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Osteogenesis from Pluripotent Stem Cells: Neural Crest or Mesodermal Origin?

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1. Introduction

Research in stem cell biology has the potential to dramatically alter the way we understand the vast complexity and coordination that is required for an organism to develop and function. The creation of therapeutic tools that will inevitably accompany these discoveries in this field of research may completely revolutionize our approach to medicine in the 21st century.

In this chapter we will examine one facet of stem cell research that holds great potential to improve the quality of life for millions of individuals; the study of osteogenesis from pluripotent stem cells. Despite its overt rigid structure, which provides mechanical support and protective functions, bone is a highly dynamic tissue that is tightly regulated to serve multiple roles in the body. Bone tissue is constantly being remodeled by the actions of the osteoblasts, the bone forming cells, and the osteoclasts, the bone resorbing cells. The improper balance of these cells can result in a number of bone-related and osteodegenerative diseases. Osteoporosis, for example, is estimated to effect 75 million individuals in Europe, Japan and the US alone, and thus the potential benefits of understanding the processes regulating osteogenesis may be quite far reaching.

Despite the similarity of the bone tissues found in the adult mammalian skeleton, there are three different sources from which bone is derived in the developing embryo (Fig. 1). Two of these bone origins are from mesodermal progenitors, where cells from either the lateral plate or paraxial mesoderm contribute to the appendicular or axial skeleton, respectively. The third origin of bone tissue can be traced back to ectodermal cells where neural crest progenitors differentiate into many of the bones within the craniofacial region. Differences in the origin in bone are also paralleled in differences seen in the bone formation process. Most bones of mesodermal origin develop via the process of endochondral bone formation, whereas the bones of ectodermal origin form by a process called intramembranous bone formation. These processes differ most generally in the series of cell differentiations that lead to the mature tissue. In endochondral bone formation the mesenchymal progenitors differentiate into chondrocytes, which lay down the cartilaginous framework that is eventually replaced by the mineralized matrix of invading osteoblasts, while the chondrocytes undergo apoptosis. In intramembranous bone formation, the progenitors differentiate directly into osteoblasts. In addition, mature bone tissues house adult stem cell niches, such as those composed of mesenchymal or hematopoietic stem cells. These cells are the source for diverse cell types throughout the life of the organism and are critical for normal maintenance and overall physiology.

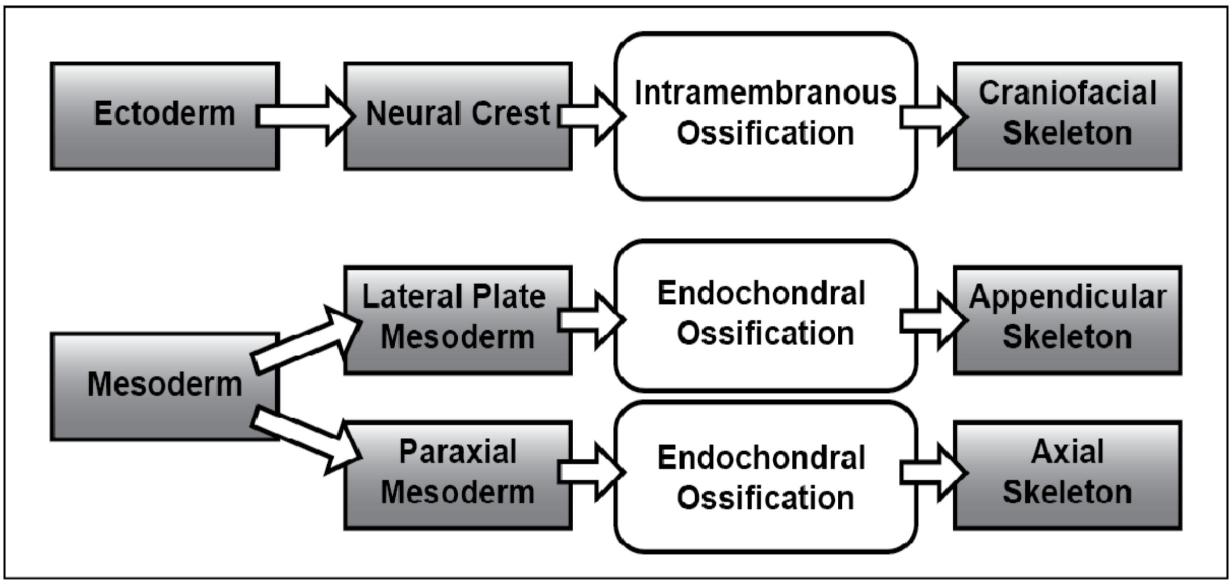

Fig. 1. Embryonic origins of bone tissue

While it is widely accepted that pluripotent stem cells have the capability to give rise to osteoblasts, it has only recently been examined whether they do so through a mesodermal route or through progenitors with neural crest characteristics. This chapter will provide a review of the current understanding of the different progenitors that contribute to the aforementioned bone formation processes and regulatory networks known to play critical roles in these cells. It will further examine the experimental manipulations in stem cell culture systems that have allowed us to derive neural crest and mesodermal type osteoprogenitors *in vitro*. However, it remains elusive whether a neural crest type progenitor and a mesodermal progenitor will have the same capacity to repair bone when transplanted or whether one will be superior to the other in a certain transplantation site. In order to systematically assess the influence of the type of progenitor and the transplantation site as well as the process of bone formation that is typically used as repair mechanism in a particular transplantation site, this chapter therefore also summarizes bone tissue engineering studies that have been undertaken using these diverse progenitors and that will bring us closer to eventual clinical applications that this exciting field of research will provide.

2. Pluripotent stem cells to bone

Both *in vitro* and *in vivo* studies continue to elucidate the developmental program that pluripotent stem cells take to their eventual differentiated states. One such program is the development of bone tissue; and research in this field has already made a positive impact on the lives of individuals in various clinical trials (Giordano et al., 2007). However, before these applications become commonplace in the medical field, further study is required to improve both our understanding and methodologies. This chapter seeks to give a broad overview of a diverse range of topics, from differentiation of pluripotent stem cells along osteogenic lineages, some current approaches in applying stem cell based bone engineering for potential clinical applications, and concluding with a discussion of different bone origins and their respective developmental pathways.

