The use of novel antibodies to identify substates within the stem cell compartment of human ES cells

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Submitted in fulfilment for the Degree of Master of Philosophy

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September 2014

Acknowledgements

Abstract

Various recent data point to the heterogeneity of undifferentiated stem cells, which appear to be able to exist in alternative states that can interconvert from one to another. The precise significance of these 'sub-states' remains to be established. It may be that they may represent different steps as a stem cell prepares to differentiate so that, while not committed to differentiate, cells in some states are more likely to differentiate than those in other states.

Another possibility is that the sub-states exhibit lineage priming so that cells in one sub-state might be more likely to differentiate into a particular lineage than cells in another sub-state at the time when they commit to differentiate. To test these hypotheses it is essential to be able to identify and isolate cells in particular sub-states. The phenomenon of 'culture adaptation' of human ES cells tends to 'trap' cells in sub-states in a way that permits their isolation. Otherwise, prior to culture adaptation it is thought that some such sub-states may exist but only in a transitory way, making it difficult to isolate them. Consequently in this project we are utilising the EC cell line NTERA2, which given its oncogenic nature it can be regarded as a culture adapted cell line. We are also employing the well characterised, culture adapted, human ES cell line, H7.S6, together with several newly derived antibodies that mark the stem cell state, in conjunction with other established markers, to define, isolate and characterise sub-states that may exist within the undifferentiated stem cell compartment of human ES cells.

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ALP	Alkaline Phosphatase
cDNA	Complementary Deoxyribonucleic Acid
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
dNTPs	Deoxynucleotide Triphosphates
DTT	Dithiothreitol
EC	Embryonal Carcinoma
EDTA	Ethylenediaminetetraacetic acid
ES	Embryonic Stem
FACS	Fluorescent Activated Cell Sorting
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
HMBA	Hexamethylene Bisacetamide
ICM	Inner Cell Mass
MMLV	Moloney Murine Leukemia Virus
OCT4	Octamer-binding transcription factor 4
PFA	Paraformaldehyde
QPCR	Quantitative Polymerase Chain Reaction
RA	Retinoic Acid
RNA	Ribonucleic Acid
SSEA(1,3,4)	Stage Specific Embryonic Antigen (1,3,4)

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Chapter 1

1 Introduction

1.1 Properties of Stem Cells

Stem cells are a type of cell that are capable of self-renewal, a process by which a cell divides and gives rise to another identical cell which retains the capacity to differentiate into progenitor cells which later develop into the specialised cells that maintain the tissues and organs of a living organism. Embryonic stem cells are derived from the inner cell mass of the blastocyst and under appropriate in-vitro conditions they exhibit unlimited undifferentiation potential (Evans and Kaufman, 1981; Martin, 1981). Non-embryonic (somatic or adult) stem cells are thought to be undifferentiated cells that are found together with differentiated cells in a specific tissue or organ. The main function of these adult stem cells is to maintain and repair the tissue or organ in which they reside (Valdes Chavarri *et al.*, 2005).

The importance of stem cells for living organisms lies not only on their ability to give rise to all the specialised cell types and organs that make up the organism. Some adult tissues, such as the brain, muscle and bone marrow, have discreet populations of stem cells that are able to generate replacements for cells that have been lost due to ageing, injury or disease (Graf and Stadtfeld, 2008). This has led to their being used in therapies for regenerative medicine.

Nevertheless, this very ability of stem cells to differentiate into any cell type has also proven to be problematic in terms of scientists not being able to control and regulate the mechanisms of differentiation. Therefore, much work remains to be done if they are to be used safely in

the clinic. In recent years, much work has been done on dissecting the role of particular signalling pathways in the differentiation and self-renewal of human pluripotent stem cells. Some strategies that have been developed focus on either maintaining cells in an undifferentiated state or controlling the particular cell fates that cells can take. An important point of focus in the lines of work carried out in the laboratory is on genetic changes in ES cells, such as mutation and selection. It has been noted that some of the common changes that take place in variant ES cells also occur in EC cells, the malignant counterpart of embryonic stem cells (Draper *et al.*, 2004a; Draper *et al.*, 2004b).

The mechanism of self-renewal is one of the key features of stem cells that may be shared by cancer stem cells. It has therefore been proposed that the signalling pathways that regulate this process might also be involved in oncogenesis. These signalling pathways include Notch, Sonic Hedgehog and Wnt signalling pathways (Taipale and Beachy, 2001). Furthermore, the stimulation of TGF-β and FGF signalling constitutes the central strategy for maintaining human embryonic stem cells in a state of self-renewal (Galvin-Burgess *et al.*, 2013). However, one important common factor for applying methods of signalling pathway activation/repression is the fact that it is assumed that every cell in the stem cell population is in the same state and thus will respond in a coordinated manner to the exogenous signals being applied. Nevertheless, it has recently become apparent that human pluripotent stem cell cultures are made up of a heterogeneous mixture of cells. This raises the issue of not being able to determine whether all cells will respond in the desired manner to the stimuli being applied.

1.1.1 Differentiation and heterogeneity

If scientists want to harness the ability of directing the differentiation of stem cells for their use in medical therapies, they must first understand how a stem cell decides when and what to transform themselves into. However, it has been shown that not all pluripotent stem cell lines display the same capacity to differentiate into a specific cell type *in vitro.* There are genetic and epigenetic factors that alter the growth and differentiation properties from one cell line to another and that in turn cause heterogeneity within clones (Cahan and Daley, 2013).

Stem cell populations, throughout development, display one common feature that defines their ability to differentiate into several phenotypes: heterogeneity. This heterogeneity is a manifestation of the fluctuations in gene expression of stem cell populations. These fluctuations are in turn the basis of the existence of distinct sub-populations of cells within the main stem cell population (Bhatia *et al.*, 2013). Another form of heterogeneity could simply be due to the presence of early spontaneous differentiated derivatives in cultures.

Stem cells have been described to fluctuate between pluripotent states in response to the expression levels of certain pluripotency genes such as *Nanog*. ES cells are able to interconvert between these states while still remaining uncommitted to differentiate (Burgess-Galvin *et al.*, 2013). It has been argued that this heterogeneity mechanism appeared as a means of allowing stem cells to respond to cues that induce differentiation while at the same time being able to maintain their self-renewal potential (Graf and Stadtfeld, 2008).

1.1.2   Regulatory networks of undifferentiation

The culture conditions required to keep ES cells in an undifferentiated state of are quite specific and they have been designed to regulate the transcription-factor regulatory networks that maintain pluripotency (Othsuka and Dalton, 2008). The main transcription factors that regulate this regulatory network are *OCT4* (POU5F1), *SOX2* and *NANOG*. The orchestrated interaction between these autoregulated transcription factors, together with other components, such as the Polycomb group (PcG) chromatin regulators are responsible for maintaining lineage specification programmes in a silent and controlled way (Boyer *et al.*, 2005).

Recently, it has been shown that the co-expression of these transcription factors in pluripotent stem cells prevents commitment to differentiation to any lineage but they promote specific fates when they act independently of each other (Thomson *et al.*, 2011). The signalling pathways that are involved in directing cells towards a mesendodermal or ectodermal fate are also in part calibrated by this pluripotency network of transcription factors (Cahan and Daley, 2013). However, perturbations that occur in the pluripotency network and that in turn cause some functional differences in pluripotent stem cells might be detectable or remain silent at the genetic expression level in the pluripotent state. For example, if there is a change in the sensitivity to differentiation signals, it may be because of a variable expression of elements of the networks responsible for lineage commitment (Cahan and Daley, 2013). Thirty years ago, Evans and Kaufmann anticipated the concept of stem cell variability by proposing that there might exist some degree of differentiation ability rather than a selective restriction as one possible reason for the different cell type

distributions found in teratomas that arise from mouse ES cell subclones (Evans and Kaufman, 1981).

1.2   Embryonal carcinoma cells

1.2.1   Basic properties of EC cells

Teratomas and teratocarcinomas are a type of tumour that contains tissue derivatives that come from all three germ layers. In humans, they represent a subtype of germ-cell tumours. Undifferentiated embryonal carcinoma cells are the elements that confer malignancy to these tumours. In mouse, when EC cells are injected to adults, they give rise to tumours, however when injected to early-embryos they form differentiated tissues. Unlike EC cells, when ES cells are injected into adult mice, they only produce teratomas but not teratocarcinomas (Damjanov, 2004).

The teratocarcinoma experimental system was described in 1967 (Pierce, 1967; Stevens, 1967). However, Stevens & Little reported in 1954 that male mice from the 129 strain developed spontaneous testicular teratomas and teratocarcinomas and these could be observed as incipient tumours described as embryoid bodies in the seminiferous tubules of the developing gonad at day 13 of embryonic development (Stevens, 1964). Inbred strains of mice with high incidence of spontaneous testicular teratocarcinomas were established in these studies. These tumours were shown to be transplantable and their origin was traced to stem from primordial germ cells in foetal testes. Furthermore, cells from tumours were shown to be able to grow in tissue culture. However, it was the demonstration that transplanting a

single cell *in vivo* could give rise to a teratocarcinoma made up of a wide variety of differentiated tissues that established the existence of pluripotent tumour (EC) stem cells (Kleinsmith and Pierce, 1964).

The establishment of EC cell cultures led to work on ES cells and offered many advantages over the use of the inaccessible mammalian embryo, as they posed a more scalable and manipulable experimental system than the mouse embryo. Studies were published in which mass cultures of cells from embryoid bodies were isolated to establish clonal cultures (Kahan and Ephrussi, 1970; Rosenthal *et al.*, 1970). However, it was two years later when clonal lines from well differentiated solid teratocarcinomas were established and they were shown differentiate even after prolonged passage in culture (Evans, 1972).

Embryonal carcinoma cells are derived from teratocarcinomas, which are a malignant type of Germ Cell Tumour (GCT). Teratocarcinomas also contain differentiated cells from the three embryonic germ layers (Sperger *et al.*, 2003). When transplanted into another tissue or organ, embryonal carcinoma cells can produce tumours.

One of the reasons for their use in research lies in the fact that they can generate normal somatic tissue after being injected into a mouse blastocyst (Papaioannou *et al.*, 1975). As such, human EC cells might give an insight into the mechanisms that drive human development.

Given the nature of EC cells, their use in research has become widely available. In many instances, human EC cell lines provide an advantage over their ES counterparts in terms or requiring less attention when growing in culture. The undifferentiated nature of human ES cell cultures is quite susceptible to the density under which they are grown. However, most

human EC cell lines are able to remain undifferentiated when kept at high density (Andrews & Goodfellow, 1980; Andrews, *et al.*, 1982; Andrews, *et al.*, 1987).

The use of EC cell lines also provides an advantage over the direct use of mouse embryos, since EC cells can be cultured as a self-renewing population, thus providing enough quantities of material in a straightforward and inexpensive way. Despite the increasing use of human ES cell lines since their derivation in 1998(Thomson *et al.*, 1998a); EC cell lines continue to be the tool of choice given the insights that have been gained as the best characterised model for early human development.

1.2.2   NTERA2/clone D1

The parental NTERA2 cell lines were derived from a mouse xenograft of the Tera-2 cell line. Clone D1 of NTERA2 constitutes a pluripotent human testicular embryonal carcinoma cell line derived from the parental NTERA2 line (Andrews *et al.*, 1984b).

The human cell line NTERA2 together with the murine line P19 are amongst the most extensively studied EC cell lines. These two cell lines undergo differentiation with retinoic acid and neuronal cells are among the most prominent cell type found in the differentiated population (Andrews, 1984; McBurney *et al.*, 1982). The Wnt and Notch signalling pathways have been shown to be responsible for the regulation of neural differentiation from uncommitted precursor cells during embryonic development (Cadigan and Nusse, 1997; Weinmaster, 1997). Recently, using NTERA2 cells, these two pathways have been proposed

in a tentative model for the maintenance of EC stem cells in an undifferentiated state and also for regulating their differentiation (Walsh and Andrews, 2003).

The ability to proliferate rapidly *in vitro* and to differentiate into at least two postmitotic cell types (Andrews, 1984; Bani-Yaghoub *et al*., 1999), confers the NTERA2 cell line great potential to be an important research tool.

NTERA2/D1 cells have been shown to undergo differentiation along neuroectodermal lineages when exposed to retinoic acid (RA) or hexamethylene bisacetamide (HMBA) (Andrews, 1984; Andrews *et al*., 1984b; Andrews *et al*., 1990). The importance of establishing how these cells respond and control the processes that take place during differentiation was highlighted by the fact that, just as with the immune system, the different cell types that are present in teratocarcinomas could be characterised by looking at the spectrum of differentiation antigens that are expressed on these cells (Andrews, 1988). Boyse and colleagues introduced the idea that different subsets of cells in a particular developing system (i.e. the immune stystem) could be distinguished by looking at the different range of cell surface antigens expressed (Boyse and Old, 1978). These differentiation antigens constitute a practical strategy for analysing the different subsets of cells that exist in a heterogeneous system and also provide a method for separating the cells in a way that allows their developmental relationships to be studied. Amongst those antigens, there may be some that play a role in mediating cell-cell interactions, which in turn are important in regulating development.

1.2.3 Cell surface antigens

The glycolipids expressed by murine and human EC cells are for the most part glycosphingolipids with a globo-series carbohydrate core structure (Eppig *et al.*, 1977; Willison *et al.*, 1982). Mouse EC cells might present a N-acetyl galctosamine moiety to produce an epitope recognised as the Forssman antigen (Stern *et al.*, 1978). Human EC cells lack this antigen and instead a globoside is further modified which in turn yields structures that bear the epitopes corresponding to the stage-specific embryonic antigens (SSEA3-4) and the globo-ABH antigens (Fenderson *et al.*, 1987; Shevinsky *et al.*, 1982).

SSEA3 was found to be strongly expressed in human EC cells (2102Ep) and soon after, SSEA4 expression was also detected in these cells (Andrews *et al.*, 1982; Kannagi *et al.*, 1983). However, mouse EC cells did not express this antigen (Shevinsky *et al.*, 1982). The positive expression of SSEA3/SSEA4 was later found to be quite common in other human EC cell lines such as NTERA2 (Andrews *et al.*, 1984b). Likewise, EC cells from human germ cell tumour specimens removed from patients during surgery displayed the SSEA3$^+$/SSEA4$^+$ phenotype (Damjanov *et al.*, 1982).

One important aspect of studying the expression of cell surface antigens in EC cells is highlighted by the fact that tumours from xeno-transplanted mice with SSEA3/SSEA4 negative human TERA-2 (cl. w1) cells, contained cells that expressed these antigens even when explanted and passaged *in vitro* for 3 months (Andrews *et al.*, 1985). It was suggested that this phenomenon might involve a type of interrelation between the expression of these antigens and EC tumour growth (Andrews, 1988).

A notable difference between mouse and human EC cells is that the latter have little or no reaction with antibodies that recognise the SSEA1 antigen (Andrews *et al*., 1982; Damjanov *et al*., 1982; Kannagi *et al*., 1982; Solter and Knowles, 1978). Nevertheless, the appearance of SSEA1 in some human EC-like cell lines has been associated with the cells being in an early stage of differentiation (Fenderson *et al*., 1987).

There are other EC cell antigens recognised by monoclonal antibodies that have been described (Andrews *et al*., 1984a; Blaineau *et al*., 1984; Rettig *et al*., 1985). Two of these, TRA-1-60; TRA-1-81, have been associated with high molecular weight polypeptides of approximately 200,000 and 400,000 respectively (Andrews *et al*., 1984a). The epitopes expressed by this family of high molecular weight protein antigens appear to be restricted to human cells, however mouse embryos and murine EC/ES cells have been found not to express them (Muramatsu *et al*., 1978).

Alkaline phosphatase (ALP) is an enzyme expressed on the cell surface of human EC and ES cells in a tissue non-specific form and can be detected by two monoclonal antibodies, TRA-2-49 and TRA-2-54 (Andrews *et al*., 1984c; Benham *et al*., 1981). Two other antigens used include TRA-1-85 and CD9. TRA-1-85 reacts with a pan-human antigen known as the blood group antigen Ok(a) (Williams *et al*., 1988). CD9 is down-regulated upon differentiation and has been useful in identifying subsets of cells that still reside in the stem cell compartment (International Stem Cell *et al.,* 2007; Laslett *et al.,* 2007).

1.3 Human ES/EC cell subpopulations

1.3.1 Stem cell substates

When stem cells respond to cues from different signalling pathways in order to either differentiate or self-renew, they express a wide array of surface antigens that can identify a cell as being ready for differentiation into a particular lineage or mark the cell for self-renewal or even death.

The decisions that stem cells make in order to differentiate into specific cell lineages is a major goal in stem cell biology. The heterogeneity found in progenitor cell populations is a reminder that stem cell subsets express different properties such as varying propensities of lineage selection upon differentiation. Stem cells are faced with a number of options which include self-renewal, differentiation, apoptosis and quiescence. Understanding how the cells select and coordinate these various pathways is a key factor in trying to elucidate the causes of cancer and also in harnessing their properties for regenerative medicine.

It is also important to be able to control the heterogeneity present in stem cell populations. In this respect, the study of the expression of specific cell surface markers has led to the general consensus that SSEA3-4, TRA-1-60, TRA-1-81 conform some of the most important and useful markers of undifferentiation. Moreover, it has been suggested that SSEA3 could be a sensitive marker of the most primitive state for human ES cells thanks to studies carried out on human EC and ES cells (Andrews *et al.*, 1996; Draper *et al.*, 2002; Fenderson *et al.*, 1987; Henderson *et al.*, 2002). Since human embryonic stem cells can spontaneously differentiate in standard culture conditions, the heterogeneous expression of SSEA3 observed

in human ES colonies is an excellent tool to study undifferentiated stem cell substates (Tonge *et al.*, 2011).

Even though SSEA3 is closely associated with the undifferentiated phenotype of human ES cells, it has also been shown that it is not necessary for human development. This antigen together with SSEA4 is expressed on red blood cells, however a small percentage of the human population have red cells that do not synthesise these globoseries antigens (Tippett *et al.*, 1986). Nevertheless, the importance of SSEA3 in undifferentiated cells was highlighted in a comparative analysis of early passage diploid human ES cells and later passage, culture adapted variants (Enver *et al.*, 2005) where it was suggested that undifferentiated human ES cells exist in two substates: SSEA3^{Positive} and SSEA3^{Negative}. It has recently been reported that undifferentiated ES cells can be separated into functionally discrete subpopulations based on their SSEA3 expression profile (SSEA3^{High}, SSEA3^{Low}, and SSEA3^{Negative}) (Tonge *et al.*, 2011).

The findings gleaned from the study carried out by Enver and colleagues in 2005 established that in the early passage diploid cells, SSEA3^{Positive} stem cell substate was speculated to be particularly unstable, hence very few undifferentiated SSEA3^{Negative} stem cells could be detected by clonogenic assays. Nevertheless, the phenomenon of culture adaptation seemed to 'trap' the cells in the stem cell compartment making the clonogenic SSEA3^{Negative} undifferentiated stem cells detectable in the variant later passage lines. The cells in the SSEA3^{Negative} subpopulation of human ES cells is less stable and closer to committing to differentiate than cells found in the SSEA3^{Positive} substate (Enver *et al.*, 2005).

Based on these findings, the study by Tonge *et al.*, in 2011 further suggested that cells with different properties with regard to differentiation potential exist in the pluripotent human ES cell compartment. The study examined the functional heterogeneity of ES cells by testing the

capacity of single cells to choose between a neural or non-neural fate in human ES and EC cells (Tonge *et al.*, 2011).

NTERA2 EC cells were used in the study described by Tonge and cols in 2011 to track and model the expression of SSEA3 in human pluripotent stem cells, further highlighting their impact on stem cell research. It was also noted that the cloning efficiency of human ES and EC cells is closely dependant on the expression of SSEA3, which further underlines the importance of this sensitive marker in assessing the health of pluripotent stem cell cultures (Tonge *et al.*, 2011).

Thanks to studies like the ones mentioned above, it has been proposed that the stem cell compartment exists as a continuum of substates where cells oscillate in their propensity to reaching a differentiation threshold (Tonge *et al.*, 2011). This, in turn, encourages the further study and characterisation of these substates by trying to identify novel antigens that define specific subsets of cells within the stem cell compartment.

1.3.2   Novel cell surface antigens

It is important to stress the need for a novel set of surface markers to identify subsets of cells. This is because as mentioned earlier, many of the strategies that are intended to be used in the clinic rely heavily on the premise of being able to isolate specific subpopulations of stem cells from the larger population in which they exist.

Being able to identify and define new stem cell substates would make the application of therapeutic strategies more readily available by providing scientists with a greater insight into the intricate mechanisms that govern cell fate choice.

Many studies have made use of commonly surface markers either alone or in combination to try and identify substates of cells. For example, human ES cells that exhibit ectodermal bias have been postulated to be identified by the use of the marker CD133 (King *et al.*, 2009). Others have proposed to look for expression of CD24, podocalyxin and GCTM2 as markers of mesoderm progenitors (Lin *et al.*, 2010).

Currently, with all of the studies that have been performed in human ES and EC cells, it would seem narrowly minded to think that the markers that exist and that have been extensively used to date are enough to define the undifferentiated state of human pluripotent stem cells. Therefore, this issue has started to be addressed by trying to derive new antibodies that are capable of defining novel stem cell substates (Wright *et al.*, 2011).

Many features of the panel of antibodies described by Wright and collaborators in 2011 were studied and characterised. However, more experiments are needed in order to establish a more definitive outline of the abilities of these antibodies to identify new possible substates, which is in turn the basic question that this present study aims to address. A summary of the general properties of these novel antibodies is presented in table 1.

Antibody	Isotype	Reactivity			Summary
		N2102Ep	NTERA2	H7.S6	
AA11	IgM	+	+	*	glycolipid/ganglioside
AG10	IgM	+	+	+	glycolipid/ganglioside
BE12	IgM	+	*	*	non-sialated; non-ceramide
BF4	IgM	+	+	+	sialated; non-ceramide
CC9	IgM	+	+	+	sialated; non-ceramide
CH8	IgG	+	+	+	non-sialated; non-ceramide
DA9	IgM	*	+	*	sialated; non-ceramide
EF12	IgM	+	+	+	glycolipid/ganglioside

Table 1. General properties of novel set of antibodies. The reactivity of the antibodies in the three different cell lines is the one reported in this study. + symbol = High reactivity. * symbol = Low reactivity

Chapter 2

2   Materials and Methods

2.1   Cell culture

2.1.1   Culture of Human ES cells

The Human embryonic stem cell line used in this study was a subline of H7, H7.S6 (Andrews, Matin, *et al*., 2005). Cells were cultured in growth media consisting of, Knockout-DMEM (Life Technologies) supplemented with 20% knockout-serum replacement (KO-SR, Life Technologies) , 1X non-essential amino acids (Life Technologies), 1mM glutamine (Sigma-Aldrich), 0.1mM β-Mercaptoethanol (Sigma-Aldrich) and 4ng/ml bFGF (Life Technologies). Cell were routinely passaged by treatment with collagenase type IV (Life Technologies) and scraping with sterile glass-beads (Sigma-Aldrich), transferred to fresh flasks containing seeded 6 x 10^3 cells/cm^2 mitomycin C-treated MF-1 mouse embryonic fibroblasts at a split ratio of 1:5 every 5-7 days. Cells were cultured at 37°C in humidified atmosphere of 5% CO_2.

2.1.2   Culture of Human EC cells

Human embryonal carcinoma cells used in this study was NTERA2 clone D1 (Fogh & Trempe, 1975; Andrews, *et al*., 1984) and N2102Ep (Andrews, Bronson *et al*., 1980; Andrews, *et al*., 1982).

Cells were grown using Dulbecco's Modified Eagle's Medium, containing 4500 mg glucose / litre, L-glutamine, $NaHCO_3$, pyridoxine HCl, (DMEM, Life Technologies) supplemented with

10% by volume foetal calf serum (Gibco BRL) and 2mM L-glutamine (Sigma-Aldrich). Tissue culture flasks were incubated in a humidified atmosphere of 10% CO_2 in air at 37°C.

2.2 Gene expression analysis

2.2.1 RNA Extraction

Both human ES and EC cells were washed once with Dulbecco's PBS (without Mg^{2+}, Ca^{2+})(dPBS) and dissociated by treatment for 1 minute at 37°C with 1ml of trypsin (0.25% w/v in EDTA). Cells were collected in FACS buffer (PBS + 5% FBS) to neutralise the trypsin and centrifuged for 3 minutes at 104g. The pellet was then re-suspended in 1ml of TRIzol reagent (Life Technologies) and left for 5 minutes at room temperature. 200μl of chloroform (Sigma-Aldrich) per 1ml of Trizol was added and samples were vortexed. The samples were then centrifuged at 10,000g for 10 minutes at 4°C.

The colourless upper aqueous phase was carefully transferred into a new 1.5ml Eppendorf tube and 500μl of Isopropanol (Sigma-Aldrich) per 1ml of TRIzol was added. The samples were incubated overnight at -20°C. The precipitate was centrifuged at 14,000g for 20 minutes at 4°C. The pelleted RNA was then washed once with 1ml of 75% Ethanol and centrifuged at 14,000g for 10 minutes. After this, the pellet was left to air-dry for 5-10 minutes and then re-dissolved in RNAse free H_2O.

