INVESTIGATING FACTORS INFLUENCING *IN VITRO* DIFFERENTIATION DURING DEVELOPMENT USING MOUSE EMBRYONIC STEM CELLS AS A BIOLOGICAL SYSTEM

BY

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A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR

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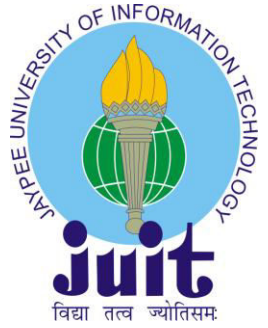

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This thesis is dedicated to my dearest Grandmother

Your guiding hand on my shoulder will remain with me forever

DECLARATION

I hereby declare that the work contained in the present PhD thesis entitled **"Investigating factors influencing *in vitro* differentiation during development using mouse embryonic stem cells as a biological system"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my original research work, carried out under the supervision of **Dr. Simran Tandon**. This work has not been submitted in part or full for the award of any other degree or diploma in any other University/Institute.

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CERTIFICATE

This is to certify that the thesis entitled, **"Investigating factors influencing *in vitro* differentiation during development using mouse embryonic stem cells as a biological system"** submitted by **Saras Jyoti (Enroll. No. – 106559)** to the **Jaypee University of Information Technology**, **Waknaghat, India** for the award of degree of **Doctor of Philosophy in Biotechnology** is a record of the candidate's own work, carried out by her under my supervision. This work has not been submitted in part or full to any other University or Institute for the award of this or any other degree or diploma.

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(Saras Jyoti)

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ABBREVIATIONS

5-FU	5-fluorouracil
AFP	Alpha fetoprotein
ANOVA	Analysis of variance
AP	Alkaline phosphatase
BSA	bovine serum albumin
CNS	Central nervous system
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
EBs	Embryoid bodies
EC	Embryonal carcinoma
EG	Embryonic germ
ES	Embryonic stem
EST	Embryonic stem cell test
FACS	Fluorescence activating cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescein-5-isothiocyanate
Flk-1	Vascular endothelial growth factor receptor-2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
hESC	Human embryonic stem cell
HIF	Hypoxia inducible factor
HMGR	β-hydroxy- β-methyl Glutaryl-Coenzyme A reducatse
IC_{50}	50% inhibition of growth
ICC	Immunocytochemistry
ICM	Inner cell mass
ID_{50}	50% inhibition of differentiation
iPSC	Induced pluripotent stem cell
KOSR	Knockout serum replacement
LIF	Leukemia inhibitory factor
MHC	Myosin heavy chain

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nes	Nestin
PBS	Phosphate buffered saline
Pen G	Penicillin G
PI	Propidium iodide
qRT-PCR	Quantitative real time polymerase chain reaction
RA	All *trans* retinoic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
VEGF	Vascular endothelial growth factor

ABSTRACT

Mouse embryonic stem (ES) cells perpetuate *in vitro* developmental potential of creator cells of the blastocyst in the developing embryo. ES cells persistently self-renew in *in vitro* culture while recreating embryogenesis impeccably, differentiating as planned to generate all aspects of the developing fetus. The ES cell emanates stability, homogeneity and immense differentiation potential. The study was based on a concept that physico-chemical factors present in the environment might influence the differentiation potential of these incredible cells during embryogenesis. This thesis provides insights into differentiation ability of ES cells, which are of great importance when it comes to future cell-based applications in regenerative medicine, drug screening, functional genomic applications and for studying early mammalian embryonic development. This study evaluated the reliability of ES cells cultured *in vitro* as a model system mirroring cells in the developing embryo upon exposure to physico-chemical factors, and examined their differentiation efficacy by analyzing the expression of key lineage markers to reveal the effect of growth environment on embryonic development. This work has thrown light on the genetic basis of physico-chemical factors influencing lineage differentiation of ES cells during embryogenesis.

The impact of exposure to chemical factors in the ES cells was evaluated initially on the basis of the validated *in vitro* embryonic stem cells test (EST). Further, in the study gene expression analysis was carried out to not only make the EST easier but also have a wider applicability. The study explored current knowledge of the EST model in governing the influence of chemical compounds, influencing lineage differentiation during embryogenesis.

In this study, after the initial standardization of EST protocol with a training set of drugs (whose *in vivo* embryotoxic potential and harmful effects are well documented) another class of drugs, statins (whose harmful effects during the fetal development are still not very well understood), were investigated for their embryotoxic potential. The expression level of key genes controlling the differentiation into ectodermal, mesodermal and endodermal lineages was examined by quantitative real time-polymerase chain reaction using two different protocols of differentiation. The systematic exposure of statins was given to ES cells during the different stages of the differentiation process. The results provided an insight into the genetic basis of developmental toxicity due to statin intake.

Further, the utility of mouse ES cells as a cellular model for developmental toxicity testing of homeopathic remedies (*Nux Vomica* and *Sepia*) at potency 30C was investigated. Firstly, cytotoxicity of homeopathic remedies on ES cells was tested and, secondly, the influence of these remedies was explored at genomic levels. The results revealed that homeopathic treatment led to non significant cytotoxicity towards embryonic and adult cells and slight modulations in the expression of certain lineage specific genes but this effect was also not significant. These observations supported the use of these homeopathic remedies during the sensitive stage of pregnancy.

Finally, the study presented a perspective on the role of a novel physiological factor *i.e.* hypocapnia (lower carbon dioxide tension) on ES cell characteristics. In this study, the gene and protein expression of pluripotent markers along with the morphological changes in ES cells were analyzed when subjected to hypocapnia. In addition, the differentiation potential of ES cells under varying carbon dioxide tensions was examined at various time periods *i.e.* day 10 (5+5d), 12 (5+7d) and 15 (5+10d). Enhanced expression of pluripotent markers (Oct3/4, Nanog and Sox2) was observed both at gene and protein levels. The differentiation propensity was particularly influenced towards the mesodermal and endodermal lineages (as evident by expression of marker genes of these lineages) in cultures maintained and differentiated at hypocapnic conditions (1.5% CO_2) at all the time periods under study. These results, which are the first of their kind, indicated that the physiological factor *i.e.* hypocapnia seems to be preferred for the maintenance of pluripotency and the subsequent differentiation in mouse ES cells.

This thesis supports a role of physico-chemical factors in lineage derangement of key genes which are specifically involved in ES cell differentiation leading to specification and patterning into highly organized tissues during embryonic development.

HYPOTHESIS

Alterations in growth environment during development results in derangement of lineage specification genes in embryonic stem cells

OBJECTIVES

Objective 1: Investigating the effect of Statins on differentiation of ES cells

Objective 2: Exploring the impact of homeopathic medicines on ES cell differentiation

Objective 3: Investigating the role of the physiological factor i.e. hypocapnia on embryonic stem cells pluripotency and differentiation

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.

1.2.1Embryonic 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].

1.2.2Adult 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].

1.2.3Why 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).

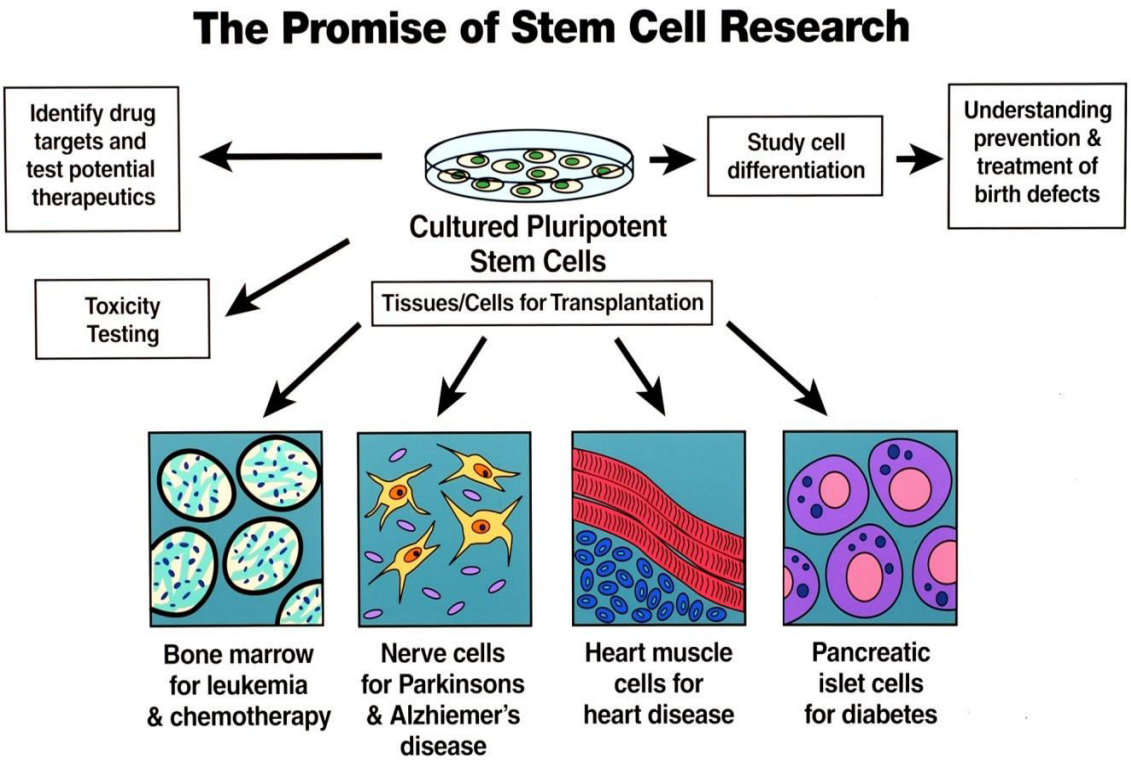

Figure 1.1: Potential of ES cells in regenerative medicine and drug discovery. (Modified image taken from department of Genetics (GENIE-Genetics Education Networking for Innovation and Excellence), University of Leicester) [4].

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].

1.2.5Historical 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).

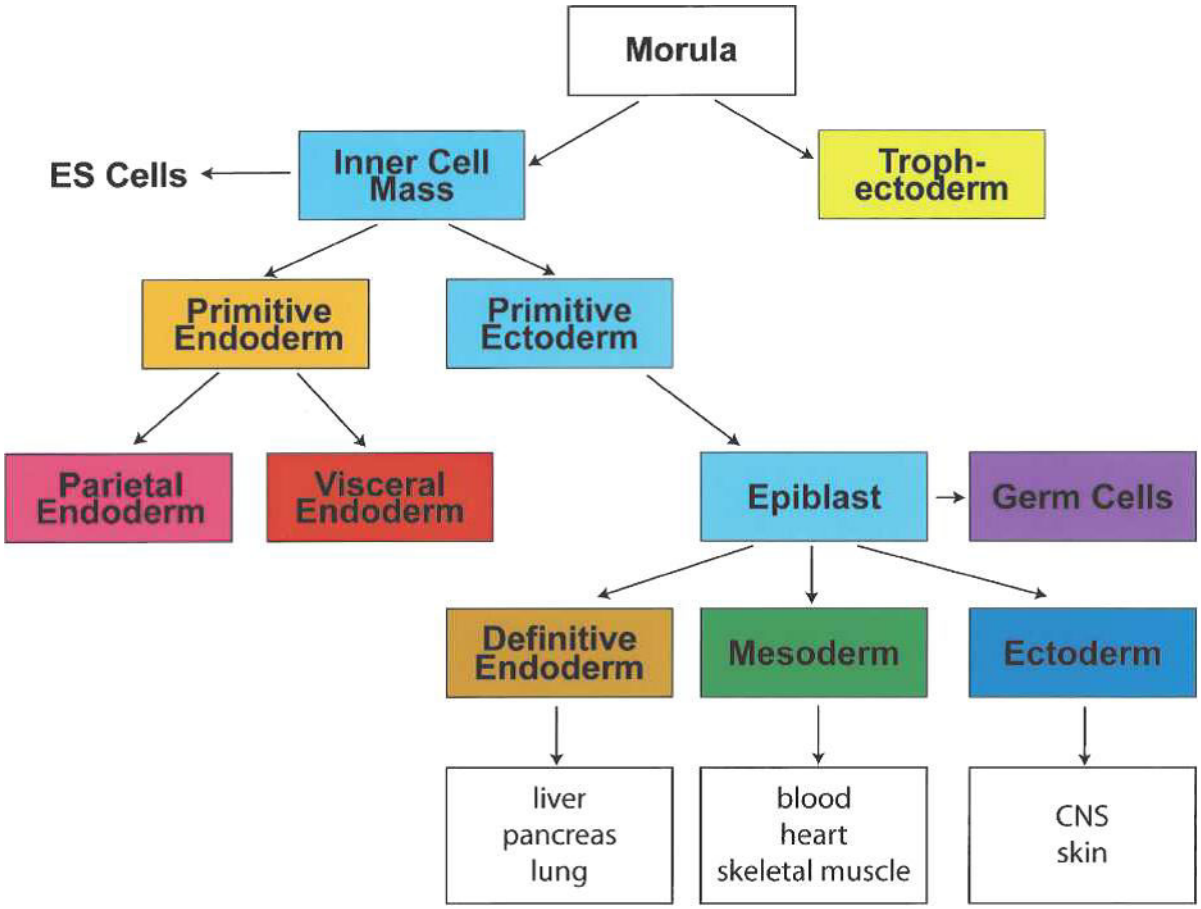

Figure 1.2: Schematic representation of early mouse embryonic development into germ layers. Image taken from review article written by Gorden K [1].

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*.

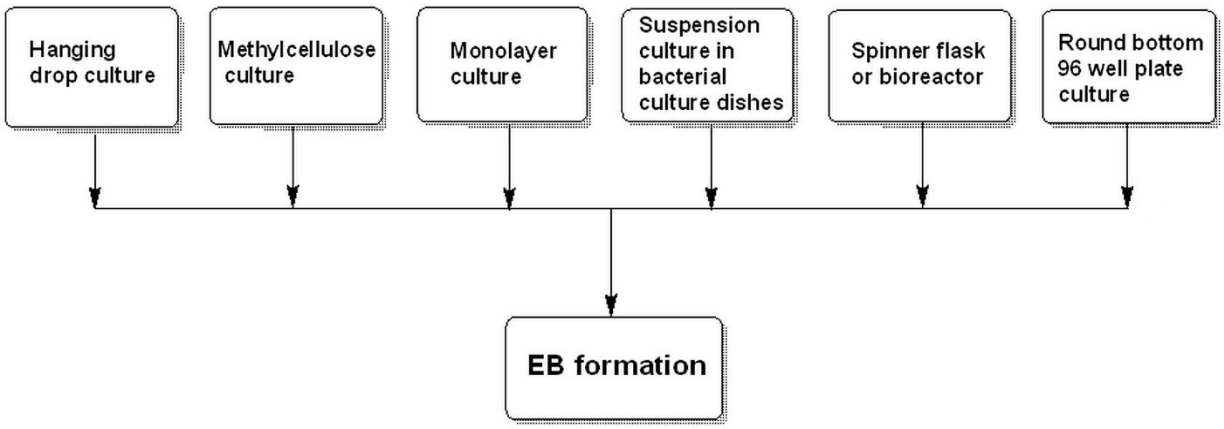

Figure 1.3: Outline of various methods for formation of EBs.

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 thefirst 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 alsofound 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 trophoectoderm[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 *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 Sox2leads to embryonic lethality shortly after implantation, signifies the essential role of Sox2inmaintaining 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 wasfound in trophectodermand later in all neuroectodermal cells and in different endodermal and epithelial tissues [52]. Overexpression of Sox2influencesES 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 *in vivo* is more limited to the naive pluripotency compartment as compared to Oct3/4 and Sox2. On the other hand, Mitsui *et al* identified Nanogand confers that is essential for establishment of pluripotency in inner cell mass and in ES cells [37]. Nanognull 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 Nanognull 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 embryogeneisis 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).

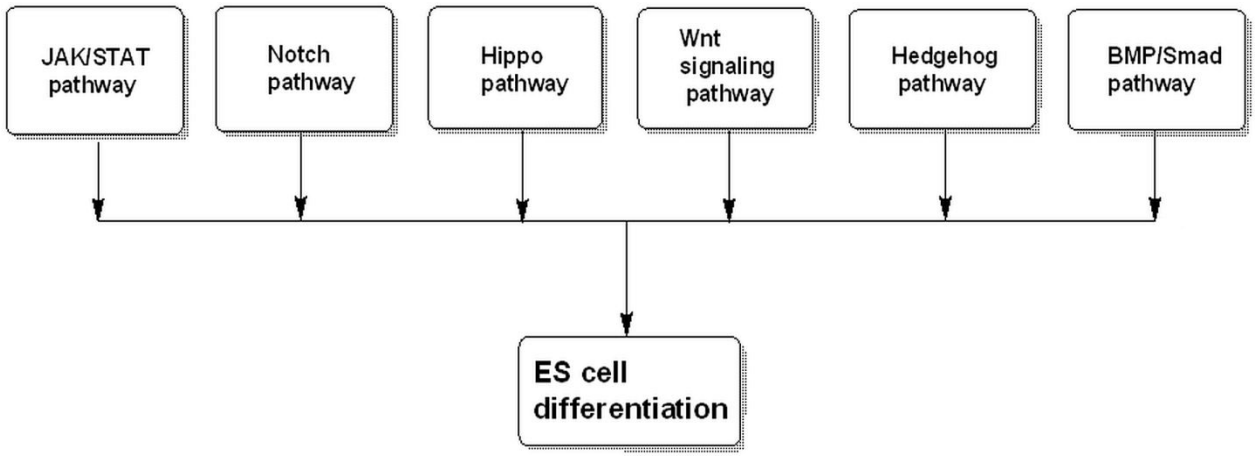

Figure 1.4: Signaling pathways known to regulate ES cell fate.

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 cellslike 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 astranscription 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 complexesattained, 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 Brachyuryin 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 amplifiedthe 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 signalingwherein 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 milieureliant, 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 vivo*necessity 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 involveauto 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 Gli^A or Gli^R 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).

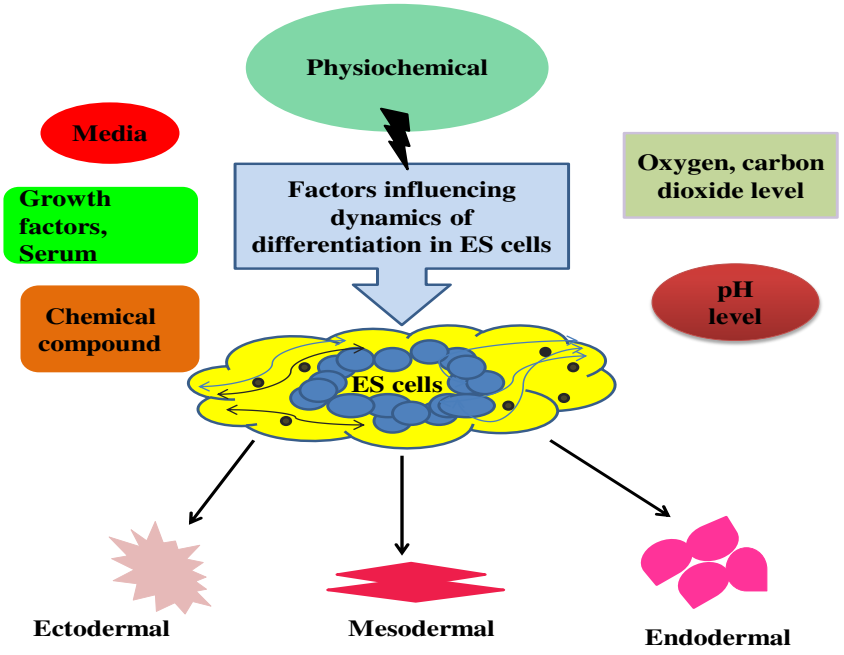

Figure 1.5: Factors influencing differentiation of ES cells.

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 [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 [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 [128]. During embryonic development, lineage commitment requires the secretion specific growth factors [129, 130]. It was reported that in the absence of serum, BMP4 was not capable of inducing cardiac differentiation [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 *in vitro* differentiation models.

1.5.3 LIF

The pluripotent colonies from inner cell mass maintained *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 [66, 132-136]. Williams *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 [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 [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

O_2 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 O_2 supply (hypoxia) [153] and purposely stimulate hypoxia inducible factor (HIF - a set of O_2 regulated genes) [154]. Adaptation to the low levels of O_2 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 O_2 concentration so as to be successful [155]. Therefore, an understanding of the levels of O_2 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 O_2 and this corresponds to the O_2 tension in the tissue. Various reports have shown that the stem cell niches show O_2 concentrations well below the atmospheric levels of 21%, suggesting that O_2 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% O_2 which actually would be a state of hyperoxia [157]. In fact, it has been reported that the *in vivo* pO_2 levels in monkey blastocyst can be as low as 11 mm Hg (1.5% O_2) [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 O_2 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 O_2 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 O_2 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 O_2 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 O_2 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 (CO_2) 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 CO_2 leads to either hypercapnia (high CO_2) or hypocapnia (low CO_2) 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% CO_2 should be reconsidered. There exists a close relationship between O_2 consumption and CO_2 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 pluripoteny [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 IC_{50}[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 CO_2 was a condition which was preferred by the ES cells for both maintenance of pluripotency as well as for differentiation. A schematic representation of thestudy design is given in Figure 1.6.

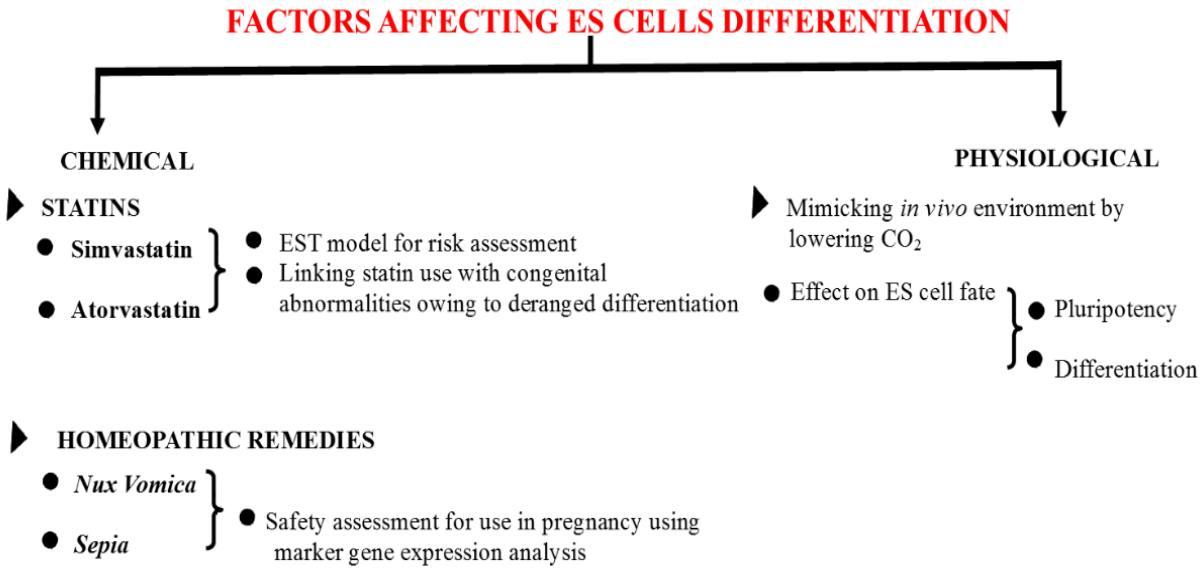

Figure 1.6: Schematic presentation of work design.

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ABSTRACT

EScells in the body have a unique ability to renew themselves and give rise to more specialized cell types having functional commitments. Under specified growth conditions, these cell types remain unspecialized but can be triggered to become specific cell type like heart, nerve, or skin cell. This ability of EScells for differentiation into different cell types makes it a prominent candidate as tool for understanding the process of embryogenesis and revealing the effect of physico-chemical factors on the growth of fetus. In addition, geneticvariations and birth defects caused by mutations and teratogen, affecting early mammaliandevelopment, could also be studied on this basis. Moreover, replacement of animal testing is needed because it involves ethical, legal, cost issues. Embryonic Stem Cell Test(EST), an *in vitro* assay system was developed for screening of various drugs to classify them based on their embryotoxic potential.Statins are a class of drugs whose deleterious effects during the fetal development are still not very well understood. Data from animal studies and retrospective studies done in pregnant women, gave conflicting reports. In this study, an*in vitro* differentiation model of EScells was used, which mimic the differentiation process of the embryo.The ES cells were systematically exposed to two lipophilic statins, simvastatin and atorvastatin, at various doses and at critical times during differentiation. The analysis of key genes controlling the differentiation into ectodermal, mesodermal and endodermal lineages was assessed by quantitative real time polymerase chain reaction.The results showed that genes of the mesodermal lineage were most sensitive to statins, leading to changes in the transcript levels of Brachyury, Flk-1, Nkx2.5 and α/β MHC. In addition, changes to endodermal marker Afp, along with ectodermal Nes and Neurofilament 200kDaimplies that during early differentiation exposure to these drugs led to altered signaling which could translate to the congenital abnormalities seen in the heart and limbs.

CHAPTER 2

2.1 INTRODUCTION

This study was based on EST, which was developed for *in vitro* embryotoxicity testing of drugs and chemicalcompounds. In contrast to other *in vitro* embryotoxicity tests, for example, the post implantation whole rat embryo culture test and the micro massassay, in the EST, no pregnant animals are needed. The EST takes advantage of ES cells to differentiate *in vitro* spontaneously into contracting cardiomyocytes cells [1-3].

The EST protocol uses two permanent mouse cell lines, the pluripotent ES cell line (D3) which represents embryonic tissue and the differentiated fibroblast cell line (3T3) which represents adult tissue [4,5]. Three endpoints were used to classify the embryotoxic potential of test chemicals into three categories of *in vivo* embryotoxicity: non, weak or strong embryotoxic. These endpoints were: inhibition of growth (cytotoxicity) of (i) 3T3 cells (IC_{50}3T3) and (ii) ES cells (IC_{50}D3) after 10 days of treatment determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and (iii) the inhibition of differentiation of ES cells into contractingcardiomyocytes (ID_{50}) after 10 days of treatment. The concentration-response relationships were noted and 50% inhibition concentrations were determined for the three endpoints.In EST, contracting cardiomyocytes were selected as an endpoint because it not only detects the mere differentiation into a certain cell type, but also revealed the intact functional interaction between several other cell types like sinusoidal, atrial and ventricular cells [6]. The test procedure and the classification into the three classes of embryotoxicity applying a biostatistician prediction model,were described in detail by Spielmann *et al* [7].

Although the ESTbased on the endpoints(IC_{50}D3, IC_{50}3T3, and ID_{50}) could categorize most of the chemicals correctly, however, there were certain drawbacks associated with EST model. Firstly, the EST was laborious because more than 300 EBs had to be individually seeded into wells of plate and then optically analyzed. Moreover, the main problem of EST was requirement of monitoring the changes in the EB differentiation caused by exposure to the test chemical,which has to be manually performed by a skilled and individual with the required expertise. Secondly, this microscopic evaluation during the 10 days of differentiation to determine whetherthese EBs were contractile, or not, was subject to human error. In addition, the EST approach used only mesodermal lineage

differentiationasan endpoint for predicting embryotoxicity of drugs during embryonic development [8]. But manychemical compoundslike valproic acid and methyl mercury are known to cause an *in vivo* impairment which is characterized by limb deformations, skeletal abnormalities or central nervous system (CNS)malformations [9-11]. As ES cells are also able to differentiate *in vitro* into the cells of three germ layers like osteoblasts, chondrocytesand neurons [12-14] under appropriate culture conditions, it was suggested that different lineage marker should be incorporated in EST to see the influence on patterning of lineages.

2.1.1 Improvement in EST using new molecular endpoints and assays

One approach was the assessment of marker genes (molecular endpoints) which get expressed when the ES cell differentiated along a specific lineage. When stem cells differentiate into embryoid bodies [EBs (a three-dimensional mass of cells of the three lineages resembling an early embryo)], many different cell types arise. Therefore, it would be possible to assess this differentiation by simultaneously measuring the expression of genes characteristic for different cell types. Gene expression profiles of human and mouse ES cells have been published [15]. Although there are several common features in EScell lines from these two species, differences are also present, e.g., expression of differentiation markers and the cell cycle. Based on thisdata, valuable markers could be used as new endpoints and help inevaluating the similarities and also the differences between different cell lines and species, thus, improving the toxicological risk assessment. Specifically, the analysis of cardiomyocytes differentiation alone as an endpoint did not suffice for the determination of embryotoxic effects. Therefore,various approaches were used for further improvement of EST to include more molecular endpoints for mesoderm, endoderm and ectoderm lineages.

As such, genetic modifications and molecular markers such as the ones detected by fluorescence-activated cell sorting (FACS) and quantitative gene expression analyses, offered significant advantages in developing a new test approach. In FACS, the expression of tissue-specific marker proteins exposed to testchemical areestimated in ES cells. In comparison to morphological evaluation, FACS may assist the adaptation of the EST to applications in high-throughput screening systems.For example, the expression of cardiac

marker proteins,*i.e.*α-actinin and MHC, were estimated under the influence of chemical compoundsby intracellular flow cytometry in ES cells [16].

The gene expression levelof different lineage markerswas also estimatedby qRT-PCRin the presence or absence of test chemicals. Gene expression analysis is important because it providesan insight into false-negative results of the classic EST by analyzingspecific effects on single endpoints, such as the strong inhibition of neurogenesis by valproic acidand the specific repression of osteogenesis following thalidomide administration [17].

For the optimization study, the test chemicals used were those for which sufficient high quality *in vivo* data wasavailable from both testing in animals and human pregnancies. The drugs were selected from a list of chemicals recommended by the US Teratology Society for *in vitro* teratogenesis test validation [18].Thetest chemicals usedfor the optimization in this study mainly consisted of drugs which have been used in human pregnancy, for example, in the case of bacterial infections or the therapy of cancer. The training set of drugs comprised of two strongly embryotoxic drugs; All *trans* retinoic acid(RA)and 5-fluorouracil(5-FU), and one non embryotoxic drug *i.e.*Penicillin G (Pen G). In addition to this, one more endpoint was studied to see whether it could enhance the sensitivity of EST.For this,the expression of α/β MHC in untreated (solvent control) and treated EBs (exposed to test chemicals) was analyzed. The atrial and ventricular cells during early embryonic heart development are known to express α/β MHC and hence thisgenewas chosen [19]as,itcould be used as apotential marker for cardiac development during ES cell differentiation.

2.1.2Statins and developmental toxicity

Having confirmed that the EST (based on molecular endpoint analysis)carried out in this study had correctly categorized thetraining set of drugs as per the reported literature, it was then applied to evaluate the developmental toxicity of statins using the modified EST which utilizes gene expression as a molecular endpoint. Statins are cholesterol lowering drugs. Elevated cholesterol level in the blood is a major predisposing factor for coronary heart disease (CHD) and cardiovascular deathand has therefore, led to the greater use of lipid lowering medications [20, 21]. For many years,hyperlipidaemia was said to be a natural

consequence of pregnancy due to the changes in the level of sex hormones and hepatic and adipose metabolism prevalent in the system. Therefore, no monitoring of cholesterol or need for regulating elevated levels was considered. However, the *in utero* environment has directbearing on fetal health and is therefore, a matter of great concern. High maternal cholesterol levels during pregnancy are now linked with increased risks of preterm delivery, gestational diabetes and preeclampsia, as well as the later development of atherosclerosis in offsprings [22-27].

"Statins," act on the key enzyme, β-Hydroxy-β-Methyl Glutaryl-Coenzyme A (HMG-CoA) reductase of the cholesterol biosynthetic pathway [28]. They have been used successfully in thetreatment of hyperlipidemia and for reducing morbidity and mortality in coronary artery disease. When these drugs were first introduced in the late 1980s, they were categorized as 'X' owing to the results from animal studies where they showed developmental toxicity on the axial skeleton, viscera and CNS. In addition, by lowering the cholesterol levels, they also interfered with development [29, 30].Statins could exert this effect as they lead to an inhibition of a critical component, mevalonic acid, which is involved in DNA replication and is also essential for the synthesis of steroids and cell membranes in the fetus [31-33].Inspiteof this, statins are still prescribed to pregnant womenbyphysicians because theyhave to weigh the risk to benefit ratio [28, 34, 35].The lack of data on the adverse effects of statins during pregnancy could be due to either lack of reporting[36] or the want of an *in vitro* model system which recapitulates the events in fetal development. Thus, over the years no concrete results have been seen regarding the safety prospect of statins use in pregnancy.

Edison and his group examined case reports of statins exposure during the sensitive first trimester of pregnancy. They found that these drugs led to CNS and limb anomalies to the fetuses [37].Simvastatin and atorvastatin were reported to cause fetal neurological damage and CNSdefects as well as impaired placental implantation [38-40]. However, a study conducted in Canada found no evidence of an increased risk of fetal anomalies among firsttrimester statins users, or any discernible pattern of congenital anomalies among live births but their conclusions were uncertain in the absence of outcome ofdata on non-live births [41].

Simvastatinand atorvastatin being lipophilic in nature,both achieve embryo placental concentrations similar to those of maternal plasma and hence pose a greater threat to the developing fetus. However, conflicting findings and the lack of scientific data on the effect of statins during fetal development with regard to the developmental toxicity risks of first trimester use of statins, prompted to carry out this systematic study using the EScell model system. Mouse EScell lines have been at the forefront of research for understanding the process of mammalian embryogenesis at both thegene and protein level. Their differentiation mimics the changes which occur during embryogenesis *in vivo,* that aretraceable according to markers of lineage specification [42, 43]. *In vivo*, early cell division stages from blastomeres to the morula stage are known as totipotent, for the reason that they can give rise to highly complex organism. Cells from the inner cell mass (ICM) can retain the potential to generate three germ layers, the endoderm, mesoderm, and ectoderm as well as the primordial germ cells (PGC), the founder cells of male and female gametes [44]. *In vitro*, mouse ES cells showed the potential to generate the different somatic and germ cell types [45-48].Therefore, to assess the developmental toxicity risk associated with the use of statins,the key marker genes for the three lineages were analyzed. Dose, timing, and duration of drug exposure are important parameters in evaluating potential developmental toxicity of statins and hence multiple time points were studied using two different methodsfor *in vitro* differentiation *i.e.* monolayer as well asEB (hanging drop method) (Table 2.1). EBs are three dimensional structures which resemble the early embryo, however owing to varying sizes, the diffusion of various chemicals and gases permeating to the cells within the EB might vary greatly and hence the non-uniformity between the EBs will impact the results. On the other hand, the monolayer method, whereby differentiation proceeds by avoiding the formation of EBs and therefore, the ease as well as the reproducibility scores over the hanging drop method. Therefore,in this study these two methods of differentiation for comparing the effects of atorvastatin and simvastatin were used. Since, a similar pattern of changes was found in the gene expressionwith both methods, therefore further studies were carried out using the monolayer method of differentiation.