2.1 Embryonic stem cells

Pluripotent stem cells can be distinguished from adult stem cells based on their nature of origin, but first and foremost based on their more versatile differentiation capability. This unsurpassed differentiation capability is known as pluripotency, the potential to generate cell types from the three embryonic germ layers: the mesoderm, the ectoderm and the endoderm. One class of pluripotent stem cells, the embryonic stem cells (ESCs) have been under fervent ethical debate since their initial derivation. The crux of this debate can be attributed to their source being a cluster of cells found in the blastocyst, an early pre-implantation embryonic stage. This cluster of cells, the inner cell mass (ICM), is established directly after the developing embryo has gone through the first fate decision, in which the trophoectoderm secedes from the ICM. While this outer trophoectodermal layer of the blastocyst eventually gives rise to the placenta, the *in vivo* fate of the ICM is to develop into the embryo proper, which contains cell types of the three germ layers. Mirroring this capability of the ICM, isolated ESCs also have the capacity to give rise to cell types of all three germ layers when differentiated *in vitro* (Itskovitz-Eldor et al., 2000).

ESCs were first derived from mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981) and since then their derivation has been reported from a number of mammals including: hamster, rabbit and rat (Doetschman et al., 1988; Giles et al., 1993; Iannaccone et al., 1994). Although these alternative rodent ESC lines have never gained recognition as model systems, their utilization continues to provide insights into stem cell biology. As for primate ESC derivation, the initial challenges that plagued the field for years were finally overcome by Thomson et al. (1995), and this study laid the ground for the establishment of human ESCs by the same team just shortly before the turn of the century (Thomson et al., 1998).

In addition to the pluripotent nature, it is their second characteristic of being capable of unlimited proliferation that ESCs first became an attractive cell source for regenerative therapies. This propagation in the undifferentiated state can be supported in culture with the addition of leukemia inhibitory factor (LIF) (Williams et al., 1988). Since LIF is inefficient in maintaining the undifferentiated state in human ESCs, the molecular cues needed maybe released by murine embryonic fibroblast feeder layers, which both human ESCs and murine ESCs can be grown on (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). In feeder-independent conditions, basic fibroblast growth factor (bFGF) is able to maintain the pluripotent state of human ESCs (Amit et al., 2003).

2.2 Differentiation of ESCs into bone cells

Historically the developmental program that pluripotent stem cells take to form bone tissue was first elucidated using murine ESCs. Buttery and coworkers were the first to show that mESCs maintained in medium supplemented with beta-glycerophosphate and ascorbic acid had mineralized in culture, a hallmark feature of bone tissue formation (Buttery et al., 2001). In the past decade, numerous protocols have then been established that allow ESC differentiation into bone and cartilage and their characteristic cell types, the osteoblasts and osteoclasts as well as the chondocytes. The studies that describe formation of osteoblasts typically all assess the ability of the cells to secrete an organic matrix composed of collagen type I (COL I) and proteoglycans, the deposition of inorganic hydroxyapatite and the expression of osteoblast-specific genes (Davis et al., 2011; Handschel et al., 2008; Shimko et al., 2004).

One difference between these protocols however, is the choice of additional osteogenic inducers. While beta-glycerophosphate and ascorbic acid are absolutely necessary for the cells to calcify, the additional supplementation of either dexamethasone, retinoic acid or 1,25alpha $(OH)_2$ vitamin D_3 (VD_3) each can significantly increase the amount of bone nodules and expression of osteogenic markers in both mouse and human ESC cultures (Buttery et al., 2001; Phillips et al., 2001; Sottile et al., 2003; zur Nieden et al., 2003).

Similar to endochondral bone formation in the embryo, osteogenesis from ESCs *in vitro* can be direct or the future bone can at first undergo a chondrocyte phase. Both processes have been described for ESCs. For example, during ESC *in vitro* intramembranous ossification, osteoblasts would be specified through a mesenchymal precursor and then directly into the osteoblastic fate. In this case, markers for hypertrophic chondrocytes should be absent or should only be minimally expressed. In turn, ESC differentiation would model embryonal endochondral ossification when ESCs would first differentiate into chondrocytes, then undergo hypertrophy and give way to osteoprogenitors that calcify. Hegert and colleagues, supported by data from our group, have shown that chondrogenic ESC cultures indeed can be manipulated to calcify, whereby such ossification results in a lower calcium content of the matrix then the direct (non chondrocyte-mediated) differentiation (Hegert et al., 2002; zur Nieden et al., 2005). This direct chondrocytic differentiation is mediated by growth factors of the transforming growth factor family, including bone morphogenic proteins and TGFβ1 (Kramer et al., 2000; Hegert et al., 2002; zur Nieden et al., 2005; Toh et al., 2007). Under such treatment the cartilage-specific transcription factors Sox9 and scleraxis are up-regulated at early stages of differentiation (Kramer et al., 2002, 2005; zur Nieden et al., 2005). The addition of BMP also increased the formation of cartilaginous matrix, comprised of collagen, proteoglycans and ECM proteins and expression of collagen mRNAs found in cartilage, such as collagen type II and collagen type X, the latter being indicative of chondrocytes undergoing hypertrophy (Kielty et al., 1985). ESC cultures containing such hypertrophic chondrocytes also initiate expression of osteoblast-specific mRNAs. This overlap of the chondrocyte-specific and the osteoblast- specific differentiation program suggest that ESCs may be undergoing the endochondral bone formation process.

In addition to growth factors and chemicals that direct differentiation through the endochondral or intramembranous route, different physical means have also been utilized to induce ESC differentiation into bone. While murine ESCs are typically grown into small (i.e. approximately 300-400 μm) agglomerates of differentiating cells called embryoid bodies (EBs) (Trettner et al., 2011), as the first stage of differentiation, human ESCs can alternatively be induced to differentiate by overgrowing colonies on a plate (Karp et al., 2006). Further osteogenic differentiation can be observed when intact EBs or dissociated EB cells are cultured in the presence of osteogenic supplements (Buttery et al., 2001; Cao et al., 2005; Chaudhry et al., 2004; zur Nieden et al., 2003). Woll and coworkers trypsinized mouse EBs into single cell suspensions and plated those at very low clonal density (Woll et al., 2006). They reported that approximately 60-80% of single-cell derived colony formation exhibited matrix mineralization as determined by von Kossa staining. Further qPCR analysis of osteoblast markers supported the potential of these cells to undergo osteogenesis, although there was heterogeneity between colonies in expression of these specific markers. Despite this heterogeneity between these individual colonies, the clonal expansion from a single cell offers an easy approach to dissect the differentiation pathway leading to bone cell formation.

This seems to be of particular importance as ESCs can be lead to differentiate from pluripotency into mesenchyme and subsequently bone, whereby mesenchyme may be specified either from a mesodermal or neural crest derived origin. More recent studies have indeed reported the generation of mesenchymal stem cell like cells from ESCs as well as the isolation of progenitors with osteogenic properties that were mesoderm or neural crest derived (Aihara et al., 2010; Olivier et al., 2006; Sakurai et al., 2006; Trivedi and Hematti, 2007).

