The role of Fibroblast Growth Factor Signalling on the Regulation of Embryonic Stem cells

Paz Freile Vinuela

A thesis submitted for the degree of Doctor of Philosopy at the University of Edinburgh

2008

Department of Gene Function and Development, Roslin Institute, Edinburgh

Declaration

I declare that this thesis has been composed, along with the work described herein, by the candidate, Paz Freile Vinuela. This work has not been submitted for any other degree or professional qualifications. All sources of information have been acknowledged.

Paz Freile Vinuela
2008

Acknowledgements

I would like to thank my supervisors Tom Burdon and Lesley Forrester for their help and guidance throughout this project. Thanks are also due to the members of both labs who give assistance and time. Martin Waterfall in particular deserves special thanks for providing much help and support. I would also like to thank Mike Clinton's lab for the helpful advice and to many others at the Roslin Institute who aided me during this project. Thanks are also due to my funding body, the MRC for their financial support, which made possible this project.

A special thank you is for my partner David for believing in me, for his encouragement and patience. Also I would like to thank to my parents for their financial and moral support.

Abstract

Fibroblast growth factor (FGF) signalling plays many fundamentally important roles during the development of the mammalian embryo. However, its effects on pluripotent stem cells derived from mouse and human embryos appear to be markedly different. FGF2 is routinely added to culture medium for propagating undifferentiated human (hES) cells, whereas in mouse (mES) cell cultures FGFs have been described as regulators of their differentiated progeny. To assess the effect of FGF signalling on undifferentiated mES cells, the effects of FGF2 and 4 were analysed in the presence of saturating and sub-saturating levels of the inhibitor of differentiation, leukaemia inhibitory factor (LIF). Mouse ES cell self-renewal was quantified by measuring the expression of the stem cell specific reporter Oct4-LacZ in biochemical and fluorometric assays. Treatment with FGF reduced the expression of the OCT4-LacZ reporter, even under saturating concentrations of LIF and this was mirrored by decreased levels of OCT4 protein. Furthermore, treatment with FGF leads to upregulation of the ectodermal differentiation marker Pax6. These results suggest that FGF signalling has a direct impact on undifferentiated mES cells, and actively promotes their differentiation. To asses the effect of FGF signalling on hES cells without the influence of undefined factors, a feeder and serum free system was developed. Cells growing in this conditions for >20 passages maintained expression of surface (SSEA3 and TRA1-60 and 81) and internal (OCT4) markers specific for undifferentiated hES cells. Expression of these markers was dependant on the continuous presence of FGF2. Indeed, withdrawal of FGF2 resulted in a rapid decrease of in hES cell growth and of the emergence of cell flattened morphology and of the surface marker SSEA1, changes typically associated with differentiation. Two important signals activated by FGF in hES cells are the ERK/MAPK and PI3K

pathways. To assess their functional relevance, hES cell cultures were treated with the drugs UO126 and LY294002, inhibitors of the MAPK and PI3K pathways respectively. Drug mediated suppression of the phosphorylation of these pathways, correlated with a reduction in cell growth, flattening of the colonies and reduction in SSEA4 expression. Use of SB431542, specific inhibitor of TGFβ/activin type I receptor kinase (Alk5) also resulted in the flattening of the colonies and the appearance of dispersed cells. Therefore, inhibition of MAPK and PI3K appears to impair growth and self-renewal in hES cells and this may be happening in conjunction with TGFβ/Activin pathway. Taken together, these results suggest that FGF signalling has opposite effects in mouse and human ES cells: inducing differentiation in mES and sustaining self-renewal in hES.

List of abreviations

Ab	Antibody (ies)
AP	Alkaline phosphatase
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
bHLH	Basic helix loop helix
BMP	Bone morphogenic protein
Bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
^{o}C	Degrees celsisus
CM	Conditioned medium
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Doxyribonuclease
dNTP	Deoxyribonucloasid triphosphate
d.p.c.	Days post coitum
EB	Embryoid body
EC	Embryonal carcinoma
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EG	Embryonic germ
EGF	Epidermal growth factor

ERK	Extracellular signal-regulated kinase
ES	Embryonic stem
ExE	Extraembryonic ectoderm
ExEn	Extraembryonic endoderm
FBS	Foetal bovine serum
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FRS	FGF receptor substrate
Gab1	Grb2-associated binder 1
GFP	Green fluorescent protein
GSK3β	Glycogen synthase kinase 3β
HSPG	Heparan sulfate proteoglycan
IP_3	Inositol triphosphate
ICM	inner cell mass
JAK	Janus kinase
Kb	Kilobase (s) or 1000 bp
KDa	Kilodalton (s)
KSR	Knock out serum replacement
LIF	Leukaemia inhibitory factor
LPA	Lysophosphatidic acid
MEF	Mouse embryonic fibroblast
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
MW	Molecular weight

NGF	Nerve growth factor
NFκβ	Nuclear factor κβ
OD	Optical density
Oligo	Oligodeoxyribonucleotide
P	phosphorylated
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGC	Primordial germ cell
PH	Pleckstrin homology
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-OH kinase
PDGF	Platelet-derived growth factor
PLCγ	Phosphoinositide-specific phospholipase C gamma
PTB	Phosphotyrosine-binding
PTK	Protein tyrosine kinase
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcription PCR
SD	Standard deviation
SDS	Sodium dodecyle sulphate
SEM	Standard error of the mean
SH	src homology
SSEA	Stage-specific embryonic antigen
STAT	Signal transducer and activator of transcription
TAE	Tris-Acetate EDTA

TBE	Tris-Borate EDTA
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with 0.1% Tween20
TDGF1	Teratoma-derived growth factor 1
TGF-β	Transforming growth factor β
TRA1	Tumour rejection antigen 1
Tris	Tris (hydroxymethyl) aminomethane
TVP	Trypsin Verene Phosphate
µl	Microlitre
µM	Micromolar
UV	Ultraviolet
U	Unit

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Preface

Understanding the mechanisms of self-renewal in human embryonic stem (ES) cells has proven to be a major challenge. Initially mouse and human ES cells were isolated and propagated in an identical manner, on inactivated mouse embryonic fibroblasts (MEFs) (Thomson *et al.,* 1998). However, the factors that mediate self-renewal in mouse and human ES cells are different. Mouse ES (mES) cell differentiation is prevented by the cytokine Leukaemia Inhibitor Factor (LIF) and the Bone Morphogenetic Protein 4 (BMP4) (Dani *et al.,* 1998; Ying *et al.,* 20003a). Significantly, LIF and BMP4 not only are unable to support human ES (hES) cell proliferation but they promote their differentiation (Xu *et al.,* 2002; Daheron *et al.,* 2004). By contrast, fibroblast growth factor 2 (FGF2) is the key component in the culture of hES cells (Levenstein *et al.,* 2005), despite also being highly expressed by the cells (Wei *et al.,* 2005). This differs with mES cells, which do not require the supplement of FGF or express FGF2. Mouse ES cells, instead, synthesise high levels of FGF4 (Rappolee *et al.,* 1994), although it does not appear to have a positive role in the propagation of mES cells (Rappolee *et al.,* 1994; Wilder *et al.,* 1997). In the murine system, paracrine FGF4 signalling is required for the development of the trophectoderm and the primitive endoderm *in vivo* (Feldman *et al.,* 1995) and appears to have a role *in vitro* because mES cells fail to form EBs in the absence of FGF signal (Chen *et al.,* 2000; Ying *et al.,* 2003a). FGF2 in turn, co-regulates later stages of development, such as the patterning of mesodermal and neural cell lineages (Burdsal *et al.,* 1998). Therefore, FGF signalling pathway seems to be important in both species but in different ways.

The divergent biology between mouse and human ES cells may be reflecting a slightly different stage of development in the cells when they were isolated, thus perhaps indicating that hES cells are originated later in development. This seems to be in line with hES cells requirement for FGF in culture, which is consistent with the growth requirements of a later epiblast stage (Camus *et al.,* 2006). A better understanding of the mechanisms in control of mouse and human ES cell self-renewal would enhance the knowledge in the early developmental biology of the two species, giving insight into conserved and divergent mechanisms.

In addition to the fundamental differences in the regulation of mouse and human ES cells, this thesis will address more specifically the role of FGF in hES cell proliferation. The biology of hES cells is relatively poorly understood. In fact the mechanisms by which FGF2 sustain the undifferentiated proliferation of hES cells are still unclear. Clarifying these mechanisms will give rise to developing improved strategies in the culture of hES cells, which would aid to the better understanding of the biology of hES cells and by association, clarifying human development and disease.

The following introduction will describe the different pluripotent cells, highlighting mouse and human ES cells and their regulation. FGFs, their signalling and functions will be also reviewed in this introduction. The roles of FGF signalling on mouse and human ES cells will be studied in chapters 3 and 5 respectively. Present problems associated with the development of culture systems to grow hES cells and the

approaches employed to tackle them will be studied in chapter 4. The main objectives of this project will be outlined at the end of this chapter.

1.2 Pluripotent embryonic cells

Pluripotency is defined as the capacity to give rise to all the three embryonic cell lineages. Pluripotent stem cells exist briefly during embryonic development in the inner cell mass (ICM) of the blastocyst of preimplantation embryos and in foetal gonads, as primordial germ cells (PGCs). These transitory cells can be maintained as established cell lines in culture and in the last few years several cell lines of murine and of human origin have been characterised. Embryonic stem (ES) cells are derived from the ICM of blastocysts (Martin, 1981; Evans and Kaufman, 1981; Thomson *et al.,* 1998). Cells derived from primordial germ (PGC) cells are designated embryonic germ (EG) cells (Martin, 1981; Evans and Kaufman, 1981; Matsui *et al.,* 1992; Resnick *et al.,* 1992; Resnick *et al.,* 1998; Shamblott *et al.,* 1998). In addition to this, pluripotent lines have been derived from tumorigenic derivatives of germinal tissues, which are designated embryonic carcinoma (EC) cells (Kleinsmith and Pierce, 1964; Andrews *et al.,* 1984). Recently, a new type of pluripotent cell line has been derived from the epiblast of early post implantation mouse and rat embryos designed epiblast stem (EpiSCs) cells (Brons *et al.,* 2007; Tesar *et al.,* 2007).

The derivation of these cell lines has been a gradual process, which started with the isolation of EC cells from mouse testicular teratocarcinomas (Kleinsmith and Pierce, 1964). This early report led to the first derivation of murine ES cells (Evans and Kaufman, 1981; Martin, 1981), followed by human EC cells (Andrews *et al.*,1984), murine EG cells (Andrews *et al.,* 1984; Matsui *et al.,* 1992) primate ES cells (Thomson *et al.,* 1995) human ES cells (Thomson *et al.*,1998) and murine and rat EpiES cells (Brons *et al.,* 2007; Tesar *et al.,* 2007).

Although these cell lines have similar properties such as immortality, their different origins are reflected in their development potential. EC and EpiES cells have a more restricted potential and it is unlikely to be transmitted through the germ line of chimeric animals (Brons *et al.,* 2007; Tesar *et al.,* 2007). EG cells normally undergo spontaneous differentiation but may be unable to support normal development due to epigenetic modifications, which have occurred during the formation of PGCs (Tada *et al.,* 1997). Thus, ES cells were shown to have the greatest developmental potential by differentiating into the widest range of cell types reviewed in (Smith, 2001).

1.2.1 Embryonic Carcinoma (EC) cells

Germ cell tumours are derived from the primordial germ cells (PGCs), the embryonic precursors of the gametes in the adult animal (Gardner, 1978), which also give rise to embryonic germ (EG) cells when cultured in the right conditions. Germ cell tumours can be broadly grouped into two types: benign teratomas, which are composed of a wide

variety of differentiated tissue types and malignant teratocarcinomas, which contain an additional undifferentiated cell population. This undifferentiated population can be isolated and propagated indefinitely in culture as EC cell lines (Kleinsmith and Pierce, 1964). Teratocarcinomas can also be induced by grafting early embryos into ectopic locations of immune-compromised mice (Finch and Ephrussi, 1967).

EC cells retain the ability to differentiate and can form teratocarcinomas when transplanted back into an appropriate mouse host (Damjanov *et al.,* 1982). The developmental potential of murine EC cell lines has also been demonstrated by injecting them into the blastocyst. The ECs incorporated into the developing embryo, contributed to a range of cell lineages, but were never transmitted through the germline (Brinster, 1974; Rappolee *et al.,* 1994). This may occur because typically EC cells have an abnormal karyotype, which is possible to prevent progression through meiosis to produce mature, functional gametes (Bradley *et al.,* 1984). The behaviour of EC cells in culture is not standardised, some EC cell lines remain undifferentiated if they are maintained in sub-confluent cultures, but differentiate spontaneously when grown to confluence. However, other EC cell lines require culture on feeder layers to prevent differentiation (Rosenthal *et al.,* 1970), which suggests that feeder cells produce factors with a positive effect on their pluripotency. In this case, differentiation could be induced by removing the EC cells from the feeder cells and then grown in suspension to form embryoid bodies (Martin and Evans, 1975).

Human EC cells have been isolated from spontaneously occurring human germ cell tumours (testicular teratocarcinomas) and maintained in culture (Andrews, 1988). Like mEC cells, hEC cells retain the capacity to differentiate *in vitro* into tissues representative of all three germ layers (Pera *et al.,* 1989) and express OCT4 (Yeom *et al.,* 1996). Furthermore, they share similar morphology and growth patterns, and both express high levels of alkaline phosphatase. Characteristically, hEC cells do not require special culture conditions such as addition of extrinsic factors. This could be due to the fact that hEC are always aneuploid and usually have a limited capacity for spontaneous differentiation. Furthermore, and in contrast with many mouse EC lines, hEC cells need to grow at high density and differentiate when grown as single cells (Andrews, 2002), which may indicate an autocrine mechanism to maintain self-renewal.

1.2.2 Embryonic Germ (EG) cells

The isolation of EC cells from teratocarcinomas (Kleinsmith and Pierce, 1964) suggested that undifferentiated cells could be derived directly from PGCs. Murine PGCs obtained before midgestation and grown on feeder cell layers in medium supplemented with FGF2, LIF and stem cell factor (SCF) could be induced to proliferate in culture (Matsui *et al.,* 1992; Resnick *et al.,* 1992). These cells were termed EG cells and had many similarities to murine EC and ES cells such as SSEA1 expression (Matsui *et al.,* 1992; Resnick *et al.,* 1992). EG cells retain the capacity to differentiate *in vitro* via EBs and *in vivo* by forming tumours in immunodeficient mice and by contributing to chimaeras following blastocyst injection (Matsui *et al.,* 1992).

(Matsui *et al.,* 1992; Shamblott *et al.,* 1998) isolated human EG cells from genital ridges after five to nine weeks of embryonic development. Human EG cells express similar antigens to human ES cells but they also express SSEA1. SSEA1 is the marker associated with mouse but not with human ES cells. Human EG cells retain the capacity to differentiate into the three germ layers in vitro via EB intermediates as well as maintaining a normal karyotype through prolonged periods of culture (Matsui *et al.,* 1992; Shamblott *et al.,* 2001).

1.2.3 Embryonic Stem (ES) cells

Since teratocarcinomas can also be formed by grafting blastocysts at ectopic sites, it was thought that pluripotent cell lines might be derived directly from blastocysts rather than from tumours. In the blastocyst, ICM and trophectoderm are formed at embryonic day 3.5 in the mouse and day 5 in human. The trophectoderm will form the trophoblast layers of the placenta while the ICM gives rise to the embryo proper (Rossant and Nagy, 1995). Pluripotent embryonic stem (ES) cells derive from the inner cell mass (ICM) of the pre-implanted blastocyst and retain the differentiation potential of cells in the ICM. Furthermore, ES cells have the capacity for unlimited self-renewal in an undifferentiated state when maintained under the appropriate culture conditions (Smith, 2001).

In 1981, two groups, Evans and Kaufman and Martin derived for the first time murine ES cells and, in 1998 Thomson and colleagues reported the isolation of hES cells. These

cells, unlike EC cells were karyotypically normal and expressed the classical markers of pluripotency: the transcription factors OCT4, SOX2, NANOG and REX1 and the enzyme alkaline phosphatase. However, the expression of surface markers was different between mouse and human ES cells. Undifferentiated human and primate ES cells shared many markers, and these markers are also expressed in the ICM of human blastocysts (Henderson *et al.,* 2002). This includes the expression of stage-specific embryonic antigen 3 (SSEA3) and SSEA4, tumour rejection antigen 1-60 (TRAl-60) and TRA1-81. Interestingly, hES cells only express SSEA1 after differentiation (Andrews, 1984; Thomson, 1998). In contrast, differentiation of mES is typically characterised by the loss of SSEA1 and could be accompanied by the appearance of SSEA3 and 4 (Solter and Knowles, 1979). Murine ES cell expression of surface markers is shared by cells of the ICM and primitive ectoderm (Henderson *et al.,* 2002). An illustration of the different expression of surface antigens in human and mouse at different stages of the embryonic development is shown in Table 1.1.

Mouse (Evans and Kaufman, 1981; Martin, 1981) and human ES cells (Itskovitz-Eldor *et al.,* 2000) can differentiate spontaneously into multiple cell types representative of all three embryonic germ layers. Both cell types can form teratomas after grafting into mice or *in vitro* through differentiation in EBs under appropriate conditions. However, only mouse ES or EG cells have been shown to contribute to the germ line when reintegrated into the normal embryonic development (Bradley *et al.,* 1984). In addition to pluripotency, another key defining characteristic of ES cells is their ability to undergo unlimited self-renewal in an undifferentiated state. The appropriate conditions to

maintain self-renewal differ between mouse and human ES cells and this will be discussed in section 1.3 of this introduction.

Antigens	2-8 cell		Morula		ICM		Trophoblast	
	Mouse	Human	Mouse	Human	Mouse	Human	Mouse	Human
SSEA1	+	-	+	+	++	-	+	+
SSEA3	+	-	+	-	-	++	-	-
SSEA4	+	-	++	-	-	++	-	-
TRA1-60	-	-	-	-	-	+++	-	-
TRA1-81	-	-	-	-	-	++	-	-

Table 1.1. Cell surface antigen expression by pre implantation stages of human and mouse embryos (adapted from Henderson *et al.,* 2002). These results reported by Henderson *et al.,* 2002 were obtained by immune fluorescence staining of embryos and ES cells for the expression of SSEA1, SSEA3, SSEA4, TRA1-60, and TRA1-81. All the antigens except SSEA1 were localized mainly on the ICM in human blastocysts whereas these markers (except TRA1-60 and 81) were absent from mouse embryos at the same stage but present at earlier cleavage stages. Conversely, SSEA1 was expressed on the trophectoderm of human blastocyst and not on ICM whilst being expressed on murine ICM.

1.2.4 Epiblast stem cells (EpiSC)

Recently two independent groups, Brons *et al,* (2007) and Tesar *et al*, (2007) reported the isolation of a new type of rodent pluripotent embryonic stem cell. These cells were termed epiblast stem cells (EpiSCs) because of their origin in the post implantation epiblast (5.75 d.p.c). EpiSCs were derived and propagated using chemically defined media containing Activin and FGF2 (CDM/AF), the same culture conditions required for growing hES cells. The use of identical culture conditions might be the cause of the similar morphology observed in EpiSCs and hES cells. Both cell types produce large, flat colonies that grow in monolayers differing from the compact colonies of mES cells. In addition to requiring the same growth factors, a similar pattern of gene expression is observed in EpiES cells and hES cells (Brons *et al.,* 2007 and Tesar *et al.,* 2007), and this differs from the gene expression found in mES cells (Cai *et al.,* 2006). For example, gene targets of OCT4 in human ES cells and EpiSCs overlap seven times more (Tesar *et al.,* 2007) than between human and mouse ES (Boyer *et al.,* 2005). This gene expression in EpiSCs (Brons *et al* and Tesar *et al.,* 2007) and a dependency on FGF and Activin signalling is characteristic of the cells in the epiblast (Camus *et al.,* 2006). Mouse ES cells, on the other hand, express genes of the pre-implantation embryo, suggesting that mES and EpiSCs cells are at two distinct pluripotent states. Interestingly, a shared gene expression and activation of equivalent signalling pathways may also imply a similar origin of epiblast between EpiSCs and hES cells. Another shared feature between EpiSCs and hES cells is their striking capacity to differentiate into cells expressing markers of primitive endoderm and trophectoderm when exposed to

BMP4 (Brons *et al.,* 2007; Xu *et al.,* 2002). Conversely, mES cells have little or no capacity for contribution to either primitive endoderm or trophectoderm lineages in chimaeric embryos (Beddington and Robertson, 1989) and only differentiate into trophectoderm by the mutation of the *Oct4* gene (Niwa *et al.,* 2000). It is logical to think that if cells in the ICM of the blastocyst cannot differentiate into primitive endoderm and trophectoderm cells, then EpiSCs which represent later stages of development should have a more restricted differentiation potential.

EpiSCs express the universal markers of pluripotency OCT4, SOX2 and NANOG, and the murine specific marker SSEA1. The pluripotency of EpiSCs was confirmed *in vitro* by the wide variety of cell types formed in EB differentiation and *in vivo* by injecting cells in the testis and formation of teratomas containing several types of tissues. However, when EpiSCs were injected into the blastocysts they did not integrate successfully with the endogenous cells and subsequently, were not transmitted into the germ line. Both groups agreed in hypothesising that the difficulty to form chimeras was a consequence of developmental asynchrony (Brons *et al* and Tesar *et al.,* 2007). This may indicate that although pluripotent, EpiSCs may represent an intermediate stage of development between mouse ES cells and committed cells.

In conclusion, the finding of EpiSCs has signified a change in the views of ES cells, since many differences between mouse and human ES cells that were previously attributed to species divergence could instead be reflecting a difference in the pluripotent state of the cells of origin. This links with the culture requirements and gene expression,

which may indicate a similar epiblast origin in hES cells and EpiSCs. However, a similar pluripotent state is not consistent with EpiSCs expression of the mES cell marker SSEA1, which is expressed on the human morula. Furthermore and as expected, the ICM marker Rex1 is not found in EpiSCs but REX1 is expressed by hES cells. In addition to this, clarification is still needed of the developmental incongruence, which is the differentiation into trophoblast of EpiSCs and hES cells. A summary of the reported differences between the profile of mES cells and EpiSCs and those of hES cells are shown in Table 1.2.

	Culture media		**Marker expression**			**Other characteristics**
	LIF/BMP4	FGF/Activin	OCT4/SOX2 NANOG	REX1	SSEA1	Trophoblast differentiation
Mouse ES cells	+	-	+	+	+	-
Rodent EpiSCs	-	+	+	-	+	+
Human ES cells	-	+	+	+	-	+

Table 1.2. Differences in culture media requirement and marker expression between mouse ES, EpiSCs and hES cells. Whilst mES cells are dependent on LIF and BMP4, EpiSCs and hES cells are dependent on FGF and Activin to maintain self-renewal, pluripotency and prevent differentiation. The three cell types are pluripotent as shown by their specific marker expression of OCT4, NANOG and SOX2.

1.3 Regulation of Embryonic Stem cells

Although much of mammalian development biology is based on studies of the mouse, there are significant differences between early mouse and human development. Differences in embryonic development could also be reflected in the ES cells, for example, in the different pattern of surface markers expressed and in the ability to differentiate into trophectoderm. Substantial differences also exist in a number of key signalling pathways that regulate mouse and human ES cell proliferation and differentiation. The LIF/STAT3 and BMP4 are the essential signalling pathways in the maintenance of mES pluripotency. Conversely, LIF and BMP4 cannot maintain hES cells and it is the FGF and Activin/TGFβ signalling pathways that have been related to hES cell self-renewal.

1.3.1 Intrinsic factors in embryonic stem cell regulation

OCT4, SOX2 and NANOG are master regulators of pluripotency by antagonising pro-differentiating signals. The traditional belief was that together, these transcription factors activated key genes in self-renewal and repressed inducer genes of differentiation (Boyer *et al.,* 2005). However, new evidence indicates that different genes may be the targets of NANOG, since ES cells deficient in NANOG express many genes involved in pluripotency (Chambers *et al.,* 2007). The findings in the report by Chambers *et al,* (2007) also question the conventional mechanisms of regulation in *Oct4, Sox2* and *Nanog*. Against the suggested reciprocal regulation supposedly occurred in mES

(Catena *et al.,* 2004; Rodda *et al.,* 2005) and hES cells (Boyer *et al.,* 2005), it was demonstrated that ES cells deficient in *Nanog,* highly express *Oct4* and *Sox2* (Chambers *et al.,* 2007). Furthermore, it was shown that NANOG and OCT4 act independently from each other. Their over-expression cannot activate each other or be able to reverse the differentiation caused by the reduction in the levels of the other (Chambers *et al.,* 2003).

1.3.1.1 OCT4

OCT4, encoded by *Pou5f1* belongs to the POU (Pit-Oct-Unc) family transcriptional regulator. OCT4 usually heterodimerize with the transcription factor SOX2 and both bind to adjacent domains when regulating gene expression. OCT4-SOX2 sites have been found contiguous to each other in regulatory regions of *Sox2, Rex1, Fgf4, Nanog,* etc (Yuan *et al.,* 1995; Nishimoto *et al.,* 1999; Tomioka *et al.,* 2002).

OCT4 is expressed throughout oogenesis and pre-implantation development. During the blastocysts stage, OCT4 becomes restricted to the ICM and later in the development it is expressed throughout the early epiblast, before being limited to the developing germ cells (Palmieri *et al.,* 1994; Yuan *et al.,* 1995; Nishimoto *et al.,* 1999). Targeting gene deletion has shown that *Oct4* deficient embryos develop only to the blastocyst stage due to the inability for normal differentiation of the ICM cells. These cells can only generate trophoblast cells and as a consequence are absorbed shortly after implantation (Yuan *et al.,* 1995; Nichols *et al.,* 1998). OCT4 expression is also exclusive and essential for

maintaining the pluripotency in undifferentiated mouse and human EC, EG and ES cells (Yuan *et al.,* 1995; Reubinoff *et al.,* 2000). To sustain ES cell self-renewal, OCT4 levels need to be on a narrow range. Mouse ES cells differentiate into trophectoderm when the levels of OCT4 are reduced to less than 50%. On the other hand, a less than 2-fold increase triggers differentiation into endoderm and mesoderm, which are the lineages produced by LIF withdrawal (Niwa *et al.,* 2000) or *Nanog* deletion (Chambers *et al.,* 2003; Mitsui *et al.,* 2003). This might suggest that although OCT4 and NANOG control lineage commitment, they may use different mechanisms. Experiments using RNAi induced depletion of OCT4 confirmed trophoblast differentiation in both mouse and human ES cells (Hay *et al.,* 2004).

1.3.1.2 NANOG

NANOG is a homeobox transcription factor isolated by Chambers *et al* and Mitsui *et al* in 2003. These groups established that NANOG was expressed in the ICM and PGC cells, in mouse ES, EG and EC and in human EC cell lines. Both groups demonstrated that over-expression of NANOG was sufficient to maintain the self-renewal of mES cells whilst its deletion seemed to trigger differentiation into primitive endoderm in ES cells. Recently it has been shown that other types of differentiation can also occur in *Nanog* null cells, but this differentiation is more restricted to the endoderm lineage (Chambers *et al.,* 2007). However, it has been also demonstrated that ES cells with both alleles of *Nanog* deleted could still proliferate undifferentiated and retain pluripotency (Chambers *et al.,* 2007). ES cells expressing different levels of NANOG also occur

naturally in ES cell cultures. These cells are in a transient state in which they can re-express NANOG and remain undifferentiated, but due to the loss of the buffering effect of NANOG against differentiating signals, they also have a major tendency to differentiate (Chambers *et al.,* 2007).

During mouse development the expression of NANOG in pluripotent cells is also dynamic. NANOG is expressed in the morula in an opposite pattern to the expression of Gata6 and Cdx2 (Hyslop *et al.,* 2005), which may indicate that Nanog maintains pluripotency by repressing these transcription factors associated with extraembryonic lineages (Hyslop *et al.,* 2005). NANOG expression continues in the blastocyst and it is down-regulated at the time of implantation (Chambers *et al.,* 2003) and expressed again in the egg cylinder (Hart *et al.,* 2004). NANOG re-expression in the egg cylinder was believed to be necessary in preventing an early commitment during gastrulation (Chambers *et al.,* 2007).

NANOG is also expressed in the ICM and the nucleus of human ES cells and its knock-down induces hES cell differentiation into extra-embryonic lineages (Hyslop *et al.,* 2005). Hyslop *et al,* (2005) reported the down regulation of OCT4 and differentiation of hES and EC cells as a consequence of NANOG silencing by small interfering (si) RNA. However, its function in hES cells is not clear since *NANOG* was not found among the 532 genes up-regulated in undifferentiated hES cells (Brandenberger *et al.,* 2004).

1.3.2 Extrinsic factors on mouse embryonic stem cell regulation

Murine ES cells can be maintained undifferentiated in culture indefinitely by the administration of leukaemia inhibitor factor (LIF) (Smith *et al.,* 1988) and bone morphogenetic protein 4 (BMP4) (Ying *et al.,* 2003a). A simplified diagram of the regulation of mES cells is shown in Figure 1.1. A recent publication also attributes heparan sulphates (HSs) a significant role in the maintenance of mES cell self-renewal (Sasaki *et al.,* 2008). Sasaki *et al,* (2008) reported that autocrine/paracrine Wnt/β catenin signalling through heparin sulphate (HS) chains is required for the regulation of Nanog expression. Therefore, in addition to LIF plus BMP4 or serum HS might be part of the extrinsic signalling required for mES cell self-renewal and pluripotency.

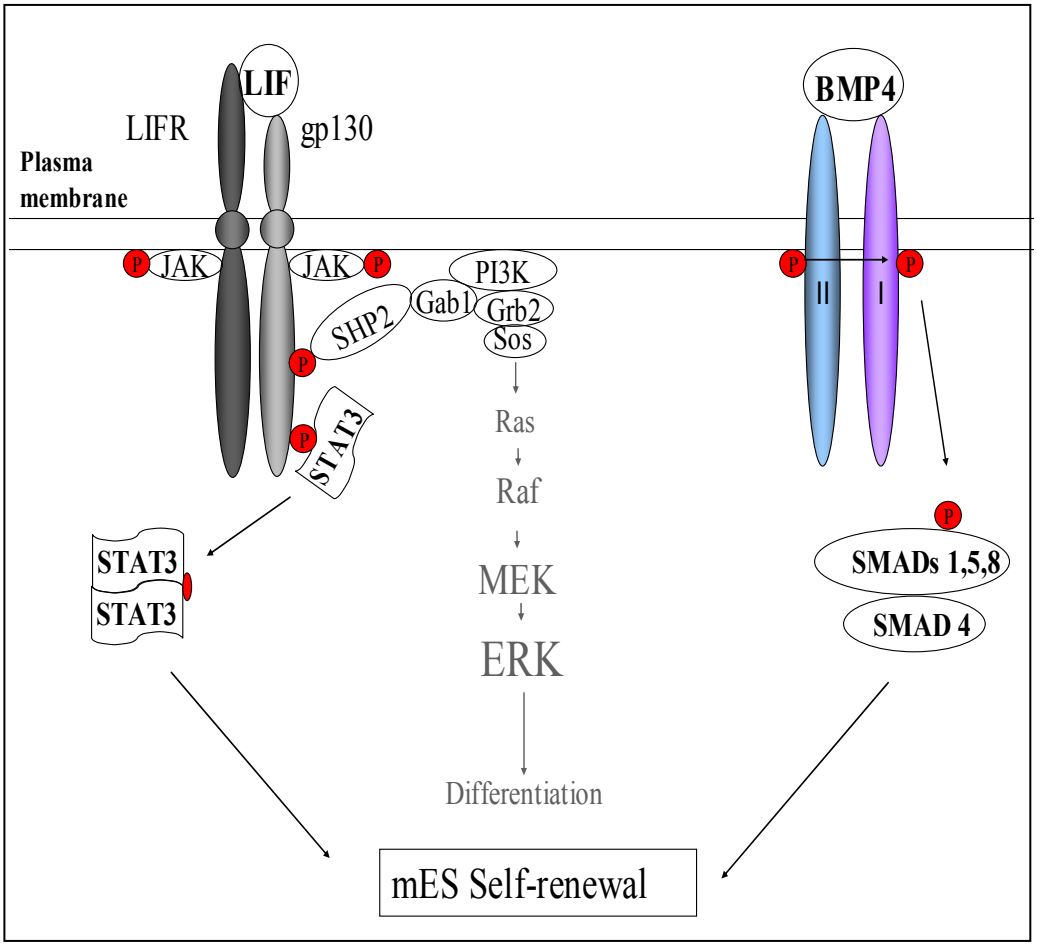


Figure 1.1. Mouse ES cells regulation. LIFR and GP130 receptor subunits exist as monomers that dimerise by LIF binding and subsequent phosphorylation of receptor-associated JAKs. JAKs phosphorylate tyrosine residues on the cytoplasmic domain of GP130, which recruits STAT3 via the SH2 domain which subsequently becomes phosphorylated. STAT3 becomes tyrosine phosphorylated and disengages from the receptor before dimerising in the cytoplasm. STAT3 dimers translocate to the nucleus where they act as transcription factors. Activated JAKs also activate SHP2, leading to activation of RAS via GRB2 or GAB1. Active Ras initiates a cascade of signals which leads to activation of ERK. LIF acts in conjunction with BMP4 to enhance mES cell self-renewal. BMP4 induces phosphorylation of the serine/threonine kinase receptors (BMPR1) and subsequent activation of the SMAD1/5 transcription factors. SMAD1/5 proteins bind to a co-transcription factor SMAD4 and together translocate into the nucleus where they induce the expression of the inhibitor of differentiation (Id) genes.

1.3.2.1 Leukaemia Inhibitor Factor (LIF)

Independent culture of mES cells from primary mouse embryo fibroblast (MEF) feeder cells is possible since the discovery that LIF is the factor released by the MEFs that prevented mES cell differentiation (Smith and Hooper, 1987). *In vivo*, *Lif* mRNA is expressed by the trophoblast cells whilst the pluripotent cells of the ICM express the specific receptors, LIFR and GP130 (Nichols *et al.,* 1996). LIF signalling is necessary for the survival of the cells in the ICM during the delay in embryo implantation or diapause (Nichols *et al.,* 2001) . This could suggest a role of LIF in promotion of the survival and propagation of undifferentiated ES cells rather than suppressing ES cell differentiation. However, Smith *et al,* (1988) showed that besides the selective proliferation and replating advantage over differentiated cells, LIF also inhibits ES cell differentiation. Therefore, LIF seems to have two major effects in mES cells: self-renewal of the undifferentiated cells (including cell proliferation and inhibition of differentiation) and survival of the undifferentiated cells (Viswanathan *et al.,* 2003). By contrast hES cell self-renewal is independent from LIF (Humphrey *et al.,* 2004). This is congruent with the low expression of the receptors LIF and GP30 in hES cells, whilst LIFR is readily detected in mouse ES cell cultures (Brandenberger *et al.,* 2004; Ginis *et al.,* 2004).

Important signal transduction molecules activated by the GP130 and LIF receptors are the signal transducer and activator of transcription (STAT), mitogen-activated protein

kinase (MAPK/ERK) and phosphoinositide-3 kinases (PI-3 kinase) (Yin and Yang, 1994; Boulton *et al.,* 1994; Ernst *et al.,* 1996).

1.3.2.1.1 Signal transducer and activator of transcription (STAT)

The signalling of LIF or the related cytokines through the GP130 receptor is critical for mES cells self-renewal (Niwa *et al.,* 1998). LIF induces GP30 hetero-dimerisation with LIFR, triggering the activation of associated Janus kinases (JAKs) by cross-phosphorylation (Yin and Yang, 1994; Ernst *et al.,* 1996; Heinrich *et al.,* 1998). JAK activation leads to the phosphorylation of the cytoplasmatic tyrosines of gp130, creating binding sites for proteins with the binding motif Src-homology 2 (SH2) domain. The principal proteins activated by the receptor are the signal transducer and activator of transcription (STAT) 1, 3, and 5 transcription factors. Following activation, the STATs dissociate from the receptor and hetero/homo dimerise through their SH2 domain (Ihle, 1996). The most abundant STAT factor in murine ES cells is STAT3, which in the main form homodimers (Burdon et al., 1999a). Once the dimer is formed, the STATS translocate to the nucleus and bind to the specific enhancer sequences of their target genes. STAT3 activity is required for mES cell self-renewal (Niwa *et al.,* 1998) possibly in association with the transcription factor MYC (Cartwright *et al.,* 2005) since it is a key effector of the LIF/STAT3 pathway. MYC has also been shown to be involved in cell immortalisation (Cartwright *et al.,* 2005) possibly through the activation of TERT, the regulatory subunit of telomerase (Wang *et al.,* 1998).

1.3.2.1.2 Mitogen-activated protein kinase (MAPK) pathway

LIF stimulation of GP130 also activates the MAPK/ERK signalling pathway in mES cells when GP130 recruits and phosphorylates the tyrosine phosphatase SHP2. SHP2 phosphorylation results in the binding of growth factor receptor bound protein 2 (GRB2). GRB2 recruits son of sevenless homologue (SOS) and thereby activates the oncogene RAS. Activation of RAS initiates a signalling cascade, which culminates in ERK activation. The MAPK pathway can also be activated through GP130 by the GRB2 associated protein 1 (GAB1) activation of RAS.

Unlike STAT3 activation, MAPK stimulation is not required for ES cell self-renewal; in fact it is likely to be important in ES cell differentiation. ES cells over-expressing a catalytically inactive SHP2 are compromised in their ability to activate MAPK and to differentiate (Burdon *et al.,* 1999b). This was demonstrated by the stable introduction of a mutated *Gp130* receptor that was unable to engage SHP2 into D027 ES cells. In D027 ES cells, the endogenous activity of both copies of the *Lif* gene had previously been ablated (Dani *et al.,* 1998). D027 ES cells containing the mutant receptor, prolonged STAT3 activation and enhanced self-renewal, indicating a negative role for MAPK activation in ES cell self-renewal. ES cells also showed an enhanced self-renewal and reduced dependency on LIF when cultured in the presence of the MAPK pathway inhibitor PD98059 (Burdon *et al.,*1999b). This supports a role for MAPK activity in ES cell differentiation downstream of GP130.

1.3.2.1.3 Phosphatidylinositol-3-OH kinase (PI3K) pathway

Phosphatidylinositol-3-OH kinases (PI3Ks) are heterodimers composed of a regulatory subunit p85 constitutively bond to the p110 catalytic subunit. The subunit p85 contains two SH2 and one SH3 domain, by which PI3K is connected with many active tyrosine kinases and with tyrosine phosphorylated docking proteins such as FRS2/IRS1 and the scaffold protein GRB2-associated binder protein1 (GAB1). Stimulation SHP2 by GP130 leads to PI3K signalling. Phosphorylated SHP2 can bind to Grb2 and Gab1. Gab1 recruits the lipid kinase PI3K to the complex formed between SHP2 and GRB2, which results in PI3K mediated phosphorylation of phospholipids in the membrane and the amplification of the signal (Takahashi-Tezuka *et al.,* 1998).

PI3Ks catalyse the phosphorylation of inositol-containing lipids, known as phosphatidylinositols (PtdIns). Activated PI3K phosphorylates $PtdInsP_2$ or PIP_2 to generate the second messenger $PtdInsP_3$ or PIP_3. The activity of PIP_3 can be negatively regulated by PTEN and SHIP, two phosphoinositide specific phosphatases that convert PIP_3 back to PIP_2 (Bolland *et al.,* 1998). PIP_3 mediates translocation to the membrane of a variety of signalling proteins such as the Ser/Thr kinases PKB or AKT and PDK1, GAB1 and PLCγ1, among many others (Bellacosa *et al.,* 1998). For example, PKB/AKT is phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) when translocates to the plasma membrane (Stokoe *et al.,* 1997).

PI3K has been implicated in mES cell proliferation and survival by regulating cyclin D1 levels (Jirmanova *et al.,* 2002). Cyclin D1 is present at low levels in mES cells,

compared to differentiating cells and may not be required for G1/S transition in mES cells (Savatier et al., 2002). The role of PI3K in proliferation and survival is related to the oncogene ES cell-expressed Ras (ERAS). ERAS binds and activates PI3K and have been shown that in its absence, ES cell proliferation rates decrease considerably However, ERAS is not essential to maintain pluripotency in ES cells, since ERAS null ES cells form fertile chimaeric mice (Takahashi *et al.,* 2003).

In addition to proliferation, PI3K has a role in regulation of self-renewal of mES cells in the presence of LIF (Paling *et al.,* 2004). Inhibition of PI3K signalling was associated with an increase in ERK phosphorylation resulting in a reduction of mES cell self-renewal (Paling *et al.,* 2004). Upon PI3K inhibition the important balance between STAT3 and ERK signals in determining cell fate (Burdon *et al.,* 2002) seems to be altered in favour of the pro-differentiation effects of ERK. Nevertheless, inhibition of ERK reversed the effects of PI3K inhibition on self-renewal independently of STAT3 activation (Paling *et al.,* 2004).

1.3.2.2 Bone Morphogenetic Protein 4 (BMP4)

LIF is only effective in medium containing serum; however LIF in combination with the Bone Morphogenetic Protein 4 (BMP4) alone are sufficient to maintain mES cells in a pluripotent state in the absence of serum (Ying *et al.,* 2003a). The TGFβ superfamily of ligands signal through two branches: the SMAD1/5 branch, which transduces BMP and GDF (growth differentiation factor) ligands and the TGFβ/Activin/Nodal branch, which

activate SMAD2/3. Murine ES cells only use the BMPs/Smads1/5 pathway since they do not express the specific receptors for TGFβ (Goumans *et al.,* 1998). BMP4, through specific serine/threonine kinase receptors (BMPR1) activates an independent pathway to the LIF/STAT3 for ES self-renewal. Subsequent to the receptor phosphorylation, the SMAD1/5 transcription factors are activated and in turn induce the expression of the inhibitor of differentiation (*Id)* genes. *Id* genes sequester the neurogenic basic/helix-loop-helix (bHLH) transcription factors, preventing their differentiating activity in mES cells. In fact, over expression of *Id* genes can bypass BMP signals in the presence of LIF and in the absence of serum (Ying *et al.,* 2003a).

1.3.3 Extrinsic factors on human embryonic stem cell regulation

Traditionally, the proliferation of undifferentiated hES cells has relied on the presence of mouse (Thomson *et al.,*1998) or human (Amit *et al.,* 2003; Richards *et al.,* 2003) fibroblast feeder layers. The feeder cells secrete factors that produce a matrix required to allow adherence and to maintain self-renewal and pluripotency of the hES cells (Thomson *et al.,* 1998). Alternatively, the feeder cells can be replaced with Matrigel, an extracellular matrix (ECM) preparation, and conditioned medium (CM) from the feeder cells (Xu *et al.,* 2001). CM provides numerous factors produced by the feeder cells to sustain the undifferentiated growth of hES cells. Among those factors, FGF2 and several members of the TGFβ family have been implicated in hES cells self-renewal. A simplified diagram of the regulation of hES cells is shown in Figure 1.2.

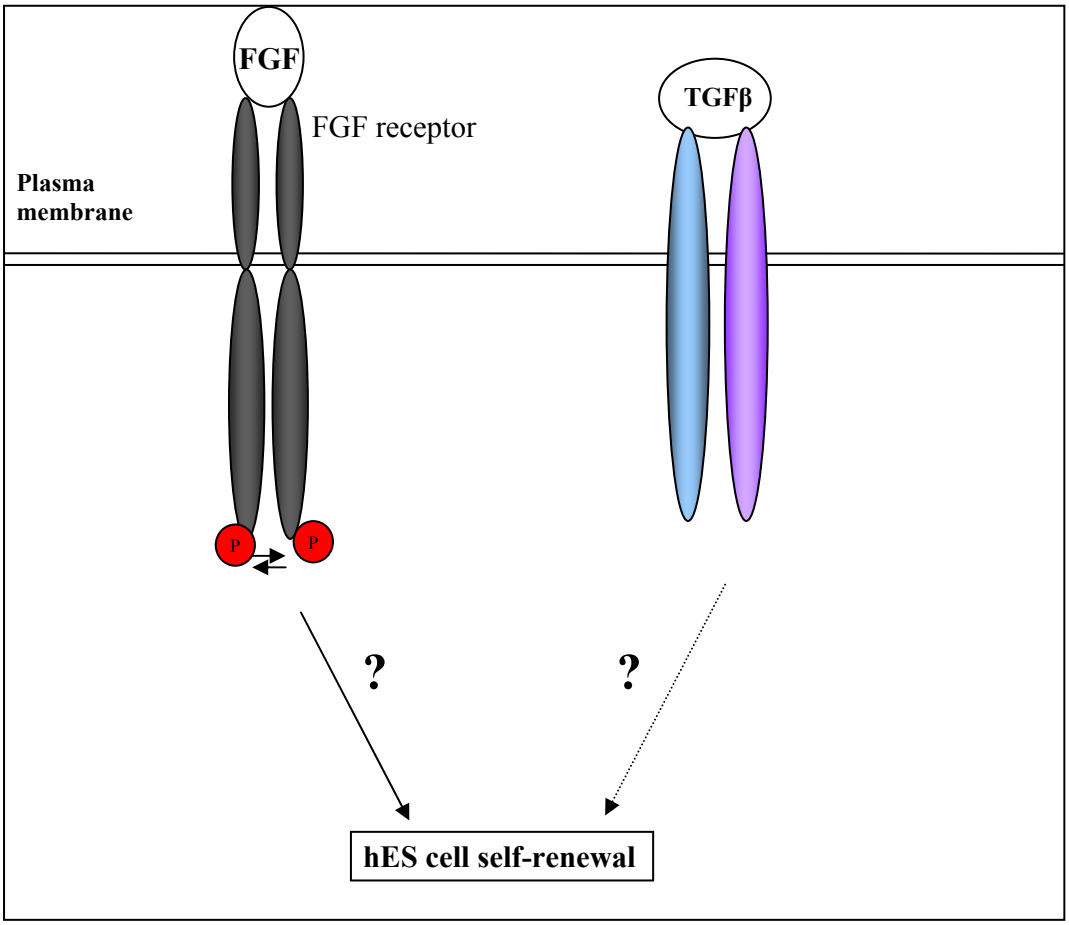


Figure 1.2. Human ES cell regulation. FGFR monomer dimerises by FGF binding resulting in receptor activation. Activated receptors recruit signalling proteins with the culmination of regulation of hES cell self-renewal. TGFβ/Activin/Nodal signalling pathway activates the TGFβ receptors.

1.3.3.1 Fibroblast growth factor 2 (FGF2)

FGF2 is the central point of study in this thesis, thus an overview of the functions, structure and signal transduction of FGFs will be discussed in the following sections. FGF2 is a mitogenic, angiogenic and neurothrophic factor that plays key roles in the development, remodelling and tumour growth in almost every organ system reviewed by Bikfalve *et al,* (1997). In hES cells, the requirement for exogenous FGF2 signalling to sustain self-renewal and retain their pluripotency is currently accepted (Levenstein *et al.,* 2005). *Fgf2* and its receptors are highly expressed in undifferentiated hES cells (Brandernberger *et al.,* 2004) suggesting the activity of autocrine, paracrine and possibly an intracrine FGF signalling on these cells. In transient hES cells, on the other hand, FGF2 has been shown to induce development of ectodermal and mesodermal cells (Schuldiner *et al.,* 2000) and to support hES cell differentiation into neural lineages (Carpenter *et al.,* 2001; Reubinoff *et al.,* 2001). By contrast, studies in mES cells have indicated that autrocrine FGF signalling is preferred for neural specification (Ying *et al.,* 2003b). *Fgf2* is not detected in mES cells (Reubinoff *et al.,* 2001; Wei *et al.,* 2005) and neither is it required for their culture. *Fgf2* knock-out mice are viable, although with neuronal and cerebral cortex defects at birth (Ortega *et al.,* 1998; Reubinoff *et al.,* 2001). This indicates that although FGF2 is not essential for embryonic development, its specific role in neurogenesis is not compensated by other FGF family members.

MAPK, PI3K and NFκβ transcripts have been found to be enriched in hES cells (Armstrong *et al.,* 2006), which suggests that there may be some of the effector

signalling pathways of FGF in hES cell self-renewal. In addition to these pathways, some reports point towards TGFβ and Wnt signalling as being responsible for undifferentiated hES cells (Sato *et al.,* 2003). However, recent reports indicate that it is FGF2 and TGFβ/Activin/Nodal factors, which may be involved in maintaining the self-renewal and pluripotency of hES cells (Reubinoff *et al.,* 2001; Amit *et al.,* 2004; Vallier *et al.,* 2005; Levenstein *et al.,* 2005; Greber *et al.,* 2006). Nevertheless, the signalling pathways downstream of FGF2 which are responsible for hES cell self-renewal have not been described. Furthermore, the possible collaboration of FGF with other signaling pathways needs to be clarified.

1.3.3.2 Transforming growth factor β/Activin/Nodal

Several members of the branch of the TGFβ/Activin/Nodal in the TGFβ family have been implicated in maintaining hES cells undifferentiated state (Reubinoff *et al.,* 2001; Vallier *et al.,* 2004; Beattie *et al.,* 2005; James *et al.,* 2005). Conversely, BMP/GDF, which activates the Smad1,5,8 branch induces hES cell differentiation (Xu *et al.,* 2002; Vallier *et al.,* 2004; Pera *et al.,* 2004; James *et al.,* 2005). Indeed, inhibition of BMP signalling promotes the undifferentiated growth of human ES cells (Pera *et al.,* 2004; James *et al.,* 2005; Xu *et al.,* 2005a). Like FGFRs, most of the TGFβ receptors are expressed in hES cells (Wei *et al.,* 2005). However, a difference in the reduction in FGFRs expression upon differentiation on hES cells, (Brandernberger *et al.,* 2004) TGFβ1 and its receptors TGFR1 and TGFR2 are all expressed throughout human EB formation (Poon *et al.,* 2006). This contrasts with the low levels or absence of TGFRs

on mES cells suggesting fundamental differences regarding the importance of FGF and TGFβ1 signalling in early development.

1.4 Fibroblast growth factors (FGFs)

FGFs are small (from 17 to 34KDa) signalling polypeptide growth factors with functions of crucial importance in all the stages of development and in the physiology and pathology of adult tissue. The times and the patterns of FGF expression differ in the distinct tissues; some FGFs are only expressed during embryonic development whereas others are expressed in embryonic and adult tissue (Ornitz and Itoh, 2001). To date 22 homologue members of the *Fgf* family have been identified in a variety of organisms from nematodes and flies to mice and humans (Zhang *et al.,* 2006). FGFs have in common a central core of 120 aminoacids and a high affinity for the transmembrane receptor tyrosine kinase, fibroblast growth factor receptor (FGFR) (Ornitz and Itoh, 2001). FGFs also interact with membrane-bound heparan sulfate proteoglycans (HSPGs) or their soluble fragments, such as heparin. Binding of FGFR to these proteins is necessary for the stable activation of the receptors and for modulation of FGF activity. Although most FGFs produce their effect in target cells by signalling through FGFRs, some members can interact with intracellular proteins by internalisation in complex with the receptor. Furthermore, internal activation can occur because some FGFs are sited in the cytoplasm or nucleus of the producing cells due to poor or no classical secretion signal motifs reviewed by Goldfarb, (2001). The high expression of FGF2 and FGF4 in

human and mouse ES cells respectively, make them a focus of study in this project. The functions of these FGFs in ES cells as well as in development will be more thoroughly discussed in chapters 3 and 5 but their general features are outlined below.

1.4.1 Fibroblast growth factor 2 (FGF2; basic FGF, bFGF)

FGF2 was identified based on its mitogenic activity on the Bal/c3T3 cell line (Gospodarowicz *et al.,* 1975). FGF2 is the prototypic FGF since it is the most extensively studied FGF and has a high (>90%) sequence homology across a wide range of species. FGF2 contains four cysteine residues with intramolecular disulfide bonds, a large number of basic residues and two sites that can be phosphorylated by protein kinases A and C (Bikfalvi *et al.,* 1998). FGF2 is encoded by a single copy gene that is alternatively translated to produce one low (18KDa) and four high (22-34KDa) molecular mass isoforms (Abraham *et al.,* 1986). The 18KDa low molecular mass FGF2 lacks the secretion signal sequence and it is released associated with other molecules by an exocytotic mechanism independent of the endoplasmatic reticulum/Golgi pathway (Piotrowicz *et al.,* 1999). It has also been reported that FGF2 is released as a result of cell damage, such as membrane disruptions and death (Conrad *et al.,* 1998). Subsequently in a paracrine or autocrine manner FGF2 binds and activates its specific FGFRs. Cytosolic 18KDa and all the high molecular mass isoforms of FGF2 containing a nuclear localisation sequence (NLS) are targeted to the nucleus (Sheng *et al.,* 2004). This form of FGF2 produces receptor independent intracrine activities, for

example activation of MAPK, by induction of the PLC/PKC signalling pathways (Gaubert *et al.,* 2001).

1.4.2 Fibroblast growth factor 4 (FGF4; hst-1/kFGF)

FGF4 was described as a human stomach cancer transforming factor by (Taira *et al.,* 1987). The role of *FGF4* as oncogene has been established by finding that FGF4 is expressed in human teratoma cell lines and in surgically removed human testicular germ cell tumours including seminomas and embryonal carcinomas (Yoshida and Morii, 1998). In addition to this, FGF4 is found in stomach and breast cancer and in Kaposi sarcoma (Adnane *et al.,* 1991). This expression indicates that FGF4 expression in adult organisms seems to be associated with malignancies. By contrast, in murine early development stages, FGF4 is an essential factor expressed as early as the 1 cell stage (Rappolee *et al.,* 1994) and its deletion results in early embryonic death (Feldman *et al.,* 1995). Crucially, FGF4 is also highly expressed in mES cells (Wei *et al.,* 2005) under the regulation of OCT4 and SOX2 (Yuan *et al.,* 1995). This may suggest a role of FGF4 in mES cell self-renewal but it has been demonstrated that mES cells do not require FGF4 (Wilder *et al.,* 1997). Indeed, a recent report appoints FGF4 as inducer of neuroectoderm differentiation in mES cells (Kunath *et al.,* 2007). FGF4 unlike FGF2 contains a secretion signal sequence indicating that FGF4 acts predominantly through cell surface receptors. Contrasting with the high expression of *Fgf4* in mES cells, mRNA expression in human ES cells appears to be approximately 30-fold lower (Ginis *et al.,* 2004; Brandernberger *et al.,* 2004).

1.4.3 Biological functions of FGFs

FGFRs activate numerous downstream signalling proteins with a wide range of roles at the cellular level, such as migration, survival, death, proliferation, self-renewal, differentiation, adhesion, and alterations in the cell cytoeskeleton. As a result of some of these functions, FGFs are essential in all the stages of development as well as having a role in inflammation, haematopoiesis and the repair process of the adult tissue. All functions need to be tightly regulated otherwise they can contribute to disease and cancer (Ornitz and Itoh, 2001).

1.4.3.1 FGF functions in cells

FGFs have a wide range of roles in cell behaviour. The same FGF can generate different responses on the same cell when they are at different stages of differentiation. For example, components of the FGF/FGFR signalling pathway (including FGFR1, 2, 3 and 4 as well as FGF2, 11 and 13) are found at significantly higher levels in undifferentiated hES cells than in their differentiated progeny and mES cells (Sato *et al.,* 2003; Sperger *et al.,* 2003) In addition to this, the same stimulus can produce a different response in different cell types. For instance, FGFR1 stimulation in fibroblasts leads to cell proliferation whilst in neuronal cells it induces cell survival and differentiation. The different responses are likely to be the consequence of the expression of cell type-specific effector proteins and transcription factors in different cells (Schlessinger, 2000).

FGFs can also stimulate a range of cellular responses even in the same cell type. For example, FGF2 induce both proliferation and differentiation in cerebelar granule neurons, chondrocytes, and osteoblasts (Bikfalvi *et al.,* 1997). A reason for the different responses in the same cell has been shown to be related to differences in FGF concentration. Low and high concentrations of FGF2 rescue cells from apoptosis induced by serum starvation. Conversely, in the same conditions, intermediate concentrations of FGF2 increase apoptosis (Garcia-Maya *et al.,* 2006). In addition to FGF concentration, different FGFR activation, the effector pathway used and the strength and duration of signalling are other factors responsible for the divergent responses to the same FGF (Schlessinger, 2000). The level of FGFR activation would depend on its binding capacity. This would be reflected on the rate of FGF binding to the cell and in turn may result in different cell response to FGF (Richardson *et al.,* 1999). The cell cycle phase or the level of confluency of the cell population may also be factors influencing the cell choice in transduction pathway (Schlessinger, 2000). Furthermore, different duration and strength of signal can activate diverse transduction pathways with, consequently, different outcomes. For example, transient FGF stimulation can activate the SRC pathway leading to a migratory phenotype, whilst sustained stimulation appears to correlate with cell proliferation through MAP kinase activation (LaVallee *et al.,* 1998). However, the opposite can also occur and the signal duration and strength can generate different outcomes through the same pathway. For instance, a transient stimulation of MAPK stimulates PC12 cell proliferation whilst a sustained and robust MAPK response results in cell differentiation of the same cells (Marshall, 1995).

1.4.3.2 FGF functions in development

Fgf knock-out studies together with studies of the expression patterns of *Fgfs* in different tissues and cells demonstrate that these factors are critical during most stages of mouse development. Significantly, their functions at cellular level, which include proliferation, differentiation, survival, apoptosis and migration, indicate a critical role in early development.

Some FGFs have a much specialised biological role resulting in a highly specific phenotype whilst the loss of other FGFs can be compensated by related members of the FGF family, resulting in no obvious phenotypes. For example, *Fgf4* $^{-/-}$ and *Fgf8*$^{-/-}$ cause embryonic lethality, whereas *Fgf2*$^{-/-}$, *Fgf1*$^{-/-}$ and *Fgf6*$^{-/-}$ have apparently a normal phenotype (Powers *et al.,* 2000). Furthermore, targeted disruption of the four *Fgfrs* shows a wide range of phenotypes. Firstly, *Fgfr1* knock-out causes embryonic lethality before or during gastrulation (E9.5-E12) as a result of defects in cell migration (Deng *et al.,* 1994). *Fgfr2*$^{-/-}$ mice die after implantation of blastocyst (E10.5) due to defects in endoderm differentiation from the ICM (Arman *et al.,* 1998). Furthermore, disruption of the *Fgfr3* gene results in bone overgrowth whereas no evident pheonotype was observed in *Fgfr4*$^{-/-}$ mice (Eswarakumar *et al.,* 2005) suggesting that *Fgfr3* and *4* operate at later stages of embryonic development.

FGF4 is required from the earliest stages of development possibly by regulating cell division (Chai *et al.,* 1998). FGF4 and its specific receptor FGFR2 are expressed in the

blastocyst: *Fgf4* in the ICM and *Fgfr2* in the trophectoderm (Rappolee *et al.,* 1994; Haffner-Krausz *et al.,* 1999). After implantation, *Fgf4* is expressed throughout the epiblast (Niswander and Martin, 1992) whilst *Fgfr2* is restricted to the extraembryonic ectoderm (Orr-Urtreger *et al.,* 1991). This pattern of expression is consistent with the role of paracrine FGF4 signal in the proliferation of trophectoderm (Nichols *et al.,*1998) and extraembryonic endoderm (ExEn) (Wilder *et al.,* 1997). This is shown by the absence of extraembryonic endoderm in *Fgf4* and *Fgfr2* mutant embryos and null ES cells (Wilder *et al.,* 1997). Consequently, the null embryos do not survive after implantation and null blastocysts do not survive in culture (Feldman *et al.,* 1995; Arman *et al.,* 1998). The effect of dominant negative FGFR (dnFGFR) was the blockage of embryo division before implantation (Chai *et al.,* 1998), rather than the generation of the *Fgf4* null embryo after implantation (Feldman *et al.,* 1995). The phenotypes of dnFGFR embryos come into view earlier than those of *Fgf4* null mutant (Chai *et al.,* 1998), possibly due to maternal FGF4 (Rappolee *et al.,* 1994) or other FGFs.

FGF2 is also involved in early mouse development, and *Fgf2* together with its specific receptor *Fgfr1* (Ornitz *et al.,* 1996) are expressed in mouse blastocyst (Campbell *et al.,* 1992). *Fgf2* is expressed in the primitive ectoderm and is detected in pregnant mouse uterus (Jirmanova *et al.,* 1999). FGF2 signalling has a role in regulating the patterning of mesodermal and neural cell lineages (Burdsal *et al.,* 1998). This has been confirmed by the failure of differentiation into visceral endoderm and primitive ectoderm found in EBs derived from ES cells with a targeted disruption of *Fgfr1* (Esner *et al.,* 2002). A more extreme outcome was observed by the effects of a dnFGFR in ES cells in which

FGF repression of signalling completely inhibited the differentiation of EBs (Chen *et al.,* 2000).

In humans, developmental disorders normally occur when a copy of the *Fgf* gene is lost or by altered activity of the FGFR rather than null mutations, which are rare. Developmental defects due to mutations in FGFR genes involve achondroplasia or short limb dwarfism, craniosynostosis or premature fusion of the skull bone, Apert Syndrome or fusion of digits and many other types and degrees of malformations (Burke *et al.,* 1998).

1.4.3.3 FGF functions in adult tissue

FGFs have essential roles in the control of the nervous system, in hematopoiesis, in angiogenesis and wound healing. In wound repair FGFs play a role in inflammation by inducing the migration of inflammatory cells. FGFs also have significant roles in repair and regeneration due to their proliferative effects on other cells reviewed by Powers *et al,* (2000).

1.4.3.4 FGF functions in cancer

FGFs and their receptors can contribute to cancer when their functional regulation is impaired. Neoplastic transformations, especially haematological malignancies, are often related to abnormal activation of FGFRs. For example, in multiple myeloma (MM), a

translocation results in the constitutive activation of FGFR3 causing MM cell proliferation and survival (Chesi *et al.,* 2002). Other types of cancer are characterised by the over-expression of FGFRs, such as breast cancer in which FGFR1 is over-expressed. In pancreatic adenocarcinoma, an abnormal expression of FGFR1 and FGFR4 is also found, in thyroid carcinoma there is an over expression of FGFR3, etc. Over-expression of secreted FGFs is another mechanism by which FGFs can promote cancer progression. This is due to the mitogenic, angiogenic and migratory effects of FGFs on tumour and endothelial cells, which result in tumour metastasis (Powers *et al.,* 2000).

An example that links cancer, stem cells and FGFs is the non-seminomatous testicular cancer, which has been associated with FGF4 and 2. For example, the F*gf2* gene is highly expressed in human testicular carcinomas. FGF produces tumour proliferation or migration at low and high concentrations respectively (Granerus *et al.,* 1993). *FGF4* is also over-expressed in different human types of germ cell tumours and in murine EC cells. FGF4 and FGFR1 are associated with the most aggressive types of testicular germ cell tumours (Suzuki *et al.,* 2001). Moreover, FGF2 and FGF4 are expressed in the undifferentiated human EC cells and both genes are down regulated during their differentiation (Alanko *et al.,* 1996).

1.5 Fibroblast growth factor receptors (FGFRs)

1.5.1 Structure and isoforms of FGFRs

Like all receptor tyrosine kinases, FGFRs are composed of an extracellular ligand binding domain, a transmembrane domain and a cytoplasmic domain containing the catalytic protein tyrosine kinase core as well as additional regulatory sequences (Schlessinger, 2000). There are four mammalian *Fgfr* genes *(Fgfr1-4)*, which by alternative mRNA splicing, produce a variety of isoforms (Plotnikov *et al.,* 1999). The four receptor types are present in hES, principally FGFR1, which is the main target of exogenous FGF2 and whose expression decreases as the ES cells differentiate (Brandernberger *et al.,* 2004). By contrast, undifferentiated mES cells weakly express FGFRs 1, 2 and 4 and their expression increases upon differentiation (Esner *et al.,* 2002).

The different *Fgfr* splicing, results in insoluble secreted FGFRs as well as variants with missing fragments. In the full length FGFR, the extracellular ligand binding domain is composed of three immunoglobulin-like domains that are designated D1-D3. They are linked by short segments; a stretch of acidic residues in the linker connection D1 and D2, designated the "acid box" and a positively charged region in D2 that serves as a binding site for heparin (Schlessinger *et al.,* 2000) (Figure 1.3). Alternative splicing of the D3 domain confers binding specificity to the receptor (Johnson *et al.,* 1991). In other splice variants the receptors are either missing the first D1 domain or missing both D1 and the acid box region (Burke *et al.,* 1998). This does not affect FGFR function

because neither D1 nor the acid box are required for FGF binding to the FGFR. In fact, their deletion enhances binding of the receptor to FGF and heparin (Wang *et al.,* 1995). D1 and the acid box have an auto-inhibitory function by competing with FGF and heparin respectively (Plotnikov *et al.,* 1999). This occurs when FGFRs are in equilibrium and display a close configuration, which opens when they are ready to dimerise. FGF and heparin will bind to the open configuration and will shift the equilibrium towards the dimeric form of FGFR (Olsen *et al.,* 2004).

The cytoplasmic catalytic domain contains a protein tyrosine kinase (PTK) core and additional regulatory sequences that are subjected to autophosphorylation and phosphorylation by heterologous protein kinases (Hunter, 1998). Crystal structures of the PTK domain of FGFR in an inactive or low activity state shows a kinase residue interfering with the substrate binding site in the activation loop. In order to maintain the catalytic domain in an active state, two tyrosine residues in the activation loop must be autophosphorylated (Mohammadi *et al.,* 1996) (Figure 1.3).

1.5.2 FGFR dimerisation and activation

The key event in transmembrane signalling is the ligand-induced dimerisation of FGFR monomers in the cell membrane. Dimerisation triggers the activation of the receptors and consequently, activation of downstream signalling proteins (Ullrich and Schlessinger, 1990). FGFRs can form heterodimers and thus exhibit heterologous transphosphorylation (Bellot *et al.,* 1991). For this reason over-expression of any type

of receptor with a non-functional tyrosine kinase domain inhibits FGF signalling via all FGFRs (Chen *et al.,* 2000). Crystal structures of activated receptors have shown FGFs interacting with D2, D3 and the linker connecting between these two domains in the FGFR. The FGFRs/FGF/HS complex is stabilised by interactions between FGF and D2 domain in the second receptor, receptor:receptor interactions and by binding of heparin to the D2 domains of the two FGFRs in the dimmer and the bound FGF molecules (Plotnikov *et al.,* 2000). Receptor dimerisation produces an increase in the concentration of protein tyrosine kinases (PTK), which leads to the phosphorylation of specific tyrosine residues on their own and each others cytoplasmic region (Lemmon and Schlessinger, 1994). The activation of the tyrosines induces a conformational change in the activation loop of the catalytic PTK domain, which adopts an open configuration permitting access to ATP and substrates. This enables the phosphotransfer from ATP to tyrosines on the receptor and on cellular proteins involved in signal transmission (Hubbard *et al.,* 1998) (See Figure 1.3).

In addition to the autophosphorylation sites within the catalytic domain, there are other autophosphorylation sites in the noncatalytic regions of the cytoplasmic domain. These sites are involved in the recruitment of proteins by creating docking sites for molecules containing Src-homology 2 (SH2) or phosphotyrosine binding (PTB) domains (Pawson and Schlessinger, 1993). Many of the recruited molecules are signalling proteins containing intrinsic enzymatic activities, in addition to protein modules, which are able to interact with other proteins, with phospholipids or with nucleic acids (Schlessinger, 2000).

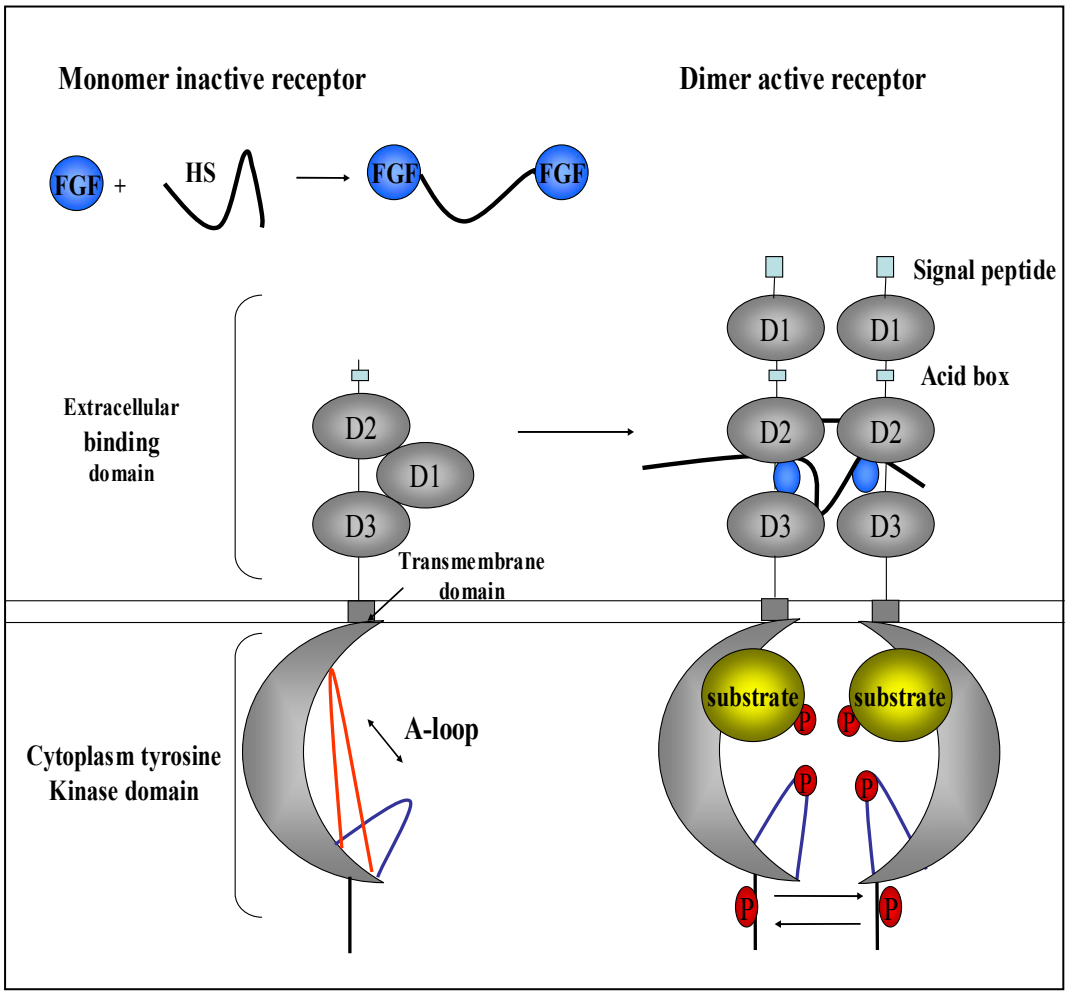


Figure 1.3. FGFR activation. Monomers of FGFR in equilibrium display a close configuration with D1 and the acid box occupying the binding sites of FGF and heparin in the receptor (Olsen *et al.*, 2004). In the cytoplasmatic domain during the equilibrium state of FGFR, the inactive catalytic A-loop, adopts a conformation (red line) that interferes with the protein substrate and ATP binding. FGFR monomer dimerises by ligand-heparin (FGF-HS) binding resulting in the phosphotyrosine autoactivation of the receptor (Plotnikov *et al.*, 1999). By ligand binding of the extracellular domain, the local concentration of kinases increases leading to the transphosphorylation of the kinase residues of the A-loop. This produces a change in conformation of the loop, allowing the binding of proteins and their consequent activation. Autophosphorylation of additional tyrosines of the receptor serves as binding sites for downstream signalling proteins (Schlessinger, 2000).

1.5.2.1 The role of FGF-heparin interaction

Heparin, heparan sulphate (HS) or heparan sulphate proteoglycans (HSPGs) are heterogeneously sulphated linear polymers, which contain repeating disaccharide subunits. The saccharide sequence and sulfatation pattern are variable, providing high specificity in binding to the ligands (Ornitz, 2000). The principal function of these sugars in FGFR activation is the stabilisation and enhancement of the half-life of FGF/FGFR dimmers (Plotnikov *et al.,* 1999). In addition to this, HSs have an earlier role protecting FGF from denaturation and proteolysis (Gospodarowicz and Cheng, 1986) by binding free FGFs. This would also induce FGF oligomerisation and subsequent FGF/HS binding to FGFRs (Ornitz *et al.,* 1992). Furthermore, FGF binding by HSs facilitates activation of the receptors by increasing FGF concentration on cell surface (Folkman *et al.,* 1988).

