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1α, 25-dihydroxyvitamin D_3 induced osteogenesis in murine embryonic stem cells: the

role of Wnt5a

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "1α, 25-dihydroxyvitamin D$_3$ induced osteogenesis in murine embryonic stem cells: the role of Wnt5a" submitted by Lesley Anne Margaret Davis in partial fulfilment of the requirements of the degree of Master of Science.

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Abstract

This thesis explores the role of Wnt5a during *in vitro* differentiation of murine embryonic stem cells to osteoblasts. It shows for the first time that Wnt5a gain of function increases mineralization, specifically calcification, during osteoblast differentiation. This increase in mineralization is dependent on Wnt5a concentration upon osteoinduction. In addition, Wnt5a acts with 1α, 25-dihydroxyvitamin D_3 to increase the expression of bone specific genes, such as alkaline phosphatase, osteopontin and osteocalcin. The increase in alkaline phosphatase and osteopontin expression occurred earlier than previously reported; suggesting that the addition of Wnt5a could accelerate osteogenesis *in vitro*. The reason for the synergistic relationship between Wnt5a and 1α, 25-dihydroxyvitamin D_3 could be due to an overlap in signalling pathways, as both molecules may be able to translocate β-catenin to the plasma membrane during osteogenesis.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AER	Apical Ectodermal Ridge
ALP	Alkaline Phosphatase
APC	Adenomatous Polyposis Coli
BMP	Bone Morphogenic Protein
BSP	Bone Sialoprotein
CamKIIα	Calmodulin dependent protein kinase II alpha
cAMP	Cyclic adenosine monophosphate
Cbfa1	Core binding factor alpha 1
CC	Control Conditioned media
ctrl	ES media
Dkk1	Dickkopf 1
Dsh	Dishevelled
EB	Embryoid Body
EC	Embryonic Carcinoma
ECM	Extracellular Matrix
ERK	Extracellular signal Regulated Kinase
ES	Embryonic Stem
FACS	Fluorescence Activated Cell Sorting
FGF	Fibroblast Growth Factor
Fzd	Frizzled
GPAA	β-Glycerophosphate and Ascorbic Acid

HEK	Human Embryonic Kidney
HLH	Helix Loop Helix
ICC	Immunocytochemistry
IFC	Immunofluorescence
IGF	Insulin Growth Factor
Ihh	Indian Hedgehog
Lef	Lymphoid enhancer factor
LRP	Lipoprotein related protein
MAPK	Mitogen Activated Protein Kinase
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cell
NLK	Nemo-Like Kinase
OCN	Osteocalcin
OPN	Osteopontin
PI3K	Phosphoinosital 3 Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PTHrP	Parathyroid hormone related Protein
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Sfrp1	Secreted Frizzled related protein 1
TAK1	Transforming Growth Factor β Activated Kinase 1
Tcf	T-cell Factor

TGFβ	Transforming Growth Factor β
VD3	GPAA, 1α, 25-dihydroxyvitamin D_3
VDR	1α, 25-dihydroxyvitamin D_3 receptor
Vitamin D_3	1α, 25-dihydroxyvitamin D_3

Chapter One: Introduction

1.1 Overview

Bone degeneration is the major cause of fractures in middle aged and elderly adults. One bone degenerative disease, osteoporosis, is defined by the loss of bone mass and effects 8 million women and 2.5 million men in the United States (Surgeon General Report; www.surgeongeneral.gov). The loss of bone mass increases a patient's susceptibility to bone fracture. After the first fracture, mortality increases three fold (Center, Nguyen *et al.* 1999). Osteoporosis pseudoglioma syndrome and high bone mass disorders have been linked specifically to the Wnt pathways and the Wnt receptor LRP5 (Gong, Slee *et al.* 2001; Boyden, Mao *et al.* 2002; Little, Carulli *et al.* 2002). In addition Wnt proteins are known to be involved in skeletal development and may play an important role in therapeutics for bone degenerative disorders.

Much of the Wnt pathway's role in bone development has been deciphered using pre-osteoblastic cell lines or mesenchymal stem cell lines. These cell lines are predetermined for the mesodermal lineage and therefore do not answer some of the fundamental questions regarding early bone differentiation. Using embryonic stem cells as a model for embryo development and inducing the cells toward an osteoblast fate, we can better understand the roles of proteins early in osteogenesis.

This project uses murine embryonic stem cells to determine the role of the protein Wnt5a in endochondral bone formation. In this chapter, the history and use of murine embryonic stem cells as a model system as well as the known mechanism of bone development *in vivo* will be discussed. In addition, key molecules involved in bone differentiation and formation are discussed with particular attention to 1α, 25-

dihydroxyvitamin D$_3$. Finally, background information on Wnt, specifically Wnt5a signalling and their role in bone formation *in vivo* and *in vitro* will be introduced.

1.2 Embryonic stem cells

The derivation of embryonic stem (ES) cells began with the investigation of teratocarcinomas in the 129 strain of mice in 1958. Teratocarcinomas are malignant tumors containing cells of many different tissues as well as a large population of undifferentiated cells (Spangrude 2003). When cells derived from teratomas were re-injected into developing mouse embryos, the teratocarcinoma cells or embryonal carcinoma cells (EC) were able to differentiate into all cells types of the body without loss of their euploid chromosomal complement, a phenomenon termed pluripotency (Spangrude 2003). It was proved that the cells could be expanded and maintained in their pluripotent state by co-culturing with mitotically inactive embryonic fibroblasts (Smith 2001). It was concluded that these fibroblasts were secreting a critical molecule that would maintain pluripotency and thus were referred to as feeder cells.

ES cells were first isolated from the inner cell mass (ICM) of a mouse blastocyst 25 years ago (Evans and Kaufman 1981; Martin 1981). They are a model system for the study of early molecular events of mammalian embryogenesis as well as the mechanisms for maintaining pluripotency (Smith 2001). They closely resemble EC cells in morphology, growth, and gene expression as well as the ability to produce teratomas when injected into an adult mouse. The biological use for ES and EC cells is in the ability of researchers to create transgenic animals. This use has led to our current understanding of embryonic cell proliferation and differentiation into endodermal, ectodermal, and mesodermal cell types (Smith 2001).

The pluripotent potential of a murine ES cell is maintained with the addition of Leukemia Inhibitory Factor (LIF) (Metcalf, 1990), or when cultured on embryonic fibroblasts (Smith 2001). Differentiation is therefore initiated by removing the cells from their feeder layer or from LIF treatment. LIF is a member of the IL-6 cytokine family and binds to the low affinity LIF receptor (LIFR) which is expressed in the inner cell mass of mouse embryos (Nichols, Davidson *et al.* 1996). This binding creates the formation of a high affinity receptor complex with the cytokine receptor gp130. This results in the activation of many signalling pathways including: Ras, ERK 1/2, Jak non-receptor kinases, and Stat3 (Ernst, Oates *et al.* 1996; Boeuf, Hauss *et al.* 1997; Tighe and Gudas 2004). The removal of LIF will activate differentiation and when the ES cells are transferred to a LIF-free non-adherent culture dish, they will form aggregates known as embryoid bodies (EBs). Embryoid bodies will form endoderm from day 2-4 of differentiation on the outer surface of the aggregates, by day 4 columnar epithelium and a basal lamina as well as a central cavity occurs. Mesodermal and endodermal cells appear with continued growth in suspension (Leahy, Xiong *et al.* 1999). Expression of the mesoderm marker T-brachyury is first detectable on day 3 in suspension culture, with an increase on day 4 (Leahy, Xiong *et al.* 1999) As osteoblasts form out of the mesoderm, these are the first days of osteogenesis *in vitro*.

The use of ES cells as a treatment for disease requires an understanding of the differences between mouse and human ES cells. There is significant divergence between mouse and human ES cell expression of markers and responsiveness to intrinsic signals, such as LIF. Using microarray it has been determined that out of 918 transcripts found in human ES cells there are 541 mouse orthologs, which after statistical analysis indicated

that this is not coincidental overlap (Sato, Sanjuan *et al.* 2003). However, there are some key differences between mouse and human ES cells particularly in morphology, immunophenotype, and growth properties. Mouse ES cells grow in attached masses in which single cells are difficult to identify, whereas human ES cells grow in a monolayer of flat colonies that have distinct cell borders (Verfaillie, Pera *et al.* 2002). As many of the genes between mouse and human are similar (Sato, Sanjuan *et al.* 2003), the mouse model is an good model system for studying cell replacement therapy (Guasch and Fuchs 2005).

1.3 General bone development

Pluripotent cell lines are used regularly as models to study osteoblast formation and thus bone development. This section focuses on skeletogenesis spanning the overall mechanism of bone development and molecules involved.

Bone development begins in the limb bud of the mouse on day 9.5 post coitum with mesenchymal condensations. By day 11.5 post coitum the condensations have become chondrocytes for future endochondral bone formation (Yang and Karsenty 2002) or have formed directly into bone as in intramembraneous bone formation. A small number of bones, such as craniofacial bones, form through intramembraneous bone formation. However, the majority of the mammalian skeleton develops through endochondral bone formation. Mesenchymal condensations start the process of skeletogenesis and the size, shape and number is determined by patterning signals (Johnson and Tabin 1997; de Crombrugghe, Lefebvre *et al.* 2001). This patterning information is conveyed through Wnt, Ihh, and FGF families, as well as the TGFβ superfamily. The transcription factors responsible for this process are Hox, Pax,

Forkhead, and basic helix loop helix (HLH) families (Karsenty 1999; Olsen, Reginato *et al.* 2000; de Crombrugghe, Lefebvre *et al.* 2001).

1.3.1 Mechanism of endochondral ossification

Most of the vertebrate skeleton is formed through a cartilage precursor and therefore is considered endochondral bone. The process involves the early embryo creating a limb bud that contains cells undergoing a series of differentiation steps, with the end product being a complex tissue with many different cell types.

Endochondral ossification requires mesenchymal cells to differentiate first into pre-chondrocytes then chondrocytes. The pre-chondrocytes begin by proliferating in one direction creating columns of cells (de Crombrugghe, Lefebvre *et al.* 2001). These cells will secrete a cartilaginous matrix and will eventually become encapsulated in this matrix (Baron 2006). When the cells have become embedded in this matrix and are located in the lacunae, they are referred to as chondrocytes. At the periphery of cartilage formation *in vivo*, the perichondrium, mesenchymal cells continue to differentiate and proliferate. At the centre of the cartilaginous mass of cells, chondrocytes will stop proliferating, differentiate and become hypertrophic. Hypertrophy is characterized by the depositing of a mineralized matrix initiated by matrix vesicles. Once this matrix is calcified, it is partially resorbed as blood vessels bring nutrients and osteoclasts to the developing limb (de Crombrugghe, Lefebvre *et al.* 2001). In addition to nutrients and osteoclasts, the blood vessels also provide oxygen tension to influence differentiation (Pacicca, Patel *et al.* 2003). The resorption of the hypertrophic cartilage matrix is due to osteoclasts that are deposited in the bone from the blood along with the matrix metalloproteinases (MMPs). The cleavage of the two main matrix proteins, aggrecan and collagen type 2, is

accomplished by MMP13 and MMP9. These two MMPs synergize to cleave the matrix proteins in the most terminal hypertrophic zone of the growth plate (Stickens, Behonick *et al.* 2004).   MMP13 is the major collagenase for collagen type 2 cleavage and is located in the developing long bone is found at the cartilage bone interface. The cartilage matrix separates invading capillaries from hypertrophic chondrocytes (Lee, Murphy *et al.* 1999; Stickens, Behonick *et al.* 2004). Osteoblasts will begin differentiating in this area forming a layer of woven bone on top of the cartilage (Baron 2006).

Woven bone is created through the mineralization of the growth plate cartilage *in vivo*. It starts with high levels of Ca^{2+} and PO^{4-} ions that will precipitate into calcium phosphate which leads to hydroxyapatite crystal formation. This precipitation is achieved by membrane bound matrix vesicles, which originate inside the cytoplasm and are deposited within the matrix. The osteoblast or chondroblast is rich in alkaline phosphatase (ALP) at this phase as well as acid phospholipids which hydrolyze inhibitors of calcification, such as pyrophosphate and ATP, allowing for condensation of apatite crystals. Once in the matrix the crystals will grow and coalesce to completely calcify the matrix, filling in spaces between and within the collagen fibres. The process of ES cells differentiating into bone involves the cells secreting an extracellular matrix consisting mostly of collagen type I and calcium (Baron 2006).

In vivo, osteoblasts invade the extracellular matrix scaffold laid down by chondrocytes and begin differentiating and secreting an extracellular matrix typical of bone. This process starts in the middle of the limb bud and will proceed toward the ends of the long bones to establish two growth plates that will separate the distal cartilaginous epiphysis and the medial bony diaphysis (Stickens, Behonick *et al.* 2004).

1.3.2 Molecules involved in endochondral bone formation in vivo

There are a large number of proteins and signalling pathways involved in the creation of the vertebrate skeleton. These molecules are needed to create the body plan, as well as differentiate and elongate the bone. This section lists and describes a few key molecules necessary to understand the molecular formation of endochondral bone.

Endochondral bone formation is regulated by key transcription factors and signalling molecules. Hox genes determine the overall pattern and shape of the skeletal body. After Hox genes have established a basic body plan, the transcription factors Sox9 and Cbfa1 will determine chondrocyte and osteoblast cell fate (de la Fuente and Helms 2005).

1.3.2.1 Sox and Cbfa1

Sox9 belongs to the sox family of transcription factors, characterized by a high mobility group box DNA binding domain (de Crombrugghe, Lefebvre *et al.* 2001). Sox9 is involved in early chondrocyte differentiation as well as endochondral bone formation. During limb formation Sox9 expression is first detected in mesenchymal condensations, followed by chondroprogenitor cells, and all non-hypertrophic chondrocytes. Its role is to regulate chondrocyte differentiation by inhibiting hypertrophy (de Crombrugghe, Lefebvre *et al.* 2001; Chung 2004). Sox9 is activated through phosphorylation in the pre-hypertrophic zone of the growth plate cAMP dependent PKC, this event will enhance its ability to act as a transcription factor (Huang, Chung *et al.* 2001) Specific for chondrogenesis, Sox9 along with other Sox proteins such as, L-Sox5 and Sox6, regulate the expression of the cartilage specific gene, specifically, Collagen type 2a1, Collagen type 1a2, and aggrecan (de Crombrugghe, Lefebvre *et al.* 2001; Boland, Perkins *et al.*

2004). Sox9 is also involved in endochondral osteogenesis. Cartilage that will be replaced by bone, secretes factors necessary for osteogenesis, and Sox9 is necessary for this process (de la Fuente and Helms 2005). Bone morphogenic proteins (BMPs) and Indian Hedgehog have been shown to induce and maintain Sox9 expression.

Core binding factor 1 alpha (Cbfa1) is a member of the Runt-domain family of transcription factors and is expressed, like Sox9, in mesenchymal condensations and hypertrophic chondrocytes. However it is also expressed by osteoblasts (de Crombrugghe, Lefebvre et al. 2001). Cbfa1 is required for osteoblast formation and chondrocyte differentiation and therefore is involved in both endochondral and intramembraneous bone formation (Schroeder, Kahler et al. 2004). Cbfa1 expression starts on day 9.5 post coitum in the notochord and from 10.5 days post coitum in all mesenchymal condensations. Later this transcription factor is expressed exclusively in osteoblast and pre-hypertrophic chondrocytes (Yang and Karsenty 2002). The necessity of Cbfa1 in limb and bone formation was found through the observations made of Cbfa1 null mice. These mice have no bone formation due to the cartilage matrix failing to be degraded and cells of the periosteum therefore being unable to invade the matrix left by the hypertrophic chondrocytes. In addition, the maturation of chondrocytes to hypertrophic chondrocytes in the proximal limb bones, such as the humerus or femur, is inhibited (de Crombrugghe, Lefebvre et al. 2001). Although the proximal limbs are unable to form hypertrophic chondrocytes, the hypertrophic chondrocytes in the distal limb bones, such as tibia and radius are formed but fail to mature. (Chung 2004). Haploinsuffiency of Cbfa1 causes the autosomal dominant bone disorder cleidocranial dysplasia (Schroeder, Kahler et al. 2004) a disease characterized by a delay in osteoblast

differentiation in intramembraneous formed bones (Yang and Karsenty 2002). In contrast, over-expression of Cbfa1 will cause osteopenia (Schroeder, Kahler *et al.* 2004) a disorder characterized by accelerated endochondral ossification (de Crombrugghe, Lefebvre *et al.* 2001). The phenotypic changes caused by varying levels of Cbfa1 suggest that the effect of this protein on osteogenesis is dose dependent (de Crombrugghe, Lefebvre *et al.* 2001; Chung 2004; Schroeder, Kahler *et al.* 2004). Cbfa1 expression is regulated by TAZ, a transcriptional co-activator that binds directly to Cbfa1 enhancing its transcriptional ability (Cui, Cooper *et al.* 2003). In contrast histone deacetylase 3 interacts with Cbfa1 inhibiting its ability to enhance the expression of its target genes (Schroeder, Kahler *et al.* 2004). The gene targets for Cbfa1 include osteocalcin, bone sialoprotein, osteopontin, and collagen type 1a1, all of which are regulated by Cbfa1 binding OSE2, a cis-acting regulatory element within the respective promoters (Ducy, Zhang *et al.* 1997). Sox9 and Cbfa1 are transcription factors that are regulated and regulate a number of other molecules involved in the formation of endochondral bone. Both are targets for parathyroid hormone related protein (PTHrP) (Huang, Zhou *et al.* 2000; Chung 2004), FGF2 (Murakami, Kan *et al.* 2000) and BMPs (de Crombrugghe, Lefebvre *et al.* 2001)

1.3.2.2 Parathyroid hormone related protein

Parathyroid hormone related protein (PTHrP) regulates transcription factors as well as the time in which cells mature and differentiate. PTHrP signalling is mediated by cAMP (Huang, Zhou *et al.* 2000) and is responsible for maintaining chondrocytes in their proliferating state. This is achieved by inhibiting the rate in which chondrocytes exit the cell cycle and become hypertrophic (de Crombrugghe, Lefebvre *et al.* 2001). By

regulating the maturation of chondrocytes to hypertrophic chondrocytes PTHrP therefore regulates Sox9 expression in the developing limb. During osteogenesis PTHrP regulates the PKA dependent phosphorylation of Cbfa1 for degradation (Tintut, Parhami *et al.* 1999; Chung 2004). PTHrP expression is stimulated by the presence of Indian hedgehog (Ihh) in pre-hypertrophic chondrocytes that are not being inhibited by PTHrP. (de Crombrugghe, Lefebvre *et al.* 2001).

1.3.2.3 Indian hedgehog

Indian hedgehog is also a key regulator of chondrocytes, and thus endochondral bone formation, by connecting a number of different signalling pathways. Ihh stimulates PTHrP and therefore is involved in chondrocyte proliferation and the establishment of columns of chondrocytes in the growth plate (de Crombrugghe, Lefebvre *et al.* 2001). This protein signals through the receptor Smoothened, a G-protein coupled receptor that is repressed by the protein Patched 1 (Long, Zhang *et al.* 2001). Ihh binds Patched 1 and thus smoothened is no longer repressed and the signalling pathway is activated (Long, Zhang *et al.* 2001). This pathway directly, or indirectly, promotes cyclin D1 transcription (Long, Zhang *et al.* 2001), offering an explanation of how it promotes chondrocyte proliferation.