A few days into the differentiation, ESCs will express T-Brachyury, a gene that is typically transcribed in the primitive streak when the early embryo undergoes gastrulation to establish the three germ layers (Beddington et al., 1992). The primitive streak contains cells with mesendodermal character, a subpopulation of cells that can later become osteoblasts. T-Brachyury expression is often used to characterize the output of differentiating mesendoderm (Gadue et al., 2006; Nakanishi et al., 2009) and is thus also informative to the very early differentiation events of osteogenesis. Similarly, modeled after the early lineage decisions *in vivo*, activin and nodal induction may be used to enhance the percentage of mesendodermal cells positive for Goosecoid (Gsc), E-cadherin, and platelet-derived growth factor receptor alpha (PDGFRα) (Tada et al., 2005), which are a combination of markers expressed by organizer cells in the primitive streak region. During subsequent development, this triple-positive cell population diverges to Gsc+ and either E-cadherin or PDGFRα positive intermediates that later differentiate into definitive endoderm and mesodermal lineages, including calcified osteopontin expressing osteoblasts (Tada et al., 2005).

While the mesendodermal progenitors are being established in the process of gastrulation *in vivo*, neurulation has already initiated in the anterior part of the embryo. Therefore, specification of neural crest populations may occur *in vitro* during ESC differentiation at around the same time or slightly after T-Brachyury+ or Gsc+ populations are found. In the embryo, neural crest cells emerge from the dorsal epithelium of the neural tube after it has formed, undergo epithelial-to-mesenchymal transitions and become highly migratory. These cells later disperse to and incorporate within skin tissue (i.e. melanocytes) as well as neurons and glia in the peripheral nervous system (Chung et al., 2009; Dupin et al., 2007; Weston, 1991). Due to the multitude of cell types that arise from the highly specific population of neural crest cells, it is sometimes regarded as the fourth germ layer. In addition, the neural crest is generally considered to be the source of a population of cells deemed the ectomesenchyme, which produces a variety of mesenchymal tissues including craniofacial cartilage and bone (Morrisskay et al., 1993; Smith and Hall, 1990). More recently however, the view that mesenchymal cell types are established from progenitor populations of neural crest origin was challenged by Weston and colleagues, who suggest that neural crest and ectomesenchyme are developmentally distinct progenitor populations, possibly distinguishable by the expression of E-cadherin and PDGFRα (Weston et al., 2004).

While it seems widely established that ESCs have the capacity to differentiate into osteoblasts from these various origins, other questions related to the feasibility of their clinical use are still under investigation. As pluripotent cells, ESCs are particularly attractive for the treatment of critical size bone defects that require large numbers of cells as an illimitable source of progenitors, be it mesoderm or neural crest derived MSCs or even more committed osteoprogenitors. More recently, a new less ethically controversial source of pluripotent cells has been discovered in the artificial creation of induced pluripotent stem cells (iPSCs). In this method mature, fully differentiated cells are reprogrammed to a pluripotent state. Explicitly, pluripotency-associated genes are shuttled into somatic cells, e.g. fibroblasts or keratinocytes (Aasen et al., 2008; Okita et al., 2007; Takahashi and Yamanaka, 2006), and brought to expression before they are silenced, which is just enough to turn the differentiated cells into ESC-like cells with a pluripotent pheno- and genotype. Only five years after their discovery, iPSCs have been recently exploited to study osteogenesis and have already been shown to possess comparable differentiation capacity (Bilousova et al., 2011).

3. Bone tissue engineering

The current gold standard for bone tissue replacement is the autologous graft, which utilizes bone tissue that has been extracted from another site within the patient's own body. However, there is only a limited amount of bone tissue that can feasibly be harvested without inducing considerable donor site morbidity (Rose and Oreffo, 2002). On the other hand, surgical procedures using an allograft, where the bone is harvested from a cadaver, can provide enough material to correct large-scale bone defects. However, this approach carries its own disadvantages including potential immunorejection and pathogen transmission. Techniques involving synthetic materials such as metals and ceramics are continually being used and explored as alternatives to these approaches, but these substitutes continually fall short of bone grafts in areas such as host site integration and tensile strength (Rezwan et al., 2006; Yaszemski et al., 1996). Thus, the attractive features and potential versatility of stem cells offers the investigator an exciting source to improve and develop new technologies that may significantly enhance the efficacy of these procedures.

Currently a popular approach in applying stem cells to *de novo* bone synthesis is the *in vitro* culturing or 'seeding' of cells onto scaffolding materials that can be used for subsequent implantation. In order for this approach to be successful there are a number of essential properties that a researcher must keep in mind when designing the appropriate scaffolding material. These properties will have a direct effect on both the colonization of the scaffold and its successful incorporation into host bone tissue. To achieve an optimal scaffold design a number of considerations such as biocompatibility, porosity, pore size, osteoinductivity and conductivity (including biomolecule incorporation), biodegradability, and mechanical properties must be accounted for (Salgado et al., 2004). Thus, reaching this goal will be a challenge that requires the coordinated efforts of researches across the diverse disciplines of material and biological sciences.

3.1 Mesenchymal stem cells in bone tissue engineering

Beyond the type of scaffold used in a particular study, the choice of seeded cell type will also play a critical role in the creation of *de novo* bone tissue. Starting with the most differentiated cell type, seeding a scaffold with harvested autologous osteoblasts superficially seems attractive because of their inherent cellular program to develop new bone. However, using this cell type is problematic because of low initial concentrations following harvest and relatively poor proliferation capacity *in vitro*. Also, if these treatments are designed to not only amend bone defects, but also to alleviate bone disorders, it is unlikely that harvested osteoblasts will have the suitable characteristics to be effective. Another possible cell type is the multipotent adult mesenchymal stem cell.

Mesenchymal stem cells (MSCs) are unspecialized adult stem cells that reside in mature somatic tissues, predominantly the bone marrow in the long bones. There they share the niche with hematopoietic stem cells, but differ from them in the array of specialized daughter cells that they can generate. MSCs were first described forty five years ago by

Friedenstein and colleagues, when they first found this heterogeneity in differentiation capacity between cells isolated from bone marrow. While they described the cells as ossific progenitor cells of stromal origin in rats in this first study, subsequent studies proved the multilineage differentiation potential of these cells into fibroblasts, chondrocytes and other cells of connective tissue coining the term mesenchymal stem cell (Friedenstein et al., 1966, 1976, 1987; Tondreau et al., 2004a, b; Johnstone et al., 1998; Young et al., 1998; Niemeyer et al., 2004).

