CHAPTER 2

ABSTRACT

ES cells 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 ES cells for differentiation into different cell types makes it a prominent candidate as a tool for understanding the process of embryogenesis and revealing the effect of physico-chemical factors on the growth of fetus. In addition, genetic variations and birth defects caused by mutations and teratogen, affecting early mammalian development, 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 ES cells 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 200kDa implies 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.
2.1 INTRODUCTION

This study was based on EST, which was developed for in vitro embryotoxicity testing of drugs and chemical compounds. In contrast to other in vitro embryotoxicity tests, for example, the post implantation whole rat embryo culture test and the micro mass assay, 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 contracting cardiomyocytes (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 EST based 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 whether these EBs were contractile, or not, was subject to human error. In addition, the EST approach used only mesodermal lineage
differentiation as an endpoint for predicting embryotoxicity of drugs during embryonic development [8]. But many chemical compounds like 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, chondrocytes and 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 ES cell lines from these two species, differences are also present, e.g., expression of differentiation markers and the cell cycle. Based on this data, valuable markers could be used as new endpoints and help in evaluating 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 test chemical are estimated 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 compounds by intracellular flow cytometry in ES cells [16].

The gene expression level of different lineage markers was also estimated by qRT-PCR in the presence or absence of test chemicals. Gene expression analysis is important because it provides an insight into false-negative results of the classic EST by analyzing specific effects on single endpoints, such as the strong inhibition of neurogenesis by valproic acid and 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 was available 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]. The test chemicals used for 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 this gene was chosen [19] as it could be used as a potential marker for cardiac development during ES cell differentiation.

2.1.2 Statins and developmental toxicity

Having confirmed that the EST (based on molecular endpoint analysis) carried out in this study had correctly categorized the training 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 death and 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 direct bearing 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 offspring [22-27].

“Statins,” act on the key enzyme, \( \beta \)-Hydroxy-\( \beta \)-Methyl Glutaryl-Coenzyme A (HMG-CoA) reductase of the cholesterol biosynthetic pathway [28]. They have been used successfully in the treatment 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]. Despite this, statins are still prescribed to pregnant women by physicians because they have 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 CNS defects 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 first trimester statin users, or any discernible pattern of congenital anomalies among live births but their conclusions were uncertain in the absence of outcome of data on non-live births [41].
Simvastatin and 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 the gene and protein level. Their differentiation mimics the changes which occur during embryogenesis in vivo, that are traceable 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 methods for in vitro differentiation i.e. monolayer as well as EB (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 expression with both methods, therefore further studies were carried out using the monolayer method of differentiation.
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 modifications which 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 hanging drop 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 easier as 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.

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

<table>
<thead>
<tr>
<th>Dosing day</th>
<th>EB differentiation</th>
<th>Monolayer differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Day 3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Day 5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Day 10</td>
<td>RNA isolation</td>
<td>RNA isolation</td>
</tr>
</tbody>
</table>

+ indicates dosing day
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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°C and were fed every day and routinely passaged after every 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°C and were routinely passaged after every 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°C. Further dilutions were made in DMEM to obtain a concentration range of 125-2000µg/ml. The final concentration of PBS was ≤ 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°C. Further dilutions were made in DMEM to obtain a concentration range of 0.0000375-0.007µg/ml. 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.

**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°C. 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 concentration was used as the upper limit and serial 2-fold 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°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°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.

### 2.2.3 Determination of cytotoxicity using MTT assay

Cytotoxicity of chemical compounds was 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 hemocytometer 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. For this, 30µl of MTT solution (5mg/ml in PBS) was added to each well containing the cells and incubated for 4 hours at 37°C. 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 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 given below:

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

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) were placed 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°C under 5% CO₂ and 95% humidity in the presence of different concentration 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, was expressed as the concentration of the test chemical that decreases the development into contracting cardiomyocytes by 50% (ID₅₀) 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 $5 \times 10^4$ cells/ml in 6 well 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), 0.1 mM β-mercaptoethanol.

