CHAPTER 1

1 INTRODUCTION

1.1 Significance

The notion that physico-chemical factors are key players in controlling stem cell fate is an established fact. Differentiating embryonic stem cells into a specific lineage or cell type is one of the most investigated areas of stem cell research; however, it is wrought with hurdles. The differentiation ability of ES cells is an important and focal point for researchers to understand the specification process, which is panoply of numerous factors. This differentiation process during embryogenesis is greatly influenced by physico-chemical factors. These factors direct key genes for lineage commitment, which in turn is responsible for patterning into highly organized tissues resulting in an organ formation. This thesis focuses on the influence of physico-chemical factors on differentiation of ES cells which mimics the embryogenesis in vivo.

1.2 Stem cells

Stem cells are the cornerstone cells for development of every organ and tissue in our body. These specialized cells have their origin in an initial pool of cells i.e. embryonic stem cells, which are formed shortly after fertilization. All throughout our lives, we rely upon another set of stem cells i.e. adult stem cells in order to continually divide to replace the dying and injured cells/tissues that are lost every day, such as those in our skin, hair, blood and the lining of gut.

1.2.1 Embryonic stem cells

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of an early developing embryo at the blastocyst stage. They are characterized by the ability to self-renew i.e. proliferate indefinitely in vitro while maintaining pluripotency; the later one is the potential to differentiate into germ cells and various cell types that emanate from the three primary germ layers viz. endoderm, mesoderm and ectoderm. Definitely, mouse ES cells could simply be the product of an in vitro culture system or be truly representative of pluripotent stem cells those are naturally present in the early embryo. With these two attributes, ES cells have immense importance as a model system to mimic early mammalian development along with their further use in clinical applications [1].
1.2.2 Adult stem cells

Adult stem cells are also known as tissue-specific stem cells, which are sometimes referred to as “somatic” stem cells. Although, these cells are unspecialized but their differentiation capacity is restricted with respect to ES cells and they are said to be multipotent rather than pluripotent *i.e.* they can produce some or all of the mature cell types found within the specific tissue or organ in which they are located. For example, stem cells found within the adult brain are capable of making neurons and two types of glial cells, astrocytes and oligodendrocytes. Adult stem cells are located at particular place in organs known as niche. For example, bone marrow is niche for hematopoietic stem cells. The idiosyncrasy of adult stem cells is that one type of adult stem cells can differentiate into another type of tissue depending upon growth or differentiation conditions, a property known as ‘plasticity’ [2, 3].

1.2.3 Why are ES cells so precious?

Unlike adult stem cells, ES cells have the capacity to produce every cell type found in the body. Based on this unique and powerful property, these cells can be grown and expanded indefinitely in this unspecialized or “undifferentiated” state under the favorable conditions. The path breaking studies on ES cells lays down the foundation for understanding the voyage of how a cluster of cells in the ICM lead to the formation of a multicellular organism *via* the differentiation process. ES cells are new toolkits for researchers to learn about early mammalian developmental processes that are otherwise difficult to identify. Moreover, use of these ES cells had open up a window of research to study birth defects, progression of various diseases and providing foundations for establishing novel strategies that could ultimately lead to cell based regenerative therapy design and drug discovery (Figure 1.1).
1.2.4 Timeline of ES cells discovery

**1878:** First time reports of fertilization of mammalian eggs outside the body [5].

**1960s:** Reports from teratocarcinomas in the testes of several inbred strains of mice indicated they stem from embryonic germ cells. Further work establishes embryonal carcinoma (EC) cells as a kind of stem cell [6, 7].

**1968:** First report of fertilizing the first human egg *in vitro* [5].

**1970s:** EC cells injected into mouse blastocysts produce chimeric mice. Cultured stem cells were represented as models of embryonic development [8].

**1978:** First IVF baby was born in England [5].

**1981:** Evans and Kaufman and Martin, derived mouse ES cells from the inner cell mass of blastocysts. They established *in vitro* culture conditions to grow pluripotent mouse ES cells. The established ES cell lines showed normal, diploid karyotypes and produced cells of all the three
primary germ layers as well as primordial germ cells. Injecting ES cells into mice induced the formation of teratomas [9, 10].

1984-88: Andrews et al developed pluripotent, genetically identical (clonal) cells called embryonal carcinoma (EC) cells from Tera-2, a cell line of human testicular teratocarcinoma [11]. Exposure of cloned human teratoma cells to retinoic acid resulted in their differentiation into neuron-like cells and other cell types [11, 12].

1989: Pera et al derived a clonal line of human embryonal carcinoma cells, which yielded tissues from all the three primary germ layers. The cells were aneuploid (fewer or greater than the normal number of chromosomes in the cell) and with limited potential to differentiate spontaneously in vitro. The nature of human EC cell clones were differed from that of mouse ES or EC cells [13].

1994: Human blastocysts generated using IVF technology for reproductive purposes and donated by patients for research, were produced from the 2-pronuclear stage. The inner cell mass of the blastocyst was maintained in culture and aggregates were generated with trophoblast-like cells at the periphery and ES-like cells in the center. The cells retained a complete set of chromosomes (normal karyotype); most cultures retained a stem cell-like morphology, although some inner cell mass clumps differentiated into fibroblasts [14, 15].

1995-96: Non-human primate ES cells were derived and maintained in vitro. The first ES cell line was from inner cell mass of rhesus monkeys [16], and then from marmosets [17]. These ES cells were diploid and had normal karyotypes. They were pluripotent and differentiated into cells types of all the three primary germ layers. The primate ES cells look like human EC cells and suggested that it could be possible to derive and maintain human ES cells in vitro.

