IGF axis expression and activity in differentiating dental pulp cells

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Declaration

‘The PGR confirms that the work submitted is his own, except where work which has formed part of jointly authored publications has been included. The contribution of the PGR and the other authors to this work has been explicitly indicated below. The PGR confirms that appropriate credit has been given within the thesis where reference has been made to the work of others’.

“Data in Figures (20,25,26 and 27) were produced in collaboration with Dr.Claus Oxvig at the University of Arhus in Denmark. ”

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Abstract

The insulin-like growth factor (IGF) axis comprises two growth factors (IGF-I and IGF-II), two cell surface receptors (IGF1R and IGF2R), six high affinity soluble IGF binding proteins (IGFBP1-6) and various families of IGFBP proteases. The IGF axis acts co-ordinately to control several cellular processes including mitogenesis, apoptosis, cell migration and differentiation. In this latter respect the IGFs are the most abundant growth factors present in bone matrix and the IGF axis is believed to play an important role in the differentiation of osteoblast precursors and also to control bone accretion and resorption which occurs throughout adult life. There is much interest in the use of mesenchymal stem cells (MSCs) as a resource for tissue engineering approaches in the restoration of bone and other hard tissue lesions. As such a thorough knowledge of the effects of the IGF axis on osteogenic differentiation is essential to the success of such tissue engineering approaches. In our laboratories we use dental pulp/stromal cells (DPCs) as a source of precursor cells which can be differentiated to an osteogenic phenotype by culture under appropriate conditions. In order to address some of the issues raised above we have examined the expression and activity of the IGF axis in DPCs which have been induced to differentiate to an osteogenic/matrix mineralising phenotype *in vitro.* We found that DPCs express all components of the IGF axis (except IGF-I and IGFBP-1) under both basal and osteogenic conditions. With respect to IGFBPs we report that IGFBP protein concentrations in DPC conditioned medium closely follow mRNA levels prepared from DPCs. IGFBP-4 is the most abundant IGFBP in both basal and osteogenic DPCs and levels are not altered between basal and osteogenic cultures. IGFBP-4 and -5 inhibit IG-I and IGF-II stimulated osteogenic differentiation in DPCs. Of some interest although IGFBP-4 expression is not altered by differentiation of DPCs IGFBP-4 proteolysis is enhanced under osteogenic conditions. Subsequently we found that pregnancy associated plasma protein-A (PAPP-A) is the sole IGFBP-4 protease in DPC conditioned medium and its concentration is increased under osteogenic conditions. Further IGF-II (an activator of PAPP-A) and stanniocalcin-2 (STC2-

an inhibitor of PAPP-A) concentrations are respectively increased and decreased during osteogenic differentiation of DPCs. We suggest that our data may represent a series of co-ordinated changes in IGF axis expression and activity which represent a novel osteogenic “signature” of differentiating DPCs.

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Abbreviations

ALS: acid labile subunit

ALP: alkaline phosphatase

BMD: bone mineral density

BSA: bovine serum albumin

Ca-P: inorganic calcium-phosphate

CM: conditioned medium

DFSCs: dental follicle stem cells

DPCs: dental pulp/stromal cells

DPSCs: dental pulp stromal/stem cells

ECM: extra cellular matrix

FBS: Foetal bovine serum

GAGs: glycosoaminoglycans

HBD: heparin binding domain

hDPSCs: human dental pulp stromal/stem cells

hOB: human osteoblast

HRP: horseradish peroxidase

IGF-I: insulin-like growth factor-1

IGF-II: insulin-like growth factor-2

IGF1R: type 1 insulin-like growth factor receptor

IGF2R: type 2 insulin-like growth factor receptor

IGFBPs: insulin-like growth factor binding proteins.

IR: insulin receptor

MAPK: mitogen activated protein kinase

MMP: Matrix metalloproteinase

MSCs: mesenchymal stem cells

OCN: osteocalcin PAPP-A: pregnancy-associated plasma protein-A

PDLSCs: periodontal ligament derived stem cells

PI3K: phosphoinositide 3 kinase

pNPP: para-nitrophenylphosphate

PTM: post-translation modifications

PTEN: Phosphatase and tensin homolog

PI3K: phosphatidylinositol-3-kinase

proMBP: proform of eosinophil major basic protein

RUNX-2: runt-related transcription factor

STC2 stanniocalcin-2

TGF-β: Transforming growth factor-beta

Chapter 1 Introduction

1.1 General Introduction

The current study is concerned with some aspects of the biology of cells derived from dental pulp tissue (dental pulp cells – DPCs). In particular we will discuss a series of studies which examines the differentiation of DPCs into cells displaying characteristics of an osteogenic phenotype with the ability to secrete a mineralising matrix. Our focus will be to extend our previous studies on the role of the insulin-like growth factor (IGF) axis on the regulation of this process [1-3] and to describe how novel co-ordinated transcriptional and post-transcriptional changes in the expression and activity of the genes associated with the IGF axis affect the osteogenic differentiation of DPCs. We propose that such changes represent an "osteogenic signature" characteristic of differentiating DPCs and discuss how further understanding of such local growth factor signalling cues may assist in the development strategies for the use of DPCs in hard tissue engineering programmes. We begin our literature review with a discussion of some relevant aspects of dental pulp and DPC biology.

1.2 Dental Pulp Biology

1.2.1 Development of dental pulp tissue

Dental pulp tissue is derived from cells of both ectodermal (neural crest) and mesodermal (mesenchymal) origin [4] During the "bell" stage of tooth development (**Fig 1**) neural crest derived cells develop into an odontoblast (O) layer towards the periphery of a condensed mesenchymal dental papilla tissue (P) which will form the remainder of the mature dental pulp tissue. Under the influence of humoral factors secreted by the outer enamel epithelium (OEE) the closely opposed odontoblast layer secretes pre-dentin (PD) which in due course will develop into mature tertiary dentin. Pre-ameloblasts (A) derived from the OEE at this stage secrete an early form amelogenin matrix which is juxt-opposed to the pre-dentin layer. The outer and inner enamel epithelial layers meet at the cervical loop (CL) and downward growth of this structure as Hertwig's sheath represents the precursor of the root structure.

Anatomical relationships amongst the various cell types at this stage of tooth development are represented in **Fig 1.**

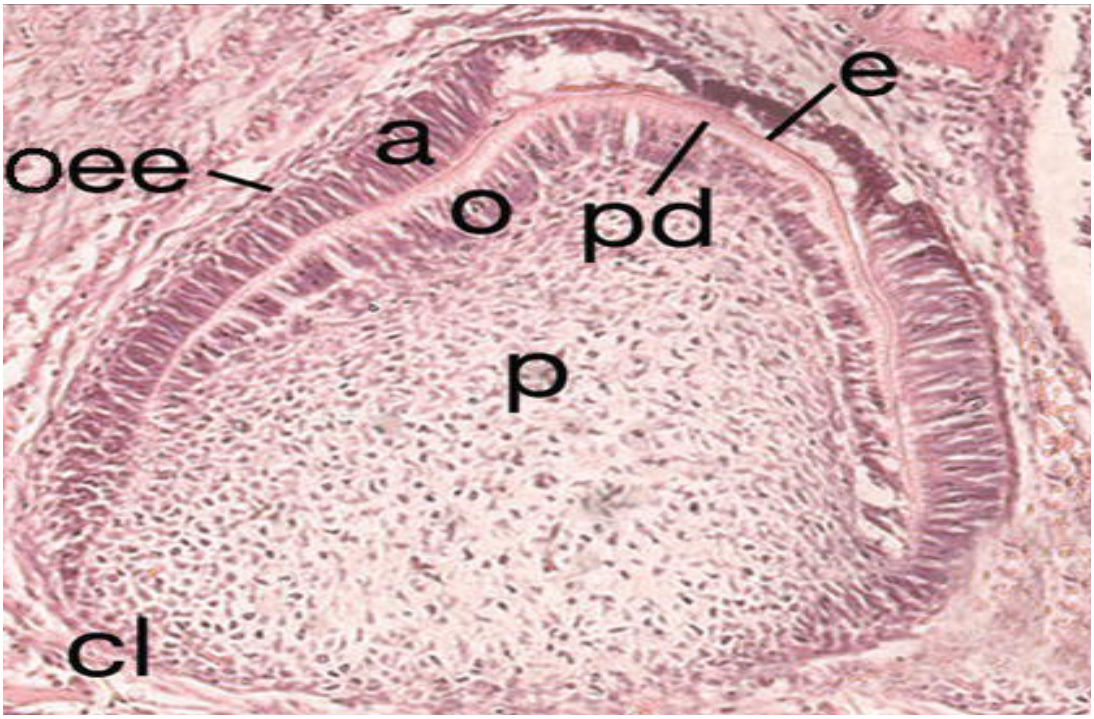


Figure 1 Cell types and anatomical relationships during the bell stage of tooth development

oee - outer enamel epithelium; a – ameloblast layer; o – odontoblast layer; pd – predentin; p-pulp; cl – cervical loop See text for further details. Adapted from https://pocketdentistry.com/wp-content/uploads/285/c03f00412.jpg.

1.2.2 Dental pulp stem cells (DPSCs)

The presence of stem cell populations within the dental pulp was first described at the beginning of this century when transplantation of DPSCs into immunocompromised mice along with hydroxyapatite/tricalcium phosphate (HA/TCP) carrier led to the formation of dentin like structure *in vivo* [5]. Encouragingly these structures recapitulated some of the structural details of dentinal pulp present *in situ* including a peripheral odontoblast-like layer surrounding a rudimentary dental pulp. Following these initial observations DPC populations were further characterised by our group and others in respect of the expression of typical stem cell surface markers [2, 6, 7]. This characterisation was typically obtained through fluorescence activated cell sorting (FACS) analysis with +ve selection for markers such as

CD29, CD90, CD105, CD146, CD166 and CD271 and –ve selection of haematopoietic stem cell specific markers CD34 and CD45. On occasion expression of pluripotent stem cell markers such as NANOG and OCT4 in DPC populations has also been confirmed by techniques such as qRT-PCR [8, 9]. Although the proportion of putative stem cells in heterogeneous populations of DPCs has varied widely amongst different studies [10-12] DPSCs are perceived to have advantages over stem cells derived from other tissue compartments. For example, they can be easily isolated from the pulp of extracted teeth (typically discarded in the clinic) grown then expanded and stored for future use. On isolation DPCs demonstrate a fibroblast-like morphology and proliferate quickly during culture and following passage (**Fig 2**) although as a caveat there are very few studies which have systematically examined retention of pluripotency during serial passage of cells. However, in combination with tooth banking programme such an approach provides a source of cells for autologous regenerative strategies. In addition to adult dental pulp stem cells have been isolated from other dental tissues including periodontal tissue (PDLSCs) [13], apical papilla (SCAP) [14], dental follicle (DFSCs) [15], pulp tissue isolated from and deciduous teeth (SHED) [16] see **Fig 3**. As all of these tissues share a mixed ectoderm/mesoderm embryonic

origin, they share many of the properties of pluripotent embryonic stem cells and can differentiate into several different phenotypes under appropriate culture conditions [17, 18].

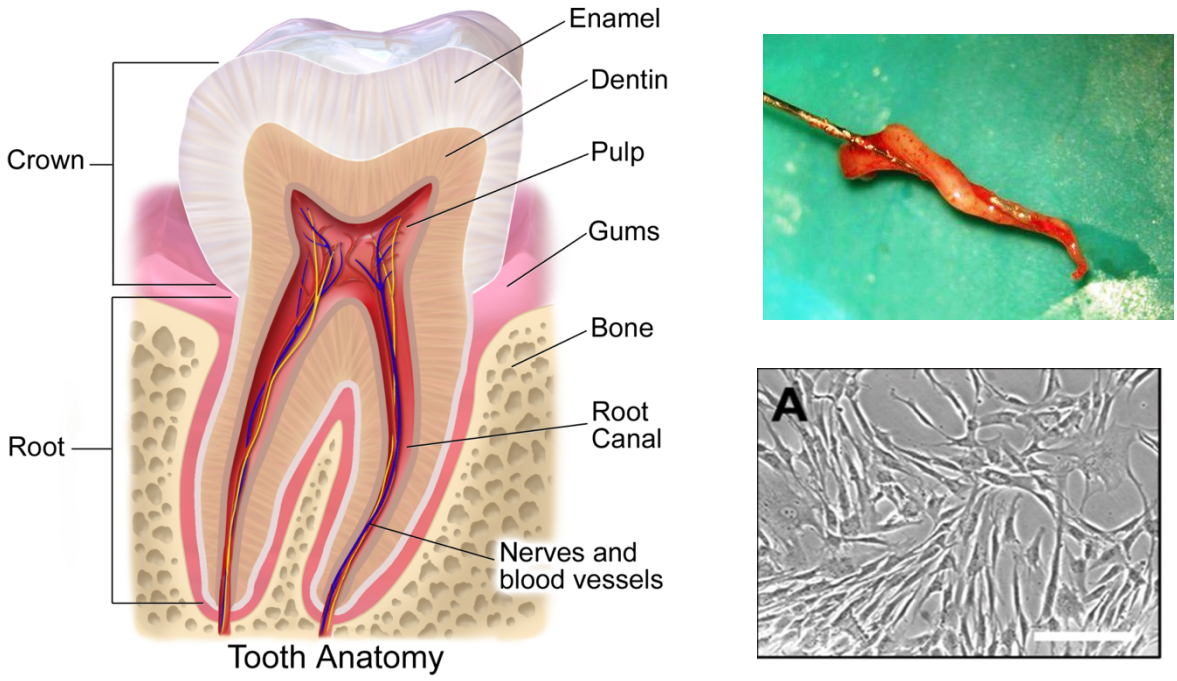


Figure 2 Anatomical relationship of dental pulp with other structures in adult tooth

(left) Appearance of dissected dental pulp tissue.

Adapted from https://www.capstonedental.com.au/blog/ask-the-dentist-sensitive-teeth

(upper right) appearance of fibroblast-like undifferentiated DPCs.

Adapted from https://imgur.com/gallery/Q2Qrq/comment/546728917

(lower right) isolated following tissue digestion.

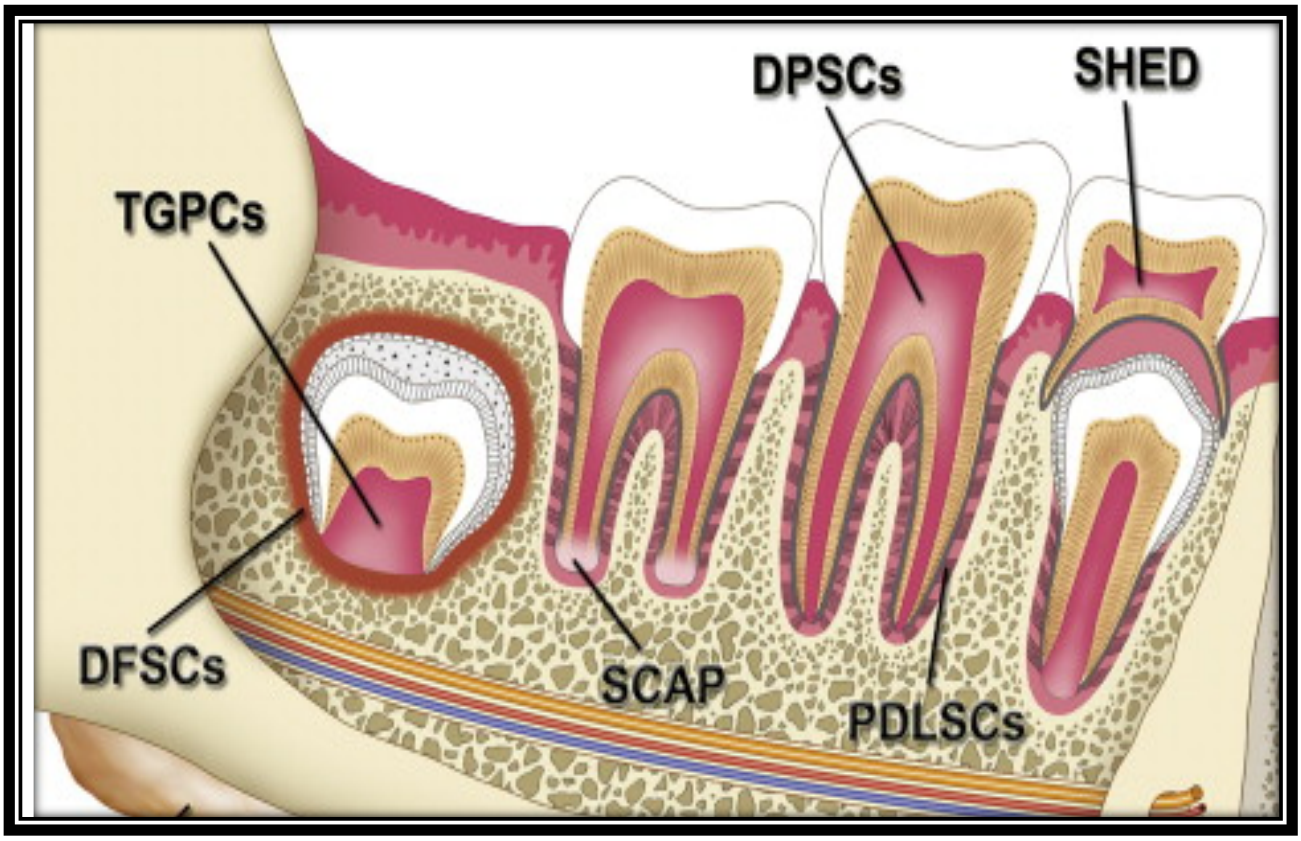


Figure 3 Location of stem cell niches in the oral cavity

DPSCs- dental pulp stem cells; PDLSCs - periodontal ligament derived stem cells; SHED - stem cells from exfoliated dental pulp; SCAP- stem cells from apical papillae; DFSCs - dental follicle stem cells; TGPCs - tooth germ progenitor derived stem cells.

(Adapted from: http://www.slideshare.net/hamedgholami104/dental-stem-cells-18007596)

1.2.3 Differentiation of DPSCs

The embryonic origin of dental tissues is from both oral ectoderm and neural crest derived mesenchyme and because of this these tissues (including dental pulp) contain populations of stem cells with a pluripotent potential similar to that that displayed by embryonic stem cells. Accordingly under appropriate culture conditions they can differentiate into many different phenotypes [17, 18] see **Fig** 4. Similarly we and others have identified the cell surface expression of stem cell markers of the surface of DPCs [19-21].This work also characterised these cell populations as adherent to plastic with a high proliferative potential and an ability to self-renew which is characteristic of typical stem cell populations. Although the literature contains studies describing the differentiation of DPCs down adipogenic [22-24], angiogenic [25, 26],neurogenic [15, 27] and myogenic [28] lineages by far the majority of studies have examined the differentiation of DPCs to odontogenic or osteogenic phenotypes [29-31]. This reflects the fact that the formation of dentin (odontogenesis) or bone (osteogenesis) from DPCs may be a route for *in situ* repair of various dental tissue injuries or even provide the means for reconstruction of hard tissues from other anatomical locations. Accordingly several *in vitro* studies have shown that DPCs can functionally differentiate into osteoblasts and secrete a mineralizing matrix [32, 33], Further when such cells are transplanted into immunocompromised mice they form bone like structures *in vivo* [34, 35]. The formation of appropriate dentin structure *in vivo* is only described in a few studies [36, 37] and for both dentin and bone formation from transplanted DPCs spectrophotometric or microscopic confirmation of appropriate 3D tissue structure has not been universally confirmed. Nonetheless for dentin physiological differentiation occurs following trauma/injury where DPCs differentiate into odontoblast-like cells which secrete reparative dentin in an attempt to minimize tissue degradation.

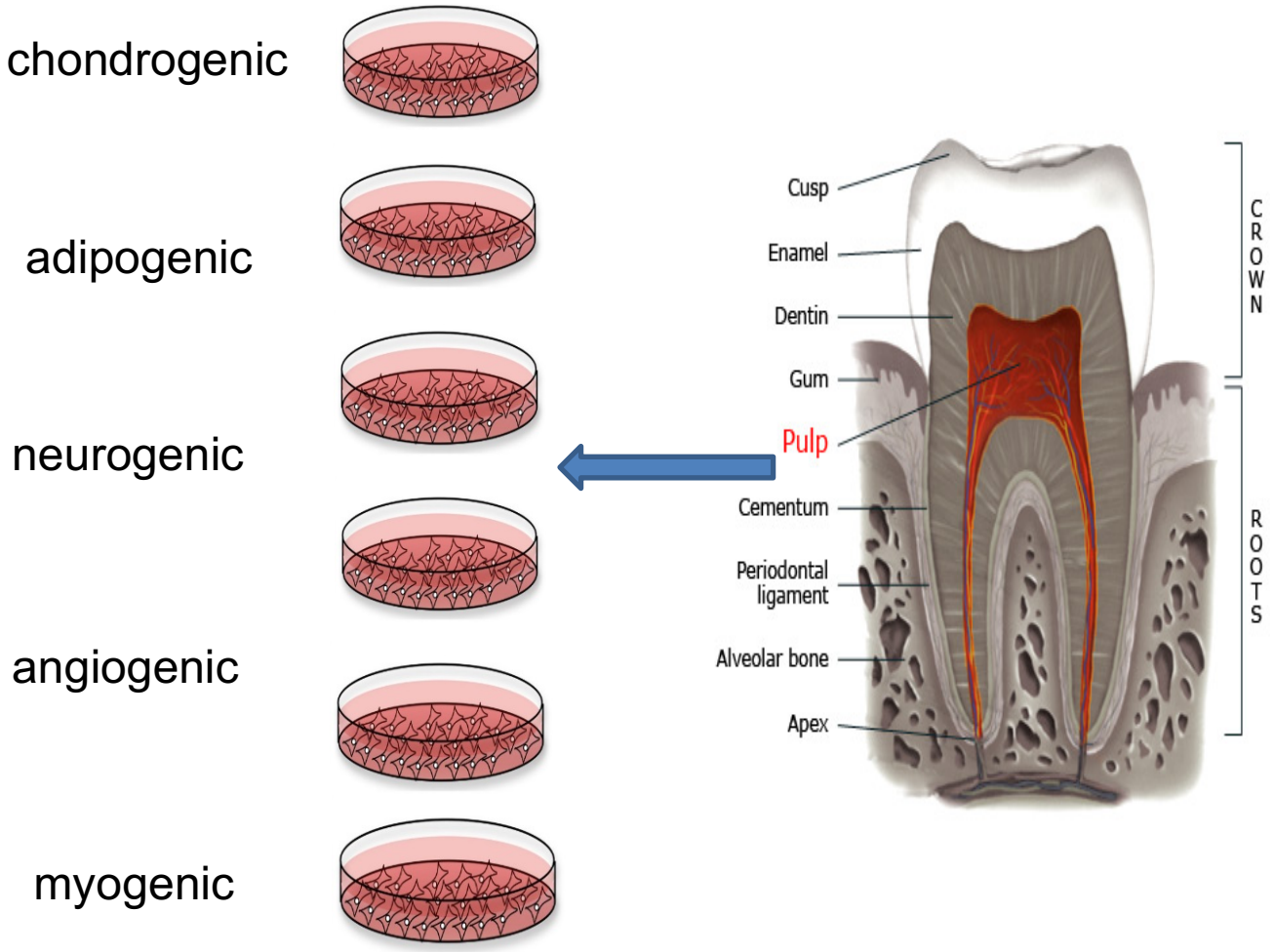


Figure 4 Dental pulp stem cells can be differentiated down numerous phenotypic lineages

Right photo is adapted from

https://rickwilsondmd.typepad.com/rick_wilson_dmds_blog/2010/09/dental-pulp-calcification-spelunking-in-the-dentin.html see [7, 38-42].

1.3 Growth factor regulation of osteogenesis

In our laboratory and in other groups the osteogenic differentiation of MSCs (including DPCs) is achieved by the addition of glucocorticoid (typically dexamethasone) and ascorbic acid (vitamin C) [2, 43, 44]. In some occasions the nucleation factor β-glycerophosphate is also used although this may lead to non-physiological deposition of mineral in cell cultures [45]. However, *in vivo* the differentiation of DPCs into a matrix mineralising phenotype most likely occurs through more physiologically relevant routes. This is typically associated with expression of osteogenic markers including alkaline phosphatase (*ALP*), osteocalcin (*OCN*) and *runt*-related transcription factor 2 (*Runx2*) along with an extracellular protein matrix which is characteristic of mineralised tissues and leads ultimately to the deposition of inorganic calcium-phosphate (Ca-P) within this matrix. It is important in these circumstances that appropriate 3D structure of hard tissue is achieved and although some successful studies using xenograft transplantation in immunocompromised mice have been reported [10] the appropriate 3D structure of this tissue is often not rigorously confirmed. One reason for this may be the absence of appropriate physiological signals in the form of growth factor or other cytokine gene expression and signalling *in situ.* Further research is needed in this area and initially work has been reported on the bone morphogenetic protein (BMP) family. These proteins belong to the Transforming growth factor-Beta (TGF-β) superfamily and are extensively reported to play an important roles in the development and maintenance of skeletal tissues including tooth development -see [46] for recent review. Therefore various lines of evidence have shown that individual BMPs will stimulate the differentiation of mesenchymal stem cells (MSCs) into osteoblast progenitor cells [47-49] However in human bone tissue matrix by far the most abundant growth factor is insulin-like growth factor-1 (IGF-I) and this polypeptide growth factor acts in concert with pituitary growth hormone to regulate long bone accretion in childhood [50].As our experimental work examines the involvement of the IGF axis in osteogenic differentiation at this stage we provide a short review of the

literature which describes the role of IGFs in skeletal maintenance and development. This is followed by a more detailed analysis of the components of the IGF axis

1.3.1 IGF axis and bone physiology

Genetically engineered mice provided some of the first evidence for the importance of the IGF axis in the development and maintenance of skeletal tissues. IGF-I heterozygous or homozygous null mice showed reduced post-natal growth although homozygous null mice showed a large degree of perinatal death [51, 52]. In terms of bone structure authors have described a “mixed phenotype” in IGF-I null mice with a reduction in cortical but increased trabecular bone density and connectivity. It has been suggested that this may be due to the fact that IGF1 has effects on both osteoblast and osteoclast cell biology. Studies with *igf1-r* knock out mice have proved more problematic as these animals invariably die perinatally [52]. However, cre-lox specific deletion of the *igf-1r* in osteoblasts resulted in several alteration of bone trabecular structure including reduced trabecular number and volume [53]. In contrast to the above studies over expression of IGF1 from a metallothionein promoter resulted in increased body weight gain but very little discernible phenotype in skeletal structure. In an attempt to dissect out the contribution of local versus systemic circulating IGF to body growth several sophisticated tissue specific KO transgenic animals have been generated [54-58]. In summary these studies have shown that endocrine IGF-I derived from the liver is not required for normal body growth but this endocrine source of IGF-I can compensate for a lack of locally produced IGF-I in skeletal tissue. It is therefore highly likely that both circulating and locally produced IGF-I are required for normal development and maintenance of skeletal tissues.

Many lines of evidence confirm the importance of the IGF axis in development and maintenance of skeletal tissues in humans. In cases of pituitary growth hormone (GH) deficiency-where IGF-I levels are also decreased, reduced bone mineral density is apparent

and this can be countered by GH administration leading to increased serum IGF-I [56, 59, 60]. Similarly mutations of the GH receptor as seen in Laron syndrome [61] lead to low serum IGF-I levels and severe growth restriction in affected individuals. In addition mutations in the IGF-I gene leading to lowered plasma levels of the growth factor [62-64] or mutations in the IGF-I gene itself [65-67] are associated with intrauterine and postnatal growth defects. This area has been the subject of an excellent recent review [68].