Table 2.1: Schematic outline of experimental steps for two different protocols used.

Dosing day	EB differentiation	Monolayer differentiation
Day 0	+	+
Day 3	+	+
Day 5	+	+
	↓	↓
Day 10	RNA isolation	RNA isolation

+ indicates dosing day

2.1.3 Hanging drop method

This method is used for the differentiation of ES cells and its foundation is based on the method originally published by Wobus *et al*[49], with various modificationswhich are basically dependent on the initial cell number for aggregation.Hanging drop culture is a widely used protocol for EB formation. The number of ES cells aggregated in a hanging drop can be optimized by changing the number of cells in the initial cell suspension to be hung as a drop on the inner lid of petri dish. Using this method, homogeneous EBs from a predetermined number of ES cells were produced. However, the hangingdrop method is time consuming and cost intensive. In addition to this, the volume of a drop is limited to less than 50µl due to maintaining hanging drops on the lid by surface tension. Furthermore, the hanging drop method consists of two steps, which may be troublesome for testing effect of chemicals.

2.1.4 Monolayer differentiation

The monolayer approach offers advantages for detailed *in vitro* characterizations and potential mechanistic and therapeutic screening [50]. This method is far easieras it requires less skill and time as compared to the hanging drop method. Monolayer differentiation in mouse ES cells was achieved by withdrawal of LIF (anti-differentiation) which allows cells to aggregate for differentiation.

2.2 MATERIALS AND METHODS

2.2.1 Maintenance of D3 ES and 3T3 cell lines

Undifferentiated mouse ES cell line D3 was cultured in high glucose (4.5g glucose/l)Dulbecco's modified Eagle's medium (DMEM; Gibco LifeTechnologies) containing 15% fetal calf serum (Hyclone), 2mM glutamine (Gibco),antibiotics (50U/ml penicillin and 50µg/ml streptomycin; Gibco),1% non-essential amino acids (Gibco), 0.1mM mercaptoethanol (Sigma) and 1000U/ml murine leukemia inhibitory factor (mLIF; Chemicon). LIF was added directly to the culture dish. Cells were maintained in 35mm cell culture dishes (Corning, Germany) under 5% $CO_2$and 95% humidity at 37^oC and were fed every day and routinely passaged afterevery 2 days so as to prevent differentiation. Early passage numbers were cryopreserved and experiments were done on passage numbers between 3 and 10.

3T3 cells were maintained in DMEM supplemented with 10% fetal calf serum, 2mM glutamine, 50U/ml penicillin, and 50µg/ml streptomycin. Cells were maintained in T-25 cell culture flask (Corning, Germany) under 5% $CO_2$and 95% humidity at 37^oC and were routinely passaged afterevery 3-4 days.

2.2.2 The concentration range of the chemicals used

Pen G: The stock of Pen G (100mg/ml) (Sigma) was made in phosphate buffer saline (PBS) and sterile filtered through a 0.22µm filter(Millipore). Aliquots were made and stored at -20^oC. Further dilutions were made in DMEM to obtain a concentration range of 125-2000µg/ml.The final concentration of PBS was $\leq$ 1% in the test concentrations and thus PBS at 1% concentration was used as a solvent control.

RA: The stock of RA (30.05mg/ml) (Sigma) was made in absolute ethanol and sterile filtered through a 0.22µm filter (Millipore). Aliquots were made and stored at -80^oC. Further dilutions were made in DMEM to obtain a concentration range of 0.0000375-0.007µg/ml.The final concentration of ethanol was $\leq$ 0.5% in the test concentrations and thus ethanol at 0.5% concentration was used as a solvent control.

5-FU: The stock of 5-FU (1mg/ml) (Sigma) was made in PBS and sterile filtered through a 0.22µm filter (Millipore). Aliquots were made and stored at -20^oC. Further dilutions were

made in DMEM to obtain a concentration range of 0.00625-1.78µg/ml. PBS at a final concentration of 1% was used as the solvent control.

Statins: Simvastatin and atorvastatin (Sigma Aldrich) were applied in a concentration of 0.003, 0.006, 0.012, 0.024, 0.048, and 0.096µM. The rationale for choosing these concentrations for cytotoxicity analysis was based upon approximate peak plasma concentrations of statins reached during treatment of hypercholesterolemia which was found to be 0.1µM [51].Therefore, this concentrationwas used as the upper limit and serial 2fold dilutions done to give a total of 6 concentrations to be analyzed. Simvastatin was dissolved in 0.1M NaOH which was then further diluted with the DMEM. The aliquots of stock solution were stored at 4^{o}C. The final concentration of NaOH was 0.1mM and used as solvent control. Atorvastatin was dissolved in DMSO (0.25% DMSO final concentration and used as solvent control). The aliquots of stock solution were stored at 4^{o}C. The control used for comparing data was the solvent control. To study the effect on differentiation, the doses were selected based on the cytotoxicity analysis and were 0.003, 0.006, 0.012 and 0.024µM.

2.2.3Determination of cytotoxicity using MTT assay

Cytotoxicity of chemical compoundswas determined by using MTT assay according to the EST protocol using 3T3 adult cells and D3 ES cells [18]. In brief, trypsinized cells were counted using heamocytometer and seeded in each well of 96 well plates at density of 500 cells/50µl culture media. After 4 hours of incubation, 150µl culture media containing the chemical compounds at the concentrations mentioned above were added to the cells. The media containing the specific test concentrations was replenished on days 3 and 5. Cytotoxicity was assessed using the MTT dye on day 10. Forthis,30µl of MTT solution (5mg/ml in PBS) was added to each well containing the cells and incubated for 4 hours at 37^{o}C. Then the media was replaced with 150µl of DMSO and incubated for next 20 minutes. Absorbance of the formazan products due tothe viable cells was measured at a wavelength of 570 nm with reference wavelength of 630nm in microplate reader (BIO-RAD model 680)and the concentration-response curve was obtained. The experiment was performed four times in triplicates.

The cytotoxicity was calculated as per the formula give below:

$$\% \text{ Cytotoxicity} = \frac{\text{O.D. of Solvent control} - \text{O.D. of Test sample}}{\text{O.D. of Solvent control}} \times 100$$

2.2.4 Assessment of differentiation

To detect effects of various drugs on the differentiation potential of ES cells into cardiomyocytes, a hanging drop differentiation assay [52] was performed in which, 1000 cells in 20 µl differentiation medium (media without LIF) wereplaced on the inner lid of a 60mm petri dish filled with PBS (Sigma,St. Louis, MO, USA) and then incubated for 2 days at 37^{o}C under 5% CO_2 and 95% humidity in the presence of differentconcentration range of test chemicals. During this period, the cells form aggregates referred to as EBs. After 2 days of "hanging drop" culture, the EBs were transferred to bacterial (Non tissue culture treated (Corning) 60mm petri dishes containing the appropriate concentration of test chemicals for another 3 days. Bacterial Petri dishes were used to avoid adherence and outgrowth of the EBs during this stage of the culture. On day 5, EBs were plated separately into wells of a 24well plate (containing the test concentration of chemical) to allow adherence and outgrowth of the EBs and development of spontaneously contracting cardiomyocytes. Differentiation was determined by microscopic inspection of contracting cardiomyocytes at day 10 of differentiation.

For every test concentration, one 24well plate was used. The differentiation was judged by microscopic evaluation of the contracting cardiomyocytes on day 10 using CKX31 Inverted microscope(Olympus) at 10X magnification. The percentage of wells per plate in which contracting cardiomyocytes developed was determined and compared to the percentage that was found for the solvent control. An experiment was considered to be valid if the solvent control contained contracting cardiomyocytes in a minimum of 21 out of 24 wells. The differentiation inhibition, evaluated through contraction of the cardiomyocytes,wasexpressed as the concentration of the test chemical that decreases the development into contracting cardiomyocytes by 50%(ID_{50}) compared to solvent

control.The ID_{50} value was calculated from dose–response curves.This experiment was done four times in triplicates.

2.2.5 Monolayer differentiation

The monolayer differentiation was also adopted to mimic differentiation of ES cells [53]. The cells were seeded at density of 5 x 10^4cells/mlin 6well plates (Thermo scientific) in Dulbecco's modified Eagles Medium (Invitrogen), complemented with 15% heat inactivated fetal bovine serum (FBS), 2 mM glutamine (Invitrogen), 50U/ml penicillin and 50μg/ml streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1mM β-mercaptoethanol.

2.2.6 Immunocytochemistry

EBs were obtained from hanging drop methodology and transferred ontogelatin coated 24 well plate at day 5. After culture for next 5 days in 24 well plate, EBs were fixed in paraformaldehyde solution (4% in PBS) for 30 minutes at room temperature.After a PBS wash, EBs were permeabilized with Triton X-100 (0.25% in PBS) added for 15minutesfollowed by washingwith wash buffer [1% bovine serum albumin (BSA) in PBS].This was followed by incubation for 1 hour with 5% BSA in PBS at room temperature to block non-specific binding and again washed with wash buffer.EBs were thenincubated with primary antibody against α/β MHC (Abcam) diluted at 1:250ratio, andkept overnight at 4^{o}C. After washing with wash buffer, cells were incubated with secondary antibody: Fluorescein-5-isothiocyanate(FITC)-conjugatedrabbit anti-mouse (Sigma) diluted in the ratio 1:500 and again washed with wash buffer. Then cells were treated with 2μg/ml of Hoechst staining for next 25 minutes and washed three times with wash buffer. The EBs were viewed under the Nikon eclipse T*i* microscope at 10X magnification using the appropriate filters excitation at 530nm for FITC staining.The ICC experiment was performed three times in triplicates.

2.2.7 RNA isolation, cDNA synthesis and quantitative real time PCR (qRT-PCR) for analyses of gene markers associated with differentiation

The RNA was isolated with RNeasy Mini Kit (Qiagen) which also included DNA digestion.Theconcentration and quality of RNA was measured with a Nano drop 2000

spectrophotometer (Thermo Scientific). Synthesis of cDNA from RNAwas carried out by using an oligo dT (15) primer in the presence of M-MULV Reverse Transcriptase (Genetix). PCR was performed with 0.5 µg cDNAof each sample using gene specific primers in order to determine the expression level for target gene. qRT-PCR was performed on CFX-96 real time PCR (Bio-Rad laboratories) using SYBR Green real time PCR dye (Bio-Rad laboratories). The conditions were: initial denaturation at93^{o}C for 4 minutes, followed by 39 cycles each of denaturation(95^{o}C for 15 seconds), annealing (54.5-60.2^{o}C for 20 seconds) and extension (72^{o}C for 1 minute). The relative quantitative expressions of lineage specific markers were calculated after normalization against GAPDH, a housekeeping gene.The present study analyzed a total of seven gene markers which are associated with the formation of three different lineages. The gene markers associated with the formation of mesodermal lineage included; Brachyury, Flk-1, Nkx2.5 and α/β MHC while Alpha fetoprotein (Afp) was selected due to its association with the development of endoderm. For studying the expression of ectodermal lineage, nestin (Nes) and Neurofilament 200 kDa (ND 200) gene markers were analyzed. The details of the primers with annealing temperatures are provided in the Table 2.2. The gene expression analysis was performed three times in triplicates.

Table 2.2:Details of the primers used in this study

Genes	Primer sequence (Forward primer-FP and Reverse primer-RP)	Annealing temperatures (^{o}C)
GAPDH	FP 5'-GCACAGTCAAGGCCGAGAAT-3'	58.5
	RP 5'-GCCTTCTCCATGGTGGTGAA-3'	
Afp	FP 5'-GCTGCAAAGCTGACAACAAG-3'	58.7
	RP 5'-GGTTGTTGCCTGGAGGTTTC-3'	
Flk-1	FP 5'-CAGCTTCCAAGTGGCTAAGG-3'	54.5
	RP 5'-CAGAGCAACACACCGAAAGA-3'	
Nes	FP 5'-GCTTTCCTGACCCCAAGCTG-3'	60.2
	RP 5'-GGCAAGGGGGAAGAGAAGGA-3'	
ND 200	FP 5'-TGGACATTGAGATTGCCGC-3'	62.4
	RP 5'-GAGAGAAGGGACTCGGACCAA-3'	
Nkx2.5	FP 5'-CAAGTGCTCTCCTGCTTTCC-3'	56.5
	RP 5'-GGCTTTGTCCAGCTCCACT-3'	
α/β MHC	FP 5'-ACCTGTCCAAGTTCCGCAAG-3'	58.5
	RP 5'-CTTGTTGACCTGGGACTCGG-3'	
Brachyury	FP 5'-TTCTTTGGCATCAAGGAAGG-3'	57.0
	RP 5'-TCCCGA GACCCAGTTCATAG-3'	

2.2.8Dose and time frame analysis of ES cells in monolayer culture

To assess thechanges in differentiation of ES cells exposed to statins in dose, time and duration dependent manner, ES cells were exposed to the statins at different time

intervals during differentiation process. The exposure of statins was started at day 0 and RNA was isolated on day 10. The experimental design for exposure time and duration to ES cells is shown in Table 2.3. The dose and time frame analysis was performed three times in triplicates.

Table 2.3: **Schematic presentation of experimental steps**

Dosing day	**A**	**B**	**C**	**D**	**E**	**F**
Day 0	**+**	**+**	**+**	**X**	**X**	**X**
Day 3	**X**	**+**	**X**	**+**	**+**	**X**
Day 5	**X**	**X**	**+**	**X**	**+**	**+**
	↓	↓	↓	↓	↓	↓
Day 10	RNA isolation	RNA isolation	RNA isolation	RNA isolation	RNA isolation	RNA isolation

+ indicates dosing day, X indicates no dosing

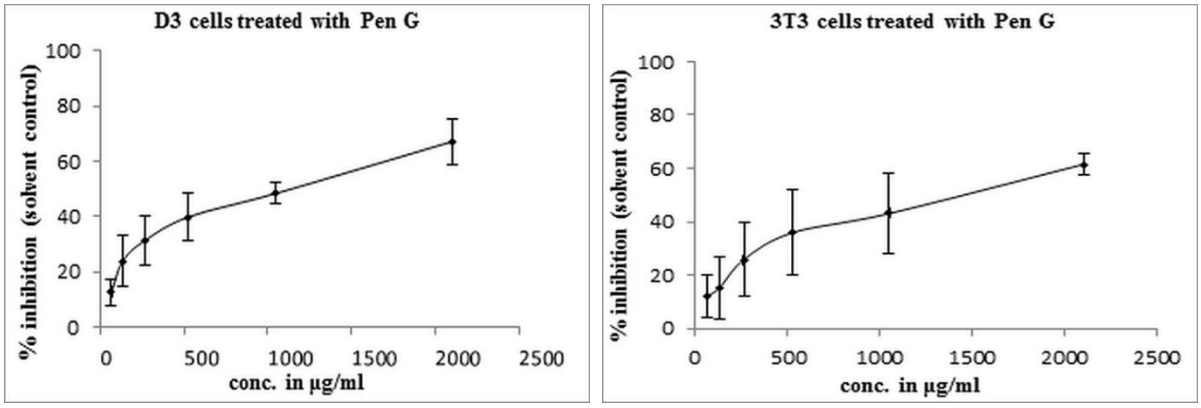
2.2.9Statistical analysis

The cytotoxicity data was obtained by calculating the mean±SEM (standard error mean) from four individual experiments done in triplicates. The IC_{50} values were calculated using dose-response curves. The inhibition of differentiation was obtained by calculating the mean ± SD (standard deviation) from four individual experiments done in triplicates. The ID_{50} values were calculated using dose-response curves. The ICC experiment was performed three times in triplicates. The effects of simvastatin and atorvastatin on the expression levels of the lineage specific markers were analyzed using the $2^{-\Delta\Delta}$Ct method. The gene expression analysis was obtained by calculating the mean ± SD from ≤ 4individual experiments done in triplicates. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.

2.3 RESULTS

2.3.1 Determination of endpoints

The cytotoxicity owing to the treatment with the various chemicals, Pen G, 5-FU and RA after 10 days of exposure to D3 and 3T3 cells was evaluated by the MTT test for obtaining endpoints *i.e.* IC_{50}D3 and IC_{50}3T3, which were required for predicting the embryotoxicity of chemicals according to EST model. The cytotoxicity results revealed that Pen G was not toxic to both thecell types at its clinically relevant dose *i.e.* 250-500µg/ml. In this study, it was seen that the 3T3 adult cells were more sensitive to Pen G in comparison to ES cells. The IC_{50}3T3was found to be 910µg/ml and IC_{50}D3 was found to be 1259µg/ml for Pen G (Figure 2.1).



(a) **(b)**

Figure 2.1: Concentration–response curves showing effects of Pen G. Cytotoxicity was assessed by the MTT assay in (a) undifferentiated D3 cells and (b) differentiated 3T3 cells. Bar graphs show mean±SEM. All values are average of four experiments done in triplicates.

The EBs treated at the IC_{50}D3 value resulted in a decrease in size and slight alteration (Figure 2.2) in its shape as compared to those of solvent control (Figure 2.2). However, there was no alteration in their ability to attach to the dishes at day 5 and led to differentiation which was similar to the solvent controls.

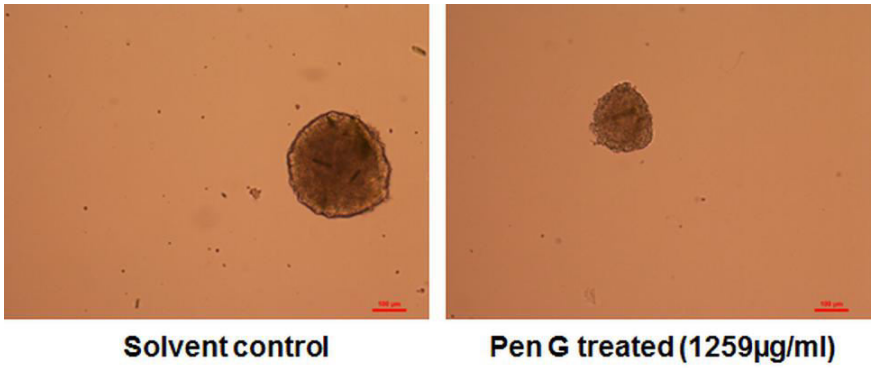


Figure 2.2: Phase contrast pictographs showing formation of EB at day 3 using hanging drop method. Magnification 10X. Bars: 100µm.The experiment was performedfour times in triplicates.

For the determination of the ID_{50} which resulted in 50 percent inhibition of contracting cardiomyocytes in the EBs, individual wells of the 24 well plate treated at each concentration, were visualized under the inverted microscope. The percentage of wells per plate in which contracting cardiomyocytes developed was determined and compared to the percentage that was found for the solvent control.From the plotted curve (Figure 2.3), the ID_{50} for Pen G was found to be 1215µg/ml.

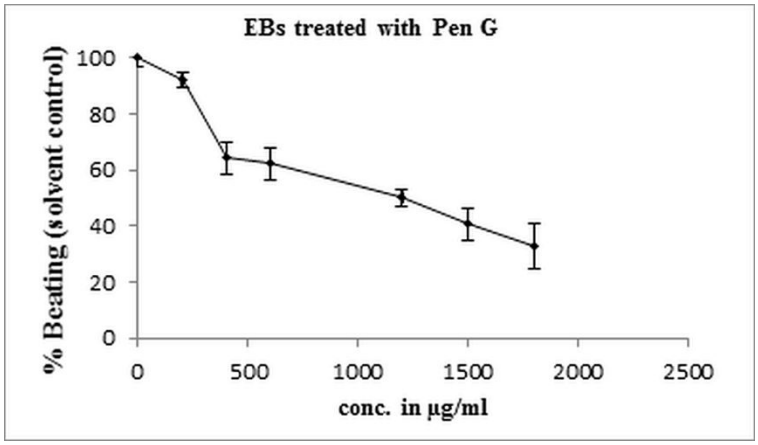
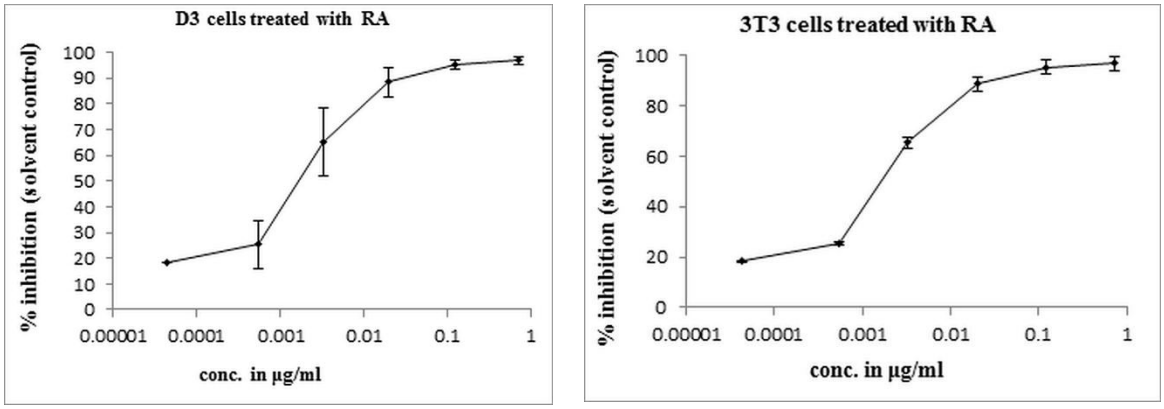


Figure 2.3: Concentration–response curve obtained from microscopical evaluation showing inhibitory effects of Pen G on differentiation of EBs into contracting cardiomyocytes. Bar graphs show means±SD.All values are average of four experiments done in triplicates.

In contrast, RA was highly toxic towards both the cell lines and the sensitivity towards ES cells was greater. The IC_{50}3T3was found to be 0.069µg/ml and IC_{50}D3 was 0.032µg/ml for RA (Figure 2.4).



(a) **(b)**

Figure 2.4: Concentration–response curves showing effects of RA. Cytotoxicity was assessed by the MTT assay in (a) undifferentiated D3 cells and (b) differentiated 3T3 cells. Bar graphs show means ± SEM. All values are average of four experiments done in triplicates.

The higher concentrations of RA lead to diminution in size and shape of the EBs but also most of the EBs at higher concentrations failed to differentiate properly (Figure 2.5)as compared to solvent control (Figure 2.5).

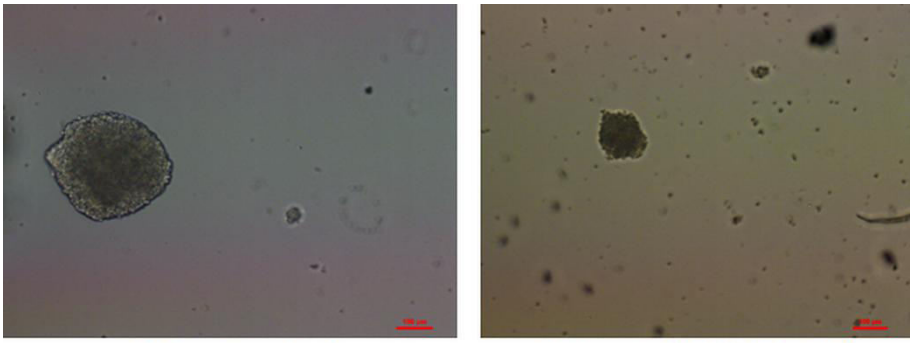


Figure 2.5: Phase contrast pictographs showing formation of EB at day 3. Magnification 10X. Bars: 100µm.The experiment was performedfour times in triplicates.

For the determination of the ID_{50} in RA treated EBs, individual wells of the 24 well plate treated with different concentrations of RA, were seen under the inverted microscope. The percentage of wells per plate in which contracting cardiomyocytes developed was determined and compared to the percentage that was found for the solvent control (Figure 2.6).

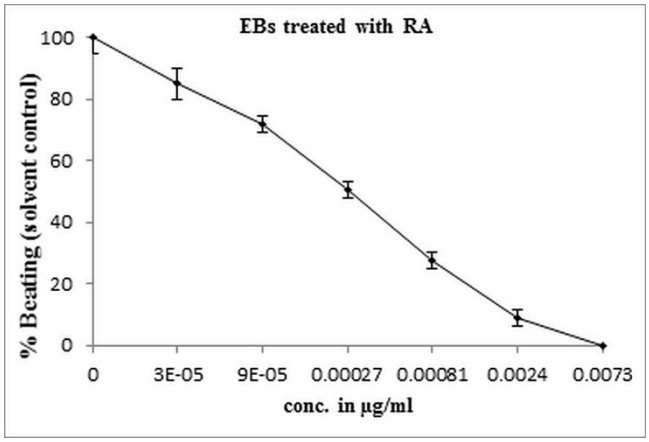
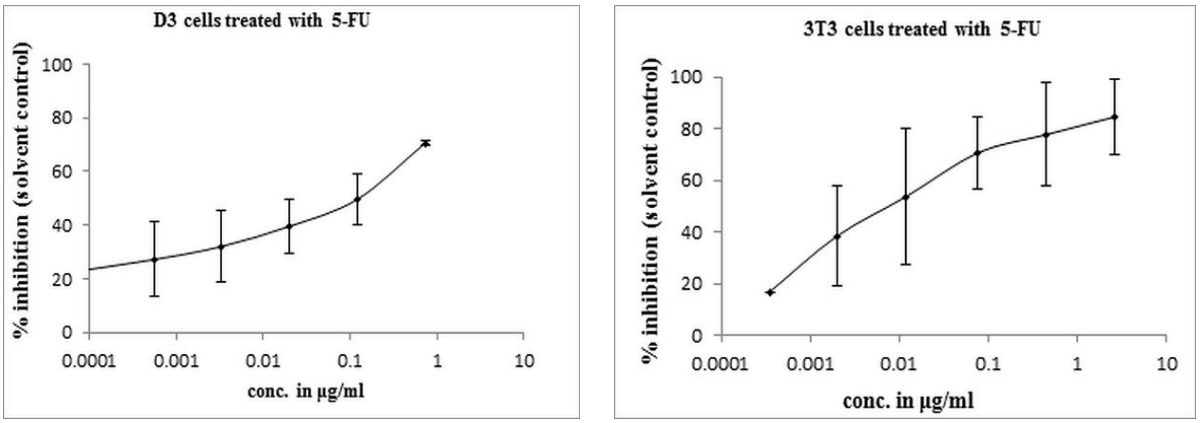


Figure 2.6: Concentration–response curves obtained from microscopic evaluation showing inhibitory effects of RA on differentiation of EBsinto contracting cardiomyocytes. Bar graphs show means ± SD. All values are average of four individual experiments done in triplicates.

Similarly, 5-FU showed an intense cytotoxicitywhich was evident by the IC_{50}D3 and IC_{50}3T3 values achieved at much lower concentrations. The IC_{50}3T3was found to be 0.006µg/ml and IC_{50}D3 was 0.0002µg/ml for 5-FU (Figure 2.7).



(a) (b)

Figure 2.7: Concentration–response curves showing effects of 5-FU. Cytotoxicity as assessed by the MTT assay in (a) undifferentiated D3 cells and (b) differentiated 3T3 cells. Bar graphs show means ± SEM. All values are average of four individual experiments done in triplicates.

It was observed that when the ES cells were treated with different concentrations of 5-FU during the formation of EBs, at the highest concentration not only the EBs were very small in size but also there was no differentiation (Figure 2.8)as compared to solvent control (Figure 2.8).

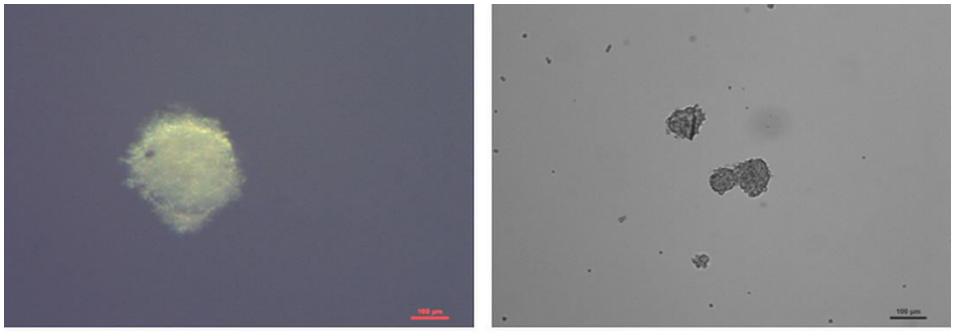


Figure 2.8: Phase contrast pictographs showing formation of EB at day 3. Magnification 10X. Bars: 100µm.The experiment was performedfour times in triplicates.

The results of inhibition of differentiation showed that 5-FU in a dose dependent manner inhibited the spontaneous differentiation of the EBs into beating cardiomyocytes. From the curve (Figure 2.9), the ID_{50} for the ES cells was found to be 0.054µg/ml.

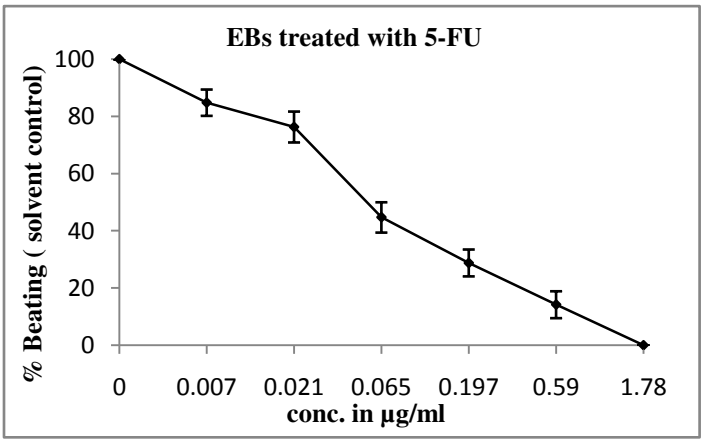


Figure2.9: Concentration–response curves obtained from microscopical evaluationshowing inhibitory effects of 5-FU on differentiation of EBsinto contracting cardiomyocytes. Bar graphs show means ± SD. All values are average of four individual experiments done in triplicates.

2.3.2 Gene expression analysis of molecular endpoint α/β MHC by qRT-PCR

It was observed that alterations upon exposure to test compound could be assessed by quantifying changes in expression of the marker gene for cardiomyocytes differentiation *i.e.* α/β MHC. The ID_{50} α/β MHC value obtained for the training set of drugs was more sensitive and more reliable than conventional ID_{50} value which was obtained by microscopic evaluation(Figure 2.10).

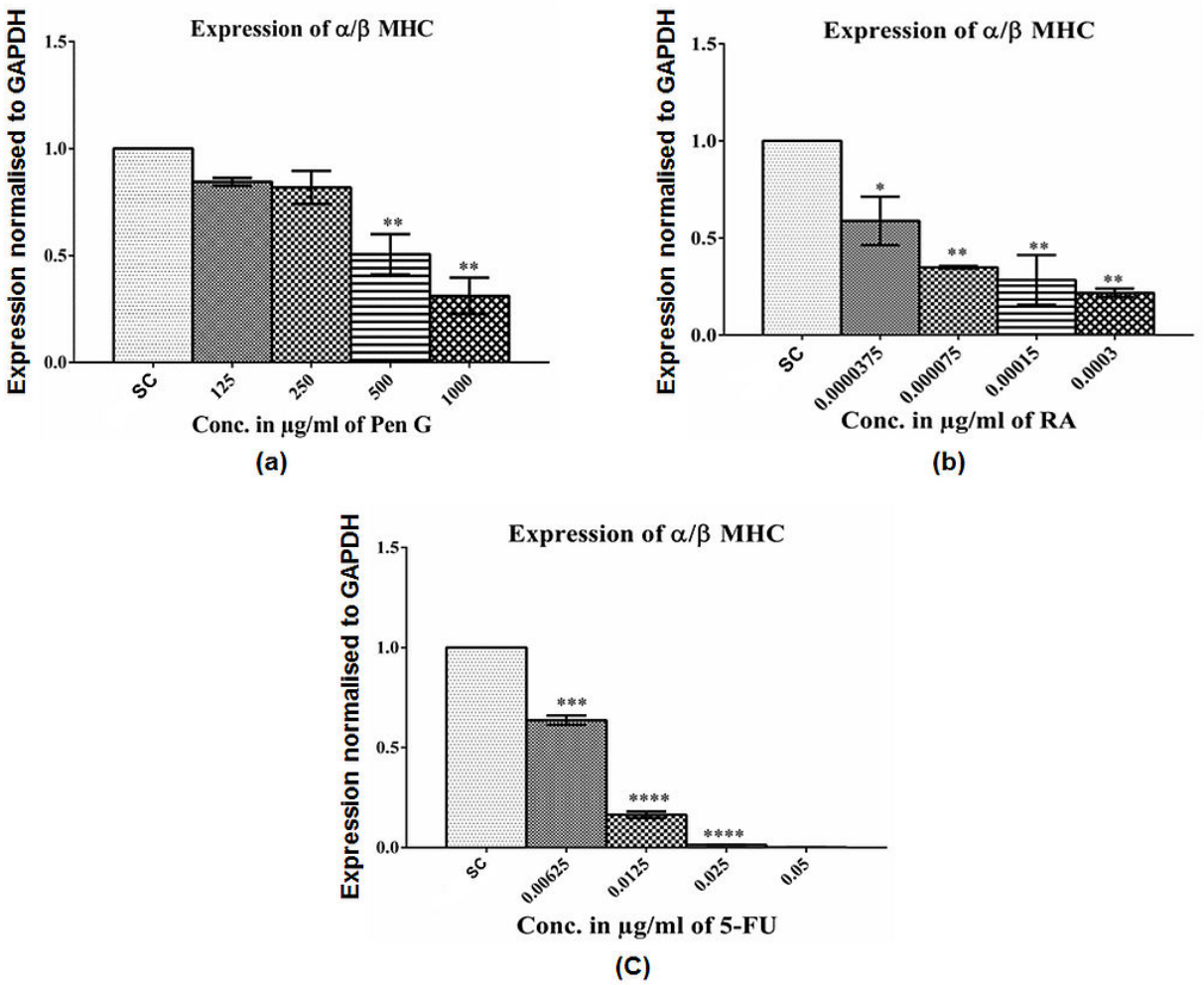


Figure 2.10:Expression levels of cardiomyocytes differentiation marker gene of α/β MHC upon treatment with (a) Pen G; (b) RA and (c) 5-FU analyzed by qRT-PCR, normalized to housekeepinggene*i.e.*GAPDH expression. The y-axis represents fold changesin expression of α/β MHC. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show means ± SD. All values are average of four individual experiments done in triplicates. Significant changes between treated and solvent control cells (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

It was evident that the molecular endpoint *i.e.*ID_{50} α/β MHC was more sensitive as compared to conventional ID_{50}. The endpoints obtained with training set of drugs are given in Table 2.4.

