plated on tissue culture plastic, collagen I or i3T3 cells (see **Table 2.3a** for details of plating substrata and initial medium used). The first three biopsies (VM436, VM694 and VM695) taken were cultured as pure melanocytes on tissue culture plastic in maintenance medium A. Unfortunately the melanocyte number obtained was extremely low for VM436 and VM695 and melanocyte proliferation was not observed after several weeks. VM694 did yield many melanocytes and the culture was successfully passaged twice but after 3 months became infected with yeast. Biopsies from patients VM1, 2, 4, and 5 were taken primarily for culturing cells as CEA sheets for grafting but surplus cells obtained from the biopsies were set up for pure culture in Green's medium. This medium was then changed to maintenance media B or C after a few days to a week in culture. Additional pure cultures of melanocytes were set up from the CEA sheets of VM2 and VM4. Surplus cells from the CEA sheet of patient VM2 were frozen for 6 months before being cultured in maintenance medium B and then C on tissue culture plastic, while surplus cells from the CEA sheet of patient VM4 were cultured in

Table 2.5 Fibroblast and HaCaT growth medium.

	Normal Fibroblasts	3T3 Fibroblasts	HaCaT
Basal Medium	DMEM[a]	DMEM[a]	DMEM[b]
Foetal Calf Serum	10%	-	5%
Newborn Calf Serum	-	10%	-
$NaHCO_3$	45mM	45mM	[b]
L-Glutamine	2mM	2mM	2mM
Pyruvate	-	-	[b]
Penicllllin/streptomycin	100IU/ml:100µg/ml	100IU/ml:100µg/ml	100IU/ml:100µg/ml
Fungizone	1.25µg/ml	1.25µg/ml	-

[a] Dulbecco's modified Eagle's Medium, bought as a x10 stock, final concentration x1.
[b] Dulbecco's modified Eagle's Medium, bought already made up and suplemented with $NaHCO_3$ and pyruvate.

Table 2.6 Human endothelial cell line growth medium.

Supplement	Final Concentrations
Basal Medium	MCDB 131[a]
Foetal calf serum	10%
Epidermal growth factor	10ng/ml
Hydrocortisone	1µg/ml
Penicillin/streptomycin	100IU/ml:100µg/ml
Fungizone	1.25µg/ml

[a] An endothelial basal medium supplemented with L-glutamine.

maintenance medium C on tissue culture plastic straight after the dissociation of the CEA sheet. During cell culture, cells were incubated at 37^0C in 5% CO_2

The experimental use of each pure culture of vitiligo melanocytes is listed in **Table 2.3a**. Normal (listed in **Table 2.3b**) and vitiligo melanocyte cultures which were approximately 70% confluent at P3 were paired together 1-2 weeks before experimentation for comparative studies. Normal and vitiligo melanocytes were not paired from the initial isolation because a low yield of vitiligo melanocytes was obtained from small biopsies and there was a wide variety in proliferation rates between normal melanocytes as well as vitiligo melanocytes.

2.3 CELL CULTURE OF FIBROBLASTS, HaCaT AND ENDOTHELIAL CELL LINES.

2.3.1 Fibroblast cell culture.

Normal dermal fibroblasts were isolated from sections of human dermis. The dermis was cut up finely and then incubated at 37^0C in 0.05% collagenase A (prepared in DMEM containing 10% FCS) for between 24-48 hours. The mixture was centrifuged at 200g for 5 minutes (as described in **Section 2.2.1**) and the pellet resuspended and plated out into a T25 tissue culture flask in fibroblast growth medium (see **Table 2.5**) and cells were incubated at 37^0C in 5% CO_2. The primary cultures were set up by various members of staff in the laboratory, but I was involved in the maintenance of cultures used for the experiments. Cells were passaged by aspirating the spent medium, washing twice with PBS and then incubating for 5 minutes with trypsin/EDTA. The action of the tryspin was halted by addition of fibroblast medium containing serum (at 10%) and the cells were centrifuged at 1000rpm for 5 minutes as described. The resuspended pellets were generally split 1 in 4 to 1 in 8, depending on requirements.

The maintenance of the 3T3 Swiss mouse fibroblasts (J2 clone) (a kind gift from Professor H Green, Harvard Medical School, Boston, USA) was by various members of the laboratory, notably, Ms L Gould and Ms R Dawson. Frozen stocks were quickly thawed in a water bath and fibroblast medium (containing 10% newborn calf serum (NCS) instead of 10% FCS, as detailed in **Table 2.5**) added. The cells were centrifuged

as described, counted and then taken to the Blood Transfusion Service to be irradiated with 6000 rads $_{60}$CO source (γ-irradiation). Following irradiation cells were recounted and plated out at 2 x 10^6 cells per petri dish (or the equivalent in a T25 flask). This was done the day prior to epidermal cell isolation for the culture of CEA sheets and cells were incubated at 37^0C in 5% CO_2.

2.3.2 Human endothelial cell line culture.

The human endothelial cell line (CDC.HEMC.-1) (a kind gift from the Centre for Disease Control, Atlanta, USA) was maintained in a MCDB 131 medium supplemented as described in **Table 2.6** and flasks of cells were incubated at 37^0C in 5% CO_2. Cells were passaged using trypsin/EDTA and new flasks were inoculated with 10^5 cells/flask (T75).

2.3.3 HaCaT cell line culture.

The immortalized keratinocyte cell line, HaCaT cells (a kind gift from Professor NE Fusenig, Institute of Biochemistry, German Cancer Research Centre, Heidelberg), was cultured in DMEM and flasks of cells were incubated at 37^0C in 5% CO_2 (supplementation described in **Table 2.5** based on the methods of Boukamp *et al.* (1988)). Cells were passaged when confluent, by aspirating the medium, and washing once with PBS. The flasks were then incubated for 20 minutes at 37^0C with 25mM HEPES/0.05% EDTA solution. This was carefully removed and trypsin/EDTA was added for 5 minutes to fully detach the cells. A sterile glass pipette was used to mix the cells and obtain a single cell suspension. Medium containing 10% FCS was added to halt the action of the trypsin/EDTA. The flask was washed with PBS and all contents were collected in a universal tube and spun at 300g for 5 minutes (same centrifuge as described in **Section 2.1.1**). The cells were split 1 in 20 at each passage.

2.4 SKIN COMPOSITE MODEL.

2.4.1 Preparation of the de-epidermised dermis.

The de-epidermised dermis was prepared as described in Ghosh *et al.* (1997) by Ms R Dawson. Split thickness skin grafts from routine plastic surgery specimens (same

source as in **Section 2.1.1**) were placed in 50% glycerol for at least 4 hours, then 85% glycerol for 4 hours and finally 98% glycerol for at least 1 month so that the specimen was sterilised. The skin was rehydrated for at least 4 hours, with several changes of PBS before being incubated in 1M sodium chloride for 4 days. This produces the epidermal/dermal split. To remove cells from the dermis, it was placed in distilled water (dH_2O) for a month with twice weekly changes of dH_2O. Small biopsies were taken to confirm sterility and acellularity (using formalin fixed tissue) and then the de-epidermised dermis (DED) was stored in PBS with antibiotics until it was required. If required, basement membrane proteins were removed by treating sections of the dermis with 0.25% dispase at 37^0C for 3 hours.

2.4.2 Preparation of the epidermal/dermal construct and the experimental procedure.

Ms Dawson cultured the fibroblasts for these experiments and isolated the keratinocytes. P4 fibroblasts were added to the reticular surface (underside) of the dermis at 1×10^5 cells into a 1cm diameter stainless steel ring placed on the dermis, on day 1 (in some experiments fibroblasts were not added). Throughout the experiments, the composites were incubated at 37^0C in 5% CO_2. 24 hours later the ring was removed and the composite flooded with medium. Freshly isolated keratinocytes (characteristically contain a small proportion of melanocytes (1-2%)) and melanocytes (P1 or P2) were added to the papillary surface (upper) of the dermis in a 95%:5% ratio respectively in Green's medium on day 3. In initial experiments, melanocytes and keratinocytes were added at the same time, but from the histological examinations it was shown that the melanocytes were not reaching their normal position along the basement membrane. In the experiment described in **Section 3.4.10**, melanocytes (P2) were added a few hours before the keratinocytes and this method ensured melanocytes reached the basement membrane. The ring was removed 24 hours following seeding and on day 5 the composites raised to the air liquid phase. The composites were refed twice more with Green's medium over a 7 day period. On the 15^{th} day, the medium was changed to (a) normal Green's medium, (b) Green's deficient in CT in the presence 10^{-8}M α-MSH or (c) Green's deficient in CT (in the experiment reported in **Section 3.4.10**). Composites were refed twice more during the 5 day experimental period. On the 19^{th} day, 24 hours following the final feed, the composites were photographed by Mr R Salthouse or Ms A Reed (Medical Illustration Department, Northern General Hospital). The composites

were then prepared for histology and immunohistochemistry and stained (see **Sections 2.4.3-2.4.7**) by Mr C Layton (Department of Histopathology, Northern General Hospital).

2.4.3 Preparation of the composites in paraffin wax for sectioning.

The composites were trimmed to remove excess dermis and half the region populated with cells was processed in paraffin wax and the other half was fresh frozen (as described in **Section 2.4.6**). The tissue was fixed in 4% buffered formalin for a minimum of 24 hours before being dehydrated through ascending grades of alcohol. The specimen was impregnated with paraplast (a plasticised paraffin wax) using a Miles Scientific VIP3000 tissue processor. The process takes approximately 17 hours. Following this, the tissue was then embedded in plasticised paraffin wax in a tissue cassette using a Miles Scientific Tissue-Tek embedding centre. From this tissue block, 4μm thick sections were cut on an Anglis Scientific 0325 rotary microtome.

2.4.4 Histology:-Carazzi's Haematoxylin and eosin (H and E).

The H and E staining was performed on a Shaudon Linistain GLX. Sections were deparaffinized in xylene, washed in alcohol and then in water. Haematoxylin solution was added for up to 5 minutes. Sections were briefly washed in water and excess cytoplasmic stain removed with acid-alcohol (1% hydrochloric acid in 70% alcohol) before washing well in water and blueing agent (2% aqueous sodium bicarbonate). At this stage, the sections were checked microscopically to confirm that the nuclei were exhibiting a deep blue colour whilst the background should show a weak residual haematoxylin colouration. After another wash in water, the sections were stained with eosin solution (1%) for 5 mins. Before mounting, sections were washed quickly in water, excess cytoplasmic stain removed as before and dehydrated in alcohol.

2.4.5 Histology:- Masson Fontana stain for melanin.

Sections were deparaffinized as before (**Section 2.3.4**) and then placed in silver solution (10% silver nitrate) at full power for 75 secs. The sections were then rinsed with distilled water several times before they were treated for 10 minutes with 0.2% gold

chloride. A two minute incubation with 3% sodium thiosulphate followed a further rinse with distilled water. Sections were then counterstained with 1% safranin for 1 minute, washed in water, dehydrated, cleared and mounted. The melanin stains black while the rest stains red. A control section of malignant melanoma was used as a reference.

2.4.6 Preparation of composites for fresh frozen sections.

OCT Mounting Medium was added to a plastic mould (avoiding air bubbles) and the composite placed on end into the OCT. The mould is placed on the surface of the liquid ntirogen until the OCT changed from clear to white and then the mould was submerged until the liquid nitrogen ceased bubbling. Everything is removed from the liquid nitrogen and the mould removed to leave the sample frozen in the OCT block. The block was stored in a cryovial in a liqiud nitrogen dewar until sections were to be cut for staining.

2.4.7 Immunhistochemistry:- S100 staining for melanocyte detection and collagen IV staining for basement membrane protein detection.

Sections were picked-up on poly-L-lysine coated slides, air dried for 30 minutes and fixed in acetone for 15 minutes. Sections were then air dried and stored in slide trays, wrapped in cling film to prevent desiccation, and stored at -80^{0}C or liquid nitrogen until staining.

The embedding medium was removed by washing in buffer and 0.1% polyoxyethylenesorbitan monolaurate (Tween; used at 0.1% throughout this section) for 10 minutes, then blocking serum was used to decrease background staining (1:25 dilution of horse or swine serum in Tris((hydroxymethyl)methylamine buffered saline (TBS)/Tween for mono- or polyclonal antibodies respectively) for 10 minutes. The blocking serum was tipped off, and primary antibodies (diluted in buffer and 0.1% Tween) were added for 60 minutes. For S100 staining, a rabbit anti-human polyclonal antibody was used at a dilution of 1:20000 while for collagen IV staining, a mouse anti-human monoclonal antibody was used at a dilution of 1:200. Sections were washed for 10 minutes in TBS and Tween before the secondary antibodies (1:200 dilution) were

added for 30 minutes (mouse and rabbit secondary antibodies were used for mono- and polyclonal primaries respectively). A Vectastain avidin:biotinylated enzyme complex was added to sections for 30 minutes following a 10 minute wash in TBS and Tween. The colour substrate was prepared from the Vector alkaline phosphatase colour kit and added for 10 minutes following a 10 minute wash in TBS and Tween. The sections were finally washed in water, nuclei stained with haematoxylin (20 seconds), excess cytoplasmic stained removed in acid alcohol (1 second) and treated with blueing agent (as in **Section 2.4.4**), dehydrated, cleared and mounted.

2.5 PREPARATION OF INDIVIDUAL AND CELL-DERIVED EXTRACELLULAR MATRIX PROTEINS.

2.5.1 Preparation of rat tail collagen I.

Collagen I was prepared in house by Mr M Wagner. Rat tail tendons were extracted with wire cutters and washed in sterile PBS before being placed in 100ml acetic acid (0.1M). This was stirred for 72 hours at 4^0C. Any undissolved material was removed by centrifugation and the collagen stored at 4^0C until required or freeze dried.

2.5.2 Preparation of the individual ECM proteins.

Stock solutions of ECM components, collagen I (in acetic acid), collagen IV (in acetic acid), fibronectin (in PBS), laminin (in PBS), were prepared at 0.2mg/ml, and then 50μl/well (96 well plates) were added to each well to obtain 10µg per well. After a 2-3 hour incubation at 37^0C, the wells were washed several times with PBS. The incubation period was based on a protocol described in Morelli *et al.* (1993).

2.5.3 Preparation of cell-derived ECM.

Fibroblast-derived ECM, endothelial cell-derived ECM and HaCaT cell-derived ECM were obtained by plating the cells at specific cell densities and then feeding the cells daily for up to 5 days. On 24 well plates, cells were seeded at 5 x 10^4cells/well for fibroblasts, and at 4 x 10^4cells/well for endothelial cells, while, on 96 well plates, the

seeding densities were 2 x 10^4 and 1 x 10^4cells/well, for fibroblast and endothelial cells, respectively.

Fibroblasts were initially lysed by incubating in water for up to an hour and then they were washed several times with PBS to remove cell debris. However a technique using 0.1% sodium deoxycholate was employed in later experiments. Cells were incubated for up to 10 minutes and then washed with PBS, before being air-dried. The plates could then be stored for a few weeks at room temperature. On the day of use, they were washed again in PBS.

Endothelial cells were incubated with 10μM cytochalasin D for up to an hour while HaCaT cells were incubated for up to 10 minutes with 0.5% t-octylphenoxypoly-ethoxyethanol (Triton X-100). The plates were then washed with PBS. A commercially produced bovine subendothelial matrix multiwell plate was also used for some experiments.

2.6 ULTRAVIOLET IRRADIATION (UVR).

2.6.1 UVR source.

Four UVB Helarium lamps (40W) (now known as Arimed B lamps) were the UVR source. These lamps had a spectral output of approximately 80% in the ultraviolet A range (UVA 320-400nm) and 20% in the UVB range (290-320nm). Insignificant amounts of UVC are emitted. The lamps were housed in a custom made unit that held the lamps in a parallel horizontal plane, with a 2.5cm separation along their length. The lamps were raised from the culture dishes by 20cm. The output of the lamps was monitored by a meter provided by Professor B Diffey (Department of Medical Physics, University of Newcastle-upon-Tyne). As output from the lamps tended to reduce with time, before any plates were irradiated, a reading was taken and the time required to deliver the desired UV dosage was calculated.

2.6.2 Protocol for UVB irradiation.

The UVB irradiation schedule was based on the protocol by Friedmann *et al.* (1990b) for normal human melanocytes in which cells received 180mJ/cm^2 of UVB daily for 6 days. In the protocol used in this study, melanocytes were irradiated with 143mJ/cm^2 UVB for 1, 4 and 7 days daily. This dose was based on a spectral output reading of 305 and cells being irradiated for 6 minutes (as described in Dr J Buffey's thesis, 1993). Parameters were measured 24 hours after the final UV irradiation.

Melanocytes were plated on to tissue culture plastic or ECM proteins (fibroblast-derived or HaCaT-derived) in melanocyte maintenance media A, B or C. 24 hours following seeding, the medium was changed to PBS and cell were irradiated. Following each irradiation, melanocytes received experimental media 1, 10 or 24. The parameters investigated were cell proliferation (in terms of cell number, **Section 2.7**), pigmentation (**Section 2.8**) and ICAM-1 expression (**Section 2.9**). In the ICAM-1 experiments, 100U/ml IFN-γ was added 24 hours before ICAM-1 analysis as a positive control (only in medium 10).

2.7 ASSESSMENT OF CELL NUMBER.

2.7.1 Cell counts.

Cells were pooled by trypsinization, washed in PBS and counted in a known amount of . solution (media or PBS) using a haemocytometer.

2.7.2 MTT-eluted stain assay.

The suitability of this assay, which measures cell metabolism as a proliferation assay was first examined. Melanocytes were seeded into a 96 well plate over a range of cell numbers from 5 x 10^3 to 4 x 10^4cells per well (n=4 replicates). Cells were allowed to adhere for 4 hours. Medium was changed to one lacking FCS (but BPE was present). 10µl of 0.5mg/ml MTT ([4,5-dimethylthiazol-2-y]-2,5-dipheylterazolium bromide) was added to each well and cells were incubated for 1 hour. The medium was removed and

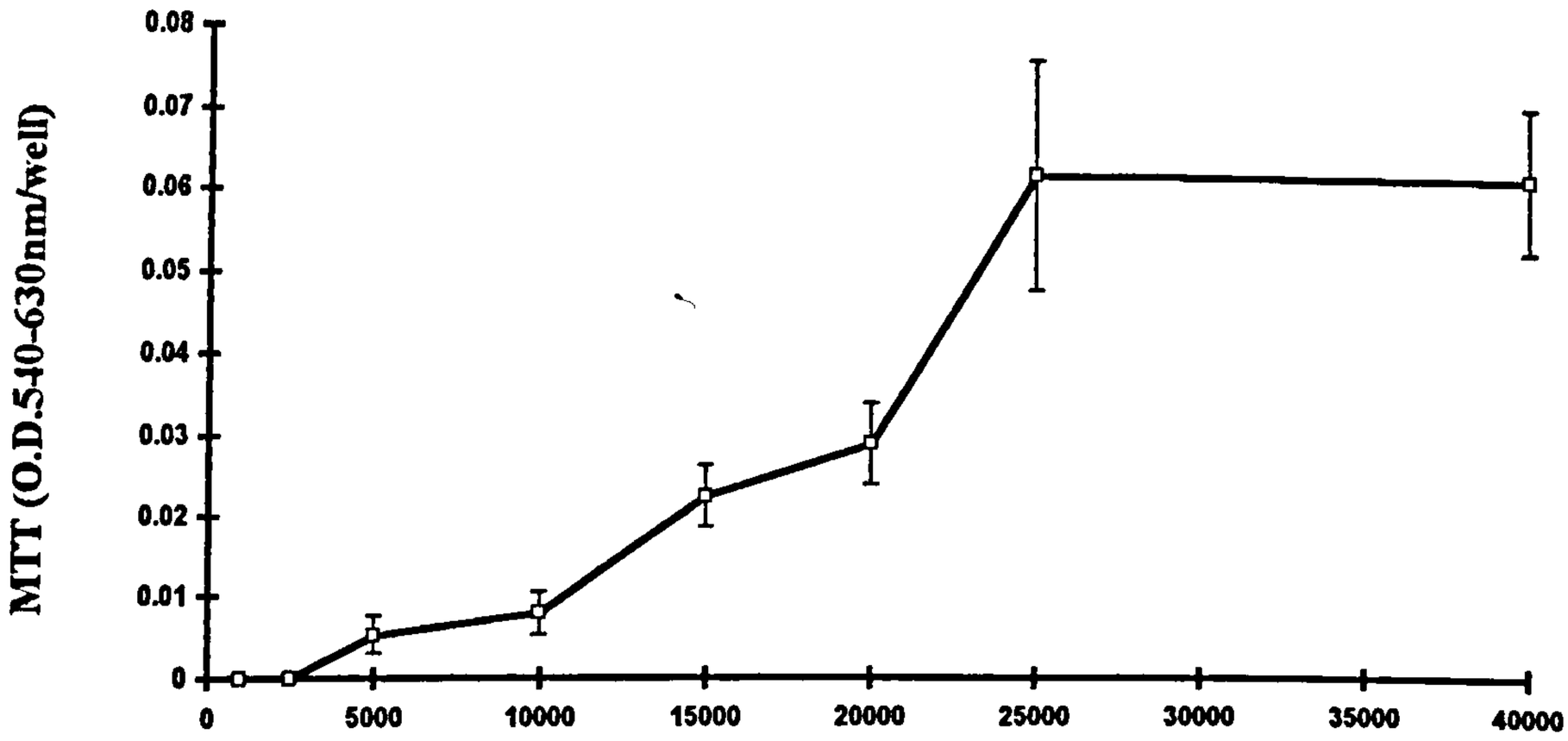


Figure 2.1 Standard curve for the MTT-ESTA assay. Melanocytes from one donor were plated at densities of 0.5 to 4 x 10^4 cells/well (in a 96 well plate) and allowed to adhere for 4 hours prior to a 1 hour treatment with MTT (as described in **Section 2.7.2**). The stain was eluted and the plate read at an absorbance of 540nm with a reference wavelength of 630nm. The graph indicated for each cell density, the mean ± standard error of mean (SEM) of 4 replicate wells.

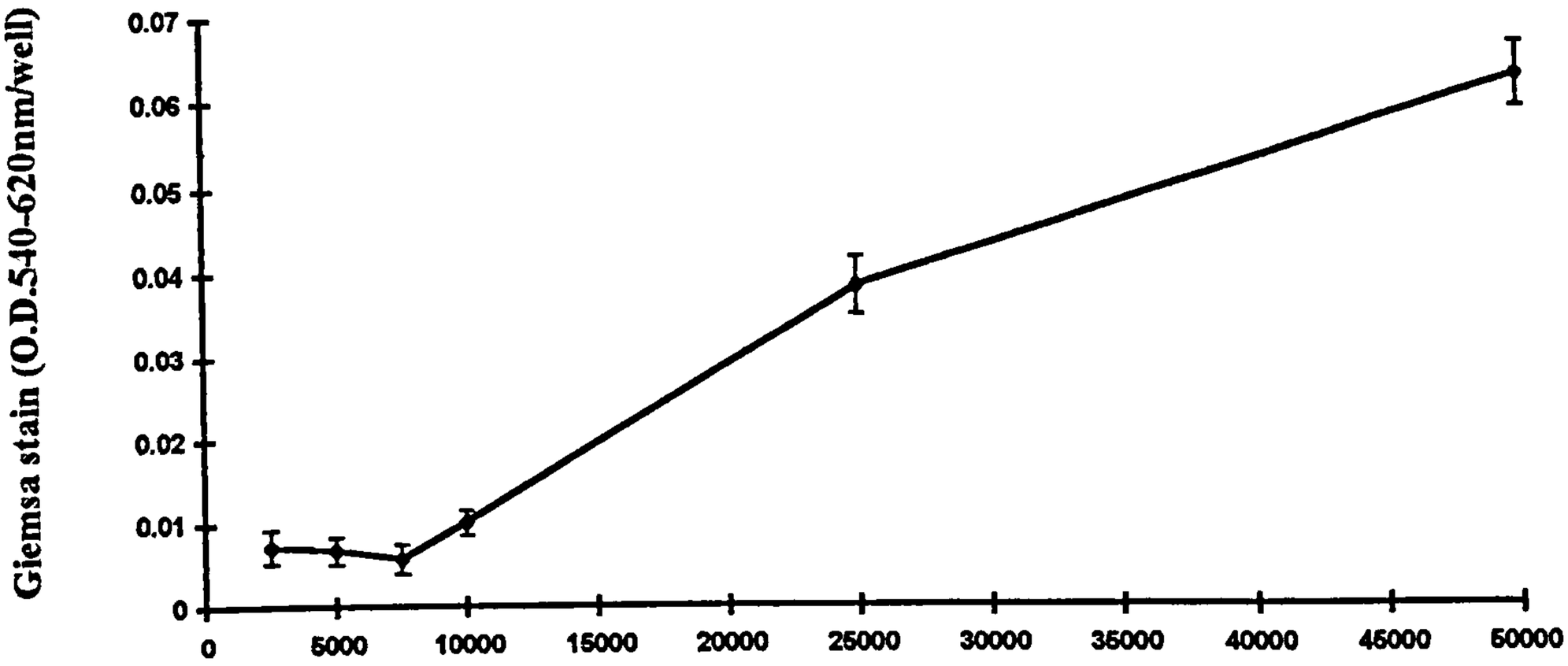


Figure 2.2 Standard curve for the Giemsa stain. Melanocytes from one donor were plated at densities of 0.25 to 5 x 10^4 cells/well (in a 96 well plate) and allowed to adhere for 4 hours prior to the treatment with Giemsa stain (as described in **Section 2.7.3**). The plates after being rinsed in water and air dried were read at an absorbance of 540nm with a reference wavelength of 620nm. The graph indicates for each cell density the mean ± SEM of 4 replicate wells.

100µl of acidified isopropanol (24.6µl of hydrochloric acid to 20mls isopropanol) was added to elute the stain. The plate was then read on a Dynatech platereader at filter 540nm with a reference wavelength of 630nm. However the results obtained (**Figure 2.1**) indicated that cell number showed a linear increment in MTT optical density (O.D.) only over a comparatively small range of cell densities. Additionally, melanocytes cultured in various different media would have different metabolic rates and this could affect the results, therefore this method was not used experimentally.

2.7.3 Giemsa stain.

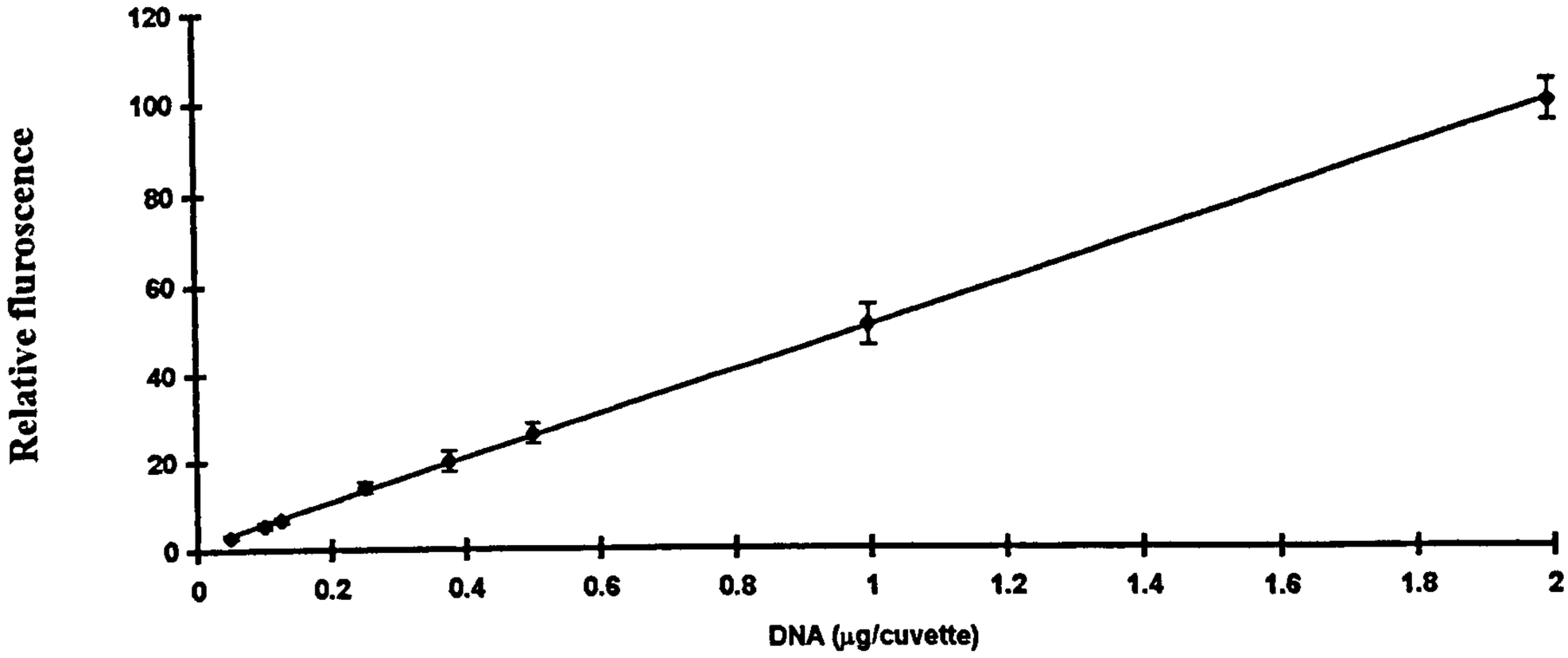
Giemsa stains the nucleus of the cells. A cell standard curve was set up, and melanocytes after adhering for 4 hours were rinsed in water. 100µl of 33% aqueous Giemsa solution was added for 5 minutes. After several rinses with water the plates were air dried for 5 minutes and then read on a Labsystems iEMs microplate reader at 540nm with a reference wavelength of 620nm. **Figure 2.2** illustrates the result. Although a standard curve was obtained with reasonable linearity between cell number and Giemsa O.D., cytoplasmic staining was also noted and this would make it difficult to compare melanocytes cultured in different media where they exhibit different morphologies. This method was therefore discarded.

2.7.4 DNA analysis.

Cell number was next assessed by calculating the DNA content, based on a method described by Rao & Otto (1992). At the end of the experiments, parallel plates of varying numbers of cells, cultured in identical conditions, were harvested after half an hour to one hour incubation in 0.04% sodium dodecyl sulphate plus 8M urea, made up in sodium citrate (0.015M), and sodium chloride (0.154M). The contents were collected into cuvettes and 100μl of 1μg/ml Hoechst 33258 (in sodium citrate and sodium chloride) was added directly before reading. The fluorescence of each cuvette was read at excitation λ355nm and emission λ460nm. The fluorimeter was calibrated to a DNA standard curve (DNA concentrations from 0.05μg/cuvette - 2µg/cuvette) (**Figure 2.3a**) with cell numbers calculated using a cell number standard curve (cell numbers ranging from $1x10^3$ to 1 or 2 x 10^5 cells/cuvette) (**Figure 2.3b-c**). This method was successful and used subsequently.

Figure 2.3 Standard curves for (a) DNA, (b) cell number and (c) DNA versus cell number, for the DNA assay. A range of DNA concentrations (0.05 - 2μg/cuvette) were dissolved in 0.04% sodium dodecyl sulphate in 8M urea prepared in a salt solution (as described in **Section 2.7.4**). 100μl of 1μg/ml Hoechst 33258 was added directly before a fluorescent reading was taken at excitation λ355nm and emission λ460 (**a**). The fluorimeter was calibrated to this standard curve. Cell standards (0.01 to 2 x 10^5 cells/cuvette) were harvested in the same sodium dodecyl sulphate/urea solution and fluorescence readings taken following Hoechst addition (**b**). The fluorescent values for cell number were converted in DNA and a graph representing cell number versus DNA (**c**). From these graphs, unknown cell numbers could be calculated. The data points illustrated in each graph represent the mean ± SEM of 5 different trials for this assay using melanocytes from a different donor for each trial.

a


Relative fluroscence
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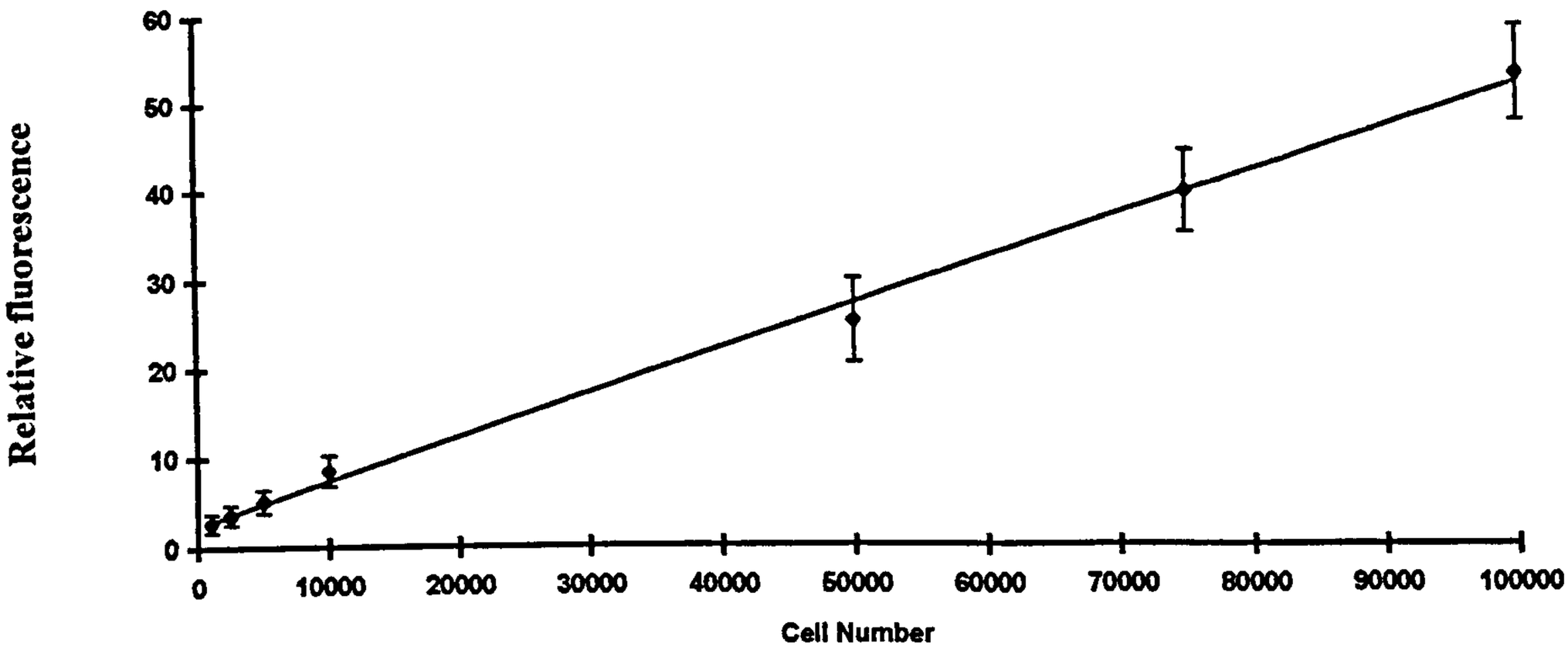
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
DNA (µg/cuvette)

b


Relative fluorescence
60
50
40
30
20
10
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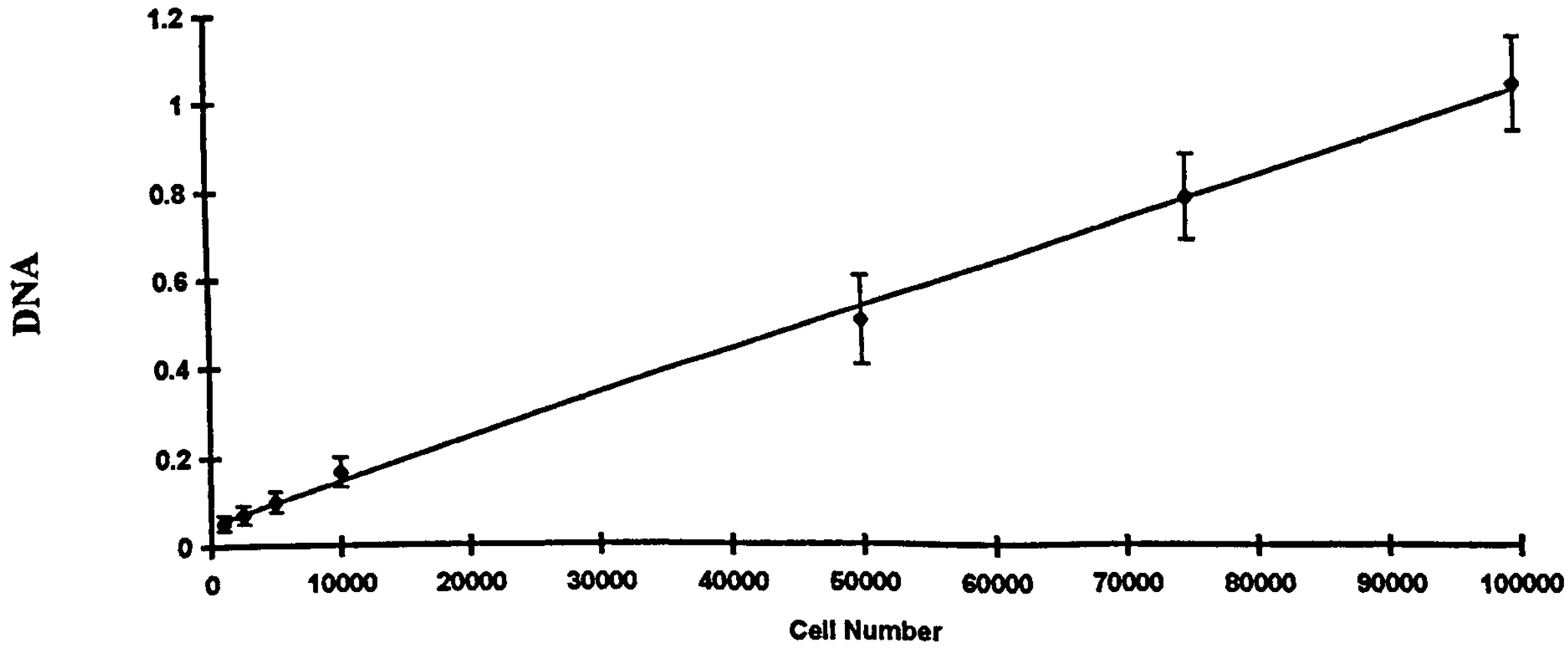
0
10000
20000
30000
40000
50000
60000
70000
80000
90000
100000
Cell Number

c


DNA
1.2
1
0.8
0.6
0.4
0.2
0
0
10000
20000
30000
40000
50000
60000
70000
80000
90000
100000
Cell Number

2.8 DETERMINATION OF MELANOCYTE PIGMENTATION

2.8.1 Dopa staining for the detection of melanocytes.

Melanocytes were washed in PBS three times then fixed in 4% formaldehyde for 20 minutes at 37^{0}C. Cells were washed again in PBS and 10mM L-dopa added (prepared in PBS). Cells were incubated in this solution for up to 10 hours in the dark at 37^{0}C in 5% CO_2. Control cells received only PBS. The effects of dopa staining are illustrated in **Figure 2.4**.

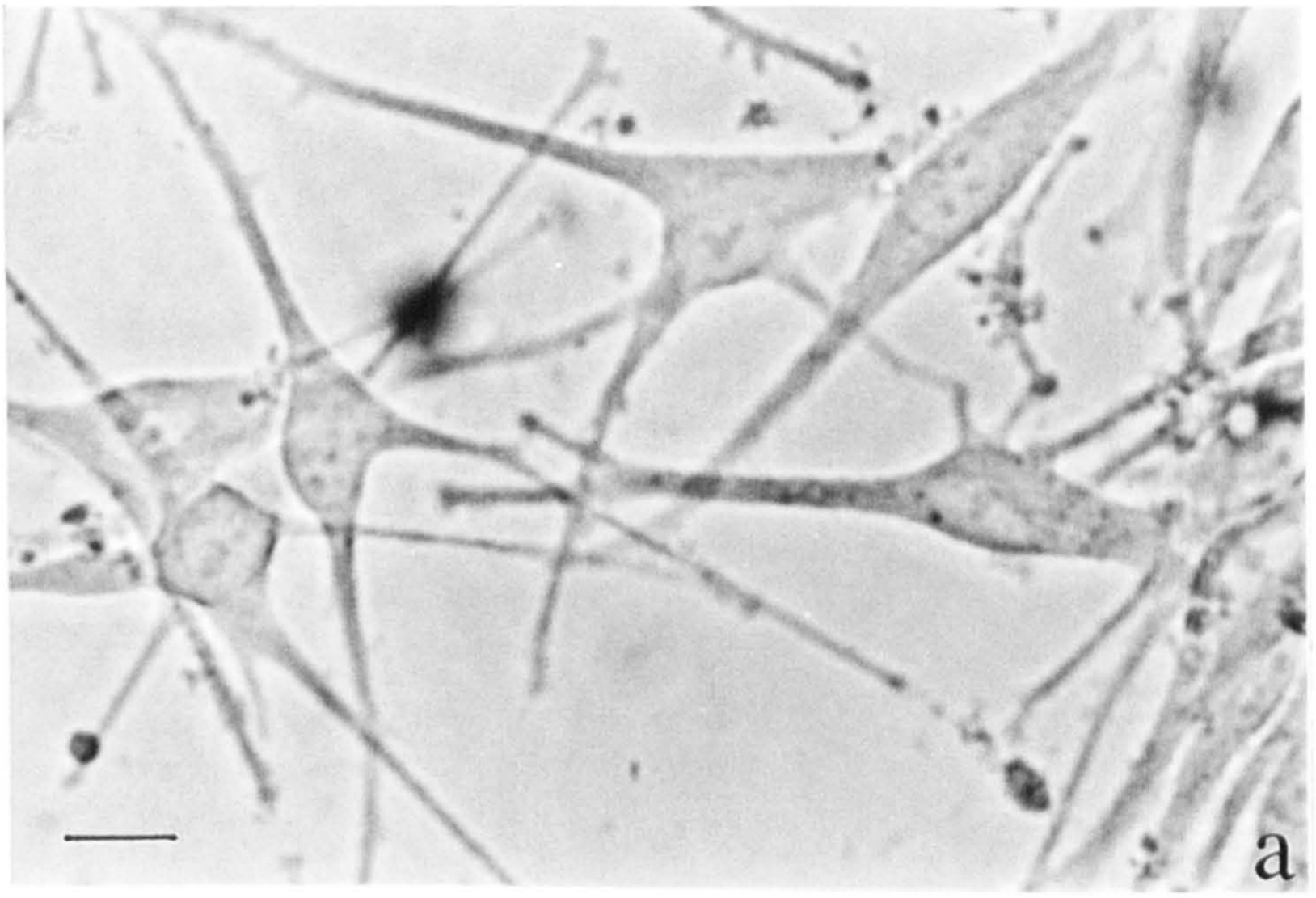
2.8.2 Tyrosinase assay.

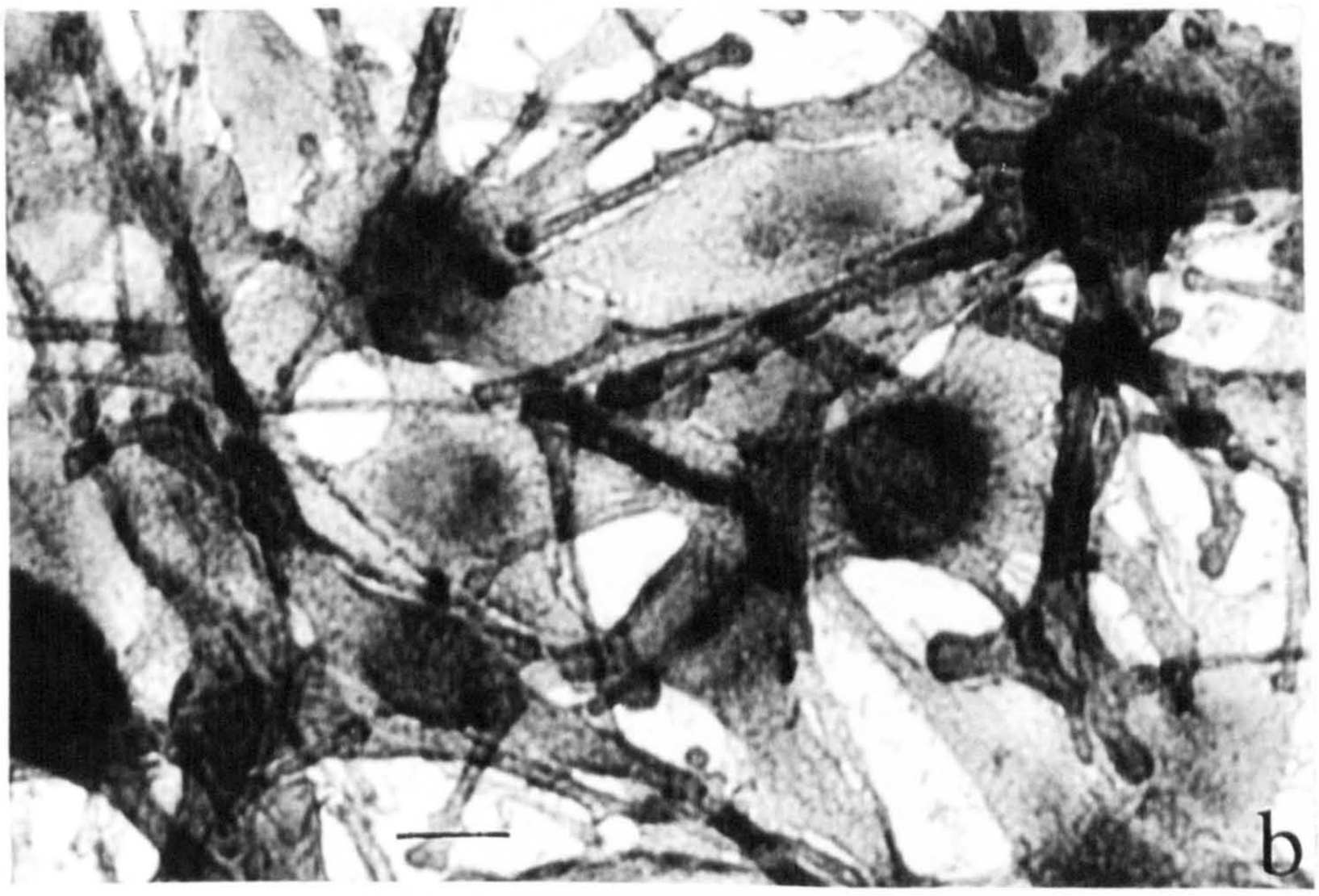
The dopa oxidase activity of the melanocyte tyrosinase enzyme was measured by taking the O.D. readings at 492nm (on a Labsystems iEMs microplate reader) of dopachrome as it was formed by the enzyme from the substrate L-dopa. The measurements were taken over a 2-4 hour time period. Several slightly different procedures were employed depending on the culture details of the experiments (modified from Jara *et al.* (1988)).

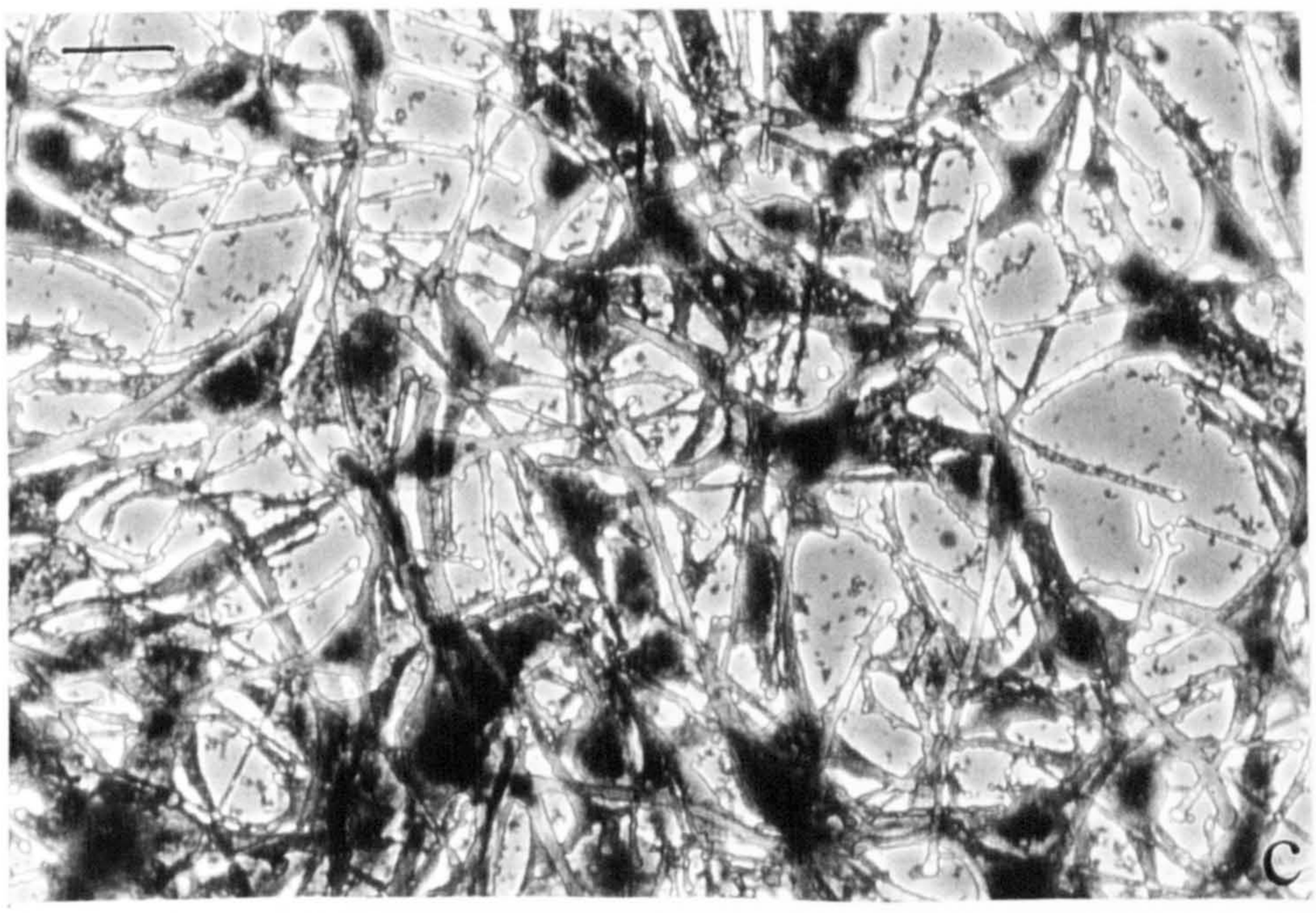
(a) Melanocytes cultured in a 96 well microtitre plates were washed 3 times with PBS and 45µl of 0.1% Tween 20 (made up in a 0.1M phosphate buffer). The cells were frozen at -80^{0}C for half an hour and then thawed at room temperature. After warming at 37^{0}C for 15 minutes, 5µl of 10mM L-dopa was added to start the reaction. The cells were then incubated at 37^{0}C throughout the experimental timeperiod. As with all of the procedures, a reading was performed immediately and then at specified timepoints for up to 4 hours. **Figure 2.5a** illustrates the kinetics graph for melanocytes from one donor at cell densities ranging from 0.5 - 2.5 x 10^4 cells/well, and the standard curve of dopa oxidase activity (described as ΔO.D.492nm/min/well x 10^{-4}) is illustrated in **Figure 2.5b**.

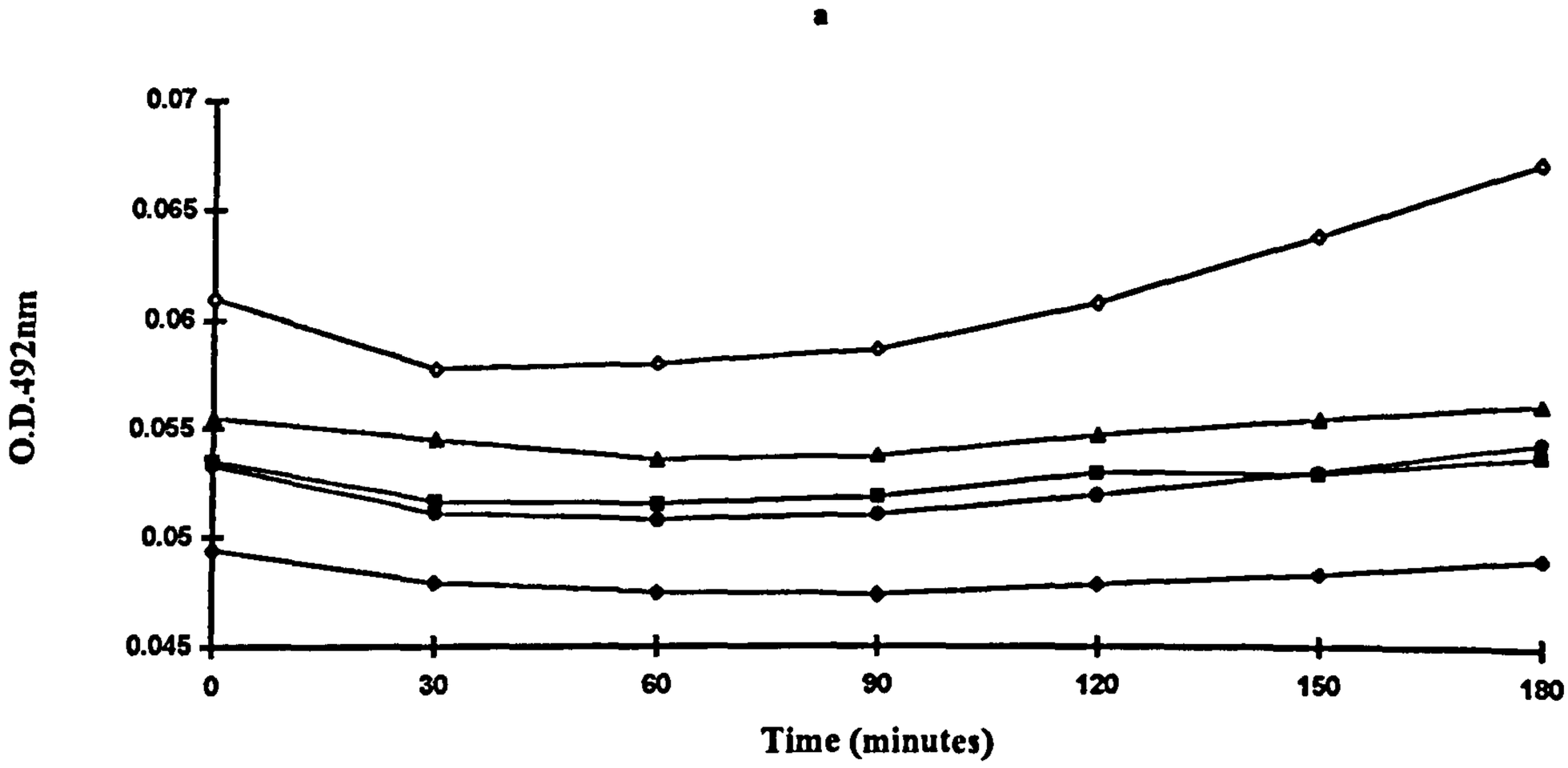
(b) 0.1% Tween 20 and L-dopa were added to 24 well plates at a proportionally increased amount in the experiments investigating ECM effects on melanocyte functions. Measurements were made by transferring 50µl aliquots into 96 well plates so that O.D. readings could be made at specific timepoints.

Figure 2.4 Dopa stained melanocytes in pure culture. Melanocytes from donor M417 were fixed and the stained with 10mM L-dopa for a period of up to 10 hours (as described in **Section 2.8.1**). Melanocytes (P1) are shown in the absence of L-dopa **(a)**, and in the presence of L-dopa **(b-c)**. Scale bars represent 7.5µm **(a-b)** and 15µm **(c)**.


a


b


c



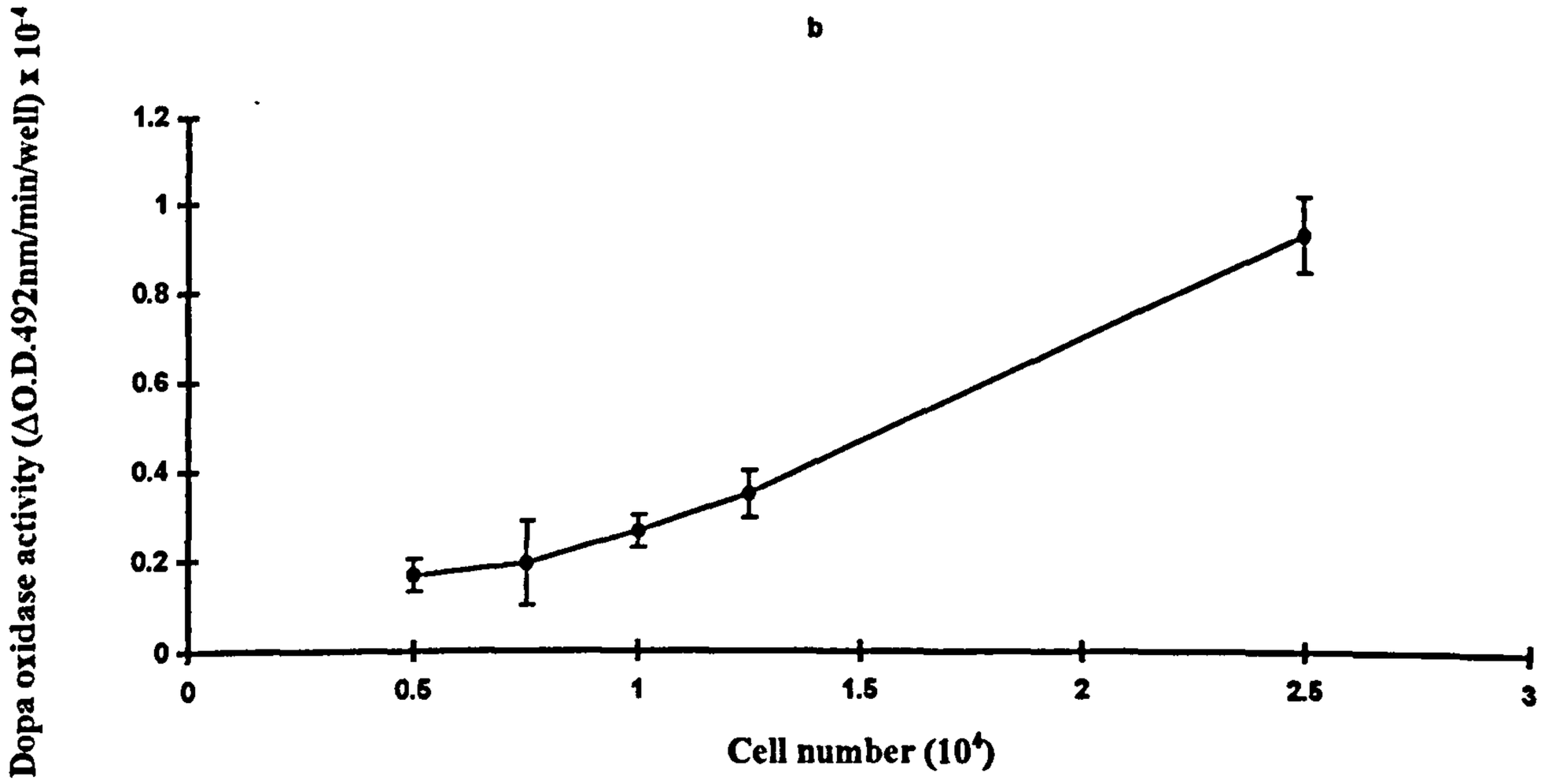
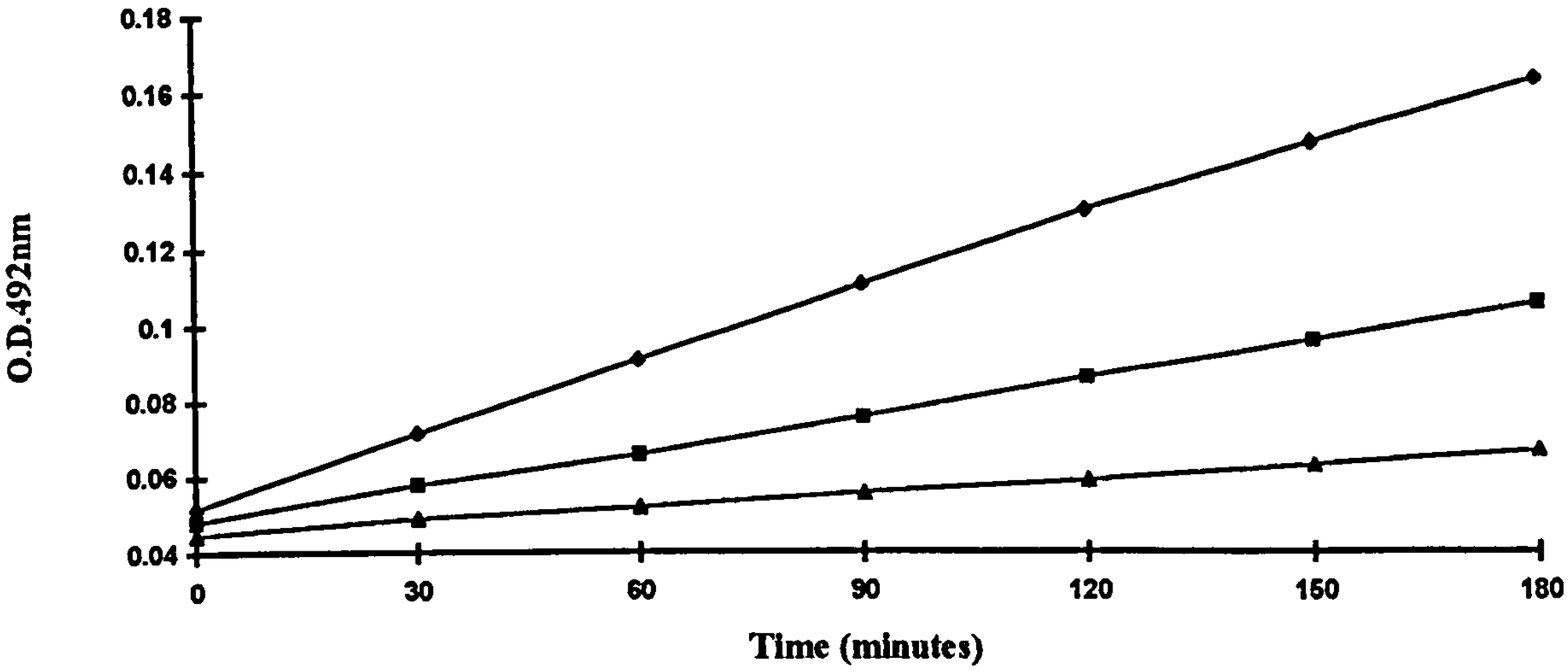


Figure 2.5 Enzyme kinetic (a,c) and standard curves (b,d) for dopa oxidase activity. Panel A. Melanocytes were plated at 0.5 (closed diamond), 0.75 (closed circle), 1 (closed square), 1.25 (closed triangle) or 2.5 (open diamond) x 10^4 cells/well (in a 96 well plate) and allowed to adhere for 4 hours prior to being freeze-thawed in 0,1% Tween. 10mM L-dopa was added to initiate the reaction (as described in **Section 2.8.2a)** and readings made every 30 minutes for up to 3 hours at O.D.492nm. The kinetics graph is illustrated in **(a)** and the standard curve for dopa oxidase activity described as ΔO.D.492nm/min/well x 10^{-4} is shown for the cell densities plated **(b)**. Each data point represents mean ± SEM of 8 replicate wells.

c



d

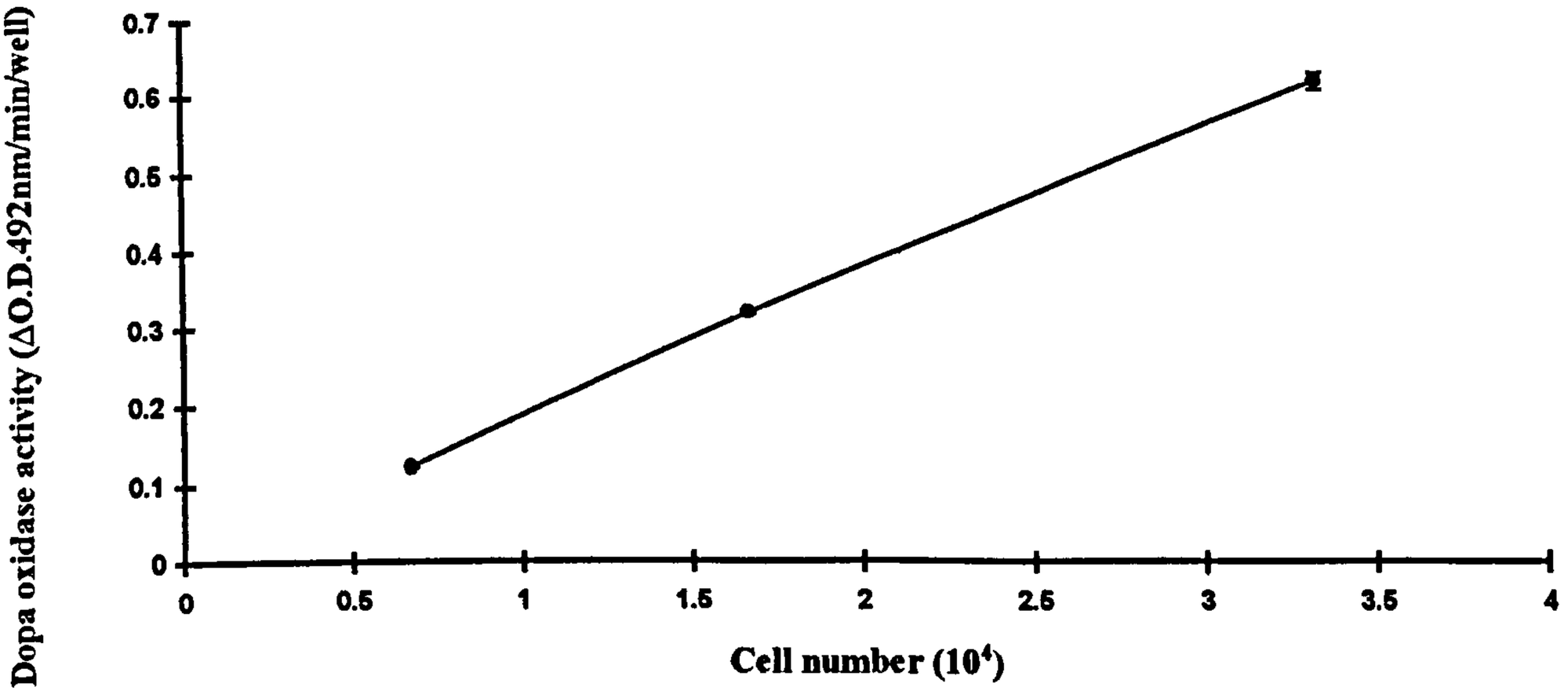


Figure 2.5 Panel B Melanocytes plated at densities 0.2 to 1 x 10^5 cells/well (in a 24 well plate in triplicate) were allowed to adhere for 4 hours and then frozen. The cells were then freeze-thawed in 0.1% Tween and aliquots transferred (as described in **Section 2.8.2d**) to 96 well plates to obtain cell lysates from 0.67 (closed triangle), 1.67 (closed square) and 3.33 (closed daimond) x 10^4 cells. The assay was conducted as previously described in Panel A, and the enzyme kinetics (**c**) and standard curve (**d**) are illustrated. Each data point represents the mean ± SEM of 9 replicate wells.

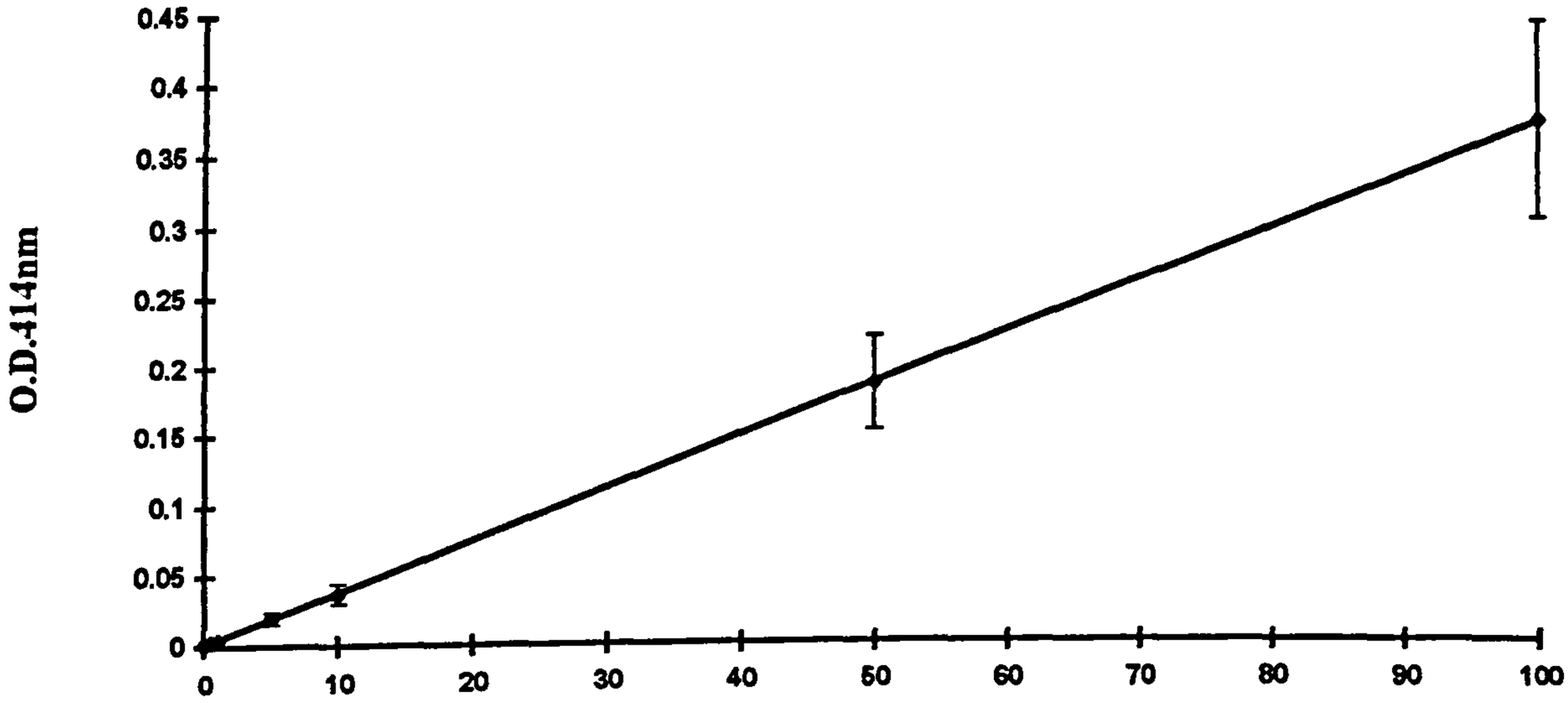


Figure 2.6 Standard curve of synthetic melanin at an absorbance of 414nm. A range of concentrations of synthetic melanin (0.1 - 100µg/ml) was prepared in 1M sodium hydroxide and the absorbance measured at 414nm. The graph indicates the mean ± SEM of 5 individual standard curves. For each experiment a standard curve was prepared.

(c) Melanocytes were pooled for each condition by trypsinization, PBS washed and cell counted. This procedure was used mainly with the UV experiments and the α-MSH experiments run in 12 well plates. In some cases the cell suspension was split for both melanin and tyrosinase assessment. Cells that were used for tyrosinase were pelleted and resuspended in an appropriate amount of 0.1% Tween 20 to obtain between 0.5 and 1.5 x 10^4 cells/96well when plated out in 90µl aliquots based on the standard curve shown in **Figure 2.5b**. Once plated out, the cells were freeze-thawed as before and 10µl of 10mM L-dopa was added to start the reaction.

(d) The final method involved washing the melanocytes with PBS then freezing the plates at -20^{0}C until cell number assessment had been conducted on the parallel plates. After cell number assessment, 0.2-0.3mls of 0.1% Tween was added to each well and cells were freeze-thawed to aid lysis. Appropriate amounts of 0.1% Tween were added to obtain a cell lysate that came from between 0.5 and 1.5 x 10^4 cells/well on a 96 well plate (**Figure 2.5c-d**). This method was used for the α-MSH pigmentation experiments conducted in 24 well plates, as the number of wells was too great for one person to handle by either methods 2 or 3.

A time course of tyrosinase activity versus incubation time was used to calculate enzyme activity over the linear part of the time course (usually between 60 and 180 minutes). Results were expressed as the increase in O.D.492nm/min/well and corrected for cell number unless stated.

2.8.3 Analysis of total melanin content.

Between 0.35 to 0.5mls of 1M sodium hydroxide was used to dissolve melanocytes, whether the melanocytes were pelleted following a cell count or in plates of 24 or 12 wells. 100µl aliquots were plated in to 96 well plates for O.D. determination at 414nm (Labsystems iEMs microplate reader). Melanin content (µg/ml/10^4cells) was calculated from a standard curve of synthetic melanin (prepared by oxidation of tyrosine with hydrogen peroxide) digested in 1M sodium hydroxide (0.01µg/ml to 100µg/ml, see **Figure 2.6**).

2.9 CELL BASED ELISA METHOD FOR INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1).

Cells were cultured for the appropriate amount of time and incubated with cytokines, hormones (if applicable) and fresh medium for 24 hours before the ELISA was run.

Cells were washed three times in PBS (with calcium and magnesium) and fixed in 1% paraformaldehyde for 20 minutes at room temperature. This was increased to 2% paraformaldehyde in later experiments to try and prevent cells washing away in later stages. Cells were washed 3 times in PBS (no calcium and magnesium) before 10% goat serum (GS + PBS) was added to block non-specific binding sites for 30 minutes. The incubation times were as detailed in Foster *et al.* (1994). Mouse anti-human ICAM-1 monoclonal antibody or mouse IgG_{2a} isotype control monoclonal antibody were added at a concentration of 0.4μg/ml in 5% GS. Plates were either incubated overnight at 4^0C or for 2 hours at room temperature (all the MSH/ICAM-1 experiments were run this way as were some of the comparison studies between normal and vitiligo). The cells were washed 3 times in PBS and incubated for 1 hour at room temperature with 10% GS before the horse radish peroxidase (HRP) conjugated goat $F(ab')_2$ anti-mouse IgG_1 secondary antibody was added at 2% volume to volume (v/v) in 5% GS. The cells were incubated for 1 hour at room temperature and then washed 3 times with PBS. The substrate was added for 1 hour and consisted of 3-amino-9-ethyl carbazole, N,N-dimethyl formamide, 50mM sodium acetate buffer (pH 5.0) and 30% hydrogen peroxide. This substrate was prepared and filtered through a 0.22μm filter immediately prior to use. The cells were washed once with PBS and ethanol (0.4ml per well) added for 20 minutes to elute the characteristic red colour. Aliquots of the eluted stain (100μl) were transferred into 96 well plates for O.D. determination (Labsystems iEMs microplate reader). ICAM-1 was expressed as O.D.492nm-620nm/well/10^4cells after blank and isotype control values were subtracted.

2.10 FLOW CYTOMETRY ANALYSIS OF IMMUNE-RELATED CELL SURFACE MOLECULES.

2.10.1 Principle for flow cytometry.

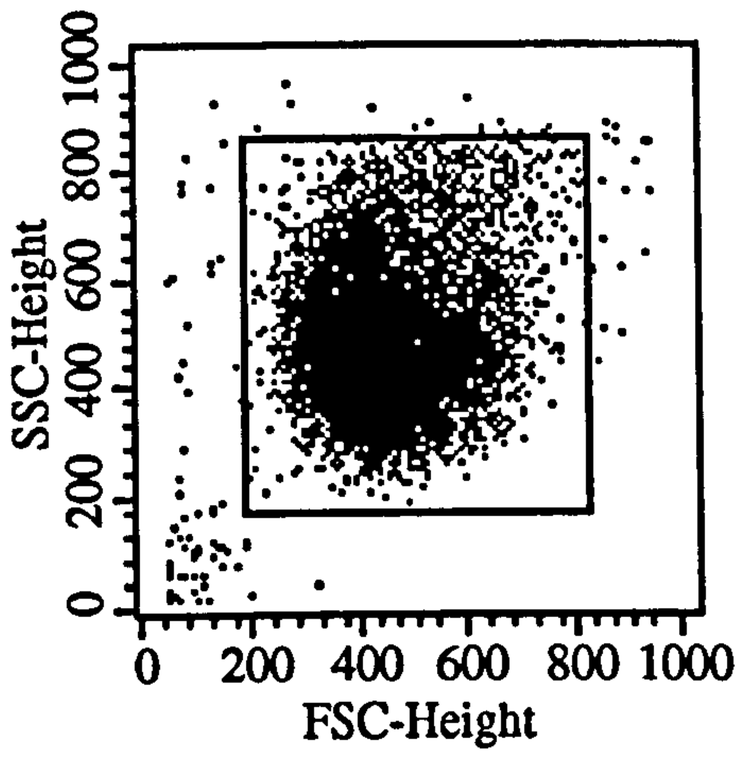
In flow cytometry, a single file of cells from a suspension passes through a LASER beam, causing light to be scattered in all directions from each cell. The light is collected by objective lenses and photo detectors convert the light to electronic signals. Light scattered in the forward position, known as the forward angle light scatter (FSC), is proportional to cell size. The side angle light scatter (SSC) is the light scattered at 90^0 to the excitation beam and is proportional to the granularity of the cell i.e. the refractive degree of the cell's internal structure. A dot plot is drawn up from the FSC/SSC combination of each cell (illustrated in **Figure 2.7a**).

Staining the cells with a fluorochrome or labelling the cells with an antibody-fluorochrome complex allows for a wide range of parameters such as DNA content, enzyme activity, pH, membrane potential and the expression of biological molecules in the cytoplasm or on the plasma membrane to be measured. Fluorescently labelled cells emit longer light wavelengths when they pass through the excitation beam. The SSC collected allows for a frequency histogram to be plotted (**Figure 2.7b-c**), where the y axis represents the number of cells and the x axis (a log scale) represents the intensity of the fluorescence which is emitted from the fluorochrome. Dual (using quadrant plots as shown in **Figure 2.7d**) and triple labelling can be performed, as different fluorochromes emit light at different wavelengths.

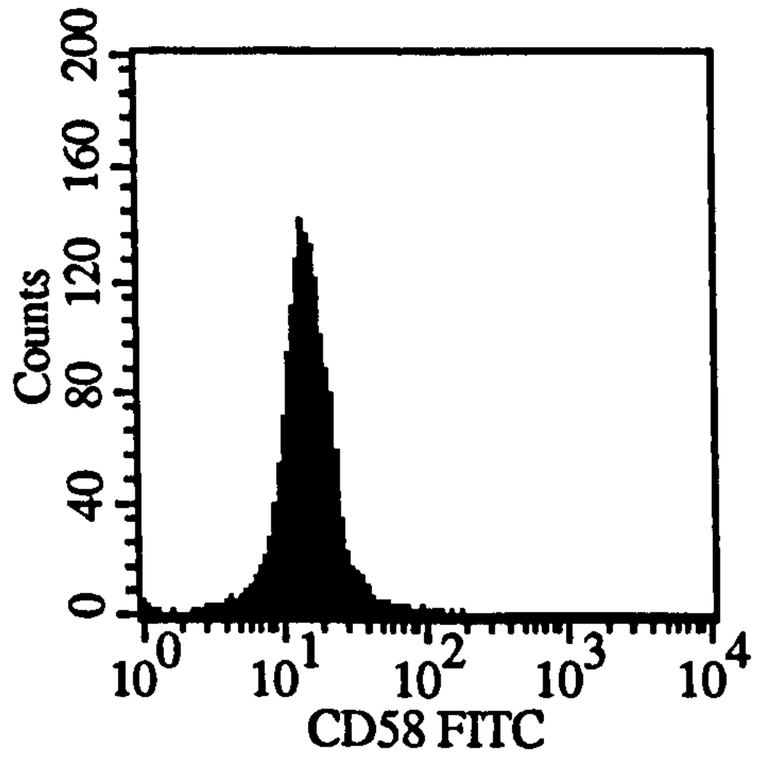
Statistical analysis was performed using computer software. Using the SSC/FSC dot plot, a subset of cells were selected (gated) to exclude possible cell debris (low FSC- and SCC-height) and these data could be analysed for the percentage of cells expressing the parameter of interest and for the mean fluorescent intensity of the histogram.

Figure 2.7 Flow cytometry data plots. A single file of cells in suspension pass through a laser bean and the forward angle light scatter (FCS) and side angle light scatter (SSC) are collected to form a dot plot (**a**). This allows the user to select a region and discard low FCS/SCC cells which are likely to be debris. When cells are labelled with fluorochromes or antibody-fluorochrome complexes, the SCC collected allows for a frequency histogram to be plotted (**b-c**). This allows for the intensity of staining to be calculated as for a fluorescein isothiocyante (FITC) (**b**) labelled or a r. phycoerythrin (PE) labelled (**c**) cell. When cells are dual labelled the quadrant plot is plotted (**d**). The upper left and upper right quadrants represent the number of cells expressing the PE fluorochrome (in this example) and the upper right and lower right quadrants represent the number of cells expressing the FITC fluorochrome (in this example). (The software package also allows for individual histograms to be plotted from these quadrant plots as shown in (**b-c**)).

a


SSC-Height
0
200
400
600
800
1000
FSC-Height

b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120
160
200
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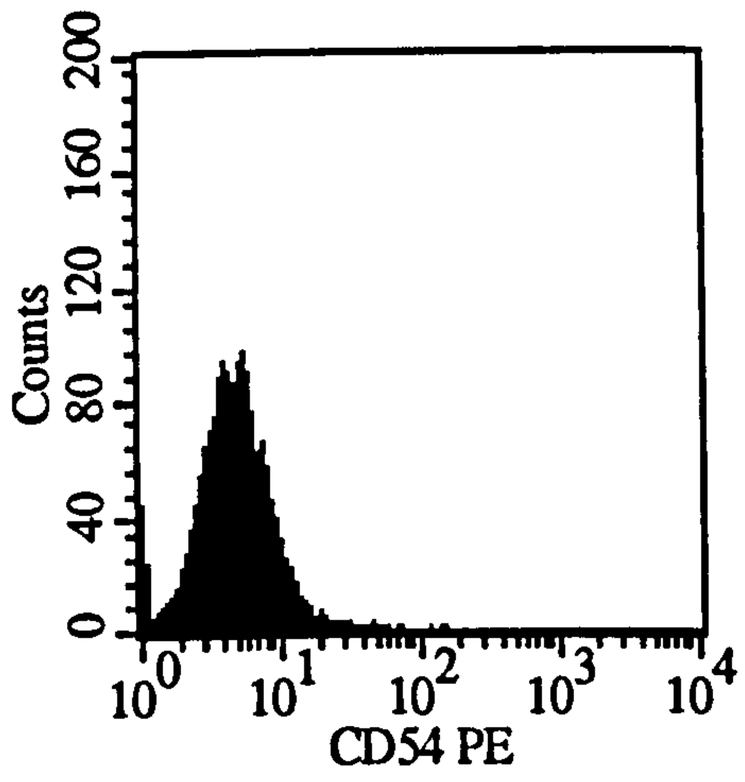
10^1
10^2
10^3
10^4
CD58 FITC

c


Counts
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10^1
10^2
10^3
10^4
CD54 PE

d

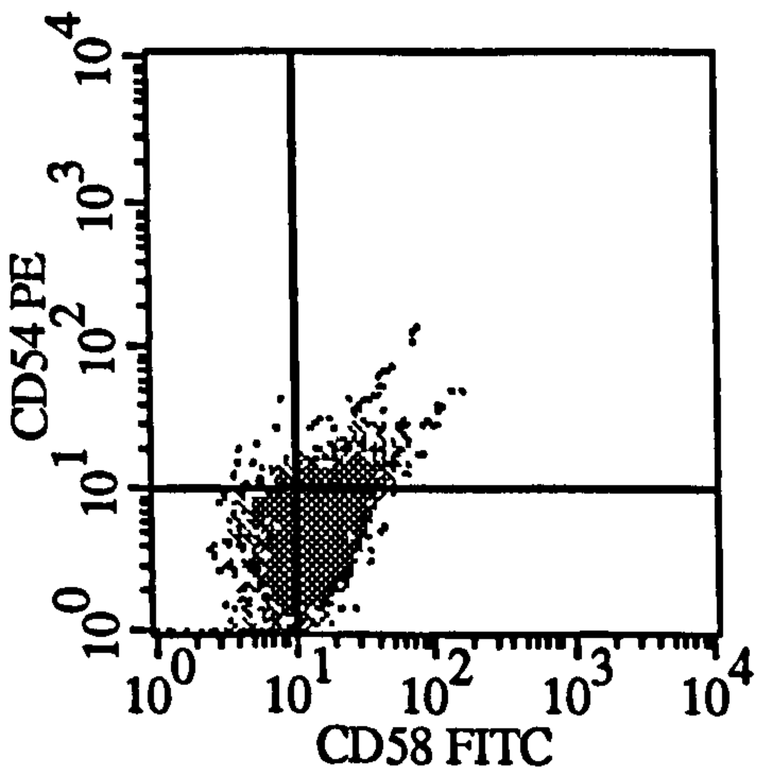

CD54 PE
10^0
10^1
10^2
10^3
10^4
CD58 FITC

Table 2.7 Flow cytometry antibodies.

Cell surface molecule	Immunoglobulin type	Clone	Fluorochrome labelling
CD40	IgG_1	B-B20	PE
CD54 (ICAM-1)	IgG_1	B-C14	PE
CD58 (LFA-3)	IgG_{2a}	BRIC5	FITC
HLA-A,B,C[a]	IgG_{2a}	W6/32	FITC
HLA-DR,DP,DQ[b]	IgG_{2a}	WR18	PE

All antibodies were mouse anti-human for the specific cell surface moelcues. Antibodies were labelled with R. phycoerythrin (PE) or fluorescein isothiocyanate (FITC). In dual labelling studies, anti-CD54 and anti-CD58 were paired, and anti-HLA-A,-B,-C and anti-HLA-DR,-DP,-DQ were paired. The appropriate labelled mouse IgG_1 and IgG_{2a} negative control antibodies were used in all studies.

[a] HLA-A,-B,-C was a monomorphic antibody for MHC class I.

[b] HLA-DR,-DP,-DQ was a monomorphic antibody for MHC class II.

2.10.2 Flow cytometry analysis of immune-related cell surface molecules on melanocytes.

Melanocytes, 24 hours after experimental treatments, were carefully trypsinized, 10% FCS added and pelleted. The cells were PBS washed and resuspended in 200-300µl of 1% bovine serum albumin/0.01% sodium azide in PBS. The samples were split into 2 to 3 tubes (1 tube for isotype antibodies and the other tubes for specific cell surface antibodies) and labelled with the appropriate antibodies (listed in **Table 2.7**) for half an hour on ice in the dark. All antibodies were used at a 1 in 40 v/v dilution. Excess antibodies were washed away with 2 PBS washes and then 10^4 cells from each sample examined on a Becton Dickinson FACS machine. CellQuest software was used to analyse the data. Quadrant plots were used to determine the percentage of cells in the gated region expressing the appropriate antigen, while histograms were used to examine the mean fluorescence values. Mr R Metcalfe (Department of Medicine, University of Sheffield), kindly helped with the setting of parameters on the FACscan machine and running the flow cytometry analysis on the MHC class I, MHC class II and CD40 work. The work on expression of ICAM-1 and LFA-3 (details in **Sections 4.3.6** and **4.5.1**) was performed by myself.

2.11 T CELL BINDING ASSAY.

2.11.1 Preparation of T cells.

Venous blood was collected from myself, and 100U/ml heparin added (9 parts blood: 1 part heparin) to prevent coagulation. An equal volume of PBS was added. 6mls of diluted blood was carefully layered over 3mls of lymphoprep (which contains 9.6% sodium metrizoate and 5.6% polysaccharide) in universal tubes and centrifuged at 940g for 20 minutes. The sodium metrizoate, a high density compound, prevents the blood mixing with the polysaccharide. The polysaccharide causes aggregation of erythrocytes at the interface and these sediment at the bottom of the tube during centrifugation. The mononuclear cells form a layer on the interface and were carefully removed using a pasteur pipette (**Figure 2.8**) and diluted with RPMI-1640 containing 5% FCS. This suspension was centrifuged at 280g for 10 minutes and cells were

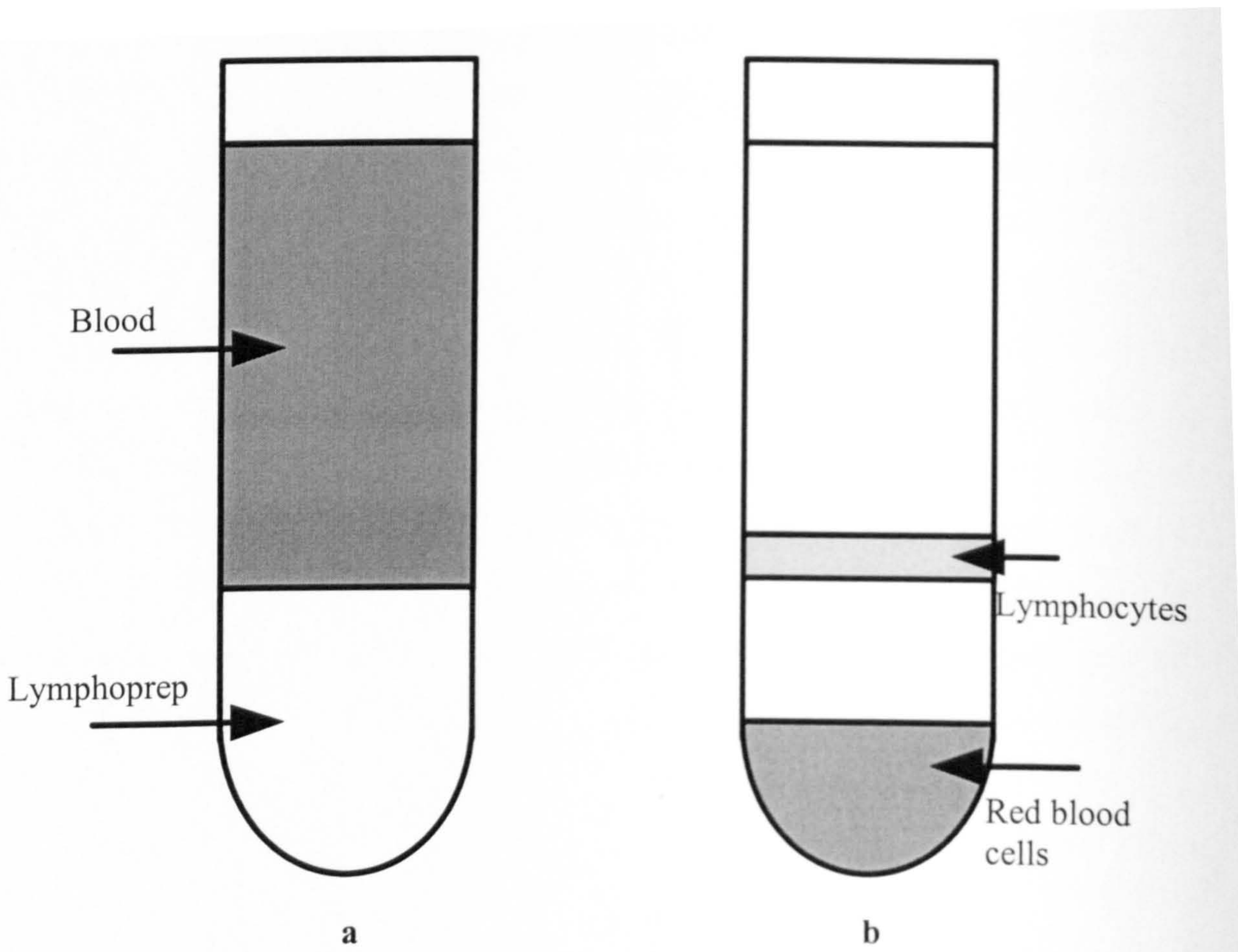


Figure 2.8 The centrifugal separation of lymphocytes from venous blood. Heparinized blood was layered over lymphoprep (**a**) and centrifuged as described in **Section 2.11.1**. The positioning of the various cells from the blood is indicated in (**b**).

resuspended at 10^8cells per 7mls of medium. This cell suspension was added to a Uni-Sorb T + B nylon wool column to obtain an enriched T cell population free from adherent cells such as monocytes, macrophages and B cells. The column was incubated for 1 hour at 37^0C, before medium (2 x 6mls) was washed through to release the T cells. A previous study by Little *et al.* (1998) (from this laboratory), demonstrated that approximately 80% of the cells washed through the column were CD3 positive and hence T cells. As the current study was only preliminary, analysis of the cells washed through the column was not carried out. Cells were resuspended at 2 x 10^6 cells/ml in RPMI-1640 with 5%FCS and cooled on ice for 5 minutes.

2.11.2 Activation of T cells.

PMA at a concentration of 50ng/ml was added to the lymphocyte suspension and cells remained on ice for 10 minutes. The sample was then diluted and cells spun at 280g for 10 minutes (0^0C). 100µl of T cell suspension was added to each well to give 2 x 10^5 cells/well (although this was not possible with the three experiments carried out in the thesis; see **Table 4.12**). The melanocytes to be assessed for T cell binding were washed with RPMI-1640 with 5% FCS prior to T cell addition.

2.11.3 Enzyme linked immune cell adhesive assay.

The determination of the number of bound T cells by a coloured end-point was based on a method by Bruynzeel *et al.* (1990) and used by Dr MC Little, a former member of the laboratory to study T cell interactions with keratinocytes.

Prior to the T cell binding assay, melanocytes had been cultured under experimental media for 5 days and received cytokine treatments for the final 24 hours. The plates of melanocytes were incubated at 4^0C for 20 minutes and then at 37^0C for 1 hour. The plates were then carefully washed with assay medium (100µl/well) 4 times, rotating the plate each time. The complexes were fixed with 0.25% glutaraldehyde for 10 minutes at 4^0C before being washed again 4 times in RPMI-1640 + 5% FCS. 1% human serum albumin (HSA) (200µl/well) was added for 1 hour at 37^0C to block non-specific antibody binding, before the antibodies, anti-CD45 (leukocyte common antigen) polyclonal antibody conjugated to HRP and Ig/HRP negative control were added at a 1

in 25 dilution (in a 1% HSA/0.1% Tween solution prepared in PBS). The antibodies were present for 30 minutes at room temperature. Plates were washed with the HSA/Tween/PBS solution before addition of 100µl per well of substrate (o-phenylenediamine in a phosphate citrate buffer). After a 10 minute incubation in the dark at room temperature, the reaction was stopped with 50µl/well of 2M sulphuric acid and the plates read at 492nm with reference 630nm (Labsystems iEMs microplate reader).

CHAPTER 3

INVESTIGATION OF THE INFLUENCE OF MEDIA, α-MELANOCYTE STIMULATING HORMONE, EXTRACELLULAR MATRIX PROTEINS, ULTRAVIOLET IRRADIATION AND CYTOKINES ON NORMAL AND VITILIGO HUMAN MELANOCYTE MORPHOLOGY, PROLIFERATION AND MELANOGENIC ACTIVITY.

3.1 INTRODUCTION.

Human cutaneous melanocytes originate from the neural crest and reside in a complex enviroment in the adult. In the skin, they are located in the basal layer of the epidermis, adjacent to the basement membrane, they interact with keratinocytes to form a highly regulated integrated pigmentary unit. A keratinocyte-Langerhans' cells-melanocyte triad has been proposed (Pelc & Nordlund, 1993) and recently the innervation of normal melanocytes has been suggested (Hara *et al*, 1996).

In vivo, UVR remains one of the best known physiological stimulator of melanocytes (Miller-Milinska & Staricco, 1963; Pathak *et al*, 1965; Quevedo *et al*, 1965; Rosen *et al*, 1987; Stierner *et al*, 1989). Melanocytes in shielded skin, adjacent to UV exposed skin are also affected (Stierner *et al*, 1989). Skin organ cultures primarily using perilesional vitiligo skin have shown melanocytes to be responsive to pulses of UV light (Iyengar, 1992a&b, 1994a&b, 1996 & 1998). The mechanisms by which UVR affects melanocytes remains unclear. Indirect mechanisms are most likely to occur through the production of growth factors and cytokines by keratinocytes and possibly fibroblasts. The importance of keratinocytes in the biology of melanocytes has been illustrated in co-cultures studies (De Luca *et al*, 1998a & b; Vayli-Nagy *et al*, 1990; Haake & Scott, 1991; Scott & Haake, 1991; Donatien *et al*, 1993; Valyi-Nagy *et al*, 1993; Nakazawa *et al*, 1995a; Tenchini *et al*, 1995; Kippenberger *et al*, 1996; Nakazawa *et al*, 1997; Kippenberger *et al*, 1998a; Nakazawa *et al*, 1998), the use of keratinocyte-conditioned medium (KCM) (Gordon *et al*, 1989, Lacour *et al*, 1992; Hara *et al*, 1995; Tenchini *et al*, 1995) and keratinocyte-derived ECM (Nakazawa *et al*, 1995b). Additionally, fibroblast-conditioned medium (FCM) was recently illustrated to influence melanocytes (Imokawa *et al*, 1998). UVB and UVA have been shown to directly influence *in vitro* cultured melanocyte melanogenesis by several groups (Friedmann & Gilchrest, 1987; Libow *et al*, 1988; Tomita *et al*, 1988; Friedmann *et al*, 1990a&b; Ramirez-Bosca *et al*, 1992; Kao & Yu, 1992; Aberdam *et al*, 1993; Abdel-Malek *et al*, 1994b; Im *et al*, 1994; Roméro *et al*, 1994; Mengeaud & Ortonne, 1996; Duval & Schmidt, 1997). The intracellular signalling pathway for UVB-induced melanogensis has not yet been fully elucidated. DNA damage (Eller *et al*, 1996), 1,2-diacylglycerol (DAG) acting via a PKC pathway (Carsberg *et al*, 1994 & 1995b), guanosine 3':5' cyclic monophosphate

(cGMP) pathway activation (Roméro-Graillet *et al*, 1996) and just recently the PKA pathway activated by α-MSH (Im *et al*, 1998) have been implicated in the regulation of UV-induced pigmentation in man.

α-MSH, a POMC-derived peptide, is known to have a broad spectrum of biological activities (as reviewed in Eberle, 1988). As a pigmentary hormone it has well-defined effects, for example, in the mouse it produces increased hair follicular eumelanogenesis (Burchill *et al*, 1986; Burchill *et al*, 1993). However, the role of α-MSH as a pigmentary hormone in man remains controversial. *In vivo*, subcutaneous injections of α- and β-MSH (Lerner & McGuire, 1961), ACTH (Lerner & McGuire, 1964) and NDP-MSH (Levine *et al*, 1991) increased skin darkening in human subjects. Hypersecretion of α-MSH has been implicated in hyperpigmentation in skin (Pears *et al*, 1992), and increased plasma levels of ACTH have been implicated in hyperpigmentation in endocrine disorders such as Addison's disease and Cushing's syndrome. *In vitro*, early studies with cultured human melanocytes failed to demonstrate pigmentary responses to MSH peptides (Halaban *et al*, 1983; Ranson *et al*, 1988b; Friedmann *et al*, 1990a; De Luca *et al*, 1993), although α-MSH (De Luca *et al*, 1993) and β-MSH (Halaban *et al*, 1983) binding to melanocytes was displayed and cells showed an increase in cAMP in response to α-MSH (Ranson *et al*, 1988b; Friedmann *et al*, 1990b). More recently, four groups demonstrated a moderate effect of POMC-derived peptides on pigmentation in human melanocytes (Hunt *et al*, 1994a, b & c; Abdel-Malek *et al*, 1995; McLeod *et al*, 1995; Maeda *et al*, 1996). In skin biopsies of varying skin types, α-MSH failed to stimulate tyrosinase activity (Burchill *et al*, 1990) with similar results obtained in human hair bulbs (Burchill *et al*, 1991), while in a foreskin organ culture, mixed results were reported in response to α-MSH (Iwata *et al*, 1990b).

Melanocytes possess the ability to bind POMC peptides (Halaban *et al*, 1983; Donatien *et al*, 1992; De Luca *et al*, 1993; Thody *et al*, 1993; Suzuki *et al*, 1996). The melanocortin receptor family has been cloned (Mountjoy *et al*, 1992; Chhajlani & Wikberg, 1992; Chhajlani *et al*, 1993; Gantz *et al*, 1993a & b; Gantz *et al*, 1994; Roselli-Rehfuss *et al*, 1995) and melanocytes are defined as possessing the melanocortin 1 receptor (MC1R). Several studies support expression and production of POMC-derived peptides in skin with both keratinocytes and melanocytes implicated in

this local production (Thody *et al*, 1983; Farooqui *et al*, 1993; Schauer *et al*, 1994; Iyengar, 1995; Kippenberger *et al*, 1995; Slominski *et al*, 1995; Chakraborty *et al*, 1996; Wintzen *et al*, 1996; Wakamatsu *et al*, 1997; Can *et al*, 1998). Recently, Langerhans' cells and human fibroblasts were reported to express POMC-peptides (Morhenn, 1991; Teofoli *et al*, 1997; respectively). Levels of α-MSH and ACTH released from normal skin cells are also increased following UV exposure (Schauer *et al*, 1994; Iyengar, 1995; Chakraborty *et al*, 1996) and the MC1R is modulated by UVB in humans (Funasaka & Ichihashi, 1997). Thus, the current picture suggests that melanocytes *in vivo* will be involved in both the production of POMC peptides and in the response to them.