RNA concentration was measured by using a nanophotometer (Geneflow) by absorbance at 260nm.

2.2.2 Synthesis of complementary DNA (cDNA)

cDNA was synthesized from total purified RNA using Oligo dT$_{(15-18)}$ and random hexamers primers using the following recipe. The following were added to a 500µl tube:

- 1µg of purified total RNA

- 1µl of oligo dT primer (1µg/µl)

- 1µl hexaprimers (0.5µg/µl)

- 9µl of DEPC-treated H$_2$O

To prime the RNA for reverse transcription primers were annealed by heating up the mixture for 5 minutes at 70°C. Then the following was added to the sample:

- 4µl 5x RT buffer (Life Technologies)

- 2µl of 0.1 M Dithiothreitol DTT (Life Technologies)

- 1µl of 10mM dNTPs (Life Technologies)

The samples were pre-incubated at 25°C for 5 minutes before 1µl of 200 units MMLV reverse transcriptase (Life Technologies) was added. The reverse transcription reaction was then carried out with the following specifications:

25°C for 5 minutes; 42°C for 60 minutes; 70°C for 10 minutes; 4°C hold

The completed reaction samples were then used directly for PCR or stored at -20°C.

2.2.3   Quantitative Polymerase Chain Reaction (QPCR)

For analysis of gene expression levels a master mix containing QPCR 10ng of cDNA, AmpliTaq Gold DNA polymerase (Life Technologies), Uracil-DNA glycosylase (Life technologies) and dNTP's (including dUTP) was made up using the following recipe.

Amplification Master Mix	
Taqman Gene expression Master Mix (Life Technologies, cat no 4369016)	125µl
H_2O	120µl
cDNA	5µl
Total	250 µl

For each QPCR reaction, 1.5 µl of the following TaqMan Assay-on-demand primer pairs (Life technologies) was added to 28.5µl of amplification master mix.

Gene	Assay-on demand
ACTB	Hs01060665_g1
POU5F1(OCT4)	Hs03005111_g1
GATA6	Hs00232018_m1
SOX17	Hs00751752_s1
Brachyury (T)	Hs00610080_m1

MIXL1	Hs00430824_g1
OTX2	Hs04332701_m1
SOX1	Hs01057642_s1
PAX6	Hs002240821_m1

QPCR reactions were performed in in 96 well PCR amplification plates (Life Technologies) using an Applied Biosystems QuantStudio™ 12K Flex real-time PCR system (Life Technologies). For each set of QPCR reactions an ACTB amplification that replaced cDNA with H_2O was amplified as a negative control. Each QPCR reaction was analysed in triplicate with the specific gene expression values normalized to the ACTB threshold cycle for each sample.

2.3   Immunofluorescence

2.3.1   In-situ Immunostaining

Cells were cultured on 12 well plates for 5 days after seeding at clonogenic densities (200 cells per cm^2) and medium removed. Cells were washed once with PBS and then fixed with 4% Paraformaldehyde (PFA) for 15 minutes at room temperature. PFA was washed with PBS and plates were then stored at 4°C or immediately subjected to antibody staining.

Cells were dual-stained with SSEA3/OCT4 and were first incubated for 30 minutes in block solution (PBS + 5% FBS). Anti-SSEA3 (1:10) was then added for 1 hour and left at room temperature. Cells were then washed with PBS + 5%FBS (wash buffer) and incubated with the secondary antibody for 1 hour. After washing the cells again, triton detergent solution was

added for 1 hour and left at 4°C. Cells were washed and anti-OCT4 was added to the wells and left at 4°C overnight.

After washing the cells, the secondary antibody was added together with Hoechst 33342 for nuclei staining. Plate were stored at 4°C before imaging with an InCell Analyser 1000 (GE Healthcare) and analysis by Developer Toolbox Software (GE Healthcare).

2.3.2   Flow Cytometry/Fluorescence Activated Cell Sorting analysis

Confluent T25 flasks of human ES and EC cells were treated with 1ml trypsin EDTA for 2 minutes until in single cell suspension. Nine ml of wash buffer were added to the cell suspension to neutralise trypsin. The suspension was then transferred to a sterile 15ml tube and cells were counted and centrifuged at 800 rpm for 5 minutes. Supernatant was discarded and the pellet resuspended in wash buffer in a volume of 10^7 cells per ml. The cell suspension (200µl) was added to sterile FACS tubes and the primary antibody was diluted to a working volume of 1:10. The tubes were then placed on a shaker at 4°C for 30 minutes. After this, cells were centrifuged at 1200 rpm for 3 minutes. Supernatant was discarded and cells were washed 2 more times doing the same procedure. Secondary antibody was added at a working dilution of 1:100 and placed on shaker for 30 minutes. Cells were spun down and washed as before and the cells were resuspended in 1ml of wash buffer for each sample. Samples that were to be sorted using FACS were done so using a Dakocytomation MoFlo prior to being replated into 12 well plates at clonogenic density (1000 cells per well). Normal flow cytometry was performed with a Dakocytomation CyAN.

2.3.3   Quantitative Data Analysis

For flow cytometry, the data was plotted on histograms showing FITC intensity on a logarithmic scale against cell number. The positive and negative populations of cells stained for a particular antibody were discriminated by isolating a region of the histogram using a P3X negative control as a cut-off point.

In order to extract cell and colony information from in-situ stained samples collected by the In Cell Analyser 1000 high-content microscope system the Hoechst 33342 nuclei in each image were segmented using the Developer Toolbox software (GE Healthcare). The segmented nuclei were used to (1) Directly to count the number of cells in a field (2) to mask an area within the antibody staining image channel that would allow unambiguous identification of the cells and measurement of the fluorescent intensity of the antigen being probed in each cell, (3) by expanding the area assigned to each nuclei the closely spaced nuclei found in colony merge together thus demarking the position of colonies within a field.

Colonies could be identified and quantified both in terms of total cells contained within a colony, the fluorescent intensity associated with each cell within a colony and thus whether a cell was positive or negative and total area in μm^2 associated with each colony. Data was then exported to an Excel spreadsheet divided by well (summarising information on the total number of cells and positive cells) or by colony. Scatter and histogram plots were then created using SPSS Statistics software by IBM.

Table of antibodies:

Primary Antibody	Reference	Concentration
P3X	Kohler & Milstein, 1975	1:10 (flow cytometry)
MC-480 (SSEA1)	Solter & Knowles, 1978	1:10 (flow cytometry)
MC-631 (SSEA3)	Shevinsky, *et al.*, 1982	1:10 (flow cytometry/in-situ)
MC-813-70 (SSEA4)	Kannagi, *et al.*, 1983	1:10 (flow cytometry)
TRA-1-60	Andrews, *et al.*, 1984	1:10 (flow cytometry)
TRA-1-81	Andrews, *et al.*, 1984	1:10 (flow cytometry)
TRA-1-85	Williams, *et al.*, 1988	1:10 (flow cytometry)
Novel antibodies (AA11; AG10; BE12; BF4; CC9; CH8; DA9; EF12)	Wright, *et al.*, 2011	1:10 (flow cytometry)
OCT4	Cell Signaling Technology	1:100 (in situ)
Secondary Antibody	**Supplier**	**Concentration**
Anti-Rat IgM Dylight-594	Jackson Immunoresearch	1:100
Anti-Rabbit IgG Dylight 488	Jackson Immunoresearch	1:100
SSEA3/AF647 conjugate antibody	Made in-house	1:100
TRA-1-60/AF488 conjugate antibody	Made in-house	1:100

2.3.4 Antibody Conjugation

The antibody conjugation of SSEA3 and TRA-1-60 with an Alexa Fluor 488 (FITC) and Alexa Fluor 647 (C_y5) respectively was performed in-house. Four different fractions of purified primary antibodies were measured in a spectrophotometer and based on their concentration, one particular fraction was chosen for conjugation.

Zeba desalt spin columns (Fisher Scientific) were first washed three times with Sodium Bicarbonate ($NaHCO3$) buffer. 5ml of antibody and 5ml of sodium bicarbonate buffer were added to separate columns and spun down at 2,500 rpm for 2 minutes. The buffer filtrate was discarded and the antibody transferred to the next column. After the centrifugation steps, the respective fluorochrome in DMSO was added to the concentrated antibody, wrapped in foil and placed on a shaker set to low rocking for 1 hour. Three further washes were done after this and the sample was filtered through a 0.2µm filter.

Chapter 3

3 Characterisation of the ability of a novel set of antibodies to identify subsets of human EC and ES cells

3.1 Introduction

A considerable amount of research has been done on the expression patterns of surface antigens displayed by ES and EC cells over the years. The stage specific embryonic antigen-1 (SSEA1) was detected on mouse EC cells and in the ICM of early embryos from a monoclonal antibody (MC480) from lymphocytes of a mouse immunized with F9 EC cells in 1978 (Solter and Knowles, 1978). This antigen was found to be down-regulated on differentiation (Solter *et al.*, 1979). Soon after, Shevinsky and collaborators produced another monoclonal antibody (MC631) by immunizing rats with four-cell cleavage stage mouse embryos. The antigen, SSEA3, is absent from mouse EC and ES cells, however it is present in human EC and ES cells (Shevinsky *et al.*, 1982) (Damjanov *et al.*, 1982) (Andrews *et al.*, 1982) (Thomson *et al.*, 1998b) (Reubinoff *et al.*, 2000) (Draper *et al.*, 2002). The antibody MC813-70 was later produced by immunizing with human EC cells and this antibody recognized the antigen SSEA4 (Kannagi *et al.*, 1983). These studies paved the way for later characterization of other molecules that have been used as markers, such as the proposition that CD133 is useful in identifying human ES cells with ectodermal bias (King *et al.*, 2009).

A novel set of monoclonal antibodies that might provide a new way of identifying subpopulations of cells within the stem cell compartment was recently described (Wright *et al.*, 2011). These antibodies were produced after immunisation of mice with human EC cells (2102Ep) and human ES cells (Shef1). Eight antibodies in total were derived and they all recognise surface antigens. Seven of these antibodies showed developmental regulation and most of them appear to detect epitopes different from the ones recognized by the previously mentioned and widely used markers of human ES cells. They offer the capability of increasing the resolution of the stem cell state. In this project, these antibodies were further analysed in order to see whether they were capable of identifying subsets of cells with distinct properties.

Although the long term aim of this project is to provide tools for identifying substates within the undifferentiated compartment of human ES cells, EC cells such as the pluripotent line, NTERA2 (Andrews *et al*., 1984b) present some features that make them easier to control and analyse. For example, they can be grown without feeders in a simpler medium, with minimal spontaneous differentiation. This allows easier production of standardised cultures of cells for sorting by FACS to provide sufficient cells from minor set populations for subsequent analysis. In this study, we therefore used NTERA2 EC cells together with the culture adapted human ES cell line, H7.S6, which also grows well and was previously shown to trap cells in substates that are transitory and otherwise difficult to detect (Enver *et al*., 2005). The novel set of antibodies described by Wright *et al* 2011 were compared with well-known markers of human ES cells (SSEA3, SSEA4, TRA-1-60 and TRA-1-81) for their ability to detect sets of cells with differential characteristics of self renewal, differentiation and gene expression.

3.2   Results

3.2.1   Antibody Titration

In order to have a better understanding of how this novel set of antibodies reacts in human EC and ES cell lines, basic Fluorescent Activated Cell Sorting analysis was carried out in N2102Ep cells along with a titration assay to ensure the optimal concentration for all of the antibodies used in the experiments. The first group of antibodies, the standard set, comprised MC480, MC631, MC813-70, TRA-1-60, TRA-1-81 and TRA-1-85. The second group is the panel described by Wright *et al* 2011 comprising AA11, AG10, BE12, BF4, CC9, CH8, DA9 and EF12 (Figure 1).

The histograms in figure 1 are representative of three separate analyses for each antibody to show their distribution in N2102Ep cells. The first antibody, SSEA1, has a unimodal and rather defined distribution. This antibody is absent in undifferentiated cultures and becomes positive upon differentiation of cells. SSEA3 showed a broader, bimodal distribution and this antibody is a good marker for the undifferentiated state of stem cells. Its expression gradually decreases upon differentiation. SSEA4, another marker of the undifferentiated cells presented broad distributions when used at higher concentrations, whereas at lower concentrations, its distribution became more compact which suggests a loss in its capacity to mark cells. The expression of TRA-1-60 was positive throughout the different dilutions, but its distribution shifted towards the negative side as it became less concentrated. TRA-1-81 is a glycoprotein which is expressed on the surface of human EC and ES cells, just like the others and the antibody recognises a carbohydrate epitope. Its expression hardly fluctuated in N2102Ep cells at different concentrations, although its distribution did become broader as its

concentration decreased. TRA-1-85 showed a sharply peaked distribution with just a slight shift to the left of the histogram in the least concentrated sample.

Next, antibodies from the panel described by Wright and colleagues were titrated. The first antibody, AA11, displayed a clearly bimodal distribution where most of the cells were on the positive side of the spectrum. AG10 showed a unimodal distribution with a long tail extending towards the negative side of the histogram. BE12 had a more compact distribution pattern which shifted to the negative side as the concentration of the antibody decreased. BF4 and CC9 showed a similar shaped histogram with a slight shift towards the negative side as less antibody was used. CH8 did not show much variation in its expression pattern and its distribution was quite similar to that of TRA-1-85. Both antibodies recognise a protein-based antigen (Williams *et al*., 1988). DA9 was the only antibody that did not mark a great percentage of cells. It was only at high concentrations that the antibody had a positive presence which quickly dropped as the concentration of the antibody was lowered. Lastly, EF12 showed a wide distribution across the different concentrations used and marked mostly positive cells within the population. Based on the histograms and the titration assays, the subsequent experiments were all performed using the antibodies at a 1:10 dilution (Figure 1 & 1A). Although some antibodies showed a slightly better response when used at different dilutions (e.g. BF4), the majority of the responses from the antibodies stayed in the 1:10 range (Figure 1.1).

Titration Assay in N2102Ep with Standard set of antibodies

Figure 1 – FACS histogram profiles from titration assays in N2102Ep with Standard antibodies. The titration assays were performed using different dilutions of antibody to determine the concentration to be used in subsequent experiments. **Continued on page 29**.
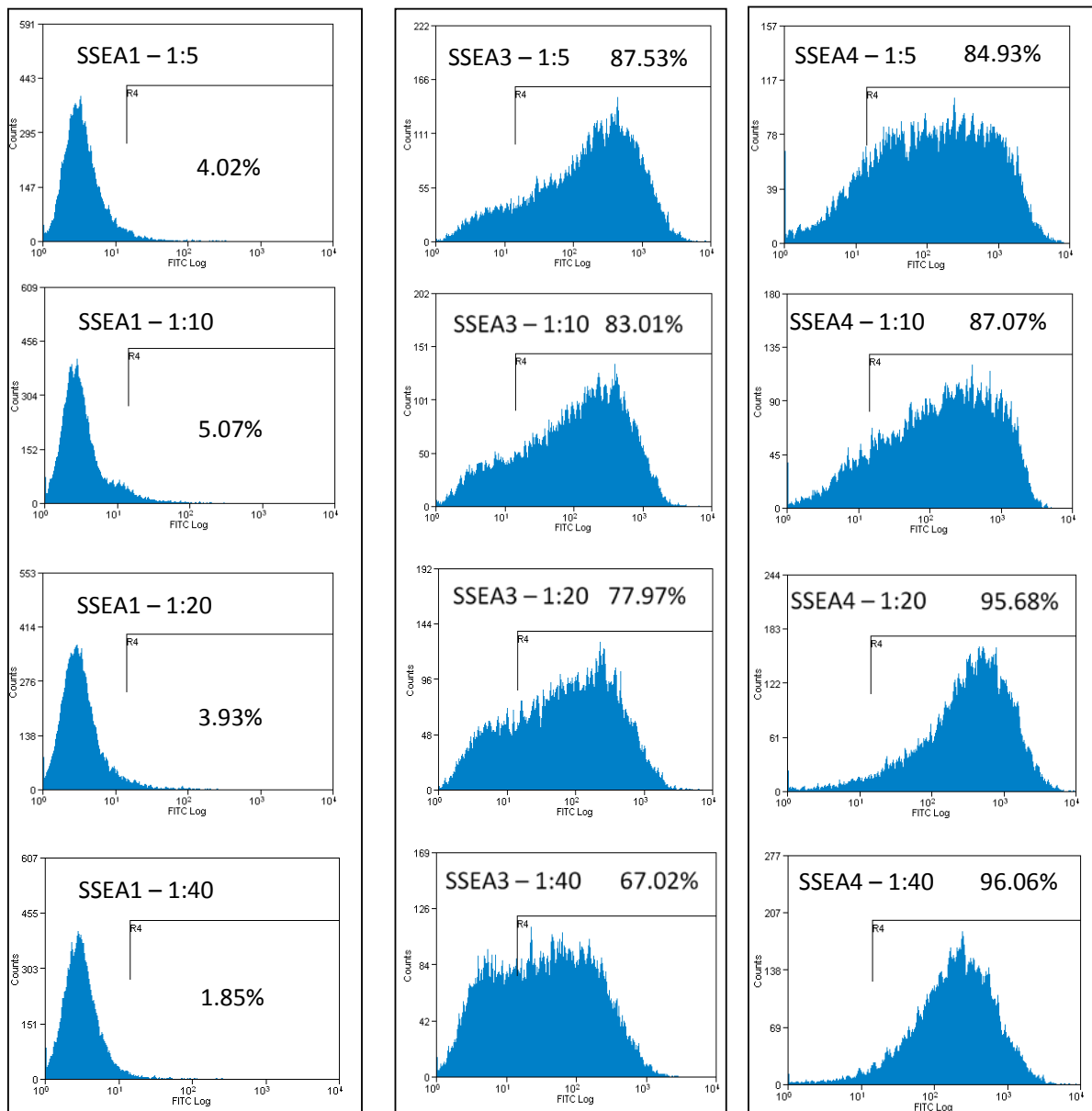
Figure 1 – FACS histogram profiles from titration assays in N2102Ep with Standard antibodies. The titration assays were performed using different dilutions of antibody to determine the concentration to be used in subsequent experiments.

Titration curves for standard and novel set of antibodies in N2102Ep cells.

Median Fluorescent Intensity

Median Fluorescent Intensity

Median Fluorescent Intensity

Median Fluorescent Intensity

Median Fluorescent Intensity

Median Fluorescent Intensity

Median Fluorescent Intensity

Median Fluorescent Intensity

Median Fluorescent Intensity

Median Fluorescent Intensity

Median Fluorescent Intensity

Median Fluorescent Intensity

Median Fluorescent Intensity

Median Fluorescent Intensity

Figure 1a – Titration assay curves. The median fluorescent intensity was plotted against different dilutions for each antibody of the standard set of antigens and the novel set.

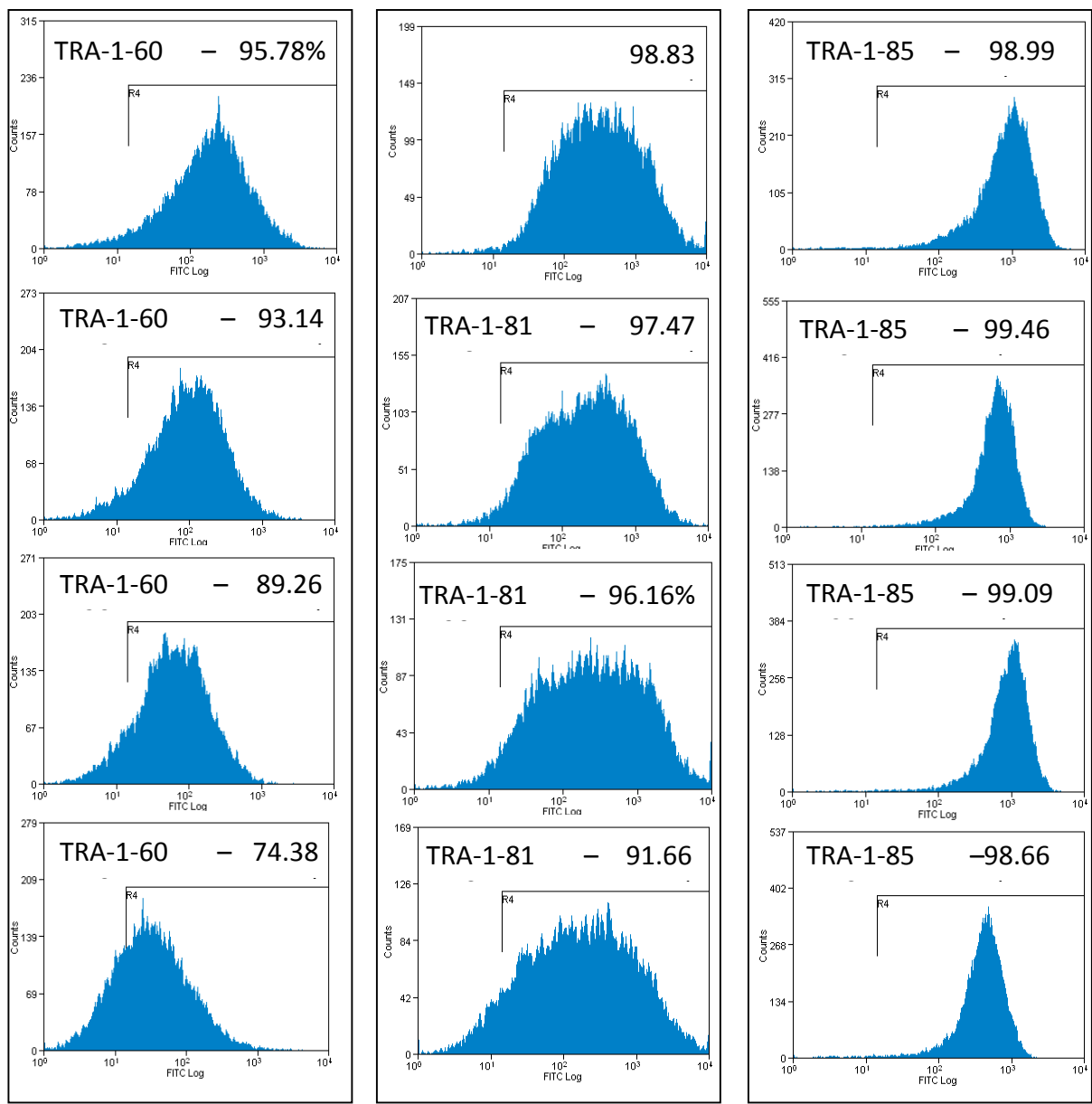**Titration assay in N2102Ep cells with novel set of antibodies.**