Despite the fact that the scientific community has long exploited MSCs to understand the processes of osteogenic and chondrogenic differentiation as well as for the study of adult stem cell maintenance (Bruder et al., 1990; Gazit et al., 1993; Grayson et al., 2006; Hong and Yaffe, 2006), the isolation of the non-hematopoietic mesenchymal stem cell from bone marrow or other tissue sources remains complex. Initially, Friedenstein isolated the MSCs by their tight adherence to plastic (Friedenstein et al. 1976). Yet, newer studies suggest that by isolating MSCs based on their plastic adherence, a portion of mesenchymal stem cells are lost (Zhang et al. 2009). Unfortunately, the fibroblast-like MSCs show a variable profile of surface marker expression (Simmons und Torok-Storb, 1991; Jiang et al. 2002; Vogel et al. 2003), which makes it difficult to isolate them based on a specific marker set. A few years ago, a group of investigators with extensive track records in MSC research has agreed on specific characteristics that need to be met by a cell in order to be called an MSC (Dominici et al., 2006, the International Society for Cellular Therapy position statement). For example, CD14, CD34 or CD45 mark hematopoietic cells and are therefore considered negative markers for MSCs. The most commonly used markers for the detection and purification of MSCs are CD90 (Thy-1 cell surface antigen), CD105 (endoglin) and CD73 (ecto-5'-nucleotidase) (Pittenger et al., 1999; Dominici et al., 2006). Both CD105 and CD73 are constitutively expressed by MSCs, however are also expressed by endothelial cells (Gougos und Letarte, 1988; Airas et al., 1995). Therefore, a combinatorial approach using CD106 (vascular cell adhesion molecule 1) is also recommended in the literature to identify MSCs, as CD106 is only expressed on the MSC surface, but not on endothelial cells (Pittenger et al., 1999; Osborn et al., 1989). Stro1 (Stenderup et al., 2001), glycophorin A (Pittenger et al., 1999; Reyes et al., 2001; Jones et al., 2006), D7-fib (Jones et al., 2002) and p75 (Quirici et al., 2002) have also been associated with MSCs recently, but are not contained in the International Society for Cellular Therapy position statement.

Currently, the use of bone marrow derived mesenchymal stem cells (BDMSCs) to study bone tissue generation is popular because these can be harvested from the patient's own body, thereby removing concerns of immunorejection and disease transmission. Because the transition of BDMSC studies to clinical applications is currently more direct, and not enveloped in ethical considerations, there have been many studies looking at the differentiation capacity of BDMSCs *in vivo* (Arinzeh et al., 2003; Bruder et al., 1998; Gao et al., 2001; Kotobuki et al., 2008). BDMSCs are already used in preclinical trials for treatment of osteogenesis imperfecta and non-union bone fractures (Le Blanc et al., 2005; Tuch, 2006).

However, this does not exclude the necessity to examine ESCs as a potential source of bone engineered cells. In fact, improvements in the techniques of somatic nuclear transfer (Byrne et al., 2007) and creating iPSCs (Nakagawa et al., 2008; Yu et al., 2007), make it quite plausible that the protocols derived from the study of ESCs may someday become more applicable to the future of regenerative medicine than their adult stem cell counterparts. In addition, there are drawbacks from using BDMSCs, including the limited number that can be obtained, more restricted proliferation and differentiation capacities when compared to

ESCs, and they may also harbor undesirable characteristics when harvested from unhealthy bone. So although the use of MSCs has progressed further in clinical applications of bone tissue engineering, the examination of ESCs as a potential source for repairing bone defects and disorders still merits a great deal of attention.

3.2 Embryonic stem cells for bone tissue engineering

Since Levenberg and colleagues (2003) demonstrated the potential to create complex tissue structures on 3D scaffolds using differentiating human ESCs, a number of investigations sought to refine and optimize the conditions required to engineer specific tissue types within 3D scaffolds. In 2004, Chaudhry and colleagues (2004) were the first to demonstrate the feasibility of inducing mineralization of murine ESC derived cells within 3D poly L-lactic acid (PLLA) scaffolds. To accomplish this goal the team initially differentiated murine ESCs into osteoblast progenitor cells in 2D culture. EBs were initially formed, which were then subsequently transferred into suspension dishes for 3 days in the presence of retinoic acid, and then were grown in the presence of β-glycerophosphate and ascorbic acid. EBs were trypsinized and seeded onto PLLA scaffolds. After four weeks of subsequent culture in osteogenic media, the scaffolds showed extensive bone nodule formation on the surface of the scaffold and evidence of cell invasion/mineralization with the interior, as demonstrated by electron microscopy and von Kossa staining. Molecular characterization of the cells that had colonized the scaffold also revealed expression of the osteoblast specific markers osteocalcin, osteopontin and alkaline phosphatase (Alk Phos).

When discussing synthetic scaffolds for tissue engineering it is important to realize that not only the composition of the material itself is important, but that the nano-scale architecture can also play a critical role in the successful colonization of the material. Smith and colleagues (2009) developed a fabrication method of producing a nanofibrous PLLA scaffold in an attempt to mimic a collagen matrix. These were compared to traditional 'solid-walled' PLLA scaffolds in both 2D and 3D osteogenic culture systems. It was found that the 3D nanofibrous matrices expedited differentiation of mouse ESCs as revealed by markers runx2, an osteoblast-specific transcription factor (5 times greater), bone sialoprotein (8.5 times greater) and osteocalcin (2.9 times greater). These scaffolds were also found to contain greater amounts of COL I (5.5 times) and calcium (3 times) when cultured for 28 days. Another point of interest from this study showed that the nanofibrous scaffold, unlike all the other materials tested, was also able to support osteogenesis without the addition of osteogenic supplements. Although, the osteogenic output was not as robust as when cultured with media supplemented with ascorbic acid, β-glycerophosphate, and dexamethasone. Thus, it appears that the nano-scaled architecture of these scaffolds mimics the endogenous ECM.

These differences in geometry presumably create a more appropriate spatial context to facilitate cell-cell interactions and communication for bone tissue development. In addition, it was previously found that this nanofibrous scaffold absorbed four times the amount of serum proteins than their traditional solid walled counterparts (Woo et al., 2003). Thus, the ability of this nano-scaled architecture to both improve the spatial arrangement of cells and to absorb more growth factors, demonstrates how attention to microscopic manufacturing of materials can greatly enhance the potential and success of these scaffolds.