The positioning of the melanocytes adjacent to the basement membrane has led to the investigation of the influence of ECM proteins on melanocyte function. A variety of melanocyte parameters, including melanocyte morphology and/or attachment (Gilchrest *et al*, 1985; Ranson *et al*, 1988a; McClenic *et al*, 1989; Scott *et al*, 1992; Danen *et al*, 1994; Zambruno *et al*, 1993; Nakazawa *et al*, 1995b; Searles *et al*, 1995; Mengeaud *et al*, 1996, Le Poole *et al*, 1997), melanocyte proliferation (Ranson *et al*, 1988a; Mortarini *et al*, 1995), melanocyte migration (Morelli *et al*, 1993), and melanocyte pigmentation (Ranson *et al*, 1988a; Buffey *et al*, 1994; Nakazawa *et al*, 1995b) are influenced by cell-derived and individual ECM proteins. Melanocyte attachment to ECM proteins and migratory properties are regulated through integrins (Scott *et al*, 1992; Danen *et al*, 1994; Morelli *et al*, 1993; Zambruno *et al*, 1993). α-MSH (Hunt *et al*, 1993; Scott *et al*, 1997), retinoic acid (Situ *et al*, 1993), stem-cell factor (SCF) (Scott *et al*, 1994), cations (Hara *et al*, 1994; Searles *et al*, 1995), nitric oxide (NO) (Ivanova *et al*, 1997) and endothelin-1 (Scott *et al*, 1997) have been reported to be involved in the regulation of these properties. The differentiation of melanocytes from their embryonic precursors, neural crest cells, can be achieved *in vitro* by dermal ECM derived from embryonic tissues (Derby, 1982). Fibronectin probably has a role in directing melanocytes from the neural crest to their final destination in the epidermis (Scott *et al*, 1992). Le Poole *et al*. (1997) reported that increased tenascin, an ECM protein that melanocytes do not attach to, may be a causative factor in the start of vitiligo while NO has also been proposed to have a role in contributing to the loss of melanocytes in vitiligo through reducing *de novo* attachment to ECM proteins (Ivanova *et al*, 1997).

3.1.1 Growth of melanocytes *in vitro* and *ex vivo*.

The complex environment of the melanocyte, the low population and the low mitotic index *in vivo* inherently make the cutaneous melanocyte a difficult cell to culture once isolated. Eisinger & Marko (1982) were the first group to report successful culture of melanocytes using a medium supplemented with PMA and CT (**Table 3.1a**). The success of their media was due to inhibition of keratinocytes by PMA and the plastic culture substrata which was selective towards melanocyte adhesion. Neonatal melanocytes were better suited to growth in this medium. Extracts from melanoma cells, astrocytoma cells, and embryonic lung fibroblasts were found to have growth-promoting effects on melanocytes in this medium (Eisinger *et al*, 1985). A variant of this medium improved the culture of neonatal melanocytes in the presence of IBMX or placental extract (Halaban *et al*, 1986; Halaban *et al*, 1987) (**Table 3.1a**). Unwanted fibroblasts were removed by geneticin (Halaban *et al*, 1984) although another group described the use of epidermis from suction blisters as a good way to obtain a fibroblast-free epidermal cell suspension (Tomita *et al*, 1985). Recently, Horikawa *et al*. (1996b) reported that alteration of calcium levels with a low dose of geneticin for 48 hours removed unwanted fibroblasts from melanocyte cultures. However, McLeod & Mason (1995) reported that enriched cultures of melanocytes could be obtained by initially co-culturing skin cells in mitogen rich media before sub-culturing as pure melanocytes.

Tsuji & Karasek (1983) employed 5-fluorouracil to remove keratinocytes from melanocyte cultures established in McCoy's 5A medium supplemented with CT and IBMX. A hormone supplemented medium for melanocytes reduced the requirement for serum and pituitary extract replaced PMA (Gilchrest *et al*, 1984; Wilkins *et al*, 1985) (described in **Table 3.1a**) while Nielson & Don (1984) described an unusual culture method using fibroblast-conditioned medium (further described in **Table 3.1**). bFGF was first described as a mitogen for melanocytes when present in a PC-1 medium (Halaban *et al*, 1987). Since then bFGF has been regularly employed in many culture media and can replace the requirement for pituitary extract as exemplified in the defined MCDB153 medium described by Pittelkow & Shipley (1989) (see **Table 3.1a**). A novel chemically defined medium was described by Herlyn *et al*. (1988) in which

Table 3.1a Culture media for normal human melanocytes.

Reference	Eisinger & Marko, 1982[a]	Gilchrest *et al*, 1984[b]	Nielson & Don, 1984[c]	Halaban *et al*, 1986[d]	Herlyn *et al*, 1988[e]	Pittelkow & Shipley, 1989[f]	Donatien *et al*, 1993[g]	Ikeda *et al*, 1994b[h]	Swope *et al*, 1995
Basal medium	EMEM	M199	FIB41B	Ham's F10	W489	MCDB153	MGM	MM-4	MCDB153
BPE[i,j]		150µg/ml				25µg/ml	47µg/ml	150µg/ml	
FCS/FBS[i,j]	5%	2%					1-3%		5%
HSSP[i,j]			15%						
NCS[i,j]				8%					
Nu-serum[j]				8%					
cholera toxin[k]	10nM	1nM		25nM				10ng/ml	
IBMX[i,k]				0.1mM					
α-MSH[i,k]					10ng/ml				10nM
vitamin E[l]									1µg/ml
bFGF[i,m]					40ng/ml				0.6ng/ml
EGF[i,m]		10ng/ml				10ng/ml	3.3ng/ml		
endothelin-1[m]									10nM
L-glutamine[m]	2mM								
hydrocortisone[m]		50µM				50µM	0.94µM		
insulin[m]		10µg/ml			5µg/ml	5µg/ml			5µg/ml
PMA[i,m]	16nM			85nM	100nM	16nM		16nM	
transferrin[m]		10µg/ml							1µg/ml
T/T[i,m]		1nM							
ethanolamine[n]						0.1mM			
antibiotics	✓	✓	✓	✓					✓

Table 3.1b Culture media for vitiligo melanocytes.

Reference	Medrano & Nordlund, 1990[o]	Im *et al*, 1992	Jee *et al*, 1993 [p]	Olsson & Juhlin, 1993[q]	Le Poole *et al*, 1997	Maresca *et al*, 1997
Basal medium	MCDB153	MCDB153	F12	PC-1	Ham's F10	Ham's F10
BPE[i,j]	30µg/ml	0.4%				
FCS/FBS[i,j]	2%		1%			10%
human AB[j]		10%				
Ultroser G[j]					1%v/v	
cholera toxin[k]			40ng/ml			
dbcAMP[i,k]				0.5mM		
IBMX[i,k]					0.1mM	
catalase[l]	20µg/ml					
vitamin E[l]	1µg/ml					
bFGF[i,m]	0.6ng/ml	1ng/ml		5ng/ml		
ECGS[i,m]			15mg/ml			
EGF[i,m]						
EPGF[i,m]			20ng/ml			
L-glutamine[m]				2mM	2mM	
hydrocortisone[m]	0.5µg/ml	0.5µg/ml	1mM			
insulin[m]	5µg/ml	5µg/ml	5µg/ml			
PMA[i,m]	8nM	16nM	85nM		16nM	16nM
retinol[m]			0.1µM			
transferrin[m]	5µg/ml		5µg/ml			
antibiotics				✓	✓	✓

Table 3.1: Important media conditions that have been used to propagate and serial culture normal (**a**) and vitiligo (**b**) melanocytes are illustrated.
[a] Puri *et al.* (1987) used this media in an early attempt to culture vitiligo melanocytes.
[b] The concentration of BPE for this hormone supplemented medium was taken from Wilkins *et al.* (1985). FCS was added only for the initial isolation period and during passage.
[c] The $FIB_{41}B$ medium was conditioned by fibroblasts and melanocytes were cultured in this medium following the initial 24-48 hours in a calcium and magnesium free $FIB_{41}B$ medium supplemented with chelated FCS.
[d] Kaufmann *et al.* (1998) used a similar medium to culture vitiligo melanocytes for the treatment of vitiligo patients (see **Table 5.1**).
[e] The basal W489 medium was a 4:1 mix of MCDB153 and L15 media. Although this experimental medium could not propagate the culture of melanocytes from freshly isolated epidermal cells, once melanocytes were established, proliferation was good.
[f] The BPE component could be replaced by 10ng/ml bFGF for successful melanocyte growth. Schallreuter-Wood *et al.* (1996) used a similar medium to this to culture vitiligo melanocytes.
[g] The MGM basal medium was a mix of MCDB153 and Iscoves's modified Dulbecco's medium (IMDM). Bessou *et al.* (1997a) used this medium to culture normal and vitiligo melanocytes.
[h] The MM-4 basal medium was specially formulated to replace MCDB153 basal medium. The BPE component could be replace by 5ng/ml b-FGF with heparin.
[i] The abbreviations are basic fibroblast growth factor (bFGF), bovine pituitary extract (BPE), dibutryl cAMP (dbcAMP), endothelial cell growth supplement (ECGS), epidermal growth factor (EGF), Eagle's modified essential medium (EMEM), epithelial growth factor (ETGF), foetal calf/bovine serum (FCS/FBS), horse serum with spermidine, spermine and putrescine (HSSP), 3-isobutyl-1-methylxanthine (IBMX), α-melanocyte stimulatng hormone (α-MSH), newborn calf serum (NCS), phorbol 12-myristate 13-acetate (PMA), triiodothyronine (T/T).
[j] Supplements which are serum or pituitary.
[k] Supplements which are cAMP elevating agents.
[l] Supplements which are antioxidants. Catalase was added only for the initial isolation period.
[m] Supplements which are growth factors, mitogens, or survival agents.
[n] Ethanolamine was added with phosphoethanolamine; both at a final concentration of 0.1mM.
[o] First medium in which successful growth of vitiligo melanocytes was reported. Zachariae *et al.* (1993) used a similar method (see **Table 5.1**) for growing melanocytes to treat patients with vitiligo.
[p] This medium could not stimulate the proliferation of melanocytes from patients with generalised vitiligo.
[q] This medium was used to culture melanocytes to treat patients with vitiligo (see **Table 5.1**). PC-1 medium for the growth of normal melanocytes was described by Halaban *et al.* (1987).

melanocytes were cultured in a mixed basal medium of MCDB153 and L15 supplemented with insulin, PMA and bFGF (**Table 3.1a**). While this medium was very mitogenic in the presence of various cAMP elevating agents, in particular α-MSH, it was unable to propagate melanocytes from freshly isolated epidermal suspensions unless EGF, BPE and foetal bovine serum (FBS) were used instead of bFGF and α-MSH. Donatien *et al.* (1993) described a medium that did not require artifical mitogens such as PMA or CT, while Ikeda *et al.* (1994b) used a medium that would retain melanocyte physiological properties similar to the *in vivo* situation during long-term culture including dendricity. bFGF could replace the BPE component in this medium in the presence of heparin, while the addition of hepatocyte growth factor (HGF) increased the mitogenic capacity of this medium. Swope *et al.* (1995) recently described the long term culture of melanocytes in a low serum MCDB153 medium containing the physiological mitogens, endothelin-1, bFGF and α-MSH. Endothelin-1 and α-MSH replaced the requirements for pituitary extract and phorbol esters (**Table 3.1a**)

Medrano & Nordlund (1990) first described the successful growth of vitiligo melanocytes from the uninvolved skin of patients with vitiligo, using an MCDB153 based medium (as described in **Table 3.1b**) following initial attempts by Puri *et al.* (1987). Catalase was apparently the vital component in the isolation and early stages of these melanocyte cultures. Several groups now culture melanocytes using a wide range of media (Im *et al*, 1992; Jee *et al*, 1993; Olsson & Juhlin, 1993; Zachariae *et al*, 1993; Schallreuter-Wood *et al*, 1996; Le Poole *et al*, 1997; Maresca *et al*, 1997; Kaufmann *et al*, 1998) (described in **Table 3.1b**).

It is difficult to examine melanocyte biology and compare results because of the wide range of media used, and the difference in melanocytes used. Eisinger & Marko (1982) first recognised that foreskin and skin melanocytes behaved differently in their culture media, and Abdel-Malek *et al.* (1994a) also reported that age and anatomical site influenced the growth of melanocytes. In the current study, adult non-foreskin melanocytes were used to compare normal and vitiligo melanocytes. Three main experimental media were used, referred to as media 1, 10 and 24 (refer to **Table 2.2** for further details) which varied in their mitogenic composition, to investigate the effects of ECM proteins (both individual and cell-derived) and UVR on melanocyte morphology,

proliferation and pigmentation. A much more comprehensive survey of media conditions was undertaken to try to establish experimental conditions under which a reproducible pigmentary response to α-MSH could be demonstrated in human melanocytes.

Pigmentation of melanocytes was also investigated by returning melanocytes to a 3-dimensional environment which mimicked several aspects of normal skin. The development of epidermal/dermal reconstructs (also described as skin composites) is very important with respect to learning about the interactions of melanocytes with keratinocytes in a more "natural" environment. Such composites may also serve a greater purpose as models for the study of various diseases. The behaviour of naevocytes (Cooper *et al*, 1991; Bessou *et al*, 1997b), melanoma cells (Bizik *et al*, 1997; personal communication, S Mac Neil, 1998) and vitiligo keratinocytes and melanocytes (Bessou *et al*, 1997a) are already underway. Additionally, the use of these skin equivalents may provide vital treatment for burns patients (Boyce *et al*, 1995; Harriger *et al*, 1995). Recently the addition of Langerhans' cells was described (Regniér *et al*, 1997) and endothelial cells have been added to composites within this laboratory (personal communication, S Mac Neil, 1998). The preliminary study presented in this chapter examined the use of an epidermal/dermal reconstruct developed in Dr S Mac Neil's laboratory as a model in which to examine pigmentation and monitor the addition of extra melanocytes with respect to future experimentation with vitiligo cells. Pigmentation in response to UVB has been widely studied in these models (Todd *et al*, 1993; Bessou *et al*, 1995 & 1996; Nakazawa *et al*, 1997) but the effect of other pigmentary stimuli appears to have been reported in only one study (Bessou *et al*, 1997b). With the recent interest in α-MSH as a pigmentary hormone in humans (Hunt *et al*, 1994a, b &c; Abdel-Malek *et al*, 1995; McLeod *et al*, 1995; Maeda *et al*, 1996) and the recent finding of elevated levels of α-, β- and γ-MSH in the epidermis of vitiligo lesions (Liu *et al*, 1995), it was decided that α-MSH should be tested in the epidermal/dermal reconstruct model.

3.2. MODULATION OF THE MORPHOLOGY OF NORMAL AND VITILIGO MELANOCYTES.

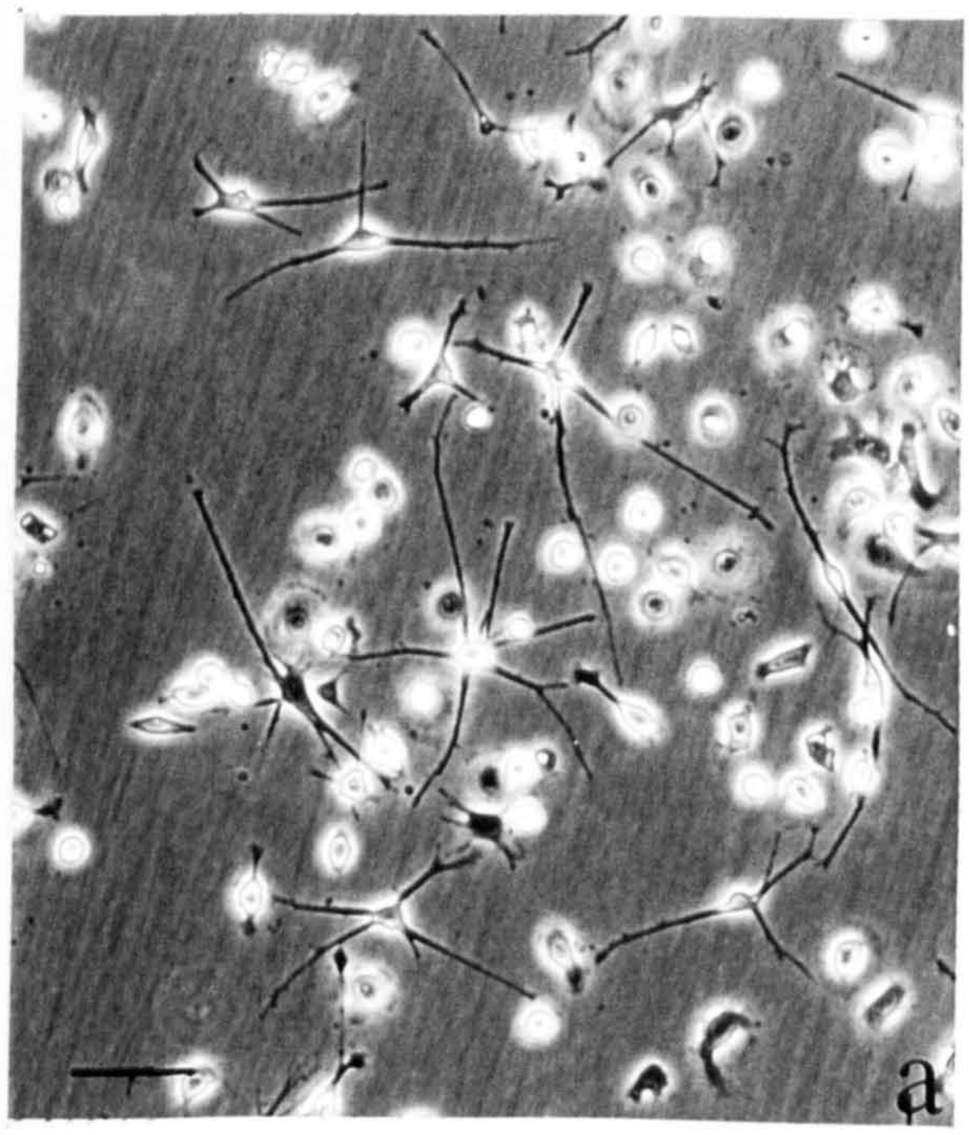
In vivo melanocytes interact with keratinocytes to form a tightly regulated pigmentary unit. The melanocytes are generally non-proliferative and are highly dendritic. Taken out of their natural environment, the study of melanocyte function becomes difficult as the cells require several mitogenic drives to survive and/or proliferate. In this section, the influence of commonly used non-physiological mitogens and serum/pituitary extracts were examined in addition to the effects of UVR and other factors on melanocyte morphology. In **Sections 3.3** and **3.4** experiments which examined proliferation and pigmentation in response to these factors are described.

3.2.1. Effect of media on normal and vitiligo melanocyte morphology.

When melanocytes were initially isolated in either of the three maintenance media, they were small, with many dendritic (pin thin) processes (**Figure 3.1a-d**). As melanocytes were further cultured, the melanocytes would become longer and larger with cells exhibiting 2 to 3 dendrites (described as bipolar and tripolar) predominating as colonies (**Figure 3.1e**). Some very dendritic cells would remain (**Figure 3.1f**). Generally by P1 and onwards, cells were less dendritic. This was essentially the same for both normal and vitiligo melanocytes (**Figures 3.2a-b** and **3.2f-g** illustrating P3 melanocytes from M577 and VM4 respectively). VM2 melanocytes however retained a large population of very large multidendritic cells throughout their culture period (**Figure 3.3a-f**). Melanocytes from VM5 and VM3 (from the same vitiligo patient, **Figures 3.3g-k**) are also shown to illustrate melanocyte morphology. VM3 at P3 (**Figures 3.3j-k**) were large and quite multidendritic and demonstrated a different morphology to melanocytes from normal donor M577 at P3 (**Figure 3.3l**) which were more bipolar and shorter. Therefore, donor-to-donor variations were seen.

Three experimental media (1, 10 or 24) were commonly used throughout the thesis. The morphology obtained in these media after 5 days of culture for normal and vitiligo melanocytes are illustrated in **Figure 3.2** (summary of morphology in all media variants in **Table 3.2**). In medium 1, cells exhibited a bi/tripolar morphology with elliptical

Figure 3.1 Morphological appearance of normal melanocytes in culture medium. Normal melanocytes were isolated (as described in **Section 2.1.1**) and plated into T25 flasks in maintenance medium C. Melanocytes from donor M983 were photographed at the first (**a-b**) and second change of medium (**c-d**) (3 and 6 days after seeding respectively) while melanocytes from donor M969 were photographed at 1 month after seeding (**e-f**). Melanocytes also exhibited these characteristics when isolated in maintenance media A and B (**Table 2.1** details maintenance media compositions). Scale bars represent 30μm (**a, c** and **e-f**) and 15μm (**b** and **d**).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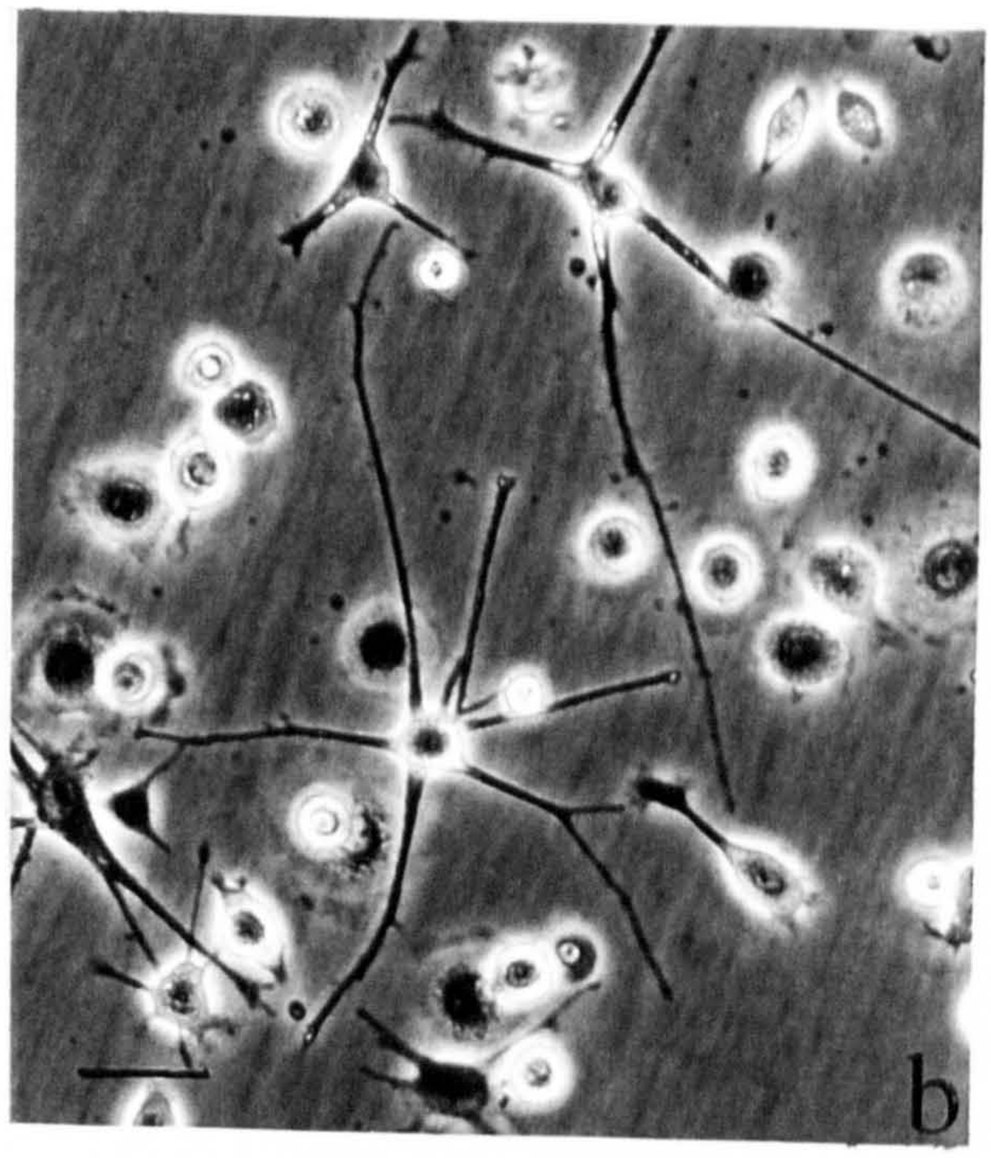
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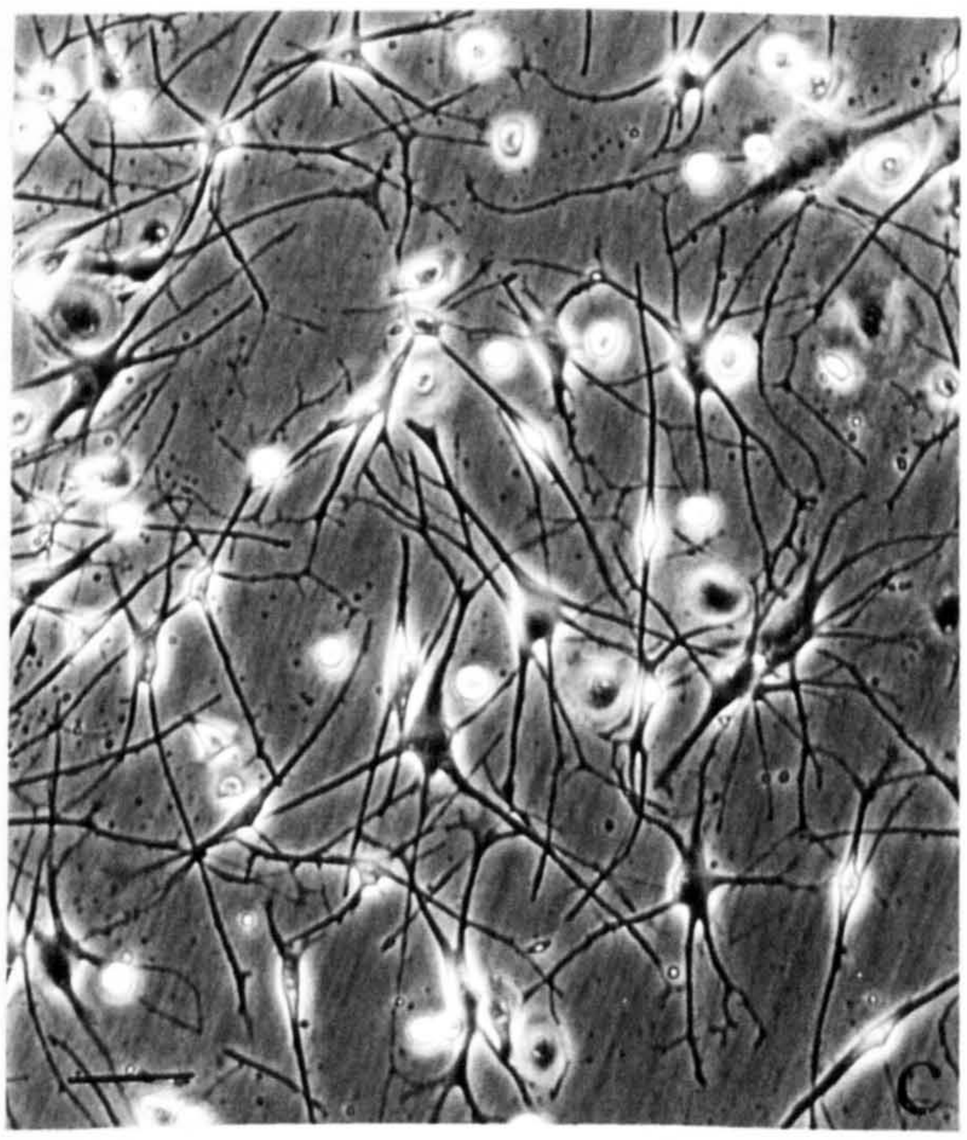
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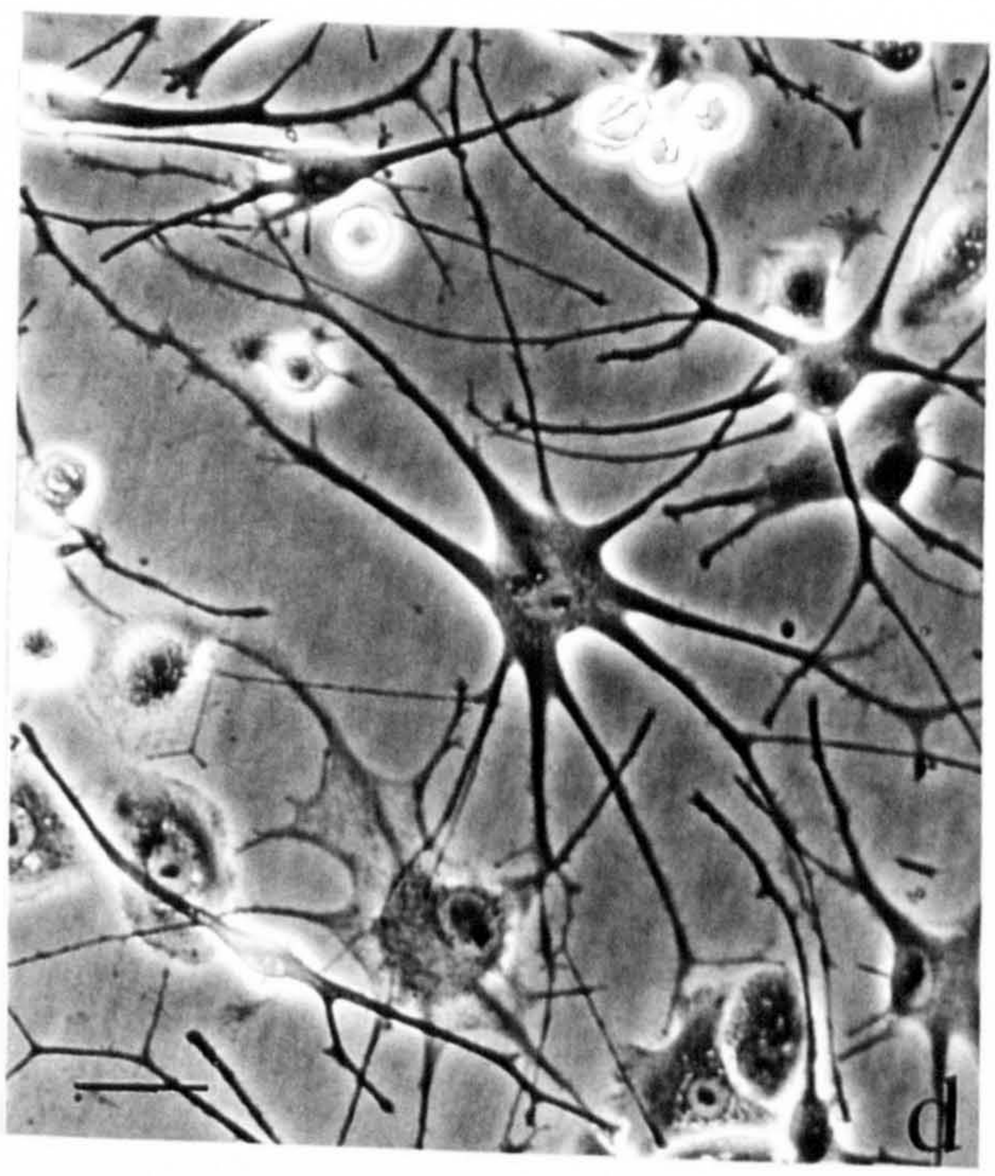
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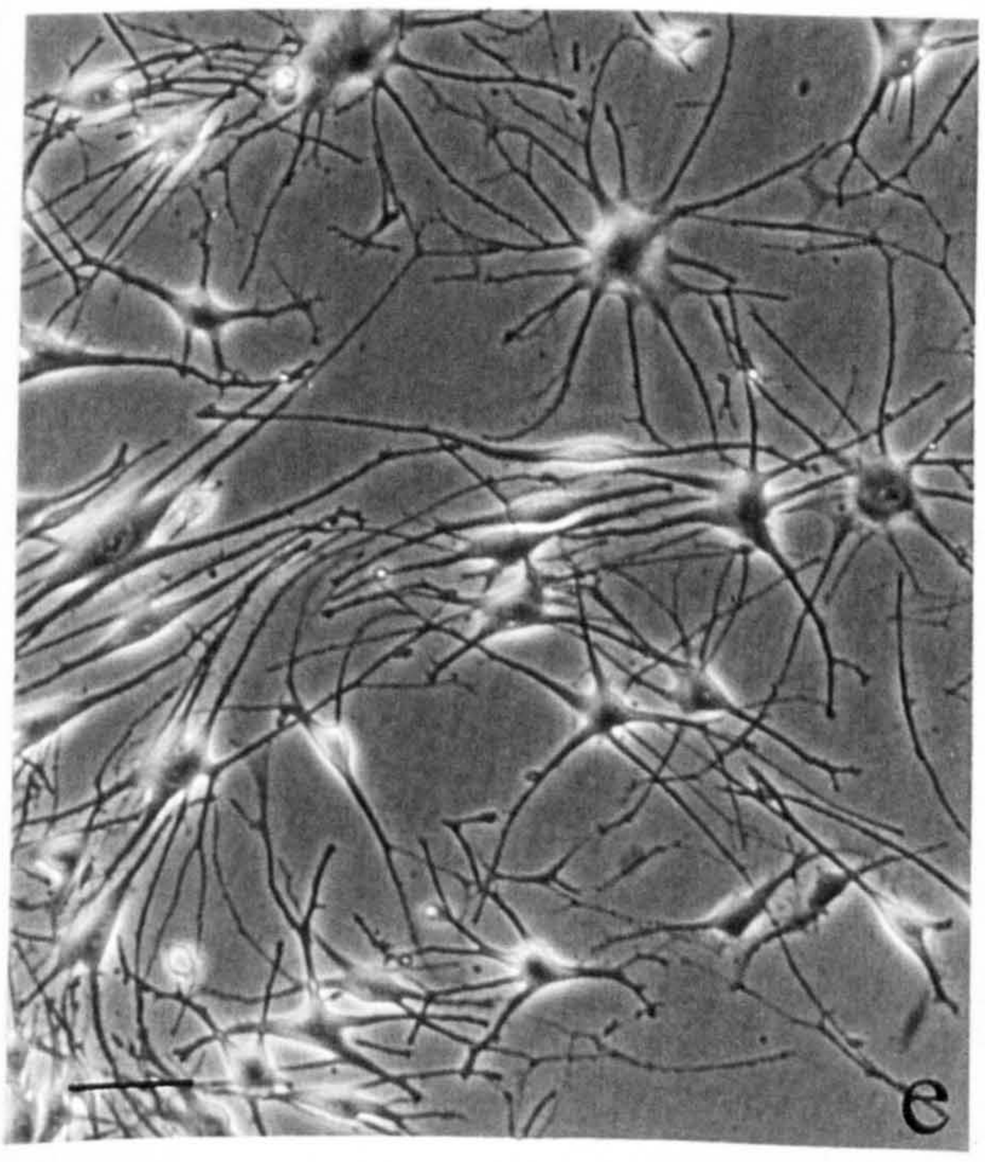
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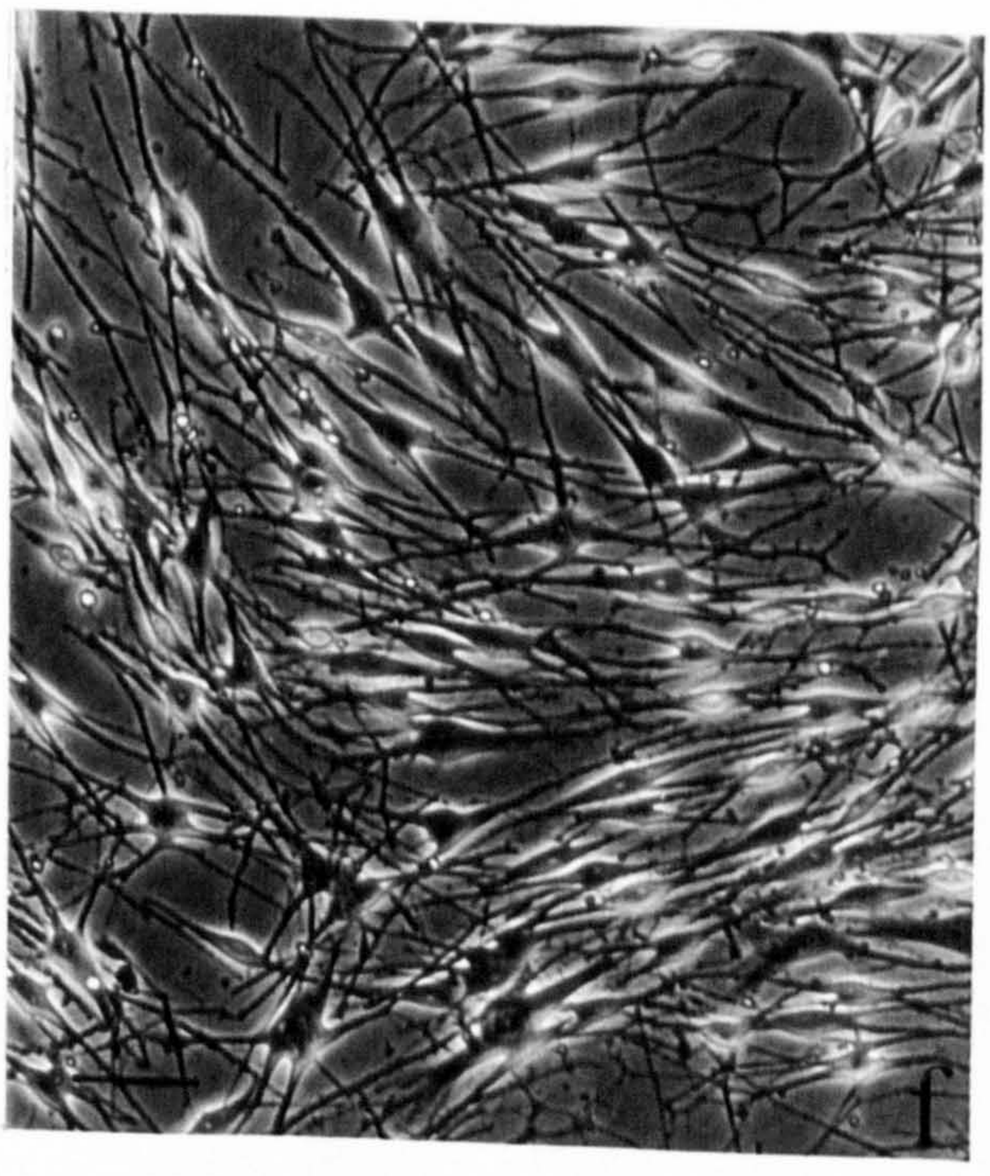
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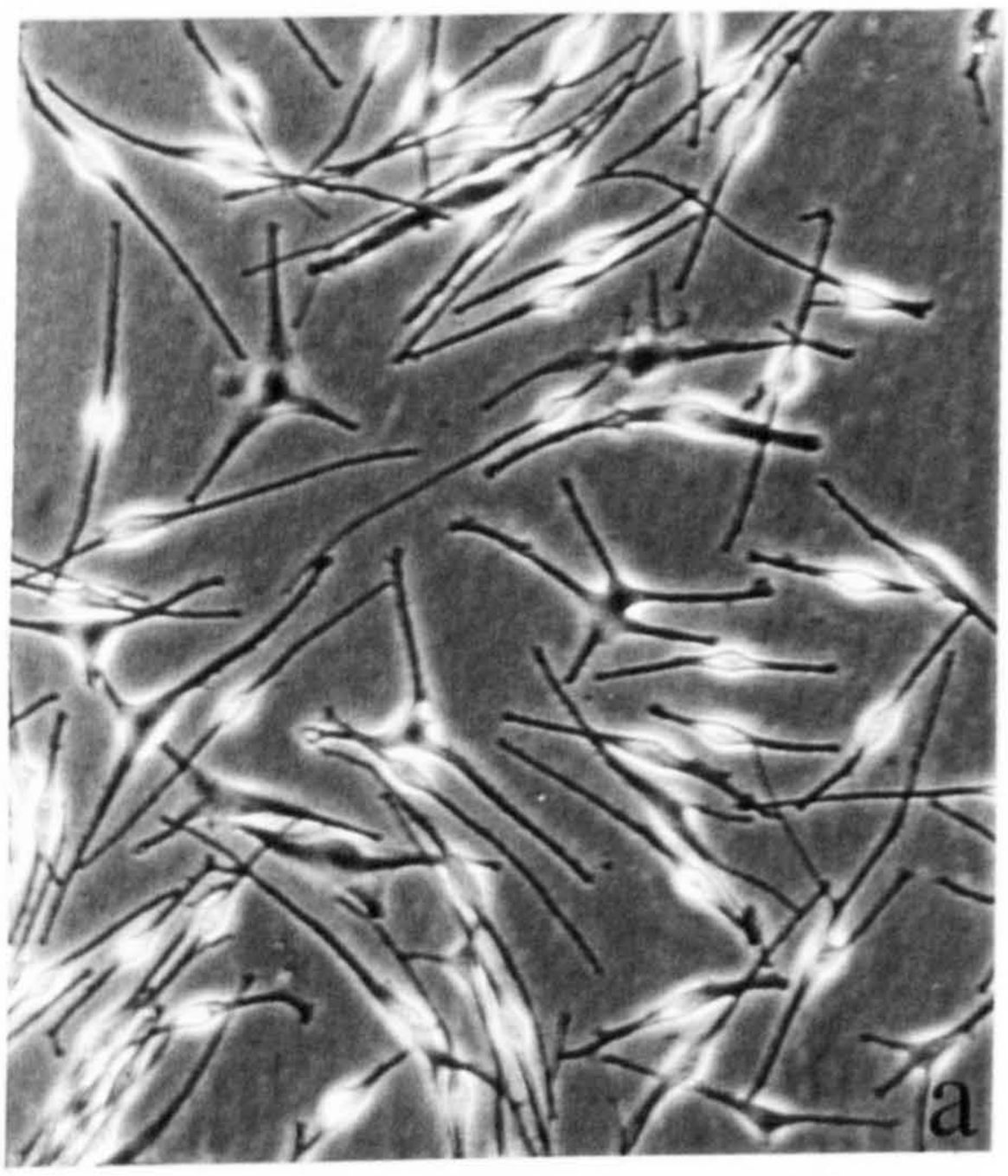
Figure 3.2 Comparison of normal and vitiligo melanocytes at P3 and P4 under 4 different media variants. Melanocytes from normal donor M843 (**a-e**) were isolated and cultured on plastic in maintenance medium C (as described in **Section 2.1.1**) while melanocytes from vitiligo donor VM4 (**f-j**) were isolated and cultured in Green's medium on collagen I for the first week then swapped into maintenance medium C (as described in **Section 2.2** and **Table 2.3a**). From passage 1, VM4 melanocytes were cultured on plastic in maintenance medium C. Melanocytes from donor M843 (**a-b**; both photomicrographs taken at different points in the same T25 flask) and melanocytes from donor VM4 (**f-g**; both photomicrographs taken at different points in the same T25 flask) were paired at passage 3. Both cultures were then passaged, allowed to adhere for 24 hours in maintenance medium C before being cultured for a further 5 days in media 1, 10 or 24 (**c-e** respectively for M843, and **h-j** respectively for VM4) prior to photography. Scale bars represent 30μm.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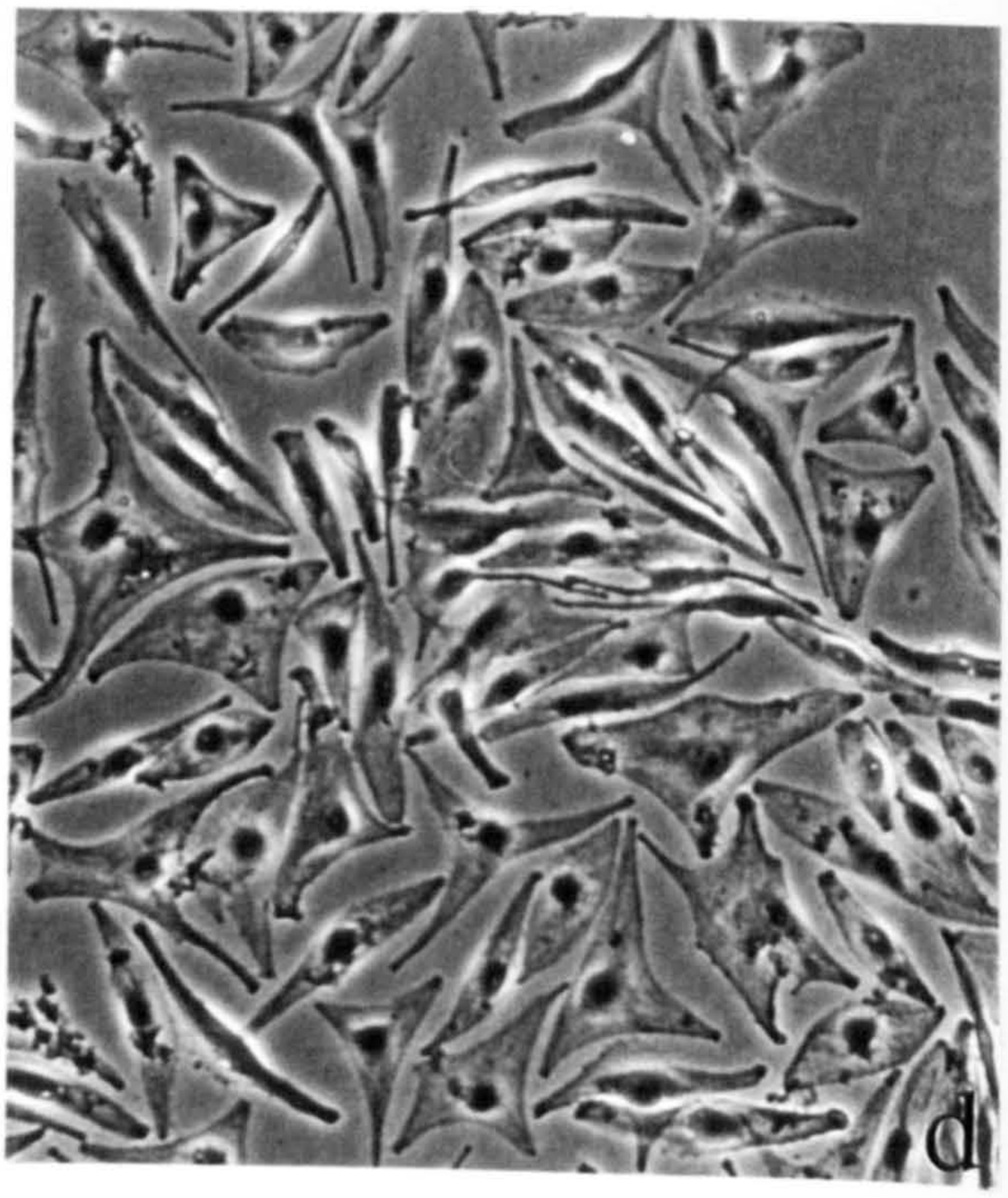
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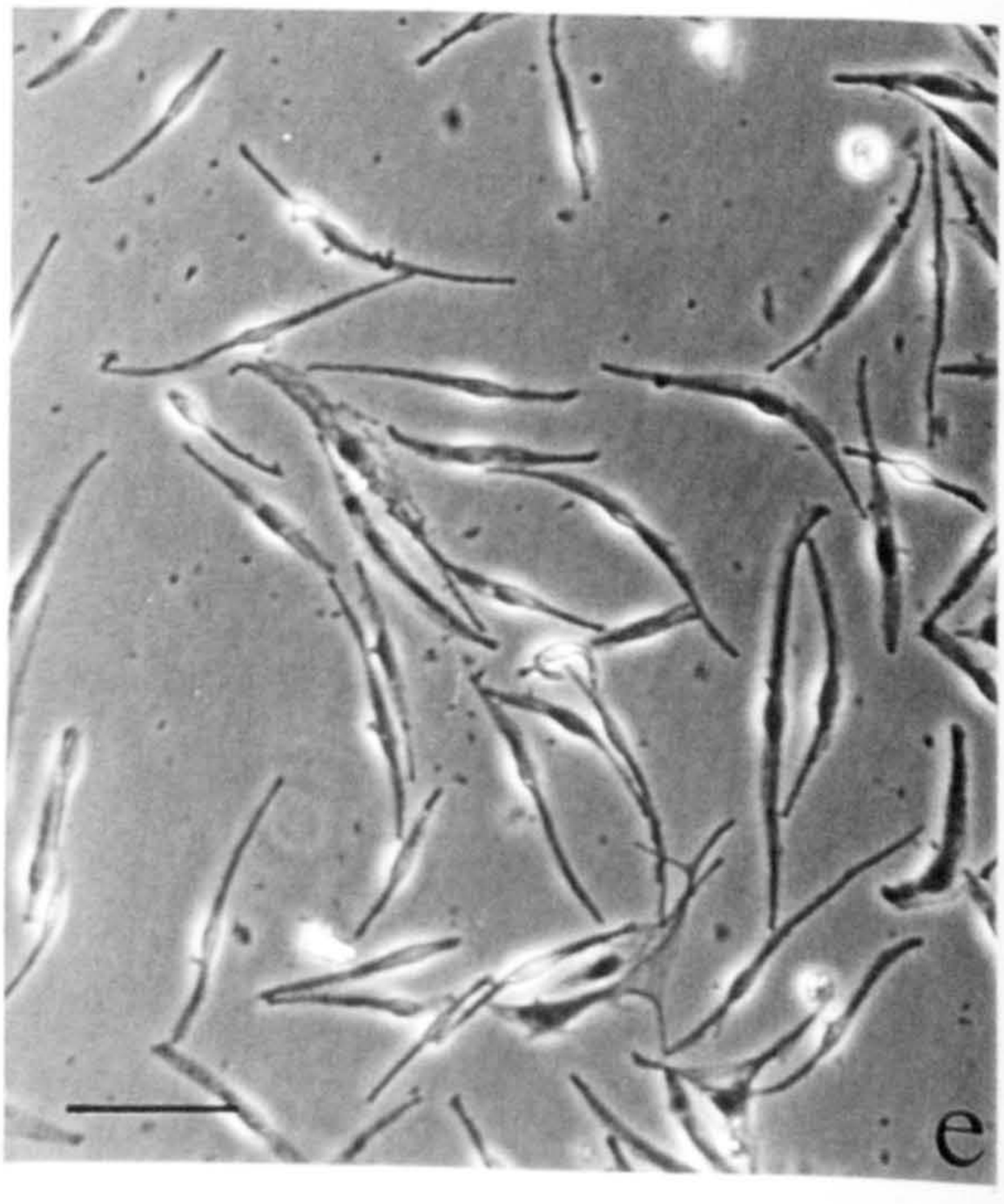
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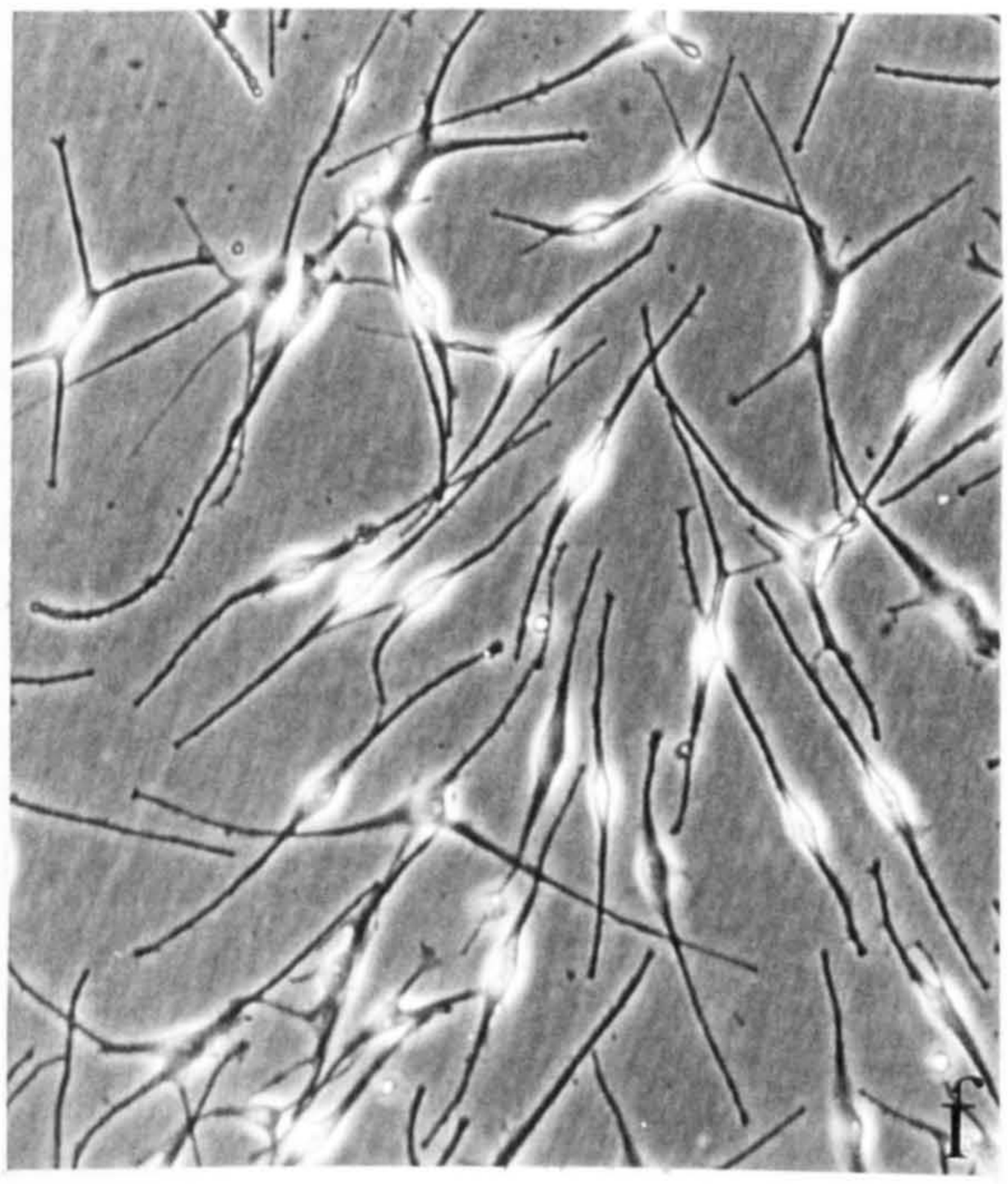
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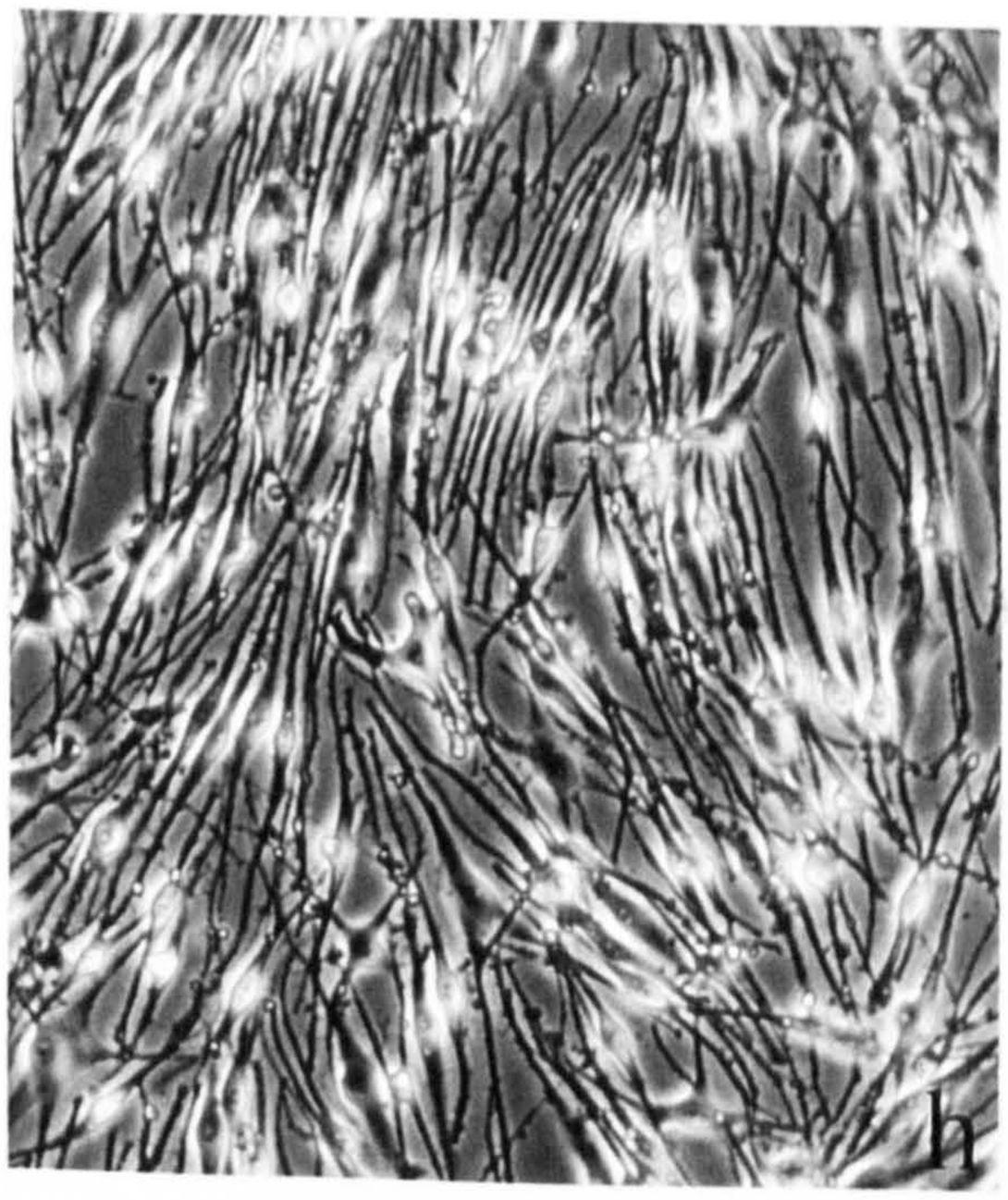
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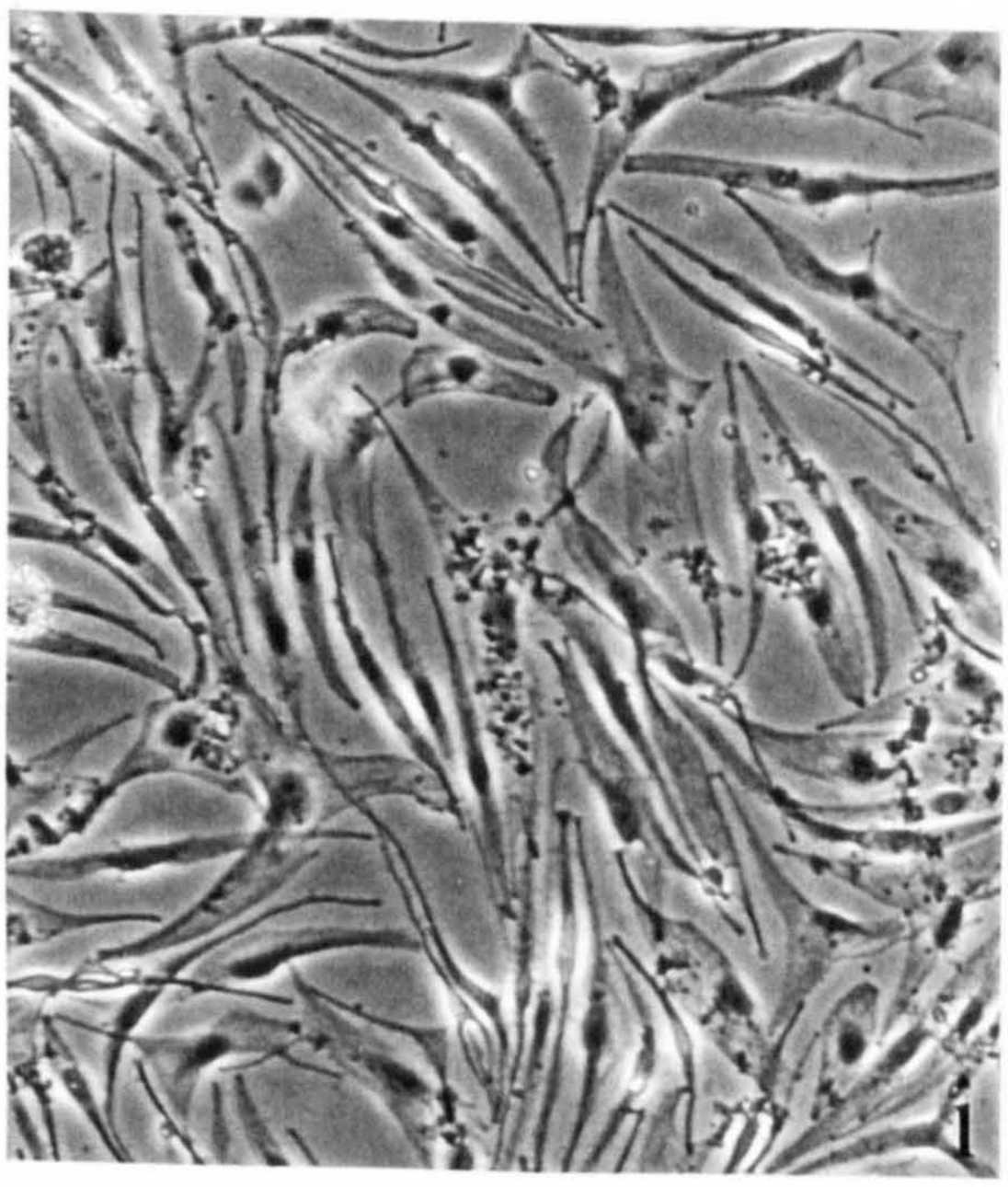
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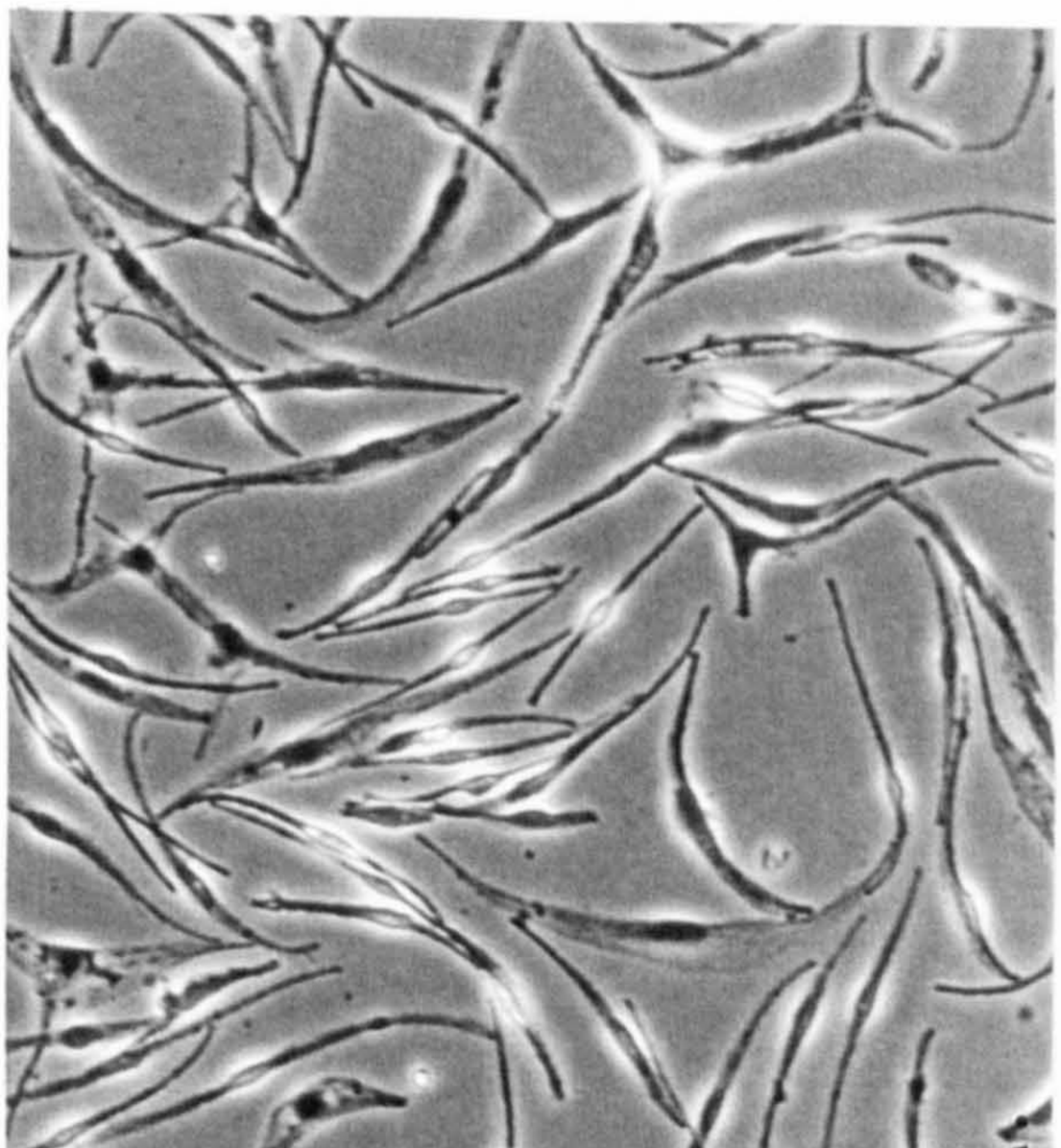
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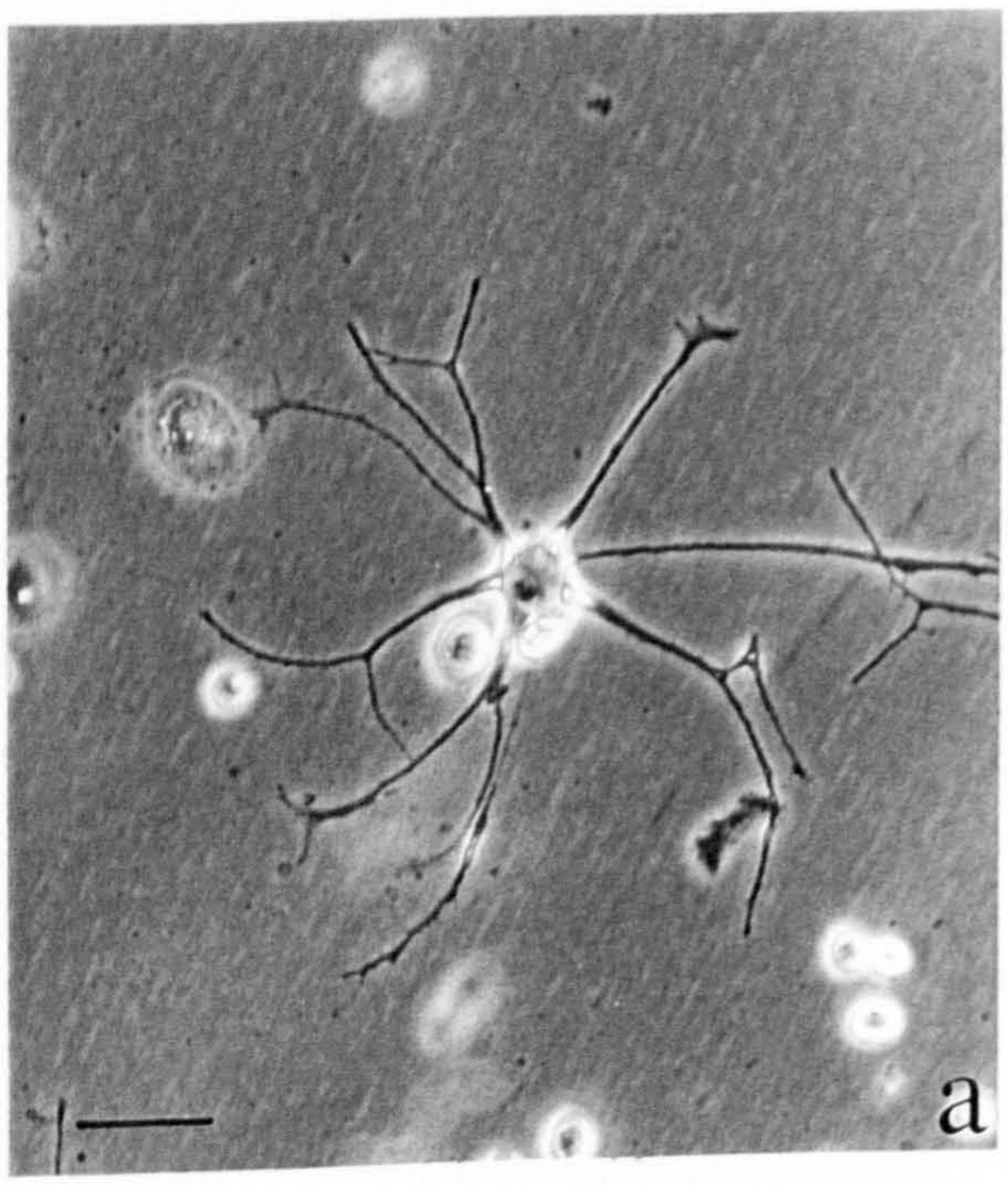
Figure 3.3 Morphological appearance of vitiligo melanocytes in culture medium. **a-f** illustrate the morphology of vitiligo melanocytes from donor VM2, while **g-k** illustrate the morphology of vitiligo melanocytes from 2 biopies (VM3 and VM5) which were taken at different times from the same donor (see **Table 2.3a** for further details). VM2 melanocytes shown in this figure were initially cultured as a CEA sheet (as described in **Section 2.2.1**). The cells were then frozen at time of passage for 6 months, then plated onto plastic and cultured in maintenance medium B, before being cultured at later stages in maintenance medium C. VM2 melanocytes after 5 days in culture at P1 (**a-b**; both photomicrographs taken at different points in the same T25 flask), VM2 melanocytes after 21 days in culture at P1 (**c-d**; both photomicrographs taken at different points in the same T25 flask) and VM2 melanocytes at P3 (**e-f**; both photomicrographs taken at different points in the same T25 flask) are shown. VM5 melanocytes were seeded into Green's medium for the first few days and then transferred to maintenance medium C. Primary melanocytes from VM5 are illustrated after 5 and 21 days (**g** and **h** respectively) in culture. Melanocytes from VM5 at passage 2 are also illustrated (**i**). Melanocytes from biopsy VM3 (same donor as VM5 biopsy) were cultured on plastic in maintenance medium B and then maintenance medium C prior to experimentation. VM3 melanocytes at P3 (**j-k**) and melanocytes from normal donor M577 (which were used as comparative melanocytes to VM3) at P3 (**l**) are illustrated. Additional morphological prints of VM3 and M577 under experimental conditions are illustrated in **Figure 3.8**. Scale bars represent 30μm.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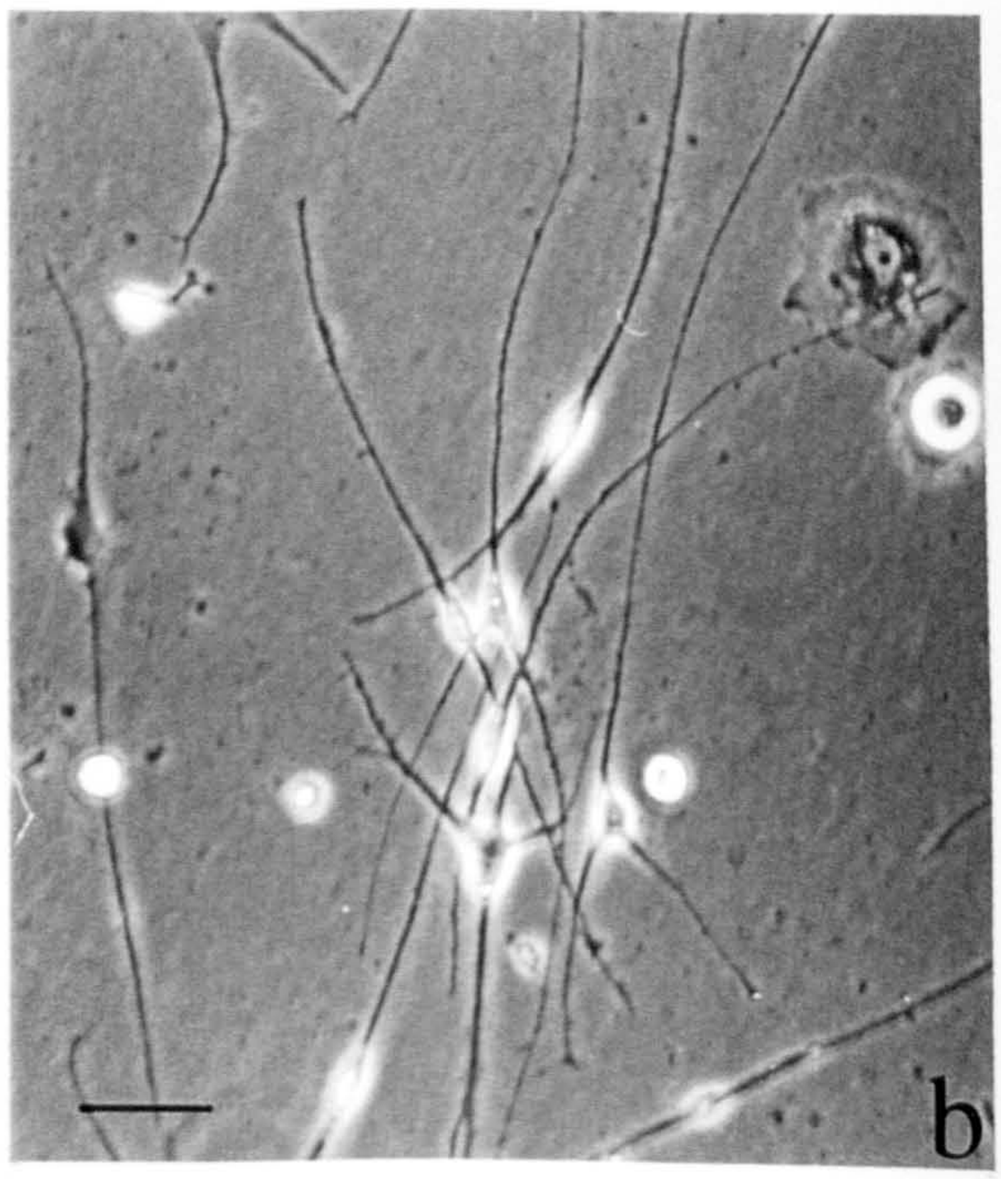
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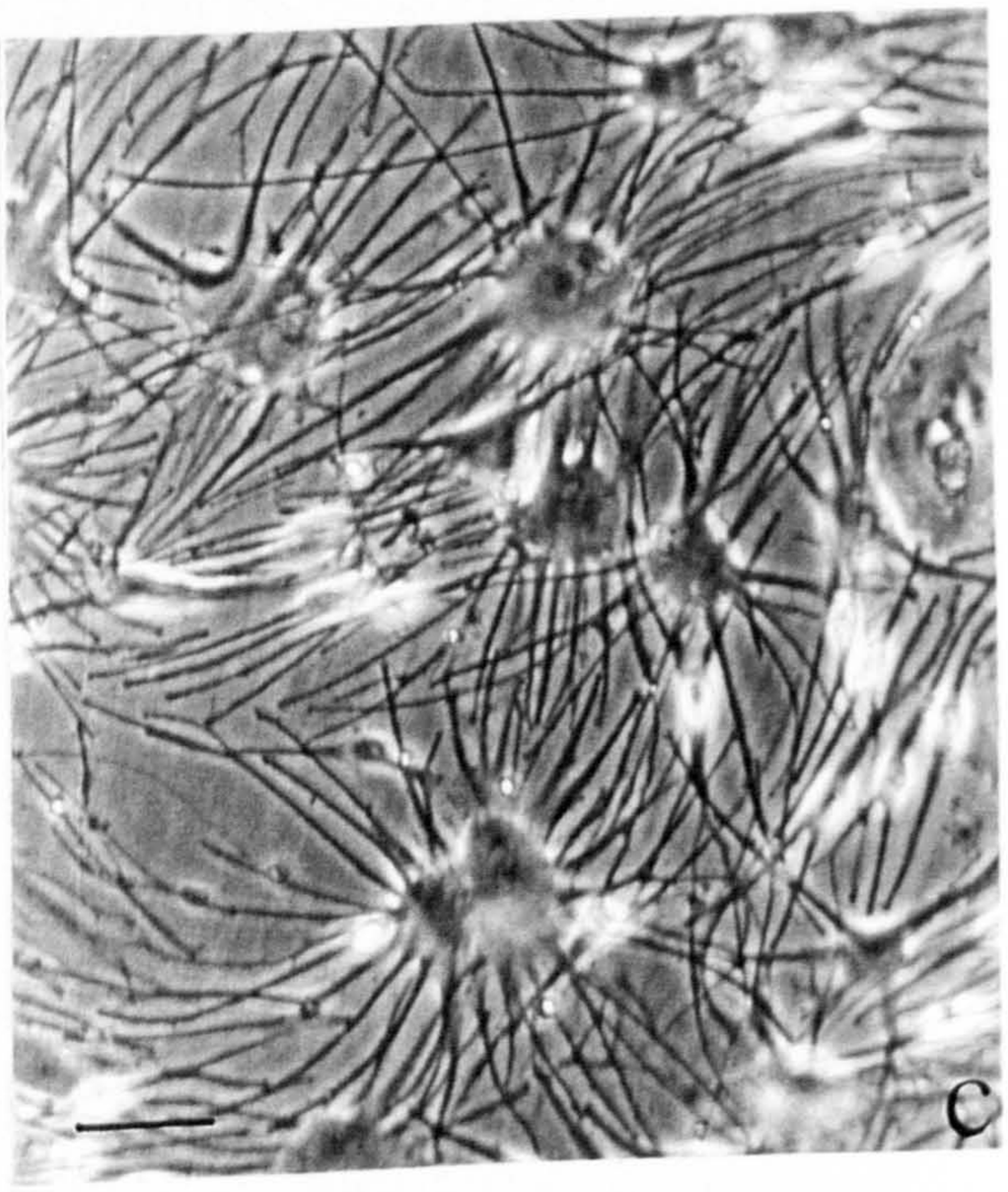
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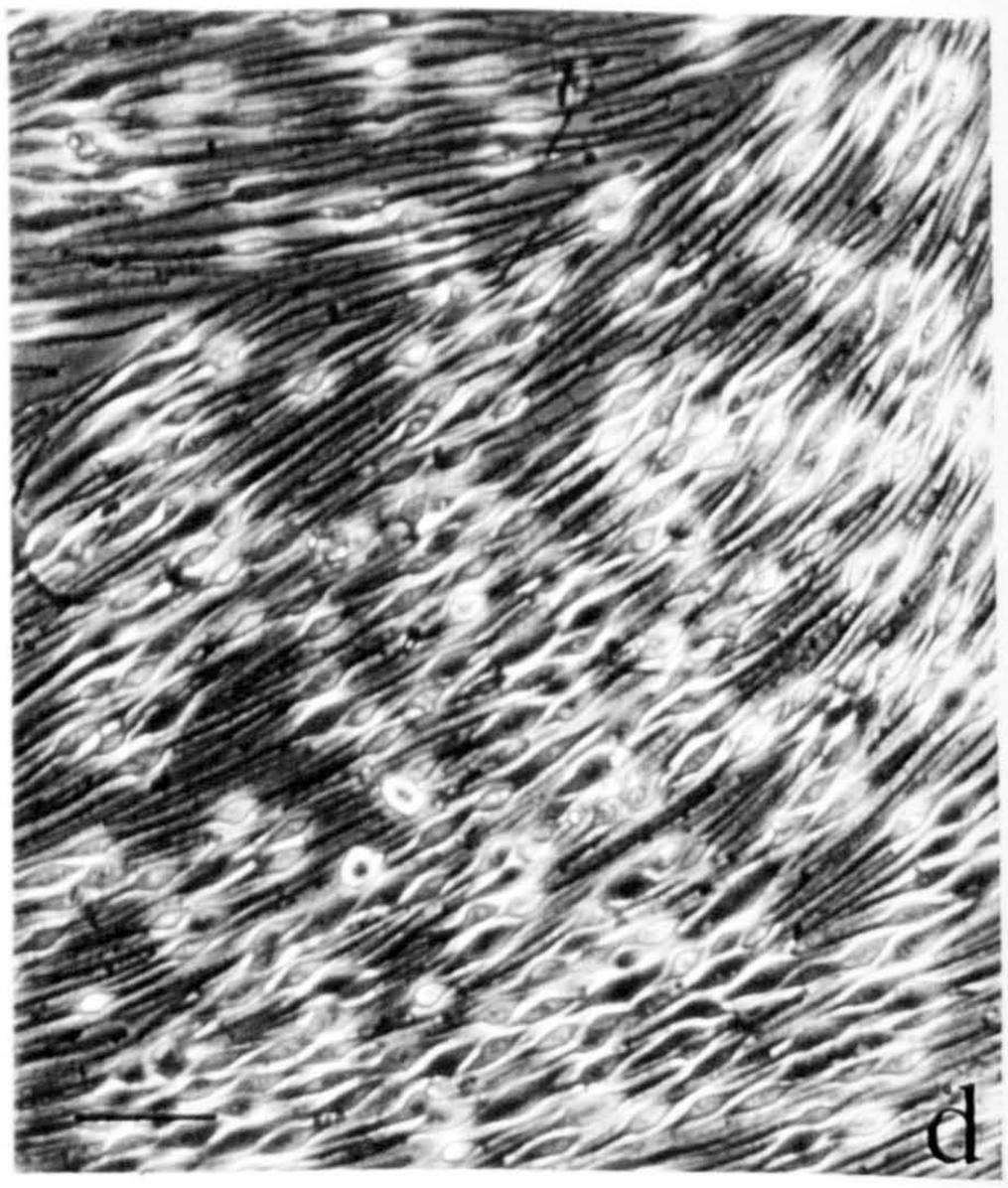
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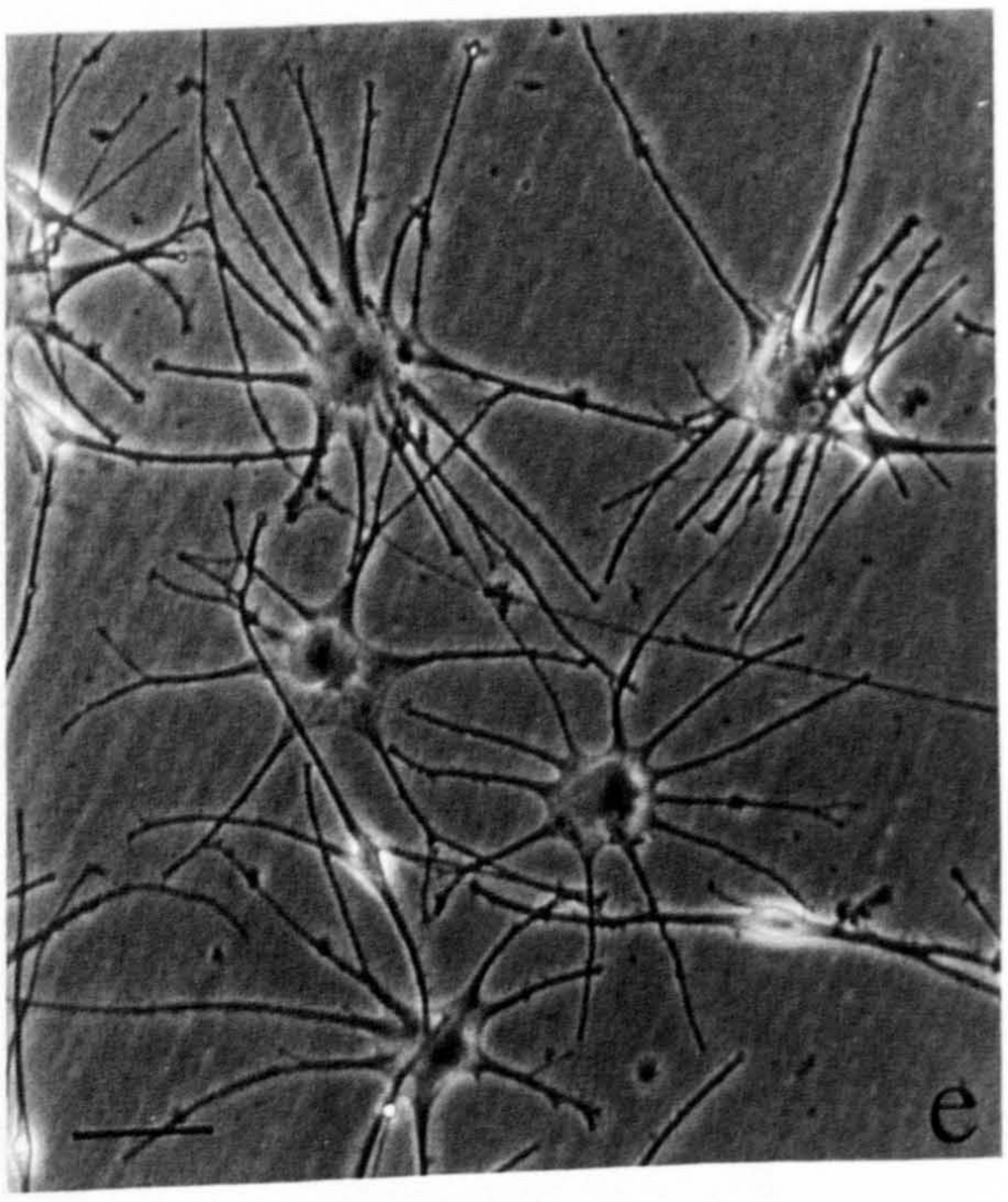
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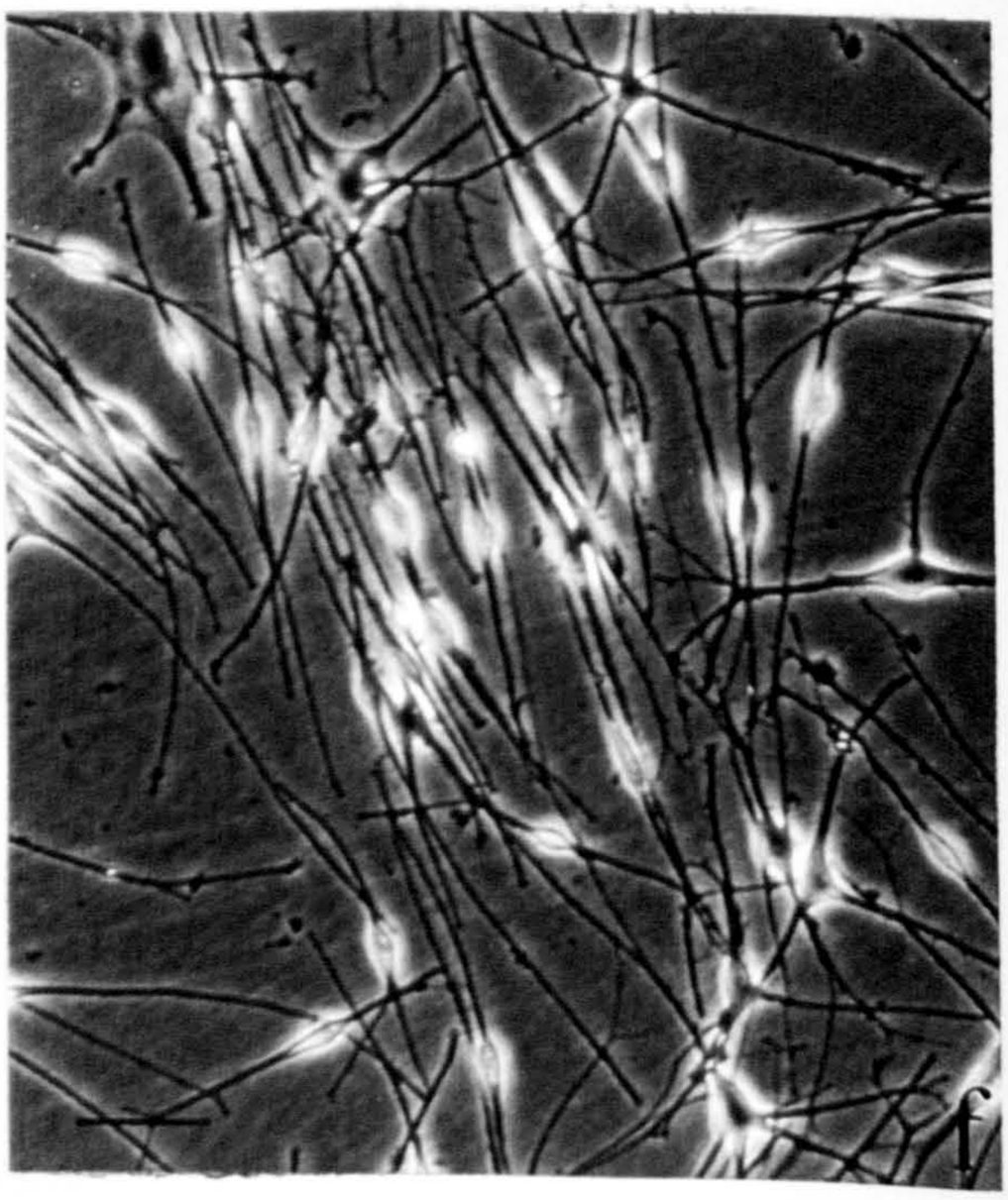
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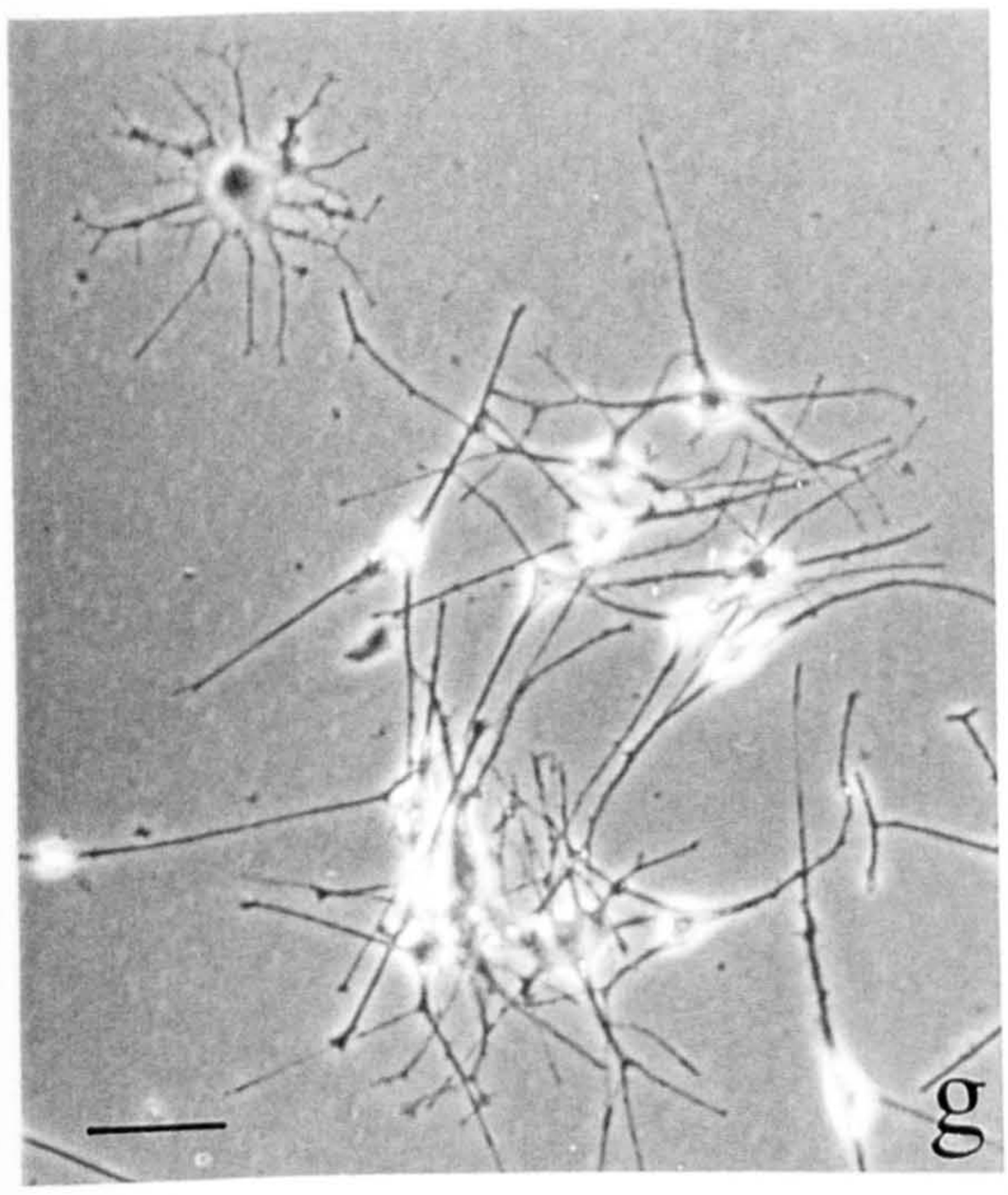
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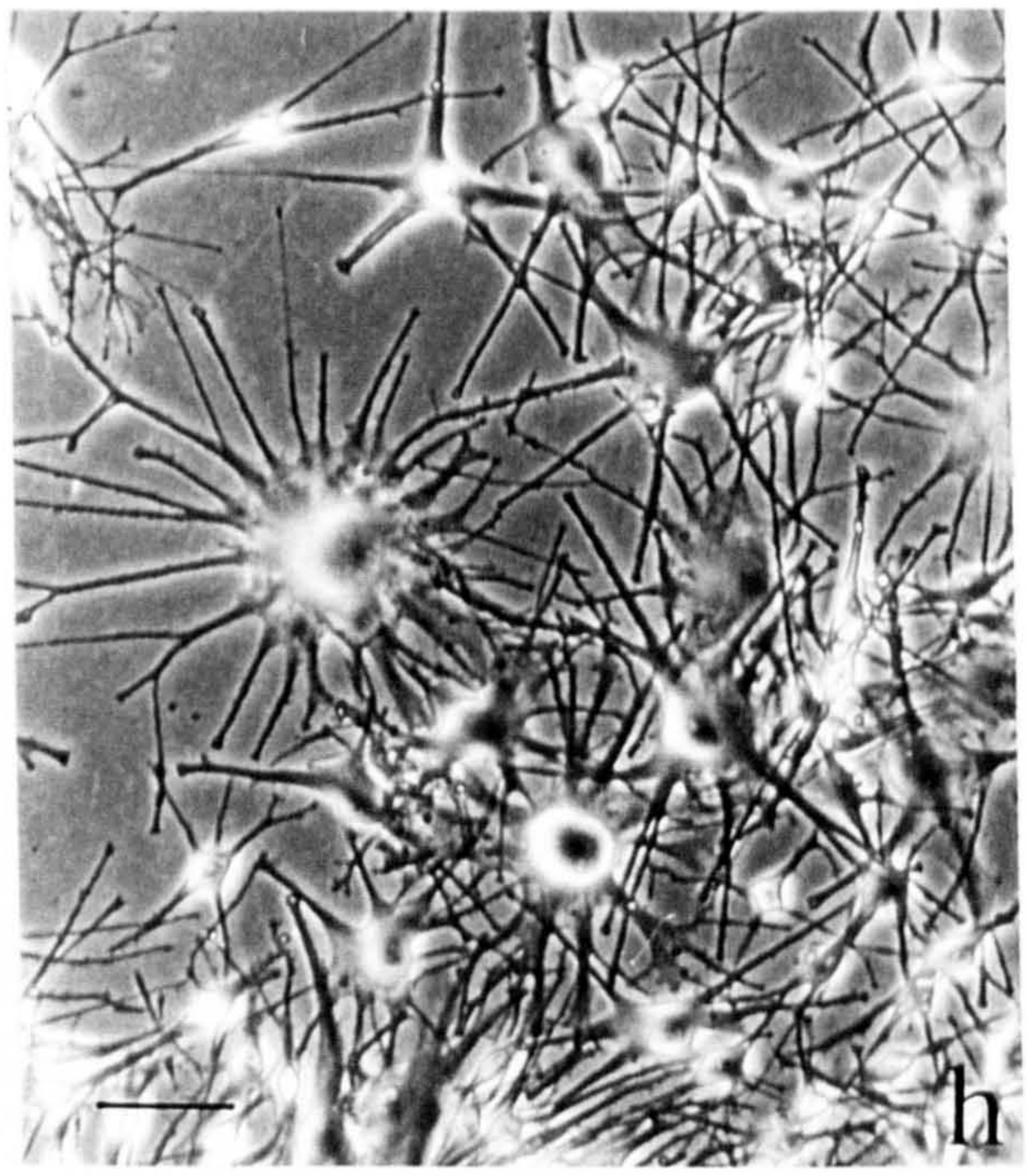
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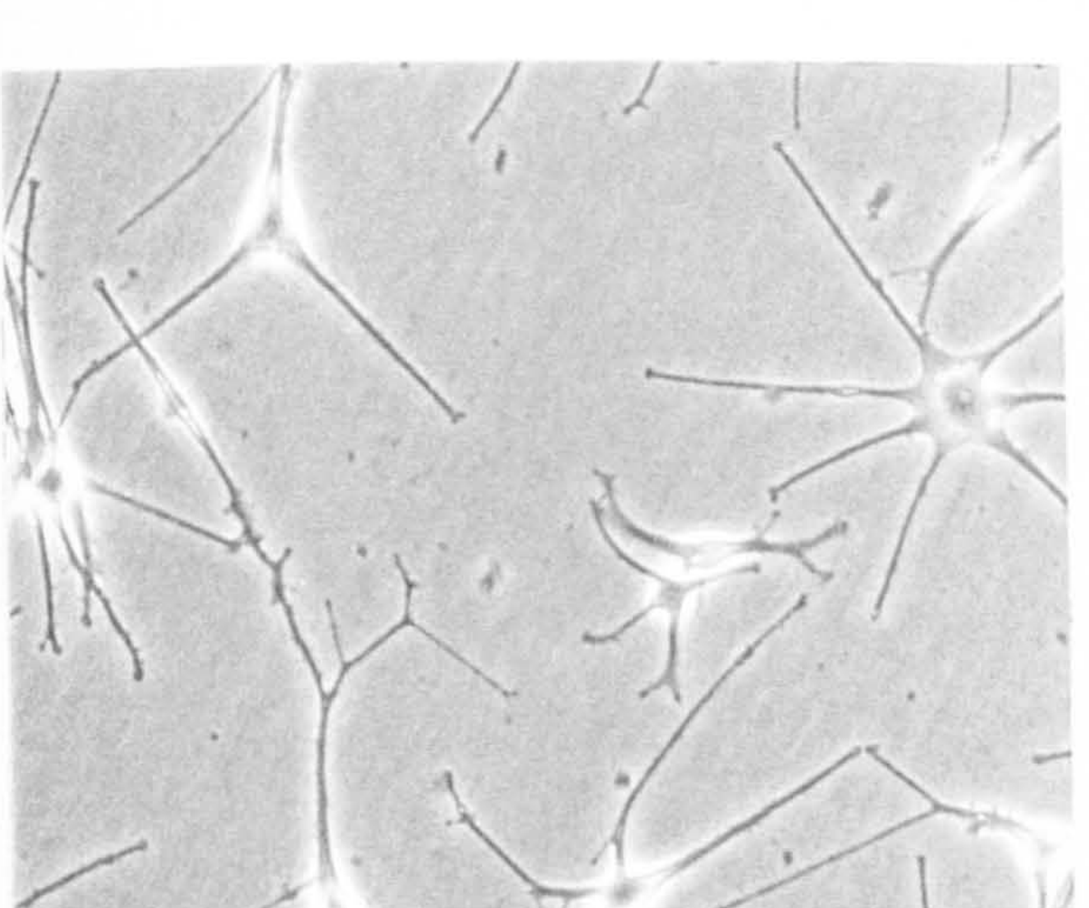
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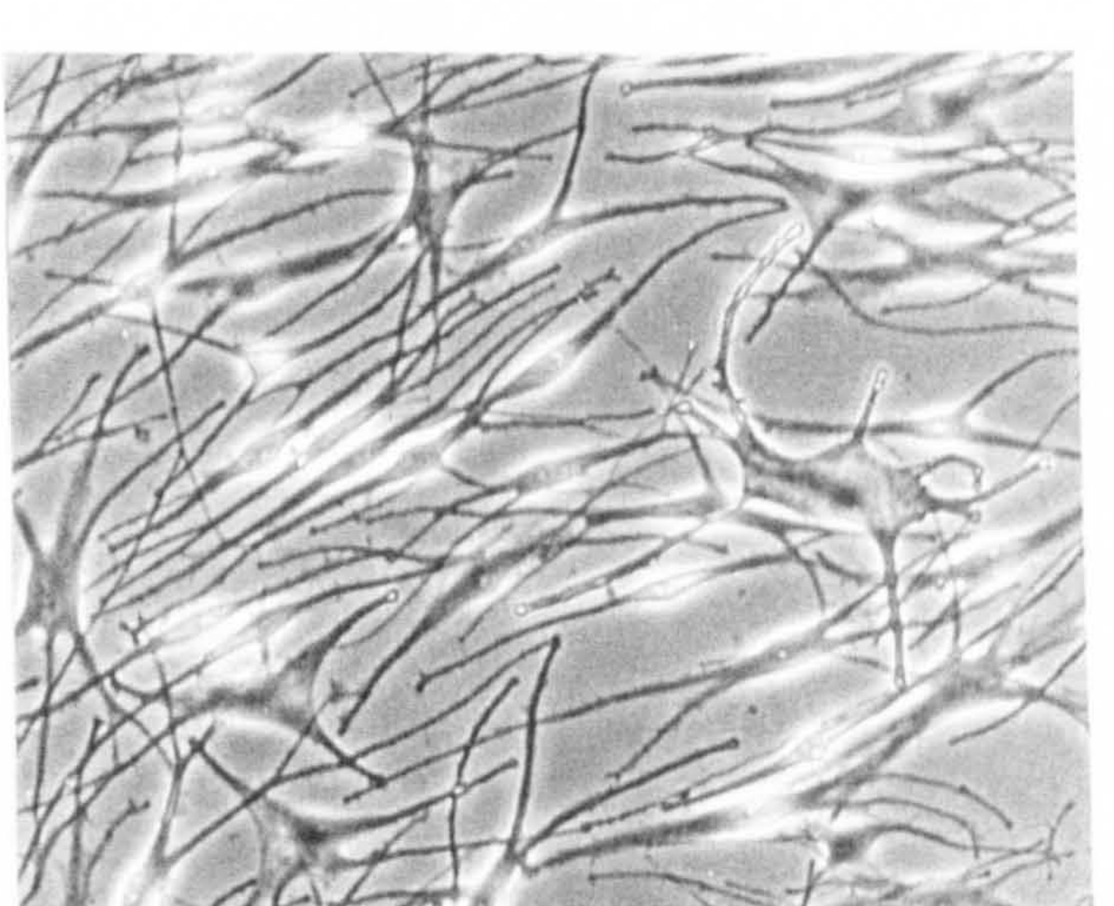
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Table 3.2 Influence of media composition on melanocyte morphology.