In mature adult bone IGF-I and IGF-II regulate the differentiation and activity of osteoblasts and osteoclasts and as such are critically important in the process of bone remodelling [53]. The activity of IGFs in bone physiology is regulated by six soluble high affinity IGF binding proteins (IGFBP 1-6). IGFBPs have the ability to enhance or inhibit the action of IGFs in a tissue specific manner (including bone) and also display IGF independent effects [69]. However, importantly in the context of tissue engineering, IGFBPs are present at a high concentration within bone matrix where they are believed to form tripartite complexes with IGFs and extracellular matrix molecules (collagen, fibronectin, laminin). As such they may form a low affinity sink for IGFs within bone tissue from which growth factor can be released into the pericellular environment to act through cell surface IGF-I and IGF-II receptors (IGF1R and IGF2R) [70, 71]. This naturally occurring regulation of bone metabolism may provide a route for more realistic approaches to hard tissue engineering where delivery of active growth factor is achieved through normal cell physiology. It is clear from the above that the IGF axis is multicomponent and as the main experimental section of this thesis deals with the potential role of the IGF axis in the osteogenic differentiation of DPCs it is appropriate that we provide some more detail with respect to the IGF axis genes and proteins and discuss further their role in hard tissue metabolism including tissues in the oral cavity. This area has been reviewed recently by our group and others [70, 71]

1.4 IGF axis – molecular components

The IGF axis comprises two polypeptide growth factors (IGF-I and IGF-II), two cell surface receptors (IGF1R and IGF2R) and six soluble high affinity IGF binding proteins (IGFBP-1-6)

[72, 73]. In addition to this a number of ancillary proteins also interact with the main IGF axis gene products – see below. A diagrammatic representation of the main IGF axis proteins is provided in **Fig 5.**

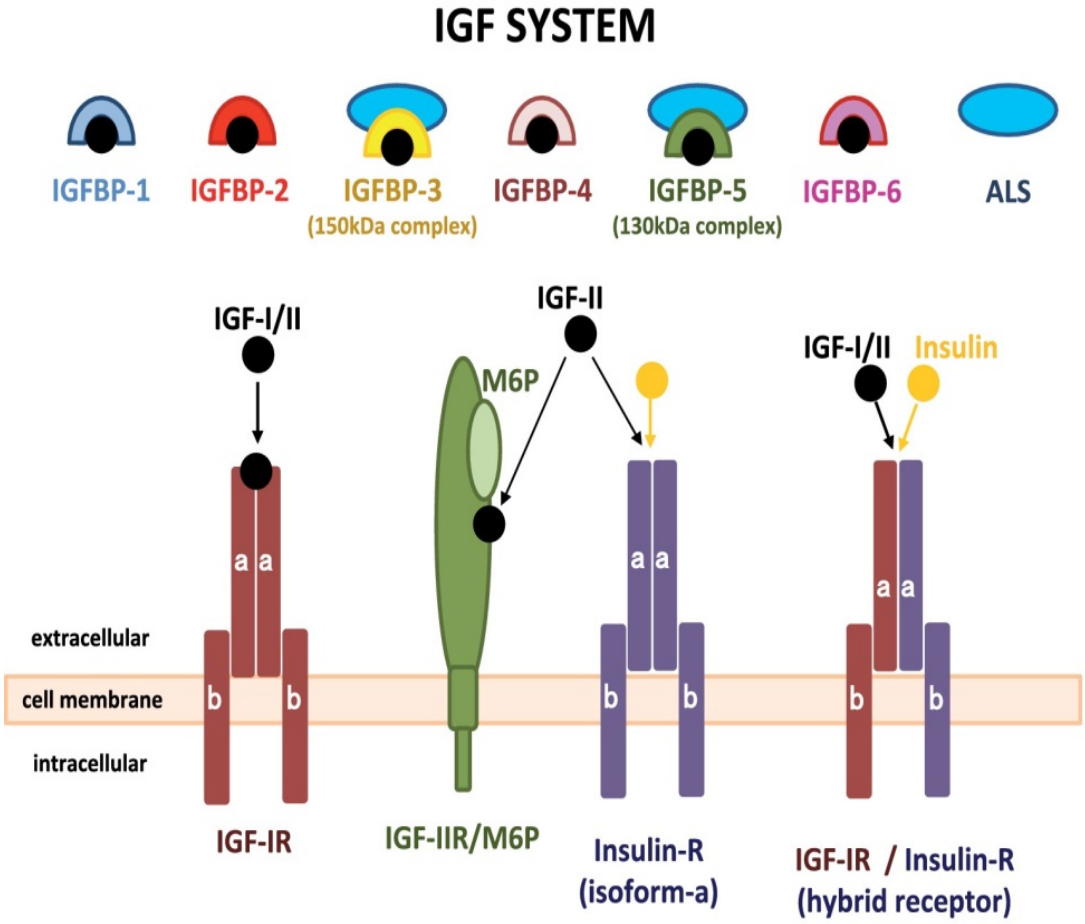


Figure 5 Components of the IGF axis

The IGF axis comprises 2 growth factors (IGF-I and IGF-II); cell surface cognate IGF receptors IGF1R and IGF2R and 6 soluble high affinity IGF binding proteins (IGFBP1-6). An acid labile subunit (ALS) is able to bind to IGF: IGFBP-3/5 binary complexes to form a tripartite complex. IGF-I and IGFII both bind to the IGF1R although affinity of IGF-I for IGF1R is 10-fold higher than IGF-II. IGF-II also binds to the insulin receptor isoform a (IR-A) and both growth factors also bind to hybrid receptors formed between IGF1R and IR-A. Note IGF-II = IGF2. Adapted from Yau *et al* [74].

1.4.1 IGFs

IGF-I is a 70 amino acid polypeptide (7.5 KDa) hormone encoded on chromosome 12 (human) with structural homology to insulin. In biological fluids a large majority of IGF-I is bound to the high affinity IGFBPs such that only around 1% of total IGF-I is present in the free form. Total concentration (free and bound) in plasma is of the order of 150-400ng/ml. Liver is the main source of IGF-I and in this tissue expression is positively regulated by pituitary growth hormone (GH). However, IGF-I is expressed by several other tissues - including skeletal tissues (muscle, bone, and cartilage) and transgenic mice with tissue specific knock out of hepatic IGF-I display a normal growth phenotype indicating the importance of peripheral and local synthesis of the growth factor in whole animal physiology. IGF-I has pleiotropic actions regulating cell development, proliferation, differentiation and migration as well as exhibiting anti-apoptotic actions [75, 76]. and many of these activities occur through autocrine and paracrine mechanisms [77]. IGF-II is of a similar size to IGF-I (67 residues) but is a distinct gene product encoded on human chromosome 11. IGF-II concentrations peak during foetal growth and then decline in adult life. IGF-II expression is not GH dependent [78]

1.4.2 IGF receptors

IGF-I activity is mediated at the cell surface by IGF1R which is a heterotetrameric transmembrane tyrosine kinase receptor [79]. It comprises two extracellular α-subunits containing the IGF binding domain and these are connected by disulphide bonds to two transmembrane β-subunits that contain tyrosine kinase activity [80-82]. When IGF-I binds to the α subunits, the β subunits undergo conformational changes which stimulates tyrosine kinase activity causing activation of downstream signalling molecules by further protein phosphorylation [77]. Among these are the SRC homology 2 domain-containing protein (SHC) and insulin receptor substrate family of proteins (IRS1-4). These signalling

intermediates play an important role in activation of the phosphoinositide 3 kinase (PI3K) and mitogen-1 activated protein (MAPK) kinase pathways [82]**.** The biological effects of IGF-II are also believed to be transduced through the IGF1R although IGF-II binds to IGF1R with a lower affinity than IGF-I. The cognate receptor for IGF-II (IGF-2R) is a mannose- 6-phosphate receptor and binds both IGF-I and IGF-II although with a higher affinity for IGF-II compared with IGF-I [83]. Although no biological action as yet been definitively assigned to the IGF-2R it is a key player in IGF-II turnover and degradation [84]. In addition by diverting IGF-II from binding to the IGF1R it has been suggested that IGF2R may act as a tumour suppressor [85] although further evidence is required to substantiate this suggestion.

There is a high degree of similarity between insulin receptor (IR) and the type I IGF receptor (IGF-IR). IR activation involves stimulation of intracellular mediators that results in the regulation of cell metabolism, proliferation and survival. Generally, IR has anabolic effects, while IGF-IR has antiapoptotic, mitogenic, and transforming effects. The IR can be stimulated by IGF-II, IGF-II sends a proliferative signal through the IR. Insulin receptor has two isoforms: isoform A (IR-A) and isoform B (IR-B). IGF-II binds with higher affinity to IR-A than IR-B. IR-A, produced by exon 11 skipping, is characterized by the absence of 12 amino acid residues at the carboxyl terminus of the IR α -subunit and is predominantly expressed in fetal tissues and cancer cells. On the other hand, IR-B, containing the 12 amino acid residues encoded by exon 11, binds insulin well and IGF-II poorly, and is usually expressed in adult differentiated cells [86].

1.4.3 IGF binding proteins (IGFBPs)

The 6 IGFBPs are secreted in a cell and tissue specific manner and are soluble high affinity IGF binding proteins[77].IGFBPs are 24-42 kDa proteins and share a generic structure comprising highly conserved N- and C-terminal domains connected by less well conserved flexible linker domain. The N- and C-terminal domains are characterised by a highly

conserved intra-domain disulphide bonding pattern and both domains contribute to IGF binding. Most IGFBPs contain heparin binding sites within the C-terminal domain and in some instances a cryptic heparin binding site in the central domain [87]. IGFBP-1 and -2 also have RGD integrin binding motifs in the C-terminus. Figure 7 shows a highly stylised representation of the generic IGFBP domain relationships. To date there has been no report of a 3-dimensional structure for an entire IGFBP protein. This is most likely due to the highly flexible nature of the central IGFBP linker region which bridges the N- and C-terminal domains of the protein which makes crystallisation of proteins difficult. However partial solved structures for isolated domains reveal a largely β-barrel like structure and homology modelling as ben used to infer this conserved 3D structure amongst all the IGFBPs. Figure 8 shows a representation of solved N- and C-terminal domain structures for IGFBP-4 in complex with bound IGF-I IGFBP domain and tertiary structure. IGFBPs are subject to post-translation modifications (PTM). Therefore IGFBP-3 is extensively glycosylated and IGFBP-1 is subject to serine phosphorylation which reduces its affinity for binding IGFs. However, in the context of our report the most important PTM of IGFBPs is that of proteolysis. We will discuss this extensively for IGFBP-4 and IGFBP-5. Because of the fact that IGFBPs have a 10-fold higher affinity for IGFs comprising than the cell surface IGFRs, this has important physiological consequences and is relevant to the Experimental section of this thesis. In biological fluids 99% of circulating IGFs are bound to IGFBPs [88] and this may represent a reservoir depot of IGFs [77, 89]. In addition association of IGFs with IGFBPs may represent a buffering of IGF activity in that IGFs are present at around x1000-fold higher concentration than insulin and the presence of large concentrations of free IGFs in the circulation may induce hypoglycaemic shock [90]. IGFBPs affinity for IGFs can be affected by post-translational modifications of these proteins and particularly by proteolysis [89, 91]. As this is also an important part of the Experimental section of this thesis we discuss this topic at greater length below. Finally, IGFBP-3 and -5 are commonly found in a ternary complex with an acid labile subunit (ALS) which has the effect of inhibiting trans endothelial passage of IGF: IGFBP complexes [92, 93]. A short description the function of each of the IGFBPs

follows with special emphasis on IGFBP-4 – the main IGFBP species investigated in this work.

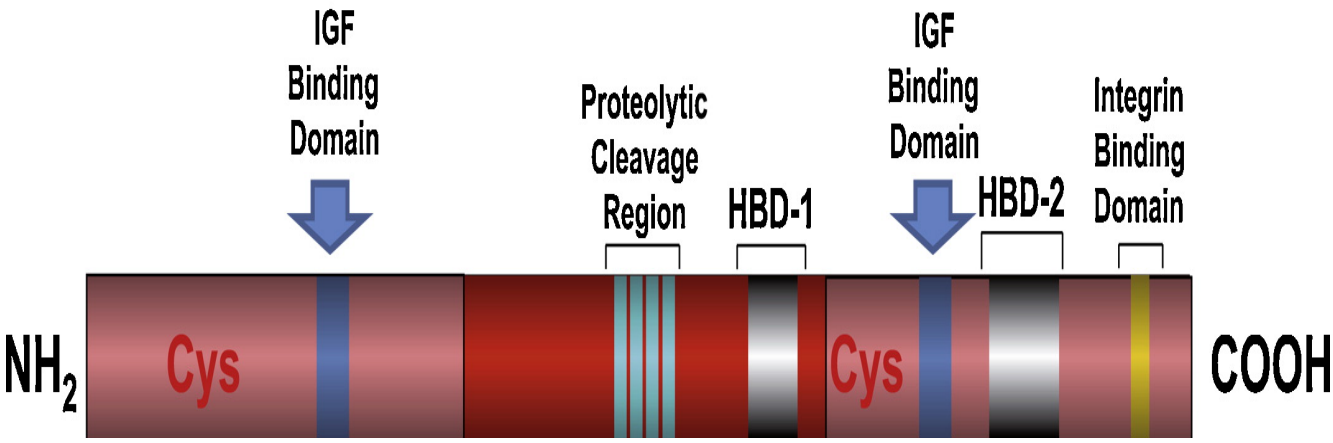
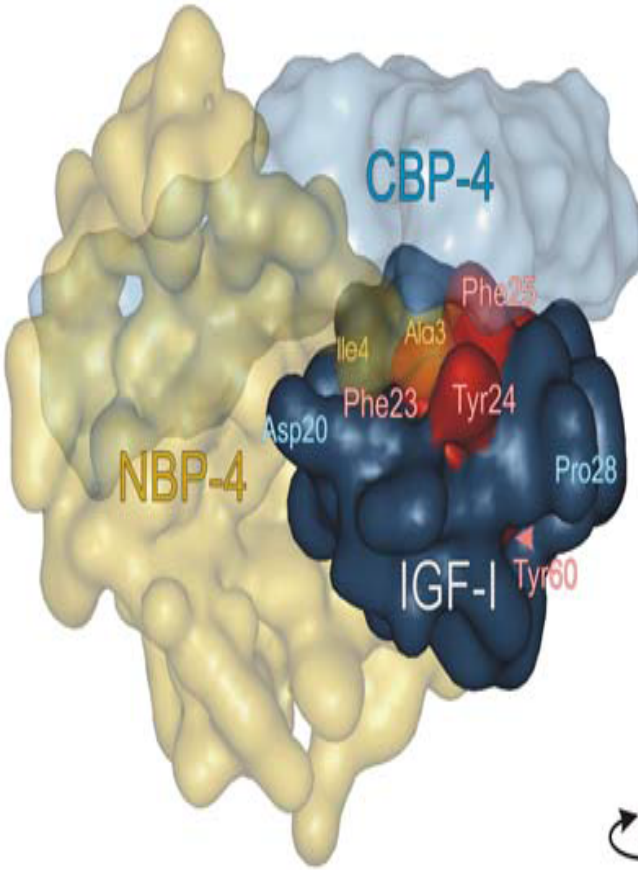


Figure 6 The generic 3 domain structure of IGFBPs shows IGF binding elements in the N- and C-terminal domains

Also shown are the heparin binding domains in the C-terminal domain (HBD-2) and a cryptic heparin binding domain (HBD-1) in the central domain. Integrin binding domains (RGD) are also present in the C-terminal domains of some IGFBPs. Finally, IGFBPs are subject to proteolysis by several families of protease enzymes. As indicated these sites are located mainly in the central domain. Figure is adapted from Russo *et al* [94].



Affinity ~ 0.2 nM

Cf. IGF1R ~ 2nM

Figure 7 Space filling 3D model of IGF-I

(blue) interaction with the solved structures for the N-terminal (yellow) and C-terminal (grey) domains of IGFBP-4 Residues in IGF-I which are involved in binding the N- and C-terminal domains are highlighted. Note as indicated that affinity of IGF-I for IGFBP is over 10-fold higher than for cell surface IGF1R (see text for further details). Figure adapted from Siwanowicz *et al* [95].

IGFBP-1

IGFBP-1 stimulates cell motility, adhesion and migration. It binds specifically to the $\alpha_5\beta_1$ integrins [89, 96], and is present at very high concentrations in placenta. There are higher IGFBP-1 concentrations in female plasma compared to male plasma [97]. IGFBP-1 expression is inversely regulated by insulin and phosphorylation of IGFBP-1 reduces IGF affinity [89]. . IGFBP-1 is expressed at low levels in primary hOB cultures under regulation of glucocorticoid and insulin although the physiological relevance of this effect in bone tissue has not been established [98]. A recent prospective study (10 year follow up) in a cohort of elderly women reported a positive correlation between serum IGFBP-1 and osteoporotic fracture, suggesting an IGF-independent osteopenic effect of IGFBP-1[99]. Further data are required on IGFBP-1 and its effects (if any) on bone physiology.

IGFBP2

There is a literature describing IGF-dependent and IGF-independent effects of IGFBP-2 in osteoblast cultures and bone tissues. An early study using a unilateral disuse osteoporosis model in the rat showed that osmotic minipump delivery of IGF-II/IGFBP2 complexes prevented the decrease in BMD in affected femurs associated with this model [100]. A subsequent report from the same group showed that IGF-II/IGFBP2 complexes bound to heparin-sepharose and it was suggested that such complexes may associate with ECM components in bone tissue potentially increasing the local concentration of IGFs [101]. In agreement with the above findings, IGFBP-2 potentiated IGF-II-induced increases in ALP activity in cultures of rat tibial osteoblasts [102], and we have demonstrated the same effect of IGFBP-2 on IGF-I stimulated ALP activity in differentiating DPCs [1]. Studies in IGFBP-2 KO mice indicated gender specific differences in osteogenic phenotype with increased cortical thickness and periosteal circumference in female mice but reduced cortical bone area and trabecular volume in male KOs [103]. Although difficult to rationalise, it clearly

suggests interplay between the IGF axis and other hormone systems. This same group also reported impaired osteoclastogenesis in bone marrow cells derived from *igfbp2 -/- mice* and a transfection study in these cells indicated that both the IGF and heparin binding domain of IGFBP-2 was required for osteoclast generation [104] This description of IGF-independent effects of IGFBP-2 *in vitro* was confirmed in concurrent studies demonstrating restoration of osteogenic phenotype in *igfbp2 -/-* bone marrow cells by addition of a heparin binding domain (HBD) peptide derived from IGFBP2. In addition, *in vivo* administration of HBD peptide restored osteoblast number in *igfbp2 -/-* mice [105]. Recently studies in the mouse MC-3T3 pre-osteoblast cell line showed that IGFBP-2 can bind and inhibit the activity of receptor phosphotyrosine phosphatase β (RPTP β) causing increased levels of phosphorylated PTEN, activation of Akt and stimulation of osteogenesis [105, 106]. Further reports from this laboratory highlight the importance of the scaffold/adaptor protein IRS-1, PKCζ and early activation of AMP dependent protein kinase (AMPK) in the osteoblast differentiation of primary rat calvarial cells and the differentiating MC-3T3 cell line [107, 108]. It should be noted that IGFBP-2 is also a Pregnancy Associated Plasma Protein -A (PAPP-A) substrate, although this IGFBP is cleaved less efficiently than IGFBP4 and IGFBP-5 [109].

IGFBP-3

IGFBP-3 is the most abundant IGFBP in serum and reaches highest levels during puberty. IGFBP-3 provides 75-80% of the IGF carrying capacity in serum and binds IGFs in high affinity complexes. The IGF-I-IGFBP3 complex forms a tripartite complex with a third protein known as acid labile subunit (ALS) – see page 28 and this prolongs the circulating half-life (~16 hours) of bound IGF-I compared to free growth factor (<15 minutes) [77] . Around 90% of IGFBP-3 and 55% of IGFBP-5 circulate in a trimeric complex with ALS during adult life [110]. Interestingly and pertinent to the current study, IGF action on osteoblasts is enhanced by IGFBP-3 [111]. A very early study reported inhibition of IGF-I stimulated DNA synthesis in

two osteoblast cell lines by intact IGFBP-3 [112]. This inhibitory effect on both IGF-I and IGF-II stimulated DNA synthesis was confirmed in cultures of rat calvarial cells [113]. Although this data suggest an inhibitory role for IGFBP-3 in bone metabolism, other *in vivo* data [114] and cross-sectional studies in a cohort of female patients with postmenopausal osteoporosis suggest an anabolic role for IGFBP-3 in maintaining bone density [115].

IGFBP-5

IGFBP-5 is also present at high concentrations in bone matrix and has been associated with both inhibitory and stimulatory activities in bone cells and tissues. IGFBP-5 was reported to have IGF-dependent and IGF-independent effects in bone tissue although the literature is conflicted in this area. IGFBP-5 was shown to enhance IGF-stimulated mitogenesis in hOB cultures [116, 117] and to stimulate the differentiation of two osteoblast cell lines in an IGF-independent fashion [118, 119]. In ovariectomised rats, daily subcutaneous injection of IGFBP-5 increased osteoblast proliferation [120] and enhanced the association of IGF1 with bone cells possibly via specific cell-surface binding sites for IGFBP-5 [121, 122] or through a specific IGFBP-5 receptor on osteoblast membranes [123, 124]. Disappointingly however a specific IGFBP-5 receptor has not been isolated or characterised further. Signalling studies suggest that the actions of IGFBP-5 in osteoblasts involve Ras association family isoform C (RASSF1C) activation of Erk-1/2 [125]. The association of IGFBP-5 with four and a half lim domain protein (FHL2) within the nucleus of U2 osteosarcoma cells has also been reported although the functional significance of this observation remains unknown [126]. Although all the above findings are consistent with a stimulatory role for IGFBP-5 action in bone tissue (IGF-dependant or independent), some authors have reported contrary findings. For example, IGFBP-5 was reported to inhibit IGF-I stimulated proliferation in the U2 human osteosarcoma cell line [127] and transgenic mice expressing IGFBP-5 from the osteocalcin promoter showed decreased trabecular bone formation and reduced rates of mineral deposition during the first few weeks of post -natal life [128]. Stromal cells isolated from

transgenic animals also showed decreased levels of osteogenic markers. Constitutive over expression of IGFBP-5 in the mouse osteoblast precursor cell line MC3T3-E1 also decreased osteogenic marker expression and delayed formation of mineralised nodules under osteogenic culture conditions [129]. Finally, addition of exogenous wtIGFBP-5 or expression of IGFBP-5 from an adenovirus promoter inhibited osteoblast differentiation and growth of mouse metatarsal bones in short term culture [130].

Although IGFBP-5 is cleaved in an IGF-independent fashion by PAPP-A and PAPP-A2, it is also a substrate for other proteolytic enzymes. Matrix metalloproteinase -1 and -2 (MMP-1 and MMP-2) were shown to degrade IGFBP-5 in a time-dependent fashion in medium conditioned by the mouse MC-3T3-E1 cell line [131], and the complement component C1s was identified as an IGFBP5 specific protease in human dermal fibroblast conditioned media [132]. Following on from this, Mohan *et al* described ADAM-9 (a disintegrin and metalloprotease -9) as an IGFBP-5 protease expressed the U2 human osteosarcoma cell line [133]. Although the importance of IGFBP-5 proteolysis may (as for IGFBP4 proteolysis) lie with the regulation of free pericellular IGF concentrations, this is somewhat complicated by the observations of IGF-independent actions of IGFBP-5 described above. Clearly, these may also be impacted by IGFBP-5 proteolysis. Further work is required to establish the role of IGFBP-5 in osteoblast differentiation and in bone tissue metabolism in general. Finally, there are reports of broad-spectrum proteolytic enzyme families which degrade IGFBP-5 (and other IGFBPs). Among these are the serum proteases plasmin [134] and thrombin[135], and other tissue serine proteases including cathepsin G and elastase [136]. However, issues in regard to the specificity and physiological role of these proteases remain to be answered.

IGFBP-6

IGFBP-6 mRNA was expressed in primary osteoblast cultures derived from foetal rat calvaria [137] and both mRNA and protein expression was up regulated in a dose-dependent fashion by cortisol or retinoic acid treatment [138, 139] cultures. Conversely IGFBP-6 expression was negatively regulated by TGFβ1 in the same cell culture system [140]. IGFBP-6, uniquely amongst the IGFBP family shows a higher affinity for IGF-II than IGF-I. Accordingly, it was shown to be a more potent inhibitor of IGF-II stimulated DNA and glycogen synthesis in hOB cells than IGF-I [141]. This inhibitory effect of IGFBP-6 was confirmed in the SaoS2 human osteosarcoma cell line using a stable antisense transfection strategy to demonstrate that the anti-differentiative activity of all-trans retinoic acid (at-RA – Vitamin D) was at least partly mediated via IGFBP-6 [142].More recently IGFBP-6 has been shown to interact with the thyroid hormone receptor alpha1 (TR alpha1) and to inhibit the tri-iodothyronine (T3) induced increase in osteoblast marker expression in the human U2-OS osteosarcoma cell line [143]. In contrast to these reports the inhibitory effect of IGFBP-6 attenuated by intracellular interaction with the LIM mineralising protein (LIMP) in both human and mouse osteoblastic cells [144] and one study reported a stimulatory effect of IGFBP-6 on DNA synthesis and mitogenesis in the human osteosarcoma Saos-2/B-10 cell line [145]. As for IGFBP-1 and IGFBP-3, the role of IGFBP-6 in osteogenesis and bone tissue physiology has been underreported and further studies are required to elucidate the role of these 3 IGFBPs in osteogenesis and bone physiology.

1.4.4 IGFBP-4

IGFBP-4 was first identified as an inhibitory IGFBP in medium conditioned by the TE89 human osteosarcoma cell line [146] and then cloned from cDNA libraries of various tissues in human and rat [147, 148]. It is a 237-residue protein sharing the 3-domain structure previously described for other IGFBPs – see Fig 8 for details of IGFBP-4 primary structure.

Early studies showed that IGFBP-4 inhibited IGF-II stimulated thymidine uptake in primary cultures of human osteoblasts [121] and in the MC3T3-E1mouse osteoblast cell line [149] and inhibited IGF-I stimulated amino-isobutyrate uptake in bovine fibroblasts and in the rat neuronal B104 cell line [150, 151]. This inhibitory activity *in vitro* led to the hypothesis that IGFBP-4 generally displayed anti-anabolic and anti-proliferative effects. In confirmation of this, overexpression of IGFBP-4 in a malignant prostate epithelial cell line decreased the proliferative response to IGF-I and delayed tumour development when transfected cells were transplanted into nude mice [152]. *In vivo* data also supported an inhibitory role for IGFBP-4. Tissue specific overexpression of IGFBP-4 in smooth muscle cells using an α-actin promoter caused smooth muscle hypoplasia [153] and a similar strategy using a protease resistant form of IGFBP-4 (see 1.4.5 IGFBP4 proteolysis) resulted in transgenic mice with decreased internal smooth muscle mass in stomach, bladder and aorta [154]. Importantly with respect to this review, IGFBP-4 over expression in osteoblasts decreased bone formation and compromised global skeletal growth [155]. Some epidemiological data also supported an inhibitory role for IGFBP4 with increased levels in a cohort of female patients with age-related osteoporotic fractures of the hip and spine [156]. Although this evidence suggested an inhibitory role for IGFBP4, other reports indicated an anabolic role for IGFBP4. Therefore, systemic administration of IGFBP-4 to mice increased bone tissue markers (osteocalcin and alkaline phosphatase) in serum and skeletal tissue [157]. Additionally, IGFBP-4 knock out (KO) mice exhibited prenatal growth retardation, suggesting that IGFBP-4 may be required for full growth promoting effects of IGF-II in the foetus [158]. IGFBP-4 KO mice also showed gender dependent changes in skeletal phenotype with female mice having reduced BMD along with other features associated with osteopenia [159]. Clearly further research is required to definitively establish the role of IGFBP-4 in bone tissue physiology. In this respect, the observation of IGFBP4 proteolysis by fibroblast and bone cell cultures have attracted much interest as a means of regulating the activity of IGFs in bone and other tissues and we provide a short summary of this area in the following section.