1.6 Signal transduction from FGFRs

Signalling pathways downstream of FGFRs form a complex network with multiple positive and negative feedback mechanisms. The principal pathways activated by FGF are going to be reviewed in this section and a schematic representation is shown in Figure 1.4. The pathways are going to be divided into those activated by the docking protein FRS2 and those directly recruited and activated by FGFRs. Docking and signalling proteins bind specific phosphotyrosine residues on the activated receptor by their Src-homology 2 (SH2) or phosphotyrosine binding (PTB) domains (Pawson and Schlessingert, 1993; Pawson et al., 1993). The role of docking proteins such as FRS2, GRB2, CRK and SHC is recruiting signalling proteins to the FGFRs and the membrane when they become tyrosine phosphorylated in response to FGF stimulation (Pawson, 1995). The most important docking protein in FGFR signalling is FGF receptor substrate2 (FRS2) and in particular FRS2α (Hadari *et al.,* 2001). Conversely, signalling proteins such as the tyrosine kinase activity containing SRC or tyrosine phsosphatase activity SHPs are directly activated by the catalytic domain of FGFRs (Pawson *et al.,* 1993).

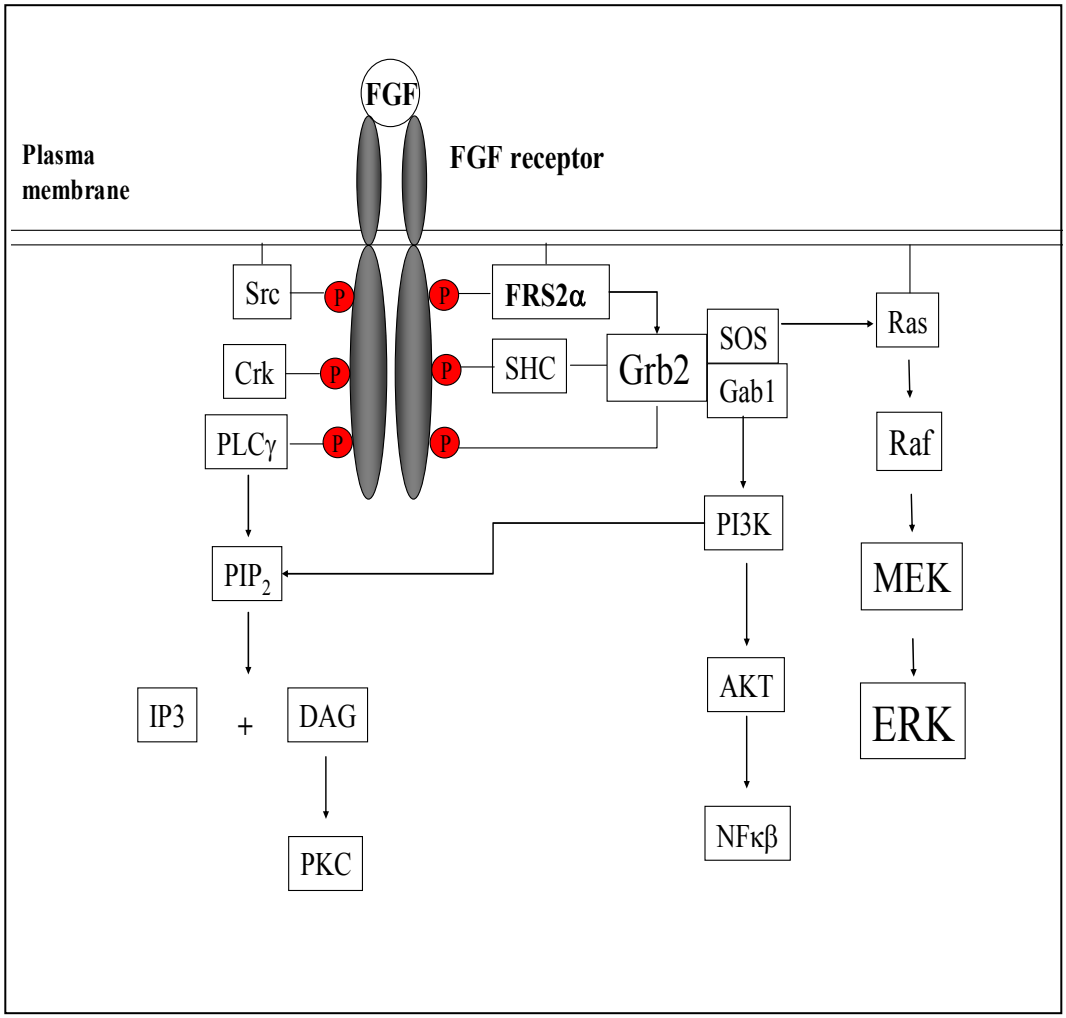


Figure 1.4. Principal signalling pathways activated by FGFR. FRS2α activates both the RAS/MAPkinase and PI3Kinase pathways. In addition to this, the SRC, CRK and PLCγ signalling pathways can be activated directly by FGFR.

1.6.1 Role of the docking protein FRS2α

FRS2 (α and β) are part of the family of docking or adaptor proteins, which also includes IRS1,2,3 and 4 and GAB1 and 2. The function of docking proteins is to recruit signalling proteins to the receptor when they become activated (Schlessinger, 2000). For this purpose docking proteins contain multiple tyrosine phosphorylation sites for the SH2 domains of signalling proteins and a phosphotyrosine binding (PTB) domain to connect with specific RTKs (Kouhara *et al.,* 1997). In addition to this, docking proteins are linked to the cell membrane by transmembrane domains such as is myristyl achor in FRS2 (Kouhara *et al.,* 1997) and the pleckstrin homology domain (PH) in GAB1 (Rocchi *et al.,* 1998).

The critical role of FRS2α in early embryo development was established by the lethality (E7-7.5) caused by the targeted disruption of Frs2α (Hadari *et al.,* 2001). Since FRS2β is expressed exclusively in the embryo beyond E10-10.5 time point in development, this member of the family is unable to compensate for the loss of FRS2α earlier than E10 (Gotoh *et al.,* 2004). Interestingly, FRS2 is expressed in hES cells but could not be detected in mES (Wei *et al.,* 2005). This suggests that human and mouse ES cells may use different signalling pathways downstream of FGFR and this might account for the different roles of FGF in these two cell types.

Tyrosine phosphorylated FRS2α forms a complex with the adaptor protein GRB2, which in turn binds to SOS, linking FGFR with MAPK signalling pathway (Kouhara *et al.,*

1997). Furthermore, because GRB2 binds constitutively to GAB1, this protein can be phosphorylated by the GRB2/FRS2α complex. GAB1 phosphorylation is followed by recruitment of SH2 domain containing signalling proteins including PI3K (Hadari *et al.,* 2001). Experiments with embryonic fibroblasts from Frs2α $^{-/-}$ mice have confirmed the critical role of FRS2α in FGF induced MAP and PI3 kinase activation, with the consequences of cell motility and proliferation (Hadary *et al.,* 2001).

1.6.1.1 RAS/MAP kinase pathway

Hadari *et al,* (2001) demonstrated that FRS2α is required for MAPK sustained activation, whereas in the absence of FRS2α, only a transient activation of MAP kinase was achieved, even at high concentrations of FGF. Furthermore, a sustained activation of MAPK was associated with cell proliferation even at low concentrations of FGF. Therefore, the duration and strength of MAP kinase activation determined the biological response in ES cells (Hadari *et al.,* 2001). This indicated that FGF induced stimulation of MAPK is mediated by FRS2α-dependent and FRS2α-independent mechanisms.

FGFR activation of MAPK is always mediated through the phosphorylation of the oncogene RAS (Kouhara *et al.,* 1997; Schlessinger, 2000) RAS is activated by SOS in complex with the adaptor protein GRB2. GRB2/SOS complex are activated by FGFR through the SH3 domain in GRB2, in association with the protein SHC linked to the FGFR (Klint *et al.,* 1995) or in association with FRS2 (Kouhara *et al.,* 1997). Once it is phosphorylated, RAS activates several effector proteins such as the serine/threonine

kinase RAF. RAF activation triggers a cascade of transphosphorylation involving the mitogen-activated protein kinase-kinase (MAPKK, MEK) and ending in the activation of the extracellular signal regulated protein kinase (ERK) (Kosako *et al.,* 1993). Activated ERK can activate diverse transcription factors by translocating to the nucleus. In addition to this, ERK can phosphorylate various cytoplasmatic and membrane linked substrates (Karin and Hunter, 1995).

FGF activation of MAPK pathway stimulates many intracellular processes in ES and somatic cells, predominantly MAPK controls metabolic processes, cell cycle, cell migration and cell shape (Schlessinger, 2000) as well as proliferation and differentiation (Marshall, 1995; Armstrong *et al.,* 2006). In order to regulate the wide range of functions of MAPK, positive and negative feedback loops operate in this pathway. *Spry* and *Sef* are target genes of FGF and both are negative regulators of MAPK. Sprouty (*Spry*) proteins antagonise the MAP kinase pathway by interfering at GRB2 or RAF levels (Yusoff *et al.,* 2002; Sasaki *et al.,* 2003). On the other hand, SEF acts at receptor level, since ectopic SEF expression blocks the phosporlation of FRS2 (Kovalenko *et al.,* 2003).

1.6.1.2 Phosphoinositide 3- Kinase (PI3K) /AKT pathway

Downstream of the FGFR, PI3K is indirectly phosphorylated by activated docking proteins FRS2α, SHC or SHP2. FRS2α and GAB1 combine via GRB2 resulting in the

tyrosine phosphorylation of GAB1 which in turn activates the PI3K/AKT pathway (Ong *et al.,* 2001).

AKT or protein kinase B (PKB) functions as an anti-apoptotic enzyme by inhibiting different pro-death proteins such as caspase-9, BAD and FAS ligand. In addition to this, AKT has a positive role in proliferation by influencing the balance of the cell cycle by the inhibition of cyclin D1 kinase glycogen synthase kinase-3beta (GSK3β). This prevents cyclin D1 degradation, resulting in a more rapid G1/S transition. AKT can also negatively regulate the expression of cyclin inhibitors with similar results. Dysregulation of any of these functions is the cause of many human cancers (Vivanco and Sawyers, 2002).

Components of the PI3K pathways have been shown to be enriched in hES cells, indicating a connection of this pathway with proliferation and maintenance of pluripotency in hES cells (Armstrong *et al.,* 2006) and monkey cells (Watanabe *et al.,* 2006). This was also confirmed by the down regulation of AKT and PDK1 phosphorylated in the differentiation of hES cell to EBs (Armstrong *et al.,* 2006). Armstrong *et al.,* (2006) proposed that MAPK and NFκβ were down stream of PI3K, collaborating in hES cells self-renewal. In contrast with hES cells, FGF activation of PI3K/AKT pathway is required for early epithelial differentiation of mES cells (Chen *et al.,* 2000). Conversely, activation of PI3K by gp130, in the presence of LIF is required for mES cell self-renewal (Paling *et al.,* 2004).

1.6.2 Direct activation of SRC, PLCγ, and CRK

Primary embryonic cells lacking the FRS2 binding site on the FGFR1 are still able to activate MAPK in response to FGF, indicating the presence of other effectors for FGFR1 signal (Hoch and Soriano, 2006).

1.6.2.1 SRC pathway

The SRC family of non-receptor tyrosine kinases are proteins broadly expressed which includes SRC, FYN and YES. SRC proteins contain a myristyl anchor to the membrane, followed by a specific sequence that differs between SRC family members, SH3 and SH2 domains and a catalytic protein tyrosine kinase (PTK) domain (Thomas and Brugge, 1997). SRC kinases are activated by growth factor receptor stimulation of cell surface receptors and by integrins (Schlessinger, 2000). Once activated, Src initiates a signal transduction cascade involving the adaptor protein SHC, which culminates in the transcriptional activation of the transcription factor MYC (Courtneidge, 2002). It has been shown that activation of SRC kinases by FGFs is involved in migration and proliferation of fibroblasts (Kilkenny *et al.,* 2003). In addition to this, SRC has been connected to FGF regulated adhesion, survival and the production of autocrine growth factors (Courtneidge, 2002). Interestingly, it has been reported that this family of kinases and in particular cYES, could be a common pathway for human and mouse ES cells in preventing differentiation (Anneren *et al.,* 2004).

1.6.2.2 Phosphoinositide-specific phospholipase C gamma (PLCγ) pathway

Phosphoinositide-specific phospholipase C gamma (PLCγ) is a phosphoprotein found associated through the SH2 domain to a specific phosphotyrosine (766) of the FGFR (Mohammadi *et al.,* 1991). Once activated, PLCγ is recruited to the cell membrane by the binding of its Pleckstrin homology (PH) domain to the phosphoinositide 4,5 biphophate (PtdIns4, 5) (PIP_2), which is a product of PI-3 kinase activation. Hydrolysis of PIP_2 by PLCγ generates inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to specific intracellular receptors that result in a calcium release from intracellular stores. Calcium then binds to calmodulin, which in turn activates a family of calcium/calmodulin-dependent kinases. Moreover, both, DAG and calcium activate protein kinase C (PKC), stimulating a variety of intracellular responses. Activation of PLCγ could be connected to cytoskeletal alteration, although it seems that it is not involved in FGF-induced proliferation, differentiation or cell mortality (Schlessinger, 2000).

1.6.2.3 CRK-mediated signalling

CRK is an SH2/SH3 containing adaptor protein able to form stable complexes with FGFR1 by tyrosine phosphorylation (463). CRK is critical for propagating FGF2 induced endothelial cell proliferation possibly by CRK activation of MAP kinase (Larsson *et al.,* 1999). However, this could be a cell specific effect as tyrosine 463 has not an effect in mitogenesis of fibroblasts (Mohammadi *et al.,* 1996).

1.7 Project objectives

The aim of this thesis was to determine and compare the role of FGF signalling in the regulation of human and mouse ES cells. The specific objectives were:

1. To establish FGF activation of mouse ES cells

2. To determine the effect of FGF signalling on mES cell self-renewal

3. To develop a serum and feeder free culture system in which to study the effects of specific factors

4. To characterise the effect of FGF2 on hES cell self-renewal and proliferation

5. To clarify the role of the principal signalling pathways downstream of FGFR, on hES cell self-renewal

6. To identify other factors in collaboration with FGF2 involved in hES cell self-renewal.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 General Materials

All cells were maintained under sterile conditions in a humidified incubator in a 5% CO_2 atmosphere at 37^{o}C. Centrifugation of the tubes was performed in a BIOFUGE primo R, Heraeus. Plates were from Costar Corning, centrifuge tubes from Fisher, filter units from Millipore, Pipettes from Merck, aspirating pipettes from Becton Dickinson, filter cap flasks (human) from Nunc and flasks for mouse ES and fibroblast culture from Corning. All the cells were viewed and the photos taken at x10 and x4 magnification.

2.1.2 Culture of Murine Embryonic Stem (mES) Cells

2.1.2.1 Solutions and Media

Phosphate Buffered Saline PBS (Oxoid/Unipath)

0.16M NaCl, 0.003M KCl, 0.008M disodium hydrogen orthophosphate (Na2HPO4), 0.001M potassium dihydrogen orthophosphate (KH2PO4)). Supplied in tablet form, it was reconstituted in distilled water (dH_2O) and autoclaved prior use.

L-Glutamine solution (GibcoBRL/Invitrogen; 200mM)

2mM. Aliquoted and stored at -20^{o}C

Non essential aminoacids (NEAA; GibcoBRL/Invitrogen; 10mM)

0.1mM. Aliquoted and stored at 4^{o}C

TVP (Trypsin Verene Phosphate)

1 ml Trypsin (2.5%) (Invitrogen), 0.0037g Na_2EDTA (Sigma), 1ml Chicken serum (Invitrogen) made up to 10 ml with PBS (Dulbecco). Filtered and stored at -20^{o}C.

Murine ES cell medium

Components	**Concentration**	**Supplier**
Glasgow's Minimum Essential Medium (GMEM)	95%	Sigma
Foetal Calf Serum (FCS)	5%	Globepharm
L-glutamine	2mM	Invitrogen (Gibco)
Sodium piruvate	1mM	Invitrogen (Gibco)
Non essential aminoacids	0.1mM	Invitrogen (Gibco)
β-mercaptoethanol	0.1mM	Invitrogen (Gibco)
Leukaemia Inhibitory Factor (LIF)	100U/ml	ESGRO; Chemicon

To the Glasgow's Minimum Essential Medium (GMEM; Sigma) was added the components specified above by filtering then with a 50ml syringe through a sterile Minisart 0.20μM filter unit (Sartorius). The final media was stored at 4^{o}C for a maximum period of 4 weeks since L-glutamine is converted to glutamic acid after 4 weeks, which is toxic to the cells.

Cryopresevation medium

Cryopreservation medium was prepared by filtering through a syringe, 60% mES cell medium, 20% FCS, 20% dimethyl sulfoxide (DMSO).

2.1.2.2 Cell lines

IOUD2 C2 cells (Dani *et al.*,1998) derived from E14TG2a, (Hooper *et al.*, 1987) carried an *Oct4* βgeo stem cell reporter. Y118F (Burdon *et al.*,1999b) cell line was a subclass of the cell line D027, which have both copies of the *Lif* gene inactivated by homologous recombination and additionally carry an IREs-βgeo reporter gene inserted within one allele of the *Oct4* gene (Dani *et al.*,1998). The HPRT deficient cell line, HM1 was provided by J.McWhir (Roslin Institute, Scotland). All the cell lines had been derived from strain 129 mouse blastocysts. The passage number of IOUD2, Y118F and HM1 cells ranged from 16 to 40.

2.1.2.3 Murine ES cell maintenance

All plastic ware was coated with gelatine (Sigma) at least 5 minutes before use. Culture medium was replaced daily and cells were passaged when they reached 80-90% confluency, usually every 2 days. The vented caps were left loose to ensure good circulation of CO_2 in the flask.

2.1.2.4 Passage of mES Cells

A medium change was carried out 1-2 hours prior to passaging to prime the cells for the procedure. The cells were washed with sterile PBS, before adding the TVP solution (2ml to a 25cm^2 flask). The flask was placed in an incubator 37^{o}C for 2 minutes for the trypsin to break intercellular protein bonds between the cells. To loosen cells and break the cell aggregates to a single cell suspension, the flask was tapped. The TVP was inactivated by the addition of 5ml of ES cell medium to the flask. The cells were collected from the flask to a 15ml tube and centrifuged at 166g (1000rpm) for 5 minutes. The medium with the TVP was removed from the pellet, followed by the re-suspension and mixed by pipetting of the cells in fresh medium.

2.1.2.5 Count of mES Cells

When cell numbers needed to be calculated, a haemocytometer (Assisten) was used. Cells were processed as above and mixed well in 10ml of medium. 10µl of the cell suspension were dropped under either side of the haemocytometer coverslip. 2 of the square grids, consisting of 25 squares and whose area is 1cm^3 each were counted and the mean taken of the 2 counts. This count multiplied by 10^4 represented the number of cells present in 1ml of the cell suspension.

2.1.2.6 Freeze of mES Cells

To freeze flasks of cells in vials, a medium change was carried out on the flask 1-2 hours prior to freezing to induce growth in the cells. The cells were trypsinised in the conventional manner and centrifuged to form a pellet. The cells were re-suspended in 50% mES cell medium and 50% freezing mix and placed in pre-chilled cryovials. From a $25cm^2$ flask, 4 cryovials were obtained (1ml per tube). The vials were placed at -80^oC overnight and then transferred to -150^oC for long-term storage.

2.1.2.7 Thaw of mES Cells

ES cells kept frozen require rapid thawing as they are sensitive once thawed to the cryopreservant DMSO, present in the freezing mix. A vial was removed from a -150^oC freezer and rapidly immersed at 37^oC water. Once thawed the content of the vial was mixed with medium in a 15ml tube and centrifuged at 166g (1000rpm) for 5 minutes. The medium was aspirated off the tube and 5ml of fresh medium added in order to re-suspend the pellet before being added to a gelatinised flask.

2.1.3 Culture of Human ES cells

2.1.3.1 Solutions and Media

PBS/EDTA EDTA 0.5mM EDTA (Ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid) in Ca2+/Mg+ free phosphate-buffered saline (PBS)

Collagenase IV solution (GibcoBRL/Invitrogen; 200units/ml=1mg/ml).

This dissociation solution was prepared by dissolving 20,000 units of collagenase IV in 100ml Knockout ^{TM} DMEM. All the components were added to a 250 ml filter unit (0.22µM, Corning, cellulose acetate, low protein-binding) and filtered. This was aliquoted and stored at -20^{o}C until used.

Growth factor-reduced Matrigel ® (Becton Dickinson)

Matrigel was required to be thawed at 4^{o}C for at least 2 hours to avoid polymerisation, and then diluted 1:2 in cold KO-DMEM . Whilst keeping the mixture on ice it was mixed and aliquoted into pre-chilled tubes for storage at -20^{o}C.

Human basic fibroblast growth factor, recombinant (FGF2) (GibcoBRL/Invitrogen; 10µg/ml)

10 µg of FGF2 were dissolved in 1ml PBS with 0.2% BSA (Fraction V, Sigma). The solution was filtered using a 0.22µM, Corning, cellulose acetate, low protein-binding filter. The stocks were stored at -20^{o}C and when thawed they were kept at 4^{o}C for up to 1 month.

MEF Medium

Components	**Concentration**	**Supplier**
Dulbecco's modified Eagle's medium (DMEM)	90%	Invitrogen (Gibco)
Foetal Calf Serum (FCS)	10%	Globepharm
L-glutamine	2mM	Invitrogen (Gibco)
Non essential aminoacids	0.1mM	Invitrogen (Gibco)

MEF medium was prepared by filtering in a 500 ml filter unit (0.22 μm, Corning, cellulose acetate, low protein binding) the components above described. The medium was then stored at 4º C for up to one month.

Human ES medium

Components	Concentration	Supplier
Knockout ^{TM} Dulbecco's modified Eagle's medium (KO-DMEM)	80%	Invitrogen (Gibco)
Knockout ^{TM} Serum Replacement (KO-SR)	20%	Invitrogen (Gibco)
β-mercaptoethanol	0.1mM	Invitrogen (Gibco)
L-glutamine	2mM	Invitrogen (Gibco)
Non essential aminoacids	0.1mM	Invitrogen (Gibco)

Human ES cells was prepared by filtering in a 500 ml filter unit (0.22 μm, Corning, cellulose acetate, low protein binding) the components above described.

Conditioned Medium

Conditioned medium (CM) was collected from the feeder flasks the day after human ES medium, supplemented with 4ng/ml FGF2 was added to flasks of MEFs. For an illustration see Figure 2.1. Collected CM was either stored at –20º C or filtered and supplemented with 8 ng/ml of FGF2 to be used for daily maintenance of human ES cultures.

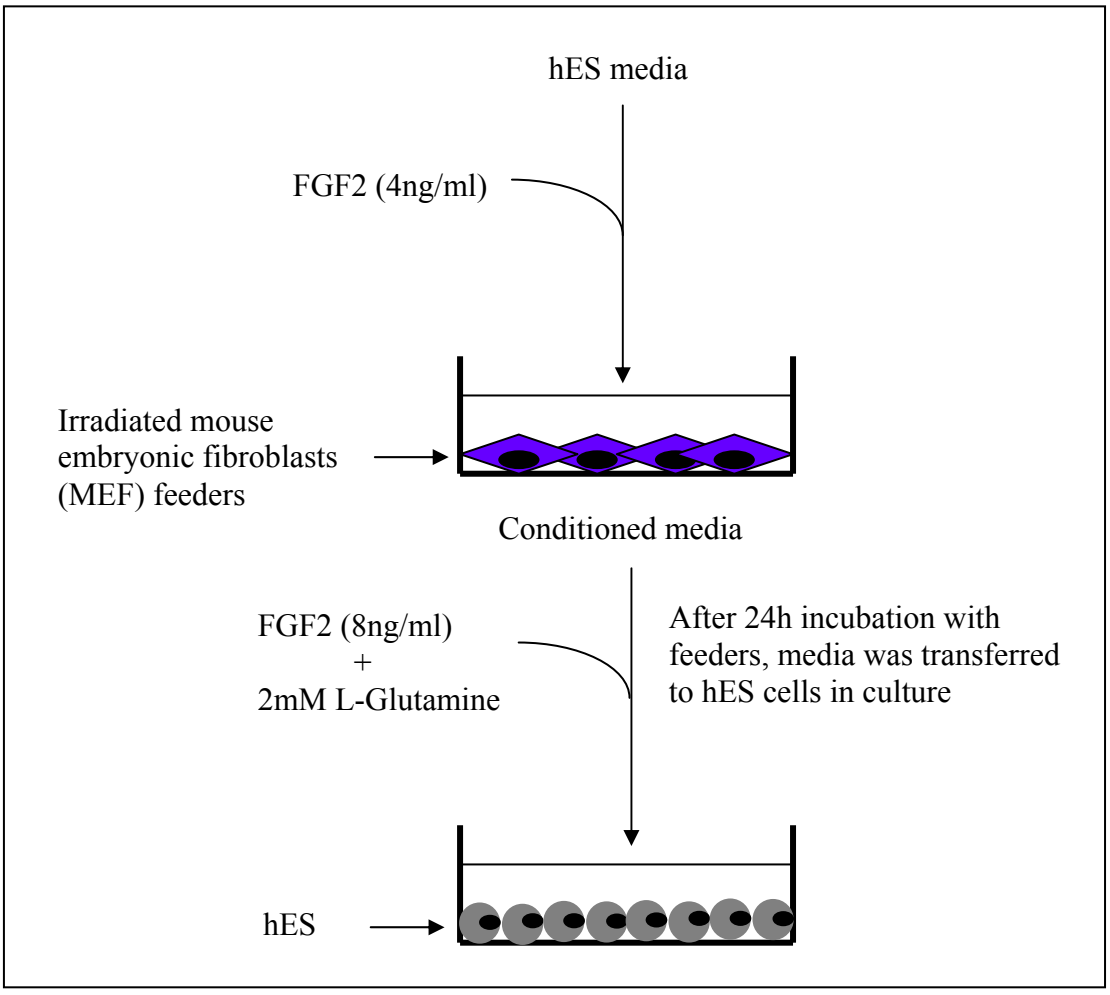


Figure 2.1 Production of Conditioned medium by mouse embryonic fibroblasts (MEFs) and culture of hES cells. See text for details.

Serum free Medium

Components DMEM/F12-N2 medium	**Concentration**	**Supplier**
DMEM/F12	100%	Invitrogen (Gibco)
Insulin	25µg/ml	Sigma
Transferrin	100µg/ml	Sigma
Progesterone	6ng/ml	Sigma
Putrescine	16µg/ml	Sigma
Sodium Selenite	30nM	Sigma
Bovine Serum Albumin (BSA)	50µg/ml	Invitrogen (Gibco)

Components Neurobasal/B27 medium	**Concentration**	**Supplier**
NeurobasalTMMedium	98%	Invitrogen (Gibco)
B27	2%	Invitrogen (Gibco)
L-Glutamine	2mM	Invitrogen (Gibco)

The DMEM/F12-N2 medium was 50:50 mixed with Neurobasal/B27 medium by filtration producing the serum free N2B27 medium.

Cryopresevation medium

Human cryopreservation medium was prepared by filtering in a 500 ml filter unit (0.22 µm, Corning, cellulose acetate, low protein binding), 3 parts of KO-Serum replacement, 1 part of DMSO and 1 part of medium (CM or N2B27).

Differentiation medium

Human differentiation medium was prepared by filtering in a 500 ml filter unit (0.22 μm, Corning, cellulose acetate, low protein binding) 400ml KO-DMEM and 20% FCS.

2.1.3.2 Cell lines

H1 and H9 cell lines were obtained from Geron (San Francisco, USA). T5 (Gerrard *et al.,* 2006) was an *OCT4*-EGFP transgenic cell line whereby a plasmid containing a selectable marker and the EGFP reporter under control of the OCT4 promoter was introduced into H1 cell line to generate clonal cells lines and was obtained from Dr Wei Cui (Roslin Institute, Scotland). The passage number of H1 and T5 cells used in this thesis ranged between 41 and 65. Mouse Embryonic fibroblasts (MEFs) were generated in the lab from 13 days post coitum mouse embryos following a protocol from Geron.

2.1.3.3 Isolation of primary MEFs

Pregnant female mice were killed on the 13-14 day of pregnancy. The abdomens were swabbed with 70% ethanol and the abdominal cavities were dissected to expose the uterine horns. Uterine horns were then placed into a 10cm bacteriological Petri dish containing 10ml PBS supplemented with 0.25ml penicillin (5000 U/ml) / streptomycin (5000 μg/ml; Gibco-BRL); (PBS+ P/S). The embryos were released into the saline while placenta, heads, membrane and soft tissues removed from the embryos and these passed to a fresh dish containing PBS. The embryos were washed in two further

changes of PBS P/S and placed individually into bijou tubes containing 2ml of TVP. Tubes were incubated for 10 minutes at 37ºC and the content was vortexed, incubated for a further 10 minutes at 37ºC and vortexed one more time. 3 ml of MEF medium was added to inactivate the trypsin, the tubes were vortexed for a few seconds and individual supernatants containing the cells were transferred into 75 cm^2 flasks containing 12 ml MEF medium. Flasks were incubated at 37ºC and the medium was changed the following day to remove the cellular debris. The cells were split at 1:2 when cells were 80-85% confluent and when this confluency was reached again the MEFs were used as feeders. MEFs could be expanded for up to 5 passages before being discarded.

2.1.3.4 Irradiation of MEFs

MEFs were harvested in a similar way to mES cells and placed as a single cell suspension in a 50ml tube. The cell containing tubes were irradiated at 40-80 Gy in a Gamma cell irradiator, in order to stop the MESs cell division whilst still alive. The cells were centrifuged, re-supended and plated in a previously gelatinised flask at 56,000cells/cm^2 for cultures to be used for CM. Irradiated MEFs were used up to 7 days for CM.

2.1.3.5 Matrigel Coating for Human ES Cultures

Matrigel was thawed at 4ºC for at least 2 hours to avoid polymerisation, and then diluted 1:15 of 1:50 in cold KO-DMEM using a chilled pipette to obtain a final dilution of 1:30

or 1:100 depending on the use with CM or N2B27 respectively. Typically 25 cm^2 flasks were coated with 3ml of the solution. Flasks were either incubated at RT for 1-2 hours before use or stored at 4ºC for no longer than 2 weeks. The Matrigel solution was removed from the flasks before use.

2.1.3.6 Passage of hES cells

For both conditions CM or N2B27 the cells were passaged when they reached 80-90 % confluence.

CM was removed and cells were washed once with KO-DMEM. Typically, 1ml of 200 units/ml collagenase IV per well of 6 well plate was added to incubate at 37 ºC for 5-10 minutes. The collagenase was aspirated and gently the cells were washed with KO-DMEM. The cells were detached from the surface by scraping and dissociated into clusters (50-500 cells) by gentle pipetting. The Matrigel (1:30) was removed from the plates and the cells in CM supplemented with 8 ng/ml FGF2 seeded into the Matrigel coated wells. In this system, the hES cells were maintained in a high density with an optimal split ratio of 1:3.

N2B27 was removed and cells were washed once with PBS. Typically, 2ml of PBS/EDTA per well of 6 well plates was added to incubate at 37 ºC for 2-3 minutes. The PBS/EDTA was aspirated and gently the plate was tapped and fresh medium poured in order to loosen the cells. In this system, the hES cells were able to be seeded at low

densities in small clusters of less than 50 cells, with a typical split ratio of 1:6. The Matrigel (1:100) was removed from the plates and the cells in N2B27 supplemented with 8 ng/ml of FGF2 seeded onto the Matrigel coated wells.

2.1.3.7 Freeze of hES cells

The cells were harvested as above with the only variation being leaving larger clusters of cells, when mixed with the medium, to facilitate their thaw. The cells were centrifuged and the pellet gently re-suspended in 50% of their usual medium of growth and 50% freezing mix and placed in pre-chilled cryovials. The vials were placed at -80^{o}C overnight and then transferred to -150^{o}C for long-term storage.

2.1.3.7 Thaw of hES cells

The procedure was comparable to the mES thaw. In the case of hES cells the gentleness should be increased. In the case of thawing cells in CM, two weeks in culture were needed before cells could be expanded.

2.1.3.8 Cell count

Human ES cells harvested in PBS/EDTA were counted in a Nucleocounter^{TM} (Chemometec). In order to count the total number of cells in a suspension, a sample of the suspension (100µl) was first treated with lysis buffer (100µl) and then stabilising buffer (100µl). The lysis buffer disaggregated the clusters of cells and permeated the

plasma membrane allowing the nuclei to be stained with propidium iodide, which is coated on the inside of the NucleoCassette™. The stabilising buffer increases the efficiency of nuclear staining by raising the pH of the mixture. A sample of the mixture was loaded into the NucleoCassette™ and this placed into the Nucleocounter that would give the number of viable cells in an optimal range of 10^5-$2x10^6$ cells/ml, which should be multiplied by the multiplication factor (x3) to obtain the number of cells/ml.

2.1.3.9 Formation of Embyroid Bodies

The cells were harvested using collagenase as the cells should be collected in clumps. 2 ml per well (into 6 well plate) of differentiation medium was added. The cells were scraped and transferred to one well of low attachment plate (1:1 split).

2.2 RNA Methods

2.2.1 Extraction and Analysis of RNA

2.2.1.1 RNA Isolation from cells

All equipment was autoclaved twice or thoroughly cleaned with RNase Erase (Q Biogen) before use. Cells grown in monolayer were lysed directly in the culture dish by the addition of RN-Bee (ams biotechnology) (1ml of reagent for 10cm^2) passing the cell lysate several times through a pipette. 200µl of chloroform were added to the homogenised material, shaked vigorously for 15-30 seconds and incubated on ice for 5 minutes. The samples were centrifuged at 12000g for 15 minutes at 4^{o}C. After centrifugation the upper aqueous phase, containing the RNA was transferred to a new tube, being careful to avoid protein and DNA contamination by accidental carry over of the interphase. For RNA precipitation, 500µl of cold isopropanol was added to the sample, which was well mixed and incubated for 5-10 minutes at room temperature. The sample was then centrifuged (12000g; 5 minutes; 4^{o}C), the supernatant removed and the pellet washed with 75% ethanol. The washed pellet was centrifuged (7500g; 5minutes; 4^{o}C) and dried by incubating in a heat block for 2 minutes at 45^{o}C. To improve the solubilisation, the pellet was mixed in nuclease free water (Ambion) and incubated for 10-15 minutes at 55-60^{o}C. To avoid degradation in long-term storage, RNA was stored as an ethanol precipitate. To solubilised RNA 1/10 volumes of 3M sodium acetate (NaOAc) were added to two and a half volumes of absolute ethanol before mixing and storage at -20^{o}C.

2.2.1.2 Quantitation and assessment of RNA quality

To assess the quality of RNA it was used electrophoresis of total RNA and then ethidium bromide staining. This relies on the fact that ribosomal RNA (rRNA) quality and quantity reflect that of the underlying messenger RNA (mRNA). rRNA makes >80% of total RNA samples with the majority of that comprised by the 28S and 18S rRNA species. Therefore, two bands 5 and 2Kb corresponding to 28S and 18S are indicative of intact RNA. The spectophotometric measurement of the amount of ultraviolet irradiation absorbed by the bases was used to measure the amount of RNA present in the samples. In addition to this, the more sensitive method Agilent bioanlyser was used in combination with the RNA 6000 Nano LabChip®kit (Agilent Technologies). This system uses a combination of microfluidics, capillary electrophoresis and fluorescence to evaluate both RNA concentration and integrity.

2.2.1.3 Complementary DNA (cDNA) Synthesis

Reverse transcriptase is a RNA-dependent DNA polymerase, which is encoded by retrovirus. Their viral function is to copy the viral RNA genome into DNA prior to its integration into host cells. This can be exploited to allow production of DNA (cDNA) from any RNA template and is known as reverse transcription PCR. After RNA was precipitated, quantitated and diluted in nuclease free water to a final concentration of 5µg of RNA in 8µl, the RNA was denatured (65^{o}C; 10 minutes). Two systems were used to synthesising DNA from RNA and the cDNA synthesised was used as a template in the Polymerase Chain Reaction (PCR).

MMLV Reverse Transcriptase (Epicentre Biotechnologies)

MMLV (Moloney Murine Leukemia Virus) is an RNA-dependent DNA polymerase that synthesises the complementary DNA (cDNA) first strand from a single-stranded RNA template to which a prime has been hybridised. MLV-RT will also extend primers hybridised to single stranded DNA. Second strand cDNA synthesis can be achieved from some mRNA templates without an additional DNA polymerase.

The following reagents were added to a microcentrifuge tube at room temperature containing 25µl of 2.5 µg of RNA.

- 12 µl RNase free H2O
- 5 µl 10x MMLV RT buffer
- 5 µl 0.1 M DTT
- 1 µl dNTP mix (2.5 µM each)
- 1 µl Oligo (dT; 10pM)
- 1 µl (10 U) MMLV RT (added the last)

The reaction was incubated at 37^{o}C for 60 minutes

First Strand cDNA Synthesis kit (Amersham)

The cDNA synthesis was also catalysed by the M-MuLV reverse transcriptase. The preassembled bulk firs-strand cDNA reaction mixes required only the addition of DTT, RNA and the primer Not I-d$(T)_{18}$. The RNA sample was placed in a microcentrifuge tube and RNAse-free water added to bring the RNA to the appropriate volume, 5µg in 5µl. The RNA solutions were heated at 65^{o}C for 10 minutes, and then chilled on ice.

Gently pipette the bulk first-strand cDNA reaction mix to obtain a uniform suspension. The appropriate volume of the bulk first-strand cDNA reaction mix (5µl) was added to a sterile 0.5ml microcentrifuge tube. To this tube was added 1 µl of DTT solution, 1 µl of the primer at 1:25 dilution and the heat denatured RNA. After mixing, the tubes were incubated at 37^{o}C for 1 hour followed by 10 minutes at 90^{o}C in order to inactivate the enzyme. The cDNA was aliquoted and stored at -20^{o}C.

2.3 DNA Methods

2.3.1 Solutions

10x TBE buffer

0.89M Tris-HCL, 0.89M borate and 0.02M EDTA, pH 8.0

Genomic DNA resuspension solution

400mM Tris-HCL (pH 8.0), 60mM EDTA, 150mM NaCl

Type III loading buffer (6x)

0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol

2.3.2 Polymerase Chain Reaction (PCR)

PCR was performed on the cDNA produced from the reverse transcription of mRNA. This method allowed the enzymatic synthesis of specific segments of DNA using two

oligonucleotide primers that hybridise to opposite strands and flank the region of interest in the target DNA. All the primers (Table 2.1) were kindly supplied by Dr Lesley Forrester from the Queens Medical Research Institute, University of Edinburgh. The primers were design spanning an intron to avoid co-amplification of genomic DNA. A repetitive series of cycles, involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. Because the primer extension products synthesised in one cycle serve as a template in the next, the number of target DNA copies approximately doubles at every cycle (Erlich, 1989).

The system used in this report was the HotStarTaq Master Mix Kit (Qiagen). HotStart DNA polymerase is activated by a 15 minute 95^{o}C incubation step, which was incorporated into the routine thermal cycling programs. The HotStartTaq Master Mix contains HotStarTaq DNA Polymerase, PCR Buffer (with 4mM MgCl2), and 400 μM each dNTP. The PCR reaction mix was prepared by adding 10μl HotStartTaq master mix, 1 μl of each primer (at a final concentration 0.2 μM) and 6 μl of RNAse free H2O to 2 μl of the DNA template. The PCR products were then run on an agarose gel, as outlined in section 2.3.3.

2.3.3 Agarose Gel Electrophoresis

Agarose gels were prepared by dissolving agarose (Sigma) at 0.8-1.5% in 1xTBE by boiling in a microwave and allowed to cool down. Ethidium bromide (Sigma) was added to a final concentration of 0.5 μg/ml before pouring the gel into a horizontal electrophoresis gel kit (RunOne^{TM} System, Embi Tec) and inserting the gel combs. Once set, the gel was submerged in 1X TBE buffer in a gel tank and the DNA samples, containing 20% DNA loading buffer, were loaded into the wells. One well was reserved for loading molecular weight markers (1 Kb plus DNA ladder; Invitrogen). Electrophoresis was carried out either for 2 hours (80-120 Volts) or overnight (15-30 Volts). DNA was visualised by illumination on a long wave UV light box and photographed. The size of the DNA fragments were examined by a comparison of their mobility to that of restriction fragments of known size, typically DNA Hyperladder 100 lanes (Bioline).

2.3.4 Reverse Transcription (RT-PCR) Analysis

RT-PCR reactions were carried out as the PCR reactions. To ensure no contamination with genomic DNA, a "no-RT" control was always included. To normalise the concentration of template used in subsequent PCR reactions, a PCR reaction of a housekeeping gene, typically Clathrin was also included. A band of the correct size was expected whe the cDNA was synthesised with RT, whereas a pure cDNA sample shoul not show any bands in absence of RT.

Gene	Primer sequences	Anneal. T^a ^oC	Product (bp)	Cycles
Oct4	5'-GGCGTTCTCTTTGGAAAGGTGTTC-3' 5'-CTCGAACCACATCCTTCTCT-3'	58	320	20
Nanog	5'-ATGCCTGCAGTTTTTCATCC-3' 5'-GAGGCAGGTCTTCAGAGGAA-3'	58	110	30
Rex1	5'-TGGCTTCCCTGACAGATACC-3' 5'-CCTTCGAACGTGCACTGATA-3'	58	490	30
Akp	5'-GCACCTGCCTTACCAACTCT-3' 5'-TTTCAGGGCATTTTTCAAGG-3'	57	110	28
Gata6	5'-GCAATGCATGCGGTCTCTAC-3' 5'-CTCTTGGTAGCACCAGCTCA-3'	59	570	35
Afp	5'-ATGTATGCCCCAGCCATTCTGTCC-3' 5'-GAGATAAGCCTTCAGGTTTGACGC-3'	55	470	30
E-cadherin	5'-CGTGATGAAGGTCTCAGCC-3' 5'-ATGGGGGCTTCATTCAC-3'	60	610	30
Pax6	5'-TGCCCTTCCATCTTTGCTTG-3' 5'-TCTGCCCGTTCAACATCCTTAG-3	55	190	30
Brachyury	5'-TGCTGCCTGTGAGTCATAAC-3' 5'-TCCAGGTGCTATATATTGCC-3'	57	120	28
Fgf5	5'-ACCCTTTGAGCTTTCTACCC-3' 5'-CCGTCTGTGGTTTCTGTTGAGG-3'	58	188	32

Table 2.1. PCR primers used and their conditions.

2.4 Protein methods

2.4.1 Solutions

SDS loading buffer

10% Glycerol, 3% SDS, 62.5mM TrisHCL pH 6.8, 0.005% Bromophenol blue, 3% β-mercaptoethanol

4x lysis buffer

To make 100ml: 40ml glycerol, 12gr SDS, 25ml 1MTris-HCl (pH 6.8), 0.04% bromophenol blue, 20ml β-mercaptoethanol.

RIPA lysis buffer

10mM Tri-HCl at pH 7.4, 1mM EDTA, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.1% Sodium Deoxycholate

Wash buffer Tris-buffered saline-Tween (TBS-T)

140mM NaCl, 20mMTris-HCL at pH 7.6, 0.1% Tween20

Running buffer (5x)

30grTris, 188gGlycine, 10gSDS (sodium dodecyl sulphate) to make 2 litres with dH2O

Transfer buffer (10x)

29.3g Glycine, 58g Tris, 18.8 ml 20% SDS to make 1 litre. To prepare 1X solution used 100ml of 10x, 700ml dH2O and 200ml Ethanol 98%

Stripping buffer

62.5 mM Tris-HCl at pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol

2.4.2 Western Blotting

Western Blotting or Immunoblot is a sensitive technique for detecting specific protein molecules in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate denatured proteins by mass. These proteins are then transferred (blotted) out of the gel onto a membrane (nitrocellulose or nylon sheet), where they are "probed" using antibodies specific to the protein of interest. The membrane is then incubated with the specific antibody for the particular protein, followed by the washing away of any

unbound antibody. Those proteins in the gel that bind the antibody are detected either by autoradiography (if the specific antibody was radiolabeled) or by using a second labelled antibody that binds to the primary antibody (Hames and Hooper, 2000).

2.4.2.1 Cell lysis, protein isolation and gel electrophoresis

Following the pertinent treatment, the cells were washed once with ice-cold PBS and lysed in 100 µl (per 100mm dish) 1x SDS sample buffer. The samples were homogenised by sonication and before being used, they were boiled for 5 minutes in order to unfold the proteins completely. 5-10µl aliquots of the lysate were taken and fractionated on a 10% SDS-polyacrylamide. The reducing agent β-mercaptoethanol breaks all the disulfide bonds and the strong anionic detergent SDS disrupts nearly all the non-covalent interactions in the protein, thereby unfolding the polypeptide chain. SDS binds to the amino acid residues, which gives the denatured protein a large net negative charge that is proportional to its mass (Hames and Hooper, 2000). The electrophoresis was carried out at 100V for about 3 hours, which ended with a gel of separated proteins on the basis of their mass.

2.4.2.2 Determination of protein concentration

When normalisation of the protein content was required, the RIPA lysis buffer was used because it is compatible with the colorimetric Pierce BCA (bicinchoninic acid) Protein Assay. The RIPA buffer enables the extraction of cytoplasmic, membrane and

particularly nuclear proteins. This buffer used in conjunction with the BCA assay permitted an accurate measurement of protein concentration in the samples.

Protease and phosphatase inhibitor cocktails (Sigma) were added to the RIPA Buffer before lysing of the cells. The cells were washed with cold PBS and scraped off in 100µl of RIPA buffer (per 100mm dish), whilst always maintaining the samples in ice. The samples were spun at 14,000 rpm for 5 minutes at 4^{o}C and the supernatant containing the solubilised proteins was transferred to a plate to determine the concentration of the protein. The BCA (bicinchronic acid) assay (see Figure 2.2) was carried out following Pierce indications: 10µl of lysate were mixed with 190µl of reagent B+A (BCA quit), incubated at 37^{o}C for 10-15 minutes and read at 562nm. Once the protein concentration was determined, all the different concentrations were adjusted using the RIPA buffer. Equal volumes of the SDS sample buffer were added to each sample prior to being stored at -20^{o}C or being boiled for use.

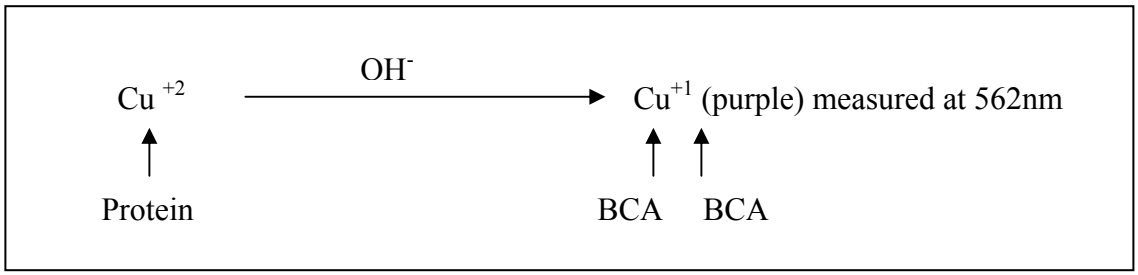


Figure 2.2. Protein estimation by BCA (bicinchoninic acid) protein assay. Proteins reduced Cu^{2+} to $Cu1^{+}$ in an alkaline medium. Two molecules of BCA reagent were chelated by one cuprous cation (Cu^{1+}) producing a purple product that exhibits a strong absorbance at 562nm that is nearly linear at increasing concentrations of protein (BCA protein assay kit, Pierce).

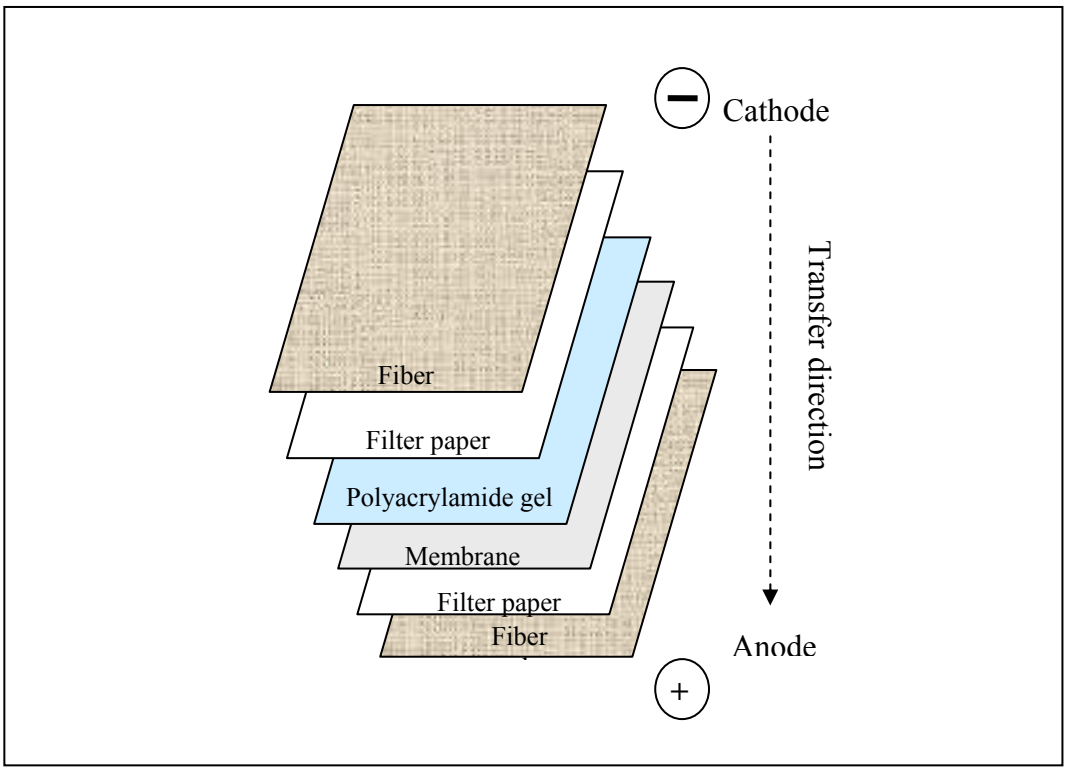


Figure 2.3. Blotting in a wet transfer unit. See text for details

2.4.2.3 Electrophoretic Transfer

In order to make the proteins accessible to antibody detection, they are passed from the gel onto a nitrocellulose membrane. Firstly the nitrocellulose membrane(s) were soaked in dH_2O for 5 minutes and equilibrated in transfer buffer for at least 10 minutes. For each gel, two fibre pads and two pre-cut Whatman 3MM filter papers saturated in transfer buffer were assembled on a cassette in the order shown in Figure 2.3. The cassette was inserted into the electrode module along with a stir bar and a Bio-Ice cooling unit (stored at -20°C) placed in the buffer tank and filled with transfer buffer. The buffer tank was placed on a magnetic stir plate and stirred at medium speed. The electrodes were attached and electrophoresed at 100 V for 90 minutes. When the transfer was finished the membrane was washed and incubated for 1 hour at room temperature with a blocking buffer (5% non-fat dried milk in TBS-T). The incubation with the protein (in this case casein) was carried out to prevent interactions between the membrane and the antibody used for detection of the target protein, since the membrane has a high ability to bind protein. This reduced "noise" and eliminated false positives.

2.4.2.4 Antibody detection of protein

The membranes were then rinsed with TBST and probed with the primary antibody, diluted in TBS-T, for 1 hour at room temperature on an orbital shaker. The membrane was washed with TBS-T, 3x15 minutes. This was followed by the incubation of the membrane with a secondary antibody at room temperature for 1 hour. This was a Hoseradish peroxidise (HRP) conjugated antibody with the capacity of recognising and

binding to the primary antibody. An illustration of the antibodies used during this thesis is shown in Table 2.2.

Antibody	Isotype	Working dilution	Supplier
Phospho-p44/42 Map Kinase (Thr202/Tyr204)	Rabbit IgG	1:1000	Cell Signalling
Phospho Stat3 (Tyr705)	Rabbit IgG	1:1000	Cell Signalling
Phospho-AKT (Thr308)	Rabbit IgG	1:1000	Cell Signalling
ERK2	Mouse IgG1	1:1000	BD Transduction
STAT3	Mouse IgG1	1:2500	BD Biosciences
SMAD2/3	Mouse IgG1	1:500	BD Biosciences
OCT4	Mouse IgG1	1:1000	Santa Cruz
SH-PTP2, SHP2	Rabbit IgG	1:500	Santa Cruz

Table 2.2. Antibodies used in Western Blotting. Rabbit IgG and Mouse IgG1 were purchased from Amersham Biosciences. Both secondary antibodies were used at 1:5000 dilutions.

2.4.2.5 Enzymatic Chemiluminescence (ECL) detection

Chemiluminescence is the emission of light as the result of a chemical reaction. The HRP catalyzes the conversion of Luminol into an excited intermediate, which emits light when it decays to ground label and can be detected by a short exposure to blue-light sensitive auto-radiography.

Following the incubation of the blots with HRP-coupled anti-rabbit IgG or anti mouse IgG1, the membranes were washed and the reaction prepared using Amersham ECL reagents used according to the directions provided by the supplier. After 1 minute of incubation with the reagents, the membranes were placed in an X-ray film cassette in a suitable detection pocket. A sheet of auto-radiography film (Hyperfilm ECL) was placed on top of the membrane and exposed for times varying from 30 seconds to 1 hour

to get an optimuml exposure. The light produced by this chemiluminescent reaction peaks after 5-20 minutes and decays slowly thereafter with a half life of approximately 60 minutes. The exposed film was developed in a Konica SRX-101A X-ograph machine.

2.4.2.6 Reprobing membranes

Membranes were washed with TBS-T and blocked in 5% non-fat dried milk in TBS-T for 1 hour at room temperature. The removal of primary and secondary antibodies from the membrane is possible by submerging the membrane in stiripping buffer for 30 minutes at 50^{o}C with occasional agitation. After washing of the membrane, the immunodetection protocol was repeated.

2.4.2.7 Quantification of Protein Concentration

To quantify the concentration of protein in relation to the total protein loaded, the Quantity One densitometry programme was used on the developed X-ray films of the blots, using a BIO-RAD Fluor-S^{TM} MultiImager.

2.5 Other Analytical Methods

2.5.1 Flow Cytometry

Flow cytometry is a method of quantitation of components or structural features of cells, by optical means. The cells may be alive or fixed at the time of measurement, but must be in single cell suspension as they are passed through a laser beam by continuous flow. Each cell scatters some of the laser light, and also emits fluorescent light excited by the laser. The cytometre measures up to five parameters for each cell: forward light scatter intensity (approximately proportional to cell diameter), side light scatter (which is approximately proportional to the quantity of granular structures within the cell) and three fluorescence intensities at several wavelengths (FL1 (emissions wavelength 515-545 nm), FL2 (564-606 nm), FL3 (650nm)) and the pulse height and width of each fluorescence parameter.

Forward size and side scatter plots were used to exclude dead cells and debris from the fluorescence data. At least 10,000 "live" cells were acquired from each sample. Fluorescent antibodies were used to report the densities of specific surface markers and intracellular components, thus distinguishing populations of differentiated cell types. Flow cytometry was performed on a 488 nm laser FACScan (Becton Dickinson). A 530/30nm band pass filter was used to measure EGFP fluorescent intensity or FITC-conjugated antibodies. A 585/42 nm band pass filter was used to measure phycoerythrin-conjugated antibodies. For cell surface marker analysis, detector settings

were adjusted with both untreated and isotype controls. Analysis of the data was performed on CellQuesPro software (BD Biosciences).

2.5.1.1 Evaluation of Cell-Surface Markers

Flow cytometry was used to determine the expression of protein on the surface of a cell as a marker of the self-renewal status of the cells. An antibody was incubated with the cells and a secondary carrying a fluorescent dye was attached to the first antibody. The amount of cell surface marker was proportional to the level of fluorescence.

After harvesting, the clumps of cells were dissociated by pipetting or using TVP. They were washed with FACS PBS (PBS, 0.1% BSA fraction V, 0.1% NaN_3). $5x10^6$-$2x10^7$ cells/ml was re-suspended in FACS-PBS and 50µl aliquoted per sample tube. The primary or isotype control antibody was added to the FACS tubes (Greiner) and incubated for 30-40 minutes at 4^oC in the dark. The primary antibody was washed on FACS PBS by spinning at 250g for 5 minutes and discarding the supernatant. The pellet was re-suspended in 50µl, and the secondary antibody added to a final dilution of 1:100. After 20-30 minutes incubation at 4^oC in the dark the antibody was washed. 300-500µl FACS PBS was added for the acquisition of the data by flow cytometry. A list of the antibodies used in flow cytometry during this thesis is shown in Table 2.3.

Antibody	Working dilution	Supplier	Isotype	Working dilution	Supplier
SSEA1	1:50	Santa Cruz	Mouse IgM	1/100	Jackson
SSEA3	1:50	Santa Cruz	Rat IgM	1/100	Sigma
SSEA4	1:100	Santa Cruz	Mouse IgG_3	1/100	Sigma
TRA1-81	1:25	Chemicon	MouseIgM	1/100	Jackson

Table 2.3. Antibodies used in Flow cytometry.

2.5.1.2 Cell Cycle Analysis by Propidium Iodide (PI) Staining

Propidium Iodide is a typical cell cycle stain, because it can pass through a permeabilised membrane and intercalate into cellular DNA. The intensity of the PI signal is then directly proportional to DNA content. One important aspect of the DNA analysis is the ability of exclusion of dead/apoptotic cells and cell clumps. The histogram pulse width versus pulse area was used to exclude cell clumps and dead cells. The selection of live cells and the cell cycle analysis was done with FlowJo software from Treestar. Once the single cell population was identified, the percentage of cells in G1, S phase and G2/M can be estimated by the software. Quiescent and G1 cells will have one copy of DNA and will therefore have 1X fluorescence intensity. Cells in G2/M phase of the cell cycle will have two copies of DNA and accordingly will have 2X intensity. Since the cells in S phase are synthesising DNA, they will have fluorescent values between the 1X and 2X populations. Histograms of the PI staining of the whole population is shown in figure 2.3 A, and a histogram produced after exclusion of dead and the agregates is shown in figure 2.3 B.

The samples containing 0.5 to $1x10^6$ were centrifuged at 400g for 5 minutes and the supernatant discarded. The pellet was fixed overnight in 0.3ml 50% FCS in FACS PBS and 0.9 ml cold 70% ethanol. The fix was washed off and the pellet re-suspended in 0.3 ml PI solution (50μg/ml PI + 100 μg/ml RNAse in FACS PBS). The cells were stained for 1 hour at room temperature in the dark and stored in ice until analysis. To avoid cell aggregates that could lead to a mistake in the analysis, the cells were harvested using TVP in order to produce single cell suspension.

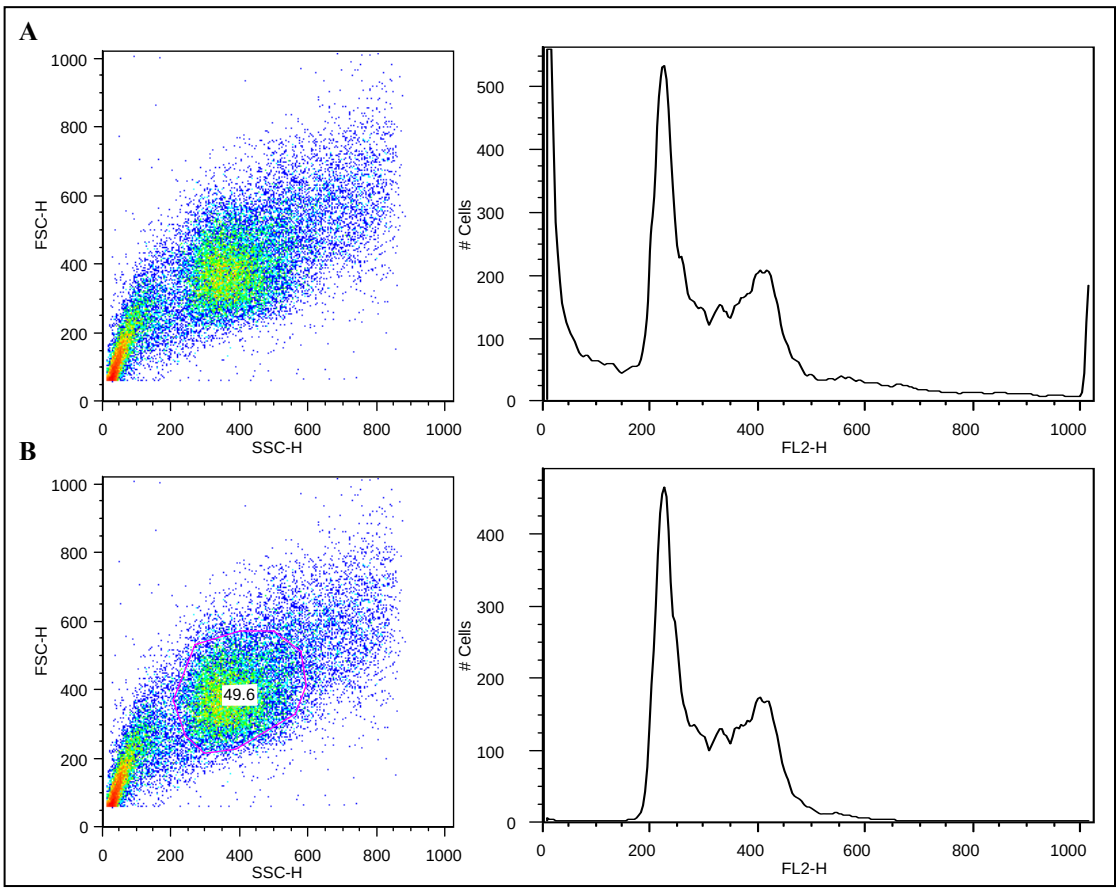


Figure 2.3. PI staining for cell cycle. A: PI staining profile of the whole population. **B: PI profile of single live cells.** A dot plot was used to exclude the dead cells and aggregates from the analysis. The cell cycle analysis was done with FlowJo software from Treestar, which is based on the PI staining and thus the copies of DNA.

2.5.2 Induction experiments

In experiments where cells were induced with growth factor, $1x10^6$ cells per 100-mm dishes were plated. The following day the medium was changed to serum and LIF free GMEM medium in the case of the mES cells, and N2 without insulin or B27 in the hES cells, for 3 hours prior to stimulation with the cytokines or growth factors of interest. Inductions were carried out with 50µl at 25ng/ml of FGF, (in the case of FGF4 also was added 1µg/ml of heparin) and 10ng/ml of LIF. Subsequent induction, the reaction was stopped simultaneously in all the samples by removing the stimulus; washing with PBS and placing the samples in ice. The biochemical analysis of the effect of the induction was carried out by western blotting assays.

2.5.3 Inhibition experiments

The inhibitors were titrated before being used in the biological assay. 10^6 cells/well were plated on N2B27 containing FGF2. After 24 hours the medium was changed to a control medium (N2 without B27, insulin and FGF2). Following 4 hours of incubation the medium was changed to the control medium with the different concentrations of the inhibitor. After incubating for 1 hour the cells were stimulated with 25ng/ml of FGF2 or 10ng/ml of TGFβ. Subsequent inductions followed the procedure as described above. A list of the inhibitors used in this thesis is shown in table 2.4.

Inhibitor	Pathway	Working concentration	Supplier
LY294002	PI3K/AKT	2.5 μM	Calbiochem
UO126	MAPK/ERK	5 μM	Promega
SU5402	FGF	20 μM	Calbiochem
SB 431542	TGFβ	20 μM	Tocris

Table 2.4. List of inhibitors used in this thesis.

2.5.4 Proliferation assay

This assay is based on the incorporation of the radioactive^{3}H (tritium) into the Hydrogen of the Thymidine during DNA replication. Therefore, the amount of ^{3}H-Thymidine incorporated into the DNA relates to cell proliferation**.** Cells were plated at different cell densities ($2.42x10^{4}$-$3x10^{3}$) in the presence of FGF2. Once the cells were attached to the matrix (4-5hours) the medium was changed to + or - FGF2 and after 24 hours of incubation in these conditions the thymidine assay was performed. Each well was labelled with 1μCi (0.037MBq) ^{3}H-Thymidine at a dilution 1:20 in PBS. After 6 hours of incubation of the plates with the label, the samples were harvested. Once the plates have been harvested the filters were incubated for 1 hour at 65^{o}C. The dry filter was placed in a cassette, which was inserted to be analysed in a β-counter.

2.5.5 Self-renewal assays in ES cells

ES cells are defined by their ability of self-replicating whilst maintaining their undifferentiated state. The self-renewal of the ES cells is directly correlated to the amount of the transcription factor OCT4, whose levels can be detected and quantified by

different methods. The cell line IOUD2 (Dani *et al.*,1998) used in this project, carries an E. coli *lacZ* gene encoding β-galactosidase (β-gal) reporter gene (Beckwith, 1980), inserted within one allele of the *Oct4* gene. Therefore, β-galactosidase, serves as a very useful and sensitive reporting tool for the *Oct4* gene expression and consequently for the self-renewal status of the cell population. The quantitation β-galactosidase activity was carried out using Chromogenic assays such as the X-gal staining and the ONPG assay and Fluorometric assays such as fluorescein di-β-D-galactopyranoside (FDG).

2.5.5.1 Chromogenic assays

2.5.5.1.1 X-Gal staining

The β-galactosidase reporter can be detected using a variety of substrates, all of which have a galactose link through a β-D-glycosidic linkage to a moiety whose properties change upon liberation from galactose. The most common substrate used is an indole derivative, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Holt and Sadler, 1958). When β-gal cleaves the glycosidic linkage in X-gal, a soluble, colourless indoxyl monomer is produced. Subsequently, 2 of the liberated indoxyl moieties form a dimmer which is oxidized and whose resultant halogenated indigo is a very stable and insoluble blue compound, which is easily detected (Holt and Sadler, 1958).

The cells were washed with PBS and fixed (0.2ml 50% Glutaraldehyde and 0.5ml 0.5M EGTA (Ethylene Glycol Bis (2-aminoethyl ether) –N'N'N'N' Tetraacetic acid) in 50ml of wash buffer (84mM Na_2HPO_4, 16mM NaH_2PO_4, 0.091.3mM $MgCL_{12}$ $6H_2O$) for 5

minutes at room temperature). The cells were washed 3 times with the wash buffer and then stained (6.4mM $K_3Fe(CN)_6$, 4.45mM $K_4Fe(CN)_6$ 50mg/ml X-Gal (Promega) in dithyl formamide). The stained cells were incubated for 1 hour to overnight depending on the intensity of the expression of the lacZ. After staining the cells were washed and stored at 4°C.

2.5.5.1.2 Orto-nitrophenyl-β-D galactopyranoside (ONPG)

Stem cell specific expression of β-galactosidase from the *Oct4* locus was quantified by ONPG assay. This assay provides a quantitative estimate of lacZ expression by measuring enzyme activity directly. ONPG is the artificial colourless chromogenic substrate that when cleaved released a yellow product, orthonitrophenol (ONP). Therefore activity of the enzyme can be measured by the rate of appearance of yellow colour using a spectrophotometer (λmax = 420nm). Duplicate assays were performed on triplicate samples for each treatment. On day 6 of the treatment, the cells were washed once with PBS and lysed in 0.4ml of 0.25 M Tris, pH 7.5, 0.5 mM DTT, 0.5% NP40. 40 µl of the lysate were mixed with 100µl of ONPG buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM $MgCl_2$, 50mM 2-mercaptoethanol, 1.2 mM ONPG) in a microlitre plate and incubated at 37°C for 2-4 hours. The absorbance was read at 420 nm at every hour to get readings of the different points of the reaction. The results obtained for β-gal were adjusted for the protein values. The quantification of protein was obtained with the BCA kit that was prepared as indicated by the manufacturer

(Pierce) and measured at 562nm. Once the concentration of protein was determined the values of the ONPG absorbances were normalised with the protein values.

2.5.5.2 Fluorogenic assay

2.5.5.2.1 Fluorescein di-β-D-galactopyranoside (FDG) assay

In this method the β-galactoside analogue FDG was used to carefully distinguish *LacZ* positive cells from the negative ones, allowing time-dependent fluorescence activated cell analysis. FDG is cleaved by β-galactosidase in L*acZ* positive cells to yield fluorescein (Nolan *et al.*,1988) along with galactose. Fluorescein emits light at a maximum of 514 nm when excited with light at 491 nm, whereas the precursor molecule FDG does not. Therefore, the activity of the enzyme is directly proportional to the rate of fluorescent accumulation at these wavelengths and can be detected and measured by fluorescence activated cell sorter (FACS) (Nolan *et al.,* 1988). Compared to the ONPG, this assay had the advantages of not requiring to kill, fix or to lyse the cells to allow product formation. Further advantages were shorter incubation times, smaller sample volume and the possibility of including propidium iodide for the identification of damaged cells.

The cells were harvested as protocol, collected and centrifuged. The pellet was re-suspended in PBS supplemented with 10% FCS, giving a total volume of 100µl. The cells were carefully mixed and 40µl taken into a FACS tube for the FDG staining. In order to accelerate the pass of the fluorescein through the cell membrane, the cell

solution was placed into a water bath at 37°C. After 5 minutes the tubes were taken from the water and 0.5 mM FDG (prepared in H_2O, which is pre-warmed at 37°C, at 50% of the isotonic concentration) was loaded into the tubes, mixed gently and rapidly placed back into the 37°C water bath. After 1 minute, the hypotonic shock was terminated by placing the cells in ice and adding 1:9 dilution of ice-cold isotonic medium and placed at 4°C to allow fluochrome generation and prevention of FDG leakage. After 60 minutes LacZ positive cells generated a peak of fluorescence that was measured by flow cytometry.

2.5.6 Statistical Analysis

Analysis of the variance (ANOVA) was performed to determine the significance of a given result. For the FDG assay, the data was log transformed to equalise variances. Then, the data was subjected to a 2-way ANOVA to investigate the effects of the treatment. All data are expressed as the mean ± SD of observations within each experiment and usually of three independent experiments. Statistical analysis was performed using Minitab 14. Statistical significance was accepted at $p<0.05$.

CHAPTER 3

CHARACTERISATION OF THE EFFECT OF FGF ON MOUSE ES CELLS

3.1 Introduction

FGF signalling is a conserved coordinator of neural development (Yamaguchi *et al.,* 1992; Wilson *et al.,* 2000; Delaune *et al.,* 2005; Zhang *et al.,* 2006; Willems and Leyns, 2008). Among the 22 members of the FGF family (Zhang *et al.,* 2006), FGF2, and particularly FGF4 are essential factors during the development of the mouse embryo.

Ffg2 is expressed in the primitive ectoderm, which suggests a possible role in the formation of the ICM. However, FGF2 is not expressed in undifferentiated mES cells, and it is only after 5 days in the absence of LIF that FGF2 is expressed (Jirmanova *et al.,* 1999). Nevertheless, mES cells (Esner *et al.,* 2002) and the mouse blastocyst (Campbell *et al.,* 1992) display FGFR1 and FGFR2, the specific binding receptors for FGF2. Esner et al, (2002) found that FGFR1 expression was necessary for the maturation into visceral endoderm of EBs. This established the critical role of FGF2 in the regulation of EBs and led them to the conclusion that FGF2 did not have an effect in mES cell proliferation. Studies *in vivo* showed that FGF2 deficient mice, although viable, suffered severe defects in the formation of the cerebral cortex due to a delayed differentiation (Dono *et al.,* 1998). Taken together, these studies indicate that FGF2 is a decisive factor in the process of embryonic mouse differentiation and may suggest a role of FGF2 as inducer of differentiation in mES cells. This role in differentiation would be the counter effect that FGF2 produces on the human system. Indeed, FGF2 is a critical factor in derivation and propagation of hES cells. Opposing effects of FGF2 in these two species could be associated with the different levels of FGF2 expression: high in

hES cells and low in mES cells. For this reason, it was chosen to asses the effect of FGF2 on mES cells and in later chapters this is compared to the effect of FGF2 in hES cells.

Fgf4, on the other hand, is expressed at multiple stages of mouse development; at the RNA level it has been detected as early as the 1 cell stage and its expression increases during the period of blastocyst growth (Rappolee *et al.,* 1994). In the pre-implantation blastocyst, *Fgf4* expression is restricted to the cells of the ICM and coincides with *Oct4* expression (Niswander and Martin, 1992). In the post implantation embryos *Fgf4* expression becomes restricted to the primitive streak and in later development, *Fgf4* remains in only a few cell types (Niswander and Martin, 1992). Mouse ES and EC cells express *Fgf4* and its receptors at the RNA and protein levels, whereas this expression is repressed when they differentiate (Rizzino *et al.,* 1988). This is a consequence of *Fgf4* gene expression in ES and EC cells being controlled by the pluripotency-associated transcription factors OCT4 and SOX2 (Curatola and Basilico, 1990). OCT4 binding and activation of the *Fgf4* has also been confirmed in mouse embryos, in which deletion of *Oct4* resulted in a reduced or absent expression of *Fgf4* (Nichols *et al.,* 1998).