Table 2.4: Endpoints obtained for ES cells treated with training set of drugs.

Test compound	**IC_{50}D3 (MTT)**	**IC_{50}3T3 (MTT)**	**ID_{50} D3**	**ID_{50} α/β MHC**
Pen G	1259±190.7	910±149.2	1215±50.5	450±19****
RA	0.032±0.0021	0.069±0.0012	0.0003±0.00003	0.0001±0.00007**
5-FU	0.0002±0.00005	0.006±0.0008	0.054±0.01	0.007±0.0012****

Values are expressed in µg/ml. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.All values are the average of four individual experiments done in triplicates. Significance was determined between ID_{50}D3 and ID_{50} α/β MHC (p<0.01, ****p<0.0001)**

2.3.3 ES cell derived cardiomyocytes treated with training set of drugs characterized by ICCfor α/β MHC

To identify the cardiomyocytes within the EBs, ICCfor the marker protein α/β MHC was performed (Figure 2.11). EBs which showed contracting cardiomyocytes were positively stained with α/β MHC and this staining was visible in solvent control and Pen G treated EBs.ICC analysis also elucidated the loss of protein expression of α/β MHCupon**t**reatment with strongly embryotoxic drugs.

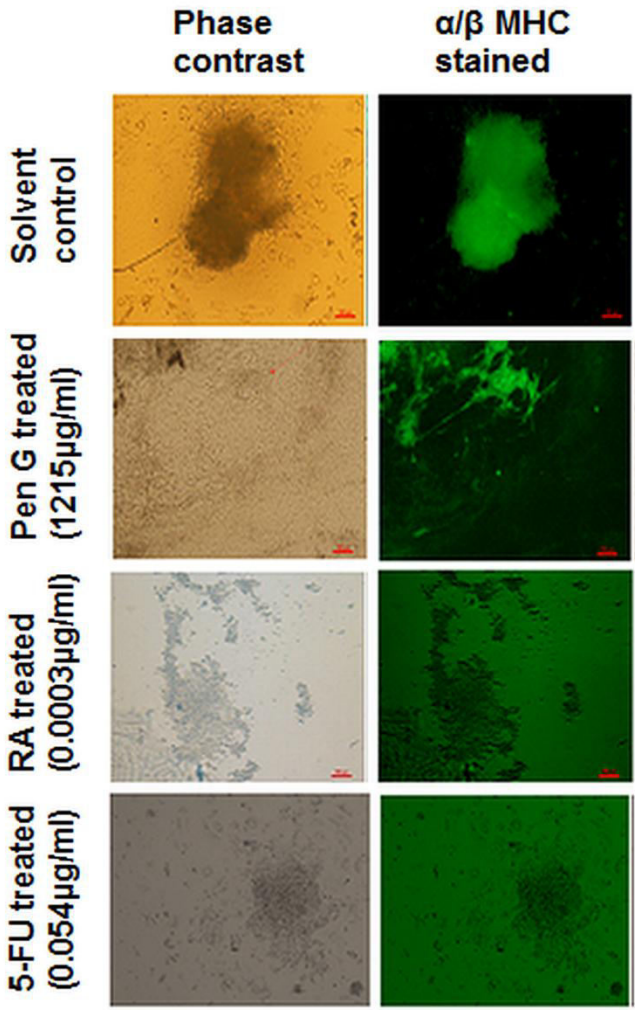


Figure 2.11:ICCanalysis of EBs stained with cardiomyocytes differentiation marker α/β MHC at day 10. Magnification 10X. Bars: 100µm. The experiment was performedthree times in triplicates.

2.3.4 EST prediction model

To predict the embryotoxicity of test compound, a prediction model for EST was developed by Spielmann and his co-workers in 1995 which was subsequently redefined using data from ZEBET lab during pre-validation study in 1997 [7]. After obtaining the endpoints for test chemicals, the values were inputted in EST prediction model to see whether the test compound was correctly classified for their teratogenic potential into three

classes (Table 2.5).The molecular endpoint *i.e.* ID_{50} α/β MHC was used instead of conventional ID_{50}endpoint for evaluating the teratogenic potential of training set of drugs.

Table 2.5: Classification of training set of drugs according to prediction model of EST

DRUG	FUNCTION I	FUNCTION II	FUNCTION III	CLASSIFICATION
	5.92 log (IC_{50}3T3) +3.50 log (IC_{50}D3) – 5.31(IC_{50}3T3 -ID_{50}) /IC_{50}3T3 -15.7	3.65 log (IC_{50}3T3) +2.93 log (IC_{50}D3) – 2.03(IC_{50}3T3 -ID_{50}) /IC_{50}3T3 -6.85	- 0.125 log (IC_{50}3T3) -1.92 log (IC_{50}D3) +1.5 (IC_{50}3T3-ID_{50}) /IC_{50}3T3 -2.67	Class 1: Non embryotoxic (If I>II and I>III) Class 2: Weak embryotoxic (If II>I and II>III) Class 3: Strong embryotoxic (If III>I and III>II)
PEN G	5.92 log (910) +3.50 log (1259) – 5.31(910-450) /910 -15.7	3.65 (910) +2.93 log (1259) – 2.03(910 -450) /910 -6.85	0.125(910)-1.92 log (1259) +1.5(910-450)/910-2.67	Non embryotoxic
RA	5.92 log (0.069)+3.5 log (0.032)-5.31(0.069-0.0003/0.069)-15.7	3.65 (0.069) +2.93 log (0.032) – 2.03 (0.069-0.0001)/0.069 -6.85	-0.125 log (0.069) -1.92 log (0.032) +1.5 (0.069-0.0001)/0.069 -2.67	Strong embryotoxic
5-FU	5.92 log(0.006) + 3.5 log (0.0002)-5.31(0.006-0.007)/0.006)-15.7	3.65log (0.006) +2.93 log (0.0002) – 2.03(0.006-.007) /0.006 -6.85	-0.125 log (0.006) -1.92 log (-0.0002 -1.5(0.006-0.007)/0.006-2.67	Strong embryotoxic

After having experimentally confirmedthat the EST had correctly categorised the training set of chemicals into the respective categories,and thatthe analysis of key gene controlling lineage commitment was a more sensitive endpointas well as reliable,this model system was appliedto statins, aclass of drugs whose use in pregnancy gives conflicting reports. The major focus of this workwas to assess genetic association between statinsand developmental toxicity.

2.3.5Determination of endpoints of statins concentration through validated EST

For cell viability analysis, MTT assay according to EST model was performed to study the cytotoxicity effect on D3 and 3T3, which represents embryonic tissues and adult fibroblasts, respectively. In both the cell types, statins exhibited cytotoxicity in a dose-dependent manner. The sensitivity of statins towards D3 was more as compared to 3T3 cells as evidenced by the greater cell death observed. The cytotoxicity was calculated using dose–response curves. In case of simvastatin, IC_{50} D3 was 0.014µM and IC_{50} 3T3 was 0.083 µM. The IC_{50} D3 was 0.020µM and IC_{50} 3T3 was 0.079µM upon treatment with atorvastatin (Table 2.6). The molecular endpoint *i.e.*ID_{50} α/βMHC was also analyzed in ES cells upon treatment with statins using qRT-PCR. The ID_{50} α/βMHC of simvastatin was found to be 0.012µM and in case of atorvastatin, it was 0.018 µM.

Table 2.6: Endpoints obtained for D3 and 3T3 cell lines after treatment with statins.

Statin	IC_{50} ES	IC_{50} 3T3	ID_{50} α/βMHC
Simvastatin	0.014±0.0075	0.083±0.0063	0.012±0.003
Atorvastatin	0.020±0.0033	0.079±0.016	0.018±0.0057

The values are expressed in µM. All values are the average of four individual experiments done in triplicates.

2.3.6 Prediction of embryotoxic potential of statins using the EST model.

The endpoint obtained for simvastatin was put into the PM formula;

Function I: 5.92 log (IC_{50}3T3) +3.50 log (IC_{50}D3) – 5.31(IC_{50} 3T3 -ID_{50})/IC_{50}3T3 -15.7

5.92 log (0.083) +3.50 log (0.014) – 5.31(0.083 -0.012)/0.083 -15.7

5.92 (-1.080) +3.50 (-1.853)-5.31(0.0855)-15.7

= -9.98

Function II: 3.65 log (IC_{50}3T3) +2.93 log (IC_{50}D3) – 2.03(IC_{50} 3T3 -ID_{50})/IC_{50}3T3 -6.85

3.65 log (0.083) +2.93 log (0.014) – 2.03(0.083 -0.012)/0.083 -6.85

3.65 (-1.080) +2.93(-1.853)-2.03(0.0855)-6.85

=-3.64

Function III: -0.125 log (IC_{50}3T3) -1.92 log (IC_{50}D3) +1.5(IC_{50} 3T3 -ID_{50})/IC_{50}3T3 -2.67

-0.125 log (0.083) -1.92 log (0.014) +1.5(0.083-0.014)/0.083 -2.67

-0.125(-1.080)-1.92(-1.853) +1.5(0.0855)-2.67

= -3.43

It was observed that Function III>Function II and Function III> Function I. Based on the above calculations,simvastatin was found to be strongly embryotoxic drug as it fell into class 3.

The endpoint values obtained for atorvastatin was also inputted into PM to determine its embryotoxic potential.

Function I: $5.92 \log (IC_{50}3T3) + 3.50 \log (IC_{50}D3) - 5.31(IC_{50}\ 3T3 - ID_{50})/IC_{50}3T3 - 15.7$

$5.92 \log (0.079) + 3.50 \log (0.020) - 5.31(0.079 - 0.018)/0.079 - 15.7$

$5.92 (-1.102) + 3.50(-1.698) - 5.31(0.772) - 15.7$

$= -13.179$

Function II: $3.65 \log (IC_{50}3T3) + 2.93 \log (IC_{50}D3) - 2.03(IC_{50}\ 3T3 - ID_{50})/IC_{50}3T3 - 6.85$

$3.65(0.079) + 2.93(0.020) - 2.03(0.079 - 0.018)/0.079 - 6.85$

$3.65(-1.102) + 2.93(-1.698) - 2.03(0.772) - 6.85$

$= -4.637$

Function III: $-0.125 \log (IC_{50}3T3) - 1.92 \log (IC_{50}D3) + 1.5(IC_{50}\ 3T3 - ID_{50})/IC_{50}3T3 - 2.67$

$-0.125(0.079) - 1.92(0.020) + 1.5(0.079 - 0.018)/0.079 - 2.67$

$-0.125(-1.102) - 1.92(-1.698) + 1.5(0.772) - 2.67$

$= -2.709$

Similarly, in case of atorvastatin, Function III>Function II and Function III> Function I which concluded that atorvastatin also fell under the category 3 of strongly embryotoxic drugs and therefore as per this model its use in pregnancy is contradicted.

2.3.7Statins globally altered expression of ES cell differentiation genes in monolayer as well as EB culture

To track the effect of statins on ES cells two protocols *i.e.* monolayer differentiation and EB differentiation were performed. The schematic representation for the experiment is given in Table 2.1. Genes involved in differentiation of mesodermal, endodermal and ectodermallineages mimicking embryonic tissue development were analyzed by qRT-PCR. Statins treated cells were then evaluated against the ES cells treated with solvent control (there were no significant difference between untreated controls and respective solvent controls). The cells were given an exposure to simvastatin and atorvastatin at various time periods. The rationale was to mimic exposure of the fetus to these drugs at various stages of embryonic development and studied the same by using mouse ES cells undergoing differentiation. The exposure was started at the onset of experiment, taken as day 0, with media changes along with test concentration on day 3, 5 and

differentiation was continued until day 10. The RNA was isolated on day 10 to evaluate statins effect on differentiation. It was observed that the continuous exposure of statins from day 0 onwards, has a direct bearing on differentiation and this effect was directly proportional to the dose. On comparing the results of monolayer versus EB differentiation, similar pattern was observed with respect to their effect on gene expression during critical stages of embryonic development. Keeping this in mind, the monolayer method was selected for further analysis of time, dose and duration of exposure to statins, owing to the ease as well as the reproducibility over the hanging drop method.

2.3.7.1Effect of statins on the mesodermal marker genes during EB differentiation

The expression levels of Brachyury, Flk-1, Nkx2.5 and α/β MHC were altered in EBs treated with statins. The expression of Brachyury, an early mesodermal marker, was unexpectedly increased in dose dependent manner when exposed to simvastatin. At a concentration of 0.003µM, the fold change in comparison to solvent controls was 102 ($p<0.01$) and at the highest concentration *i.e.* 0.024µM there was a highly significant ($p<0.0001$) alteration in the fold change expression (381 fold change). A similar pattern was observed in atorvastatin exposed EBs *i.e.* Brachyury was enhanced in dose dependent manner. Atorvastatin as compared to simvastatin, led to greater fold changes at all doses tested and this increased expression of Brachyury was highly significant ($p<0.0001$). A 145 fold at 0.003µM concentration was seen upon treatment with atorvastatin, which jumped to 478 fold at 0.024µM concentration (Figure 2.12A, B).The enhanced expression of Flk-1 was observed in EBs after application of simvastatin (Figure 2.12C). It was found that at a lower dose of simvastatin *i.e.* 0.003µM, no significant change in gene expression was seen in the exposed EBs (Figure 2.12C) but at a higher concentration (0.024µM), the expression was elevated to 35 fold ($p<0.0001$). As observed in Figure 2.12D, the Flk-1 expression also increased upon treatment with atorvastatin, and this expression was directly proportional to the concentration. A 14 fold ($p<0.05$) at 0.006µM, 34 fold ($p<0.001$) at 0.012µM and 73 fold ($p<0.0001$) at 0.024µM concentration as compared to the solvent control.

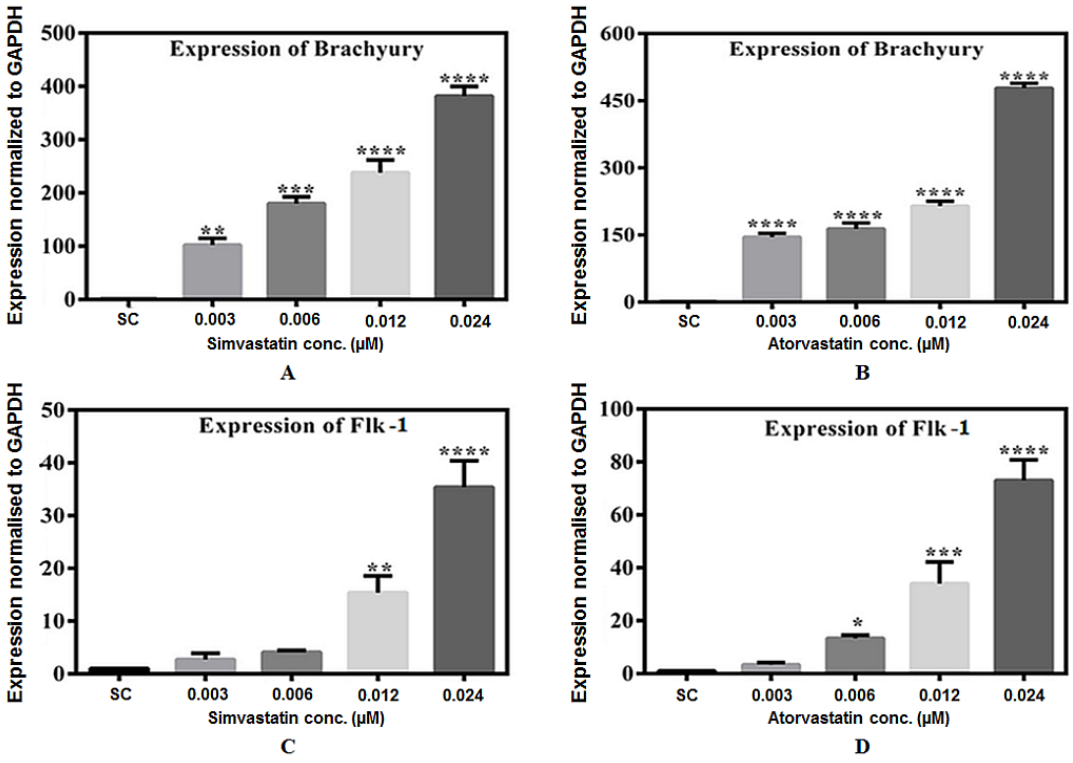


Figure 2.12: Early mesodermal marker genes, Brachyury and Flk-1 assessed by qRT-PCR in EBs treated with statins.(A); (B) Brachyury expression after exposure to simvastatin and atorvastatin, respectively. (C); (D) Flk-1 expression after exposure to simvastatin and atorvastatin, respectively. Expression level was normalized to housekeeping gene *i.e.* GAPDH. The y-axis represents fold changesin the expression of the gene under study. The data was analyzed by using the $2^{-\Delta\Delta}$Ct method.The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show mean±SD (n=3). Significance was evaluated between exposed and solvent control treatment (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

The expression of Nkx2.5 which is an early cardiac marker was also found to increase in dose dependent manner when exposed to simvastatin (Figure 2.13A). Similar to the changes as induced by statins treatment to Brachyury expression, atorvastatin as compared to simvastatin also enhanced the expression level of Nkx2.5 at similar doses. At 0.003μM, the fold change by simvastatin was 5 fold as compared to 15 fold by atorvastatin ($p<0.05$). Similarly, the alteration by 0.024μM simvastatin was 24 fold ($p<0.0001$) in comparison to 53 fold ($p<0.0001$) induced by atorvastatin (Figure 2.13B). Interestingly, the expression levels of α/β MHC showed a down-regulation in expression with increase in dose of both the statins (Figure 2.13C, D) at 0.003μM

of simvastatin, a 4 fold ($p<0.05$) increase as compared to solvent control was seen, which inversely decreased upon treatment at higher concentrations. Atorvastatin was also seen to depress expression of α/β MHC in a dose dependent manner. At 0.003µM, the fold increase was 11($p<0.0001$) which decreased to 0.5 fold at 0.024µM concentration (Figure 2.13D).

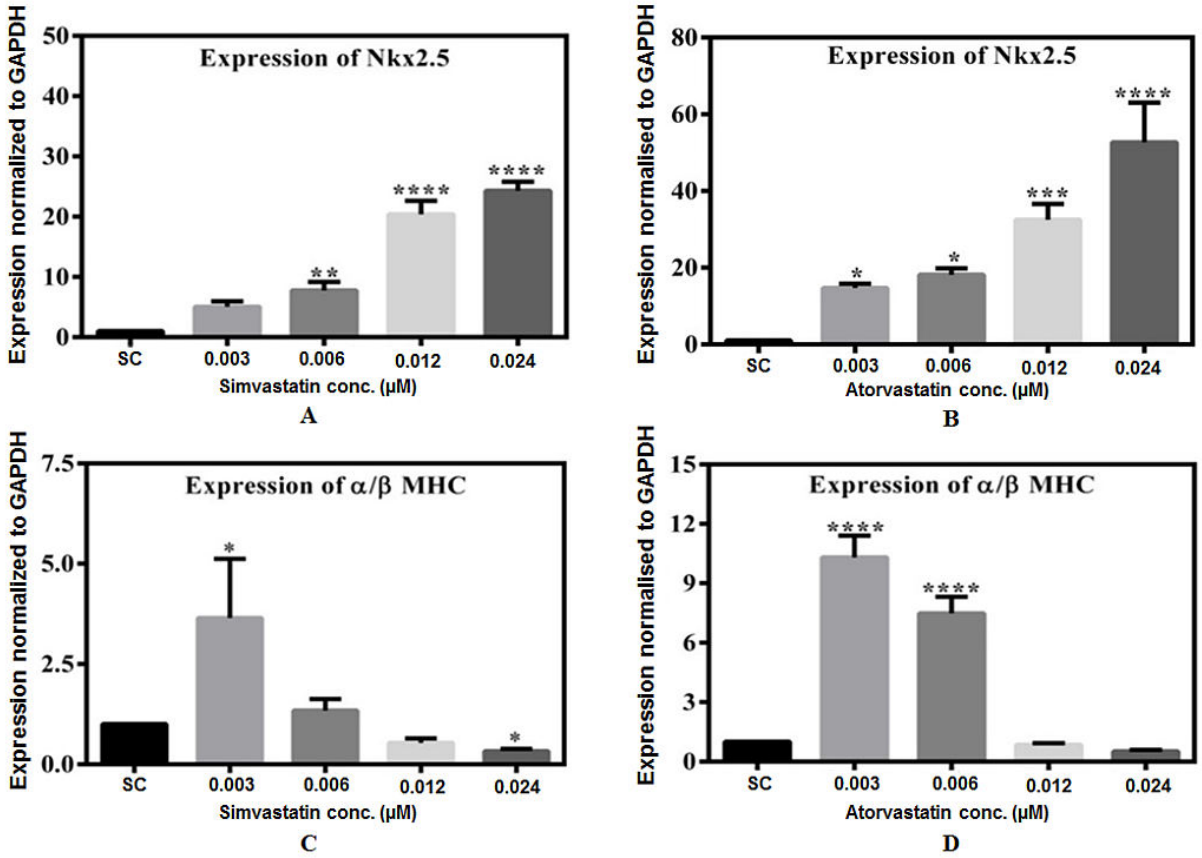


Figure 2.13: Mesodermal cardiac marker genes, Nkx2.5 and α/β MHC assessed by qRT-PCR in EBs treated with statins. (A); (B) Nkx2.5 expression after exposure to simvastatin and atorvastatin, respectively. (C); (D) α/β MHC expression after exposure to simvastatin and atorvastatin, respectively. Expression level was normalized to housekeeping gene *i.e.* GAPDH. The y-axis represents the fold changes in the expression ofthe gene under study.The data was analyzed by using the $2^{-\Delta\Delta}$Ct method.The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show means±SD (n=3). Significance was evaluated between exposed and solvent control treatment (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

2.3.7.2 Effect of statins on the endodermal gene markers during EB differentiation

To study the effect of statins on endodermal lineage in EBs, the effect of the simvastatin and atorvastatin on the expression level of Afp gene was evaluated. Expression of Afp is responsible for the differentiation of visceral endoderm. This gene which is expressed in early embryogenesis has also been seen to be expressed in EBs and therefore, makesit a target gene for a good *in vitro* model system. Afp was significantly up-regulated in EB differentiation during simvastatin treatment as seen in Figure 2.14A.Simvastatin at 0.003µM led to 11 fold ($p<0.05$), 15 fold at 0.006µM ($p<0.01$), 46 fold at 0.012µM ($p<0.0001$) and 60 fold at 0.024µM concentration($p<0.0001$). The expression pattern was according to the dose concentration indicating the potential of simvastatin in altering the gene expression during differentiation. Similar effect was observed upon atorvastatin treatment on the expression of Afp (Figure 2.14B).There was again a dose-dependent enhancement in expression. It was found that with treatment at 0.003µM of atorvastatin a 12.7 ($p<0.05$) fold increase, 14.5 ($p<0.05$) fold at 0.006µM, at 34.9 ($p<0.001$) fold at 0.012µM and 74.7 ($p<0.0001$) fold at 0.024µM concentration was seen in the EBs.

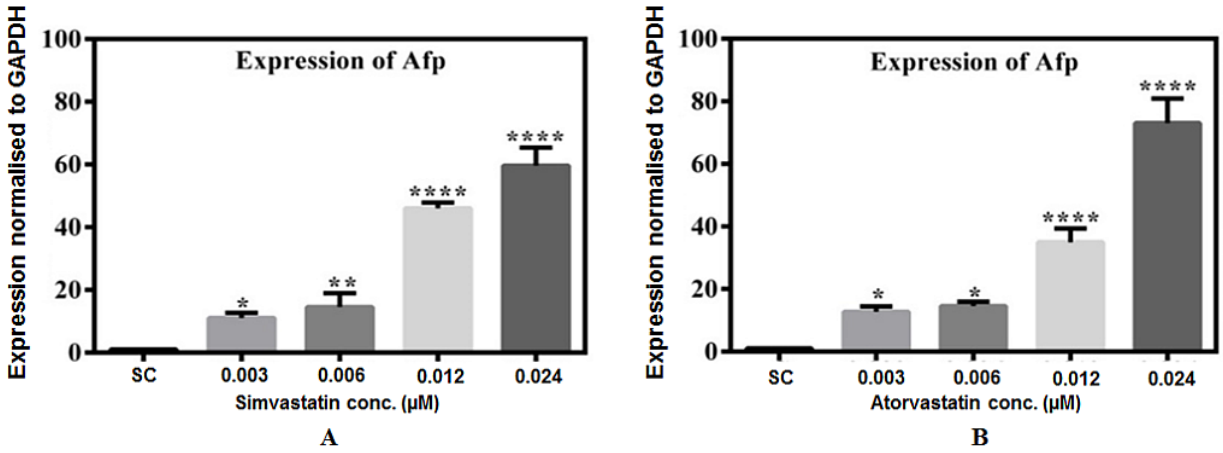


Figure 2.14: Endodermal marker gene, Afp assessed by qRT-PCR in EBs treated with statins. (A); (B) Afp expression after exposure to simvastatin and atorvastatin, respectively.Expression level was normalized to housekeeping gene *i.e.*GAPDH. The y-axis represents fold changes in the expression of Afp. The data was analyzed by using the $2^{-\Delta\Delta}$Ct method. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show means±SD (n=3). Significance was evaluated between exposed and solvent control treatment (*p<0.05, **p<0.01, ****p<0.0001).

2.3.7.3 Effect of statins on the ectodermal gene markers during EB differentiation

To assess the changes in ectodermal lineage, Nes (primitive neural stem cell marker) and Neurofilament 200kDa gene expression in EBs were analyzed. It was found thatthe expression level of both the genes was inversely proportional to the statins concentration to which the EBs were exposed. As observed in Figure 2.15, the expression of Nes was up-regulated 8 fold ($p<0.01$) upon treatment with 0.003μM simvastatin, however, treatment with a similar concentration of atorvastatin led to only 1.6 fold increase. Statins exposure at highest concentration (0.024 μM) led to decreased expression of Nes ($p<0.05$). A similar trend was observed in the expression of Neurofilament 200kDa upon simvastatin and atorvastatin treatment (Figure 2.15C, D). The expression was seen to be decreasing in dose dependent manner.

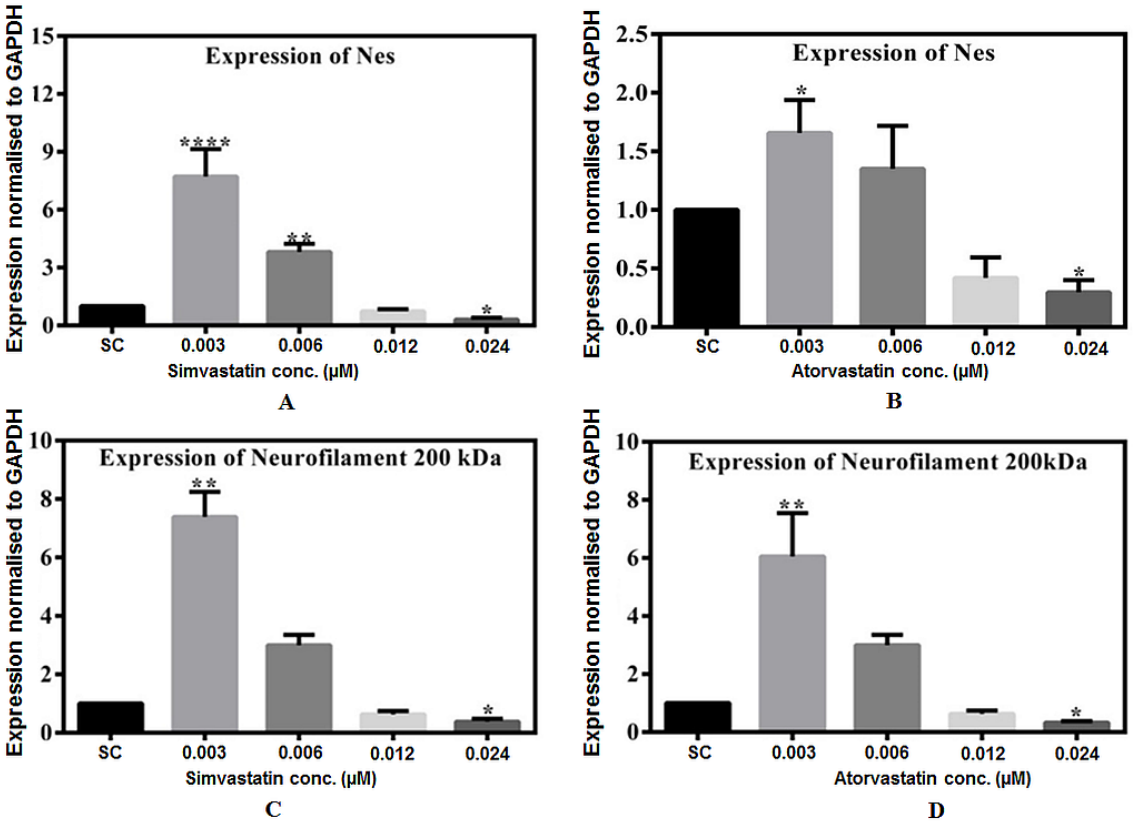


Figure 2.15: Expression of ectodermal marker genes,Nes and Neurofilament 200kDaassessed by qRT-PCR in EBs treated with statins.(A); (B) Nes expression after exposure to simvastatin and atorvastatin, respectively. (C); (D) Neurofilament 200 kDa expression after exposure to simvastatin and atorvastatin, respectively. Expression level was normalized to housekeeping gene *i.e.*GAPDH. The y-axis represents fold changes in the expression of the gene under study.The data was analyzed by using the $2^{-\Delta\Delta}$Ct method. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show mean±SD (n=3). Significance between exposed andsolvent control treatment (*p<0.05, **p<0.01, ****p<0.0001).

2.3.7.4 Monolayer differentiation: mesodermal expression in the presence of statins

To study whether the model system used for differentiation had an impact on the lineage differentiation of ES cells when exposed to statins, the study was replicated in ES cells which weremade to undergo differentiation using the monolayer method and exposed them to similar concentrations of simvastatin and atorvastatin.

The gene expression analysis of the statins exposed monolayer cultures of ES cells, when compared with the solvent control revealed differentially expressed genes. The qRT-PCR revealed the expression of mesodermal markers*i.e.*Brachyury, Flk-1, Nkx2.5 and α/β MHC,whichwere disturbed during monolayer differentiation whenexposed to statins. Brachyury gene expression in the presence of simvastatin was enhanced in a dose dependent manner (0.003µM, 62 fold, $p<0.01$; 0.006µM, 87 fold, $p<0.001$; 0.012µM, 208 fold, $p<0.0001$ and 0.024µM, 343 fold, $p<0.0001$). The expression in monolayer differentiation revealed no drastic alterations in pattern of up-regulation of Brachyury expression (Figure 2.16A) as compared to EB differentiation (Figure 2.12A). Similarly,atorvastatin also showed molding in expression in monolayer cultures as shown in Figure 2.16B. The alteration in expression was significant at all the concentrations tested. Flk-1 which is the receptor for VEGF, upon treatment with atorvastatin resulted in a highly significant dose dependent increase (0.003µM, 5 fold; 0.006µM, 14.4 fold, $p<0.01$; 0.012µM, 38 fold, $p<0.0001$ and 0.024µM, 62 fold, $p<0.0001$) whereas simvastatin although showed a similar pattern but to a slightly lesser degree (Figure2.16C, D).

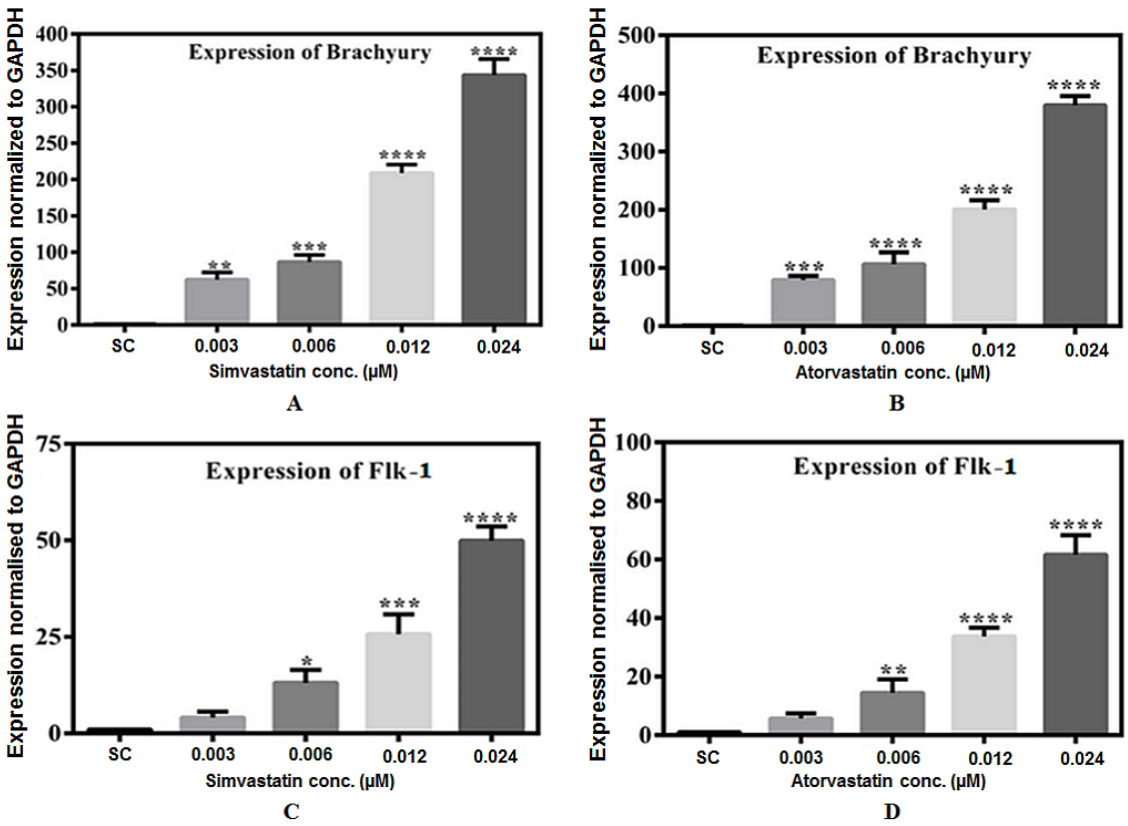


Figure 2.16:Early mesodermal marker genes, Brachyuryand Flk-1assessed by qRT-PCR in monolayer cultures exposed to statins. (A); (B) Brachyury expression after exposure to simvastatin and atorvastatin, respectively. (C); (D) Flk-1 expression after exposure to simvastatin and atorvastatin, respectively. Expression level was normalized to housekeeping gene *i.e.*GAPDH. The y-axis represents fold changes in the expression of the gene under study .The data was analyzed by using the $2^{-\Delta\Delta}$Ct method. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show mean±SD (n=3). Significance was evaluated between exposed and solvent control treatment (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Nkx2.5 showed up-regulated expression in monolayer cultures exposed to simvastatin (0.003μM, 2 fold; 0.006μM, 4 fold; 0.012μM, 15 fold, p<0.001; 0.024μM, 27 fold, p<0.0001)(Figure 2.17A). Atorvastatin also regulated Nkx2.5 in similar manner as observed in Figure 2.17B. Again, this trend was similar to that observed during EB differentiation. The specific reduction of α/β MHC expression in a dose dependent manner was also observed in D3 monolayer cultures exposed to simvastatin and atorvastatin treatment(Figure 2.17C, D). A

maximum reduction was observed at 0.024µM concentration in treatment with simvastatin (0.34 fold) ($p<0.01$) and atorvastatin (0.4 fold) ($p<0.05$), that again reflected the alterations which took place inEBs. This alteration in mesodermal marker expression in the monolayer differentiation model mimicked that of EB differentiation indicating similar interaction of statins in both differentiation protocols.