Although Ihh has a role in both maintaining chondrocytes in their proliferative state, as well as aiding in their actual proliferation, Ihh is also required for osteoblast differentiation. Mice lacking Ihh have no osteoblasts and their limbs are severely shortened (St-Jacques, Hammerschmidt *et al.* 1999; Chung 2004). In summary, this protein is not required for intramembraneous bone formation but is needed for endochondral bone formation (de Crombrugghe, Lefebvre *et al.* 2001; Chung 2004)

1.3.2.4 Transforming Growth Family β

This family of proteins specifically affects differentiation at the cellular level, rather than overall bone and cartilage formation. The TGFβ superfamily includes the BMP family which is known to increase osteogenesis (Bain, Muller *et al.* 2003) and chondrogenesis (Tuan 2003). BMP proteins signal through transmembrane receptors that have serine/threonine kinase activity and ultimately activate Smad proteins involved in transcription (Attisano and Wrana 2002; Bain, Muller *et al.* 2003). BMP2 treatment of the pre-osteoblast cell line C3H10T1/2 can induce both osteoblast and chondrocyte differentiation and leads to the activation of β–catenin signalling (Bain, Muller *et al.* 2003; Nakashima, Katagiri *et al.* 2005). In early limb development, BMPs regulate mesoderm proliferation, control the growth and regression of the apical ectodermal ridge (AER), specify the anterioposterior axis, initiate chondrogenesis and regulate apoptosis (Zhang, Schwarz *et al.* 2003). During endochondral ossification *in vivo*, BMP2,4,5,7 are expressed in the perichondrium whereas BMP6 is expressed in pre-hypertrophic chondrocytes (Zhang, Schwarz *et al.* 2003). In culture, BMPs can stimulate osteoblast differentiation, however, this same role has not been proven *in vivo* (Chung 2004). However, bone formation resulting from an increase in BMP expression does occur during endochondral bone formation (Chung 2004). One BMP specifically studied with regard to its role in limb formation is BMP2. BMP2 was first identified as a protein that could convert C2C12 myoblasts to the osteoblast lineage (Katagiri, Yamaguchi *et al.* 1994). Its role appears to involve the stimulation of Cbfa1, and Osterix through the transcription factor Dlx5 (Lee, Kwon *et al.* 2003). Osterix is novel zinc-finger transcription factor, specifically expressed in developing bones (Nakashima, Zhou *et al.*

2002) and is involved in terminal differentiation of osteoblasts and fate decision for osteo-chondro progenitors toward the osteoblast lineage (Nakashima, Zhou *et al.* 2002).

1.3.2.5 Markers of Osteogenesis

A differentiating cell goes through a number of stages as it develops into a mature osteoblast. Each stage is defined by the expression of specific marker genes. Alkaline phosphatase (ALP) is known to be up-regulated in undifferentiated cells, pre-osteoblasts, and its expression is decreased as mineralization occurs (Aubin 1998; zur Nieden, Kempka *et al.* 2003). Its expression is controlled through the canonical Wnt pathway (Rawadi, Vayssiere *et al.* 2003). It is a membrane bound enzyme that cleaves an inorganic phosphate off β–glycerophosphate, which then enters the cell and induces osteopontin expression (Beck, Zerler *et al.* 2000).

Osteopontin (OPN) is a cytokine-like molecule that is rich in sialic-acid. It is considered to be part of the small integrin binding ligand N-linked glycoprotein (SIBLING) family of genes that are expressed predominantly in bone. OPN is expressed in many different tissues besides bone, such as dentin, cementum, hypertrophic cartilage, kidney, vascular tissues, activated macrophages, lymphocytes and the epithelia of mammary glands (Rangaswami, Bulbule *et al.* 2006) The expression of OPN is in the early stages of bone differentiation and is associated with cell adhesion. Its role is as a substrate for Matrix metalloproteinases (MMPs) which cleave OPN. These fragments derived from OPN enhance cell adhesion. In addition to β-glycerophosphate, vitamin D_3 stimulates OPN expression and OPN in turn inhibits apatite crystal formation and subsequent mineral resorption. An additional role of OPN is to inhibit apoptosis by causing the activation of phosphoinositide 3 kinase (PI3K). PI3K signals through Akt,

which in turn regulates cell cycle, cell migration, and cell survival (Rangaswami, Bulbule *et al.* 2006). During bone differentiation *in vitro*, OPN expression is known to peak twice, during proliferation and again just prior to Bone sialoprotein (BSP) and osteocalcin (OCN) expression. BSP and OCN gene expression are both up-regulated in the mature osteoblast (zur Nieden, Kempka et al. 2003).

The chondrocyte lineage is marked by the expression of Sox9, collagen type 2a1 and collagen type 1a as well as aggrecan and link protein. The collagen proteins are the major molecules found in the ECM of chondrocytes. Aggrecan is a large aggregating chondroitin sulphate proteoglycan. It is the major component of cartilage that binds to hyaluronic acid via an amino-terminal globular region. This binding is stabilized by link protein (Doege, Sasaki *et al.* 1991), and is bound within the collagen network of the ECM.

1.4 1α, 25-dihydroxyvitamin D$_3$ and its role in bone formation

One symptom of osteoporosis is the lack of active 1α, 25-dihydroxyvitamin D$_3$ (vitamin D$_3$) in the body. This hormone will increase bone mass and osteoblast formation *in vivo* and *in vitro*. This section focuses on the role of vitamin D$_3$ in bone formation and the signalling cascade that it activates.

During bone development in the mammalian skeleton, vitamin D$_3$ harvests Ca^{2+} from the intestine, kidney, and bone to increase serum calcium levels to indirectly mineralize bone. It is also responsible for cell growth, differentiation, hormone secretion, T-cell proliferation, cytokine production, sterol metabolism, and cardiovascular function (Christakos, Raval-Pandya *et al.* 1996). It works with parathyroid hormone and calcitonin to regulate serum calcium homeostasis (Suda, Ueno *et al.* 2003) and thus move Ca^{2+} to

areas of bone growth. Although vitamin D_3 is known to stimulate osteoblast specific bone formation, whether it is directly responsible for the mineralization of bone is still controversial (Suda, Ueno *et al.* 2003). Physiological doses of vitamin D_3 inhibit parathyroid hormone induced bone resorption. However, doses exceeding physiological levels will induce bone resorption (Suda, Ueno *et al.* 2003).

The effect of vitamin D_3 on osteoblast differentiation is biphasic; either inhibiting or stimulating normal development and gene expression depending on whether treatment is during proliferation or differentiation (Gurlek, Pittelkow *et al.* 2002). Vitamin D_3 is known to inhibit proliferation of osteoblasts and induce them toward a more differentiated state (Gurlek, Pittelkow *et al.* 2002). This inhibition was observed when vitamin D_3 was given to rat calvaria osteoblast cultures; it not only hindered proliferation it also reduced collagen synthesis and ALP activity. Moreover, osteocalcin (OCN) expression was inhibited and nodule formation was blocked (Owen, Aronow *et al.* 1991; Ishida, Bellows *et al.* 1993). In contrast, in another study, vitamin D_3 given to mature osteoblasts resulted in the increased expression of OCN and OPN as well as stimulate the accumulation of calcium (Matsumoto, Igarashi *et al.* 1991). Although there is still controversy surrounding the effect of vitamin D_3 on osteoblast growth *in vitro*, there is a general agreement that it has anti-proliferative and pro-differentiative properties (Gurlek, Pittelkow *et al.* 2002).

1.4.1.1 The signalling pathway of 1α, 25-dihydroxyvitamin D_3

Vitamin D_3 regulates gene expression and differentiation by binding to specific vitamin D receptors (VDR) of the nuclear receptor superfamily. Activated VDRs enter the nucleus of the cell and bind to specific nucleotide sequences called vitamin D

response elements in target genes to activate or repress their expression (Palmer, Gonzalez-Sancho *et al.* 2001).

Nuclear receptors bind small lipophilic ligands such as steroids, thyroid hormone, retinoids and vitamin D_3. They can be divided into two subfamilies: type I which form homodimers such as the androgen and estrogen receptors and type II which form retinoid X receptors (RXR) heterodimers such as the VDR, thyroid receptor, retinoic acid and peroxisome proliferator activated receptor (PPAR) (Mulholland, Dedhar *et al.* 2005). All nuclear receptors will interact with ligands, cause gene activation and post translational events with many different signalling pathways including MAPK, PI3K/AKT and Wnt (Mulholland, Dedhar *et al.* 2005).

The Wnt pathway molecules such as Wnts, β–catenin, Tcfs, cyclin D1 and GSK3β alter nuclear receptor function through transcriptional activation, repression and phosphorylation (Mulholland, Dedhar *et al.* 2005). In contrast, the activation of the nuclear receptor VDR by vitamin D_3 can effect the expression of genes linked to the Wnt pathway, such as E-cadherin (Palmer, Gonzalez-Sancho *et al.* 2001). Vitamin D_3 causes a VDR and β–catenin interaction which is hypothesized by Palmer *et al.* 2001 to decrease the binding of Tcf4 to β–catenin thus inhibiting β–catenin nuclear activity. Palmer *et al.* showed that VDR activation by vitamin D_3 will cause changes in the integrity of adheren junctions, increase differentiation and decrease oncogenic cell signalling (Palmer, Gonzalez-Sancho *et al.* 2001). In addition, vitamin D_3 does effect proliferation and cell adhesion, which are both functions of β–catenin, cyclin D1, a target gene of β–catenin, is

unaffected by vitamin D_3 treatment either at the RNA or protein level (Palmer, Gonzalez-Sancho *et al.* 2001).

Other pathways associated with vitamin D_3 and osteoblast formation include the phosphoinositol kinase (PI3K) and PKC signalling pathway in many cell types (Gniadecki, Gajkowska *et al.* 1997; Hmama, Nandan *et al.* 1999). The activation of PKC involves a negative feedback loop involving parathyroid hormone, VDR and PKC. First, cAMP elevation increases parathyroid hormone expression. This in turn increases VDR expression which up-regulates PKC activation which down-regulates VDR expression (Krishnan and Feldman 1992; Krishnan, Cramer *et al.* 1995). The activation of PKC by VDR will increase OPN expression which activates the PI3K pathway (Chang and Prince 1993). The PI3K pathway inhibits GSK3β activity through phosphorylation events by AKT thus allowing β–catenin to accumulate in the nucleus and increase proliferation of the cell.

The TGFβ pathway stimulates collagen matrix production and proliferation in human osteoblasts. Its been suggested that vitamin D_3 increases TGFβ production (Finkelman, Linkhart *et al.* 1991), as well as TGFβ type I and type II receptors (Wu, Haugen *et al.* 1997). Vitamin D_3 will induce cells to terminate proliferation and express OCN (Gurlek, Pittelkow *et al.* 2002), a mature bone marker. The two molecules together synergistically increase ALP in a dose and time dependent manner (Ingram, Bonde *et al.* 1994). However, the increase in ALP production can also be shown with the addition of Insulin growth factor 1 and vitamin D_3 in a synergistic manner (Kurose, Seino *et al.* 1989).

Of note, dexamethasone, a pharmacological osteoblast inducer used in many laboratories to study osteogenesis inhibits β–catenin nuclear activity through GSK3β. In osteoblast cultures, dexamethasone activates GSK3β promoting β–catenin degradation (Mulholland, Dedhar *et al.* 2005). Therefore dexamethasone and vitamin D_3 ultimately achieve the same result of decreasing the levels of β–catenin within the nucleus.

In conclusion, vitamin D_3 and the nuclear receptor VDR inhibit the action of β–catenin in the nucleus, thereby terminating cell proliferation.

1.5 **Osteogenesis** *in vitro*

This project is the continuation of Dr. N.I. zur Nieden's PhD thesis work, where ES cells were induced to become osteoblasts under the influence of β-glycerophosphate, ascorbic acid and vitamin D_3 (Figure 1) (zur Nieden, Kempka *et al.* 2003). β-glycerophosphate and ascorbic acid have been shown to trigger mineralization in embryoid bodies undergoing osteogenesis (Buttery, Bourne *et al.* 2001). Ascorbic acid has been shown to induce matrix formation, and ALP activity (Chentoufi, Hott *et al.* 1993). This study outlined the specific days of the 30 day protocol on which osteoblast marker genes were up-regulated in their expression. The stages of *in vitro* osteogenesis start with proliferation of osteoprogenitor cells which will differentiate into osteoids and finally proceed to mature osteoblast formation. The first five days of osteoblast induction shows a small increase in Cbfa1 accompanied with OPN, and collagen type I (zur Nieden, Kempka *et al.* 2003). However, ALP, the first marker of osteogenesis known to up-regulate in early osteogenesis, is found to peak its RNA expression and enzyme activity on day 15 (zur Nieden, Kempka *et al.* 2003). This enzyme precedes the osteoblast

phenotype and is thought to play a role in the initiation of mineralization. ALP expression is followed by OPN, which is found in mineralized tissue bound to hydroxyapatite (Somerman, Prince *et al.* 1987). This marker is known to up-regulate its expression starting on day 18 and maintain its expression to day 23 (zur Nieden, Kempka *et al.* 2003). The final week of osteogenesis is marked by an increase in Cbfa1 followed by a second peak of ALP, then with OCN and BSP. The time in which ALP and OPN are expressed is thought to be near the end of the proliferative state during matrix deposition (zur Nieden, Kempka *et al.* 2003). Therefore, through the expression of certain genes, the stage of osteoblast differentiation *in vitro* can be determined. The expression of these marker genes are in part also known to activate and cross-react with other pathways. One such pathway is the Wnt signalling pathway, which is known to be involved in osteogenesis.

1.6 Wnt signalling

Osteogenesis is thought to be regulated, in part, by the Wnt signalling pathways. There are a number of disorders for which the symptoms are due to mutations in molecules that are members of these pathways. This section outlines what is controversial and known in the Wnt signalling pathways with regard to signalling and bone formation.

1.6.1 General

The Wnt family consists of a number of highly conserved, secreted, glycoproteins involved in many developmental processes such as cell differentiation, polarity, cell migration, and cell proliferation (Moon, Bowerman *et al.* 2002). The pathway was first discovered in *Drosophila melanogaster* as a mutation in the gene Wingless. Both mice and humans have 19 Wnt genes (Miller 2002) and the structure of Wnt molecules is not

yet resolved. However, they are hydrophobic, and highly insoluble. All Wnts contain 23 or 24 cysteine residues and palmitoylation is necessary for Wnt activity (Willert, Brown *et al.* 2003). The vertebrate Wnts historically have been divided into two functional groups; those that induce secondary axis formation in *Xenopus* embryos, such as Wnt 1, 2, 3, 3a, 8, and 8b, and those that do not induce an axis, such as Wnt 4, 5a, 5b, 6, 7a and 11. The axis-inducing Wnts activate Frizzled receptors (Fzd), which signal through the transcription factor, β-catenin. This pathway is now called the canonical pathway. The evidence that some Wnts can operate in a β-catenin-independent manner introduced a less characterized alternate pathway, dubbed the non-canonical pathway. The latter has been split into 3 pathways, 2 of which stimulate intracellular calcium release. The pathways that stimulate intracellular calcium release are involved in cell adhesion and cell shape. Those that do not stimulate intracellular calcium release are involved in cell polarity. Many downstream targets of the non-canonical pathway have not been completely described, and none are unique to that pathway (Yang, Topol *et al.* 2003). Neither the canonical and non-canonical Wnt signalling groups are absolutely distinct. In fact, both pathways can signal through the protein dishevelled (Dsh) (Yang, Topol *et al.* 2003), and some non-axis forming Wnts are involved in axis formation, and vice versa (Figure 2) (Kuhl, Sheldahl *et al.* 2000).

1.6.2 The Canonical Pathway:

The canonical pathway is defined by the stabilization or degradation of β-catenin, and the translocation of this protein to the nucleus, where it acts as a transcription factor. Each component of this pathway is evolutionarily conserved and fundamental to the pathway's involvement in the control of cell proliferation and differentiation (Topol,

Jiang *et al.* 2003) and survival (Almeida, Han *et al.* 2005). The canonical pathway begins

with a Wnt protein binding a Frizzled receptor and a co-receptor low-density lipoprotein

5/6 (LRP5/6). There are ten known members of the Fzd gene family in humans and mice.

Little is known about the Wnt ligand specificity or affinity of vertebrate Fzds. It has been

suggested that there is some redundancy in Wnt-Fzd binding as there are half the number

of Fzds as there are Wnt proteins. Wnts interact with frizzled receptors through a

cysteine rich domain which folds into a structure similar to serpentine receptors and

therefore thought to interact with heterotrimeric guanine nucleotide binding proteins or

G-proteins (Malbon, Wang et al. 2001; Logan and Nusse 2004). The intracellular tail of

Fzds contains the motif KTxxxW which will recruit phosphorylated dishevelled (Dsh) to

the membrane (Umbhauer, Djiane et al. 2000). Dsh is activated and suppresses the

phosphorylation activity of glycogen synthase kinase 3 beta (GSK3β). β-catenin can also

be regulated in a non-GSK3β manner by recruitment of axin to the membrane by

LRP5/6, which causes axin degradation. As a consequence, β-catenin is no longer bound

to the axin-GSK3β complex and β-catenin degradation is reduced. The phosphorylation

of β-catenin and subsequent degradation is thus prevented, and it accumulates in the

nucleus where it binds to the transcription factor lymphoid enhancer factor (Lef) or T-cell

factor (Tcf) and triggers downstream gene transcription (Logan and Nusse 2004). In the

absence of Wnt signal, the multiprotein complex involving Axin, casein kinase 1,

GSK3β, APC and Dsh mediate β–catenin degradation. GSK3β phosphorylates

β–catenin creating binding sites for β-TrCP, an F-box protein in the E3 ubiquitin ligase

complex, to attach and mark β-catenin for proteasome-mediated degradation (Figure 2) (Topol, Jiang *et al.* 2003).

1.6.3 The non-canonical pathway:

The non-canonical Wnt pathway regulates a wide range of developmental activities such as heart induction, tissue separation, and neuronal migration. This alternate method of Wnt signalling was first discovered and proven by studying the mechanisms and regulation of gastrulation movements and involvement of the planar cell polarity (PCP) signalling pathway in *Drosophila melanogaster*. There are three different non-canonical pathways, each characterized by a unique set of proteins. Two pathways cause a calcium flux that activates calmodulin dependent protein kinase II alpha (CamKIIα) and/or protein kinase C (PKC) (Kuhl, Sheldahl *et al.* 2000). The pathway going through CamKIIα may signal through a heterotrimeric G protein, TGFβ activated kinase (TAK1) and Nemo-like kinase (NLK) to inhibit β–catenin in the nucleus (Ishitani, Kishida *et al.* 2003) or it may also signal through PKC and the transcription factor NF-AT (Veeman, Axelrod *et al.* 2003). The other pathways involve protein Jun-kinase (JNK) and both small monomeric and large heterotrimeric G proteins (Veeman, Axelrod *et al.* 2003). The non-canonical pathway is well known as many of the associated proteins can interact and signal through other developmental pathways (Figure 2).

1.6.4 Wnt pathways and bone development

The involvement of the Wnt signalling pathway during osteogenesis is complex, as many molecules show contradictory phenotypes depending on dose, time of treatment, cell line, and model organism. Most of the information known about the Wnt family of proteins and their involvement in osteogenesis stems from pluripotent precursor cell lines

such as; C2C12, C3H10T1/2, MC3T3, and ST2. All of these cell lines develop the osteoblast phenotype in the presence of BMP2 or other osteogenic stimuli (Aubin 1998). The roles of both the canonical and non-canonical pathway during osteogenesis both *in vivo* and *in vitro* are controversial. It has been suggested that the Wnt5a non-canonical pathway is predominant during endochondral bone formation specifically with regard to chondrocyte differentiation whereas the canonical pathway is predominant in osteogenesis (Yates, Shortkroff *et al.* 2005).

The role of the Wnt pathway in chondrocyte differentiation in the mouse, as in other models, revolves around the location and expression of the protein β–catenin. Constitutively active β–catenin inhibits both chondrocyte differentiation and proliferation in the growth plate (Akiyama, Lyons *et al.* 2004). However, forced expression of Wnt5a will delay chondrocyte maturation to hypertrophic stages (Yang 2003). As many other pathways involve β–catenin, including cell adhesion, TGFβ superfamily, and Wnt, it can be concluded there is considerable cross-talk between the pathways.

Mesenchymal condensations are characterized by increased cell density and cell-cell adhesion. N-cadherin is a calcium dependent protein involved in cell-cell adhesion (Oberlender *et al.* 1994; reviewed in Tuan 2003) and it directly interacts with β–catenin at the plasma membrane. This interaction occurs specifically at the time of mesenchymal condensations before differentiation begins (Tuan 2003). During cellular differentiation, N-cadherin and β–catenin expression decreases while collagen type 2a expression increases (Tuan 2003). Therefore, the interaction of β–catenin and N-cadherin for cell-cell adhesion is necessary for mesenchymal condensations but not differentiation.

Lithium chloride treatment is known to increase β–catenin expression by inhibiting serine/threonine phosphorylation activity of GSK3β. When treating C3H10T1/2 cells with lithium chloride, there is a decrease in the ability of BMP2 to stimulate chondrogenesis. Also there is a decrease in the level of N-cadherin mRNA expression and nuclear β–catenin. In addition, the interaction of N-cadherin with β-catenin and GSK3β was reduced (Fischer, Boland *et al.* 2002; Hwang, Yu *et al.* 2005). Although the canonical Wnt pathway is involved in mesenchymal condensations, other proteins such as Sox9 are also involved. In fact, β–catenin and Sox9 negatively regulate each other and it has been suggested that β–catenin binds Sox9 at its transactivation domain, thus effectively inhibiting its activity (Akiyama, Lyons *et al.* 2004). The role of the canonical Wnt pathway during bone development requires chondrocyte differentiation and specifically cartilage matrix gene expression (Zhou, Eid *et al.* 2004).