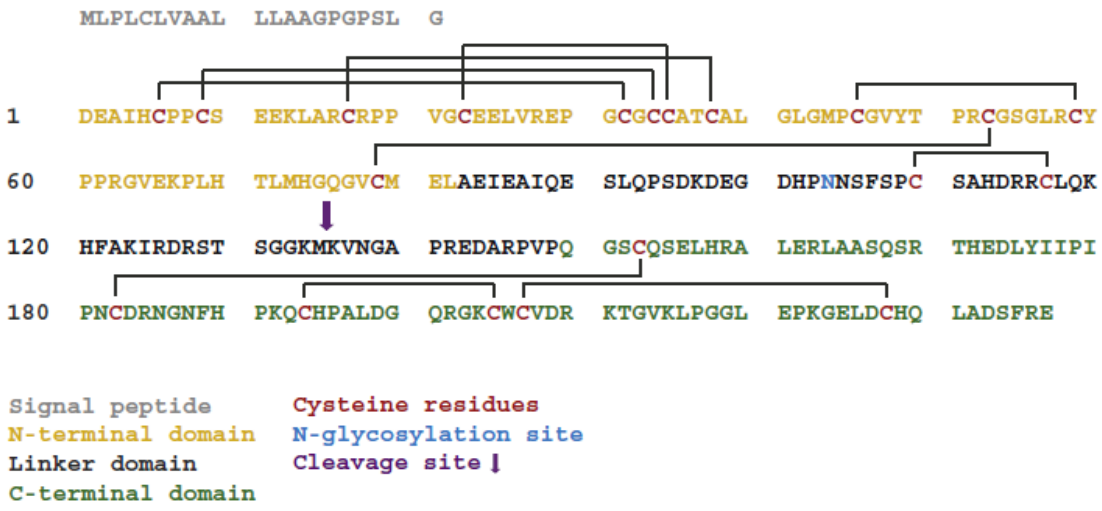


Figure 8 The primary sequence of IGFBP-4

The 21 residue signal peptide sequence is displayed in grey above the N-terminus of the mature protein. Residues comprising the N-terminal (yellow), C-terminal (green) and central (black) domains are highlighted. The location of 6 N-terminal and 3 C-terminal intra-domain disulphide bridges are indicated. These are conserved in all IGFBP species. A disulphide bridge unique to IGFBP-4 is also present in the central domain. The location of the N-linked glycosylation site at N104 is indicated in blue and the PAPP-A cleavage site between M135 and K136 is indicated by an arrow. Adapted from [160]. For further details see http://www.uniprot.org/uniprot/P22692

1.4.5 IGFBP-4 proteolysis

Addition of IGF-I to cultures of human fibroblasts reduced the levels of a 24 kDa IGFBP in conditioned medium and development of specific antibodies confirmed this species as IGFBP4 [161, 162]. IGF-I dependent down regulation of IGFBP4 occurred independently of IGF1R activation and was not associated with changes in IGFBP4 mRNA levels suggesting a direct post-translational regulation of IGFBP4 by IGF-I. Shortly thereafter, IGF-induced decreases in IGFBP-4 protein were shown to be due to the presence of a proteolytic activity in fibroblast conditioned medium which in cell free assays was activated by IGF-I or IGF-II [163]. IGFBP-4 was cleaved into two discrete fragments by this protease, suggesting a specific cleavage point within the protein [164]. The cleavage site was identified at the peptide bond M135-K136 within the central domain of IGFBP4 producing 14 and 18kDa protein fragments [151]. This data was used to engineer protease resistant IGFBP4 mutants which have proven useful in the further study of the biological significance of IGFBP4 proteolysis [151, 165] and became apparent when intact, but not cleaved IGFBP-4, was shown to inhibit [^{3}H] aminoisobutyric acid uptake into bovine fibroblasts with the inference that cleaved IGFBP-4 fragments did not bind IGF-I. Further study indicated that IGF-II was a more potent activator of IGFBP-4 proteolysis than IGF-I and IGF-II pre-treatment of human dermal fibroblast cultures increased sensitivity of cell cultures to IGF-I. The concept of IGF-II-mediated IGFBP4 proteolysis as a route for increasing sensitivity to IGF-I [166] may be significant as IGF-I and IGF-II are usually present together in the pericellular environment suggesting a complex interaction between the growth factors to regulate anabolic responses.

Primary cultures of human osteoblasts (hOB) expressed IGFBP-4 protease activity identical to that described for fibroblasts [167], and pre-treatment of osteoblast cultures with IGF-II also increased sensitivity to IGF-I stimulated [^{3}H] thymidine incorporation [168]. Subsequently, IGFBP-4 protease activity has been reported in human endometrial stromal cells [169] and in porcine aorta derived smooth muscle cells [170], suggesting that proteolysis of IGFBP-4 may have widespread biological significance. At around this time a

landmark study identified pregnancy-associated plasma protein-A (PAPP-A) as the enzyme responsible for IGF-dependent cleavage of IGFBP-4 in fibroblast conditioned medium [171] . PAPP-A was also shown to proteolyse IGFBP-5 although in this instance proteolysis occurred independently of IGF [172] . Identification of PAPP-A as the IGF-dependent IGFBP-4 protease expressed caused a paradigm shift in this area of IGF research. Whereas previously IGFBP-4 had been viewed mainly as an inhibitory IGFBP in tissue culture studies, co-expression of PAPP-A in cell culture could negate this inhibitory effect. Furthermore, the activation of PAPP-A by IGFs suggested possible positive feedback loop whereby growth factor action could be enhanced. Further aspects of function, structure and regulation of PAPP-A activity are discussed below.

1.5 Pregnancy associated plasma protein-A (PAPP-A)

1.5.1 Structural Aspects

Although PAPP-A was isolated over four decades ago from pregnancy serum [173] it was only after the cloning and expression of this large (1547-residue) protein that detailed work on protein structure began [174]. PAPP-A belongs to the metzincin superfamily of metallo-endopeptidases containing a Zn binding motif and highly conserved methionine residue [175]. PAPP-A associates with cell membranes through two of five short consensus repeat (SCR) modules within the C-terminus of the protein and membrane-bound PAPP-A remains catalytically active. This may ensure release of IGF from IGFBP-4 in the vicinity of cell surface IGF1R [176]. Under reducing conditions, PAPP-A migrates as a 200kDa protein although in pregnancy serum (and some other biological fluids) it is primarily present as a disulphide-bound dimer associated covalently with another disulphide bound dimer of the proform of eosinophil major basic protein (proMBP) in a 2:2 heterotetrameric complex [177, 178]. The structure of the heterotetrameric PAPP-A: proMBP complex identifies a disulphide bridged dimer of PAPP-A covalently bound to a disulphide bridged dimer of proMBP via two interchain disulphide bridges [179]. In this configuration, PAPP-A is inactive with the proMBP

dimer binding at or close to the active site of PAPP-A, suggesting that steric inhibition of enzyme activity may result. Both PAPP-A and proMBP are extensively glycosylated and under native gel electrophoresis conditions the complex runs as a large (>500 KDa) molecular weight species. A mutagenic analysis of the substrate IGFBP-4 suggested that the C-terminal domain of IGFBP-4 conferred the IGF dependence for PAPP-A hydrolysis of IGFBP-4 [180]. In addition this same study showed that the region between the Zn binding domain and the Met turn motif of PAPP-A was important for proteolytic activity towards the IGFBP-4:IGF-I complex. Availability of purified PAPP-A allowed confirmation that the rate of IGFBP-4 proteolysis is enhanced by binding of IGFs to IGFBP-4 [172] and detailed kinetic analysis confirmed IGF-II as a more potent activator of proteolysis than IGF-I. The effect of IGFs on IGFBP4 proteolysis was associated with changes in both affinity (K_m) and turnover rate (K_{cat}). This study also confirmed IGFBP-5 as a PAPP-A substrate although proteolysis of IGFBP5 was not IGF dependent [181]. Further mutational analysis suggested that the Lin12-Notch repeat (LNR) modules within PAPP-A are responsible for the differential requirement of IGFBP-4 and IGFBP-5 for IGF during PAPP-A mediated proteolysis [182, 183].

1.5.2 Functional Aspects

PAPP-A was partially purified from human fibroblast conditioned medium by Lawrence *et al* [184] and its identity confirmed by mass spectroscopy. By using polyclonal anti-PAPP-A antibodies, IGFBP4 protease activity in fibroblast conditioned medium could be completely inhibited, suggesting that PAPP-A may be the only IGFBP-4 protease expressed by these cells. PAPP-A isolated from fibroblast cultures was found to be identical to the enzyme described in pregnant serum [184-186], showing both IGF dependency and the same site of proteolytic cleavage in the central hinge domain of IGFBP-4 (see 1.4.5 IGFBP4 proteolysis). Identification of PAPP-A allowed some elegant transgenic studies highlighting the importance of this enzyme. Mice made transgenic with a col I (collagen I) promoter-PAPP-A

construct overexpressed PAPP-A specifically in osteoid tissue causing increased calvarial BMD [187]. In double transgenic mice overexpressing PAPP-A and a protease resistant form of IGFBP-4 (pr IGFBP-4) bone phenotype was similar to single pr IGFBP-4 transgenics, showing decreased calvarial thickness and BMD compared to WT mice. This provided strong evidence that *in vivo* anabolic effects of PAPP-A were due to IGFBP-4 proteolysis, most likely resulting in an increase in local IGF concentrations [188]. In confirmation of this, PAPP-A KO mice showed reduced femur BMD and blunted responses to the anabolic actions of parathyroid hormone (PTH) [189].In a clinical context, PAPP-A has been proposed as a target for anti-proliferative therapies for cancer. Studies in an ovarian cancer tissue model [190] and using xenografts of adenocarcinoma A549 cells [191] showed that antibody mediated inhibition of PAPP-A activity decreased tumour growth presumably because pericellular IGF remains associated with IGFBPs leading to a reduction in free IGF in the local tumour environment. This may be important as current anti-IGF based strategies have proved disappointing in clinical trials. Anti-IGF1R strategies are hampered by hyperinsulinemia secondary to elevated GH levels as a result of impaired IGF-I feedback at the level of the pituitary [192]. This may lead to increased mitogenic signalling by elevated insulin levels through the insulin receptor (IR). IGF-I receptor blockade may also result in IGF-I signalling through the insulin receptor (IR) or through hybrid IGF1R/IR isoforms which are known to exist in many tissues [193] and which may not be blocked by anti-IGF1R directed monoclonal antibodies (Mabs). See Yee *et al* [194] for an excellent review of the above arguments. In contrast, the use of anti-PAPP-A directed antibodies would not be associated with these complications acting only to inhibit IGF-I release from pericellularly proteolysed IGFBP: IGF complexes. The potential role(s) of PAPP-A in the aetiology of different cancers has been reviewed very recently [195].

1.5.3 Regulation of PAPP-A activity

Relatively few agents have been shown to influence PAPP-A activity. IGFBP-4 proteolysis was inhibited following treatment of fibroblast cultures with phorbol esters. The attenuation of this effect by prior treatment with actinomycin D or cycloheximide suggested Protein Kinase C (PKC) regulated expression of an inhibitor of IGFBP-4 proteolysis [196] Such an inhibitory activity was also reported in SV40 transformed hOB cells, suggesting that the process of cellular transformation may be associated with inhibition of IGFBP-4 proteolysis [197]. The finding that phorbol esters and/or SV40 mediated transformation increased the expression of proMBP – a covalent inhibitor of PAPP-A – suggested at least one route by which these agents may act to inhibit IGFBP-4 proteolysis in fibroblast cultures [198].

An early study reported stimulation of IGFBP-4 proteolytic activity in the rat neuronal B104 cell line by glucocorticoids [199] and following identification of PAPP-A as an IGFBP-4 protease, the synthetic glucocorticoid dexamethasone was shown to increase enzyme activity in primary cultures of rat vertebral osteoblasts [200]. PAPP-A mRNA levels were not altered by dexamethasone treatment, suggesting a post-transcriptional mechanism by which enzyme activity was increased. In contrast to the above, TGFβ increased PAPP-A mRNA levels approximately 12-fold in hOB cultures and this was associated with increased PAPP-A activity in conditioned medium [201]. The demonstration of increased IGF-II mediated IGFBP-4 proteolysis following TGFβ treatment of hOB cultures [202] may be of particular significance given the fact that IGF-II and TGFβ are two of the most abundant growth factors present in bone matrix and a co-ordinated action of TGFβ and IGF-II in bone matrix to increase local availability of IGF may occur. Osteoblasts secrete IGF peptides endogenously (IGF-II > IGF-I) and, despite the fact that IGFBP-4 levels in osteoblast conditioned media are typically an order of magnitude higher that IGF-II levels, endogenous IGF-II can stimulate the proteolysis of concurrently expressed IGFBP-4 protein in osteoblast cultures [165, 203].

Recently some novel protein inhibitors of PAPP-A activity have been described. These are members of the stanniocalcin family (STC1 and STC2) and were first identified as regulators of Ca homeostasis in teleost fish [204, 205]. However, in the context of the mammalian IGF axis, their status as PAPP-A inhibitors means that these proteins are negative growth regulators. Overexpression of STC1 or STC2 resulted in growth retardation in transgenic mice [206, 207], whereas KO of STC2 causes increased growth [208]. Molecular mechanisms of STC1 and STC2 inhibition of PAPP-A differ with STC2 forming a disulphide-bonded covalent complex with PAPP-A and STC1 forming a high affinity non-covalent complex with the enzyme. Nonetheless, both STC1 and STC2 potently inhibit PAPP-A which may cause an increased concentration of IGF bound in complex with IGFBP-4 (and IGFBP-5) and hence less bioavailable IGF in the pericellular environment. In agreement with this, STC2 inhibited PAPP-A stimulated IGF1R phosphorylation in transfected cells exposed to IGF-I:IGFBP4 complexes [204]. A recent study using whole exome sequencing of a large human cohort reported two separate single amino acid mutations of STC2 leading to compromised inhibition of PAPP-A. The fact that these alleles strongly associated with increased height in the sampled population is of particular interest [209]. A diagrammatic representation of the IGFBP-PAPP-A-STC axis is presented in Fig 9.

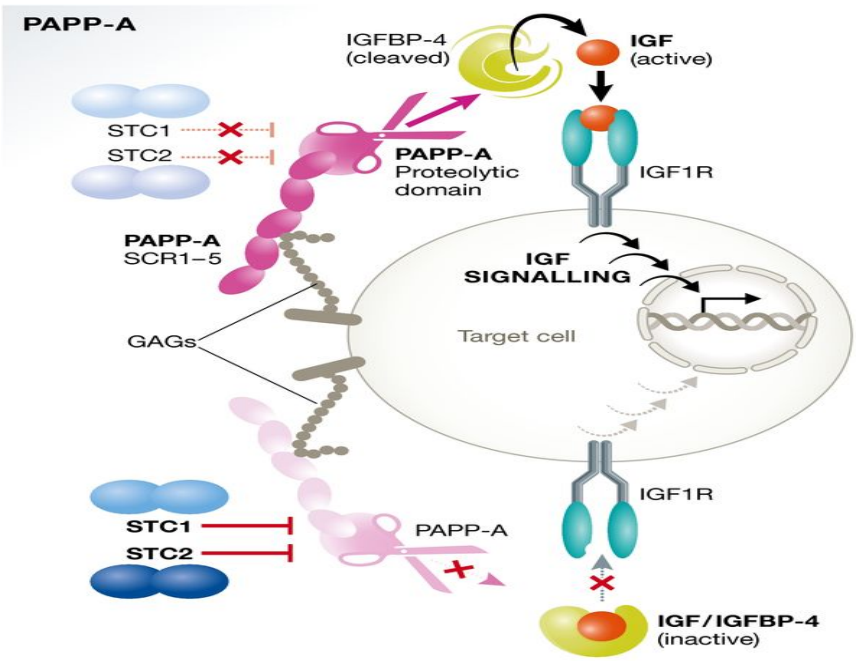


Figure 9 The PAPP-A: IGFBP-4: STC axis.

In the absence of STCs (upper part of Figure) cell membrane associated PAPP-A cleaves IGFBP-4 to release pericellular IGF-I allowing interaction of growth factor with IGF1R and stimulation of IGF-I mediated gene transcription. In the presence of STC1 and/or STC2, PAPP-A activity is inhibited, IGF-I remains bound to IGFBP-4 and IGF-I interaction with IGF1R is inhibited. PAPP-A is believed to associate with cell surfaces via glycosaminoglycan (GAG) anchors although the molecular details of this remain unresolved.

1.5.4 PAPP-A in other species

PAPP-A has also been cloned from a mouse cDNA library [210]. Although murine (m) PAPP-A shares 91% homology with the human enzyme and cleaves IGFBP-4 in an IGF-dependent manner, mPAPP-A activity is not elevated in pregnant serum or in placenta. In addition, a variant mPAPP-A containing a 29 residue insert (PAPP-Ai) was also isolated. Interestingly this PAPP-A isoform was a less efficient IGFBP-4 protease than the shorter variant of the enzyme. The significance of these differences between murine and human PAPP-A remains to be resolved, although PAPP-A null mice are 40% smaller than littermates, suggesting a role for PAPP-A during embryogenesis [211]. This may be due to diminished IGFBP-4 proteolysis and, therefore, reduced IGF availability in the developing foetus. In agreement with this IGFBP-4 proteolysis is absent in fibroblast cultures derived from these null mice. PAPP-A is present in multiple other species, including zebrafish and interestingly the absence of PAPP-A in this species causes a developmental delay which is independent of proteolytic activity [212].

1.5.5 PAPP-A2

Overgaard *et al* described the cloning of a metzincin protease from placental cDNA libraries with homology to PAPP-A. This protein, which cleaves IGFBP-5 (and IGFBP-3), was named PAPP-A2 [213] and is present in human pregnancy serum where it releases IGF-I from IGF-I:IGFBP-5 complexes [214]. PAPP-A2 appears as a monomer of 200kDa in non-reducing gel electrophoresis and in contrast to PAPP-A does not bind to proMBP or associate with cell surfaces. Cleavage of IGFBP-5 by PAPP-A2 is not IGF-dependent, but as IGFBP-5 has been reported to have both IGF-dependent and IGF-independent effects in hOB cultures, PAPP-A2 activity may also have major relevance in bone cell physiology. In agreement with this, homozygous PAPP-A2 KO mice show decreased post-natal growth along with reduced body length [215]. Similarly, conditional PAPP-A2 knockout in

osteoblasts decreased body mass and bone length, although other tissue sources of PAPP-A2 may be involved in appropriate post-natal growth [216]. *PAPP-A2* may represent a quantitative trait locus (QTL) regulating body shape in mice [217, 218], Recently, two separate families (of Palestinian and Spanish ancestry) were found to have two different inactivating PAPP-A2 mutations which result in growth retardation in homozygous children [219]. Further analysis of affected individuals indicated significant increases of IGF-I in ternary ALS complexes with reduced free serum IGF-I. In addition, affected individuals showed moderate microcephaly, mild BMD effects and thin long bones. This phenotype was presumably associated with the inability of mutant PAPP-A2 to proteolyse IGFBP-3 and IGFBP-5 substrates. More recent *in vitro* and *in vivo* experiments in PAPP-A2 deficient human subjects suggests that PAPP-A2 treatment increases free IGF-I levels in these subjects and may constitute a novel therapeutic route for treatment of idiopathic short stature [220]. In this context and in analogy to PAPP-A, PAPP-A2 is also inhibited by STC-2 [205] and the role of the PAPP-A2: STC-2 axis in the regulation of IGF action has been recently reviewed [221].

1.6 The IGF axis in dental tissue

The limited literature in this area suggests that most components of the IGF axis are expressed in human dental pulp stem cells – reviewed in [222]. In a functional sense IGF-I has been reported previously to promote the differentiation of human dental pulp stem cells via mTor [223] and MAPK/Stat-3 [224] signalling pathways and there is evidence that IGFs which are trapped in the matrix during dentin formation can be released into dental pulp following demineralisation of dentin [225-227]. In addition, IGF-I has been used as a pulp capping material in rat molars and enhanced reparative dentinogenesis in this experimental model. IGF-I increased extra-cellular matrix secretion by dental-pulp derived fibroblasts [228] possibly via the induction of bone morphogenetic protein (BMP)-2 expression [229]. Caviedes *et.al* demonstrated higher expression of IGF-1R in dental pulp derived from human teeth having incomplete roots compared with complete roots. This might be as a result of

increased cell proliferation during root formation and suggest a role for IGFs in this process [230]. In agreement with this, a very recent study used *let-7c* microRNA based knock down of IGF1R expression in DPCs to demonstrate the importance of IGF-I stimulated signalling pathways in the osteogenic differentiation of DPCs [231]. IGF-II was highly expressed in dental pulp cells isolated from deciduous teeth where the pulp was exposed during the removal of proximal caries compared to the level of expression in healthy premolar teeth [232]. IGF-II also stimulates osteotypical matrix deposition and osteodentin formation [80]. Both IGF-I and IGF-II (acting via the IGF-1R) can induce ALP activity in canine dental pulp cells [233] and IGF-II secretion was reported during matrix mineralisation of human dental pulp derived fibroblasts

With respect to other cells and tissues of the oral cavity, a detailed study of IGF axis expression in differentiating ameloblasts reported position specific expression of IGF-I, IGF-II, IGF-1R and IGF-2R toward the outer enamal layer and away from pulp facing ameloblasts was reported arguing for the importance of the IGF axis in development of this tissue [234, 235] and an elegent study demonstrating the developmental stage-dependent expression of IGF-I in the contnually erupting rat incisor model [236] also argues strongly for a role of the IGF axis in the development of dental tissues. IGF-I induces the accumulation of amelogenin and ameloblastin suggesting that this growth factor plays a role in enamel bio-mineralization as ameloblasts also express IGF-1R [237]. A very recent study using stem cell populations isolated from apical papillae (SCAP) reported the stimulation of cell proliferation, ALP expression and mineralisation activity by IGF-I. Simultaneously, expression of odontogenic markers (dentin sialoprotein and dentin sialophosphoprotein) was down regulated arguing for a bias in IGF-I action toward bone formation and away from odontogenic differentiation in this tissue niche [238, 239]. Gotz *et al.* (2001) demonstrated the expression of IGF axis components during reparative processes in periodontal connective tissue with IGF axis proteins found in both the cellular and acellular compartments of extrinsic fibres of cementum. In addition cementoblasts and periodontal ligament fibroblasts express IGF-1R

and these findings confirm to some extent previous reports of an association between mineralised nodule formation and increased expression of IGF-1R [240]. As IGFII has been reported to be expressed at moderate to high levels in our experiments and in previous reports [241] this suggests that both IGF-I and IGF-II may be available locally to act through the IGF1R [242, 243]. This is also in accord with the observation that IGF-II and IGF-II along with all six IGFBPs are present in the ECM of the periodontal ligament and IGF-1R is present on the surface of periodontal ligament derived fibroblasts [244]. Finally, microarray analysis indicated an 8-fold increase in IGFBP-5 expression in DPCs derived fron non-carious wisdom teeth following 10 days treatment with mineralisation medium although this group did not confirm IGFBP-5 protein expression [39, 245].

Dental tissues develop from oral ectoderm and neural crest derived mesenchyme and contain pluripotent stem cell populations which display a developmental potential similar to embryonic stem cells (ESCs)[18, 246] . Dental pulp tissue represents an accessible source of such cells and hDPCs have been extensively used in various cell differentiation studies [29, 47, 247] . Undifferentiated cells display a fibroblast-like morphology with associated high efficiency for adherent colony formation and high proliferative potential[248]. These properties suggest that adult dental tissues may provide a source of material (often discarded in the clinic) to provide multipotent cells for subsequent tissue engineering studies. To date the main use of such cells has been in hard tissue engineering programmes with a view to treatment of dental trauma. As such hDPCs have been differentiated down osteogenic/odontogenic lineages to generate appropriate 3-dimensionalbone and or dentin structure [245, 249]. Although such strategies have met with some limited success, they are hampered by an inadequate understanding of the effects of local growth factors on dental pulp cell differentiation. In the context of osteogenesis, the IGF axis is known to play a crucial role in both the maintenance and differentiation of bone tissue [1]. Similarly, some recent studies have suggested a role for IGFs in regulating osteogenic/ odontogenic differentiation of hDPCs [223, 250] although the majority of these studies have given little attention given to the role of IGFBPs in this process. Therefore, in the current study we have

used the hDPC culture model to report comprehensively on IGF axis expression and activity during the osteogenic differentiation of these cells. In addition, we investigate the activity of the specific IGFBP-4 proteinase (PAPP-A), which is expressed as the sole IGFBP-4 protease by hDPCs, and the potential regulation of PAPP-A activity by stanniocalcin-2 (STC2) a recently described proteinase inhibitor of PAPP-A. We describe a series of co-ordinated changes in IGF axis expression which we suggest may represent part ofan “osteogenic signature” associated with differentiating hDPCs.

1.7 Aims & Objectives

Aim

To investigate the basal expression of components of the IGF-axis which are potentially implicated in regulating dental pulp cell osteogenic differentiation.