1998: Thomson et al derived human ES cells from the inner cell mass of normal human blastocysts donated by couples undergoing treatment for infertility. The cells when maintained for longer passages, were able to maintain their normal karyotypes, retained high levels of telomerase activity, and presented a panel of markers typical of human EC cells [18]. Gearhart and colleagues derived human embryonic germ (EG) cells from the gonadal ridge and mesenchyma of 5- to 9-week fetal tissue that resulted from elective abortions. When EG cells were grown in vitro for approximately 20 passages, they showed normal karyotypes. These cells immediately formed aggregates which then spontaneously differentiated and expressed...
derivatives of all the three primary germ layers. The EG cells did not form teratomas when injected into immune-deficient mice [19]

2007: Generation of induced pluripotent stem cells was shown by Takashi and Yamanaka [20].

2007: Induced pluripotent stem cells were shown to form germ line chimerism [21].

1.2.5 Historical background of ES cell differentiation

If development is scaled from conception to death, then it evokes a key question that what it means for a cell to be “functionally mature” or “differentiated”? In the embryogenesis process, cell differentiation is a fundamental requirement as it not only leads to the development of the fetus but also produces the different cell types in their innumerable quantity which are required for sustaining present as well as future growth. The seeding of the principles for the differentiation process dates back to the 1970’s when embryonic carcinoma (EC) cell lines were established from teratocarcinomas [22]. These clonally originated EC cells had the differentiation ability to generate the cells of mesodermal, endodermal and ectoderm germ layers; however, the EC cells showed loss of differentiation potential indicating that they did not retain their pluripotent potential in long term culture. Taking cues from the culturing conditions of EC cells, establishment for the first murine ES cells line in early 1980’s became a reality and with this the desire to differentiate these ES cells into various mammalian cell types became the focus of intensive research [10, 11, 23-28].

1.2.6 ES cells ability to translate embryogenesis

Embryonic development is a dynamic process. During mouse embryogenesis, the three germ layers originate from primitive ectoderm of the epiblast. These three germ layers formed during embryogenesis are mesoderm, endoderm and ectoderm (Figure 1.2).
The interactions between these germ layers generate all tissues and organs of the developing embryo (Figure 1.2). The multifaceted interactions which regulate the switching between ectoderm to visceral and parietal endoderm in the post implantation embryo pursued by the formation of mesoderm at the gastrulation stage (days 3 to 7 post coitum), are only initiated to be clear. These *in vivo* processes are by unveiled by *in vitro* differentiation potential of ES cells. Differentiation is triggered by ES in absence of culturing ES cells as aggregates known as embryoid bodies (EBs), in dearth of signals for self-renewal given by feeder layers or LIF, either in hanging drops [29-32] in liquid “mass culture” [27] or in methylcellulose [33]. Further, co-culture with stromal cell line [34], and in monolayer cultures in the dearth of LIF [35] have been resulted into differentiated of ES cells *in vitro* (Figure 1.3). The sequential expression pattern of tissue and organ specific genes and proteins in ES-derived cells during *in vitro* differentiation...
signifies that early processes of *in vivo* development into ectoderm, mesoderm, and endoderm lineages are recapitulated *in vitro*.

![Diagram of various methods for formation of EBs]

**Figure 1.3: Outline of various methods for formation of EBs.**

### 1.3 Intrinsic factors regulating ES Cells self-renewal

ES cells maintain their ability for pluripotency via interactions of intrinsic factors in the form of transcription factors. Three transcription factors have been demonstrated as major regulators of ES cells pluripotency which are comprised of the POU domain-containing transcription factor Oct3/4, the homeobox transcription factor Nanog and a member of the HMG-domain DNA-binding-protein family Sox2 [36-38]. Oct3/4 and Nanog are known to play key roles in the formation and maintenance of the inner cell mass in mouse embryogenesis [39-41].

The POU domain transcription factor Oct3/4 is the master regulator of ES cells pluripotency during development and was the first transcription factor identified as controlling ES cell fate[42, 43]. Oct3/4 is expressed in oocytes and early embryos through to early somite stages and is thereafter maintained exclusively in the germ cell lineage. Oct3/4 is also found in EC and EG cells [40]. In established ES cells, triggered erasure of Oct3/4 leads to loss of self-renewal and differentiation. [41]. However, overexpression of Oct3/4 leads to differentiation of ES cells into endoderm and mesoderm. Thus, the levels of Oct3/4 seem to be paramount in sustaining ES cells self-renewal and to prevent differentiation of cells into trophectoderm[41]. It was noted that enforced expression of Oct3/4 does not firm or increase ES cell self-renewal. In contrast, even modest increase in expression triggers differentiation [41]. This finding suggested that Oct3/4 plays a dual role in self-renewal and differentiation and that its expression level must be accurately controlled to maintain pluripotency. In the contrary, low levels of Oct3/4 have been
shown to disturb ES cell differentiation without affecting self-renewal [44, 45]. A probable role for Oct3/4 in the implementation of lineage commitment \textit{in vivo} is constant with its continual expression in the postimplantation epiblast during admittance through the primitive streak [46-48].

The Sry-related transcription factor Sox2 is known to act interdependent with Oct3/4 to induce Oct-Sox enhancers, which control the expression of pluripotent stem cell-specific genes, including Nanog, Oct3/4 and Sox2 itself [39, 49, 50, 51]. The knockout of Sox2 leads to embryonic lethality shortly after implantation, signifies the essential role of Sox2 in maintaining cells within the epiblast in an undifferentiated state [39]. The deletion of Sox2 leads to trophoblast formation [39, 51]. Sox2 is more broadly expressed than Oct3/4 [39]. In addition, its expression was found in trophectoderm and later in all neuroectodermal cells and in different endodermal and epithelial tissues [52]. Overexpression of Sox2 influences ES cells to differentiation, indicating that, like Oct3/4, Sox2 expression levels should be constrained for efficient self-renewal of ES cells [53, 54].