The non-canonical Wnt pathways are essential for chondrogenesis in the same way the canonical pathway is essential for osteogenesis (Hu, Hilton *et al.* 2005). The inactivation of β–catenin will cause chondrocyte formation in an osteoinducing medium, thus reinforcing the idea that β–catenin regulation is key to determining the fate of an osteo-chondro precursor (Day, Guo *et al.* 2005). Further evidence shows that the up-regulation of β–catenin precedes osteoblast differentiation during intramembraneous ossification, and during endochondral bone formation β–catenin is down-regulated in the chondrogenic condensations and up-regulated in the surrounding cells (Day, Guo *et al.* 2005). In addition, maturation of chondrocytes is accelerated by canonical over-expression (Day, Guo *et al.* 2005). In conclusion, the canonical pathway inhibits

chondrocyte differentiation, enhances endochondral bone formation and promotes chondrocyte maturation. In contrast, canonical signalling has been shown to inhibit osteogenesis in dexamethasone treated human mesenchymal stem cells (MSCs) (de Boer, Siddappa *et al.* 2004). This study found that Wnt3a conditioned media from L-cells reduced ALP expression, mineralization, and increased proliferation of mesenchymal stem cells treated with dexamethasone and β-glycerophosphate (de Boer, Siddappa *et al.* 2004).

β-catenin induces early, not late osteogenesis, as shown through ALP mRNA expression and activity; it has no effect on OCN expression. β–catenin also stops osteoblasts from transdifferentiating to chondrocytes (Hill, Spater *et al.* 2005). The role of the canonical pathway in proliferation extends to a role that also abrogates apoptosis by Wnt3a inducing the phosphorylation of ERK which, in turn inactivates GSK3β and up-regulates the anti-apoptotic protein bcl-2 (Almeida, Han *et al.* 2005). β–catenin binds Lef/Tcf transcription factors to induce the expression of many genes including cyclin D1. Lef1 expression decreases Cbfa1 activation of OCN promoter in undifferentiated cells (Kahler and Westendorf 2003). Lef1 has been shown to delay osteoblast differentiation in MC3T3 pre-osteoblast cells and regulate the expression of extracellular matrix proteins (Kahler, Galindo *et al.* 2006). The reduction of Lef1 with short hairpin RNAs resulted in accelerated differentiation, but did not effect cell proliferation as these cells are already committed to the osteoblast lineage (Kahler, Galindo *et al.* 2006). The concept that canonical signalling is suppressed in mature osteoblasts (Boland, Perkins *et al.* 2004) is

supported by the increase in Dickkopf 1 (Dkk1) and other Wnt antagonists in mature osteoblasts (Kalajzic, Staal *et al.* 2005).

1.7 Wnt5a

Wingless-type MMTV integration site family, member 5A (Wnt5a) was first discovered by Gavin *et al.* 1990 in the mouse using a PCR based method to screen for genes involved in fetal development (Gavin, McMahon et al. 1990). It is a member of the Wnt family of proteins, cysteine rich, with a predicted molecular weight of approximately 41 KiloDaltons. It contains a hydrophobic signal sequence, is palmitoylated (Willert, Brown *et al.* 2003) and acts in a number of developmental systems. The developmental niches it affects include the female reproductive system and the neural system. The parts of the reproductive system that is effected by Wnt5a consists of the genital tubercle, external genitalia development (Heikkila, Peltoketo *et al.* 2001), and uterus (Mericskay, Kitajewski *et al.* 2004). The effected areas of neural development includes anterior-posterior axis formation, and the cerebral cortex (Shimogori, VanSant *et al.* 2004). In addition, it is known to regulate convergent extension movements, adhesion and migration through collagen, activation of the collagen binding discoidin domain receptor 1 (Jonsson and Andersson 2001) and proliferation of progenitor cells (Yamaguchi, Bradley *et al.* 1999) including the hematopoietic system (Murdoch, Chadwick *et al.* 2003). Wnt5a also acts as a tumor suppressor (Liang, Chen *et al.* 2003) and is involved in rheumatoid arthritis (Sen, Lauterbach *et al.* 2000; Jonsson and Andersson 2001) The mechanism of Wnt5a signaling was first studied in zebrafish and xenopus and it was with these studies that they found that Wnt5a over-expression gave the same phenotype as the overexpression of 5HT1c serotonin receptor in xenopus embryos (Slusarski, Corces *et al.*

1997; Kuhl, Sheldahl *et al.* 2000). As 5HT1 receptors are serpentine, stimulate an intracellular calcium release and bind G-proteins, it was thought that Wnt5a would activate a similar pathway. The Fzds found to be activated by Wnt5a included Fzd2 based on the ability to trigger intracellular calcium release (Kuhl, Sheldahl *et al.* 2000). Further study using G-protein inhibitors, such as pertussis toxin, inhibited the release of intracellular calcium. Also, the addition of the biphosphonate compound, L690330, which inhibits inositol monophosphatase, blocked intracellular calcium release. Therefore, Wnt5a signals through Fzd2, a G-protein and the phosphatidylinositol pathway. This non-canonical pathway is thought to activate genes involved in cell adhesion and cell shape. Wnt5a has been proven to signal through the non-canonical Wnt pathways (Slusarski, Corces *et al.* 1997; Kuhl, Sheldahl *et al.* 2000; Ishitani, Kishida *et al.* 2003; Kinoshita, Iioka *et al.* 2003); whether it can also signal through the canonical pathway is controversial.

Nonetheless, Wnt5a has been shown to interact with the canonical pathway in a manner that reduces β–catenin nuclear activity. There are two theories regarding how Wnt5a reduces β-catenin activity in the nucleus. The first involves β–catenin reduction in a GSK3β independent manner involving the protein Siah2 (Topol, Jiang *et al.* 2003). Here, Wnt5a mediated β–catenin degradation did not go through either CamKIIα or NF-AT (Topol, Jiang *et al.* 2003). Nor did it involve a G-protein or calcium influx (Mikels and Nusse 2006). In contrast, Ishitani *et al.* 2003 showed signalling through a G-protein, involving a calcium influx and the proteins CamKIIα, TAK1 and NLK. Other groups have suggested, and shown, that Wnt5a can induce a secondary axis, and therefore induce

the canonical Wnt pathway, depending on which Fzd it binds (He, Saint-Jeannet *et al.* 1997). Here, it was shown that human Fzd5, mouse Fzd8 and xenopus Fzd8 produced a secondary axis when stimulated by Wnt5a (He, Saint-Jeannet *et al.* 1997). Recently, Mikels and Nusse (2006) proved that Wnt5a activates or inhibits β–catenin in the nucleus depending on whether it signals through Fzd4, enhancing the canonical pathway or Ror2, inhibiting the canonical pathway in HEK293T cells. Ror2 was chosen to study as it is a single-pass transmembrane tyrosine kinase and its expression pattern overlaps with Wnt5a, their knockout phenotypes are similar, and they act synergistically to activate Jun kinase. However, other groups have shown that Wnt5a cannot activate nuclear β–catenin induced transcription with any of the ten frizzled proteins (Liu *et al.* 2005). In addition, Wnt5a cannot bind LRP6, only Fzd, and both proteins are required for canonical signalling (Liu, Bafico *et al.* 2005). Mikels and Nusse (2006) found similar results to Liu *et al.* 2005 in regard to the ability of Wnt5a to bind LRP and the need for both Fzd and LRP receptors to signal through the canonical pathway; however, they studied LRP5. They indicated that when Fzd4 and LRP5 are co-expressed together on the cell surface, then Wnt5a could stabilize β–catenin with Fzd4 alone. However, transcription was not activated (Mikels and Nusse 2006). The effect of Wnt5a on the cell will depend on the receptor, and the expression of the receptor will differ depending on how the cells are cultured. When Wnt5a and its receptor Fzd2 were cultured either as a monolayer or as an aggregate there was a marked decrease in Fzd2 mRNA expression when cells were grown in aggregate but Wnt5a expression remained (Nishioka, Dennis *et al.* 2005). This suggests that the role of Wnt5a in mesenchymal condensations does not involve the Fzd2 receptor.

The pathway Wnt5a signals through has also been thought to be regulated by the protein Dsh. This protein is comprised of three domains, DEP, PDZ and DIX and, upon Wnt signalling, is recruited to the cell membrane and will activate either the PCP or β−catenin pathway. The absence of any of the three domains will activate the PCP pathway, especially the absence of DIX (Veeman, Axelrod *et al.* 2003). This same deletion activates PKC and intracellular calcium flux in a G-protein independent manner (Sheldahl, Slusarski *et al.* 2003).

Wnts that signal through the nuclear receptor PPARγ, specifically Wnt5a, regulate the growth, differentiation, apoptosis and insulin sensitivity of differentiating cells (Debril, Renaud *et al.* 2001; Michalik, Desvergne *et al.* 2004). The heterodimers PPARγ/RXR promote the differentiation of the cells in the mesenchymal lineage. Specifically this has been shown with the differentiation of pre-adipocytes to adipocytes (Gerhold, Liu *et al.* 2002). Here, in the absence of adipogenic stimuli, canonical Wnt proteins will promote the cell growth and cell proliferation by acting on cyclin D1 and c-myc, while inhibiting PPARγ. Cyclin D1 and c-myc inhibit by binding directly to PPARγ and the CCAAAT enhancer binding protein (C/EBPα) transcription factor respectively (Shtutman, Zhurinsky *et al.* 1999; Tetsu and McCormick 1999; Ross, Erickson *et al.* 2002). The expression of C/EBPα coincides with the phosphorylation of β−catenin and subsequent degradation.

1.7.1 Wnt5a and Bone

Wnt5a has now been implicated in the differentiation of the mesodermal lineages, chondrocyte, adipocytes and osteoblasts. The Wnt5a knockout embryo shows a

phenotype of caudal truncation, shortened anterior-posterior axis, truncation of the snout, tongue and mandible, shortened fore- and hindlimbs which lack digits, absent genital tubercle and lung abnormalities (Yamaguchi, Bradley *et al.* 1999). Its role in arthritis and the resulting phenotype of limb truncation in the knockout mouse started an investigation into its role in limb development. Ectopic over expression of Wnt5a, in the chicken model, delayed chondrocyte differentiation (Hartmann and Tabin 2000). Using the mouse model, studies of the mechanism of Wnt5a in endochondral bone formation resulted in Wnt5a being implicated in regulating differentiation and proliferation in articular cartilage and growth plate (Yang, Topol *et al.* 2003). Its role in differentiation was suggested as the expression pattern of Wnt5a in the ectoderm and distal mesenchyme (Gavin, McMahon *et al.* 1990) overlaps with Ihh and Cbfa1 (Yang, Topol *et al.* 2003). In addition, Wnt5a knockout mice lacked Sox9 expression in mesenchymal condensation, Cbfa1 expression was reduced along with OCN expression, and PTHrP expression was normal (Yang, Topol *et al.* 2003). Its second function with regard to the regulation of proliferation has been shown by its ability to stop articular cartilage from becoming proliferative (Yang, Topol *et al.* 2003). Cyclin D1, a transcriptional target of β−catenin, is expressed in proliferating chondrocytes, whereas p130, a cycle cycle regulator that inhibits cell division, is expressed in articular cartilage. It was suggested that Wnt5a up-regulates p130 and thus promotes hypertrophy (Yang, Topol *et al.* 2003; Daumer, Tufan *et al.* 2004). The delay in chondrogenesis has been linked to decreased levels of cellular β−catenin in Wnt5a transfected cells (Daumer, Tufan *et al.* 2004). In fact, Wnt5a over-expression in chick limb bud mesenchymal cells resulted in phenotype opposite of β−catenin over-expression. Also, Wnt5a over- expressing cells also increased N-

cadherin which is up-regulated in hypertrophic chondrocytes and osteoblast cells when cultured with βGP and AA (Daumer, Tufan *et al.* 2004).

Human marrow stromal cells induced to become chondrocytes through the ectopic addition of TGFβ1 to the culture media increase Wnt5a and LRP5 expression and nuclear β−catenin (Zhou, Eid *et al.* 2004). Additionally, TGFβ stimulated extracellular matrix accumulation in chondrocytes synergistically with increased canonical Wnt signal. In contrast, Wnt-LRP5 signalling increases TGFβ signalling in osteoblasts (Yao, Chen *et al.* 2002). Other genes from the TGF superfamily have been shown to interact with Wnt5a. One gene from the TGF superfamily, TGFβ3, will increase Wnt5a expression in chick limb buds (Jin, Park *et al.* 2006). This paper suggested that Wnt5a up-regulated chondrocyte differentiation through the PKC and p38 MAPK pathway.

Wnt5a has been implicated in osteogenesis specifically in regard to endochondral ossification. In human bone marrow derived MSCs, Wnt5a has been shown to increase osteogenesis and Ror2 was also shown to increase its expression during osteogenesis (Boland, Perkins *et al.* 2004). It was suggested that the non-canonical pathway was directly involved in osteogenic differentiation and the canonical pathway regulated proliferation (Boland, Perkins *et al.* 2004). During osteogenesis, Wnt5a has also been shown to inhibit apoptosis (Daumer, Tufan *et al.* 2004; Almeida, Han *et al.* 2005). The anti-apoptotic effect has been shown to be the result of the Src/ERK and PI3K/AKT pathways (Almeida, Han *et al.* 2005).

In conclusion, Wnt5a is known to be involved in chondrogenesis and one study looked at Wnt5a in osteogenesis. Wnt5a is known to decrease chondrocyte proliferation

and regulate Sox9; however the signalling pathway and the exact role it plays in bone formation is still controversial.

1.8 Hypothesis and Objectives of this thesis

Wnt5a will increase endochondral osteogenesis in differentiating murine embryonic stem cells by signalling through the non-canonical Wnt pathway.

The objectives of this thesis include: the determination of the effect of Wnt5a on differentiating murine embryonic stem cells, specifically with regard to mineralization and calcification; the determination of the functional role Wnt5a has on the Wnt signalling pathway during *in vitro* osteogenesis.
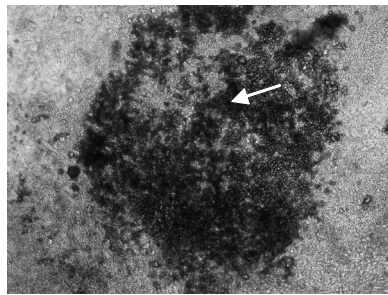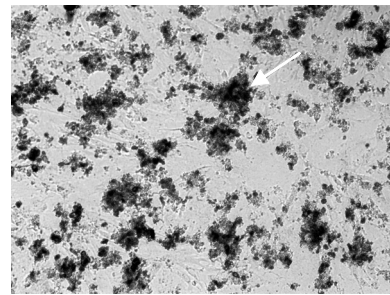
Embryoid body   Monolayer

Figure 1: Images of day 30 osteoblasts. ES cells were either plated as embryoid bodies (left) or as a monolayer (right). Each culture was induced through the addition of β-glycerophosophate, ascorbic acid and 1α, 25-dihydroxyvitamin D_3. The embryoid body was magnified 2x under phase contrast microscopy whereas the monolayer was magnified 10x. White arrows indicate mineralized osteoblasts which appear jet black.
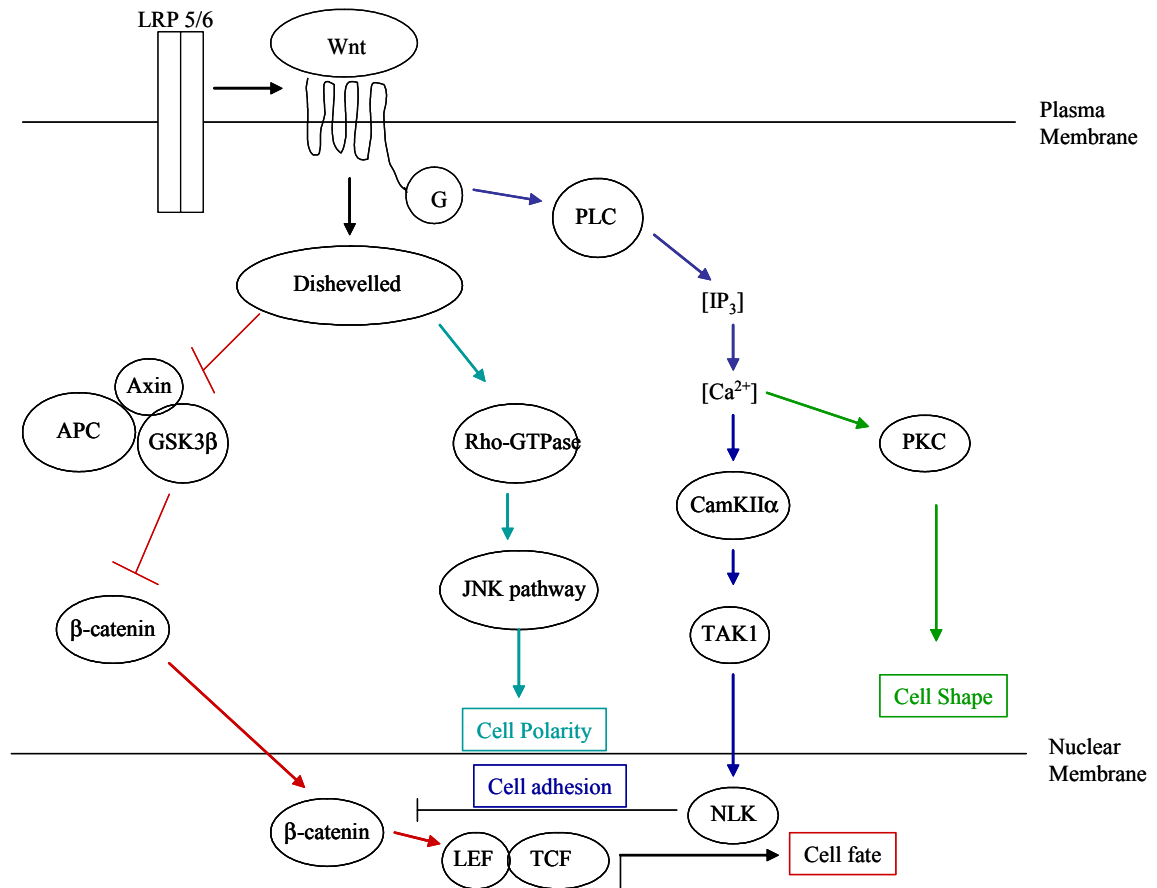
Figure 2: Simplified cartoon of the canonical and three non-canonical Wnt pathways. Canonical pathway is thought to play a role in cell fate (red box), while the JNK pathway acts on cell polarity (teal box), CamKIIα pathway plays a role in cell adhesion (blue box) and PKC on cell shape (green box). The PKC and CamKIIα are the two non-canonical pathways activated by intracellular calcium.

Chapter Two: Materials and Methods

2.1 Cell lines

The D3 embryonic stem cell line was obtained from the American Type Culture Collection (ATCC: Cat. No.CRL-11632). The Wnt5a over expressing cell line L-M(TK⁻) and its corresponding parental line were also obtained from ATCC (Cat. No, CRL-2814, CRL-2648).

2.2 Disposables, chemicals and media additives

Disposables for cell culture were purchased through VWR from Falcon (Becton Dickinson). RNase and DNase-free disposables, such as filter tips, pipette tips and reaction vials were ordered from VWR. Media additives were reconstituted or dissolved as follows and stored in aliquots at –20°C with the exception of β-glycerophosphate which was weighed and dissolved for immediate use.

Ascorbic acid	5 mg/ml stock in Phosphate Buffered Saline (PBS)
β-glycerophosphate	1 M in PBS, filter sterilized
1α, 25-OH$_2$ vitamin D$_3$	50 μg/ml stock in dimethyl sulfoxide (DMSO), Calbiochem
Wnt5a	10 μg/ml stock in PBS, 0.1% Bovine Serum Albumin, R&D Systems

All water-based solutions were generated using Millipore water (Milli-Q Synthesis system). Alcohol-based solutions and all other chemicals were of tissue culture quality or >98% pure.

2.3 Cell culture media

All basic tissue-culture media were ordered from Gibco (Invitrogen) and supplemented

with the respective additives.

