Histone and Runx2 Gene Expression During Embryoid Body Differentiation of Human Embryonic Stem Cells

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Abstract

Human embryonic stem cells can be induced to form embryoid bodies which contain all three germ layers of the human body. Histone expression is tightly coupled to cell growth while Runx2 is essential for bone differentiation. This project tested the hypothesis that when stem cells cease to proliferate and differentiate into embryoid bodies, the Runx2 gene is induced.
Acknowledgements

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I. Introduction

The reciprocal relationship between cell growth and differentiation is a well established concept of cell biology. Histone gene expression is tightly coupled to cell growth, and used as a marker of proliferation. The $\text{Runx2}$ transcription factor is expressed in mesenchymal osteoprogenitor cells and is essential for osteoblast differentiation. Human embryonic stem cells can be cultured in vitro to form a structure composed of the 3 germ layers (ectodermal, mesodermal, endodermal). In this project we tested the hypothesis that when human embryonic stem cells form an embryoid body and mesodermal cells commit to a bone phenotype, $\text{Runx2}$ is induced. Experiments were done to test exactly when $\text{Runx2}$ is induced in human embryonic stem cells and if there is a relative change in cell growth by monitoring the Histone H4 levels when they do so. In doing these experiments, more information will be obtained in the area of bone development and embryonic gene expression.

Research in human embryonic stem cells has been largely focused in the area of tissue replacement therapy. Human embryonic stem cells have been shown to be promising in treating damaged tissue due to trauma as well as other disorders and diseases (Stem Cells, 2001). Some of the disorders human embryonic stem cells many help treat in the future include: Parkinson’s disease, diabetes, traumatic spinal cord injury, Duchennes muscular dystrophy, heart failure, Alzheimer’s disease, cancer, osteogenesis imperfecta and other genetic disorders of the skeleton (Stem Cells, 2001). There is still much research that must be done in the area of stem cell therapy. The research done in this project may lead to future developments and advances in the areas of bone disorders and cancer.
1.1 Stem Cells

Stem cells are defined as non-differentiated cells that give rise to differentiated, specialized cells. Stem cells are self renewing and continue to proliferate undifferentiated until there are certain in vitro growth conditions (factors added to culture medium) present which appear to induce the cells to develop into a differentiated cell type (Bodnar, et al., 2004). Human embryonic stem cells originate from the human blastocyst and are pluripotent, which means having the ability to differentiate into any of the three embryonic germ layers of the body (Bodnar, et al., 2004).

In addition to human embryonic stem cells there are adult stem cells. Adult stem cells are undifferentiated cells in an environment of differentiated and specialized tissue. Adult stem cells are located within the tissue into which they will develop, and are a source of precursor cells. It has been observed that these cells can develop from one type of cell to another type. For example, under appropriate conditions hematopoietic stem cells from bone marrow which are capable of developing into blood/immune cells have been found to be able to develop into cells which have many characteristics of neurons. Furthermore, mesenchymal stem cells can differentiate into many tissue type cells (muscle, bone, cartilage, nerve). This characteristic is known as adult stem cell plasticity. In addition, adult stem cells are multipotent, rather than pluripotent; they provide a source of limited types of precursor cells (Stem Cells, 2001).
1.2 Development

The development of the human embryo begins with the zygote which then develops into the blastocyst and further develops into the gastrula (Figure 1).

Figure 1 Development of Human Cells (Stem Cells, 2001)
The blastocyst, where trophoblasts define a hollow space, contains within it a mass of cells, the inner cell mass. The inner cell mass consists solely of embryonic stem cells. Every cell type of the human body is derived from the inner cell mass of the blastocyst. Embryonic stem cells are found in the inner cell mass of a 4-5 day blastocyst (Figure 2). Inside the blastocyst is the inner cell mass of approximately 30 stem cells (Stem Cells, 2001).

