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Modulating the Transcriptional Activity of Beta-Catenin During the Osteogenic Differentiation of Embryonic Stem Cells

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular, and Developmental Biology

by

Kevin Clinton Keller

December 2014

Dissertation Committee:
Dr. Nicole zur Nieden, Chairperson
Dr. Morris Maduro
Dr. Jin Nam

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The Dissertation of Kevin Clinton Keller is approved:

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ABSTRACT OF THE DISSERTATION

Modulating the Transcriptional Activity of Beta-Catenin During the Osteogenic Differentiation of Embryonic Stem Cells

by

Kevin Clinton Keller

Doctor of Philosophy, Graduate Program in Cell, Molecular and Developmental Biology
University of California, Riverside, December 2014
Dr. Nicole zur Nieden, Chairperson

Because of their pluripotent nature, as defined by their self-renewal and differentiation capacity, mouse embryonic stem cells (mESCs) have become an invaluable resource for unraveling the underlying mechanisms that control developmental programs. Understanding the formation and maintenance of bone tissue will undoubtedly facilitate the creation of new therapeutic approaches that address bone-related pathologies. Numerous reports have demonstrated the importance of the Wnt signaling pathway in regulating osteogenesis, however, given the revealed complexity of this regulation further elucidation of the time- and cell-specific response is needed. The *in vitro* culture of mESCs allows investigators to examine multiple stages of differentiation while simultaneously manipulating this signaling pathway in a time-dependent manner. Through the use of known modulators and activity reporters, studying beta-catenin (CTNNB1) (i.e. the central effector of canonical Wnt signaling) will provide invaluable insights towards a greater understanding of osteogenic processes. For instance, we

have found that supplementation of the non-canonical Wnt ligand Wnt5a, during a narrow time window of osteoblast precursor formation, was able to improve osteogenic differentiation at later stages. This supplementation resulted in increased activation of multiple downstream effectors and was also correlated with a decrease in CTNNB1-dependent LEF/TCF transcriptional activity. Interestingly, when studying the impact of high concentrations of glucose within culture media, we found a decrease in osteogenic differentiation, which corresponded to an upregulation of CTNNB1-dependent LEF/TCF reporter activity within the same time period. This change in transcriptional activity was linked to differential activation of the AKT/FOXO signaling pathway. Thus, our work continues to reinforce the dynamic role of CTNNB1 in regulating *in vitro* osteogenesis. With this knowledge we employed multi-stage supplementation of either canonical or non-canonical Wnt ligands to direct osteogenic differentiation of mESCs in serum-free cultures. We found that these ligands, or corresponding chemical modulators, could yield measures of osteogenic output that was comparable to cultures containing fetal bovine serum. With these advancements in our understanding of the role of CTNNB1 in both developmental osteogenesis and the dysregulated processes underlying bone-related pathologies, novel strategies can be developed that improve differentiation protocols and therapeutic applications.

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Chapter 1: Introduction

The stem cell

Since the derivation of embryonic stem cells (ESCs) from the inner cell mass of mouse blastocysts in 1981 (Martin, 1981; Evans and Kaufman, 1981), the field of vertebrate developmental biology has immeasurably benefitted from their study. ESCs have two key characteristics that make them particularly attractive for research: (1) their practically limitless self-renewal potential while maintaining a stable genome and (2) their inherent ability to differentiate into any cell type of the adult organism (i.e. pluripotency). Formal demonstration of this differentiation capacity came in 1984 when Bradley and colleagues (1984) created chimeric mice by injecting developing blastocysts with cultured ESCs. The injected ESCs were able to give rise to every tissue of the adult mice including germline cells. Since that time, myriad investigations have demonstrated the *in vitro* differentiation potential of ESCs and corresponding protocols have been produced to direct these differentiations along specific lineages. The work presented herein will critically depend on the knowledge gained from differentiating ESCs along osteogenic lineages (Buttery et al., 2001; zur Nieden et al., 2003). For a more detailed review of osteogenic differentiation from pluripotent stem cells the reader is referred to chapter 1 of this thesis.

Experimental investigation of ESCs has become invaluable towards unraveling the underlying mechanisms that control developmental programs. Furthermore, employing ESCs gives the researcher a unique opportunity to study the etiology of diseases without impractical, or invasive *in vivo* examinations. Here, through genetic and therapeutic manipulation of differentiating ESCs, I intend to reveal previously

uncharacterized molecular mechanisms regulating developmental osteogenesis and to gain a greater understanding of bone disease manifestation.

Bare bones

Despite its overt rigidity bone tissue is considerably dynamic, constantly being remolded throughout the lifetime of the organism. This remolding process is mediated by the balanced actions of three cell types: the osteoblast, the osteocyte, and the osteoclast. Osteoblasts are the 'bone-forming' cells. They secret the extracellular matrix (ECM) proteins that constitute the organic component of bone (i.e. the osteoid) as well as alkaline phosphatase (ALP), an enzyme that mineralizes the secreted ECM. The inorganic component of bone is a calcium-phosphate mineral in the form of hydroxyapatite crystals. The osteocytes, which are the most abundant cell type in bone tissue, help regulate tissue homeostasis via sensing metabolic and mechanical signals. They are embedded in the bone mineral matrix and communicate via long thin projections of the plasma membrane (Dudley and Spiro, 1961). Osteocytes are terminally differentiated osteoblasts that have become encased within the bone matrix. Lastly, the osteoclasts are multinucleated cells that are responsible for breaking down bone tissue. This degradation occurs by acidification via the action of vacuolar ATPase proton pumps within resorptive pits between the osteoclast plasma membrane and bone surfaces (Väänänen et al., 1990). Unlike the osteoblast and osteocyte, which differentiate from mesenchymal stem cell (MSC) precursors, the osteoclast are of hematopoietic stem cell (HSC) origin (Ash et al., 1980). The dysregulation of these cell types has been implicated in the pathophysiology of multiple bone diseases, such as osteoporosis; a prevalent low bone density disorder that is reportedly found in 9% of

adults over the age of 50, as defined by the World Health Organization (Looker et al., 2012).

Bone tissue is also intimately associated with cartilaginous tissue that provides the compressive force resistance required for the proper functioning of skeletal systems. Additionally, chondrocytes (i.e the cells which produce cartilage) share a common developmental lineage with osteoblasts, being both specified from MSC precursors (Ashton et al., 1980). This close relationship between these cell types is particularly evident during embryonic skeletal establishment. Briefly, chondrocytes are responsible for laying down the initial extracellular protein framework for many putative bones in a process called endochondral ossification. Here, proceeding mesenchymal condensation, ECM secreting chondrocytes continue through a stage of hypertrophy and eventually undergo apoptosis. The established ECM is then invaded by vasculature and osteoblasts, which begin to mineralize the tissue. Most mesoderm-derived bone proceeds through this intermediate chondrocyte phase, whereas most of the neural crest-derived bone (i.e. of ectoderm origin) develops through intramembranous ossification process in which condensed mesenchyme differentiates directly into osteoblasts (Hall and Miyake, 1992). For a more detailed examination of these bone development processes *in vivo*, the reader is directed to the following chapter of this thesis.

Outlining the osteoblast

To gain a greater understanding of the pathology and etiology of bone-related disorders it is necessary to elucidate the developmental progression of osteoblast specification and to characterize marker expression. Although there are numerous,

critical cell specification steps that are required for a fertilized egg to develop adult bone tissue, we will begin our consideration following the formation of MSCs. The first step towards an osteoblast from a MSC is the formation of an osteo-chondroprogenitor cell, which is characterized by the expression of the transcription factor Sox9. A conditional knockout of Sox9 within the limb mesenchyme of developing mice fail to differentiate into both chondrocytes and osteoblasts (Akiyama et al., 2005). Although, expression of Sox9 appears to be absolutely required for chondrocyte formation, its role in osteoblast development is less clear and the resulting lack of bone formation in Sox9 conditional mutants may be a secondary consequence of absent chondrocytes (Long, 2012). For instance, removal of Sox9 from cranial neural crest cells resulted in a complete loss of endochondral craniofacial elements, however, there was ectopic osteoblast-specific marker expression within the nasal area, which is normally designated to become cartilage (Mori-Akiyama et al., 2003).

Following the osteo-chondroprogenitor stage, activation of the canonical Wnt signaling pathway facilitates osteoblast lineage specification and suppresses chondrogenic cell fate (Day et al., 2005). The formation of immature pre-osteoblasts is accompanied by the expression of runt-related transcription factor 2 (RUNX2, formally known as CBFA1). In mice, haploinsufficiency of *Runx2* causes hypoplastic clavicles and a delay in the closure of the fontanelles (Mundlos et al., 1997). Whereas a complete lack of RUNX2 expression results in the absence of skeletal ossification (Otto et al., 1997; Komori et al., 1997). RUNX2 binds promoter elements in multiple genes for proteins found in bone ECM such as bone sialoprotein (*Bsp*), collagen type 1 (*Col1*), and osteopontin (*Opn*) (Ducy et al., 1997). Mice lacking RUNX2 also lack expression of the transcription factor Osterix (OSX), which is additionally required for osteoblast formation

(Nakashima et al., 2002). Since OSX null mice do not appear to have altered RUNX2 expression, its activity appears to be downstream and dependent on RUNX2. Transcriptional regulation by these two transcription factors leads to the formation of mature osteoblasts, which are typically characterized by high expression of the ECM protein osteocalcin (OCN) and their ability to mineralize the osteoid matrix (Liu et al., 1997). OSX and RUNX2 will continue to function as important regulators of mature osteoblasts throughout postnatal life (Ducy et al., 1999; Zhou et al., 2010).

Knowledge of these lineage markers, as well as a number of biochemical assays to examine mineral content and ALP activity, the differentiation potential of various cell types can be examined. For example, cells with the capacity to differentiate into osteoblasts have been isolated from adipose tissue, (Zuk et al., 2002), peripheral blood (Zvaifler et al., 2000), fetal blood (Campagnoli et al., 2001), umbilical cord (Romanov et al., 2003), placenta (Fukuchi et al., 2004), and amniotic fluid (De Coppi et al., 2007). Furthermore, researchers can use marker expression and endpoint analyses to study the consequence of pharmacological and genetic manipulation of these cells in order to improve differentiation protocols and disease modeling. The continual investigation of osteogenesis has revealed a growingly complex field of integrated signaling pathways that regulate bone development.

Who, What, When, Where, Wnts

One of the signaling pathways of particular importance for osteogenesis is the Wnt signaling pathway. This pathway is known to regulate diverse aspects of animal development such as cell differentiation, cell polarity, cell migration, body axis formation, and organogenesis; all of which are critically dependent on the lipid-modified secreted

Wnt glycoproteins. The first *Wnt* gene was characterized by Roel Nusse and Harold Varmus when screening integrated MMTV proviruses that caused mammary tumors in mice (Nusse and Varmus, 1982). To clarify the nomenclature the initial gene name *int1* was later changed to incorporate the name of the *Drosophila* homologue *Wingless,* resulting in the portmanteau "*Wnt.*"

Wnt genes are found in every metazoan examined and are speculated to be instrumental for the emergence of complex body plans characteristic of the animal kingdom (Holstein, 2012). In both human and mice there are 19 Wnt proteins, which can elicit diverse cellular responses. The Wnt proteins were initially divided into two subclasses based on their ability to induce a secondary body axis in developing *Xenopus* embryos (Du et al., 1995) or their ability to morphologically transform C57MG mammary epithelial cells (Wong et al., 1994). The Wnt proteins that were able to either induce ectopic body axes or transform these epithelial cells were deemed the 'canonical' Wnts, whereas the remaining Wnts were placed in the 'non-canonical' subclass. Later, the definition of a canonical or non-canonical Wnt was amended to account for their dependence or independence on the effector protein β-catenin (CTNNB1), respectively.

CTNNB1 is a multifunctional protein that was originally characterized through its binding to the cell adhesion molecule E-cadherin (Takeichi, 1990). Shortly after this finding CTNNB1 was identified to be the vertebrate homologue to the segment polarity gene, *Armadillo* (McCrea et al., 1991). In 1993, CTNNB1 was found to associate with the protein Adenomatous polyposis coli (APC) (Rubinfeld et al., 1993), which stirred intrigue since mutations in *APC* had been previously linked in familial colon cancer (Groden et al., 1991). In fact, most colorectal carcinomas (63%) and adenomas (60%) have mutations in the tumor suppressor gene *APC* (Powell et al., 1992).

APC is one of the core components of a complex, which targets CTNNB1 for proteolytic degradation. Additional proteins of the CTNNB1 destruction complex include the scaffolding protein Axin and the serine/threonine kinases: glycogen synthase kinase 3 (GSK3), and casein kinase 1 (CK1) (Stamos and Weis, 2013). Following phosphorylation by GSK3 and CK1, CTNNB1 becomes a target for the E3-ubiquitin ligase β-TrCP (Polakis, 2002). Ubiquitinylation by β-TrCP results in the recognition and degradation of CTNNB1 by the 26S proteasome (Aberle et al., 1997). Interestingly, there is no signal that activates this destruction complex, and by default cytoplasmic CTNNB1 is constitutively targeted for destruction. Signal transduction through canonical Wnt signaling, on the other hand, inhibits this complex and thereby stabilizes CTNNB1 (Figure 1).

To initiate canonical Wnt signaling a Wnt ligand binds to the cysteine-rich domain of a frizzled (FZD) receptor (Bhanot et al., 1996), along with either co-receptor LDL-receptor-related protein 5 or 6 (LRP5/6) (Tamai et al., 2000). The 10 characterized seven-pass transmembrane Fzd receptors in both human and mouse are within the G-protein coupled receptor superfamily (Fredriksson et al., 2003). Mutations in the *Fzd* genes were originally implicated in their role in regulating planar cell polarity *Drosophila* (Gubb and García-Bellido, 1982), but have since been shown to act as receptors for both canonical and non-canonical Wnt signaling cascades.

Following the formation of the WNT receptor complex, the proteins dishevelled and axin are recruited to the plasma membrane. In a manner that is not completely understood, this recruitment displaces or inhibits the CTNNB1 destruction complex (MacDonald and He, 2012). As a result CTNNB1 accumulates in the cytoplasm and can subsequently translocate into the nucleus where it binds to members of the lymphoid

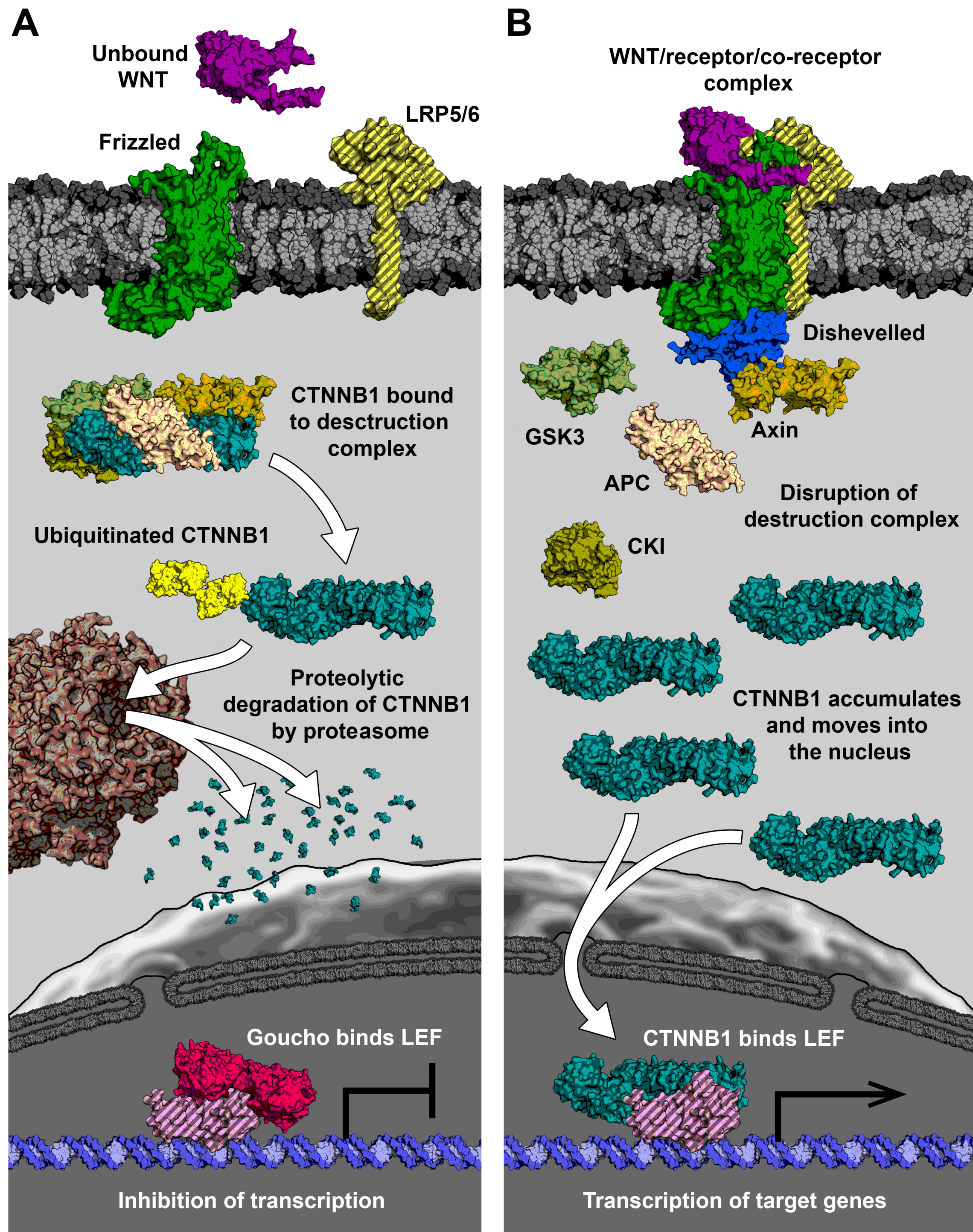

Figure 1.1: The canonical WNT signaling pathway. (A) In the absence of WNT signaling, CTNNB1 is targeted for destruction. (B) Activation of the canonical WNT signaling cascade results in CTNNB1 stabilization and subsequent nuclear translocation and transcriptional activity.

enhancer factor (LEF) and T-cell factor (TCF) family of transcription factors (Behrens et al., 1996; Molenaar et al., 1996). Generally these transcription factors can function either as transcriptional repressors or transcriptional activators based on the absence or presence of CTNNB1 binding, respectively (Cadigan and Waterman, 2012). Although in vertebrates, the specific activity of the four members of the LEF/TCFs vary; where TCF3 most often acts a repressor (Merrill et al., 2004), LEF1 acts as a activator, and TCF1 and TCF4 alternate depending on cellular or tissue context (Liu et al., 2005). These transcription factors mediate repression when bound to members of the Groucho family of corepressors (Brantjes and Roose, 2001). Although CTNNB1 lacks a DNA binding domain, its association with the LEF/TCFs causes the displacement of bound corepressors. The resulting CTNNB1/LEF/TCF complex recruits a multitude of coregulators, which then remodel chromatin causing target gene expression (For a full list of Wnt/CTNNB1 target genes see: http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes).

Non-canoncal Wnt signaling

The non-canonical WNT pathways, classically defined to transduce signals through CTNNB1-independent mechanisms, are less understood than the extensively studied canonical WNT signaling pathway. Functionally, non-canonical WNT pathways are known to regulate cell polarity and migration through changes in cytoskeletal elements. One such function is the regulation of planar cell polarity (PCP) (i.e. the process through which a cell orients itself within the plane of a tissue). Examples of non-canonical WNT signaling directing PCP include hair organization on the wings of *Drosophila* and alignment of inner ear hair cells within the cochlea of mammals

(Dabdoub and Kelley, 2005; Maung and Jenny, 2011). Although the upstream components of these signaling pathways such as the frizzled receptors and dishevelled are shared between non-canonical and canonical Wnt pathways, there are many downstream effectors that are not shared. For instance c-Jun NH2-terminal kinase (JNK) and small GTPases of the Rho family were uncovered as non-canonical effectors when loss-of-function mutations were able to rescue PCP defects associated with overexpression of FZD and dishevelled (Strutt et al., 1997; Boutros et al., 1998).

Following the logic that the FZD receptors are within the GPCR superfamily of receptors, evidence has suggested that the FZD activation can switch on G-proteins in both canonical and non-canonical Wnt signaling events (van Amerongen, 2012). Activated G-proteins commonly elicit the release of calcium ions from endoplasmic reticulum stores. Wnt5a (i.e. the prototypical non-canonical Wnt) was first linked to downstream calcium signaling when the frequency of calcium transients in zebrafish embryos increased following Wnt5a treatment (Slusarski et al., 1997). Following an increase in cytoplasmic calcium, a number of downstream effectors are subsequently activated such as: protein kinase C (PKC) (Sheldahl et al., 1999) and calmodulin-dependent protein kinase II (CAMKII) (Kühl et al., 2000). These effectors can then activate the transcription factors nuclear factor κB (NFκB) and cAMP response element binding protein (CREB) (De, 2011), which are known to regulate many developmental processes.

Aside from the FZD receptors, the tyrosine kinase receptors ROR1, ROR2, and RYK have been shown to bind to Wnt ligands and mediate non-canonical signaling (Oishi et al., 2003; Yoshikawa et al., 2003). Adding to the complexity of WNT signaling, a growing body of evidence has demonstrated cross-talk between non-canonical and

canonical signal transduction cascades. For instance, Ishitani et al. (2003) found that non-canonical Wnt5a/CAMKII pathway activated the TGF-beta activated kinase 1 (TAK1)/nemo-like kinase (NLK) pathway, which suppressed canonical Wnt signaling in human embryonic kidney cells. Other investigators have also supported a model of mutual antagonistic functions between the canonical and non-canonical Wnt pathways (Westfall et al., 2003; Topol et al., 2003; Baksh and Tuan, 2007). Further blurring the lines between canonical and noncanoncial signaling van Amerongen and colleagues (2012) reported that the quintessential non-canonical WNT5a, was able to elicit CTNNB1 transcriptional activity based on the receptor context of cells in both *in vitro* and *in vivo* examinations.

Other regulators of Wnt signaling

There are various endogenous regulators of WNT signaling, which target multiple nodes of these signal transduction cascades. At the level of ligand-receptor binding, the secreted frizzled-related proteins (SFRPs) sequester WNT ligands in the extracellular space via cysteine-rich domains that are highly similar to the binding sites found on the FZD family of receptors (Bafico et al., 1999). Through a binding domain found on the RYK tyrosine kinase receptor, Wnt inhibitory factor 1 and 2 (WIF1/2) similarly suppress WNT ligand-receptor interaction by reducing the concentration of free WNT proteins outside of the cell (Hsieh et al., 1999; Cruciat and Niehrs, 2013). Sclerostin and the dickkopf (DKK) proteins target the LRP5/6 transmembrane coreceptors; effectively inhibiting their functions (Bafico et al., 2001; Li et al., 2005). Interestingly, antagonism of WNT signaling by DKK has been demonstrated throughout the animal kingdom including

the freshwater polyp, Hydra (Guder et al., 2006), indicating the ancient nature of this mode of signal regulation.

Pharmacological targeting of WNT signaling pathway is of particular interest for biomedical research because of myriad pathologies associated with dysregulation of these signaling cascades. Recent high-throughput screens have yielded multiple compounds which can either inhibit or activate these pathways (For review see: Zimmerman et al., 2012). Commonly, increasing CTNNB1 concentration *in vitro* is achieved through lithium-mediated inhibition of the negative regulator GSK3 (Klein and Melton, 1996). While lithium is widely prescribed as a mood disorder medication (i.e. whose mode of action may be partly attributed to CTNNB1 regulation in the CNS), its use as a specific agonist of CTNNB1 *in vivo* is impeded by off-target effects and the diverse function of GSK3 (Young, 2009). Another small molecule of note is 6-bromoindirubin-3′-oxime (BIO), which as additional been shown to inhibit GSK3. Stabilization of CTNNB1 through BIO treatment of ESCs has been shown to improve pluripotency maintanence (Sato et al., 2004).

Additional signaling events have been shown to sequester CTNNB1 within the nucleus and abrogate the canonical WNT signaling response at the level of gene expression. For instance, activation of the retinoid nuclear receptors have been shown to compete with the TCF transcription factors for CTNNB1 and reduce LEF/TCF reporter activity (Xiao et al., 2003; zur Nieden et al., 2007). Additionally, activation of the nuclear receptor for 1,25-dihydroxyvitamin D_3 (VD_3), a known osteogenic inducer (zur Nieden et al., 2003), has been shown to downregulate LEF/TCF reporter activity (Palmer, 2001).

Wnt signaling and bone

The role of Wnt signaling in bone development had long been suspected, but clear evidence did not appear until mutations in the LRP5 co-receptor were found to be responsible for the autosomal recessive disorder osteoporosis pseudoglioma syndrome (OPPG); a disease characterized by a low bone density phenotype (Gong et al., 2001). Soon afterward Kato and colleagues (2002) showed that *Lrp5*$^{-/-}$ mice exhibited an analogous defect, where post-natal bone accrual was attenuated. Conversely, gain-of-function mutations in *Lrp5* have been show to result in high bone-density disorders (Boyden et al., 2002; Little et al., 2002). High bone-density phenotypes associated with Sclerosteosis and van Buchem disease have also been linked to mutations in the secreted Wnt antagonist *sclerostin* (*SOST*) (Balemans et al., 2002; Gardner et al., 2005). Furthermore, genetic ablation of *Sost* and the secrected Wnt antagonist, *Sfrp1,* results in similar bone pathologies in mice (Bodine et al., 2004; Li et al., 2008).

To determine whether CTNNB1, was involved in osteogenesis Bain and colleagues (2003) expressed a stabilized form of this protein within the multipotent mesenchymal cell line C3H10T1/2 and found an increase in ALP expression and characteristic changes in cellular morphology analogous to BMP2-induced osteogenesis. When examining the *in vivo* role of CTNNB1 in developmental osteogenesis Day et al. (2005) created conditional deletions of CTNNB1 in head and limb mesenchyme. When these precursors lacked CTNNB1 there was an inhibition of osteoblast formation and presumptive skeletal tissue consisted mainly of cartilage. Thus, it appears that Wnt/CTNNB1 signaling at the MSC stage directs these cells towards an osteogenic lineage instead of chondrocyte one. Following the same demonstration of the necessity for CTNNB1 during endochondral and intramembranous osteogenic differentiation, Hill

et al. (2005) next asked whether stabilized CTNNB1 would result in the opposite phenotype of increased osteoblast specification from mesenchymal precursors. Interestingly, gain-of-function CTNNB1 expression resulted in a similar impairment in osteogenic differentiation, reminiscent of the loss-of-function phenotype. This initially counterintuitive result makes sense in light of the current view of CTNNB1 during developmental osteogenesis. It has become increasingly apparent that the impact of CTNNB1 on differentiation outcome is highly dependent on the spatial-temporal context of individual cells.

When Boland et al. (2004) treated human MSCs at different differentiation stages, they found that Wnt3a strongly inhibited osteogenic differentiation of naïve MSCs, whereas the same treatment only had a moderate inhibitory effect on MSCs that had already started an osteogenic induction program. This study also demonstrated an inverse correlation between the rates of differentiation and proliferation. This same result was reported when lithium, instead of Wnt3a treatment, was employed to stabilize CTNNB1 (de Boer et al., 2004) or when adipose-derived MSCs were used (Cho et al., 2006). Quarto et al. (2010) examined multiple cell types including juvenile and adult mouse calvarial osteoblasts and found that high concentrations of Wnt3a or stabilized CTNNB1 could have opposite effects when employed on cell types of different developmental histories. Here, Wnt/CTNNB1 signaling promoted osteogenic differentiation of the adult osteoblasts yet resulted in a dose-dependent inhibition of differentiation when applied to the juvenile osteoblasts. This result was recapitulated *in vivo*, where calvarial fracture repair was either inhibited or accelerated based on the age of the mice following Wnt3a treatment. Paralleling these findings, lithium treatment could significantly improve tibia fracture repair within mice, but only when supplied

subsequent to the fracture generation (i.e. presumably during the later stages of osteoblast differentiation) (Chen et al., 2007). An interesting retrospective study comparing patients whom received lithium for treatment of mood disorders showed a statistical decrease in bone fracture risk as compared to matched controls (Vestergaard et al., 2005).