 ES medium – see Table 1

Dulbecco's Modified Eagle medium (1x) (high glucose, cat. No. 11995-073)
15% Fetal Calf Serum (FCS) (Gibco - selected batches)
1% non-essential amino acids 10mM solution (Cat.No. 11140-050)
50 U/ml Penicillin (Cat. No 15070-063)
0.1 mM β-mercaptoethanol (Cat. No. 21985-023)

VD3 medium – see Table 1

ES medium
10 mM β-glycerophosphate (Sigma-Aldrich, Cat. No. G9891)
50 µg/ml ascorbic acid (Sigma-Aldrich, Cat. No. A92902)
5×10^{-8} M 1α, 25 OH$_2$ vitamin D$_3$ (Calbiochem, Cat. No. 679101)

Freeze medium

90% FCS (Gibco)
10% DMSO (sigma)

L-cell conditioned medium

ES medium
0.6mg/ml G418 (Biochemika – Fluka Cat.No.48753)

Culture media was stored at 4°C and used within two weeks. Directly before the
experiment, media was warmed up to 37°C.

2.4 Buffers and solutions

If not otherwise noted, chemicals were purchased from Sigma-Aldrich.

DEPC-H$_2$O   Millipore-water
                         0.1% (v/v) DEPC
                         Incubate overnight at room temperature
                         autoclave at 121°C for 60 min before use

1x TE	10 mM Tris-HCl
	1 mM EDTA, pH 8.0

1xPBS	137mM NaCl
	2.7mM KCl
	10mM Na_2HPO_4
	2mM KH_2PO_4
	Millipore Water
	pH 7.4
	autoclave at 121°C for 60 min before use

RIPA	10 mM NaCl
	10 mM Tris, pH 7.2
	0.1% SDS
	1% triton X-100
	1% Deoxycholate
	5 mM EDTA
	Protease Inhibitors: 1mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 2 µg/ml leupeptin, 100 µM sodium orthovanadate and 10 mM p-nitrophenylphosphate (freshly added)

2.5 Antibodies

Antibodies	Dilution	Isotype	Manufacturer
Monoclonal anti-mouse β-catenin	200	Mouse IgG1-Kappa	Zymed Cat. No. 18-0226
Polyclonal anti-mouse Wnt5a	100	goat IgG	R&D systems Cat. No. AF645
Secondary anti-goat HRP	5000	donkey IgG	R&D systems Cat No. HAF109
Secondary anti-mouse Alexa Fluor 546	500	rabbit IgG	Molecular Probes Cat. No.A-11060
Secondary anti-goat Alexa Fluor 488	500	donkey IgG	Molecular Probes Cat. No. A-11055

2.6 Oligonucleotides

The sequences of the genes under investigation were taken from an internet based database (www.ncbi.nlm.nih.gov/Entrez/Nucleotide) and inserted into a primer design program (www.primer3.com). Primer sequences were generated with primer3 and the oligonucleotides ordered from Operon. Sequence specificity was verified using a sequence comparison tool on the basis of the BLAST (Basic local alignment tool) algorithm (Altschul, Gish *et al.* 1990) as well as with an electronic reverse PCR (ePCR) that finds possible amplicons from two primer sequences in a database (http://www.ncbi.nlm.nih.gov/sutils/e-pcr/reverse.cgi) Lyophilized primers were reconstituted in 1x TE to a stock concentration of 100 pmol/µl.

Primer Sequences

Name	Forward	Reverse	Amplicon
ALP	GTGCCCTGACTGAGGCTGTC	GGATCATCGTGTCCTGCTCAC	81
β-catenin	GTGCAATTCCTGAGCTGACA	CTTAAAGATGGCCAGCAAGC	209
Bone sialoprotein	CAGAGGAGGCAAGCGTCACT	CTGTCTGGGTGCCAACACTG	80
Cbfa1	CCGAGTCATTTAAGGCTGCAA	TGCGCTGAAGAGGCTGTTT	101
CamKIIα	ATCGCCTATATCCGCATCAC	ACATTCCACGGACAAAGAGC	215
Dvl2	CTCATGACCAGTGAGCTGGA	CTTCTCCATGTTGAGCGTGA	231
Fzd2	CCGCGCCACCTTCTGGGCTG	CATACCACGCGCTTTTGGAG	482
Fzd4	GTCAAGATCGGGGTCTTCTC	CAAGCTGTGACCTGCTAAAG	558
GAPDH	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA	151
Link Protein	TTCTGGGCTATGACCGCTG	AGCGCCTTCTTGGTCGAGA	80
GSK3β	TGGCGTGTGATGTCAGGTAT	TAAGCTGGCATCTGCAACAC	176
Osteocalcin	CCGGGAGCAGTGTGAGCTTA	TAGATGCGTTTGTAGGCGGTC	80
Osteopontin	GATGCCACAGATGAGGACCTC	CTGGGCAACAGGGATGACAT	81
Osterix	GGAAAGGAGGCACAAAGAAG	TGAGGGAAGGGTGGGTAGTC	124
PKCα	CCCATTCCAGAAGGAGATGA	TTCCTGRCAGCAAGCATCAC	212
PKCβ	TCCCTGATCCCAAAAGTGAG	AACTTGAACCAGCCATCCAC	229
Wnt1	ACCTGTTGACGGATTCCAAG	GGAATTGCCATTTGCACTCT	159
Wnt3a	ATTGAATTTGGAGGAATGGT	CTTGAAGTACGTGTAACGTG	318
Wnt4	GCGCTAAAGGAGAAGTTTGAC	GGATGTCCTGCTCACAGAAGT	157
Wnt5a	CAAATAGGCAGCCGAGAGAC	CTCTAGCGTCCACGAACTCC	217
Wnt8a	TCGTGGACAGTTTGGAGAAA	AGATGCCATGACACTTGCAG	120
Wnt11	CAGGATCCCAAGCCAATAAA	CCCCATGGCATTTACACTTC	99

2.7 Cell culture

2.7.1 Passaging

ES cells were routinely cultured in 75cm^2 (T75), primaria coated, tissue culture flasks at 37°C, a humidity of 95% and a CO_2 content of 5%. When the cells reached approximately 80% confluence, they were passaged by trypsinization and distributed into new tissue culture flasks. Medium was removed by aspiration and the cells were overlaid with pre-warmed 0.25% Trypsin/EDTA (Invitrogen). After a five minute incubation period at 37°C, the cells were collected and dissociated into a single cell suspension by pipetting up and down. After centrifugation at 630g for 5 min in a Mistral 1000 centrifuge, the supernatant was decanted and the cell pellet re-suspended in fresh ES medium. One tenth of the cells were transferred into a new tissue culture flask. Leukemia inhibitory factor (Gibco) was directly added to the culture flask at a final concentration of 1000 U/ml.

2.7.2 Determining cell numbers

All cell counts were performed using a hemocytometer. Two 10 µl cell suspension samples were taken from the tissue culture flasks after trypsinization and added to the hemocytometer. The number of cells from each of the four quadrants was counted and averaged.

2.7.3 Cryoconservation of cells

ES cells were cryopreserved using freeze medium (section 2.3). After trypsinization (section 2.7.1), the ES cells were pelleted. The pellet was re-suspended in freeze medium and transferred to a cryovial. Cells were slowly frozen in a styrofoam

container at −80°C for 24 hours before being transferred into a liquid nitrogen tank for long term storage.

2.7.4 Thawing of cells

All appropriate cell culture materials were prepared prior to thawing the cells to minimize the time between thawing and inoculation. The cryovial containing the frozen cells was then removed from the liquid nitrogen tank and thawed quickly by immersing it half-way in a 37°C water bath. Care was taken not to submerse the cap in order to prevent contamination. When the liquid was completely thawed, the cryovial was sprayed down with 70% ethanol. The cell suspension was added to a prepared 15ml centrifuge tube containing 10 ml ES medium and the cells were pelleted at 630g for 5 min. The supernatant was discarded and the cell pellet re-suspended in 1 ml of ES medium and added to the T75 primaria coated tissue culture flask. Another 24 ml of ES medium, with a final concentration of 1000 U/ml of leukemia inhibitory factor was added. Cultures were discarded after 25 passages post-thawing.

2.7.5 Differentiation

Differentiation was induced with the removal of LIF and starting the "hanging drop" protocol as described (Wobus, Wallukat *et al.* 1991) (Figure 3). Here, ES cells were trypsinized (section 2.7.1) and suspended at a concentration of 3.75×10^4 cells per ml in ES media, as described above. The suspended culture of cells was pipetted in 20ul droplets onto the lids of petri dishes. The lids with the droplets were placed on dishes containing 5ml of PBS and were placed in an incubator set at 37°C and 5% CO_2 for three days. Gravity will cause the ES cells to aggregate forming an embryoid body (EB). The EBs were then transferred to a bacterial petri dish containing culture media and grown in

suspension for two additional days. On day 5 either the intact EBs were placed in primaria coated tissue culture flasks (VWR), or they were trypsinized (section 2.7.1) and plated as a monolayer of cells at 50000 cells per cm^2. Osteogenesis was induced with 10mM β-glycerophosphate, 50ug/ml ascorbic acid, and 5×10^{-8} M 1α,25-dihydroxy vitamin D_3 (VD3 media; see Table 1), which was added to regular ES media (section 1.3). ES media without supplements was used for spontaneously differentiating controls. Fresh media was replenished every second day. After 30 days, approximately 60% of EBs or cells induced to undergo osteogenesis will have differentiated into mature mineralized osteoblasts (Figure 1).

2.7.6 Conditioned media

Wnt5a conditioned media was prepared from Wnt5a over expressing L-M(TK-) cells following the manufacturer's protocol. Cells were cultivated in ES media (section 1.3) with 0.6mg/ml of G418 and subcultured by trypsinization (section 2.7.1 followed by re-suspension of cells in ES media at a 1:6 ratio. Wnt5a conditioned media was made by first splitting the cells at 1:10 ratio in ES media, and then plating them out onto primaria coated tissue culture plates without G418. They were allowed to grow for four days before the media was collected and filter sterilized. Fresh ES media was added to the L-cells for another three days. The conditioned media was once again collected and filter sterilized and added to the first batch of media. The conditioned media was kept at $4^{\circ}C$ and used within 2 weeks. Control conditioned media was prepared in same manner but using the untransfected parental line.

2.7.7 Lithium chloride treatment

Cultures were differentiated as stated in section 2.7.5 and given 4mM (De Boer, Wang et al. 2004) of lithium chloride from a stock solution of 400mM. The lithium chloride powder was resuspended in ES media (section 1.3) and stored at 4°C.

2.8 Isolation, quantification and characterization of nucleic acids

2.8.1 RNA isolation

Cultures of differentiating osteoblasts were harvested at various time-points throughout the 30 day osteoblast differentiation period. Intact EBs, or monolayer cultures, were washed with PBS and trypsinized for 5 min at 37°C (section 2.7.1). Cells were collected by centrifugation (630g, 5 min) and the cell pellet lysed with guanidine-isothiocyanate-containing RNA lysis buffer (RNeasy mini kit, Qiagen) and stored at −80°C for long term storage. For isolation of total RNA, the samples were thawed on ice and the remaining cell clumps were removed by adding the lysate through a QIAshredder cs Branch, National Institutes of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892-8022, USA. tuanr@mail.nih.gov</AUTkit according to the manufacturer's protocol. Cell lysates were added to the RNAeasy spin column (Qiagen). The isolation was based on RNA binding to the silica membrane of that column. Contaminants were removed via three wash steps with provided wash buffers and centrifugation at 10000rpm in a table top centrifuge (2 min). Any DNA on the column was digested with addition of DNase I to the column. The RNA was eluted in 2x 30 µl of DEPC-water through centrifugation.

2.8.2 RNA quantification

The amount of isolated total RNA was determined with the RiboGreen™ reagent and kit (Molecular Probes). Aliquots of each sample were diluted 1:800 in a 96-well plate and mixed 1:1 with 100 µl of a 1:200 dilution of RiboGreen™ reagent. Both RNA and RiboGreen™ reagent were diluted in 1x Tris-EDTA buffer (20x stock provided with the RiboGreen™ reagent). Fluorescence of incorporated dye was measured in a fluorescent plate reader (Fluoroskan Ascent FL, Thermo Electron Corp.) at 495/520nm. A ribosomal RNA standard was measured along with the samples for the generation of a standard curve.

2.8.3 cDNA synthesis

Complementary DNA was synthesized from 625ng RNA per reaction with 40U Superscript II RNase H$^+$ reverse transcriptase (Invitrogen). The first strand reaction contained 50ng random hexamers, 2.5 mM MgCl$_2$, 10 mM DTT and 0.5 mM dNTPs in 5x first strand buffer (Invitrogen). The reaction was activated for 15 min at 25°C, followed by elongation of the strands at 42°C for 50 min. Lastly, the synthesis was terminated by inactivating the enzyme at 70°C for 15 min. Contamination from remaining RNA was eliminated by incubation with 2U RNase H at 37°C for 20 min.

2.8.4 Polymerase Chain Reaction

PCR reactions consisted of 5 µl aliquots of the first strand reaction and amplification was performed with specific primers (see 2.6). Each reaction was composed of 0.8µM of each of the primers, 2 mM MgCl2, 0.1 mM of each of the dNTPs and 2.5U of Taq Polymerase in 1x PCR buffer (Invitrogen) in a total reaction volume of

25 µl. Primer sequences for osteoblast-specific genes and PCR conditions have been described previously (zur Nieden, Kempka *et al.* 2003). After an initial 5 minute denaturation step, reactions were cycled through 35 rounds of 45 seconds at 94°C and 45 seconds at the corresponding annealing temperature. PCR reactions for GAPDH were terminated after 25 cycles. PCR products were visualized on 3% agarose gels containing 0.1 µg/ml of ethidium bromide under a VWR M-20E UV-light source and photographed.

2.8.5 Quantitative PCR

Quantitative real-time PCR (qPCR) analysis was performed in an iCycler IQ system (BioRad, Hercules). The accumulation of reaction products during PCR was monitored by measuring the increase in fluorescence caused by the binding of SYBR® Green (BioRad cat.no.PA11) to double-stranded DNA. Reaction mixtures were set up as suggested by the manufacturer containing 500nM of each of the primers and 40ng of cDNA. Following a 10 min Taq Polymerase activating step at 95°C, the reactions were cycled by denaturing at 94°C for 30 seconds and annealing and elongation for 45 seconds at the corresponding annealing temperatures (section 2.6). Target gene C_T-values were standardized against GAPDH expression and induction of expression in treated cells was normalized to control cells. GAPDH was chosen as a house-keeping gene as it has been previously shown to not alter expression during embryonic stem cell differentiation (Murphy and Polak 2002). Melting curve analyses were conducted after each run to ensure that there was no contamination, mis-priming or primer dimers, which could result in erroneous C_T values for the DNA sequence of interest.

2.8.6 Microarray analysis

The rationale for the choice of time points during differentiation was based on marker gene expression at those time points as shown previously by qPCR (zur Nieden, Kempka *et al.* 2003). Total RNA was derived as described above and sent to the Stem Cell Network Facility (Ottawa) for probe preparation and hybridization to Affymetrix chips. First, RNA quality was checked with the Agilent 2100 Bioanalyzer system. Double stranded cDNA was generated from samples only that passed the quality control whereupon a 5' T7 RNA polymerase promoter was affixed to the cDNA during second strand synthesis. Biotinylated cRNA was derived by T7 transcription in the presence of biotinylated UTP. Resulting biotinylated cRNAs were fragmented and hybridized to individual chips. The chips used were M430A mouse genomic chips. After extensive washing, signals were detected using a two-step signal amplification protocol (phycoerythrin-streptavidin, biotinylated anti-streptavidin antibody, and phycoerythrin-streptavidin) and scanning at 488nm. Raw Affymetrix data files that were generated by the Stem Cell Network Facility were examined using Affymetrix Data Mining Tool (DMT) and the GeneSpring software package. Three independent runs were performed for each biological time point. Intensity signals were normalized across chips and a mean was calculated for each biological triplicate. Signals from VD3 (Table 1) treated cultures were compared to untreated controls for the d6, 8, 16, 25 and day 30 time point. Cultures from day 4 and day 5 were only treated with control ES medium and thus compared to each other. A probe list was generated from genes that were showing a 2-fold or higher regulation in at least one of the time points with a p-value lower than 0.05 (t-test and Mann-Whitney).

2.9 Histochemical methods and biochemical characterization

2.9.1 Determination of calcium deposition

Calcium is the major mineral secreted by mature osteoblasts. Therefore the amount of calcium laid down by the cells will correlate with the number of osteoblast that formed. Calcium deposition was quantified using the "calcium detection reagent" (Diagnostic Chemicals Limited) containing 0.15mmol arsenazo III. Arsenazo III or 2, 2'-bisbenzene-arsonic acid reacts with calcium ions in an acid solution and forms a blue/purple complex. Each culture was washed 3 times with PBS and topically treated with calcium detection reagent. The calcium detection reagent was removed and diluted 2-4 times to stop the reaction and the absorbance was read at 650nm. In order to calculate the amount of calcium in culture dish, the standard (positive control) and calcium detection reagent alone (negative control) were also read. A solution of 10mg/dl of calcium was used as the standard: 10ul of the standard was added to 2ml of culture media, for a final concentration of 500ng/ml of calcium. The average reading from the negative control was stubtracted from the average reading from the standard. This number is defined as "x". All readings were divided by "x" to get ng/ml of calcium. That number is multiplied by the amount the calcium detection reagent was originally added to the flask and then multiplied again by the amount it was diluted before the reading. This number is then divided by the total concentration of protein in the culture (section 2.9.3).

2.9.2 Determination of alkaline phosphatase activity

Alkaline phosphatase activity was determined on day 11 and day 30, of the osteogenesis protocol. The cell culture was washed three times in PBS and lysed in Radioimmunoprecipitation (RIPA) buffer. After gentle rocking for 30 min, the lysates

were collected and alkaline phosphatase activity, as well as total protein content, determined. Alkaline phosphate activity was determined by adding 10ul of the cell lysate to 200ul of the substrate for alkaline phosphatase, p-nitrophenol (Sigma, cat.no. P7998). The lysate-substrate solution was incubated at 37°C for 30 min. The absorption was read at 450nm at both 0 and 30 min. The difference in absorption was subtracted and units per ml were calculated using the following formula: [U/ml] = ((final absorbance – initial absorbance)/20) x 5/18.45, where, 18.45 is the millimolar extinction coefficient of p-nitrophenol.

2.9.3 Determination of total protein content

As the total number of cells present in each sample was not known, calcium content as well as alkaline phosphatase activity was normalized to the total protein content of the sample. A 5 µl aliquot of the lysate was mixed with DC protein assay reagents (Biorad, 500-0116). After 15 min incubation at room temperature, absorbance was measured at 750nm in a Benchmark Plus microplate spectrophotometer (Biorad). Protein quantities in the samples were taken from a BSA standard curve.

2.9.4 Immunocytochemistry and Immunofluorescence

The localization of protein expression of several possible Wnt5a signalling pathway members was assessed through immunocytochemistry. Cells were washed 3 times with PBS and fixed using ice-cold methanol: acetone at a ration of 7:3. The cells were washed another three times in PBS and permeabilized in 0.1% triton-X 100 in PBS for 15 min at room temperature. After additional three washes in PBS, the ES cells were incubated at 37°C for 30 min in blocking solution which consisted of PBS, 10% goat serum and 0.5% bovine serum albumin. The cells were then treated with the respective

primary antibody (see Table 2) diluted in blocking solution overnight at 4°C. The ES cells were washed with PBS and treated with the appropriate AlexaFluor conjugated secondary antibody in PBS, 10% fetal calf serum, and 0.5 µg/ml Hoechst 33342. After 2 hours of incubation at room temperature, the cells were washed and stored in PBS at 4°C.

2.9.5 Fluorescence Activated Cell Sorting

ES cells were trysinized with the addition of 10ng of DNase I per ml of 0.25% of trypsin/EDTA (Invitrogen) at 37°C for 5 minutes (section 2.7.1). Once the cultures were dissociated into single cells, the cells were collected by centrifugation. The cell pellet was washed twice with PBS and fixed in 2% paraformaldehyde for 30 min at 4°C. After three washes in PBS, the cells were put in blocking solution (1xPBS, 1% BSA, 10% goat serum, 0.15% saponin) at 37°C for 30 minutes. Therefore non-specific binding was blocked. The cells were pelleted and re-suspended in PBS to a total volume of 100 µl. A goat anti-donkey Wnt5a primary antibody was added at the appropriate dilution and incubated at 4°C for 1 hour to overnight. Cells were centrifuged and washed with PBS and treated with an anti-goat Alexa Fluor 488 conjugated secondary antibody for 2 hours, then washed and re-suspended in PBS. As a control, ES cells induced to become osteoblasts with VD3 and those allowed to spontaneously differentiate, were treated with just the secondary antibody alone. Flow cytometry was conducted using a FACS Calibur instrument and the CellQuest software (Becton Dickinson) by the University of Calgary FACS Core Facility. For each sample, 10000 events were registered. The samples were gated between a forward scatter (FSC-H) of 200 and 800 to exclude cellular debris and aggregates. Only events that showed >99% fluorescence intensity, as compared to cells that were incubated with the secondary only, were registered.