![Blastocyst Diagram](image)

**Figure 2 Blastocyst** (Stem Cells, 2001).

### 1.3 Embryoid Body Properties

Human embryonic stem cells *in vitro* can be induced to differentiate into embryoid bodies, which will further differentiate into the three main embryonic germ layers (Bodnar, *et al.*, 2004). Embryoid bodies contain cells from all three germ layers. The three embryonic layers include the endoderm, the mesoderm and the ectoderm. The endoderm is the innermost layer of the gastrula and gives rise to the formation of the respiratory tract, the gastrointestinal tract, and the endocrine glands. The mesoderm is the middle layer of the gastrula and gives rise to the formation of the muscular, bone, blood
and vascular systems, and the reproduction systems. The outermost layer of the inner cell mass of the blastocyst, the ectoderm, gives rise to the epidermis and the nervous system (Stem Cells, 2001). All three germ layers are represented in an embryoid body in a disorganized manner and ‘clumped’ together in a spherical mass (Figure 3a, b).

Figure 3 Embryoid bodies derived from the H1 embryonic stem cell line and grown in differentiation medium for five weeks. A) An early stage of the formation of an embryoid body photographed using a 40X objective B) A more mature, older embryoid body photographed using a 10X objective.

1.3.1 Characterization of Human Embryonic Stem Cells

Human embryonic stem (ES) cells can undergo an unlimited number of cell divisions without differentiation. Human embryonic stem cells are also clonogenic, which is the ability of single ES cell to give rise to a genetically identical colony of cells, all with the same genetic properties as the original cell. This is a feature unique to embryonic stem cells. Human embryonic stem cells have also been found to express a transcription factor known as Oct-4, which is a marker for the undifferentiated cells (Bielby, et al.,
2004). Embryonic stem cells can be induced by exogenous factors to either continue proliferating, or to differentiate. ES cells have a very short G1 cell cycle phase, due to their rapid proliferation. The cells spend a majority of their time in S phase synthesizing DNA. Human ES cells also maintain a diploid set of chromosomes (Stem Cells, 2001).

The undifferentiated state of the embryonic stem cells is maintained under certain growth conditions. It has been found that embryonic stem cells grown on an adherent surface, such as glass or plastic, differentiate spontaneously without external factors being added. To prevent differentiation, mouse embryonic fibroblasts (MEFs) are often used as a feeder layer for the human embryonic stem cells. The MEFs are usually isolated from 12-14 day mid-gestation mouse embryos (Borros, et al., 2005). The MEFs produce bFGF2 to provide a sufficient stimulus for the proliferation of the embryonic stem cells. Thus, MEF generated factors prevent embryonic stem cell differentiation (Borros, et al., 2005, Stem Cells, 2001).
1.3.2 Qualitating Growth and Differentiation Properties of Embryonic Stem Cells

Several techniques have been used to confirm the identity of the undifferentiated human embryonic stem cell. These techniques are listed below (Bodnar, et al., 2004).

1. Markers of undifferentiated human embryonic stem cells. The markers that are used are Oct-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and can be detected by immunofluorescence microscopy or quantitatively analyzed by mRNA or protein expression (Table 1).

2. The ability to form embryoid bodies. The presence of all three germ layers is confirmed by using the AFP, FLK-1, and NCAM markers to detect the presence of the endoderm, mesoderm, and ectoderm, respectively, via RT-PCR.

3. The ability to form teratomas when injected into nude mice. Teratomas also show tissue derived from all three germ layers.
### Table 1. Embryonic Stem Cell Markers

<table>
<thead>
<tr>
<th>Factor</th>
<th>Full Name</th>
<th>Reference</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct-4</td>
<td>POU transcription factor family- POU domain,</td>
<td>Protein Design Group</td>
<td>Marker for hESC</td>
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<tr>
<td></td>
<td>class 5, transcription factor 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSEA-3</td>
<td>Stage Specific Embryonic Antigen 3</td>
<td>Stem Cells, 2001</td>
<td>Glycoprotein expressed in early embryonic development and undifferentiated pluripotent stem cells (PSCs)</td>
</tr>
<tr>
<td>SSEA-4</td>
<td>Stage Specific Embryonic Antigen 4</td>
<td>Stem Cells, 2001</td>
<td>Glycoprotein expressed in early embryonic development and undifferentiated PSCs</td>
</tr>
<tr>
<td>TRA-1-60</td>
<td>tumor recognition antigen 1-60</td>
<td>Stem Cells, 2001</td>
<td>Marker for extracellular matrix molecule synthesized by undifferentiated PSCs</td>
</tr>
<tr>
<td>TRA-1-81</td>
<td>tumor recognition antigen 1-81</td>
<td>Stem Cells, 2001</td>
<td>Marker for extracellular matrix molecule normally synthesized by undifferentiated PSCs</td>
</tr>
<tr>
<td>AFP</td>
<td>Alpha Fetoprotein</td>
<td>Ersoy, O., 2005</td>
<td>Marker for endoderm in embryoid bodies</td>
</tr>
<tr>
<td>FLK-1</td>
<td>Fetal Liver Kinase 1</td>
<td>Protein Design Group</td>
<td>Marker for mesoderm in embryoid bodies</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
<td>Sinanan, et al., 2004</td>
<td>Marker for ectoderm in embryoid bodies</td>
</tr>
</tbody>
</table>
H4 tetramer. Histones have a net positive charge due to the large number of lysine and arginine residues they contain (Camporeale, et al., 2004).