### 2.2.6 Immunocytochemistry

EBs were obtained from hanging drop methodology and transferred on gelatin 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 15 minutes followed by washing with 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 then incubated with primary antibody against α/β MHC (Abcam) diluted at 1:250 ratio, and kept overnight at 4°C. 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. 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 Ti 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. The concentration and quality of RNA was measured with a Nano drop 2000
spectrophotometer (Thermo Scientific). Synthesis of cDNA from RNA was 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 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°C for 4 minutes, followed by 39 cycles each of denaturation (95°C for 15 seconds), annealing (54.5-60.2°C for 20 seconds) and extension (72°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

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (Forward primer-FP and Reverse primer-RP)</th>
<th>Annealing temperatures (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>FP 5’-GCACAGTCAAGGCCGAGAAT-3’ RP 5’-GCCTTCTCCATGGTGTTGAA-3’</td>
<td>58.5</td>
</tr>
<tr>
<td>Afp</td>
<td>FP 5’-GCTGCAAAGCTGACAACAG-3’ RP 5’-GGTTGTTGCTGGAGGTTC-3’</td>
<td>58.7</td>
</tr>
<tr>
<td>Flk-1</td>
<td>FP 5’-CAGCTTCCAAGTGGCTAACC-3’ RP 5’-CAGAGCAACACACCCGAAAG-3’</td>
<td>54.5</td>
</tr>
<tr>
<td>Nes</td>
<td>FP 5’-GCTTTCCTGACCCCAAAGCTG-3’ RP 5’-GGCAAGGGGGAAGAGAAGG-3’</td>
<td>60.2</td>
</tr>
<tr>
<td>ND 200</td>
<td>FP 5’-TGGACATTGAGATTGCCGC-3’ RP 5’-GAGAGAAGGGGGAAGAGAAGG-3’</td>
<td>62.4</td>
</tr>
<tr>
<td>Nkx2.5</td>
<td>FP 5’-CAAGTGCTCTCCTGCTTCCC-3’ RP 5’-GGCTTTGTCCAGCTCCACT-3’</td>
<td>56.5</td>
</tr>
<tr>
<td>α/β MHC</td>
<td>FP 5’-ACCTGTCAAAGTCCGCAGCAAG-3’ RP 5’-CTTGTGACCTGGGACTCGG-3’</td>
<td>58.5</td>
</tr>
<tr>
<td>Brachyury</td>
<td>FP 5’-TTCTTTGGCATCAAGGAAGG-3’ RP 5’-TCCGA GACCCAGTTCATAG-3’</td>
<td>57.0</td>
</tr>
</tbody>
</table>

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

To assess the changes 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**

<table>
<thead>
<tr>
<th>Dosing day</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Day 3</td>
<td>X</td>
<td>+</td>
<td>X</td>
<td>+</td>
<td>+</td>
<td>X</td>
</tr>
<tr>
<td>Day 5</td>
<td>X</td>
<td>X</td>
<td>+</td>
<td>X</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
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<td>↓</td>
<td>↓</td>
<td>↓</td>
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</tr>
<tr>
<td>Day 10</td>
<td>RNA isolation</td>
<td>RNA isolation</td>
<td>RNA isolation</td>
<td>RNA isolation</td>
<td>RNA isolation</td>
<td>RNA isolation</td>
</tr>
</tbody>
</table>

+ indicates dosing day, X indicates no dosing

### 2.2.9 Statistical analysis

The cytotoxicity data was obtained by calculating the mean±SEM (standard error mean) from four individual experiments done in triplicates. The IC₅₀ 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 ID₅₀ 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 $2^{\Delta \Delta \text{Ct}}$ method. 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 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 the cell 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}3T3 was found to be 910µg/ml and IC_{50}D3 was found to be 1259µg/ml for Pen G (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.](image)

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.
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Figure 2.2: Phase contrast pictographs showing formation of EB at day 3 using hanging drop method. Magnification 10X. Bars: 100µm. The experiment was performed four 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 $IC_{50}$ of 3T3 was found to be 0.069µg/ml and $IC_{50}$ of D3 was 0.032µg/ml for RA (Figure 2.4).