In 2003, two different groups identified Nanog as an important regulator of pluripotency. Nanog is a homeodomain-containing transcription factor [36, 56]. Its expression \textit{in vivo} is more limited to the naive pluripotency compartment as compared to Oct3/4 and Sox2. On the other hand, Mitsui \textit{et al} identified Nanog and confers that is essential for establishment of pluripotency in inner cell mass and in ES cells [37]. Nanog null embryos resulted in embryonic lethality, with embryos at E5.5 comprised of disturbed organization of extra-embryonic tissue without an epiblast or extra-embryonic ectoderm. It was suggested that ES cells could not be isolated from Nanog null pluripotent epiblast [37]. It was observed that deletion of Nanog results in the differentiation of cells into trophoblast in the inner cell mass and, in addition, resulted in the lack of hypoblast development. On the other hand, Sox2 and Oct3/4 are expressed in the morula and in all the cells of the ICM and until the hypoblast has been separated [39, 56, 57]. Hence, Nanog restricted expression in the epiblast, in comparison with a wider expression of Oct3/4 and Sox2, suggests that Nanog may signify epiblast in cells that already express Oct3/4 and Sox2 [55-59]. Hence, it looks like that Nanog is essential during embryogenesis to identify pluripotent epiblast and later for proper development of germ cells [55, 61].
1.4 Signaling pathways in regulation of stem cell fate

Stem cells maintain pluripotency and the ability to self-renew through the interplay of various signaling pathways acting in both in vivo and in vitro conditions. The events in early embryogenesis of all vertebrate embryos are similar and involve the differentiation and specification of the dorsoventral and anteroposterior axes along with the accurate spacing of the three germ layers (ectoderm, mesoderm and endoderm) during gastrulation. This is achieved primarily by cell-cell interactions mediated by the JAK/STAT pathway, bone morphogenetic protein (BMP), hedgehog, nodal, Hippo and Wnt pathways, which comprise the major embryonic signaling pathways [62-65] (Figure 1.4).

![Figure 1.4: Signaling pathways known to regulate ES cell fate.](image)

1.4.1 LIF/gp130/STAT pathway

Communication between cells through the secretion of cytokines is an established fact and the subsequent binding of cytokines with their cognate receptors results in the phosphorylation of tyrosine residues of the bound receptor. The LIF/gp130/STAT pathway plays essential part in regulating future of stem cells like differentiation and proliferation, in reply to growth stimulating factors and cytokines. This pathway in mouse ES cells plays a key role in maintaining the pluripotent state. The in vitro culture of mouse ES cells requires the addition of LIF that binds to the cytokine receptor gp130 and LIFR [66]. The heterodimeric complex of gp130 and LIFR is a receptor for LIF [67]. Then constitutively binding of tyrosine kinase Janus kinase (JAK) to the intercellular domain of this receptor complex takes place in its inactive form. Consequent to LIF binding, JAK kinase phosphorylates tyrosine residues of gp130 and LIFR.
Phosphorylation of the intracellular domain of gp130 and LIFR recruits signal transducers and activators of transcription (STAT) 1 and STAT3 through their SH2 domains [68]. STATS are activated by JAK-mediated tyrosine phosphorylation which forms homodimers and/or heterodimers and leads to nucleus translocation, and there they act as transcription factors [69].

1.4.2 BMP/Smad pathway

Bone morphogenetic proteins (BMPs) belong to the family of transforming growth factor (TGF). BMPs are secreted ligands which bind to heterodimeric complexes of type I (ALK2, ALK3, ALK6) and type II (BMPRII, ActRII, ActRIIB) receptor tyrosine kinases. BMPs binding provoked twisted formation of the receptor components and assists phosphorylation of Smads. The Smads are intracellular signal transduction molecules; they are divided into three categories: receptor-regulated Smads (R-Smads (Smad 1, Smad 5 and Smad 8), cooperating Smad (Co-Smad) and inhibitory Smads (I-Smads (Smad 6 and Smad 7). Upon BMPs binding, R-Smads are phosphorylated and form heteromeric complexes with Smad 4 (the single Smad known in mammals) [70, 71]. Nucleus translocation of Smad complexes attained, and they act as transcription factors. Ying et al explained that BMP4 and LIF were associated with each other in the maintenance of pluripotency of mouse ES cells [72]. In serum-free conditions, LIF alone triggered neural differentiation in ES cells. Though, addition of BMP4 suppressed neural differentiation and maintained the undifferentiated state of mouse ES cells, even in the absence of serum [73]. It was shown that BMP4 induced the expression of inhibitor of differentiation (Id), an inhibitor for basic helix-loop-helix transcription factors known to be involved in many cell fate determinations, including neural differentiation and represses the neuroectodermal marker Pax6 and up regulates Brachyury in a concentration-dependent fashion, independent of cell survival or proliferation [74-76]. BMP4 promotes differentiation of cellsakin to posterior–ventral embryonic mesoderm, while dorsal–anterior mesodermal cell kinds are provoked by one more TGFβ-related molecule, Activin A [74]. The efficiency of BMP4 in shifting cell fate is limited to a period prior to the onset of neural differentiation in culture [77]. Under different conditions, BMP4 induces the differentiation of ES cells toshell of ectoderm at the cost of neuroectoderm, emphasizing the properties of BMP4 as a suppressor of neuroectodermal differentiation.
1.4.3 Wnt pathway

Wnt signaling pathway regulates lineage specification in embryogenesis and pluripotency in ES cells, but how the equilibrium between progenitor self-renewal and differentiation is controlled during axis specification and tissue patterning is still not clear. The stage-specific effects of the different Wnt pathways results in multifaceted effects during development [78]. In report by Jeanisch and co-workers, it was shown that over expression of Oct-4, an additional pluripotent gene causes bruises of progenitor cells and amplified the β-catenin transcriptional activity [79]. Another data reported that establishment of Wnt signaling, by genetically deleting the task of the negative regulator APC, enhances the undifferentiated phenotype of mouse ES cells [80]. The importance of Wnt signaling for embryonic mesoderm induction is reflected in ES cell mesodermal differentiation [81]. Using EBs as a biological system of the embryo, Keller and his coworkers have shown that Wnt3a in coordination with TGF β signaling to induce mesoderm as identified by Brachyury expression, in differentiating EBs [82]. Though, these experiments involved the addition of growth factors to a serum-free culture medium for stimulation of mesoderm lineage, when EBs were cultured in serum containing medium, spontaneous mesoderm induction occurred. Dkk1 has been shown to antagonize spontaneous mesoderm formation, while BMP4, a member of the TGF β superfamily, can also rescue the induction of mesoderm [83, 84]. Both studies confirm that Wnt and TGF β pathways cooperate in mesoderm differentiation of ES cells.