Media number	Mitogen composition[a]	Morphology	Dendrite No	Figure
1	FCS/BPE/PMA/CT	thin melanocytes, with long dendrites, glowing eliptical bodies	2-4	shown[b]
2	FCS/BPE/PMA/bFGF	similar to medium 1	2-4	**3.4a**
3	FCS/BPE/PMA	similar to medium 1	2-4	not shown
4	FCS/BPE/CT	epitheloid cells, dark central region	2-4	**3.4c**
5	FCS/BPE/bFGF	similar to medium 10	2-4	not shown
6	FCS/PMA/CT	similar to medium 13	2-4	not shown
7	FCS/PMA/bFGF	similar to medium 13	2-4	not shown
8	BPE/PMA/CT	similar to medium 1	4 or more	**3.5c**
9	BPE/PMA/bFGF	similar to medium 1	3-4	**3.4b**
10	FCS/BPE	similar to medium 4	2-4	shown[c]
11	FCS/CT	similar to medium 4	2-4	**3.4d**
12	FCS/bFGF	quite thin bipolar cells, bright cell bodies	2^{-3}	**3.4e**
13	FCS/PMA	thin bipolar cells	2^{-3}	**3.5e**
14	BPE/PMA	thin melanocytes, bright cell bodies	3 or more	**3.4f**
15	BPE/CT	large bodied cells, quite thin dendrites	3 or more	**3.4g**
16	BPE/bFGF	similar to medium 24	2^{-3}	not shown
17	PMA/CT	similar to medium 13	2^{-3}	not shown
18	PMA/bFGF	similar to medium 13	2^{-3}	not shown
19	FCS	similar to medium 24	2^{-3}	not shown
20	BPE	similar to medium 15	3 or more	not shown
21	PMA	similar to medium 13	2^{-3}	not shown
22	CT	mix of thin and slightly fattened cells	2-4/5	**3.4h**
23	bFGF	similar to medium 24	2^{-3}	not shown
24	no mitogens	slightly fattened bi/tripolar cells	2^{-3}	shown[d]
25	FCS/BPE/PMA/bFGF	similar to medium 1	2-4	not shown

Table 3.2: Melanocytes of various passages from normal and vitiligo donors were seeded into culture vessels (generally 24 or 12 well plates) in one of three maintenance media (A, B or C). Cells were allowed to adhere for 24 hours before cells were cultured for a further 5 days in one of the above media (composition of media also detailed in **Table 2.2**). Photomicrographs were taken and morphology under both melanocyte mainetenance media and experimental media are illustrated in **Figures 3.1-3.9** (indivdiual figures for experimental media are denoted). Generalized morphologies for the experimental media are listed in the above table. It should be noted that experimental media 1, 2 and 24 closely resemble maintenance media A, C, and B respectively and that morphologies obtained are all very similar.

[a] The mitogens used in these media are foetal calf serum (FCS), bovine pituitary extract (BPE), phorbol 12-myristate 13-acetate (PMA), cholera toxin (CT) and basic fibroblast growth factor (bFGF).

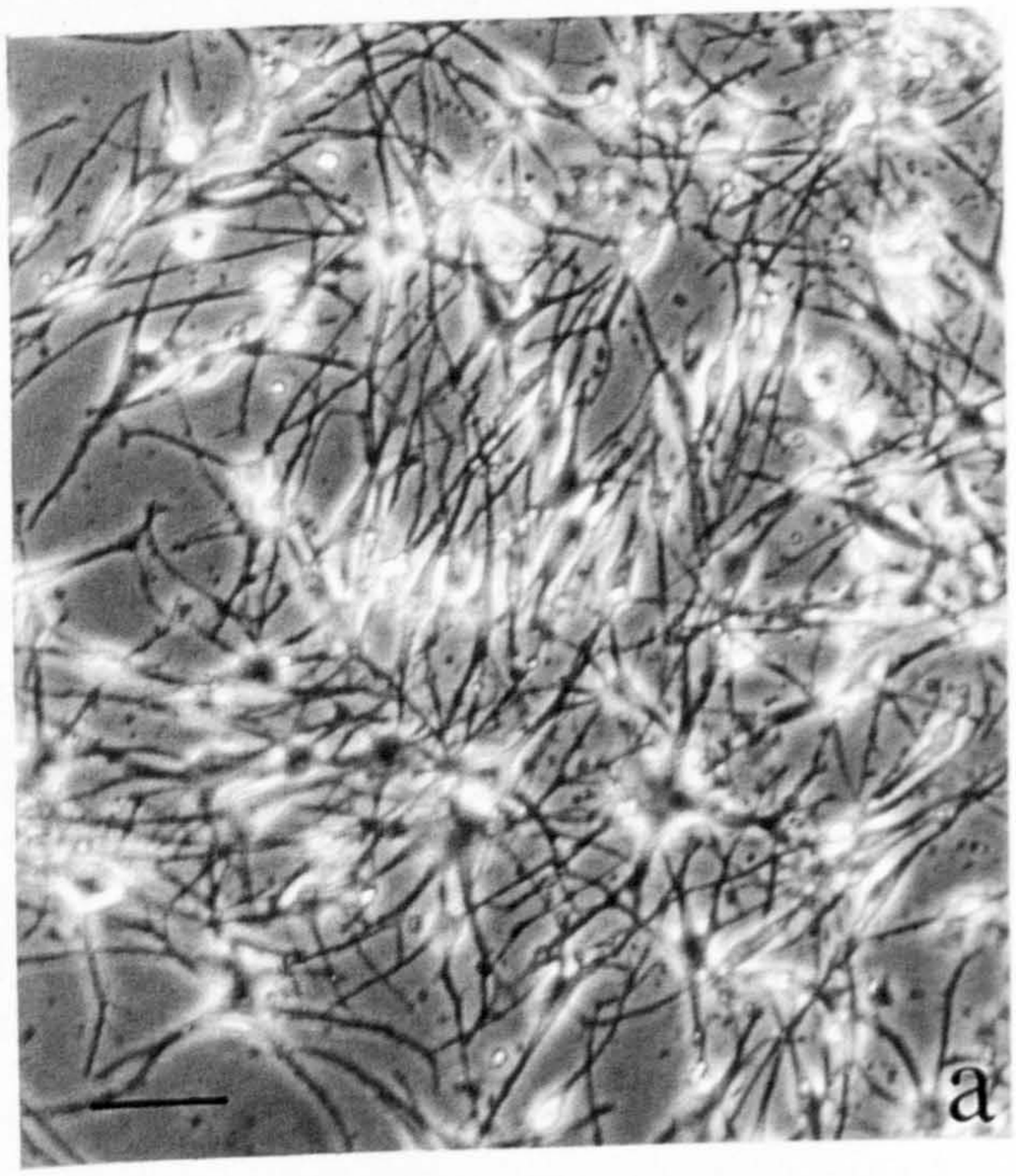
[b] Representative photomicrographs for melanocytes grown in medium 1 are shown in **Figures 3.3c, 3.3h, 3.6a, 3.7a, 3.8a, 3.8g, 3.9a** and **3.9i.**

[c] Representative photomicrographs for melanocytes grown in medium 10 are shown in **Figures 3.3d, 3.3i, 3.5a, 3.6c, 3.7b, 3.8b, 3.8h, 3.9d** and **3.9l.**

[d] Representative photomicrographs for melanocytes grown in medium 24 are shown in **Figures 3.3e, 3.3j, 3.6e, 3.7c, 3.83.8c, 3.8i, 3.9g** and **3.9o.**

bodies that often glowed (**Figures 3.2c** and **3.2h** for normal and vitiligo melanocytes respectively; additional representative photomicrographs are shown in **Figures 3.3c, 3.3h, 3.6a, 3.7a, 3.8a, 3.8g, 3.9a** and **3.9i** for melanocytes from various normal and vitiligo donors). The dendritic processes were long and thin. Melanocytes of a tetrapolar or multidendritic morphology, characteristic of early stage primary cultures, were in a small minority. Medium 1 closely resembles maintenance medium A. The morphology obtained in the other two experimental media (2 and 25) that resemble maintenance medium C and B respectively is similar to that obtained in medium 1 (**Figure 3.4a** illustrates the morphology obtained in medium 2). In medium 10 (containing FCS and BPE), melanocytes fatten and become epithelioid and the central region often darkens (**Figures 3.2d** and **3.2i** for normal and vitiligo melanocytes respectively; additional representative photomicrographs are shown in **Figures 3.3d, 3.3i, 3.5a, 3.6c, 3.7b, 3.8b, 3.8h, 3.9d** amd **3.9l** for melanocytes from various normal and vitiligo donors). In medium 24 (**Figures 3.2e** and **3.2j** for normal and vitiligo melanocytes respectively; additional representative photomicrographs are shown in **Figures 3.3e, 3.3j, 3.6e, 3.7c, 3.8c, 3.8i, 3.9g** amd **3.9o** for melanocytes from various normal and vitiligo donors) lacking all mitogens but containing insulin, transferrin, hydrocortisone and L-glutamine, cells are generally bi-tripolar, but they quickly lose their thinness with the central region darkening. The addition of CT (**Figure 3.4h,** medium 22) or BPE (medium 20) slightly increases dendricity. The morphology seen in medium 20 resembled that seen in medium 15 (containing BPE and CT, **Figure 3.4g**), although the cells were slightly thinner. The addition of FCS (medium 19) and bFGF (medium 23) had very little effect on morphology (not shown) while PMA induced thinness in the cells (media 21, not shown). Cell morphology in medium 21 resembled that shown in medium 13 (containing FCS/PMA, **Figure 3.4e**). In all media containing PMA (media 1-3, 6-9, 13, 14, 17, 18, 21 and 25), cells were thin (for example in media 8 and 9 shown in **Figures 3.5c** and **3.4b** respectively), but dendrite number varied. Melanocytes cultured in the absence of PMA, generally fattened and often became epithelioid, with a dark central region (for example in medium 4, **Figure 3.4c**). The contrast between adding PMA and CT to media is illustrated for medium 11 (containing FCS/CT, **Figure 3.4d**) and medium 13 (containing FCS/PMA, **Figure 3.4e**) for melanocytes from donor M741 and for medium 14 (containing BPE/PMA, **Figure 3.4f**) and medium 15 (containing BPE/CT, **Figure 3.4g**) for melanocytes from donor M835,

Figure 3.4 Effect of media composition on melanocyte morphology. Primary, P1 and P4 melanocytes were cultured under 25 different media variants (composition of the media variants is described in **Tables 2.2** and **3.2**) for 5 days prior to photography. Morphology obtained in the three most commonly used experimental media, 1, 10 and 24 are illustrated in **Figures 3.2**, and **3.7-3.9**. Media 2 (**a**) and 9 (**b**) are illustrated for melanocytes from donor M746 at passage 4; media 4 (**c**), media 11 (**d**) and media 13 (**e**) are illustrated for melanocytes from donor M741 at passage 4; media 14 (**f**), media 15 (**g**) and media 22 (**h**) are illustrated for melanocytes from donor M835 at passage 1. While different melanocyte donors are shown, morphology obtained for each media variant was very similar between donor to donor and the morphology for each media is summarised in **Table 3.2**. Scale bars represent 30μm.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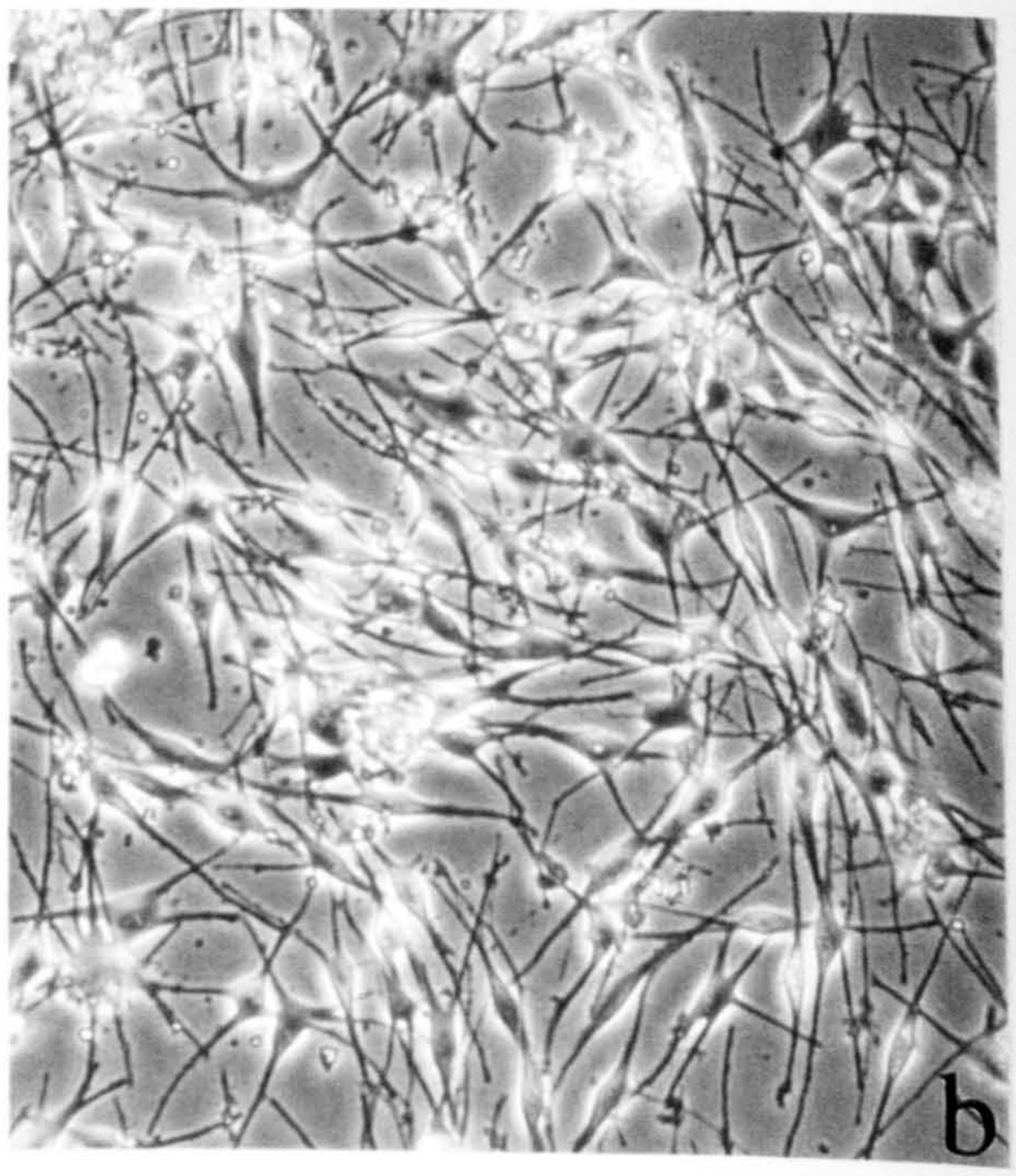
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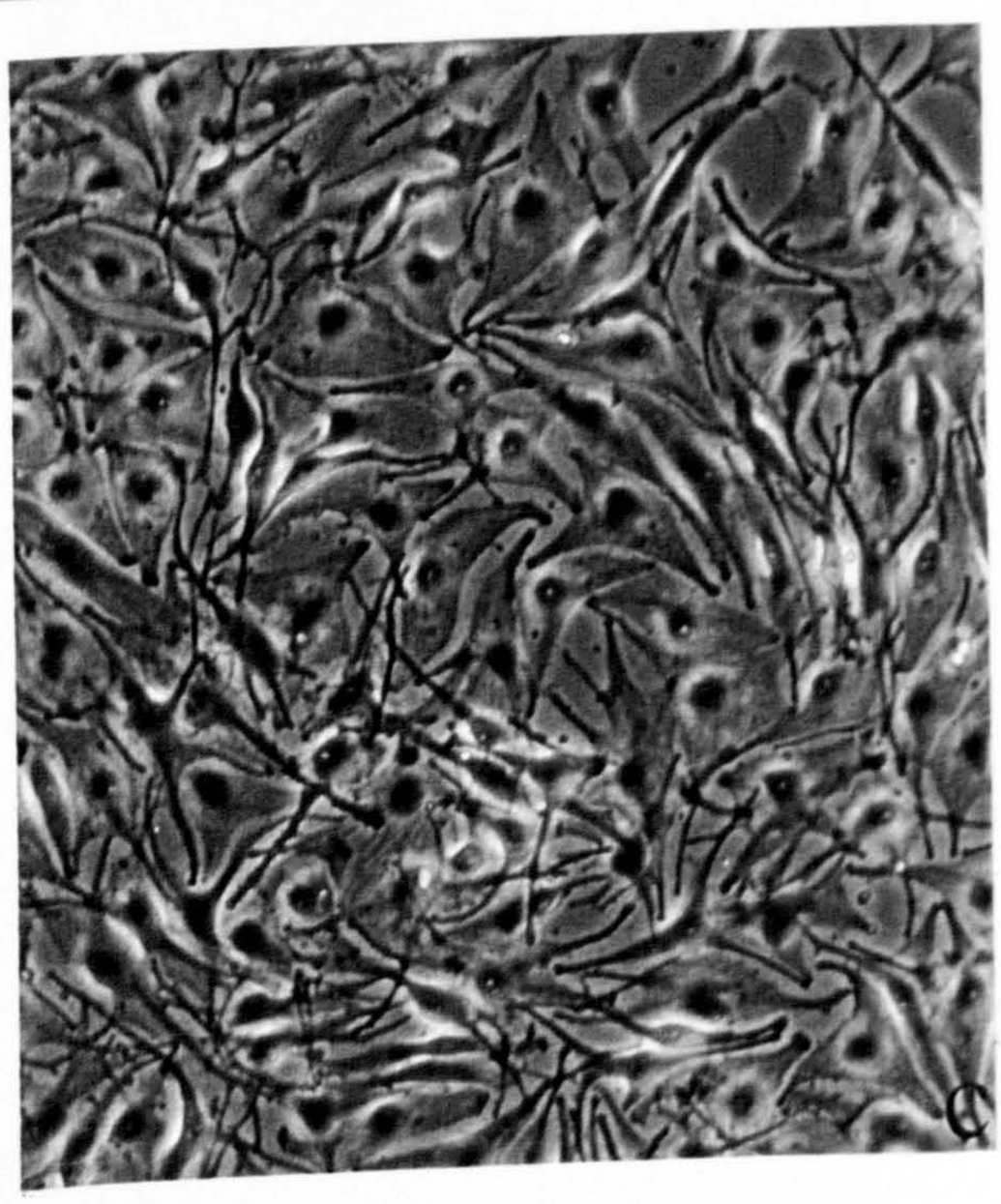
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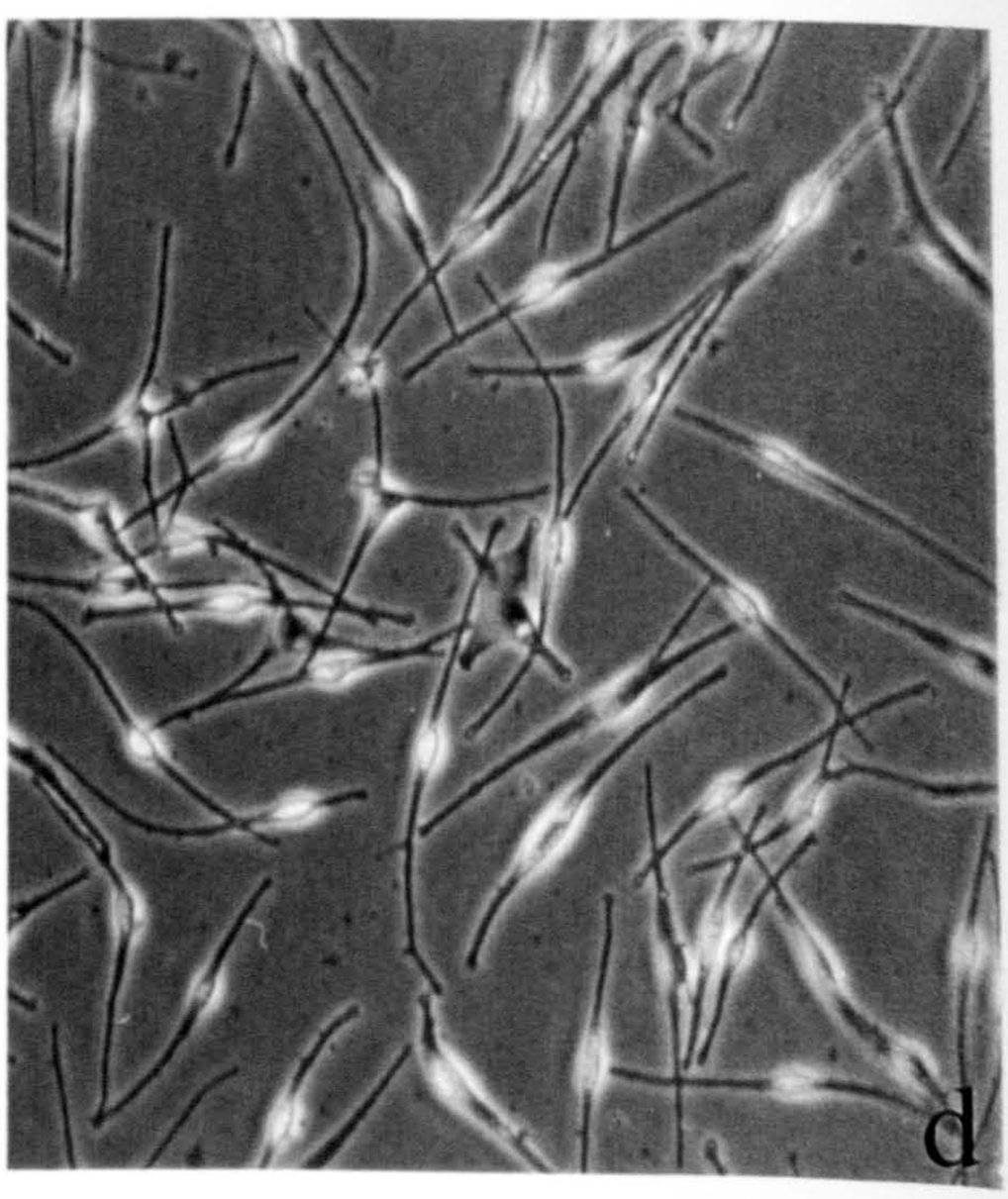
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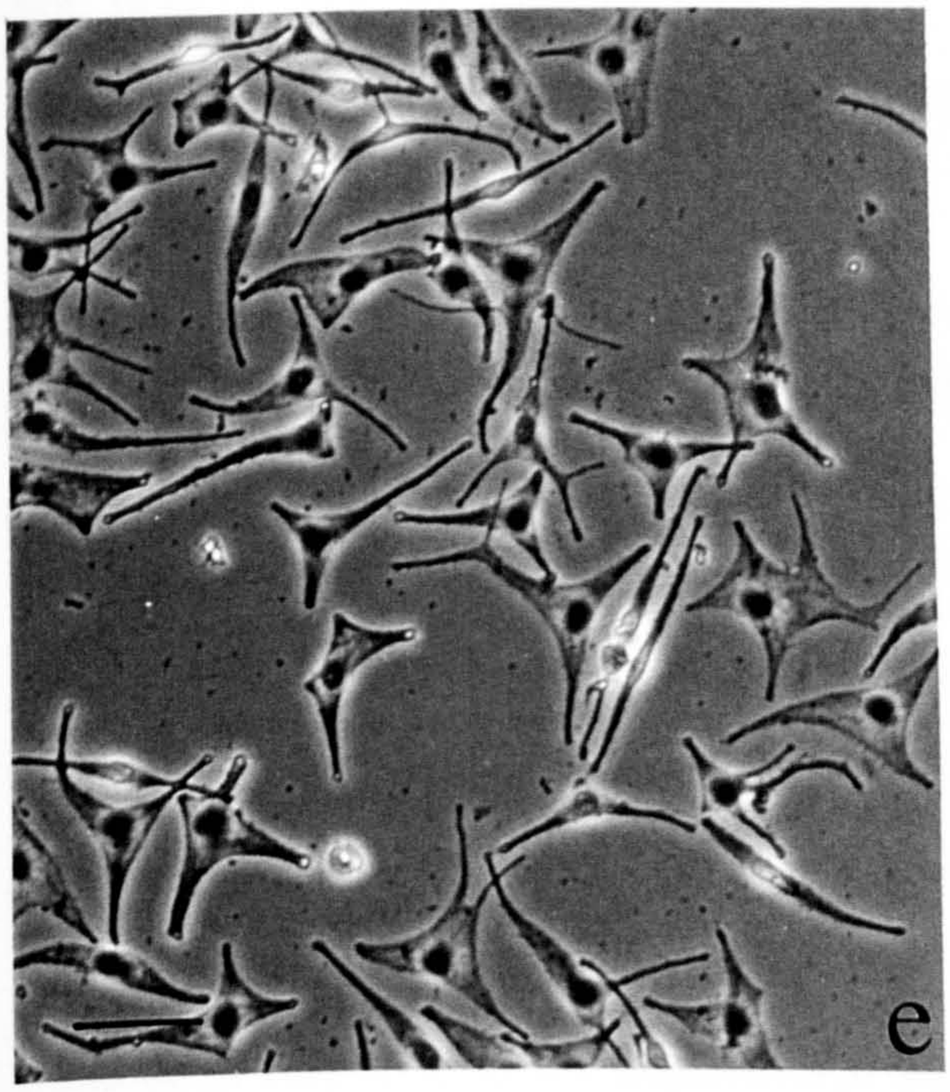
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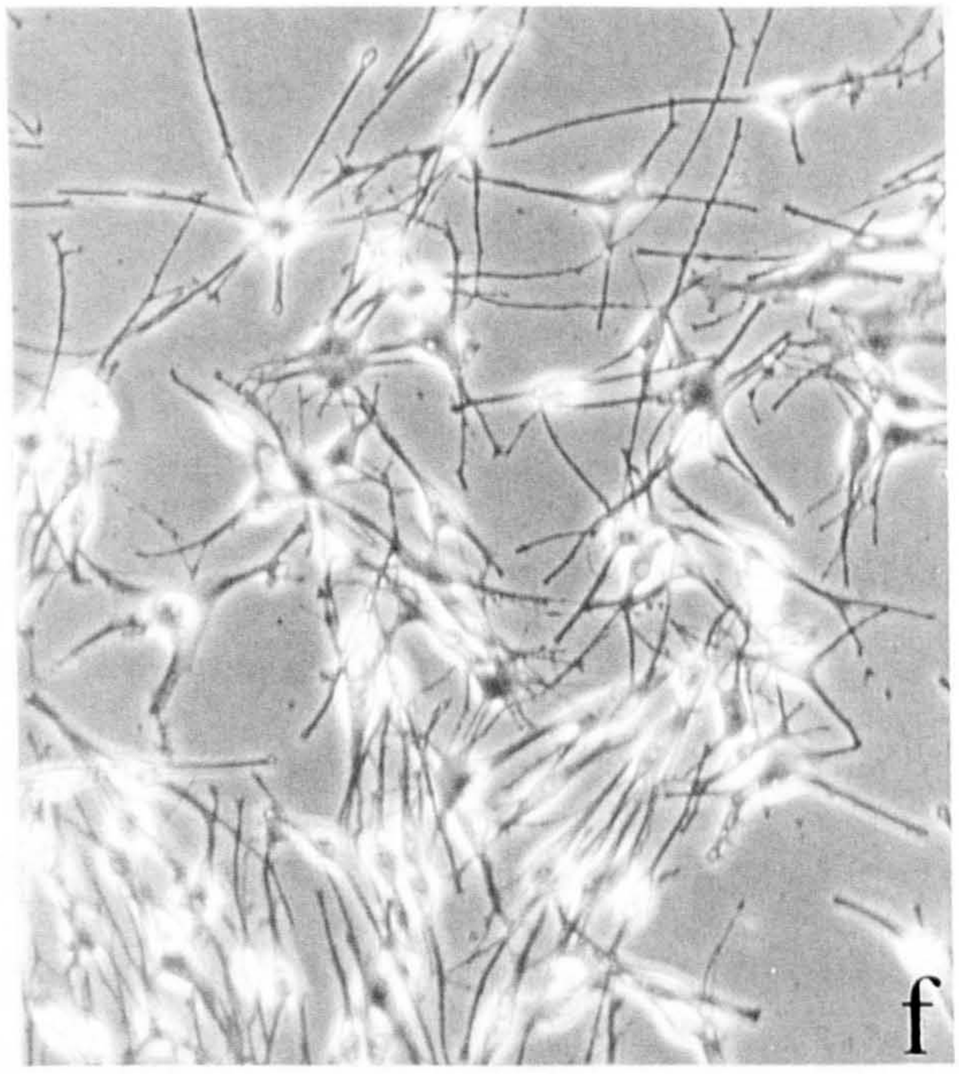
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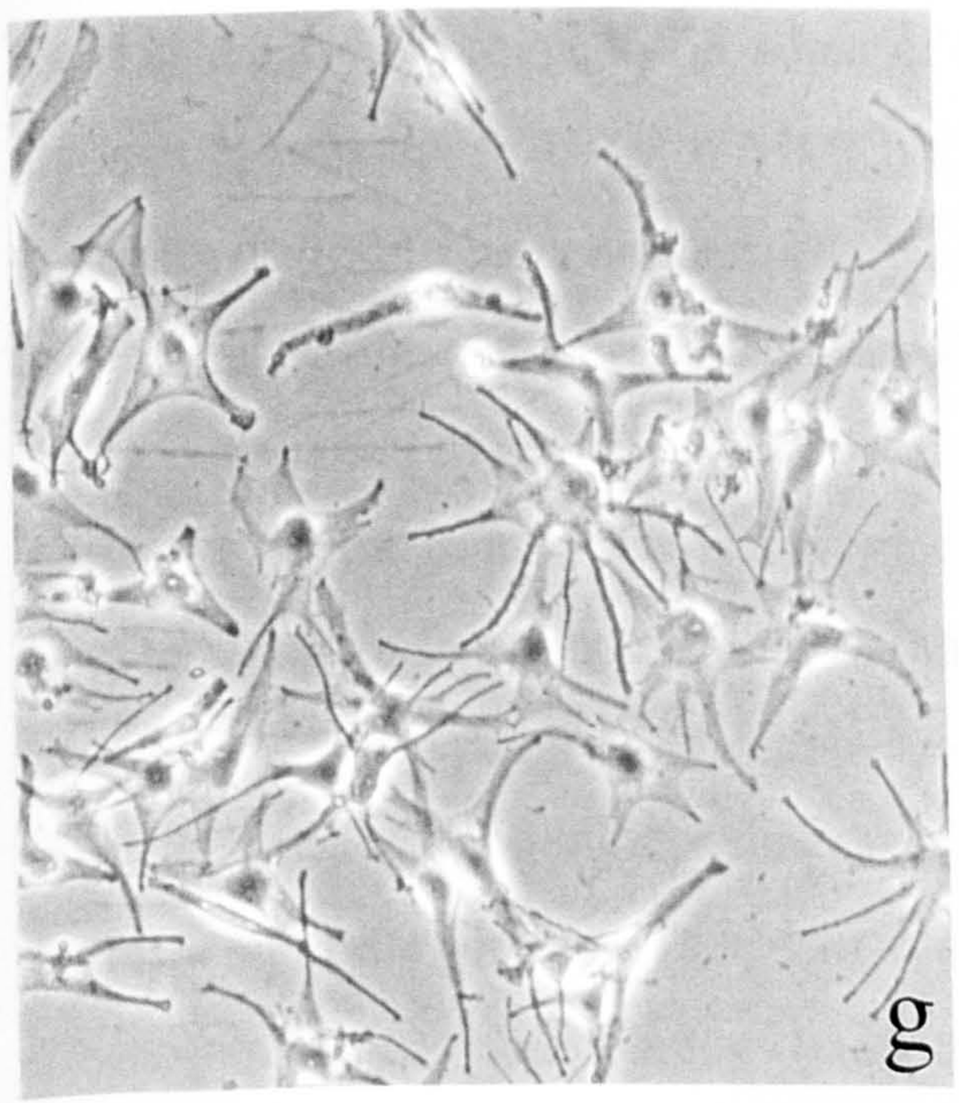
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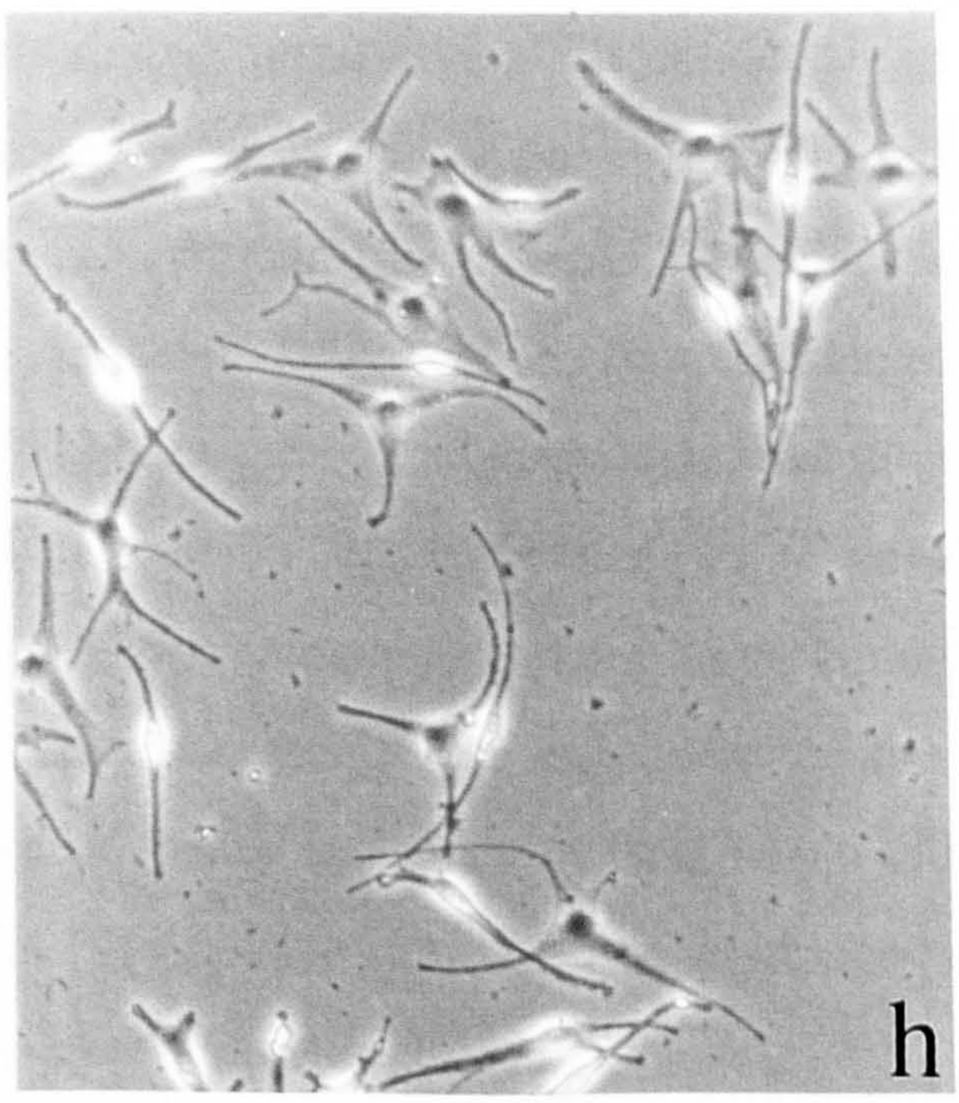
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where melanocytes were thin if PMA was present, but fattened if CT was present instead. Morphologies obtained were reproducible amongst cultures.

The presence of PMA induces thinness in melanocytes and the cells are generally bipolar. BPE and CT induce an increase in dendrite number while FCS and bFGF did not play a role in altering melanocyte morphology. Adult melanocytes are very sensitive to changes in melanocyte media, and alter their morphology within 5 days, even at a passage number of 4.

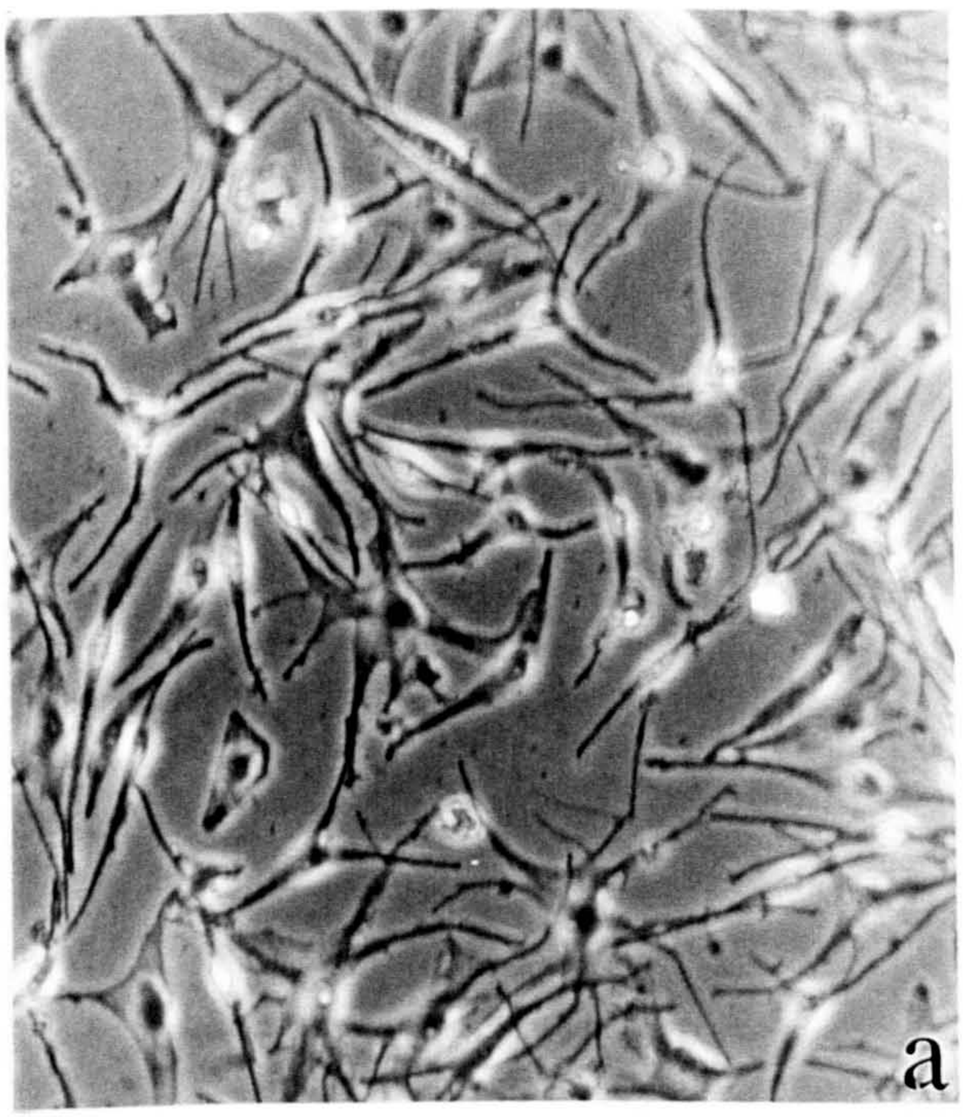
3.2.2 Effect of α-MSH on melanocyte morphology.

The effect of 10^{-10}M or 10^{-8}M α-MSH on melanocyte morphology was examined under the 25 experimental media variants. Under the majority of media conditions examined melanocyte morphology was rarely altered in response to the addition of α-MSH (10^{-10}M or 10^{-8}M) as illustrated in medium 10 for melanocytes from donor M832 (**Figure 3.5a**, no MSH; **Figure 3.5b**, plus MSH). From these two figures it can be seen that cell number in response to the peptide was slightly reduced in this medium as discussed in **Section 3.3.2**. Occasionally in some of the cultures, melanocytes did exhibit slight changes in response to 10^{-8}M α-MSH but these were limited to one or two media and generally the results were not reproducible between cultures. For example, the dendrites of melanocytes from donor M741 cultured in medium 8 (**Figure 3.5c**, control), started to shorten slightly in response to 10^{-8}M α-MSH (**Figure 3.5d**). One notable exception was melanocytes from donor M746 which were initially thin in medium 12 (**Figure 3.5e**) but fattened in response to the 10^{-8}M α-MSH with a dark central region becoming prominent (**Figure 3.5f**). Cell number was also increased in response to the peptide under this medium condition. However, in other media, melanocytes from M746 did not respond to the peptide. Therefore, α-MSH did not have significant or reproducible effects on melanocyte morphology under the media variants examined.

3.2.3 Effect of IBMX on melanocyte morphology.

IBMX, a cAMP modulator was used as a comparative reference to α-MSH throughout this chapter. Whereas 10^{-10}M or 10^{-8}M α-MSH had little or no consistent effect on

Figure 3.5 Effect of 10^{-8}M α-MSH on melanocyte morphology. Primary, P1 and P4 melanocytes were cultured under 25 different media variants (composition of the media variants is described in **Tables 2.2** and **3.2**) in the absence or presence of 10^{-8}M α-MSH (added on 3 days during the experimental period) for 5 days prior to photography. The effect of 10^{-8}M α-MSH in medium 10 (**a**, no α-MSH; **b**, plus α-MSH) is illustrated for melanocytes from donor M832 at passage 1, in medium 8 (**c**, no α-MSH; **d**, plus α-MSH) is illustrated for melanocytes from donor M741 at passage 4 and in medium 12 (**e**, no α-MSH; **f**, plus α-MSH) is illustrated for melanocytes from donor M746 at passage 4. Scale bars represent 30μm.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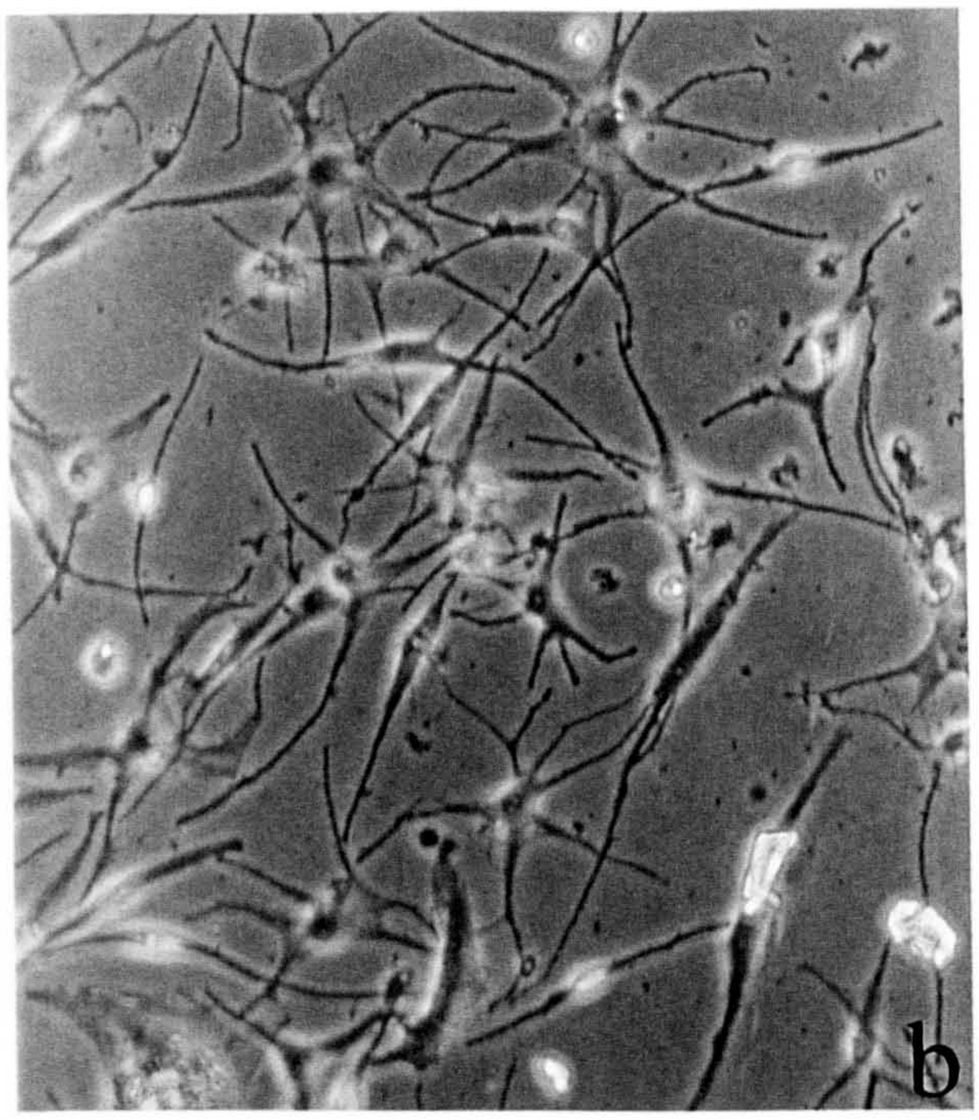
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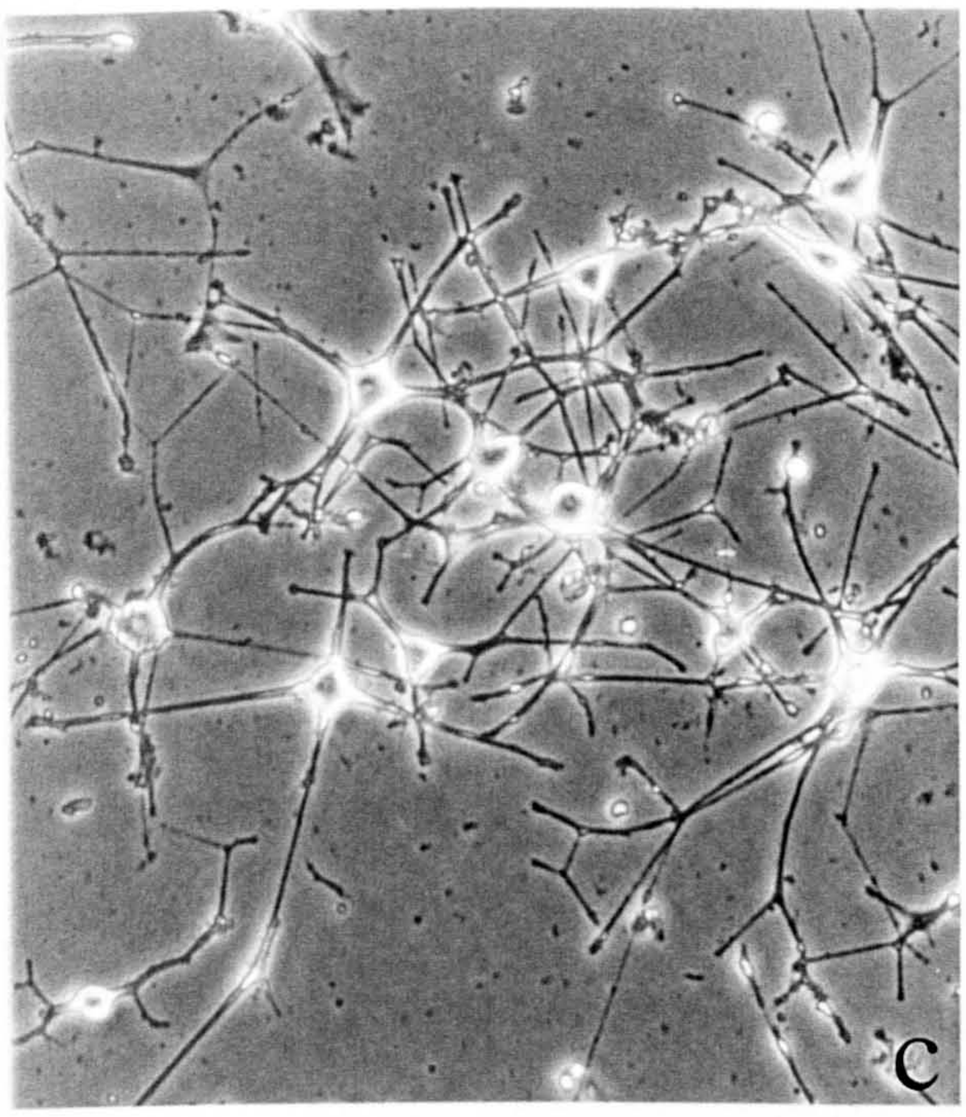
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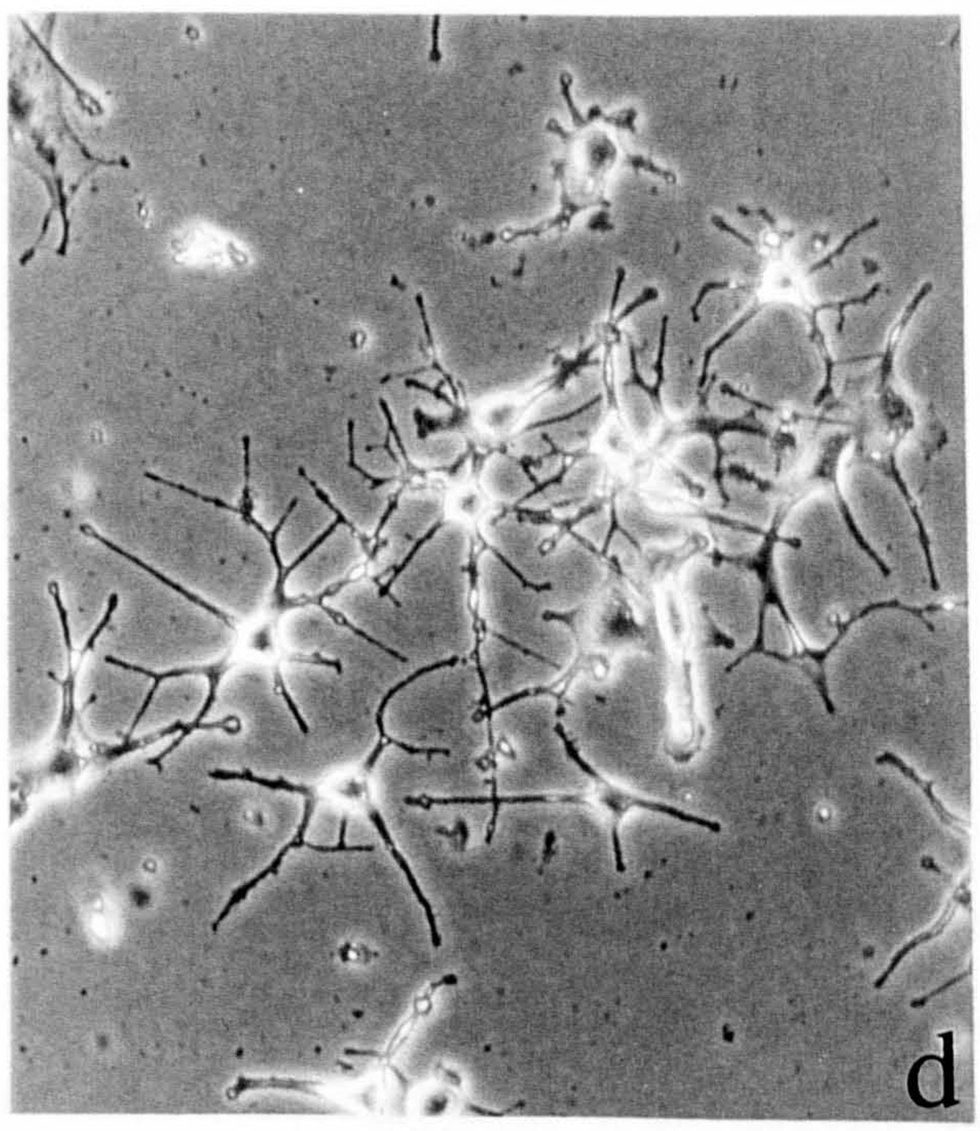
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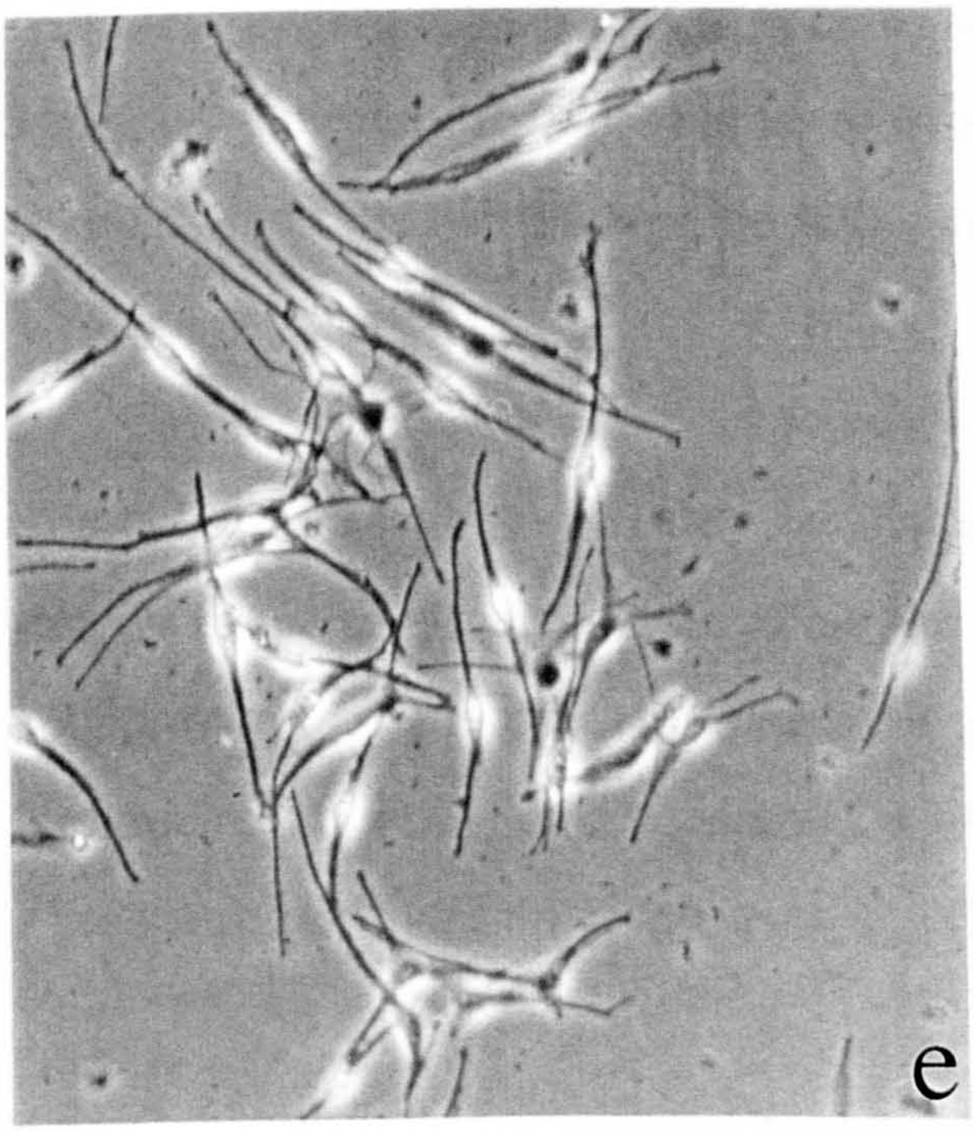
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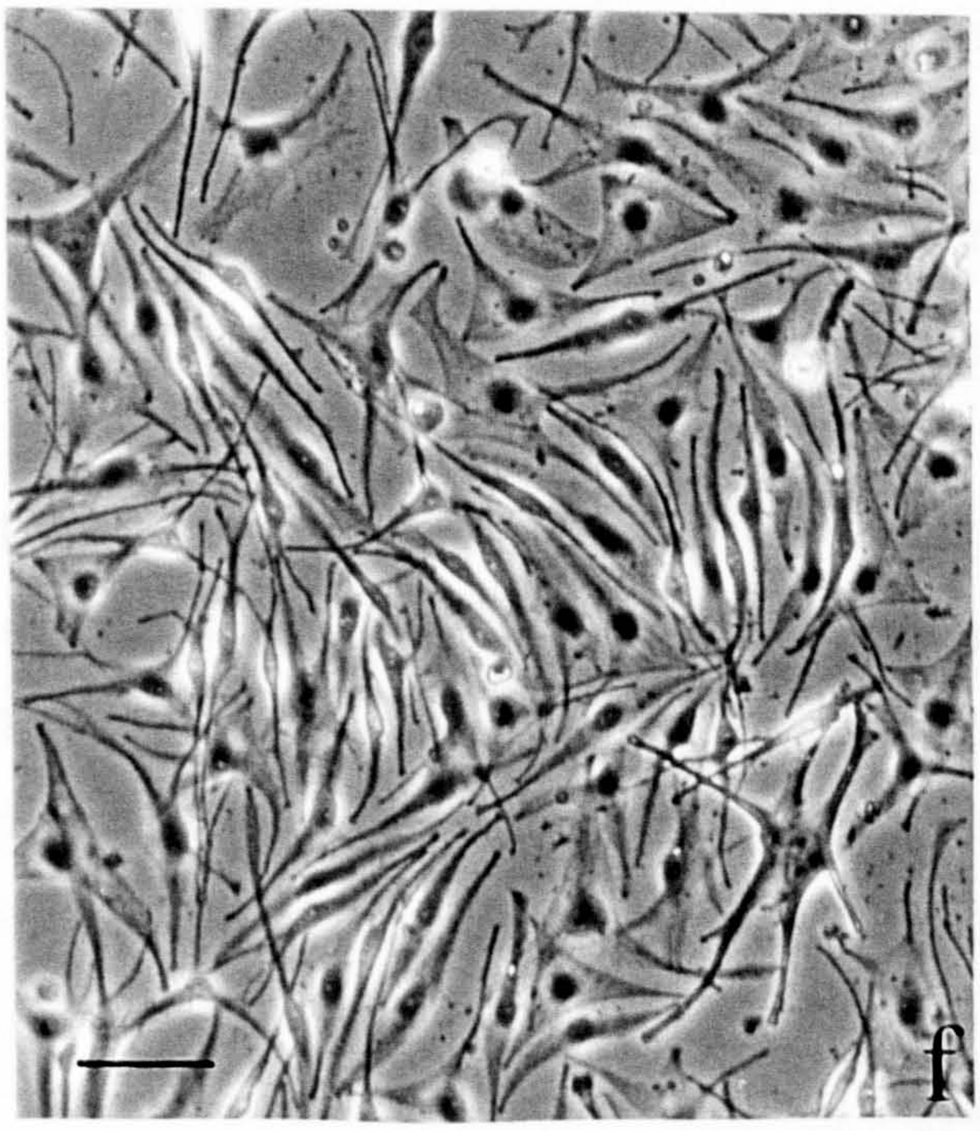
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melanocyte morphology, 10^{-3}M IBMX had a striking and reproducible effects on melanocyte morphology under the five media conditions in which it was examined (representative photographs are illustrated in **Figure 3.6** for melanocytes from donor M741). In media 1 (**Figure 3.6b**), and 10 (**Figure 3.6d**), melanocytes often had distinct bright glowing cell bodies and were multidendritic in comparison to the control (**Figures 3.6a** and **3.6c** respectively for media 1 and 10). In medium 10, the dendrites also showed extensive ramification. In medium 24 (**Figure 3.6f**), cells again had bright bodies, exhibited slightly more dendrites but rarely showed ramification in comparison to the control (**Figure 3.6e**). The melanocytes also became slightly thinner. In media 2 and 12, the morphologies obtained in response to IBMX (not shown) are the same as in media 1 and 10 respectively.

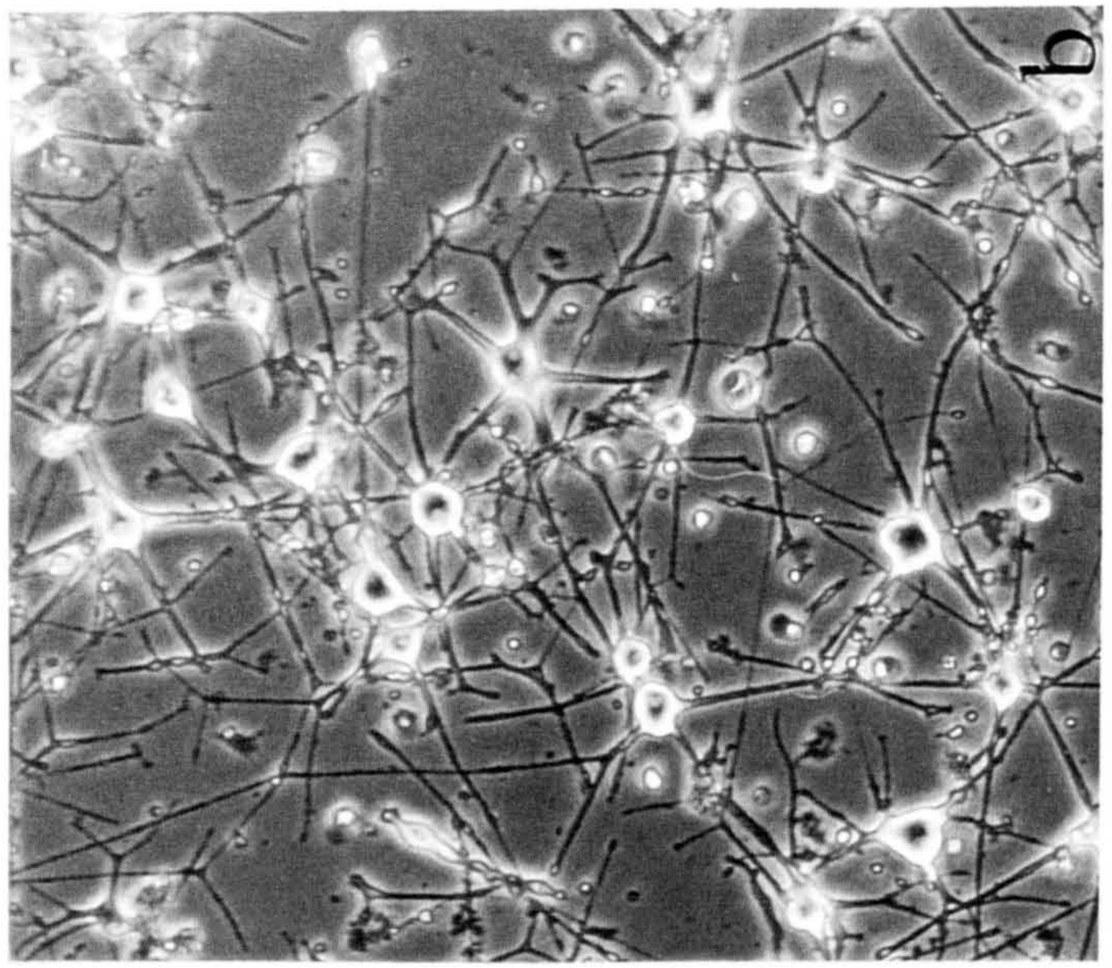
IBMX had a strong influence, like other mitogenic additives (**Section 3.2.1**), on melanocyte morpholgy with dendrite formation increased. In media already containing BPE and/or CT, the effect was at least additive.

3.2.4 Effect of media composition and substrate choice on melanocyte morphology

Melanocytes used in this study were cultured in maintenance medium A. Media again were shown to play a significant role in influencing melanocyte morphology. Melanocytes cultured on plastic in medium 1 (**Figure 3.7a**) and medium 10 (**Figure 3.7b**) generally assumed the characteristic morphologies of these media. In medium 24, cells had bi/tripolar morphology as previously noted but several cells in this experiment and other experiments from this section were rounded, appeared poorly attached to the substrate and often, within a matter of a few days, appeared heavily vacuolated (**Figure 3.7c**). Most cells did not survive long-term culture in medium 24, and often died after 10 days.

The culture of cells on cell-derived matrix proteins, and on individual ECM proteins, usually resulted in melanocytes of similar morphologies to those cultured on plastic for cells grown in medium 1 (**Figures 3.7d** and **3.7g** for cells cultured on fibronectin and laminin respectively) and medium 10 (**Figures 3.7e** and **3.7h** for cells cultured on fibronectin and laminin respectively). **Table 3.3** summarises the morphology of the

Figure 3.6 Effect of 10^{-3}M IBMX on melanocyte morphology. Primary, P1 and P4 melanocytes were cultured under 5 different media variants (composition of the media variants is described in **Tables 2.2** and **3.2**) in the absence or presence of 10^{-3}M IBMX (added on 3 days during the experimental period) for 5 days prior to photography. The effect of IBMX on melanocyte morphology is illustrated for melanocytes from donor M741 at passage P4 in medium 1 (**a**, no IBMX; **b**, plus IBMX), medium 10 (**c**, no IBMX;**d**, plus IBMX) and in medium 24 (**e**, no IBMX;**f**, plus IBMX). The effect of IBMX in medium 2 and medium 12 (the additional 2 media in which the effects of IBMX were examined) are not shown but melanocytes demonstrate the same morphology as illustrated in media 1 (**b**) and 10 (**d**) respectively. Scale bars represent 30μm.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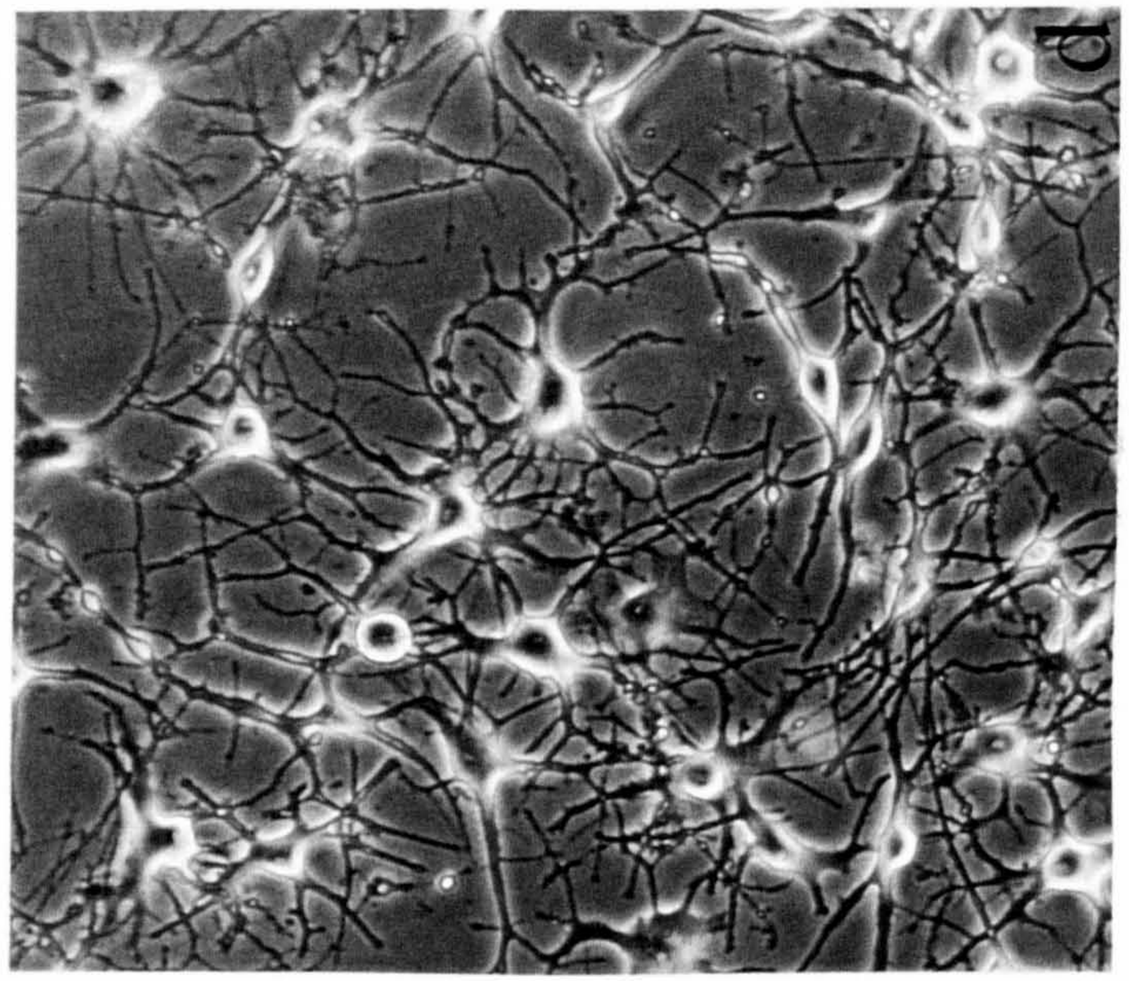
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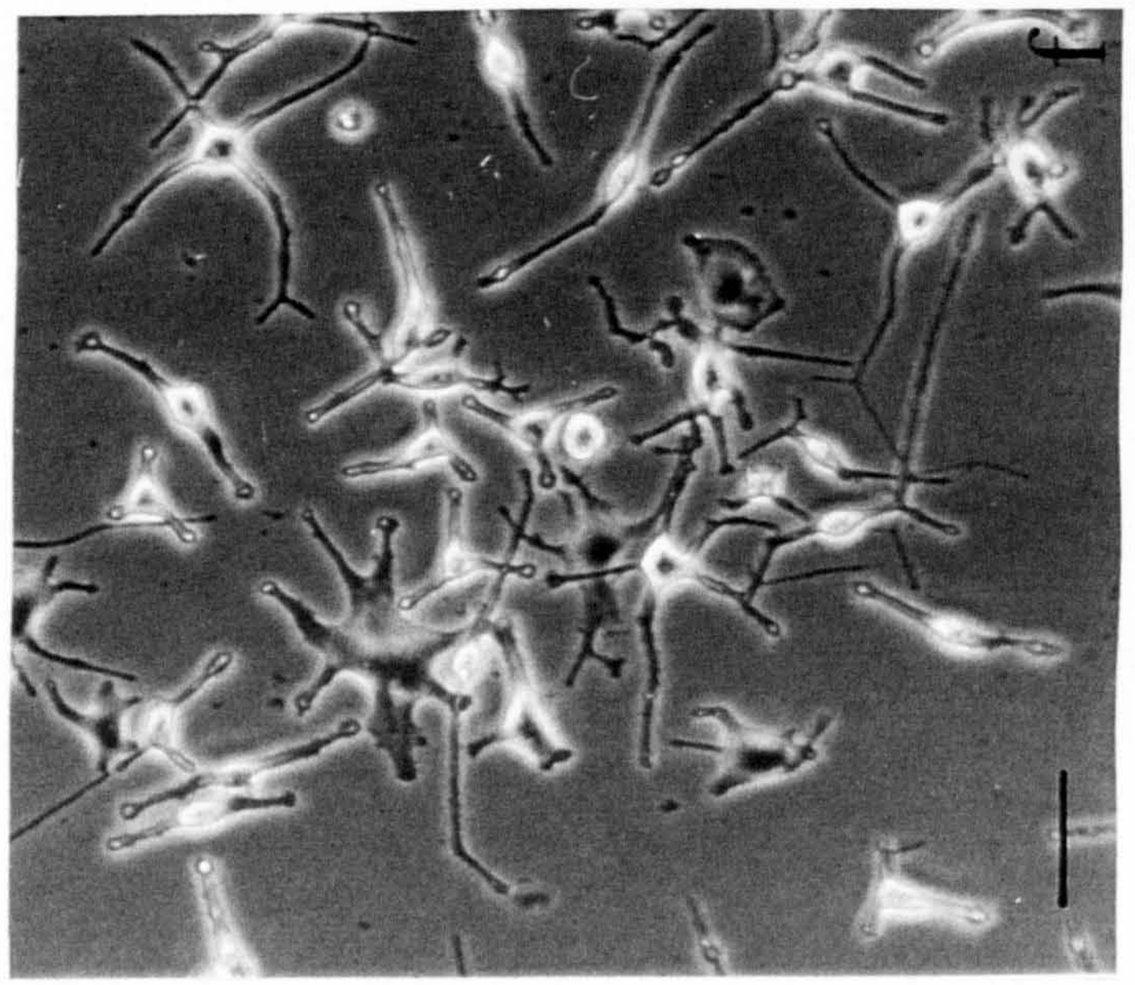
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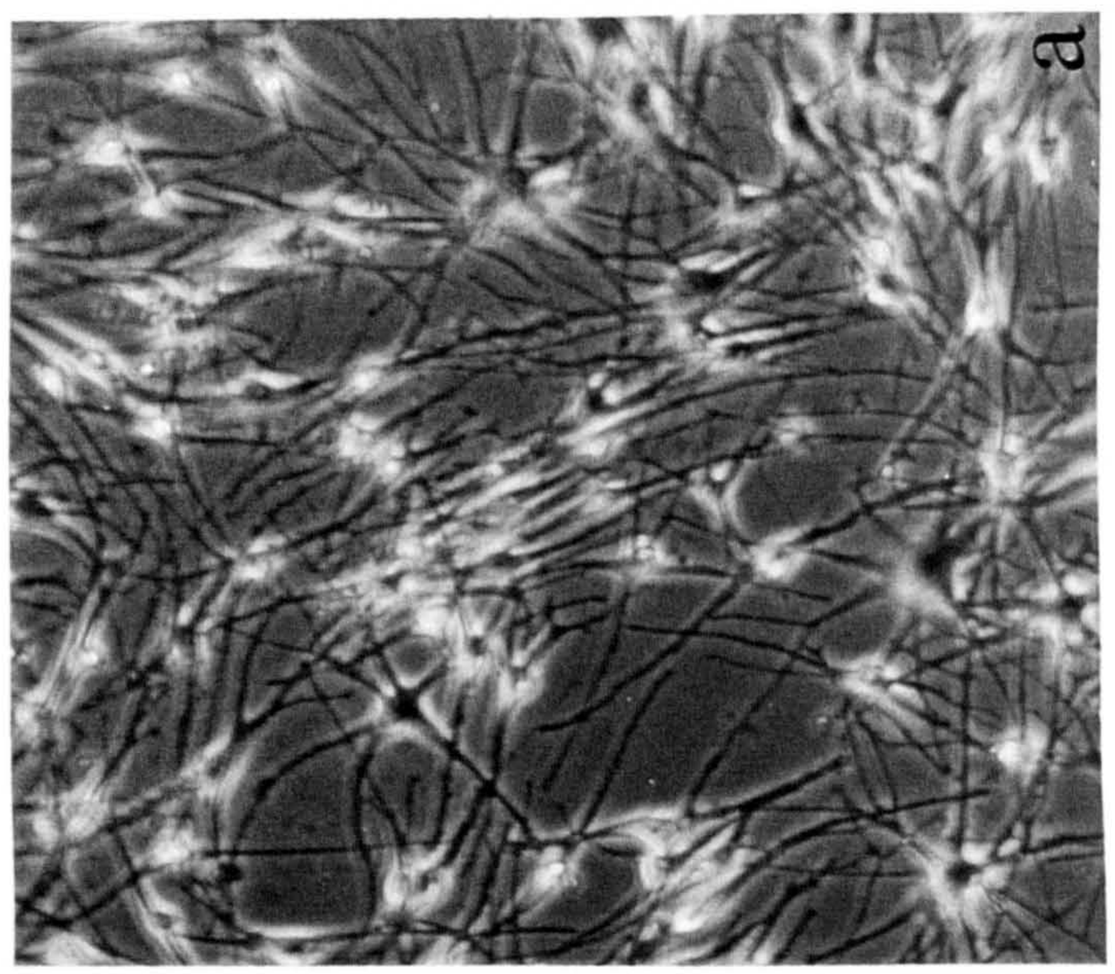
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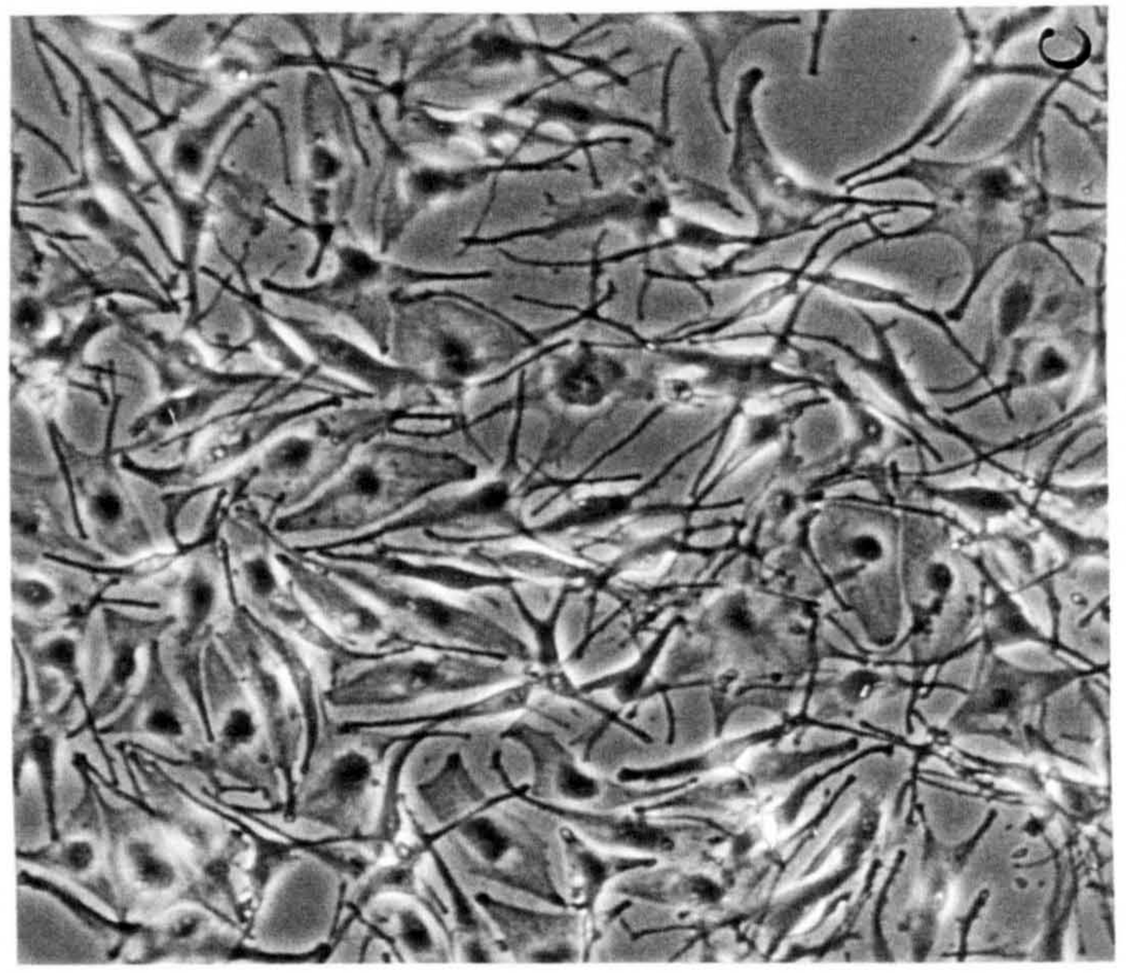
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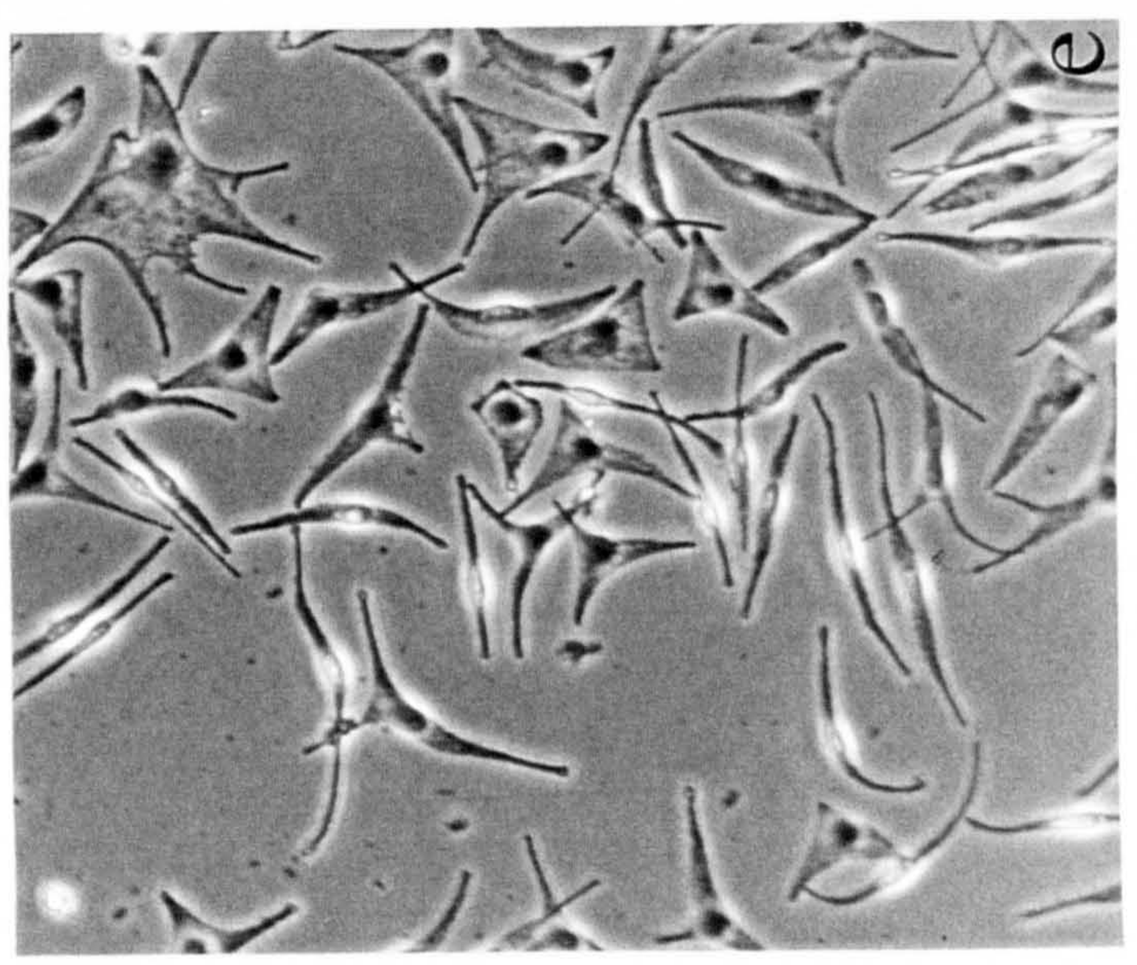
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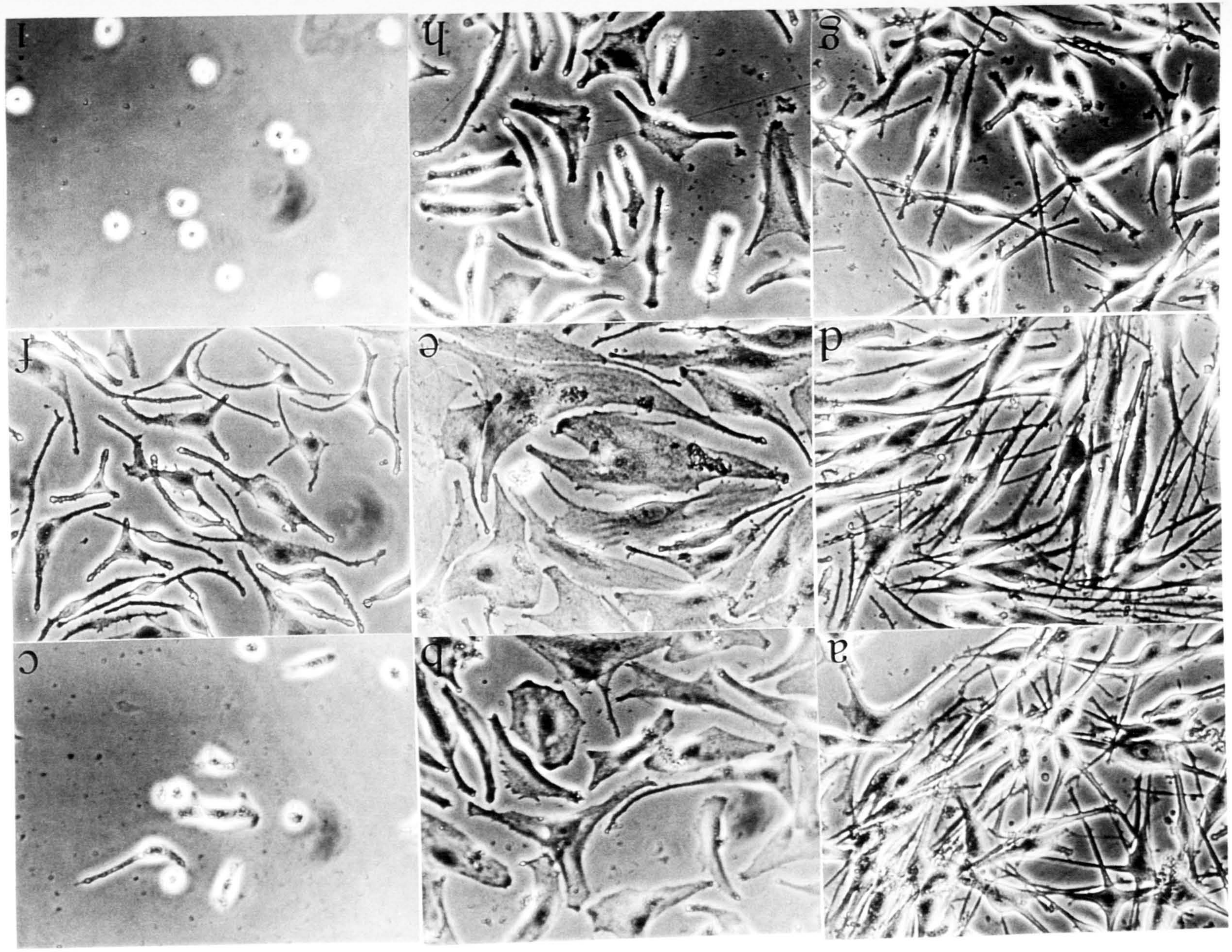
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Figure 3.7 Influence of substrate choice on melanocyte morphology under 3 media variants. Melanocytes at passage 4 were cultured on plastic (**a-c**), fibronectin-coated plastic (**d-f**) or laminin-coated plastic (**g-i**) in media 1 (**a,d,g**), 10 (**b,e,h**) or 24 (**c,f,i**) for 5 days. This is a representative example of 4 such experiments. Morphology of melanocytes cultured on a range of individual and cell-derived ECM proteins are summarised in **Table 3.3**. Photomicrographs were taken on a 20x objective, but scale cannot be calculated as the original negatives were lost.

Table 3.3 Morphology of melanocytes in culture on individual and cell-derived ECMs.

Substrate	Media		
	Medium 1	Medium 10	Medium 24
Plastic	mainly bi/tripolar, thin processes (**Figure 3.7a**)	epitheloid bi/tripolar (**Figure 3.7b**)	small bi/tripolar, majority rounded and vacuolated (**Figure 3.7c**)
Collagen I	as for plastic	as for plastic	bipolar or rounded
Collagen IV	as for plastic	as for plastic	as for collagen I
Fibronectin	as for plastic (**Figure 3.7d**)	as for plastic (**Figure 3.7e**)	bi/tripolar (**Figure 3.7f**)
Laminin	as for plastic (**Figure 3.7g**)	as for plastic (**Figure 3.7h**)	all rounded (**Figure 3.7i**)
Fibroblast ECM	as for plastic	as for plastic	as for fibronectin
Bovine endothelial ECM	as for plastic	as for plastic	as for fibronectin
Human endothelial ECM	as for plastic	as for plastic	as for fibronectin

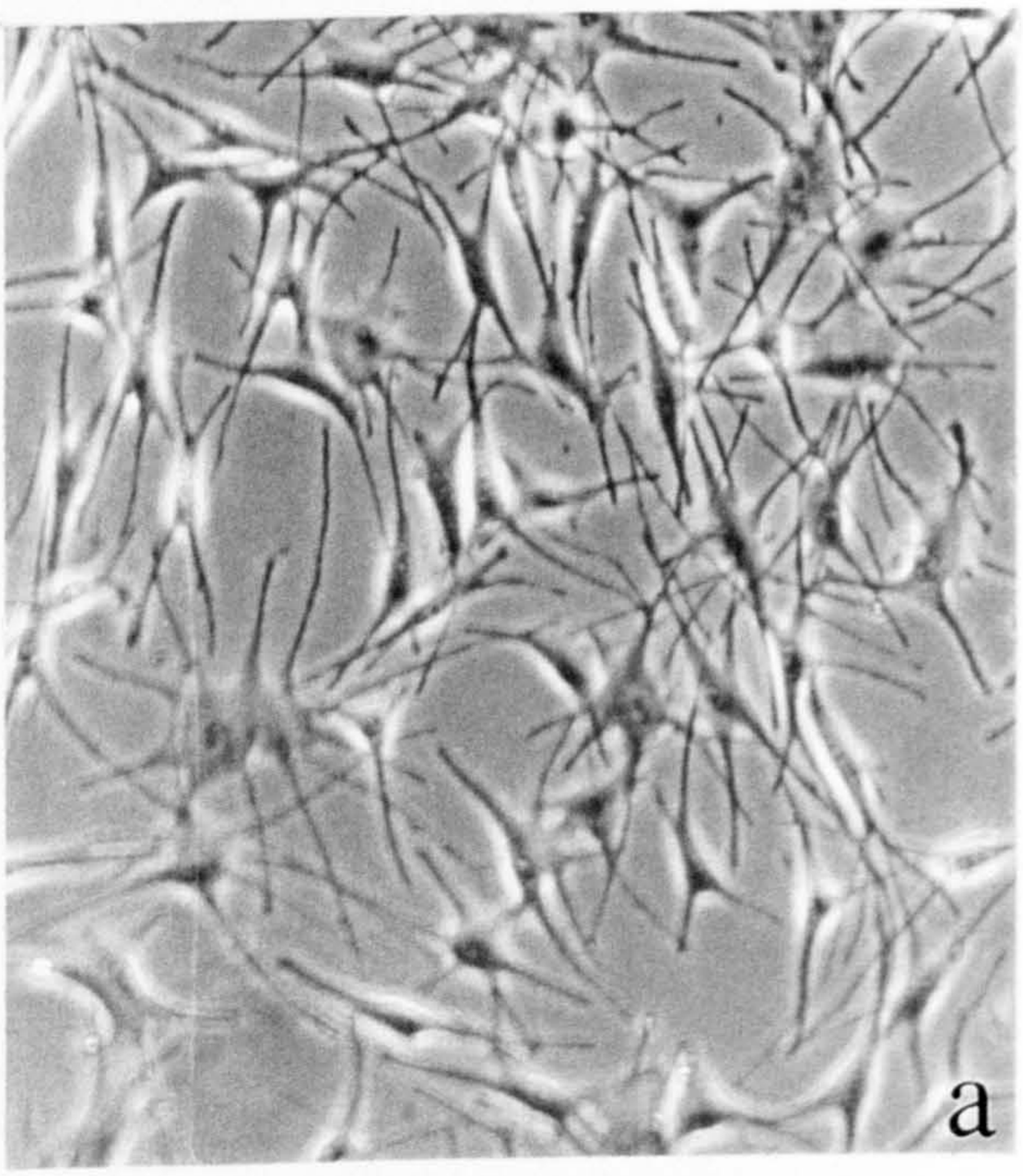
Normal adult human cutaneous melanocytes were plated out on the different substrates (substrates prepared as described in **Section 2.5**) in maintenance medium A and allowed to attach for 24 hours before the medium was changed to either media 1, 10 or 24. Cells were then cultured for a further 5 days in these media prior to morphology assessment.

cells grown on the different substrates. The contribution of the ECM proteins was most evident when the cells were cultured in medium 24, where the presence of the substrate was sufficient to maintain a greater proportion of melanocyte survival over 7-10 days. Here, cells tended to be bi/tripolar and even tetrapolar and were smaller with relatively long processes that were often thicker than those of cells cultured in medium 1 on ECM proteins (**Figure 3.7f** for cells cultured on fibronectin). Essentially, the presence of the ECM substrate appeared to prevent cell vacuolation and death, when melanocytes were grown in the absence of all mitogenic drives. The only exception was laminin (**Figure 3.7i**), on which the cells were all rounded, and were not saved from cell death when cultured in the incomplete medium.

3.2.5 Effect of media composition and UVB on normal and vitiligo melanocyte morphology

UVB is one of the most well known activators of melanocytes and induces increased dendricity *in vivo*. This study compared the effects of UVB on normal and vitiligo melanocyte morphology. Normal melanocytes from donor M577 and vitiligo melanocytes from VM3 were cultured for 7 days under 3 experimental media (1, 10 or 24). On each day, they received a dose of 143mJ/cm^2 of UVB (during irradiations melanocytes were in PBS) (as described in **Section 2.6**). In medium 1, cells were not quite as thin as some cultures from other donors and had quite large bodies (**Figures 3.8a** and **3.8g** for normal and vitiligo melanocytes respectively). In response to UVB, the cell bodies became enlarged, and cells increased dendrite number (**Figures 3.8d** and **3.8j** for normal and vitiligo melanocytes respectively). In medium 10, both normal and vitiligo cells exhibited the epitheloid shape that is characteristic of this medium. Melanocytes from vitiligo donor VM3 were more bipolar (**Figure 3.8h**) than the melanocytes from donor M577 (**Figure 3.8b**). UVB induced melanocytes to enlarge their size (**Figures 3.8e** and **3.8k** for normal and vitiligo melanocytes respectively). In medium 24, both cells are bi-tetrapolar, but melanocytes from VM3 were larger (**Figure 3.8i**) than the melanocytes from M577 (**Figure 3.8c**). UVB did not affect melanocyte morphology in this medium (**Figures 3.8f** and **3.8l** for normal and vitiligo melanocytes respectively).