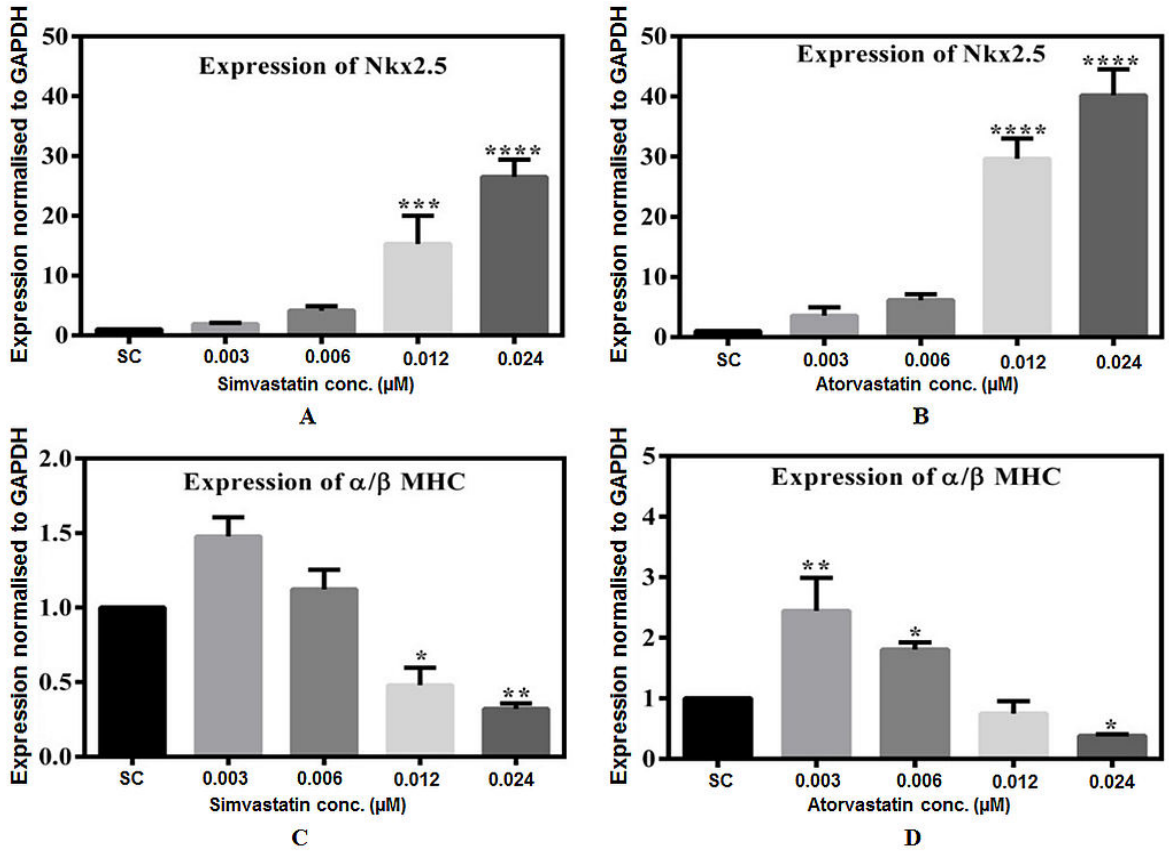


Figure 2.17: Mesodermal cardiac marker genes, Nkx2.5and α/β MHCassessed by qRT-PCR in monolayer cultures exposedtostatins. (A); (B)Nkx2.5 expression after exposure to simvastatin and atorvastatin, respectively.(C); (D)α/β MHC expression after exposure to simvastatin and atorvastatin, respectively. Expression level was normalized to housekeeping gene *i.e.*GAPDH.The y-axis represents fold changes in the expression of the gene under study. The data was analyzed by using the $2^{-\Delta\Delta}$Ct method. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show mean±SD (n=3). Significance evaluated between exposed and solvent control treatment (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

2.3.7.5 Endodermal expression inmonolayer cultures in the presence of statins

In ES cell monolayer cultures, the endodermal marker,Afp was strongly expressed upon treatment with statins. The expression of Afp was up-regulated in dose dependent manner with both simvastatin (Figure 2.18A) and atorvastatin (Figure 2.18B) with maximum up-regulation of 30.5 fold ($p<0.0001$) in the treatment with atorvastatin (0.024µM). Furthermore, on comparing endodermal expression in monolayer cultures with EB culture, similar effects of statins were observed.

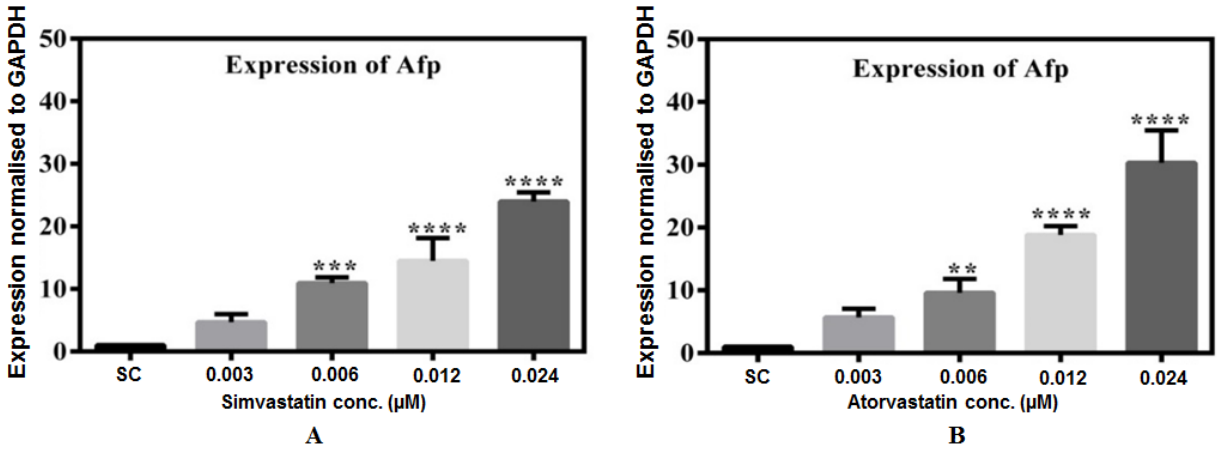


Figure 2.18: Endodermal marker gene, Afp assessed by qRT-PCR in monolayer cultures exposed to statins. (A); (B) Afpexpression after exposure to simvastatin and atorvastatin, respectively.Expression level was normalizedto housekeeping gene *i.e.*GAPDH. The y-axis represents fold changes in the expression of Afp.The data was analyzed by using the $2^{-\Delta\Delta}$Ct method. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show mean±SD (n=3). Significancebetween exposed and solvent control cells (p<0.01,***p<0.001, ****p<0.0001).**

2.3.7.6 Ectodermal expression in monolayer cultures in the presence of statins

Theexpression of ectodermal genes, Nes and Neurofilament 200kDadisplayed compromised differentiation upon treatment with increasing concentrations in monolayer differentiation(Figure 2.19). The early ectodermal lineage marker, Nes, showed significant down-regulation with increasing concentration of simvastatin *i.e.* 0.34 fold ($p<0.05$) at 0.024µM compared to 1.62 fold at 0.003µM concentration (Figure 2.19A). Neurofilament 200kDa showed

significant up-regulation *i.e.* 4 fold at 0.003µM,$p<0.05$, while the higher concentration resulted in down-regulation which was statistically non significant(Figure 2.19C). Atorvastatin showed similar although more potentiated affects at 0.003µMconcentration in both Nes ($p<0.01$) and Neurofilament 200kDa ($p<0.0001$), while no significant alterations at higher concentration was observed(Figure 2.19B, D).

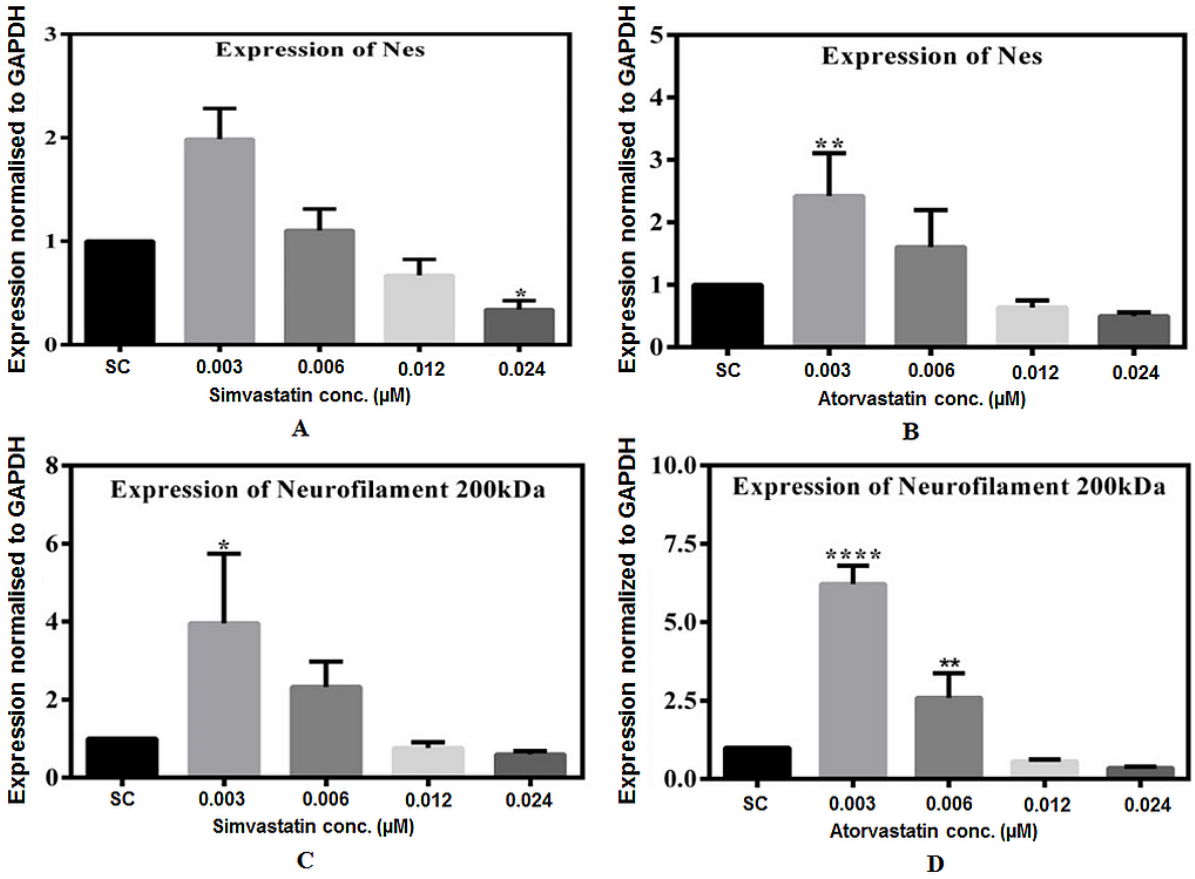


Figure 2.19: Ectodermal markers genes, Nes and Neurofilament 200kDa assessed by qRT-PCR in monolayer cultures exposed to statins.(A); (B)Nesexpression after exposure to simvastatin and atorvastatin, respectively. (C); (D)Neurofilament 200 kDa expression after exposure to simvastatin and atorvastatin, respectively. Expression level was normalized tohousekeeping gene *i.e.*GAPDH. The y-axis represents fold changes in the expression of the gene under study. The data was analyzed by using the $2^{-\Delta\Delta}$Ct method. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show mean±SD (n=3). Significance between exposed and solvent control cells (*p<0.05, **p<0.01, ****p<0.0001).

2.3.8 Duration and time frame analyses of statins on ES cells differentiation

In addition to the continuous dose dependent effects, the study also revealed, that at what stage of development statins altered the gene expression. In order to carry out this objective, the expressions of lineage specific markers were analyzedin monolayer cells undergoing differentiation in specific exposure-duration windows. The experimental outline shown previously in Table 2.3 (section 2.2.8) explains the manner in which various concentrations of the statins were given at specific times, followed by RNA isolation at day 10 and have been categorized as: 'A' single exposure at day 0; 'B' exposure at day 0 and day 3; 'C' exposure at day 0 and day 5; 'D' single exposure at day 3; 'E' exposure at day 3 and day 5; 'F' single exposure at day 5. The rationale for this workwas to ascertain whether the statins were interfering at the initial stage when ES cells start to aggregate (day 0 exposure) or during the process of EB formation (day 3 exposure) or when the EBs were established and were in the process of differentiation (day 5 exposure).

2.3.8.1 Time frame analysis of statins on ES cell differentiation: mesodermal lineage expression

The effect of simvastatin on the early mesodermal gene, Brachyury, suggested that the window of influence is crucial and exposure at the time when the EBs were established and differentiating (day 5),are most sensitive which resulted in highly deranged expression. However, exposure to atorvastatin from day 3 onwards led to significant alterations in expression, thereby suggesting that differentiating ES cells are more susceptible to this form of statins (Figure 2.20b). Similarly, the results for Flk-1 showed that simvastatin led to dose dependent alterations in the expression (Figure 2.20c), however,atorvastatin exposure at the various concentrations did not lead to noticeable alterations but were constant with respect to fold changes, except at day 5 exposure ('F') where, 0.012μM and 0.024μM concentrations caused sudden increases in fold expression(Figure 2.20d).

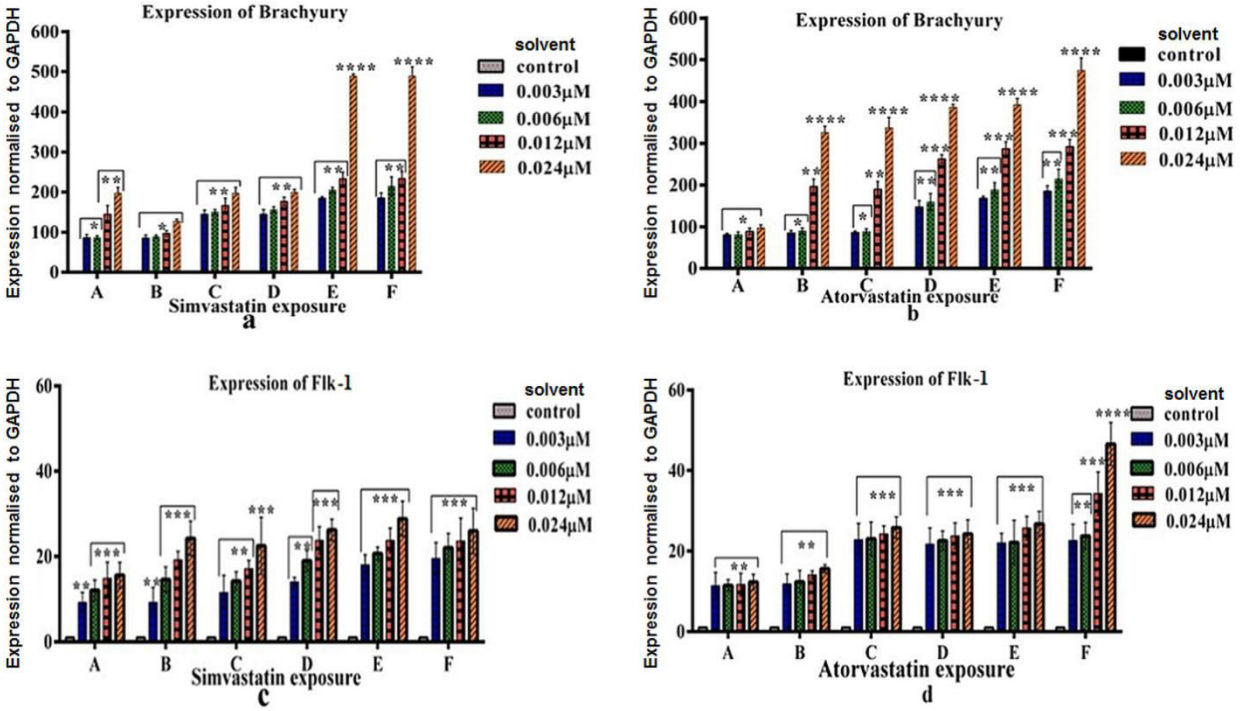


Figure 2.20: Expression pattern of early mesodermal marker genes, Brachyury and Flk-1during window exposure to statins.(a); (b)Brachyuryexpression after exposure to simvastatin and atorvastatin, respectively. (c); (d)Flk-1 expression after exposure to simvastatin and atorvastatin, respectively.Expression levels were normalized to housekeeping gene *i.e.*GAPDH. The y-axis represents fold changes in the expression of the gene under study. The data was analyzed by using the $2^{-\Delta\Delta}$Ct method.The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show mean ± SD (n = 3). Significance between exposed and solvent control cells (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

The cardiac precursor Nkx2.5 also showed an increased expression in ES cell differentiation when exposed to simvastatin at day 0 and day 5 as shown in Figure 2.21a, with the least effect seen at single exposure at day 3. On the other hand, ES cells treated with atorvastatin during time frame analysis showed near constant effects; resulting in up-regulation at all the exposure windows (Figure 2.21b). Interesting was the expression level of α/β MHC (this gene express both isomers) which was increasing at lower concentration (0.003µM) and remain unchanged with higher concentration (0.24µM) of simvastatin (Figure 2.21c). Another point observed in this time course analysis was that the expression of α/β MHC with simvastatin increased during initial exposure *i.e.* with onset of experiment for differentiation and this

expression was not significant during exposure at day 5. Atorvastatin showed significant increase in the α/β MHC expression at lower dose only in the initial phase of differentiation(Figure 2.21d). Moreover, the α/β MHC expression was less down-regulated during this time course analysis as compared to continuous exposure in EB and monolayer culture.

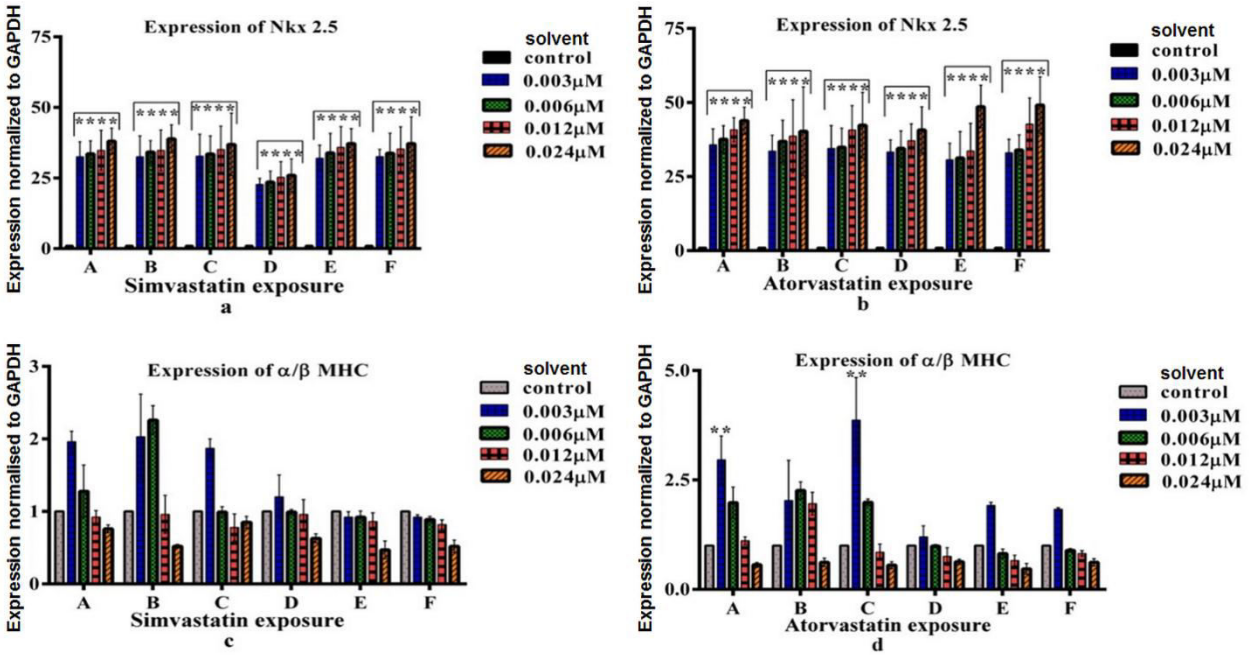


Figure 2.21: Expression pattern of the cardiac marker genes, Nkx2.5 and α/β MHC during window exposure to statins.(a); (b)Nkx2.5 expression after exposure to simvastatin and atorvastatin, respectively. (c); (d)α/β MHC expression after exposure to simvastatin and atorvastatin, respectively. Expression levels were normalized to housekeeping gene *i.e.*GAPDH. They-axis represents the fold changesin the expression of the gene under study. The data was analyzed by using the $2^{-\Delta\Delta}$Ct method. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show mean ± SD (n = 3). Significanceevaluated between exposed and solvent control treatment (p<0.01, ****p<0.0001).**

The down-regulation of α/β MHC protein upon exposure to the IC_{50}D3 of statins was further confirmed by ICC analysis asshown in Figure 2.22.

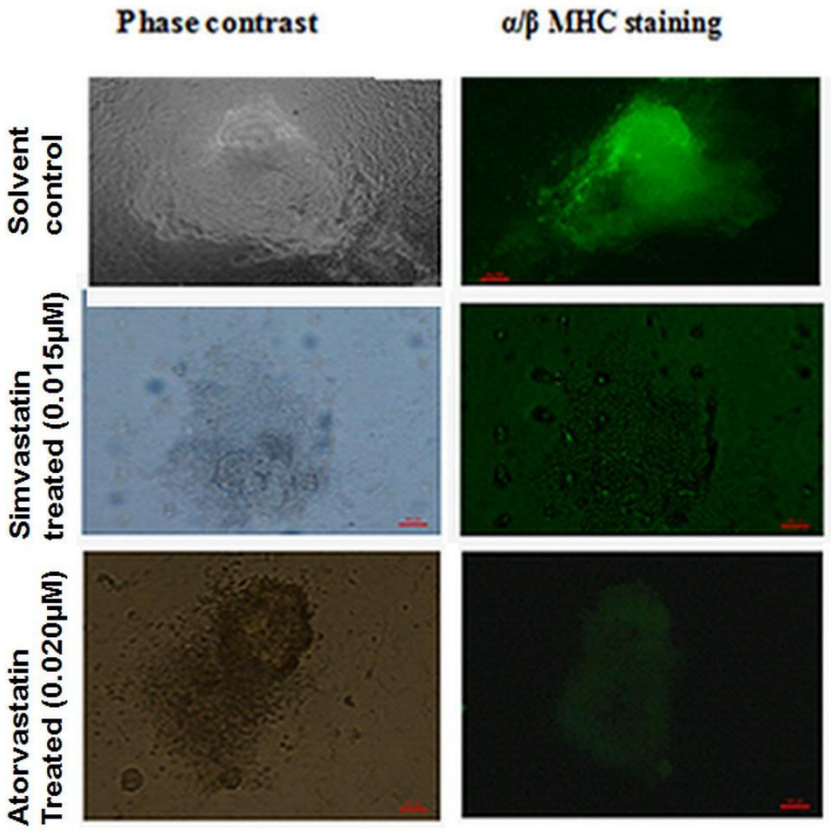


Figure 2.22: Representative images of differentiation of EBs exposed to statins stained with cardiac lineage marker α/β MHC. Magnification 10X. Bars: 100μm.The experiment was performedthree times in triplicates.

2.3.8.2 Time frame analysis of statins on ES cell differentiation: endodermal lineage expression

The expression of Afp exhibited the same pattern with varying conditions*i.e.* it was increased in dose dependent fashion with simvastatin (Figure 2.23a)whereas the Afp expression reached maximum increase in fold change with atorvastatin when the exposure was given at day 3, 5–10 (Figure 2.23b). This expression concluded that middle phase of differentiation was more influenced by atorvastatin exposure. The time frame analysis indicated that simvastatin and atorvastatin have different sensitivity on particular stage of differentiation for endodermal lineage.

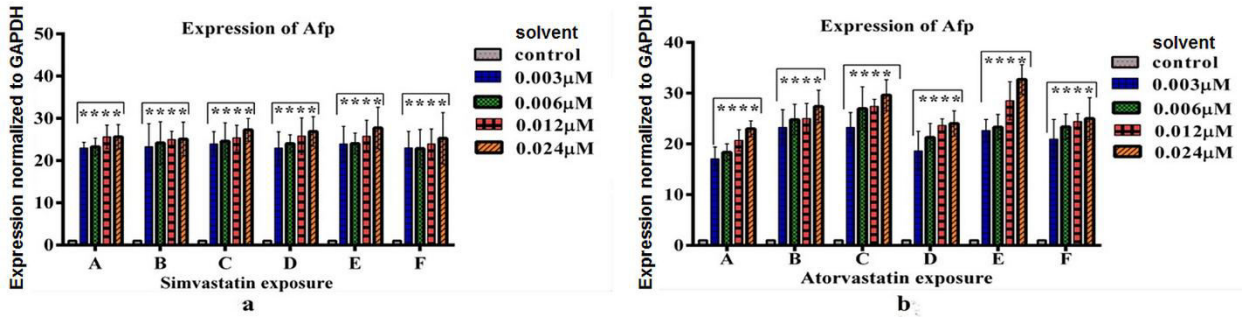


Figure 2.23: Expression patterns of the endodermal marker gene, Afp during window exposure to statins.(a); (b)Afpexpression after exposure to simvastatin and atorvastatin, respectively.Expression levels were normalized to housekeeping gene *i.e.*GAPDH. The y-axis represents fold changes in the expression of Afp. The data was analyzed by using the$2^{-\Delta\Delta}$Ct method.The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show mean ± SD (n = 3). Significance evaluated between exposed and solvent control treatment (**p<0.0001).**

2.3.8.3 Time frame analysis of statins on ES cell differentiation: ectodermal lineage expression

Ectodermal expression markers were regulated differently after exposure to statins during the time course analysis. The results showed extremely low expression levels of ectodermal markers at higher concentration of statins in EB and monolayer cultures (Figure 2.15 and 2.19).The expression level in time courserevealed the enhanced expression of ectodermal maker *i.e.* Nes (marker of neural progenitor cells) and Neurofilament 200kDa (mature neural cell marker) at lower concentrationsof statins (Figure 2.24). It was found that during the time course analysis,the expression level of Nes with simvastatin showed significant increase in fold expression at lower concentration when dose was given at days 3 and 5 (Figure 2.24a). Like simvastatin, atorvastatin produced similar effects on Nes *i.e.* the expression reached maximum change when exposure was given at days 3 and 5 and in this case, the expression was significant even at higher concentration of atorvastatin (Figure 2.24b). The expression of Neurofilament 200kDa showed significant increase in expression when treated with simvastatin on days 3 and 5 (Figure 2.24c). This indicated that simvastatin has interacted more strongly with EBs formation and during their differentiation.On the other hand, the expression of Neurofilament 200kDa with atorvastatin was significantly increased with dose given at onset of experiment and the expression level reached peak point at days 3 and 5 of exposure(Figure 2.24d). The results from

this study coveredthat the early EB development and their differentiation was effected by statins which behaved differently in altering the expression of ectodermal markers.

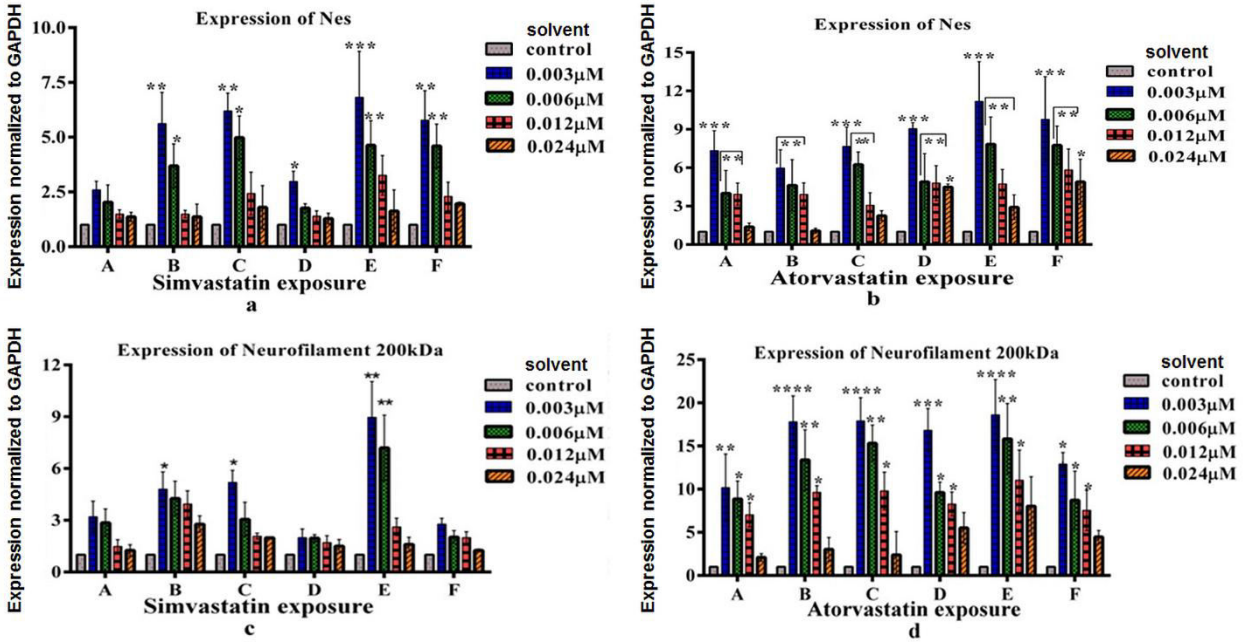


Figure 2.24: Expression patterns of the ectodermal marker genes, Nes and Neurofilament 200 kDa during window exposure to statins. (a); (b)Nesexpression after exposure to simvastatin and atorvastatin, respectively. (c); (d)Neurofilament 200 kDa expression after exposure to simvastatin and atorvastatin, respectively.Expression levels were normalized to housekeeping gene *i.e.*GAPDH. The y-axis represents fold changes in the expression of gene under study.The data was analyzed by using the $2^{-\Delta\Delta}$Ct method. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show mean ± SD (n = 3). Significance evaluated between exposed and solvent control treatment(*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

2.4 DISCUSSION

EScells are undifferentiated cells which are capable ofboth self-renewal and differentiation. ES cells play a critical and fundamental role in the formation of different tissues and organs of fetus during embryonic development. However, it has been reported that the exposure of fetus to statins during the critical periods of embryogenesis couldresult in congenital abnormalities [54, 55]. The studies contributed for exploring the relationship between statins and fetal exposures which have not been elucidated completely because lack of information and the available data are contradictory. Therefore, statins use remains contradicted in pregnancy. Information garnered from various studies has shown that in addition tothe inhibition of HMG-CoA reductase, statins have wide ranging actions. The major pleiotropic effects in broad way are, improving endothelial function, increased expression of VEGFandincreased mobilization of stem cells (endothelial progenitor cells) regulated *via* molecular mechanism [56]. Statins have also been shown to affect various pathways which play a critical role in embryogenesis such as Wnt canonical pathway, NO (nitric oxide) signaling, PI3/AKT and notch pathway [57-60]. The major focus of the present work was to assess genetic association between statins and congenital defects, revealing interaction of genes in signaling pathways in cellular processes that are essential for embryonic development.

This study used two differentiation protocols: monolayer and EB differentiation. The results uncovered a set of genes involved in critical stages of embryonic development whose expression was deregulated by statins and thus expands the findings of developmental toxicity of statins. Further,the dose with the time of exposure was also evaluated to substantiate the available information about the developmental toxic effects of statins on ES cells using monolayer differentiation protocol. Statins caused alteration in the expression of genes of mesodermal, ectodermal and endodermal lineage differentiation. The ability of statins to alter gene expression might throw some light on developmental toxic behavior of statins during embryonic development thereby resulting in congenital deformities.