Objectives

- to investigate expression of IGFs, IGFRs and IGFBP mRNA in DPCs.
- to investigate IGF and IGFBP expression at protein level
- to investigate changes in IGF and IGFBP expression during osteogenesis
- to investigate activity of IGFs in differentiating DPCs
- to investigate the action of selected IGFBPs on IGF activity
- to examine IGFBP-4 and IGFBP-5 proteolysis in DPCs
- to examine the potential role of PAPP-A in IGFBP-4 proteolysis in DPCs
- to examine a potential regulation of PAPP-A activity by STC-2 in DPCs
- to examine membrane association of PAPP-A in DPCs

Chapter 2 Materials & Methods

2.1 Materials

Table 1 Material used in all experimental workTable

	Material	supplier	Catalogue no
General laboratory material	Alpha-Modified Minimum Essential Medium (α-MEM)	BioWhittaker, UK.	BE17-516F
	Phosphate buffered saline (PBS)	BioWhittaker, UK.	BE12-169F
	Penicillin/Streptomycin (Pen/Strp)	Sigma-Aldrich, UK.	P4333
	foetal bovine serum (FBS)	Sigma-Aldrich, UK.	F9665
	L-glutamine	Sigma-Aldrich, UK.	G7513
	0.25% (w/v) Trypsin-EDTA solution	Sigma-Aldrich, UK.	T4049
	Trypan blue solution, 0.4% (w/v)	Sigma-Aldrich, UK.	T8154
	Dimethylsulfoxide (DMSO)	Sigma-Aldrich, UK.	276855
	dexamethasone,	Sigma-Aldrich, UK.	31375
	L-ascorbic acid	Sigma-Aldrich, UK.	A4403
	Naphthol AS-MX phosphatase solution 0.25% (w/v) 20mL	Sigma-Aldrich, UK.	855
	Collagenase type I	ThermoFisher scientific	17100-017

	Dispase II neutral protease, grade II	Sigma-Aldrich, UK.	4942078001
	15mL centrifuge tubes	Corning®, UK.	430790
	50mL centrifuge tubes,	Corning®, UK.	430828
	T-25 cm^2 tissue culture flasks	Corning®, UK.	430639
	T-75 cm^2 tissue culture flasks	Corning®, UK.	430641
	T-175 cm^2 tissue culture flasks	Corning®, UK.	#431080
	10cm Petri dishes	Corning®, UK.	353803
	6 well tissue culture plates	Starlab UK	18341
	Syringes	TERUMO, UK	05SE1
	Syringe filters	Sartorius - UK	16532
	70µm strainer	Falcon, USA	352350
	Pasteur pipettes	SS Scientific lab supply, UK	PIP4105
Alizarin Red staining	Alizarin Red staining quantification assay	ScienCell, UK	8678
Qrt-PCR	RNeasy® mini kit	Qiagen, UK	74104
	β-mercaptoethanol	Applichem, UK	A4338
	Absolute ethanol (200 Proof, Molecular	Scientific Laboratory Supplies Ltd, UK	BP2818-500

	Biology Grade)		
	TaqMan probes (see Supplementary 1 for further information)		
	Ultrapure DNAse/RNase-free distilled water	Thermo Fisher Scientific, UK	10977035
	RNase-free tubes	Thermo Fisher Scientific, UK	AM12400
	Lens cleaning tissue	Thermo Fisher Scientific, UK	FB13067
	DNase I Amplification	Invitrogen, UK	18068015
	Optical adhesive seal	Geneflow Ltd, UK	P3-0300
	High Capacity RNA to cDNA kit	Applied Biosystems (ABI), UK	4387406
	master mix	Applied Biosystems (ABI), UK	4369016
	PCR tubes (0.2mL flat cap)	Bio-Rad, UK.	TFI0201
	Non-stick RNA-free 1.5mL microfuge tubes	Ambion ®, Mexico	AM12450
	96 well PCR microplates	Starlab, USA	I1402-9909
	PTC-100 Peltier -version 9	MJ Research	2030882
	Roche LC480 Light Cycler was used to generate data	Roche	05015243001
Western blot And ELISA	Precision Plus Protein™ WesternC™ Blotting Standards, 250 µl	BioRad, UK	1610376

	Precision Protein Streptavidin-HRP, 125 ul	BioRad, UK	1610381
	Pierce LDS Sample Buffer (non-Reducing) (4X)	BioRad, UK	84788
	10x Tris-buffered saline (TBS)	BioRad, UK	1706435
	10x Tris/Glycine/SDS, 1 L	BioRad, UK	1610732
	Restore™ PLUS Western Blot Stripping Buffer	BioRad, UK	46430
	Mini-protein® TGX stain-free gels, 8-16% (30 µl)	BioRad, UK	4568103
	Mini-protean® TGX stain-free™ gels 4 -15% (30 µl)	BioRad, UK	456-8083
	Trans-Blot Turbo transfer pack (PVDF) 7x 8.5cm	BioRad, UK	1704156
	Trans-Blot® Turbo transfer system (transfer pack mini format 69 BR007547)	BioRad, UK	1704150
	ChemiDoc imager	BioRad, UK.	Version 6
	Tween® 20	Fisher Scientific Ltd, UK	BPE337-500
	Bovine albumin Faction V	MP Biomedicals, UK	160069
	Tergitol solution type NP-40 70% solution	Sigma-Aldrich, UK	NP40S-1
	Corning gel-loading tips (0.2MM)	Sigma-Aldrich, UK	CLS4884-400EA

	Human IGFBP-4 Antibody (Antigen Affinity-purified Polyclonal Goat IgG)	R&D Systems, UK	AF804-SP
	Human IGFBP-5 Antibody (Antigen Affinity-purified Polyclonal Goat IgG)	R&D Systems, UK	AF875-SP
	Donkey Anti-Goat IgG HRP Affinity purified Secondary Antibody	R&D Systems, UK.	HAF109
	Anti-STC2 antibodies at 1 µg/mL.	R&D Systems, UK.	AF2830
	Polyclonal swine anti-rabbit IgG-HRP	R&D Systems, UK.	P0217
	Polyclonal rabbit anti-goat IgG-HRP	Dako	P0160
	Super-Signal® West Femto Maximum Sensitivity Substrates	Fisher scientific, UK.	34095
	Round gel-loading tips	Starlab, UK	I1022-0810
	3-8% Tris-acetate gradient SDS-PAGE gels were from Novex, Life Technologies.	Novex, Life Technologies.	**EA0375PK2**
	PVDF membranes	Millipore	IPVH00010
	ImageQuant LAS 4000 instrument (Images were captured and quantitated)	GE Healthcare	LAS 4000
	IGFBP-2 (DuoSet) ELISA	R&D Systems, UK.	DGB200

	IGFBP-3 (Quantikine) ELISA	R&D Systems, UK.	DGB300
	IGFBP-4 (DuoSet) ELISA	R&D Systems, UK.	DY804
	IGFBP-5 (DuoSet), ELISA	R&D Systems, UK.	DY875
	IGFBP-6 (DuoSet), ELISA	R&D Systems, UK.	DY876
	IGF-I (Quantikine) ELISA	R&D Systems, UK.	DG100
	IGF-II Quantikine ELISA	R&D Systems, UK.	DG200
	STC2 (DuoSet) ELISA	R&D Systems, UK.	AF2830
	Human PAPP-A (Quantikine) ELISA	R&D Systems, UK.	DPPA00
	Wash buffer	R&D Systems, UK.	WA126
	Reagent Diluent	R&D Systems, UK.	DY004
	normal goat serum	R&D Systems, UK.	DY005
	streptavidin- horseradish peroxidase (HRP)	R&D Systems, UK.	890803
	Substrate Solution: colour reagent A (H_2O_2), colour reagent B (Tetramethylbenzidine)	R&D Systems, UK.	DY999
	stop solution -2 N H_2SO_4	R&D Systems, UK.	#DY994
	The 96 well ELISA plates	Starlab, UK.	S1837-9600
	Varioskan Flash type 300 spectrophotometer (for absorbance determination)	Thermo-Scientific	300 spectrophotometer

Alkaline phosphatase assay	p-nitrophenol Alkaline buffer solution 100mL	Sigma-Aldrich, UK.	100-02-7
	p-Nitrophenyl Phosphate Liquid	Sigma-Aldrich, UK.	A9226
	Substrate System	Sigma-Aldrich, UK.	N7653

2.2 Methods

2.2.1 Isolation of dental pulp stromal cells

Healthy third molar teeth were used to study the expression of IGF axis components under basal conditions and during differentiation of dental pulp stromal/stem cells (DPSCs) into osteogenic lineages. Freshly extracted healthy fully erupted third molars were collected from adult patients (20-40 years of age) at the outpatients' dental clinic of Leeds Dental Institute. Teeth were obtained through Leeds Dental and Skeletal tissue bank (LDI Research Tissue Bank; 200116/1/a), with patients' informed consent. The age and gender of patients were recorded (Table 2). External tooth surfaces were washed using sterile PBS and surrounding soft tissue attachments were removed using a sterile scalpel. External tooth surfaces were washed again with sterile PBS and were cracked open using a decontaminated device to access the pulp tissue. Pulp tissue was gently separated by sterilized tweezers from the crown and root chambers, avoiding the apical third of the pulp tissue to prevent cross contamination with periodontal tissues. Isolated tissues were minced using a sterile scalpel before being digested in a solution of 5mL α-MEM, 3mg/mL collagenase type I and 4mg/mL dispase. The tissue- enzyme mixture was incubated for 1 h at 37° C and was continuously mixed using a shaker inside the tissue culture incubator. The tissue-enzyme mixture was regularly checked at 15 min intervals to avoid over-digestion. The enzymatic reaction was stopped with 20% (v/v) FBS after complete dissociation of the pulp tissue. Cell pellets were obtained by centrifugation at 1000 g for 10 minutes. The supernatant was carefully aspirated and discarded. The pelleted cells were re-suspended in proliferation medium consisting of α-MEM, supplied with 20% (v/v) FBS, 100 unit/mL Pen Strep, and 200mM L-glutamine. The cell suspension was passed through a 70µm strainer and seeded into T-25 cm^2 flask and 15cm Petri dish. The cultures were incubated at 37°C and 5% CO_2 in proliferation medium.

Table 2 Details of all donors used in the current study

Donor	Age	Gender	tooth Type
H1	20	Female	Third molar
H2	35	Female	Third molar
H3	24	Female	Third molar

We were obviously restricted in teeth selection by those donors which were available at the Leeds Dental Institute during the time of this study.

2.2.2 Cell culture and expansion

Isolated DPSCs were sub-cultured in T-175 cm² flasks, and media were changed every 5 days until cells reached 80% confluence. Monolayers were then washed with sterile cold PBS and detached using 0.25% (w/v) Trypsin-EDTA solution for 5 minutes. Twenty percent FBS was added to neutralize the trypsin, and then the cell suspension was transferred to a 50mL universal tube and centrifuged at 1100 g for 5 minutes. The supernatant was discarded, and the cell pellet was re-suspended in fresh proliferation media and cultured in the tissue culture flasks or plates according the experimental requirements. Cells were counted using a haemocytometer after staining with 0.01% (w/v) Trypan blue to exclude dead cells. Cells were seeded with the required densities according to the experimental plan.

2.2.3 Osteogenic differentiation of DPSCs

hDPSCs at passage 4 were cultured in 6-well plates at $1X10^5$ cells/well under basal conditions (α-MEM supplemented with 20% (v/v) FBS, 200mM L-glutamine, and 100unit/mL Pen Strep). When the cells reached 80% confluence, they were cultured in triplicate under basal or osteogenic conditions (basal medium + 10nM dexamethasone and 100μM of L-

ascorbic acid). Cultures were terminated at 1 and 3 weeks for further investigation of changes in gene expression using qRT-PCR for detection of relative changes in the expression of changes in the IGF axis gene expression. Histological staining (Alkaline Phosphatase (ALP) and Alizarin red stains), as well as IGF protein expression were also investigated. Experiments were performed on cells derived from three healthy donors and triplicate wells were used for each time point and each culture condition; basal and osteogenic.

2.2.4 Gene expression

2.2.4.1 mRNA extraction

DPCs cultured for 1 and 3 weeks under basal and osteogenic conditions were detached as described above and counted to determine the amount of cell lysis buffer required. mRNA extraction was performed according to manufacturer's instructions using Qiagen RNAeasy® min kit summarised in Table 3.

2.2.4.2 mRNA quantification

A NanoDrop spectrophotometer (ND 1000) was used to quantify the yield and purity of mRNA. 2 µl of the extracted mRNA was used and quantities were recorded as ng/µl. A260/280 ratios were also recorded as an indication of mRNA purity and were typically 1.8 - 2.0 indicating acceptable purity.

Table 3 RNA extraction using RNAeasy mini kit

<table>
<tr><th>Reagents</th><th colspan="2">The amount needed to perform the reaction</th></tr>
<tr><td rowspan="4">Buffer RLT
1- Add sufficient buffer RLT and mix.
2- Transfer lysed cells to nuclease free Eppendorf and mix on the vortex for 1 min.
3- Add 70% ethanol at 1:1 (v/v) with buffer RLT
4- Transfer up to 700µL of the mix to RNAeasy spin column placed in 2mL collection tube</td><td>Number of pelleted cells</td><td>Volume of buffer RLT (µl)</td></tr>
<tr><td>< 5 X 10^6</td><td>350</td></tr>
<tr><td>5 X 10^6 to 1 X 10^7</td><td>600</td></tr>
<tr><td>More than 1 X 10^7</td><td>Not suitable</td></tr>
<tr><td>5- Add RW1, centrifuge, and discard the flow through.</td><td colspan="2">700µL</td></tr>
<tr><td>6- Add buffer RPE, centrifuge, and discard the flow through.
7- Repeat step no. 6 and use new collection tube.</td><td colspan="2">500µL</td></tr>
<tr><td>8- Add nuclease free water, centrifuge and collect the RNA elutes in a new tube.</td><td colspan="2">30µL</td></tr>
</table>

2.2.4.3 DNase purification

To ensure that the mRNA was pure and clear of any genomic DNA, DNase I amplification grade kit was used according to the manufacturer's instructions. Briefly, a mixture of DNase I buffer, enzyme, and mRNA sample (up to 1µg) were mixed in a 10µL reaction volume and incubated at room temperature for 15 minutes. The reaction was stopped by adding 1µL EDTA to the mix, then incubating at 65°C for 10 minutes in the PTC-100 thermal cycler.

2.2.4.4 Reverse transcription

Reverse transcription was carried out to generate single stranded cDNA from the mRNA by preparing 20µL reaction volume using the ABI high capacity RNA to c-DNA kit. Briefly, 10µL of buffer were added to 1µL of enzyme, and 9µL of mRNA sample, and then incubated in the PTC-100 thermal cycler for 1 hour at 37°C followed by 5 minutes at 95°C. Negative controls for the experiment were generated during this step, by removing the mRNA template in one reaction and reverse transcriptase enzyme in the other.

2.2.4.5 qRT- PCR

Quantitative real time PCR was performed using a Roche LC480 light cycler. The experiment was performed in a 20µL reaction volume composed of 10µL gene expression master mix, 1µL Taqman gene expression assay specific for each gene, 8µL nuclease free water and 1µL c-DNA sample. The 20µL mix was added into each well of 96 well PCR reaction plate in triplicate. In addition, triplicates of non-template negative control and RT negative control were included in each plate. The plate was sealed securely and centrifuged for 10 second before starting the amplification procedure using the light cycler. Amplification was carried out according to Applied Biosystems (AB) recommendations for Taqman® probes.

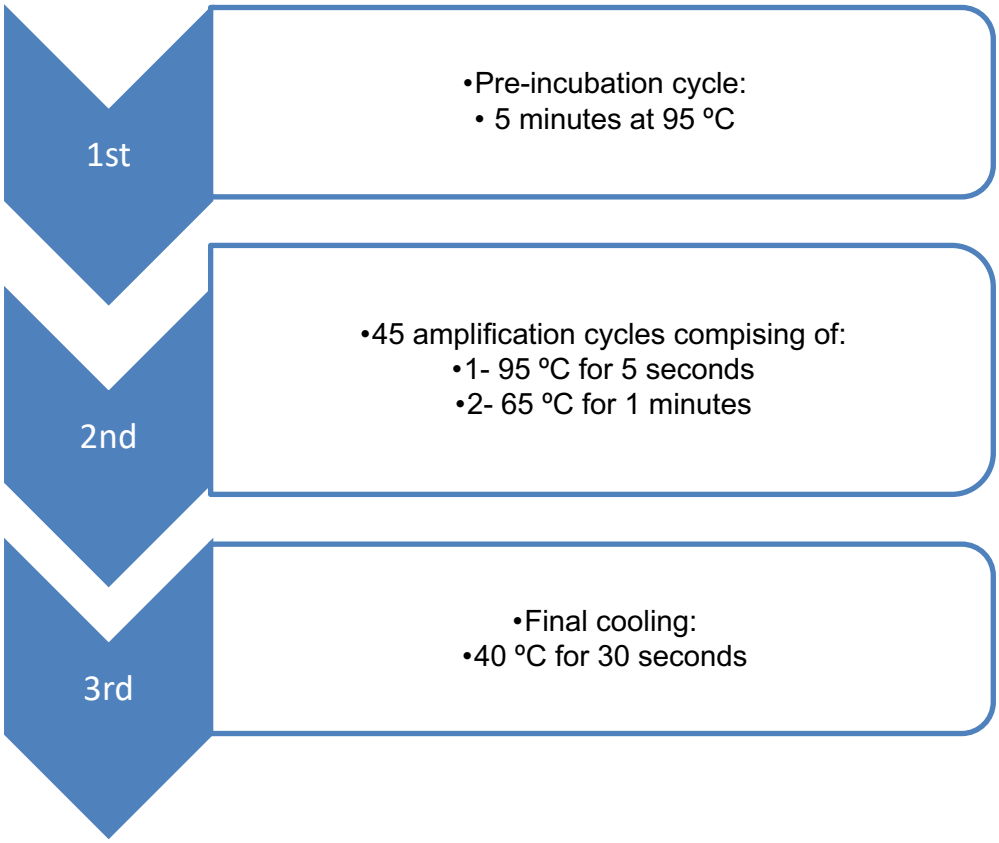


Figure 10 Details of qRT-PCR amplification program

Taqman gene expression assay identifiers are presented in Appendix Table I.

2.2.4.6 Data analysis

For each gene, relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [251, 252]. In brief, the threshold cycle (Ct) value was determined for each gene of interest in triplicate. Technical replicates were averaged, and then normalized to that of the house-keeping gene GAPDH. This is referred to as the ΔCt value. The ΔΔ Ct values were determined by normalizing the ΔCt value for each treated sample (under osteogenic conditions) to the appropriate control samples (under basal conditions) at each time point to be able to determine the relative changes in gene expression of IGF axis following osteogenic induction of DPCs. The relative changes in gene expression were calculated using the $2^{-\Delta\Delta ct}$ equation. In all cases, the $2^{-\Delta\Delta ct}$ values for all markers were compared in DPCs. Changes in the gene expression levels were plotted as $2^{-\Delta\Delta ct}$ ± SD.

2.2.5 Protein expression

DPCs were cultured under both basal and osteogenic conditions for 1 and 3 weeks as described previously, changing media was performed once every week. One mL of conditioned medium was collected from each medium change and was either freeze-dried and stored at -20ºC or directly stored at -80ºC without freeze-drying.

2.2.5.1 Western blotting

In western blotting, 1mL of medium conditioned by DPCs cultured under basal and osteogenic conditions was collected, and freeze-dried. Freeze-drying is a process used to remove the water from the sample by freezing then drying it under a vacuum at very low temperatures. Powdered media were then dissolved in 4X SDS-polyacrylamide (SDS-PAGE) sample buffer containing β-mercaptoethanol (1:20, v/v) and loaded onto 4-15% (w/v) SDS-polyacrylamide pre-cast gels (30μL /well). Suitable molecular weight markers (5μL of dual colour standards, 10 -250 kDa) were loaded in a lane next to the samples to be able to

determine molecular weight of the target proteins. Gels were run at 120V for 1 hour then proteins were transferred to PVDF semi dry membranes using the Trans-Blot Turbo device (BioRad) for 10 minutes. After blotting, the membrane was rinsed three times with distilled water (dH_2O) and incubated for 1hour with gentle agitation at room temperature in blocking solution, which comprised 5% bovine serum albumin (BSA) in Tris-buffered saline containing Tween-20 (TBS-T, 0.05%, v/v). After blocking, the membrane was washed three times for 5 minutes using 0.05 (v/v) TBS-T then incubated overnight at room temperature with one of the following primary antibodies: anti- IGFBP-4 or anti-IGFBP-5 reconstituted (1:1000, v/v) in 0.05% (v/v) TBS-T. Next day, the membrane was washed three times for 5 minutes using 0.05% (v/v) TBS-T then incubated with the appropriate streptavidin horseradish peroxidase (HRP) conjugated secondary antibody reconstituted (1:10000, v/v) in 5% (w/v) BSA in 0.05% (v/v) TBS-T for 1 hour with gentle agitation at room temperature. After that, the membrane was washed three times, each for 15 minutes using 0.05% TBS-T, and was developed with ultra-sensitive enhanced chemiluminescent (ECL) substrate (A and B, 1:1 v/v) and images were obtained and recorded on the ChemiDoc imager.

2.2.5.2 Enzyme-linked immunosorbent assay (ELISA)

DPCs were grown under basal and osteogenic conditions for 1 and 3 weeks. Conditioned medium (1mL) was collected from each culture at 1 and 3 week time points under basal and osteogenic conditions. IGFBP(2,3,4,5,6), IGF-I, IGF-II, STC2 and PAPP-A concentrations in conditioned media were determined by ELISA using human IGFBP-2 Duo Set ELISA kit , human IGFBP-3 Quantikine ELISA Kit, human IGFBP-4 Duo Set ELISA kit, human IGFBP-5 Duo Set ELISA kit, human IGFBP-6 Duo Set ELISA kit, human IGF-I Quantikine ELISA Kit, human IGF-II Quantikine ELISA Kit, human stanniocalcin 2 ELISA kit and Human PAPP-A Quantikine ELISA kit according to the manufacturer's protocol. Briefly, capture antibody (mouse anti-human) was diluted at 2µg/mL for IGFBP-2 and at 4µg/mL for IGFBP-4, 5 and 6) and for STC2 was diluted at 7.5µg/mL in PBS was plated in 96 well microplates (100µL/well) and incubated overnight at room temperature. On the following day, the plate was washed with 300µL of 1:25 (v/v) diluted washing buffer (0.05% (v/v) Tween-20 in PBS) four times. Plates were blocked using 300µL of the 1:5 (v/v) regent diluent (5% (v/v) Tween -20 in PBS, 0.2µm filtered) and incubated for 1 hour at room temperature. After washing four times with 400µL washing buffer, 100µL of the samples, and appropriately diluted standards were added to the previous 96 well plate and incubated for 2 hours at room temperature. Plates were washed as above and 100µL of the detection antibody (biotinylated goat anti-human IGFBP2, 4, 5, 6 and STC2) were added at 200ng/mL in reagent diluent with 2% (v/v) heat activated goat serum for 2 hours at room temperature. After washing, 100µL of working dilution of streptavidin-HRP (1:200, v/v) was added for 20 minutes and plates were incubated at room temperature in the dark. Finally, plate was washed and 100µL of substrate solution (colour reagent A: H_2O_2 and colour reagent B: Tetramethylbenzidine, 1:1 v/v) were added and the plate was incubated for 20 minutes at room temperature in dark. 50µL of stop solution (2 N H_2SO_4) was added and absorbance was determined immediately using a microplate reader (Thermo-Scientific Varioskan Flash type 300 spectrophotometer) at 450nm.

For IGFBP-3, IGF-I, IGF-II and PAPP-A, the Quantikine ELISA kit was used. The assay was performed following the manufacturer's instructions. In brief, 100µL of assay diluent for (IGFBP-3: RD1-62, IGF-I: RD1-53 and PAPP-A: RD1-15) was plated in a 96 well plate. Next, 100µL of the samples, and appropriately diluted standards in calibrator diluent for (IGFBP-3: RD5P, IGF-I: RD5-22, IGF-II: RD5-42 and PAPP-A: RD6-14) were added to the assay diluent and incubated for 2 hours at 2-8ºC. The plate was washed with 400µL of 1:25 (v/v) washing buffer (buffered surfactant) three times. Then for IGFBP-3, 200µL of chilled IGFBP-3 polyclonal anti-IGFBP-3 HRP conjugate was added per well and incubated for 2 hours at 2-8ºC, for IGF-I, It was 200µL of chilled human IGF-I conjugate added per well and incubated for 2 hours at 2-8ºC, for IGF-II, 200µL of Human IGF-II Conjugate to each well and covered with a new adhesive strip then Incubate for 2 hours at room temperature on the shaker, for PAPP-A, 200µL of Human PAPP-A Conjugate added to each well and covered with adhesive strip then Incubate for 2 hours at room temperature. The plate was washed as above and 200µL of substrate solution were added per well (colour reagent A: stabilized H_2O_2, colour reagent B: stabilized Tetramethylbenzidine, 1:1, v/v) and incubated for 30 minutes at room temperature in the dark. Finally, 50µL of stop solution (2 N H_2SO_4) was added and absorbance was determined immediately using a microplate reader at 450nm. All ELISA assays were specific as reported on suppliers product information sheets (see the following websites for the following products from R&D Systems, UK.)

https://www.rndsystems.com/products/human-igfbp-2-quantikine-elisa-kit_dgb200

https://www.rndsystems.com/products/human-igfbp-3-quantikine-elisa-kit_dgb300

https://www.rndsystems.com/products/human-igfbp-4-duoset-elisa_dy804

https://www.rndsystems.com/products/human-igfbp-5-duoset-elisa_dy875

https://www.rndsystems.com/products/human-igfbp-6-duoset-elisa_dy876

https://www.rndsystems.com/products/human-igf-i-igf-1-quantikine-elisa-kit_dg100

https://www.rndsystems.com/products/human-igf-ii-igf2-quantikine-elisa-kit_dg200

https://www.rndsystems.com/products/human-stanniocalcin-2-stc-2-antibody_af2830

https://www.rndsystems.com/products/human-pappalysin-1-papp-a-quantikine-elisa-kit_dppa00

2.2.6 *In vitro* bioassay

DPCs were grown to 80% confluence to examine the effect of IGF by incubating cells with and without adding (100nM) of IGF-I for 14 days in both basal and osteogenic conditions. Then, cells incubated in basal and osteogenic medium with a fixed concentration of IGFBP-4 and IGFBP-5 at (10nM), and varying concentrations (0nM, 1nM, 10nM, 100nM) of IGF-I. Medium was changed at day 4, 7, 10, 13, 17 and cultures were terminated at day 21.

ALP enzyme activity has been examined as an osteogenic marker. Alkaline phosphatase activity was measured by the ability of alkaline phosphatase enzyme to convert the colourless substrate para-nitrophenylphosphate (pNPP) into yellow para-nitrophenyl (pNP) as previously described [253]. Briefly, Cells were washed twice with PBS, and lysed by 200µL of 0.1% (v/v) Triton™x-100 followed by three cycles of freezing and thawing. Lysates were centrifuged (1000g) for 5 minutes and 20µL of the supernatants were used in ALP activity assay. Standards were prepared by diluting pNP in buffer, provided with the pNPP substrate as a kit, at concentrations of 5, 25, 50, 100, 150 and 200 nmol/mL. A 100µL of each standard was added to the wells of a 96 well plate (n=3). A 90µL of pNPP substrate was then added to 10µL of sample lysates in triplicates in 96 well plate. The plate was then incubated at 37 ºC for 30 minutes in dark. The reaction was stopped by adding 100µL of 1M NaOH. The sample absorbance was measured at 405nm using a microplate reader. Alkaline phosphatase specific activity was expressed as nanomoles of p-nitro phenol/µg DNA.

For Alizarin red s staining quantification assay, calcium deposits were evaluated in cell culture by examining the effect of IGF through incubating cells in both basal and osteogenic conditions with and without adding (100nM) of IGF-I for 14 days. Cells

were washed 3 times with PBS. 4% formaldehyde was used to fix cells for 15 minutes at room temperature. Then cells were washed 3 times with distilled water. After removal of distilled water, 1mL of Alizarin red stain was added for each well and incubated for 20-30 minute at room temperature with gentle shaking. The cells were washed 5 times with dH2O after dye removal and stored at -20°C before dye extraction. 200 µL of 10% acetic acid were added to each well and incubated for 30 min at room temp with shaking then cells were collected using scraper and transferred to a 1.5-mL microcentrifuge tube and vortexed for 30 sec. Samples were heated at exactly 85°C for 10 minutes and incubated for 5 min on ice then centrifuged for 15 min. After centrifuging, 200µL of supernatant were transferred to new tubes and 75µL of 10% ammonium hydroxide were added to neutralize the acid. Finally, 50 µL/well of samples and standards were added in triplicate on a 96-well plate (opaque-walled, transparent-bottomed plates) and then, the absorbance at 405 nm with a plate reader was recorded.

2.2.7 *In vitro* IGFBP proteolysis

I-IGFBP-4 (10nM) and IGF-I (100 nM) were incubated with 20 pM recombinant PAPP-A (rPAPP-A) or basal/osteogenic CM containing an equivalent concentration of PAPP-A (determined by ELISA see **Fig20**) at 37C in a medium of 2mM $CaCl_2$/50 mM Tris pH 7.5. Total assay volume was 25ul. Inhibitory anti-PAPP-A monoclonal antibody (1/41) or control monoclonal IgG1 (both 20 nM) were present when required. Reactions were terminated after 6hr by the addition of x1 non-reducing SDS sample buffer. Following electrophoresis through 12% polyacrylamide SDS-PAGE gels levels of intact and fragmented IGFBP-4 determined by direct autoradiography as previously described [184]. Details of ^{125}I-labelled IGFBP-4 proteolysis assay have been published previously [181].

2.2.8 Immunoprecipitation and WB

Immunoprecipitation (IP) of PAPP-A and STC2 from human SMC conditioned media was performed by incubation (4°C, 16 h) of 500 µl medium with 15 µl protein G-Sepharose 4 Fast Flow beads (GE Healthcare) cross-linked to PAPP-A-specific mAb PA11 or mAb STC221 respectively, in a total volume of 1 mL. Beads cross-linked to irrelevant IgG2a served as a control. Antibodies were immobilized at 2 mg/mL by using dimethyl pimelimidate dihydrochloride (Sigma) cross-linking [184] After three washes in 20 mM NaH2PO4, 500 mM NaCl, 0.01% Tween 20, pH 7.4, precipitated protein was eluted with 0.1 M glycine pH 2.0. At the end of all steps, beads were centrifuged at 3,000 ×g for 5 min. Bound protein was eluted into non-reducing SDS sample buffer. Eluted protein was analyzed by Western blotting. Briefly, proteins were separated by 3-8% Tris-acetate or 12% Tris-glycine SDSPAGE and blotted onto PVDF membranes. After blocking with 2% Tween 50mM Tris-HCl,500 mM NaCl (pH7.5) for 5 min, membranes were incubated overnight at rom temp with polyclonal rabbit anti-PAPP-A [178]or polyclonal goat anti-STC2 at 1 µg/mL. Antibodies were diluted in 2% skimmed milk powder in 50 mMTris-HCl,500 mM NaCl, 0.1% Tween 20, pH 9.0 (TST). Membranes were subsequently incubated (30 min) with polyclonal swine anti-rabbit IgG-HRP or polyclonal rabbit anti-goat IgG-HRP diluted 1:2000 in 2% skimmed milk powder in TST, and then developed by enhanced chemiluminescence (ECL Prime, GE Healthcare). Images were captured using an ImageQuant LAS 4000 instrument (GE Healthcare). Between the steps, membranes were washed in TST three times. Recombinant PAPP-A, STC2, and the PAPP-A: STC2 complex used for control were generated as described previously [204, 254]. Details of the IP/WB methods have been published previously in the supplementary material to ref [255].

