

The support of undifferentiated human embryonic stem cell lines by different matrices

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Abbreviations

aCGH	array Comparative genomic hybridisation
bFGF	Basic Fibroblastic Growth Factor
BSA	Bovine Serum Albumin
CO ₂	Carbon dioxide
Ct	Threshold cycle
CY3 or CY5	Cyanine 3 or 5
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl Sulphoxide
EB's	Embryoid Bodies
EC	Embryonal Carcinoma
EDTA	ethylenediaminetetraacetic acid
EG	Embryonal germ cell
FCS	Foetal calf serum
FGF	Fibroblast growth Factor
FITC	fluorescein isothiocyanate
g	Gram
HDFn	Human dermal neonatal fibroblasts
hESC	Human embryonic stem cell
hiPSC	Human induced pluripotent stem cell
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
ICM	Inner cell mass
IF	Immunofluorescence
IVF	In vitro fertilisation

KOSR	Knockout serum replacement
LIF	Leukaemia inhibitory factor
LN2	Liquid nitrogen
MEF	Mouse embryonic fibroblast
mESC	mouse embryonic stem cells
ml	Millilitre
µl	Microlitre
MRC	Medical Research Council
ng	Nanogram
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT- PCR	Reverse transcription polymerase chain reaction
SSEA	Stage specific embryonic antigen
TGFβ	Transforming growth factor beta
TLDA	Taqman Low density array
UKSCB	UK Stem Cell Bank
Wnt	wingless-type MMTV integration site

Abstract

The future of human embryonic stem cell (hESC) research with regards to their applicability in a therapeutic setting, relies on the development and standardisation of consistent and robust methods to demonstrate their defining characteristics; their pluripotent ability to form all three germ layers and their capacity for self-renewal. Although much research has been carried out to investigate new methods of culturing hESCs, many of these studies have not robustly concluded the impact of prolonged culture on genetic and genomic stability nor have they examined in any comparative detail the impact of the culture conditions such as differences in feeders used or the media composition in which the stem cells are cultured in. The aim of this thesis therefore was to investigate and evaluate methods for improving the uniform and robust culture and characterisation of hESCs over prolonged periods in culture.

Four hESC lines (RH5, HUES9, SHEF1 and NCL5) were chosen on the basis that they had not previously been well characterised and therefore could potentially benefit the wider stem cell community by increasing diversity, rather than continue to use the already small subset of well publicised lines. The RH5, HUES9, SHEF1 and NCL5 cells were subjected to long term passaging using recombinant enzyme TrypLE™ Express, on human feeders, mouse feeders and feeder free matrix Matrigel in combination with defined media mTeSR1, for uniform scale up. Changes in characteristic stem cell surface markers were compared using two techniques; flow cytometry and quantitative *in situ* fluorescence microscopy. Genomic stability was assessed by real time PCR. Chromosomal integrity was monitored using array genomic hybridisation (aCGH).

Array genomic hybridisation analysis of cells cultured for 20 passages by enzymatic passaging revealed changes in copy number variations in all the stem cell lines. Aberrations on chromosomes 12, 17 and 20, appeared most commonly as a result of long term culture. Although no significant differences were seen between hESCs cultured on mouse and human feeders, cultures on Matrigel showed fewer detected chromosomal aberrations. Expression of cell surface stemness markers SSEA3, SSEA4, TRA1-60 and TRA1-81 were maintained by hESC cultured on all matrices and confirmed by the use of flow cytometry and high throughput quantitative immunofluorescence imaging using the TissueFaxs™ cell analysis microscopy system. In depth imaging revealed subtle but important differences in the way in which hESCs attach and proliferate on different matrices. Genetic profiling of each of the stem cell lines using Taqman Low density array cards to assess the expression of 96 genes by Real Time PCR, demonstrated the continued expression of stemness genes

at late passage, and low level expression of differentiation genes, inherent to particular stem cell lines.

Although both mouse and human feeders and Matrigel support the undifferentiated growth of hESCs, subtle differences from the hESCs were seen as a result of their use, most obviously, changes in morphology and how they proliferate. This was further explored in the stem cell line NCL5, as it demonstrated a readiness to adapt to new matrices, better chromosomal stability and higher expression of cell surface markers compared with the other hESC lines. Using *in vitro* differentiation assays to all three germ layers, NCL5 cultured to late passage (p+20) on human feeder iMRC5, mouse feeder iMEF and feeder free matrix Matrigel, demonstrated the ability to differentiate to ectoderm, endoderm and mesoderm progenitors after induction using three 7 day flat based directed differentiation protocols. Altered differentiation patterns were detected by Real Time PCR and TissueFaxs™ imaging and quantitative analysis, as a consequence of the prolonged culture on the specific matrices used. Such key findings allude to the strong influences of microenvironment and will help to improve the standardisation of *in vitro* differentiation assays. From these studies, chromosomal changes had no impact on NCL5 stem cell lines' ability to form progenitors, however small genetic instabilities may still play a role in terminal differentiation of germ lineage specific cell types.

The findings of the programme of work described has led to the successful culture methods and characterisation testing validated in this project being incorporated into routine culture and banking of research grade hESCs at the UK Stem Cell Bank. These protocols will now be made more widely available and should assist stem cell researchers in adopting the most suitable and optimum conditions for culturing stem cells in the undifferentiated and stable state. With the huge surge in stem cell research over the past decade, the development of robust characterisation and culture methods will undoubtedly have significant impact on the exploitation of these cells for regenerative medicine and to assist with this a future aim of the stem cell bank will be to standardise methodologies for clinical grade banking.

Chapter 1

General Introduction

1. Introduction

Historically, traditional treatments for human disease had been focussed on the repair of damaged tissue and organs or the pharmacological management of disease and injury through the use of drugs. Fundamental advances in biology impacting on treatments have been rare. However, three in particular have resulted in radical progression during the last 50 years.

The human genome project has forwarded the understanding of disease and opened up new areas of biomedical research, resulting in gene therapies. Molecular pathology has propelled the concept of personalised medicine, unveiling many possibilities, including the use of genetic screening for diseases such as chronic leukaemias and breast cancer in order to treat specific mutations within individuals (Holleman et al., 2004; Apostolou et al., 2013). Nonetheless, these remain extremely costly thus limiting their use for routine treatment of injury and diseases.

Secondly, the concept of regenerative medicine has been introduced. This term was initially used as an umbrella to cover organ transplant and replacements such as joints (Tayton., 2012) and artificial tracheal replacement (Baiguera et al., 2010). These utilise biomedical scaffolds on which replacement tissues are built. The increase in sophistication of technology in the surgical setting has resulted in many such procedures becoming routine.

The term regenerative medicine has also been expanded to include the use of stem cells in various settings such as bone marrow transplantation for the treatment of leukaemias. Stem cells have a unique pluripotential and can give rise to all the cell types in the body. It was in the 1900s that scientists became fascinated with the potential use for stem cells when it was first recorded that progenitor cells gave rise to immature blood cells (Lord & Dexter., 1995). Further studies resulted in their use in bone marrow cell transplants in the late 1950s (Lorenz et al.,1952; Jacobson et al.,1950) as stem cells present in the bone marrow are able to produce all the different blood cell types from a single stem cell. Adult stem cells are present in various parts of the body, notably the bone marrow and conjunctiva and can be harvested, grown and used to repair injury and disease for personalised individual therapies without the concern of host rejection. This is an advantage over organ transplantation. However, it is the discovery of embryonic stem cells by Thomson et

al. (2008) which has ushered in a new era of regenerative medicine possibilities in the form of 'cell therapies' and is the third major advance in biology to fundamentally effect the treatment of injury and disease.

1.1 What are Stem Cells?

Stem cells are unspecialised cells of the embryo, foetus or adult and are defined by two main characteristics: they are capable of becoming any cell type in the body and they have the ability of self-renewal through cell division to continue producing progeny of unspecialised cells (Morrison & Kimble, 2006). Each new 'daughter' cell may remain the same as its progenitor or become a more specialised cell type (differentiate). The majority of adult stem cells in the human body are lineage restricted. Stem cells become restricted by a process defined as differentiation. Cell differentiation is the process by which a cell becomes fully mature, non-dividing with a specialised gene expression profile needed to carry out a specific tissue function (Reubinoff et al., 2000). It is this special characteristic that gives stem cells the unique potential to replace any cell type in the body.

1.2 Stem cell differentiation potential

On the basis of their developmental or differentiation potential ES cells can be further subdivided into the following types: totipotent, pluripotent, multipotent and unipotent. Totipotent stem cells are those which have the ability to form any cell type in the body. These stem cells have the ability to form the entire organism for example, the embryo and the trophoblast of the placenta. Multipotent cells have a more limited range of potential. Multipotent cells can form multiple lineages of differentiated cell lineages for an entire tissue or tissues and tend to be specific to their location for example, haematopoietic cells. Cells with unipotential capacity (e.g. spermatogenic stem cells) only have the potential to form cells of a single lineage (Smith., 2006).

Pluripotent cells have the ability to form any cell type of all three of the germ layers, mesoderm, endoderm and ectoderm (Figure 1) and also include germ cells. It is this unique capacity to differentiate into numerous, varied cell lineages that makes human embryonic stem cells an exceptional tool to treat a host of illnesses and neurodegenerative disorders as well as an ideal model to understanding the intricate biology and various disease pathways in humans.

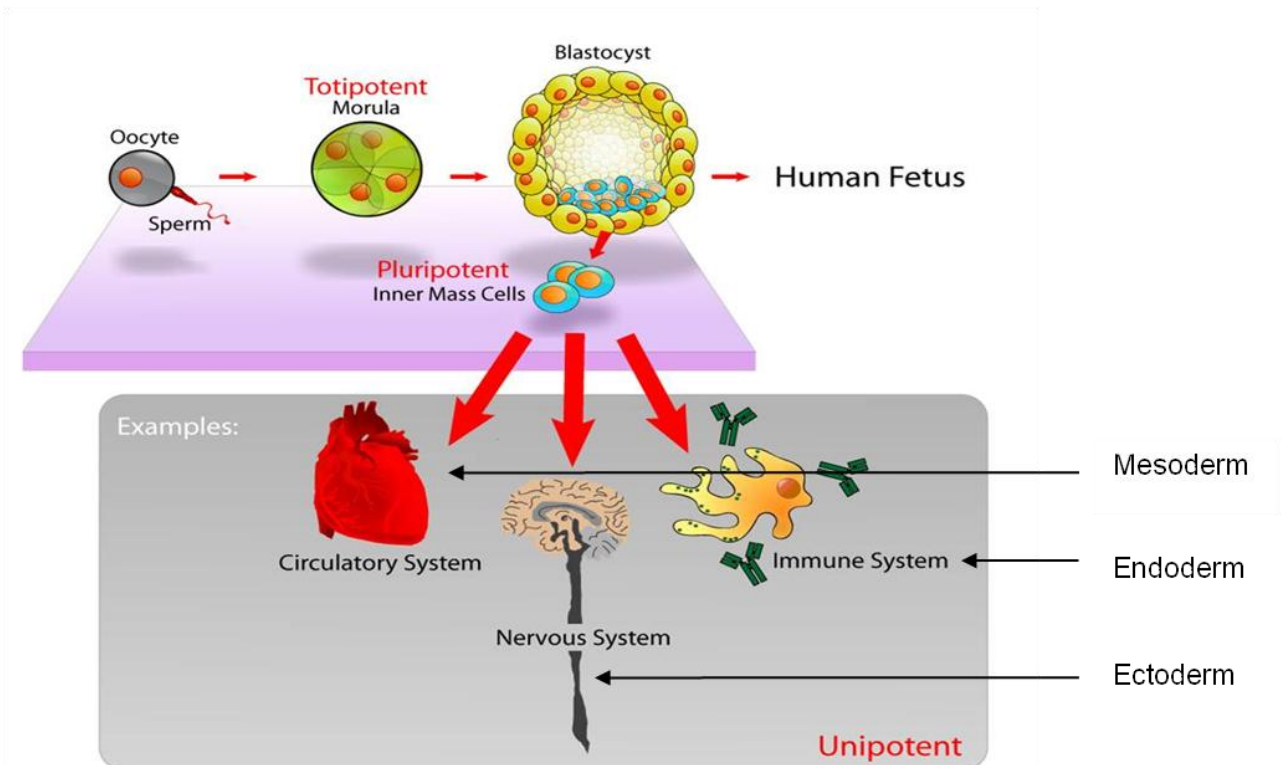


Figure 1. Origin and potential of pluripotent stem cells. Examples of stem cell differentiation into three germ layers mesoderm (heart), ectoderm (nervous system) and endoderm (immune system).

(Image obtained by free link to

http://en.wikipedia.org/wiki/File:Stem_cells_diagram.png#file)

1.3 Types and sources of stem cells

1.3.1 Adult stem cells are found throughout the body. They have been identified and characterised in the skin, blood, gut, and bones. Their main role is maintenance through replacement and repair of the organ/tissue type from which they originate. Adult stem cells maintain their ability to divide throughout life, from early development through to late adulthood and they also give rise to specific cell types. For example, promyeloblasts in blood have the ability to form basophils and eosinophils (Schroeder, 2008). Tissue specific stem cells maintain a careful balance of proliferation, differentiation and cell death at a steady-state.

1.3.2 Foetal and neonatal stem cells have huge therapeutic potential but were originally very difficult to obtain due to ethical issues concerning the use of foetal material. Foetal stem cells were used for the treatment of neural diseases in the late 1990s (Kordower et al., 1995; Shambloott et al., 1998; Deacon et al., 1999) including Parkinson's disease. It is during foetal development at approximately 5-6 weeks gestation, when primitive organs begin to form. This is thought to be a stem cell rich stage in development and has much potential for scientists to study developmental pathways that are crucial to organogenesis as well as other therapeutic potentials. Following on from the research of various groups (Andrews et al., 1991) a source of stem cells was found in the gonadal ridge of the aborted fetus. These cells are known as embryonic germ cells (EG) as they go on to develop germ cells. They have similar properties to embryonic stem cells (Shambloott et al., 1998) and have aided scientists with many important studies into the function of germ cells and embryology.

Much foetal stem cell research has been conducted in mice (Martin, 1980; Liu et al., 1997) and these types of studies may be the key to unlocking the secrets to promoting the regeneration of stem cells reserves, in order to repopulate damaged adult tissue. Foetal tissue had also been used in research into regenerating areas of neuronal cell growth within the brain to aid healing of damaged cells due to Parkinson's Disease and other mid brain trauma. Nonetheless, the use of foetal cells and tissue is an ethically sensitive area of research and has many barriers to overcome before being classed as acceptable tools for research.

More recently cord blood has proved to be an easily obtainable source of concentrated neonatal stem cells. Its use in a clinical setting has been well translated and many companies have been set up to bank cord blood stem cells due to their potential use (Bertram & Shearer., 2007; Thornley et al., 2009).

1.3.4 Embryonic Germ cells (EG) are cells found in the specific part of the embryo known as the gonadal ridge that normally develops into mature gametes. These cells share properties similar to those of ESCs (Geijsen et al., 2004; Hua et al., 2009).

1.3.5 Embryonic carcinoma cells (EC) are pluripotent stem cells derived from teratocarcinomas. These are malignant germ cell tumors that include a mixture of various differentiated cells (Andrews, 2002). Before the derivation of embryonic stem cells both EG and EC cells were used as in vitro models of mammalian differentiation (Solter, 2006). However, their value is limited as EC cell lines are well known to carry severe chromosomal abnormalities and have a relatively limited differentiation potential. Therefore, these cell lines are better served as reference lines to the ES cell lines (Josephson et al., 2007).

1.3.6 Embryonic stem cells (ESC) are derived from the blastocysts stage of an embryo. In humans, the majority of embryos are obtained from fertility clinics from patients undergoing IVF treatment. Specialist consent is given by the donors who allow the use of their embryos for research. These embryos would otherwise be discarded (Mitalipova et al., 2003).

1.4 Deriving embryonic stem cells

The first embryonic stem cell derivation came from mouse models, almost 30 years ago in 1981 when scientists Kaufman and Evans and Martin detailed the derivation culture process from mouse embryos (Evans, 1981). Previously, research on embryonic development and differentiation of cells was based around embryonic germ cells and embryonic carcinoma cells. Based on previous studies of early morula formation (Evans, 1972) and using delayed implantation, mimicked in laboratory mice through the administration of hormones, Kaufman and Evans extracted the blastocysts of pregnant mice. The blastocysts were recovered and carefully cultured. After a few days it was possible to dissociate the blastocysts into individual cells and transfer them to mitotically inactivated fibroblast feeder layers. The difference between Kaufman and Evans and Martins' work was that Martin chose to grow the cells using conditioned media from EC cells, whereas Evans and Martin used mitotically inactivated mouse feeders. From both culturing conditions mouse embryonic stem cells were successfully derived and mass-cultured in an undifferentiated state.

Following on from this work almost ten years later, Thomsons' group made the remarkable breakthrough of propagating human embryonic stem cells (Thomson et al., 1998) following the derivation of primate embryonic stem cells (Thomson et al., 1996). The progression of Thomsons' work fuelled a number of labs to begin deriving human embryonic stem cell (hESC) lines in order to explore the many possibilities that these cells may hold for disease treatment.

The inner cell mass (ICM) of the human blastocyst is isolated from the trophectoderm layer via immunosurgery at day five to six post fertilisation, and plated onto a mitotically inactivated fibroblast layer. After approximately two weeks in culture the ICM-derived cells are carefully dissociated by manual dissection and replated, as outlined by Figure 2 (Bongso et al., 1994). Characteristic undifferentiated hESC morphology can be distinguished by a high nuclear: cytoplasmic ratio and many prominent nucleoli (Sathananthan et al., 2002).

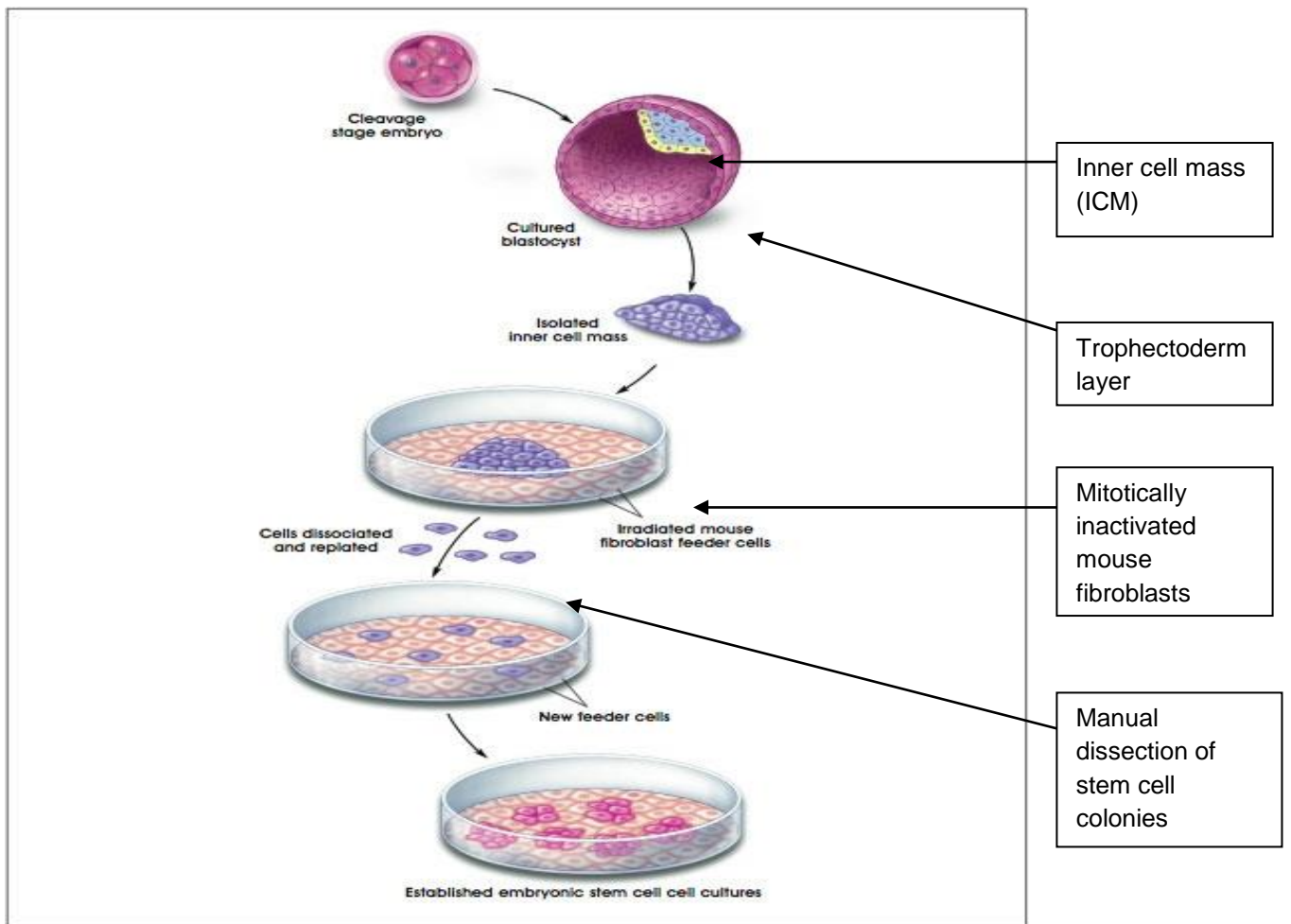


Figure 1.2. The derivation process of human embryonic stem cells, as carried out by Thomsons' research group. (Obtained from website on 10th august 2010) obtained from:

<http://stemcells.nih.gov/StaticResources/info/scireport/images/figurec1.jpg>

1.5 Induced pluripotent stem cells

Stem cell research achieved another breakthrough in 2006, when Yamanaka *et al* successfully reprogrammed adult mouse fibroblasts to ES-like cells called induced pluripotent stem cells (IPSCs) using 4 pluripotency associated genes: OCT4, Sox2, Klf4 and c-Myc (Takahashi and Yamanaka, 2006). This resulted in the reprogramming of cells which have the characteristics of pluripotent 'ES-like' cells and thus capable of differentiating into multiple lineages. Although reprogramming of cells is well practiced and documented (Li., 2002; Maherali et al., 2008; Kim et al., 2009) the process of reprogramming to give rise to stem cells had not been achieved or previously attempted as scientists assumed that the adult stem cell pathway was finite and could not possibly be reversible.

Induced pluripotent stem cells have an advantage over hESCs as they can be derived from the same person requiring differentiated cells/tissue, thus eliminating potential rejection (Zhao et al., 2009; Knoepfler., 2009). The creation of IPSCs is a significant advancement towards personalised medicine and removes the ethical and political issues surrounding the use of embryos. However, to realise the full potential of IPSCs, their detailed molecular characterisation is necessary to decide how comparable they are to human embryonic stem cells (Chin et al., 2009; Bock et al., 2010; Chen et al., 2011), as little is known about their cellular reprogramming and stability and many scientists are concerned that the epigenetic memory of IPSC is retained by the cells from which they were reprogrammed (Kim et al., 2010). More recently the derivation of IPSCs using non integrating methods such as the StemGent™ mRNA kit, hold much greater promise for the progression of IPSCs into therapeutic applications (Warren et al., 2010; Yakubov et al., 2010) as they remove the use of viruses which present safety and biocontamination issues.

1.6 Undifferentiated stem cell characteristics

To be able to exploit the potential of hESCs their characteristics need to be fully understood. The characterisation of a stem cell is assessed through a number of different techniques. The first and most obvious is morphology. Distinct tight clusters of cell colonies with prominent bright nuclei and a high nuclear to cytoplasmic ratio, as shown in Figure 1.3, gives a clear indication of undifferentiated stem cells under a microscope (Sathananthan., 2001). The method of passaging and substrate used to support hESC growth can have an effect on their growth rate. The process of

dissociation and potential expansion of colonies is known as subculturing or passaging. Although they are mostly reported to proliferate as distinct round colonies, hESCs can be maintained as monolayers, particularly when passaged by enzymatic methods. hESCs can also be cultured on immortalised human feeders, but this can change their morphology. Such changes can make determining their state of pluripotency difficult if they do not exhibit typical morphological features. Therefore the expression of key 'stemness genes and cell surface markers, which are the genes involved in maintaining pluripotency and promoting self-renewal becomes crucial when attempting to predict stem cell fate (Draper et al., 2002). These genes can be detected using Real Time Polymerase chain reaction (RT-PCR) and phenotypically using fluorescence conjugated antibodies to cell surface proteins by '*in situ*' staining and flow cytometry.

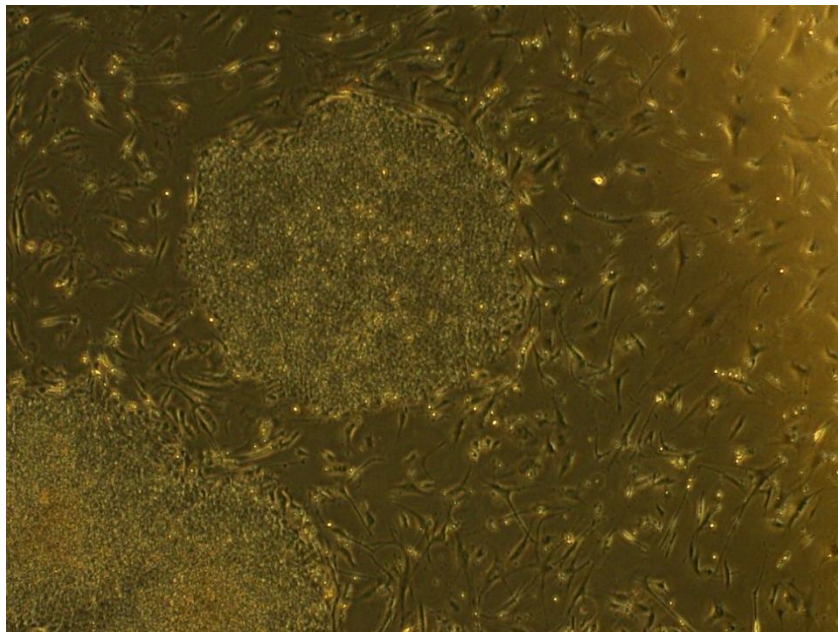


Figure 1.3. Typical morphology of hESCs cultured on inactivated mouse embryonic fibroblasts MEFs. hESCs typically proliferate in tight discrete colonies supported by MEFs, inactivated using Mitomycin C.

The pluripotency of hESCs is associated with the expression of key transcription factors such as Oct4 (POU5F1), from the family of POU genes, an important regulator of self-renewal in ESCs (Nichols et al., 1998). Its expression is important when maintaining cultures of stem cells in the undifferentiated state over extended passages. The suppression of Oct4 leads to loss of pluripotency and differentiation (Atlasi et al., 2008). Other transcription factors of importance include Nanog (Chambers et al., 2007) and SOX2, which are both essential for determining self-renewal in hESCs. Similarly, MYC, located on chromosome 8, plays a key role as one of the main four factors used in reprogramming of fibroblasts to IPS cells (Takahashi and Yamanaka, 2006). In addition to transcription factors, other molecules such as fibroblast growth factor 2 (FGF2) may also be involved in maintaining pluripotency (Vallier et al., 2005).

Apart from the above, glycolipid cell surface antigens such as SSEA3 and SSEA4 (Kannagi, 1983), and TRA160 and TRA181 (Draper et al., 2002; Schopperle & DeWolf, 2007) are also important when assessing undifferentiated hESCs and are easily detected by flow cytometry and immunostaining.

To assess the pluripotent potential of hESCs *in vivo*, teratoma forming assays are still currently employed as the gold standard as they demonstrate the ability to form all three germ layers. hESCs are injected into immunocompromised mouse models and then left to form teratomas, which are assessed for the presence of all three germ layers by histological methods (Muller et al., 2010). This method has however been criticised for being too variable between laboratories and extremely difficult to standardise (Muller et al., 2011; Buta et al., 2013). Thus, attempts have been made to introduce new methodologies that meets the needs of researchers, especially where scientists want to refrain from using animal models (Muller et al., 2011; Buta et al., 2013). These include using *in vitro* differentiation assays to demonstrate the presence of germ lineage specific genes using PCR (Bock et al, 2010).

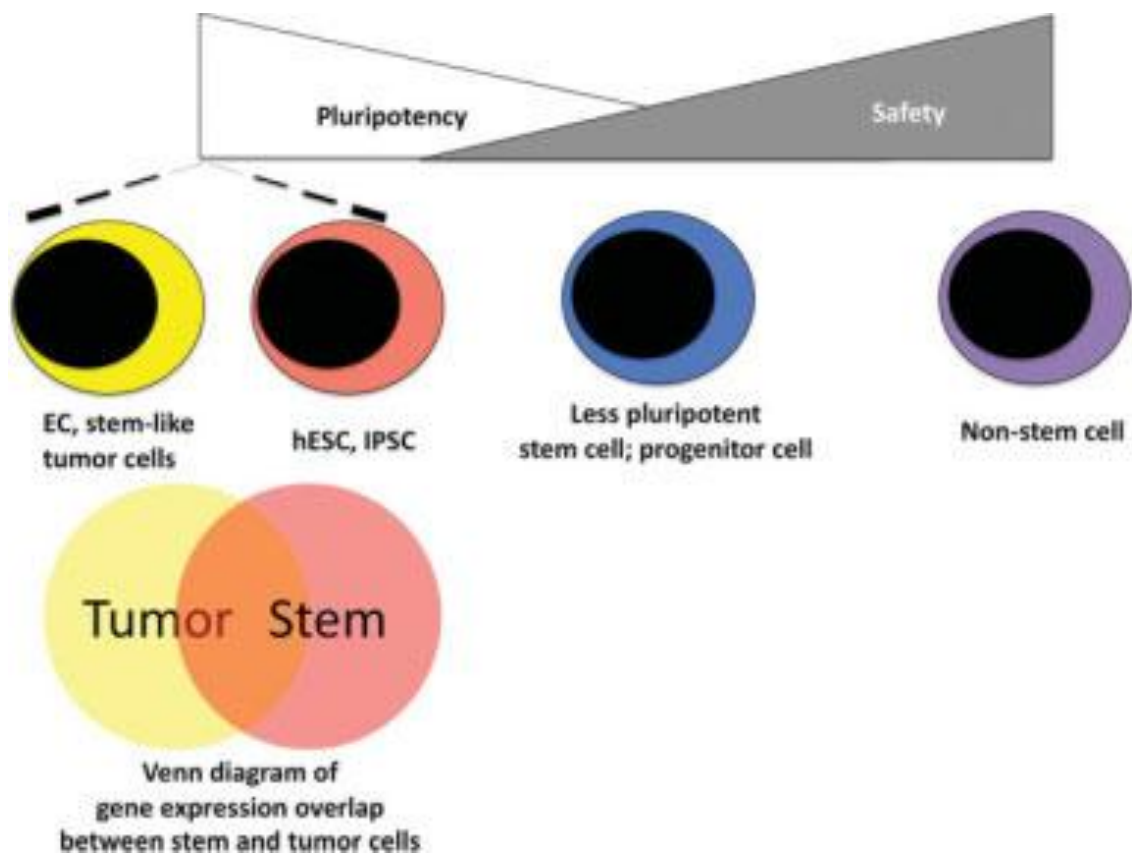


Figure 1.4. Diagram outlining the relationship between pluripotency and tumorigenicity. Taken from (Knoepfler., 2009).

1.7 Ethics surrounding the use of stem cells

The UK and much of Europe have long debated the ethical and political issues surrounding derivation and research using human embryonic stem cells. This is due to founding religious beliefs in the use of unwanted embryos and fertility treatment. Although guidelines surrounding the use of hESCs have been developed (Sugarman, 2008), much scrutiny has been put forward by modern society, in that people should be given a choice of which technologies they choose to improve their lives. The majority of the medical and scientific communities support the use of stem cells due to their immense potential.

1.8 Regulation and governance of stem cell use

Following the derivation of many stem cell lines worldwide the UK government put in place certain measures to ensure the regulation of hESCs used for research purposes certified the collection of consent from donor embryos. In 2004 the Medical Research Council (MRC) agreed that all stem cell lines derived in the UK must be deposited and stored in the UK Stem Cell Bank (UKSCB) (White paper reference, House of Lords, 2004). It is the role of the UKSCB to provide quality controlled stocks of these cells to researchers worldwide. To obtain cells, formal applications have to be made and the bank, along with guidance from the Steering Committee, who determine the eligibility of the project and whether it would benefit the stem cell community, before deciding whether the application is successful. The UKSCB also acts as a hub for hESC information (Stacey and Hunt, 2006) and provides support through various collaborations including the International stem cell initiative (ISCI). The latter comprises of participating stem cell laboratories worldwide, whose overall aim is to provide consistent consensus relating to best methods of practice in relation to the culture of hESCs (Stacey et al., 2009). The National institute for health (NIH) has reviewed the derivation of hESCs regarding donor consent and has so far issued 22 lines from the UK as having sufficient supporting evidence and consent before derivation (http://grants.nih.gov/stem_cells/registry/current.htm). The derivation, storage and manufacture of hESCs are governed by the Human Tissue Authority (HTA) who issue various licences based on the type stem cell work being conducted. They ensure that premises are suitable for such activities by auditing. The UKSCB currently holds a HTA licence for the storage and distribution of research grade hESCs.

1.9 The potential of stem cells in translational medicine

In order to fully support the potential that hESCs hold for regenerative medicine, standard laboratory methods for stem cell culture and maintenance is critical. This includes suitable clinics obtaining embryos with consent for the derivation of stem cell lines, alongside advice from regulatory authorities to govern the use and storage of hESCs with the potential for research and later, clinical applications (Coecke et al., 2005; ISCBI 2009). Also, the transplantation of differentiated stem cells provides challenges that need to be overcome as stem cells move towards clinical application. As hESCs differentiate they express markers on their cell surfaces which are recognised by the body's immune system, which could lead to the rejection of implanted differentiated cells without the use of strong immunosuppressive drugs. Although many scientists see iPSCs as a solution to this problem as reprogrammed cells can be taken directly from a patient and HLA matched (Nakatsuji et al., 2008), research is still being conducted to demonstrate that iPSCs are equivalent and have the same capabilities as hESCs. In addition, the epigenetic memory of the iPSCs' original cell type that they were programmed from is a cause for concern, as this could have implications for their differentiation potential. Furthermore, the way in which these cells are derived is also important as it has been documented that viral vectors can leave a 'molecular footprint' in reprogrammed cells. This could have an effect on the potential application of these cells used for therapy (LakshmiPathy et al., 2010).

The need to develop robust culturing methods using well defined components is of paramount importance. Some clinical applications require 10×10^9 cells in order to begin scale up. Uniform, qualified and well tested banks of hESCs need to be produced under GMP which can be easily reproduced and cultured rapidly without compromising the end use/product.

For stem cells to be accepted for clinical therapy trials, scientists must be able to demonstrate that the cells are non-tumorigenic (Gropp., 2012). Research has shown links between pluripotency and tumorigenicity (Dressel., 2011) and methods for overcoming the challenges of stem cell tumorigenicity have been explored (Schuldiner et al., 2003). The tumorigenic nature of hESCs has been repeatedly discussed (Dalebar et al., 2007; Baker et al., 2007; Ben-David and Benvenisty., 2011) and culture methods have been identified as a means for selection, as it has

been well documented that stem cells acquire a greater number of genetic alterations through prolonged culture (Draper et al., 2004; Amps et al., 2012). Attempts to yield and accurately identify purer populations of hESCs which are homogenous appear to be the aim of such studies, however more work needs to be done on predicting the nature of smaller, undifferentiated hESC populations. Furthermore, the profound effects of reprogramming for iPSC production have yet to be determined (Knoepfler., 2009) as the elementary concepts that govern stem cell biology are shared with tumorigenesis, for instance, the roles that Myc and KLF4 play in regulating pluripotency and differentiation pathways. Figure 1.4 outlines the relationship between pluripotency and tumorigenicity.

Techniques used to characterise human embryonic stem cells are abundant and well documented. However, there is little consensus as to which methods are most appropriate in terms of robustness and sensitivity and also, none of these methods have been assured for the use of clinical Stem cell banking applications. Much work is needed to validate and standardise these techniques as the field rapidly progresses towards clinical applications. In particular, it is important that cell culture methods are simple, robust and reproducible. Characterisation testing must demonstrate that the cells still exhibit the key characteristics of undifferentiated hESCs. Addressing these concerns has been within the core remit of the UK Stem Cell Bank.

1.10 Characterisation and quality control of banked stem cells at the UKSCB

The UKSCB aims to produce banks of undifferentiated stem cells through standardised methods for thawing, subculturing, scale-up, cryopreservation and well defined quality control testing release criteria, to ensure the stem cells being provided to other research and industry-based organisations are of the highest standard. Banking facilities such as the UKSCB ensure that this is the case, thus saving the end users a considerable amount of time involved with the laborious, time consuming and sometimes costly process of producing their own cells from source.

In fulfilling its role, the UKSCB ensure each hESC bank meets predefined acceptance conditions for release criteria QC tests to ensure that the stem cells provided are safe and free of contamination from microorganisms or other cell lines.

Each cell bank is tested for viability, mycoplasma contamination, sterility, mandatory viral markers and identity by DNA profiling as a minimum. This is also in line with good cell practice guidelines (Coecke et al., 2009) and cell banking codes of practice (ISCBI, 2006). As a rule therefore, newly deposited hESC lines are maintained under quarantine conditions until the first post-thaw mycoplasma and sterility tests are completed to demonstrate that the lines are free of contamination. Following expansion to give a pre master and master cell bank, stem cell lines are held in an 'In-process' status, in a liquid nitrogen vessel, until the sterility, mandatory viral markers and DNA profile indicate that it is safe for the cells to be moved to the distribution vessel following review of necessary paperwork by quality assurance personnel. On completion and successful review of its cell line master file, which contains all the necessary production paperwork and data on quality and sterility testing, a certificate of analysis is issued, and the distribution cell bank stem cell line is made available for release. Such procedures ensure that the banks of cells produced are of high quality, and do not run the risk of compromising the reproducibility of cutting edge research. It has been estimated that 30% of cell lines reported in published work have been misidentified, cross contaminated with another cell line or contaminated with Mycoplasma (Capes-David et al., 2010; Uphoff et al., 1992).

Sterility testing is performed as soon as the cells are in culture and then subsequently every three to four weeks and before and after a bank is frozen down and thawed out again, to confirm that the cells are free of microbial contamination. This also demonstrates that the cells have been handled carefully with a good aseptic technique. Microbiological broths are used to screen for the presence of bacteria and moulds. Once inoculated, the broths are incubated and the appearance recorded after several days.

Mycoplasma testing is carried out as soon as the cells are in culture, and before each of the banks are frozen down. This ensures that the cell lines banked for distribution are free of contamination. Mycoplasmas are free living organisms that can detrimentally affect cell cultures and can spread quickly through a shared laboratory facility. Mycoplasma can also be extremely difficult to remove once cultures are contaminated, therefore consistent and sensitive testing methods is the best way of assuring that cultures remain mycoplasma free. Testing consists of both

PCR test against well-known strains of mycoplasma species and the mycoplasma culture test using selective agar plates, which are inoculated with cell culture sample and cultured for 6 weeks before being read.

Cell line identity testing by DNA profiling is used to ensure that the hESC banks have the same DNA profile as the original starting material. This demonstrates that no cross contamination or misidentification has occurred during the transfer, thawing or during the banking process. This test has historically been carried out externally by specialised laboratories such as The Doctors Laboratory (TDL) using Short tandem repeat analysis which uses a standard set of forensic markers. Viral PCR may also be performed to demonstrate that the cells banked are free from viruses that may cause harm in humans or have the potential to alter the genome of the host. The UKSCB employs pass/fail criteria; therefore stem cell lines which fail these tests will either be discarded, or quarantined for further investigation.

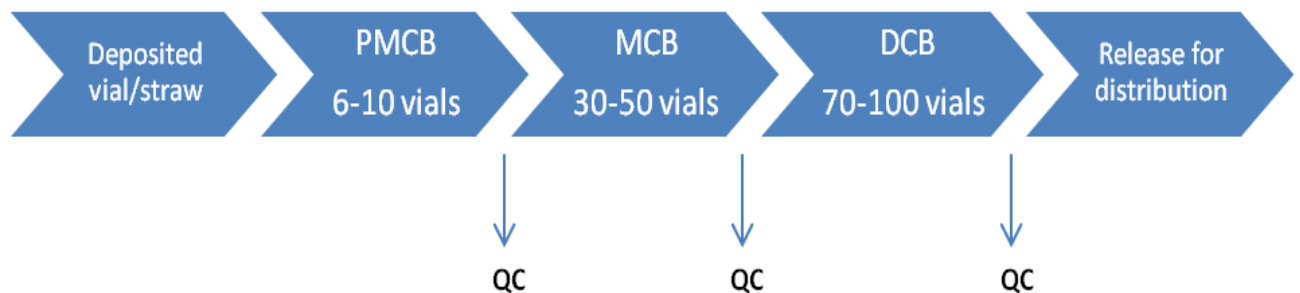


Figure 1.5. Diagram outlining the process of banking hESCs at the UKSCB. Samples are deposited as vials/straws and quarantined until suitable testing confirms that the line is free of contamination. The banking process consists of the production of three banks; Pre-Master Cell Bank (PMCB), Master Cell Bank (MCB), and Distribution Cell Bank (DCB). Between each bank stem cells are expanded, then frozen down by standard freezing methods. Cells are tested at each banked level to show that they are suitable for release however only the DCB is available for external/worldwide distribution.

Apart from providing banked hESCs, The UKSCB supports the wider stem cell community by giving advice on standardising methods for culture, characterisation and safety testing of hESCs and through participation in a number of collaborations and research programmes. For example the use of hESCs for toxicology screening (ESNATs European project) requires their ability to differentiate towards neuronal lineage (Pistollato et al., 2012). Previous well established collaborations such as the international Stem Cell Initiative (ISCI), immensely improved the characterisation of hESCs in 2007. As part of a 21 worldwide collaborative study, the UKSCB characterised 60 hESC lines using gene expression by Real Time PCR, cell surface antigen expression of pluripotency markers by flow cytometry, DNA profiling, tumorigenicity studies, imprinting and X chromosome inactivation to address epigenetic status and microbiology to examine viral and bacterial presence within feeder and hESC culture (Adewumi et al., 2007). This body of work was aimed at standardising methodologies used by scientists to characterise hESCs, as there is much variation in the type and techniques used to test stem cell lines for expression of pluripotency markers, chromosomal stability and ability to differentiate. The UKSCB also participated in ISCI 2, who published a study comparing eight culture systems and from this concluded that only two media, STEMPRO and MTeSR1 maintained consistent undifferentiated growth (Akopian et al., 2010).

The studies in this thesis are focused on developing and optimising culture conditions to standardise the culture and maintenance of hESCs for banking and for world-wide distributions. Although some of the techniques discussed below and in the project have already been established, most have not been optimised for stem cell culture in a way that supports culture of the different cell lines available. Moreover most protocols are have only been used for a limited number of stem cell lines and have not taken into account emerging lines and heterogeneity in these lines. Thus, to be able to standardise culture conditions the Bank embarked on developing and optimising various protocols required for culture including investigating the use of enzymes for improving the speed and scale up of passaging hESCs.

In addition, techniques not previously used in stem cell research have been adopted to improve the characterisation of hESC lines and include the use of newer molecular based techniques such as array comparative genomic hybridisation for

genetic stability studies and TissueFacs™ image analysis to quantitatively demonstrate the expression of cell surface markers. Such techniques have the added advantage of improving the sensitivity of testing, once optimised for use with stem cells.

1.11 The development of stem cell culture systems and methodologies

Embryonic stem cells are renowned for being difficult to culture long term and are extremely sensitive to environmental change including accumulation of excess toxins, CO₂ and O₂ levels, changes to media composition and dissociation methods. All these factors, if wrongly altered, have the potential to permanently induce unwanted differentiation. This has resulted in research into new culturing methods to better regulate these processes.

Stem cells are routinely cultured at 37°C, 5% CO₂, on inactivated murine embryonic fibroblasts (MEF) also known as mouse feeder layers. Studies have demonstrated that feeders enhance attachment by allowing stem cells to anchor themselves, and are important for growth, due to the secretion of multiple growth factors including FGFs, activin, Wnts, TGFβ and antagonists of BMP signalling (Unger et al., 2008; Yoon et al., 2010; Hongisto et al., 2011; Lee et al., 2012). Co-culture systems using feeder layers have been used extensively for isolating and growing other types of cell lines i.e. stroma cells for the culture of haematopoietic stem cells (Funk et al., 1995; Bramono et al., 2010).

Inactivated MEFs were originally used in the derivation of mouse embryonic stem cells (Evans & Kaufman, 1981), and for the derivation of the first hESC lines (Reubinoff et al., 2000; Zhang et al., 2006). Nonetheless, using MEFS, as with bovine serum, introduces animal derivatives into the culture system and can lead to possible contamination by mouse retroviruses. For instance, hESCs cultured on MEFs have been found to express immunogenic non-human sialic acid (Martin et al., 2005; Wang et al., 2011). Unfortunately such a finding compromises the ability for stem cells to act as therapeutic tools (Amit et al., 2004), as it may provoke an immune response and lessen the benefits of autologous transplant therapies (Wang et al., 2011; Padler-Karavani, 2011). It also raises the need for the derivation of clinical grade hESCs, free of animal product contamination.

In addition to using feeders, another progression in stem cell culture has been the development of suitable media which will maintain hESCs in the undifferentiated state and/or keep the cultures free of contaminating factors that could limit their potential exploitation. Originally stem cells were cultured in bovine serum albumin supplemented with glutamine, amino acids and growth factors. This still however requires the use of animal components which carry the risk of potential contamination. The use of human serum (Stojkovic et al., 2005), and subsequently serum-free media has been a considerable step forward towards establishing conditions that would limit contamination and aid towards the production of clinical grade hESCs (Inzunza et al., 2005; Skottman and Hovatta, 2006).

The function of mouse feeder cells has been documented and translated to the use of human feeder cells. The use of human feeders is well documented and many different sources of human feeders have been shown to support undifferentiated hESC growth. Human feeders derived from foetal muscle (Richards et al., 2002), foetal skin (Richards et al., 2003), adult fallopian tube epithelial cells, foreskin fibroblasts (Hovatta et al., 2003), adult marrow cells (Cheng et al., 2003) and adult endometrial cells (Lee et al., 2005) have all demonstrated the supportiveness of hESC growth and are easy to obtain and from a variety of human tissues and cell sources. Many laboratories have proved that their suitability to support stem cells is as proficient as mouse feeders (Eiselleova et al., 2008). They have also been shown to support iPSCs (Unger et al., 2009). The use of MEFs however, continues to be the gold standard method for culture.

1.11.1 Feeder free culture of hESCs

Derivation of stem cells on extra-cellular matrix (ECM) from human feeders is a well-documented method (Amit et al, 2004; Klimanskaya et al, 2005; Stojkovic et al., 2005; Escobedo-Lucea et al., 2012) and has been adopted from the use of ECM derived from mouse feeders. The use of ECM in cultures is not novel but has continually shown to be supportive. The usefulness of feeder free matrices such as Matrigel and Laminin have been documented (Xu et al., 2001) (Rodin et al., 2010). Although the use of Matrigel was not novel (Amit et al., 2003) it has been extremely successful and led to many other laboratories developing and testing new feeder free methods to support stem cell growth in order to move away from feeders. Matrigel (BD) matrix is a soluble basement membrane extract from Engelbreth-Holm-

Swarm mouse tumour that forms a basement membrane; a continuous sheet of specialised extra cellular matrix. It has been successfully used in a number of laboratories, has shown to maintain undifferentiated cells for prolonged culture periods and has demonstrated its use as a suitable matrix for the derivation of hESCs (Ludwig et al., 2006).

The work of Ludwig et al (2007) was one of the first studies to list the components used in defined media, mTeSR1. This led to the development of well-defined media and matrices which contain the essential growth factors and nutrients to sustain undifferentiated hESC proliferation. However, there are varying opinions of how well these matrixes can support stem cells for extended culture and whether their robustness is sufficient to support a variety of different hESC lines in different hands. Much recent research has been emphasised on investigating new support matrices for the growth of hESCs and many laboratories have reported the successful derivation of hESCs cell lines with conditioned media and on feeder free, synthetic matrices (Yoon et al., 2009; Klim et al., 2010; Hannoun et al., 2010). Furthermore, the use of synthetic matrices has been shown to aid differentiation studies (Tate et al., 2009). However these biomaterials and synthetic matrices have proved too expensive for most laboratories, and have slowed its development.

Despite all the progression of culture methods within the field, scientists still use mouse feeders as the gold standard for maintaining undifferentiated cultures of hESCs. This is most likely due to the method being well established and widely accepted in the majority of laboratories. Still, the use of feeders is not a simple process and requires time and effort to prepare, inactivate and test banks of feeders to ensure they are supportive. It can also be difficult to adjust stem cell lines onto new feeder batches as there can be much variation due to the type of mouse strain used, whether consistency is maintained when preparing banks, plating density etc. Opportunities to focus the needs of new research and revisit the use of human feeders, ECM and synthetic matrices such as Matrigel always arise. The need for xeno free grade hESCs for clinical applications has also encouraged the development of xeno free media and related culturing products, for the production of such cells (Ellerström et al., 2006; Unger et al., 2008; Hua et al., 2009). The progression of xeno free, defined media and matrices serve as valuable tools for

standardising the culture of hESCs to benefit both research and clinical application of these cells (Villa-Diaz et al., 2010).

As the requirement for xeno-free media increases, increased effort must be made to standardise alternative support matrices to successfully culture hESCs, without altering their gene expression (Stephenson et al., 2010). This is especially important to the UKSCB, as the move towards banking clinical grade stem cell lines approaches, it is important to find ways of substituting animal products for xeno free products but with a need to ensure that their function and quality is not compromised. Studies Such as the ISCI 2 (Akopian et al., 2010) are important for demonstrating the use of new culture systems, as a standardised example to encourage change within the stem cell community.

1.11.2 Passaging methods

The first established hESC lines were passaged using manual cutting procedures under a dissection microscope (Thomson et al., 1998; Reubinoff et al., 2001). This is the process of dissecting confluent colonies of hESCs and transferring them onto new feeders, to expand and give rise to new colonies, continuing their culture *in vitro*. Although these methods clearly demonstrated how hESCs could be maintained in an undifferentiated state for long culture periods, they are not suitable for the scale up and banking of large numbers of hESC lines as they are laborious, time consuming and extremely operator variable.

Literature has shown that researchers are keen to adapt enzymatic passaging methods in order to speed up culturing times and scale up cell culture production (Oh et al., 2005; Couture., 2010), as manual dissection is time-consuming and labour-intensive. hESCs must be cultured by methods which give rise to uniform, undifferentiated colonies in large quantities, to be beneficial for pre-clinical and clinical studies. In particular the research conducted by Ellerström et al (2007) demonstrated the ease with which hESCs could be expanded by single cell dissociation, and that their clonal survival was enhanced through culture on human foreskin fibroblasts in contrast to MEFs. It has however been argued that this type of passaging can induce differentiation and it can be difficult to recover cells to form colonies once made into single cells. Furthermore, the long term use of enzymes has

been shown to incur chromosomal instability (Brimble et al., 2004; Buzzard et al., 2004).

1.11.3 Expansion and Scale up

The expansion and scale up of hESC cultures in the form of gel beads (Phillips et al., 2008) and bioreactors on a small scale has been successful (Dang et al., 2004; Kehoe, 2010). Researchers have also investigated the suitability of scaling up stem cell cultures in suspension (Steiner et al., 2010). Also, the use of scaffolds for scale up has been successfully documented (Dellatore et al., 2008). Robotic automated systems have been successful in large scale up of hESCs but are expensive to obtain. However novel and interesting these methods are, they have not yet been applied in laboratories needing to maintain robust 'research scale' volumes of hESCs, therefore most scientists still employ the use of more simplified solutions, for the scale up of stem cell cultures, such as using enzymes.

Many commercial enzymes are available for the dissociation of cells and up until recently the most widely used enzyme for cell culture was Trypsin. This enzyme proved to be harsh and when used with embryonic stem cells induced karyotypic instability (Draper et al., 2004; Amit et al., 2003). Other commercial enzymes were developed including Collagenase 2 and 4, TrypLE select, Dispase, and more recently, Versene. TrypLE™ Express is a recombinant trypsin-like enzyme that is faster, more gentle on cells and boasts better clonal cell survival (Gray et al., 2009). Previously used enzymatic treatments including porcine Trypsin and Dispase have been known to damage cell membranes, decrease clonal survival and reduced attachment ability (Ellerstrom et al., 2007). The importance of using a recombinant Trypsin preparation has been stressed as more of hESC culture moves away from animal derived products (Ellerstrom et al., 2006). In order for newer enzymes such as TrypLE™ Express to be used routinely as part of the banking procedure it must be validated and confirm that there are no karyotypical changes associated with its long term use. It also needs to demonstrate that it doesn't change other fundamental characteristics of the hESCs. Although there is no well-established criteria for how many passages a single stem cell line should be cultured through before it is deemed 'unacceptable' for use, it is advised that hESCs go through minimal manipulation and passaging during the cell culture and banking process, as advised by the International Stem Cell Banking Initiative (ISCBI, 2009). Further advice has

been provided by documents such as Guidance on good cell culture practice (Coecke et al., 2005). The wide range of enzymes available have made it difficult to standardise the use of one or two within a robust culture system, as laboratories culturing hESCs tend to adhere to in-house methods which are well practiced, rather than test out new methods which is time consuming.

Important issues still remain to be addressed if hESCs are to be used to their full therapeutic potential. These include optimising protocols for efficient and reproducible scale-up and scale out, thereby producing purified populations of both undifferentiated and differentiated stem cells as well as developing robust differentiation protocols. Opportunities to focus the needs of new research and revisit the use of human feeders, ECM and synthetic matrices such as Matrigel always arise. As the requirement for xeno-free media increases, increased effort must be made to standardise alternative support matrices to successfully culture hESCs, without altering their gene expression (Stephenson et al., 2010). This is especially important to the UKSCB.

1.12 Characterisation testing of hESCs

Techniques for the characterisation of banked hESC lines are recommended by the International stem cell banking initiative ISCBI (2009). Currently there are over 700 hESC lines used in published studies (International Stem Cell Registry, ISCR). Consensus on techniques for characterising hESCs is becoming more important as the applications of hESCs move closer to the clinic. Although not part of release criteria testing, the UKSCB does characterise banked lines of hESCs by employing methods such as karyology, gene expression profiling by real time PCR, immunofluorescence by flow cytometry and *in situ* staining. The results are included in the cell line master files as information only, to potential customers requesting the lines. Information on banked hESC line characteristics is important to researchers as they give key information on the stability and genetic state of hESC lines when they are banked, which can have a significant impact on their research.

1.12.1 Karyology

Long term passaging, culture conditions and epigenetic changes can all contribute to karyotypic changes of hESCs, which can lead to the selection of certain populations of stem cells that have enhanced self-renewal over other populations. Particular populations within long term cultures gain copies of extra chromosomes, for example

chromosome 17 and 20q. These populations of stem cells continue to proliferate with an increased growth advantage over other populations (Draper et al., 2003), and are therefore selected and passaged on. Detection of these populations is crucial if we are to examine their stability in culture. The karyology of hESCs is tested by metaphase spread analysis and conventional chromosomal analysis in the form of G banding, to look for chromosomal deletions or additions, mosaicisms and balanced translocations at low resolutions. Karyology provides important information as cell lines with significant chromosomal aberrations may affect the reproducibility and reliability of experimental results and can reduce the potential for clinical application (Josephson et al, 2007). Although G banding has been the preferred method of choice by most researchers wanting to assess the chromosomal stability of their hESC lines, novel molecular methods have been developed, which are far more sensitive and can detect copy number variations (CNVs) in whole populations of cell cultures. Comparative genomic hybridisation has been shown to be useful in detecting a number of different known aberrations in hESCs (Lefort et al., 2008; Spits et al., 2008). Clinical applications of aCGH outlined by Shinawi and Cheung (2008) describe how routine karyotype analysis is not sensitive enough to detect subtle chromosomal rearrangements of less than 4MB. However aCGH has been a sensitive and useful tool in the detailed detection of chromosomal aneuploidies and structural aberrations which are an underlying cause of congenital anomalies, dysmorphism, autism, miscarriages and several other genetic syndromes (Shaffer et al., 2007; Bejjani & Shaffer, 2006).

1.12.2 Flow cytometry

Another technique used to characterise hESCs is flow cytometry analysis. Flow cytometric techniques are used to quantify and separate sub populations of cells within complex mixed cell samples. The technique works by using the basic properties of each cell type; its ability to absorb fluorescence and scatter light, based on reaction with specific cell surface markers. This information is translated into forward scatter, side scatter, which is captured and transmitted to a screen where it can be analysed. Human embryonic stem cells characteristically express a well-known panel of cell surface markers for pluripotency. The most frequently used are stage specific embryonic antigens SSEA-1, SSEA-3, SSEA-4 (Draper et al., 2002). Another group of commonly used cell surface antigens include high molecular mass

glycoproteins TRA1-60, TRA1-81 (Schopperle and DeWolf., 2007). These antigens are shared with mouse ESCs, but the expression of SSEA-1 and SSEA-4 is reversed. *In situ* immunofluorescent staining provides a qualitative method of assessing undifferentiated hESC growth, by staining for the cell surface markers outlined above, and for intracellular transcription markers OCT4 and Nanog.

1.12.3 Gene expression

The regulation of gene expression for pluripotency and some early differentiation markers are important for establishing gene profiles, which can help to predict their pluripotent capacity as well as demonstrating expression of key stemness genes (Abeyta et al., 2004; Liu et al., 2006). Gene expression can be measured using traditional Reverse transcriptase PCR or quantitative PCR (qPCR). Although many laboratories use PCR for the detection of stemness genes, the type of PCR used tends to be qualitative and therefore only demonstrates the presence or absence of a small selection of genes. More recently real time PCR has demonstrated to be a more accurate and sensitive method, particularly when relative quantification is performed, with reference to endogenous controls samples and using stable housekeeping genes (Derveaux et al., 2010; Veasey et al., 2011). The application of real time PCR has led to the development of Taqman low density arrays, which work as customisable 384-well microfluidic cards. This enables hundreds of real time PCR reactions to occur simultaneously and can accommodate 1-8 samples to be run in parallel. LDA card technology utilises novel gene signature arrays designed especially to detect 16 housekeeping genes specific to hESCs, using endogenous control selection. Ensuring hESCs maintain a relatively stable genetic profile is of importance particularly at late passage, as this property demonstrates their applicability in a therapeutic environment. The studies in this thesis will also aim to investigate this particular question using TLDA cards, to monitor whether there is a difference in genetic stability over long term passaging and possibly reveal which genes (stemness or germ lineage specific) are most affected.

1.12.4 Differentiation studies

In order to fully assess the pluripotency of hESCs they must be able to differentiate. Although the majority of lines will do so spontaneously in culture, their therapeutic potential can only be reached by the application of robust, efficient and controlled differentiation protocols. There are many different methods to directly differentiate

hESCs and most have the aim to mimic signalling pathways *in vivo*. However their efficiency to produce pure populations of differentiated cells are extremely variable. Figure 1.6 demonstrates the process of gastrulation and subsequent formation of all three germ layers.

Neuroectoderm differentiation

The successful differentiation of hESCs to neural lineage is an important step towards the use of hESCs for treatment of neurological diseases (Reubinoff et al., 2001). The commitment of cells to one of the three germ layers marks the second major step towards embryonic development. As multipotent cells begin to organise themselves, some cells undergo neurulation. This is the development of the neural plate for the formation of nervous system, which sequentially leads to the formation of the neural tube. Neural differentiation commences with the formation of the notochord from mesodermal cells can be identified by gene expression of the regulatory protein brachyury. Neural progenitors can be characterised by the expression of PAX6, frequently seen in neuroectoderm differentiation, FOXG1 and OXT2.

Mesoendoderm

Endoderm and mesoderm differentiation is induced by nodal signalling by TGF- β molecules. Higher nodal levels specify endoderm and lower levels specify mesoderm. Although no source of nodal protein is known, Activin A from the TGF- β family binds to the same receptors, triggering intracellular events which, in the presence of low serum and high Wnt3a or BMP4 (bone morphogenetic protein 4) (D'Amour et al., 2006), which is known to play a pivotal role in formation of primitive streak, endoderm and mesoderm, lead to the induction of endoderm formation. Endoderm differentiation can be seen after 5 days and demonstrated by the expression of definitive endoderm marker SOX17 and FOXA2 (McLean et al., 2007) and alpha fetoprotein (AFP), known for early stage specification to hepatic lineage. Early mesoderm/mesoendoderm can be detected by the presence of GSC gene encoding the homeobox protein goosecoid, Brachyury and PTF1a, a gene encoding for pancreas specific transcription factor.

Differentiation to mesoderm and, in particular, cardiomyocytes is one of the most dramatic and difficult transformations. Although many laboratories report spontaneous differentiation and formation of visible beating cardiomyocytes, robust

protocols that report high yield are far and few between (max 20-30%). Recently research has focused on the directed differentiation of hESCs to hematopoietic-mesoderm lineage specification (Cerdan et al., 2012).

Recent publications have enabled faster and far more reliable differentiation methods, due to a varied choice of starting material in the form of embryoid bodies (Iskovitz-Eldor et al., 2000) or adherent cultures using conditioned media (D'Amour et al., 2006), small molecules and growth factors coupled with specific matrices including Poly-D lysine for Neural differentiation (Reubinoff et al., 2001), co-culture of hESCs with visceral endoderm like cells for cardiomyocyte differentiation (Mummery et al., 2003) and Matrigel (Ludwig et al., 2006) designed to enhance lineage specific cell production in purer populations in under a month. However each protocol recommends optimisation of their method when using different stem cell lines, which is costly, time consuming and ultimately such variability in protocols will limit stem cell fields' progression with regards to demonstrating the production of progenitors or terminally differentiated cell types. Also, the methods used to confirm differentiation have not been standardised, with many labs self-selecting one or two genes and cell surface markers to confirm differentiation towards a particular germ layer.

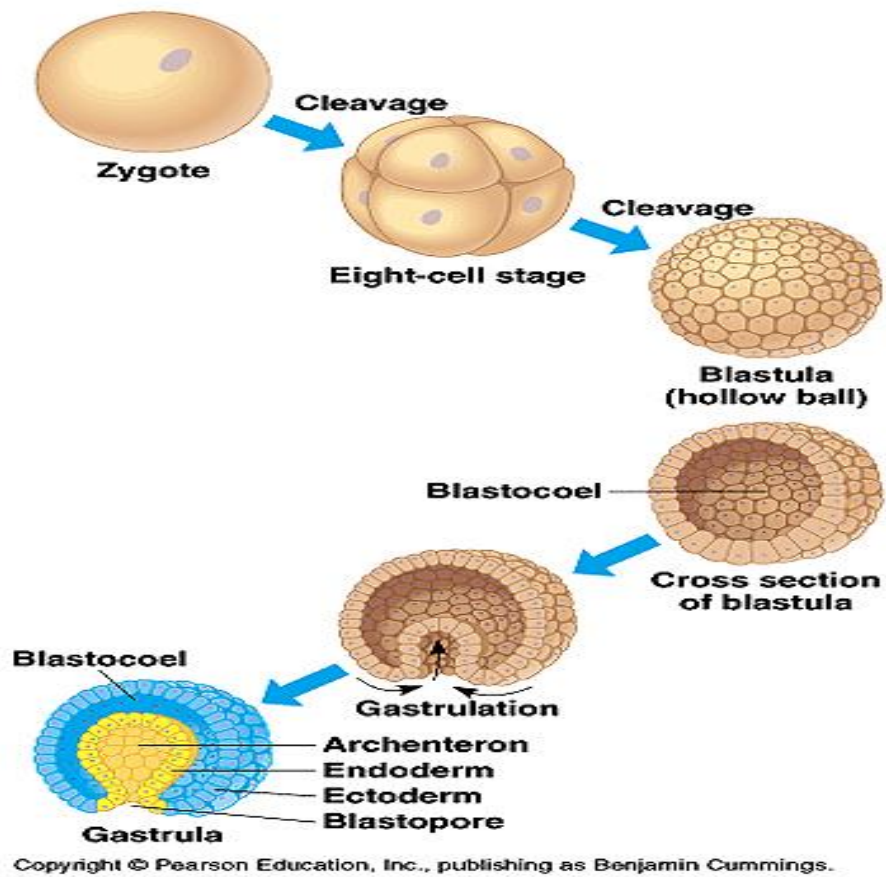


Figure 1.6. Diagram showing process of gastrulation and subsequent formation of the three germ layers. Image obtained from http://www.bio.miami.edu/dana/106/106F05_4.html.

1.13 Stem cells: Research to clinical use

In order to support the potential that hESCs hold for regenerative medicine, standard laboratory methods for cell culture passaging and cryopreservation have to be developed and need to be reproducible and robust. The need to develop robust culturing methods using well defined components is of paramount importance. Clinical applications require 10×10^9 cells in order to begin scale up. Uniform, qualified and well tested banks of hESCs need to be produced under GMP which can be easily reproduced and cultured rapidly without compromising the end use/product. hESCs have the potential to revolutionize medicine and healthcare. Research has begun into the applications of iPSCs for the production of mature HSCs to help with the growing number of blood transplants (Migliaccio et al., 2012; Wagner et al., 2013). However such applications will require significantly large numbers of robustly specialised and functional HSCs to fully fulfil this goal.

Techniques used to characterise human embryonic stem cells are abundant and well documented however, none of these methods have been assured for the use of clinical Stem cell banking applications. Much work is needed to validate and standardise these techniques as the field rapidly progresses towards clinical applications. Such techniques will be of importance to demonstrating the progression of hESCs as they are differentiated into lineage specific cells for transplantation into diseased and damaged organs such as cardiomyocytes (Zwi-Dantsis et al., 2013) and for the replacement of skin grafts derived from patient specific iPSCs using bioscaffolds (Bi & Jin, 2013).

Many researchers have demonstrated their ability to maintain the undifferentiated growth of hESCs, but with huge variations in culture methods. It is important that these methods are standardised as well as simple, robust and reproducible. Characterisation testing must demonstrate that these hESCs still exhibit the key characteristics of undifferentiated human embryonic stem cells. Furthermore the same stem cell lines are continually characterised, which although demonstrates their ease of culture, can be seen as biased as these lines are clearly easy to grow and manipulate. It does not add to the diversity of hESCs available for use which may precede the benefits of hESCs currently trending use. The approaches and developments described should help the UKSCB to achieve its objectives and meet its commitments to the wider stem cell community.

1.14 Aim of thesis

The aim of this thesis is to conduct a comparative study of different mouse and human feeders and feeder-free Matrigel (together with mTeSR1) in supporting long term culture of hESCs in the undifferentiated state, and to further explore the chromosomal stability of cultures under these conditions. Studies on the different matrices will be coupled with the use of TrypLE™ Express to assess its suitability of the latter as a clump passaging method that would be better tolerated by cells and thus enable uniform scale up of hESC cultures. Matrices will also be compared to demonstrate their ability to support the differentiation of hESCs to progenitor cells by *in vitro* methodologies, as a measure of their pluripotency following long term passage. Furthermore, the use of TissueFacs™ for image analysis of cell surface markers and aCGH for assessing chromosomal stability of stem cells in routine culture will also be validated. This is considered important since these methods have not been qualified for use in the host laboratory and currently the field of stem cell research requires robust and sensitive methodologies for routine characterisation, particularly for the prolonged culture of cells. Chromosomal stability, gene expression, detection of cell surface pluripotency markers will be determined using aCGH, real-time PCR, immunofluorescence (IF) using flow cytometry and *in situ* staining, respectively.

Chapter 2

Materials and Methods

Materials

The tables below show details of the materials used for the culture of cell lines including list of feeder line and stem cell lines used in this project. The hESC lines HUES9 (Harvard, USA), RH5 (University of Edinburgh), SHEF1 (Pfizer, originally from Sheffield University) and NCL5 (University of Newcastle) were all obtained through deposit agreements within the UK Stem cell Bank.

Table 2.1 Details of feeder lines and fibroblasts used to grow hESCs

Name	Organism	Origin	
MEF	MF1 mouse	Embryo	Fibroblast
3T3-J3	Swiss Albino mouse	Embryo	Mouse cell line
HDFn	human	Neonatal	fibroblast
MRC-5	human	Fetal lung	Cell line
11235	human	Fetal skin	fibroblast
HUVEC _{JR2}	human	Umbilical cord vein	Cell line and ECM epithelial cells
MRC-5 _{JR}	human	Fetal lung	ECM

Table 2.2. Details of Stem cell lines used in this project

Stem cell line	Cell type	Depositor	Country
HUES-9 p24	Human embryonic	Harvard	USA
HUES-3 p22	Human embryonic	Harvard	USA
SHEF-1 p27	Human embryonic	Pfizer	UK
RH5 p35	Human embryonic	University of Edinburgh	UK
NCL-5 p27	Human embryonic	Newcastle upon Tyne	UK
NCL-2 p22	Human embryonic	Newcastle upon Tyne	UK
H9 p26	Human embryonic	WiCell	USA

Table 2.3. Details of media used in this project

Knockout hESC media	Components	Volume	Final concentration	Cat number	Supplier
	Knockout serum replacement	100ml	20%		Invitrogen
	Knockout DMEM	388ml	77%	10829-018	Invitrogen
	Non-essential amino acids (NEAA) 100x	5ml	1%	11140-035	Invitrogen
	Glutamax 100x	5ml	1%	35050-038	Invitrogen
	bFGF	1ml	0.1%		Invitrogen
	Betamercaptoethanol (BME)	1ml	0.1%	313350-010	Invitrogen
	Total volume		500ml		
MEF media	DMEM	445ml	89%	D5456	Sigma
	Foetal Calf Serum	50ml	10%		Biosera
	Glutamax	5ml	1%	35050-038	Invitrogen
	Total volume		500ml		
mTeSR1 Media	Stem cell technologies Basal media	400ml	80%	05850/05896	Stem Cell Technologies
	5x supplement	100ml	20%	05850	Stem Cell Technologies
	Total volume		500ml		
	DMEM/F12	500mL		36254	Stem Cell Technologies
Matrix	Matrigel	Made up to 342 µl per 14 vials		354277	BD Biosciences

Table 2.4. Enzymes used for subculture

Name of enzyme	Supplier	Cat number	Derivative	System used with
TrypLE™ Express (25mM)	Invitrogen	12604-013	recombinant	Stem cell subculture
Trypsin (0.25mM)	Invitrogen		Porcine	Original feeder bank preparation
Dispase in F12	BD Biosciences	07923		Matrigel/mTeSR1

Table 2.5. Reagents used for inactivation of feeder cells

Reagent	Supplier	Cat number	Used for	Concentration/dilution
Mitomycin C	Sigma-Aldrich	50-07-7	Inactivation of feeders	2mg/ml
DMSO	Fisher Bioreagents	67-68-5	Component of freezing media	10% of final solution
FCS	Biosera	5170G	Component of freezing media	90% of final solution
Cryovials				N/A
T175 filtered flasks	Falcon		Culturing feeders	N/A
Mr frostie HandiFreeze supplemented with Isopropanol	Fisher Scientific		Slow freezing of cells	100% Isopropanol

Table 2.6. Reagents and supplies used for PCR

Reagent	Supplier	Cat number	Used for	Concentration/dilution
1.5ml sterile v bottom tubes with screw cap lids	Starsted		Storing DNA/RNA pellets at -80°C	N/A
PCR tubes	Life Technologies		Performing PCR reactions	N/A
RNAse free H ₂ O	Invitrogen		Dilution of RNA/DNA and Mastermix component	N/A
Ethanol	Fisher Scientific		RNA extraction	70% solution made up with distilled H ₂ O
TBE	NIBSC, SSS	Made in house	Buffer for running gel	
Agarose			Gel electrophoresis	
Syber Safe DNA stain			DNA stain	
BME/RT buffer solution	UKSCB, SK	n/a	Used to store RNA pellets at -80°C	10ul BME into 1ml RT buffer solution.
cDNA kit	Applied Biosystems		Components used to make cDNA	As instructed see method
Gene expression Mastermix for RT PCR	Applied Biosystems		Used for PCR reaction	2x concentration

Table 2.7. Reagents and supplies used for immunofluorescence staining and flow cytometry

Reagent	Supplier	Used for	Concentration/ dilution
Distilled H ₂ O	NIBSC, SSS	Plate washing	
Washbuffer solution	NIBSC, SSS	Plate washing/diluting antibodies	0.1% PBS, sodium azide and 0.1%BSA
PBS	NIBSC, SSS	Plate washing	
96 well flat bottomed plate	Falcon	Processing stem cells for flow cytometry	N/A
96 well v bottomed plate	Falcon	Preparation of stem cells for flow cytometry	N/A
Flat bottomed 24 well plate	Falcon	Culturing stem cells for in situ fluorescence staining	N/A
Paraformaldehyde (PFA)	Fisher	fixative	16% (10ml vial) diluted to 4% final solution in PBS
Triton X	Invitrogen	Permeabilisation of stem cells	1/100 dilution in PBS
Acetone/ methanol solution	Fisher Scientific	Permeabilisation of stem cells	1:1 final concentration

Table 2.8a. Materials used for aCGH preparation of samples and slides.

Reagent	Lot number	Supplier
H ₂ O	559788	Perkin Elmer
0.5M EDTA	0711004	Ambion
0.5M NaCl	0710004	Ambion
Primers	554056	Perkin Elmer
Klenow	549120	Perkin Elmer
Cye 5 dye	549395	Perkin Elmer
Cye 3 dye	549396	Perkin Elmer
70% ethanol	531893	Sigma
Isopropanol	559787	Sigma

Table 2.8b. Washbuffer solutions for washing slides

Reagent	Prewash (100 ml)	Washbuffer 1 (250 ml)	Washbuffer 2 (250 ml)	Washbuffer 3 (350 ml)
SSC (20x)	10	17.5	1.25	1.75
SDS (10x)	1	3.5	2.5	
dH ₂ O	89	329	246	348

2.1 Culture method overview

Traditionally, hESCs are cultured directly onto a layer of inactivated MEF and passaged by manual dissection under a microscope. An alternative mouse feeder line 3T3 was also used in these studies. The mouse cell line 3T3 was established by Todaro and Green in 1963 from disaggregated Swiss mouse embryos. It has already been shown to support growth of stem cell lines as well as being used in clinical applications for skin transplantation (Dadheech et al., 2013; Petek et al., 2010; Supp et al., 2000).

Other culture methods have been explored, including the use of inactivated human fibroblasts, human cell lines, MRC-5 derived from foetal lung tissue and used in vaccine production and HDFn, human dermal fibroblasts, which have also shown to support undifferentiated hESC growth. Furthermore the use of enzymes to passage hESCs as a faster method for scale up has been explored.

