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Stem Cell Derived Osteoprogenitors and their Role in Bone Repair Using Morphogenetic Activators

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Abstract

Bone constantly cycles through a dynamic process of breakdown and remodeling. Osteoblasts are the specialized mesenchymal stem cells that have a major role in bone formation and the remodeling process whereas their counterpart osteoclasts, handle bone resorption. Embryonic stem cells can be partially differentiated into Progenitor cells, and we worked with #18, a candidate for being an osteoprogenitor that has the potential to respond to morphogenic activators. In the case of bone remodeling, TGF-β 2, BMP-2 and an abundance of CA++ have been shown to be potential activators of differentiation into osteoblasts. Eight different trials were conducted with the cells using different combinations of the three morphogenic activators. After inducing the cells with the activators, we performed Immunohistochemistry (IHC) to analyze the expression of osteocalcin, which is the enzyme that binds calcium to mineralize bone. The cells with varying activator combinations showed different physiology with a variance in the cell shape, structure, and spacing. The greatest results were from the combination of TGF-β 2 and BMP-2, which is consistent with #18 operating as an osteoprogenitor. A 3D construct model of #18 seemed to have a similar structure to that of an osteon, possibly indicating the formation of bone. We took slices of the model and performed an IHC staining for Osteocalcin, Prolyl Hydroxylase (5B5), and Collagen I. We saw a strong positive signal for Col I and 5B5, and a slight positive signal for Osteocalcin. This information confirmed that #18 is an osteoprogenitor and is able to assemble bone.

1 Introduction

Bone cells, like all other types of cells, originate from progenitor stem cells and can form bone by either endochondral ossification or intramembranous ossification. These two types of bone formation differ in that endochondral ossification allows bone formation by replacing a cartilage intermediate and intramembranous ossification forms bone by interacting directly with mesenchymal cells (Lopes, 1994), (Erlebacher, 1998). This research focused on these cells and their unique ability to work together to synthesize and calcify collagen into a tightly knit organic matrix that ultimately composes bone tissue. Osteoblasts work on the surface to continually synthesize new bone while their counterpart, osteoclasts, work to dissolve bone so that the osteoblasts have a platform to reabsorb and reconstruct the bone (Hu, 2016). Together, these
cells maintain and balance the process of bone formation. These osteoblasts are specialized mesenchymal stem cells that are responsible for bone formation and the remodeling process, which can be seen as osteoprogenitors. Stem cells are body’s raw materials (Gallagher 2010) (King 2012). Under the right circumstances, stem cells can divide to form more cells, whether it be in the body or in a lab. In many tissues, they serve as an internal repair system that can divide without limit. When these cells divide they have the potential to remain a stem cell or become a specialized cell, such as a muscle cell, brain cell, or mesenchymal cell. Mesenchymal stem cells that are responsible for bone formation and remodelling, differentiation is sensitive to the timing of growth factor presentation and bone formation. Bone repair and remodeling are regulated through various expressions of vascular endothelial growth factor (VEGF). VEGF is a signaling protein that promotes the growth of new blood vessels. These expressions of VEGF are regulated by various growth factors such as a BMP2 and TGF-B2 (Deckers, 2000), (Wang 2007). Discovery of a technique for remodeling bone tissue would require customizability and variance as recent trials for cell therapies have proven to range on a case by case basis. Developing a drug administration for such medication would open a new field to the pharmaceutical industry and establish a solution toward the 6 million yearly cases of individual suffering from bone degenerative symptoms (Bruder 1994). There are many ways in which human stem cells can be used in research and the clinic. The primary goal of this research is to identify how undifferentiated stem cells become the differentiated cells that eventually form tissues, organs, or bone (Newman, 2016).