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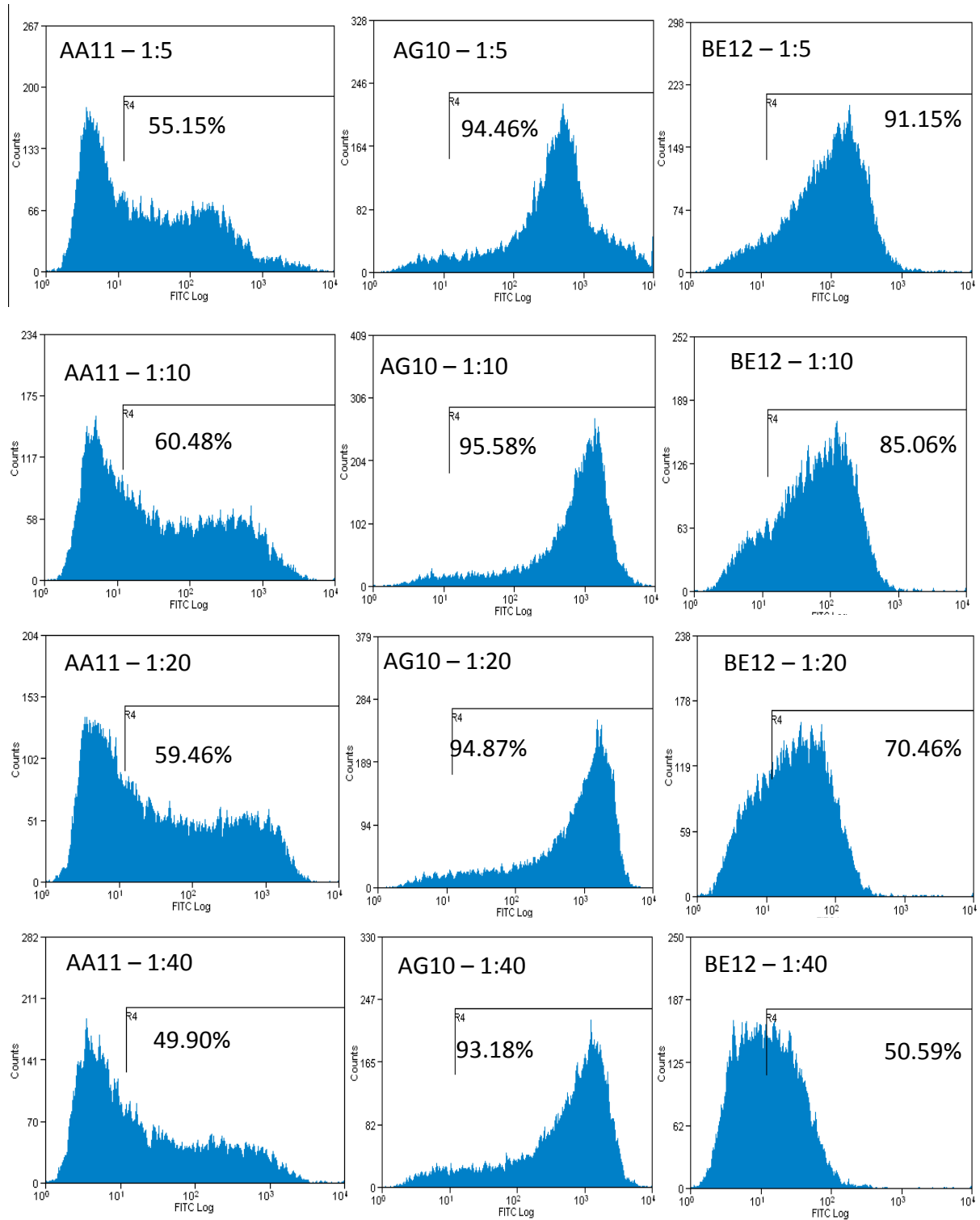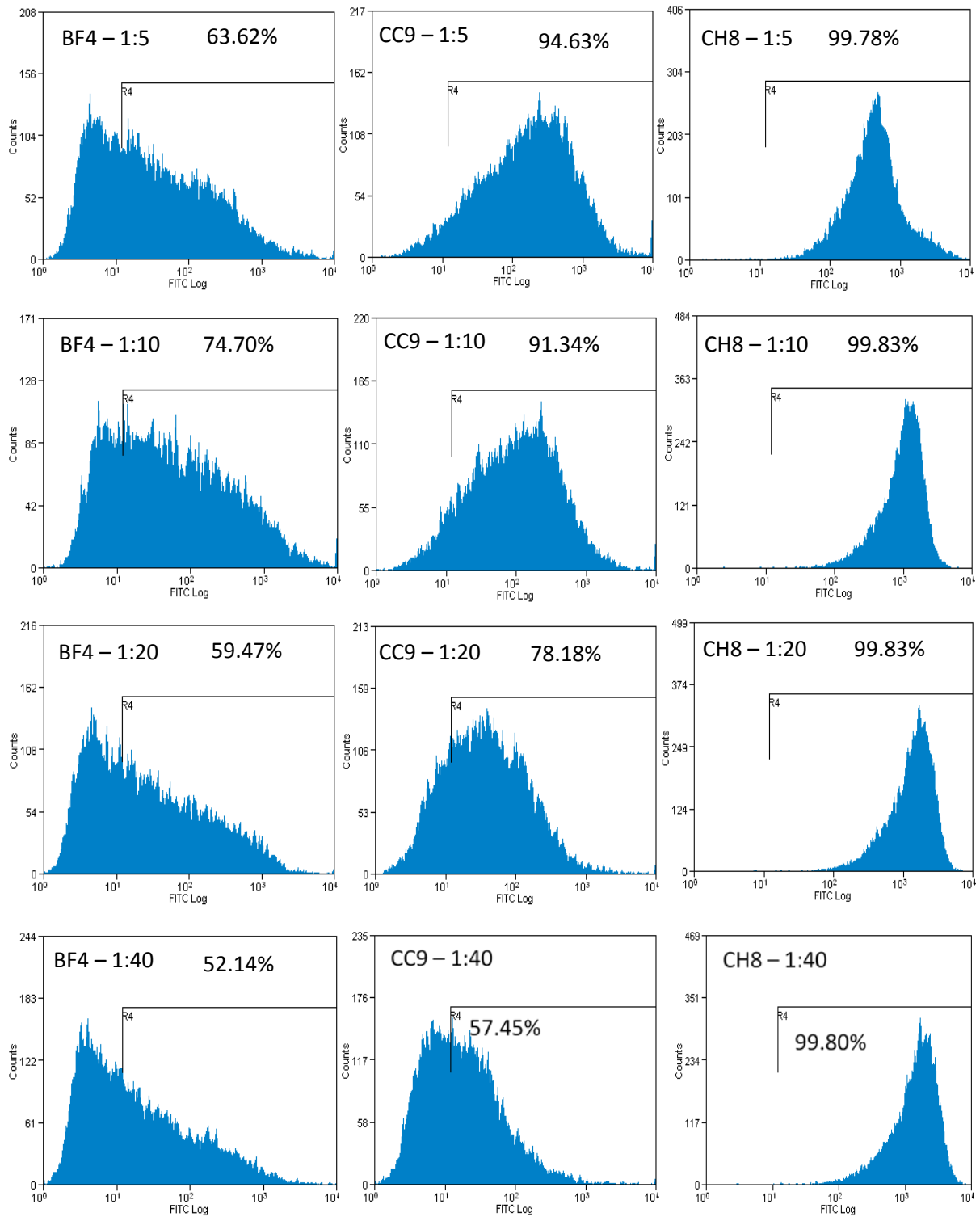
Figure 1.1 FACS histogram profiles from titration assays in N2102Ep cells with the novel set of antibodies. The titration assays were performed using different dilutions (1:5; 1:10; 1:20; 1:40) of antibody to determine the concentration to be used in subsequent experiments.
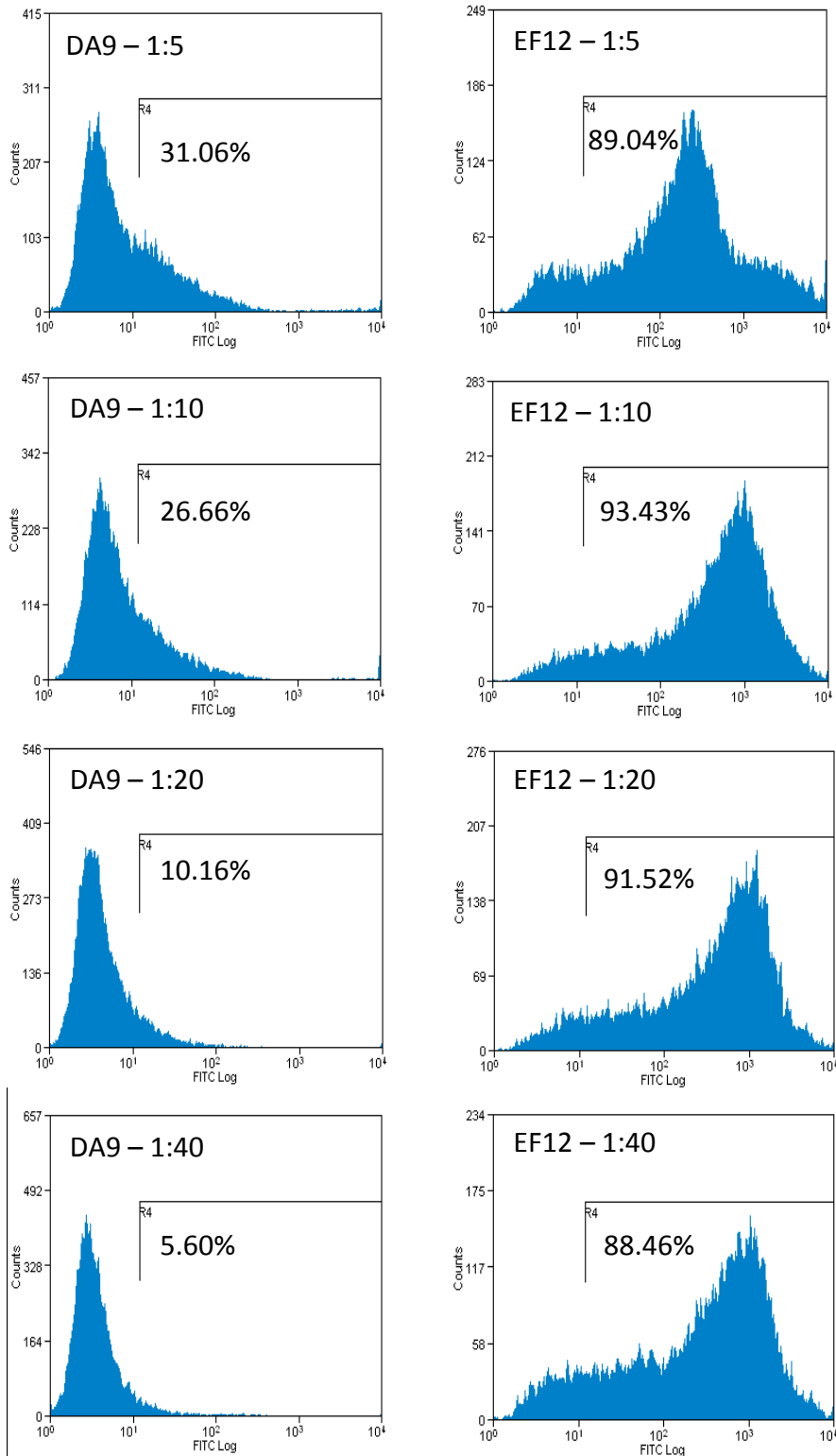
3.2.2 Antigen Expression Patterns in human Pluripotent Stem Cells, NTERA2 and H7.S6

To assess whether the new antibodies may identify substates of human ES cells distinct from those detected by the standard set of commonly used antibodies, we compared the fluorescence flow cytometry profiles obtained following their binding to 2102Ep nullipotent EC cells, NTERA2 pluripotent EC cells and H7.S6 human ES cells (Figures 2 and 3; Table 1). Three biological replicates were carried out for each antibody and cell line, generally giving comparable results. A representative histogram for each is shown in Figures 2 and 3; the data for all replicates are summarised in Table 1.

The first difference found on these figures is that the expression of SSEA1 is slightly higher in ES cells than in EC cells. The population in this fraction seems to be rather defined with a unimodal distribution in the three cell lines. SSEA3 and SSEA4 showed good positive reactivity in the three cell lines, especially in NTERA2. The distributions for the SSEA3 fraction are bi-modal in NTERA2 and H7.S6 but unimodal in N2102Ep. Likewise, SSEA4 showed a more defined unimodal distribution in NTERA2 and H7.S6.

The expression of TRA-1-60; TRA-1-81 and TRA-1-85 was positive in all cells and they showed unimodal distributions with the exception of H7.S6 in which TRA-1-60 and TRA-1-81 displayed broad bi-modal distributions. The expression profile of TRA-1-85 seems very similar to that of CH8 possibly due to their relationship in both recognising protein antigens on the cell surface (Wright *et al.,* 2011) (Williams *et al*., 1988).

Next, the expression profile patterns of the novel panel of antibodies were analysed. The first antibody, AA11, reacted in both EC cell lines. However, AA11 was mostly negative in ES cells where only 24% of the cells showed a positive expression. AG10 was positive in the three cell lines and its expression was quite similar between H7.S6 and NTERA2, where the distribution

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was clearly bimodal, whereas in N2102Ep cells AG10 exhibited a unimodal distribution. BE12 was only present in N2102Ep cells with 85% of them being positive for this antibody. This antibody, however, showed very low expression in ES cells and in NTERA2 cells. BF4 displayed a very wide distribution in all three cell lines. CC9 was also present in the three cell lines and its distribution was rather different across all three. In ES cells, its distribution was bimodal whereas in NTERA2 cells the distribution was more unimodal. Although still unimodal in N2102Ep cells, the distribution of CC9 reactivity was not as defined, but was spread more across the spectrum of positive and negative cells. CH8 had a single distinctive sharp peak that resembled that of TRA-1-85. Of all the eight Axordia Antibodies, DA9 was the only one that showed very low reactivity in N2102Ep cells, less than 30% of them were positive. However, NTERA2 cells showed a good response to this antibody showing a bimodal distribution. In ES cells the expression of DA9 was practically non-existent. Finally, EF12, was present in the three cell lines and its distribution was quite wide and bimodal, although a little less defined in N2102Ep cells. By looking at these FACS expression profiles of the antibodies in three cell lines it is evident that there is heterogeneity within the stem cell population. Therefore, the next step was to have a closer look at how these antibodies might help dissect subpopulations of cells by performing further experiments such as clonogenic assays and looking at the varying influence that the antibodies might have on the expression of markers characteristic of the undifferentiated state, such as OCT4 and SSEA3.

Figure 2. Representative flow cytometric histograms for standard antibodies. FACS histogram profiles showing a representative expression for each antibody of the standard set in N2102Ep, NTERA2 and H7.S6. Filled histogram = Antibody staining. Blank histogram = P3X negative control
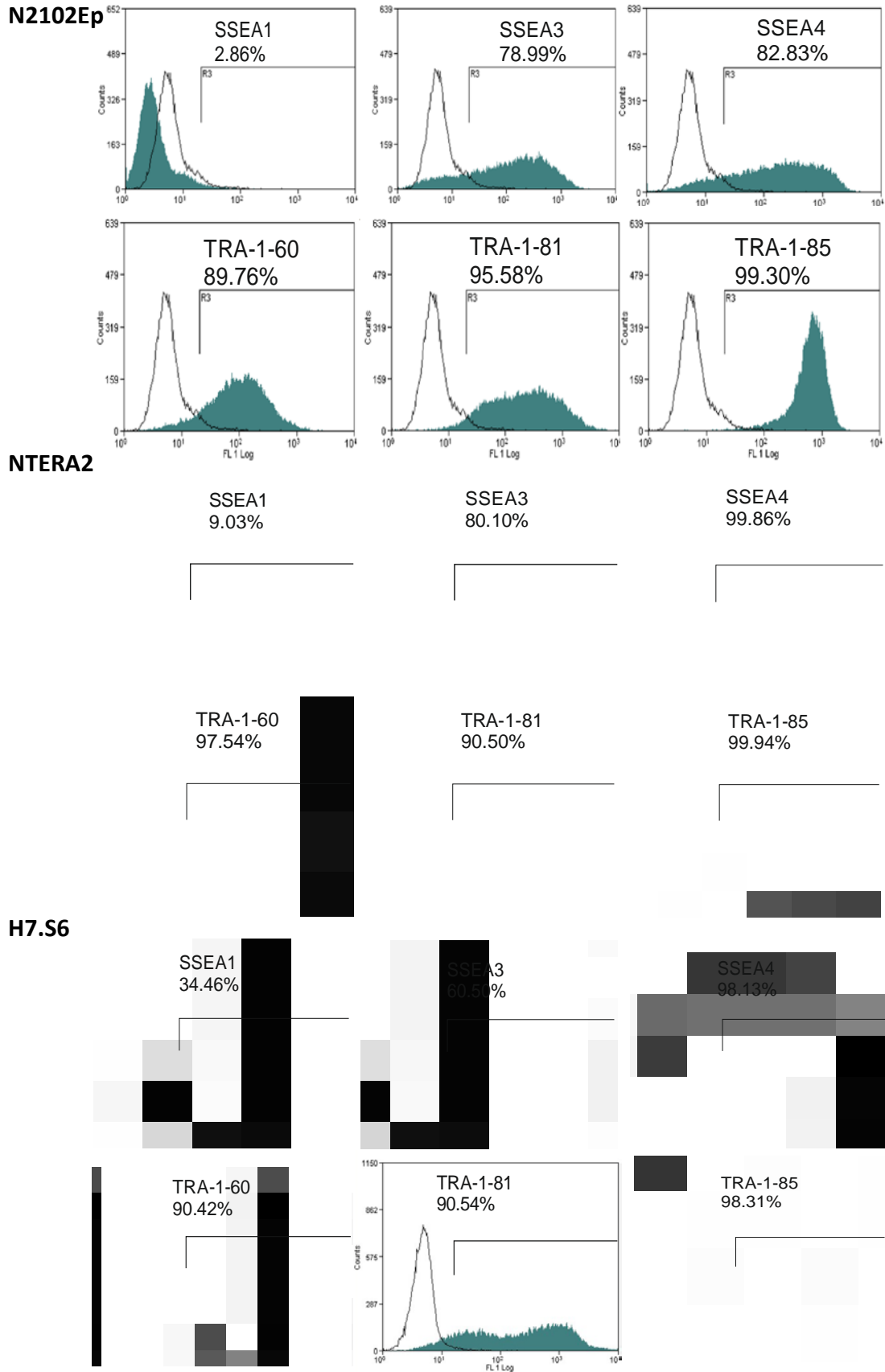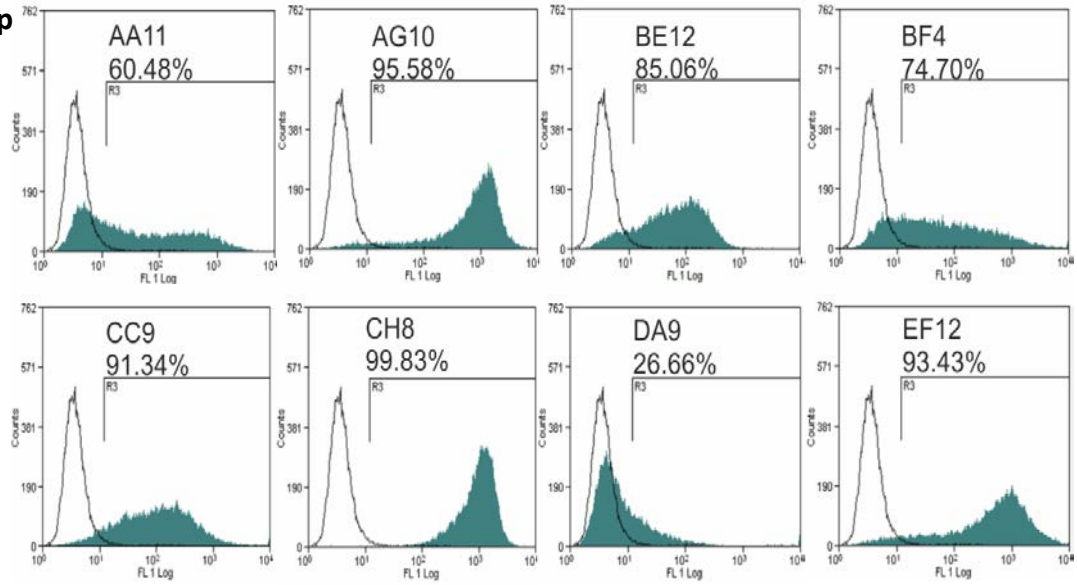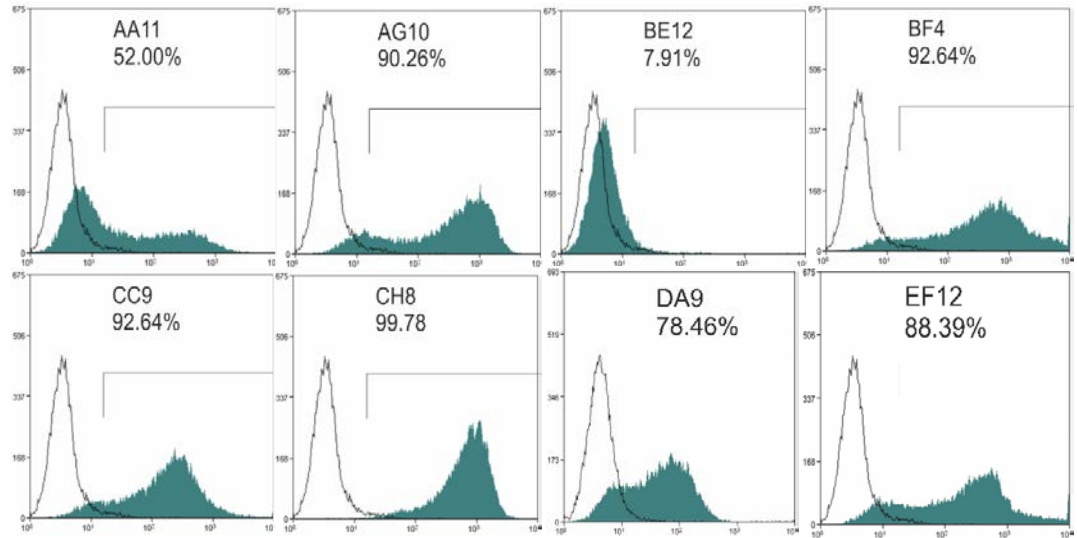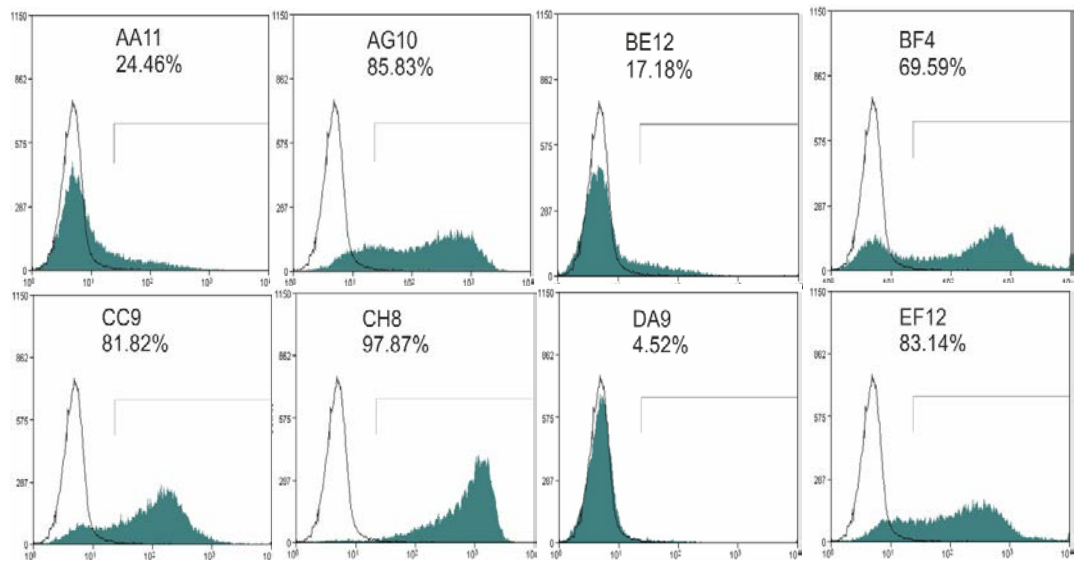
Figure 3. Representative flow cytometric histograms for novel antibodies. FACS histogram profiles showing a representative expression for each antibody of the standard set in N2102Ep, NTERA2 and H7.S6. Filled histogram = Antibody staining. Blank histogram = P3X negative control

N2102Ep			NTERA2			H7.S6		
Antibody	Median Flourescent Intensity			Median Flourescent Intensity			Median Flourescent Intensity		
SSEA-1	3.29	2.85	3.06	4.73	5.27	8.73	7.03	1.85	9.73
SSEA-3	209.69	126.46	123.36	202.25	195.07	279.94	44.37	24.01	37.04
SSEA-4	131.11	168.83	273.72	464.16	447.69	449.44	556.03	481.23	432.15
TRA-1-60	202.25	101.82	163.67	481.23	464.16	317.65	416.49	666.08	498.21
TRA-1-81	323.45	225.39	242.28	51.26	49.44	43.35	233.68	827.27	889.24
TRA-1-85	921.95	955.86	890.63	666.08	680.6	598.93	821.95	464.16	135.94
AA11	16.73	22.34	21.54	13.47	12.99	9.12	15.01	6.31	19.33
AG10	447.69	458.96	521.36	431.81	416.49	432.23	170.95	162.84	536.31
BE12	105.57	96.39	66.01	5.08	5.27	3.81	4.56	5.27	1.66
BF4	23.16	42.79	19.33	447.69	431.81	444.37	55.1	88.15	53.5
CC9	188.15	105.57	118.46	195.07	181.48	105.57	33.23	61.82	55.1
CH8	431.81	403.52	413.25	769.62	762.7	635.94	195.07	189.24	270
DA9	5.66	5.87	3.67	46	56	77	4.9	2.65	3.06
EF12	202.25	576.48	517.28	242.28	233.68	215.98	185	131.11	279.11

N2102Ep			NTERA2			H7.S6		
Antibody	Percentage Positive Cells			Percentage Positive Cells			Percentage Positive Cells		
SSEA-1	3.29	2.85	3.06	9.03	22.5	29.13	33.53	28.48	16.8
SSEA-3	87.53	83.01	77.97	80.1	79.62	87.62	74.18	62.02	70.73
SSEA-4	84.93	87.07	95.68	99.86	99.61	86.9	98.22	92.46	93.14
TRA-1-60	95.78	93.14	89.26	97.54	97.24	97.3	91.59	85.52	90.18
TRA-1-81	98.83	97.47	96.16	90.5	90	91.7	91.62	86.09	92.94
TRA-1-85	98.99	99.46	99.09	99.94	99.72	99.98	98.56	96.84	96.01
AA11	55.15	60.48	59.46	52	51.28	34.06	56.35	37.7	62.88
AG10	94.46	95.58	94.87	90.26	90.07	88.7	82.72	87.31	82.14
BE12	91.15	85.06	70.46	7.91	8.37	7.54	22.5	18.46	8.68
BF4	63.62	74.7	59.47	92.64	92.38	90.57	76.56	73.06	77.11
CC9	94.63	91.34	78.18	92.64	92	95.84	83.34	84.26	83.55
CH8	99.78	99.83	99.35	99.78	99.59	99.29	98.58	98.09	93.32
DA9	31.06	26.66	10.16	78	77	62.85	4.51	17.32	5.55
EF12	89.04	93.43	91.52	88.39	87.92	92.59	83.86	84.93	79.83

Table 2. Median Flourescent Intensity and Percentage of positive cells. Replicate data for the three cell lines (N2102Ep, NTERA2 and H7.S6).