The methods used in these studies include the preparation and culture of hESCs by enzyme using TrypLE™ Express to dissociate hESCs into single cells or small clumps on human and mouse feeders TrypLE™ Express (25mM) is a xeno-free, gentler version of Trypsin, and is thought not to lead to genetic changes of cells during extended culture use. Also, the use of Matrigel (BD Biosciences) and mTeSR1 (Stem Cell Technologies) with Dispase (Stem Cell Technologies); a commercially available enzyme were tested. The use of extra cellular matrix (ECM) derived from human cell lines to support the undifferentiated growth of hESCs was also investigated and its preparation from human feeders is described below.

2.2.1 Preparation of primary mouse feeder cells and mouse fibroblast cell lines

Mouse embryonic fibroblasts (MEFs) were obtained from pregnant MF1 mice (Harlan) at 13.5 days using a standard preparation technique employed by UKSCB scientific staff. The Murine embryonic fibroblast line 3T3-J3 was originally obtained from CellTran, Sheffield (UKSCB, Acc. No. R-05-004). See Table 2.1.

Using a Class II safety cabinet (Labconco purifier), one vial of frozen MEF p0 was thawed, centrifuged for 5 minutes at 1200rpm (Sorvall Legend RT) and resuspended into a T75cm flask in MEF media consisting of DMEM (Invitrogen D6546), 1% Glutamax (Gibco, ref:35050-38) and 10% FCS. Cells were incubated at 37°C, 95% O₂ 5% CO₂ (Heraeus, HeraCell 240) until culture was at 70-80% confluency.

Flasks were then passaged/expanded enzymatically at 1:6 ratio. Media was removed from flasks, cells were gently washed with PBS, replaced with 10 mLs of TrypLE™ Express (Gibco, ref: 12604-013) and incubated at 37°C for 3-5 minutes until a few cells were starting to detach and appear bright under a phase contrast microscope. TrypLE™ Express was gently removed and cells resuspended in fresh media. Flasks were tapped to dislodge cells which were gently pipetted and transferred into clean T175 flasks containing fresh MEF media (30-50 mLs). Cells were passaged every 4 to 5 days in T175 flasks until enough cultures were obtained to make a large enough bank (70-100 vials).

2.2.2 Preparation of primary human feeder cells and human fibroblast cell lines

Human dermal fibroblast cells (neonatal) HDFn (cat. no. C-004-5C) and human fetal lung fibroblast line MRC-5 were obtained from Cascade Biologics® and NIBSC (Acc. No. 660902). The cells were thawed and subcultured as above.

2.2.3 Inactivation of fibroblasts using Mitomycin C and Cryopreservation

Fibroblast cell cultures were inactivated using Mitomycin C treatment for 2-4 hours to arrest their growth and provide a support matrix for the hESCs to attach and grow. Mitomycin C is a compound isolated from *Streptomyces Caespitosis* and is an alkylating agent that targets guanine nucleosides and produces oxygen free radicals that are preferentially toxic to hypoxic cells. It works by inhibiting DNA synthesis and nuclear division.

Mitomycin C at a concentration of 2 mg/ml was dissolved using 20-50 ml MEF media (DMEM, 10% FCS and 1% Glutamax), then filtered using a 0.2um filter and a 20ml syringe. The filtered solution was then diluted in 200ml of MEF media, which was equally distributed between the T175 flasks contained 70-80% confluent feeders and incubated for 2-4 hours at 37°C, 5% CO₂. Following incubation, the media containing mitomycin C solution was poured off each T175 flask and the feeder cultures within the flasks were washed three times using phosphate buffered saline (PBS, NIBSC). Each flask was then treated with 10ml of TrypLE™ Express (25 Mm, Invitrogen) and incubated for 5 minutes to allow the cells to dissociate. The TrypLE™ Express was then removed carefully by pipetting and 10 ml MEF media was added to each flask. The cells were dislodged by tapping sharply on each side of the flask. The flasks were swirled and the cell solution pipetted carefully into 50ml conical

tubes (BD biosciences). The cells were then centrifuged (Sorvall Legend) at 300g for 5 minutes. The supernatant was discarded and the cell pellet re-suspended in 10 ml MEF media and pipetting up and down several times. A cell count was performed using Trypan Blue (Invitrogen, 0.4% solution, T8154) and a disposable haemocytometer (C-Chip). The cells were centrifuged again and the pellet, re-suspended in FCS containing 10% DMSO at final concentration of 1×10^6 cells/ml. These cells were then pipetted into labelled cryovials (1ml per vial). Each vial was frozen slowly at -1°C per minute using a Mr Frostie HandiFreeze at -80°C freezer, overnight. The vials were then transferred to liquid nitrogen (-196°C) for long term storage.

2.2.4 Examination of feeders for confluency

A vial of each feeder line was thawed out quickly and resuspended into 1 ml of MEF media, then diluted into 10 ml of MEF media in a 15 ml conical tube (BD, Falcon) and centrifuged for 5 minutes at 300g to remove the cryopreservant. The supernatant was carefully removed and the cell pellet was resuspended in fresh MEF media. 2 ml of media was added to each well of a 6 well plate (BD, Falcon), previously coated with 0.1 % gelatin for a minimum of 1 hour at room temperature (Porcine, NIBSC). The plate was gently swirled to ensure even distribution of feeder cells across each well.

After 24 hour incubation at 37°C each feeder plate was examined and photos taken using a phase contrast microscope at x4 objective (Olympus CKX41), to ensure the feeder layer density was confluent enough for the growth of all the stem cell lines (at least 80 % of the well surface covered with feeders).

2.2.5 Preparation of feeders from Fibroblasts

Vials of inactivated feeder cells were thawed by fast thawing at 37°C . Cells were pipetted out of their cryovials and placed in 7 mls of pre-warmed MEF media. Each vial was then washed with 1-2 ml MEF media and then centrifuged at 300g for 5 mins to remove the cryopreservation media. The supernatant was carefully removed by pipetting, and the feeder cells re-suspended into 2 mls of fresh MEF media and transferred to pre gelatin coated FB6. Plates were gently swirled to evenly distribute cells, and placed in an incubator to settle for 24 hours before observation. After 24 hours, the confluency was assessed and if suitable for seeding with hESCs, MEF

media was replaced by KO-HES (see Table 2.3 for composition) media consisting of Knockout serum replacement, Nonessential amino acids (NEAA), Beta-mecaptoethanol (BME), DMEM and Foetal Calf Serum (FCS), and incubated for a minimum of 1 hour to equilibrate the media and condition the MEFs, prior to the addition of HESCs.

2.3. Thawing of hESCs

Each stem cell line was thawed from cryovials by fast thawing at 37°C. Cells were gently mixed and pipetted from their cryovials into 7 mls pre-warmed KO-HES media. Each vial was then washed using 1-2 ml KO-HES media and then centrifuged at 200g for 5 mins to remove the cryopreservant. The supernatant was carefully removed by pipetting, and the stem cells re-suspended into 2 mls of fresh KO-HES media and transferred into 1-2 wells/FB6 of prepared inactivated feeder layers. Stem cells were left to settle for 3-4 days before observation and partial media change.

2.3.1 Routine culture of hESCs

Culture media was changed every other day (2mls per well). Once the stem cell line had successfully thawed and re-established, the colonies were manually passaged by cutting, using disposable mini Pasteur pipette under a dissection microscope (Olympus) and transferred onto fresh inactivated feeders once a week for 1-2 passages.

All four hESC lines were adapted to enzymatic passaging using TrypLE™ Express (GIBCO), and were subcultured every 5-7 days. Morphology was assessed when media was changed and when passaging. Images were taken to document adaptation onto different feeders using TrypLE™ Express. As each stem cell line was received at a different passage number by the depositors of derivation laboratories, stem cell cultures were collected at time points p+5 for early passage, and p+20 for late passage.

2.3.2 Manual passaging of hESCs

Fresh Hes media was used to replace old media using an aspirating pipette or 10 ml pipette, at least 1 hour before passaging. At the point where the stem cell colonies were deemed 'confluent' and a sufficient number of colonies were confluent, the cells were placed under a dissection microscope (Vision Lynx, 029986, ISIS Ergohead) for manual passaging. A sterile 'hook' or thin Pasteur pipette tip was used to 'cut' around the edges and across the stem cell colony (Figure 2.1). The colony pieces were then gently lifted at the edges and gently pipetted so they could float up into the media. A 20 µl pipette tip was used to quickly collect the colony pieces, which were transferred to a plate of fresh feeders which had been incubated with Hes media for a minimum of 1 hour. The colony pieces were carefully pipetted onto a well of the 6 well plate, then evenly distributed across the well. The whole process was repeated again for a number of colonies until sufficient amount of material had been dissected and transferred to new feeders.

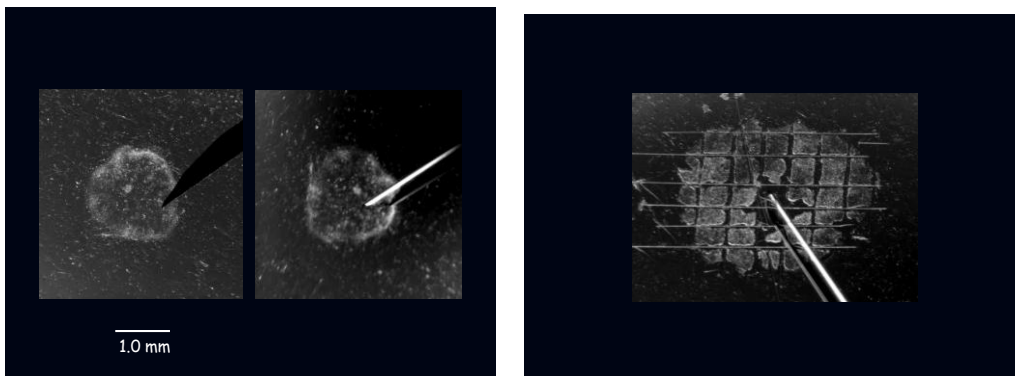


Figure 2.1. Diagram showing manual passaging of hESCs using mini Pasteur pipette.

2.3.3 Enzymatic passaging

After about a week of growth confluent stem cell colonies were passaged. Media from the 6 well plate of stem cells was removed and replaced with 1 ml per well of TrypLE™ Express, and left for 3-4 minutes at room temperature. The TrypLE™ Express was then carefully removed and 2 ml of HES media added to the well. The cells were gently dissociated to small clumps using a 1 ml pipette tip and transferred to a fresh 6 well plate containing inactivated mouse (i3T3 and iMEF) or human feeders (iMRC5 and iHDFn). The plate was gently swirled to evenly distribute the

stem cells, then carefully placed at 37°C, 5% CO₂ until the media required replacing (on average every 2 days). Stem cells were passaged for 20 passages (p+20).

2.3.4 Cryopreservation of human embryonic stem cell lines

2 to 3 vials of each stem cell line cultured on each feeder line or on Matrigel, were frozen down at early (p+5) and later passage (p+15 to p+17) in order to provide a small stock to repeat culture experiments at a later date. 1 or 2 confluent wells (70-80%) of each stem cell line were TrypLE™ Express treated for 3-4 minutes to allow dissociation to small clumps, then 1ml freezing media consisting of 10% DMSO (Fisher Scientific) and 90% foetal calf serum (FCS) added. The hESCs were pipetted to resuspend cultures, then immediately transferred to cryovials, placed in a Mr Frostie Handifreeze (submerged in isopropanol) and slow-frozen at -80°C. After 24 hours, cryovials were transferred into liquid nitrogen vessels for long term storage.

2.3.5 Culture of hESCs using human fibroblasts 11235.

Human fetal skin tissue was used to prepare banks of human feeders 11235, which were mitotically inactivated using Mitomycin c, at various passages and then plated onto 6 well plates. Stem cell lines NCL2, SHEF1, H9 and NCL5 were then cultured on i11235 for 5 passages and their morphology observed.

2.4.0 Feeder free culture of hESCs

2.4.1 Culture of hESCs by Extracellular matrix (ECM) derived from human cell lines

Human feeder cell line MRC-5, at various passages (p9 and p11), were cultured using commercial medium supplemented with 2 % and 10 % FBS respectively, in 6-well plates and 25 cm² flasks to 100 % confluency. To remove the cells, the cell layer was rinsed with PBS, then incubated in H₂O, agitated vigorously and further rinsed with H₂O. Plates/flasks were stored immediately at -70° C or air-dried in an MSC and stored at room temperature. Additional preparation conditions tested included incubation in 2M NaCl prior to, or 70 % ethanol after the final rinse with H₂O.

hESCs lines SHEF1, NCL2, HUES3, H9, RH5, NCL5, TRANS1, MEL1 and an IPS line were all cultured on ECM preparations, by transferring hESCs cultured on mouse feeders using manual passaging, and assessed for expression of pluripotency markers, morphology, karyology, embryoid body formation and

expression of differentiation markers.

2.4.2 Culture of hESCs using Matrigel (BD).

Human embryonic stem cell lines HUES9, SHEF1, NCL5 and RH5 were cultured on Matrigel coated plates using mTeSR1 (Stem Cell sciences). Matrigel was prepared following manufacturer's instructions. 300 µl aliquots of Matrigel was carefully mixed into 24 mls of DMEM/F12 media, then equally distributed amongst 4 flat bottomed 6 well plates and left to coat for 1 hour at room temperature (minimum). Plates that were not used immediately were wrapped in parafilm and stored at 4°C for up to 2 weeks. After 1 hour the Matrigel coating was removed and wells were washed in 1-2 mls of DMEM/F12. mTeSR1 media was made up by adding the x5 supplement and mTeSR1 basal media together and mixing well. 2 ml of mTeSR1 media was transferred into each well of FB6, and plates were then ready to use immediately.

Stem cells were transferred from feeder cultures onto Matrigel/mTeSR1 and allowed 1-2 passages to adjust to the matrix/media before being counted as (n+1) from their original passage, for the study. Media changes were performed on cultures every 2 days and passaged every 5 to 7 days. Stem cells were cultured for 20 passages. Morphology was assessed at each passage. Cell pellets for gene expression and flow cytometry were obtained from early p+5 and late passage p+20. Microbiological monitoring and mycoplasma testing was also routinely carried out on cultures as described below.

2.5.0 Release criteria testing

2.5.1 Sterility and mycoplasma testing

The stem cell cultures were all routinely tested to ensure sterility was maintained using a European pharmacopeia method. 0.5-1 ml of cell suspension to inoculate agar broths Tryptone soya broth (TSB) and Fluid thioglycollae medium (FTM) at 37°C and Sabourauds liquid medium (SAB) at 25°C. TSB is used to detect a broad range of microorganisms, including Bacilli, Staph, Strep, E. coli, Pseudomonas, Clostridia and non-sporing anaerobes. FTM is used to detect a range of microorganisms, including Bacilli, Staph, Pseudomonas & Clostridia. SAB detects fungal species. Testing was completed by NIBSC Microbiology department and assessment carried out at 14 days to check for turbidity/contaminants before the results were given back to UKSCB staff.

2.5.2 Mycoplasma screening by direct PCR

Samples were also tested to ensure that the cultures were negative for mycoplasma contamination. This test was completed in-house by PCR. Reference numbers were assigned to the negative control sample (H₂O), followed by consecutive numbers to each test sample and the positive control, which consisted of a sample of mycoplasma-contaminated cell line or a standard mycoplasma organism e.g. *Acholeplasma laidlawii*, which must produce a positive result i.e. a clear band visible on an agarose gel. A number of PCR tubes were labelled with sample reference numbers and the PCR master mix prepared as outlined in the table below. Then 39 µl of master mix was added to each tube/well and the samples were centrifuged at 200g for 3 minutes. 1 µl of supernatant was added to the appropriate tube/well and the contents mixed well. The tubes were then placed in the Bio-Rad DNA Engine Peltier Thermal cycler and the programme set to run as follows:

- 95 °C for 5 min.
 - 94 °C for 30 sec.
 - 57 °C for 30 sec
 - 72 °C for 1 min.
 - 72 °C for 10 min.
- } 40 cycles

A 2% gel was made up by dissolving 3g of ultra-pure agarose in 150 ml of 1x TBE made from a 10x TBE stock. The agarose was dissolved by heating in a microwave on full power for 2 minutes. This was allowed to cool slightly before adding 15 µl of SYBR Safe DNA gel stain and mixing well. The mixture was then poured into the gel preparation tank. A size 30 well comb was placed into the gel mix and the gel allowed to set for approximately 30 minutes. After the gel had set the comb was carefully removed and the gel placed in the electrophoresis tank. 1x TBE was poured into the tank to cover the gel. 4 µl of 100bp DNA ladder was pipetted into the first well and 18 µl of each test sample and positive controls into the appropriate wells. The gel was then run at 100V for approximately 60 to 80 minutes, after which it was photographed using the UV illuminator and camera.

Table 2.9. Details of PCR Mastermix

PCR MASTER MIX	For 1 reaction	Master Mix (e.g. for 10 reactions)
HotStarTaq <i>Plus</i> Master Mix, 2x	20 µl	200 µl
MGSO primer (10 µM)	1 µl	10 µl
GPO-3 primer (10 µM)	1 µl	10 µl
CoralLoad Concentrate, 10x	4 µl	40 µl
DNase-free water	13 µl	130 µl
Total	39 µl	390 µl

2.5.3 Collection of stem cell pellets for DNA profiles.

DNA profiling, also known as DNA fingerprinting, is a method of distinguishing one cell type from another by PCR. Although 99.9% of human DNA is the same in everyone, 0.1% is variable and easily detected using PCR. The most widely used PCR based method is STR. It is a comparable test which matches the base pairs of a sample of DNA and can be used to distinguish one sample from another. The technique works by using short tandem repeat regions of sequences which are repeated side by side, usually between 2-6 base pairs. It is very similar in closely related humans, but different enough that it can be used to distinguish between individuals of no relation.

Confluent colonies of stem cells cultured on feeders as per the culture methods above, were scraped using a Falcon cell scraper and pipetted into a 1ml Eppendorf tube, centrifuged for 1 minute to pellet the cells (Eppendorf centrifuge 5415D) and the supernatant removed. The cell pellet was stored at -80 °C until sufficient numbers had been collected to be sent off for external DNA profiling at TDL (Doctors Laboratory, London). This process was carried out at P+5 and P+20 and compared to ensure there was no cross-contamination amongst the 16 cultures being studied.

2.6. Characterisation of hESCs

2.6.1 Classical cytogenetics: karyotyping by g banding

Human embryonic stem cells are routinely karyotyped to evaluate chromosomal abnormalities. Cytogenetics is currently the easiest method for detecting chromosomal abnormalities. Basic conventional cytogenetic method involves chromosome harvest, slide preparation, banding of chromosomes, analysis of Banding patterns and interpretation of the results. Chromosome harvest consists of arresting the cell cycle at metaphase, hypotonic treatment of the cells and their fixation, after which the chromosomes are spread onto glass slides, dried and stained before banding. Bands are defined as part of a chromosome that is clearly distinguishable from its adjacent segments by appearing darker or lighter (Loring et al,2006).

G banding is a commonly used method of banding patterns using Giemsa stain for the analysis of metaphase spreads. Each band has a specific number assigned to indicate its location on the human chromosome. The nomenclature of band assignment and chromosome aberrations is endorsed by the International system of human cytogenetic Nomenclature ISCN 2005.

For quality control of banked hESCs the UKSCB routinely prepares samples for chromosome spreads, which are screened on glass slides which are Giemsa stained to observe a minimum of 20 metaphase spreads. The samples are then stored in fixative, and sent out to an external company, The doctors Laboratory (TDL), for G banding.

2.6.2 Preparation of samples for metaphase spreads

Samples for metaphase spread analysis were prepared using stem cell cultures between 3 to 5 days old. 60-70% confluent wells of a 6 well plate (Falcon) were incubated using 10ug/ml colcemid (GIBCO KaryoMax) for 1 hour, then enzymatically treated using TrypLE™ Express (Gibco) to dislodge cells The cell suspension was then transferred to a 15ml Falcon tube and spun down for 5 minutes at 200g.

The supernatant was removed and the remaining cell pellet was swollen using a hypotonic solution of equal volumes (1:1) PBS and H₂O for 15 minutes at 37°C. The cell solution was centrifuged again, the supernatant removed, and the cells fixed slowly using acetone/methanol (3:1) by adding drops of fixative while continuously

vortexing the solution. The fixed cell solution was then centrifuged, resuspended and fixed again using acetone/methanol (3:1 ratio). The sample was subsequently stored at 4°C in 4 ml fixative or at -20°C for up to 6 months (if unable to send for Gbanding assessment immediately) until ready for staining.

2.6.3 Examination of metaphase spreads

The fixed cell solution was further diluted in 1-2 ml of fixative if the suspension was too cloudy, then spread onto a methanol dipped-glass slide (VWR, superfrost plus, cat: 631-0108) by angling the slide at 45° and using a 2 ml pasteur pipette to place a few drops of solution onto the slide from a height of 2 or 3 metres. The slide was allowed to air dry completely before undergoing staining using Giemsa stain 1:20 (Invitrogen, Karyomax) with PBS.

2.6.4 Giemsa staining

The prepared slides with samples were dipped into coplin jars of 0.25% Trypsin (Invitrogen) for 2 seconds, then straight into saline for 10 seconds, saline again for 30 seconds, then into Giemsa stain (1:20) diluted in Gurr's buffer for 10 minutes (Invitrogen). The excess Giemsa was washed off in PBS and the slides were allowed to dry on a hot plate before being examined under a Phase contrast microscope at x20 objective.

Between 15/20 metaphase spreads were examined before the fixed sample was deemed qualitatively good enough to be sent to TDL for external G-banding analysis.

2.7. Array Comparative genomic hybridisation (aCGH)

Molecular karyotyping by array comparative genomic hybridisation was set up in-house. Array comparative genomic hybridisation (aCGH) is a cutting edge tool used for detection of deletions and duplications in chromosomes with a detection range of 1MB-500KB. This method involves differential labelling and hybridisation of sample DNA and (normal) reference DNA to an array of genetic probes covering the whole genome. The detection of unbalanced gains or losses is revealed by the comparison of sample DNA to reference DNA. This method enables sub-microscopic chromosomal aberrations to be detected as well as enabling the analysis of the genome at a higher resolution than that achieved by conventional G-banding.

Each of the four stem cell lines was cultured on four feeder lines (16 samples in total) up to P+20 on 0.1% gelatine-coated flat bottomed 6 well plates. Cell pellets were collected at p+5 and p+20.

DNA from all four stem cell lines on each different feeder layer was obtained by scraping cells from 6 well plates into 1ml eppendorf tubes using a Falcon cell scraper. The cells were centrifuged in a microcentrifuge, the supernatant removed and then stored at -80°C. DNA extraction was carried out using Qiagen DNeasy blood and tissue kit (cat number 69504) as per instructions. DNA was then quantified using the Nanodrop™. Normal male and female reference DNA was obtained from Promega.

2.7.1 NanoDrop™ DNA Quantification

DNA quantification was carried out using a NanoDrop (Labtech, ND-1000 spectrophotometer and software ND-1000 v3.3.0) so as to enable the correct quantity of DNA to be used for aCGH. The NanoDrop is a spectrophotometer that does not require the use of cuvettes, and is capable of measuring large concentrations, and so eliminating the need for dilutions that may cause inaccuracies. The prepared DNA sample (1µl) was loaded onto the fibre optic measurement surface directly, and the arm lowered. The sample meets the upper fibre optic tip and a liquid column results. The path length of the resultant column of sample is controlled. Absorbance measurements were made automatically. Between each sample the surfaces were wiped with a tissue, and after each measurement, a blank (water) was loaded to ensure correct readings were being obtained and there was no carryover of sample. NanoDrop results were obtained as a measurement of ng/µl.

2.7.2 Array preparation

The Perkin Elmer (PE) Constitutional Chip 4.0 kit was used as per instructions. The kit includes the arrays, labelling reagents, and hybridisation buffers. NaCl, EDTA, Isopropanol and reference DNA. Reference and sample DNA were labelled with labelling dye and incubated at 70°C for 10 minutes, then snap-cooled on ice-slurry. The reference and sample DNA was then mixed together and allowed to hybridise for 20 minutes before the precipitation step in 70% alcohol. The reaction was stopped using EDTA (0.5Mm). The samples were then carefully pipetted onto a cover slip which was very carefully placed onto a new clean array.

2.7.3 Hybridisation of arrays

The arrays were placed in hybridisation chambers, on top of moistened wipalls and into plastic boxes. The arrays were then incubated at 56°C for 16 hours (minimum) in a hybridisation oven. This step was always carried out overnight. The following day the arrays were carefully disassembled from hybridisation chambers and carefully washed in copping jars using a series of 3 buffers (SDS buffer details). Finally excess buffer was removed using an alcohol wash in 70% w/w isopropanol and the slides spun-dry (Labnet slide spinner) and stored in a desiccators until ready to be scanned.

The arrays were scanned using a ScanArray Gx Scanner (Perking Elmer) and analysed using Scanarray Express. Spectral views of the chromosomes were viewed using SpectraWare Molecular Karyotype Analysis v2.3.3. Analysis was completed using the OneClick aCGH software (version 4.3.3). Figure 2.2 demonstrates an overview of aCGH method.

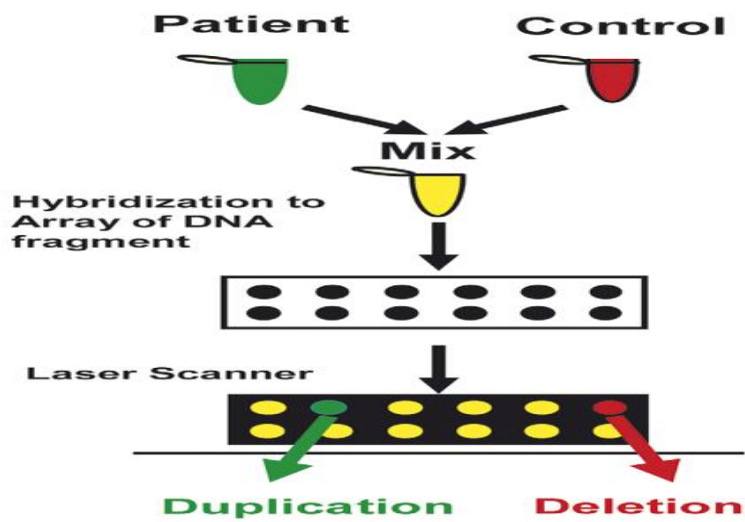


Figure 2.2. Method overview of aCGH obtained from Perkin Elmer Constitutional Chip 4.0 User manual.

2.8.0 Flow cytometry using the Guava™ for the expression of pluripotency markers

Cells were cultured on their respective feeders using culture method outlined above. Cells were enzymatically treated using TrypLE™ Express for 3-4 minutes and gently pipetted using 2 mls Hes media, transferred to 15 ml Falcon tubes and centrifuged for 5 minutes at 200 g. The supernatant was removed and the cell pellet resuspended in 500 µl PBSa and fixed in 4% PFA for 15 minutes. 5 ml PBSa was added to the cell culture suspension and the tube was centrifuged for a further 5 minutes at 200 g. The cell solution was then stored at 4°C in 10ml WashBuffer (PBSa+0.1% BSA and 0.1% Sodium azide) until ready for staining (maximum 6 months).

Stem cell suspension was pelleted and cells counted using the NucleoCounter (Chemotec). The cell suspension was then resuspended in Washbuffer and 20 ul pipetted onto 96 well plates at a dilution of 4×10^6 /ml. Cells were incubated with designated antibodies and their respective isotype controls for each cell surface marker at optimised dilutions for at least 30 minutes at room temperature and covered with foil to avoid exposure to light. Pluripotency cell surface antibodies Oct 4 SSEA1, SSEA3, TRA160 and TRA181 and their respective isotype controls (Table 2.10) were all grown and filtered in-house (UKSCB, NIBSC) using hybridomas. Hybridoma technology is the production of monoclonal antibodies using hybrid cells that are specifically selected for their ability to grow in cell culture with an absence of antibody chain synthesis. The antibodies produced by the hybridoma are of single specificity (monoclonal).

The plates were then washed twice in Washbuffer and centrifuged at 600 g for 5 minutes. The cells were finally resuspended in wash buffer and the stained cells transferred to a flat bottomed 96 well plate. The final volume was adjusted to 300µl using wash buffer and the plate was loaded onto the Guava™ Flow cytometer (Millipore, Guava EasyCyte W/SSC) for the cells to be processed. Control carcinoma cell line n2102EP (CSCB, Andrews et al, 2002) was used and routinely set up and stained on the same plate. The analysis was completed on the Guava using CytoSoft 3.6.1 ExpressPlus programme and the results exported to a Word Excel spreadsheet to produce bar charts and line graphs in order to visually compare expression of cell surface markers between the 16 samples.

Table 2.10. Details of primary antibodies and isotype controls used for Flow cytometry and *in-situ* fluorescence staining.

Antibody	Subtype	supplier	Cat number	Lot number	Dilution
IgM	Mouse IgM	Caltag	MGM00	1000A	1/100
Tra-1-60	Mouse IgM	Hybridoma	Made in house	Made in house	1/5
Tra-1-81	Mouse IgM	Hybridoma	Made in house	Made in house	1/5
SSEA1	Mouse IgM	Hybridoma	Made in house	Made in house	1/5
IgG3	Mouse IgG3	Abcam	AB18392	305108	33ug/ml (1/50)
SSEA4	Mouse IgG3	Abcam	Ab16287	339164	33ug/ml (1/50)
Rat IgM	Rat IgM	Caltag	RGM004	0503	1/50
SSEA3	Rat IgM	Hybridomas	Made in house	Made in house	1/5

Table 2.11. Details of secondary antibodies used for flow cytometry and *in-situ* fluorescence staining

Antibody	supplier	Cat number	Lot number	dilution
Mouse Igm + IgG FITC	Caltag	LM30801	30801	1/100
Goat anti- mouse (PE) IgM	Caltag	M31504	488000A	1/100
Alexafluor 647 (CY5) Goat anti rat IgM	Invitrogen	A21248	764793	1/200
Alexafluor 488 (FITC) Goat antimouse IgG	Invitrogen	A11017	796017	1/200
4',6-diamidino- 2-phenylindole, dihydrochloride (DAPI) nucleic acid stain	Invitrogen	D1306		1/1000

2.9.0 TissueFacs™ quantitative Imaging system

Stem cells cultured in 6 well plates on each of the four feeder types were transferred into flat bottomed 24 well plates (BD Biosystems) using TrypLE™ Express and

cultured as above until 70% confluent and containing <5% differentiated cells. The cells were fixed in-situ using 4 % PFA for 20 minutes, then washed three times in PBSa. 3-4 mls of wash Buffer was carefully pipetted into each well and each plate was sealed using parafilm and stored at 4°C until ready for staining and imaging.

Plates were stained using SSEA-3, TRA1-60, TRA1-81 and their respective isotype controls (Hybridomas, UKSCB, NIBSC) at 1:5 dilution for 1 hour at room temperature or 4°C overnight. Primary antibodies were washed off using WashBuffer and the secondary antibody (1:100 dilution) pipetted into each well and incubated for a minimum of 1 hour at room temperature. The secondary antibody was washed three times using Washbuffer before staining each well with DAPI (1:100, Invitrogen) for 5 minutes at room temperature. Once washed off and resuspended using Washbuffer, the plate was set-up for imaging using the TissueFacs™ imaging system (x20 magnification). Analysis of successfully stained wells was carried out using the TissueQuest analysis software (version 3.02) to give accurate quantitation of the expressed cell surface markers in each stem cell sample.

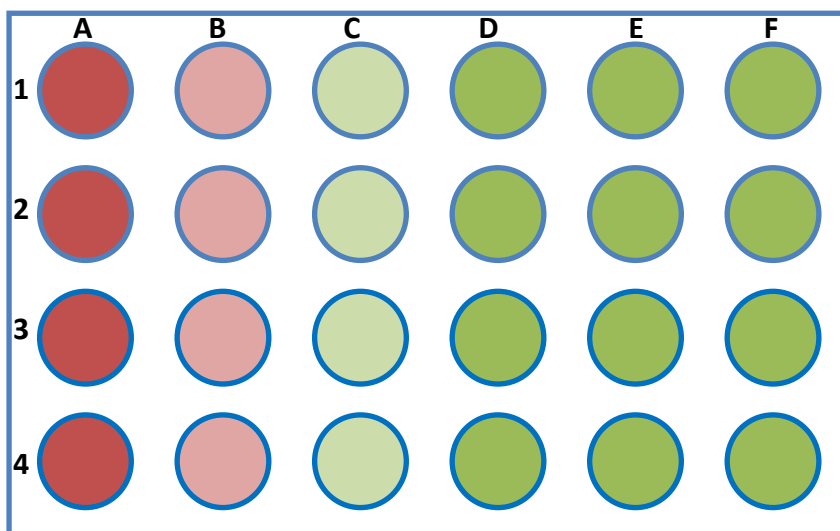


Figure 2.3. Plate layout for *in-situ* staining. The wells in column A were used as an isotype control. Column B was used as a negative control with only secondary antibody staining. All plates fixed for this project were stained in the following order to allow for easy and comparable analysis and combinations of antibody expression:

Wells C2, C3 D4, F2, F3:**SSEA4**

Wells C1, F2-F4, E4, D4, C4:**SSEA3**

Wells C4, D4, F1, E2, E3:**TRA1-81**

Wells D2, D3, E1, E4: **TRA1-60**

2.10. Taqman Low Density Array (TLDA) cards for relative expression of pluripotency genes

Stem cells were cultured using the same culture methods stated above. Cell pellets were collected at passaging intervals p+5, p+20. Confluent cell cultures containing >5% differentiated stem cell colonies were carefully scraped using Falcon cell scrapers and pipetted into 1ml eppendorf screw cap tubes. The cell pellets were spun down in an Eppendorf microfuge, supernatant removed and stored at -80°C until ready for RNA extraction using Qiagen Rneasy spin column as per leaflet instructions. An extra step was added to this method using Qiagen QiaShredder, to increase RNA purity.

2.10.1 RNA Extraction

Total RNA isolation was carried out using a Qiagen Rneasy mini kit according to the protocol provided (Qiagen, Cat. No 74104). This method combines the selective binding properties of a silica based membrane with spin technology. Samples for RNA extraction were obtained by harvesting the cells using trypsin/EDTA passaging. After centrifugation for 2 minutes at 200 g (Eppendorf table top centrifuge 5415D) the cell pellet was re-suspended in 350 µl of RLT buffer (a highly denaturing guanidine-thiocyanate containing buffer, which inactivates RNases to ensure purification of intact RNA) and stored at -80°C. To each sample, 350 µl of 70% ethanol was added, which created appropriate binding conditions to the silica membrane. The sample was then transferred to an Rneasy column in a 2ml collection tube, and centrifuged for 15s at 8000g and the flow-through discarded. To each tube, 700 µl of buffer RW1 was

added, and the tubes centrifuged for 15s at 8000g, with the flow-through once again being discarded. 500 µl of buffer RPE (made up with 4 volumes of 100% ethanol added to the stock bottle) was added to each tube, and centrifuge for 15s at 8000g, again discarding the flow-through. This step was repeated. The columns were then transferred to new 2 ml tubes, and centrifuged for 2 minutes at full speed. The Rneasy columns were placed into clean 1.5 ml tubes, and 50 µl of PCR grade RNase-free water added to each column to elute the RNA. The columns were centrifuged for 1 minute at 8000g. The optional step of re-using the flow-through in order to elute more RNA, with 50 µl of RNase-free water was also carried out, using a fresh volume of water, and the tubes centrifuged for 1 minute at 8000g, giving a total volume of 100 µl containing RNA.

The prepared RNA was measured using NanoDrop™ to assess 260/280 ratio integrity, using the method outlined above. The RNA was then examined further using Agilent Nano Chip (Agilent Technologies) to decide whether the extracted RNA was of good enough quality to use for LDA cards. This was performed according to Agilent technology instructions.

2.10.2 Preparation of samples for Agilent Assay

RNA integrity was assessed by RIN number (RNA integrity number). RNA with a RIN measurement of 8-10, calculated as a result of 28s:18s ribosomal RNA ratio, was processed further to make cDNA. 1-10, with 1 being poor and 10 being excellent. RIN 10: Intact RNA, RIN 5: Partially degraded RNA, RIN 3: Strongly degraded RNA.

First the Agilent RNA 6000 Nano kit was taken from 4°C and left at room temperature for 30mins. The RNA samples of interest and one aliquot of RNA ladder (already aliquoted into 1 µl per sample) were denatured at 70°C for 2 mins using a heating block, then snap cooled on ice. 550 µl of RNA 600 nano gel matrix was spun down in a spin column provided in the Agilent kit at 1500g for 10 mins. The filtered gel was aliquoted (65µl) into 0.5ml RNase-free microfuge tubes (Agilent RNA free tubes). Filtered gels can be stored at 4°C for up to four weeks. The RNA 6000 Nano dye concentrate was vortexed for 10 seconds, then spun down and 1µl of dye added to the 65µl aliquot of filtered gel. The solution was vortexed and centrifuged at 13000g for 10 minutes. The prepared gel was used within one day.

Using a new Agilent Chip 9.0 µl of gel-dye mix was pipetted in the well-marked (C). The chip was then carefully placed into the chip priming station and the plunger

placed at position 1ml. The chip priming station was then closed and the plunger pressed so it was held in place by the clip. Using a timer the plunger was left in this position for exactly 30 seconds, before being released. After 5 seconds, the plunger was slowly and carefully returned to the 1ml position by hand. 9.0 μ l of gel-dye mix was carefully pipetted in the 2 wells marked (G). 5 μ l of RNA 6000 Nano marker in all 12 sample wells and in the well-marked ladder, avoiding pipetting bubbles and touching the bottom of the chip so as not to pierce the chip. Pipette 1 μ l of prepared ladder in the well-marked ladder. 1 μ l of sample was pipetted in each of the 12 samples well. RNA 6000 Nano Marker was pipetted into each unused well (All wells must be full). The chip was carefully placed horizontally in the adapter of the IKA vortexer and vortexed for 1 minute at 2400rpm. The Chip was then run on the Agilent 2100 Bioanalyser within 5 minutes on the Eukaryotic total RNA assay selection. Figure 2.7 below shows an electrogram of a hESC RNA sample. Two peaks are shown from which the RIN ratio is calculated (28s:18s).

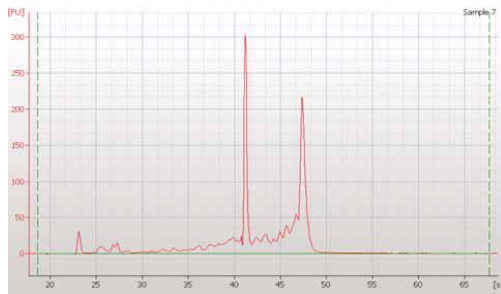


Figure 2.4. Electrogram showing example of RNA integrity from a stem cell sample.

2.10.3 cDNA preparation

cDNA was prepared from suitable RNA on ice using Applied Biosystems High capacity cDNA Reverse Transcription kit with random primers for use with the TaqMan LDA cards. The RNA samples were mixed with PCR master mix (see concentrations/dilutions in table) and loaded into a pre-set thermocycler and amplified using the following cycle. The cDNA was then stored at -20°C until ready to be loaded onto a TLDA card.

Table 2.12. Preparation for 2XRT master mix (20 µl per reaction)

Component	Volume/Reaction (µ/L)
10XRT Buffer	2.0
25XdNTP Mix (100mM)	0.8
10xRT Random Primers	2.0
MultiScribe Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H ₂ O	3.2
Total per reaction	10.0

Table 2.13. Reverse Transcription program for Thermocycler (Applied Biosystems 7900HT).

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10min	120min	5min	∞

2.10.4 Preparation of TLDA cards

cDNA samples were prepared by adding RT PCR Mastermix (Applied Biosystems) and loaded into a TaqMan array card which has been pre-filled with TaqMan gene expression assays prepared by Applied Biosystems for the UKSCB. These cards contain 96 genes specific to embryonic stem cell markers including 6 endogenous controls and 16 house-keeping genes. Once carefully loaded with 100 µl sample-specific PCR reaction the cards were centrifuged at 300 g for 2x1 minute.

The cards were then sealed to remove the fill reservoirs using an AB card sealer. The fill reservoirs were then subsequently cut off carefully using scissors and the prepared card loaded onto a 7900HT fast real time PCR analyser (Applied Biosystems) for relative quantification using comparative Ct. Once the cycles were complete the data was transferred into RQ manager software (version 2.1) for analysis. Further analysis was carried out using ExpressionSuite software version 1.1 (Life Technologies).

Chapter 3. Assessment of human embryonic Stem Cell morphology on different matrices and release criteria testing.

3.1 Introduction

In keeping with the stringent quality testing that the UKSCB needs to adhere to, it was necessary to ensure that the stem cell lines used for these studies were subjected to quality control testing. This is particularly important since the protocols developed will be incorporated into improving the culturing procedures for a more standardised banking process. Rigorous 'release' criteria is employed by the UKSCB to test banked stem cell lines, consisting of viability, sterility, mycoplasma testing, viral PCR and DNA profiling along with detailed records of the banking procedure. Such testing ensures that the banks of cells produced are of high quality, and do not run the risk of compromising the reproducibility of cutting edge research. It is this type of quality assurance testing that ensures scientists accessing banked hESC lines that they are reliably cultured, free from contamination and furthermore, it distinctly separates the UKSCB apart other research organisations and providers of research grade hESC lines.

Thus in this thesis, a panel of screening protocols have been adopted for testing the quality of the cells used in all the studies and include screening for bacterial, fungal, yeast and mycoplasma contamination. More importantly, since the characteristics, morphology and differentiated status of hESCs can be regulated by the matrix on which they are cultured as well as the method of passaging and media used, experiments were carried out to preliminary evaluate the suitability of an ECM source MRC5, derived from human feeder lines. These were compared with fibroblasts derived from foetal skin, neonatal skin and mouse fibroblast line 3T3 with the current standard culture matrix, MEFs, to determine whether hESCs can adapt to and maintain their undifferentiated state on these matrices.

The use of better defined media and matrices for the undifferentiated culture of hESCs is becomingly increasingly routine, especially as researchers move away

from using feeders. Often, such media is used in combination with defined media components and a non-feeder matrix such as Matrigel, a basement membrane matrix derived from Engelbreth Holm-Swarm (EHS) mouse sarcoma cells. Matrigel, in combination with defined media such as mTeSR1 has been shown to support the growth of a variety of stem cell lines (Ludwig et al., 2005), for many different applications. Moreover, it is batch tested, which again, adds another level of quality assurance to its benefits, compared with using mouse feeders which have huge variation due to the use of different mouse strains, preparation methods and variation due to the number of viable cells and method of inactivation (Stacey et al., 2006). In this thesis, additional comparative studies were therefore carried out examining the morphological effects of stem cell growth ECM derived from human lung fibroblast line MRC5, Laminin and Matrigel and mTeSR1 media.

The investigations in this first results chapter were essential before any further studies are carried out, to ensure that the quality of the cells used and the basic conditions that determine their undifferentiated status are defined.

3.2 Methods

3.2.1 Preparation of mouse, human feeders and Matrigel/mTeSR1 and for culture assessment using hESCs

Preparation of mitotically inactivated feeder banks from two mouse cell lines, 3T3 and MEF, and two human cell lines MRC5 and HDFn, and the subsequent culture adaption of hESCs onto different feeder types were as described in the general methods section (chapter 2.1). Matrigel and mTeSR1 media was prepared according to manufacturer's protocols, as described in Methods chapter section 2.4.2. The stem cell lines HUES9, NCL5, SHEF1 and RH5 were all cultured on feeder free matrix Matrigel using mTeSR1 media, for extended passage, up to p+20. Stem cells were assessed for morphology throughout the culture process. Throughout the culture process the cells were closely observed and images taken to document their adaption from mouse to human feeder layers and Matrigel, and for two of the hESC lines, their adaption from mechanical dissection passaging (cutting) to enzymatic passaging using TrypLE™ Express.

3.2.2 Assessment of undifferentiated hESC growth using human feeders derived from foetal skin

Foetal skin tissue obtained from ethical sources was prepared by standard methods to give foetal fibroblast line 11235. The cell line was cultured using MEF media (DMEM, 10% FCS and 1% Glutamax) and inactivated according to standard methods described in methods chapter 2.3.5. Stem cell lines H9, H7, NCL5, and NCL2 were cultured on i11235 using KO HES media for 5 passages, and subcultured every 5-7 days using traditional cutting method for the first 1-2 passages, then adapted to passaging using TrypLE™ Express for the remaining passages. The cells were assessed for typical undifferentiated hESC morphology.

3.2.3 Preparation of extracellular matrix (ECM) from human feeders for culture assessment using hESCs

The human cell line MRC5 was cultured for up to 5 passages using standard MEF media as described in chapter 2.2.1, and ECM prepared from MRC5 as described in section 2.4.1. The stem cell lines NCL5 (P31), HUES9 (P23), and H9 (P27) were culture on ECM using KO he's media and passaged using TrypLE™ Express as described in methods chapter 2.3.3.

3.2.4 DNA profile, sterility and mycoplasma PCR testing

Samples for DNA profile and viral PCR testing were prepared from stem cells cultured on i3T3s, iMEFs, iHDFns, iMRC5s and Matrigel at early and late passages (P+5, P+20) to ensure that cross contamination of cell lines had not occurred through the extended culture process, and that the cultures were free of viral contamination. Collection of samples for DNA profiling for PCR analyses were carried out as described in chapter 2. Mycoplasma broths were also inoculated with cell suspension, for potential detection by culture method, as described in the general methods chapter (section 2.5.3).

Samples (spent media) for sterility testing were taken throughout the culture process, in particular, at thawing and freezing down stages, and subjected to the various tests as described in sections 2.5.1 of the general methods chapter.

3.3 Results

3.3.1 Effects of human and mouse feeders on growth and morphology of hESCs

Figures 3.1 to 3.8 shows the morphology observed from RH5, HUES9, NCL5 and SHEF1 cultured on human and mouse feeders, at late passage (p+20). Post thaw, following two weeks of culture using iMEFs, all cell lines appeared to be healthy, with typical morphology. Both RH5 and HUES9 quickly adjusted and appeared to proliferate well on human feeders, and required passaging twice a week with TrypLE™ Express. Passage ratios were adjusted from 1 in 6 wells, to 1 in 12 wells to allow for all the different stem cell lines/conditions to be passaged at the same time once a week. At early passage (P+5) HUES9 grew in between the human feeders and pushed the feeders either side of their colonies as they became larger and more confluent. At later passage the morphology of these cells changed as a result of culture on human feeders. Figures 3.1 A and 3.2 B showed much larger colonies with angular borders. Slightly more differentiation was also seen from cultures grown on iHDFn human feeders than iMRC5 overall. When compared to cultures on human feeders, HUES9 colonies on mouse feeders (Figures 3.2 C and D) became much smaller in size, with more differentiation around the edges of the colonies.

Stem cell line RH5 showed some signs of differentiation and cell death at early passage as the cells adapted to different feeders, as demonstrated by Figure 3.3 (A, B, black arrows). Distinct morphology changes were observed with early passage (p+5) cultures of the RH5 cells on human feeders which appeared to grow not in a typical round shape as they did on mouse feeders, but in elongated, more angular colonies, which followed the patterns produced by the plated inactivated human feeders. At late passage RH5 stem cells grew on top of mouse fibroblast line i3T3 as shown in Figure 3.4 (image B), and on iMEF, in distinct colonies (Figure 3.4 image A). RH5 also proliferated with distinct morphology on human fibroblast iHDFn, as they grew in between the feeders, as shown by Figure 3.4 (image C) and in swirls (image D). RH5 appeared to adapt better to iMRC5 and iHDFn human feeders, in comparison to iMEF and i3T3 mouse feeders, as there were a lower number of

attached colonies and more differentiation (denoted by arrows) observed on mouse feeders (Figure A, B) compared with colonies on both human feeders.

Considerable differentiation and cell death was observed when SHEF1 were initially enzymatically passaged on iHDFn human feeders. As a result this stem cell line was manually passaged for 1-2 passages on human feeders before switching to enzymatic passaging. This approach enabled this cell line to grow undifferentiated on iMEFs but not on human feeders or on the mouse fibroblast line i3T3, with more spontaneous differentiation occurring between passages, despite adjustments made to passage ratios, from 1 to 6 wells to 1 to 3 wells. SHEF1 maintained typically small distinct colonies of hESCs, with clear defined edges, with the exception of iHDFn cultures and iMRC5, which were elongated (Figure 3.5 image C) and more angular (Figure 3.5 image D, E). Figure 3.5 (D and E) also demonstrates how the stem cells cultured on iMRC5 human feeders proliferated, as they pushed the feeders to the sides, as shown by the thickened edges of the stem cell colonies (denoted by arrows). The culture of SHEF1 cells using TrypLE™ Express resulted in more confluent cells, as their colonies became large and began to merge and grow as a monolayer. This pattern of growth was seen in Figure 3.6 by all feeder types at later passage (p+11).

Stem cell line NCL5 required more time to adapt to culture on human feeders (1-3 passages) and in particular, on iHDFn, as more spontaneous differentiation was observed. Following 5 passages, NCL5 demonstrated better adaptation and was consistently growing with mostly undifferentiated colonies on all four feeder types. Colonies on both human feeders appeared angular and elongated and grew in between the feeder layers. As observed by SHEF1 cell line, NCL5 pushed the feeders aside of the colonies as the cells proliferated (Figure 3.7, denoted by arrows). As shown by Figure 3.8 (C, D) much larger, more elongated colonies with irregular borders were observed compared with those observed on mouse feeders, which were round, tight and distinct colonies (image A, B). Slightly more differentiation was observed from cultured on i3T3 and iHDFn (Figure 3.8 image B, C). The morphologies observed at late passage were mostly consistent with images at early passage (p+5) on human and mouse feeders, suggesting that the culture procedure using TrypLE™ Express did not alter the morphology of the cells in culture.

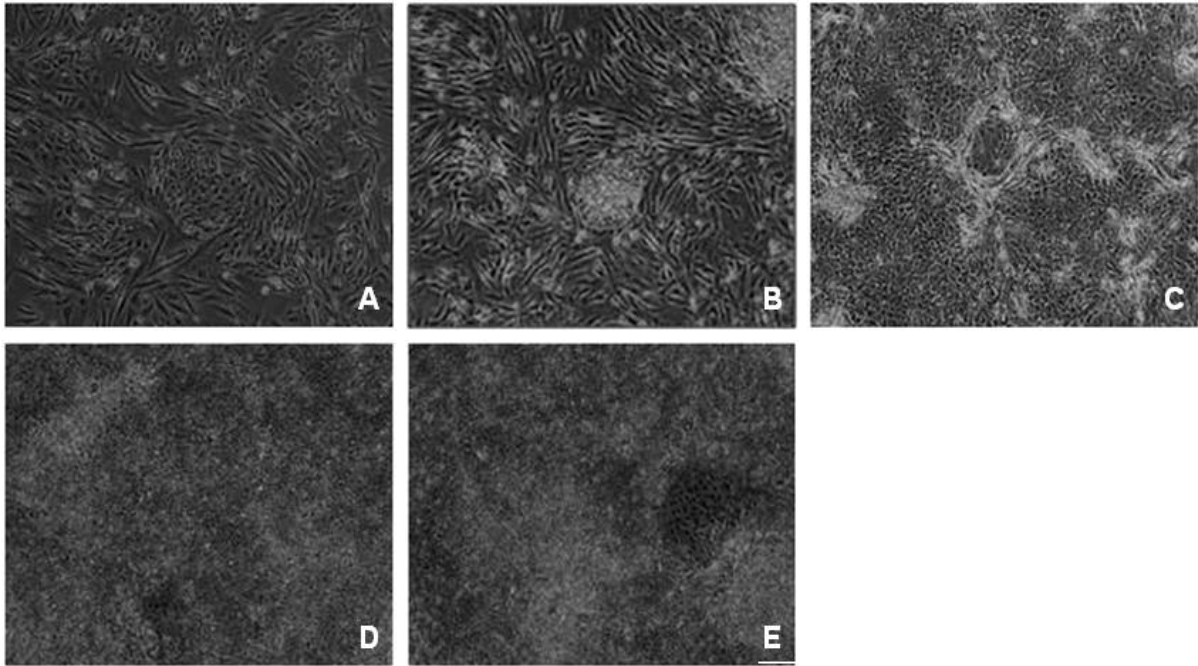


Figure 3.1. Morphology showing HUES9 stem cells cultured on human feeder iMRC5 (A), iHDFn (B), i3T3 (C), and on iMEF mouse feeders (D, E) at early passage (p+5). Cells were dissociated from monolayers using TrypLE™ Express and seeded onto the respective feeders at a seeding density of 2×10^5 cells/ml. The photographs were taken with a light microscope at x4 magnification. Scale bar: 100 μ m.

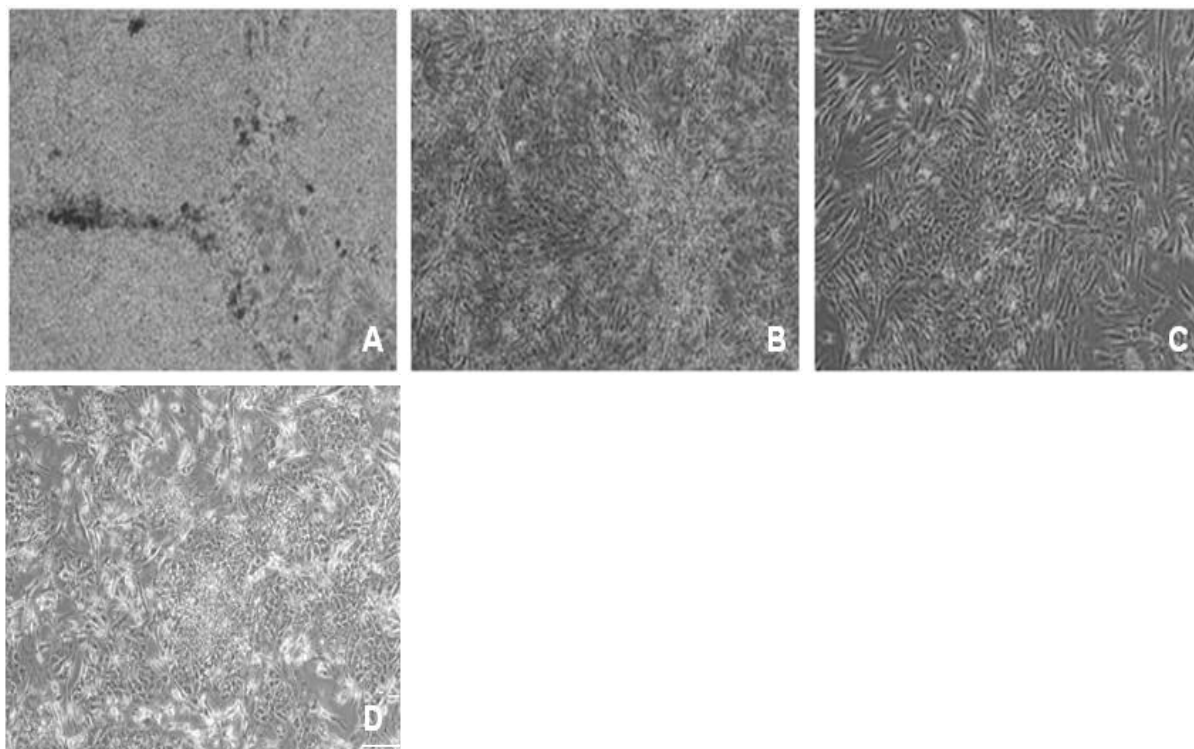


Figure 3.2. Morphology showing HUES9 stem cells cultured on human feeder iMRC5 (A), iHDFn (B), i3T3 (C), and on iMEF mouse feeders (D) at late passage (p+20). Cells were dissociated using TrypLE™ Express and seeded onto the respective feeders at a seeding density of 2×10^5 cells/ml. The photographs were taken with a light microscope at x4 magnification. Scale bar: 100 μ m.

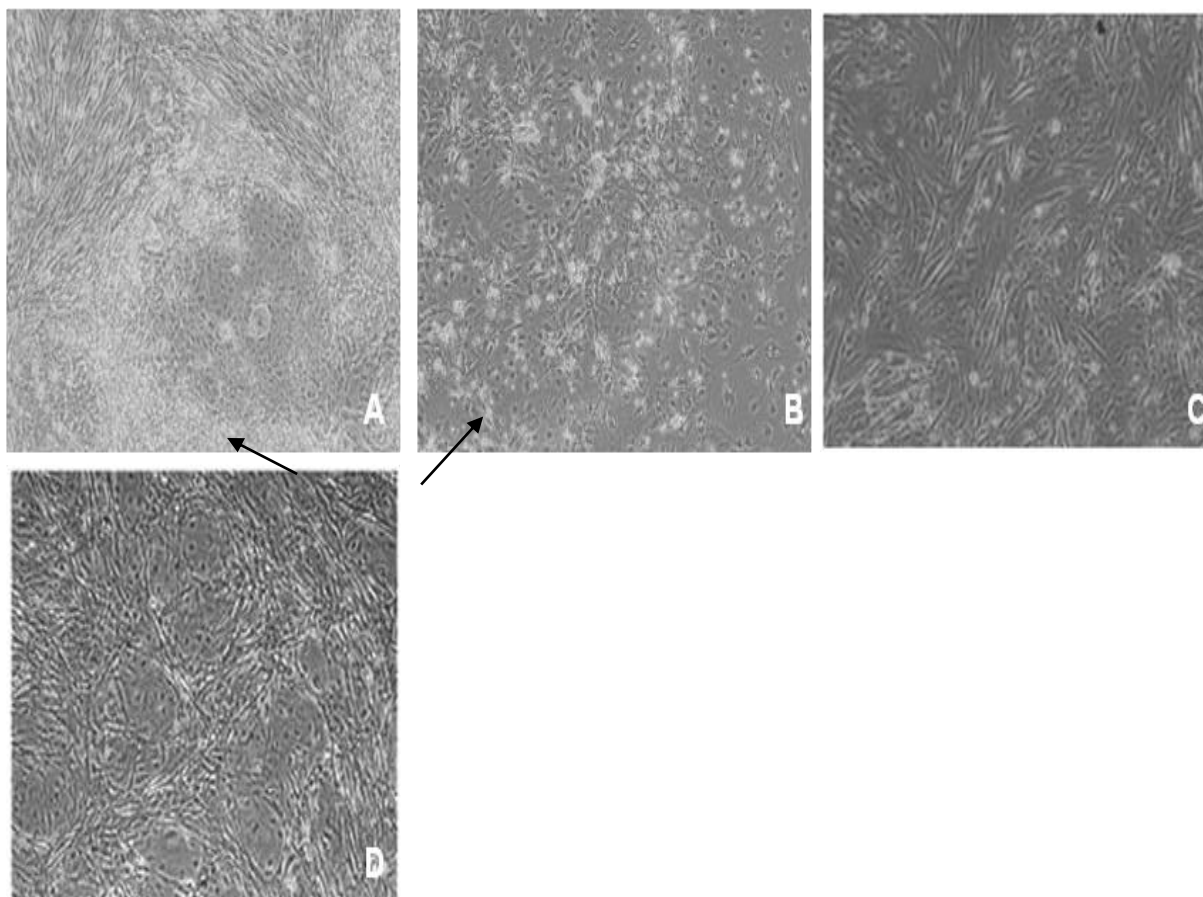


Figure 3.3. Morphology showing RH5 cultured on iMEF (A), i3T3 (B), iHDFn (C) and iMRC5 (D) at early passage (p+5). Cells were dissociated using TrypLE™ Express and seeded onto the respective feeders at a seeding density of 2×10^5 cells/ml. The photographs were taken with a light microscope at x4 magnification. Scale bar: 100 μ m. Arrows indicate areas of cell differentiation and cell death.

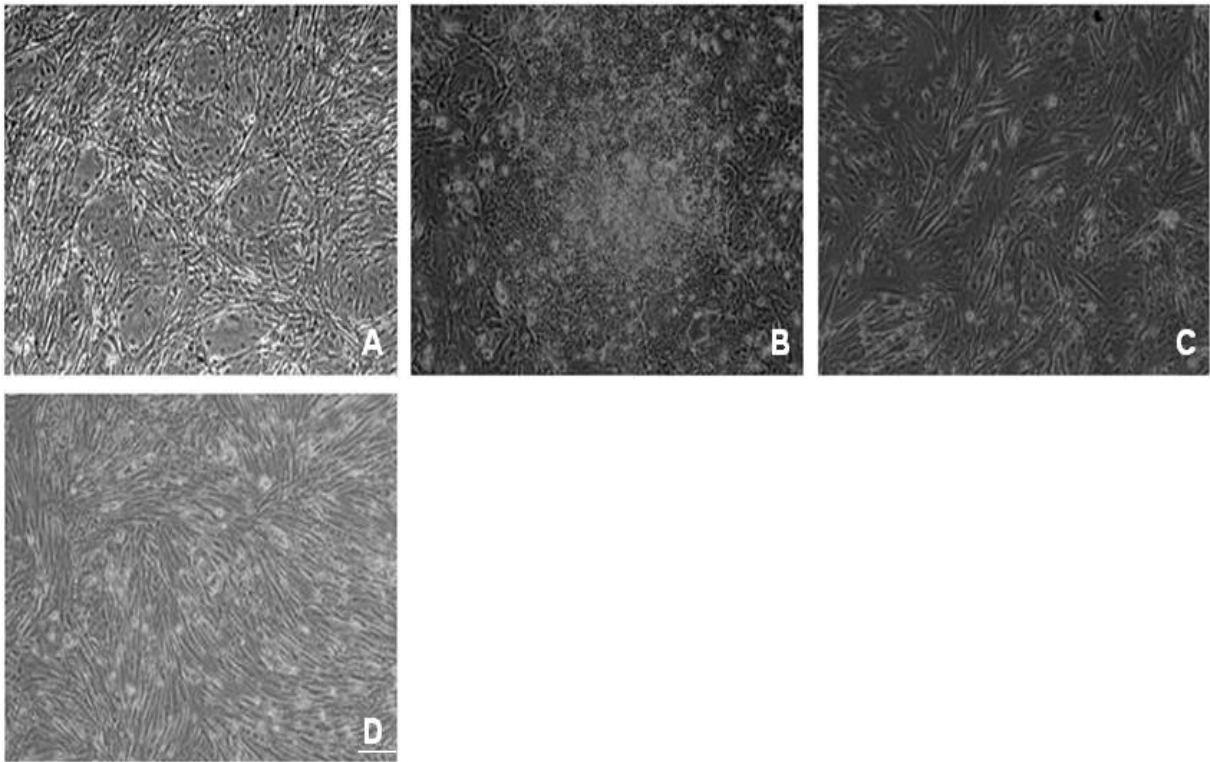


Figure 3.4. Morphology showing RH5 cultured on iMEF (A), i3T3 (B) and iHDFn (C), iMRC5 (D) at late passage (p+20). Cells were dissociated using TrypLE™ Express and seeded onto the respective feeders at a seeding density of 2×10^5 cells/ml. The photographs were taken with a light microscope at x4 magnification. Scale bar: 100 μ m.

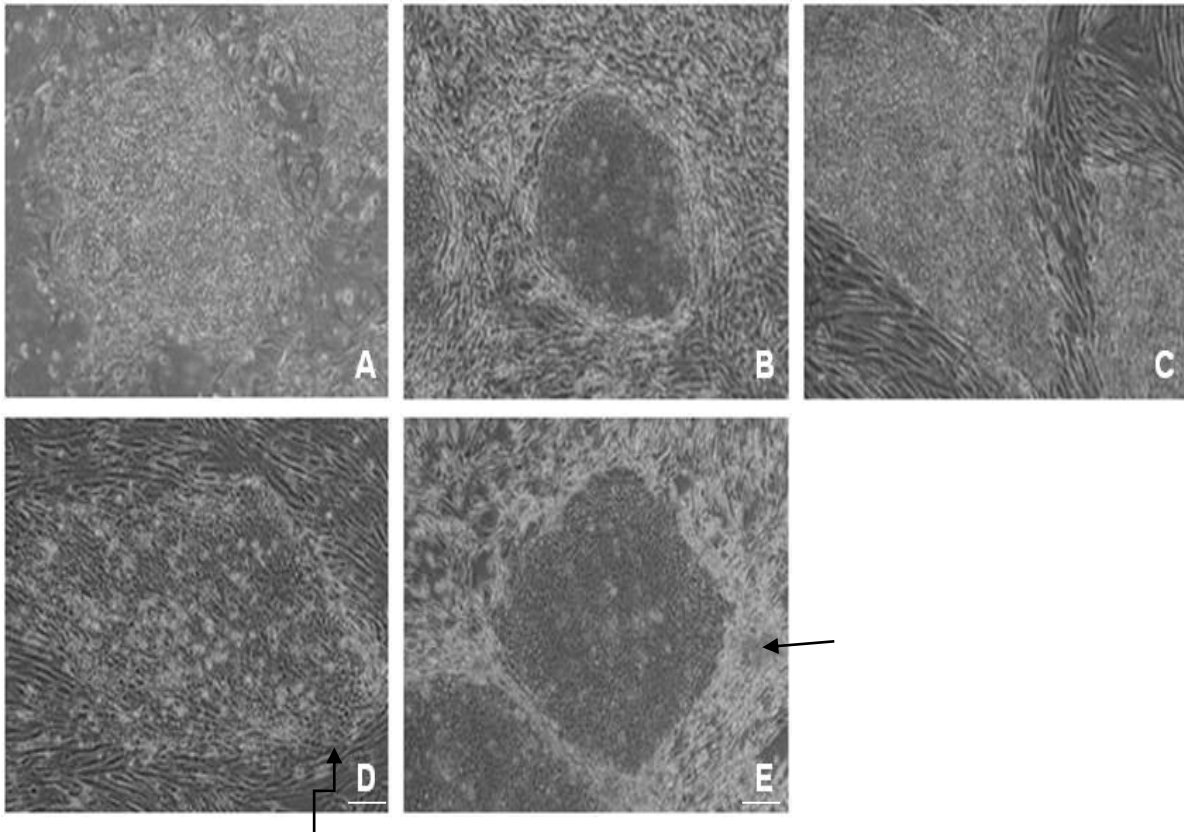


Figure 3.5. Morphology showing Stem cell line SHEF1 cultured on iMEF (A) i3T3 (B), iHDFn (C), iMRC5 (D,E) at early passage (p+5). Cells were dissociated using TrypLE™ Express and seeded onto the respective feeders at a seeding density of 2×10^5 cells/ml. The photographs were taken with a light microscope at x4 magnification. Scale bar: 100 μ m. Arrows indicate areas where inactivated feeder cells have been pushed to the sides of the stem cell colonies to allow for further proliferation.

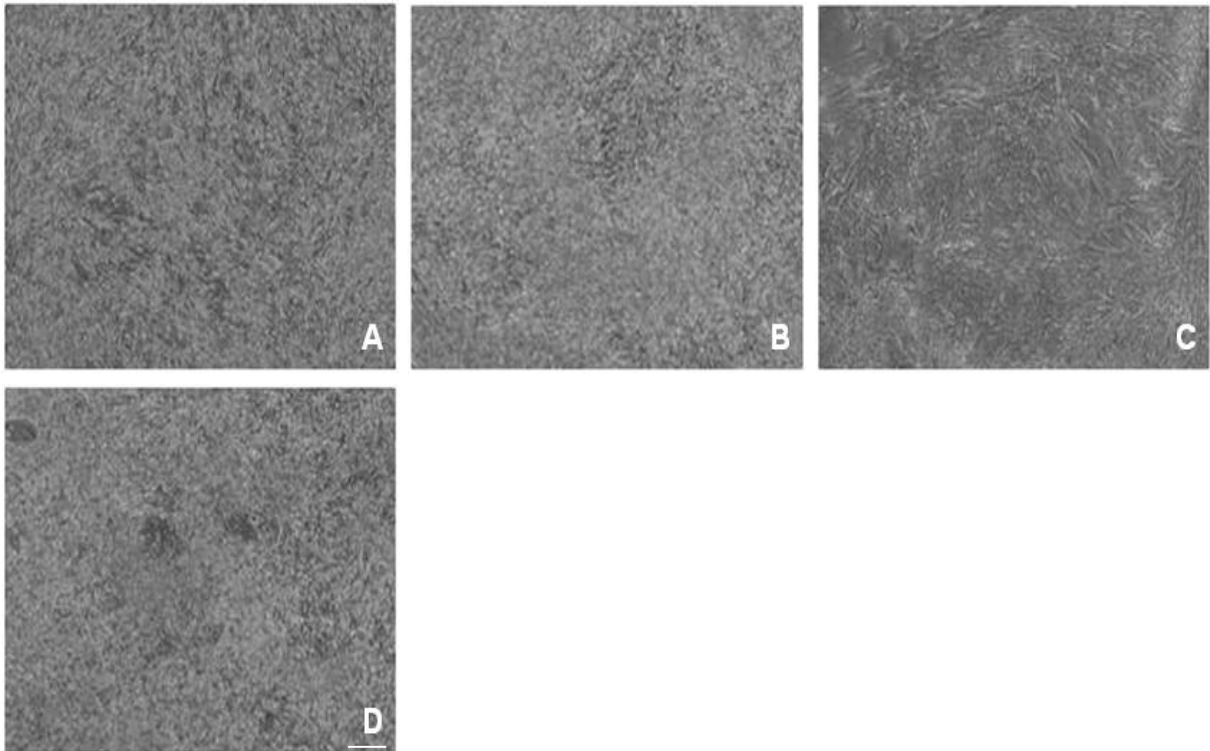


Figure 3.6. Morphology showing stem cell line SHEF1 cultured on iMEF (A), i3T3 (B), iHDFn (C), iMRC5 (D) at late passage (p+11). Cells were dissociated using TrypLE™ Express and seeded onto the respective feeders at a seeding density of 2×10^5 cells/ml. The photographs were taken with a light microscope at x4 magnification. Scale bar: 100 μ m.

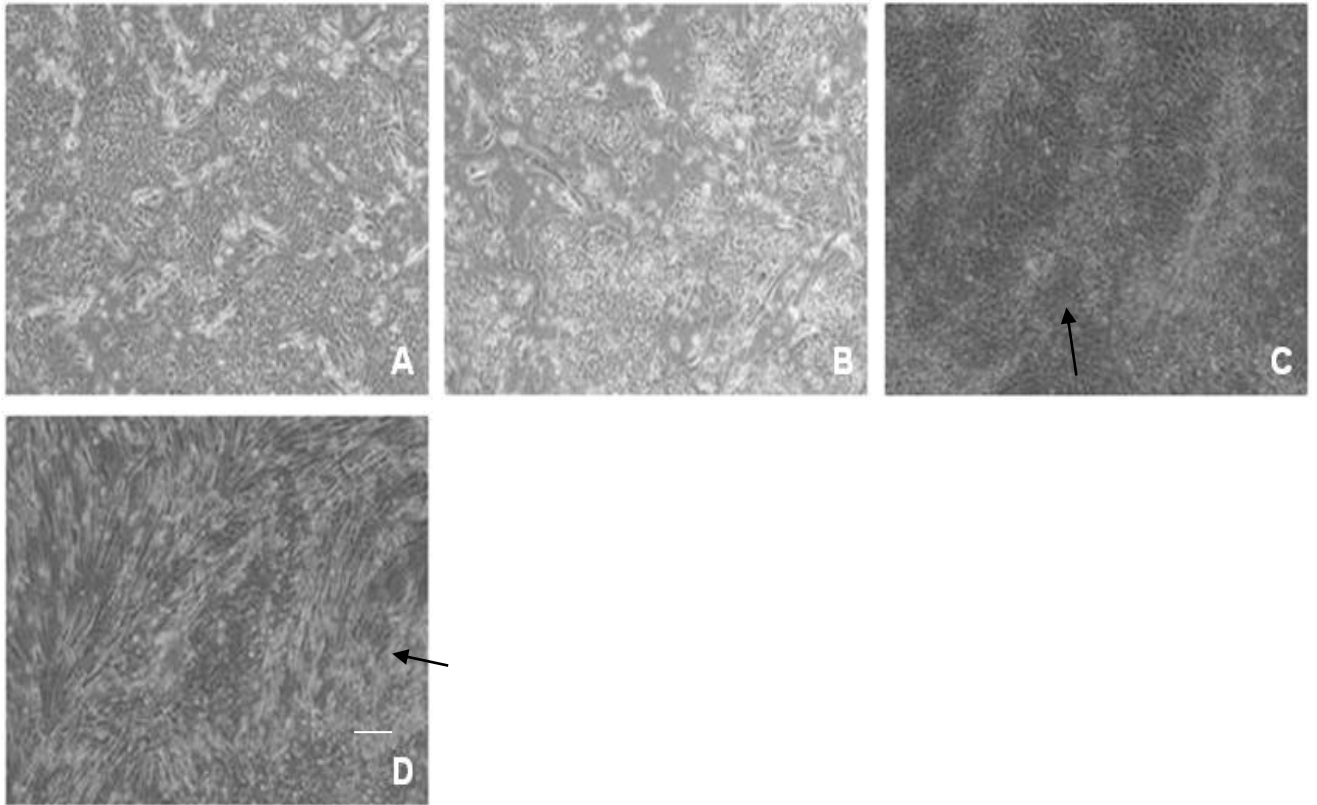


Figure 3.7. Morphology showing NCL5 cultured on iMEF (A), i3T3 (B), iHDFn (C), iMRC5 (D) at early passage (p+5). Cells were dissociated using TrypLE™ Express and seeded onto the respective feeders at a seeding density of 2×10^5 cells/ml. The photographs were taken with a light microscope at x4 magnification. Scale bar: 100 μ m. Arrows indicate areas where inactivated feeder cells have been pushed to the sides of the stem cell colonies to allow for further proliferation.