The most important finding was the altered expression level of mesodermal lineage markers. Mesodermal lineage is responsible for cardiogenesis which is a complex biological process requiring the combination of stem cell commitment and multifarious communication from developing embryo. Various studies have revealed the involvement of key genes in controlling cardiac development.Congenital heart diseases are major cause of neonatal morbidity and mortality in humans [61]. The genetic basis for many of heart defects remains elusive,

however alterations in gene expression encoding core cardiac transcription factors has been shown to be a great contributor for congenital heart diseases [62]. In the present study, genes controlling cardiogenesis were shown to be deregulated upon statins exposure, which might be linked to morphological and functional forms of congenital heart defects. Mainly, four key mesodermal genes were focused: Brachyury, Flk-1, Nkx2.5, and α/β MHC.Statins stimulated a highly significant change in the expression pattern of Brachyury which is expressed during the primitive streak as well as in the developing mesoderm [63] and is necessary for normal mesoderm formation during gastrulation. In this study, Brachyury expression was sharply elevated at lower concentration of statins during the 10 days of differentiation. It has been studied that mouse ES cells without Brachyury expression were unable to undergo orchestrated gastrulation movements due to the non expression of the key cell adhesion molecules [64].However, till date the significant up-regulation in expression of Brachyury with statins exposure has not been elucidated and linked to the developmental toxicity of drugs. Simvastatin has been seen to have effects on oesteoblastic viability and on differentiation of osteoprecursor cells [65]. It has also been reported that in the ES cells, the patterning of the mesoderm and the endoderm are due to signaling centers formed in aggregated EBs and dense monolayer cultures [66]. Studies by Ding *et al* have shown that NO might be involved in early differentiation through the regulation of β-catenin and Brachyury by controlling the specification of the primitive streak [67]. The results of derangement in the key genes modulating mesodermal differentiation could therefore explain the limb abnormalities seen in infants exposed to statins *in utero.*

Flk-1(vascular endothelial growth factor receptor-2) is associated with distinct mesoderm restricted progenitors according to a biphasic expression profile during embryogenesis in which early expression marks hematopoietic lineages and delayed expression identifies cardiovascular progenitor cell potential[68].It has also been reported that this growth factor plays a critical role in cardiac by inducing the pre-endocardial mesenchyme to become endocardial epithelium morphogenesis, although the mechanisms are not fully understood(endocardial vasculogenesis) [69]. However, even a modest increase in VEGF (vascular endothelial growth factor) levels during embryonic development resulted in an abnormal signalling which has been shown to lead to severe anomalies [70]. Flk-1 has been also implicated in endothelial cell migration leading to angiogenesis by forming a complex with VE-cadherin [71].It has also been

reported that over expression of VEGF receptors in mouse embryos resulted in an excess production of blood vessels and malformed hearts [72].This up-regulated Flk-1 expression upon statins treatment in this study could thus throw light on the genetic basis of congenital heart defects.

It was also observed that the Nkx2.5 showed significant up-regulated expression. Nkx2.5 is an important transcription factor that regulates various aspects of cardiac development starting with specification and proliferation of cardiac progenitor cells [73]. Nkx2.5expression in cardiac tissues continues throughout development and into adult life [74] but the functions regulated by its continued expression is unknown. In this study, effects of statins were detected on this particular gene and resulted in its increased expression. The homeobox gene Nkx2.5is the earliest known marker of vertebrate heart development [75]. Lyons *et al* found that targeted interference of murine Nkx2.5 ended in an early embryonic lethality and cardiac arrest at the linear heart tube stage prior to looping [76]. Another important finding was that the statins led to decreased α/β MHC expression at higher concentrations (0.012μM and 0.024μM) which was correlated with poor differentiation observed in the EBs upon ICC analysis. These results suggested that the overall disruption by simvastatin and atorvastatin of the regulatory framework of early transcription factors in early embryonic cardiac development played a vital role in the manifestation of the cardiac defects. In addition to this, *in vitro* embryotoxicity analysis using ES cells have revealed the Nkx2.5 and α/β MHC genes as sensitive marker for predicting the teratogenicity of compounds.

Afpis considered as a marker for the visceral endoderm in both the early embryo development and *in vitro* EB differentiation [77, 78]and has also been shown to be a very sensitive molecular endpoint to detect embryotoxicants [79]. In accordance with its embryotoxic nature, the elevated expression levels in ES cells were found after treatment with statins at all concentrations and at each time window of exposure. The probable physiologic functions of Afp in directing the developmental events such as erthyropoiesis, histogenesis/organogenesis, fetal growth and differentiation, and the fetal defects associated with a symptom of elevated levels of Afp are very well explained by Mizejewski *et al*[80]. The establishment of patterning of the endoderm during development is interplay of various signals which arise from the surrounding mesoderm derived tissue and it has been seen that this specification is reciprocal. These results therefore suggested that changes in the *in utero* environment quickly translated to a rapid

increase in Afp which would then impact differentiation leading to the defects associated with raised levels of Afp such as fetal neural tube defects; anencephaly and spina bifida [81].

Nes is expressed in cells during ectodermal development and in neural progenitor cells [82]. As neurogenesis proceeds, Nes is replaced by specific intermediate filaments (Neurofilament in neurons and GFAP in astrocytes). In these results,statins affected the expression of Nes and Neurofilament200 kDa, in a dual manner, wherein the expressions were up-regulated at lower concentrations (0.003µM and 0.006µM) but down-regulated with increasing dose (0.012µM and 0.024µM). The Nes expression has shown to be enhanced by neurotoxic compounds such as methyl mercury chloride and valproic acid, in studies reported by Hogberg *et al*[83]. The more specific neural marker Neurofilament 200 kDa showed down-regulatedexpression in a dose dependent manner. More specifically, atorvastatin had more impact on ectodermal lineage. The up-regulation of ectodermal genes driven by statins at lower doses, observed in this study, could contribute to the congenital anomalies related with exposure to statins seen in the CNS of the developing embryo.

Hence, it was hypothesized that during differentiation, up-regulation of lineage specific genes due to statins might stem from their pleiotropic effects. Up-regulation of genes with statin exposure can be correlated to various signaling pathways such as Wnt canonical pathway, NO signaling, and PI3/AKT and notch pathway. In this study, Brachyury which is a target gene of Wnt pathway was up-regulated and is expressed during primitive streak formation. It has been shown that NO–β-catenin involved in modulating primitive streak formation in ES cell *via* enhancing the expression of Brachyury and β-catenin which might contribute to osteogenic differentiation [67].The role of NO in cardiogenesis during embryonic development is well known [84, 85]. In a study done by Mujoo and co-workers, they found an increased expression of Nkx2.5 in murine ES cells upon treatment with NO [86]. Similarly, the potent angiogenic growth factor Flk-1was also up-regulated in mice models upon statins treatment [87]. Their study explained that binding of VEGF to flk-1 directed the receptor phosphorylation upon atorvastatin exposure [88] and subsequent activation of PI3K/Akt and other downstream signaling proteins [89]. However, as reported earlier [90], down-regulation of the PI3K pathway is required for the formation of the definitive endoderm, therefore continued signaling by this pathway would result in aberrant patterning of the endoderm. It was studied that simvastatin through notch signaling pathway promoted endothelial differentiation from bone marrow stromal cells (BMSCs) and

induced arteriogenesis [91]. If statins interacts with these signaling components during embryogenesis, the effect could result in abnormal expression in fetus. These effects mightbe associated with dose and kind of statins taken during pregnancy.

In the past few years, ES cells have been the backbone of basic as well as advanced biomedical research for e.g. drug discovery and cell based therapy. Invaluable and limitless insights into normal and abnormal cellular changes occurringduring embryogenesis have been gleaned by studying the expression of these cell lines *in vitro*. These findings demonstrated that each stage of development is critical and sensitive to statins in its own way. Changing the time, concentration and duration of statins exposure resulted in a varying pattern of lineage gene expressions which could impact the developing embryo *in utero.*

Furthermore, since signals from one lineage could impact the differentiation of the others, the results obtained in this study suggested that statins treatment plays a pivotal role in the signaling crosstalk between the endoderm,ectoderm and mesoderm lineages during differentiation which regulate stem cell fate and throw light on the potential genetic basis for congenital anomalies.

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ABSTRACT

Homeopathic medicines have garnered much attention and debate in recent years. However, well documented studies are required for their potential effects and safety issues regarding their use in pregnancy. In this study, mouse embryonic stem (ES) cells were used as a model system and exposed to 30C potency of *Nux Vomica* and *Sepia*, which are medicines prescribed for the management of pregnancy related symptoms. Cytotoxicity studies were done using a modified ES cells testing (EST). The expression level of key genes and proteins expressed during differentiation was analyzed using real time polymerase chain reactionand immunocytochemistry, respectively. The results showed that homeopathic treatment led to slight modulations in the expression of certain lineage specific genes but this effect was not significant and showed normal differentiation as demonstrated by the expression of α/β MHC and α-actinin proteins in the differentiated ES cells.This study for the first time showed the use of ES cells in the developmental toxicity testing of homeopathy and the results obtained further support the use of these medicines in pregnancy.

CHAPTER 3

3.1 INTRODUCTION

Homeopathic medicines are considered to be safe because of the belief that at dilutions which are conventionally used for treatment were associated with less adverse effects [1,2]. The popularity of homeopathy amongst the believers in this system of medicine is not based on hearsay but on rational, systematic studies and observation of the effects of the remedies on people. Moreover, homeopathic medicines are prepared by the process of dynamization, in which a miniscule quantity of the original natural medicinal essence is necessary when manufacturing these medicines. Being incalculably small in concentration, these medicines have not shown to induce any adverse effects even when taken for extended periods and can therefore be safely given even to drug sensitive group like pregnant women, babies and elders. Conventional medicine generally suppresses symptoms rather than treating the underlying cause, and when taken during pregnancy does have a threat of various side effects on the developing fetus. Homeopathy balances the whole system and thus allowing the body to heal itself. Since homeopathy works with the body's natural defense system, it is advocated by the homeopaths that homeopathic medicines pose no harmful effect on developing embryo.

The foundation for the homeopathic system of medicine was systematized by the German physician Samuel Hahnemann in 1796. The mainprinciple behind the action of homeopathy is the *Similarity (or Similia) Principle: 'Similia similibus curentur' ('Let like be cured by like')*, stated by Samuel Hahnemann [3]. Simply put, this means that a substance which has potential of causing disease in healthy organisms can be used as medicines for treatment of similar patterns of disorder experienced by patients.

A second principle of homeopathy is *individualization of treatment for the patient*. The properties of the chosen medicine must be as similar as likely to the symptoms of the disease in the patient. This most compatible match is called the 'simillimum'. Resemblance may be at the 'whole person' level, counting the symptoms and signs of the disease, the patient's physical built, personality, temperament and genetic predisposition. This great level of individualization is however not always needed: 'similarity' may be at a more particular, local level, especially in the curing of severe conditions [3].

A third principle of homeopathy is the *use of the minimum dose*. The dilutions used in treatment ranges from those which are similar in concentration to some conventional medicines

to very high dilutions containing no material trace of the starting substance. Vigorous shaking of the solution along with impact or 'elastic collision' (known as 'succussion') is an established manufacturing process and is a fundamental element in the production of homeopathic medicines [4, 5].

Dilutions are designated by numbers and letters, for example,30X or 30C. These designated letters and numbers indicate the potency of the homeopathic remedy. 'X' potency is made by taking one drop of a natural material substance (like ink from squid-*Sepia*) and diluting it in 10 drops of water/alcohol (X is 10 potency). 'C' potency is taking the same substance and diluting it in 100 drops of water/alcohol (C is 100^{th} potency). Then one drop is taken from that X or C potency after succussion and diluted in another 10 or 100 drops, respectively. Each stage produces the next potency as in 10X to 11X or 12C to 13C. Potencies can be diluted and succussed thousands of times to produce M potency (1000), 10M, 50M, CM (100M) and MM (1000M). It should be noted that these high potencies are not to be used lightly and should be given by experienced homeopaths. The most common potencies used in pregnancy are 30C and 200Cas they are considered safe during first trimester in pregnancy by CCRH,(Central Council for Research in Homeopathy), India [6].

Homeopathy is a controversial science. The elucidation of how homeopathic medicines work, has evoked the cynicism from the scientific community[7, 8]. The most serious criticisms related to homeopathy are the lack of transparency of mechanisms behind these remedies [9, 10]. It is said that homeopathy works at the cellular level as all the biological and physiological activities occur at the cellular level. Researchers need high quality cells/model systems to prove the efficiency of homeopathy and herein liesthe dilemma of homeopathy's potential because of the lack of scientific data [11]. Thus, the present work was doneto suggest a model system to study the effects of homeopathic remedies taken during pregnancy using ES cells as the model which mimics *in vivo* development of the embryo. The two homeopathic remedies *Nux Vomica* and *Sepia* under study were chosen as they are routinely prescribed in early pregnancy to counteract many of the symptoms encountered during this stage [12].

Nux vomica is the dried, ripe seed of *Nux Vomica L.* (Family,Loganiaceae), a native tree of Burma, China, eastern India, Thailand, and northern Australia[13]. As homeopathic remedy, *Nux Vomica* is given for allergies, back pain, colds, constipation, digestive problems, emotional

stress, flu, hangovers, headaches, hemorrhoids, and menstrual problems. *Nux vomica* isa popular homeopathic remedy used in pregnancy and is given for nausea. Although*Nux Vomica* appeared as a treatment in 19th Century medical publications, there is a very little documentation on its therapeutic effectiveness in today's standard medical journals. *Nux Vomica* is a common homeopathic remedy and is given to counter symptoms of nausea and constipation during pregnancy. It is therefore,necessary to carry out research on this remedyto generate sufficientexperimental data for its safe use in pregnancy [13-15].

Sepia is a product from the fresh ink of *Sepia officinalis*whichis commonly known as cuttlefish. When this cuttlefish sense danger it releases out ink which serves for bothdefensive and aggressive purposes. This ink has been used as a source for making the homeopathy remedy *Sepia* [15-17].*Sepia* is a popular homeopathy remedy prescribed to counteract morning sickness, constipation and headache duringpregnancy.But the safety use of *Sepia* in pregnancy is not well documented. Moreover, these homeopathic remedies are regulated as over the counter drugs.

ES cells are the source of all organs and tissues of the body. Being pluripotent in nature, they have the potential to differentiate into all cells found in the body. In their undifferentiated state, they can be maintained in indefinitely and with capable of differentiation *in vitro* - represent an unlimited source of somatic cells. ES cells research contributes to a fundamental understanding of how organisms develop and grow, and how tissues are maintained throughout adult life.The development of ES cell lines hasprovided researchers with the reliable tool to study developmental toxicity and birth defects [18]. The effects of conventional medicine taken during pregnancy have been studied using this*in vitro* EST model.Currently, there are no methods for testing developmental toxicity of homeopathy medicines to prove their safety use in pregnancy. In this study,various lineage specific genes were studied which could serve as biomarkersfor specification of these three lineagesto predict the safety of homeopathic remedies using mouse ES cells.

3.2 MATERIALS AND METHODS

3.2.1 Cell lines and culture conditions

The mouse ES cell line D3 was cultured at 37^0C and 5% CO_2 and routinely passaged three times a week. ES cells were cultured in Dulbecco's modified Eagles Medium (Invitrogen), supplemented with 15% heat inactivated fetal bovine serum (FBS), 2mM glutamine (Invitrogen), 50U/ml penicillin and 50µg/ml streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1mM β-mercaptoethanol and 1000U/ml LIF (leukemia inhibitory factor, ESGRO., Chemicon International Inc., Temecula, CA).

NIH 3T3 cells were procured from National Center for Cell Sciences, Pune. NIH 3T3 was maintained in complete medium, which was composed of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Sigma, USA), 50U/ml penicillin and 50µg/ml streptomycin (Gibco). When the cell were approximately 80–90% confluent, they were subcultured (2 times a week).

3.2.2 Homeopathy remedies used for testing

The 30C potency of the homeopathic remedies *Nux Vomica* and *Sepia* were tested at concentrations of 1µl/ml and 5µl/ml. These remedies were obtained from Wilmar Schwabe, Germany. The concentration was selected on the basis of review of homeopathic database citing its use in pregnancy. As homeopathic remedies are prepared in 90% ethanol,it was needed to limit the maximum concentration of ethanol to below 0.5% v/v so as to negate the effect of the solvent. So, the final concentration of ethanol was ≤ 0.5% in the test concentrations and thus ethanol at 0.5% concentration was used as a solvent control.

3.2.3 MTT assay

Cytotoxicity determination of the homeopathic remedies was done on the basis of MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay according to the modified shortened EST protocol using NIH 3T3 fibroblast and D3 ES cells, as previously described [18, 19]. In brief, trypsinized cells were counted using heamocytometer and seeded in each well of 96 well plates at density of 20,000 cells/50 µl media. After 4 hours of incubation, the cells were treated with 150µl of culture media containing homeopathic remedies at concentration of 1µl/mland 5µl/ml.The media was replaced on day 3 with fresh media containing the same

concentrations of the remedies. Cytotoxicity was assessed using the MTT on day 5. Following this,20µl of MTT solution (5mg/ml in phosphate buffer saline) was added to each well containing the cells and incubated for 4 hours at 37^0C. Then the media was replaced with 150µl of DMSO and incubated for next 20 minutes. Absorbance of the formazan products due to the viable cells was measured at a wavelength of 570nm with reference wavelength 630nm in microplate reader (BIO-RAD model 680) andthe concentration-response curve was obtained.The assay was performed four times in triplicates.The cytotoxicity was calculated as per the formula given below:

$$\% \text{ Cytotoxicity} = \frac{\text{O.D. of solvent control} - \text{O.D. of test sample}}{\text{O.D. of solvent control}} \times 100$$

3.2.4 Flow cytometry analysis for cell viability

Flow cytometry was performed using an Accuri C6 flow cytometer (BD Sciences) to analyze cell viability using propidium iodide (PI) staining dye. For PI cell viability analysis,$1.5x10^5$ cells were seeded in6 well plates. The seeding day was considered as day zero. After 24 hours, old media was replaced with fresh media along with homeopathic remedies (*Nux Vomica* and *Sepia*) in different concentrations (1µl/ml and 5µl/ml) and kept for a period of 3 days. At day 3, the media was again replaced with fresh media containing the homeopathic remedies at the specific concentrations. On day 5, cells were trypsinized and the pellet was collected after centrifugationat 80 *g*for 5 minutesand PI (5µg/ml) treatment was given for 30 minutes. This was followed by the analysis of live and dead cell population in Accuri C6 flow cytometer. PI is excited at 488nm and emits at a maximum wavelength of 617nm. The experiment was done three times in triplicates.

3.2.5 Differentiation of ES cells

To analyze the effects of homeopathic remedies on differentiation of ES cells, two different differentiation protocols were performed: the hanging drop method and the monolayer differentiation method. The choice of method was dependent on the assay that had to be performed.

3.2.5.1 EB differentiation

The EB assay was selected for the differentiation experiments as EBs mirror the early steps of embryogenesis for both mouse and human ES cells differentiation protocols [20, 21]. Differentiation was carried out in hanging drops according to a modified method done byHeuer *et al*[21]. In brief, a drop of 20µl from ES cell suspension (5 x 10^4 cells/ml) was placed onto the inner side of the lid of a Petri dish (Corning) filled with phosphate buffered saline (PBS) (Himedia) and then incubated at 37^0Cwith 5% CO_2. After culturing for 3 days, the formed aggregates (EBs) were transferred into bacteriological petri dishes (non-treated). At day 5, EBs were plated separately onto gelatin coated coverslips in 6 well plates (Thermo Scientific) for ICC analysis at day 10.

3.2.5.2 Monolayer differentiation

The cells were seeded at density of 5 x 10^4/mlin 6 well plates (Thermo scientific) in Dulbecco's modified Eagles Medium (Invitrogen), supplemented with 15% heat inactivated fetal bovine serum (FBS), 2mM glutamine (Invitrogen), 50U/ml penicillin and 50µg/ml streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1mM β-mercaptoethanol without LIF (leukemia inhibitory factor). The RNA was isolated on day 5.

3.2.6Immunocytochemistry (ICC)

D3 (EBs) were obtained from hanging drop methodology and transferred onto gelatin coated coverslips in 12 well plate at day 5. After culture for next 5 days, the EBs were fixed in paraformeldehyde solution (4% in PBS) for 30 minutes at room temperature.After a PBS wash, EBs were permeabilized with Triton X-100 (0.25% in PBS) added for 15 minutes followedby washing with wash buffer [1% bovine serum albumin (BSA) in PBS]. This was followed by incubation for 60 minutes with 5% BSA in PBS at room temperature to block non-specific binding and again washed with wash buffer. EBs were then incubated with primary antibodies againstα/β MHC (Abcam) and α-actinin (Sigma) which were diluted at 1:250 and 1:200, respectively, and kept overnight at 4^0C.After washing with wash buffer, cells were incubated with secondary antibody:Fluorescein-5-isothiocyanate (FITC)-conjugated rabbit anti-mouse (Sigma) diluted in the ratio 1:500 and again washed with wash buffer. The EBs were viewed under the Nikon eclipse Ti microscope at 10X magnification using the appropriate filters

excitation at 530nm for FITC staining. The ICC experiment was performed three times in triplicates.

3.2.7RNA isolation, cDNA synthesis and quantitative real timePCR (qRT-PCR) for analyses of gene markers associated with differentiation

The RNA was isolated with RNeasy Mini Kit (Qiagen) and included DNA digestion. The concentration and quality of RNA was measured with a Nano drop 2000 spectrophotometer (Thermo Scientific). Synthesis of cDNA from RNAwas carried outby using an oligo dT (15) primer in the presence of M-MULV Reverse Transcriptase (Genetix). PCR was performed with 0.5µg c-DNA (cDNA) of each sample using gene specific primers in order to determine the expression level for target gene. qRT-PCR was performed on CFX-96 real time PCR (Bio-Rad laboratories) using SYBR Green real time PCR dye (Bio-Rad laboratories). The conditions were: initial denaturation at 93^0C for 4 minutes, followed by 39 cycles each of denaturation (95^0C for 15 ~~s~~ seconds), annealing (54.5-60.2^{0}C for 20 seconds) and extension (72^0C for one minute). The relative quantitative expressions of lineage specific markers were calculated after normalization against GAPDH, a housekeeping gene.The present study analysed a total of seven gene markers which are associated with the formation of three different lineages. The gene markers associated with the formation of mesodermal lineage included Brachyury, Flk-1, Nkx2.5 and α/β MHC while (Afp) Alpha fetoprotein wasselecteddue to its association with the development of endoderm. For studying the expression of ectodermal lineage, nestin (Nes) and Neurofilament 200 kDa (ND200) gene markers were analyzed. The details of the primers with annealing temperatures are provided in the Table 3.1. The gene expression analysis was performed three times in triplicates.

Table 3.1: **Detail of the primers used in this study**

Genes	Primer sequence (Forward primer-FP and Reverse primer-RP)	Annealing temperatures (^{0}C)
GAPDH	FP 5'-GCACAGTCAAGGCCGAGAAT-3'	58.5
	RP 5'-GCCTTCTCCATGGTGGTGAA-3'	
Afp	FP 5'-GCTGCAAAGCTGACAACAAG-3'	58.7
	RP 5'-GGTTGTTGCCTGGAGGTTTC-3'	
Flk-1	FP 5'-CAGCTTCCAAGTGGCTAAGG-3'	54.5
	RP 5'-CAGAGCAACACACCGAAAGA-3'	
Nes	FP 5'-GCTTTCCTGACCCCAAGCTG-3'	60.2
	RP 5'-GGCAAGGGGGAAGAGAAGGA-3'	
ND 200	FP 5'-TGGACATTGAGATTGCCGC-3'	62.4
	RP 5'-GAGAGAAGGGACTCGGACCAA-3'	
Nkx2.5	FP 5'-CAAGTGCTCTCCTGCTTTCC-3'	56.5
	RP 5'-GGCTTTGTCCAGCTCCACT-3'	
α/β MHC	FP 5'-ACCTGTCCAAGTTCCGCAAG-3'	58.5
	RP 5'-CTTGTTGACCTGGGACTCGG-3'	
Brachyury	FP 5'-TTCTTTGGCATCAAGGAAGG-3'	57.0
	RP 5'-TCCCGA GACCCAGTTCATAG-3'	

3.2.8Statistical Analyses

The cytotoxicity data was obtained by calculating the mean±SEM (standard error mean) from four individual experiments done in triplicates. The cytotoxic effects of homeopathic remedies were statistically analyzed with one-way ANOVA followed by Tukey test using

GraphPad Prism version 6.0. The ICC experiment was performed three times in triplicates.The effect of these remedies on the expression levels of the lineage specific markers were analyzed using the $2^{-\Delta\Delta}$Ct method.The gene expression analysis was obtained by calculating the mean ± SD (standard deviation) from 3 individual experiments done in triplicates. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad Prism software version 6.0.

3.3 RESULTS

3.3.1 Determination of the cytotoxicity of homeopathy remedies using ES cells (D3 cells) and adult fibroblast cells (3T3 cells)

For cytotoxicity analysis, MTT assay according to a modified EST model was performed to study the cytotoxicity effects on D3 and 3T3, cells which represented embryonic and adult cells, respectively. In both cell types, the homeopathic remedies namely *Nux Vomica* and *Sepia*, were tested at their different concentrations.

Sensitivity of D3 cells showed varied responses upon treatment with the homeopathic remedies. Cytotoxicity in D3 cells treated with 30C potency of*Nux Vomica* showed a killing of 15.47±2.5% at 1µl/ml concentration and 20.36±4% at 5µl/ml concentration while cytotoxicity in D3 cells treated with 30C potency of*Sepia* showed a killing of 17.71±3.0% at 1µl/ml concentration and 18.73±4.0% at 5µl/ml concentration, with respect to the solvent control of D3 cells (Figure 3.1a). The percent cytotoxicity of D3 cells exposed to homeopathic remedies was found to be non significant with respect to the solvent control.Although slightly higher cytotoxicity was observed at the higher concentration(5µl/ml) as compared to 1µl/ml, however,this was a non significant difference in the observed cytotoxicity in the D3 treated cells which had been exposed to the two different concentrationsof both *Sepia* and *Nux Vomica* used in this study. The statistical analysis was done by using one-way ANOVA followed by Tukeytest.

3T3 cells also showed increased cytotoxicity with increasing concentration of 30C potency of *Nux Vomicai.e.* 4.70±1.2% at 1µl/ml concentration and15.9±2.7% at 5µl/ml concentration. Similarly,upon treatment with 30C potency of *Sepia*, 3T3 cellsshowed increased cytotoxicity with increase in concentration *i.e.* 8.07±1.9% at 1µl/ml concentration and 13.77±3.50% at 5µl/ml concentration, with respect to the solvent control of 3T3 cells (Figure

3.1b).The statistical analysis which was done by using one-way ANOVA followed by Tukey test revealed that percent cytotoxicity observed in the 3T3 cells treated with the homeopathic remedies wasnonsignificant with respect to both the solvent control as well as between the two concentrations under study and this held true for both *Sepia* and *Nux Vomica*.

Although marginal cytotoxicity was observed upon treatment of ES and 3T3 cells with the homeopathy remedies, however, these results were not statistically significant. Although, the sensitivity of homeopathic remedies towards D3 was more as compared to 3T3 cells as evidenced by increased cell death observed in the D3 cells. The cytotoxicity data was represented as concentration–response curves as shown in Figure 3.1.

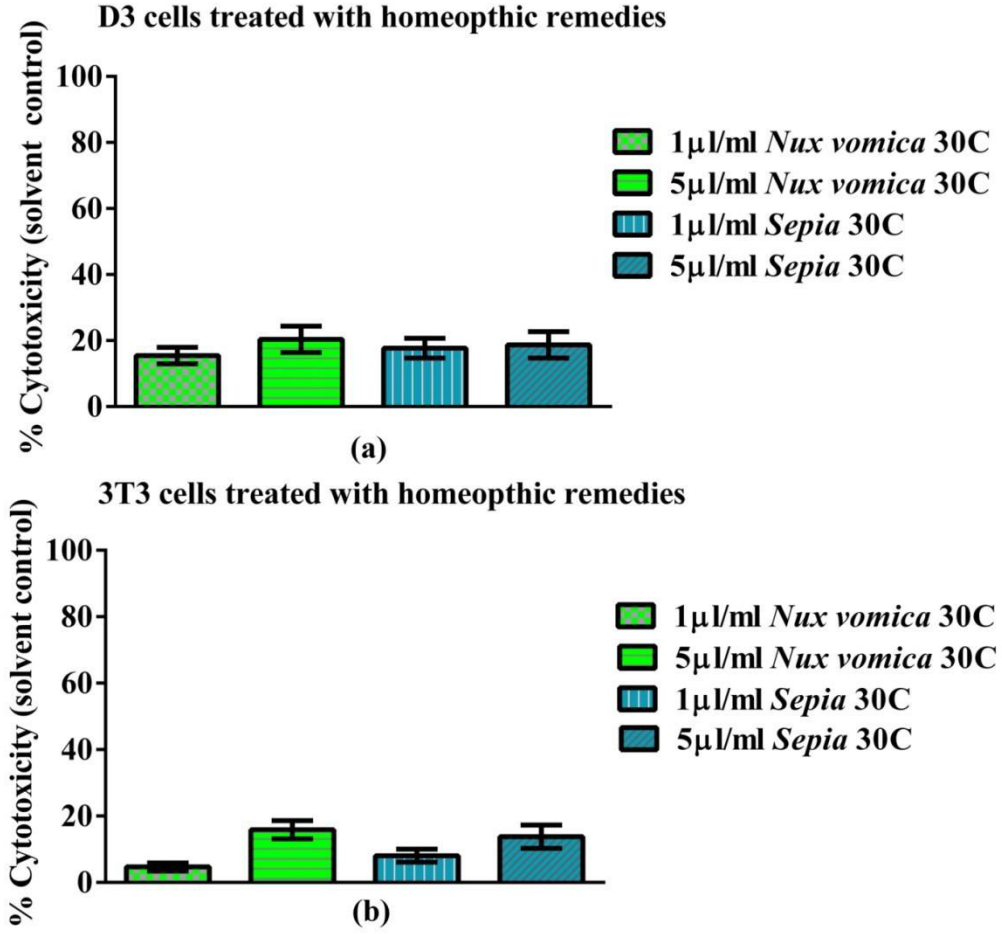

Figure 3.1:Concentration–response curves obtained for homeopathic remedies. Cytotoxicity was assessed by the MTT assay in (a) undifferentiated D3 cells and (b) differentiated 3T3 cells. Bar graphs show means ± SEM. All values are average of four experiments done in triplicates.

3.3.2Analysis of cell viability using flow cytometry

The flow cytometry data revealed that the homeopathic remedies had no effect on cell viability of both cell types. It was observed that D3 cells after exposure to *Nux Vomica* 30C potency at 1µl/ml concentration had 81.7% live cell population and at 5µl/ml concentration, the live populationwas 85.9%. This was not statistically significant as compared to the control (untreated D3 cells which had 86.6% and solvent control had 86.8% live cell population). Treatment of 30C potency of*Sepia*on the D3 cells followed a similar trend and was also found to be non significant as compared to the control *i.e.* 1µl/ml concentration resulted in 85.9% of live population and 5µl/ml concentration resulted in 84.3% of live population (Figure 3.2). 3T3 cells also showed a similar trend as the data revealed that treatment of 30C potency of *Nux Vomica* had 78.1% and 70.1% live cell population upon treatment with 1µl/ml and 5µl/ml concentration, respectively.In contrast to this, the control cells had 84.4% and solvent control had 82.5% of live cell population.

Similarly, 30C potency of *Sepia* also showed no sensitivity on 3T3 cells as compared to the controls (untreated 3T3 cells which had 84.4% and solvent control had 82.5% live cell population)*i.e.* they exhibited 73.8% live population upon treatment with 1µl/ml concentration and 75.8% live cell population upon treatment with 5µl/ml concentration. These results further confirmed that the both the cells types were not harmed by homeopathic remedies because the effects were similar to controls and moreover were not significantly different (Figure 3.3).

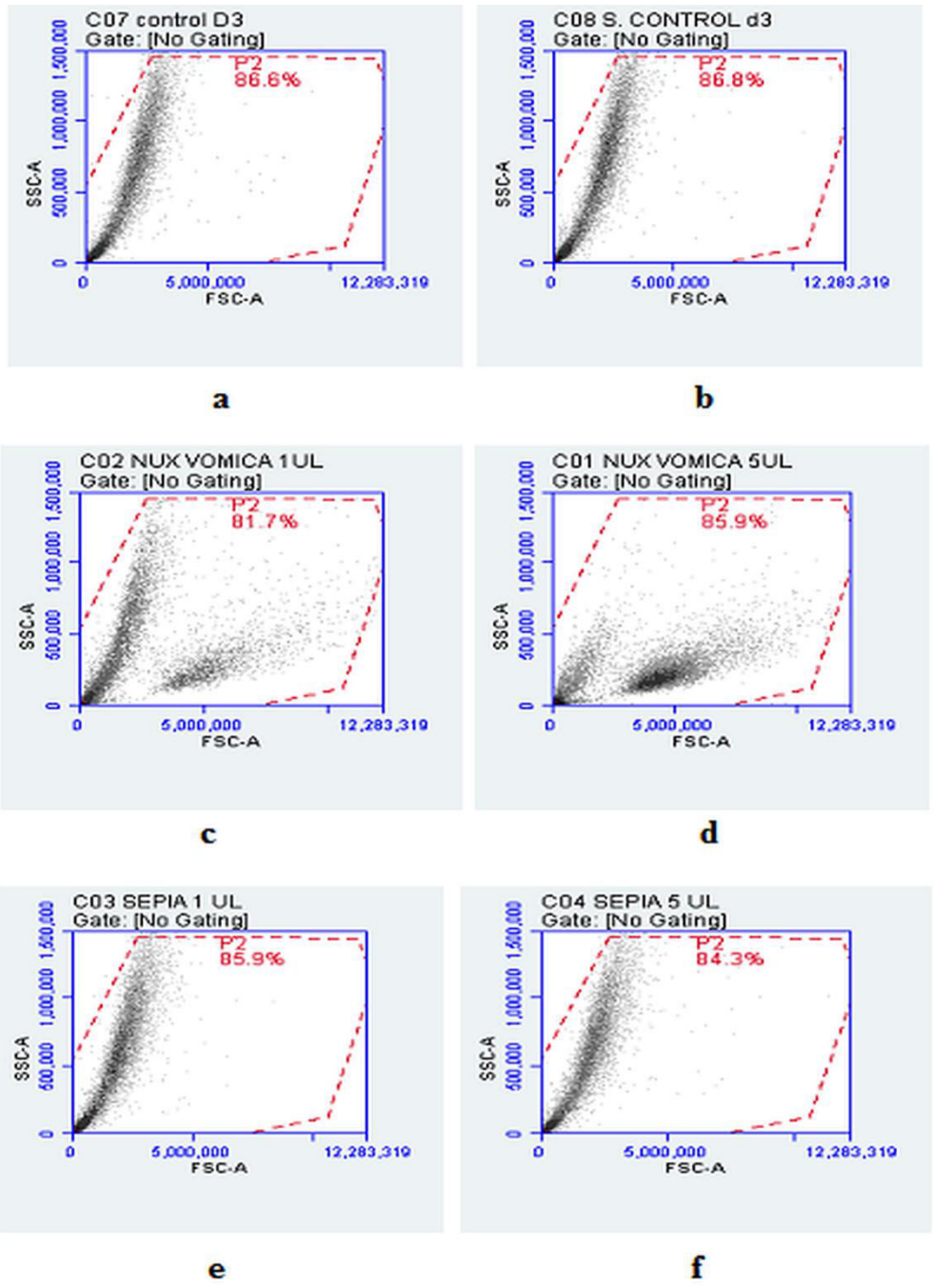

Figure 3.2:Flow cytometry analysis showing the cell viability of D3 cells after treatment with homeopathic remedies on day 5. (a) untreated control D3 cells; (b) Solvent control treated D3 cells; (c) D3 cells treated with 1µl/ml concentration of *Nux Vomica*30C potency; (d) D3 cells treated with 5µl/ml concentration of *Nux Vomica*30C potency;(e) D3 cells treated with 1µl/ml concentration of *Sepia*30C potency; (f) D3 cells treated with 5µl/ml concentration of *Sepia* 30C potency.