Thus, it appears that canonical Wnt signaling has a positive effect on osteogenesis in mature osteoblasts. Supporting this model, Glass and colleagues (2005) created a conditionally expressed stable form of CTNNB1 under the control of an osteoblast-specific collagen promoter and found a high bone mass phenotype. This was an opposite phenotype from the aforementioned gain-of-function CTNNB1 expressed in the mesenchymal precursor stage (Hill et al., 2005). Interestingly, the higher bone mass found in this study appeared to be a consequence of CTNNB1-mediated transcription of the osteoclast differentiation inhibitor osteoprotegerin (Glass et al., 2005).

Recent evidence has additionally supported the role of CTNNB1 in the regulation of osteoclasts. Wei and others (2011) have shown that canonical WNT signaling promotes osteoclast progenitors to switch from a quiescent to a proliferative state. However, canonical WNT signaling must be downregulated to proceed from the proliferative stage to the terminally differentiated osteoclast.

Non-canonical WNT signaling, in particular by WNT5A, has also been found to be an important regulator of osteogenesis. Although several WNT proteins are expressed in the developing mammalian limb, WNT5A is the only known WNT protein to be expressed in the underlying mesenchymal cells of the progress zone (Gavin et al., 1990; Parr et al., 1993). Additionally, WNT5A is expressed in the neural crest-derived mesenchyme of the frontal-nasal processes during early stages of mouse development.

Wnt5a$^{-/-}$ embryos are characterized by significantly shortened limbs, truncated craniofacial structures, and missing distal digits (Yamaguchi et al., 1999), which may be partly attributed to a delay in chondrocyte hypertrophy and endochondral ossification (Yang et al., 2003). *In vitro*, Wnt5a treatment of human MSCs promoted osteogenic differentiation (Boland et al., 2004). Additionally, Takada et al. (2007) found that within the mesenchymal precursor cell line (ST2), Wnt5a supplementation could suppress the adipocyte specific transcription factor peroxisome proliferator-activated receptor-gamma (PPAR-γ) and induce *RUNX2* expression via the non-canonical Wnt/CamKII pathway. Since adipocytes arise from the same MSC precursors as osteoblasts, it is suggested that the Wnt5a signaling promotes osteoblast specification at the expense of adipogenesis. For a more comprehensive list of skeletal defects arising from genetic manipulation of Wnt signaling components the reader is directed to the review by Regard et al., 2012.

AKT signaling

The serine/threonine kinase AKT (also known as protein kinase B) has been implicated in diverse roles during mammalian development and tissue maintenance. This kinase is primarily studied in the context of cell survival, proliferation, and growth. The signaling pathway that activates AKT begins with growth factors binding to receptors of the tyrosine kinase family (RTK) including fibroblast growth factor receptors (FGFRs), insulin-like growth factor receptors (IGFRs) and the insulin receptor (Hemmings and Restuccia, 2012). Ligand binding elicits RTK dimerization and autophosphorylation of tyrosine residues within the cytosolic domain of these receptors. These phosphorylated residues then serve as docking sites for adaptor proteins, such as the insulin receptor

substrate-1 (IRS1), to mediate downstream signaling. IRS1 then recruits the p85 subunit of the heterodimeric phosphoinositide 3-kinase (PI3K) (Butler et al., 1998). When the catalytic p110 subunit of PI3K is in close proximity to the plasma membrane it will phosphorylate membrane-bound substrates including phosphatidylinositol-4,5 diphosphate (PIP_2). Phosphorylation of PIP_2 produces phosphatidylinositol-3,4,5 triphosphate (PIP_3) (Auger et al., 1989), which serves as a docking site for pleckstrin homology (PH) domain-containing proteins, including AKT and 3-phosphoinositide dependent protein kinase-1 (PDK1) (James et al., 1996). Recruitment to the plasma membrane through this domain is essential for AKT activation (Lemmon and Ferguson, 2000).

Fully active AKT results from its phosphorylation by both PDK1 (Alessi et al., 1997) and mTOR Complex 2, which is composed of the mammalian target of rapamycin (mTOR) and the rapamycin-insensitive companion of mTOR (RICTOR) (Sarbassov et al., 2005). Termination of AKT signaling commences with the action of the protein phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome ten). PTEN removes a phosphate from PIP_3 and converts it back to PIP_2 (Maehama and Dixon, 1998), thereby limiting the substrate needed for Akt recruitment and activation. When AKT is active it phosphorlyates many targets including tuberous sclerosis complex 2 (TSC2) (Cai et al., 2006). TSC2 is a negative regulator of mTOR complex 1 (mTORC1), which includes mTOR and regulatory-associated protein of mTOR (RAPTOR). Since mTORC1 is an important regulator of ribosome production and translation initiation (reviewed by Wullschleger et al., 2006), increasing its activity upregulates protein production, which is necessary for the continued growth and proliferation that is associated with AKT signaling. Accompanying these events, AKT

additionally inhibits pro-apoptotic processes through multiple methods (Marte and Downward, 1997). For instance, AKT can inhibit the pro-apoptotic factor BAD (Peso, 1997), enhance the activity of MDM2 (i.e. the key regulator of the tumor-suppressor p53) (Ogawara et al., 2002), and suppress the transcriptional activity of the forkhead box (FOXO) family of transcription factors.

The FOXO proteins are a set of highly conserved transcription factors that are instrumental to oxidative stress response, apoptosis initiation, inhibition of cell proliferation, and regulation of metabolism (reviewed by Calnan and Brunet, 2008). Some of the target genes facilitating these responses are: *FasL*, *Bim*, *G6Pase*, *Pepck*, *Gadd45*, *MnSOD*, and *catalase* (Greer and Brunet, 2005). In vertebrates there are four FOXO transcription factors: FOXO1, FOXO3, FOXO4, and FOXO6. AKT-mediated suppression of FOXO transcriptional activity arises from increased affinity of the phosphorylated FOXOs for 14-3-3 proteins, resulting in their exclusion from the nucleus (Brunet et al., 1999; Medema et al., 2000; Brownawell et al., 2001).

Interestingly, in 2005 it was suggested that FOXO-mediated transcription requires CTNNB1 as a coactivator in mammalian cells (Essers et al., 2005). Furthermore, work in our lab has recently demonstrated that CTNNB1 and the FOX transciption factors co-occupy the same promotors (manuscript under submission). Although prior to this discovery, there was evidence that demonstrated crosstalk between the AKT and WNT signaling pathways. For instance, the first identified target of AKT was GSK3 (Cross et al., 1995). Later, it was shown that AKT could directly phosphorylate CTNNB1. Both of these actions by AKT result in the stabilization of CTNNB1 and its subsequent transcriptional activity (Fang et al., 2007; He et al., 2007; Baksh et al., 2007). Additionally, the oxidative stress response has been shown to divert

CTNNB1 away from the LEF/TCF transcription factors to increase FOX-mediated transcription (Hoogeboom et al., 2008; Almeida et al., 2007; Hoogeboom and Burgering, 2009).

Aside from its role in mediating cell survial, AKT signaling has been implicated in the maintenance of stem cell populations. The conditional loss of PTEN within primordial germ cells, was shown to dysregulate cell proliferation and cause the formation of testicular teratomas (Kimura et al., 2003). A similar deletion PTEN within neural stem cells resulted in increased brain size characterized by unregulated proliferation, a decrease in apoptosis, and increase individual cell volume (Groszer et al., 2001). With regards to mESCs, inhibition of PI3K, and the resulting suppression of AKT activity, has been shown to reduce the ability of LIF to maintain pluripotency (Paling et al., 2004). Conversly, the expression of a myristoylated, active form of AKT is sufficient to maintainin pluripotency in the absence of LIF (Watanabe et al., 2006). Interestingly, this active form of AKT also could support the self-renewal and differentiation capacity of primate ESCs, which do not respond to LIF as a pluripotency factor.

AKT signaling in bone

Since AKT is intimately involved in processes regulating cell survival, it has been difficult to tease apart these predominant effects from those that may specifically govern bone development and maintenance. However, researchers have recently begun to uncover the role of AKT signaling in bone biology through tissue-directed genetic manipulation. For example, conditional removal of PTEN from osteo-chondroprogenitors in developing mouse embryos showed disorganized growth plates, excessive ECM production, extensive trabeculation, and an increase in cortical bone thickness (Ford-

Hutchinson et al., 2007). When PTEN was conditionally removed from mature osteoblasts (i.e. cre recombinase under control of the osteocalcin promoter), mice did not demonstrate a statistical increase in bone mineral density until 6-weeks after birth. However, this density progressively increased as the mice aged (Liu et al., 2007). On the other hand, knock out of the AKT1 isoform in mice resulted in shortened bones and delayed ossification (Ulici et al., 2009). Double knockouts of AKT1 and AKT2 isoforms resulted in dwarfism and minimal ossified tissue within the skeleton (Peng et al., 2003). Recent evidence has also shown that the sensing of mechanical strain by osteocytes appears to be dependent on AKT-mediated GSK3 inhibition and CTNNB1 transcriptional activity (Sunters et al., 2010).

A putative role for glucose

A number of bone-related pathologies are associated with altered glucose regulation. For example, patients with diabetes mellitus type 1 have a significantly enhanced risk of osteoporosis and bone fractures (Hofbauer et al., 2007). While dysregulation of hormonal factors and the inflammatory response are cited as possible mechanisms for diabetic bone disorders, the mechanisms underlying these pathologies still remain largely unknown (Hamann et al., 2012).

In addition to the multiple classes of growth factors that can activate AKT, exposure to high glucose concentrations have also been found to turn on the AKT signaling events (Xin et al., 2005; Wu et al., 2009). A growing body of evidence has also indicated that canonical Wnt signaling and CTNNB1 can be modulated by processes governed by glucose concentrations (Ni et al., 2003; Fujino et al., 2003; Anagnostou and Shepherd, 2008). Interestingly, a genome-wide SNP analysis has revealed a strong

association with polymorphisms in the *TCF4* gene with a predisposition for type 2 diabetes (Grant et al., 2006; Cauchi et al., 2007). The linkage between glucose dysregulation and its effect on both the AKT and WNT signaling pathways has been explored recently, however these investigations been restricted to models of cancer progression and maintenance (Chocarro-Calvo et al., 2013; Liu et al., 2013; García-Jiménez et al., 2014). Given the critical roles of these signaling pathways regulating osteogenesis and the demonstration that glucose can modulate of these pathways, it is evident that investigations attempting to illuminate the mechanisms controlling glucose-related bone disorders would benefit from examining these pathways.

Aims and Rationale of the Following Chapters

The work presented in the following chapters will encompass an extensive analysis of how modulation of CTNNB1 influences osteogenic differentiation of mESCs. The following chapter will be a more comprehensive review of *in vivo* osteogenesis and will highlight some recent advances in bone tissue engineering. Then the three subsequent chapters will put forth an experimental examination of the consequences of altered or dysregulated CTNNB1 within our osteogenic induction protocol.

First, despite the previous reports of the role of WNT5A in promoting osteogenesis within *in vitro* systems (Baksh and Tuan, 2007; Bilkovski et al., 2010), its effect has only been explored on cells will a limited differentiation potential (i.e. MSCs or osteoblast cell lines). Thus, to the best of our knowledge, these will be the first experiments to examine the role of WNT5A during the differentiation of pluripotent stem cells. As a consequence, the knowledge gained from this work can be applied to broaden our understanding of non-canonical WNT signaling during the early stages of *in*

vivo osteogenesis. Furthermore, it has become increasingly clear that the traditional view of CTNNB1 independence from non-canonical WNT signaling mechanisms is not universally true (Topol et al., 2003; Westfall et al., 2003; Mikels and Nusse, 2006). Thus, we can address the possible crosstalk between noncanoncial signaling and CTNNB1 activity as a possible mechanism through which WNT5A facilitates osteogenesis within our system. Studying this aspect of CTNNB1 regulation will help clarify the increasingly complex WNT signaling pathways and there roles in development and disease manifestation.

Secondly, in addition to the increased risk of bone disorders found with diabetic patients, newborns of diabetic mothers exhibit low bone-mineral density (Mimouni et al., 1988; Verhaeghe et al., 1988; Lampl and Jeanty, 2004). Even though it is reported that these newborns have an increased risk of neonatal mortality and the development of future health complications (CDC, 2014), it is surprising how little research has focused on the mechanisms behind this developmental consequence. From the limited number of studies that have attempted to explore this dysregulated osteogenesis there has emerged two theories: (1) Maternal calcium supply is reduced in diabetic mothers, through a possible increased calcium excretion in the urine (Garland et al., 1997), or inefficient mineral transport through the placenta (Husain et al., 1994); and (2) bone tissue development in the embryo is itself directly impaired, as indicated by reduced ossification centers and OCN expression (Verhaeghe et al. 1995). From an *in vitro* standpoint, the latter of these theories can be addressed. By excluding the possible differences in placental mineral supply through controlled media supplementation, questions addressing tissue differentiation efficacy can be directly asked. Thus, any changes in osteogenic differentiation capacity, through a hyperglycemic environment,

will be attributed to the intrinsic cellular response to these elevated glucose concentrations. The work presented herein is the first study of its kind that aims to uncover the disrupted signaling mechanism by which hyperglycemia attenuates osteogenic differentiation of ESCs *in vitro*.

Lastly, as investigations into the progressive steps of lineage specification are explored, it has become apparent that the response to WNT/CTNNB1 signaling is dependent on the developmental context of individual cells (Regard et al., 2012). The knowledge gained from the study of osteogenic specification and the stage-specific response to WNT/CTNNB1 signaling will be invaluable towards the development of bone engineering protocols. While differential responses to either canonical or non-canonical WNT signaling during *in vitro* differentiation have recapitulated the responses found *in vivo* (Boland et al., 2004; de Boer et al., 2004; Quarto et al., 2010), a multistage CTNNB1 modulation approach to osteogenic differentiation of mESCs has yet to be performed. Furthermore, a number of chemical agents have recently been shown to regulate CTNNB1 transcriptional output (Zimmerman et al., 2012). Thus, replacing recombinant WNT proteins with alternative regulators of CTNNB1 may offer a cheaper and potentially more effective option in the development of osteogenic differentiation protocols. Additionally, the positive effects on differentiation by these alternative regulators can support their prospective use as therapeutic agents to treat bone-related disorders.

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Chapter 2: Osteogenesis from Pluripotent Stem Cells: Neural Crest or Mesodermal Origin?

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 (Figure 1). Two of these bone origins are from mesodermal progenitors, where cells from

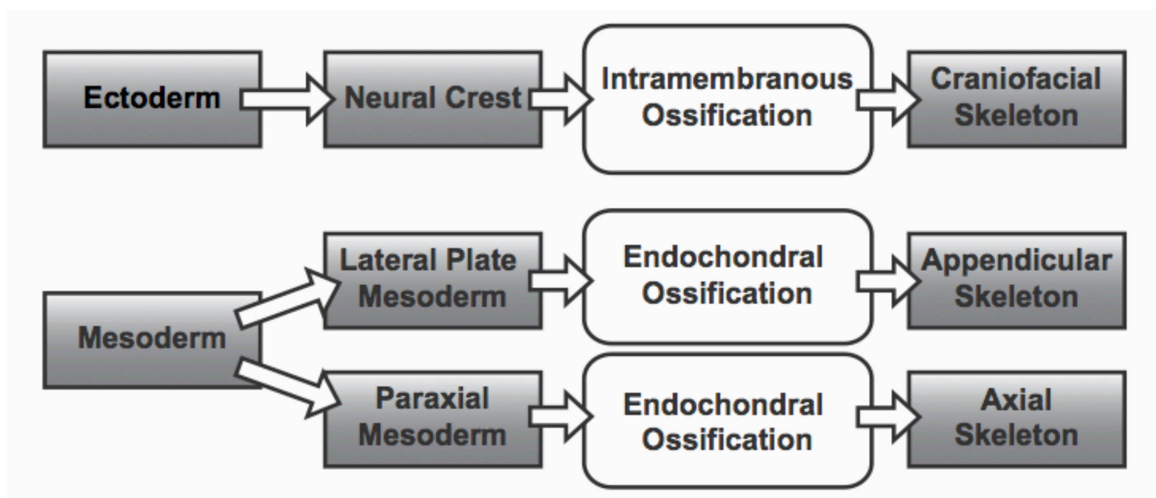

Figure 2.1. Embryonic origins of bone tissue

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.

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.

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.

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).

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.

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.

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.

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.

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.

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.

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. The following sections will give an overview of the developmental origins and the process through which bone is formed in a intramembranous manner.

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).

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.

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 within *in vitro* culture conditions can recapitulate the neural crest osteogenic pathways found in the developing embryo.

Conclusion

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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Chapter 3: Wnt5a supports the formation of ectomesenchyme-derived osteoprogenitors in embryonic stem cells

Abstract:

The specification of pluripotent stem cells into the mesodermal lineage and the involvement of Wnt/beta-catenin signaling has been explored in a number of studies. However, the current body of literature has yet to adequately address the role of the non-canonical Wnt glycoproteins in the differentiation and specification of pluripotent stem cells. During mouse embryonic stem cell (ESC) *in vitro* osteogenesis, the non-canonical Wnt5a is expressed at an early differentiation stage. Cells either sorted by their positive Wnt5a expression or when supplemented with recombinant WNT5a (rWNT5a) during a two-day window showed significantly enhanced osteogenic yield. We further characterized the Wnt5a positive cells to be additionally expressing markers of ectomesenchymal origin; suggesting that non-canonical Wnts may play a role in neural crest-derived osteogenesis, which is in addition to the previously identified role for canonical Wnts in mesodermal differentiation. Additionally, rWNT5a supplementation up-regulated PKC, CamKII and JNK activity while antagonizing the key effector of canonical Wnt signaling: beta-catenin. Chemical inhibitors of PKC, CamKII and JNK reduced the ability of rWNT5a to serve as an osteogenic inducer. Likewise, supplementation with factors that enhance canonical Wnt signaling, during the time window of Wnt5a action, suppressed osteogenic differentiation. Ultimately, elucidating the role of Wnt5a in directing osteogenic differentiation has the potential to considerably improve tissue engineering protocols and applications for regenerative medicine.

Introduction:

Bone development is a complex process involving the crosstalk of multiple tissue types and morphogens. One of the signaling pathways that regulate the specification of bone-matrix-secreting osteoblasts, from mesoderm-derived trunk and limb mesenchyme, is the canonical Wnt signaling pathway. Previous studies have cemented a role for beta-catenin (CTNNB1), the main effector of the canonical Wnt pathway, in the maturation of osteoblasts within long bones (Day et al., 2005; Hill et al., 2005; Hu et al., 2005). However, the same studies have also hinted to the fact that early osteoblast differentiation in the limbs may occur independently of CTNNB1, potentially via non-canonical Wnt signaling. In these early states, the non-canonical Wnt5a is indeed the only known mouse Wnt protein to be expressed (Gavin et al., 1990; Parr et al., 1993) and *Wnt5a*$^{-/-}$ embryos are characterized by significantly shortened limbs and missing distal digits (Yamaguchi et al., 1999).

In contrast, the osteoblasts in the facial bones and jaw are derived from an cranial neural crest (NC) origin, the development of which is also thought to be mainly regulated by canonical Wnt signaling. Specifically, the induction and specification of NC cells in mammals by canonical Wnt signaling is believed to occur while the neural tube is still closing (Nichols, 1986; García-Castro et al., 2002; Stuhlmiller and Garcia-Castro, 2012). However, recent studies are now beginning to unravel a dependence of NC delamination and migration on non-canonical Wnt signaling as well (Mayor and Theveneau, 2014).

In the embryo, developing cells exploit the Wnt signaling network because of its integral role in regulating lineage decisions (Reya and Clevers, 2005; Lyashenko et al., 2011). Because of the importance of this pathway in fate determination it is therefore not surprising that CTNNB1 affects osteogenesis of stem cells *in vitro*. For

example, at specific stages during the *in vitro* differentiation of pluripotent embryonic stem cells (ESCs) CTNNB1 controls gastrulation-like events, (Ueno et al., 2007; Davis and zur Nieden, 2008) which supports the subsequent specification of mesenchymal cells with osteoprogenitor character (Ding et al., 2012).

Since ESCs are pluripotent, they are ultimately capable of differentiating into mature matrix-mineralizing osteoblasts from mesenchymal precursors of either mesodermal or NC origin (Sottile et al., 2003; zur Nieden et al., 2003). Although both NC and mesodermal populations have been isolated from differentiating ESCs (Tada et al., 2005; Lee et al., 2007), it is still unclear how Wnts contribute to these specifications and whether there are specific stages that depend on non-canonical Wnts, as is suggested *in vivo*. Thus, because of the aforementioned activity of WNT5a in both the limb mesenchyme and migrating NC cells, we sought to explore these relationships during the osteogenic differentiation of ESCs. Ultimately, we hope to apply the knowledge gained from this study towards numerous questions facing cell and developmental biology.

Results:

Wnt5a is expressed upon VD_3 induction in ESCs

In agreement with our previous transcriptional profiling (zur Nieden et al., 2007), cells treated with the osteogenic inducer VD_3 demonstrated a significant increase of *Wnt5a* mRNA and WNT5a production on day 7 of the differentiation (Figure 3.1A, B). After establishing this correlation between *Wnt5a* upregulation with VD_3 induction, we next sought to explore the effects of exogenous addition of WNT5a upon the differentiation outcome. Initially, we found recombinant WNT5a (rWNT5a)

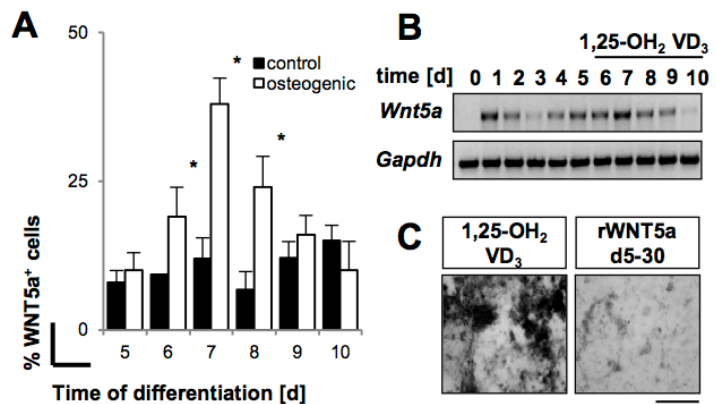

Figure 3.1: Wnt5a is expressed during ESC osteogenesis. (A) Percentage of WNT5a+ cells determined with an anti-Wnt5a antibody and flow cytometry. *$p<0.05$ compared to d5, One-Way ANOVA. (B) RT-PCR uncovered stage-specific expression of *Wnt5a*. (C) Lack of calcification in cultures supplemented with 50 ng/ml recombinant Wnt5a (rWNT5a) between days 5-30. Bar = 100 µm. Gapdh, glyceraldehyde 3-phosphate dehydrogenase; VD_3, vitamin D_3.

treatment at 50 ng/ml, for the duration of the osteogenic induction period (i.e. days 5-30), resulted in reduced ECM mineralization as compared to controls (Figure 3.1C).

Stage-specific WNT5a supplementation augments mineralization

In light of previous studies, where *Wnt5a* expression correlated to early stages of osteogenic differentiation (Gavin et al., 1990; Parr et al., 1993), prolonged rWNT5a treatment in our *in vitro* system may potentially mask pro-osteogenic effects. To test this hypothesis, ESCs were treated with different concentrations of rWNT5a, with and without VD_3, from days 5-7 (i.e. corresponding to the period of endogenous mRNA expression as seen in Figure 3.1B). Treatment with rWNT5a at a concentration of 100 ng/ml without VD_3 resulted in a 2.6-fold increase in Ca^{2+} content as compared to treatment with mineralizing agents β-glycerophosphate (GP) and ascorbic acid (AA) alone (Figure 3.2A). This result demonstrated that rWNT5a could functionally mimic the VD_3-induction of osteogenesis in regards to calcium deposition when supplemented during this time window. A synergistic enhancement of Ca^{2+} content was further noted in rWNT5a+VD_3 groups ($p<0.01$). In addition, rWNT5a supplementation was able to rescue the calcification defect found in *Wnt5a*$^{-/-}$ ESCs

when applied between days 5-7 of the differentiation (Figure 3.2B). Furthermore, we found a significant increase in the expression of the osteoprogenitor markers alkaline phosphatase (*Alk Phos*) and osteopontin (*Opn*) just 24 hours after initial rWNT5a

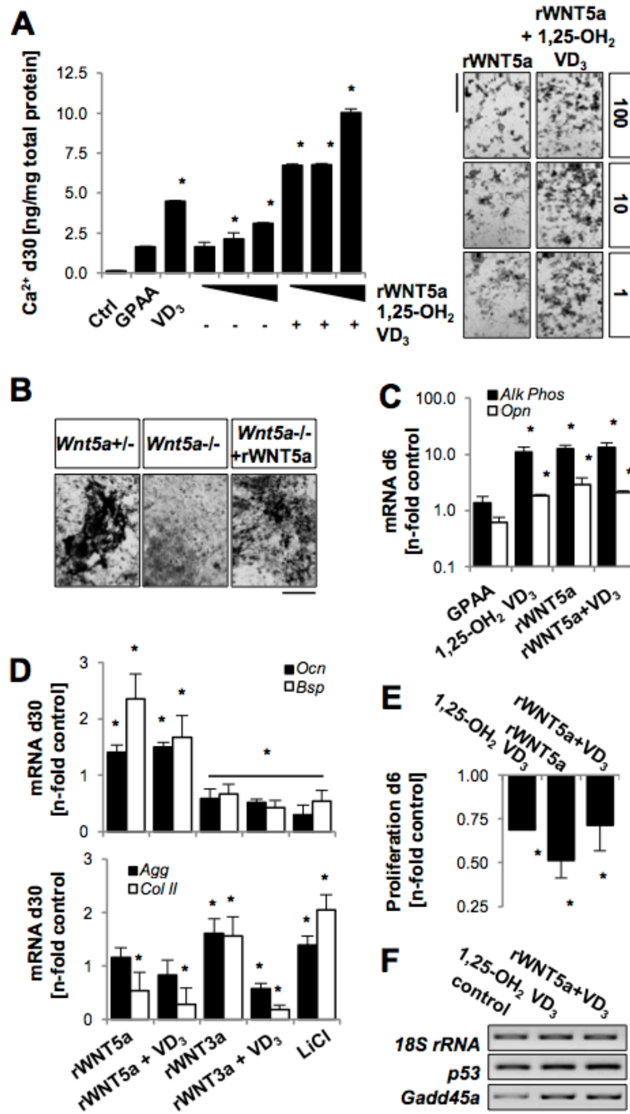

Figure 3.2: Recombinant Wnt5a enhances osteogenic differentiation of murine ESCs. (A) Timed supplementation with rWNT5a (concentrations 1 ng/ml, 10 ng/ml and 100 ng/ml) between days 5-7 led to more deposition of calcium on differentiation day 30. Photomicrographs of cultures in right panel of (A), bar = 100 µm. *p< 0.05 compared to GPAA only, One-Way ANOVA. (B) Treatment with rWnt5a [50 ng/ml] from d5-7 restored calcification in *Wnt5a*$^{-/-}$ ESCs. Bar = 250 µm. (C) Expression levels of bone and cartilage specific genes were measured on differentiation day 30 with quantitative PCR after medium supplementation with rWNT5a or rWNT3a [50 ng/ml] (with or without VD_3) or LiCl [10 mM] between differentiation days 5-7. N-fold changes in expression were calculated with the $\Delta\Delta C_T$ method and correction for PCR efficiency, n=3 ± SD; *p<0.01 compared to VD_3, One-Way ANOVA. (D) Quantitative PCR revealed that pre-osteoblast markers *Alk Phos* and *Opn* were up-regulated over GPAA with rWNT5a either alone or in combination with VD_3. Calculations were performed as described under (C), *p< 0.01 compared to GPAA. (E) Wnt5a effects on proliferation were measured with a BrdU assay 24h after supplementation. *p<0.01 over non-osteogenically induced control cultures, One-Way-ANOVA, n = 6. (F) RT-PCR revealed that rWNT5a and rWNT5a+VD_3 up-regulated mRNAs for cell cycle regulatory genes 24h after supplementation. Alk Phos, alkaline phosphatase; Agg, aggrecan; Bsp, bone sialoprotein; Col II, collagen type II; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; GPAA, glycerophosphate and ascorbic acid; Ocn, osteocalcin; Opn, osteopontin; VD_3, vitamin D_3.

supplementation (Figure 3.2C). We also found a significant increase in osteocalcin (*Ocn*) and bone sialoprotein (*Bsp*) mRNA expression at later stages of the differentiation (Figure 3.2D). Concurrent to the increase in these osteoblast-specific markers, there was also a significant drop in the condrocyte-specific *Aggrecan* (*Agg*) and *collagen type II* (*Col II*) expression (Figure 3.2D). In contrast, treatment with agents that act in a canonical Wnt fashion, such as rWNT3a or lithium chloride (LiCl), resulted in the opposite pattern of marker expression.