2.2.9 Statistical analysis

All experiments were carried out in triplicate from three different donors in each group; DPCs. ELISA and qRT-PCR results were analysed using students t-test and one way analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests. P-values were determined, and $P<0.05$ was considered significant. The statistical analyses were carried out using the GraphPad Prism software (v-6).

Chapter 3: IGF axis expression and activity in DPSCs

3.1 Introduction

We have previously partially characterised IGF axis expression and activity in DPCs. in this section we confirm our previous observations and extend our studies to accurately quantify IGF axis genes at both the mRNA and protein level using qRT-PCR and ELISA respectively [1, 2].

3.2 qRT-PCR

We used TaqMan based qRT-PCR to quantify relative expression of IGF axis genes. Previous studies have established that 7 day and 21 day time points were appropriate to study IGF axis gene expression in hDPCs (see figure 2 in [1]).

Expression was expressed relative to GAPDH housekeeping gene using the ΔCt method (see Methods 2.2.4.6). In Fig 10 we show data obtained for IGF axis expression following culture of DPCs for 1wk under basal conditions. These data show that all IGF axis genes, with the exception of IGF-I and IGFBP-1 were expressed in DPCs under our culture conditions. The fact that IGF-II is the main growth factor expressed by DPCs has important implications and we return to subject in the Discussion section of this Chapter. In terms of abundance of IGFBP expression we found that the following order BP4>BP5>BP-6 ~ BP-2 > BP-3 was repeatedly seen – note the logarithmic axis of Figure 10. We found that IGF1R and IGF2R were expressed at approximately the same level, although we present evidence later in this section that in common with most cell types IGF1R is the active IGFR in DPCs. With raw Ct values for those genes expressed at 15-25 (Table 4) these represent moderate to highly expressed mRNAs. As indicated above with Ct values >30 IGFBP-1 is expressed at a very low level and with Ct values typically >35, IGF-I is most likely not expressed by DPCs.

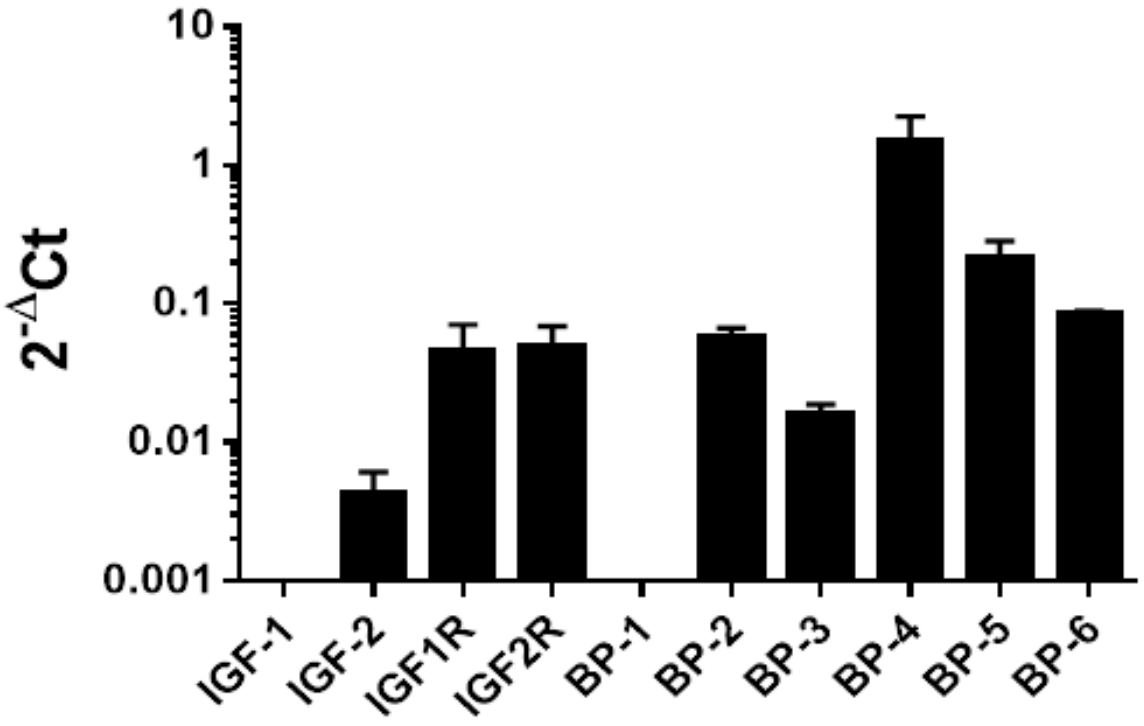


Figure 11 qRT-PCR analysis of IGF axis expression in DPCs grown under basal conditions for 1wk

Data are expressed as $2^{-\Delta Ct}$ relative to GAPDH and represent triplicate technical replicates of three separated cell cultures (mean ± SD; n=3). Note logarithmic scale of y-axis. IGF-I and IGFBP-1 displayed Ct values > 30 indicating very low expression of these genes.

Table 4 A representative set of raw Ct values for IGF axis gene expression in DPCs grown under basal conditions indicates reproducibility of data

GAPDH	15.81	17.13	17.71
IGF-I	35.66	36.46	38.20
IGF-II	24.41	24.48	25.89
IGF1R	20.65	22.32	21.65
IGF2R	20.69	21.74	21.50
IGFBP-1	31.16	30.63	30.89
IGFBP-2	20.90	21.27	20.85
IGFBP-3	23.21	22.96	22.59
IGFBP-4	15.65	16.64	17.12
IGFBP-5	18.85	18.89	19.89
IGFBP-6	20.39	20.56	20.43

Also shown are values for the house keeping gene (HKG) GAPDH. Individual triplicate values are shown. Note that Ct values vary inversely with mRNA abundance (see Methods section).

As this current work is mainly concerned with the osteogenic differentiation of DPCs initially we wished to examine how IGF axis expression altered during differentiation. Previous work had shown no consistent change in the expression of IGF1R and IGF2R in DPCs following differentiation (H. Al-Kharobi PhD Thesis 2016, University of Leeds). Similarly, IGF-I expression remained undetectable during osteogenic differentiation of DPCs and we return to the issue of IGF-II gene and protein expression later in this thesis (see Section 5.3). For the present we focussed on how the expression of IGFBPs was affected by differentiation of DPCs. Fig 11 shows the results of such and experiment where the fold change in IGFBP expression is reported using the $2^{-\Delta\Delta Ct}$ method (see 2.2.4.6) and represents the increase (>1) or decrease (<1) change in IGFBP expression following incubation of DPCs under osteogenic conditions (see Methods). As shown in Fig 12 our data confirm previous observations of reciprocal changes in IGFBP-2 and IGFBP-3 expression and we have previously reported the potential biological significance of these alterations in IGFBP expression [1]. However, of more relevance to the current study we found no significant change in the expression of IGFBP-4, -5 or -6 during osteogenic differentiation of DPCs. Therefore, our previous studies and this current work have comprehensively detailed the changes in IGFBP mRNA expression in DPCs during osteogenic expression. Note that the expression of IGFBP-1 remains very low under osteogenic conditions. In order to complete our analysis of IGFBP profile in these cells it was important to examine whether the mRNA profiles reported above were replicated at the level of IGFBP proteins. As IGFBPs are essentially secreted proteins to achieve this we assayed by ELISA the concentrations of IGFBP proteins in medium which had been conditioned by DPCs under basal or osteogenic conditions.

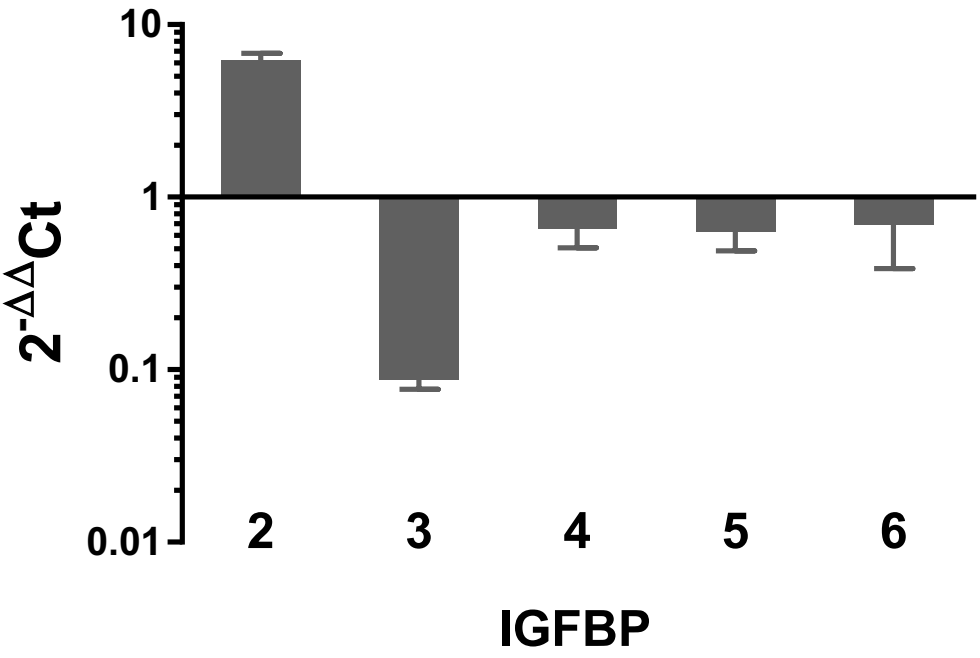


Figure 12 Fold change in IGFBP expression following osteogenic differentiation of DPCs for one week.

IGFBP expression in DPCs grow under basal and osteogenic conditions was analysed using the $2^{-\Delta\Delta Ct}$ method (see Methods 2.2.4.6 Data analysis) and represent technical triplicates for triplicate cell cultures grown under basal or osteogenic conditions (mean ± SD; n=3). Values >1 represent increased expression osteogenic v basal and <1 decreased expression osteogenic v basal. IGFBP-1 was expressed at a very low level under both basal and osteogenic conditions and is not shown in the Figure (see Fig11 and Table 4). Note logarithmic scale for Y-axis.

3.3 ELISA of IGFBPs

3.3.1 Assay conditions

Prior to undertaking these experiments, it was important to investigate and establish the appropriate conditions for Elisa. For IGFBP-2 and IGFBP-3 these had already been established (H Al-Kharobi PhD thesis; University of Leeds 2016) therefore we focussed our attention on IGFBP 4-6. Initial experiments for ELISA of IGFBP-4 and IGFBP-5 examined the appearance of standard curves for both analytes in a background of different buffer/culture media conditions (see Methods 2.2.5.2 for further details). Representative results of such an experiment for both IGFBPs are shown in Fig 13. In these experiments dilutions of standard were made in buffer containing different ratios of reactant diluent (RD - supplied in ELISA kit) and unconditioned serum free medium (M). For IGFBP-4 concentrations of standard were diluted 2-fold between 0.5-32 ng/ml and for IGFBP-5 standards were diluted 2-fold between 0.625-40 ng/ml. For both IGFBP-4 and IGFBP-5 assay interference was apparent as the ratio RD: M increased. In practice this meant that assays of cell conditioned medium were conducted in samples which were diluted at least 1:10 in RD. At this level of dilution concentration values for both IGFBP-4 and IGFBP-5 fell within the standard curve which was also constructed using standards which were diluted in a 1:10 (RD:M) buffer background. It is important to note that failure to perform such preliminary experiments would lead to an underestimation of IGFBP-4 and -5 levels if inappropriately large volumes of cell conditioned medium were assayed. Fortunately for IGFBP-6 no issues of assay interference were evident although in interests of consistency IGFBP-6 ELISA and standard curves were also performed at 1:10 (RD:M) dilutions and under these conditions IGFBP-6 concentrations also fell within the assay range. After establishing these assay conditions, we then assayed DPC conditioned medium for IGFBP content.

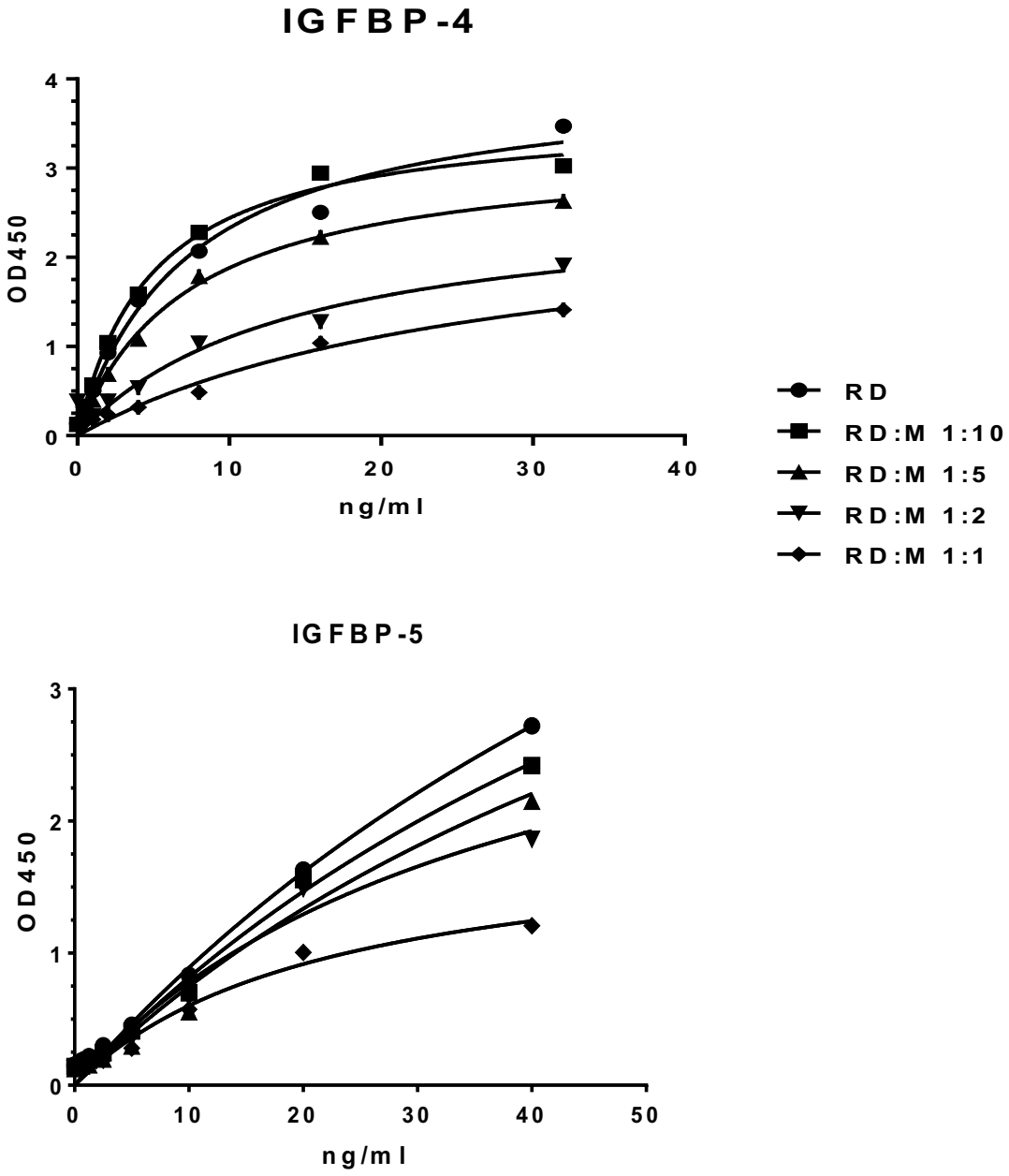


Figure 13 Culture medium interferes with assay of IGFBP-4 and IGFBP-5 present in conditioned medium

Standard curves for IGFBP-4 (0-32 ng/ml) and IGFBP-5 (0-40 ng/ml) were constructed in varying ratios of reactant diluent (RD) to culture medium (M) as indicated. Standards were assayed as duplicates although for clarity only mean values are shown. Increasing RD: M ratios led to increased interference for both IGFBP-4 and IGFBP-5 ELISA.

3.3.2 IGFBP concentration in conditioned medium

Using the conditions established above for IGFBP4-6 and those previously described for IGFBP-2 and IGFBP-3 (H.Al-Kharobi, PhD Thesis; University of Leeds 2016) we assayed IGFBP concentrations in DPC conditioned medium under both basal and osteogenic conditions **(Fig 14).** We confirm previous observations of reciprocal alterations in IGFBP-2 and IGFBP-3 protein concentrations following osteogenic differentiation of DPCs. However, we show that for IGFBP-4, -5 and -6 there is no change in protein concentrations in conditioned medium following differentiation of DPCs. We would also highlight that the relative IGFBP protein abundance closely mirrors that for IGFBP mRNA expression (compare **Fig 11** and **Fig 14**). In accordance with this IGFBP-4 (~ 80 ng/ml) is more abundant than the combined concentrations of IGFBP-5 (~12ng/ml), IGFBP-6 (~3 ng/ml), IGFBP-2 (~2 ng/ml) and IGFBP-3 (~0.25 ng/ml). Therefore, although we have previously reported on the effects of IGFBP-2 and IGFBP-3 as an enhancer and inhibitor respectively of IGF-I action in DPCs the fact that IGFBP-4 and IGFBP-5 are more abundantly expressed in these cells led us to investigate the potential biological activity of these IGFBPs in our DPC cultures. Prior to this we wished to confirm independently in our hands that IGFs were biologically active in DPC cultures.

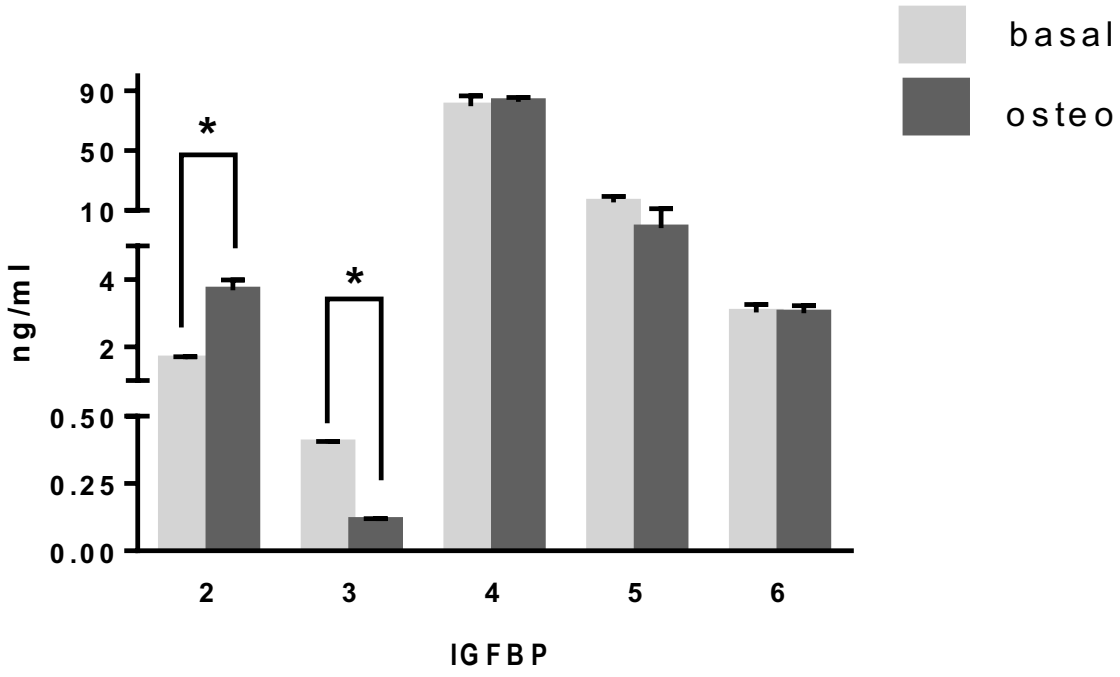


Figure 14 Elisa determination of IGFBP-2, 3, 4, 5 and 6 protein concentrations in DPC

conditioned medium in cells grown under basal or osteogenic conditions. Data are derived from triplicate technical replicate assays of conditioned medium derived from 3 separate cell cultures grown under basal or osteogenic conditions and represent mean ± SD (n=3). Based on qRT-PCR data IGFBP-1 ELISA was not performed. * $p < 0.05$ Student's t-test basal v osteogenic. Note split Y-axis scale.

3.4 IGF activity in DPCs

As indicated above the biological end point for most of our studies was the differentiation of DPCs into matrix secreting osteoblast like cells. Amongst assays used to measure the differentiation of mesenchymal cells into pre-osteoblast or osteoblast cells are colorimetric detection of matrix in cells culture (usually Alizarin Red) [256] and /or detection of osteogenic specific markers such as RunX2, Osteocalcin (OCN) or alkaline phosphatase (ALP) and our group has routinely used such assays to monitor osteogenic differentiation of DPCs [257]. ALP in particular lends itself to a moderately high throughput assay in that ALP enzyme activity is easily measured in cell lysates following exposure of intact cell cultures to various osteogenic conditions [258, 259]. Initially therefore we examined both Alizarin Red and alkaline phosphatase activity in DPCs under basal and osteogenic conditions and the effect of IGF-I at a single concentration of 100nM under both these conditions. The results of this experiment are shown in **Fig 15.** For Alizarin Red based assay addition of 100 nM IGF-I increased staining in cultures under both basal and osteogenic conditions. It is believed that even in the absence of osteogenic agents a proportion of MSCs in confluent cultures may be able to partially differentiate. In our hands only low levels of Alizarin Red staining was apparent under basal conditions although this was stimulated by addition of IGF-I (**Fig 15** - first two columns upper panel). Under osteogenic conditions Alizarin Red staining was clearly increased (compare columns 3 and 1) and this effect was further enhanced under osteogenic conditions in the presence of IGF-I (compare columns 3 and 4). Very similar data were seen when ALP activity was measured in DPC cells treated under conditions identical to those for Alizarin Red assay with IGF-I enhancing ALP enzyme activity under osteogenic conditions. Because the ALP assay appeared to provide a greater signal to noise ratio in terms of the IGF-I enhancement of osteogenic activity and also due to the fact that ALP assay lends itself to a higher throughput of samples we decided to concentrate on developing this assay in our future studies. The ALP assay described in **Fig 15** was conducted over a 14 day incubation period. However, in order to examine the time course of

IGF-I stimulation of ALP activity we carried out the experiment described in **Fig 16.** This experiment showed that a significant stimulation of osteogenesis in the absence of IGF-I occurred at day 14 of incubation. However, in the presence of 100nM IGF-I a significant stimulation of osteogenesis was evident at day 10 of incubation. Although this significant effect of IGF-I persisted at days 14, 17 and 21 of incubation the greatest effect of IGF-I in the presence of osteogenic medium versus osteogenic medium alone (i.e. O v O+IGF-I) was apparent at day 10. Therefore, as this was also the earliest time point at which significant differences were seen (O v O+IGF-I) we used this incubation time point in subsequent studies. After these preliminary experiments we then went on to examine the effects of the two most abundantly expressed IGFBPs – IGFBP-4 and IGFBP-5 – on the action of IGF-I (and independently of IGF-I) in DPCs.

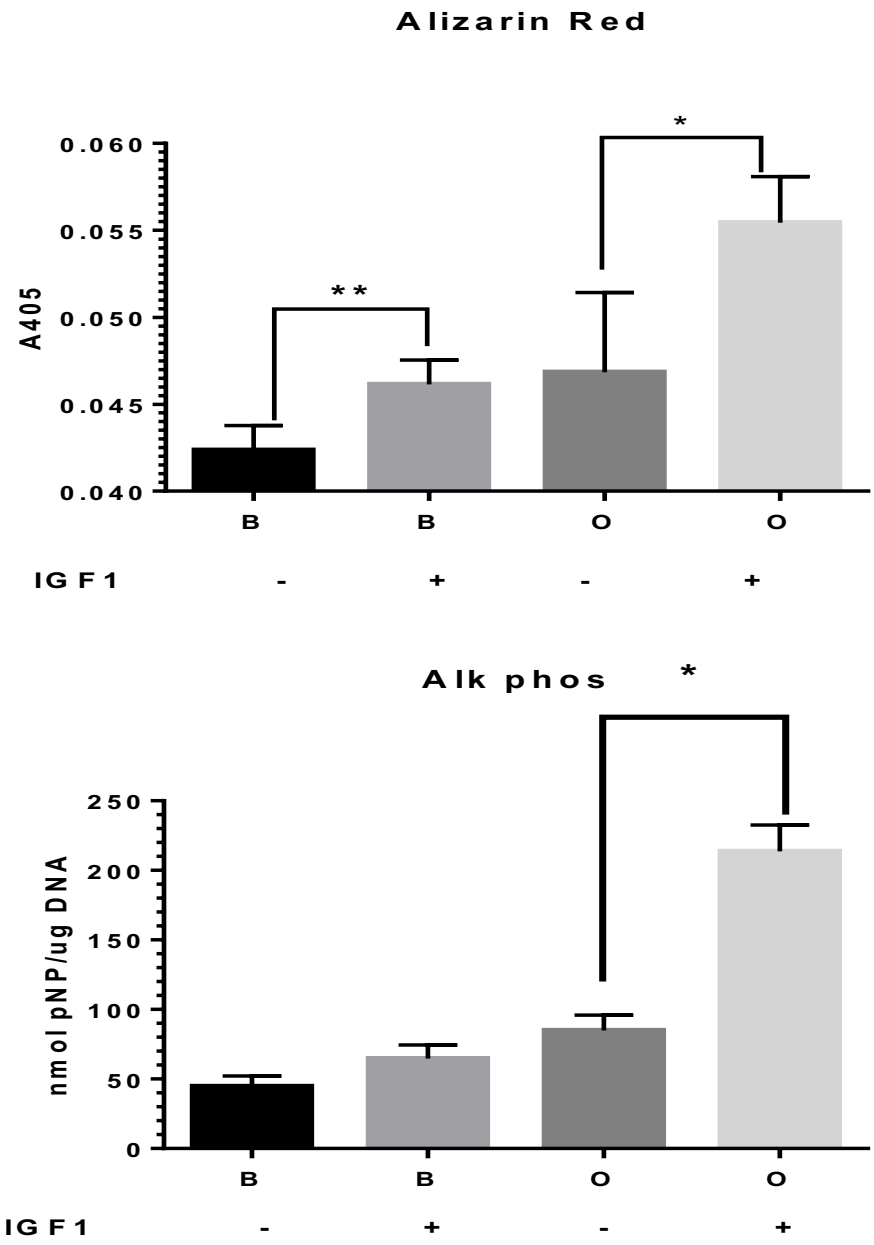


Figure 15 Semi-quantitative assay for Alizarin Red staining and Alkaline phosphatase activity

Semi-quantitative assay for Alizarin Red staining (upper panel) and alkaline phosphatase activity (lower panel) in DPCs grown under basal (B) or osteogenic (O) conditions in the absence (-) or presence (+) of 100 nM IGF-I for 14 days. Data are derived from triplicate cultures assayed as technical duplicates and are presented as mean ± SD (n=3). *$p<0.05$, **$p< 0.01$. See Methods section 2.2.6 for further details.