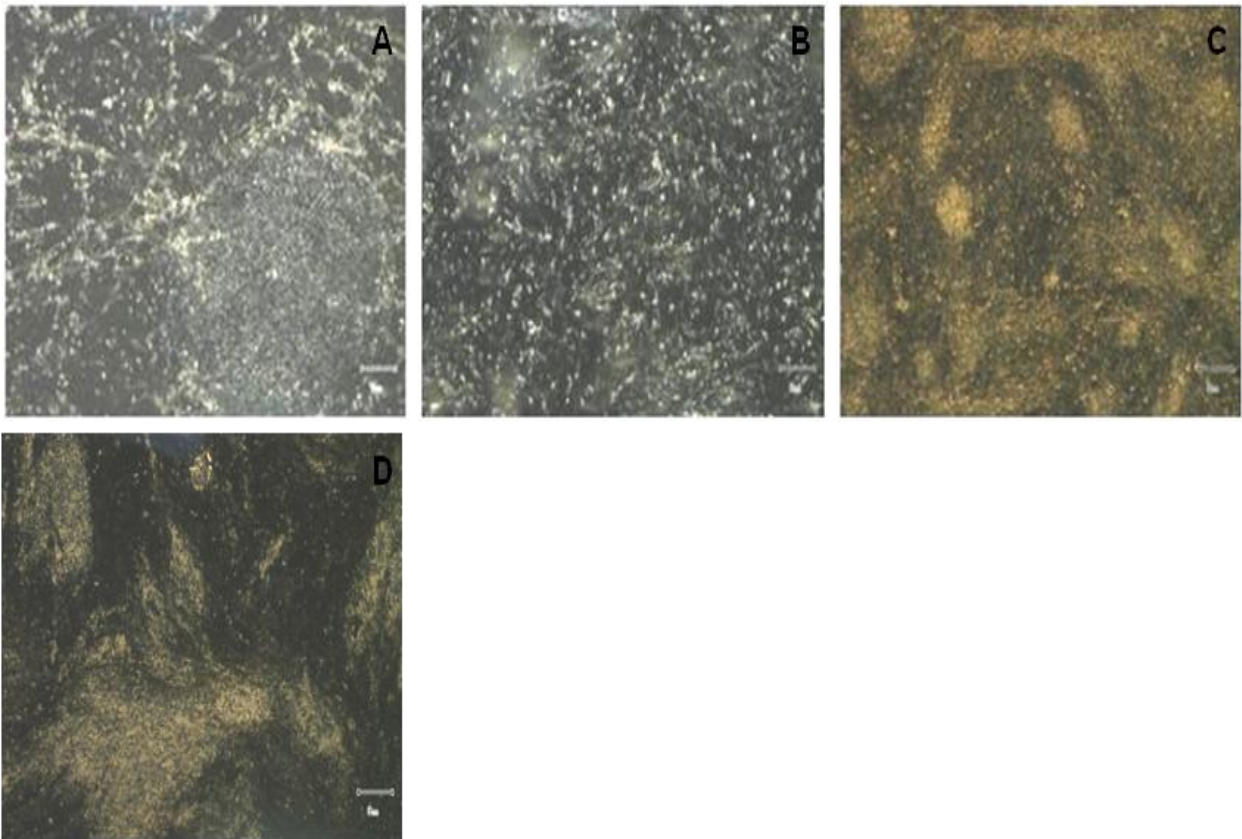


Figure 3.8. Morphology showing stem cell line NCL5 cultured on iMEF (A), i3T3 (B), iHDFn (C) and iMRC5 (D) at late passage (p+20). Cells were dissociated using TrypLE™ Express and seeded onto the respective feeders at a seeding density of 2×10^5 cells/ml. The photographs were taken with a light microscope at x4 magnification. Scale bar: 100 μ m.

3.3.2 Morphology of stem cells cultured on human foetal fibroblast line i11235

The hESC lines H9, NCL2, NCL5 appeared to readily adapt to culture on in-house derived fibroblast line i11235 with little differentiation observed. Although cells at p+1 did exhibit some irregular colony borders (as shown by Figure 3.9 A-C), morphology was consistent with cultures on MEFs, particularly at p+5 (Figure 3.13 D-E.).

Very small areas of differentiation were seen from all hESC lines, consistent with their culture on mouse and human feeders. Later passage stem cells lines (p+5) exhibited very little morphological difference from earlier passages and were easily adapted to enzyme passaging using TrypLE™ Express.

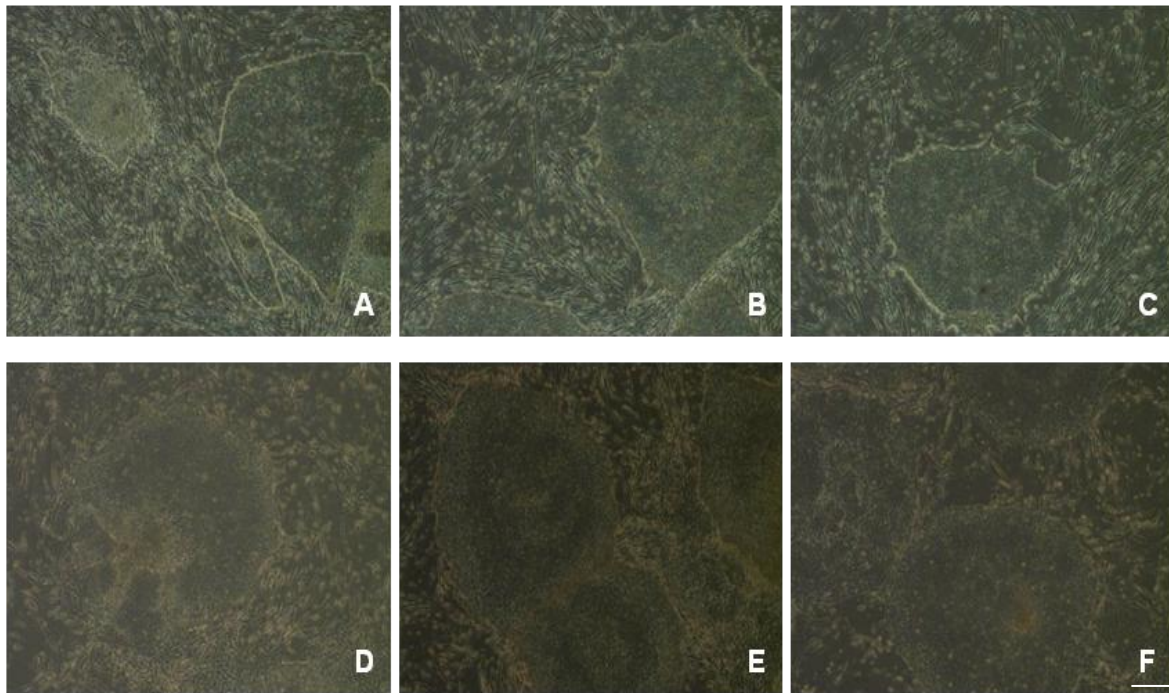


Figure 3.9. Morphology of stem cell lines NCL5, NCL2, and H9 cultured on human foetal fibroblast line i11235. Images A-C at p+1 and D-E at p+5. Stem cells were dissociated using TrypLE Express™ and seeded onto the respective human feeders at a seeding density of 2×10^5 cells/ml. The photographs were taken with a light microscope at x4 magnification. Scale bar: 100 μ m.

3.3.3 Morphology of hESCs cultured on feeder-free matrix Matrigel with mTeSR1™ media

Although each stem cell line appeared to adapt to Matrigel/mTeSR1™ there were significant changes in morphology, particularly for the two stem cell lines which were traditionally enzyme passaged, RH5 and HUES9. Both lines appeared to grow in large colonies and eventually as sheets at 80% confluency, whereas NCL5 and SHEF1 appeared to be growing in smaller defined colonies. This may be due to the absence of feeders, as cell colonies grew in flatter, larger colonies, as they required contact with the Matrigel surface for support. The colonies also appeared thinner, which also may have been due to the absence of feeders. When left for more than 8 days without subculturing, spontaneous differentiation was visible in all the stem cell lines cultured on Matrigel. This was similar to observations seen from hESCs cultured on feeders. NCL5 appeared to adapt quickly and grew well on Matrigel for 20 passages with little differentiation. NCL5s also maintained discrete tight compact morphology as seen from feeder based cultures (Figure 3.10 A). Large flat colonies were also observed when confluent, which would merge together to form large sheets if left unpassaged. NCL5 proliferated much faster and maintained healthy colonies with very little differentiation. They also required passaging after 4 to 5 days instead of 5-7 days, at early passage. More differentiation was seen from HUES9 colonies which required passaging every 3 to 4 days and proliferated in large sheets. RH5 also proliferated in large colonies, with smaller colonies only observed 1 day after they had been passaged. .

The SHEF1 cells could not be cultured further than passage n+12. This was thought to be due to insufficient cell number from the original project bank thawed or poor quality of banked cells. Clear morphological differences were seen when NCL5, SHEF1, HUES9 and RH5 were cultured on feeder free matrix Matrigel. NCL5 and SHEF1 (3.10 A and B) proliferated as tight, round distinct colonies whereas HUES9 formed very small colonies (Figures 3.10C and 3.11C), which merged into large flat sheets as they became more confluent. RH5 also grew into large colonies; however they were more elongated than the other stem cell colonies (Figure 3.10 D).

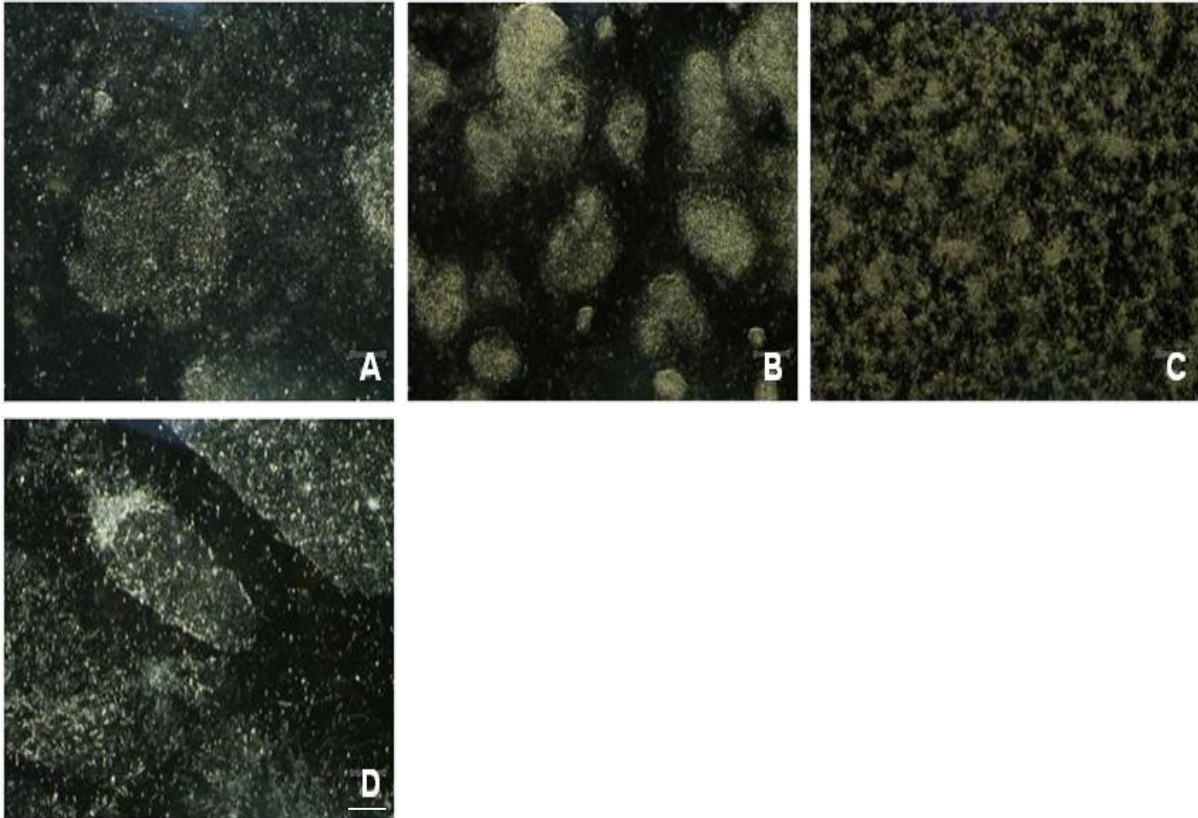


Figure 3.10. Morphology of stem cell lines NCL5 (A), SHEF1 (B), HUES9 (C) and RH5 (D) on Matrigel at early passage (p+5). Cells were dissociated using TrypLE Express™ and seeded onto Matrigel coated plates at a seeding density of 2×10^5 cells/ml. The photographs were taken with a light microscope at x4 magnification. Scale bar: 100 μ m.

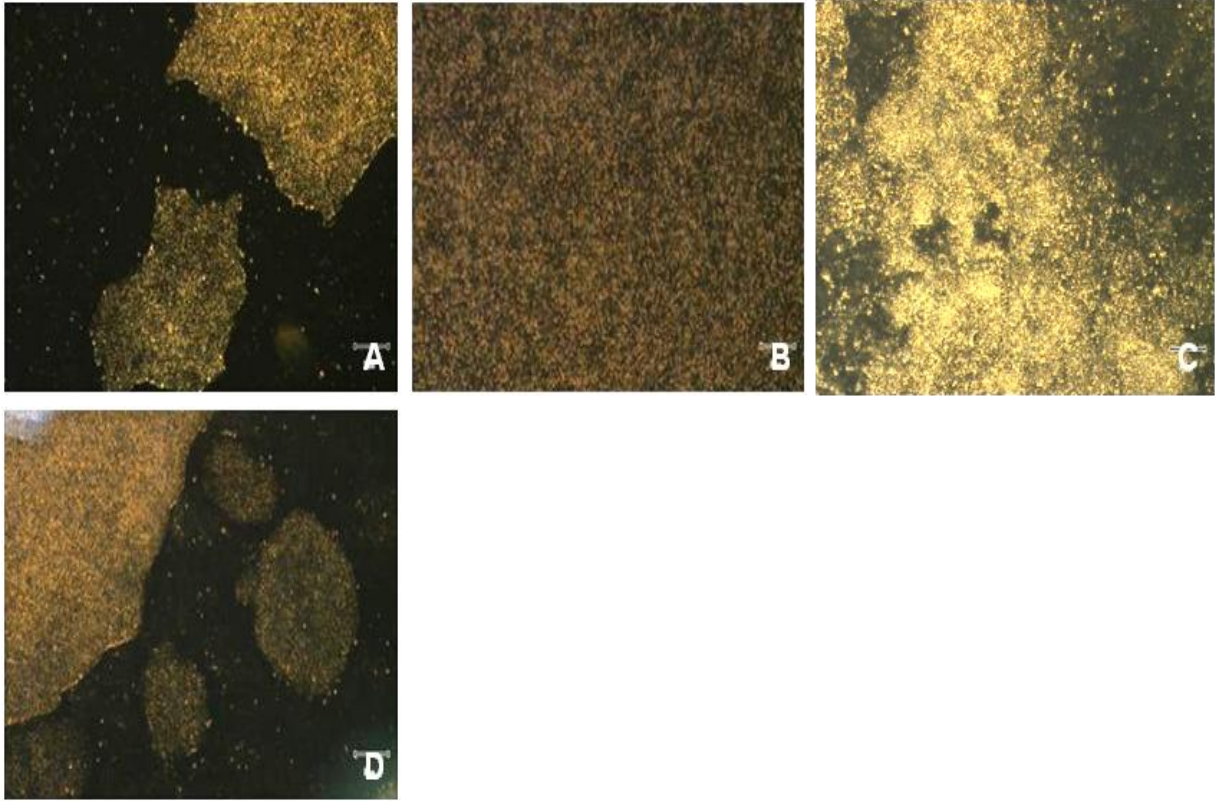


Figure 3.11. Morphology of stem cell lines NCL5 (A), SHEF1 (B), HUES9 (C) and RH5 (D) on Matrigel at late passage (p+20). Cells were dissociated using TrypLE™ Express and seeded onto Matrigel coated plates at a seeding density of 2×10^5 cells/ml. The photographs were taken with a light microscope at x4 magnification. Scale bar: 100 μ m.

3.3.4 Morphology of hESCs cultured on ECM of human feeders

Cells transferred for culture on ECM did display some changes in morphology. Initially, cells proliferated slowly and colonies remained small in size with irregular borders, as demonstrated by all three hESC line HUES3, H9 and NCL5 (Figure 3.12 image A-C). A considerable amount of cell death occurred from all three hESC lines, and NCL5 also exhibited some differentiation around the edges of the colonies. As cells adapted, they became larger in colony size. Care was taken not to over trypsinise the cells when passaging with TrypLE™ Express. After 5 passages, cells had clearly adapted to the ECM and proliferated well forming large colonies with more typical rounded, hESC morphology from all three hESC lines (Figure 3.12 D-F).

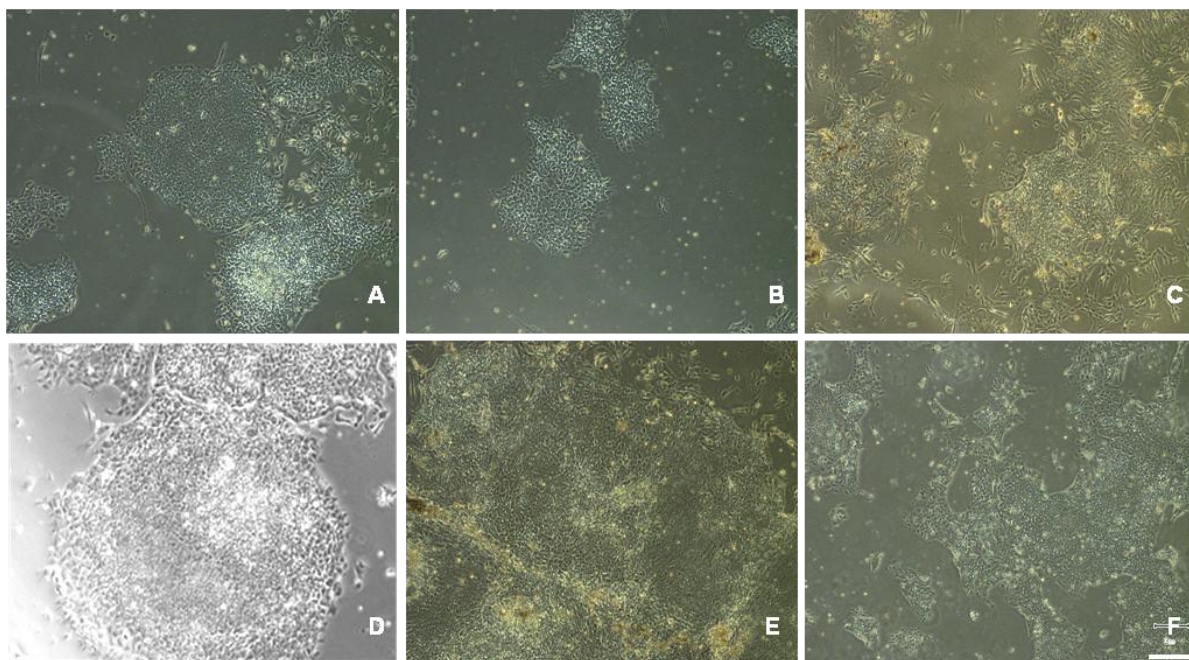


Figure 3.12. Morphology of stem cell lines HUES3, H9 and NCL5 cultured on ECM from human feeder line MRC5. Images A-C at p+1 and D-E at p+5. Cells at passage p+5 were dissociated using TrypLE™ Express and seeded onto the respective human feeders at seeding density of 2×10^5 cells/ml. The photographs were taken with a light microscope at x4 magnification. Scale bar: 100 μ m.

3.3.5 DNA profile, Viral, mycoplasma and sterility testing

Results obtained from the DNA profiling studies were manually checked against the previous DNA profiles from the same samples to confirm that there has not been any cross contamination with other stem cell lines during the extended culture process. As can be seen in Table 3.10, the summary data shows no cross contamination as all cell lines were found to match their original DNA profiles provided by the depositors and did not display additional alleles.

Results for mandatory viral markers are given in Table 3.2. The method of reporting is denoted by 'detected' or 'not detected' and the data obtained show clearly that all cell lines analysed were free of viral contamination. Similarly, no microbial or mycoplasma contamination was found through the extended culture process in any of the stem cell or feeder lines used in this project, as demonstrated by the sterility test reports summarised in Table 3.2. The results demonstrate that good aseptic techniques were effectively used throughout the culture process.

Unfortunately, stem cell line SHEF1 failed to grow beyond P+12 on either human feeders or mouse feeders. This was apparent by slowed growth, small colony formation and differentiation upon further attempted passage.

Cell line	DNA Profile at early passage	DNA profile at late passage
HUE9 on iMEF	Match with DCB	Match with early passage
HUES9 on i3T3	Match with DCB	Match with early passage
HUES9 on iMRC5	Match with DCB	Match with early passage
HUES9 on iHDFn	Match with DCB	Match with early passage
RH5 on iMEF	Match with DCB	Match with early passage
RH5 on i3T3	Match with DCB	Match with early passage
RH5 on iMRC5	Match with DCB	Match with early passage
RH5 on iHDFn	Match with DCB	Match with early passage
NCL5 on iMEF	Match with DCB	Match with early passage
NCL5 on i3T3	Match with DCB	Match with early passage
NCL5 on iMRC5	Match with DCB	Match with early passage
NCL5 on iHDFn	Match with DCB	Match with early passage
SHEF1 on iMEF	Match with DCB	Match with early passage
SHEF1 on i3T3	Match with DCB	Match with early passage
SHEF1 on iMRC5	Match with DCB	Match with early passage
SHEF1 on iHDFn	Match with DCB	Match with early passage

Table 3.1. Summary of DNA profile results of stem cell lines SHEF1, HUES9, RH5 and NCL5 cultured on mouse feeders iMEF at early and late passage.

DNA profiling was carried out by TDL, using an AMP/STR identifier kit (Applied Biosystems), analysing 15 polymorphic autosomal DNA markers. The cell line gender has been identified as male. No contamination with other cell lines was detected, as SHEF1 had the same DNA profile as that obtained from the Distribution cell bank (DCB).

Table 3.2. Summary of sterility, viral and mycoplasma test results for Stem cell lines HUES9, RH5, NCL5 and SHEF1 cultured on mouse, human feeders and Matrigel/MTeSR1 at early and late passage.

Cell line	Sterility test results at early passage	Viral and Mycoplasma test results at early passage	Sterility test results at late passage	Viral and Mycoplasma test results at late passage
HUES9 on iMEF	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
HUES9 on i3T3	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
HUES9 on iMRC5	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
HUES9 on iHDFn	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
HUES9 on Matrigel	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
RH5 on iMEF	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
RH5 on i3T3	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
RH5 on iMRC5	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
RH5 on iHDFn	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
RH5 on Matrigel	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
NCL5 on iMEF	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
NCL5 on i3T3	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
NCL5 on iMRC5	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
NCL5 on iHDFn	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
NCL5 on Matrigel	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected

SHEF1 on iMEF	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
SHEF1 on i3T3	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
SHEF1 on iMRC5	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
SHEF1 on iHDFn	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected
SHEF1 on Matrigel	Contamination not detected	Contamination not detected	Contamination not detected	Contamination not detected

Overall results from three broths TSB, FTM and SAB are reported back from Microbiology after day 14. Results are denoted by a measurement of turbidity. Mycoplasma PCR testing was carried out in-house by NIBSC Cell Supply. These results confirm that no microbial or mycoplasma contamination was detected from any of the cell lines cultured on four feeder types iMEF, i3T3, iMRC5 and iHDFn.

3.4 Discussion

The UKSCB produces and distributes hESC banks for worldwide use under a HTA licence. Thus it is of paramount importance that best practice methods are adhered to. This is partly achieved by following guidelines outlining the minimum standards required for cell and tissue culture known as Good Cell Culture Practice, GCCP (Stacey et al., 2006). Testing includes assessing maintenance of essential characteristics as well as traceability, reporting, quality assurance, training and education and safety. The stem cell lines cultured for this project were subjected to release criteria testing as part of best practice methods (Coecke et al., 2009) and in accordance to UKSCB banking procedures for research grade stem cell lines. Such testing adds value to a cell line, as it assures the end user that a considerable amount of care has been taken when culturing these cell lines. Quality assurance testing is not frequently carried out in academic labs, where a number of cell lines can be cultured at one time, and incubators and safety cabinets are shared. This type of testing is mandatory for cell lines that are to be provided for worldwide distribution.

Contamination is the largest cause to loss of cell cultures and to start with, all the cell lines used were first checked for contamination, focusing not only on bacterial, fungal and mycoplasma but also on identifying cross-contamination with other cell lines. Testing by broth inoculation confirmed that all the cell lines cultured for these studies were free of contamination from bacteria and fungi throughout the culturing process. This provided assurance that the different cell lines cultured on different feeders and feeder free matrices had been cultured with good aseptic technique. The testing was also sufficient to ensure that low levels of microbial contamination could be detected, as the tests are carried out by a Microbiologist as part of a service agreement for the release of the stem cell lines for worldwide distribution. This type of testing is easy to set up, implement perform and is relatively low cost. Many research labs do not routinely carry out microbial testing and instead rely on antibiotics which can mask low level infections.

Mycoplasmas are also a frequent problem for scientists culturing cells and can be detrimental as, once introduced in a laboratory, they spread quickly and can sometimes be difficult to detect at low levels. They are also extremely difficult to remove from contaminated cultures without adversely affecting the cell line.

Contamination with mycoplasma can go unnoticed for many months if routine testing is not carried out. The only sure method is adequate and regular culture testing by at least two methods. The results obtained from both tests confirmed that stocks obtained and subsequent cultures were mycoplasma free. This finding together with the lack of detectable bacterial or fungal infections and maintenance of cell line identity fulfil important criteria for scale-up and banking of cells.

All stem cell lines with the exception of SHEF1 were deemed viable under each of the culture conditions. SHEF1 differentiated extensively after p+12 on both human feeders and mouse feeders and on Matrigel. Further investigation by repeat thaw and culture found this to be due to poor banking of the original project line, and was confirmed by the subsequent thawing of 3 more vials, which had poor viability on thawing and then failed to grow after 3 or 4 passages. Furthermore, a vial of SHEF1 from the distribution cell bank was thawed and found to be viable, confirming the poor viability of the project cell bank. Therefore the SHEF1 line was excluded from further analysis and instead the thesis focused on the hESC lines RH5, HUES9 and NCL5, which all maintained healthy cultures on mouse and human feeders to late passage.

The results concluded that the established hESC cultures were contamination free and could be maintained in culture in a viable state up to high passages. hESCs cultured on human feeders did proliferate well, which has also been confirmed by previous findings (Richards et al., 2002; Hovatta et al., 2006). Distinct morphology changes of hESCs HUES9, NCL5 and RH5 were seen on human feeders as colonies became elongated and elliptical in shape. This was consistent with previous reports (Amit et al., 2003), however, no apparent morphological advantage for culturing hESCs on human feeders compared to mouse feeders such as increased attachment or proliferation was observed. There were no negative observations, such as differentiation or loss of attachment, seen on stem cell growth following the initial 1-2 passages when adaptation occurred. Further characterisation testing will be carried out to confirm these findings. Observations did reveal that the hESCs required contact with the feeder cells to proliferate, either by attachment on top of the mouse feeders, or by attachment in between the human feeders. Further investigation will establish whether human feeders induce genotypic or genomic changes, or negatively affect protein marker expression associated with

undifferentiated hESC culture. Differences in morphology may be due to the way in which each different feeder type supports the growth of different stem cell lines in relation to adhesion, attachment and release of cytokines and growth factors. In this regard, the observations from these studies are consistent with published work, as comparable levels in BMP-4, TGF β 1 and FGF-2 were found in varied amounts from MEF mouse embryonic fibroblasts and HFF human foreskin fibroblasts (Eiselleova et al., 2008). Also mouse feeder cells secreted larger quantities of Activin A than human feeder cells (Eiselleova et al., 2008).

All hESC lines cultured on ECM displayed undifferentiated morphological hESC characteristics. Further characterisation testing is required to confirm that these cells were indeed undifferentiated. The use of ECM from human feeders to support the undifferentiated growth of hESCs has been previously described (Escobedo-Lucia et al., 2010). However, with any cell based culture system, there are heterogeneous differences between batches of prepared ECM. Thus, although preliminary observations from these studies have demonstrated that ECM does support the differentiation of hESCs this would need to be further validated with characterisation testing and until this can be produced on a larger scale with better standardised methodologies, the use of in house ECM was not be continued in this project. For the same reason, the use of i11235 foetal derived fibroblasts will not be continued, however the ease of producing such feeders will allow for the potential creation of clinical grade banks, should appropriate consent for use be allowed. This is also within the remit of UKSCB research work and has been previously discussed (Stacey et al., 2006).

The move to culturing hESCs on defined matrices is important for the progression of stem cells for clinical applications. The results from these experiments demonstrate that there were varying degrees of success when culturing hESCs on Matrigel/mTeSR1, however the ease of adapting hESCs to this culturing system using TrypLE™ Express was apparent. In the first instance, some differentiation was detected in some of the stem cell lines, and they were slower to proliferate when compared with cultures on mouse feeders. However, after a few passages the stem cell lines began to adapt and proliferate steadily, with much less differentiation. As Matrigel/mTeSR1 is a commercial preparation and can be purchased as hESC qualified preparation, further culture and characterisation of undifferentiated hESCs

will be conducted, and will provide a robust comparison against the use of both mouse and human feeder systems. It is however worth noting that there are reports of varying success of culturing different stem cell lines on Matrigel/MTeSR1. For instance there are issues with adapting cell lines onto Matrigel when they have previously been cultured on feeders. Often the cells fail to establish and this has been confirmed at the UKSCB with a number of different stem cell lines. Even when attempting to thaw stem cells directly onto Matrigel there is often significant cell death when compared with thawing onto feeders. Explanations for this may be due to considerable differences in the components within mTeSR1 and Matrigel, such as higher levels of FGF, compared to KO-HES media and MEFs (in house observations).

Local research groups have reported problems with differentiating stem cells once they have been cultured in MTeSR1/Matrigel for long periods of time (in house communication with NIBSC Biotherapeutics and CBI departments). Other groups report the successful differentiation to specific germ lineages using Matrigel (Ludwig et al., 2007; Ma et al., 2008; Lawton et al., 2013). To overcome some of these issues, certain protocols have developed a stepwise process of adapting stem cells to feeder layer free culture by first using Laminin coated plates combined with media such as MEF conditioned media (Hongisto et al., 2011). Nonetheless, these methods can take time and involves the hESC line having to adapt twice, adding to instabilities and time/increase in passage, for the line to adapt. Newer stem cell manuals explore much faster routes to adaptation, which do not include additional steps and instead instruct the thawing of hESCs straight onto Matrigel (Loring and Peterson., 2012; Stem Cell Technologies Handbook 2012) but have yet to be tested with different hESC lines. In these studies, adaption appeared to occur within a few passages. What was notable was that selection of healthy cultures to transfer onto new matrices/media tended to result in successful adaption, compared with those cells which had visible differentiating colonies. Although hESC lines with a larger proportion of differentiated cultures (20- 30%) were manually removed by scraping, positive attempts were made to ensure that entire wells of culture were enzymatically passaged on, to ensure minimal biased and selection occurred from passage to passage. The practice of removing differentiated areas of hESCs before passaging is commonly adopted in many laboratories and has been recommended by Stem

Cell Technologies, where large areas of differentiation occur (personal communication with Stem Cell Technologies).

The successful adaption of cultures on feeder free matrices was achieved simply by passaging at lower ratios using TrypLE™ Express. This allowed greater cell survival on new matrices, and proved to be consistently successful. Greater clonal survival as a result of TrypLE express usage over Trypsin had been previously demonstrated by Ellerstrom et al (2006) in the derivation of Xeno free hESC line. It also removed the step wise process which has been recommended which in turn, streamlined the transition onto different feeder types and Matrigel. hESCs cultured on human feeders were first manually passaged for the first 1-2 passages. As soon as they had settled they were passaged using TrypLE™ Express, to assess the suitability of the enzyme to efficiently expand cells for banking. Previously the standard method for culturing hESCs in-house had been by manual cutting methods. Although different cutting tools have been tested in-house including the StemPro Ezy passage tool™ (Invitrogen) and Biopsy pipettes by Hunter Scientific, little consistency was gained due to operator variability. Also, different enzymes have been tested in house, including Collagenase IV and Dispase. Although these enzymes did provide greater scale up of cell culture, differences in banked vials of cells were observed due to differences in the time that cells were enzymatically treated, which resulted in greater cell death as a result of colony dissociation to single cell (usually due to over trypsinisation of cells, in-house observations). The use of glass beads with Collagenase has also been a recommended method (Andrews and Moore, CSCB, 2008 Handbook). However this method increases time taken to passage cells and introduces variability and potential contamination, as the beads need to be kept sterile.

The use of TrypLE™ Express significantly standardised the method and reduced the time taken to passage stem cells (3-5 minutes per well of a 6 well plate) in comparison to manual dissection which can be time-consuming (up to 30 minutes per plate) and extremely variable, depending on the scientists' experience and tool used for dissection. Manual dissection also requires a lot of precision and patience as bad dissection can lead to poor clump size, which can result in differentiated colonies. Furthermore, when compared with other enzymes, TrypLE™ Express is not as harsh on cultures as Trypsin, even at low concentrations of 0.25% (In house

observations, Invitrogen, product information sheet). In addition it does not require neutralisation with media, which speeds up passaging time and reduces cell death. In addition to the above advantages, passaging of stem cells with TrypLE™ Express did not compromise viability and allowed for the cells to be passaged as clumps rather than single cells which is desirable (Gray et al., 2009). Thus, the use of TrypLE™ Express provides an easy, rapid and robust method for scaling up stem cell cultures. TrypLE™ Express could also be used in combination with manual dissection for difficult or sparse cultures, as differentiated areas can be removed by manual dissection and the remaining undifferentiated areas could then be passaged by enzyme. More recently TrypLE™ Express has been used to culture Neural stem cells (et al., 201) and hESC cells for *in vitro* differentiation experiments (Chetty et al., 2013).

Although research has already demonstrated the successful use of enzymes to passage and scale up hESCs (Ellerstrom et al., 2007; Oh et al., 2005), as well as the use of human feeders to support the undifferentiated growth of hESCs (Eiselleova et al., 2008; Lee et al., 2004; Inzunza et al., 2005) it was of particular importance to the UKSCB to carry out this work, in order to assess, and hopefully progress its own methods used in-house, for banking stem cells.

The widespread use of well-defined media such as mTeSR1™ had led to the development of other better defined media and culture components such as mTeSR2™, E8™, which contains eight essential components (Invitrogen). However reports have yet to confirm the widespread success of much more novel matrices and xeno free media on different hESC lines. This is important to establish because these culture systems can be very costly as they are produced in much smaller batches compared to standard cell culture media.

Despite the emergence of feeder-free systems, culturing stem cells on feeders remains the chosen method for many researchers. This may be because it is not always within a groups' best interest to attempt to use new methods if it has no overall benefit to the endpoint of their research projects, for instance, differentiation of stem cells to mature cardiomyocytes for functional testing. Such projects are often time limited so scientists tend to use methods that work and are routinely used in house rather than testing new systems.

3.5 Conclusion

From the results shown in this chapter, the stem cell lines used for this project were deemed consistent with their previous DNA profiles therefore no cross-contamination had occurred during the stem cell culture process and confirmed to be not contaminated with Mycoplasma, viruses or bacteria/fungi by sterility broth inoculation.

The findings from these experiments demonstrate the suitability of human feeders and Matrigel/mTeSR1 to support the growth of hESCs. Differences in morphology were observed as a result of culture on different feeders and Matrigel. Further characterisation testing by gene expression and protein/cell surface marker studies are required to confirm whether these cells were truly undifferentiated.

These studies have led to the inclusion of Matrigel as an alternative to mouse feeders in the banking of research grade hESCs for worldwide distribution however it has not yet been deemed appropriate to replace MEFs with Matrigel. This is an important and positive step towards the standardisation of banking procedures and an example of the UKSCBs' aim to work with the wider stem cell community by supplying banks of well tested hESCs on feeder free conditions. As a result of this culture experiment, the use of TrypLE™ Express has also been adopted into routine passaging at the UKSCB. Further characterisation studies will help to determine whether human feeders and Matrigel/mTeSR1 maintain hESC growth as well as mouse feeders.

Chapter 4: Characterisation of human embryonic stem cells using immunofluorescence (IF).

4.1 Introduction

Immunofluorescence (IF) is a widely accepted technique, used for the identification of nuclear and cell surface antigens in many different cell types, and is a continually developing field. It is a useful way to identify specific cell populations, particularly in heterogeneous cell cultures by detection of antigens, conjugated to fluorescent antibodies. The improvement of antibodies and fluorophores has enabled dual and triple staining with greater specificity alongside sophisticated microscopes. Immunofluorescence (IF) is frequently used to characterise human embryonic stem cells (Damjanov et al., 1984; Draper et al., 2002; Henderson et al., 2002).

Undifferentiated, pluripotent human embryonic stem cells can be characterised by their unique expression of cell surface markers. The most frequently used were established by the International Stem Cell Initiative (ISCI), which involved 12 participating labs worldwide, with the UKSCB acting as a 'hub' (initiative, 2007). Sixty stem cell lines were characterized using the following stem cell markers for flow cytometry including the glycolipid stage specific embryonic antigens, SSEA3, SSEA4 (Kannagi, 1983; Henderson et al., 2002) keratin sulphate associated proteins TRA1-60, TRA1-81 (Draper et al., 2002; Schopperle and DeWolf, 2007) and nuclear markers octamer binding transcription factor 4, Oct-4 (Nichols et al., 1998; Martin et al., 2004) and homeobox transcription factor Nanog (Chambers et al., 2003). Over or under expression of either one of these transcription factors results in differentiation (Chamber et al., 2007; Korkola et al., 2006). These transcription factors interact directly with DNA targets and additional genes which are also highly expressed in pluripotent cell populations. Alkaline Phosphatase (AP) a hydrolase enzyme responsible for removing phosphate groups from many types of molecules is found in most tissues/organs in the body. It is expressed at elevated levels in hESCs and therefore used as a test of undifferentiated cells. Glycan cell surface antibody SSEA1 is used as an early indicator of differentiation. These markers are also shared by mouse embryonic stem cells, but the expression of SSEA1 and SSEA4 are reversed (as demonstrated by Table 4.1).

Flow cytometry employs the technique of measuring cellular properties as they move through a steady fluid stream past stationary detectors and works by using the basic properties of each cell type; its ability to absorb fluorescence and scatter light, based on how they react with specific cell surface and nuclear markers. This information is

translated into forward scatter and side scatter, which is captured and transmitted to a screen where it can be analysed. Flow cytometry is based on the detection of fluorescence; therefore fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a higher wavelength than the light-source.

Currently, most IF staining of stem cells *in situ* is carried out on fixed cultures on cover slips or on 8/16 well glass chamber slides (Turksen et al., 2010). In the past, such techniques were used as they do not require large numbers cells and are more cost effective due to smaller amounts of antibody. However, it was found that these fixed cultures were very fragile and had a tendency to disassociate during staining and/or washing steps. Therefore a new in-house method for IF staining, using fixed cultures on 6 or 24 well plates was developed. It was found that this gives greater representation of how stem cells grow *in situ* and allows for more statistically relevant data.

Stem cells are assessed qualitatively using IF and quantitatively using flow cytometry (Draper et al., 2002; Carpenter et al., 2003; Menendez et al., 2006; Ungrin et al., 2007). However, significant information about the morphology and co-location of cells within a heterogeneous population is not captured. Such information can give significant insight into the way in which hESCs proliferate and express stemness cell surface markers as a result of prolonged culture on different feeder types and Matrigel. Studies of hESCs have demonstrated the importance of understanding the relationship of cells within populations and their inherent differences at single cell level has been discussed (Enver et al., 2005; Stewart et al., 2006; Hough et al., 2009). The significance of such events for whole cell populations have been demonstrated through the formation of clones from a single hESC line and shown that the clones expressed different germ layer specific markers when differentiated (Sidhu & Tuch 2006). This highlights the importance of producing homogenous populations of hESCs. However, methods for accurately detecting and quantifying heterogeneous populations in different hESC lines have yet to be developed. Furthermore the use of such techniques to determine the consequences of long term hESC culturing using different matrices has not been demonstrated. New high throughput imaging and analysis technology has emerged to fill this void.

4.1.2 The TissueFaxes™ fluorescence image acquisition and analysis

The TissueFaxes™ is an imaging system consisting of a Zeiss fluorescent microscope, computer hardware with scanning software and high resolution screens combined with TissueQuest™ software which allows for quantitative image analysis. The system uses either a plate or slide format from which tissue sections or fixed cell samples can be scanned as a preview (x5 objective lens), and regions of interest captured through image acquisition by multichannel colour technology, to allow for accurate visualisation of cells/tissue (x20 objective lens). Scanned images are imported into TissueQuest™ analysis software and quantitatively analysed using a number of different cell/tissue based parameters. The software allows for forward and back gating of individual cells/tissue sections and the setting of cut offs for fluorescence intensity, to give accurate analysis, similar to flow cytometry (Streit et al., 2006). The TissueFaxes™ has been used for a number of different applications including the investigation of stromal-epithelial cells in prostate cancer (Massoner et al., 2008), the cerebroprotective effect of T cells in experimental Stroke (Liesz et al., 2009), the effects of macrophages on chemotherapeutic response in breast cancer (Shree et al., 2012).

The TissueFaxes™ allows for microscopy based fluorescent cytometry of fixed colonies by automated processing of multi-labelled samples *in situ*. Thousands of images can be imported into the TissueQuest™ software, and using the software's algorithms, can be set up to recognise patterns of cells which can be analysed, quantified and represented by dot plots. The system is able to capture not only the morphology of the colonies, but the location and number of colonies present. This type of technique has not previously been used to characterise the differences between cell surface marker expression of hESCs following long term culture on mouse and human feeders and Matrigel/mTeSR1™ cultures. Obtaining detailed information from such culture conditions will emphasise the need for better standardisation of techniques, and give insight into how best to achieve homogenous populations of hESC lines, a key objective when producing uniform banks of hESCs. This has therefore been the focus of the studies described in this chapter.

Table 4.1. The similarities and differences between expression of cell surface markers in mouse embryonic stem cells, hESCs Embryonic Carcinoma cells and Embryonic Germ cells.

Cell-type	AP	SSEA-1	SSEA-4	TRA-1-60	TRA-1-81
Mouse ES Cell	✓	✓	--	--	--
Mouse EG Cell	✓	✓	--	--	--
Human ES Cell	✓	--	✓	✓	✓
Human EG Cell	✓	✓	✓	✓	✓
Human EC Cell	✓	--	✓	✓	✓

ES cell = Embryonic stem cell
 EG cell = Embryonic germ cell
 EC cell = Embryonic carcinoma cell

4.2 Methods

4.2.1 Stem cell culture

The four stem cell lines, NCL5, HUES9, RH5 and SHEF1 were cultured as described in Chapter 2 over 20 passages on the two mouse feeder's i3T3 and iMEF two human feeders, iMRC5 and iHDFn in standard KO-HES media. In parallel experiments, Matrigel was also used together with mTeSR1 media.

4.2.2 Preparation of cells for flow cytometry

Stem cells were cultured in flat bottomed six well plates, and treated with TrypLE™ Express to dissociate cells for approximately 3 to 4 minutes. Remaining hESC clumps were broken up and feeder cell clumps removed by careful pipetting. Stem cells were centrifuged at 300g for 5 minutes, supernatant removed and cells fixed in 4 % PFA for 20 minutes at room temperature, washed 3 times using wash buffer and stored in wash buffer at 4°C, in 15 ml tubes until ready for staining.

Stem cells were stained with primary and secondary antibodies according to the antibody list and dilutions in Table 4.2 and Table 4.3. SSEA-1, SSEA-3, TRA-160 and TRA1-81 were grown and purified in-house (UKSCB, NIBSC) using hybridoma cell lines obtained from the Centre for Stem Cell Biology, (CSCB) Sheffield by fusing a specific antibody producing B cell with a non-antibody producing cancer cell (usually myeloma or lymphoma). The antibodies produced by the hybridoma are of single specificity (monoclonal). Hybridomas were initially thawed and cultured in T175 flasks using Dmem/F12 media, for 1-2 weeks and passage once a week at 1:20 ratio. Scale up culture of hybridomas was carried out using roller bottles (Corning), until 5 litres of each antibody had been produced within the spent media. Media was carefully twice filtered and aliquoted into 1ml aliquots and stored at -80°C. Each hybridoma was tested for suitability using various hESC lines and n2102EPs, against a commercial antibody to ensure detection by flow cytometry and IF staining was equal.

All staining experiments were carried out in a v bottomed 96 well plate, then transferred to flat bottomed 96 well plates, and the final volume adjusted to 300 µl using Wash buffer. The plate was loaded onto the Guava™ Flow cytometer (Millipore, Guava EasyCyte W/SSC) for the cells to be processed. The embryonal carcinoma cell line n2102EP (CSCB, Andrews et al, 2002) was routinely set up as a

positive control (Shevinsky et al., 1982; Josephson et al., 2007) and stained on the same plate. The analysis was completed on the Guava using CytoSoft 3.6.1 ExpressPlus programme. The results were exported to an Excel spread sheet to produce bar charts, to visually compare expression of cell surface markers between the different samples.

4.2.3 Preparation of cells for *in-situ* staining

Stem cells for *in situ* staining were cultured on flat bottomed 24 well plates (FB24), until 70-80% confluent. Plates of cells were then fixed in 4% PFA for 20 minutes at room temperature, washed 3 times using wash buffer and stored in wash buffer at 4°C until ready for staining.

Cells were stained with primary antibody listed in Table 4.3 for 1 hour minimum at room temperature in the dark (maximum overnight at 4°C) then washed in Washbuffer before secondary antibody staining according to Table 5.4 for 1 hour minimum at room temperature in the dark (maximum overnight at 4°C). The plates were then carefully washed and stained using DAPI nuclear stain for 5-8 minutes, washed using DH₂O and topped up with 2 mls of Washbuffer before being placed onto the microscope for imaging.

The diagram below (Figure 4.1) shows the similar workflow for both techniques with the exception that TissueFacs™ analysis included image selection of field of views (FOVs).

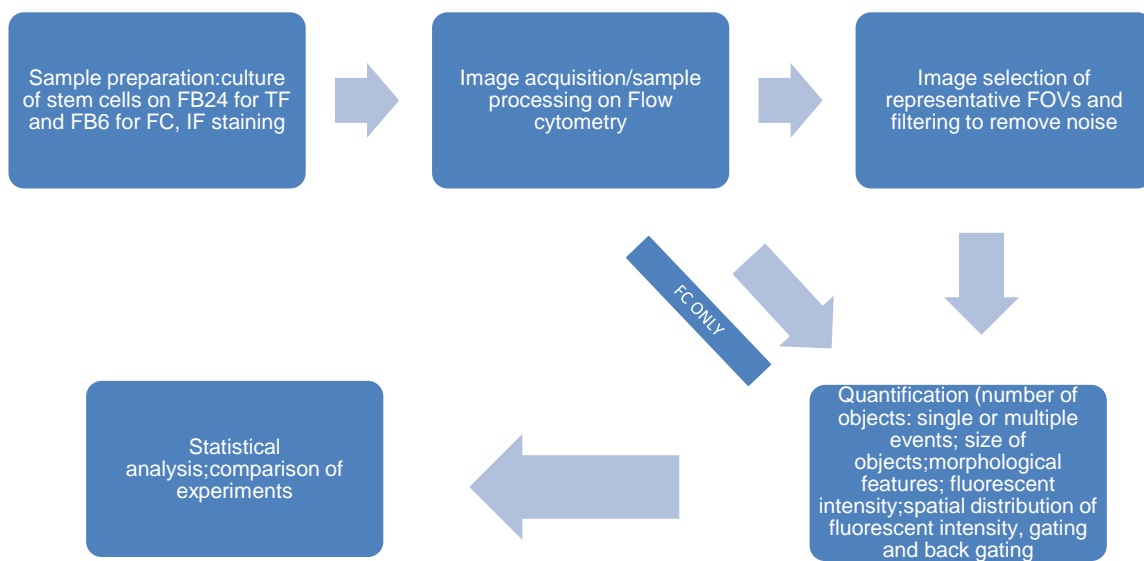


Figure 4.1. Schematic showing process of preparing samples for IF by Flow Cytometry (FC) and TissueFaxs™ (TF).

Table 4.2 Details of primary antibodies and Isotype controls used for Flow cytometry and *insitu* fluorescence staining.

Antibody	Subtype	supplier	Cat number	Dilution for TissueFaxes™	Dilution for Flow Cytometry
IgM	Mouse IgM	Caltag	MGM00	1/100	1/100
TRA-1-60	Mouse IgM	Hybridoma	Made in house	1/5	1/5
TRA-1-81	Mouse IgM	Hybridoma	Made in house	1/5	1/5
SSEA1	Mouse IgM	Hybridoma	MC-480	1/5	1/5
IgG3	Mouse IgG3	Abcam	AB18392	1/50	33µg/ml (1/50)
SSEA4	Mouse IgG3	Abcam	Ab16287	1/30	33µg/ml (1/50)
Rat IgM	Rat IgM	Caltag	RGM004	1/50	1/50
SSEA3	Rat IgM	Hybridoma	MC-631	1/50	1/5

For the majority of IF staining, hybridomas were used, as they were cultured in abundance at low cost (in house preparation), and could be used at the same concentration for both types of techniques. From the eight antibodies (and isotype controls) used, four were commercial, SSEA4 (Abcam), Isotype controls Mouse IgM (Caltag), Rat IgM (Caltag) and Mouse IgG3 (Abcam).

Table 4.3. Secondary antibodies used for flow cytometry and in-situ fluorescence staining.

Antibody	Supplier	Cat number	Dilution
Mouse IgM + IgG FITC	Caltag	LM30801	1/100
Goat anti-mouse (PE) IgM	Caltag	M31504	1/100
Alexafluor 647 (CY5) Goat anti rat IgM	Invitrogen	A21248	1/200
Alexafluor 488 (FITC) Goat anti-mouse IgG	Invitrogen	A11017	1/200
DAPI nucleic acid stain	Invitrogen	D1306	1/1000 NB: only used for <i>insitu</i> staining

SSEA3, TRA-1-60 and TRA-1-81 and their respective isotype controls (see table) were all grown and purified in-house (UKSCB, NIBSC) using Hybridoma cell lines obtained from the Centre for Stem Cell Biology, (CSCB) Sheffield. All the secondary antibodies used were consistent for each technique throughout this study. Only PE and FITC conjugated secondary antibodies could be used for flow cytometry on the Guava EasyCyte, as it has lasers for the detection of only PE and FITC channels.

4.2.4 Analysis of flow cytometry results using Guava EasyCyte flow cytometer

Raw data from guava results, were exported from the Cytosoft software v.0.6 into an excel file and an average calculated from two technical repeats for each sample. With the exception of NCL5 stem cell line which was repeated 3 times (n=3), all other samples were repeated only twice (n=2) as a screening process to determine whether the Guava flow cytometer was a suitable tool to test a number of different stem cell lines.

4.2.5 Statistical analysis of flow cytometry data

Differences between cell lines and feeders were assessed by fitting a general linear model (GLM) using Duncan's method for pairwise comparisons and Two way analysis of variance (2 way ANOVA).

Table 4.4. List of antibodies and secondary fluorophores used for *in situ* staining of hESCs by TissueFacs™ using three different colour channels.

ANTIBODY	FLOUROPHORE CHANNEL
NUCLEAR	DAPI-BLUE
SSEA4	CY5 RED
SSEA3	FITC GREEN
TRA-1-60	PE YELLOW
TRA-1-81	PE YELLOW

4.2.6 TissueFaxes™ analysis for quantitative imaging

Raw TissueFaxes™ image files were imported into TissueQuest™ analysis software. DAPI fluorescent channel was selected and applied as a cell identifier to initially recognise the cells. The in-built algorithms calculated the amount of fluorescence signal attached to each cell. Masks were created to identify cells, using a set of user-defined parameters to label areas of cells; comparison to background, by area and grey, size of cell, overall background threshold. Parameters used to create masks are shown in Table 4.5 and Table 4.6. The parameters for each stem cell line cultured on each feeder type had to be adjusted to accurately mask, and capture the cells. The software then calculated the amount of fluorescence in each colour channel and produced dot plots which were checked to ensure that every population of stem cells were in the correct quadrant on the scatterplot. This was done by back-gating to locate single cells, to correctly identify positive/negative stained cells and then set cut offs, gate relevant populations (as carried out in flow cytometry) and remove cells which were not of interest and may have skewed the results (debris, doublets, bright artefacts). The TissueFaxes™ images were then copied from each PDF project (see Figure 4.2 for example) and their corresponding scatterplots with tables showing quantitative data which relate to the amount of cell surface expression from each image as a percentage. Each of the scatterplots and tables displayed are an average of the most appropriate two 'Field of views' (FOVs) for each fluorescent channel/antibody used. Other parameters such as the size of the nucleus, and the number of cells/mm² are also measured by the software.

Tables 4.5 and 4.6 show the parameters chosen to create the cell masks for the fluorophores DAPI (nuclear), CY3, CY5 and FITC used in TissueFax analysis, for Stem cell line RH5 cultured on mouse fibroblast line i3T3 at late passage p+20. Unfortunately the same parameters could not be used for each sample, therefore parameters for each stem cell line/feeder type had to be optimised to accurately mask and gate the cells imaged. Figures 4.3 and 4.4 show an example of the types of scatterplots produced from the analysed TissueFaxes™ results. Many images were produced as a result of using a high throughput imaging system, therefore the best images were selected on a basis of their clarity and how accurately they reflected the other images taken from the culture conditions (i.e. stem cell line cultured on mouse/human feeder or Matrigel).

HUES9 on iHDFN late passage - Region 024 SSEA3 TRA181

Institution: , ,
 User: Administrator
 Date of analysis: Monday, 27 August 2012, 17:17:54
 Detection method: Various Shapes

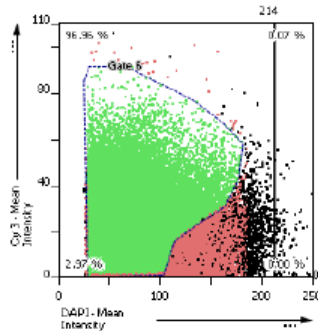


DAPI - nuclei size: 7
 - discrimination by area: 2
 - discrimination by gray: 0
 - background threshold: 23

Cy 3 - ring mask: (-1, 30)
 - max growing: 100
 - offset from nuclei: 1
 - background threshold: auto [5, 255]
 - nuclei mask: no

Cy 5 - ring mask: (-1, 2)
 - max growing: 70
 - offset from nuclei: 2
 - background threshold: auto [5, 255]
 - nuclei mask: yes

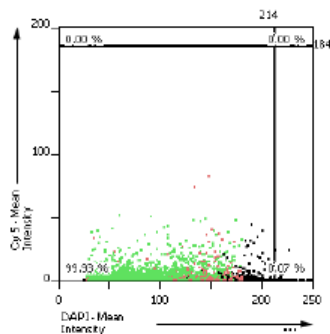
FITC - ring mask: (-1, 5)
 - max growing: 87
 - offset from nuclei: 2
 - background threshold: auto [5, 255]
 - nuclei mask: yes



All events in the scattergram					
	X-Mean	Y-Mean	Events	No./mm ²	%
UL	69.00	16.35	109612	67187.80	96.96
UR	223.84	18.40	79	48.42	0.07
LL	54.61	0.00	3355	2056.48	2.97
LR	0.00	0.00	0	0.00	0.00
SUM	68.68	15.86	113046	69292.71	100.00

Gate 5					
	X-Mean	Y-Mean	Events	No./mm ²	%
UL	67.27	16.29	107162	65686.05	96.97
UR	0.00	0.00	0	0.00	0.00
LL	54.56	0.00	3348	2052.19	3.03
LR	0.00	0.00	0	0.00	0.00
SUM	66.89	15.79	110510	67738.25	100.00

Gate 6					
	X-Mean	Y-Mean	Events	No./mm ²	%
UL	62.08	18.99	87757	53791.56	100.00
UR	0.00	0.00	0	0.00	0.00
LL	0.00	0.00	0	0.00	0.00
LR	0.00	0.00	0	0.00	0.00
SUM	62.08	18.99	87757	53791.56	100.00



All events in the scattergram					
	X-Mean	Y-Mean	Events	No./mm ²	%
UL	0.00	0.00	0	0.00	0.00
UR	0.00	0.00	0	0.00	0.00
LL	68.58	0.08	112967	69244.29	99.93
LR	223.84	0.36	79	48.42	0.07
SUM	68.68	0.08	113046	69292.71	100.00

Gate 5					
	X-Mean	Y-Mean	Events	No./mm ²	%
UL	0.00	0.00	0	0.00	0.00
UR	0.00	0.00	0	0.00	0.00
LL	66.89	0.08	110510	67738.25	100.00
LR	0.00	0.00	0	0.00	0.00
SUM	66.89	0.08	110510	67738.25	100.00

Gate 6					
	X-Mean	Y-Mean	Events	No./mm ²	%
UL	0.00	0.00	0	0.00	0.00
UR	0.00	0.00	0	0.00	0.00
LL	62.08	0.09	87757	53791.56	100.00
LR	0.00	0.00	0	0.00	0.00
SUM	62.08	0.09	87757	53791.56	100.00

Figure 4.2. Screen print of PDF results from TissueQuest software of analysed result of the HUES9 cell line on iHDFn feeders at passage 20. This PDF shows the corresponding Scattergram and table of all acquired events and demonstrate SSEA3/TRA-1-81 co-staining. A large gated area of TRA-1-81 positive events is visible in the first scatterplot (top, left plot), denoted by the green gated region. Other parameters such as the size of the nucleus, and the number of cells/mm² are also measured by the software and displayed in the results.

Table 4.5. Parameters used to create cell masks for DAPI nuclear stain for TissueFaxs™ analysis.

Fluorophore/Parameter	DAPI
Nuclei size	10
Discrimination by area	6
Discrimination by grey	0
Background threshold	12

Table 4.6. Parameters used to create cell masks for CY3, CY5, and FITC Cytoplasmic stains for TissueFaxs™ analysis.

Fluorophore/Parameter	CY3	CY5	FITC
Ring mask	-1,15	-1,15	-1,5
Max growing	75	75	75
Offset from nuclei	5	1	1
Background threshold	Auto detect	Auto detect	Auto detect
Nuclei mask	yes	yes	yes

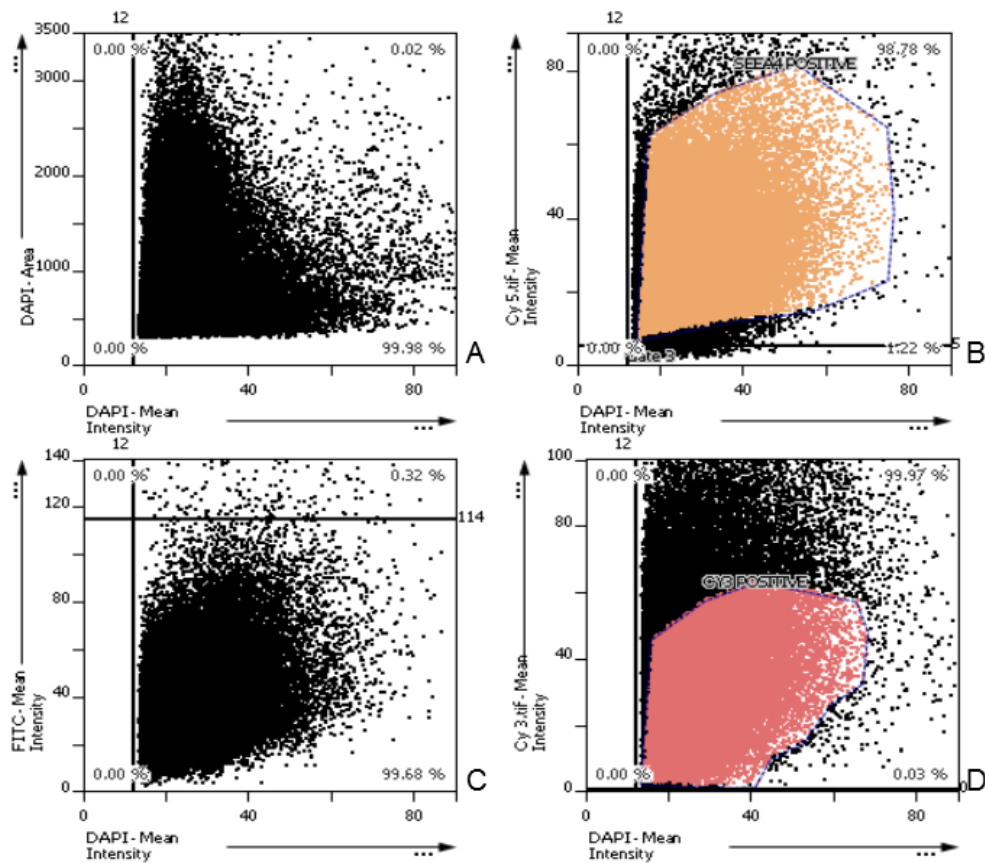


Figure 4.3. Example of scattergrams showing distribution of stem cells by their size and staining intensity for each channel; A: Dapi nuclear marker, B: CY5 SSEA4, C: FITC SSEA3, D: CY3 TRA-1-60 and TRA-1-81. Following staining, image acquisition and detection by the TissueQuest software, DAPI nuclear marker was selected as the Master channel as it was easy to identify all the imaged cells. Each cell represents a single event (dot) on the scatterplot. All events are gated against the master channel for mean intensity of fluorescence staining, then subsequent fluorescent channels are gated against their mean intensity of fluorescence staining, for example FITC mean intensity (Y axis), and Dapi mean intensity (x axis) to form the scattergram. Data from each quadrant region, formed as a result of creating cut-offs and gates, is also displayed as a table. The scatterplots displayed above show the variations in fluorophore intensity obtained. Gating and backgating are used to group the events based on their similarities of size and intensity, thus allowing reliable detection of informative events, which can then be accurately quantified.

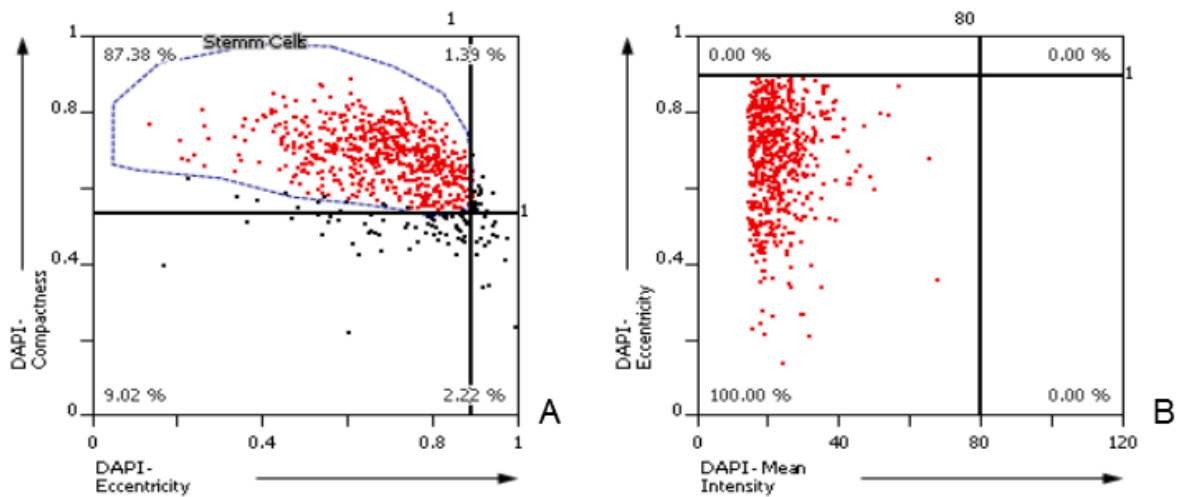


Figure 4.4. Example of scattergrams showing distribution of stem cells gated by unique parameter settings DAPI eccentricity and DAPI compactness ratio. Stem cells were stained with DAPI and gated using parameters such as DAPI eccentricity and DAPI compactness ratio, which were calculated based on the shape of the cell, compared to a perfect circle (giving a value of 1) or if the cell is elongated, (value of 0). The parameters were used to distinguish different cell types on the basis of their shape, for example doublets, clumps of cells and feeder cells. They were also used to detect stem cells cultured on different feeder types, as it has been observed that some hESC lines proliferate into much larger colonies than others, when cultured on human feeders.

4.3 Results

4.3.1 Changes in expression of undifferentiated hESC markers for the RH5 stem cell line by flow cytometry

Initially, changes in the expression profiles of stemness markers (SSEA3, SSEA4, TRA-1-60, and TRA-1-81) under the different culture conditions (RH5 cultured on iMEF, i3T3, iMRC5, iHDFn and Matrigel) were detected using the standard, well established flow cytometry method. As shown in Figures 4.5 A and B, hESC undifferentiated markers TRA-1-60 and TRA-1-81 were more highly expressed than SSEA3 and SSEA4, which, although were expressed in lower abundance, were still detectable. By comparison TF analysis showed much greater sensitivity across all samples with much higher expression levels of all the markers. In particular SSEA3 and SSEA4 which showed relatively low levels of expression by FC, were found to be almost 8 or 9 times more highly expressed by TF analysis (Table 4.7). This clearly supports the claim that TF would be a much more sensitive and thus useful technique for detecting cell surface marker expression in hESCs. The Figures demonstrate that human feeders and Matrigel maintained higher expression of undifferentiated markers compared with mouse feeders. TRA-1-60, TRA-1-81 and SSEA3 expression increased when cultured on human feeders from early to late passage. A decrease in SSEA4 expression was shown from all feeder types and Matrigel at late passage. Expression of differentiated marker SSEA1 was highest from cultured on the iHDFn human feeders at early passage, but this decreased at late passage. Expression of undifferentiated markers from cultures on mouse feeder i3T3 was low, in comparison to the other feeder types.

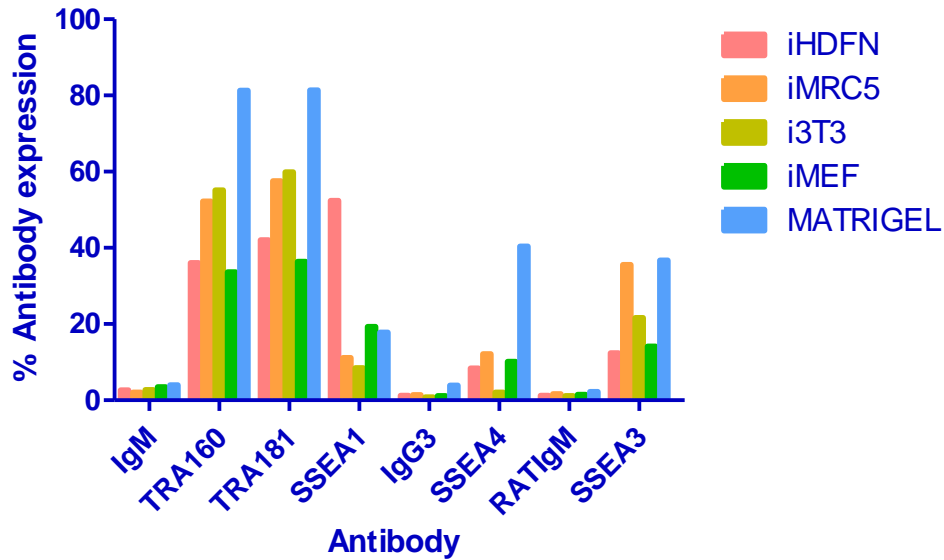
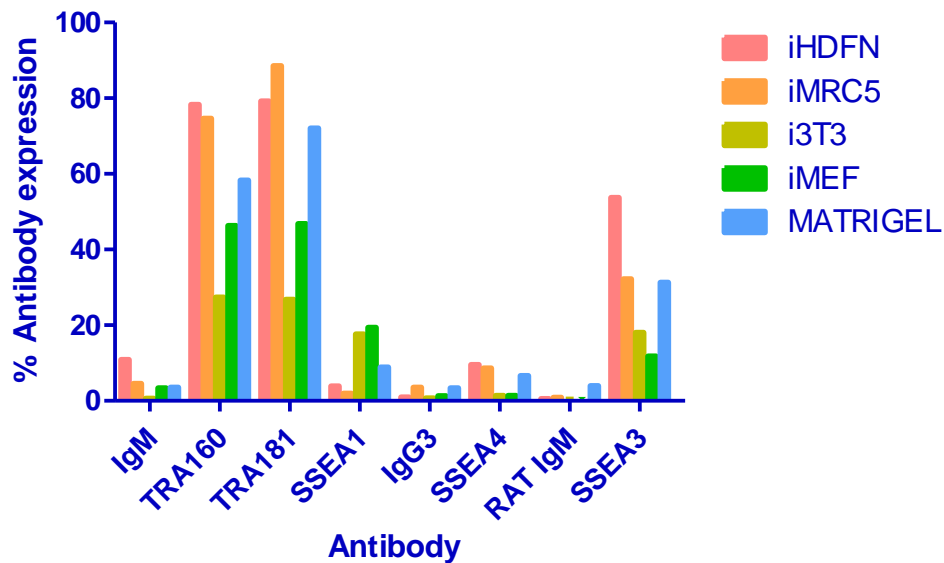
A**B**

Figure 4.5. Effect of Matrigel and different feeder types on undifferentiated marker expression in RH5 cell line at early (A) and late (B) passage. (n=2). RH5 stem cells were cultured at early (P+5) and late passage (P+20) on mouse and human feeders and Matrigel, and prepared for flow cytometry as described by the methods (section 2.8). The data is the mean of 2 separate experiments.

4.3.2 Changes in expression of undifferentiated hESC markers for the RH5 stem cell line by TissueFaxs™ image analysis

Positive undifferentiated hESC marker expression was observed from RH5 cultured on iMEF mouse feeders as shown by the TissueFaxs™ images in Figure 4.6. Differences in morphology were clearly observed by RH5 cultured on iMEFs compared with human feeders (as discussed in chapter 3). As demonstrated in Figure 4.6D colonies of RH5 formed a doughnut shaped appearance when stained for SSEA3. The cells on the outside of the colony are clearly proliferating as they are positively stained and form a visible ring, whereas the cells within the ring may be older and no longer proliferating. Smaller, less visible rings of SSEA4 proliferating cells can also be seen in Figure 4.6C. Figures 4.6E and 4.6F show positive expression of TRA-1-60 and TRA-1-81, respectively. This pattern of staining was not observed from RH5 cultured on human feeders where the latter were better distinguished from stem cells. The iHDFns, unlike the mouse feeders were larger and more elongated (Figure 4.7A and 4.7B). Consequently, RH5 stem cells took on a different morphology when cultured on human feeders and grew in swirls and elongated colonies. Although positive SSEA4 staining was similar on mouse and human feeders (Table 4.7), staining patterns for undifferentiated markers SSEA3 (Fig.4.6D and Fig.4.7D) and both TRA-1-60 (Fig. 4.6E and Fig 4.7.E) and TRA-1-81 (Fig.4.6F and Fig.4.7F) are very different. This is most probably due to the different feeder types.

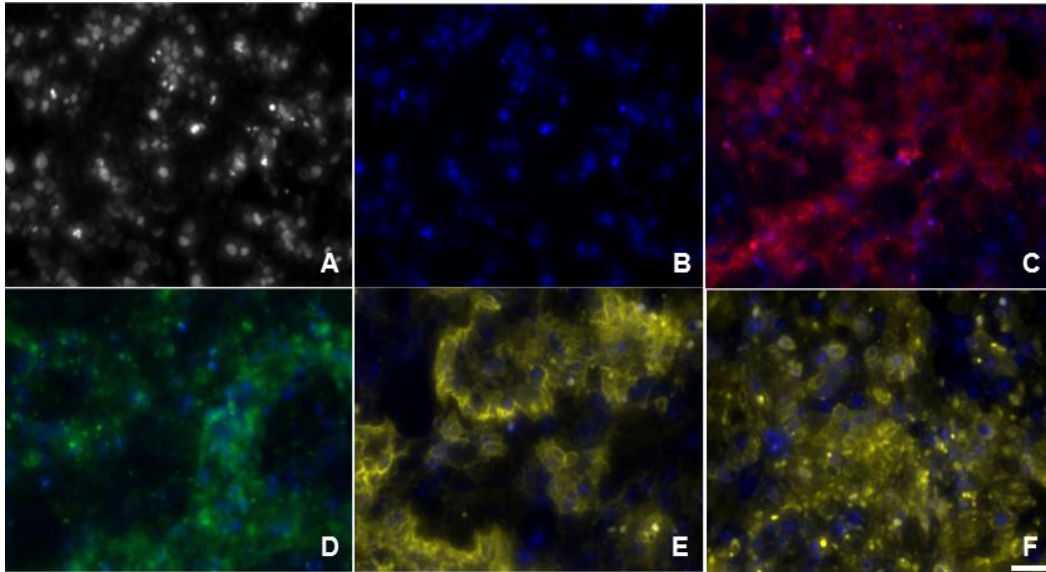


Figure 4.6. IF TissueFaxes™ images for RH5 stem cell line cultured on iMEF mouse feeders demonstrating expression of stemness markers at late passage. RH5 stem cells were cultured on iMEF mouse feeders at late passage on 24 well plates until confluent. Plates were fixed with 4% PFA for 20 minutes, then stained as described in the methods (2.9). Images taken by TissueFaxes™ imaging system show A: Grey scale DAPI, B: colour DAPI and positive expression of stemness markers C: SSEA4, D: SSEA3, E: TRA-1-60, F: TRA-1-81. All images were captured at x20 magnification. Scale bar = 100µm.