Differentiation is the process of manipulating unspecialized cells so that they create specialized cells. Human embryonic stem cells were the unspecialized cells that were manipulated to form osteoblasts. Within the realm of stem cells there are two main types: human embryonic stem cells (hESCs), and adult stem cells (somatic stem cells). Human embryonic stem cells and adult stem cells differ in that hESCs are pluripotent, meaning they can differentiate into all cell types of the body whereas adult stem cells are not and can only differentiate into cell types of their tissue of origin. Because a large number of cells were needed for potential differentiation into osteoblasts, hESCs were utilized because they are easier to differentiate than adult stem cells (Pittenger 1999). When first working with these hESCs, the cells would clump together and differentiate amongst themselves into unwanted muscle and nerve cells. In order to avoid this problem and allow the opportunity to differentiate the cells into osteoblasts, the chemical composition of the culture medium was altered and growth factors were added.

The focus of this research was to discover whether or not Progenitor #18 cells could be differentiated into osteoblast cells. Differentiating these cells would greatly increase the probability of calcium phosphate crystallization, a positive result when attempting to view osteoblast differentiation. With this advance, it could be proven that Progenitor #18 cells have bone forming characteristics. By identifying a pattern between the development and efficiency of osteoblasts and osteoclasts, and a successful combination of morphological activators, it could provide the most affinity toward this process. TGF-β 2, Bone Morphogenic Protein (BMP-2), and Ca++ were the morphogenic activators used and were combined to see what combination of
these created the best process towards osteoblast differentiation. Furthermore, this research explores the differences in adaptability and productivity between bone regeneration/remodeling and bone repair. This was an important comparison because unlike bone formation, bone repair utilizes already existing cells in an attempt to restore bone function and structure without regenerating any new cells or increase bone volume. Bone remodeling depends on the spatial and temporal coupling of bone formation by osteoblasts and bone resorption by osteoclasts; however, the molecular basis of these inductive interactions is unknown (Newman, 2016).

The combination of various plates of stem cell derived osteoprogenitors and morphogenic activator counterparts has the potential to be successful in the formation and repair of bone. This type of research, if continued, could promote new insights regarding the formation of bone. Two of the morphogenic activators used, TGF-β 2 and BMP, have already been reported to play significant roles in bone formation during mammalian development (Information on Stem Cell, 2016). Mesenchymal stem cells, whether isolated from an embryo or an adult, have been reported to provide a basis for therapeutic remedies regarding the self-repair of cells (Stem Cell Basics, 2008). Diseases such as diabetes, spinal cord injuries, hearing and vision loss, and heart diseases could be potentially treated if research regarding the differentiation of embryonic stem cells was pursued. A potential problem that this type of research will face involves the rejection of the transplanted embryonic stem cells. Genes have to be turned on or off in order to display specific results and when working with stem cell derived osteoprogenitors there is a risk that the specific genes will not properly turn on or off. Creating a course of treatment that revolves around the differentiation of stem cell derived osteoprogenitors into specific cell types could be a revolutionary step for bone repair (Information on Stem Cells, 2016).

2 Methods and Materials

2.1 Cell Culture

Human embryonic stem cell line Progenitor #18, was received from BioTime. The cells were plated on p-60 culture dishes and grown in DMEM 10% FBS medium. An 8-well slide was set up in order to split the cells from the p-60 plate. The cells were rinsed with 5mL total of PBS and removed from the dish by trypsinizing. After centrifuging the cells, they were evenly split into the 8-well slide. Upon confluency the cells were induced with a different combination of the three morphogenic activators: Ca++, BMP 2, and TGF-β 2. The wells were labelled as followed: Well 1 as the control; Well 2 induced with Ca++; Well 3 induced with Ca++ and TGF-β 2; Well 4 induced with Ca++, TGF-β 2, and BMP 2; Well 5 induced with Ca++ and BMP 2; Well 6 induced with only TGF-β 2; Well 7 induced with only BMP 2; and lastly, Well 8 induced with TGF-β 2 and BMP 2. After four days of incubation at 37°C, images were taken of their morphology. Once the images were taken the cells were placed back in the incubator at 37°C.