Figure 3.8 Influence of UVB on normal and vitiligo melanocyte morphology under 3 media variants. P4 normal and vitiligo melanocytes from donors M577 (**a-f**) and VM3 (**g-l**) were irradiated with 143mJ/cm^2 of UVB on 7 consecutive days (as described in **Section 2.6**) After each irradiation, fresh experimental media (1, 10 or 24) was added. Sham irradiated cells are shown for medium 1 (**a** and **g** for M577 and VM3 respectively), for medium 10 (**c** and **i** for M577 and VM3 respectively) and for medium 24 (**e** and **k** for M577 and VM3 respectively) while irradiated cells are shown for medium 1 (**b** and **h** for M577 and VM3 respectively), for medium 10 (**d** and **j** for M577 and VM3 respectively) and for medium 24 (**f** and **l** for M577 and VM3 respectively). This is a representative example of 3 such experiments. Scale bars represent 30μm.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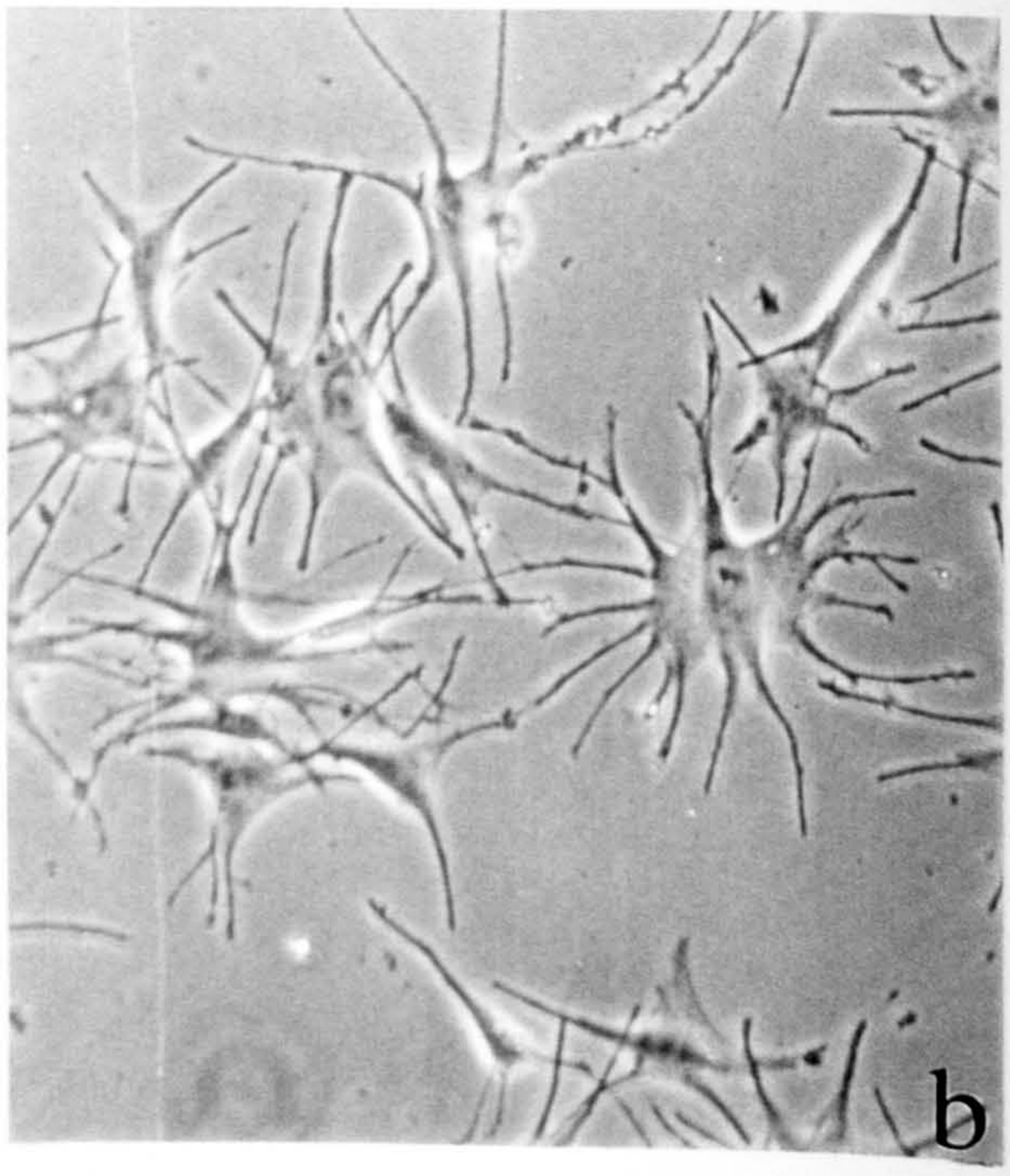
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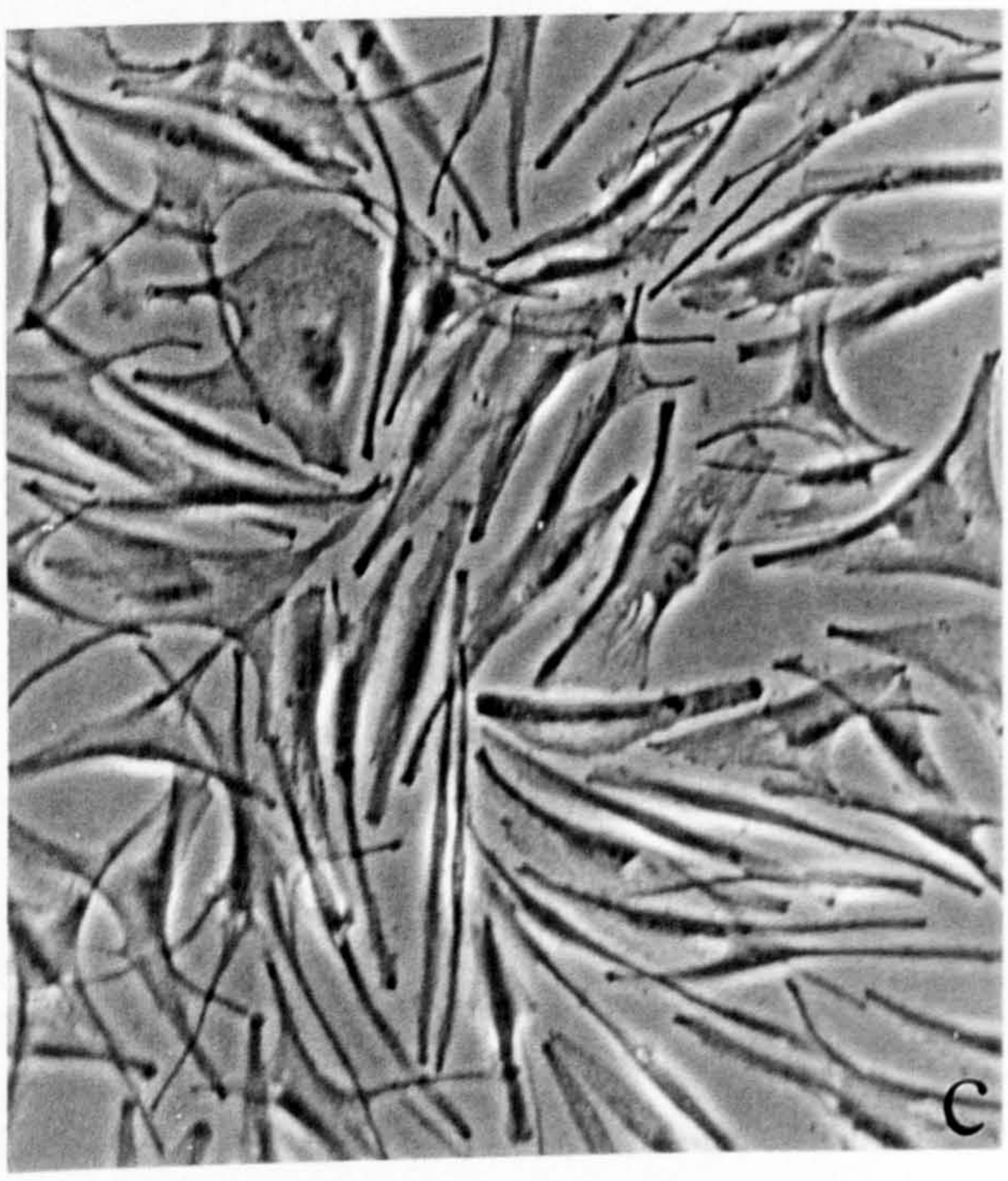
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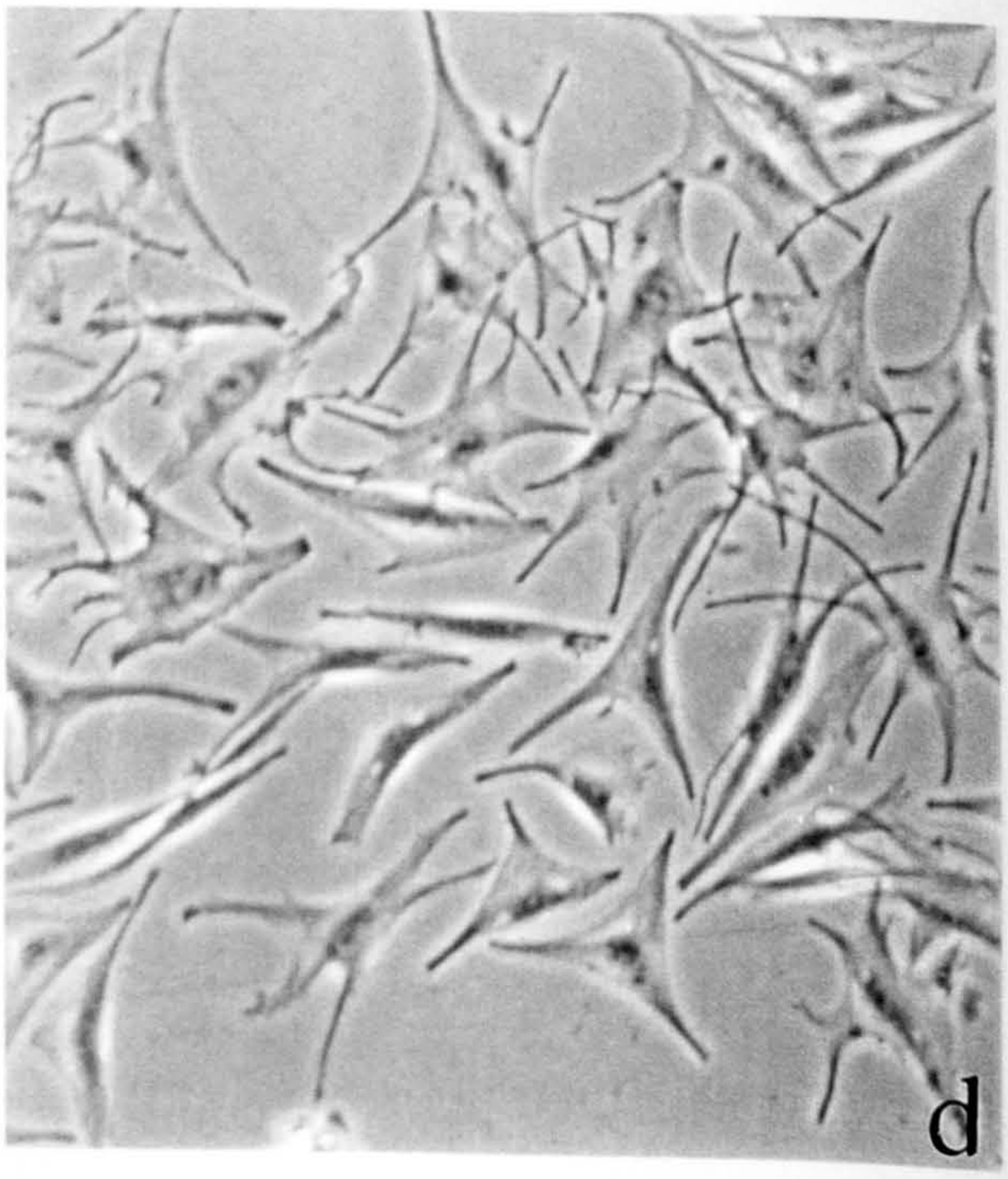
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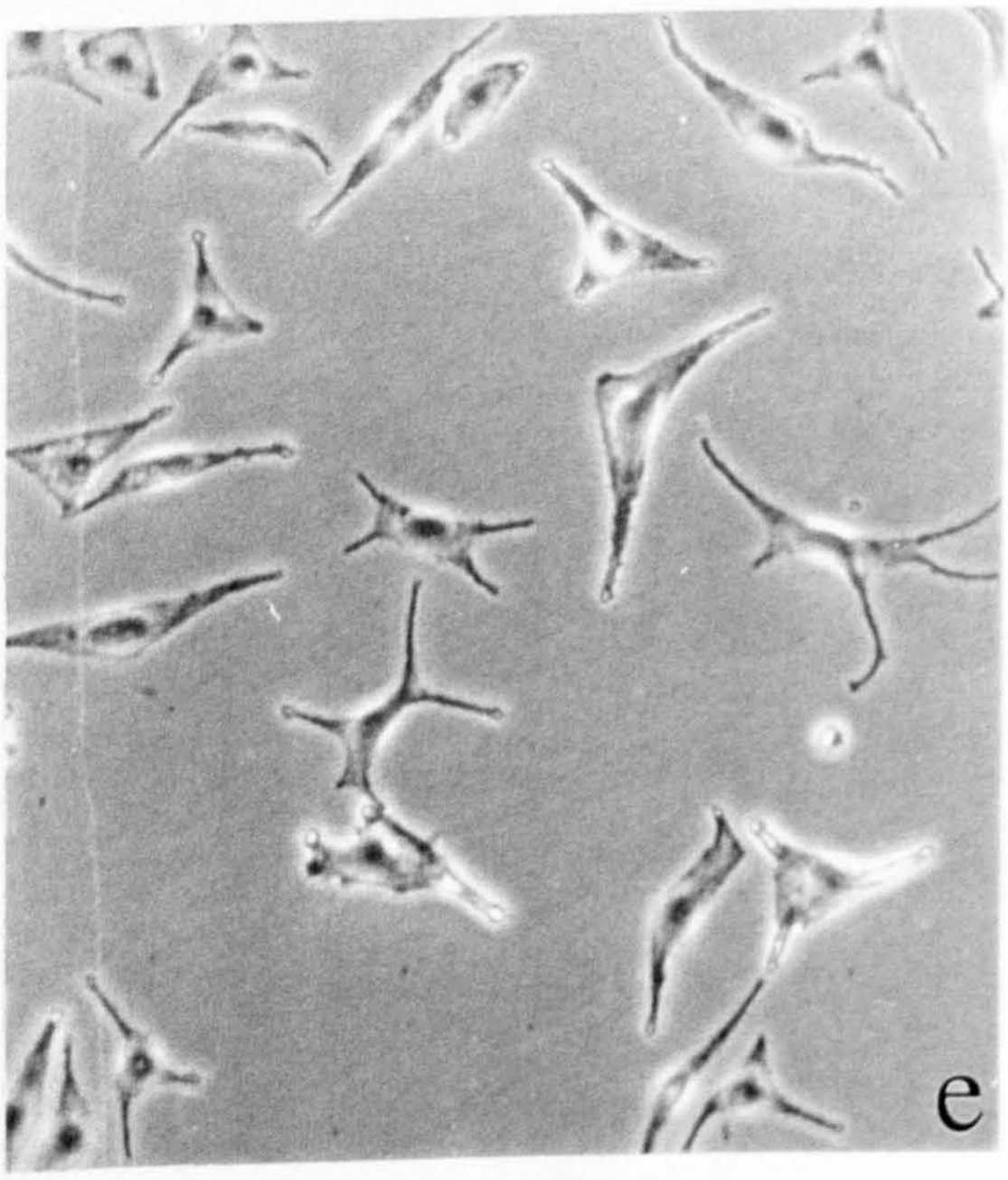
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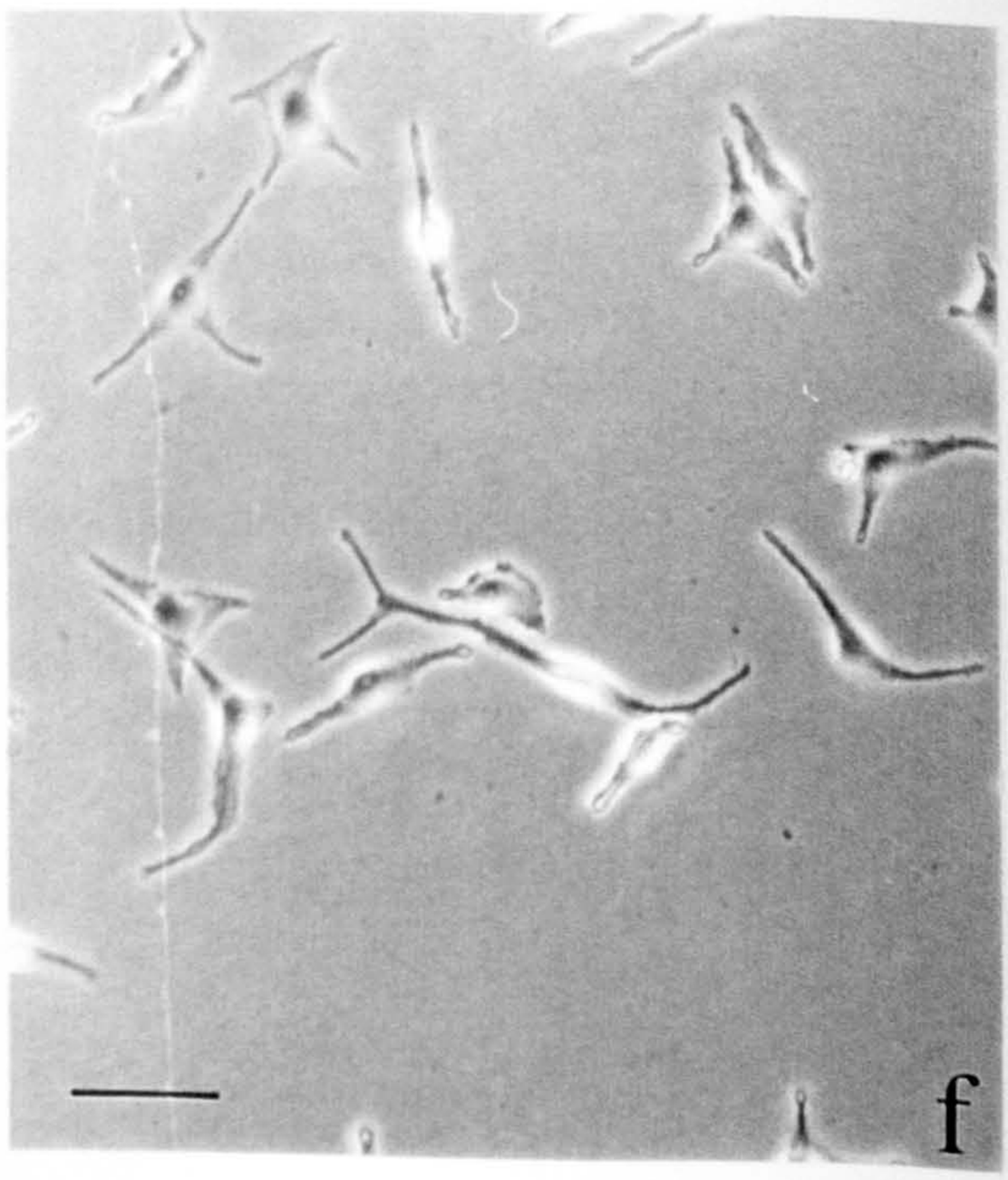
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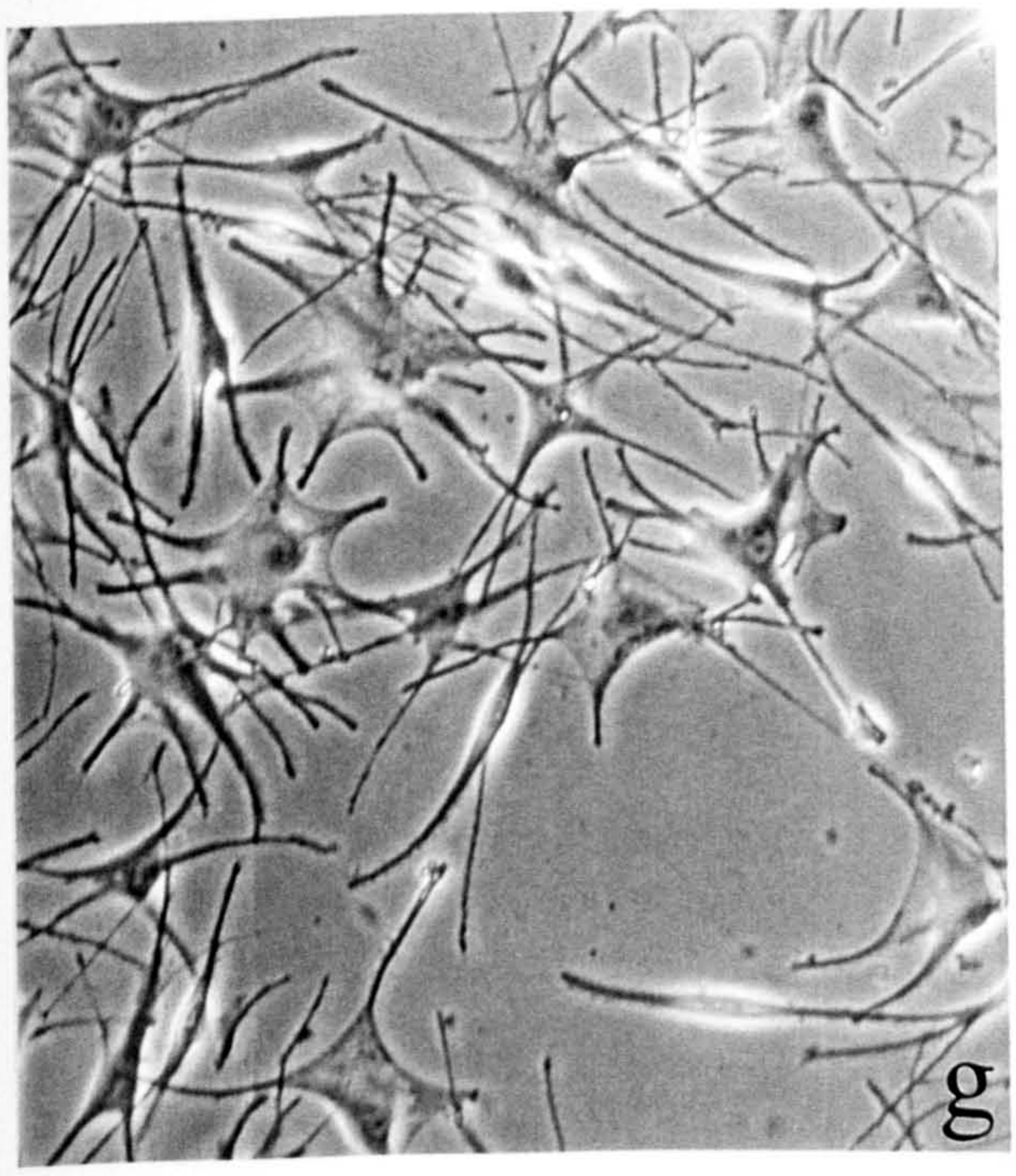
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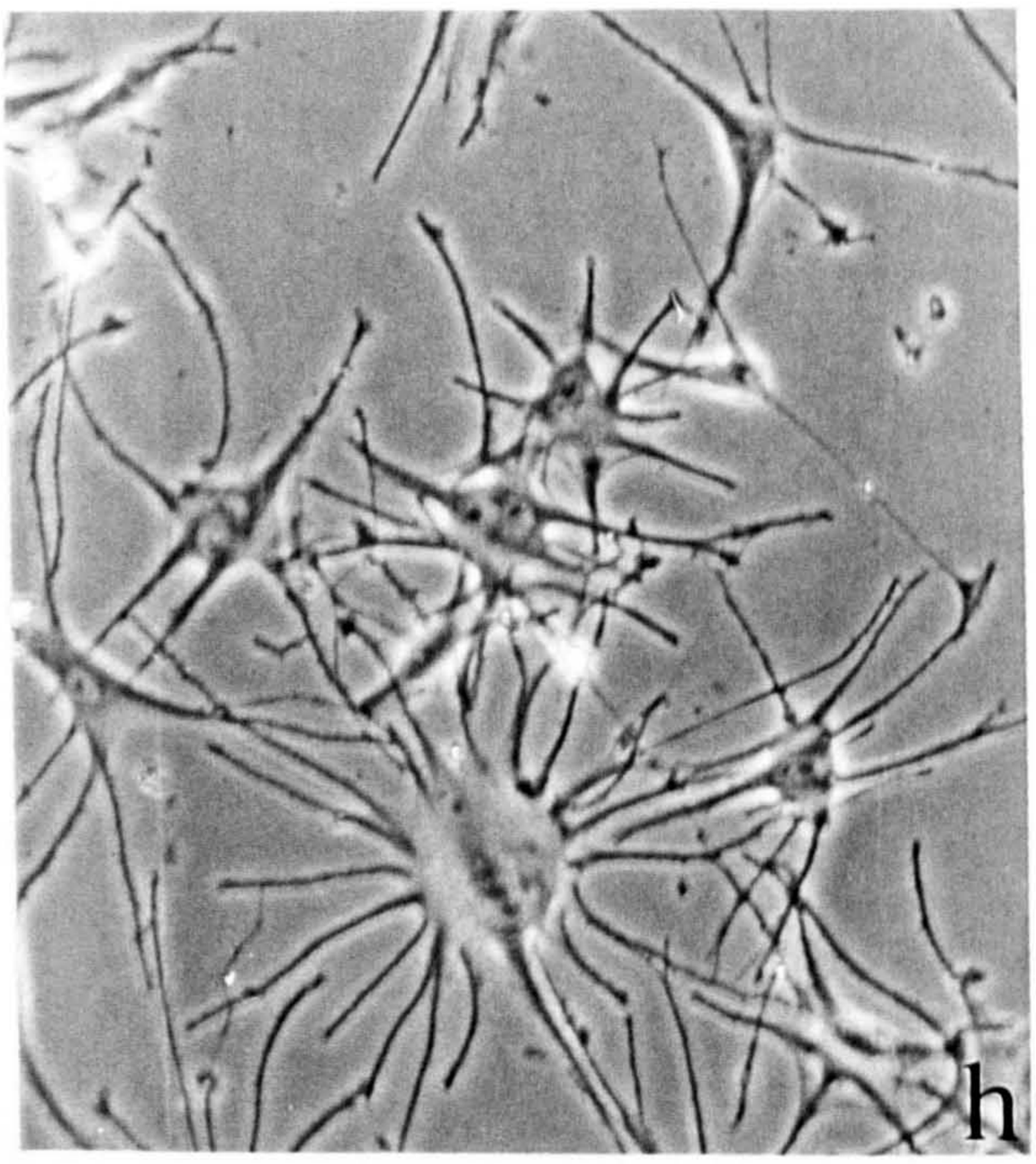
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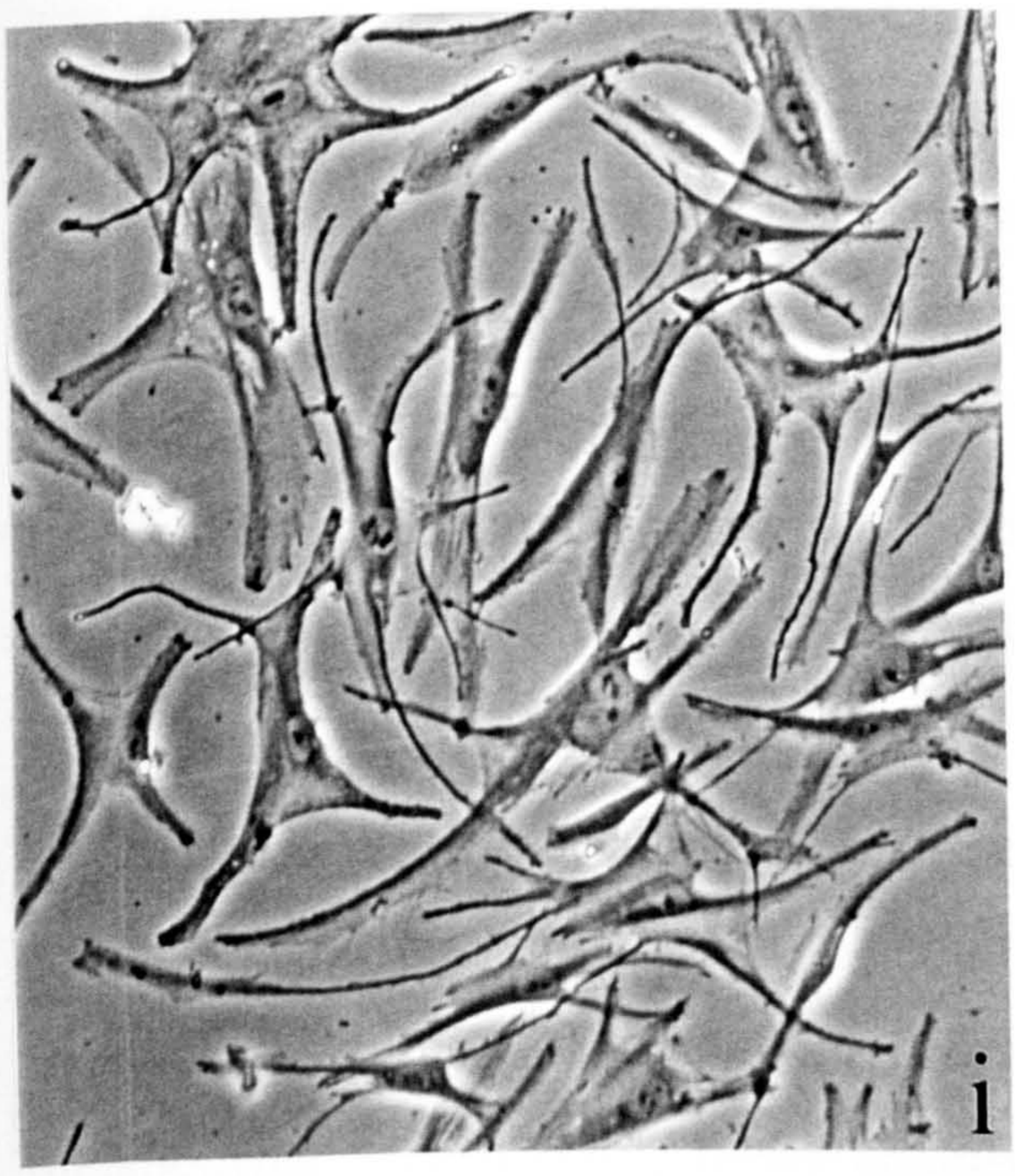
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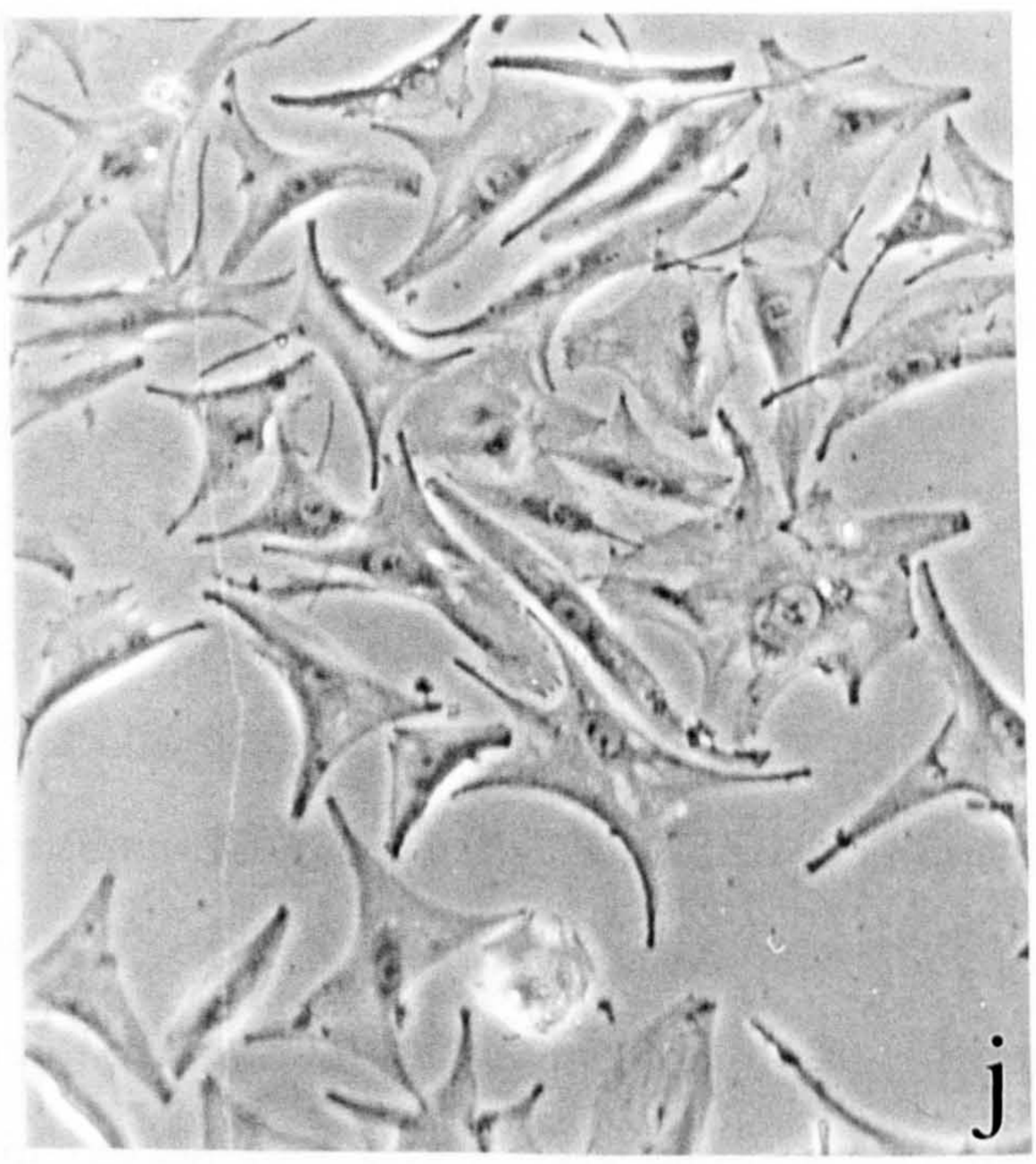
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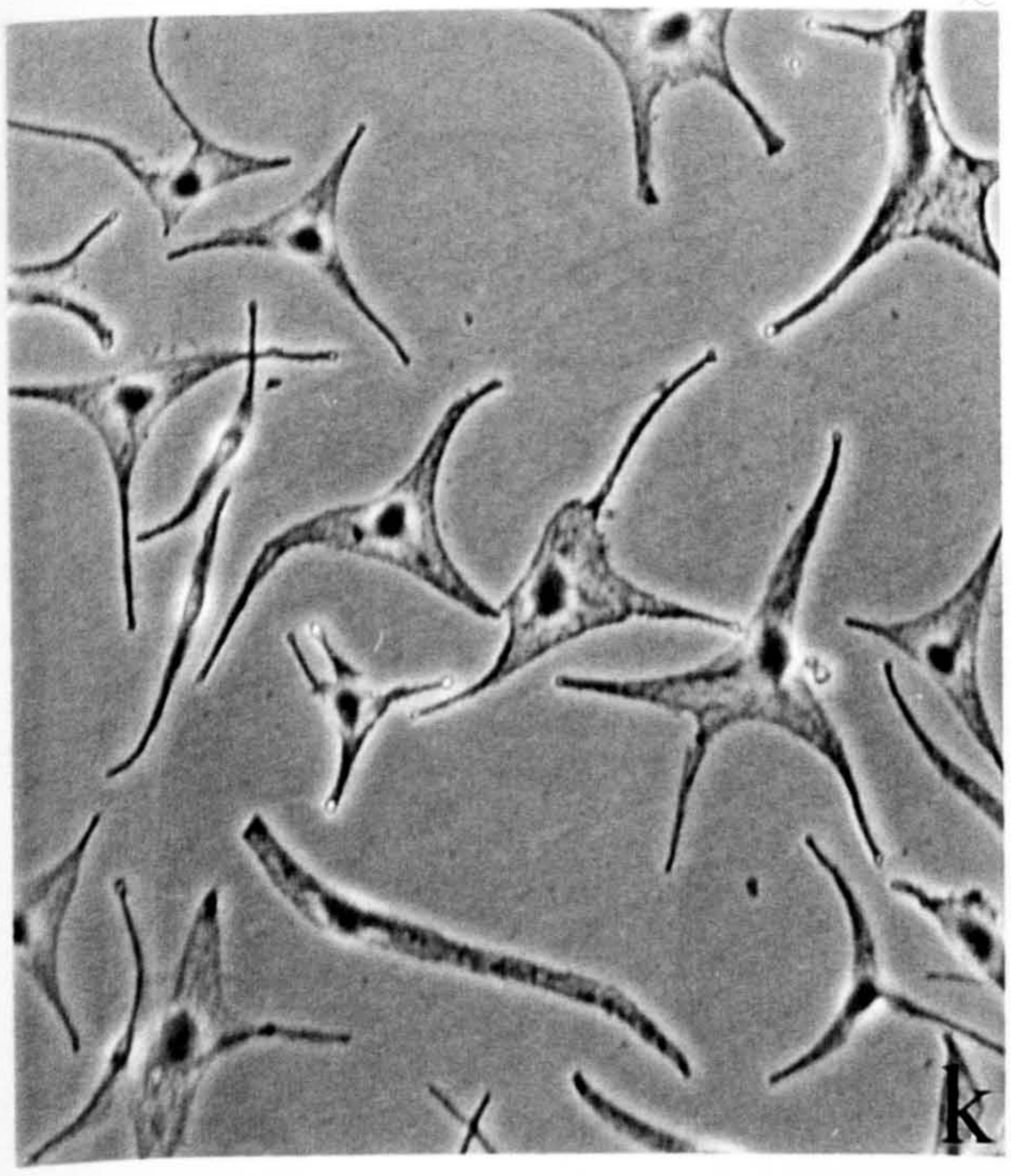
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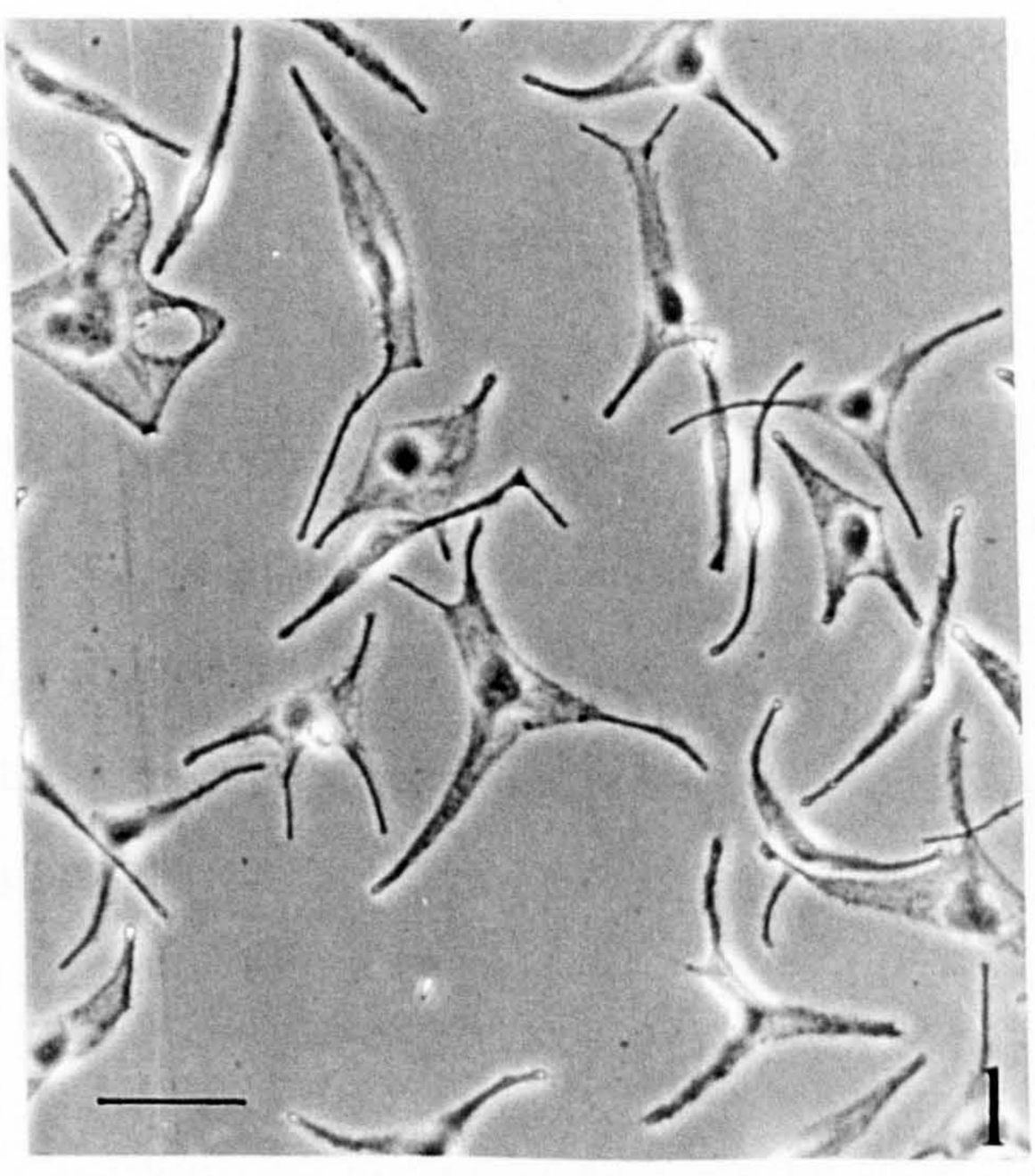
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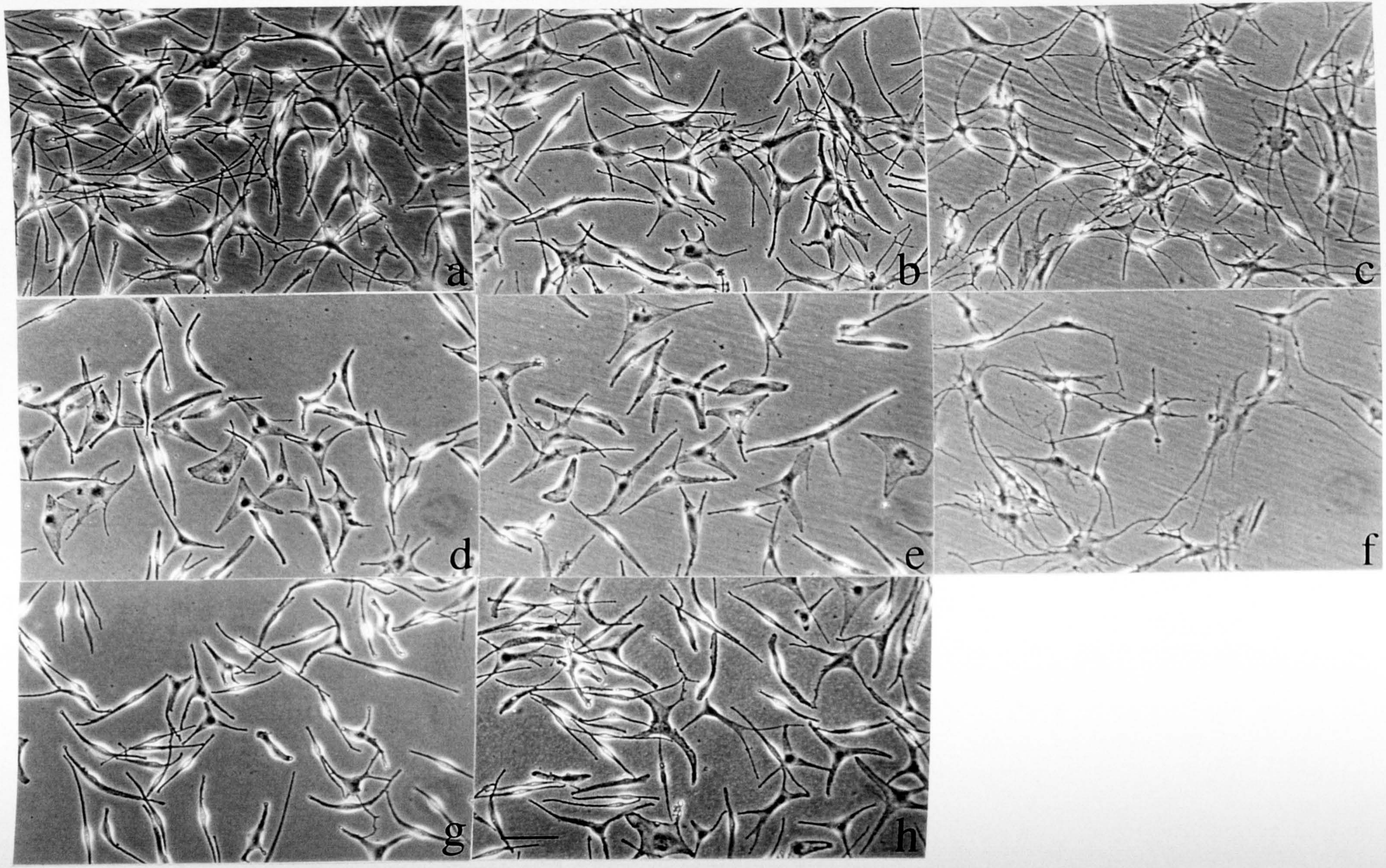
UVB increased the size of both normal and vitiligo melanocytes in the more proliferative media and dendrite number was also increased in medium 1. The melanocytes from donor VM3, were slightly larger than their paired normal compatriots (particularly in medium 24), but the vitiligo melanocytes were longer in size, in culture prior to experimentation (as illustrated in **Figures 3.3j-k** and **3.3i** for melanocytes from donor VM3 and M577 respectively).

3.2.6 Effect of media composition and cytokines on normal and vitiligo melanocyte morphology

The effect of cytokines (present for the final 24 hours of a 5 day experimental period) was examined in normal and vitiligo melanocytes from donors M843 and VM4 respectively to check that at the concentrations used, they were not harming the melanocytes. Normal and vitiligo melanocytes exhibited characteristic morphology in experimental media 1 (**Figures 3.9a** and **3.9i** for normal and vitiligo melanocytes respectively), 10 (**Figures 3.9d** and **3.9l** for normal and vitiligo melanocytes respectively) and 24 (**Figures 3.9g** and **3.9o** for normal and vitiligo melanocytes respectively). The vitiligo melanocytes were slightly larger than the normal melanocytes in all the media conditions. The addition of 100U/ml IFN-γ to media 1 slightly increased the cell body size of both normal (**Figure 3.9b**) and vitiligo (**Figure 3.9j**) melanocytes. In media 10 (**Figures 3.9e** and **3.9m** for normal and vitiligo melanocytes respectively) and 24 (**Figures 3.9h** and **3.9p** for normal and vitiligo melanocytes respectively), IFN-γ slightly increased cell size. The addition of 25% lymphocyte conditioned medium (LCM) to medium 1 and 10 induced a raggedness to both normal (**Figures 3.9c** and **3.9f** for media 1 and 10 respectively) and vitiligo (**Figures 3.9k** and **3.9n** for media 1 and 10 respectively) melanocytes.

Typical morphology was seen for both normal and vitiligo melanocytes under the 3 media conditions with IFN-γ increasing melanocyte size slightly. The LCM (which would have contained a mixture of cytokines) induced a large morphological difference, in which the cells looked more ragged and stressed. TNF-α did not have any influence on melanocyte morphology at 100U/ml (not shown).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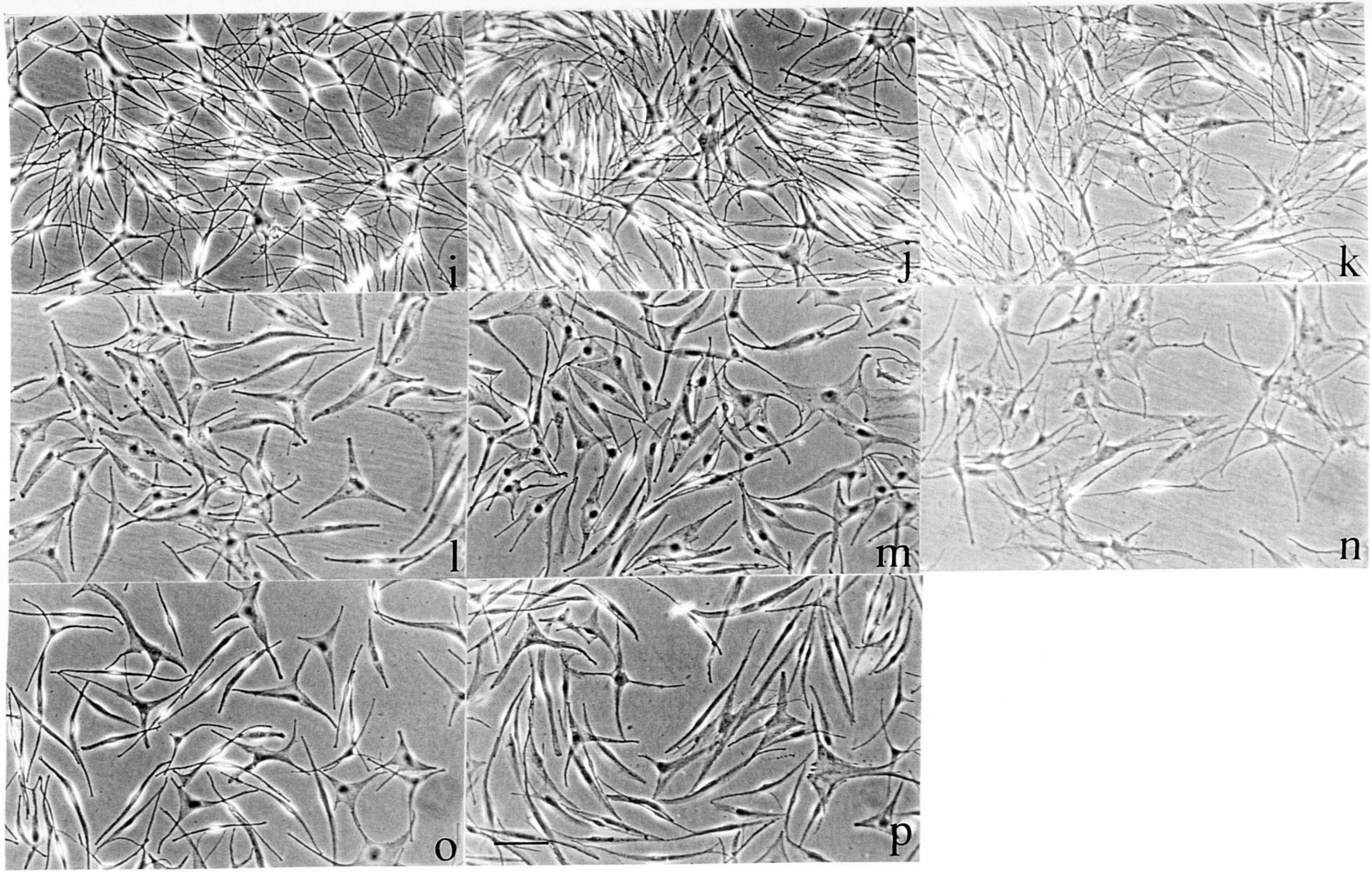
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3.3 MODULATION OF NORMAL AND VITILIGO MELANOCYTE PROLIFERATION

3.3.1 Influence of 25 media varying in mitogenic potency on normal melanocyte proliferation.

Commonly used mitogens in melanocyte culture media are generally non-physiological (i.e. PMA or CT) or undefined (i.e. serum or pituitary extracts). In **Section 3.2** it was seen that they can have a strong influence on melanocyte morphology. However mitogen rich melanocyte media may mask the effects of natural mitogens or melanogens such as α-MSH. This section contains data from melanocytes examined for the effects of α-MSH on proliferation and pigmentation (results described in **Sections 3.3.2** and **3.4.2**). Cell number attained in the various media variants (described in **Table 2.2**) after a 5 day culture period was assessed by either cell counts (as described in **Section 2.7.1**) or by the DNA assay (as described in **Section 2.7.4**).

Medium 1 which closely resembled maintenance medium A, induced the greatest increase in cell number by 3.5 fold (mean value, $p<0.001$) from the day of plating (in total 6 days) and therefore was used as the control medium (individual data for cell number obtained in this medium for melanocytes from each donor are illustrated in **Table 3.4**). Results for all other media were expressed as a percent of medium 1 for graphic representation (**Figure 3.10a**). Medium 2 (9% less cells than medium 1 ($p<0.05$)) and medium 25 (10% less cells) are maintenance media C and B respectively. The removal of mitogens (either 1 at a time or more) significantly reduced the proliferative potential of all other media examined in comparison to medium 1. All media containing BPE (of which there were 13), were in the top 15 most mitogenic media. Therefore, in this MCDB153 culture system, BPE was a vital component for good proliferation. Media 12 (containing FCS/bFGF), 18 (containing PMA/bFGF) and 23 (containing bFGF) were the three least proliferative media with proliferation rates 3.1 fold, 3.2 fold and 3.8 fold less than medium 1, respectively. Surprisingly, all three contained bFGF, a well known melanocyte mitogen.

Table 3.4 Investigation of proliferation, dopa oxidase activity and melanin content obtained in melanocytes from 13 different donors cultured in medium 1 for 5 days.

	Cell number x 10^4		Dopa oxidase activity (ΔO.D.492nm/min/well/10^4cells x 10^{-4})		Melanin (μg/ml/10^4 cells)	
Donor	-α-MSH	+α-MSH	-α-MSH	+α-MSH	-α-MSH	+α-MSH
M765 Prim[a]	3.31	3.44	0.69	1.21	ND[b]	ND
M759 P1[a]	3.13	3.44	0.11	0.08	3.9	3.14
M740 P4[a]	7.61	5.15	1.21	2.3	4.51	5.06
M759 P4[a]	2.58	2.45	ND	ND	5.26	5.01
M833 Prim[c]	3.98	5.14	ND	ND	0.63	0.75
M837 Prim[d]	2.35	1.86	0.57	1.52	0.22	0.76
M823 P1[d]	3.22	3.74	2.8	2.84	1.85	2.69
M829 P1[d]	6.59	6.45	2.15	2.62	0.98	1.35
M830 P1[c]	9.03	12.17	2.69	2.2	0.43	2.3
M832 P1[c]	4.48	4.58	4.2	3.84	0.34	1.08
M835 P1[c]	3.33	4.03	1.36	1.66	0.96	0.89
M840 P1[d]	7.37	7.64	0.92	1.03	0.33	0.48
M741 P4[c]	9.08	9.29	1.88	1.78	0.61	0.86
M746 P4[d]	4.33	4.9	0.92	1.02	0.45	0.58

Melanocytes from 13 different donors (primary, P1 and P4; M759 was used at P1 and P4) were plated at 2 to 4 x 10^4 cell/well (12 wells in duplicate for melanocytes from donors M759 and M740) in maintenance medium B or 1.5 to 2 x 10^4 cells/well (24 well in triplicate) in maintenance medium C and allowed to adhere for 24 hours before medium was changed to experimental medium 1. Primary cells were seeded at 1 x 10^6 epidermal cells/well (12 well for melanocytes from donor M765) or 0.75 to 1 x 10^5 cells/well (24 well) and cultured for 2^{-3} weeks before experimental procedure. Cells were cultured for a further 5 days in the presence or absence of 10^{-10}M α-MSH (for cells cultured in 12 wells) or 10^{-8}M α-MSH (for cells cultured in 24 wells) before **(a)** cell number was analysed by counts (as described in **Section 2.7.1** for cells cultured in 12 wells) or the DNA assay (as described in **Section 2.7.4** for cells cultred in 24 wells) **(b)** dopa oxidase activity was analysed (as described in **Sections 2.8.2c** and **2.8.2d** for cells cultured in 12 and 24 wells respectively) and **(c)** melanin content was analysed (as described in **Section 2.8.3**). This table is to represent the donor to donor varability and statistical analysis on the correlation between cell number and pigmentation are shown in **Figures 3.21** and **3.22**.

Table 3.4:

[a] In addition to being cultured in medium 1 melanocytes from donors M759, M740 and M765 were cultured in experimental media 3, 4, 6, 8, 10, 11, 13, 14, 15, 17, 19, 20, 21, 22, 24 and 25 (refer to **Table 2.2** for composition of media) for 5 days in the presence or absence of 10^{-10}M α-MSH (data not shown).

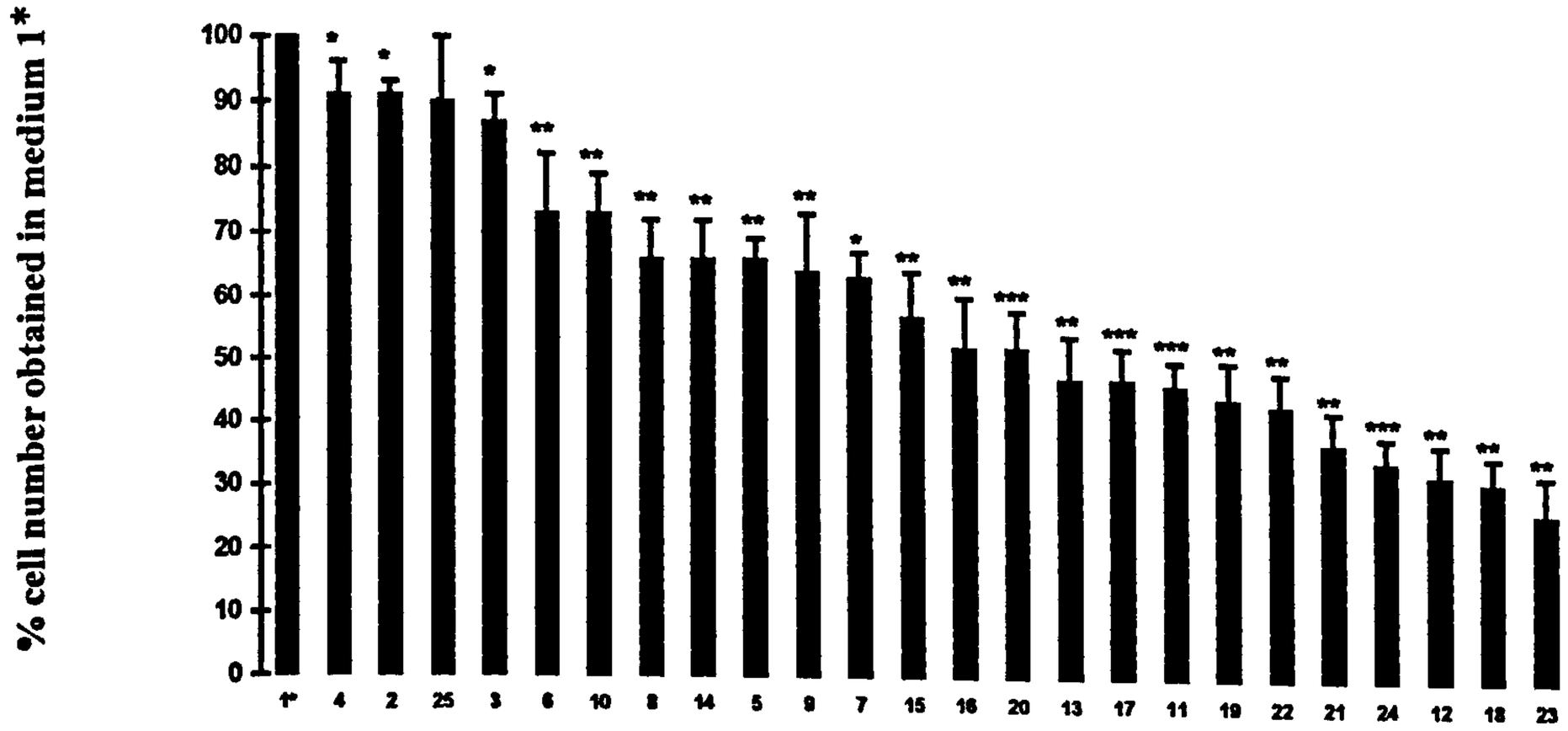
[b] ND - not done.

[c] In addition to being cultured in medium 1 melanocytes from donor M741, M830, M832, M833 and M835 were cultured in experimental media 3, 4, 6, 8, 10, 11, 13, 14, 15, 17, 19, 20, 21, 22 and 24 (refer to **Table 2.2** for composition of media) for 5 days in the presence or absence of 10^{-8}M α-MSH (data not shown).

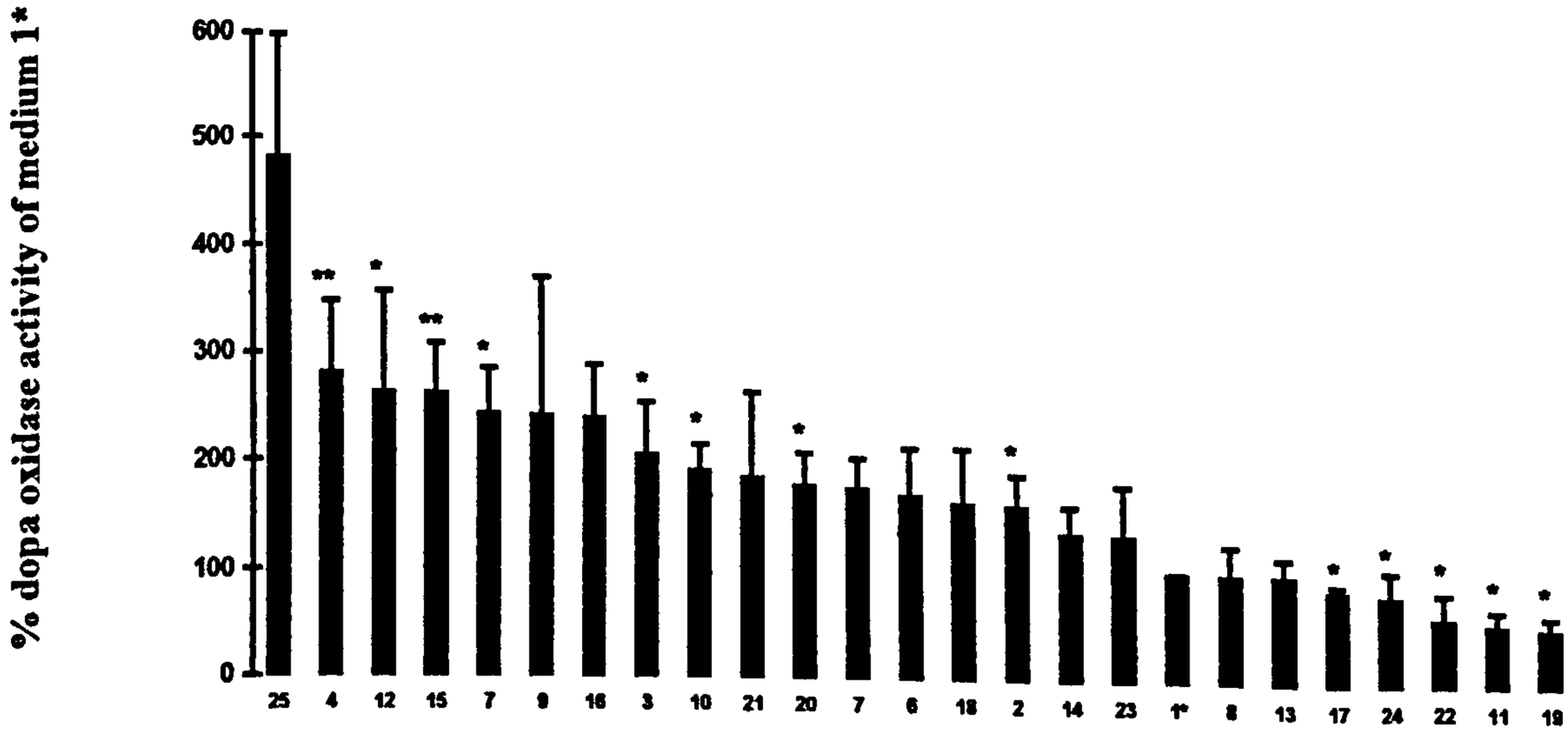
[d] In addition to being cultured in medium 1 melanocytes from donor M746, M823, M829, M837 and M840 were cultured in experimental media 2, 5, 7, 9, 12, 16, 18, 23 and 24 (refer to **Table 2.2** for composition of media) for 5 days in the presence or absence of 10^{-8}M α-MSH (data not shown).

Figure 3.10 Effect of media on melanocyte proliferation (a), dopa oxidase activity (b) and melanin content (c). Melanocytes from 13 different donors (primary, P1 and P4) were plated (as described in **Table 3.4**) and then cultured in experimental media as indicated by the numbers (composition of media variants described in **Table 2.2**) for 5 days. Cell number, dopa oxidase activity and melanin were analysed (as described in **Table 3.4**). The results are expressed as % of parameter response in medium 1 and are means ± SEM of 5-14 experiments (individual results for medium 1 of each parameter for all melanocyte donors are illustrated in **Table 3.4**). Parameter functions that differed significantly from medium 1 are indicated by *=$p<0.05$, **=$p<0.01$, and ***=$p<0.001$ based on Student's paired t-test (performed on data before normalisation).

a



b



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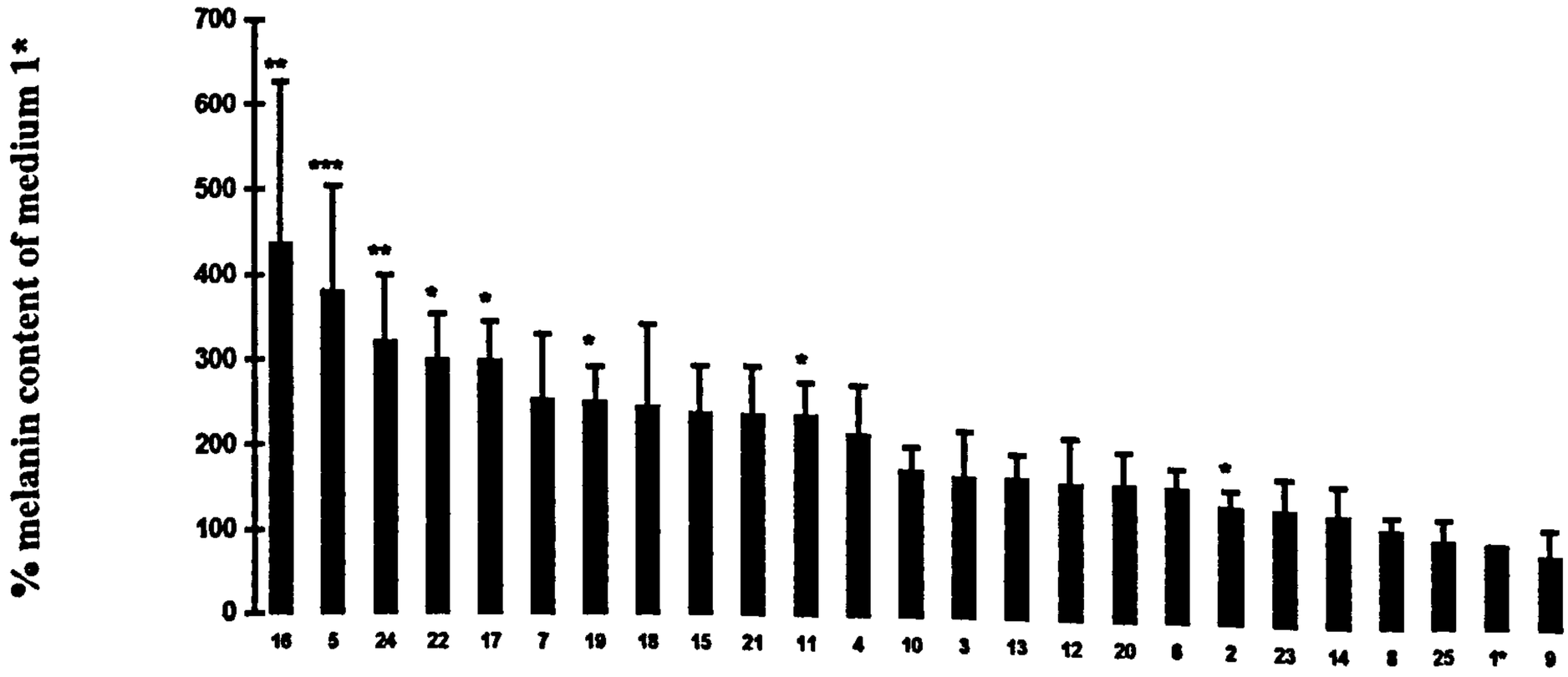


Media variants

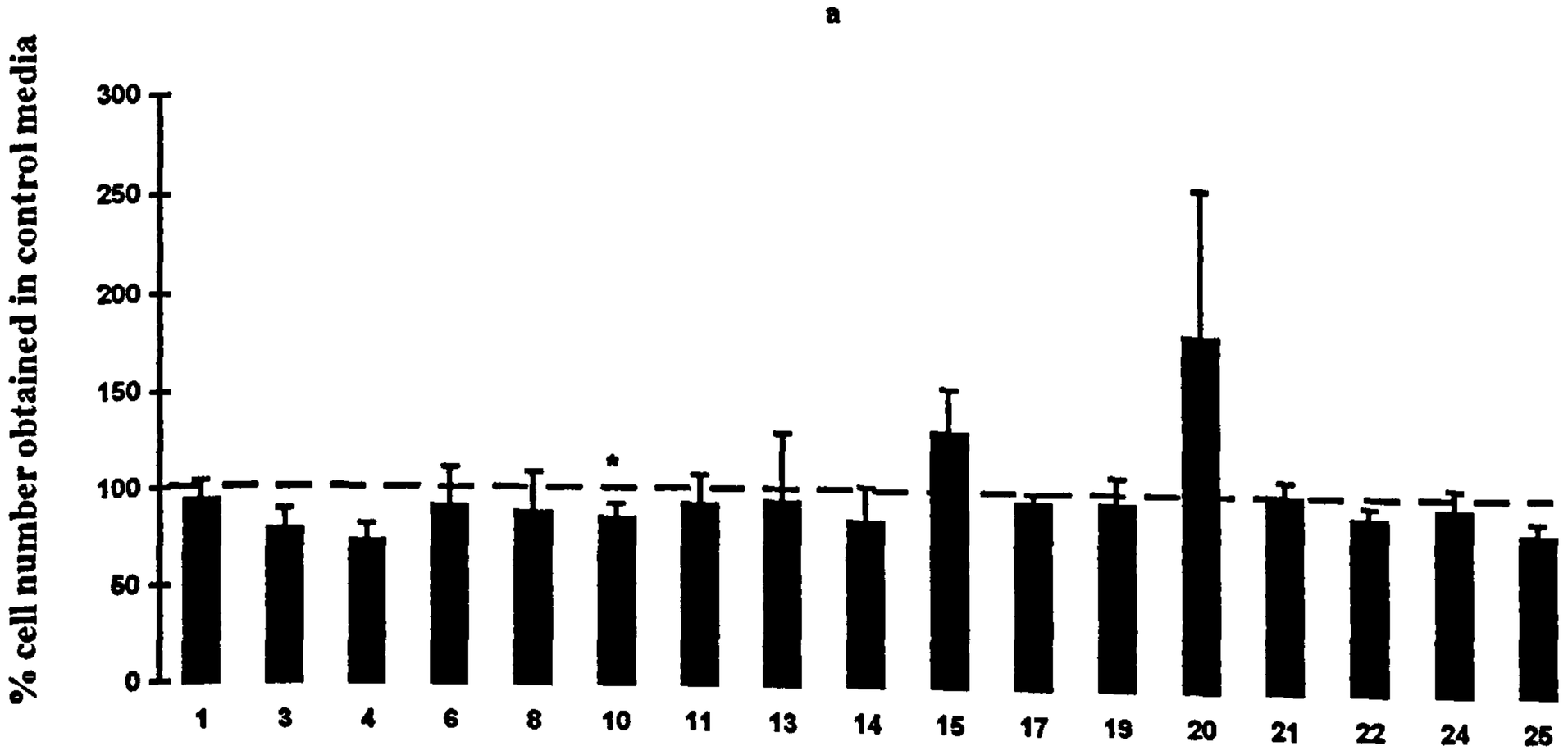
3.3.2 Effect of α-MSH on melanocyte proliferation in 25 different media variants.

In an initial study to examine the effects of α-MSH on pigmentation (described in **Section 3.4.7**) there was little response and responses were not reproducible. Accordingly, a wide range of media were employed to investigate which culture conditions were best to see reproducible proliferative and pigmentary responses to α-MSH. Culturing cells with 10^{-10}M α-MSH for 5 days (**Figure 3.11a**) generally had little or no effect on cell number, except in medium 10 (containing FCS/BPE) where α-MSH significantly reduced cell number by 15% ($p<0.05$). α-MSH at 10^{-8}M significantly increased cell number in only 4 of the 24 media by 10% (medium 1, containing FCS/BPE/CT/PMA), by 61% (medium 12, containing FCS/bFGF), by 47% (medium 15, containing BPE/CT) and by 45% (medium 18, containing PMA/bFGF) (all $p<0.05$) (**Figure 3.12a**). Generally, in media containing two, one or no mitogens, cell proliferation was increased in the presence of 10^{-8}M α-MSH but due to donor to donor variation, statistical significance was not achieved in all cases. These increases ranged from 11% (medium 11, containing FCS/CT) to 61% (medium 12, containing FCS/bFGF, $p<0.05$).

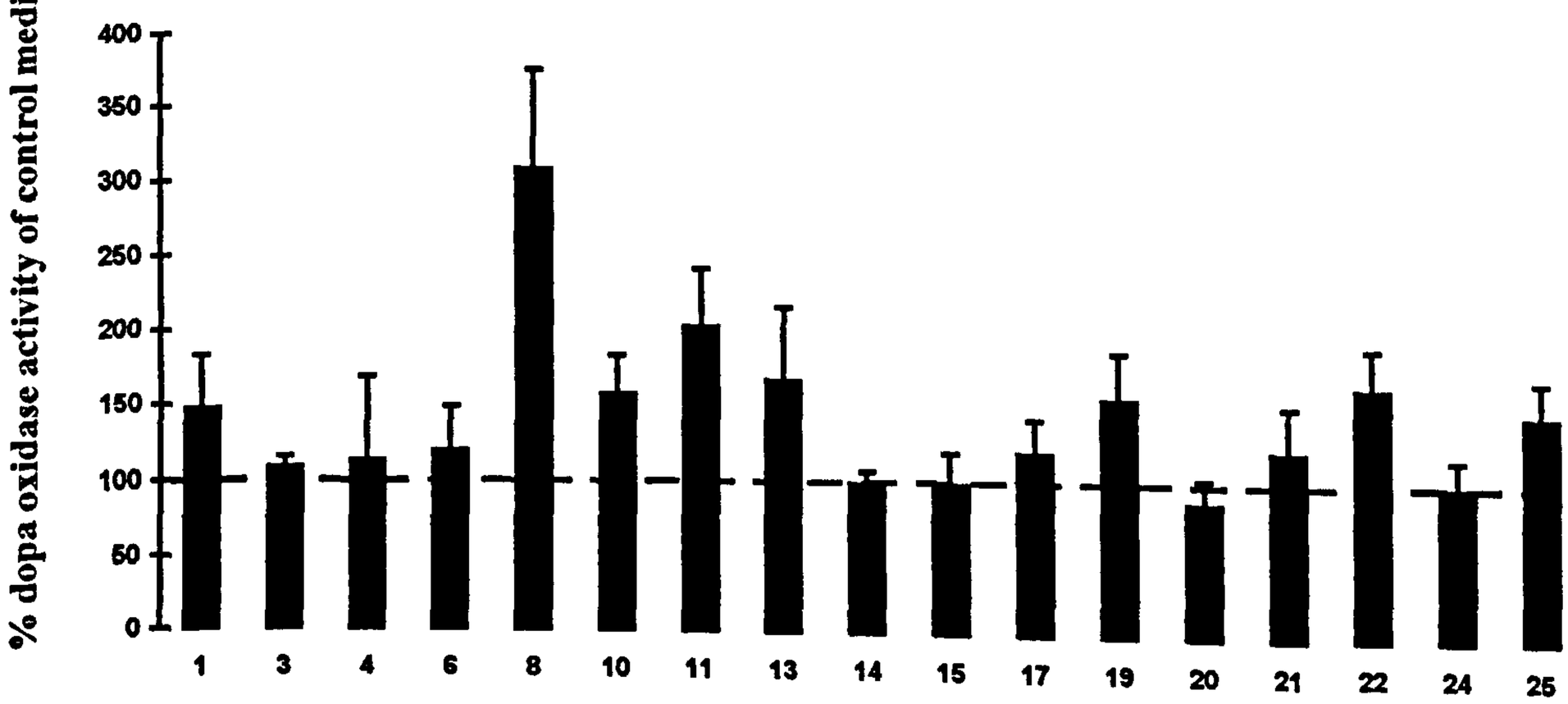
Cell proliferation was also increased by 38% in medium 23 (only containing the mitogen bFGF) but just failed to reach statistical significance ($p<0.051$). However including data from a further 6 donors, a significant increase in proliferation of 59% ($p<0.01$) was obtained in medium 23 in response to 10^{-8}M α-MSH. Further work in medium 23 (due to the ICAM-1 study described in **Section 4.4**) showed that α-MSH increased cell proliferation in a dose dependent manner irrespective of the presence of TNF-α (**Figure 3.13a** and **Tables 3.5a-b**). In the absence of TNF-α, the cell number was significantly increased by 42% at 10^{-10}M, by 75% at 10^{-9}M and by 76% at 10^{-8}M α-MSH (all $p<0.05$) while in the presence of TNF-α, the cell number was significantly increased by 50% at 10^{-10}M, by 56% at 10^{-9}M and by 59% at 10^{-8}M α-MSH (all $p<0.01$). No significant effect on melanocyte proliferation was seen in response to addition of α-MSH for the final 24 hours irrespective of the presence of TNF-α. When cells were cultured with 10^{-8}M α-MSH for 5 days, increasing concentrations of TNF-α

Figure 3.11 Effect of 10^{-10}M α-MSH on cell number (a), dopa oxidase activity (b), and melanin content (c). Melanocytes from 3 different donors were plated on plastic in maintenance medium B, and allowed to adhere for 24 hours (P1 and P4) before medium was changed to those indicated by the numbers, in the absence or presence of α-MSH (as described in **Table 3.4**). Primary cells were cultured for 2-3 weeks before experimental procedure. Cells were cultured for a further 5 days in these media before cell counts, dopa oxidase activity and melanin were analysed (as described in **Sections 2.7.1, 2.8.2c** and **2.8.3** respectively). For each parameter, the results are expressed as % of control in response to α-MSH and are means ± SEM of 2-4 experiments. Parameter function significantly altered by α-MSH is indicated by *=$p<0.05$ based on Student's paired t-test performed on the data before normalisation.

a



b



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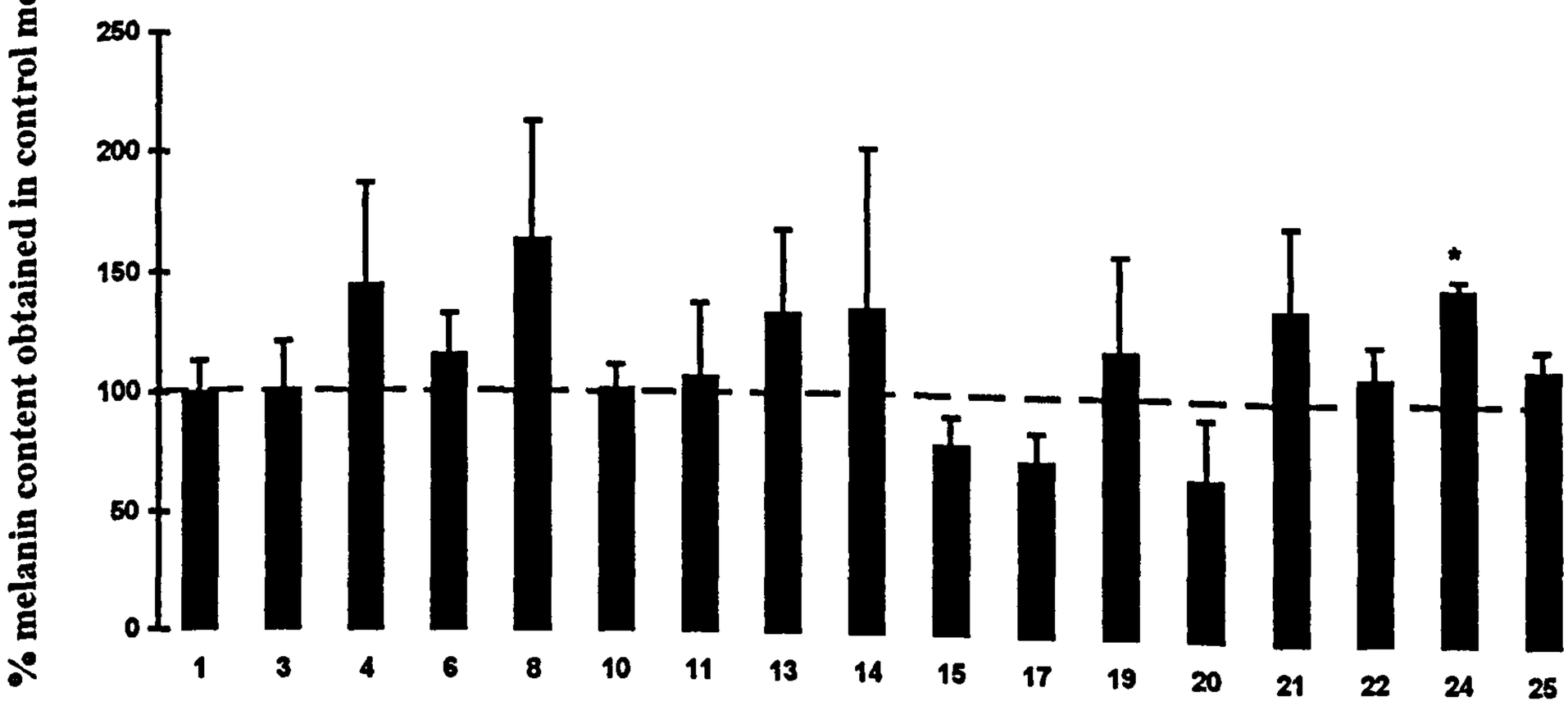
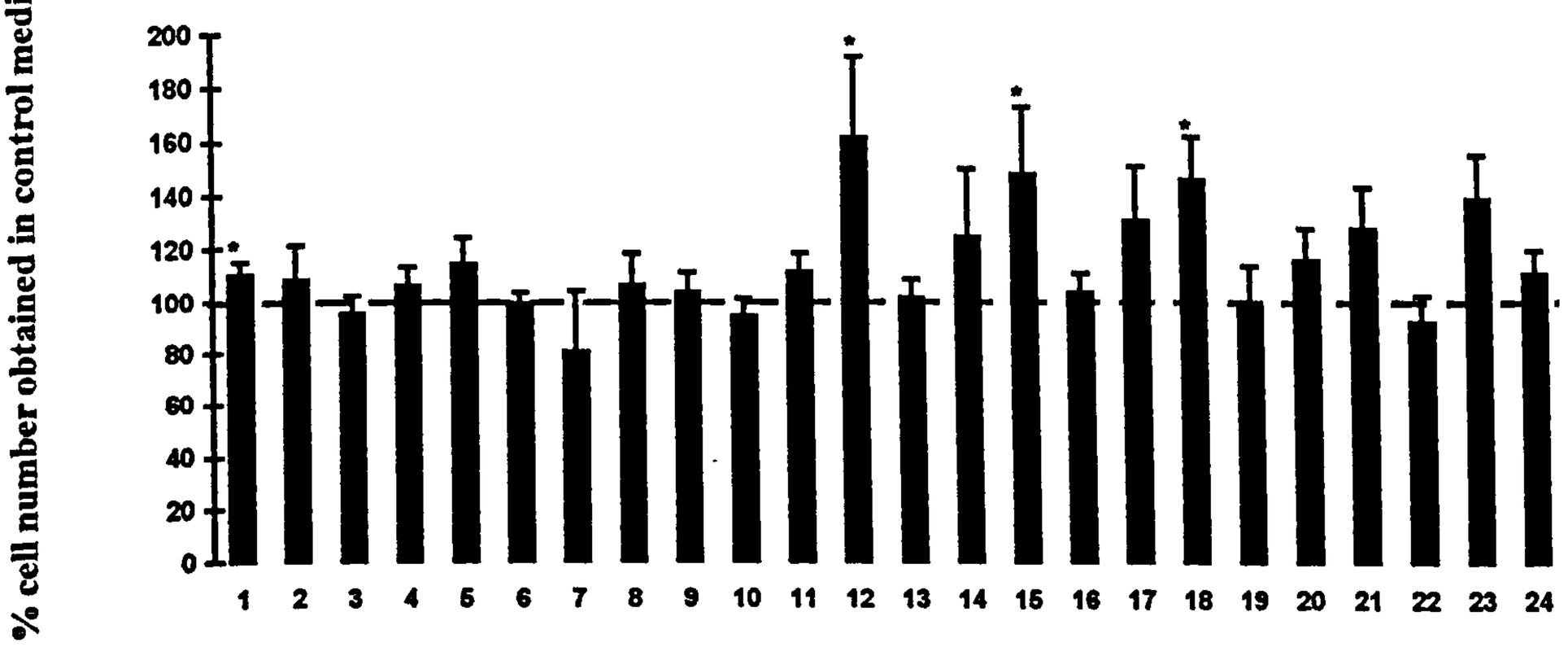


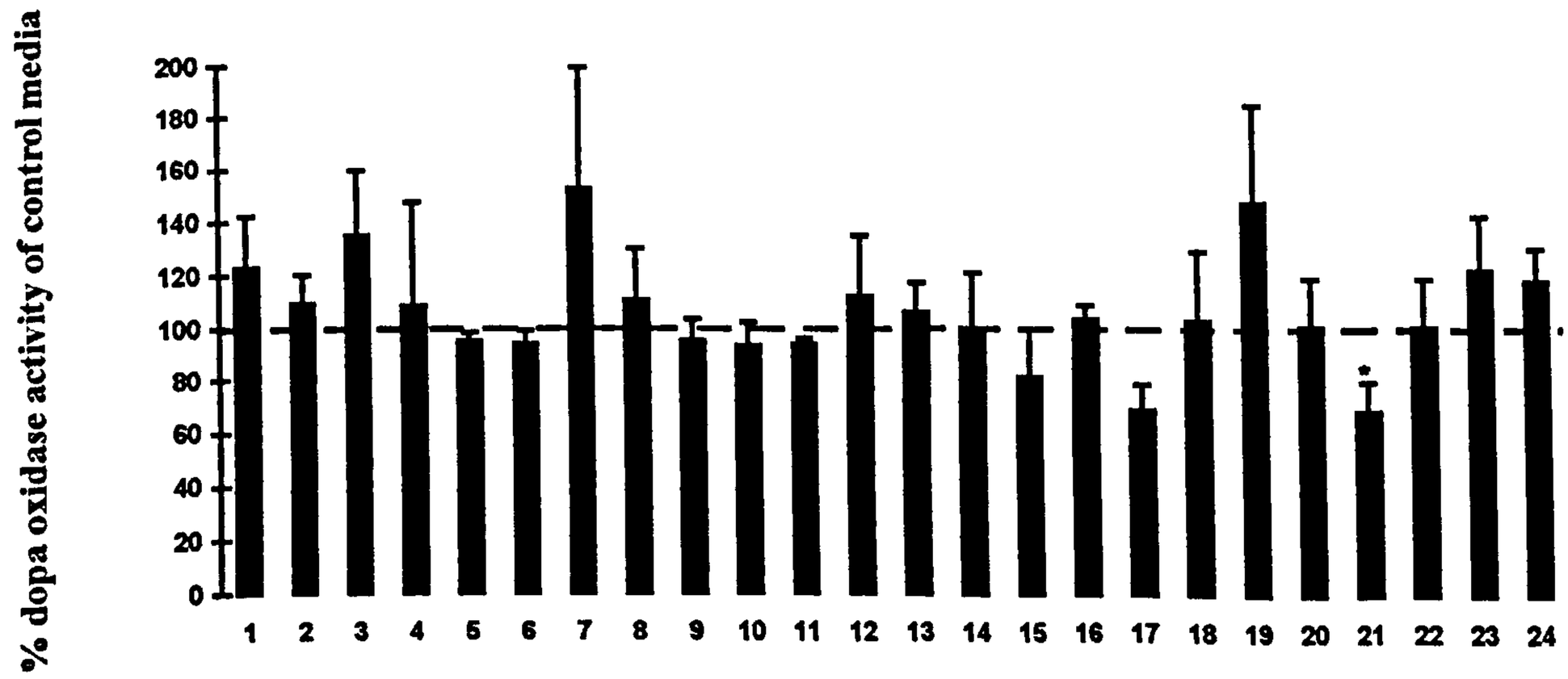
Media variants

Figure 3.12 Effect of 10^{-8}M α-MSH on cell number (a), dopa oxidase activity (b), and melanin content (c). Melanocytes, primary, P1 and P4 were plated on plastic in maintenance medium C, and allowed to adhere for 24 hours (P1 and P4) before medium was changed to those indicated by the numbers (composition of media described in **Table 2.2**), in the absence or presence of α-MSH (as described in **Table 3.4**). Primary cells were cultured for 2-3 weeks before experimental procedure. Cells were cultured for a further 5 days in these media before DNA, dopa oxidase activity and melanin analysis (details in **Sections 2.7.4, 2.8.2d** and **2.8.3**, respectively). For each parameter, the results are expressed as % of control in response to α-MSH and are means ± SEM of 5 experiments (except media 1 and 24, where n=10 experiments). Parameter function significantly altered by α-MSH is indicated by *=$p<0.05$ and **=$p<0.01$ based on Student's paired t-test performed on the data before normalisation.

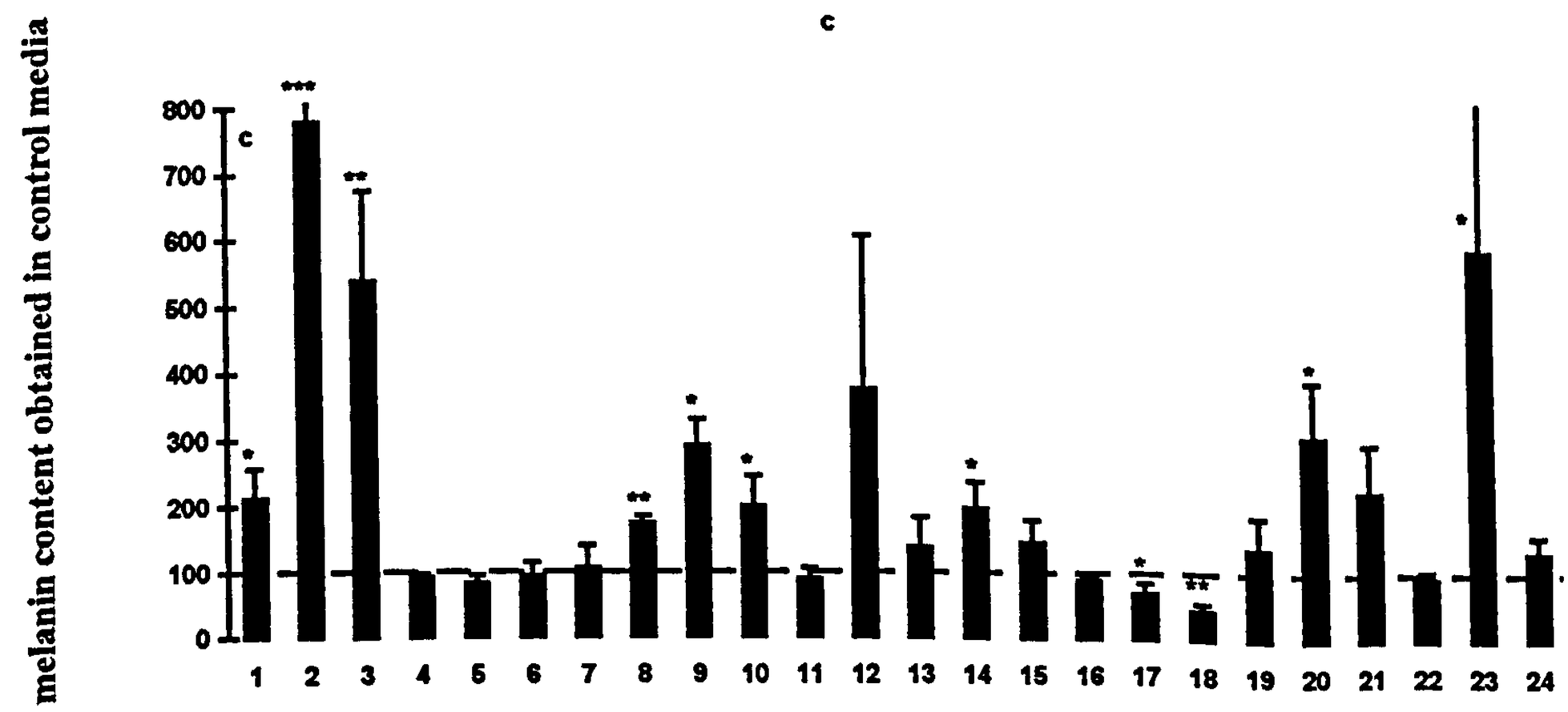
a



b



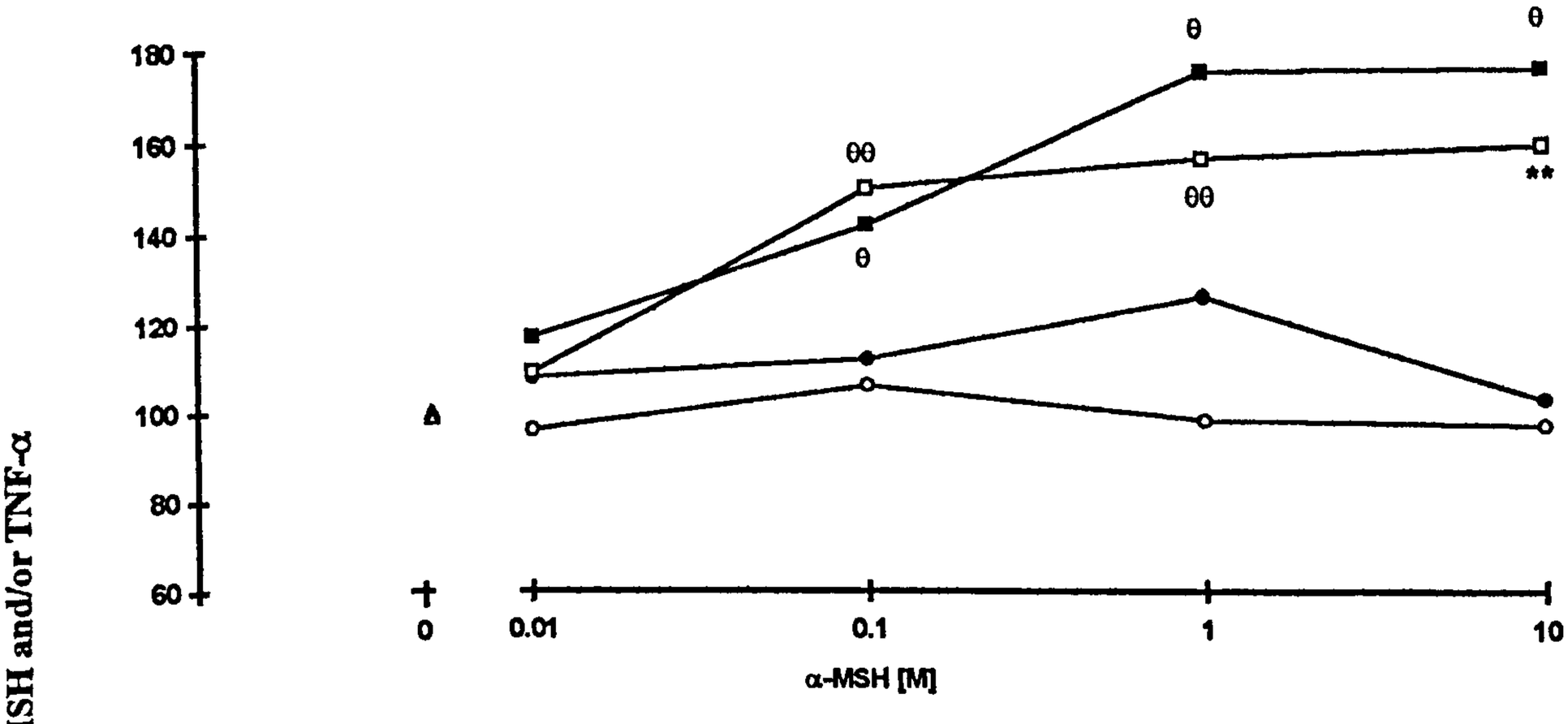
c



Media variants

Figure 3.13 Comparison of the effects of α-MSH and TNF-α on melanocyte cell number. P4 melanocytes were cultured in medium 23 for 5 days (as described in **Table 3.4**). α-MSH was added either for the final 24 hours or at the 3 changes of feed throughout the experimental period, while TNF-α was added for the final 24 hours. Cell number was analysed by the DNA method (as described in **Section 2.7.4**) or cell counts (as described in **Section 2.7.1**). The data has been expressed as percentage of the control (untreated cells) in response to α-MSH and/or TNF-α and are means ± SEM of (3-8) experiments. (Individual data for each experiment is represented in **Table 3.5**). A dose response curve to α-MSH (10^{-11} - 10^{-8}M) is illustrated in (**a**). Cells were untreated (control, closed triangle), treated with 100U/ml TNF-α for the final 24 hours (open triangle), preincubated with MSH without TNF-α (closed square) or with 100U/ml TNF-α for the final 24 hours (open square), or treated with α-MSH for the final 24 hours without TNF-α (closed circle) or with 100U/ml TNF-α (open circle). A dose response curve to TNF-α (1U/ml to 500U/ml) (closed diamond) is illustrated in (**b**). Untreated control cells are represented as a open circle. The influence of preincubation with 10^{-8}M α-MSH (closed triangle) or the presence of 10^{-8}M α-MSH for the final 24 hours (closed square) on the dose response to TNF-α is also shown. Significant differences in cell number in response to α-MSH are indicated by *=$p<0.05$ and **=$p<0.01$ or by $^{\theta}$=$p<0.05$ and $^{\theta\theta}$=$p<0.01$ based on Student's paired and unpaired t-test respectively performed on the data before it was normalised.

a


% control in response to α-MSH and/or TNF-α
180
160
140
120
100
80
60
A
θ
θθ
θθ
θ
θ
**
0
0.01
0.1
1
10
α-MSH [M]

b

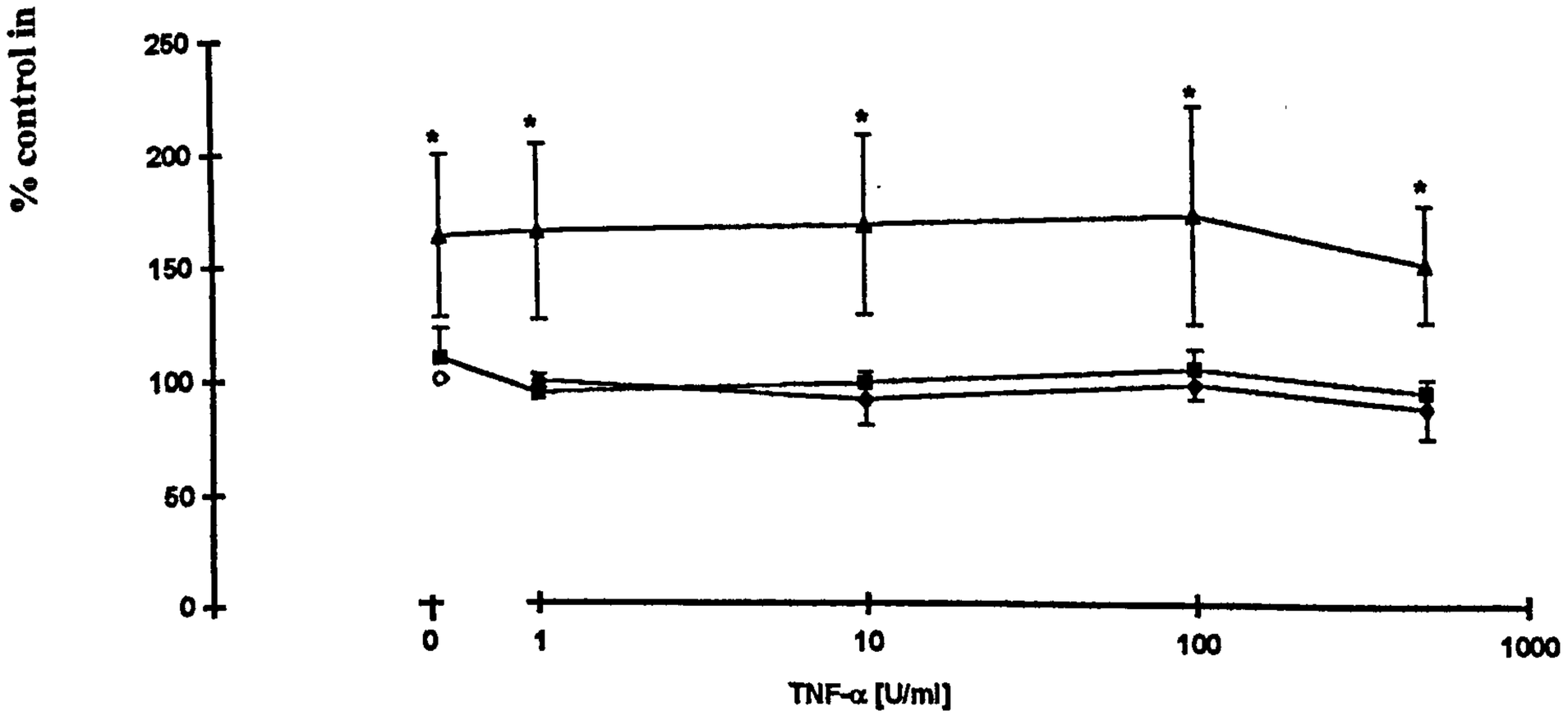

250
200
150
100
50
0
*
*
*
*
*
0
1
10
100
1000
TNF-α [U/ml]

Table 3.5a The effect of α-MSH and IBMX on melanocyte proliferation.

		α-MSH								IBMX	
		10^{-11}M		10^{-10}M		10^{-9}M		10^{-8}M		10^{-3}M	
	Control	24 hours	5 days	24 hours	5 days	24 hours	5 days	24 hours	5 days	24 hours	5 days
M741	4.75							4.80	5.6		
M746	1.21							1.62	2.82		
M823	2.84	2.99	3.02	2.84	3.35	2.79	4.15	2.35	4.83		
M829	3.27							3.05	4.44		
M830	4.43	5.46	6.43	5.70	6.85	5.74	8.23	5.50	9.15	4.48	3.08
M840	3.17										
M852	3.92	3.83	3.94	4.17	5.96	5.86	7.62	3.14	7.43	5.38	5.31
M857	2.33										
Mean	3.4	4.09	4.46	4.24	5.39*	4.8	6.67*	3.41	5.71*		
SEM	± 0.53	± 0.72	± 1.02	± 0.83	± 1.05	± 1	± 1.27	± 0.6	± 0.92		
Mean		108	117	112	142	126	175	103	176		
SEM		± 8	± 14	± 9	± 12	± 15	± 15	± 9	± 18		

Melanocytes were seeded at 1.64 to 2.8 x 10^4 cells/well (24 well plates, in triplicate) in maintenance medium C, and allowed to adhere for 24 hours. The cells were then cultured in medium 23 (containing bFGF) for 5 days. α-MSH (10^{-11}M to 10^{-8}M) or IBMX (10^{-3}M) was added either at each feed or for the final 24 hours. TNF-α (at the stated concentrations in **Tables 3.5b-c**) was added to the appropriate cells for the final 24 hours. Cell number (x 10^4) was then analysed by the DNA assay (as described in **Section 2.7.4**). **Table 3.5a** shows the results just for melanocytes treated with α-MSH or IBMX, while **Table 3.5b** shows the results for melanocytes treated with α-MSH + TNF-α or IBMX + TNF-α. **Table 3.5c** shows the results for melanocytes treated with 10^{-8}M α-MSH and increasing concentrations of TNF-α. The data represents the mean ± SEM of n=3 wells for each individual experiement and the mean ±SEM of all the experiments for each condition. The mean of the normalised data is also illustrated (individual data for each experiment omitted for clarity) and is represented in **Figure 3.14**. Cell numbers, differing significantly from the control, are indicated as *$p<0.05$ and **$p<0.01$ based on Student's unpaired t-test and $^{\uparrow}=p<0.05$ and $^{\uparrow\uparrow}=p<0.01$ based on Student's paired t-test.

Table 3.5b The effect of α-MSH, IBMX and TNF-α on melanocyte proliferation.

		TNF-α	α-MSH + 100U/ml TNF-α								IBMX + TNF-α	
		100U/ml	10^{-11}M		10^{-10}M		10^{-9}M		10^{-8}M		10^{-3}M	
	Control	24 hours	24 hours	5 days	24 hours	5 days	24 hours	5 days	24 hours	5 days	24 hours	5 days
M741	4.75	4.15							5.05	5.71		
M746	1.21	1.3							1.42	3.23		
M823	2.84	3.21	2.74	3.4	1.85	4.29	1.96	4.92	2.07	4.73		
M829	3.27	3.17							1.42	3.23		
M830	4.43	4.35	3.97	5.33	4.19	7	4.06	6.94	3.89	7.55	5.34	5.47
M840	3.17	2.83	2.17	1.75					3.33	3	2.42	2.5
M852	3.92	4.4	5.13	5.57	6.23	5.52	5.27	5.95	4	5.97	6	5.49
M857	2.33	2.08	2.17	2.5					2.17	4.08		
Mean	3.4	3.19	3.23	3.71	4.09	5.61**	3.76	5.93**	2.92	$4.69^{\uparrow\uparrow}$	4.59	4.49
SEM	± 0.53	± 0.39	± 0.58	± 0.76	± 1.27	± 0.78	± 0.97	± 0.58	± 0.47	± 0.57	± 1.1	± 0.99
Mean		99	96	109	106	150	98	156	97	159	117	114
SEM		± 4	± 10	± 15	± 28	± 5	± 19	± 2	± 5	± 18	± 22	± 18

Table 3.5c The effect of TNF-α and α-MSH on melanocyte proliferation.

	TNF-α													
	0			1U/ml			10U/ml			100U/ml			500U/ml	
	No	+ α-MSH		No	+ α-MSH		No	+ α-MSH		No	+ α-MSH		No	+ α-MSH	
	MSH	24 hrs	5 days	MSH	24 hrs	5 days	MSH	24 hrs	5 days	MSH	24 hrs	5 days	MSH	24 hrs	5 days
M741b	4.75	4.8	5.6	4.9	5.07	5.56	4.87	5.17	5.53	4.15	5.05	5.71	4.89	5	5.4
M746b	1.21	1.62	2.82	1.23	0.98	2.93	0.84	1.13	2.98	1.3	1.42	3.23	0.74	1.03	2.42
M829	3.27	3.05	4.44	3.04	3.08	4.37	3.31	3.01	4.58	3.17	2.83	4.16	3.14	3.03	4.48
Mean	3.07	3.16	$4.29^{\uparrow}$	3.06	3.04	$4.29^{\uparrow}$	3.01	3.1	$4.3^{\uparrow}$	2.87	3.1	$4.37^{\uparrow}$	2.93	3.02	$4.1^{\uparrow}$
SEM	±1.03	± 0.92	± 0.81	± 1.06	± 1.18	± 0.76	± 1.17	± 1.17	± 0.69	± 0.84	± 1.06	± 0.73	± 1.2	± 1.15	± 0.88
Mean		109	163	99	94	165	91	98	168	97	104	172	87	94	151
SEM		±13	±36	±3	±8	±39	±11	±5	±40	±6	±9	±48	±13	±6	±26

Table 3.6a The effect of 10^{-8}M α-MSH on melanocyte proliferation.

	Cell number x 10^4								
	Medium 1			Medium 10			Medium 24		
	No MSH	10^{-8}M α-MSH		No MSH	10^{-8}M α-MSH		No MSH	10^{-8}M α-MSH	
	Control	24 hours	5 days	Control	24 hours	5 days	Control	24 hours	5 days
M741	11.69	9.28	11.25	10.51	7.67	8.44	4	3.97	4.24
M746	9.33	9.41	10.27	8.47	7.68	8.26	3.79	2.91	2.63
M760				3.72	4.5	3.11			
M771				5.55	4.57	4.25			
Mean				7.06	6.11 (92)	6.01* (85)			
SEM				± 1.51	± 0.91 (10)	± 1.37 (5)			

Table 3.6b The effect of 10^{-8}M α-MSH and TNF-α or IFN-γ on melanocyte proliferation.

	Cell number x 10^4											
	Medium 1				Medium 10				Medium 24			
	No MSH		10^{-8}M α-MSH		No MSH		10^{-8}M α-MSH		No MSH		10^{-8}M α-MSH	
	Control	TNF	24 hours	5 days	Control	TNF	24 hours	5 days	Control	TNF	24 hours	5 days
M746	9.33	9.93	8.07	9.75	8.47	8.19	7.97	8.18	3.79	3.87	2.89	2.53
M760					3.72	1.64	3.68	3.76				
M771					5.55	4.29	5.15	4.85				
Mean					5.91	4.7 (73)	5.6 (95)	5.6 (95)				
SEM					± 1.38	± 1.9 (15)	± 1.26 (2)	± 1.33 (4)				

P4 melanocytes were plated at 3.44 x 10^4 cells/well (M760, in 12 well plate) or at 2.5 x 10^4 cells/well (24 well plates, in triplicate) in maintenance medium A (M746) or maintenance medium B (M760, M771) and allowed to adhere for 24 hours. Medium was then changed to experimental media 1, 10, or 24 and changed twice more during the 5 day experimental period. Cells were treated with 10^{-8}M α-MSH at each change of feed (3x), or for the final 24 hours, or untreated. Melanocytes under the appropriate conditions were treated with 100U/ml TNF-α for the final 24 hours. Cell number was then analysed by cell counts (M760) (as described in **Section 2.7.1**) or the DNA assay (as described in **Section 2.7.4**). (Results for M760 were calibrated for 24 well). The % of the control result in shown in brackets for each condition. Cell number, differing significantly from control, is indicated as *$p<0.05$ based on Student's paired t-test.

had a slight dose dependent effect on cell proliferation (**Figure 3.13b** and **Table 3.5c**). Cell proliferation was significantly increased by 63% ($p<0.05$, n=3 different donors) by 10^{-8}M α-MSH in the absence of TNF-α, while in the presence of TNF-α, cell proliferation was increased by 65% (1U/ml TNF-α), 65% (10U/ml TNF-α) and 72% (100U/ml TNF-α). In response to 500U/ml TNF-α and 10^{-8}M α-MSH (present for 5 days) there was a significant increase in proliferation by 51% ($p<0.05$) but this was lower than in response to 10^{-8}M α-MSH alone. TNF-α alone and in combination with 10^{-8}M α-MSH (added just for the final 24 hours) had no significant effects on melanocyte proliferation.

In medium 10 (containing FCS/BPE), 10^{-8}M α-MSH decreased the proliferation of melanocytes by 10% ($p<0.05$, based on the combined results of the different donor melanocytes used in both pigmentation and ICAM-1 studies). This was the only medium containing 2 mitogens in which a significant decrease was seen. While the decrease was only 6% in the pigmentation study (**Figure 3.12a**), 4 out of the 5 cultures showed decreases in proliferation in response to 10^{-8}M α-MSH. A significant decrease of 15% ($p<0.05$) was seen in the proliferation of melanocytes from 4 different donors used in the ICAM-1 study (**Table 3.6a**) in response to 10^{-8}M α-MSH.