2.9.6 Morphometric analysis

General mineralization was quantified through morphometric analysis of photomicrographs taken of the cell cultures under phase contrast microscopy. As mineralized osteoblasts appear black under phase-contrast (Figure 1), the number of pixels per image that were black was determined using the software IMAGE J 1.33u available from the website of the National Institutes of Health (http://www.rsb.info.nih.gov/ij/). Photographs were taken with the SPOT Advanced Imaging system (Diagnostic Instruments) mounted to an Olympus IX70 inverted microscope. One EB, or the monolayer culture, was captured on each picture with a 2x objective resulting in an image size of 1600 x 1200 pixels (number of columns x number of rows of pixels). A gray-scale picture was taken with a user-defined exposure with a brightness of 0.91 and a gamma adjustment to a value of 1.8. The pixel bit depth was set to a monochromatic value of 8 bpp. To each pixel, an intensity value in the range between 0 (white) and 255 (black) was assigned automatically. The mean black value per image was taken from a histogram generated by IMAGEJ from n=3 images of each treatment group.

2.9.7 ELISA capture assay

To determine the amount of Wnt5a active protein in the conditioned media, an ELISA capture assay was preformed using goat anti-donkey Wnt5a primary antibody as the capture antibody and streptavidin-HRP with p-nitrophenol as the substrate. ELISA plates (Costar) were coated with goat anti-donkey Wnt5a primary antibody diluted in sterile PBS and incubated overnight. The wells were washed repeatedly with 0.05% Tween 20 in PBS and blocked for 2 hours in 1% bovine albumin in PBS. The standard

curve used to calculate the amount of Wnt5a in each sample started at 2000pg of recombinant Wnt5a protein was added and allowed to incubate for an additional 2 hours. Antigoat –HRP secondary (1:5000) was added to the plate and allowed to incubate for 2 hours. Streptavidin-HRP (1:200) was consequently added and allowed to react for 20 min. The substrate solution consisting of Tetramethylbenzidine was added to cause the color change. The reaction was halted with the addition of 2N H_2SO_4. A standard curve was charted and the quantity of Wnt5a active protein in the conditioned media was calculated using the linear coefficient.

2.10 Biostatistics

2.10.1 Determination of specific gene expression

N-fold expression of the gene of interest was calculated using the delta-delta C_T method (Livak and Schmittgen 2001). Primers were tested first by RT-PCR that only one product was amplified. Then the primers were tested against GAPDH via qPCR to ensure that the increase in product by the primers was at the same rate as GAPDH. In each qPCR run, the expression of the gene of interest as well as GAPDH expression was determined in triplicate. For each sample, a C_T value was assigned by the software. The C_T value describes the amplification cycle in which the fluorescent signal first exceeds the detection limit. The smaller the C_T value the higher the expression of a particular gene. By employing this formula, the n-fold expression level of the gene of interest in the control culture equalled 1:

$$2^{-\Delta\left(\Delta C_T^{\text{ gene of interest}} - C_T^{\text{ GAPDH}}\right)\text{treated} - \left(\Delta C_T^{\text{ gene of interest}} - C_T^{\text{ GAPDH}}\right)\text{control}}$$

Applying this calculation, interpretation errors resulting from differences in the amounts of cDNA used were prevented.

2.10.2 Statistical comparison

The statistical significance of the results were determined with a one way ANOVA using the web-based program http://www.statcrunch.com. If overall significance was determined then either an unpaired student's t-test was performed or Tukey's posthoc test. The student's t-test was executed using a web-based program from the physics department of the College of Saint Benedict/Saint John's University (http://www.physics.csbsju.edu/stats/t-test.html). The Tukey's posthoc test was calculated using the following formula: Q=Mi-Mj/(square root MSE/n): where Mi and Mj are the two means, MSE = the mean of the variances and n= the number of scores in each group. The "p" value was calculated using the studentized range calculator at http://psych.rice.edu/online_stat/chapter10/pairwise.html.

Media abbreviation	Contents of Media
ctrl (ES medium)	DMEM, non-essential amino acids Penicillin, streptomycin, fetal bovine serum, β-mercaptoethanol
GPAA	Ctrl media + β-glycerophosphate, Ascorbic acid
VD3	GPAA + 1α, 25-dihydroxyvitamin D_3
Wnt5a	GPAA + Wnt5a conditioned media or Wnt5a recombinant protein
Wnt5a+VD3	Wnt5a + 1α, 25-dihydroxyvitamin D_3 GPAA
CC	GPAA + Control conditioned media
CC+VD3	CC + 1α, 25-dihyroxyvitamin D_3 + GPAA

Table 1: List of media abbreviation and contents of media. Each media is given for the full 30 day differentiation, with the exception of media with conditioned media or recombinant protein additives that are given from day 5 through day 10.

Day 0 — 750 ES cells per 20ul droplet

Day 3 — EBs transferred to petri-dish to grow in suspension

Day 5 – Day 30 — Transferred to tissue culture plate
Given: β-gycerophosphate, ascorbic Acid and 1α, 25 dihydroxyvitamin D_3
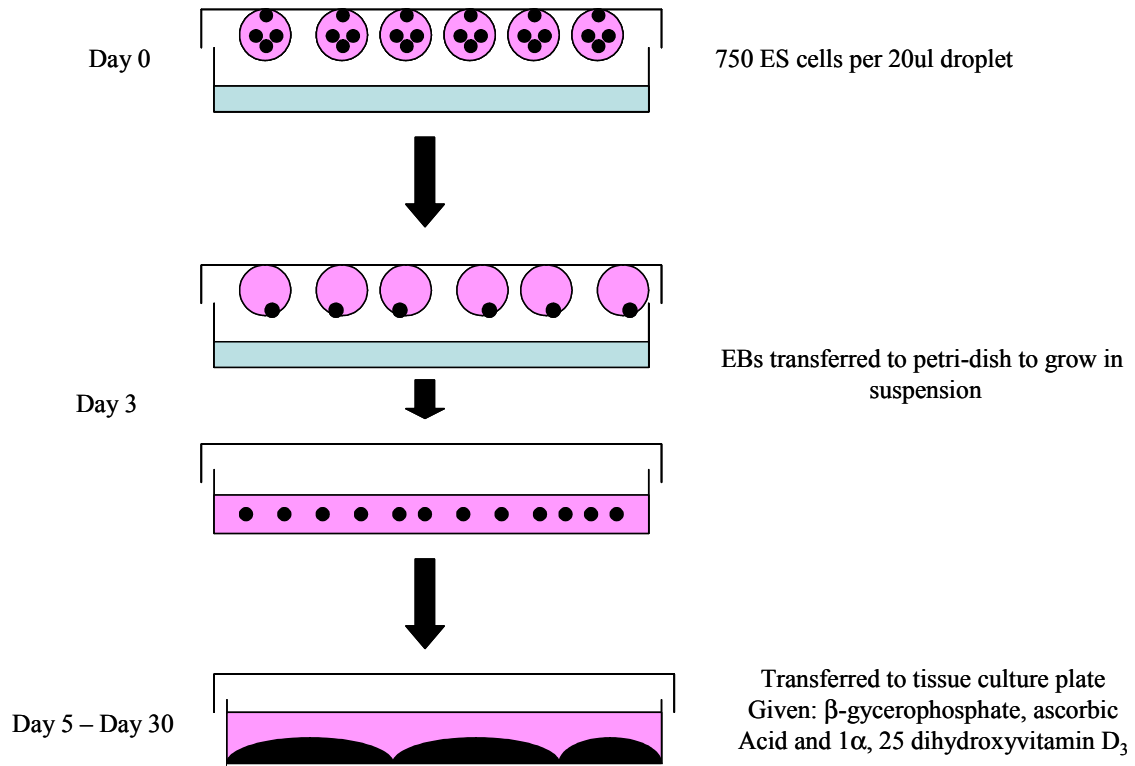
Figure 3: Cartoon depicting the hanging drop protocol used to differentiate D3 ES cells to osteoblasts. This picture has been modified from (Wobus, Wallukat *et al.* 1991). The differentiation begins with the removal of LIF and 750 ES cells placed in hanging drops from the lid of a petri-dish. Over three days, the cells will accumulate at the bottom of the droplet forming and embryoid body (EB). On this day, the EBs are transferred to another petri-dish to grow in suspension. On day 5, the EBs are either trysinized or plated directly onto tissue culture plates and given osteoblast inducing media (section 2.7.5).

Chapter Three: Results

The objective of this thesis was to determine the roles of Wnt5a during *in vitro* osteogenesis using murine embryonic stem (ES) cells. These were assessed through the degree of mineralization, gene expression, and localization of β-catenin in the differentiating cell during osteogenesis. This chapter will start by describing the microarray experiment that led to the discovery that Wnt5a is involved in *in vitro* ES cell mediated osteogenic differentiation. This description will be followed by the study of mineralization when additional Wnt5a protein was added to the media: either as a recombinant protein or in conditioned media. Finally, this chapter will discuss data that suggests the signalling pathway that Wnt5a activates during osteogenesis.

Table 2, Figures 5,6,16 and 17 as well as Appendix A: Table 1 were the work of Dr. N.I. zur Nieden. However, the conclusions from these tables and Figures made in this thesis are my own.

3.1 Microarray

The differentiation of ES cells to osteoblasts requires the coordinated expression of a number of different molecules. In order to identify molecules that could enhance osteoblast differentiation, a large scale microarray experiment was performed comparing ES cells that were allowed to spontaneously differentiate with those that were induced to become osteoblasts. The experiment was repeated in triplicate and gene expression was compared on days 0, 4, 5, 6, 8, 16, 25, 30 of differentiation. These time-points were

chosen based upon time-points where the culture was manipulated during the hanging drop protocol (Figure 3) and days where osteoblast specific genes are known to be up-regulated (zur Nieden, Kempka *et al.* 2003). The analysis from this microarray experiment revealed nine different gene families that were up-regulated at one or more of these time-points throughout differentiation, compared to ES cells that were allowed to spontaneously differentiate. These nine signalling families include; Retinoic acid, BMP, Nitric oxide, Sox, TGFβ, Follistatin, Prostaglandin, Insulin Like Growth Factor, and Wnt. This microarray analysis yielded the preliminary data which indicated that the Wnt family, specifically Wnt5a, could be involved in osteogenesis (Table 2).

The Wnt family of genes found to up-regulate expression during *in vitro* osteogenesis included Wnt4, Wnt5a, Wnt8a, Sfrp1, and axin (Table 2). Most of these molecules are specific to the canonical Wnt pathway, except for Wnt5a, a non-canonical Wnt protein. As Wnt5a was up-regulated the day after the osteoinducing factors were added to the media (day 6) and as it is already thought to be involved in limb development (Yang *et al.* 2003), the role of Wnt5a in osteogenesis was pursued.

ES cells are thought to differentiate through an osteoblast-chondrocyte progenitor stage of differentiation when given osteoblast inducing media (Hartmann 2006). Although Wnt5a has been shown to be involved in chondrogenesis (Yang *et al.* 2003), the microarray analysis suggested that Wnt5a may have a role in osteogenesis. As current protocols will induce approximately 60% of a given culture toward the osteoblast fate, Wnt5a treatment may increase the number of osteoblasts *in vitro*. Wnt5a expression was

	day 6	day 8	day 16	day 25	day 30
Wnt5a	2.57	1.43	2.47	-2.27	-1.01
Wnt4	1.62	2.81	1.39	1.05	1.11
Wnt8a	1.66	1.38	-3.3	1.86	1.01
Sfrp1	1.41	1.96	1.59	-1.52	1.09
axin	2.67	1.4	-1.42	1.51	-1.07

Table 2: The normalized hybridization signal of five Wnt signalling genes during osteoblast differentiation. The signal was normalized to the signal of ES cells that are spontaneously differentiating on each day of differentiation. The mean n-fold change in expression is shown where n=3. Decreasing expression is indicated with a "-". Numbers in red represent significant increase over spontaneously differentiating controls where P<0.05 using both Mann-Whitney and t-test were performed. This table has been modified from the work of Dr. N.I. zur Nieden.

validated through RT-PCR (Figure 4), FACS analysis (Figure 5), and ICC (Figure 6). RT-PCR of Wnt5a mRNA expression indicates that in repeated experiments Wnt5a is expressed on day 6, however it has been shown to also expresson day 4 (Figure 4). In Figure 4 the second RT-PCR shows some Wnt5a expression in spontaneously differentiating cells that can be explained by the fact that the cDNA is made with random hexamers on a mixed cell population, therefore the presence of Wnt5a could be due to cells other than those forming osteoblasts. FACS and ICC expression profile of Wnt5a indicated that the expression of Wnt5a starts to increase on day 5, peaks day 6 and returns to background levels by day 10. This data confirmed that during osteogenesis, Wnt5a does increase its expression during the first days following VD3 treatment. Due to this data, culture media was enriched with Wnt5a protein from day 5 through day 10 for each differentiation experiment.

3.2 Wnt5a gain of function on osteogenesis:

To determine the effect of additional Wnt5a on osteogenesis, Wnt5a was added exogenously to the culture media from day 5 through day 10 of differentiation. The amount of mineralization was quantified by calculating the amount of calcium in the extracellular matrix (ECM) after 30 days (Chapter 2, Sections 2.9.1 and 2.9.6). Recombinant Wnt5a protein did cause a significant increase in calcification when given to ES cells grown as a monolayer. In this section, I will present the results that led to the finding that Wnt5a gain of function does increase calcification of differentiating osteoblasts.
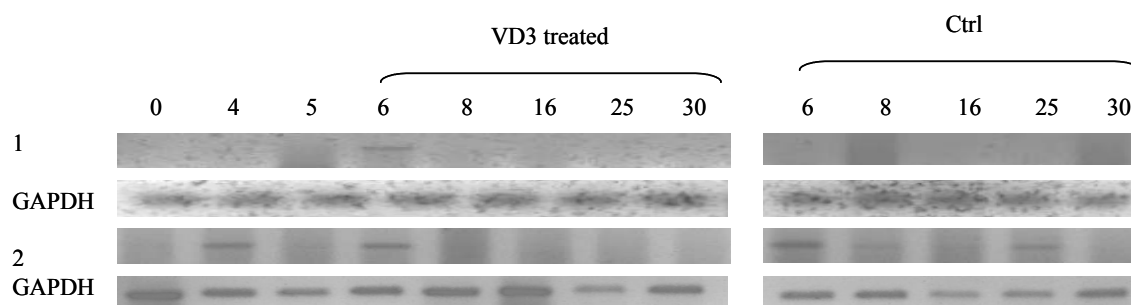
Figure 4: RT-PCR showing the variation in Wnt5a gene expression. Expression of Wnt5a on day 6 remains consistent. 1) the original total RNA used for the microarray and 2) replicate using the same total RNA as microarray but new cDNA. VD3: ES cells treated to become osteoblasts – see Table 1 for complete list of reagents in solution. Ctrl: ES cells allowed to spontaneously differentiate.   Numbers over each well indicates day of differentiation. Bands were confirmed to be Wnt5a through DNA sequencing.
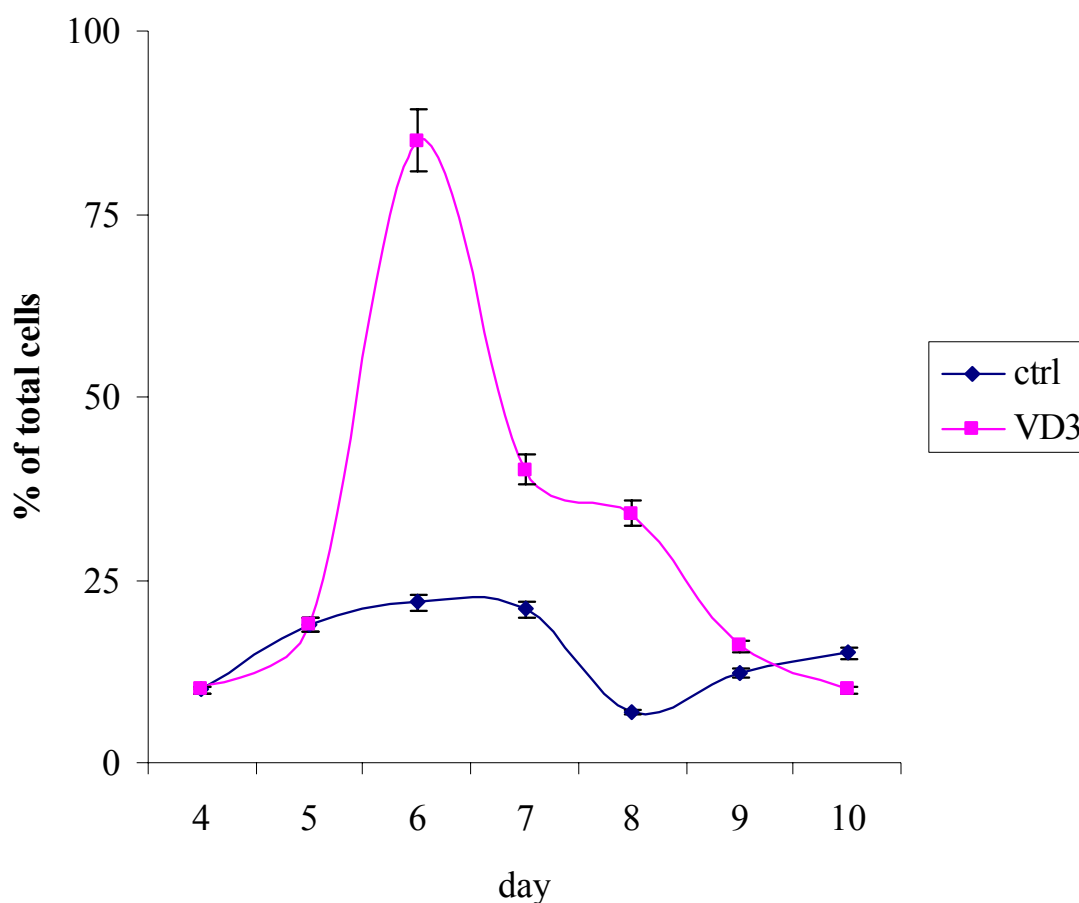
Figure 5: Fluorescence Activated Cell Sorting (FACS) of Wnt5a expression. This graph depicts FACS analysis measuring the mean percentage of total cells expressing the Wnt5a protein when treated with VD3 media compared to those treated with ctrl media (see Table 1 for contents of media). Both treatments were normalized to cells given both treatments and probed with the anti-goat secondary antibody alone. The data is represented as the mean % of total cells +/- SD with n = 3 showing the Wnt5a protein expression at its peak on day 6. This Figure is the work of Dr. N.I. zur Nieden.
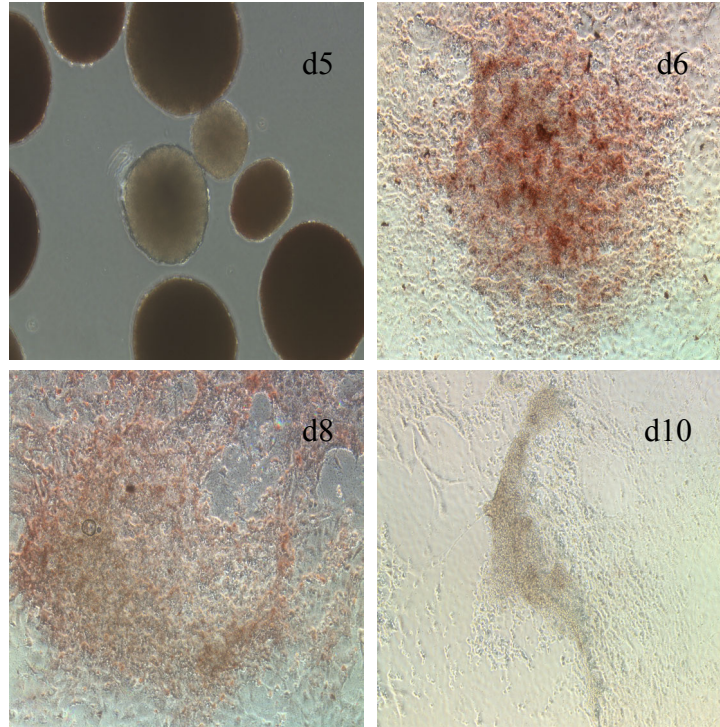
Figure 6: Microarray validation using Immunocytochemistry (ICC). EBs on day 5, 6, 8, and 10 were stained for Wnt5a protein. EBs on day 5 were treated with ctrl media and those on day 6, 8, and 10 treated with VD3 media (see Table 1 for contents of media). There is increased expression of Wnt5a protein on day 6. The EBs were probed with goat anti-donkey Wnt5a primary and anti-goat secondary antibody. This Figure is the work of Dr. N.I. zur Nieden.