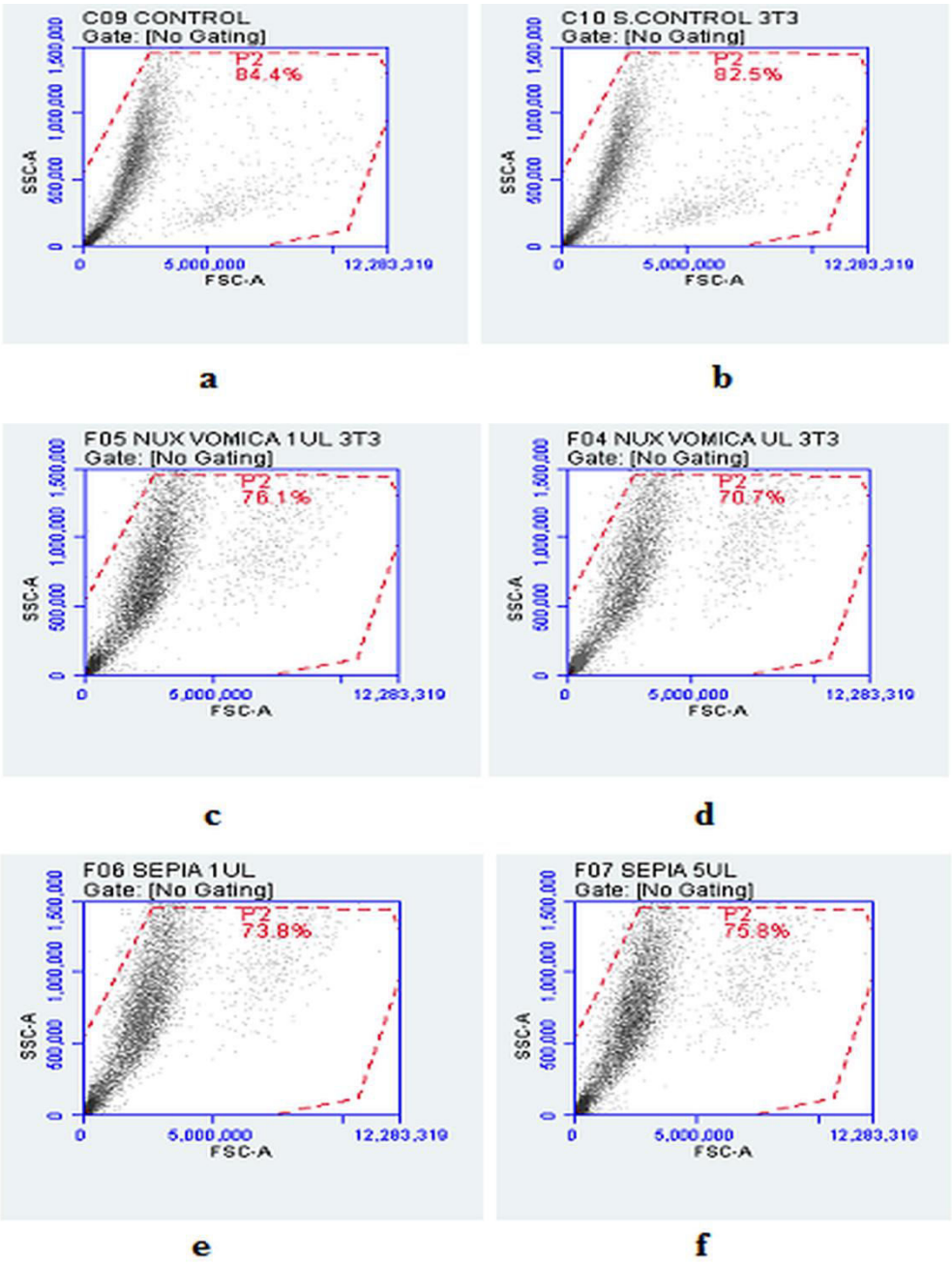

Figure 3.3:Flow cytometry analysis showing the cell viability of 3T3 cells after treatment with homeopathic remedies on day 5. (a) untreated control of 3T3 cells; (b)Solvent control treated 3T3 cells; (c) 3T3 cells treated with 1µl/ml concentration of *Nux Vomica* 30C potency; (d) 3T3 cells treated with 5µl/ml concentration of *Nux Vomica*30C potency; (e) 3T3 cells treated with 1µl/ml concentration of *Sepia*30C potency; (f) 3T3 cells treated with 5µl/ml concentration of *Sepia* 30C potency.

3.3.3Immunocytochemistry of mesodermal markers of EBs exposed to homeopathic remedies

ICC analysis of α/β MHC and α-actinin revealed that remedies had no effect on differentiation of the EBs when tested at the highest concentration under study *i.e.* 5µl/mlconcentration of 30C potency. On observing Figure 3.4, untreated control and solvent control EBs (without homeopathic treatment) showed expression of α/β MHC as well as α-actinin which were comparable to that of the treated EBs (5µl/ml concentration of homeopathic remedies). Treatment with5µl/ml concentration of 30C potency*Nux Vomica*and *Sepia* represented EBs stained with antibodies against α/β MHC and α-actinin, whichshowed normal growth and differentiation. In addition, there was no variation in the size of the EBsas compared to solvent control (Figure 3.4).

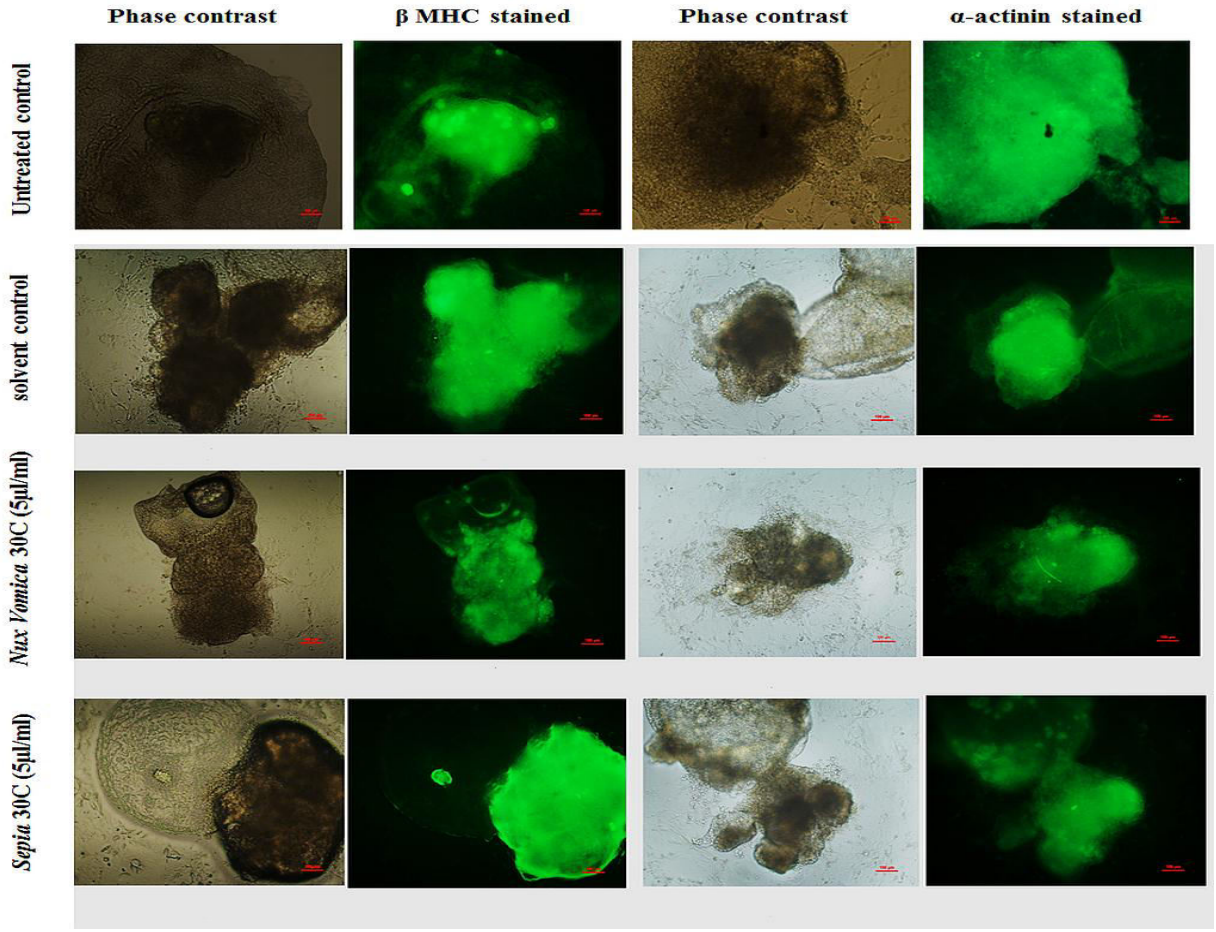

Figure 3.4:ICC analysis of EBs stained with cardiomyocytes differentiation marker α/β MHC and α-actinin after treatment with homeopathic remedies at day 10. Magnification 10X. Bars: 100µm. The experiment was performed three times in triplicates.

3.3.4Expression of marker genes of differentiation of ES cells exposed to *Nux Vomica* (30C potency)

To compare the changes in the expression of genes responsible for lineage commitment, the untreated and treated ES cellswere subjected to monolayer differentiation. The expression level of genes considered as the markers of differentiation during embryogenesis was analyzed to identify the changes caused by exposing the ES cells to homeopathic remedies *Nux Vomica* and *Sepia* (potency 30C).

It was found that the cells exposed to *Nux Vomica* 30C potency (1µl/ml and 5µl/ml, concentrations) for 5 days down-regulated the expression of early mesodermal marker, Brachyury to 0.52±0.11 fold and 0.50±0.08 fold, respectively. The Flk-1 gene which is also an early mesodermal marker was also down-regulated to the same extent *i.e.* 0.55±0.07 fold for 1µl/ml concentration and 0.38±0.05 at concentration of 5µl/ml. The markers for cardiomyocytes *i.e.* α/β MHC and Nkx2.5 showed negligible down-regulation in expression upon treatment with 30C potency of *Nux Vomica*. 1µl/ml concentration of 30C potency*Nux Vomica* resulted in the decreased expression of α/β MHC to 0.92±0.05 fold and Nkx2.5 to 0.82±0.11 fold. Similarly, 5µl/ml concentration also resulted in the down-regulation of α/β MHC expression to 0.55±0.07 fold and Nkx2.5 to 0.53±0.09 fold. All the changes observed were not significant with respect to the solvent control (Figure 3.5a).

Afp which represents the endodermal lineage was also down-regulated to 0.61±0.14fold in case of 1µl/mlconcentration and 0.53±0.05 fold after exposure to 5µl/mlconcentration(Figure 3.5b).

Ectodermal markers, Nes and ND200 were also found to be down-regulated to the same extent *i.e.*0.63±0.07 fold at 1µl/ml concentrationand at 5µl/ml concentration,the change was 0.60±0.09 fold (Nes) and 0.32±0.05 fold (ND200) (Figure 3.5c).

Although down-regulation for all the genes of the 3 lineages was observed but this effect was not significant.

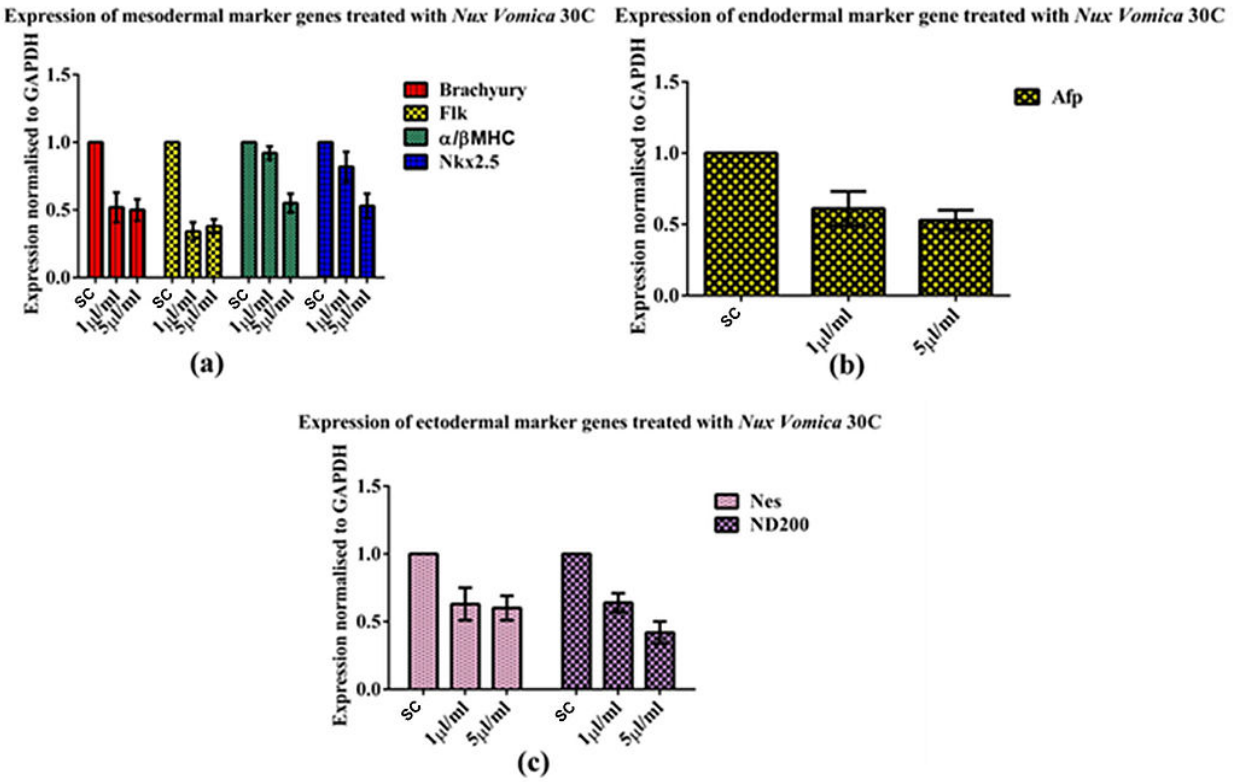

Figure 3.5:Gene expression analysis of three lineages of ES cells exposed to homeopathic remedy *Nux Vomica*30C (a)mesodermal; (b)endodermal; (c) ectodermal.Expression level was normalized to housekeeping gene *i.e.* GAPDH. The y-axis represents the fold changes in the expression of the gene under study. The data was analyzed by using the $2^{-\Delta\Delta}$Ct method. Bar graphs show means ± SD (n = 3). Significance between treated cells and untreated cells was calculated by applying two-way ANOVA.

3.3.5Expression of markers genes of ES cells differentiation after exposure to *Sepia* 30C potency remedy

Sepia, homeopathic remedy at 30C potency decreased the expression of genes in concentration-dependent manner. Brachyury and Flk-1, genes expression level after exposure to 1µl/ml concentration was reduced to 0.52±0.06 and 0.82±0.12,fold, respectively. After exposure to 5µl/ml concentration, the expression level was found to be 0.41±0.07 and 0.66±0.09 fold, respectively. The cardiac marker, α/β MHC showed 0.74±0.11 fold and Nkx2.5 showed 0.72±0.1 fold expression after exposure to 1µl/ml concentration of 30C potency and 0.61±0.09 and 0.60±0.09, fold after exposure to 5µl/ml concentration of 30C potency (Figure 3.6a). Similarly, the Afp was also decreased and showed 0.65±0.14 and 0.48±0.05,fold change with 1µl/ml and

5µl/ml, concentration as compared to normal expression (Figure 3.6b). The ectodermal markers were also influenced by the 30C potency*Sepia* (Figure 3.6c). The Nes expression was 0.58±0.08 fold and ND200 expression was 0.69±0.05 fold, after exposure to 1µl/ml concentration. The expression of Nes at 5µl/ml concentration was 0.55±0.07 fold. The expression of ND200 was found to be 0.53±0.08 fold after exposure to 5µl/ml concentration.

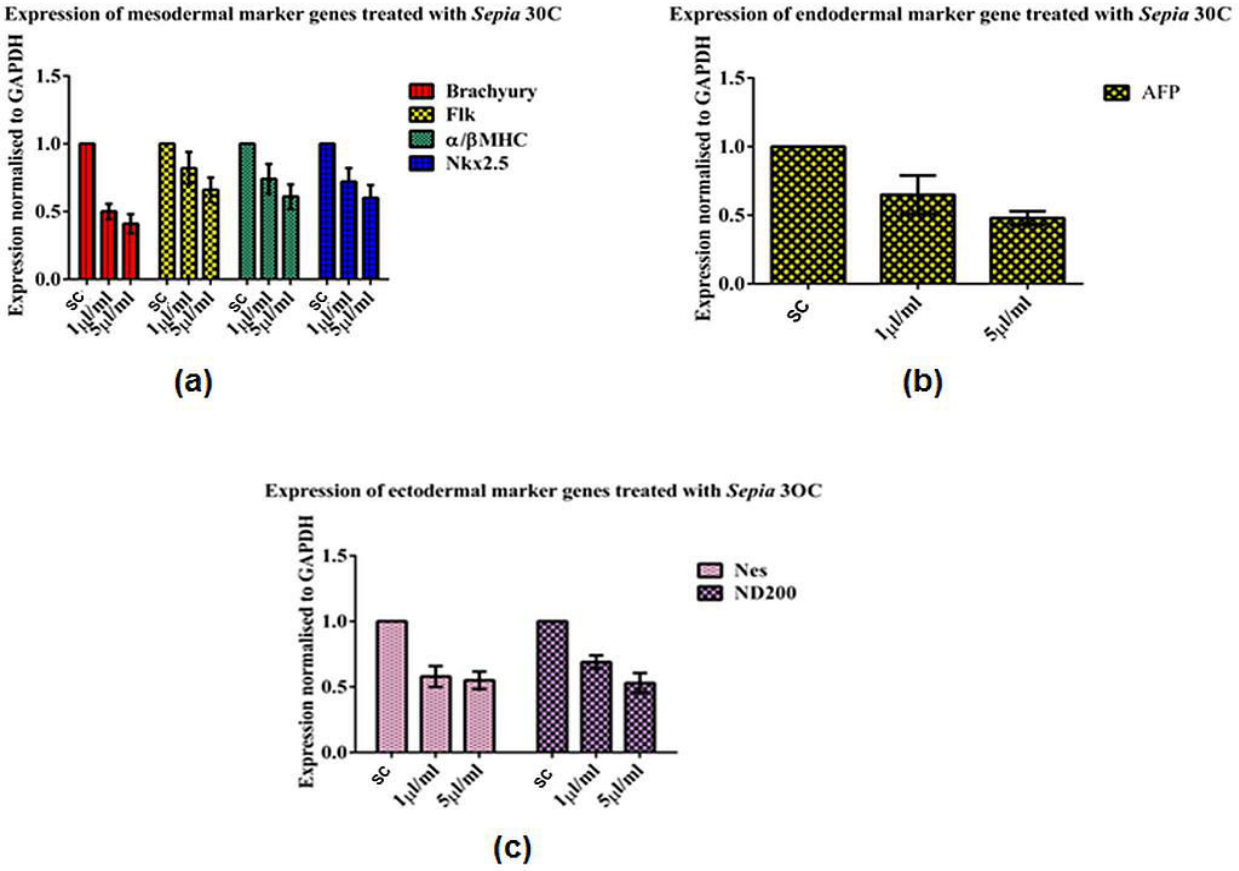

Figure 3.6:Gene expression analysis of three lineages of ES cells exposed to homeopathic remedy*Sepia* 30C (a) mesodermal; (b) endodermal; (c) ectodermal.Expression level was normalized to housekeeping gene *i.e.* GAPDH. The y-axis represents the fold changes in the expression of the gene under study. The data was analyzed by using the $2^{-\Delta\Delta}$Ct method. Bar graphs show means ± SD (n = 3). Significance between treated cells and untreated cells was calculated by applying two-way ANOVA.

The results from the gene expression analysis during the differentiation of mouse ES cells provided new insights to the effects of this homeopathic remedy. It is clear from these results

that remedies which were advocated as safe during pregnancy have been validated by experimental data.

3.4 DISCUSSION

Over the last decade, the use of homeopathy remedies has grown exponentially. However, knowledge regarding harmful effects of these remedies by users is limited and available data is conflicting. This is of particular concern in pregnancy, where need of considering their potential effects on fetal development and drug interactions are of extreme importance. The mechanism of action of these remedies and factors related to their use in pregnancy need extensive studies for safety assessment during this sensitive stage of life. Thisresearch work provides the first step in evaluating and providing scientific evidences for effects of homeopathic remedies during embryogenesis.

In order to know the causes of congenital abnormalities due to drug intake, ES cell has been the first choice as a model system, to identify toxicities that may potentially be encountered in the embryos. Therefore, in this study the potential of ES cells was used in exploring the effects of homeopathic remedies taken during pregnancy.

Homeopathic remedies are commonly used for common problems in pregnancy e.g. nausea, constipation, indigestion by a large section of society in Asia and certain regions of Europe [23, 24]. The focus of this study was to evaluate the possible effects of the 30C potency on the differentiating ES cells at an early stage of the developmental process which mimics the growing embryo. In this study, the cytotoxic effects of *Nux Vomica* and *Sepia* homeopathic remedies on both adult cells and ES cells were analyzed. The analysis of key genes which mark the onset of differentiation of the 3 lineages was studied using qRT-PCR analysis which revealed nonsignificant alterations in the genes upon treatment with these remedies. These results will help in convincing the skeptics that these medicines prescribed during pregnancy are indeed safe and do not contribute to any developmental toxicity at the potency of 30C. These results are in conformity with other well documented studies with regard to the safety assessment of homeopathic remedies on normal cell lines [25-27].

Nux Vomica is a popular homeopathic remedy prescribed for symptoms such as morning sickness and constipation, which are frequently encountered during pregnancy. In this study, it was observed that *Nux Vomica* had a marginal effect on cell viability and gene expression. It was

seen that the early mesodermal biomarkers, Brachyury and Flk-1 were not significantly sensitive to 30C potency and this was true for the cardiac markers α/β MHC and Nkx2.5 as well which showed similar sensitivity to 30C potency. The normal expression of α/β MHC and α-actinin which are key proteins involved in the differentiation towards cardiomyocytes and are required for the proper development and functioning of heart, were also documented. These effects on gene and protein expression upon treatment with 2 different concentrations revealed that although changes were not significant as compared to controls, however, the remedies did act in a concentration-dependent manner leading to the effects on expression of genes and that embryonic cells were more sensitive to the treatment as compared to adult cells.

Sepia is derived from ink of the cuttlefish (is the pigment Melanin), which is comprised of sulphur, calcium, and magnesium, among other compounds and it appears bilious like a dark stain[15, 16]. The effect of *Sepia* 30C potency on differentiation of key lineage genes was not significant. For both adult as well as EScells, the results followed a similar trend as that of *Nux Vomica* exposure. The non cytotoxic nature of the homeopathic medicines was reiterated by the fact that the ES cells which were allowed to aggregate in the form of EBs showed a size comparable to untreated ES control cells and differentiated to express key proteins α/β MHC and α-actinin,that are present in cardiomyocytes. This demonstrated no impact on the differentiation capability of the ES cells which were exposed to either *Nux Vomica* or *Sepia*.

In this study, a key question wasraised whether the homeopathic remedies werehaving effects on the growing embryo which could lead to abnormalities? In order to study this, a panel of genes representing the three lineages was chosen. The rationale was that if there were significant changes in the expression of these key genes then that would indicate adverse effects to the embryo. The choice of the genes to be assessed was based upon previous studies in which various researchers had used these genes to demonstrate the developmental toxicity upon exposure to chemical compounds [22, 28, 29]. In the mouse ES cellbased differentiation model, Brachyury is known determinant of meso-endoderm lineage, and cells which are positive in Brachyury expression are able to differentiate into mesodermal and definitive endodermal lineages[30]. Flk-1 gene has biphasic potential in embryogenesis in which early expression leads to hematopoietic lineages and later on the expression is positive for cardiovascular progenitor cell potential [31]. In embryogenesis, Flk-1 is expressed by the primitive endoderm, embryonic angioblasts and in the blood islands as well as in angiogenic vessels. The aimed disruption of

gene Flk-1 ended in mice lethality at E8.5-E9.5 due to lack of development of the blood islands, embryonic vasculature and hematopoietic cells [32, 33].The expression of α/β MHC in mammalian hearts can be expressed in two isoforms; α-MHC is solely expressed in cardiac tissue, whereas β-MHC expression is observed in both cardiac and slow skeletal muscle. The expression of α/β MHC plays an important function in the development of contraction and adaptation in cardiac muscle [34, 35]. The normal expression of this α/β MHC gene was clearly observed in ES cells after treatment with homeopathic remedies. Nkx2.5 which is a dynamic transcription factor functions in various aspects of early cardiac development leading to specification and proliferation of cardiac progenitor cells and its expression is constant during development and in adult life [36, 37].This homeobox gene, Nkx2.5 [38] is the most primitive known marker of mammalian cardiac development. It was reported that targeted disruption of murine Nkx2.5 resulted in embryonic lethality and cardiac arrest at the linear heart tube stage prior to looping [39]. In this study, homeopathic remedies did not alter the expression of mesodermal genes under study.

Afp gene expression leads to endoderm specification during embryogenesis[40]. Its expression is used extensively as biomarker of endoderm lineage. Its sensitivity is also used for studying embryotoxicity of drugs [41]. Altered levels of Afp which are not within normal limits in pregnancy, have been reported for multitude of congenital malformations of the fetus [42-44]. Although, this study did observe that the expression of Afp was decreased, this was not significant~~ly~~ and therefore, cannot be linked to the developmental effects of homeopathic remedies.

It has been reported that as neurogenesis proceeds, Nes (neural stem cell marker) is replaced by specific intermediate filaments[45, 46]. From this study, it was observed that homeopathy remedies could affect mouse ES cells but as the effects were non significant, they probably do not contribute to the developmental toxic effects on developing embryos.

With appropriate toxicity data sets, acceptable exposure levels and actual safety of prescription and nonprescription drugs as well as environmental chemicals could be established for individuals that are more vulnerable to chemical exposure, such as pregnant women and their unborn children. To reduce the spending of live animals, an assortment of *in vitro* assays has been proposed. By using ES cells and adult cells in testing system, differential effects to

embryonic and adult tissues can be assessed. Homeopathy has been regarded as an alternative system of medicine and its entry into mainstream conventional practice has been restricted owing to limited experimental data regarding safety and efficacy.

In conclusion, this study proposes that the expression of different lineage biomarker genes can be useful in examining the side effects of homeopathy which is an important but neglected issue in pregnancy. Implementing analysis of the expression of these biomarkers of differentiation for risk management of homeopathy use for a select section of users who are more susceptible to any drug exposure, such as pregnant women and their unborn children can go a long way in not only ensuring safety but would also lead to the promotion of homeopathic medicines to control various symptoms prevalent during pregnancy.

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ABSTRACT

The efficient utilization of ES cells for applications in cell based therapy, transplantation and drug discovery largely depend upon the culturing conditions of these cells. In this study,the gene andprotein expression levels along with the morphological changes of ES cells,were analyzed when subjected tophysiological factor*i.e.*hypocapnia (lowered carbon dioxide levels). The quantitative expression of pluripotent genes, Oct3/4, Nanog and Sox2 and genes involved in the differentiation of the threelineages, under varying carbon dioxide (CO_2) levels showedenhanced expression at cultures maintained at 1.5% CO_2 as compared to those maintained at 5% CO_2.The cells exposed to hypocapnic conditions when subjected to immunocytochemical analysis stained positive for pluripotent transcription factors;Oct3/4,Nanogand Sox2.Further, flow cytometry and western blot showed that the pluripotent proteins in the 1.5% CO_2 maintained cultures showed higher levels of expression as compared to the ES cells maintained at 5% CO_2. In addition, there was enhanced differentiation particularly towards the mesodermal and endodermal lineages in cultures maintained and differentiated at 1.5% CO_2at all the time periods analyzed*i.e.* day 10 (5+5d),12(5+7d) and 15(5+10d).Theseresults, which are the first of their kind, indicated that the novel physiological factor *i.e.*hypocapnia seems to be preferred for the maintenance of pluripotency and the subsequent differentiation.

4.1 INTRODUCTION

MouseES cells have been widely used as model systems for experimental studies. The use of ES cells in research lies in their ability to maintain pluripotency and if the conditions so permit then, to undergo differentiation. Moreover, the selection of stem cells resource relies upon the ability to isolate and culture the cells. Therefore,standardizing the basic culturing conditions for improving and maximizing the experimental informationwhile maintaining the viability, pluripotency and genetic stability of the daughter cell population is of prime importance.

Theideal stem cell culture system should have essential as well as balanced physiological and chemical properties such as pH, osmolality, viscosity, temperature, extracellular matrix proteins, O_2 and CO_2 tension in such a way that it should mimic the *in vivo* conditions that support growth and proliferation of parent and daughter cell population. The chemical and physical environment so provided for maintenance is basedupon the belief that these cells are located in regions similar to the other somatic sells of the body, therefore, would thrive just as well as the conditions routinely used for culturing cell lines, the exception usually being the addition of LIF to maintain pluripotency conditions. However, the niches, in which certain stem cells are located, for example, the bone marrow,usually have different tensions of two essential gases, namely oxygen (O_2)and CO_2.It is logical to assume that optimal *in vitro* culture conditions for maintaining precursor cells in the desired state of differentiation probably reflect the physiological O_2 levels that these cells encounter in either the embryo or the adult. A large body of work has been done on the effect on ES cells of varying the O_2 levels and reports stressed that low O_2 tensions led to ES cells with characteristics different from that of those cultured under 21% O_2[1-4].

CO_2 is important in regulating the metabolism of the body and chemoreceptors of CO_2quickly sense the concentration of CO_2 and help in maintaining the pH and acid base reactions. Changes in CO_2 levels in the body tissues result in either hypercapnia or hypocapnia, which are associated with pulmonary, metabolic and neuronal responses. Taking into account a study published in 1987, Carney and Bavisterhighlighted the role of CO_2 on hamster embryo development. They hypothesized the valuable effects of CO_2 on blastocyst development. Their

study demonstrated that hamster embryos,cultured in 10% CO_2 hada remarkable effect on *in vitro* development[5]. A study reported by Liang *et al* showed that hypercapnia regulated cultured brainstem neurons synaptic interactions[6].Another study also demonstrated that hypercapnia suppressed the NF-*K*b signaling in mouse embryonic fibroblast and in other mammalian cells[7]. Hypocapniawas used as a curativestrategy for brain injury linked with increased intracranial pressure[8]. In the light of this,it was interesting to see whether alterations in the CO_2 levels had any role to play in terms of maintenance of ES cell characteristics. The rationale was that if in the stem cell niche there was hypoxia, was it possible that hypocapnia could also be playing a role and the alterations in the pluripotent gene expression could be a sum of these two factors?

Physiologically, our blood contains CO_2equivalent to 40mm Hg, which is close to the 5%concentration used in the incubators for culturingof cells [9]. Hence, exposure to low CO_2 could possibly results in genomic and physiological changes. From an academic point of view, it was worthwhile to characterize mouseES cellscultured under varying CO_2 levels, with respect to their undifferentiated state and pluripotent gene expression. It is a well-known fact that the physico-chemical environment influences the cells in culture.The quality of fetal bovine serum (FBS) affecting pluripotency of ES cell is also well documented, with a lot of importance given to serum which is ES qualified[10]. In addition, short term effects(24-48hrs) of exposing human embryonic stem cells (hESC) to low temperature, has shown hESC possess relatively high tolerance which could have useful implications for the salvage of hESC culture during infrequent occurrences of incubator break-down and power failure [11,12]. Therefore, if mouseES cells can also tolerate low CO_2 exposure then the option of maintaining them in case of a shortage/breakdown of CO_2 supply would mean that the cultures need not to be discarded and can be used for experimental purposes.

In this study,the effectsof hypocapnia onmouseES cells exposed to varying CO_2levels were analyzed and studied the morphological changes, expression of marker proteins for pluripotency by immunocytochemistry, western blot, flow cytometeryand the expression of pluripotency genes, Oct3/4, Sox2 and Nanog.In addition, the spontaneous differentiation into three lineages was also analyzed at various time periods under varying CO_2 levels.

4.2 MATERIALS AND METHODS

4.2.1 Culture conditions

The mouse ES cell line D3 wasmaintainedroutinely at 37^{o}C and 5%CO_2 and the cells were passaged three times a week. Undifferentiated mouse ES cell line D3 was cultured in high glucose (4.5g glucose/l) Dulbecco's modified Eagles Medium (DMEM; GibcoLifeTechnologies).The media was supplemented with 15% heat inactivated FBS (Gibco), 2mM glutamine (Invitrogen), 50U/ml penicillin and 50µg/ml streptomycin (Sigma), 1% non-essential amino acids (Invitrogen), 0.1ymM β-mercaptoethanol (Sigma) and 1000U/ml LIF (leukemia inhibitory factor, ESGRO., Chemicon International Inc., Temecula, CA). To create the hypocapnia condition of 1.5%CO_2 level, theCO_2 level in the incubator (New Brunswick Galaxy 170 R)was reprogrammed. The cultureswere maintainedfor at leastten passages at 1.5%CO_2. ES cells maintained at 5%CO_2 conditions served as control. The media was changed daily for the hypocapnia as well as the control cultures.