Wnt5a treatment increases osteogenic commitment at the expense of proliferation

The rise of bone-specific marker expression following rWNT5a treatment may be attributed to these markers being direct downstream targets of activated signaling cascades, or supplementation may act through more indirect means, such as altering progenitor population specification. Given that Wnt5a has previously been demonstrated to negatively affect cell proliferation (Goodwin et al., 2007), BrdU incorporation in differentiating ESCs was measured following 24 hours of treatment. Both VD_3 and rWNT5a treatment inhibited proliferation, independently as well as when supplemented simultaneously (Figure 3.2E). This inhibition of proliferation was supported by the upregulation of the cell cycle regulatory genes *p53* and *Gadd45* (Figure 3.2F). In conjunction with the upregulation of bone-specific markers, these data suggest a shift from proliferation towards differentiation following treatment with VD_3, rWNT5a, or when used simultaneously. To further investigate whether endogenous Wnt5a expression marks a population with enriched potential to differentiate into osteoblasts, we utilized an ESC line that expressed GFP under the control of the

Wnt5a promoter, GFP detection was found to temporally correlate to *Wnt5a* mRNA levels (Figure 3.3A).

GFP$^+$ cells, sorted on day 7, showed enhanced calcification and Alk Phos activity compared to the GFP$^-$ fraction when measured at the end of the differentiation period (Figure 3.3B, C, and D). Conversely, GFP$^+$ cells sorted on day 5 exhibited reduced calcification when compared to GFP$^-$ cells sorted on the same day.

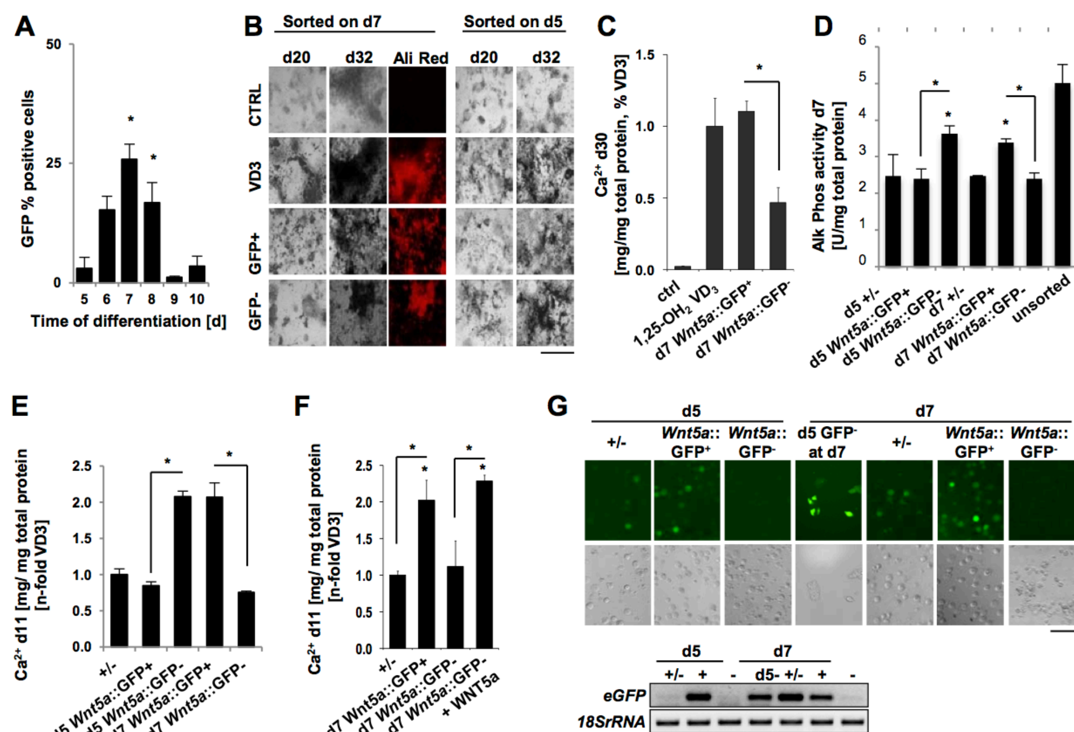

Figure 3.3: A Wnt5a positive population is enriched for cells with osteogenic potential. (A) Percentage of GFP$^+$ cells was determined with flow cytometry in a *Wnt5a*::GFP ESC line that was subjected to osteogenic induction, *p<0.05 compared to d5, One-Way ANOVA. (B) GFP$^+$ cells were flow sorted either on d5 or on d7 and placed back in culture. Osteogenic phenotype was captured in photomicrographs and with Alizarin Red S stain. Bar = 100 µm. (C) Calcium deposition in d7 sorted cells after an additional 23 days in culture. (D) Alk Phos activity of d5 and d7 sorted cells on d7. (E) Early mineralization of flow sorted cultures was determined by quantification of calcium deposited into the extracellular matrix on d11 of culture. (F) Calcium deposit in d7 *Wnt5a*::GFP- cells was rescued with addition of rWNT5a [50 ng/ml]. (G) Fluorescent pictures and RT-PCR for GFP to follow GFP expression in d5 sorted cells over time. Cells that were negative for GFP on d5 acquired GFP expression by d7. Bar = 50 µm. *p<0.05, One-Way ANOVA. Alk Phos, alkaline phosphatase; VD_3, vitamin D_3.

In both cases, these differences in mineralization could be detected as early as day 11 (Figure 3.3E). Furthermore, the degree of mineralization in day 7 GFP^- cells could be rescued if cultures were additionally supplemented with rWNT5a (Figure 3.3F). Day 5 GFP^- cells could acquire GFP expression by day 7, potentially explaining their increased capacity for osteogenesis (Figure 3.3G). Thus, it appeared that the varying capacity of these GFP reporter cells to differentiate along the osteogenic lineage was dependent on endogenous *Wnt5a* expression.

Osteogenic induction proceeds through mesenchymal specification of cells with ectodermal character

Following the time course of osteogenic differentiation, an upregulation of the mesenchymal markers *CD105* and *CD73* during the period of increased Wnt5a expression was noted, which also corresponded to a secondary wave of T-brachyury (*Tbra*) expression (Figure 3.4A). *Tbra*, although generally recognized as an early mesoderm marker, has been demonstrated to regulate mesenchymal tissues in mice during later development (Liu et al., 2003) and is expressed in trunk NC cells. Since mesenchymal cells can arise from multiple developmental origins, we next sought to determine if VD_3- or rWNT5a-induction of osteogenesis revealed expression patterns indicative of particular progenitor cell types.

Both treatments up-regulated mRNA levels for *Wnt5a* and down-regulated a LEF/TCF::GFP reporter, possibly indicating that this non-canonical Wnt supports a feed-forward loop to enhance non-canonical signaling (Figure 3.4B). In addition, mRNA levels for the NC specific genes *Sox1* and *Sox10* were present, but unchanged following treatment with either VD_3 alone or in combination with rWnt5a. Although, *Slug*, *Snail* and *p75* expression increased, indicating a potential NC origin of these

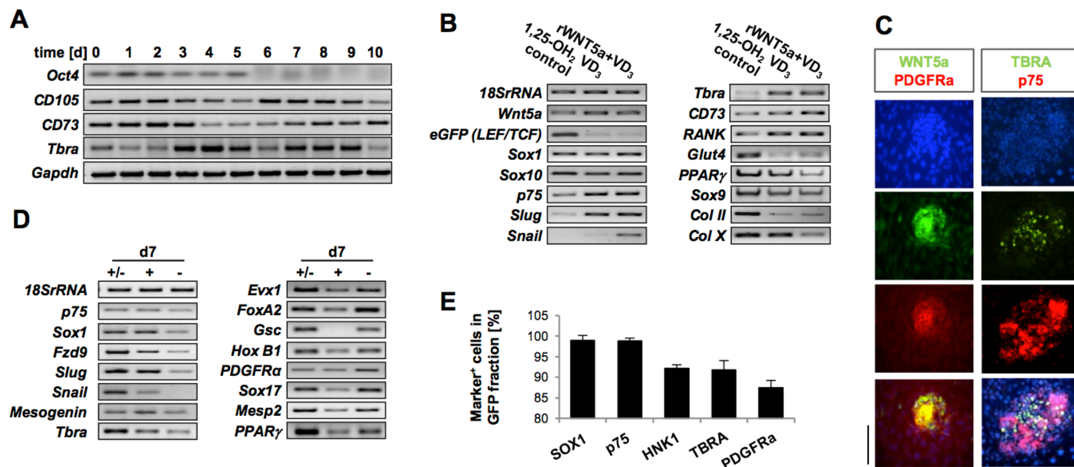

Figure 3.4: The Wnt5a$^+$ population shares markers of mesenchymal cells with neural crest identity. (A) RT-PCR for pluripotency-associated and mesenchymal genes. (B) Differentiating cultures were either supplemented with VD_3 alone or in combination with rWNT5a [50 ng/ml] on d5 and profiled for expression of mRNAs specific for neural crest induction and migration, mesenchymal identity and transcription factors as well as extracellular matrix genes associated with early osteoclast-, adipogenic and chondrogenic lineages on d7. (C) Staining with specific antibodies revealed co-localization of WNT5a$^+$ cells in clusters with cells of neural crest and mesenchymal nature. Bar = 100 µm. (D) d7 *Wnt5a*::GFP$^+$ cells were flow sorted and profiled for expression of mRNAs typically found in cells of neural crest, mesenchymal, endodermal or mesodermal origin. (E) Percentage of cells positive for neural crest and mesenchymal markers in the *Wnt5a*::GFP$^+$ fraction determined with flow cytometry. Alk Phos, alkaline phosphatase; Col, collagen; Fzd, frizzled; Glut4, glucose transporter type 4; Gsc, goosecoid; HNK1, human natural killer-1; Mesp, mesoderm posterior; PDGFR, platelet derived growth factor receptor; PPAR, peroxisome proliferator activated receptor; RANK, receptor activator of nuclear factor-kB ; Sox, sex-determining region Y (Sry) box-containing; Tbra, brachyury; VD_3, vitamin D_3.

osteoprogenitors. From the lack of *Sox1* and *Sox10* induction, it appeared that Wnt5a mediated its pro-osteogenic effect after NC cells had been specified. We also found that both treatment groups demonstrated an increase in mesenchymal marker (e.g. *CD73*) expression on differentiation day 7, which was concomitant with both a reduction of adipocyte markers (e.g. *Glut4* and *PPARγ*) and chondrocyte markers (e.g. *Sox9, Col II*, and *Col X*) (Figure 3.4B). Collectively, these results suggest that

Wnt5a may participate in the transition from NC cells towards mesenchymal cells, which then follow a primarily osteoprogenitor fate at the expense of chondro- and adipogenic progression.

The detected changes in marker expression upon rWNT5a supplementation led us to next examine whether cells that endogenously express *Wnt5a* also express defined lineage markers under osteogenic differentiation conditions. Cell-dense foci were found to concurrently expressed *Wnt5a*, neuroectodermal, and mesenchymal markers (Figure 3.4C). RT-PCR analysis of sorted Wnt5a^{+} cells on day 7 revealed an expression profile of NC-specific markers, which was unique from cells lacking Wnt5a expression on this day (Figure 3.4D). Further quantification via flow cytometry of day 7 Wnt5a^{+} cells demonstrated a high degree of neuroectodermal and mesenchymal marker expression (Fig 4E), thereby implicating a NC origin of the Wnt5a^{+} cells.

Both VD_3 and Wnt5a decrease nuclear levels of CTNNB1

Despite the fact that it is typically the canonical Wnts and CTNNB1 that are thought to determine the fate of NC cells (Brault et al., 2001; Hasegawa et al., 2002), it appeared that in ESCs Wnt5a, a non-canonical Wnt, contributed to the specification of osteoprogenitor cells which had NC character. While non-canonical Wnts, such as Wnt5a, have been classically viewed to act independently of canonical Wnt signaling, a growing body of evidence has uncovered numerous instances of cross-talk between these pathways (Topol et al., 2003; Westfall et al., 2003). Reports indicate that cells expressing a profile of specific Frizzled (Fzd) receptors, co-receptors, and cytoplasmic regulators of CTNNB1 were able to activate canonical Wnt signaling cascades in response to Wnt5a (van Amerongen et al., 2012; He et al., 2004; Mikels and Nusse, 2006). Thus, we next aimed to examine the relationship between Wnt5a

and CTNNB1 during the time period in which this non-canonical Wnt has a pro-osteogenic effect.

First, we examined the expression of Wnt antagonists and receptors. Expression of *Fzd4*, a receptor that has been reported to mediate Wnt5a activation of canonical Wnt signaling (Katoh and Katoh, 2007), was decreased during the time window of Wnt5a action (Figure 3.5A). Concurrently, *Fzd2*, a related receptor known to mediate non-canonical signaling by Wnt5a (Sato et al., 2010), and the secreted canonical Wnt antagonist Dickkopf1 (*Dkk1*) were highly expressed. Low nuclear CTNNB1 localization and activity of the LEF/TCF::GFP reporter were also detected at that time (Figure 3.5B).

Analyzing the LEF/TCF::GFP reporter line as a functional readout of CTNNB1 transcriptional activity, we detected a decrease in *GFP* mRNA expression following VD_3, rWNT5a, and rWNT5a + VD_3 treatment, which corresponded to: a decrease in *CtnnB1* expression (Figure 3.5C), an increase of *GSK-3β* mRNA expression (Figure 3.5C), a decrease in nuclear CTNNB1 levels (Figure 3.5D), and low GFP protein levels (Figure 3.5E). Conversely, LiCl and rWNT3a supplementation caused an increase in GFP expression (Figure 3.5E). Thus, it appears that modulation of CTNNB1 localization, at this time point, could lead to predicable outcomes of osteogenic output. We tested this further by artificially elevating nuclear CTNNB1 with 6-bromoindirubin-3′-oxime (BIO), a GSK-3β inhibitor. Treatment with BIO dramatically reduced subsequent mineralization of the cultures (Figure 3.5G). Additionally, when compared to the day 5-7 treatment group, both shorter and longer BIO treatments were not as effective in reducing mineralization; highlighting the importance of this time window and low CTNNB1 nuclear levels in directing osteogenic differentiation.

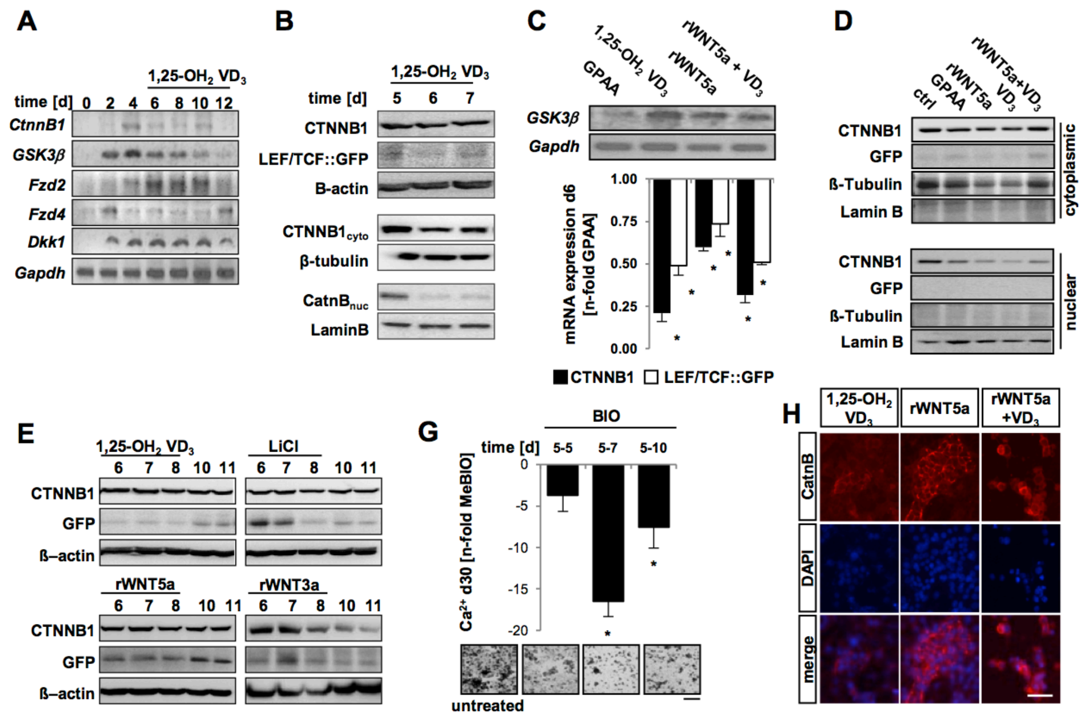

Figure 3.5: The time window and action of Wnt5a coincides with low levels of nuclear CTNNB1. (A) RT-PCR for endogenous expression of Wnt pathway associated mRNAs. (B) Western blot analysis on whole cell lysates of a LEF/TCF::GFP ESC line (top) and on fractionated lysates (bottom). (C) Cells were treated with BIO [1 μm] to artificially elevate CTNNB1 levels at time points indicated while being induced to osteoblasts. Calcium deposition was measured on d30 and normalized to calcium content in cultures treated with the kinase inactive BIO derivative MeBIO. *p<0.01, One-Way ANOVA. Photomicrographs in bottom panel, bar = 100 μm. (D) RT-PCR for *GSK3β* and qPCR for total *CtnnB1* and *GFP* (LEF/TCF::GFP ESCs) 24h after Wnt5a addition. N-fold changes in expression were calculated with the $\Delta\Delta C_T$ method and correction for PCR efficiency, n=3 ± SD; *p<0.01, One-Way ANOVA. (E) RT-PCR on cultures treated with VD_3 alone and in combination with Wnt5a showed enhanced expression of Wnt inhibitors *Sfrp3* and *Dkk1* as well as induction of expression of *Fzd9*, a neural-crest associated Fzd receptor, 24h after treatment initiation. (F) CTNNB1 immunostaining (d6) in VD_3 and Wnt5a treated cells either alone or in combination, bar = 100 μm. (G) Western blot on fractionated protein lysates from LEF/TCF::GFP ESCs 24h after treatment initiation. (H) Time course Western blots on total protein lysates using a LEF/TCF::GFP cell line following treatment between d5-8. BIO, 6-BromoIndirubin-3′-Oxime ; CTNNB1, beta-catenin; CF, cytoplasmic fraction; Dkk, dickkopf; Fzd, frizzled; GSK, glycogen synthase kinase; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; GPAA, glycerophosphate and ascorbic acid; MeBIO, methyl-BIO; NF, nuclear fraction; Sfrp, secreted frizzled related protein; VD_3, vitamin D_3.

We also noted a plasma membrane localization of CTNNB1 following 24 hours of VD_3, rWNT5a and rWNT5a + VD_3 treatment (Figure 3.5H). These results further support the pro-osteogenic role for both VD_3 and rWNT5a through the downregulation of nuclear CTNNB1 and its correlated reduction in transcriptional activity.

Inhibition of downstream effectors of Wnt5a suppress osteogenesis

In order to address how Wnt5a signaling specifically affected CTNNB1 transcriptional activity, we examined three known downstream effectors of Wnt5a signaling: Ca^{2+}/calmodulin-dependent protein kinase II alpha (CAMKIIα), c-Jun N-terminal kinase (JNK) and protein kinase C (PKC). All three of these downstream effectors were detectable during the window of pro-osteogenic action of Wnt5a (Figure 3.6A). An upregulation of the alpha isoform of *PKC* was noted, and to a lesser extent the beta isoform, following treatment with VD_3 or rWnt5a+VD_3 (Figure 3.6B). In a PKC activity assay, rWNT5a treatment produced a significant increase of activity above GPAA controls following analysis 15min, 45min and 12h post-treatment (Fig 6C). However, a statistical difference in PKC activity was only detected at the 12h period following VD_3 supplementation. Thus, although both osteogenic inducers achieve an increase in PKC activity, they appear to do so with different kinetics. Consequently, the combinatorial treatment of rWNT5a+VD_3 achieved a significant upregulation of PKC activity at all time points examined. We also detected a clear upregulation of *CamKIIα* as well as *Jnk1* and *Jnk2* expression following both VD_3 and rWNT5a treatment (Figure 3.6B). In addition, supplementation with both of these osteogenic inducers resulted in an increase in active CAMKIIα and JNK1/2 as determined by the presence of activating phosphoryl groups after 45 minutes, although JNK1 was only minimally changed (Fig 6D).

However, in contrast to PKC activation, this effect did not persist when examined at 12 hours post-treatment (data not shown).

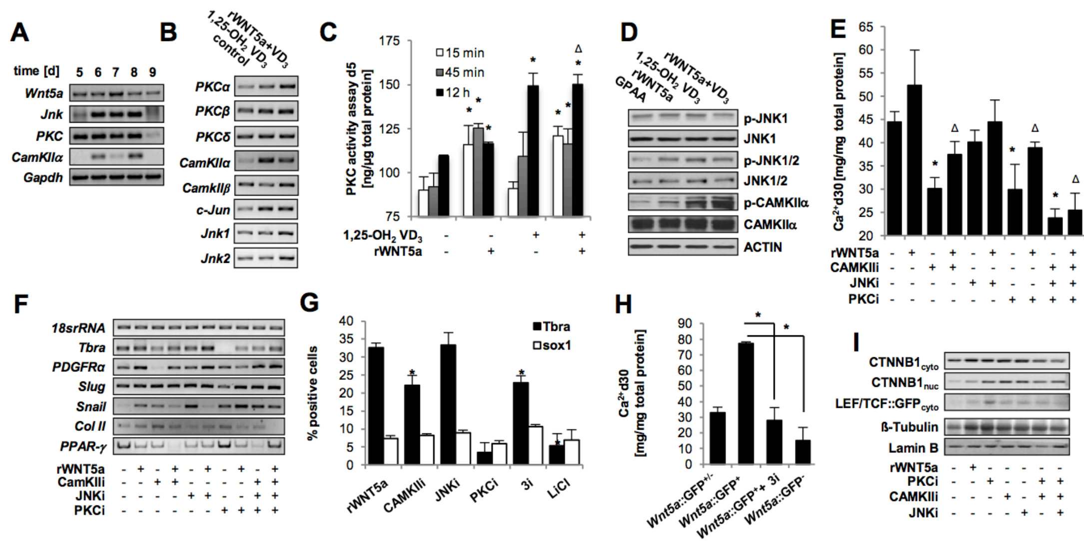

Figure 3.6: Wnt5a signals through CAMKII, JNK and PKC to enhance osteogenesis. (A) Elevated mRNAs for *CamKII, Jnk*, and *PKC* coincide with peak *Wnt5a* expression. (B) RT-PCR of isoform-specific expression of these downstream components following media supplementation for two days with indicated treatments. (C) A PKC activity assay revealed changes in activation kinetics in this kinase after rWNT5a supplementation. (D) Western blot analysis of activated JNK (p- T183 Y185) and CAMKIIα (p-T286) levels after rWNT5a treatment with or without VD_3 induction. (E) Osteogenic yield as quantified by determination of extracellular matrix calcium content after treatment with $CAMKII_i$ [400 nM], JNK_i [40 nM] or PKC_i [100 nM]; *$p<0.01$ as compared to VD_3 induction only (first column), $\Delta p<0.01$ compared to VD_3 + Wnt5a. (F) Expression analysis was done with semi-quantitative PCR on d7 of differentiation to characterize changes in progenitor identity upon inhibition of potential Wnt5a sub-pathways. (G) Flow cytometry was performed on cultures treated as indicated and stained with anti-Tbra or anti-Sox1 antibodies, n=3 ± SD, *$p<0.01$ compared to Wnt5a alone. (H) Calcium content in cultures flow sorted for Wnt5a::GFP expression on d7 and returned to culture with $CamKII_i$, JNK_i and PKC_i (3i) in concentrations as stated in (E), *$p<0.01$, One-Way ANOVA. (H) Ca^{2+} content in cultures sorted for Wnt5a and treated with 3i, n=5±SD, *$p<0.01$, One-Way ANOVA. (I) Analysis of CTNNB1 levels and transcriptional activation through Western blotting on fractionated protein lysates using a LEF/TCF::GFP ESC line with or without inhibitor treatment. CamKII, calmodulin kinase II; $CamKII_i$, CamKII inhibitor KN-93; CTNNB1, beta-catenin; Col, collagen; cyto = cytoplasmic protein lysate; JNK, c-jun kinase; JNK_i, JNK inhibitor II; nuc, nuclear protein lysate; PDGFR, platelet-derived growth factor receptor; PPAR, peroxisome proliferator activated receptor; PKC, protein kinase C; PKC_i, PKC inhibitor Calphostin-C; Tbra, brachyury; Tbx, T-Box.

If these downstream effectors mediated the effect of rWNT5a on osteogenic differentiation and CTNNB1 localization, then their inhibition should abrogate these effects. Initial morphological examination of our cultures revealed that chemical inhibition of all three effectors reduced mineralized ECM. Individually each inhibitor treatment during days 5-7 significantly reduced calcium deposition, with PKC and CAMKIIα inhibition resulting in the strongest impact on ECM calcification (Figure 3.6E). We also detected a down-regulation of *Slug* and *Tbra* following PKC inhibition and a dampened *Pdgrf*α and *Snail* expression following CAMKII inhibition (Figure 3.6F). At the same time, treatement with both inhibitors correlated to an up-regulation of adipogenic and chondrogenic genes. CAMKII and PKC inhibition also resulted in a reduction of TBRA protein, with the PKC inhibitor being approximately six times more potent (Figure 3.6G), while Sox1 levels were unaffected. With *Tbra* and *Pdgrf*α being associated with mesenchymal progenitors and *Slug* and *Snail* being involved in NC migration through the regulation of E-cadherin (Peinado et al., 2004), these immediate changes suggest that PKC and CAMKII regulate the migration and mesenchymal potential of NC cells derived from osteogenically differentiating ESCs. The described changes in early mesenchymal and migratory gene expression in response to the inhibitors indeed correlated with an inhibition of calcification in the Wnt5a^{+} population (Figure 3.6H). Furthermore, we found an upregulation of CTNNB1 in the nuclear fraction of cells treated with each inhibitor; further supporting the role of WNT5a and its effectors as negative modulators of nuclear CTNNB1 and its subsequent transcriptional activity (Figure 3.6I).

Discussion:

The importance of the canonical Wnt signaling pathway and CTNNB1 in relation to *in vivo* and *in vitro* osteogenesis has been explored in a number of studies; however, the current body of literature has yet to fully address the role of non-canonical Wnts in specific stages of osteogenesis. By using an *in vitro* pluripotent stem cell model, with the potential of generating osteoblasts from both mesoderm and NC origin, we explored the endogenous expression of the non-canonical *Wnt5a* and the consequence of rWNT5a supplementation in culture.