*3.2.1 The effect of dose of Wnt5a on osteogenesis*

In order to test the effect of Wnt5a on differentiating ES cells induced to become osteoblasts, Wnt5a conditioned media was added to ES cells from day 5 through day 10 mimicking the time of expression as determined through FACS (Figure 5) and ICC (Figure 6). Wnt5a recombinant protein was added to ES cells grown as a monolayer at increasing concentrations. Here, we see that increasing quantities of Wnt5a recombinant protein added from day 5 to day 10 along with VD3 media does cause a variation in the amount of calcification (Figure 7). The effect of Wnt5a on calcification appears to vary with increases in calcification occurring between 1ng and 30ng, 50ng to 70ng and 80ng to 100ng, with treatment at 40ng/ml having little effect on mineralization (Figure 7). This result suggests that differentiation of ES cells to osteoblasts is sensitive to dosage of Wnt5a and the effect may be triphasic.

To determine the optimal amount of Wnt5a needed to induce the most mineralization, recombinant Wnt5a protein was added to ES cells treated with VD3 in logarithmically increasing quantities (Figure 8). The amount of calcification increased in a linear fashion until 10000ng/ml when Wnt5a recombinant protein becomes toxic (Figure 8). Toxicity was presumed, as only a few cells remained attached to the culture dish, and they were small in size (Figure 8). Wnt5a recombinant protein without VD3 did not increase mineralization in a linear fashion. However, at 1000ng/ml, it did induce a similar amount of mineralization as VD3 alone (Figure 8). Therefore, Wnt5a does increase calcification and mineralization in a dose dependent manner.
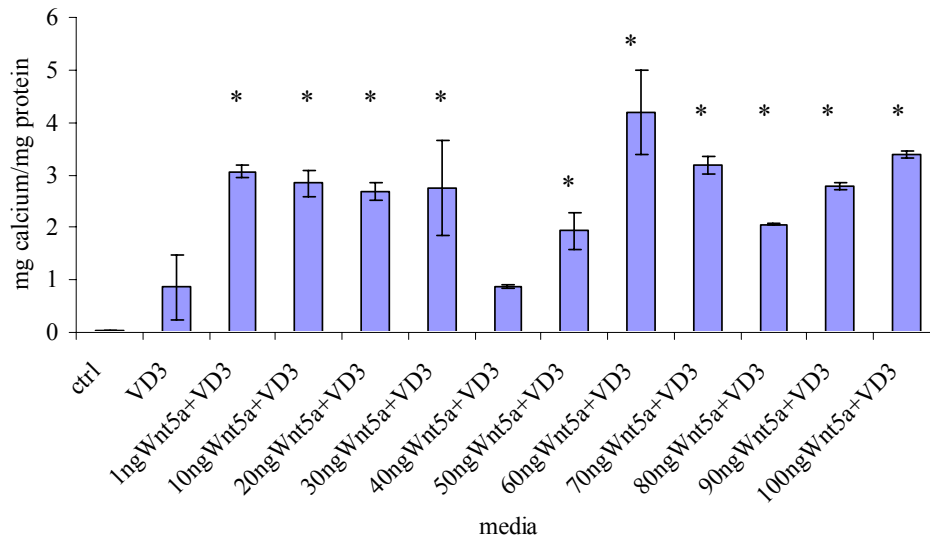
Figure 7: Wnt5a recombinant protein concentration test on ES cells grown as a monolayer. Most cells treated with Wnt5a recombinant protein increase calcium deposition over VD3 alone. ES cells treated with low or higher levels of recombinant protein increased calcium deposition, however ES cells treated with 40ng/ml of recombinant Wnt5a showed no increase in deposition when compared to VD3 alone. The quantity of recombinant protein is indicated before media on graph. * indicates a significant increase in calcification over VD3 media where n=3 separate differentiations and $P < 0.05$. Data is represented as a mean +/- SD. Differences were first analyzed by ANOVA then by Tukey's posttest procedure.
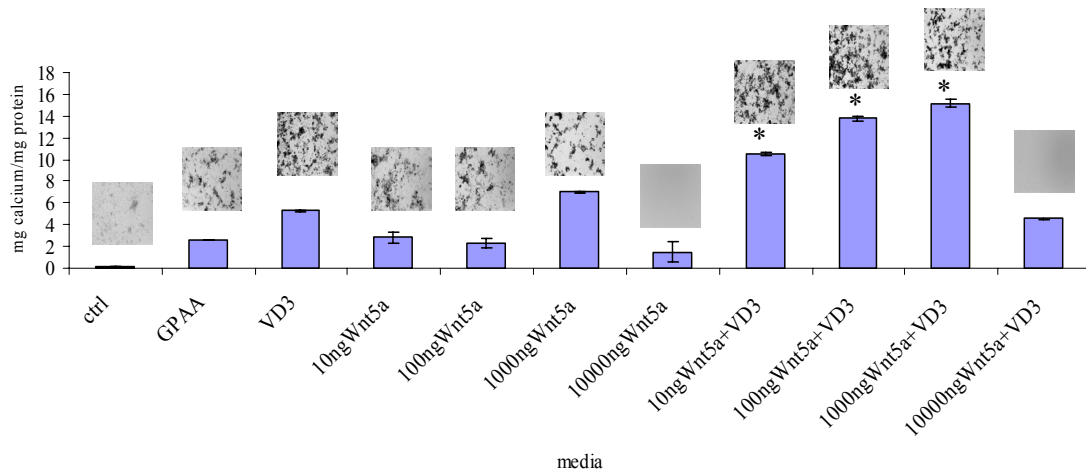
Figure 8: **Calcium deposition of ES cells grown in monolayer given different concentrations of Wnt5a recombinant protein**. Sample pictures of mineralized cultures on day 30 are above their respective treatment. There was an increase in calcium deposition when ES cells were given recombinant Wnt5a (ng/ml) with VD3 media (Table 1). There is a linear increase between 10ng/ml and 1000ng/ml of Wnt5a when cells are cultured in VD3 media, with $R^2 = 0.949$. * indicates a significant increase in calcification over VD3 media where n=3 separate differentiations and P<0.05. Data is represented as a mean +/- SD. Differences were first analyzed by ANOVA to determine overall significance then by Tukey's posttest procedure.

3.2.2 The effect of Wnt5a gain of function on osteoblast specific gene expression:

Calcification of ES cells is only one indication that the cultured ES cells are differentiating to osteoblasts. The gene markers alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OCN) are known to increase their expression during osteoblast differentiation. These molecules can be used to determine the cell culture's stage of differentiation.

This thesis was the continuation of Dr. Nicole I. Zur Nieden's doctoral work; therefore most differentiations were carried out in a similar fashion as her thesis, with ES cells being grown as embryoid bodies (EBs) rather than as a monolayer. In addition Wnt5a conditioned media was used due to the limited availability of Wnt5a recombinant protein. Therefore, using Wnt5a conditioned media, the n-fold increase in bone specific gene expression was determined through quantitative PCR. There was an increase in the pre-osteoblast marker ALP, and the mature osteoblast marker OCN on day 30 of embryoid bodies (EBs) treated with Wnt5a + VD3 (Figures 9 and 11). However, OPN, a gene marker of pre-osteoblasts that have started mineralizing, increased expression on day 30 when EBs were treated with Wnt5a over GPAA alone (Figure 10). In conclusion, Wnt5a conditioned media increases osteoblast specific gene expression in differentiating ES cells.
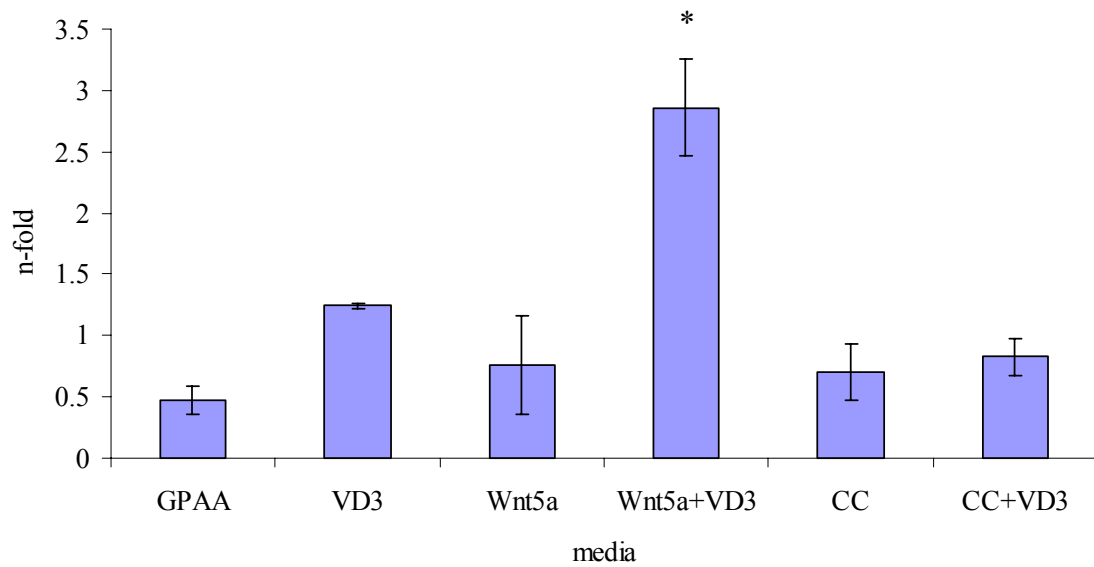
Figure 9: Quantitative Real-Time PCR of alkaline phosphatase mRNA expression on day 30 of differentiation. The n-fold expression of alkaline phosphatase, normalized to GAPDH with spontaneously differentiating cells set to an n-fold of 1. EBs treated with Wnt5a + VD3 increased alkaline phosphatase expression on day 30 over VD3 or Wnt5a alone. * indicates a significant increase over VD3 media where n=3 separate differentiations and $P<0.05$. Data is represented as a mean +/- SD. Differences were first analyzed by ANOVA to determine overall significant differences then by Student's t-test.
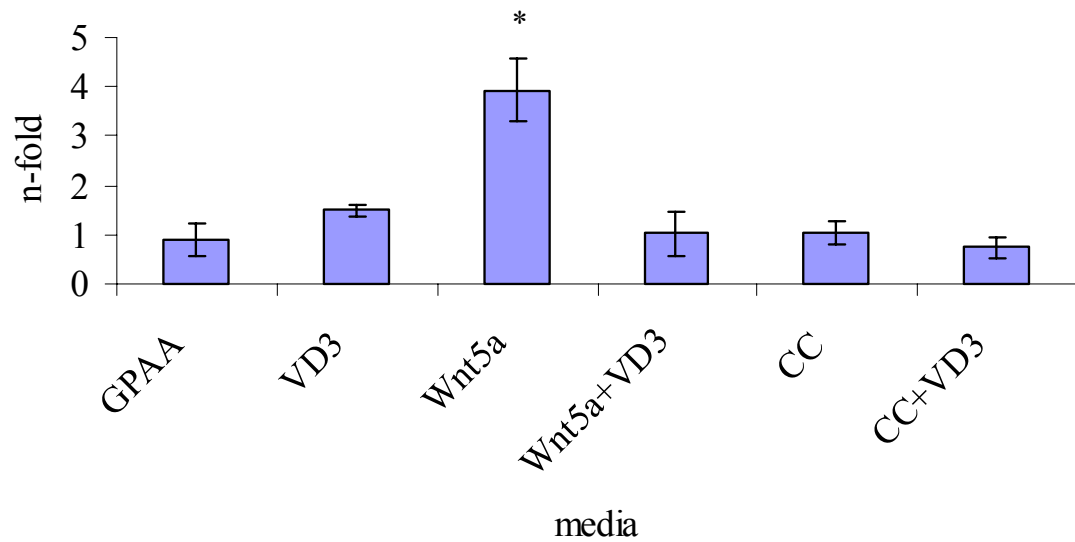
Figure 10: Quantitative Real-Time PCR of osteopontin mRNA expression on day 30 of differentiation. The n-fold expression of osteopontin, normalized to GAPDH with spontaneously differentiating ES cells set to an n-fold of 1. EBs treated with Wnt5a (Table 1) increased the expression of osteopontin over all other media. * indicates a significant increase over VD3 media where n=3 separate differentiations and $P<0.05$. Data is represented as a mean +/- SD. Differences were first analyzed by ANOVA to determine overall significant differences then by Student's t-test.
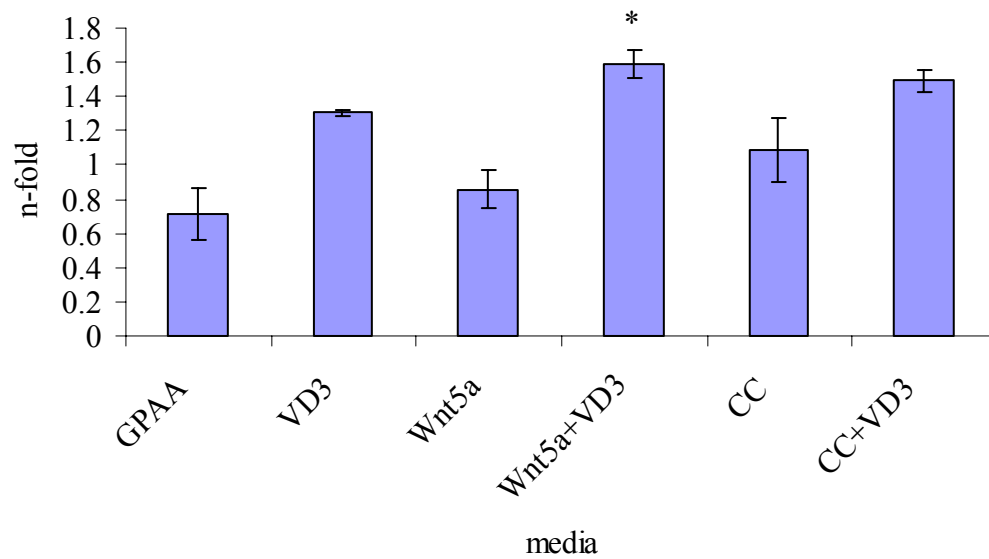
Figure 11: Quantitative Real-Time PCR of osteocalcin mRNA expression on day 30 of differentiation. The n-fold expression of osteocalcin, each treatment was normalized to GAPDH and spontaneously differentiating cells were set at n-fold of 1. EBs treated with Wnt5a +VD3 (Table 1) increase the expression of osteocalcin over all other media. * indicates a significant increase over VD3 media where n=3 separate differentiations and $P<0.05$. Data is represented as a mean +/- SD. Differences were first analyzed by ANOVA to determine overall significant differences then by Student's t-test.

3.2.3 Early Osteogenesis and the effect of Wnt5a gain of function

Early osteogenesis is marked by changes in gene expression toward a mesodermal fate indicating that these cells will have the ability to differentiate into myocytes, adipocytes, chondrocytes and osteoblasts. With the addition of VD3, the ES cells will be induced toward an osteoblast fate. The effect of Wnt5a occurs early in osteoblast differentiation as reflected with its expression increasing between day 5 and day 10 of the 30 day protocol (Figures 5 and 6). In order to understand the immediate effect Wnt5a has on osteogenesis, the day following Wnt5a treatment, day 11 was chosen to study osteoblast gene expression. Also on day 11, ES cells given just VD3 generally have not started mineralizing yet and it is the time-point just prior to the start of ALP expression (zur Nieden, Kempka *et al.* 2003). Therefore it can be determined from gene expression on day 11 if Wnt5a is inducing more cells toward an osteoblast fate immediately after treatment.

As day 11 is still early in the differentiation process, most of the cells differentiating toward an osteoblast should be at the pre-osteoblast or mineralization stages. Before mineralization has begun, ALP expression and activity indicates pre-osteoblast formation, while OPN expression marks the start of mineralization. ALP or OPN expression was determined through quantitative PCR (Figures 12 and 13). ES cells treated with Wnt5a + VD3 show increased ALP and OPN expression on day 11. This expression of ALP and OPN mRNA suggests that cells treated with both Wnt5a and VD3 are actively secreting an ECM by day 11. If ALP is active, then it is cleaving inorganic phosphate from βGP for internalization, which is followed by induction of OPN expression (Beck, Zerler *et al.* 2000). To determine if the enzyme ALP was active and

therefore the production of the ECM had begun, an ALP activity test was performed. Wnt5a + VD3 treated EBs showed an increase ALP activity over other media treatments on day 11 (Figure 14). This result suggests that Wnt5a + VD3 treatment pushes more ES cells toward an osteoblast fate earlier than does treatment with VD3 alone.

3.3 The signalling pathway of Wnt5a during osteogenesis

The signalling pathway as well as components of the proposed pathways Wnt5a activates is controversial. It is accepted by many, but not all researchers, that Wnt5a is exclusively a non-canonical Wnt that initiates a signalling pathway that will ultimately inhibit β-catenin nuclear activity (Ishitani, Kishida *et al.* 2003; Topol, Jiang *et al.* 2003; Mikels and Nusse 2006). As Wnt5a must be added with vitamin D_3 to differentiating ES cells in order to see a significant effect on osteogenesis (Figures 8,9,11,12,13 & 14), the Wnt signalling pathway in response to vitamin D_3 and Wnt5a was explored.