2.2 Cell culture immunohistochemistry
Seven days after the addition of morphogenic activators to the 8-well slide of line #18, immunohistochemistry (IHC) was done on all eight wells. Each well was separated and marked off using a pap-pen. Cells were fixed to the slide with an Acetone-Methanol solution for 10 minutes at room temperature. The cells were stained for 50 minutes with a 1:200 dilution of primary antibodies, either Osteocalcin or Prolyl Hydroxylase. The secondary antibody used was a 1:200 dilution of goat anti-mouse IgG 594 Red. The wells were examined with a conventional fluorescence microscope (Axiovert 40 CFL) and images were taken using a Nikon D600.

2.3 3D construct immunohistochemistry
After observing the cell culture, a 3D construct, created by #18, was observed and put under IHC. The 3D model was observed under a microscope and images were taken. Slices were taken from the model using a cryostat. Three different slices were put on glass slides in order to observe under a microscope. The slides were labelled 1-3. Each slide was stained for 50 minutes with a primary antibody. Slide 1 was stained with Osteocalcin, slide 2 was stained with Collagen I, and slide 3 was stained with Prolyl Hydroxylase. The secondary antibody used was goat anti-mouse IgG 488 Green. The slides were examined under a fluorescence microscope (Leica DMI 4000B) and images were taken.

3. Results

3.1 Activator induced morphological changes
Under light microscope analysis, culture of line 18 in the presence of morphological activators resulted in clear morphological changes. The control line did not show any signs of morphology change. Cells induced with BMP-2 began to spread out and become confluent, while cells induced with TGF-β 2 began to cluster more after induction. The cells treated with a combination of BMP-2 and TGF-β 2 presented the greatest morphological change. Its physiology had the most unique development when comparing the wells.

![Figure 1: Changing morphology in response to morphogenic activators in #18 (40X)](image)
P60 plate of #18 induced with different combinations of BMP-2 and TGFβ-2. (A) #18 not treated with any Morphogenic Activators (the control). (B) #18 treated with BMP-2. (C) #18 treated with TGFβ-2. (D) Progenitor cell line treated with a combination of BMP-2 and TGFβ-2.

3.2 Expression of Osteocalcin under separate treatment of BMP-2 and TGF-β 2
After culturing line 18 for seven days, immunohistochemistry was done in order to characterize the expression of Osteocalcin. Although this line did not show major morphological changes upon the separate addition of BMP-2 (Figure 2) and TGF-β 2 (Figure 3) it is possible to see an increase in the expression of Osteocalcin. Quantification of osteocalcin expression confirmed the

![Figure 2: Progenitor cells treated with BMP-2 express Osteocalcin I (40X)](image)
P60 plate of #18 stained for Osteocalcin (A) DAPI stain shows nuclei and where living cells reside. (B) IHC stain shows that BMP-2 induced cells express Osteocalcin. (C) Merge of results from (A) and (B).

increase in expression of bone specific proteins.

3.3 Expression of Osteocalcin under combined treatment of BMP-2 and TGF-β 2
Once again immunohistochemistry was performed to characterize the expression of Osteocalcin. The combined addition of BMP-2 and TGF-β 2 presented the greatest morphological change (Figure 4). It also appeared to show the greatest increase in the expression of Osteocalcin. It is

![Figure 3: Progenitor cells treated with TGF-2 express Osteocalcin I (40X)](image)
P60 plate of #18 stained for Osteocalcin (A) DAPI stain shows nuclei and where living cells reside. (B) IHC stain shows that TGF-2 induced cells express Osteocalcin. (C) Merge of results from (A) and (B).
possible to see that the addition of both activators combined will give the best expression in a bone specific protein, which is produced by osteoblasts.

3.4 Expression of Collagen I on a 3D osteon model
After taking slices of the 3D model, immunohistochemistry was performed to characterize the expression of Collagen I. The IHC stain presents a great expression of Collagen I (Figure 5). It shows that Collagen I does not completely overlap the nuclei. This is because Collagen I is secreted as an extracellular protein, and not internal to the cells. It would not be expected that this protein completely overlaps the nuclei. The positive expression shows that collagen is made in the vicinity of the cells.