As mentioned above, FGF4 is expressed in the ICM in early epiblast (Niswander and Martin, 1992; Rappolee *et al.,* 1994) and FGF receptors are up-regulated in the trophectoderm and extraembryonic ectoderm (Rappolee *et al,* 1994; Arman *et al.,* 1998). Nichols *et al*, (1998) suggested that OCT4 directed the secretion of FGF4 from the ICM/epiblast and FGF4 promoted the expansion of trophectoderm. FGF4 in

conjunction with heparin is also required for the proliferation of trophoblast stem (TS) cells (Tanaka *et al.,* 1998). TS cells are derived from blastocysts or early post-implantation trophoblast and can contribute to the trophoblast lineages of the placenta when re-introduced into the early embryo (Tanaka *et al.,* 1998). Therefore, FGF4 plays a key role in the growth and survival of extra embryonic tissues (Wilder et al., 1997; Nichols *et al.,* 1998; Tanaka *et al.,* 1998). The essential role of FGF/FGFRs during development was demonstrated when mES cells expressing a dominant negative (dn) FGFR2 failed to form EBs (Chen *et al.,* 2000). Furthermore, mouse embryos with both *Fgf4* alleles inactivated *($Fgf4^{-/-}$)*, died soon after implantation, confirming *in vivo* the critical role of FGF4 during embryogenesis (Feldman *et al.,* 1995).

Nevertheless, in culture, the addition of FGF4 does not seem to affect the proliferation of the mES cells (Rizzino *et al.,* 1988; Wilder *et al.,* 1997). Wilder *et al,* (1997) demonstrated that both, wild type *($Fgf4^{+/+}$)* and knockout *($Fgf4^{-/-}$)* mES cells gave rise to a similar mixture of differentiated tissues in teratomas. However, they found that inactivation of the *Fgf* gene severely compromised survival and proliferation of differentiated cells *in vitro.* This observation led them to propose that FGF produced by ES cells operated in a paracrine manner on the differentiated progeny, which form spontaneously at low density. This conclusion is consistent with other studies which demonstrated an increase in extraembryonic endoderm cells derived from the ICM when FGF4 was added to *in vitro* blastocysts (Rappolee *et al.,* 1994). These reports appointed FGF4 as a survival factor and a promoter of their proliferation for the differentiating progeny of the mES cells.

However, it is also possible that FGF4 directly promoted the differentiation of ES cells, as in its absence mES cells were not capable of differentiation. In the report by Wilder *et al,* (1997) the addition of FGF4 to *Fgf*$^{-/-}$ cells seemed to induce more differentiated cells than when the wild type mES cells spontaneously differentiated. Moreover, when FGF4 was supplemented to *Fgf*$^{-/-}$ cells, the size of the undifferentiated colony appeared smaller than when the wild type cells were maintained in absence of this factor (Wilder *et al.,* 1997). Taken together, these observations could support a pro-differentiative role of FGF4 on mES cells. In addition to this, results presented by Rappolee and colleagues, (1994) could also be interpreted to support this hypothesis. They demonstrated that the addition of FGF4 to cells derived from the ICM cells (small sized nuclei) resulted in an increase in the number of cells with medium sized nuclei. These cells had the morphology and markers of parietal and/or primitive endoderm and were negative for SSEA1 (Rappolee *et al.,* 1994). Therefore, if small and medium/large nuclei are associated with undifferentiated and differentiated cells respectively, FGF4 seemed to increase the amount of differentiation in culture and the differentiated cells could have developed from the ES cells. This was supported by the lack of mitogenic responses to the reduction of endogenous FGF4 in embryos (Rappolee *et al.,* 1994).

In conclusion, previous work has suggested that the proliferation of mES cells is not influenced by FGF4 and FGF2 signalling (Rappolee *et al.,* 1994; Wilder *et al.,* 1997). However, this lack of response in the growth of mES cells does not exclude the possibility of an effect of FGF on the self-renewal of these cells. Murine ES cells

express the appropriate receptors for both FGF2 and FGF4, and FGF4 is highly expressed in mouse ES and EC cells.

3.2 Hypothesis

That FGF signalling promotes differentiation of mES cells, growing in presence of LIF, as well as sustains the growth of already differentiated cells. A diagram of the hypothesis proposed is shown in Figure 3.1.

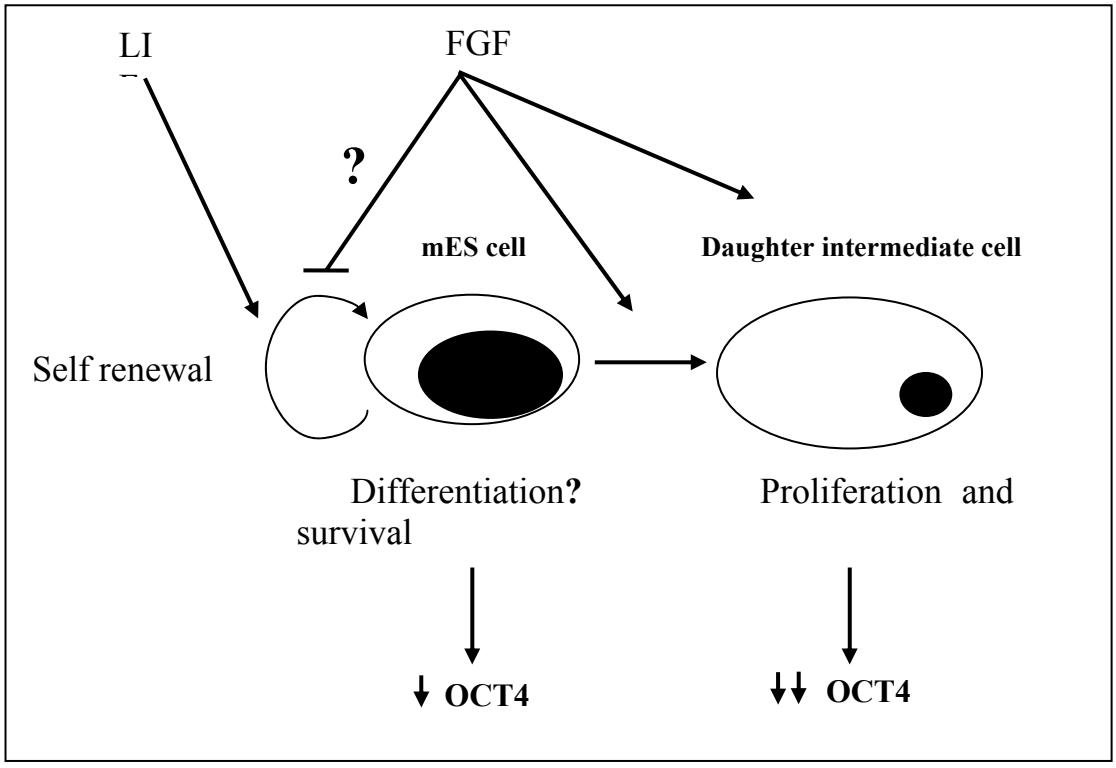


Figure 3.1. Schematic representation of the hypothesis proposed in this chapter.

3.3 Aims

I. To establish the biochemical activation of mES cells treated with FGF2 and FGF4

II. To characterise the effects of FGFs on undifferentiated mES cells

III. To characterise the phenotype induced by FGF

3.4 Results

3.4.1 FGF activation of Mouse ES cells

It has been shown that FGFs induce a strong stimulation of the mitogen-activated protein kinase pathway (MAPK) in different cell types and species as for example, in mouse endothelial cells (Pintucci *et al.,* 2002), *in vivo* mouse embryo (Marshall, 1995), mouse fibroblasts (Hadari *et al.,* 2001) etc. Therefore, the analysis of the phosphorylation in the key downstream effectors of FGFRs, extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Ornitz and Itoh, 2001) is an effective readout of the activation of the MAPK pathway.

To determine whether mES cells are activated by FGF, the phosphorylation of ERK was evaluated by western blotting after stimulating the cells with 25ng/ml of FGF2 or FGF4.

In addition to the FGFs, mES cells were stimulated with 10ng/ml of LIF, which has been shown to significantly activate ERK (Burdon *et al.,* 1999b).

ERK was phosphorylated when mES cells were treated with LIF and to a lesser degree with FGF4 and FGF2 (Figure 3.2). This was confirmed by densitometric quantification of the immunoblots, which also showed a higher level of activation in FGF4 than in FGF2 (Figure 3.3). ERK phosphorylation in FGF2 treated cells was slightly higher over the basal level when loading was taken into account. LIF strongly activated STAT3 in mES, which is consistent with previous reports (Burdon *et al.,* 1999a). Neither FGF2 nor FGF4 induced STAT3 activation. In conclusion, mES cells respond to FGF signalling.

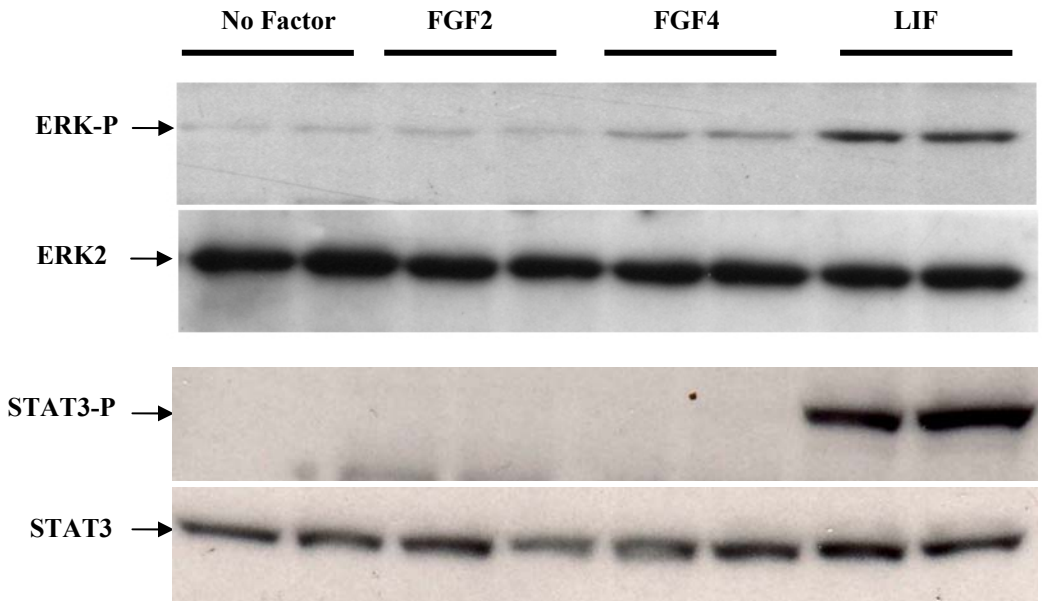


Figure 3.2. Western blotting of ERK and STAT3 activation on mES cells. Cells were induced with FGF or LIF and their stimulation was analysed by examining the activation of ERK protein or the STAT3 protein. Cell lysates from cells that have been starved of signal were used as controls. The immunoblots of the cell lysates were probed with an antibody specific for the active phosphorylated forms of ERK1/2-P or STAT3-P. After stripping, the membranes were reprobed with antibodies that recognise ERK2 or STAT3 protein as controls for protein loading.

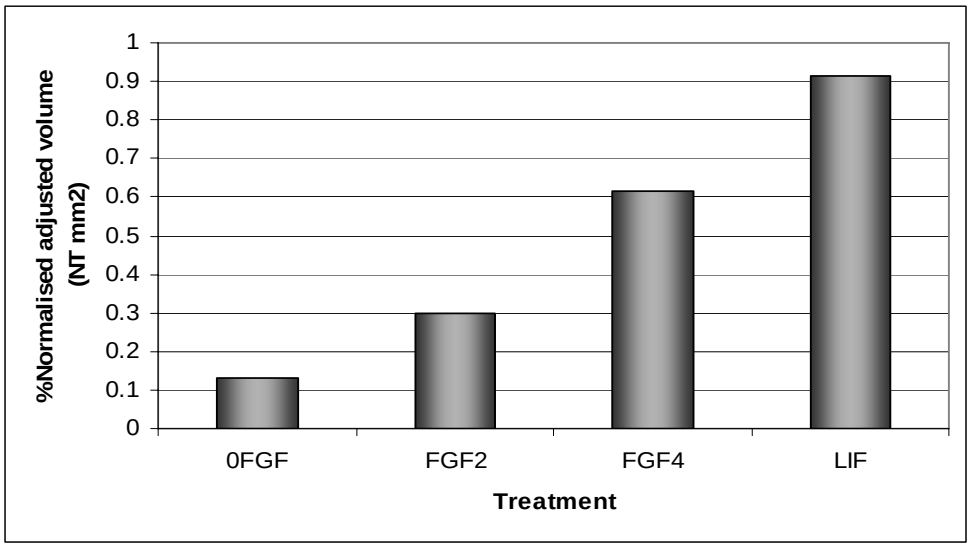


Figure 3.3. Quantification and Normalisation of the adjusted volume of ERK-P to ERK levels. The immunoblots ERK-P and ERK were scanned, quantified by densitometry and normalised. Results confirmed the increased induction of ERK by FGF4. The results shown here are mean values of two independent wells.

3.4.2 Effect of FGFs on mES cell self-renewal

To determine whether the addition of FGF to mES cells affects their self-renewal or differentiation, two cell lines (IOUD2 and Y118F) with a *lacZ* insertion within the stem cell specific gene *Oct4* were used (see section 2.1.2.2 for details). The β-galactosidase activity resultant was monitored *in situ* by X-gal staining and quantified biochemically using the ONPG or FDG assays. β-galactosidase expression is restricted to undifferentiated cells expressing the reporter from within the *Oct4* locus, thus the level of activity of this enzyme relates to the potential for self-renewal in the ES cells (Figure 3.4).

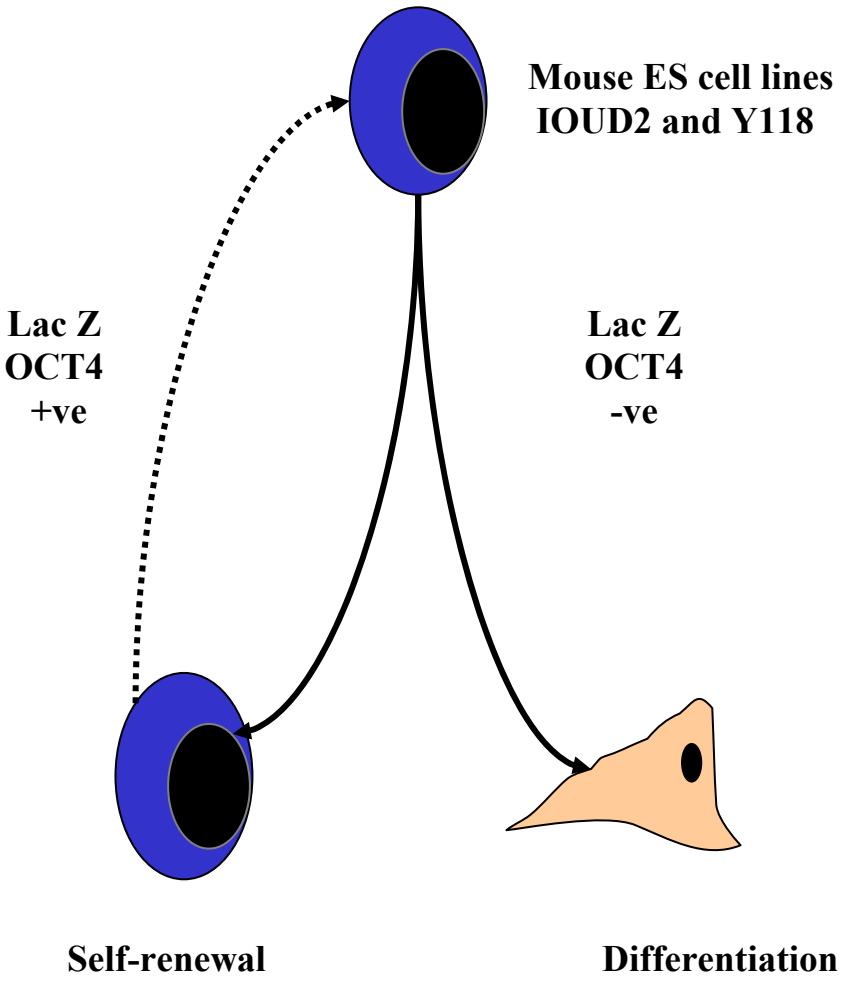


Figure 3.4. Schematic representation of the experimental system used to measure the biological response of mES cells to the different growth factors. The self-renewal assays make use of *lac Z* insertion within the stem cell specific gene *Oct4* in IOUD2 and Y118F cell lines. Expression of this *Oct4* reporter is restricted to undifferentiated mES cells. The resulting β-galactosidase activity (shown as blue), therefore, provides a measure of stem cell self-renewal.

3.4.2.1 LIF titration

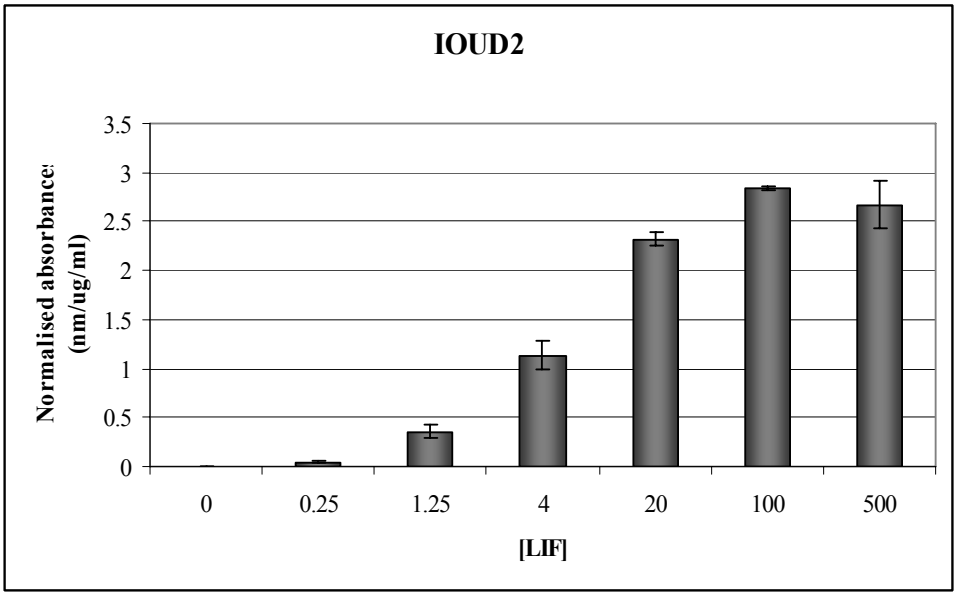
The biochemical quantification of the β-galactosidase product of the *LacZ–Oct4* reporter was determined using the ONPG assay. ONPG is the colourless chromogenic substrate that when cleaved by β-galactosidase releases a yellow product - orthonitrophenol (ONP), the absorbance of which can be measured on spectrophotometre. Therefore, the activity of the enzyme is reflected by the intensity of the absorbance, which in turn represents the levels of OCT4 and the state of the self renewal of the mES cells.

Previous studies into the effects of FGF on mES cells from other groups have utilised a single standard concentration of LIF (10ng/ml in the case of Wilder *et al.*, 1997 and 1000 U/ml in Esner et al., 2002). In order to establish if there is any interaction between LIF and the FGF, different concentrations of LIF were assessed. To establish a range of concentrations of LIF to apply in the self-renewal assays, ONPG assays were performed. To carry out the selection, seven different concentrations of LIF were titrated covering a range from 0 to 500 Units/ml. The resultant absorbances were normalised against the protein content and the results for IOUD2 and Y118F cell lines are shown in Figure 3.5. β-galactosidase activity peaked at 100Units/ml and progressively decreased proportionally to the concentration of LIF.

On the basis of these results (Figure 3.5) the concentrations: 0, 4, 100 and 500 Units/ml of LIF were selected to be used in conjunction with the titration of FGFs. These concentrations represented specific points in the distribution of β-galactosidase activity

at which to identify any interactive effect with the FGFs in the self-renewal experiment. 500U/ml of LIF was chosen as a near saturating concentration, 100U/ml was used as a control, because it is the concentration of LIF at which mES cells are routinely grown in the laboratory. 4U/ml of LIF was selected because this concentration corresponded to approximately half-maximal self-renewal. It was hypothesised that at this sub-optimal concentration of LIF the mES cells should be more susceptible to any effects of FGF on self-renewal. In addition to this, the effects of FGF in the absence of LIF were studied.

A



B

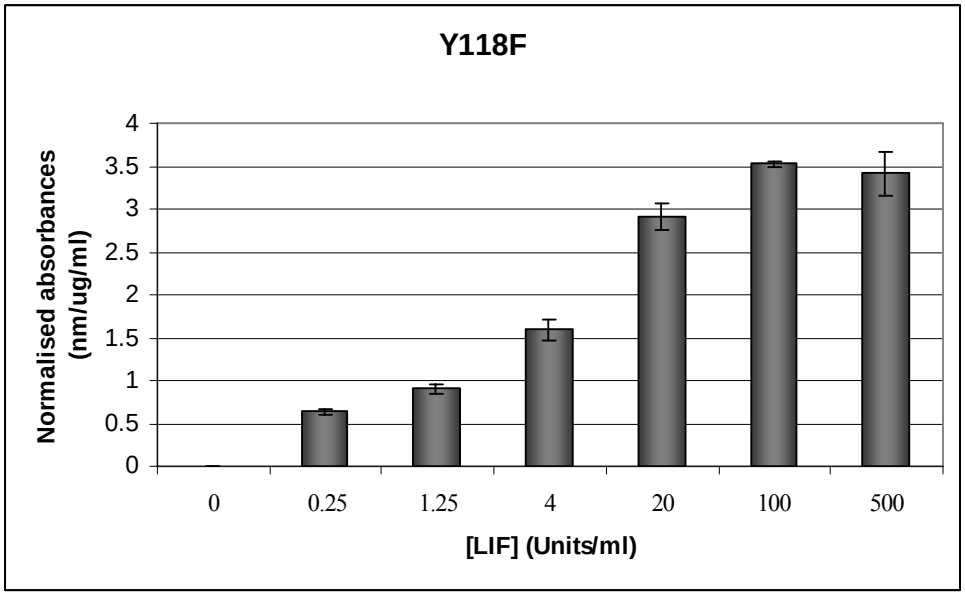


Figure 3.5 Effect of different LIF concentrations on self-renewal and growth of mES cells. Six days after plating on the different concentrations of LIF, the cells were lysed and the β-galactosidase activity measured by ONPG assay. The resultant absorbance values were normalised against protein concentration in cell lysates. Values of the mean of triplicate samples and three different experiments are shown as ± SD in cell lines IOUD2 **(A)** and Y118F **(B).**

3.4.2.2 Effect of FGF in conjunction with LIF

Once the selection of the LIF concentrations were established, their effect in conjunction with 8 different concentrations of FGF2 or FGF4 titrated from 0 to 250ng/ml was tested. The data presented in Figure 3.6 represents the means of four independent experiments for both FGF2 and FGF4. Each experiment consisted of duplicate determinations of β-galactosidase activity from triplicate samples. The mean results of the absorbance determinations were normalised against the total protein concentration of the sample. The final result is presented normalised against to the response in the absence of FGF at 100U/ml. The ONPG assays confirmed a direct relationship between LIF concentration and the level of β-galactosidase expressed by the cells and, as expected, self-renewal was reduced at the lower concentrations of LIF. The addition of FGF did not increase the levels of β-galactosidase activity. In fact, it resulted in a slight reduction at high LIF concentrations, indicating that FGFs do not enhance the self-renewal of mouse ES cells (Figure 3.6). Intriguingly, β-galactosidase was reduced after the addition of high concentrations of FGF, at 100 and 500 U/ml LIF, which are concentrations used to inhibit differentiation in mES cells (Smith *et al.,* 1992). Figure 3.7 shows the slight decrease in the β-galactosidase at 100U/ml of LIF in the presence of 25ng/ml of FGF. In conclusion, there was not an increase in β-galactosidase activity, and consequently ES cell self renewal, when FGF was added to high concentrations of LIF.

A

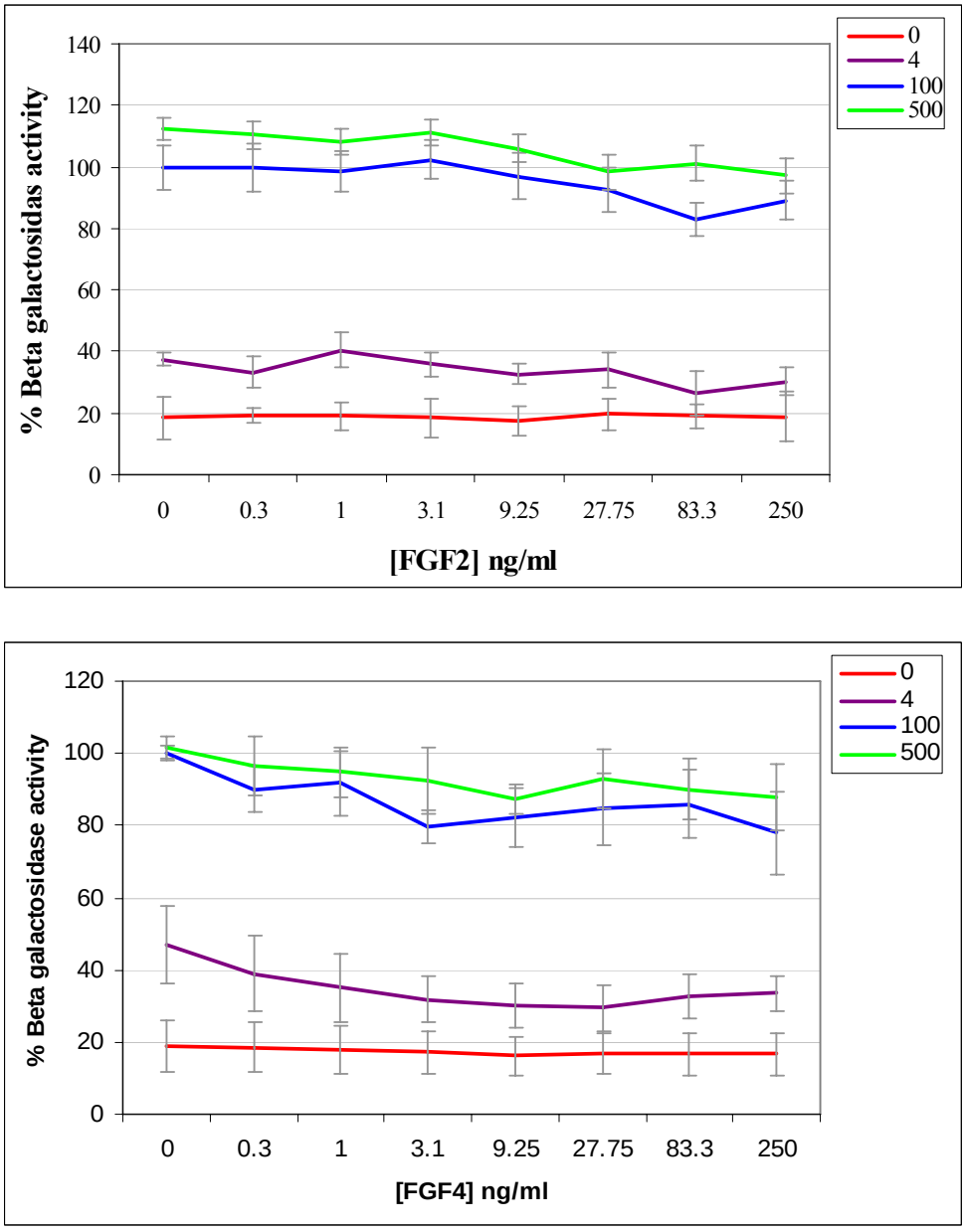


Figure 3.6 Effect of different concentrations of FGF and LIF on the normalised β-galactosidase activity in IOUD2 (A)

B

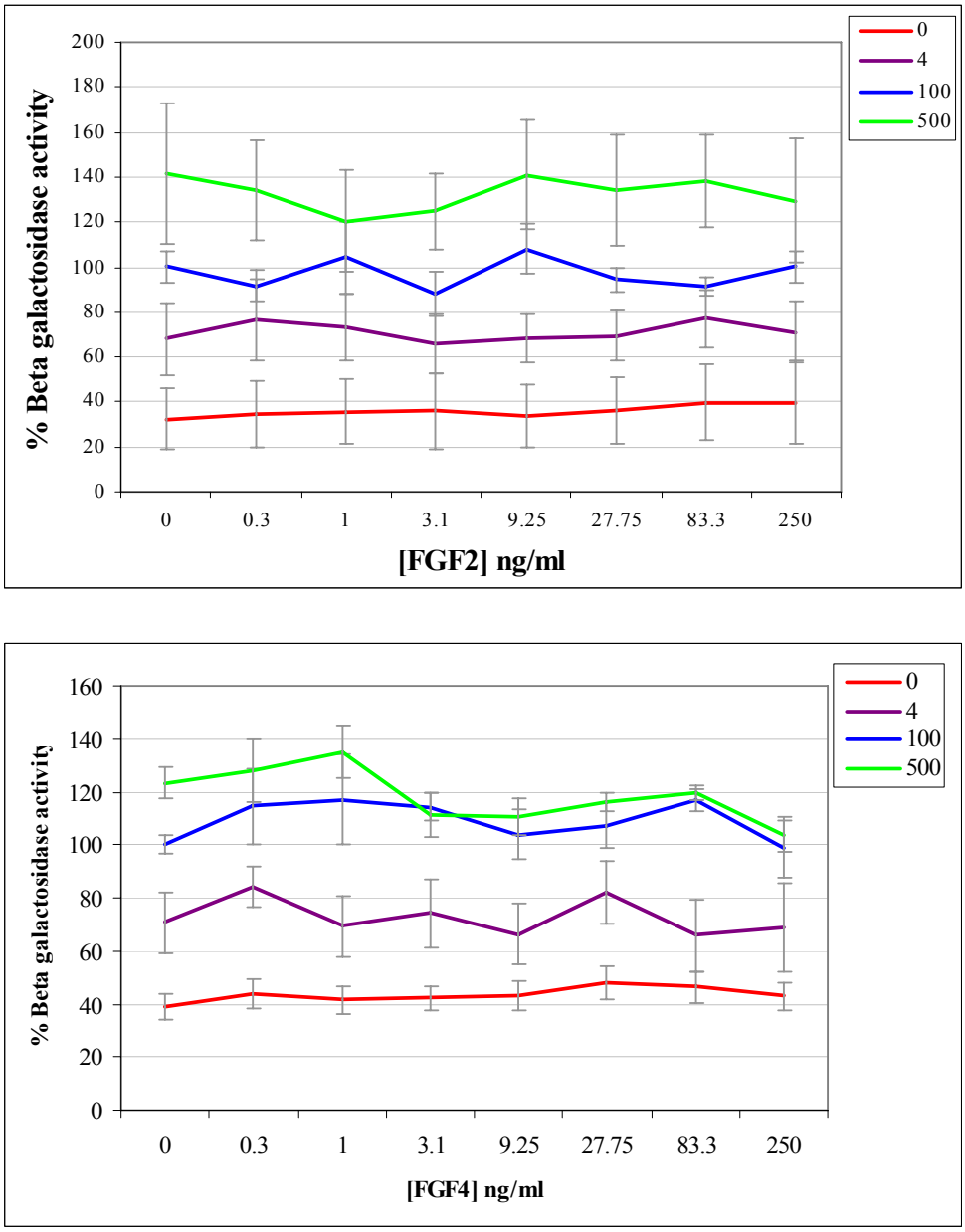


Figure 3.6 Effect of different concentrations of FGF and LIF on the normalised β-galactosidase activity in IOUD2 (A) and Y118F (B) mES cells. Six days after plating the cells in the different concentrations of LIF and FGFs, β-galactosidase activity was measured by an ONPG assay and normalised against the protein concentration in cell lysates. The concentrations 0, 4, 100 and 500 U/ml, were used as negative control, minimal, optimal and semi-saturating LIF concentrations for mES self-renewal respectively. The data presented has been normalised against 100% β-galactosidase activity at 100 Units of LIF and 0ng/ml of FGF. The mean of four independent

experiments is shown. The error bars denote the standard error mean (S.E.M) between the values in the independent experiments.

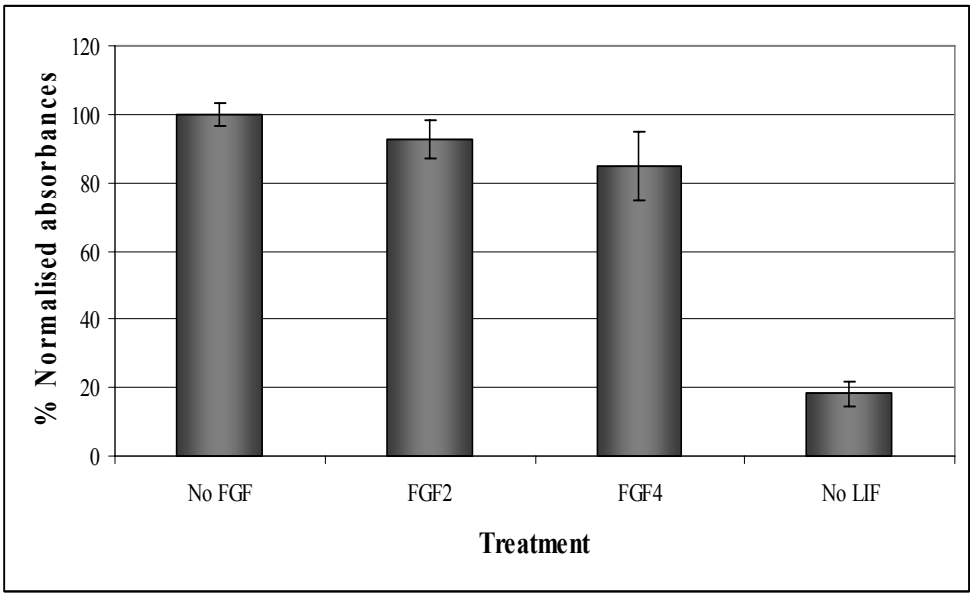


Figure 3.7. Percentage of β-galactosidase activity in IOUD2 cells treated with 25ng/ml of FGF in the presence of 100U/ml of LIF. Results from the ONPG assay were normalised and presented as an effect of 25ng/ml of FGF in the presence of 100U/ml of LIF.

3.4.2.3 Statistical analysis

With the intention of determining whether the reduction in the β-galactosidase activity by FGF (Figures 3.6 and 3.7) was statistically significant, an ANOVA test was carried out (Figure 3.8). To isolate any change in the β-galactosidase's activity of FGFs, the effect of exogenous LIF and any possible noise were excluded for this analysis.

Results from the ANOVA test confirmed an overall decrease in the β-galactosidase levels by FGF2 and FGF4 (Figure 3.8). This effect was more pronounced for FGF4, which induced the reduction of the enzyme activity at all the concentrations tested. In the case of FGF2, the results showed a slight increase in the β-galactosidase levels at 250ng/ml. This was not a consistent effect; it was caused by the results from one of the experiments.

In conclusion, FGF addition seemed to induce the decrease in the levels of the OCT4 reporter in a proportional manner to the concentration of growth factor added. The ANOVA test seemed to indicate a greater effect than previous data presented in Figures 3.6 and 3.7, which may be caused by the exclusion of the concentrations of protein from the analysis. Normalisation of β-galactosidase levels to the protein concentration is standardising the results in the culture. In this case the decrease in enzyme activity (Figure 3.6) could be caused by either differentiation or by a decline in cell growth. Since a less significant effect was observed when the results were normalised, it was

indicated that the levels of protein were not reduced, suggesting a reduction in the self-renewal.

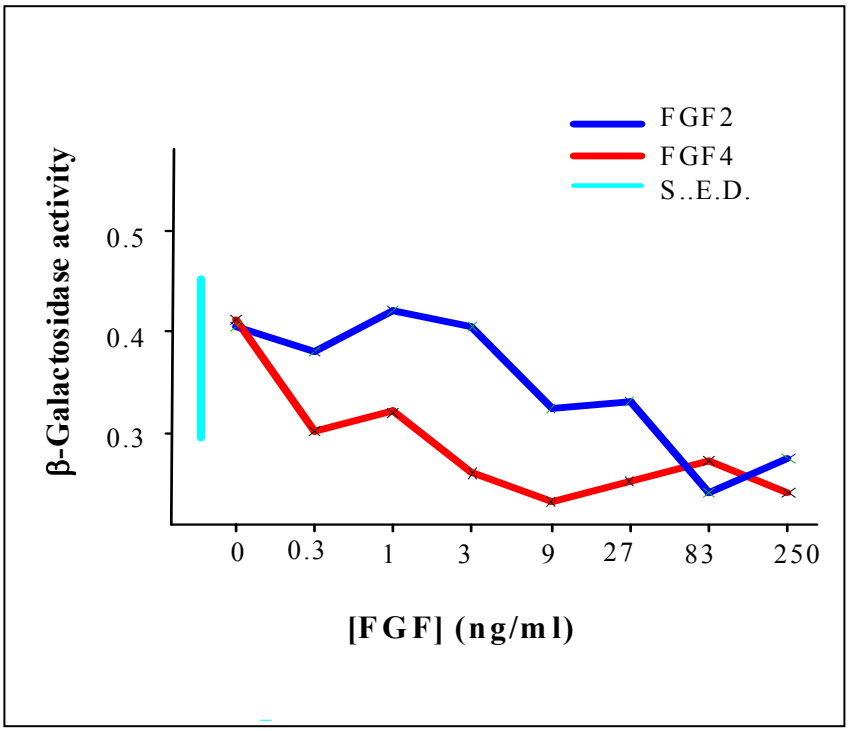


Figure 3.8. ANOVA analysis into the effect of different concentrations of FGF in mES cell self-renewal when LIF and protein levels were excluded. The points in the plot are the means for the responses at the various levels of each factor plotted against the β-galactosidase value. The S.E.D. shows the standard error of a difference between the means. There was a decrease in β-galactosidase activity when the concentration of FGF was increased. FGF4 produced a higher decrease than FGF2. Data is presented as a mean of four independent experiments.

3.4.3 Determination of the specificity of FGF on mES cells

Previous results indicate a positive effect of FGF in mES cell differentiation. This could occur by either proliferation of the already differentiated cells or, as suggested by the effect of FGF and at high concentrations of LIF, by promoting differentiation. To further examine the possibility of FGF producing a specific induction of mES cell differentiation, the effects of FGF were examined at the cellular level. For this purpose the FDG assay was used, which allows the relative quantification of β-galactosidase enzymatic activity per cell.

3.4.3.1 Conditions for the Fluorescein di-β-D-galactopyranoside (FDG) assay

FDG was used to sensitively distinguish *LacZ* positive and negative cells, allowing time-dependent fluorescence activated cell analysis. FDG is cleaved by β-galactosidase in *LacZ* positive cells to yield fluorescein along with galactose (Nolan *et al.,* 1988). The emission of light by fluorescein can be detected and measured by fluorescence activated cell sorter (FACS) and the activity of the enzyme is directly proportional to the rate of fluorescence accumulation (Nolan *et al.,* 1988).

Before performing this assay, some key issues needed to be taken into account. Firstly, the number of cells should be sufficient to provide enough enzymatic activity to cleave the substrate supplied and to provide the cells with a sufficient concentration of substrate for the enzyme. This issue was addressed by performing titrations of the substrate on a standard number of cells. Secondly, the fluorochrome produced by the cleavage of the

FDG must be retained within the cells, avoiding any transfer from the positive to the negative cells. With the purpose of preventing FDG leakage, following the loading of the FDG substrate by hypotonic shock at 37^{o}C, the samples were rapidly cooled with ice-cold PBS, which sealed the cells. This was followed by incubation at 4^{o}C to allow accumulation of the fluorescent product at the same time that cell leakage was prevented.

3.4.3.2 Titration of the substrate (FDG)

A titration was carried out with the aim of selecting the optimal concentration of FDG to use in the experiment. To facilitate the examination of the fluorescence emission, the *LacZ*$^{+}$ cell line (IOUD2) was mixed 50:50 with a *LacZ*$^{-}$ line (HM1). This procedure had the advantage of allowing the assessment of any transfer of the fluorochrome from the positive to the negative cells. The volume of FDG and cells loaded was adjusted from 100µl (which was the amount used in the protocol described by Nolan *et al,* (1988) to 40µl. It was reasoned that loading a lower volume of FDG, the variability between samples, would be minimised by decreasing the time in manipulation of the samples. Different concentrations of FDG (2-0.1mM) were loaded into 40µl of cells at 10^{7}cells per ml for 2 minutes under hypotonic conditions at 37^{o}C to improve the uptake of substrate through the membrane. FDG loaded cells were incubated for 60 minutes at 4^{o}C at standard osmolarity. The rate of fluorescence increment, at that temperature, is linear and directly proportional to β-galactosidase activity. Fluorescence reaches a plateau when the hydrolysis of the substrate is completed.

Results presented in Figure 3.9 indicate that the concentrations 2-0.25 mM gave a peak ratio of 50:50 indicative of an accurate generation of fluochrome. At these concentrations and cell number (10^7/ml), the intracellular enzyme seems to have enough substrate. Moreover, the negative peaks have shifted to the right at 2 and 1mM. At the concentrations of 0.25mM and 0.1mM the resolution of the peaks were unsatisfactory, indicating sub-optimal concentrations of FDG since they did not provide sufficient substrate for the enzymatic reaction. As a result of this titration, the concentration of 0.5mM FDG was selected in the subsequent experiments.

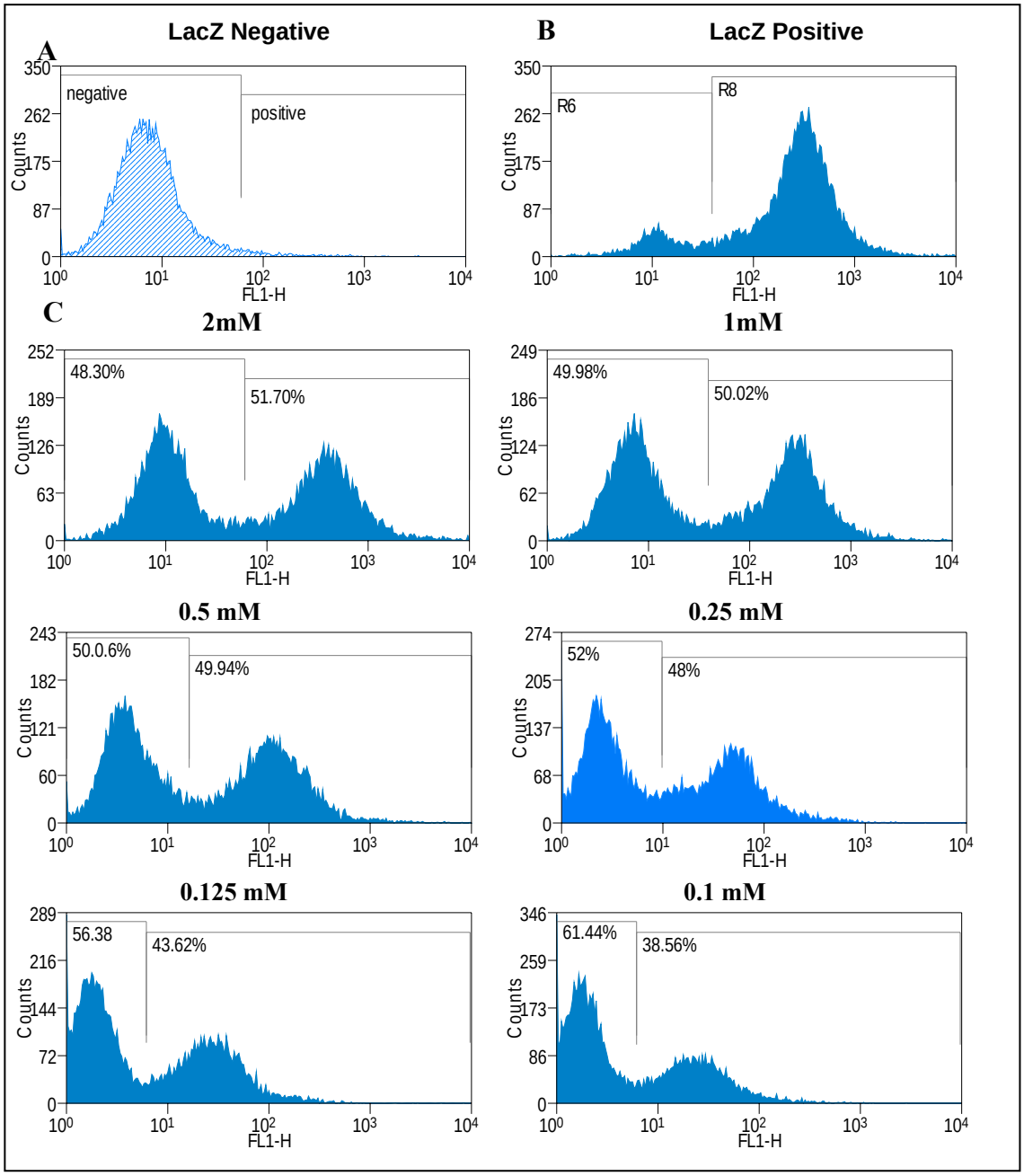


Figure 3.9. Specific staining of *LacZ*+ and *LacZ*- cells with a titration of FDG. All cells were uniformly loaded at 37°C and allowed to generate fluorescein at 4°C for 60 minutes. **A:** *LacZ*- cells stained alone did not develop appreciable fluorescence above the auto-fluorescence background. **B:** *LacZ*+ cells developed considerable fluorescence with a minor population of cells, which have lost *LacZ* expression. **C:** The 50:50 mixtures treated with concentrations of FDG (2-0.1 mM) showed two discrete peaks, *LacZ*+ clearly resolved from *LacZ*- cells. FDG concentrations below 0.5mM were suboptimal and at 0.5 mM and above there was sufficient substratum for the 10^7 cells/ml.

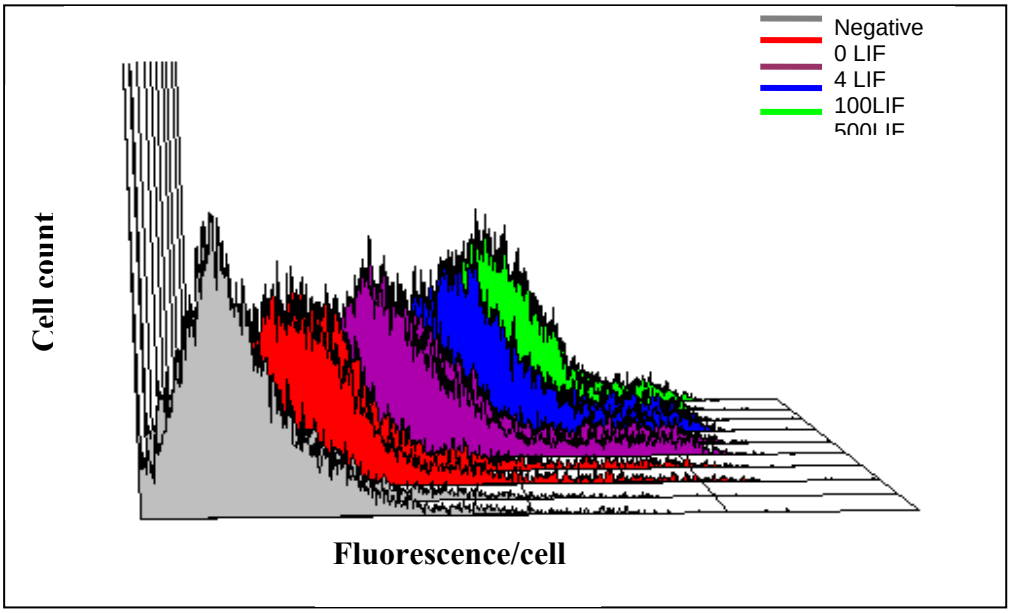
3.4.3.3 Cell number

The protocol developed by Nolan and *et al*, (1988) suggested 10^7cells/ml to be used with 2mM FDG. The results in Figure 3.9 show that 0.5mM provides a sufficient concentration of substratum to saturate the enzyme present at that cell number.

3.4.3.4 Validation of the FDG assay

To asses the efficiency of the FDG assay, the 4 concentrations of LIF (0, 4, 100 and 500 U/ml) used in the previous experiments were tested. After 6 days of growth in the different concentrations of LIF, the plates were harvested and 10^7cells/ml FDG stained. Flow cytometry results were equivalent to the results obtained by the ONPG assay. Accordingly, flow cytometry analysis showed an increase in the fluorescence emission relative to concentrations of LIF (Figure 3.10). The fluorescence emission corresponds to the conversion of FDG and thus it is related to the activity of the β-galactosidase.

A



B

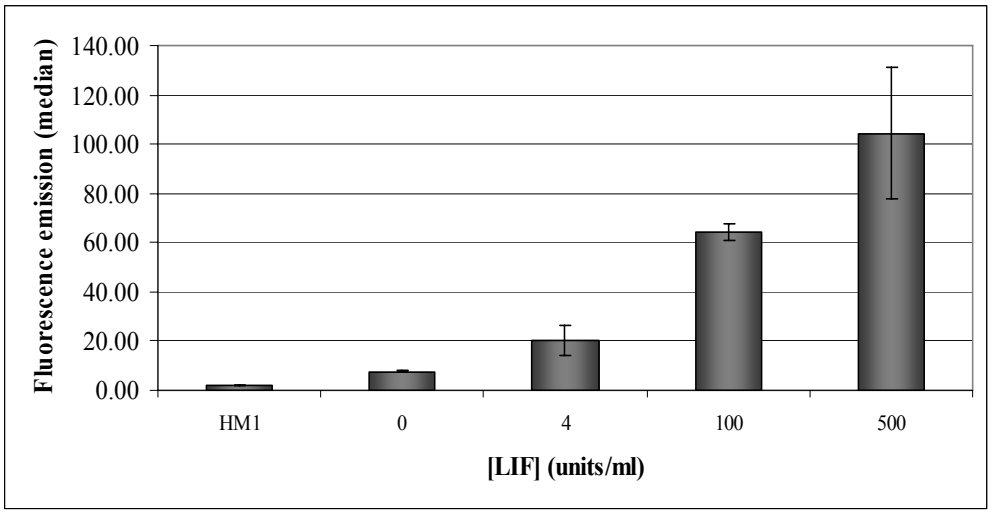


Figure 3.10. Effect of LIF concentration on the fluorescence emission. Six days after plating, β-galactosidase activity was measured by flow cytometry. 0.5μM of FDG was added to the mixture and incubated for 1 hour at 4°C. **A**: The histograms show duplicate samples representing the fluorescence distribution of *LacZ*- cells (control, grey) and *LacZ*+ that have been maintained in 0 (red), 4 (purple), 100 (blue) and 500 (green) Units/ml of LIF. **B**: Plotted values for the median of three independent experiments show that increasing concentrations of LIF are associated with a significant increased in fluorescence emission ($p<0.001$). Two way analysis of variance used for generation of p value.

3.4.3.5 Effect of FGF addition on FDG conversion

To determine the specificity of the effect of FGF, FDG assays were carried out since they can report the effect of FGF at the cellular level. The cells were maintained for 6 days in the following conditions: LIF alone, FGF2 or FGF4 with LIF or in absence of factors. LIF was used at a concentration of 100U/ml because it was reasoned that if FGF induced a decrease in β-galactosidase levels at 100U/ml of LIF (concentration used to grow undifferentiated mES cell) then the undifferentiated ES cells could be the target of FGF. The concentration of FGF was 25ng/ml, since at this intermediate concentration a decrease in enzyme activity by the ONPG assay was observed and it is the concentration used to induce ERK activity. Figure 3.11 is representative of the results obtained in the FDG assay.

Four independent experiments consistently showed a decrease in the fluorescence emission when FGF2 was added and more noticeably with FGF4 (Appendix 1). The loss of brightness was concomitant with a decrease in the number of bright cells, which suggests that the reduction in the reporter occurs in the whole population. In conclusion, FGF produced a decrease in enzymatic activity, and the shift of the whole population indicates that also the undifferentiated cells are targeted.

To allow a comparison with data from hES cells grown in a defined medium, the analysis of FGF effects in mES cells was also carried out in the N2B27 medium (Ying *et al.,* 2003b). The FDG experiment was repeated in triplicate in independent wells four

times in the standard mES cells medium GMEM (Figure 3.12, A) and twice in a serum free medium, N2B27 (Figure 3.12, B). Results from the FDG analysis were statistically analysed and the values for the medians were presented as a percentage of FDG conversion. The values were normalised, taking the FDG conversion obtained in the absence of FGFs and in the presence of 100U/ml of LIF as 100% of substrate conversion.

Averaged results showed a decrease in the percentage of the FDG conversion in response to FGF supplementation. This decrease of β-galactosidase activity was more pronounced in the normal culture conditions for mES cells (Figure 3.12A) than when the defined medium was used (Figure 3.12B). The fact that in the absence of LIF the enzyme levels were not dramatically reduced could suggest that N2B27 sustained enzyme activity and may mitigate the effect of FGF. In conclusion, the effect of FGF was independent of the culture conditions and was promoting a reduction in the β-galactosidase activity. The decrease in the value of the medians indicates that FGF affects the entire distribution, since the whole peak shifted.

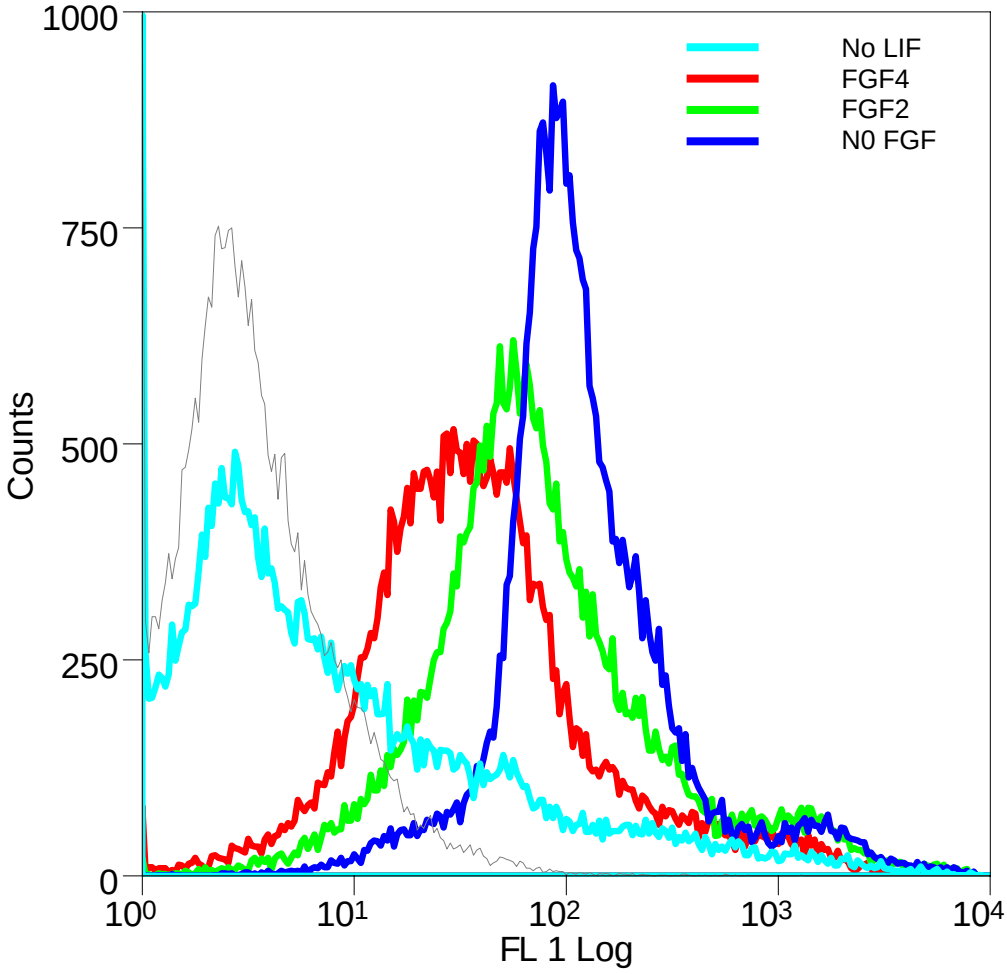
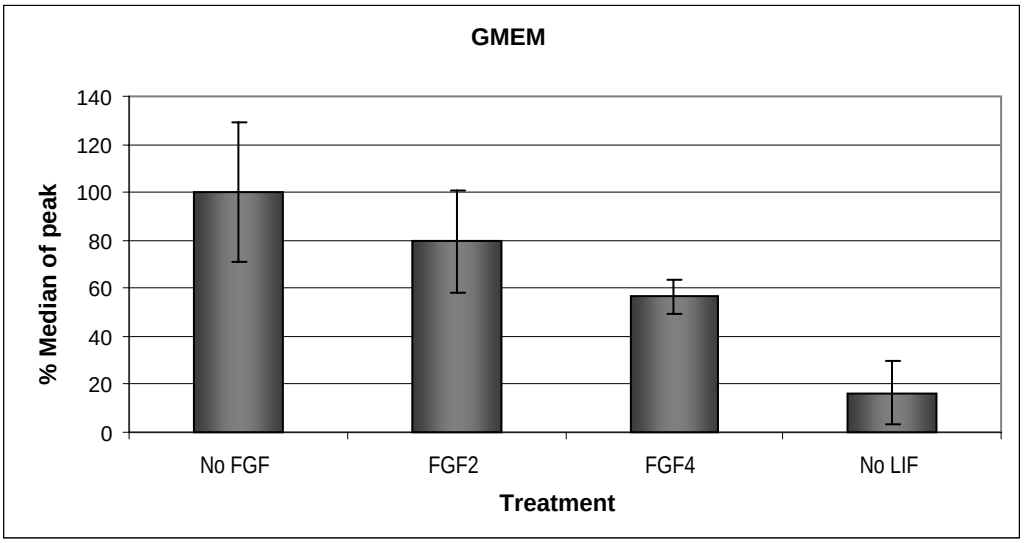


Figure 3.11. Effect of FGF addition on FDG conversion. The *LacZ+*, cell line IOUD2, was maintained for 6 days in the presence of LIF alone (blue), LIF and FGF2 (green) or FGF4 (red) or LIF absence (light blue). The HM1, a *LacZ-* cell line (grey), did not convert the FDG substratum. A decrease in the fluorescence emission was observed in the presence of FGF2 and more evidently in the presence of FGF4. As expected the reduction was major in the absence of LIF. The histogram is formed with data from one experiment and is representive of the results obtained in 4 independent experiments which are shown in appendix 1.

A



B

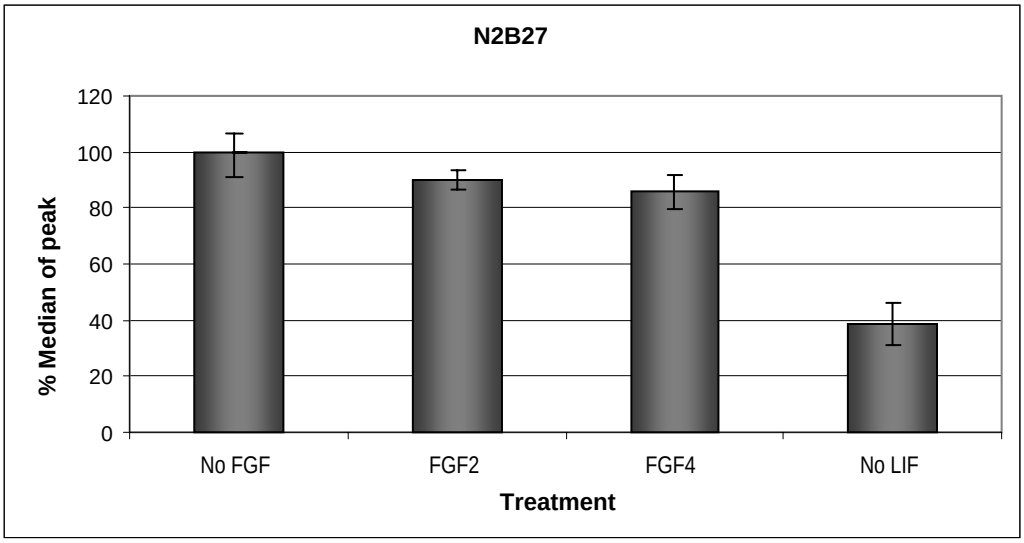


Figure 3.12. Normalised percentage of the FDG conversion in GMEM (A) and N2B27 (B) in the presence of FGF and LIF. The values were normalised at 100% with the cells in the presence of LIF alone. Error bars represent the S.D. of 3 replicates in 4 different experiments **(A)** and 3 replicates in 2 experiments **(B).** Both cases showed a more distinct decrease in the FDG conversion in the cells treated with FGF4, which in the case of the standard conditions was significantly lower ($p<0.001$) than in the other treatments. Two way analysis of variance used for generation of the p value.

3.4.4 Validation of the *LacZ* as reporter of OCT4

3.4.4.1 Western Blotting

In order to verify that *LacZ* was reliably reporting the changes occurred in *Oct4* levels, the expression of the OCT4 protein was tested directly by immunoblotting (Figure 3.14). As a control of the sensitivity of this assay, series dilutions of the lysate protein of the samples corresponding to 100U/ml of LIF, in the absence of FGF, was firstly immunoblotted (Figure 3.13A). This showed that the limit of detection of OCT4 was about 20% of normal levels. The quantification by densitrometry of the level of protein (Figure 3.13B) showed a direct proportional increase of protein to the increase amount of cell lysate.

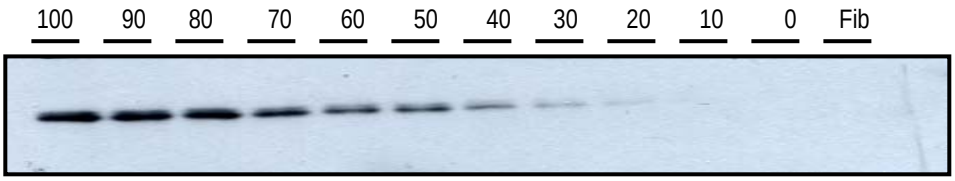
Once the conditions were established, to directly assess the levels of the OCT4 protein, a western blot was carried out. The cells were maintained in the following conditions: LIF alone (100U/ml), LIF and FGF (25ng/ml) and in the absence of FGF and LIF. In addition to this, a sample of fibroblast lysate was added as a negative control of OCT4 expression. After 6 days in culture the cells were lysed with Ripa buffer and the protein quantified and normalised. The immunoblot was probed with OCT4 antibody and reprobed with SHP2 antibody as a loading control (Figure 3.14A).

The western blot showed a reduction in OCT4 in response to FGF, which confirms the decrease of the reporter shown in the ONPG and FDG assays. OCT4 as expected was not detected in mouse fibroblasts. The levels of protein were quantified by densitometry

and the results normalised to SHP2 levels (Figure 3.14B). The results were normalised against the control cells (LIF alone). FGF2 treatment produced a decrease of 20% and FGF4 a reduction of 44% in the OCT4 levels. The OCT4 protein expression in the cells in the absence of LIF dropped 85.5%.

These results were comparable to the ONPG and FDG assays in terms of relative levels between treatments. However, the lower decrease in the ONPG assay reflects a reduction in the effect by the protein normalisation. Therefore, the cell population presents high protein levels implying that FGF is not reducing cell growth. In conclusion, the western blotting shows that FGF4 and to a lesser extent FGF2, caused the reduction in the levels of OCT4 protein correlating with the results in the reporter assays. Reduction in OCT4 protein is consistent with the differentiating effect of FGF.

A



B

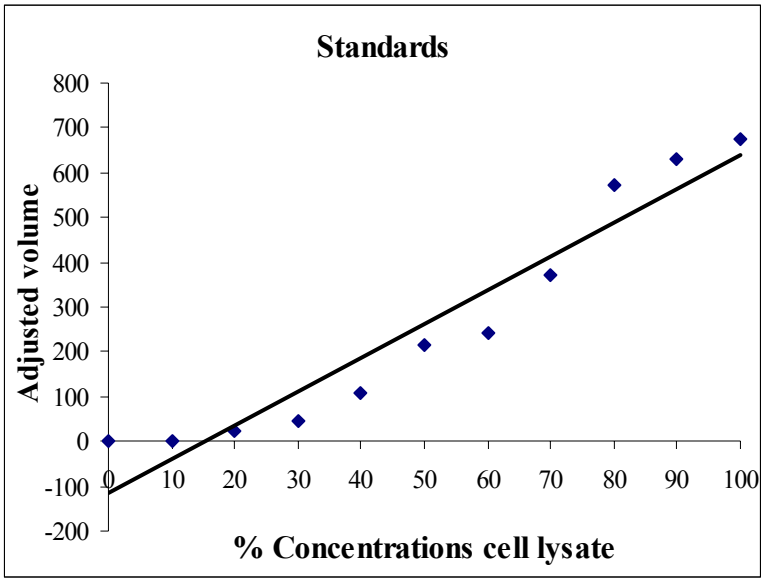


Figure 3.13 Immunoblotting and densitometric quantification of series dilution of mES cell lysate grown in 100U/ml LIF and without FGF. A: The series dilutions of the samples were immunoblotted and probed with an antibody that recognises OCT4 protein. Fibroblast lysate was included as a negative control. **B:** The levels of protein were quantified by densitometry showing a directly proportional increase in the levels of protein to the percentage of cell lysate loaded. This was linear for the concentrations between 30% and 80% of cell lysate.

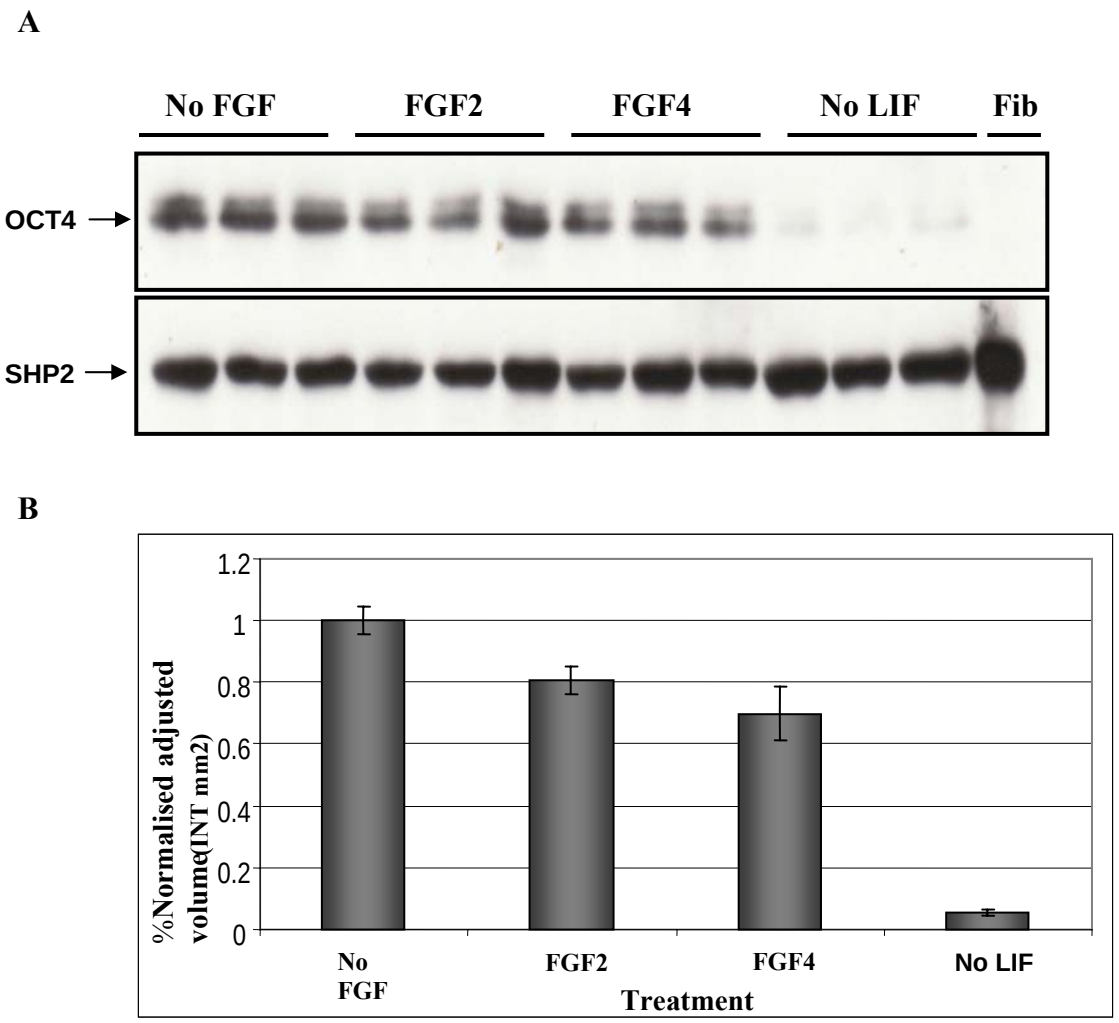


Figure 3.14. Effect of FGF on the levels of OCT4 protein assessed by immunoblotting (A) and quantification and normalisation of the adjusted volume of OCT4 to SHP2 levels (B). Cells were maintained in LIF alone (No FGF), with FGF2, FGF4 and LIF in the absence of growth factors. Fibroblast lysate (Fib) was used as a negative control of OCT4 expression. After 6 days in these conditions, the cells were lysed and the protein levels normalised. **A:** The immunoblots were probed with a specific antibody for OCT4 and after stripping, the membrane was reprobed with an antibody that recognises the total SHP2 protein, as a control of an equivalent amount of loaded protein. The triplicate samples correspond to 3 independent wells with the treatment specified above. **B:** After scanning the film, the levels of OCT4 and SHP2 were quantified by densitometry and normalised. The results shown here are mean values of three independent wells and the error bars representing the standard deviation.

3.4.5 Effect of FGF on the morphology of mES cells

Preceding results demonstrated a suppressive effect of the FGFs on the OCT4 levels of mES cells. To establish the effect of OCT4 reduction in mES cell morphology, FGF at 25ng/ml and LIF at 100U/ml were added to the cells and after 6 days, the cells were fixed and X-gal stained (Figure 3.15A). Cell staining showed that colonies of mES cells looked more compact and darker stained in the presence of LIF alone. FGF supplemented cultures produced flattened and more diffuse colonies with a paler staining, principally in the presence of FGF4. In confirmation of the previous assays, these effects were evident at two high concentrations (100U/ml) of LIF, a routinely used and a near saturating concentration of LIF respectively.

In addition to this, cells maintained in the absence of LIF with or without FGF4 supplement were X-gal stained (Figure 3.15B). In the absence of factors a few dark small colonies of ES cells remained, whereas in the presence of FGF4 the staining appeared lighter and in a diffuse manner. This could be indicative of the fact that even in the absence of LIF some ES colonies still remained, whereas FGF4 addition induced an overall differentiation. A larger number of pale stained colonies in the presence of FGF4 could indicate the presence of intermediate cells in the process of differentiation. These X-gal results are in agreement with the findings of ONPG, FDG, and western blotting and could be indicative of a direct effect of FGF on the mES cells.

In all the experiments, FGF was supplemented with heparin (1μg/ml), which has been reported to support oligomerisation of the growth factors and stabilisation of the complexes with FGFRs (Plotnikov *et al.,* 1999). To exclude heparin as the reason in the morphological changes and decrease of OCT4, the effect of FGF4 was assessed in the presence or absence of heparin by X-gal staining (Figure 3.16).

The addition of heparin alone did not decrease the activity of the enzyme, which contrasts with the inhibitory effect of the FGFs alone. X-gal staining showed that heparin addition seems to have an effect on the compactness of the colonies but the principal factors are the FGFs.