We propose that endogenous expression of WNT5a *in vitro* can mark a progenitor population with osteogenic potential. Those cells positive for *Wnt5a*::GFP co-expressed a number of markers, which indicated an NC origin. However, since rWNT5a does not appear to alter Sox1 and Sox10 levels, we believe that WNT5a is not involved in NC induction, but rather later stages of NC development. This hypothesis seems fitting given that the timeline of events during ESC osteogenesis coincides with the suggested sequence of events during embryogenesis and *in vivo* NC induction. For instance, Basch and colleagues (2006) have put forth the idea that *in vivo* NC induction may occur simultaneously with gastrulation, thereby making it a very early embryonic event. During ESC differentiation, gastrulation-like events as indicated by presence of TBRA have been described between day 2.5 and 5 after differentiation induction (Fehling et al., 2003; Ding et al., 2012), right before the time window of our reported action of Wnt5a. By extension this would suggest that NC induction in ESCs could indeed occur as early as the first up-regulation of *Tbra* expression, but right before specification of the Wnt5a^{+} progenitors. In fact, we have previously shown that activation of canonical Wnt signaling at a time of gastrulation in ESCs can enhance the osteogenic output (Ding et al., 2012). We have attributed this

effect to the up-regulation of *Tbra*, and hence a CTNNB1-dependency on mesodermal osteogenesis. In the light of our newer findings, these past results may also suggest a dependency of NC induction by CTNNB1, which has been put forth for avian and amphibian embryos, but is still debated in mammalian systems (Jones and Trainor, 2005). The propensity of Wnt5a$^+$ cells to generate osteogenic derivatives suggests that this NC population contains cranial NC cells, but the additional detection of *Tbra* also suggest that this population additionally contains trunk NC cells.

Upon rWNT5a treatment and in Wnt5a:GFP$^+$ cells, we found altered *Slug* and *Snail* expression, which are typically associated with epithelial to mesenchymal transitions (EMT), which are required for NC cells to delaminate, primarily in the cranial NC (Nieto al, 1994; Kalcheim and Burstyn-Cohen, 2005). In a multitude of other cells, normal and cancerous, Wnt5a contributes to the regulation of these EMT events (Ford et al., 2014). Given this link among Wnt5a, EMT, and NC delamination the altered expression of *Slug* and *Snail* could be indicative of Wnt5a regulating NC cell migration during ESC osteogenesis. Although the migratory behavior of Wnt5a$^+$ cells was not directly examined here, irregular distribution of calcified regions in Wnt5a$^-$ cells and CTNNB1 redistribution to the plasma membrane would underscore the plausibility of this explanation.

Based on the alterations in mRNA expression and cellular identity upon rWNT5a supplementation, we also forward the hypothesis that Wnt5a may—at least partially—contribute to the specification of NC cells to adopt a mesenchymal-osteoprogenitor fate. For example, the decrease in expression of the adipocyte-specific transcription factor peroxisome proliferator-activated receptor-gamma *(PPARγ)* in rWNT5a treated cells goes hand in hand with the reported suppression of *PPARγ* and induction of runx2 expression in the ST2 mesenchymal precursor cell

line by Wnt5a (Takada et al., 2007). Since adipocytes and osteoblasts arise from the same mesenchymal precursors, it is plausible that rWNT5a treatment promotes osteogenesis at the expense of adipogenesis through the regulation of lineage-specific transcription factors.

Despite this evidence for the involvement of Wnt5a in the specification of NC-derived cells, it is typically the canonical Wnts and CTNNB1 that are thought to determine the fate of NC cells. As such, targeted inactivation of CTNNB1 in the dorsal neural tube of mouse embryos results in severe defects in cranial NC derivatives including the craniofacial skeletal elements (Brault et al., 2001; Hasegawa et al., 2002). Since Wnt5a may signal through canonical cascades, depending on the receptor make-up of the cell, it is therefore possible that the pro-osteogenic role of Wnt5a was mediated by CTNNB1. However, in the present work, multiple lines of evidence support the negative regulation of CTNNB1 by Wnt5a including: increased expression of canonical Wnt antagonists following rWNT5a treatment, a redirection of CTNNB1 from the nucleus to the plasma membrane, and attenuated activity of a LEF/TCF::GFP reporter. Ishitani and co-workers (2003) found that suppression of canonical Wnt signaling was achieved by WNT5a through CAMKII activation, a Wnt5a effector whose activation was also found here.

In addition to CamKII activation, we found activation of JNK and PKC upon rWNT5a, which was also found in cells supplemented with VD_3. JNK, specifically, has been implicated in the migration of cranial NC cells (Li et al., 2001) providing another link between Wnt5a and VD_3 and its potential role in NC migration. Although VD_3 has been classically characterized to act through the vitamin D receptor (VDR) (Jurutka et al., 2007), VDR seems to contribute to bone formation postnatally (Kato et al., 1999). Instead, the membrane associated protein protein disulfide isomerase A3 (PDIA3) has been suggested to promote early osteogenesis through rapid activation

of PKC (Boyan et al., 2006; Olivares-Navarette et al., 2012). Therefore, PKC may be a common node for which the osteogenic induction by VD_3 and Wnt5a is mediated.

In summary, modulation of CTNNB1 by WNT5a through its effectors can enhance *in vitro* osteogenic differentiation of ESCs, potentially from the ectomesenchyme, and by extension, may regulate normal NC development *in vivo*.

Materials and Methods:

ESC culture. Murine D3 embryonic stem cells (American Type Culture Collection, Rockville, MD, USA) were expanded as described (zur Nieden et al., 2003). Differentiation was initiated through embryoid body formation and osteogenic lineage commitment was induced with active vitamin D3 (1,25-OH_2 VD_3) as described (zur Nieden et al., 2003).

Genetically modified ESC lines. A Wnt5a::GFP reporter ESC line was generated by transfecting an ApaL1 linearized and gel purified Wnt5a-eGFP reporter plasmid into the D3 ESCs, which uses the pEGFP-1 backbone from Clontech to drive GFP expression from the murine Wnt5a promoter (generously provided by Dr. D. Rancourt, University of Calgary). Transfection of ESCs and identification of clones were performed as previously described (zur Nieden et al., 2005). LEF/TCF::GFP ESCs were donated by Dr. I. Weissmann (Stanford University) and *Wnt5a*$^{-/-}$ as well as *Wnt5a*$^{+/-}$ ESCs were kindly provided by Dr. Kang-Yell Choi (Yonsei University, Korea).

Analytical flow cytometry and FACS sorting. For analytical flow cytometry, cells were harvested at days indicated. Single cell suspensions were generated with

trypsin and cell numbers and viabilities were determined using a CASY cell counter. Two million cells per sample group were run through a Cytomics™ FC500 instrument (Beckman Coulter) against a control sample from time-corresponding wildtype cells or IgG controls. Appropriate scatter gates were employed during analysis with the CXP software (Beckman Coulter) to avoid cellular debris and cell aggregates. For sorting experiments, cultures were harvested on d5 and d7. Cells were resuspended in PBS/1% FCS and sorted into 6-well culture plates containing complete osteogenic medium using a BD FACSaria. Cultures were phenotypically analyzed on d25 with the GelCount™ system from Oxford Optronics.

RNA isolation, semi-quantitative and real-time quantitative PCR. Total RNA was isolated and transcribed into cDNA as described (zur Nieden et al., 2003). Quantitative PCR was performed essentially as described using a SYBR green PCR master mix (Abgene) (Dienelt and zur Nieden, 2011). A melting curve was obtained for each PCR product after each run to confirm the presence of a single amplicon. Primers were designed with primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and run through the reverse e-PCR mask on the NCBI website to determine their specificity (http://www.ncbi.nlm.nih.gov/sutils/e-pcr/reverse.cgi) (Table 1). Primer sequences were designed to have an annealing temperature of 60°C and can be found in the supplemental data. Primer sequences for bone and cartilage specific genes as well as for lineage markers have been described elsewhere (Cormier et al., 2006; zur Nieden et al., 2003, 2005, 2007; Ding et al., 2012).

Biochemical determinations. Cells were rinsed in PBS and lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5

mM EDTA) containing protease inhibitors (P-8340; Sigma-Aldrich, St. Louis, MO). After gentle rocking for 30 min, lysates were collected, centrifuged and an aliquot of the supernatant mixed with DC protein assay reagents from Biorad. After 15 min incubation at room temperature, absorbance was read in a Safire II (Tecan) at 750 nm. Protein quantities in samples were taken from a BSA standard curve. Alk Phos enzyme activity and calcium measurements were performed as described (Davis et al., 2011).

Protein expression analysis. Cells were harvested in 1xPBS (pH 7.4) containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (1:100, Sigma), sodium fluoride (10 mM, Sigma) and sodium vanadate (1 mM, Sigma). Protein concentrations were measured as described above and boiled samples were run on a 12% SDS-polyacrylamide gel. Blotted proteins were probed with either a mouse monoclonal anti-CTNNB1 (Invitrogen), a goat polyclonal anti-GFP (Abcam), a mouse monoclonal anti-β-actin (Santa Cruz), a mouse monoclonal anti-CamKIIα (Abcam), rabbit polyclonal anti-phospho-CamKIIα (T286), anti-JNK1, anti-phospho-JNK1 (T183, Y185), anti-JNK1/2 or anti-phospho JNK1/2 (T138, Y185) antibody (all Abcam) followed by horseradish peroxide-conjugated polyclonal anti-mouse (Santa Cruz), anti-goat (Invitrogen), or anti rabbit (Cell Signaling) IgG. Detection was performed with the ECL Western Blotting detection kit (Amersham). Blots were then incubated with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50°C for 1 h with occasional agitation and reprobed.

Immunocytochemistry. Cultures were fixed with 2% PFA in PBS for 20 min at 4°C. Unspecific binding was blocked with 10% FCS in PBS for 30 min at room temperature. CTNNB1 protein was detected with either mouse anti-CTNNB1

(Zymed) at 10 µg/ml, goat anti-Wnt5a (R&D Systems) at 1:80, goat anti-Tbra (R&D Systems) at 1:80, mouse anti-PDGFRα (abcam) at 200 µg/ml and rabbit anti-p75 (abcam) at 1:250. Cells were overlaid with the first antibody in PBS, 10% FCS and incubated at 4°C over night. The secondary antibodies, a goat anti mouse IgG (H+L) Alexa Fluor 546-conjugated (Invitrogen), a goat anti-rabbit Alexa Fluor 546 (Invitrogen), and a donkey anti-goat Alexa Fluor 488 (Invitrogen) were diluted to 1:2000 with 5% FCS in PBS and applied to the cells for 2 hours at 4°C. Cells were imaged with a Leica fluorescence microscope.

Proliferation assay. The proliferative capacity of the cells was determined using BrdU incorporation (BrdU labeling & detection kit, Roche) according to the manufacturer's instructions. 50.000 cells per treatment group were inoculated on differentiation day 5 and treated as indicated. After three hours, BrdU labeling solution was added and proliferation measured after an 18h incubation period.

PKC activity assay. Cells were harvested in buffer containing 20mM MOPS, 50 mM β-glycerolphosphate, 50Mm sodium fluoride, 1mM sodium orthovanadate, 5mM EGTA, 2mM EDTA, 1% NP40, 1mM dithiothreitol (DTT), 1mM benzamidine, 1mM phenylmethanesulphonylfluoride (PMSF) and 10 µg/mL leupeptin and aprotinin. PKC activity was measured using the Enzo Life Sciences PKC kinase activity kit as per the manufacturer's instructions.

Statistical analysis. Data are presented as mean ± SD. Comparison of two groups was made using Student's t-test for unpaired data. Comparison of more than two groups was conducted using ANOVA. A p-value of above or equal to 0.05 was considered significant.

Table 3.1: Primers

Name	Forward sequence	Reverse sequence
18S	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC
AGG	GATCTGGCATGAGAGAGGCG	GCCACGGTGCCCTTTTTAC
ALP	GTGCCCTGACTGAGGCTGTC	GGATCATCGTGTCCTGCTCAC
BSP	AAAGTGAAGGAAAGCGACGA	GTTCCTTCTGCACCTGCTTC
CamKIIα	ATCGCCTATATCCGCATCAC	ACATTCCACGGACAAAGAGC
CamKIIβ	GGACACCGTTACTCCTGAA	CCCACTGAGAAATTCCGTGT
CatnB	CCCTGAGACGCTAGATGAGG	TGTCAGCTGAGGAATTGCAC
CD105	GATTGCCCAGTGATTCTCC	GAGGATGCGGACACTTTTG
CD73	GAACCCAACGTGCTGTTTTT	GGGATCAATCAGTCCTTCCA
c-jun	TCCCCTATCGACATGGAGTC	GCTTAAGCTGTGCCACCTGT
Col II	GCTGCTGACGCTGCTCATC	GGTTCTCCTTTCTGCCCCTT
Col X	CAAGCCAGGCTATGGAAGTC	AGCTGGGCCAATATCTCCTT
Dkk1	GTCCAAGATCTGTAAACC	GAGTCAAGACAATCAACC
evx-1	GCACACAGCCTGATTAGCAA	AGAGGCGCTGAAGGTTTGTA
FoxA2	GACATACCGACGCAGCTACA	GGCACCTTGAGAAAGCAGTC
Fzd2	CCGCGCCACCTTCTGGGCTG	CATACCACGCGCTTTTGGAG
Fzd4	GTCAAGATCGGGGTCTTCTC	CAAGCTGTGACCTGCTAAAG
Fzd9	AAGACGGGAGGCACCAATAC	AACCATAACTCACAGCCTAG
Gadd45a	GCTCAACGTAGACCCCGATA	GTTCGTCACCAGCACACAGT
GAPDH	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA
GFP	CCCACCCTCGTGACC	GCGGATCTTGAAGTT
Glut4	ATGGCTGCTGCTGGTTTCTC	ACCCATAGCATCCGCAACAT
Gsc	CTCGGAGGAGTCAGAAAACG	TCGACTGTCTGTGCAAGTCC
GSK-3β	TGGCGTGTGATGTCAGGTAT	TAAGCTGGCATCTGCAACAC
HoxB1	GGCAGGAGTTGGGAAATGTA	GGCTGACTCCAGATCAAAGC
JNK	GGCATGGGCTACAAGGAGAA	CCACTGATCAATATAGTCCCTTCC
JNK1	GTGTGCAGCTTATGATGCTATTCTTGAA	GGATTTTGTGGCAAACCATTTCTC
JNK2	GGAGCTGGTGAAAGGTTGTGTG	GCGGGGTCATACCAAACAG
LRP5	CTGTGGCTGTGCTTCACACT	TGGCTGAACAGCAAGAAGGT
Mesogenin	AACCTGGGTGAGACCTTCCT	AGAGAAGGAGCTGGGGAGAG
Mesp2	ACACCCCCGCTTCTAGGTAT	ATGGAACGACCCTCTCACAG
OCN	CCGGGAGCAGTGTGAGCTTA	TAGATGCGTTTGTAGGCGGTC
Oct4	GCCTTGCAGCTCAGCCTTAA	CTCATTGTTGTCGGCTTCCTC
OPN	GATGCCACAGATGAGGACCTC	CTGGGCAACAGGGATGACAT
p53	CACAGCGTGGTGGTACCTTA	CTTCTGTACGGCGGTCTCTC
p75	CAGAGCGAGACCTCATAGCC	TGCAGCTGTTCCATCTCTTG
PDGFRα	TTGGTGCTGTTGGTGATTGT	AGCATCTTCACAGCCACCTT
PKCα	CCCATTCCAGAAGGAGATGA	TTCCTGTCAGCAAGCATCAC
PKCβ	TCCCTGATCCCAAAAGTGAG	AACTTGAACCAGCCATCCAC
PKCδ	CAGACCAAGGACCACCTGTT	CGTCCCTGTCTAGCATCACA
PPARγ	ATCATCTACACGATGCTGGCC	CTCCCTGGTCATGAATCCTTG
RANK	GCTGGCTACCACTGGAACTC	GTGCAGTTGGTCCAAGGTTT
Sfrp3	CAAGGGACACCGTCAATCTT	CGATCCTTCCACTTCTCAGC
Slug	GCACTGTGATGCCCAGTCTA	TTGGAGCAGTTTTTGCACTG
Sox1	CACAGTTCAGCCCTGAGTGA	AGGCCACAACAACACAACAACA
Sox10	AGGCAGGAAGGGTTAGGGTA	GCGGAGAAAGGATCAGAGTG
Sox17	GACTGCGGAGTGAACCTCTC	AGGATTTCCTTAGCGCTTCC
Sox9	GCAGACCAGTACCCGCATCT	CTCGCTCTCGTTCAGCAGC
Snail	GAGGACAGTGGCAAAAGCTC	TCGGATGTGCATCTTCAGAG
Tbra	CCCTGCACATTACACACCAC	GTCCACGAGGCTATGAGGAG
Wnt5a	CAA ATAGGCAGCCGAGAGAC	CTCTAGCGTCCACGAACTCC

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Chapter 4: Hyperglycemia Impairs Osteogenic Differentiation of Embryonic Stem Cells by Altering Beta-Catenin Transcriptional Activity

Abstract:

Diabetes, which is characterized by an increase in blood glucose concentration, is accompanied by low bone turnover, increased fracture risk, and the formation of skeletal system malformations of the embryo if persistent during pregnancy. There are few studies elucidating the underlying alterations in signalling pathways leading to these osteogenic defects during prenatal development. A key component of osteogenic differentiation of embryonic stem cells (ESCs) is beta-catenin (CTNNB1). The transcriptional activity of CTNNB1 is precisely regulated during development to direct differentiation decisions leading to mature osteoblasts. Additional to its function in osteogenesis, dysregulated Wnt/CTNNB1 signalling has been implicated in the development of diabetes. Thus, we hypothesized that the bone formation deficiencies in the embryos of diabetic mothers results from altered CTNNB1 activity. To test this hypothesis we used a previously established ESC model of differentiation that mimics the diabetic environment of the developing embryo. We differentiated murine ESCs within osteogenic-inducing media containing either high (diabetic) or low (physiological) levels of D-glucose and performed time course analyses to study the influence of hyperglycemia on early and late bone cell differentiation. We found a clear reduction of endpoint measures for ostegenic differentiation as well as altered expression of precursor-specific markers at multiple time points. Furthermore, we found a significant difference in the transcriptional activity of the LEF/TCF and FOXO transcription factors

during precursor formation. Modulation of the AKT protein, a known upstream regulator of both LEF/TCF and FOXO transcriptional activity, could be employed to rescue some of the reductions in osteogenic output seen in the high glucose condition. Thus, within our *in vitro* model we found a clear involvement of LEF/TCF and FOXO signalling pathways in the regulation of osteogenic differentiation, which may account for the skeletal deficiencies found in newborns of diabetic mothers.

Introduction:

With an estimated 347 million people worldwide affected with diabetes mellitus, the morbidity and mortality rates associated with group of metabolic disorders is staggering (Danaei et al., 2011). While elevated blood glucose levels is believed to be the cause of many of the adverse health effects associated with diabetes, the mechanisms connecting hyperglycemia to these debilitating complications is not well understood. Individuals with diabetes are more likely to have osteoporosis and, as a consequence, are more susceptible to bone fractures (Räkel et al., 2008). Furthermore, newborns born of diabetic mothers exhibit a higher rate of birth defects including skeletal system malformations (Schwartz and Teramo, 2000; Mimouni et al., 1988).

Transcriptional profiling of developing embryos in diabetic mothers have revealed multiple alternatively expressed components of the WNT/beta-catenin (CTNNB1) signaling pathway (Pavlinkova et al., 2008, 2009). Additionally, genetic variants in TCF7L2 (i.e. a downstream component of this pathway) has been linked to an increased risk of developing type 2 diabetes (Grant et al., 2006). Canonical WNT/CTNNB1 signaling, regulates numerous processes in the formation of the mammalian skeleton

such as limb bud initiation, early limb patterning, late limb morphogenesis, embryonic bone development, and the maintenance of adult bone tissue (Westendorf et al., 2004; Krishnan et al., 2006; Bodine and Komm, 2006; Liu et al., 2008; Chen and Alman, 2009). Furthermore, dysregulation of CTNNB1 signaling has been implicated in multiple bone disorders (Gong et al., 2001; Boyden et al., 2002; Little et al., 2002). Despite evidence of modulation of this signaling pathway by glucose in multiple cell types (Lin et al., 2006; Anagnostou and Shepherd, 2008; Cognard et al., 2013), the connection between the hyperglycemic environment and the manifestation of bone disorders through altered WNT/CTNNB1 signaling has not been adequately explored.

The protein kinase AKT targets many proteins, including the O subclass of the forkhead box (FOXO) family of transcription factors. It's activity integrates cellular responses to changing environmental conditions, such as glucose and nutrient availability (Plas and Thompson, 2005; Tzivion et al., 2011). Recently, the AKT signaling pathway and the FOXO proteins have emerged as regulators of developmental osteogenesis (Rokutanda et al., 2009; Teixeira et al., 2010; McGonnell et al., 2012). Interestingly, the FOXO transcription factors depend on CTNNB1 as a co-activator to transcribe target genes (Essers et al., 2005). This binding between CTNNB1 and the FOXOs has additionally been shown to sequester CTNNB1 away from the lymphoid enhancer factor (LEF) and T-cell factor (TCF) family of transcription factors, which mediate canonical WNT/CTNNB1 transcriptional output (Hoogeboom et al., 2008). Thus, given the intimate relationship between these signaling components and their role in osteogenic differentiation, we hypothesized that the reported osteogenic deficiencies associated with diabetes is attributed to the dysregulation of these pathways by the hyperglycemic environment.

To test our hypothesis we differentiated murine embryonic stem cells (mESCs) in the presence of high (HG) or low glucose (LG), which correspond to a hyperglycemic or physiological concentration of D-glucose, respectively. Other investigations have commonly employed multipotent or differentiated cell lines to explore osteogenic differentiation potential, however these experiments do not encompass all of the developmental stages that cells proceed through to become mature osteoblasts. Furthermore, it has been shown that the role WNT/CTNNB1 signaling in directing osteogenesis is highly dependent on the developmental context of the cells receiving these signals (Boland et al., 2004; Hill et al., 2005; Quarto et al., 2010a). Thus, the ESC model is better suited to explore the potential dysregulation of this and other pathways along each cell specification step. Previously, we have shown that the hyperglycemic culture environment impairs the osteogenic differentiation of both mouse and primate ESCs (Dienelt and zur Nieden, 2011), recapitulating the defects found *in vivo*. Thus, by using an ESC model for developmental osteogenesis, we can begin to explore the possible mechanisms through which hyperglycemia impairs osteogenesis *in vitro* and thereby give insight into the *in vivo* bone pathophysiology resulting from diabetes.

Results:

Glucose regulates differentiation of ESCs into osteoblasts

To characterize the effect of glucose on osteogenic differentiation of mESCs we followed cells differentiating in both HG and LG conditions of 4.5 g/L and 1.0 g/L D-glucose, respectively. We initially observed a clear difference in the calcification of extracellular matrix, as indicated by black areas within bright field microscopy and

Alizarin Red staining (Figure 4.1A). Qualitative differences could be discerned as early as day 11 and persisted throughout the differentiation period. Calcium within the extracellular matrix was quantified and demonstrated a significant reduction within the HG condition at each time point examined (Figure 4.1B). The largest difference seen was on day 30 where there was a 318-fold reduction in calcium in the HG condition. Unlike the LG condition, it also appeared that calcium incorporation had ceased within the HG condition by day 20.

Following these differences in mineral deposition, we next examined the expression of known osteoblast lineage markers. Expression of alkaline phosphatase (*ALPL*), encoding the secreted enzyme that facilitates matrix minerialization, was consistently maintained thoughout differentiation in the LG condition (Figure 4.1C). Whereas, cells in the HG condition expressed *ALPL* at significantly lower levels on day 20 and 30. The secreted extracellular matrix (ECM) protein osteopontin (*OPN*) increased in both conditions, but only on day 11 was the expression significantly lower in the HG condition. Interestingly, collagen1 (*COL1A1*) was found to be significantly higher in the HG condition on day 30, potentially indicating a change in the differentiation kinetics (Figure 4.1C). This difference in the rate of differentiation progression was also supported by the difference in expression of transcription factors that regulate osteoblast precursor cells (Ducy et al., 1997; Nakashima et al., 2002). Both osterix (*OSX*) and runt-related transcription factor 2 (*RUNX2*) were significantly reduced during early time points in the HG condition, but were significantly increased at later time points when compared to the LG condition (Figure 4.1C).

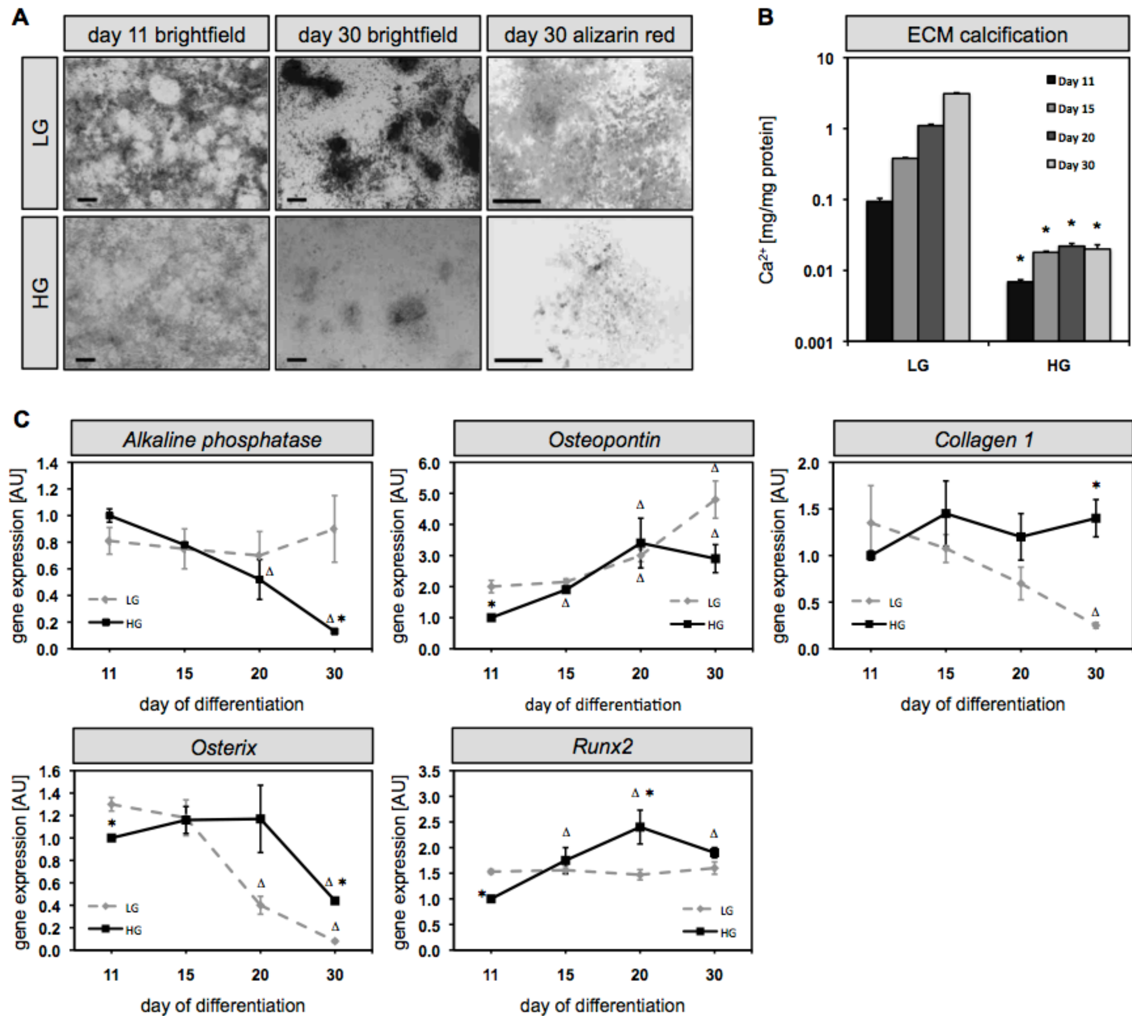

Figure 4.1: Osteoblast differentiation. (A) Osteoblast differentiation was visualized by brightfield microscopy on day 11 and day 30. Calcification of extracellular matrix was revealed through alizarin red staining; bar = 200 µm (B) Calcium deposition in the ECM was quantified at day 11, 15, 20 and 30 of differentiation and normalized to measured protein concentration for each sample. (C) Gene expressions for osteoblast markers were analyzed in a multiplex Array. Significance was determined by comparison to day 11 within the same condition (Δ$p<0.05$), or in comparison to LG condition (*$p<0.05$).