![Graph](image1)

![Graph](image2)

**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).

![Image](image3)

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

![Graph showing concentration-response curves](image)

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

Similarly, 5-FU showed an intense cytotoxicity which was evident by the IC$_{50}$D3 and IC$_{50}$3T3 values achieved at much lower concentrations. The IC$_{50}$3T3 was found to be 0.006$\mu$g/ml and IC$_{50}$D3 was 0.0002$\mu$g/ml for 5-FU (Figure 2.7).
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 performed four 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.
Figure 2.9: Concentration–response curves obtained from microscopical evaluations showing inhibitory effects of 5-FU on differentiation of EBs into 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 housekeeping gene i.e. GAPDH expression. The y-axis represents fold change in 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 \( i.e. \text{ID}_{50} \alpha/\beta \text{ MHC} \) was more sensitive as compared to conventional \( \text{ID}_{50} \). 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.**

<table>
<thead>
<tr>
<th>Test compound</th>
<th>( \text{IC}_{50} \text{D3 (MTT)} )</th>
<th>( \text{IC}_{50} \text{3T3 (MTT)} )</th>
<th>( \text{ID}_{50} \text{ D3} )</th>
<th>( \text{ID}_{50} \alpha/\beta \text{ MHC} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pen G</td>
<td>1259±190.7</td>
<td>910±149.2</td>
<td>1215±50.5</td>
<td>450±19****</td>
</tr>
<tr>
<td>RA</td>
<td>0.032±0.0021</td>
<td>0.069±0.0012</td>
<td>0.0003±0.00003</td>
<td>0.0001±0.00007**</td>
</tr>
<tr>
<td>5-FU</td>
<td>0.0002±0.00005</td>
<td>0.006±0.0008</td>
<td>0.054±0.01</td>
<td>0.007±0.0012****</td>
</tr>
</tbody>
</table>

Values are expressed in \( \mu \text{g/ml} \). 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 \( \text{ID}_{50} \text{D3} \) and \( \text{ID}_{50} \alpha/\beta \text{ MHC} \) \((**p<0.01, ****p<0.0001)\)

**2.3.3 ES cell derived cardiomyocytes treated with training set of drugs characterized by ICC for \( \alpha/\beta \text{ MHC} \)**

To identify the cardiomyocytes within the EBs, ICC for the marker protein \( \alpha/\beta \text{ MHC} \) was performed (Figure 2.11). EBs which showed contracting cardiomyocytes were positively stained with \( \alpha/\beta \text{ MHC} \) and this staining was visible in solvent control and Pen G treated EBs. ICC analysis also elucidated the loss of protein expression of \( \alpha/\beta \text{ MHC} \) upon treatment with strongly embryotoxic drugs.
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 \textit{i.e.} ID_{50} \alpha/\beta MHC was used instead of conventional ID_{50} endpoint for evaluating the teratogenic potential of training set of drugs.
<table>
<thead>
<tr>
<th>DRUG</th>
<th>FUNCTION I</th>
<th>FUNCTION II</th>
<th>FUNCTION III</th>
<th>CLASSIFICATION</th>
</tr>
</thead>
</table>
|      | 5.92 log (IC$_{50}$T3) +3.50 log (IC$_{50}$D3) - 5.31(IC$_{50}$T3 - ID$_{50}$) /IC$_{50}$T3 -15.7 | 3.65 log (IC$_{50}$T3) +2.93 log (IC$_{50}$D3) - 2.03(IC$_{50}$T3 - ID$_{50}$) /IC$_{50}$T3 -6.85 | - 0.125 log (IC$_{50}$T3) -1.92 log (IC$_{50}$D3) +1.5 (IC$_{50}$T3-ID$_{50}$) /IC$_{50}$T3 -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(0.006-0.0007)/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 confirmed that the EST had correctly categorised the training set of chemicals into the respective categories, and that the analysis of key gene controlling lineage commitment was a more sensitive endpoint as well as reliable, this model system was applied to statins, a class of drugs whose use in pregnancy gives conflicting reports. The major focus of this work was to assess genetic association between statins and developmental toxicity.