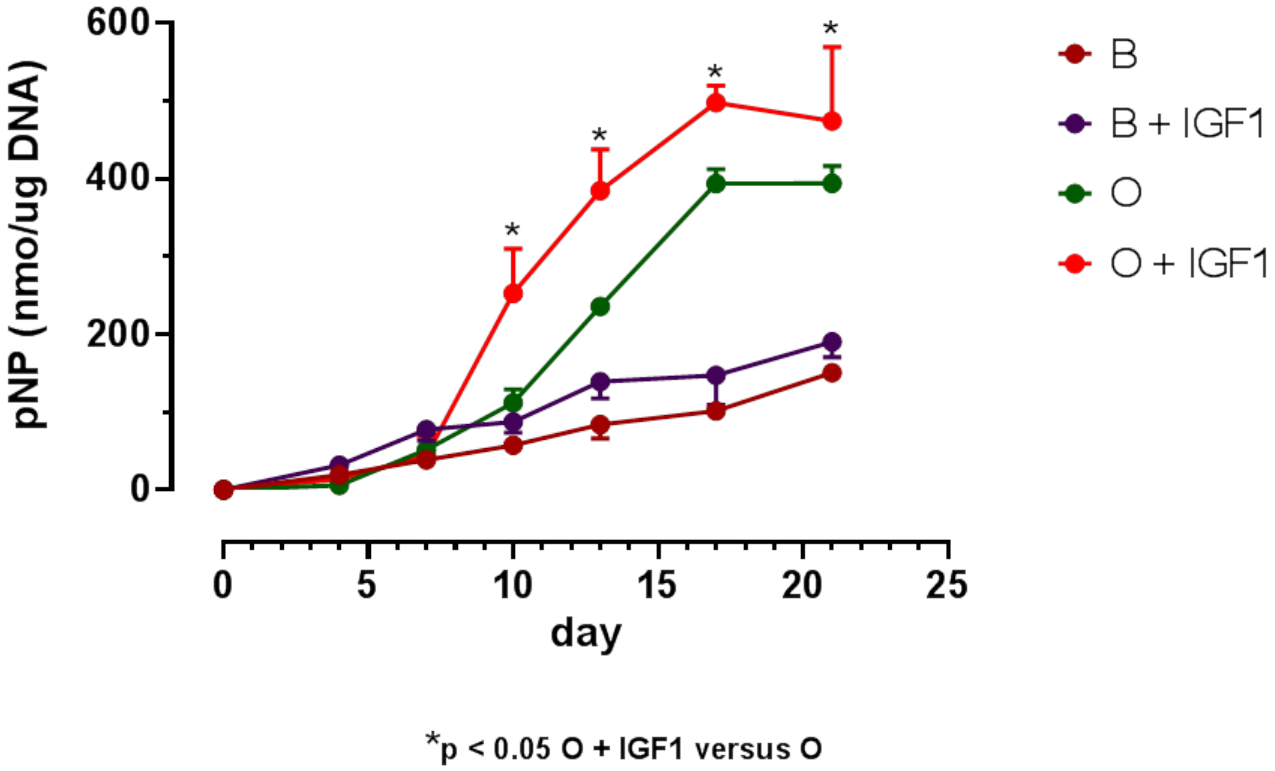


Figure 16 Time course for AP activity in DPCs grown over the period 0-21 days

DPCs were grown under basal or osteogenic conditions as indicated in the presence or absence of 100 nM IGF-I. Cultures were terminated on days 4, 7, 10, 13, 17 and 21 and assayed for AP activity. Assays were performed in triplicate and are presented as mean ± SD (n=3). In some instances, SD values are lower than symbol size. * p<0.05 O+IGF-I v IGF-I. This experiment was performed twice with similar results in each instance.

3.5 Activity of IGFBP-4 and IGFBP-5 in DPCs

In order to examine the effect of IGFBP-4 and IGFBP-5 in DPCs we conducted the experiments described in **Fig 17**. In this experiment we conducted a dose-response experiment over the range of 0-100 nM IGF-I in the presence of a fixed concentration (10 nM) of IGFBPs using ALP activity as an end point of biological activity. As indicated in the data below both IGFBP-4 and IGFBP-5 inhibited the activity of IGF-I over the concentration range of IGF-I which was used. It is important to note that at a concentration of 10nM, IGFBP is present at a ratio of equimolar, sub-equimolar and supra-equimolar with respect to IGF-I (1, 10 and 100). It is also important to note that this experiment involved the addition of exogenous IGFBP-5 and IGFBP-4 together with exogenous IGF-I after a 30 min pre-incubation period. Note also that in the presence of IGFBP-4 or IGFBP-5 alone (zero IGF-I **Fig 17**) there is no effect on ALP activity. We discuss both of these features below (Section 3.3.5).

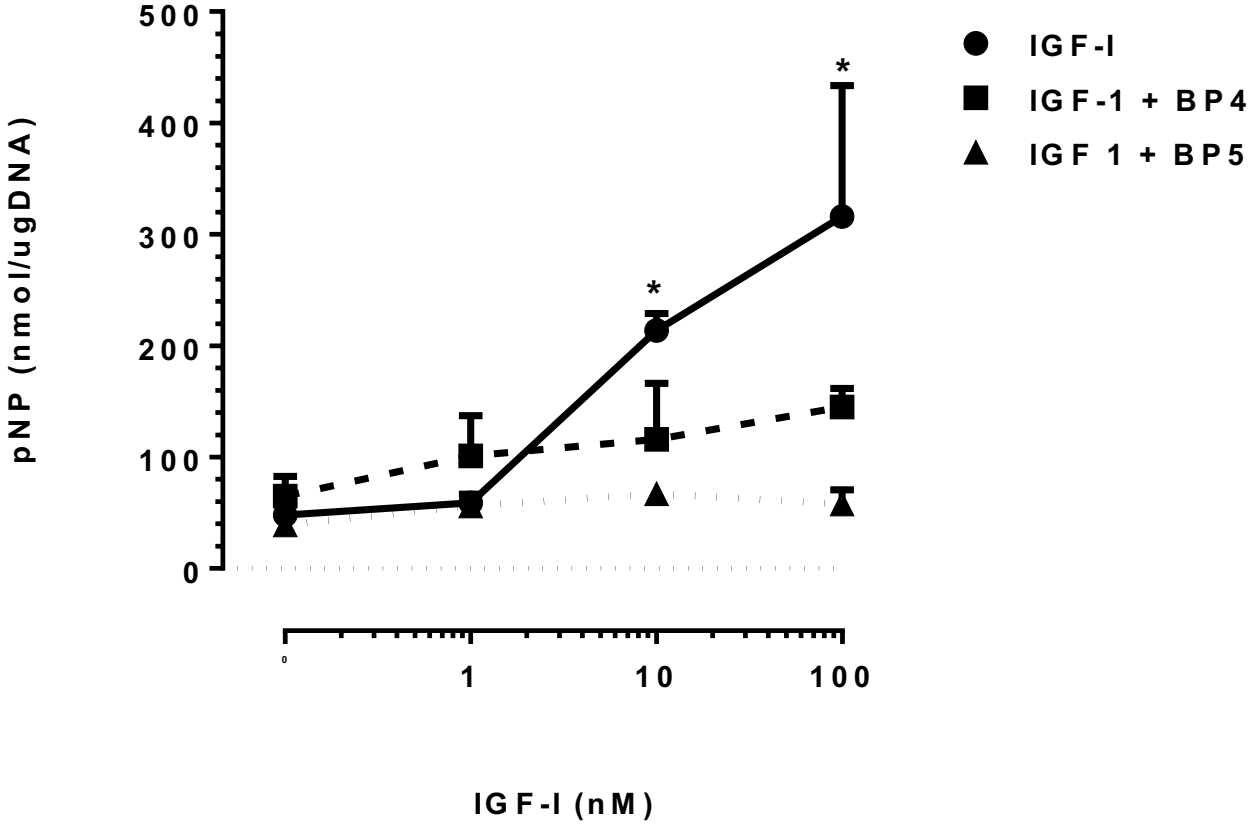


Figure 17 Effect of inhibitory action IGFBP-4 and IGFBP-5 on IGF-I in DPC grown under osteogenic conditions

Cells were incubated for 10 days in the presence of varying concentrations (0-100 nM) of IGF-I in the presence of a fixed concentration (10 nM) of IGFBP-4 or IGFBP-5. Data are derived from triplicate cultures of DPCs each assayed in technical triplicates and are presented as mean ± SD (n=3). * $p < 0.05$ IGF-I v IGF-I+IGFBP-4/5. Note that in the absence of IGF-I (0 nM) neither IGFBP-4 nor IGFBP-5 showed any effect on AP activity.

3.6 Discussion

We have quantified IGFBP mRNA and protein expression in DPCs and shown that with the exception of IGFBP-1 DPCs express each of the well characterised high affinity IGFBPs. IGFBP-4 is the most abundant IGFBP expressed under both basal and osteogenic conditions. Surprisingly very few studies have reported quantitative data for all IGFBP species expressed in a given cell culture system. This is rather surprising given that a complete picture of IGFBP profile (qualitative and quantitative) is required for the interpretation of both IGF-dependent and IGF-independent effects of IGFBPs. In general terms IGFBPs are thought to be expressed at higher concentrations than IGFs in osteoblast and fibroblast cultures [203]. This is the case in our DPC cultures under both basal and osteogenic conditions where global IGFBP concentration is approximately 4 nM and IGF-II is present at 0.1nM (see **Appendix Table II and Chapter 5**). Therefore, under basal conditions this represents an IGFBP: IGF ratio of 40 and suggests that the majority of IGF-II in DPC CM is bound to IGFBPs. Later in this thesis we also show the IGF-II protein concentration in osteogenic conditioned medium is higher than under basal conditions. We return to this topic later in this thesis. Although we did not detect IGF-I mRNA or protein in DPC cultures, exogenous IGF-I was shown to stimulate osteogenic differentiation of DPCs **(Figs 15-17)** confirming reports from previous groups [223, 224]. In this regard it is important to note that dental pulp is a well vascularised tissue and IGF-I therefore may access DPCs through the systemic circulation or via paracrine expression in neighbouring cells. Our finding of higher expression of IGF-II than IGF-I in DPCs has also been reported previously in osteoblast cultures [102, 260] and in DPCs [238].

We have recently shown that IGFBP-2 enhanced and IGFBP-3 inhibited the pro-osteogenic effects of IGF-I in DPCs. This was co-ordinated with increased and decreased expression of IGFBP-2 and IGFBP-3, respectively, during differentiation of these cells [1]. The expression of IGFBP-4 and IGFBP-5 did not change during DPC differentiation **(Figs 12 and 14)**, but as these IGFBPs are 10-100 fold more abundant than IGFBP-2 or IGFBP-3 **(Figs 11 and 14),**

we investigated the effect of IGFBP-4 and IGFBP-5 on the osteogenic activity of IGF-I. **Fig 17** shows that both IGFBPs at equimolar concentrations inhibit the pro-osteogenic action of growth factor. For IGFBP-4, this inhibitory effect is in agreement with most *in vitro* studies in cultured osteoblasts [121, 149]. For IGFBP-5, both inhibitory [127-130] and stimulatory [117-119, 123] effects on osteoblast activity have been described. We found no evidence of enhancement of IGF-I activity by exogenous IGFBP-5 added to DPC cultures. In addition, neither IGFBP-4 nor IGFBP-5 stimulated ALP-activity independently of IGF-I. Interestingly the inhibitory effect of IGFBP-4 and -5 was not overcome by increasing concentrations of IGF-I. Although we have no ready explanation for this, we would emphasise that the affinity of IGFBPs for IGFs is 10-fold higher that of IGF1R and it may be that IGFBP-4 and-5 can concentrate IGFs within the abundant extracellular matrix secreted by DPCs. Investigations into this phenomenon are continuing in our laboratory. It is also the case that the activity of IGFBP-4 and IGFBP-5 can be modified by proteolysis in cell culture. In the next Chapter we examine this phenomenon in DPC cultures.

Chapter 4 IGFBP proteolysis in DPC

4.1 Introduction

Although we have shown that IGFBP-4 and IGFBP-5 mRNA and protein levels did not change during osteogenic differentiation of DPCs **(Chapter 3)** as indicated above we wished to test the hypothesis that IGFBP-4 and IGFBP-5 proteolysis may be differentially regulated between basal and osteogenic conditions.

4.2 Western blot (WB) analysis of endogenous IGFBP-4 and IGFBP-5 proteolysis

In order to examine this possibility, we conducted Western blotting experiments of medium conditioned under basal and osteogenic conditions by DPC cultures. In **Fig 18** we show the results of a representative blot from this series of experiments. For IGFBP-4 **(left panel Fig 18)** the intact protein runs in SDS-PAGE with a molecular weight (Mr) of 28 kDa. It is evident that there is extensive proteolysis of IGFBP-4 under both basal and osteogenic conditions to produce two distinct fragments of approximate Mr of 18 and 14 kDa. At this stage we would draw attention to the fact that at the 24hr incubation point intact IGFBP-4 has almost disappeared from the osteogenic conditioned medium. In the basal conditioned medium at 24hr incubation some intact IGFBP-4 is still present. However, at the 48hr time point very little intact IGFBP-4 is evident. For IGFBP-5 **(right panel Fig18)** the intact protein runs in SDS-PAGE with an Mr of 32 kDa. Under all conditions osteogenic + basal, 24 and 48 hr incubations very little intact IGFBP-5 is apparent. However, unlike IGFBP-4, for IGFBP-5 a broad band of reactivity is apparent between the15 and 20 kDa Mr markers. This implies that whereas IGFBP-4 appears to be proteolysed at a discrete site within the protein, IGFBP-5 may be a substrate for several different proteases.

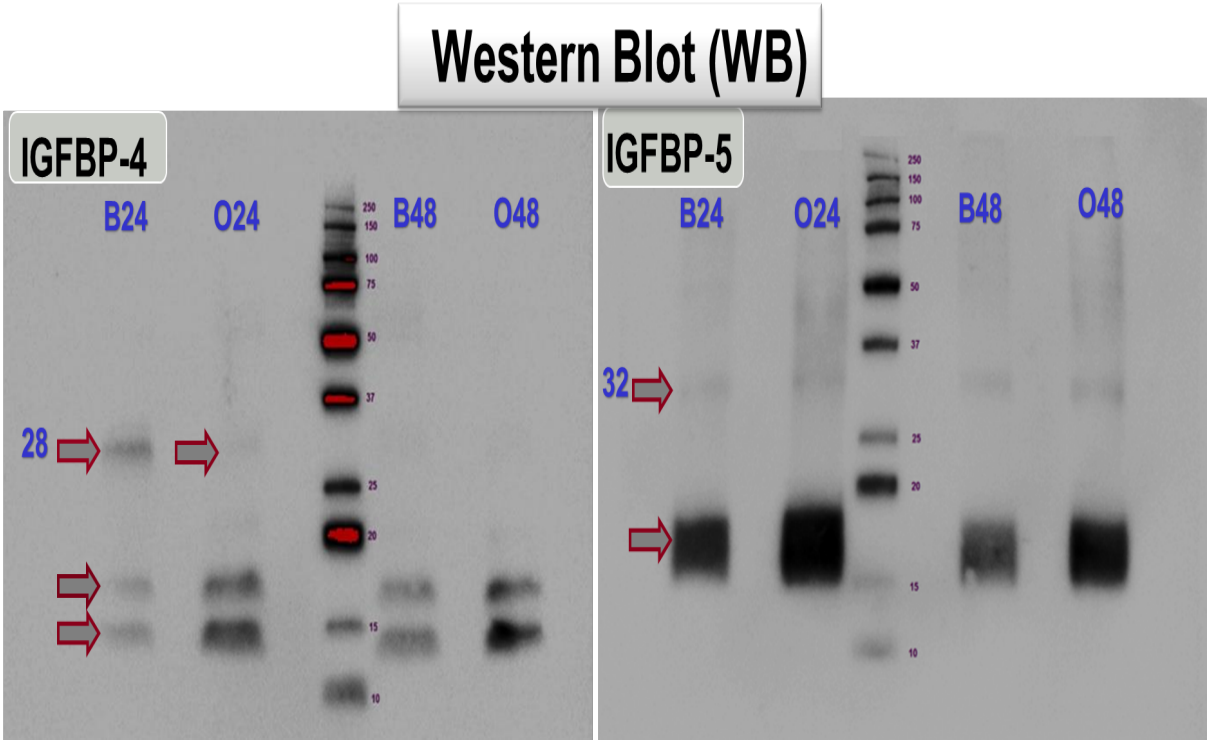


Figure 18 Western blot analysis of endogenous IGFBP-4 and IGFBP-5

Western blot analysis of endogenous IGFBP-4 (left panel) and IGFBP-5 (right panel) in DPC medium conditioned under basal (B) or osteogenic (O) conditions for 24 or 48hr. The expected locations for intact IGFBP-4 (28kDa) and the presence of 18 and 14 kDa IGFBP-4 fragments is indicated (left panel). Similarly, the expected location of intact IGFBP-5 (32kDa) along with a broad smear of reactive IGFBP-5 fragments is also indicated (right panel). This experiment was repeated with different batches of DPCs on at least 5 occasions and similar results were obtained in each instance. A representative blot is shown.

Given the appearance of intact IGFBP-4 under basal but not osteogenic conditions, we decided to explore further the potential differential proteolysis of IGFBP-4 under basal or osteogenic conditions. Therefore, we performed several WBs for endogenous IGFBP-4 in medium conditioned by DPCs under basal and osteogenic conditions. A representative of such a blot is shown in **Fig 19 upper panel.** It is clear from this data that IGFBP-4 is proteolysed to a greater extent under osteogenic conditions compared to basal conditions. This is confirmed by densitometric analysis of such data which indicates that 82.7±3.3% remains intact under basal conditions while in osteogenic cultures 38.2±5.4% of IGFBP-4 remains intact. We discuss the potential implications of these findings below.

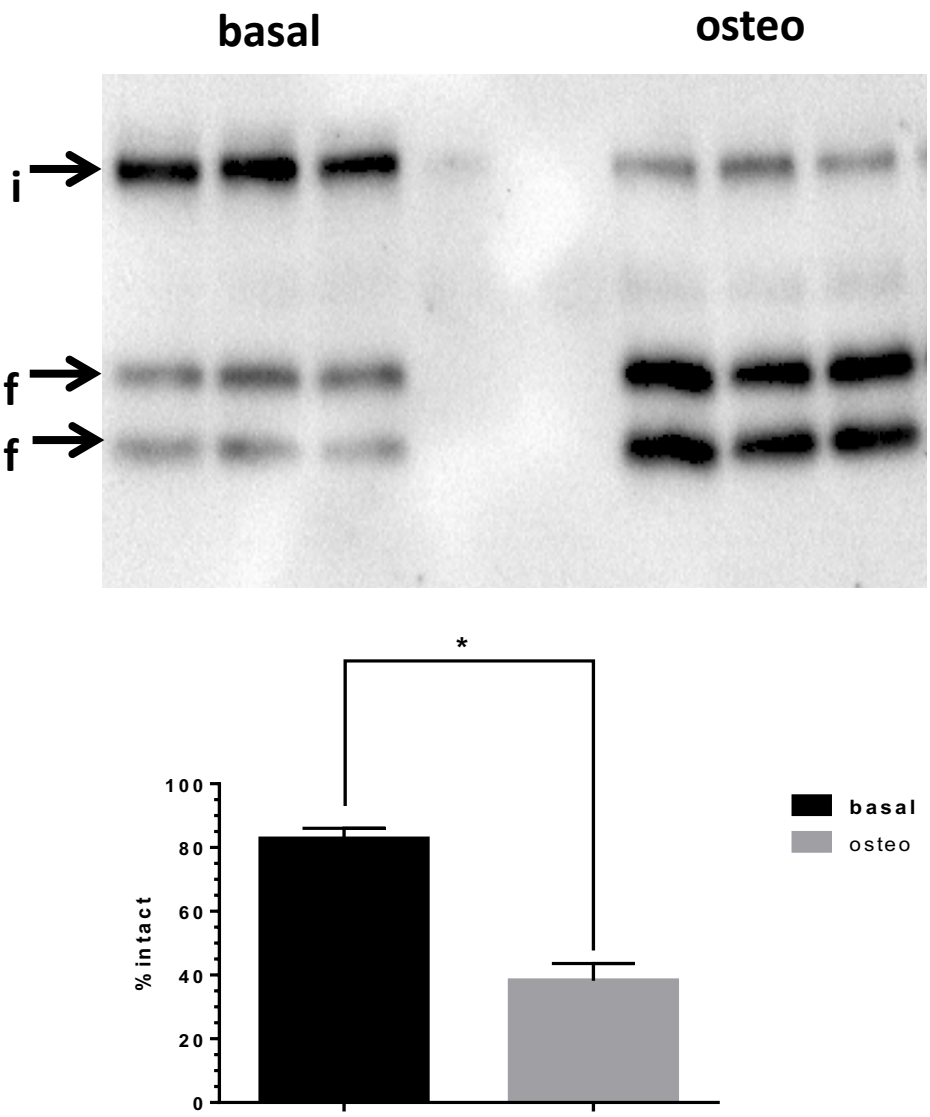


Figure 19 WB analysis of endogenous IGFBP-4 profile

Cells were treated under basal (B) or osteogenic (O) conditions for 7 days. CM was subsequently collected over a 24hr time period, freeze dried, reconstituted at x5 concentration in dH_2O then stored at -80C prior to analysis. Location of intact (I) and fragmented (f) IGFBP-4 are indicated (arrows). Triplicate lanes were run for both basal and osteogenic CM. The central two lanes contain unconditioned medium controls. This experiment was repeated on 5 occasions with similar results in each instance. Bottom panel densitometric analysis of %intact protein * $p< 0.05$ basal v osteogenic mean ± SD (n=3).

4.3 PAPP-A activity in DPCs

As indicated above PAPP-A is reported to cleave IGFBP-4 into two discrete fragments similar to those we report above for DPCs. In order investigate whether PAPP-A was involved in IGFBP-4 proteolysis in DPC conditioned medium we conducted the experiment described in **Fig 20** in collaboration with Dr Claus Oxvig at the University of Aarhus, Denmark. These *in vitro* experiments use ^{125}I-labelled IGFBP-4 as a substrate for PAPP-A. Under these assay conditions recombinant PAPP-A proteolyses radio-labelled IGFBP-4 into a lower Mr species **(lane 2 Fig 20).** In the presence of an inhibitory PAPP-A antibody (1/41) this proteolysis is inhibited whereas incubation with a control IgG antibody has no effect **(compare lanes 3 and 4 Fig 20).** Importantly medium conditioned by DPCs under basal (B) or osteogenic (O) conditions displays a proteolytic activity towards ^{125}I-IGFBP-4 and this activity can be completely inhibited by the anti-PAPP-A antibody 1/41 **(lanes 5-8 Fig 20).** Again, importantly this suggests that **all** of the IGFBP-4 proteolytic activity in DPC conditioned medium is represented by PAPP-A

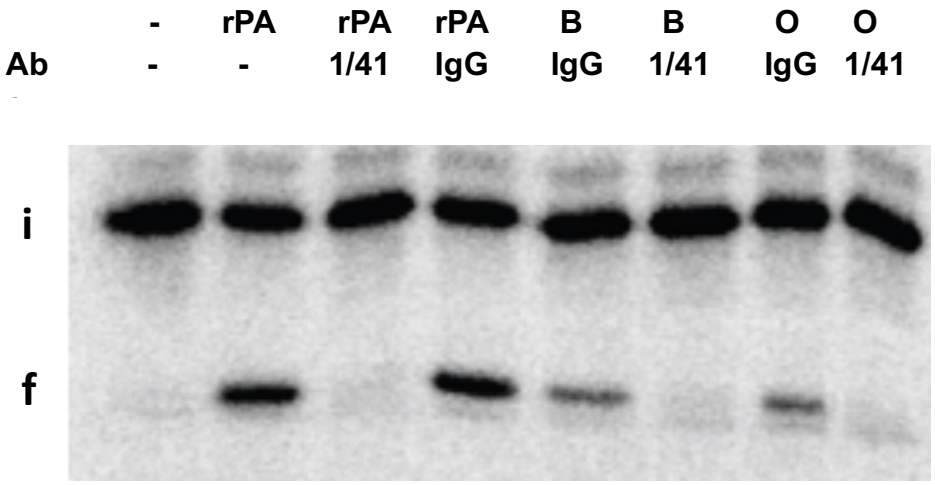


Figure 20 Identification of IGFBP-4 proteolytic activity in DPC CM

^{125}I-IGFBP-4 was incubated under cell free conditions with the following additions: Lane 1 control; lane 2 rPAPP-A only; lane 3 = rPAPP-A + inhibitory antibody (1/41 - PA Ab); lane 4 = rPAPP-A + control Ab; lane 5 = basal CM + control Ab; lane 6 = basal CM + inhibitory antibody; lane 7 = osteogenic CM + control Ab; lane 8 = osteogenic CM + inhibitory antibody. Incubations were conducted over 6 hours period. The location of intact (i) and cleaved (f) IGFBP-4 is indicated. See Methods 2.2.7 for further details.

4.4 Discussion

Although we show that IGFBP-4 concentrations are not altered following osteogenic differentiation of DPCs, it is well established that the activity of IGFBP-4 in fibroblast and osteoblast cultures can be modified by IGF-dependent proteolysis [166, 168]. We therefore examined whether there was any difference in IGFBP-4 proteolysis between basal and osteogenic DPC cultures. To investigate proteolysis of endogenous IGFBP-4, we conducted WB analysis of DPC CM. We found that IGFBP-4 was proteolysed into fragments which migrated at 18 and 14 kDa in reducing SDS-PAGE **(Fig 18).** This is similar to previously described endogenous IGFBP-4 proteolysis in osteoblast cultures [167]. However, in medium conditioned for 24hr under basal conditions, we repeatedly observed that a higher proportion of intact IGFBP-4 was present compared to CM from osteogenic cultures **(Fig18).** As IGFBP-4 protein concentrations are equivalent by ELISA between basal and osteogenic media **(Fig 14 and Appendix Table II),** this suggests increased IGFBP-4 proteolysis under osteogenic conditions (note that the IGFBP-4 ELISA detects both intact and fragmented IGFBP-4 species. Repeated Western blot experiments confirmed an increased proteolysis of IGFBP-4 under osteogenic conditions **(Fig 19).** PAPP-A has been characterised as an IGFBP-4 protease expressed by several cell types (reviewed in [261]) and our experiments in cell free assays suggested that most, if not all IGFBP-4 proteolysis was due to the activity of PAPP-A present in CM **(Fig 20)** and that the difference between co-migration of IGFBP-4 fragments in cell free assay **(Fig 20)** compared with the resolvable IGFBP-4 fragments evident during endogenous IGFBP-4 proteolysis **(Fig 19)** is explained by the use of a tagged recombinant IGFBP-4 species for cell free assay. Note that in (**Fig 20)** there is less difference in intensity between IGFBP-4 fragments in the basal and osteogenic media (lanes 5 and 7) treated cultures than that seen in the tissue culture experiments described in **(Fig 18).** Note however, that the invitro assays described in **(Fig 20)** were conducted over a 6 hour time course as opposed to 24 and 48 hour incubation periods for the tissue culture experiments described in **(Fig 18)**. therefore, the shorter time period used for the invitro

proteolysis experiments may not allow so obvious differences in IGFBP-4 proteolysis to be apparent. We also performed WBs for endogenous IGFBP-5 in DPC CM. In this instance, we found that little intact 32 kDa IGFBP-5 was present in either basal or osteogenic DPC CM with most protein running as a broad band between 15-20 KDa. This may represent the proteolysis of IGFBP-5 by several proteinases known to act on this IGFBP each with different cleavage sites [131, 132]. Time pressure precluded further analysis of IGFBP-5 proteolysis in this experimental model. Furthermore, IGFBP-2 is also known to be proteolyzed by PAPP-A and is expressed by DPCs. Again, pressure of time precluded further studies of IGFBP-2 proteolysis.

At this stage of our studies we began to formulate the idea of an **"integrated IGF axis signature"** associated with the pro-osteogenic activity of the IGF axis in DPCs. In this model the inhibitory action displayed by IGFBP-4 **(Fig17),** the most abundant IGFBP in DPC cells **(Fig14)** is attenuated by increased proteolysis of this IGFBP (**Fig19**) to a non-IGF binding species thus allowing the osteogenic activity of IGFs **(Fig15-17)** to act in DPC cells in culture. We investigate this phenomenon further in the next Chapter where we provide additional evidence for the existence of this novel signature of IGF axis expression and activity during osteogenic differentiation of DPCs.