Osteoclast formation is diminished in the presence of high glucose

ESCs have been shown to additionally differentiate into osteoclasts (i.e. the cells responsible for bone resorption) when maintained in osteogenic induction media containing vitamin D_3 (Dienelt and zur Nieden, 2011). To examine the specification of

this cell type we first stained for the osteoclast-specific tartrate resistant acidic phosphatase (TRAP). Here, we found a decrease of TRAP staining within the HG condition when visualized on both day 20 and 30 (Figure 4.2A). Biochemical analysis of TRAP activity revealed a 1.3-fold decrease in enzyme activity of cells differentiating in HG at day 30 (Figure 4.2B). Despite, what appeared to be differential staining on day 20 in these conditions, the measured enzymatic activity was not significantly different. We were unable to detect TRAP staining or activity at day 11 or day 15 in either condition

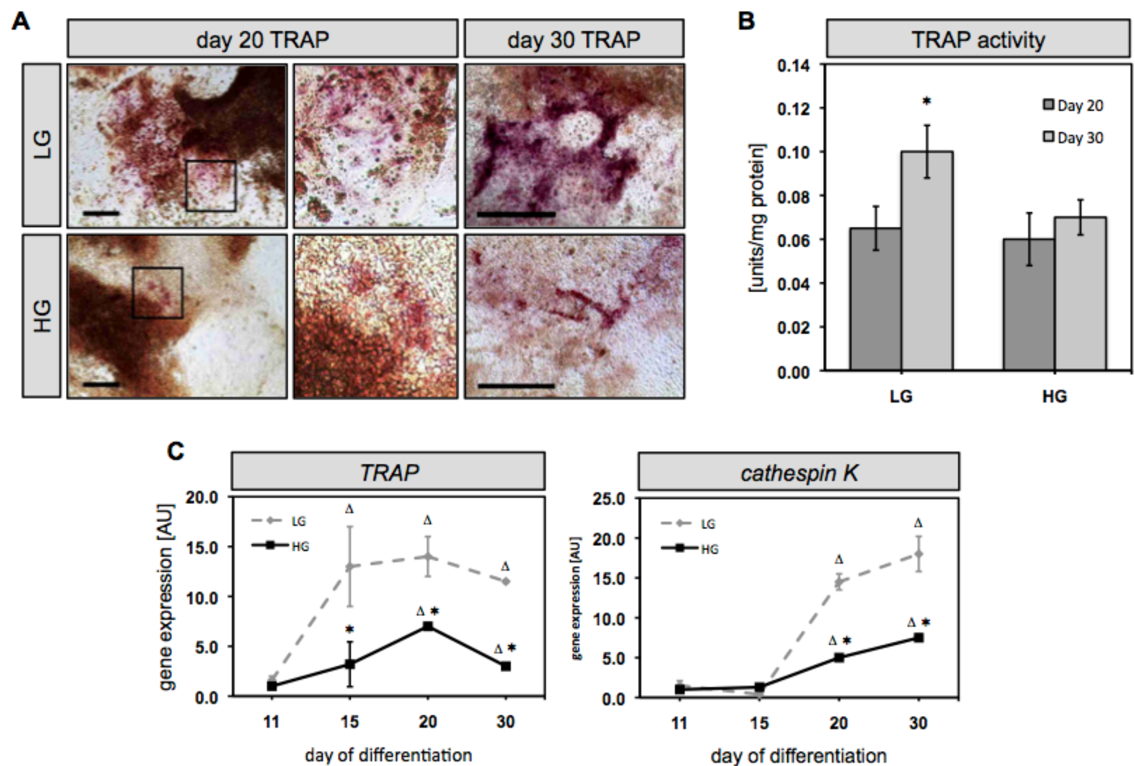

Figure 4.2: Osteoclast differentiation. (A) Osteoclast differentiation was visualized by TRAP staining on day 20 and day 30; bar = 200 µm (B) TRAP activity was quantified at day 20 and 30 of differentiation (C) Gene expressions for osteoclast markers were analyzed quantitatively in a multiplex approach; Significant differences for the expression of *TRAP* and *cathespin K* were detected between day 15 and day 30 of differentiation. Significance was determined by comparison to day 11 within the same condition (Δp<0.05), or in comparison to LG condition on the same day (*p<0.05).

(data not shown). However we were able to detect *Trap* mRNA expression on day 15 and later in HG but at levels that were statistically lower than the expression seen in LG (Figure 4.2C). Similarly, the expression of the osteoclast-specific marker cathepsin K (*Ctsk*) was significantly reduced in the HG condition during the later stages of differentiation.

Spontaneous differentiation into other cell types

Although *in vitro* differentiation protocols are improving, the current methods to induce osteoblast specification are still accompanied by the formation of additional cell types. To address whether the HG environment alters the degree to which these other cell types are produced we first examined early germ layer marker expression. On day 4 neurofilament 68kD (*NF68*) and α-fetoprotein (*Afp*) were used as markers for ectodermal and endodermal cell specification, respectively (Dziadek, 1979; Krumlauf et al., 1985; Julien et al., 1986; Kim et al., 2011). Although we did not detect significant differences in *NF68*, we did see a 5.6-fold increase in *Afp* expression in the HG conditions (Figure 4.3A). Thus, prior to osteogenic induction, the population of cells that have an osteogenic potential already appears to be diminished by day 4 when cells are exposed to a HG environment. Subsequently, when examining mesenchymal stem/stromal cell markers (MSC) (e.g. *CD90*, *CD105*, and *PDGFRα)* during the osteogenic induction phase of the differentiation (Davis & zur Nieden, 2008) we found either a reduction or delay in the expression within the HG condition (Figure 4.3B).

To further characterize changes in cell specification, we next turned our attention to the differentiation progression leading to both osteoblasts and osteoclasts. Although osteoclasts arise from hematopoetic stem cells (HSCs) (Boyle et al., 2003) and

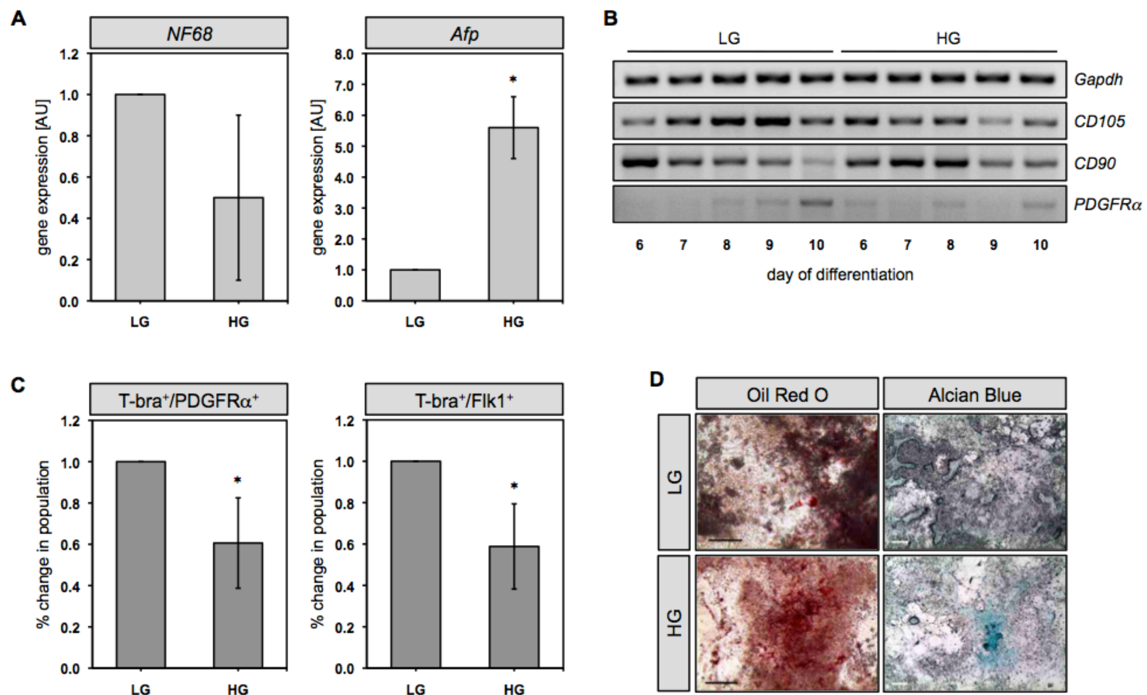

Figure 4.3: Formation of other osteoblast precursors and other MSC cell derivatives. (A) Gene expression of lineage markers for ectodermal (*NF68*) and endodermal (*Apf*) cells was analyzed by qPCR. (B) RT-PCR of MSC markers during days 6 – 10 in both glucose conditions. (C) Flow cytometric analysis of mesodermal and hematopoetic marker expression. Percentage of cells co-stained with T-Bra and either PDGFRα or Flk1 in the HG condition, were normalized to the percentage found in the LG condition. (D) Spontaneous differentiation into adipocytes and chondrocytes was observed following staining with Oil Red O and Alcian Blue, respectively; bar = 200 μm. Significant differences between the HG and LG conditions are indicated (*p<0.5).

osteoblasts arise from MSCs (Caplan, 1991), they share an early mesendodermal precursor, which is characterized by elevated expression of T-brachyury (T-bra) (Kubo et al., 2004). To distinguish between these cell populations we used the previously described markers PDFGRα and FLK1 to identify MSCs and HSCs, respectively (Huber et al., 2004; Kouskoff et al., 2005; Tada et al., 2005). In both cases T-bra co-expression with either PDFGRα or FLK1 was significantly reduced in cells differentiating in HG on day 7 (Figure 4.3C). These results corresponded to both the decrease in osteoclast

specification and activity seen in figure 4.2, as well as the aforementioned decrease in MSC marker expression.

Since MSCs have the potential to differentiate into adipocytes and chondrocytes (Caplan, 1991) we next examined the cultures for biochemical markers of these cell types. We observed a marked increase in Oil Red O staining for fat vesicles in the HG condition, which indicates a higher degree of adipogenesis (Figure 4.3D). We also found an increase in proteoglycan staining, via Alcian blue, which designates the presence of more chondrocytes in HG cultures (Figure 4.3D). Thus, despite the smaller percentage of cells exhibiting markers of MSCs in the HG condition, there is greater overall cell specification along the adipogenic and chondrogenic lineages.

Differential transcriptional activity between glucose conditions

After describing differential marker expression during multiple stages of the differentiation within the HG condition, we next began to examine possible differences in transcriptional control, which could lead to these altered cell populations. Given the integral role of WNT/CTNNB1 signalling in both *in vitro* and *in vivo* osteogenesis, we employed a LEF/TCF::GFP reporter as a readout of CTNNB1/LEF/TCF transcriptional activity. When differentiating this reporter line within these glucose conditions we found differences in GFP production during days 7 – 9, with day 8 being statistical significant (Figure 4.4A). Other investigations have reported that activation of WNT/CTNNB1 signalling can suppress osteogenic differentiation during precursor cell differentiation (Boland et al., 2004; de Boer et al., 2004; Quarto et al., 2010b). Thus, elevated CTNNB1/LEF/TCF transcriptional activity in the HG condition could account for the decrease in osteogenic precursor formation.

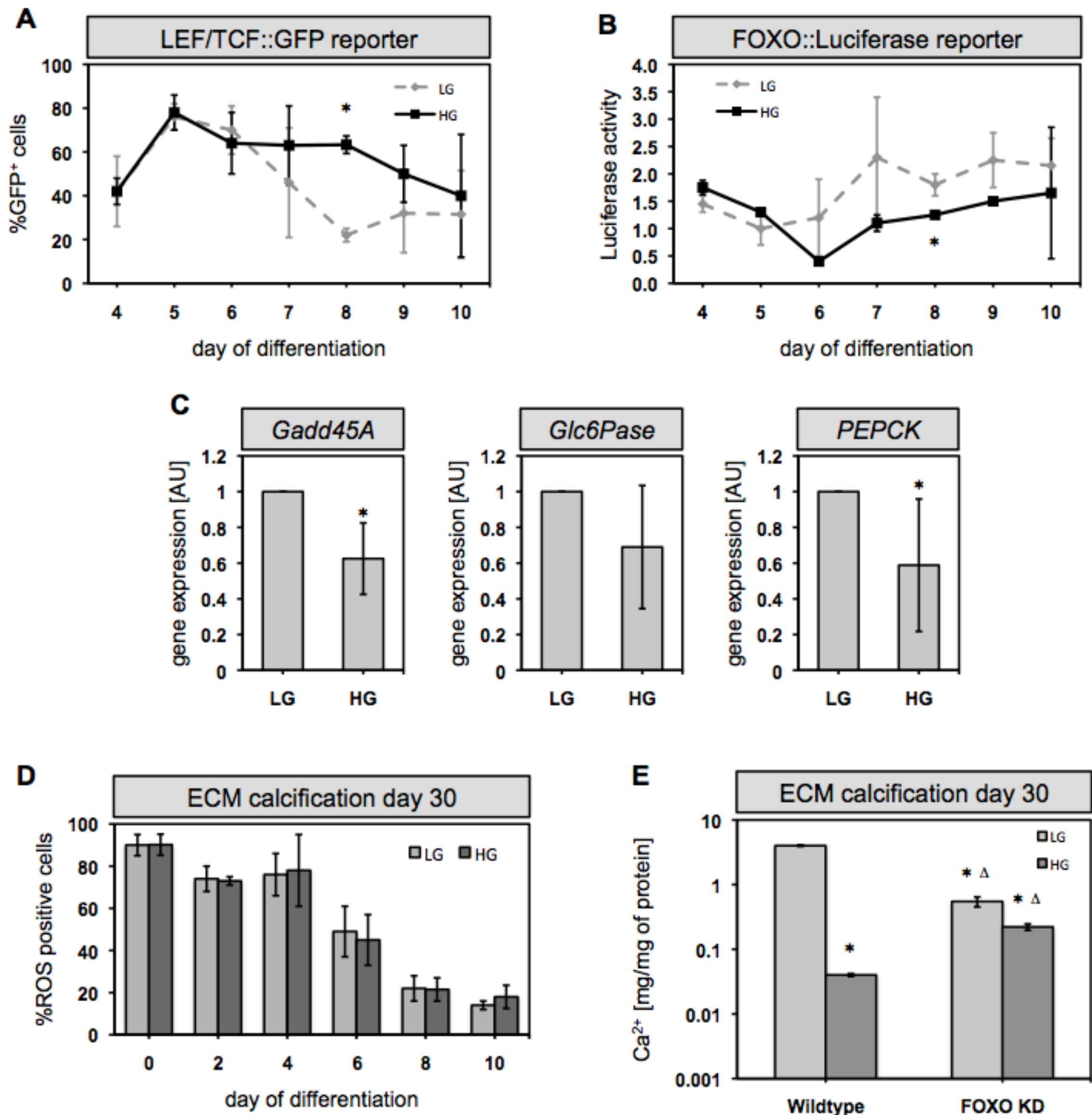

Figure 4.4: LEF/TCF and FOXO transcriptional activity. (A) LEF/TCF transcription factor activity was determined with the use of a GFP reporter cell line. GFP expression was assessed in flow cytometric measurements and represented as the percentage of cells that surpassed threshold levels of fluorescence. (B) Nuclear activity of the FOXO transcription factors was analyzed by the use of a FOXO-Luciferase reporter cell line. (C) Gene expression of FOXO target genes were analyzed by qPCR. (D) Flow cytometry assay using dihydrorhodamine to detect reactive oxygen species (ROS) beyond threshold levels. (E) Calcium deposition was normalized to protein concentration in both wildtype and a FOXO knockdown line (KD) on day 30. Significance was determined by comparison to the LG condition (*$p<0.05$), or between the two glucose conditions within the FOXO KD line ($\Delta p<0.05$).

The FOXO transcription factors are also important regulators of metabolism and glucose homeostasis and have recently been implicated in the formation and regulation of bone tissue (Gross et al., 2008; Ambrogini et al., 2010). Thus, we employed a luciferase reporter under the control of FOXO promoter elements to examine changes in FOXO transcriptional activity. Here, we saw a reduction in reporter activity in the HG condition, which was statistically significant on day 8 (Figure 4.4B). Interestingly, this period of decreased FOXO reporter activity was directly correlated with the increase in LEF/TCF reporter activity within this condition. Furthermore, when examining the endogenous expression of FOXO transcriptional targets, we found a significant decrease in the expression of *Gadd45a* and *PEPCK*, but not in *Glc6Pase* in the HG condition (Figure 4.4C). Since target genes of the FOXO transcription factors are known to play a role in oxidative stress response, we performed a dihydrohodamine flow cytometric assay to indicate the presence of reactive oxygen species (ROS). Despite, increased FOXO transcriptional activity in HG, we did not detect any differences in ROS between these cells and the LG control cells (Figure 4.4D). However, we did notice an overall reduction in ROS-positive cells in both groups from day 0 to day 10.

To explore whether FOXO-mediated transcription during this time period is facilitating osteogenic differentiation we employed a FOXO knockdown (KD) line. Within the LG condition we found significantly less calcium deposition in the FOXO-KD cell line, thereby implicating the FOXO transcription factors during *in vitro* osteogenic differentiation (Figure 4.4E). We also noted a further decrease in calcium deposition with FOXO-KD cells differentiating in the HG condition, suggesting that the decrease in osteogenic output of wild-type cells within the HG condition is not solely attributed to changes in FOXO transcriptional activity.

Differential activation of AKT

The demonstrated decrease in FOXO transcriptional output within the HG condition led us next to examine the key regulator of FOXO localization and activity, AKT. To determine if this upstream regulator is differentially activated in these glucose conditions we examined relative AKT protein expression and the presence of a phosphorylated residue that is indicative of its activation (i.e. Ser473). Interestingly, we found an overall decrease of AKT protein in HG, however, levels of active AKT appeared to be similar to or greater than the LG condition at the days examined (Figure 4.5A). Thus, there is a striking difference in the percentage of AKT that is active in the HG condition during the period of decreased FOXO transcriptional activity.

Given that both the FOXO and LEF/TCF transcription factors depend on CTNNB1 as a cofactor for transcription, it is plausible that the HG environment is shifting CTNNB1 away from the FOXO transcription factors towards the LEF/TCF transcription factors. To explore this potential shift in binding partners we performed a co-immunoprecipitation and found a noticeable reduction in FOXO3A/CTNNB1 binding on this day (Figure 4.5B). If AKT activity is responsible for the nuclear exclusion of FOXO3A in the HG condition, then inhibition of AKT could restore CTNNB1/FOXO3A co-immunoprecipitation. When using 10 μM of AKT inhibitor we see a modest increase in the pulldown of FOXO3A by CTNNB1. Similarly, we see a modest but significant increase in deposited calcium on day 30 when employing the AKT inhibitor during the time window of ostegenic precursor formation (Figure 4.5C). At a higher concentration of AKT inhibitor (i.e. 50 μM) there was a striking change in culture growth and morphology by day 11 (Figure 4.5D), where there appeared to be a strong suppression of proliferation within the HG condition. Interestingly, we observed individual cells, which

appear to be depositing calcium. When we quantified the relative calcium deposition in the HG condition treated with AKT inhibitor, we found no significant difference from the deposition levels measured in the LG condition (Figure 4.5E).

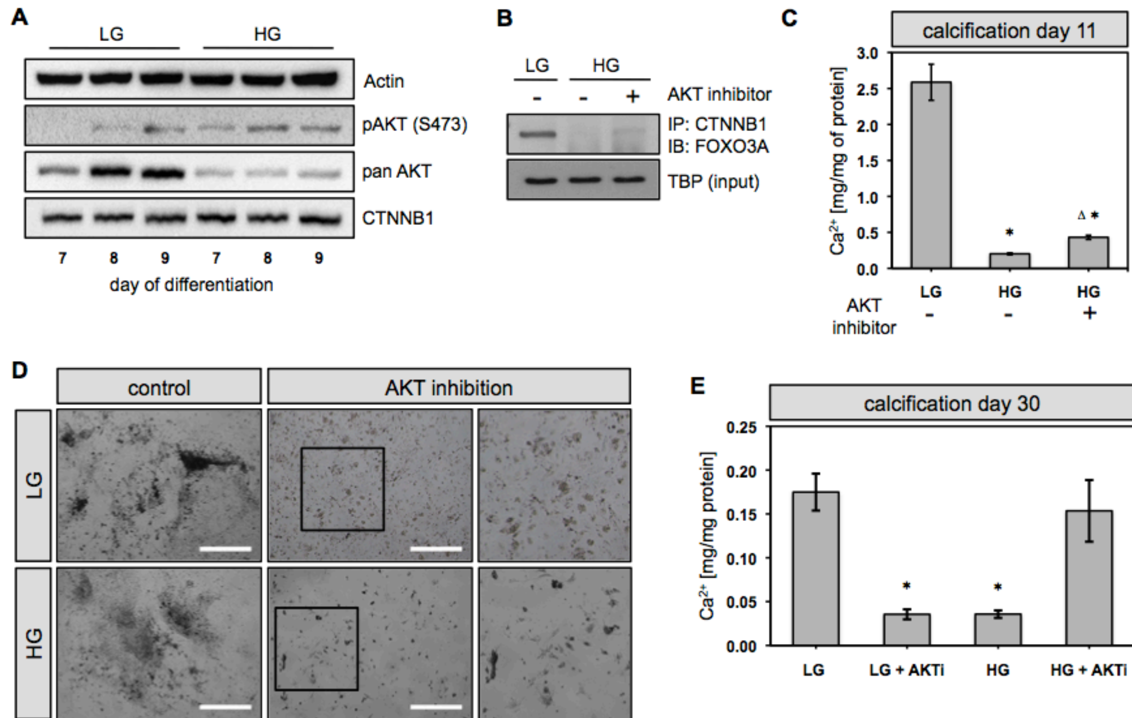

Figure 4.5: Differential AKT activation and its inhibition. (A) Western blot analysis of lysates obtained from cells differentiation in both glucose conditions during days 7 – 9. (B) Co-immunoprecipitation was performed by collecting nuclear fractions on day 7, capturing CTNNB1 and probing for FOXO3A, in both glucose conditions. An additional HG group was additionally treated with 10 µM of AKT inhibitor for 24 hours. (C) AKT inibitor was supplemented to a HG group from days 8-10 and compared to untreated LG and HG groups on day 30. (D) 50 µM of AKT inhibitor was employed from days 8-10 of the differentiation in both glucose groups and compared to untreated controls on day 11 with brightfield microscopy; size bar = 500 µm. (E) Quantification of calcium deposition relative to protein content on day 11. Significance was determined by comparison to the LG condition (*p<0.05), or AKT inhibitor treatment within the same glucose condition (Δp<0.05).

Discussion:

Suppression of osteogenic differentiation of ESCs and other cell types by HG conditions has been previously reported by our group and others (Verhaeghe et al., 1986, 1999; Wang et al., 2010; Zhen et al., 2010; Dienelt and zur Nieden, 2011), however, little is known about the mechanism through which elevated glucose disrupts osteogenesis. Given the myriad roles of CTNNB1 signalling in the developmental progression of the osteogenic lineage, we sought to explore whether a hyperglycemic environment could dysregulate this signalling pathway within our ESC model and, as a consequence, impair osteogenic differentiation.

Similar to reported results *in vivo*, showing reduced ossification centers and mineral content within fetuses of diabetic mothers (Verhaeghe et al., 1986, 1999), we found a clear reduction in ECM mineralization through staining and quantitative analyses of calcium deposition. Thus, by utilizing this ESC model of HG-induced disruption of osteogenesis we began to explore the potential mechanisms causing this effect. First, we asked whether the HG condition impairs the formation or function of mature osteoblasts. Here, we found differential expression of multiple osteogenic markers, such *Osx* and *Runx2* (i.e. transcription factors important in osteoblast precursor specification), were significantly reduced on day 11 in cells differentiating in the HG condition. Interestingly, expression of these markers at later time-points was significantly higher when compared to the LG condition. These results suggest that elevated glucose may cause a delay or deceleration in differentiation progression. When Boland et al. (2004), induced canonical WNT signaling in cultured MSCs, they found an increase in proliferation at the expense of osteogenic differentiation. In light of our reported increase

in LEF/TCF reporter activity within the same time window as MSC marker detection, it is quite plausible that a similar inhibition of differentiation is occurring in the HG condition through the induction of a proliferative program by CTNNB1/LEF/TCF.

Until now it was unclear, whether hyperglycemia dependent embryonic skeletal malformations are due to defective precursor formation or whether the effect alters mature cell function. We reported here for the first time that precursor formation (i.e. on day 7 of the differentiation) is already disturbed in hyperglycemic conditions leading to a 40% reduction in progenitor cell population. The fact that the precursor formation is disturbed in hyperglycemic glucose concentrations is also supported by the observation that a diminished ECM calcification is already visible in high glucose conditions on day 11 of the differentiation (i.e. before osteoblast maturation is completed). Thus, the osteogenic defects seen in newborns of diabetic mother may arise at earlier stages of development then previously suspected.

Other investigators have reported an *in vitro* reduction in ECM calcification and bone-specific markers expression in primary calvarial osteoblasts and the osteoblastic MG-63 cell line within HG conditions (Zhen et al., 2010; Wang et al., 2010). However, given the limited differentiation potential of these cell types, these investigations could not explore possible impacts on osteoclast formation in varying glucose conditions. Previously, we have reported the formation of osteoclasts in our ESC osteogenic induction protocol (Dienelt and zur Nieden, 2011). We initially considered the HG condition might cause an increase in the osteoclast differentation, which could account for the decrease in detected calcium deposition. However, we found a reduced expression of osteoclast-specific genes and a functional reduction in TRAP activity as seen on day 30. Thus, we can attribute the reduction of ECM mineral deposition seen in

the HG condition from impared osteoblast differentiation and not the result of an increased specification of osteoclasts. These results agree with other studies specifically examining osteoclastogenesis of more terminally differentiated cell types isolated from bone marrow (e.g. monocytes and macrophages) (Kim et al., 2007; Wittrant et al., 2008) .

Pathway analyses were performed to elucidate the negative impact of HG on the development of osteogenic precursors in more detail. Previous studies by our group revealed that tight control of canonical Wnt/CTNNB1 activity is a prerequisite for effective osteogenic differentiation of ESCs (zur Nieden et al., 2007). Here we report elevated CTNNB1/LEF/TCF activity in the HG condition during the period of osteoblast precursor formation. Furthermore, given the close relationship between energy metabolism and AKT signalling, we decided to examine this pathway in our differentiation model. Others have reported an increase in AKT activity within ESCs and other cell types (Xin et al., 2005; Kim et al., 2006; Liu et al., 2013). We report here that despite lower levels of AKT protein overall, the relative amount of AKT that harbored a phosphorylation indicative of its activation was considerably greater in the HG condition. Thus, since the FOXO transcription factors are direct targets for AKT, its differential activity could account for the differences seen in FOXO reporter activity between these conditions. Interestingly, reports have demonstrated a FOXO dependence on CTNNB1 in the transcription of target genes (Essers et al., 2005; Hoogeboom et al., 2008). It is also well established that AKT targets CTNNB1 and its negative regulator, GSK3; both events result in increased LEF/TCF transcriptional output (Cross et al., 1995; Weston and Davis, 2001; Fang et al., 2007). Given that we did not find differences in CTNNB1 protein levels between these treatment groups, we propose a model where competition

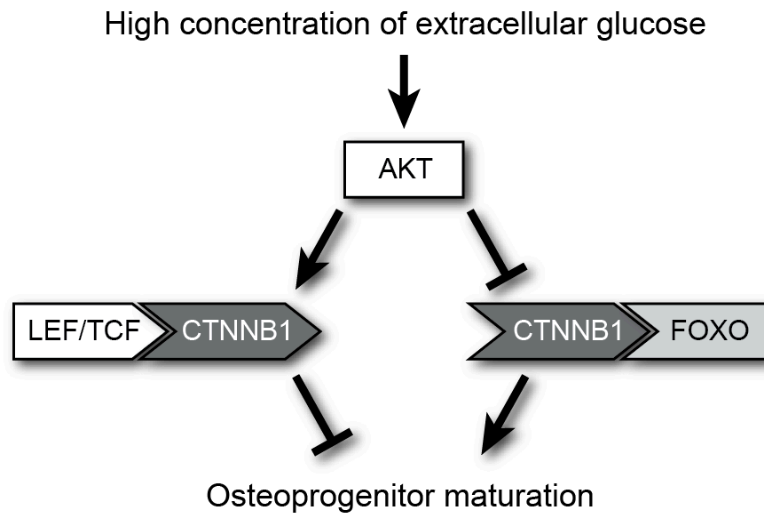

Figure 4.6: Model for the glucose induced changes during osteogenesis.

for the binding of available CTNNB1, by either the LEF/TCF or FOXO transcription factors, is modulated by the concentration of glucose (Figure 4.6). Specifically, glucose levels mediate changes in AKT activity, which directly alters the availability these transcription factors.