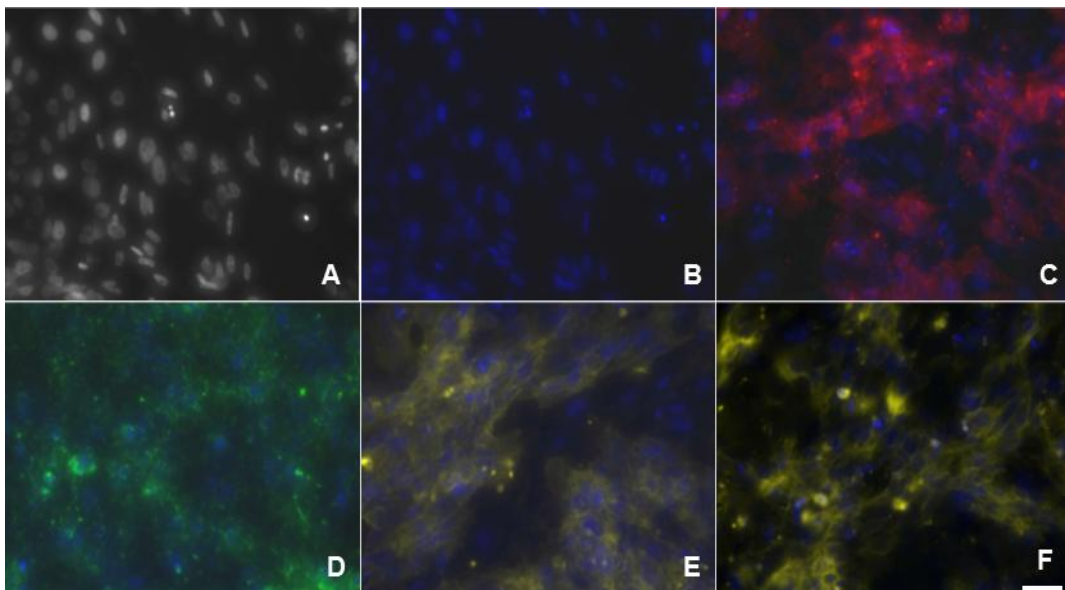


Figure 4.7. IF TissueFaxes™ images for RH5 stem cell line cultured on iHDFn human feeders demonstrating expression of undifferentiated hESC markers at late passage. RH5 stem cells were cultured on iMEF mouse feeders at late passage on 24 well plates until confluent. Plates were fixed with 4% PFA for 20 minutes, then stained as described in the methods (2.9). Images taken by TissueFaxes™ imaging system show A: Grey scale DAPI, B: colour DAPI and positive expression of stemness markers C: SSEA4, D: SSEA3, E: TRA-1-60, F: TRA-1-81. All images were captured at x20 magnification. Scale bar = 100µm

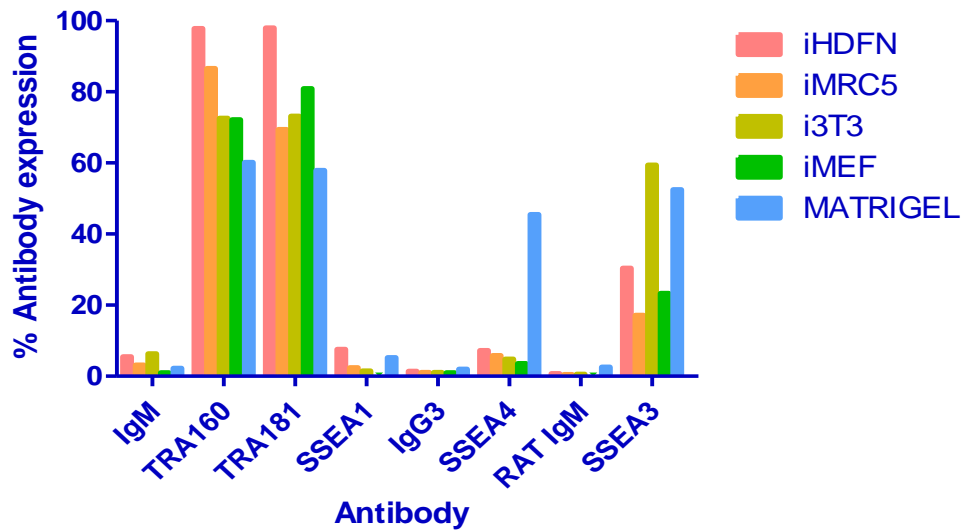
Table 4.7. Comparison of percentage expression of undifferentiated markers for RH5 stem cell line at late passage by flow cytometry (FC) and by TissueFaxs™ image analysis (TF).

STEM CELL LINE/FEEDER	SSEA3 by TF	SSEA3 BY FC	SSEA4 BY TF	SSEA4 BY FC	TRA-1-60 BY TF	TRA-1-60 BY FC	TRA-1-81 BY TF	TRA-1-81 BY FC
RH5/iMEF	89.7±1.43	11.8	83.7±1.27	1.4	53.3±1.33	46.3	93.0±16.00	46.7
RH5/i3T3	99.6±1.56	18.0	88.7±2.44	1.4	74.7±1.27	27.3	82.9±1.28	26.8
RH5/iMRC5	84.6±0.14	32.1	94.6±0.13	8.6	76.9±0.21	74.6	99.7±0.14	88.5
RH5/iHDFn	99.8±0.17	53.6	99.0±0.17	9.52	99.3±0.72	78.3	93.1±0.71	79.1
RH5/Matrigel	87.25±1.20	31.3	72.90±0.35	6.61	77.35±2.50	58.3	81.54±4.33	72.0

4.3.3 Changes in expression of undifferentiated hESC markers for HUES9 stem cell line by flow cytometry

Detection of positive stemness markers from HUES9 confirmed the difference in sensitivity between FC and TF (Figures 4.8 A and B). FC showed relatively low expression of the markers: SSEA3 and SSEA4, from all feeder types. But these were much more significantly expressed when detected by TF (Table 4.8). TRA-1-60 and TRA-1-81 expression was high, particularly from cultures on iHDFn at early passage, although cultures on Matrigel and i3T3 mouse feeders had the highest TRA-1-60 and TRA-1-81 expression at late passage. HUES9 cultured on i3T3 had the highest expression of SSEA3 at early passage. Expression of early differentiation marker SSEA1 was less than 10% on all four feeder types. Cultures on Matrigel also maintained higher expression of all undifferentiated markers at late passage.

A



B

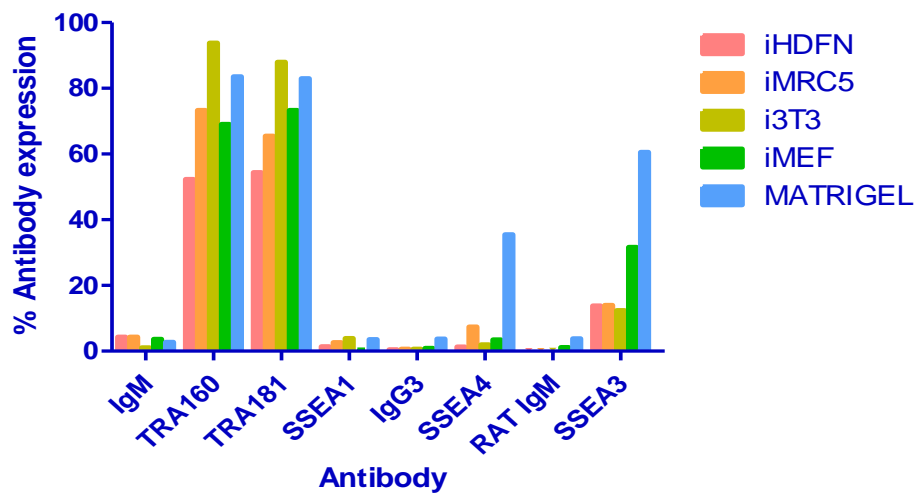


Figure 4.8. Effect of Matrigel and different feeder types on undifferentiated marker expression in HUES9 cell line at early (A) and late (B) passage. (n=2). HUES9 stem cell line were cultured at early (p+5) and late (p+20) passage on mouse and human feeders and Matrigel and prepared for flow cytometry as described by the methods (section 2.8). The data is the mean of 2 separate experiments.

4.3.4 Changes in expression of undifferentiated hESC markers for HUES9 stem cell line by TissueFaxs™ image analysis

Images from Figure 4.9 and 4.10 clearly demonstrate positive stemness marker staining from HUES9 cultured on iMRC5 human feeders and i3T3 mouse feeders. A very typical HUES9 morphology is shown in Figure 4.10 as this stem cell line grew in confluent monolayers on both feeder types. HUES9 cultured on iMRC5 human feeders grew in swirly monolayers and were also larger than the stem cells which grew on i3T3s. This affected the quality of the images which were poor, as the camera could not detect or accurately focus the stem cells cultured on a bi-layer. It also affected the type of masking required to detect and distinguish HUES9 from human feeders to that used to detect HUES9 grown on mouse feeders, which was difficult and time consuming.

The IF images shown in Figures 4.11 and 4.12 demonstrate positive stemness marker staining for HUES9 cell line cultured on iMEF and iHDFn. The morphology observed suggest that the newly proliferating stem cells push the older cells to the top of the monolayer, which may explain why the top layer of cells are strongly DAPI stained, while those underneath stain for undifferentiated markers, as shown by SSEA4 the positive staining in Figure 4.12C. The monolayer culture of proliferating stem cells does however appear to be peeling as shown in Figure 4.11.C and 4.11.E, despite continued expression of SSEA4 and TRA-1-60.

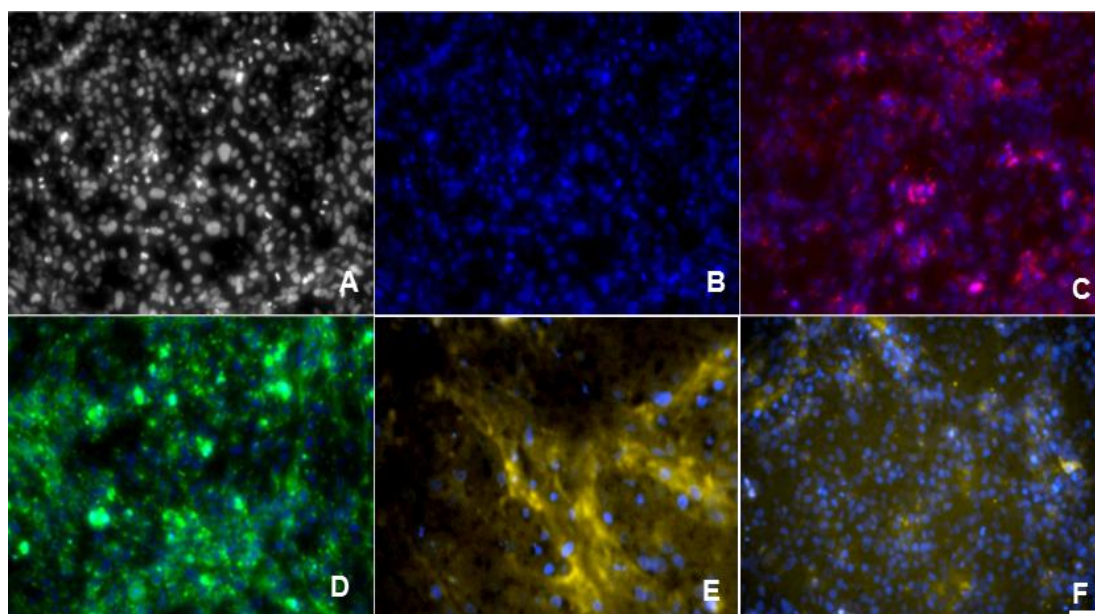


Figure 4.9. IF TissueFaxes™ images for stem cell line HUES9 cultured on iMRC5 showing expression of undifferentiated markers at late passage. HUES9 stem cells were cultured on iMRC5 mouse feeders at late passage on 24 well plates until confluent. Plates were fixed with 4% PFA for 20 minutes, then stained as described in the methods (2.9). Images taken by TissueFaxes™ imaging system show A: Grey scale DAPI, B: colour DAPI and positive expression of stemness markers C:SSEA4, D:SSEA3, E:TRA-1-60, F:TRA-1-81. All images were captured at x20 magnification. Scale bar = 100µm.

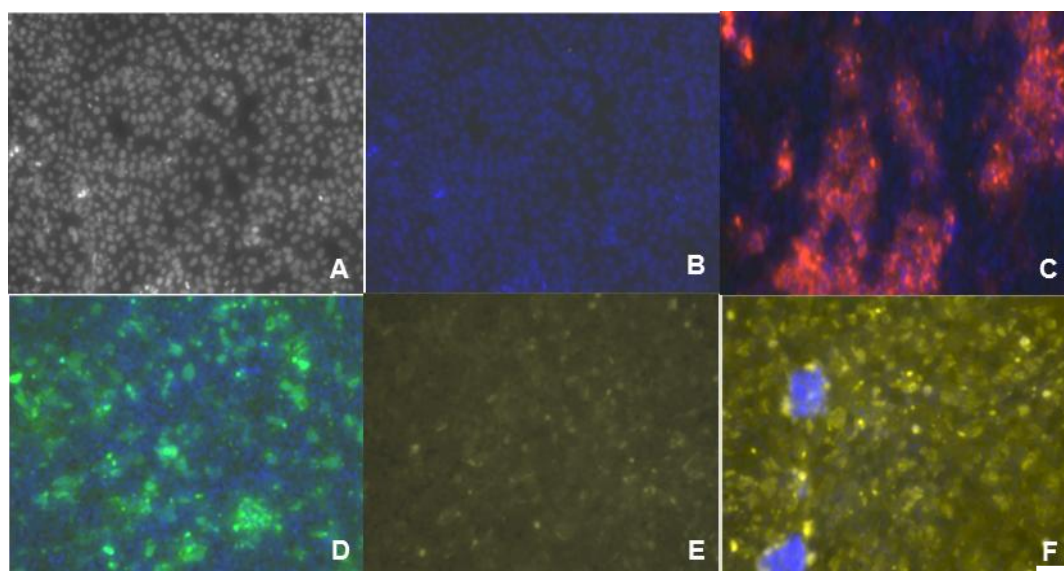


Figure 4.10. IF TissueFaxes™ images for stem cell line HUES9 cultured on i3T3 showing expression of undifferentiated markers at late passage. HUES9 stem cells were cultured on i3T3 mouse feeders at late passage on 24 well plates until confluent. Plates were fixed with 4% PFA for 20 minutes, then stained as described in the methods (2.9). Images taken by TissueFaxes™ imaging system show A: Grey scale DAPI, B: colour DAPI and positive expression of stemness markers C: SSEA4, D:SSEA3, E:TRA-1-60, F:TRA-1-81. Scale bar = 100µm.

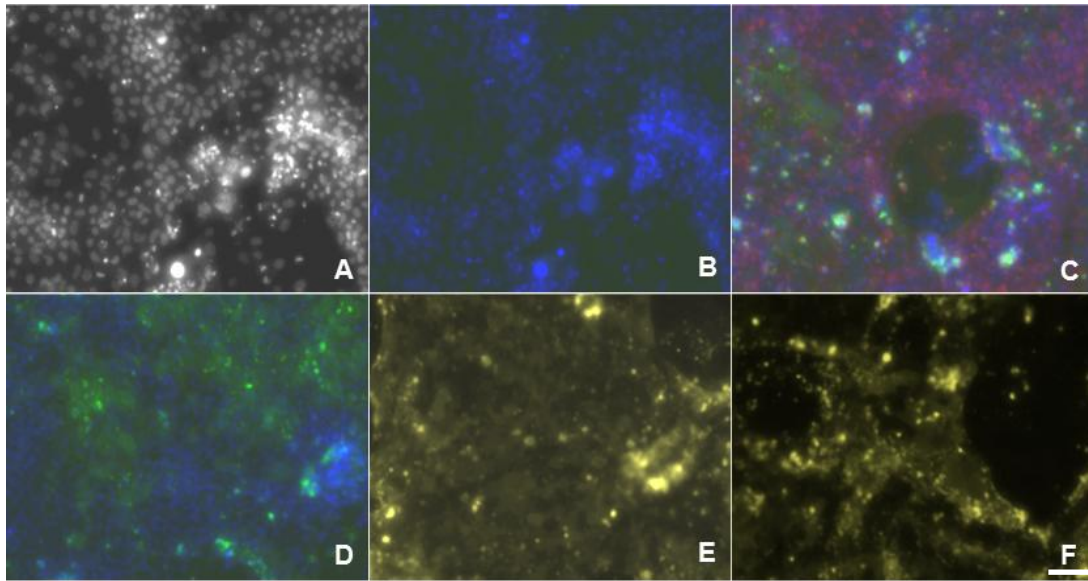


Figure 4.11. IF TissueFaxes™ images for stem cell line HUES9 cultured on iMEF showing expression of undifferentiated markers at late passage. HUES9 were cultured on iMRC5 mouse feeders at late passage on 24 well plates until confluent. Plates were fixed with 4% PFA for 20 minutes, then stained as described in the methods (2.9). Images taken by TissueFaxes™ imaging system show A: Grey scale DAPI, B: colour DAPI and positive expression of stemness markers C: SSEA4, D: SSEA3, E: TRA-1-60, F: TRA-1-81. All images were captured at x20 magnification. Scale bar = 100µm.

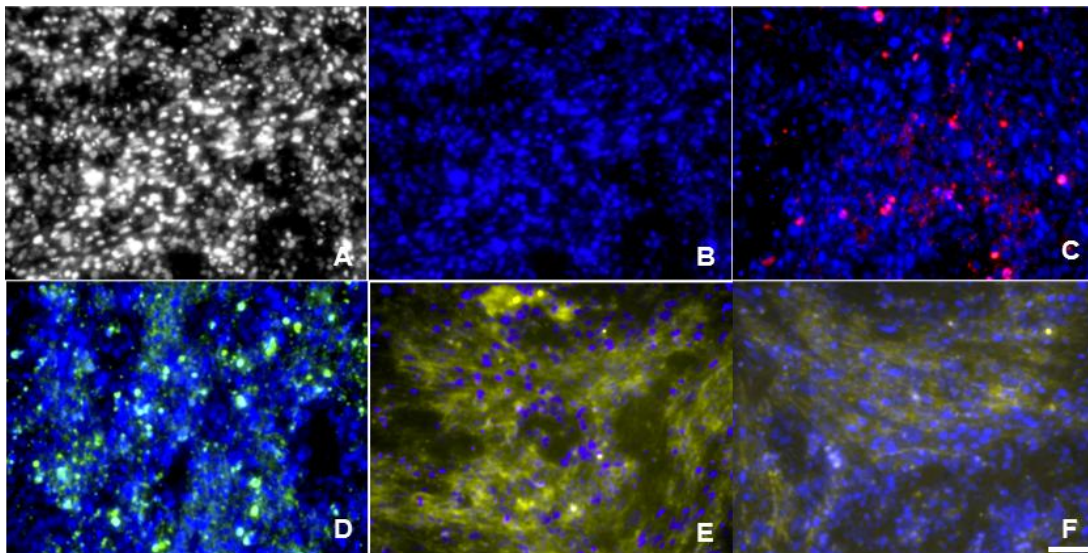


Figure 4.12. IF TissueFaxes™ images for stem cell line HUES9 cultured on iHDFn human feeders showing expression of undifferentiated markers at late passage. HUES9 were cultured on iHDFn human feeders at late passage on 24 well plates until confluent. Plates were fixed with 4% PFA for 20 minutes, then stained as described in the methods (2.9). Images taken by TissueFaxes™ imaging system show A: Grey scale DAPI, B: colour DAPI and positive expression of stemness markers C: SSEA4, D: SSEA3, E: TRA-1-60, F: TRA-1-81. All images were captured at x20 magnification. Scale bar = 100µm.

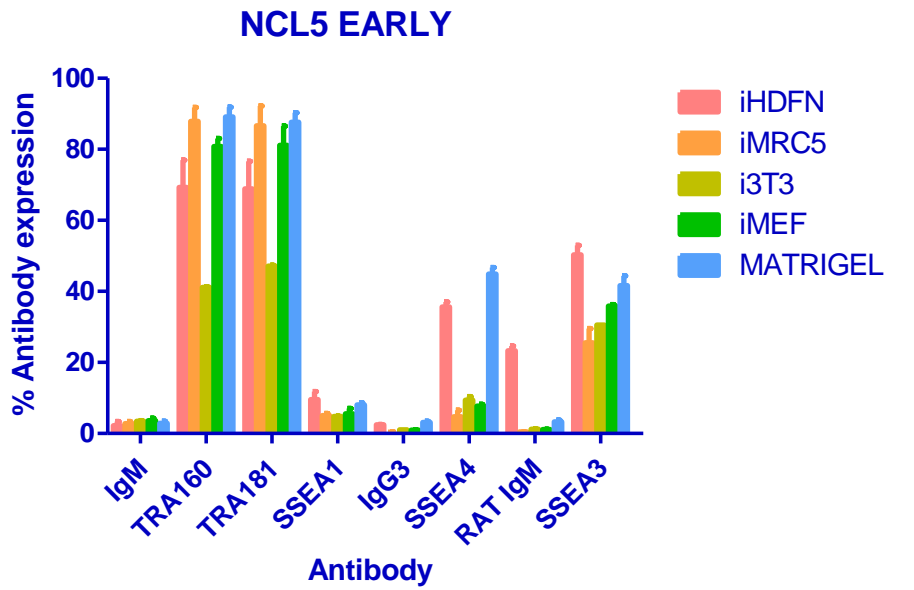
Table 4.8. Comparison of percentage expression of undifferentiated markers for stem cell line HUES9 at late passage by flow cytometry (FC) and TissueFaxs™ image analysis (TF).

Stem cell line/feeder	SSEA3 BY TF	SSEA3 BY FC	SSEA4 BY TF	SSEA4 BY FC	TRA-1-60 BY TF	TRA-1-60 BY FC	TRA-1-81 BY TF	TRA-1-81 BY FC
HUES9/iMEF	77.9±3.2	31.9	84.1±0.70	3.4	62.1±1.40	69.0	92.4±2.7	73.3
HUES9/i3T3	83.6±1.54	12.3	79.2±0.91	1.9	85.5±1.11	93.7	85.1±1.11	87.9
HUES9/iMRC5	93.2±0.5	13.9	94.2±6.2	7.3	98.6±3.1	73.3	95.7±3.1	65.4
HUES9/iHD Fn	99.5±0.39	13.8	93.8±0.30	1.3	97.0±6.2	52.2	80.0±3.03	54.3
HUES9/Matrigel	69.2±4.11	60.5	78±2.79	35.4	82±1.37	83.5	86±0.82	82.9

4.3.5 Changes in expression of undifferentiated hESC markers for stem cell line NCL5 by flow cytometry

Results in Table 4.9 demonstrated clear differences in expression between the markers. SSEA3 and SSEA4 expression was higher from cells cultured on both human feeders compared to mouse feeders, whereas expression of TRA-1-60 and TRA-1-81 were highly expressed in cells from mouse feeders compared with human feeders. Cells on Matrigel maintained high levels of expression of all four markers at late passage. Worth noting is the fact that there was considerable variation in the levels of markers detected between the two methods used, i.e. FC and TF. The latter appeared more sensitive, giving overall much higher detected levels of expression when compared to FC. Only expression of TRA-1-60 and TRA-1-81 from all samples appeared to be comparable with both techniques.

When comparing overall undifferentiated marker expression, levels were highest in NCL5 cells cultured on iMEF mouse feeders, iMRC5 human feeders or feeder free matrix Matrigel (Figure 4.13). The highest levels of expression were however with NCL5 cultured on iMEF at late passage (Figures 4.13 A and B). From initial observation of the results, NCL5 had higher, more consistent expression of all markers from early to late passage compared to the other stem cell lines. Therefore, biological replicates were carried out and included in these results (n=3), and showed consistencies for this experimental set for NCL5 cell line. There was a decrease in expression from early to late passage from NCL5 cultured on iMRC5 (18%). Culture of NCL5 on Matrigel was also comparable with human feeders, with high expression of TRA-1-60, TRA-1-81, SSEA3 and the highest expression of SSEA4 from all the conditions, at early and late passage.



B

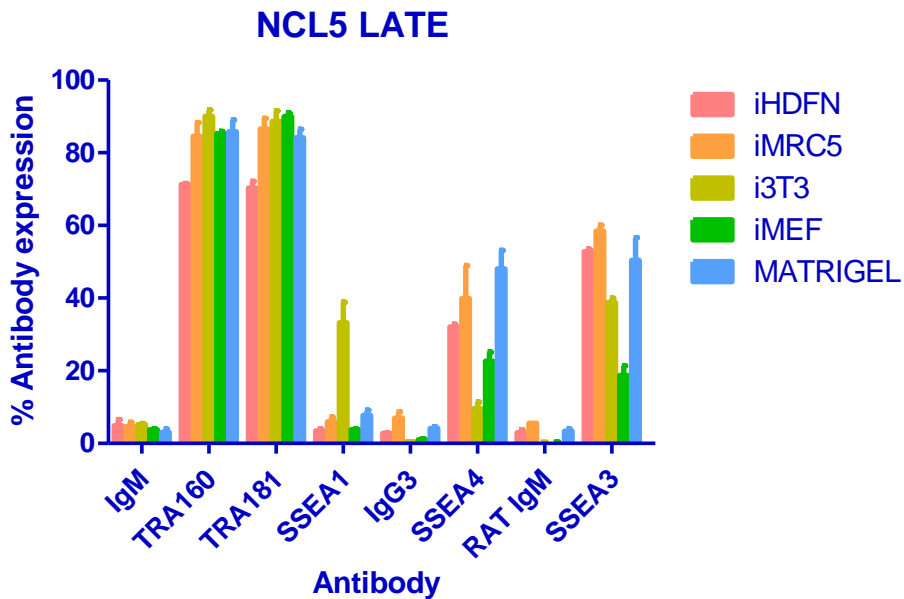


Figure 4.13. Effects of Matrigel and different feeder types on undifferentiated marker expression in NCL5 stem cell line at early (A) and late (B) passage (n=3). NCL5 were cultured at early (p+5) and late (p+20) passage on mouse, human feeders and Matrigel until confluent. Cells were prepared for flow cytometry as described in the methods (section 2.8). The data is the mean of 3 separate experiments.

4.3.6 Changes in expression of undifferentiated hESC markers for NCL5 stem cell line detected by TissueFaxs™ analysis

Figures 4.14 and 4.15 demonstrate positive expression of markers for NCL5 cultured on mouse fibroblasts iMEF (Figure 4.14) and human feeder's iHDFn (Figure 4.15). However, some of the images produced from NCL5 on iMEF at late passage were out of focus. As well as difficulties encountered as a result of imaging stem cells on feeders, considerable cell piling was also observed (Figure 4.14.A and 4.14.B) despite adjustments to seeding density (passaging 1:6 adjusted to 1:12). As cells pile on top of each other, the camera could not be easily focused onto the cell layers to identify clear morphologies. Although tools to allow the re-acquirement of small areas were applied, the images obtained were of poor visual quality. This also made analysis difficult. Nonetheless, positive expression of all four stemness markers was detected, as shown in Figure 4.14 (C-F).

Imaging NCL5 cells cultured on human feeders was also challenging. The cells grew as elongated colonies (demonstrated by Figure 4.15.D-F), which could not be imaged clearly due to piling of cells in the middle of the colonies, and thinning of cells at the edges where the colonies tapered off. Imaging hESCs NCL5 on iMEF mouse feeders was more straightforward and produced brighter colonies, as shown in Figure 4.14. In this case, the cells grew in recognisable patterns and were easily detected by the microscope with less piling. This may result in lower levels of fluorescence intensity, which meant that SSEA3 expression was not as high on iHDFn (65.00% \pm 3.02) compared with NCL5 on iMEF (98.64% \pm 5.81). Tight, distinctly-shaped stem cell colony formation was observed with NCL5 cultures on mouse feeder layer i3T3 as shown in Figure 4.16A and 4.17B. The images obtained were clear and fluorescence was easy to visualise. This is probably due to the stem cells proliferating on top of the mouse feeder layer rather than between the monolayers, making them easier to detect and image.

NCL5s cultured on human feeders iMRC5 grew in waves and swirls between the human feeders, as observed with the other hESC lines. From the IF images, it is apparent that imaging populations of stem cells on human feeders is a more challenging task than on mouse feeders. Figure 4.16 of NCL5s on i3T3 feeders

shows much clearer and better defined pictures than images of NCL5 cultured on iMRC5 (Figure 4.17). In particular, expression of TRA-1-60 and TRA-1-81 on the 3T3 mouse feeders was much brighter than those obtained on human feeders (Figure 4.16.E and F). Therefore, the human feeder population was not easy to locate with the scatterplot purely on the basis of cell size. Thus, the human feeders could not be eliminated from the overall analysis, which was much easier for mouse feeders as they are much smaller than the stem cells and could be eliminated using size and shape parameters. This discrepancy most likely affected the intensities and influenced the overall mean percentage of expression of stem cell markers, as these cells may have been too bright or too dim and probably gated into bright artefacts or baseline cut-offs.

Figures 4.15 and 4.17 also highlight the changes in morphology seen with hESCs on human feeders. As the colonies elongated they proliferated on the larger human feeder cells. Both human and mouse feeder types maintained expression of undifferentiated stem cell markers at late passage, although again, the images were not very clear and difficult to analyse as a result of culture on a feeder layer. Once again, the main issues faced with producing higher quality images was imaging on a bilayer of feeders, which could not be improved without removing the feeders.

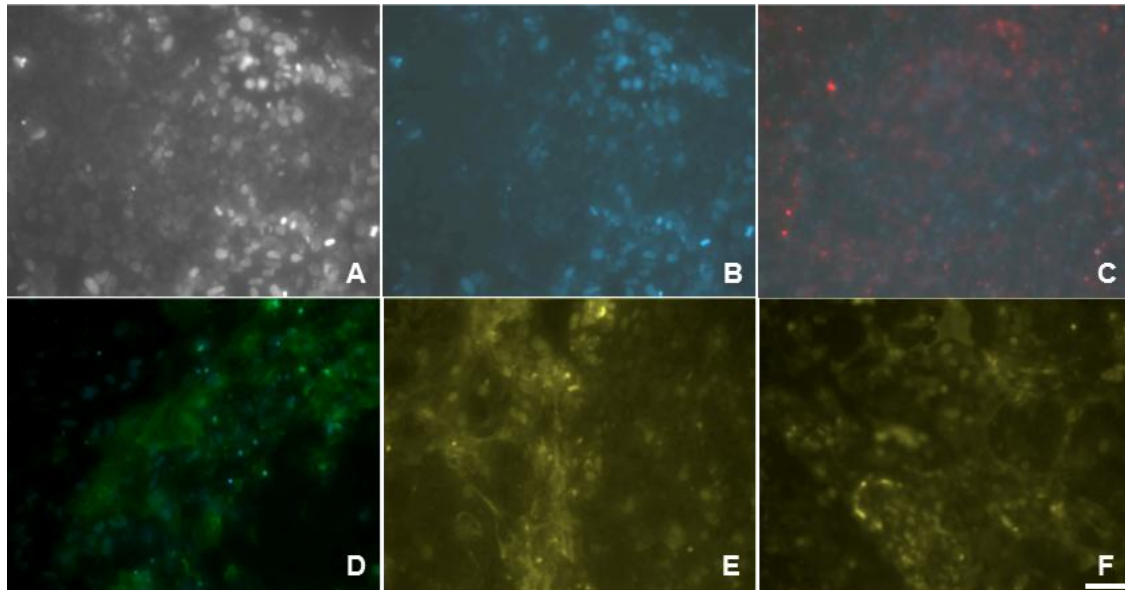


Figure 4.14. IF TissueFaxes™ images for stem cell line NCL5 cultured on iMEF mouse feeders showing expression of undifferentiated markers at late passage. NCL5 were cultured on iMEF mouse feeders at late passage on 24 well plates until confluent. Plates were fixed with 4% PFA for 20 minutes, then stained as described in the methods (2.9). Images taken by TissueFaxes™ imaging system show A: Grey scale DAPI, B: colour DAPI and positive expression of stemness markers C: SSEA4, D: SSEA3, E: TRA-1-60, F: TRA-1-81. All images were captured at x20 magnification. Scale bar = 100µm.

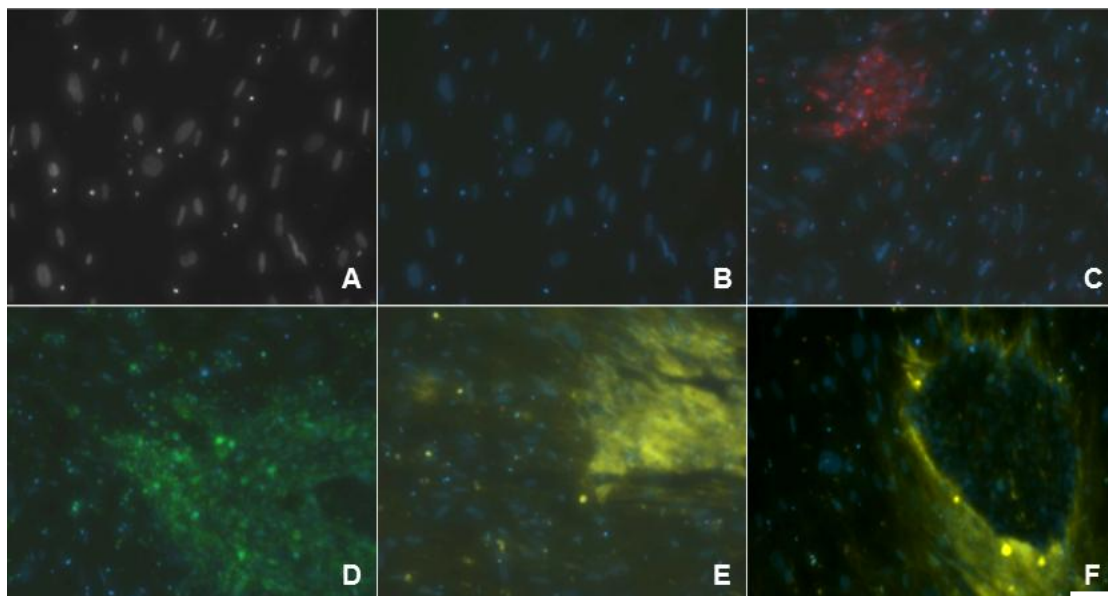


Figure 4.15. IF TissueFaxes™ images for stem cell line NCL5 cultured on iHDFn human feeders showing expression of undifferentiated markers at late passage. NCL5 were cultured on iHDFn human feeders at late passage on 24 well plates until confluent. Plates were fixed with 4% PFA for 20 minutes, then stained as described in the methods (2.9). Images taken by TissueFaxes™ imaging system show A: Grey scale DAPI, B: colour DAPI and positive expression of stemness markers C: SSEA4, D: SSEA3, E: TRA-1-60, F: TRA-1-81. All images were captured at x20 magnification. Scale bar = 100µm.

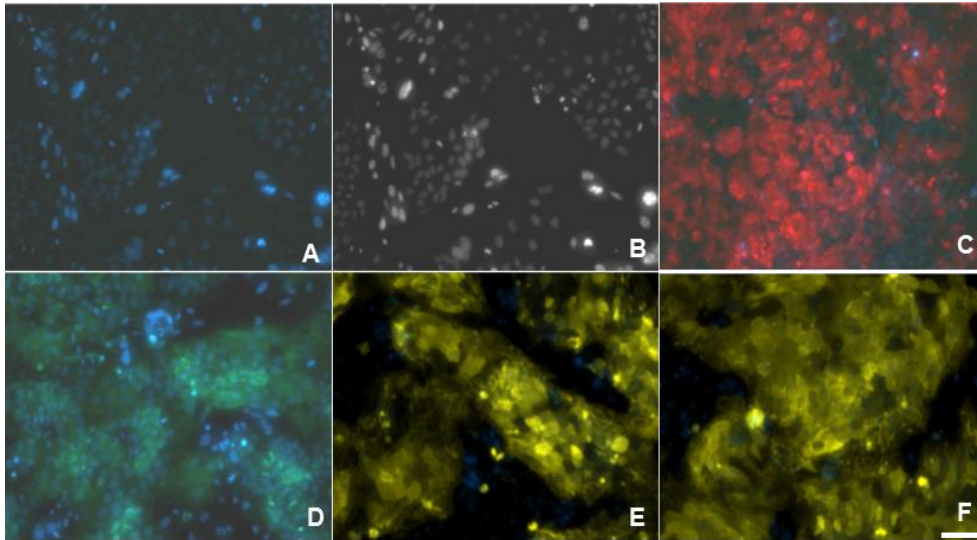


Figure 4.16. IF TissueFaxes™ images for stem cell line NCL5 cultured on i3T3 mouse feeders showing expression of undifferentiated markers at late passage. NCL5 were cultured on i3T3 mouse feeders at late passage on 24 well plates until confluent. Plates were fixed with 4% PFA for 20 minutes, then stained as described in the methods (2.9). Images taken by TissueFaxes™ imaging system show A: Grey scale DAPI, B: colour DAPI and positive expression of stemness markers C:SSEA4, D:SSEA3, E:TRA-1-60, F:TRA-1-81. All images were captured at x20 magnification. Scale bar = 100µm.

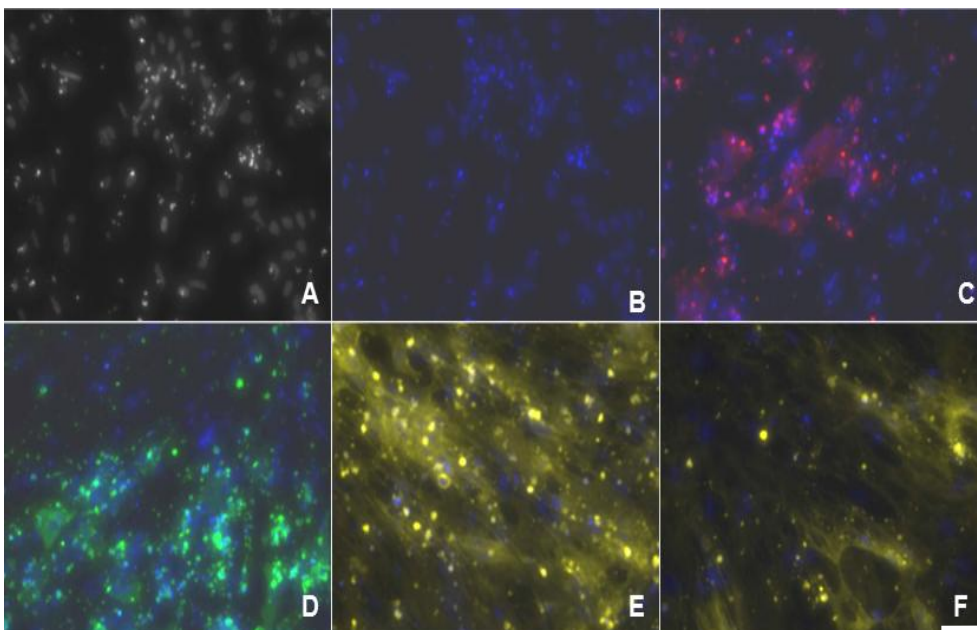


Figure 4.17. IF TissueFaxes™ images for stem cell line NCL5 cultured on iMRC5 human feeders showing expression of undifferentiated markers at late passage. NCL5 were cultured on iMRC5 human feeders at late passage on 24 well plates until confluent. Plates were fixed with 4% PFA for 20 minutes, then stained as described in the methods (2.9). Images taken by TissueFaxes™ imaging system show A: Grey scale DAPI, B: colour DAPI and positive expression of undifferentiated markers C:SSEA4, D:SSEA3, E:TRA-1-60, F:TRA-1-81. All images were captured at x20 magnification. Scale bar = 100µm.

Table 4.9. Comparison of expression (%) of undifferentiated markers for stem cell line NCL5 at late passage by flow cytometry (FC) and TissueFaxs™ image analysis (TF).

Stem cell line/feeder	SSEA3 BY TF	SSEA3 BY FC	SSEA 4 BY TF	SSEA 4 BY FC	TRA-1-60 BY TF	TRA-1-60 BY FC	TRA-1-81 BY TF	TRA-1-81 BY FC
NCL5/iMEF	98.6	18.7	98.32	22.6	96.74	85.28	96.75	89.9
NCL5/i3T3	84.9	38.8	81.69	9.58	86.40	90.0	94.92	88.58
NCL5/iMRC 5	94.61	58.4	97.35	40.0	94.05	54.5	86.40	86.5
NCL5/iHDFn	65.0	52.8	96.17	32.1	91.9	71.3	97.82	70.2
NCL5/Matrigel	97.17	50.37	99.45	40.0	75.37	85.8	92.65	84.2

4.3.7 Changes in expression of undifferentiated hESC markers for stem cell line RH5, HUES9 and NCL5 cultured on Matrigel detected by TissueFaxs™ image analysis

As described in Chapter 3, morphological changes of hESCs were demonstrated as a result of their culture on the feeder free Matrigel/mTeSR1 system. TissueFaxs™ results (Figure 4.18) show a selection of images that confirm all three hESC lines expressed undifferentiated hESC markers (full PDF of results for each hESC line shown in appendix). Qualitative and quantitative assessment of marker expression was significantly improved by feeder free culture. The time taken to image these colonies was reduced in comparison to imaging feeder dependant cultures as it was easy to focus the microscope to obtain good quality images. However, as already described from culture on feeders, hESCs do tend to grow on top of each other when passaged enzymatically which can cause piling. This resulted in slightly blurry imaging as shown in Figure 4.18; F, G and I.

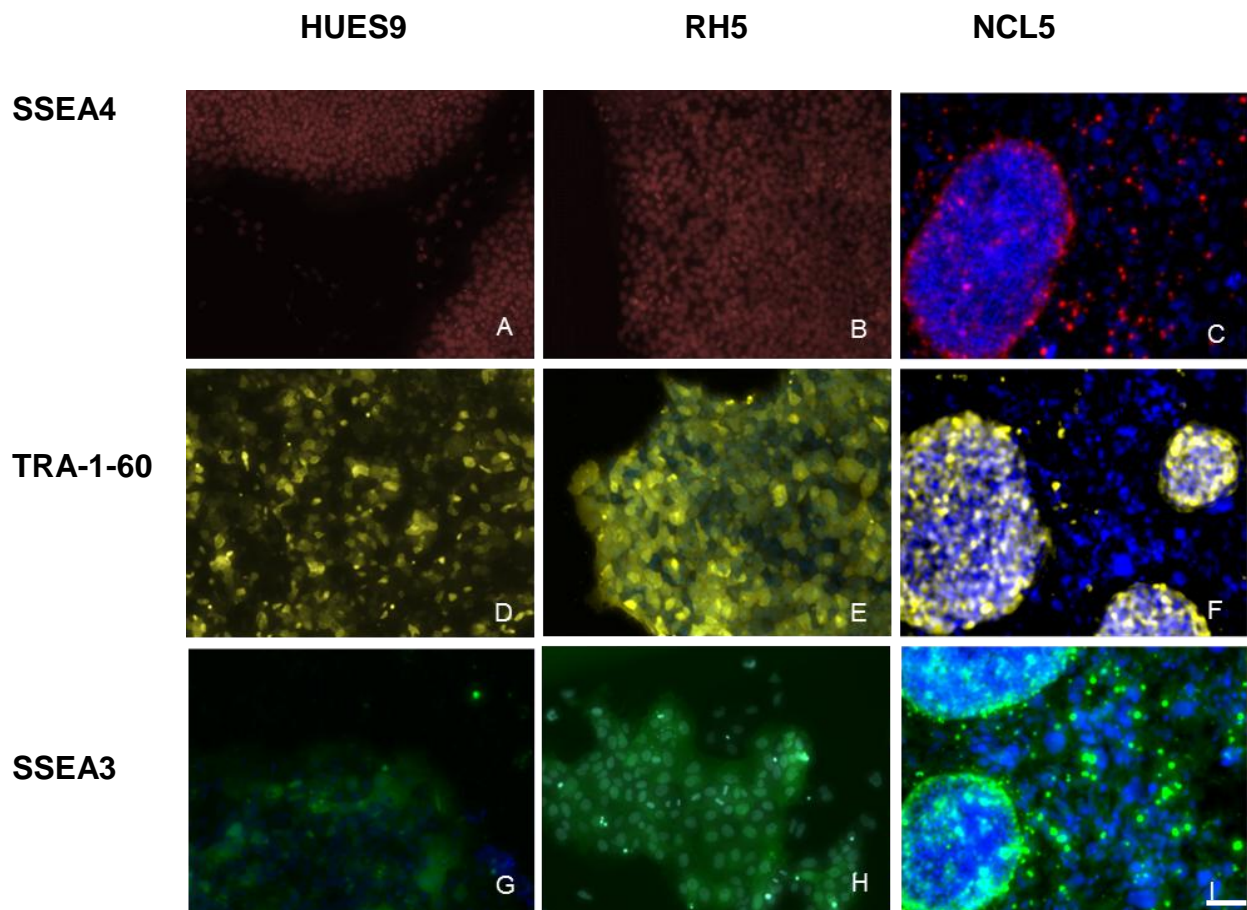


Figure 4.18: IF TissueFaxes™ images for stem cell lines HUES9, RH5 and NCL5 cultured on Matrigel showing expression of undifferentiated markers at late passage. All three hESC lines were cultured at late passage on Matrigel coated 24 well plates until confluent. Plates were fixed with 4% PFA for 20 minutes, then stained as described in the methods (2.9). Images taken by TissueFaxes™ imaging system show A: Grey scale DAPI, B: colour DAPI and positive expression of stemness markers C:SSEA4, D:SSEA3, E:TRA-1-60, F:TRA-1-81. All images were captured at x20 magnification. Scale bar = 100µm.

Table 4.10. Comparison of percentage expression of undifferentiated markers for stem cell line RH5, HUES9 and NCL5 at late passage by TissueFacs™ image analysis (TF).

STEM CELL LINE	SSEA3 (FITC)	SSEA4 (CY3)	TRA-1-60 (CY3)	TRA-1-81 (CY3)
RH5	87.25±1.20	72.90±0.35	77.35±2.50	81.54±4.33
HUES9	69.27±4.11	78±2.79	82±1.37	86±0.82
NCL5	97.17±2.8	99.45±1.36	75.37±0.98	92.65±2.35

4.3.8 Detection of co-expressed stemness markers

The co-expression of the stemness markers SSEA4/TRA-1-81 was assessed in all three stem cell lines cultured on the four feeders and on Matrigel, at late passage. This was to establish better defined populations of undifferentiated hESCs. Both markers were detectable in RH5 cultured on iMRC5 (Figures 4.19 A and corresponding scattergram Figure 4.19C at 85.74%) and in RH5 cultured on iMEF (Figure 4.19 B and scattergram D, at 90.29%). These markers were also co-expressed in NCL5 cultured on iMRC5 (Figure 4.21 A and corresponding scattergram C at 92.09%) and in NCL5 cultured on iMEF (Figure 4.20 B and scattergram D, at 98.02%). Again, clearer imaging was obtained from NCL5 cultured on mouse feeders, compared with images on human feeders. Comparison of these results to the single fluorescent channel results showed similar percentages of detection (Figure 4.6 and Table 4.10). This demonstrates that the results obtained are reliable and consistent.

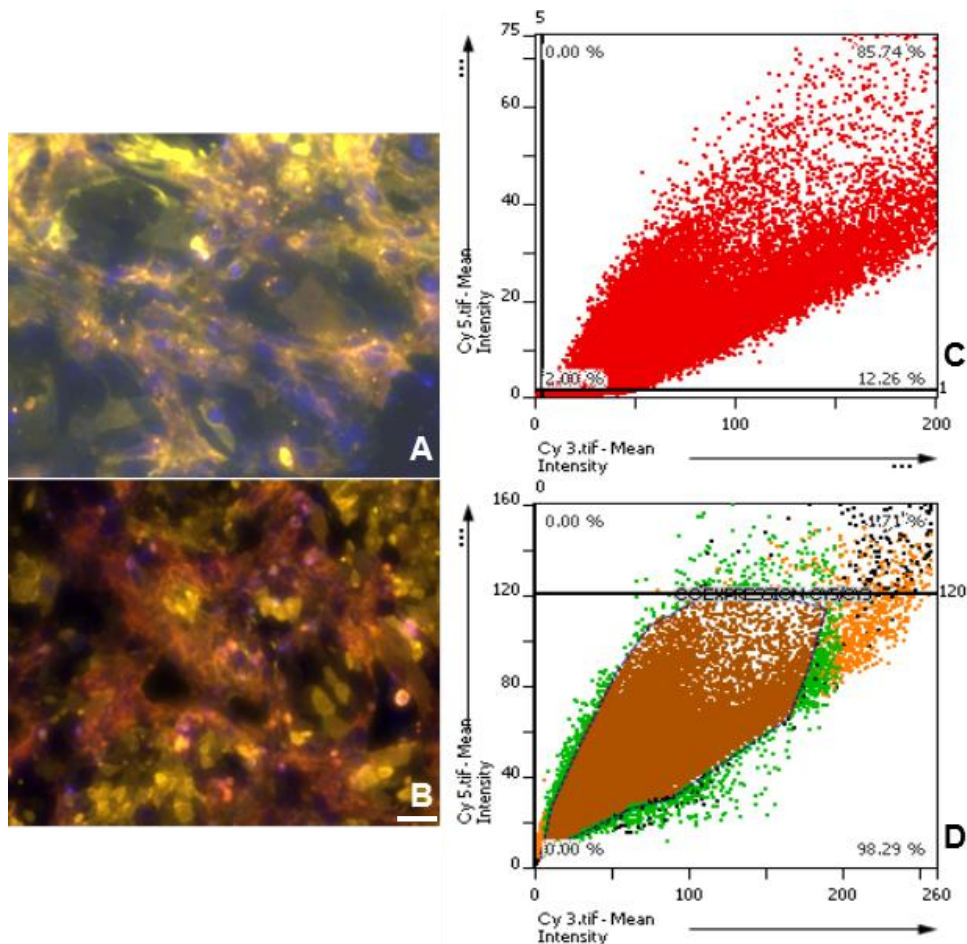


Figure 4.19. Co-expression of SSEA4/TRA-1-81 by TissueFaxs™ image and corresponding scatterplot in RH5 cultured on iMRC5, (A and C) and RH5 cultured on iMEF, (B and D) at late passage. TissueFaxs™ image analysis was able to identify 85.74% of RH5 stem cells cultured on human feeder's iMRC5 as both SSEA4 and TRA-1-81 positive, at late passage, in comparison with culture on mouse feeder iMEFs, at 90.29%. The higher expression in mouse feeders was most likely due to difficulties in detection of positive stem cells cultured on human feeders, which grow as swirls, and therefore it is trickier to distinguish and detect stem cells from human feeders using masks as the stem cells tend to grow in between the feeder layers. All images were captured at x20 magnification. Scale bar = 100µm.

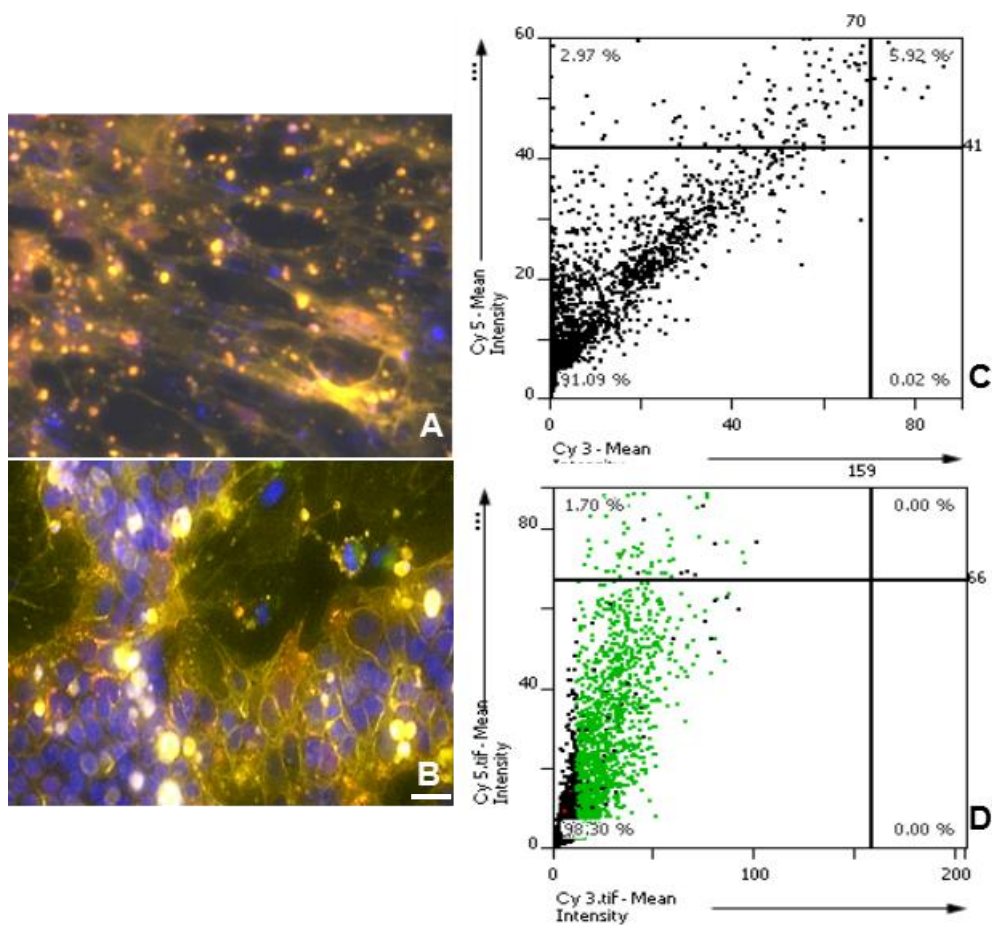


Figure 4.20. Co-expression of SSEA4/TRA-1-81 by TissueFaxs™ image and corresponding scatterplot in NCL5 cultured on iMRC5 (A and C) and NCL5 cultured on iMEF late passage (B and D). In concurrence with Figure 4.18, stem cells cultured on mouse feeders showed higher levels of stemness marker expression (98.30%) compared with those cultured on human feeders (91.09%). Co-expression image B, obtained from NC5 cultured on mouse feeder at late passage was much clearer than image A, from human feeders.

4.3.9 Expression of stemness markers by TissueFaxs™ image analysis

The results in the Table 4.11 are based on the average fluorescent intensity obtained from its corresponding Field of View (FOV) images. For the majority of these results, the events/cells have been gated by parameters Dapi area and Dapi intensity, to appropriately detect Dapi stained single cells. The results were then further gated to detect accurate positively stained events for each stemness marker. Where no specific gated population was detected, an accurate percentage of expression was calculated by dividing the overall number of events/cells obtained from one scatterplot/table from Dapi (Master channel parameters) by the events sub-gated in the fluorescent channel. Confidence intervals were calculated using events gated from commercial isotype controls, and ensure the reliability of results obtained.

Table 4.11 demonstrates that while expression of undifferentiated hESC markers was detected in all late passage hESC line cultured on all four feeder types and Matrigel there were differences in expression, which were ranked as HIGH/LOW (cut off: 60% expression for all cell surface markers). Overall, SSEA3 expression was low for cells cultured on i3T3 and iHDFn compared with iMRC5 and iMEF. Furthermore, HUES9 cultured on both mouse feeders had between 10-15% lower expression compared with cultures on human feeders. RH5 also had slightly lower expression on mouse feeders when compared to both human feeders for overall stemness expression. Results for TRA-1-60 cell surface marker expression were slightly lower than the other three markers. The Table also clearly shows that overall SSEA3 and SSEA4 expression was low from FC compared with TF.

Table 4.11. Summarised comparison of stemness marker expression by each stem cell line detected by TissueFacs™ image analysis (TF) and flow cytometry (FC) at late passage.

STEM CELL LINE/FEEDER	SSEA3 BY TF	SSEA3 BY FC	SSEA4 BY TF	SSEA4 BY FC	TRA-1-60 BY TF	TRA-1-60 BY FC	TRA-1-81 BY TF	TRA-1-81 BY FC
RH5/iMEF	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW
RH5/i3T3	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW
RH5/iMRC5	HIGH	LOW	HIGH	LOW	HIGH	HIGH	HIGH	HIGH
RH5/iHDFn	HIGH	LOW	HIGH	LOW	HIGH	HIGH	HIGH	HIGH
RH5/Matrigel	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH	HIGH
NCL5/iMEF	HIGH	LOW	HIGH	LOW	HIGH	HIGH	HIGH	HIGH
NCL5/i3T3	HIGH	LOW	HIGH	LOW	HIGH	HIGH	HIGH	HIGH
NCL5/iMRC5	HIGH	LOW	HIGH	LOW	HIGH	HIGH	HIGH	HIGH
NCL5/iHDFn	HIGH	LOW	HIGH	LOW	HIGH	HIGH	HIGH	HIGH
NCL5/Matrigel	HIGH	LOW	HIGH	LOW	HIGH	HIGH	HIGH	HIGH
HUES9/iMEF	HIGH	LOW	HIGH	LOW	HIGH	HIGH	HIGH	HIGH
HUES9/i3T3	HIGH	LOW	HIGH	LOW	HIGH	HIGH	HIGH	HIGH
HUES9/iMRC5	HIGH	LOW	HIGH	LOW	HIGH	HIGH	HIGH	HIGH
HUES9/iHDFn	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW
HUES9/Matrigel	HIGH	LOW	HIGH	LOW	HIGH	HIGH	HIGH	HIGH

4.3.10 Statistical analysis using 2 way variance and Duncans grouping to compare Stem cell lines and human versus mouse feeders from early and late passage.

Flow cytometry

Using 2 way ANOVA to compare the differences in expression between cell surface markers, feeders and hESC lines, overall significant differences were found for SSES3 expression, between the stem cell lines ($P = 0.004$) however, not as a result of culture on the different feeders ($P > 0.05$). No significant difference were noted between early and late passage cultures ($P = 0.449$). Similarly SSEA4 expression also showed overall significant differences between cell lines ($P < 0.0001$) but not the feeders ($P = 0.449$) and there was no overall significant differences between the early and late passages ($P = 0.326$).

TissueFaxes™

Results from TissueFaxes™ analysis of undifferentiated markers SSEA3 and TRA-1-81 were also subject to 2 way ANOVA and GLM, as described above. Statistical analysis revealed no significant difference from early to late passage, and between feeder types ($P=0.386$, $P=0.846$), but significant differences between stem cell lines ($P=0.001$).

4.4 Discussion

The characterisation of hESCs is important to the stem cell community. Flow cytometry is a useful tool to assess the undifferentiated state of hESCs by cell surface and nuclear marker expression. Although the molecular identities are unknown, cell surface stemness markers TRA-1-60, TRA-1-81 and SSEA3, SSEA4 are routinely tested for in many laboratories (Adewumi et al, 2007; Chambers et al., 2007; Jonathan S. Draper, 2004). Visual assessment of cell cultures alone, although useful, cannot determine the outcome of the potential use of a cell line. High content image-based cell screening marks a positive advancement to precisely quantify the IF staining within cell cultures. Although its use within the stem cell field is relatively new, its potential applications are endless. For example, optimising cell density for plating efficiencies, viability assessment, cell counting, assessing well to well variation between cultures and the prospects of acquiring large number of individual cell measurements or searching for rare cell events are all potential applications.

From the initial results, both flow cytometry and TissueFaxs™ analysis confirmed the expression of undifferentiated hESC markers in all the stem cell lines cultured on feeders and Matrigel at late passage. However detection by flow cytometry gave markedly lower sensitivity, when compared with marker expression from every cell line processed by TissueFaxs™ analysis. SSEA3 and SSEA4 expression were particularly low across all stem cell lines and feeder types by Flow cytometry with less than 50% expression detected. Variability of SSEA3 expression in hESCs by flow cytometry and *in situ* staining has been previously reported (Adewumi et al., 2007), however the results were qualitative (*in situ* fluorescence staining) and therefore subject to variability. Variability of FACs analysis can occur due to other factors such as fixation methods, which can affect the expression of antibody binding and could be further investigated to establish whether a more appropriate fixation method could improve the detection of SSEA3 and SSEA4. Also, operator error and gating of cell populations can affect overall flow cytometry results. This is difficult to standardise, and well defined cut offs have to be put in place in order to apply this technique as a QC test. The use of commercially available isotype controls does improve this variability, as gating was more reliably performed and consistent between experiments. However this task was difficult to perform as the same gates could not be applied to all the different stem cell lines used, due to variations in

growth and size of the stem cell lines themselves. Also, hESCs cultured on human feeders appeared to grow much larger than those cultured on mouse feeders. This also made gating difficult, as different gate templates had to be created for each stem cell line, and sometimes for each stem cell line cultured on each feeder type, due to the large variations in size. Clumping and cell death may also have contributed to low expression of SSEA3 and SSEA4 results by FC. It was noted that a number of hESC lines on feeder cultures were much clumpier when compared to Matrigel cultures. Manual separation of stem cells from their feeders is difficult as stem cells are frequently lost as a result of clump removal, making it difficult to prepare single cells suspensions for testing such as flow cytometry. The use of magnetic separation kits (MACs™, Miltenyl Biotech) does solve this problem for mouse feeders however at the time of writing there was no kit developed for the separation of human feeders from hESCs. Mouse feeders are much smaller than stem cells, so subsequently it was relatively easy to gate out this population from the main stem cell population.

In addition, the Guava™ could only process and analyse a small number of cell events (3000) in comparison to other instruments, for example, BD FACS Canto™, which can process an average of 10, 000 events. At the time that these studies were carried out the UKSCB only had access to The Guava™ flow cytometer, which is additionally limited in its capabilities compared with other flow cytometers. It only has the ability to detect PE and FITC fluorophores, and the software cannot back-gate.

As demonstrated by the results in this chapter, flow cytometry is a useful technique to characterise undifferentiated stem cells, particularly when a number of antibodies can be used together to make an informed decision on whether a stem cell line has maintained its undifferentiated characteristics over a number of passages. Flow cytometry using the Guava EasyCyte™ has the advantage that it is easy to set up and execute reliable experiments. In addition, Flow cytometry should not be used as a single characterisation test, but instead used in conjunction with immunofluorescent staining to give information on spacial analysis of stem cells as typically, they grow as colonies.

The results demonstrate the precision by which the TissueFacs™ is able to detect single cells as events. The sophisticated gating analysis software allows the user to

back-gate and specifically critique and remove/re-gate individual events with much accuracy, compared to a flow cytometer, which is subject to false positive and false negative results due to debris from apoptotic cells, cell artefacts and unconjugated fluorescent antibody, all of which can be removed by the TissueFacs™. The results obtained by TissueFacs™ are considerably higher than the results obtained from the Guava however, the number of cells/events analysed is also significantly higher than the guava. As demonstrated by these studies, the TissueFacs™ has demonstrated its ability to analyse between 15,000-40,000 events. This is probably due to a greater level of sensitivity and accuracy when detecting a true stem cell population from dead cell cells/debris/feeders etc. Improved analysis functions enabled the successful identification and gating of feeder populations, to allow for much more accurate quantification of positive staining. TissueFacs™ quantitative imaging has a significant advantage over this as the results are quantitative and remove discrepancies in reporting results associated with qualitative fluorescence imaging. TissueFacs™ demonstrated 60-90% positive SSEA3 and SSEA4 expression over flow cytometry. It was reported that SSEA4 staining patterns were typical of integral membrane components compared to TRA-1-81 and TRA-1-60, which showed a granular pattern of staining around the cells, characteristic of proteins deposited in the pericellular matrix (Adewumi et al., 2007). The use of visual and spacial analysis when assessing undifferentiated hESCs using TissueFacs™ has clear advantages over traditional flow cytometry, as different cell populations can be better identified with the correct use of antibodies.

The biggest disadvantage of the TissueFacs™ is the inability to image in brightfield, to compare morphology with fluorescent images. The TissueFacs™ is a very sensitive piece of equipment and slight adjustments can only be made by experienced staff. The microscope also struggled to image on a bilayer of cells which for some cell lines resulted in poor quality images. hESCs cultured on Matrigel significantly improved this issue and gave much clearer fluorescent images, as demonstrated by the results.

Co expression of cell surface markers

The co expression of stem cell surface markers using two antibodies was carried out to try and obtain a better defined population of undifferentiated hESCs following long

term culture on human and mouse feeders. TRA-1-81/SSEA4 positive populations of stem cells were detected from all three stem cell lines, and were noticeably higher from stem cell line NCL5 and RH5. Furthermore, differences in co-expression were seen as a result of culture on different feeder types. Expression of TRA-1-81/SSEA4 was higher (8-10%) from stem cells cultured on mouse feeders compared to stem cells cultured on human feeders, at late passage (Figures 4.19 and 4.20). However, this was most likely due to the limitations in detecting stem cells cultured on human feeders, as they tend to grow in between the feeder layers, and therefore difficult to mask and detect by TissueFacs™ analysis. Furthermore, TRA-1-81/SSEA4 co expression was not detected from all stem cell lines cultured in-house and human feeders, perhaps indicating that these markers were not co expressed in late passage stem cell lines. Previously, research has been conducted to demonstrate the role of SSEA3/SSEA4 positive cells and their importance in promoting self-renewal within a stem cell population (Mantel et al., 2007; Stewart et al., 2006).

The significance of co-expression of these proteins is not yet known, but may be useful in the identification of subset colonies and better defined colonies of pluripotent stem cells which typically grow in a heterogeneous population. Definition and ability to sort accurate undifferentiated phenotypes of stem cells from mixed populations potentially harbouring nullipotent or early differentiated populations, will be crucial for progression towards stem cell based therapies. As demonstrated by the results of this thesis, stem cells at late passage maintain high expression of TRA cell surface proteins. The selection of both SSEA3/SSEA4 positive and OCT4/SSEA1 (early differentiation marker) positive stem cells for example, coupled with the use of a cell sorter, may result in the selection of stem cells with greater pluripotent potential. Although the accurate identification of co expressed markers was not fully achieved by these studies, this highlights a limitation that requires more time to investigate and refine methods to successfully achieve the accurate identification of subpopulations of hESCs.

The ability to isolate purer stem cell populations and eliminate early differentiating cell populations has great importance to cell based therapy. Such assays are already widely used for clinical application in other disciplines, such as the selection of CD4/CD8 counts, when assessing haematological markers for HIV drug dosage and selection of CD144/CD34 cells from bone marrow, intended for patients undergoing

chemotherapy cycles. This is of particular importance to the UKSCB as its focus is quality control, hence robust testing is a high priority.

Differences in marker expression have previously been described and SSEA3 and SSEA4 pinpointed as better indicators of stemness, (Ramirez et al., 2011). This is because the expression of other markers, including TRA-1-81 and TRA-1-60, has been found in long term cultures which also co-expressed early differentiation markers at low levels (Enver et al., 2005; Stewart et al., 2006; Ramirez et al., 2011). These reports concur with the results obtained in these studies and further emphasize the heterogeneous nature of hESCs, as well as the need to use a combination of cell surface markers to confirm their phenotype changes as a result of long term passaging and changes in culture conditions. Flow cytometry and TissueFacs™ image analysis demonstrated that NCL5 had the highest percentage of positive stem cell marker expression in comparison to the other stem cell lines. This was first observed during initial trend analysis of results from the stem cell lines cultured and confirmed in repeat experiments.

RH5 cultures gave higher marker expression on Matrigel and human feeders, at early passage, however, this decreased at late passage. HUES9 cultured on Matrigel demonstrated improved marker expression from early to late passage. In both stem cell lines, cultures on Matrigel produced higher than average results when compared with feeders. These differences demonstrate the successful ability of hESC cell lines to adapt to feeder free culturing over 20 passages, when compared with i3T3 and iHDFn feeders.

Statistical analysis using 2 way variance and Duncans' grouping for pairwise comparisons demonstrated that overall, significant differences of expression of markers by flow cytometry were seen between stem cell lines, but not between feeders (mouse versus human). There was no significant difference of marker expression from early to late passage (only compared from flow cytometry results). Two way analysis of variance, carried out using the TissueFacs™ results, concluded that there was no significant difference between the feeder types (mouse versus human), only the stem cell lines themselves, which concurred with the flow cytometry results.

Differences in marker expression as a result of culture on different matrices

iMRC5 human feeders, iMEF mouse feeders and feeder free matrix Matrigel cultures gave higher expression of markers when compared with iHDFn and i3T3. The highest expressing markers from all stem cell lines on feeders were TRA-1-60 and TRA-1-81, indicating that the expression of TRA cell surface markers are maintained over extended passaging.

It also confirms other findings, that human feeders provide a suitable alternative to mouse feeders, as they support the undifferentiated growth of hESCs as well as mouse feeders and have the advantage of cultures without potential animal pathogen contamination (Richards et al., 2002; Amit et al., 2003; Hovatta et al., 2004).

The results here are also in contrast to previous reports which concluded that hESCs cultured on human feeders expressed lower levels of SSEA3 compared with mouse feeders (Eiselleova et al., 2008). It has been argued that not all human feeders are suitable to support undifferentiated hESC growth (Richards et al., 2003). In particular MRC5 feeder cells have been described as unsupportive as they did not support hESC growth (Richards et al., 2003). This is also in contrast to the results obtained here, which confirm that MRC5 cells were indeed able to support hESC growth. These discrepancies could be due to the flow cytometry technique used to detect marker expression, as shown by the results obtained in these studies. The use of a more sensitive technique like the TissueFacs™ may help to close the gap between such inconsistencies, and provide more reliable and sensitive assessment of stemness marker expression.

The subtle differences in feeder supportiveness may indicate a species specific preference for human feeders. The differences in the way in which the stem cells appear morphologically i.e. stem cells grew and proliferated between the human feeder layers in contrast to on top of mouse feeders, demonstrate the contrasting mechanisms that stem cells use to attach and grow. It may also be an indication as to the type growth factors secreted within their extracellular matrix that each feeder type provides to effectively support proliferating hESCs (Eiselleova et al., 2008).

4.5 Conclusion

Although previous literature has shown that the comparisons between cultures on mouse and human feeders revealed morphological differences in the way that stem cells proliferate on different matrices, these findings do not favour the use of one feeder type over another. Both IF techniques confirm that all three stem cell lines maintained expression of markers when cultured on the four feeder types and Matrigel using enzymatic passaging. The only significant variable in marker expression over extended passage were due to the inherent difference within the Stem Cell lines themselves ($p=0.001$).

Flow cytometry is a useful technique to assess the expression of cell surface markers in hESCs. The Guava EasyCyte is ideal as a quick screening tool as it doesn't require complex parameter set up. Its ease of use has been demonstrated by these studies. However the TissueFaxs™ provides considerably more information on spacial analysis of marker expression in addition to accurate and more sensitive quantitative analysis, with comparison to flow cytometry. The culture plate platform allows high throughput imaging combined with effective analysis that is quantitatively comparable to a flow cytometer, with the added benefit of being able to visually assess the cells *in situ* and back-gate to a cell on an image, and collect large data sets for accurate statistical analysis.

The use of Matrigel clearly has an advantage when applied to these techniques as it removes the variability and inaccuracies faced as a result of using feeders, both in flow cytometry and image analysis. The use of better standardised matrices in combination with defined media mTeSR1, and TrypLE™ Express will aid progression to achieving reproducible, uniform cultures for scale up methods and improve QC assessment methods including high throughput image analysis. Standardisation of these methods will better enable the field to progress, closing the gaps formed as a result of lab to lab variation.

Chapter 5.

Real time PCR analysis of gene expression profiles in human embryonic stem cells cultured on different matrices

5.1 Introduction

The long term culture of human embryonic stem cells relies on maintaining undifferentiated populations of cells. Gene expression patterns underline the basic differences that define the transcription of genes in human pluripotent stem cells (Tanaka et al., 2002; Abeyta et al., 2004; Bhattacharya et al., 2004; Korkola et al., 2006). Many different studies have assessed the gene expression signatures of different hESCs (Abeyta et al., 2004; Sperger et al., 2003; Bhattacharya et al., 2005), (Chin, et al., 2009; Barbet et al., 2011) and the majority conclude that although each stem cell line does have a unique genetic signature, they do share commonalities. These genes have been shown to be linked to maintenance of self-renewal, stemness markers i.e. present in undifferentiated cultures and genes required for attachment, support and growth.

Real time PCR allows the assessment of pluripotency genes by detecting changes in gene expression using relative quantification when compared to a calibrator (control sample), and in reference to 'housekeeping' control genes. These are genes which are required for the maintenance of basic cellular functions, and are therefore always expressed at a relatively constant level across many conditions.

There is much variability between laboratories in their choice of genes to screen for presence of stemness, pluripotency and differentiation, although transcription factors OCT4 (POU5F1) and Nanog appear most commonly (Chambers et al., 2003; Matin et al., 2004). Other variables include number of cells analysed, appropriate selection of calibrator and housekeeping gene(s), quality and amount of RNA/cDNA for reverse transcription (RT) step. Although research has demonstrated the continued expression of genes for self-renewal in long term cultures of hESCs (Sato et al., 2003; Sperger et al., 2003; Richards et al., 2004), and that the effects of maintaining undifferentiated cultures of hESCs on different feeder types is due to their inherent ability to express growth factors FGF2, BMP4 and Activin A (Eiselleova et al., 2008) there is little robust and conclusive data to indicate whether the type of matrices used for co-culture influence gene expression in hESCs over long term passaging.

A few studies have discussed the low level expression of differentiation genes, particularly at late passage (Tanaka et al., 2002). Yoon et al (2010) showed that three hESC lines cultured on feeder free matrices, Vitronectin, CellStart with

StemPro media or Matrigel/mTeSR1, expressed similar global gene patterns to those cultured on iMEF mouse feeders. Furthermore, studies assessing the use of different feeder types and Matrigel cultured hESCs on gene expression tend to be focussed on already well characterised hESC lines such as H1, H7, H9 and H14 (Carpenter et al., 2004) and only demonstrate the presence of a select few genes.

Advances in high throughput genomic screening have led to the development of TaqMan low density array cards (TLDA, Applied Biosystems), to screen for pluripotency and differentiation genes. These array cards have been carefully designed to avoid common errors associated with setting up and conducting large numbers of individual PCR assays manually, as demonstrated by the international stem cell initiative study, ISCI (Adewumi et al., 2007). TLDA card technology utilises novel gene signature arrays designed especially to detect genes specific to hESCs, using endogenous control selection. Each well is sealed therefore the individual reactions are both reliable and robust, as they allow for minimal cross contamination between samples. TLDA cards are cycled on an Applied Biosystems 7900HT real time PCR system and the relative quantification software gives accurate and reproducible gene expression data. The cards are designed to screen for 96 genes (inclusive of 6 housekeeping genes) carefully chosen for hESCs. The use of TaqMan probe technology ensures the highest probe specificity and easy loading of sample with minimal preparation. A recent study by Barbet et al (2011) demonstrated the sensitivity and robustness of TLDA cards to detect differences in gene expression between hESCs cultured on iMEFs and Mesenchymal stem cell lines (MSCs) committed towards mesoderm lineage.

The aim of these studies was to construct and compare gene expression profiles using TLDA card data from the four stem cell lines RH5, HUES9, SHEF1 and NCL5 cultured over 20 passages on the two mouse fibroblast lines iMEF and i3T3, the two human fibroblast lines iMRC5 and iHDFn, and the feeder free Matrigel matrix with mTeSR1 used as media. Data from early and late passage samples were compared, to examine if any changes in the pattern of gene expression occurred in individual stem cell lines as a consequence of prolonged culture on different matrices. The expression of germ layer specific differentiation genes was also included in the analysis of results. This information can be of use when profiling stem cells as they

can express such genes endogenously, at low levels, which can be used to indicate whether a stem cell line has begun to differentiate at a transcription level.

5.2 Methods

5.2.1 Preparation of hESCs for detection of early differentiation marker SSEA1 by flow cytometry

Stem cell lines NCL5, RH5, HUES9 and SHEF1 were cultured on iMRC5, iHDFn, iMEF and i3T3 using standard KO-HES media or Matrigel with defined media mTeSR1, as described by methods (Chapter 2). Samples were collected at early and late time points using TrypLE™ Express and fixed in 15 ml tubes using 4% PFA for 20 minutes, then pelleted and stored in WashBuffer as fixed pellets at 4°C until ready for flow cytometry staining as described in chapter 2 and chapter 5. Cells were assayed on Guava Easycyte flow cytometer and analysed using Easycyte software and Microsoft Excel. SSEA1 expression in NCL5 cells was determined in three individual experiments but repeated only twice for RH5, HUES9 and SHEF1 due to time limitations.