3.2.3 Clonogenic Assays in EC cells (NTERA2)

To assess whether cells expressing or lacking the new antigens exhibited differentiated functional properties, cells were isolated by FACS according to their level of antigen expression and plated out at clonal densities. The resulting colonies were then assessed by a high content colony screening.

Each sorted population (positive and negative) was analysed for different aspects of colony growth. First, the cloning efficiency of each fraction was analysed. Then, I looked at the percentages of positive SSEA3 and OCT4 cells per colony. Next, the size of colonies was assessed both in terms of number of cells per colony and the physical area occupied by each colony. This is represented by scatter plots of these parameters against the percentage of OCT4 and SSEA3 positive cells per colony. And finally, the relationship between the percentage of positive SSEA3 and OCT4 cells per colony was also determined with a scatter plot. Both positive and negative fractions of each antibody were taken into account in these graphs.

The first antibody to be assessed for all these features was AA11. Cells negative for AA11 had good cloning efficiency, followed by its positive counterpart (Figure 4A). However, this high cloning efficiency could simply be due to the cells being analysed on different days. However, because of a lack of time, no biological replicates were obtained for this antibody.

The percentage of SSEA3 and OCT4 positive cells per colony was low and there was no considerable difference between the positive and the negative fractions (Figure 4.1A). The size of colonies expressing either SSEA3 or OCT4 positive cells per colony was relatively the same, where the smallest colonies consisted of four cells and the largest ones had just over four thousand cells in the positive fraction (Figure 4.1B). In terms of area occupied by the

colonies, again their size was relatively comparable between colonies positive for SSEA3 and colonies positive for OCT4 (Figure 4.1C). The correlation between OCT4 positive cells per colony with SSEA3 positive cells per colony was low in general, although there were a few instances where the cells that grew back where positive for both OCT4 and SSEA3 (Figure 4.1D). The low expression of both SSEA3 and OCT4 in these colonies could mean that the cells come from differentiated cells.

The next antibody, AG10, had a cloning efficiency of less than 10% for both fractions (Figure 4A) and the positive fraction had a better cloning efficiency than its negative counterpart. AG10, showed a notorious difference with respect to AA11 between the percentage of positive SSEA3 and OCT4 cells per colony amongst fractions (Figure 4.2A). The positive fraction of AG10 had a greater number of cells that were positive for both SSEA3 and OCT4 than the negative fraction (Figure 4.2B). The colonies that reformed from both fractions were small overall in terms of surface area when measuring either SSEA3 or OCT4. There were also similar numbers of SSEA3 positive and negative cells, likewise OCT4 positive and negative cells (Figure 4.2C). The positive fraction of AG10 showed a slight advantage in terms of its cells being positive for both SSEA3 and OCT4 and in general, less than 80% of the two fractions were positive for both SSEA3 and OCT4 (Figure 4.2D). AG10 positive colonies are likely to come from undifferentiated cells since they had high levels of expression of both SSEA3 and OCT4.

The clonogenicity of the BF4 sorted populations was less than 10% and the positive fraction had better cloning efficiency than the negative fraction (Figure 4A). The BF4 positive fraction showed a slightly higher number of cells for OCT4 than the negative fraction. However, the percentage of SSEA3 positive cells per colony was very low in both fractions (Figure 4.3A). The

size of colonies was small in terms of surface area for both fractions in general when looking at the percentage of OCT4 positive cells per colony. The number of cells per colony for OCT4 positive cells was low as well (Figure 4.3B). When looking at the percentage of SSEA3 positive cells per colony, the size of the few SSEA3 positive cells was also small both in terms of number of cells per colony and the area of the colony (Figure 4.3C). There was little correlation between OCT4 and SSEA3 positive cells per colony in amongst fractions of BF4 (Figure 4.3D). BF4 positive colonies are also likely to come from undifferentiated cells since they exhibited high levels of OCT4.

The cloning efficiency of the CC9 sorted populations was lower than 5% and there was little difference between the positive and the negative fractions (Figure 4A). CC9 showed some significant difference across fractions of sorted cells, just like AG10. The number of cells that were positive for SSEA3 after reseeding was higher in the positive fraction compared to the negative counterpart. However, for OCT4 there was not much of a difference between the fractions (Figure 4.4A). When measuring the size of the colonies by looking at the percentage of OCT4 positive cells per colony against the number of cells per colony and the surface area of the colony, the positive fraction had a higher presence in the scatter plots compared to the negative fraction. The size of colonies was consistent with the size of the other antibodies and when all the cells in the colony expressed OCT4 their size ranged from 4 to 30 cells per colony for the positive fraction (Figure 4.4B). There was no great difference in the size of colonies when looking at the percentage of SSEA3 positive cells per colony (Figure 4.4C). There was no strong correlation between the positive and negative fractions for the percentages of OCT4 and SSEA3 positive cells per colony (Figure 4.4D). In the case of CC9 positive colonies, the fact

that they showed high levels of SSEA3 suggested that these colonies formed from undifferentiated cells.

CH8 positive cells cloned better than the negative fraction (Figure 4A). The positive fraction of CH8 had more cells that expressed OCT4 after seeding. The percentage of SSEA3 positive cells per colony however was very low in both fractions (Figure 4.5A). This may be indicative that the positive colonies formed from undifferentiated cells. More than a hundred cells per colony were positive for OCT4 and the size of the colonies overall in terms of number of cells per colony consisted of 60 or less cells. The area occupied by OCT4 positive colonies was consistent with the size for the previous antibodies (200000μm^2) (Figure 4.5B). The size of the colonies positive for SSEA3 was very similar to that of OCT4 (Figure 4.5C). The positive fraction of CH8 had a good correlation between cells positive for SSEA3 and OCT4 and some of the negative CH8 cells also had a good correlation of OCT4 and SSEA3 positive cells (Figure 4.5D).

The DA9 negative population had a slightly better cloning efficiency than the positive fraction (Figure 4A). DA9 did not show much difference between fractions in terms of percentage of positive SSEA3 cells per colony or OCT4 positive cells (Figure 4.6A) but the high expression of OCT4 in positive colonies suggests they come from undifferentiated cells. The number of cells positive for OCT4 was slightly higher than for SSEA3. The size of colonies that were positive for OCT4 was very similar to the other antibodies and there was practically no difference in size between those colonies with no OCT4 positive cells at all and colonies with 100% OCT4 positive cells. They had a size no bigger than 40 cells per colony overall. Their surface area was also quite similar and consistent with the other antibodies (Figure 4.6B). The size of SSEA3 positive colonies showed a difference between those that had few or no SSEA3 positive cells at all and those with a hundred percent of their cells positive for SSEA3. The latter were

slightly bigger with a few colonies having more than 50 cells per colony. Colonies negative for SSEA3 had less than 20 cells per colony. The area occupied by SSEA3 positive colonies had a maximum value of $400000\mu m^2$ but their size overall was consistent with the other antibodies (Figure 4.6C). There was also a high correlation of OCT4 and SSEA3 positive cells per colony (Figure 4.6D).

Lastly, EF12 positive cells cloned better than the negative population (Figure 4A) and the EF12 sorted fractions showed a difference between them in the percentage of SSEA3 positive cells per colony. The positive fraction yielded more cells with a high percentage of SSEA3 expression and both fractions were positive for OCT4 (Figure 4.7A). The size of colonies positive for OCT4 was consistent with the rest of the antibodies. There was not much difference in size between cells positive for SSEA3 and the negatives. The area of the colonies was also consistent with the rest of the antibodies (Figures 4.7 B-C). There was a greater correlation between OCT4 and SSEA3 positive cells in the positive fraction of EF12 compared to the EF12 negative fraction (Figure 4.7 D). Both fractions (positive and negative) had high expression of OCT4 which suggests that the colonies grew from undifferentiated cells.

SSEA3 was used to compare the response of the novel antibodies with a more common marker. The cloning efficiency of the sorted populations of SSEA3 was not far off from that of the rest of the antibodies (Figure 4 A). Overall, it was very similar in terms of percentage of cells that grew back after seeding and the positive sorted fraction of SSEA3 cloned slightly better than the negative population. The positive fraction of SSEA3 showed a slight advantage in terms of percentage of SSEA3 positive cells per colony compared to the negative population. The percentage of OCT4 positive cells per colony between sorted fractions was very similar (Figure 4.8 A). In terms of size, the colonies with 100% of OCT4 positive cells in

both sorted fractions had between 4 and 50 cells per colony and the area occupied by them was around 100000μm^2. The colonies positive for SSEA3 were also very similar in this aspect of size (Figure 4.8 B-C). The colonies with 100% SSEA3 and OCT4 positive cells were not many but there was more correlation with increasing percentages (Figure 4.8 D).
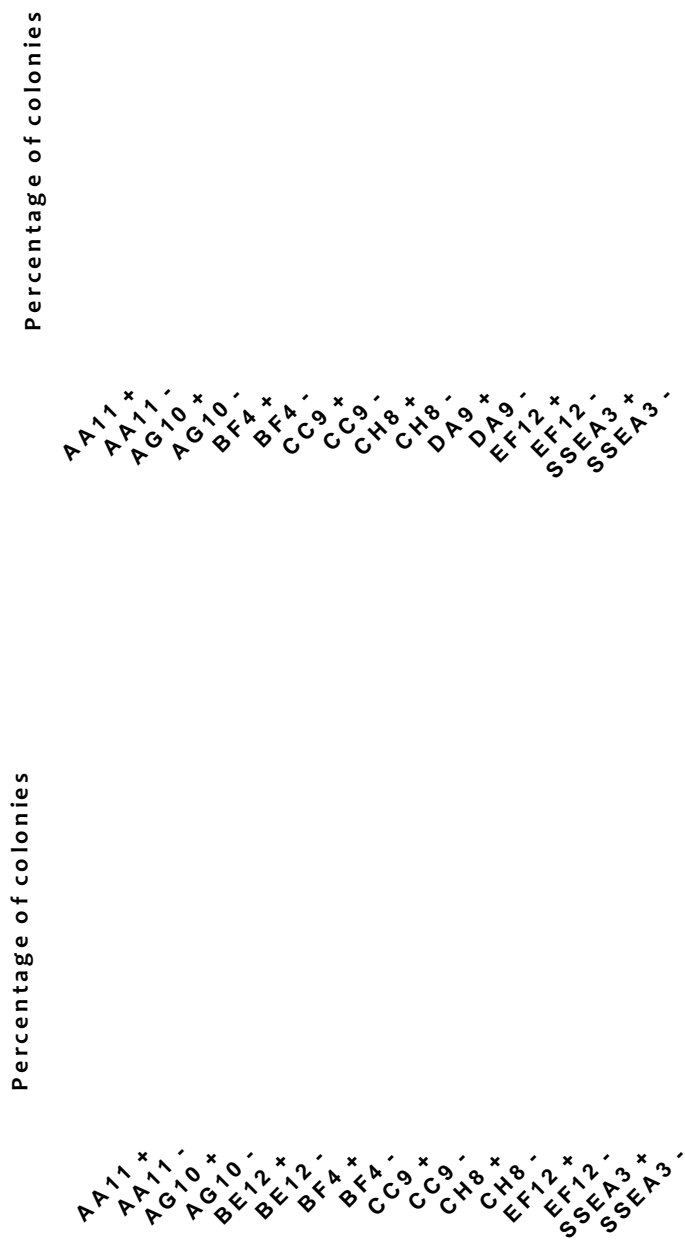
Figure 4. Cloning efficiency of sorted populations in EC (A) and ES (B) cells. Grey bars represent biological replicates. All other samples were from single biological samples.
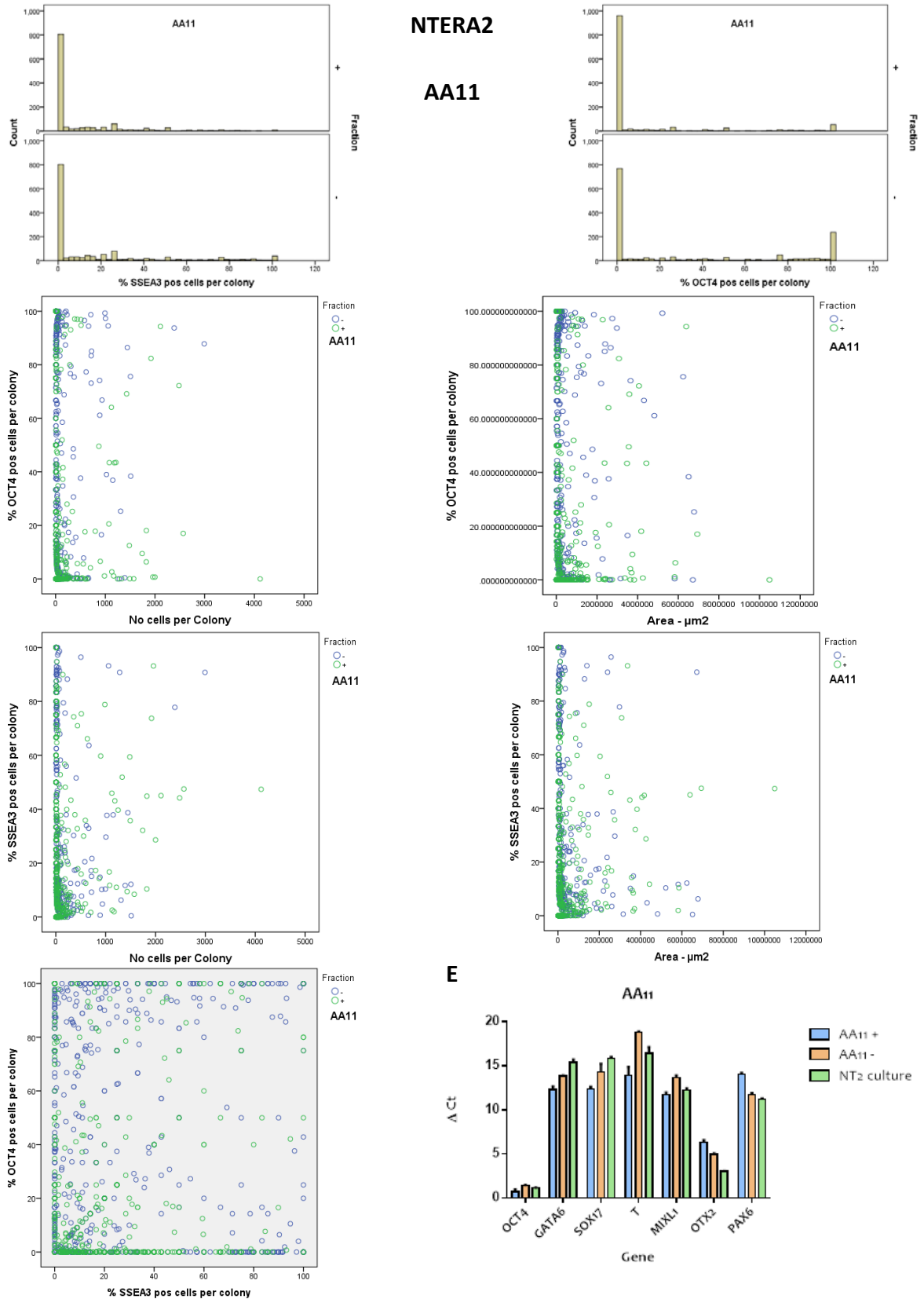
Figure 4.1 A) **Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony**. **B, C**) **Size of colonies** - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D) Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony**. **E) qPCR –** Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (OTX2, PAX6).

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Figure 4.2 A) **Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony**. **B, C**) **Size of colonies** - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D**) **Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony**. **E) qPCR –** Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (OTX2, PAX6).
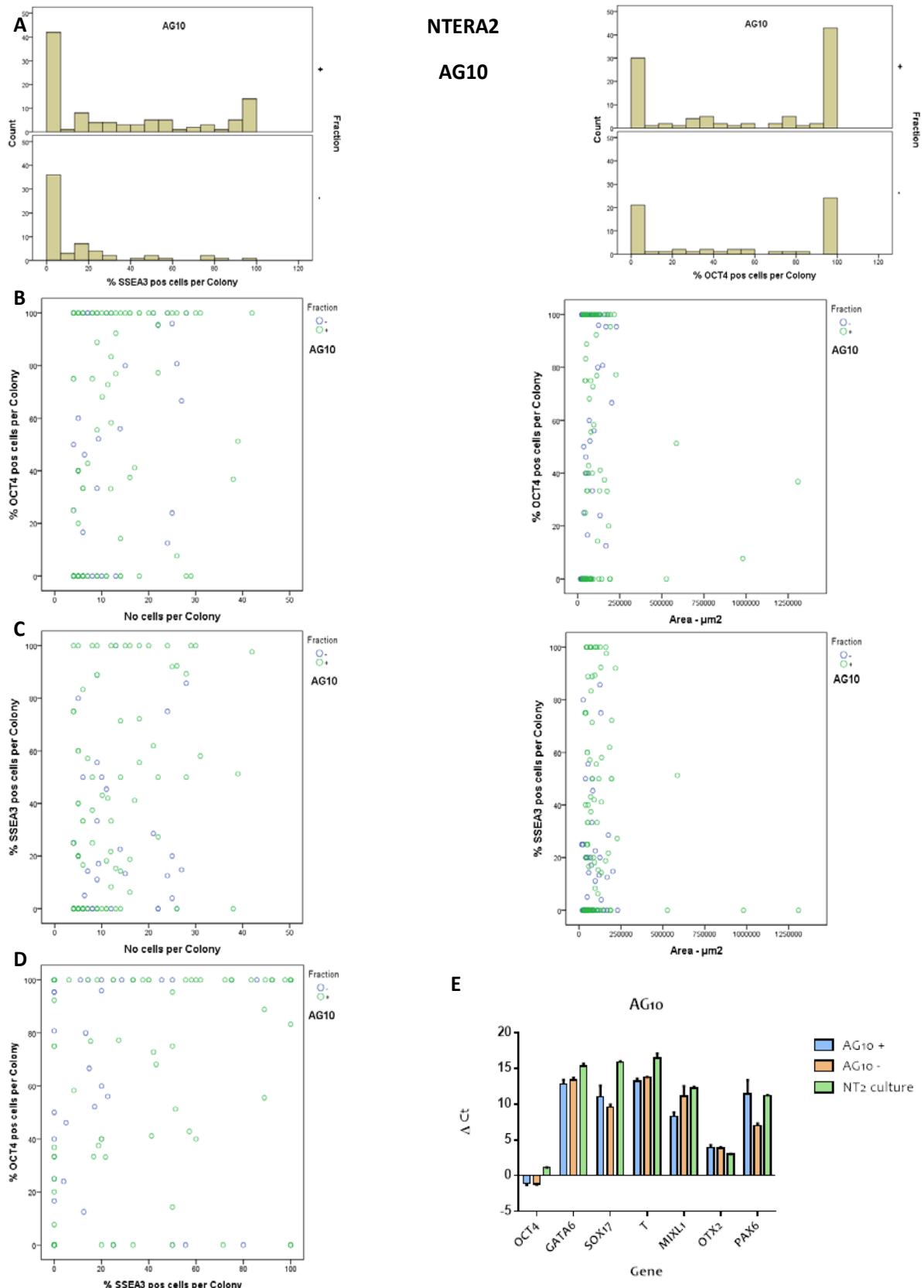
Figure 4.3 A) **Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony**. **B, C)** **Size of colonies** - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D) Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony**. **E) qPCR** – Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (OTX2, PAX6).
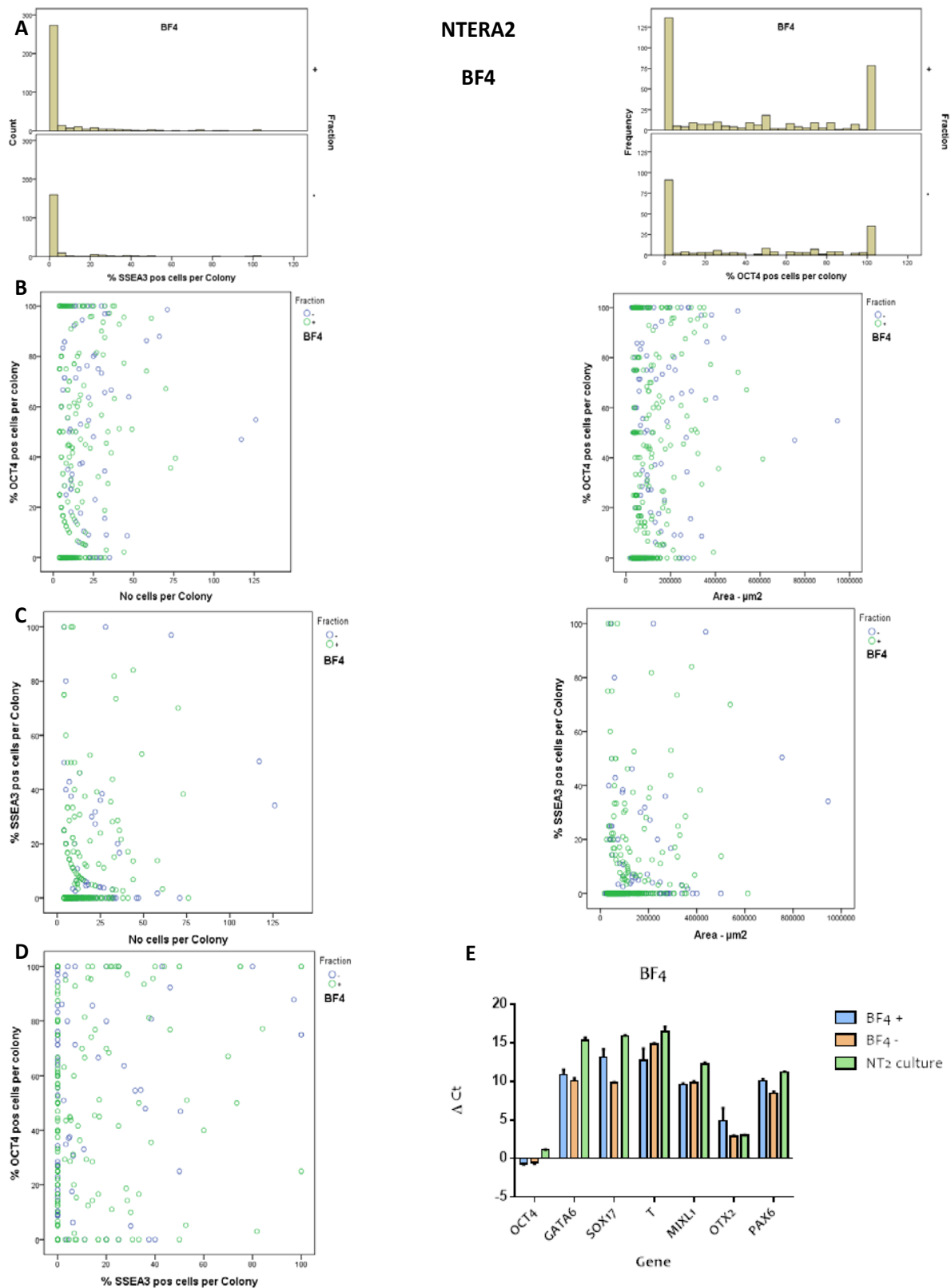
Figure 4.4 A) Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony. B, C) Size of colonies - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D) Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony. E) qPCR –** Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (OTX2, PAX6).
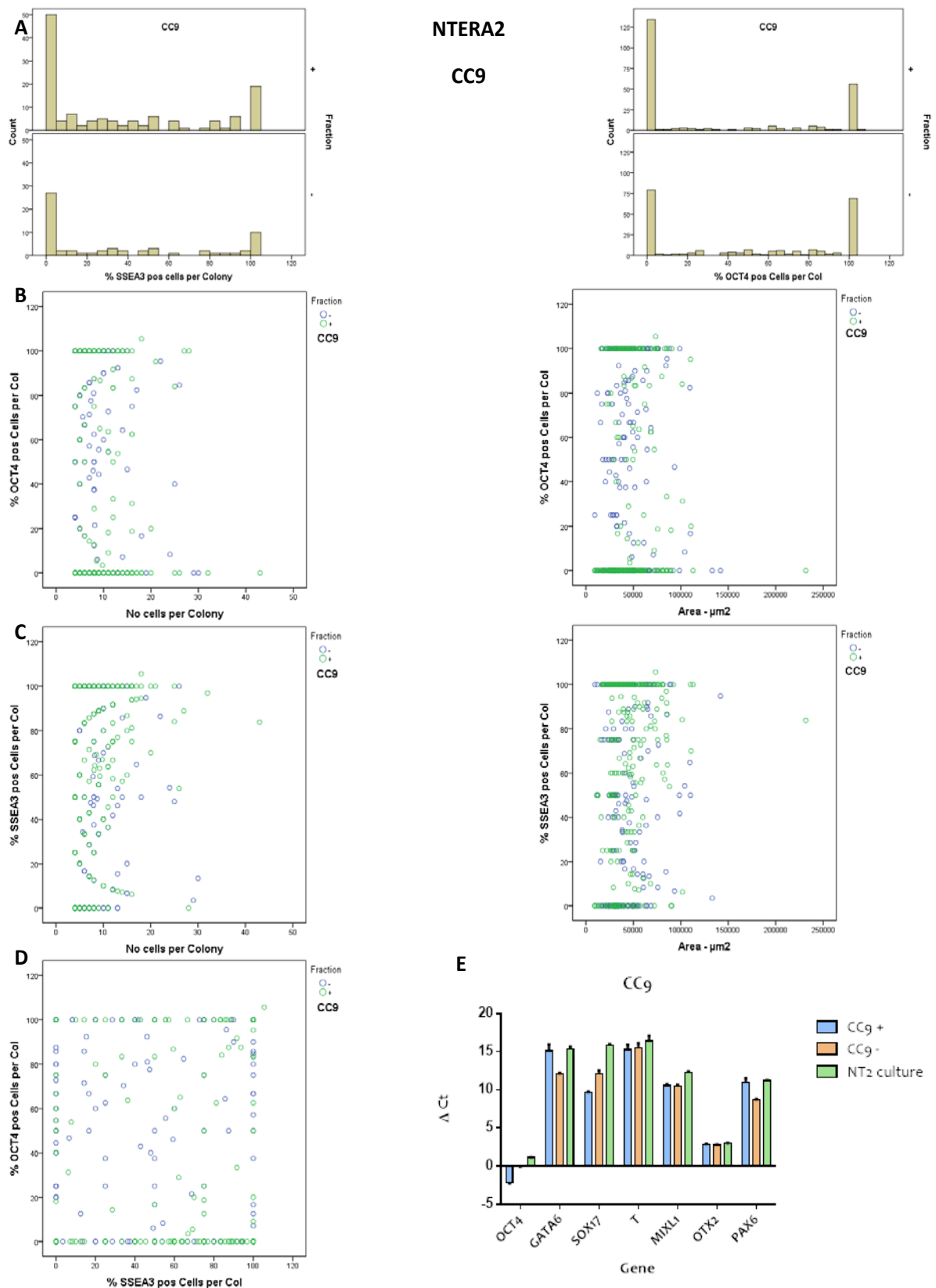
Figure 4.5 A) Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony. B, C) Size of colonies - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D) Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony. E) qPCR –** Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (OTX2, PAX6).
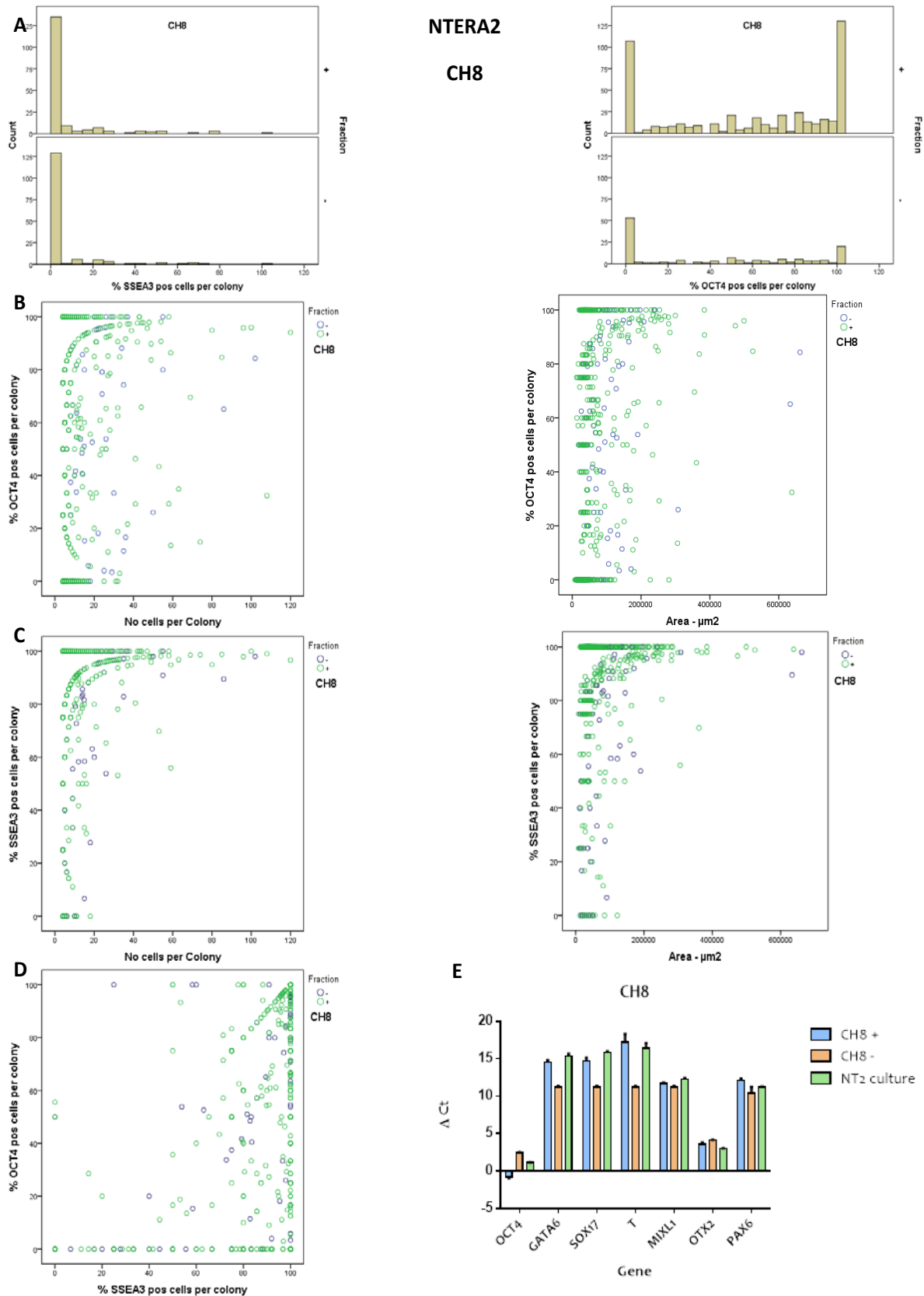
Figure 4.6 A) **Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony**. **B, C**) **Size of colonies** - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D**) **Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony**.