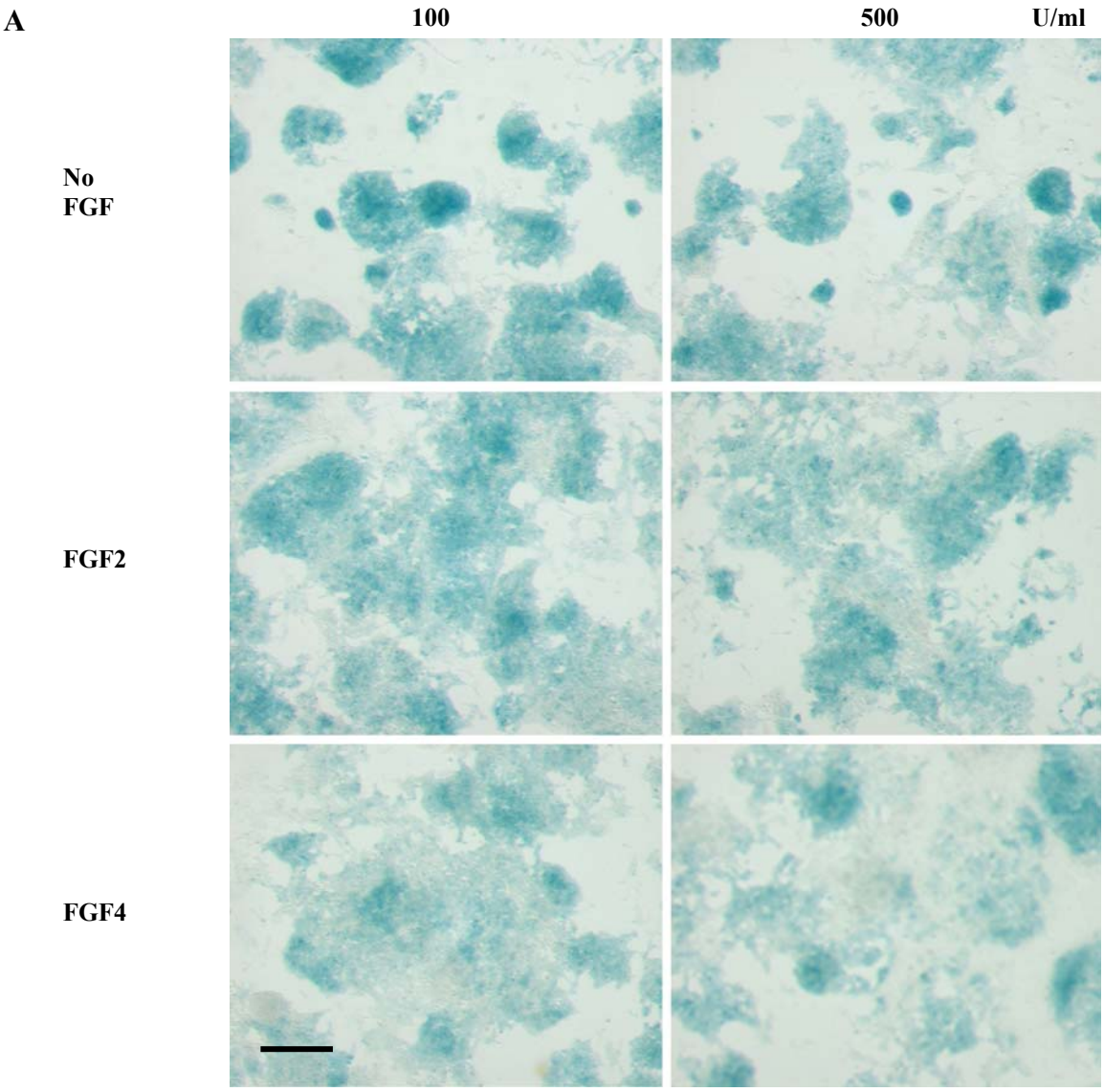


Figure 3.15 The effect of FGF on *LacZ+* mES cells in the presence/absence of LIF. A: IOUD2 cells were X-gal stained following 6 days incubation of the cells with LIFalone (100U/ml) or with 25ng/ml FGF2 or FGF4 (scale bar = 200μm).

B

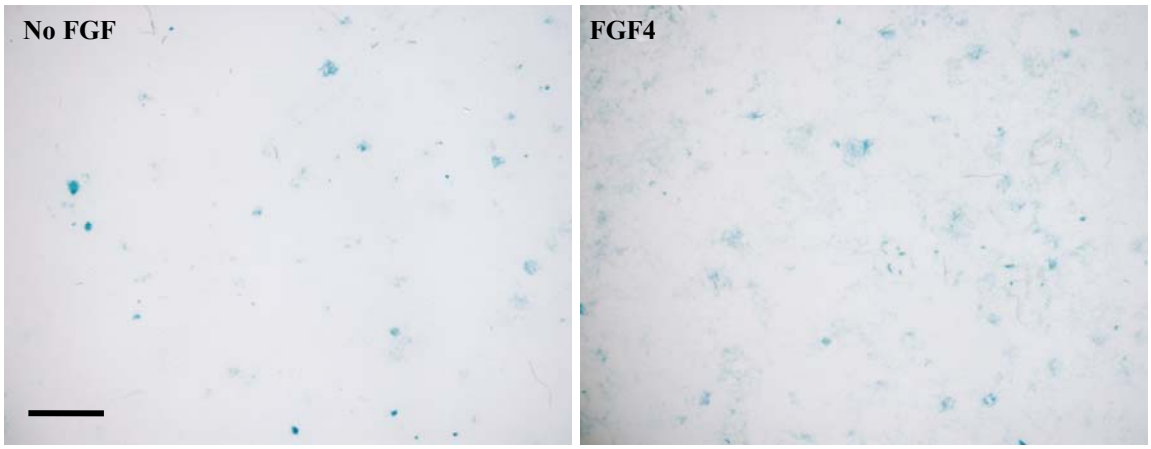


Figure 3.15 The effect of FGF on *LacZ+* mES cells in the presence/absence of LIF. B: Cells were grown for 6 days in the absence of LIF with or without FGF4 (25ng/ml) (scale bar = 500μm).

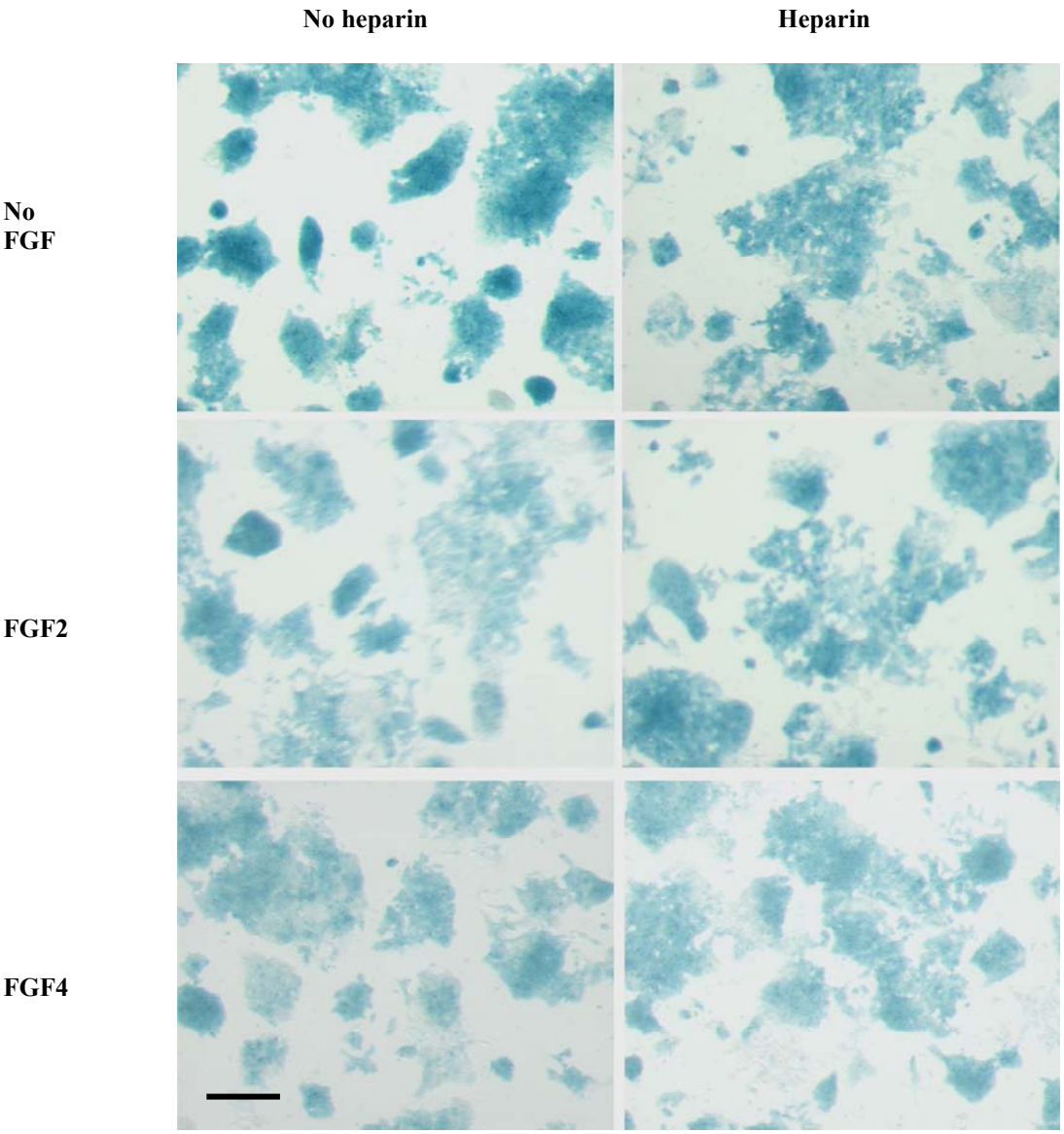


Figure 3.16 Effect of FGF on *LacZ*+ mES cells in the presence/absence of heparin. IOUD2 cells were X-gal stained following 6 days incubation of the cells with or without heparin in the presence of LIF (100U/ml), in the absence of growth factor or in conjunction with FGF2 and FGF4. The decrease in the β-galactosidase activity was more evident in the presence of FGF (scale bar = 200μm).

3.4.6 Effect of FGFR inhibition by SU5402

It has been shown that SU5402 inhibitor decreased activated phospho ERK1/2 in mES cells (Kunath *et al.,* 2007). Furthermore, another inhibitor of the FGFRs, PD173074 has been shown to block the effect of the addition of FGF to $FGF4^{-/-}$ cells, therefore preventing differentiation of these cells (Kunath *et al.,* 2007). To confirm the differentiating role of FGF on mES cells, the inhibitor SU5402, was used. This specifically interacts with intracellular catalytic domain of FGFRs (Mohammadi *et al.,* 1997). Cells grown in GMEM supplemented with LIF (100U/ml) and in the presence of FGF (25ng/ml) were exposed to the inhibitor SU5402 (20μM). Cells cultured in standard GMEM medium with LIF and the equivalent maximum concentration of the diluent, dimethylsulfoxide (DMSO) served as a control (Figure 3.17).

After six days of continuous exposure to SU5402 the colonies appeared compact and rounded with well defined boundaries contrasting with the more disperse and flattened morphology of those maintained with only the FGFs. This effect was observed from early stages of the treatment. Interestingly, the phenotype of the cells in presence of the inhibitor was different than that of the cells without exogenous FGF. This indicated the action of autocrine FGF in spite of the daily media change. The effect of DMSO is shown by the presence of some loose cells, which suggested that the diluent is opposing the effect of the inhibitor. In conclusion, inhibition of FGF signal prevented the emergence of the typical morphology of differentiating cells in mES cells and confirmed the activity of autocrine FGF4 on mES cells (Rathjen et al., 1999).

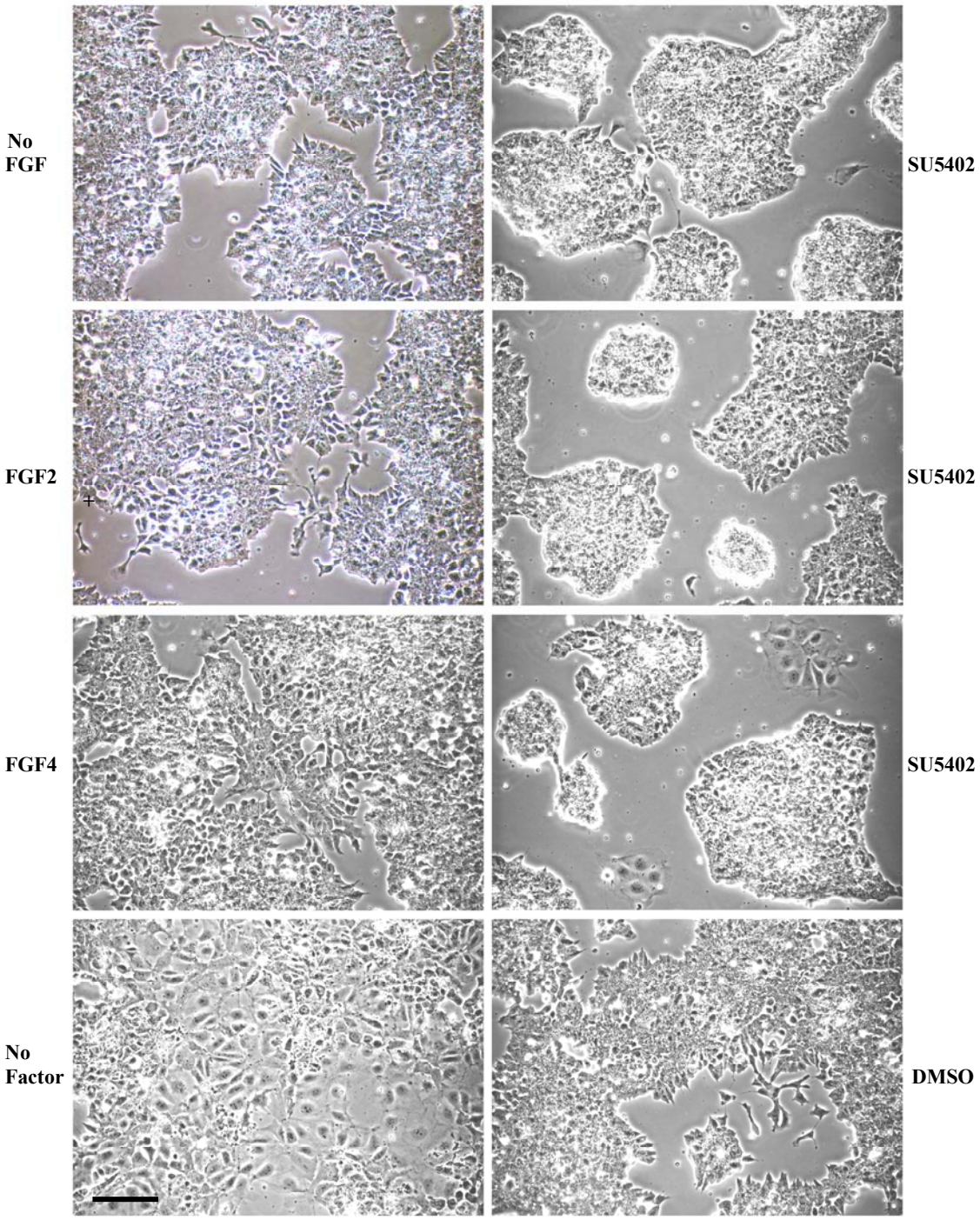


Figure 3.17 Effect of the inhibition of FGFR by SU5402 (SU) on mES cell morphology. IOUD2 cells were cultured in a standard medium with LIF (except "No factor") and 25ng/ml of FGF2 or FGF4 were added (except to "DMSO"). After six days of cell exposure to 20μM of the FGFR inhibitor SU5402, the colonies were tight and well defined contrasting with the flattened morphology in the presence of FGF. Cells in the absence of LIF, as expected, appeared differentiated (scale bar = 200μm).

3.4.6.1 Is FGF signalling required for mES cell differentiation?

To establish whether FGF was a required signal for mES cell differentiation, the inhibitor of the FGFR, SU5402 was used on IOUD2 cells maintained in the absence of LIF. In addition to this, cells were also grown in the absence of factor, with SU5402 and LIF, and LIF and FGF4 at the concentrations described above. The cells were maintained in these conditions for three days with a daily media change.

Inhibition of FGF signal, in the absence of LIF, resulted in a compact colony morphology, which contrasted with the stretched and flattened colonies that formed when the cells were starved of LIF and the FGF signal was not blocked (Figure 3.18). The morphology associated with differentiation observed in the absence of LIF was more noticeably induced when FGF4 was added to the LIF starved cells. In the presence of LIF, FGF4 seemed to loosen the colonies. In conclusion, FGF signalling seemed to be required for mES cell differentiation and therefore, it might have a role in early differentiation in the murine system.

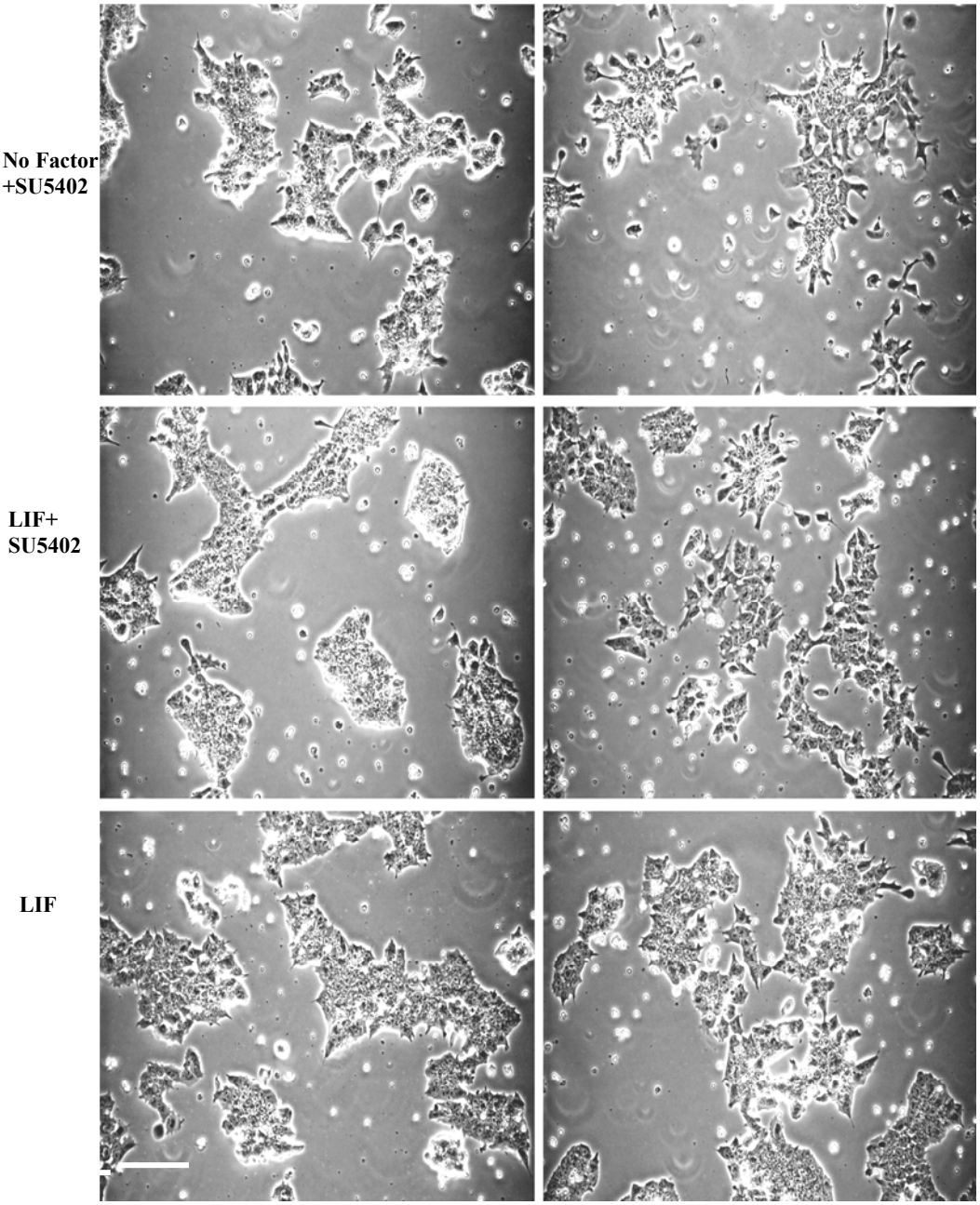


Figure 3.18. Effect of inhibition of FGF signal in the absence of LIF. IOUD2 cells were supplemented with SU5402 in the absence of LIF (No factor+SU5402) and in the presence of LIF (LIF+SU5402). Cells were also maintained in the absence of factor, in only the presence of FGF4 (No LIF+FGF4), only LIF and in the presence of LIF and FGF4 (LIF+FGF4). Cells without FGF signal seem to lose the capacity for differentiation in the absence of LIF (scale bar = 200μm).

3.4.7 Characterisation of the cells treated with FGF

To establish whether the FGF associated reduction of OCT4 observed in the previous experiments, resulted in the differentiation of the IOUD2 cells into a particular cell lineage, the expression of specific genes to the three different lineages was analysed. In addition to this, the expression of genes related to pluripotent stem cells was also examined. *Clathrin* was the housekeeping gene used as a loading control and a minus RT control was used to test genomic DNA contamination. The results shown in Figures 3.18 and 3.19 are representative of repeated experiments.

3.4.7.1 Expression of differentiation-associated genes

To examine changes in gene expression, cDNA from mES cells maintained for six days in the previously reported conditions (100U/ml LIF, LIF and FGF2 or FGF4 (25 ng/ml) or no factors) were analysed by PCR. All the conditions were analysed in duplicate samples from independent wells. In addition to this, a cDNA sample from day nine embryoid body (EB) cultures was added as a control for differentiation. The expression of the genes *Fgf5* and *Pax6* (ectoderm), *Brachyury* (mesoderm), *Afp* and *Gata6* (endoderm) were evaluated (Figure 3.19).

Fgf5 (primitive ectoderm marker) expression was detected in all the conditions and there was a slight increase in its expression in the samples cultured in the presence of FGF4 or in the absence of LIF. The other ectoderm marker, *Pax6* was up regulated in the presence of FGF4, which suggested an induction towards the ectoderm lineage by FGF4.

Expression of these two genes, *Fgf5* and *Pax6* was also detected in the control cells (LIF alone). Up regulation of these ectodermal markers could be a consequence of the expontaneous differentiation caused by the plating of the cells at low density. The expression of the other genes, *Brachyury, Afp* and *Gata6* was not affected by the action of the FGFs. LIF withdrawal induced the up regulation of *Gata6*, *Brachyury* and *Fgf5*. In the EB sample, up regulation of four of the five genes analysed was detected: *Fgf5, Brachyury, AFP* and *Gata6*. By contrast, *Pax6* appeared to be expressed at a lower degree in the EB and the samples without any factor than in the controls. This could be due to an earlier temporal expression of *Pax6* in differentiation. *AFP* was exclusively expressed in the EBs, as it has been shown that *AFP* is not expressed until day 7 of differentiation (Cai *et al.,* 2006). In conclusion, the addition of FGF4 to mES cells seemed to induce markers of the ectodermal lineage, even in the presence of LIF. Withdrawal of LIF resulted in non-selective differentiation into all the lineages.

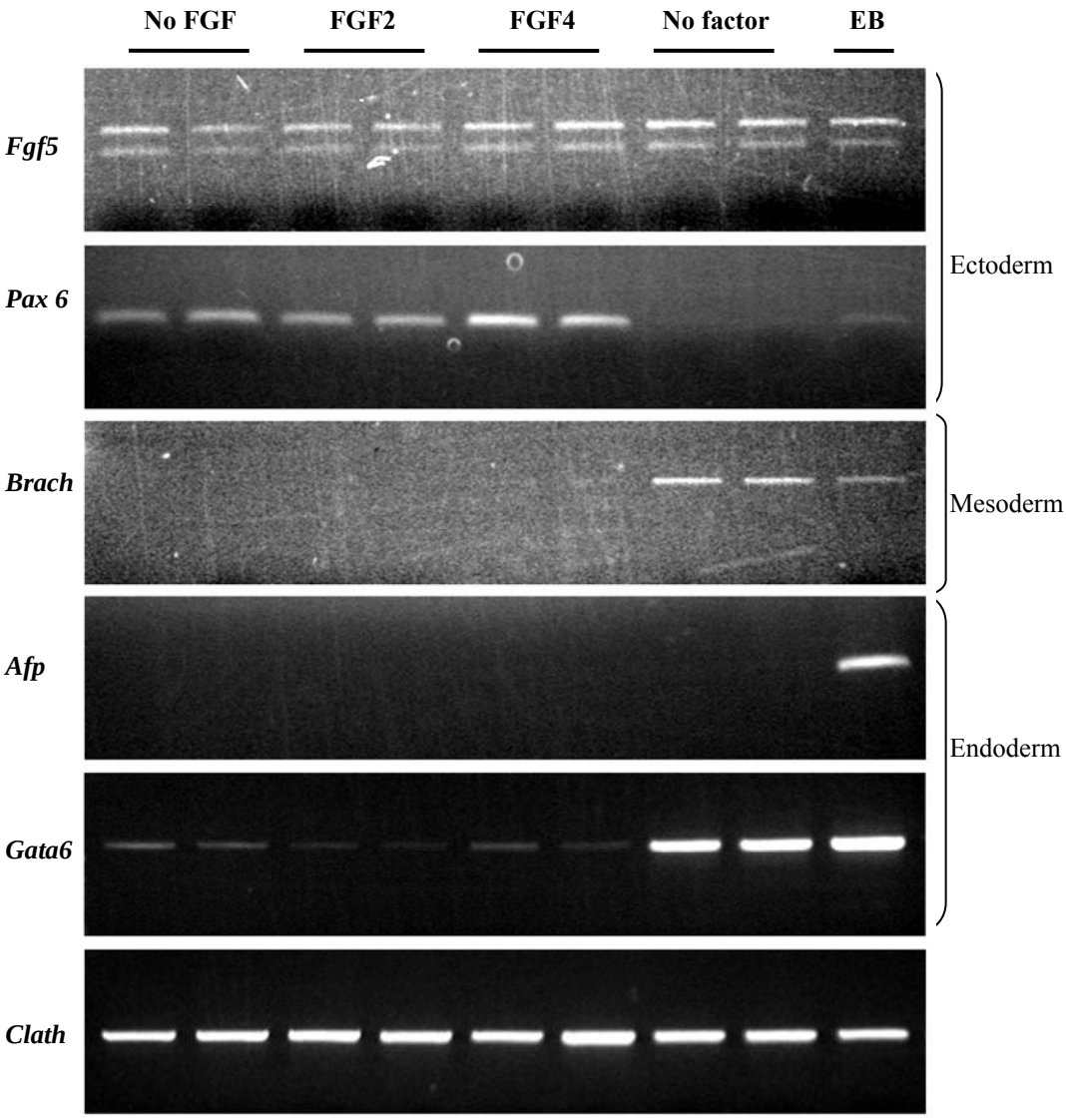


Figure 3.19. RT-PCR analysis of gene expression in IOUD2 cells. RNA was extracted from IOUD2 cells following 6 days of culture treatment. cDNA was prepared and amplified with primer pairs specific for the genes shown. PCR products for the same marker were all run on the same gel. Cells were maintained for 6 days with LIF alone (100U/ml), with LIF and FGF2 or FGF4 (25ng/ml) and in the absence of factors. Sample EB corresponds to the control amplification with day 9 embryoid body. The housekeeping gene Clathrin was used as a loading control. Samples were obtained in duplicate from independent wells.

3.4.7.2 Expression of ES-associated genes

In addition to the differentiation-associated genes (Figure 3.19) the expression of genes related with pluripotency such as *Oct4, Nanog* and *Rex1* and the gene for the adhesion molecule *E-Cadherin* were assessed using the samples as above (Figure 3.20).

The RT-PCR results in Figure 3.20 show the reduction in expression of *Oct4*, *Nanog* and *Rex1* in the samples without any factor and in the EBs, but not when FGF was added. The regulation of *E-Cadherin* did not seem to be significantly altered by the addition of FGFs although the levels appeared slightly reduced in the absence of factor and in the EB samples.

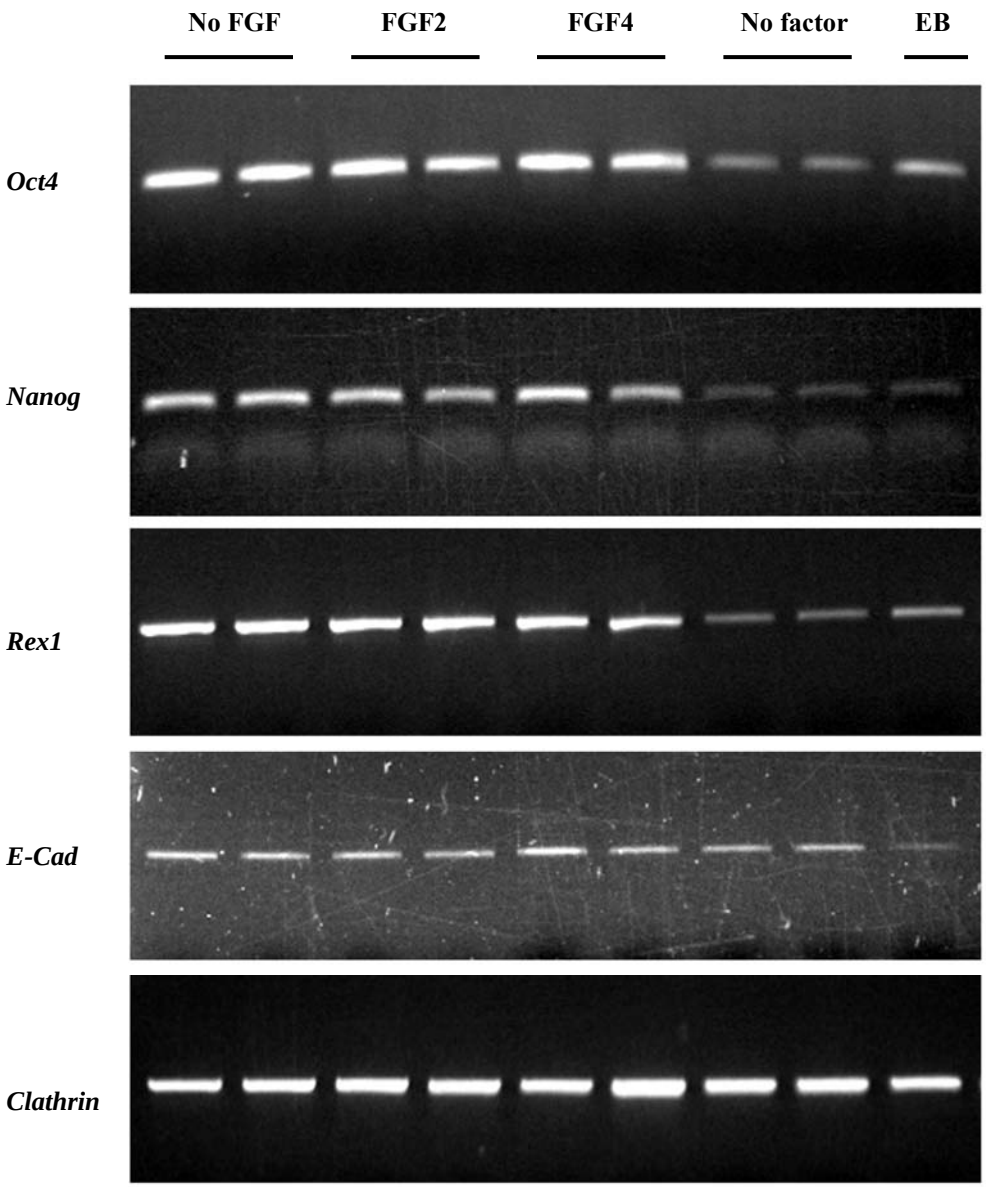


Figure 3.20. RT-PCR analysis of gene expression in IOUD2 cells. Following 6 days of treatment of the IOUD2 with the FGFs, the RNA was extracted; the cDNA was prepared and amplified with primer pairs specific for the genes shown. Sample EB corresponds to control amplification with day 9 embryoid body.

3.5 DISCUSSION

The findings reported here suggested that FGF4 promoted the reduction of OCT4 in mES cells; more specifically, treatment with FGF4 seemed to lead to up regulation of the marker of ectodermal differentiation *Pax6,* although this would have to be confirmed by quantitative RT-PCR. The role of FGF as a promoter of differentiation in mES cells differed from previous reports suggesting that FGF supported the growth and survival of the differentiated progeny without affecting the undifferentiated mES cells (Rappolee *et al.,* 1994; Wilder *et al.,* 1997; Rizzino *et al.,* 1988). Wilder *et al,* (1997) based this argument on the results obtained by comparing the differentiating capacity of mES cells with the *Fgf4* inactivated ($Fgf4^{-/-}$) against the wild type. Experiments *in vivo* showed that $Fgf4^{-/-}$ and $Fgf4^{+/+}$ gave rise to a similar mixture of differentiated tissues, indicating that the host animal could provide the necessary FGF. However, *in vitro*, the survival and proliferation of the differentiated cells was severely compromised by the inactivation of the *Fgf4* gene. Additionally, the data presented by Wilder *et al,* (1997) may imply a lack of the appropriate signals for differentiation in $Fgf4^{-/-}$ cells. The $Fgf4^{-/-}$ colonies presented a compact homogeneous undifferentiated morphology, even when the cells had been plated at clonal density. Furthermore, $Fgf4^{-/-}$ cells were only capable of differentiation when FGF4 or retinoic acid was added to the medium. This is in line with the necessity for FGF signals to induce the differentiation reported in this chapter. The maintenance of tight colonies in the absence of LIF when FGFR was blocked by the chemical inhibitor SU5402, suggested the requirement of FGF signal for mES cell differentiation. This contrasted with the stretched colonies associated with

differentiation when the inhibitor and LIF were not added. The flattening of the colonies and loose cells could also be observed when FGF4 was added in the presence of LIF. A quantitative RT-PCR analysis of these cells treated with the FGFR inhibitor, along with the samples to which FGF had been supplemented, would have highlighted the specificity of the effect of FGF on differentiation, such as was the case with the up regulation of Pax6.

X-Gal staininig showed that FGF produced a flattened morphology of the colonies in association with a decrease in the levels of the stem cell reporter OCT4**.** Interestingly, this reduction also happened in the presence of the inhibitor of differentiation LIF, which could be indicative of the direct role of FGF4 as a promoter of differentiation of mES cells. Mouse ES cells express FGF and LIF receptors implying that these two signals can happen simultaneously. Further support for this direct role, was in the loss of strong *LacZ*-expressing colonies when FGF4 was supplemented to a LIF free medium. Conversely, some of those strong *LacZ* colonies were observed in the absence of LIF when FGF4 was not added. It would be of interest to further analyse whether the morphological changes observed were the cause or the consequence of the differentiating process that the cells were undergoing. For example, further studies would clarify whether the observed flattening of the cells induced by FGF have a role in the process of differentiation. Among the adhesion molecules, which could be involved in this process (fibronectin, laminin and heparins), only *E-Cadherin* levels were assessed and the morphological changes observed in the presence of FGF could not be related to changes in the levels of *E-Cadherin*. Furthermore, FGF4 stimulation of the secretion of

specific enzymes (gelatinase and plasminogen activator) (Rappappolee *et al.*,1994) associated with the differentiation of embryonic stem cells (Behrendtsen *et al.,* 1992) could have a role in the observations reported in this chapter.

The levels of the *Oct4-LacZ* reporter were further analysed by chromogenic and fluorogenic methods of quantification, ONPG and FDG respectively. Using a reporter of the stem cell marker OCT4 was a powerful tool to quantify self-renewal and therefore they were chosen to the alkaline phosphatase staining. Moreover, ONPG and FDG are standard methods used for assessing the levels of mES cell self-renewal by measuring the levels of β-galactosidase activity. The ONPG assay has been used to establish that inhibition of ERK and SHP-2 signalling promoted mES cell self-renewal (Burdon *et al.,* 1999b). In the case of FDG, this assay has been applied recently to determine the differentiation and self-renewal properties of subpopulations of mES cells with a heterogeneous expression pattern of NANOG (Singh *et al.,* 2007). The validity of the reporter was confirmed by direct measurement of the levels of the OCT4 protein by western blotting. Western blotting was preferred to the analysis of OCT4 by flow cytometry because when this method was applied, unspecific staining of the negative control fibroblast was detected. Further stainings were performed on the mES cell line ZHBtc4.1 cells that carry two null Oct4 alleles modulated by a doxycycline-responsive Oct4 transgene (Niwa *et al.,* 2000). In this case, both the treated and untreated cells showed similar levels of OCT4 staining although western blotting established that only the cells that had not been treated with the drug expressed OCT4.

In this thesis, ONPG and FDG methods confirmed that β-galactosidase activity in ES cells was dependent on the dose of LIF, which is consistent with a previous report by Smith *et al,* (1988). More significantly was the reduction in the expression of the OCT4 reporter when FGF was added. This was consistently quantified by the ONPG and FDG assays. In fact, the FDG assay indicated that the decrease in the levels of β-galactosidase occurred in the entire population rather that in a more differentiated fraction of the cells. This result suggested a direct effect on the ES cells, which is consistent with the reduction in enzymatic activity observed at high concentrations of LIF. Therefore, ONPG and FDG assays confirmed the morphological studies and contrasted with previous reports (Rappolee *et al.,* 1994; Wilder *el al.,* 1997; Rizzino *et al.,*1998; Viswanathan *et al.,*2003). It should be noted, results from the FDG assay revealed that a reduction in the OCT4 reporter was caused by a decrease in self-renewal rather than a reduction in cell proliferation, since an equal number of cells were used in all the FDG experiments. FGF induced reduction of OCT4 protein is consistent with the decrease in the OCT4 reporter activity. Densitometry quantification verified a significant decrease of 30% between the FGF4 samples and the controls.

This reduction in protein levels, however, was not reflected by the *Oct4* gene expression, which indicates the limited sensitivity of the non quantitative RT-PCR. Down-regulation of *Oct4* and the other ES cell associated genes analysed *Nanog* and *Rex1,* was only detected when LIF was withdrawn. On the other hand, the effect of FGF in the morphological changes and in the reduction of OCT4 did correlate with a slight increase in *Pax6*. The up regulation of *Pax6*, a marker associated with ectoderm lineage,

contrasts with reports of FGF4 promoting primitive endoderm (Wilder *et al.,*1997; Esner *et al.,*2002) and mesoderm (Ciruna and Rossant, 2001) differentiation in mES cells. This discrepancy in the reports could be a consequence of the different approaches used, that is exogenous FGF was added in this thesis, instead of the inhibition of endogenous FGF signal performed in the reports above. However, more recently other groups have revealed the association of FGF and the ectodermal lineage in mES cell differentiation. It has been reported that FGF4 promoted mES cell neuroectodermal differentiation when the inductive signals for alternative fates were removed (Kunath *et al.,* 2007). Furthermore, a gene identified in the chicken embryo induced by FGF, Churchill (*chch*), has been found to inhibit the expression of the mesoderm marker *Brachyury* and to facilitate the ectoderm induction to neural fate (Sheng *et al.,* 2003).

The control cells also expressed the two ectodermal markers, *Fgf5* and *Pax6*, which could be a response to the low density at which the cells were seeded at the beginning of the experiment. Cell exposure to autocrine differentiating signals, as a consequence of their maintainance for 6 days in the same medium, could also induce the expression of these markers. Expression of *Fgf5* is used as an early marker of ES cell differentiation since it is up regulated by LIF withdrawal, and is associated with the formation of a population corresponding to the egg cylinder in the epiblast (Rathjen *et al.,* 1999). *Fgf5* expression has been shown in pluripotent cells of the primitive ectoderm from 4.75 d.p.c (Pelton *et al.,* 2002) and also low levels have been found in ES cell cultures (Kunath *et al.,* 2007). This could be due to the progressive appearance of different pluripotent populations in the transition from ICM to primitive ectoderm (Pelton *et al.,* 2001).

In contrast to the up-regulation of *Pax6,* the addition of FGF had no effect on *Brachyury, AFP* and *Gata6* expression. This indicates the restriction in the choice to the ectoderm lineage when the mES cells were supplemented with FGF4. As mentioned above, the addition of FGF could have different outcomes than just the inhibition of FGF. For example, the absence of FGF signalling reduces the expression of *Afp* (Esner *et al.,* 2002), but supplementation of FGFs did not promote the expression of this endoderm specific gene. Furthermore, the differentiation method used in this chapter could have an impact on differentiation. For example, FGF was added in the presence of LIF, which by activating STAT3 resulted in the suppression of neurodermal differentiation. In addition to this, the adherent culture used in this report may be limiting the differentiation capacity to only one lineage, since it does not fully reflect embryonic differentiation. In addition to this, an adherent model perhaps results in the slower regulation of the genes and therefore, a wide range of different points in time would need to be assessed. Furthermore, it needs to be taken into account that maintained in culture, some active factors may be altering or promoting certain markers and/or restricting others.

The results of the PCRs seem to be in line with previous reports suggesting that O*ct4* and *Nanog* regulate differentiation by transcriptional repression of genes that promote differentiation (Mitsui *et al.,* 2003). However, this appears to occur only for certain lineages since down regulation of *Oct4* and *Nanog* correlated with the increased expression of *Gata6* and *Brachyury*. However, also within the pluripotent O*ct4* positive

cells, distinct genes associated with differentiation were detected. Cells with a strong expression of O*ct4* and R*ex1* also expressed *Pax6* and *Fgf5* and in lower level expressed *Gata6*, but did not express *Brachyrury* and *Afp.* This co-expression of typical ES cells and differentiation markers may be denoting a state of transition in the pluripotent cell population. A model for the transition from ICM to primitive ectoderm would be in agreement with the regulation of peri-implantation in pluripotent cell development by FGF signalling (Feldman *et al.,* 1995, Armand *el al.,* 1998). However, the resultant alteration of *Rex1* expression *in vivo* was not confirmed in this project.

The expression of the *Fgf4* gene in ES is controlled by OCT4 and SOX2 activation of *its* enhancer (Curatola and Basilico, 1990) and all the three factors are expressed simultaneously in pluripotent cells. It has been established that the maintenance of ES cells depends on the tight regulation of the levels of OCT4 (Niwa *et al.,* 2000). FGF4 has an essential role in the development of the mouse embryo and thus it should be also closely regulated. Therefore, when differentiating signals such as FGF4 are stronger than those promoting self-renewal such as STAT3, differentiation would occur. As shown by immunoblotting in this chapter, FGF induced ERK activation but did not activate STAT3. This contrasts with LIF, which activated both STAT3 and ERK. Activation of STAT3 is essential in the self-renewal of mES by possibly blocking ERK signalling (Burdon *et al.,* 1999b). Therefore, when both LIF and FGF act simultaneously, this balance is lost. This hypothesis could also be associated with the differentiation caused by OCT4 over expression, which is a similar differentiation to LIF withdrawal (Niwa *et al.,* 2000) or NANOG deletion (Chambers *et al.,* 2003; Mitsui *et al.,* 2003). This could

be caused by the over stimulation of FGF4 by high levels of OCT4, which in turn could increase FGF autocrine signalling, promoting differentiation. Furthermore, this could be related to a recent report which attributes to FGF a role in NANOG regulation (Hamazaki *et al.,* 2006). FGF through the activation of MAPK/ERK pathway repressed NANOG expression, resulting in endoderm differentiation (Hamazaki *et al.,* 2006). The divergence in the lineages up-regulated by FGF in the report by Hamazaki et al, (2006) and this thesis could be caused by the different culture conditions. The high concentrations of LIF used here could be repressing an endodermal differentiation. It would have been of interest to assess whether the down-regulation of OCT4 by FGF was through the modulation in NANOG expression. In particular, assessment of different points in time in *Gata6* expression may clarify the involvement of NANOG in the process.

At the time of writing this thesis, two reports (Stavridis *et al.,* 2007; Kunath *et al.,* 2007) regarding the functions of FGF in mES cells were published. Both reports confirm the role of FGF4 and to a lesser extent FGF2 in the induction of mES cell differentiation into ectoderm lineage. More specifically, Kunath *et al.,* and Stavridis *et al.,* (both 2007) established that FGF4 is the initiator of neuroectodermal commitment, indicating the role of FGF4 in earlier stages of differentiation. In addition to this, and in agreement with this thesis, Kunath *et al,* (2007) established the differentiative role of FGF. *Fgf4*$^{-/-}$ mES cells remained in an undifferentiated state, even upon LIF withdrawal, instead of selecting an alternative commitment program when FGF4 was not added (Kunath *et al.,* 2007). Kunath *et al,* (2007) also demonstrated that the requirement for FGF was

selective, since the addition of FGF5 failed to restore the neural differentiation of *Fgf4*$^{-/-}$ mES cells. Among the three pathways activated during neural specification (PI3K, phospholipase C gamma (PLCγ) and MAPK/ERK) (Stavridis *et al.,* 2007), it was established that it is the FGF/ERK pathway that initiates neural specification in mES cells (Kunath *et al.,* 2007) and in the embryo (Stavridis *et al.,*2007). In addition to ERK, it would be interesting to get further insight into the role of PI3K in the process of differentiation. FGF/PI3K pathway has been shown to be necessary for differentiation of EBs (Chen *et al.,* 2000). In addition to this, PI3K/AKT regulates numerous cellular processes such as cytoskeletal rearrangement that could also be associated to differentiation. Finally, Kunath *et al,* (2007) proposed that FGF activation of ERK induced a transitory state in the mES cells in which pro differentiating signals could specify the cell commitment. This implied the necessity for controlling FGF signals in order to sustain pluripotency. The transcription factors OCT4, SOX2 and NANOG are the suppressors of those inductive signals (Silva and Smith, 2008). The results from this chapter are in line with this model, in that a FGF4 reduction of OCT4 caused a decrease in resistance to differentiation and those cells with reduced OCT4 were "open" to signal inducers of lineage commitment. A schematic representation of this model is shown in Figure 3.21.

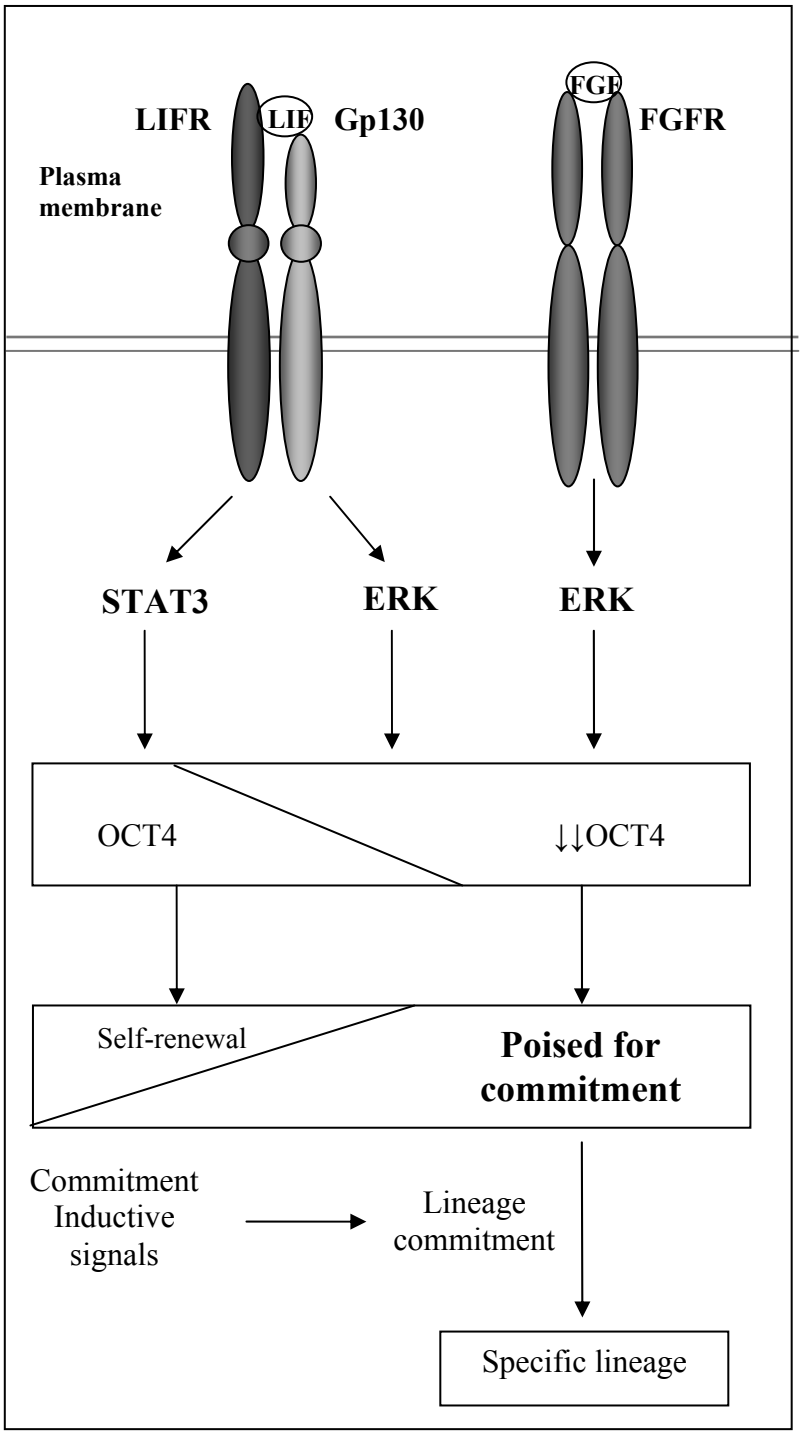


Figure 3.21 Regulation of mES cell self-renewal. OCT4 is maintained during LIF activation of STAT3. OCT4 in these conditions maintains self-renewal by suppressing differentiative signals. FGF increased ERK activity, causes the reduction in OCT4 levels and provides the signal to prepare the mES cells to accept inductive signals for commitment.

CHAPTER 4

CHARACTERISATION OF THE CONDITIONS IN WHICH TO ANALYSE THE EFFECTS OF FGF2 ON HUMAN ES CELLS

4.1 Introduction

Human (Thomson *et al.,* 1998) and mouse (Evans and Kuffman, 1981) ES cells were initially isolated and propagated on irradiated mouse embryo fibroblast (MEFs) feeder cells, in a medium that contained serum. In mouse ES cell propagation the principal function of the feeder layer was to supply the cytokine LIF. LIF signalling activates the transcription factor STAT3, which is the key event to sustain mES cell self-renewal (Niwa *et al.,* 1998; Matsuda *et al.,* 1999). The identification of LIF as the required cytokine in mES cell self renewal was a significant development in mES cell knowledge (Smith *et al.,* 1988). Consequently, mES cells could be propagated and maintained in a serum containing media to which the appropriate amount of LIF is added (Williams *et al.,* 1988). However, the requirements for hES cells propagation are more complex and although LIF can also activate the gp130/STAT3-signalling pathway, this mechanism cannot support the growth of the hES cells (Thomson *et al.,* 1998; Daheron *et al.,* 2004). The different signalling requirements for the self-renewal of mouse and human ES cells imply the existence of alternative factors to LIF released by the mouse embryonic fibroblasts (MEFs) and/or present in the serum to support hES cells self-renewal.

MEFs provide the substratum and the microenvironment for the maintenance and growth of undifferentiated hES cells. However, MEFs also add unknown factors to the culture system (Lim and Bodnar, 2002). This is a critical issue for the studies of this thesis, since the complexity of the system could cause confusion in the analysis of the effects of individual factors. Furthermore, the large amount of factors in this system, all

potentially highly variable, complicates the consistency of the growth conditions. In addition to this, the heterogeneity of the culture complicates the study of hES cells biology and biochemistry at the same time that contact with the MEFs can increase the risk of hES cells contamination with pathogens. Therefore, to minimise the effects of undefined factors in the growth of the hES cells, different culture systems have been developed. In the first feeder free method elaborated, hES cells were maintained on Matrigel coated plates with a culture medium that had been conditioned from mitotically inactivated MEFS and supplemented with FGF2 (Xu *et al.,* 2001). This system eliminated direct contact of hES cells with MEFs but still required the use of conditioned medium (CM) from MEFs. Therefore, the possibility of pathogen transmission caused by the mouse feeders when generating MEF-CM remained. Furthermore, it did not provide a solution to the complexity and variability.

Human feeders including human foreskin fibroblasts (Amit *et al.,* 2003) and human adult marrow cells (Chen *et al.,* 1999) were demonstrated to support hES cell growth. However, human serum also contained undefined components and it is variable in quality. Consequently, a serum free medium (SFM) medium produced by conditioning with human feeder cells and containing recombinant proteins has also been used to culture hES cells in a feeder free system (Li *et al.,* 2005). However, the use of an additional cell line still implied variability in performance. For these reasons it was necessary to develop a culture medium that was defined, contained only recombinant proteins and did not require conditioning with feeder cells.

The objective when developing a serum-feeder free medium is to replicate the conditions in which hES cells are propagated when maintained on the feeder cells through the addition of growth factors to a basal medium. Feeder cells are believed to support hES proliferation because they release factors that promote the growth and/or remove toxic or differentiating components from the medium. However, at the time of preparing this thesis, with the exception of FGF2, the active/s factor and specific mechanisms that sustained hES cells growth had not yet been identified. Feeder cells also secrete either protease inhibitors or binding proteins that adjust FGF2 stability, as suggested by the enhanced stability of FGF2 in CM (Levenstein *et al.,* 2005). Finally, another essential function of the feeders is the promotion of the extracellular matrix formation (Lim and Bodnar, 2002).

Culture systems have been developed to reproduce the culture conditions attained in conditioned media. The first serum free media containing only human sourced or recombinant proteins were developed for culturing human progenitor cells, mainly hematopoietic stem cells (Lam *et al.,* 2001). One of the earliest feeder and serum free culture system for hES cells made use of serum replacement (SR) supplemented with FGF2 and TGFβ1 on plates coated with fibronectin (Amit *et al.,* 2004). This system did not require the conditioning of the medium, but undefined bovine sourced materials were still included. More recently another feeder free and defined system has been reported in which the growth of the hES cells was achieved by adding high concentrations of hFGF2 (80ng/ml). However, the total composition of the medium was not published (Li *et al.,* 2005). During the preparation of this thesis, other groups have

reported increasingly refined feeder free culture systems for hES cells. All these systems included FGF2 or/and growth factors from the TGFβ family in order to achieve hES cell self-renewal (Wang *et al.,* 2005; Beattie *et al.,* 2005; Xu *et al.,* 2005a; Xu *et al.,* 2005b). However, in these systems the serum has been substituted by knockout serum replacement (KSR) which is still undefined and of animal origin. More recently further developed chemical defined and animal free culture mediums have been reported (Beattie *et al.,* 2005; Yao *et al.,* 2006; Lu *et al.,* 2006; Liu *et al.,* 2006; Ludwig *et al.,* 2006b) These newly described systems have been demonstrated to support the growth of undifferentiated hES cells but still are not used routinely for hES cell culture. Nevertheless, they can help to clarify the requirements for hES cell self-renewal, pointing towards the significant factors and signalling pathways involved in the propagation of hES cells. For example, the medium developed by Ludwig *et al,* (2006), is a combination of FGF2, LiCl, GABA, pipecolic acid and TGFβ. LiCl (lithium chloride) activates the Wnt pathway and together with the presence of FGF2 and TGFβ, highlights the importance of activation of this three signalling pathways in hES cells (Amit *et al.,* 2004; Sato *et al.,* 2004; Xu *et al.,* 2005b; Yao *et al.,* 2006). In addition to this, GABA (γ-aminobutiric acid) was used because microarray assays indicated that GABA receptor is highly expressed in hES cells (Sperger *et al.,* 2003; Sato *et al.,* 2004) and stimulates proliferation of different tissues (Watanabe *et al.,* 2006). Pipecolic acid was included as it has been reported to enhance the response to GABA (Takahama *et al.,* 1986). Therefore, the use of these or similar factors can serve as a starting point for further optimisation of the culture conditions of hES.

4.1.1 Limitations of conditioned medium

The standard conditions for hES cells culture make use of conditioned medium (CM), which is highly complex due to the secretion of multiple growth factors by the fibroblasts (Wei *et al.,* 2005). In addition to the factors secreted by the fibroblasts, hES cells highly expressed and released many factors including FGF2 (Dvorak *et al.,* 2005) and FGF4 (Mayshar *et al.,* 2008). The broad expression of FGFRs in hES cells strongly suggested that there was an autocrine activity of these factors occurring (Dvorak *et al.,* 2005). The complexity of the CM is aggravated by multitude of proteins and soluble factors contained in the serum which can be both beneficial and detrimental to hES cells culture. The effects of CM complexity on hES cell culture is indicated by the difficulty in showing an unambiguous differentiated phenotype upon FGF2 withdrawal. This suggests FGF2 presence in the medium and is illustrated by the tight colonies indicative of hES cells after twelve days in FGF2 withdrawal (Figure 4.1). FGF2 presence in CM is not sufficient to maintain the proliferation of hES cells and routinely 4-8 ng/ml needs to be added to the CM. However, in spite of FGF2 supplement, hES cell cultures appear heterogeneous with great amount of stromal cells surrounding the hES cells (Figure 4.1). This suggests the presence of factors inducer of differentiation in the CM implying that the growth conditions in the CM are not ideal. A further consequence of the use of CM is the high variability in the ability to support hES cell proliferation throughout the different batches of serum and MEFs. The quality of the MEFs is also highly variable throughout the days when the medium is collected implying a high variability in the quality of CM. For these reasons and in particular because the presence of FGF2 in the

CM, any results obtained in a system including CM and serum could be questionable in the study of FGF2.

To be able to define the contribution of FGF signalling to hES cell self-renewal in short-term experiments using a define culture media was crucial. Such a medium would reduce any inconsistency in experiments associated with serum or MEF variability would allow defined and reproducible differentiation studies. This chapter reports the use of a semi defined culture system for hES cells composed of a serum free medium, which did not require conditioning with feeder cells and Matrigel. The use of this medium permitted preliminary studies characterising the effect of FGF2 on hES cells. It should be noted that there is a high expression of endogenous FGF2 in undifferentiated and differentiated hES cells. This endogenous FGF2 signal acts in an autocrine and intracrine fashion in undifferentiated hES cells and its inhibition results in differentiation (Dvorak *et al.,* 2005). In addition to FGF2, it has recently been reported that FGF4 and a new splice isoform (FGF4si) are secreted from undifferentiated hES cells as well as from mES cells but not from MEFs (Mayshar *et al.,* 2008). Mayshar et al, (2008) proposed that FGF4 promoted the undifferentiated growth of the hES cells, whilst FGF4si opposed its effect. This agrees with the pattern of expression, that is, whilst FGF4 is not expressed in late differentiation, FGF4si remains in those stages.

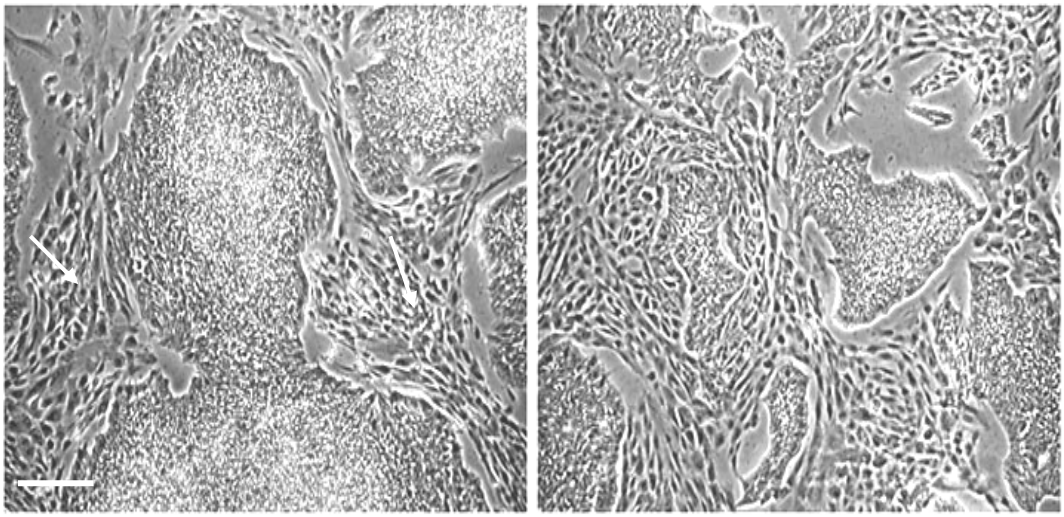


Figure 4.1. Human ES cells growing on Matrigel and CM. Morphology of the cell line H1 grown in CM in the presence or absence of FGF2. The withdrawal of FGF2 did not result in a complete differentiation after 12 days in culture, which indicates the presence of factors that support hES cell self-renewal. Asterisks highlight the colonies with a tight morphology associated with undifferentiated hES cells. Even when FGF2 is added to the culture there is a great amount of flat stromal cells (highlighted by arrows) surrounding the compact colonies of the hES cells, suggesting the inadequacy of the culture system (bar scale = 500 μm).

4.2 Hypothesis

That human ES cells could be propagated in semi-defined conditions and in this environment, hES cells still depend on FGF2 for their growth in an undifferentiated state.

4.3 Objectives

I To define the contribution of FGF signalling on hES cell self-renewal

II To develop a defined medium suitable for the study of FGF signalling on hES cells

III To characterise the hES cells growing under defined conditions

4.4 Experimental design

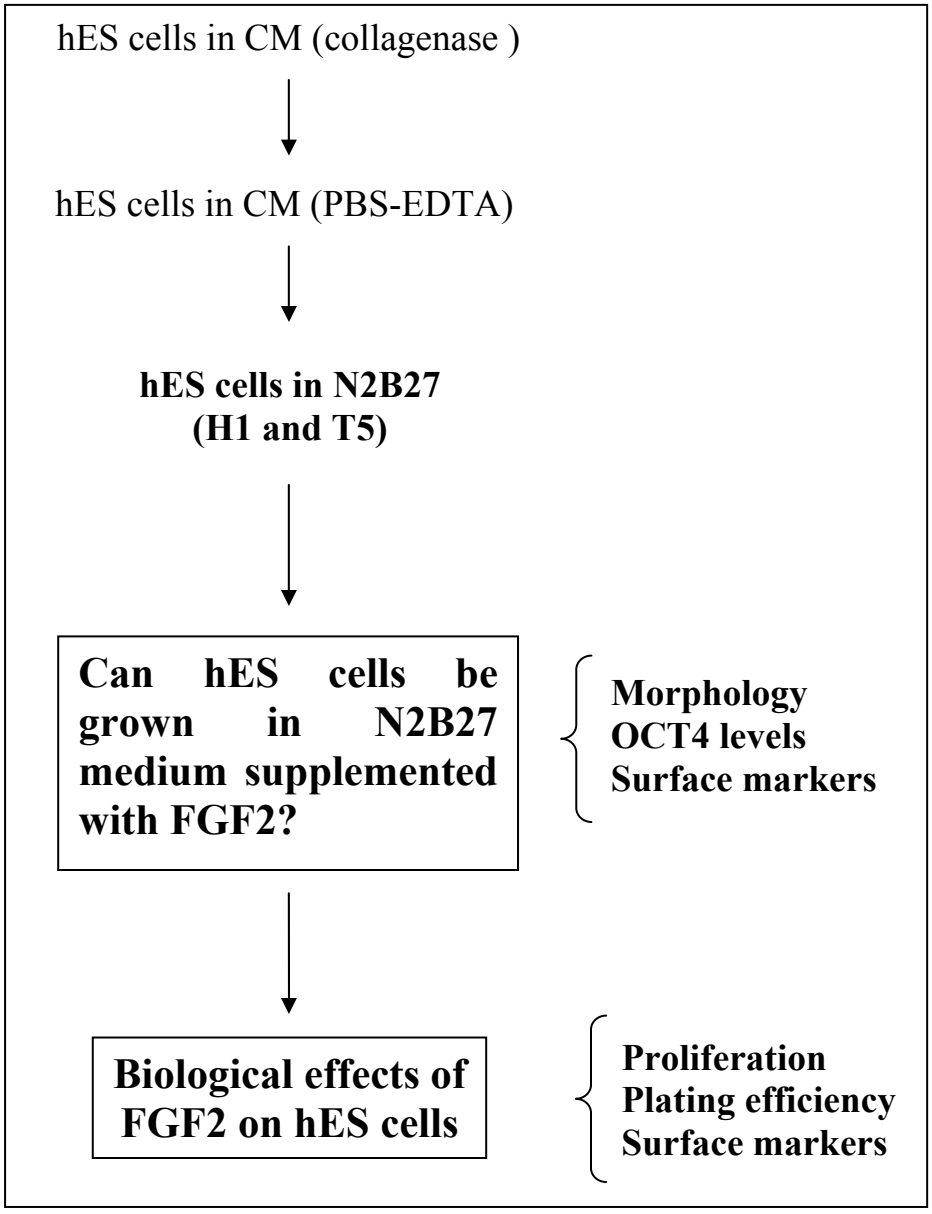


Figure 4.2. Diagram of the experimental steps followed in this chapter. Two cell lines T5 and H1 were transferred from CM to a defined culture condition where the effects of FGF in morphology, cell growth and self-renewal were studied.

4.4.1 The dissociation methods

In this study the hES cells were not grown on feeders but, because they require adhesion to an extracellular matrix for survival and growth, the hES cells were seeded on Matrigel. The cell culture was initially carried out in CM, using collagenase as method of harvesting. However, this typical method for the dissociation of hES cells produced low number of cells over long time. Because the experiments planned for this thesis depended largely on routine availability of moderate to large number of cells, the harvesting system was changed from collagenase to PBS-EDTA. PBS-EDTA has being used previously to transfections (Gerrard *et al.,* 2005) and is chemically defined. PBS-EDTA is used prior to transfection because this method produces flattened colonies in a monolayer, which makes easier the direct visualisation of any morphological change result of the treatments studied. Observation of the morphology is also improved by the clearer appearance of the colonies (Figure 4.3). Collagenase harvesting requires the manual scraping of the cells from the plate surface, generating big clumps of cells, whereas PBS-EDTA dissociaton is in smaller groups of 4-8 cells. This provided enough cells to prevent the differentiation caused by single cell dissociation that occurs when trypsin is used (Draper *et al.,* 2004). Furthermore, the use of PBS-EDTA improved the plating efficiency, enhancing the growth rate from the start and therefore larger number of cells was obtained (own observations).

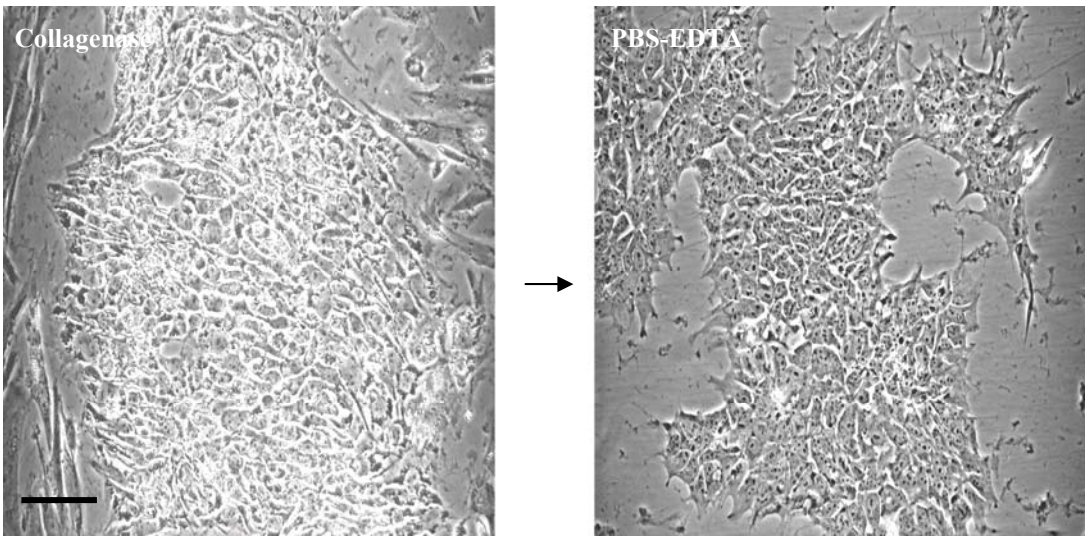


Figure 4.3. Morphology differences between hES cells harvested by collagenase and PBS-EDTA. The method of harvesting the hES cell line H1 was changed from the standard collagenase to PBS EDTA, in order to facilitate the visualisation of any changes in the cell morphology and accelerate the cell growth (scale bar = 200μm).

4.4.2 Cell lines

The experiments described here were performed on two hES cell lines, H1 (Thomson *et al.,* 1998) and the transgenic T5 (Gerrard *et al.,* 2005) cell lines. T5 cells had been derived from the H1 cell line by the transfection of an enhanced green fluorescence protein (EGFP) vector under the control of the *Oct4* promoter (Gerrard *et al.,* 2005). The insertion of the *Oct4* promoter upstream of EGFP strongly drives its expression and therefore the levels of the reporter provide a measurement of stem cell self-renewal in the T5 cells (Gerrard *et al.,* 2005). The system of the *OCT4*-EGFP reporter is equivalent tool to the OCT4-*LacZ* in the IOUD2 and Y118F cell lines but it has the advantage over OCT4-*LacZ* in that allows direct checking of *OCT4*-EGFP in living cells. Expression of the autofluorescent protein can be directly observed by confocal microscopy and quantified by flow cytometry, and fluorimetric assays. Therefore, T5 cell line was a powerful tool to study the effects of the culture conditions on the hES cells (Figure 4.4).

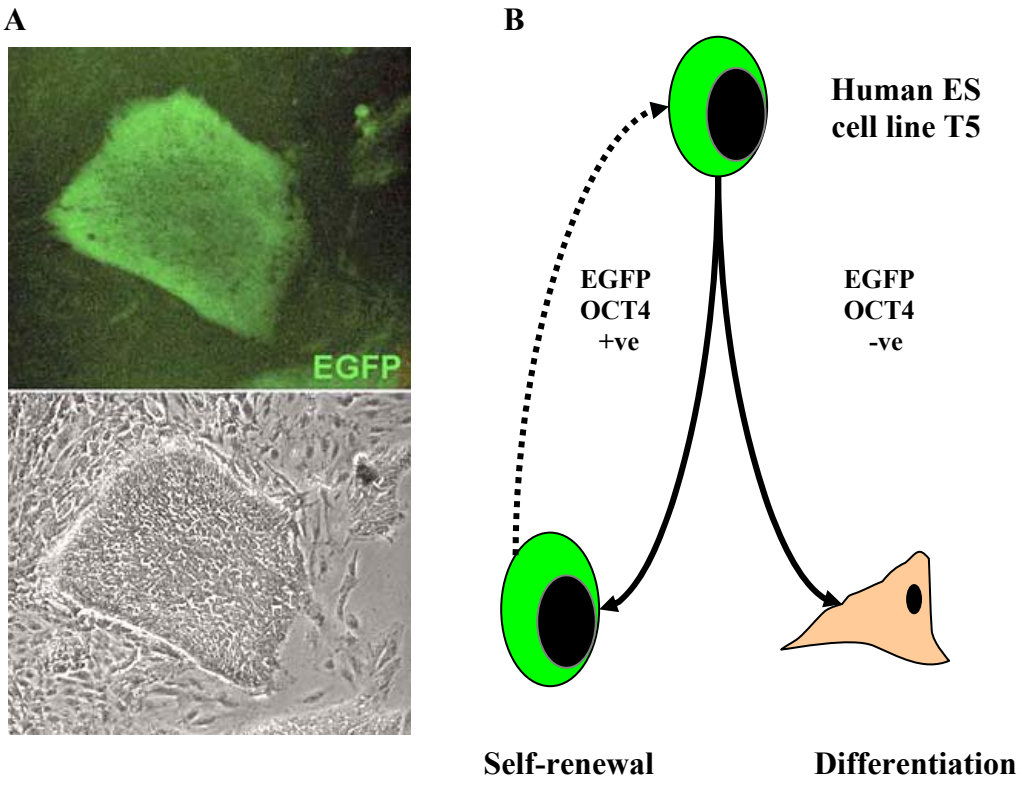


Figure 4.4. OCT4-EGFP clonal cell line T5 derived by (Gerrard *et al.*, 2005) A: EGFP image and corresponding phase-contrasting image of T5 (taken from Gerrard *et al.*,2005). Only the hES cell colony expressed OCT4-EGFP. **B**: Experimental system. T5 cell line carries the EGFP reporter of the stem cell specific marker, OCT4. Expression of the OCT4-EGFP reporter in hES cells is restricted to undifferentiated ES cells (green) and down-regulated upon differentiation.

4.5 Results

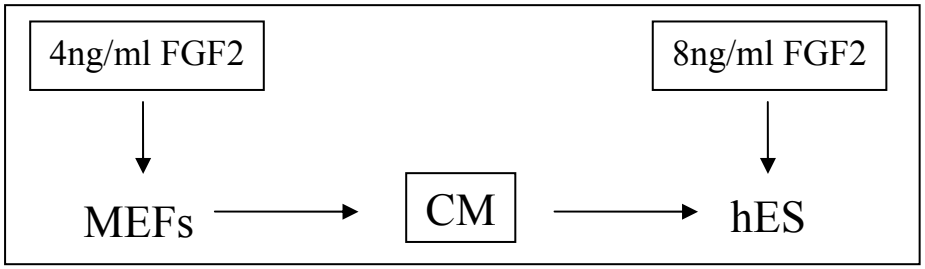
4.5.1 Cell growth in un-pre-treated CM

The routine maintenance of hES cells involved a conditioned medium (CM) prepared by incubating the basal medium (Xu *et al.,* 2001) on MEFs to which 4 ng/ml of FGF2 is added. To the CM an extra 8ng/ml of FGF2 is added at the time of feeding the hES cells (CM+FGF2). To reduce FGF2 from the culture medium, CM to which FGF2 had not been added at any point was intended to be used in early experiments. H1 cells were plated in medium (untreated), in untreated medium with FGF2 added to the hES cells (untreated+FGF2), medium treated with 4g/ml of FGF2 alone (CM) and the medium routinely used (CM+FGF2). A diagram of the conditions is shown in Figure 4.5.A.