The availability of a blood supply, especially to large bone grafts, is critical for engineered tissue transplant efficacy. The creation of a flap for transplantation is one surgical approach to address this issue. A flap is tissue that already has a vasculature system in place to support nutrient and gas exchange. Although not explored in ESC-derived grafts, studies performed with BDMSCs demonstrate the feasibility of this approach. Warnke and colleagues performed an interesting clinical demonstration of this technique in 2004. Here, a seeded scaffold intended to repair a large resection of the patient's mandible was first implanted within the patient's latissimus dorsi muscle. This *in vivo* incubation period allowed time for the graft, a titanium mesh cage filled with hydroxyapatite blocks coated with recombinant human BMP7, to develop vasculature. The graft was initially seeded with solution containing autologous bone and natural bovine bone-mineral extract. After seven weeks the implant was removed along with the muscle tissue containing the thoracodorsal artery and vein, which had provided the circulation to the implant, and was transplanted into the patient's jaw. Bone mineral density was measured using non-invasive 3D chromatography and revealed continuous improvement for the duration of 38 weeks (Warnke et al., 2004). Due to ethical considerations, a biopsy of the implant was not undertaken. However, mineralized scar tissue in areas of implant overgrowth was histologically examined and showed young cancellous bone formation containing viable osteoblasts and osteocytes. The patient's continual smoking and alcohol abuse compromised the initial favorable prognosis of the treatment, and unfortunately the patient had passed away 15 months following the operation (Warnke et al., 2006). Also due to the nature of the procedure, which precluded the use of control implants, statistical analyses were not performed. However this study provides at least an initial demonstration of principle within a human subject, and may eventually serve as a model to vascularize engineered bone tissue *in vivo*.

To examine the differences in the *in vivo* osteogenic capacity of between BDMSCs and ESCs, Tremoleda and colleagues (2008) implanted chambers that were cell-impermeable. These chambers contained either BDMSCs or ESCs that had been cultured *in vitro* for 4 days in standard osteogenic media. Since the pore size of these chambers precluded the passage of cells but allowed the diffusion of growth factors and other macromolecules, comparison of the intrinsic capacity for differentiation of these cell types became more straightforward. After 79 days post-implantation within nude mice, the authors reported no qualitative differences between the bone tissue formation between the BDMSCs, H7, and H9 embryonic stem cells. Although an interesting finding revealed that the ESC lines used did not require the *in vitro* osteogenic culture prior to implantation to form de novo bone tissue, which was unlike the BDMSCs, which required this pretreatment. Thus, although a significant difference between these cell types with the same osteogenic treatment was not uncovered in this study, the fact that ESCs required less coaxing and were more primed to respond to the bone tissue environment may be capitalized upon in future studies.

However, one of the major concerns when using ESCs for reparative medicine is the potential for residual undifferentiated cells to form teratomas following *in vivo* introduction. As such, undifferentiated murine ESCs form teratomas when injected into a healthy knee joint (Wakitani et al., 2003). The rate at which the teratomas grew in the knee joint however was slower than upon subcutaneous injection, suggesting that the microenvironment in the knee joint is not as favorable for ESC proliferation as for example a subcutaneous injection site. Surprisingly, if cells were injected into an inflammatory environment caused by a full-thickness osteochondral defect, the cells integrated and repaired the defect even in an allogenic setting (Wakitani et al., 2004).

Also, our group was recently able to show that ESCs lose their teratoma formation capacity with progressing osteogenic differentiation and maturation *in vitro*, whereby the *in vitro* microenvironment used to steer differentiation influences their teratoma formation capacity

in vivo. Whereas spontaneously differentiated cells formed teratomas in 16% of the cases when taken from day 10 old cultures, 30-day osteogenic cultures did not show any sign of teratoma formation upon subcutaneous injection (Taiani et al., 2009).
Highlighting the concern of teratoma formation further, Nakajima and colleagues (2008) seeded mouse ESCs embedded in a collagen matrix into osteochondral defects within the knee joints of mice. Their investigation focused on the differentiation potential of these cells when the joint was either free to move or physically immobilized. They revealed that the mechanical environment appears to have a dramatic effect on the differentiation outcome of these implanted cells. Three weeks post operation, the defects were examined and the free-moving joints were shown to contain cartilaginous tissue formation with favorable histological characteristics. Surprisingly, when the joint was immobilized a teratoma formed in every instance of study. Thus, considering the close link between chondrogenesis and osteogenesis (to be discussed further in next section), it is important to note the results here and recognize that the mechanical environment into which undifferentiated stem cells are placed can have important consequences.

3.3 ESC-derived MSCs

Another cell type that has been recently gained attention as a possible therapeutic source is the embryonic stem cell-derived mesenchymal stem cell (ESC-MSC) in which ESCs are induced along the mesenchymal stem cell lineage. For a more detailed overview of the markers and techniques used to isolate such mesenchymal stem cell like cells from ESCs, the reader is referred to two recent reviews by Hematti (2011) and zur Nieden (2011). In one study of this cell type Barberi and colleagues (2005) demonstrated that cells initially differentiated along a paraxial mesoderm lineage were able to undergo osteogenesis *in vitro*. They found that this induced and sorted cell type (i.e. using the mesenchymal stem cell marker CD73) was able to undergo osteogenesis, by various staining assays and expression of bone specific markers. Similarly, Hu and colleagues derived human ESC-MSCs, and examined their capacity to differentiate into bone forming cells (Hu et al., 2010). When these cells were cultured in the presence of dexamethasone and BMP-7, they found that both Alk Phos levels and calcium deposition was statistically higher in dishes containing both supplements. This improvement found with both supplements was a synergistic one, as revealed through the modest effect when BMP-7 was used independently. When these cells were grown on 3D PLLA nanofibrous scaffolds, similar to that of Smith and colleagues (2009) discussed earlier, they exhibited growth throughout the scaffold and demonstrated extensive mineralization.
The *in vitro* osteogenic capacity between isolated human MSCs and derived human ESC-MSCs, was directly compared by de Peppo and others (2010). In this study they designated human ESC-MSCs as human embryonic stem cell-derived mesodermal progenitors hES-MPs and used a similar approach to that of Hu et al. (2010) to derive this cell type (Karlsson et al., 2009). Here they demonstrated that *in vitro* culture of hES-MPs resulted in faster ECM mineralization as compared to human MSCs. These results were contrary to their Alk Phos assays, which showed significantly greater activity of Alk Phos in human MSCs at every point during the first five weeks of differentiation. This apparent discrepancy may reflect a differential dependence of Alk Phos to mineralize the ECM between these cell types. In addition, this study examined the osteogenic capacity of cells in relation to their passage number. In every assay performed the osteogenic capacity decreased as passage number increased for all cell types examined. Although they reported that the hES-MPs were more buffered against this diminishing capacity, it brings attention to the problem with serial