Figure 4.7 A) Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony. B, C) Size of colonies - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D) Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony. E) qPCR** – Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (OTX2, PAX6).
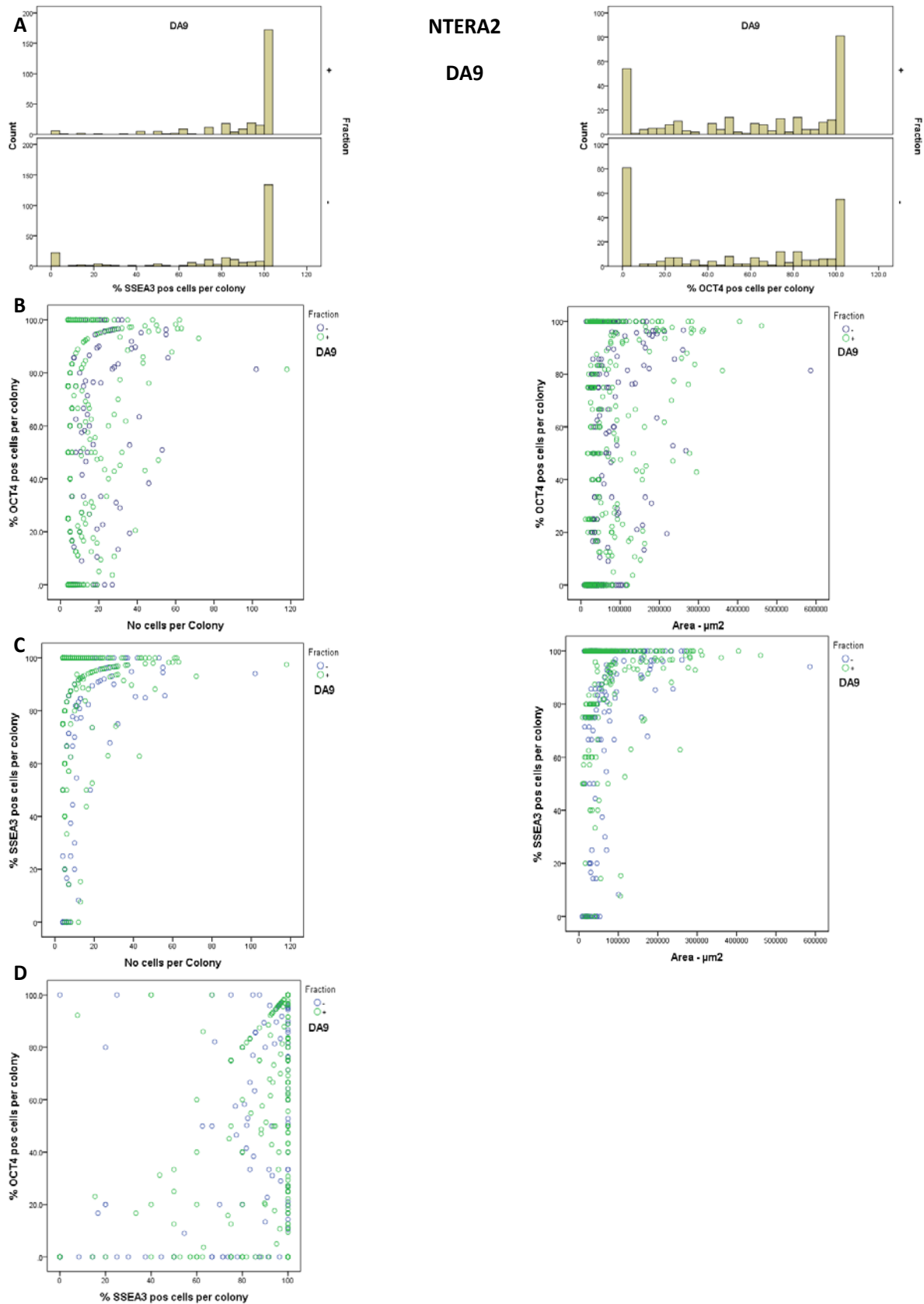
Figure 4.8 A) Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony. B, C) Size of colonies - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D) Percentage of OCT4 positive cells per colony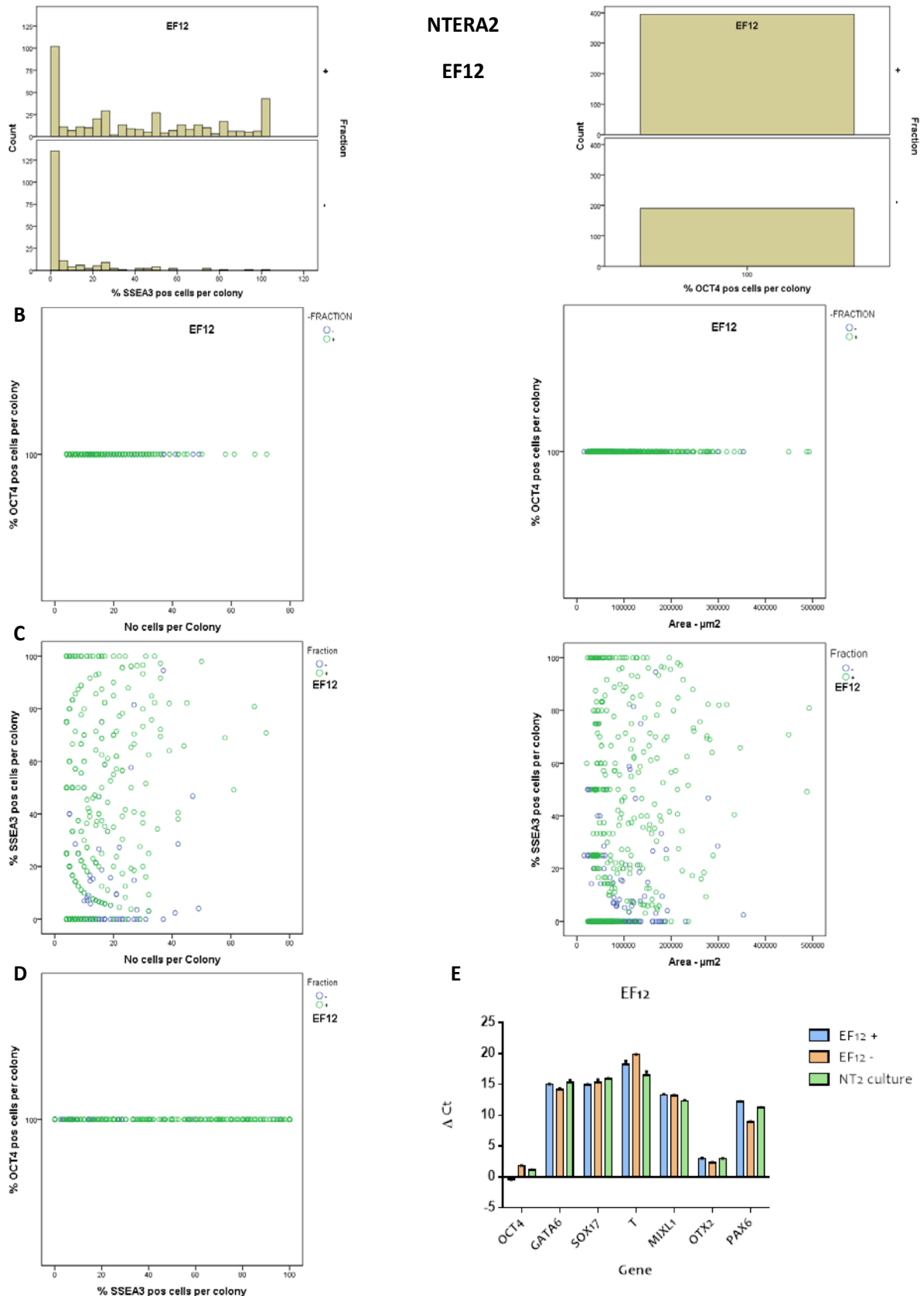 vs percentage of SSEA3 positive cells per colony. E) qPCR –** Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (OTX2, PAX6).
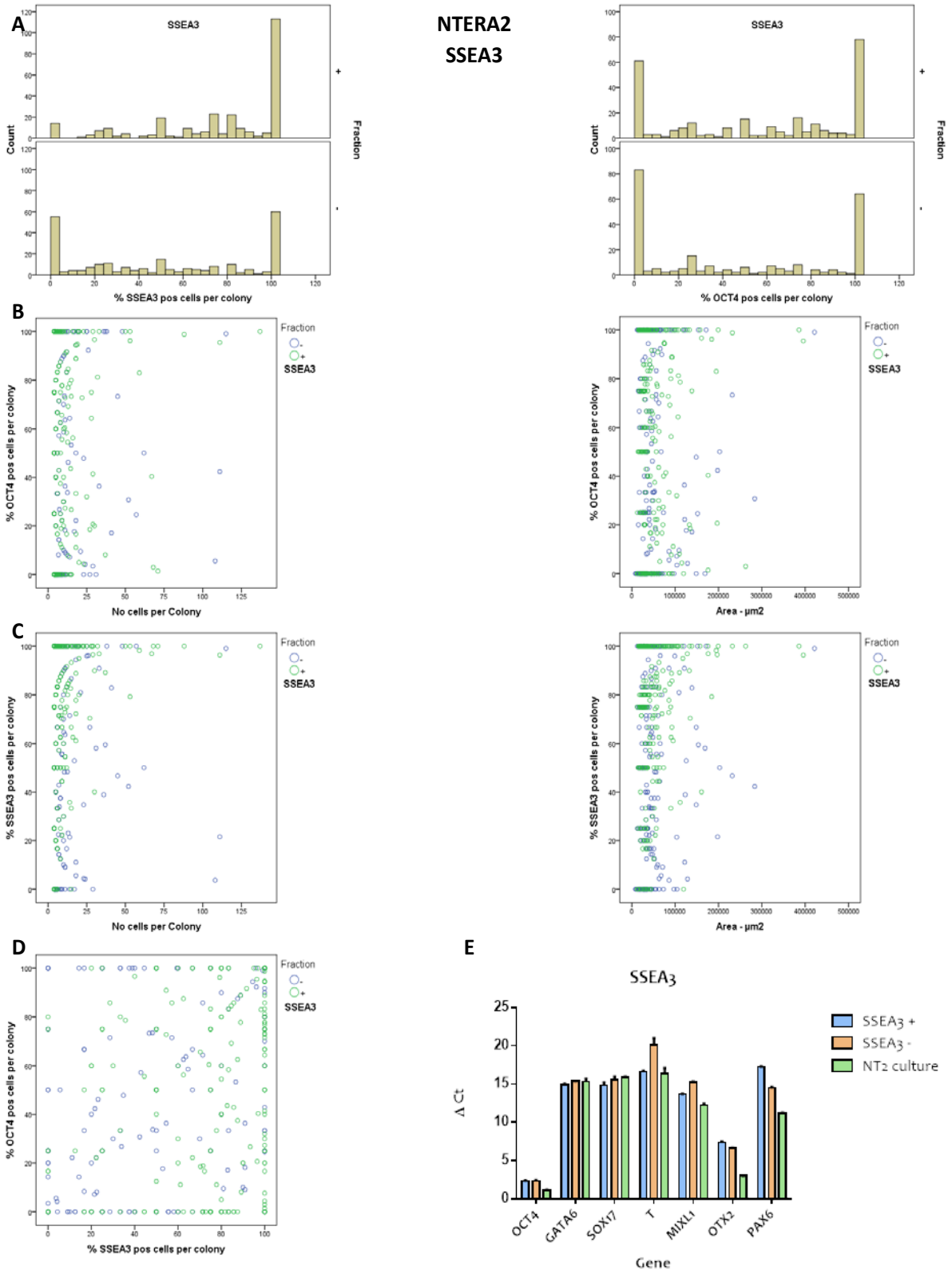
3.2.4 Clonogenic Assays in ES cells (H7.S6)

The cloning efficiency for ES cells was lower overall compared to that of EC cells. AA11 showed less than 2% cloning efficiency for both positive and negative fractions (Figure 4B). AA11 did not show much difference between fractions in the expression for SSEA3 and OCT4 in ES cells (Figure 5A). The size of the colonies was smaller than in EC cells both in terms of number of cells per colony and in surface area when looking at the percentage of OCT4 positive cells (Figure 5B). The size of colonies when looking at the expression of SSEA3 was also small and comparable to that of OCT4 (Figure 5C). There was low correlation between OCT4 and SSEA3 positive cells as well (Figure 5D).

The positive fraction of AG10 had a slightly better cloning efficiency than the negative fraction but both were lower than 2% (Figure 4B). AG10 fractions were similar in their expression of SSEA3 and OCT4 (Figure 5.1A). Neither fraction displayed an advantage over the other when looking at the percentage of both SSEA3 and OCT4. The size of colonies with OCT4 positive cells was small overall, although there were some colonies which were at least 50% positive for OCT4 with as many as 600 cells in the negative fraction of AG10. The largest colony of AG10 positive cells that expressed OCT4 (~40%) had about 450 cells. The area of the few OCT4 positive colonies was less than 100000µm^2 (Figure 5.1B). The size of SSEA3 positive colonies was very similar to the OCT4 population. Very few cells that were positive for SSEA3 were also positive for OCT4, only the positive fraction of AG10 showed some correlation in this respect (Figures 5.1 C-D).

BE12 showed better cloning efficiency in the negative fraction compared to the positive population (Figure 4B). The negative fraction of BE12 displayed an increased number of cells expressing SSEA3 than the positive fraction. The positive fraction only had up to 70% positive

expression of SSEA3 whereas the negative fraction did have expression of 100% of SSEA3 in some cells. There was little difference in OCT4 expression between fractions but overall, the negative fraction had slightly more positive OCT4 cells (Figure 5.2A). The size of colonies in which there were some positive OCT4 cells was small for both fractions, these colonies consisted of less than 40 cells. As the percentage of OCT4 positive cells in the colonies decreased, their size increased. However, this was more noticeable in the negative fraction. The biggest colony, in terms of number of cells per colony, consisted of 250 cells in the negative fraction and only around 30% of those cells in the colony were positive for OCT4. The size of SSEA3 positive colonies was very similar and the area occupied by the colonies was also relatively the same in OCT4 and SSEA3 positive colonies of both fractions (Figure 5.2 B-C). Only a few colonies of the negative BE12 fraction with 100% of SSEA3 expression were also 100% positive for OCT4. In general, there was little correlation regarding this feature of the colonies (Figure 5.2D).

BF4 showed a big increase in cloning efficiency in one of the replicates in both fractions. The positive fraction reported more than 6% efficiency whereas the negative fraction had an efficiency of 3.68% (Figure 4B). The cells from the BF4 fractions did not have a marked expression of SSEA3 after re-seeding and there was only a small difference in the expression of OCT4 (Figure 5.3A). The size of colonies was comparable between colonies with some expression of OCT4 and SSEA3. Overall, their size did not reach beyond 50 cells per colony. The largest colonies had 120 cells and the expression of OCT4 and SSEA3 was around 50% and they were found in the negative fraction of BF4. The area of the colonies was very similar as well (Figure 5.3 B-C). Only the positive fraction of BF4 showed a complete correlation between SSEA3 and OCT4 positive cells (Figure 5.3 D).

CC9 had a fairly similar cloning efficiency between replicates and the positive fraction cloned better than the negative fraction (Figure 4B). The sorted populations of CC9 showed some difference in the expression of SSEA3 positive cells per colony. The positive fraction had a higher number of cells with a high percentage of SSEA3 expression compared to the negative fraction. Also, there was a difference in the expression of OCT4 between the positive and the negative fraction (Figure 5.4 A). The size of the colonies with a high percentage of OCT4 and SSEA3 in terms of number of cells per colony was similar and the positive fraction displayed the largest colonies with a size of nearly 800 cells per colony, although their expression was very low for OCT4 but very high for SSEA3. In terms of surface area, there was a similarity between OCT4 and SSEA3 positive colonies. The positive fraction of CC9 occupied most of the spectrum in the plot. The largest colonies occupied an area of roughly $500000\mu m^2$ and they belonged to the CC9 positive fraction (Figure 5.4 B-C). There was slightly more correlation when looking at the relationship between OCT4 and SSEA3 positive cells per colony and it was only the positive fraction that had 100% of its cells in a colony expressing OCT4 and SSEA3 (Figure 5.4 D).

There was a clear difference in the cloning efficiency of the CH8 sorted fractions, with the positive population having a higher percentage of cloning than the negative fraction (Figure 4B). The expression of SSEA3 in the CH8 positive fraction was also higher than in the negative population. However, the expression of OCT4 was practically the same between fractions (Figure 5.5 A). The size of CH8 positive colonies that expressed OCT4 was small in terms of number of cells per colony, although there was an instance in which a colony with around 90% of OCT4 positive cells had more than 300 cells. The area of the majority of these colonies was less than $100000\mu m^2$. The colonies that had SSEA3 expressing cells were somewhat larger

overall both in terms of number of cells and surface area (Figure 5.5 B-C). There was some correlation in the expression of both OCT4 and SSEA3 (Figure 5.5D).

The EF12 positive fraction had a high cloning efficiency compared to the rest of the antibodies, whereas the negative population had a low percentage of clones (Figure 4B). The positive fraction of EF12 also displayed greater expression of both SSEA3 and OCT4 than the negative fraction (Figure 5.6A). The size of colonies from both fractions with OCT4 positive cells was very similar. The majority of the colonies did not surpass 200 cells per colony. The biggest colonies were found in the positive fraction with as much as 800 cells per colony. The surface area of the colonies was also similar between fractions. Colonies with SSEA3 positive cells had a similar size to those that only expressed OCT4 both in terms of number of cells per colony and surface area. Again, the positive fraction presented bigger colonies than the negative fraction (Figure 5.6 B-C). There was a high correlation of SSEA3 and OCT4 positive cells for the positive fraction, whilst the negative population had lower levels of this feature (Figure 5.6D).