Within 5 days of culture in the conditions described above, the morphological differences became evident. The cells growing in CM+FGF2 presented the typical morphology of hES cells cultured in CM. This contrasted with the less compact colonies growing in the untreated medium +FGF2, which were surrounded by cells with a differentiated morphology. As expected, the growth of the cells in CM alone was slower and highly differentiated. This contrasted with the complete cell loss in the untreated medium alone (Figure 4.5B).

These results highlighted the necessity for a medium independent of the conditioning process. They also suggested the importance of the addition of FGF2 as early as the conditioning step to perhaps stimulate the secretion of other factor/s.

A



B

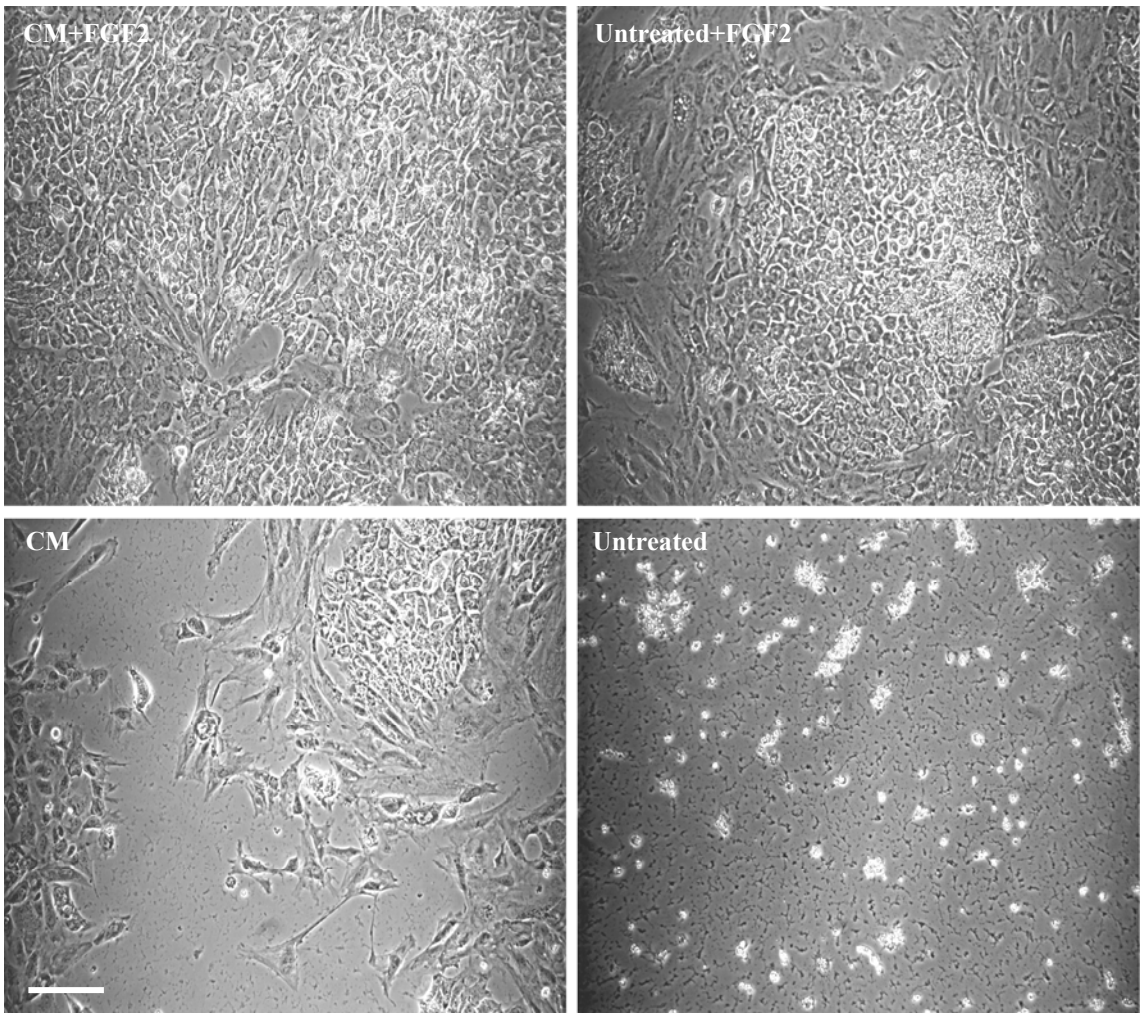


Figure 4.5. A: Process of media conditioning (for more details see 2.1.3.1) **B: Morphology of hES cells in CM in presence/absence of FGF2.** H1 cells grown in treated conditioned medium with FGF2 addition before and after the conditioning step (CM+FGF2), in CM supplemented with 8ng/ml after conditioning (untreated+FGF2), in CM supplemented with 4ng/ml FGF2 before the conditioning (CM) and in CM without FGF2 (untreated). Adequate hES cell proliferation was attained only with CM produced by MEFs to which FGF2 had been added.

4.5.2 Transition from CM to serum free medium

The chemical defined medium N2B27 has been shown to sustain mES cells (Ying *et al.,* 2003b). At the outset, it was intended to use the N2B27 medium, supplemented with FGF2 and in conjunction with Matrigel, only to analyse the short term effects of FGF on hES cells. However, surprisingly, hES cells (T5 and the parental H1 cell lines) could also be maintained in the N2B27 for several passages.

4.5.2.1 Morphological changes

The fact that there was not an increase in death during the transition of H1 and T5 hES cell lines to the new serum free conditions suggested that the signalling activated by the N2B27 were not significantly different from CM. However, some changes were noted in the morphology of the cells during the first hours of the transition into the serum free medium N2B27 (illustrated by H1 cell line in Figure 4.5). N2B27 seemed to induce a rapid increase in cell size accompanied by a flattened morphology. This morphology reversed in the next few hours and during the next day in N2B27 the cells proliferated rapidly. Once the cells have reached confluence (80-85% of the surface area covered) the hES cells were harvested and plated at a 1:3 dilution. After a few hours following cell plating, the hES cells in N2B27 had attached to the Matrigel and after 24 hours, the cells were spreading, maintaining dense and compact cell morphology (Figure 4.7). This was observed in H1 and T5 cell lines, which became confluent after two days in N2B27, contrasting with the slower cell attachment and proliferation in CM. Furthermore, both cell lines presented clear defined boundaries, unlike the cells in

conditioned medium, (Figures 4.1 and 4.3,A) with a dramatic decrease in the stromal differentiated cells that normally surround the hES cells colonies (Figures 4.7 and 4.8). T5 cells grown in N2B27 maintained the expression of OCT4-*EGFP* reporter (Figure 4.8). This expression was associated specifically with colonies of undifferentiated hES cells and was absent in the differentiated cells highlighted with arrows in Figure 4.8.

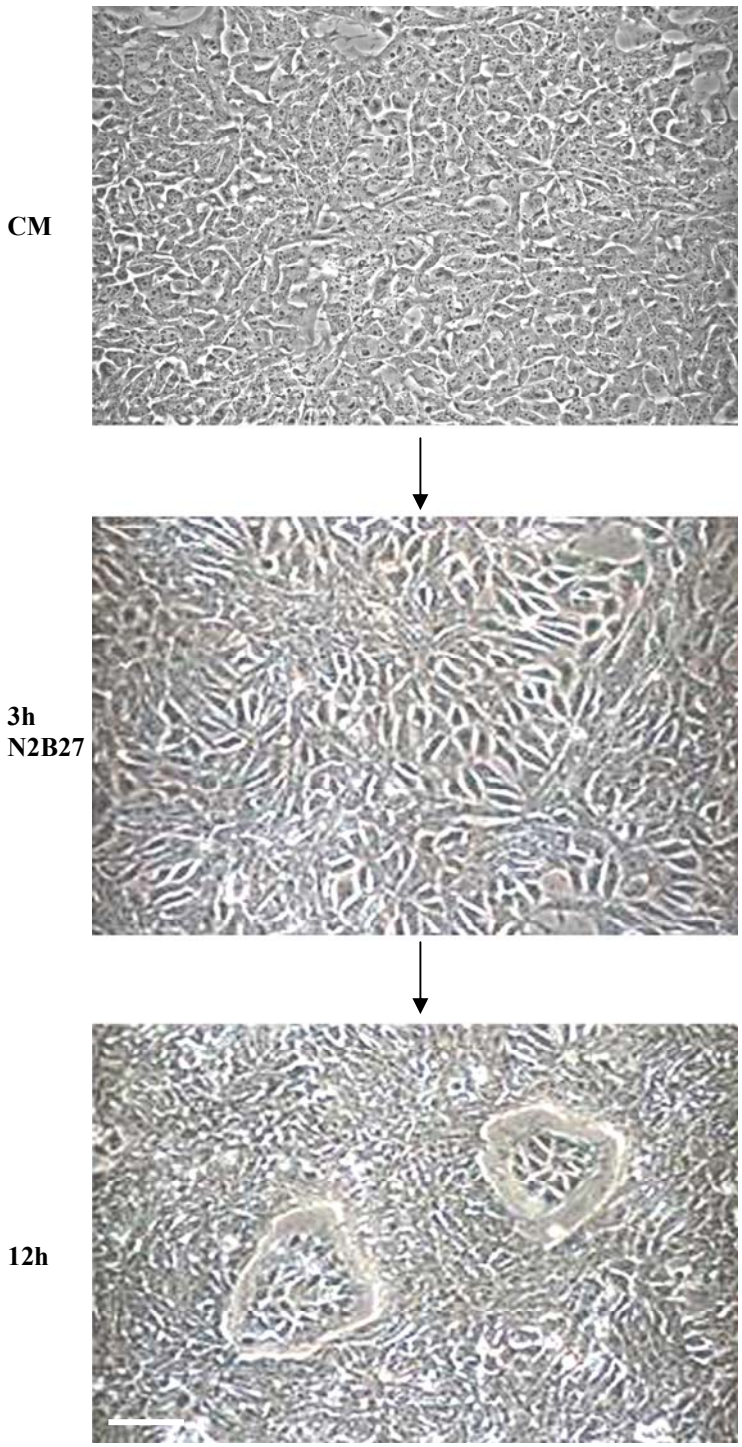


Figure 4.6. H1 transition from CM to N2B27 defined medium. H1 cells maintained in CM were passaged into N2B27. Initially, N2B27 induced flattening and enlargement of the cells. After 12 hours the cells formed a compact monolayer, in a similar manner to that of their sisters in CM. (Scale bar = 200μm).

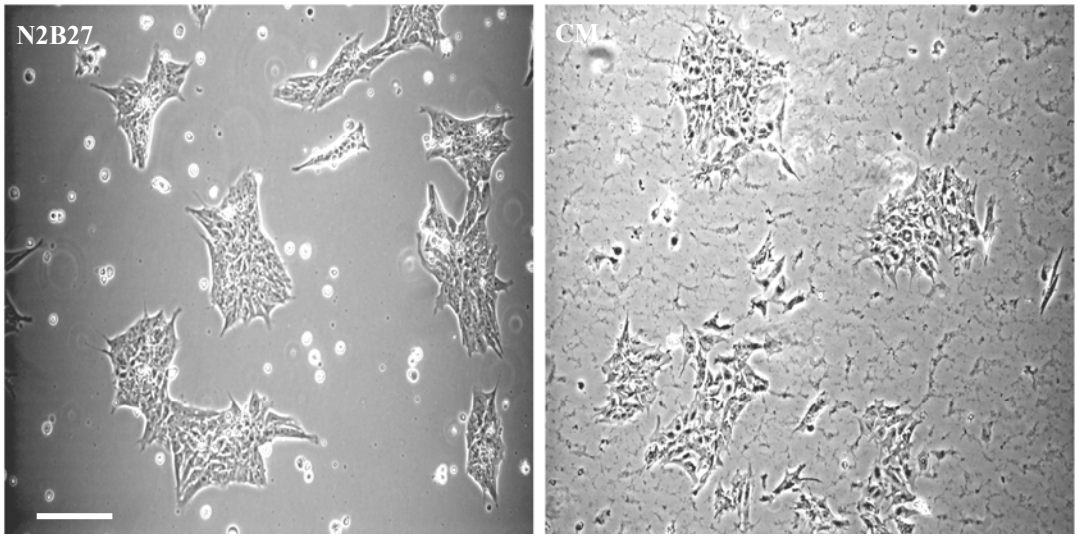


Figure 4.7. Cell morphology of H1s following first passage in N2B27 and how it compares to the CM conditions. Following an effective cell attachment in the new conditions, H1s showed neat and compact colonies after 24 hours from the first passage in N2B27. The morphology of H1p66 1EDTA colonies in CM appeared flatter and less compact and defined (scale bar = 200μm).

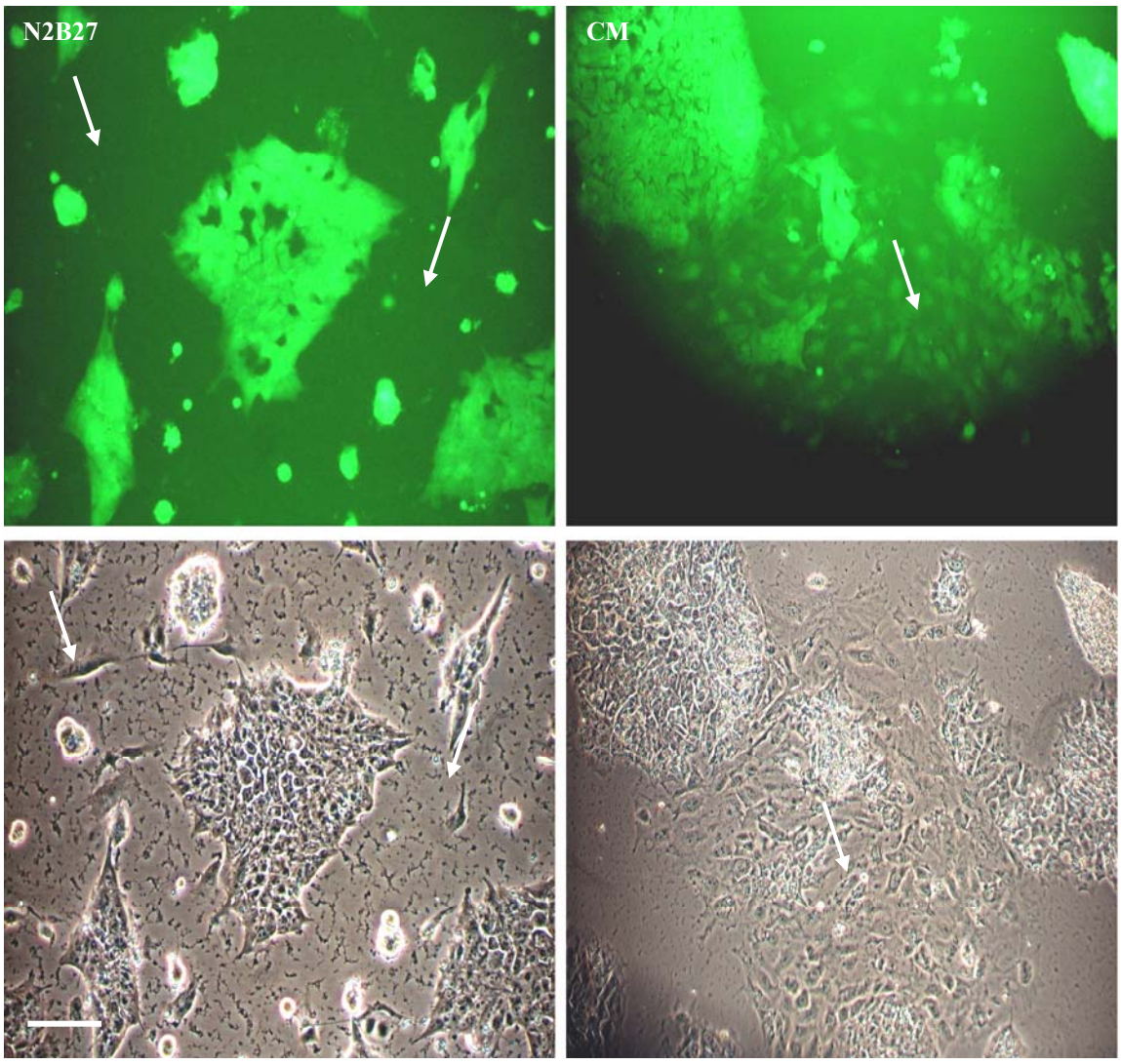


Figure 4.8. Live *OCT4*-EGFP clonal cell line T5 in serum free conditions and CM. EGFP image and corresponding phase contrast image of T5 in N2B27 and CM (PBS-EDTA) show sharper and better defined colonies in serum-free medium compared to CM. Live images show that the hES *OCT4*-EGFP clonal cell line T5 growing in N2B27 for several passages maintained the *OCT4* EGFP reporter. EGFP is expressed uniquely and specifically in the undifferentiated hES colonies. The arrows highlight differentiated cells that have lost their EGFP expression. Note that in the EGFP image (N2B27) there are some cells in the centre of the colony not expressing EGFP. These cells do not present the morphology of differentiated cells but have possibly silenced the reporter, which could be due to epigenetic changes resulting from the integration site (scale bar = 200μm).

4.5.2.2 Expression of OCT4

To determine whether hES cells growing in N2B27 maintained the expression of the internal standard marker for pluripotent cells a Western Blot was carried out. Samples containing equivalent numbers of cells were prepared from H1 and T5 cell lines cultured in serum free conditions for over 10 passages. The immunoblots were probed using an antibody against OCT4 and the membranes stripped and probed for SHP2 to ensure equality in loading.

High levels of OCT4 protein were detected in both cell lines after several passages in the defined conditions when analysed by immunoblotting (Figure 4.9). The levels of OCT4 in the serum free medium were comparable to those of the cells maintained in CM, establishing the maintenance of the stem cell marker OCT4. In addition to this, the *OCT4*-EGFP (Figure 4.8) was validated as an effective reporter of the endogenous expression of OCT4 in the T5 cell line.

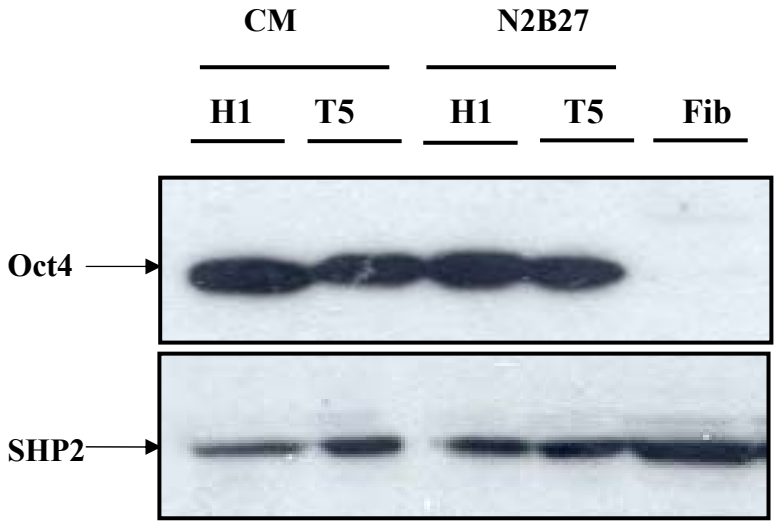


Figure 4.9. Expression of OCT4 in H1s and in the subclone T5. After harvesting, an equivalent number of cells were lysed and the immunoblots probed using an antibody against OCT4. After stripping the membranes, they were reprobed with an antibody against SHP2, as a control. Fibroblast lysate (Fib) was used as a negative control.

4.5.2.3 Expression of surface markers

H1 and T5 cell lines propagated in serum free conditions maintained the expression of the marker of pluripotency OCT4. To get an independent confirmation that hES cells growing in N2B27 retained qualities of cells in CM, the expression of cell surface markers was compared by flow cytometry. Expression of SSEA3 and 4 and TRA1-81 as markers of undifferentiated hES cells and SSEA1 as a marker of early differentiation was assessed. It is widely reported that human ES and EC cells typically express SSEA3 and SSEA4 but not SSEA1 whilst their differentiation is characterised by down-regulation of SSEA3 and SSEA4 and an up-regulation of SSEA1 (Andrews *et al.,* 1984).

Regular assessment of surface markers was carried out in hES cells and Figure 4.10 shows a representative outline of the expression of surface markers on the H1 cell line. In N2B27 and CM conditions the surface markers associated with undifferentiated hES cells SSEA4, SSEA3 and TRA1-81 were highly expressed. This expression was slightly higher on the cells maintained in N2B27, whilst the expression of early differentiation marker: SSEA1 was lower than on those cells in CM. This could be reflecting the decrease in the amount of cells with differentiated morphology observed in N2B27.

The expression of surface marker was also assessed on the T5s growing in CM (Figure 4.11 A) and after 12 passages in N2B27 (Figure 4.11.B) by flow cytometry. TRA1-81 and SSEA3 expression were analysed as markers of undifferentiated hES cells and

SSEA1 as a marker for differentiation. T5s in CM showed a high expression of TRA1-81 (97%) with a high percentage of cells co-expressing this hES cell marker with EGFP (62.4%). SSEA1 expression was low (6.7%) with a small percentage of cells co-expressing EGFP and this early marker of differentiation (3.8%). This could be reflecting cells in transition to differentiation that still retained EGFP expression (24 hours half-life). The high percentage of unstained cells (US) may give account of cells which have silenced the reporter and are negative for SSEA1 expression. Analysis of surface markers expression on T5s in N2B27 (Figure 4.11B) showed high levels of expression of the specific surface marker for undifferentiated hES cells SSEA3 (96.8%) simultaneously with a low expression of SSEA1 (2.7%). SSEA1 expression was lower than in CM, confirming the decrease in the differentiated cells in the serum free conditions detected by direct observation of the cell morphology.

The maintenance of surface markers on H1s and T5s associated with undifferentiated hES cells and a reduction of the expression of the early differentiation marker SSEA1 was established by flow cytometry. Surface marker expression was related to the high expression of OCT4 protein illustrated in Figure 4.9. Flow cytometry also confirmed the expression of EGFP by ultraviolet microscopy in the T5s maintained in the serum free medium (Figure 4.8).

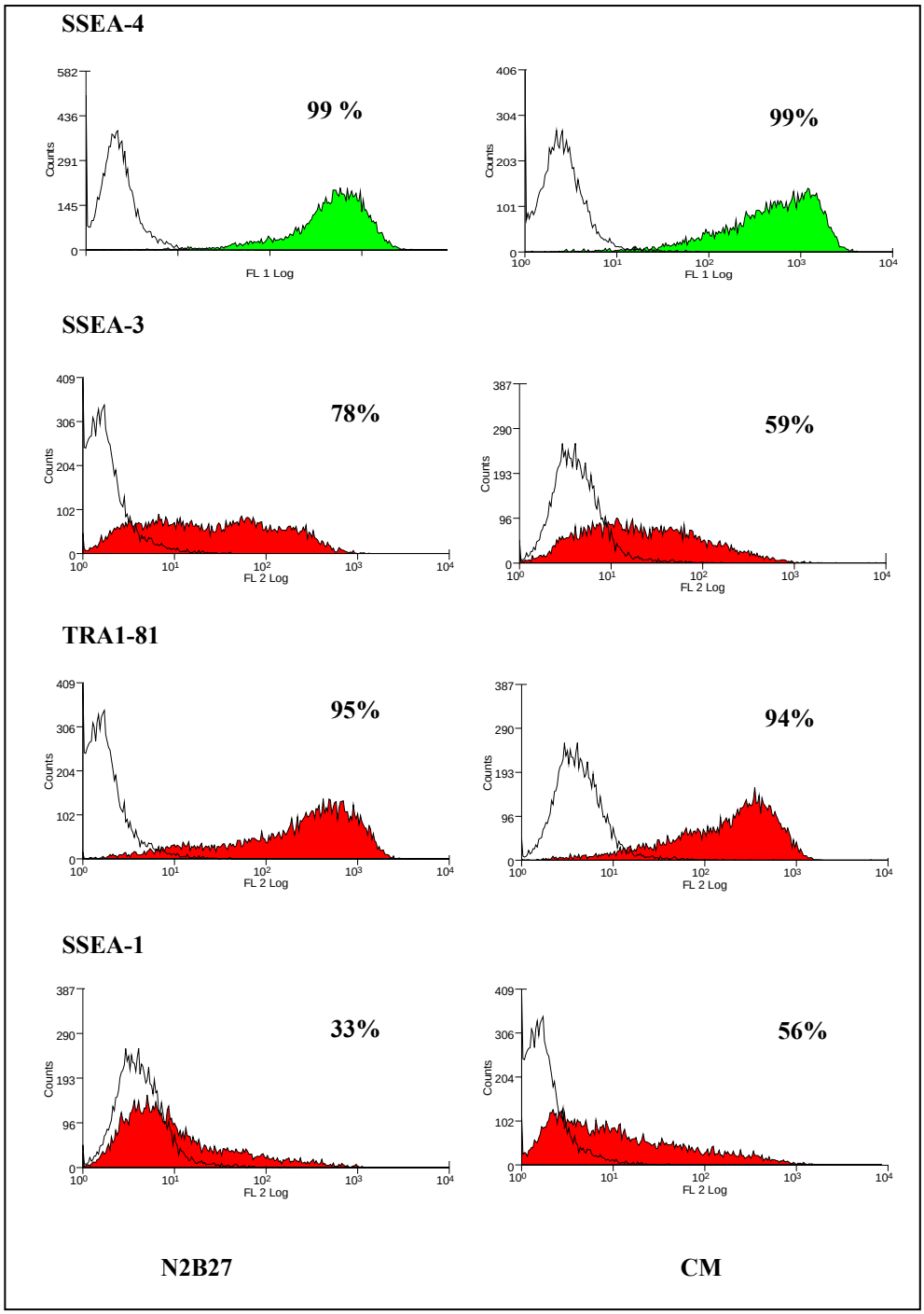
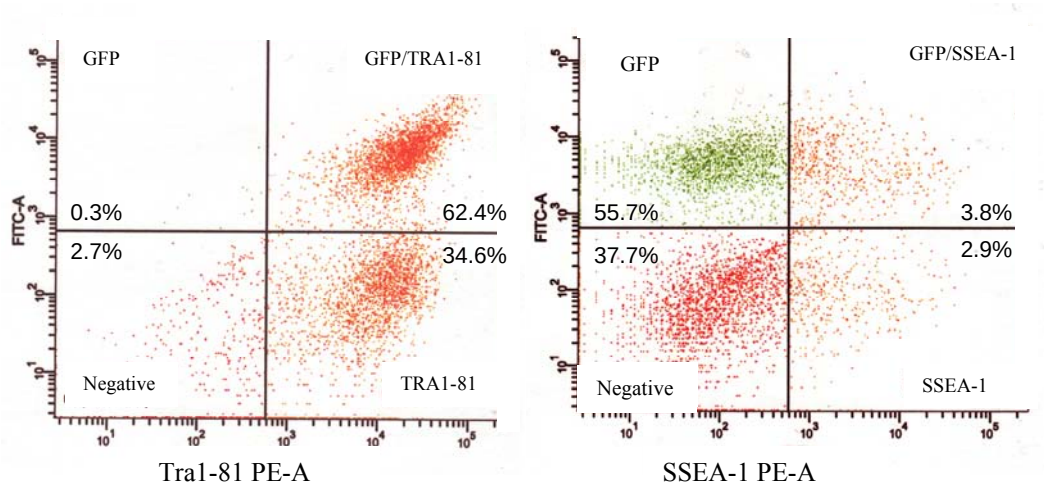


Figure 4.10. Flow cytometry analysis of surface markers expression on H1 maintained in N2B27 vs CM. In the defined conditions H1s showed a higher expression of surface markers associated with undifferentiated hES cells and a lower expression of the early marker of differentiation SSEA1 than on the cells in CM

A



B

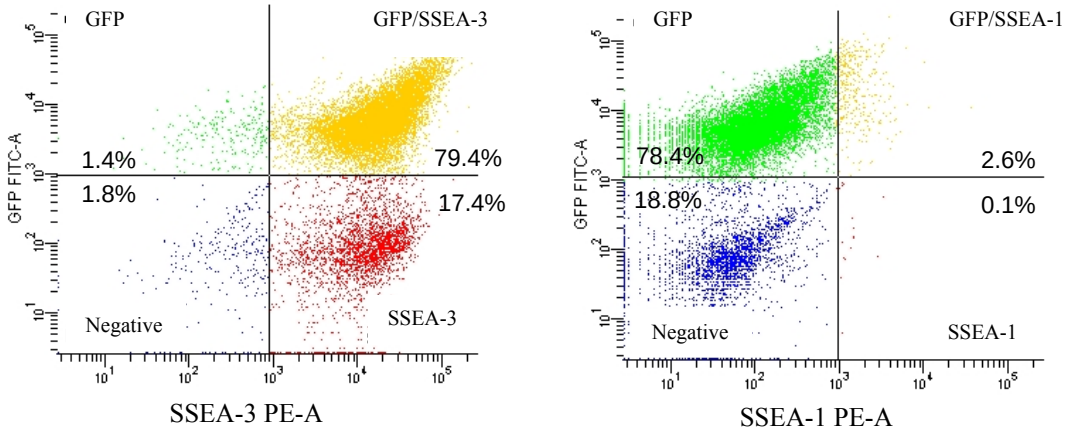


Figure 4.11. Flow cytometry analysis of surface markers expression on *OCT4*-EGFP cell line T5 maintained in CM (A) and N2B27 (B). EGFP expression and surface antigens, TRA1-81, SSEA3 and SSEA1 were analysed by flow cytomety. In both conditions EGFP and surface markers associated with self-renewal were expressed in CM and N2B27. SSEA1, as early differentiation marker, was also detected at low levels in the two conditions.

.

4.5.3 Effect of FGF2 on hES cells growing in serum free conditions

4.5.3.1 Effect of FGF2 cell growth

After establishing that H1 and T5 cell lines grown in N2B27 maintained the expression of internal and cell surface markers associated to hES cells, the effects of FGF2 were studied. To establish the role of FGF2 on cell growth, H1s and T5 cells ($5x10^5$cells/well) were seeded with and without FGF2 and the media changed daily.

Observation of the plates showed larger colonies in the presence of FGF2, indicative of a positive effect of FGF2 on the growth of the H1 (Figure 4.13) and the T5 (Figure 4.15) cell lines. However, in the absence of FGF2 the T5s maintained a strong *OCT4*-EGFP expression as well as a similar morphology to the cells growing in FGF2 (Figure 4.15). It could also be observed that in the early stages of the experiment (Figure 4.13 (A), D1) there was more debris in the absence of FGF2. This could be indicative of cell death and might suggest a possible role of FGF2 on cell viability or cell adhesion. The effect of FGF2 on H1 cell growth was confirmed by quantification of the cell number. During the 6 days of the experiment, cells from 3 independent wells were counted and the mean results used to produce a growth curve (Figure 4.14). The results showed an increase in cell number in the presence of FGF2 and similar results were obtained in two independent experiments (appendix 2). The lower number of cells harvested in day 1 (D1) was possibly a consequence of the low density plated. In conclusion, the absence of FGF2 from the medium produced a decrease in cell growth in the two cell lines T5 and the parental H1.

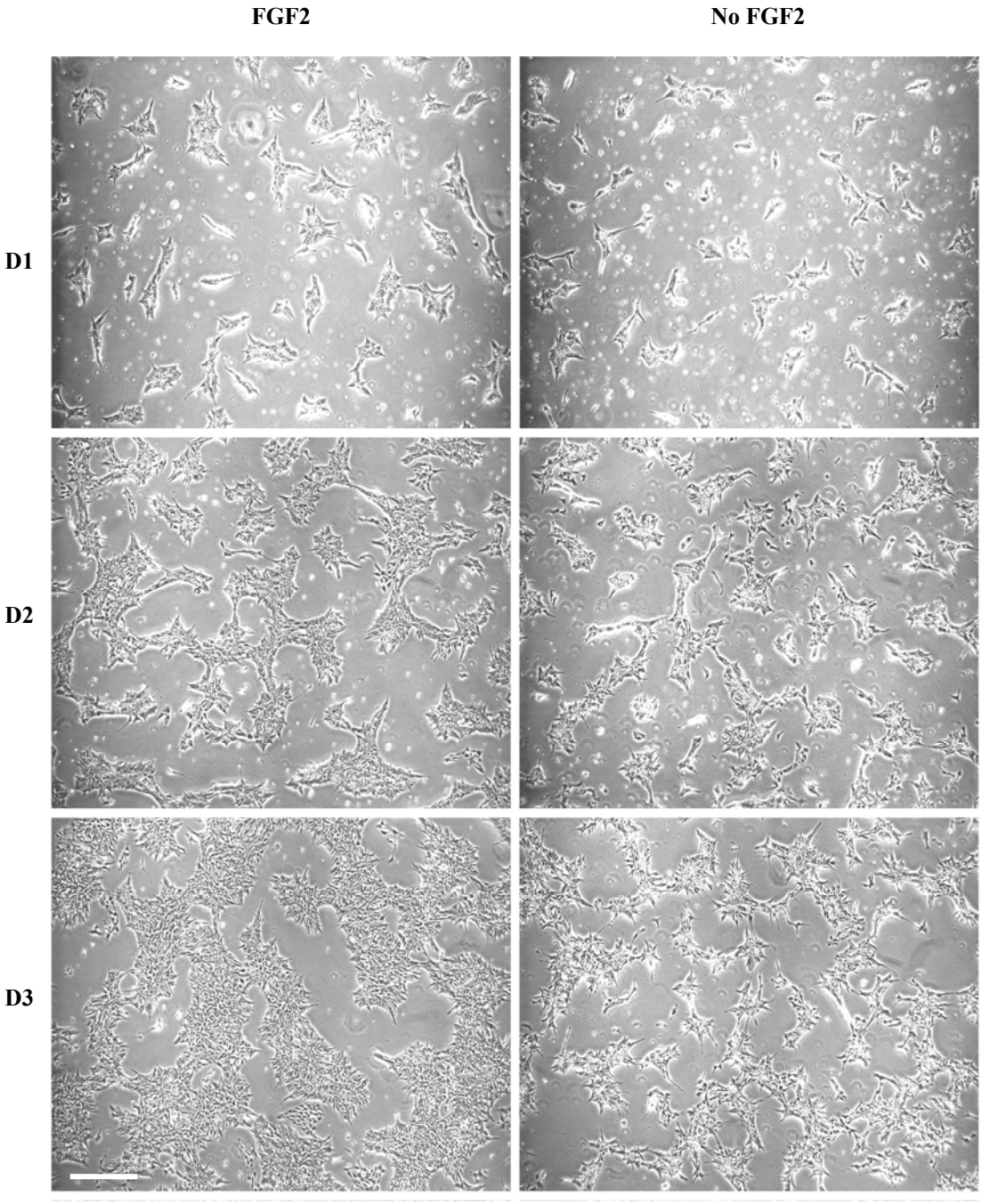


Figure 4.13. A. Effect of FGF2 on H1 cell morphology (days 1-3). The H1 cell line was seeded at $5x10^5$ cells/well in 6 well dishes and maintained for 6 days in the presence/absence of FGF2 with daily media change. These images were representative of the wells (scale bar = 500μm).

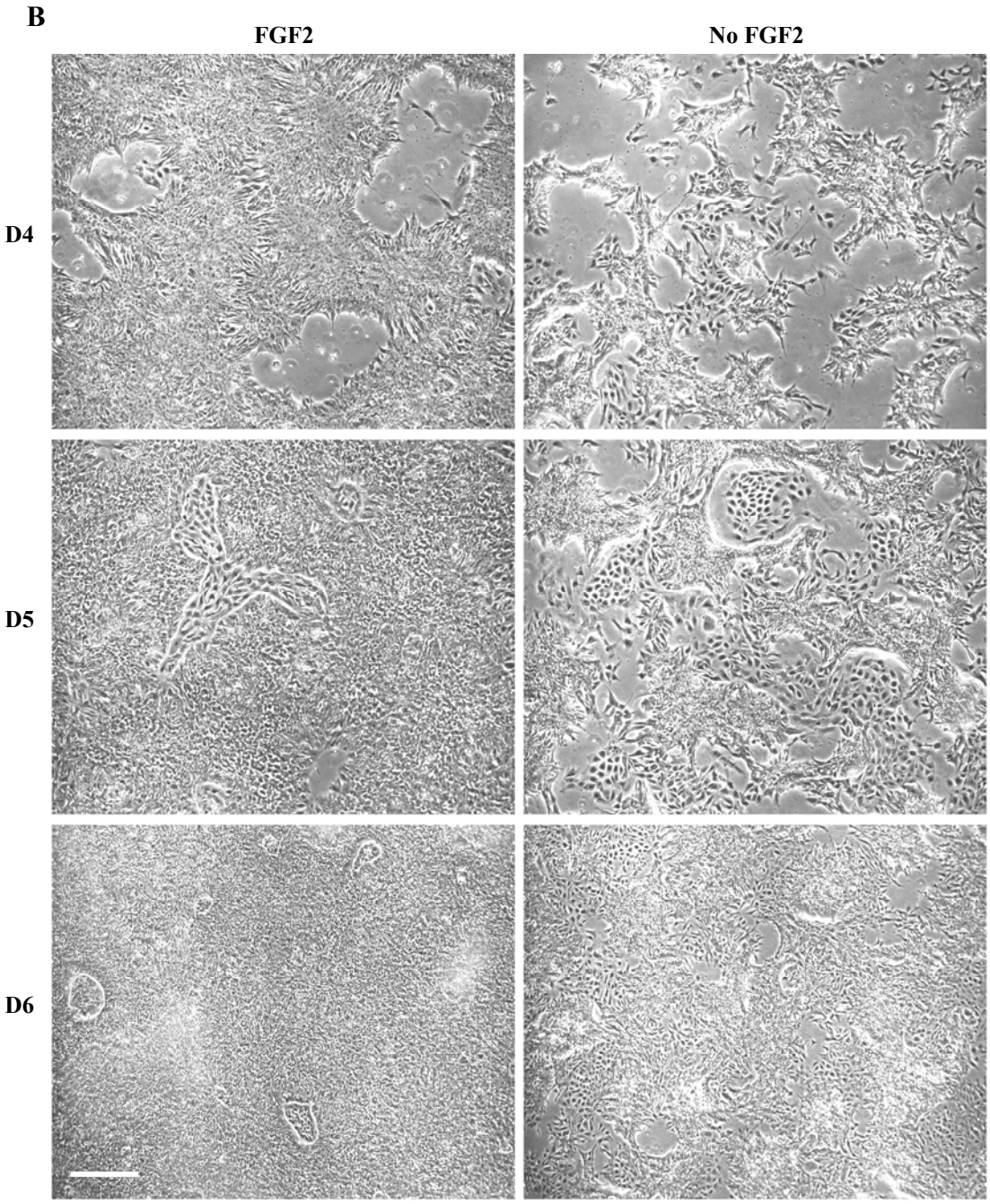


Figure 4.13. B. Effect of FGF2 on H1 cell morphology (days 3-6). The H1 cell line was seeded at $5x10^5$ cells/well in 6 well dishes and maintained for 6 days in the presence/absence of FGF2 with daily media change. These images were representative of the wells ((scale bar = 500μm).

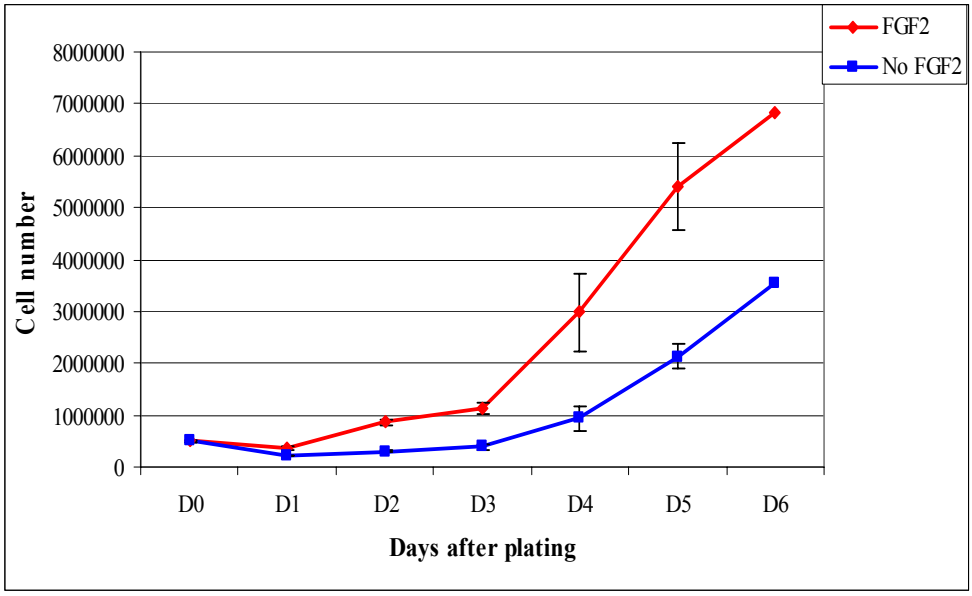


Figure 4.14. Effect of FGF2 on H1s cell numbers. The H1cell line was seeded at $5x10^5$ cells/well in 6 well dishes and maintained for 6 days in the presence/absence of FGF2 with daily media change. The cell number from three independent wells was counted daily and from the results a growth curve was produced. Cell numbers increased significantly ($p<0.001$) when FGF2 was present in the cell culture. Data is presented as the mean ± S.E.M of 3 counts. Two way anlysis of variance used for generation of p value.

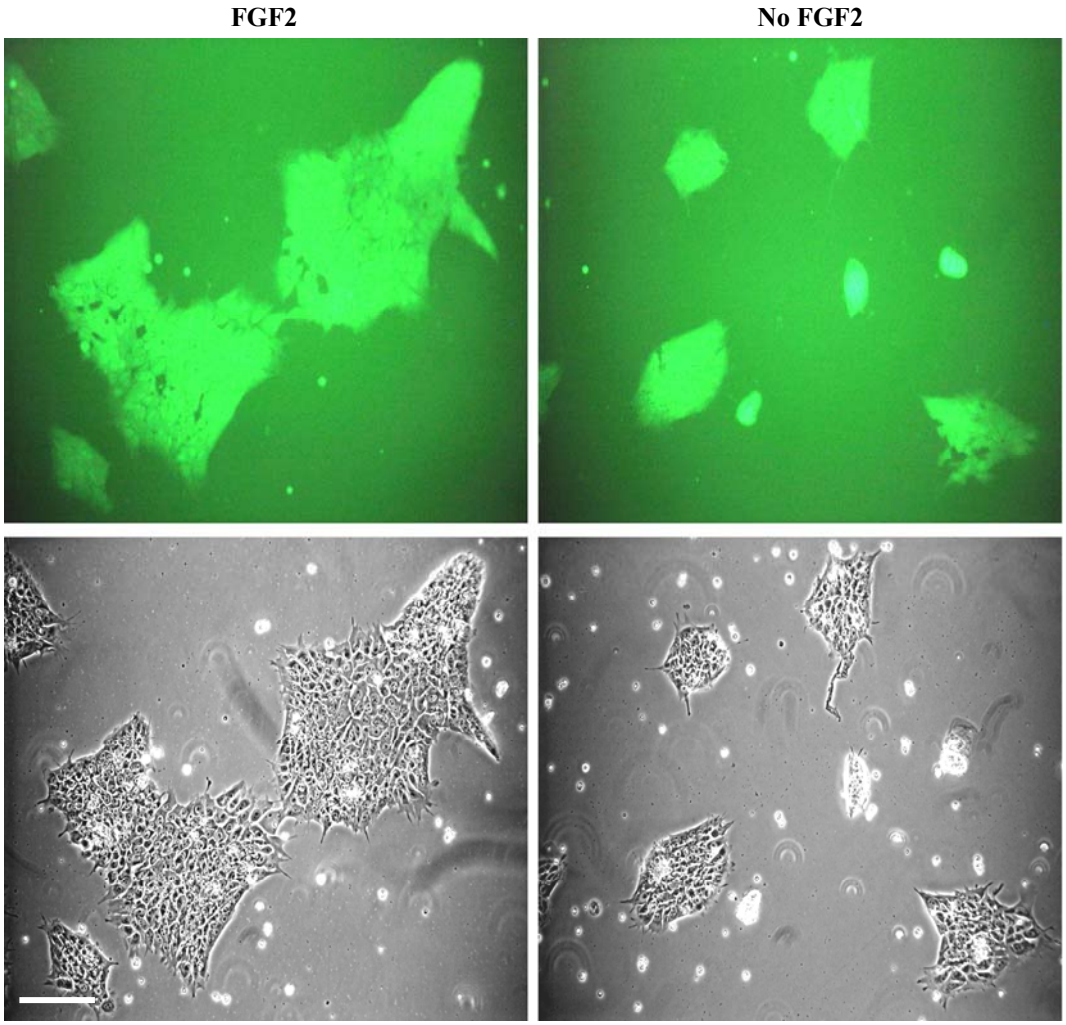


Figure 4.15. Effect of FGF2 on hES *OCT4*-EGFP. The T5 cell line was seeded at $5x10^5$ cells/well in 6 well dishes and maintained for 6 days in the presence/absence of FGF2 with daily media change in order to prevent any autocrine effect. These images were taken on the 4^{th} day of the experiment and were representative of the wells (Scale bar = 200μm).

4.5.3.1.1 Effect of FGF2 on plate efficiency

The experiments outlined in section 4.5.3.1 indicated an increased in cell number due to the addition of FGF2. FGF2 could be enhancing cell attachment to the substratum or improving the survival and proliferation once the cells got attached. Studies carried out in different cell types have indicated that FGF2 can modulate cell attachment and spreading in feeder free conditions by the induction of adhesion molecules (Debiais *et al.,* 2001; El-Hariry *et al.,* 2001; Jang and Chung, 2002). To examine if FGF2 has an effect on cell plating efficiency a 34 well dish was seeded with different cell densities in the presence or absence of FGF2. After 24 hours of being plated the fluorescence emission of EGFP was measured. The EGFP was excited (489nm) and the fluorescence emission (508nm) was detected and measured in a fluorescence plate reader (Figure 4.16). To relate these results to any possible changes in cell morphology, $5x10^5$ cells were plated (equivalent cell density to the higher cell number plated for the fluorescence emission assessment) with or without FGF2. The effect of FGF2 to T5s cell morphology after 24 hours of seeding is shown in Figure 4.17.

Quantification of the fluorescence emission showed that the presence of FGF2 at the time of plating resulted in a slightly higher expression of the EGFP reporter than in its absence (Figure 4.16). This increase in *OCT4*-EGFP seemed to correlate with an increase in the amount of cells in the presence of FGF2 at the time of plating (Figure 4.17). FGF2 seems to have a positive effect on the plate efficiency, which could be due to an improvement in cell attachment to the Matrigel, although an enhancing in cell proliferation and survival might also be involved.

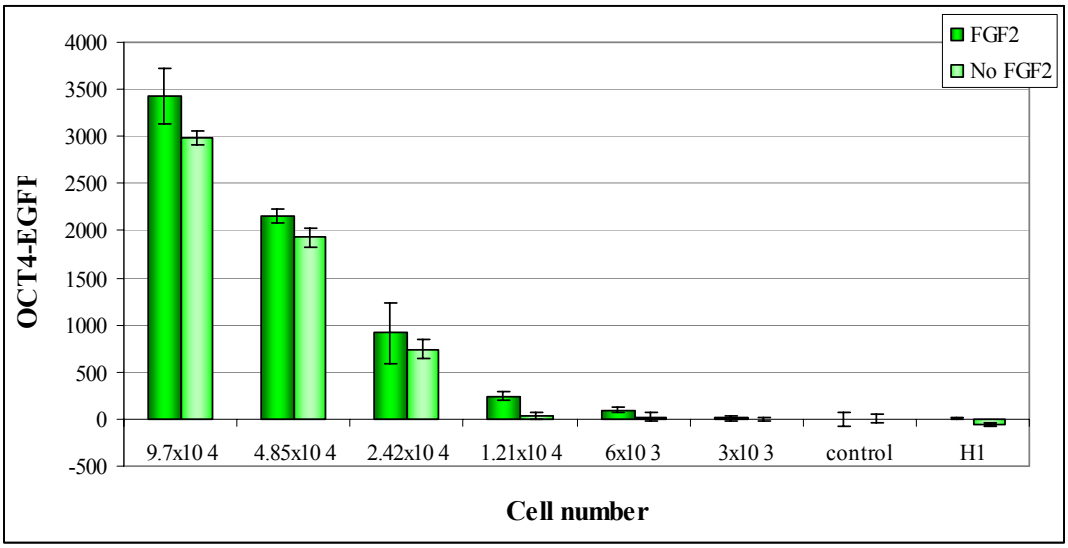


Figure 4.16. EGFP fluorescence emissions from T5s plated in the presence/absence of FGF2. T5 cells were plated at different cell densities in the presence/absence of FGF2 and the EGFP emission measured in a Multilabel Counter 24 hours after plating. The fluorescence emission was slightly higher in the presence of FGF2 than in its absence. H1, the parental line did not produce any fluorescence. In the control the wells were coated with Matrigel and optimem was used for the readings. Data is presented as the mean ± S.E.M.of six replicates from two independent experiments. The results have been normalised relative to the control: Matrigel + Optimem.

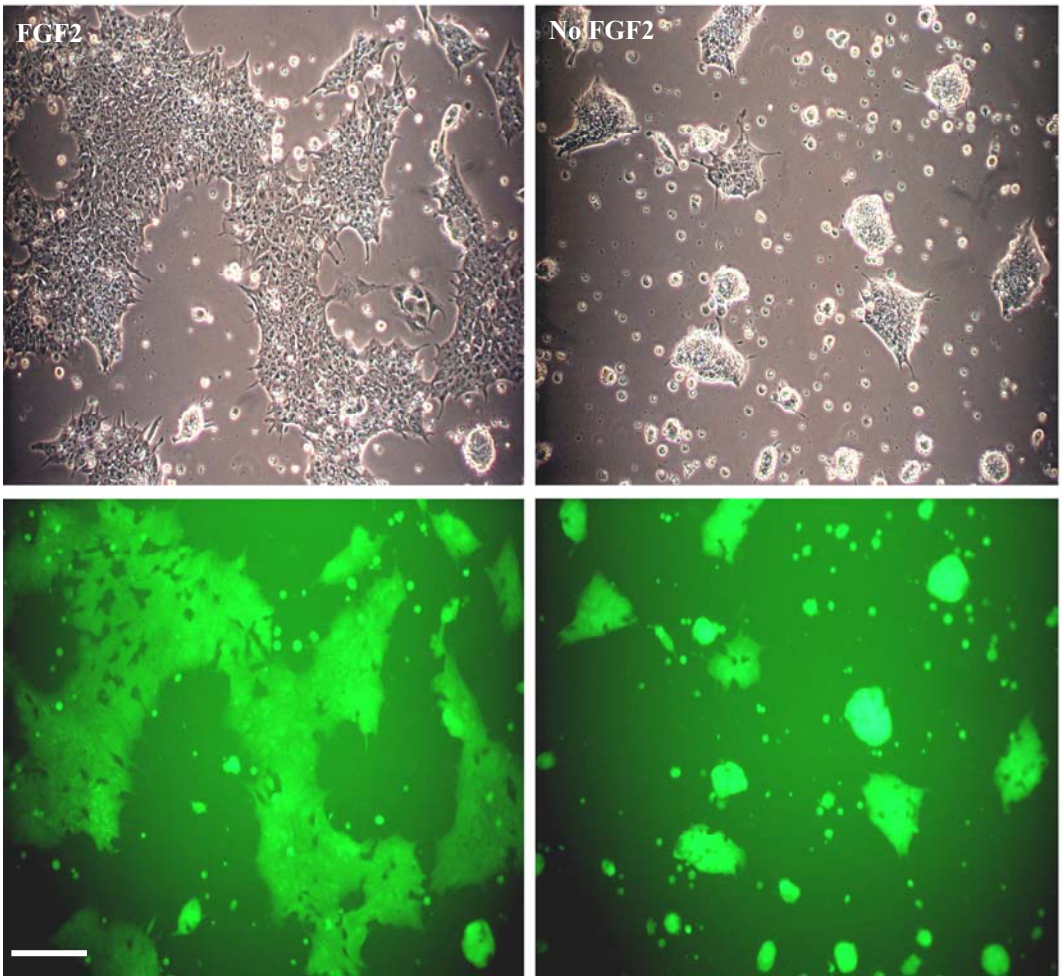


Figure 4.17. Effect of FGF2 on T5 cell line plating efficiency. $5x10^5$ cells were plated in the presence or absence of FGF2 and 24 hours later the effects on their morphology was observed**.** After 24 hours in the presence of FGF2, the cells seem to be spreading, whereas without FGF2 the cell colonies appear tight and rounded. Floating dead cells could also be observed when FGF2 was not supplemented (Scale bar = 200μm).

4.5.3.1.2 Effect of FGF2 in cell proliferation

To establish if the reduction of *OCT4*-EGFP in the presence of FGF2 was due to an increase in cell proliferation, the thymidine assay was carried out. This assay is based on the incorporation of the radioactive ^{3}H (tritium) into the Hydrogen of the Thymidine during DNA synthesis. The amount of ^{3}H-Thymidine incorporated into the DNA can be quantified by measuring the total amount of labelled DNA in a population and this is directly proportional to the amount of cell division occurring in the culture.

T5 cells were plated at different cell densities in the presence of FGF2. Once the cells were attached to the matrix (4-5 hours), the medium was changed to + or - FGF2 and after 24 hours the thymidine assay was performed. The results presented in Figure 4.18 were obtained by measuring cell filtrates in a β-counter after incubating the cells with the radioactive tritium for 6 hours. Thymidine assay showed that FGF2 produced a significant increase in cell proliferation.

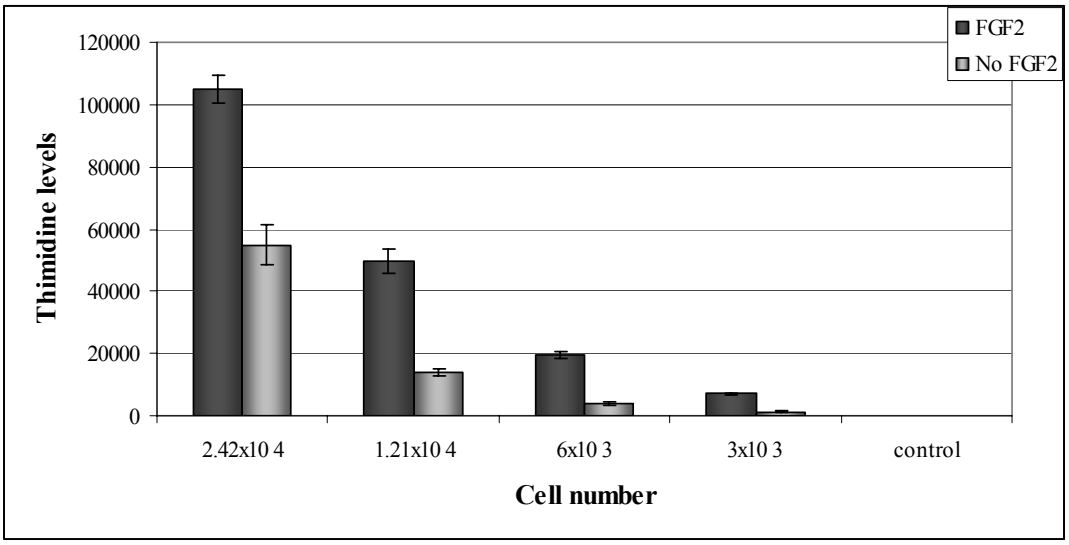


Figure 4.18. Effect of FGF2 on the ^{3}H-Thymidine incorporation. T5 cell line proliferation in the presence/absence of FGF2 was measured by ^{3}H-Thymidine incorporation and read in a β-counter after 6 hour labelling. The increase in cell proliferation was significantly higher ($p<0.001$) for all the cell densities. Paired T-test analysis of variance was used for the generation of a p value. Data is presented as the mean ± S.E.M.of six replicates. The results have been normalised relative to the control: cells without the label.

4.5.3.1.3 Effect of FGF2 on the cell cycle

Previous experiments showed that the addition of FGF2 to the culture produced an increase in cell proliferation. A likely mechanism to increase cell proliferation is by FGF2 having a positive effect on the cell cycle. Mouse (Savatier *et al.,* 2002) and rhesus monkey (Fluckiger *et al.,* 2006) embryonic ES cells follow an unusual cell cycle with a short or non-existent G1 phase in contrast with somatic cells, which remain a longer time in G1. A diagram representing the cell cycle is shown in Figure 4.19.

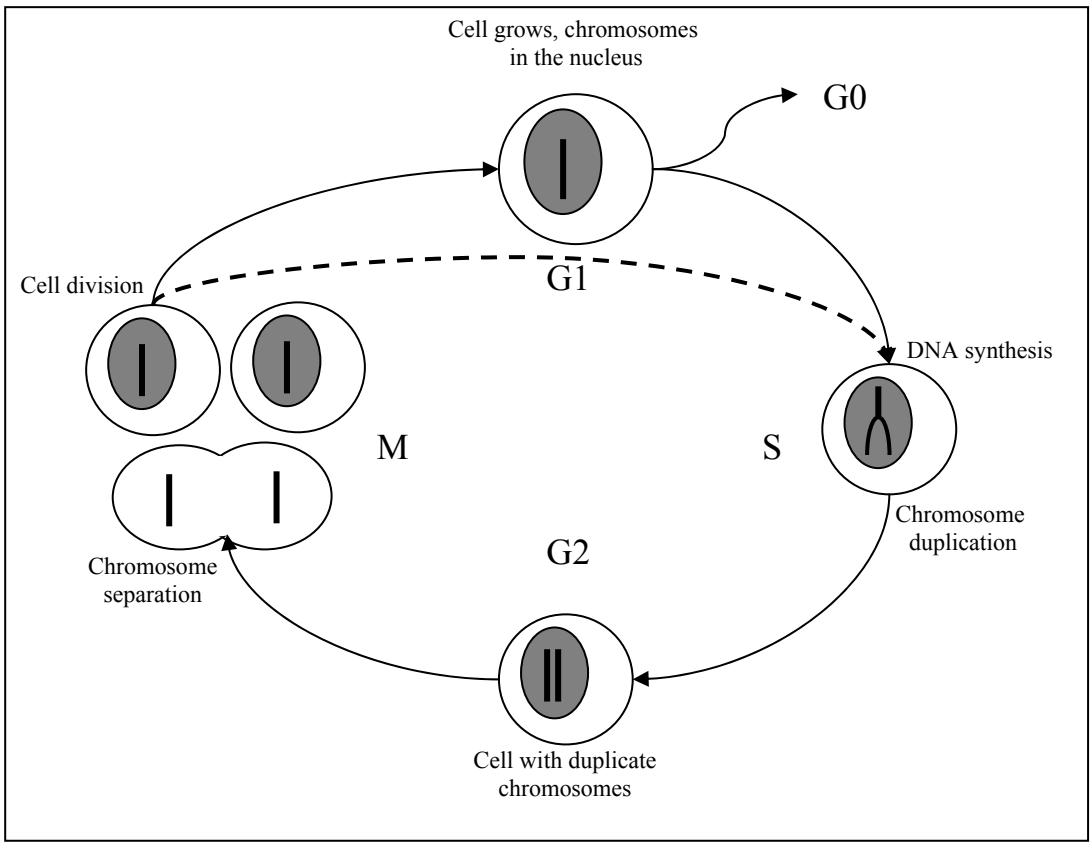


Figure 4.19. The cell cycle in mammalian cells. In the first phase (G1) the cell grows, transcribes RNA, synthesises proteins and when a certain size is reached, the cell enters the phase of DNA synthesis (S) where the chromosomes duplicate. In G2 the cell prepares for division by duplicating its chromosomes. During mitosis (M) the chromosomes are separated from the daughter cells. The cycle is completed when the cells start again in G1. The cells that are resting (quiescent) are said to be in the G0 phase. Mouse and primate ES cells have a peculiar cell cycle with a short or non existent G1 phase as the cells pass rapidly from mitosis to synthesis (broken line).

To study the role of FGF2 in the cell cycle of hES cells, the pattern of cycling in H1 cell line was examined in the presence and absence of FGF2. $5x10^5$ cells were plated in N2B27 containing FGF2, and once the cells were attached to the matrix the medium was changed to + or - FGF2. For six days the medium was changed daily and each day 10^6 cells were fixed in cold ethanol and PBS. At the end of the experiment the fixed cells were stained with propidium iodide, whose fluorescent emission is proportional to the amount of DNA present in the cell and can be detected by flow cytometry. The results obtained by flow cytometry produced a histogram which was used to analyse the cell cycle. This assessment was only performed on single live cells (For details see section 2.5.2 in materials and methods).

The effect of FGF in the cell cycle in day two of the experiment is shown in Figure 4.20. After only one day in the presence of FGF2, a slight increase in the amount of cells cycling was observed compared with the cells in the absence of FGF2. At this early point, however, the overall profile was similar in the two conditions. An overlay for the cell cycle in day 1 of cells with/without FGF2 is shown in Appendix 3. A more striking difference between treatments was observed in the second day of treatment (Figure 4.20). The addition of FGF2 resulted in a greater amount of cells in the S and G2 phases. FGF2 promotion of cell division in the second day of treatment corresponds to the first evident effects on cell number shown in Figures 4.13 and 4.14. Furthermore, a high proportion of cells in S phase was consistent with the higher percentage of cells incorporating BrdU in FGF2 treated cells following pulse labeling (Figure 4.18). However, there was a progressive increase of cells in the G1 phase for both conditions

during the experiment, which is perhaps a consequence of the high confluencies reached. This produced a similar overall cell cycle profile after day four of plating and it was difficult to make further conclusions. An overlay for the cell cycle in day 5 of cells with/without FGF2 is shown in Appendix 3.

In conclusion, the results of the cell cycle analysis (Figure 4.20) indicated that FGF2 promotes cell division. This effect may be due to FGF2 acting as a mitogenic factor, thereby promoting a rapid synthesis and cell division, which is consistent with FGF2 enhancement of cell growth and in DNA synthesis as illustrated in Figure 4.13 and 4.18 respectively.

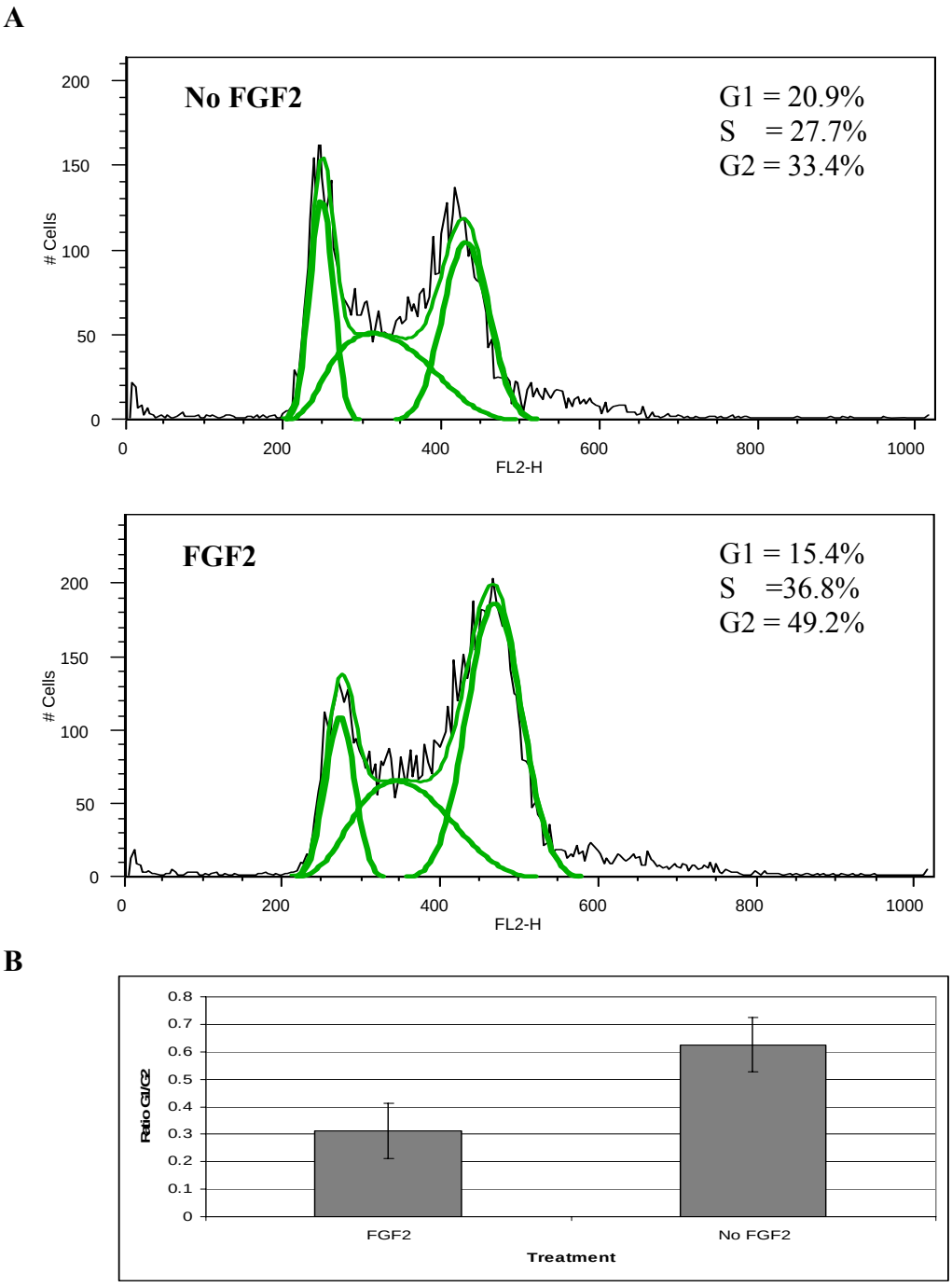


Figure 4.20. Effects of FGF2 on hES cell cycle analysed by flow cytometry. Following two days with or without FGF2 the cells were stained with Propidium iodide and analysed by flow cytometry. **A**: Cells without FGF2 displayed a larger G1 than those cells maintained in FGF2. **B:** Comparison of the ratio of cell percentage between G1 and G2 phases in presence/absence of FGF2. Data here presented was representative of two independent experiments and the error bars represent the S.D.

4.5.3.2 FGF2 effect on self renewal

4.5.3.2.1 FGF2 effect on the self renewal of the cell line T5

The absence of FGF2 from the N2B27 medium diminished cell growth in the two cell lines, T5 and the parental H1. However, in the absence of FGF2 the morphology of the T5 cells was similar to those growing in FGF2 and a strong *OCT4*-EGFP was expressed (Figure 4.15). This suggested that T5 cell self-renewal was not dependeding on FGF2, which conflicts with the reported role of FGF2 in the maintenance of the hES cells self-renewal (Levenstein *et al.,* 2005). To establish whether T5 cell self-renewal responded to the effect of FGF2, the surface markers SSEA3 and SSEA1 were analysed by flow cytometry. The expression of these surface markers and EGFP was compared in the cells growing in the presence or absence of FGF2 for 6 days (Figure 4.22).

Gerrard *et al,* (2005) established that the down-regulation of the *OCT4*-EGFP marker during differentiation correlated with endogenous *OCT4* and hES cell surface markers. However, flow cytometry analysis indicated that T5 cells maintained high co-expression of *OCT4*-EGFP with SSEA3 in absence of FGF2 as well as a low expression of SSEA1 (Figure 4.22). This might suggest that the T5 cells may have lost their capacity to differentiate when they were transferred into the N2B27 medium.

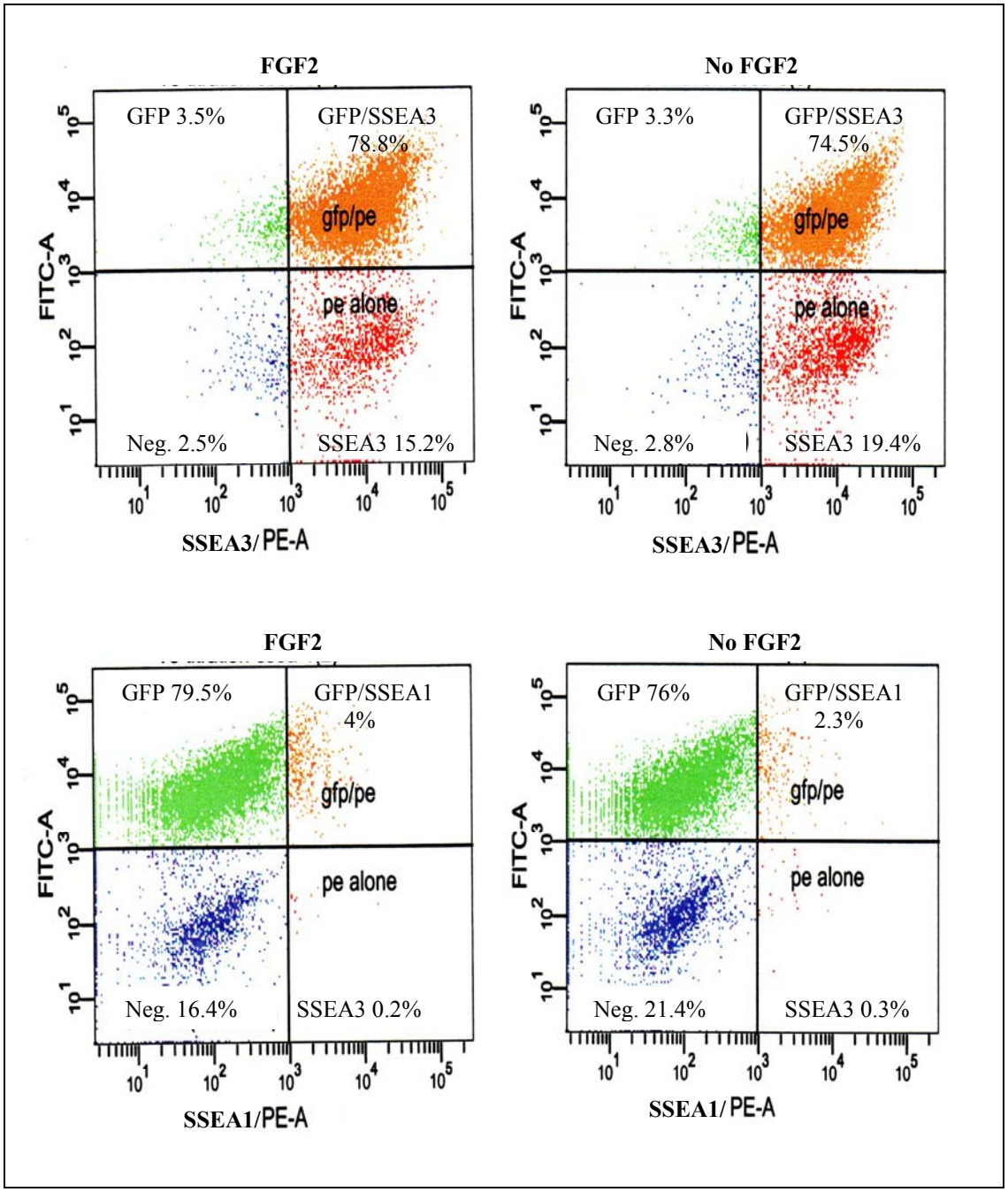


Figure 4.22. Analysis of *OCT4*-EGFP and the surface markers SSEA3 and SSEA1 on T5 cells growing in serum free medium for 6 days in +/- FGF2. EGFP and SSEA3/1 expressions were analysed by flow cytometry. After 6 days in culture in the absence of FGF2, the T5s maintained high levels of SSEA3, which were associated with a strong EGFP expression. Furthermore, an increase in SSEA1 was not noted.

4.5.3.2.2 Has the T5 cell line lost the capacity for differentiation?

To determine whether the T5 cell line was unable to differentiate as a result of potential changes generated by the serum free conditions, BMP4 was added to the N2B27 to induce differentiation. Contrasting with the role of BMP4 in suppressing mES differentiation, BMP4 cannot maintain hES cells propagation but can promote hES cells differentiation into trophoblasts (Xu *et al.,* 2002). T5 cells were seeded at 1:3 density in N2B27 supplemented with FGF2 (8ng/ml) and BMP4 (5ng/ml) or N2B27 alone or in N2B27 with FGF2 alone (8ng/ml) (Figure 4.23).