The histone which was studied in this project is H4. Histone mRNAs are present in cells mostly during the S phase and can be markers for cell proliferation. Histone mRNA is tightly coupled to cell growth and levels of DNA synthesis. This histone also plays a large role in the organization of the DNA-histone complex (Camporeale, et al., 2004). RT-PCR can determine the level of expression of histone H4 mRNA at different time points in the growth and differentiation of human ES cells and is related to cell proliferation and growth. Histone expression is expected to be elevated during stem cell proliferation and reduced when the cells cease to proliferate and differentiate into embryoid bodies. There are several genes in the histone H4 family (H4/a, H4/b...). In this project H4 family members H4/b, H4d+e and H4/n+o were examined because those are most related to cell growth and proliferation.

1.5 Bone Development and Runx2 Gene Expression

Skeletal formation is a process initiated by endochondral and intramembranous ossification (Lengner, et al., 2002). Endochondral ossification is the process by which cartilage is replaced with bone. It is during intramembranous ossification when bone formation occurs (Figure 4). In humans, bone formation begins at about the 6-7th week after conception and is formed through the process of ossification of cartilage formed from the mesenchyme (Hill, et al., 2005) (Figure 4). This process initially begins with an
undifferentiated mesenchymal cell. This mesenchymal cell will then develop into a multipotent stem cell that will be committed to skeletal lineage cells (chondrogenic & osteogenic phenotype), and further develop into a defined osteoprogenitor cell, an osteoblast and finally the cell will terminally differentiate into an osteocyte once the osteoblasts become trapped in the matrix they form (Figure 5). Thus, osteoblasts are cells involved in the formation of bone while osteocytes are mature bone cells which help to maintain bone tissue, and osteoclasts are cells involved in the resorption of bone (Hill, 2005).
Figure 5 Osteoblast Differentiation Pathway (Ducy et al., 1997). Once the multipotent mesenchymal stem cell becomes committed to the osteoblast lineage, the stem cell goes through a series of developments maturing from an osteoprogenitor cell to the terminally differentiated osteocyte which is involved in bone formation.
1.5.1 Runx2

Runx2 is a member of the runt family of transcription factors and is a key regulatory protein for promoting osteogenesis. Runx proteins are part of a group of gene regulatory master-switches which function to regulate the transcription of genes necessary for the differentiation of osteoblast lineage cells. During embryogenesis Runx2 is expressed very early in mesenchymal condensations forming the skeleton (Lengner et al., 2002)

Runx2 is essential for bone formation and therefore expressed at very early stages of embryonic development (Otto et al., 1997). Runx2 has been found to be an essential factor for the differentiation of human embryonic stem cells into osteoblasts. There are two different isoforms of Runx2 which differ only in their amino terminal sequences. Type-I contains MRIPV while type-II has MASNSLFSAVTPCQSFFW as the amino terminus (Figure 6). It is the Runx2 promoter P1 which controls expression of the type II isoform, which is increased during osteoblast differentiation (Lengner, et al., 2005).

While type-II Runx2 is considered osteoblast specific (Ducy, et al., 1997), it has been found that osteoblast-like cells express both type I and type II Runx2 protein. For example, it has been determined that both type-I and type-II Runx2 proteins are expressed in cells with a mature osteoblast phenotype (Sudhakar et al., 2002). Importantly, they are both expressed in early somites in the embryo that will form the skeleton (Lengner 2002, Smith 2000). There is usually a low level of Runx2 in the undifferentiated stem cell and this protein appears to represent a different isoform from the isoform expressed at high levels in mature bone cells (Smith, 2000). The type I isoform is ubiquitous and remains at
a low level. Both however may be required for bone formation. In this project embryoid bodies were assayed for both isoform I and isoform II Runx2.