The cloning efficiency of both sorted populations for SSEA3 was the same and overall it was very similar when compared to the rest of the novel antibodies (Figure 4 B). The percentage of SSEA3 positive cells per colony was very similar across the sorted fractions, although the positive fraction had two times more number of colonies containing 100% SSEA3 positive cells than the negative population. In the case of OCT4 positive cells per colony, the negative fraction showed just a little more number of colonies with 100% OCT4 positive cells than the positive population (Figure 5.7 A). The colonies with 100% OCT4 positive cells had less than fifty cells and accordingly, their surface area was also small. The biggest colony with a high percentage of OCT4 positive cells (> 90%) was found in the positive fraction of SSEA3 and consisted of almost 500 cells, with an area of nearly 600000μm^2. The colonies positive for

SSEA3 in both fractions were also small, similar to those with OCT4 positive cells (Figure 5.7

B-C). There was a high correlation of colonies with high percentages of SSEA3 and OCT4

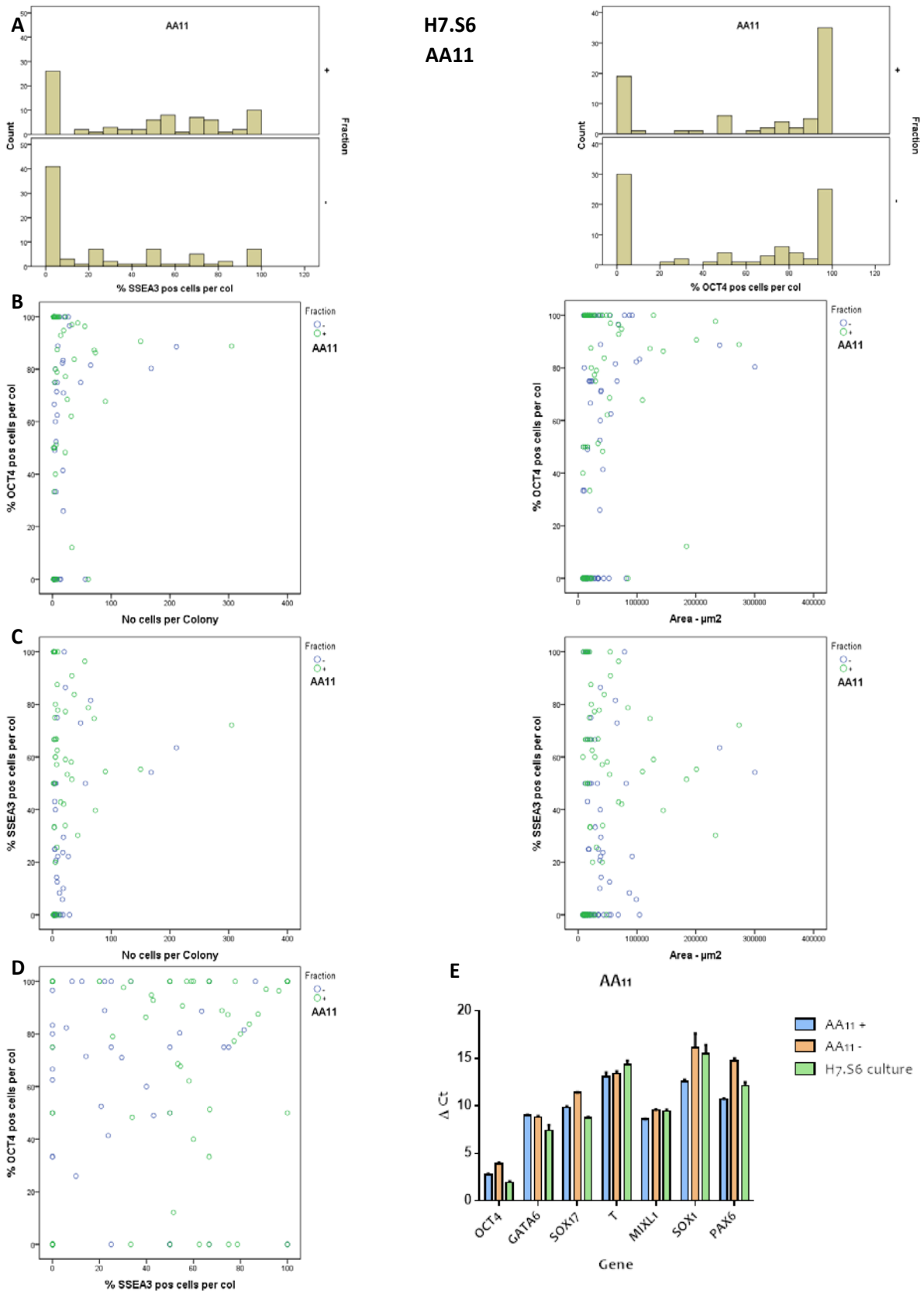positive cells for both sorted fractions (Figure 5.7 D).

Figure 5 A) Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony. B, C) Size of colonies - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D) Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony. E) qPCR –** Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (SOX1, PAX6).

Figure 5.1 A) **Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony**. **B, C**) **Size of colonies** - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D**) **Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony**. **E) qPCR –** Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (OTX2, PAX6).

Figure 5.2 A) **Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony**. **B, C**) **Size of colonies** - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D**) **Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony**. **E) qPCR** – Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (OTX2, PAX6).
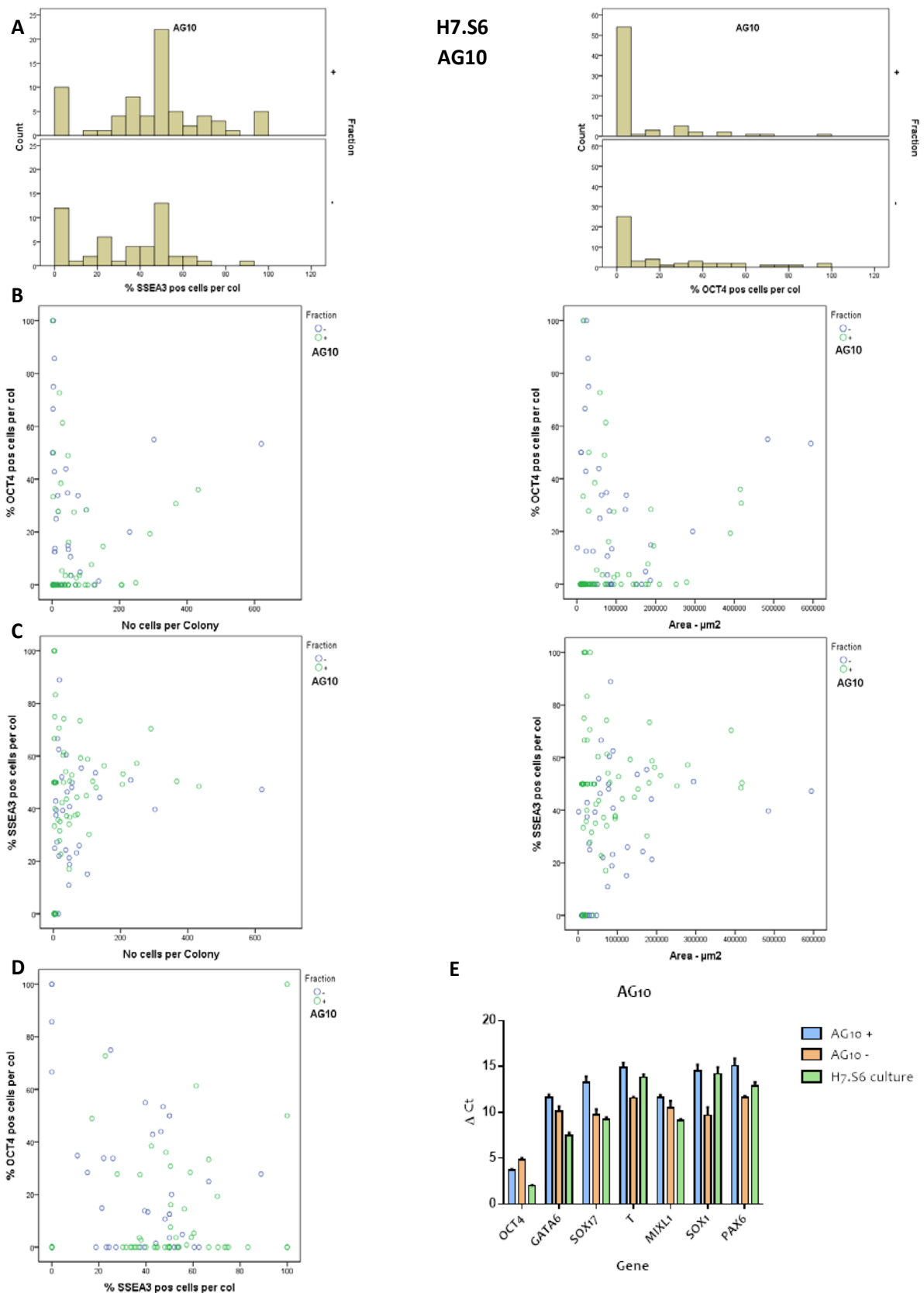
Figure 5.3 A) **Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony**. **B, C**) **Size of colonies** - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D**) **Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony**. **E) qPCR –** Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (OTX2, PAX6).
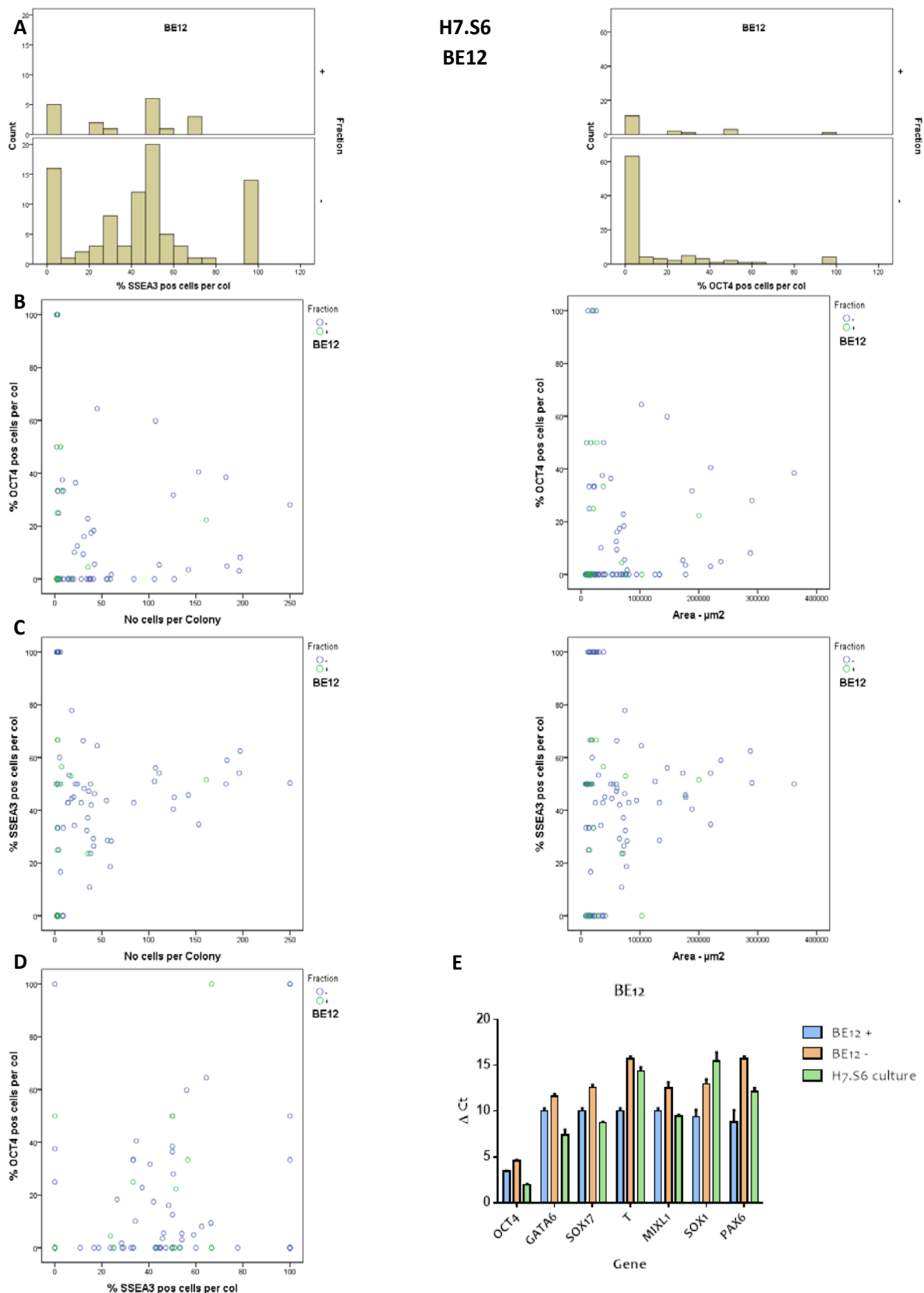
Figure 5.4 A) **Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony**. **B, C**) **Size of colonies** - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D**) **Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony**. **E) qPCR –** Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (OTX2, PAX6).
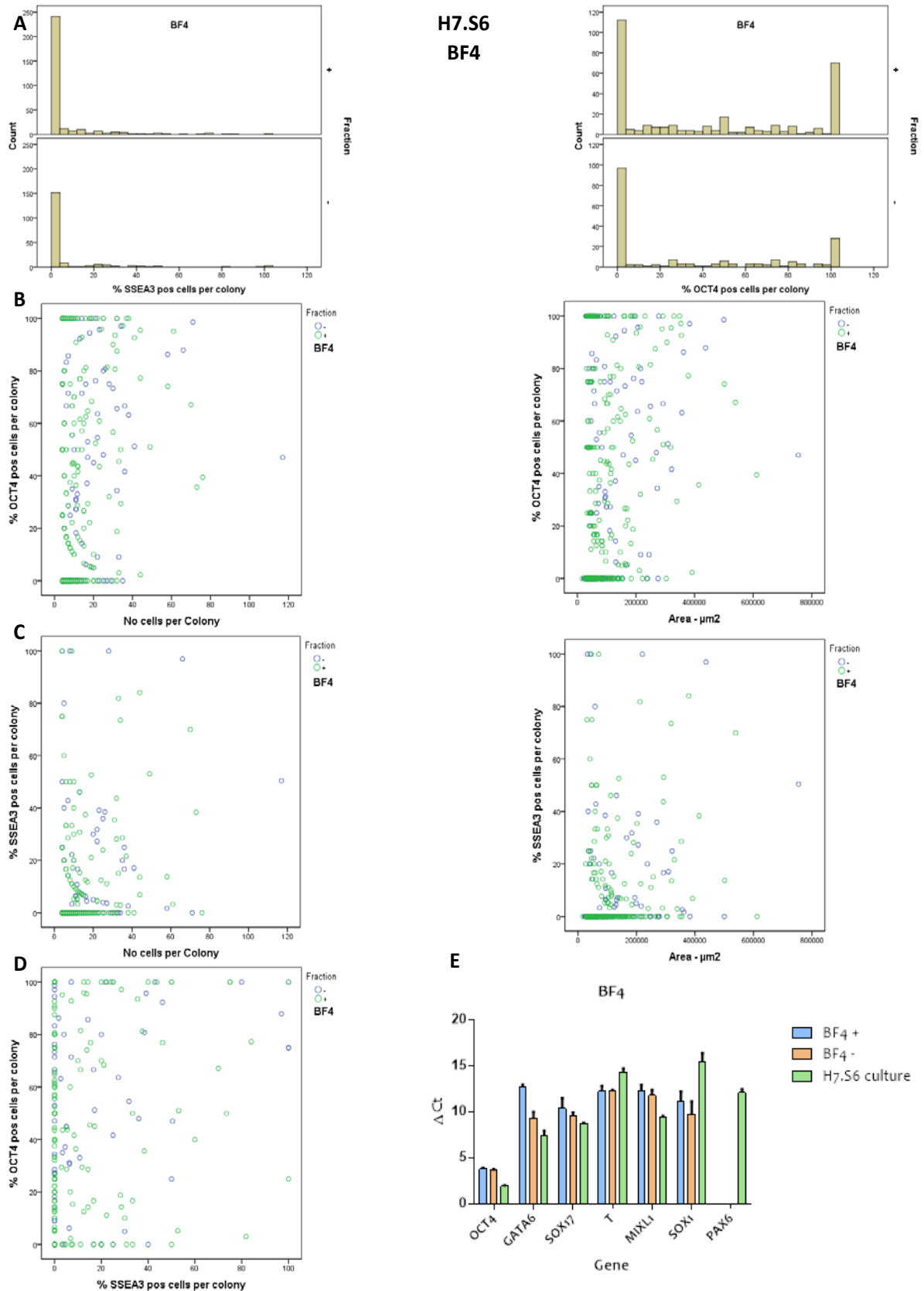
Figure 5.5 A) Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony. B, C) Size of colonies - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D) Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony.**
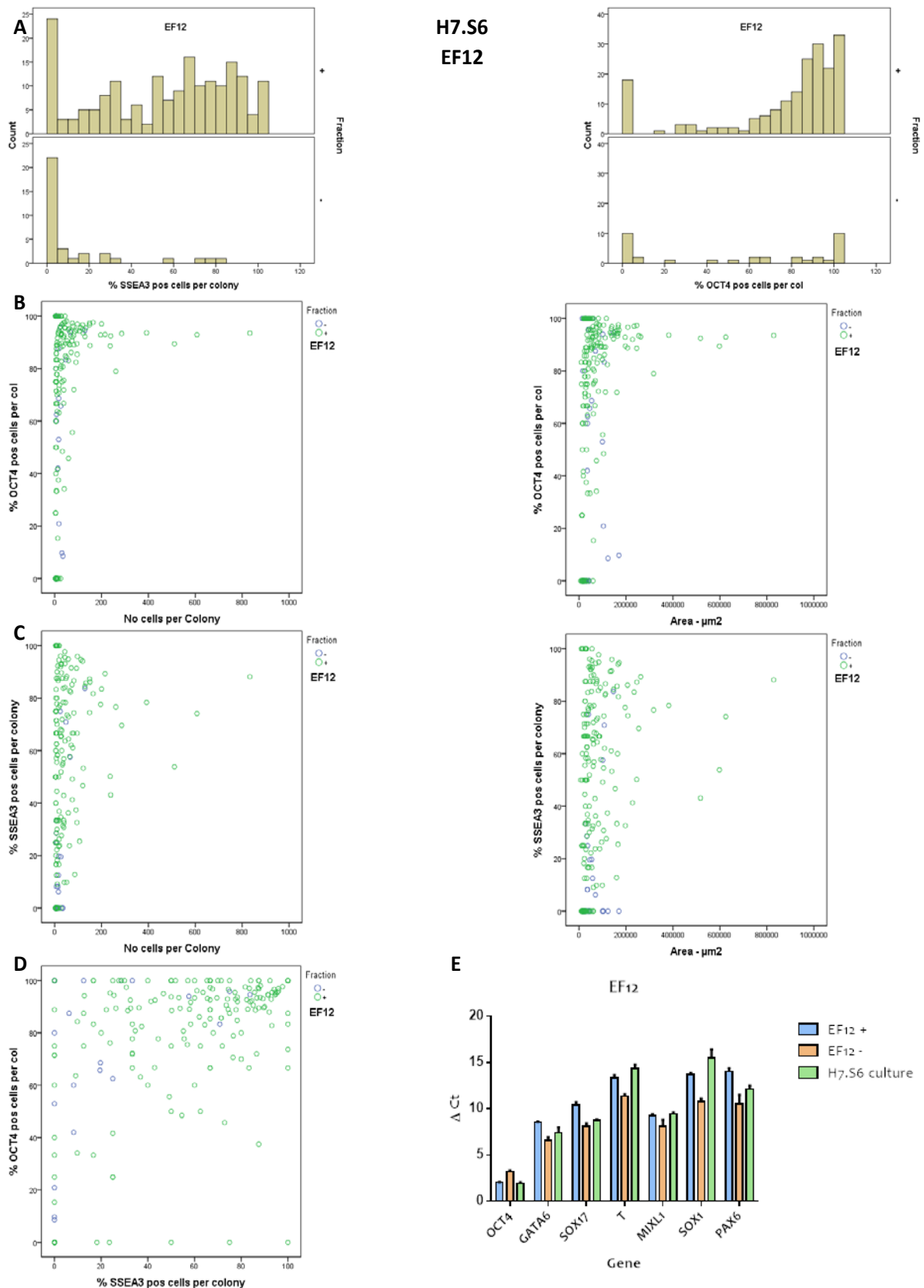
Figure 5.6 A) Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony. B, C) Size of colonies - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D) Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony. E) qPCR –** Positive fraction (Blue), negative fraction (Orange) and unsorted culture (Green). Genes of endoderm (GATA6, SOX17), mesoderm (T, MIXL1) and ectoderm (OTX2, PAX6).
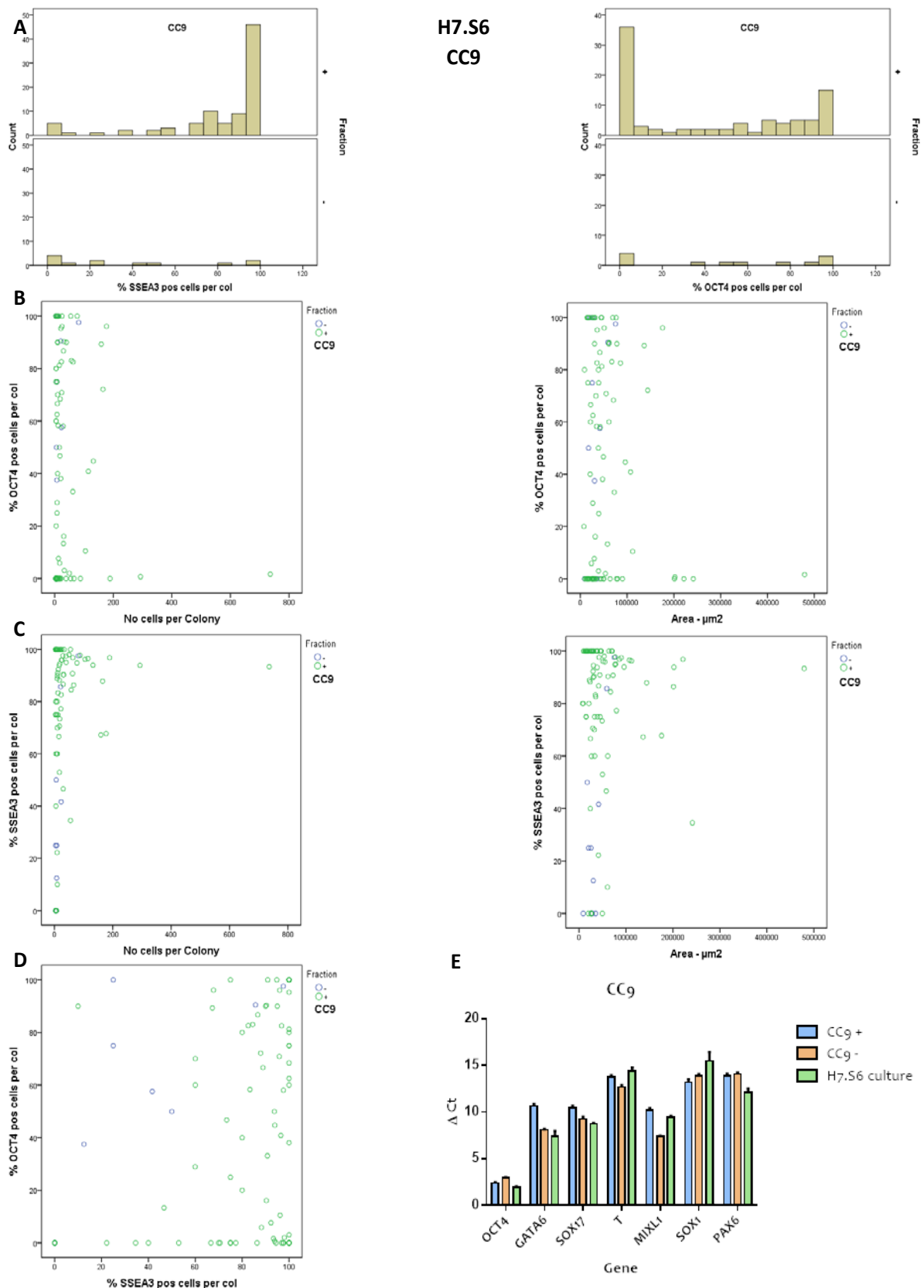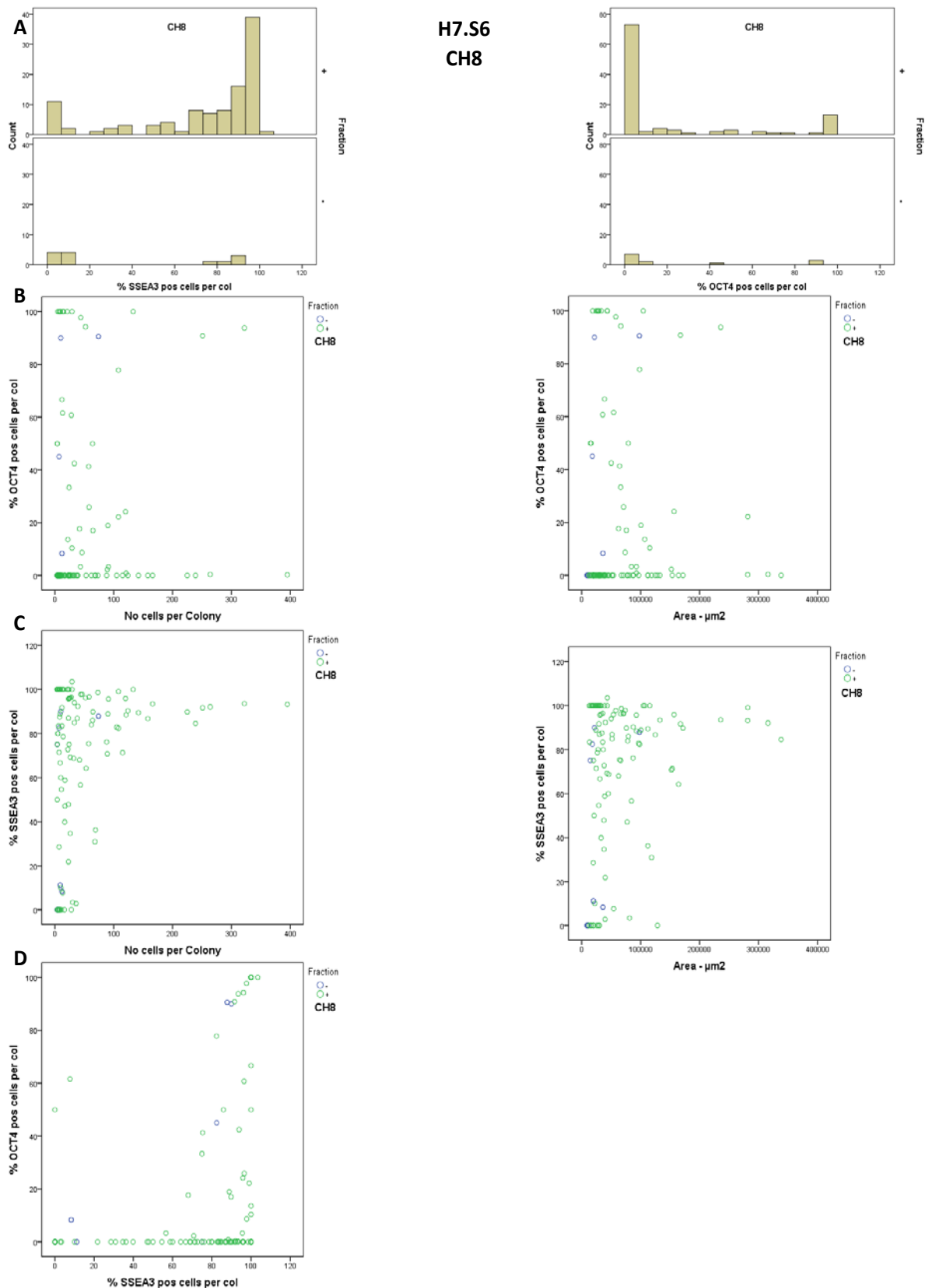
**Figure 5.7 A) Frequency histograms for the percentage of SSEA3 and OCT4 positive cells per colony. B, C)
Size of colonies** - in terms of number of cells per colony and area for OCT4 and SSEA3 positive cells. **D)
Percentage of OCT4 positive cells per colony vs percentage of SSEA3 positive cells per colony.**
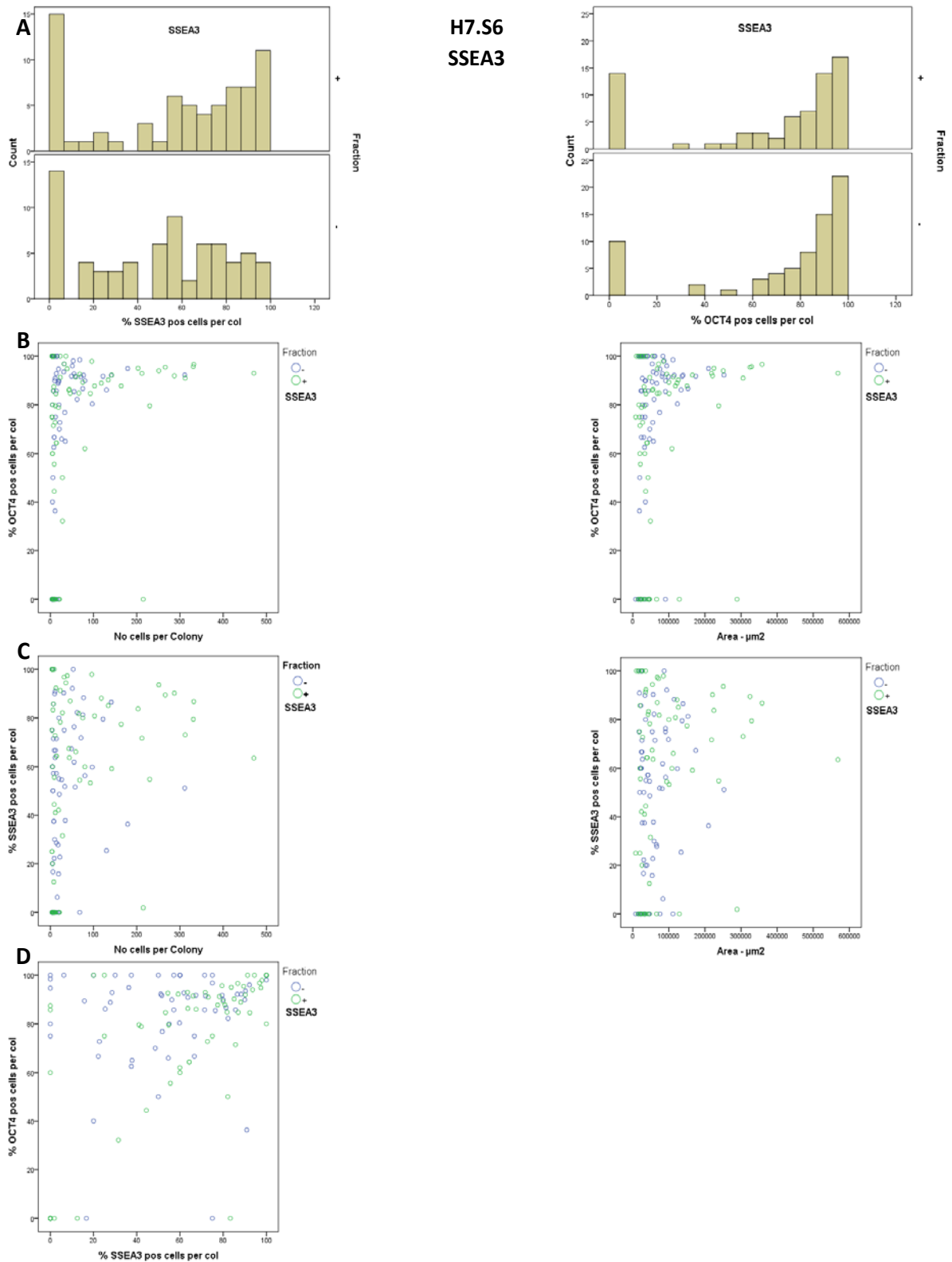
3.2.5 Gene expression analysis in EC (NTERA2) cells