5.2.2 Preparation of hESCs for gene expression studies using TLDA cards

In parallel to flow cytometry, RNA was extracted from samples collected at early and late time points using Rneasy kit (Qiagen) following manufacturers' instructions as outlined in general methods chapter 2. RNA samples were subsequently assessed for integrity using the Agilent Bioanalyser (see methods section chapter 2). Low quality RNA can affect the downstream processes in PCR reactions therefore RNA with RNA integrity number (RIN) of 7 and above was used in the production of cDNA. Agilent RNA integrity chip technology gives a measure of RNA integrity RIN number, incorporating 18/28s and 260/230 ratios which indicate protein contamination and RNA degradation. Figure 5.1 shows an example of results obtained from Agilent Bioanalyser. cDNA was prepared using The Reverse Transcription Kit (Applied Biosystems) and amplified as described in general methods chapter 2. The TLDA cards were prepared according to manufacturer's guidelines and results were analysed using SDS2.4 and RQ manager v2.1 for plate to plate comparison of datasets.

The SHEF1 cells were only cultured to a maximum of P+15 as it failed to grow well and maintain typical undifferentiated stem cell morphology. Results from stem cell lines HUES9 and RH5 were from a single sample (n+1) as this formed part of an initial experimental screen to demonstrate whether any correlations in gene

expression data were apparent. Stem cell line NCL5 grew particularly well and samples from this stem cell line were assayed three times (n=3) to give more detailed results on the specific genomic integrity of the stem cell line. NCL5 cultures were frozen down at early (p+5) and late passage (p+20), then re-cultured, expanded and fresh RNA samples obtained. Care was taken to ensure only 1-2 passage difference between biological repeat samples.

5.2.3 Data analysis

Results were first examined in SDS2.4 software and gene expression reports with errors for example, high standard deviation or poor amplification, were omitted, to allow for better statistical measurements in analysis software RQ Manager. This also allowed for easier data handling when comparisons of data sets were performed, using ExpressionSuite software. Genes across all samples which were consistently not amplified (Ct value of 40) were also removed to allow for more accurate analysis of results. Samples were then grouped by germ layer/gene (Table 5.2) and compared in RQ Manager. Selection of reference genes (also known as a housekeeping gene) was also carried out by examining amplification of potential candidate reference genes (i.e. GAPDH and 18S) for each plate, then selecting which was most consistent across all the plates (GAPDH used for all samples). An endogenous control is a gene that is expressed constitutively and at the same level in all samples to be analysed. The calibrator sample for each plate and each study was assigned to be the stem cell line for that particular study cultured on MEFs at early passage as this is currently the standard culturing method (example: for NCL5 stem cell line, NCL5/iMEF early passage). Each calibrator study was examined in SDS2.4 software to ensure there were no erroneous results produced for the main genes of interest. Normalisation of the data against a calibrator allowed for the correction of PCR efficiency, which when carefully selected, can be applied to number of result plates and therefore allowed the comparison of the results from different PCR runs which was used to track changes over time (early and late passage).

A single preparation of the Embryonal teratocarcinoma (EC) cell line n2102EP was run on each card to ensure consistency across all the plates, and to allow for plate to plate comparison in RQ Manager. It has been demonstrated that hESCs demonstrate similar properties to EC cell lines (Sperger et al., 2003) and

consequently have been used as reference cell lines (Josephson et al., 2007). cDNA from the same n2102EP RNA sample was used across all plates in this experiment. The data produced from n2012EP was examined in SDS2.4 and RQ Manager to ensure consistency of results, but was excluded from data sets before analysis as these samples were not comparable. Samples were then analysed by normalising the data against the endogenous control and the calibrator sample to give delta ct values and relative quantification (RQ) values.

A TLDA card containing cDNA prepared from iMEF, iMRC5, i3T3 and iHDFn was also run to allow for genetic profile information of the feeders used in this project. This information was also used to compare against stem cell lines and as a negative control for the majority of the genes, as feeders do not express stem cell markers. Further analysis was carried out using gene expression analysis software ExpressionSuite (v.1.01, Applied Biosystems). Table 5.2 displays all the genes found on a TLDA card. The genes were organised by expression in undifferentiated cells, maintenance of pluripotency, differentiation markers and controls.

5.2.4 Statistical analysis

ExpressionSuite Software was used for analysis, which contains a number of statistical methods to normalise the data (GeNorm coupled with Pearson's correlation) and for outlier detection, Grubbs test was used. ExpressionSuite enabled the comparison of multiple plates and selection of endogenous controls from all genes analysed, to allow for accurate and reliable consistency across plates/different stem cell lines. Visualization tools to appropriately display fold change, biological significance, and cluster plots for establishing relationships between the stem cell samples on a per-plate basis were also applied. Results were deemed significant if they resulted in at least a 3 fold change. The overall significance of genes from each group in Table 5.2, were subject to T tests. 2 way ANOVA was also used to compare each stem cell line and results grouped at early and late passage, and by feeder type (mouse and human) to give P values significant to 0.05.

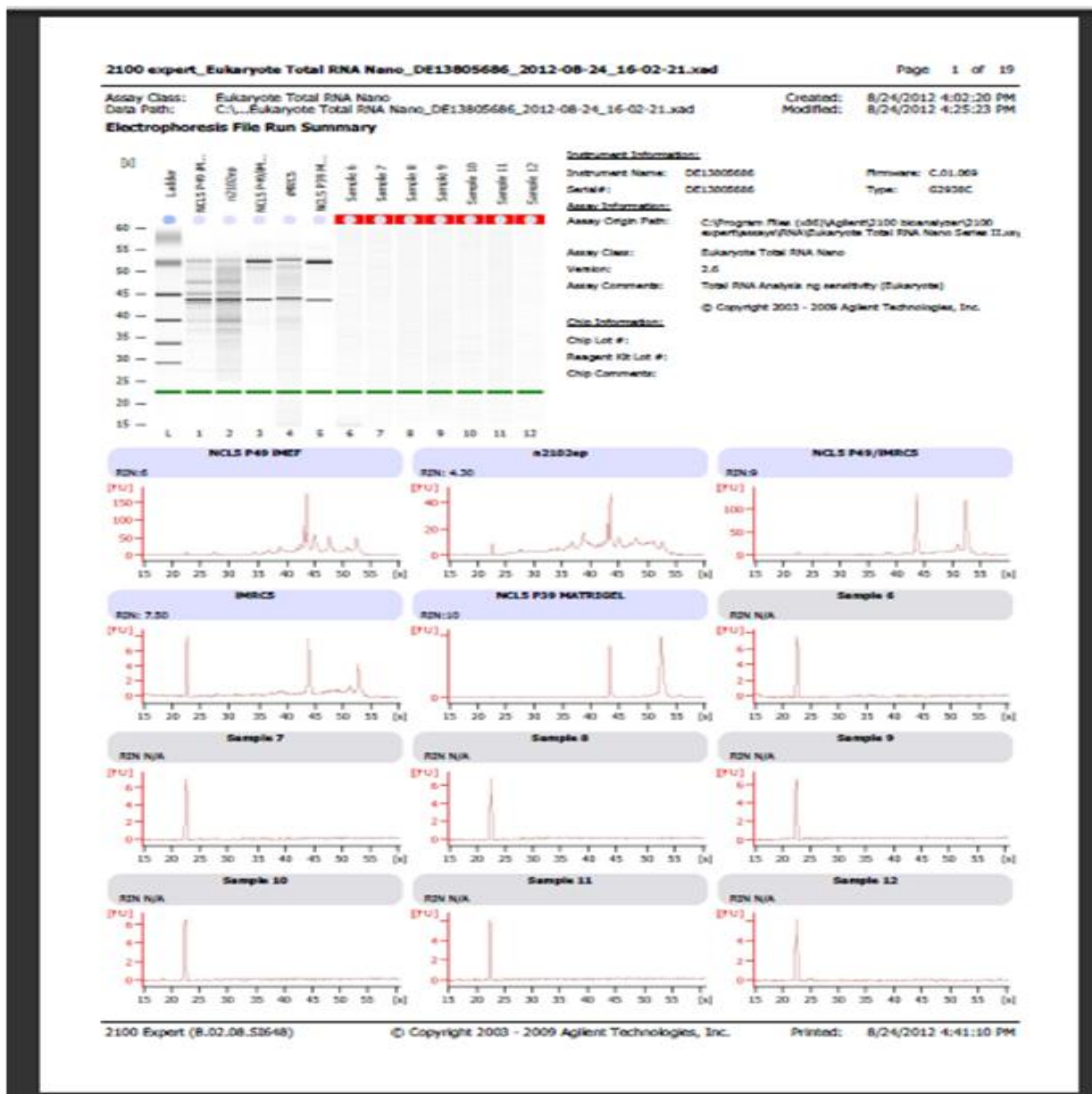


Figure 5.1. PDF printout of Agilent Bioanalyser results for RNA extracted from NCL5 on iMEF, n2102EP and NCL5 on iMRC5. The gel image in the top left hand corner displays the actual sample run, including the RNA nano marker ladder (first sample). Each graph shows the electrogram for the individual samples. Typically, two large peaks are seen for each sample, corresponding to 18s/28s ratio. The smaller peaks found between the large peaks relate to contamination by proteins or chemicals, for example ethanol carryover, as shown by the second graph for n2102EP sample. RNA samples with an overall RIN of less than 7 were not used to make cDNA for RT PCR.

Table 5.1: List of samples collected for RT PCR

Cell line/feeder type	Time point for sample collection
NCL5/IHDFN	P+5, P+25
NCL5/IMRC5	P+5, P+25
NCL5/I3T3	P+5, P+25
NCL5/IMEF	P+5, P+25
NCL5/Matrigel	P+5, P+25
HUES9/IHDFN	P+5, P+25
HUES9/IMRC5	P+5, P+25
HUES9/I3T3	P+5, P+25
HUES9/IMEF	P+5, P+25
HUES9/Matrigel	P+5, P+25
RH5/IHDFN	P+5, P+25
RH5/IMRC5	P+5, P+25
RH5/I3T3	P+5, P+25
RH5/IMEF	P+5, P+25

Table 5.2 Genes on TLDA Stem cell pluripotency card

Reference genes	Expression in undifferentiated cells	Correlation to stemness	Endoderm	Ectoderm	Mesoderm
ACTB	NANOG	BRIX	AFP	NEUROD1	ACTC
RAF1	POU5F1	CD9 COMMD3 CRABP2	SERPINA1	NES	Brachury (T)
CTNNB1	SOX2	DDX4	LAMC1	PAX6	EOMES
GAPDH	TDGF1	EBAF	LAMA1	GFAP	WT1
EEF1A1	DNMT3B	FGF4	FN1	ISL1	MYF5
18S	GABRD3	FGF5	LAMB1	TH	MYOD1
	GATA6	FOXD3	SOX17	SYP	DES
	GDF3	GAL	PTF1A	SYCP3	NPPA
		GBX2	FOXA2	HLXB9	HBB
		GRB7	GATA4	OLIG2	HBZ
		IFITM1 IFITM2	TAT	FOXD3	RUNX2
		IL6ST	PAX4	NR6A1	COL1A1
		IMP2	SST	NR5A2	COL2A1
		KIT	INS		PECAM1
		KRT1	IPF1		CDH5
		LEFTB	GCG		CD34
		LIFR	IAPP		
		LIN28	FLT1		
		NODAL	INS		
		NOGGIN			
		PODXL			
		PTEN			
		REST SEMA3A			
		SERPINA1			
		SFRP2			
		TERT TFCP2L1 UTF1			
		Xist			
		ZFP42			

5.3 Results

5.3.1 Expression of differentiation marker SSEA1 on gene expression in hESCs

As the expression of undifferentiated genes in hESCs cultured long term can vary from passage to passage, it was important to demonstrate that the hESCs studied still maintained the ability to proliferate in a mostly undifferentiated homogenous population. The expression of early differentiation cell surface marker SSEA1 was therefore examined by flow cytometry at early and late passage. The expression of this cell surface marker is of particular importance when assessing gene expression from early to late passage, as this could affect the quantity of differentiation genes detected. Table 5.3 demonstrates that for the hESC lines HUES9 and NCL5, less than 10% differentiation was detected by the relatively low expression levels of SSEA1 at early and late passage under the different experimental conditions. The RH5 cells demonstrated higher levels of SSEA1 expression at early passage but cells cultured on human feeders and Matrigel appeared to lose SSEA1 expression at later passage compared with those on mouse feeders, possibly indicating an increased ability to adapt on human feeders compared with mouse. NCL5 cultured on i3T3 at late passage also demonstrated greater than 10% differentiation (33.21%). Increases in SSEA1 expression at late passage will be correlated with any detected upregulation in genes associated with differentiation.

Table 5.3. Detection of early differentiation cell surface marker SSEA1 determined by flow cytometry.

STEM CELL LINE/FEEDER	SSEA1 expression by FC at early passage (%)	SSEA1 expression by FC at late passage (%)
RH5/iMEF	19.29	19.34
RH5/i3T3	8.49	17.62
RH5/iMRC5	11.09	1.92
RH5/iHDFn	52.35	3.89
RH5/Matrigel	17.81	8.85
HUES9/iMEF	0.27	0.39
HUES9/i3T3	1.50	1.34
HUES9/iMRC5	2.40	2.59
HUES9/iHDFn	1.45	0.52
HUES9/Matrigel	5.20	3.58
NCL5/iMEF	4.33	3.81
NCL5/i3T3	4.81	33.21
NCL5/iMRC5	6.15	4.54
NCL5/iHDFn	5.23	3.43
NCL5/Matrigel	8.07	7.71

5.3.2 Changes in gene expression in the RH5 stem cell line as a result of long term culture on different feeders and Matrigel

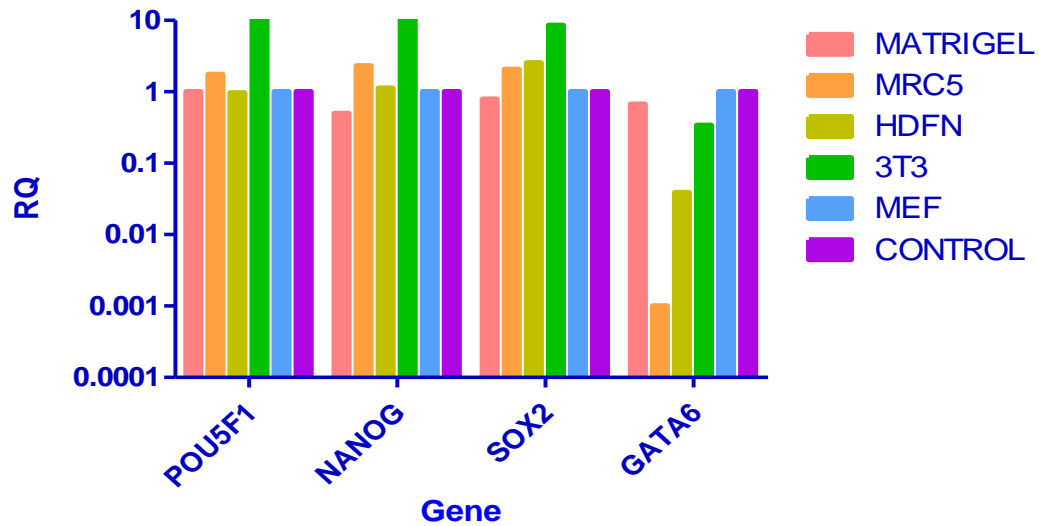
Figure 5.2 demonstrates that expression of undifferentiated genes were all maintained and the majority upregulated from early to late passage. Expression of OCT4 and NANOG increased on all feeders and Matrigel cultures at late passage, with the exception of RH5 cultured on i3T3 at early passage which showed unusually high RQ expression of OCT4, NANOG and SOX2. These were however downregulated at late passage. GATA6 expression was lower in cultures on both human feeders at early passage compared with mouse feeders and Matrigel, and was further downregulated at late passage. RH5 on iMEF (mouse feeder) and iMRC5 (human feeder) demonstrated the greatest stability of self-renewal and differentiation markers compared to the other feeder types.

Differences in expression of differentiation markers were seen with RH5 cultured on i3T3, by significant upregulation (greater than 3 fold) of SOX17 and FOXA2 and downregulation of AFP and GATA4 from early to late passage. Downregulation of SOX17, AFP and FOXA2 was observed for all other cultures from early to late passage. PAX6 and OLIGO2 expression were upregulated at late passage on all four feeder types. Expression of Nestin, ISL1, and HLXB9 was upregulated by cultures on feeder lines iMRC5, iHDFn and iMEF and downregulated on i3T3s. Relative quantity of mesoderm genes from RH5 cultured on Matrigel was low in comparison to the four feeder types. RH5 cultured on iHDFn and iMRC5 human feeders demonstrated significant upregulation of Mesoderm genes RUNX2, DESMIN and COL1A1 from early to late passage. RH5 cultures on i3T3 showed downregulation of RUNX2, COL1A1 and Desmin.

Overall, RH5 cultured on human feeders appear to indicate upregulation of mesoderm and ectoderm genes (Figures 5.4 and 5.3), whereas RH5 cultured on mouse feeder i3T3 showed a preference towards endoderm as shown by upregulation of SOX17 and FOXA2, from early to late passage. However this could be specifically correlated with cell surface marker SSEA1 expression, which was greater than 10% from RH5 cultured on both mouse feeders. The upregulation of PAX6, OLIGO2, ISL1, NESTIN and GFAP as seen in Figure 5.3 may indicate a preference of RH5 to differentiate towards early neuronal lineage. Further differentiation studies are required to confirm this observation.

RH5

A



B

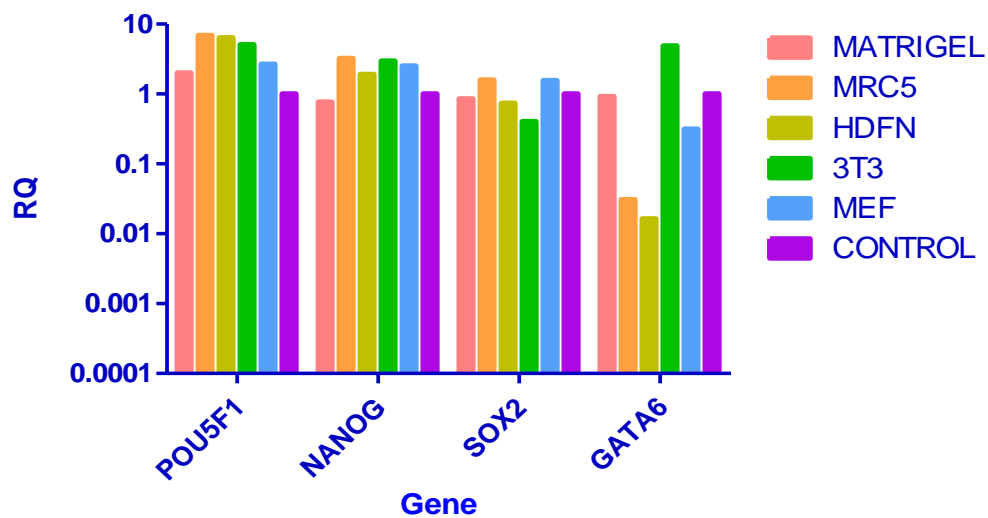
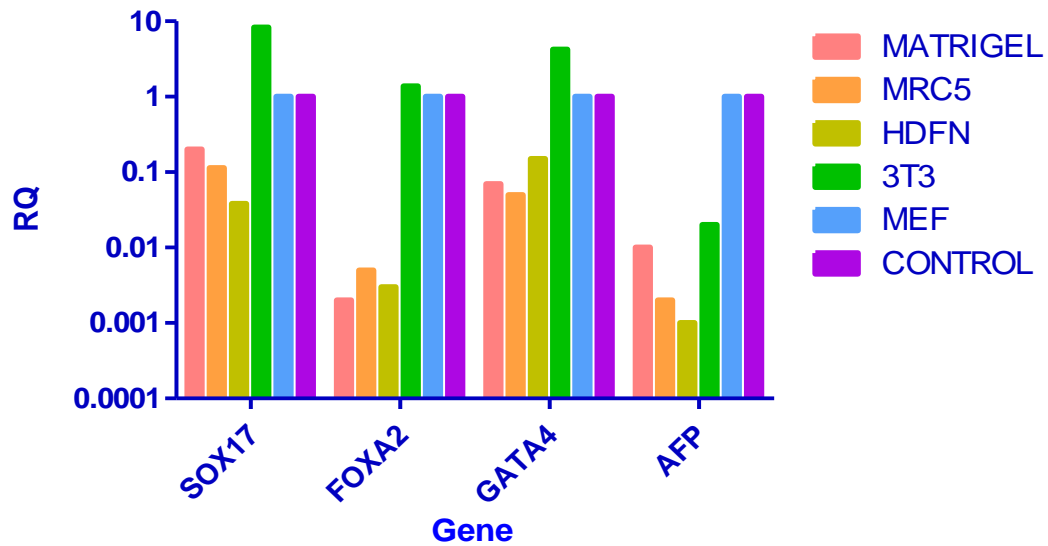


Figure 5.2. Changes in undifferentiated gene expression profile from RH5 stem cells induced by long term culture on mouse feeders, human feeders or Matrigel using TLDA cards. Relative Quantity (LOG10RQ) of selected undifferentiated hESCs genes from stem cell line RH5 cultured on four feeder types and Matrigel at early passage A and late passage B were obtained from TLDA card real time PCR. The data was obtained from one experiment.

RH5

A



B

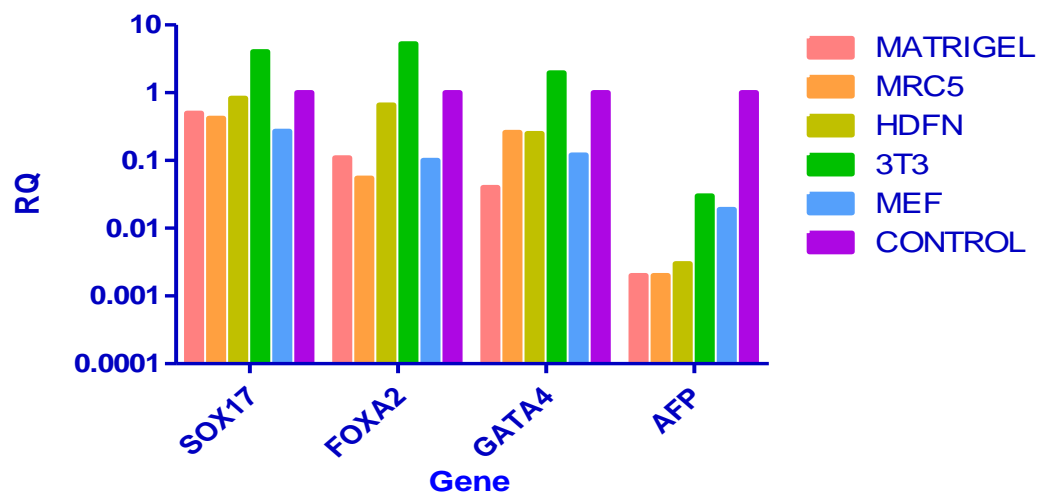


Figure 5.3. Changes in Endoderm gene expression profile from Stem cell line RH5 as a result of long term culture on mouse, human feeders and Matrigel using TLDA cards. Relative Quantity (log₁₀ RQ) of selected endoderm genes from stem cell line RH5 cultured on four feeder types at early A and late B passage demonstrated downregulation of SOX17, AFP and FOXA2 was observed from all cultures from early to late passage, with the exception of 3T3 mouse feeders, which showed significant upregulation. Data is representative of one experiment.

RH5

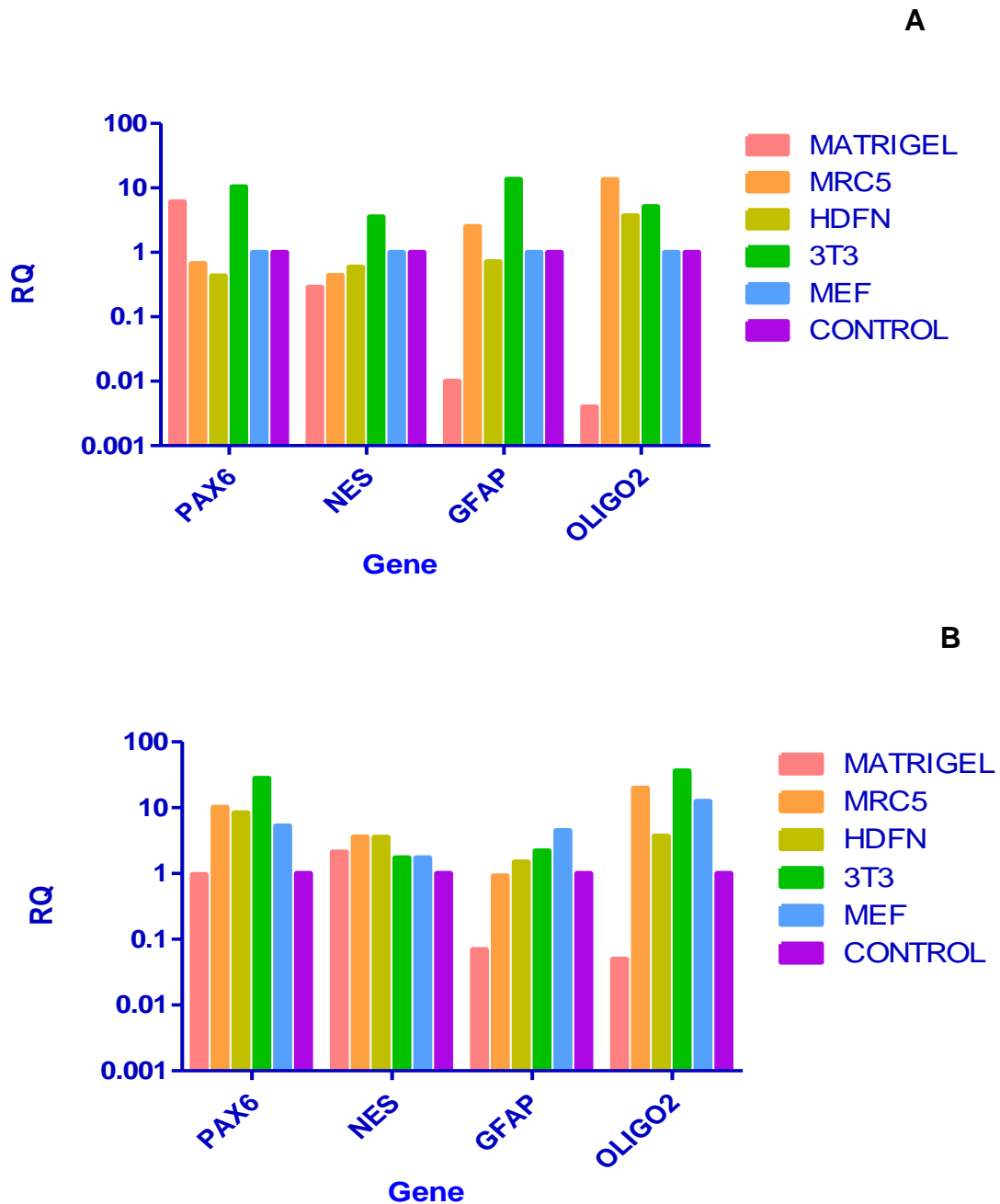


Figure 5.4. Changes in ectoderm gene expression profile from Stem cell line RH5 as a result of long term culture on mouse, human feeders and Matrigel using TLDA cards. Relative Quantity (LOG10 RQ) of selected ectoderm genes from stem cell line RH5 cultured on four feeder types at early A and late B passage show that higher levels of PAX6, OLIGO2 and NESTIN were detected from RH5 cultured on i3T3s and iMRC5, from early to late passage. Expression of ectoderm genes decreased from cultures on Matrigel, at early to late passage. Data is representative of one experiment.

RH5

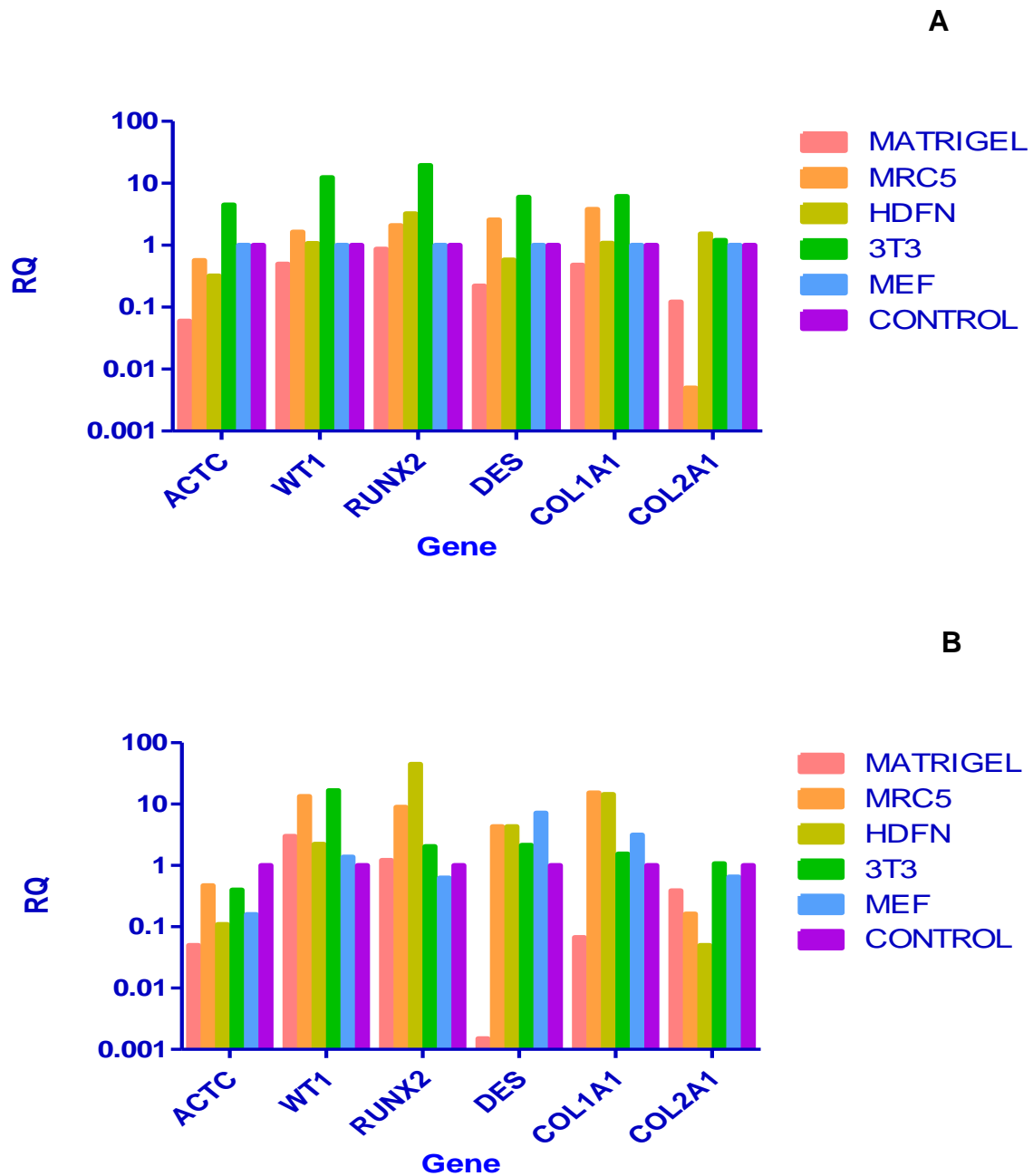


Figure 5.6. Changes in mesoderm gene expression profile from Stem cell line RH5 as a result of long term culture on mouse, human feeders and Matrigel using TLDA cards. Relative Quantity (LOG10 RQ) of selected mesoderm genes from stem cell line RH5 cultured on four feeder types at early A and late B passage show that expression of all mesoderm genes was higher from RH5 cultured on 3T3s and MRC5 compared to the other feeder types and control. Matrigel cultures gave consistently low expression of mesoderm genes from early to late passage. Data is representative of one experiment.

5.3.3 Changes in gene expression in HUES9 stem cell line as a result of long term culture on different feeders and Matrigel

Expression of undifferentiated genes SOX2, POU5F1, Nanog, and GATA6 were maintained from HUES9 cultured on four feeder types at early and late passage (Figure 5.6). Slight downregulation of SOX2 and GATA6 was seen for HUES9 cultured on iMRC5, iHDFn and iMEFs from early to late passage. Significant upregulation of NANOG from HUES9 cultured on iMEF at late passage was detected. Furthermore, significant downregulation of POU5F1 and NANOG was seen on both human feeders.

Overall, low expression of ectoderm genes were detected on HUES9 cultures (Figure 5.8). HUES9 cultured on iMRC5 had slightly higher expression of these genes compared to iHDFn and iMEF cultures, at early passage. Significant downregulation of endoderm genes AFP and GATA4 from iMRC5 and iMEF cultures occurred, from early to late passage. A significant upregulation of SOX17, FOXA2 and GATA4 was observed at late passage for HUES9 cultured on i3T3s at late passage (<0.1 to >1), relative to the control

Higher levels of all mesoderm genes ACTC, WT1, RUNX2, DES and COL1A1 were found on both human feeders compared to mouse feeders at early passage, although significant downregulation of RUNX2, COL1A1 and Desmin was observed at late passage (Figure 5.9). Higher expression of mesoderm genes was detected in HUES9 cultured on human feeders compared with other germ layer specific genes. However these values do not indicate that the cells were differentiated as these RQ values were still low as was SSEA1 cell surface marker expression. This may provide insight that these cells could have greater potential to differentiate towards mesoderm in comparison to ectoderm and endoderm, if left to spontaneously differentiate. Cultures on Matrigel appeared to have the lowest expression of mesoderm genes (<1.0) from early to late passage.

HUES9

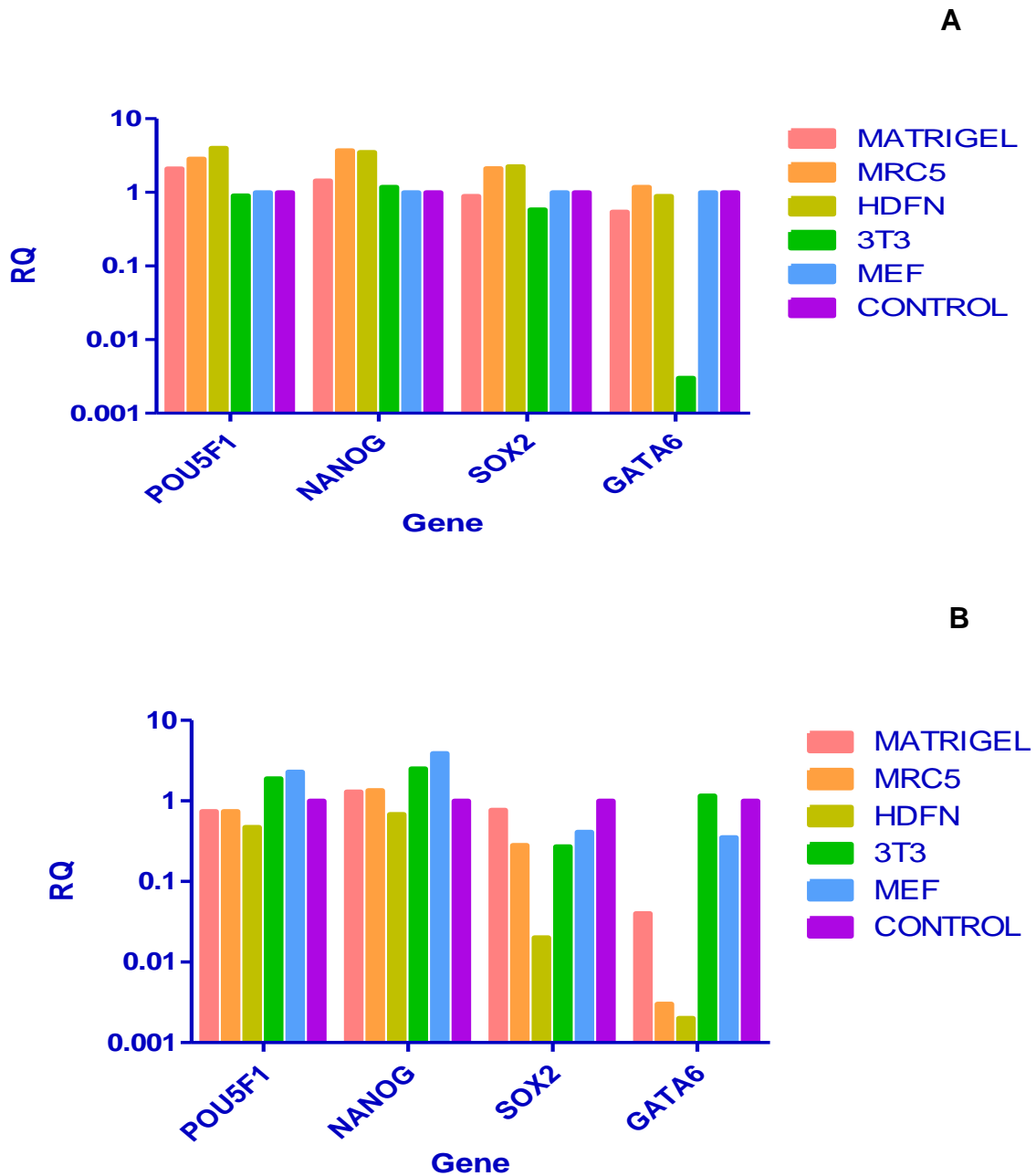
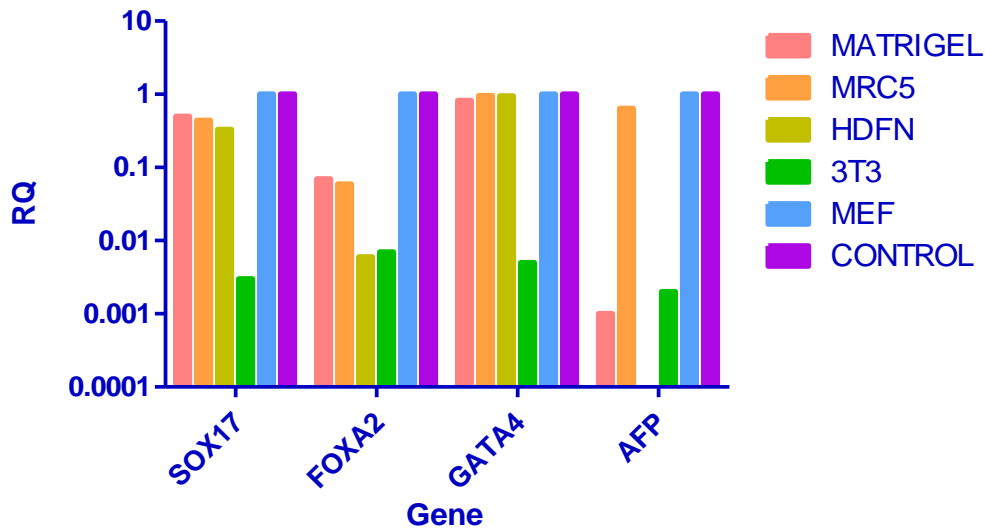


Figure 5.6. Changes in undifferentiated gene expression profile from Stem cell line HUES9 as a result of long term culture on mouse, human feeders and Matrigel using TLDA cards. Relative Quantity (LOG10 RQ) of selected undifferentiated genes from HUES9 cultured on four feeder types and Matrigel at early A and late B passage show that undifferentiated gene expression was maintained from early to late passage. Cultures on human feeders gave higher expression of undifferentiated genes at early passage. Data is representative of one experiment.

HUES9

A



B

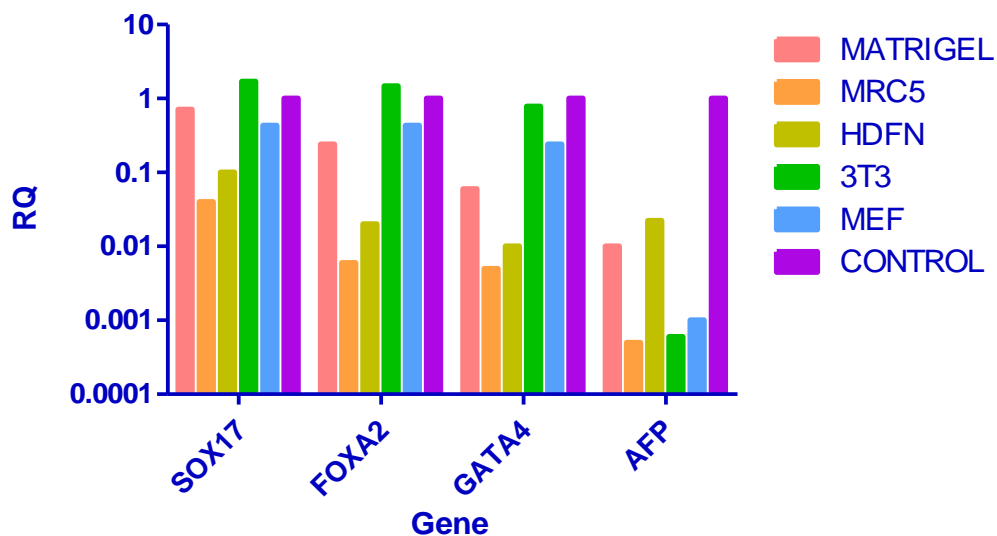
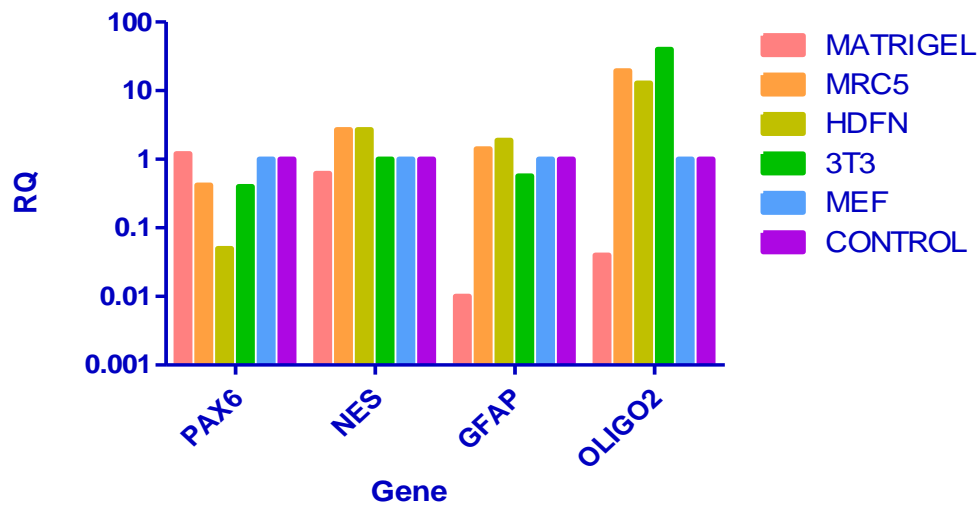


Figure 5.7. Changes in endoderm gene expression profile from Stem cell line HUES9 as a result of long term culture on mouse, human feeders and Matrigel using TLDA cards. Relative Quantity (LOG10 RQ) of selected endoderm genes from HUES9 cultured on four feeder types at early A and late B passage showed demonstrated low levels of endoderm genes were detected at both early and late passage for HUES9. Expression of AFP for RH5 on iMEF and iMRC5 cultures significantly decreased from early to late passage. Data is representative of one experiment.

HUES9

A



B

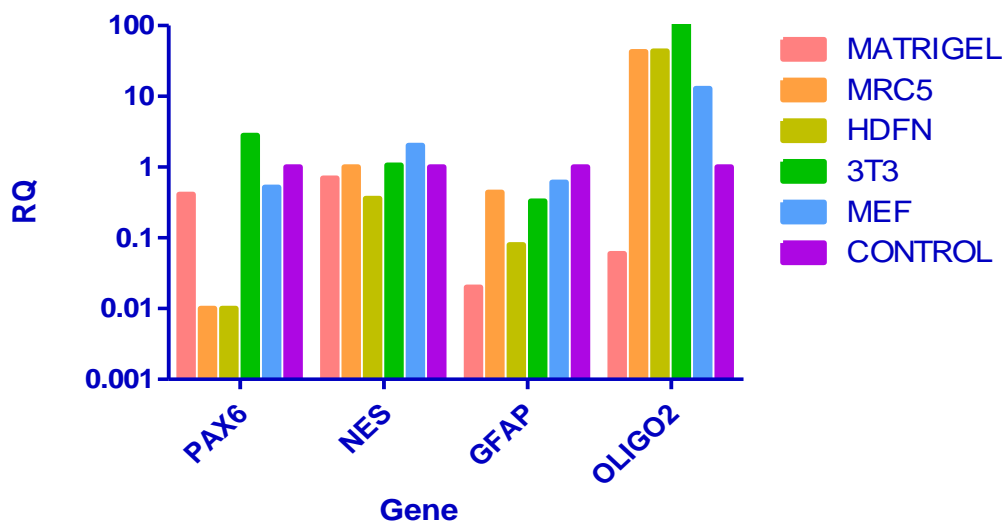
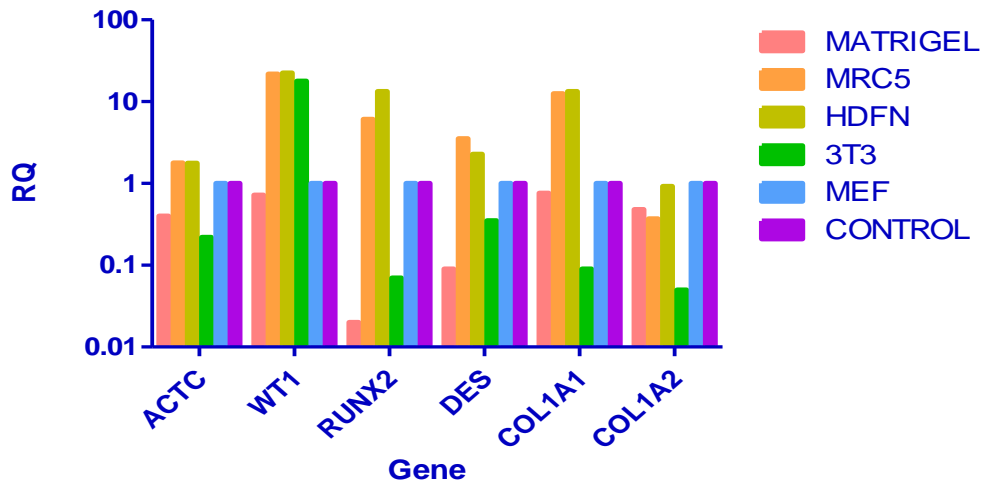


Figure 5.8. Changes in endoderm gene expression profile from Stem cell line HUES9 as a result of long term culture on mouse, human feeders and Matrigel using TLDA cards. Relative Quantity (LOG10RQ) of endoderm genes from RH5 cultured on four feeder types at early A and late B passage showed that expression of OLIGO2 was significantly higher for HUES9 cultured on i3T3s, iMRC5 and iHDFn at early passage, in comparison to iMEF and Matrigel cultures. Expression of ectoderm genes was consistently low for Matrigel, at both early and late passage (<1.0). Data is representative of one experiment.

HUES9

A



B

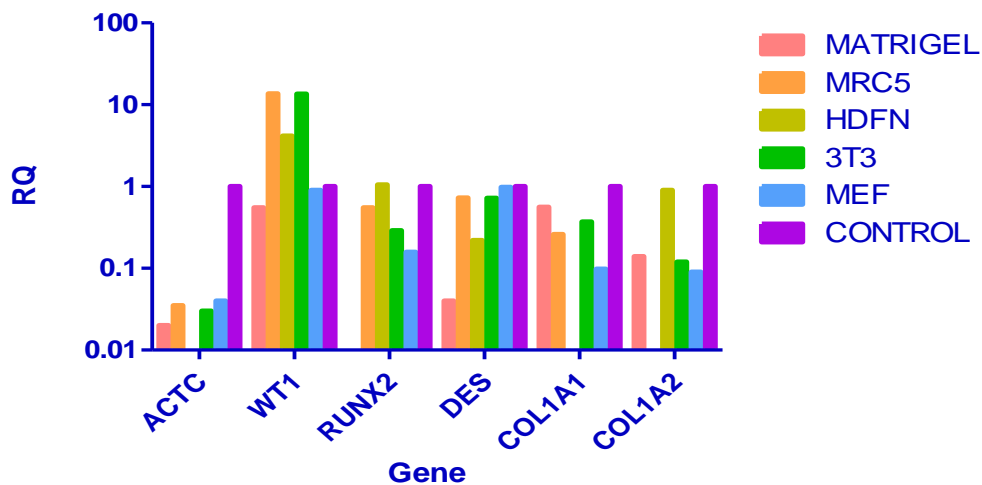


Figure 5.9. Changes in mesoderm gene expression profile from Stem cell line HUES9 as a result of long term culture on mouse, human feeders and Matrigel using TLDA cards. Relative Quantity (LOG10RQ) of mesoderm genes from HUES9 cultured on four feeder types and Matrigel at early A and late B passage showed upregulation of mesoderm genes ACTC, WT1, RUNX2, DES and COL1A1 at early passage for HUES9 cultured on iMRC5 and iHDFn, however these were markedly downregulated at late passage. Cultures on Matrigel appeared to have the lowest expression of mesoderm genes (<1.0) from early to late passage. Data is representative of one experiment.

5.3.4 Changes in gene expression in NCL5 stem cell line as a result of long term culture on different feeders and Matrigel

Results for the NCL5 stem cell line were subject to biological repeats, as this stem cell line maintained healthy cultures at late passage (n=3). Results show that expression of undifferentiated genes was maintained at early and late passage (Figure 5.10). Fluctuations of GATA6 expression were detected from early to late passage in that levels in cells cultured on Matrigel appeared downregulated, whereas those cultured on iHDFn and iMEF were upregulated. Overall, GATA6 expression was low in comparison to the other genes.

Upregulation of endoderm genes FOXA2, SOX17 and GATA4 was seen from early to late passage for NCL5 cultured on iMEF, iMRC5 and Matrigel (Figure 5.11). Endoderm expression was lowest for NCL5 cultured on i3T3 at early and late passage. Overall, a significant increase in endoderm expression was detected for NCL5 cultured on iMEF and iMRC5 (Figure 5.11 B). Differences in ectoderm expression were apparent due to different feeder types, from early to late passage. NCL5 cultured on mouse feeders iMEFs and i3T3s caused significant upregulation of ectoderm genes, whereas a slight downregulation of NESTIN and GFAP was detected from iMRC5 and iHDFn, although iMRC5 also demonstrated upregulation of PAX6 at late passage. NCL5 cultured on Matrigel also showed upregulation of ectoderm genes at late passage (Figure 5.12).

Mesoderm expression was highest for iMRC5 and Matrigel cultures, which increased significantly at late passage (Figure 5.13B). The lowest expression of mesoderm genes was detected for NCL5 on mouse feeders, iMEF and i3T3, at early and late passage. Expression of ACTC and RUNX2 significantly decreased at late passage from NCL5 cultured on all iMEF, i3T3 and iHDFn.

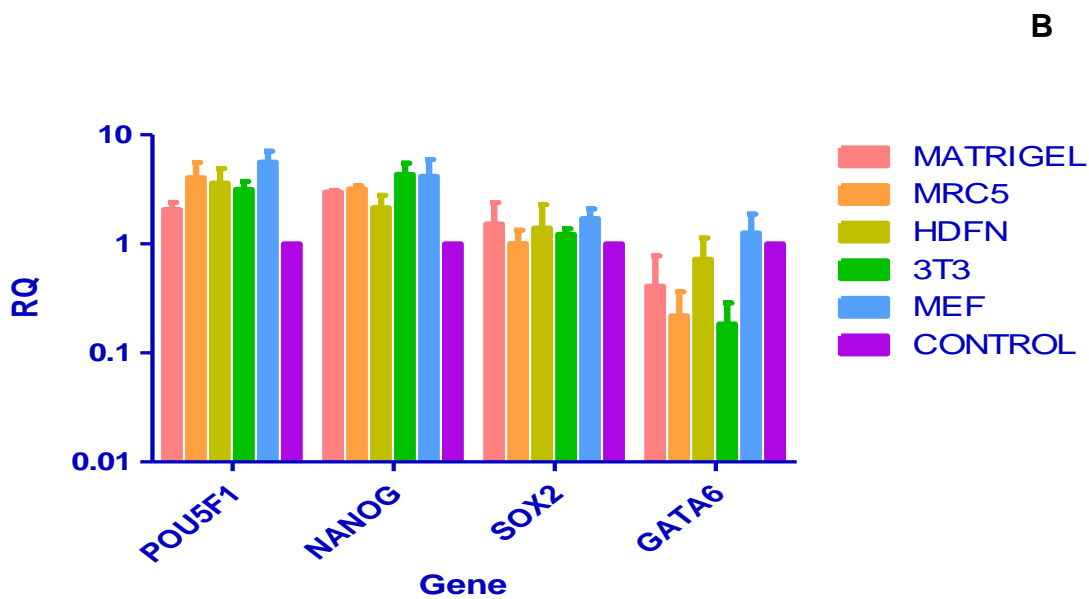
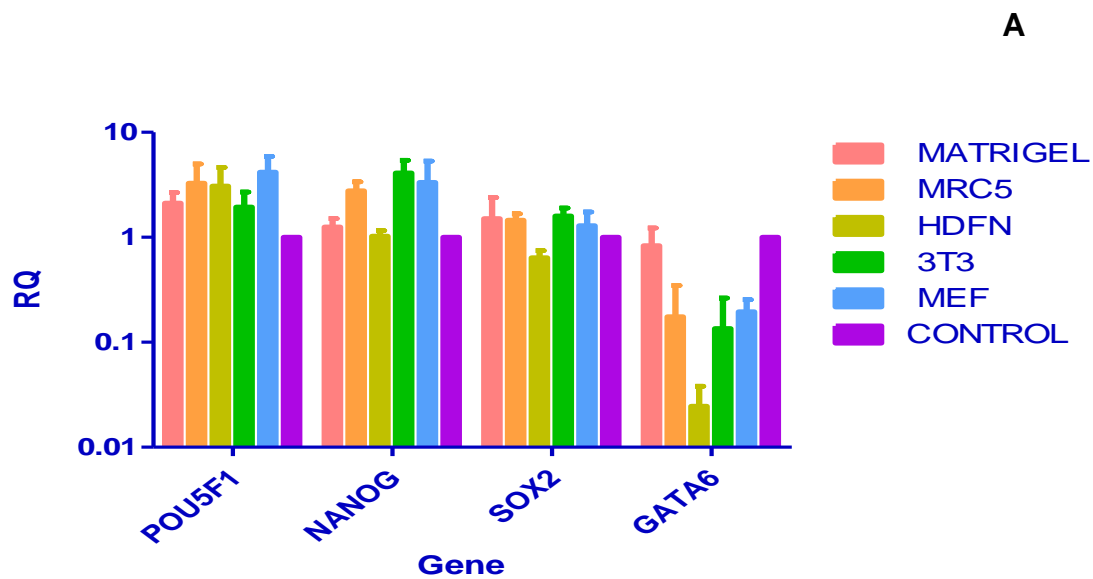


Figure 5.10. Changes in undifferentiated gene expression profile from Stem cell line NCL5 as a result of long term culture on mouse, human feeders and Matrigel using TLDA cards. Average Relative Quantity (LOG10 RQ) of selected undifferentiated genes from NCL5 cultured on four feeder types and Matrigel at early A and late B passage demonstrated that expression of stemness genes were maintained at early and late passage. A slight increase in POU5F1 and NANOG was detected at late passage. Data is representative of three experiments (n=3).

NCL5

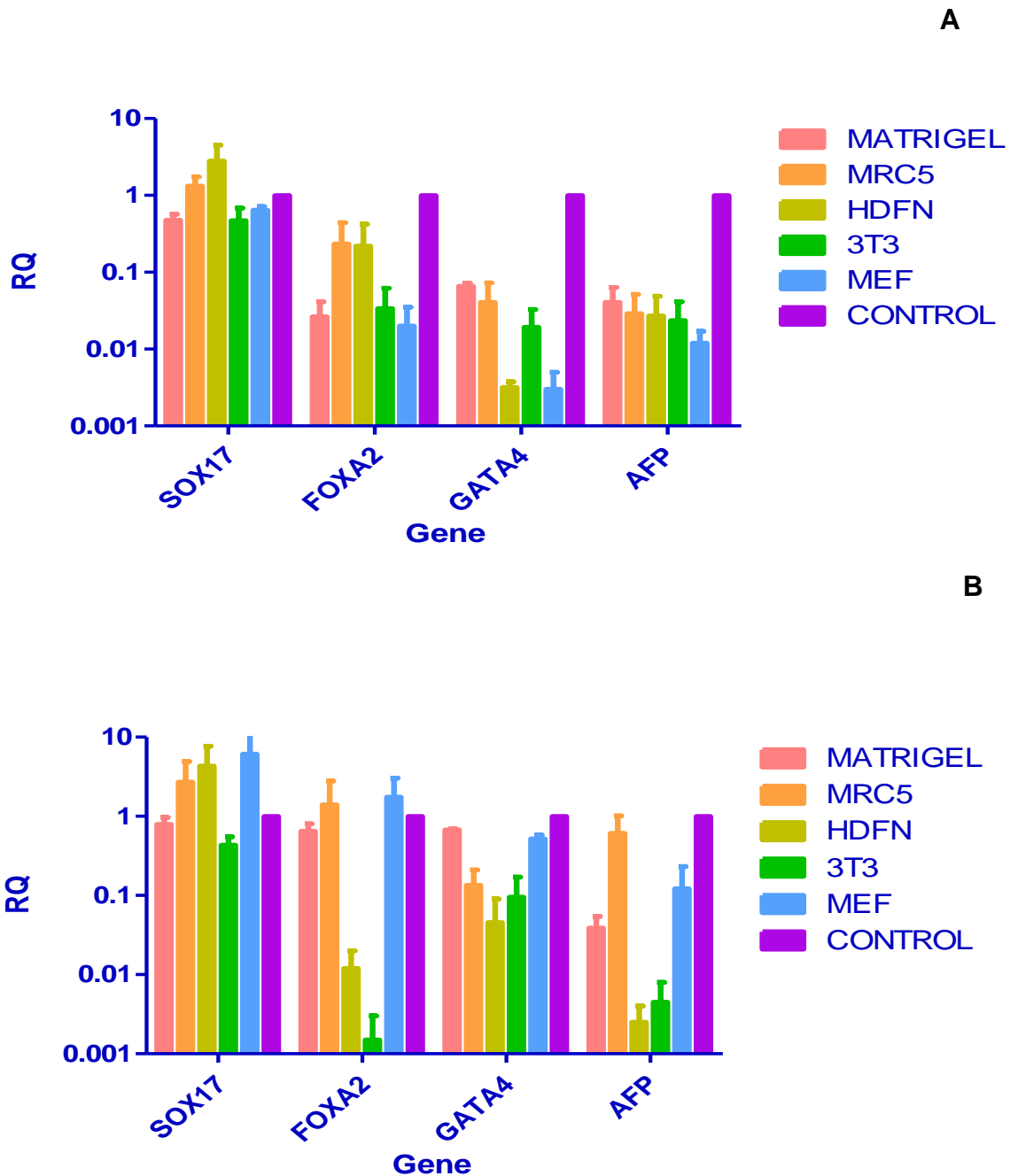
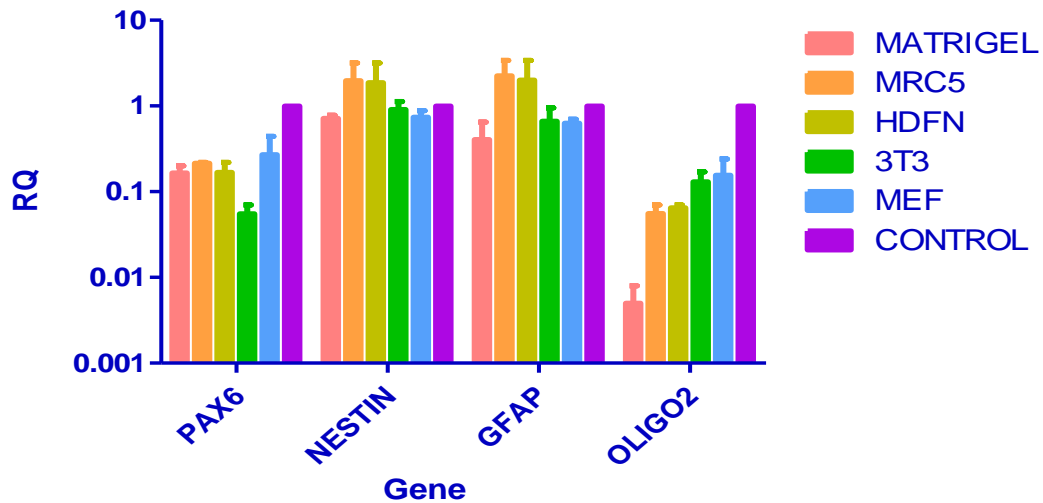


Figure 5.11. Changes in endoderm gene expression profile from Stem cell line NCL5 as a result of long term culture on mouse, human feeders and Matrigel using TLDA cards. Relative Quantity (LOG10 RQ) of selected Endoderm genes from NCL5 cultured on four feeder types and Matrigel at early A and late B passage show that expression of endoderm genes were generally low at early passage and increased for NCL5 cultures on iMEF, iMRC5 and Matrigel from early to late passage. Significant upregulation of SOX17 was seen from NCL5 cultured on iHDFn at late passage. Data is representative of three experiments (n=3).

NCL5

A



B

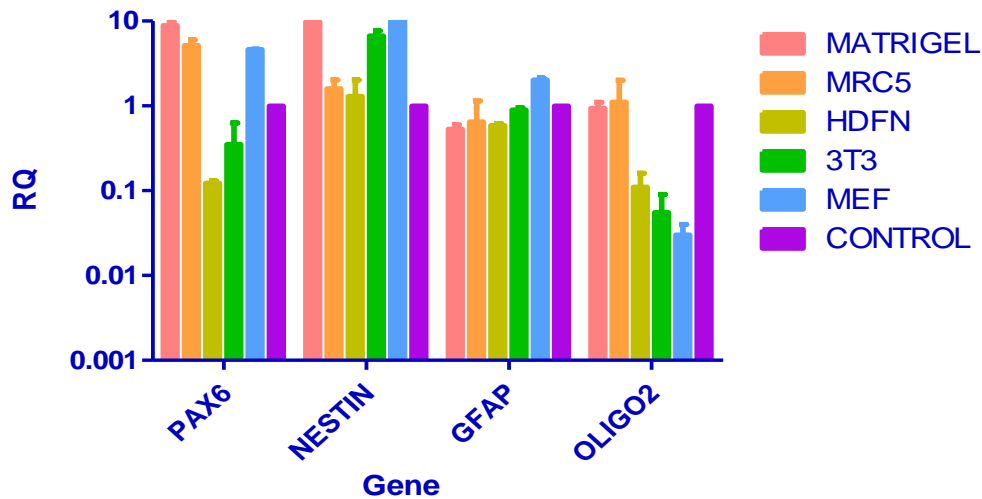


Figure 5.12. Changes in ectoderm gene expression profile from Stem cell line NCL5 as a result of long term culture on mouse, human feeders and Matrigel using TLDA cards. Relative Quantity (LOG10 RQ) of selected ectoderm genes from NCL5 cultured on four feeder types and Matrigel at early A and late B passage showed significant upregulation of ectoderm genes was seen from cultures on Matrigel, iMEFs and i3T3s, from early to late passage. Downregulation of NESTIN and GFAP were observed in NCL5 cultured on human feeders' iMRC5 and iHDFn. Data is representative of three experiments (n=3).

NCL5

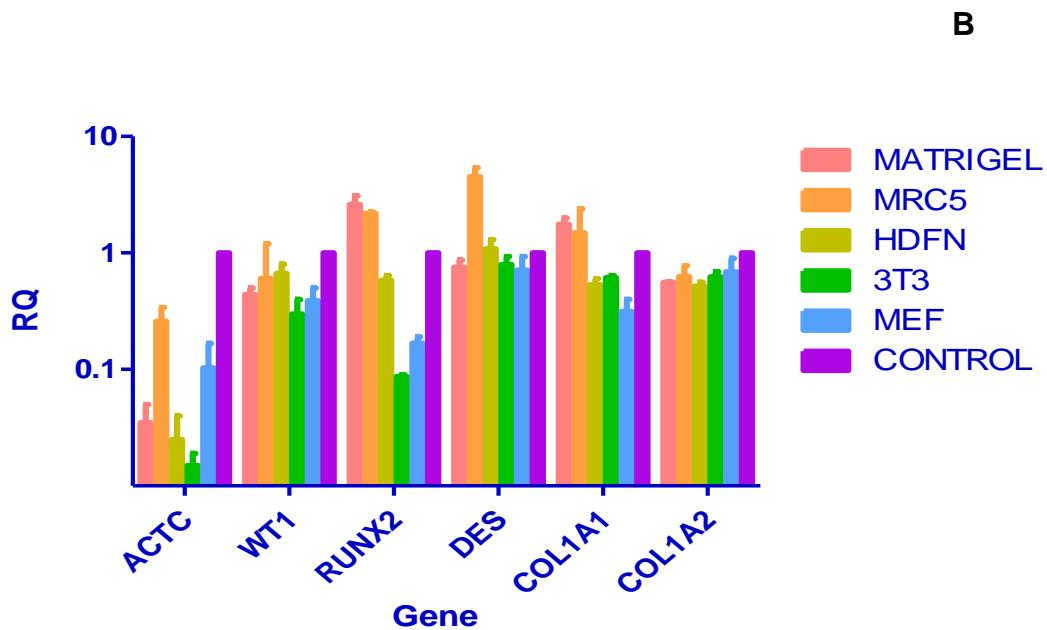
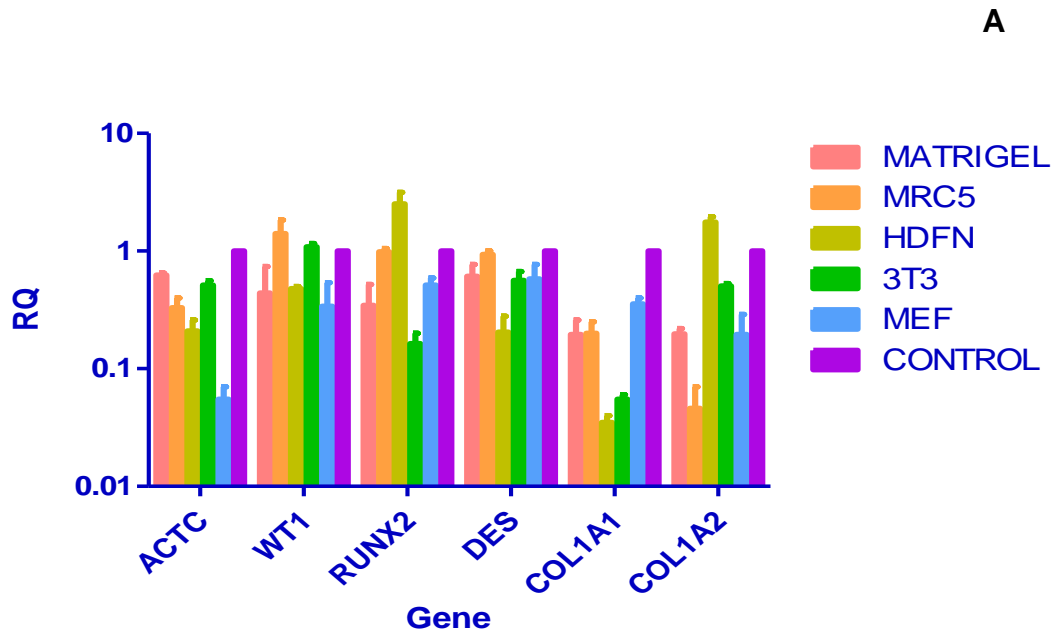


Figure 5.13. Changes in mesoderm gene expression profile from Stem cell line NCL5 as a result of long term culture on mouse, human feeders and Matrigel using TLDA cards. Relative Quantity (LOG10 RQ) of selected Mesoderm genes from NCL5 cultured on four feeder types and Matrigel at early A and late B passage demonstrated upregulated expression of mesoderm genes for iMRC5 and Matrigel cultured cells from early to late passage. Expression of ACTC was very low from all conditions at late passage. Data is representative of three experiments (n=3).

5.3.5 Comparison of gene expression between stem cell lines cultured on human, mouse feeders at early and late passage.

ExpressionSuite Software allowed the selection of appropriate reference genes by permitting all targets (genes) to be candidate reference genes (Figure 5.14A). This enabled the selection of a number of stable reference genes across all samples, with less than 2 fold change; therefore accurate hierarchical cluster analysis could be performed across all the stem cell samples. Figure 5.14B shows the Ct value for reference gene 18s, which demonstrated considerable variability across the majority of stem cell samples; therefore more stable genes such as ACTB were selected.

The box plot shown by Figure 5.15 demonstrates a wide variation in Ct values across three different stem cell lines cultured on four different feeders at early and late passage. Data for the embryonal carcinoma cell line n2102EP is also shown (purple bars) as it was originally included as a control cell line and shows a wide range in Ct values. The box plot also showed that NCL5 and RH5 cultured on iMEF demonstrated an increase in Ct value from early to late passage. Cultures on both human feeder types appeared to be less variable compared to both mouse feeders. The widest variation in Ct value was seen from both human feeders iMRC5 and iHDFn compared to iMEF, which showed the least variation in Ct value.

Figure 5.16 shows the delta Ct values for the three stem cell lines cultured on four feeder types at early and late passage, which have been 'heat mapped' into arbitrary ranges to give an indication of high (green) and low (red) expression. Genes for Brachury (T), SERPINA, NPPA, NEUROD, MYOD, MYF5, KRT1, INS, HBB and GCG were removed due to inconsistencies with expression. Human feeders, early passage stem cells and the majority of NCL5 clustered to the left of the heat map, whereas the majority of HUES9, RH5, and stem cells cultured at late passage, on iMEFs, iMEF feeder line and n2102EP grouped together to the right of the heat map, due to the increased expression of differentiation genes.

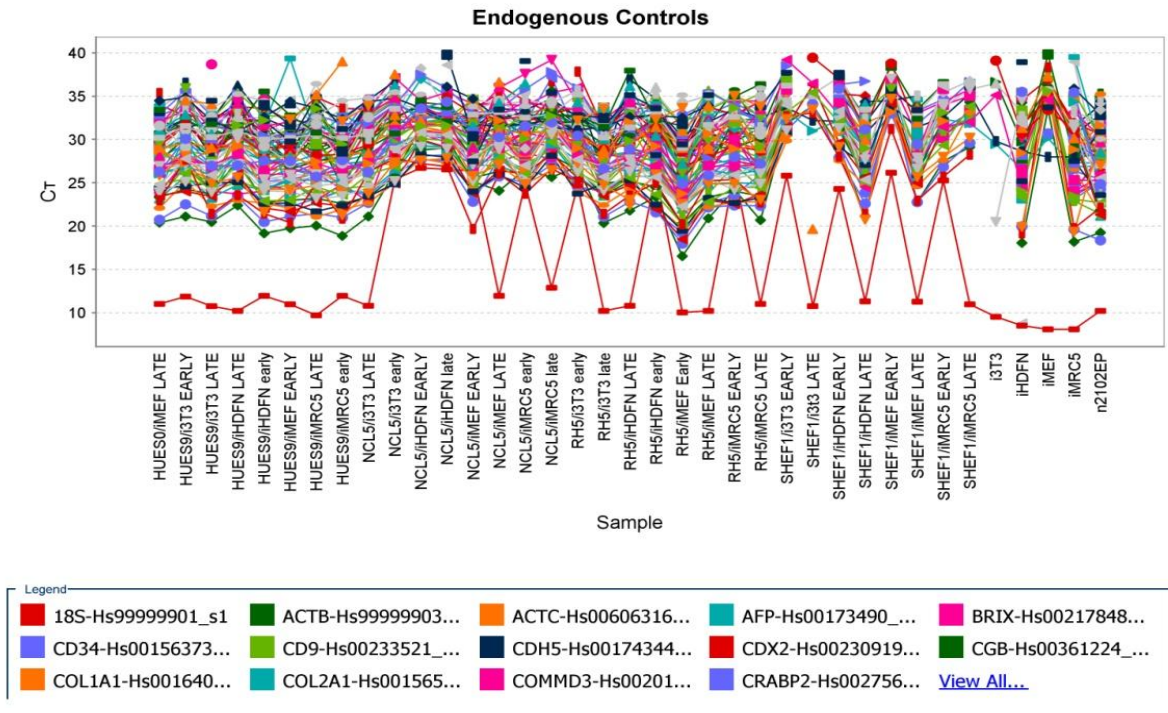
Stem cell lines NCL5 and HUES9 grouped together, whereas RH5 appeared to group by feeder type with the other stem cell lines, instead of correlating as a stem cell line. RH5 and HUES9 cultured on mouse feeder's iMEF and i3T3 at late passage grouped together. Early passage samples also grouped together, at the far left side of the heat map and included NCL5 cultured on iMEF, NCL5 cultured on iMRC5, NCL5 cultured on i3T3 and RH5 cultured on iHDFn. Stem cell line HUES9

appears in the middle of the heat map, whereas RH5 appears to be scattered across the heat map, indicating its spread in expression of genes as a result of the influence of different feeder types, from early to late passage. Stem cell line NCL5 appeared to be more consistent in comparison with the other stem cell lines, cultures on iHDFn and i3T3s grouped together at early and late passage.

Genes that grouped together from all samples includes those commonly associated with support, growth and maintenance of pluripotency i.e. COL1A2, POU5F1 (OCT4), LAMB1, LAMA1 NOGGIN, KIT and SOX2. Differentiation genes such as RUNX2, COL1A1, FLT1, and GFAP also grouped together as they were upregulated. Self-renewal and maintenance genes Nanog, CRAPB and PODXL did not appear to correlate strongly amongst the stem cell lines. Interestingly, CDX2 was found to be highly expressed for stem cells cultured on iHDFn human feeder.

Gene expression levels were also analysed in all four feeder cells alone, to determine whether expression could be detected at low levels, which could have contributed to increased expression within the stem cell lines they were cultured on. Unsurprisingly, the mouse feeders grouped together, to the far right of the heat map as they expressed the least amount of genes. iMEF did not express any genes, whereas iHDFn and iMRC5 grouped together at the far left of the heat map, separate from the stem cell lines, as they were found to express low levels of GATA6, KIT, NOGGIN and SOX2. Human feeders also expressed low levels of differentiation genes GFAP, ISL1, COL1A1, FOXD3, NESTIN, REST and FGF5. iHDFn expressed low levels of NODAL, LIFR and PAX4. With the exception of RH5, overall, the results demonstrate that feeder type did not contribute significantly to the difference in grouping. The biggest difference appears to be the stem cell lines themselves.