A similar competition model has been proposed by Almeida et al. (2007), however their model depends on differences in oxidative stress in order to shift CTNNB1 away from the LEF/TCF transcription factors and towards the FOXO transcription factors. Although, glucose metabolism and the production of ROS is linked in other systems, we did not detect any differences in ROS production between our two glucose concentrations. We suspect any differences in ROS production between our conditions is quickly restored to homeostatic levels. Additionally, the experiments performed by Almeida et al. (2007) employed mature osteoblasts and is thus more applicable to understanding age-related osteoporosis disorders. Importantly, the developmental context of our cells within our study respond to LEF/TCF transcriptional activity in an

opposite manner to that of mature osteoblasts when examining differentiation outcomes (Boland et al., 2004; Quarto et al., 2010a).

By shifting the transcriptional activity between these sets of transcription factors within our model, we can make predictions about osteogenic outcome. By targeting AKT with a chemical inhibitor we sought to divert CTNNB1 away from LEF/TCF and towards FOXO binding in the HG condition, recapitulating the transcriptional activity seen in LG. When applying 10 μM of inhibitor during the time window of osteogenic precursor formation we found a modest, but significant increase in calcium deposition. This treatment also showed a similar increase in the co-immunoprecipitation of CTNNB1 with FOXO3A. At 50 μM of AKT inhibitor, we found levels of calcium deposition on day 11 in the HG condition that was the same as untreated cells within the LG condition. However, examination of the culture revealed a dramatic reduction in cell density, indicating that the inhibitor is suppressing proliferation or may be inducing apoptosis. However, the population of cells that remain in culture are more likely to deposit calcium within the extracellular matrix. Interestingly, in chapter 3 of this thesis I put forth model that rWnt5a supplementation during a similar time window of differentiation is able to improve osteogenic differentiation through the inhibition of CTNNB1/LEF/TCF transcriptional activity. Thus, multiple lines of evidence demonstrate that downregulation of these signalling events during precursor formation can improve measures of osteogenic differentiation at later stages.

In summation, our results indicate that high glucose concentrations adversely affect osteogenic differentiation. Precursor formation and maturation of osteoblasts and osteoclasts are diminished in diabetic environments, leading to reduced ECM mineralization. By highlighting the interconnectivity of LEF/TCF and FOXO dependent

signalling as a function of the glucose metabolism, it is becoming increasing clear that the regulation of energy metabolism and skeletal system development are tightly linked to each other.

Materials and Methods:

ESC growth and differentiation. Murine embryonic stem cells (D3; ATCC, Manassas, VA, USA) were routinely cultured in High Glucose DMEM with addition of Penicillin/Streptomycin, fetal calf serum, glutamax, β-mercaptoethanol and the presence of leukemia inhibitory factor as described previously (Catalano et al., 2003). Differentiation was performed as described previously (zur Nieden et al., 2003). Briefly, differentiation was initiated by the hanging drop protocol. On day 5 the embryoid bodies were trypsinized and 50,000 single cells per cm^2 were plated on gelatin-coated dishes. The cells were grown for up to 25 additional days in medium supplemented with ascorbic acid (25 µg/mL), β-glycerophosphate (10 mM) and $1\alpha,25\text{-}(OH)_2$ vitamin D_3 ($5x10^{-8}$ M). Differentiation was performed in medium containing either 1.0 g/L D-glucose or 4.5 g/L D-Glucose.

Reporter cell lines. A murine D3 ESC line carrying a GFP reporter under the control of four LEF/TCF binding sites was kindly provided by Dr Irving Weismann (Stanford University). The FOXO reporter cell line was created by lentiviral transduction of D3 cells with a FOXO luciferase reporter construct according to the manufacturer's instruction (Cignal Lenti FOXO reporter; Qiagen, Hilden, Germany). Transduced cells that integrated the reporter into their genome were selected via puromycin resistance

(1µg/ml). Subsequently, stable expressing clones were selected, analyzed for their functionality and used for further experiments. Differentiation of the reporter cell lines was induced as mentioned above. At distinct time points of differentiation the cells were trypsinized, collected and analyzed via flow cytometry for GFP expression or for luciferase activity respectively. Flow cytometric analyses were performed as described below. Luciferase activity was measured with a luciferase assay system according to the manufactures instruction (Promega, Madison, WI, USA) and normalized to the protein content of the cell lysates.

Morphological analyses. Differentiation cultures were analyzed for the manifestation of distinct cell types at day 20 and 30 of the differentiation. Mineralized matrix was visible by a black appearance in bright field microscopy. Additional, calcium ions incorporated into the matrix were stained with 2% Alizarin Red S solution on methanol/acetone (7:3) fixed cells. TRAP staining was performed on cells fixed with 10% glutaraldehyde for 10 minutes at 37°C. Afterwards cells were incubated in TRAP staining buffer containing 50mM Sodium-Acetate Buffer pH 5.0, 30mM sodium tartrate, 0.1% Triton X-100, 100µg/ml Naphtol AS-MX phosphate, 300µg/ml Fast Red Violet LB. Adipocyte differentiation was shown by staining lipid vacuoles with 0.3% Oil Red O within a 60% isopropanol solution. Deposition of chondorgenic matrix was visulaized by proteoglycan staining with 1% Alcian Blue. Pictures were taken with a Leica DM IL microscope (Leica Microsystems, Wetzlar, Germany).

Biochemical assays. Calcium and protein determination, as well as TRAP activity assays were performed as previously reported (Dienelt and zur Nieden, 2011).

Immunoprecipitation. Immunoprecipitation of the transcription factors FOXO3A and CTNNB1 were performed on differentiation day 7. Intracellular proteins were cross-linked with the use of DSP (200 µg/ml in PBS) prior cell lysis in IP-lysis-buffer supplemented with protease- and phosphates inhibitors (50 mM Tris pH 7.4, 200 mM NaCl, 1% (v/v) TritonX-100, 0,5% SDS, 2 mM EDTA). 500 µg protein solution was incubated overnight with 1 µg FOXO3A antibody (Table 1) at 4°C. Protein G-agarose beads were equilibrated in IP-lysis-buffer. The protein/antibody mix was added to the protein G-agarose beads following the incubation of the protein solution with the respective antibodies. Antibody bound proteins were capture by the beads for 6 hours at 4°C under constant mixing of the sample. Following washes, proteins were eluted in 50 µl SDS-PAGE buffer (314 mM Tris-HCl pH 6.8, 10% SDS, 50% Glycerin, 25% ß-mercaptoethanol, 0.05% bromophenol blue).

Western Blot. Proteins from whole cell lysates (50 µg) or immunprecipitates (20 µl of elutant) were separated by SDS-Polyacrylamide gelelectrophoresis. Proteins were blotted subsequently on a PVDF membrane. Protein abundance was verified with antibody staining corresponding to the antigen of interest (Table 1). Afterwards, the membrane was incubated with horseradish peroxidase-labeled secondary antibodies. Visualization was performed with a gel imaging system (GelLogic 1500, Kodak, Rochester, New York, USA) following the addition of ECL substrate solution.

Flow Cytometry. Cells were harvested at the time points indicated and trypsinized to yield a single cell suspension, washed two times in PBS before the cell number was

determined. 1-2x10^6 cells were used for each analysis. Surface markers (FLK1, PDGFRα) were stained by incubating the living cells in 1% BSA in PBS supplemented with an adequate concentration of the first or respectively secondary antibody (Table 1) for 30 min at 4°C. After antibody incubation, cells were washed three times with PBS. For measurements of LEF-TCF GFP reporter activity cells were fixed with 4% formaldehyde for 10 minutes at 4°C. Afterwards fixed cells were incubated in a 1% BSA, 0.1% Saponin, 0.05% NaN_3 PBS solution containing the diluted antibody (Table 1). To get rid of unbound antibody, cells were washed three times with staining buffer. Cells were stored in PBS at 4°C until measurement. 5x10^4 cells of the stained population were measured on the same day of harvesting in a FC500 Flow Cytometer, (Beckman Coulter, Brea, CA, USA). Analysis was performed with the corresponding CXP Analysis software. IgG isotype control treated cells were used for gate settings.

ROS detection. To demonstrate the formation of reactive oxygen species, cells were incubated with 500 nM dihydrorhodamine 123 (Invitrogen) for 20 minutes at the differentiation time points indicated. Intracellular ROS formation was proven by flow cytometric measurements detecting green fluorescence of the oxidized reagent rhodamine 123 as described above.

RNA-isolation and quantitative PCR. Total RNA was isolated at the time points indicated using an RNeasy kit (Qiagen, Germany). For single qPCR analyses, cDNA was synthesized from 625 ng RNA in a total volume of 25 µL with Superscript II as indicated by the manufacturer (Invitrogen, Carlsbad, CA, USA). One microliter of cDNA was used for qPCR, which was performed in a Roche Light Cycler 480 system using a

SYBR Green PCR master mix (F. Hoffmann-La Roche AG, Switzerland), with previously quoted cycle conditions (Räkel et al., 2008). Primer sequences were generated with primer 3 (http://frodo.wi.mit.edu/primer3) and tested for specificity with reverse ePCR (http://www.ncbi.nlm.nih.gov/projects/e-pcr/reverse.cgi). Primer sequences are listed in Table 2. Expression of each gene was normalized to the expression detected in the LG condition. For multiplex quantitative PCR 150 ng of total RNA per sample were analysed using the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, USA). Analyses were performed according to manufacturer's instruction with slight modifications. Reverse transcription was performed in a reduced volume of 10 µL by using sequence specific reverse primers including a flanking universal reverse sequence. Subsequently PCR was executed in a total volume of 10 µL using a mixture of sequence specific forward primers (Table 2) tagged with a flanking universal reverse sequence, universal forward and universal reverse primers. Primer design and PCR analysis were done using of the corresponding system specific software. Obtained results were normalized to the expression of *Tbp* and the expression detected on day 11 in LG conditions.

Statistical analyses. Statistical analyses were performed with SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA). ANOVA using Bonferroni's method or Student's t-test was used to detect differences between the various treatment groups. P-values ≤ 0.05 were considered to be statistically significant. All experiments were performed from three biological samples in minimum. Data are displayed as the mean ± standard deviation, except the multiplex qPCR time course data, which are shown as mean ± standard error for better visualization.

Table 4.1: List of used antibodies

Antigen	Manufacturer
beta-Catenin, monoclonal mouse, clone CAT-5H10	Invitrogen, 138400
T-Brachyury, polyclonal goat	Santa Cruz, sc-17743
eGFP, polyclonal rabbit	Abcam, ab290
Flk1-Alexa488, monoclonal rat, clone 89B3A5	Biolegend/Biozol, 121907
FOXO3a, polyclonal rabbit	Acris, AP00319-PU-N
PDGFRα, monoclonal mouse, clone SPM473	Abcam, ab54452
Akt kinase, monoclonal rabbit, clone AW24	Merck Millipore, 05-796
Akt kinase (p-Ser473), monoclonal mouse, clone 11E6	Merck Millipore, 05-669
Alexa-Fluor 488 goat anti mouse IgG	Invitrogen, A11017
Alexa-Fluor 546 donkey anti goat IgG	Invitrogen, A11056
Alexa-Fluor 488 goat anti rabbit F(ab')2 IgG	Invitrogen, A11070
HRP goat anti mouse IgG	Santa Cruz, SC-2060
HRP donkey anti rabbit IgG	GE Healthcare, NA934
HRP rabbit ant goat IgG	Zymed, 61-1620

Table 4.2: Primer sequences

gene	Sequence forward primer	Sequence reverse primer
Col1a1	GACGTCCTGGTGAAGTTGGT	CAGGGAAGCCTCTTTCTCCT
Runx2	CAGACCAGCAGCACTCCATA	CAGCGTCAACACCATCATTC
OPN	GAAGCTTTACAGCCTGCACC	TCCATCGTCATCATCATCGT
Osx	CTGGAGAGGGAAAGGGATTC	TGGGGATCTTAGTGACTGCC
ALP	AACCCAGACACAAGCATTCC	CCAGCAAGAAGAAGCCTTTG
CTSK	GGGAAGCAAGCACTGGATAA	CACCAACGAGAGGAGAAATGA
TRAP	GGGTCACTGCCTACCTGTGT	AGCAGGACTCTCGTGGTGTT
Tbp	AACAGCAGCAGCAACAACAG	ATGATGACTGCAGCAAATCG
NF68	AGTGGCTTTCTGGCTTGCTG	TCTGTGTGATTCACATTGCCATAG
AFP	GAAGCAAGCCCTGTGAACTC	GGCATAGGTTTCATCCCTCA
T-Bra	CCCTGCACATTACACACCAC	GTCCACGAGGCTATGAGGAG
18s rRNA	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC
Gadd45A	GCTCAACGTAGACCCCGATA	GTTCGTCACCAGCACACAGT
Glc6Pase	GACCTCCTGTGGACTTTGGA	CTTCCGGTACATGCTGGAGT
PEPCK	CTGGCACCTCAGTGAAGACA	TCGATGCCTTCCCAGTAAAC
Runx2	ATCCCCATCCATCCACTCCA	TGCTTCGTGGGTTGGAGAA
Tbp	CAGCCTTCCACCTTATGCTC	CCGTAAGGCATCATTGGACT

Reference:

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Chapter 5: Canonical and Non-Canonical Wnts Specifically Steer Differentiation of Osteoblasts in Serum-Free Conditions.

Abstract:

The efficient differentiation of pluripotent stem cells along specific differentiation lineages is of fundamental importance towards developing regenerative medicine applications. However, the translation of many currently established protocols is hindered by their dependence on fetal bovine serum. Recently, serum replacement approaches have been explored in the osteogenic differentiation of stem cells, however, a standardized methodology has yet to be developed. Given the integral position of the Wnt/beta-catenin (CTNNB1) signaling pathway during developmental osteogenesis *in vivo*, we sought to improve a preexisting *in vitro* differentiation protocol by recapitulating the dynamic role of this signaling pathway. It is known that individual precursor stages, leading to mature osteoblasts, differentially respond to the Wnt/CTNNB1 pathway. Thus by correctly manipulating this signaling pathway in a time-dependent manner we can direct cell fate decisions. Here we report that stage-specific treatment with recombinant Wnt proteins during the differentiation of mouse embryonic stem cells (mESCs) in a serum-free environment is able to direct osteogenic differentiation to levels comparable to that of serum-containing cultures. Furthermore, we found that we could substitute the recombinant proteins with chemical modulators of CTNNB1 and yield the same differentiation outcome. Thus, we find that modulation of CTNNB1 at specific stages offers a viable strategy towards the improvement of existing methodologies of serum-free osteogenic differentiation.

Introduction:

The use of animal sera in culture media presents a number of concerns in regards to basic research and downstream applications. For instance, the heterogeneity of serum components is a major hurdle towards consistency and standardization required for the progression of stem cell-based therapies. In addition, because of potential transmission of zoonotic pathogens, animal sera in cell culture media precludes the cells grown in these conditions to be directly applied to human therapeutics. The collection of animal serum itself also has ethical implications. Thus, with the ever-growing demand of sera paired with the desire to reduce or eliminate potential animal suffering, the need for serum replacement with defined components is necessary for both the sustainability and reliability of future research.

In order to elucidate vertebrate developmental programs *in vitro* so that knowledge gained can be applied to *in vivo* systems, we differentiated mouse embryonic stem cells (mESCs) while simultaneously manipulating the Wnt signaling pathway in defined media conditions. This signaling pathway has been implicated in the regulation of numerous developmental programs such as body axis specification (Hikasa and Sokol, 2013), cell migration (Nelson and Nusse, 2004), cancer progression (Reya and Clevers, 2005), as well as stem cell maintenance (Wend et al., 2010; Sokol, 2011) and differentiation (Clevers, 2006; Wang et al., 2012). In canonical Wnt signaling the effector protein beta-catenin (CTNNB1) acts as a co-transcription factor to the LEF/TCF family of transcription factors (Logan and Nusse, 2004). Non-canonical Wnt signaling, on the other hand, has been traditionally viewed to act through CTNNB1-independent mechanisms. However, this paradigm has shifted in recent years following reports of

non-canonical signaling acting antagonistically to the transcriptional activity of CTNNB1 (Ishitani et al., 2003; Topol et al., 2003; Westfall et al., 2003; Baksh et al., 2007); a result we have also seen in our work (See Chapter 3).

Wnt signaling is involved in a continuum of differentiation decisions ranging from the exit from pluripotency to the specification of terminal cell types arising from mesenchymal stromal/stem cells (MSCs) (e.g. osteoblasts, chondrocytes, and adipocytes) (Ling et al., 2009). Although many studies have focused on a particular Wnt ligand's effect on directing lineage decisions, it has become increasingly apparent that differentiation outcome is highly dependent on the temporal-spatial context of the cell receiving the signal (zur Nieden et al., 2007b). First, canonical Wnt signaling has been implicated in maintaining pluripotency of ESCs (Sato et al., 2004; Ogawa et al., 2006; Merrill, 2012). Interestingly, canonical Wnt signaling is also required for the early differentiation decisions and for the initiation of gastrulation (Liu et al., 1999; Barrow et al., 2007). Furthermore, the development of mesoderm as well as neural crest induction and migration are dependent on Wnt signaling (Haegel et al., 1995; Ikeya et al., 1997; Hari et al., 2002; Lindsley et al., 2006). An important regulator of mesoderm formation MSC specification, T-brachyury (T-bra), is direct target for canonical Wnt signaling (Yamaguchi et al., 1999b). Following MSC specification from either mesoderm or ectodermal origin, both inhibition and activation of the Wnt/CTNNB1 signaling have been shown to promote osteogenic differentiation in a cell context-specific manner (See Chapters 1 and 2 for overview). For instance, the same Wnt ligand can have opposite effects on differentiation outcome when applied at different times during both *in vitro* and *in vivo* differentiation (Boland et al., 2004; Ueno et al., 2007; Quarto et al., 2010). Thus,

in our current study we employed different Wnt treatment strategies to account for this potential cell context specific response.

Aside from Wnt ligands, other molecules have been found to regulate CTNNB1 activity. For instance nitric oxide (NO), an essential signaling molecule in diverse physiological and pathological processes, mediates many of its effects through the post-translational modifications of proteins (Gow et. al, 2004; Monteiro et al., 2005) including CTNNB1 (Prévotat et al., 2006; Williams et al., 2011). Furthermore we have shown that CTNNB1 regulation by the NO donor SNAP, and NO acceptor PTIO, alters differentiation outcome of early primitive streak cells *in vitro* (Ding et al., 2012). Additionally, treatment with LiCl is frequently employed to stabilize CTNNB1 through inhibition of the negative regulator GSK-3 (Stambolic et al., 1996), whereas retinoic acid (RA) treatment has been shown to downregulate LEF/TCF reporter activity (Easwaran et al., 1999); effects that we have previously demonstrated in mESCs (zur Nieden et al., 2007b).

Despite numerous studies regarding CTNNB1 influence on cell lineage decisions, we are far from a complete understanding of this protein's role throughout development. Thus, we sought to explore the temporal influence of CTNNB1 regulation in defined media conditions. Through various treatment types and schemes we have shown that mESC differentiation can be significantly altered via modulation of CTNNB1 in regards to osteoblast, chondrocyte and adipocyte outcomes.

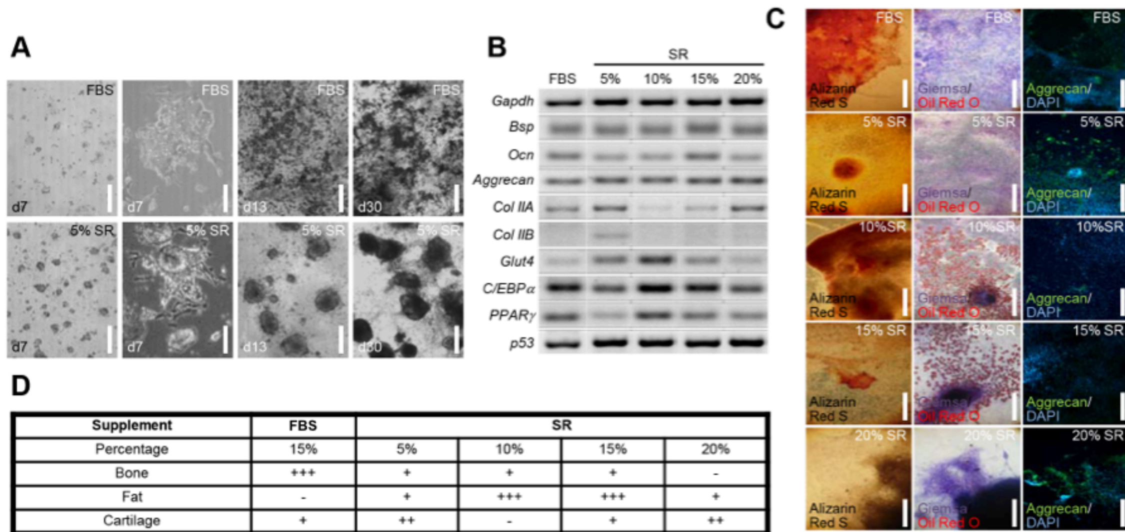
Results:

Concentration of serum replacer alters differentiation outcome

We initially performed an osteogenic differentiation of mESCs (as outlined in (zur Nieden et al., 2003)), to examine the impact of serum replacer (SR) as compared to fetal bovine serum (FBS). We noticed a number of changes in the morphology of the cell aggregates and overall culture appearance following 30 days of differentiation (Figure 5.1A). As early as day 7 colonies that formed in 5% SR appeared to have a distinct rounded center, whereas cells grown in FCS had a more uniform colony morphology. The differences between these groups was even more apparent at later time points, where cells grown in SR displayed distinct cell clusters with flat cells resembling fibroblasts in the intervening space. Cells grown in FBS appeared more uniformly distributed, with a greater degree of calcification (i.e. as indicated by the black areas of the photomicrographs).

We acknowledged that the concentration of SR could be a direct factor in altering differentiation potential. Thus, we next compared cultures differentiating for 30 days in 5%, 10%, 15%, and 20% SR. The differential expression of markers for osteoblasts, chondrocytes and adipocytes demonstrated that each condition affected differentiation (e.g. Glut4, C/EBPα, and PPARγ) (Figure 5.1B), which corresponded to the highest Oil Red O staining for lipid droplets in the 10% and 15% SR groups (Figure 5.1C). SR concentration of 5% and 20% appeared to promote chondrocyte differentiation as indicated by Col2A expression (Figure 5.1B) and Aggrecan protein staining (Figure 5.1C). We found similar levels in the expression of the osteoblast markers, *Bsp* and *Ocn*, between the FBS and SR cultures (Figure 5.1B), however, the FBS cultures

showed clearly more calcium deposition as visualized by Alizarin Red S staining (Figure 5.1C). Thus, varying the concentration of SR could itself directly impact differentiation outcome. After compiling these results into a table (Figure 5.1D), we decided to proceed with all further differentiations with 5% SR when comparing to 15% FCS.



Supplement	FBS	SR			
Percentage	15%	5%	10%	15%	20%
Bone	+++	+	+	+	-
Fat	-	+	+++	+++	+
Cartilage	+	++	-	+	++

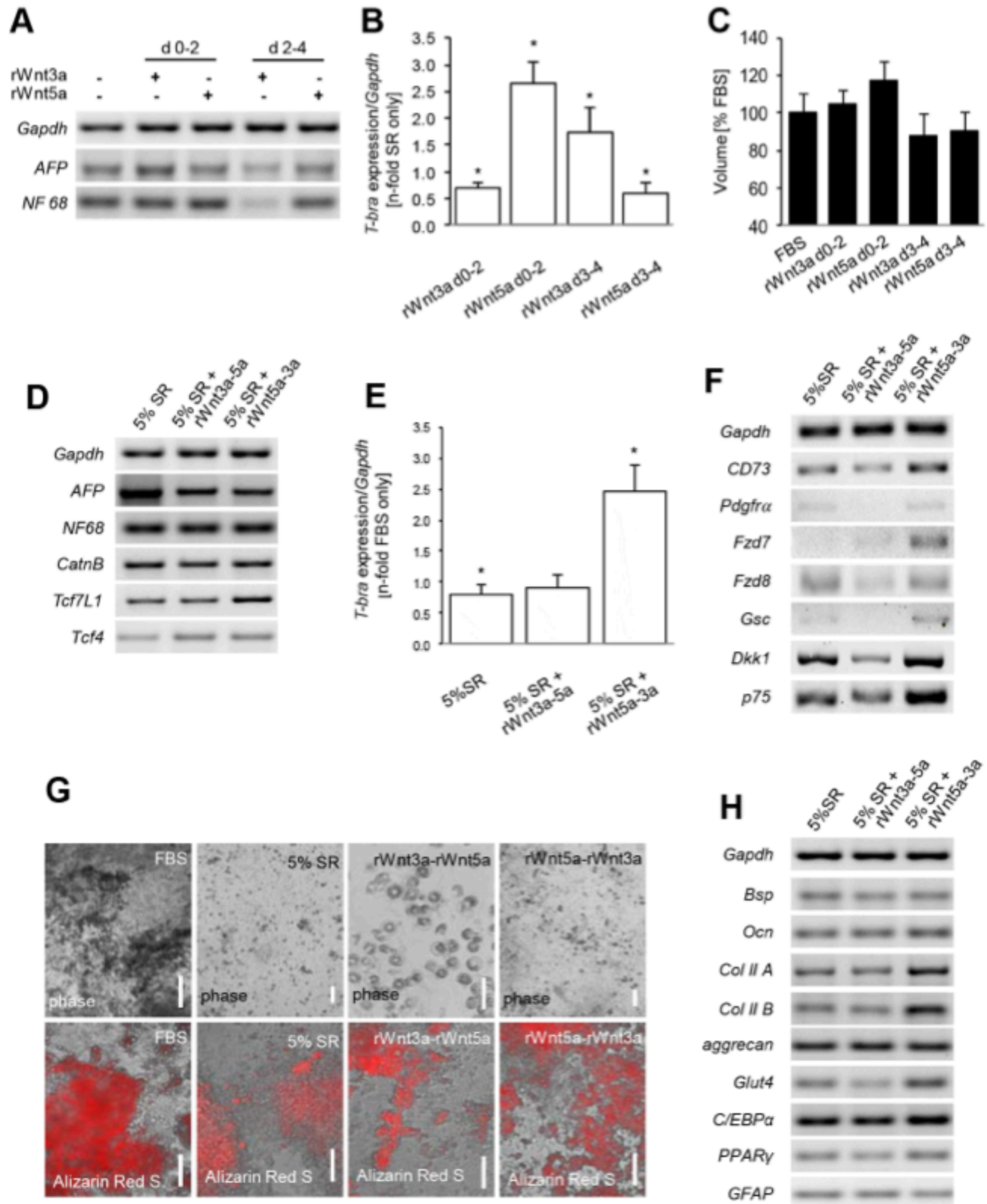
Figure 5.1. Concentration of serum replacer alters differentiation outcome. (**A**) Phase contrast photomicrographs of differentiating mESCs in the presence of 15% FBS or 5% SR on the indicated day. Size bar = 200 µm. (**B**) RT-PCR results comparing the expression of known markers of osteoblasts, chondrocytes, and adipocytes in various concentrations of SR and 15% FBS. (**C**) The same groups were compared visually via histochemical and immunocytological examination of calcium deposition via Alizarin Red S staining, lipid droplets via Oil Red O and Giemsa Staining, as well as the presence of Aggrecan via immunocytochemistry. (**D**) Double-blind scoring of staining degree or antibody signal was performed on cultures grown in the indicated conditions and was tabulated.