BMP4 supplement resulted in a rapid onset of a flattened morphology, which corelated with the loss of the expression of the *OCT4*-EGFP reporter. Therefore, the T5 cell line was able to differentiate. This might indicate that autocrine and/or intracrine mechanisms could have been activated and maintained these cells in a constant self-renewal state.

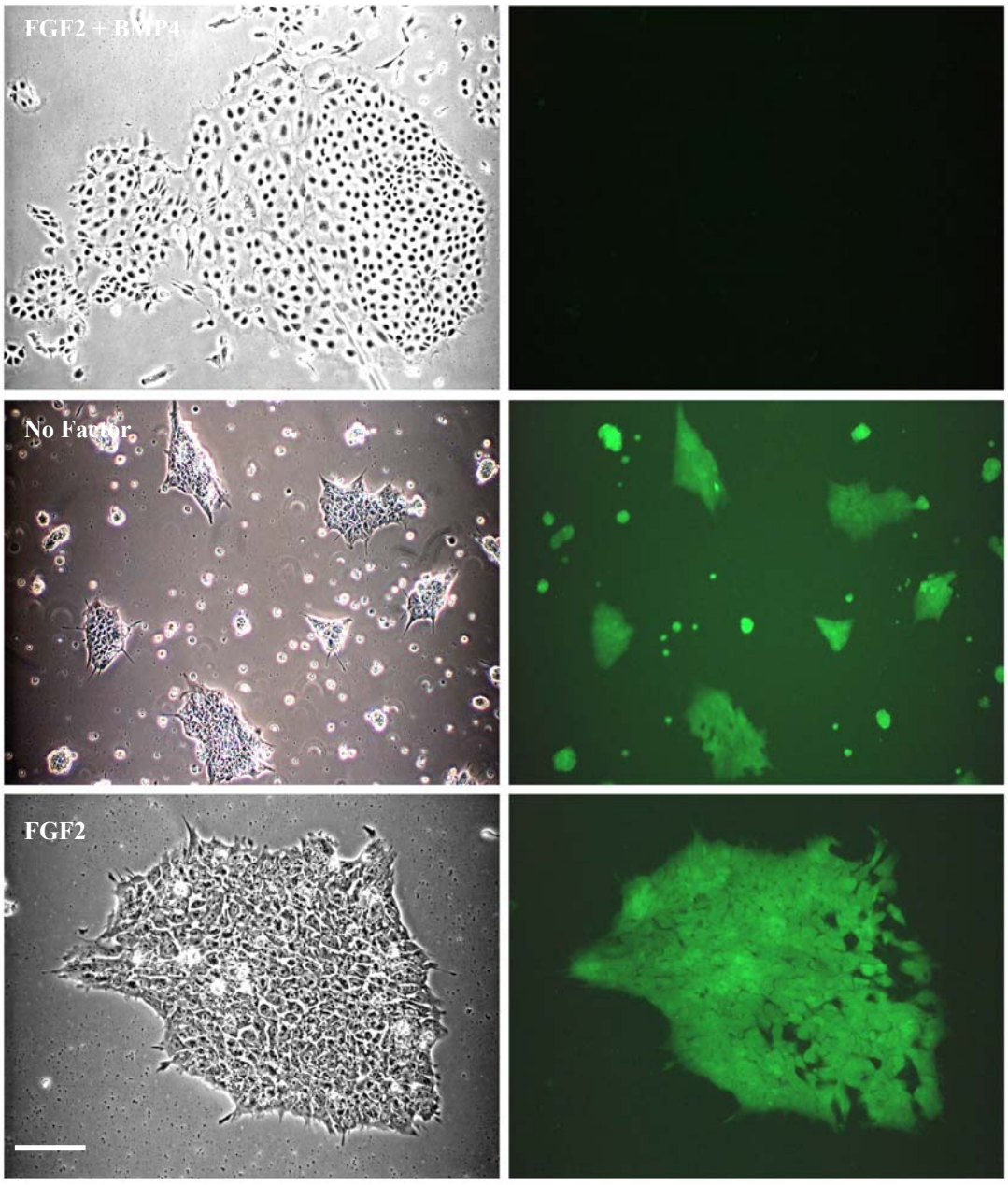


Figure 4.23. Induced differentiation of T5s in serum free medium. T5s differentiated when the serum free medium was supplemented with BMP4 but not upon FGF2 withdrawal (scale bar = 200μm).

4.5.3.2.3 FGF2 effect on the self-renewal of the parental cell line H1

The expression of surface and internal markers characteristic of hES cells suggested the normal behaviour of the H1 and T5 cells lines in the serum free conditions. However, T5 cells failed to differentiate upon FGF2 withdrawal. To test whether the self-renewal of the parental line H1 was dependent on FGF2 in the serum free conditions, the expression of cell surface markers was assessed in the presence and absence of FGF2. $5x10^5$ cells were seeded in the presence of FGF2 and once attached to the substratum the medium changed to +/- FGF2. Following six days in these conditions and a daily media change (Figure 4.24) the expression of TRA1-81 and SSEA1 was assessed (Figure 4.25). The expression of these markers was also analysed before setting the experiment to monitor any possible plating effect.

The effect of the absence of FGF2 (Figure 4.24) could be observed as early as day two in the differentiation process. At this early stage the lack of FGF2 resulted in the elongation and flattening of the cells, which correlated with the larger G1 of the cell cycle (Figure 4.20C). Further flattening of the colonies and an increase in the amount of loose cells appeared progressively throughout the experiment. These morphological changes associated with differentiation, were concurrent with a decrease in cell proliferation as discussed previously.

Flow cytometry results showed a comparable expression of surface markers before and after the experiment only in the presence of FGF2 (Figure 4.25). Strikingly, when FGF2 was not added the expression of the marker associated with differentiation SSEA1

increased from 6.5% to 68.2%. The up-regulation of SSEA1 was related to a decrease in TRA1-81 expression. The change in TRA1-81 expression was not as dramatic as that in SSEA1, possibly because induction of a marker was more readily detected than the loss of one already expressed. Interestingly, the withdrawal of FGF2 resulted not only in the decreased expression of TRA1-81 but also in the number of cells expressing this surface protein. This suggested a reduction in the expression of the marker on individual cells as well as on the whole population.

Results compiled from the analysis of the expression of surface markers from 3 independent experiments are presented in Figure 4.25. These experiments consistently showed an increase in SSEA1 and a decrease in TRA1-81 expression in the absence of FGF2. This indicated that H1 cell self-renewal was dependant on an FGF2 supplement to the N2B27 medium.

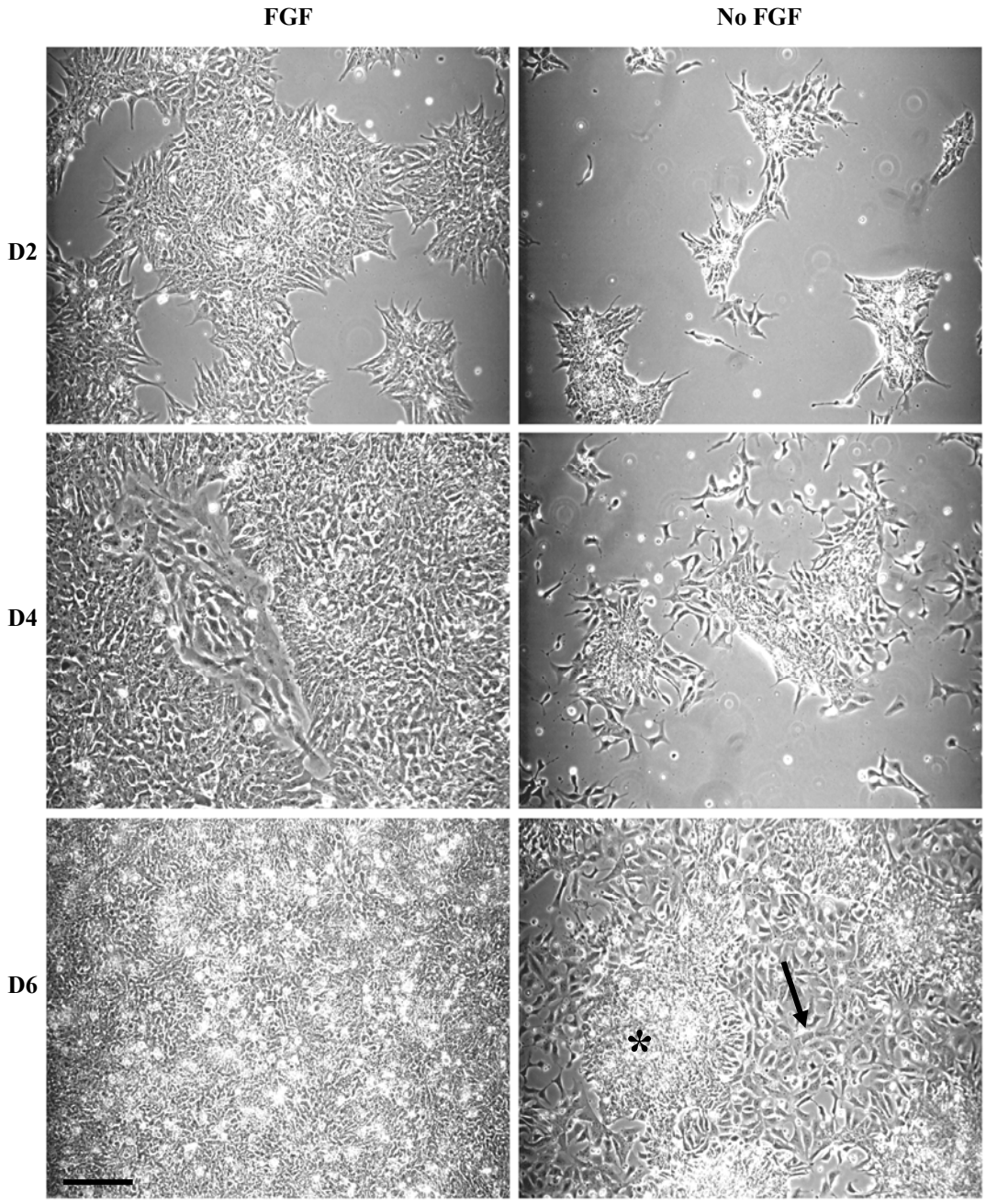


Figure 4.24. Morphological effect of FGF2 on H1's self renewal. The H1 cells were maintained in the presence or absence of FGF2 for 6 days with a daily media change. Morphology associated with hES cell differentiation was appreciated early (D2-3). At the end of the time course the cells grown with FGF2 had formed a homogeneous layer, whereas their counterparts without FGF2 were reduced to small areas of tight colonies (highlighted by the asterisk) surrounded by flat cells with differentiated morphology (highlighted by the arrow). (Scale bar = 500µm)

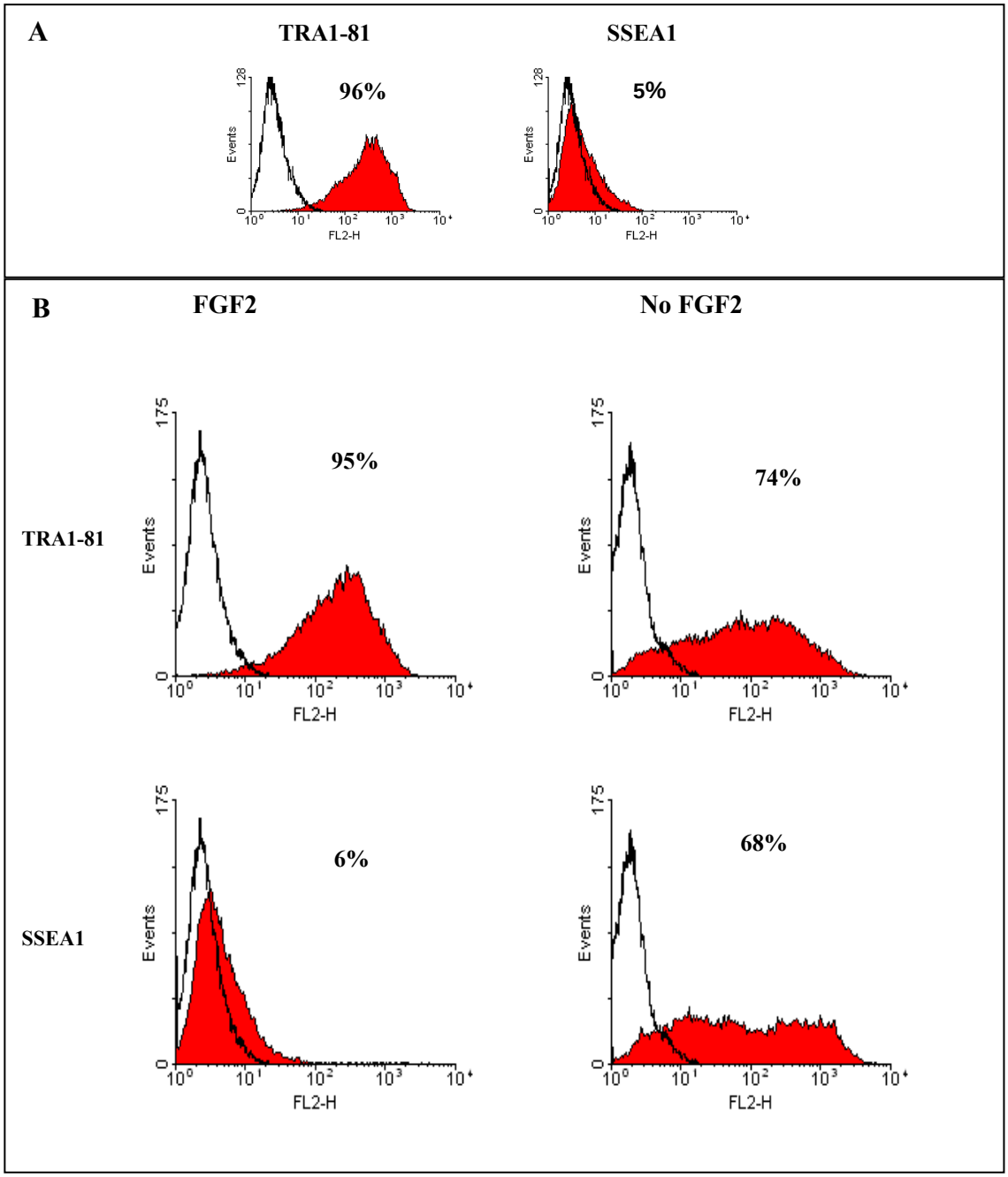


Figure 4.25. Effect of FGF2 on the expression of H1 surface markers analysed by flow cytometry. **A:** Surface markers expressed on H1 before being plated for the differentiation experiment. **B**: expression of surface markers with or without FGF2 at day 6 of the experiment. The isotype antibody control appears as an empty histogram plot and cells stained with SSEA1 or TRA1-81 antibody as red fill. Results here presented are representative of three independent experiments.

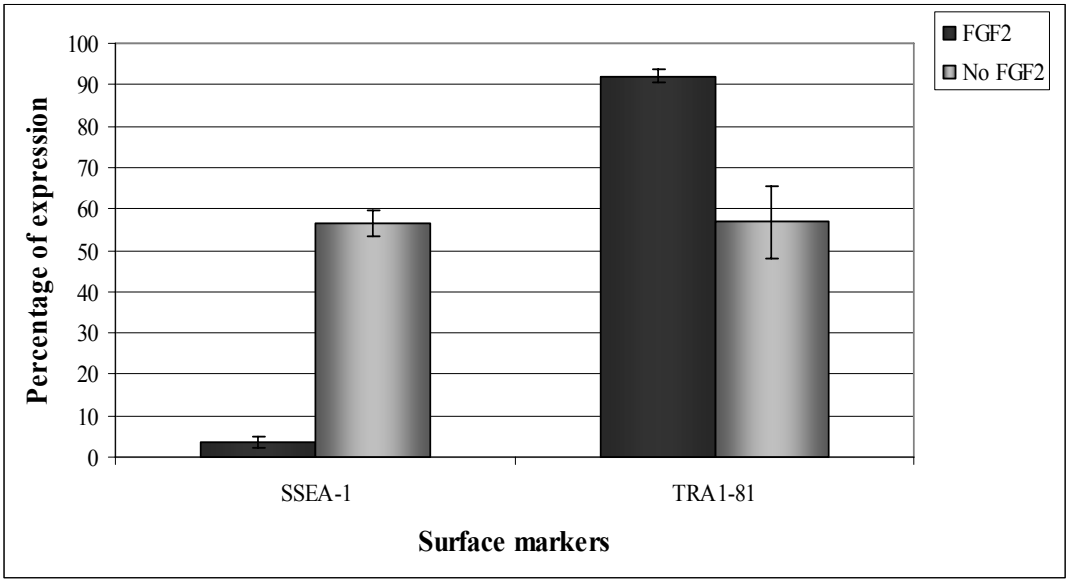


Figure 4.26. Effect of FGF2 on the expression of H1 surface markers. The expression of the surface markers SSEA1 or TRA1-81 on H1 was assessed by flow cytometry. SSEA1 expression increased in the absence of FGF2 and TRA1-81 was down-regulated. This effect was highly significant ($p<0.001$). A two-way test of variance was applied to generate a p value. The results were represented as an average of the percentage of cells expressing these markers in three independent experiments.

4.6 Discussion

Typically hES cell proliferation depends on the presence of a mouse (Thomson *et al.,* 1998) or a human (Amit *et al.,*2003) fibroblast feeder layer to produce the matrix required for adherence of the cells and to secrete factors to maintain their self-renewal and pluripotency potential. Recently the culture techniques are moving from the use of feeder cell layers towards more defined systems. However, hES cell culture still in the main involves the use of the ill-defined conditioned media from the feeder cells. During conditioning of the medium, undefined factors are secreted into the culture. This produces a highly variable system, thereby complicating the replication of the culture conditions and the quality control. FGF2 is one of the factors released by the feeder cells (Lim and Bodnar, 2002), which creates complications in evaluating the effect of the supplemented FGF2. In addition to this, hES cells tend to spontaneously differentiate in CM causing a mixture of cells at different stages of differentiation. For these reasons a defined culture system within which to propagate hES cells was essential for the study of FGF2. However, at the time of preparing this thesis there was not a commercially available serum free media specifically produced for hES cells. Furthermore, there was no conclusive data published clarifying the highly complex requirements of hES cells. The defined medium N2B27 (Ying *et al.,* 2003b) was chosen to study the short term biological and biochemical effects of FGF2 on hES cells. N2B27 had been developed for the study of mES cells, but surprisingly also supported hES cells propagation when supplemented with FGF2 and when used in conjunction with Matrigel. Matrigel is a basement membrane preparation extracted from a murine Englebreth-Holm-Swarm

sarcoma. As it is composed of murine proteins and growth factors, Matrigel potentially adds some variability to the system. To minimise this variability a growth factor-reduced Matrigel at a dilution of 1:100 was used.

This thesis reports the propagation of two cell lines: T5 (Gerrard *et al.,* 2005) which carries an EGFP reporter under the control of the OCT4 promoter, and the parental line H1 (Thomson *et al.,* 1998) in the N2B27 medium. After several passages in N2B27, the two lines maintained a high expression of the OCT4 protein and of the surface markers characteristic of hES cells. These cells formed aggregates with the characteristics of embryoid bodies in a similar manner to the cells in CM (data not shown). However, the expression of the three embryonic markers was not analysed, which would be necessary to confirm pluripotency. Two groups have stablished though the pluripotency of H1 hES cells maintained in N2B27 by differentiation into the three germ layers. Yao *et al*, (2006) established pluripotency through the injection of the hES cells into nude mice followed by the histological characterisation of the formed teratomas. In addition to this, Liu *et al,* (2006) demonstrated that the H1s growing in the N2B27 medium readily formed embryoid bodies, which when plated differentiated into the three layers and trophoblast in a similar manner to those cells in CM.

The addition of FGF2 to the hES cells growing in these semi-defined conditions produced an enhancement in the cell number and a lower amount of floating cells. Therefore, FGF2 seemed to have a positive effect on cell proliferation and a role in plating efficiency. However, it needs to be noted that FGF2 might also be supressing

cell death. FGF2 improvement in plating efficiency might be achieved by inducing the expression of cell adhesion molecules (Debiais *et al.,* 2001) or forming complexes with FGFR along with Matrigel associated heparin sulfates (Richard *et al.,* 2000). Analysis of the effect of FGF in the expression of cell adhesion molecules (e-cadherin, integrins α4 and α5, ICAM 1 and 3) would give an indication of their interactions and their role on cell attachment and plate efficiency. The enhancement in cell proliferation in the presence of FGF2 seemed to be related to the increase in cell synthesis and mitosis as indicated by the BrdU incorportation and the cell cycle analysis. This is consistent with the reported role of FGF2 in the regulation of genes associated with hES cell proliferation (Greber, 2006). FGF2 seems to up- regulate more than 30 genes involved in cell cycling as well as inducing the expression of oncogenes such as *Fos, Jun, Myc, Ets2* (Greber, 2006). In addition to the mitogenic effect, cell cycle analysis also might suggest that FGF induced a more rapid transit of the hES cells through the G1 phase. However, a synchronisation of the cells should be carried out to confirm if the short G1 phase was enhanced by the effect of FGF2. A short G1 phase would concur with the reduced time of this phase that has been reported to be characteristic of ES cells (Savatier *et al.,* 2002; Fluckiger *et al.,* 2006; Becker *et al.,* 2006). This brief G1 phase is the cause of the shortened cell cycle in hES cells (15.8h against 24-32h in proliferating somatic cells) as determined by the proliferative index (Becker *et al.,* 2006). The report presented by Becker *et al,* (2006) also strongly suggested that high levels of cyclin D2/CDK4 complexes were to account for this rapid proliferation of hES cells. Another possible candidate in the stimulation of entry in the S phase is the protooncogene *c-Myc*.

It has been reported that *c-Myc* collaborates with the retinoblastoma pathway in the activation of *Cyclin* genes required to entry in the S phase (Bartek and Lukas, 2001). In addition to this role in the cell cycle, it has been suggested that *c-Myc* is a key regulator of mES self-renewal since it is a common target of LIF/STAT3 and/or Wnt (Cartwright *et al.,* 2005). Therefore expression levels of the *c-Myc* gene in the presence/absence of FGF2 could demonstrate whether this gene is activated by FGF2 and maybe identify possible connections between the cell cycle and self-renewal.

The negative effect in cell proliferation when FGF2 was not supplemented was bypassed in T5s by maintaining the medium in which the cells were plated (Appendix 4). This suggested the presence of FGF2 and/or other autocrine factor/s were able to maintain T5 cell proliferation in the absence of exogenous FGF2. It was believed that autocrine signalling could also account for the unexpected maintenance of T5's self-renewal when FGF2 was not added to the medium. However, addition of BMP4 resulted in the rapid differentiation of the T5 cells. This indicated that these cells have not lost the capacity for differentiation and suggested there could be activity of FGF and/or others signals. A noticeable differentiation in the absence of FGF2 was by contrast suggested in the parental line H1. Withdrawal of FGF2 resulted in the emergence of the flattened cell morphology and of the surface marker SSEA1 as well as the down regulation of TRA1-81, which are changes associated with hES differentiation.

In conclusion, although intended to be used only for the short term experiments, hES cells could be propagated in the N2B27 defined medium for several passages and

provided a consistent tool for the study of FGF2 on hES cells. However, it needs to be taken into account that this is a semi defined system since the Matrigel used can be variable and it also includes growth factors and proteins. This implies that a cooperation of FGF with other signal cannot be excluded.

CHAPTER 5

THE EFFECT OF FGF SIGNALLING AND CO-OPERATING FACTORS ON HUMAN ES CELL SELF-RENEWAL

5.1 Introduction

It was shown in the previous chapter that FGF2 could sustain the proliferation of the hES cells. However, in later batches of medium it was seen an increase in the background differentiation. Matrigel is a variable component in the N2B27 culture system, which implies that other elements might be involved in the regulation of self-renewal besides FGF2. This chapter dealt with the possible factors included in Matrigel, which could collaborate with FGF2. In addition to this, the principal pathways down-stream of FGFR were studied.

Self-renewal of hES cells is sustained when the cells get the appropriate signals to prevent spontaneous differentiation. In conventional culture systems, these signals are provided by the feeder cells, either by direct contact of hES cells with the feeder layer and/or by soluble factor/s secreted by the feeders and present in the CM (Brivanlou *et al.,* 2003). Recent systems have tried to replicate these signals by adding specific growth factors to basal medium and several groups have reported the growth of hES cells in feeder free conditions (Amit *et al.,* 2004; Sato *et al.,* 2004; James *et al.,* 2005; Dravid *et al.,* 2005; Xu *et al.,* 2005a; Xu *et al.,* 2005b). However, at the time of these publications the numerous signals from the feeders were mostly yet undefined. FGF2 was the exception, since it has been identified early as the key factor that is necessary for hES cell propagation. Accordingly, FGF2 has been used consistently to derive and maintain hES cell lines (Amit *et al.,* 2000) and has been shown to support hES cell growth in the absence of CM (Amit *et al.,* 2004; Wang *et al.,* 2005; Xu *et al.,* 2005a; Xu

et al., 2005b). Therefore, substantial evidence indicates the significance of FGF signalling in hES cell proliferation. However, this conclusion has been reached through the use of complex culture conditions in which other factors may be acting.

Recent transcriptional profiling has confirmed the significance of the FGF pathway, but it has also been suggested that Wnt and TGFβ pathways may play relevant roles in the regulation of hES cells (Sato *et al.,* 2003; Levenstein *et al.,* 2005; Wei *et al.,* 2005). It has been shown that the sustained activation of Wnt by the GSK-3 specific inhibitor BIO was sufficient to maintain hES cell self-renewal (Sato *et al.,* 2004). Conflicting with this report, (Dravid *et al.,* 2005) demonstrated, by using Wnt antagonists, that Wnt activation is not sufficient to maintain undifferentiated and pluripotent hES cells in the long-term. Dravid *et al,* (2005) suggested that Wnt signalling promotes hES cell proliferation only when other self-renewal signals, both intrinsic and extrinsic are present. As discussed above, significant evidence indicates that FGF2 is a promoter signal for hES cell self-renewal, but other factors could also be co-operating in this role. It has been proposed that Activin and TGFβ, two factors produced by MEFs (Beattie *et al.,* 2005) could be involved in sustaining the undifferentiated state of hES cells. More specifically (James *et al.,* 2005) suggested that Activin was the factor, which collaborated with Wnt in the maintenance of pluripotency. In this model the TGFβ/Activin/Nodal branch of the pathway through Smad2/3 needed to be activated and the BMP4/GDF branch repressed to prevent differentiation of hES cells (James *et al.,* 2005). It has been shown that BMP4 activation of Smad 1/5 induces trophoblast differentiation in hES cells (Xu *et al.,* 2002), which can be prevented by the antagonic effect of FGF2 (Pera *et al.,* 2003).

Consistent with this, it has been suggested that inhibition of BMP4 activity (Wang *et al.,* 2005; Xu *et al.,* 2005b) is the mechanism by which significantly high concentrations of FGF2 (100ng/ml) (Levenstein *et al.,* 2005; Xu *et al.,* 2005b; Liu *et al.,* 2006) sustain the undifferentiated state in hES cells.

FGF2 autocrine and possibly intracrine signalling have to be added to the complex extrinsic factors acting on hES cells (Dvorak *et al.,* 2005; Xu *et al.,* 2005b; Liu *et al.,* 2006). Autocrine/intracrine mechanisms was hypothesised in Chapter 4 might be sustaining T5 cell proliferation and self-renewal in the absence of exogenous FGF2. The high expression of FGF2 by hES cells (Brandenberger *et al.,* 2004; Xu *et al.,* 2005b; Liu *et al.,* 2006) suggests the potential role of this factor in autocrine and paracrine signalling. This is consistent with the difficulty of clonal growth of hES cells, which indicates the necessity for FGF2 autocrine signalling. However, this could also suggest the involvement of other autocrine factors, such as Nodal, which are also produced by hES cells, (Xu *et al.,* 2001).

In conclusion, FGF2 is the common key component of the many different systems used to grow hES cells. FGF2 as shown in Chapter 4 is required for proliferation and self-renewal of hES cells in serum free conditions. However, the presence of Matrigel includes critical factors to the system suggesting co-operating activity. The activity of co-operating factors with FGF2 was also suggested by the observation that CM which had not been produced in presence of FGF2 could not sustain the hES cells. This would be in line with recent, increasingly popular, suggestions that signals, principally those

activators of SMAD2/3 might be involved in hES cells self-renewal. Therefore, to identify the key signalling pathways downstream of FGF2 and to establish whether these signals are sufficient in sustaining self-renewal it is necessary to understand the regulation of hES cells.

5.2 Hypothesis

That FGF2 signalling plays the key role in maintaining hES cell self-renewal and that other signalling pathways may contribute to this outcome.

5.3 Aims

I. To establish the biochemical activation of the MAPK/ERK signalling pathway in hES cells when treated with FGF.

II. To identify the key signalling pathways downstream of FGF2 involved in hES cell self-renewal.

III. To describe the possible interactions between the FGF pathway and other significant signalling pathways such as TGFβ in deciding the fate of the hES cells.

5.4 Experimental design

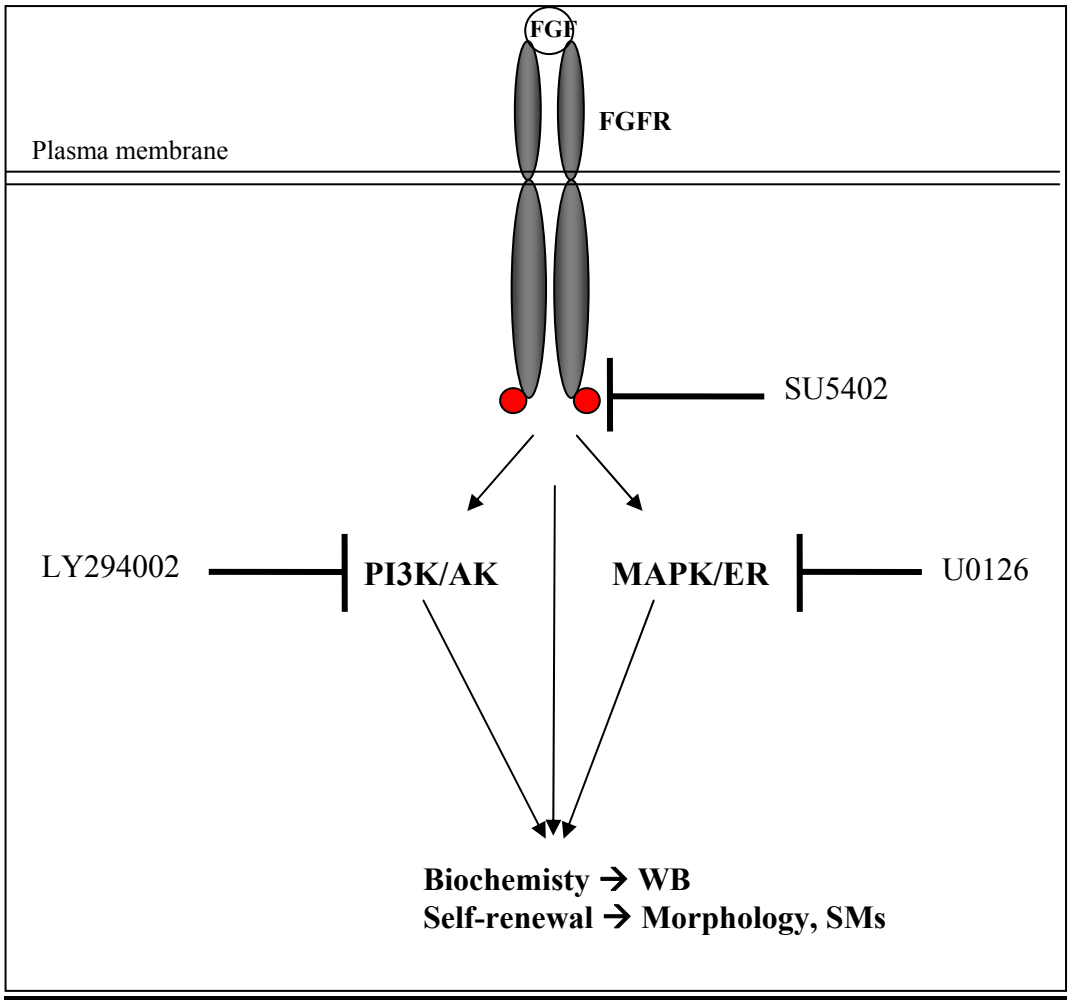


Figure 5.1. Diagram of the experimental steps followed in this chapter. Western Blotting (WB) was used to asses the induction of H1 cell line with FGF2 or FGF4. Also WB was used to assess the biochemical effect of blocking FGFR, and the signalling pathways downstream of the receptor PI3K and MAPK. These inhibitions would be performed by the use of the inhibitors SU5402, LY29400 and UO126 respectively. The effect of these inhibitions was monitored by observation of the morphological changes and analysis of surface markers (SMs) on the hES cells.

5.5 Results

5.5.1 Activation of hES cells by FGF

To determine whether hES cells maintained in N2B27 are activated by FGF, the phosphorylation of the extracellular signal-regulated kinase (ERK) was evaluated by western blotting after stimulating the cells with 25ng/ml of FGF2 or FGF4. LIF, which has been shown to activate ERK in mES cells (Burdon *et al.,* 1999b; Kang *et al.,* 2005) was used to stimulate hES cells at a concentration of 10ng/ml. STAT3 as well as ERK phosphorylation was analysed in the cells treated. FGF2 and 4 strongly activated ERK1 and 2 (ERK-P) in the hES cells (Figure 5.2). Immunoblotting of the samples with anti-ERK2 antibody, revealed an additional band above the inactive ERK2 indicative of a motility shift. When compared with FGF activation of ERK on mES cells (Figure 3.2), this "mobility shift" suggested that FGF activation of ERK in the hES cells was stronger. This strong activation is likely to be consequence of the higher expression on the hES cells of all four FGF receptors (Brandenberg *et al.,*2004) compared with the mES cells (Esner *et al.,* 2002).

On the other hand, FGF did not activate STAT3 on hES cells, although it was strongly phosphorylated by LIF. This contrasts with LIF activation of ERK in mES cells, which was not phosphorylated by FGF.

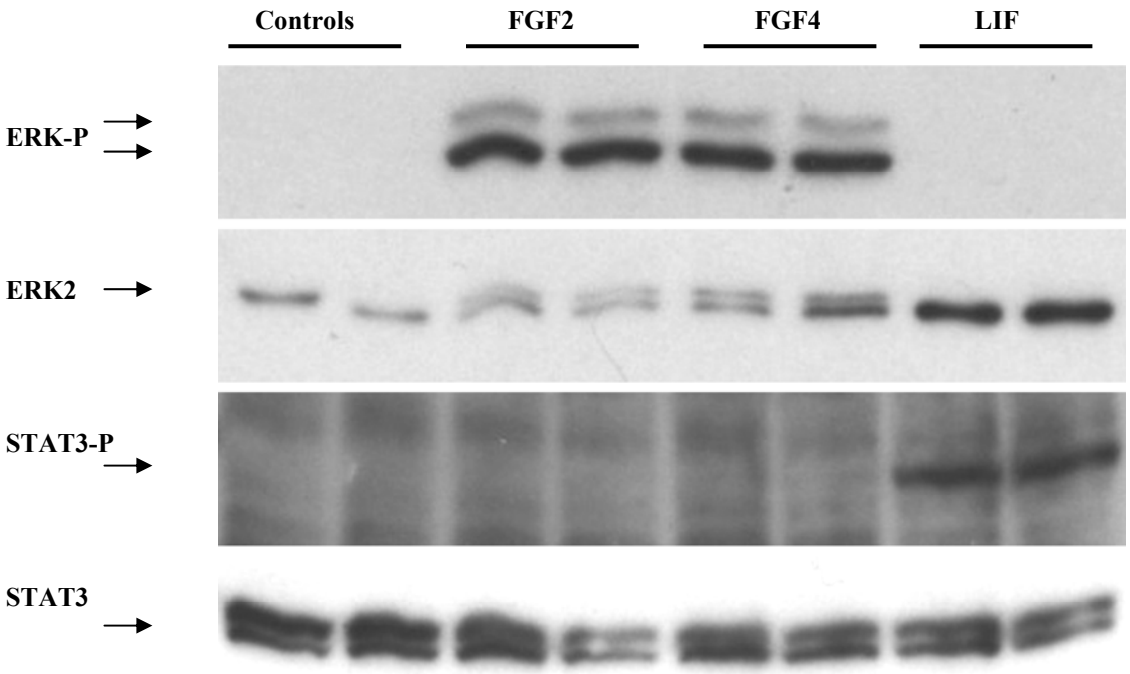


Figure 5.2. Western Blotting of ERK and STAT3 activation on hES cells. Human ES cell stimulation by FGF or LIF (10 minutes) was analysed by examining the activation of ERK or STAT3 proteins. Cell lysates from cells that have been starved of signal were used as controls. The immunoblots of the cell lysates were probed with an antibody specific for the active phosphorylated forms of ERK1/2-P or STAT3-P. After stripping, the membranes were re-probed with antibodies that recognise ERK2 or STAT3 protein as controls for protein loading. This pattern of activation was consistent in two other immunoblot repeats in H1 and H9 cell lines.

5.5.1.1 FGF2 titration

To determine a range of concentrations that activate hES cells, different doses (0, 2, 4, 8, 16 and 32ng/ml) of FGF2 were used in a 10 minutes induction. Following the blotting, the nitrocellulose membrane was probed with a specific antibody for the phosphorylated form of ERK and re-probed with an antibody against the total ERK2 protein as a control (Figure 5.3). ERK was activated in hES cells even at the lowest concentration tested of FGF2 (2ng/ml).

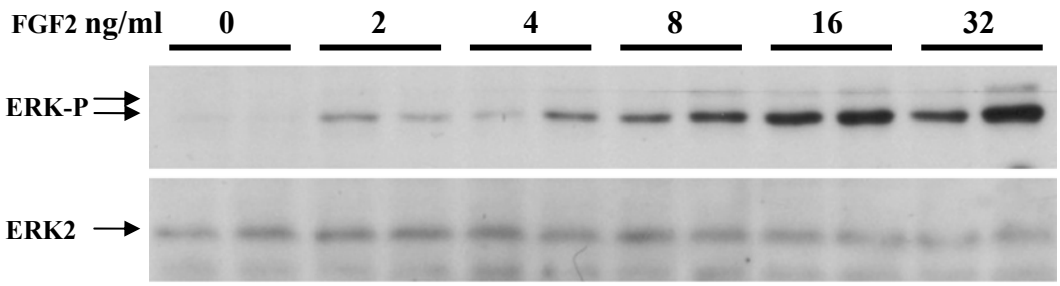


Figure 5.3. Western Blotting of ERK activation by FGF2 titration. Stimulation of hES cells with titrated concentrations of FGF2 were analysed by examining the activation of ERK protein. Cell lysates from cells that have been starved of signal were used as controls. The immunoblots of the cell lysates were probed with an antibody specific for the active phosphorylated forms of ERK1/2-P. After stripping, the membranes were re-probed with antibodies that recognise ERK2 protein as controls for protein loading. FGF2 titration has been repeated with identical results.

5.5.2 Effect of FGF2

To establish the effect of the different concentrations of FGF2 on the cell biology, duplicated wells with $5x10^5$ cells were plated and different concentrations of FGF2 (from 0 to 32ng/ml) were added into the N2B27media (Figure 5.4). To prevent major autocrine effect, the media was changed daily. After 6 days of treatment the expression of SSEA1 and TRA1-81 was assessed by flow cytometry (Figure 5.5).

Cell morphology (Figure 5.4) indicated that the effect of FGF2 on hES cells was dose dependent. In the absence of FGF2 the colonies appeared flattened with large cells, a morphology associated with differentiation. At only 2ng/ml of FGF2 the effect of the factor could be observed by a few tight colonies indicative of perhaps remaining ES cells. This change in cell morphology was associated with the increased phosphorylation of ERK shown in the western blotting (Figure 5.3).

After 6 days of continuous exposure to the different doses of FGF2, the expression of cell surface markers was assessed by flow cytometry (Figure 5.5). As expected by the flattened cell morphology (Figure 5.4) observed in the absence of FGF2, the marker of differentiation SSEA1 was highly expressed in contrast to the low expression of TRA1-81. Coinciding with the few remaining tight colonies at the lowest concentration of FGF2 (2ng/ml), TRA1-81 levels increased and SSEA1 decreased in relation to 0ng/ml of FGF2. From 4ng/ml, TRA1-81 expression is higher than SSEA1.

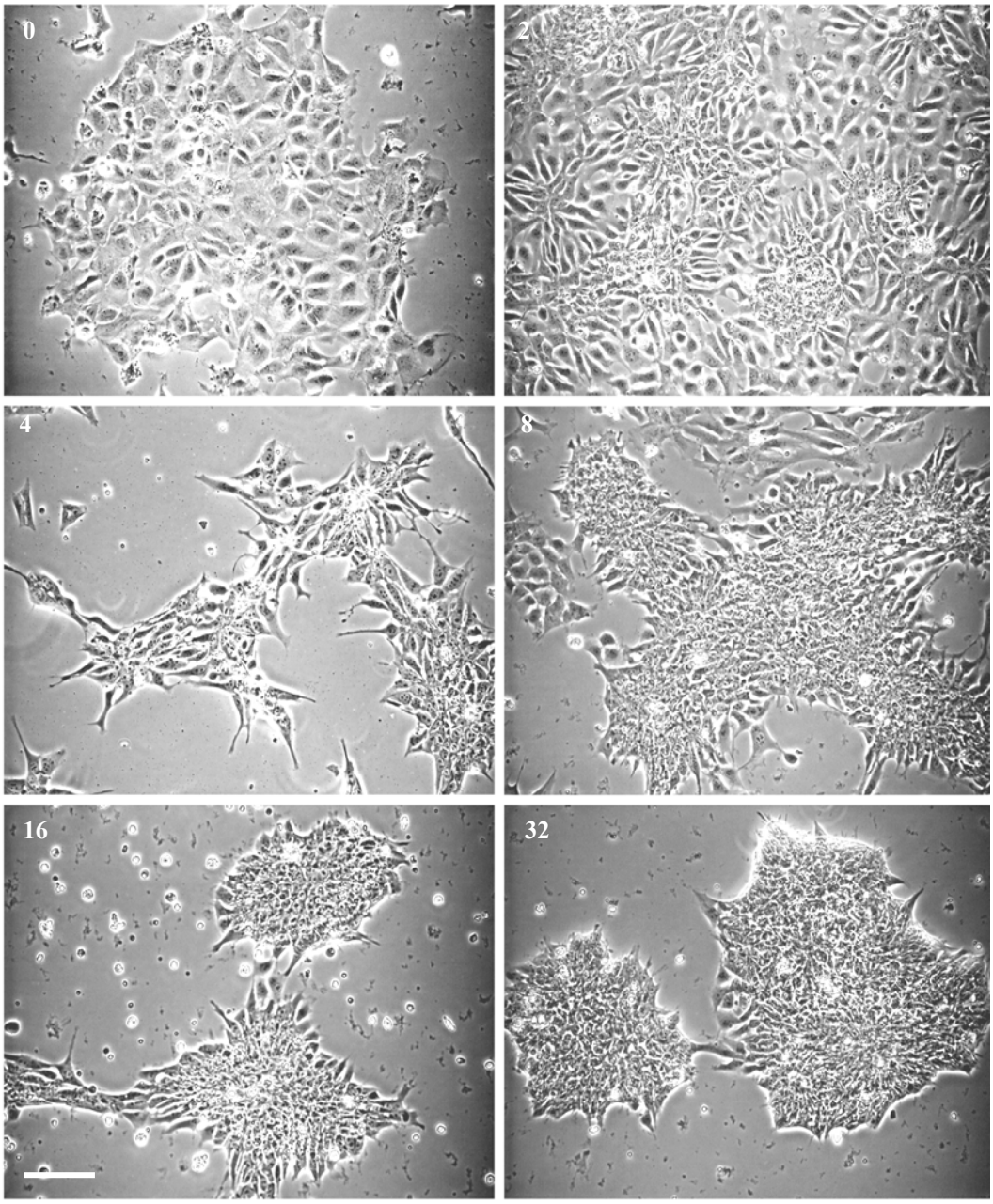


Figure 5.4. Effect of FGF2 titration on hES cells morphology. Increased concentrations of FGF2 (0-32ng/ml) were added to the hES cells. The lower doses of FGF2 (2 and 4 ng/ml) were unable to support the undifferentiated state of the cells, which is shown by the flattened, spicky morphology of the colonies. At higher concentrations, the colonies appeared progressively more compact with more defined edges (scale bar = 200μm). FGF2 titration has been repeated on cell morphology with matching results.

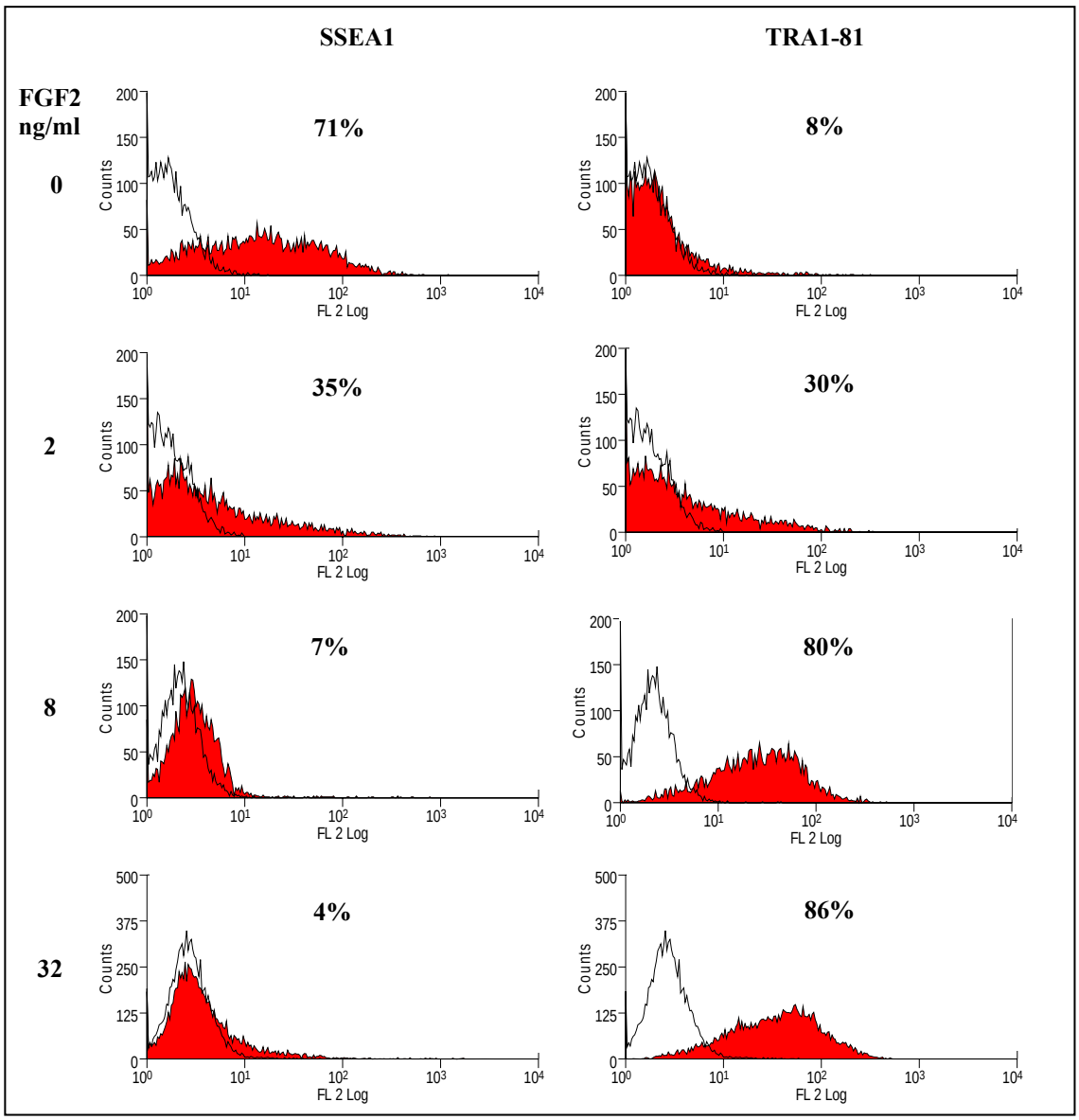


Figure 5.5. Effect of FGF2 titration on hES expression of surface markers Increased concentrations of FGF2 (0-32ng/ml) were added to the hES cells. After 6 days in those conditions, the expression of the surface markers SSEA1 and TRA1-81 were analysed by flow cytometry. The compact colony morphology at increased FGF2 concentration was associated with a stronger TRA1-81 and a decrease in SSEA1 expression. FGF2 titration has been repeated on cell morphology with matching results.

5.5.3 Effect of FGF4 on hES cells

FGF4 significantly activated hES cells at a similar level than FGF2 (Figure 5.2). In addition to this, a recent report attributes an important role to FGF4 in hES cell signalling with the consequences of growth and differentiation (Mayshar *et al.*, 2008). To determine whether the effect of FGF2 on hES cells is specific, the effect of FGF4 on the morphology of hES cells was assessed. 10^5 cells were plated in N2B27 and FGF4 (8ng/ml) was added into the media daily. After 6 days of treatment, morphological observation suggested that FGF4 was not compensating for FGF2 (Figure 5.6). However, a positive effect of this factor on cell growth could be indicated by the larger colonies formed in the presence of FGF4 compared with N2B27 alone. This could mean that the effect of FGF2 on hES cells is specific and even when hES cells clearly respond to FGF4 (Figure 5.2) it could not sustain hES cell self-renewal.

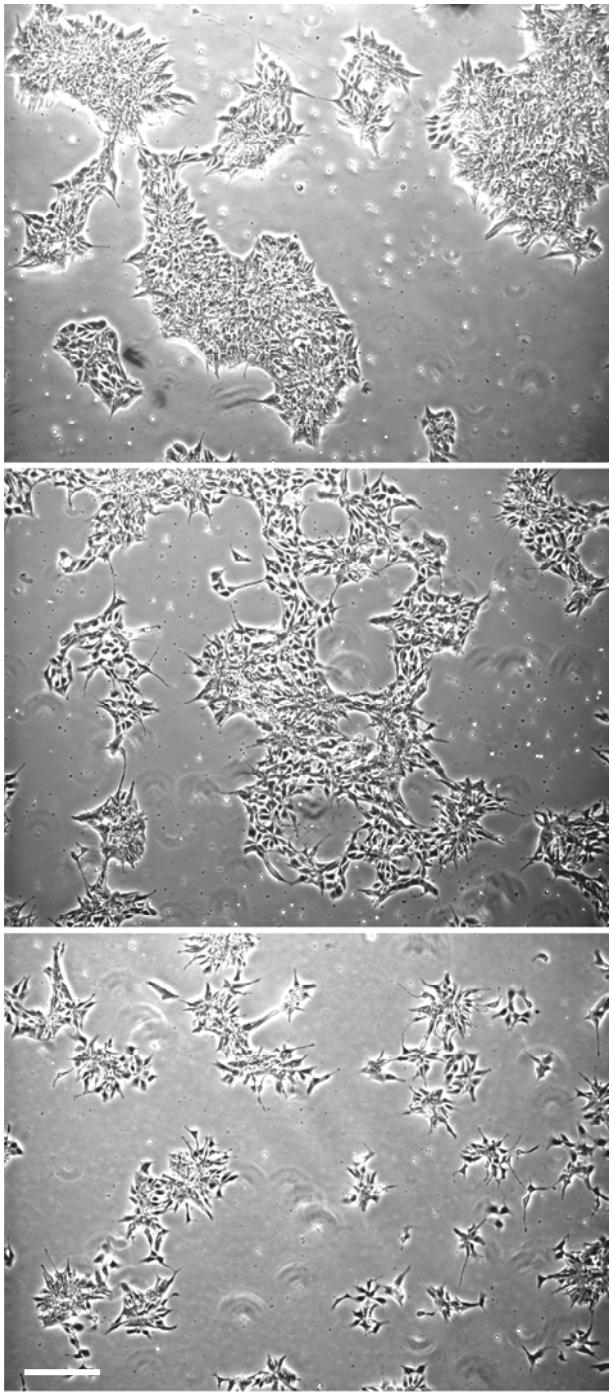


Figure 5.6. Effect of FGF4 on the morphology of hES cells. FGF4 supplement seemed to enhance cell growth but it could not sustain hES cell self-renewal indicating the specificity of the FGF2 effect. However, this should be repeated to confirm. Scale bar = 500μm

5.5.4 Effect of FGFR inhibition by SU5402

To confirm the promotion of FGF2 on hES cell self-renewal and to test whether autocrine FGF signalling is playing any role in the proliferation of undifferentiated hES cells, FGFR signalling was inhibited. SU5402 selectively inhibits the tyrosine kinase activity of FGFR1 by interacting with the catalytic domain of this receptor without inhibiting PDGF, EGF or insulin receptors (Mohammadi *et al.*, 1997).

To determine the concentration of inhibitor to use in the biological assessment, 10^6 cells/well were plated on N2B27 containing FGF2. After 24 hours the medium was changed to a control medium (N2 without B27, insulin and FGF2). Following 4 hours incubation, the medium was changed to the control medium with the inhibitor. After incubating for 1 hour the cells were stimulated with 25ng/ml of FGF2.

High levels of phosphor-ERK were observed in the absence of SU5402 following FGF2 simulation (Figure 5.7). ERK phosphorylation was potently inhibited when SU5402 was present. ERK phosphorylation was observed at low levels in 20μM SU5402 and was detectable in 30 μM SU5402. The membrane was stripped and re-probed with antibody that recognises ERK2 protein as a control. This indicated that total ERK was unaffected by SU5402. SU5402 inhibited FGF2 mediated phophorylation in the H1 cell line hES cells.

To assess the biological effects of inhibiting FGFR, $5x10^5$ cells/well were plated in a 6 well dish with FGF2 and 20μM of SU5402. This concentration was chosen as the increased concentration of 30μM resulted in too high a cell death to analyse. As controls, the cells were treated with a concentration of DMSO, which is equivalent to the maximum used with the inhibitors. Cells with or without FGF2 were also included.

After 2 days of continuous exposure to SU5402, the cells acquired a differentiated morphology in the centres of the colonies, in addition to slower proliferation. After 6 days, very few small flattened colonies remained in the presence of the inhibitor. (Figure 5.8). This morphology characteristic of differentiation of H1 cells in N2B27 was associated with an increase in SSEA1 and a decrease in TRA1-81 expression (Figure 5.9). In conclusion, morphology and surface marker expression indicated that H1 hES cell self-renewal is attained by activation of FGFRs. Interestingly, a more dramatic effect was observed by the use of the inhibitor than when FGF2 was withdrawn, suggesting an autocrine activation of FGFR.

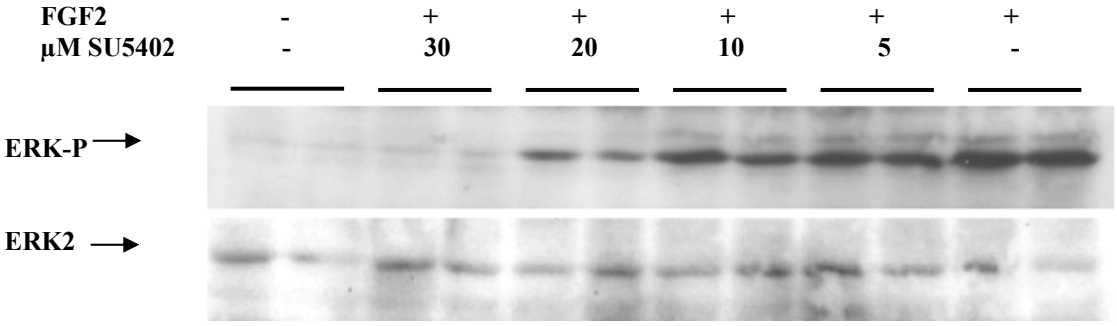


Figure 5.7. Inhibition of FGFR signal by SU5402 on H1s. Cells were cultured in a control medium (medium without FGF, insulin or B27) for 4 hours before changing to a medium with the inhibitor SU5402 at different concentrations (30 to 5 μM). After 1 hour of treatment with the inhibitor, the cells were stimulated with FGF2 and lysates were blotted and probed with an antibody specific to phosphorylated ERK (ERK-P). SU inhibited FGF2 mediated phosphorylation of ERK1 and 2 in H1s hES cells. SU5402 had no effect on the overall level of ERK2 as revealed by re-probing with an antibody that recognised total ERK2. The results shown here are representative of three independent experiments.

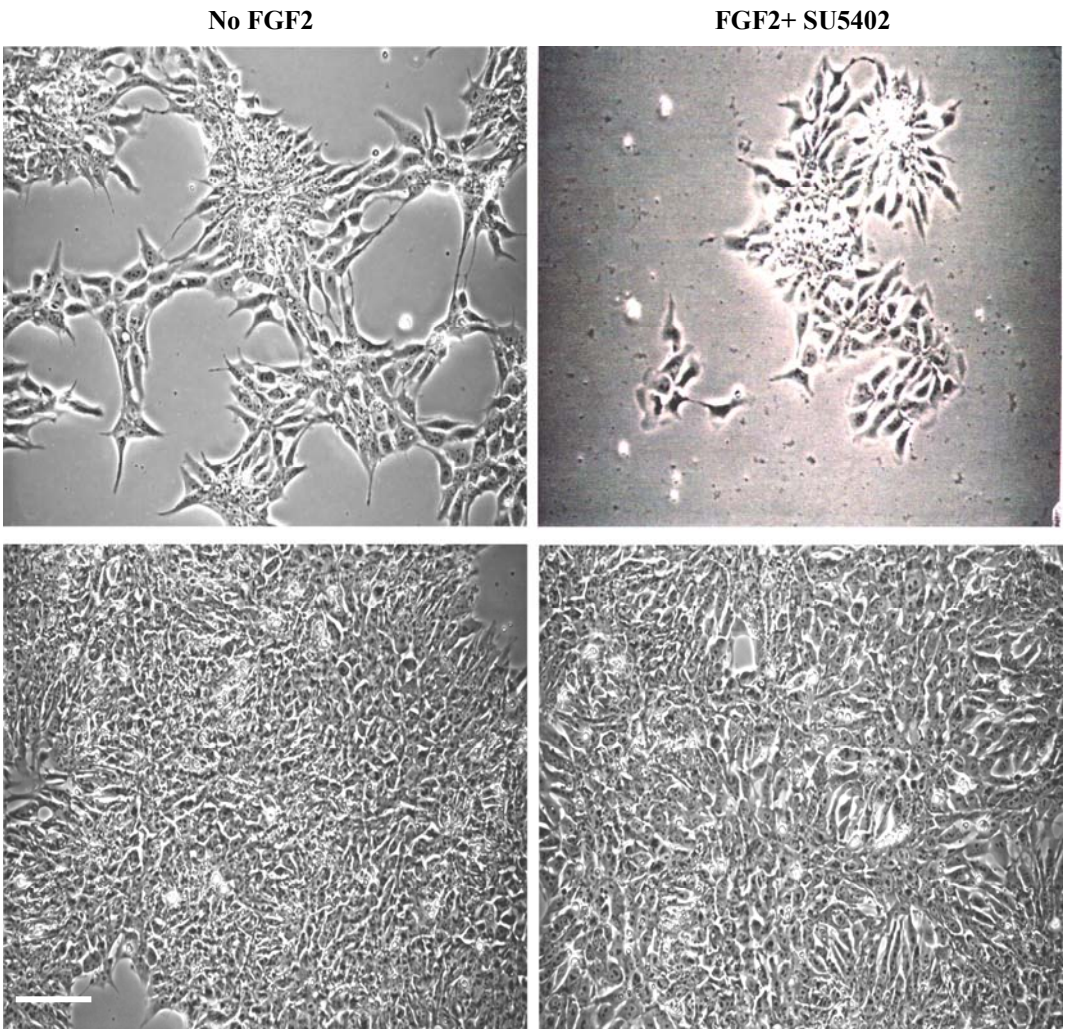


Figure 5.8. Effect of SU5402 on cell morphology. Duplicate wells were plated with 10^5 cells/well in the absence of FGF2, FGF2 and the FGFR inhibitor Su5402 (20ng/ml), FGF2 alone and FGF2 and DMSO as a control. After 6 days in these conditions, cells in the absence of FGF2 presented a differentiated morphology in addition to a lower number of cells when compared with the controls. The inactivation of FGFR resulted in a similar morphology to that produced by FGF2 withdrawal, which indicated that FGFR activation was key in hES cell self-renewal. The more striking morphology in the presence of the inhibitor than in the absence of FGF2 could be indicative of FGF autocrine signalling (scale bar = 200μm). The results shown here are representative of three independent experiments.

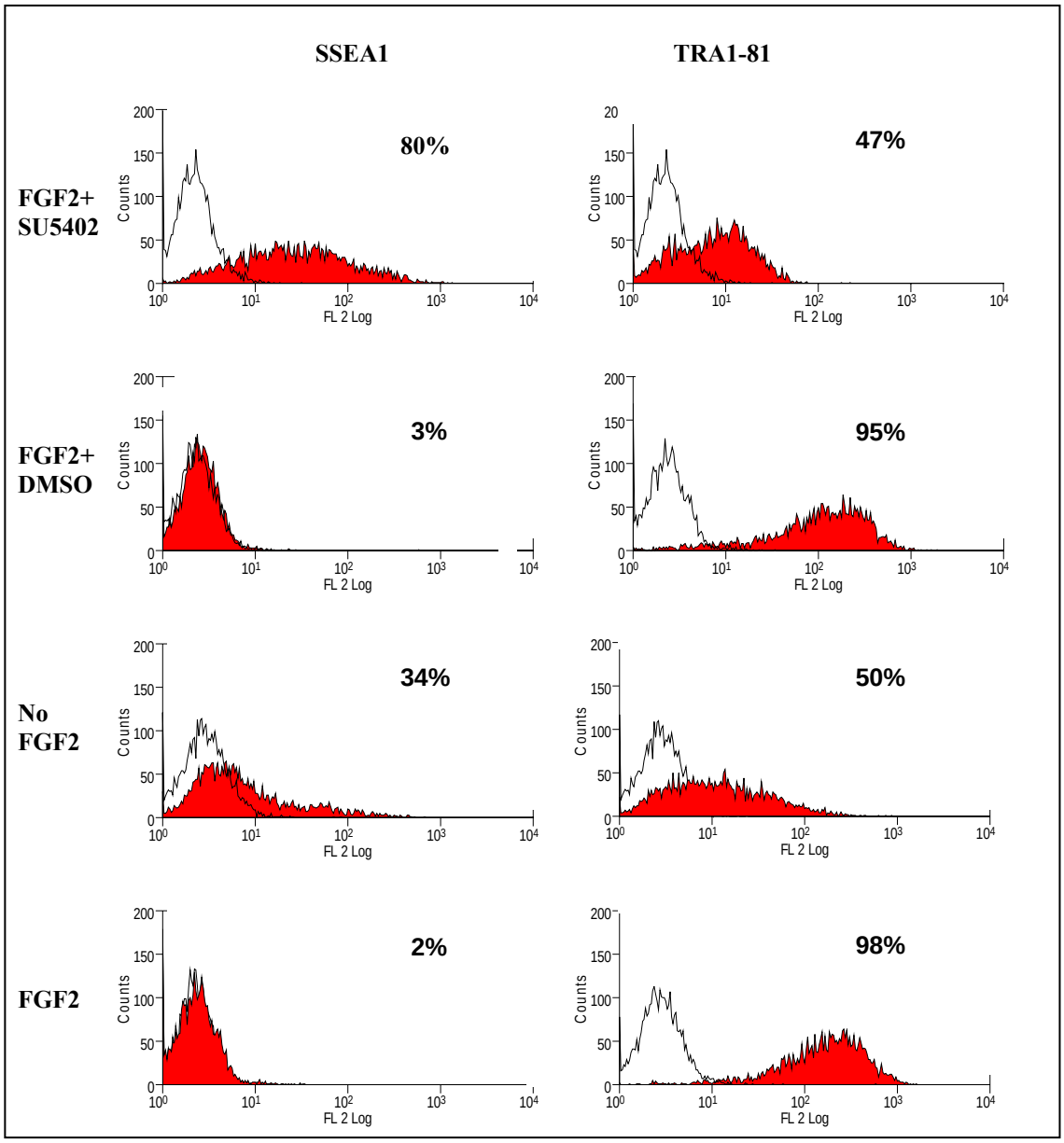


Figure 5.9. Effect of SU5402 on the expression of cell surface markers. Duplicate wells were plated with 10^5 cells/well in the absence of FGF2, FGF2 and the FGFR inhibitor SU5402 (20ng/ml), FGF2 alone and FGF2 and DMSO as control. After 6 days in these conditions, the cells exposed to the inhibitor dramatically up-regulated SSEA1 and down-regulated TRA1-81. The results shown here are representative of three independent experiments.

5.5.5 Signalling pathways downstream of FGF, which may contribute to self-renewal of hES cells

The previous section confirmed that FGF2 is a key factor in the propagation of hES cells in serum free conditions. The question is which pathways downstream of FGF2 contribute to hES cell self-renewal. Two important signals activated by FGF in hES cells are the ERK/MAPK (Kouhara *et al.,* 1997; Hadari *et al.,* 2001; Armstrong *et al.,* 2006) and PI3K/AKT (Hadary *et al.,*2001; Armstrong et al,. 2006) pathways. To assess their functional relevance, H1 cells were treated with the drugs UO126 and LY294002, inhibitors of the MAPK and PI3K pathways respectively.

To determine the minimum effective dose of inhibitor, a titration was carried out and assessed by immunoblotting. Duplicated wells with 10^6 cells were treated with different concentrations (5, 10, 20μM) of the inhibitor. Following one hour incubation, the cells were stimulated with 25ng/ml of FGF2 for 10 minutes and the cell lysates analysed by western blotting (Figure 5.10). Immunoblots from lysates treated with UO126 were probed with an antibody specific for the active phosphorylated forms of ERK1 and 2. Immunoblots from lysates treated with LY294002 were probed with an antibody specific for the active phosphorylated forms of AKT, downstream in the PI3 Kinase pathway.

5.5.5.1 Effect of MAPK and PI3K inhibition in hES cell biochemistry

The inhibitor UO126 was used to block the MAPK signalling pathway. UO126 inhibits the kinase activity of MAP Kinase I and II (MAPKK or MEK) by directly inhibiting the

action of phosphorylated MEKs (Kouhara *et al.,* 1997; Favata *et al.,* 1998) thereby preventing phosphorylation of ERK1 and 2. The inhibitor LY294002 was used to inhibit the PI3K pathway. This is a cell permeable, potent PI3K inhibitor, which acts on the ATP binding site of the enzyme and in the catalytic subunit of DNA-activated protein kinase. LY294002 is specific in its function and does not affect the activity of EGF receptor kinase, MAP kinase, PKC, S6 kinase, PI 4-kinase and C-SRC (Baumann and West, 1998).

High levels of activated ERK1 were observed in the absence of UO126 following FGF2 simulation. ERK phosphorylation was potently inhibited when UO126 was present. ERK phosphorylation was observed at low levels in 5μM UO126 and was detectable in 10μM UO126 at a similar intensity than the basal level of activation. This basal level of ERK could be due to the activity of intrinsic FGF2, which has been previously reported to activate ERK (Dvrorak *et al.,* 2005). The membrane was stripped and re-probed with antibody that recognises ERK2 protein as control. In conclusion, UO126 inhibited FGF2 mediated phophorylation of the H1 cells in serum free conditions.

AKT phosphorylation was observed in the absence of LY294002 following FGF2 simulation. AKT phosphorylation was potently inhibited at all the doses tested of LY294002. The membrane was stripped and re-probed with antibody that recognises SHP2 protein, confirming that wells were evenly loaded. In conclusion, LY294002 inhibited FGF2 mediated phophorylation of the H1 cells in serum free conditions.

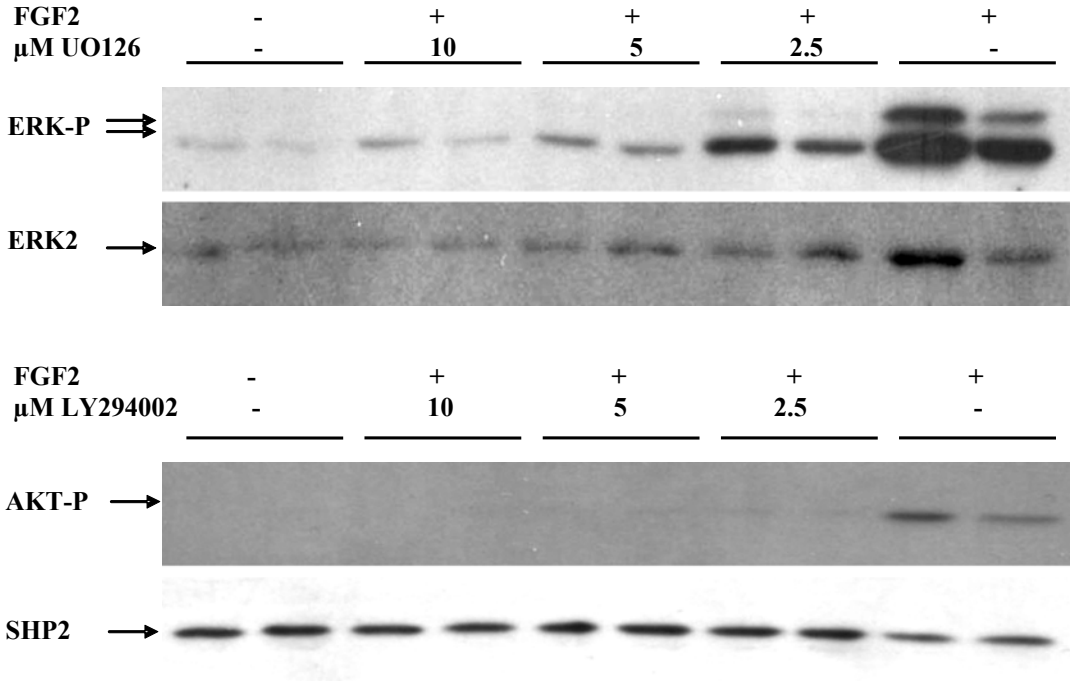


Figure 5.10. MAPK and PI3K inhibition by UO126 and LY294002 in hES cells. Cells were cultured in control medium (medium without FGF, insulin or B27) for 4 hours before changing to medium with the inhibitors (10 to 2.5µM). After 1 hour of treatment with the inhibitor, the cells were stimulated with FGF2 and lysates were blotted and probed with an antibody specific to phosphorylated ERK (ERK-P) or phosphorylated AKT (AKT-P). UO126 inhibited FGF2 mediated phosphorylation of ERK1 and 2 in H1s hES cells. LY294002 inhibited FGF2 mediated phosphorylation of AKT at all the doses. The membranes were stripped and re-probed with antibodies that recognise ERK2 and SHP2 proteins respectively. The results shown here are representative of three independent experiments.

5.5.5.2 Effect of MAPK and PI3K inhibition on hES cell biology

To asses the biological effects of the inhibition of a specific pathway, $5x10^5$ cells were plated in N2B27+FGF2 (8ng/ml) + inhibitor. The inhibitors were added at a concentration of 5μM (UO126) and 2.5μM (LY294002) and 20 μM for SU5402, which was included as control. In addition to this, early passage cells with FGF2 were included. After 5 days of continuous exposure to the inhibitor, the morphological effects (Figure 5.11) and the expression of surface markers (Figure 5.12) were examined.

The effect of MAPK inhibition on cell morphology was noticed after 2 days in culture by the appearance of flattening cells in the centre of some colonies. After 5 days the colonies appeared completely flattened. The effect of PI3K pathway inhibition was more rapidly revealed. From the first day of treatment it could be observed by the flattening in the middle of the colonies and the stretched and detached cells. This effect increased progressively, and in the 5^{th} day of treatment PI3K inhibition resulted in a general morphology of "star-shaped" colonies. This experiment has been repeated three times with similar results and Figure 5.11 shows a representation of these.

To asses the effects of the inhibition of MAPK and PI3K pathways in self-renewal, the expression of SSEA1 and TRA1-81 were analysed (Figure 5.12). TRA1-81 expression decreased and SSEA1 levels increased when MAPK and PI3K pathways were inhibited. However, these changes were not as pronounced as when FGFR was inhibited, which

could indicate the role of both pathways MAPK and PI3K in self-renwal, or the contribution of a complementary pathway.