3.5 Expression of Prolyl Hydroxylase on a 3D osteon model
An immunohistochemistry was performed to characterize the expression of Prolyl Hydroxylase on the 3D model (Figure 6). The stain expressed the presence of Prolyl Hydroxylase located near the expression of collagen. The location of this expression is relevant because Prolyl Hydroxylase is a processing enzyme for collagen. The expression of this enzyme appears to
overlap between the cells themselves and the collagen because it is secreted by cells in order to create collagen.

3.6 Expression of Osteocalcin on a 3D osteon model
An immunohistochemistry was performed to characterize the expression of Osteocalcin on the 3D model. The stain showed a positive expression for Osteocalcin (Figure 7). Although this stain does not express the greatest presence, it is the most important for implication of bone formation. Osteocalcin, secreted solely osteoblasts, acts to mineralize bone. The expression of this protein combined with Collagen 1, implicates that osteoblasts are forming along with bone mineralization.
**Diagram 1**: Depiction of cell types that make up a single osteon.

**Diagram 2**: Depiction of a single 3-D osteon.

4. Discussion

One approach to advancing the field of regenerative medicine is to manipulate progenitor cells derived from stem cells instead of working with stem cells independently. Progenitor cells were propagated in vitro in a skeletal medium and were treated with a combination of factors to see if they could be further triggered to function as osteoblasts. For example, a calcifying matrix was secreted and mediated by osteocalcin as used in bone repair and remodeling. The results were helpful in gauging whether there were changes in cell morphology and if cells were able to form larger cell masses with a higher order structure. With each set of experiments, markers were set up to signal for a presence of osteoblasts and the morphogenetic activators, BMP-2, TGF-B2, and CA++ were used in order to see the effects they had on progenitor cell line, #18.

The timing of introduction regarding morphogenic activators could potentially affect the success rate and when introduced immediately after the cells were split into the wells, it proved to be successful. In Figure 1, the gross morphology of the cell cultures were affected by the introduction of the morphogenetic activators. Plate D showed that the cells had completely coalesced whereas in plates A, B, and C, there was less cell adhesion. This consolidation was due to the introduction of BMP2 and TGF-B2 separately which, in figures 2 and 3, triggered some expression of osteocalcin. The best result, however, came from when we combined BMP2 and TGF-B2 which is shown in figure 4. The combination of the morphogenic activators created unique spacing and structure while also giving the clearest results of fluorescence. This clear result allowed us to see a ball of clustered nuclei and a cell body stained for osteocalcin. Results showed a positive stain for osteocalcin which proves that Progenitor line #18 is a type of osteoprogenitor cell. This result is significant because the cells were derived originally from human embryonic stem cells and by adding BMP2 and TGF-B 2 there was a differentiation to create osteoblasts.

When using hESC for regenerative medicine, differentiation is required so that usable cell types can be created to reconstruct organs. In vitro, the 3D model was employed to see if #18 could also form some aspect of bone morphology. Figure 5 showed a positive stain for collagen I expression in a spongy arrangement that is consistent with bone morphology. Figure 6
confirms that the positive collagen signal is produced by #18 because the staining of propyl hydroxylase overlaps with the presence of the cells and collagen. Figure 7 showed a positive expression for Osteocalcin which is important in the research of bone formation because Osteocalcin acts to mineralize bone. The expression of this protein combined with Collagen 1, implicates that osteoblasts are forming along with bone mineralization.

While there are clearly more than just these factors at play, these experiments may further the understanding of what exact conditions are needed for the regeneration of bone tissue. Different areas of the medical field have already been using drug administration techniques with both TGF-B2 and BMP to treat osteoporosis, which only further supports that the two are important cofactors. Whether or not osteoclasts are developed along with the osteoblasts is unknown and further study must be conducted in order to know for sure as osteoclasts develop from a fusion of osteoblasts with macrophages and monocytes. The exact molecular formula for what stimulates bone remodeling and repair is currently unknown (Erlebacher 1998). What is known, however, is that TGF-B and BMP have been closely associated with bone remodeling and repair. This research paper supports the conclusion that stem cell derived osteoprogenitors and their morphogenic activator counterparts play a critical role in bone repair and remodeling.

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6. References


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