A



B

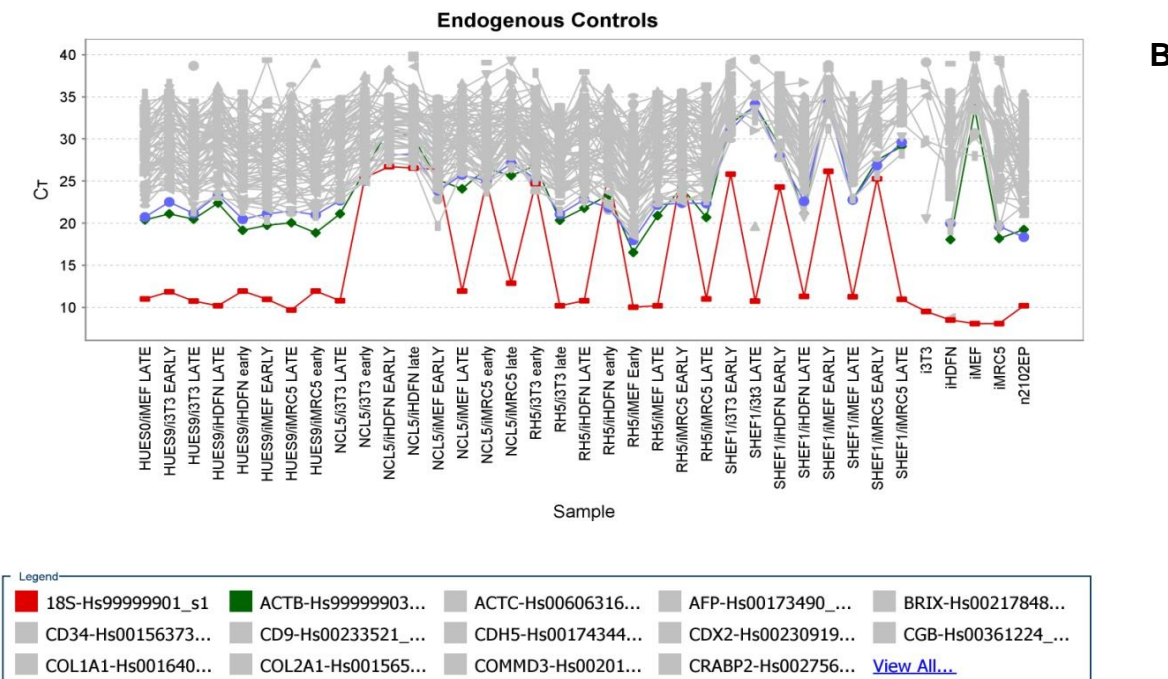


Figure 5.14. Global expression plot demonstrating the average Ct values for the selection of potential reference genes (endogenous control). Reference genes were selected from the global gene expression plot based on their stability (less than 2 fold change) across all samples, from all available targets in ExpressionSuite Software (A). Commonly used housekeeping gene 18S was not suitable due to large variations in Ct values from the global expression plot (B).

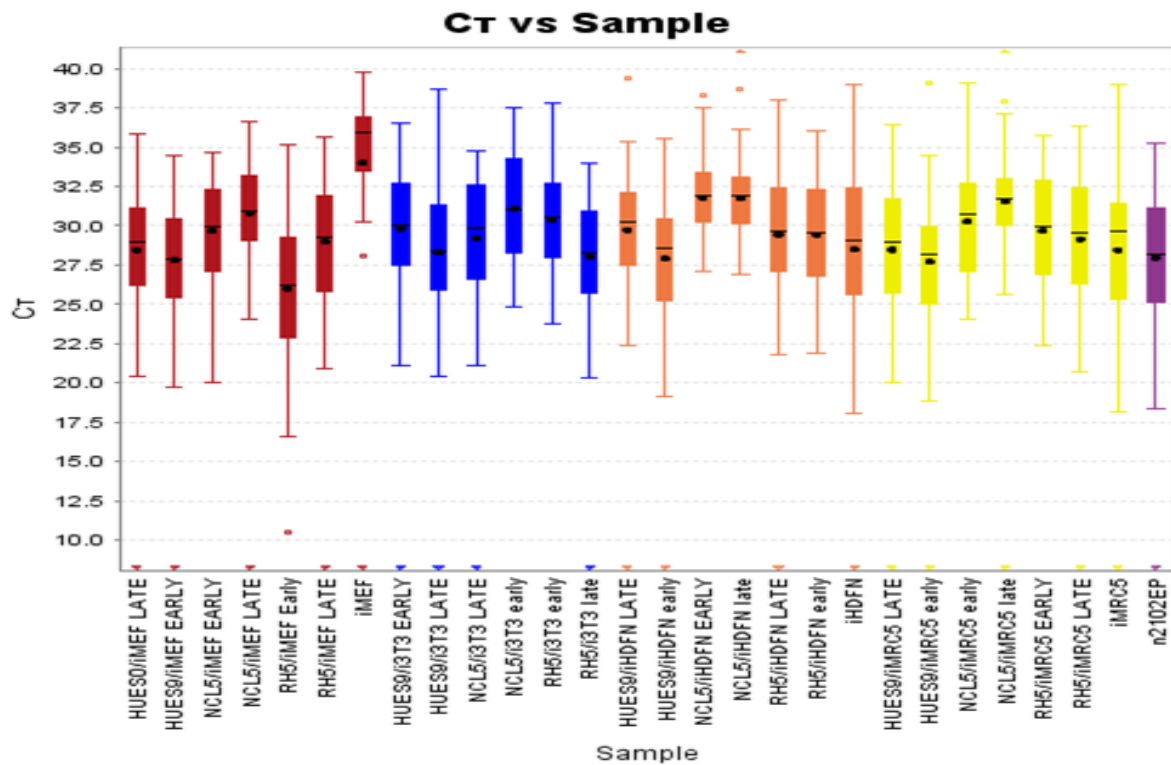
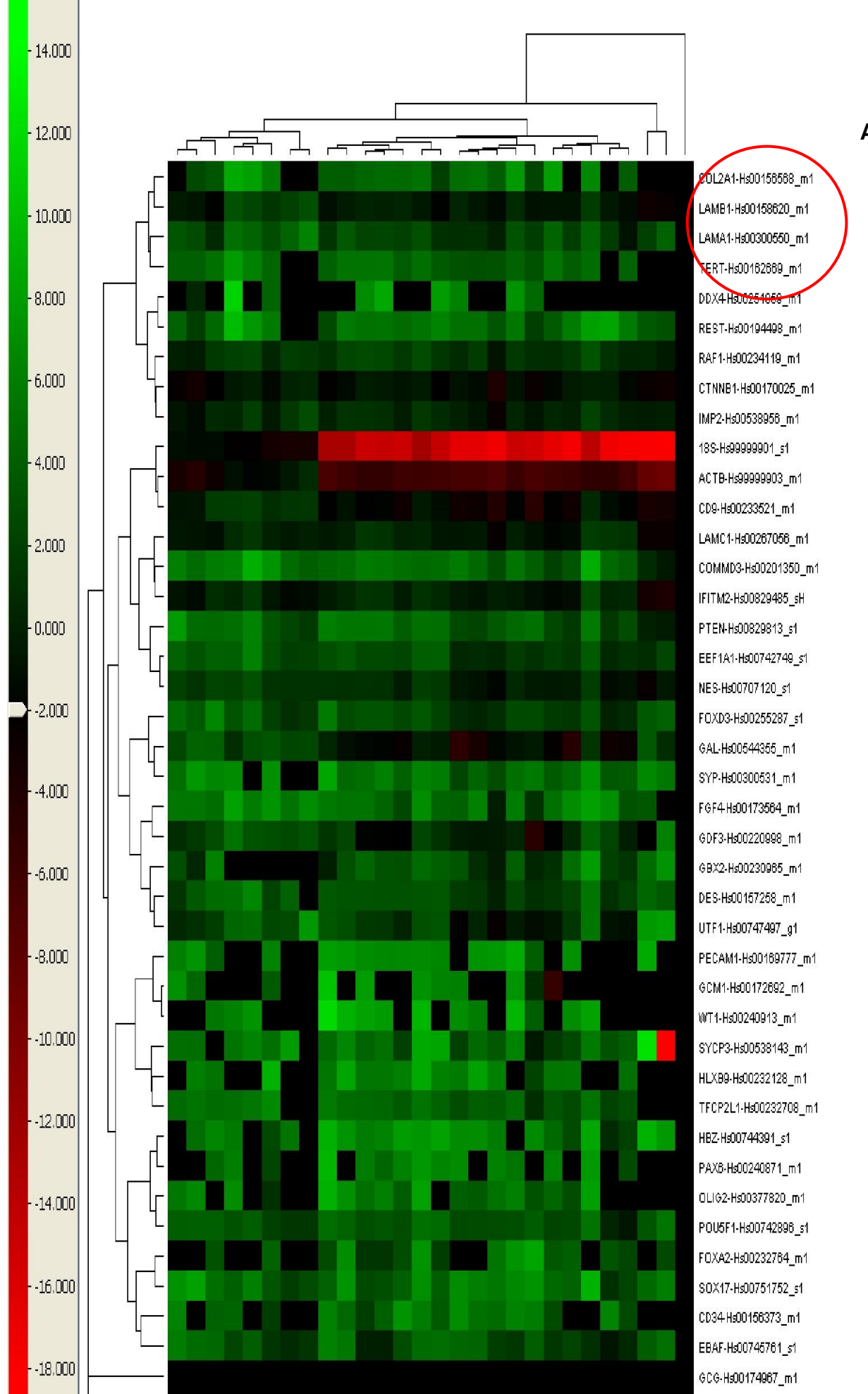
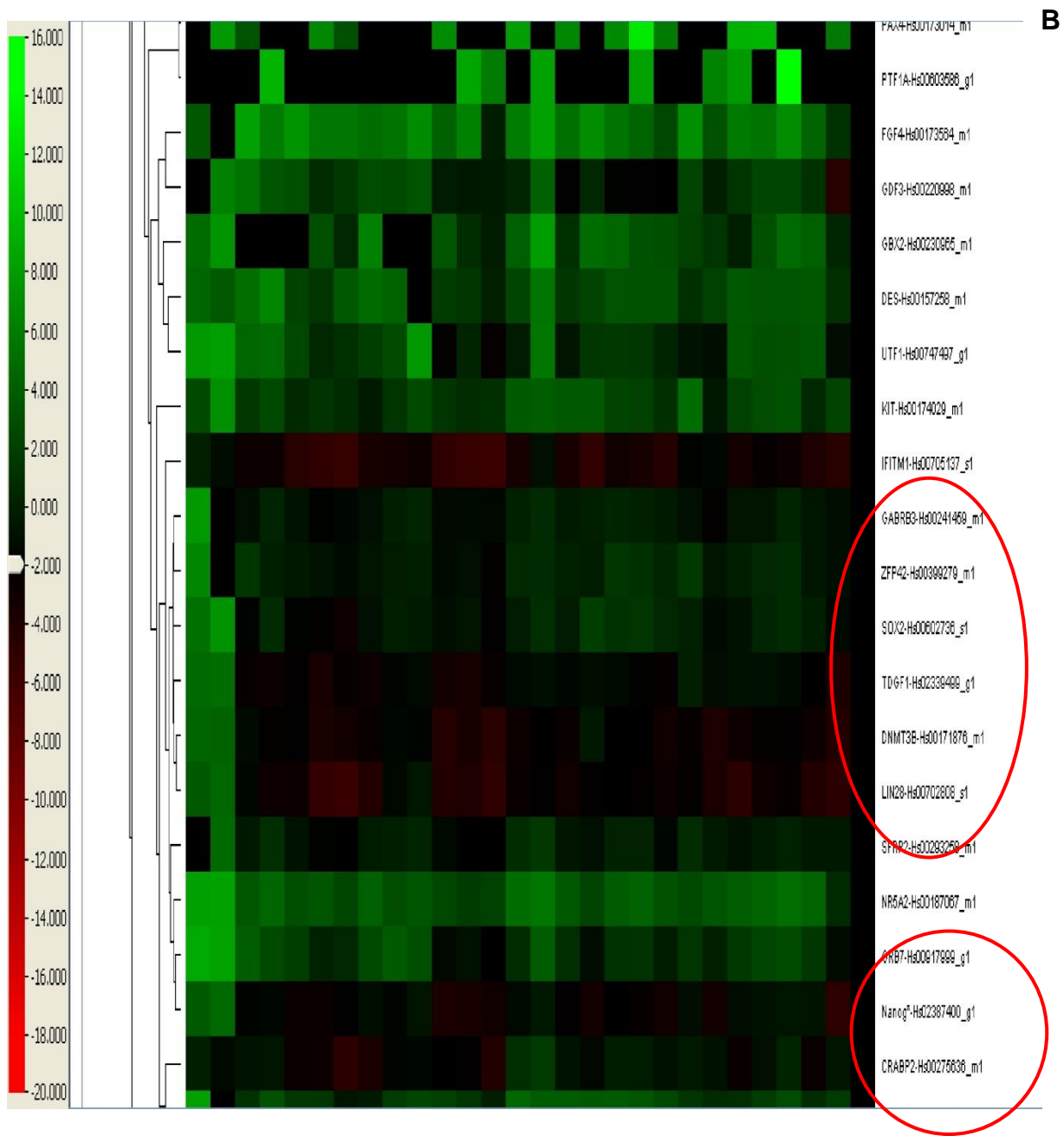


Figure 5.15. Box plot showing Ct values for stem cell lines RH5, HUES9 and NCL5 cultured on four different feeder types at early and late passage. Box plot analysis was obtained following the selection of appropriate reference gene(s) across all the stem cell samples, as a quality control measure to allow the visualisation of Ct values across all samples, grouped by stem cell line. The correlation between the average Ct values and each stem cell sample and the feeder types shows that a wide variation of Ct values was detected from all the samples analysed for gene expression. The samples have been colour grouped by their culture on specific feeder types; red for MEFs, blue for i3T3, orange for iHDFn and yellow for iMRC5.

Distance Measure: Pearson's Correlation, Clustering Method: Complete Linkage, Map Type: Global (ΔC_T)



A



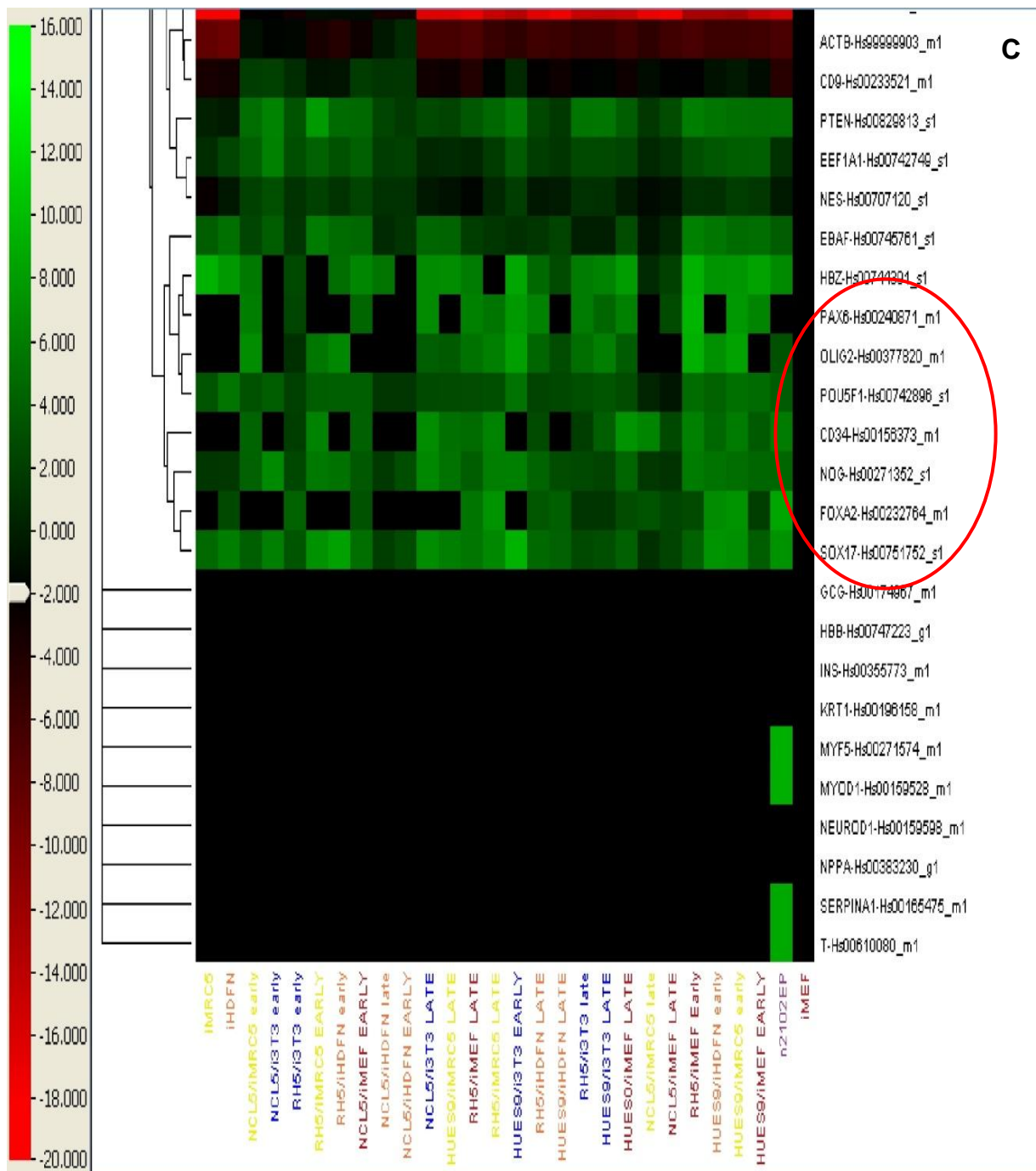


Figure 5.17. Heat map showing correlation of genes expressed by three hESC lines cultured on mouse and human feeders and four individual feeder types. Gene expression studies from HUES9, RH5 and NCL5 cultured on four feeder types, iMRC5, iHDFn, i3T3 and iMEFs at early and late passage, were grouped to form a heat map, then analysed and compared using 2 way cluster analysis (Pearson's correlation) to show trends in gene expression. Delta Ct values have been plotted to represent correlations between genes expressed and stem cell lines/samples cultured at early and late passage, significant by 2 fold expression. The heat map has been divided into three parts for easy visualisation. Figure **A** shows the top of the heat map and **B** shows the middle and **C** shows the base.

5.4 Discussion

Quantitative Real time PCR allows for the quantification of gene expression over time when compared to a suitable reference gene, irrespective of culture conditions. The sensitivity of the ExpressionSuite software (Applied Biosystems) enabled the detection of changes as low as 2 fold, which is important when attempting to construct detailed genetic profiles for hESCs which included the low level expression of germ lineage specific markers, as demonstrated by these studies.

Use of the MIQE guideline document, the standard for outlining the minimum information required to report the experimental details for PCR experiments, including details of instrumentation, requirements for assessing RNA integrity, and selection of reference genes, was also applied to these studies (Bustin et al., 2009; Taylor et al., 2010). Such details are not only useful for publishing, but will help provide guidelines for researchers when selecting reproducible protocols within publications. The selection of appropriate reference genes for normalisation of gene expression data sets has been discussed (Andersen et al., 2004), (Veazey & Golding, 2011) (Derveaux et al., 2010). For the individual stem cell lines results, GAPDH was chosen as a reference gene, as its values were consistent across each particular stem cell line cultured on the four feeder types from early to late passage. For global gene expression analysis, three reference genes were selected which provided consistency across the three different stem cell lines cultured on four feeder types and Matrigel. Interestingly, frequently used reference genes GAPDH and 18s, were not suitable for normalising the data, as they were particularly inconsistent across all the hESC lines. Such quality assurance measures are important to the UKSCB when attempting to introduce standardised testing of banked hESC lines which are produced for worldwide distribution. These studies also compared multiple genes across a number of biological variables/stem cell lines. Assaying greater numbers of genes allows for better statistical significance as well as more accurately defining the state of a population of hESCs in long term culture.

Although early literature characterising hESC gene expression patterns using microarray techniques were extremely useful, and gave good insight into the genes necessary for maintenance of an undifferentiated phenotype, the studies drew close comparison on germ cell tumours and mouse ES cells which have since been well characterised and understood to have quite different gene expression patterns

(Sperger et al., 2003). Furthermore the studies unveiled a large number of genes which clustered together, but at the time had poorly defined functions (Sperger et al., 2003). It is now accepted that stem cell cultures are heterogeneous by nature (Graf and Stadtfeld., 2008; Narsinh et al., 2011) and understanding their characteristics and the genes that govern the switch to early differentiation will be key to identifying how to better maintain hESCs in an undifferentiated state and control the differentiation of cells by environmental factors which is important for developing and standardising robust culture conditions and testing methods.

Differences between hESC cultured on mouse, human feeders and Matrigel

From initial screening, overall, there was no difference in maintenance of undifferentiated hESC gene expression from RH5, HUES9 and NCL5 when comparing cultures on mouse and human feeders and feeder free matrix Matrigel, at early and late passage. In general, differentiation markers were absent or at very low levels in the hESCs cultures, therefore demonstrating that stemness was still present at late passage. Statistical analysis (simple t-test) of each of the data sets organised by stem cell line and germ layer determined that overall, there was no significant difference between expression of both undifferentiated and germ layer specific differentiation genes and the type of matrix used to culture the stem cells from early to late passage (stats data). This was further confirmed by 2 way ANOVA and General linear model (GLM, $p=0.4$). This is in concurrence with previous studies which have compared the undifferentiated gene expression of hESC growth by mouse and human feeder lines (Richards et al., 2003) and feeder and feeder free support matrices (Yoon et al., 2010). However, both studies did not employ the sole use of enzymes for passaging, and in particular, Richards et al. used a combination of mechanical cutting and Dispase to achieve clump passaging, which is more time consuming, labour intensive and more difficult to standardise. Also, the studies only compared the growth of two similarly derived hESC lines from the same research institute (HES-3 and HES-4), whereas the studies described here are less biased in this respect, as the three hESC lines are more diverse and from different institutes. In addition, although the gene expression studies reported positive results of undifferentiated hESC genes, they were not quantitative, signifying that the gene expression studies described here are far superior as they are not only quantitative but also very sensitive.

The studies outlined here demonstrated no significant differences of gene expression between both human feeder's iMRC5 and iHDFns, in contrast to previous studies, which reported that MRC5 foetal fibroblasts were not as supportive as foreskin fibroblasts HFF (Richards et al., 2003). Although the studies did report that human and mouse feeders vary in their ability to support undifferentiated hESC growth, which was subtly observed in chapters 3 and 4, this may have been due to variations as a result of mechanical/Dispase passaging, which can certainly result in morphological changes of hESCs from passage to passage. hESC cultures on Matrigel/mTeSR1 also proved to maintain the expression of undifferentiated genes in all three stem cell lines, and overall, gave low expression of differentiation genes. Although a slight increase in ectoderm gene expression was detected, this was not significant.

Some less apparent differences between expression of differentiation genes were seen from cultures on mouse and human feeders, as RH5 cultures on both human feeders appear to designate low level preference of mesoderm and ectoderm expression whereas RH5 cultured on mouse feeders i3T3 showed a preference towards endoderm, as demonstrated by the upregulation of SOX17 and FOXA2, from early to late passage. Upregulation of endoderm genes FOXA2, SOX17 and GATA4 was seen from early to late passage for NCL5 cultured on iMEF, iMRC5 and Matrigel. Overall, a significant increase in endoderm expression was detected for NCL5 cultured on iMEF and iMRC5. Interestingly, slight differences in low level ectoderm expression were apparent due to different feeder types, from early to late passage. Although these observations demonstrate the subtle effects of culturing hESCs on different feeders, they could not be correlated with changes detected from all the stem cell lines, and would require directed differentiation studies to confirm whether these effects could be sustained.

Additionally, low level expression of mesoderm genes was highest from NCL5 cultured on iMRC5 and Matrigel, which increased significantly at late passage (greater than 3 fold increase). This may further indicate inherent preferences of stem cell lines to act towards specific pathways when cultured in specific way i.e. on human feeders which promote attachment, increase in growth factors, and even contribute towards the expression of stemness and self-renewal genes. Such consistent external events, sustained over long passage could influence gene

expression even at the pluripotent state (Guilak et al., 2009) and provide an advantage towards specific lineages when differentiation occurs. Proteomic studies of mouse and human feeders have revealed differences in expression of FGF2, FGF4, BMP4, activin A and TFGB1 (Greber et al., 2007; Eiselleova et al., 2008). Insulin like growth factor binding proteins (IBFBP4) is expressed by mouse embryonic feeders STO (Lim and Bodnar, 2002). It has also been reported that IGFBP3 and IGFBP6 are expressed in human foreskin fibroblasts (Prowse et al., 2005). Furthermore, Kueh et al., 2006 reported high expression of FGF-2 and gremlin (a BMP4 inhibitor) from fetal skin feeder cells and not from MRC5. This is in contrast to the studies here, from which both HDFn and MRC5 demonstrated the expression of FGF4, LAMAC1, LAMB1, OCT4, NANOG and brings into question the sensitivity of the PCR experiments and analysis parameters used by these groups, as the studies here clearly demonstrate the robust and reliable real time PCR platform, which further validates these results.

Gene expression levels were also analysed in individual samples of each feeder type used for the long term culture of the hESC lines. This was to provide information on whether the feeders were potentially influencing the expression of genes within the stem cell lines over long term culture. Interestingly a number of pluripotency and differentiation genes were found to be expressed in low levels by both human feeders, including OCT4, NANOG, NOGGIN, SOX17 LAMA1, LAMB1 and LAMC1. iHDFn also expressed very low levels of COL2A1 which is found in connective tissue. Such findings maybe of importance when deciding which feeders to use for iPSC derivation studies. Some may provide better starting material than other feeder sources, as they exhibit a greater ability for overall attachment, survival and self-renewal.

Upregulation of laminins, collagen and fibroblast growth factors in human feeders compared to mouse feeders could be significant as it may account for reasons why differences in undifferentiated hESC gene expression occurred. It also relates to similarities of integrin expression with human cells by providing a better environment to mimic hESC growth *in vivo*, as demonstrated by the co-culture of other cell types using human fibroblasts (Shi et al., 2002; Cheng, 2003; Bramono et al., 2010). More recent publications have demonstrated the expression of hESC-like properties from mouse embryonic feeder line i3T3 (Dadheech et al., 2013) which displayed very low

basal expression (between 0.001-0.01) of OCT4, NANOG and SOX2 by q-PCR. However, the samples were normalised to mesenchymal stem cells, therefore results would not be comparable with hESC lines. In contrast, the studies in this project were normalised to early passage hESC lines cultured on iMEF feeders and concluded that both the sole preparations of human feeders (without stem cells) expressed genes associated with stemness and self-renewal. As demonstrated by the heat map, neither mouse feeder iMEF nor i3T3 expressed genes associated with stemness or self-renewal. This information does demonstrate that human feeders may be contributing to the long term maintenance of undifferentiated hESCs through the expression of stemness gene. Although the effects are not significant, they may play a role in differentiation studies.

Differences in genes expressed for self-renewal, maintenance of undifferentiated cultures and potential differentiation towards specific germ lineages

From the results obtained in these studies, expression of Nanog and OCT4 was found to be generally higher than for SOX2. This observation is consistent with one other report by Adewumi et al. (2007). SOX2 is thought to play a role in undifferentiated hESCs and is closely linked in a regulatory loop with NANOG and OCT4 (Kuroda et al., 2005; Boyer et al., 2005) but is also expressed in the neurectoderm lineage (Uwanogho et al., 1995). A recent publication suggests the involvement of OCT4, NANOG and SOX2 in lineage specific commitments when differentiated; therefore each factor controls specific cell fates (Wang et al., 2012). Low levels of OCT4 can induce ectoderm differentiation, whereas higher levels give rise to mesoderm in the presence of BMP4 (Wang et al, 2012). This may indicate a process of selection occurring in the three hESC lines investigated in these studies, as a result of long term culture and demonstrates the importance of monitoring the changes in expression of these master regulator genes when attempting to maintain undifferentiated hESC cultures for long periods of time.

Furthermore, the expression of GATA6 was consistently low across all hESC lines used in these studies, GATA6 expression has been associated with early blastocyst formation (Koutsourakis et al., 1999) and maintenance of pluripotency and self-renewal alongside OCT4, NANOG and SOX2 (Adewumi et al., 2007), as well as

being upregulated during visceral endoderm formation (Fujikura et al., 2002). Although these studies suggest that this gene was not useful in determining the undifferentiated state of hESCs cultured in long term passaging using different matrices, it does illustrate that using a number of different genes to characterise hESCs provides greater confidence when attempting to determine overall cell state. Such detailed characterisation is crucial to the consensus as to which specific detectors should be used when characterising hESC lines as they are all quite individual, and will help to better standardise the use of PCR.

Correlations in gene expression profiles between stem cell lines

Expression of early differentiation marker SSEA1 was higher overall, from RH5 compared with the other cell lines, indicating that a higher proportion of these cultures were spontaneously differentiated (overall greater than 10% but less than 20%). Early indications from the initial gene expression studies may suggest that RH5 stem cell line show a preference to differentiate towards early neuronal lineage, as demonstrated by upregulation of OLIGO2 and PAX6, and ISL1. Further differentiation studies are required to confirm this observation, although this stem cell line has already been shown to differentiate to neural lineage through semi directed differentiation (M.Gillet, PhD thesis, UCL, 2010). Such suggestions demonstrate that some cell lines have inherent preferences to differentiate towards specific germ lineages, even when maintained on different feeder types in a mostly undifferentiated state, which has been described by other reports (Osafune et al., 2008); however without correlation to what percentage of cells were actually differentiated, either by cell surface marker SSEA1 or other marker of differentiation.

Global gene expression analysis by heat mapping revealed that NCL5 and HUES9 grouped together on both mouse and human feeders, whereas RH5 appeared to group by feeder type. Early passage samples also grouped together, at the far left side of the heat map and included the majority of NCL5 cultures. HUES9 stem cell lines appear in the middle of the heat map, whereas RH5 appears to be scattered across the heat map, indicating its spread in expression on different feeder types, from early to late passage. Overall comparison of all three hESC lines show that NCL5 appeared to demonstrate more consistent gene expression profile, as cultures from both human and mouse feeders (iHDFn and i3T3) grouped together at early

and late passage. This further demonstrated slight correlation as a result of cultures from mouse and human feeders.

Genes that grouped together at the top of the heat map included those related to adhesion, attachment and housekeeping genes i.e. LAMB1, LAMC1, ACTB, 18S, CTNNA1. Genes that clustered in the middle included those involved in regulation of self-renewal, pluripotency and housekeeping genes DNMT3B, ZFP42, SOX2, and LIN28. NANOG clustered with CRABP2. This demonstrates the shared intrinsic properties of different hESCs which has previously been reported (Adewumi et al., 2007; Sperger et al., 2003). Differentiation genes SOX17, FOXA2, CD34 clustered at the base of the heat map and were more strongly expressed by RH5 and HUES9 cultured on i3T3s, iHDFns and iMEFs, as well as late passage cultures. The differences in gene expression between stem cell lines have been described as stochastic and heterogeneous by nature (Abeyta et al., 2004; Liu et al., 2006; Allegrucci & Young, 2007; Osafune et al., 2008). Hierarchical cluster analysis allowed the comparison of such events, particularly to correlate the expression of genes as a result of culture on different matrices which may potentially influence their microenvironment, which in turn can affect their genetic profiles. The results from this study do confirm that early passage stem cell lines group separately to late passage stem cell lines, suggesting changes in gene expression as a result of long term passaging. Although all the stem cell lines continued to express undifferentiated genes at late passage, even when cultured on different feeder types, they were also beginning to express low levels of differentiation genes, shared by other late passage stem cell lines cultured on mouse and human feeders (HUES9 and RH5 cultured on iMEFs, iHDFn and i3T3s at late passage), however this was not statistically significant. This was consistent with morphological and cell surface marker expression results obtained from previous chapters, as all matrices and cell types suggested undifferentiated stem cell growth over extended passage.

Real time PCR is useful for comparing genetic differences between stem cell lines, as it is sensitive enough to detect changes as low as 2 fold, which may be useful for determining the genetic profile of a hESC line that may be primed to differentiate towards a particular germ layer. However, if carrying out PCR on single stem cell line, TLDA cards may not be the most cost effective option, and standard, single assay PCR in 96 well plate formats would be much more appropriate. A paper by

Bock et al (2010) details the genetic characterisation of twenty hESCs and twelve iPSCs through the establishment of genome wide reference maps of DNA methylation and gene expression. The data was then used to develop score cards specifically to indicate whether a stem cell line has differentiated towards a specific germ layer. This information would be extremely useful, as with the TLDA cards it removes the guess work required to predict which genes to screen for, and is a much more standardised and robust method of comparing many PCR assays. The paper also suggests the development of an ES reference cell line, which could significantly enhance the standardisation and robustness of gene expression assays by real time PCR. In these studies early hESC cultures from iMEF were used as control lines. Although this is the best representative of what is currently used as a gold standard culture in most laboratories, and also takes into consideration the heterogeneity of each hESC line, iMEFs do express low levels of certain genes, as mentioned earlier, which could bias the overall results. Reassuringly, screening of iMEFs and i3T3 mouse feeders by TLDA cards revealed very little expression of genes present on the TLDA card, therefore demonstrating that hESC cultures on iMEFs were a better choice than using cultures from human feeders or Matrigel as an alternative control.

The studies carried for this project conclude that late passage cultures of hESCs do exhibit changes in gene expression profiles. Such changes were more consistently demonstrated across the different stem cell lines in this chapter, in comparison with the previous chapters, where differences in morphology and stemness marker expression by immunofluorescence were more inherent to the specific cell line and/or matrices. Cultures should therefore be regularly screened for a range of genes specific to stem cell maintenance as well as those genes involved in transcription. This detailed characterisation will allow for the sensitive and robust detection of better defined undifferentiated cultures for future studies, particularly when deciding which tests to use for potency assays and assuring mechanisms of actions (MOAs) when assessing stem cells for therapeutics in early clinical trial studies (Bravery et al., 2013).

5.5 Conclusion

Although slight differences were seen between the expression of pluripotency genes from individual stem cell lines, there was no significant difference between mouse and human feeders and Matrigel. Both feeder types and Matrigel maintained stemness marker expression in three stem cell lines from early to late passage. This is consistent with data obtained from the previous chapters, which confirmed undifferentiated hESC cultures by morphology and expression of stemness cell surface markers at early and late passage. Overall, the expression of differentiation genes from undifferentiated stem cell cultures was lowest from Matrigel cultures. Differences involving the expression of genes between different stem cell lines appear to be inherent. This is further demonstrated by the early detection of low level germ layer specific markers in undifferentiated cultures, at late passage. This would suggest that even when undifferentiated, there may be a predetermined preferential cell fate within each stem cell line, and would need to be confirmed by directed differentiation studies. This preference could be used to the advantage of stem cell researchers, with the selection of stem cell lines for particular differentiation studies.

TLDA cards are easy to prepare and run. They remove the inconsistencies frequently found when performing large PCR experiments as the same amount of cDNA is loaded onto each card giving robust and reliable data which significantly reduces processing error, in comparison to setting up 96 individual PCR reactions per sample. They provide a large amount of information on the genetic status of a stem cell line using small amounts of cDNA (50 ng), and have proved to be particularly sensitive and useful for comparing the genetic integrity of hESCs over extended passaging, and identifying trends in gene expression, as demonstrated box plot diagrams and global gene expression heat maps. The analysis of gene expression profiles between different stem cell lines on different matrices, help to provide a detailed insight into their characteristics as a result of changes in their environments over long term passaging. A better understanding of the impact of these changes will help to develop more robust culture methods.

Chapter 6.

***In vitro* directed differentiation of human embryonic stem cells to early endoderm, mesoderm and ectoderm progenitors.**

6.1 Introduction

The ability of cells to differentiate into the three germ layers endoderm, mesoderm and ectoderm, is key to demonstrating the pluripotency of human embryonic stem cell lines. The pluripotent capacity of cultured human embryonic stem cells has traditionally been assessed *in vivo*, by teratoma formation assays (Damjanov, 2005; Pal et al., 2007). Although still the gold standard method of testing, this technique is notoriously variable, costly and has caused much debate in the stem cell field (Muller et al., 2010). *In vitro* directed differentiation assays, which work by using growth factors and small molecules in nutrient enriched media, to drive differentiation towards a particular germ lineage and blocking pathways to the other germ layers, have gained prominence in stem cell research and suggested as a suitable alternative, particularly when trying to progress research without the use of animal models (Dressel et al., 2013).

An extensive amount of research has been focused towards the development of differentiation protocols with varying success. Most protocols have been focused towards the differentiation of therapeutically relevant derivatives such as hepatocytes, cardiomyocytes and dopaminergic neurons. Many different methods of starting differentiation have been reported for instance, differentiation by aggregates including embryoid body formation (Kurosawa, 2007), and on adherent plates (D'Amour et al., 2005). These assays try to mimic differentiation pathways *in vivo* to give a greater yield and purity of differentiated cell types. It has been argued that EB formation is supposed to be a better method of differentiation as it is thought to mimic processes *in vivo*., The selection of appropriately sized EB aggregates has, however, been shown to influence differentiation (Bauwens et al., 2008; Hong et al., 2010) and therefore requires standardising.

Other protocols have been developed to give rise to specific cell types by stage specific processes, for example, dopaminergic neural differentiation of human pluripotent stem cells by dopaminergic neuron induction, differentiation, specification and maturation (Chambers et al., 2004) and cardiomyocyte differentiation by careful, stepwise cell signalling using BMP4 and Wnt/Activin A (Sa & McCloskey, 2012; Kattman et al., 2011; Burridge et al., 2011).

The use of novel growth factors and small molecules alongside new information on differentiation pathways play a significant role in the development of new differentiation protocols (Chambers et al., 2009; Surmacz et al., 2012; Song et al., 2012). Some protocols describing differentiation to neuroectoderm are heavily influenced by dual inhibition of SMAD signalling (Chambers et al., 2009) which uses a drug, SB431542, and Noggin, both of which have been previously used for the neural conversion of hESCs.

A publication by D'amour et al. demonstrated the *in vitro* differentiation of hESCs to endoderm by Wnt signalling from adherent cultures, which is differentially expressed at critical stages during liver development *in vivo* (Hay et al., 2008) and Activin A growth factor, which works by suppression of signalling from phosphatidylinositol 3-kinase (PI3K) protein (Mclean et al., 2006; D'amour et al., 2005). Both protocols utilise mechanisms to block signalling pathways that are important to both maintaining pluripotency and promoting differentiation and report dramatic changes in gene expression profiles (D'amour et al.; Chambers et al., 2009). However, both reports show only upregulation of very select lineage specific genes following *in vitro* directed differentiation of adherent flat cultures.

Scientists are aware of the effect that microenvironment can contribute in promoting differentiation, and although it is too early to control all the variables, many published techniques describing *in vitro* differentiation methods detail the use of specific matrices that aid the induction of differentiation through cell-matrix interactions. Poly-L-ornithine (PLO), vitronectin, and fibronectin (Prowse et al., 2010; Hu et al., 2009; Chen et al., 2012; Passier & Mummery, 2005; Lecina et al., 2010) have all been reported to give rise to the formation of specific progenitor types including glial-like neurons and visceral endoderm (Iskovitiz-Eldor, 2002; Reubinoff et al., 2001, Braam et al., 2009). Feeders have also been shown to aid differentiation in hESCs. The co-culture of hESCs with stromal cells to promote differentiation to haematopoietic cells has been used for many years (Vodiyani et al., 2005). Visceral endoderm (VE) like cells from mouse has been used for cardiomyocyte induction (Mummery et al., 2003), and although the co-culture proved successful in the production of cardiomyocytes from hESCs, the exact function of the mouse VE was not clarified, as effects of FGFs were not discussed, and it was thought that activation of BMP4

signalling, important for formation of mesoderm, was also not the sole contributor of the VE cells (Mummery et al., 2003).

The establishment of robust and standardised differentiation methods to efficiently demonstrate pluripotency is important to all research labs maintaining cultures of undifferentiated hESCs on a routine basis. Currently there is no published work describing the direct comparison of *in vitro* differentiation assays from adherent plate based methods and embryoid body formation. Moreover, it is not clear how differentiation may be regulated following prolonged culture of stem cells on specific matrices. The studies outlined in this chapter therefore demonstrates three simplified directed differentiation protocols that use an adherent plate method, for the formation of early neural, mesoderm and endoderm progenitors, to efficiently establish the pluripotent capacity of NCL5 stem cell line pre-cultured on human feeders iMRC5, mouse feeders iMEF and feeder free matrix Matrigel following extended passaging using TrypLE™ Express (20 passages). These directed differentiation studies are the first to be developed in the host laboratory. To give a reliable account of the differentiation potential of NCL5, non-directed differentiation studies by embryoid body (EB) formation from Matrigel cultures was also assessed, following 7 day growth in Knockout serum replacement media (KSR). This method was adapted from the low adherent spin plate method described by Ng et al., 2008. The method here describes the use of aggrewell™ spin plates from Stem Cell Technologies (Antonchuk, J., 2013).

6.2 Materials and Methods

6.2.1 Formation of Embryoid bodies using Aggrewell™ and culture in KSR

Embryoid bodies were formed by single cell dissociation using TrypLE™ Express for 5 minutes, then Ro kinase inhibitor Y-27632 (RoK) at 10µM was added, before determining the optimum cell number for using the Aggrewell plates (2.4x10⁶ cells per well). RoK increases survival of single cells and improves aggregation by reducing dissociation induced-apoptosis and increasing cloning efficiency (Stem Cell Technologies Technical manual Aggrewell™, Watanabe et al., 2007). The plate was spun down to allow aggregates to form which, following 24 hour incubation, formed uniform EBs. These were then cultured in EB formation media (see Table 6.1) using non adherent 10 cm² petri dishes for 7 days. Images were taken on day 7 to assess EB health and record whether they had grown in size or changed in morphology. The images in Figure 6.2 demonstrate the successful formation of NCL5 into Embryoid bodies using aggrewell 800 plates, giving 3000 EBs per well. Embryoid bodies were collected by careful pipetting and transferred through a 20µm reversible sieve (Stem Cell Technologies) to remove single cells, then centrifuged at 300 g for 3 minutes to pellet cells. Supernatants were removed and RNA extracted as described in the general methods chapter (section 2.10.1). The cDNA was produced as described in general methods chapter (section 2.10.3). RT-PCR was prepared in 96 well plate format, and ran on the Quantstudio Thermocycler (Life Technologies) using germ lineage specific Taqman probes (Life Technologies, see appendix).

Table 6.1 Reagents used for the culture of EBs

Reagent	Supplier	concentration
KRS knockout	Invitrogen	
Glutamax	Invitrogen	1% OF 5Mm
FCS	Biosera	2%
NEAA	Invitrogen	0.1%

6.2.2 *In vitro* directed differentiation of NCL5 using flat based cultures

Stem cell line NCL5 at late passage (p50 to p53) were cultured on Matrigel using MTeSR1 Media, iMEF mouse feeders and iMRC5 human feeders, to 70-80 % confluency over 5-7 days. Two wells from each culture were TrypLE™ Express treated to give single cells (yielding on average 5×10^5 cells/mL), then plated onto Matrigel coated plates in KSR media supplemented with RoK. Cells were incubated and allowed to reach 70-80% confluency (approximately 2 days) before treatment with lineage specific media (growth factors and small molecules). Media was changed every 2 days, and cells were imaged to record changes in morphology throughout the differentiation process. Cells were collected for RT PCR at day 7, by scraping using Falcon cell scraper, then centrifuging at 300 g for 3 minutes to pellet. The supernatant was removed and cells underwent RNA extraction and cDNA production (general methods section). RNA from NCL5 cultured on its original matrix at early passage (generally, p35) were used as controls and stored at -80°C until used for RT PCR. PCR plates were sealed and centrifuged at 300g for 5 minutes then loaded onto Vii7 Real time PCR Thermocycler.

6.2.5 *In vitro* Endoderm differentiation

Endoderm differentiation was carried out using hESCs transferred to Matrigel (as described above) in the presence of Activin A and Wnt3a, as described by D' Amour et al. (2005). At 70-80% confluency their standard media KSR was replaced with KSR supplemented with 1%, Activin A and Wnt3a for 3 days, as shown in Table 7.2. After 3 days Wnt3A was removed. The cells were cultured for 7 days, then collected for real time PCR to assess for the upregulation of SOX17, FOXA2, Brachyury, AFP, CXCR4, ISL1, GSC and DCN.

Table 6.2 Reagents used for *in vitro* endoderm differentiation

Reagent	Supplier	concentration
KRS knockout	Invitrogen	
Glutamax	Invitrogen	1% OF 5Mm
FCS	Biosera	0.2%, 2%
Activin A	R & D systems	100ng/ml
Wnt3A	R & D systems	25ng/ml

6.2.4 *In vitro* neuroectoderm differentiation

Neuroectoderm differentiation was carried out using adherent plate method based on the publication by Chambers et al. (2009). The stem cells were seeded at 2×10^5 cells/mL and reach 70-80% confluency (1-2 days) on Matrigel in KSR and then cultured in neural media containing KSR, noggin, Dorsmorphin and SB431542 made up as indicated in Table 7.3. The media was changed every other day, for 7 days. Stem cells were monitored each day for morphology changes and collected for Real Time PCR on day 7 to assess for the upregulation of neural genes SOX1, PAX6, Hes5, OXT2, FOXG1, NEUROD1 and down regulation of stemness markers OCT4 and Nanog.

Table 6.3. Reagents used for *in vitro* neuroectoderm differentiation

Reagent	Supplier	concentration
KRS knockout	Invitrogen	
SB431542	Tocris	10 μ M
Dorsmorphin	Tocris	600 nM
Noggin	Invitrogen	50 ng/ml

6.2.6 *In vitro* Mesoderm differentiation

hESCs were prepared for *in vitro* differentiation as described above. At 70-80% confluency, cells were treated with BMP4/Activin A in KSR for 7 days (So et al., 2011). The media was changed every 2 days. Cells were assessed for morphological changes and images recorded on day 0, 3, and 5. Samples for RT PCR were collected at D7. RT PCR plates were prepared as described above, with a defined set of specific probes. HAND1, Pecam1, Col1a1, Brachyury, FGB, BMP4, GATA4, Desmin, Vimentin, PDGFRa and PITX1 were chosen by preliminary screening of other early *in vitro* differentiated hESCs in house, and recent literature searches. cDNA was made as described in general methods chapter. Samples were prepared for Real time PCR with relevant TaqMan probes and gene expression Master Mix (Applied Biosystems) as shown in table 6.4.

Table 6.4. Reagents used for *in vitro* mesoderm differentiation

Reagent	Supplier	concentration
KRS knockout	Invitrogen	
Glutamax	Invitrogen	1% OF 5Mm
BMP4	R & D systems	40 ug/ ml
Activin A	R & D systems	10 ng/ml

6.2.3 Analysis of real time PCR results

Visualisation and pre-analysis was carried out using the QuantStudio software. PCR runs were normalised to the reference gene GAPDH, and calibrator sample, to determine delta delta Ct values and results visually examined to remove non amplified genes/genes above 40 cycle limit. Further analysis was completed on Gene Expression software (Life Technologies, version 1.1). Samples were grouped and normalised by three stable reference genes, then analysis of results carried out by heat map using two way Pearson's correlation, to show trends in gene expression, organised by germ lineage, significant to 3 fold change. Results were also imported into GraphPad Prism (version 5) software and organised into bar graphs to give (geometric) mean relative quantity of gene expression (RQ), comparable to a calibrator sample which was automatically set to a value of 1, by the QuantStudio software (normal i.e. NCL5 cultured on iMEF feeders, early passage).

Table 6.5. Gene expression Mastermix

Gene expression Mastermix	10
H ₂ O	7
cDNA	2
Probe	1
Total volume per well	20 µL

6.2.7 Data analysis

Relative quantification values (RQ) were determined by comparison of delta Ct values with a calibrator sample. This was taken as the undifferentiated hESC line cultured on feeder type/matrix at early passage i.e. NCL5 cultured on Matrigel p35 and was set to a value of 1. Initial data was collected from Quantstudio software (Life Technologies). RQ values were exported into Prism and SD/MEAN RQ values calculated and displayed in bar graphs. Results were also imported into ExpressionSuite (Life Technologies) for the comparison of gene expression from different matrices, and for the construction of heat maps and volcano plots, to give visual comparisons of the different matrices and expression of germ lineage specific genes.

6.2.8 IF staining using TissueFaxs™

SOX17, DCN, Vimentin, Hand1, PAX6 and Nestin were all used to confirm the presence of early progenitor cells following directed differentiation to all three germ layers (Abcam, 1/100 dilution).

Late passage NCL5 from the three different culture conditions were cultured in 24 well Matrigel coated plates until confluent. Each plate was treated with endoderm, mesoderm and ectoderm specific media, as described above, for 7 days. The media was changed every 2 days. After 7 days, spent media was carefully removed, cells washed in PBS and fixed/permeabilised in cold acetone: methanol solution (1:1) for 8 minutes. Cells were then carefully washed in PBS, and then blocked with 10% FCS (in PBS) for 10 minutes. Cells were carefully washed again, before incubation with germ layer specific primary antibody, overnight at 4°C. Following primary antibody incubation, cells were carefully washed twice with PBS then incubated with 1/200 dilution of goat anti-mouse FITC conjugated secondary antibody, Abcam for Vimentin and Nestin, (CY5) rabbit anti-mouse, DCN and Hand1 for 2-3 hours at room temperature. Cells were again carefully washed in PBS, counter-stained with DAPI nuclear stain for 3-5 minutes before washing in H₂O, then carefully resuspended in 200 µL of WashBuffer. Images were acquired on the TissueFaxs™ fluorescence imaging system and analysed using TissueQuest™ software to give quantitative assessment of germ lineage specific cell markers.

6.3 Results

6.3.1 Non directed differentiation of stem cell line NCL5 from Embryoid bodies

The photographs in Figure 7.1 demonstrate the successful formation of EBs from Aggrewwells, following 7 days of culture in KSR media. The EBs obtained at day 1 appeared well formed and mostly symmetrical (Figure 6.1A). However, after 5 days in culture EBs attached as small aggregates to the dish and displayed signs of proliferation, becoming slightly larger in size (Figure 6.1B). On day 7 the EBs were beginning to disassociate and appeared shrunk (Figure 6.1C).

RT PCR gave interesting results, as expression of all endoderm genes were downregulated. Only ectoderm genes NEUROD1 and SOX1 and mesoderm gene HAND1 were upregulated after 7 days but there was consistent expression of stemness genes OCT4 and Nanog over this period (Figure 6.2). This may indicate that the cells did not fully differentiate to all three germ layers as aggregates. The EB experiments were eventually discontinued and flat based *in vitro* directed differentiation experiments set up to assess the robust formation of all three germ layers in NCL5.

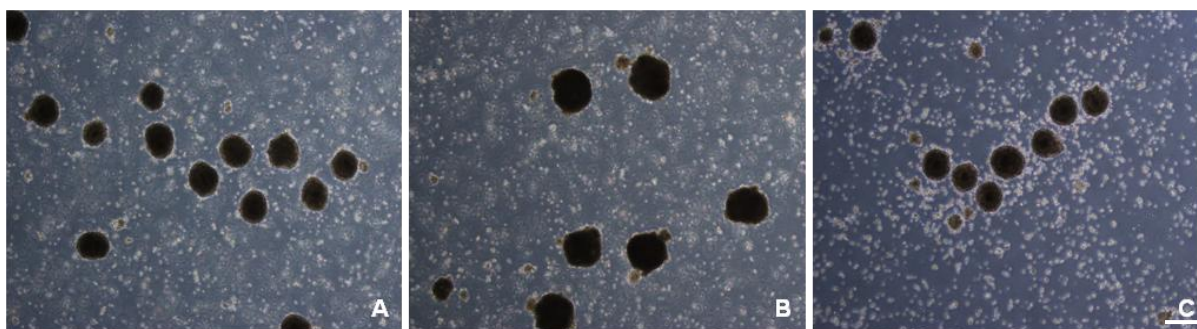


Figure 6.1. Morphology of EBs in KSR media from the stem cell line NCL5 at day 1 (A), 5 (B) and 7 (C). Late passage NCL5 cultured on Matrigel were subjected to EB formation in AggreWell plates as described in the methods sections. EBs were transferred to 10 cm² dishes for 7 days of culture in KSR media. Images were taken at x4 magnification using a phase contrast light microscope. Scale bar = 50 μ m.

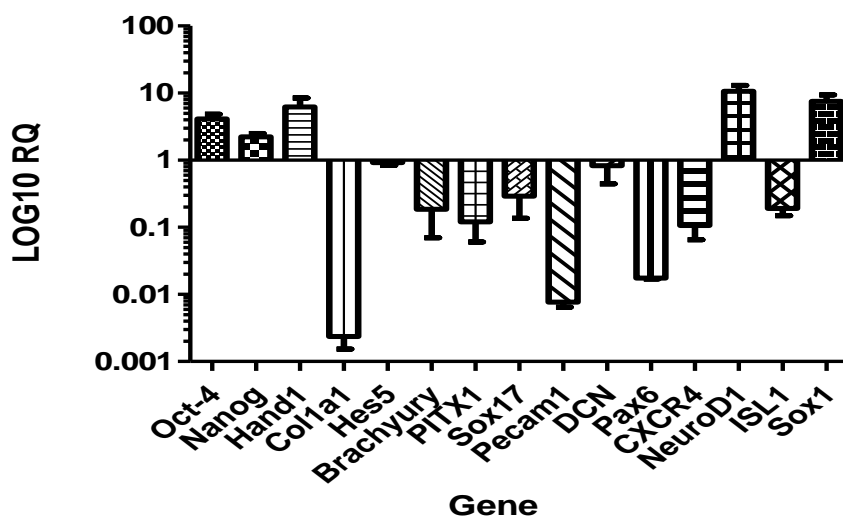


Figure 6.2. Gene expression profile of EBs derived from the NCL5 stem cell line after 7 days of culture in KSR media. EBs made from NCL5s cultured on Matrigel at late passage were assessed for the expression of germ layer specific differentiation genes by real time PCR. The results are the geometric average of three individual experiments.

6.3.2 Flat based *in vitro* directed differentiation of NCL5 stem cells to early endoderm

Differences in morphology as shown in Figure 6.3 at day 3, 5 and 7 from late passage NCL5, which had previously been cultured from iMEF mouse feeders (A), iMRC5 human feeders (B) and Matrigel (C). Considerable cell death was observed from all cultures at day 1 and 2 when initially subjected to the differentiation media. By day 3 cultures appeared to have recovered and were proliferating. Although hESCs continued to proliferate mostly as discrete colonies, changes around the edges of colonies were apparent, as individual cells became more angular and square in shape. The morphological changes were more obvious at day 5. hESCs also appeared to proliferate as large flat colonies, becoming sheets at day 7 with considerable cell piling and some cell death. Morphological changes were still visible and cells were much more angular from all three culture conditions. More piling and differentiation was seen from NCL5-iMRC5 (Figure 6.3 A) in comparison to the other two conditions, potentially indicating increased proliferation.

Gene expression studies demonstrated upregulation of the majority of endoderm genes by NCL5 cultures from all three matrices. The highest upregulated genes on all three matrices were GSC, FOXA2 and AFP but ISL1 and SOX17 were significantly upregulated (Figure 6.4) in cells cultured on both feeder types (100 log₁₀ fold or greater). Endoderm gene expression was lower in Matrigel cultures when compared to cultures from both human and mouse feeders, with the only exception being the expression of Brachyury which was significantly higher. Interestingly, transcription factors POU5F1 and NANOG were downregulated but only in iMEF mouse feeder cultures. Upregulation was found from iMRC5 human feeder and feeder free Matrigel hESC cultures. This may indicate a heterogeneous population of hESCs therefore not all cells underwent directed differentiation.

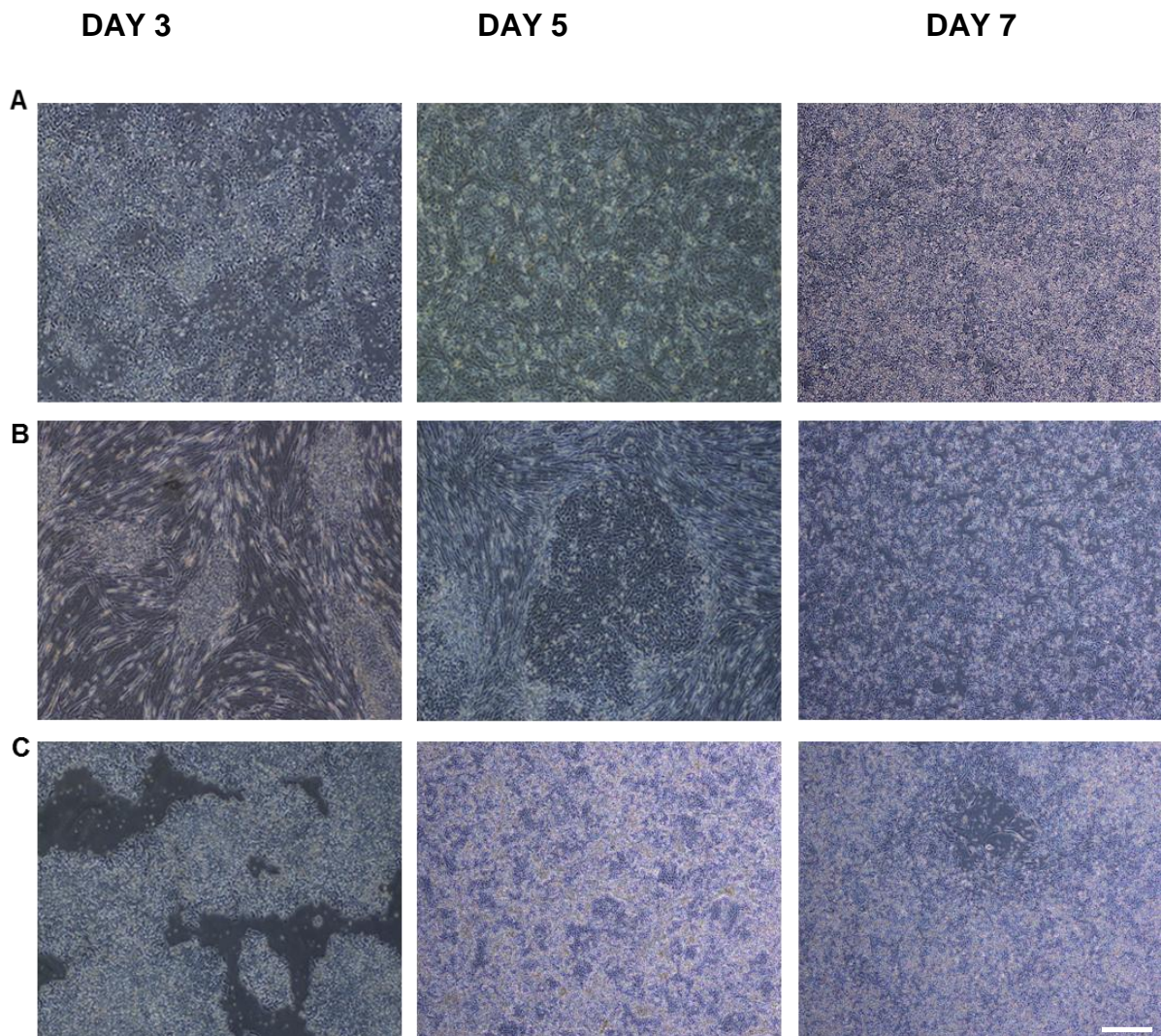


Figure 6.3. Morphological changes of stem cell line NCL5 following *in vitro* directed differentiation to endoderm from prolonged culture on iMEF mouse feeders (A), iMRC5 human feeders (B) and Matrigel (C) at day 3, 5 and 7. Images are representative of 3 independent experiments and were taken with a light phase contrast microscope at x4 objective. Scale bar =50 μ m.

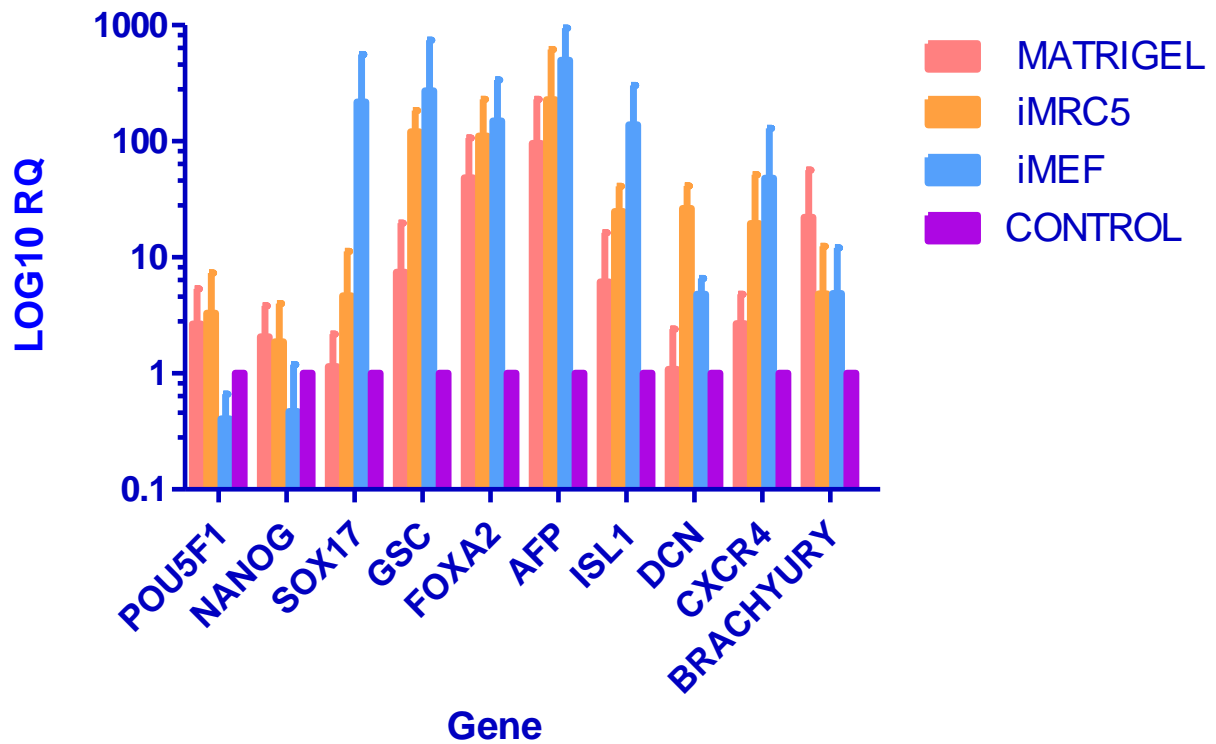


Figure 6.4. Gene expression profile in the NCL5 stem cell line following *in vitro* directed differentiation to early endoderm. NCL5s cultured on iMEF mouse feeders, iMRC5 human feeders and feeder free Matrigel at late passage were plated as single cells onto Matrigel coated plates, then subjected to flat based *in vitro* directed differentiation for 7 days. Cell pellets were collected for real time PCR analysis using germ layer specific probes as described in the methods. The results are the geometric average of three individual experiments. The results are representative of 3 independent experiments.

6.3.3 Flat based *in vitro* directed differentiation of NCL5 stem cells to early mesoderm

Striking morphological changes were seen as a consequence of *in vitro* directed differentiation over 7 days (Figure 6.5). NCL5s from both feeders appeared as discreet colonies at day 3, with observed changes at the edges. Matrigel cultures on the other hand grew as single cells, with areas of large flattened sheets of cells forming. At day 5 changes were very apparent with populations of cell colonies forming. Some individual cells were almost squamous in appearance with very prominent nuclei, others grew in swirls and piled, forming thick long differentiated structures. Matrigel and NCL5-iMEF cultures appeared to proliferate much faster than NCL5-iMRC5 cultures. At day 7, differences between all three culture conditions were seen. Small, thickened structures were visible from Matrigel cultures. NCL5-iMEF cultures were much flatter but overgrown with small darkened areas of differentiation which appeared to be forming 3D structures. NCL5-iMRC5 cultures were more overgrown than the other two culture conditions with ring like structures forming as a result of cells proliferating and piling to form structures.

The gene expression data showed upregulation of DESMIN, BMP4 and significantly high upregulation of HAND1 and PTX1 (greater than 100 log fold) were detected from all culture conditions, relative to the control (Figure 6.6). Almost all other genes were upregulated from both feeder cultures. Significant upregulation of FGB was also seen from both feeder cultures. Slight downregulation of Nanog was detected from all culture conditions. Matrigel and iMRC5 cultures maintained expression of OCT4, relative to the control.

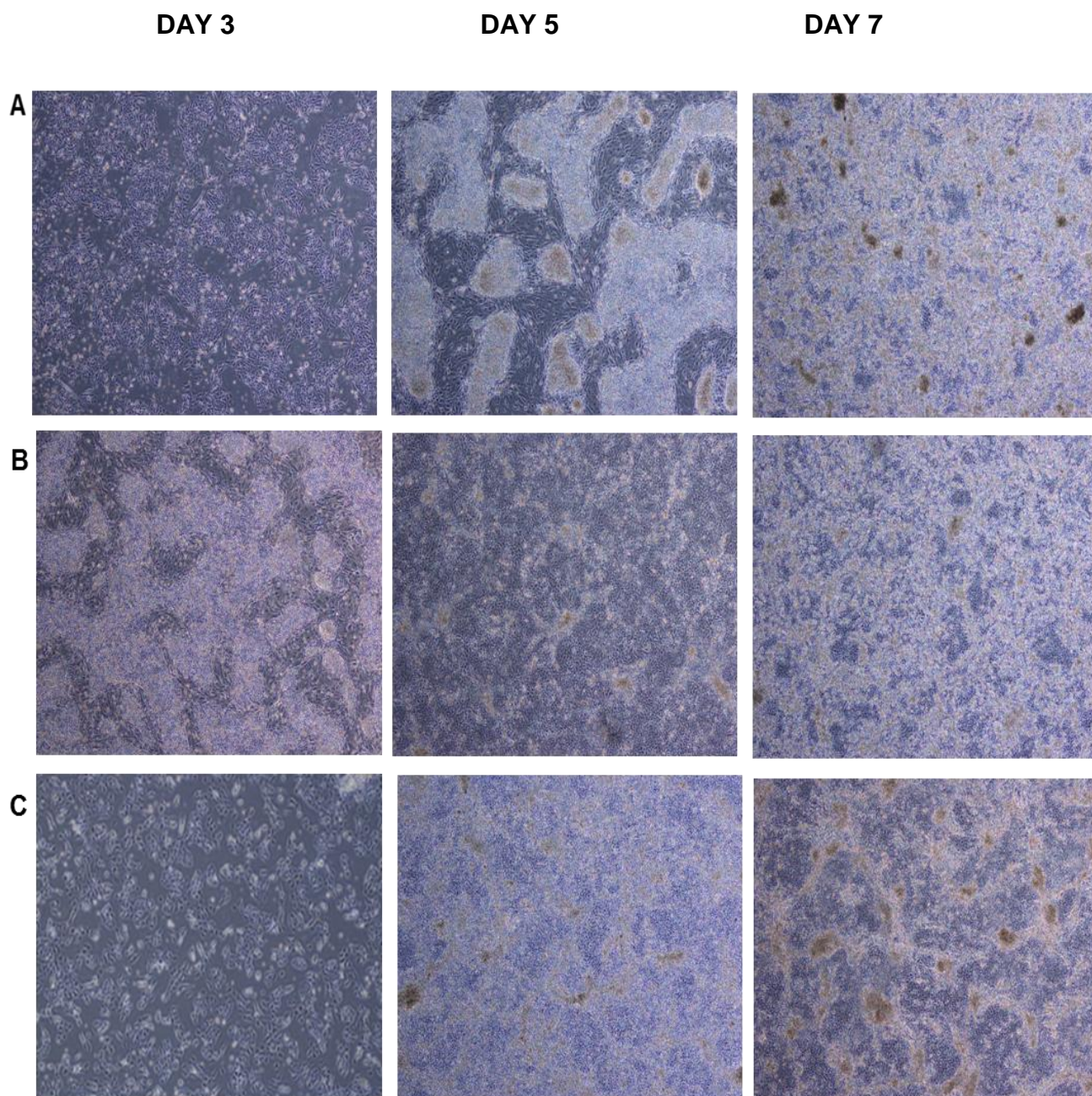


Figure 6.5. Morphological changes of stem cell line NCL5 following *in vitro* directed differentiation to mesoderm from prolonged culture on iMEF mouse feeders (A) iMRC5 human feeders (B) and Matrigel (C) at day 3, 5 and 7 . Images are representative of 3 independent experiments and were taken with a light phase contrast microscope at x4 objective. Scale bar = 50 μ m.

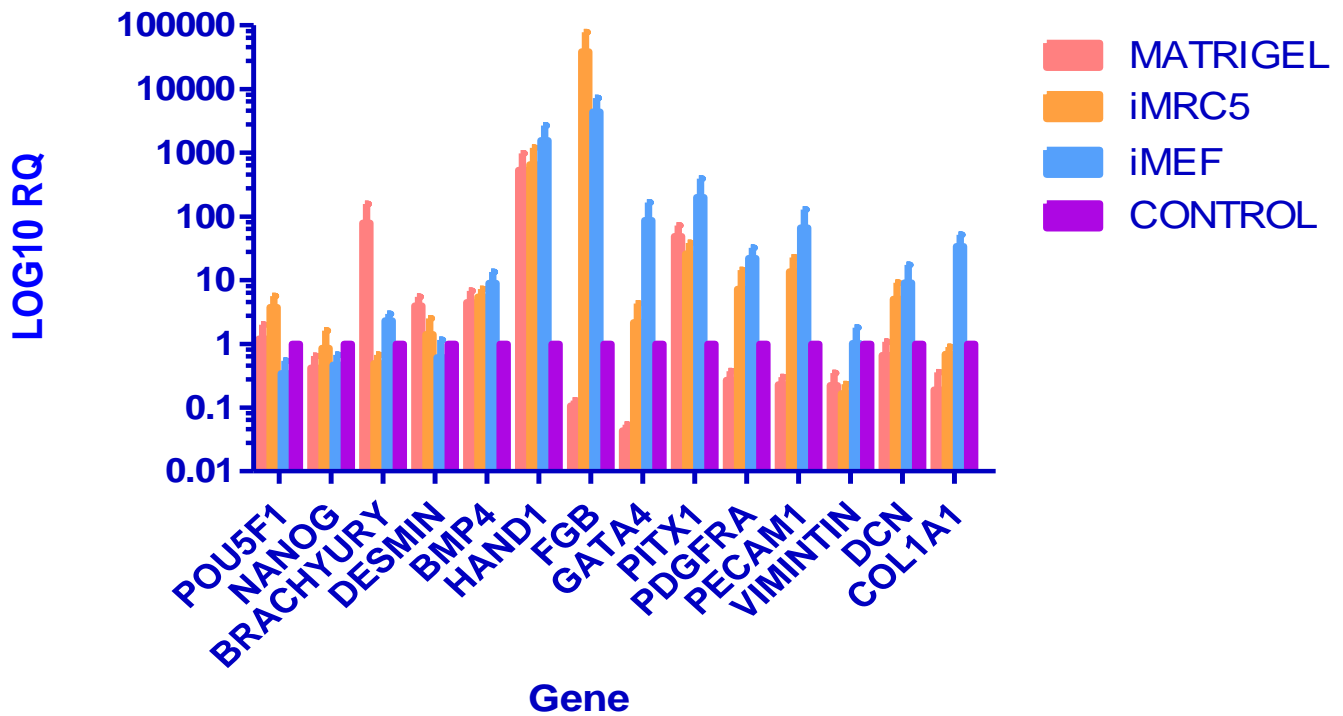


Figure 6.6. Gene expression of stem cell line NCL5 following *in vitro* directed differentiation to early mesoderm. NCL5 at late passage cultured on iMEF mouse feeders, iMRC5 human feeders and feeder free Matrigel were plated as single cells onto Matrigel coated plates, then subjected to flat based *in vitro* directed differentiation for 7 days. Cell pellets were collected for real time PCR analysis using germ layer specific probes, as described in the methods. The results are the geometric mean of three individual experiments. Variations in mesoderm gene expression were detected from late passage NCL5 cultured on three matrices, at day 7. Cultures on Matrigel had lower expression compared with both feeders. Significant upregulation of FGB, HAND1 was seen from all cultures. iMEF mouse feeders and iMRC5 demonstrated upregulation of most mesoderm genes (n=3).

6.3.4 Flat based *in vitro* directed differentiation of stem cell line NCL5 to early neuroectoderm

Images from Figure 6.7 show that NCL5 from iMRC5 feeder cultures appeared much sparser at day 3 (B), in comparison to the other two matrices. NCL5-Matrigel cultures displayed less defined colonies (C), in comparison to NCL5-iMEF colonies, which still appear as discreet colonies (A) comprised of small flat cells. By day 5 changes in morphology were more apparent in all cultures. hESCs were more visible as single cells, smaller in size compared with individual stem cells and with larger nuclei. Cultures from Matrigel appeared more settled than feeder cultures, proliferating well with little cell death. More cell death was seen from NCL5- iMEF cultures. On day 7 cell piling was visible from NCL5-iMRC5, though proliferating cells underneath still displayed characteristic changes. NCL5-iMEF cultures appeared to have stabilised, with not much death visible. Stem cell cultures from Matrigel had begun to detach in some areas, with others displaying early neural rosette formation.

Quite varied gene expression was detected across the three matrices, with upregulation of FOXC1 in all cultures (Figure 6.8). Upregulation of NESTIN, PAX6, B3T, NEUROD1 and HES5 were detected from both feeder cultures. Differences between human feeder and mouse feeder cultures unveiled the highest upregulation of ectoderm genes FOXC1 and NESTIN from NCL5-iMEF cultures, whereas NeuroD1 and SOX1 were further upregulated from NCL5-iMRC5 cultures. Expression of POU5F1 and Nanog were only downregulated from Matrigel cultures.

Matrigel cultures gave significant upregulation of FOXC1, with little upregulation of other genes. Whereas both feeder cultures displayed upregulation of all ectoderm genes with iMRC5 cultures showing higher upregulation in comparison to iMEF cultures. Downregulation of OCT4 and NANOG was only demonstrated by Matrigel cultures.