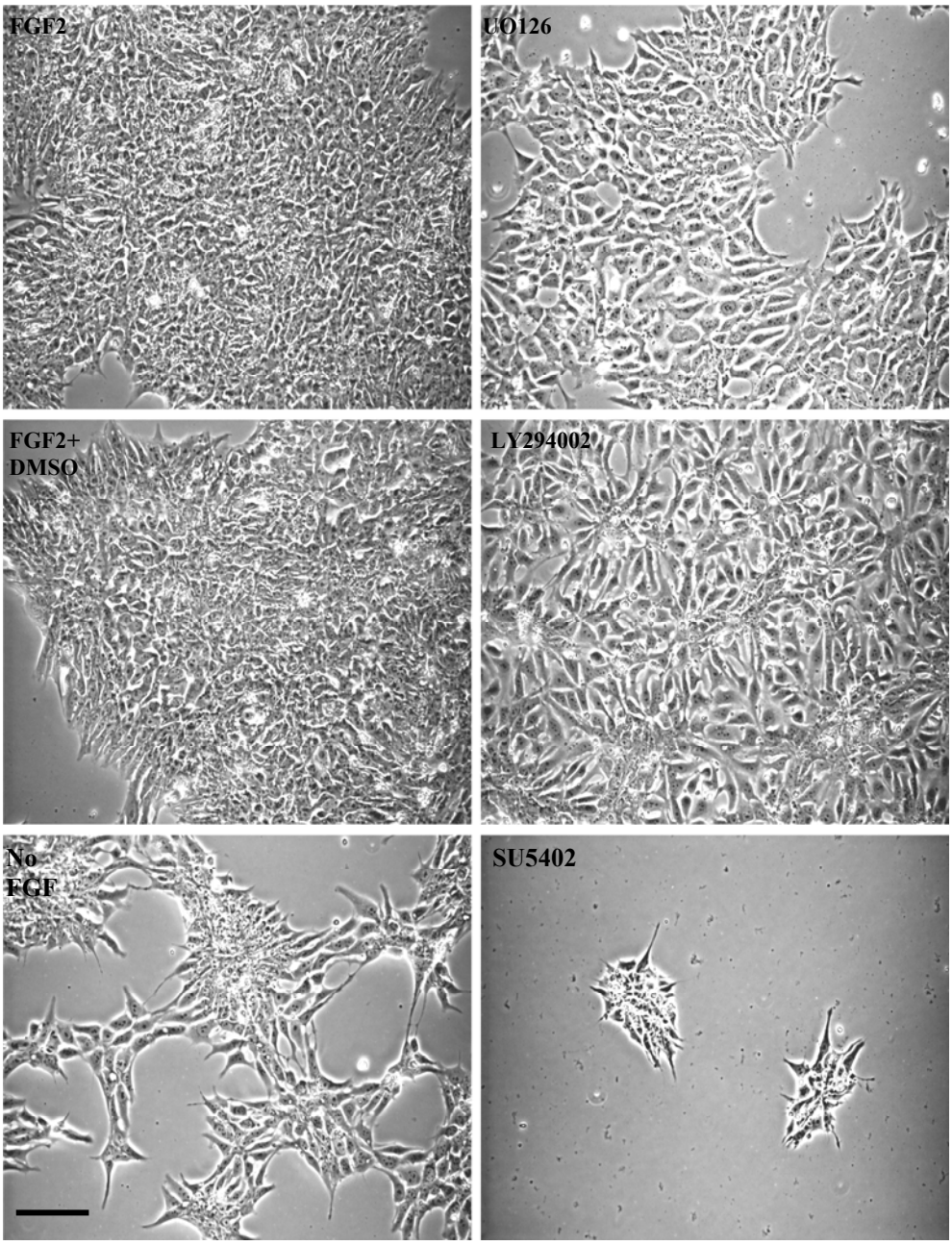


Figure 5.11. Effect of inhibition of MAPK and PI3K pathways on H1s morphology. Cells were supplemented continuously for 5 days with UO126 (5µM) or LY294002 (2.5 µM). SU5402 (20 µM) and cells with and without FGF2, and cells supplemented with DMSO were included as controls. Scale bar = 200µm. The results shown here are representative of three independent experiments.

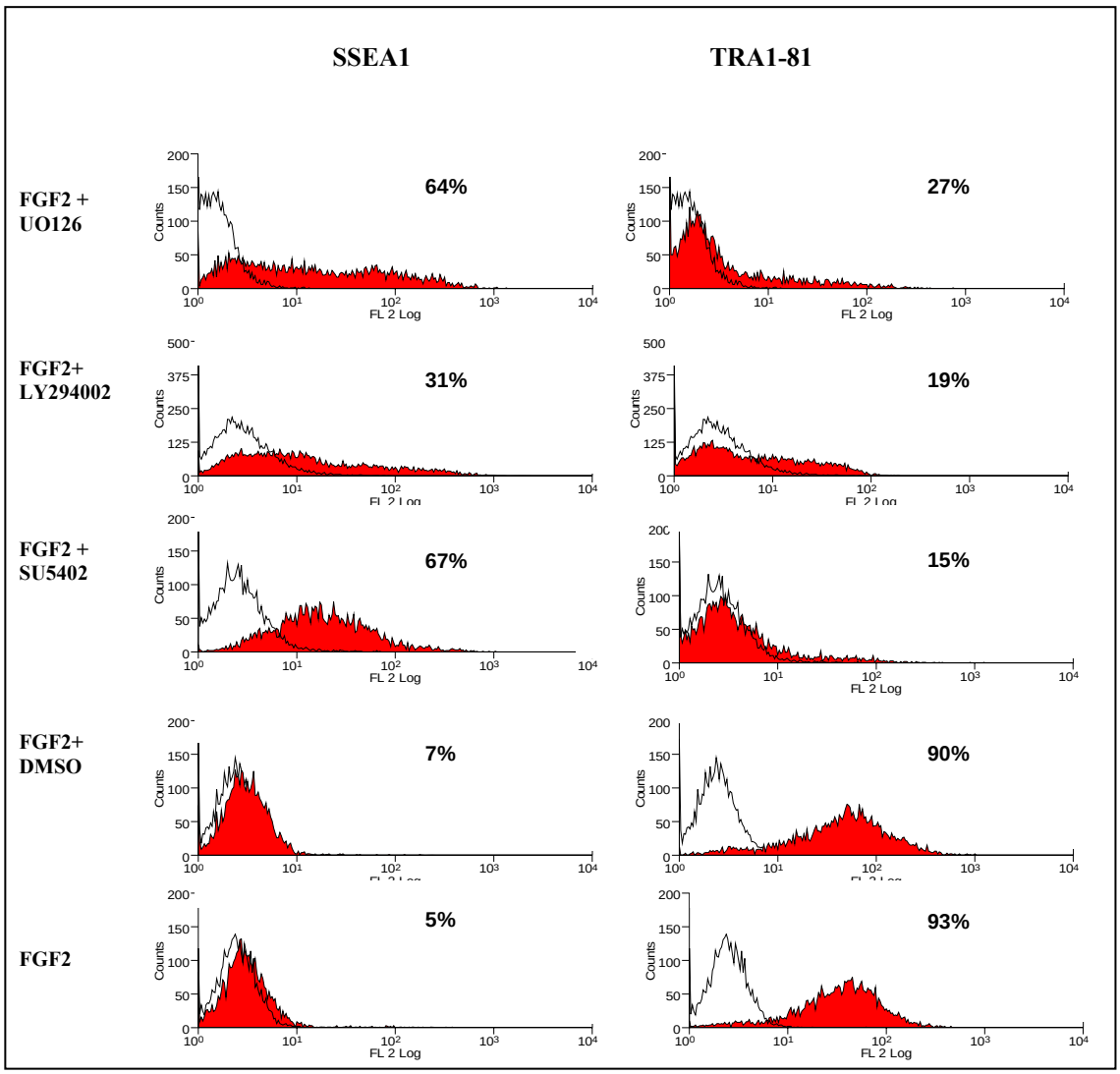


Figure 5.12. Effect of inhibition of MAPK and PI3K pathways on H1s surface markers. Cells were supplemented continuously for 5 days with UO126 (5μM) or LY294002 (2.5 μM). SU5402 (20 μM) and cells with FGF2 and cells supplemented with DMSO were included as controls. MAPK and PI3K inhibition resulted in the increase in SSEA1 and decrease of TRA1-81. This effect was not produced by the DMSO. The results shown here are representative of three independent experiments.

5.5.5.3 Is FGF2 sufficient for hES cell self-renewal?

Two observations have suggested the participation of additional factors to FGF2 in the maintenance of hES cell self-renewal. Firstly, the requirement of FGF2 addition to the MEFs to produce effective CM indicated the induction of paracrine factors. Secondly, the observation that although initially FGF2 sustained hES cell proliferation in N2B27, in later batches of the components of the system, an increase in the background differentiation appeared (Figure 5.13). This was accompanied by the progressive increase in SSEA1 and loss of TRA1-81 (Figure 5.14). Matrigel is an undefined component of the system and therefore any change in its composition may account for FGF2 no longer being sufficient and other signals might be necessary for self-renewal. Even in the presence of the growth factor-reduced form of Matrigel used in this thesis, heparan sulphate proteoglycan, laminin, collagen IV and entactin are all present. In addition to this, a large number of growth factors including TGFβ (1.7ng/ml), FGF2 (0-0.1pg/ml), EGF (<0.5ng/ml), IGF1 (5ng/ml), PDGF (<5pg/ml) and NGF (<0.2ng/ml) are included. This may suggest that any inbalance in the Matrigel composition, either by the increased presence of a pro-differentiating factor or alternatively the reduction of a pro-self-renewal factor could result in the loss of self-renewal.

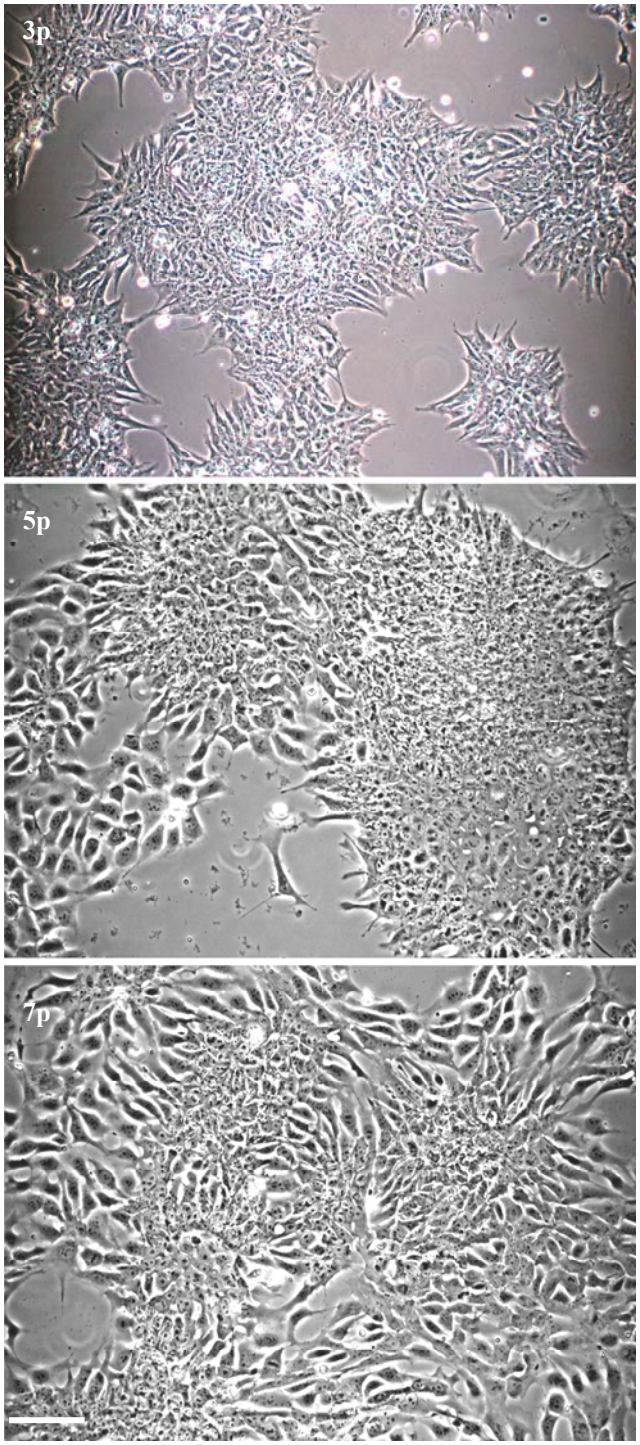


Figure 5.13. Spontaneous differentiation of H1 cells. Cell morphology indicated the progressive onset of differentiation with every passage. Shown above are passages 3, 5 and 7 (Scale bar = 200μm).

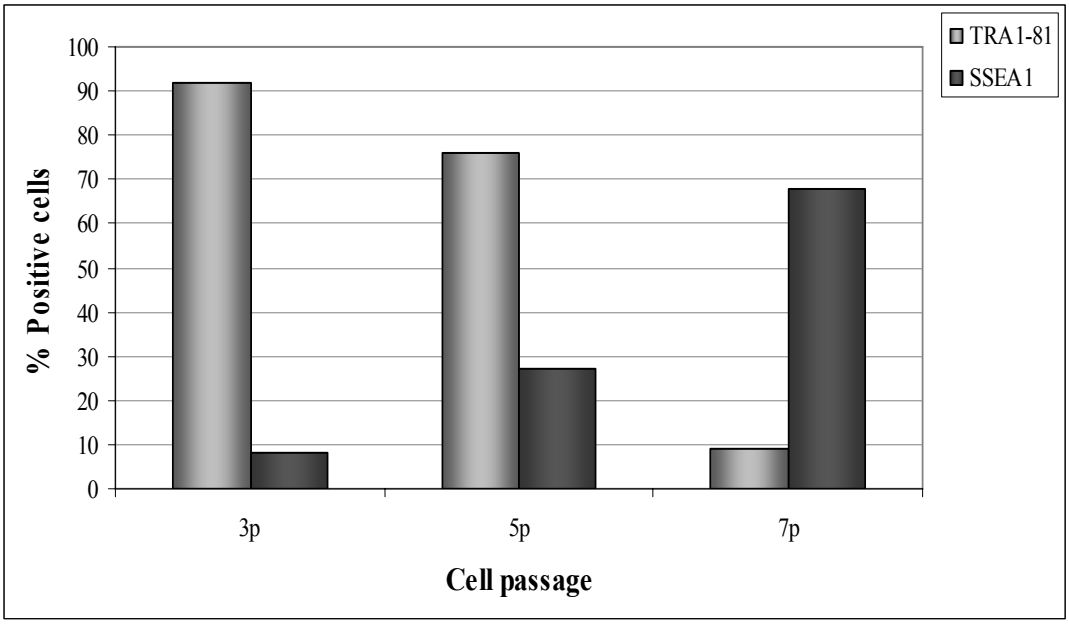


Figure 5.14. Spontaneous differentiation of H1s. Flow cytometry assessment of surface markers showed an increase in expression of SSEA1 along the passages. This related to the down-regulation of TRA1-81 expression from 90% of expression in the first passages to under the 10% of expression. The results shown here are representative of several repeated analyses.

5.5.5.4 Can high doses of FGF2 sustain H1 self-renewal?

To determine whether FGF2 could compensate for any deficiency and/or excess in Matrigel, high concentrations of FGF2 were added to the cultures of the H1 cells.

Cell morphology observation (Figure 5.15) and flow cytometry analysis (Figure 5.16) suggested that a high dose of FGF2 is sufficient in sustaining the self-renewal of the H1 cells. The mechanism could be the inhibition of promoter signals of differentiation or compensating for a missing factor. However, the costs limit the experimental design and therefore, routine use of extremely high concentrations of FGF2 was not possible.

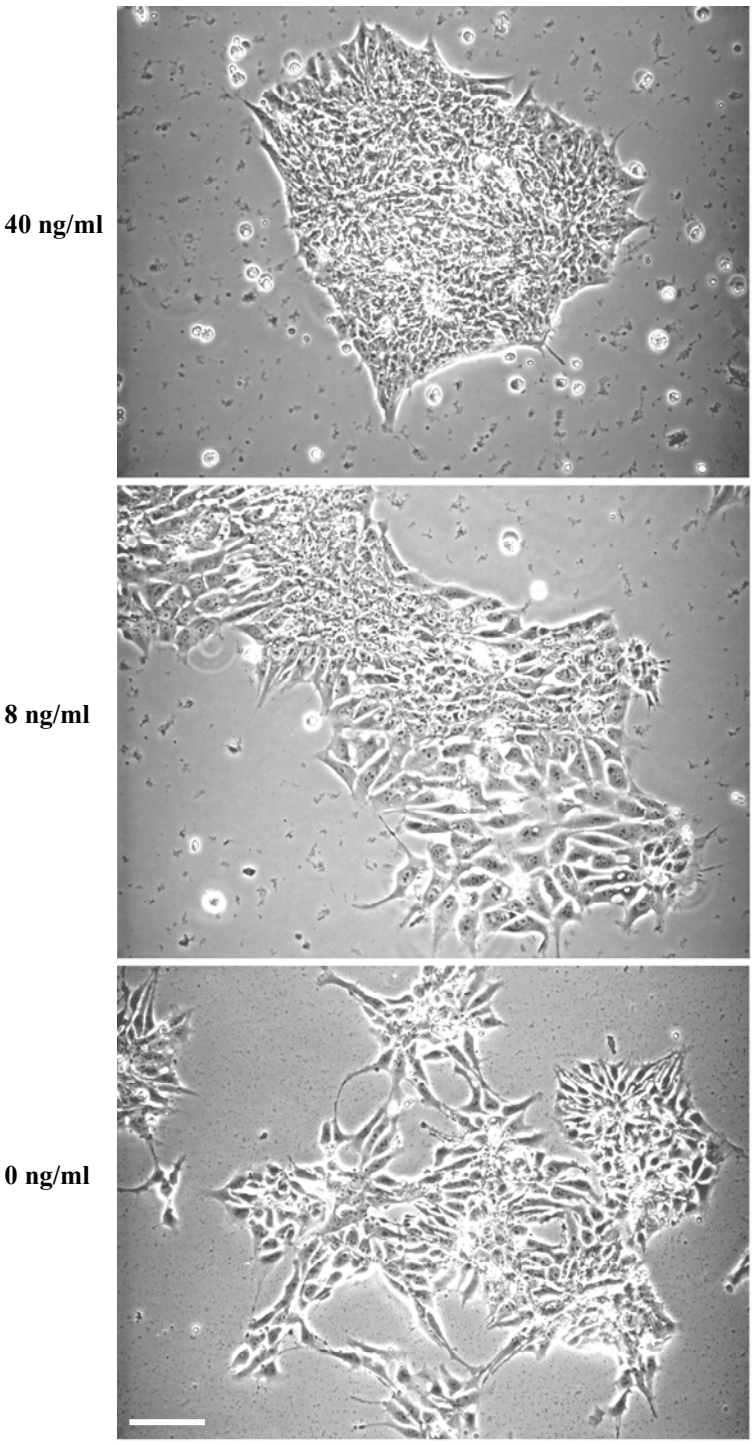


Figure 5.15. Effect of high doses of FGF2 on the morphology of H1 cells that are spontaneously differentiating. Increased concentration of FGF2 to 40ng/ml sustained the cell morphology associated with self-renewal (scale bar = 200μm). The results shown here are representative of two independent experiments.

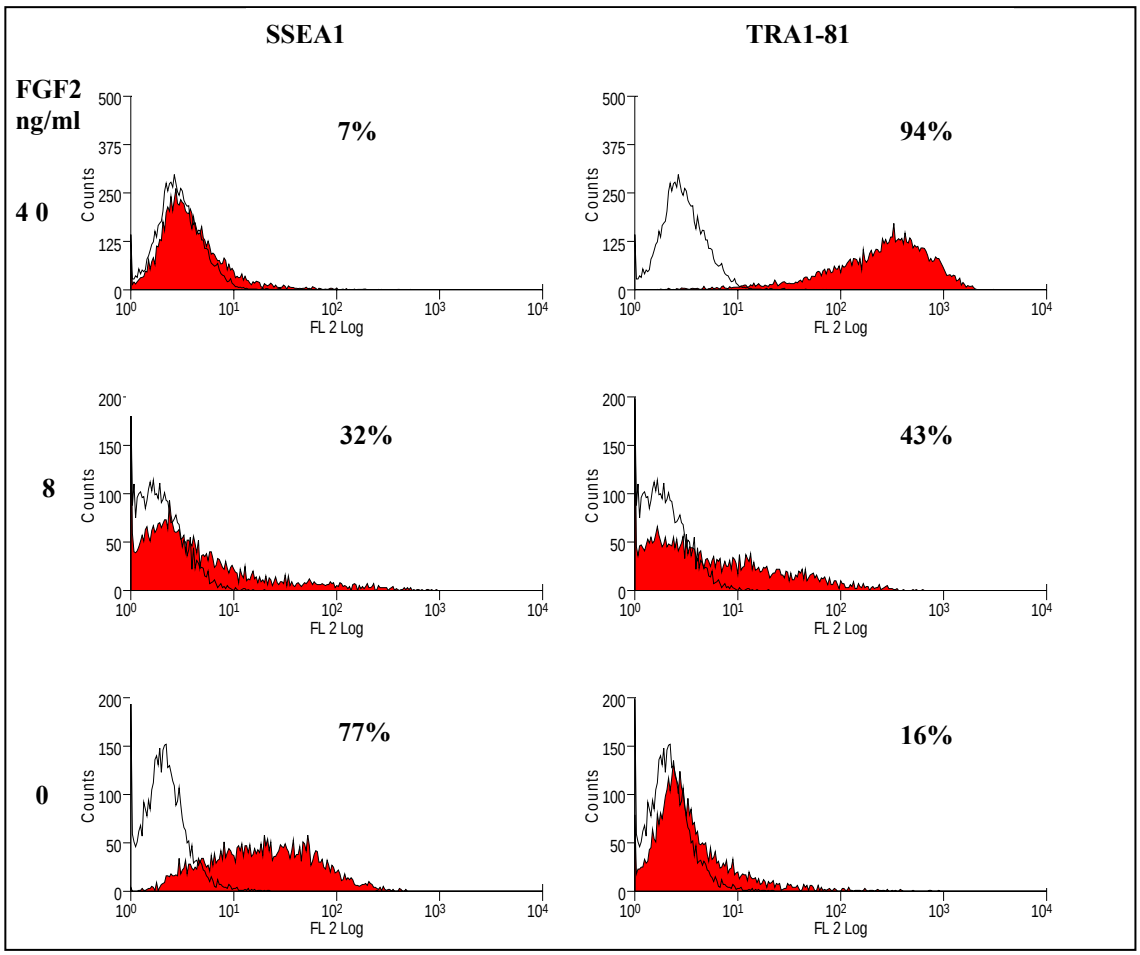


Figure 5.16. Effect of FGF2 on the expression of cell surface markers of spontaneously differentiating H1 cells. Increased concentration of FGF2 to 40ng/ml sustained the expression of surface cell markers associated with self-renewal. The results shown here are representative of two independent experiments.

5.5.6 Possible factors co-operating with FGF2

High doses of FGF2 were sufficient to sustain the proliferation of H1 cells; however this did not exclude the potential requirement for other factor/s. Two components of Matrigel are heparin and TGFβ and they could have a role as regulators of hES cells.

5.5.6.1 Heparin

Heparin is a significant component of Matrigel and as discussed in the introduction, this is a co-factor in FGF signalling. Consequently, it was thought that heparin could have a role in the enhancement of FGF activity, indicating that a decrease in its levels in Matrigel would result in cell differentiation.

To establish whether heparin addition could enhance the effect of FGF2 on H1 cell self-renewal, 1µg/ml of heparin was supplemented with FGF2 to the N2B27 medium. Cell morphology (Figure 5.17) and surface marker expression (Figure 5.18) indicated that the addition of heparin to the cell culture resulted in an increase in differentiation. This suggested that a decreased amount in the heparin content of Matrigel was not causing differentiation. However, the possibility of an increase in heparin content cannot be excluded.

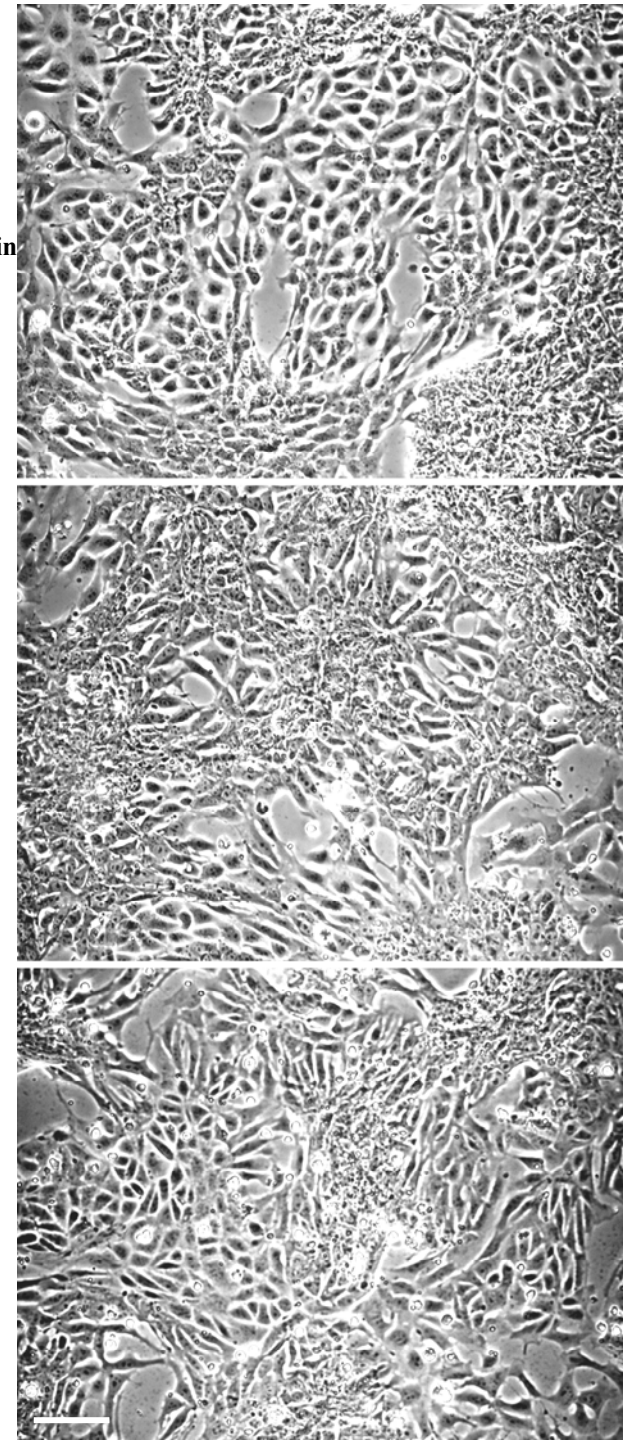


Figure 5.17. Effect of heparin on the morphology of spontaneously differentiating H1 cells. Supplement of 1μg/ml of heparin induced the flattened morphology associated with differentiation. The results shown here are representative of three independent experiments. (Scale bar = 200μm).

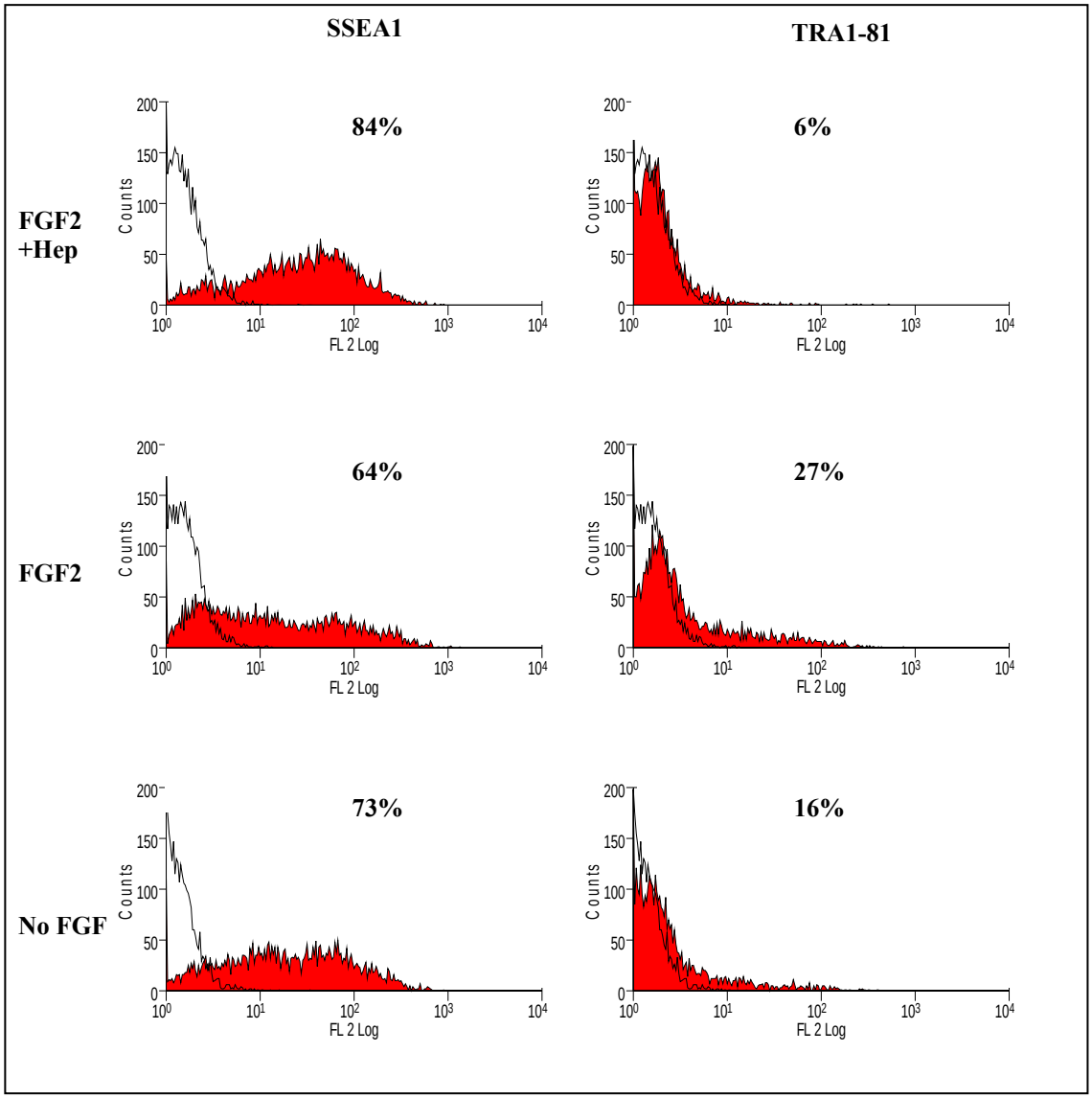


Figure 5.18. Effect of heparin on the morphology of spontaneously differentiating H1 cells. Supplement of 1μg/ml of heparin induced the increase in SSEA1 and the decrease in TRA1-81. The results shown here are representative of three independent experiments.

5.5.6.2 TGFβ

TGFβ is included in growth factor-reduced Matrigel at the significant concentration of 1.7ng/ml. It has been reported that hES cells with TGFβ alone remained undifferentiated, although their proliferation was poor (Amit *et al.,* 2004). Furthermore, TGFβ1 has been included in defined culture systems for the growth of hES cells (Ludwig *et al.,* 2006a). To determine whether TGFβ was the "missing" factor, 5ng/ml of TGFβ was added in addition to FGF2 to the N2B27 medium. TGFβ was added from the 5th passage, when the spontaneous differentiation started.

TGFβ supplement maintained the compact colony morphology and prevented the flattening of the cells, which occurred when FGF2 alone was added (Figure 5.19). This suggested a positive effect of TGFβ on the cell self-renewal, which was confirmed by flow cytometry analysis of the surface markers (Figure 5.20). Expression of SSEA1 rapidly increased in FGF2 alone, but it decreased when TGFβ was added. Equivalent effects were observed on TRA1-81, whose expression was recovered by TGFβ addition to the medium. In conclusion, adding TGFβ rescued cell morphology and surface marker expression of undifferentiated hES cells. These results indicated that TGFβ co-operated with FGF2 in the maintenance of hES cell self-renewal. The effect of TGFβ in conjunction with FGF2 was also assessed from the early passages, and the effect on surface marker expression was analysed at passages 3, 5, 7 and 9 (Figure 5.21). This confirmed the positive effect of TGFβ addition.

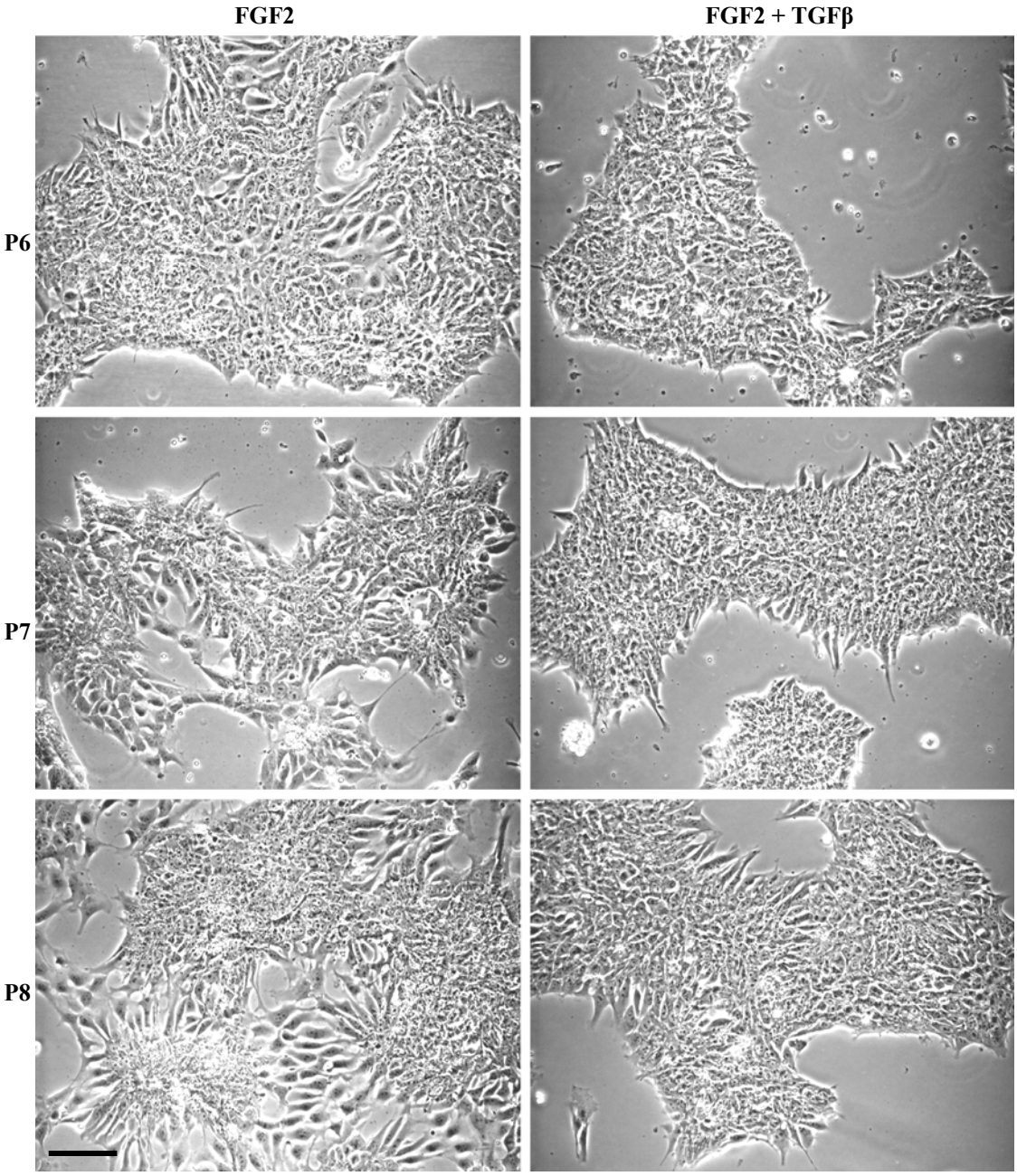


Figure 5.19. Effect of adding TGFβ on the morphology of spontaneously differentiating H1 cells. Cells were maintained for 8 passages with FGF2 alone or FGF2 and TGFβ (5ng/ml). Addition of 5ng/ml of TGFβ prevented the flattening of the colonies, which is associated with ES cell differentiation. The results shown here are representative of three independent experiments. (Scale bar = 200μm).

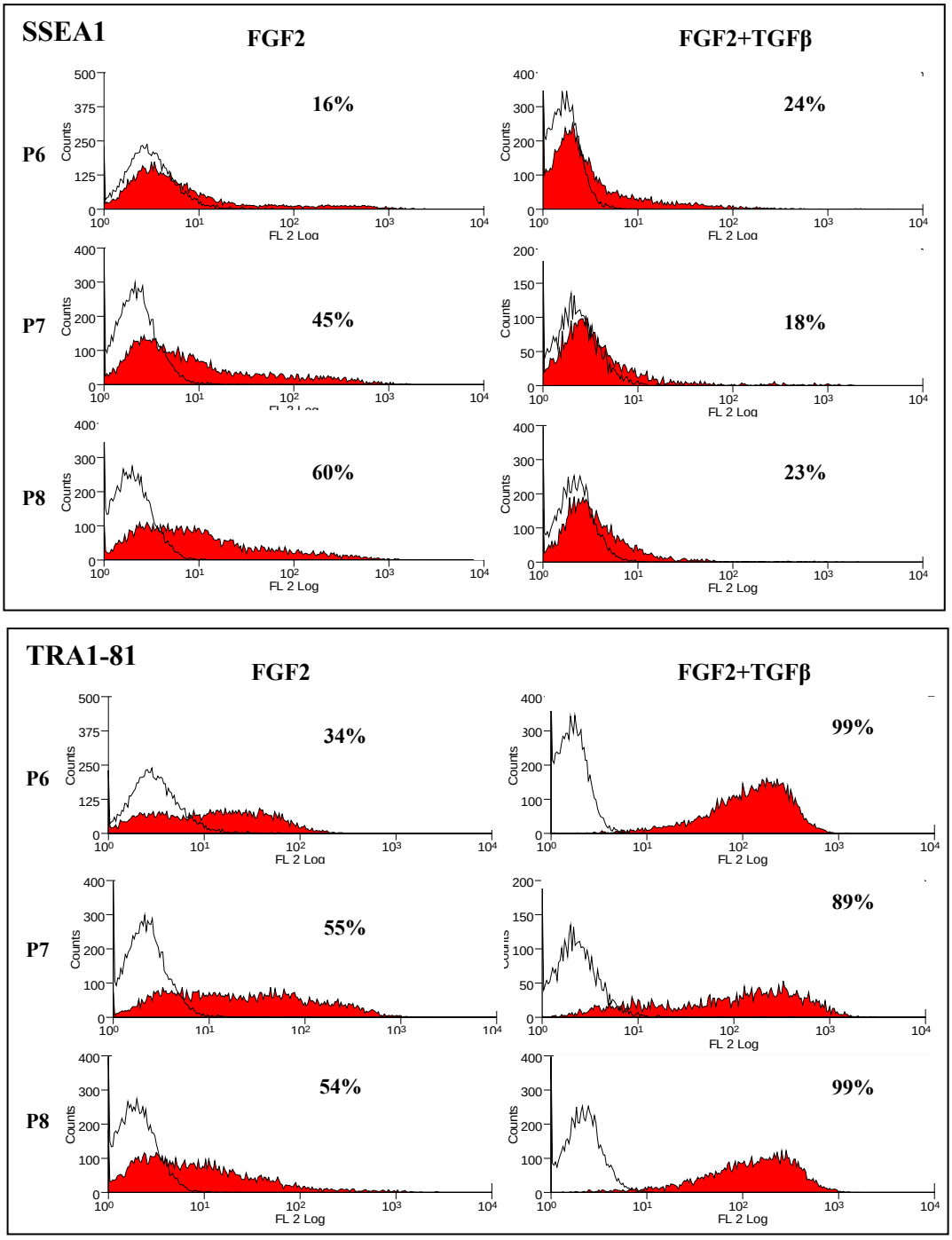


Figure 5.20. Effect of adding TGFβ on the surface markers of spontaneously differentiating H1 cells. Cells were maintained for 8 passages with FGF2 alone or FGF2 and TGFβ (5ng/ml). Addition of TGFβ prevented the increase of SSEA1 and loss of TRA1-81. The results shown here are representative of three independent experiments.

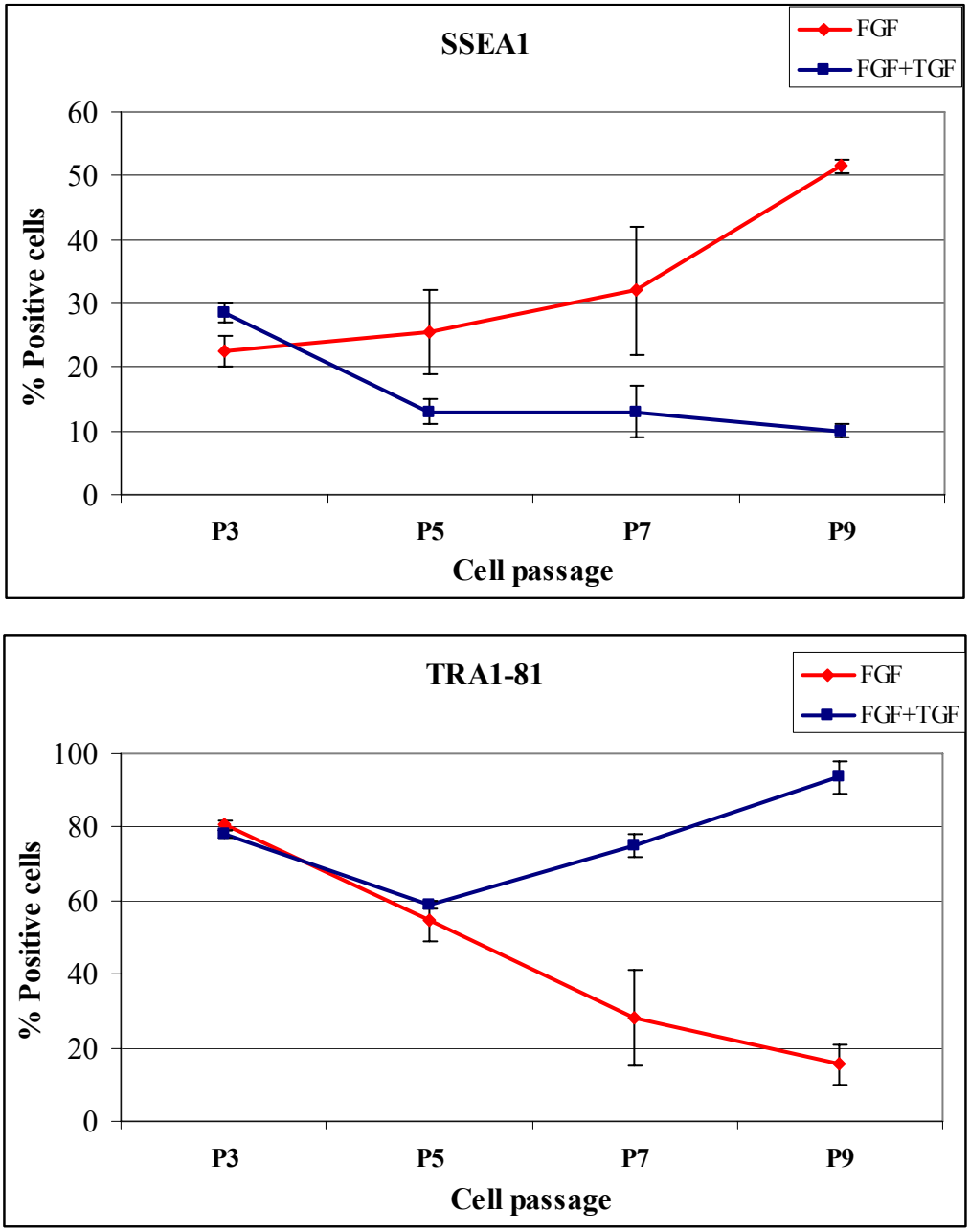


Figure 5.22 Effect of adding TGFβ to the surface markers of early passages of spontaneously differentiating H1 cells. Cells were maintained from passages 3 to 9 passages with FGF2 alone or FGF2 and TGFβ (5ng/ml). Addition of TGFβ prevented the increase of SSEA1 and loss of TRA1-81. Error bars represent the S.E.M. of 3 independent experiments.

.

5.5.6.2.1 TGFβ role in hES cell self-renewal

The results above indicated that the morphology and surface marker expression was recovered when TGFβ was added in conjunction with FGF2. However, this did not exclude the possible support of self-renewal by TGFβ alone. To establish whether TGFβ co-operated with FGF2 or could sustain self-renewal alone, the H1 cells were plated in the presence of FGF (8ng/ml) and TGFβ (5ng/ml) and TGFβ alone. The cells plated in TGFβ alone have been maintained with the two factors: FGF2 and TGFβ for eight passages.

After five days, the cell morphology indicated that TGFβ was not sufficient to maintain the growth of undifferentiated hES cells (Figure 5.22). It was only when the combination of the two factors was used that a compact cell morphology was adopted. Interestingly, some tight colonies with ES morphology remained in the absence of FGF2, contrasting with the complete differentiation observed with TGFβ alone. TGFβ could not compensate for FGF2 and in fact in the absence of FGF2 the effect of TGFβ seemed to promote differentiation.

To further assess the effect of TGFβ signalling, SB431542 inhibitor of the TGFβ type I receptor-like kinase ALK5 (Inman *et al.,* 2002) was used. SB31542 also inhibits ALK 4 and 7 receptors for Activin and Nodal respectively, therefore the inhibition of the TGFβ/Activin/Nodal signalling pathway was expected to occur. Firstly, a titration of the inhibitor was carried out and 10ng/ml of TGFβ used to stimulate the cells. The activation of SMAD proteins downstream of TGFR were examined by western blotting

(Figure 5.23A). 20μM of inhibitor was chosen in subsequent experiments despite detectable SMAD activation. SMAD basal level of phosphorylation suggested TGFβ/Activin/Nodal activity either from the Matrigel or in an autocrine fashion.

To examine the cell morphology, $5x10^5$ cells were plated in the presence of 20μM of the SB431542 inhibitor. As controls the following were included: first passage of cells in FGF2, cells treated with SU5402 (20μM) and cells with FGF2 and DMSO. After 5 days of continuous exposure, the effect of the inhibition was shown by the spreading of the cells (Figure 5.23B) and the maintenance of low levels of SSEA1 (Figure 5.24). Although high levels of TRA1-81 were detected, the amount of cells expressing this marker had decreased in comparison with the controls in FGF2. In conclusion, TGFβ was not sufficient to sustain hES cell undifferentiated proliferation.

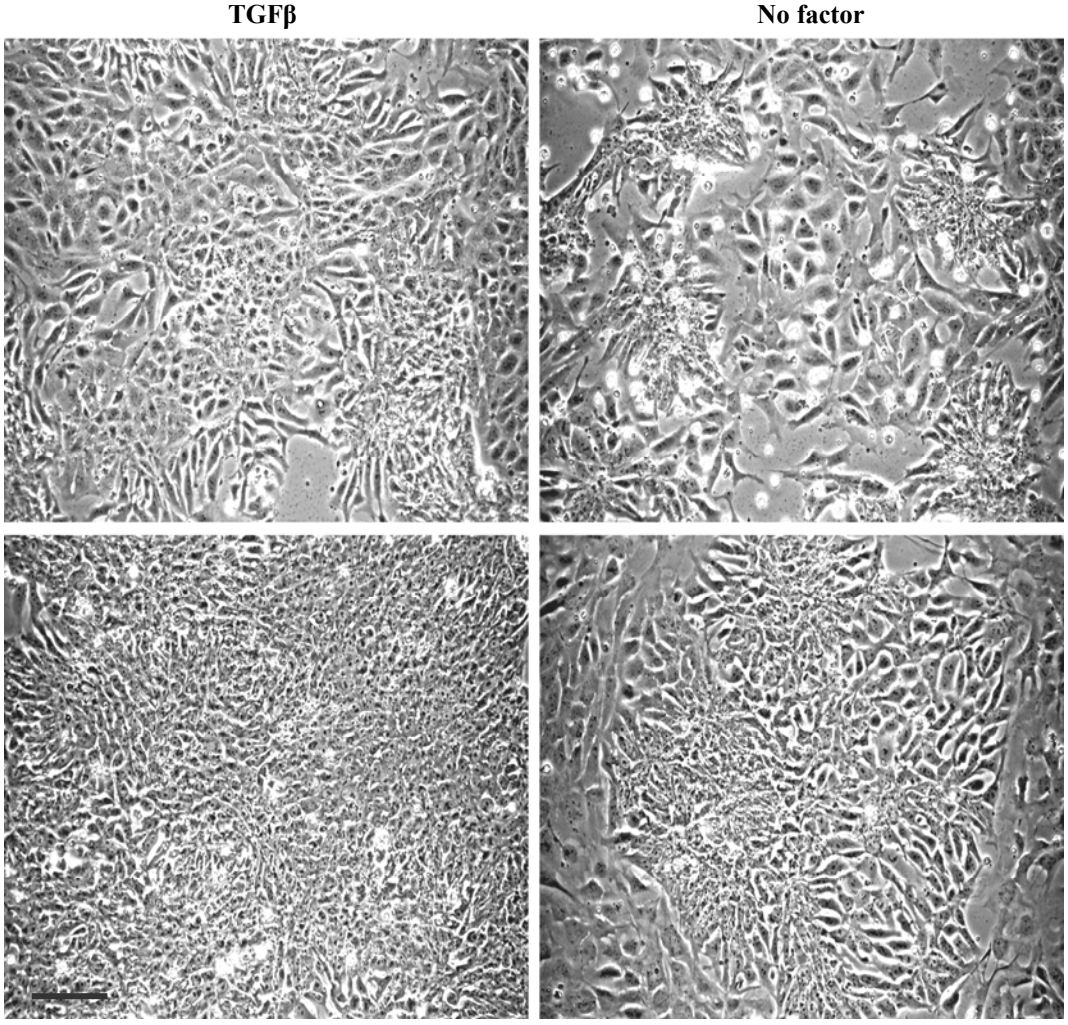


Figure 5.22. Effect of TGFβ alone on the cell morphology of spontaneously differentiating H1 cells. H1 cells were maintained for 8 passages in FGF2 and TGFβ were plated in TGFβ alone and in the absence of factors. Cells in FGF2 and FGF+ TGFβ (as passaged) were also included. The addition of TGFβ alone was not capable of compensating for FGF in the self-renewal of the hES cells. The results shown here are representative of two independent experiments. (Scale bar = 200μm)

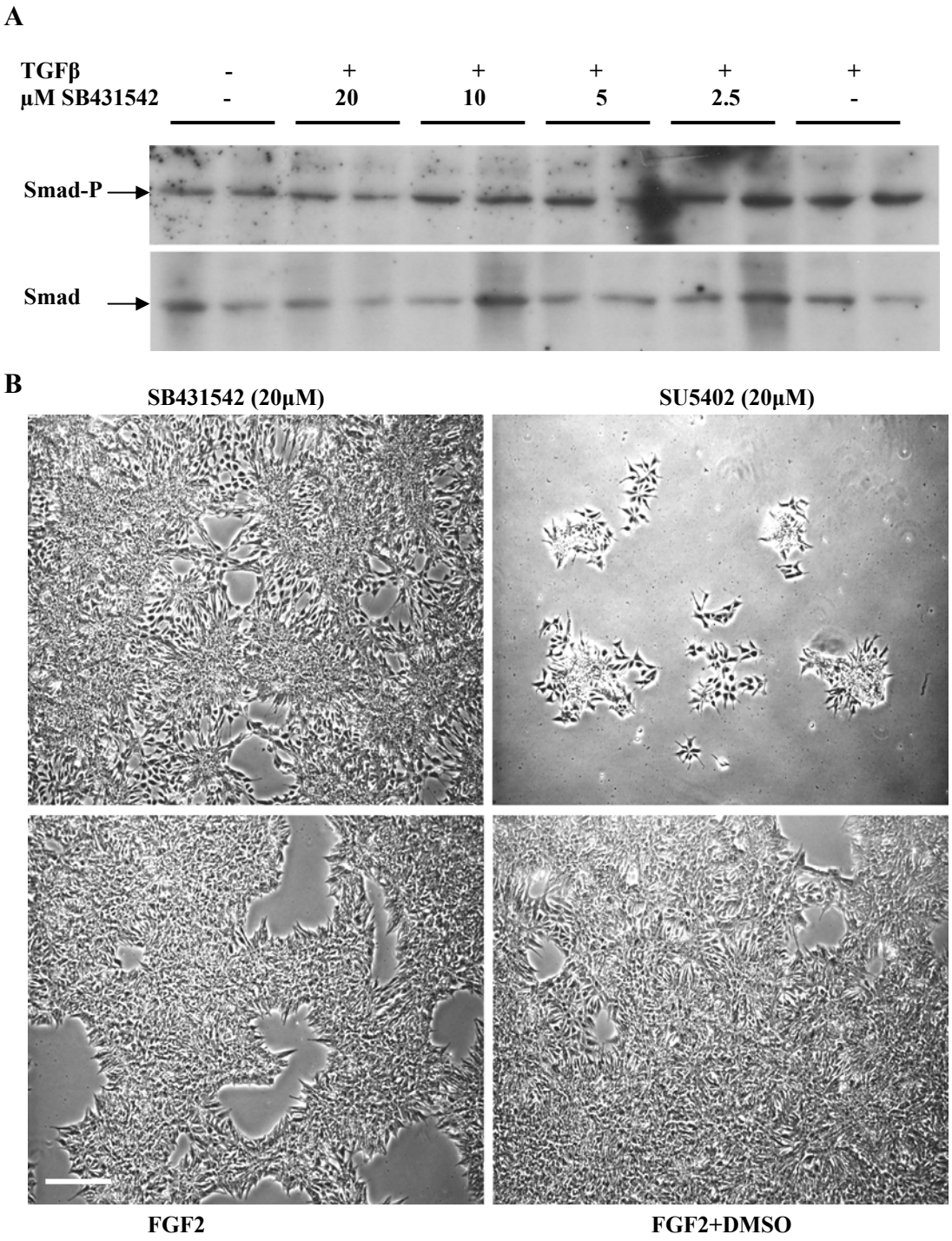


Figure 5.23. Inhibition of TGFβ/Activin/Nodal signal by SB431542. A: Titration of the inhibitor. After 1 hour of incubation with the inhibitor the cells were stimulated with 10ng/ml of TGFβ and the lysates were blotted and probed with an antibody specific to phosphorylated Smad (Smad-P). **B**: **Effect of TGFβ inhibition on cell morphology.** $5x10^5$ cells were plated in the presence of 20μM of inhibitor. Scale bar = 500μm.

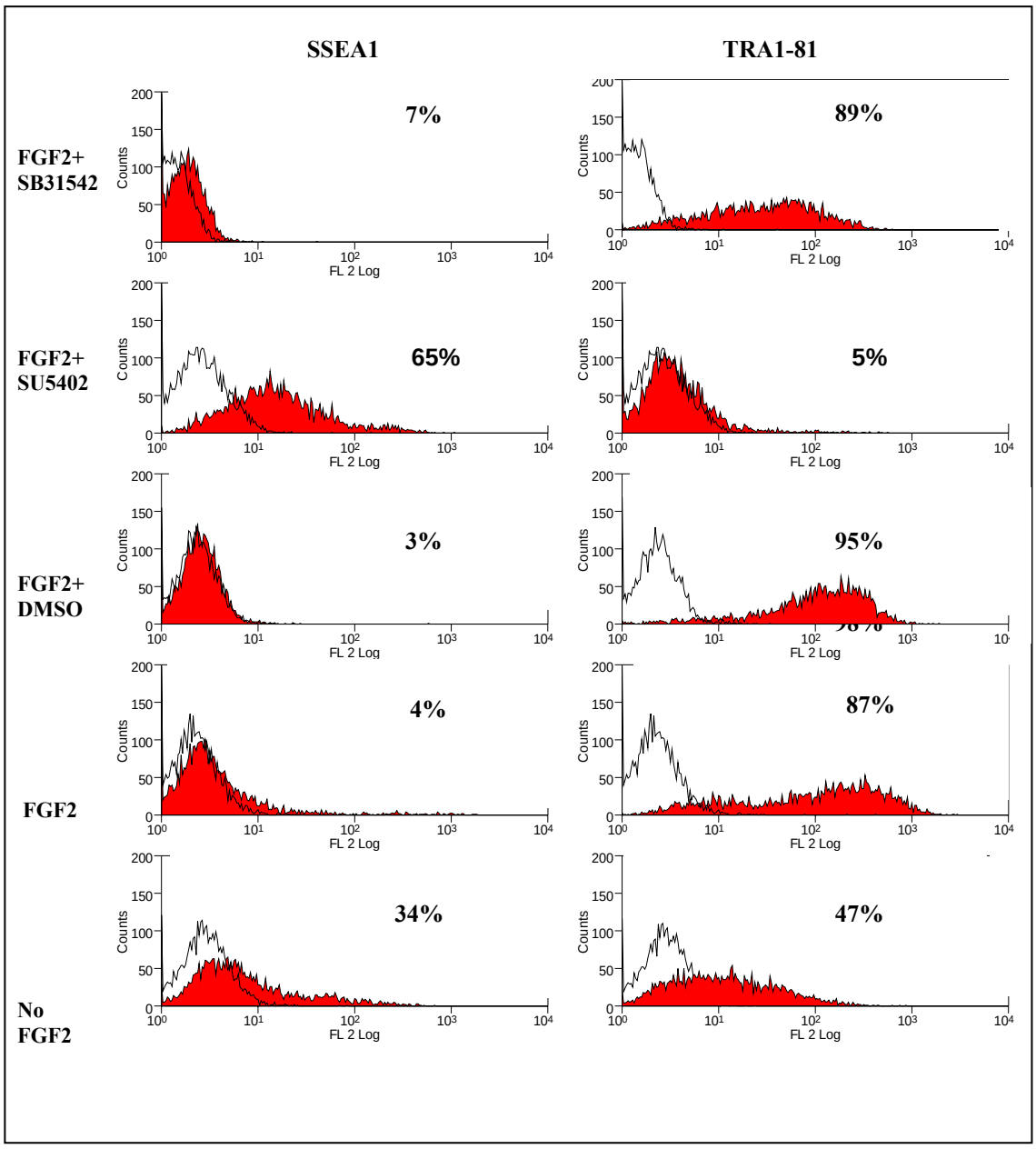


Figure 5.24. Effect of SB31542 on the expression of cell surface markers. After 5 days of continuous exposure to the inhibitor SB431542, low levels of SSEA1 and high levels of TRA1-81 were observed showing a maintenance of the markers when TGFβ signalling was inhibited. This contrasted with the effect of SU5402, which increased SSEA1 and decreased TRA1-81 expression. The results shown here were performed once.

5.6 Discussion

In addition to the different roles of FGF in the biology of human and mouse ES cells, the biochemistry of FGF in these two cell types also seemed to differ. In chapter 5 has been shown that FGF can sustain hES cell self-renewal and highly activated the MAPK/ERK pathway. This contrasts with FGF activation of MAPK/ERK, which is an initiator of lineage commitment (Kunath *et al.,* 2007). On the other hand, LIF addition to the hES cells did not result in the phosphorylation of ERK, although LIF activated STAT3. FGF activation of STAT3 had been reported (Schlessinger, 2000), however, this was not observed in the repeated inductions performed in this study and neither has it been confirmed by Dvorak *et al,* (2005). LIF phosphorylation of STAT3 is consistent with an intact LIF/STAT3 signalling pathway suggested by Daheron *et al,* (2004). Despite its phosphorylation, it is recognised that STAT3 activation is not sufficient in maintaining hES cells in an undifferentiated state (Humphrey *et al.,* 2004; Daheron *et al.,* 2004). Data presented in this chapter indicated that it is through the activation of FGFRs that hES cell self-renewal is sustained. This was supported by the chemical inhibition of FGFR, which resulted in a very dramatic loss of proliferation and self-renewal. FGF2 maintained self-renewal in a dose dependent manner. Comparison of cell morphology and surface markers of cells treated with FGFR inhibitor and FGF withdrawal suggested the activity of autocrine FGF signalling. Furthermore, the requirement for FGF2 seems to be specific since FGF4, which also highly activates ERK, did not sustain hES cell self-renewal. Inhibition of the MAPK and PI3K pathways by UO126 and LY294002 respectively result in the loss of morphology and surface markers associated with hES

cells. This indicated that both pathways may be involved in hES cell self-renewal and perhaps proliferation. In addition to these, other signals could be activated downstream of FGF2, for example, the SRC pathway could also be involved in hES cell self-renewal. These results are supported by a recent report indicating the key function of MAPK and PI3K in maintaining the pluripotency and viability of hES cells (Armstrong *et al.,* 2006).

Furthermore, FGF2 may be also co-operating with other factors, either in an autocrine manner or provided by Matrigel, which is an undefined component of the system. The effects of two significant factors included in Matrigel, heparin and TGFβ were tested. Heparin has an important role in FGF signalling by stabilising the FGF:FGFR binding complexes (Pantoliano *et al.,* 1994), which could suggest a positive effect on self-renewal. However, heparin did not improve the cell self-renewal in the presence of FGF2 and in fact, cell morphology and surface markers indicated an increase in differentiation. The explanation could be associated with the reported ability of heparin in activating the FGFR independently from the growth factors (Roghani *et al.,* 1994). Absence of the high affinity ligand would result in a weaker FGFR activation and consequently a reduction in transmembrane signalling. This could also result in a dominant negative effect, increasing differentiation. In addition to engaging with the receptors, an excess of heparin could occupy all the binding sites on the FGF molecule, restricting the effect of FGF on receptor activation. A differentiating role of heparin in conjunction with FGF2 has been indicated by the induction of differentiation of neural stem cells into functional neurons (Ren *et al.,* 2006). On the other hand, a recent publication attributes heparan sulphates (HSs) a significant role in the maintenance of

mES cell self-renewal (Sasaki *et al.,* 2008). Sasaki et al, (2008) reported that a reduction in the levels of HS chains resulted in a decrease of mES cell proliferation and differentiation into extraembryonic lineages. One of the mechanisms responsible for this outcome was the HS regulation of BMP/Smad signalling and consequently the blocking of neural differentiation (Sasaki *et al.,* 2008). As discussed previously, an increase in BMP signalling could result in hES cell differentiation. In contrast with heparin, TGFβ addition to the medium in conjunction with FGF2 had a positive effect on hES cell proliferation. When an increase in spontaneous differentiation occurred in the cultures, TGFβ supplement with FGF2 restored the undifferentiated cell proliferation. This suggested that the variation in the TGFβ concentration in Matrigel could affect differentiation and more importantly, it indicated the collaboration of FGF2 and TGFβ in sustaining self-renewal. A co-operation between these two signalling pathways in hES cells was reported at the time of printing this thesis. The report by Wu *et al,* (2008) implied that the TGFβ/Activin/Nodal pathway was necessary to determine pluripotency in early human development. They showed by RT-PCR and western blotting that the expression of FGF2, FGF4 and FGF8 was highly repressed when TGFβ/Activin/Nodal pathway was inhibited (Wu *et al.,* 2008). Based on the observations that both, inhibiton of TGFβ/Activin/Nodal and activation of BMP were required for trophoblast differentiation they proposed that trophoblast separation from the ICM is controlled by TGFβ/Activin/Nodal signalling in human embryonic development. In this way, in the morula, the cells that did not receive sufficient TGFβ/Activin/Nodal signal form the trophoblast, and only those that receive the signal develop into ICM. Significantly, when they inhbited TGFβ/Activin/Nodal pathway in the 8-cell stage mouse embryo, the

ICM and trophoblast formed normally, demonstrating that this pathway was not involved in the regualation of pluripotency in mouse (Wu *et al.,* 2008). Nevertheless, it should be noted that although abnormalities were not detected at d.p.c. 4.5 they may have appeared later in development. A later effect would be consistent with the requirement for Activin and FGF signalling by the cells in the mouse epiblast (Camus *et al.,* 2006). This dependency on these two signalling pathways is also maintained by the epiblast stem cells from post implantation epiblast (5.75 d.p.c) (Brons *et al.*,2007 and Tesar *et al.*,2007). In other animal models such as Xenopus, it was demonstrated that when FGF signalling was blocked the levels of many Activin-responsive genes (Cornell and Kimelman, 1994a) decreased implying the co-operation of TGFβ/Activin/Nodal and FGF signalling pathways.

Therefore, the results reported in this chapter 5 indicated that TGFβ and FGF2 must operate together. However, whilst FGF inhibition resulted in differentiation, the blockage of TGFβ/Activin/Nodal pathway did not result in differentiation. This was consistent with the inability of TGFβ to sustain self-renewal alone. Taken together, these results indicated that FGF2 is the principal factor in maintaining hES cell self-renewal. This was supported by the restoration of self-renewal when high concentrations of FGF2 were applied to differentiating hES cells. It is believed that high concentrations of FGF2 can inhibit the differentiating effect of BMP4 (Wang *et al.,* 2005) in unconditioned medium (Xu *et al.,* 2005b). BMP activity could be suggested by the trophoblast-like morphology presented in spontaneous differentiating cells. Therefore, high doses of FGF2 or activation of the TGFβ/Smad2/3 branch of the

pathway might be compensating for these pro-differentiating signals and therefore prevent differentiation. It would be of great interest to assess the signalling pathways activated in the cells that were spontaneously differentiating. In the case of activation of the Smad1/5 proteins it would demonstrate that BMP-like signals were active and inducing differentiation. Once it was established whether FGF2 alone or in conjunction with TGFβ could inhibit Smad1/5 activation, the mechanisms for restoring self-renewal would be demonstrated. Furthermore, the analysis of gene expression in the differentiated cells would indicate whether the cells were differentiating into a specific cell lineage.

In CM, inhibitors of BMP4 activity such as Gremlin and Cerberus are provided by the MEFs when stimulated by FGF2 (Greber *et al.,* 2006). In addition to these, several other members of the TGFβ family including TGFβ and Activin are released in response to FGF2 at a concentration dependent manner. The secretion of these factors is not only induced from the MEFs but also from the hES cells themselves, suggesting the activity of autocrine signalling (Greber *et al.,* 2006). Other components of the TGFβ pathway activators of Smad2/3 found specifically expressed in hES cells are Nodal (Brandenberger *et al.,* 2004; James *et al.,* 2005) and Cripto (Sato *et al.,* 2003; James *et al.,* 2005). Amongst all of them, special significance has been given to Nodal and Activin, to which some groups attribute the central role in maintaining hES cells (Xiao *et al.,* 2005; Vallier *et al.,* 2005; Beattie *et al.*,2005). However, this conclusion has been based on the activation of the downstream effectors of the branch SMAD 2/3, which can also be activated by TGFβ1. TGFβ1 has recently been shown to contribute

with a combination of growth factors, which included FGF2 to maintain hES cells in a feeder free culture (Ludwig *et al.,* 2006a). TGFβ mechanism is suggested to be the direct repression of differentiation since its addition resulted in reduced cell-specific transcripts in hES cells (Schuldiner *et al.,* 2000).

In conclusion, data from this chapter indicates that FGF2 is an upstream factor in human ES cell self-renewal and that FGF2 can sustain hES cells through the activation of the MAPK/ERK and PI3K/AKT pathways. In addition to FGF2, TGFβ seems to contribute to the maintenance of hES cell self-renewal, possibly by compensating for BMP4-like signalling in unconditioned medium. The presence of TGFβ in Matrigel is likely to be critical for hES cell growth since in serum and feeder free conditions this is the main source of the factor. Therefore, any variance in TGFβ concentrations could unbalance the signalling and consequently the undifferentiated proliferation will be lost. On the whole, these conclusions were reached using flow cytometry. Other methods such as immunocytochemistry, immunoblotting and RT-PCR could be used to confirm the results for the assessment of markers of pluripotency. A model of the co-operating cell signalling proposed in this chapter is shown in Figure 5.25.

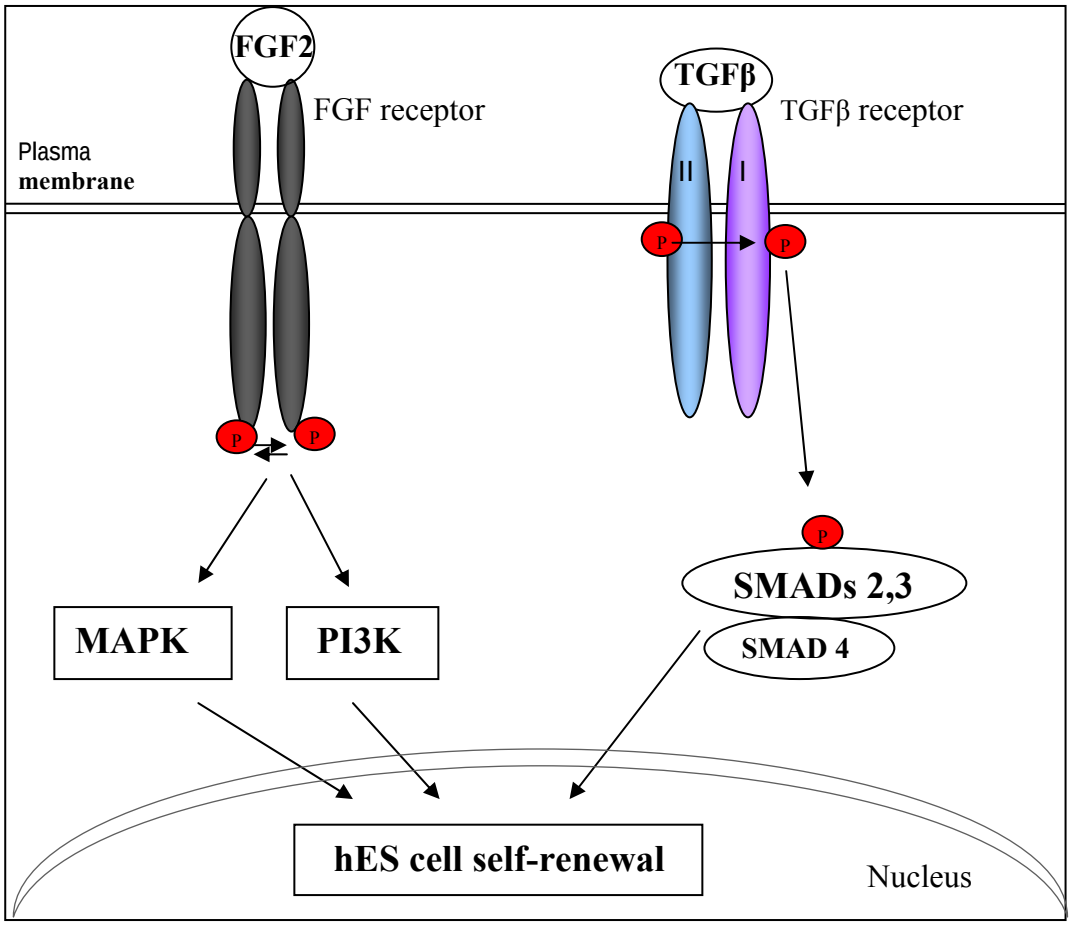


Figure 5.25. Human ES cell regulation. Activation of FGFR by FGF binding resulting in the phosphorylation of MAPK/ERK and PI3K/AKT signalling pathways, which resulted in the regulation of hES cell self-renewal. TGFβ activates the TGFβR and subsequently activates the SMAD2/3 transcription factors. SMAD2/3 bind to a co-transcription factor SMAD4 and together they translocate into the nucleus. FGF2 and TGFβ signalling co-operate to maintain the undifferentiated proliferation of hES cells.

CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE WORK

6.1 General discussion

Mouse and human ES cells propagate on inactivated murine embryonic fibroblast (MEF) feeders or in conditioned medium (CM) derived from MEFs. In these conditions, both populations of cells express markers characteristic of pluripotent cells, such as OCT4, and retain the potential to differentiate into the three germ layers (Brook and Gardner, 1997; Thomson *et al.,* 1998; Amit *et al.,* 2000). However, the signals required to maintain self-renewal and pluripotency are different in these two cell types: LIF and BMP4 signalling sustain mES cells self-renewal (Smith, *et al.,*1988; Ying *et al.,*2003a), whereas LIF cannot maintain hES cells (Daheron *et al.,* 2004) and BMP4 induces their differentiation (Xu *et al.,* 2002). FGF2 is the factor used routinely to grow hES cells whereas the media to grow mES cells is not supplemented with FGF. Furthermore, significant levels of *Fgf2* transcripts have been detected in hES cells, whilst FGF2 is undetected in mES cells. Mouse ES cells express significant levels of FGF4, whereas in hES cells this had not been detected (Wei *et al.,* 2005) until a recent study claiming that FGF4 was also expressed by hES cells (Mayshar *et al.,*2008). This difference in the expression of FGF4 have been attributed to different stages in the early embryonic development from which mouse and human ES cells are derived (Wei *et al.,* 2005). However, divergence in the factors for propagation and the distinct phenotypes of these two cell populations, such as expression of surface markers and specific gene expression, have also been thought to be characteristic of the human and mouse species. Therefore, pluripotent stem cells derived from mouse and human embryos appear to be markedly different and this has been attributed either to species specificity, or to

different developmental properties or to a combination of both. In addition to this, a different role of FGF signalling seemed to be suggested. Consequently one of the principal aims of this study was to determine the role of FGF signalling in human and mouse ES cells.