3.3.1 The signalling pathway of Wnt5a and 1α, 25-dihydroxyvitamin D₃

The canonical pathway is characterized by the activation or degradation of β-catenin. This protein has a dual role as either part of the cell adhesion apparatus or as a transcription factor within the nucleus within the cell. β-catenin is regulated by GSK3β, which will phosphorylate β-catenin; β-catenin will consequently be degraded by the proteasome. Literature has indicated that the vitamin D_3 receptor, VDR, will bind β-catenin in the nucleus, preventing nuclear activity and translocate the protein to the plasma membrane for its role in cell-cell adhesion (Palmer, Gonzalez-Sancho *et al.* 2001). The dual role of β-catenin was visualized through the use of immunofluorescence (IFC). ES cells were grown as a monolayer and given 1000ng/ml of recombinant Wnt5a,
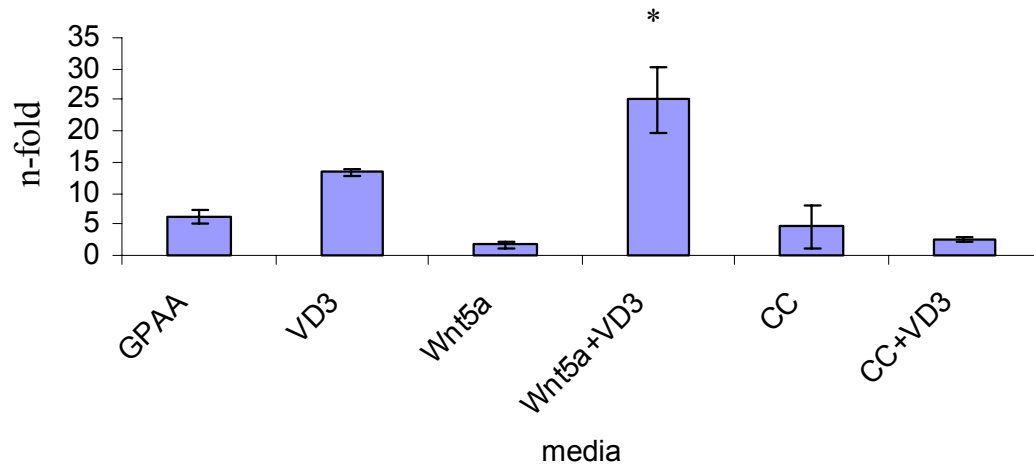
Figure 12: Quantitative Real-Time PCR of alkaline phosphatase mRNA expression on day 11. The relative expression of alkaline phosphatase normalized to GAPDH. Wnt5a + VD3 treated EBs increase alkaline phosphatase expression on day 11 synergistically over Wnt5a or VD3 alone. * indicates a significant increase over VD3 media where n=3 separate differentiations and P<0.05. Data is represented as a mean +/- SD. Differences were first analyzed by ANOVA to determine overall significant differences then by Student's t-test.
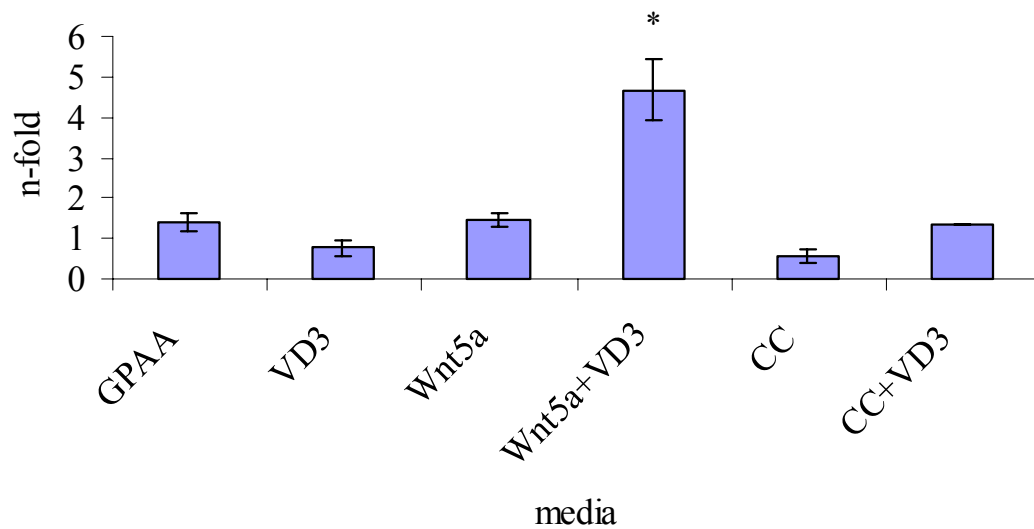
Figure 13: Quantitative Real Time PCR of osteopontin mRNA expression on day 11.
The relative expression of osteopontin normalized to GAPDH. Wnt5a + VD3 treated
EBs increase osteopontin expression on day 11 synergistically over Wnt5a or VD3 alone.
* indicates a significant increase over VD3 media where n=3 separate differentiations and
$P<0.05$. Data is represented as a mean +/- SD. Differences were first analyzed by
ANOVA to determine overall significant differences then by Student's t-test.
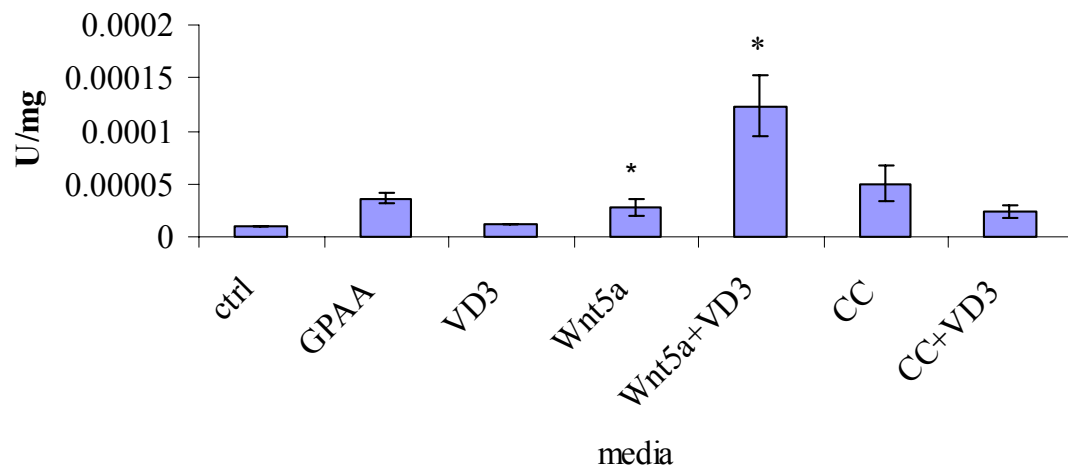
Figure 14: Alkaline phosphatase activity on day 11. This graph depicts the activity of alkaline phosphatase on day 11 of the 30 protocol of osteoblast differentiation. EBs were treated with Wnt5a conditioned media with VD3 increased alkaline phosphatase activity per milligram of protein over other media (See Table 1). * indicates a significant increase over VD3 media. Data is represented as a mean +/- SD where n=3 separate differentiations and p<0.05. Differences were first analyzed by ANOVA to determine overall significant differences then by Student's t-test.

and the localization of β-catenin was determined. Before Wnt5a or VD3 treatment on day 5, β-catenin appears to be located in the nucleus (Figure 15). After osteoblast inducing treatment begins, β-catenin appears at the plasma membrane (Figure 15). Both VD3 and Wnt5a treatments have β-catenin localized to the plasma membrane on day 8. When these two treatments, Wnt5a and VD3, are combined and β-catenin locates to the plasma membrane two days earlier, day 6 (Figure 15). This result suggests that Wnt5a may have a similar role to vitamin D_3 during osteogenesis.

3.3.2 Wnt5a and β-catenin

Wnt5a has been known to inhibit β-catenin by acting through CamKIIα, PKC and NLK, phosphorylating Lef/Tcf and thus inhibiting β-catenin from binding to the DNA in HEK293 cells (Ishitani, Kishida *et al.* 2003). In contrast, it has been suggested that Wnt5a acts to inhibit β-catenin through other processes that do not involve CamKIIα, PKC or NLK (Mikels and Nusse 2006). RT-PCR could support Ishitani *et al.* 2003 as mRNA expression of CamKIIα is up-regulated on day 6 (Figure 16) along with Wnt5a (Figure 4 and 16). In addition, PKCβ mRNA expression is increased on day 6 if Wnt5a is added to the media (Figures 17). Therefore, Wnt5a could indirectly cause the translocation of β-catenin from the nucleus to the plasma membrane by initiating a signalling cascade that inhibits β-catenin activity in the nucleus. Therefore, β-catenin could be regulated in the early stages of osteogenesis.
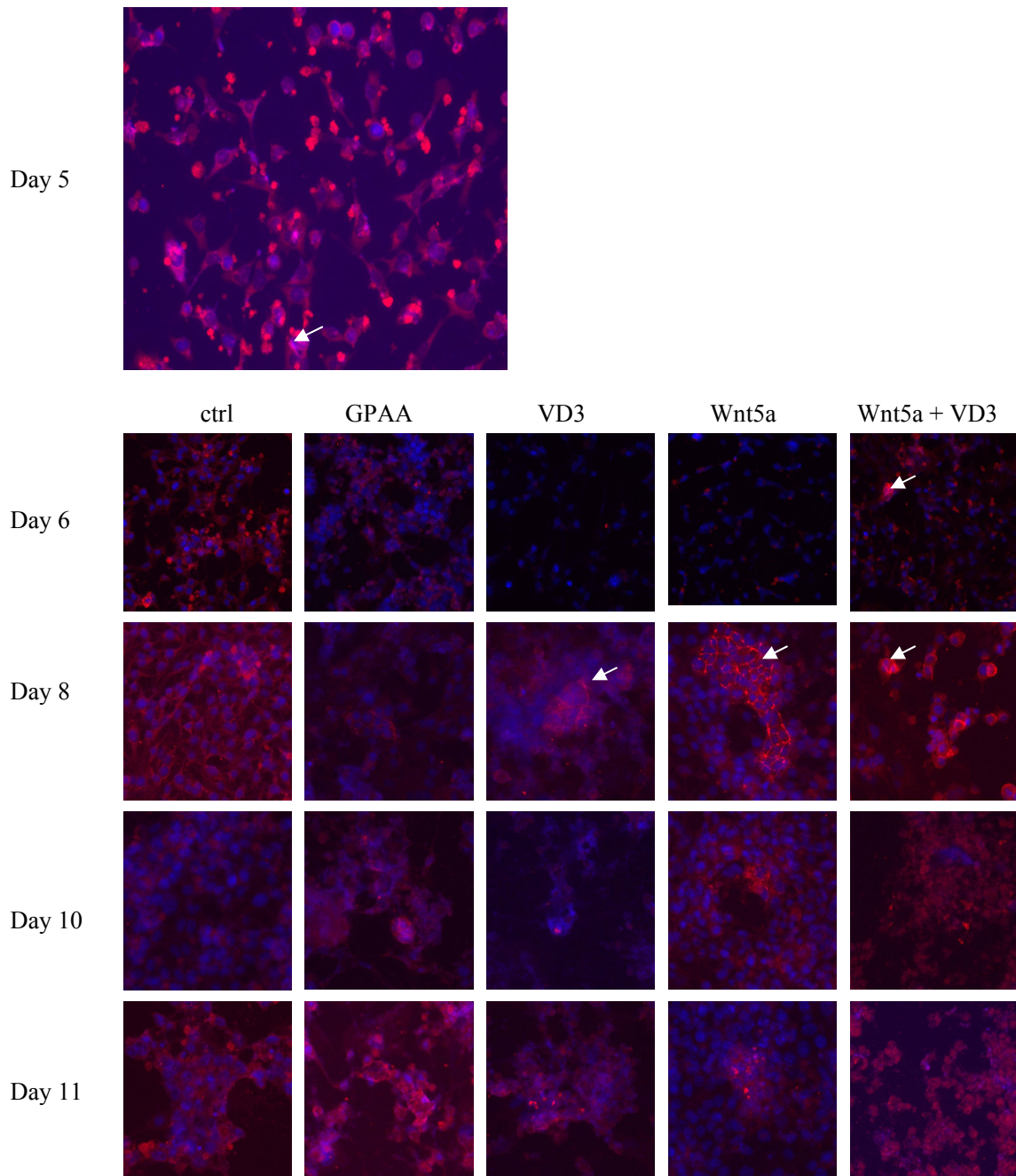
Figure 15: **Immunofluorescence of β-catenin stained ES cells cultured as a monolayer**. B-catenin is detected on the cell surface when cells are treated with VD3, Wnt5a or Wnt5a+VD3 as shown with the arrows. Arrow on Day 5 indicates the nuclear location of β-catenin. Cells were probed with anti-mouse β-catenin primary antibody and anti-mouse alexa fluor 546 secondary. Nuclei were stained with Hoechst 33342.

3.3.3 Wnt5a and Frizzled receptors

Wnt5a has recently been shown to have the ability to act through both the canonical and non-canonical pathway depending on the receptor to which it binds (Mikels and Nusse 2006). If Wnt5a binds to Fzd4, it will signal through the canonical pathway. However, binding to the receptor Ror2 will cause non-canonical pathway signalling in HEK 293T cells (Mikels and Nusse 2006) and Wnt5a is traditionally known to signal through Fzd2, initiating the non-canonical pathway (Kuhl, Sheldahl *et al.* 2000). RT-PCR shows Fzd2 mRNA expression on day 4 through day 10, the same time-points as Wnt5a (Figure 16). However, Fzd4 is increasingly expressed on days 2, 12, 14, 22, and 24 before and after Wnt5a increases expression. If mRNA expression is indicative of immediate protein expression, this could mean Wnt5a would have more opportunity to bind Fzd2, rather than Fzd4. However, the ability of Wnt5a to signal through Fzds to initiate the canonical pathway is dependent on the presence of the co-receptor LRP5/6 (Liu, Bafico *et al.* 2005).

3.3.4 Wnt5a and other Wnts

The expression of other Wnt molecules already shown to be involved in osteogenesis, as well as those Wnts observed to be up-regulated in the microarray were compared. Certain Wnts are known to be either specific to the canonical or non-canonical Wnt pathways and can cause different phenotypes in the cell when expressed. The appearance of certain Wnts at particular time-points can suggest which of the Wnt pathways are active and the cell's stage of differentiation. The canonical Wnts, Wnt3a, Wnt4 and Wnt8a express before or after Wnt5a, during times of cell proliferation (zur Nieden, Kempka et al. 2003). Wnt3a increases expression after mineralization has started

from day 14 through day18 (Figure 16). Wnt4 increases its expression on day 4 through day 6, 12 through 14 and on day 18. Wnt8a increases expression on day 2 suggesting it may be involved with early mesoderm formation. Wnt11, a non-canonical Wnt, has been suggested to have a role in osteogenesis (Lako, Strachan *et al.* 1998; Boland, Buitrago *et al.* 2005). Its expression starts when the expression of Wnt5a ends, day 8 through day 22. This data suggests that both the canonical and non-canonical pathways could regulate each other and are overlapping throughout osteogenesis.

3.3.5 Wnt5a and GSK3β

GSK3β is considered a pivotal molecule connecting the Src/ERK pathway and the canonical Wnt pathway. Specifically, this molecule is known to inhibit the canonical pathway in favour of the non-canonical pathway (Almeida, Han *et al.* 2005). RT-PCR indicates that GSK3β gene expression is present from day 2, and steadily decreases expression until day 16 (Figure 16). As β-catenin has been implicated in osteogenesis (Hill, Spater *et al.* 2005), lithium chloride treatment was used to increase cytosolic β-catenin levels by inhibiting GSK3β. β-catenin will then either be routed to the nucleus or the plasma membrane, but not degraded. Lithium chloride treatment had no effect or decreased the amount of calcium deposited compared to VD3 treatments (Figure 18). Lithium chloride increased mineralization only when the treatment spans day 5 ending day 7 (Figure 18). Day 5 through day 7 are also the days in which Wnt5a expression is the highest according to FACS and ICC (Figures 5 and 6). Wnt5a is thought to inhibit β-catenin activity in the nucleus (Ishitani, Kishida et al. 2003) and lithium chloride is known to inhibit the degradation of cytosolic β-catenin (De Boer, Wang et al. 2004).
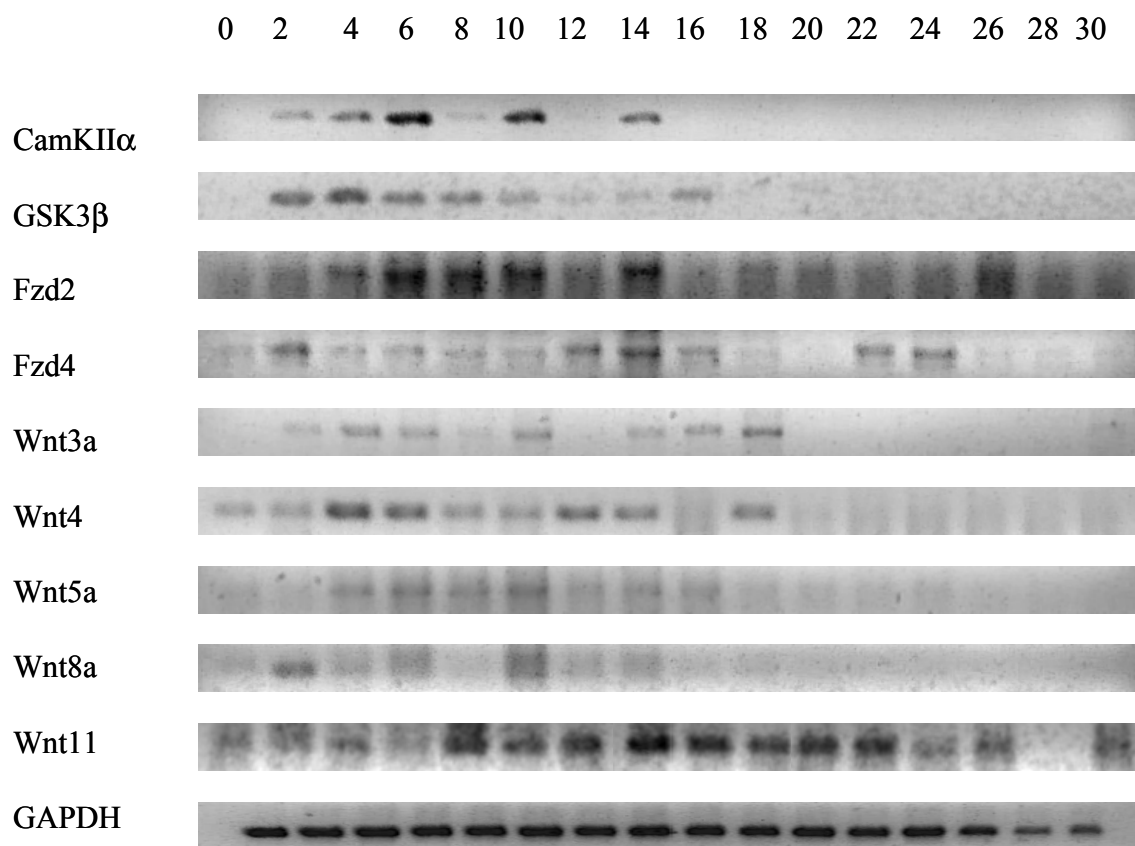
Figure 16: RT-PCR timecourse of various Wnt associated molecules. While most Wnt related gene expression occurs early between day 0 and day 16, Fzd4 and Wnt11 signal later in osteogenesis day 18 through day 30. CamKIIa: Calmodulin kinase II alpha, GSK3β: g-protein serine kinase 3 beta, Dsh2: Dishevelled 2, Fzd2: Frizzled 2, Fzd4: Frizzled 4, LRP5: Lipoprotein related protein 5. This Figure is the work of Dr. N.I. zur Nieden.
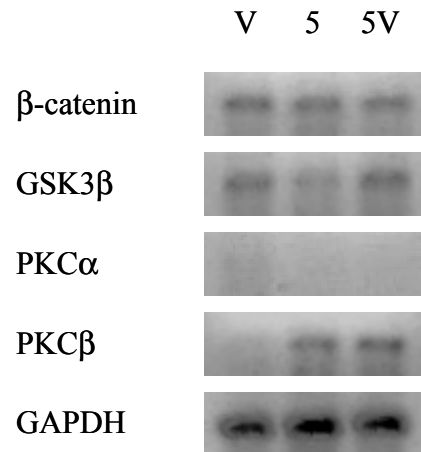
Figure 17: RT-PCR of Wnt signalling molecules on day 6. Comparison of Wnt signalling molecules on day 6, the day after osteoinduction begins. Symbols: V = VD3, 5 = Wnt5a, 5V = Wnt5a + VD3 media. Media with Wnt5a were given 1000ng/ml of recombinant Wnt5a protein. PKCα is not expressed on day 6. However, PKCβ is expressed exclusively in media where Wnt5a is added. This Figure is the work of Dr. N.I. zur Nieden.

If Wnt5a does inhibit β-catenin activity in the nucleus and lithium chloride is causing an increase in β-catenin in the cytosol, the non-degraded cytosolic β-catenin could be routed to the plasma membrane. The increase in plasma membrane bound β-catenin would increase cell adhesion which could be contributing to the increased mineralization. The fluctuation in calcium deposition due to lithium chloride treatment indicates that GSK3β may be needed to regulate the Wnt pathway, specifically cytosolic β-catenin levels.