1.4.4 The Notch pathway

Notch signaling is an evolutionarily preserved pathway in multicellular organisms which control cell fate during development and maintains adult tissue homeostasis. The Notch pathway facilitates juxtacrine cellular signaling wherein both the signal sending and receiving cells are affected through ligand-receptor crosstalk by which congregating of cells cell fate determinations in neuronal, cardiac, immune, and endocrine development were regulated. Notch receptors are single-pass transmembrane proteins comprised of functional extracellular (NECD), transmembrane (TM), and intracellular (NICD) domains. Through interaction with Notch ligands such as Deltalike1 (Dll1) and Jagged1 (Jag1), the transmembrane protein Notch is sliced by γ-secretase, releasing NICD. In stem cell biology, Notch signaling is highly milieu-reliant, and the biological consequences of pathway initiation can be range from stem cell maintenance or
expansion to promotion of stem cell differentiation [85-88]. Momentary activation of Notch signaling during distinct stages of ES cell differentiation has been proposed to up-regulate and or direct the generation of specific, therapeutically relevant tissue precursor cells [85]. Time specific studies on the activation of Notch/RBP-J signaling at 1, 2, or 3 days after initiation of ES cell differentiation into mesodermal cell lineages pointed out that production of Flk-1 mesodermal cells was abridged by activated Notch, signifying that Notch/RBP-J signaling may block the generation of Flk-1 cells at several stages of mesoderm induction [89, 90]. In contrast, activated Notch appears to enhance the neural commitment of ES cells when cultured in the absence of self-renewal factors [90, 91]. Collectively, these reports might suggest that Notch signaling plays a role in mesodermal development, in cardiomyogenesis, and in balancing the generation of endothelial cells versus vascular smooth muscle cells of blood vessels. As a result, although Notch signaling can amend the outcome of ES cells differentiation, so appears to be no in vivonecessity for this pathway until all the three germ layers are formed [92].

1.4.5 Hippo pathway

Hippo signaling is an evolutionarily conserved pathway that controls organ size by regulating cell proliferation, apoptosis, and stem cell self-renewal. Center to the Hippo pathway is a kinase cascade, in that way Mst1/2 (ortholog of *Drosophila* Hippo) kinases and Sav1 form a complex to phosphorylate and switch on LATS1/2. LATS1/2 kinases in turn phosphorylate and restrict the transcription co-activators YAP and TAZ, two important downstreamassemblers of the Hippo pathway. When dephosphorylated, YAP/TAZ was translocated into the nucleus and the interaction between TEAD1-4 and other transcription factors lead to the expression of genes whichsupported cell proliferation and inhibited apoptosis. In mammals, YAP and TAZ are phosphorylated by LATS1/2 in vitro and in vivo [91-93]. The mechanism of inhibition by Hippo signaling comprises phosphorylation of Ser 127 in YAP or the corresponding sites in TAZ and Yki, which enhanced 14-3-3 binding and subsequent cytoplasmic sequestration and inactivation [92-98]. YAP and TAZ regulate ES cell self-renewal in response to TGFβ/BMP signaling [99-100]. Further, two studies have shown that, during ES cells differentiation, YAP was inactivated, and the knockdown of YAP or TEAD proteins resulted in loss of pluripotency [101, 102]. In ES cells, YAP relates with Smad1 to direct Id gene transcription for ES maintenance in reply to BMP stimulation [103]. The reports have demonstrated the link among YAP/TAZ-dependent BMP/TGFβ (BMP/TGFβ) transcriptional output, ES cells maintenance and differentiation. On
the other hand, YAP is activated in induced pluripotent stem cells, enhanced the reprogramming competence, and inhibited differentiation in ES cells when it is overexpressed in derange manner [101]. These studies also indicated that in ES cells YAP- TEAD binds to and enhances the transcription of genes which maintain (Oct3/4, Sox2, Nanog, LIF targets, and BMPs targets) [104].

1.4.6 Hedgehog pathway

The naturally conserved Hedgehog pathway plays a major role in a time and position-dependent fashion during development by regulating patterning and maintenance of proliferative niches. Hedgehog-family ligands (Sonic, Desert, and Indian Hedgehog) require accurate secretion and gradient distribution, and involve auto processive cleavage as well as cholesterol and palmitate lipid modifications [105-108]. Signaling in this pathway is provoked when the secreted protein Hedgehog binds to its membrane receptor, Patched. Patched blocks the seven-spanner transmembrane protein Smoothened (Smo) and binding of Hedgehog inhibit Patched consequential in Smo derepression. Active Smo then promotes the activation of the cytoplasmic steps of the signaling pathway [105, 108]. However, through the analysis of Gli2 and Gli3 mutant mice, a complex and differential use of Gli in various Hh-dependent processes during embryonic development was revealed. For example, Shh is a mitogen and leads to cell proliferation in different embryonic and adult tissues. In addition to cell proliferation and its regulation, Shh signaling has a major role in the survival and patterning of neural progenitors. It enhances survival and proliferation of neural progenitor cells in the ventral spinal cord by blocking Gli3[109]. Moreover, Shh signaling variously utilizes GliA or GliR to control the expression of distinct sets of homeodomain proteins in different progenitor cell populations [110].