In summary, α-MSH generally stimulated melanocyte proliferation in media containing a low number of mitogens. The effect was concentration dependent as illustrated in medium 23 (**Figure 3.13a**). α-MSH also significantly inhibited melanocyte proliferation in medium 10, but this was to a modest degree. TNF-α had no effects on melanocyte proliferation in media 23 (further details on cytokine effects on melanocyte proliferation are described in **Section 3.3.5**).

3.3.3. Effect of IBMX on melanocyte proliferation in 5 different media variants.

The effect of 10^{-3}M IBMX in comparison to 10^{-8}M α-MSH on melanocyte proliferation was examined in 5 media variants varying in mitogenic potency. 10^{-3}M IBMX significantly reduced cell number in medium 1 (containing FCS/BPE/PMA/CT) by 58% ($p<0.05$), in medium 2 (containing FCS/BPE/PMA/bFGF) by 59% ($p<0.01$), and in

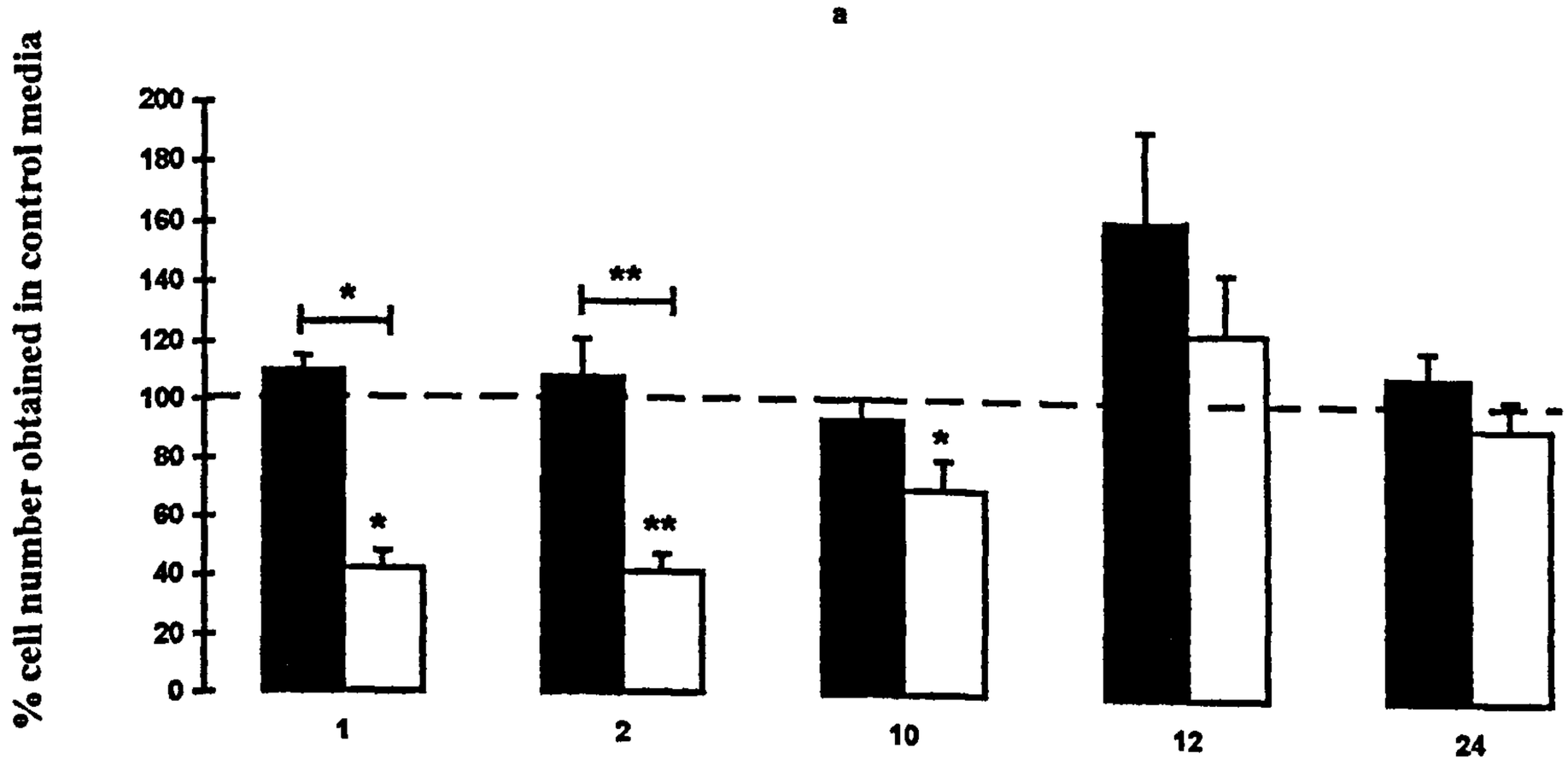
medium 10 (containing FCS/BPE) by 30% ($p<0.05$) (**Figure 3.14a**). In medium 12 (containing FCS/bFGF), IBMX increased cell number by 24% (not achieving statistical significance). No effect of IBMX was seen in medium 24. **Figure 3.14a** illustrates that the effects of IBMX and α-MSH on melanocyte proliferation often differed. Thus IBMX inhibited melanocyte proliferation in very mitogenic media, but had less effect in media containing fewer mitogens.

3.3.4 Influence of UVB, media and ECM on normal melanocyte proliferation.

In vivo UV irradiation increases melanocyte proliferation and activation. The effects of UVB on melanocyte proliferation and pigmentation (**Section 3.4.8**) were examined in addition to the effects on morphology (**Section 3.2.5**). Melanocytes were additionally cultured on fibroblast- and HaCaT-derived ECM to examine the role of ECM proteins in providing a protective role against possible UVB damage. The effects of UVB on melanocyte proliferation were examined in cells that had been cultured on plastic or cell-derived ECM proteins under experimental media 1, 10 and 24. In medium 1, the most mitogenic medium, cell number increased 2.4 fold from day 2 to 8 day ($p<0.01$) (**Figure 3.15b**). However, if only the three cultures that were examined at each time point were analysed, a significant increase in cell number over this time period was not seen. Cell number in medium 10, increased almost linearly over the time period, with a 1.6 fold increase from day 2 to day 8 ($p<0.05$) (**Figure 3.15c**). Cell number was suprisingly 20% greater in medium 10 compared to medium 1 at day 5 ($p<0.01$) (**Figure 3.15a**). This is not in agreement with the results seen in **Section 3.2.1** where medium 1 was the most mitogenic medium at a similar time point. By day 8, cell number was significantly lower by 24% ($p<0.01$) indicating the greater mitogenic potency of medium 1 (**Figure 3.15a**). Melanocytes did not proliferate in medium 24 and by day 8 cell number was reduced by 13% (not achieving statistical significance) in comparison to day 2 (**Figure 3.15d**). However, when comparing the 3 cultures that were examined at all three time points there was a significant reduction of cell number by 22% in this medium ($p<0.05$). This indicates that long term culture of melanocytes on plastic in medium 24 is not feasible. In medium 24, cell number was lower by 18% at day5 (just failing to achieve statistical significance, $p<0.0565$) and by 68% at day 8 ($p<0.001$) in comparison to the cell number seen in medium 1 (**Figure 3.15a**).

Figure 3.14 Comparison of the effects of 10^{-8}M α-MSH and 10^{-3}M IBMX on cell number (a), dopa oxidase activity (b), and melanin content (c). Melanocytes, primary, P1 and P4 were plated on plastic in maintenance medium C, and allowed to adhere for 24 hours (P1 and P4) before medium was changed to those indicated by the numbers (composition of media described in **Table 2.2**), in the absence or presence of α-MSH (closed bar) or IBMX (open bar) (as described in **Table 3.4**). Primary cells were cultured for 2-3 weeks before experimental procedure. Cells were cultured for a further 5 days in these media before DNA, dopa oxidase activity and melanin analysis (details in **Sections 2.7.4, 2.8.2d** and **2.8.3**, respectively). For each parameter, the results are expressed as a percentage of control in response to 10^{-8}M α-MSH or 10^{-3}M IBMX and are means ± SEM of 5 experiments using melancoytes from different donors. Parameter function significantly altered by 10^{-8}M α-MSH or10^{-3}M IBMX is indicated by *=$p<0.05$ and **=$p<0.01$ based on Student's paired t-test performed on the data before it was normalised. Significant differences between responses to 10^{-8}M α-MSH and 10^{-3}M IBMX for each parameter are indicated by ⊢*=$p<0.05$ and ⊢**=$p<0.01$ based on Student's paired t-test performed on the data before it was normalised.

a



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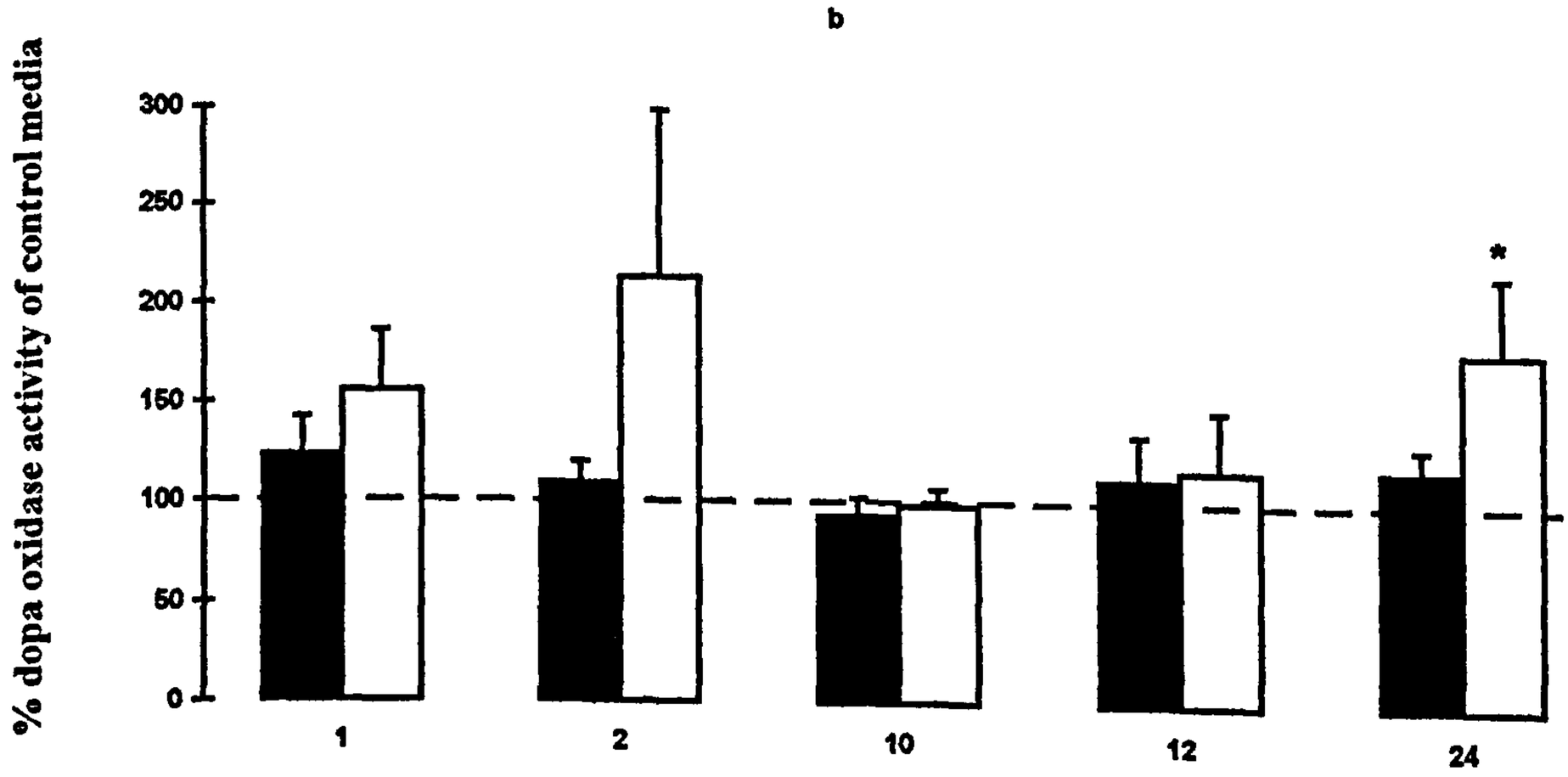

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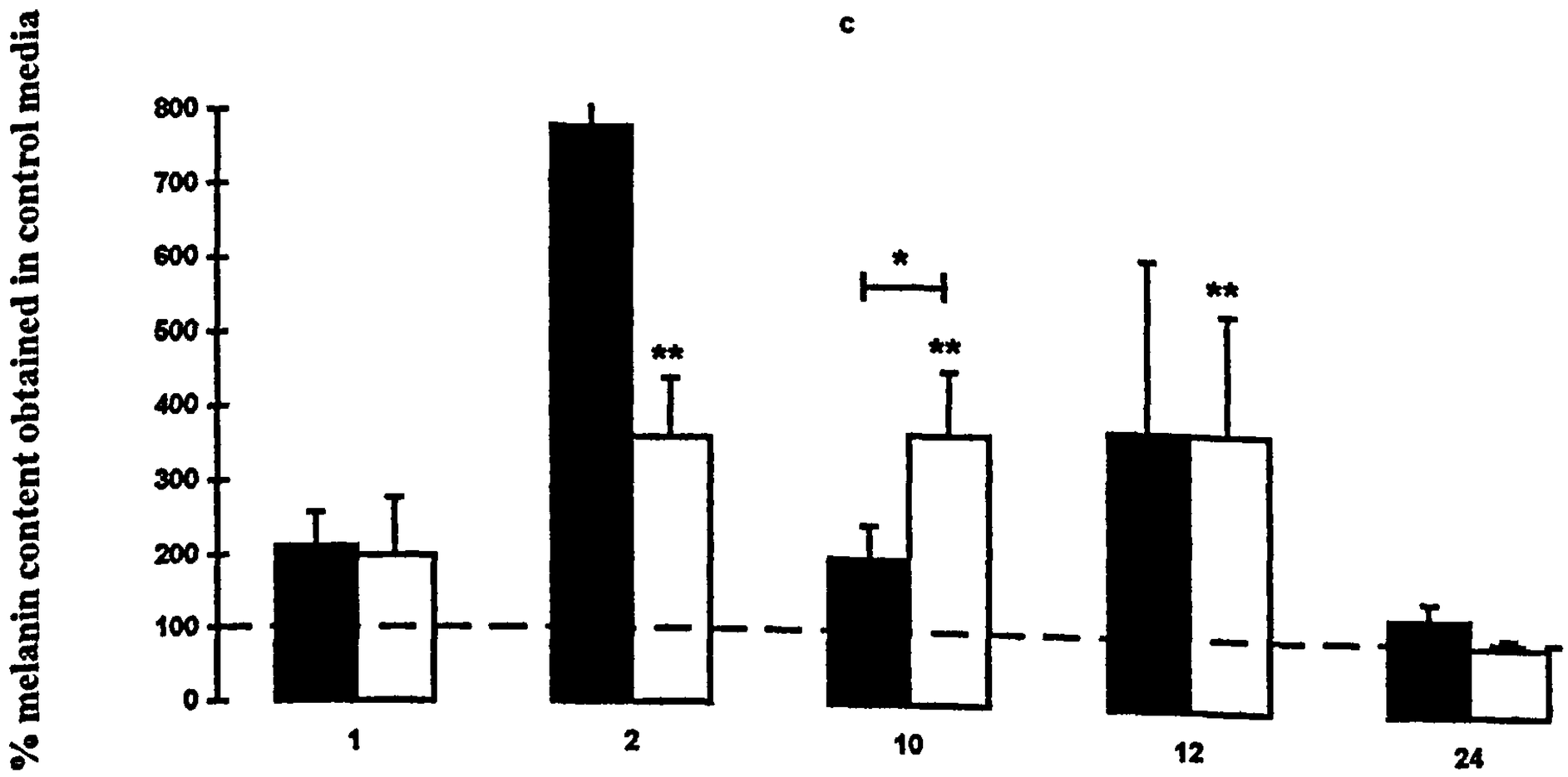


Media variants

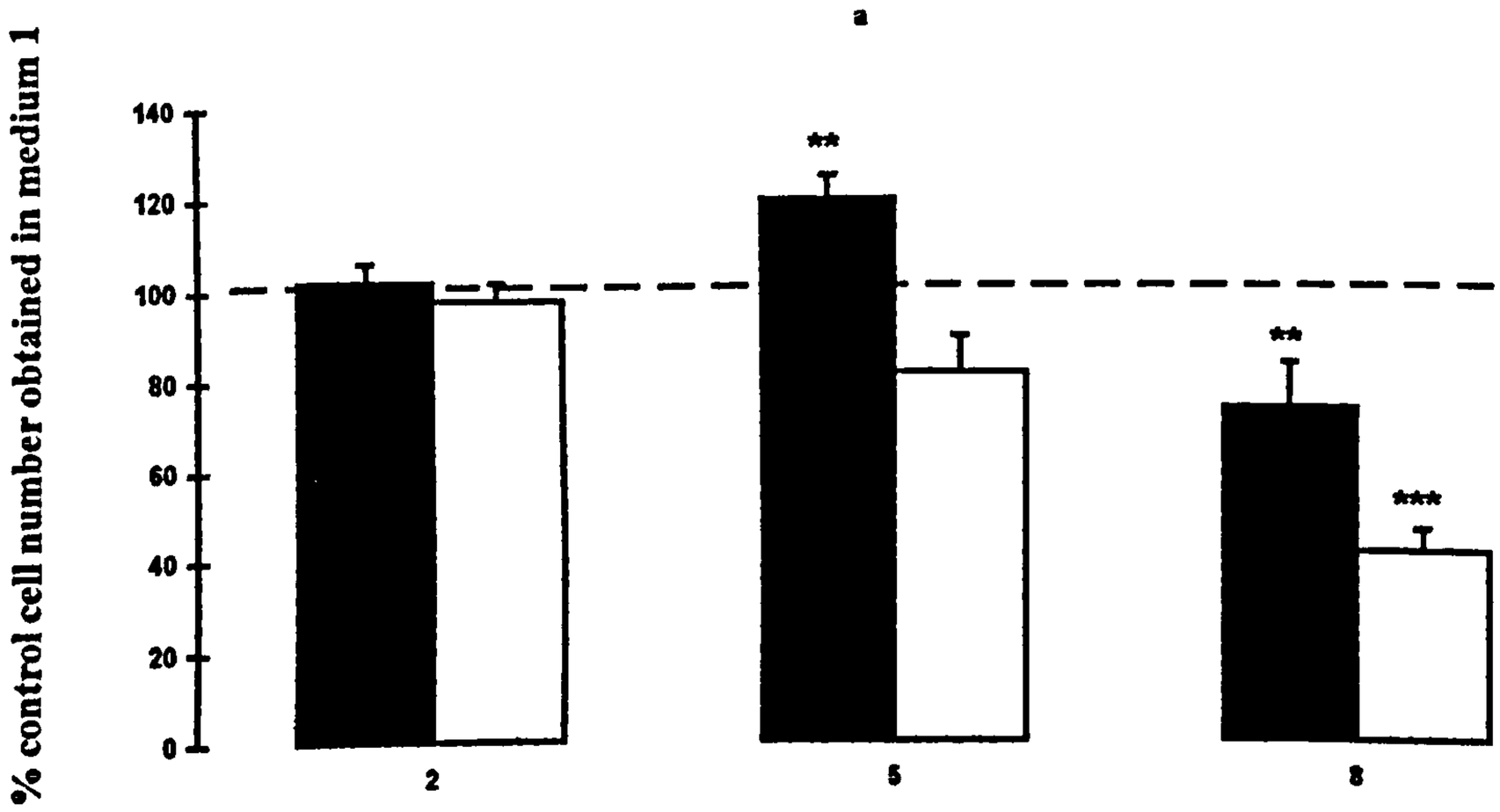
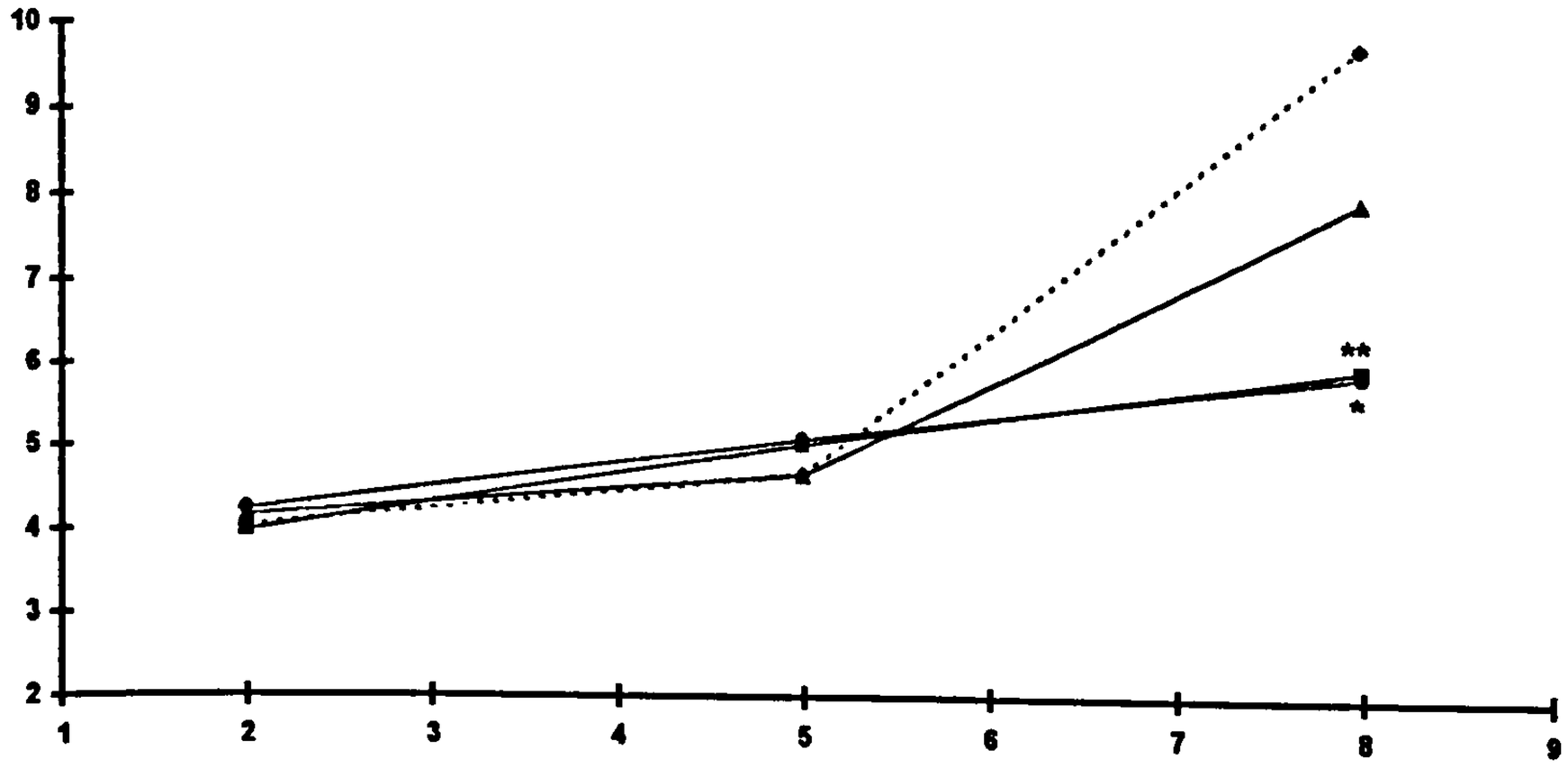


Figure 3.15 Effect of media, UV and ECM on normal melanocyte proliferation. Melanocytes from 8 different donors were used in these experiments, with melanocytes from one donor being used in two experiments but using different cell derived-ECMs. Only three cultures were examined at each timepoint but the data is an amalgamation of all results obtained for each timepoint. Melanocytes were seeded at 2.44 to 7.6 x 10^4 cells/well (12 well) or 1.5 x 10^4 cells/well (24 well) in maintenance media A, B or C (for cells in 24 well plate), in triplicate and allowed to adhere for 24 hours. Plating substrata was plastic, fibroblast-derived ECM or in 1 experiment HaCaT-derived ECM (ECM production detailed in **Section 2.5.3**). Melanocytes were irradiated with 143mJ/cm^2 of UVB for either 1, 4 or 7 days (as described in **Section 2.6**). PBS was present during the irradiation period and experimental media (1, 10 or 24) replaced the PBS following each irradiation. Cell number was analysed by cell counts (as described in **Section 2.7.1**) after day 2, day 5 and day 8. Cell number was calculated per 12 well for all experiments (including those run in 24 well plates, which were converted). Cells were then used for pigmentation analysis (as described in **Figures 3.27-3.28** and **Table 3.15**). The effects of media 10 (closed bar) and media 24 (open bar) on cell number over the three timepoints are illustrated in (**a**). The data represents the mean normalised values ± SEM of 4 (timepoint I), 5 (timepoint II) and 8 (timepoint III) experiments using melanocytes from different donors (some melanocyte cultures were used at all timepoints). Significant cell reduction in response to a particular media in comparison to medium 1 is indicated as **=$p<0.01$ and ***=$p<0.001$ using Student's paired t-test performed on data prior to normalisation. The effect of UV and ECM (alone or in combination) on cell number (x 10^4) are shown for medium 1 (**b**), medium 10 (**c**) and medium 24 (**d**). The mean data is shown for sham irradiated cells on plastic (closed diamond, dashed line), UV irradiated cells on plastic (closed square), sham irradiated cells on ECM (closed triangle) and UV irradiated cells on ECM (closed circle) for all timepoints. Data differing significantly from the sham plastic (for each media) are indicated by *=$p<0.05$ and **=$p<0.01$ based on Student's paired t-test.

b



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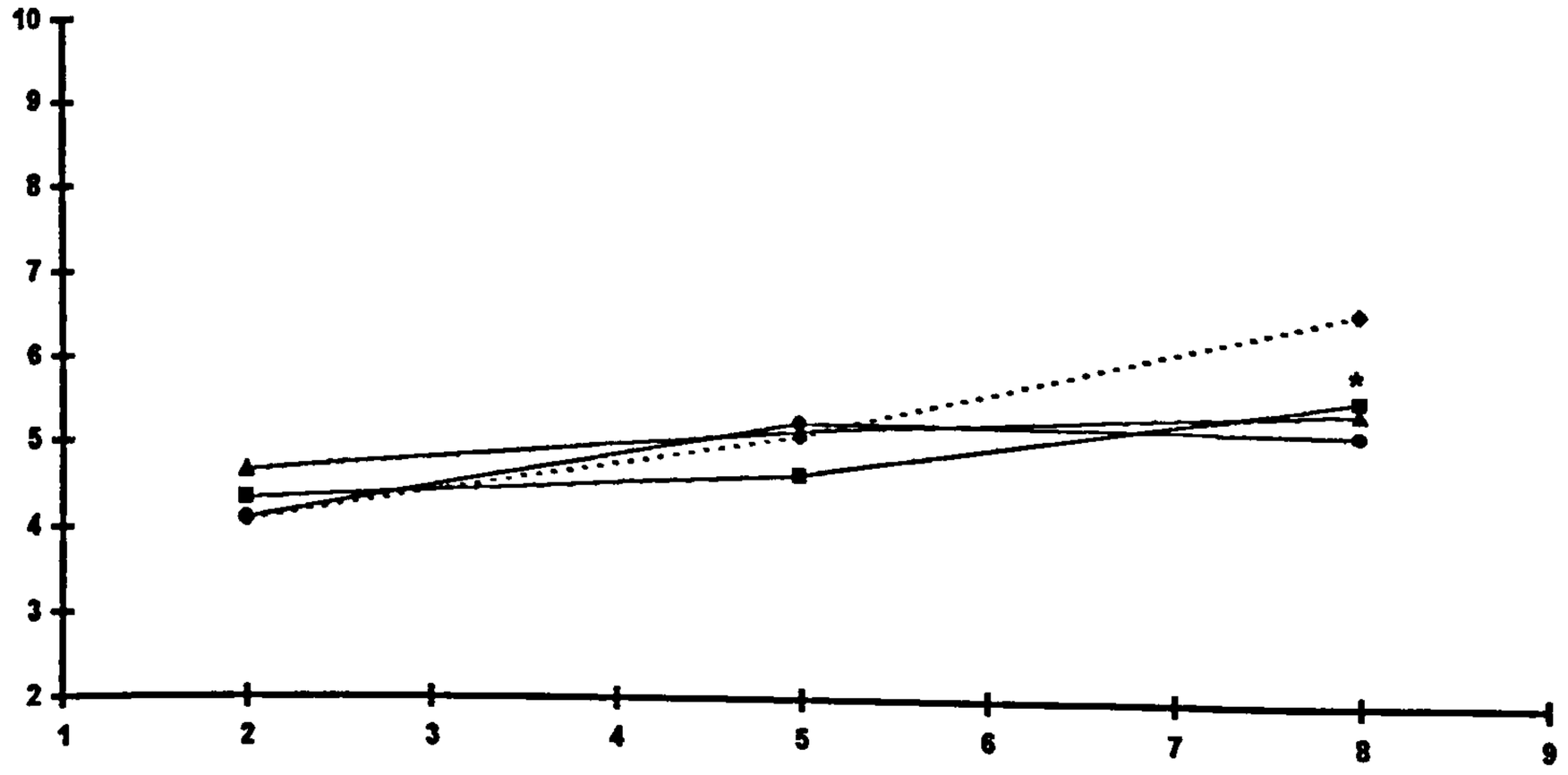

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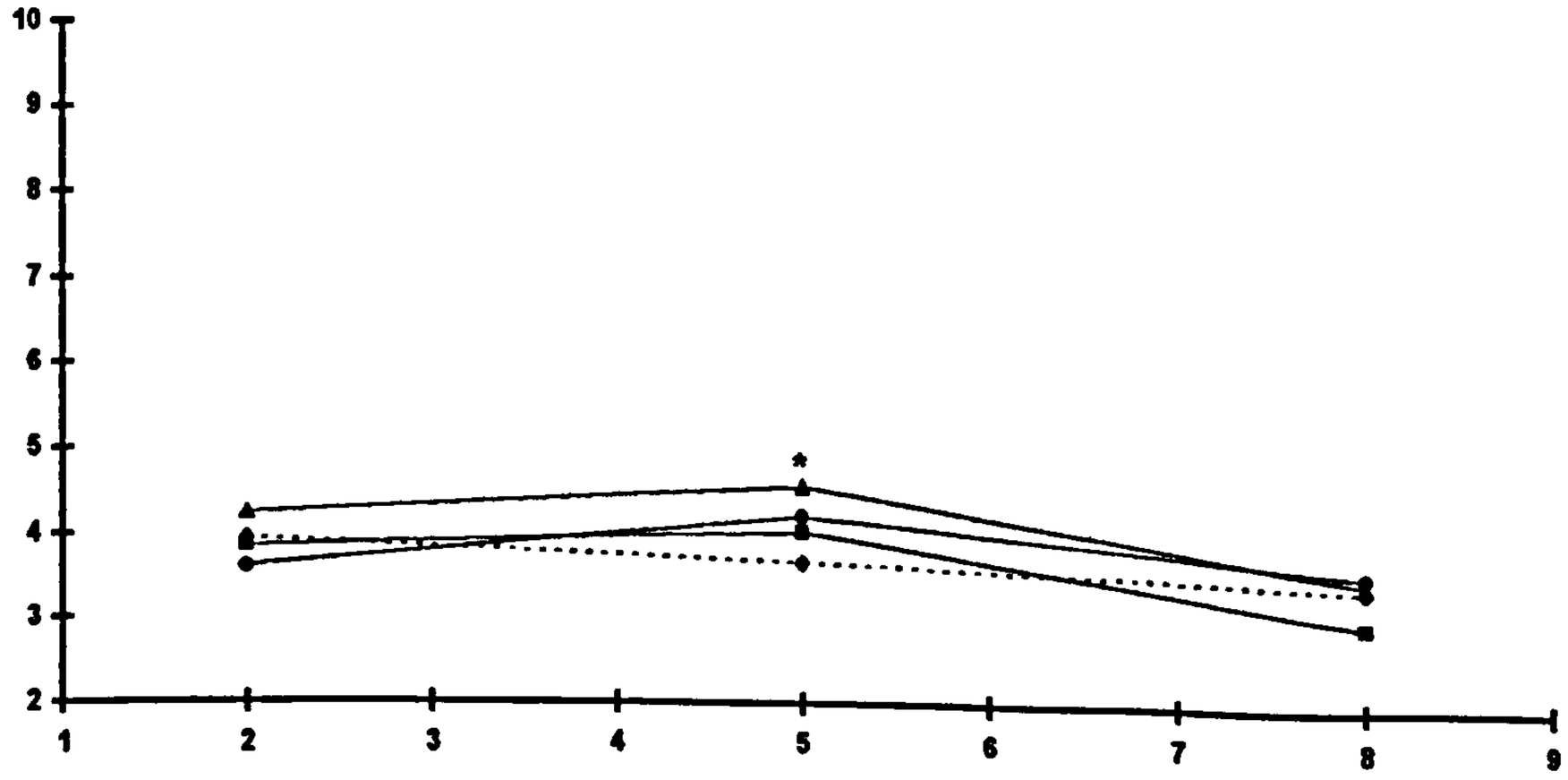


Time (days)

Table 3.7a Comparison of melanocyte cell number of sham-irradiated cells on plastic (at day 2) and UV-irradiated cells on plastic (at day 8).

	Cell Number x 10^4					
	Medium 1		Medium 10		Medium 24	
	Sham plastic day 2	UV plastic day 8	Sham plastic day 2	UV plastic day 8	Sham plastic day 2	UV plastic day 8
M541	5.25	4.83	5.17	6.58	5	4.25
M575	4.38	4.19	4.46	4.42	4.17	2.48
M593	2.92	2.48	3.31	3.23	2.67	1.88
Mean	4.18	3.83*	4.31	4.74	3.95	2.87*
SEM	± 0.68	± 0.7	± 0.54	± 0.98	± 0.68	± 0.71

Table 3.7b Comparison of melanocyte cell number of sham-irradiated cells on ECM (at day 2) and UV-irradiated cells on ECM (at day 8).

	Cell Number x 10^4					
	Medium 1		Medium 10		Medium 24	
	Sham ECM day 2	UV ECM day 8	Sham ECM day 2	UV ECM day 8	Sham ECM day 2	UV ECM day 8
M541	5	9.333	5.92	9	4.25	5.5
M575	4.13	5.06	4.58	5.13	4.46	4.21
M593	2.98	3.1	2.9	3.06	3.67	2.1
Mean	4.04	5.83	4.47	5.73	4.13	3.94
SEM	± 0.59	± 1.84	± 0.87	± 1.74	± 0.24	± 0.99

Melanocytes were seeded at 2.44 to 6 x 10^4 cells/well (12 well, in triplicate) in maintenance medium A or B and allowed to adhere for 24 hours. Cells were cultured on plastic or fibroblast-derived ECM. Cells were irradiated daily for 7 days with 143mJ/cm^2 (as described in **Section 2.6**) and cultured in media 1, 10 or 24. Cells were pooled for each of the conditions described in the above tables and cell counted (as described in **Section 2.7.1**). Mean cell number for UV irradiated cells differing significantly from sham irradiated cells (both cultured on plastic) are indicated by *=$p<0.05$ based on Student's paired t-test.

UVB irradiation did not affect cell proliferation at day 2 and day 5 but after 7 doses, cell number was lower in all three media by 39% in medium 1 ($p<0.01$), by 16% in medium 10 ($p<0.05$) and by 13% in medium 24 (not achieving statistical significance) when compared to sham irradiated cells at this point (**Figure 3.15b-d** respectively). The reduction in cell number in response to UVB could be due to a halt in the cell cycle or due to cell death. To examine this in a crude way, cell number obtained in response to 7 doses of UVB was compared to the cell number in the sham plastic cells at day 2. Only the 3 melanocyte donors that were utilised throughout the full time-course were examined (**Table 3.7a**). In medium 10, there was no significant difference in cell number, while in medium 1, there was a significant but small 8% ($p<0.05$) decrease in cell number. This would suggest that in these two media, UVB was actually halting the cell cycle with perhaps a small amount of cell death in medium 1. In medium 24, there was a significant decrease of 27% ($p<0.05$) in cell number over this time period suggesting cell death results from UVB irradiation.

ECM had no significant effects on cell proliferation in media 1 (**Figure 3.15b**) and 10 (**Figure 3.15c**), but enhanced melanocyte survival in medium 24 with a 24% difference in cell number at day 5 ($p<0.05$) (**Figure 3.15d**). At day 8, ECM significantly enhanced melanocyte survival in 5 out of the 7 cultures examined ($p<0.01$, based on the paired data of the 5 cultures) in medium 24. In a separate study using a further 2 different donor melanocytes (**Figure 3.16a**) to examine the effects of fibroblast-derived ECM alone, ECM had the greatest effect on melanocyte proliferation in medium 24 and the effect became progressively less in media 10 and 1 where the mitogen content increased. ECM initially provided protective measures against UVB irradiation up to day 5 (under all 3 media conditions), but by day 8, cell number was reduced by 31% in medium 1 ($p<0.07$) and by 22% in medium 10 ($p<0.05$), while in medium 24, ECM still provided protection against UVB irradiation. When comparing the cell number obtained at day 8 in response to UVB irradiation on ECM to the cell number obtained with sham-irradiated cells on ECM at day 2 (**Table 3.7b**), there was actually a 45% and 28% increase in cell number (not achieving statistical significance), in media 1 and 10 respectively whereas in medium 24, there was no change. This data further suggests a protective role for ECM against UVB irradiation.

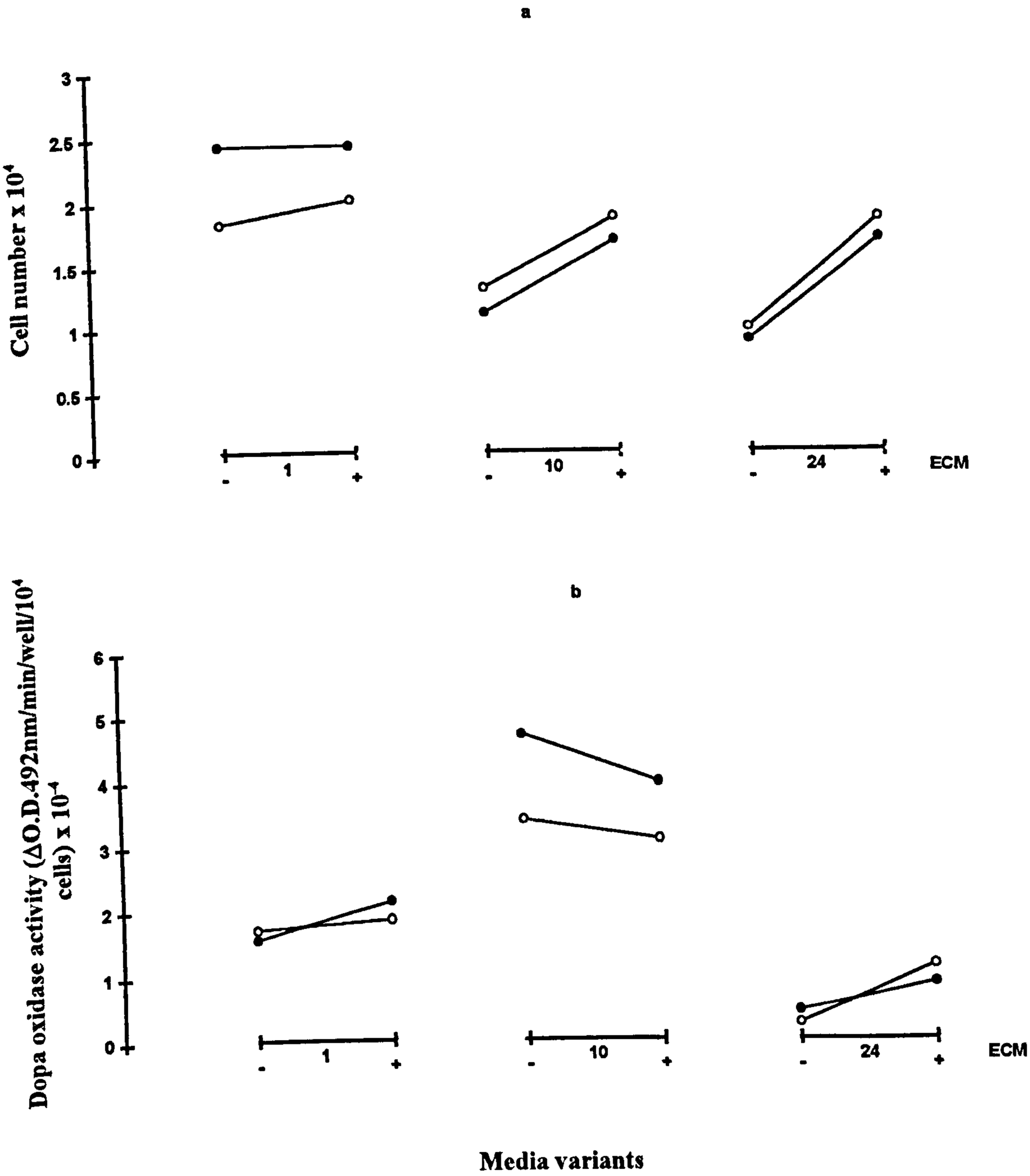


Figure 3.16 Effect of fibroblast-derived ECM on melanocyte proliferation and dopa oxidase activity. Melanocytes from two different donors (passage 4) were plated out at 1 x 10^4 cells/well (96 well plate) in maintenance medium A on either plastic or fibroblast-derived ECM (ECM production as described in **Section 2.5.3**). 24 hours later, culture medium was replaced with media 1, 10 or 24 and cells cultured for a further 5 days. At this point dopa oxidase activity per well was determined (as described in **Section 2.8.2a**) and parallel cultures were used to determine cell number by the DNA assay (as described in **Section 2.7.4**), shown in (**a**). Dopa oxidase activity was corrected for cell number as shown in (**b**).

The effect of UVB was assessed in melanocytes from a further 3 donors, which had been used for ICAM-1 studies (**Section 4.2.4**). The proliferation of melanocytes again was greatly influenced by media; less so by UVB and IFN-γ. Media 10 and 24 inhibited melanocyte proliferation with reductions in cell number of 30% and 43% respectively, at timepoint 2 and of 41% and 74% respectively, at timepoint 3, (**Figure 3.17**) but statistical significance was not achieved due to donor to donor variation (**Table 3.8**). UVB irradiation did not have any significant effects at timepoints I and II (timepoint III was not examined because the cells in one experiment were lost due to infection). IFN-γ significantly decreased cell number by 11% ($p<0.05$, **Figure 3.17b**) after the cells had been cultured in media 10 for 6 days prior to cytokine addition, but had no other effects at the earlier timepoints and had no synergistic/additive effects with UVB.

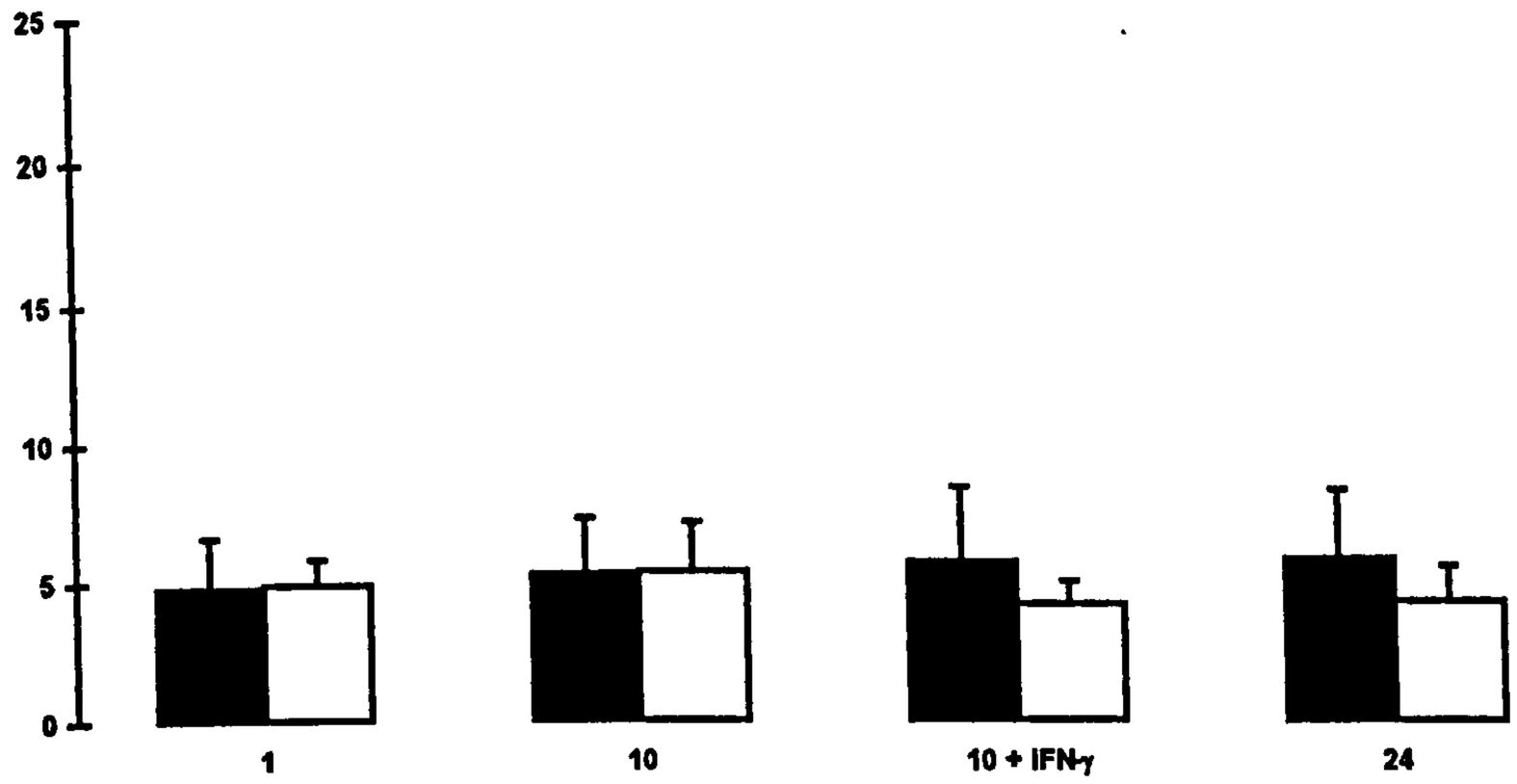
In conclusion, medium composition alone was a strong modulator of melanocyte proliferation (as previously discussed in **Section 3.3.1**), but also had strong effects on the proliferation response to UVB and ECM. UVB inhibited melanocyte proliferation in all media. The greatest effect of ECM on proliferation was seen in the less mitogenic media and reduced as the mitogenic content of the media increased. ECM was also able to provide some protection against UVB irradiation.

3.3.5 Effect of cytokines on the proliferation of normal melanocytes cultured on plastic or fibroblast-derived ECM.

The effects of IFN-γ and TNF-α (both at 100U/ml) were examined in melanocytes from 9 different donors, under three media conditions (**Table 3.9a** and **Figures 3.18a-c**); 3 of these cultures were plated on fibroblast-derived ECM in addition to plastic, with a fourth separate culture also plated onto ECM (results shown in **Table 3.9b** and **Figures 3.18d-f**). Melanocytes from a further 3 different donors were used to examine TNF-α under a wider range of media (**Table 3.9** and **Figure 3.19**). Melanocytes were cultured for 5 days in the appropriate experimental media with the cytokines added for the final 24 hours of this experimental period.

Figure 3.17 Effect of UVB and IFN-γ on normal melanocyte proliferation. P4 melanocytes were sham irradiated (closed bar) or irradiated (open bar) daily for 1 (**a**), 4 (**b**) or 7 (**c**) days with 143mJ/cm^2 of UVB (as described in **Table 3.8** and **Section 2.6**) when cultured in media 1, 10 or 24. 100U/ml IFN-γ was added to the appropriate cells in medium 10 for the final 24 hours after the final irradiation in each protocol and then cells were cell counted (as described in **Section 2.7.1**). The data shows the mean cell number (x 10^4) ± SEM for melanocytes from 3 different donors (individual data shown in **Table 3.8**). Significant changes in melanocyte proliferation in response to IFN-γ are indicated by ⊢ *=$p<0.05$ based on Student's paired t-test.

a



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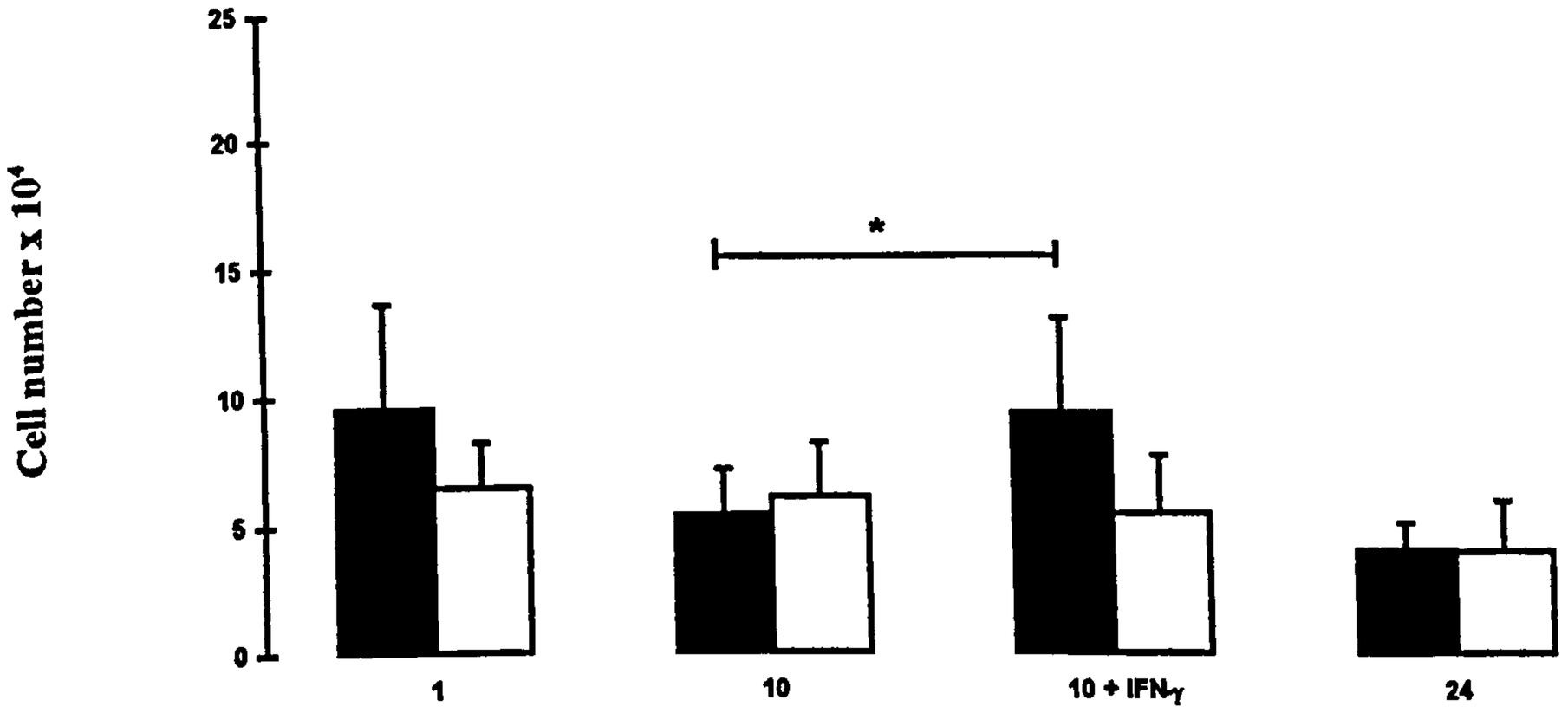

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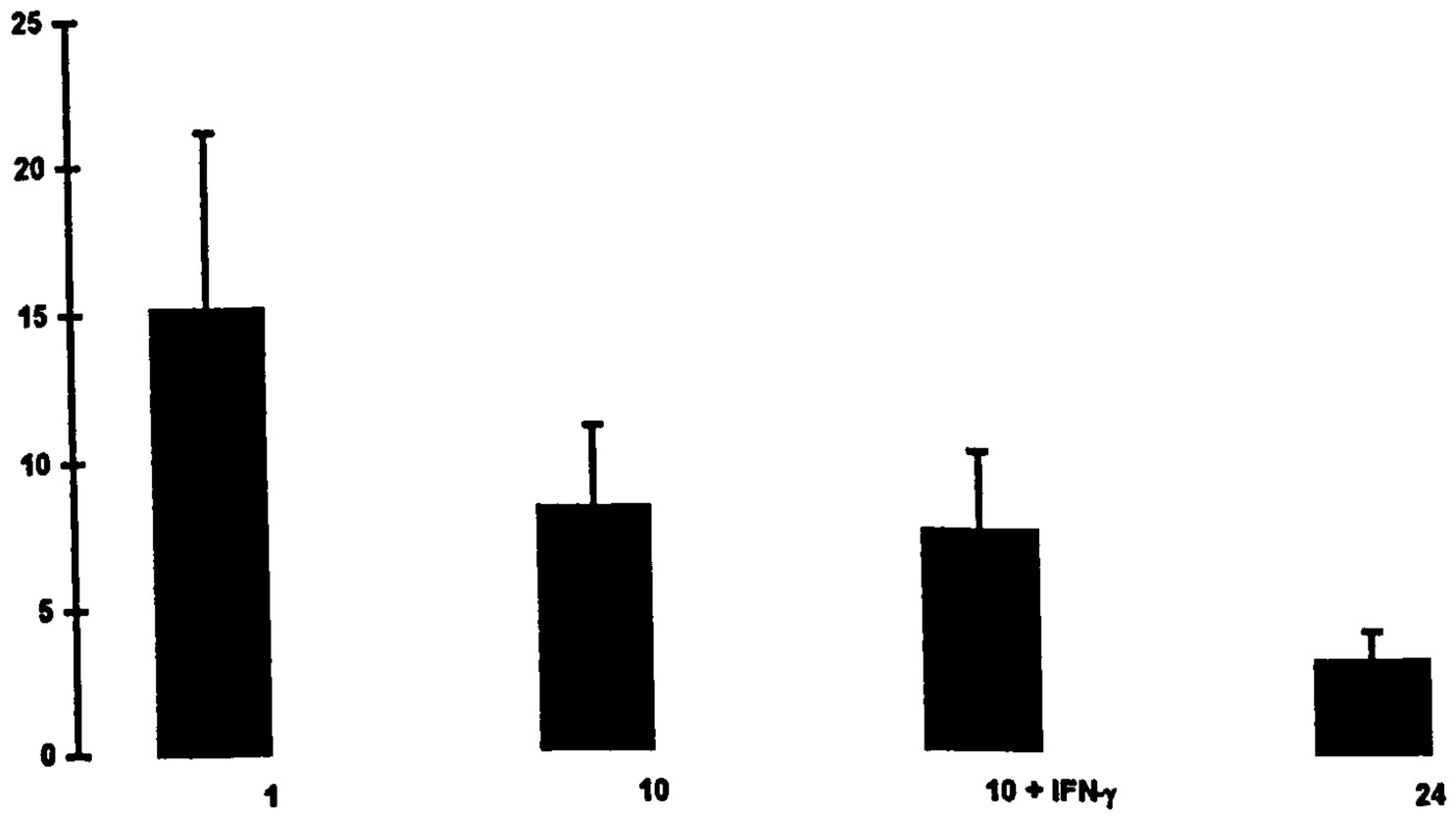


Media variants

Table 3.8a Effect of 1 dose of UVB on melanocyte proliferation under 3 different media conditions.

	Cell Number x10^4							
	Medium 1		Medium 10		Medium 10 + IFN-γ		Medium 24	
	Sham	UVB	Sham	UVB	Sham	UVB	Sham	UVB
M671	1.88	3.25	2.19	3.25	3.31	2.94	2.31	2.38
M687	4.5	5	4.75	4	2.75	3.75	4.5	3.75
M729	8	6.38	9	8.88	11	5.75	10.5	6.63
Mean	4.79	4.88 (*121*)	5.31 (112)	5.38 (*111*)	5.69 (110)	4.15 (*92*)	5.77 (118)	4.25 (*83*)
SEM	± 1.77	± 0.9 (*27*)	± 1.99 (3)	± 1.76 (*20*)	± 2.66 (27)	± 0.84 (*24*)	± 2.45 (9)	± 1.25 (*12*)

Table 3.8b Effect of 4 doses of UVB on melanocyte proliferation under 3 different media conditions.

	Cell Number x10^4							
	Medium 1		Medium 10		Medium 10 + IFN-γ		Medium 24	
	Sham	UVB	Sham	UVB	Sham	UVB	Sham	UVB
M671	2.75	3.38	2.94	3	2.94	2.5	3.75	2
M687	8.75	6.75	4.5	5.25	9.25	4	3.25	2
M729	17	9.25	8.75	10	15.75	9.75	6	7.75
Mean	9.5	6.46 (*85*)	5.4 (70)	6.08 (*111*)	9.31 (162)	5.42 (*63*)	4 (57)	3.92 (*88*)
SEM	± 4.13	± 1.7 (*20*)	± 1.74 (19)	± 2.06 (*5*)	± 3.7 (32)	± 2.21 (*12*)	± 1.01 (21)	± 1.92 (*21*)

Table 3.8c Effect of 7 doses of UVB on melanocyte proliferation under 3 different media conditions.

	Cell Number x10^4							
	Medium 1		Medium 10		Medium 10 + IFN-γ		Medium 24	
	Sham	UVB	Sham	UVB	Sham	UVB	Sham	UVB
M671	4.38	3.25	2.88	2.94	2.44	2.5	1.75	1.19
M687	15.75	6.75	9.75	8.25	8.5	7	2.5	2.5
M729	25.25	ND	12.25	ND	11.5	ND	5.25	ND
Mean	15.13		8.29 (59)		7.48 (89)*		3.17 (26)	
SEM	±6.03		± 2.80 (5)		± 2.67 (3)		± 1.06 (7)	

Table 3.8: P4 melanocytes were seeded at 2.4 to 7.6 x 10^4 cells/well (12 well) in maintenance medium A or B and allowed to adhere for 24 hours. Cells were irradiated daily for 1, 4 or 7 days with 143mJ/cm^2 UVB in PBS (as described in **Section 2.6.2**). Experimental media (1,10 or 24) was added following each irradiation. 100U/ml IFN-γ was added (only in medium 10) for 24 hours following the final irradiation in each protocol and then cells were cell counted (as described in **Section 2.7.1**). The mean cell number ± SEM of melanocytes from 3 different donors is shown for each condition in addition to the individual experimental data. In brackets, mean normalised data are illustrated (individual data not shown for clarity). The data for the effects of media have been normalised to sham medium 1, while UVB data has been normalised to appropriate media sham result (italics). Significant differences in response to IFN-γ are indicated by *=$p<0.05$ based on Student's paired t-test (performed on data prior to normalisation).

[a] ND = not done.

Table 3.9a Influence of IFN-γ or TNF-α in three media of varying mitogenic potency on normal melanocyte proliferation.

	Cell Number x10^4								
	Medium 1			Medium 10			Medium 24		
	Control	IFN-γ	TNF-α	Control	IFN-γ	TNF-α	Control	IFN-γ	TNF-α
M729	12	12.06	15.38	10.25	9.13	11.19	10.69	7.25	7.13
M731	20.12	17.88	19.38	12.63	10.75	12.25	6.75	7.88	8.38
M739	6.5	8	10	4.25	6.25	4.5	3.5	3.75	4.25
M740	6.25	8.75	10.25	6	8	7.5	4.5	2.75	3.25
M749	7.25	6.5	6.5	6	8.25	5	4	2	3.75
M756	17	22.75	23.25	13	12.25	12.75	10.75	8.5	9.5
M767	5.21	4.8	5.3	3.98	3.51	3.96	2.19	1.84	1.74
M833	8.2	8.27	8.47	4.9	5.63	5.35	3.77	4.07	4.02
M843	18.62	15.28	15.63	10.79	9.64	11.45	2.45	4.05	2.4
Mean	11.24	11.59 (106)	12.68 (118)	7.98 (73)**	8.16 (109)	8.22 (104)	5.4 (52)**	4.68 (93)	4.94 (95)
SEM	± 1.96	± 1.98 (7)	± 2.03 (10)	± 1.22 (4)	± 0.9 (8)	± 1.22 (4)	± 1.1 (7)	± 0.85 (12)	± 0.91 (7)

Table 3.9b Influence of IFN-γ or TNF-α and fibroblast-derived ECM, in three media of varying mitogenic potency on normal melanocyte proliferation.

	Cell Number x10^4								
	Medium 1			Medium 10			Medium 24		
Donor	Control	IFN-γ	TNF-α	Control	IFN-γ	TNF-α	Control	IFN-γ	TNF-α
M716	12.5	13.5	ND	9.88	7.5	ND	9.75	6.88	ND
M731	13.38	15.13	18.66	8.88	9.75	11	7.63	7.25	6.25
M739	6.5	ND	5.75	5.25	ND	3.25	4.25	ND	2
M740	7	8.25	6	3.5	5.25	5.75	3.25	5	4.25
Mean	9.85	12.29 (113)	10.14 (104)	6.88 (69)**	7.5 (112)	6.67 (117)	6.22 (62)**	6.38 (107)	4.17 (87)
SEM	± 1.8	± 2.08 (3)	± 4.26 (18)	± 1.5 (7)	± 1.3 (21)	± 2.28 (30)	± 1.5 (7)	± 0.7 (25)	± 1.23 (24)

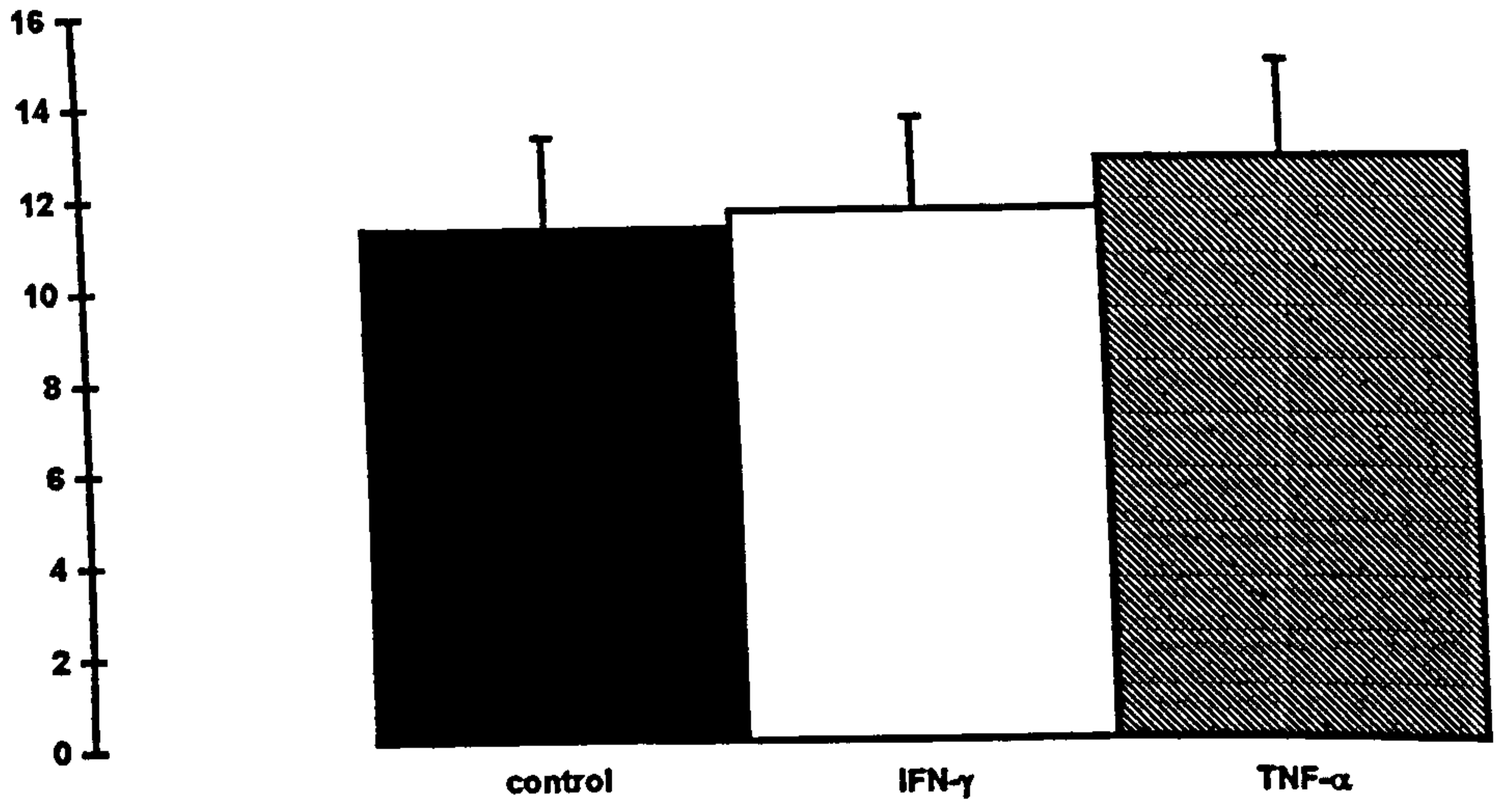
Table 3.9c Influence of IFN-γ or TNF-α in three media of varying mitogenic potency on vitiligo melanocyte proliferation.

	Cell Number x10^4								
	Medium 1			Medium 10			Medium 24		
Donor	Control	IFN-γ	TNF-α	Control	IFN-γ	TNF-α	Control	IFN-γ	TNF-α
VM1 (M756)	8.25	9.5	6.25	6.25	6	5.25	5	4.25	5
VM2 (M767)	3.28	3.69	3.91	3.69	3.51	3.55	2.33	2.17	2.28
VM4 (M843)	23.65	20.32	20.5	15.04	12.68	13.88	6.39	6.67	5.67
VM5 (M833)	6.87	7.21	7.31	6.95	7.84	7.48	6.17	6.2	6.82
Mean	10.51 (80)	10.18 (81)	9.5 (78)	7.98 (69)	7.51 (67)	7.54 (66)	4.97 (46)	4.82 (45)	4.94 (47)
SEM	± 4.5 (17)	± 3.58 (11)	± 3.74 (15)	± 2.45 (11)	± 1.94 (12)	± 2.26 (13)	± 0.93 (10)	± 1.03 (11)	± 0.96 (13)
Mean[a]	12.25	12.78 (102)	15.71 (106)	8.14 (67)*	7.76 (65)	8.37 (69)	4.79 (41)*	4.62 (39)	4.41 (38)
SEM	± 3.28	± 3.98 (11)	± 3.02 (11)	± 2.22 (5)	± 1.96 (4)	± 2.18 (4)	± 2.02 (10)	± 1.4 (7)	± 1.76 (10)

Normal and vitiligo melanocytes (P4) were seeded at 2.54 to 5.99 x 10^4 cells/well (in 12 well plates with one well for cell number) or at 1.33 to 3 x 10^4 cells/well (in triplicate in 24 well plates) in maintenance medium B or C. The cells were allowed to adhere for 24 hours and then changed in to one of the experimental media (1, 10 or 24) for a 5 day experimental period. 100U/ml IFN-γ or 100U/ml TNF-α were added for the final 24 hours, and cell number calculated either by cell counts on trypsinized cells (12 wells, as described in **Section 2.7.1**) or by the DNA assay (24 wells, as described in **Section 2.7.4**). The results show cell number per 12 well (results from 24 wells were converted by a factor of 2.04 as calculated from the areas of a 12 (3.83cm^2) and 24 (1.88cm^2) well). **Table 3.9a**, illustrates the results of normal melanocytes from 9 different donors, when cultured on tissue culture plastic. **Table 3.9b**, illustrates the results of normal melanocytes from 4 different donors cultured on fibroblast-derived ECM (ECM prepared as described in **Section 2.5.3**). The mean normalised data is illustrated in brackets and for **Tables 3.9a-b**, data have been normalised as a % response to control cell number in medium 1 for plastic (**Table 3.9a**) or ECM (**Table 3.9b**). **Table 3.9c**, illustrates the results of vitiligo melanocytes cultured on tissue culture plastic. The paired normal melanocyte cultures are indicated in brackets, with the mean[a] of these 4 normal melanocytes cultures presented in this table. The mean normalised data in this table has been expressed as percent response to control cell number in medium 1 for normal melanocytes. Significant effects of media 10 or 24 on cell number in comparison to to medium 1 are indicated by *=$p<0.05$ or **=$p<0.01$, based on Student's paired t-test (performed on data prior to normalisation).
[a] ND - not done.

Figure 3.18 Influence of cytokines and ECM on the proliferation of normal melanocytes under 3 different media variants. P4 normal melanocytes were cultured for 5 days in experimental media 1 (**a,d**), 10 (**b,e**) or 24 (**c,f**) (as described in **Table 3.9**) on plastic (**a,b,c**) or fibroblast-derived ECM (**d,e,f**). Cells were untreated (closed bar) or treated with 100U/ml IFN-γ (open bar) or TNF-α (hatched bar) for the final 24 hours. Cell number (x 10^4) was determined by cell counts (as described in **Section 2.7.1**) or by the DNA assay (as described in **Section 2.7.4**). The results are the mean ± SEM of 9 experiments using melanocytes from different donors for cells cultures on plastic or 3 to 4 experiments using melanocytes from different donors for cells cultured on ECM (individual results shown in **Table 3.9**). Medium 10 or medium 24 control values differing significantly from controls in medium 1 for plastic and ECM are indicated by *=$p<0.05$ or **=$p<0.01$ based on Student's paired t-test.

a



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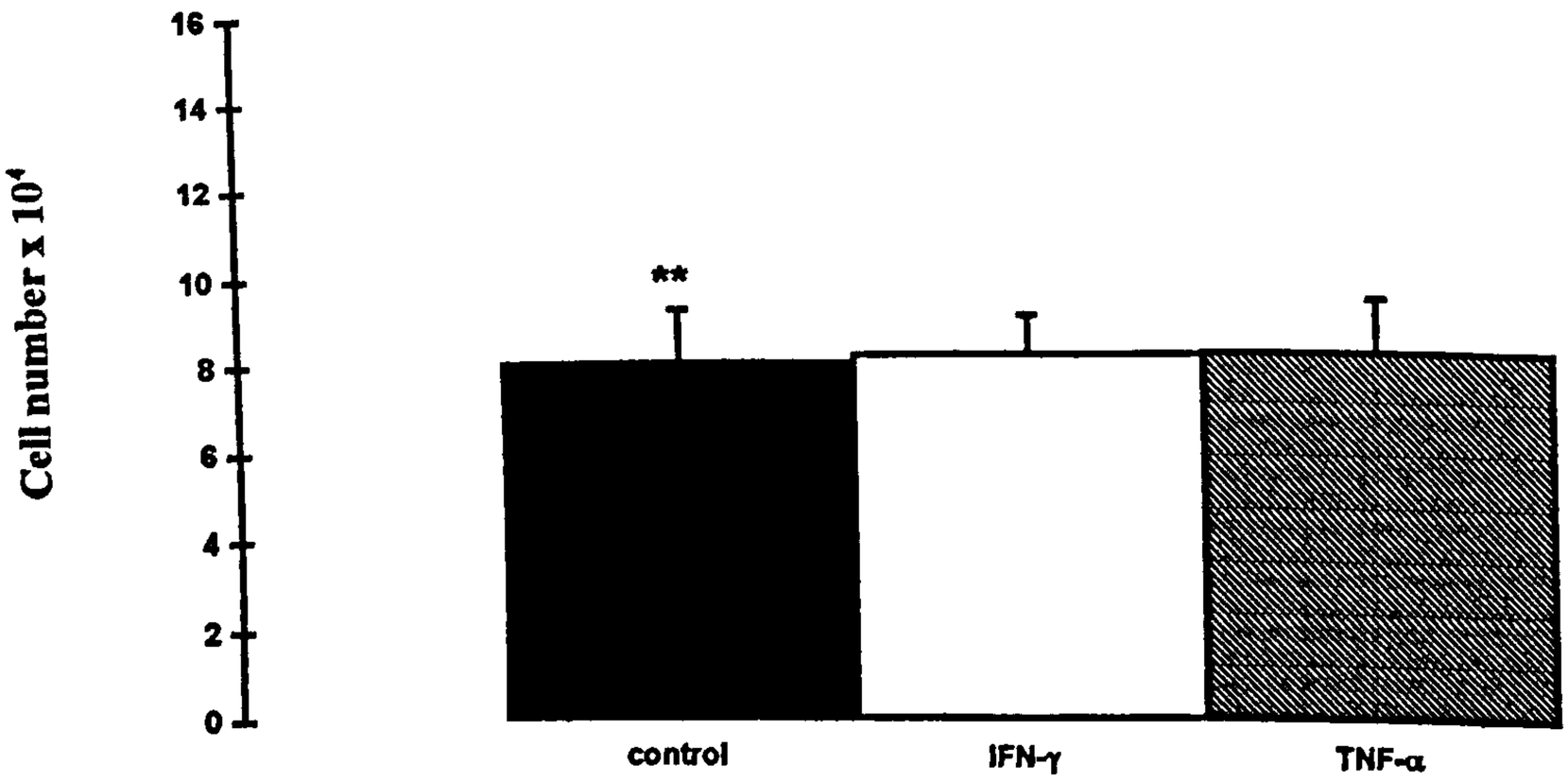

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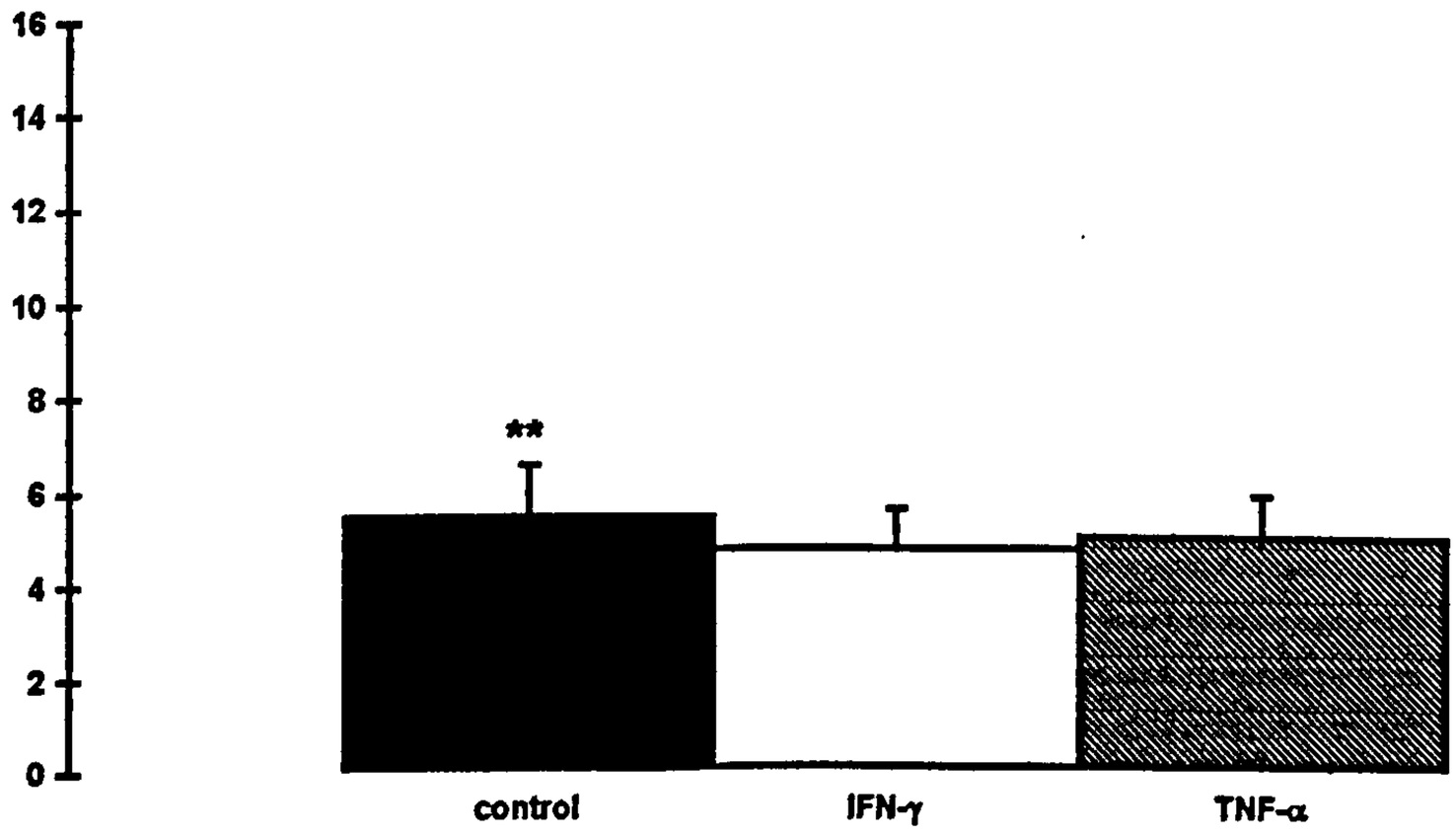

Cytokine additions

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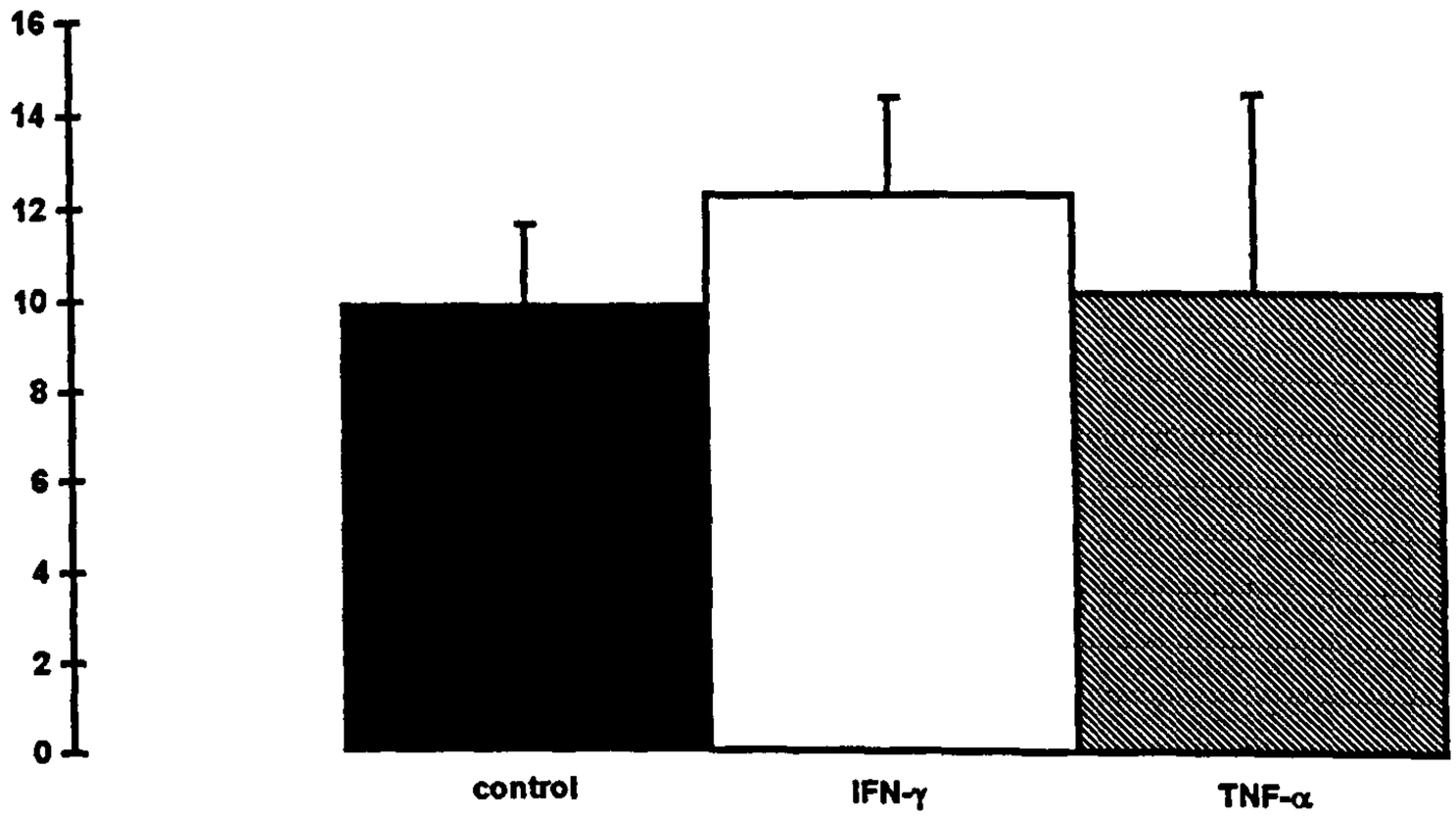

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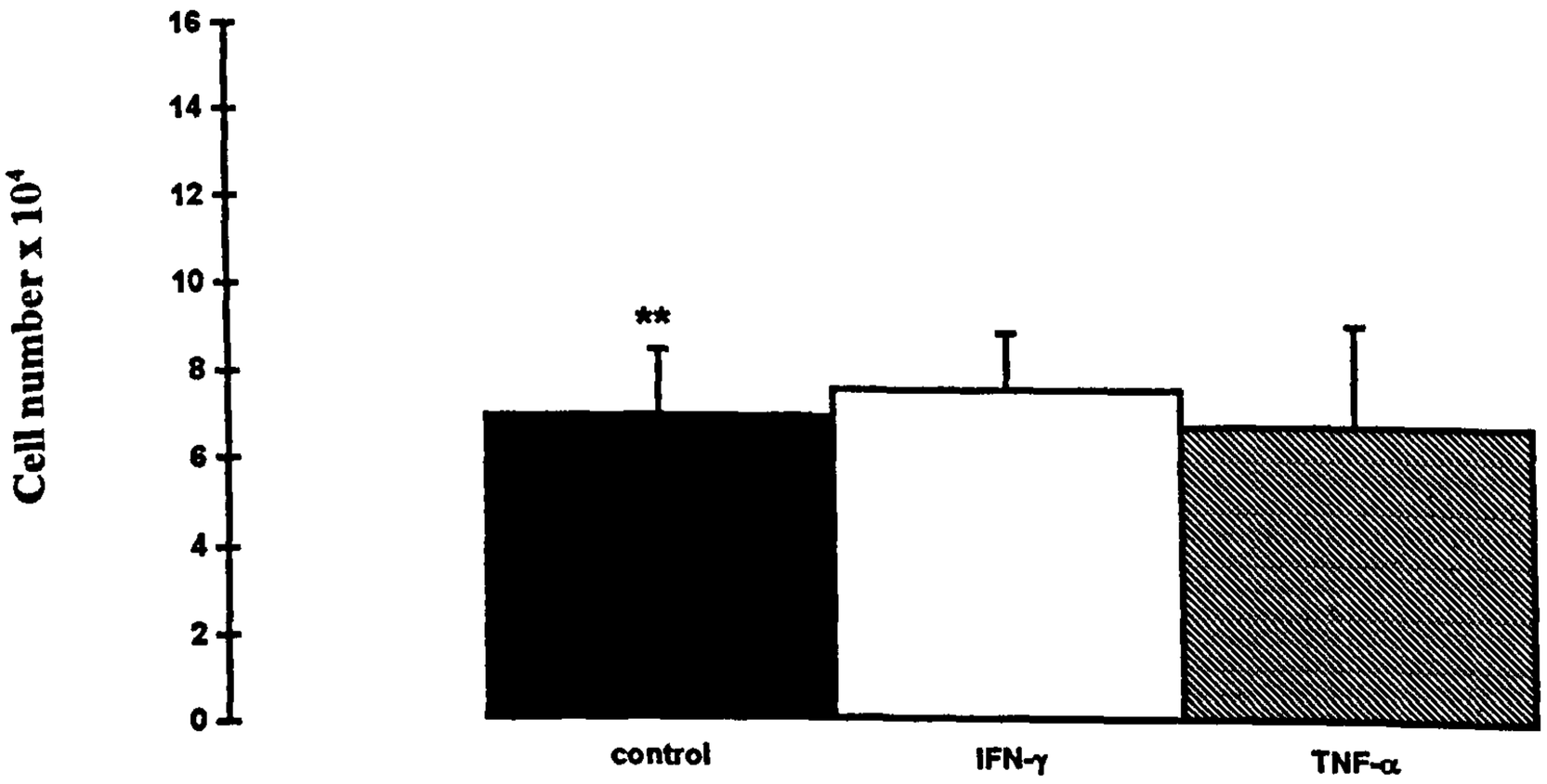

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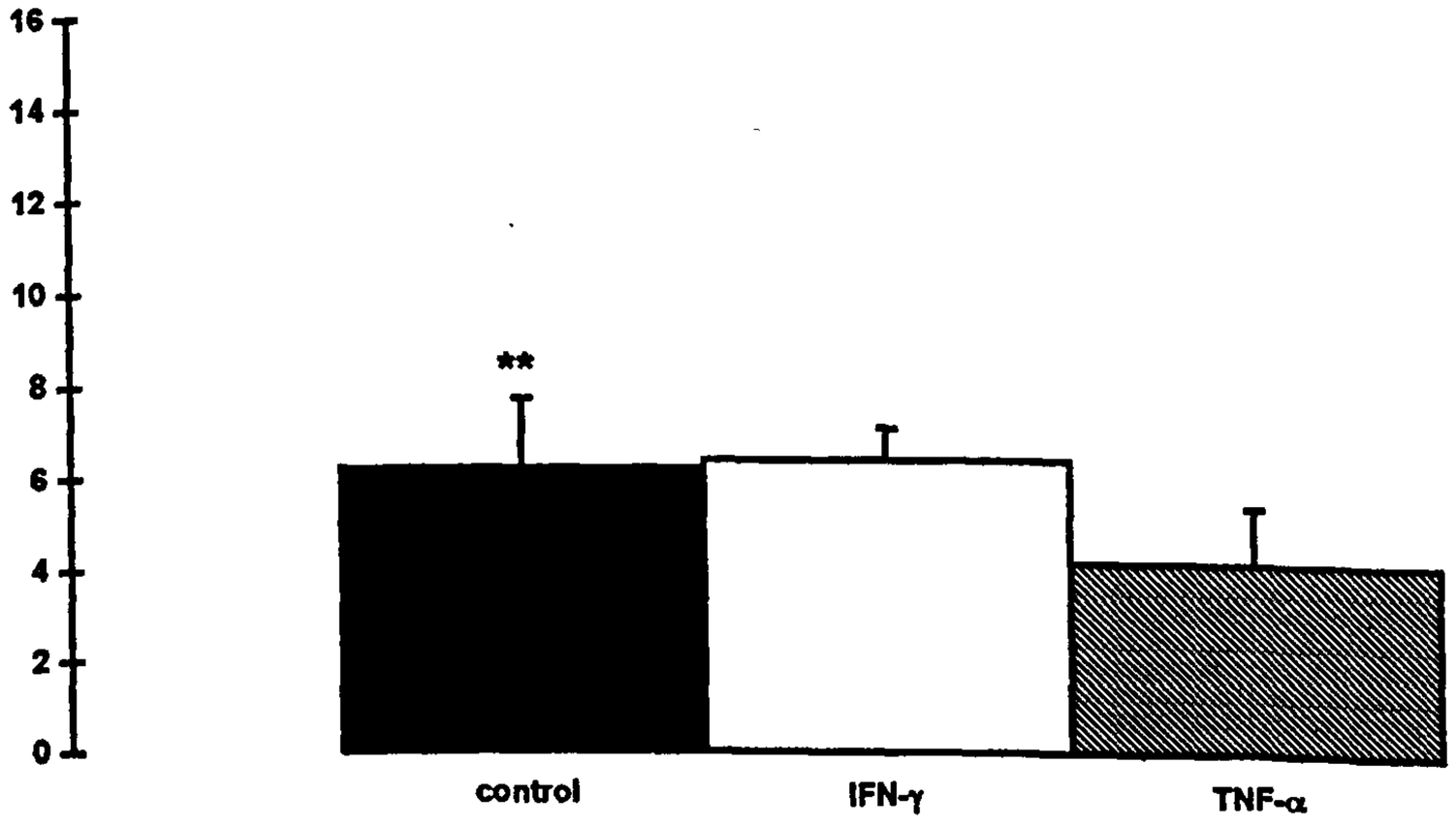


Cytokine additions

IFN-γ and TNF-α failed to exert any significant effects under all conditions irrespective of culture substrata, except in one medium, 14 (containing BPE/PMA), where TNF-α reduced cell number by 14% ($p<0.05$) (**Table 3.10**). Media composition (as previously discussed in **Section 3.3.1**) played a significant role in regulating melanocyte proliferation as indicated in **Figures 3.18-3.19** and **Tables 3.8-3.9**. Cell proliferation was reduced in all of the media examined with significant reductions in cell number ranging from 9% (medium 4 containing FCS/BPE/CT, $p<0.05$) to 67% (medium 21 containing PMA, $p<0.01$). **Figure 3.19b** illustrates that the mean % control response to different media (control being medium 1) were very similar ($r= 0.93$) to those seen in the population of cells analysed in **Section 3.3.1** (where n=4-14) for these 17 media variants.

ECM did not influence cell number (unlike in **Section 3.3.4**). (The ECM had been produced by a sodium deoxycholate method in this section instead of water as described in **Section 2.5.3**). As with cells cultured on plastic, significant decreases in cell number of 31% ($p<0.05$) and 38% ($p<0.01$) were seen for melanocytes cultured on ECM in media 10 and 24 respectively when compared to melanocytes cultured on ECM in medium 1 (**Table 3.9b**).

In summary, cytokines and ECM did not influence melanocyte proliferation whereas media composition played a strong role in influencing melanocyte proliferation.

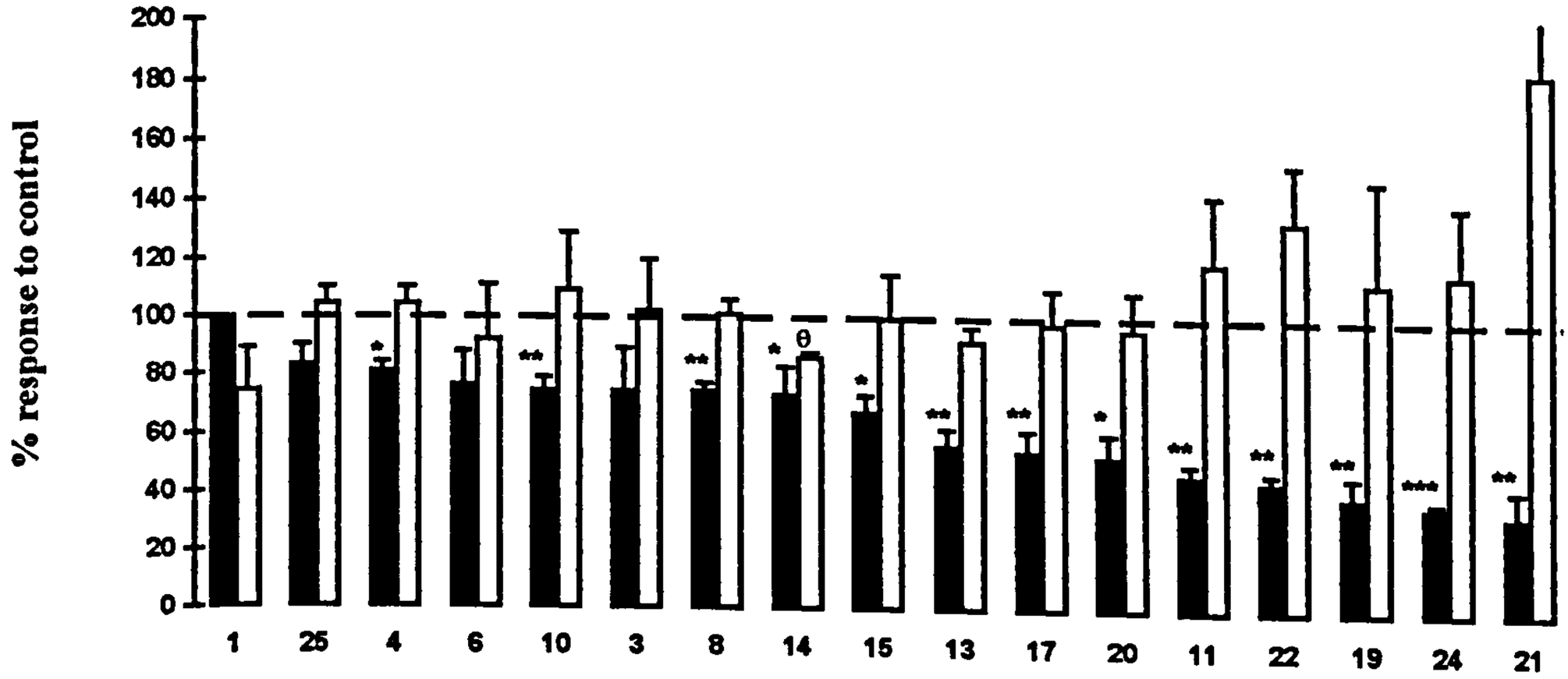
3.3.6 Comparison of normal and vitiligo melanocyte proliferation.

A comparison of the growth of normal and vitiligo melanocytes was undertaken by (a) determining the length of time the cells were in culture prior to experimentation and (b) assessing cell numbers obtained in an experimental period where cells had been plated out at the same time and cultured under identical conditions.

The culture of melanocytes from small biopsies was inherently difficult whether the skin source was from a normal subject or a vitiligo patient. Unfortunately, all vitiligo cultures of melanocytes were set up from small biopsies. Accordingly, in some cases, the freshly isolated cells were cultured as epidermal sheets or on collagen I to improve

Figure 3.19 Influence of 17 different media variants and TNF-α on the proliferation of normal melanocytes. P4 melanocytes were cultured in the appropriate media variant as indicated by the numbers (refer to **Table 2.2** for media compositions) for 5 days (as described in **Table 3.9**). 100U/ml TNF-α was added for the final 24 hours and cells were counted. **(a)** The data for untreated cells (closed bar) has been expressed as a % of the cell number obtained in medium 1 while the data for cells treated with TNF-α (open bar) has been expressed as a % of the appropriate media control. Results are the mean ± SEM of n=3 different melanocytes donors (individual data prior to normalisation is shown in **Table 3.10**). Significant effects of media on melanocyte proliferation are indicated by *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ based on Student's paired t-test while significant effects of TNF-α are indicated as $^{\theta}$=$p<0.05$ based on Student's paired t-test (performed on the data prior to normalisation) **(b)** illustrates the influence of media on two series of experiments using melanocytes from different donors. Results are expressed as a % of medium 1 and are the mean ± SEM of melanocytes from 3 different donors (closed bar) (these melanocytes, cell number results shown in **a**, were used in the investigation of the effects of cytokines on ICAM-1 expression further described in **Section 4.2.3**) and melanocytes from 13 different donors (open bar) (these melanocytes were used in the investigation of the effects of α-MSH as described in **Section 3.3.1** and shown in **Figure 3.10**). Significant effects of media are indicated by *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ based on Student's paired t-test test (performed on the data prior to normalisation).

a



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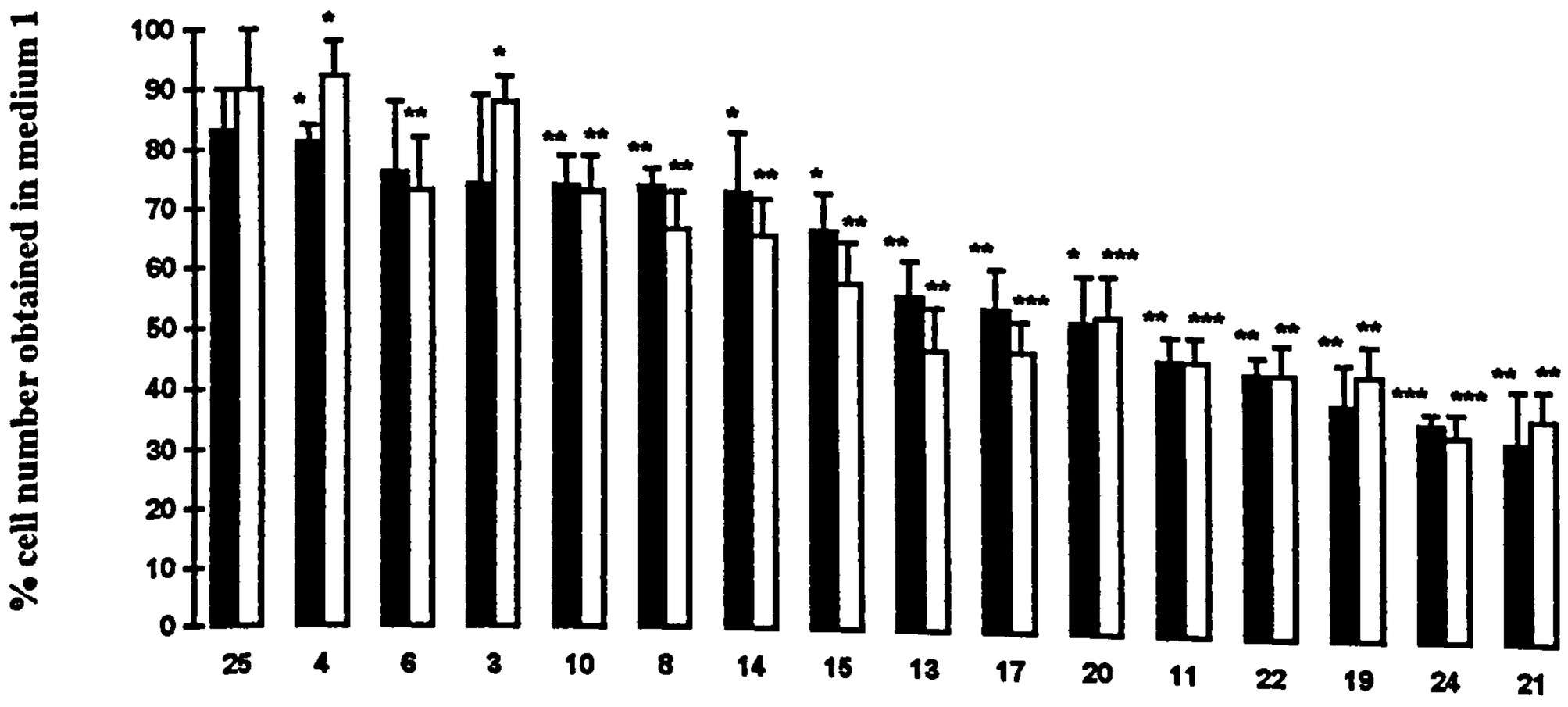


Media variants

Table 3.10 Effect of media and TNF-α on melanocyte proliferation.

	Cell number ($x10^4$)										Mean normalised data to			
	M684P4		M738P4		M764P1		Control		TNF-α		medium 1		TNF-α	
Media	Control	TNF	Control	TNF	Control	TNF	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1	10.75	9	10	4.5	12.75	11.75	11.17	0.82	8.42	2.11	100	0	74	15
3	9	7.5	4.5	6.25	11.75	10	8.42	2.11	7.92	1.10	74	15	102	18
4	9.25	10.75	8.25	7.75	9.5	9.75	9	0.38	9.42	0.88	81*	3	104	6
6	5.75	7	9.25	5.5	10.5	10	8.5	1.42	7.5	1.32	76	12	92	19
8	7.25	8	7.75	7.5	9.75	9.25	8.25	0.76	8.25	0.52	74**	3	101	5
10	8	7	6.5	9.75	10.5	9.5	8.33	1.17	8.75	0.88	74**	5	109	20
11	5.75	6	4	3.5	5.75	9.5	5.17	0.58	6.33	1.74	46**	4	119	23
13	4.75	4.75	6	5.5	8	6.75	6.25	0.95	5.67	0.58	56**	6	92	5
14	7.25	6.5	6	5	11.75	10.25	8.33	1.75	7.25	1.56	73*	10	86*	2
15	8.5	6.75	6	7.75	7.75	7.25	7.42	0.74	7.25	0.29	67*	6	100	15
17	5.25	6.25	4.5	4.5	8.5	6.5	6.08	1.23	5.75	0.63	54**	7	98	12
19	4.75	3.75	2.5	4.5	6.25	5	4.5	1.09	4.42	0.36	39**	7	113	34
20	4.5	4.75	6.75	4.75	5.75	6.5	5.67	0.65	5.33	0.58	52*	8	96	13
21	4.25	4.25	1.5	5.25	5.5	5,5	3.75	1.18	5	0.38	33**	9	183	83
22	4.25	6.25	5	4.75	5.25	8.25	4.83	0.3	6.42	1.01	44**	3	133	19
24	3.75	4.5	3.25	5	5	3.75	4	0.52	4.42	0.36	36***	2	116	23
25	7.75	8.5	8	7.25	12.25	13.5	9.33	1.46	9.75	1.91	83	7	104	6

Melanocytes from 3 different donors were plated at 2.5 to 3.18 x 10^4 cells/well (in 12 well plates with 1 well for cell number) in maintenance media A or B, and allowed to adhere for 24 hours. Cells were then cultured for 5 days in the appropriate experimental media (as designated by the numbers in the tables, refer to **Table 2.2** for compostition). 100U/ml TNF-α, was added for the final 24 hours. Cell number was assessed by counts (as described in **Section 2.7.1)** . The mean data ± SEM is illustrated for control and TNF-α treated cells for cell number in addition to the mean normalised data (individual normalised data not shown for clarity) (mean normalised data shown in **Figure 3.19**). The normalised data is expressed as the percent control in response to medium 1 or TNF-α in the individual media. Values differing significantly in response to either media changes or in response to TNF-α are indicated by *=$p<0.05$, **=$p<0.01$ or ***=$p<0.001$ based on Student's paired t-test (performed on data prior to normalisation).

yields. Melanocyte cultures from different donors proliferate at a different rate and, therefore, it was much easier to pair up normal and vitiligo cultures closer to the time of experimentation. The mean yield of 0.34 ± 0.07 x 10^5 cells/T25 flask, obtained for normal melanocytes was not significantly different to the mean yield of 0.32 ± 0.07 x 10^5 cells/T25 flask obtained for vitiligo melanocytes which indicates that the pairing was done quite successfully. The length of time that was required to obtain sufficient melanocytes from each donor for experimentation are listed in **Table 2.3**. The mean culture period of 59.5 ± 10.5 days prior to experimentation for normal melanocytes was a significantly shorter period ($p<0.05$) than the mean culture period of 91.5 ± 8.4 days for vitiligo melanocytes. Using the length of time in culture to obtain sufficient numbers for experimentation as a guide to proliferation it can be seen from **Table 2.3a** that the most proliferative vitiligo melanocytes cultured were from the youngest donor (VM4, age 34 at time of donation), while the least proliferative vitiligo melanocytes were from oldest donor (VM2, age 77 at the time of donation). Six of the normal donor melanocytes were more proliferative than the vitiligo melanocytes (**Table 2.3b**). Melanocytes from donor M813 had the slowest proliferation of the normal melanocytes although the donor was quite young. The median age of the normal melanocyte donors was 30.5 years, and this was significantly younger ($p<0.01$, Mann Whitney test), than the median age of 67.75 years for the vitiligo donors.