passages, which are inexorably tied to the requirements of tissue engineering, and their resulting potential to undergo osteogenesis.
The apparent discrepancy in relative Alk Phos activity was also found by Bigdeli and others (2010) when they compared the osteogenic capacity of human MSCs and a derived human ESC line (Bigdeli et al., 2008), which could be expanded on culture plastic without the support of feeder layers or other dish coatings such as Matrigel. Utilizing this cell line allowed the investigators to perform more direct comparison of the two cell types, since the typical differences between culture conditions were eliminated. Like the aforementioned study (de Peppo et al., 2010), they found that although Alk Phos expression was significantly lower at each time point examined, the derived human ESC line was better able to mineralize the extracellular matrix when compared to human MSCs. These results were further supported by ion mass spectrometry of the mineralized ECM, which demonstrated the signature of natural hydroxyapatite.
A study comparing osteogenesis of murine MSCs and murine ESCs derived from the same mouse strain (Shimko et al., 2004) also revealed this pattern where the mineral content was not directly correlated to Alk Phos activity. Thus, although Alk Phos activity is used frequently in studies of osteogenic differentiation, the level of enzyme activity may not directly correspond to the potential of the cells to mineralize the extracellular matrix. In addition, diverse Alk Phos levels may not necessarily suggest that more or less osteoblasts were formed, but may simply reflect different maturation kinetics of the different cell types.
Shimko et. al, (2004) went further in characterizing the mineralized matrix between murine MSCs and murine ESCs derived from the same mouse strain and cultured in the same conditions. As compared to natural hydroxyapatite found in bone, where the ratio of calcium to phosphorous is: 1.67:1; murine ESCs exhibited a ratio far closer (1.26:1) than murine MSCs (0.29:1). Mouse ESC cultures also contained, on average, a mineral content 50 times greater than mouse MSCs. However, once again reflecting distinct differentiation kinetics, pathways, or inherent differences in mineralization capacity, Alk Phos activity was significantly higher in MSCs throughout the course of the experiment. In addition, expression of osteocalcin and COL I in mouse ESCs was delayed relative to mouse MSCs. Thus, murine MSC differentiation appeared to be more reflective of natural osteogenesis, when examining organic matrix components and gene expression. On the other hand, the quantity and quality of the mineralization found in murine ESCs significantly surpassed what was exhibited by murine MSCs.
Although transferring the techniques of osteogenic induction of ESCs from flat culture dishes towards 3D scaffolds have demonstrated initial success, there continues to be the need for method refinement in order for these approaches to bone engineering become widely accepted. One such area of study where current knowledge is lacking is an understanding of the possible differentiation pathways that are normally found in vertebrate development these cells take in attempts at bone tissue engineering.

4. Different embryonic bone origins

Both *in vitro* and *in vivo* studies continue to elucidate the developmental program that pluripotent stem cells take to their eventual differentiated states, among them the osteoblast. Because of their capacity to differentiate into any cell type of the body, pluripotent stem cells may differentiate through the neural crest route or the mesodermal route, followed by mesenchymal specification. Similarly, ossification from pluripotent stem cells may occur through intramembranous bone formation or endochondral bone formation. In regard to the *in vivo* source of mesenchymal cells, which differentiate into bone in the appropriate developmental context, there also appears to be multiple developmental origins. The earliest MSCs appear to arise from Sox1+ neuroepithelium through a neural crest intermediate stage (Takashima et al., 2007) and not from mesoderm progenitors as previously believed.

The process of fracture healing also occurs through both intramembranous and endochondral means, which is dependent on the mechanical conditions at the fractured site (Claes et al., 1998). When dissecting the steps of bone development, far more is known about the endochondral pathway than the intramembranous process. The most overt difference between these two pathways is that either chondrocytes will arise from mesenchymal condensations, which subsequently apoptose and are replaced by invading osteoblasts, or there is a direct differentiation into osteoblasts themselves. Thus the differential influence and the necessity of chondrocytes highlight the most apparent differences between these bone-forming pathways. Thus, in order to optimize bone tissue-engineering procedures there is a need to understand the molecular basis underlying different bone formation processes. However, a current review of the literature demonstrates large holes in our understanding of these multiple routes in which bone naturally forms and how they are recapitulated in experimental systems. The remaining part of this chapter will be devoted towards our preliminary understanding of these processes with particular emphasis to their roles in bone tissue engineering.

4.1 Endochondral ossification

In the endochondral process mesenchymal cells condense and differentiate into proliferating chondrocytes, which take on the general shape of the future bone. These chondrocytes eventually fall out of the cell cycle and these post-mitotic chondrocytes undergo hypertrophy. In this stage of development the mature hypertrophic chondrocytes lay down cartilage-specific proteins into the surrounding matrix. This cartilaginous framework provides molecular cues, which attracts invading vasculature along with osteoblasts, which will replace the cartilage intermediate. Osteogenesis occurs directly adjacent to hypertrophic chondrocytes. It appears that both the parathyroid hormone (PTH)-related peptide (PTHrP) and its receptor PPR are critical in the process osteogenesis via the endochondral pathway. In mice, upon disruption of the either PTHrP or PPR, the formation of ectopic hypertrophic chondrocytes is accompanied by ectopic bone collar formation (Karaplis et al., 1994). To determine if the hypertrophic chondrocytes induce osteogenesis in adjacent cells and is not a spatial/temporal coincidence, Chung and colleagues (2001) studied transgenic mice that express constitutively active PPR under the control of a chondrocyte specific promoter. This constitutive action resulted in suppression of hypertrophic chondrocyte formation and concurrent suppression of bone collar and primary spongiosa development. In addition, when these transgenic mice were mated to PTHrP-/- mice the resulting rescue of the ectopic bone formation, supported the conclusion that hypertrophic chondrocytes are responsible for the induction of osteogenesis in adjacent tissue.

Regulation of the PTHrP/PPR signal appears to be controlled by one of the members of the hedgehog family of paracrine factors, Indian hedgehog (Ihh). Members of this signaling family are found throughout the animal kingdom and take on a number of critical roles in the developing organism. Here, Ihh is expressed by both prehypertrophic and hypertrophic chondrocytes. This signal mediates the expression of PTHrP by cells of the perichondrium, which in turn binds to PPR on chondrocytes. Ihh and PTHrP signaling thereby creates a negative feedback loop which suppresses differentiation of the proliferating chondrocytes into hypertrophic ones (Lanske et al., 1996; Vortkamp et al., 1996). Thus, this balance of signals dictates the spatial positioning of the hypertrophic chondrocytes. However, the role of Ihh appears to have a broader impact on osteogenesis than its PTHrP-dependent regulation of chondrocyte maturation.

St-Jacques and colleagues (1999) demonstrated that Ihh also plays a role in chondrocyte proliferation and the direct development of osteoblasts in endochondral bones. Previous studies have demonstrated a critical role of Wnt signaling and β-catenin localization as well (Gong et al., 2001; Kato et al., 2002). Hu and others (2005) found nuclear β-catenin localization within the cells of perichondrium indicating an upstream role of Ihh signaling to facilitate proper Wnt signaling. Furthermore, Ihh null mice do not exhibit osteocalcin expression within endochondral bones, whereas this expression is readily detected within the intramembranous bones of the skull and clavicle. This differential dependence of Ihh signaling underscores one of the differences between endochondral and intramembranous bone formation.