The next step in the analysis of sorted fractions of the different Axordia antibodies was to look at the gene expression profile for each sorted population in order to see if any fraction possessed cells with a higher expression of a particular marker from the three germ layers (ectoderm, endoderm and mesoderm).

The positive and negative populations of SSEA3 had lower expression of OCT4 than unsorted NTERA2 cells (Figure 4.8 E). There was no expression of the GATA6 and SOX17 markers in any population, either sorted or unsorted. The rest of the markers showed similar results, with the exception of OTX2 which is expressed in NTERA2 cells.

OCT4 was more expressed in the positive fraction of AA11 and the other markers did not show considerable expression (Figure 4.1E). There was considerably more expression of OCT4 in the AG10 positive and negative fractions compared to a normal culture of NTERA2 cells. There was practically no expression of the rest of the markers in any culture, normal or sorted, except for OTX2. The negative fraction of AG10 did show some expression of the ectoderm marker PAX6 (Figure 4.2E).

The positive and negative sorted populations of BF4 and CC9 displayed higher expression of OCT4 than normal NTERA2 cells (Figure 4.3; 4.4 E). Similar to the previous antibodies, there was no significant expression of any other markers of the three germ layers. However, CC9 negative cells expressed PAX6 to some extent.

The sorted fractions of CH8 had very low expression of any gene of interest (Figure 4.5 E) and the negative fraction of EF12 displayed some expression of PAX6 (Figure 4.6 E).

3.2.6   Gene expression analysis in ES cells (H7.S6)

The expression of the genes of interest in ES cells was a little different from that found in EC cells. Markers of endoderm lineage seemed to be a little more expressed in some sorted populations, as well as some mesoderm markers.

The positive fraction of AA11 had higher expression of OCT4 than the negative fraction. However, unsorted H7.S6 cells were found to express more OCT4 than both AA11 positive and negative populations (Figure 5 E). Likewise, the endoderm markers GATA6 and SOX17 had a higher expression in unsorted ES cells. Mesoderm markers had similar expression across all three populations and the ectoderm markers SOX1 and PAX6 were more expressed in the positive fraction of AA11 compared to the negative and unsorted populations.

The sorted fractions of AG10 displayed a similar pattern of expression of OCT4 as AA11 sorted cells and unsorted ES cells had higher expression of OCT4 than both sorted populations of AG10 (Figure 5.1 E). A similar pattern of expression of endoderm markers in AA11 sorted fractions was also seen in AG10, with the highest expression of these genes (GATA6, SOX17) found in unsorted ES cells. However, the negative fraction of AG10 had a higher expression of the mesoderm marker T than positive or unsorted cells. The same was observed for both ectoderm markers (SOX1 and PAX6). MIXL1 was more expressed in unsorted ES cells.

In the case of BE12 sorted populations, very similar results to AA11 and AG10 were seen for the expression of OCT4 and the endoderm markers (Figure 5.2 E). However, it was the positive fraction of BE12 that displayed a higher expression of the mesoderm marker T. MIXL1 was similarly expressed in both the positive fraction and unsorted ES cells and higher than the negative population. In the case of the expression of ectoderm markers, the positive fraction displayed higher levels of SOX1 and PAX6 than the negative fraction and unsorted cells.

The positive and negative sorted populations of BF4 expressed less OCT4 than unsorted cells (Figure 5.3 E). However, BF4 negative cells had higher levels of SOX1 and T than unsorted ES cells. The positive population of CC9 expressed more OCT4 than the negative cells but was lower than unsorted ES cells (Figure 5.4 E). The endoderm markers had higher expression in the negative population of CC9 compared to its positive counterpart but lower than unsorted cells. CC9 negative cells also displayed more expression of MIXL1 than the positive fraction and unsorted cells. Expression of the ectoderm markers was very low in the three populations.

The expression of OCT4 in EF12 positive cells was almost the same as in unsorted cells and the negative fraction had lower expression of this pluripotency marker (Figure 5.6 E). Endoderm expression was higher in the negative fraction and the same was also observed in the expression of mesoderm and ectoderm genes, although their expression was lower than GATA6 and SOX17.

3.3   Discussion

The titration of the antibodies showed that overall the best response from standard and novel antibodies was seen when they were used at a 1:10 dilution. Many of the antibodies showed similar expression patterns (BF4 and CC9; CH8 and TRA-1-85) which suggested the possibility that the antibodies could be recognising similar antigens on the cell surface.

In order to gain more insights into this possible scenario, analysis of the antigen expression patterns in human pluripotent stem cells was carried out. The data showed that all antibodies responded uniformly after each replicate and some differences could be seen from these experiments. The expression of SSEA1 seemed slightly higher in ES cells than in EC cells. However, AA11 had a lower expression in ES cells. Furthermore, BE12 had very low expression in NTERA2 and H7.S6 cells but it was highly expressed in nullipotent cells (N2102Ep). CC9, although present in all three cell lines tested, displayed a different distribution in each one of them. The only antibody that had very low reactivity in N2101Ep cells was DA9, where less than 30% of the cells were positive for this antibody. Moreover, DA9 showed a good response in NTERA2 cells but was practically non-existent in ES cells. The FACS expression profiles showed that there is heterogeneity in the stem cell population and so further analyses were carried out, such as clonogenic assays and gene expression assays.

Overall, the cloning efficiency of the sorted fractions of the novel antibodies was similar to that of SSEA3 both in EC and ES cells (Figure 4 A-B). Also, the SSEA3 positive fraction cloned better in EC cells than its negative counterpart, whereas in ES cells both positive and negative fractions had the same cloning efficiency. Generally, the colonies from the sorted fractions of EC cells were bigger and had more content of SSEA3 and/or OCT4 than those of ES cells. When looking at the features of the colonies, such as their size and content of either SSEA3 or OCT4,

there were some novel antibodies that showed big differences when compared to SSEA3 sorted fractions in both EC and ES cells. In EC cells, EF12, CH8, BF4 and AG10 were generally more dissimilar to the cells sorted for SSEA3 than the cells sorted for rest of the antibodies. Whereas in ES cells, the negative fractions of novel sorted antibodies tended to have low content of SSEA3 positive cells in their colonies compared to the negative fraction of SSEA3, with the exception of BE12.

In EC cells, some colonies that formed from positive fractions of antigens had high levels of OCT4 expression, this suggests that such colonies were formed from undifferentiated cells. Furthermore, assessing the colonies for different features together with gene expression analysis helped in determining whether some novel antigens are useful in identifying different subsets of cells. For example, AG10, CC9 and EF12 showed interesting patterns of SSEA3/OCT4 expression after replating. They displayed high levels of these markers, suggesting that the resulting colonies emerged from undifferentiated cells. Moreover, the genetic expression of lineage markers in these sorted fractions showed that there might be a bias towards specific lineages. AG10 positive cells, for example, had higher levels of MIXL1 than the unsorted and negative population, whereas the negative fraction of AG10 expressed SOX17 in higher levels. In the case of CC9, the negative fraction could potentially be biased towards an ectoderm lineage, and the positive fraction may be biased towards an endoderm lineage. Likewise, in EF12, the negative fraction might be biased to the ectoderm lineage.

As speculated, there were many similarities but also quite a few differences between the colonies of sorted novel antibodies and those of SSEA3 sorted fractions. Hence, in order to further investigate how similar these novel antibodies might be to the already established set of standard antibodies and how they might help us further our understanding and power of

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resolution of subsets of stem cells, the next step was to perform antigen competition assays to see if any of the novel antibodies recognised the same epitope on the surface of cells.

Chapter 4

4 Competition assays between the novel set of antibodies and standard markers of cell differentiation

4.1 Introduction

Stage specific embryonic antigens are expressed as cell-surface molecules that display patterns of expression in a lineage-restricted manner during development. They are useful tools that help identify embryonic stem cells and their differentiated derivatives. Glycan epitopes such as SSEA1, SSEA3-4 and the glycoprotein antigens TRA-1-60; 1-81 have been used to identify and isolate specific cell types from heterogeneous populations and have been shown to undergo changes upon differentiation (Andrews *et al.*, 1990) (Lanctot *et al.*, 2007).

The structure of SSEA3 and SSEA4 consists of 5-6 monosaccharides attached to a ceramide lipid tail (Figure 7) and they are one of the most commonly used markers to identify embryonic stem cells (Kannagi *et al.*, 1983) (Muramatsu and Muramatsu, 2004). Their expression on the plasma membrane sees a rapid down-regulation upon differentiation (Fenderson and Andrews, 1992). Another commonly used marker of ES cells is the family of glycoprotein antigens (TRA-1-60; 1-81) which have been shown to recognise a keratan sulphated proteoglycan in a neuraminidase-sensitive and insensitive fashion, respectively (Badcock *et al.*, 1999) (Andrews *et al.*, 1984a).
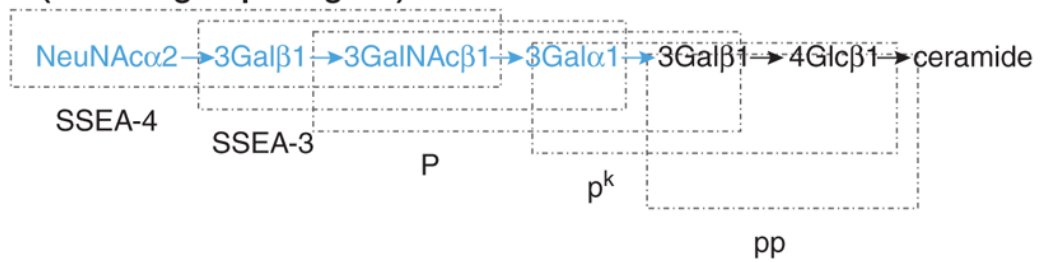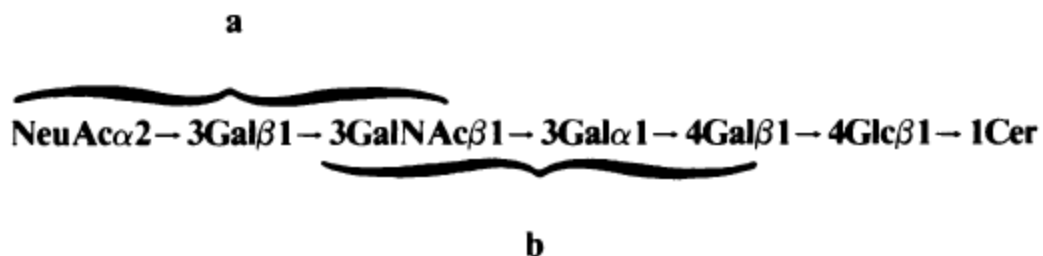
Figure 7. Carbohydrate structure of SSEA3 & SSEA4. Modified from Andrews, P.W. 2011

There is a relationship in the expression of surface molecules of the mouse embryo and human teratocarcinoma cells. In mouse, the changes that occur during the course of murine embryonic development have been well documented using immunological methods (Jacob, 1977) (Solter and Knowles, 1979). Many of the antigens that are developmentally regulated are expressed by pre and peri-implantation mouse embryos and these in turn have also been found on the surface of murine teratocarcinoma cells (Kannagi *et al*., 1983). Human teratocarcinoma cell lines have been shown to express an antigen that is expressed on cells of the F9 murine teratocarcinoma cell line (Hogan *et al*., 1977) (Holden *et al*., 1977). Likewise, the monoclonal antibody defined antigen SSEA1, which is found on the 8-cell stage mouse embryo, was also found to be expressed on some cells of different human teratocarcinoma-derived cell lines (Andrews *et al*., 1980). Nevertheless, SSEA1 is not explicitly found on the surface of human EC cells; instead it is majorly expressed on some differentiated derivatives of EC cells (Andrews *et al*., 1982) (Damjanov *et al*., 1982). SSEA3, however, is expressed on human EC cells and it is also an antigenic determinant found on zygote and cleavage-stage mouse embryos (Shevinsky *et al*., 1982).

The identification of SSEA3 came about after immunizing a rat with 4 to 8 cell-stage mouse embryos. The monoclonal antibody derived from this immunization reacted with all preimplantation mouse embryos up to the early blastocyst stage. The reactivity of SSEA3 was

found to be restricted to endoderm in early post-implantation embryos and to the kidney in adult mice (Fox *et al*., 1982). The subsequent analysis of the immunoprecipitates from murine embryos and human EC lines showed that molecules bearing SSEA3 were both glycolipid and glycoprotein, which suggested a carbohydrate antigenic determinant (Shevinsky *et al*., 1982). The derivation of an antibody recognising SSEA3 confirmed that immunization with embryos rather than with teratocarcinomas is also useful to define embryo-specific antigens.

Soon after the derivation of SSEA3, another related stage-specific embryonic antigen (SSEA4) was reported and like SSEA3, was shown to be an epitope of a globolipid unique to human teratocarcinoma cells (Kannagi *et al*., 1983). Both of these antibodies were reactive with a unique globo-series ganglioside with the following structure:

$$\underbrace{NeuAc\alpha2\rightarrow3Gal\beta1}_{a}\rightarrow\underbrace{3GalNAc\beta1\rightarrow3Gal\alpha1\rightarrow4Gal\beta1}_{b}\rightarrow4Glc\beta1\rightarrow1Cer$$

The terminal 'a' structure is recognised by MC813-70 (SSEA4), whereas the internal 'b' structure is recognised by the antibody MC631 (SSEA3). Further analysis of the reactivity of these two antibodies by solid-phase radioimmunoassay (RIA) determined that these antibodies recognise different antigenic determinants present on the same molecule and

despite their similar reactivity with cells and embryos tested by Kannagi and cols., MC813-70 defines a novel stage-specific embryonic antigen (Kannagi *et al*., 1983).

There are also several high molecular weight glycoprotein antigens expressed on human EC cells that are down-regulated upon differentiation. TRA-1-60 is one of these antigens that shares properties with other monoclonal antibodies like TRA-1-81, GCTM2 and K21 (Badcock *et al*., 1999). Whilst the EC cell marker antigens SSEA3 and SSEA4 are glycolipids, TRA-1-81, GCTM2 and K21 are monoclonal antibodies that recognise proteins of similar size to that identified by TRA-1-60; and they all display similar patterns of expression on tumour-derived cell lines (Andrews *et al*., 1984a) (Andrews *et al*., 1996). This family of high molecular weight protein antigens display some epitopes that appear to be human specific, but they have also been found in mouse embryos and mouse EC/ES cells (Muramatsu *et al*., 1978).

Other commonly used markers include alkaline phosphatase (ALP) which is expressed in its tissue non-specific form in human EC and ES cells and is recognised by two antibodies, TRA-2-49 and TRA-2-54 (Andrews *et al*., 1984c; Benham *et al*., 1981). Another couple of antigens used in the study of human EC and ES cells are CD9 and TRA-1-85. CD9 is typically expressed by human ES cells and is down-regulated upon differentiation. CD9 has also proved to be a valuable marker for identifying subsets of cells that still reside in the stem cell compartment (International Stem Cell *et al*., 2007; Laslett *et al*., 2007). TRA-1-85 recognises a pan-human antigen, also known as the blood group antigen Ok(a) (Williams *et al*., 1988). This antibody has been found to be expressed in several human cell lines and due to this widespread distribution has not been found to be useful in terms of identifying ES cell differentiation (Wright and Andrews, 2009).

Some of the novel antibodies described by Wright, *et al*., in 2011 have been shown to share similarities with some of the antibodies just described. For example, CH8 was shown to recognise CD9 (Wright *et al*., 2011). The other antibodies from the new panel have the potential to serve as new tools to help in the identification of subsets of stem cells. In the previous chapter, it was shown that sorting for specific populations that either expressed or lacked a particular antibody yielded patterns of gene expression that might insinuate that such sorted populations might be biased to a particular lineage. However, it might also be that these novel antibodies are recognising antigens already detected by one of the standard antibodies. Thus, in order to answer this question, competition assays were carried out against SSEA3 and TRA-1-60 in particular since they represent the most likely candidates for being duplicated.

4.2 Results

SSEA3 was conjugated with a secondary antibody in order to carry out competition assays against the novel panel of antibodies. An initial titration curve of the conjugate antibody (SSEA3/AF647) was carried out and the FACS expression profiles were compared to standard SSEA3 (Figure 6 A-B). The concentration used was 1:10, the same used for the standard antibody in indirect staining. This initial titration assay showed that the optimal concentrations were in a similar range as the ones used for the standard SSEA3 antibody alone. Based on this first characterisation, the conjugation of the antibody with AF647 proved to have worked and a concentration of 1:50 seemed to be the ideal.

A second titration curve was carried out where the dilutions used were less compared to the first one (Figure 6.1 A-B). In this case, the dilution at 1:100 for the conjugate antibody still gave a good response on N2102Ep cells and it was the concentration used for the subsequent competition assays against the novel antibodies.

Next, the competition assay using the conjugate antibody (SSEA3/AF647) against SSEA4 and the novel panel of antibodies was performed (Figure 6.2). The median fluorescent intensity value was plotted against the dilution used for each competing antibody. In this first competition assay the dilutions used were 1:100; 1:50 and 1:10. SSEA4 displayed a notable drop in the value of median fluorescent intensity, suggesting that the antibody is recognising the same epitope as SSEA3 and out-competing it. This further establishes the existence of the reported similarity discussed previously between these two antibodies.

In the case of the novel set of antibodies AA11, AG10 and BE12 did not alter the binding of SSEA3 in any form. The same was observed for the rest of the novel antibodies. EF12, however, showed a similar response to SSEA4, but the median fluorescent intensity suggests

that it did not outcompete SSEA3. Nevertheless, it is interesting to note that this is the only antibody from the novel panel that showed such a response. Thus, this first analysis suggests that the other antibodies are indeed recognising novel antigens on the cell surface.

In a second assay performed against the same panel of antibodies, it was more evident that SSEA4 outcompeted SSEA3 and unlike the first assay, it did not take a great concentration of SSEA4 to displace SSEA3 from its binding site (Figure 6.3 & 6.4). The percentage of median fluourescent intensity dropped below 10% for all dilutions used of SSEA4 (Figure 6.4). On the other hand, the novel set of antibodies did not show such a decrease in their MFI (Median Fluorescent Intensity) values, except for EF12, whose MFI value did decrease to 15% when used at dilutions of 1:50 and lower (Figure 6.4). This raises the question of whether there might be some recognition of the same epitope as SSEA3 or perhaps just a segment of the epitope. In any case, the novel nature of the rest of the antibodies is further established by looking at this results.