2.3.5 Determination 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.**

<table>
<thead>
<tr>
<th>Statin</th>
<th>IC$_{50}$ ES</th>
<th>IC$_{50}$ 3T3</th>
<th>ID$_{50}$ α/βMHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simvastatin</td>
<td>0.014±0.0075</td>
<td>0.083±0.0063</td>
<td>0.012±0.003</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>0.020±0.0033</td>
<td>0.079±0.016</td>
<td>0.018±0.0057</td>
</tr>
</tbody>
</table>

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

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

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

$$= -9.98$$

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

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

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

$$= -3.64$$

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

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

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

$$= -3.43$$

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.
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Function I:  
\[ 5.92 \log (IC_{50}^{3T3}) + 3.50 \log (IC_{50}^{D3}) - 5.31(\frac{IC_{50}^{3T3} - ID_{50}}{IC_{50}^{3T3}}) - 15.7 \]

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

\[ 5.92 (-1.102) + 3.50(-1.698)-5.31(0.772)-15.7 \]

\[ = -13.179 \]

Function II:  
\[ 3.65 \log (IC_{50}^{3T3}) + 2.93 \log (IC_{50}^{D3}) - 2.03(\frac{IC_{50}^{3T3} - ID_{50}}{IC_{50}^{3T3}}) - 6.85 \]

\[ 3.65(0.079) + 2.93(0.020) - 2.03(0.079-0.018)/0.079 - 6.85 \]

\[ 3.65(-1.102) + 2.93(-1.698)-2.03(0.772)-6.85 \]

\[ = -4.637 \]

Function III:  
\[ -0.125 \log (IC_{50}^{3T3}) - 1.92 \log (IC_{50}^{D3}) + 1.5(\frac{IC_{50}^{3T3} - ID_{50}}{IC_{50}^{3T3}}) - 2.67 \]

\[ -0.125(0.079)-1.92(0.020) + 1.5(0.079-0.018)/0.079 - 2.67 \]

\[ -0.125(-1.102)-1.92(-1.698) + 1.5(0.772) - 2.67 \]

\[ = -2.709 \]

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.

2.3.7 Statins 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 ectodermal lineages 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.

### 2.3.7.1 Effect 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 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).

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 of the gene under study. The data was analyzed by using the 2^ΔΔCt 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, makes it 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 $2^{ΔΔCt}$ 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 that the 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 200 kDa assessed 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 and solvent 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 were made to undergo differentiation using the monolayer method and exposed them to similar concentrations of simvastatin and atorvastatin.
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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, which were disturbed during monolayer differentiation when exposed 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 (Figure 2.16C, D).
Figure 2.16: Early mesodermal marker genes, Brachury and Flk-1 assessed by qRT-PCR in monolayer cultures exposed to statins. (A); (B) Brachury 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).
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 in EBs. 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, Nkx2.5 and α/β MHC assessed by qRT-PCR in monolayer cultures exposed 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 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^ΔΔCt 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 in monolayer 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) 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^{-ΔΔCt} 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<0.01, ***p<0.001, ****p<0.0001).

2.3.7.6 Ectodermal expression in monolayer cultures in the presence of statins

The expression of ectodermal genes, Nes and Neurofilament 200kDa displayed 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 \textit{i.e.} 4 fold at 0.003\text{µM}, \textit{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 0.003\text{µM}concentration in both Nes (\textit{p}<0.01) and Neurofilament 200kDa (\textit{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 \textit{i.e.} GAPDH. The \textit{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 (\textit{n}=3). Significance between exposed and solvent control cells (*\textit{p}<0.05, **\textit{p}<0.01, ****\textit{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 analyzed in 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 work was 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-1 during window exposure 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 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 change 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<0.01, ****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 as shown 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 performed three 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.
CHAPTER 2

Figure 2.23: Expression patterns of the endodermal marker gene, Afp during window exposure to statins. (a); (b) Afp 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 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 course revealed the enhanced expression of ectodermal maker i.e. Nes (marker of neural progenitor cells) and Neurofilament 200kDa (mature neural cell marker) at lower concentrations of 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 covered that 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)Nes expression 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
ES cells are undifferentiated cells which are capable of both 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 could result 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 to the inhibition of HMG-CoA reductase, statins have wide ranging actions. The major pleiotropic effects in broad way are, improving endothelial function, increased expression of VEGF and increased 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]. Nkx2.5 expression 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 Nkx2.5 is 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.

Afp is 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 erythropoiesis, 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-regulated expression 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-1 was 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 might be 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 occurring during 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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