1.5 Factors influencing the ES cells fate

The development of stem cell–based application is totally dependent on an understanding of the underlying factors which regulate stem cell responses. Although the mechanism of differentiation is unlikely to be conserved during embryogenesis and in all adult tissues, some important comparable parameters can be made between various stem cell differentiation strategies [111, 112]. Differentiation of ES cells is the specification into the three germ layers, i.e. ectoderm, mesoderm, and endoderm [113-117]. These processes in vivo are regulated by
numerous signaling pathways which are considered as key regulators of stem cells fate. Understanding the crosstalk within these signaling pathways and the available physico-chemical cues are essential to explain how unique attributes of ES cells mediate the construction of tissues and organs which are three-dimensional entities. Although it is difficult, characterizing the cell signaling along with physico-chemical factors which are critically required in directing stem cell differentiation is very important to understand and replicate the differentiation process. This can be studied using the popularly recognized method of EB formation wherein the ES cells cultured in suspension are allowed to self-aggregate and spontaneously differentiate into various cell types. The 3D structures of the EB resemble the early embryo in which cell-cell and cell-matrix interactions abound and lead to the formation of the germ layers as well as their various cell types. A number of physico-chemical factors have been identified which direct the tissue and organ formation both in vivo and in vitro. In vitro, the physico-chemical factors include; composition of media, fetal bovine serum (FBS), chemical compounds, growth factors, cytokines, oxygen, carbon dioxide and pH level, all of which have shown varied sensitivity and selectivity towards differentiation of ES cells (Figure 1.5).

**Figure 1.5: Factors influencing differentiation of ES cells.**
CHAPTER 1

1.5.1 Media

ES cells are undoubtedly precious because of their origin and potential application in the health industry. Till date there is no universal stem cell culture system that can effectively reproduce in vivo conditions and therefore, this is a topic of intense research. It is imperative to provide the ideal culturing environment so as to keep them healthy and at the same time ensure the fidelity of their differentiation potential so as to be certain of the credibility of experimental data. Keeping in mind their origin, various types of media with different compositions have been introduced for ES cell culture over the years which allow researchers to expand stem cells without compromising on their ability to differentiate into the three germ layers.

In 1981, the first ES cell line was established from mouse [9, 10] and these cells were cultured on plates coated with mitotically inactivated mouse embryonic fibroblast (MEF) called as ‘feeder layers’. The media used to support these cells was supplemented with FBS (specially chosen from batches) or conditioned media derived from teratocarcinoma stem cell cultures. The feeder coating provided a matrix environment which allowed the ES cell attachment and exuded a variety of growth factors which supported the maintenance and propagation of ES cell populations [118], whereas the supplemented FBS [119, 120] was a storehouse for hormones and vital nutrients required for the growth and maintenance of the ES cells. Inadequacy of these culturing systems due to their undefined compositions was attributed for inconsistent effects on the growth and differentiation of ES cells. Thus, need of the hour was to provide a chemically defined media which could efficiently and consistently support the growth and maintenance of these ES cells. In 2004, Knockout serum replacement (KSOR), a chemically defined substitute for FBS was introduced. This media was able to maintain the mouse ES cells but still required the presence of the feeder layers [121]. Problems, however, persisted with the KSOR and batch to batch variations were still prevalent [122]. In 2008, it was observed that DMEM/F12 supplemented with N2 which comprised of transferrin, insulin, progesterone, putrecine and selenite along with LIF and basic fibroblast growth factor (bFGF) was able to maintain the cells without the support of either serum or feeder cells [123-124].
1.5.2 FBS/FCS (fetal bovine serum/ fetal calf serum)

FBS/FCS is one of the most important components of cell culture medium and provides growth factors, hormones and supports binding of cells with factors present in the extracellular matrix, moreover it shows minimal effect as a growth inhibitory factor \cite{119,120}. FBS is a standard requirement of cell culture and is used at concentration ranging from 10\% to 20\% for culturing stem cells. The application of FBS in regenerative medicine and drug discovery is still controversial as the composition of serum is not accurately defined and also harbors the threat of transferring viral, bacteria and other infective agents to the stem cells \cite{125,126}. Moreover, various researchers use treatments such as heat inactivation of the serum prior to use and the consequence of this on stem cell maintenance and differentiation is debatable as very few reports are available \cite{128}. During embryonic development, lineage commitment requires the secretion specific growth factors \cite{129,130}. It was reported that in the absence of serum, BMP4 was not capable of inducing cardiac differentiation \cite{131}. Since serum itself has pleitropic effects, hence in the presence of serum, the specific role of growth factors in directed differentiation is confounding and hence remains elusive. Therefore to get a clear picture, the expression of specific lineage markers needs to be explored in the presence or absence of serum in \textit{in vitro} differentiation models.

1.5.3 LIF

The pluripotent colonies from inner cell mass maintained \textit{in vitro}, require feeder layers of fibroblast or the presence of DIA (inhibition of differentiation activity) which is a soluble product obtained from many sources \cite{66,132-136}. Williams \textit{et al} were the first to see the similarities between DIA and a hematopoietic regulator myeloid LIF which is a member of the Interleukin 6 family and reported that cells treated with recombinant LIF were able to resist the differentiation of ES cells \cite{137}. LIF is routinely used in labs for maintaining ES cells in feeder free cultures and plays multifunctional roles in ES cell biology which includes cell survival, proliferation and surprisingly even differentiation. It was suggested that the distribution of DIA/LIF and other cytokines during ES cell differentiation might play an important part in early embryo development \cite{133,138}. For example, it was observed that during EB differentiation, the presence of LIF inhibited the development of visceral and parietal endodermal cells, without altering the differentiation of the primitive endodermal cell precursors of these extra embryonic
cell lineages. Moreover, the decreased expression of FGF-5, muscle, and neuronal markers indicated that the differentiation of primitive ectodermal cells was also inhibited by LIF. Further, Nicholas et al reported that mRNA of LIF and its counterparts LIF-R (LIF-receptor complex) and gp130 were untraceable in 2 cell embryos, but traceable in blastocyst stage during early mouse embryogenesis [139, 140]. A complementary relationship was found in an inner cell mass where the LIF transcripts were missing but LIFR transcripts were localized.