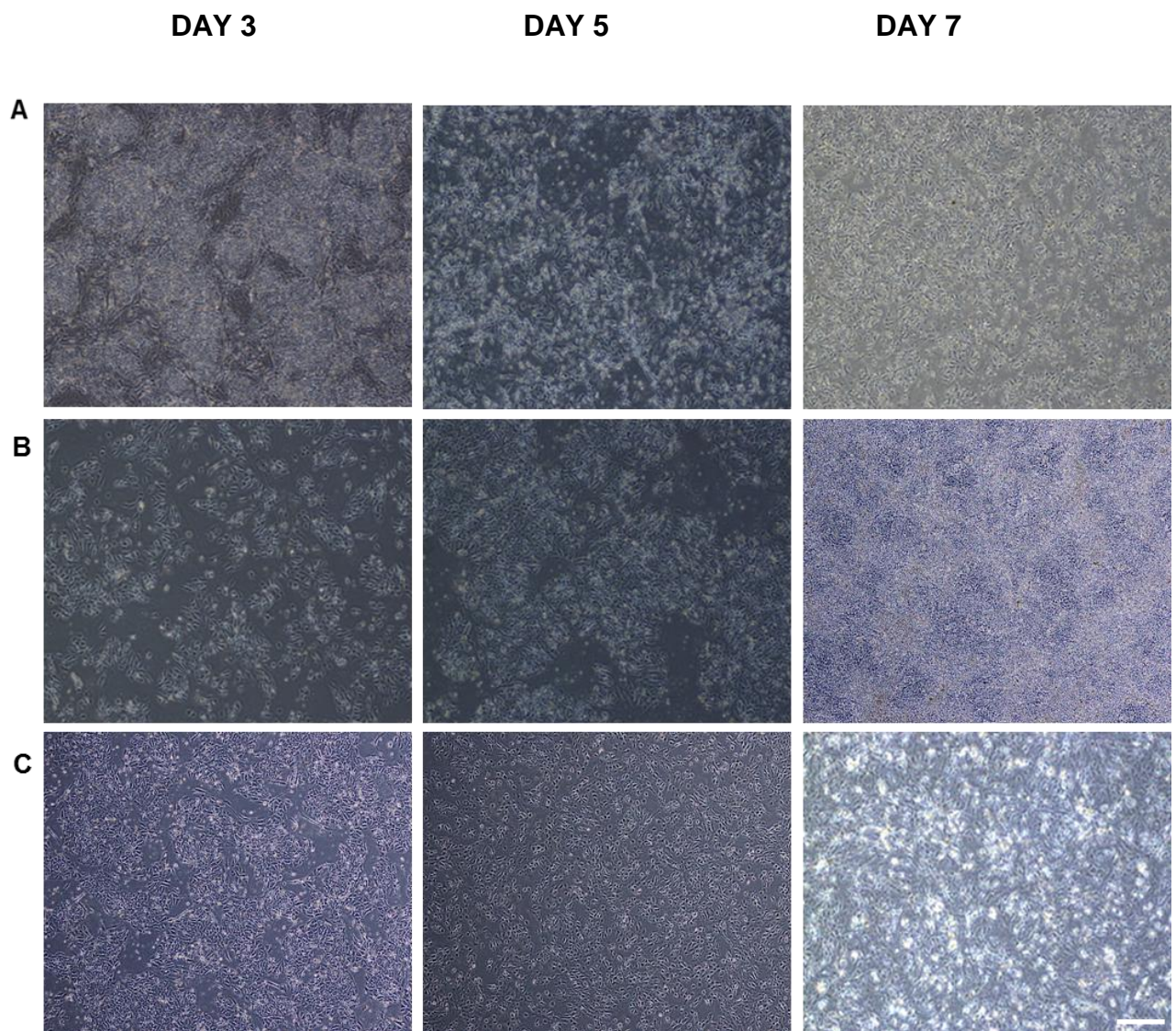


Figure 6.7. Morphological changes in stem cell line NCL5 following *in vitro* directed differentiation to ectoderm from iMEF mouse feeders (A) and iMRC5 human feeders (B) and Matrigel (C) at day 3, 5 and 7. Images were taken with a light phase contrast microscope at x4 objective. Scale bar = 50 μ m.

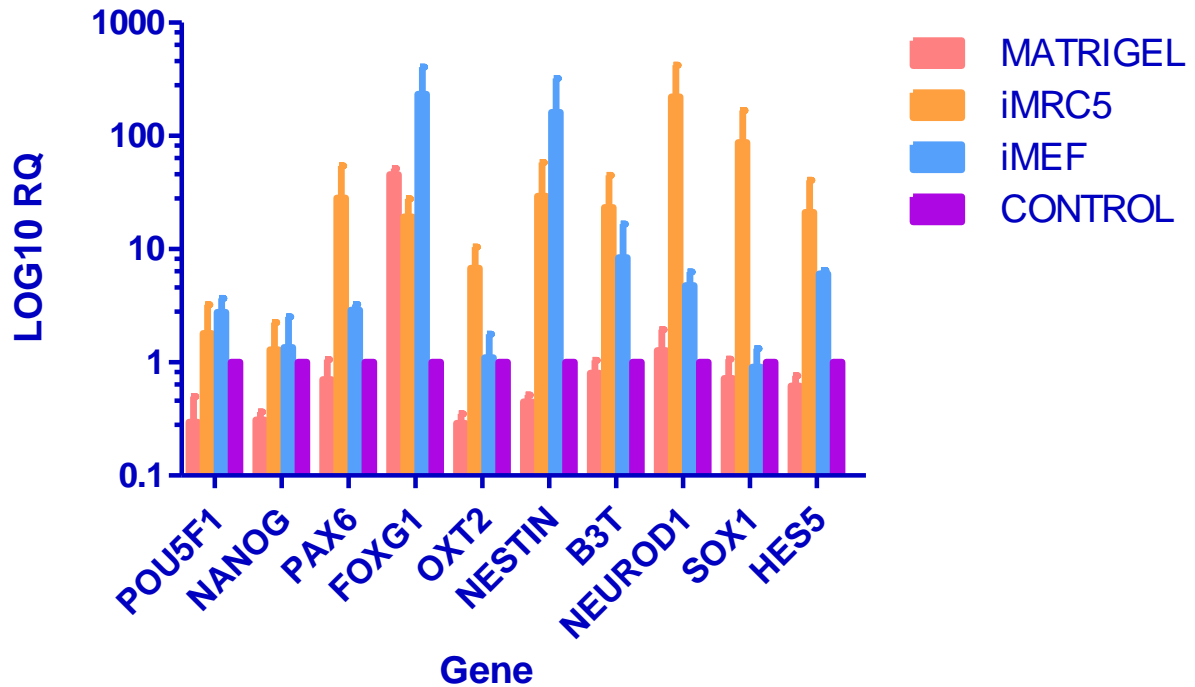


Figure 6.8. Gene expression of stem cell line NCL5 following *in vitro* directed differentiation of NCL5 to early ectoderm. NCL5 at late passage cultured on iMEF mouse feeders, MRC5 human feeders and feeder free Matrigel were plated as single cells onto Matrigel coated plates, then subjected to flat based *in vitro* directed differentiation for 7 days. Cell pellets were collected for real time PCR analysis using germ layer specific probes, as described in the methods. Clear differences in upregulation of ectoderm genes were detected as a result of culture on different matrices. (n=3).

6.3.5 Effects of different matrices for prolonged culture of NCL5 stem cells on gene expression from three germ layers using Heat map analysis

Comparison of genes expressed from each germ lineage from differentiated NCL5 previously cultured on mouse feeders iMEF, human feeders, iMRC5 and feeder free matrix Matrigel was performed using ExpressionSuite software. Gene significance was set to <3 fold, based on the average change between endogenous controls from each sample type. Figure 6.9 A reveals different expression profiles from all three differentiated samples at day 7 (D7). All endoderm genes were upregulated across all three samples, with stronger upregulation of the genes FOXA2 and GSC from NCL5-iMEF and NCL5-Matrigel samples.

Expression of ectoderm genes revealed slight correlation across the samples from different matrices, demonstrated by upregulation of SOX1, HES5 and PAX6. Expression of OTX2 was downregulated in NCL5-iMEF and NCL5-Matrigel, with NCL5-iMRC5 showing very slight upregulation (Figure 6.9 B).

Expression of mesoderm genes showed greatest correlation compared with the other germ layers. Pearson's' correlation revealed all three samples showed linked expression of mesoderm genes, with NCL5 from both feeder types iMRC5 and iMEF showing closer correlation than NCL5-Matrigel, which had a more distinct gene expression profile from the other two samples. Expression of Brachyury was strongly upregulated in all samples, followed by upregulation of FGB, Pecam1, PDGFRA, Desmin and DCN. Differences in HAND1, Vimentin and COL1A1 were detected, with downregulation of the latter two genes.

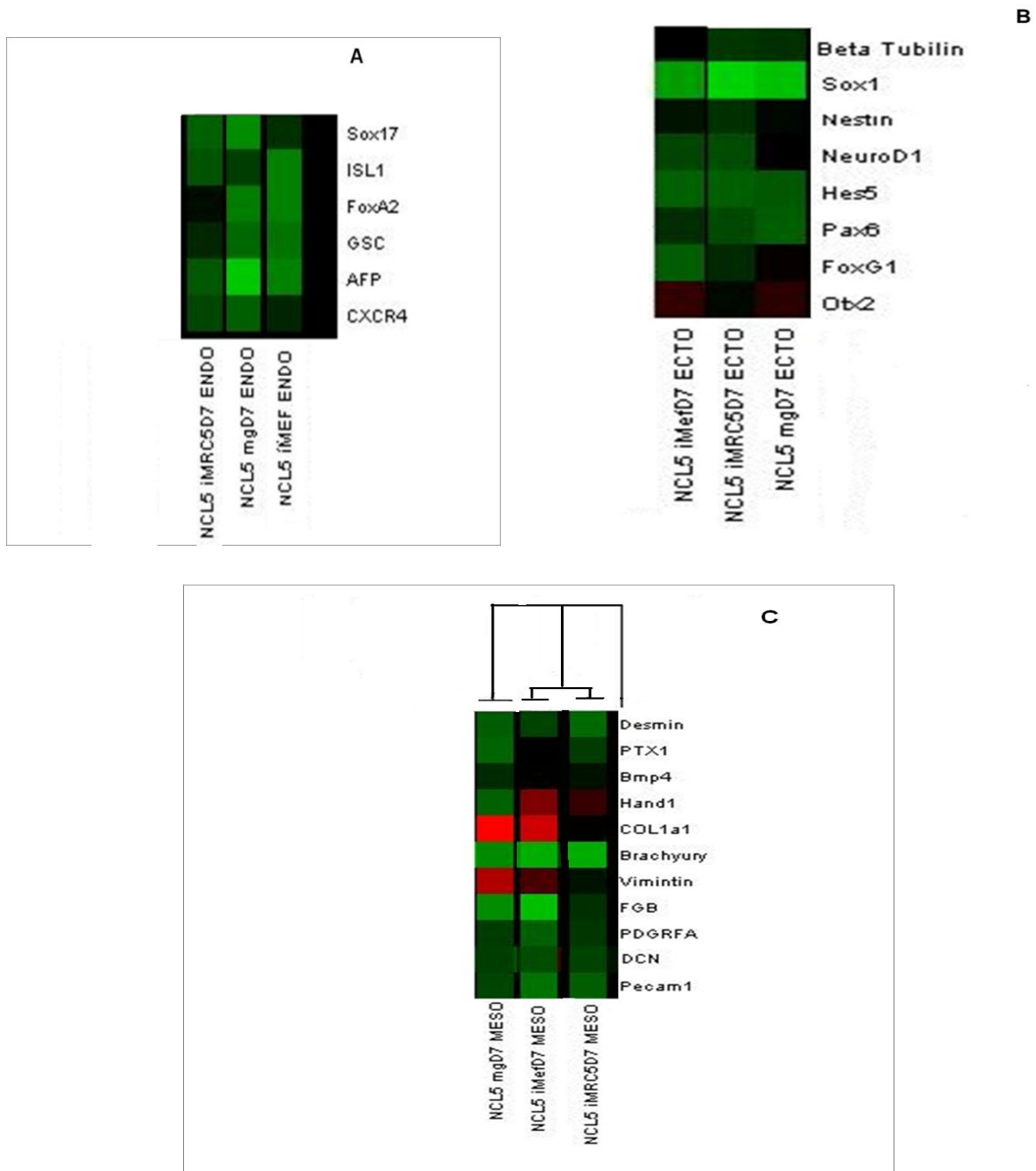
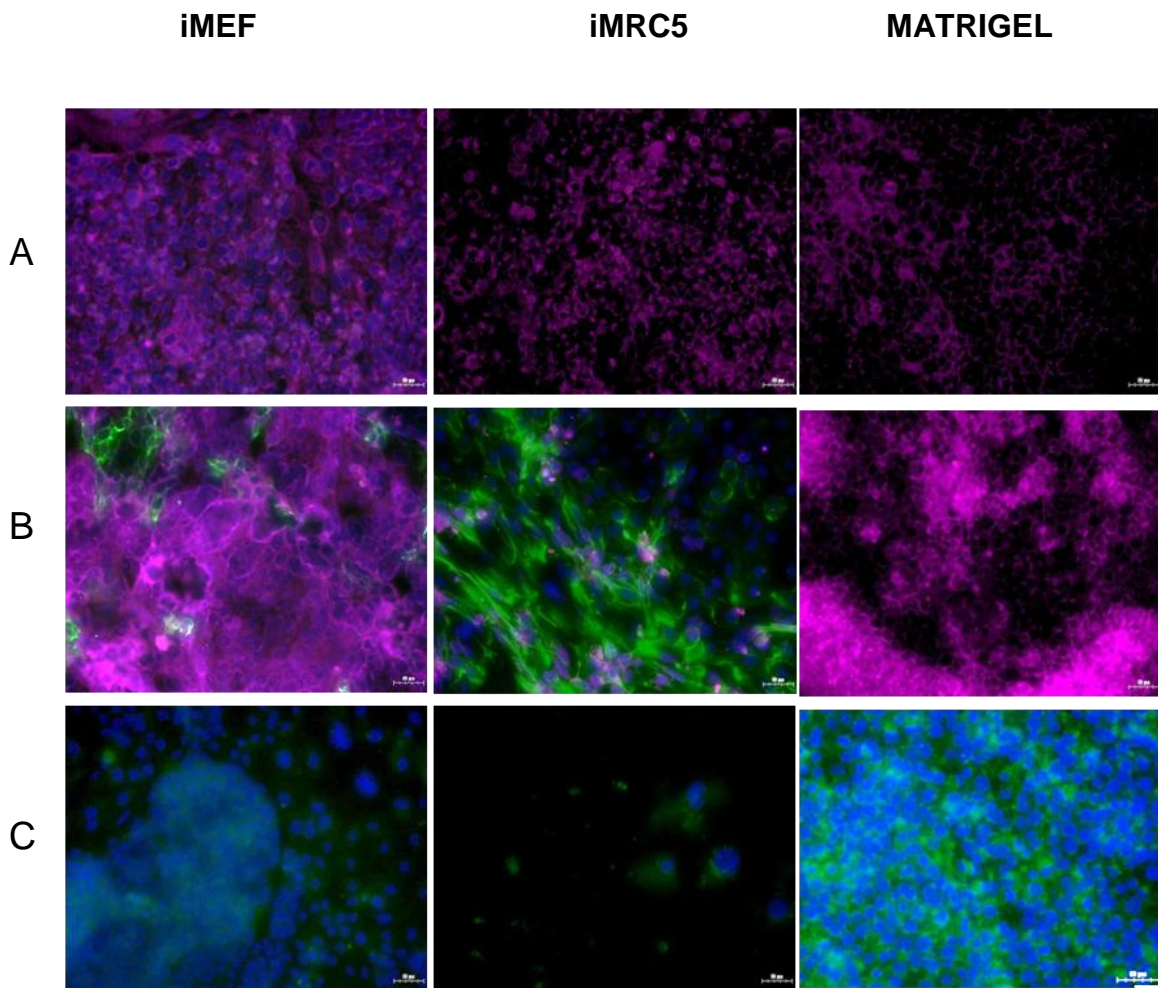


Figure 6.9. Heat map demonstrating correlation of differentiated gene expression to endoderm (A), ectoderm (B), and mesoderm (C), from late passage NCL5 stem cells cultured on Matrigel, iMEF and iMRC5.

6.3.6 TissueFaxes™ analysis of expression of protein markers for each germ lineage following *in vitro* directed differentiation

Fluorescent images of nuclear and cell surface markers were assessed following *in vitro* directed differentiation to all three germ layers. Positive expression of markers was observed from all cultures. Expression of mesoderm markers Vimentin and Hand1 and endoderm markers DCN, FOXA2 and SOX17 were strongest. Expression of PAX6 and NESTIN were detected (Figure 6.10), but with weaker expression from NCL5-iMRC5 cultures. Although Nestin gene expression was found to be low in Matrigel cultures, it was expressed at protein level. Very obvious changes in morphology were observed. However, piling from all cell cultures was detected, which made image acquisition and analysis difficult.

TissueFaxes™ images also allowed the comparison of morphology across the three different matrices. Initially no obvious differences could be seen when imaging. However, differences in the parameters applied to detect each matrix type had to be applied during TissueFaxes analysis, indicating that subtle changes were apparent as a result of influences from their culture environments. Figure 6.10 showed that NCL5-iMEF and NCL5-iMRC5 differentiated cells were slightly larger and elongated, whereas overall, Matrigel cells were smaller. Furthermore, the amount of differentiated cells for each germ lineage marker was quantified using TissueQuest. Figure 6.10 also revealed that all three matrices displayed greater than 60% positive expression of germ lineage specific markers. NCL5-iMRC5 exhibited the lowest expression of SOX17, HAND1 and NESTIN and NCL5-Matrigel, the highest expression.



hESC-Matrix	Endoderm % SOX17 (A)	Mesoderm % HAND1 (B)	Ectoderm % NESTIN (C)
NCL5-iMEF	84.60	83.30	82.19
NCL5-iMRC5	58.11	60.88	75.15
NCL5-MATRIGEL	91.20	78.80	94.20

Figure 6.10. TissueFacs analysis of differentiation markers expressed in NCL5 stem cells pre-cultured on iMRC5 human feeders, iMEF mouse feeders and feeder free matrix Matrigel. Panel A shows early endoderm marker SOX17, Panel B the mesoderm marker HAND1 (purple) (Green) and Panel C the ectoderm marker Nestin. The results are representative of two independent experiments. Cells imaged at x20 objective. Scale bar: 100µm.

6.4 Discussion

In vitro differentiation assays are useful and important for demonstrating the pluripotent nature of hESCs by formation of early progenitor cells from all three germ layers. Quantitative methods real time PCR and TissueFaxs™ image analysis proved successful for the assessment of human feeders' iMRC5, mouse feeders' iMEF and feeder free Matrigel, to support the pluripotent nature of NCL5 at late passage (p58) as they were still able to differentiate. Following a 7 day *in vitro* directed differentiation assay, stemness genes were down regulated and differentiation genes upregulated for endoderm, mesoderm and ectoderm. This demonstrated that that all conditions supported the directed differentiation of NCL5 towards each germ layer, which is in concordance with the papers from which these methods were derived (D'amour et al., 2005; Chambers et al., 2009; Sa et al., 2011).

Results confirmed that NCL5 formed consistently good EBs. Non directed differentiation in KSR media after 7 days showed upregulation of ectoderm transcription factor genes Neurogenic differentiation 1 (NEUROD1) and Sex determining region Y-box 1 (SOX1). Neurod1 is involved in regulating expression of the insulin gene. SOX1 expression is restricted to neuroectoderm. Mesoderm gene Heart and neural crest derivatives-expressed protein 1 (HAND1), was also significantly upregulated (<3 fold). HAND1 is uniquely expressed in trophoblasts. Upregulation of ectoderm genes was consistent with results from chapter 6 in the undifferentiated NCL5 cultures from Matrigel at early and late passage. However, all endoderm genes were down regulated. Furthermore OCT4 expression was not downregulated and Nanog only slightly downregulated. This demonstrated that EBs were not a suitable starting material for these differentiation studies. This may have been due to NCL5 lack of ability to form aggregates at such late passage, due to acquirement of chromosomal aberrations, as shown in chapter 4. Longer EB studies may demonstrate greater upregulation of germ lineage specific markers, as effects of high FGF levels from Matrigel/mTeSR1 and feeders may also slow or lessen the effects of non-directed differentiation as aggregates. Many differentiation protocols use spontaneous differentiation experiments to demonstrate a heterogeneous differentiation population following the formation of EBs (Xu, C., et al, 2001). However

some reports have shown that expression of germ lineage specific genes can vary up to 400 fold between EBs formed for non-directed differentiation experiments at day 16 from different hESC lines (Osafune et al., 2008).

Differences between the gene expression of differentiated NCL5 pre cultured on feeder and feeder free matrix were observed. Although gene expression studies showed most ectoderm, mesoderm and endoderm genes were upregulated, each NCL5 condition exhibited individual gene expression profiles as a result of prolonged culture on different feeder type.

Differences in the correlation of samples from different matrices were dependant on the expression of germ layer specific genes. Only mesoderm gene expression proved to show a greater correlation across the three samples, possibly indicating that they all utilise signalling mechanisms which act on the same pathway, or that the strength of the inducing factors used were sufficient to overcome the inherent differences between the NCL5 cultures as a result of prolonged culture on different matrices. Statistical analysis demonstrated stronger correlation of mesoderm gene expression from mouse and human feeder cultures, when compared with Matrigel cultures. This is probably due to the secretion of growth factors from feeders which could be enhancing the differentiation towards early mesoderm. Profiling of feeders from chapter 6 revealed the expression of FGFs, and laminins by human feeders when compared to mouse, which clearly demonstrates the difference in ability to support undifferentiated hESC growth and also influence *in vitro* differentiation. It is not surprising that interactions with FGFs and Activin A secreted from feeder sources can have varied effects on gene expression profiles, as both types of growth factors are known to have close involvement in Wnt and SMAD signalling pathways (Rajasekhar and Vemuri., 2009). The results demonstrated that the intrinsic effects of prolonged culture on different feeders were sustained, even when the cells were transferred to Matrigel for one passage. To our knowledge this has not been reported before and is a novel finding.

The use of feeders in co culture has previously been explored and suggested as a better alternative to synthetic matrixes as they are thought to more closely mimic *in vivo* interactions as a result of stronger integrin binding and better reorganisation of ECM proteins. This results in both faster upregulation of germ layer specific gene

expression and morphological changes, when compared to synthetic matrices, as shown in Figure 6.6. This may also account for the differences in germ layer specific gene expression between NCL5 cultured on three different matrices, as NCL5s cultured on feeders showed a much wider range of germ lineage specific marker expression as well as overall higher expression, compared to Matrigel. This demonstrates that differences in differentiation propensity are not limited to differentiation into germ lineage. Such findings have already been shown by differences in frequency of EB formation between HUES3 and HUES1 cell lines (Osafune et al., 2008). Striking differences in morphology and of germ layer specific genes expression were confirmed as a result of consistencies between biological repeats, and the decision to use a greater number of differentiation genes than those which are commonly published for such an assay. The results show that iMRC5 human feeders and iMEF mouse feeders demonstrated upregulation of a greater number of mesoderm, endoderm and ectoderm genes, in comparison to Matrigel cultures. Matrigel cultures gave stronger upregulation of mesoderm and endoderm genes with a greater number of endoderm genes expressed in comparison to the other germ layers. Ectoderm genes were relatively low, with the exception of FOXP1 which was highly expressed. When compared to chapter 6, undifferentiated hESCs on Matrigel demonstrated slight tendency toward ectoderm differentiation, as these genes were slightly upregulated.

The influence of using different matrixes to induce and enhance differentiation towards specific germ layers has been explored. Matrixes such as Vitronectin (Prowse et al., 2010, Mummery et al., 2008) and co-culture with stromal cells have been shown to increase differentiation potential to haematopoietic lineage. To our knowledge, this the first study to demonstrate the influence of iMRC5 human feeders and iMEF mouse feeders in comparison to Matrigel, to increase differentiation ability to different germ layers.

Proteomic analysis of human feeders has revealed the expression of col1a1 and BMP4, both of which have been shown to be upregulated in mesoderm differentiation. Furthermore, analysis of mouse feeders has shown higher levels of activin A compared with human feeders, which expressed higher levels of FGF2 (Eiselleova et al., 2008). Such differences over long term culturing have resulted in the difference in genes expressed following *in vitro* differentiation in the experiments

described here as these growth factors play synergistic roles in blocking and enhancing specific germ lineages. Understanding the signalling effects of co-culture cells on differentiating hESCs *in vitro* is vitally important for understanding the mechanisms that hESCs differentiate *in vivo*, so the properties of the feeders can be better exploited. The process of differentiation is thought to be stage specific, therefore by mimicking the supporting or co-culture cells, a better understanding of the signalling required to induce such changes can be more accurately repeated *in vitro*, helping to progress this area of research. Such research has been recently revisited, to investigate the differentiation of hESCs to mature pancreatic islet cells using endothelial coculture (Jaramillo & Bannagi., 2012). This illustrates the benefits of co-culture in spite of consistent efforts to use feeder free matrices, as scientists clearly recognise that fibroblasts can be used to mimic external micro environmental signalling cues for successful differentiation.

The studies performed here demonstrate the sustained benefits of coculture achieved by pre-co-culture of hESCs with feeders, even after transfer to Matrigel for one passage. This reduces the method time as it removes the need for adjusting cultures onto feeder free matrices before initiating differentiation. More simplistic and straight forward culturing methods need to be progressed for standardisation and clinical application, particularly when screening a number of different hESC lines. NCL5s cultured on Matrigel did exhibit changes in morphology that were typical for early endoderm and neural differentiated cells. However, at mRNA level this expression was low, particularly when compared to NCL5 cultured on mouse and human feeders.

hESCs cultured on Matrigel have been reported to take longer to differentiate compared to those cultured on mouse feeders (Xu et al., 2005). Some degree of this observation was demonstrated in these studies. Although NCL5s from pre-cultured Matrigel did exhibit both morphological and genetic changes consistent with directed differentiation, the cultures did not show as increased upregulation of differentiation markers compared with pre-cultured hESCs from both mouse and human feeders. Other explanations as to why Matrigel cultures were not as successful in promoting differentiation reside with the components within Matrigel/mTeSR1 media. High levels of bFGF have been shown to inhibit differentiation (Xu et al., 2005). Although Matrigel does consist of ECM, there may be smaller, but more important growth

factors and proteins which are lost during commercial preparation, which although are not necessary for maintaining undifferentiated cultures, may play an important role in supporting the transition towards specific differentiation pathways. Cell-cell interactions and adhesion and anchoring molecules found in fibroblasts also influence self-renewal (Kueh et al., 2006). A lack of these interactions as a result of culturing hESCs long term on Matrigel may account for such differences in gene expression profiles, observed during progenitor formation. Recommendations would be to adjust the time in differentiation media, with perhaps longer wnt3a/Activin A treatment to allow for greater upregulation of endoderm genes SOX17, FOXA2. This protocol may require finer adjustments to allow for better success of endoderm differentiation on hESCs cultured on Matrigel in comparison to feeders.

Heat map analysis also revealed differences in expression level from NCL5 cultured on different Matrices. For neural differentiation, overall, greater up regulation of differentiation genes was seen from NCL5 cultured on iMRC5 human feeders compared to iMEF mouse feeders and feeder free Matrigel. More consistent results were seen from NCL5 on iMEF after endoderm differentiation compared with NCL5 iMRC5 and Matrigel. However, NCL5 cultured from iMRC5 demonstrated upregulation of Brachyury indicating that mesoendoderm differentiation was occurring. This may explain the much lower levels of early endoderm gene expression compared to NCL5 cultured on iMEF mouse feeders. Altered endoderm gene expression observed from NCL5 cultured on both mouse and human feeders may indicate differences in pathway regulation when undergoing endoderm differentiation. This would require further investigation to establish the cause of these differences.

In vitro differentiation to mesoderm progenitor consistently demonstrated upregulation of HAND1 and vimentin by gene upregulation and IF staining, in comparison with other genes. Vimentin has also been used as a marker of epithelial to mesenchymal transitions (EMTs) that take place during embryogenesis and metastasis. It codes for an intermediate filament protein and has been shown to influence shape transitioning, increase in cell motility, and increase in focal adhesion dynamics (Mendez et al., 2010). Increased expression of vimentin by both feeder cultures compared with Matrigel may be an important characteristic of feeders which aid differentiation by reorganisation of the cell matrix and cells themselves. This

again suggests that feeders provide contact and communication with hESCs that is vital for their adhesion.

These studies demonstrate the ability to simplify and better standardise three *in vitro* differentiation methods and translate its use in other hESCs on different feeders/ matrices at late passage following a 7 day protocol. The adherent method provided an easy method to differentiate cells and its success may be due to better penetration of cells with growth factors, in comparison to forming EBs. The reduced ability to differentiate as EBs may be due to the age of the cell line. Though prolonged passaging had not reduced the ability to form aggregates as they readily form EBs, it may have decreased their ability to function as aggregates. Although these studies successfully validate the adherent plate method, which is fast and cost effective, decreased ability to form EBs may have important implications for use in clinical applications as aggregate formation has been widely adopted, and therefore warrants further investigation.

Improvements to the protocols to increase homogenous yields of early germ layer specific differentiated hESCs would undoubtedly be useful to researchers, particularly if this can be achieved over a shorter time span than current methods, which require around 21-28 days. The use of quantitative methods to evaluate the success of differentiation protocols was demonstrated by TissueFaxes™ and quantitative real time PCR. The progression of such an outdated and inconsistent test will undoubtedly propel the field into establishing robust studies such as the ones described here. As the UKSCB plays an important role in supporting the wider stem cell community, the success of this work will be further implemented into routine characterisation testing of banked hESCs to give researchers more information of hESCs obtained, as well as increasing their value as research tools.

6.5 Conclusion

The standardisation of differentiation methods is important for demonstrating the pluripotent capacity of hESCs used in long term studies, by different laboratories. Late passage NCL5 cells from all three matrices demonstrated successful *in vitro* differentiation using a straight forward adherent plate method, to all three germ layers after 7 days. Such work is highly robust and reproducible, with the benefit of using media and matrices commonly used in the majority of laboratories.

Differences between mouse and human feeders and Matrigel were apparent upon directed differentiation *in vitro*. Overall, cultures from both feeder types better supported differentiation towards all three germ layers in comparison to Matrigel cultures, with particular attention to early ectoderm. These studies reveal how the effects of using different feeder types contribute to the differentiation of hESCs towards early progenitors of all three germ layers *in vitro*. Furthermore it highlights the variability of using different matrices for long term culture of hESCs on pluripotency. Contribution of this work is important to the wider stem cell community as currently there is no recommended method of differentiating cells *in vitro*.

Establishing robust differentiation assays is not a simple task. Microenvironment plays an important but variable role which is key to the success of producing early progenitor cells. The continuation of this work using other hESC lines will help to strengthen the robustness and reproducibility of these relatively simple but effective assays to form progenitor cells from all three germ layers in a reliable way. As these studies are the first of their kind, it will also serve to demonstrate that prolonged culture of hESCs on different matrices can be sustained through *in vitro* directed differentiation assays.

Cell health has been a key contributor to the accomplishment of this work. The use of Matrigel, defined components, small molecules, growth factors all provide better starting materials towards the development, standardisation and overall success of these assays.

Chapter 7.

Analysis of chromosomal changes in hESCs using comparative genomic hybridisation: effects of long term passaging using mouse, human feeders and Matrigel.

7.1 Introduction

Human embryonic stem cells (hESCs) carry normal diploid karyotypes at early passage (Baker et al., 2007; Mayshar et al., 2010) but this may change in prolonged culture and affect their defining characteristics as stem cell lines, including their potential to differentiate. The precise chromosomal changes may vary between cell lines and with the culture conditions, including the matrix on which the cells are grown, the process by which they are propagated or their duration in culture. For instance, a gain on chromosome 17q has been shown in three hESCs (H1, H7 and HES3) as a result of long term culture over 30 passages (Allegrucci et al., 2007). The gain was observed in cells grown on MEF feeders or on a fibronectin feeder free matrix, passaged either with EDTA free trypsin or manually using a glass pipette (Draper et al., 2004). A similar gain on chromosome 17q as well as chromosome 20 has also been reported in eight out of nine hESCs lines including H1 and HES3 cultured over 20 passages by both feeder (irradiated MEFs) and feeder free methods (Matrigel) (Maitra et al., 2005).

In contrast, studies with SHEF1, SHEF3 and HS181 continuously propagated with Collagenase IV and cultured on Matrigel have shown that these cells maintain a normal karyotype up to passage 10, after which the SHEF3 and HS181 gained an extra chromosome 12, as detected by Spectral Karyotyping (SKY). The same hESC lines cultured on human feeders, HFF by both manual and enzymatic passaging in KO-HES media, maintained a stable karyotype for 30 passages (Catalina et al., 2008). In another study using feeder free conditions the hESCs H1, H7 and H9 maintained a normal karyotype on Matrigel and Laminin for up one year (approximately 42 passages) (Xu et al., 2001; Stojkovic et al., 2005). The reasons for these discrepancies are currently unclear but raise serious concerns about establishing the chromosomal stability of stem cells from different culture methods and the implications this may have on experimental data obtained using these cells. It is however clear that karyotype changes may occur, but the precise changes or how these relate to the cell type and/or the culture conditions remains to be established in a comprehensive and comparative study.

In addition to the above, the method for assessing chromosomal stability needs to be standardised and suitable protocol established which can be exploited routinely in

both specialist and non-specialist laboratories. Currently, the majority of research into chromosome stability of stem cells has been investigated by traditional cytogenetic methods including G banding (Bongso et al., 1994; Brimble et al., 2004; Hanson & Caisander, 2005). This technique involves the preparation of metaphase spreads from proliferating cultures, which are then fixed and stained with Giemsa stain. The metaphase spreads are identified, examined and counted (at x40 objective). Typically, fixed cell suspensions (from different cell types, but mostly from whole blood samples) are sent to a specialist cytogenetic laboratory where G banding is performed on a small number of cells by a trained cytogeneticist under high magnification (x100 objective). The adoption of this method from a clinical setting involves the partial digestion of chromosomes using trypsin and staining with Giemsa to reveal dark bands on the chromosomes that are rich in Adenine and Tyrosine, which are paired and analysed for overall chromosomal stability. The availability of participating clinical cytogenetic laboratories to process stem cell samples for G banding is not readily available in most laboratories, making it difficult for scientists to routinely determine changes in the stability of stem cell lines. Also, the preparation of metaphase spreads from stem cell lines has been problematic, leading to inconsistent results.

Although other methods such as spectral karyotyping, single nucleotide polymorphism (SNP) analysis and fluorescent in situ hybridisation (FISH) have been assessed for routine karyotyping (Catalina et al., 2007; Meisner & Johnson, 2008), these techniques are limited in resolution, particularly for the detection of small changes less than 50kB. Furthermore, such techniques only report detailed analysis on a few cells which are supposed to be reflective of a whole culture. Thus, developing a sensitive and robust protocol which could be used routinely for karyotyping would prove invaluable, and this has been one key objective at UKSCB which was addressed as part of this thesis focusing on optimising the routine and reproducible use of Array Comparative Genomic Hybridisation (aCGH). The latter is a much more sensitive technique which has been applied in clinical settings (Cleide et al., 2008) for comprehensive assessment of chromosome stability. This technique, coupled with accurate information on specific chromosome aberrations, can link to gene databases to give detailed assessment of the clinical significance and consequences of specific losses or gains of chromosome(s).

The studies described in this chapter were therefore aimed at detecting changes in copy number variations (CNVs) and in establishing how such changes might be affected by the culture conditions. Furthermore, the far-reaching nature of the experiments carried out should also validate aCGH for in-house use as an established method for routine detection of changes in chromosomal stability in stem cells.

In these studies stem cell lines NCL5, RH5, HUES9 and SHEF1 were selected as they are not well publicised lines. Therefore information on their basic characteristics may help to diversify the hESC lines currently in use, which would prove useful to the wider stem cell community. Each cell type was cultured on mitotically inactivated human feeders (iHDFn and iMRC5) and mouse feeders (iMEF and i3T3s) as well as on feeder free matrix Matrigel in mTeSR1 media, and cultured for 20 passages. Samples were prepared for G banding analysis, outsourced testing by The Doctors Laboratory (TDL) and aCGH using the Perkin Elmer spectral BAC array, specific for the detection of chromosome changes within the whole genome. The results from both techniques would provide a useful comparison of which is more sensitive, reliable and robust.

7.2. Method

7.2.1 Stem cell culture

Stem cell lines RH5, HUES9, NCL5 and SHEF1 were routinely maintained in continuous culture and when required, plated onto two inactivated mouse feeder lines iMEF and i3T3, two inactivated human feeder lines iMRC5 and iHDfn with standard KO-HES media, and on synthetic matrix Matrigel with mTeSR1 media. Each line was cultured over twenty passages using TrypLE™ Express, as described in general methods sections 2.4.

7.2.2 Sample preparation for metaphase spread analysis

Samples were prepared from the cell lines mentioned above for metaphase spread analysis (in-house) as described in general methods, section 2.4

7.2.3 DNA extraction

DNA was extracted for each condition from confluent early (p+5) and late passage (p+25) cultures using DNeasy extraction kit according to manufactures' protocol as described in general methods, section 2.4.

7.2.4 aCGH

aCGH was carried out as described in the methods (section 2.0) and involved five main steps: labelling, hybridisation, washing, scanning and data analysis. The arrays used were Perkin Elmer constitutional BAC 4.0 consisting of 1200 bacterial artificial chromosomes (BAC) clones spaced at one Mega Base intervals on a clear glass slide (compared with traditional karyotyping at 10MB intervals). This system has been validated for research use and has whole genome coverage, which is important for detecting novel karyotype changes. The array preparation was set up in a clean room to minimise exposure of the dyes to contaminants.

The 'reference sample' used was the early passage of each stem cell line on their respective feeder/matrix. Therefore, the 'test' sample was the culture pellet collected at p+20 (extended passage). Thus, a more accurate assessment of chromosome change could be determined over the 20 passage culture process. The samples were also subject to dye-swap wherein the test and reference dyes were prepared in a reverse reaction and swapped, which further ensures validity of the results.

A maximum of 6 arrays were prepared during one experiment, to minimise exposure to light and ozone and also minimise the number of human errors due to complex processing and long incubation steps.

The prepared arrays were scanned using the Perkin Elmer Scan array and corresponding Perkin Elmer software. Figure 7.1.A shows an example of a prepared array, enlarged tile of individual BAC arrays (7.1.B) and a ratio plot (7.1.C).

7.2.5 Data analysis

The fluorescent scanner captured data from the array and SpectralWare® software converted the scanner output data into an intensity ratio profile. The software analysed copy number changes and displays the location of the changes within the genome.

A number of analysis parameters were used to ensure the results obtained were reliable, including normalisation of the array spots, background correlation of the arrays, and threshold detection with lower and upper limits to ensure ratio-metric analysis for both dyes and standard deviation of the average spot intensity ($2.5 \times \text{STDEV}$).

The results obtained from aCGH analysis were exported as word documents, and diagrams for visualisation of karyotypes. The term karyotype refers to a display of the chromosomes of a cell by lining them up, beginning with the largest and with the short arm oriented toward the top of the karyotyping sheet. The fluorescent scanner captures data from the array and SpectralWare® software converts the scanner output data into an intensity ratio profile. The software analyses copy number changes and displays the location of the changes within the genome. The spot fluorescence intensity is averaged by adjusting the PMT voltage for each dye colour. The spots were then aligned and tagged as good or bad. Any misaligned spots, bad spots or debris/dye artefacts were manually adjusted or removed from the analysis. The aligned arrays were then converted into GPR files and analysed as ratio plots (Figure 7.1C) using the One Click™ software for CNV analysis version 4.0. Visualisation of individual chromosomes and adjustment of ratio plots were performed using this software, as well as creating tables of results with specific details of cell line karyotypes.

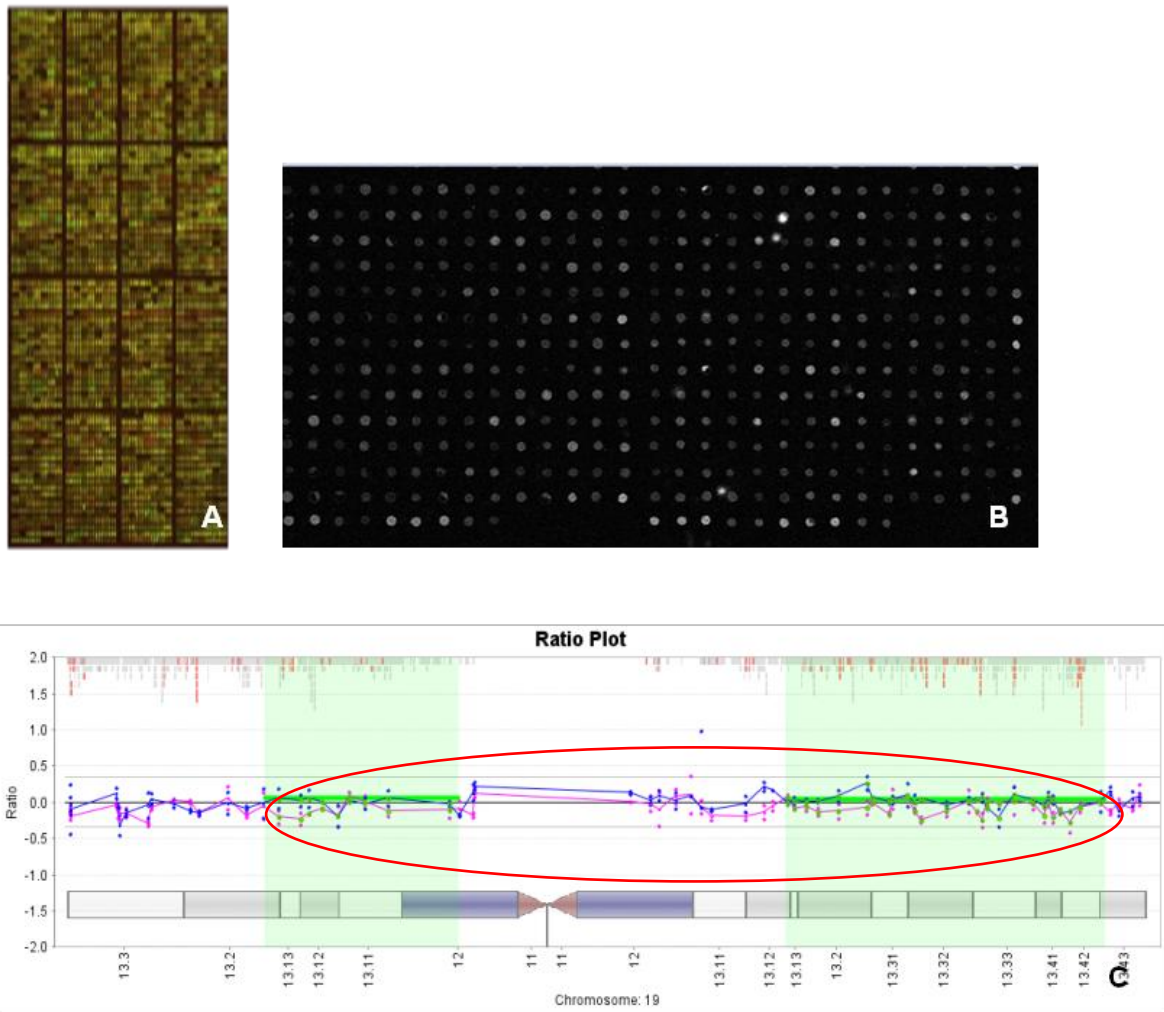


Figure 7.1. (A) Example of a prepared and scanned BAC array containing 1200 clones, (B) enlarged image of array tile showing individual BAC spots which correspond to specific genes and (C) example of a ratio plot for chromosome 19 from One click CNV software.

Sample and reference DNA were labelled in a two colour, ratiometric experiment and carefully hybridised to an array slide. The clones were covalently coupled to glass microscope slides, and spotted in duplicate. Once each slide had been scanned the corresponding sample which has been dye swapped was overlaid (CY5 and CY3), as shown in Figure 7.1. (A). The changes observed were for samples from original stem cell cultures at early passage compared with samples at late passage to give a karyotype over twenty passages. A red line on top and blue line on the bottom demonstrates a loss in chromosome number. A blue line on top and red line on the bottom indicates a gain in chromosome number, as demonstrated for chromosome 19 (Figure 7.1.C) as a subtle gain, from 13.3 of the P arm to 13.42 of the q arm. Chromosomes are arranged by short arm, p and long arm q, and divided by the centromere.

7.3. Results

7.3.1 Karyology results by G banding analysis

Results for karyotyping by G banding analysis (outsourced by The Doctors' Laboratory, TDL) are displayed in Table 7.1. Samples originally prepared from all 20 conditions (i.e. NCL5, HUES9, RH5 and SHEF1 cultured on iMEFs, i3T3s, iHDFns, iMRC5 feeders and Matrigel) were reported as 'not analysed' by G banding despite metaphase spreads being successfully visualised in-house (see list below Table 7.1). Previous karyology results from G banding analysis of distribution cell banks (DCB) and master cell banks (MCB), where available, were also included to look for consistencies in results reported. Although most of the results displayed in Table 7.1 conclude a normal karyotype, smaller aberrations were detected from many of the samples, but were assumed to be artefacts, despite the reporting of several chromosomal aberrations from SHEF1 MCB (7 aberrations in 5 cells) to SHEF1 DCB (1 aberration in 1 cell). Some increased chromosomal counts were reported. For example stem cell line RH5 cultured on iHDFn human feeders was reported as tetraploid (cells with four sets of chromosomes), however, only 4 cells were analysable. RH5 cultured on iMEF mouse feeders was also found to have gains on 9 chromosomes. RH5 samples taken from project banks i.e. for these studies, cultured on iMEF and human feeders iHDFn and iMRC5 (all highlighted in bold), were all reported as having isochromosomes, a chromosome that has lost one of its arms and replaces it with an exact copy of the other arm. This was observed on the long arm (q arm) of chr.17. The inconsistencies found as a consequence of using this technique highlighted greater focus on the aCGH studies to more reliably characterise the stem cell lines cultures on different matrices, to give complete data sets.

Table 7.1. Stem cell line karyotypes by G banding analysis.

Cell line/feeder type	karyotype	comment
RH5/iHDFn, late passage	Complex tetraploid	Only 4 cells analysable, varied chromosome counts. Isochromosomal on long arm of 17.
RH5/iMEF late passage (Project bank)	77,XXX, Gain on 1, 3, 6, 10q, 12, 13, 17, 20, 21	Isochromosomal on long arm of 17.
RH5/iMRC5 late passage	Not possible	Increase chromosomal count. Isochromosomal on long arm of 17.
SHEF1/iMEF MCB	Normal, karyotype 46, XY	5 abnormal cell anomalies detected, thought to be artefactual, loss of chr.1, 3, 5, 6, 13, 19, 21.
SHEF1/iMEF DCB	Normal karyotype, 46, XY	8 cells available for analysis, 7 cells displayed model 'normal' karyotype, 1 cell displayed loss of chr.21.
HUES9/iMEF DCB	Normal karyotype, 46, XX	17 cells displayed model 'normal karyotype,' 3 cells displayed translocation 2;18 and break on 21q, 13p, loss of chr.13 and chr.18 (44, XX), assumed to be artefactual anomalies.
HUES9/iMEF (project bank created 3 passages after DCB)	Normal Karyotype, 46, XX	6 cells displayed model 'normal karyotype,' 2 abnormal cells displayed aberrations attributed to harvest artefact: loss of chr.10 and loss of chr.4 and 14.

The following samples did not show any analysable results:

NCL5-iMRC5, NCL5-iHDFn, NCL5-i3T3, NCL5-iMEF

SHEF1-iMRC5, SHEF1-iHDFn, SHEF1-i3T3, SHEF1-iMEF

HUES9-iMRC5, HUES9-iHDFn, HUES9-i3T3

RH5-i3T3

7.4.0 Chromosomal changes detected by aCGH in the SHEF1 stem cell line

Despite sufficient sample preparation being carried out, no results were available for SHEF1 stem cell line cultured on feeders, due to the inability to properly scan the arrays. All the arrays for this cell line had extremely high background. SHEF1 cultured on Matrigel only sustained healthy cultures to passage p+11, which resulted in karyological changes detected on chromosomes 3, 6, 9, 10, 11, 12, 14, 15, 16, 17, by aCGH. Morphological indications from chapter 3 suggest that this stem cell line was not as healthy and viable in comparison to other three stem cell lines, which may explain the large number of detected aberrations. Therefore no further studies were carried out using this stem cell line.

7.4.1 Chromosomal changes detected by aCGH in stem cell line NCL5 cultured on iMRC5 human feeders.

The columns in Table 7.2 display the type of aberration detected (loss or gain), the chromosome number, the exact position of the aberration on the chromosome (number of base pairs from Start to End), the standard deviation, copy number variations (CNVs) linked to the aberration detected, the number of corresponding publications and the number of known genes found within the chromosome region. The results show two gains on chromosome 19 on the q and p arm and a loss of chromosome 20 which spans the length of the whole chromosome, from 20p13 to 20q13.33. All changes detected were associated with greater than 99 genes, indicating their potential significance. These changes are further represented by the ideogram (Figure 7.2), which highlights the difference in sizes of the two small gains on chromosome 19 in comparison to the whole chromosome loss on chromosome 20.

Table 7.2. Chromosomal changes in stem cell line NCL5 cultured on iMRC5 human feeders.

Type	Chr	Start	End	SD	Start Cyto	End Cyto	CNVs	Genes
Gain	19	11,841,463	23,197,810	0.061	19p13.2	19p12	22 Pub. G:128 L:377	>99
Gain	19	42,638,332	61,437,252	0.063	19q13.1 2	19q13.43	29 Pub. G:569 L:1067	>99
Loss	20	35,996	62,463,816	0.093	20p13	20q13.33	28 Pub. G:490 L:1318	>99

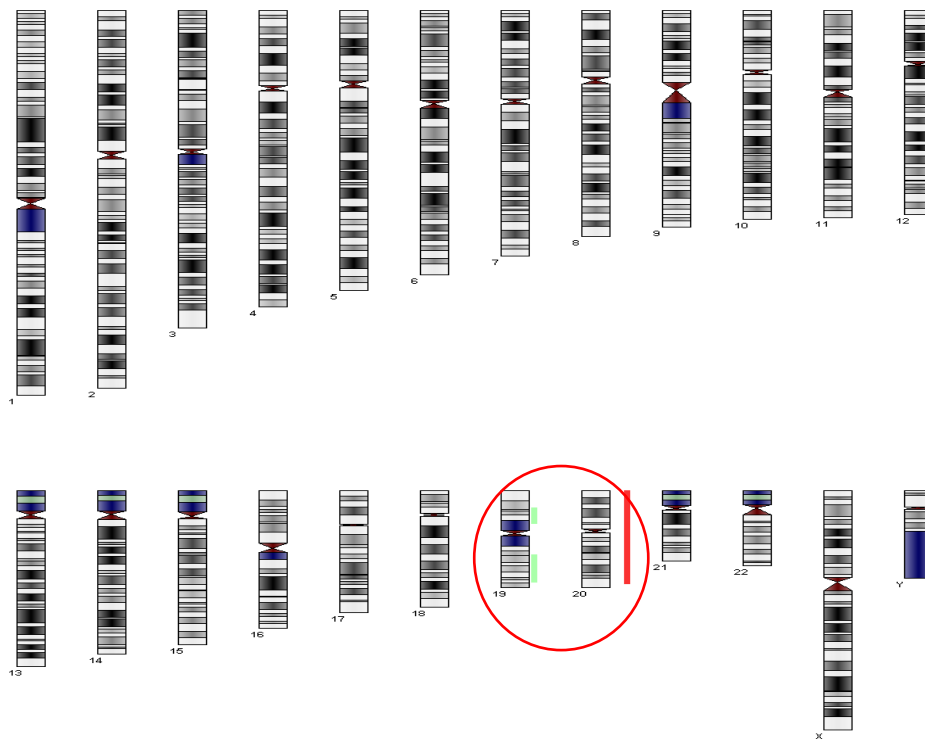


Figure 7.2. Karyotype of chromosomal changes in stem cell line NCL5 cultured on iMRC5 human feeders. NCL5 were cultured on mitotically inactivated human fibroblasts derived from foetal lung iMRC5, for twenty passages using TrypLE™ Express. DNA samples were taken from early and late passage. A whole chromosome loss was detected on chromosome 20 (red vertical line) over twenty passages. This change is denoted by the continuous red line and two small gains (green vertical lines) detected on the p and q arm of chromosome 19. No other chromosomal changes were detected from this sample.

7.4.2 Chromosomal changes detected by aCGH in stem cell line HUES9 cultured on iMRC5 human feeders

Culture of HUES9 on the human feeder iMRC5 resulted in seven changes, including one partial chromosome loss at chr.7 q arm and six whole chromosome gains at chr.12, 13, 16, 17, 19 and 20 as shown in Table 7.3. This is further depicted by the karyotype shown in Figure 7.3 and 7.4 which demonstrates the spread of changes across the chromosomes. The changes in chromosomes 12, 17 and 19 all occur at position p13.3, as shown in the table below. However, the size of the chromosome change differ slightly, with gains on chromosomes 19 and 20 ending on the q arm position 13 and gains on chromosomes 12 and 16 ending on the q arm at position q24.3.

Table 7.3. Chromosomal changes in stem cell line HUES9 cultured on iMRC5 human feeders.

Type	Chr	Start	End	SD	Start Cyto	End Cyto	CNVs	Genes
Loss	7	98,027,648	159,179,376	0.168	7q22.1	7q36.3	30 Pub. G:789 L:2699	>99
Gain	12	2,047,581	133,096,600	0.221	12p13.33	12q24.33	30 Pub. G:1029 L:3973	>99
Gain	13	17,768,610	114,890,800	0.184	13q11	13q34	29 Pub. G:615 L:2467	>99
Gain	16	1,030,472	88,783,720	0.228	16p13.3	16q24.3	31 Pub. G:1816 L:2475	>99
Gain	17	-675,750	79,079,008	0.201	17p13.3	17q25.3	30 Pub. G:1334 L:2503	>99
Gain	19	-156,484	64,003,664	0.207	19p13.3	19q13.43	30 Pub. G:1236 L:2424	>99
Gain	20	-156,484	63,297,896	0.165	20p13	20q13.33	28 Pub. G:490 L:1318	>99

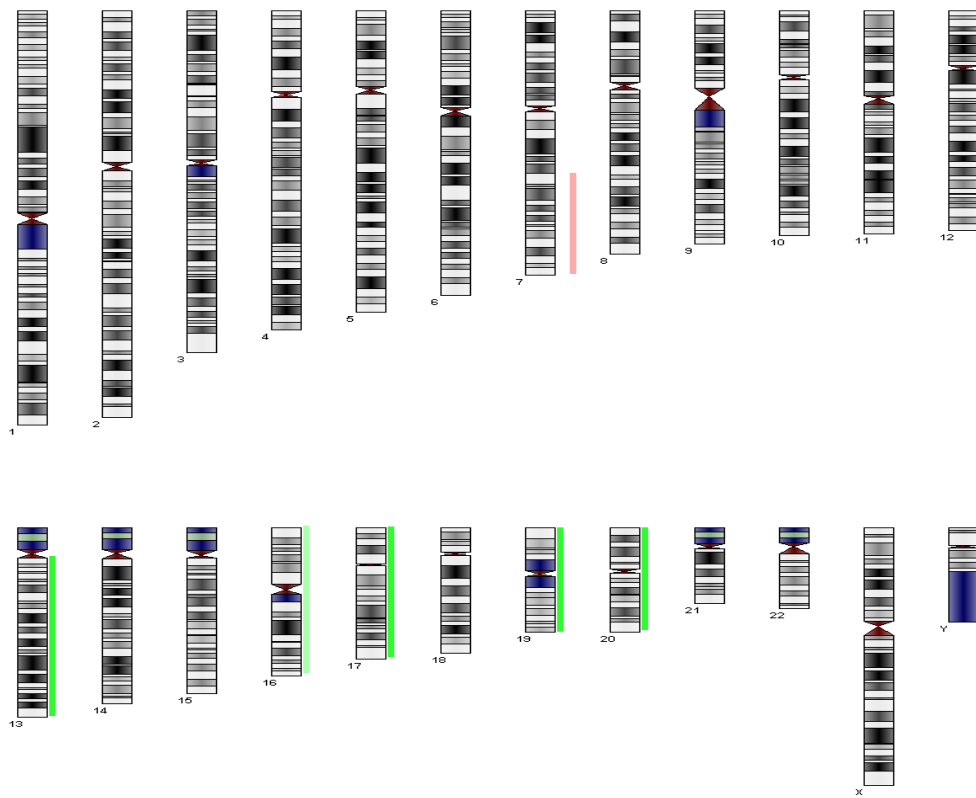
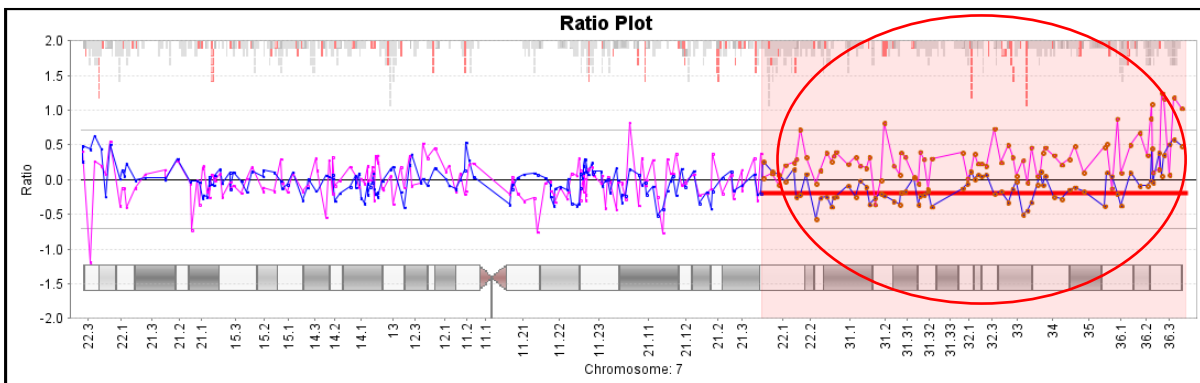


Figure 7.3. Karyotype of chromosome changes detected in stem cell line HUES9 cultured on iMRC5 human feeders. The diagram shows that six whole chromosome gains were detected by aCGH analysis, as shown by the solid green lines. A single, partial loss on chromosome 7 was detected on q arm. This loss was found to span the q arm from 7q22.1 to 7q36.3, as also demonstrated in Table 4.2.

(a)



(b)

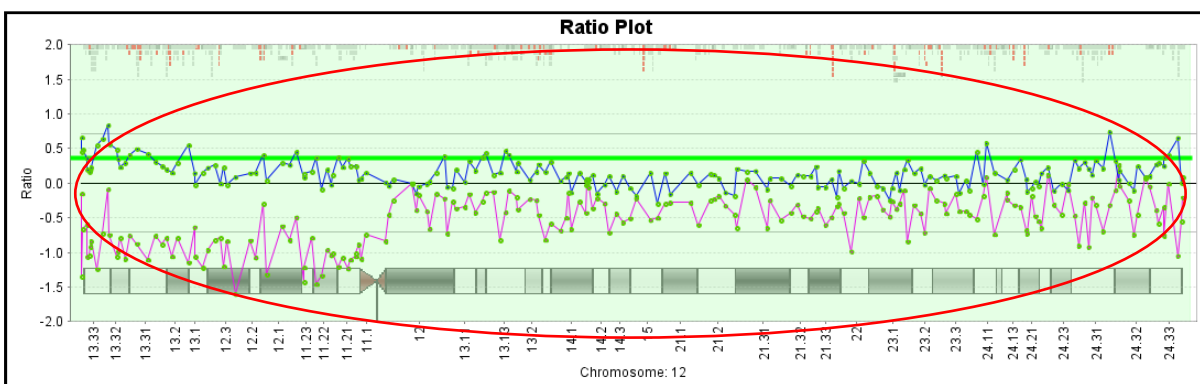


Figure 7.4. Spectral ratio plots for individual chromosomes 7 (a) and 12 (b). These figures highlight the significant differences in the ratio of dye swap (blue and red line), which correlate to the magnitude of change on the individual chromosomes. For normal chromosomes, both lines should be indistinguishable. The closer the lines are to the centre, the more balanced the ratio, the less chromosome change detected. A loss or partial chromosome loss is seen if the red line is detected on the top of the spectral ratio view, as demonstrated by the spectral view result for chromosome 7 from stem cell line HUES9 cultured on iMRC5 (Figure a). The p arm of chromosome 7 is relatively discreet and can be described as balanced, however, the q arm is clearly different, as the red line moves to the top of the ratio line and the blue line is clearly separated. A gain or partial chromosome gain is apparent if the blue line is seen on the top of the spectral ratio view, as demonstrated by spectral views for chromosome 12 (Fig 7.4 (b)), where the red and blue lines are both visibly distinguishable and the red line can also be seen below the ratio line, spanning the entire length of the chromosome. This spectral ratio plot was marked as significant.

7.4.3 Chromosomal changes detected by aCGH in stem cell line RH5 cultured on iMRC5 human feeders

A number of different changes on various chromosomes were detected for RH5 stem cells cultured on iMRC5 feeders. The data shown in Table 7.4 highlights a loss of Chromosome 7, 17, 19 (whole chromosome), and 22 (partial loss) and gains of chromosome 12 (q arm) and 20. An example of a whole chromosomal loss on chromosome 19 is shown in Figure 7.5(a). The thick red continuous line indicates a whole chromosome loss, as determined by the analysis parameters mentioned above (methods, data analysis). The corresponding ratio plot figure (7.5b) further highlights the size of the chromosomal change and the specific points at which the loss occurred; 19.p13.3 to 19q13.43. This loss spans across almost the entire chromosome.

Table 7.4. Chromosomal changes in stem cell line RH5 cultured on iMRC5 human feeders.

Type	Chr	Start	End	SD	Start Cyto	End Cyto	CNVs	Genes
Loss	7	105,512,368	158,860,864	0.188	7q22.2	7q36.3	30 Pub. G:660 L:2388	>99
Gain	12	56,970,240	100,205,776	0.092	12q14.1	12q23.2	27 Pub. G:140 L:742	>99
Loss	17	32,235,100	46,968,912	0.176	17q12	17q21.33	26 Pub. G:524 L:738	>99
Loss	19	100,156	63,490,380	0.192	19p13.3	19q13.43	30 Pub. G:1236 L:2400	>99
Gain	20	5,168,808	22,556,208	0.075	20p12.3	20p11.21	26 Pub. G:168 L:303	62
Loss	22	35,381,776	49,322,644	0.18	22q12.3	22q13.33	23 Pub. G:158 L:471	>99

(a)

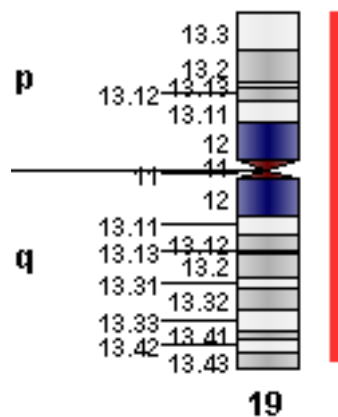


Figure 7.5 (a). Diagram showing chromosome 19 in stem cell line RH5 cultured on iMRC5 human feeders. This diagram was obtained from one click software for CNV changes and its corresponding ratio plot was prepared, marked as 'significant' and selected by the same software (b). The thick continuous red line denotes a significant change and shows the loss of a whole chromosome.

(b)

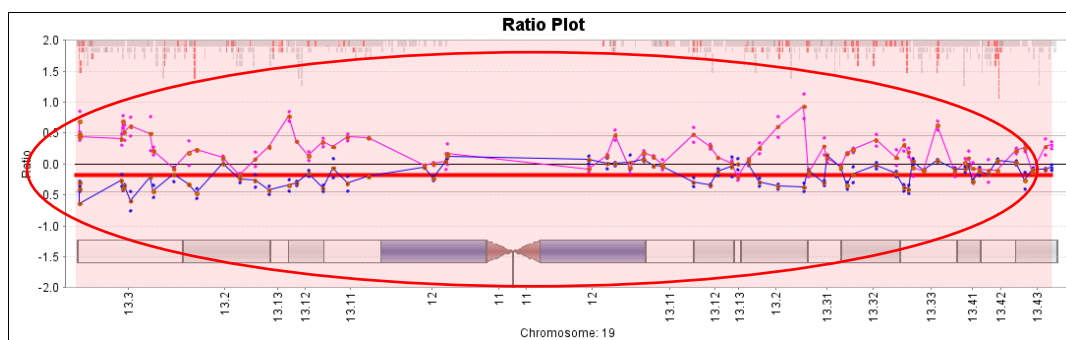


Figure 7.5 (b). Spectral ratio plot of Chromosome 19 in stem cell line RH5 cultured on iMRC5 human feeders. For a normal chromosome, the red and blue lines are normally indistinguishable. The lines demonstrate the differences in the ratio of dye swap (blue and red line), which correlate to the magnitude of change on the individual chromosomes. The closer the lines are to the centre, the more balanced the ratio, therefore the less chromosome change detected. From the spectral ratio plot Chromosome 19 has undergone a significant loss, as both red and blue corresponding dye lines are clearly distinguishable from the ratio line, with the red line predominately above the blue line.

7.4.4 Chromosomal changes detected by aCGH for Stem cell line NCL5 cultured on iMEF mouse feeders

The results in Table 7.5 shows 11 chromosome changes detected in NCL5 cultured on iMEF over 20 passages. These included chromosome gains on chr.3, 16, 17, 19, 22 and losses on chr.4 (partial), 5, 10, 12 (partial, q arm),13 and 20. Correlation of sizes and position of changes detected vary, as further demonstrated by Figure 7.6, with very small deletions (less than 15MB) on chromosomes 4, 5, 10 (all associated with less than 40 genes which suggests these changes were less significant), and a small gain on chromosome 3 (9MB) associated with greater than 99 genes, indicating its potential significance. Furthermore, losses on chromosomes 5 and 12 start at the same position on the q arm, q14.1. Gains on chromosome 19 and 22 both end on the q arm 13.3 and 13.4.

Table 7.5. Chromosomal changes detected by aCGH for stem cell line NCL5 cultured on iMEF mouse feeders

Reg.	Type	Chr	Start	End	SD	Incl.	Start Cyto	End Cyto	CNVs	Genes
1	Gain	3	46,693,784	55,311,864	0.111	100%	3p21.31	3p14.3	20 Pub. G:45 L:496	>99
2	Loss	4	43,043,788	55,502,968	0.115	100%	4p13	4q12	23 Pub. G:75 L:161	38
3	Loss	5	91,099,200	106,325,872	0.095	100%	5q14.3	5q21.3	25 Pub. G:119 L:473	33
4	Loss	10	105,458,376	115,223,536	0.078	100%	10q24.33	10q25.3	19 Pub. G:185 L:164	27
5	Loss	12	58,826,980	99,277,400	0.087	100%	12q14.1	12q23.1	27 Pub. G:126 L:720	>99
6	Loss	13	52,554,504	112,608,792	0.115	100%	13q21.1	13q34	29 Pub. G:252 L:1507	96
7	Gain	16	55,812,812	74,910,712	0.157	100%	16q13	16q23.1	23 Pub. G:320 L:323	>99
8	Gain	17	32,875,702	79,719,616	0.178	100%	17q12	17q25.3	29 Pub. G:786 L:1496	>99
9	Gain	19	-413,124	64,709,420	0.211	100%	19p13.3	19q13.43	30 Pub. G:1236 L:2424	>99
10	Loss	20	10,879,061	24,994,294	0.091	100%	20p12.2	20p11.21	25 Pub. G:158 L:274	63
11	Gain	22	15,623,854	49,723,820	0.154	100%	22q11.1	22q13.33	29 Pub. G:1042 L:1137	>99

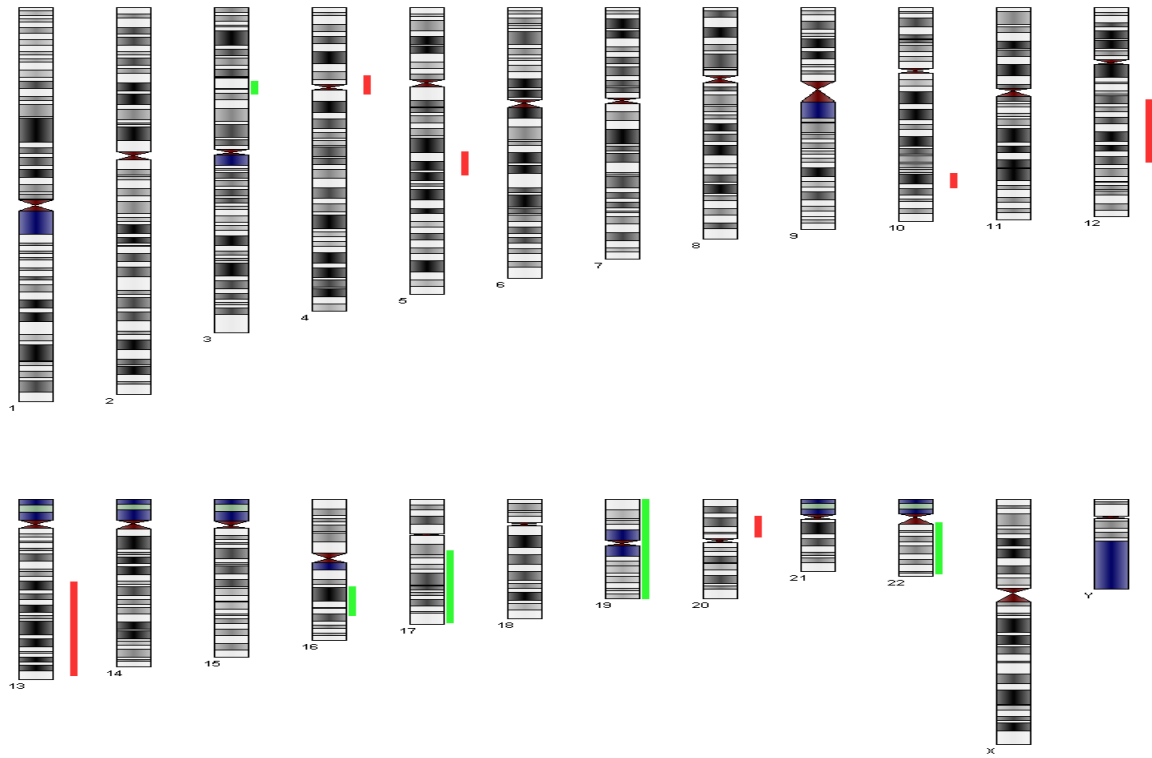


Figure 7.6. Karyotype of chromosomes for stem cell line NCL5 cultured on iMEF mouse feeders.

The diagram displays the distribution of changes across the chromosomes. Mainly losses (red bars) were seen across chromosomes 1-13 and gains (green bars) across chromosomes 16-22. Indications by size and visual display of position by ideogram may suggest a number of small balanced translocations (an even exchange of chromosomal material without the loss/gain of genetic information).

7.4.5 Chromosomal changes detected by aCGH in Stem cell line HUES9 cultured on iMEF mouse feeders

Table 7.6 shows a partial loss of the q arm of chromosome 7, a partial gain of p arm on chromosome 12 and whole chromosome gains seen on chromosomes 14, 16, and 17 in HUES9 cells cultured on MEF mouse feeders over 20 passages. These changes are also reflected in the ideograms and spectral ratio plots in Figures 7.7 and 7.8 which demonstrate specifically the magnitude of the changes detected in chromosomes 7 and 12. All changes detected were associated with greater than 99 genes, suggesting that they were significant.

Table 7.6. Chromosomal changes in stem cell line HUES9 cultured on iMEF mouse feeders.

Reg.	Type	Chr	Start	End	SD	Incl.	Start Cyto	End Cyto	CNVs	Genes
1	Loss	7	83,058,216	159,497,872	0.224	100%	7q21.11	7q36.3	30 Pub. G:849 L:3119	>99
2	Gain	14	19,848,820	106,576,224	0.098	100%	14q11.2	14q32.33	27 Pub. G:843 L:1921	>99
3	Gain	16	- 1,390,810	89,594,480	0.113	100%	16p13.3	16q24.3	31 Pub. G:1818 L:2475	>99
4	Gain	17	- 1,308,105	78,935,112	0.138	100%	17p13.3	17q25.3	30 Pub. G:1334 L:2503	>99

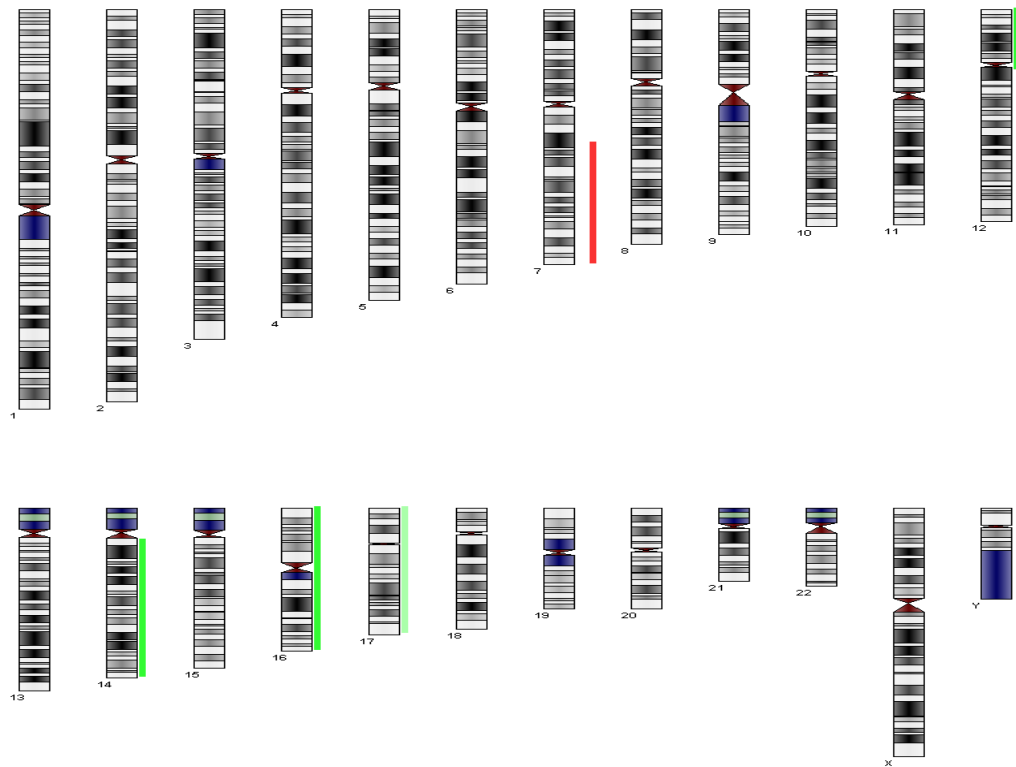
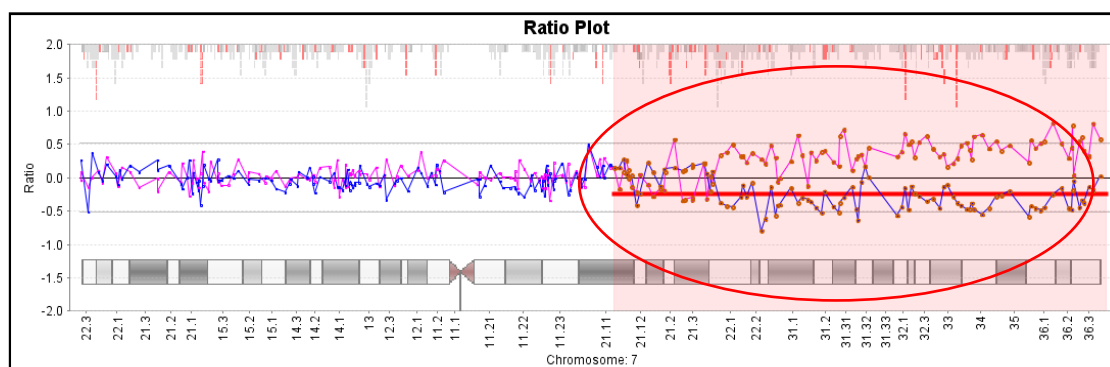


Figure 7.7. Karyotype of chromosomes for stem cell line HUES9 cultured on iMEF mouse feeders.