Induction of canonical and non-canonical Wnt signaling alters cell fate

Previous work in our lab and others has shown that Wnt signaling is critical for controlling early differentiation decisions of mESCs (Davis and zur Nieden, 2008). To examine how Wnt signaling could direct differentiation outcome we began supplementing our cultures during the early stages of our protocol (i.e either on days 0-2

or on days 2-4). We utilized recombinant Wnt3a (rWnt3a) or recombinant Wnt5a (rWnt5a) to induce canonical or non-canonical Wnt signaling cascades, respectively. We found that treatment with rWnt3a during days 2-4 lowered both the expression of the primitive endoderm marker *Afp* and the ectodermal marker NF 68 as measured on day 4 (Figure 5.2A). This effect was not found when rWnt3a was supplemented during the earlier time window. On the other hand, NF 68 showed higher expression following rWnt5a treatment during days 0-2. Furthermore, the mesoderm marker *T-bra* was also differentially regulated depending on the treatment type and time period (Figure 5.2B). rWnt5a treatment significantly increased *T-bra* expression when supplemented from days 0-2, and had the opposite affect when added on days 3-4. Conversely, rWnt3a significantly increased expression of *T-bra* when supplemented on days 3-4, and decreased expression when used at the earlier time window. Since Wnt signaling is known to modulate the cell cycle (Massagué, 2004), we examined embryoid body (EB) volume on day 4 following the various treatments as an indirect measure of cell proliferation (Figure 5.2C). Although there were some differences measured between groups they were not statistically significant.

Following the pattern of *T-bra* expression seen in Figure 5.2B, we began combinatorial rWnt supplementation to induce a ‘pro-mesoderm’ condition (i.e. rWnt5a followed by rWnt3a on days 0-2 then on days 2-4, respectively) or an ‘anti-mesoderm’ condition (i.e. rWnt3a followed by rWnt5a on days 0-2 then on days 2-4, respectively). We initially noticed that both treatment schemes reduced the endoderm marker AFP as compared to SR alone, and resulted in minimal changes in the expression of Wnt signaling components on day 4 (Figure 5.2D). Following our ‘pro-mesoderm’ treatment we found a significant elevation in the expression of *T-bra* over cells both cultured in



A
d 0-2
d 2-4
rWnt3a
rWnt5a
Gapdh
AFP
NF 68
B
T-bra expression/Gapdh [n-fold SR only]
rWnt3a d0-2
rWnt5a d0-2
rWnt3a d3-4
rWnt5a d3-4
C
Volume [% FBS]
FBS
rWnt3a d0-2
rWnt5a d0-2
rWnt3a d3-4
rWnt5a d3-4
D
5% SR
5% SR + rWnt3a-5a
5% SR + rWnt5a-3a
Gapdh
AFP
NF68
CatnB
Tcf7L1
Tcf4
E
T-bra expression/Gapdh [n-fold FBS only]
5%SR
5% SR + rWnt3a-5a
5% SR + rWnt5a-3a
F
5%SR
5% SR + rWnt3a-5a
5% SR + rWnt5a-3a
Gapdh
CD73
Pdgfrα
Fzd7
Fzd8
Gsc
Dkk1
p75
G
FBS
5% SR
rWnt3a-rWnt5a
rWnt5a-rWnt3a
phase
Alizarin Red S
H
5%SR
5% SR + rWnt3a-5a
5% SR + rWnt5a-3a
Gapdh
Bsp
Ocn
Col II A
Col II B
aggrecan
Glut4
C/EBPα
PPARγ
GFAP

Figure 5.2. Biphasic Wnt treatment alters differentiation outcome. (**A**) RT-PCR examination of early differentiation markers on day 4 in cultures that were supplemented with rWnt3a or rWnt5a at 50 ng/ml for the indicated duration. (**B**) Relative expression of the mesoderm marker *T-bra* on day 4 as determined by qPCR. (**C**) The mean volume of embryoid body formation on day 4 in each condition was determined from the diameter of 50 embryoid bodies as measured with image analysis software (ImageJ, National Institutes of Health). (**D**) Early differentiation and Wnt pathway marker expression was examined via RT-PCR on day 4 in biphasic treatment groups (e.g. rWnt3a-5a = day 0-2 treatment with rWnt3a followed by day 2-4 treatment with rWnt5a is indicated by rWnt3a-5a). (**E**) qPCR of *T-bra* on day 4 with biphasic Wnt treatment schemes. (**F**) RT-PCR examination of Wnt signaling and MSC markers on day 7. (**G**) Phase contrast photomicrographs and corresponding Alizarin Red S staining of cultures on day 30 in the indicated condition. Size bar = 200 μm. (**H**) Day 30 expression analysis of known markers of osteoblasts, chondrocytes, and adipocytes via RT-PCR. $^{*}p < 0.05$.

FCS or SR alone (Figure 5.2E). Although, the anti-mesoderm treatment did not significantly reduce *T-bra* expression on day 4, multipotent mesenchymal stromal cell (MSC) markers CD73 and PDGFRAα were found to be reduced when examined on day 7 (Figure 5.2F). Interestingly, we found that on this day components of Wnt signaling such as Fzd 7, Fzd 8, and Dkk1 were also differentially expressed between two treatment groups, indicating that the treatment groups are likely to respond differently to further Wnt signals beyond this differentiation stage. Detection of endoderm marker Gsc, and ectodermal marker p75 expression also indicate that the cell population in our 'pro-mesoderm' treatment group still contain cell types of different developmental origin.

Following our 30-day osteogenic differentiation, we found an increase in Alizarin Red S staining in our 'pro-mesoderm' treatment over SR alone (Figure 5.2G), however the degree of staining observed did not reach the same level seen with cells differentiating in FCS. We next took a closer look at lineage markers arising from MSC precursors on day 30 (Figure 5.2H). We found an increase in *Ocn*, *Col II A*, *Col II B*, *Glut4*, *C/EBP*α, and *PPAR*γ indicating a higher degree of specification of chondrocytes, adipocytes and osteoblasts in our pro-mesoderm condition. These results complement

our detected increase in MSC marker expression that we found on day 7 within this treatment group (Figure 5.2F).

Wnt signaling can augment MSC lineage differentiation following our 'pro-mesoderm' treatment

Since it is well established that Wnt signaling modulates MSC specification and lineage commitment (Yang et al., 2012; Krishnan, 2006; Prestwich and Macdougald, 2007), we began expanding our treatment times to examine the effects of prolonged Wnt signaling on differentiation outcomes. Figure 5.3A outlines the treatment schemes we employed. Heretofore all treatments following the timetable depicted in this figure will be listed sequentially and separated with a hyphen (e.g. '5a-3a-5a' is supplementation with rWnt5a from days 0-2 followed by rWnt3a from days 2-4 which is then followed by rWnt5a from days 4-7). We first observed a clear difference in lineage-specific staining when our 'pro-mesoderm' was additionally followed by either rWnt5a or rWnt3a supplementation (Figure 5.3B). For instance, Col II detection was dramatically increased when rWnt treatment was extended to day 7 of the differentiation. Similarly, we saw a significant increase in calcium deposition and alkaline phosphatase activity over the 5a-3a treatment and SR alone (Figure 5.3C). Interestingly, these improvements in the osteogenic endpoints occurred regardless of which rWnt protein used between days 4 and 7. MSC marker expression also appears to improve with both triphasic rWnt treatment schemes, with an increase in *CD73* and *CD105* expression (Figure 5.3D). The mesoderm-derived MSC marker PDGFRα is higher in the 5a-3a-3a, whereas neural crest markers Slug and Snail were upregulated in the 5a-3a-5a treatement group.

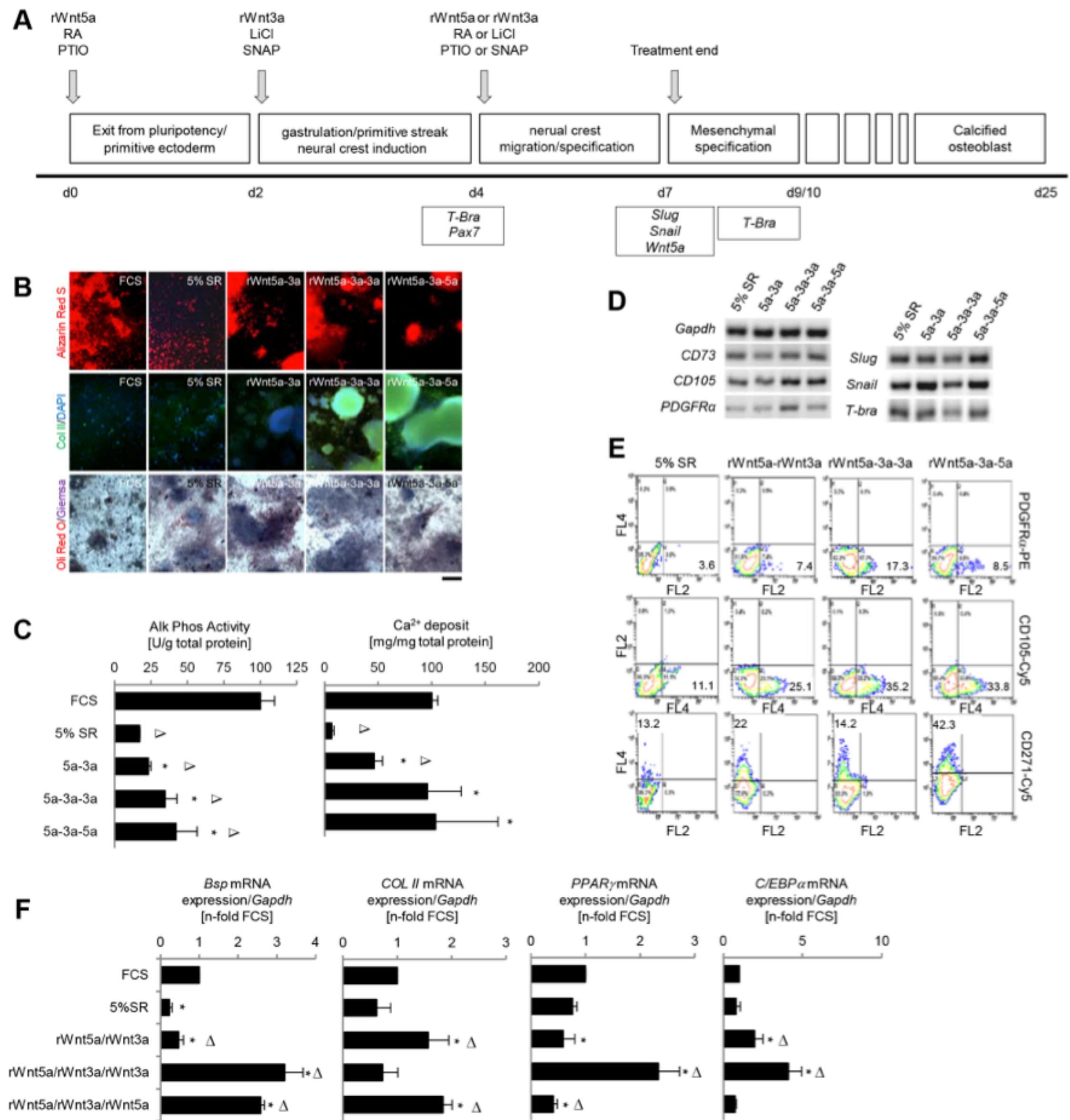

A
rWnt5a
RA
PTIO
rWnt3a
LiCl
SNAP
rWnt5a or rWnt3a
RA or LiCl
PTIO or SNAP
Treatment end
Exit from pluripotency/ primitive ectoderm
gastrulation/primitive streak neural crest induction
nerual crest migration/specification
Mesenchymal specification
Calcified osteoblast
d0
d2
d4
d7
d9/10
d25
T-Bra
Pax7
Slug
Snail
Wnt5a
T-Bra
B
Alizarin Red S
Col II/DAPI
Oli Red O/Giemsa
FCS
5% SR
rWnt5a-3a
rWnt5a-3a-3a
rWnt5a-3a-5a
C
Alk Phos Activity [U/g total protein]
Ca2+ deposit [mg/mg total protein]
FCS
5% SR
5a-3a
5a-3a-3a
5a-3a-5a
D
5% SR
5a-3a
5a-3a-3a
5a-3a-5a
Gapdh
CD73
CD105
PDGFRα
Slug
Snail
T-bra
E
5% SR
rWnt5a-rWnt3a
rWnt5a-3a-3a
rWnt5a-3a-5a
PDGFRα-PE
CD105-Cy5
CD271-Cy5
FL2
FL4
3.6
7.4
17.3
8.5
11.1
25.1
35.2
33.8
13.2
22
14.2
42.3
F
Bsp mRNA expression/Gapdh [n-fold FCS]
COL II mRNA expression/Gapdh [n-fold FCS]
PPARγ mRNA expression/Gapdh [n-fold FCS]
C/EBPα mRNA expression/Gapdh [n-fold FCS]
FCS
5%SR
rWnt5a/rWnt3a
rWnt5a/rWnt3a/rWnt3a
rWnt5a/rWnt3a/rWnt5a

Figure 5.3. Triphasic Wnt treatment alters differentiation towards terminal cell types. (**A**) The 3-phase treatment scheme is depicted here along with the corresponding differentiation stage as determined by marker expression. (**B**) Histochemical and Immunocytological examination of cultures treated with indicated Wnt-treatment scheme. Size bar = 200 µm. (**C**) Calcium deposition was measured on day 30 and normalized to protein concentration for each sample. Alkaline phosphatase activity was also examined in each replicate and normalized to protein content. (**D**) RT-PCR examination of MSC marker and neural crest marker expression on day 7. (**E**) Cells undergoing each specified treatment were probed with MSC marker-specific antibodies on day 7 and analyzed via flow cytometry. Following ten thousand events, the percent of cells exceeding the negative control threshold signal (i.e. secondary antibody probing only) are presented in each graph. (**F**) Relative expression of known osteoblast, chondrocyte, and adipocyte markers were determined via qPCR on day 30. $^*p < 0.05$ as compared to 5% SR control, $\Delta p < 0.05$ as compared to FCS control.

These results suggest that induction of canonical Wnt signaling during the third stage of treatment supports MSC specification from mesoderm precursors, whereas non-canonical signaling at this stages supports MSC specification from neural crest precursors. These results were supported by FACS analysis of these markers, where we found a concurrent increase in CD105 detection in both triphasic treatment types, an increase in PDGFRα in the 5a-3a-3a group, and increase in neural crest marker CD271 in the 5a-3a-5a group (Figure 5.3E).

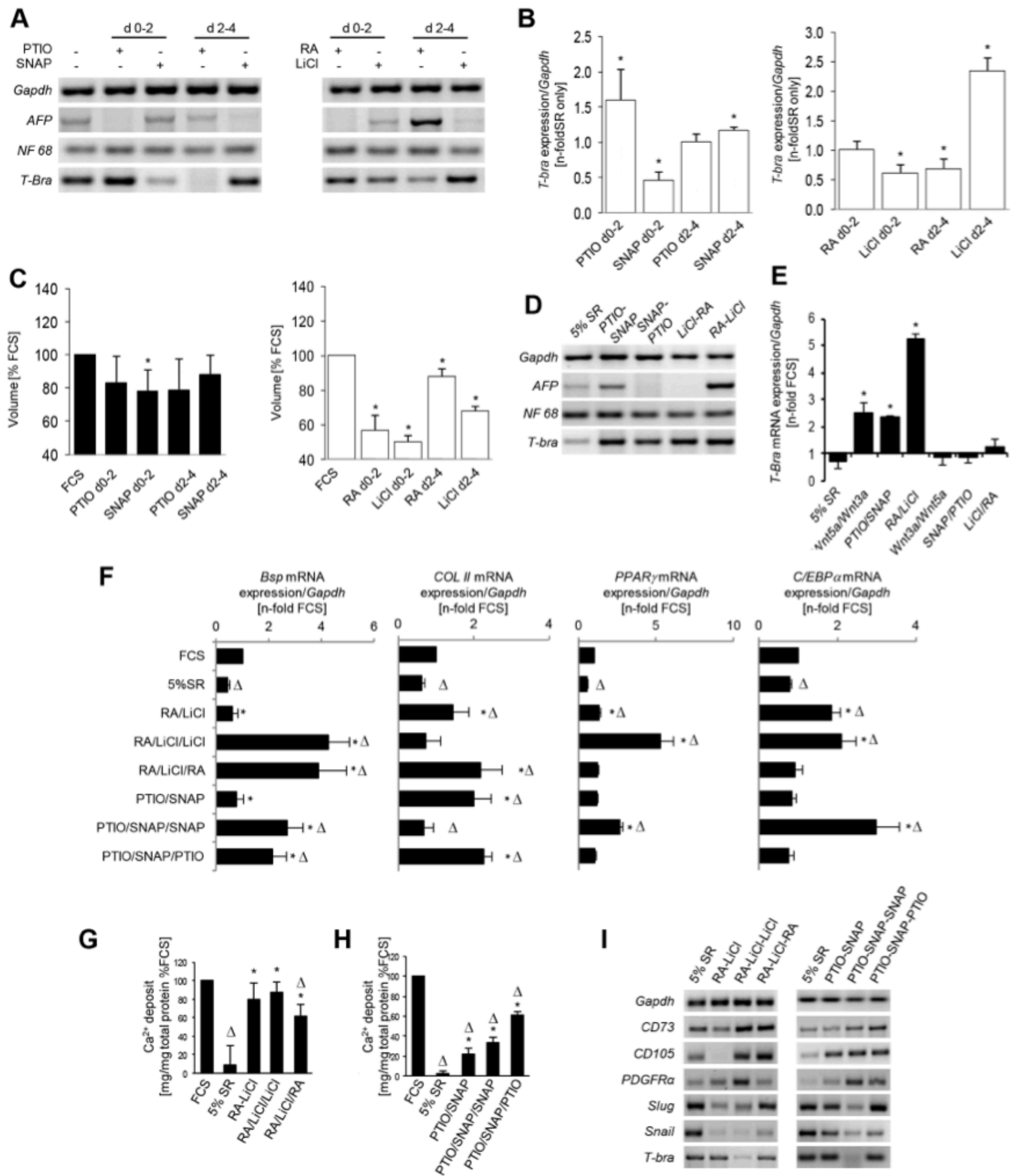
Following an increase in MSC marker expression as measured on day 7, we next asked whether this altered cell population has a higher potential to differentiate into MSC derived lineages when measured on day 30. Expression of the secreted bone-specific marker Bsp showed a significant increase following both 5a-3a-3a and 5a-3a-5a treatments, whereas the 5a-3a treatment and SR alone showed a decrease of Bsp as compared to the FCS control (Figure 5.3F). For the chondrocyte marker ColII we found a significant increase in expression following the 5a-3a and 5a-3a-5a treaments. The adipocyte markers PPARγ and C/EBPα showed an inverted expression pattern when compared to ColII, where the 5a-3a-3a treatment showed the highest marker expression

relative to the 5a-3a and 5a-3a-5a treatments. Thus, the specific modulation of Wnt signaling during early stages of differentiation of mESCs can alter differentiation outcome.

Other modulators of CTNNB1 have similar impacts on differentiation outcome

Although we have shown that rWnt proteins, when supplemented at specific time points, can induce MSC lineage differentiation, the monetary cost of these recombinant proteins makes their reasonable incorporation into differentiation protocols prohibitive. Previous work in our lab has demonstrated that known modulators of nitric oxide (NO): PTIO and SNAP, were able to regulate CTNNB1 activity (Ding et al., 2012). Specifically, the NO scavenger PTIO decreased CTNNB1 transcriptional activity, whereas the NO donor SNAP was able to increase CTNNB1 transcriptional activity, which is analogous to rWnt5a and rWnt3a signaling, respectively. Additional work in our lab had also demonstrated that mESC treatment with lithium chloride (LiCl), through inhibition of GSK3β, increases CTNNB1 transcriptional activity, whereas retinoic acid (RA) decreases this activity (zur Nieden et al., 2007). Thus, we began employing these alternative supplements in order to manipulate CTNNB1 in the same directed manner that we have established with rWnt proteins.

When employing the treatment scheme as outlined in Figure 5.3A with rWnt alternatives, expression of the primitive endoderm marker AFP showed notable differences between treatment groups (Figure 5.4A). Here, we found a decrease in expression when using the CTNNB1 antagonists PTIO and RA from d0-2, an effect that was not maintained when supplemented at days 2-4. In fact, the strongest expression of AFP was detected when cultures were treated with RA from days 2-4. On the other



A
d 0-2
d 2-4
PTIO
SNAP
RA
LiCl
Gapdh
AFP
NF 68
T-Bra
B
T-bra expression/Gapdh [n-foldSR only]
PTIO d0-2
SNAP d0-2
PTIO d2-4
SNAP d2-4
RA d0-2
LiCl d0-2
RA d2-4
LiCl d2-4
C
Volume [% FCS]
FCS
D
5% SR
PTIO-SNAP
SNAP-PTIO
LiCl-RA
RA-LiCl
E
T-Bra mRNA expression/Gapdh [n-fold FCS]
Wnt5a/Wnt3a
PTIO/SNAP
RA/LiCl
Wnt3a/Wnt5a
SNAP/PTIO
LiCl/RA
F
Bsp mRNA expression/Gapdh [n-fold FCS]
COL II mRNA expression/Gapdh [n-fold FCS]
PPARγ mRNA expression/Gapdh [n-fold FCS]
C/EBPα mRNA expression/Gapdh [n-fold FCS]
FCS
5%SR
RA/LiCl
RA/LiCl/LiCl
RA/LiCl/RA
PTIO/SNAP
PTIO/SNAP/SNAP
PTIO/SNAP/PTIO
G
H
Ca2+ deposit [mg/mg total protein %FCS]
I
RA-LiCl
RA-LiCl-LiCl
RA-LiCl-RA
PTIO-SNAP
PTIO-SNAP-SNAP
PTIO-SNAP-PTIO
CD73
CD105
PDGFRα
Slug
Snail
T-bra

Figure 5.4. Chemical modulators of CTNNB1 mimic Wnt-treatment schemes. (**A**) RT-PCR examination of early differentiation markers on day 4 in cultures that were supplemented with 100 μM PTIO, 100 μM SNAP, 100 nM RA or 20 mM LiCl for the indicated duration. (**B**) qPCR was employed to determine the relative expression of the mesoderm marker *T-bra*. (**C**) The mean volume of embryoid body formation on day 4 in each condition was determined from the diameter of 50 embryoid bodies as measured with image analysis software (ImageJ, National Institutes of Health). (**D**) Early differention marker expression was examined via RT-PCR on day 4 in biphasic treatment groups. (**E**) A quantitative comparison *T-bra* expression was made between each biphasic treatment group using qPCR. (**F**) Relative expression of known osteoblast, chondrocyte, and adipocyte markers were determined via qPCR on day 30. (**G**) Calcium deposition was measured on day 30 and normalized to protein concentration for each sample in cultures treated with either RA and LiCl or (**H**) PTIO and SNAP. (**I**) MSC marker and neural crest marker expression was examined via RT-PCR on day 7. $*p < 0.05$ as compared to 5% SR control, $\Delta p < 0.05$ as compared to FCS control.

hand, SNAP and LiCl, when supplemented during days 2-4, demonstrated a decreased in AFP expression, which parallels the result seen with rWnt3a treatment during this time period (Figure 5.2A). When examining the ectodermal marker NF68, we detected minimal changes in expression; although we did note that RA treatment during either time period resulted in the highest degree of expression. In contrast, *T-bra* expression was dramatically different depending on the type of treatment. *T-bra* expression increased following PTIO and RA treatment during the early time point. This effect was also seen when SNAP and LiCl was supplemented during days 2-4. We noted that these results followed modulation of *T-bra* expression with rWnt proteins (Figure 5.2B). Furthermore, quantification of *T-bra* expression with qPCR (Figure 4B) correlated to the detected mRNA levels found with standard PCR techniques in Figure 5.4A.

When measuring the EB volume following treatments we found that EBs were generally smaller but a significant reduction in size was only observed for the day 0-2 SNAP treatment (Figure 4C). Whereas treatment with either RA or LiCl, at any time

period, resulted in a significant decrease in EB size, potentially indicating an impact on cell proliferation.

We next began supplementing these factors to direct the differentiation of ESCs following the combination schemes outlined in Figure 5.3A. Interestingly, we found that all double treatment groups showed an increase in *T-bra* expression, and the treatments that mimicked our aforementioned 'pro-mesoderm' supplementation additionally showed and increase in AFP expression when examined on day 4 (Figure 5.4D). Quantification of *T-bra* expression on day 7 revealed a significant increase in expression following our 'pro-mesoderm' conditions, with RA-LiCl resulting in the largest increase in marker expression (Figure 5.4E).

At the end of 30 days of differentiation the endpoints of MSC derived cell types were examined in a similar fashion as indicated in Figure 5.3F. In terms of relative marker expression pattern, we found that in each case, PTIO was interchangeable with RA, and SNAP was interchangeable LiCl (Figure 5.4F). These same patterns also corresponded to the results found when using rWNT supplementations (Figure 5.3F). Interestingly, the treatment groups that resulted in the highest bone-specific marker *Bsp*, also showed the lowest expression of the chondrocyte-specific marker *Col II*. These groups also showed the highest expression of the adiopocyte markers *PPAR*γ and *C/EBP*α. Chondryocyte marker expression was highest following either the 'pro-mesoderm' day 0-4 treatments or when these treatments were additionally followed with RA or PTIO. To further quantify the impact on osteogenic differentiation potential, extracellular matrix calcium deposition was measured on Day 30 (Figure 5.4G/H). Here, every treatment group showed an increase in calcium deposition over 5% SR alone, with

RA-LiCl-LiCl and PTIO-SNAP-PTIO treatments showing the highest levels of detected calcium within their respective groups.

Previous triphasic treatment schemes with rWNT supplementation suggested MSC specification from either mesoderm or neural crest precursors depending on the third stage treatment with either canonical or noncanonical rWNTs, respectively (Figures 3D/E). When employing alternative CTNNB1 modulators we saw similar pattern of expression (Figure 5.4I). Here we find that the modulators known to increase CTNNB1 transcriptional activity are able to concurrently increase *PDGFR*α, *CD73*, and *CD105* expression, indicating mesoderm-derived MSC specification. Whereas, chemical modulators known to antagonize CTNNB1 were found to increase *Slug*, *Snail*, *CD73*, and *CD105* indicating neural crest-derived MSC specification. These results further support the model where CTNNB1 activity during days 4 – 7 favors different precursor cells to differentiate into MSCs.

Discussion:

The ability to efficiently direct the lineage decisions of undifferentiated cell types into specific terminally differentiated cell types is of paramount significance towards developing stem cell based-therapeutics. In order to rigorously test the efficacy of individual media components in modulating specific signaling pathways and directing differentiation, we believe investigators should avoid the variability inherent with the use of animal serum. When we began incorporating serum-replacer (SR) into our differentiation protocol, we discovered that the concentration we used had noticeably altered osteogenic, chondrogenic, and adipogenic outcome. Although we presume that

individual labs similarly optimize their differentiation conditions, they seldom report their findings. Here we found that 5% SR was just as, if not more, effective in producing these individual cell types when compared to higher SR concentrations commonly found in other protocols. In a similar study Chaudry et al. (Chaudhry et al., 2008), reported that 5% SR was more effective in the production of embryoid bodies when compared to 15% SR; highlighting the importance of examining SR concentration in directing cell differentiation.