1.5.4 Other growth factors and cytokines

During embryogenesis, the growth factors secreted by microenvironment or by cells, are firmly controlled in space and time [129,141- 142]. Differentiation is a result of expression of a specific subset of genes in which the cell is determined or forced to commit into a functional cell lineage. Combinations of cytokines and their presence in different concentrations along with their associated receptors are key diverging points of stem cell fate and a significant body of work has been done to correlate cytokine and growth factor with their addition at specific time periods in culture. Taking cues from such studies would be an important tactic in directing differentiation of ES cells [143-145].

To obtain functionally differentiated cells, an understanding about the cocktail of cytokines and time point of delivery in culture is a particularly challenging factor for the optimization of differentiation, as various cytokines in parallel are influencing stem cell fate directly or indirectly. Since ES cells recapitulate the early stages of development, they provide a very useful model system to critically assess the role of growth factors and cytokines on the differentiation process. Research over the past few years has focused on numerous factors having the ability to induce directed differentiation of mouse ES cells. It was seen that cytokine IL-3 directed cells to the myeloid lineage specifically macrophages, mast cells or neutrophils [146], whereas IL-6 lead to differentiation into the erythroid lineage [147]. TGFβ1 was shown to induce myogenesis [148, 149], while VEGF-A or BMP4 directed differentiation of ES cells to endothelial cells [150, 151]. As it is apparent from these reports, each growth factor has its unique influence which can lead to directed differentiation. Studies carried out by Schuldiner et al in human ES cells were landmarks in the sense that they threw light upon factors influencing directed differentiation [130]. They were able to broadly categorized a set of eight growth factors that they studied according to their effects on differentiation, as assessed by the
expression of cell specific markers that covered all the three lineages: Activin-A and TGFβ1 lead to the induction of the mesodermal lineage; retinoic acid, EGF, BMP4, and bFGF were able to induce ectodermal as well as mesodermal lineage while NGF and HGF had a universal effect and lead to the differentiation into the three embryonic germ layers [130, 149-152]. From the study, it was evident that although different factors affected the differentiation in their own subtle manner, however, there was no one factor that resulted in the differentiation to one particular cell type.

1.5.5 Oxygen

O₂ is one of the major factors influencing cell fate. It is the prime source of metabolic energy; almost every cell type can perceive and sense limited O₂ supply (hypoxia) [153] and purposely stimulate hypoxia inducible factor (HIF - a set of O₂ regulated genes) [154]. Adaptation to the low levels of O₂ during embryogenesis is a consequence of the fact that the stem cells naturally occupy hypoxic niches and therefore, the differentiation has to directly correlate with the O₂ concentration so as to be successful [155]. Therefore, an understanding of the levels of O₂ in the micro milieu will significantly contribute to more efficient differentiation of ES cells in vitro [156]. Evidently, tissues and organs have different requirements for O₂ and this corresponds to the O₂ tension in the tissue. Various reports have shown that the stem cell niches show O₂ concentrations well below the atmospheric levels of 21%, suggesting that O₂ concentrations of 1 to 8% are more suitable to mimic the in vivo environment and can be referred to as physiologic normoxia rather than hypoxia, in contrast to culturing at 21% O₂ which actually would be a state of hyperoxia [157]. In fact, it has been reported that the in vivo pO₂ levels in monkey blastocyst can be as low as 11 mm Hg (1.5% O₂) [158].

It appears that the ultimate effect of hypoxia or physiologic normoxia depends upon several factors, including the stem cell line, degree and duration of hypoxia, as well other variables present in the culture media. Intensive research over the past few decades has focused on the role of varied O₂ tensions to which the cells are exposed and their response on differentiation of embryonic, neural, hematopoietic and trophoblast stem cells [159-164]. Contradictory reports exits on the role of hypoxia in stem cell biology. Whereas on one hand hypoxia related studies have shown a marked reduction in differentiation capability but enhancement in pluripotency of human ES cells [165-166] and refined the clonal survival of
mouse ES cells [167]. Others studies had reported that the hypoxic environment promotes differentiation of ES cells to mesoderm and the generation of hemato-endothelial progenitor cells [168], differentiation into the three germ layers [169] and furthermore, have revealed that low O₂ tension assists differentiation into specific lineages like chondrocytes and cardiomyocytes [170]. Moreover, cells cultured under the hypoxic environment improved the generation of the induced pluripotent stem cells (iPS cells) of both mouse and human [171]. Hypoxic priming speeds up the vascular differentiation via HIF1-regulated inverse control of Oct4 and VEGF in mouse ES cells [172]. In 2010, Rodrigues et al reported the enhanced proliferation of neural stem cell derived from mouse ES cells [173]. The hypoxia supported the enhanced proliferation and survival of ES cells derived neural stem cell as well as dopaminergic differentiation [174]. Moreover, derivation of neural precursor cells from human ES cells at lower O₂ tensions lead to enhanced survival with no impact on regional specification and functional differentiation [175]. Differentiation into endodermal and hepatic progenitor cells was also efficiently enhanced under hypoxic condition [176]. The role of hypoxia during embryogenesis in stimulating angiogenesis has also been reported [177]. The O₂ tension, to which ES cells have adapted themselves, is an important factor which influences differentiation propensity. Moreover, the behavior of mouse and human ES cells is different and might vary with different cell lines, for example, low O₂ levels retard the spontaneous differentiation in human ES cells [178].