4.2.2 Assessment of differentiation

The embryoid body (EB) assay was selected for the differentiation experiments owing to its comparatively rapid response and because EB formation is a common early step in both mouse and human ES cells differentiation protocols[13].Differentiation was carried out in hanging drops [14] according to a modified method of Heuer*et al* [15]. In brief, a drop of 20µl from ES cells suspension (5 x 10^4 cells/ml) was placed onto the inner side of the lid of a petridish filled with phosphate buffered saline (PBS) (Himedia) and then incubated at 37^{o}C with either 5% CO_2or 1.5 % CO_2levels. After culture for 2 days, the formed aggregates (EBs) weretransferred into bacteriological petridishes. At day 5, EBs were plated separately into 24 well plates (Thermo Fischer) for immunocytochemistry (ICC) analysis and also in 60 mm (Thermo Fischer) treated dishes for RNA isolation.

4.2.3Cryopreservation and revival of mouseES cells

Cryopreservation was done using dimethylsulphoxide (DMSO)as a cryoprotectant. Trypsinized cells were centrifugedat ~80*g* for 5 minutes and cryopreserved in freezing media

having a composition of 80% FBS and 20%DMSO. Cell suspension containing approximately 1x 10^6cells and freezing media waskept in the ratio of 1:1. For revival, cellswere taken from liquid nitrogen tank and thawed quickly at 37^{o}C, mixed gently with complete media containing 15% heat inactivated FBS,centrifuged at ~80*g* for 5 minutes, supernatant was discarded and the pellet was gently resuspended in complete media and then kept back in the incubator at 37^{o}C and5% or 1.5% CO_2.

4.2.4Morphology observation of mouse ES cells

The morphological changes in ES cell colonies exposed to different levels of CO_2wereobserved after 48 hours of culture by phase contrast microscopy at each passage (Nikon eclipse T*i*).

4.2.5Population doubling time and cell viability

To assess changes in proliferation at low CO_2level (1.5%) exposed cell cultures, the population doubling time was recorded by using equation PDT=CT/log N/N_O x3.31 [16]. In this report, the growth rate of low CO_2exposed cells was calculated and compared to the controls (5% CO_2). The cells were seeded at 2x10^5 per well in 6 well plates, cultures were incubated to achieve confluency,following which trypsinization and cell count using hemocytometer was performed. By inputtingthe values in equation PDT=CT/log N/N_O x 3.31,PDT was calculated where CT stands for culture time, N_O stands for cell number at time of seeding and N stands for cell number calculated after trypsinization. The population doubling time and cell viability experiments were performed three times in triplicates.

4.2.6Alkaline phosphatase (AP) staining

AP is a stem cell marker and increased expression of this enzyme is associated with pluripotency. Approximately, 2x10^5cellswere seeded onto a cover slip and kept in 6 well plates at the appropriate CO_2 levels for 24 hours. The media was then removedand the ES cells were washed withPBS twice. The cells were fixed with 4% paraformaldehyde (Sigma) for 10 minutesand then washed with PBS. Staining was donewith a mixture of two parts of fast red violet (1mg/ml), one part of napthol(1mg/ml) (Sigma) and one part of sterile water for 10 minutes followed by PBS washings.The stained cells were viewed under phase contrast

microscope using a 10X objective (Nikon eclipse T*i*). The AP staining wasperformed three times in triplicates.

4.2.7Immunocytochemistry (ICC)

ICC of ES cell colonies was done at passage 2 and passage 10 to evaluate the long term effect of hypocapnic condition on the expression of pluripotent markers. Approximately, $2x10^5$cellswere seeded onto a cover slip kept in 6 well plates at the appropriate CO_2 levels for 24 hours.Thecells were fixedwith 4% paraformaldehyde (Sigma) for 30 minutes at 37^{o}C and then gently washedwith wash buffer [1% bovine serum albumin (BSA)inPBS].Followed this,0.25% Triton X-100 (Sigma) was added for 10 minutes and then washed with wash buffer. The cells were then exposed to blocking buffer (5% BSA in PBS) for a further 40 minutes at 37^{o}Cin order to block non-specific binding of the antibodies. The cells were again washedwith wash buffer and incubated witha specific primary antibody diluted in ratio of 1:200 againsteither Oct3/4 (Sigma), Nanog (Santacruz) or Sox2 (Santacruz) overnight at 4^{o}C temperature. Following this, thecellswere washed with wash bufferand incubated for a further 2 hours at room temperature with species specific secondary antibodies: Fluorescein-5-isothiocyanate(FITC)-conjugated rabbit anti-mouse (Sigma) diluted in the ratio 1:500 and rabbit anti goat IgG-FITC (Santacruz) diluted in ratio 1:200 for Nanog and Sox2 and again washed with wash buffer.Following this,the cells were treated with Hoechst dye (1µg/5 ml) for 30 minutes and washed with wash buffer. For the ICC negative controls, 1% BSA was used for overnight incubation instead of primary antibody following the same protocol. The cells were viewed under the Nikon eclipse T*i* microscope at 10X magnification using the appropriate filters excitation at 530nm for FITC staining. For confocal microscopy, secondary antibody used was TRITC(TetramethylrhodamineIsothiocyanate) (Santa Cruz) labeled,diluted in the ratio 1:500 and the cells were imaged using the Olympus confocal laser scanning microscope(Fluoview FV10i) at 60 X magnification. The ICC experiment was performed three times in triplicates.

For ICC of EBs, the EBswere fixed in 4% paraformeldehyde (Sigma)for 30 minutes at room temperature in the 24 well plates where they had been grown. After a PBS wash, cells were permeabilized using 0.25% Triton X-100 (Sigma) in PBS for 30 minutes and then washed with wash buffer (1% BSA in PBS) and kept in blocking buffer consisting of 5% BSA in PBS. After

1hour of incubation in blocking buffer, cells were incubated with primary antibody againstα-actinin (Sigma) diluted in a ratio of 1:200 with wash buffer and against α/β MHC (Abcam) diluted at 1:250, kept for overnight at 4^{o}C. After incubation with primary antibodies, the cells were washed 3 times with wash buffer for 5minutes. After washing, cells were incubated with secondary antibody: FITC-conjugated rabbit anti-mouse (Sigma) diluted in the ratio 1:500 and again washed with wash buffer. For negative control, 1% BSA was used for overnight incubation instead of primary antibody following the same protocol. The cells were viewed under the Nikon eclipse T*i* microscope with a 10X objective using the appropriate filters. The ICC experiment was performedthree times in triplicates.

4.2.8RNA isolation and purification, reverse transcription and real-time PCR

RNA was isolated from EBs at different days of differentiation (upto 10 days) for gene expressionanalysis. The EBs were maintained for the three time periods under study *i.e.* day 10 (5+5d), 12 (5+7d) and 15 (5+10d). For qRT-PCR, the total RNA was extractedwith RNeasy Mini Kit (Qiagen) and included DNA digestion. RNA concentration and quality was measured with a Nanodrop 2000 spectrophotometer (Thermo scientific). RNA (1µg per sample) was reverse transcribed into cDNA using an oligodT (15) primer by M-MULV Reverse Transcriptase (Genetix). PCR was performed using 0.5 µg of cDNA for each sample using gene specific primers to determine the expression GAPDH and for target gene.PCR amplification was performed using the following thermal cycles: initial denaturation for 3 minutes at 95°C followed by 35 cycles of: denaturation at 95°C for 15seconds, annealing at 56.5-60°C for 15seconds and extension at 72°C for 30seconds and cooled to 4^{o}C. Bands were then viewed on a 1.2% agarose gel with ethidium bromide using a Fluor-S Multi-Imager densitometer (Bio-Rad laboratories).

qRT-PCR was performed on CFX-96 real time PCR (Bio-Rad laboratories) using SYBR Green real time PCR dye (Bio-Rad laboratories). The amplification conditions comprised an initial denaturation at 95°C for 5 minutesfollowedby 39 cycles eachof denaturation at 94^{o}C for 15seconds,annealing at 56.5-60°Cfor 40 seconds and elongation at 72^{o}C for 1 minute. The gene expression analysis was performed three times in triplicates. The list of the primers used in this study is shown in Table 4.1.

Table 4.1: Details of the primers used in this study

Genes	Primer sequence (Forward primer-FP and Reverse primer-RP)	Annealing temperatures (^{o}C)
GAPDH	FP 5'-GCACAGTCAAGGCCGAGAAT-3'	58.5
	RP 5'-GCCTTCTCCATGGTGGTGAA-3'	
Oct3/4	FP 5'-GGAGAGGTGAAACCGTCCCTAGG-3'	60.0
	RP 5'-AGAGGAGGTTCCCTCTGAGTTGC-3'	
Sox2	FP 5'-GTGGAAACTTTTGTCCGAGAC-3'	57.5
	RP 5'-TGGAGTGGGAGGAAGAGGTAAC-3'	
Nanog	FP 5'-CGTTCCCAGAATTCGATGCTT-3'	57.0
	RP 5'-TTTTCAGAAATCCCTTCCCTC-3'	
Differentiation markers		
Afp	FP 5'-GCTGCAAAGCTGACAACAAG-3'	58.7
	RP 5'-GGTTGTTGCCTGGAGGTTTC-3'	
Nes	FP 5'-GCTTTCCTGACCCCAAGCTG-3'	60.2
	RP 5'-GGCAAGGGGGAAGAGAAGGA-3'	
Nkx2.5	FP 5'-CAAGTGCTCTCCTGCTTTCC-3'	56.5
	RP 5'-GGCTTTGTCCAGCTCCACT-3'	
α/β MHC	FP 5'-ACCTGTCCAAGTTCCGCAAG-3'	58.5
	RP 5'-CTTGTTGACCTGGGACTCGG-3'	

4.2.9Intracellular immunofluorescence staining for flow cytometry analysis

ES cell colonieswere dissociated into single cell suspension and then fixed in 4% paraformeldehyde (Sigma) solution in PBS for next 20 minutes. After washings, the cells were permeabilized with 0.4% (v/v)Triton X-100 (Sigma) solution in PBS for next 10 minutes, followed by blocking non-specific binding by incubating with 5% BSA in PBS for 15 minutes. The cells were incubated with the primary antibodies (Pierce Scientific) against Oct3/4 diluted in 1:50 ratio, Sox2 at dilution of 1:100 and Nanog at dilution of 1:50 for 1 hour at 37^{o}C. Secondary antibody,FITC-conjugated goat anti-rabbit IgG (Pierce Scientific), diluted in 1:200 ratio in 1%BSA in PBS, was then added to the cells after washing, and incubated for 1 hour at room temperature. Cells incubated with appropriate secondary antibody alone were used as negative control. After washing, the acquisition of the samples was performed in a C6 Accuri flow cytometer and analyzed using the Cell Quest software. Dead cells and debris were excluded from the analysis which was based on electronic gates using forward scatter (cell size) and side scatter (cell complexity) criteria. A minimum of 10,000 events was collected for each sample. The experiment was performedthree times in triplicates**.**

4.2.10Western Blotforpluripotent marker

Cells were washed with PBS, lysed in radioimmune precipitation assay (RIPA)buffer consisting of 50mMTris, pH 8.0, 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, along with protease inhibitor cocktail mixture (BioRad), and incubated for 30 minutes on ice. The whole cell lysatewas centrifuged at 12000rpmfor 12 minutes (Eppendorf 5804R).The protein concentration was estimated by Bradford assay and the equal amounts of protein(25µg) was subjected to electrophoresis on 10% polyacrylamide gel containing 0.1% SDS. After electrophoresis, the protein was transferred onto a PVDF membrane using a Bio-Rad western blot apparatus. The membrane was blocked with 5% skimmed milk in Tris-buffered saline containing 0.05% Tween 20 (TPBS) for 1 hour andthenprobed with anti-Oct3/4, Nanogor Sox2 (Pierce Scientific)antibody overnight at 4^{o}C.2µg/ml concentration of anti-Oct3/4 and anti-Nanogprimary antibodywas used and 1.5µg/ml concentration was used for anti-Sox2 primary antibody. The loading control was anti-β-actin antibody (Santa Cruz), dilutedin 1:500ratio. The membrane was washed with TPBS and then incubated with appropriate anti rabbit secondary

antibodies conjugated with horseradish peroxidase. The membrane then was washed, followed with incubation of an anti rabbit peroxidase-conjugated secondary antibody(1:2,000 Santa Cruz) at room temperature for 1 hour and the antigen-antibody complex was visualized using the DAB method. The western blot analysis was performed three times in duplicates.

4.2.11Statistical analysis

The expression of pluripotent genes in control and low CO_2 exposed mouse ES cell culture was normalized to GAPDH and analyzed with unpaired Student's 't' test. The gene expression analysis was obtained by calculating the mean ± SD from ≤ 4 individual experiments done in triplicates. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using graph pad prism software version 6.0.

4.3 RESULTS

4.3.1 Morphology observed under hypocapnic conditions

AllmouseES cells cultures under low CO_2 conditions showed attached colonies.The ES cells, which were maintained at 1.5%CO_2,looked significantly better than cultures at 5%CO_2 conditions in terms of their compact and undifferentiated appearance as shown in Figure 4.1.Revived cells appeared healthy and there was no change in morphology after long-term exposure to hypocapnic conditions with viability rate of 97±1%upon revival (Table 4.2). The control cells (5%CO_2) showed viability rate of 98±1% (Table 4.2).There was no difference in the pH of the media between the hypocapnia exposed and normal CO_2 exposed cultures.

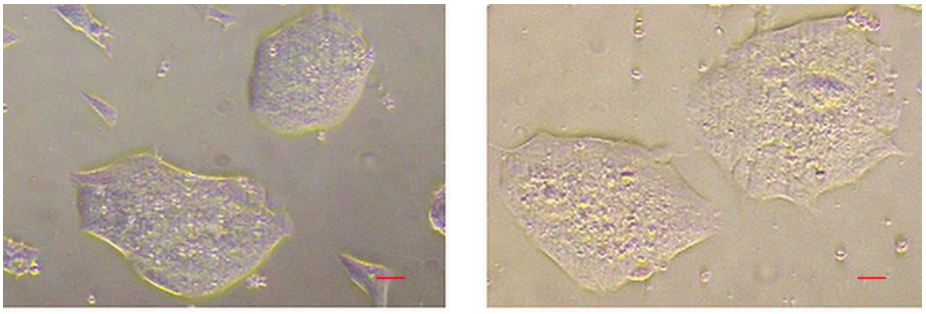

Figure 4.1: ES cells under varying CO_2 conditions. Magnification 20X. The morphological observations were recordedafter 48 hours. Bars: 100 px.

Table 4.2: Population doubling time and cell viability of mouse ES cells under hypocapnia conditions

Parameter	Cells at 5% CO_2	Cells at 1.5% CO_2
Population doubling time	22.22±1.1(hours)	27.90±1.8* (hours)
Cell Viability	98±1%	97±1%

The experiments were performed three times in triplicates for upto five passages. Statistical significance was tested by using student's 't' test (* p < 0.05) by using GraphPad prism software version 6.0.

4.3.2 Increase in cell population doubling time

The population doubling time values for mouse ES cells were significantly higher at 1.5%CO_2 when compared with 5%CO_2 indicating the slower rate of proliferation. Based on population doubling time values, 5%CO_2 cultured cells tended to double on an average of 22.22±1.1hours while 1.5%CO_2 cells cultures exhibited 27.90±1.8 hours doubling time. This change was statistically ($p<0.05$) significant (Table4.2).

4.3.3 AP activity

To see whether the undifferentiated state of the colonies was maintained throughout the serial passages, the colonies were stained with AP.The colonies at both the CO_2 levels were seen to be positively stained and expression of AP was best seen at 1.5%CO_2(Figure 4.2), wherein rounded compact colonies with homogeneous staining were visible as compared to ES cells maintained at 5%CO_2(Figure 4.2).

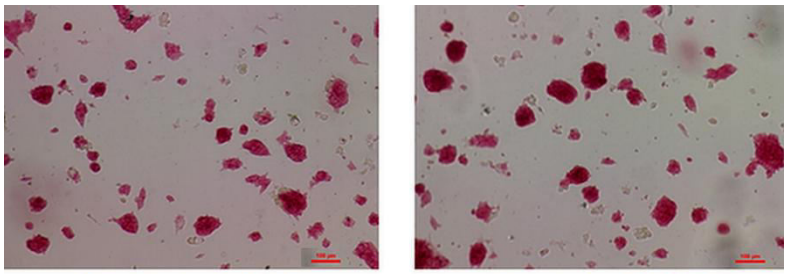

Figure 4.2: Expression of alkaline phosphatase in ES cells under varying CO_2 conditions. Magnification 10X. Bars: 100µm.

4.3.4 ICC analysis of pluripotent factors: Oct3/4, Nanog and Sox2

In addition to staining positive for alkaline phosphatase, the cells were also positive for the pluripotency markers Oct3/4,Nanog and Sox2as shown in the Figure4.3. Immunocytological results showed that the low CO_2 treated cells confirmed their pluripotent nature by expressing Oct3/4, which is member of POU family along with,Nanog and Sox2 which are core regulatory proteins, all of which known to be involved in ES cell fate determination. This result emphasizes that lowered CO_2 levels in no way inhibits the expression of the transcription factors required for the maintenance of pluripotency(Figure 4.3). The pictographs of negative control were given in Figure 4.4.

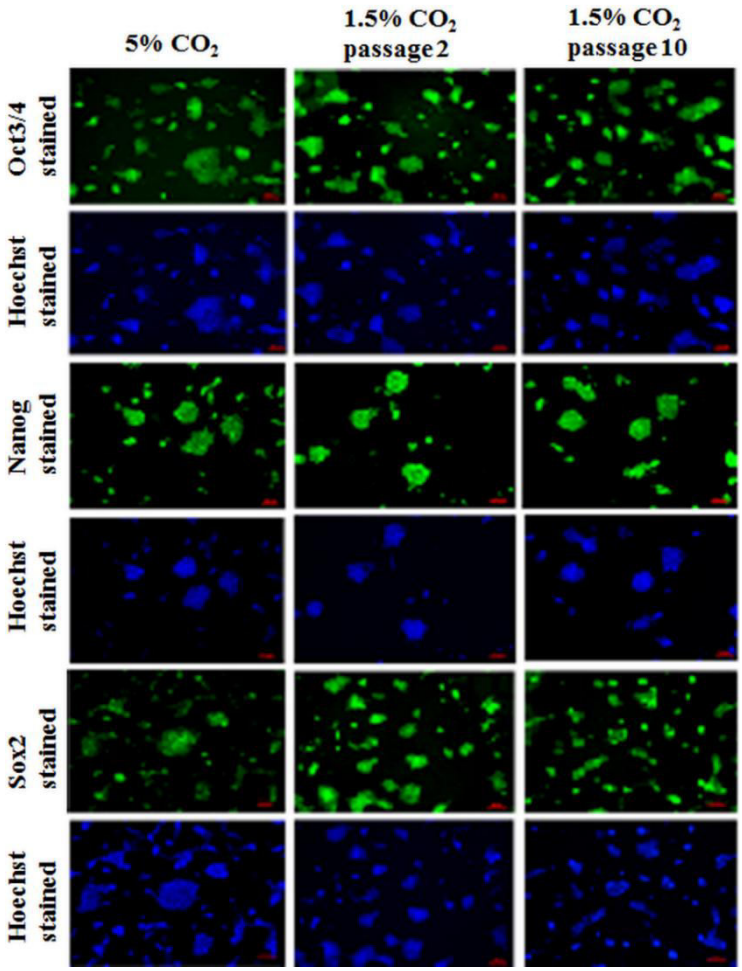

Figure 4.3: ICC of pluripotent markers (Oct3/4, Nanog and Sox2) of ES cells under hypocapnia condition. Magnification 10X. Bar: 100µm.

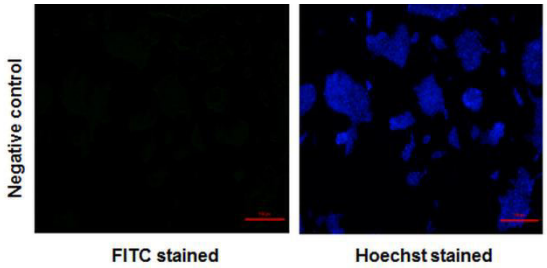

Figure 4.4: ICC of mouse ES cells using 1% BSA as primary antibody and FITC as secondary antibody.Magnification 20X.Bar: 100 px.

ICC was also done using TRITC labeled secondary antibody for analysis of pluripotent markers using the Olympus confocal laser scanning microscope (Fluoview FV10i) at 60X magnification. It was observed that more population of cells expressed Oct3/4, Nanog and Sox2 under hypocapnia (Figure 4.5). This further validates the hypothesis of hypocapnia favoring the pluripotent nature of ES cells.

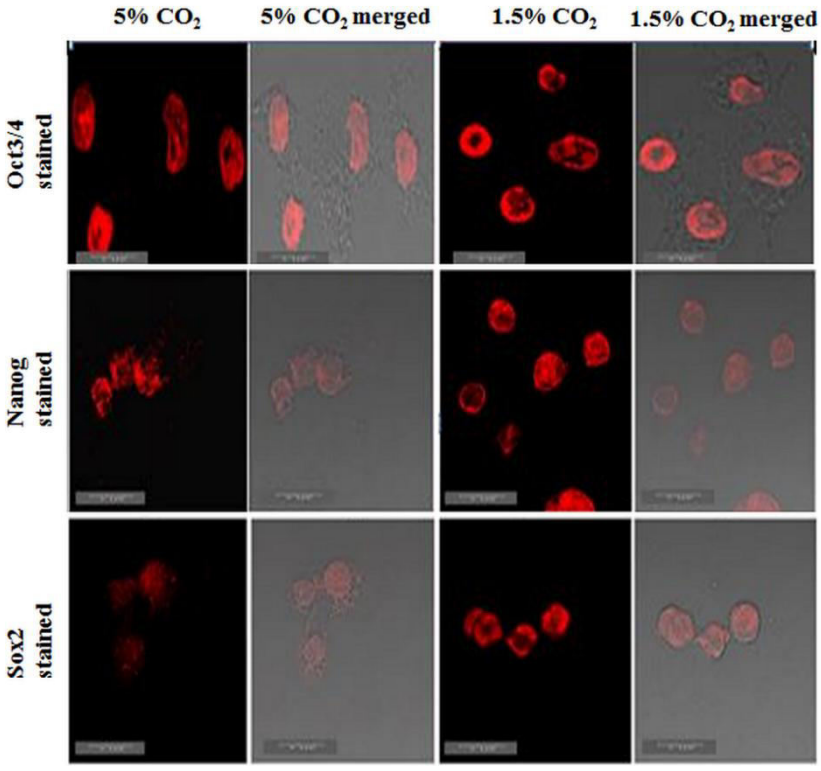

Figure 4.5: Confocal images of pluripotent markers (Oct3/4, Nanog and Sox2) by ICC of mouseES cells under control (5%) and hypocapnic conditions (1.5%).Magnification 60X.Bar: 5µm.

4.3.5 Enrichment of pluripotent marker genes after hypocapnia treatment

Gene expression analysis was carried outwith both RT-PCR and qRT-PCR. RT-PCR showed enhanced expression in genes responsible for pluripotency, namely Oct3/4, Sox2 and Nanog in 1.5%CO_2 exposed cells (Figure 4.6). qRT-PCRanalysis showed increased fold changes in Oct3/4, Sox2 and Nanog under hypocapnic condition. The expression of Oct3/4 and Sox2 increased 4.5fold and 3.6 fold, respectively, and a 7.2fold increase was seenin Nanog expression in 1.5% CO_2 exposed cell cultures when compared with 5% CO_2. These results are in concordance with the appearance of the colonies at the low CO_2 tension (Figure 4.7) which were more compact and less differentiated.

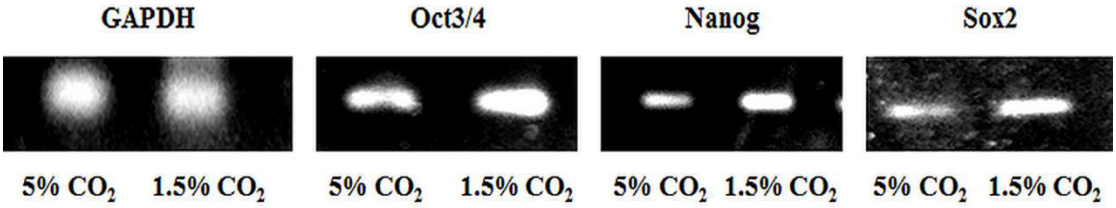

Figure 4.6: Gel images of RT-PCR analysis for pluripotent marker genes (Oct3/4, Nanog and Sox2) in mouse ES cells under varying CO_2 conditions. GAPDH was used as loading control. The experiment was performed three times in triplicates.

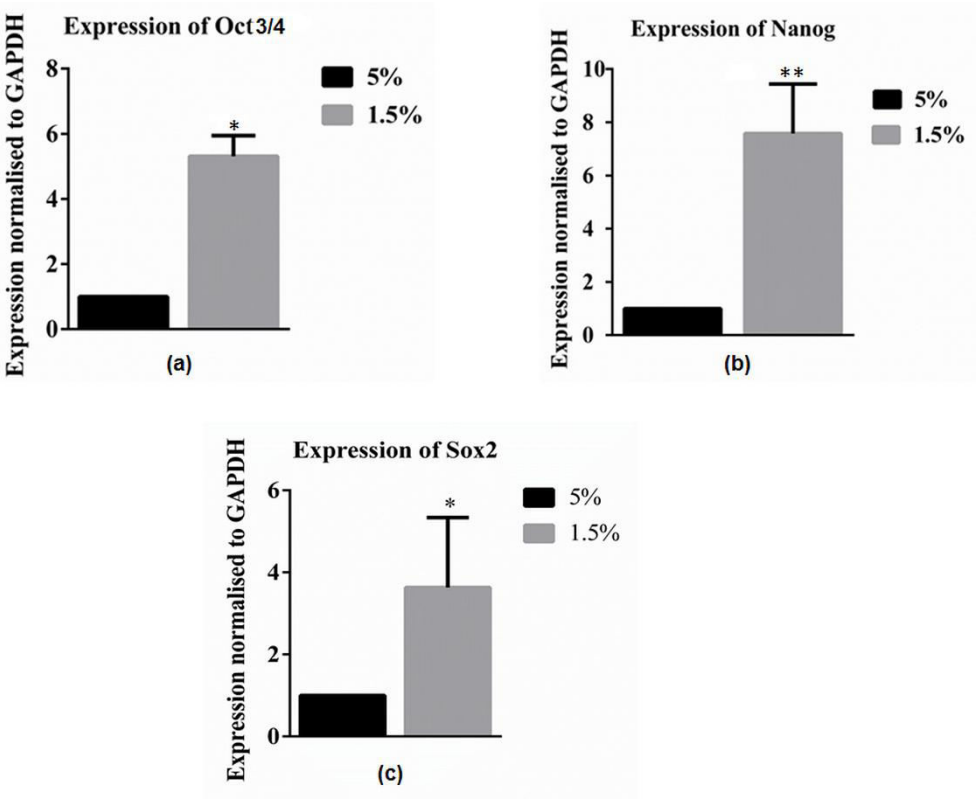

Figure 4.7:Gene Expression analysis by qRT-PCR, Oct3/4(a),Nanog (b) and Sox2 (c) in mouse ES cells under varying CO_2 conditions using SYBR Green dye.Values werenormalized to housekeeping gene *i.e.* GAPDH expression. The y-axis represents fold changes in expression of studied gene. The statistical analysis was done using one-way ANOVA followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show means ± SD. All values are average of three individual experiments done in triplicates. Significant changes between hypocapnic (1.5% CO_2) and control cells (5% CO_2) (*p<0.05, **p<0.01).

4.3.6 Flow cytometric Analysis

Flow cytometry of mouse ES cells was carried out to evaluate the changes in the protein expression exhibited by mouse ES cells under hypocapnia (1.5% CO_2) and 5% CO_2 for Oct3/4, Nanog and Sox2. Results from flow cytometry revealed increased percentage of Oct3/4, Nanog and Sox2 positive populations of mouse ES cells under hypocapnia (1.5% CO_2) in comparison to

cells maintained at 5% CO_2.This further supports the hypothesis that hypocapnia results in increased levels of pluripotency markers (Figure 4.8).

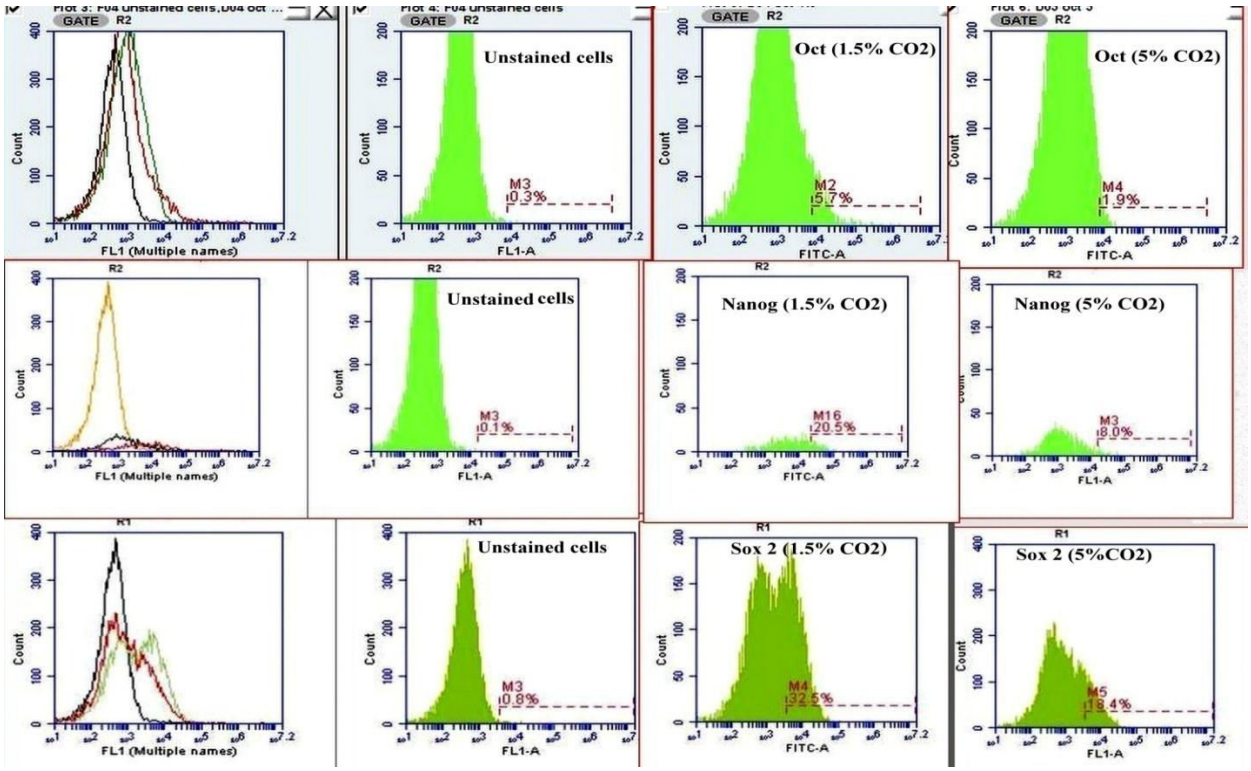

Figure 4.8: Flow cytometry histogram plots representing percentage of mouse ES cells expressing pluripotency markers; Oct3/4, Nanog and Sox2. These plots represent percentage of ES cells under hypocapnia condition (1.5% CO_2) and control (5% CO_2). The two populations were plotted against unstained cells. The experiment was performedthree times in triplicates.

4.3.7 Western blot analysis for pluripotent markers

Effect of hypocapniawas further examined on pluripotency factors using western blot analysis. The results showed that the protein expression of Oct3/4, Nanog and Sox2 present in 1.5% CO_2 maintained cultures of mouse ES cells was increased as compared to 5% CO_2 cultured cells (Figure 4.9).

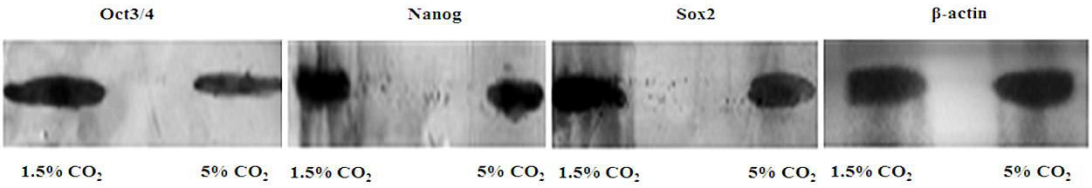

Figure 4.9: Western blot analysis of pluripotent marker proteins (Oct3/4, Nanog and Sox2) in mouse ES cells under varying CO_2 conditions. β-actinwas used as loading control. 25µg/ml of protein was loaded in each well. The experiment was performed three times in triplicates.

These results along with the flow cytometry data are in accordance with the gene expression data, which pointed towards hypocapnia favoring pluripotency.

4.3.8 Hypocapnia exposed EBs efficiently differentiated into mesodermal lineage

The differentiation of the ES cells under control and hypocapnia conditions resulted in similar size and yield of EBs (Figure4.10).

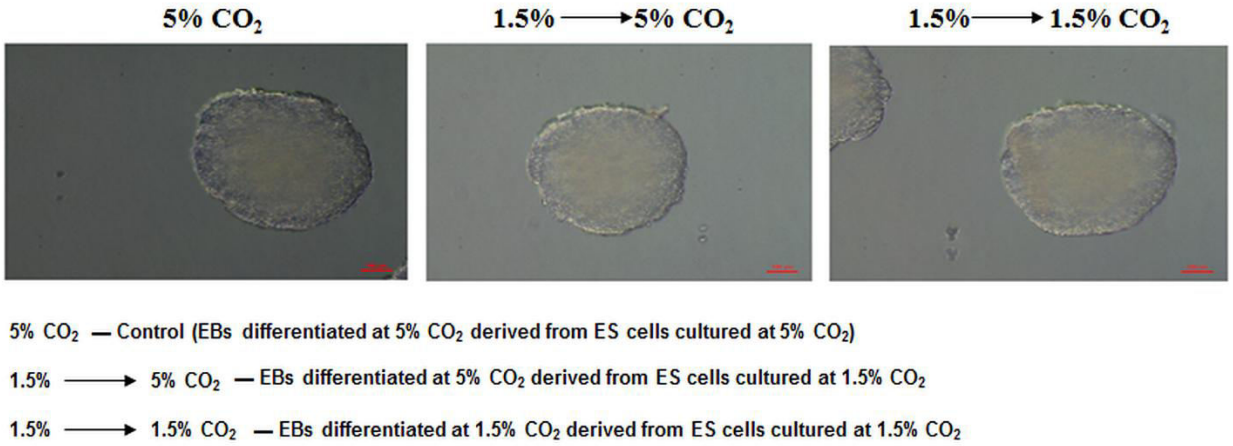

Figure 4.10: Phase contrast pictograph showing EBs differentiated under varying CO_2conditions at day 2.Magnification 10X. Bars: 100µm.