However, once vitiligo melanocytes from donors VM1, 2, 4 and 5 were established under the three experimental media conditions there was no significant differences in the mean proliferation when compared to the normal melanocytes over a 5 day period (illustrated by the normalised data in **Figure 3.20**, statistical analysis performed on the data before normalisation as shown in **Table 3.9c**). However, in medium 1, 3 of the 4 cultures of vitiligo melanocytes were less proliferative than their respective paired normal melanocyte cultures. The mean decrease of 20% did not achieve statistical significance as the vitiligo melanocytes from the youngest donor (VM4) were more proliferative than the respective normal melanocyte pair (M843) in this medium (**Table 3.9c**). Both media 10 and 24, as expected, reduced the proliferative potential of the normal and vitiligo melanocytes but significant reductions in cell numbers of 23% ($p<0.05$) and 59% ($p<0.05$) respectively were only seen with the normal melanocytes. The vitiligo melanocytes were less responsive to media 10 and 24 and hence no

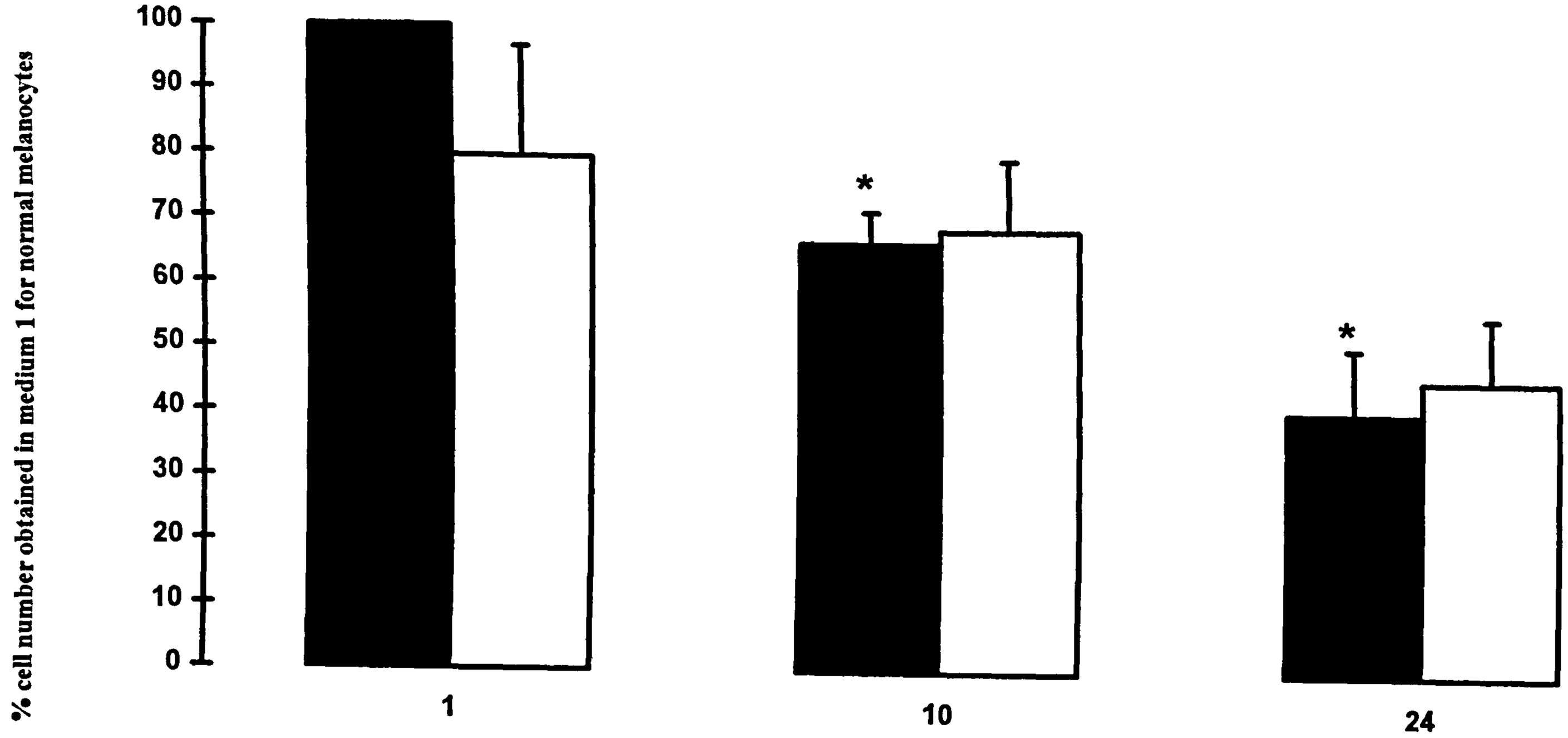

% cell number obtained in medium 1 for normal melanocytes
100
90
80
70
60
50
40
30
20
10
0
*
*
1
10
24
Media variants

Figure 3.20 Comparison of the proliferation of normal and vitiligo melanocytes under 3 different media variants. P4 normal melanocytes (closed bar) and vitiligo melanocytes (open bar) were cultured for 5 days in media 1, 10 or 24 (as described in **Table 3.4**). Cell number was determined by cell counts (as described in **Section 2.7.1**) or by the DNA assay (as described in **Section 2.7.4**). Results have been normalised to the proliferation of normal cells cultured under medium 1 to illustrate the effect of media more clearly. The data represents the mean cell number (x 10^4) ± SEM of 4 experiments using different donor melanocytes. Melanocyte cell number differing significantly from proliferation of normal cells in medium 1 is indicated by *=$p<0.05$ based on Student's t-test (statistical analysis performed on values prior to normalisation).

significant reduction in vitiligo cell number was seen under these media conditions. The effect of IFN-γ and TNF-α was also examined in these cultures but there was no effect on cell number (**Table 3.9c**).

The effect of UVB and cell-derived ECM was examined on normal and vitiligo melancoyte proliferation over a longer time period of 8 days when cells were cultured in media 1, 10 or 24 (**Table 3.11a**). Media 10 and 24 reduced the proliferative capacities of all the cells studied in this section (in agreement with **Section 3.3.1**). UV alone, ECM alone and UV and ECM in combination reduced the proliferative capacities under most of the conditions studied. The ECM results are different to that seen in most cultures studied in **Section 3.3.4**. This may be due to the fact that the lysis of the fibroblasts was by sodium deoxycholate rather than water as in the previous sections. Melanocytes from donor VM1 were generally less proliferative than melanocytes from the paired culture M756 under all media conditions (statistical analysis was not performed as the experiments were not performed in triplicate). Melanocytes from VM3 were significantly less proliferative than melanocytes from M577 when just cultured on plastic by 29% in medium 1 ($p<0.01$) and by 18% in medium 10 ($p<0.01$). However, melanocytes from VM3 were more proliferative than melanocytes from M748 in medium 1 and medium 24 by 16% ($p<0.05$) and by 28% ($p<0.05$) respectively.

Vitiligo melanocytes were generally less proliferative than normal melanocytes in mitogen rich media. However, in less mitogenic media, vitiligo melanocyte proliferation was not reduced to the same extent as normal melanocytes.

3.4 MODULATION OF NORMAL AND VITILIGO MELANOCYTE PIGMENTATION.

3.4.1 Influence of media components on melanocyte pigmentation.

Melanocyte pigmentation was assessed generally by the dopa oxidase activity of the cells and on some occasions by melanin content. In this section it was of interest to see how melanocyte pigmentation varied in response to commonly used mitogens of melanocyte culture media. From all the studies in **Section 3.4**, it was clear that

Table 3.11a The effect of UV and ECM on normal and vitiligo melanocyte cell number.

		Cell number x 10^4			
		sham plastic	UV plastic	sham ECM	UV ECM
VM 1	1	3.76	0.57	0.82	0.9
	10	1.55	0.57	0.82	0.57
	24	0.74	0.53	0.33	0.33
M756	1	5.11	1.8	1.15	2.41
	10	1.35	1.02	0.37	1.39
	24	1.27	0.57	0.21	1.27
VM 3	1	4.63	4.72	3.10*	3.32**
	10	3.79†	4.46	3.23	3.71
	24	2.43†††	2.58	1.46**	1.46*
M577	1	6.56	5.62	4.4**	3.44***
	10	4.61††	5.48	2.67**	3.4**
	24	2.52†††	2.33	1.57***	1.57***
M748	1	3.99	3.46*	ND[a]	ND
	10	3.32††	3.19	ND	ND
	24	1.89†††	1.99	ND	ND

Table 3.11b Effect of UV and ECM on the melanin content of normal and vitiligo melanocytes.

		Melanin (µg/ml/10^4 cells)			
		sham plastic	UV plastic	sham ECM	UV ECM
VM 1	1	1.64	5.34	2.34	3.71
	10	2.6	3.85	2.43	6.2
	24	3.48	3.47	7.34	5.64
M756	1	1.3	2.33	1.98	2.69
	10	2.3	4.37	2.65	4.38
	24	2.39	3.46	3.32	3.47
VM 3	1	1.65	1.97	3.28*	3**
	10	2.74†	2.96	3.13	3.15
	24	1.81	2.31	3.68	4.76**
M577	1	0.89	0.82	0.84	1.46**
	10	1.56†	0.43**	1.96	1.35
	24	0.54	0.64	1.48	0.49
M748	1	0.73	1.63*	ND	ND
	10	2.42†	3.5*	ND	ND
	24	0.59	1.46**	ND	ND

Table 3.11: Normal and vitiligo melanocytes were seeded at 3.15 x 10^4 cells/well (12 well, cultures VM1 and M756) in maintenance medium B or at 1.5 x 10^4 cells/well (24 well, cultures VM3, M577 and M748) in maintenance medium C. Cells were cultured on plastic or fibroblast-derived ECM (fibroblasts were lysed by sodium deoxycholate in these experiments as described in **Section 2.5.2**), in triplicate and were irradiated with 143mJ/cm2 of UVB daily for 7 days (as described in **Section 2.6.2**). After the first irradiation cells were cultured in the experimental media 1, 10 or 24. Cell number was calculated by cell counts on pooled wells for each condition (cultures VM1 and M756) (as described in **Section 2.7.1**), or assessed by the DNA assay (as described in **Section 2.7.4**). **(a)** Cell number results are indicated for the area of a 24 well for all cultures. For VM 3, M577 and M748 data represents mean of n=3 wells x 10^4 therefore statistical analysis was performed. SEMs (of these three culture results) are omitted for clarity but ranged from 0.11 to 0.53 in medium 1, from 0.04 to 0.45 in medium 10 and from 0.03 to 0.23 in medium 24. **(b)** Parallel wells were set up for melanin analysis in cultures VM3, M577 and M748, whereas in VM1 and M756, melanin was analysed on the cells which had been cell counted. Melanin was assessed in melanocyte sodium hydroxide digests (as described in **Section 2.8.3**). Statistical analysis was only performed for cultures VM3, M577 and M748 as the data represents the mean of n=3 wells (with O.D. analysis for 3 aliquots per each well). SEMs are omitted for clarity but ranged from 0.03 to 0.63 in medium 1, from 0.05 to 0.47 in medium 10 and from 0.04 to 0.86 in medium 24. UV and ECM cell number/melanin data differing significantly from sham plastic for each media are indicated by *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ based on Student's unpaired t-test. Cell number or melanin data differing significantly from sham plastic in medium 1 for cultures VM3, M577 and M748 are indicated by $^{\uparrow}$=$p<0.05$, $^{\uparrow\uparrow}$=$p<0.01$, $^{\uparrow\uparrow\uparrow}$=$p<0.001$ based on Student's unpaired t-test.

[a] ND - not done.

melanocytes were capable of pigmentation even in the highly proliferative medium 1 and this was used as the control medium.

Most media variants stimulated melanocyte dopa oxidase activity in comparison to medium 1 (**Figure 3.10b**). Significant increases in dopa oxidase activity ranged from 60% (medium 2 containing FCS/BPE/PMA/bFGF, $p<0.05$) to 181% (medium 4 containing FCS/BPE/CT, $p<0.05$). Seven of the 11 media that caused significant increases in enzyme activity contained BPE. Additionally, medium 16 (containing BPE/bFGF) increased dopa oxidase activity by 139% but just failed to achieve statistical significance ($p<0.0541$). The 9 media containing bFGF also enhanced melanogenesis in melanocytes (not all achieved statistical significance), with increases in enzyme activity ranging from 34% (medium 23 containing bFGF) to 381% (medium 25, Sigma medium). Significant reductions in dopa oxidase activity compared to medium 1 ranged from 14% (medium 17 containing PMA/CT, $p<0.05$) to 48% (medium 19 containing FCS, $p<0.05$).

For cells cultured in medium 1, there was no correlation between cell number and dopa oxidase activity ($r=-0.21$, **Figure 3.21a**) or between dopa oxidase activity and melanin ($r=-0.34$, **Figure 3.22a**). However, there was a slight correlation between melanin content and cell number ($r=0.53$, **Figure 3.21b**) for all the cultures in medium 1. When examining the mean cell number and mean dopa oxidase activity for each of the media variants, there was no correlation ($r=0.49$, **Figure 3.21c**).

Unlike the dopa oxidase activity, there was no pattern to what stimulated melanin except that the majority of media stimulated an increase in the level of melanin in comparison to medium 1 (**Figure 3.10c**). Significant increases in melanocyte melanin content ranged from 40% (medium 2 containing FCS/BPE/PMA/bFGF, $p<0.05$) to 336% (medium 16 containing BPE/bFGF, $p<0.01$). As with the dopa oxidase results, there was no correlation between the mean cell number and the mean melanin content for each media variant ($r=-0.32$, **Figure 3.21d**).

Overall, there was no correlation between melanin and dopa oxidase activity for all media conditions ($r=-0.19$, **Figure 3.22b**). However, 6 media (11, 17, 19, 22, 24 and

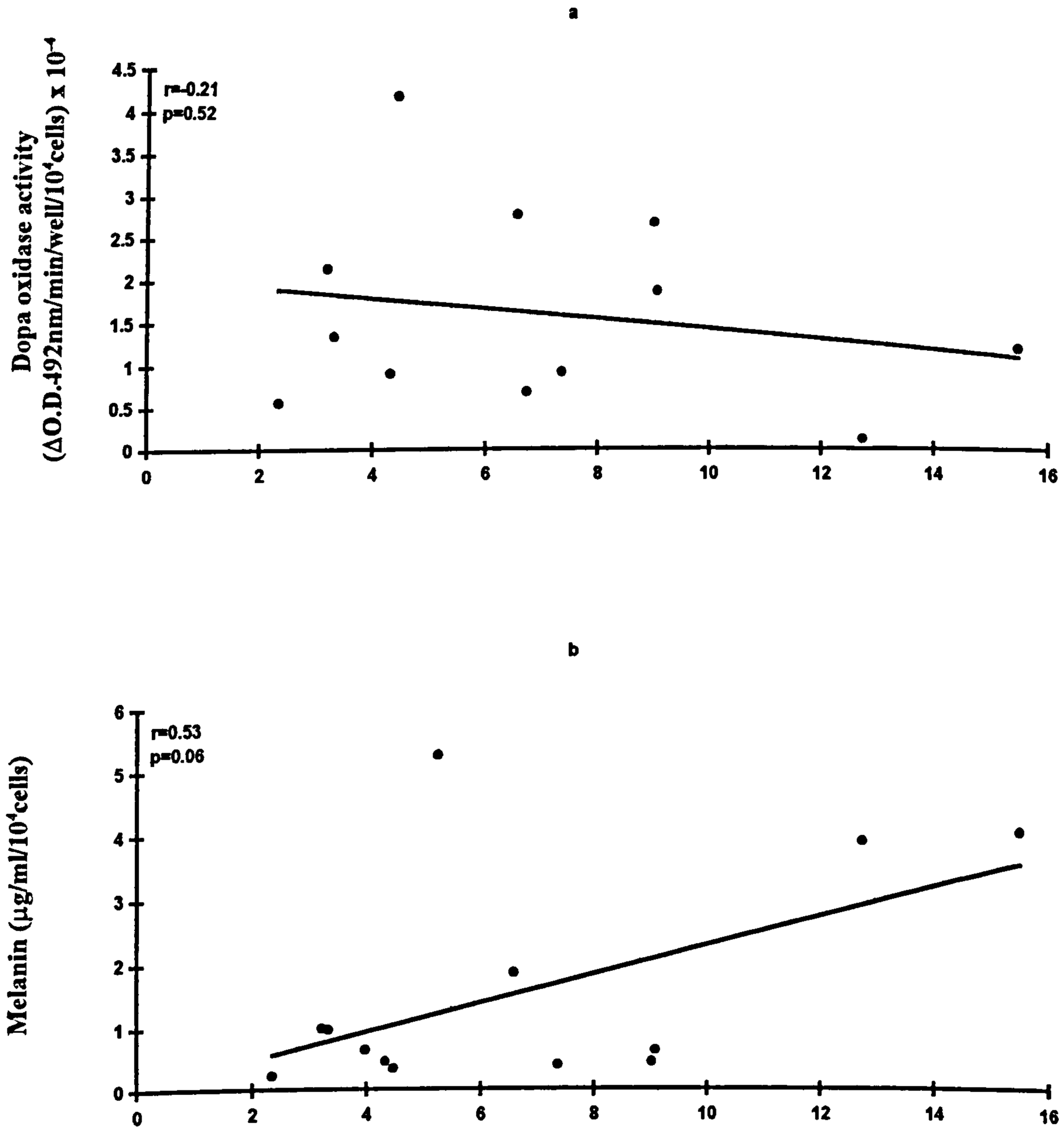
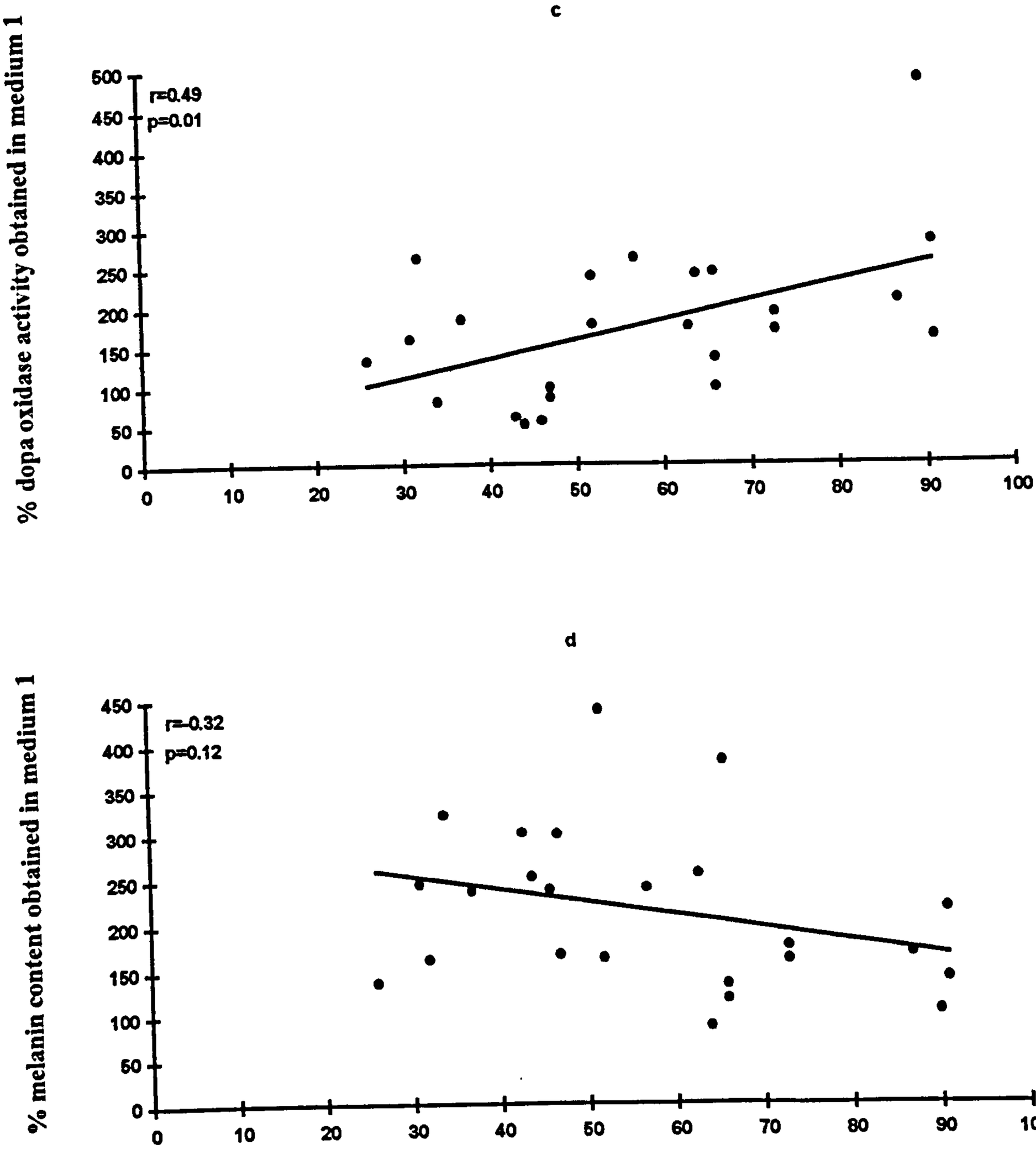
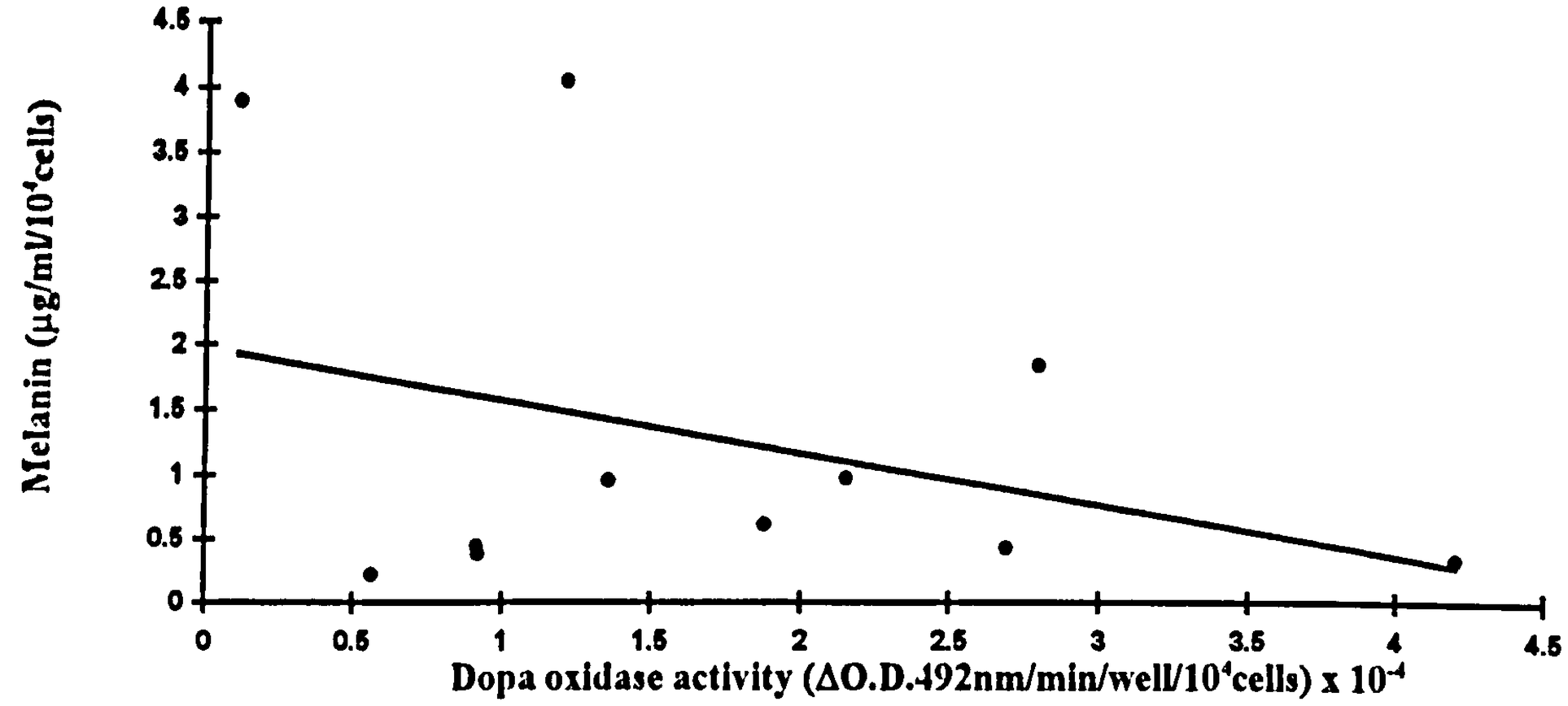


Figure 3.21 Investigation of the relationship between melanocyte proliferation and pigmentation. Melanocytes from 13 different donors were cultured for 5 days under 25 different media conditions (as described in **Table 3.4**). Linear regression analysis was examined between cell number (x 10^4) and dopa oxidase activity (described as ΔO.D.492nm/min/well/10^4 cells x 10^{-4}) for the different melanocyte donors cultured in medium 1 (**a**) and cell number and melanin (described as μg/ml/10^4 cells) for the different melanocyte donors cultured in medium 1 (**b**). The cell number data from **Figure 3.10a** was combined with the dopa oxidase activity data from **Figure 3.10b** (**c**) or the melanin data from **Figure 3.10c** (**d**) as scatter plots



to indicate the correlation between cell number and pigmentation in the 25 different media variants. All data has been normalised for the results in medium 1 for each parameter for **c-d**. The correlation coefficient r, (shown on the graphs) indicates significant correlation between the parameters if $r \geq \pm 0.5$. The p value indicates if the slope of the line is significantly greater than zero.



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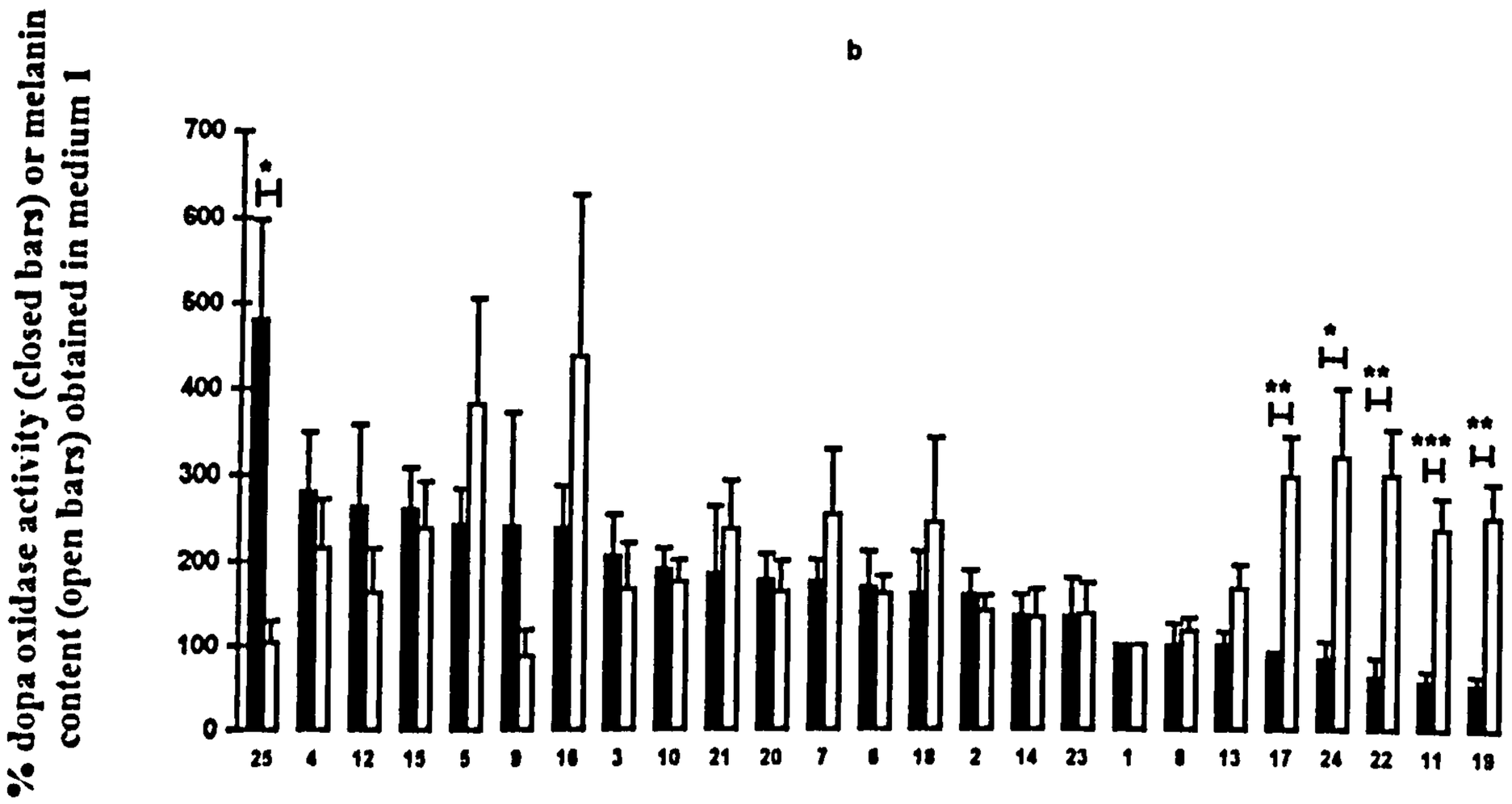


Figure 3.22 Investigation of the relationship between dopa oxidase activity and melanin in melanocytes cultured under 25 different media variants and in response to α-MSH. **Panel A.** Melanocytes from 13 different donors were cultured for 5 days under 25 different media conditions (as described in **Table 3.4**). Linear regression analysis was examined between dopa oxidase activity and melanin (expressed as ΔO.D.492nm/min/well/10^4cells x 10^{-4} and µg/ml/10^4cells respectively) in medium 1 for all the different melanocyte donors (**a**) in the absence of MSH. Data from **Figures 3.10b** and **3.10c** were combined to illustrate the differences in dopa oxidase activity and melanin in the different media (**b**) in the absence of MSH. Both sets of data were normalised to the results obtained in medium 1. Dopa oxidase activity and melanin content that differed significantly once normalised are indicated as ┼*=p<0.05, ┼**=p<0.01, ┼***=p<0.001 based on the ANOVA test (results using ANOVA agreed with those using Student's unpaired t test).

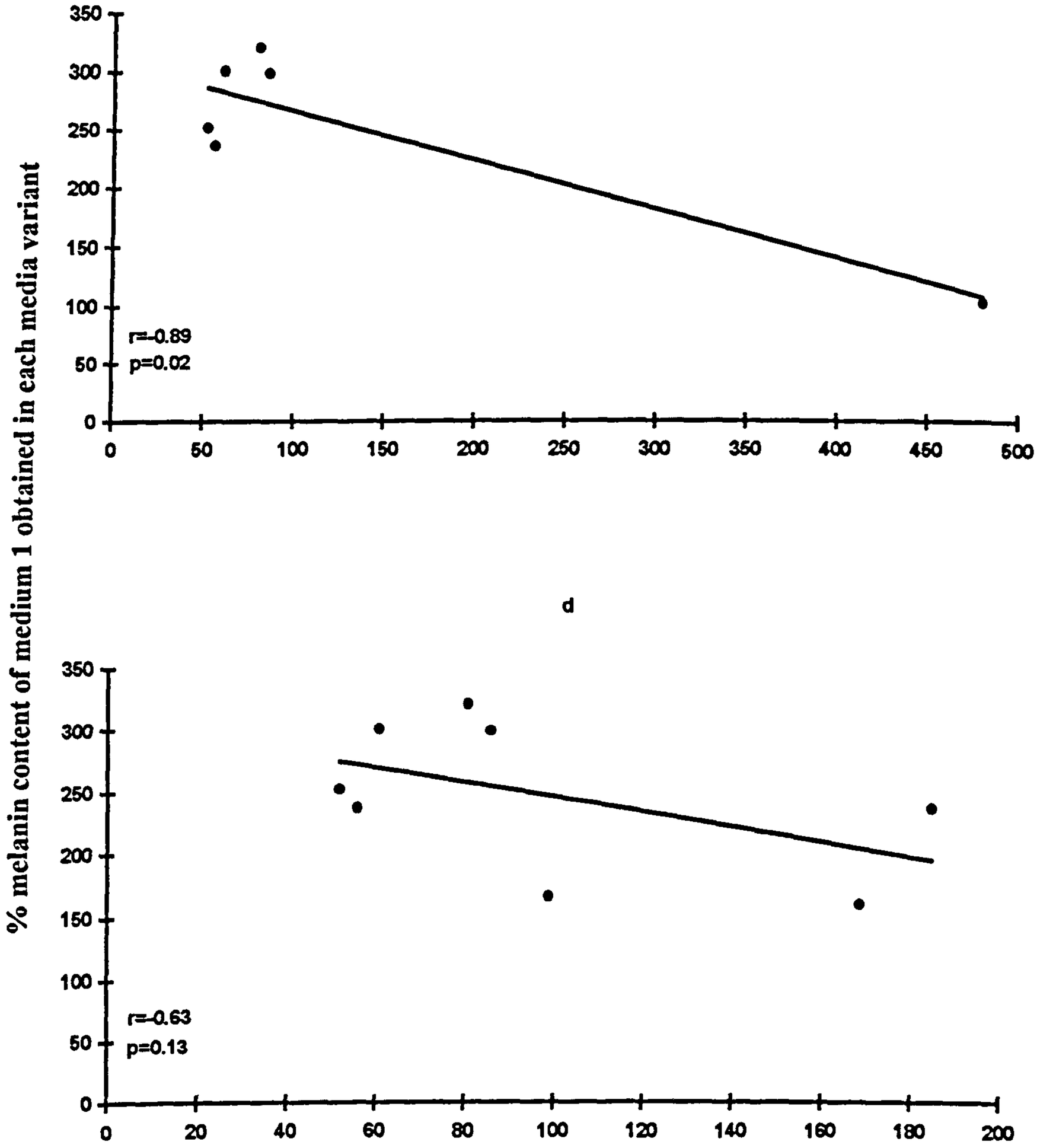
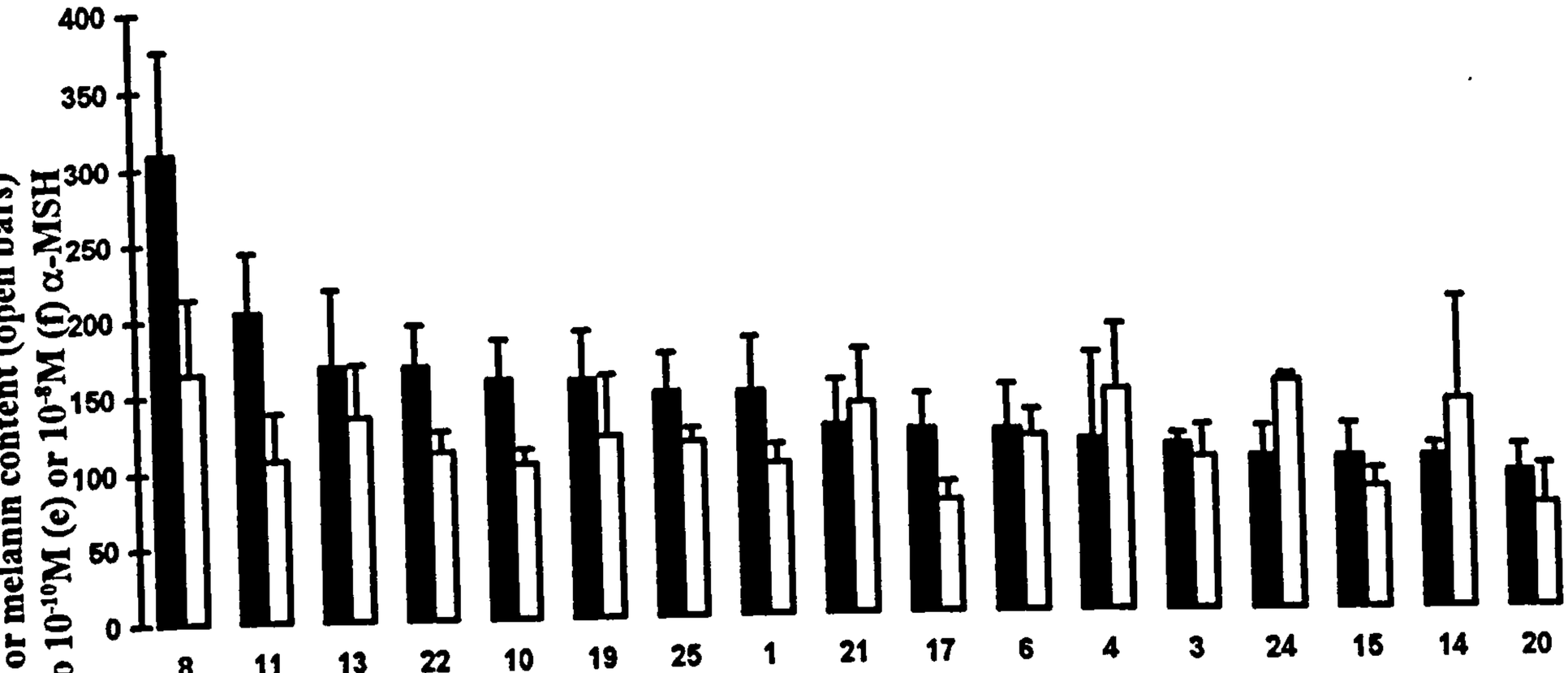


Figure 3.22 Panel B The two scatter graphs represent melanin versus dopa oxidase activity for media (11, 17, 19, 22, 24 and 25) in which the dopa oxidase activity and melanin content differed significantly as (shown in **b**) (**c**) and melanin versus dopa oxidase activity for all media devoid of BPE and/or bFGF media (6, 11, 13, 17, 19, 21, 22, and 24) (**d**) in the absence of MSH. The correlation coefficient r, (shown on the graphs) indicates significant correlation between the parameters if $r \geq \pm 0.5$. The p value indicates if the slope of the line is significantly greater than zero.

e



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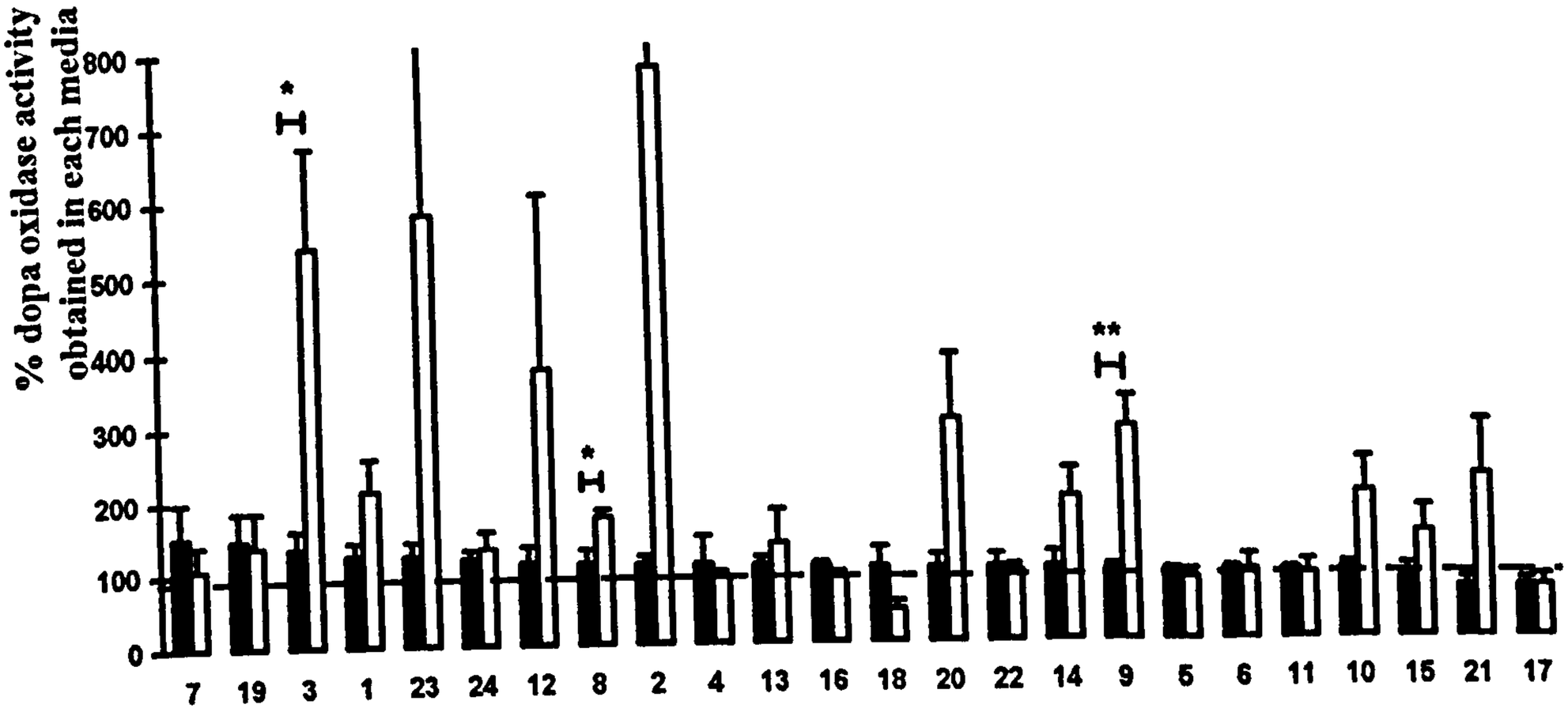


Media variants

Figure 3.22 Panel C. The data from **Figures 3.11b** and **3.11c** are combined (e) and the data from **Figures 3.12b** and **3.12c** (f) are combined to show the similarity and differences in percentage of control in response to α-MSH (at 10^{-10}M and 10^{-8}M respectively) for dopa oxidase activity and melanin under the different media examined. Significant differences in the dopa oxidase activity and melanin are indicated by ⊢* =$p<0.05$ and ⊢** =$p<0.01$ based on the ANOVA test, using the percentage values.

25) caused significant differences in dopa oxidase activity and melanin content of the melanocytes (using the ANOVA test) with an inverse correlation between these media (r=-0.89, **Figure 3.22c**). In 5 out of the 6 cases, the dopa oxidase level was lower than the melanin. None of these 5 media contained BPE and/or bFGF but there was no correlation between the melanin and dopa oxidase activity of these media (r=0.26). However, there was an inverse correlation between the melanin content and dopa oxidase activity of melanocytes cultured in all 8 media (6, 11, 13, 17, 19, 21, 22, and 24) devoid of BPE and/or bFGF (r=-0.63, **Figure 3.22d**).

Reducing the mitogenic capacity of the media generally increased the melanogenic capacity of the media although not in a reciprocal manner. The presence of BPE and/or bFGF in the media appeared to be important to having high melanogenic activity (BPE was also mitogenic as shown in **Section 3.3.1**). Several media, in particularly those lacking BPE and/or bFGF, there was no similarity between the level of dopa oxidase activity and the melanin content with an inverse correlation occurring.

3.4.2 Effect of α-MSH on dopa oxidase activity and melanin content in several media varying in mitogenic potency.

The effect of α-MSH was examined in such a wide range of media in an attempt to find media in which reproducible effects could been seen. Responses to α-MSH were expressed as a % of control reponses in each medium. Melanocyte donor variability in response to α-MSH was such that in all of the 17 media examined, there was no significant alteration in dopa oxidase activity to 10^{-10}M α-MSH (**Figure 3.11b**). While 10^{-8}M α-MSH (**Figure 3.12b**) decreased dopa oxidase activity in medium 17 (containing PMA/CT) by 31% (not acheiving statistical significance) and in medium 21 (containing PMA) by 31% ($p<0.05$), there was no significant increases in enzyme activity in response to 10^{-8}M α-MSH. There was no relationship between cell number responses to MSH and dopa oxidase activity responses to MSH when examining the mean results for each media for either 10^{-10}M α-MSH (r=-0.29) or 10^{-8}M α-MSH (r=-0.39).

The lower concentration of α-MSH (10^{-10}M) studied (**Figure 3.11c**) failed to significantly alter melanin content except in medium 24 (an increase of 49%, $p<0.05$). However, 10^{-8}M α-MSH increased melanin content under several media conditions (**Figure 3.12c**). The increase in melanin content ranged from 74% in medium 8 (containing BPE/PMA/CT) ($p<0.05$) to 677% in medium 2 (containing FCS/BPE/PMA/bFGF) ($p<0.001$). There was a slight relationship between cell number responses to 10^{-10}M α-MSH and melanin response to 10^{-10}M α-MSH ($r=-0.57$) when examining the mean results for each media but there was no relationship in response to 10^{-8}M α-MSH ($r=0.12$).

There was no relationship between dopa oxidase activity and melanin content responses to either 10^{-10}M α-MSH ($r=0.42$) or 10^{-8}M α-MSH ($r=0.23$), when examining the mean results for each media (**Figures 3.22e** and **3.22f** respectively). The % response of dopa oxidase activity in response to 10^{-10}M α-MSH was not significantly different to the % response of melanin content to 10^{-10}M α-MSH under any of the media conditions examined. In media 3 (containing FCS/BPE/PMA), 8 (containing BPE/PMA/CT) and 9 (containing BPE/PMA/bFGF), melanin content was significantly increased compared to dopa oxidase activity in response to 10^{-8}M α-MSH (statistical significance were ($p<0.05$),($p<0.05$) and ($p<0.01$) respectively using the ANOVA test).

α-MSH at either concentration did not have reproducible stimulatory effects on dopa oxidase activity whereas 10^{-8}M α-MSH did significantly stimulate the increase in melanin content under several media conditions. The % responses of melanin and dopa oxidase activity to 10^{-8}M α-MSH were significantly different in only 3 media variants (3, 8 and 9) but not under any of the other conditions. However a good correlation between the two parameters was not seen as in **Section 3.4.1.**

3.4.3. Effect of IBMX on dopa oxidase activity and melanin content in 5 media varying in mitogenic potency.

The effect of IBMX on melanocyte dopa oxidase activty (**Figure 3.14b**) and melanin content (**Figure 3.14c**) was examined in the same cells used in **Section 3.4.2.** In

medium 24, IBMX increased dopa oxidase activity significantly by 78% ($p<0.05$) which was clearly greater than the response to 10^{-8}M α-MSH. IBMX significantly increased the melanin content by 259% in medium 2 ($p<0.05$), by 265% in medium 10 ($p<0.01$), and by 273% in medium 12 ($p<0.05$).

3.4.4 Time dependent effect of cell-derived matrix proteins on melanocyte dopa oxidase activity

Melanocytes are situated in the basal layer adjacent to basement membrane proteins. It is conceivable that these proteins interact with the melanocyte to influence the melanocyte pigmentary behaviour (examined in **Sections 3.4.4-3.4.9**). Melanocytes from two different donors were used to examine a time dependent effect of fibroblast-derived ECM on melanocyte dopa oxidase activity. Melanocytes were cultured either on plastic or on fibroblast-derived matrix under two media conditions for up to 13 days (experiment 1, **Figure 3.23a**) and under three media conditions for up to 14 days (experiment 2, **Figure 3.23b-d**). The dopa oxidase activity per well of the cells was analysed at the indicated timepoints.

The dopa oxidase activity per well of experiment 1 melanocytes increased over the 13 day time period when cells were cultured in medium 1 (irrespective of plating substrata, **Figure 3.23a**). In medium 24, enzyme activity decreased during this time period and melanocytes had significantly lower enzyme activity in medium 24 than for those cells cultured in medium 1. The dopa oxidase activity of melanocytes cultured on plastic and ECM in either media was very similar except on day 10 when melanocytes cultured on ECM, in medium 1, had significantly lower dopa oxidase activity than the melanocytes cultured on plastic.

In experiment 2, melanocytes cultured on plastic in medium 1 attained a maximal enzyme rate by day 11 (**Figure 3.23b**) while corresponding melanocytes cultures on fibroblast-derived ECM achieved maximal enzyme activity by day 8. Hence, fibroblast-derived ECM accelerated the rate at which maximal dopa oxidase activity per well was attained. In medium 10 (**Figure 3.23c**), the maximal enzyme rate was attained by day

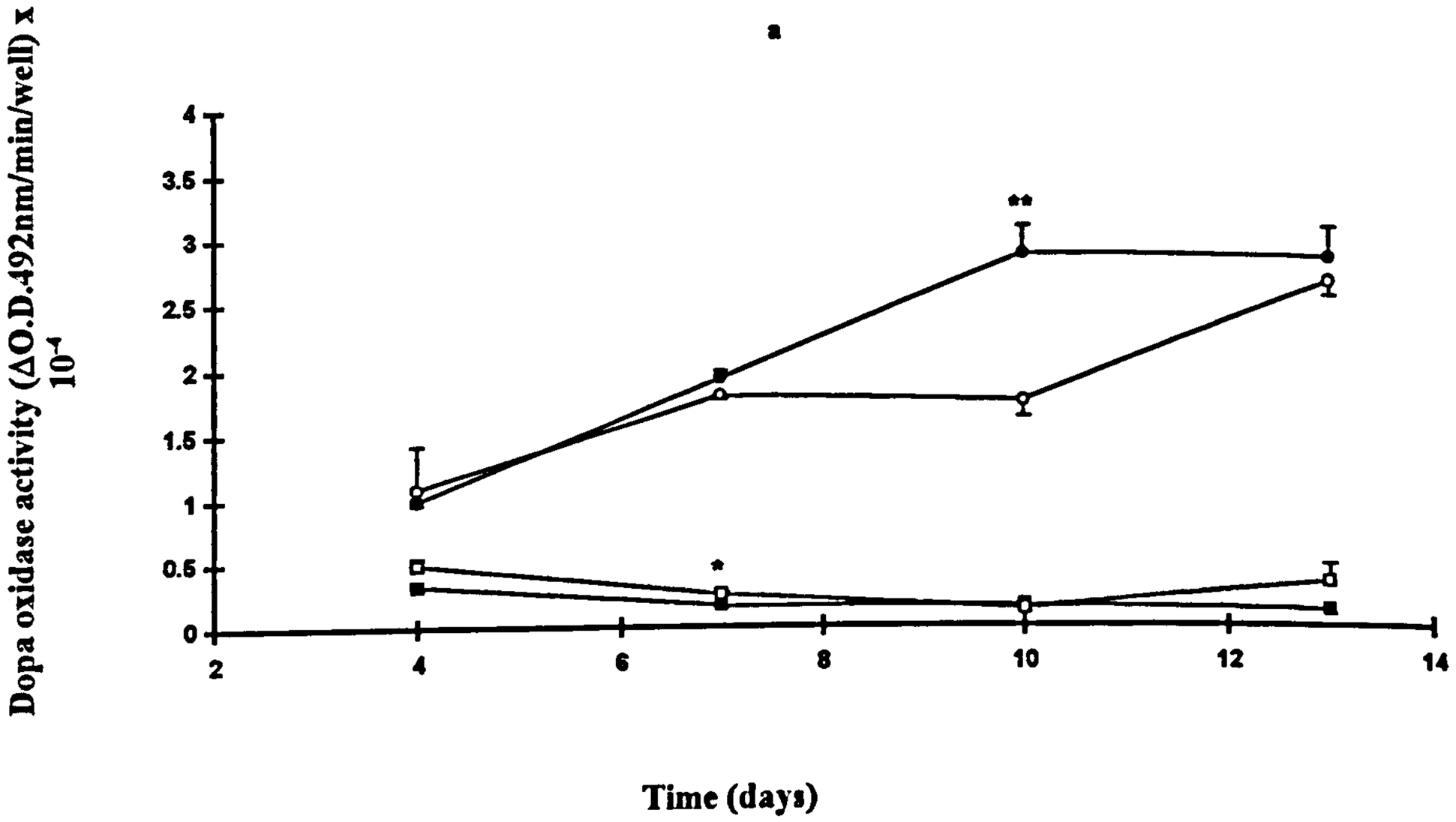
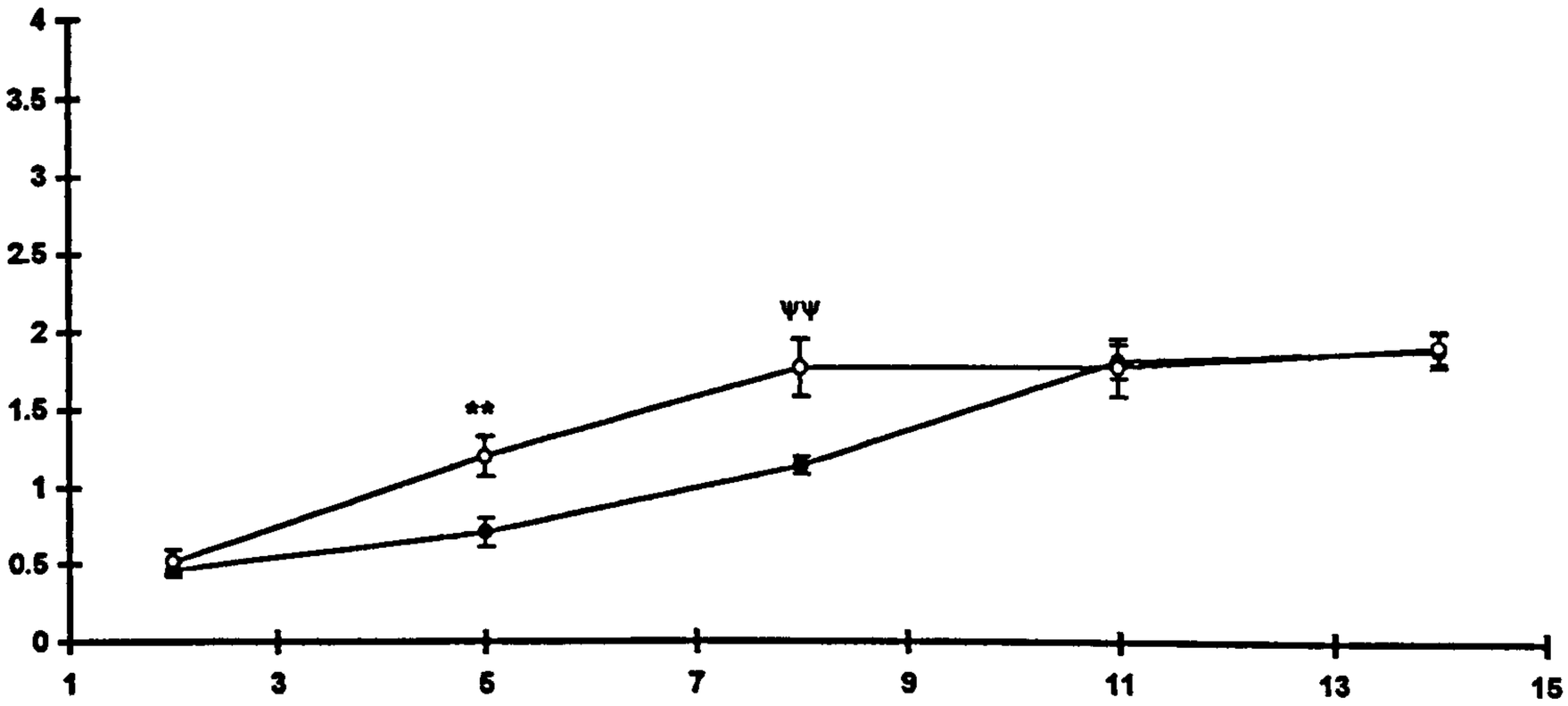


Figure 3.23 Time course for the effect of fibroblast-derived matrix proteins on melanocyte dopa oxidase activity (indicated as rate of increase in O.D.490nm/min/well x 10^{-4}). Passage 4 melanocytes were seeded at 1 x 10^4 cells/well (96 well plate) in medium 1 on either plastic (closed circle) or fibroblast-derived ECM (open circle), or in medium 24 on either plastic (closed square) or fibroblast-derived ECM (open square). (ECM production detailed in **Section 2.5.3**) **(a).** Passage 3 melanocytes were seeded at 1 x 10^4 cells/well (96 well plate) in maintenance medium A on either plastic (closed circle) or fibroblast-derived ECM (open circle). After 24 hours, medium was changed to medium 1 **(b)**, medium 10 **(c)**, or medium 24 **(d)**. Medium was changed every 3 days. Cultures were terminated and analysed for dopa oxidase activity per well at the time points indicated (as described in **Section 2.8**). Enzyme activity of cells cultured on ECM that differed significantly from that of cells cultured on plastic is indicated by *=$p<0.05$, **=$p<0.01$ ***=$p<0.001$ based on Student's unpaired t-test or by $^{\Psi}$=$p<0.05$, and $^{\Psi\Psi}$=$p<0.01$, based on the Mann Whitney test. Results shown are means ± SEM of 8 replicate wells for cells cultured on plastic and of 5 wells for cells cultured on ECM **(a)** or six replicate wells **(b-d)**.

b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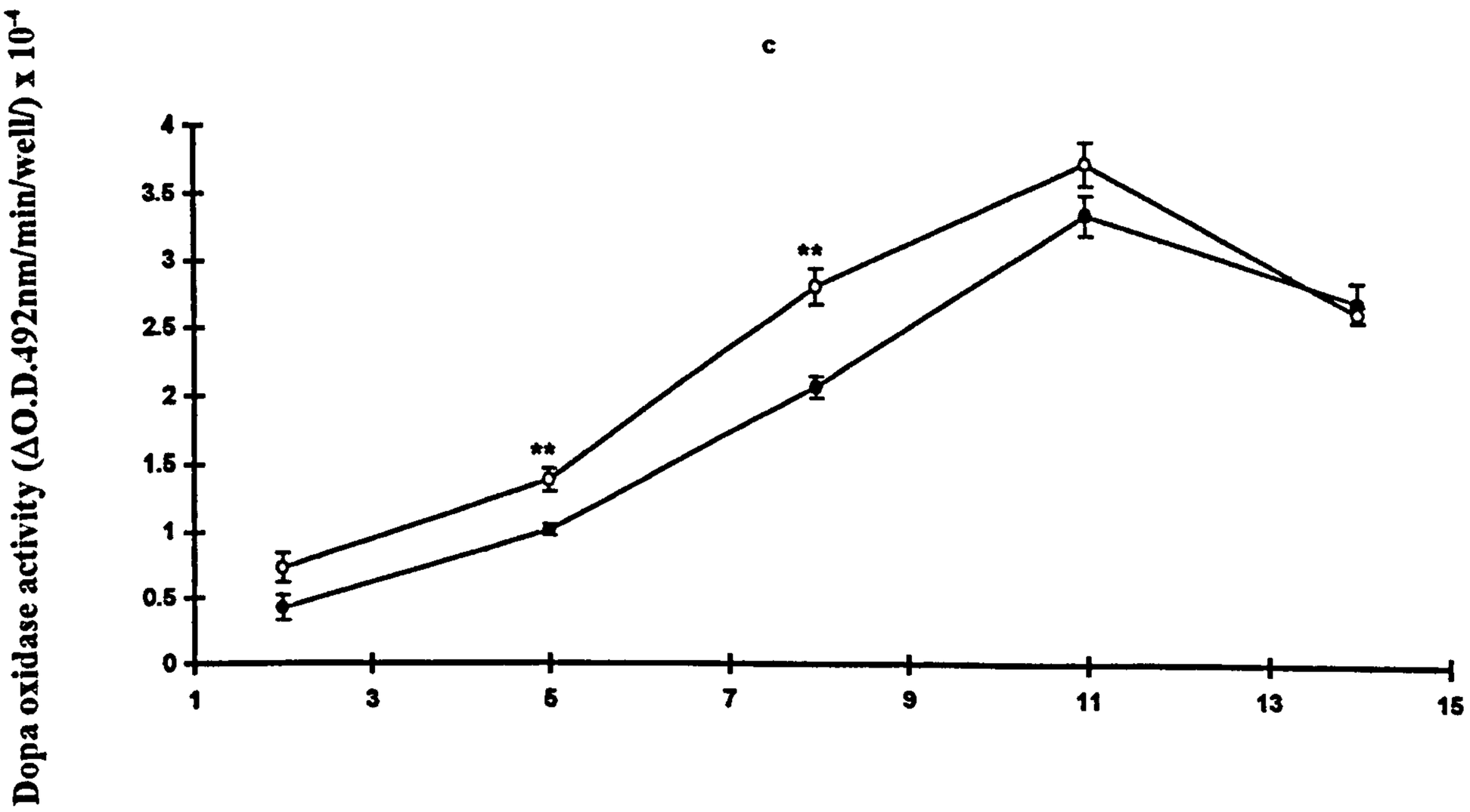
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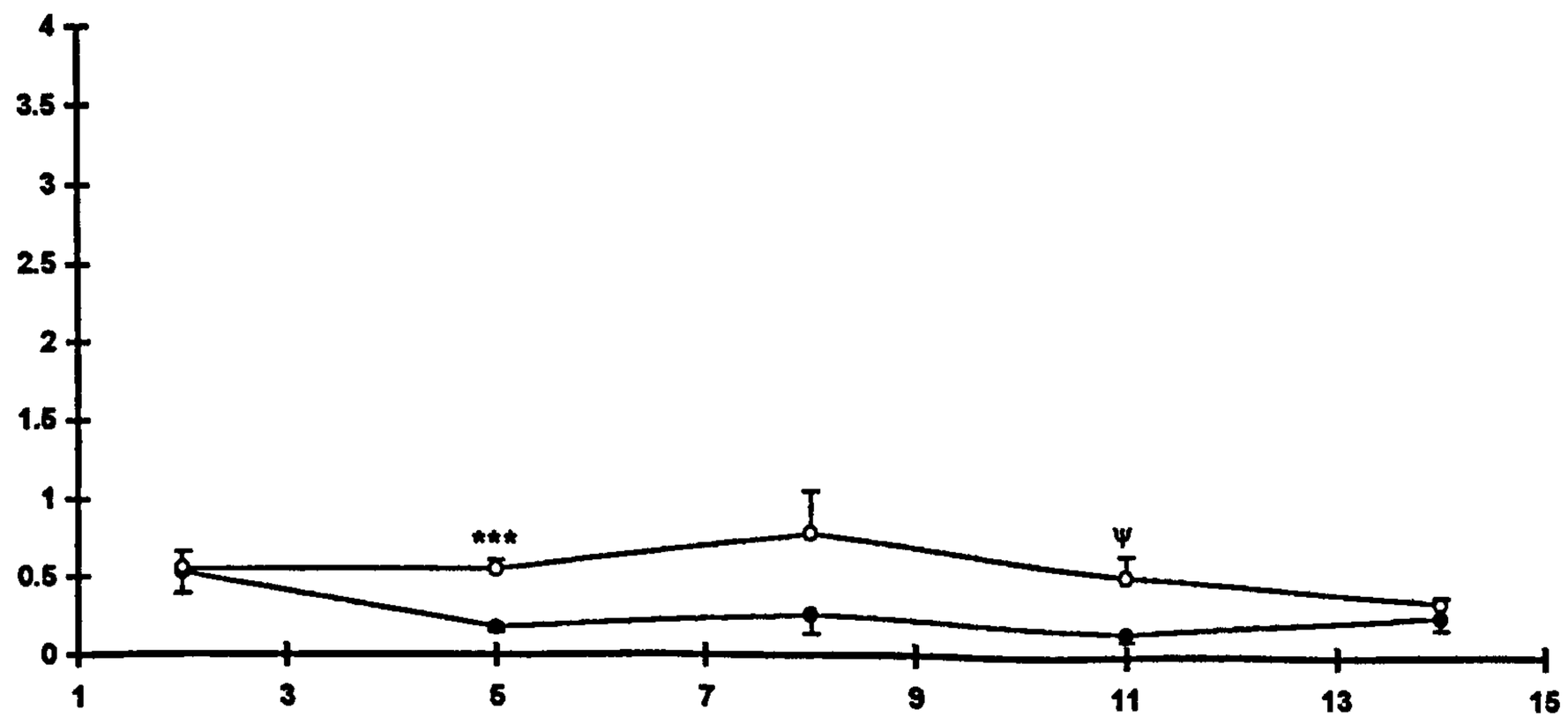
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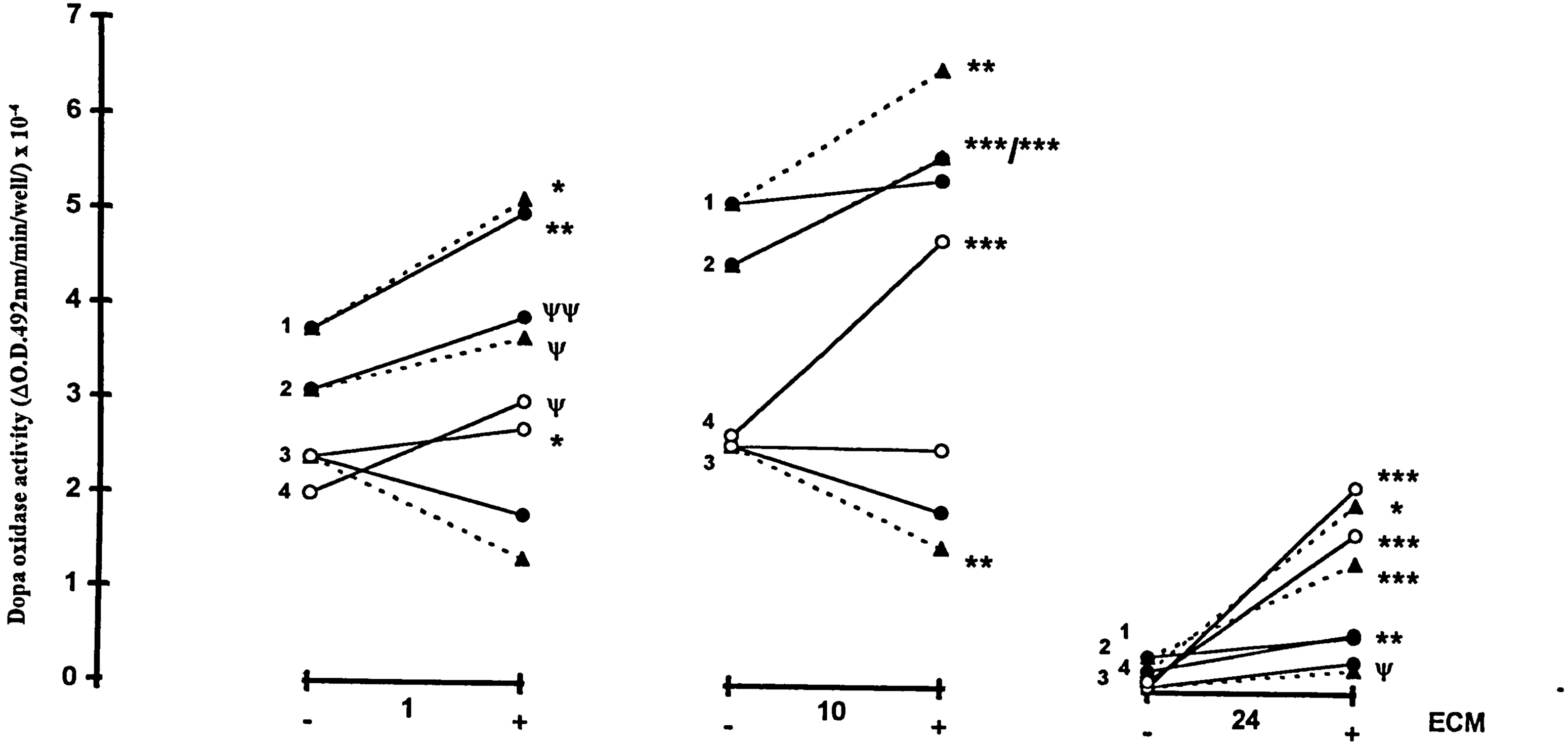
11. The dopa oxidase activity was slightly greater for those cells cultured on ECM than on plastic. There was a slight decline in enzyme activity after this point.

The dopa oxidase activity per well of melanocytes cultured in medium 24 dropped from day 2 to day 5 (consistent with progressive loss of cells under these conditions) (**Figure 3.23d**). A very low level of enzyme activity was maintained up to 14 days by which time relatively few cells remained. By day 11, a 92% decrease in dopa oxidase activity per well was observed when compared to the medium 1. A low but steady rate of dopa oxidase activity was maintained throughout 11 days when the cells were cultured on fibroblast-derived ECM. Cells grown on ECM had a 3.4 fold increase in enzyme activity compared to cells cultured on plastic on day 11, under this medium condition. ECM would appear to be able to provide at least some of the signals necessary to maintain dopa oxidase activity under this medium condition, which was not itself adequate to maintain melanocyte morphology or dopa oxidase activity.

In experiment 1, fibroblast-derived ECM had no effect on melanocyte dopa oxidase activity but in the second experiment fibroblast-derived ECM enhanced dopa oxidase activity under all three media. The effect of fibroblast-derived matrix on dopa oxidase activity per well was most evident around days 5 to 8, for cells cultured in the presence of some mitogens, and around days 5 to 11 for cells cultured in the absence of mitogens. The greatest dopa oxidase activity overall was seen in cells cultured in medium 10 while the lowest enzyme activity was seen in cells cultured in medium 24. From these results, it was decided that all experiments were to be conducted in cells cultured for a 5 day experimental period unless stated otherwise.

3.4.5. Comparison of matrices derived from fibroblasts, human endothelial cells and bovine endothelial cells on melanocyte dopa oxidase activity

The effects of fibroblast-derived ECM, human endothelial cell line-derived ECM and bovine corneal sub-endothelial-derived ECM on melanocyte dopa oxidase activity were examined (the preparation of the cell-derived ECMs are described in **Section 2.5.3**) on melanocytes from several different donors (**Figure 3.24**). When cells were cultured in media 1 or 10, the cell-derived ECM increased dopa oxidase activity per well in some,



Media variants

Figure 3.24 Comparison of the effect of cell-derived matrices on dopa oxidase activity in normal cutaneous melanocytes. Melanocytes, passage 3 or 4, were cultured on either plastic or cell-derived matrices for 5 days in media 1, 10 or 24 as indicated. The figure shows data of 4 experiments (each using a different donor skin for melanocyte culture). Experiment 4 cells had initially been plated out at 6 x 10^4 cells/well in the experimental media while the other 3 experiments cells had been seeded at 1 x 10^4 cells/well (96 well plates, experiments 1 and 2) or at 2.8 x 10^4 cells/well (24 well) in maintenance medium A for 24 hours. The dopa oxidase activity of cells cultured on cell-derived ECMs is indicated by +, or for cells cultured on plastic as -. The cell-derived matrices were fibroblast-derived ECM (closed triangle), bovine corneal endothelial cell-derived ECM (open circle), and human endothelial cell line derived ECM (closed circle). Values shown are means of n=8 (experiments 1 and 2), n=4 (experiment 3), and n=7 (experiment 4) replicate wells. SEMs (which are omitted for ease of showing data) averaged 5% of the mean for cells cultured in media 1 and 10, while in medium 24, they averaged 36% of the mean. Where the presence of cell-derived ECM significantly increased dopa oxidase activity/well this is indicated by *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ based on Student's unpaired t-test or by $^{\Psi}$=$p<0.05$ and $^{\Psi\Psi}$=$p<0.01$ based on the Mann Whitney test.

Table 3.12 The effect of cell-derived ECM on dopa oxidase activity of melanocytes.

	Dopa oxidase activity (ΔO.D.492nm/min/well) x 10^{-4}					
	Medium 1		Medium 10		Medium 24	
ECM presence	-	+	-	+	-	+
Fibroblast -derived ECM	3.08 ± 0.39	3.39 ± 1.11	4.06 ± 0.77	4.57 ± 1.56	0.22 ± 0.92	1.23 ± 0.52
Human endothelial-derived ECM	3.08 ± 0.39	3.56 ± 0.94	4.06 ± 0.77	4.33 ± 1.22	0.22 ± 0.92	0.53 ± 0.1*
All cell-derived ECM	2.86 ± 0.24	3.32 ± 0.49	3.7 ± 0.43	4.24 ± 0.69	0.19 ± 0.048	1.15 ± 0.28**

Melanocytes, passage 3 or 4 were cultured on either plastic or cell-derived matrices for 5 days in media 1, 10 or 24 as indicated in the table (as described in **Figure 3.24**). The presence of ECM is indicated by +, the absence by -. The results are mean ± SEM of n=3 experiments for fibroblast-derived ECM,and human endothelial-derived ECM or n=8 for all ECM types (this includes the bovine endothelial-derived ECM, as well as the two other previously mentioned cell-dervied ECMs). Data significantly differing from control is indicated by *=$p<0.05$ and **=$p<0.01$ based on Student's paired t-test.

but not all experiments. One of the donors (experiment 3) in fact had significant reduction of dopa oxidase activity in response to ECM proteins derived from any cells in these two media. In medium 1, the range of increase in enzyme activity/well in response to ECM was 30% ($p<0.01$, Mann Whitney test) for experiment 2, human endothelial matrix) to 50% ($p<0.05$, Mann Whitney) for experiment 4, bovine endothelial matrix); in medium 10 this increase was 26% ($p<0.001$ for experiment 2, fibroblast and human endothelial matrix) to 79% ($p<0.001$ for experiment 4, bovine endothelial matrix). Medium 10 significantly enhanced dopa oxidase activity per well by 29% ($p<0.05$, based on the mean value for all the experiments shown in **Table 3.12**, Student's paired t-test). In contrast, when cells were cultured on plastic in medium 24, dopa oxidase activity per well was reduced by 93% ($p<0.001$, based on the mean value for all the experiments shown in **Table 3.12**, using Student's paired t-test). There was significant enhancement of enzyme activity in 6 out of the 8 conditions when the cells were cultured on cell-derived ECM in medium 24. The level of enhancement ranged from 2.7 fold ($p<0.001$ for experiment 2, human endothelial matrix) to 39.4 fold ($p<0.001$ for experiment 3, bovine endothelial matrix). When mean data for the above experiments are examined (**Table 3.12**), it is seen that cell-derived ECM did not have a significant effect on dopa oxidase activity for cells cultured in media 1 and 10. In medium 24, fibroblast-derived ECM increased enzyme activity per well by 5.52 fold (not achieving statistical significance) while human endothelial-derived ECM and all cell-derived ECM's significantly increased enzyme activity by 2.38 fold ($p<0.05$) and 6.05 fold ($p<0.01$) respectively.

Cell-derived ECM proteins were able to enhance melanocyte dopa oxidase activity per well with the most consistent effects seen in the mitogen reduced medium, medium 24. There was a slight difference between the results obtained in fibroblast and human endothelial-cell derived ECMs, but there was a lot of donor to donor variability.

3.4.6. Effect of individual ECM proteins on dopa oxidase activity

Melanocytes from 3 different donors (for one donor cells were used at P3 and P4) were cultured on individual ECM proteins for 5 days under media 1, 10 or 24. Dopa oxidase activity per well was examined (as described in **Section 2.8.2a**). Media alone had

effects on melanocyte dopa oxidase activity, with cells cultured in medium 10 exhibiting increased dopa oxidase activity of 45% (not achieving statistical significance), while dopa oxidase activity per well was significantly reduced by 82% ($p<0.01$) in melanocytes cultured in medium 24 (statistics performed on values prior to normalisation as shown in **Table 3.13**).

The contribution of the ECM proteins varied considerably in these four experiments (illustrated in **Table 3.13**). All ECM proteins were capable of stimulating dopa oxidase activity in some of the experiments, but the reproducibility of these substrate effects was poor from experiment to experiment. Melanocytes from the same donor, M536, also showed varibility in results. Stimulatory effects were seen most consistently in media 1 and 24, except for laminin. Significant effects due to the ECM in the individual experiments are indicated in **Table 3.13**. Melanocytes from donor M514 were plated onto the individual substrates in the experimental media (unlike the other experiments where cells were plated out in maintenance medium A). The substrates appeared to give a plating advantage in medium 24, where mitogens are absent. Therefore, enhancement of dopa oxidase activity per well could be in part due to cell number differences. In response to individual ECM proteins significant increases in dopa oxidase activity per well for the individual experiments ranged from 21% (experiment 3 collagen I, $p<0.001$) to 79% (experiment 4 fibronectin, $p<0.001$) for medium 1; from 16% (several conditions) to 29% (exp 4 laminin, $p<0.001$ Mann Whitney) in medium 10 and from 26% (exp 1 collagen I, $p<0.05$) to 376% (exp 4 collagen IV, $p<0.01$, Mann Whitney) in medium 24. Combining the data from the four experiments (**Figure 3.25**), it was seen that collagen I, collagen IV and fibronectin all significantly enhanced dopa oxidase activity by 21% ($p<0.05$), 14% ($p<0.05$) and 31% ($p<0.05$) respectively in medium 1. In medium 24 dopa oxidase activity was significantly increased by 29% on collagen I, 41% on collagen IV, 38% on fibronectin, and 5% on laminin (all $p<0.05$). The effects of the individual ECM proteins varied from donor-to-donor, but the most consistent effects of ECM proteins were seen in medium 1 and medium 24.

Table 3.13a The effect of individual ECM proteins on melanocyte dopa oxidase activity in medium 1.

	Dopa oxidase activity (ΔO.D.492nm/min/well) x 10^{-4}				
	Plating surface				
	Plastic	Collagen I	Collagen IV	Fibronectin	Laminin
M514 P4	2.44	3.71***	3.04**	4.35***	2.76
M521 P4	3.62	4.38***	3.86	4.81***	3.34
M536 P3	4.62	5.72♦	5.73♦	6.35♦♦♦♦	5.77♦♦
M536 P4	6.1	6.41	6.43	6.53	5.36♦
Mean	4.2	5.06*	4.77*	5.51*	4.31
SEM	±0.78	±0.62	±0.79	±0.55	±0.74

Table 3.13b The effect of individual ECM proteins on melanocyte dopa oxidase activity in medium 10.

	Dopa oxidase activity (ΔO.D.492nm/min/well) x 10^{-4}				
	Plating surface				
	Plastic	Collagen I	Collagen IV	Fibronectin	Laminin
M514 P4	5.92	7.4***	5.79	6.88***	7.63♦♦♦
M521 P4	4.2	4.43	3.56*	5.04***	4.35
M536 P3	7.71	8.97**	8.57*	7.9	8.91*
M536 P4	6.47	6.19	6.17	6.3	5.97
Mean	6.08	6.75	6.02	6.53	6.71
SEM	±0.73	±0.96	±1.03	±0.6	±0.99

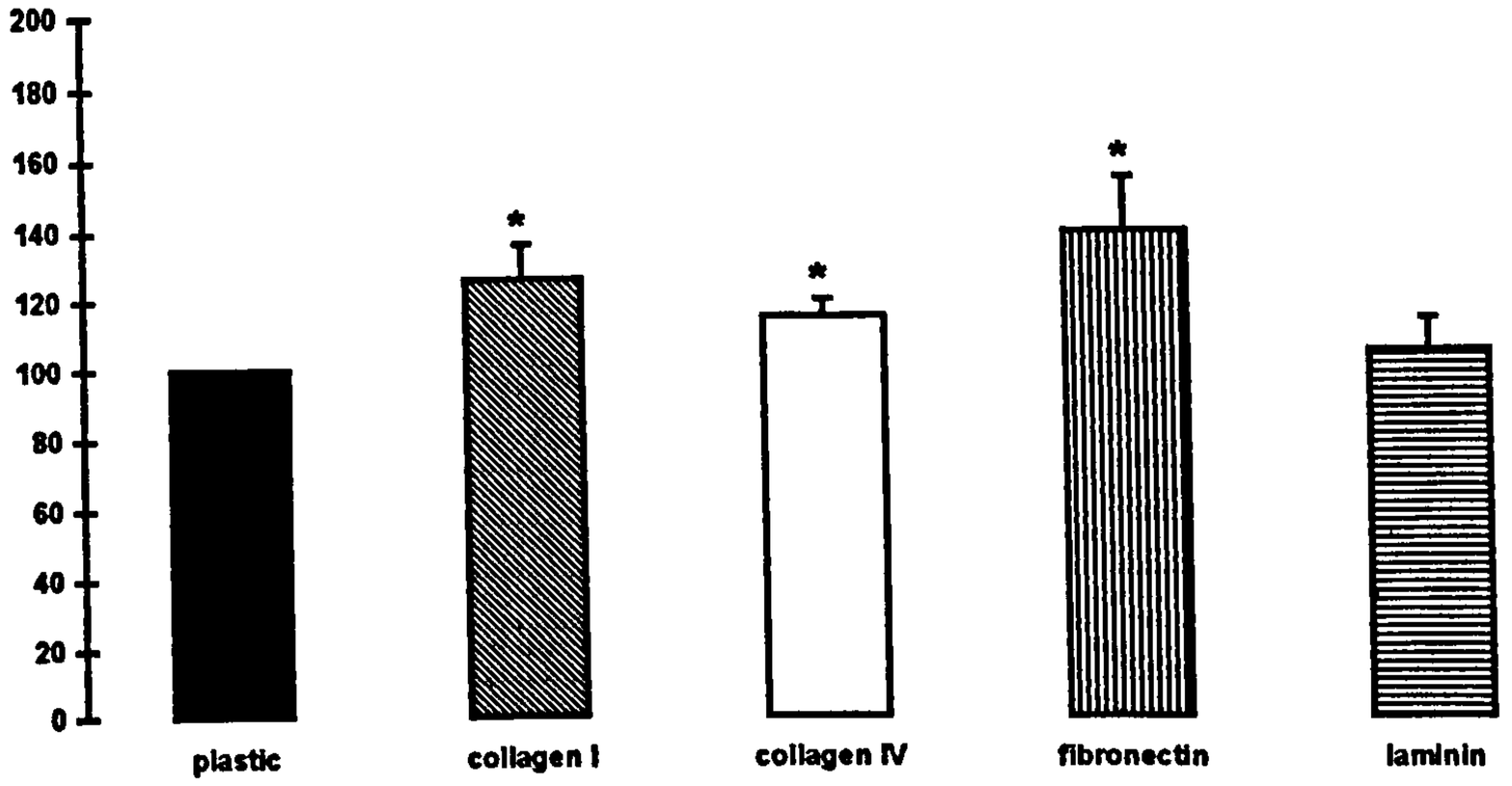
Table 3.13c The effect of individual ECM proteins on melanocyte dopa oxidase activity in medium 24.

	Dopa oxidase activity (ΔO.D.492nm/min/well) x 10^{-4}				
	Plating surface				
	Plastic	Collagen I	Collagen IV	Fibronectin	Laminin
M514 P4	0.17	0.33**	0.63♦♦	0.46♦	0.21
M521 P4	0.5	0.73*	0.47	0.55	0.56
M536 P3	1.58	1.99*	1.93	2	1.61
M536 P4	0.81	0.9	1.27	1.21♦♦	0.83
Mean	0.76	0.99*	1.08*	1.06*	0.8*
SEM	±0.3	±0.35	±0.33	±0.36	±0.3

Table 3.13: Melanocytes (passage 3 or 4) were plated out at 1 x 104 cells/well (96 well plate) on either plastic, collagen I, collagen IV, fibronectin or laminin in maintenance medium A and then transferred to media 1 (**a**), 10 (**b**), or 24 (**c**) 24 hours later. Melanocytes were then cultured for a further 5 days. Melanocytes from donor M514 were plated out at 1 x 104 cells/well (96 well plate) in experimental media and then cultured for a further 5 days. The data for each of the different melanocyte donors is the mean ± SEM of n=8 replicates and the mean ± SEM for dopa oxidase activity of the 4 separate experiments is shown. The data was normalised to cells cultured on plastic in each individual media and the mean normalised data is shown in brackets (illustrated in **Figure 3.25**). Significant changes in dopa oxidase activity in response to the individual ECM proteins are indicated by *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ based on Student's unpaired t-test for individual experiments or based on Student's paired t-test for the mean data or by $^{\phi}$=$p<0.05$, $^{\phi\phi}$=$p<0.01$ and $^{\phi\phi\phi}$=$p<0.001$ based on the Mann-Whitney test.

Figure 3.25 Comparison of effect of individual ECM proteins on dopa oxidase activity in normal human cutaneous melanocytes. Melanocytes (passage 3 or 4) were cultured on either plastic, collagen I, collagen IV, fibronectin or laminin in media 1 (**a**), 10 (**b**), or 24 (**c**) for 5 days. The data represents the mean normalised dopa oxidase activity data ± SEM for 4 individual experiments (as described in **Table 3.13**) where the data has been expressed as a percent of the result obtained for plastic for the 3 different media. Data of melanocytes cultured on ECM proteins differing significantly from those of cells grown on plastic are indicated by *=$p<0.05$ based on Student's paired t-test (performed on data prior to normalisation).

a



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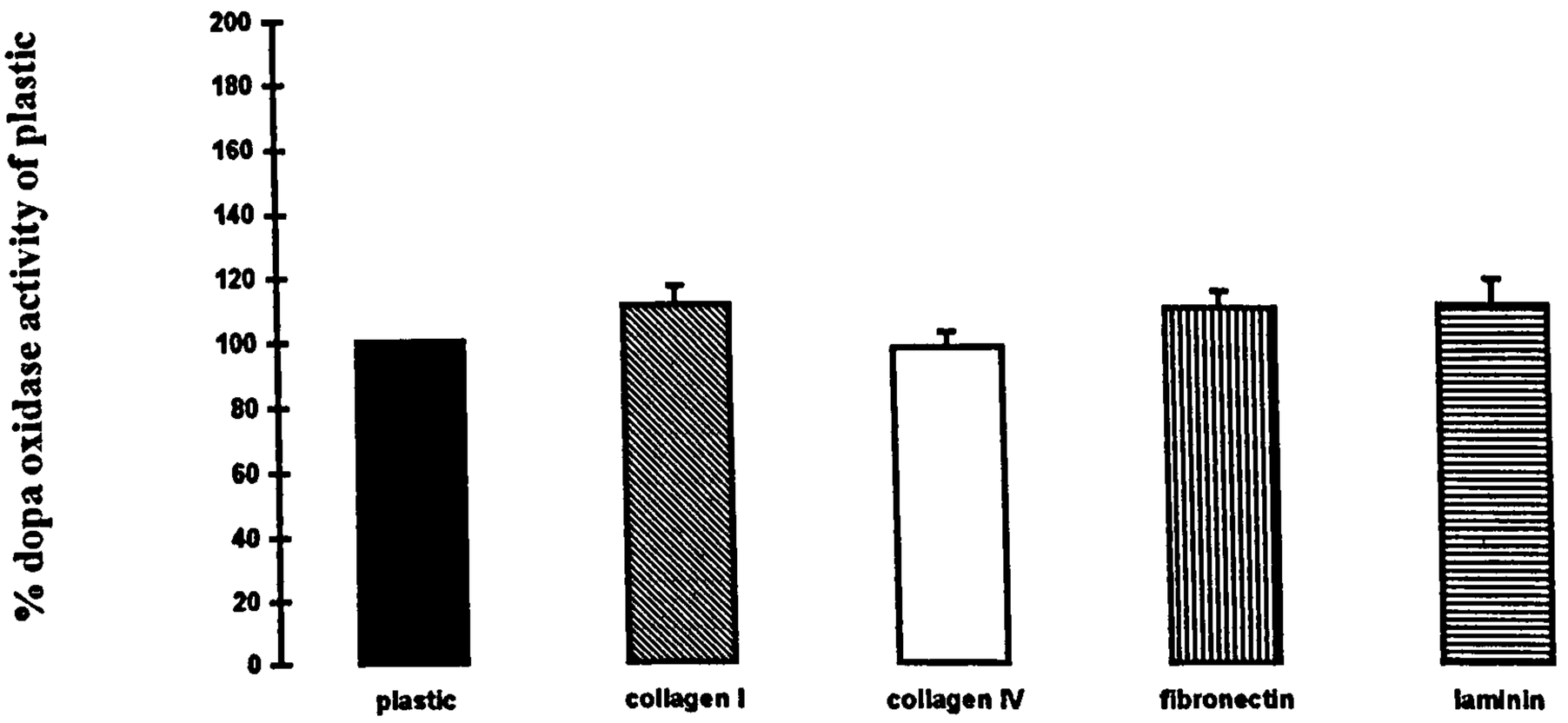

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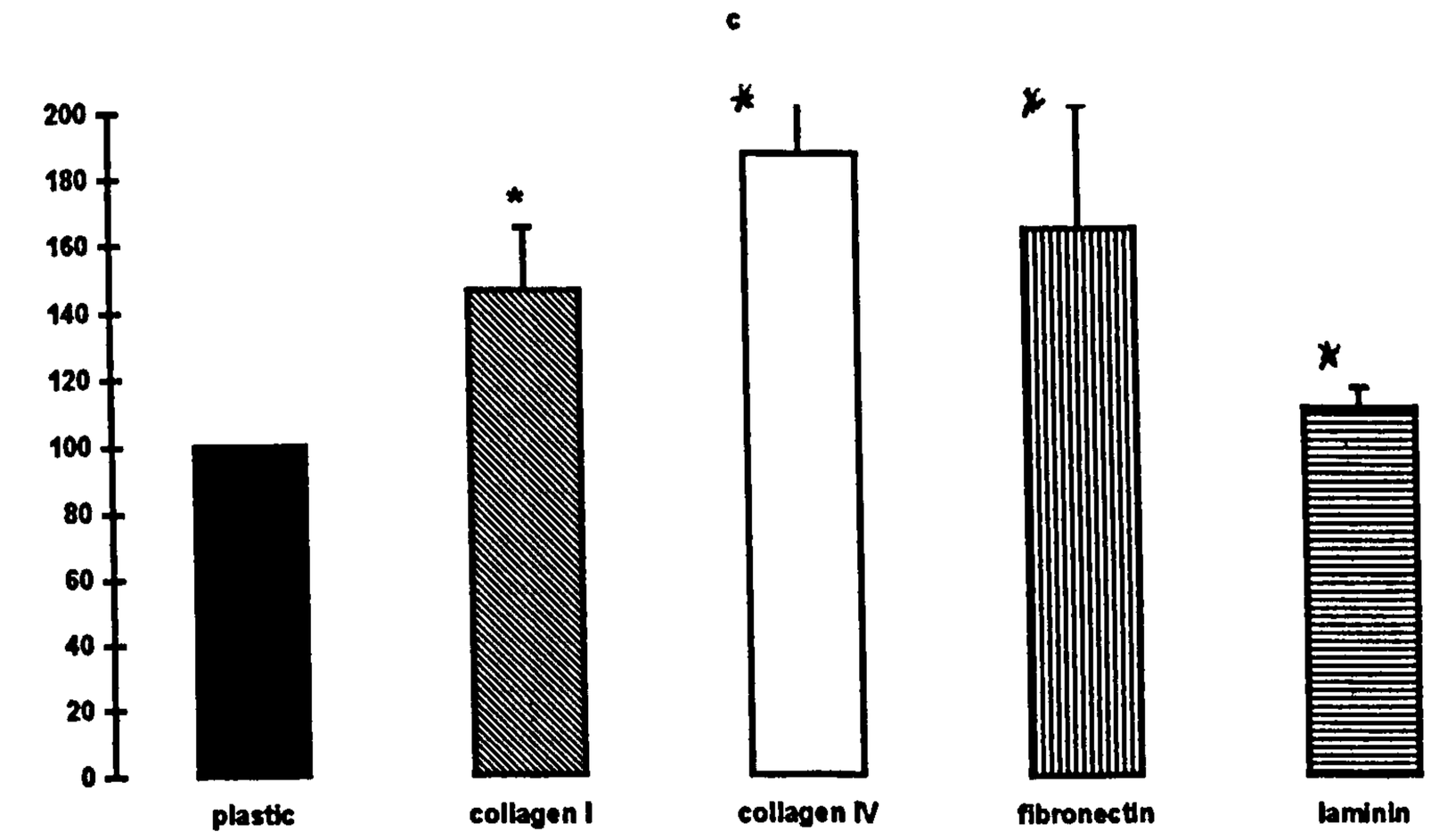


Plating substrata

3.4.7. Effect of α-MSH on dopa oxidase activity of melanocytes cultured on plastic and on fibroblast-derived ECM

Initial experiments to investigate the effect of α-MSH were carried out on melanocytes from three different donors. Cells were expanded in maintenance media A, and then cultured for 5 days on plastic (**Figures 3.26a-c**) or fibroblast-derived ECM (**Figures 3.26d-f**) in one of three experimental media (media 1, 10 or 24). Dopa oxidase activity was performed on cells that had been counted (as described in **Section 2.8.2c**). As several cells in many wells were washed away during the first feed, statistical analysis was not performed for cell number. Additionally statistical analysis was not performed on individual experiments as cells has been pooled for each condition (2 wells in 12 plates had been set up for each condition), then replated out in replicates for the analysis of dopa oxidase activity.

Intra-donor variability was evident in the basal levels of dopa oxidase activity (e.g. 1.68 to 2.52 OD492nm/well/10^4cells x 10^{-4} for cells cultured in medium 1). There was no consistent stimulatory effect of α-MSH on dopa oxidase activity irrespective of whether cells were cultured on plastic or on ECM proteins. Indeed, when data were normalised (combined means presented in **Table 3.14**), inhibition of dopa oxidase activity was seen more consistently. Cells cultured on plastic in medium 10 (**Figure 3.26b**) showed significant decreases in dopa oxidase activity of 47% at 10^{-11}M α-MSH ($p<0.05$) and of 58% at 10^{-7}M α-MSH ($p<0.05$). The levels of dopa oxidase activity were generally higher in cells cultured on ECM than those cultured on plastic (**Figure 3.26** and **Table 3.14**) but there was no synergism between the effects of ECM and α-MSH on dopa oxidase activity. ECM stimulated enzyme activity by 55% ($p<0.01$) in medium 1 and by 75% and 253% in medium 10 and medium 24 respectively (not achieving statistical significance). In a separate experiment using melanocytes from 2 different donors (**Figure 3.16b**), ECM had no overall effect on dopa oxidase actvity for cells cultured in medium 1 although in one culture there was a 40% increase ($p<0.05$) in dopa oxidase activity in this medium. In medium 10, dopa oxidase activity was actually decreased whereas in the mitogen deficient medium, (24), ECM increased dopa oxidase activity by 90% and by 360% ($p<0.001$) in the two different melanocyte cultures.

Figure 3.26 Effect of ECM and α-MSH on dopa oxidase activity. P4 cells were cultured on plastic (**a,b,c**) or fibroblast-derived ECM (**d,e,f**) in medium 1 (**a,d**), medium 10 (**b,e**), or medium 24 (**c,f**) for 5 days in presence or absence of α-MSH (10^{-11}-10^{-7}M) before dopa oxidase was analyzed (as described in **Section 2.8.2c**). The results are expressed as dopa oxidase activity/ΔO.D.492nm/well/10^4cells x 10^{-4}. Each line respresents a different donor of melanocytes.

Table 3.14 Effect of ECM and α-MSH on melanocyte dopa oxidase activity.

		Control	α-MSH [M]		
	ECM		10^{-11}	10^{-9}	10^{-7}
Medium 1	-	2±0.26	99%±18	87%±38	92%±25
	+	3.09±0.37**	68%±29	41%±32	73%±15
Medium 10	-	2.76±0.81	53%±11*	65%±43	42%±5*
	+	4.83±1.11	87%±29	73%±25	79%±19
Medium 24	-	0.65±0.4	90%±18	134%±48	60%±33
	+	2.29±1.36	108%±6	86%±10	124%±21

Melanocytes were seeded onto plastic (-) or fibroblast-derived ECM (+) in maintenance medium A and allowed to adhere for 24 hours before cells were cultured under experimental media conditions for 5 days (as described in **Figure 3.26**). α-MSH (10^{-11} to 10^{-7}M) was added at each feed (x 3 during the experimental period). The above data are combined means of the experiments shown in figure (n=3 different melanocyte donors). Control values are expressed as dopa oxidase activity (ΔO.D.492nm/min/well/10^4cells) x 10^{-4} while the α-MSH values have been normalised. Where dopa oxidase activity has been significantly altered by either ECM or α-MSH, this is indicated by *=$p<0.05$ and **=$p<0.01$, based on Student's paired t-test conducted on the data before normalisation.

Dopa oxidase activity (ΔO.D.492nm/min/well/10^4cells) x 10^{-4}

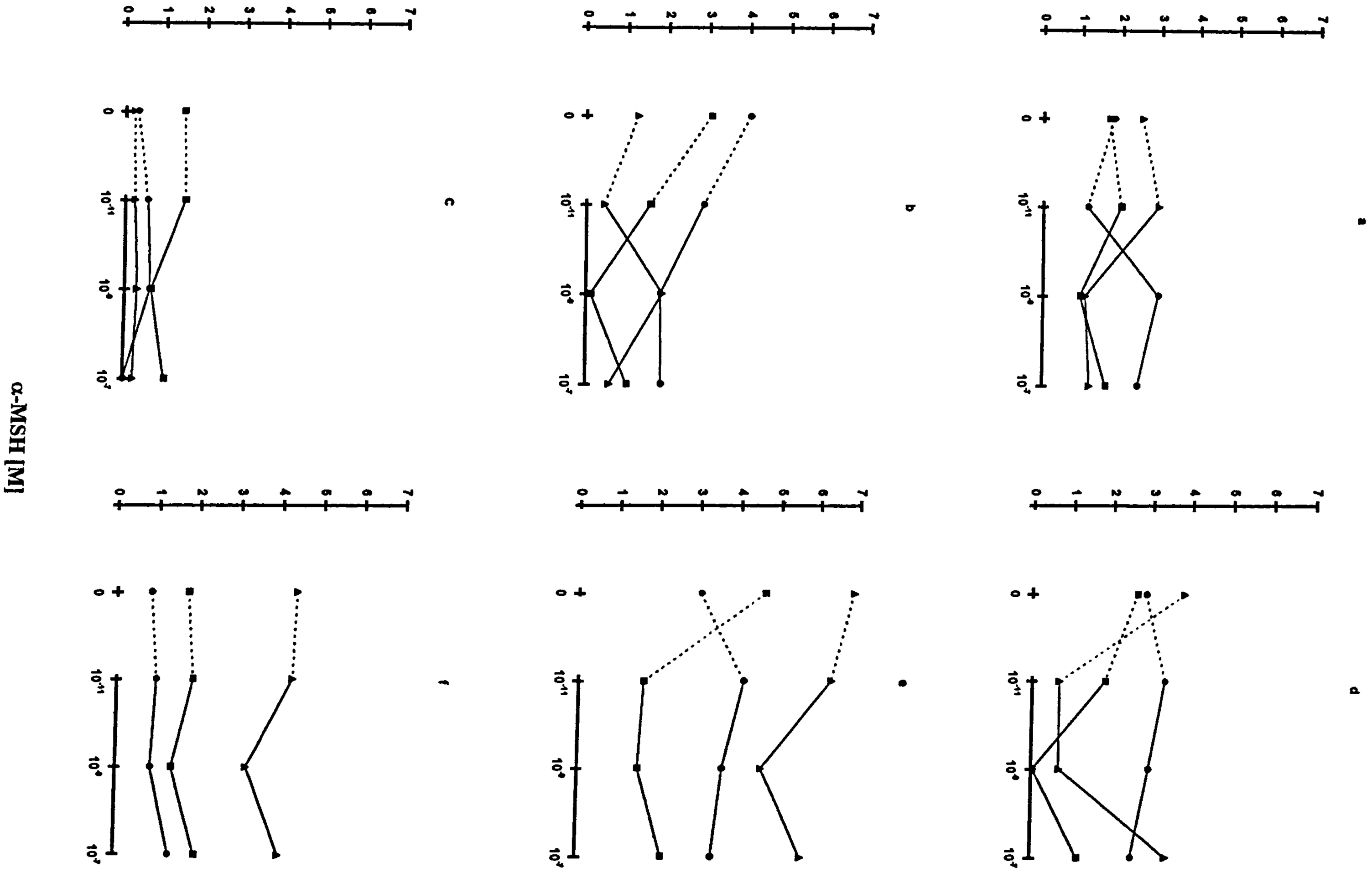


Table 3.15 The effect of UV and ECM on the dopa oxidase activity of normal melanocytes in medium 1.

	Dopa oxidase activity (ΔO.D.492nm/min/well/10^4cells x 10^{-4})				
	ECM type	sham plastic	UV plastic	sham ECM	UV ECM
M536 P3	fibroblast	1.66	3.95	2.68	1.82
M541 P4	fibroblast	2.93	3.52	3.83	4.48
M593 P4	HaCaT	2.93	3.81	2.91	2.13
M593 P4	fibroblast	3	4.02	5.36	3.32
M757 P4	fibroblast	5.36	3.83	4.78	3.65
M671 P4	fibroblast	1.82	2.77	ND[a]	ND
	Mean 1	2.948	3.65	3.91	3.08
	SEM	±0.54	±0.46	±0.52	±0.49
	Mean 2	2.47	3.61**	3.69*	2.94
	SEM	±0.3	±0.23	±0.61	±0.61

Melanocytes were seeded at 2.44 to 6 x 10^4 cells/well (12 well, in triplicate) in maintenance medium A or B and allowed to adhere for 24 hours. Cells were cultured on plastic or fibroblast-derived ECM. One culture of melanocytes, M593 was used in two separate experiments utilising both fibroblast-derived ECM and HaCat-derived ECM as indicated in the tables. Cells were irradiated daily for 7 days with 143mJ/cm^2 (as described in **Section 2.6**) and then pooled for each condition and cell counted (as described in **Section 2.7.1**). Dopa oxidase activity was then analysed on these cells (as described in **Section 2.8.2c**). Mean 1 represents all the data while mean 2 represents all the data except for M757. This culture was the only culture examined where dopa oxidase activity was reduced in response to UVB irradiation. Mean data differing significantly from the sham plastic results are indicated by *=$p<0.05$ (based on Student's unpaired t-test) and **=$p<0.01$ (based on Student's paired t-test).

[a] N.D. = not done.

The maintenance media contained cholera toxin and it was suggested by Abdel-Malek *et al.* (1995) that cells cultured in media containing CT would be less able to respond to α-MSH even when the cholera toxin had been removed for several days. Following the lack of response to α-MSH in these melanocytes on plastic or fibroblast-derived ECM, melanocytes were cultured in media lacking CT for the experiments described in **Sections 3.3.1-3.3.3** and **Sections 3.4.1-3.4.3**.