In the light of all the above findings, in depth research is required to explore the function of hypoxia and the associated genes, which might regulate differentiation in ES cells. Moreover, the differential effect of hypoxia with other factors also needs to be addressed in a standardized fashion.

1.5.6 Carbon dioxide and pH

Carbon dioxide (CO₂) is an important component of environmental gases and plays a key role in mammalian physiology. This gas is found at low levels in the atmosphere but its level within tissues and organs is comparatively higher. Changing the levels of CO₂ leads to either hypercapnia (high CO₂) or hypocapnia (low CO₂) which might be associated with a number of physiological conditions in humans [179, 180]. During pregnancy, a state of hypocapnia exists within the uterus [181, 182] and therefore, culturing of ES cells in vitro under 5% CO₂ should be reconsidered. There exists a close relationship between O₂ consumption and CO₂ production, and
the ability of ES cell to adapt in low O$_2$ is likely to be closely linked to its ability to adapt to low CO$_2$. However, the nature of this crosstalk is poorly understood and it is an area which needs further investigation. Although, a number of reports on O$_2$ tension and its effect on differentiation are being explored, the role of CO$_2$ in a similar context has been ignored. Recently, role of hypocapnia in mouse ES cells was investigated wherein it was shown that hypocapnia enhanced mesodermal and endodermal lineage differentiation with no untoward effect on pluripotency [183].

The pH of the culture medium is an important factor which plays a significant role in maintaining the vitality of cultures and therefore might influence differentiation. The sensitivity of mouse ES cells to changes in pH was demonstrated by Teo et al which showed that reducing the pH to 6.8 resulted in reduced viability as well as down-regulation of cardiac marker gene expression but supported pluripotency marker expression of ES cells. They also reported that cardiomyocyte differentiation of ES cells was also sensitive to pH changes [184]. Another study reported that mouse ES cells derived EBs were more sensitive to pH and osmolality as compared to medium glucose, glutamine, ammonium and lactate [185].

1.6 ES cells as a toolkit for drug research and studying mammalian development

The influence of chemicals during embryogenesis can irreversibly interfere with the normal development of an embryo, creating malformations and other birth defects. ES cells are the only available cells which have the differentiation potential to go through all developmental stages when cultured in vitro, from a pluripotent cell to a differentiated mature cell i.e. somatic cell/tissue. Thus, this in vitro model comprises most stages of embryonic development. Exposure of ES cells during in vitro differentiation with the physico-chemical factors of interest may be very useful to predict effects on embryonic development and also providing insights on underlying mechanism. Stem-cell based assays and technology has the potential to revolutionize drug discovery. Models of differentiated cells that are derived from mouse ES cells are already in use in drug discovery, and are beginning to find uses in high throughput screens. Embryonic stem cell test (EST) is one of the assays which have been validated to predict the influence of chemical compounds on embryonic development. This test was proposed by Spielmann et al (1995, 1997) [186, 187]. The other embryotoxicity tests require killing of pregnant animals.
whereas the EST uses two permanent cell lines of mouse origin, ES cells and differentiated adult 3T3 fibroblasts cells.

1.6.1 Embryotoxicity and cytotoxicity

Embryotoxicity tests are currently conducted with OECD (Organization for Economic Co-Operation and Development) guidelines. In vivo tests used are more time consuming, expensive and require a lot of skill and expertise. Moreover, these experiments require a number of laboratory animals to be sacrificed which raises ethical concerns and to lower down the animal experimentation, many in vitro methods have been developed. These include whole embryos from whole embryo culture (WEC), Xenopus (FETAX) test or chicken embryotoxicity screening test (CHEST). However, all these assays have been used rarely because their predictive value is only 70-80% [188-190].

According to stringent testing measures, 30,000 chemicals that are currently in the market will have to be re-evaluated over the next ten years within the European Union with an estimated use of 10 million animals for in vivo teratogenicity testing. Therefore, in vitro developmental toxicity tests need to be established in order to reduce the number of test animals and expenses without compromising the safety of consumers and patients. Furthermore, such in vitro methods would be better suited to test a larger number of chemicals as compared to in vivo tests [191-192].

More than 30 in vitro assays using invertebrates or vertebrates to predict the embryotoxic potential of test compounds have been developed. For the prediction of reproductive effects in humans, mammalian in vitro assays are the first choice. Three assays based on ontogenesis have been validated by an international study namely, the micromass test systems which use dissociated cells from the limb buds and brains of rat embryos, whole frog embryos (the Frog Embryo Teratogenesis Assay) and whole rat embryos culture test [189, 193-196].

1.6.2 Aggregates and micromass (MM) test

Different aggregates and MM cultures have been used in embrotoxicity testing. One of these systems aggregates of primary cultures makes use of chick embryo neural retina cells (CERC) [189, 197]. When these cells were cultured in small volumes at high density, they formed numerous small foci of differentiating chondrocytes. In the MM, the inhibition potential
of chemicals on the differentiation of limb bud cells and chondrocytes was evaluated. Dissociated cells from cultures of the limb buds of rat embryos at gestational day 14 (approximately 45 somites) were studied [198]. The seeding density was kept high and cultured in the presence or absence of test chemicals for 5 days, and growth and differentiation of the cells into chondrocytes were evaluated [199]. Alcian blue was used to specifically stain cells which have developed into chondrocytes and the cytotoxic effect was detected after which ID50 (50% inhibition of differentiation) and IC50 (50% inhibition of growth) values were calculated [199]. Besides this, the MM is still dependent on animals for the derivation of cells which turn this assay more of a refinement than a replacement of animal testing.