Hypocapnia exposed EBs promoted differentiation into mesodermal lineage as assessed by the expression of mesodermal markers by ICC. EBs were differentiated under 5% CO_2 or

hypocapnic condition (1.5%) for 10 days and their differentiation potential towards mesodermal lineage was confirmed by staining for α/β MHC and α- actinin. EBs under both conditionswerefoundpositively stained for α/β MHC and α- actinin (Figure 4.11).

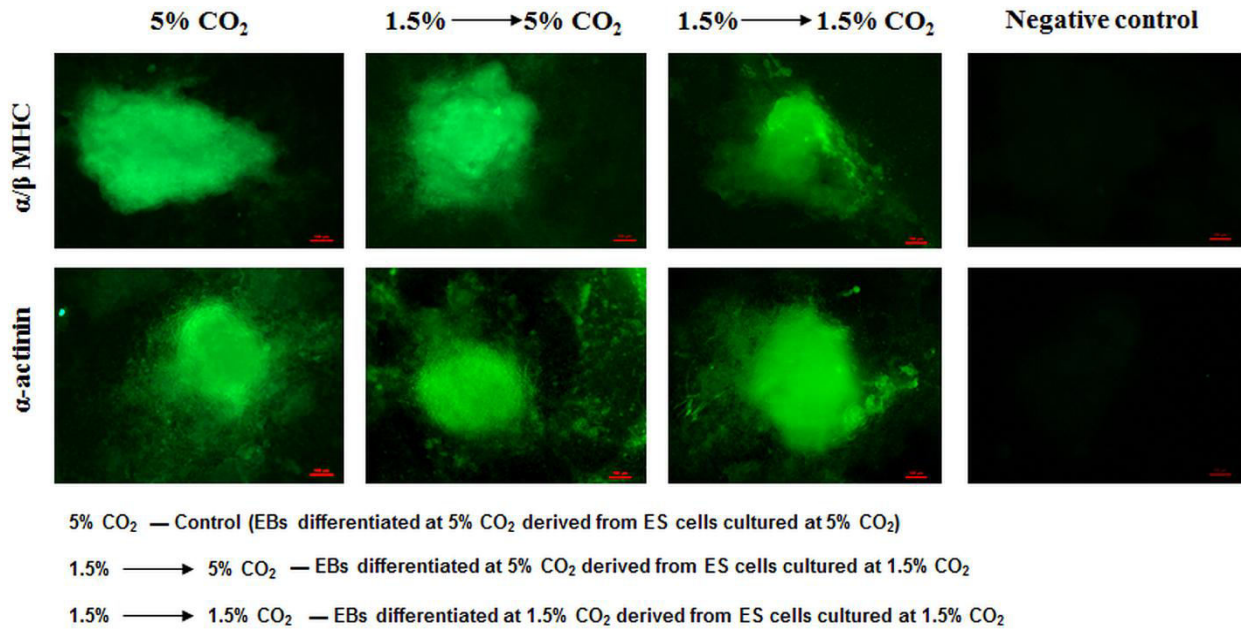

Figure 4.11: Representative images of differentiation of EBs exposed to hypocapnia stained with mesodermal lineage markers. Magnification 10X. Bars: 100µm.

4.3.9Hypocapniainduced differential expression of lineagemarker genes

Toexplore the role of hypocapnia on mouse embryonic differentiation, the differentiation of EBswas analyzed by qRT-PCR at different time intervals after EB plating. It was found that EBs under the influence of extended period of hypocapniashowed enhanced expression of differentiation marker genes.Hypocapnic condition had the ability to differentiate ES cells *via* EBs into three germ layers and to accelerate mesodermal and endodermal differentiation with significant differences between early (passage 2) and later passage populations (passage 10). The fold changesin the expression of genes (in comparison to control EBs differentiated at 5% CO_2, derived from ES cells maintained at 5% CO_2) for endodermal, mesodermal and ectodermallineageswere normalized against GAPDH expression.A significant increase in the expression of the mesodermal lineage marker was observedin hypocapnia at both passage 2 and 10.Following the initial studies on differentiation at day 10 for early and late passages, further

EBs cultures were maintained and RNA was isolated at different days *i.e.* day 12 (5+7d) and day 15 (5+10d) to see if this trend of enhanced expression in differentiation was sustained in the 1.5% maintained and differentiated cells. Since increased gene expression was seen at cells maintained under hypocapnia conditions at later passages, the subsequent studies for longer periods of differentiation were done at later passages only. The expression of mesodermal marker genes, Nkx2.5 and α/β MHC were analyzed. The Nkx2.5 expression at day 10, increased 1.6 fold at passage 2 and 31.2 increase at passage 10 (Figure 4.12), when EBs were differentiated at 5% CO_2 derived from ES cells under hypocapnia (1.5% CO_2) conditions. The expression was 26.6 fold at day 12 (5+7d) and 28.4 fold on day 15 (5+10d) for the same (Figure 4.13). The expression of Nkx2.5 was increased further when these EBs were differentiated under hypocapnia condition (1.5% CO_2) and a 1.7 fold increase at passage 2 and 48.4 fold increase at passage 10 was seen at day 10 (Figure 4.12). Notably, the expression was 58.1 fold higher at day 12 and 67.5 at day 15 as compared to the control EBs (Figure 4.13). The expression α/β MHC was increased in a similar pattern. There was a 2.4 fold increase at passage 2 and 11.02 at passage 10 (Figure 4.12) at day 10 when EBs were differentiated at 5% CO_2 and derived from ES cells under hypocapnia condition (1.5% CO_2). The results of the differentiation done at the later time periods showed 10.3 fold expression at day 12, which increased to 30.4 fold at day 15 for the same condition (Figure 4.13). The α/β MHC expression was seen to be supported further in the EBs derived from ES cells cultured at 1.5% CO_2 were differentiated at 1.5% CO_2 ; 19.6 fold increase at passage 2 and 57.4 fold increase at passage 10 at day 10 (Figure 4.12). The enhanced differentiation of α/β MHC expression was seen further in EBs under hypocapnia. A 58.6 fold change in expression at day 12 and 78.2 fold at day 15 was observed (Figure 4.13). These results clearly indicate that lowered CO_2 is beneficial for the cells in terms of differentiation of mesodermal lineage (Figure 4.13).

Similarly, significant increase in the expression of endodermal lineage marker was observed. Afpincreased 1.9 fold at passage 2 and 5.7 fold at passage 10,when EBs were derived from 1.5% CO_2maintained ES cells differentiated at 5% CO_2at day 10 (5+5d).When the EBs were differentiated at 1.5% CO_2*i.e.*under low CO_2 tensions ,the expression of Afp was increased 24.6 fold at passage 2 and 29.6 fold increase at passage 10(Figure 4.12).The fold changes at day 12 and 15 in ES cells maintained at 1.5% CO_2 and differentiated at 5% CO_2were 5.5 and 5.4,

respectively (Figure 4.13). In comparison to the 5% control EBs,the Afp expression was enhanced significantly ($p<0.0001$) at both day 12 (38.1 fold) and day 15 (58. 9 fold) in the 1.5% maintained and differentiated cells as seen in Figure4.13.

The expression of ectodermal lineage Nes gene was also altered, but not to the extent seen with the endodermal and mesodermal lineages. It was analyzed that the expression of Nes increased 1.3 fold at passage 2 and 3.1 fold at passage 10 at day 10, when EBs were differentiated at 5% CO_2 (derived from ES cells cultured at 1.5% CO_2) (Figure 4.12). The expression was increased to 2.8 fold at day 12 and 2.1 at day 15 (Figure 4.13). When these EBs were differentiated at 1.5% CO_2, the expression increased 1.6 fold at passage 2 and 3.7 fold at passage 10, when derived from ES cells at 1.5 % CO_2 at day 10 (Figure 4.12). A marginal decrease in expression of Neswas observed at day 15 under hypocapnia. An increase of 7 fold at day 12 and 6.5 fold at day 15 was observed (Figure 4.13). These results indicate that the differentiation ability was more pronounced at 1.5% CO_2 and maintaining cells for greater passages under hypocapnicconditions led to enhanced meso-endodermal differentiation of EBs at 1.5% CO_2.

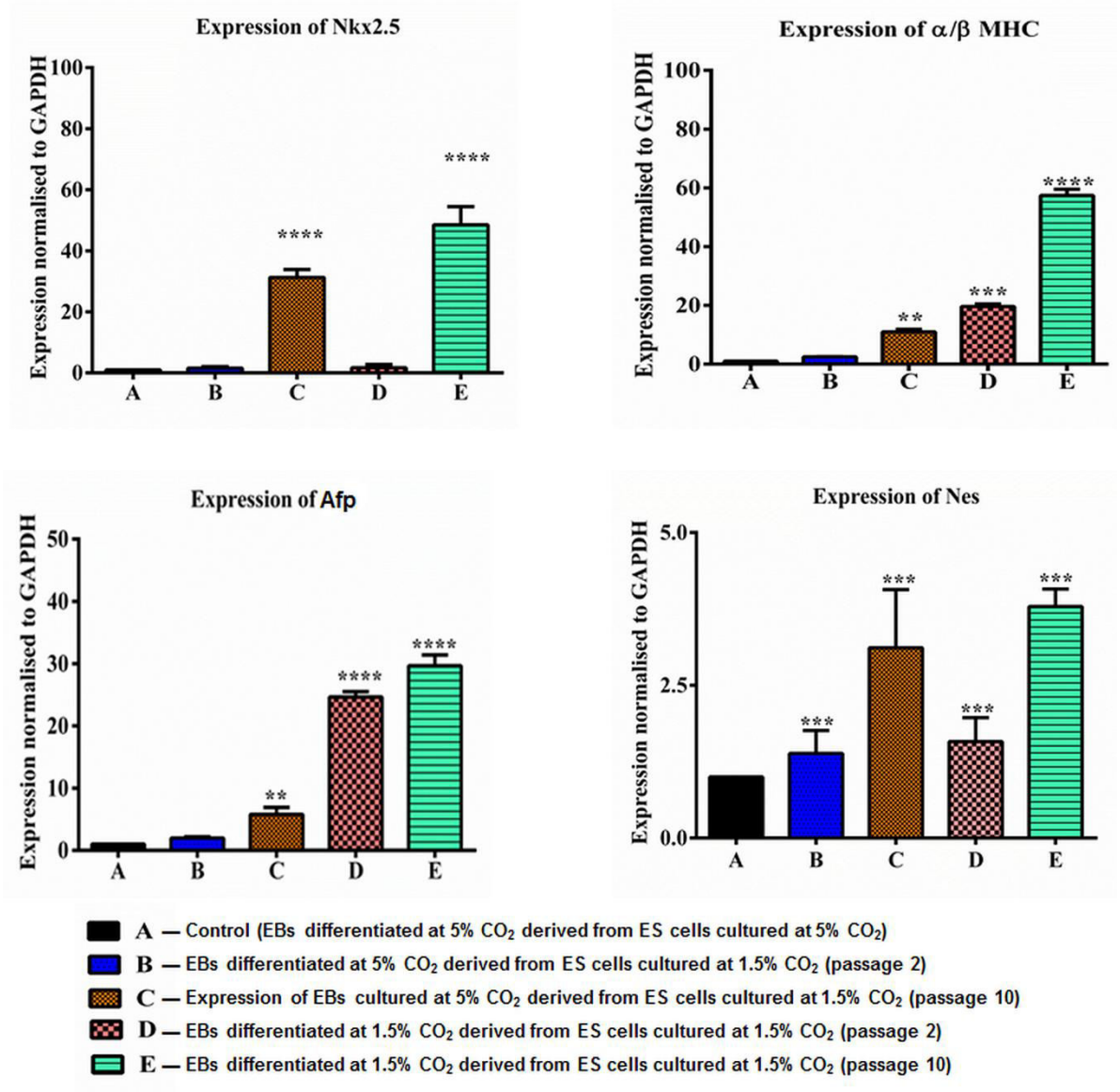

Figure 4.12: qRT-PCR analysis for differentiation markers under hypocapnia at passages 2 and 10. Expression of Nkx2.5, α/β MHC, Afp andNes were analyzed using SYBR Green dye and values were normalized to housekeeping gene *i.e.* GAPDH expression. The y-axis represents fold changes in expression of studied gene. The statistical analysis was done using one-way ANOVA followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show means ± SD. All values are average of three individual experiments done in triplicates. Significant changes between cells under varying $CO_2$and control (5% CO_2)conditions (p<0.01, ***p<0.001, ****p<0.0001).**

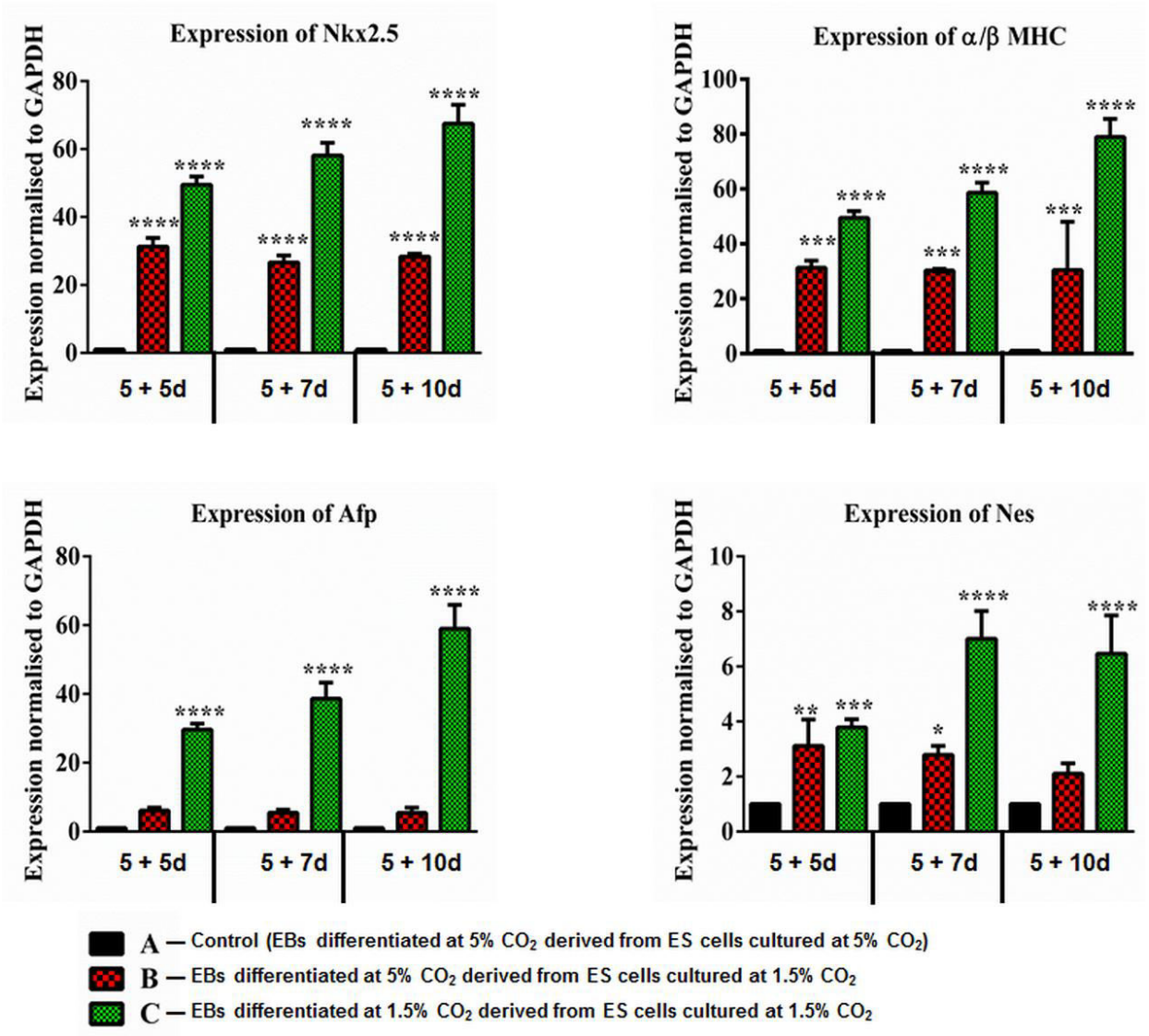

Figure 4.13:qRT-PCR analysis for differentiation markers under hypocapnia for additional periods of differentiation.Expression of Nkx2.5, α/β MHC, Afp and Nes were analyzed by using SYBR Green dye and values were normalized to housekeeping gene *i.e.* GAPDH expression. The y-axis represents fold changes in expression of studied gene. The statistical analysis was done using one-way ANOVA followed by a Bonferroni test using GraphPad prism software version 6.0.Bar graphs show means ± SD. All values are average of three individual experiments done in triplicates. Significant changes between cells under varying $CO_2$and control(5% CO_2) conditions (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

4.4 DISCUSSION

To best of our information, limited data is available on the subject of hypocapniaand mouse ES cell cultures. In this report, cells were exposedto low CO_2 conditions and studiedtheir morphology, proliferation rate, viability and expression profiling of pluripotent genesand proteins,Oct3/4, Nanog and Sox2, as well as the differentiation capability by studying the various lineage markers. Various reports have been publishedin which hypoxic conditions are created to mimic the *in vivo* conditions since it has been reported thatO_2 tension differs with respect to tissue type and the concentration of $O_2$is alsomuch lower in tissuesas compared to the atmosphere [17].The main source of CO_2 in aerobic metazoans is the electron transport chain of mitochondria.Here the molecular $O_2$is reduced to produce ATP and CO_2 is evolved as a by-product.Pluripotent blastomeres of mammalian pre-implantation embryos and ES cells are characterized by limited oxidative capacity and greater reliance on anaerobic respiration. It has been documented that there is a metabolic shift in the ES cells,leading to enhanced glycolysis and reduced mitochondrial respiration.Therefore, the main source of energy production in ES cells is glycolysis and not the TCA cycle owing to the reduced levels of O_2 in the niches where the stem cells are located [18].The Warburg effect postulates that, oxidative phosphorylation switches to glycolysis even in the presence of O_2,which promotes the proliferation of cancer cells. Many studies have demonstrated glycolysis as the main metabolic pathway in cancer cells.The same condition also holds true for EScells [19].Infact,Kondohin 2007 put forward a hypothesis that the naturally high glycolytic flux observed in mouse ES cells could be responsible for their unlimited proliferative potential [20]. Therefore, it was proposed that as oxidative phosporylation is reduced, the by product, that is CO_2, will also be lowered in these cells and hence the cells are naturally adapted to grow at lowered CO_2 levels.

It was in the late 1970s that Morriss and New clearly showed the connection between lowered O_2 levels and normal embryonic development [21]. Following this discovery, various workers tried to figure out the mechanism behind this phenomena and it was only very recently that a link between lowered O_2 and various proteins was seen. Regulation of gene expression by $O_2$is now awell-knownfactand studieshave reported that hypoxia modulates gene expression of a number of signaling pathways involved in embryonic development and stem cell fate.

HIF(hypoxia inducible factor) has shown to be essential for embryonic development and regulation of genes, which are responsible for glycolytic enzymes, can account for a greater dependence of ES cells on glycolysis for their energy requirements. Transcription factors and glucose transporters are also targets for hypoxia induced gene alterations. The HIFα protein subunit isknown to be up-regulated in response to low O_2 levels and the presence of a several putative hypoxia response element in the Oct3/4 promoter,clearly underpins the role of lowered O_2 levels in maintaining pluripotency[20,22-24]. Moreover, Forristal*et al* showed that HIF-2α regulated Oct3/4, Sox-2 and Nanog [25]. Induced pluripotent stem cell (iPSC) when exposed to hypoxic conditions increased the efficiency to reprogram the adult cells into induced pluripotent cells [26].A recently published study reported that hypoxic cultures amplify the effect of the signaling molecules that induce endodermal and early hepatic differentiation of ES cells. Accordingly, the hepatic progenitor cells maintained under hypoxic conditionswere efficiently differentiated into functional hepatocytes. They also found that the differentiating cells did not use O_2 even under normoxia, signifying that O_2 per se might play a non-constructive role on the normal endodermal and early hepatic differentiation of ES cells [27].

There exists a close association between O_2 consumption and CO_2 production and the capacity of a cell to sense changes in CO_2 is likely to be closely linked to its ability to sense changes in O_2. Therefore, it is possible that akin to a hypoxia inducible factor, there could be a protein that senses alterations in the CO_2 levels. The up-regulation of such a protein could in turn effect the expression of various genes. Indeed a report by Ana *et al* has stated that chromaffin cells of neonate rats have CO_2sensing properties, which may be of physiologic relevance, particularly for the adaptation of neonates to ex-uterine conditions [28]. So, if the niches areproposed to reside in hypoxic condition, what will be result of hypocapnic conditions on the pluripotency of these stem cells? To test the hypothesis that the ES cells are naturally adapted to grow at reduced CO_2 levels, a comparative study of ES cells maintained at 5% and 1.5% CO_2 levels was done and analyzed their growth as well as ability to maintain pluripotency and to undergo differentiation.

Results showedthat hypocapnia conditions increased the expression of pluripotent marker genes inmouseES cells. Oct3/4,Nanog and Sox2 in mouse ES cells have been shown to be key

transcriptional regulators in maintaining pluripotency and at the same time repressing differentiation. qRT-PCR of hypocapnia exposed mouseES cells showed enhanced expression of three important pluripotent genes, Oct3/4, Nanog and Sox2.After examining the shifted pattern of pluripotency genes, it was concluded that the there is possibility that similar to the hypoxic conditions there is up-regulation of pluripotency in conditions of reduced CO_2.Another remarkable observation seen in the study is the enhanced expression of Nanog as compared to Oct3/4 and Sox2.Indeed the results obtained are in agreement with Singh *et al* who correlated high nanog expressing ES cells as the "true" stem cell population capable of self-renewal,and maintenance of pluripotency [29].To examine whether this change in gene expression also correlated to enhanced expression of proteins, immunocytological, flow cytometeryand Western blot analysis wasdone for Oct3/4,Nanog and Sox2.The ES cell colonies indeed expressed increased levels of all the three transcription factors whose expression governs the pluripotency of mouseEScells, which further supportsthe present study.

Oxidative stress is a major cause of DNA damage and leads to cellular senescence.In the absence of oxidative phosphorylation for energy generation, lesser amount of reactive oxygen species will be produced and the cellular senescence can be diminished[19, 30] and cell expansion prolonged. Logically, when cells are expanded without DNA damage, the cells maintain their viability and stem cell characteristics. Earlier it has been reported that low O_2 tension leads to stem cells that cycle slowly. Previous research has also demonstrated that maintaining pluripotent state in long-term culture may lead to alterations of stability of stem cells, including enhanced proliferation that will eventually lead to epigenetic alterations in the cells, which could influence their differentiation ability.Therefore having cells, which cycle slowly, would be beneficial in maintaining pluripotency and mimicking *in vivo* conditions where stem cells are known to cycle at a slower rate[31]. It was seen that by creating hypocapnia culture conditions there was an increase in the doubling time of mouse ES cells, which showed delayed proliferation.

As stated by a Simon and Keith [32] that although most cells are maintained in culture conditions at 21% O_2, this is unlikely to be optimal for maintaining their normal proliferative or developmental state. Chaudhry *et al*[13] in their studies have stated that when optimizing cell

cultures, it is important to determine the cellular environmental tolerance ranges in order to identify critical process variables that can substantially influence the results. In this study, it was also proposed that lowered CO_2 levels should also be taken into account during culturing as, in this study, it was have demonstrated that hypocapnic conditions enhance the expression of pluripotency markers and accelerates mesodermal and endodermal lineages as seen by the results observed in the ES cultures exposed to 1.5 % CO_2.

The differentiation capability of ES cells is the key to their use in regenerative medicine. These results have shown that maintaining and differentiating cells at reduced CO_2 levels leads to enhanced differentiation as compared to the controls (5% CO_2) and this effect is sustained at all the three time points under study. Again, this supports the hypothesis that during embryonic development, the niche has lowered requirement for CO_2 and the cells are adapted for growth at these conditions. Therefore, by mimicking these conditions in this system the cells show effective differentiation as compared to cells maintained at 5% CO_2. This study may provide the baseline to increase the pluripotency of mouse ES cells under hypocapnia conditions. Therefore it was concluded that while ES cells can be maintained under atmospheric carbon dioxide tensions, lowering the CO_2 tension to 1.5% appeared to be beneficial for the propagation of this pluripotent population of cells.

Although the effects of reduced CO_2 tension have been analyzed using a single mouse ES cell line, it is reasonable to assume that it has a broader significance as the genes under study are conserved in both mouse as well as human ES cells. The mechanism by which hypocapnia leads to maintenance of pluripotency and differentiation is yet not known. However, given the key role of culture conditions in stem cell biology, future studies will need to identify the possible signaling pathways and proteins involved in CO_2 sensing along with the adaptive response to lowered CO_2 levels and impact on embryonic stem cell behavior. Such an approach could open up new vistas in the field of stem cell biology.

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CONCLUSION AND OUTLOOK

The study was conducted to investigate the factors influencing differentiation of mouse ES cells during development. The deranged expression pattern of lineage specification genes confirmed that an alteration in growth environment has direct bearing on the developing embryo. In addition, tracing the alterations in gene expression during the process of *in vitro* differentiation revealed additional target genes for identifying the developmental toxicity associated with certain chemicals, to which the fetus is exposed to during embryogenesis. Further, the results also provided insights into the association between signaling pathways of three germ layers which could be involved in causing irreversible damage to developing embryo exposed to an altered in utero environment. The results of these studies on differentiation would lead to a greater understanding of developmental toxicology and more specifically embryogenesis(Figure 5.1). The implications of such a study would require a rethinking of the conditions required for effective differentiation, which demands elucidation of key factors influencing the micro milieu *in vivo* to obtain functionally active differentiated cells to meet the demands of regenerative medicine and drug discovery.

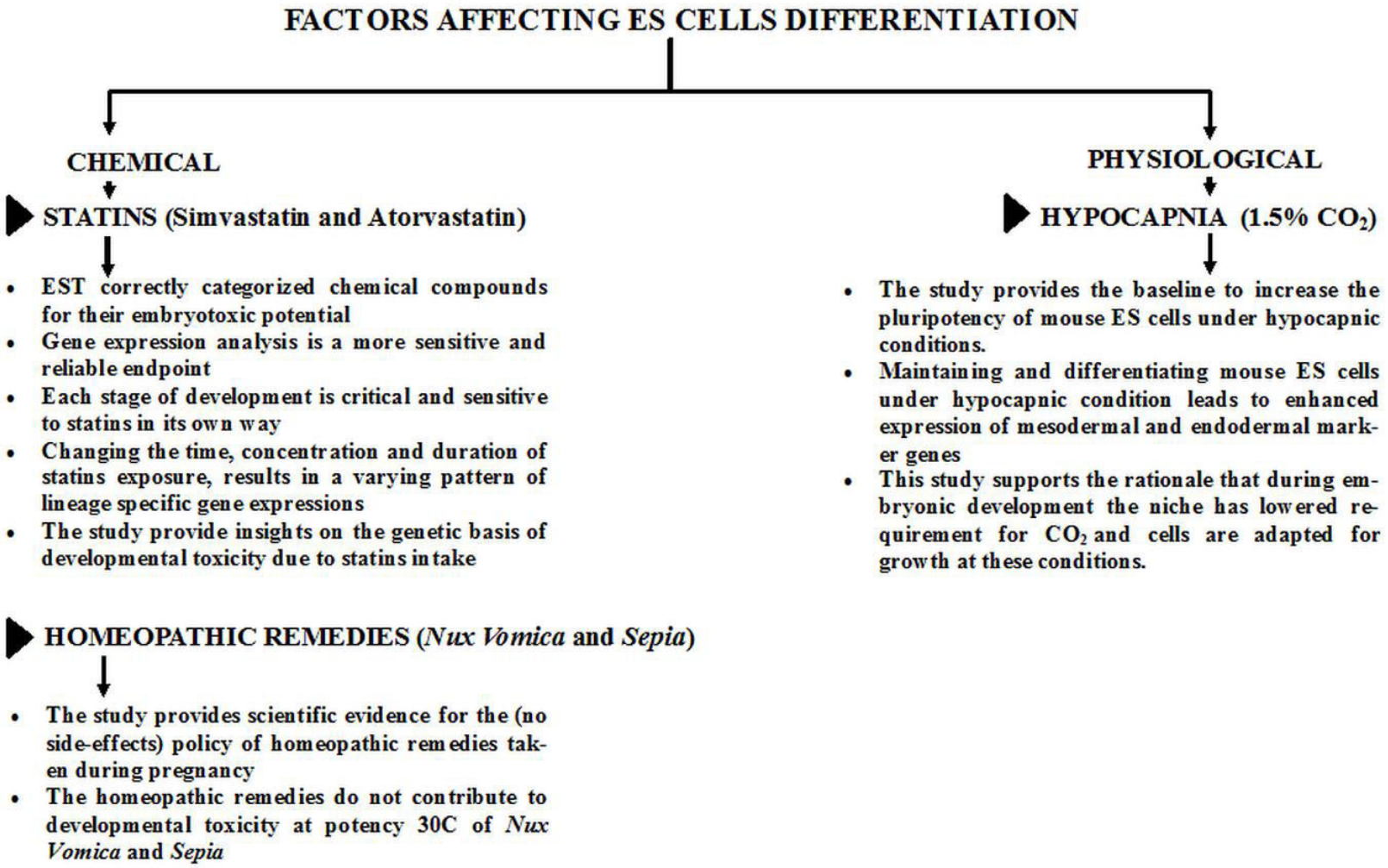

Figure5.1: Schematic representation of conclusions of the study.

The results demonstrated that:

- The exposure of ES cells to chemical compounds like 5-FU and RA down-regulated the expression of cardiac marker gene α/β MHC at both the gene and protein level.
- The exposure of ES cells to statins during the differentiation process lead to significant deregulation in mesodermal marker gene expression.
- Up-regulation of mesodermal marker genes,Brachyury, Flk and Nkx2.5 was observed upon exposure to statins indicating their possible role in congenital heart defects.
- The dose and time frame analysis of statin exposure revealed that each stage of the differentiation process *i.e.* ES cell aggregation (day 0), formation of EBs (day 3) and establishment and differentiation process of EBs (day 5) was sensitive to statins in its own way.
- The study revealed the potential of ES cells in providing scientific evidence for testing the developmental toxicity, if any, of homeopathic remedies using expression of lineage marker genes.
- The study also provided experimental evidence for the safe use of homeopathic remedies taken during pregnancy.
- The study explored the physiological factor *i.e.*hypocapniaon ES cells characteristics.
- The results proved that hypocapnia has a positive effect in maintaining the pluripotent population of ES cells.
- The ES cells maintained under hypocapnic conditions for a longer passage had a differentiation propensity towards the mesodermal and endodermal lineages.

Moving forward, it is important to recognize the influence of factors and the behavior of ES cell differentiation during development. The study of *in vitro*differentiation over the past few decades has advanced our understanding of biology in general and development in particular and has forced us to thinkabout the manner in which ES cells differentiate during development. This has not only ushered in new studies relating to the key principles of developmental biology, such as the regulation of genomic and protein expression during both embryogenesis and in adult life, but it has also given original examples for the development of therapeutic strategies and in drug discovery.

This study has provided the genetic basis of developmental toxicity of statins in mouse ES cells. An advanced knowledge of the effects of statins on differentiation will probably give insights into multiple congenital malformations occurring during pregnancy.Hence, further studies on human ES cells are necessary to firmly establish their use in pregnancy.

On the other hand, homeopathic remedies have shown no significant alteration in gene expression of lineage markers for the three germ layers. Use of these lineage specific genes as a yardstick to evaluate the effect of homeopathic drugs, will provide a unique model system to evaluate and study the developmental toxicity, if any, associated with the use of homeopathic remedies. However extensive studies should be done by using more biomarkers of differentiation to prove the safety of these remedies during pregnancy beyond a doubt. In addition, studies on human ES cells will provide more reliable experimental data for their use in pregnancy.

This study investigated the effects of reduced CO_2 tension on ES cells fate. As this study has been analysed using a single mouse ES cell line, it is reasonable to assume that it has a broader significance as the genes under study are conserved in both mouse as well as human ES cells. The mechanism by which hypocapnia leads to maintenance of pluripotency and differentiation is yet not known. The signaling mechanisms and proteins behind the hypocapnic effects on ES characteristics needs to be explored further to provideculturing conditions which truly mimic the in vivo environment.

The dynamism and potential of ES cells is based on the genetic factors operative within these cell working in a temporal manner and being influenced by the environment in which these cells are being cultured.In addition, the stability and safety of differentiated cells is also greatly influenced by various factors to which the cells are exposed during *in vitro* and *in vivo* conditions.

LIST OF PUBLICATIONS

1. **Saras Jyoti** and **Simran Tandon.** Factors influencing the dynamics of differentiation in embryonic stem cells. *Current Stem Cell Research and Therapy*. **doi**: 10.2174/1574888X10666150416113055, 2015 (Impact Factor 2.21).
2. **Saras Jyoti** and **Simran Tandon.** The genetic basis for developmental toxicity due to statin intake: an in vitro approach using ES differentiation. *Human and Experimental **To**xicolology,* 0960327114564795, doi:10.1177/0960327114564795, 2015**.** (Impact Factor 1.75).
3. **Saras Jyoti** and **Simran Tandon**. Hypocapnia leads to enhanced expression of pluripotency and meso-endodermal differentiation genes in mouse embryonic stem cells. *Experimental Cell Research 2014 April 1, 322 (2): 389-401.* (Impact factor 3.25)
4. **Simran Tandon** and **Saras Jyoti**. Embryonic Stem Cells: An Alternative Approach to Developmental Toxicity Testing. *Journal of Pharmacy and Bioallied Sciences* 4,(2), 96-100, 2012. Indexed in PubMed 'MEDLINE'

Under review

1. **Saras Jyoti** and **Simran Tandon.** The Impact of Homeopathic Remedies Taken During First Trimester on the Expression of Lineage Differentiation Genes: An *In Vitro* Approach Using Embryonic Stem Cells. Under review "*Homeopathy*".

CONFERENCES:

- 4th International Conference on Stem Cells and Cancer (ICSCC-2013): Proliferation, Differentiation and Apoptosis, 19th to 22nd October 2013, Mumbai, Maharashtra, India
- 3rd International Conference on Stem Cells and Cancer (ICSCC-2012): Proliferation, Differentiation and Apoptosis, 27th to 30th October 2012, New Delhi, Delhi, India