Data presented within this thesis suggested that in mES cells the effect of FGF2 and principally FGF4 resulted in the down regulation of OCT4. OCT4 down regulation may indicate that FGF4 is a pro-differentiative signal for mES cells. This result contrasts with previous literature suggesting that FGF function was limited to regulating the differentiated progeny (Rappolee *et al.,* 1994; Wilder *et al.,*1997; Rizzino *et al.,*1998). However, recent evidence indicates a requirement for FGF4 signalling in neuroectodermal differentiation in mES cells and in the chick embryo (Stavridis *et al.,* 2007; Kunath *et al.,* 2007). More specifically, these groups established that FGF4 is the auto inductive stimulus initiator of neural commitment, thus indicating an autocrine role of FGF4 in earlier stages of differentiation. FGF autocrine induction of differentiation in mES cells contrasts with the FGF autocrine induction of self renewal reported in hES cells (Dvorak *et al.,* 2005 and own observations). Kunath *et al,* (2007) proposed that FGF activation of ERK is necessary to induce ES cells into a receptive state towards pro-differentiating signals, rather than directly specifying a lineage. It is when the FGF/ERK signal switches the pro-self-renewal function of BMP4 (Ying *et al.,* 2003b) to a non-neural differentiation that lineage commitment could occur (Kunath *et al.,* 2007). Results presented within this thesis are in line with the argument that blocking extrinsic signals such as FGF4 is necessary to sustain the pluripotency of mES cells (Silva and

Smith, 2008). Therefore, FGF4 reduction of OCT4 levels, discussed in chapter 3, would result in a decrease in the resistance to differentiation. Consequently, cells with reduced levels of OCT4 change into a competent state in which they can accept signals that are the inducers of lineage commitment.

In addition to studying the effects of FGF signalling on mES cells, the effect of FGF was assessed on hES cells. At the start of this project, the basis for the use of FGF2 in hES cell culture had not been fully established, despite its presence in all reported hES cell culture systems. Over the course of this study the positive role of FGF2 in maintaining the self-renewal of hES cells has been reported by several groups and is now widely recognised (Levenstein *et al.,* 2005; Xu et al*.,* 2005a and b; Ludwig *et al.,* 2006). However, another factors than FGF2 also have been attributed with the main anti-differentiation and/or pro-self-renewal role in hES cells (Sato *et al.,* 2004; Beattie *et al.,* 2005; James *et al.,* 2005). Consequently, it was a key aim of this project to establish whether FGF2 signalling is sufficient in sustaining hES cell self-renewal and to determine the principal signalling pathways downstream of FGF2. In addition to this, while this thesis was carried out, any possible collaborating signals with the FGF pathway were still to be determined. The standard culture system for hES cells involves the use of the highly variable CM medium, which also contained FGF2 making it very difficult to distinguish the role of specific growth factors in self-renewal. Therefore, to define the role of FGF2 and other specific factors during hES cell self-renewal the simplified N2B27 system was established and used.

Data presented within this thesis demonstrated that FGF2 increased proliferation and decreased differentiation of hES cells. This suggested that the effect of FGF2 on hES cells is not the equivalent to that of LIF on mES cells as might be expected. The role of LIF is primarily to prevent differentiation with little effect on stimulation of proliferation (Raz *et al.,* 1999). The results presented in Chapter 4 indicate that FGF2 is not only an anti-differentiation factor but it also induced proliferation of hES cells. In addition to this, results from this thesis indicate that FGF2 acted directly on hES cells, which differs with the indirect role of FGF2 on the maintenance of hES cell self-renewal recently reported by Bendall *et al,* (2007). In their model, FGF2 induced the secretion of pro-self-renewal factors from the differentiated progeny of the hES cells (Bendall *et al.,* 2007). Among these factors, a significant role was attributed to insulin growth factor II (IGF-II) and TGFβ (Bendall *et al.,* 2007). This agrees with FGF2 induction of soluble members of the TGFβ family from fibroblasts, which has been described by Greber *et al.* (2006). However, a direct effect of FGF2 on hES cells cannot be excluded. FGF2 also enhanced the expression of promoters of self-renewal in the hES cells themselves (Greber *et al.,* 2006; Nguyen *et al.,* 2007). Furthermore, this thesis has demonstrated FGF2 activation of hES cells, in addition to their proliferation in cultures deficient in surrounding differentiation as a result of the serum-free conditions. In fact, the report of IGF-II as a factor in the regulation of hES cells (Bendall *et al.,* 2007) supports the importance of Matrigel in hES cell culture. IGF-II activates the IGF1 receptors (IGF-1R), which are also activated by the other member of the insulin like family of growth factors, IGF-I (Bendall *et al.,* 2007; Nguyen *et al.,* 2007). IGF-I is present in growth factor reduced Matrigel at a concentration of 5ng/ml. In addition to IGF-I and II, insulin

has been shown to activate the same IGF-1 receptors in mES cells (Nguyen *et al.,* 2007). Since insulin is one of the principal components of the N2B27 medium, this could indicate the cooperating role of FGF2 and the signals activated by IGF-1 receptors. FGFs have been previously described as priming factors for other signalling pathways (Cornell and Kimelman, 1994b), therefore, it was proposed that FGF2 signalling increased the competency of hES cells in receiving other signals directly involved in self-renewal in addition to directly sustaining hES cell proliferation.

Results from chapter 5 indicate that TGFβ was a cooperating factor with FGF2 in maintaining hES cells and that in feeder-free systems, TGFβ is usually provided by Matrigel. These results were in line with the suggested collaboration of FGF and TGFβ signalling pathways in sustaining the undifferentiated state of hES cells (Vallier *et al.,* 2005). However, it has been proposed that the TGFβ/Activin/Nodal is the principal signal for maintenance of hES cell self-renewal (Amit *el al.,* 2004; Vallier *et al.,* 2005; James *et al.,*2005). In contrast, results within this thesis indicated that TGFβ was not sufficient to sustain the undifferentiated state of hES. A cooperation between FGF and TGFβ/activn/Nodal signalling pathway is also required for mouse and rat EpiES cells propagation (Tesar *et al.,* 2007; Brons *et al.,* 2007) and mouse trophoblast stem (TS) cells (Guzman-Ayala *et al.,* 2004).

Further signalling studies within this thesis indicated that activation of MAPK/ERK and PI3K are pathways possibly involved in the maintenance of hES cell self-renewal. There is still somewhat limited literature about the signalling pathways responsible of

hES cell self-renewal. Data from chapter 5 agrees with the dependence of hES cells on active MAPK/ERK and PI3K/AKT downstream of FGF2 demonstrated by transcriptional profiling and functional analysis (Armstrong *et al.,* 2006). FGF activation of the MAPK/ERK pathway may be fundamental to the differences between mouse and human ES cell regulation. FGF/ERK seems to be involved in hES cell self-renewal, which contrasts with the inductive role in the differentiation of mES cells. In addition to this, the relationship between FGF and BMP signalling might be related to the conflicting function of FGF on mouse and human ES cells, in that BMP4 expression in hES cells is down-regulated by FGF signalling (Greber *et al.,* 2006) and BMP4 inhibits ERK and thereby supports mES cell self-renewal (Qi *et al.,* 2004). Therefore, it might be possible that FGF and BMP signals block each other with opposite effects in both cell populations. However, this argument is contradicted somewhat by FGF/ERK acting upstream of BMP4, rather than directly blocking BMP4 signal in mES cells (Kunath *et al.,* 2007).

6.2 Conclusions

The key finding in this thesis was the contrasting effect of FGF signalling on mouse and human ES cells. FGF signalling promoted the undifferentiated proliferation of hES cells and produced a destabilizing effect on mES cell self-renewal. This denoted fundamental differences in the regulation of ES cells propagation and differentiation, which have been attributed to different developmental properties but also to species specificity.

Arguments claiming that differences were associated to different developmental origins in mouse and human ES cells were challenged by the recent induced pluripotent stem (iPS) cells from mouse (Takahashi and Yamanaka, 2006) and human embryonic fibroblasts (Yu *et al.,* 2007; Takahashi *et al.,* 2007). Both types of iPS cells were induced by the same cocktail of transcription factors, OCT4, SOX2, KLF4 and c-MYC. However, each cell population needed to be maintained in their specific conditions: hES cells in FGF2 and mES cells in LIF. Therefore, specific requirements in the regulation of mouse and human iPS cells might be attributed to species divergence.

However, the hypothesis of species specificity disagrees with the finding that hES cells resembled the recent isolated mouse and rat EpiSCs. Similarities included gene expression and a requirement for FGF2 and the activator of Smad2/3 proteins, Activin. This gene expression in EpiSCs represents the post implantation epiblast from which these cells originate. FGF and Activin are involved in both, proliferation and differentiation of the mouse post implantation epiblast *in vivo* (Xu *et al.,* 2002; Camus *et al.,* 2006). These characteristics could suggest a similar epiblast origin in hES cells and EpiES cells, which would imply a difference in the pluripotent stages of the cells of origin for mouse and human ES cells. The different roles of FGF on the two ES cell types therefore, might be denoting the different developmental origin. Therefore, ES cells would be reflecting the in vivo roles of FGF signalling in early development. An equivalent epiblast stage of hES cells and EpiSCs also indicates that hES cells originate at a later stage of development than previously thought. However, the differentiation of hES and EpiES cells into extraembryonic trophoblast when treated with BMP4 (Xu *et*

al., 2002; Brons *et al.,* 2007) seemed to indicate otherwise. Significantly, trophoblast stem (TS) cells derived from the trophectoderm also depend on FGF and Activin signalling for self-renewal. It could be reasoned that perhaps the combination of activity of FGF and TGFβ pathways can prime hES and EpiES cells to respond to BMP4 signal by differentiating into the trophoblast lineage. Furthermore, previous views of ES cultures as homogeneous populations have been challenged by showing an inconsistent expression in the levels of Nanog (Chambers *et al.,* 2007) and Rex1 (Toyooka *et al.,* 2008) in ES cells. Supporting this, is the transcriptional activity of many genes related to lineage commitment found in ES cells (Ye *et al.,* 2003). A heterogeneous population of cells implies that there will be different responses to signals for self-renewal and differentiation. The same signals that promote self-renewal in hES cells could induce the differentiation of cells at distinct pluripotent states. For example, expression of trophoblast markers in cultures of hES and Epi cells may be caused by the increased presence of TS cells, since all of them proliferate by the same signal activation (Rossant, 2008). Another possibility is that these particular cell types in culture gain the capacity for this atypical differentiation. It has became apparent that prolonged culture of hES cells may result in genetic changes as the cells adapt to the growth under *in vitro* conditions (Draper *et al.,* 2004). The cells in the embryo from which the immortal ES originate disappear after a few days. It is therefore logical to think that some signalling pathways in ES cells might not have relevance *in vivo*. Moreover, hES cells use several intracrine or autocrine signalling pathways to retain their capacity to proliferate. Such autonomous signalling mechanisms might not have relevance *in vivo* but it may be necessary to adapt to *in vitro* conditions.

At present the causes for the differences between mouse and human ES cells are still uncertain. In support of the argument for the different developmental properties it can be said that LIF is an inhibitor of differentiation towards the post implantation epiblast (Smith *et al.,* 1988) in ICM. Therefore, cells which are in a post implantation state, such as EpiSC-like cells do not need LIF, as they are dependent on FGF and Activin in accordance with their developmental origin. However, and in support of the hypothesis of species specificity is that only murine embryos have been reported to be able to delay implantation by using LIF (Nichols *et al.,* 2001). Therefore, the requirement for LIF to sustain pluripotency seems to be exclusive of mice, indicating species specificity.

6.3 Future work

The results described within this thesis indicated the contrasting effects that activation of FGF signalling has on mouse and human ES cells. However, it is difficult to determine whether this difference could be attributed to species specificity or different timing in development. Further insight into this should be obtained by examining if mouse iPS cells could produce EpiSC-like cells in the presence of FGF and Activin.

This thesis has demonstrated the key role of FGF2 in hES cell propagation in addition to indicating the positive role of ERK and PI3K activation in the self-renewal of hES cells. However, further analysis of the exclusive role of FGF2 in the activation of these signalling pathways and the involvement of other factors would be of interest for future

research, for example, studying the role of IGF-I and II in PI3K activation. Furthermore, collaboration between FGF2 and TGFβ has been suggested here, in addition to the significant role of Matrigel as a provider of TGFβ and other potential factors in hES cell growth. The development of standardised and more favorable culture conditions for hES cells would reduce the tendency of ES cells toward spontaneous differentiation and consequently would reduce the selection pressure for mutations that inhibit those processes. Therefore, the study of the relationship between FGF2 and other factors included in Matrigel, and the effect of the combination of signals in hES cells would help to clarify the mechanisms controlling pluripotency in hES cells. This knowledge could be translated to early human development and more specifically, to the understanding of the mechanisms controlling cell fate commitment during the first events of differentiation. A combination of human and mouse ES cells thus provides a powerful approach for studying the molecular mechanisms controlling pluripotency during the first steps of mammalian development.

References

Abraham JA, Mergia A, Whang JL*, et al.* Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science 1986; **233** (4763):545-8.

Adnane J, Gaudray P, Dionne CA*, et al.* BEK and FLG, two receptors to members of the FGF family, are amplified in subsets of human breast cancers. Oncogene 1991; **6** (4):659-63.

Alanko T, Tienari J, Lehtonen E, Saksela O. FGF-2 inhibits apoptosis in human teratocarcinoma cells during differentiation on collagen substratum. Exp Cell Res 1996; **228** (2):306-12.

Amit M, Carpenter MK, Inokuma MS*, et al.* Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 2000; **227** (2):271-8.

Amit M, Margulets V, Segev H*, et al.* Human feeder layers for human embryonic stem cells. Biol Reprod 2003; **68** (6):2150-6.

Amit M, Shariki C, Margulets V, Itskovitz-Eldor J. Feeder layer- and serum-free culture of human embryonic stem cells. Biol Reprod 2004; **70** (3):837-45.

Andrews PW. Human teratocarcinomas. Biochim Biophys Acta 1988; **948** (1):17-36.

Andrews PW. From teratocarcinomas to embryonic stem cells. Philos Trans R Soc Lond B Biol Sci 2002; **357** (1420):405-17.

Andrews PW, Damjanov I, Simon D*, et al.* Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Differentiation in vivo and in vitro. Lab Invest 1984; **50** (2):147-62.

Anneren C, Cowan CA, Melton DA. The Src family of tyrosine kinases is important for embryonic stem cell self-renewal. J Biol Chem 2004; **279** (30):31590-8.

Arman E, Haffner-Krausz R, Chen Y, Heath JK, Lonai P. Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. Proc Natl Acad Sci U S A 1998; **95** (9):5082-7.

Armstrong L, Hughes O, Yung S*, et al.* The role of PI3K/AKT, MAPK/ERK and NFkappabeta signalling in the maintenance of human embryonic stem cell pluripotency

and viability highlighted by transcriptional profiling and functional analysis. Hum Mol Genet 2006; **15** (11):1894-913.

Bartek J, Lukas J. Cell cycle. Order from destruction. Science 2001; **294** (5540):66-7.

Baumann P, West SC. DNA end-joining catalyzed by human cell-free extracts. Proc Natl Acad Sci U S A 1998; **95** (24):14066-70.

Beattie GM, Lopez AD, Bucay N*, et al.* Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. Stem Cells 2005; **23** (4):489-95.

Becker KA, Ghule PN, Therrien JA*, et al.* Self-renewal of human embryonic stem cells is supported by a shortened G1 cell cycle phase. J Cell Physiol 2006; **209** (3):883-93.

Beckwith J. Industrial genetic screening. Sci People 1980; **12** (2):20-1.

Beddington RS, Robertson EJ. An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. Development 1989; **105** (4):733-7.

Behrendtsen O, Alexander CM, Werb Z. Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths. Development 1992; **114** (2):447-56.

Bellacosa A, Chan TO, Ahmed NN*, et al.* Akt activation by growth factors is a multiple-step process: the role of the PH domain. Oncogene 1998; **17** (3):313-25.

Bellot F, Crumley G, Kaplow JM*, et al.* Ligand-induced transphosphorylation between different FGF receptors. EMBO J 1991; **10** (10):2849-54.

Bendall SC, Stewart MH, Menendez P*, et al.* IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. Nature 2007; **448** (7157):1015-21.

Bolland S, Pearse RN, Kurosaki T, Ravetch JV. SHIP modulates immune receptor responses by regulating membrane association of Btk. Immunity 1998; **8** (4):509-16.

Boulton TG, Stahl N, Yancopoulos GD. Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. J Biol Chem 1994; **269** (15):11648-55.

Boyer LA, Lee TI, Cole MF*, et al.* Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 2005; **122** (6):947-56.

Bradley A, Evans M, Kaufman MH, Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 1984; **309** (5965):255-6.

Brandenberger R, Wei H, Zhang S*, et al.* Transcriptome characterization elucidates signaling networks that control human ES cell growth and differentiation. Nat Biotechnol 2004; **22** (6):707-16.

Brinster RL. The effect of cells transferred into the mouse blastocyst on subsequent development. J Exp Med 1974; **140** (4):1049-56.

Brivanlou AH, Gage FH, Jaenisch R*, et al.* Stem cells. Setting standards for human embryonic stem cells. Science 2003; **300** (5621):913-6.

Brons IG, Smithers LE, Trotter MW*, et al.* Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 2007; **448** (7150):191-5.

Burdon T, Chambers I, Stracey C, Niwa H, Smith A. Signaling mechanisms regulating self-renewal and differentiation of pluripotent embryonic stem cells. Cells Tissues Organs 1999a; **165** (3-4):131-43.

Burdon T, Smith A, Savatier P. Signalling, cell cycle and pluripotency in embryonic stem cells. Trends Cell Biol 2002; **12** (9):432-8.

Burdon T, Stracey C, Chambers I, Nichols J, Smith A. Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. Dev Biol 1999b; **210** (1):30-43.

Burdsal CA, Flannery ML, Pedersen RA. FGF-2 alters the fate of mouse epiblast from ectoderm to mesoderm in vitro. Dev Biol 1998; **198** (2):231-44.

Burke D, Wilkes D, Blundell TL, Malcolm S. Fibroblast growth factor receptors: lessons from the genes. Trends Biochem Sci 1998; **23** (2):59-62.

Cai J, Chen J, Liu Y*, et al.* Assessing self-renewal and differentiation in human embryonic stem cell lines. Stem Cells 2006; **24** (3):516-30.

Campbell WJ, Miller KA, Anderson TM, Shull JD, Rizzino A. Expression of fibroblast growth factor receptors by embryonal carcinoma cells and early mouse embryos. In Vitro Cell Dev Biol 1992; **28A** (1):61-6.

Camus A, Perea-Gomez A, Moreau A, Collignon J. Absence of Nodal signaling promotes precocious neural differentiation in the mouse embryo. Dev Biol 2006; **295** (2):743-55.

Carpenter MK, Inokuma MS, Denham J*, et al.* Enrichment of neurons and neural precursors from human embryonic stem cells. Exp Neurol 2001; **172** (2):383-97.

Cartwright P, McLean C, Sheppard A*, et al.* LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development 2005.

Catena R, Tiveron C, Ronchi A*, et al.* Conserved POU binding DNA sites in the Sox2 upstream enhancer regulate gene expression in embryonic and neural stem cells. J Biol Chem 2004; **279** (40):41846-57.

Chai N, Patel Y, Jacobson K*, et al.* FGF is an essential regulator of the fifth cell division in preimplantation mouse embryos. Dev Biol 1998; **198** (1):105-15.

Chambers I, Colby D, Robertson M*, et al.* Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 2003; **113** (5):643-55.

Chambers I, Silva J, Colby D*, et al.* Nanog safeguards pluripotency and mediates germline development. Nature 2007; **450** (7173):1230-4.

Chen QS, Wang ZY, Han ZC. Enhanced growth of megakaryocyte colonies in culture in the presence of heparin and fibroblast growth factor. Int J Hematol 1999; **70** (3):155-62.

Chen Y, Li X, Eswarakumar VP, Seger R, Lonai P. Fibroblast growth factor (FGF) signaling through PI 3-kinase and Akt/PKB is required for embryoid body differentiation. Oncogene 2000; **19** (33):3750-6.

Chesi M, Bergsagel PL, Kuehl WM. The enigma of ectopic expression of FGFR3 in multiple myeloma: a critical initiating event or just a target for mutational activation during tumor progression. Curr Opin Hematol 2002; **9** (4):288-93.

Ciruna B, Rossant J. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev Cell 2001; **1** (1):37-49.

Conrad AH, Reddy P, Conrad GW. Expression of cytoskeletal, membrane transport, and transcription factor mRNAs in embryonic and adult Ilyanassa obsoleta. Bull Mt Desert Isl Biol Lab Salisb Cove Maine 1998; **37**:2-3.

Cornell RA, Kimelman D. Activin-mediated mesoderm induction requires FGF. Development 1994b; **120** (2):453-62.

Curatola AM, Basilico C. Expression of the K-fgf proto-oncogene is controlled by 3' regulatory elements which are specific for embryonal carcinoma cells. Mol Cell Biol 1990; **10** (6):2475-84.

Daheron L, Opitz SL, Zaehres H*, et al.* LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. Stem Cells 2004; **22** (5):770-8.

Damjanov I, Bagasra O, Dominis M, Solter D. Embryo-derived teratocarcinoma. IV. The role of immune factors in the regulation of teratocarcinogenesis. Int J Cancer 1982; **30** (6):759-62.

Debiais F, Lemonnier J, Hay E*, et al.* Fibroblast growth factor-2 (FGF-2) increases N-cadherin expression through protein kinase C and Src-kinase pathways in human calvaria osteoblasts. J Cell Biochem 2001; **81** (1):68-81.

Delaune E, Lemaire P, Kodjabachian L. Neural induction in Xenopus requires early FGF signalling in addition to BMP inhibition. Development 2005; **132** (2):299-310.

Deng CX, Wynshaw-Boris A, Shen MM*, et al.* Murine FGFR-1 is required for early postimplantation growth and axial organization. Genes Dev 1994; **8** (24):3045-57.

Dono R, Texido G, Dussel R, Ehmke H, Zeller R. Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. EMBO J 1998; **17** (15):4213-25.

Draper JS, Moore HD, Ruban LN, Gokhale PJ, Andrews PW. Culture and characterization of human embryonic stem cells. Stem Cells Dev 2004; **13** (4):325-36.

Dravid G, Ye Z, Hammond H*, et al.* Defining the role of Wnt/beta-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells. Stem Cells 2005; **23** (10):1489-501.

Dvorak P, Dvorakova D, Koskova S*, et al.* Expression and potential role of fibroblast growth factor 2 and its receptors in human embryonic stem cells. Stem Cells 2005; **23** (8):1200-11.

El-Hariry I, Pignatelli M, Lemoine NR. FGF-1 and FGF-2 modulate the E-cadherin/catenin system in pancreatic adenocarcinoma cell lines. Br J Cancer 2001; **84** (12):1656-63.

Erlich HA. Polymerase chain reaction. J Clin Immunol 1989; **9** (6):437-47.

Ernst M, Oates A, Dunn AR. Gp130-mediated signal transduction in embryonic stem cells involves activation of Jak and Ras/mitogen-activated protein kinase pathways. J Biol Chem 1996; **271** (47):30136-43.

Esner M, Pachernik J, Hampl A, Dvorak P. Targeted disruption of fibroblast growth factor receptor-1 blocks maturation of visceral endoderm and cavitation in mouse embryoid bodies. Int J Dev Biol 2002; **46** (6):817-25.

Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev 2005; **16** (2):139-49.

Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981; **292** (5819):154-6.

Favata MF, Horiuchi KY, Manos EJ*, et al.* Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 1998; **273** (29):18623-32.

Feldman B, Poueymirou W, Papaioannou VE, DeChiara TM, Goldfarb M. Requirement of FGF-4 for postimplantation mouse development. Science 1995; **267** (5195):246-9.

Finch BW, Ephrussi B. RETENTION OF MULTIPLE DEVELOPMENTAL POTENTIALITIES BY CELLS OF A MOUSE TESTICULAR TERATOCARCINOMA DURING PROLONGED CULTURE in vitro AND THEIR EXTINCTION UPON HYBRIDIZATION WITH CELLS OF PERMANENT LINES. Proc Natl Acad Sci U S A 1967; **57** (3):615-21.

Fluckiger AC, Marcy G, Marchand M*, et al.* Cell cycle features of primate embryonic stem cells. Stem Cells 2006; **24** (3):547-56.

Folkman J, Klagsbrun M, Sasse J*, et al.* A heparin-binding angiogenic protein--basic fibroblast growth factor--is stored within basement membrane. Am J Pathol 1988; **130** (2):393-400.

Garcia-Maya M, Anderson AA, Kendal CE*, et al.* Ligand concentration is a driver of divergent signaling and pleiotropic cellular responses to FGF. J Cell Physiol 2006; **206** (2):386-93.

Gardner RL. The relationship between cell lineage and differentiation in the early mouse embryo. Results Probl Cell Differ 1978; **9**:205-41.

Gaubert F, Escaffit F, Bertrand C*, et al.* Expression of the high molecular weight fibroblast growth factor-2 isoform of 210 amino acids is associated with modulation of protein kinases C delta and epsilon and ERK activation. J Biol Chem 2001; **276** (2):1545-54.

Gerrard L, Zhao D, Clark AJ, Cui W. Stably transfected human embryonic stem cell clones express OCT4-specific green fluorescent protein and maintain self-renewal and pluripotency. Stem Cells 2005; **23** (1):124-33.

Ginis I, Luo Y, Miura T*, et al.* Differences between human and mouse embryonic stem cells. Dev Biol 2004; **269** (2):360-80.

Gospodarowicz D, Cheng J. Heparin protects basic and acidic FGF from inactivation. J Cell Physiol 1986; **128** (3):475-84.

Gospodarowicz D, Greene G, Moran J. Fibroblast growth factor can substitute for platelet factor to sustain the growth of Balb/3T3 cells in the presence of plasma. Biochem Biophys Res Commun 1975; **65** (2):779-87.

Gotoh N, Laks S, Nakashima M, Lax I, Schlessinger J. FRS2 family docking proteins with overlapping roles in activation of MAP kinase have distinct spatial-temporal patterns of expression of their transcripts. FEBS Lett 2004; **564** (1-2):14-8.

Goumans MJ, Ward-van OD, Wianny F*, et al.* Mouse embryonic stem cells with aberrant transforming growth factor beta signalling exhibit impaired differentiation in vitro and in vivo. Differentiation 1998; **63** (3):101-13.

Granerus M, Welin A, Lundh B*, et al.* Heparin binding growth factors and the control of teratoma cell proliferation. Eur Urol 1993; **23** (1):76-81.

Greber B, Lehrach H, Adjaye J. FGF2 Modulates TGF{beta} Signaling in MEFs and Human ES cells to Support hESC Self-renewal. Stem Cells 2006.

Guzman-Ayala M, Ben-Haim N, Beck S, Constam DB. Nodal protein processing and fibroblast growth factor 4 synergize to maintain a trophoblast stem cell microenvironment. Proc Natl Acad Sci U S A 2004; **101** (44):15656-60.

Hadari YR, Gotoh N, Kouhara H, Lax I, Schlessinger J. Critical role for the docking-protein FRS2 alpha in FGF receptor-mediated signal transduction pathways. Proc Natl Acad Sci U S A 2001; **98** (15):8578-83.

Hamazaki T, Kehoe SM, Nakano T, Terada N. The Grb2/Mek pathway represses Nanog in murine embryonic stem cells. Mol Cell Biol 2006; **26** (20):7539-49.

Hart AH, Hartley L, Ibrahim M, Robb L. Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. Dev Dyn 2004; **230** (1):187-98.

Hay DC, Sutherland L, Clark J, Burdon T. Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. Stem Cells 2004; **22** (2):225-35.

Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem J 1998; **334 (Pt 2)**:297-314.

Henderson JK, Draper JS, Baillie HS*, et al.* Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells 2002; **20** (4):329-37.

Hoch RV, Soriano P. Context-specific requirements for Fgfr1 signaling through Frs2 and Frs3 during mouse development. Development 2006; **133** (4):663-73.

Hooper M, Hardy K, Handyside A, Hunter S, Monk M. HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature 1987; **326** (6110):292-5.

Hubbard SR, Mohammadi M, Schlessinger J. Autoregulatory mechanisms in protein-tyrosine kinases. J Biol Chem 1998; **273** (20):11987-90.

Humphrey RK, Beattie GM, Lopez AD*, et al.* Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. Stem Cells 2004; **22** (4):522-30.

Hunter T. The Croonian Lecture 1997. The phosphorylation of proteins on tyrosine: its role in cell growth and disease. Philos Trans R Soc Lond B Biol Sci 1998; **353** (1368):583-605.

Hyslop L, Stojkovic M, Armstrong L*, et al.* Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages. Stem Cells 2005; **23** (8):1035-43.

Ihle JN. STATs: signal transducers and activators of transcription. Cell 1996; **84** (3):331-4.

Inman GJ, Nicolas FJ, Callahan JF*, et al.* SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol 2002; **62** (1):65-74.

Itskovitz-Eldor J, Schuldiner M, Karsenti D*, et al.* Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 2000; **6** (2):88-95.

James D, Levine AJ, Besser D, Hemmati-Brivanlou A. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development 2005; **132** (6):1273-82.

Jang JH, Chung CP. A novel splice variant of fibroblast growth factor receptor 2 in human leukemia HL-60 cells. Blood Cells Mol Dis 2002; **29** (1):133-7.

Jirmanova L, Afanassieff M, Gobert-Gosse S, Markossian S, Savatier P. Differential contributions of ERK and PI3-kinase to the regulation of cyclin D1 expression and to the control of the G1/S transition in mouse embryonic stem cells. Oncogene 2002; **21** (36):5515-28.

Jirmanova L, Pacholikova J, Krejci P, Hampl A, Dvorak P. O-linked carbohydrates are required for FGF-2-mediated proliferation of mouse embryonic cells. Int J Dev Biol 1999; **43** (6):555-62.

Johnson DE, Lu J, Chen H, Werner S, Williams LT. The human fibroblast growth factor receptor genes: a common structural arrangement underlies the mechanisms for generating receptor forms that differ in their third immunoglobulin domain. Mol Cell Biol 1991; **11** (9):4627-34.

Kang HB, Kim JS, Kwon HJ*, et al.* Basic fibroblast growth factor activates ERK and induces c-fos in human embryonic stem cell line MizhES1. Stem Cells Dev 2005; **14** (4):395-401.

Karin M, Hunter T. Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. Curr Biol 1995; **5** (7):747-57.

Kilkenny DM, Rocheleau JV, Price J, Reich MB, Miller GG. c-Src regulation of fibroblast growth factor-induced proliferation in murine embryonic fibroblasts. J Biol Chem 2003; **278** (19):17448-54.

Klint P, Kanda S, Claesson-Welsh L. Shc and a novel 89-kDa component couple to the Grb2-Sos complex in fibroblast growth factor-2-stimulated cells. J Biol Chem 1995; **270** (40):23337-44.

Kosako H, Nishida E, Gotoh Y. cDNA cloning of MAP kinase kinase reveals kinase cascade pathways in yeasts to vertebrates. EMBO J 1993; **12** (2):787-94.

Kouhara H, Hadari YR, Spivak-Kroizman T*, et al.* A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. Cell 1997; **89** (5):693-702.

Kovalenko D, Yang X, Nadeau RJ, Harkins LK, Friesel R. Sef inhibits fibroblast growth factor signaling by inhibiting FGFR1 tyrosine phosphorylation and subsequent ERK activation. J Biol Chem 2003; **278** (16):14087-91.

Kunath T, Saba-El-Leil MK, Almousailleakh M*, et al.* FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. Development 2007; **134** (16):2895-902.

Lam AC, Li K, Zhang XB*, et al.* Preclinical ex vivo expansion of cord blood hematopoietic stem and progenitor cells: duration of culture; the media, serum supplements, and growth factors used; and engraftment in NOD/SCID mice. Transfusion 2001; **41** (12):1567-76.

Larsson H, Klint P, Landgren E, Claesson-Welsh L. Fibroblast growth factor receptor-1-mediated endothelial cell proliferation is dependent on the Src homology (SH) 2/SH3 domain-containing adaptor protein Crk. J Biol Chem 1999; **274** (36):25726-34.

LaVallee TM, Prudovsky IA, McMahon GA, Hu X, Maciag T. Activation of the MAP kinase pathway by FGF-1 correlates with cell proliferation induction while activation of the Src pathway correlates with migration. J Cell Biol 1998; **141** (7):1647-58.

Lemmon MA, Schlessinger J. Regulation of signal transduction and signal diversity by receptor oligomerization. Trends Biochem Sci 1994; **19** (11):459-63.

Levenstein ME, Ludwig TE, Xu RH*, et al.* Basic FGF Support of Human Embryonic Stem Cell Self-Renewal. Stem Cells 2005.

Li Y, Powell S, Brunette E, Lebkowski J, Mandalam R. Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. Biotechnol Bioeng 2005; **91** (6):688-98.

Lim JW, Bodnar A. Proteome analysis of conditioned medium from mouse embryonic fibroblast feeder layers which support the growth of human embryonic stem cells. Proteomics 2002; **2** (9):1187-203.

Liu Y, Song Z, Zhao Y*, et al.* A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells. Biochem Biophys Res Commun 2006; **346** (1):131-9.

Lu J, Hou R, Booth CJ, Yang SH, Snyder M. Defined culture conditions of human embryonic stem cells. Proc Natl Acad Sci U S A 2006; **103** (15):5688-93.

Ludwig TE, Bergendahl V, Levenstein ME*, et al.* Feeder-independent culture of human embryonic stem cells. Nat Methods 2006a; **3** (8):637-46.

Ludwig TE, Levenstein ME, Jones JM*, et al.* Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 2006b; **24** (2):185-7.

Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995; **80** (2):179-85.

Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 1981; **78** (12):7634-8.

Martin GR, Evans MJ. Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. Proc Natl Acad Sci U S A 1975; **72** (4):1441-5.

Matsuda T, Nakamura T, Nakao K, *et al.* STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. EMBO J 1999; **18** (15):4261-9.

Matsui Y, Zsebo K, Hogan BL. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 1992; **70** (5):841-7.

Mayshar Y, Rom E, Chumakov I, *et al.* Fibroblast growth factor 4 and its novel splice isoform have opposing effects on the maintenance of human embryonic stem cell self-renewal. Stem Cells 2008; **26** (3):767-74.

Mitsui K, Tokuzawa Y, Itoh H, *et al.* The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 2003; **113** (5):631-42.

Mohammadi M, Dikic I, Sorokin A, *et al.* Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction. Mol Cell Biol 1996; **16** (3):977-89.

Mohammadi M, Honegger AM, Rotin D, *et al.* A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. Mol Cell Biol 1991; **11** (10):5068-78.

Mohammadi M, McMahon G, Sun L, *et al.* Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science 1997; **276** (5314):955-60.

Nguyen TT, Sheppard AM, Kaye PL, Noakes PG. IGF-I and insulin activate mitogen-activated protein kinase via the type 1 IGF receptor in mouse embryonic stem cells. Reproduction 2007; **134** (1):41-9.

Nichols J, Chambers I, Taga T, Smith A. Physiological rationale for responsiveness of mouse embryonic stem cells to gp130 cytokines. Development 2001; **128** (12):2333-9.

Nichols J, Davidson D, Taga T, *et al.* Complementary tissue-specific expression of LIF and LIF-receptor mRNAs in early mouse embryogenesis. Mech Dev 1996; **57** (2):123-31.

Nichols J, Zevnik B, Anastassiadis K, *et al.* Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 1998; **95** (3):379-91.

Nishimoto M, Fukushima A, Okuda A, Muramatsu M. The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. Mol Cell Biol 1999; **19** (8):5453-65.

Niswander L, Martin GR. Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. Development 1992; **114** (3):755-68.

Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev 1998; **12** (13):2048-60.

Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 2000; **24** (4):372-6.

Nolan GP, Fiering S, Nicolas JF, Herzenberg LA. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on beta-D-galactosidase activity after transduction of Escherichia coli lacZ. Proc Natl Acad Sci U S A 1988; **85** (8):2603-7.

Olsen SK, Ibrahimi OA, Raucci A*, et al.* Insights into the molecular basis for fibroblast growth factor receptor autoinhibition and ligand-binding promiscuity. Proc Natl Acad Sci U S A 2004; **101** (4):935-40.

Ong SH, Hadari YR, Gotoh N*, et al.* Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. Proc Natl Acad Sci U S A 2001; **98** (11):6074-9.

Ornitz DM. FGFs, heparan sulfate and FGFRs: complex interactions essential for development. Bioessays 2000; **22** (2):108-12.

Ornitz DM, Itoh N. Fibroblast growth factors. Genome Biol 2001; **2** (3):REVIEWS3005.

Ornitz DM, Xu J, Colvin JS*, et al.* Receptor specificity of the fibroblast growth factor family. J Biol Chem 1996; **271** (25):15292-7.

Ornitz DM, Yayon A, Flanagan JG*, et al.* Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. Mol Cell Biol 1992; **12** (1):240-7.

Orr-Urtreger A, Givol D, Yayon A, Yarden Y, Lonai P. Developmental expression of two murine fibroblast growth factor receptors, flg and bek. Development 1991; **113** (4):1419-34.

Ortega S, Ittmann M, Tsang SH, Ehrlich M, Basilico C. Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. Proc Natl Acad Sci U S A 1998; **95** (10):5672-7.

Paling NR, Wheadon H, Bone HK, Welham MJ. Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signalling. J Biol Chem 2004.

Palmieri SL, Peter W, Hess H, Scholer HR. Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. Dev Biol 1994; **166** (1):259-67.

Pantoliano MW, Horlick RA, Springer BA, *et al.* Multivalent ligand-receptor binding interactions in the fibroblast growth factor system produce a cooperative growth factor and heparin mechanism for receptor dimerization. Biochemistry 1994; **33** (34):10229-48.

Pawson T. Protein modules and signalling networks. Nature 1995; **373** (6515):573-80.

Pelton TA, Sharma S, Schulz TC, Rathjen J, Rathjen PD. Transient pluripotent cell populations during primitive ectoderm formation: correlation of in vivo and in vitro pluripotent cell development. J Cell Sci 2002; **115** (Pt 2):329-39.

Pera EM, Ikeda A, Eivers E, De Robertis EM. Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. Genes Dev 2003; **17** (24):3023-8.

Pera MF, Andrade J, Houssami S, *et al.* Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. J Cell Sci 2004; **117** (Pt 7):1269-80.

Pera MF, Cooper S, Mills J, Parrington JM. Isolation and characterization of a multipotent clone of human embryonal carcinoma cells. Differentiation 1989; **42** (1):10-23.

Pintucci G, Moscatelli D, Saponara F, *et al.* Lack of ERK activation and cell migration in FGF-2-deficient endothelial cells. FASEB J 2002; **16** (6):598-600.

Piotrowicz RS, Maher PA, Levin EG. Dual activities of 22-24 kDA basic fibroblast growth factor: inhibition of migration and stimulation of proliferation. J Cell Physiol 1999; **178** (2):144-53.

Plotnikov AN, Hubbard SR, Schlessinger J, Mohammadi M. Crystal structures of two FGF-FGFR complexes reveal the determinants of ligand-receptor specificity. Cell 2000; **101** (4):413-24.

Plotnikov AN, Schlessinger J, Hubbard SR, Mohammadi M. Structural basis for FGF receptor dimerization and activation. Cell 1999; **98** (5):641-50.

Poon E, Clermont F, Firpo MT, Akhurst RJ. TGFbeta inhibition of yolk-sac-like differentiation of human embryonic stem-cell-derived embryoid bodies illustrates differences between early mouse and human development. J Cell Sci 2006; **119** (Pt 4):759-68.

Powers CJ, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer 2000; **7** (3):165-97.

Qi X, Li TG, Hao J*, et al.* BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. Proc Natl Acad Sci U S A 2004; **101** (16):6027-32.

Rappolee DA, Basilico C, Patel Y, Werb Z. Expression and function of FGF-4 in peri-implantation development in mouse embryos. Development 1994; **120** (8):2259-69.

Rathjen J, Lake JA, Bettess MD*, et al.* Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors. J Cell Sci 1999; **112 (Pt 5)**:601-12.

Raz R, Lee CK, Cannizzaro LA, d'Eustachio P, Levy DE. Essential role of STAT3 for embryonic stem cell pluripotency. Proc Natl Acad Sci U S A 1999; **96** (6):2846-51.

Resnick JL, Bixler LS, Cheng L, Donovan PJ. Long-term proliferation of mouse primordial germ cells in culture. Nature 1992; **359** (6395):550-1.

Resnick JL, Ortiz M, Keller JR, Donovan PJ. Role of fibroblast growth factors and their receptors in mouse primordial germ cell growth. Biol Reprod 1998; **59** (5):1224-9.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000; **18** (4):399-404.

Reubinoff BE, Pera MF, Vajta G, Trounson AO. Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method. Hum Reprod 2001; **16** (10):2187-94.

Richard C, Roghani M, Moscatelli D. Fibroblast growth factor (FGF)-2 mediates cell attachment through interactions with two FGF receptor-1 isoforms and extracellular matrix or cell-associated heparan sulfate proteoglycans. Biochem Biophys Res Commun 2000; **276** (2):399-405.

Richards M, Tan S, Fong CY*, et al.* Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. Stem Cells 2003; **21** (5):546-56.

Rizzino A, Kuszynski C, Ruff E, Tiesman J. Production and utilization of growth factors related to fibroblast growth factor by embryonal carcinoma cells and their differentiated cells. Dev Biol 1988; **129** (1):61-71.

Rocchi S, Tartare-Deckert S, Murdaca J*, et al.* Determination of Gab1 (Grb2-associated binder-1) interaction with insulin receptor-signaling molecules. Mol Endocrinol 1998; **12** (7):914-23.

Rodda DJ, Chew JL, Lim LH*, et al.* Transcriptional regulation of nanog by OCT4 and SOX2. J Biol Chem 2005; **280** (26):24731-7.

Roghani M, Mansukhani A, Dell'Era P*, et al.* Heparin increases the affinity of basic fibroblast growth factor for its receptor but is not required for binding. J Biol Chem 1994; **269** (6):3976-84.

Rosenthal MD, Wishnow RM, Sato GH. In vitro growth and differetiation of clonal populations of multipotential mouse clls derived from a transplantable testicular teratocarcinoma. J Natl Cancer Inst 1970; **44** (5):1001-14.

Rossant J. Stem cells and early lineage development. Cell 2008; **132** (4):527-31.

Rossant J, Nagy A. Genome engineering: the new mouse genetics. Nat Med 1995; **1** (6):592-4.

Sasaki A, Taketomi T, Kato R*, et al.* Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. Nat Cell Biol 2003; **5** (5):427-32.

Sasaki N, Okishio K, Ui-Tei K*, et al.* Heparan sulfate regulates self-renewal and pluripotency of embryonic stem cells. J Biol Chem 2008; **283** (6):3594-606.

Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med 2004; **10** (1):55-63.

Sato N, Sanjuan IM, Heke M*, et al.* Molecular signature of human embryonic stem cells and its comparison with the mouse. Dev Biol 2003; **260** (2):404-13.

Savatier P, Lapillonne H, Jirmanova L, Vitelli L, Samarut J. Analysis of the cell cycle in mouse embryonic stem cells. Methods Mol Biol 2002; **185**:27-33.

Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2000; **103** (2):211-25.

Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2000; **97** (21):11307-12.

Shamblott MJ, Axelman J, Littlefield JW*, et al.* Human embryonic germ cell derivatives express a broad range of developmentally distinct markers and proliferate extensively in vitro. Proc Natl Acad Sci U S A 2001; **98** (1):113-8.

Shamblott MJ, Axelman J, Wang S*, et al.* Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci U S A 1998; **95** (23):13726-31.

Sheng G, dos RM, Stern CD. Churchill, a zinc finger transcriptional activator, regulates the transition between gastrulation and neurulation. Cell 2003; **115** (5):603-13.

Sheng Z, Lewis JA, Chirico WJ. Nuclear and nucleolar localization of 18-kDa fibroblast growth factor-2 is controlled by C-terminal signals. J Biol Chem 2004; **279** (38):40153-60.

Singh AM, Hamazaki T, Hankowski KE, Terada N. A heterogeneous expression pattern for Nanog in embryonic stem cells. Stem Cells 2007; **25** (10):2534-42.

Smith AG. Embryo-derived stem cells: of mice and men. Annu Rev Cell Dev Biol 2001; **17**:435-62.

Smith AG, Heath JK, Donaldson DD*, et al.* Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 1988; **336** (6200):688-90.

Smith AG, Hooper ML. Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev Biol 1987; **121** (1):1-9.

Smith AG, Nichols J, Robertson M, Rathjen PD. Differentiation inhibiting activity (DIA/LIF) and mouse development. Dev Biol 1992; **151** (2):339-51.

Solter D, Knowles BB. Developmental stage-specific antigens during mouse embryogenesis. Curr Top Dev Biol 1979; **13 Pt 1**:139-65.

Sperger JM, Chen X, Draper JS*, et al.* Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. Proc Natl Acad Sci U S A 2003; **100** (23):13350-5.

Stavridis MP, Lunn JS, Collins BJ, Storey KG. A discrete period of FGF-induced Erk1/2 signalling is required for vertebrate neural specification. Development 2007; **134** (16):2889-94.

Stokoe D, Stephens LR, Copeland T*, et al.* Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science 1997; **277** (5325):567-70.

Suzuki K, Tokue A, Kamiakito T*, et al.* Predominant expression of fibroblast growth factor (FGF) 8, FGF4, and FGF receptor 1 in nonseminomatous and highly proliferative components of testicular germ cell tumors. Virchows Arch 2001; **439** (5):616-21.

Tada M, Tada T, Lefebvre L, Barton SC, Surani MA. Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. EMBO J 1997; **16** (21):6510-20.

Taira M, Yoshida T, Miyagawa K*, et al.* cDNA sequence of human transforming gene hst and identification of the coding sequence required for transforming activity. Proc Natl Acad Sci U S A 1987; **84** (9):2980-4.

Takahama K, Hashimoto T, Wang MW*, et al.* Pipecolic acid enhancement of GABA response in single neurons of rat brain. Neuropharmacology 1986; **25** (3):339-42.

Takahashi K, Mitsui K, Yamanaka S. Role of ERas in promoting tumour-like properties in mouse embryonic stem cells. Nature 2003; **423** (6939):541-5.

Takahashi K, Tanabe K, Ohnuki M*, et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007; **131** (5):861-72.

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006; **126** (4):663-76.

Takahashi-Tezuka M, Yoshida Y, Fukada T*, et al.* Gab1 acts as an adapter molecule linking the cytokine receptor gp130 to ERK mitogen-activated protein kinase. Mol Cell Biol 1998; **18** (7):4109-17.

Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J. Promotion of trophoblast stem cell proliferation by FGF4. Science 1998; **282** (5396):2072-5.

Thomas SM, Brugge JS. Cellular functions regulated by Src family kinases. Annu Rev Cell Dev Biol 1997; **13**:513-609.

Thomson JA, Itskovitz-Eldor J, Shapiro SS*, et al.* Embryonic stem cell lines derived from human blastocysts. Science 1998; **282** (5391):1145-7.

Thomson JA, Kalishman J, Golos TG*, et al.* Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A 1995; **92** (17):7844-8.

Tomioka M, Nishimoto M, Miyagi S*, et al.* Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex. Nucleic Acids Res 2002; **30** (14):3202-13.

Toyooka Y, Shimosato D, Murakami K, Takahashi K, Niwa H. Identification and characterization of subpopulations in undifferentiated ES cell culture. Development 2008; **135** (5):909-18.

Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell 1990; **61** (2):203-12.

Vallier L, Alexander M, Pedersen RA.
Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J Cell Sci 2005; **118** (Pt 19):4495-509.

Vallier L, Reynolds D, Pedersen RA. Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. Dev Biol 2004; **275** (2):403-21.

Viswanathan S, Benatar T, Mileikovsky M*, et al.* Supplementation-dependent differences in the rates of embryonic stem cell self-renewal, differentiation, and apoptosis. Biotechnol Bioeng 2003; **84** (5):505-17.

Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002; **2** (7):489-501.

Wang F, Kan M, Yan G, Xu J, McKeehan WL. Alternately spliced NH2-terminal immunoglobulin-like Loop I in the ectodomain of the fibroblast growth factor (FGF) receptor 1 lowers affinity for both heparin and FGF-1. J Biol Chem 1995; **270** (17):10231-5.

Wang G, Zhang H, Zhao Y*, et al.* Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. Biochem Biophys Res Commun 2005; **330** (3):934-42.

Wang J, Xie LY, Allan S, Beach D, Hannon GJ. Myc activates telomerase. Genes Dev 1998; **12** (12):1769-74.

Watanabe S, Umehara H, Murayama K*, et al.* Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells. Oncogene 2006; **25** (19):2697-707.

Wei CL, Miura T, Robson P*, et al.* Transcriptome profiling of human and murine ESCs identifies divergent paths required to maintain the stem cell state. Stem Cells 2005; **23** (2):166-85.

Wilder PJ, Kelly D, Brigman K*, et al.* Inactivation of the FGF-4 gene in embryonic stem cells alters the growth and/or the survival of their early differentiated progeny. Dev Biol 1997; **192** (2):614-29.

Willems E, Leyns L. Patterning of mouse embryonic stem cell-derived pan-mesoderm by Activin A/Nodal and Bmp4 signaling requires Fibroblast Growth Factor activity. Differentiation 2008.

Williams RL, Hilton DJ, Pease S*, et al.* Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 1988; **336** (6200):684-7.

Wilson SI, Graziano E, Harland R, Jessell TM, Edlund T. An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. Curr Biol 2000; **10** (8):421-9.

Wu Z, Zhang W, Chen G*, et al.* Combinatorial signals of activin/nodal and bone morphogenic protein regulate the early lineage segregation of human embryonic stem cells. J Biol Chem 2008.

Xu C, Inokuma MS, Denham J*, et al.* Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001; **19** (10):971-4.

Xu C, Rosler E, Jiang J*, et al.* Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Stem Cells 2005a; **23** (3):315-23.

Xu RH, Chen X, Li DS*, et al.* BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat Biotechnol 2002; **20** (12):1261-4.

Xu RH, Peck RM, Li DS*, et al.* Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods 2005b; **2** (3):185-90.

Yamaguchi TP, Conlon RA, Rossant J. Expression of the fibroblast growth factor receptor FGFR-1/flg during gastrulation and segmentation in the mouse embryo. Dev Biol 1992; **152** (1):75-88.

Yao S, Chen S, Clark J*, et al.* Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. Proc Natl Acad Sci U S A 2006.

Ye M, Iwasaki H, Laiosa CV*, et al.* Hematopoietic stem cells expressing the myeloid lysozyme gene retain long-term, multilineage repopulation potential. Immunity 2003; **19** (5):689-99.

Yeom YI, Fuhrmann G, Ovitt CE*, et al.* Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development 1996; **122** (3):881-94.

Yin T, Yang YC. Mitogen-activated protein kinases and ribosomal S6 protein kinases are involved in signaling pathways shared by interleukin-11, interleukin-6, leukemia inhibitory factor, and oncostatin M in mouse 3T3-L1 cells. J Biol Chem 1994; **269** (5):3731-8.

Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 2003a; **115** (3):281-92.

Ying QL, Stavridis M, Griffiths D, Li M, Smith A. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol 2003b; **21** (2):183-6.

Yoshida S, Morii K. Brain metastasis from germinal tumors of the testis. Case report. J Neurosurg 1998; **88** (4):761-3.

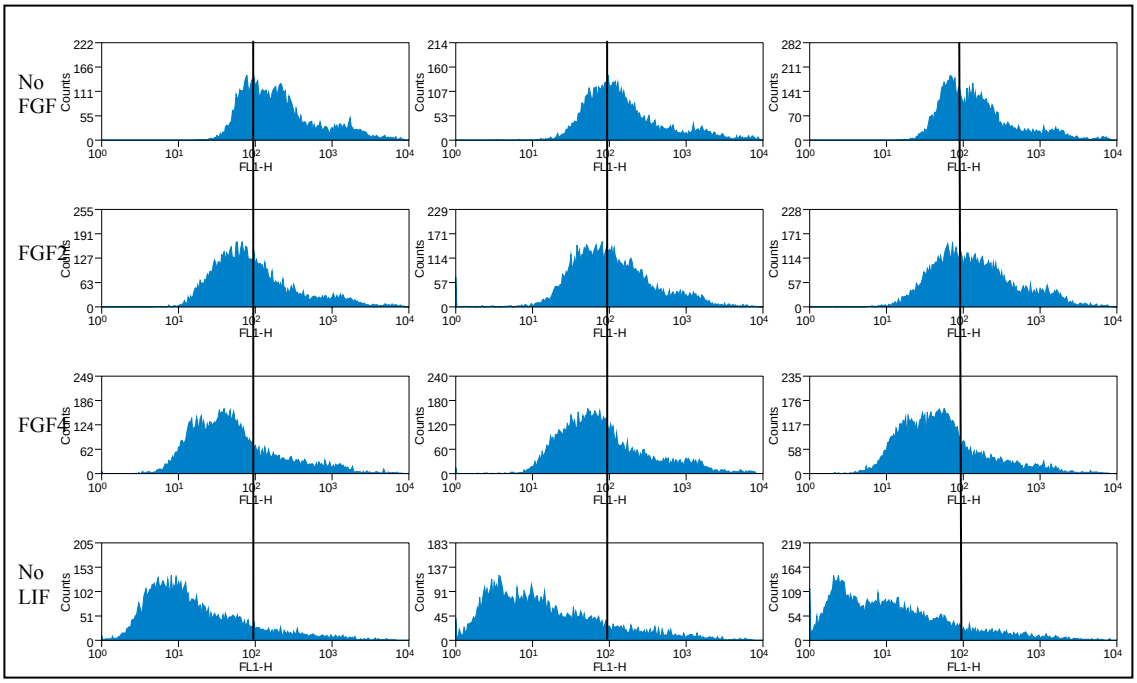
Yu J, Vodyanik MA, Smuga-Otto K, *et al.* Induced pluripotent stem cell lines derived from human somatic cells. Science 2007; **318** (5858):1917-20.

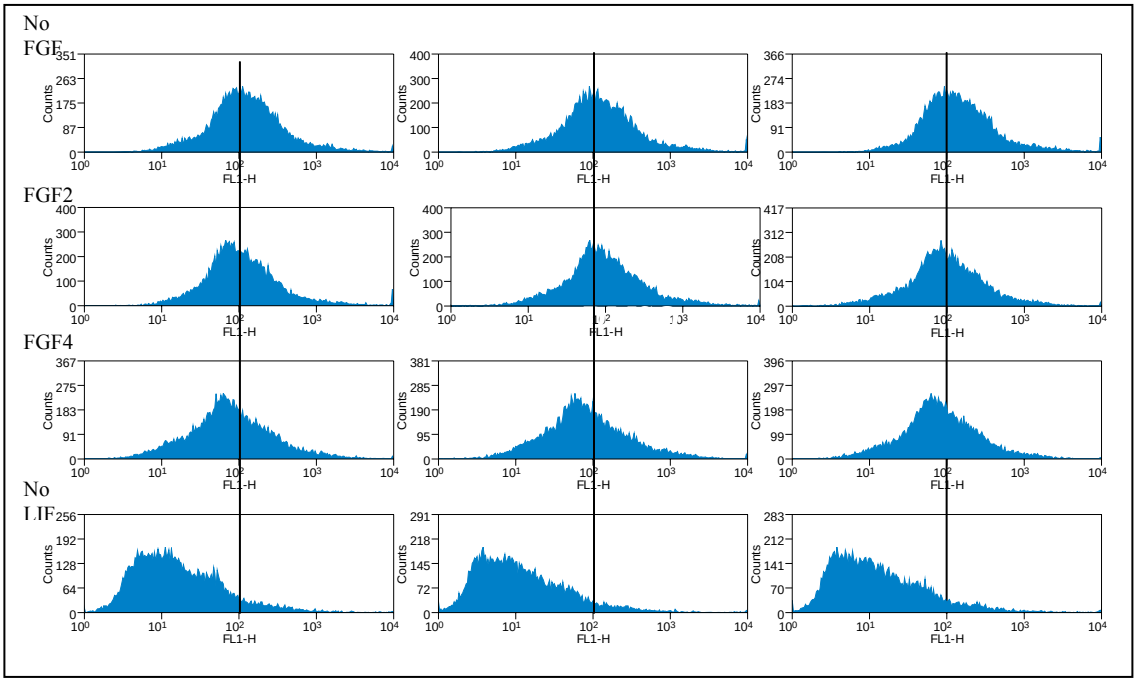
Yuan H, Corbi N, Basilico C, Dailey L. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes Dev 1995; **9** (21):2635-45.

Yusoff P, Lao DH, Ong SH, *et al.* Sprouty2 inhibits the Ras/MAP kinase pathway by inhibiting the activation of Raf. J Biol Chem 2002; **277** (5):3195-201.

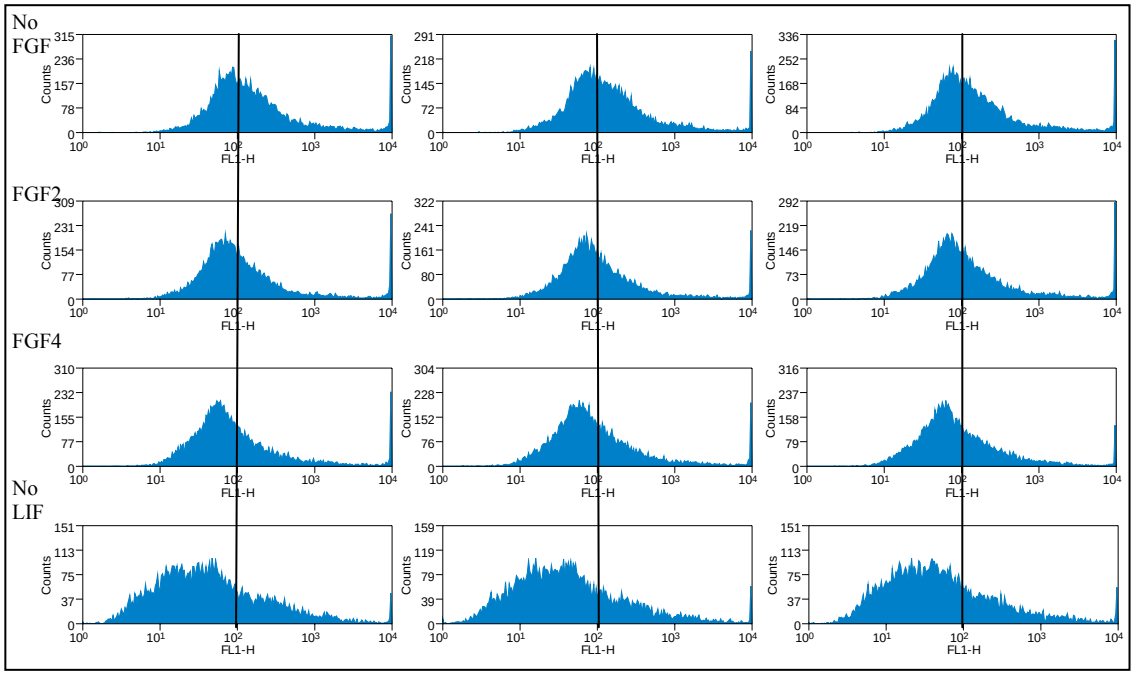
Zhang X, Ibrahimi OA, Olsen SK, *et al.* Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. J Biol Chem 2006; **281** (23):15694-700.

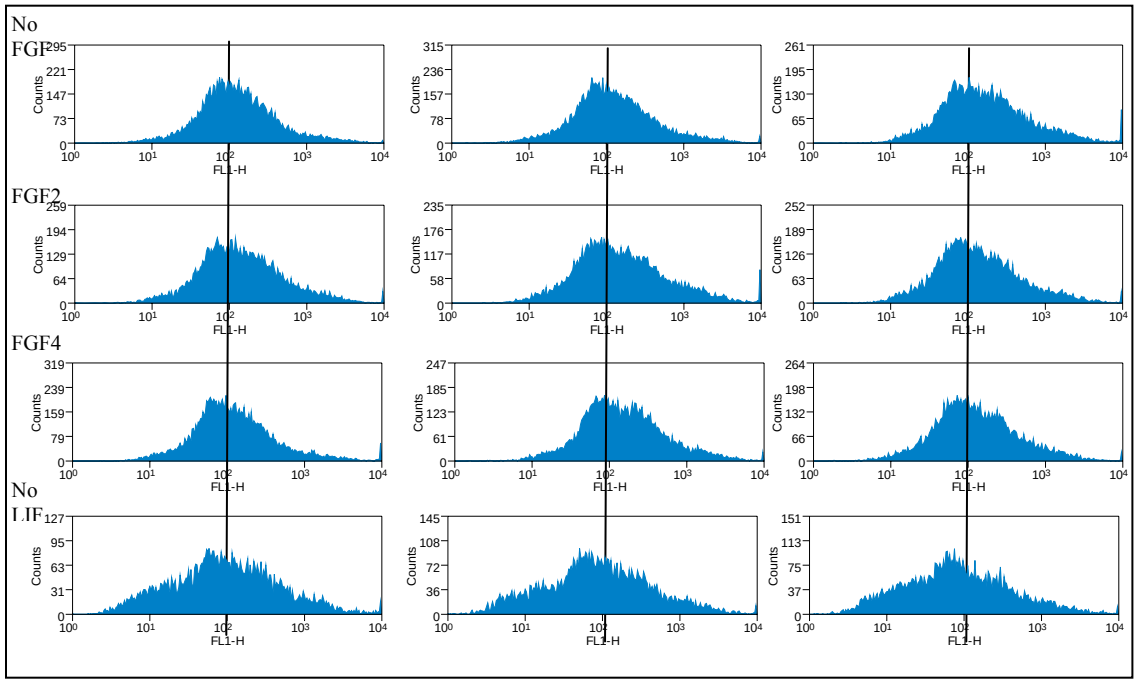
Appendix 1: Effect of FGF addition on FDG conversion.





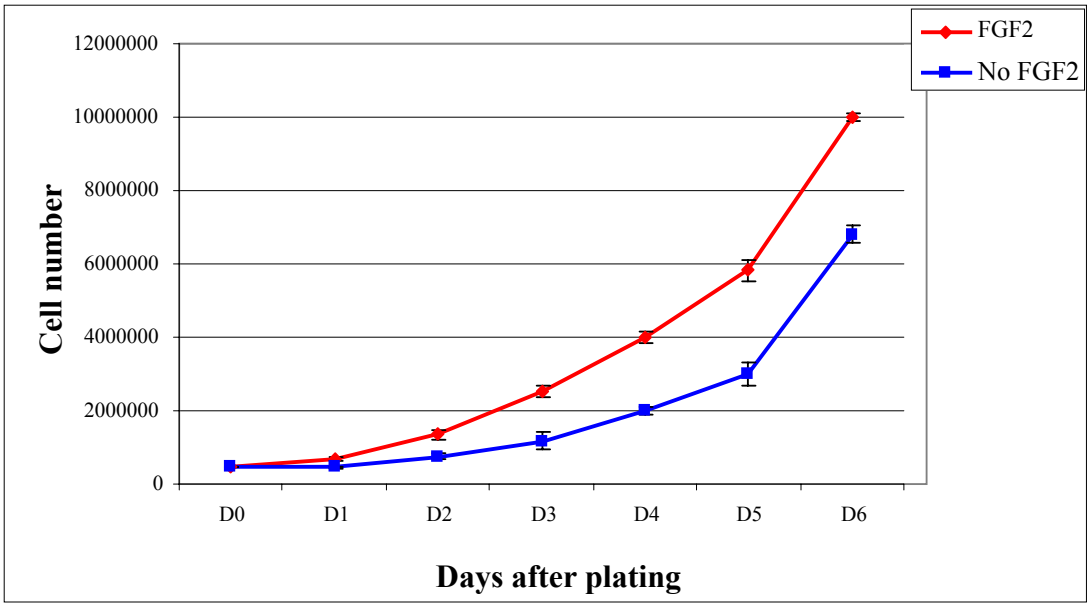
Raw data from the FDG analysis. These two experiments is part of the four independent experiments with triplicate repeats for each condition was used to produce the graphs in figure 3.12, A.

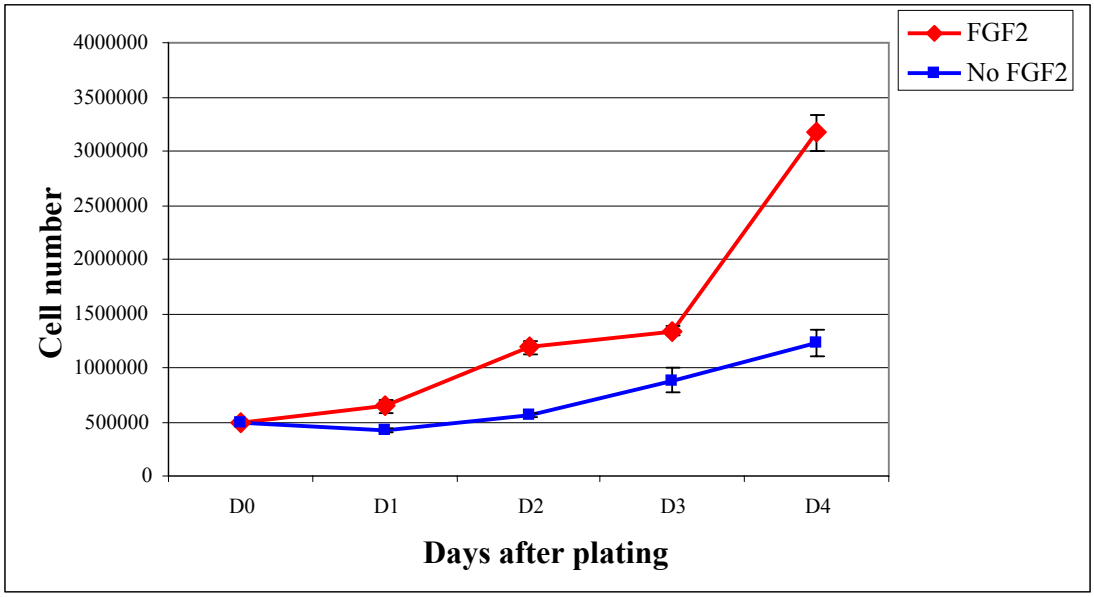




Raw data from the FDG analysis. These two experiments is part of the four independent experiments with triplicate repeats for each condition was used to produce the graphs in figure 3.12, A.

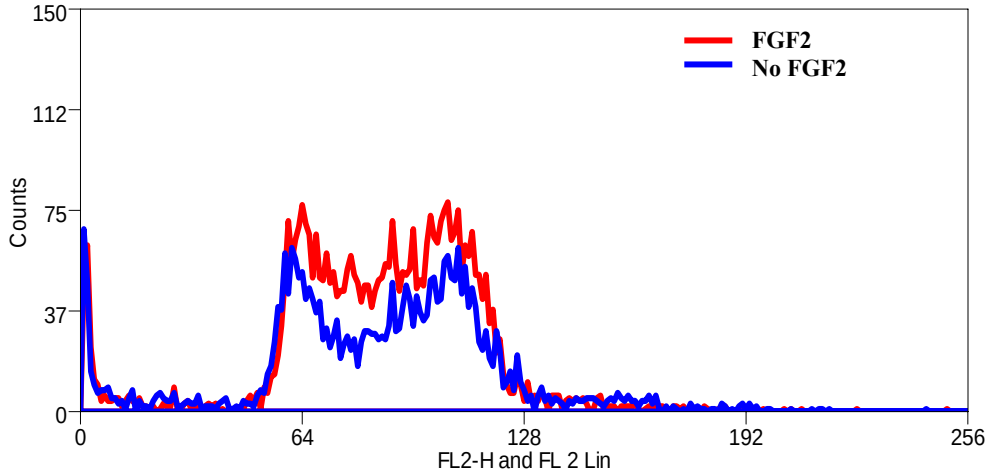
Appendix 2: Effect of FGF2 on H1 cell number



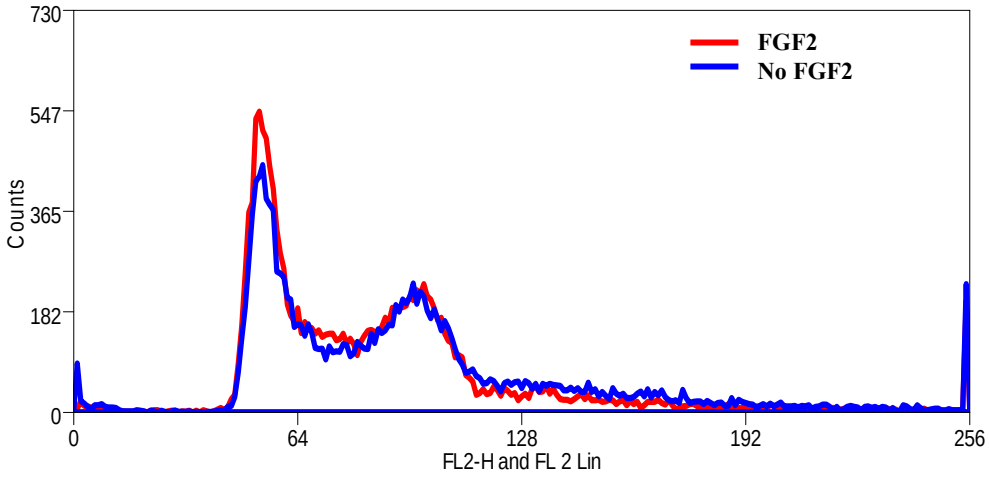


Appendix 3: Effect of cell density in cell cycle

A

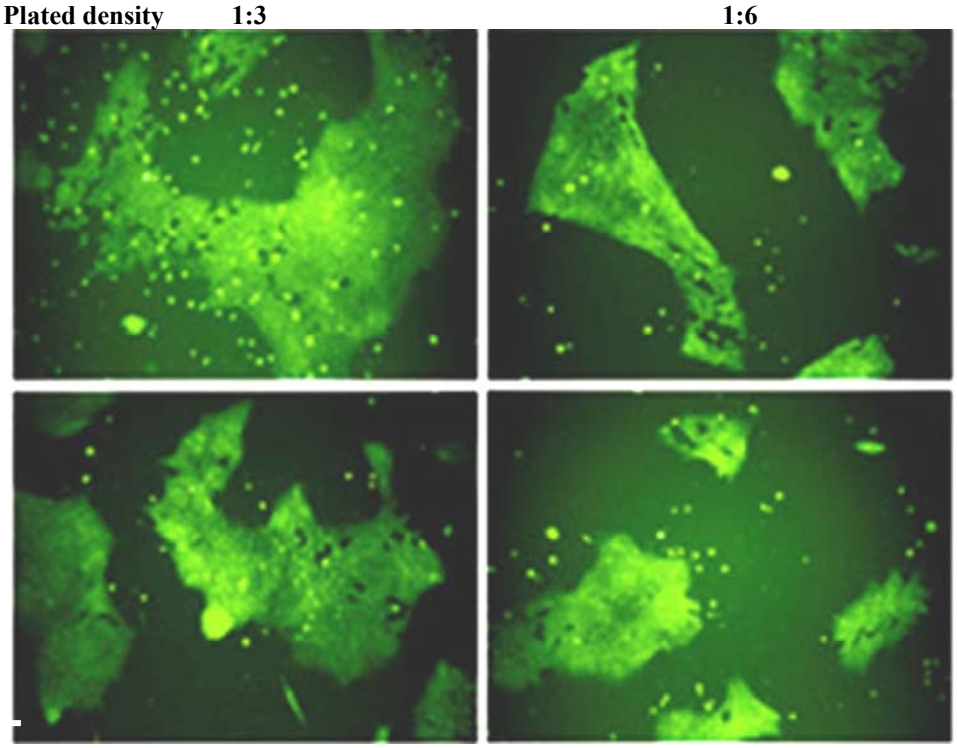


B



Effect of cell density in cell cycle. A: Cells plated for one day in presence of FGF2 showed an increased cycling. **B:** Cells plated for 5 days. Cell confluency caused an increase in G1 in the cells maintained with FGF2, which is a similar profile to that from the cells without FGF2.

Appendix 4: Autocrine regulation of T5 cells



T5 cell growth was sustained by the maintenance of the medium of plating after 5 days in culture in absence of FGF2. (scale bar = 200µm)