Growth conditions contribute to the differentiation of mesenchymal cells. When dexamethasone is added to the undifferentiated cell, Runx2 is upregulated and this upregulated gene induces osteoblast formation. Runx2 also functions in regulating osteoblast growth and proliferation. An inverse correlation has been found between Runx2 levels and growth factors; Runx2 levels are elevated when growth factors necessary for osteoblast growth and differentiation are low, and Runx2 levels are down-regulated during active proliferation of osteoblasts with the necessary growth factors are present (Figure 7), (Pratap, J., et al., 2003; Galindo, M., et al., 2005). Since Runx2 is essential for normal osteogenesis, (Komori et al, 1997, Choi, et al., 2001), and the Runx2 transcription factor promotes skeletal cell differentiation, it was experimentally
determined whether the *Runx2* gene is induced when ES cells are differentiated into embryoid bodies.

**Figure 7 Runx2 relationships to osteoblast growth and differentiation.** Runx2 levels become elevated when growth factors necessary for osteoblast growth and differentiation are low, and Runx2 levels are down-regulated during cell cycle entry and active proliferation of osteoblasts with the necessary growth factors are present (Pratap, J., *et al.*, 2003).

### 1.6 Specific Aims

There were three main goals to this project. The first was growing and propagating human embryonic stem cells in an undifferentiated state. The H1 embryonic stem cell line was used in this project, with a feeder layer of mitotically inactivated mouse embryonic fibroblasts. The presence of undifferentiated stem cell was confirmed by determining expression levels of Oct4 and Nanog through Q-PCR.
The second goal was to grow embryoid bodies. They are characterized by the presence of alpha fetoprotein (AFP) as a marker for the endoderm, FLK1 for the mesoderm, and NCAM for the ectoderm, all of which can be detected by Q-PCR.

The final goal was to determine the expression levels of the Runx2 gene during the differentiation of embryoid bodies, as well as the histone H4 expression which was determined via Q-PCR. Histone gene expression is tightly coupled to cell growth while the Runx2 transcription factor is essential for osteoblast differentiation. We tested the hypothesis that when human embryonic stem cells cease to proliferate and differentiate into embryoid bodies, the Runx2 gene is induced.
II. Methods

**MEF Preparation** - 10mL of PBS (Gibco, 14190-144) was placed in a 100mm Petri dish. The uterine horns were removed from a 13.5 day gestation mouse and the fetuses were then transferred to the fresh PBS. The head and viscera were removed and the carcasses were transferred to fresh PBS until free of blood. Each carcass was then transferred into a clean Petri dish containing 1mL of Trypsin (0.25% in 1mM EDTA (Gibco, 25200-056)). The carcasses were then cut into small pieces and minced and transferred to a 50mL centrifuge tube to be incubated for 15-20 minutes. The trypsin was neutralized with MEF feeder medium (DMEM High Glucose 90%, FCS 10%, 0.05mM Penicillin/Streptomycin (5000ug/mL), 10mM L-Glutamine [Gibco, 25030-081]) equal to two times the volume of the PBS and Trypsin and pipetted with moderate force up and down. The tube was then gently centrifuged. The pellet was resuspended in 10mL of fresh feeder medium, and this was repeated twice. The cells were plated in a gelatin coated T175 flask with 30mL of feeder medium (one fetus per flask). The flasks were incubated at 37°C and 5% CO2. The cells were then passaged every 2-6 days (or when confluent), and some passage three flasks were frozen for later usage.

**Gelatinizing Flasks and Dishes** - Enough gelatin (0.1% Porcine, Sigma G 1890) was added to each T175 flask to coat the bottom and they were left to sit at room temperature for 25 minutes. Prior to use, the gelatin was removed by aspiration.
**Passaging of Fibroblasts**- The medium was aspirated off the flasks and then rinsed with 10mL of PBS. 5mL of trypsin was added and the flask was incubated for 5 minutes at 37°C. The trypsin was neutralized with 10mL feeder medium and transferred to a 50mL centrifuge tube (1 tube per flask) and then gently centrifuged. Then the supernatant was aspirated off and the fibroblasts were resuspended in 9mL of fresh feeder medium. The flask was prepared with 27mL feeder medium, and then the suspension was plated in 3 T175 gelatin coated flasks containing feeder medium to a final volume of 30mL.