When assessing the role of local synthesis of VD_3 in transgenic mice that exhibited a chondrocyte-specific loss-of-function Cyp27b1, the enzyme that converts 25-hydroxyvitamin D_3 into the active form VD_3, it was found that the hypertrophic zone was expanded (Naja et al., 2009), thereby increasing both bone mass and trabecular size and number. The classical view that VD_3 synthesis (active form) was restricted to the kidneys and that this hormone's influence on bone tissue regulation was an indirect consequence of altering calcium and phosphate homeostasis had to be reevaluated. The authors suggest their results can be explained by a reduced osteoclast recruitment, which follows from a reported delay of vascularization that may be attributed to a reduction of VEGF found. Conversely, overexpression of Cyp27b1 under a chondrocyte-specific promoter resulted in the opposite expression profile and phenotype. These results are in accordance with chondrocyte specific VD_3 receptor ablation experiments, which showed impaired vascularization and osteoclast number in endochondral bone (Masuyama et al., 2006). As opposed to the traditional view of the role of VD_3 in bone biology as an indirect mediator of mineral uptake, these experiments demonstrate a functional role of this metabolite in regulating endochondral bone formation.

Some investigators have explored an approach to bone tissue-engineering by mimicking the development of mammalian long bones, where the creation of cartilage scaffolds *in vitro* are implanted *in vivo*. This approach hinges on the idea that the body will recognize this cartilage scaffold as an intermediate step in the endochondral bone formation process and will then proceed to ossify this construct. In the formation of endochondral bone, chondrocytes are exposed to very low oxygen levels and their survival is dependent on the expression of the transcription factor hypoxia-inducible factor-1 (For review see: Pfander and Gelse, 2007). Thus, the natural ability of chondrocytes to withstand the low oxygen supply can provide the time needed for new vasculature to develop and reach the core of the implant before widespread cell necrosis.

Jukes et al. (2008) tested whether *in vitro* differentiation of ESCs along chondrocyte lineages on scaffolds could improve *in vivo* osteogenesis following implantation. ESCs were initially induced along a chondrogenic pathway for 21 days on ceramic scaffolds. These chondrogenically-primed scaffolds were then subsequently implanted in immunodeficient mice and were found to exhibit nascent bone tissue formation when examined 21 days post-operatively. For comparison, primary chondrocytes and adult MSCs of human, goat, and bovine origin were used in lieu of the chondrocyte-induced ESCs. It was found that each cell type demonstrated differential abilities to form bone tissue *in vivo*. Interestingly, the goat MSCs resulted in the highest degree of bone tissue formation, and it appeared that this formation occurred via an intramembranous pathway.

Farrell et al. (2009) further examined the *in vitro* chondrogenic-priming of scaffolds using human MSCs and reported limited success. After the cells were cultured on collagen–GAG scaffolds for three weeks in chondrogenic media, the scaffolds were implanted subcutaneously in nude mice. Although cell survival and angiogenesis was found higher in the chondrogenically-primed scaffolds, as opposed to scaffolds that were osteogenically-primed, there appeared to be no *de novo* osteogenesis. The authors reported the chondrogenically-primed scaffolds showed evidence of the initial progression of endochondral ossification, yet were unable to proceed through the later stages of osteoblast-induced mineralization. When mineralization was induced *in vitro* prior to implantation, the nascent angiogenesis that was previously obtained was compromised. Thus, it appears that for this approach to be successful, the timed release of additional factors *in vivo* is needed to promote the osteogenic replacement of the cartilaginous scaffold.

When examining the developmental pathway of bone-tissue engineered constructs, by either endochondral or intramembranous routes, it makes sense that different cell types will mature along different pathways even when presented to the same conditions. Tortelli and others (2010) revealed how the differentiated state of implanted cells affects subsequent ossification and host cell recruitment to the graft site. They seeded hydroxyapatite scaffolds with either human MSCs or osteoblasts. When differentiated osteoblasts were used to seed the scaffolds, ossification occurred through an intramembranous pathway, as revealed by the lack of cartilage markers by immunohistological examination. This intramembranous ossification appeared to be more rapid and thus accounted for more bone deposition within the same time period when compared to the MSC-seeded scaffolds. However, MSC scaffolds, which ossified in an endochondral fashion, were able to facilitate nascent vascularization of the graft. This highlights the fact that engineered bone grafts may one day be tailored to a patient's need depending on factors such as speed of graft ossification and site incorporation. In addition this study shows that implanted MSCs can progress through the endochondral pathway, but as the aforementioned study by Farrell et al. (2009) demonstrates this process currently cannot be split into an early *in vitro* stage that can be 'picked up' later *in vivo*. However, if the process of endochondral bone formation is elucidated further and applied to tissue engineering, then it is feasible that this approach may one day become a viable avenue to repair large bone defects.

Although mimicking the development of long bone through endochondral ossification of scaffolds maybe appropriate in some contexts, intramembranous ossification may be suitable for other applications in regenerative medicine. The body utilizes both of these systems in different contexts depending on certain conditions whose reasons remain to be fully characterized. Nonetheless, it appears quite probable that bone tissue engineering need not only be tailored to the individual but also the specific bone defect or disease in order to be completely effective.

4.2 Intramembranous ossification

As for intramembranous bone formation, not only is little known about the process itself, but the developmental pathway of the cells leading to the formation of the tissues within the cranial skeleton is still not well understood.

4.2.1 Neural crest cells

As incipiently indicated, the migrating cranial neural crest cells form bone mostly through intramembranous ossification. Initially neural crest cells become committed to either an ectomesenchymal (i.e. producing tissues such as cartilage, bone and connective tissue) or a non-ectomesenchymal (i.e. producing neurons, glia and pigment cells) lineage. The ectomesenchymal tissue is also referred to in the literature as mesectoderm. Blentic and others (2008) describe how migrating neural crest cells in chick and zebrafish embryos commit to either fate. Cells that migrate into the pharyngeal arches are induced to respond to FGF signaling within these embryonic structures, resulting in the expression of the homeobox gene Dlx2. Concurrently, early neural crest markers Sox10 and FoxD2 are downregulated, which are still expressed in the neural crest cells that have not invaded the pharyngeal arches and thus are fated to become non-ectomesenchyme. Whether or not neural cells migrate into the pharyngeal arches appears to be determined by the timing of their emergence from the neural tube. Although not fully understood, it appears that early migrating cells 'fill up' the pharyngeal arches and the cells that migrate later are thus more likely to find residence outside of the arches and become non-ectomesenchyme (Blentic et al., 2008).