1.6.3 Rat whole embryo culture (WEC) Test

In the WEC, embryos were explanted and cultured for the time during organogenesis [200] More particularly, post implantation rat embryos at GD 10 (1-5 somite stage) were cultured for 48 hours in rat serum and treated with test substances [201]. In this time period, key process of the organogenesis occurred, like neural tube closure, cardiac development, as well as development of the ear and eye, and limb bud formation. Therefore, WEC made possible the evaluation of dysmorphogenesis of specific structures and general hindrance during growth and development. Function and morphology were recorded at the end of the incubation period, and the embryos were scaled according to a criteria system adapted from Brown and Fabro [202]. The mean score of total morphological value of seven embryos per concentration were then examined and weighed against to a control group [199]. Furthermore, the numbers of malformed or dead embryos were analyzed with the control group, to examine the no-effect concentration and maximal effect concentration as well as IC50[201]. Along with MM, WEC also required live animals for embryos, but since all embryos in a trash could be monitored separately, a reduce use of animals could be achieved.

Animal-free cell line based assays include the ECVAM (European Centre for the Validation of Alternative Methods ) validated ES cell test which is probably the most extensively studied test in its class as no pregnant animals have to be sacrificed, since two permanent mouse cell lines (D3 and 3T3) are used [186,187].
1.6.4 Embryonic stem cell test (EST)

Using the *in vitro* ES model, various chemical compounds were screened using inhibition of cytotoxicity (IC$_{50}$) of both undifferentiated and adult cells along with inhibition of differentiation (ID$_{50}$) of contracting cardiomyocytes values as endpoint [187, 203]. Based on their results, chemicals were classified into three major classes as "non-embryotoxic", "weakly embryotoxic" and "strongly embryotoxic" [187, 203, 204]. In order to improve the sensitivity and accuracy, this EST assay underwent several modifications by independent researchers [203, 204], but there were certain restriction flaws in EST. The quantification of contracting cardiomyocytes by morphological microscopic evaluation was complex to standardize and could only be carried out by skilled person. It was demonstrated that the EST could be tailored for more accurate and reliable quantifiable molecular endpoint [205-208]. Using this molecular EST, the alterations in expression of the cardiac marker gene, myosin heavy chain (MHC), exposed to test compound, was measured by qRT-PCR and served as an alternative endpoint for inhibition of cardiomyocyte differentiation [208]. The established EST [186] as well as the molecular EST [208] were able to screen chemical compounds for their effects particularly in cardiac development. However, the analysis of inhibition of cardiac differentiation alone as an endpoint was also not dependable because most of teratogenic compounds are known to cause birth defects like neural tube defects, congenital limb deformations and skeletal abnormalities. The use of different tissue markers as multiple molecular endpoints was developed by Zur Neiden *et al*[205]. Further, an improvement in EST was made by using quantitative FACS (fluorescence-activating cell sorting) analysis which showed the similar sensitivity for the classification of compound as the conventional endpoint along with significant reduction in test time. Tissue-specific proteins in ES cell cultures could also be studied using immune fluorescent antibody technique and FACS analysis. The expression of tissue-specific marker proteins, α-actinin and MHC was quantified by intracellular flow cytometry assay. Based on kinetic analysis, the strongest signals were observed at day 7 of differentiation indicating that a reduction in protein expression induced by embryotoxic compounds could be best monitored at day 7 of culture. To determine whether FACS with cardiac-specific marker proteins can be used as a new toxicological endpoint in the EST, selected compounds with known teratogenic potential were tested and the results were compared to those obtained with the existing EST. Almost identical dose–response curves were obtained with both methods. Based on these results it was clear that
FACS analysis could replace the microscopic evaluation of beating cardiomyocytes in the EST and α-actinin along with α/β MHC could be used as marker proteins for cardiac development. It was also suggested that tissue- or organ-specific antibodies labeled with immune fluorescent dyes could be useful in screening a high number of test chemicals in the EST. A genetically engineered mouse ES cell line expressing green fluorescent protein (GFP) under the control of cardiac α-actinin was developed which could be easily analyzed by FACS method[205-210].

Well-chosen endpoints may contribute to examining the mechanism of chemical compounds and deciding their dose concentrations in toxicology studies. In vitro testing must include homogenous cell interactions for which response for particular tissue or cell must be evaluated. These approaches will limit the questions of embryotoxicity. The ES cells could be used directly for providing insights into those events occurring during development of embryo and show us how stem cell changes their fate in vivo. This potential of ES cells could be used to identify the toxicants and show us how the differentiation of ES cells changes upon exposure to chemical compounds. This might enhance our understanding of basics of ES cell biology and factors related to them which may contribute to new way of exploring development biology. Understanding how the chemical factors influence embryo development, is an important parameter in developing new approaches for regenerative medicine and drug discovery. For this, ES cells provide a reliable in vitro model for understanding differentiation during development.

In this study, EST was used as an in vitro assay system to analyze the effects of chemical compounds on differentiation during embryogenesis and understanding as well as identifying their mechanism of action by evaluating the expression of markers of different lineages. In addition, using ES cells as a model system which reflects embryonic development the effects of hypocapnia were also studied to see whether lowered CO₂ was a condition which was preferred by the ES cells for both maintenance of pluripotency as well as for differentiation. A schematic representation of the study design is given in Figure 1.6.
Figure 1.6: Schematic presentation of work design.
REFERENCES


4. Department of Genetics (GENIE-Genetics Education Networking for Innovation and Excellence), University of Leicester.

http://www2.le.ac.uk/departments/genetics/genie/gs/law/lawembryonic


31. Wobus, A.M., Guan, K., Yang, H.T. & Boheler, K. *Embryonic stem cells as a model to study cardiac, skeletal muscle, and vascular smooth muscle cell differentiation*. In: Methods


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184. Teo, A., Mantalaris, A. & Lim, M. Influence of culture pH on proliferation and cardiac differentiation of murine embryonic stem cells. Biochemical Engineering Journal 2014; http://dx.doi.org/10.1016/j.bej.2014.05.005


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196. Rutledge, J.C. Developmental toxicity induced during early stages of mammalian embryogenesis. Mutat Res, 396 pp 113-271997