**Irradiation of Stock Fibroblasts**- First the medium from the T175 was removed and the flask then rinsed with 10mL of PBS. 5mL of Trypsin was added and the flask was incubated for 5 minutes at 37°C. The Trypsin was neutralized with 10mL of feeder medium and transferred to a 50mL centrifuge tube (1 tube per flask), and then gently centrifuged. The supernatant was then removed and the cells were resuspended in 10mL of feeder medium. Cells were then transferred to a 50mL centrifuge tube, and kept on ice until irradiation. The tubes were irradiated with 3000 rad by x-ray, or by gamma irradiation with cesium source irradiator.

**Freezing of Stock Fibroblasts**- The medium was aspirated form the T175 flasks containing the fibroblasts and the cells were rinsed with 10mL of PBS. 5mL of Trypsin was added and the flask was incubated for 5 minutes at 37°C. The Trypsin was neutralized with 10mL of feeder medium and transferred to 50mL centrifuge tube (1 tube per flask) and then gently centrifuged. The supernatant was then removed and replaced with 5mL freezing medium, which consists of 50% freezing medium (FBS characterized, Hyclone SH30071.03) 90%, DMSO [Sigma D2650] 10%. The cells were
then transferred to a 1.5mL cryogenic vial and placed in a -70°C freezer for 24 hours, and then transferred to liquid nitrogen.

**Thawing of stock fibroblasts**- A 50mL centrifuge tube was prepared with 10mL of feeder medium. A vial of stock fibroblasts was removed from the freezer and placed in a 37°C water bath until the ice was nearly thawed. The cells were slowly resuspended by adding feeder medium to the vial and then transferred to the previously prepared centrifuge tube by adding a few drops at a time until the entire suspension has been transferred to the tube. The tube was then gently centrifuged, and the pellet resuspended in 20mL of feeder medium. The cell suspension was then transferred to a gelatin coated T175 flask already containing 29mL of feeder medium, so the final volume was 30mL. The flasks were then incubated for growth.

**Propagating the Human Embryonic Stem Cells**- Two different human embryonic stem cell lines HI and H9 cells were obtained through NIH. They were propagated by plating in a 6 well plate and each well was fed 2.5mL of complete hESC media 80% DMEM/F12, 20% KSR. 10mM Glutamine Beta Mercapta Ethanol, 1% MEM non essential amino acids [Gibco, 11140-050], bFGF 0.008mM) everyday.

**Passaging of Human Embryonic Stem Cells**- The cells were passaged approximately every 6 days. 1mL of collogenase (1mg/mL Collogenase in DMEM/F12 [Gibco, 11330-032]) was placed in each well containing cells. The plate was then incubated for approximately 20 minutes. The wells were then scraped with a pipette tip to loosen the
cells, and then scraped with a cell scraper. Each well was transferred to an individual 15mL conical and gently centrifuged. The media was then aspirated off and the cells were resuspended in enough hESC medium to plate at 2.5mL per well. If the plate was very confluent, they were passages at 1:3. If the plate was at medium confluency, the cells were passaged at 1:2. If the cells were not very confluent, and needed a new MEF layer, they were passaged at 1:1.5, or 1:1.

**Freezing Human Embryonic Stem Cells**- Each well was harvested and frozen in hESC freezing medium (FBS 60%, DMSO 20%, Complete hESC Media 20%) and 50% complete hESC medium. Each well was frozen in 1 cryovial in -70°C liquid nitrogen.

**Plating for Embryoid Bodies (100mm Petri Dish)**- One confluent 6 well plate of cell line H1 hESC were plated on one 100mm Petri dish. 1.5mL of collagenase was added to each well of the 6 well H1 hESC plate, and they were left to incubate at 37°C for approximately 20 minutes. Then each well was scraped with a pipette tip, and then a cell scraper, and the medium and cells were transferred to a 50mL conical tube and centrifuged for 5 minutes at slow speed. Following this, the cells were gently resuspended in 10mL of EB medium (80% Ishchoves Dulbecco's Modified Eagle Medium [IMDM], 20% FCS, 0.05mM Penicillin/Streptomycin, 10mM L-Glutamine).