4.2.2 Neural crest and mesodermal progenitors in intramembranous bone formation

The parietal bone, which is of paraxial mesoderm origin and the frontal bone, which is of neural crest origin, both form via intramembranous ossification, thus making the study of calvarial bones an attractive platform to study the possible differences in bones of different embryonic origins. Quarto et al. (2010) examined the osteogenic capacity of first passage osteoblasts that were obtained from these respective bones in mice. Frontal bone-derived osteoblasts from post-natal day 7 and day 60 mice were found to exhibit greater mineralization capacity, as revealed by Alk Phos activity, von Kossa and Alizarin Red S staining. This was also supported by expression data of the bone-specific markers osteocalcin and runx2. These *in vitro* observations were reinforced by the relative healing capacity of these two bones. The successful healing of 2mm defects was found within the frontal bone in the majority of mice at 8 weeks post-injury, whereas complete healing was not typically found in same sized injuries of the parietal bones within the same time period.
The investigators uncovered a higher level of endogenous canonical Wnt signaling in frontal bone osteoblasts as compared to parietal bone osteoblasts that may be responsible for this differential regenerative propensity. By modulating Wnt signaling through exogenous addition of Wnt3a or transfecting osteoblasts with constructs that increase β-catenin signaling in parietal bones to frontal bone levels, and vice versa, the authors showed a reversal of osteogenic potential of these cells. Thus, providing strong evidence that that the enhanced osteogenic potential of frontal bone osteoblasts can be at least be partially attributed to these differences in endogenous Wnt signaling.
Xu and colleagues (2007) found that osteoblasts derived from the frontal bone proliferated faster and attached to culture dishes better than osteoblasts that were harvested from parietal bone. This may be linked to the fourfold greater expression of osteoblast-specific cadherin that they found within frontal bone osteoblasts. The parietal bone osteoblasts did however show double the Alk Phos activity at the time points examined. When cultured in the presence of osteogenic inducing factors, such as VD_3, the frontal bone osteoblasts showed a much more robust bone nodule formation. However, expression of osteogenic differentiation markers, such as osteopontin, Col1, and Wnt5a was significantly greater in

the parietal bone derived cells. Members of the FGF signaling cascade were also differentially expressed between these two cell types. Thus, the frontal and parietal bones appear superficially similar yet exhibit an number of different characteristics such as growth kinetics, regulation by signaling cascades and varying marker expression, all of which demonstrate that these bones are not as similar as they initially appear to be.

To further examine the regenerative osteogenic capacity of cells from different embryonic origins Leucht and others (2008) engineered mice in which developing cells of neural crest origin would irreversibly express GFP. Tissues from mesodermal origin were also induced to express β-galactosidase. Following skeletal injury in either the mandible or the tibia resulted in natural bone regeneration where the progenitor pool which became new bone tissue was derived from the same embryonic origin of the injured bone itself (i.e. cells from neural crest origin repaired mandible defects, and cells of mesodermal origin repaired tibia defects). The investigators then performed a number of transplant experiments where skeletal progenitor cells were implanted into bone of different embryonic origin. Interestingly neural crest derived progenitors were able to form more new bone when implanted ectopically into tibia injury sites, than if they were implanted back into their endogenous environment within mandible injuries. Conversely, when mesoderm derived progenitors were implanted into mandible injuries, an abundance of cartilage formed, which over time ossified via an endochondral pathway.

These results suggest a difference in the underlying reparative plasticity of cells from different origins. *In vitro* analysis demonstrated that mesoderm osteoprogenitors proliferated faster than the corresponding neural crest osteoprogenitors. However, the cells of neural crest origin were able to differentiate faster based on Alizarin Red S staining and qPCR of osteogenic markers. The authors went further to try to understand the possible molecular mechanisms underlying this difference (Leucht et al., 2008). They found that in the adult mice Hoxa11 expression was maintained in the tibia and was absent in the mandible. For neural crest osteoprogenitors, which originally lack Hoxa11 expression, they began to express Hoxa11 when ectopically placed in the tibia. This switch in expression was not found in the mesodermal osteoprogenitors, which continued to express Hoxa11 even when placed in the Hoxa11-negative environment of the mandible. This study once again reiterates how the molecular identity of cells used for transplantations can be a crucial factor in determining the success of a stem cell based bone graft. In addition, it may be true that osteoprogenitors of neural crest origin may be best suited as the stem cell source of bone grafts because of their greater plasticity to adapt to local environments.

4.2.3 ESC-derived neural crest stem cells

When ESC cultures are osteogenically induced following standard differentiation procedures, it is seldom examined which developmental progenitors are responsible for the terminally differentiated osteoblasts. Although some studies have differentiated ESCs along defined lineages and then determined their osteogenic capacity, Lee et al. (2008) reported the isolation and propagation of human neural crest cells from human ESCs. Initially they cultured human ESCs in neural induction media and then mechanically removed and replated the resulting neural rosettes. Cells that were doubly positive for the neural crest markers p75 and HNK-1 were further cultured and revealed a CD73 positive population. This marker expression indicates the presence of neural crest-derived mesenchymal stem cells. This CD73+ population could be osteogenically induced as revealed by Alizarin Red S, and Alk Phos staining, and bone sialoprotein expression.

In a similar study Jiang and colleagues (2008) also used a FACS enrichment strategy for p75 and HNK-1 positive neural crest cells after co-culture of human ESCs with PA6 stromal cells, although the osteogenic differentiation potential of such isolated neural crest cells was not determined. In another study cranial neural crest-like cells were derived from human ESCs, not by co-culture but instead though EB formation (Zhou and Snead, 2008). Here, FACS purification of neural crest cells was performed based on the expression of Frizzled3, a Wnt receptor, and cadherin11, a cadherin specifically expressed in the gastrulating embryo and migrating neural crest cells (Kimura et al., 1995). Only about 1% of cells were double positive for these selected markers and were able to self-renew and maintain multipotent differentiation potential, including runx2 positive osteoblasts with the capability to calcify. Although not definitive demonstrations of the isolation of osteoprogenitor stem cells from different germ layer-derived populations, these studies offer compelling evidence that cells existing in *in vitro* culture conditions can recapitulate the neural crest osteogenic pathways found in the developing embryo.

5. Conclusion

In summation, pluripotent stem cells are a particularly attractive source to develop new technologies and techniques to address many debilitating bone disorders and defects, and we have come far in the understanding and characterization of osteogenesis. Although more investigations and innovations are needed before regenerative bone biology becomes commonplace, the future holds great promise in this field of research.

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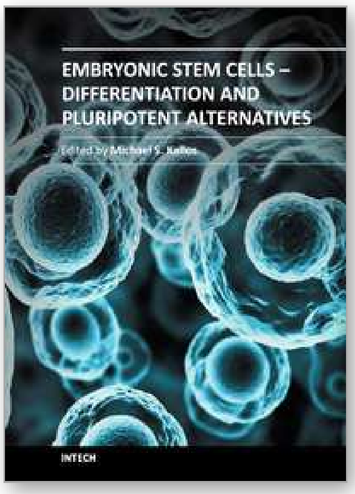

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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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