A single, partial chromosome loss was detected on chromosome 7q, with three chromosome gains found on 12p, 14q and a whole chromosome gain on 16.

(a)



(b)

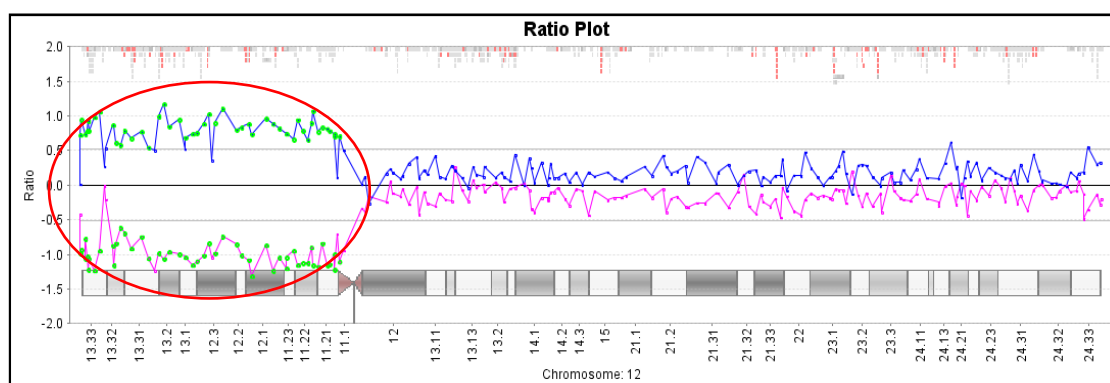


Figure 7.8. Spectral ratio plots showing chromosome 7 (a) and 12 (b) of stem cell line HUES9 cultured on iMEF mouse feeders.

The detection of a large loss on Chr.7 q arm is shown in figure (a), with the remaining spectra of the p arm appearing indistinguishable. A significant gain on Chr.12 p arm can also be seen. The spectra on the remaining q arm appears to be separating, however it is not enough to suggest a full gain of the chromosome, but may be indicative of further changes that may be occurring, particularly when comparing both part of normal chromosome spectra for chromosomes 7 and 12.

7.4.6 Chromosomal changes detected by aCGH in stem cell line RH5 cultured on iMEF mouse feeders

Table 4.7 shows the aCGH results obtained for RH5 cultured on mouse feeder iMEF. Eight aberrations were detected, and mainly consist of losses spanning from the p to q arm, correlating with greater than 99 genes. Losses detected on chromosomes 5, 10 and 11 show similarities, as all were found to start at p15.3. Figure 7.9 (a) demonstrates a gain on chromosome 12 which has been previously described in stem cell lines. Although this change was relatively small, it may indicate a culture advantage over other stem cell lines. It also demonstrates the sensitivity of the technique to detect such slight changes.

Table 7.7. Chromosomal changes in stem cell line RH5 cultured on iMEF mouse feeders.

Type	Chr	Start	End	SD	Start Cyto	End Cyto	CNVs	Genes
Gain	1	61,430	245,483,616	0.125	1p36.33	1q44	31 Pub. G:2424 L:5996	>99
Loss	5	514,438	181,737,280	0.105	5p15.33	5q35.3	31 Pub. G:1909 L:5767	>99
Loss	7	245,716	158,422,496	0.082	7p22.3	7q36.3	31 Pub. G:1628 L:5325	>99
Loss	10	-317,642	135,449,904	0.081	10p15.3	10q26.3	30 Pub. G:1161 L:4049	>99
Loss	11	-1,187,003	134,450,272	0.09	11p15.5	11q25	32 Pub. G:1098 L:4455	>99
Gain	12	67,014,816	90,372,616	0.056	12q15	12q21.33	24 Pub. G:90 L:463	75
Loss	14	19,076,912	106,598,672	0.105	14q11.1	14q32.33	27 Pub. G:1028 L:2005	>99
Loss	20	431,346	62,996,168	0.074	20p13	20q13.33	28 Pub. G:489 L:1306	>99

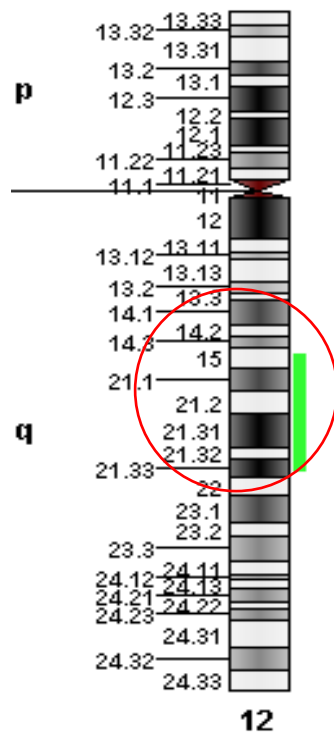


Figure 7.9. Diagram showing chromosome 12 for stem cell line RH5 cultured on iMEF mouse feeders. The green line indicates a gain on the q arm. Although this change is relatively small, it is significant to long term culture of stem cell and associated with clonal events.

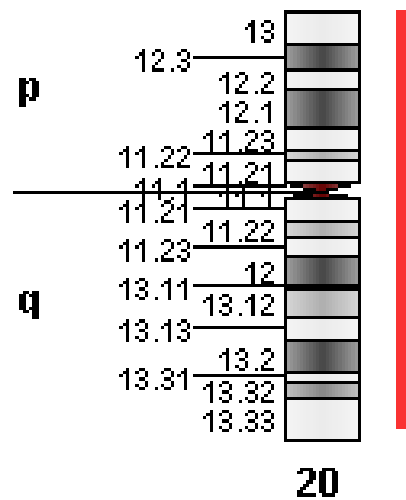
7.4.7 Chromosomal changes detected by aCGH in stem cell line RH5 cultured on mouse feeder line i3T3.

Table 7.8 shows the results obtained from RH5 cultured on i3T3 mouse feeders. Culture on this mouse feeder type resulted in the least amount of chromosomal changes from this stem cell line when compared to the changes observed from cultures on iMEF mouse feeders. A re-occurring loss of chromosome 10 and 20, and gains on chromosomes 1 and 22 was seen. Table 7.8 also shows the size and position of the loss detected on chromosome 20, which at first, appears to be the same as the size and position of the gain on chromosome 22. However when checked against the actual start and end, the size in base pairs are different. Figure 7.10b shows the specific loss on chromosome 20, also demonstrating that RH5 had generally, noisy spectra for all its chromosomes, which could cause difficulties when analysing small changes from the results. All changes were associated with greater than 99 genes, suggesting they could be significant.

Table 7.8. Chromosomal changes for RH5 cultured on mouse feeder line i3T3.

Type	Chr	Start	End	SD	Start Cyto	End Cyto	CNVs	Genes
Gain	1	-2,586,066	251,339,456	0.085	1p36.33	1q44	31 Pub. G:2549 L:6138	>99
Loss	10	-602,043	136,788,240	0.084	10p15.3	10q26.3	30 Pub. G:1161 L:4049	>99
Loss	20	-348,964	62,784,620	0.082	20p13	20q13.33	28 Pub. G:490 L:1318	>99
Gain	22	-172,453	50,425,876	0.085	22p13	22q13.33	30 Pub. G:1151 L:1180	>99

(a)



(b)

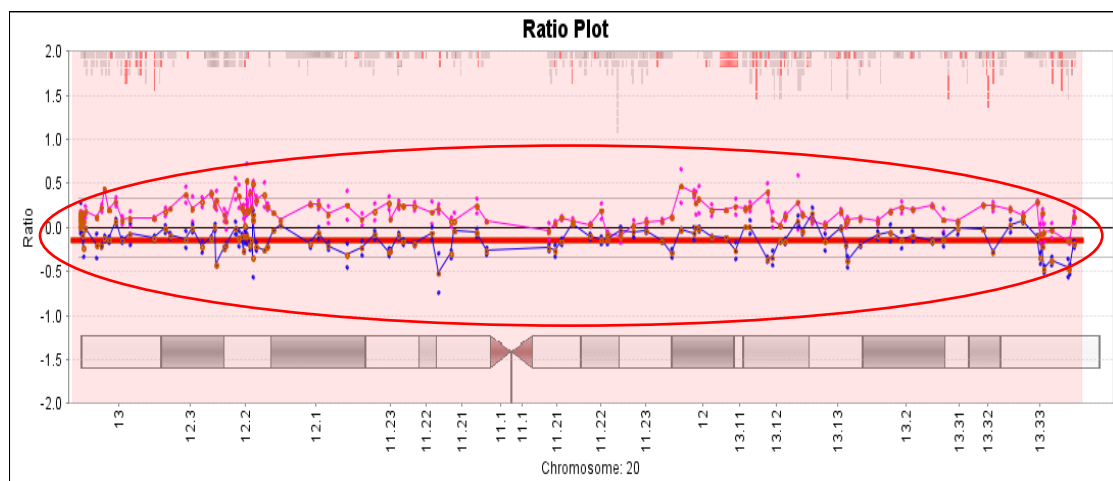


Figure 7.10. (a) and (b). Diagram and ratio plot for chromosome 20 from RH5 cultured on mouse feeder line i3T3. These cultures incurred a loss on chromosome 20, which spans the whole chromosome length. This spectra would be described as quite noisy, as the blue and red dye lines appear to come together at various positions across the chromosome e.g. p12.1 and q13.1, but with an obvious aberration. Slipping of the dye lines at the end of the chromosome may be indicative of telomere degradation.

7.4.8 Chromosomal changes detected by aCGH in stem cell line NCL5 cultured on i3T3 mouse feeders

The results from Table 7.9 show six chromosome changes consisting of a loss of the majority of chromosome 12, a whole chromosome gain on 20 and a loss of most of the q arm of chromosome 22 (2 aberrations).

7.4.9 Chromosomal changes detected by aCGH in stem cell line HUES9 cultured on i3T3 mouse feeders

Chromosome changes were seen from HUES9 cultured on i3T3 on chromosome 14 as a whole gain, a whole loss of chromosome 19 and a whole gain on chromosome 20, as shown in Table 7.9.

7.4.10 Chromosomal changes detected by aCGH in hESCs as a result of prolonged culture on i3T3 mouse feeders

A summary of all the results obtained from stem cell culture on mouse feeders i3T3s is shown in Table 7.8. Two similarities in chromosomal aberrations were found from stem cell lines NCL5, HUES9 and RH5 cultured on mouse feeders i3T3s for 20 passages; a gain on chr.20 shared by all three stem cell lines, and a loss on chr.22 shared by NCL5 and RH5. HUES9 had the least number of changes consisting of 2 gains and 1 loss. Both NCL5 and RH5 resulted in 4 aberrations, with mostly losses seen from NCL5. Both losses and gains were detected from RH5. Detailed results for Stem cell lines RH5 cultured on i3T3s are shown in Table 7.9, Figures 7.10 (a and b).

Table 7.9. aCGH results showing chromosomal changes for hESC lines NCL5, HUES9 and RH5 cultured on i3T3 mouse feeders

Red indicated a loss in chromosome and green represents a gain in chromosome.

Stem cell line	chromosome	Type of change (gain/loss)
NCL5	12, 20, 21, 22	Losses
HUES9	14, 19, 20	Gains
RH5	1, 10, 20, 22	Both

7.5.0 Chromosomal changes detected by aCGH in hESCs as a result of culture on iHDFn human feeders

A considerable number of changes were detected in the stem cell lines cultured on the iHDFn human feeders as shown by summary Table 7.10. All three stem cell lines RH5, NCL5 and HUES9 demonstrated a change on chromosome 12 which in the RH5 and HUES9 lines was detected as a gain, and in NCL5, as a loss. Other similarities in chromosomal aberrations include the changes on chr.7 and 17 shared by HUES9 and RH5, and a loss on chr.18 shared by NCL5 and RH5. The number and type of aberration detected from each stem cell line was also interesting. Both NCL5 and HUES9 cultures had 5 aberrations; RH5 resulted in mostly losses whereas mostly gains were detected from HUES9 cultures (4 gains, 1 loss). RH5 had the greatest number of changes (8) in comparison to the other stem cell lines. Interestingly changes from NCL5 cultured on iHDFn resulted in all (small) losses. Detailed results shown in Table 7.10 and Figure 7.11 further depict these very small losses in stem cell line NCL5.

7.5.1 Chromosomal changes detected by aCGH in stem cell line NCL5 cultured on iHDFn human feeders.

The results in Table 7.11 shows five chromosome losses consisting of a small loss on the q arm of chr.12, a small loss on q arm of chromosome 13, a (partial) loss of q arm of chr.16 and two small losses on chromosome 18 and 21. All aberrations were detected on the q arm of each chromosome. Three out of the five changes occurred at position q21. All the losses linked with 64 genes or less and were less than 21MB. Losses on chromosomes 13, 18, and 21 were linked with less than 10 genes, suggesting that these changes may not have been significant.

7.5.2 Chromosomal changes detected by aCGH in stem cell line RH5 and HUES9 cultured on iHDFn human feeders.

RH5 cultured on iHDFn human feeders incurred 8 chromosome changes in total over 20 passages, consisting of a number of whole losses and gains including gain on chr.6 and chr.12 and loss of Chr.7, 11, 14, 17 and 18.

HUES9 cultured on human feeders iHDFn over 20 passages revealed a loss of the q arm on chromosome 7 and small gains on chromosomes 12, 19, 22. A large gain on chromosome 17 was also seen (Figure 7.12).

Table 7.10. aCGH results showing chromosomal changes for hESC lines NCL5, HUES9 and RH5 cultured on iHDFn human feeders

Red indicated a loss in chromosome and green represents a gain in chromosome.

Stem cell line	Chromosome	Type of change (gain/loss)
NCL5	12, 13, 16, 18, 21	Losses.
HUES9	7,12,17,19,22	Mostly gains
RH5	6,7,10,11,12,14,17,18	Mostly losses

Table 7.11. Chromosomal changes in stem cell line NCL5 cultured on iHDFn human feeders.

Reg.	Type	Chr	Start	End	SD	Incl.	Start Cyto	End Cyto	CNVs	Genes
1	Loss	12	82,447,768	88,720,904	0.17	100%	12q21.31	12q21.33	16 Pub. G:21 L:215	16
2	Loss	13	61,608,488	73,527,664	0.187	100%	13q21.31	13q22.1	26 Pub. G:95 L:627	9
3	Loss	16	52,749,944	59,236,024	0.221	100%	16q12.2	16q21	17 Pub. G:126 L:116	64
4	Loss	18	20,334,480	23,550,496	0.494	100%	18q11.2	18q12.1	6 Pub. L:6	7
5	Loss	21	15,489,745	21,194,192	0.277	100%	21q21.1	21q21.1	22 Pub. G:95 L:129	7

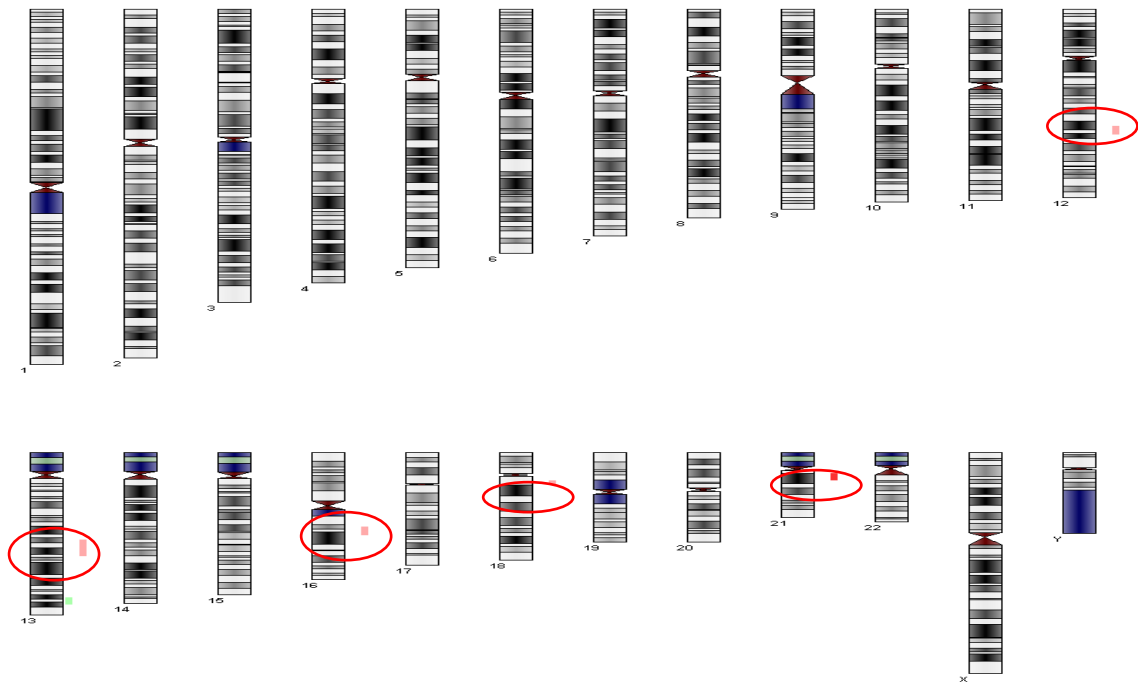


Figure 7.11. Karyotype showing chromosomes for NCL5 cultured on human feeders iHDFn.

The aberrations detected in NCL5 cultured on iHDFn were all very small losses (red bars), as reflected in Table 7.10. Also, the majority of the losses occurred between chromosomes 12 to 21. This result demonstrates the sensitivity of aCGH as a technique, as it is able to detect very small chromosomal aberrations; the largest change detected was 14KB and the smallest was 3KB. None of the changes detected were marked as significant.

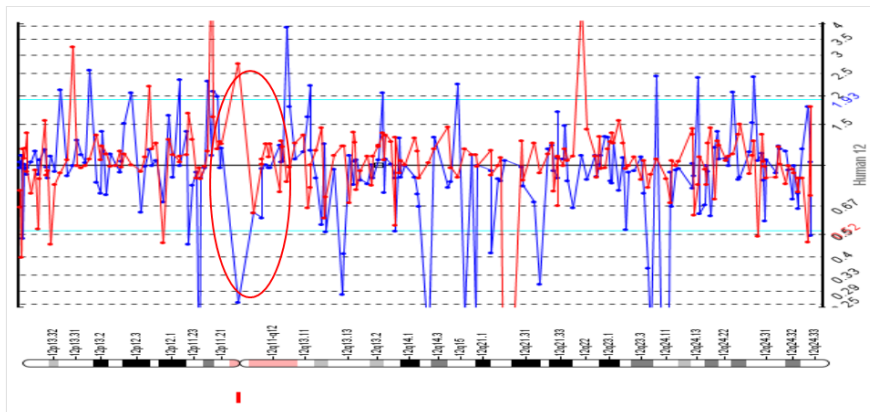
4.6.0 Chromosomal changes by aCGH in hESCs as a result of prolonged culture on Matrigel

Similarities and differences in chromosome changes were seen from stem cell lines NCL5, HUES9 and RH5 cultured on Matrigel over 20 passages. Aberrations on chromosome 4 were shared by NCL5 (gain) and HUES9 (loss). Changes on chromosome 6 were also shared by HUES9 (loss) and RH5 (gain). A small loss on chromosome 12, spanning from the p-arm to the q-arm was also shared by HUES9 and RH5 (Figure 7.12). NCL5 and HUES9 displayed the least amount of changes (4 aberrations detected) in comparison with RH5 cultured on Matrigel. However, changes from NCL5 were all partial or small changes, consisting of both losses and gains, whereas aberrations from HUES9 were mostly whole chromosome losses. RH5 also consisted of both losses and gains (7 aberrations), including loss of chromosome 17q arm, as detected by G banding analysis. These results show that NCL5 was more stable on Matrigel in comparison to the other two stem cell lines. A summary of these changes are displayed in summary Table 7.12.

Table 7.12. aCGH results showing chromosomal changes for hESC lines NCL5, HUES9 and RH5 cultured on Matrigel.

Stem cell line	chromosome	Type of change (gain/loss)
NCL5	1q23.2-24.3 4q14.1-15.1 9q23.23.1 18p24.3-22.1	Both, all partial, mostly of q arm
HUES9	4q22.1-22.3 6p12.1-q11.4 11q24.22-24.32 12p11.21-q11.12	Mostly losses
RH5	2p31.1-p46.9 5p19.6-p28.1 6p25.7-p29.9 7p22.9-31.1 9p23.1-21.1 12p11.21-q11.12 17q20.7-24.2	Both

(a)



(b)

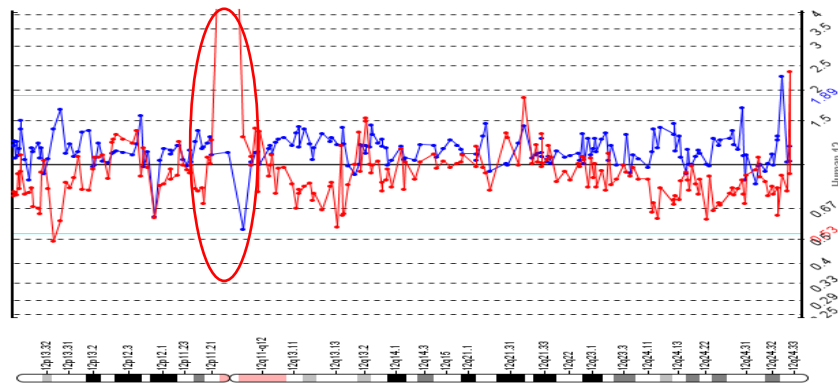


Figure 7.12. Spectral ratio plot for chromosome 12 from HUES9 (a) and RH5 (b) cultured on Matrigel. These cultures incurred a loss on chromosome 12, p arm between p11.21 and q11.12, which spans across the centromere of the chromosome. This spectrum would be described as noisy, as the blue and red dye lines appear to come together at various positions across the chromosome. This type of noise was also visible on spectra from other RH5 chromosome ratio plots.

7.7. Overall summary of aCGH results

- From the results obtained thus far, the numbers of changes detected were different for each stem cell line. The results concur that the stem cell lines with the largest number of changes were RH5 and HUES9.
- Chromosome changes detected that were common to RH5 on both mouse and human feeders included 7, 10, 12 and 20. Changes detected that were common to RH5 on mouse feeders included chr.1, 10, 12 and for RH5 on human feeders; 7, 12, and 17.
- Chromosome changes detected that were common to HUES9 cultured on both mouse and human feeders were 7, 12, 17, 19 and 20. Of these changes, 3 were common to RH5 cultured on human feeders, 7, 12 and 17. No direct correlations of changes were seen on mouse feeders.
- NCL5 had the least number of changes overall, compared with the other stem cell lines, however a high number of changes were seen from NCL5 cultured on MEF (11 changes) though many of these changes were small. Chromosome changes detected that were common to NCL5 cultured on both mouse and human feeders included chr.12, 19 and 20. No changes were common to NCL5 cultured on human feeders, and three changes were common to culture on mouse feeders; chr.12, 20 and 22. Only an aberration on chr.18 was common to NCL5 cultured on HDFN and Matrigel.
- Chromosomal changes that were common to all three stem cell lines cultured on both feeder types were chr.12 and 20. A loss on chromosome 7 was common to HUES9 and RH5. Chromosomal changes consistent with culture on Matrigel across the three stem cell lines demonstrated losses and gains on chr.4 (partial, q arm), 6 and loss on chr.9 and 12.

7.5 Discussion

Monitoring the karyotypic stability of human embryonic stem cell lines is of paramount importance to the quality assurance of stem cell lines, so their true potential in regenerative medicine can be realised (Josephson, 2007). The major concern for the use of hESCs in cell replacement therapies is safety, as there is a possibility that the cells may become tumorigenic (Fox, 2008). Therefore, as tumorigenic cells are associated with genomic alternations, it is extremely important that the chromosomal stability of hESCs is monitored during their culture, and detected appropriately. The UKSCB is working towards ensuring that the environment i.e. media and conditions used to culture, as well as methods used to propagate and expand a line are well controlled and documented. More importantly, it is also aiming to routinely determine the effects of culture conditions on chromosomal stability and has therefore embarked on researching methods to better characterise karyotypes of stem cell lines alongside the routine release criteria testing it carries out.

Karyology testing has previously been performed using traditional metaphase spread preparations and the method has been adopted from other cell types for G banding analysis in a clinical setting. However, considerable inconsistencies were found when applied to stem cell lines cultured in-house. G banding is carried out using 20 to 30 chromosome spreads, yet final banding analysis is sometimes based on as little as 3 to 5 cells which can be biased and is not representative of whole populations of cells.

Despite successful preparation of stained metaphase spreads from a number of project samples which were confirmed by a Biologist in-house, many of the metaphase spread preparation were not successfully analysable for G banding. This may have been due to incorrect processing. Most of the cell types processed at TDL are from routine clinical blood and tissue samples. Preparing metaphase spreads from hESCs requires subtle but important differences in processing methods compared to other cell types, as hESCs are more fragile (Personal communication

with cytogeneticist from Cell Line Genetics, USA). Those results which were successfully analysed by outsourced G banding analysis did give interesting results. Many of the smaller aberrations reported from the stem cells lines used in this project were considered artefacts. It is known that smaller cell aberrations can be detected by a Clinical cytogeneticist when performing G banding analysis from human blood samples, but not always reported (Barber et al., 2005), as they are such a frequent occurrence within the human population and are not considered significant (Personal communication with D Baker, Cytogeneticist, Sheffield). It is questionable whether such small aberrations may affect a small biological system such as a stem cell population, in contrast to a human. Also, it raises questions as to how to determine cut-offs for reporting small changes. There are also concerns about the sensitivity and robustness of the technique which favoured the investigation and development of alternative methods.

For this purpose, the UKSCB has assessed the suitability of aCGH which was a focus of this thesis. This method is reportedly more sensitive and provides more reliable information changes in whole cell populations and requires relatively small amounts of DNA. aCGH has already been employed in other clinical settings (Schaeffer et al., 2004; Shinawi & Cheung, 2008; Shaffer et al., 2007) and its sensitivity was much greater compared with G banding. aCGH also provides more reliable information with regards to whole population changes of stem cells in culture, even though it requires relatively small amounts of DNA.

The results show that RH5 had the highest number of detected changes over 20 passages. The observation that some stem cell lines have a tendency to acquire more karyotypic changes over time than others is consistent with other groups (Maitra et al., 2005). NCL5 exhibited the least amount of changes when comparing both human, mouse feeders and Matrigel cultures, although NCL5 cultured on iMEFs mouse feeders and iHDFn human feeders both demonstrated more than 9 changes, most of these were very small aberrations. These changes were therefore not marked as significant, as they were not linked with more than 50 genes. This method of reporting was recommended by Perkin Elmer (Personal communication).

Chromosomal changes common to all three stem cell lines cultured on feeders were found on Chr.12 and 20. Both these changes have already been reported in other

stem cell lines and claimed to be associated with long term passage (Draper et al., 2004; Lefort et al., 2008). Changes on chromosome 20 have been linked to BCL2L1, a gene thought to drive culture adaptation in ES cells (Amps et al., 2011). Furthermore, gain of chromosome 20q, as shown in these studies, has also been noted in yolk sac carcinoma and germ cell tumors which contain EC cells (Baker et al., 2007). A few interesting correlations were found as result of culture on mouse and human feeders overall, particularly between RH5 and HUES9. Changes on chromosomes 7, 12, 17 and 20 were detected from both mouse and human feeders.

Other groups have researched into the selective advantage of changes specific to chromosome 12 populations in hESCs (Draper et al., 2004; Mayshar et al., 2010) specifically trisomy 12, which has been shown to give hESCs a clonal advantage (Amit et al., 2000) (Gertow et al, 2007) and found that once transplanted *in vivo*, these cells were negatively selected. By nature, hESCs have poor clonal efficiency (>1%), therefore selective pressures give rise to increased clonal efficiency through changes in karyotype (e.g. trisomy 12) over long term passaging (Ludwig et al., 2006). Furthermore, injection of these cells lead to the development of early renal precursors, however, no conclusions were made as to why this occurred, despite the lack of trisomy 12 stem cells found *in vivo*. Also, Mayshar et al (2010) demonstrated that a human iPSC line with aneuploidies had elevated levels of Nanog, a transcription factor and regulator of pluripotency (Chambers et al., 2003). This may infer a growth advantage over other cells *in vitro*, if expressed phenotypically as high levels of Nanog have been associated with repression of specific differentiation towards neuronal crest and Neuroectoderm (Wang et al., 2013). More recently Amps et al (2011) demonstrated the tendency of an ethnically diverse group of hESCs (125 lines) to acquire changes due to prolonged culture, particularly in chr.1, 12 17 and 20 confirmed by SNP arrays. Such large studies do confer the effects of long term passaging of hESCs on chromosomal stability.

Overall, changes were detected on both feeder types and appeared to be more inherently correlated with the individual lines themselves. Stem cell line NCL5 had the least amount of changes when cultured on iMRC5 and iHDFn human feeders and Matrigel. HUES9 exhibited mostly a gain in chromosomal material overall, and had the least amount of changes when cultured on iMEF, i3T3 mouse feeders and Matrigel. RH5 exhibited both losses and gains and also had the least number of

aberrations when cultured on i3T3 mouse feeders. Subsequently the same number and similar changes were detected from RH5 cultured on iMRC5 and Matrigel (chr.20 and 22). iHDFn human feeders and iMEF mouse feeders both gave rise to hESC cultures with a greater number of chromosomal changes, whereas cultures on iMRC5 human feeders and i3T3 mouse feeders both had the least. A study by Eiselleova et al (2008) suggested that the ability of a feeder layer to promote the undifferentiated growth of hESCs is attributed to its characteristic growth factor production. Although this was interesting data, there was no information on the effect of chromosomal stability with relation to culture on feeder types. Furthermore, as with most literature confirming hESC stability, techniques such as G banding and FISH have been used, which give overall status of a hESC line based on the chromosomal status of a small number of cells, or only examine a particular set of chromosomes (Richards et al., 2003; Ellerstrom et al., 2007; Eiselleova et al., 2008). Comparison studies to demonstrate the undifferentiated growth of hESCs by mouse and human feeders Ellerstrom et al (2007) concluded that human feeders supported hESC growth better than mouse feeders but confirmed that hESCs cultured for 20 passages still remain normal with diploid karyotype. However, analysis was carried out using FISH and was specific for chromosomes 12, 13, 17, 18, 20, 21, x and y. This does not rule out changes that may have occurred due to low resolution of the technique, or discrepancies in the way results were reported for hESC lines, as shown by the G banding results obtained in these studies.

There is very little literature that robustly demonstrates the contribution of different culture on feeder types to the chromosomal stability of hESCs. These results conclude that there was no significant difference between hESCs cultured on mouse and human feeders. Although these studies cannot offer an explanation for many of the smaller aberrations detected, they do provide a reference data set for further work. Studies have demonstrated that karyotypic abnormality can affect a stem cell lines ability to differentiate (Fazeli et al., 2010) This has also been demonstrated in neural derivatives of hESCs which carried a chromosome 1q deletion and therefore could not integrate and expand when engrafted in rat models (Varela et al., 2012).

It is well known that a number of chromosomal aberrations have a known effect on humans when present in a larger proportion of CNVs. For example a loss at chromosome 17p11.2 is associated with Smith-Magenis syndrome (90% of all

detections). Clinical presentations include brachycephaly, midface hypoplasia, speech delay with or without hearing loss, psychomotor and growth retardation, and behavioural problems. Similarly, Trisomy on chromosome 21 is associated with Down's syndrome and detection of trisomy on chromosome 13 is linked with loss of life within the first month. Of the infants which survive, approximately 80% will develop congenital heart disease. Another chromosomal change often associated with disease is the loss at chromosome 13q12-13q21 which results in a loss of a gene called Rb, linked to tumor formation, Retinoblastoma.

Several chromosomal changes were detected by aCGH in a number of the cell samples analysed in these studies, and included a loss of chromosome 13, 21 and 17p. These changes may have a significant impact on the use of abnormal stem cell lines for future therapeutic applications, particularly those changes which are linked with low copy number variations, as a small alteration in chromosomal stability can have detrimental effects in a human. Thus application for clinical trials using cell lines should undergo stringent scrutiny for lines harbouring such changes and it should be reliably demonstrated that these changes are not present or any that exist would not have a profound effect on the animal/human subject (Weissbein et al., 2014 ;Ben-David and Benvenisty., 2011; Goldring et al., 2011).

Furthermore, it has been documented that many aberrations occur in humans, without phenotypic effects (Barber, 2005; Kowalczyk et al., 2007). Therefore it is important to be aware of the fact that chromosomal changes can occur and that a cytogeneticist in a clinical setting would not report these as they are known to be asymptomatic. It is also thought that such changes would not have severe consequences with regards to stem cell cultures; therefore if the same aberrations are detected they should not be reported (personal communication, D.Baker, Cytogeneticist). Guidance from cytogeneticists would surely aid in making decisions for how best to report aberrations detected by aCGH and for deciding the ultimate use of hESCs in a therapeutic setting.

Limitations to aCGH include the inability to detect changes such as balanced chromosome translocations. Although a number of samples analysed may demonstrate the presence of balanced translocations (Figure 7.6), aCGH would need to be coupled with other techniques such as single nuclear polymorphisms

(SNPs) or fluorescent in situ hybridisation (FISH) to confirm specific changes, particularly in long term cultures. aCGH also requires an experienced person to set up and run assays over 3 days which is very laborious. Both results and analysis can be operator variable due to complex preparation and it is an expensive technique to set up and maintain.

To more accurately conclude the results discussed here, further repeat testing can confirm whether some of the results obtained, particularly smaller, non-significant changes, were as a consequence of culture due to matrix or long term passaging. This study demonstrates the importance of standardising culture and passaging methods. Selection and outgrowth of advantageous population of stem cells can occur rapidly in culture (Mayshar et al., 2010; Nguyen et al., 2012). Suitable suggestions would be to pool samples from culture vessels (i.e. a 6 well plate or flask), then passage cells to ensure a heterogeneous population is selected and passaged on.

The wide variation in derivation methods of stem cell lines has also been a cause of explanation as to why some stem cell lines differ karyotypically to others (Inzunza et al., 2005; Hanson & Caisander., 2005). This does serve as a reminder that human embryonic stem cells are artefacts or transitional cells between very immature and differentiated cell types which only exist in the body for very short amounts of time. It is also a reminder of how little we understand about the similarities between *in vitro* cultured stem cells and *in vivo* stem cells, The sensitivity in detecting partial chromosome losses and by aCGH as demonstrated by these studies, has been used to investigate rates of aneuploidy and mosaicisms in preimplantation embryos, and has uncovered *de novo* changes (Mertzanidou et al., 2012). In addition, it has been reported that blastocysts (from which stem cells are derived) harbour chromosomal changes such as trisomys, tetraploidys and aneuploidys (Clouston et al., 2002; Fragouli et al., 2008). It has also been speculated that blastocysts might outgrow or 'self-correct' these changes through different mechanisms such as repair, apoptosis of aneuploid cells and preferential selection of diploid cells (Gonzalez-marrino et al., 2003; Los, et al., 2004; Robberecht et al., 2010). Such theories maybe true of what also occurs in long term cultured hESCs and does warrant investigation as to what

precisely in their microenvironment triggers these smaller aberrations, to better establish how to prevent them from occurring *in vivo*.

Some of the changes which are linked to prolonged culture may have already been established in these stem cell lines prior to their deposit and banking. All three hESC lines used for these studies were deposited at over twenty passages. Stem cell banks have no control on the passage number of a cell line deposited and are obliged to accept hESC lines with appropriate consent.

Furthermore, the number of passages it takes to bank a stem cell line is rarely consistent and therefore difficult to minimise. For these reasons, stem cell lines may already be genetically unstable before they are banked. This may have an effect on their potential for clinical applications. Some hESCs line may only be suitable for disease modelling or research based toxicity assays up to a particular passage where they have demonstrated genetic stability. Many researchers will work with lines for only 10 passages per project/experiment before returning to a small seed stock of early passage stem cells as they are concerned about such implications, and such practice has been recommended (Coeke et al., 2009). Nonetheless, cells used in therapeutic applications have already been shown to harbour aberration, for example MRC5 cells used in vaccine manufacture (Rosolowsky et al., 1998). However the aberration did not affect the cell lines ability to function as it was found in low frequency. What is important is whether the aberration is expressed in a large number of the overall population, and how the specific change(s) affects the function and ultimate use of the cell line in future studies e.g. how the cells may behave *in vivo* following transplantation. The accurate screening and detection of large and small aberrations will help to make informed decisions as to which stem cell lines and conditions are best for progressing research towards therapeutic applications.

7.6 Conclusion

The use of human embryonic stem cells as sources for cellular therapeutics is quickly approaching. When maintained in long term culture, stem cells show a loss of chromosomal integrity. Common abnormalities include whole chromosomal gains and losses. However aberrations detected as a result of hESCs cultured on different feeders did not demonstrate a difference due to mouse or human feeders. Cultures from Matrigel did however, demonstrate overall better chromosomal stability. The data show that the PerkinElmer aCGH platform was far more reliable and sensitive compared with traditional G banding, which is currently used for clinical applications. aCGH provides all of the necessary tools for the detailed analysis of the common chromosomal changes associated with the long term culture of hESCs.

Further consensus is required for reporting results with minor chromosomal changes as these may not be consistent with phenotypic changes found in humans unless it provides useful information for deciding what cell lines to use in clinical practice. Used in combination with FISH or SNP arrays to confirm specific aberrations, aCGH can provide robust and sensitive chromosomal analysis of stem cell lines.

As chromosomal stability becomes a focal point in hESC culture, the need for clear reporting guidelines will become a necessity to ensuring useful results are reported. The consequences of genetic changes in hESCs may require researchers to be selective when deciding which stem cell lines to use in a therapeutic setting. The study of stem cell lines with genetic aberrations is of interest as some have demonstrated a growth advantage over other lines. Observations of spontaneous changes in culture which disappear or reappear at later passage indicate a need to monitor smaller karyological changes more closely, particularly when assessing their suitability for clinical application.

Chapter 8

General discussion

Chapter 8

8.1 General discussion

The application of stem cells for cell therapies is promising. In order for this potential to be fully realised, researchers require access to well characterised hESCs from qualified cell sources. While there are a number of stem cell banks that are solely dedicated to research banking and distribution worldwide (Taiwan Stem Cell Bank, WISC Bank Wisconsin, USA, Spanish Stem Cell Bank, Cellartis in Sweden), their methods of culture, cryopreservation and testing all vary as a result of local requirements, research objectives and available funding. Although the time and effort required to bank hESCs is considerable and requires much experience and investment to set up and maintain, the benefits ensure that research grade and now clinical grade hESC lines can be banked and made available to the wider stem cell community, saving researchers time and money. Clinical grade hESC lines have already been derived under cGMP conditions (Stephenson et al., 2012; Ilic et al., 2012) and the move towards using human feeders and in particular clinical grade human feeders has been qualified for supporting hESC derivation and culture (Prathalingam et al., 2012).

The recent development and use of xeno free media and matrices is also a step forward to progressing the move towards better standardised culturing methods and therapeutic applications. Scale up methods and advances in the development of new substrates have also been focused towards supporting the delivery of uniform and quality control tested clinical grade hESCs (Crocco et al., 2013; kunova et al., 2013; Serra et al., 2012). However their reproducibility has yet to be demonstrated. As early clinical trials have already begun (Brindley & Mason., 2012; Schwartz et al., 2012), research has once again been focused on progressing the development of robust methods to culture and characterise hESCs, to continue their momentum towards therapeutic and pharmaceutical applications.

The UKSCB conducts focused research activities in cell banking, cryopreservation, stem cell characterisation and safety testing. This is in line with The Code of Practice developed by the Steering Committee and is key to the translation of research for clinical applications. The UKSCB has many research collaborations focused around these activities and the standardised and robust characterisation of hESCs has

played an important role in the delivery and completion of many projects e.g. ISCI and ESNATs.

hESCs require culturing using standardised methods that are both reliable and robust, and maintain phenotypic and genetic stability. This thesis was also focused on improving the current characterisation methods of hESCs. The methods chosen to assess the ability of feeders to maintain undifferentiated hESC growth were based on the ISCI study (Adewumi et al., 2007). The emphasis placed on qualifying new technologies such as TissueFaxes™ and aCGH was not an easy task, however, proved far more sensitive than previous techniques used in-house and in turn gave rise to more reliable results. Although there have been several comparative studies of mouse and human feeders to support hESC growth, the studies described here provided a much more detailed analysis of these effects on different hESC lines. The overall effects of culture on different mouse and human feeders and Matrigel from early to late passage are summarised in Table 8.1. aCGH revealed that hESCs do acquire aberrations in prolonged culture however the detailed differences in chromosomal aberrations of hESCs NCL5, RH5 and HUES9 have not previously been reported. Although no significant differences were found between cultured hESCs on mouse and human feeders, particular emphasis on the detection of novel partial losses and gains were achieved, as a result of using more sensitive techniques compared with G banding.

As the drive towards using hESCs for therapeutics increases, the thorough assessment of Matrigel and feeders provides researchers with clear reasoning of the effects of using different matrices for hESC culture. hESCs are notoriously difficult to culture and expand, and as part of the UKSCB wider remit to support the stem cell community, these findings can be appropriately communicated through the adaption of banking methods for scaling up of research and therapeutic grade hESCs. The genetic stability of hESCs was compromised by long term passaging and not the use of TrypLE™ Express. Subsequently, this enzyme has now been incorporated into routine stem cell culture and banking procedure within the UKSCB helping to speed up passaging compared with manual dissection and to better standardise one element of an already varied method. Furthermore, the use of Matrigel for hESC banking and scale up has also been validated, which will provide a global resource of feeder free hESCs, helping to give researchers more choice on starting material of

hESC lines and also, removing variability and potential risk of contamination with mouse retroviruses that could occur as a result of using MEFs.

Table 8.1. hESC self-renewal, genetic stability and differentiation from early to late passage on different matrices.

Matrices/feeder type	hESC morphology	Expression of cell surface stemness markers	Expression of stemness genes	Genetic stability (late passage only)	<i>In vitro</i> differentiation (late passage only)
iMEF mouse feeder	Same	Maintained	Maintained	4 chromosomes affected (5, 7, 12, and 20)	Differentiation to all three germ layers achieved
i3T3 mouse feeder	Same	Maintained	Maintained	2 chromosomes affected (gain on 20, loss on 22)	
iMRC5 human feeder	Changed (Elongated colonies)	Maintained	Maintained	4 chromosomes affected (losses/ gains detected on chr. 7, 12, 19, 20)	Differentiation to all three germ layers achieved
iHDfn human feeder	Changed (Elongated colonies)	Maintained	Maintained	4 chromosomes affected (losses/gains detected on chr. 7, 12 17, 18)	
Matrigel synthetic matrix	Same	Maintained	Maintained	4 chromosomes affected (loss on chr. 4, 9, 12 and gain on 6.	Differentiation to all three germ layers achieved

Morphology changes as a result of culture on human feeders and Matrigel

From very early cultures it was noticeable that all four hESCs underwent changes in morphology, as they adapted to culture on both human feeders and feeder free matrix, Matrigel. Such changes gave indications as to the way in which the cells began to adapt to new environments. This was also apparent from IF staining, as differences in the proliferation were detected by sensitive imaging system TissueFaxs™. Notably, the effects of culturing hESCs on human feeders' iHDFn and iMRC5 led to the growth of elongated colonies. This insight cannot be gained as a result of IF staining by flow cytometry, and is important when assessing suitable cultures for passaging, as most scientists are precocious when deciding which colonies to dissect/enzyme passage and will routinely regard any new changes as differentiation.

Characterisation of hESCs cultured on feeder and feeder free matrices

As summarised in Table 8.1, quantitative analysis of IF stained images for stemness markers coupled with statistical analysis, concluded that no significant difference was found between mouse and human feeders, from early to late passage. The main differences in expression were due to stem cell line differences. This provided good indications that cultures were maintained in an undifferentiated state over 20 passages on all feeder types and Matrigel. Although there are no defining criteria for percentage of cell surface marker expression that can be applied to determine a culture is truly undifferentiated, this may become an important decision, particularly when applied as a more robust QC test for clinical grade stem cell lines. The co-expression of markers by TissueFaxs™ would be better to identify purer populations of undifferentiated hESCs. This could potentially improve the selection of hESCs for differentiation studies, as stem cells with reduced heterogeneity have been shown to dominate and further inhibit the directed differentiation of whole cultures of hESCs (Stewart et al., 2006; Canham et al., 2010). This would also aid the production of uniform undifferentiated cultures required for scale up in therapies, as it is extremely important to provide good starting material before differentiating cells, as shown in the final chapter.

Real time PCR using TDLA cards demonstrated the continued expression of undifferentiated genes in all four cell lines cultured on all four feeders from early to

late passage, with only slight differences as a result of hESC culture on mouse and human feeders. Feeder free matrix Matrigel/mTeSR1 has also demonstrated consistent maintenance of undifferentiated hESC growth at early and late passage, with expression of very low level differentiation genes. Statistical analysis of results revealed that human feeders and Matrigel maintained the gene expression of stem cell markers as proficiently as mouse feeders. Furthermore, the use of hybridomas for low cost, in-house undifferentiated cell surface marker testing by IF staining using novel instrument TissueFacs™, for high throughput quantitative imaging analysis, was particularly important, as it enabled the progression of well-established but qualitative staining technique towards with a quantitative method, comparable with standard flow cytometry. These studies demonstrated that the TissueFacs™ was more sensitive, thus suggesting a robust and improved novel system for determining cell surface marker expression of undifferentiated hESCs.

As shown in chapter 5, low expression of endoderm transcription factor genes SRY-Related HMG-box (SOX17) and Forkhead Box-A2 (FOXA2), were found in undifferentiated NCL5 cultures from all three matrices. SOX17 modulates transcriptional regulation via the WNT3A and inhibits Wnt signalling. FOXA2 is found on chromosome 20p11.21 and play an important role in hepatocyte function. This may indicate that these genes were endogenous to the stem cell line and downregulated as a consequence of further directed gene expression towards endoderm lineage. The use of later endoderm genes may aid in detecting whether this suggestion may be true.

Differentiation potential of hESCs cultured on feeder and feeder free matrices

As most of the studies concluded that differences in characterisation testing were as a result of inherent differences between the hESC lines themselves, a final test of pluripotency was demonstrated by the most stable hESC line from these studies, NCL5 cultured on iMEF, MRC5 and Matrigel. Although all conditions demonstrated and confirmed the ability to differentiate towards all three germ layers, differences were shown between the conditions. NCL5 cultured on iMRC5 indicated a preference to form mesoderm by TissueFacs IF and RT-PCR and morphology, whereas NCL5 cultured on iMEFs and Matrigel showed a preference towards endoderm.

Stability of hESCs in long term culture

Chromosome changes are mostly related to disease and syndromes in humans. However, it should always be considered that although a line may contain chromosomal changes, a number of CNVs may be required for the change to be expressed as a phenotype. A number of chromosome changes have been identified in humans, with no known phenotype (Barber, 2005). In addition, stem cell lines have been shown to change as a result of prolonged culture (Maitra et al., 2005; Andrews et al., 2011). Chromosomal aberrations detected in these studies by aCGH from the three hESCs cultured on both mouse, human feeders and Matrigel include 12, 17, 19, and 20. These changes have already been documented and are associated with prolonged culturing. Additionally, a gain on Chromosome 12p-arm has been linked with maintenance of stem cell function through activation of key stem cell genes, SOX2, TDGF1, EBAF, ZFP42, FGF and NODAL (Karkalla et al., 2006). This change was detected in HUES9 cultured on iMRC5 from early to late passage (aCGH, chapter 4) and can be correlated with a slight downregulation of SOX2 gene expression, although this was not significant. The functional effects of this change would need to be confirmed through gene knockout studies, to confirm whether the change was linked with downregulation of SOX2 or whether it requires the involvement of other stemness genes.

Furthermore, the expression of GATA6 was consistently low across all hESC lines used in these studies. GATA6 expression has been associated with early blastocyst formation (Koutsourakis et al., 1999) and maintenance of pluripotency and self-renewal alongside OCT4, NANOG and SOX2 (Adewumi et al., 2007), as well as being upregulated during visceral endoderm formation (Fujikura et al., 2002). GATA6 has been linked to chromosome 18q11.2-18q.11.1, which was detected as a loss in NCL5/iHDFn from early to late passage by aCGH (chapter 7). This could potentially lead to a functional loss in development of smooth muscle which may have consequences later for the potential use of NCL5 in disease modelling if required to differentiate towards mesoderm lineage. Although the studies in chapter 6 suggest that this gene was not useful in determining the undifferentiated state of hESCs cultured in long term passaging using different matrices, it does illustrate that using a number of different genes to characterise hESCs provides greater confidence when attempting to determine overall cell state. Such detailed characterisation is crucial to

the consensus as to which specific detectors should be used when characterising hESC lines as they are intrinsically individual, and will help to better standardise the use of PCR for such applications.

Identifying the effect of chromosomal changes in hESCs for their intended purpose would be greatly beneficial to the stem cell community. Such studies have been conducted, particularly with regards to the ability of hESCs with chromosomal instabilities, to differentiate. Indeed the studies conducted in this thesis demonstrated that although stem cell line NCL5 had acquired chromosomal aberrations, this did not impede its ability to differentiate to all three germ layers and form progenitor cells (chapter 6).

Additionally, a gain on Chromosome 12p-arm has been linked with maintenance of stem cell function through activation of key stem cell genes, SOX2, TDGF1, EBAF, ZFP42, FGF and NODAL (Korkola et al., 2006). This change was detected in HUES9 cultured on iMRC5 from early to late passage (aCGH, chapter 7) and can be correlated with a slight downregulation of SOX2 gene expression, although this was not significant. The functional effects of this change would need to be confirmed through gene knockout studies, to confirm whether the change was linked with downregulation of SOX2 or whether it requires the involvement of other stemness genes.

Also, studies detailing differentiated hESCs to neural derivatives report the development of chromosomal aberrations following 50 passages (Varela et al., 2012). Following implantation into rat models, the neural stem cell cultures harbouring the chromosome 1q translocation also failed to integrate and expand, and was further demonstrated as a re-occurrence in other cultures (Varela et al., 2012). Recently, a publication demonstrated that stem cells cultured *in vivo* displayed more karyotypic changes compared with those cultured *in vitro*. Such research does warrant further investigation, but also questions the long term stability of hESCs, particularly for preclinical studies, which require hESCs to be cultured *in vitro*, potentially differentiated then engrafted or injected into hosts. These studies are also a strong reminder of the endogenous nature of hESCs, which, in their natural state, exist as transient populations *in vivo*.

Differences between feeders

Explanations as to why differences between human and mouse feeders occurred may be due to the different sources of feeders. MRC5 are derived from human foetal lung. Foetal tissue has been previously shown to support undifferentiated hESC growth (Richards et al., 2003). HDFn fibroblasts are derived from neonatal foreskin. Foreskin derived fibroblasts have also been documented to support stem cell growth (Kueh et al., 2006). MEF and 3T3 fibroblasts are derived from mouse embryos. NIH3T3 have been used to culture keratinocytes used in skin transplantation. Although MEFs and 3T3s are very similar, their slight differences are enough to produce differences in proliferation within a stem cell line. This is probably due to the secretion of different proteins, growth factors and hormones which enable and promote successful attachment and growth of stem cells (Eiselleova et al., 2008). These differences were subtle throughout the characterisation of undifferentiated cultures from early to late passage, but when late passage NCL5 were differentiated to all three germ layers *in vitro* for 7 days, the differences between each matrix was clearly demonstrated. Differences in levels of Activin A, fibroblast growth factors (FGFs), laminin and collagen, secreted by each feeder type, or in the case of Matrigel/mTeSR1, incorporated within the ECM and media, will have undoubtedly influenced the microenvironment of NCL5 over long term passaging. Although all three matrices supported *in vitro* differentiation to all three germ layers, clearly different matrices showed preferences to different germ layers. Although the co culture of hESCs to improve differentiation has been demonstrated (Pekkanen-Mattila et al., 2012), these studies represent the first comparison of the three matrices discussed here as a result of pre-culture on different matrices over 20 passages. Such differences need to be further demonstrated with other cell lines and exploited to perhaps be incorporated into the design of better substrates, to further enhance the differentiation potential to specific lineages, for example MRC5 or MEF towards mesoderm. It also highlights the point that there is still more to be uncovered in terms of how microenvironment can be better mimicked for improving *in vitro* differentiation.

Inherent differences between stem cell lines

Information about inherent differences between stem cell lines is important to researchers so they can make better informed decisions about which lines are best

to use in terms of growth rates, stability, and ability to expand and differentiate over time. At this early stage in human embryonic stem cell research it may be right to argue that all characterisation information detailing differences between hESC lines be reported, until firm decisions can be made on the criteria for hESCs to be used in therapies. The results from this thesis certainly support other reports that hESC lines are different from each other, particularly when passaged for extended periods of time. However, it may be that from the thousands of lines which have now been derived, only a select few will be taken forward for clinical use. This is more dependent on how focused the move towards personalised medicine becomes, and whether iPSCs prove to be an equal alternative to hESCs.

The sensitive QC testing used in these studies demonstrates the importance of characterising stem cell lines, as they are all individual. Most of the subtle differences arise due to their individuality and how they attach, adapt and proliferate as a result of changes in their microenvironments. These studies also provided further insight into how microenvironment influences differentiation *in vitro*. A better understanding of these changes can help to improve culturing conditions as well developing more robust characterisation testing. This is important to the wider stem cell community, as the progression to clinical application is imminent.

The future of stem cells in therapy

Studies for phase I/II clinical trials using stem cells have already begun. One major hurdle is the development of suitable potency bioassays, to confirm that the manufactured cell product is still functioning as specified. Such assays may be in the form of testing for phenotypic markers or gene expression studies (Bravery et al., 2012). Most likely they will be a combination of both. The studies carried out here demonstrate the work required to standardise such assays. Refining these culture methods/systems and incorporating them with xeno free counterparts such as mTeSR2 media will help to support the culture of clinical grade hESC lines. In combination with sensitive and robust techniques, equipment/platforms, the development of potency assays for clinical trials can be realistically achieved, and the dream of using hESCs in a therapeutic setting, a closer reality.

Final conclusion and future work

Conclusion

These studies have demonstrated the use of human feeders and Matrigel to maintain the undifferentiated growth of hESCs via robust and sensitive characterisation testing. Although the maintenance of stemness genes and cell surface markers were evident, hESCs undergo chromosomal changes in long term culture. *In vitro* differentiation studies using stem cell line NCL5 show that this did not affect their ability to form progenitors from all three germ layers. Human feeder MRC5, mouse feeder MEF and feeder free matrix Matrigel maintained the expression of stemness markers from three hESC lines and supported the differentiation of stem cell line NCL5 *in vitro*. However differences were observed in their propensity towards specific pathways. Although such insights have been demonstrated by other matrices and feeder types, this is the first comparing cultures on iMRC5 human feeders, iMEF and Matrigel.

The variations between different stem cell lines ability to adapt to new matrices highlight the intrinsic differences between the lines themselves and can provide an insight to how these cells behave *in vitro*. Unfortunately, it does make standardising culture methods difficult and even as the field progressing rapidly, with the development of many commercial media and matrices that are defined and xeno free, they are usually shown to work only with a select few stem cell lines. These studies stress the importance of exploring the suitability of different matrices/feeders and stem cell lines by comparison studies, as contributing such information to the wider stem cell community can only enhance knowledge on the selection of suitable cell lines and their conditions, as well as highlighting the importance of developing and continually improving the culture process and characterisation testing, for progressing their unique potential in disease and clinical therapies.

Future work

These studies have provided insight and considerable contribution to knowledge for the stem cell community by demonstrating 1) the smaller chromosomal abnormalities acquired in hESCs (by aCGH) as a result of long term culture, 2) the morphological changes that occur as a result of culture on human feeders in comparison to mouse and their effects on cell surface marker expression, 3) the effects of hESC differentiation on gene expression and morphology as a result of long term culture on different matrices, and 4) the potential applications of more sensitive characterisation techniques for the development of potency assays in early clinical studies. This thesis has generated new data which requires further investigation to answer important questions and conclude particular aspects of this research.

- Confirm detected aCGH changes using Single Nucleotide Polymorphism (SNP) arrays, in particular for chromosomes 7, 12q, 17q and 20, as aCGH produces huge amounts of information, it is important to determine what information is important.
- Demonstrate the ability of different hESC lines to differentiate towards all three germ layers using Matrigel and MRC5 human feeders. Further extended differentiation assays towards specific cell types may provide a better insight into the effect of culture on pathway selection using human feeders and Matrigel. This could also be combined with knockout studies to identify and confirm pathway selection as a result of secreted growth factors from feeders.
- Further QC testing to give proteomic and carbohydrate analysis of MRC5 and MEF feeders to identify the exact proteins/growth factors that support hESC growth (e.g. FGF2) could allow for the development of a combined ECM which could then be completely defined, Xeno free hESC culturing system. Demonstrating the supportiveness for such a matrix could then be validated, incorporated into routine banking of hESCs and would add considerable value to the stem cell lines banked by UKSCB, including advancing the scope for clinical applications.

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Publications

“The Effect of mouse, human feeders and Matrigel on *In vitro* directed differentiation of human embryonic stem cells to early endoderm, mesoderm and ectoderm progenitors”. S.Khadun, O.O’Shea, L.Healy, G.Stacey, A.Baydoun. Article in preparation to be submitted to *Stem Cells and Development*.

“The characterisation of human embryonic stem cells using The TissueFaxs™: high content image analysis system”. S.Khadun, O.O’Shea, S.Lees, P.Barbuti, L.Healy, G.Stacey, A.Baydoun. Article in preparation to be submitted to *PLOS1*.

Oral presentations

“Support of undifferentiated human embryonic stem cell lines by mouse and human feeder lines.” Presented at NIBSC scientific session 2012.

“Comparative study of feeders for the support human embryonic stem cell lines by feeders and other matrices”. Presented at University of Hertfordshire Postgraduate club 2012.

Poster presentations

“*In vitro* differentiation of hPSCs by standardised methods.” Orla O’Shea¹, Shalinee Khadun^{1,2}, Lyn Healy¹, Anwar Baydoun², Glyn Stacey¹. Presented at ISSCR 2013 (Boston, USA).

“Support of undifferentiated human embryonic stem cell lines by human feeder lines.” S.Khadun, P.Pipkin, L.Young, L.Healy, C.J.Robinson, M.Hamil. Abcam 4th annual meeting 2011(Cardiff).

“Support of undifferentiated human embryonic stem cell lines by human feeder lines.”S.Khadun, P.Pipkin, L.Young, L.Healy, C.J.Robinson, M.Hamil. Presented at ESTools workshop 2010 (Lisbon).

“Support of undifferentiated human embryonic stem cell lines by mouse and human feeder lines.”S.Khadun, P.Pipkin, L.Young, L.Healy, M.Hamil. Presented at UKNSCN national meeting 2011(York).

Appendix 1

Applied Biosystems, Inc. (http://www.appliedbiosystems.com)					
	Taqman Low Den	4385225			
	Appendix-1				
	Spreadsheet showing primer details of genes on TLDA card				
Assay Map:					
	Hs99999903_m1	Hs006063	Hs00173490_m1	Hs00217848_m	Hs00156373_m
	Hs00277509_m1	Hs002327	Hs00255287_s1	Hs00241459_m	Hs00544355_m
	Hs00158126_m1	Hs001740	Hs00196158_m1	Hs00300550_m	Hs00158620_m
	Hs00742896_s1	Hs008298	Hs00603586_g1	Hs00234119_m	Hs00194498_m
	Hs99999903_m1	Hs006063	Hs00173490_m1	Hs00217848_m	Hs00156373_m
	Hs00277509_m1	Hs002327	Hs00255287_s1	Hs00241459_m	Hs00544355_m
	Hs00158126_m1	Hs001740	Hs00196158_m1	Hs00300550_m	Hs00158620_m
	Hs00742896_s1	Hs008298	Hs00603586_g1	Hs00234119_m	Hs00194498_m
	Hs99999903_m1	Hs006063	Hs00173490_m1	Hs00217848_m	Hs00156373_m
	Hs00277509_m1	Hs002327	Hs00255287_s1	Hs00241459_m	Hs00544355_m
	Hs00158126_m1	Hs001740	Hs00196158_m1	Hs00300550_m	Hs00158620_m
	Hs00742896_s1	Hs008298	Hs00603586_g1	Hs00234119_m	Hs00194498_m
	Hs99999903_m1	Hs006063	Hs00173490_m1	Hs00217848_m	Hs00156373_m
	Hs00277509_m1	Hs002327	Hs00255287_s1	Hs00241459_m	Hs00544355_m
	Hs00158126_m1	Hs001740	Hs00196158_m1	Hs00300550_m	Hs00158620_m
	Hs00742896_s1	Hs008298	Hs00603586_g1	Hs00234119_m	Hs00194498_m
Gene Symbols					
	ACTB	ACTC	AFP	BRIX	CD34
	FN1	FOXA2	FOXD3	GABRB3	GAL
	ISL1	KIT	KRT1	LAMA1	LAMB1
	POU5F1	PTEN	PTF1A	RAF1	REST
	ACTB	ACTC	AFP	BRIX	CD34
	FN1	FOXA2	FOXD3	GABRB3	GAL
	ISL1	KIT	KRT1	LAMA1	LAMB1
	POU5F1	PTEN	PTF1A	RAF1	REST
	ACTB	ACTC	AFP	BRIX	CD34
	FN1	FOXA2	FOXD3	GABRB3	GAL
	ISL1	KIT	KRT1	LAMA1	LAMB1
	POU5F1	PTEN	PTF1A	RAF1	REST
Doc. PN 4385326A					

Appendix-1				
Spreadsheet showing primer details of genes on TLDA card				
Assay Map:	Hs00233521_m1	Hs00174344_m1	Hs00230919_m1	Hs00361224_gH
	Hs99999905_m1	Hs00171403_m1	Hs00232018_m1	Hs00230965_m1
	Hs00267056_m1	Hs00764128_s1	Hs00158730_m1	Hs00702808_s1
	Hs00231692_m1	Hs00173810_m1	Hs00165475_m1	Hs00293258_m1
	Hs00233521_m1	Hs00174344_m1	Hs00230919_m1	Hs00361224_gH
	Hs99999905_m1	Hs00171403_m1	Hs00232018_m1	Hs00230965_m1
	Hs00267056_m1	Hs00764128_s1	Hs00158730_m1	Hs00702808_s1
	Hs00231692_m1	Hs00173810_m1	Hs00165475_m1	Hs00293258_m1
	Hs00233521_m1	Hs00174344_m1	Hs00230919_m1	Hs00361224_gH
	Hs99999905_m1	Hs00171403_m1	Hs00232018_m1	Hs00230965_m1
	Hs00267056_m1	Hs00764128_s1	Hs00158730_m1	Hs00702808_s1
	Hs00231692_m1	Hs00173810_m1	Hs00165475_m1	Hs00293258_m1
	Hs00233521_m1	Hs00174344_m1	Hs00230919_m1	Hs00361224_gH
	Hs99999905_m1	Hs00171403_m1	Hs00232018_m1	Hs00230965_m1
	Hs00267056_m1	Hs00764128_s1	Hs00158730_m1	Hs00702808_s1
	Hs00231692_m1	Hs00173810_m1	Hs00165475_m1	Hs00293258_m1
Gene Symbols	CD9	CDH5	CDX2	CGB
	GAPD	GATA4	GATA6	GBX2
	LAMC1	LEFTB	LIFR	LIN28
	RUNX2	SEMA3A	SERPINA1	SFRP2
	CD9	CDH5	CDX2	CGB
	GAPD	GATA4	GATA6	GBX2
	LAMC1	LEFTB	LIFR	LIN28
	RUNX2	SEMA3A	SERPINA1	SFRP2
	CD9	CDH5	CDX2	CGB
	GAPD	GATA4	GATA6	GBX2
	LAMC1	LEFTB	LIFR	LIN28
	RUNX2	SEMA3A	SERPINA1	SFRP2
	CD9	CDH5	CDX2	CGB
	GAPD	GATA4	GATA6	GBX2
	LAMC1	LEFTB	LIFR	LIN28
	RUNX2	SEMA3A	SERPINA1	SFRP2

Appendix-1					
Spreadsheet showing primer details of genes on TLDA card					
Assay Map:	Hs00164004_m1	Hs99999901_s1	Hs00156568_m1	Hs00201350_m	Hs00275636_m1
	Hs00174967_m1	Hs00172692_m	Hs00220998_m1	Hs00157674_m	Hs00917999_g1
	Hs00271574_m1	Hs00159528_m	Hs02387400_g1	Hs00707120_s1	Hs00159598_m1
	Hs00751752_s1	Hs00602736_s1	Hs00174949_m1	Hs00538143_m	Hs00300531_m1
	Hs00164004_m1	Hs99999901_s1	Hs00156568_m1	Hs00201350_m	Hs00275636_m1
	Hs00174967_m1	Hs00172692_m	Hs00220998_m1	Hs00157674_m	Hs00917999_g1
	Hs00271574_m1	Hs00159528_m	Hs02387400_g1	Hs00707120_s1	Hs00159598_m1
	Hs00751752_s1	Hs00602736_s1	Hs00174949_m1	Hs00538143_m	Hs00300531_m1
	Hs00164004_m1	Hs99999901_s1	Hs00156568_m1	Hs00201350_m	Hs00275636_m1
	Hs00174967_m1	Hs00172692_m	Hs00220998_m1	Hs00157674_m	Hs00917999_g1
	Hs00271574_m1	Hs00159528_m	Hs02387400_g1	Hs00707120_s1	Hs00159598_m1
	Hs00751752_s1	Hs00602736_s1	Hs00174949_m1	Hs00538143_m	Hs00300531_m1
	Hs00164004_m1	Hs99999901_s1	Hs00156568_m1	Hs00201350_m	Hs00275636_m1
	Hs00174967_m1	Hs00172692_m	Hs00220998_m1	Hs00157674_m	Hs00917999_g1
	Hs00271574_m1	Hs00159528_m	Hs02387400_g1	Hs00707120_s1	Hs00159598_m1
	Hs00751752_s1	Hs00602736_s1	Hs00174949_m1	Hs00538143_m	Hs00300531_m1
Gene Symbols	COL1A1	18S	COL2A1	COMMD3	CRABP2
	GCG	GCM1	GDF3	GFAP	GRB7
	MYF5	MYOD1	Nanog*	NES	NEUROD1
	SOX17	SOX2	SST	SYCP3	SYP
	COL1A1	18S	COL2A1	COMMD3	CRABP2
	GCG	GCM1	GDF3	GFAP	GRB7
	MYF5	MYOD1	Nanog*	NES	NEUROD1
	SOX17	SOX2	SST	SYCP3	SYP
	COL1A1	18S	COL2A1	COMMD3	CRABP2
	GCG	GCM1	GDF3	GFAP	GRB7
	MYF5	MYOD1	Nanog*	NES	NEUROD1
	SOX17	SOX2	SST	SYCP3	SYP

Appendix-1					
Spreadsheet showing primer details of genes on TLDA card					
Assay Map:	Hs00170025_m	Hs00251859_m	Hs00157258_m	Hs00171876_m1	Hs00745761_s1
	Hs00747223_g	Hs00744391_s	Hs00232128_m	Hs00169095_m1	Hs00705137_s1
	Hs00415443_m	Hs00271352_s	Hs00383230_g1	Hs00187067_m1	Hs00265966_m1
	Hs00610080_m	Hs00356930_m	Hs02339499_g1	Hs00162669_m1	Hs00232708_m1
	Hs00170025_m	Hs00251859_m	Hs00157258_m	Hs00171876_m1	Hs00745761_s1
	Hs00747223_g	Hs00744391_s	Hs00232128_m	Hs00169095_m1	Hs00705137_s1
	Hs00415443_m	Hs00271352_s	Hs00383230_g1	Hs00187067_m1	Hs00265966_m1
	Hs00610080_m	Hs00356930_m	Hs02339499_g1	Hs00162669_m1	Hs00232708_m1
	Hs00170025_m	Hs00251859_m	Hs00157258_m	Hs00171876_m1	Hs00745761_s1
	Hs00747223_g	Hs00744391_s	Hs00232128_m	Hs00169095_m1	Hs00705137_s1
	Hs00415443_m	Hs00271352_s	Hs00383230_g1	Hs00187067_m1	Hs00265966_m1
	Hs00610080_m	Hs00356930_m	Hs02339499_g1	Hs00162669_m1	Hs00232708_m1
	Hs00170025_m	Hs00251859_m	Hs00157258_m	Hs00171876_m1	Hs00745761_s1
	Hs00747223_g	Hs00744391_s	Hs00232128_m	Hs00169095_m1	Hs00705137_s1
	Hs00415443_m	Hs00271352_s	Hs00383230_g1	Hs00187067_m1	Hs00265966_m1
	Hs00610080_m	Hs00356930_m	Hs02339499_g1	Hs00162669_m1	Hs00232708_m1
Gene Symbols	CTNNB1	DDX4	DES	DNMT3B	EBAF
	HBB	HBZ	HLXB9	IAPP	IFITM1
	NODAL	NOG	NPPA	NR5A2	NR6A1
	T	TAT	TDGF1	TERT	TFCP2L1
	CTNNB1	DDX4	DES	DNMT3B	EBAF
	HBB	HBZ	HLXB9	IAPP	IFITM1
	NODAL	NOG	NPPA	NR5A2	NR6A1
	T	TAT	TDGF1	TERT	TFCP2L1
	CTNNB1	DDX4	DES	DNMT3B	EBAF
	HBB	HBZ	HLXB9	IAPP	IFITM1
	NODAL	NOG	NPPA	NR5A2	NR6A1
	T	TAT	TDGF1	TERT	TFCP2L1

Appendix-1				
Spreadsheet showing primer details of genes on TLDA card				
Assay Map:	Hs00172872_m	Hs00173564_m1	Hs00170454_m1	Hs001765
	Hs00174360_m	Hs00538956_m1	Hs00355773_m1	Hs002368
	Hs00173014_m	Hs00240871_m1	Hs00169777_m1	Hs001936
	Hs00747497_g1	Hs00240913_m1	Hs01079824_m1	Hs003992
	Hs00172872_m	Hs00173564_m1	Hs00170454_m1	Hs001765
	Hs00174360_m	Hs00538956_m1	Hs00355773_m1	Hs002368
	Hs00173014_m	Hs00240871_m1	Hs00169777_m1	Hs001936
	Hs00747497_g1	Hs00240913_m1	Hs01079824_m1	Hs003992
	Hs00172872_m	Hs00173564_m1	Hs00170454_m1	Hs001765
	Hs00174360_m	Hs00538956_m1	Hs00355773_m1	Hs002368
	Hs00173014_m	Hs00240871_m1	Hs00169777_m1	Hs001936
	Hs00747497_g1	Hs00240913_m1	Hs01079824_m1	Hs003992
	Hs00172872_m	Hs00173564_m1	Hs00170454_m1	Hs001765
	Hs00174360_m	Hs00538956_m1	Hs00355773_m1	Hs002368
	Hs00173014_m	Hs00240871_m1	Hs00169777_m1	Hs001936
	Hs00747497_g1	Hs00240913_m1	Hs01079824_m1	Hs003992
Gene Symbols	EOMES	FGF4	FGF5	FLT1
	IL6ST	IMP2	INS	IPF1
	PAX4	PAX6	PECAM1	PODXL
	UTF1	WT1	Xist	ZFP42
	EOMES	FGF4	FGF5	FLT1
	IL6ST	IMP2	INS	IPF1
	PAX4	PAX6	PECAM1	PODXL
	UTF1	WT1	Xist	ZFP42
	EOMES	FGF4	FGF5	FLT1
	IL6ST	IMP2	INS	IPF1
	PAX4	PAX6	PECAM1	PODXL
	UTF1	WT1	Xist	ZFP42
	EOMES	FGF4	FGF5	FLT1
	IL6ST	IMP2	INS	IPF1
	PAX4	PAX6	PECAM1	PODXL
	UTF1	WT1	Xist	ZFP42

APPENDIX 2

Table showing list of genes and corresponding Taqman probes used in chapter 7.

Gapdh	CONTROL	Hs02758991_g1
Beta Actin	CONTROL	Hs01060665_g1
Pou5f	STEM CELL	Hs04260367_gH
Nanog	STEM CELL	Hs04260366_g1
Pax6	NEURAL	Hs00240871_m1
NESTIN	NEURAL	Hs04187831_g1
Otx2	NEURAL	Hs00222238_m1
Beta3 Tubulin	NEURAL	Hs00801390_s1
Neurod1	NEURAL	Hs01922995_s1
SOX1	NEURAL	Hs01057642_s1
FOXP1	NEURAL	Hs01850784_s1
DCN	ENDO	Hs00754870_s1
PDGFRa	MESO	Hs00998018_m1
Islet1	ENDO	Hs00158126_m1
PITX1	ENDO/MESO	Hs00267528_m1
Pecam1	MESO	Hs00169777_m1
Vimentin	MESO	Hs00185584_m1
Hand1	MESO	Hs02330376_s1
Col1a1	ENDO	Hs00164004_m1
Desmin	MESO	Hs00157258_m1
N-cad	MESO	Hs00983056_m1
SOX17	ENDO	Hs00751752_s1
Brachyury	ENDO/MESO	Hs00610080_m1
FOXA2	ENDO	Hs00232764_m1
GSC	ENDO	Hs00418279_m1
BMP4	MESO	Hs00370078_m1
CXCR4	ENDO	Hs00607978_s1
FGB	MESO	Hs00905942_m1