**Plating for Embryoid Bodies (T25 flask)**- 1mL of Dispase (at concentrations ranging from 0.2-0.5mg/mL) was added to 49mL of DMEM/F12. 1.5mL of this was added to each well of a confluent 6 well plate of H1 hES cells, and the plate was left to incubate at 37°C. Then the media from the plate was taken off with a pipette and placed in a 15mL conical and vortexed for approximately 20 seconds to break up the colonies. The conical
was then gently centrifuged. The cells were then resuspended in 5mL of EB medium, then add 5mL of EB medium to a T25 flask for a total of 10mL. The EB’s were incubated in 37°C

**Feeding the Embryoid Bodies**- The Embryoid bodies were fed 5mL EB medium every other day. They were fed carefully, removing 5mL of old medium, being careful not to take up any cell clumps, and then adding 5mL to the flask. The flasks were also scraped with a cell scraper right after feeding to dislodge any cells adhering to the flask.

**Osteogenic Differentiation**- The embryoid bodies from a 100mm plate were collected by gentle centrifugation. They were then resuspended in fresh EB media with an osteogenic supplement (80% IMDM, 20% FCS, 0.05mM Penicillin/Streptomycin, 10mM L-Glutamine, 50μM ascorbic acid, 10mM β-glycerophosphate, 100nM dexamethasone), and plated on a 100mm plate coated with 0.1% porcine gelatin. They were fed every 2 days. The culture medium with these additional supplements (osteogenesis-promoting medium) was changed every 2 days for a total of 24 days of in vitro culture.

**Alkaline Phosphatase Stain (ALP)**- The plate was covered with 4% paraformaldehyde for 10 minutes. The paraformaldehyde was then removed and the plate was rinsed with Cacodylic buffer and let to dry. The ALP stain (0.5mM Napthol Mx Phosphate Disodium, 0.1mM Fast Red Salt, 50% 0.2M Tris Maleate Buffer 0.2M, 3% NN dimethyl Formamide, 47 % Distilled H2O) was then added to the plate and left to incubate at 37°C for approximately ½ hour.
RNA Isolation- RNA from two 100mm Petri dishes containing embryoid bodies was isolated by adding 3mL Trizol (Invitrogen) to each plate after aspirating the media. 1mL of the Trizol and cells were added to a 1.5mL centrifuge tube and 0.2mL of Chloroform was added. The centrifuge tube was incubated at room temperature for 3 minutes and then shaken for 15 seconds. The cells were then centrifuged for 15 minutes at high speed. After this the clear aqueous portion of the sample was transferred to a new tube. 0.5mL of 70% isopropanol was added to precipitate the RNA. The tube was incubated at room temperature for 10 minutes and then centrifuged for 10 minutes at high speed. The liquid was then taken off of the resulting pellet and a couple drops of 75% ethanol was added and left to dry. The pellet was resuspended in 16ul of milliQ water and the concentration of RNA determined using the spectrophotometer. The DNA was then removed from the RNA using X kit. To do this 5ug per 20uL of RNA was used. 3uL of 10X DNAse buffer was added and then 1.5uL RNAse free DNAse I was added. 5.5uL of X amount was added to bring the total volume up to 20uL. This was incubated 15 minutes in a 37°C water bath. 150uL of 4X RNA binding buffer was then added and the centrifuge tube was placed over a 150uL auto spin column and centrifuged for 15-60 seconds at high speed. 200uL wash buffer was then added and centrifuged for 20 seconds at high speed. Another 200uL wash buffer was added and centrifuged for 60 seconds at high speed. New tubes were prepared and the columns were added to the tubes so that the RNA could be eluted 2 times each with 10uL of pre-warmed milli-Q water. The RNA was re-quantified here again. Finally cDNA was made using the Bio-Rad iscript kit. 5uL of 5X iscript reaction mix was added, and then 1uL of iscript reverse transcriptase, XuL of nuclease free water and XuL of RNA template (1ug) total RNA, to
bring the final volume to 20uL. The reaction was held for 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. The dilution for the RT-PCR was as follows: 1ug RNA input → 20uL cDNA. Dilute 1/5 with Milli-Q water (80uL Milli-Q + 20uL cDNA).