Chapter 5 An IGF axis signature in differentiating DPCs

5.1 Introduction

In the previous section we suggested that a characteristic signature of IGF axis expression and activity may be associated with osteogenic differentiation of DPCs. In the Introduction section Sections 1.3- 1.5 we discussed the importance of the IGF axis in bone physiology, introduced some of the literature describing how IGFBP-4 (1.4.4) and PAPP-A (1.5) may play a role in this process. In the previous Chapter we showed that increased IGFBP-4 proteolysis occurs in DPCs during osteogenic differentiation and that proteolysis is catalysed by PAPP-A. To further characterise this process, we first of all examined directly PAPP-A expression in DPC cultures.

5.2 PAPP-A expression in DPCs

ELISA for PAPP-A protein **(upper panel Fig 21)** indicated increased concentrations of enzyme under osteogenic culture conditions compared to basal culture conditions. In both 1wk and 3wk cultures in which serum free medium was conditioned for a 24hr period PAPP-A concentrations were approximately 2-fold higher in osteogenic cultures (18.7 ± 1.0 v 37.6 ± 2.2ng/ml – 1wk cultures and 17.7 ± 1.0 v 29.2 ± 4.4 ng/ml 3 wk cultures). Within each condition – basal or osteogenic - there was little difference in PAPP-A concentrations within the 24hr conditioning period irrespective of whether 24 hr conditioned medium was collected after 1wk or 3wk of culture. This suggests a consistent rate of PAPP-A protein expression over this period with maintenance of increased concentrations in differentiated cultures. This evidence of increased PAPP-A enzyme protein in osteogenic DPC cultures is consistent with the increased levels of IGFBP-4 proteolysis seen differentiating DPC cultures. However, examination of PAPP-A mRNA expression under the same conditions showed no significant differences between basal and osteogenic cultures at either 1 or 3 wk time point **(bottom panel – Fig 21).** We discuss this finding at length in Section 5.7

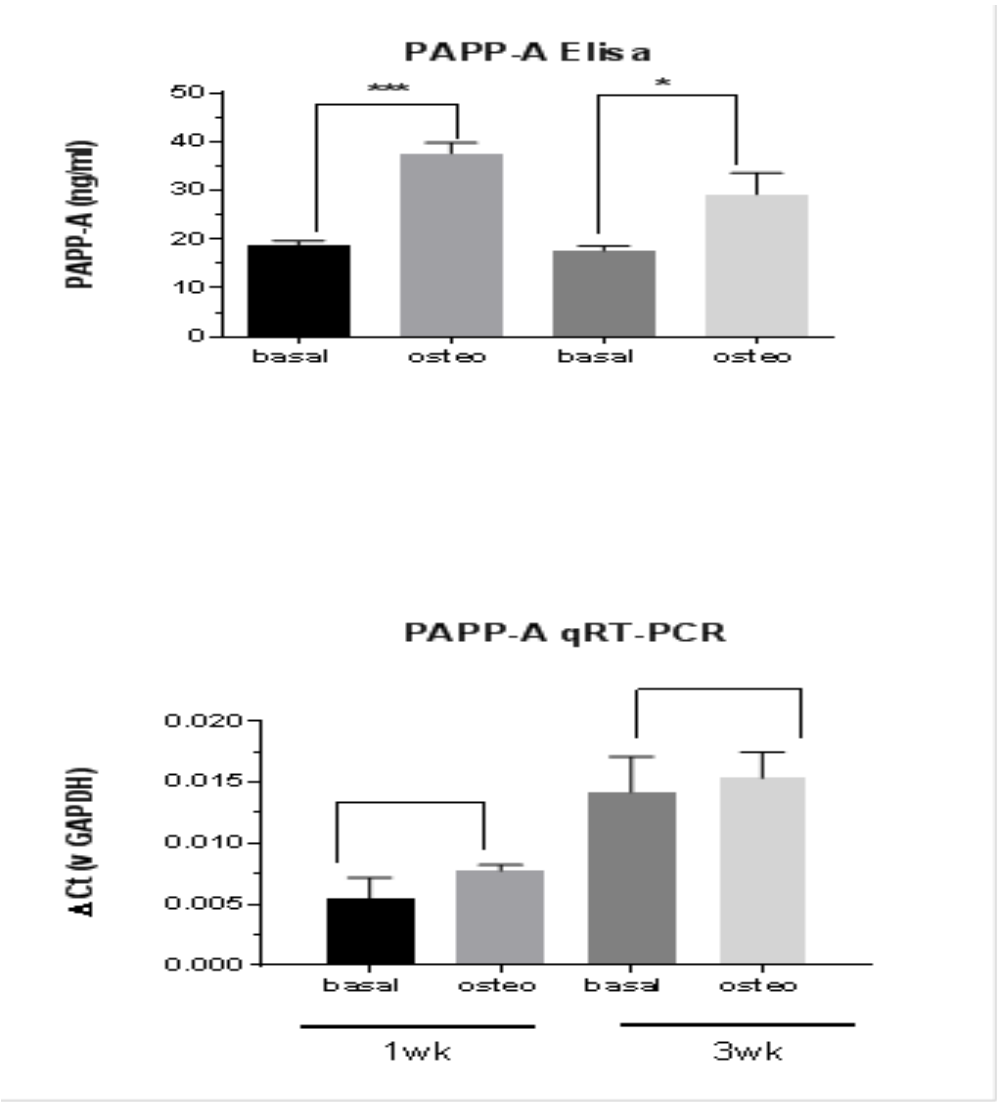


Figure 21 PAPP-A protein (upper panel) and mRNA (lower panel) expression in DPC were determined by ELISA and qRT-PCR respectively

DPC were treated under basal or osteogenic conditions for 1 or 3wk. Following this medium was conditioned over a 24hr time period and retained for PAPP-A ELISA. mRNA was prepared from the same cell cultures and PAPP-A mRNA levels were determined by qRT-PCR. Data are derived from triplicate cultures with duplicate technical replicates and are expressed as mean ± SD (n=3). * $p<0.05$; $p< 0.005$. ELISA data are expressed as ng/ml and qRT-PCR data are expressed relative to GAPDH. Triplicate technical replicates were formed on 3 separate cell preparations. Data are expressed as mean±SD (n=3). * $p<0.05$; ***$p<0.005$.

5.3 IGF2 expression in DPCs

As indicated in the Introduction of this thesis the proteolysis of IGFBP-4 by PAPP-A requires the obligatory presence of IGF. Data generated in the early part of our research indicated that IGF-I was only expressed at a very low level by DPCs **(see Fig11 and Table 4).** However, these experiments did indicate that IGF-II mRNA was expressed in DPCs. Therefore, we decided to revisit this area and determine whether IGF-II protein was present in DPC conditioned medium. **Fig 22** clearly shows that IGF-II protein is detectable in DPC conditioned medium and confirms our qRT-PCR data. However perhaps of more significance IGF-II concentrations are increased almost 10-fold under osteogenic conditions 6.4 ± 0.28 v 0.69 ± 0.17ng/ml. This also confirms previous data with respect to increased IGF-II mRNA expression in osteogenic DPCs (H Al-Kharobi 2016 PhD Thesis University of Leeds – unpublished observations). In addition, we have previously shown that IGF-II stimulates osteogenic differentiation of DPCs [1]. Therefore, the presence of IGF-II protein in DPC cultures may have significant implications and we discuss these further in **Section 5.7**.

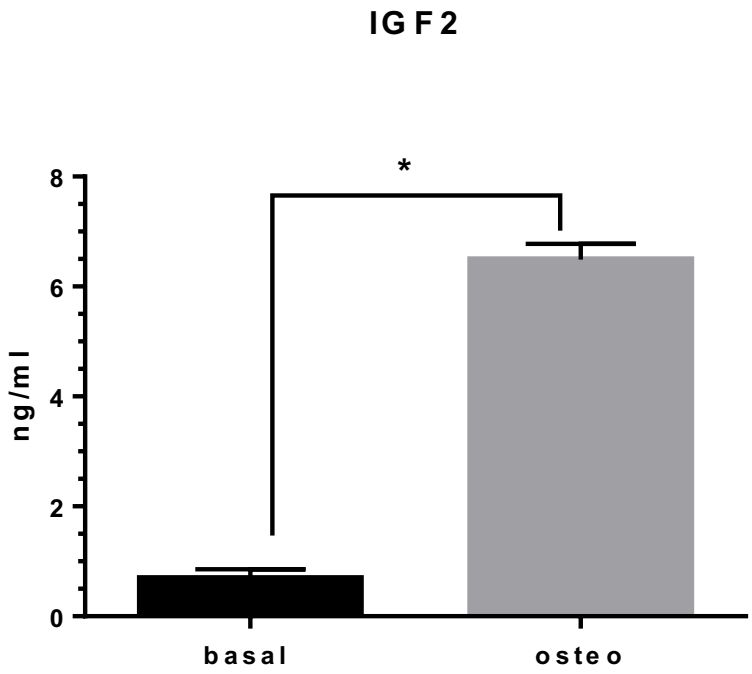


Figure 22 IGF-II protein expression in DPCs

IGF-II was assayed by ELISA under both basal and osteogenic conditions in DPC cultures grown for 1wk under basal or osteogenic conditions. Medium was conditioned for a period of 24hr after culture. Conditioned medium from triplicate cultures was assayed as technical triplicates and data is expressed as ng/ml mean ± SD (n=3). * $p<0.0001$

5.4 Stanniocalcin (STCs) mRNA expression in DPCs

Stanniocalcins (STCs) are recently described inhibitors of PAPP-A activity (see Introduction P43). There are two STC proteins (STC-1 and STC-2) which have been identified in mammalian species. However, to date there are very little data on the cellular expression of either of these proteins. To investigate whether STCs are expressed in DPCs and whether they are able to regulate PAPP-A activity (and therefore indirectly regulateIGFBP-4 proteolysis) we initially investigated both STC1 and STC2 expression in DPCs. We found that STC2 was the more abundant isoform expressed in DPCs **(Fig 23 –** note logarithmic Y-axis**)**. However, although there was a statistically significant increase in STC1 expression under osteogenic conditions in DPC cultures we found no difference in STC2 mRNA levels between basal and osteogenic cultures **(**but see **Fig 23** below**).** This figure also confirms equivalent PAPP-A expression in basal and osteogenic DPC cultures **(see also Fig21-lower panel).** As STC2 was expressed at an almost 100-fold greater level than STC1 in DPC cultures we decided to focus on STC2 expression and activity in DPCs.

5.5 STC2 protein expression in DPCs

ELISA of DPC conditioned medium indicated that STC2 protein was present **(Fig 24).** Furthermore, the concentration of STC2 was reduced over 5-fold in in osteogenic v basally conditioned medium 0.77±0.64 v 4.3±1.8 ng/ml. As STC2 is reported to be an inhibitor of PAPP-A (see **Section 1.5.3**) these reductions in STC2 protein concentrations are consistent with increased PAPP-A activity in osteogenically conditioned medium. However, note that there is an apparent disconnect between STC2 mRNA and protein levels (**Fig 23 and 24**) with no change in STC2 mRNA levels despite decreased STC2 protein in osteogenic DPC conditioned medium. We return to this topic below. STC2 forms covalent disulphide bonded complexes with PAPP-A and in this complex PAPP-A activity is inhibited (**Section 1.5.3**). Therefore, in the next series of experiments we decided to investigate whether PAPP-A/STC2 complexes could be formed in DPC conditioned medium.

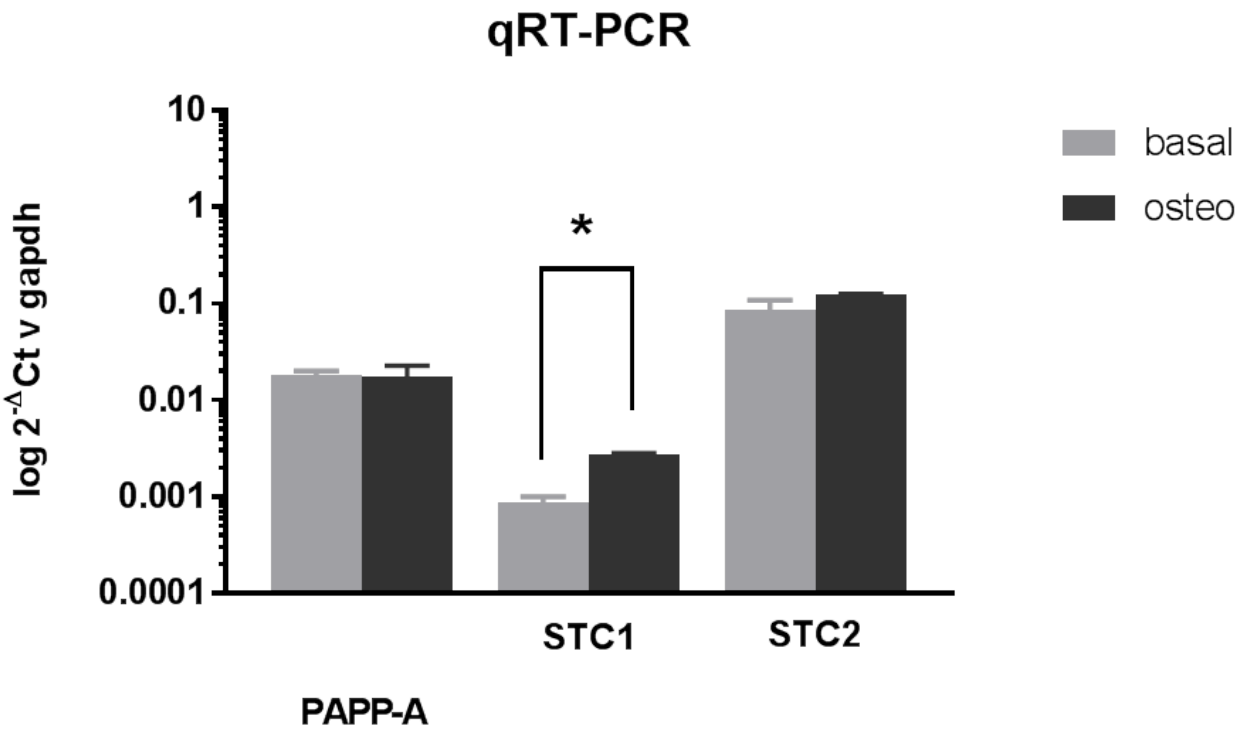


Figure 23 qRT-PCR analysis of STC expression in DPCs

Data are expressed relative to GAPDH and represents technical triplicate replicate assays on three different preparations of DPCs grown for 1wk under basal or osteogenic conditions. Mean±SD n=3. * $p < 0.05$ basal v osteogenic.

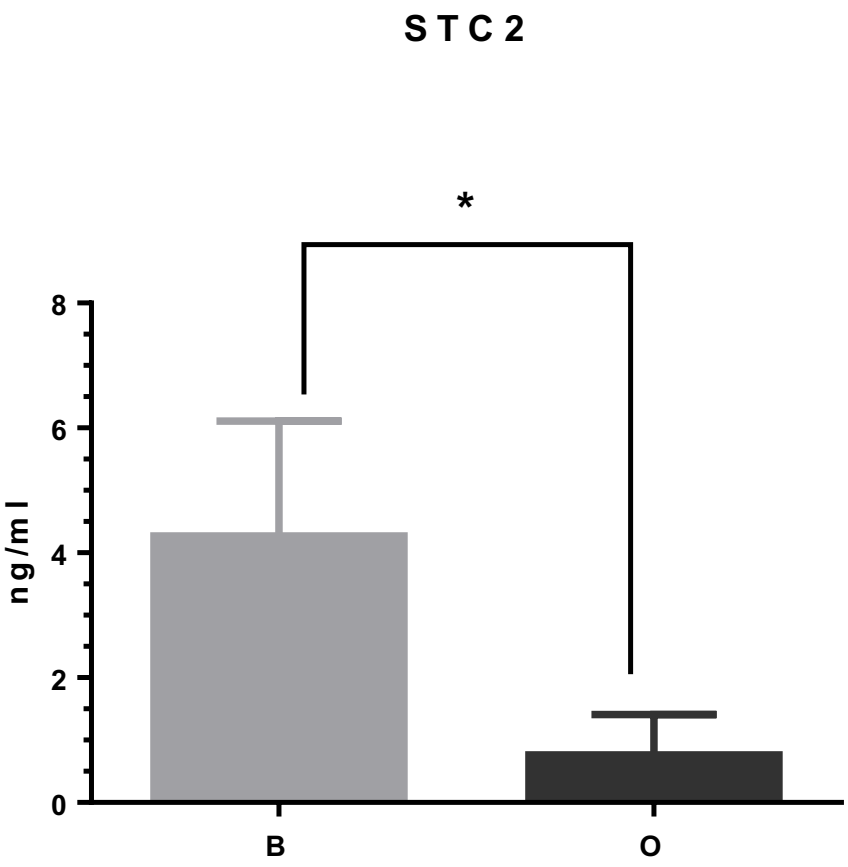


Figure 24 Stanniocalcin-2 (STC2) concentrations in basal or osteogenic CM

DPCs were treated under basal or osteogenic conditions for 7 days and serum free CM was subsequently collected over a 24hr time period. Assays were performed as technical triplicates of 3 separate preparations of CM and are presented as ng/ml mean±SD (n=3); * $p< 0.01$

5.6 PAPP-A: STC2 complexes in DPC conditioned medium

To investigate whether endogenous PAPP-A in hDPC CM can complex with STC2, we carried out the series of experiments described in Fi**gs 25-27**. In **Fig25** immuno-precipitation (IP) of DPC CM with anti-PAPP-A: protein G Sepharose followed by Western blotting (WB) with a separate PAPP-A antibody indicated the presence of a high molecular weight doublet. This reactive doublet was evident following IP/WB of both basal (lanes 4, 5, 7, 8) and osteogenic (lanes6, 9) medium. The lower molecular weight species most likely represents the PAPP-A homodimer which runs at approximately 400kDa under the non-reducing conditions used in these experiments (see also [204]). Although unidentified by this experiment the slower migrating species (~500 kDa) may represent covalent disulphide linked PAPP-A: STC2 complexes (see **Section 1.5.3** and [204]). Note that in experiments conducted on DPC CM collected from cultures after both 1wk (lanes 4-6) and 3wk (lanes 7-9) there was evidence of increased intensity of the lower molecular weight species in osteogenic v basal DPC CM (compare lane 6 v 4 and 5 in basal IP reactions and lane 9 v 7 and 8 in osteogenic IP reactions. The presence of increased uncomplexed PAPP-A under osteogenic conditions is again consistent with increased PAPP-A activity and IGFBP-4 proteolysis in osteogenic conditions. This also most likely reflects the different PAPP-A and STC2 concentrations in basal and osteogenic CM (see **Figs 21 and 24**). Also note the apparent increased reactivity for all species in 3wk v 1wk cultures (compare lanes 7-9 v 4-6 in **Fig 25**).

Although these initial experiments suggested that PAPP-A: STC2 complexes may exist in DPC CM, further evidence for this conclusion was required. **Fig 26** shows the results of an experiment similar to that described in **Fig 25.** In this instance IP of PAPP-A from CM was performed in the absence or presence of added rSTC2. It is clear that in those IP reactions which contained added STC2, there was a decrease in intensity of the faster migrating uncomplexed PAPP-A species and an increase in the intensity of the more slowly migrating PAPP-A: STC2 complex. This was particularly evident in CM from DPCs grown under basal

conditions (**lanes 2-4 Fig 26**). Although this data provided further evidence for the formation of PAPP-A: STC2 complexes in DPC CM and through this the potential regulation of PAPP-A activity, we conducted one final experiment in this series to try and obtain further confirmation of the existence of such complexes in DPC CM.

Finally, to confirm the presence of STC2 in complexes from hDPC CM, the experiments described in **Fig 27** were conducted. IP by anti-STC2: protein G Sepharose complex followed by non-reducing WB of STC2 in both basal (lanes 1 and 2) and osteogenic (lane 3) CM show STC2 present as a high Mr complex (>500 kDa) as well as free STC2 dimer and monomer migrating at 90 and 45 kDa respectively. Although the data for the basal CM is variable (lanes 1 and 2) there is evidence that in osteogenic CM there is less free STC2 present as the 70 kDa dimer (compare lane 3). This data is consistent with lower concentrations of STC2 in osteogenic compared to basal CM (**Fig 24**). Taken as a whole the data in **Figs 25-27** suggest that in hDPC cells PAPP-A activity may be regulated by formation of PAPP-A: STC2 complexes.

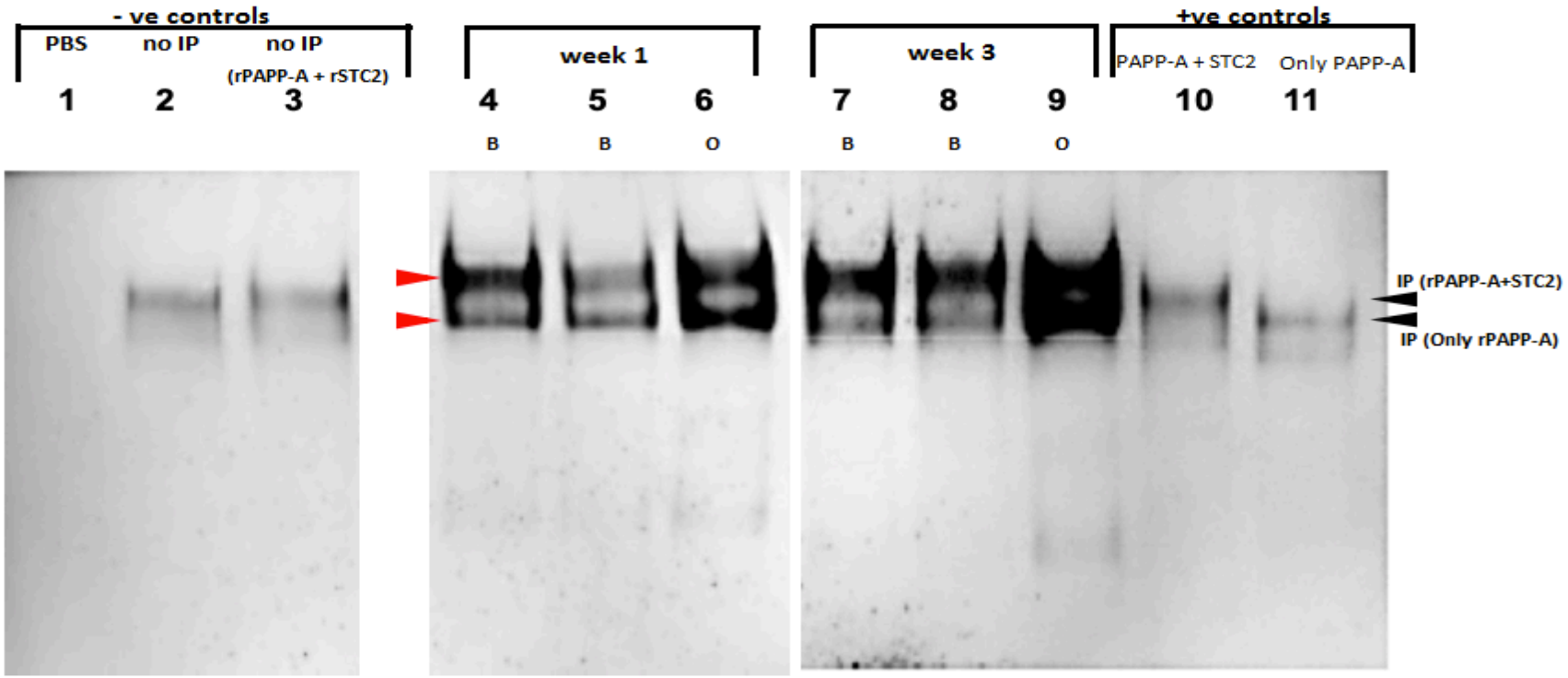


Figure 25 IP/WB detection of PAPP-A species in DPC CM

Basal (lanes 4,5,7 and 8) or osteogenic (lanes 6 and 9) CM collected over a 24hr time period from cultures grown for 1wk (lanes 4-6) or 3wk (lanes 7-9). Uncomplexed PAPP-A runs at 400 kDa (lower red arrow) and slower migrating putative PAPP-A: STC2 complexes (upper red arrow) run above this. Negative controls- lane 1 PBS; lanes 2 no IP; lane 3 no IP with rPAPP-A+rSTC2. Positive controls- lane 10 IP with rPAPP-A+rSTC2 (upper black arrow); lane 11 IP with rPAPP-A alone (lower black arrow). WBs were run under non-reducing conditions. See 2.2.8 Methods for further details

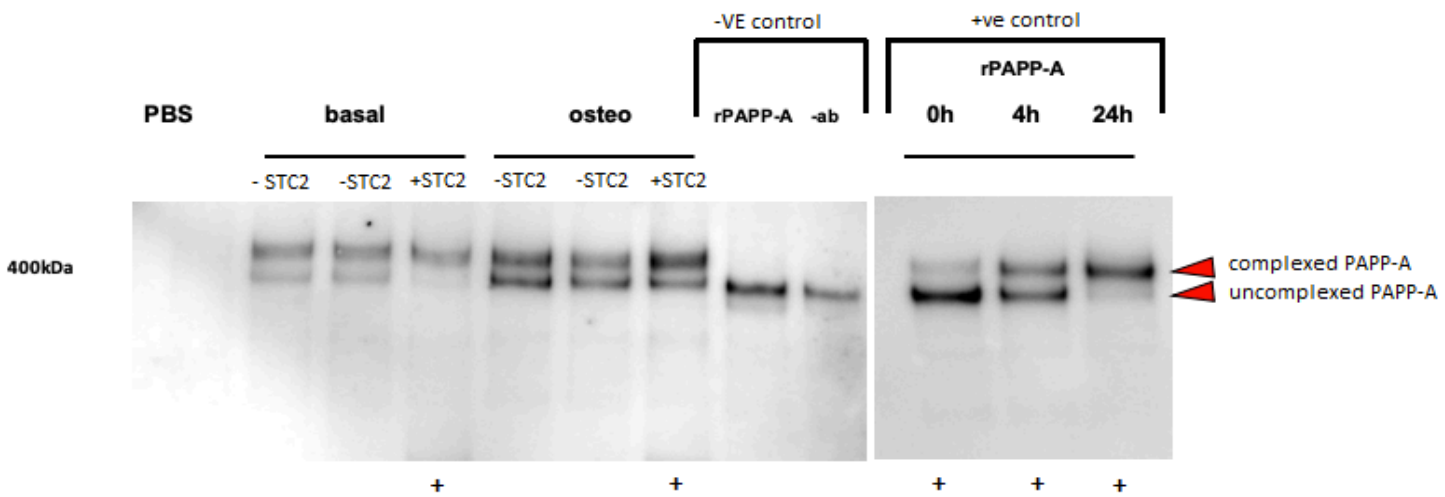


Figure 26 IP/WB analysis of PAPP-A: STC2 complexes in DPC CM.

Basal (lanes 2-4) and osteogenic (lanes 5-7) DPC CM was collected from cultures grown for 1wk. PAPP-A IP and subsequent WB was performed in the absence (lanes 2,3,5,6) or presence (lanes 4 and 7) of added rSTC2. Note decreased intensity of lower molecular weight species in the presence of added rSTC2. Negative controls lane 8-rPAPP-A only; lane 9 no IP. Positive controls lanes 10-12 show the time dependent association of PAPP-A and STC2. Lower red arrow uncomplexed PAPP-A; upper red arrow PAPP-A: STC2 complexes.