We next explored how treatment with Wnt ligands could affect early differentiation decisions. Initially, we found that the non-canonical ligand Wnt5a, when applied during days 0-2, was able to improve mesoderm specification as determined by *T-bra* expression. Although lack of Wnt5a in developing mouse embryos causes many developmental defects included anterior-posterior axis truncation, it is not required for mesoderm formation (Yamaguchi et al., 1999a). However, we suspect that the improvement in specification of mesoderm may be facilitated through antagonism of canonical Wnt signaling, which has been reported to maintain pluripotency of mESCs at this stage (Sato et al., 2004; Ogawa et al., 2006; Ying et al., 2008). Despite what appears to be a contradictory concept, canonical Wnt signaling also plays a significant role in regulating early differentiation decisions (Zhang et al., 2013). For instance, in the absence of canonical Wnt signaling mouse embryos fail to specify mesoderm (Liu et al., 1999; Huelsken et al., 2000). Here we report that Wnt3a treatment from days 2-4 significantly enhanced *T-bra* expression. Consistent with this finding, other investigators have reported a similar time window where canonical Wnt signal activation can enhance mesoderm specification in mESCs (Lindsley et al., 2006; Nakanishi et al., 2009; Gadue et al., 2006).

To the best of our knowledge, we are the first to report this biphasic Wnt supplementation to induce mesoderm formation, although other investigators have explored varying Wnt ligand supplementation during later stages of differentiation. For instance, Naito et al. (Naito et al., 2006), found that Wnt signaling had a mutually antagonistic, biphasic role in regulating cardiomyogenesis and hematopoiesis. Despite the differences between this study and ours (e.g. media components, treatment schemes, and lineage specifications) they nevertheless share our idea that modulating CTNNB1 in a stage-specific manner can result in greater differentiation efficiency.

The results from our three-stage treatment scheme yielded the highest marker expression of osteoblast, chondrocyte and adipocyte differentiation, indicating the utility of CTNNB1 modulation to direct differentiation of terminal cell-types. However, some results were initially puzzling, such as the interchangeable treatment type at the third stage in directing osteogenic differentiation. For instance, if Wnt5a and Wnt3a have presumably opposing action, how do they both improve osteogenic yield when added from days 4-7 (Figure 5.3C/F)? It is possible that during this stage of differentiation the receptor profile of these cells allows them to bind to different Wnt ligands yet elicit the same downstream signaling response, as described by Mikels and Nusse (Mikels and Nusse, 2006), who showed that Wnt5a could activate CTNNB1 transcriptional activity with the appropriate cell receptor context. However, since we detected the same response when we employed other CTNNB1 modulators in our study (Figure 5.4F, G and H), one possible explanation is that the differentiation kinetics are different between these groups despite the same differentiation outcome as measured on day 30. Boland et al. (Boland et al., 2004) found that Wnt3a increased proliferation of human MSCs at the expense of osteogenic induction, however, this effect was reversible once this Wnt

ligand was removed from the media. Similar to our findings, this group also reported osteogenic induction following Wnt5a treatment. A more intriguing explanation is that these different treatment types are causing different precursor populations to differentiate into MSCs, which subsequently have the potential to become mature osteoblasts. Although, we refer to our initial treatment scheme as 'pro-mesoderm' we find simultaneous evidence of ectoderm marker expression. Canonical Wnt signaling followed by non-canonical Wnt signaling has also been shown to be essential for neural crest induction and migration, respectively (De Calisto et al., 2005; Mayor and Theveneau, 2014). Thus, we suggest that within our triphasic treatment scheme (i.e. with non-canonical WNT signaling in the third phase) can recapitulate the *in vivo* neural crest-derived MSC specification.

To further assess whether Wnt signaling was mediating its influence on differentiation through the regulation of CTNNB1, we employed other known modulators to mimic the activating or repressive action of canonical or non-canonical Wnt signaling, respectively (i.e. SNAP or LiCl in place of Wnt3a, and PTIO or RA in place of Wnt5a). Previous work in our lab and others (Easwaran et al., 1999; zur Nieden et al., 2007b; Ding et al., 2012; Gonzalez et al., 2012) have demonstrated specific changes to CTNNB1 activity following treatment with these chemicals. With the exception of a few small differences these alternative modulators reproduced the patterns of gene expression seen with their corresponding Wnt ligands. Although we acknowledge that there are off-target effects of using PTIO, SNAP, LiCl and RA when regulating CTNNB1, the high degree of correspondence between these treatments indicates a commonality underlying their ability to influence cell fate decisions. We believe treatment with other

recently described chemical modulators of CTNNB1 (Zhang et al., 2013; Kanke et al., 2014) will recapitulate the findings we present in this report.

In this study we explore how temporal modulation of CTNNB1 through Wnt signaling and other small molecules throughout the course of differentiation can significantly alter differentiation capacity. Using the appropriate treatment scheme, we demonstrated that multiple approaches to regulate CTNNB1 increased differentiation along osteogenic, chondrogenic, and adipogenic lineages within a serum-free system. We strongly believe that further examination of lineage decisions under the control of CTNNB1 will greatly improve our understanding of developmental programs and improve stem cell-based therapeutics.

Materials and Methods:

Cell culture. Mouse ESCs of the D3 cell line (American Type Culture Collection) were maintained in monolayer cultures containing DMEM (Gibco) supplemented with 4.5 g/L D-glucose, 15% fetal calf serum (FCS) (Sigma-Aldrich), 50 U/mL penicillin, 50 µg/mL streptomycin, 1% non-essential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), and 1000 U/mL leukemia inhibitory factor (LIF) (Gibco). To initiate differentiation, cells were dispersed as a single cell suspension by treatment with 0.25% Trypsin/EDTA (Cellgro), and switched to complete media without LIF containing either 15% FCS or the indicated concentration of KnockOutTM Serum Replacement (Gibco). The hanging drop method, as described by Heuer et. al (Heuer et al., 1993), was employed during the first 5 days of differentiation. Briefly, 20 µL of media containing ~750 cells was applied as drops to the underside of a petri dish covers. After 3 days

embryoid body (EB) cell aggregates were transferred to suspension culture, where they continued to differentiate for 2 additional days. On day 5, EBs were trypsinized and replated at 50,000 cells/cm2 in the presence of osteogenic inducers: 10 mM β-glycerophosphate, 25 µg/ml ascorbic acid, and 5 x 10-8 M 1α,25(OH)2 vitamin D3, as previously described (zur Nieden et al., 2003). All maintenance and differentiation cultures were maintained at 37°C in 5% CO2.

Biochemical analysis. ALP activity and Ca^{2+} content was measured from RIPA buffer lysates containing 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 2 mg/ml leupeptin, 100 mM sodium orthovanadate. RIPA buffer was added to culture plates and incubated for 30 min at 4°C under gentle rocking before cell lysates were collected. Samples were centrifuged and the RIPA supernatant was collected for further analysis. The remaining cell pellet, which contained most of the mineral content, was lysed with 1 N HCl overnight in at 4°C.

RIPA and HCl lysates were then subjected to react with Arsenazo III reagent (DCL, Toronto, Canada) as previously described (zur Nieden et al., 2007b; Davis et al., 2011). A $CaCl_2$ standard was measured along with the samples at 650 nm in a Benchmark Plus microplate spectrophotometer (Bio-Rad). Absorbance readings for reagent only were subtracted in all instances. Amount of deposited calcium was expressed as a fraction of the total protein in the sample (Davis et al., 2011), which was measured from the RIPA lysates using DC protein assay reagent (Bio-Rad) with protocol as described therein. Samples were incubated with the reagent for 15 min and the change in absorbance was registered in a Benchmark Plus microplate

spectrophotometer (Bio-Rad) at 750 nm. Protein quantities in samples were measured against a standard curve of defined concentrations of bovine serum albumen.

ALP activity was determined from the RIPA protein lysates via incubation using p-nitrophenylphosphate (Sigma) at 37°C as described (zur Nieden et al., 2007a). The kinetic change in absorbance was determined by the change of absorbance at 405 nm between t50 sec and t520 sec using a Bio-Rad Microplate Manager 5.2. Enzyme activity was normalized to protein content and calculated as previously described (Davis et al., 2011).

Immunocytochemistry. On day 30 of the differentiation cultures were washed in PBS and fixed overnight in 4% PFA in PBS at 4°C. Cells were permeabilized in 0.5% saponin in PBS at 4°C overnight, rinsed once in PBS, and then blocked in 3% BSA at 4°C overnight. Either Aggrecan (Santa Cruz) or Collagen II (Hybridoma Bank) primary antibodies were diluted to 1:50 in 3% BSA and added to the culture wells. Following incubation overnight at 4°C, culture wells were washed 3 times with PBS and blocked again, overnight at 4°C. Following the block, the cultures were incubated with an appropriate Alexa Fluor 488 secondary antibody (Molecular Probes, Eugene, OR) and 15 mM of the DNA counterstain 49,6-diamidin-29-phenylindoldihydrochlorid (Roche), at room temperature for 2 hours. Following incubation, cultures were washed 3 times with PBS. The wells were examined using a Zeiss 510 confocal Microscope with 488, 568, and 633 nm filters. Images were prepared using Zeiss LSM image browsing software.

Histochemical staining. For Alizarin red staining, cultures were fixed overnight at 4°C in 4%paraformaldehyde, and then washed 3 times in PBS. The cultures were placed in

1% KOH solution for 48 h at 4°C and then stained with Alizarin red (1 mg Alizarin red, 100 mL of 1% KOH) for 48 h at 4°C. The aggregates were rinsed with 1% KOH several times and visualized while submerged in PBS.

For adipocyte staining, cultures were washed with PBS and without any fixation, directly overlaid with Oil-red-O working solution (0.18% Oil-Red-O dye/60% propanol) After a staining period of 15 minutes, ES cell cultures were rinsed in distilled water. Cultures were then submerged into a 10% Giemsa stain (Columbia Diagnostics) for 4 min at room temperature and washed with distilled water, then immediately visualized.

Gene expression analysis. Cells were harvested for RNA isolation in triplicate from biological duplicates at various time points as indicated. Total RNA was isolated using the RNeasy Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentration was determined with the Ribogreen RNA quantification reagent (Molecular Probes) as described (zur Nieden et al., 2004). Complementary DNA was synthesized from 500 ng of RNA with Superscript II (Invitrogen, Carlsbad, CA) as suggested by the manufacturer (Invitrogen) and described previously (zur Nieden et al., 2005). Gene expression analysis was performed using RT-PCR as previously described (zur Nieden et al., 2007a). Primer sequences were designed with primer 3 (http://www.ncbi.nlm.nih.gov/ sutils/e-pcr/reverse.cgi) and were run through reverse ePCR to bioinformatically confirm amplicon identity (Table 1). All primer annealing steps were performed at 60 ℃. PCR products were semi-quantitatively analyzed using electrophoresis in 3% agarose gels containing 2% ethidium bromide. All gels were run in the presence of 1 bp plus ladder (Invitrogen) and a cDNA template control lacking cDNA. Quantitative PCR was performed on an iCycler iQ system (BioRad) using a proprietary

SYBR Green PCR Mix. Melting curves were generated at the end of each run to ensure the presence of one single amplicon. Gene expression levels were analyzed with the $2^{(-\Delta\Delta C(t))}$ method (Livak and Schmittgen, 2001) from target gene C(t) values and respective C(t) values obtained for the reference gene GAPDH.

Flow Cytometry. On day 7 of the differentiation cells were dissociated into a single-cell suspension using 0.25% Trypsin/EDTA (Cellgro). Following antibody binding steps, cells were suspended in sorting buffer containing 1 mM EDTA, 25 mM HEPES ph 7.0, 1% Fetal Bovine Serum (FBS) in 1X PBS. Ten thousand events were registered in a Beckman Coulter FC500 and mean percentages of antibody-positive cells calculated from three independent differentiations. Untreated cells were used to gate the appropriate population of interest and to exclude cellular autofluorescence.

Statistical Analysis. Data are presented as means ± s.d. Comparison of two groups was made using Student's t-test for unpaired data. Comparison of more than two groups was conducted using ANOVA.

Table 5.1: Primer sequences

Name	Forward sequence	Reverse sequence
AFP	AGCTGCGCTCTCTACCAGAC	GAGTTCACAGGGCTTGCTTC
AGG	GATCTGGCATGAGAGAGGCG	GCCACGGTGCCCTTTTTAC
BSP	AAAGTGAAGGAAAGCGACGA	GTTCCTTCTGCACCTGCTTC
C/EBPa	CGCAAGAGCCGAGATAAAGC	GCGGTCATTGTCACTGGTCA
CatnB	CCCTGAGACGCTAGATGAGG	TGTCAGCTGAGGAATTGCAC
CD105	GATTGCCCAGTGATTCTCC	GAGGATGCGGACACTTTTG
CD73	GAACCCAACGTGCTGTTTTT	GGGATCAATCAGTCCTTCCA
Col II	GCTGCTGACGCTGCTCATC	GGTTCTCCTTTCTGCCCCTT
Dkk1	GTCCAAGATCTGTAAACC	GAGTCAAGACAATCAACC
Fzd7	CCAGGTGGATGGTGACCTAC	GACGTCCCGATGAAGAGGTA
Fzd8	CTGTTCCGAATCCGTTCAGT	CGGTTGTGCTGCTCATAGAA
GAPDH	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA
GFAP	GGGCGAGCGAGCGTG	GCTGTTCCAGGAAGCGGAC
Glut4	ATGGCTGCTGCTGGTTTCTC	ACCCATAGCATCCGCAACAT
Gsc	CTCGGAGGAGTCAGAAAACG	TCGACTGTCTGTGCAAGTCC
NF 68	AGTGGCTTTCTGGCTTGCTG	TCTGTGTGATTCACATTGCCATAG
OCN	CCGGGAGCAGTGTGAGCTTA	TAGATGCGTTTGTAGGCGGTC
p53	CACAGCGTGGTGGTACCTTA	CTTCTGTACGGCGGTCTCTC
p75	CAGAGCGAGACCTCATAGCC	TGCAGCTGTTCCATCTCTTG
PDGFRα	TTGGTGCTGTTGGTGATTGT	AGCATCTTCACAGCCACCTT
PPARγ	ATCATCTACACGATGCTGGCC	CTCCCTGGTCATGAATCCTTG
Slug	GCACTGTGATGCCCAGTCTA	TTGGAGCAGTTTTTGCACTG
Snail	GAGGACAGTGGCAAAAGCTC	TCGGATGTGCATCTTCAGAG
Tbra	CCCTGCACATTACACACCAC	GTCCACGAGGCTATGAGGAG
Tcf4	TTTCGCCTCCTGTAAGCAGT	GTGACCCAAGATCCCTGCTA
Tcf7L1	CCGAGTGTACCCTGAAGGAA	ACCCTCTGCCTCTTGGATT

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Chapter 6: Conclusion

The work presented within this thesis extensively examines how CTNNB1 modulation effects the differentiation of mESCs. Consistently, we found that singular treatment types, producing either agonistic or antagonistic effects on CTNNB1, had the most profound impact when applied during narrow time windows of the differentiation. Additionally, individual treatments frequently had opposite effects on differentiation outcome depending on the time window when they were employed. This parallels many findings *in vivo*, where the role of WNT/CTNNB1 signaling is highly dependent on the differentiation stage of osteogenic progression. It is undoubtedly clear that studying CTNNB1 regulation in the context of osteogenesis will strengthen our understanding of developmental programs and provide avenues to enhance our treatment of bone-related pathologies.

WNT5A, a short-term osteogenic inducer

When employing our osteogenic differentiation protocol, we found an upregulation of *Wnt5a* mRNA, and corresponding increase in WNT5A protein on day 7. This result validated the same finding that was seen during a genome-wide microarray analysis of gene expression (zur Nieden et al., 2007). The development of a WNT5A::GFP reporter line greatly facilitated the further exploration of the subpopulation of cells that upregulate WNT5A within our cultures. By examining the differentiation capacity of cells sorted by flow cytometry, we were able to discern whether the upregulation of this WNT was a secondary consequence of osteogenic induction or if its expression directly promoted differentiation. The higher differentiation capacity of GFP^{+}

cells on day 7, and the rescue of GFP$^-$ cells with exogenous supplementation of rWNT5A, strongly indicates a direct role of WNT5A in promoting osteogenesis of mESCs. Furthermore, supplementation of unsorted cells with rWNT5A, during days 5-7, could improve measures of osteogenic differentiation. These results support previous demonstrations of a pro-ostegenic role of WNT5A during differentiation in both in vivo and in vitro systems (Yamaguchi et al., 1999; Baksh and Tuan, 2007; Bilkovski et al., 2010), however, our work is the first to show this effect on the differentiation of pluripotent stem cells.

Further examination revealed that WNT5A treatment elicited a decrease in LEF/TCF::GFP reporter activity. Additional pro-osteogenic treatments, including the osteogenic induction factor VD_3, also were shown to reduce reported CTNNB1 transcriptional activity. Reviewing our previous examination of genome wide transcriptional changes following osteogenic induction, some of the most strongly downregulated transcripts are known targets of WNT/CTNNB1 signaling (e.g. cyclin D2, TDGF1, and plasminogen activator inhibitor-1). Furthermore, treatments that result in stabilized and transcriptionally active CTNNB1 within this period, like rWNT3A and LiCl, reduced osteogenic output. Thus, a strong argument can be made that the concurrent downregulation of WNT/CTNNB1 signaling during osteogenic induction facilitates differentiation along this lineage.

When examining the downstream effectors of WNT5A signaling, we found increased activation of JNK, PKC and CamKII, albeit at different time points and duration, following treatment with rWNT5A. To determine which of these downstream effectors were mediating the pro-osteogenic effect of WNT5A we employed specific chemical inhibitors of each protein. Interestingly, all three chemical inhibitors could

statistically lower osteogenic differentiation, and simultaneous treatment with all three resulted in the strongest effect. These data indicate that the role of WNT5A in promoting osteogenesis is multifaceted, where signal transduction propagates through multiple pathways to affect multiple targets. Although clear delineation of these contributing factors that lie downstream may be difficult to attain, analysis of upstream components may prove to be more straightforward.

In addition to the frizzled receptors, the receptor tyrosine kinase ROR2 has been shown to bind to WNT5A and elicit downstream non-canonical WNT signaling (van Amerongen, 2012). *Ror2* deficient mice exhibit the same craniofacial and skeletal abnormalities seen in *Wnt5a*-null mice (Yamaguchi et al., 1999; Schwabe et al., 2004). Additionally, the rWNT5A-mediated downregulation of LEF/TCF reporter activity, found within the HEK 293 cell line, was dependent on ROR2 (Mikels and Nusse, 2006). Thus, it appears quite plausible that in our mESC model the pro-osteogenic effect of Wnt5a is also mediated through the ROR2 receptor, and not necessarily through the traditionally studied FZD receptors. Following verification of ROR2 expression in our cell model, we could supplement the media with antibodies directed against the extracellular domain of this receptor to explore this hypothesis. A more extensive analysis employing constructs that constitutively expressed ROR2 could also be employed. Mikels and Nusse (2006) found a synergistic enhancement of LEF/TCF reporter repression following rWNT5A treatment within cells overexpressing ROR2. Thus, when employing our cell differentiation model with overexpressed ROR2 we would predict a stronger antagonistic effect on CTNNB1 and an increase in osteogenic specification following rWNT5A supplementation.

Glucose inopportunely elevates AKT and CTNNB1 signaling

Although media containing 4.5 g/L D-glucose is frequently employed during *in vitro* differentiation studies, it is seldom acknowledged that this concentration of glucose far exceeds what cells would naturally be exposed to. However, being mindful of this hyperglycemic culture environment, we can begin to explore connections between the disruption of cellular specification *in vitro* with the dysregulated development or maintenance of tissues within diabetic individuals or newborns of diabetic mothers. When using our mESC osteogenic differentiation model, we see a clear attenuation of osteogenesis within media containing 4.5 g/L D-glucose, as opposed to the physiological level of 1.0 g/L D-glucose. Thus, we set forth to uncover the mechanism behind this differential osteogenic outcome.

First we characterized the reduced osteogenic output that was observed in the hyperglycemic environment. Supporting our qualitative assessment, we found a significant reduction in the calcium deposition within in the ECM of cells growing in high glucose conditions. Additional mRNA analysis showed reduced transcription factor expression that is normally found during early stages of osteoblast specification, as well a late reduction in expression of mature osteoblast markers *Alp* and *Opn*. Despite the robust calcium deposition seen in low glucose conditions, we detected a concurrent increase in osteoclast specification via expression analysis and enzymatic activity assays; potentially indicating a suppression of hematopoietic stem cell formation or differentiation within the hyperglycemic environment.

Extensive investigation in the field of bone biology has supported the role of WNT/CTNNB1 signaling in developmental osteogenesis. Thus, we sought to explore a potential role of this signaling pathway in our hyperglycemic model of impaired

osteogenesis. When using a LEF/TCF::GFP reporter as a readout of CTNNB1 transcriptional activity, we detected a significant increase in GFP positive cells in the stage immediately following osteogenic induction. Although there is some evidence in other systems linking glucose levels to CTNNB1 regulation, the signal transduction cascades connecting these two is far from understood. Thus, we began to examine the AKT signaling pathway as a potential link between signaling that govern glucose utilization and those that regulate CTNNB1.

We detected differential activation of AKT within cells differentiating in these glucose conditions, which corresponded to the differential LEF/TCF::GFP reporter activity in these same conditions. A target of AKT, and known binding partner of CTNNB1, FOXO3A is also differentially phosphorlyated. Using a FOXO::Luciferase reporter, we detected significantly lower reported activity in the high glucose environment within the same time period of increased LEF/TCF/CTNNB1 activity. These results corresponded to an increase in expression of FOXO target genes. Furthermore, FOXO3A co-immunoprecipitated with CTNNB1 to a greater degree during this time period within the low glucose conditions as compared to the hyperglycemic condition. Chemical inhibition of AKT, resulted in an increase in CTNNB1/FOXO3A coimmunoprecipation, and could rescue the osteogenic impairment of cells differentiating in the high glucose condition.

These results link aberrant AKT and CTNNB1 signaling with the suppressed *in vitro* osteogenesis found within the hyperglycemic environment. The demonstration that these signaling pathways are similarly perturbed within *in vivo* systems would greatly support our model through which hyperglycemia results in osteogenic birth defects. To examine CTNNB1 transcriptional activity in developing mice we could utilize the

TOPGAL mouse line created by Elaine Fuchs' group. Cells within these mice express β-galactosidase under the control of concatenated TCF binding sites (DasGupta and Fuchs, 1999). CTNNB1 transcriptional activity has been characterized in the developing skeleton using TOPGAL mice and histology has revealed multiple cell types with reporter activity (e.g chondrocytes, osteoblasts, and osteocytes) (Hens et al., 2005). In the context of fetal development with high maternal glucose, a diabetic TOPGAL mouse could be created via the administration of Streptozotocin (STZ). Although, there is a reduction of fertility of STZ-treated mice (Diamond et al., 1989), developing embryos of these diabetic mothers can be recovered and then examined histologically. We would predict a higher degree of X-gal staining within skeletal cell types, which would further support our hypothesis of aberrant CTNNB1 activity in these developing embryos. Furthermore, to the best of our knowledge a study has not been performed using STZ-induced diabetes in a TOPGAL mouse background. Given the involvement of CTNNB1 in the regulation of both mesoderm and neural crest specification and subsequent endochondral and intramembranous bone formation, this in vivo model would be very informative towards linking glucose mediated dysregulation of CTNNB1 activity with the development and maintenance of multiple tissue types.

In addition, very little is known about the involvement of the FOXO proteins in regulating developmental osteogenesis. FOXO1 has been shown to be highly expressed in areas of presumptive neural-crest derived intramembranous ossification (Teixeira et al., 2010). However, attempts to knock out this transcription factor results in embryonic lethality before osteogenic precursors form (Furuyama et al., 2004). In addition, pleiotropic effects of conditional knockouts of FOXO1 withiin mesenchyme make it very difficult to study its role in osteogenic differentiation (Teixeira et al., 2010).

Thus, any potential change in FOXO1 activity or its distribution within an embryo of a STZ-induced diabetic mother, would greatly support the emerging role of this protein in the regulation of neural crest-derived bone formation.

The similarities between multistage modulators of CTNNB1

A number of qualities make the use of fetal bovine serum (FBS) attractive for the culture of eukaryotic cells. The low antibody concentration and diverse growth factors found within FBS is quite conducive for cell growth and viability. However, there are many qualities that make its use in translational studies for human therapeutics not possible. For instance, the transmission of pathogens is a serious concern when using animal products in medical treatments. For our purposes, the heterogeneity of serum lots combined with the multitude of undefined components it contains, makes for developing differentiation protocols that give consistent results particularly challenging. Thus, when we began an experimental approach to modulate CTNNB1 in a stage specific manner to control differentiation, we employed Serum Replacer to completely define our culture environment and thereby exclude unintended effects of FBS. Thus, any effects that we see in terms of differentiation outcome can be attributed directly to our treatments schemes.

During the early stages of differentiation (i.e. before osteogenic induction), we could significantly increase mesoderm specification with a biphasic treatment of rWNT5A followed by rWNT3A on days 0-2 then on days 2-4, respectively. While other studies have examined these WNT ligands individually and have reported similar results when supplemented at corresponding time periods (Gadue et al., 2006; Lindsley et al., 2006;

Nakanishi et al., 2009), our demonstration is the first of its kind showing that sequential treatment of mESCs with these WNT ligands could improve mesoderm specification.

Following biphasic CTNNB1 modulation to induce mesoderm formation we found that triphasic treatments could increase marker expression associated with MSCs, and result in a subsequent improvement in osteogenic output. Specifically, treatment with rWNT3A in the third phase could increase the expression of osteoblast specific markers whereas rWNT5a treatment increased both osteoblast and chondrocyte marker expression. Furthermore, these triphasic WNT treatments could yield calcium deposition levels within serum-free conditions that were equivalent to levels seen in well established protocols of osteogenic induction utilizing FBS. While other studies have induced osteogenesis of embryonic stem cells in culture, there work mainly employed cytokines and other growth factors to improve differentiation capacity (Heng et al., 2004; Jukes, 2010). Here, we show that the timed supplementation of rWNT proteins alone is enough to effectively differentiate mESCs into osteoblasts. Furthermore, there is strong evidence that manipulation of CTNNB1 itself can improve osteogenic specification. Although the suite of chemical treatments used (i.e. RA, LiCl, PTIO, and SNAP) are known to have additional effects aside from CTNNB1 regulation, the consistent marker expression patterns that were uncovered with there use suggest the common node of CTNNB1 regulation, and encourages their further use in this regard.

Recently Kanke and others (Kanke et al., 2014) developed a protocol to differentiate multiple pluripotent stem cell types into osteoblast with the stepwise chemical treatment, including GSK3 inhibition to increase early mesoderm specification. This report additionally modulates hedgehog (Hh) signaling at different stages to facilitate osteogenic differentiation. Specifically, they antagonized Hh during the early

stage to suppress neural-ectoderm formation, and activated Hh during later stages of osteogenic induction. Given the similarities to their work and the work presented here, it would be interesting to see the results of CTNNB1 modulation with our chemical treatments while simultaneously modulating Hh signaling as defined by their protocol.

Furthermore, results from stage-specific modulation of CTNNB1 by exogenous factors may be confounded by the endogenous expression of WNT proteins by the cells themselves. Utilizing a complete WNT-null background, a greater degree of precision can be obtained when defining the roles of each treatment type at each time point. Recently, investigators have explored targeting Porcupine (PORCN), an intracellular enzyme that exclusively pamitoylates WNT precursors, to achieve cells unable to secret functional WNT proteins. Studies have shown that when the chemical IWP-2 was used to inhibit PORCN, the disrupted effect of WNT-mediated self-renewal could be restored by rWNT supplementation (ten Berge et al., 2011). Thus, a similar chemical treatment or genetic knockout of PORCN could be employed in our system to clearly define the effects of individual treatments that modulate CTNNB1, without obstruction of endogenous WNT signaling effects.

Final summation

CTNNB1 is a fascinating protein whose ever-expanding role in development and disease continues to simultaneously confound and intrigue investigators. Although its role in regulating osteogenesis is complicated, a detailed scientific approach unraveling this complexity is of paramount significance towards a comprehensive understanding of bone biology and the development of novel therapeutics.

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