20uL of this + 300uL Milli-Q water =~ 1/16 dilution. Use 5mL of this for the RT-PCR.

Q-PCR- Using the RNA obtained form the RNA isolation the Q-PCR was performed with primers for Oct-4, Flk-1, NCAM and AFP, Nanog, a mouse Runx2, Runx2 Isoform I and Isoform II, H4/a, H4/d+e/, H4/n+o/ and 28S ribosomal RNA as a control. The primers and their functions can be seen in table 2.
Table 2. Primers used for Q-PCR and their specific functions.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Function</th>
<th>Sequence</th>
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<td>Marker for stem cell undifferentiation</td>
<td>Forward 5’ CGACCATCTGCGCGCTTTTGAG 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’ CCCCCCTGTCCCCCATTCCTTA 3’</td>
</tr>
<tr>
<td>Flk-1</td>
<td>Marker for mesoderm differentiation</td>
<td>Forward 5’ AAGGTGACAGGAAAAGACGAACT 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’ TCCCCCTATGGGCCCCGCTTAAC 3’</td>
</tr>
<tr>
<td>NCAM</td>
<td>Marker for ectoderm differentiation</td>
<td>Forward 5’ AGGAGACAGAAACGAAGCCA 3’</td>
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<td>P1</td>
<td></td>
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<td>Runx2</td>
<td></td>
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<tr>
<td>P2</td>
<td></td>
<td>Reverse 5’ TGCCTGCTGGGGCTGTA 3’</td>
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<tr>
<td>Nanog</td>
<td>Marker for stem cell undifferentiation</td>
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<tr>
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<td>Internal control</td>
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<tr>
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<td>Type II isoform of Runx2</td>
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<td>H4/d+/e</td>
<td>Histone H4 cell proliferation marker</td>
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<td>Histone H4 cell proliferation marker</td>
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III. Results

The first goal of this project was to successfully differentiate human embryonic stem cells into EB’s. Q-PCR was done on 5 week EB’s as well as on a 4 week time course of growing EB’s to analyze the germ layer marker expression. Runx2 expression during the 4 week time course was also analyzed by Q-PCR. The EB’s were induced to follow an osteogenic pathway which was confirmed by an alkaline phosphatase stain.

3.1 Characterization of Embryoid Bodies

hESC were grown in two feeder layer free 100mm Petri dishes until they formed embryoid bodies, using EB medium which promoted differentiation (see methods). After approximately 2 weeks, several early embryoid bodies could be seen. Under the microscope many individual cells could also be seen that appeared more like undifferentiated stem cells. This indicates that there was a mix of EB’s and undifferentiated hESC in the culture and that hESC were developing into EB’s at different rates. After 5 weeks approximately 20 very large and dense embryoid bodies were present. A feature of an early embryoid body is a spherical shape (Figure 8a and b), whereas more developed embryoid bodies appears to be a more clumped aggregation of cells (Figure 8c and c).
3.2 Gene Expression of Embryoid Bodies

To confirm these structures were embryoid bodies, Q-PCR was performed using several germ layer markers (Table 2, Figure 9). Germ layer marker expression was determined and compared between EB’s and undifferentiated hESC. There was very low to no expression of the lineage markers in the H9 cells, and a strong expression of the lineage markers in the embryoid bodies. There was very strong expression of Oct-4 and fairly strong expression of Nanog in the H9 and a non-detectable amount in the embryoid
bodies. These results suggest that the embryoid bodies, and not the undifferentiated H9 cells, contain all three germ layers which indicate that the embryoid bodies are further differentiated.

Q-PCR was also performed on a 4 week time course of EB’s where a sample was taken every 7 days (Figure 10). Similar results were obtained for the 4 week time course as were obtained from the EB’s analyzed at 5 weeks. The endodermal marker showed the highest expression indicating it may be expressed the earliest or developed first.