3.4.8 Influence of UVB and ECM on normal melanocyte pigmentation under 3 media varying in mitogenic potency.

The effect of UVB, media and ECM on melanocyte dopa oxidase activity was examined after 3 different UVB regimens (as described in **Sections 2.6** and **3.3.4**). In media 1 **(Figure 3.27b)** and 10 **(Figure 3.27c)**, dopa oxidase activity did not change over the time period, while in medium 24, the dopa oxidase activity of the melanocytes declined dramatically **(Figure 3.27d)**. The dopa oxidase activity was significantly increased in melanocytes cultured in medium 10 by 35% at timepoint 1 ($p<0.05$), by 34% at timepoint 2 ($p<0.05$) and by 50% at timepoint 3 ($p<0.05$) compared to melanocytes cultured in medium 1 **(Figure 3.27a)**. In medium 24, the dopa oxidase activity of melanocytes was significantly decreased by 14% at time point 1 ($p<0.01$), by 63% at time point 2 ($p<0.05$) and by 86% at timepoint 3 ($p<0.01$) when compared to melanocytes cultured in medium 1 **(Figure 3.27a)**.

In media 1 **(Figure 3.27b)** and 10 **(Figure 3.27c)**, under all conditions (i.e. UV alone, ECM alone and UV plus ECM), dopa oxidase activity of the melanocytes increased over the time course. UV had little or no effect on melanocyte dopa oxidase activity over the first two timepoints under all media conditions. In medium 1, UV increased dopa oxidase by 24% but failed to reach statistical significance at timepoint 3. Melanocytes from 1 donor failed to pigment in response to UV irradiation at day 8 and their dopa oxidase activity was inhibited by UV irradiation by 28% **(Table 3.15)**. Re-examining the data for this medium, excluding the culture that failed to pigment, UV irradiation significantly increased dopa oxidase activity by 46% ($p<0.001$). In medium 10, melanocytes from all the donors were much less responsive to UV. At time point 1, ECM alone significantly increased dopa oxidase activity by 16% in melanocytes

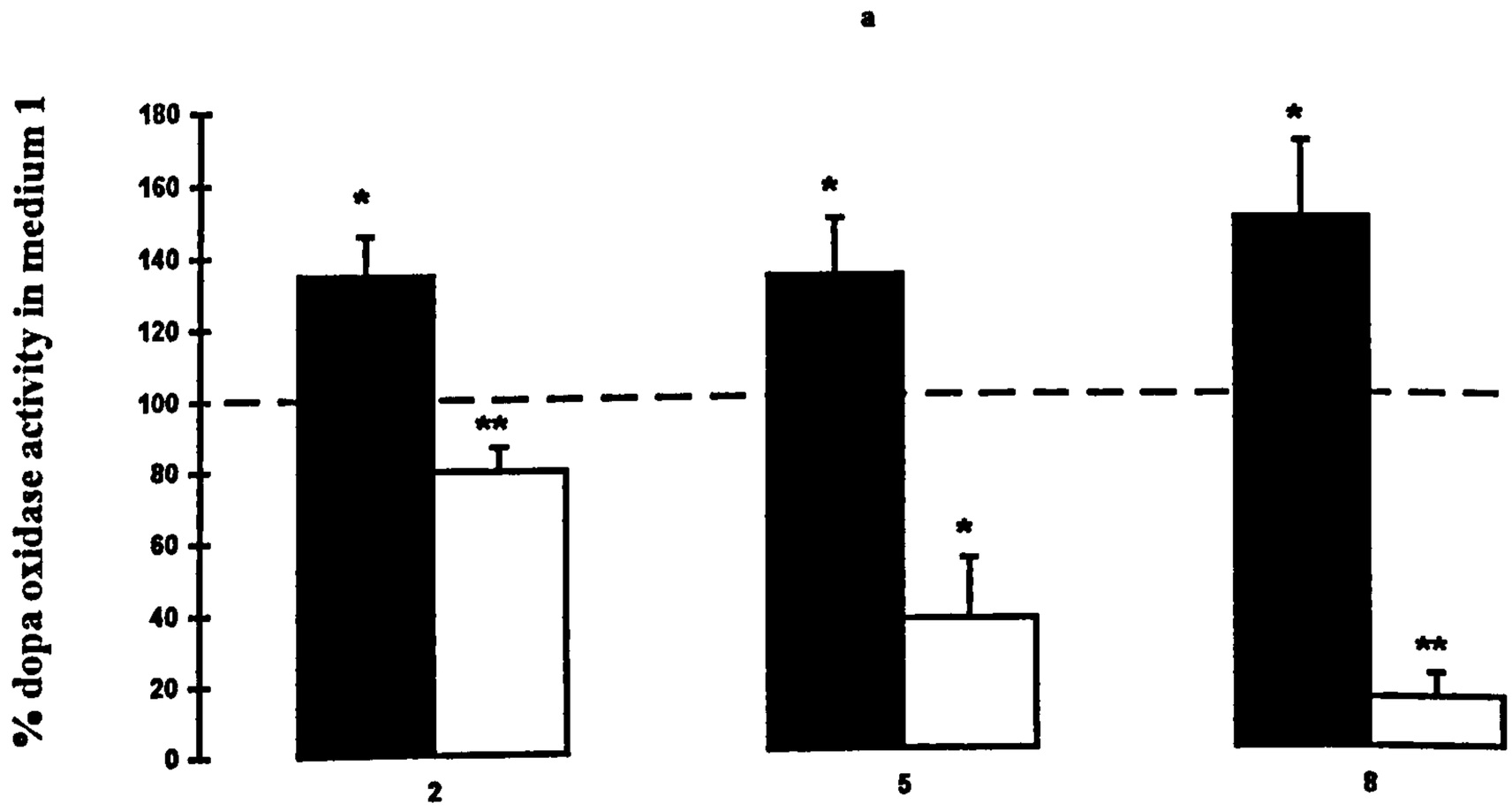
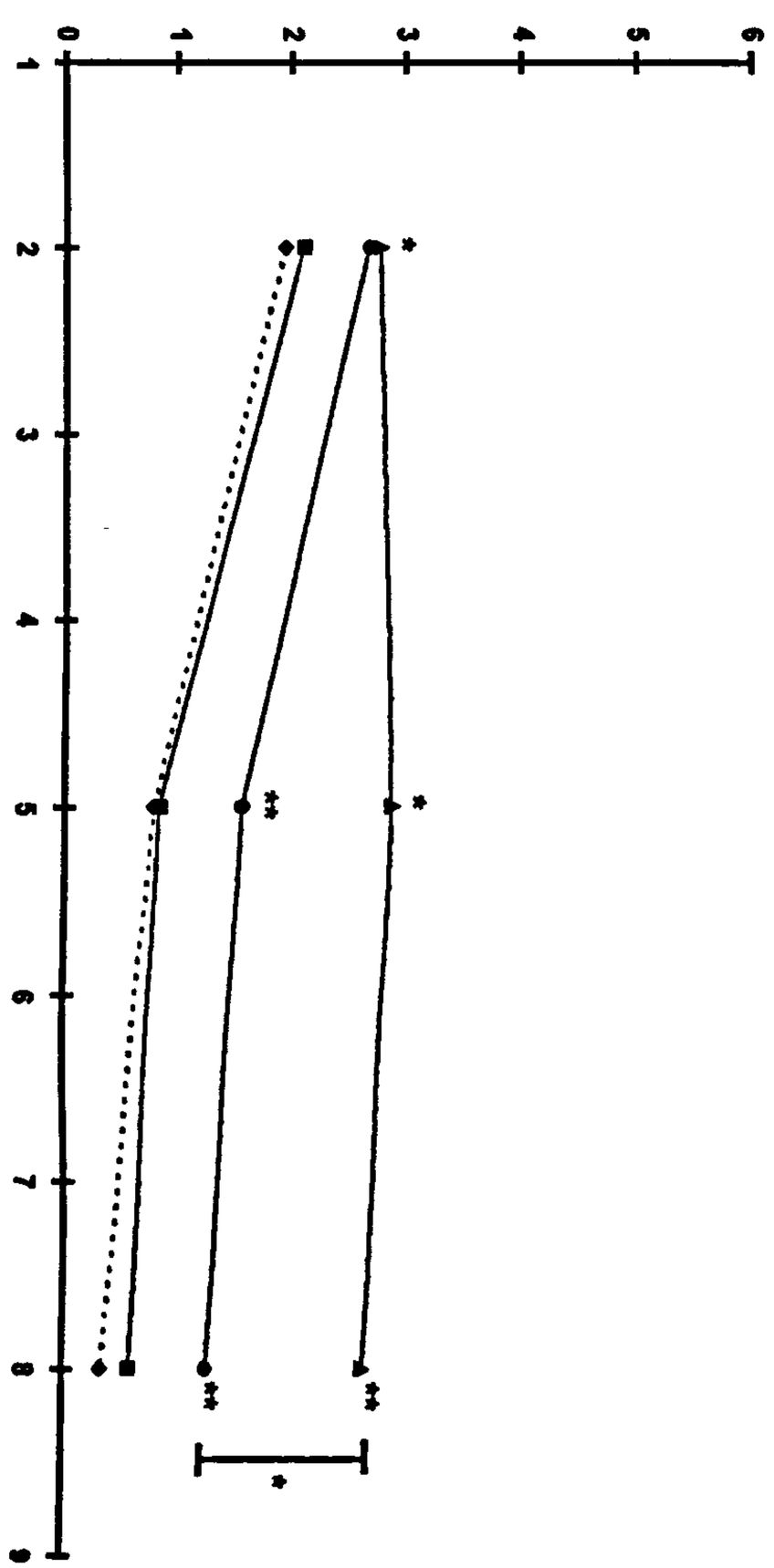


Figure 3.27 The effect of UV and ECM on normal melanocyte dopa oxidase activity. Melanocytes were sham-irradiated or irradiated with UVB and cultured in media 1, 10 or 24 (as described in **Figure 3.16**). Dopa oxidase analysis was carried out on the cells that had been cell counted (as described in Section **2.8.2c**) and the data expressed as ΔO.D.492nm/min/well/10^4 cells x 10^{-4}. The effects of media 10 (closed bar) and media 24 (open bar) on cell number over the three timepoints are illustrated in (**a**). The data represents the mean normalised values with respect to dopa oxidase activity obtained in medium ± SEM of 4 (timepoint I) and 5 (timepoint II and III) experiments. The effect of UV and ECM (alone or in combination) are shown for medium 1 (**b**), medium 10 (**c**) and medium 24 (**d**). The mean data is shown for sham irradiated cells on plastic (closed diamond, dashed line), UV irradiated cells on plastic (closed square), sham irradiated cells on ECM (closed triangle) and UV irradiated cells on ECM (closed circle) for all timepoints. Data differing significantly from the sham plastic (for each media) are indicated by *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ based on Student's paired t-test. Data differing significantly from sham ECM (i.e. UV ECM) is indicated by *=$p<0.05$ Student's paired t-test.

Dopa oxidase activity (Δ.O.D.492nm/min/well/10^4cells) x 10^{-4}



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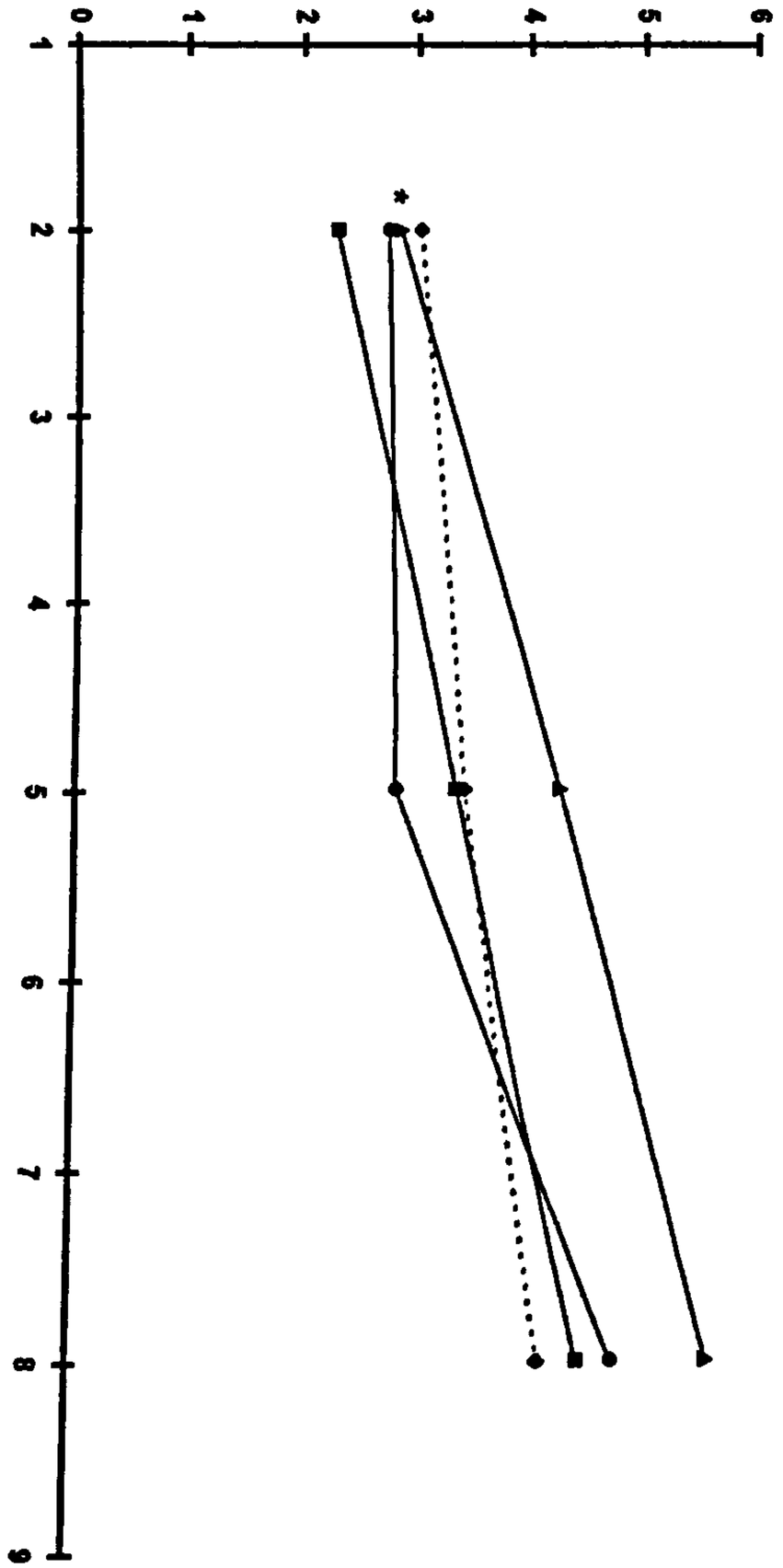

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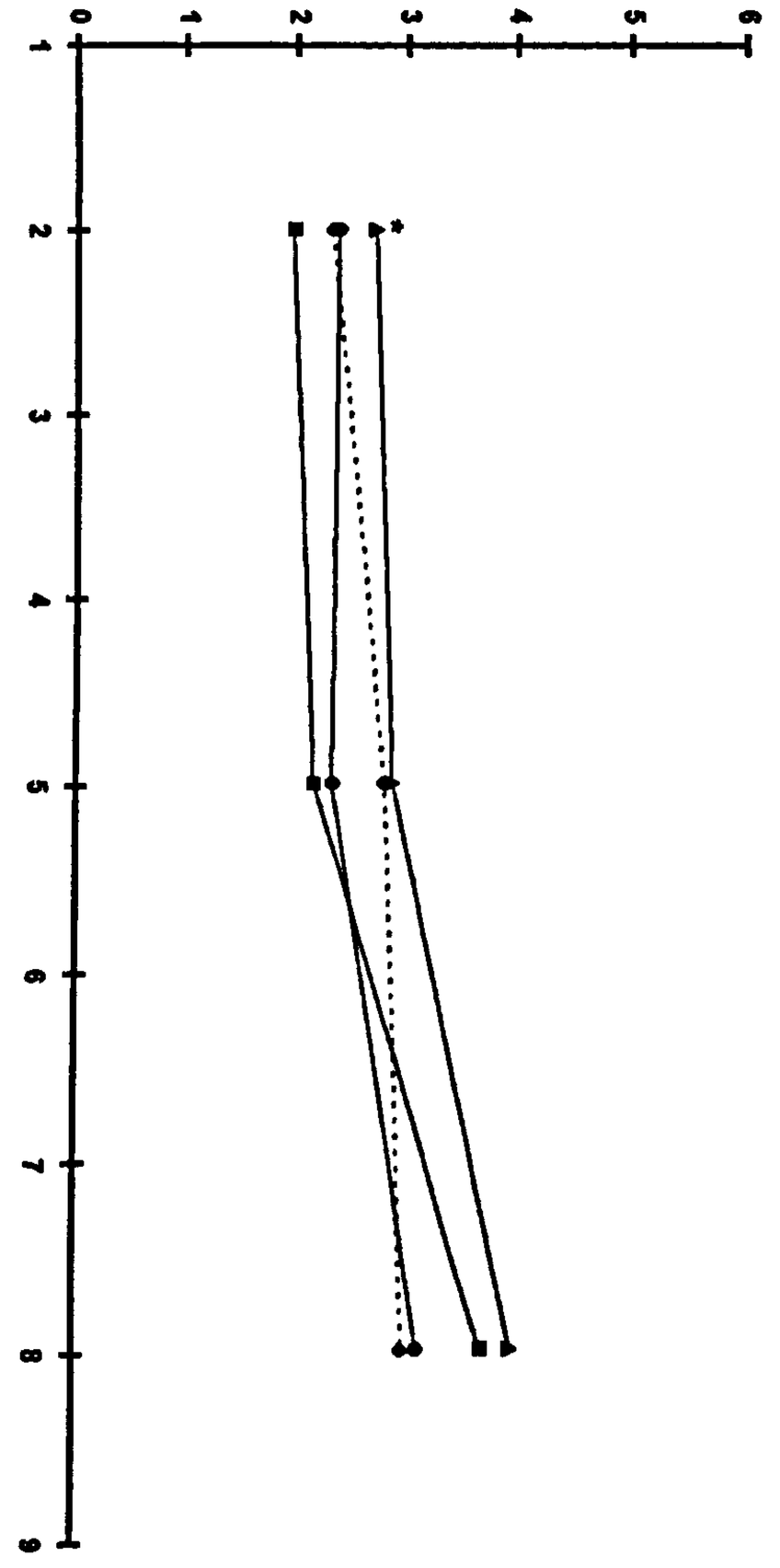


b

cultured in medium 1 ($p<0.05$) while significantly decreasing dopa oxidase activity by 6% in melanocytes cultured in medium 10 ($p<0.05$) when compared to melanocytes cultured on plastic. By 8 days, ECM had increased dopa oxidase activity by 33% in medium 1 and by 36% in medium 10 (both failing to achieve statistical significance). The combination of ECM and UV was not synergistic. In medium 1, both UV and ECM alone increased the dopa oxidase activity of melanocytes, yet when the two were combined, the stimulatory effects of both were lost and the dopa oxidase activity remained at the same level as the control. In medium 10, UV reduced the ECM effect on dopa oxidase activity.

In medium 24 **(Figure 3.27d)**, UV alone did not significantly affect melanocyte dopa oxidase activity over the time period examined. ECM maintained a steady state dopa oxidase activity level which was significantly higher by 43% at timepoint 1 ($p<0.05$), by 259% at timepoint 2 ($p<0.05$) and by 670% by timepoint 3 ($p<0.01$) than in melanocytes grown on plastic. The combination of UV and ECM saw a reduction of dopa oxidase activity over the time period although, the level of dopa oxidase activity remained significantly higher than that seen when cells were cultured on plastic alone by 96% at timepoint 2 ($p<0.01$), and by 274% at timepoint 3 ($p<0.05$). UV had significantly reduced the ECM maintained dopa oxidase level by 51% ($p<0.01$) by day 8.

The effects of UVB and ECM on melanin content after 8 days of cells cultured under media 1, 10 or 24 were examined **(Figure 3.28)**. Melanin content ranged from 0.73 to 3.36μg/ml/10^4cells for cells cultured in medium 1, from 1.13 to 3.14μg/ml/10^4 cells for cells cultured in medium 10 and from 0.54 to 3.32μg/ml/10^4cells for cells cultured in medium 24, without influence from UV or ECM. An increase of 48% was seen in the melanin content of melanocytes cultured in medium 10 compared to those cultures in medium 1 but statistical significance was not achieved ($p<0.0687$). Melanin contents were very similar between cells cultured in medium 1 and medium 24, whereas the dopa oxidase activity was very different. UV alone, ECM alone and ECM plus UV all increased melanin content under all media conditions (statistical significance not achieved under any conditions). These increases ranged from 20% in medium 1 (cells cultured on plastic in response to UV) to 100% in medium 24 (cells cultured on ECM).

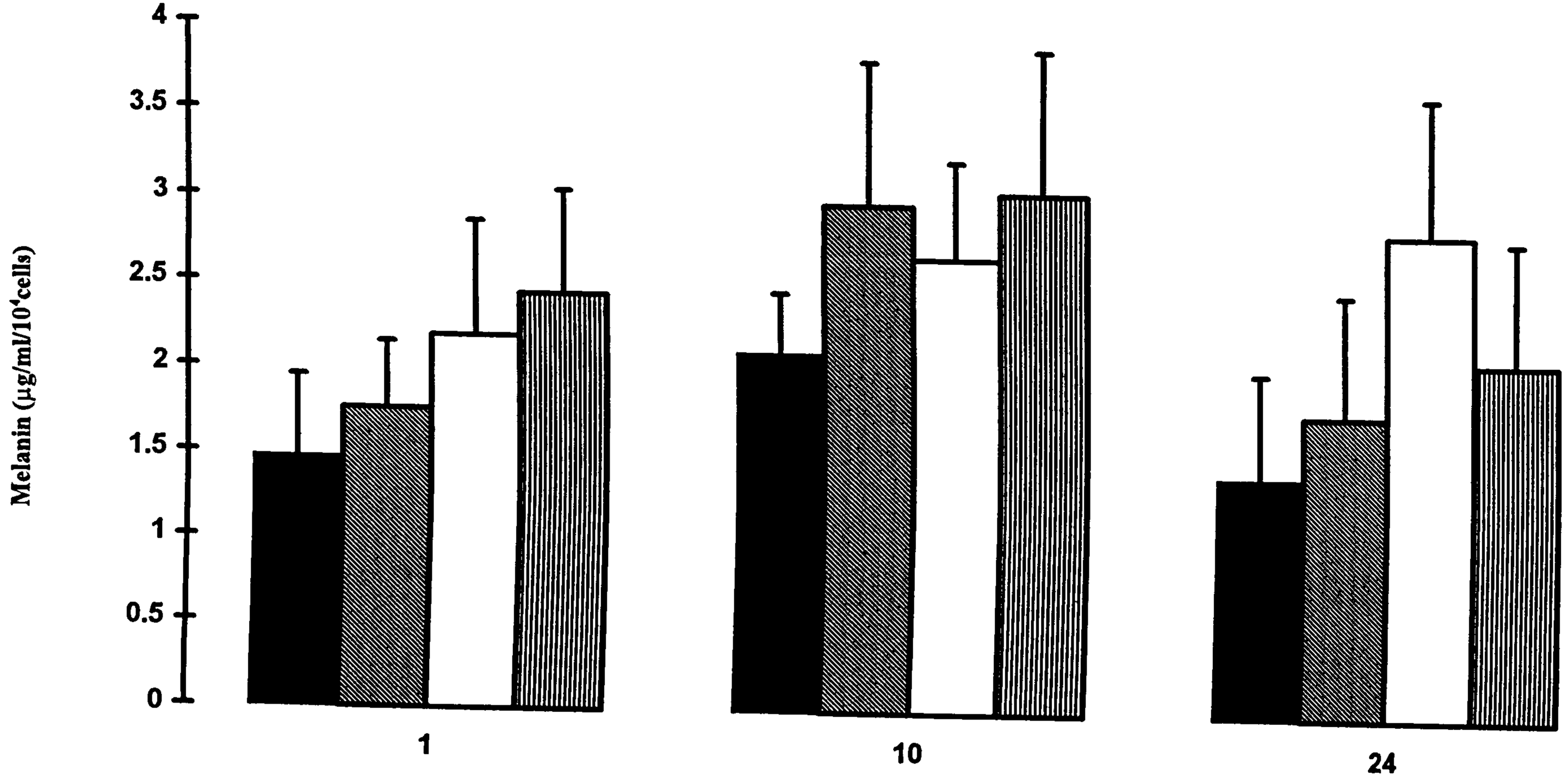

Melanin (μg/ml/10⁴cells)
4
3.5
3
2.5
2
1.5
1
0.5
0
1
10
24
Media variants

Figure 3.28 The effect of UVB and ECM on normal melanocyte melanin levels. Melanocytes were plated and treated as described in **Figure 3.15**. Melanin analysis was carried out on the cells that had been cell counted (with the exception of 2 cultures, where counts were undertaken in parallel wells). Cells were digested with 1M sodium hydroxide and plated out for O.D. determination (as described in **Section 2.8.3**). The melanin results were expressed as $\mu g/ml/10^4$ cells and the treatments were sham irradiated cells on plastic (closed bar), UV irradiated cells on plastic (hatched bar), sham irradiated cells on ECM (open bar) and UV irradiated cells on ECM (striped bar) are shown as mean ± SEM for 5 to 6 experiments using melanocytes from different donors.

UVB, over the 8 day protocol, significantly enhanced melanocyte dopa oxidase activity in medium 1 but had lesser effects in media 10 or 24. ECM, conversely, had the greatest effects on dopa oxidase activity in medium 24 with lesser effects seen in media 10 or 1. The combination of UVB and ECM gave mixed results. There were no significant effects for UVB alone, ECM alone and UVB in combination with ECM, on melanocyte melanin content, although all 3 increased melanin content slightly.

3.4.9 Influence of UVB, media and ECM on normal and vitiligo melanocyte melanin content.

The effects of UVB and ECM on normal and vitiligo melanocyte pigmentation were compared (**Table 3.11b**). In the first comparative study, between M756 and VM1, no statistical analysis could be performed as the cells were pooled for each condition, therefore invalidating any statistical examination. Statistical analysis was performed on the comparative study between M577, M748 and VM3, as illustrated in **Table 3.11b**. However, no overall conclusion can be drawn from this study as not enough cell cultures were examined.

All the cell cultures studied in this section increased their melanin content in response to being cultured in medium 10, instead of medium 1, as seen with normal melanocytes described in **Section 3.4.8**. Responses varied when cells were cultured in medium 24. Melanin was generally increased in response to UVB alone, ECM alone and in combination although naturally there were exceptions such as M577 being quite unresponsive under many of the conditions. Both vitiligo cultures proved to be just as responsive as the normal melanocytes although basal levels in all three media (i.e. when cells were cultured on plastic) of both vitiligo cultures were higher than that of the normal melanocytes they were paired with.

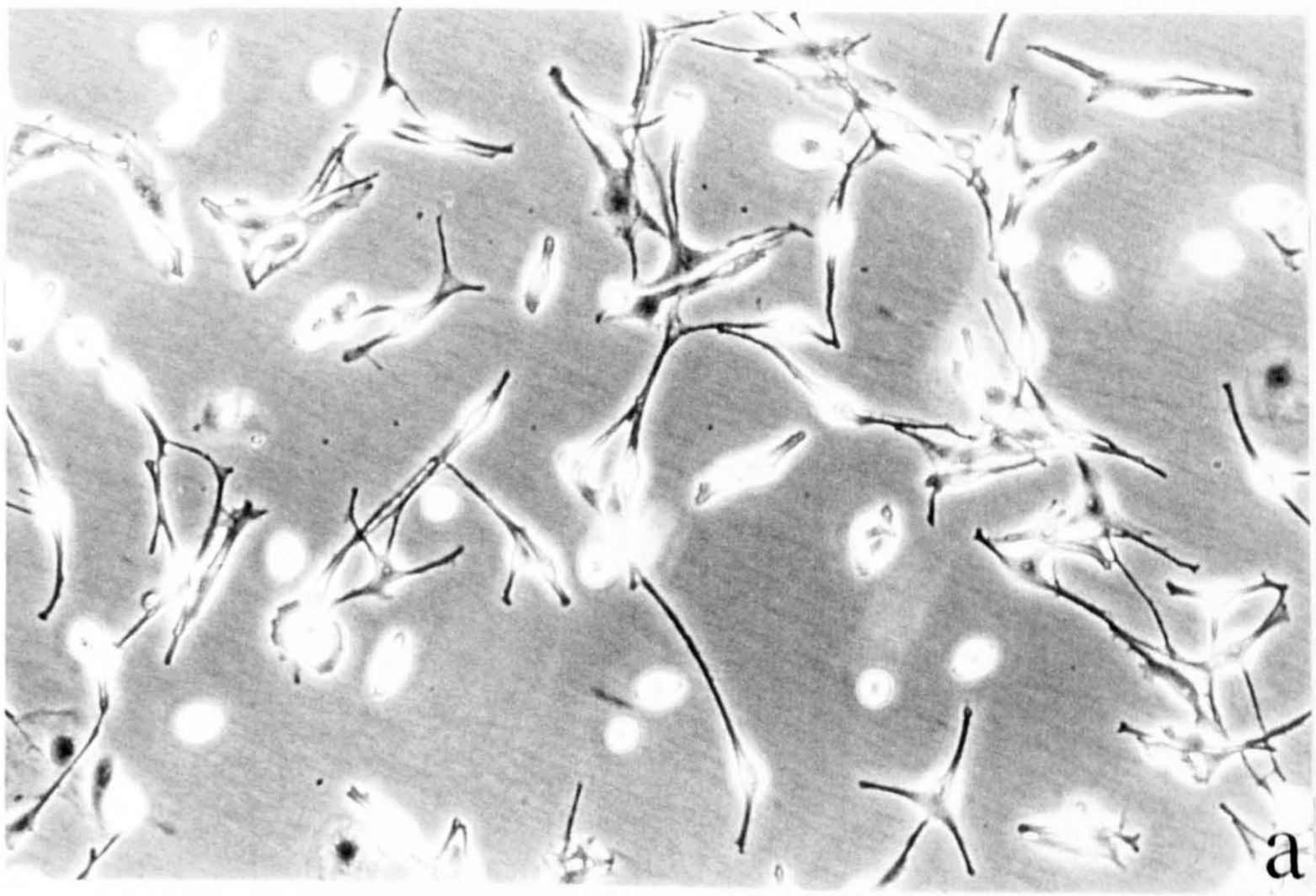
3.4.10. Effect of α-MSH, fibroblasts and basement membrane on pigmentation in an epidermal/dermal reconstruct model.

The control of melanocyte pigmentation is complex (see **Sections 3.4.1 - 3.4.9**). Skin pigmentation was examined in a 3-dimensional model consisting of a dermis,

fibroblasts, keratinocytes and melanocytes. Composites were prepared as described in **Section 2.4.1 - 2.4.2**. Initial experiments, as briefly mentioned in **Section 2.4.2**, were technically invalid as melanocytes had not reached the basal layer. This was possibly due to the melanocytes being added as a separate population a short time after the freshly isolated keratinocytes. Therefore, for the experiment described here, melanocytes at passage 2 were added to the dermis a few hours before the freshly isolated keratinocytes were added. Additionally, surplus melanocytes from M855 (**Figure 3.29a**) and melanocytes from another donor (**M856, Figure 3.29b**) were plated on to plastic in Green's medium to check that melanocytes were not affected by the media. Both cultures attached to tissue culture plastic in this medium although they exhibited different morphologies. In this experiment, the dermis was ± fibroblasts or ± basement membrane. The composites were raised to the air-liquid interface, after 48 hours of seeding. Within 3 days of raising the composites to the air liqiud interface differences in pigmentation could be seen. On day 7, the media was changed to experimental media and composites were photographed (**Figure 3.30a-c**). At this stage, it was clear that those composites which lacked basement membrane (**Figure 3.30b,** composites H, J, L) or fibroblasts (**Figure 3.30c,** composites M-P) were much darker. Pigmentation did not alter further over the next six days, when composites continued to be cultured in experimental media containing no additions, MSH or CT respectively (**Figure 3.30d**).

Histological findings (full details are given in **Table 3.16a**) indicated that α-MSH did not influence melanocyte cell number, keratinocyte layers or pigmentation (**Table 3.16b**) irrespective of whether the composites had basement membrane or fibroblasts (composites that were cultured with Green's media containing CT were excluded from **Table 3.16b**). Composites (A-G, I, K), which initially contained basement membrane and fibroblasts, had well-organised keratinocyte layers with melanocytes evenly spaced throughout the basal layer (H and E and S100 staining for melanocytes are shown in **Figure 3.31a-b** for composite B without α-MSH and CT, **Figure 3.31e-f** for composite D without CT but with α-MSH and **Figure 3.31 i-j** for composite F without α-MSH but with CT). α-MSH had no effects on the cell number, keratinocyte layers or pigmentation, while CT appeared to induce increased pigmentation, based on Masson Fontana stain for melanin, in comparison to the control composites which were not treated with α-MSH or CT (**Table 3.16c**).

Figure 3.29 Morphological appearance of melanocytes cultured in Greens medium on plastic. Melanocytes from donor M855 and M856 were initially isolated and cultured in maintenance medium B (as described in **Sections 2.1.1 - 2.1.2**), then frozen at passage 2. The cells were thawed several months later, a viability count performed using trypan blue and cells from M855 were seeded on to dermis for the composite experiment described in **Section 3.4.10**. Surplus cells were plated in Green's medium on plastic (**a**). Melanocytes from M856 (**b**) were plated in Green's medium on plastic as a comparison. Scale bars represent 30μm.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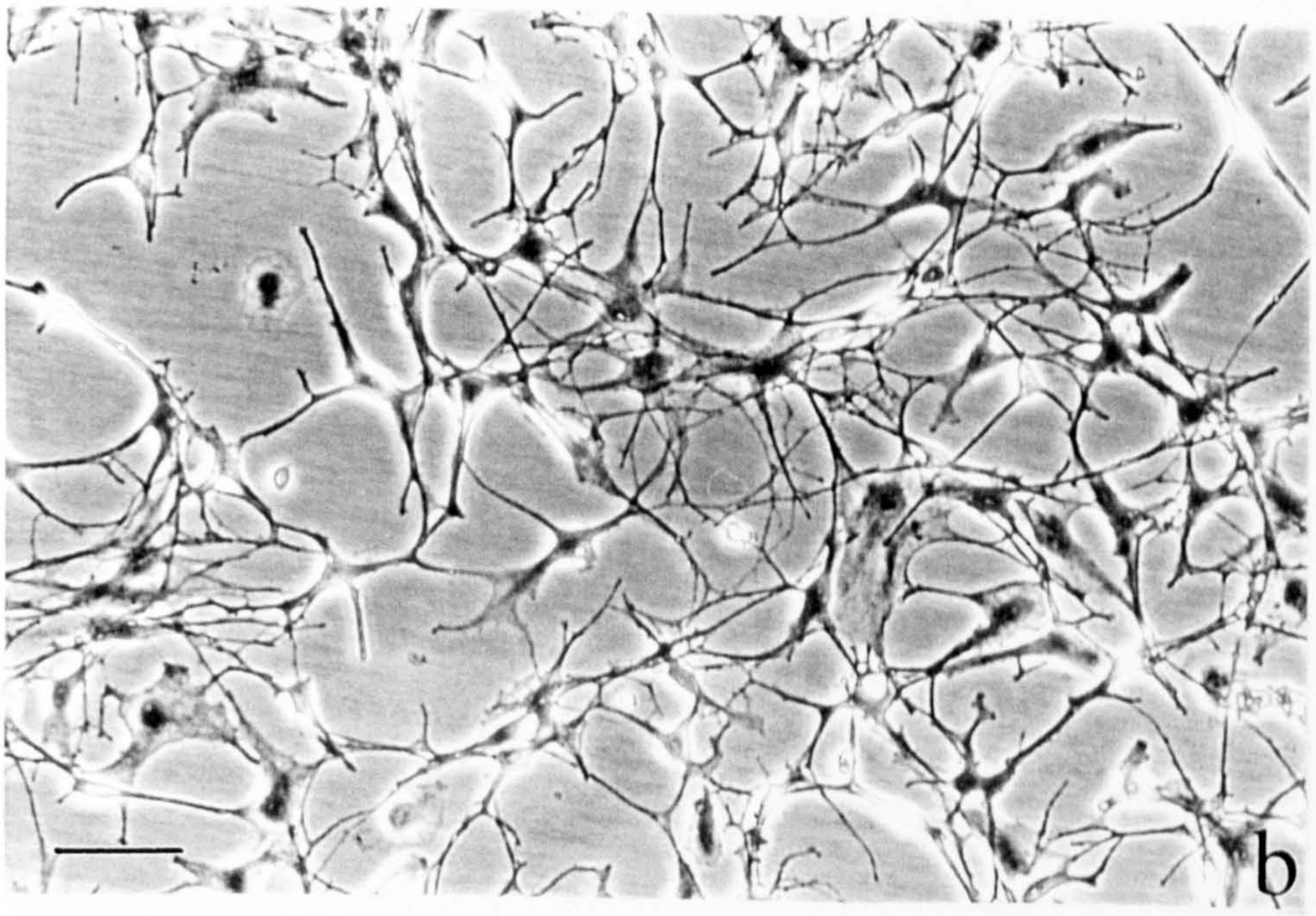
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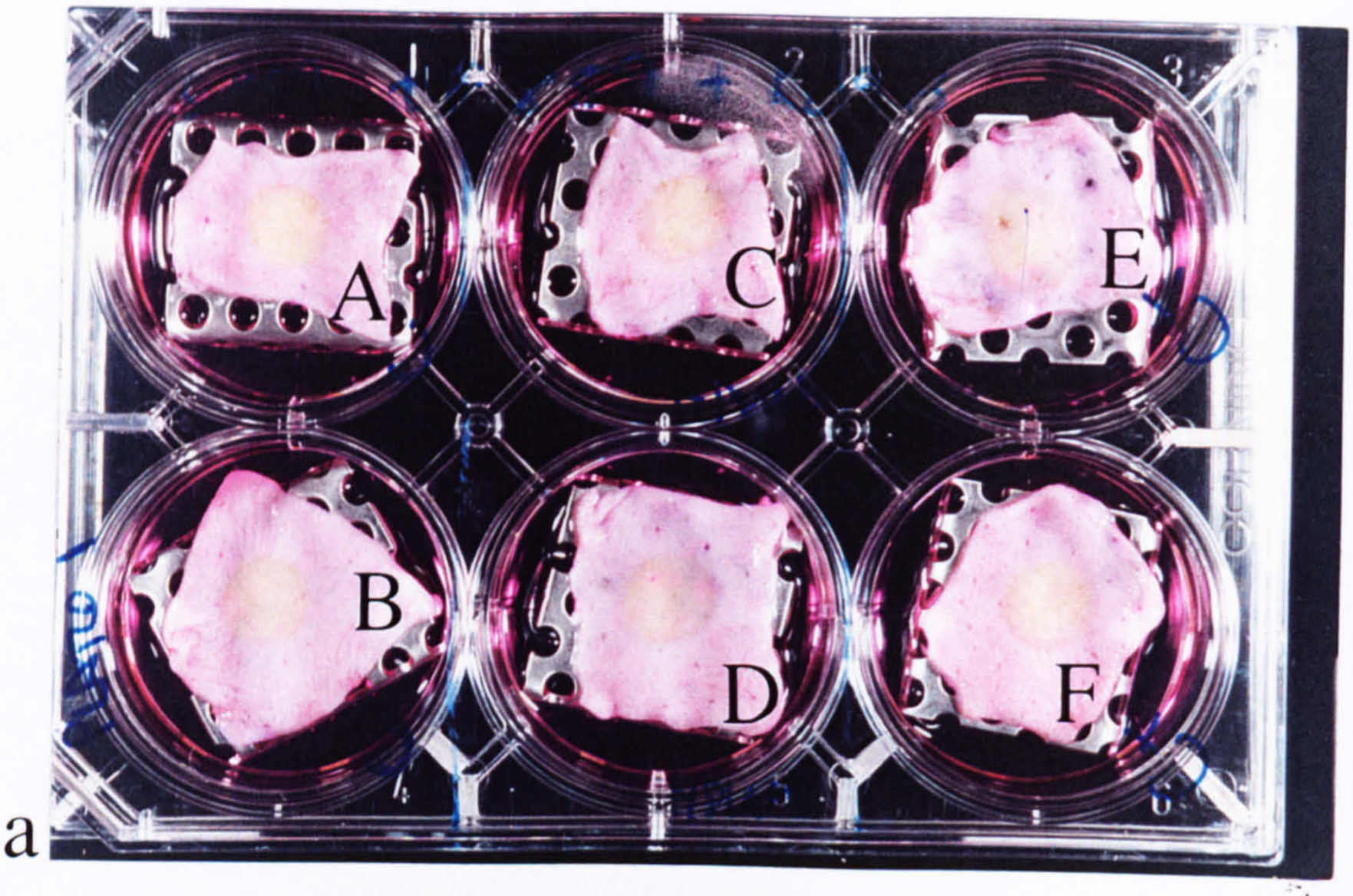

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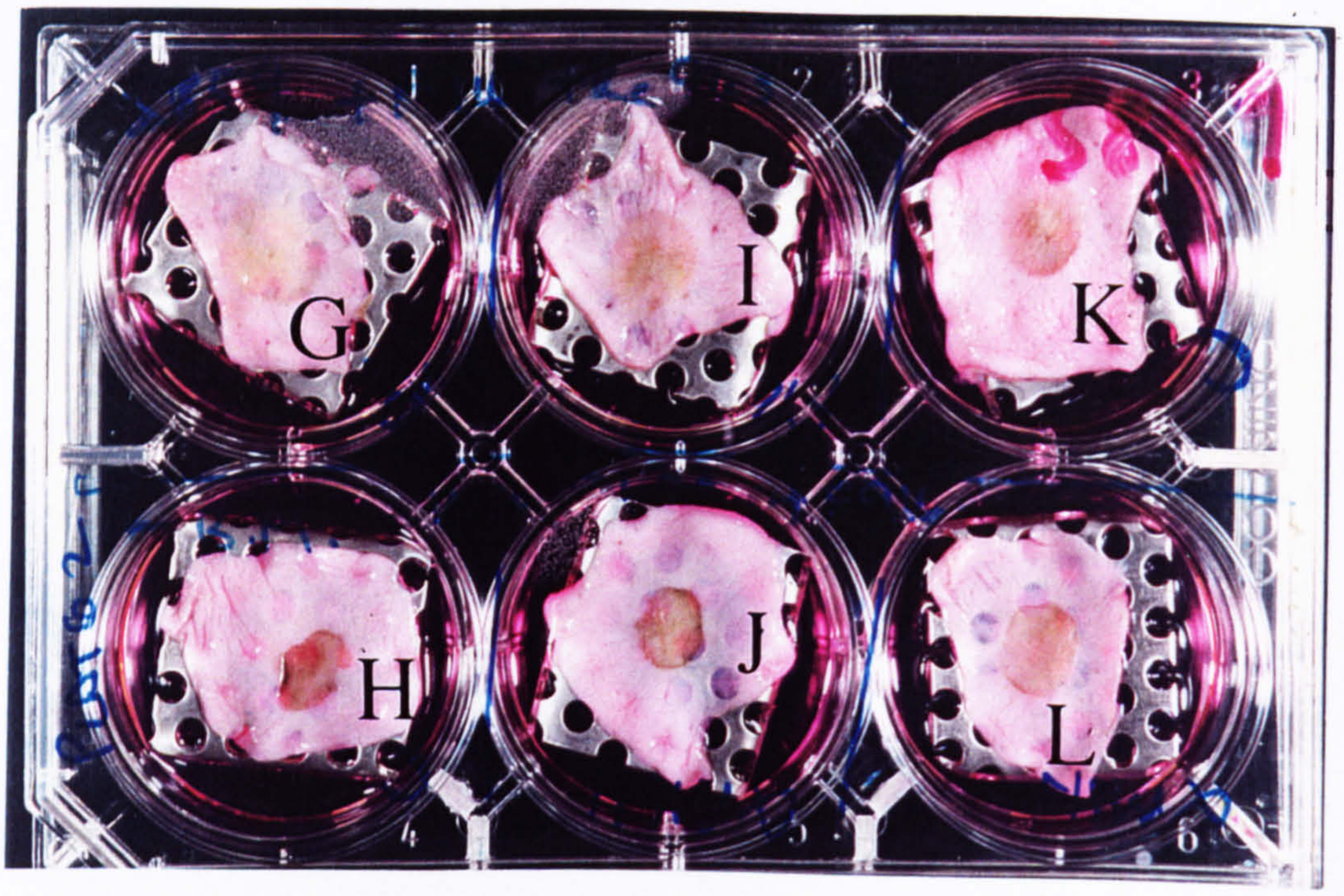

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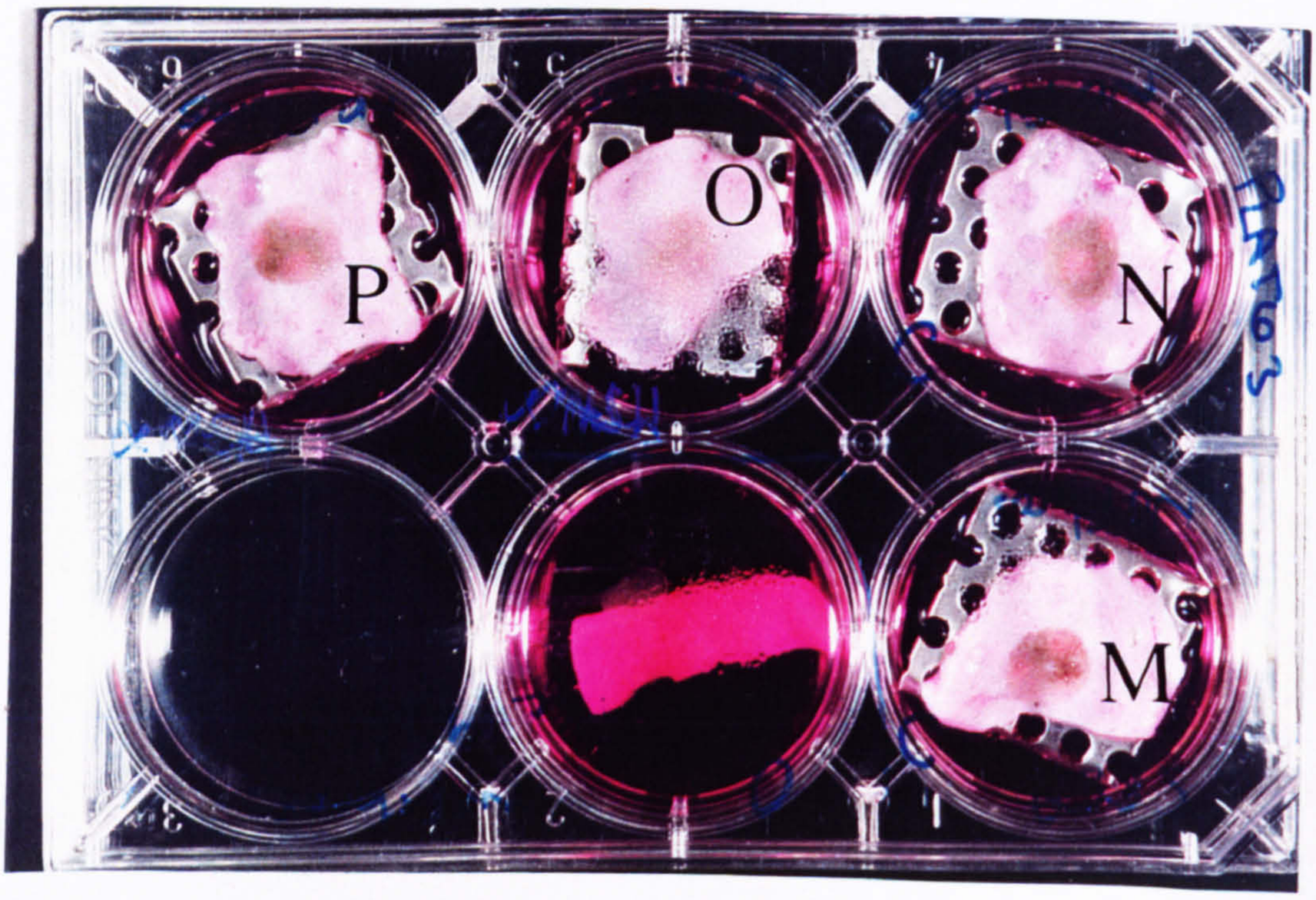

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d

Composite label	Fibs	BM	MSH	CT
A-B, K	+	+	-	-
C-D, I	+	+	+	-
E-G	+	+	-	+
H	+	-	-	-
J, L	+	-	+	-
M-N	-	+	-	-
O-P	-	+	+	-

Figure 3.30 The influence of α-MSH, fibroblasts and basement membrane on pigmentation in an epidermal/dermal reconstruct: the macroscopic examination. Melanocytes (from donor M855 at P2) and freshly isolated keratinocytes (from donor 943) were added to dermis which either retained basement membrane (BM) or was prepared with dispase to remove BM, in the presence or absence of fibroblasts (Fibs from donor 916). Composites were raised to the air liqud interface 48 hours after seeding and cultured for the first 7 days in Green's medium (as described in **Section 2.4.1-2.4.2**). Photographs of the composites were taken at this stage by Mr R. Salthouse (Medical Illustration Department, Northern General Hospital) (**Figures a-c**). Composites were then cultured in different varients of Green's medium: normal Green's medium or Green's medium lacking CT in the presence or absence of 10^{-8}M α-MSH for a further 5 days (as described in **Section 2.4.2** and **Table 3.16**). The composites (**Figure d**) were photographed 24 hours following the final feed by Ms A. Reed (Medical Illustration Department, Northern General Hospital). The labelling of the individual composites is explained by the key above and is in agreement with the key in **Table 3.16a** where the presence of Fibs (fibroblasts), BM (basement membrane), MSH (α-melanocyte stimulating hormone) and/or CT (cholera toxin) is indicated by +.

Table 3.16a Histological evaluation of the individual composite experiments.

Composite	Presence or absence				No of Melanocytes[a]	Composite quality			Collagen IV 0-5[e]	Pigmentation 0-5[f]	Location of Pigmentation[g]
	Fibs	BM	MSH	CT		KC layers[b]	Attachment[c]	Differentiation[d]			
A	+	+	-	-	39 (B)	12+	100%	good	4½	1	superficial layer only
B	+	+	-	-	29 (B)	13+	100%	good	4½	2	superficial + some basal staining
K	+	+	-	-	22 (B + S)	10+	100%	good	4	2½	superficial
C	+	+	+	-	21 (B)	14+	100%	partial	3½	1	superficial layer only
D	+	+	+	-	73 (B)	9+	100%	partial	4	1	superficial layer only
I	+	+	+	-	32 (all E)	12+	100%	poor	3½	3	throughout epidermis/dermis
E	+	+	-	+	42 (B)	10+	100%	good	4½	3	some basal mel stained
F	+	+	-	+	50 (B)	10+	100%	partial	4	3	mainly superficial
G	+	+	-	+	29 (C+U)	30+	100%	poor	4½	3	mainly superficial + in dermis
H	+	-	-	-	5 (B)	6+	100%	poor	½	½	superficial if any
J	+	-	+	-	7 (B)	9+	100%	poor	1½	3	throughout except basal region
L	+	-	+	-	12 (S)	8+	95%	partial	2	2	superficial
M	-	+	-	-	88 (all E)	2+	100%	poor	3	3½	mainly superficial
N	-	+	-	-	56 (S)	4+	100%	poor	3	2	mainly superficial + mid epidermis
O	-	+	+	-	36 (B)	2+	100%	poor	3½	3	mainly superficial
P	-	+	+	-	20 (B)	3+	100%	poor	3½	4	mainly superficial

[a] Number of melanocytes present in 4 micron sections through the centre of a 1cm diameter composite; melanocytes present in basal layer (B), in basal and superficial regions B + S), in all epidermis (E), in central and upper epidermis (C + U) or in superficial layer (S).
[b] Number of keratinocyte layers present in reconstituted epidermis.
[c] Extent to which epidermal layer was attached to underlying dermis throughout section.
[d] Good - presence of a well organised keratinocyte layer; partial - some degree of differentiation apparent; poor - no evidence of differentiation.
[e] Level of collagen IV staining at epidermal dermal junction scored on an arbitary scale of 0 (no evidence of collagen IV) to 5 (very strong level of collagen IV staining as indicated in Figure by control sample).
[f] Pigmentation scored on an arbitary scale of 0 (no evidence of pigmentation) to 5 (heavily pigmented) based on the Masson Fontana stain.
[g] Positioning of melanin granules in epidermal/dermal region based on the Masson Fontana stain.

Table 3.16b Influence of α-MSH on skin pigmentation in a skin composite model.

	-MSH	+MSH
Composite	A,B,K,H,M,N	C,D,I,J,L,O,P
Melanocyte number	40 ± 12	29 ± 8
Keratinocyte layers	7.83 ± 1.83	8.14 ± 1.65
Histological pigmentation	2.43 ± 0.43	1.92 ± 0.44

Table 3.16c Influence of MSH and CT on pigmentation in a skin composite model.

	Control	+MSH	+CT
Composite	A,B,K	C,D,I	E,F,G
Melanocyte number	30 ± 5	42 ± 16	40 ± 6
Keratinocyte layers	11.67 ± 0.88	11.67 ± 1.45	16.67 ± 6.67
Histological pigmentation	1.83 ± 0.44	1.67 ± 0.67	3 ± 0

Table 3.16d Influence of fibroblasts and basement membrane on pigmentation in a skin composite model.

	Control	-Fibs	-BM
Composite	A,B,C,D,I,K	M,N,O,P	H,J,L
Melanocyte number	36 ± 8	50 ± 15	8 ± 2*
Keratinocyte layers	12 ± 1	3 ± 1***	8 ± 1**
Histological pigmentation	1.8 ± 0.4	3.1 ± 0.4*	1.8 ± 0.7

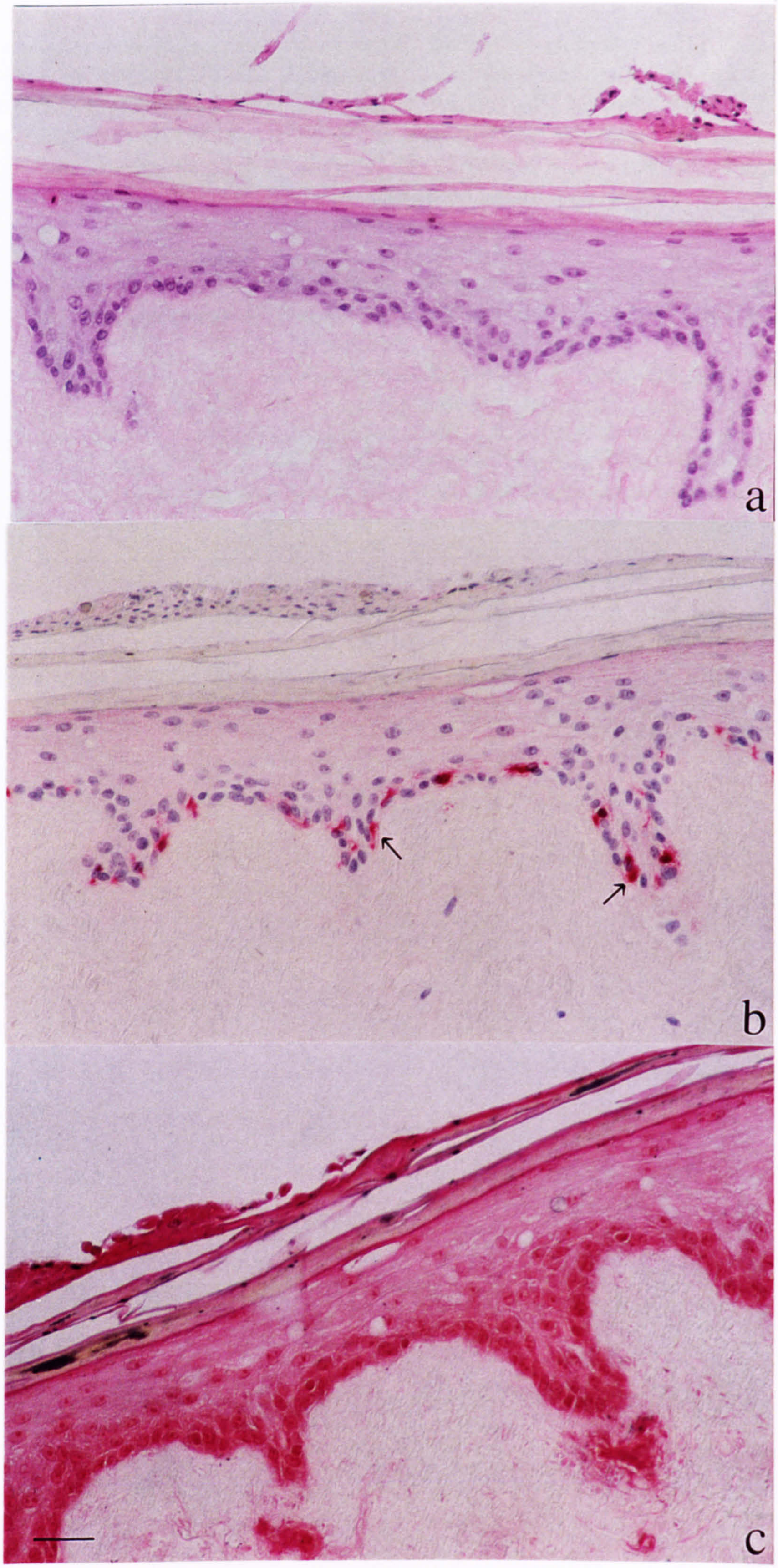
Table 3.16: Composites were prepared as described in **Section 2.4.** Melanocytes and keratinocytes (KC) were added to DED which had ± fibroblasts (Fibs) or ± basement membrane (BM). The composites were cultured at the air liquid interface for 12 days, during which they received several changes of media. Initially, while the composites were prepared and first raised to the air liquid interface, normal Green's media (containing CT) was the maintenance medium, but during the final 5 day period, the composites were fed (x 3) with Green's media -α-MSH/-CT, +α-MSH/-CT or -α-MSH/+CT. 24 hours following the last feed, the composites were photographed (**Figure 3.30,** photographed by Ms A Reed, Medical Illustration Department, Northern General Hospital) then half the composite was paraffin-fixed while the other half was fresh frozen for the various immunohistochemistry staining procedures. Melanocyte cell number was determined by S100 staining, keratinocyte layers by H&E's, while pigmentation was assessed by Masson Fontana staining for melanin and presence of basement membrane by collagen IV staining (all histological preparations and examinations were performed by Mr C Layton, Department of Histopathology, Northern General Hospital, as described in **Sections 2.4.3 - 2.4.7**). **Table 3.16a,** illustrates the individual histological evaluations for each composite (photographic examples of H&E's, S100 staining, Masson Fontana staining and collagen IV staining are illustrated in **Figure 3.31**), while **Tables 3.16b-d** illustrate combined results to examine melanocyte number, KC layers and histological pigmentation in response to various influences (composites used in these results are listed in the tables). The results in **Table 3.16b** are irrespective of the presence or absence of fibs and BM, while in **Table 3.16d,** the results are irrespective of the presence or absence of α-MSH (composites which were fed with CT were not examined in **Tables 3.16b** and **3.16d**). Values differing significantly from the controls in each table are indicated by *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ as determined by Student's unpaired t-test.

However, there were other interesting results to be gained from this experiment (**Table 3.16d**). Melanocyte number was significantly reduced in composites without basement membrane ($p<0.05$, **Figure 3.31n** for composite L) but not for composites without fibroblasts (**Figure 3.31r** for composite O). The organisation of keratinocyte layers was poorer and much reduced in composites lacking in basement membrane ($p<0.01$, **Figure 3.31m**) and fibroblasts ($p<0.001$, **Figure 3.31q**). Under histological examination, pigmentation was increased in composites without fibroblasts ($p<0.05$, **Figure 3.31s**), in agreement with the macroscopic observation. However, the level of pigmentation under histological examination was not significantly different from controls in composites that lacked basement membrane (**Figure 3.31o**). This did not agree with the result seen macroscopically (**Figure 3.30d** for composites H, J or L). At present it is difficult to explain this result other than through loss of significant keratinocyte layers containing pigment.

Collagen IV was used to stain for the presence of basement membrane in all these composites. It was necessary to ascertain whether the basement membrane had been removed from composites H, J and L by dispase, and to see if basement membrane was at all altered in the composites, in particular those without fibroblasts (composites M-P). A clear defined line of intense staining was present in all of those composites which initially had fibroblasts and basement membrane present (for composites A-G, I, K as indicated in **Table 3.16a**, and representative photomicrographs in **Figure 3.31d**, **Figure 3.31h** and **Figure 3.31l**) and this was comparable to the control dermis without added epidermal cells (**Figure 3.32a**).

The composites in which fibroblasts were absent (M-P) also showed strong staining, but it was often patchy and less defined (as indicated in **Figure 3.31t**). The three composites which had been pretreated with dispase to remove basement membrane components (H, J and L) all showed a lower level of staining (**Table 3.16a**). Composite H, which was not treated with MSH had barely detectable levels of collagen IV (**Figure 3.32b**) whereas J and L, which were treated with MSH had slightly higher levels of collagen IV (**Figure 3.31p**).

Figure 3.31 The influence of MSH, fibroblasts and basement membrane on pigmentation in an epidermal/dermal reconstruct: the histological examination. Composites were prepared and cultured as described in **Section 2.4**. Mr C Layton, performed all the histological and immunostaining work presented in this figure, while I did the photography of the sections. At the end of the experiment, half of each composite was fixed in paraffin (as described in **Section 2.4.3**) and half the composite was fresh frozen (as described in **Section 2.4.6**). Each set of four photomicrographs illustrates (i) H&E (as described in **Section 2.4.4**) **(a,e,i,m,q)**, (ii) S100 staining for melanocytes (melanocytes indicated by arrows) (as described in **Section 2.4.7**) **(b,f,j,n,r)**, (iii) Masson Fontana staining for melanin (as described in **Section 2.4.5**) **(c,g,k,o,s)** and (iv) collagen IV staining to indicate the presence or absence of basement membrane (as described in **Section 2.4.7**) **(d,h,l,p,t)**. **a-d** illustrate the histology performed on composite B (relating to **Figure 3.30** and **Table 3.16a**) which included melanocytes (M), keratinocytes (K), fibroblast (F), and basement membrane (BM) and were cultured in Green's media lacking cholera toxin. **e-h** illustrate the histological staining on composite D which included M, K, F, and BM and was cultured in Green's medium lacking CT but with the addition of 10^{-8}M α-MSH. **i-l** illustate the histological staining on composite F which included M, K, F, and BM and was cultured in Green's medium. **m-p** illustrates the histological staining on composite L which includes M, K and F but not BM, and was cultured in Green's medium lacking CT but with the addition of 10^{-8}M α-MSH. **q-t** illustrates the histological staining on composite O which included M, K, and BM but not F and was cultured in Green's medium lacking CT but with the addition of 10^{-8}M α-MSH. Scale bars represent 50μm.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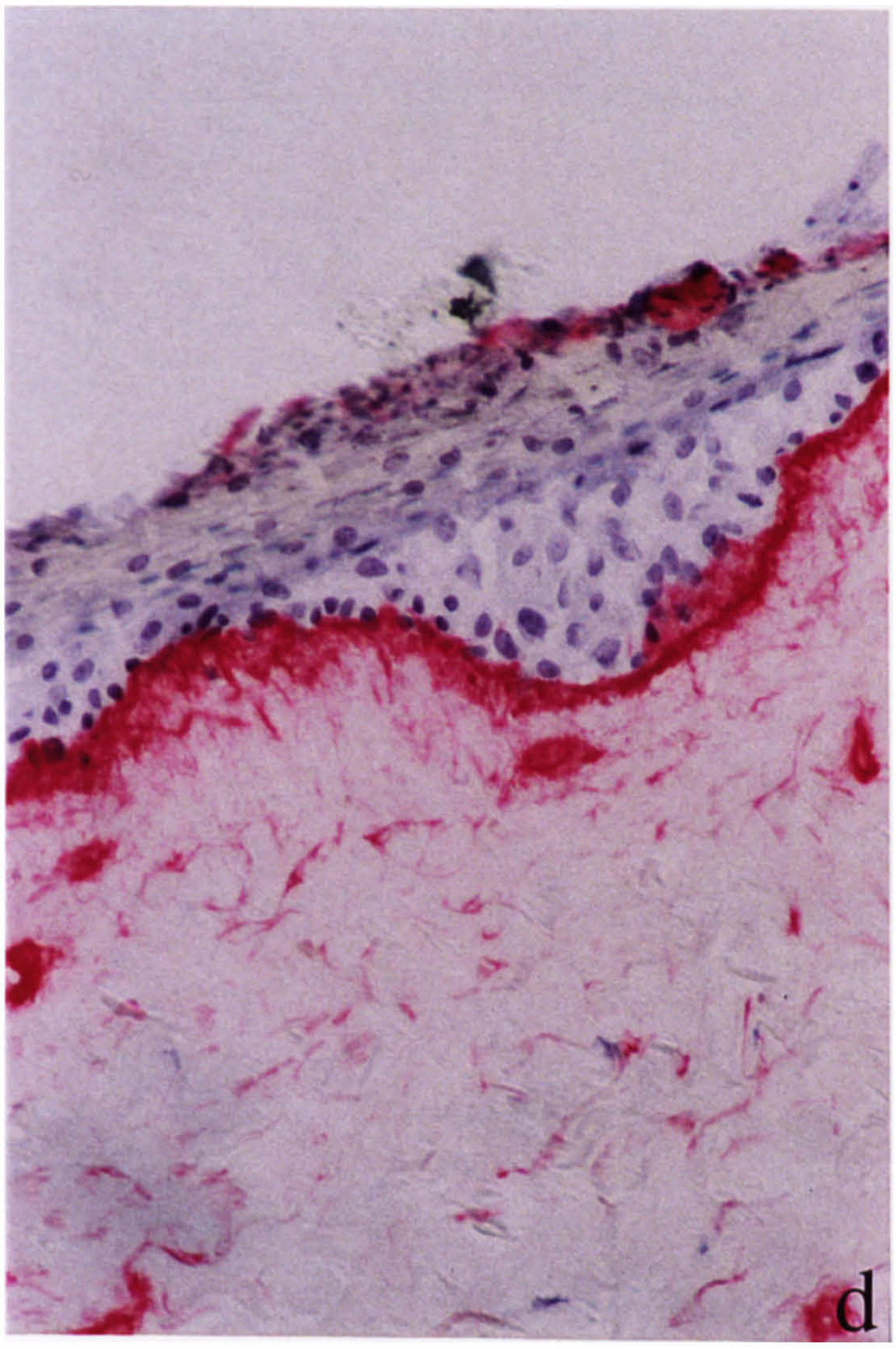
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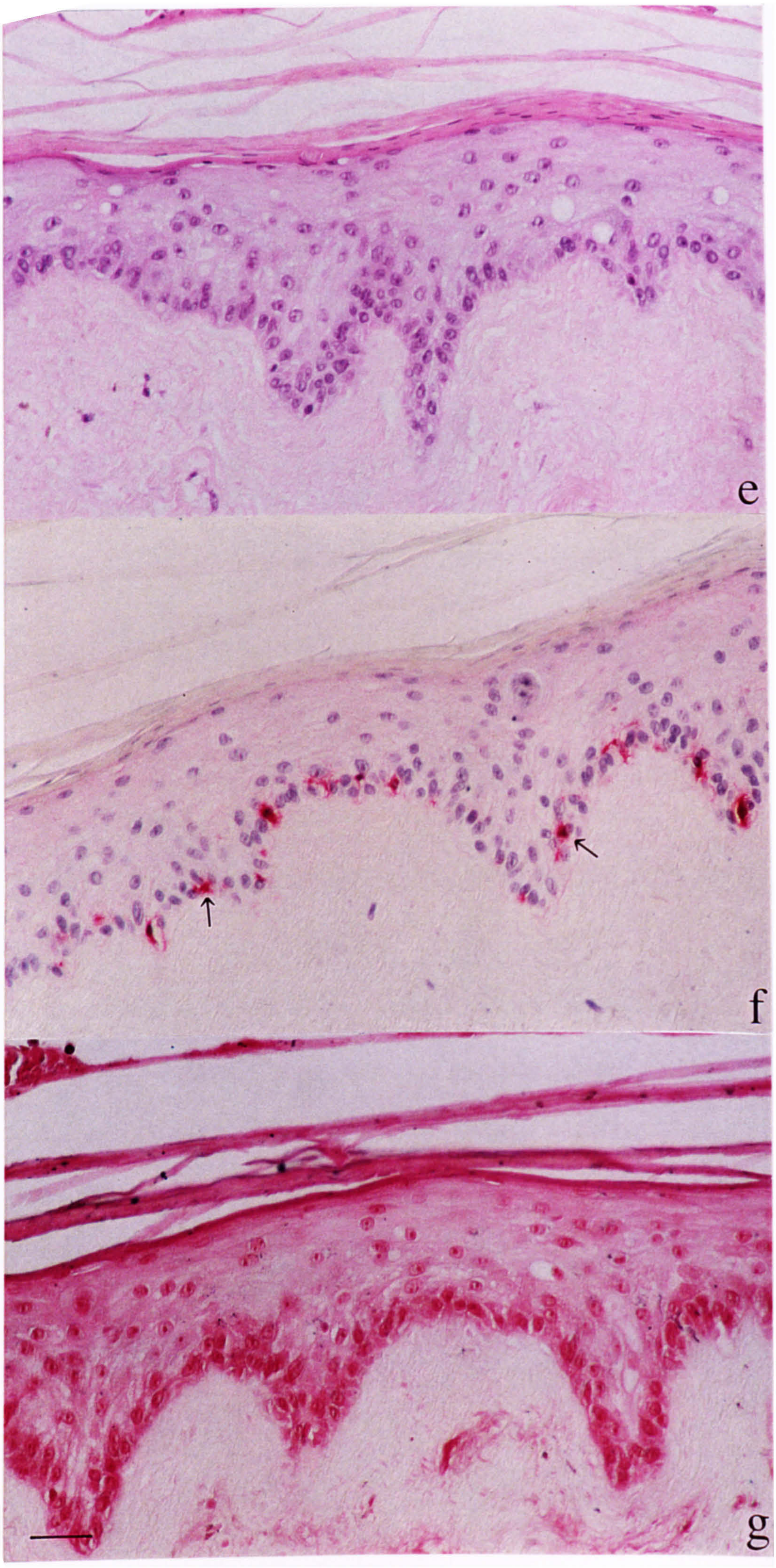
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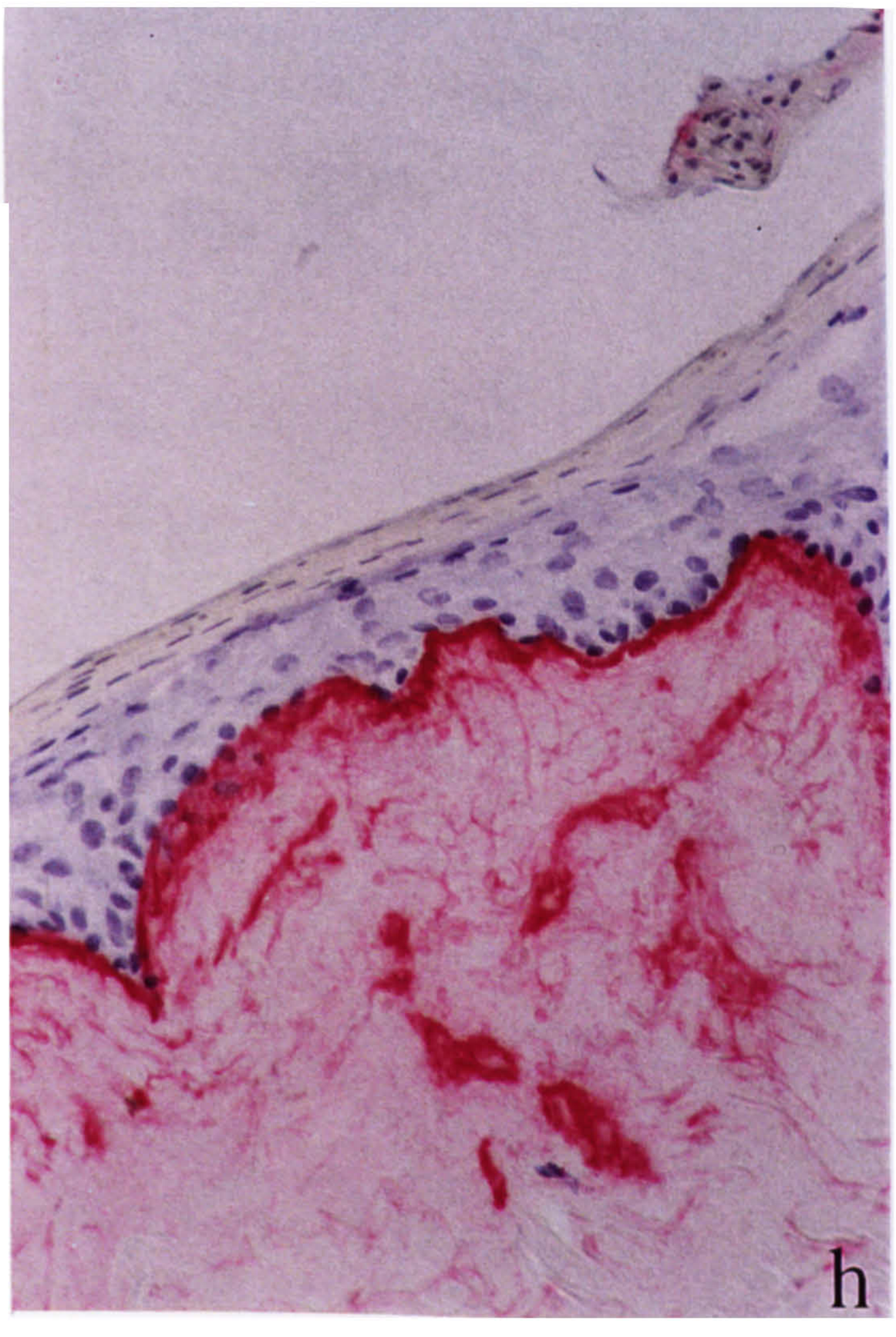
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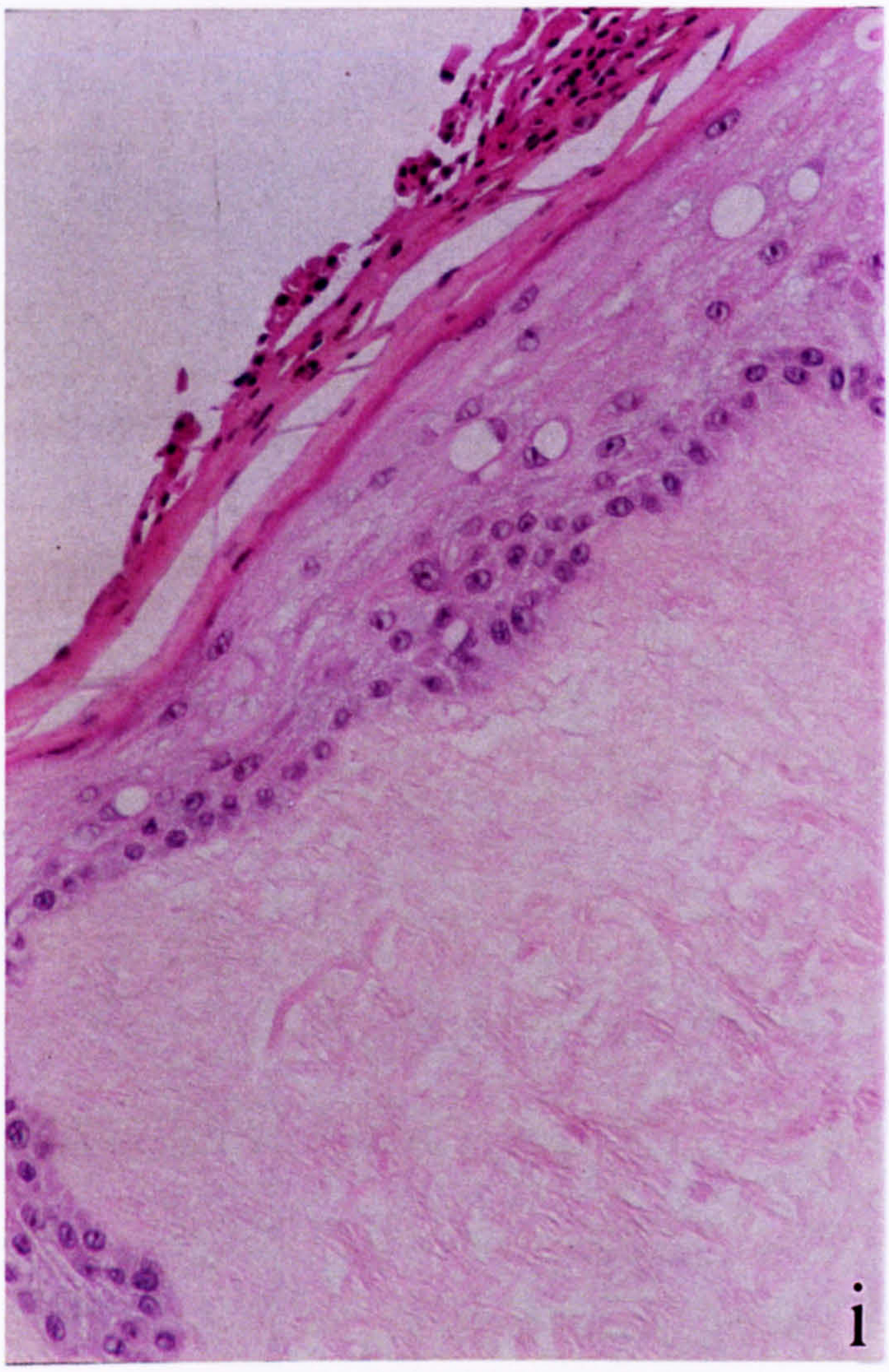
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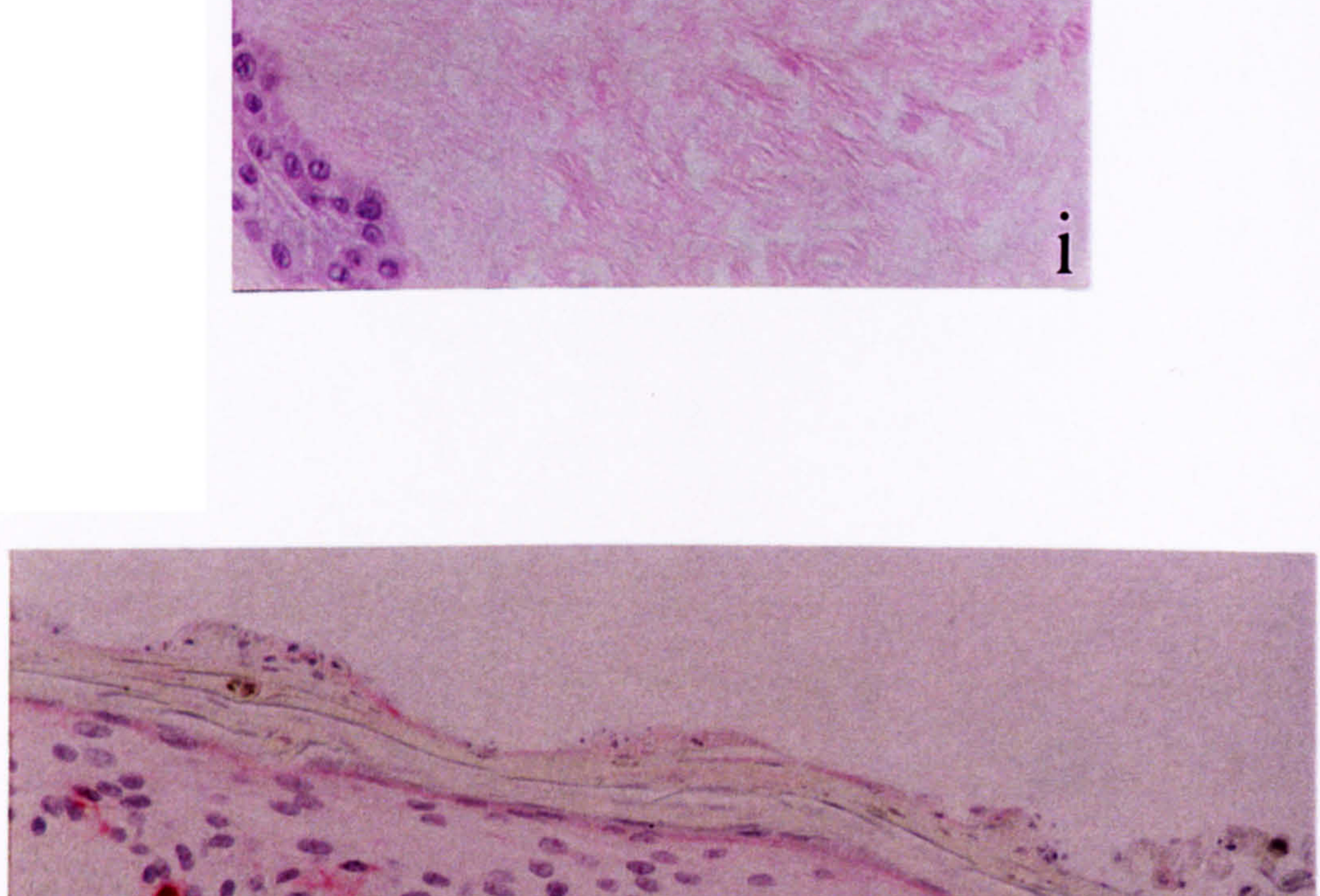
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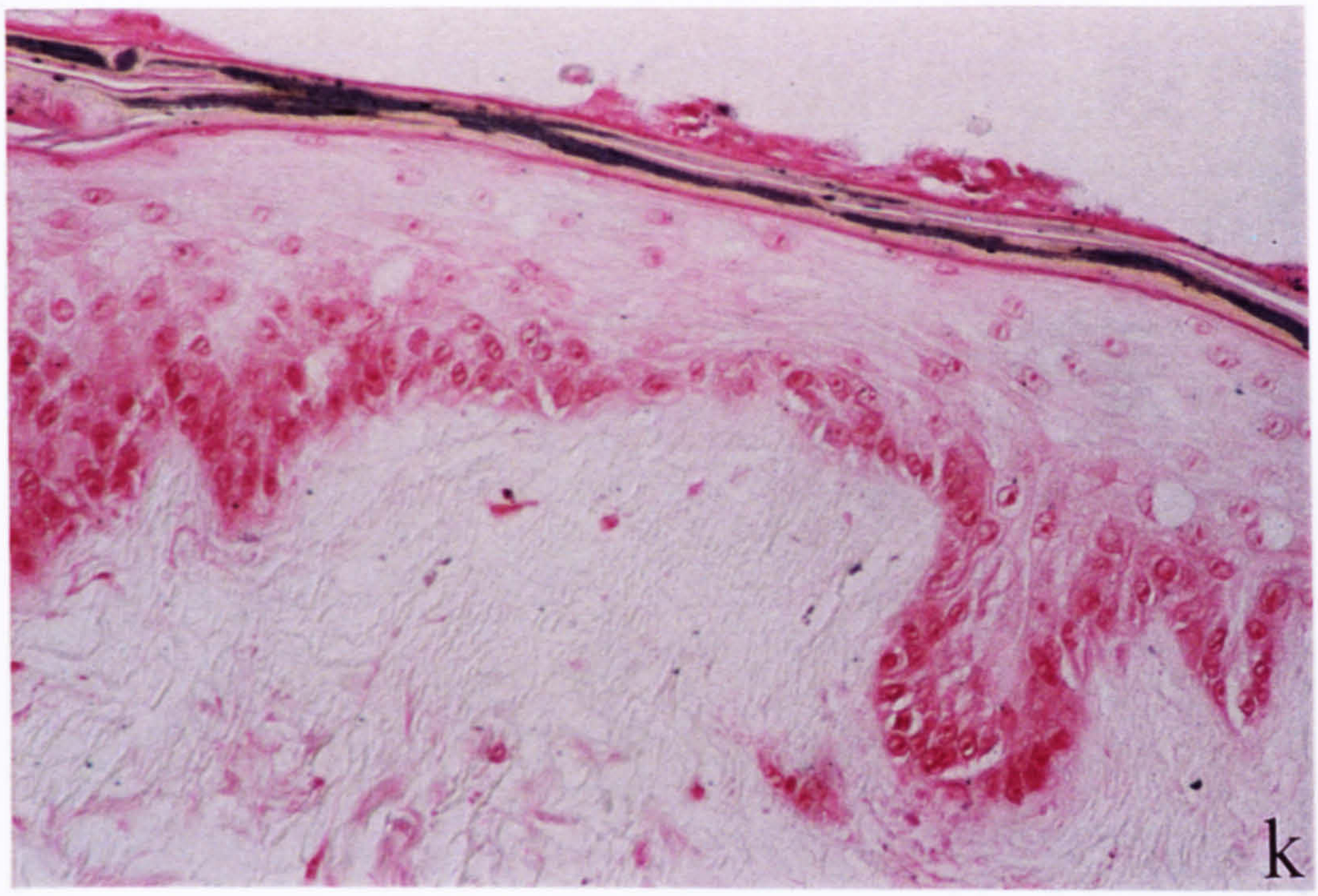
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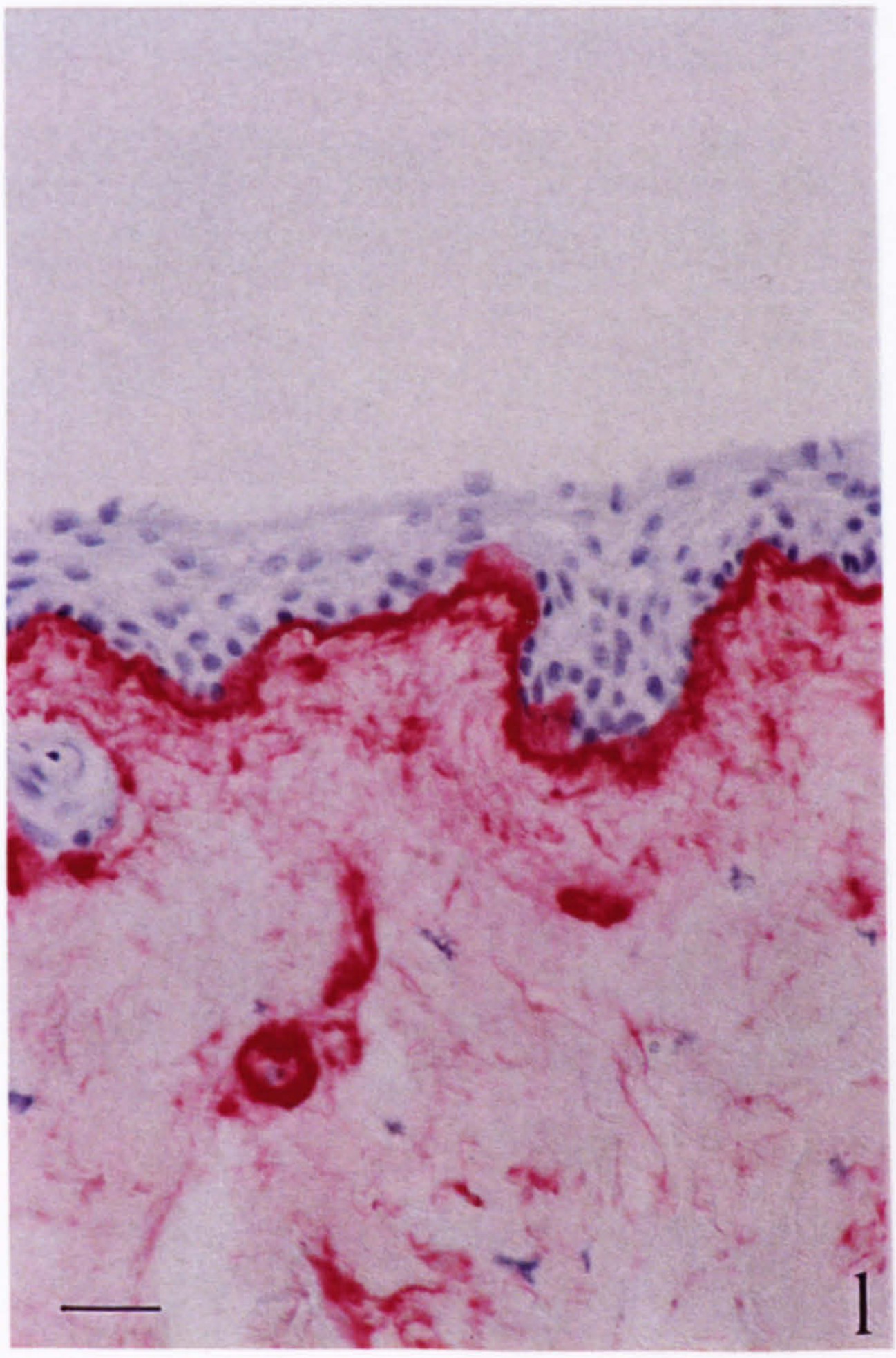
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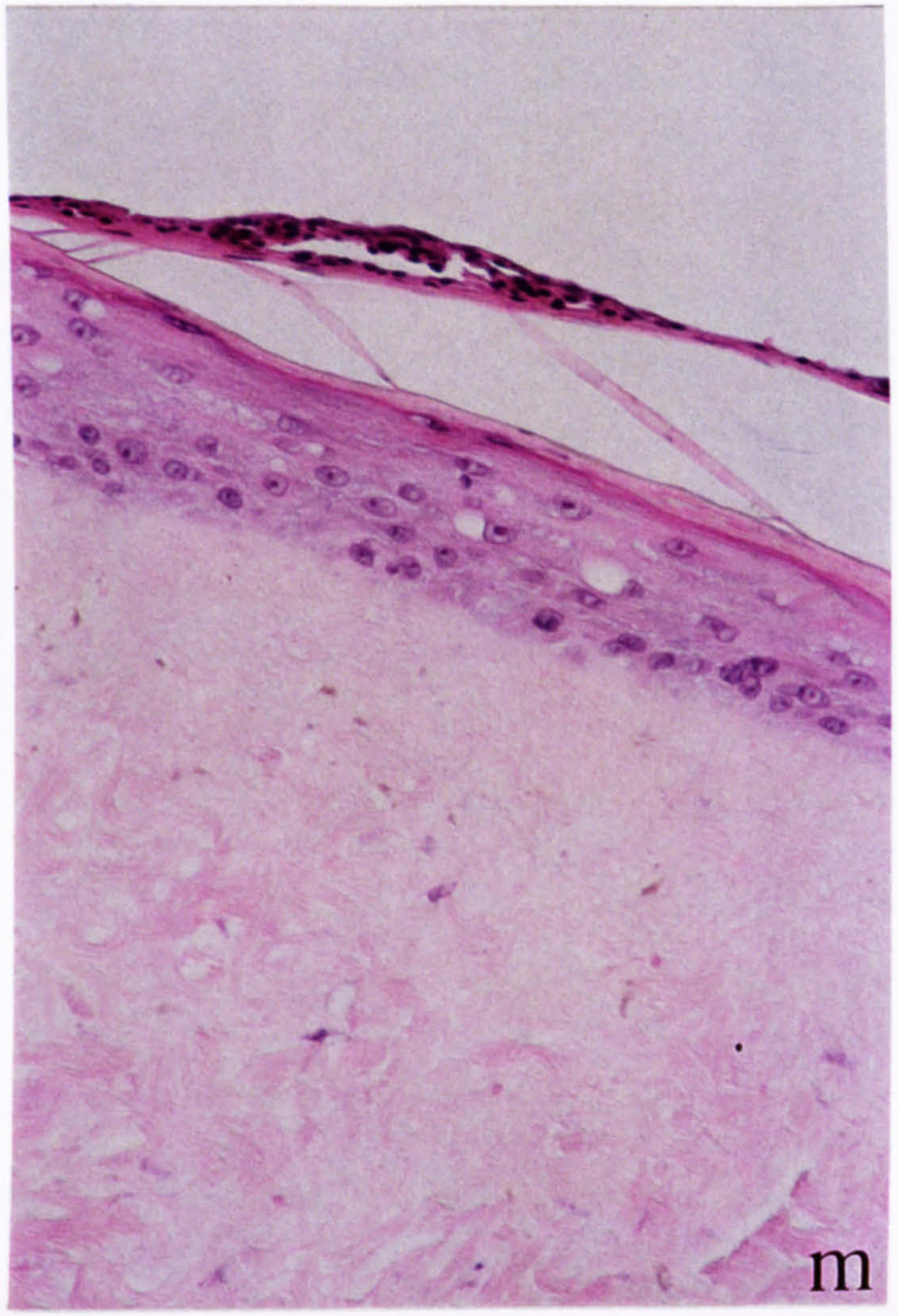
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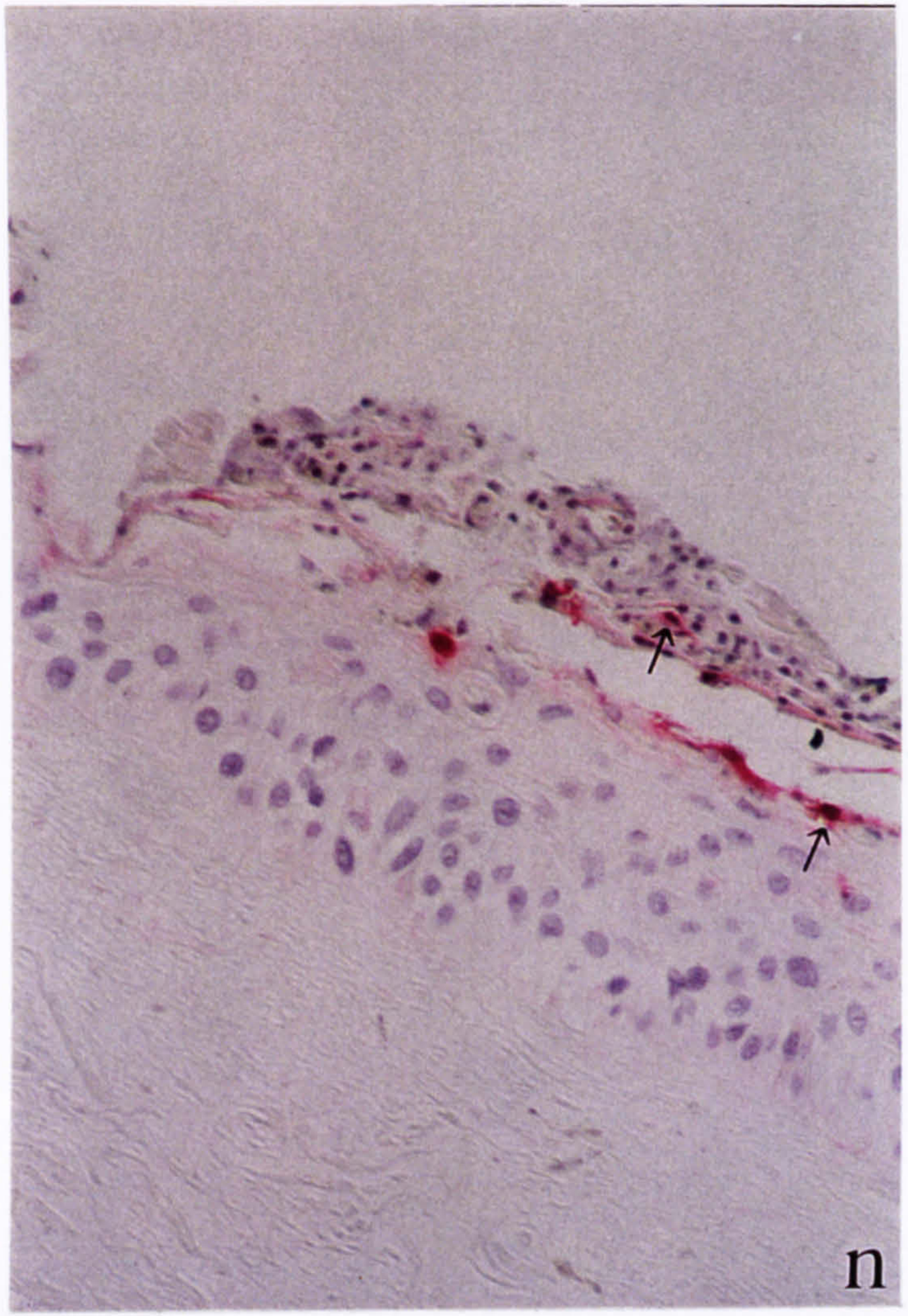
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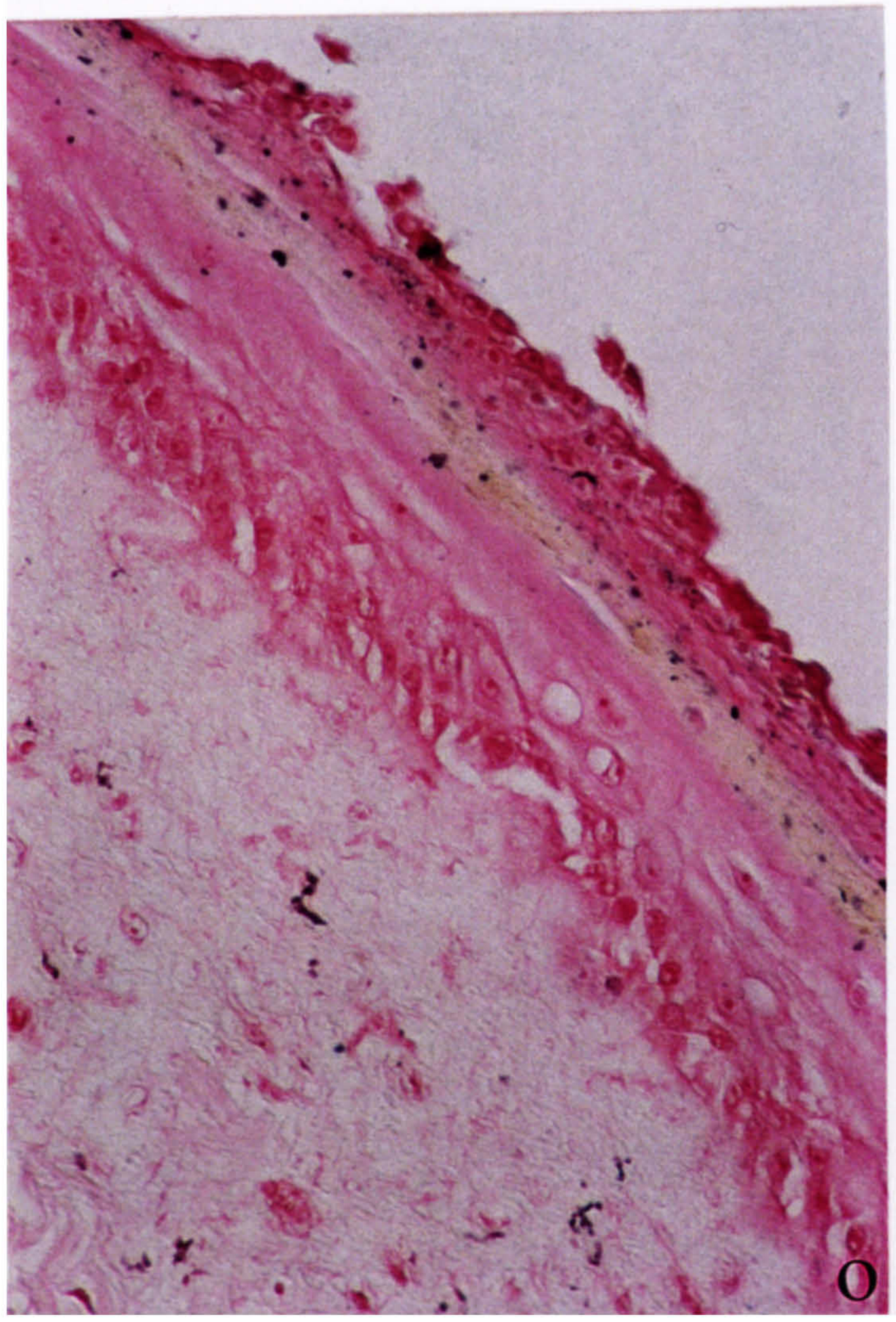
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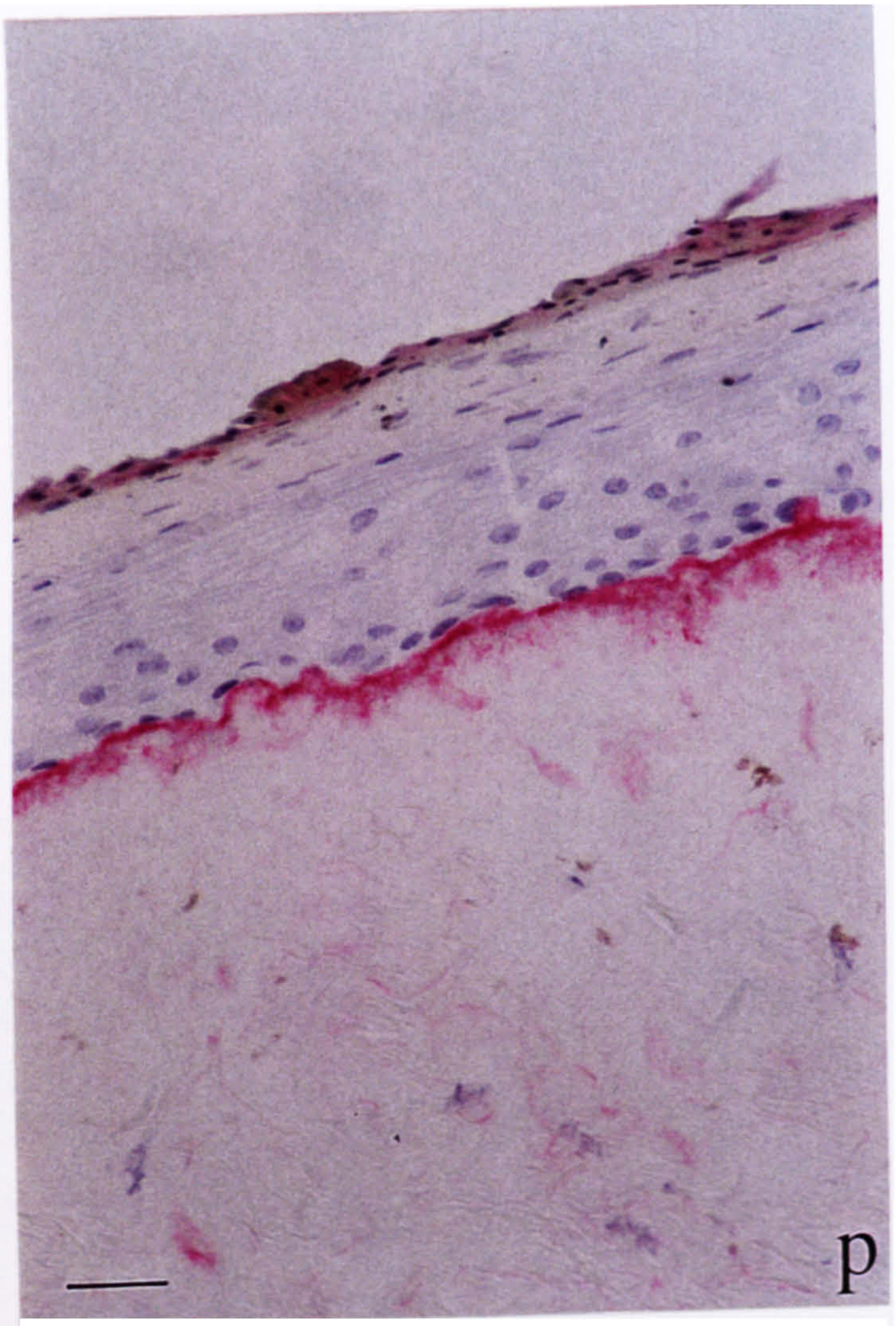
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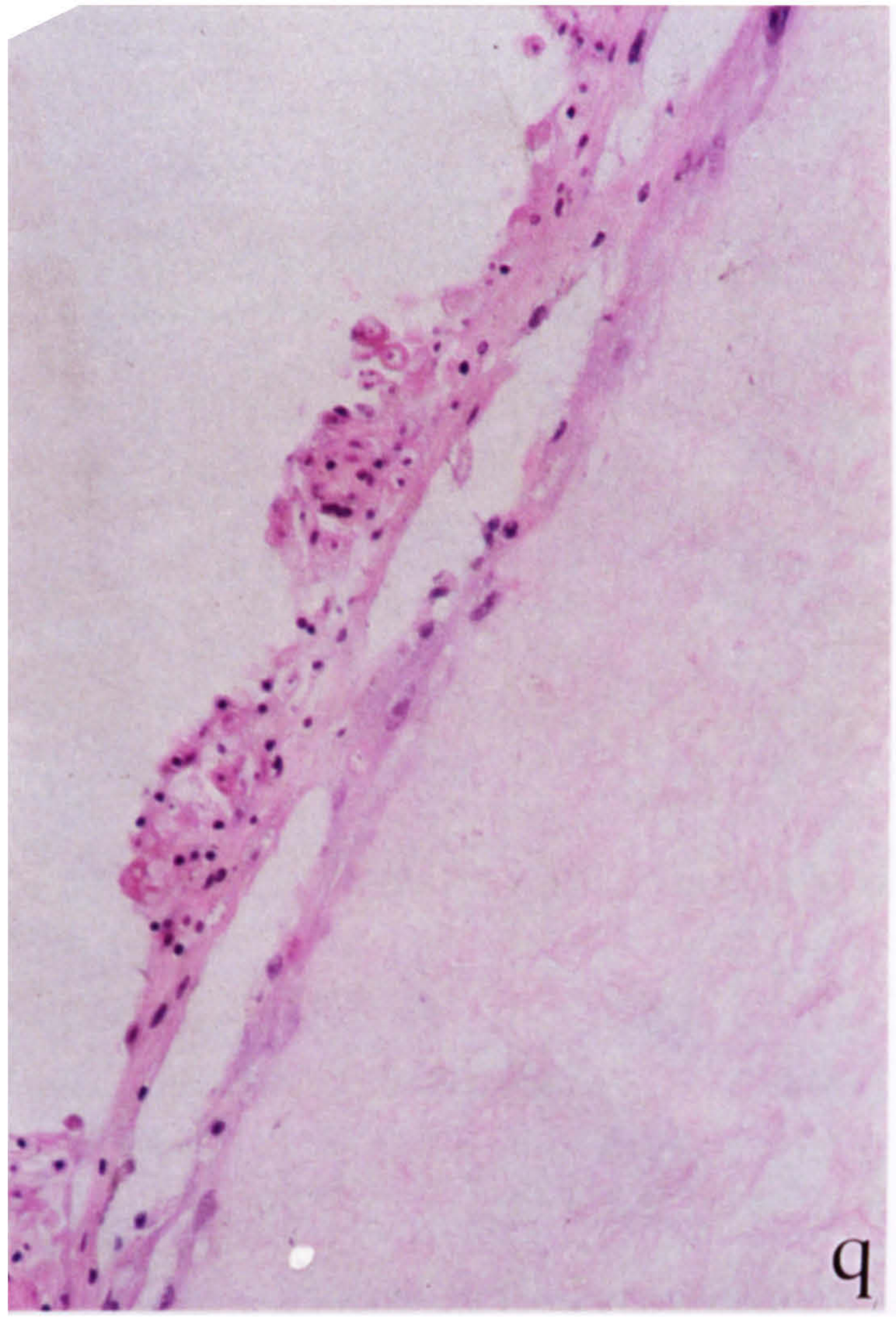