Next, competition assays with a TRA-1-60/AF488 conjugate antibody were performed (Figure 6.5 A-B). The titration assay for this conjugate antibody was done on N2102Ep cells and it showed that there was still a good response from the antibody at low concentration (e.g. 1:100), hence this was the concentration of conjugate antibody used for the subsequent competition assays.

The first antibody to be compared with TRA-1-60/AF488 was TRA-1-85. The median fluorescent intensity of this antibody displayed a decrease when used at higher concentrations (Figure 6.6). However, it was not enough to show that it is competing with the conjugate antibody (Figure 6.7). SSEA4 also displayed a decrease in its median fluorescent intensity but unlike the competition assays against SSEA3/AF647 it was not enough to

demonstrate that it may be competing with SSEA3 for a binding site (Figure 6.6). The rest of the antibodies depicted in figure 6.6 did not show any kind of sign that they may be recognising the same epitope as TRA-1-60 which further suggests that they are novel tools that might help in identifying subsets of cells within the stem cell compartment.
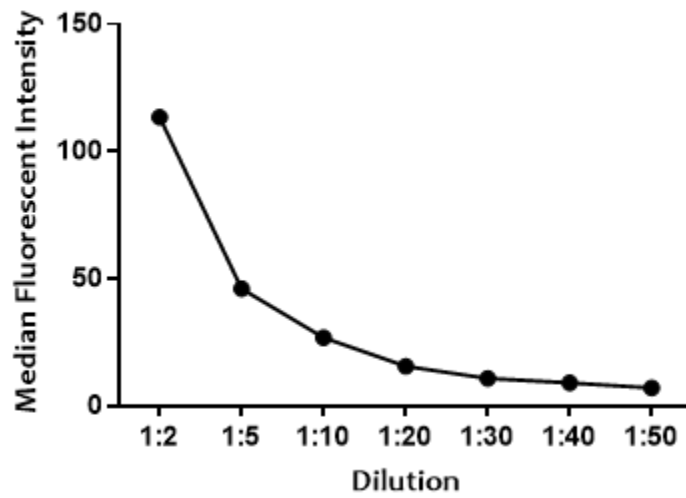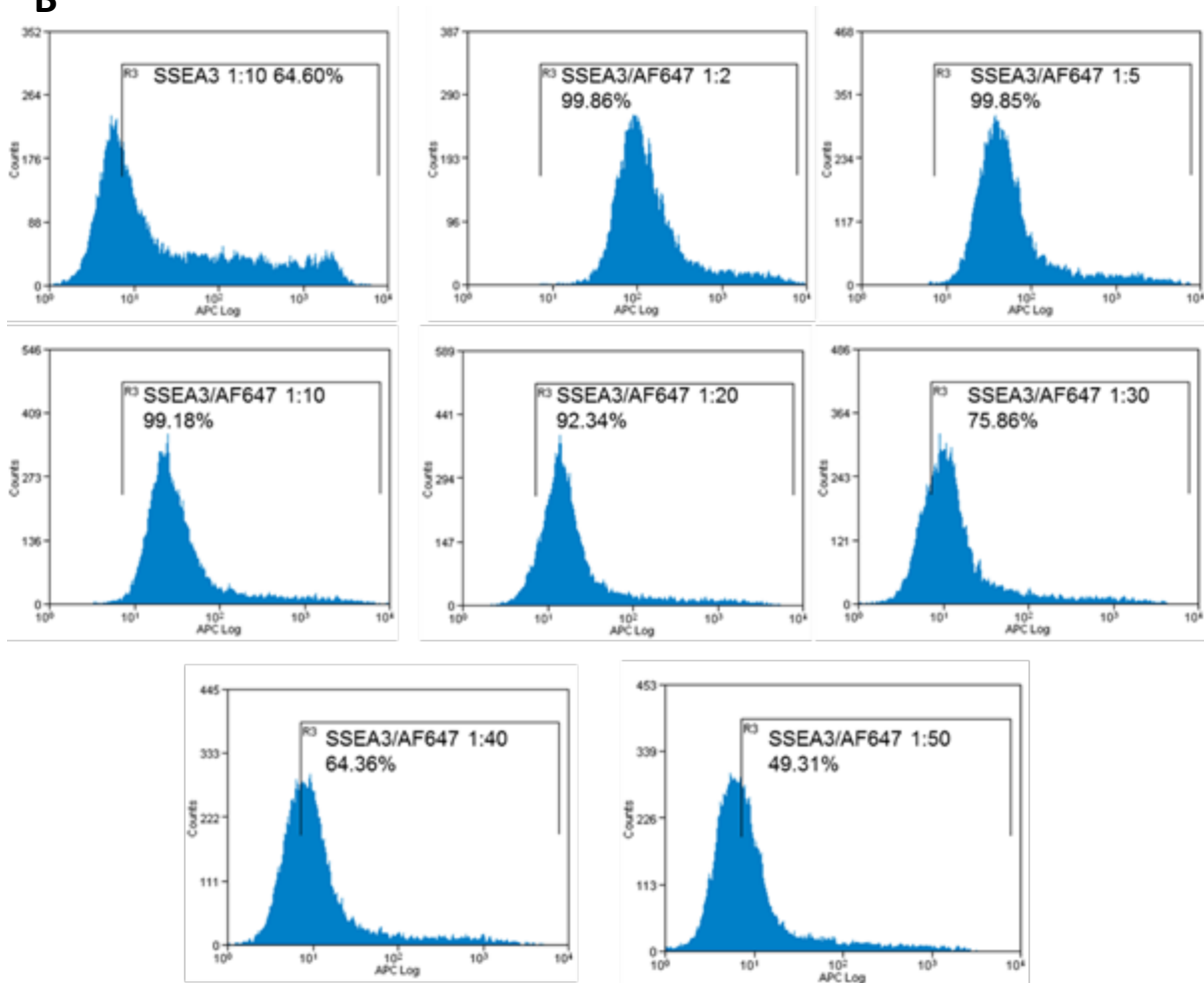
Figure 6. A) SSEA3/AF647 conjugate antibody titration curve. Titration assay was done on N2102Ep cells plotted as dilution vs median fluorescent intensity. **B) FACS expression profile histograms.** Standard SSEA3 was used at 1:10 dilution.

A

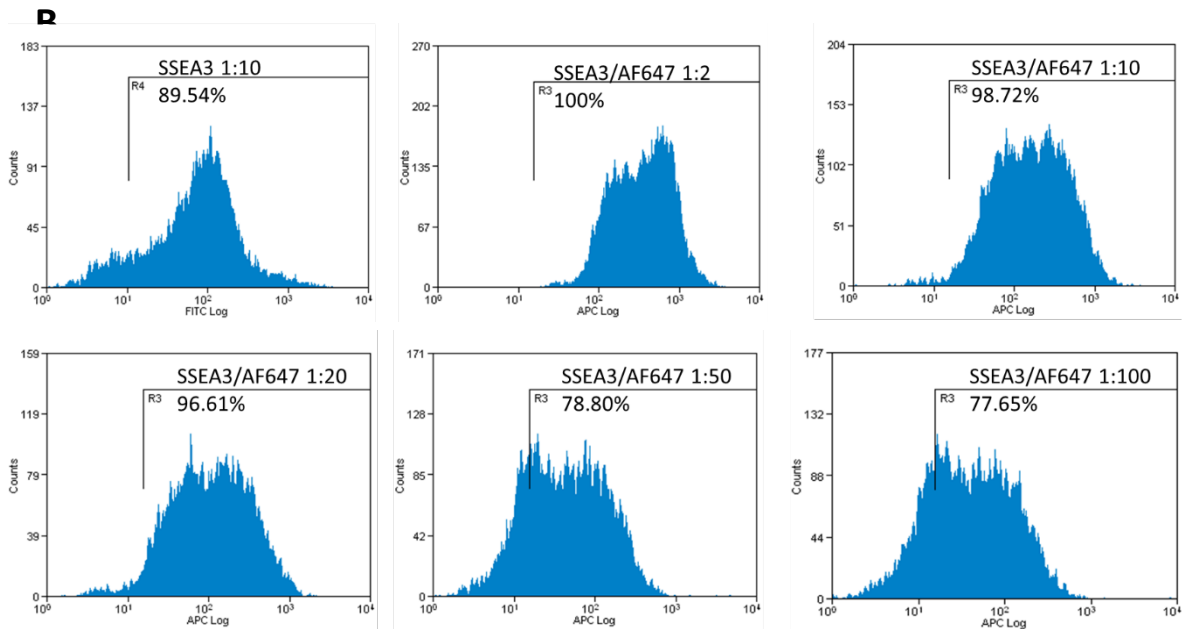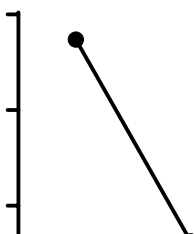Median Fluorescent Intensity

Figure 6.1 A) SSEA3/AF647 conjugate antibody titration curve. Titration assay was done on N2102Ep cells plotted as dilution vs median fluorescent intensity. **B) FACS expression profile histograms.** Standard SSEA3 was used at 1:10 dilution.

Median

Median

Median
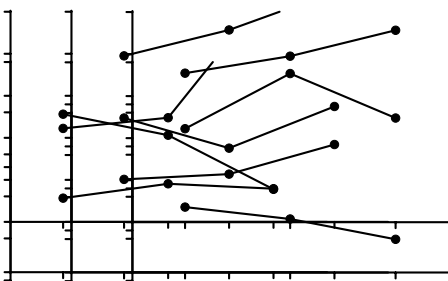
Median

Median

Median

Median

Median

Median

Figure 6.2 Competition Assay. N2102Ep cells were used and the median fluorescent intensity was plotted. AA11 1:10 (109.45); BE12 1:10 (135.94). Dilutions used were 1:10; 1:50 and 1:100. SSEA4 showed a clear competition with SSEA3 whereas none of the novel antibodies seemed to compete with SSEA3, except EF12 which showed a slight inhibition.

Median

Median

Median

Median

Median

Median

Median

Median

Median

Figure 6.3 Competition Assay of novel antibodies vs SSEA3/AF647. N2102Ep cells were used and the median fluorescent intensity was plotted for each competing antibody. Dilutions used were 1:5; 1:10; 1:50 and 1:100. Only SSEA4 and EF12 showed a more noticeable competition with SSEA3.
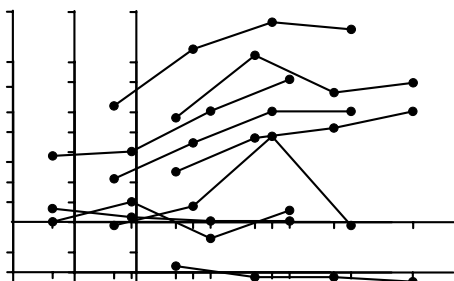
Figure 6.4 Competition Assay – Novel antibodies vs SSEA3/AF647. Median Fluorescence plotted as a percentage of median fluorescence intensity from N2102Ep cells stained with different dilutions from the competing antibody. MFI = Median Fluorescent Intensity.

A

B
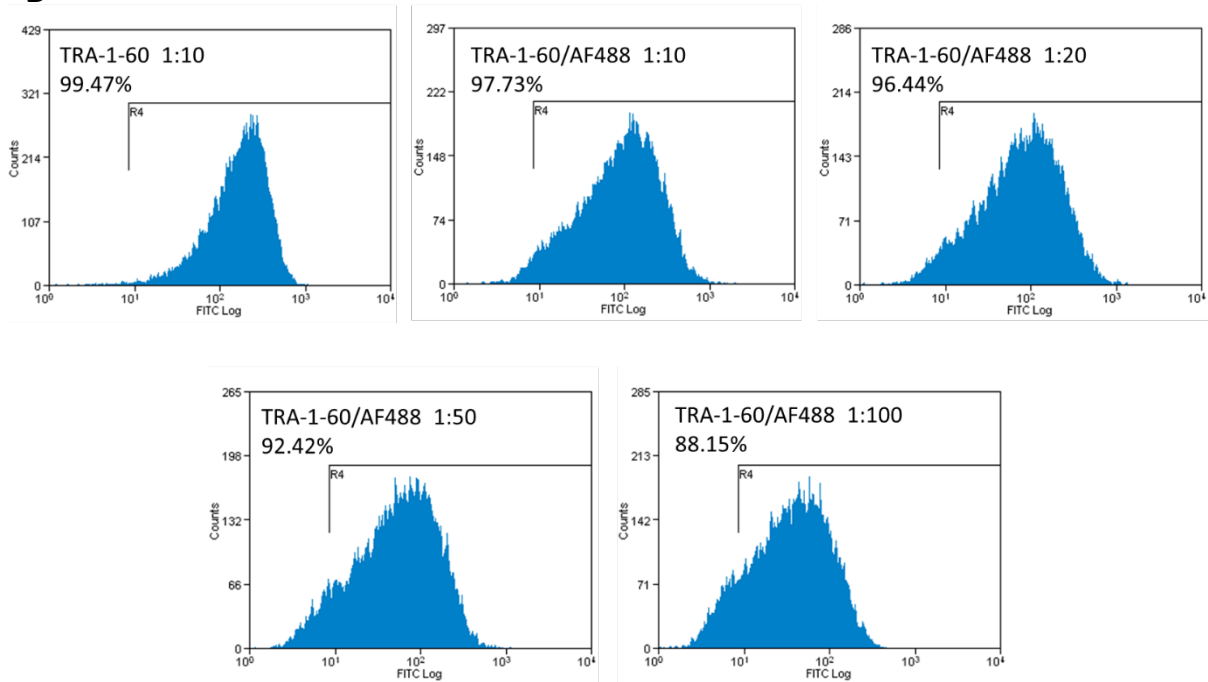
Figure 6.5 A) TRA-1-60/AF488 conjugate antibody titration curve. Titration assay was done on N2102Ep cells plotted as dilution vs median fluorescent intensity. **B) FACS expression profile histograms.** Standard TRA-1-60 was used at 1:10 dilution.

Figure 6.6 Competition Assay of novel antibodies vs TRA-1-60/AF488. N2102Ep cells were used and the median fluorescent intensity was plotted for each competing antibody. Dilutions used were 1:5; 1:10: 1:50 and 1:100. TRA-1-85 and SSEA4 showed a more noticeable competition with TRA-1-60.
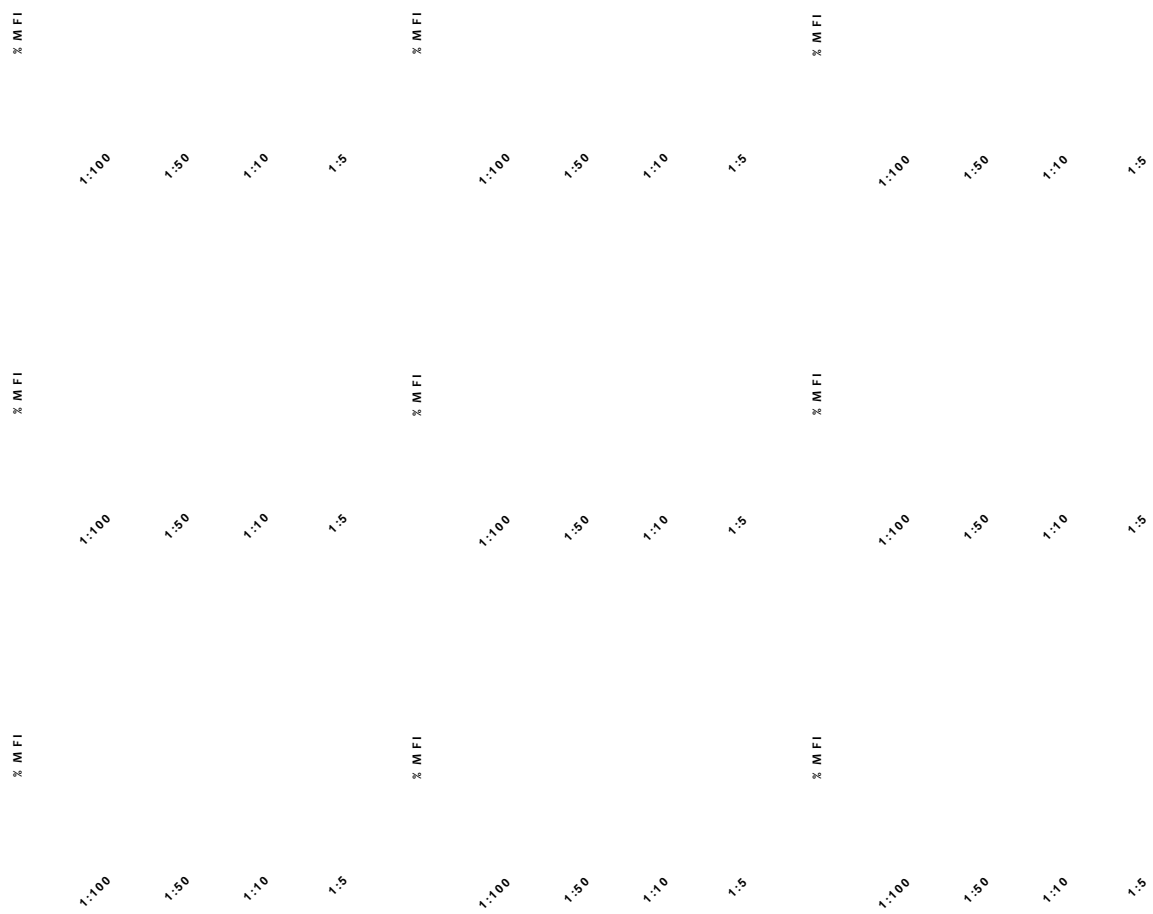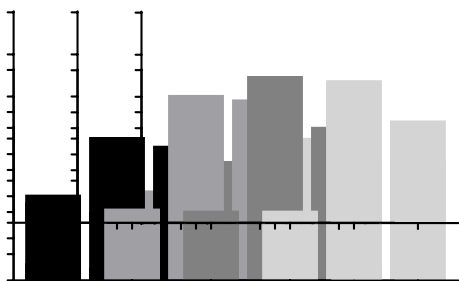
Figure 6.7 Competition Assay – Novel antibodies vs TRA-1-60/AF488. Median Flourescence plotted as a percentage of median fluorescence intensity from N2102Ep cells stained with different dilutions from the competing antibody. MFI = Median Fluorescent Intensity.
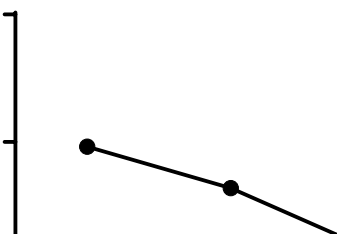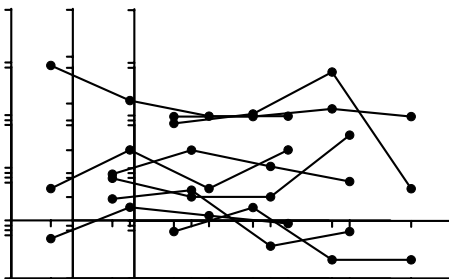
4.3 Discussion

The rationale behind these assays was to show that this novel panel of antibodies were different from previous standard set of antibodies used to identify cells in the stem cell compartment. Two of the most commonly used antibodies for this purpose are SSEA3 and TRA-1-60.

It was important to show what relationship, if any, there was between the novel set of antibodies and the standard markers because the results shown in the previous chapter suggest that some of these antibodies might be capable of recognising subsets of cells within the bigger stem cell population. Having obtained such results, the next issue to address was to establish whether or not some of these antibodies were recognising the same binding site as the standard antibodies.

Although these competition assays have shown that there is no competition for the epitope recognised by the standard antibodies and the novel panel, there was however, some degree of competition with EF12 and SSEA3. Further biochemical characterisation would be needed in order to establish a more direct relationship between these two antibodies. However, there was a clear competition between the antibodies recognising the antigens SSEA3 and SSEA4. The relationship between these two antibodies had already been suggested previously and these assays have further established such relationship.

There is no doubt that further experiments showcasing more elaborate and definitive mechanisms of novel identification of subsets of cells by the novel antibodies would be needed. Such experiments had originally been contemplated in order to be included in this work, however, due to technical and some other external factors they could not be completed in time for inclusion. Some of the experiments envisaged included looking at lineage priming

of cells by replating sorted cells in differentiation conditions and analysing the cells after a given period of growth by qPCR and immunostaining assays.

Moreover, there was a plan to assess the dynamics by which cells in different stem cell states interconvert by using high content clonogenic assays to test the capacity of subsets of cells identified with selected novel antibodies to revert to other substates within the stem cell compartment.

Nevertheless, with these competition assays it is further established that the novel set of antibodies previously described by Wright *et al.*, in 2011 might indeed be helpful in identifying subsets of cells within the stem cell compartment.

Chapter 5

5   Discussion

Nowadays, it is clear that stem cells are not homogeneous. Pluripotent stem cells are presented with a wide selection of pathways to choose from given their broad differentiation capacity. Being able to identify and mark stem cells in their undifferentiated state is crucial for selection of specific cell types. It is also important in trying to understand the particularities of cell fate choice since it would be possible to track stem cells as they make early commitment decisions. Cell surface antigens present valuable tools for marking cells, not only because of their sensitivity but also because they can be used to mark and to isolate individual viable cells for functional tests.

A number of well characterised antibodies have been widely used previously to analyse differentiation of human Pluripotent Stem Cells. In this study I have carried out a further analysis and characterisation of the novel antibodies recently described by Wright and cols. in 2011 (Wright, *et al.*, 2011). Notably, I have found that this novel group of antibodies may be helpful in identifying subsets of cells that were not being recognised by standard antibodies. For example, in EC cells, AG10, CC9 and EF12 showed interesting patterns of SSEA3/OCT4 expression after sorting for their respective positive and negative populations. They displayed high levels of SSEA3 and OCT4, suggesting that the resulting colonies emerged from undifferentiated cells.

Furthermore, gene analysis expression showed that there might be a bias towards specific lineages. The high levels of MIXL1 expression in the positive fraction of AG10 is indicative of this, since the unsorted and negative population of AG10 lacked high expression for this gene.

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Nevertheless, the negative fraction of AG10 expressed SOX17 in higher levels, suggesting an endoderm bias for the AG10 negative cells. In the case of CC9, the negative subpopulation showed a potential bias towards an ectoderm lineage, whereas the positive fraction might be biased towards an endoderm fate. EF12 negative cells had higher expression of ectoderm marker PAX6.

However, it was speculated that some of the novel antibodies could recognise similar epitopes to the ones identified by commonly used standard markers. Previous biochemical characterisation done on the novel antibodies had indeed shown a possible relationship in the structures of some antibodies, namely, AG10 and EF12 being suggested as part of the family of glycolipids related to SSEA3 and SSEA4; and CC9 being related to the TRA family of glycoproteins (Wright, *et al.*, 2011).

The competition assays performed in this study helped to further clarify whether there were any instances in which any of the novel antibodies were competing for binding sites recognised by standard antibodies for SSEA3 and TRA-1-60. Notably, it was found that there was a marked competition between the antibodies that recognise the antigens for SSEA3 and SSEA4. This is in agreement with previous studies that had established a relationship between these two markers, as the epitopes were identified as different trisaccaharide elements contained within a common glycolipd, sialyl-gal-globoside. In that case the SSEA4 epitope is dependent on the terminal sialic acid moiety, whereas SSEA3 is not (Kannagi, *et al.*, 1984). However, direct competition assays between the antibodies recognising SSEA3 and SSEA4 have not been previously reported.

The possibility that the novel antibodies recognise the same epitopes associated with the commonly used markers was questioned when they were first derived a few years ago, but it was not analysed. Therefore, by analysing their similarities with the existent set of standard antibodies, this project has helped build a more strong and coherent insight into the benefits that identifying new subsets represent. Out of the whole panel of novel markers, only EF12 appeared to be able to compete with the anti-SSEA3 antibody for binding to human EC cells, suggesting that like SSEA3 and SSEA4, the EF12 epitope is associated with the same globoseries glycolipid. It would be interesting to further investigate how closely related the antibodies for SSEA3 and EF12 are by looking at their chemical structure and identify precisely which region(s) is being recognised with the same epitope.

However, none of the novel antibodies was able to compete with TRA-1-60. Nevertheless, there was some evidence where the antibody recognising SSEA4 showed a certain degree of inhibition of the binding of the TRA-1-60 antibody, even though the SSEA4 epitope is associated with the globoseries glycolipd, sialyl-gal-globoside (Kannagi *et al*., 1984), whereas TRA-1-60 is associated with a high molecular weight proteoglycan (Badcock *et al*., 1998). Nevertheless the epitope in both cases includes a sialic acid moiety and it is possible that this is the basis for some limited cross reactivity.

The results from the current study have shown that the novel antibodies described by Wright *et al* (2011) are potentially valuable new tools, able to identify ES cell markers mostly distinct from the already well-established set of surface antigens. Further, even if there is a competition for the same binding site between the antibodies for SSEA3 and EF12, they could be useful by complementing each other just as TRA-1-81 and TRA-1-60 (Andrews, *et al*., 1984) have proved to be valuable either alone or in combination. In future these markers are likely

to be useful to define potentially lineage biased substates of human ES cells which in turn could lead to more plausible strategies for enrichment of specific cell types needed for regenerative medicine.

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