Q-PCR was also performed on the EB’s to determine if there was any expression of the bone differentiation gene, \textit{Runx2} (Figure 11). The expression of the Histone H4 gene was also monitored during the Q-PCR. Neither Runx2 isoform showed any expression and the Runx2 primers common to both isoforms also did not show any expression. These results appear to indicate that Runx2 is not present in EB’s indicating the gene is turned on later in the development process. The Histone H4 gene expression appeared to decrease as the EB time course progressed. This indicates that as the EB’s further differentiate, there is a decrease in proliferation.
Figure 9 Relative mRNA expression levels of 5 week EB’s compared to undifferentiated H9 hESC. Oct4, Nanog- stem cells markers; AFP, NCAM, Flk-1- endodermal, mesodermal, ectodermal layer markers (See Table 2 for primers).
Figure 10 Germ Layers Marker Expression in EB’s during the 4 week time course (samples were taken every 7 days). All 3 germ layers are present in the EB’s, although AFP, the endoderm marker is expressed at highest levels. Oct4 and Nanog are highest in the undifferentiated hESC (See Table 2 for primers).
Figure 11 Runx2 and Histone H4 expression in the EB’s and hESC. Histone H4 levels appear to decrease with the growth and development of the EB’s and be highest in the undifferentiated hESC. Runx2 did not appear to be present in the EB’s (See Table 2 for primers).

3.3 Bone Phenotype – Embryoid Body

3.3.1 Alkaline Phosphatase Stain

To determine whether EB’s could be induced to follow an osteogenic pathway and Alkaline Phosphatase stain was performed. Alkaline Phosphatase (ALP) is an osteogenic protein and therefore can be used as a marker for osteogenic differentiation. EB’s were grown on a 100mm plate for 24 days. Following this the EB’s received 24 days of osteogenic supplement with the ALP stain being performed on day 24 of the supplement. This was done to confirm that the embryoid bodies had osteogenic
properties. The EB’s stained positive for ALP indicating that EB’s can be induced to follow a mesodermal pathway.

![Image of Alkaline Phosphatase stain performed on EB’s](image)

**Figure 12 Alkaline Phosphatase stain performed on EB’s** A) A digital picture taken of an ALP stain done on a cell mass of EB’s after receiving 24 days of osteogenic supplement. The osteogenic supplement was added to day 24 EB’s, for a total of 48 day differentiation cells. The actual size of the cell mass was 1 cm. B) Other areas of the 100mm plate also stained red after the ALP stain. These cells appeared to have formed a film across the bottom of the plate, rather than a mass, as in A), however both stained positive. The actual width of the film is 4 cm.

### 3.3.2 Runx2

During the osteogenic differentiation of the EB’s samples were taken every 8 days to determine if *Runx2* was present by Q-PCR, although not enough RNA was able to be extracted from these samples for analysis.
IV. Discussion

Embryoid bodies have been shown here to be grown and propagated from undifferentiated human embryonic stem cells. In comparing embryoid bodies grown in 100mm plate to those grown in a T25 flask, those grown in a 100mm plate seemed to grow and differentiate more quickly and were easier to keep in suspension. In this project the embryoid bodies were differentiated to express mRNA for all three germ layers of the human body. Five week embryoid bodies had clear expression of the three germ layers, although AFP expression was much higher than the other marker expression. This indicates that these structures can be formed and express all germ layers and possibly be induced to follow a specific pathway.

Runx2 mRNA did not appear to be present in embryoid bodies in either isoform I or isoform II. These results were consistent for all time points tested for the embryoid bodies. This suggests that the Runx2 gene may be induced later in embryonic development.

Embryoid bodies differentiated along the osteogenic pathway appeared to show osteogenic markers. This indicates that embryoid bodies grown in vitro can be induced to follow a specific biological pathway, (in this case, the mesenchymal pathway).

Histone H4 expression appears to decrease with an increase in cellular differentiation in the embryoid bodies. These results suggest that the embryoid bodies are becoming more differentiated as time progresses.

Future experiments could further validate these results. These experiments include repeating the embryoid body time course and evaluating the histone H4, germ layer...
marker, and Runx2 expression. Also, to assess when in development the Runx2 gene is
induced another experiment could be done to osteogenically differentiate embryoid
bodies into osteoblasts and then measure the Runx2 expression via Q-PCR.
References


Sudhakar, S., Katz, M.S., Elango, N. Analysis of Type-I and Type-II RUNX2 Protein Expression in Osteoblasts. (2001). *Biochemical and Biophysical Research Communications*. 286:74-79