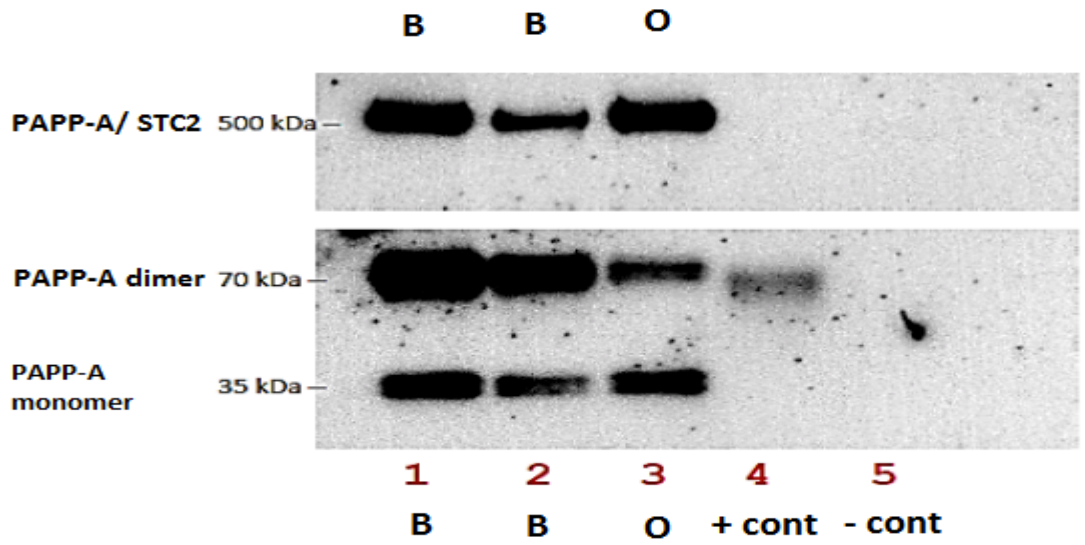


Figure 27 IP/WB of STC2 in DPC CM

Basal (lanes 1 and 2) or osteogenic (lane 3) CM were immunoprecipitated with anti-STC2: protein G Sepharose followed by WB with a different STC2 antibody. Indicated are the locations of STC2 monomer (35 kDa), dimer (70 kDa) and putative PAPP-A: STC2 complexes (500kDa). Lane 4 STC2 +ve control; lane 5 PAPP-A –ve control. Note that the central portion of this blot has been removed.

5.7 Discussion

In the previous chapter we suggested the idea of an IGF axis signature associated with the osteogenic differentiation of DPCs- see **Section 4.4**. In this Chapter we investigated this hypothesis further. Although we previously demonstrated increased IGFBP-4 proteolysis in osteogenic CM (**Fig 19**) and provided strong evidence that PAPP-A was the only IGFBP-4 protease present in DPC CM (**Fig 20**) to examine the hypothesis of increased PAPP-A activity in osteogenic medium more directly, we assayed PAPP-A protein levels in basal and osteogenic CM by ELISA (**Fig 21**). This indicated increased PAPP-A protein in osteogenic medium vs. basal medium in both 1wk and 3wk cultures. In 1 wk cultures PAPP-A concentrations were increased approximately 2-fold basal vs. osteogenic and these differences in PAPP-A protein concentrations are also evident in 3wk DPC cultures. These findings are consistent with a pattern of gene expression and/or activity which promotes IGF activity during osteogenic differentiation of DPCs. It is also worth comment that these concentrations of PAPP-A represent a rather high endogenous enzyme-to-substrate ratios (between 1:2 and 1:10) and may partly explain the extensive proteolysis of endogenous IGFBP-4 evident under both basal and osteogenic conditions. Also worthy of note in our experiments, no difference was apparent in PAPP-A mRNA expression between basal and osteogenic DPC cultures in either 1wk or 3wk cultures (**Fig 21** – **bottom panel**). This suggests that increased PAPP-A protein concentration in osteogenic CM is the result of post-transcriptional or post-translational mechanisms. Although we have no ready explanation for this at present, PAPP-A is known to associate with cell membranes [176] and we are investigating the possibility that increased release of membrane associated PAPP-A protein may occur under osteogenic conditions. Alternatively, PAPP-A ELISA may be affected by the presence of increased PAPP-A: STC2 complexes in basal CM which may not be detected by the PAPP-A ELISA. However, we provide some evidence via IP/WB experiments for increased PAPP-A protein in osteogenic CM (**Fig 26**) and although these issues may be worthy of further investigation this would not alter the conclusion of increased IGFBP-4 proteolysis under osteogenic conditions.

We demonstrate a dislocation between gene transcription and protein expression at several locations in this thesis. There may be several reasons for this. It is well known that abundance of protein target is not solely a function of corresponding mRNA abundance. Many factors including microRNA (miRNA) and non-coding RNA (ncRNA) can potentially regulate protein translation [262, 263]. Whether any of these factors operate in our experimental model is worthy of further investigation.

As PAPP-A proteolysis of IGFBP-4 is IGF dependent, this suggests that IGFs are present endogenously in DPC CM and actually bound to this IGFBP. We confirmed the expression of IGF-II in DPC cultures (**Fig 22**) and that IGF-II is active in DPCs most probably through the IGF1R **(Fig 5).** Indeed IGF-II is a more potent activator of IGFBP-4 proteolysis than IGF-I [181] and significantly the concentration of IGF-II was approximately 10-fold higher in osteogenic vs. basal medium **(Fig 22 and Appendix Table II).** This increased IGF-II concentration under osteogenic conditions would be expected to increase the fraction of IGFBPs (including IGFBP-4) associated with IGF-II. This would in turn increase substrate availability (IGFBP-4: IGF-II complexes) for PAPP-A and may contribute towards increased proteolysis of IGFBP-4 under osteogenic conditions. Importantly increased local IGFBP proteolysis may enhance the action of systemically derived IGF-I which reaches dental pulp tissue. In this instance therefore IGF-II and IGF-I are seen to act in concert to promote cell differentiation.

Recently, PAPP-A activity was shown to be inhibited by both STC1 and STC2 [204, 205]. To determine whether PAPP-A activity may be regulated by either of the STCs, we initially examined the expression of STC1 and STC2 using qRT-PCR. We found that STC2 mRNA was over 100-fold more abundant than STC1 in DPC cultures (**Fig 23**). Further to this, ELISA of CM indicated that STC2 levels in osteogenic medium were almost 6-fold lower than those in basal medium (**Fig 24**). This lower concentration of inhibitory STC2 in osteogenic compared to basal medium is also consistent with a hypothesis of co-ordinated pro-

osteogenic changes in IGF axis activity during osteogenic differentiation of DPC cultures. As a caveat to these conclusions, it is worthy of note that STC2 mRNA expression does not alter between basal and osteogenic conditions (**Fig 23**). Although we have no ready explanation for this, as for PAPP-A some consideration should be given to whether the STC2 ELISA is able to detect both free and complexed STC2. We provide limited evidence that in IP/WB experiments that STC2 protein is present at higher concentrations in basal compared to osteogenic media (**Fig 27**)**.** Comparing the concentrations of PAPP-A and STC2 under basal or osteogenic conditions indicates that the molar PAPP-A: STC2 ratio is close to 1 under basal conditions and 10 under osteogenic conditions (Appendix Table II). Although the stoichiometry of the covalent PAPP-A: STC2 complex is 2:2, we find that even under basal conditions, IGFBP-4 is proteolysed. This indicates that an active fraction of PAPP-A exists in basal CM and suggests that covalent complex formation between PAPP-A and STC2 might be a regulated process. In order to confirm that endogenous PAPP-A present in DPC CM is able to form a complex with STC2, we conducted the experiments described in **Fig 25-27**. IP and WB of basal and osteogenic CM with anti-PAPP-A antibodies showed the presence of slowly migrating 500 and 400 kDa species. The former of these represents endogenous PAPP-A: STC2 complexes and the latter uncomplexed PAPP-A. Addition of rSTC2 to basal CM decreased the intensity of the free PAPP-A species suggesting increased association of PAPP-A with added STC2 (**Fig 26**). This effect was also seen in osteogenic CM along with an increase in intensity of the more slowly migrating PAPP-A: STC2 complex. These data suggest that in DPC CM endogenous PAPP-A and STC2 can form complexes and that PAPP-A activity may be regulated by this interaction. Finally, to confirm the presence of endogenous STC2 in high-molecular weight complexes in DPC CM we carried out IP/WB experiments with anti-STC2 antibodies as described in **Fig 27**. Under these conditions a substantial proportion of STC2 is present in the form of high-molecular weight complexes in both basal and osteogenic conditions – Mr ~ 500 kDa. This provides further evidence that endogenous PAPP-A:STC2 complexes exist in medium

conditioned by DPC cultures under both basal and osteogenic conditions and may represent a mechanism by which PAPP-A and therefore IGFBP-4 proteolysis may be regulated in these culture conditions. There are very little data on the formation of PAPP-A: STC2 complexes by cell cultures. In one study primary cultures of human aortic smooth muscle cells were shown to secrete both PAPP-A and STC2 into conditioned medium [255]. In these cells the concentration of PAPP-A reached 400 ng/ml which is approximately 10-20 fold higher than we found for DPC conditioned medium (note however as conditioned medium volume was not reported by Steffensen *et al* direct comparisons are difficult). Notwithstanding this in aortic SMCs it appeared that all of the endogenous PAPP-A was associated with co-secreted STC2 such that no IGFBP-4 proteolytic activity was evident in aortic SMC conditioned medium. In contrast in both basal and osteogenically DPC conditioned medium IGFBP-4 proteolysis was clearly evident. This was supported by the presence of uncomplexed PAPP-A in DPC conditioned medium. In addition, the increased molar ratio of PAPP-A: STC2 under osteogenic conditions compare to basal conditions (10:1 see Appendix Table II) is consistent with the regulation of PAPP-A activity by complexation with STC2.

In conclusion, we have shown that co-ordinated transcriptional and post-transcriptional changes occur in the IGF axis which may facilitate the osteogenic action of IGFs during differentiation of these cells. These include increased IGF-II expression; increased PAPP-A expression; decreased STC2 expression; the presence of STC2: PAPP-A heterodimers presenting a potential regulatory action for PAPP-A. These changes culminate in increased IGFBP-4 proteolysis (the most abundant IGFBP expressed by DPCs), potentially allowing increased access of local and systemic IGFs to cell surface IGF1R which is abundantly expressed in these cells. We believe our data may be relevant in those situations where bone tissue formation and maintenance may be compromised (e.g. osteoporosis) and may provide new molecular targets for anti-osteopenic therapeutic strategies.

Whereas this thesis focuses on the PAPP-A induced cleavage of IGFBP-4 as a means of regulating IGF activity in DPCs, it should be noted that IGF-independent effects of IGFBP-4 have also been described. For example, in ovarian granulosa cell cultures IGFBP-4 inhibited steroidogenesis in an IGF-independent manner [264]. Similarly, IGFBP-4 was shown to be an IGF-independent cardiogenic growth factor both *in vitro* and *in vivo*[265]. These studies used a range of techniques – antibody blockage of IGF-1R, sequestration of IGFs with heterologous IGFBPs, non-IGF binding IGFBP-4 analogues and knock down of IGFBP-4 to dissect out IGF-independent effects of IGFBP-4. It would be interesting in future studies to use one or more of these techniques to examine IGF-independent effects of IGFBP-4.

Chapter 6 General Discussion

6.1 General Discussion

This thesis describes studies with differentiated hDPCs. These cells have been extensively characterized during differentiation to an osteogenic phenotype [1, 19-21].Our laboratories have previously confirmed the characteristics of these differentiating cells [H. Alkharobi PhD thesis].

Compared to our stated Aims and Objectives (Section 1.7) we have shown

- expression of all components of the IGF axis (except IGF-I and IGFBP-1) in DPC grown under basal and osteogenic conditions
- IGFBP protein concentrations in DPC conditioned medium closely follow mRNA levels
- IGFBP-4 is the most abundant IGFBP in both basal and osteogenic DPCs and levels are not altered between basal and osteogenic cultures
- IGF-I and IGF-II stimulate osteogenic differentiation in DPC in a dose-dependent manner
- IGFBP-4 and -5 inhibit IGF-I stimulated osteogenic differentiation in DPCs
- IGFBP-4 proteolysis is enhanced under osteogenic conditions
- PAPP-A is the sole IGFBP-4 protease in DPC conditioned medium and its concentration is increased under osteogenic conditions
- IGF-II concentrations are increased under osteogenic conditions
- STC2 concentrations are decreased under osteogenic conditions
- PAPP-A: STC2 complexes can be identified in DPC conditioned medium

We suggest that these changes in IGF axis expression and activity represent a novel IGF axis signature associated with pro-osteogenic action in DPCs. A diagrammatic summary of the main findings in this thesis is presented in **Fig 28**.

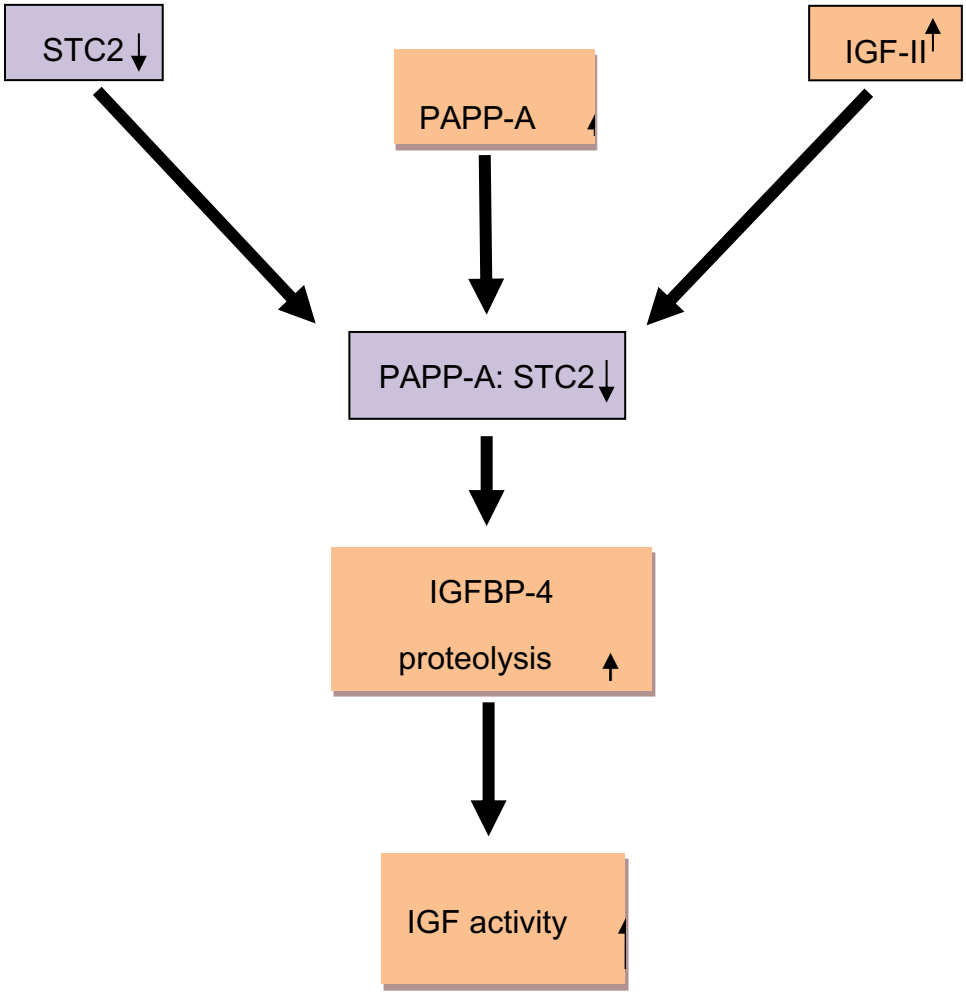


Figure 28 A summary of the experimental findings presented in the current work.

Although our study focusses on a potential role for the IGF: PAPP-A axis in the differentiation of DPCs there is a limited literature which suggests a role for PAPP-A in the differentiation of other cell types. For example during establishment of the human placenta PAPP-A mRNA and protein expression are increased 19- and 8-fold respectively during the terminal differentiation of human cytotrophoblast cells to the multinucleated invasive syncytiotrophoblast phenotype *in vitro* [266]. Similarly the addition of exogenous PAPP-A or over-expression of the protease in the C2C12 mouse myoblast cell line increased cell proliferation but also differentiation into myotube structures[267]. In this instance the authors suggested that the effects of PAPP-A were mediated through proteolysis of IGFBP-2 (see Section 1.4.3 p.31). Finally, over expression of PAPP-A in the HC11 mouse mammary cell line increased differentiation (as demonstrated by increased β-casein expression) to a lactating phenotype [268]. Note however that in this study exogenous PAPP-A was without effect and this phenomenon requires further investigation.

The PAPP-A/IGFBP-4 axis has also been proposed to play a role in other physiological/pathological biological systems. The role of this molecular axis in the atherosclerotic and cardiovascular disease has been reported. Therefore PAPP-A is expressed by both vascular smooth muscle cells (VMSCs) and endothelial cells in atherosclerotic plaques [269]. This has led to the hypothesis that increased IGF activity contributes to the formation of atherosclerotic plaques. However, the role of enhanced PAPP-A in the development of such plaques is controversial with studies reporting both protective [270-272] and deleterious [273] effects on plaque formation. Recent sophisticated transgenic approaches have suggested a role for cell surface PAPP-A activity in both the development and destabilisation of atherosclerotic plaques [274, 275].

Although not addressed in the current study, there is increasing evidence for a role of PAPP-A as a tumour promoter. Theoretically this is founded on the action of PAPP-A as an IGFBP protease releasing previously IGFBP bound IGFs into the vicinity of tumour cells and/or tissues -the implication of this being a higher paracrine concentration of mitogenic IGFs to

drive tumour cell propagation. For example Mansfield *et al* reported that PAPP-A was expressed in breast cancers displaying an aggressive phenotype [276]. Similarly in a transgenic mouse model the development of mammary tumours was associated with increased PAPP-A expression and IGFBP-4 proteolysis [277] and a subsequent report suggested that such effects may be mediated by p53 based regulation of PAPP-A expression [278].In agreement with this a very recent report showed that an engineered PAP-A resistant IGFBP-4 inhibited the invasive potential of mouse mammary carcinoma cells [279]. It should be noted however that there is some contradictory data with reports that PAPP-A was epigenetically silenced in breast cancer precursor lesions and that this lead to down regulation of PAPP-A expression and the development of more invasive tumours [280]. We would re-iterate at this point that PAPP-A presents as an attractive target for anti-tumour therapy (see p.41 Section 1.5.2) further research into off target effects is required.

Another recent study has shown the upregulation of PAPP-A on the surface of Ewing's sarcoma cells and may be associated with evasion of immune mechanisms associated with tumour suppression [281]. This has led to the suggestion that PAPP-A may represent a therapeutic target in this tumour perhaps through activation of immunological defence mechanisms [282]. A recent review has outlined the current literature in the area of PAPP-A and tumour biology [195].

There is a limited literature describing the role of the IGF axis in the development and function of dental tissues. Early studies reported the stimulation of proliferation and extracellular matrix secretion by dental pulp-derived fibroblasts following IGF-I treatment [228], leading these authors to suggest that IGF-I, together with other polypeptide growth factors (PDGF, EGF, bFGF), may play a role in differentiation of these cells. These findings have been independently confirmed, although some variation in growth factor activity related to cell passage number has been reported [283]. An elegant study used in situ hybridisation to demonstrate developmental stage-dependent expression of IGF-I in the continually

erupting rat incisor model [236] and, in related studies, GH and IGF-I acting independently were reported to increase expression of bone morphogenetic protein (BMP)-2 and -4 by up to fivefold, suggesting that the osteogenic activities of both hormones may be mediated by these BMPs [229]. Onishi et al. have shown that IGF-I and -2 increased ALP activity and proliferation in canine dental pulp-derived cells. Insulin was also effective but only at higher doses than IGFs [233], consistent with cross-reactivity but lower affinity at the IGF-1R (see above). Amongst other highly expressed genes of the IGF axis in dental pulp are IGF-2 [243] and IGFIR [284]. In an important study, high levels of IGF-2 secretion were reported during the osteogenic differentiation of human dental pulp-derived fibroblasts [238], and the same study indicated an increase in IGF-IR and IGFBP-3 expression during osteogenic differentiation of these cells. Whether any of these changes in gene expression are causally associated with the process of dental pulp stem cell differentiation remains to be established. However, our preliminary data suggest an increase in IGF-1 and IGF-1R expression after 3 weeks osteogenic differentiation of dental pulp-derived stromal cells (unpublished observations). A subsequent detailed immunohistochemical examination of IGF axis components in other dental structures reported the presence of IGF-I and -2 together with all six IGFBPs in the extracellular matrix (ECM) of the periodontal ligament, and the presence of IGF-IR on the surface of periodontal ligament- derived fibroblasts. These authors similarly concluded that the location of specific IGF axis members within dental structures may reflect distinct roles for each of these genes during processes of tooth development [285]. In a further extremely detailed in situ hybridisation/immunohistochemical analysis of IGF axis expression during the life cycle of differentiating ameloblasts, very strong reactivity for IGF-I, IGF-2, IGF-IR and IGF-2R was evident at the outer enamel epithelial layer towards the apical loop in the continuingly erupting rat incisor model. In pulp-facing ameloblasts, reactivity towards these IGF axis genes was somewhat reduced. This indicates once again position-specific expression of IGF axis components, and suggests the importance of this axis in the development of dental tissues [235, 286], IGFBP-5 is also up-regulated during

osteogenic differentiation of dental pulp stem cells [245, 287]. We and others have suggested a role for this protein in the differentiation of other cell types [288], and the role of this IGF binding protein in the differentiation of dental pulp cells warrants further investigation. Finally, an interesting recent study using stem cells isolated from apical papillae reported that IGF-I stimulated cell proliferation, ALP expression and mineralisation activity in these cells. Interestingly, expression of odontogenic markers (dentin sialoprotein and dentin sialophosphoprotein) was down regulated. This argues for a bias in IGF-I action towards bone tissue formation and away from chondrogenic structures in this particular tissue niche [289].

6.2 Limitations of current study

We were not able to resolve the issue of increased PAPP-A protein and activity in osteogenic DPC conditioned medium in the face of unaltered PAPP-A mRNA levels in basal v osteogenic conditions (Fig 21 and Section 5.7) and this is an area of ongoing investigation. As PAPP-A is known to associate with glycosaminoglycan (GAG) components of the cell membrane [176] we attempted to test the hypothesis that PAPP-A was differentially released from the membranes of DPCs undergoing differentiation resulting in higher protein concentrations in osteogenic conditioned medium. However, using a FACS based methodology we were unable to identify membrane bound PAPP-A and this area requires further investigation. Similar issues surround mRNA and protein assay for STC2 (Figs 23&24). In this instance it may be that STC2 protein levels are subject to selective post-translational control under osteogenic conditions although in section 5.7 we also highlight technical issues surrounding assays for STC2 (and PAPP-A) in the presence of PAPP-A:STC2 complexes.

In the context of DPC differentiation it is important to note dexamethasone stimulates PAPP-A activity [200]. Therefore, PAPP-A activity should be re-examined under dexamethasone-free osteogenic conditions to establish that changes in PAPP-A expression and activity are

the consequences of differentiation itself and not solely due to the effect of the synthetic glucocorticoid.

We did not investigate expression of insulin receptor (IR) A- or B (IR-A or IR-B) in DPCs .In the light of the expression of IGF-II by DPCs this may be a significant omission as IGF-II is able to bind IR-A isoform with high affinity and IGF-I binds with high affinity hybrid receptors containing an IGF1R dimer in association with an IR-A dimer [290]As both interactions are biologically productive this is an area which requires further investigation. Clearly there are limitations in extrapolation from *in vitro* studies to *in vivo* biological systems. Perhaps the most important amongst these in the IGF axis is the recreation of an appropriate matrix environment *in vitro*. As tissue matrix is known to play a crucial role in IGF axis activity (see Section 1.3.1) this is a clear limitation of many studies. In DPCs, which secrete a specific matrix during osteogenic differentiation (see Section 1.2.3) this is a particular concern in our tissue culture model and future studies should take full account of this fact.

6.3 Future Work

In addition to the issues raised above and although we clearly demonstrated increased IGFBP-4 proteolysis under osteogenic conditions in DPCs there are now commercially available ELISAs for N-terminal and C-terminal IGFBP-4 fragments [291].It may be worthwhile to obtain more accurate quantitative data on IGFBP-4 proteolysis using this technique. In addition IGFBP-4 is N-glycosylated [292] and there is evidence that PAPP-A activity may be affected by the glycosylation status of IGFBP-4[293]. It would be interesting to investigate whether this paradigm operated in DPCs. Further work is required to establish causality within our experimental model. In our view much could be gained by the establishment of transient and stable DPC cell lines transfected with osteogenic promoter constructs containing a convenient read out of activation (e.g. luciferase). Knock out and over-expression of some of the candidate genes identified in this study (particularly PAPP-A and STC2) could be used to examine potential roles for these proteins in the osteogenic

process. Some interesting recent work has reported some epigenetic mechanisms associated with IGF axis function in periodontal ligament-derived stem cells (PDLSCs) whereby the expression of IGFBP-5 is regulated by the methylation status of histone K27. Therefore down-regulation of the histone demethylase KDM6B is associated with increased K27 tri-methylation (K27Me3) in the IGFBP-5 promoter [294] and subsequent decreased IGFBP-5 expression. As the same group demonstrated that IGFBP-5 had positive stimulatory effects on the osteogenic differentiation of PDLSCs [295] these findings suggest an epigenetic route by which the IGF axis may be manipulated to enhance differentiation potential of dental tissue derived stem cells. However, whether such mechanisms exist for the PAPP-A: IGFBP-4 axis discussed in this thesis is unknown but is worthy of further investigation.

6.4 Final Conclusions

Under osteogenic conditions, increased expression of IGF-II and PAPP-A, together with decreased STC2 expression lead to a putative decrease in inhibitory PAPP-A: STC2 complex formation. The resulting increase in PAPP-A activity causes increased IGFBP-4 proteolysis which has the potential to increase pericellular IGF activity thus stimulating osteogenesis in DPCs.

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Supplementary Table 1 TaqMan expression assay identifiers for genes analysed in current study

Gene Name	***TaqMan®Gene expression assay identifier***
GAPDH	**Hs99999905_m1**
ALPL	**Hs01029144_m1**
OCN	**Hs00609452_g1**
Runx2	**Hs00231692_m1**
IGF-I	**Hs01547656_m1**
IGF-II	**Hs04188276_m1**
IGF1R	**Hs00609566_m1**
IGF2 R	**Hs00974474_m1**
IGFBP 1	**Hs00236877_m1**
IGFBP 2	**Hs01040719_m1**
IGFBP 3	**Hs00426289_m1**
IGFBP4	**Hs01057900_m1**
IGFBP 5	**Hs00181213_m1**
IGFBP 6	**Hs00181853_m1**
PAPP-A	**Hs01032307_m1**
STC1	**Hs00174970_ m1**
STC2	**Hs01063215 _m1**

Supplementary Table 2 Molar concentrations of selected IGF axis components in basal and osteogenic conditioned medium

Analyte	Basal (pM)	Osteo (pM)
IGF-II	**92 ± 22**	**863 ± 37**
IGFBP-4	**3072 ± 257**	**3180 ± 111**
IGFBP-5	**542 ± 136**	**346 ± 48**
PAPP-A	**47 ± 2.5**	**92 ± 4**
STC2	**49 ± 24**	**8.5 ± 7.2**

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Publications

- **Al-Khafaji H**, Noer PR, Alkharobi H, Alhodhodi A, Meade J, El-Gendy R, Oxvig C and Beattie J (2018) A characteristic signature of insulin-like growth factor (IGF) axis expression during osteogenic differentiation of human dental pulp cells (hDPCs): potential co-ordinated regulation of IGF action. GH & IGF Res (2018) 42-43, 14-21.

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Presentations

Oral

The IGF Axis Regulates Osteogenic Differentiation of Dental Pulp Cells
University of Leeds, Faculty of Medicine and Health Postgraduate Research Conference, 11 July 2018

A novel insulin like-growth factor (IGF) axis signature associated with differentiating dental pulp cells. London 2018 IADR/APR General Session

Poster

The role of IGFBP-4 and IGFBP-5 during Osteogenic Differentiation of Human Dental Pulp Cells.

University of Leeds, School of Dentistry Research Day July 2017