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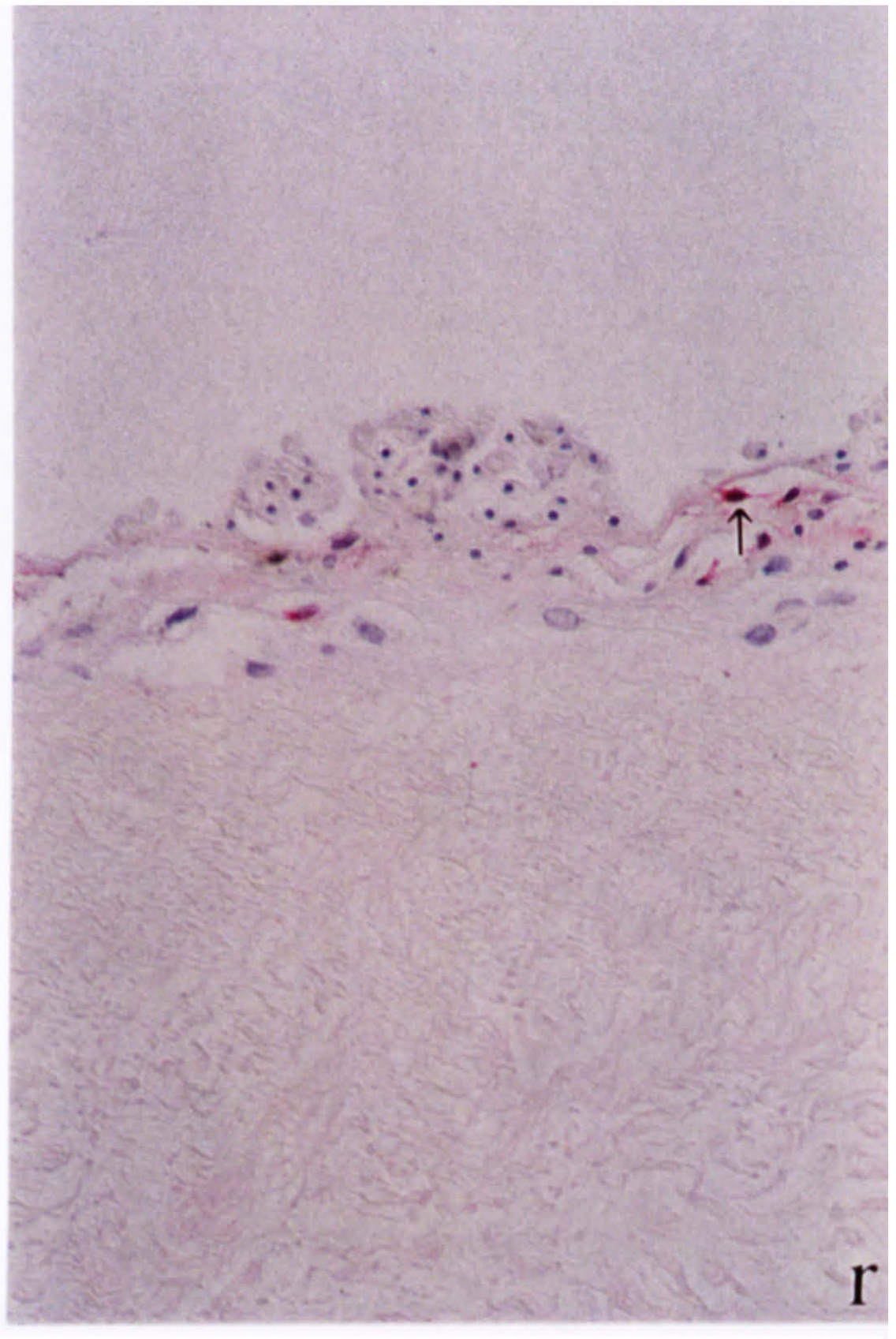

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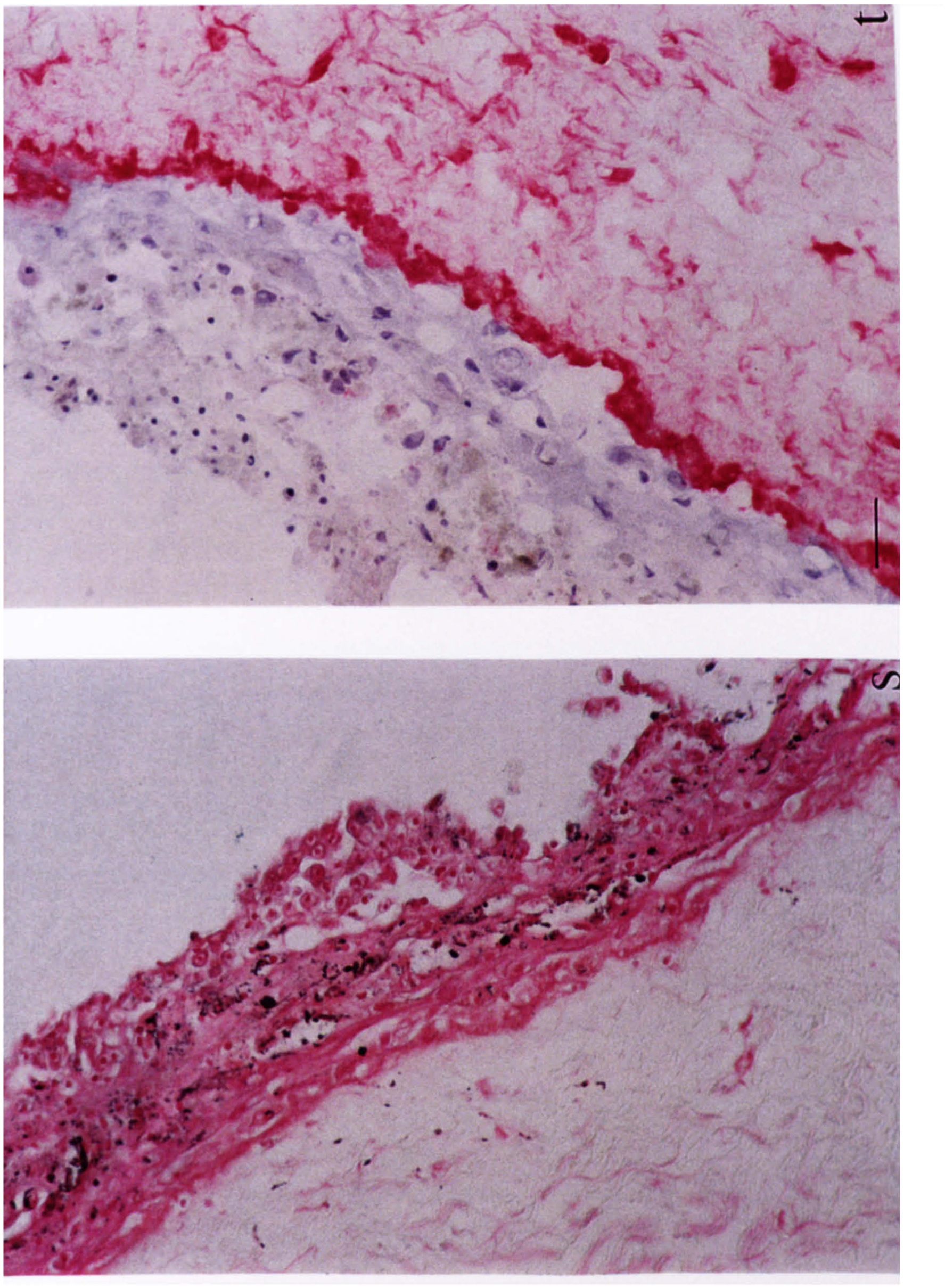

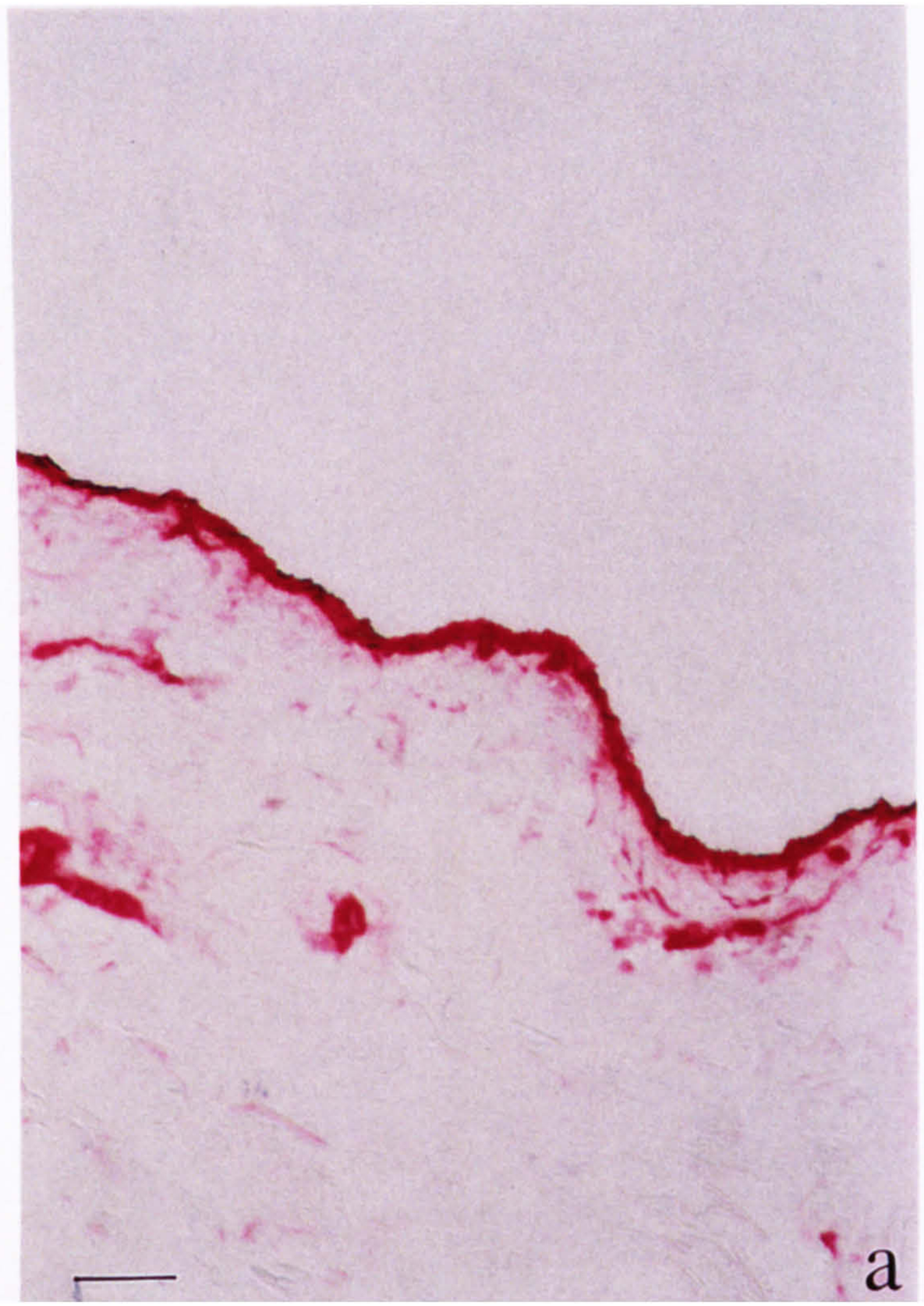
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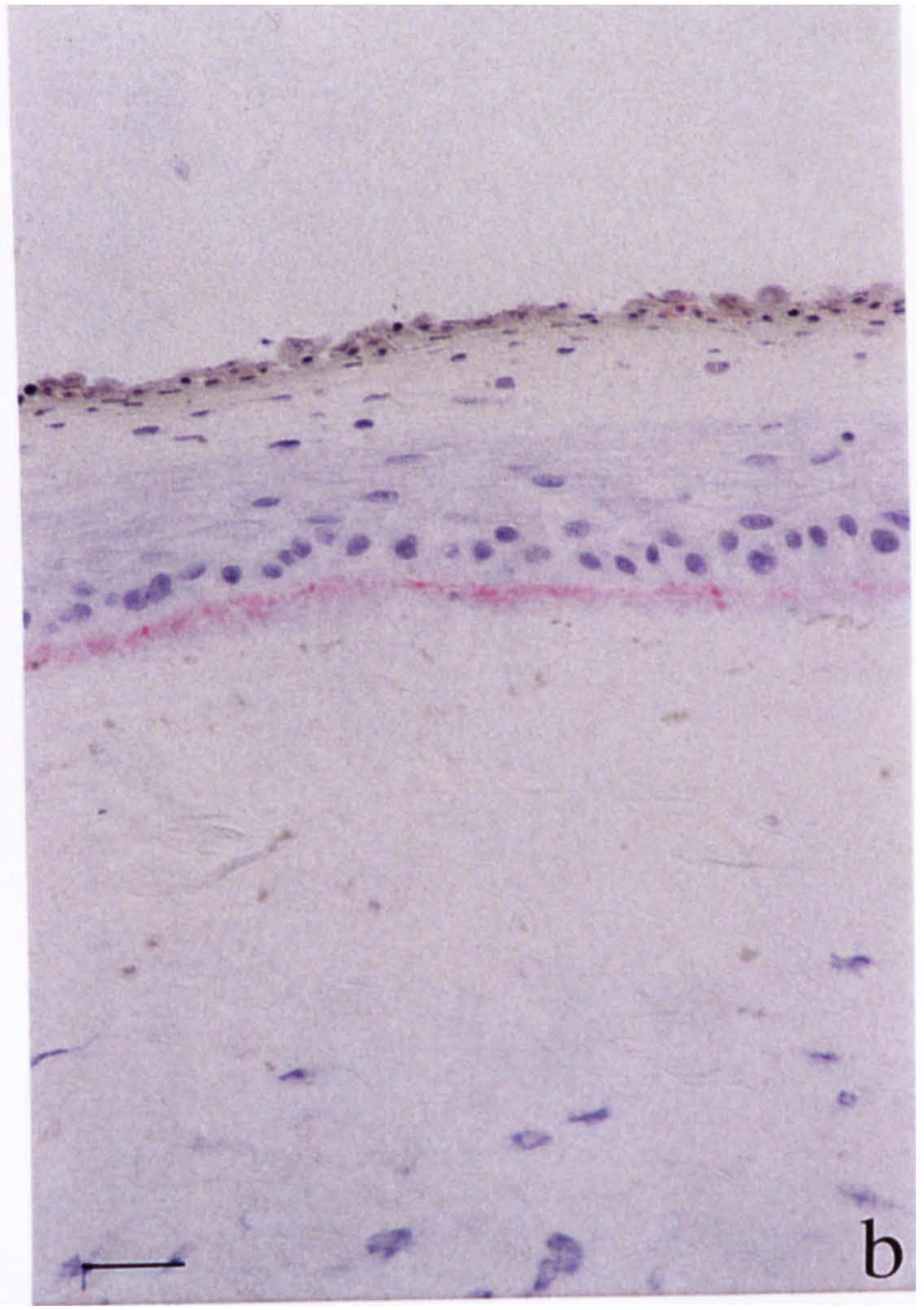
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Figure 3.32 The presence of collagen IV in a composite initially stripped of basement membrane. Composites were prepared and cultured as described in **Section 2.4** and **Figure 3.30**. Collagen IV staining was performed by Mr C Layton as described in **Section 2.4.7**. Dermis which received no cell additions is shown as a control for collagen IV staining **(a)**. Collagen IV staining is shown for composite H (see **Table 3.16a)** which was initially stripped of basement membrane proteins **(b)**. Scale bars represent 50μm.

In these composites, pigmentation was seen under all conditions. Composites that lacked fibroblasts had darker levels of pigmentation, while those without basement membrane showed darker pigmentation at the surface level but this did not agree with the results of the Masson Fontana staining. Composites without basement membrane or fibroblasts were not as well defined in terms of keratinocyte layers and melanocyte number was reduced in composites without basement membranes. Collagen IV was present in all composites although it was much reduced in composites that had been pretreated with dispase. Composites without fibroblasts had patchy collagen IV staining.

3.5 DISCUSSION.

The human cutaneous melanocyte resides in a complex environment and the importance of keratinocytes in the regulation of melanocyte function has been illustrated in numerous co-culture studies (De Luca *et al*, 1998a & b; Valyi-Nagy *et al*, 1990; Haake & Scott, 1991; Scott & Haake, 1991; Donatien *et al*, 1993; Valyi-Nagy *et al*, 1993; Nakazawa *et al*, 1995a; Tenchini *et al*, 1995; Kippenberger *et al*, 1996; Nakazawa *et al*, 1997; Kippenberger *et al*, 1998a; Nakazawa *et al*, 1998). Keratinocytes produce several growth factors and cytokines such as α-MSH (Schauer *et al*, 1994; Kippenberger *et al*, 1995; Chakraborty *et al*, 1996; Wintzen *et al*, 1996), endothelin-1 (Yohn *et al*, 1992; Imokawa *et al*, 1992), bFGF (Halaban *et al*, 1988), GM-CSF (Imokawa *et al*, 1996b), NGF (Yaar *et al*, 1991), NO (Roméro-Graillet *et al*, 1997), IL-1 (Kupper *et al*, 1987; Kramer *et al*, 1993) and TNF-α (Köck *et al*, 1990) which can modulate various aspects of melanocyte biology (as discussed further in **Section 3.5**). These factors are generally induced or upregulated by UV irradiation. Recently though, FCM had been reported to increase melanocyte proliferation through SCF and HGF (Imokawa *et al*, 1998). Gene transcripts of HGF have been found in dermal fibroblasts (Chan *et al*, 1991). HGF and SCF factors have been previously shown to induce mitogenic effects in melanocytes (Matsumoto *et al*, 1991; Funasaka *et al*, 1992; Halaban *et al*, 1992). The importance of SCF in normal adult melanocyte homeostasis has recently been illustrated in a human skin xenograft study (Grichnik *et al*, 1998). In the absence of keratinocytes, though, highly mitogenic media are required to maintain melanocyte survival and proliferation (as discussed in **Section 3.1.1**). In turn this has led to problems as these culture media

are perhaps unnaturally mitogen rich. Melanocytes retain a regulated number in the presence of unstimulated keratinocytes (De Luca *et al*, 1988a & b; Staiano-Coico *et al*, 1990; Scott & Haake, 1991). The consensus opinion formed over the past decade is that melanocytes are strongly modulated by the growth medium composition and the response to more natural melanogens may be masked. Only one study has examined the use of a more physiological supplemented medium (Swope *et al*, 1995) but this still relies on the presence of foetal calf serum. This present study illustrates the strong influence of serum and pituitary extracts and non-physiological mitogens such as CT, PMA and IBMX on morphology (**Sections 3.2.1** and **3.2.3**), proliferation (**Sections 3.3.1** and **3.3.3**) and pigmentation (**Sections 3.4.1** and **3.4.3**).

UVR remains the only well defined physiological stimulator of melanocyte function. Several studies have shown that UVB and UVA/PUVA (Friedmann & Gilchrest, 1987; Libow *et al*, 1988; Tomita *et al*, 1988; Friedmann *et al*, 1990a&b; Ramirez-Bosca *et al*, 1992; Kao & Yu, 1992; Aberdam *et al*, 1993; Abdel-Malek *et al*, 1994b; Im *et al*, 1994; Roméro *et al*, 1994; Mengeaud & Ortonne, 1996; Duval & Schmidt, 1997) can directly influence melanocyte biology *in vitro* (see **Table 3.17** for a summary of some of the pigmentary results). Many different UV protocols were used but, in accordance with these studies, melanocytes in this present study responded with increased pigmentation and alteration in morphology to UVB (**Sections 3.2.5**, and **3.4.8-3.4.9**). Melanocytes cultured in less mitogenic media were noticeably less responsive to UVB. Indirect mechanisms may result through the stimulation of keratinocytes and fibroblasts (as discussed in **Section 3.5**).

Perhaps one of the most controversial pigmentary regulators in man is α-MSH. While *in vivo* studies have clearly demonstrated that the administration of supraphysiological concentrations of α- and β-MSH (Lerner & McGuire, 1961), ACTH (Lerner & McGuire, 1964) and the potent α-MSH analogue, NDP-MSH (Levine *et al*, 1991) can induce skin darkening (particularly in sun exposed regions) in man, *in vitro* studies using pure cultures of human melanocytes have not generally shown consistent pigmentary responses to α-MSH. Only four groups (Hunt *et al*, 1994a, b, c; Abdel-Malek *et al*, 1995; McLeod *et al*, 1995; Maeda *et al*, 1996) have reported *in vitro* melanogenic responses to α-MSH/ACTH. In the initial stages of the present study,

Table 3.17 A summary of UV irradiation sources, protocols and the results obtained using pure culture melanocytes from several different investigators.

Reference	UV source	UV irradiation protocol No of doses (mJ/cm^2)	Melanocyte type	Pigmentation[a]		Level of melanogenic enzymes[b]		
				Tyrosinase activity	Melanin	Tyrosinase	TRP-1	TRP-2
Friedmann & Gilchrest, 1987[c]	solar simulator (Kratos LH153) 93%UVA/7%UVB	7 x 2.4 (UVB)	neonatal foreskin	ND[d]	3 fold or more	ND	ND	ND
Libow *et al*, 1988	Phillips TL20W/12 lamps (6) 60% UVB	1 x 200 (UVB)	neonatal balck or Hispanic foreskin	ND	2 fold	ND	ND	ND
Tomita *et al*, 1988	FL20SE lamp peak 305nm	2 x 30 (UVB)	oriental adult	1.44fold	ND	ND	ND	ND
Kao & Yu, 1992	V2-15L Vilber Lourmat, 365nm	1 x 0-12 (UVA)[e]	adult foreskin	1.35 fold	ND	ND	ND	ND
Ramirez-Bosca *et al*, 1992	Psorilux 3050A lamp, wide range of UV, no UVC	1 x 50-200 (UVB)	adult foreskin	2 fold[f]/ 1.5 fold[g]	5 fold[f]/ 2 fold[g]	ND	ND	ND
Abdel-Malek *et al*, 1994b[h]	Westinghouse FS-20, peak 312nm, 75%UVB/25%UVA	3 x 15.5 (UVB)	neonatal Caucasian/African American foreskin	2 fold[i]/ 1.7 fold[j]	1.5 fold[i]/ 1.4 fold[j]	=	=	↓
Im *et al*, 1994	FS72T12-UVB-HO lamp, peak 310-312nm, no UVC	6 x 5 (UVB)	adult Korean foreskin[k]	2.5 fold	ND	ND	ND	ND
Roméro *et al*, 1994[l]	Vilber Lourmat Stimulator, UVB only	6 x 100 (UVB)	neonatal Caucasian foreskin	2.2 fold	1.8 fold	↑	↑	↓
Mengeaud & Ortonne, 1996[j]	Vilber Lourmat T-20.L, 365nm, no UVB/C	1 x 100-800 (UVA)[m]		1.5-3 fold	1.5-3 fold	↑	=	↓
Hedley, this study	Helarium fluorescent lamps (Wolff B1.01.40W) 75%UVA/25%UVB	7 x 143 (UVB)	adult Caucasian non-foreskin	1.46 fold[n]	1.2 fold[n]	ND	ND	ND

Table 3.17:

[a] The results shown are all increases in tyrosinase activity and melanin content/synthesis.

[b] The level of protein for each of the melanogenic enzymes was measured.

[c] Two additional studies showed that using 6 doses of 260mJ/cm^2 increased melanin by (Friedmann *et al*, 1990a) and using 6 doses of 180mJ/cm^2 increased melanin by 46% (Friedmann *et al*, 1990b) using Helarium fluorescent sunlamps (Wolff B1.01.40W). They have a spectral output of 75%UVA and 25%UVB with negligible UVC emittance.

[d] ND-not determined.

[e] Melanocytes were treated with 8-MOP in addition to UVA.

[f] The results are from melanocytes cultured in a F10 nutrient medium supplemented with PMA in addition to FCS, IBMX and CT.

[g] The results are from melanocytes cultured in a keratinocyte serum free medium supplemented with BPE in addition to FCS, IBMX and CT.

[h] Barker *et al.* (1995) found that one dose of UVB significantly increased heavily pigmented melanocytes but not lightly pigmented melanocytes.

[i] The results are from melanocytes cultured from Caucasian donors.

[j] The results are from melanocytes cultured from American African donors.

[k] Melanocytes were from normal and vitiligo donors.

[l] Aberdam *et al.* (1993) obtained a 2-5 fold increase in melanin formation using the same protocol.

[m] 5-MOP was added in addition to UVA irradiation.

[n] The results are from melanocytes cultured in medium 1.

melanocytes from 3 different donors expanded in culture with a CT containing growth medium did not respond with increased pigmentation to α-MSH (**Section 3.4.7**). However melanocytes expanded in culture using a CT free medium (based on Abdel-Malek *et al*, 1995) showed some reproducible increases in proliferation and pigmentation in response to 10^{-8}M α-MSH in a few of the 25 different media variants (detailed in **Table 2.2**). Until recently, POMC-derived peptides were thought only to be produced in the pituitary. Several authors (as discussed in **Section 3.1**) now report that the skin is one of the major non-pituitary tissues to show POMC gene expression and these observations strongly suggest that α-MSH and other POMC-derived peptides play a role in skin physiology with pigmentation being only one aspect of its role in man. Recent papers by Luger and colleagues suggest a wider role of α-MSH in skin (Luger *et al*, 1997; Luger, 1998), particularly in immunomodulation.

Melanocytes lie adjacent to the basement membrane and there is evidence from a number of *in vitro* studies (Gilchrest *et al*, 1985; Ranson *et al*, 1988a; Buffey *et al*, 1994; Nakazawa *et al*, 1995b) that ECM proteins can influence melanocyte behaviour such as morphology and pigmentation. In this present study various individual and cell-derived ECM proteins were examined and it was found that melanocytes cultured in mitogen-free medium were most responsive to the different ECMs. A preliminary study in an epidermal/dermal model highlighted some intriguing results (**Section 3.4.10**). While α-MSH did not appear to influence pigmentation, the absence of fibroblasts or basement membrane proteins significantly increased the level of pigmentation.

3.5.1 MORPHOLOGY.

In early stages of adult cutaneoeus melanocyte culture, melanocytes resemble their *in vivo* multidendritic state but, within a few weeks of culture in mitogen-rich medium (i.e. maintenance media A, B or C), the melanocytes assumed a bi/tripolar spindle shape with the occasional multidendritic cell remaining (**Figure 3.1**) in accordance with data from Abdel-Malek *et al.* (1994a). Ikeda *et al.* (1994b) reported that melanocytes could retain their dendritic appearance in long-term culture if a modified medium described as MM-4 was used instead of MCDB153 (**Table 3.1a**). Normal and vitiligo melanocytes were generally very similar in morphology (**Figures 3.2, 3.8,** and **3.9**), as reported by

several authors (Mojamdar *et al*, 1987; Puri *et al*, 1987; Medrano & Nordlund, 1990; Im *et al*, 1992; Jee *et al*, 1993; Bessou *et al*, 1997a), although vitiligo melanocytes often appeared slightly larger than their normal counterparts. Melanocytes from donors VM2 and VM3 did, however, retain a larger proportion of multidendritic cells throughout their culture period (**Figure 3.3**). The melanocytes cultured from limb skin were initially multidendritic with a large cytoplasm, unlike the spindle shaped foreskin melanocytes which have few dendrites (Esinger & Marko, 1982; Im *et al*, 1992). Melanocytes from perilesional skin were larger in size and multidendritic than from uninvolved regions of skin from vitiligo patients (Mojamdar *et al*, 1987; Puri *et al*, 1987). Cytokines, except for LCM which would have a strong mix of cytokines (**Figure 3.9**), did not have strong influence on normal or vitiligo melanocyte morphology. In the presence of the LCM, both normal and vitiligo melanocytes appeared ragged and perhaps stressed (**Figure 3.9**). Other studies have indicated that cytokines influence melanocyte morphology (Varani *et al*, 1989; Krasagakis *et al*, 1991; Swope *et al*. 1991), when the cytokines were introduced to the culture media for several days.

Morphological responses to melanocytes were highly influenced by media contents, PMA, CT and pituitary extract (as summarised in **Table 3.2**) and melanocytes were significantly altered within 5 days. Melanocytes cultured in the presence of PMA were spindle shaped but dendrite number could vary (illustrated in **Figure 3.4e** and **Figure 3.4f**). In the absence of PMA, cell bodies spread to form epithelioid shapes (**Figure 3.4c**) as previously noted by Nakazawa *et al*. (1996). In the mitogen-deficient medium (medium 24), cells on plastic were much smaller and often rounded. BPE and CT had mild effects on inducing melanocyte dendricity (**Figures 3.4g** and **3.4h**) while IBMX was a potent inducer of dendricity in all 5 media conditions investigated (**Figure 3.6** illustrates the effects for 3 media variants). In contrast, α-MSH did not appreciably affect melanocyte morphology in any of the 25 conditions examined in this study. Herlyn *et al. (1988) showed that, in a defined medium containing PMA and bFGF, the* addition of α-MSH failed to effect morphology, whereas dibutryl cAMP (dbcAMP) was able to induce cells to extend up to 28 dendrites per cell. Nakazawa *et al*. (1993) also showed that modulators of intracellular cAMP induced melanocyte dendricity while PMA reduced dendricity. Hunt *et al*. (1994b & c) showed that, while approximately

66% of their cultures responded to NDP-α-MSH and α-MSH with increased cell dendricity, all their cultures responded to 8-bromo-cAMP with increased dendricity. Maeda *et al.* (1996) demonstrated that melanocytes (in culture for 7 days) became enlarged and dendritic following treatment with β-MSH, NDP-MSH or ACTH for 2 days. Scott *et al.* (1997) showed that melanocytes treated with NDP-MSH exhibited increased dendricity which related to alteration in the actin organisation.

The choice of ECM substrates was less effective in altering melanocyte morphology in comparison to the influence of media composition (**Figure 3.7** and **Table 3.3**). In the non-mitogenic medium (medium 24) the cells appear more bi/tripolar in shape instead of being rounded as when cultured on all types of ECM proteins except for laminin (**Figure 3.7i**). None of the conditions here described induced melanocytes to exhibit increased dendricity *in vitro*. Ranson *et al.* (1988a) and McClenic *et al.* (1989) also reported that dendricity was not increased, but that the cells became more epithelioid. However, Gilchrest *et al.* (1985) demonstrated that melanocytes in a hormone supplemented culture medium (described in **Table 3.1**) and on some substrates such as collagen I, appeared spindle-shaped with two or three thin long dendrites; on fibronectin, cells tended to assume a stellate/multidendritic morphology, while on laminin, they appeared rounded. Calcium additions further promoted dendrite outgrowth of melanocytes attached to ECM proteins (Hara *et al*, 1994). Keratinocyte-derived ECM induced melanocyte dendricity (Nakazawa *et al*, 1995b) with the most significant increase (219%) seen on ECM derived from UVB irradiated differentiated keratinocytes. Differentiated keratinocytes also induced longer length dendrites. Similar results were seen in a co-culture model used by Nakazawa *et al.* (1995a). It was hypothesised that the state of differentation of the keratinocyte therefore played a significant role in the regulation of melanocyte morphology (Nakazawa *et al*, 1995b).

The formation of melanocyte dendrites is promoted in the presence of keratinocytes (De Luca *et al*, 1998a & b; Valyi-Nagy *et al*, 1990; Haake & Scott, 1991; Scott & Haake, 1991; Donatien *et al*, 1993; Valyi-Nagy *et al*, 1993; Nakazawa *et al*, 1995a; Tenchini *et al*, 1995; Nakazawa *et al*, 1997 & 1998) and further illustrated using time-lapse photography (Kippenberger *et al*, 1998a). De Luca *et al.* (1988a) found that the type of keratinocyte also influenced melanocyte morphology. Melanocytes were stellate or

multidendritic when cultured with cutaneous keratinocytes, while they were spindle shaped in the presence of oral epithelial cells. KCM increased melanocyte dendricity (Gordon *et al*, 1989; Lacour *et al*, 1992; Hara *et al*, 1995; Tenchini *et al*, 1995) although Valyi-Nagy *et al.* (1993) reported no effect of KCM in the model they used. KCM was shown to influence the arrangement of actin and tubulin microfilaments (Lacour *et al*, 1992). Endothelin-1 (Hara *et al*, 1995; Scott *et al*, 1997), NGF (Peacocke *et al*, 1988; Yaar *et al*, 1991) and NO (Roméro-Graillet *et al*, 1997) also increased melanocyte dendricity although Tenchini *et al.* (1995) saw no effect of NGF in their system.

In vivo melanocytes generally become more dendritic in response to UV irradiation (Pathak *et al*, 1965; Rosen *et al*, 1987; Stierner *et al*, 1989). This phenomenon has been replicated in *ex vivo* studies (Todd *et al*, 1993; Archambault *et al*, 1995; Bessou *et al*, 1995) and organ skin studies (Iyengar 1992a&b; 1994a&b; 1996 & 1998). In this study, melanocytes from both normal and vitiligo donors exhibited increased dendricity in response to UVB when cultured in medium 1 (**Figure 3.8**), as described in many other studies (Friedmann & Gilchrest, 1987; Ramirez-Bosca *et al*, 1992; Aberdam *et al*, 1993; Abdel-Malek *et al*, 1994b; Im *et al*, 1994; Nakazawa *et al*, 1998). Tomita *et al.* (1988) showed that melanocytes irradiated twice increased their cell area, but not their dendrite number, which was seen in cells isolated from UV irradiated skin. PUVA did not alter the morphology of *in vitro* cultured melanocytes (Kao & Yu, 1992).

3.5.2 PROLIFERATION.

The proliferation of melanocytes requires highly mitogenic media (see **Section 3.1.1**). In the early attempts at culturing vitiligo melanocytes, it was reported that vitiligo melanocytes showed defective growth (Puri *et al*, 1987) but that extracts from a fetal lung fibroblast cell line could partially correct this (Majumder *et al*, 1987; Puri *et al*, 1989). Medrano & Nordlund (1990) first demonstrated that vitiligo melanocyte growth comparable with normal melanocytes could be obtained if a MCDB153-based medium was used (described in **Table 3.1a**). Catalase supplementation in the initial isolation and early culture period appeared vital in this medium. Im *et al.* (1992) and Bessou *et al.* (1997a) using slightly different media (see **Table 3.1**) found no

difference in population doubling between normal and vitiligo melanocytes. Jee *et al.* (1993) from that melanocytes from patients with segmental vitiligo grew similarly to normal melanocytes but melanocytes from patients with gneneralised vitiligo could not be propagated. Foreskin melanocytes had a shorter doubling time than adult melanocytes irrespective of whether cultures were from normal or vitiligo donors (Im *et al*, 1992). Other groups which culture vitiligo melanocytes did not compare the growth with normal melanocytes (Olsson & Juhlin *et al*, 1993; Zachariae *et al*, 1993; Schallreuter-Wood *et al*, 1996; Le Poole *et al*, 1997; Maresca *et al*, 1997; Kaufmann *et al*, 1998).

In this study vitiligo melanocytes required a significantly longer culture period than normal melanocytes to obtain adequate numbers for experimentation (**Section 3.3.6**), but once under experimental conditions where cultures were seeded at the same densities and cultured for 5 days, there was no significant difference in the mean proliferation of the 4 cultures examined (**Figure 3.20**). However, 3 of the 4 cultures were not as proliferative as their paired counterparts (**Table 3.9c**). The vitiligo patients were in their seventh or eight decade at the time of donation, whereas the age of the normal melanocyte donors ranged from 28-50 years (at time of donation). Similarly, over a longer experimental period of 8 days, the vitiligo melanocytes were generally less proliferative than the normal melanocytes in medium 1 (**Table 3.11a**). Only melanocytes from donor VM4, who had a similar age to the median age of the donors for normal melanocytes (34 years vs 30.5 years respectively), had similar proliferation to normal melanocytes. Age and anatomical site have been reported to play a role in melanocyte growth (Abdel-Malek *et al*, 1994a). The smaller biopsy size used for initiating vitiligo cultures, the short period of time in culture with Green's medium which is not primed for specific melanocyte growth and the age of the melanocyte donor may therefore be contributing factors to the differences seen in the reduced proliferation in vitiligo melanocytes. Interestingly, Jee *et al.* (1993) reported that melanocytes isolated from patients with generalised vitiligo did not proliferate, but all the donors in this study had a generalised form of vitiligo and their melanocytes were capable of proliferation.

The MCDB153 based medium used in this study (described in **Table 2.1**) was most mitogenic for melanocytes with the addition of FCS, BPE, PMA and CT (referred to as maintenance medium A or experimental medium 1). Naturally, as the mitogenic

content was reduced, melanocyte proliferation was reduced (**Figure 3.10a**). BPE was the most mitogenic component added as medium 20 containing just BPE was more mitogenic than media 19, 21, 22, or 23 which just contained FCS, PMA, CT and bFGF respectively. Additionally, all the media containing BPE were in the top 15 mitogenic media.

With respect to the effect of α-MSH on melanocyte proliferation, a low concentration of MSH (10^{-10}M) had little or no effect on cell number (**Figure 3.11a**). This was in accordance with the findings of Abdel-Malek *et al.* (1995) who demonstrated that this was the minimal effective dose for α-MSH. However, melanocytes exposed to 10^{-8}M α-MSH showed significant increases in cell number under only 4 media conditions. A fifth medium containing bFGF (medium 23), just failed to achieve a significant increase of 38% ($p<0.051$), but when additional studies were conducted, a signficant increase of 59% was obtained (**Section 3.3.2**). The effect of α-MSH was dose-dependent, as illustrated in medium 23 (**Figure 3.13a**). In order to obtain good melanocyte proliferation *in vitro*, at least 2 intracellular signalling pathways require activation. Several groups have shown that α-MSH can be mitogenic when combined with other intracellular signalling pathway activators (Herlyn *et al*, 1988; De Luca *et al*, 1993; Swope *et al*, 1995; Abdel-Malek *et al*, 1995; Suzuki *et al*, 1996). Most of these authors have combined α-MSH with PMA and bFGF. This study also showed that cells cultured in a medium containing PMA and bFGF (medium 18) exhibited enhanced cell proliferation with the addition of α-MSH. Swope *et al.* (1995) have additionally shown that a physiological medium containing α-MSH, bFGF and endothelin-1 sustained melanocyte proliferative growth. Endothelin-1 was found to be mitogenic for melanocytes and binds to specific receptors on melanocytes (Yada *et al*, 1991; Yohn *et al*, 1993; Imokawa *et al*, 1992). It is reported to exert the mitogenic effect through receptor mediation of the tyrosine kinase pathways utilising cross talk with the PKC pathway (Imokawa *et al*, 1996a). In a more recent study it was suggested that endothelin-1 may influence melanocyte physiology through an endothelin-B receptor mediated calcium signalling pathway (Kang *et al*, 1998).

In this present study, α-MSH stimulated cell proliferation in 4 media where bFGF was present either as the only mitogen (medium 23) or in combination with one other

mitogen (media 12, 16, and 18). Therefore, α-MSH and bFGF in combination were potent effectors of melanocyte proliferation when the media was mitogen poor. However, in the absence of α-MSH, bFGF was a poor mitogen and present in the three least proliferative media (**Figure 3.12a**). bFGF was first reported to be mitogenic in a PC-1 medium (Halaban *et al*, 1987) and Olsson & Juhlin (1993) have illustrated PC-1 medium as a proliferative medium for vitiligo melanocytes, when supplemented only with insulin, bFGF and dbcAMP. Puri *et al.* (1996) also showed that bFGF was capable of increasing melanocyte proliferation in a medium containing IBMX and PMA. Two unusual results demonstrated that α-MSH increased cell proliferation in media 1 (only 10% increase) and 15. Both media contained CT. Generally α-MSH is unable to elicit proliferative effects in media containing CT and preliminary results with melanocytes cultured long term in medium containing CT did not respond to α-MSH (**Section 3.4.7**). However, Hunt *et al.* (1994b) noted that α-MSH was able to increase melanin and tyrosinase activity slightly in the presence of CT. Additionally CT and IBMX both of which affect cAMP, have been combined to enhance melanocyte proliferation by Halaban *et al.* (1986) and Abdel-Malek *et al.* (1992). Hence these results might not be quite as unusual as they seem. In other studies using different media conditions not containing CT, α-MSH was not mitogenic (Ranson *et al*, 1988b; Hunt *et al*, 1994a and b). McLeod *et al.* (1995) and Maeda *et al.* (1996) did not comment on the effects of α-MSH on cell proliferation.

IBMX was inhibitory to cell proliferation in 3 of the 5 media studied, but was stimulatory in medium 12 which contained FCS and bFGF (**Figure 3.14a**). Medrano & Nordlund (1990) noted that CT and IBMX actually inhibited growth in their MCDB153 medium which was very similar to the one in this study. However, 0.1mM IBMX has been routinely used in culture of neonatal foreskin melanocytes, and reported to aid proliferation (Halaban *et al*, 1986; Abdel-Malek *et al*, 1992) but adult non-foreskin melanocytes only proliferated for a few passages in the presence of 0.1mM IBMX (Abdel-Malek *et al*, 1994a). It has also been suggested that CT and IBMX can induce adult non-foreskin nelanocytes to undergo terminal differentation (Medrano *et al*, 1993). A higher concentration of 1mM IBMX was used with the non-foreskin adult melanocytes and it is possible that under the more proliferative media combinations (media 1, 2 and 10; **Figure 3.10a**) terminal differentiation was being initiated. Under

the microscope, it was obvious that there were less cells in these three media with IBMX, and that they were more dendritic (**Figure 3.6**). Nakazawa *et al.* (1993) also showed that agents capabable of elevating cAMP reduced cell growth whereas Herlyn *et al.* (1988) found that cAMP modulating agents generally increased melanocyte growth.

Cytokines, IFN-γ and TNF-α generally did not affect melanocyte proliferation (**Section 3.3.5**). They were only present for 24 hours and cell number was assessed to check that melanocyte survival was not compromised and the cause for effects seen in the modulation of immune-related cell surface molecules (**Chapter 4**). However, other studies have indicated that a wide range of cytokines can reduce melanocyte proliferation (Krasagakis *et al*, 1991; Swope *et al*, 1991; Krasagakis *et al*, 1995) when present for several days. Other inflammatory mediators such as LTC_4 and LTD_4 are effective mitogens and do not require the addition of other mitogens to exert their effects (Morelli *et al*, 1989).

The number of dopa positive (Quevedo *et al*, 1965; Mishima & Widlan, 1967; Quevedo *et al*, 1969; Rosen *et al*, 1987; Stierner *et al*, 1989; Tobin *et al*, 1994) and TRP-1 positive melanocytes (Tobin *et al*, 1994) have been shown to be increased i*n vivo* by UV irradiation. Stierner *et al.* (1989) found that melanocyte number was increased in shielded skin next to the UVB exposed skin. Single and multiple doses of UVA and UVB resulted in similar increases in the number of melanocytes (Rosen *et al*, 1987). In *ex vivo* studies using reconstituted epidermal/dermal models, the number of dopa positive melanocytes have been reported to be increased (Berteaux *et al*, 1988; Todd *et al*, 1993; Bessou *et al*, 1995 & 1996; also see **Table 3.18**). Nakazawa *et al.* (1997) reported the number of TRP-1 positive cells were unchanged in their model. UVB increased melanocyte cell number in co-culture models (Staiano-Coico *et al*, 1990; Nakazawa *et al*, 1998). The increase in dopa-positive melanocytes may represent activation of inactive/silent melanocytes rather than proliferation (Mishima & Widlan, 1967). These may be derived from the precursor population of melanocytes which has been indicated with the use of c-KIT (Grichnik *et al*, 1996). Jimbow *et al.* (1975) reported that unstimulated melanocytes can undergo proliferation *in vivo* but that this is very rarely. In the mouse melanocyte, mitosis occurs in reponse to UVB irradiation

Table 3.18 Summary of UV sources, irradiation protocols and results obtained using reconstructed skin composites.

Reference	UV source	UV irradiation protocol No of doses (mJ/cm^2)	Dermis type/ melanocyte type	Melanocyte			Pigmentation		
				Number[a]	Staining[b]	Dendricity	Melanin synthesis[c]	Melanin transfer	Surface level[d]
Todd *et al,* 1993	Philips TLO1 40-W lamp, peak 312nm, 50% emission UVB or Helarium lamps	3 x 158 (UVB)	Prunerias type dermis[e], adult skin melanocytes from different racial donors	↑	↑	↑	NA[f]	=[g]	↑
Archambault *et al,* 1995	Kratos solar simulator, metered at 285 ± 5nm	7 x 10 (UVB)	collagen/fibroblast dermal equivalent, neonatal foreskin melanocytes	NA[h]	NA	↑	↑	↑	NA
Bessou *et al,* 1996	Biotronic lamp (Vilbert Lourmat) with peak 312nm	5 x 150 (UVB)	Prunerias type dermis, adult or neonatal foreskin - skin types I-VI examined	↑[i]	NA	NA[j]	NA[k]	↑	↑[i]
Duval & Schmidt, 1997[l]	Spectronics fluorescent tubes for UVB Oriel Corp Solar simulator for UVA	3 x 80-120 (UVB) 3 x 5-20 (10^3) (UVA)	reconstructed epidermis	NA	NA	NA	↑	NA	↑
Nakazawa *et al,*1997	Spectronics Corp. BLE-8T 312 lamp, peak emission at 312	7 x 100 (UVB)	collagen/fibroblast dermal equivalent, foreskin melanocytes	=[m]	↑	NA[n]	NA	NA	=

Table 3.18:

[a] The number of melanocytes was assessed by TRP-1 and dopa staining in Todd *et al.* (1993), by dopa staining in Bessou *et al.* (1996) and by TRP-1 staining in Nakazawa *et al.* (1997).

[b] The intensity of TRP-1 or dopa staining was assessed as a marker of functional activity (see [a] for which groups used which markers).

[c] Melanin synthesis was assessed by ^{14}C-dihydroxyphenylalanine incorporation in Archambault *et al.* (1995) and by ^{14}C-thiouracil incorporation in Duval & Schmidt (1997).

[d] Surface pigmentation of the epidermal/dermal reconstructs was noted by eye.

[e] The Prunieras type dermis was not supplemented with additional fibroblasts.

[f] NA - parameter was not assessed.

[g] The melanin quantity and distribution remained unchanged although the surface level of pigmentation was increased in response to UVB irradiation.

[h] Melanocyte survival was greatly increased in the presence of live fibroblasts and keratinocytes in response to UV irradiation.

[i] Only skin types II-VI showed increased melanocyte numbers and increased pigmentation at the surface level.

[j] An increase in melanocyte dendricity in response to UVB irradiation was noted in Bessou *et al.* (1995).

[k] An increase of 1.5-5 fold in Causcasians and 1.3 fold in black skin was seen in melanin synthesis (Bessou *et al.* 1995).

[l] This was a poster abstract from the 1997 European Society of Pigment Cell Research, and the details were taken from the poster and abstract.

[m] Although melanocyte number was not increased in this study, a further study by Nakazawa and co-workers indicated that UVB or heat was capable of increasing melanocyte number (Nakazawa *et al*, 1998).

[n] Melanocytes were already dendritic prior to UVB irradiation and further increases in dendricity were not examined.

(Rosdahl, 1978; Rosdahl & Szabo, 1978) but such quantitative data has not yet been achieved for humans.

In vitro, proliferation of melanocytes in response to UVB was halted/reduced and often accompanied with a reduction in cell viability (Friedmann & Gilchrest, 1987; Ramirez-Bosca *et al*, 1992; Abdel-Malek *et al*, 1994b; Barker *et al*, 1995; Nakazawa *et al*, 1998). In this present study, cell number was significantly lower in all three media when cells were irradiated with UVB (**Figure 3.15b-d, Section 3.3.5**). From a comparison of cell number at day 2 in response to sham irradiation and at day 8 in response to UV irradiation, it was concluded that in medium 10, proliferation was halted while in medium 1 and in particular medium 24, cell survival was significantly reduced (**Table 3.7**). ECM aided survival over the first five days only in medium 24 but the effects were reduced following further irradiations. This may be due to remodelling of the ECM by melanocytes (McClenic *et al*, 1989; Varani *et al*, 1989), or detrimental effects of UVB on the ECM proteins. Archambault *et al*. (1995) reported that melanocytes cultured on dermal equivalents in the presence of live fibroblasts had a significantly better survival rate than monolayer cultures of melanocytes. NGF and neurotrophin 3, both produced by fibroblasts (Young *et al*, 1975; Yaar *et al*, 1994 respectively), can act as survival factors when melanocytes are deprived of growth factors (Yaar *et al*, 1994) and may play a role in this effect. Keratinocytes appeared to play a minor role in this process. Fibroblast-derived ECM proteins alone could stimulate proliferation with the greatest effects seen in medium 24 (**Figure 3.15a** and **Figure 3.16a**). However, when fibroblasts were removed with sodium deoxycholate and not water, ECM did not influence proliferation under any of the media conditions (as in **Section 3.3.5**). Very few authors have reported on the effect of ECM proteins on proliferation. Mortarini *et al*. (1995) describe that laminin was not mitogenic for neonatal melanocytes, while bovine endothelial cell-derived ECM increased melanocyte proliferation in the presence of PMA and CT, while it enhanced the adhesiveness of non-proliferating melanocytes (Ranson *et al*, 1988a). Attachment and migration are important parameters, as these relate to melanoma and the initial development of melanocytes from precursor cells although ECM proteins may be seen as a survival signal as noted in this study and by Ranson *et al*. (1988a).

Only one study has shown that UVB at a low dose of 25mJ/cm^2 can stimulate *in vitro* melanocyte proliferation (Libow *et al*, 1988) based on the incorporation of ^{3}H-thymidine. However, the high DNA activity could be due to repair in DNA damage. Physiological doses of UV irradiation induce pyrimidine dimer formation in DNA (Schothorst *et al*, 1991; Noz *et al*, 1994) and DNA strand breaks (Wenczl *et al*, 1997) in cultured human melanocytes. The melanocyte cell cycle was halted in G_2 phase in response to three doses of UVB (Abdel-Malek *et al*. 1994b), while the melanocyte cell cycle was halted in G_1 phase in response to one dose of UVB (Barker *et al*, 1995) although melanocytes eventually resumed proliferation. Tumour suppressor p53 protein which causes arrest at the G_1 phase (Kastan *et al*, 1991; Ullrich *et al*, 1992; Hall *et al*, 1993), was induced (Barker *et al*, 1995; Nakazawa *et al*, 1998) and other proteins involved in the contol of cell cycle progression for example p21 and retinoblastoma protein (pRB) are affected by UVB irradiation (Medrano *et al*, 1995; Pedley *et al*, 1996). The number of cyclobutane pyrimidine dimers in lightly pigmented melanocytes is significantly higher than in darkly pigmented melanocytes (Barker *et al*, 1995) and the prolonged induction of the tumour suppressor p53 seen in lightly pigmented melanocyte would allow more time for the greater amount of DNA damage to be repaired. Heavily pigmented melanocytes had the greater capacitiy to resume proliferation than their lightly pigmented counterparts although the percent survival rate was similar (Barker *et al*, 1995). The DNA repair enzyme, T4 endonuclease V enhanced melanocyte survival rate in response to UVB irradiation (Gilchrest *et al*, 1993). α-MSH was reported to encourage melanocyte proliferation (Abdel-Malek *et al*, 1994b; Im *et al*, 1998) by pushing the cells into the S phase of the cell cycle but did not stop cells from dying as a result of UVB irradiation (Im *et al*, 1998). α-MSH was unable to modulate the UVB-induced levels of cell cycle related proteins (Im *et al*, 1998). Similarly, endothelin-1 treatment of melanocytes enabled them to overcome G_1 growth arrest but was unable to modulate cell-cycle related proteins (Tada *et al*, 1998). Additionally, cell survival following UVB irradiation was enhanced by NGF by increasing the level of bcl-2 protein (Zhai *et al*, 1996) which protects cells from apoptosis. Apoptotic death was induced in melanocytes if antisense bcl-2 was added to UV irradiated cells (Ohashi *et al*, 1996).

UVA was reported at a low dose (1 or 5J/cm^2) to be mitogenic for neonatal foreskin melanocytes of both white and black donors (Yohn *et al*, 1992) although PUVA decreased cell number in UVA dose dependent manner (Mengeaud & Ortonne, 1996). DNA and protein synthesis were decreased (Kao & Yu, 1992). The cytotoxic effects of UVA were less effective in black melanocytes which was correlated to the higher level of melanin (Yohn *et al*, 1992). *In vivo*, PUVA stimulated melanocyte migration (Ortonne *et al*, 1979 & 1980; Cui *et al*, 1991) and proliferation (Cui *et al*, 1991) in vitiligo patients while increased the number of epidermal melanocytes in normal skin (Zaynoum *et al*, 1977; Blog & Szabo, 1979; Zelickson *et al*, 1979). In areas of PUVA induced repigmentation of vitiligo lesions, the number of melanocytes was significantly increased but not to the level of unaffected skin (Kao & Yu, 1992).

3.5.3 MELANOGENESIS.

Dopa oxidase activity and melanin content were the two melanogenic parameters studied in this thesis. An apparent discrepancy between dopa oxidase activity and melanin production was found throughout this study and highlighted in **Sections 3.4.1** and **3.4.2** with changes in dopa oxidase activity generally lower than the changes in the total melanin content. However, only 6 out of the 25 media variants displayed a significant difference between dopa oxidase activity and melanin production when the results were normalised to the values obtained in medium 1 **(Section 3.4.1, Figure 3.22b)**. In response to 10^{-8}M α-MSH, only 3 media variants showed a significant difference between the changes in dopa oxidase activity and changes in melanin content **(Section 3.4.2, Figure 3.22f)**, while in response to 10^{-10}M α-MSH there was no significant difference between the change in dopa oxidase activity and melanin content in 17 media variants **(Section 3.4.2, Figure 3.22e)**. While several authors find good correlation between various methods of measuring melanogenic responses (e.g. Halaban *et al*, 1983; Iwata *et al*, 1990a; Abdel-Malek *et al*, 1993; Abdel-Malek *et al*, 1994a) others have noted discrepancies. Ramirez-Bosca *et al.* (1992) noted tyrosinase activity was lower than the change in melanin content in response to UVB irradiation (see **Table 3.17**) which was similar to this study. Naeyaert *et al.* (1991) reported that tyrosinase mRNA levels did not correlate with the pigment content of cultured human melanocytes (using melanin content as a parameter). Tyrosinase activity *in situ* was less than that in

cell homogenates (Iozumi *et al.* 1993) or reported to differ, in particular when melanocytes were stimulated with CT and IBMX (Zhao *et al.* 1994a). Hunt *et al.* (1994b) found that melanin content was less than tyrosinase activity in response to α-MSH. Additionally, there was no definitive reciprocal relationship between proliferation and melanogenesis reported (**Figure 3.21**) which was less surprising as melanogens such as α-MSH (Abdel-Malek *et al*, 1995), endothelin-1 (Imokawa *et al*, 1996a) and bFGF (Puri *et al*, 1996) have been reported to be both mitogenic and melanogenic.

When examining the effect of media composition on melanogenesis, it was seen that in comparison to the dopa oxidase activity obtained in medium 1 (the most mitogenic medium), media containing BPE and/or bFGF were likely to have a higher dopa oxidase activity (**Figure 3.10b**). The majority of media conditions increased the melanin content of melanocytes (**Figure 3.10c**). Media 20 (containing BPE), 21 (containing PMA) and 23 (containing bFGF) had higher dopa oxidase activity than medium 24 where no mitogens were added, but none of these media had a higher melanin content than medium 24. Media 19 (containing FCS) and 22 (containing CT) had both lower dopa oxidase activity and melanin content than medium 24. It would appear that BPE and bFGF are important melanogenic regulators in this medium. bFGF alone has been shown to increase melanin content and further potentiated with IBMX or CT (Puri *et al*, 1996).

α-MSH at 10^{-10}M had little or no effect on melanocyte pigmentation except in one or two media (**Figure 3.11b-c**). α-MSH at 10^{-8}M and IBMX at 10^{-3}M had little effect on dopa oxidase activity but increased melanin content significantly in several media (**Figures 3.12b-c** and **3.14b-c**). Of the previous authors who have demonstrated melanogenic responses to α-MSH in normal melanocytes, Hunt *et al.* (1994a, b & c) found that melanocytes cultured in the absence of non-physiological mitogens i.e. PMA and CT, responded to NDP-MSH and α-MSH with a sigmoidal dose response curve, while the response to ACTH was biphasic. However, these authors noted that not all cultures responded and suggested that the lack of responsiveness may be linked to hair and skin phenotypes, with melanocytes from red-haired individuals being less likely to respond to α-MSH with a melanogenic response (Hunt *et al*, 1996). The discrepancy

between melanin production and dopa oxidase activity, where α-MSH had a greater effect on tyrosinase activity than on total melanin production (Hunt *et al*, 1994a), was suggested to be due to α-MSH increasing the eumelanin to phaeomelanin ratio (Hunt *et al*, 1995). The skin phenotypes of all melanocytes used in this study were I or II and this may well be relevant to the poor melanogenic response to α-MSH which we noted.

Abdel-Malek *et al.* (1995) using culture methods similar to those described here, showed that, in the presence of 5% FCS, PMA and bFGF, 100nM α-MSH or ACTH were able to increase tyrosinase activity by 70 -450% at maximum. Their experimental medium was very similar to medium 7 in this study, in which a 74% increase in dopa oxidase activity was seen although this was not significant. They found, unlike Hunt *et al.* (1994a, b & c), that both peptides produced sigmoidal dose response curves. The tyrosinase, TRP-1 and TRP-2 levels were also increased (Abdel-Malek *et al*, 1995) in agreement with the results of tyrosinase and TRP-1 reported by Hunt *et al.* (1994a). Maeda *et al.* (1996) using an MCDB153 based medium, supplemented with epidermal growth factor, insulin and 5% FBS found that dopa oxidase activity was increased by approximately 50% by either β-MSH, NDP-MSH or ACTH. McLeod *et al.* (1995) found that their melanocytes only responded to di-MSH (a 123% increase) and ACTH (a 220% increase) when cultured on ECM proteins, but did not respond when cultured on plastic. Medium 21 in the present study produced a very unusual result in that PMA plus α-MSH significantly reduced dopa oxidase activity but significantly enhanced melanin levels. At present we are unable to conclude anything from this result. PMA was found to block or reduce pigmentation by the POMC peptides (Hunt *et al*, 1994b; McLeod *et al*, 1995). In medium 17 containing PMA and CT both dopa oxidase activity and melanin content were reduced. The initial culture medium used by McLeod *et al.* (1995) contained CT and, in several earlier reports where melanocytes failed to show a pigmentary response (Halaban *et al*, 1983; Ranson *et al*, 1988a; Friedmann *et al*, 1990a; De Luca *et al*, 1993) CT was present as a mitogen along with PMA. It is quite feasible that the inclusion of CT and PMA will mask potential melanogenic response to POMC peptides. Certainly, the findings of our study are that only under few, very defined conditions was there any evidence of a melanogenic response to α-MSH but under no medium condition was there a significant increase in *both dopa oxidase* activity and melanin content due to donor to donor

variability. Accordingly, it would be particularly relevant to examine the response to α-MSH under conditions where melanocytes are in contact with keratinocytes in a 3-dimensional architecture (further discussed in **Section 3.5.4**).

α-MSH does not appear to have been used in a skin composite model prior to this study but in a foreskin organ skin culture (Iwata *et al*, 1990a), the responses to α-MSH were mixed and did not depend on the colour of the skin, while Burchill *et al.* (1990) showed that basal levels of tyrosinase were not altered by α-MSH but were by 8-bromo-cAMP or PUVA treatment. Additionally, tyrosinase activity was not altered by α-MSH in human hair bulbs (Burchill *et al*, 1991). However, hypersecretion of α-MSH has been shown to increase skin colour (Pears *et al*, 1992).

From this study, all individual ECM proteins investigated (i.e. collagen I, collagen IV, fibronectin and laminin) were capable of increasing tyrosinase with the most consistent effects seen in medium 1 and medium 24 (**Figure 3.25**) although there was a lot of donor to donor variability (**Table 3.13**). Dopa oxidase activity per well (**Figure 3.24**) or cell (**Sections 3.4.7-3.4.8**) was consistently stimulated by various cell-derived ECM matrices, particularly in medium 24. Fibroblast-derived ECM appeared to have a stronger effect than human endothelial derived ECM. Dermal fibroblast and dermal papilla ECM (Buffey *et al*, 1994) and bovine corneal subendothelial matrix (Ranson *et al*, 1988a) have previously been reported to significantly increase melanocyte pigmentation in media low in mitogen content. Additionally matrix derived from proliferating and differentiated keratinocytes, which would be of relevance to melanocyte *in vivo* behaviour, increased melanocyte pigmentation (Nakazawa *et al*, 1995b). Gospodarowicz *et al.* (1980) noted that proliferation and differentiation of adrenal cortex and granulosa cells was influenced more by cell-derived ECM than by the individual ECM proteins which was seen in this study.

UVB can directly influence melanocyte pigmentation when melanocytes are cultured as one cell population (Friedmann & Gilchrest, 1987; Libow *et al*, 1988; Friedmann *et al*, 1990a&b; Ramirez-Bosca *et al*, 1992; Aberdam *et al*, 1993; Abdel-Malek *et al*, 1994b; Im *et al*, 1994; Roméro *et al*, 1994; Duval & Schmidt, 1997) with increases in pigmentation of up to 3 -5 fold (as summarised for some of these studies in **Table 3.17**).

As can be seen, different UVB/UVA ratios were apparent in the studies, media varied and generally neonatal or adult foreskin were the source of melanocytes. In the present study, dopa oxidase activity was increased by 46% (**Table 3.15**) and melanin content by 20% (**Figure 3.28**), when normal melanocytes cultured in medium 1, were irradiated with 7 doses of 143mJ/cm^2 of UVB. The VM1 melanocyte culture had 3 fold and 1.5 fold increases in melanin in response to the UVB irradiation protocol when cultured in media 1 and 10 respectively, whereas the VM3 melanocytes had a much poorer response (**Table 3.11b**). Similarly, the normal melanocytes had mixed responses and no firm conclusions could be drawn. Only one study has compared the response of normal and vitiligo melanocytes to UVB irradiation in pure culture (Im *et al*, 1994). Adult foreskin melanocytes from normal and vitiligo Korean subjects increased pigmentation by approximately 2.5 fold in response to a repeated dose of 5mJ/cm^2 UVB, which is the mininal erythemal dose for Korean skin.

It is not clear why melanocytes in this study had a low increase in pigmentation. Friedmann *et al.* (1990b) demonstrated a similar increase in melanin content of 46% following 6 irradiations with 180mJ/cm^2 using foreskin melanocytes from children with skin types I and II. However, in two separate studies, melanin was increased by 2.7 fold and up to 4 fold when melanocytes were irradiated with 6 doses of either 260mJ/cm^2 (Friedmann *et al*, 1990a) or 2.4mJ/cm^2 (Friedmann & Gilchrest, 1987) respectively. The change in dose between the three papers by Friedmann and colleagues could be due to a change in the UV source and changes in UVA/UVB ratios (reported in **Table 3.17**). Aberdam *et al.* (1993) documented that repeated doses of UVB did not hold the same potential in increasing pigmentation. They showed that the tyrosinase activity of melanocytes irradiated with 5 and 7 doses of 100mJ/cm^2 was lower than that of 4 and 6 doses. This might explain why the melanocytes in the present study had such a low increase in pigmentation. However, the tyrosinase activity of our melanocytes was higher in response to UVB after 7 doses than 4 doses (as indicated by **Figure 3.27b-d**). Another explanation is the use of skin type I and II melanocytes, which perhaps have a lower level of melanogenesis in response to stimuli, or produce more phaeomelanin which is the lighter pigment. Comparative studies of melanocytes from white and black or Hispanic donors have been reported to have similar n-fold increases in response to UVB (Friedmann & Gilchrest, 1987; Abdel-Malek *et al*, 1994b) but Barker *et al.* (1995)

showed that the melanin content was significantly increased in heavily pigmented melanocytes but not in lightly pigmented melanocytes in response to 1 dose of UVB (11.7-70.1mJ/cm^2). In a composite model, skin type I melanocytes remained weakly stained with dopa following UVB irradiation (Bessou *et al*, 1996).

The effect of UVB on pigmentation has been investigated in the epidermal/dermal reconstruct (Todd *et al*, 1993; Archambault *et al*, 1995; Bessou *et al*, 1995 & 1996; Duval & Schmidt, 1997; Nakazawa *et al*, 1997) and as with the pure culture studies various irradiation protocols are employed (summarised in **Table 3.18**). In this study, the effect of UV induced melanogenesis was not enhanced by the culture of melanocytes on fibroblast-derived ECM proteins (**Section 3.4.8**). However, Nakazawa *et al.* (1995b) reported that slightly different effects in the levels of pigmentation were seen in response to ECM proteins derived from UVB irradiated keratinocytes than sham irradiated keratinocytes. One earlier study by Topol *et al.* (1986) demonstrated melanosome transfer in a skin equivalent model (using a collagen/fibroblast type dermal equivalent). Archambault *et al.* (1995) and Nakazawa *et al.* (1997), using collagen/fibroblast dermal equivalents demonstrated that melanin production and TRP-1 staining increased respectively. Bessou and co-workers, have demonstrated that skin types I-VI can be reconstructed *ex vivo* using a de-epidermised dermis, and that UVB increases macroscopic pigmentation in phototypes II-V (Bessou *et al*, 1995 & 1996). Melanin transfer was increased and the amount of melanin was increased although there appeared to be abnormal melanosome transfer in skin types III-V (Bessou *et al*, 1996). Todd *et al.* (1993) also used a de-epidermised dermis and demonstrated that dopa staining and MEL-5 staining were increased in response to UVB irradiation although melanin was not transferred. Melanin synthesis was increased and corresponded with increased pigmentation at the surface level of the composites in response to varying doses of UVB and UVA (Duval & Schmidt, 1997). The intensity of dopa staining increased in skin exposed to single (Pathak *et al*, 1965; Rosen *et al*, 1987) and multiple doses of UVB or UVA (Rosen *et al*, 1987). The presence of tyrosine positive melanocytes were only detected following UVA or UVB stimulation (Pathak *et al*, 1965; Rosen *et al*, 1987).

PUVA causes repigmentation in many vitiligo patients. *In vivo* studies indicate that PUVA works indirectly or directly on a melanocyte reservoir in the hair follicle (Ortonne *et al*, 1979 & 1980, Cui *et al*, 1991; Kao & Yu, 1992). *In vitro*, cutaneous melanocyte melanogenesis was increased (Kao & Yu, 1992; Mengeaud & Ortonne, 1996) by PUVA treatments (see **Table 3.17**). Kao & Yu (1992) noted that the increase in tyrosinase activity was not UVA dose dependent with a maximal increase of 35% seen at 62mJ/cm^2 and not at the highest dose used of 124mJ/cm^2. The alteration in protein levels of tyrosinase, TRP-1 and TRP-2 (shown in **Table 3.17**), indicated that PUVA favoured the pathway of the darker, more protective eumelanin (Mengeaud & Ortonne, 1996). This might explain why PUVA was reported to provide a more protective tan than UVB (Gschnait *et al*, 1978). PUVA may be working through the activation of PKC by psoralen-fatty acid adducts (Anthony *et al*, 1997). PUVA also increased pigmentation in skin organ cultures from patients with skin types III and IV (Burchill *et al*, 1990). When 8-bromo-cAMP was added, there was futher increase in the pigmentation achieved. No studies of PUVA effects on *in vitro* cultured hair follicle melanocytes appear to have been carried out yet. However, as PUVA treatment seems to work on hair follicle melanocytes, it would seem appropriate that for vitiligo, UV irradiation studies are important. Successful culture of hair follicles has only been reported in the past few years (Tobin *et al*, 1995; Tobin & Bystryn, 1996) but the basics are there for these studies. The presence of amelanotic/inactive melanocytes in hair follicles was first reported by Staricco (1959) but confirmed by several groups (Ortonne *et al*, 1979 & 1980; Cui *et al*, 1991; Horikawa *et al*, 1996b; Tobin & Bystryn, 1996) and can be stimulated to pigment via a variety of mechanisms such as dermabrasion (Staricco, 1961) and UV irradiation (Staricco & Miller-Milinska, 1962).

The mechanism behind UV-induced melanogenesis has still not been fully elucidated. It is clear from the pure culture studies that UV irradiation can influence melanocyte behaviour directly. The treatment of melanocytes with the DNA repair enzyme T4 endonuclease V enhanced UV-induced melanogenesis measured through melanin content, tyrosinase activity and ^{14}C-dopa incorporation (Gilchrest *et al*, 1993), the addition of thymidine dinucleotides, which mimic products that would be excised during DNA repair, stimulates melanocyte pigmentation (Eller *et al*, 1994; Pedeux *et al*, 1998) and DNA damage itself is known to increase pigmentation (Eller *et al*, 1996).

DAG derivative 1-oleoyl-2-acetyl-glycerol (OAG) had been implicated in the regulation of UV induced pigmentation (Friedmann *et al*, 1990b; Carsberg *et al*, 1994; Carsberg *et al*, 1995b) due to its ability to stimulate melanogenesis (Gordon & Gilchrest, 1989, Friedmann *et al*, 1990b). The production of DAG is biphasic in reponse to UV irradiation in both melanocytes and keratinocytes (Carsberg *et al*, 1995b), and may exert the effects via a PKC independent pathway (Carsberg *et al*, 1994) which may be modulated in part by intracellular calcium (Carsberg *et al*, 1995a). Im *et al*. (1998) confirmed that PKC pathways were not involved in regulating UVB-induced melanogenesis. Vitamin D_3 (cholecalciferol) but not pro-vitamin D_3 (7-dehydrocholesterol) or activated vitamin D_3 (1α, 25-hydroxy-vitamin D_3) increased immunoreactive tyrosinase and dendricity (Tomita *et al*, 1986) and was proposed to play a role regulating melanogenesis following UV irradiation. However other studies also using human melanocytes were less convincing (Mansur *et al*, 1988; Ranson *et al*, 1988b).

Media composition has been previously noted to play a role in the level of pigmentation achieved in response to UVB irradiation (Friedmann & Gilchrest, 1987; Ramirez-Bosca *et al*, 1992). Friedmann & Gilchrest (1987) found that when the basal medium was M199, melanocytes did not respond with increased pigmentation, but did if the basal medium was DME. Ramirez-Bosca *et al.* (1992) reported that melanocytes cultured in a MCDB/BPE medium achieved a higher tyrosinase activity than melanocytes cultured in a F10/PMA medium although the level of melanin content was similar. Both these media contained IBMX, CT and FCS. In the present study, melanocytes had the greatest tyrosinase response in a mitogen rich medium (medium 1) that contained BPE, FCS, PMA and CT. In media where CT was removed (media 10 and 24) the response was much reduced. Im *et al*. (1998) recently noted that if a cAMP modulator was not present in the media, UVB irradiation actually inhibited melanocyte pigmentation. α-MSH or dbcAMP increased melanogenesis which was enhanced by UVB irradiation. This was the first report that UV-induced melanogenesis may be modulated through the PKA pathway in human melanocytes. A previous report had found no effect of α-MSH on UV induced melanogenesis (Friedmann *et al*, 1990a). α-MSH has been reported to be produced in the epidermal cells with increases due to UV-irradiation (see **Section 3.1**). MC1R expression and the binding capacity of the receptor was increased by UVB

(Funasaka & Ichihashi, 1997; Funasaka *et al*, 1998) and in a parallel manner by adult T cell leukemia-derived factor (ADF), an antioxidant which is produced in keratinocytes in response to UVB (Funasaka & Ichihashi, 1997). KCM increased melanin production by 7 fold (Gordon *et al*, 1989) and keratinocyte-derived factors increased by UV such as endothelin-1 (Imokawa *et al*, 1992; Imokawa *et al*, 1995; 1996), and GM-CSF (Imokawa *et al*, 1996b) are known to increase melanogenesis in cultured melanocytes. The expression of the MC1R was recently shown to be increased by endothelin-1 (Funasaka *et al*, 1998; Tada *et al*, 1998). The melanogeneic effects of endothelin-1 are accomplished through complex cross-talking of the PKA and tyrosine kinase pathways (Imokawa *et al*, 1996a). The level of tyrosinase and TRP-1 mRNA are increased in response to endothelin-1 and *in vivo* endothelin-1, IL-1α and tyrosinase mRNA signals were increased in response to UVB irradiation (Imokawa *et al*, 1995).

A new evolving theory is that NO regulates UV-induced melanogenesis through the cGMP pathway (Roméro-Graillet *et al*, 1996; Roméro-Graillet *et al*, 1997). This work was begun following observations of UVB induced erythema through NO release (Warren, 1994). NO was increased in UVA and UVB irradiated keratinocytes (Roméro-Graillet *et al*, 1997) and both NO and cGMP were capable of increasing melanogenesis (Roméro-Graillet *et al*, 1996). cGMP and NO synthase inhibitors have been shown to inhibit UV induced pigmentation (Roméro-Graillet *et al*, 1996) and UV KCM (Roméro-Graillet *et al*, 1997). The protein levels of tyrosinase and TRP-1 were increased (Roméro-Graillet *et al*, 1997). Abnormal increases in NO production could lead to an imbalance in the skin antioxidant status and hence a destruction in melanocytes in UV-exposed regions.

3.5.4 THE EPIDERMAL/DERMAL RECONSTRUCT MODEL.

An epidermal/dermal reconstruct (described as a skin composite) has been developed in Dr S Mac Neil's laboratory over the past 5 years for use in the treatment of patients with burns and leg ulcers. It was noted that spontaneous pigmentation was extremely rare and in many of the sections examined from these composites, the number of melanocytes detected was minimal (i.e. ranging from 1 to 10 for the 4 micron section across a 1 cm diameter of composite) (personal communication, S Mac Neil and C Layton, 1998). Skin types I and II were used in composite construction. As this model

was being further prepared to study the interactions of keratinocytes and melanocytes with respects to pigmentation and vitiligo, additional melanocytes were added at a ratio of 20 keratinocytes to 1 melanocyte. Previous studies have shown that melanocyte number is regulated within composites (Haake & Scott, 1991; Scott & Haake, 1991; Todd *et al*, 1993; Franchi *et al*, 1994). Spontaneous pigmentation of varying degrees was achieved within 7 days with the three control composites obtaining a mild pigmentation (**Figure 3.30a-c**, composites labelled A, B, K). This spontaneous pigmentation did not further increase over the following 6 days (**Figure 3.30d**). Bessou *et al.* (1995) reported that spontanous pigmentation was achieved with Caucasian donors when melanocytes were seeded at 1 to 10 keratinocytes, but not at the lower ratios of 1:20 and 1:40. In a second study, composites prepared with skin type III-VI showed spontaneous pigmentation resembling the *in vivo* situation (Bessou *et al*, 1996). Regniér *et al.* (1997) noted that their composites were pigmented using melanocytes at 1:10 ratio. Melanocytes stained with TRP-1 and other melanogenic markers without additional stimuli indicated that they were functionally active (Todd *et al*, 1993; Bessou *et al*, 1995, 1996, 1997a & c; Nakazawa *et al*, 1997 & 1998), while dopa oxidase activity and melanin synthesis were noted in control composites (Franchi *et al*, 1994). The predictability of composite pigmentation had been investigated with regard to obtaining improved cosmetic results in wound healing situations. This involved the separation of melanocytes from keratinocytes and then adding melanocytes back at a specific ratio (Swope *et al*, 1997).

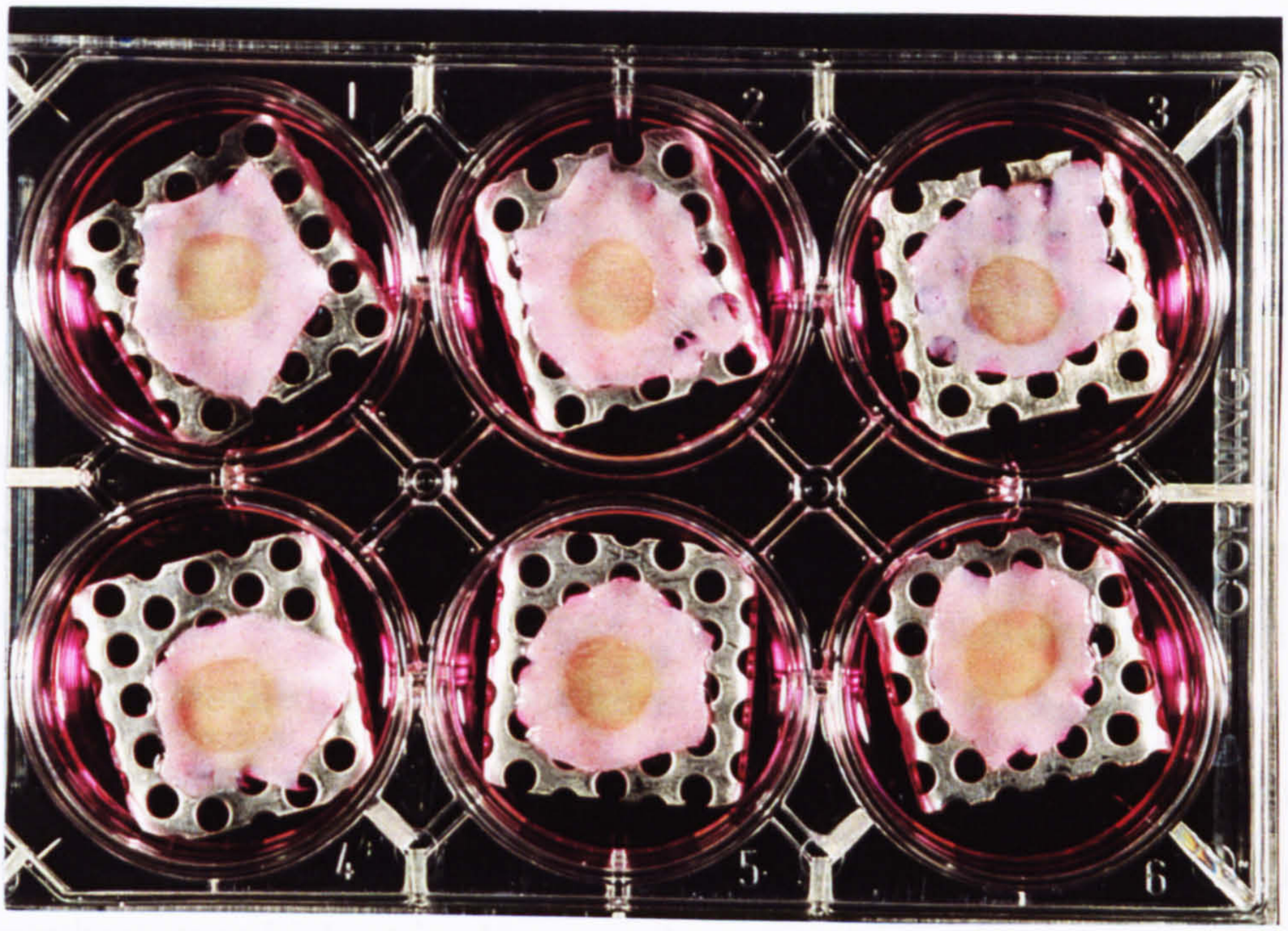
α-MSH had no effect on the pigmentation of the composites in this study (**Table 3.16b&c; Figure 3.30d**, composites C, D, I). While the level of melanin was higher in composites cultured in the presence of CT (**Table 3.16c**) there was no appreciable alteration in the surface pigmentation (**Figure 3.30d**, composites E, F, G). A similar result was noted by Nakazawa *et al.* (1997), who found that in response to UVB irradiation there was no appreciable change in pigmentation at the surface of their composites, but that the intensity of TRP-1 staining increased, indicating an increase in melanocyte activity. The lack of response in any of the parameters for α-MSH could be due to Green's medium containing CT for the initial period of culture although it was removed during the period of α-MSH addition. Additionally, there might be a change in the type of melanin produced rather than in the level of melanogenesis in response α-

MSH. Hunt *et al.* (1995) noted that melanocytes cultured *in vitro* altered from eumelain to phaeomelanin production in response to α-MSH. This may also be a reason why no change was seen at the surface of the composite in response to CT. Other groups that work with these composite type models use media that do not contain CT and in future this is to be explored. The structure of the nine composites which had fibroblasts, keratinocytes, melanocytes and a basement membrane present were well defined, with good keratinocyte layers, and cornified layer (**Table 3.16a**, composites A-G, I, K). The melanocytes were evenly spaced throughout the basal layer and the presence of a well-defined basement membrane was indicated by staining for collagen IV (**Figures 3.31d, h, l**).

While several groups have studied the effect of UVB on pigmentation in the composite models (Todd *et al*, 1993; Archambault *et al*, 1995; Bessou *et al*, 1995 & 1996; Duval & Schmidt, 1997; Nakazawa *et al*, 1997 & 1998) (discussed further in **Section 3.5.3**) only one study has reported the use of the model to to explore known pigmentary and depigmentary chemicals (Bessou *et al*, 1997c) with expected results. IBMX and OAG increased the surface pigmentation, with correlations in increased number of dopa-positive melanocytes and increased melanin transfer. IBMX also increased melanocyte dendricity. Kojic acid and mequinol led to decreased pigmentation with decreased melanin transfer. Melanocytes treated with kojic acid were non dendritic and severely damaged, while mequinol reduced the number of dopa-positive melanocyte counts. It was concluded that the model was important for the examination of such agents.

Fibroblasts were not present in some of these epidermal/dermal composites (Todd *et al*, 1993; Bessou *et al*, 1995, 1996 & 1997b & c; Regniér *et al*, 1997) while fibroblasts were added to the dermal substitute models of Archambault *et al.* (1995) and Nakazawa *et al.* (1997). The importance of fibroblasts in melanocyte survival in response to UV irradiation has been illustrated (Archambault *et al.* 1995). In the model from Dr S Mac Neil's laboratory, an interesting pigmentary phenomenon was noted by chance while the composites were being examined for contraction in the presence and absence of fibroblasts (additional melanocytes had not been added to these composites). The level of pigmentation was increased as the number of fibroblasts was decreased or completely absent (illustrated in **Figure 3.33,** results shown with permission from Dr K

Figure 3.33 Influence of fibroblasts on pigmentation in an epidermal/dermal reconstructs in the absence of added melanocytes. Composites were prepared as described in **Section 2.4,** but no additional melanocytes were added. Composites were cultured at the air-liquid interface for 10 days before photographs were taken by Mr R Salthouse (Medical Illustration Department, Northern General Hospital). Composites with (**a**) and without (**b**) fibroblasts are shown. This work was carried out by Mr K Chakrabarty who kindly allowed these findings to be presented.



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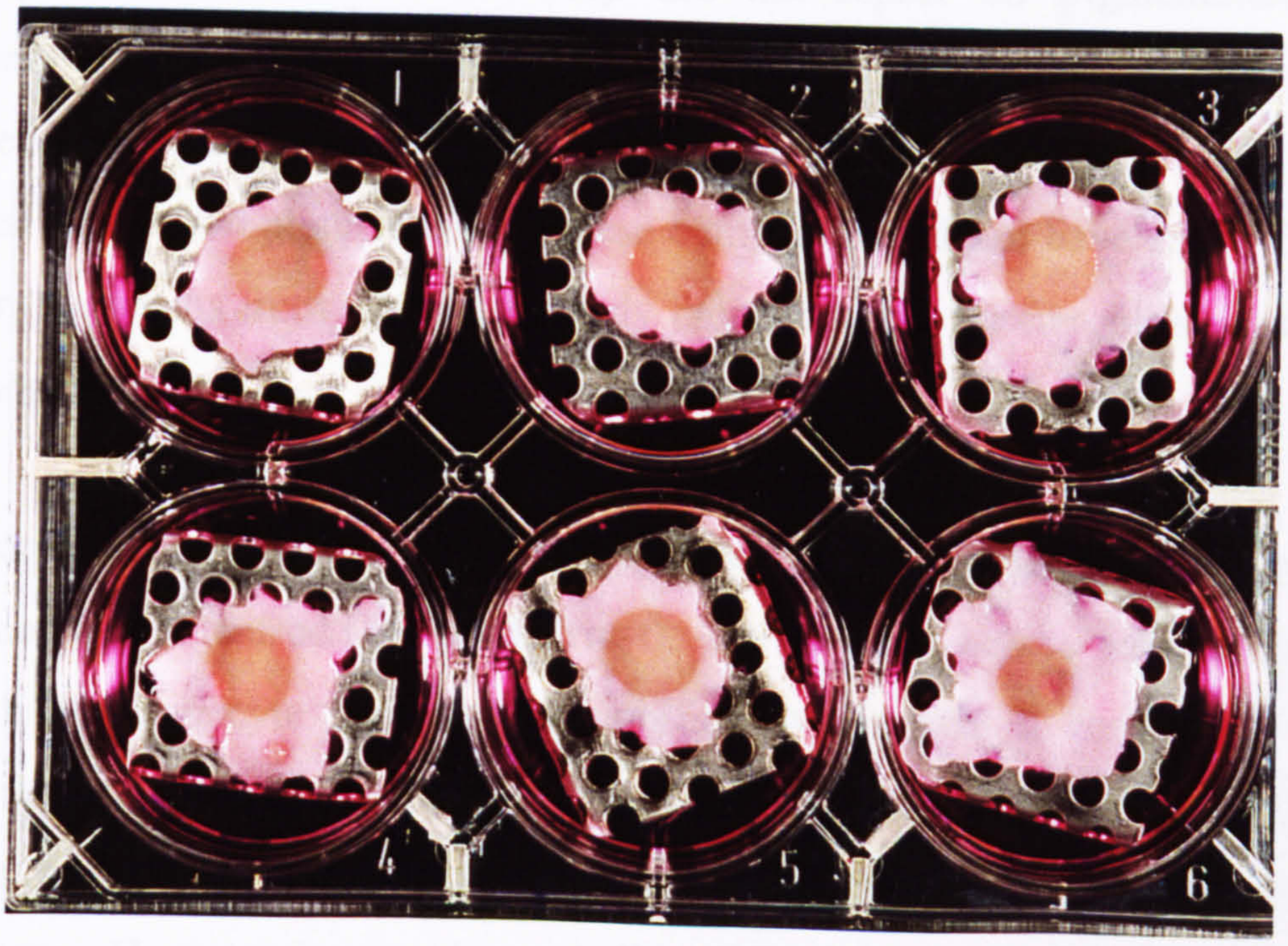


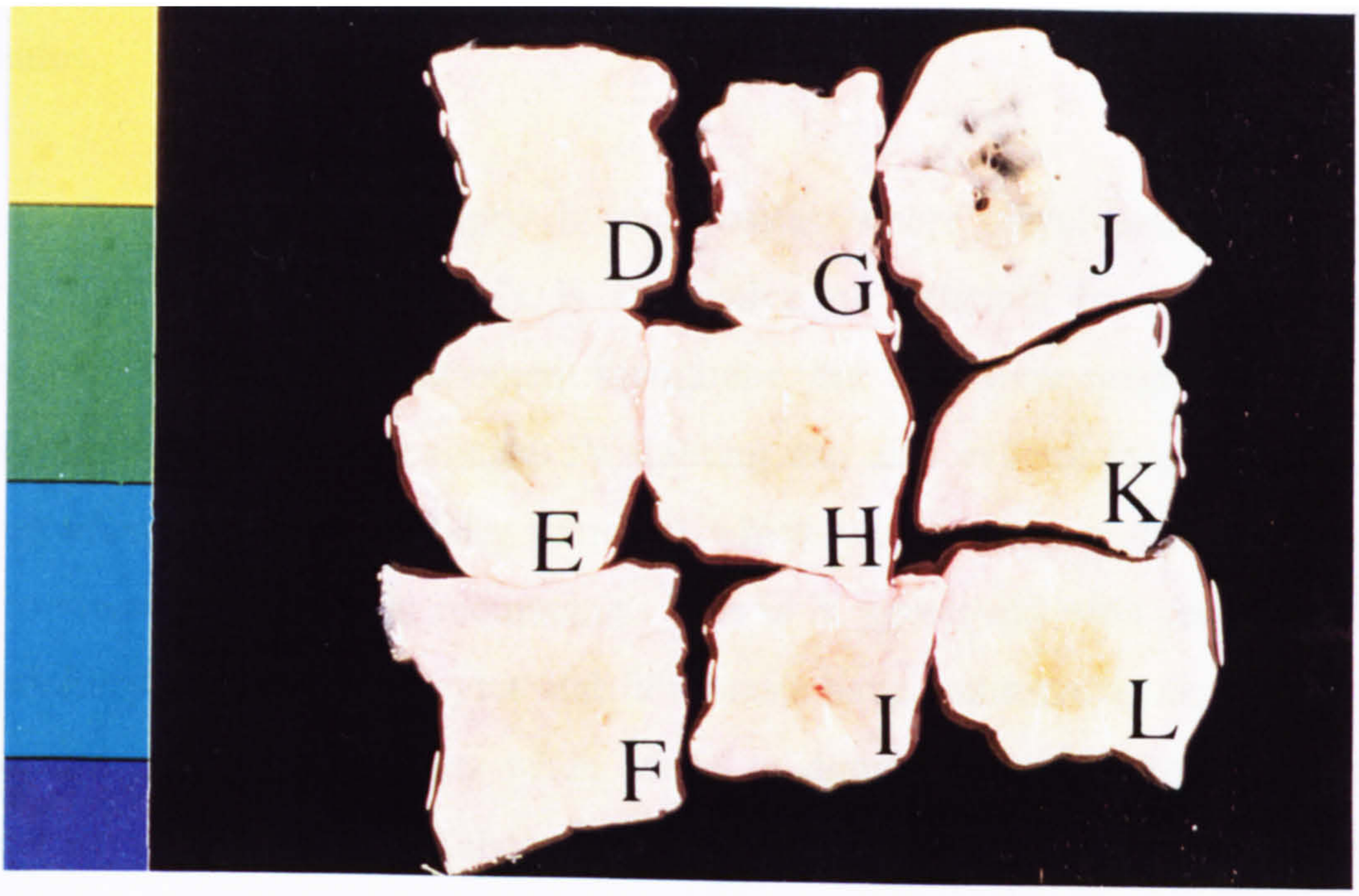
b

Chakrabarty, who was conducting the contraction study). These results have been repeated in 4 experiments (personal communication, K Chakrabarty, 1998). In the present study the level of pigmentation was dramatically increased when fibroblasts or the basement membrane were absent in composites with added melanocytes (**Figure 3.30**, composites labelled M-P in the absence of fibroblasts; H, J, L in the absence of basement membrane). The influence of basement membrane was examined as fibroblasts contribute to this. The histological detection of melanin however was not increased in comparison to the control in the three composites where the basement membrane was initially removed with dispase (**Table 3.16d**). This could be explained by the shedding of layers containing pigmentation during preparation for histological evaluation. In a recent experiment conducted during the writing-up period, the pigmented layer became detached as the experiment was ended in epidermal/dermal reconstructs lacking basement membrane. The surface pigmentation is shown in **Figure 3.34** (composites labelled) and composites in the absence of fibroblasts also showed a slight increase in pigmentation (composites labelled) (histological evaluation not completed in time for submission of thesis). Archambault *et al.* (1995) reported that melanin synthesis was highest in skin equvalents containing melanocytes in addition to keratinocytes and fibroblasts, but they did not report on pigmentation in skin equivalents with lysed fibroblasts or in the absence of fibroblasts. As previously mentioned when Bessou *et al.* (1996) prepared composites from skin types I-VI these resembled the *in vivo* pigmentation. These composites did not have added fibroblasts. However, the results from the present study indicate a role for fibroblasts in the control of melanogenesis.

The structures of the composites that lacked fibroblasts or basement membrane were less defined and organised (**Figure 3.31m-t**). Keratinocyte layers were reduced in both types, while melanocyte number was significantly reduced in composites that were initially stripped of basement membrane (**Table 3.16d**). This would imply that the basement membrane proteins are important in controlling the positioning of the melanocytes. However in co-culture studies of De Luca *et al.* (1988a & b), it would seem that the keratinocytes were also important in controlling the positioning of the melanocytes. i3T3 cells were present in these studies and therefore may in part be controlling the positioning of the melanocytes. However, in the cultured epithelial

Figure 3.34 The influence of fibroblasts and basement membrane on pigmentation in an epidermal/dermal reconstruct: macroscopic examination. Composites were prepared as described in **Section 2.4**, but cholera toxin was omitted from the Greens medium. Composites were cultured at the air liquid interface for 14 days before photographs (i) and (ii) were taken by Mr R Salthouse (Medical Illustration Department, Northern General Hospital). A key is shown below to illustrate the presence (+) and absence (-) of melanocytes, keratinocytes (K), fibroblasts (FIB) and basement membrane (BM).

Composite label	Melanocytes	Keratinocytes	Fibroblasts	Basement membrane
A-C	+	-	+	+
D-F	-	+	+	+
G-I	+	+	+	+
J-L	+	+	-	+
M-O	+	+	+	-
P-R	+	+	-	-



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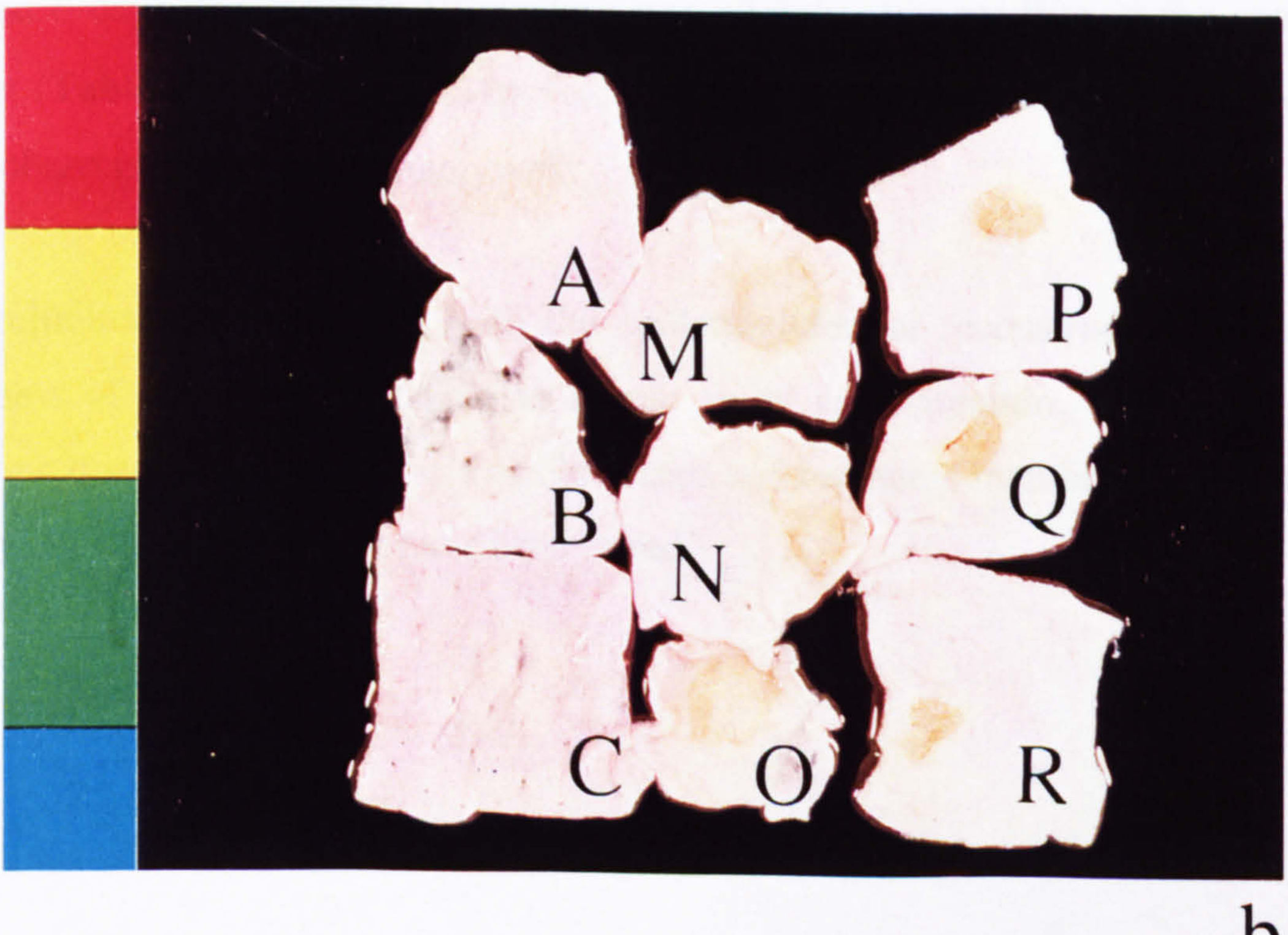

b

autografts (CEA) composed of mucosal epithelial cells, melanocytes were not located in the basal layer, but were in the presence of cutaneous keratinocytes (De Luca *et al*, 1988a). Additionally, Haake & Scott (1991) demonstrated that fetal and neonatal keratinocytes played an important role in the positioning of melanocytes in epidermal/dermal constructs. Basement membrane would be present in these composites.

Collagen IV staining was patchy in composites where fibroblasts were removed (**Figures 3.31** and **3.32b**). This is suggestive of attempts by keratinocytes and melanocytes to remodel the basement membrane, but they were unable to achieve the normal morphology. Some collagen IV staining was also evident in composites where basement membrane was initially removed which would suggest that the cells were trying to renew the basement membrane. This is in agreement with recent work from this laboratory where keratinocytes and fibroblasts have commenced *de novo* synthesis of basement membrane proteins when added to dermis lacking basement membrane (personal communication, D Ralston and S Mac Neil, 1998). In the two composites without an initial basement membrane to which α-MSH was added to the medium for the final 6 days, the collagen IV staining was slightly stronger than in the composite without (**Table 3.16a, Figure 3.31p** +α-MSH, **Figure 3.32b** -α-MSH). However this initial observation requires further work.

The preliminary data obtained with the epidermal/dermal reconstructs illustrates the usefulness of the model in trying to mimic an *in vivo* situation. From this study, intriguing questions about the role of fibroblasts and basement membrane in melanocyte positioning and pigmentation have been raised.