3.4 Day 11 Wnt signalling

The period directly following Wnt5a treatment (day 11) showed increased ALP and OPN expression (Figures 12 and 13) suggesting that Wnt5a is responsible in part for enhancing an osteoblast fate. To look more closely at Wnt signalling and the role of Wnt5a on this time-point, quantitative PCR of β-catenin was performed (Figure 19). β-catenin mRNA expression was decreased in the presence of VD3, supporting Palmer *et al.* 2001 (Figure 19). β-catenin mRNA expression also showed lowered expression when treated with conditioned media (Figure 19). However, there was a slight increase in β-catenin expression at this time-point with Wnt5a and CC + VD3 treatment compared to VD3 treatment (Figure 19). As conditioned media contains many other proteins, it is impossible to conclude from Figure 19 the cause of decreased β-catenin expression. Nonetheless, vitamin D_3 does decrease the mRNA expression of β-catenin on day 11.
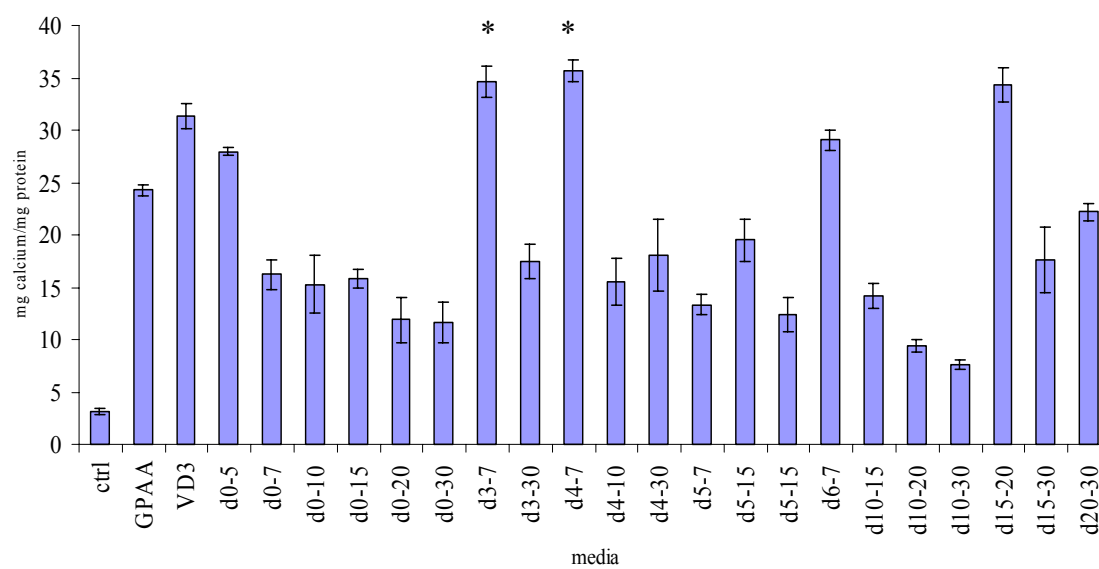
Figure 18: **Calcification of EBs treated with lithium chloride**. Lithium chloride treatment from day 3 through day 7, and day 4 through day 7 increased calcification of VD3 treated EBs. Lithium chloride treatment starting from day 0, day 10 and day 20 decreases mineralization. d: day mg: milligram calcium per embryoid body. * indicates a significant increase in calcification over VD3 media where n=3 separate differentiations and P<0.05. Data is represented as a mean +/- SD. Differences were first analyzed by ANOVA to determine overall significant differences then by Tukey's posttest procedure.
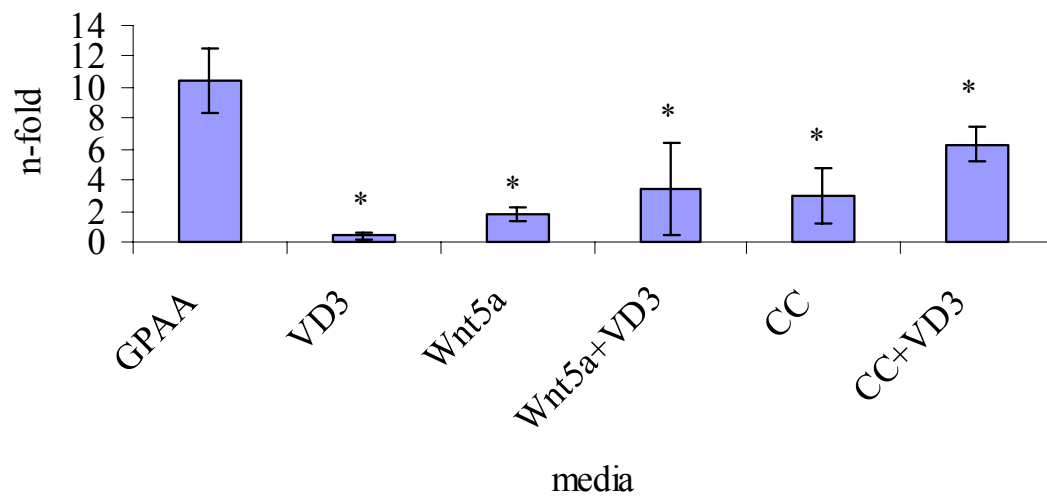
Figure 19: Quantitative Real-Time PCR of B-catenin mRNA expression on day 11.
β-catenin has less expression when treated with VD3 over GPAA. EBs treated with conditioned media with VD3 increased β-catenin expression slightly over VD3 alone whereas those treated with conditioned media (Wnt5a and CC) decreased expression over GPAA alone. The relative expression of β-catenin was normalized to GAPDH. * indicates a significant change in expression compared to GPAA. Data is represented as a mean +/- SD where n=3. Differences were first analyzed by ANOVA to determine overall differences then by Student's t-test.

3.5 Overall Results

In summary, Wnt5a gain of function does increase calcification in an osteoblast induced culture containing 1α, 25-dihydroxyvitamin D_3. In addition, these differentiating osteoblasts are sensitive to the dose of Wnt5a. Wnt5a acts early in osteogenesis, directly after VD3 treatment begins and may be directly or indirectly increasing osteoblast gene expression during osteogenesis. The signalling pathway activated by Wnt5a involves the increased expression of PKC and the translocation of β-catenin to the cell membrane.

Chapter Four: Discussion

This thesis has explored the role of Wnt5a in $1\alpha,25$-dihydroxyvitamin D_3 (vitamin D_3) induced osteogenesis. A large scale microarray project showed Wnt5a to be up-regulated during the early stages of osteoblast differentiation. The effect of Wnt5a on mineralization, specifically calcification of differentiating ES cells, as well as the signalling pathway during osteogenesis was studied here. As this thesis is based on the comparison of vitamin D_3 induced ES cells and those treated with Wnt5a with, and without vitamin D_3, the mineralization of ES cells and the pathways affected by vitamin D_3 will be examined in the following discussion. This will be followed by discussion of the effect of Wnt5a gain of function on mineralization and its signalling pathway during osteogenesis. I will then propose a model of *in vitro* osteogenesis that includes the protein Wnt5a, and end by suggesting future experiments and directions.

4.1 Microarray

A large scale microarray experiment performed in the Rancourt lab showed that the Wnt pathway was up-regulated during *in vitro* osteogenesis. There were a number of different Wnts and Wnt related molecules up-regulated during osteogenesis; Wnt5a, Wnt4, wnt8a, Sfrp1 and axin. Both Sfrp1 and axin are up-regulated early between day 6 and day 8 of *in vitro* osteogenesis (Table 2). Secreted frizzled receptor protein (Sfrp1) is a canonical Wnt inhibitor that binds Wnts before they can attach to a Frizzled receptor (Fzd). Axin is involved in the canonical pathway, regulating the degradation of β-catenin. This suggests that the canonical Wnt pathway is being regulated and possibly inhibited in the first few days that the ES cells are induced to become osteoblasts. Both Wnt4 and Wnt8a are known canonical Wnt proteins. Wnt4 is known to be involved in limb development as it

is expressed in developing joints, some bones and a subset of hypertrophic chondrocytes. Specifically it is known to block chondrogenesis *in vitro* (Church, Nohno *et al.* 2002). Little is known about Wnt8a with regard to bone and limb formation. However, it is known to be involved in mesoderm and neural crest formation (Lewis, Bonner *et al.* 2004; Ramel, Buckles *et al.* 2005). Wnt5a was chosen as it is up-regulated in early osteogenesis, on day 6 of the differentiation protocol (Table 2). In addition, it is thought to be involved in chondrogenesis and limb development (Yang, Topol *et al.* 2003). From this data, it appears that early *in vitro* osteogenesis may involve a chondrocyte precursor expressing Wnt5a. Also, the canonical pathway could be regulated early during *in vitro* osteogenesis (Table 2).

In support of the conclusion that osteogenesis, specifically endochondral ossification, is occurring upon use of the hanging drop protocol and the addition of VD3 media, a number of endochondral specific genes were found to be up-regulated (Appendix A: Table 1). Cartilage Link Protein was up-regulated early on day 6, the same day as Wnt5a (Appendix A: Table 1) and also on day 11 (Appendix A: Figure 1). This suggests an osteoblast-chondrocyte progenitor early in the differentiation process. The up-regulation of MMP13 and vitamin D-24-hydroxylase on day 16, the end of the proliferative period of osteogenesis (zur Nieden, Kempka *et al.* 2003), supports the current literature which states that while osteogenesis is occurring any chondrocyte ECM formed is being broken down for osteoblasts to enter the ECM and start mineralization (Baron 2006).

4.2 The role of 1α 25 dihydroxyvitamin D3 during *in vitro* osteogenesis

4.2.1 General

Vitamin D$_3$ induces ES cells to osteoblasts *in vitro* when added along with β-glycerophosphate and ascorbic acid (designated VD3 medium, see Table 1) (zur Nieden, Kempka *et al.* 2003). The protocol introduced by zur Nieden *et al.* 2003 for *in vitro* osteoinduction was followed closely in this project. In addition, the times in which osteoblast markers were found to increase expression were used as guidelines to determine stage of differentiation, particularly when given Wnt5a. In order to ensure protocols used by zur Nieden *et al.* 2003 could be reproduced in my hands, I compared degrees of mineralization induced between VD3 media and GPAA media (Table 1). In addition, the effect that this hormone has on Wnt associated molecules, and on the expression of specific osteoblast markers, was confirmed.

4.2.2 Mineralization analyses

Using murine ES cells as a model for *in vitro* osteogenesis, aids in the understanding of bone development and the role of key proteins. In the developing embryo, vitamin D$_3$ increases bone mass; *in vitro* it will help induce osteoblast formation (Matsumoto, Igarashi *et al.* 1991). Vitamin D$_3$ recruits calcium and phosphate ions from the intestine and kidneys to the serum and ultimately these minerals will aid in the mineralization of bone (Christakos, Raval-Pandya *et al.* 1996). This hormone also acts as a transcription factor for genes such as OPN and OCN (Matsumoto, Igarashi *et al.* 1991) and possibly ALP (Ingram, Bonde *et al.* 1994). *In vitro* studies of D3 ES cells differentiating to osteoblasts indicate that vitamin D$_3$, along with GPAA, increases osteogenesis (zur Nieden, Kempka *et al.* 2003). This thesis confirms the findings shown

in zur Nieden *et al.* 2003 that vitamin D3 plus GPAA increases osteoblast formation (Figures 8,9,10,11,12,18 and Appendix A: Figure 2,3,4,5). Therefore this project supports Matsumoto *et al.* 1991 and zur Nieden *et al.* 2003 in claims that vitamin D_3 does increase calcification and mineralization.

In addition to the increase in mineralization, zur Nieden *et al.* showed that VD3 treatment increases the expression of osteoblast specific markers, although the effect may not be direct. These osteoblast markers include ALP, OPN, and OCN (zur Nieden, Kempka *et al.* 2003). This result was confirmed, as the treatment of undifferentiated ES cells with VD3 media over a 30 day period also increases ALP, OPN, and OCN gene expression (Figures 9, 10 and 11). It has been suggested that vitamin D_3 will inhibit or induce osteogenesis depending on the time of treatment (Gurlek, Pittelkow *et al.* 2002). One protein proposed to be directly or indirectly expressed in the presence of vitamin D_3 is the pre-osteoblast marker ALP. ALP has been shown to be initiated on day 12 of ES cell differentiation and peak at the end of the proliferative period on day 18 (zur Nieden, Kempka *et al.* 2003). This thesis shows that VD3 treated ES cells on day 11 have increased ALP but not OPN expression (Figures 12 and 13). Therefore, on day 11 of osteogenesis, the gene expression for mineralization is about to begin and ES cells can be considered at the pre-osteoblast stage. In conclusion, VD3 treatment of ES cells will increase the number of cells that calcify and increases osteoblast gene expression. This confirms the results from zur Nieden *et al.* 2003 and hence this protocol can be used to determine the change in osteoblast differentiation with the addition of Wnt5a. As this thesis also examines the signalling pathway of Wnt5a, the signalling of vitamin D_3 was examined so that a comparison could be made.

4.3 1α, 25-dihydroxyvitamin D$_3$ targets

The increased expression of genes involved in VD3-induced osteogenesis in ES cells is directly and indirectly the result of the pathways that this hormone activates and through which it signals. As vitamin D$_3$ directly affects proliferation and differentiation during osteogenesis, the molecules it interacts with and activates are crucial to bone formation. ALP has been reported to express in at the same time as both the TGFβ pathway and the production of the collagen matrix during chondrogenesis (Finkelman, Linkhart *et al.* 1991; Ingram, Bonde *et al.* 1994; Wu, Haugen *et al.* 1997; Gurlek, Pittelkow *et al.* 2002). Moreover, ALP is known to be involved in early osteogenesis and is a marker for pre-osteoblast formation (Gurlek, Pittelkow *et al.* 2002; zur Nieden, Kempka *et al.* 2003). In conclusion, the results show that on day 11, VD3 treated cultures when compared to GPAA treatments increase ALP expression (Figure 12). Therefore, VD3 treated cells have differentiated into pre-osteoblasts or an osteoblast-chondrocyte progenitor cell line. The increase in ALP could be due to the ability of vitamin D$_3$ to act as a transcription factor as well as activate and crosstalk with signalling pathways that will affect osteogenesis such as the Wnt pathway.

The canonical Wnt pathway affects proliferation through activation of β−catenin. Vitamin D$_3$ is thought to inhibit β−catenin in the nucleus by the direct binding of the receptor, VDR to β−catenin and subsequently blocking β−catenin from interacting with Tcf4 (Palmer, Gonzalez-Sancho *et al.* 2001). We were able to show that the regulation of β−catenin at the protein level by vitamin D$_3$ can be detected using IFC. On day 5, before osteoinduction, β−catenin is localized to the nucleus, whereas with vitamin D$_3$ treatment,

β–catenin appears to be re-located to the plasma membrane on day 8 (Figure 15). This suggests that β–catenin is routed to the plasma membrane in the presence of vitamin D_3 supporting the claim by Palmer *et al.* 2001, that vitamin D_3 will increase the number of cell-cell junctions by transporting β–catenin out of the nucleus.   Quantitative PCR indicates that the ectopic treatment of differentiating ES cells with vitamin D_3 interrupts the transcription of β–catenin on day 11 (Figure 19).   Therefore vitamin D_3 could regulate β–catenin at both the protein and RNA level.

Not only does vitamin D_3 inhibit the canonical Wnt pathway, but it is also thought to activate molecules seen in the non-canonical Wnt pathway, such as PKC. The vitamin D_3 receptor (VDR) and PKC form a negative feedback loop, where VDR increases the expression of PKC which in turn will inhibit VDR (Krishnan and Feldman 1992; Krishnan, Cramer et al. 1995). In relation to osteogenesis, PKC will increase the expression of OPN, the gene marker for mineralization (Rangaswami, Bulbule *et al.* 2006), after activation (Gniadecki, Gajkowska *et al.* 1997; Hmama, Nandan *et al.* 1999). Quantitative PCR reveals an increase in OPN expression on day 30 with vitamin D_3 treatment (Figure 17). Therefore there may be some crosstalk between the non-canonical Wnt pathway and vitamin D_3 signalling.

In conclusion, treatment with VD3 does increase mineralization in differentiating ES cells as reported by zur Nieden *et al.* 2003. In addition, its role within osteogenesis appears to include activation of osteoblast specific genes, the inhibition of β-catenin in the nucleus and possibly the re-routing of β-catenin to the cell membrane.   In the

remainder of this chapter, the effect of Wnt5a on this system of VD3 induced osteogenesis will be discussed.

4.4 Wnt5a

Wnt5a is the traditional non-canonical Wnt signalling molecule with known involvement in chondrogenesis (Yang, Topol *et al.* 2003). The Rancourt lab performed a large scale microarray experiment in which Wnt5a showed peak expression 24 hours after VD3 treatment (Table 2 and Figure 4, 5 and 6). This suggested that Wnt5a may also play a role in the differentiation of ES cells to osteoblasts *in vitro*. The following section discusses the result of Wnt5a being ectopically added to mineralizing ES cells from day 5 through day 10 mimicking the expression time, as determined through FACS and ICC (Figure 5 and 6). In addition, this thesis starts to address the signalling pathway that Wnt5a activates upon binding its receptor during ES cell differentiation to osteoblasts.

4.4.1 Mineralization

The study of the non-canonical pathway in osteogenesis has focused primarily on its role in endochondral bone formation, as Wnt5a has been shown to be involved in regulating proliferation and differentiation in articular cartilage *in vivo* (Yang, Topol *et al.* 2003). Conversely, *in vitro* studies have shown that when mesenchymal stem cells are transfected with Wnt5a there is an increase in osteogenic gene expression, however mineralization was not effected (Boland, Perkins *et al.* 2004).

In contrast to Boland *et al.* 2004, this thesis provides evidence that Wnt5a will increase mineralization of osteoinduced murine ES cells when ectopically added from day 5 through day 10 of a 30 day differentiation protocol (Figure 7 and 8). A more detailed study of the effect of Wnt5a recombinant protein from 1ng to 100ng on

calcification indicated that between 40ng/ml and 50ng/ml calcification decreased (Figure 7). As this only occurs at two quantities, this could be an artefact. However, the experiment was repeated in triplicate. Nonetheless, the result could also indicate a triphasic effect of Wnt5a, that it acts both at low or high doses on the mineralization of the extracellular matrix.

When recombinant Wnt5a was added along with VD3 media over logarthimically increasing concentrations starting at 10ng/ml up to 1000ng/ml, there was a significant increase in mineralization over VD_3 or Wnt5a alone. The most calcification was seen at 1000ng/ml of Wnt5a + VD_3 media (Figure 8). This experiment showed that calcification increased in a linear fashion when the dose of Wnt5a was increased in a logarithmic manner (Figure 8). Of note, D3 cells carrying a Lef/Tcf-GFP reporter (kind gift of I. Weissman, Standford University) died when given Wnt5a doses exceeding 100ng/ml suggesting that transfected cells are more sensitive to the amount of Wnt5a used in the treatment (Appendix A: Figure 5). Cell death was observed as the cells become round, no longer attached to the culture dish or one another. Untransfected D3 ES cells died upon treatment with 10000ng/ml of Wnt5a recombinant protein.

In order to compare the addition of Wnt5a recombinant protein and conditioned media, the amount of Wnt5a in the conditioned media had to be determined. Wnt5a conditioned media was prepared from L-cells and an ELISA analysis was performed (Appendix: Figure 6). The results from this analysis indicate that there was active Wnt5a in all conditioned media.

In conclusion, additional Wnt5a in the culture media of differentiating ES cells will increase mineralization, specifically calcification of the ECM. In addition to

mineralization, the effect of Wnt5a on osteoblast specific markers and the Wnt signalling pathways in relation to VD3 media will be discussed.

4.4.2 Wnt5a and its pathway in osteogenesis

4.4.2.1 General

Based on results indicating that Wnt5a increases mineralization, the effect of Wnt5a gain of function has on osteoblast specific gene expression was studied. Wnt signalling has been implicated in many different developmental processes, specifically in bone development. As much of the literature focuses on cell lines that have already started to differentiate and therefore considered to be in a pre-osteoblast state, the early stages of osteogenesis and the determination of fate is not well understood. In order to understand early osteogenesis, differentiation starting from the ES cell is required. In this thesis, osteogenesis in ES cells is explored with respect to the protein Wnt5a.

4.4.2.2 Day 0 to Day 11

In the first five days of differentiation, induced through the hanging drop protocol (chapter 2: section 2.7.5), the ES cells are physically forced to interact through gravity resulting in cell aggregates. By using this protocol, at this early time in differentiation, the cells will increase their expression of Brachyury, a marker of mesenchymal gene expression (Leahy, Xiong *et al.* 1999). The canonical pathway is thought to be more involved in proliferation at this stage while the non-canonical pathway is involved in differentiation (de Boer, Siddappa *et al.* 2004) that occurs later on day 5. At the differentiation stage, vitamin D_3 will stop cells from proliferating and induce differentiation (Gurlek, Pittelkow *et al.* 2002). It is at this same stage that Wnt5a starts to increase its expression (Figure 5 and 6). Wnt5a could be acting as an anti-apoptotic

factor (Almeida, Han *et al.* 2005), keeping the cells alive while they start to differentiate or it could be acting on β–catenin signalling (Ishitani, Kishida *et al.* 2003). In a recent study, it was found that both the canonical and noncanonical pathways prolong the survival of osteoblast progenitors and osteoblast cells via GSK3β. This study indicated that high levels of active β–catenin will inhibit the anti-apoptotic effect of Wnt proteins through a negative feedback mechanism involving Axin2 (Almeida, Han *et al.* 2005). Wnt5a is thought to be involved in mesenchymal condensation and prevention of proliferating chondrocytes from entering hypertrophy, the role of Wnt5a during calcification could be due to its role in prevention of apoptosis. This could induce more cells toward an osteoblast or chondrocyte fate depending on the hormones and other factors present in the immediate environment. In the first 24 hours after osteoinduction, the ES cells show an increase in expression of genes characteristic of both the canonical and non-canonical pathway. Wnt5a, β-catenin, GSK3β, CamKIIα and PKC all have up-regulated mRNA expression (Figures 16, 17 and Appendix A: Figure 8). If mRNA expression is an indicator of protein expression, this suggests that both the canonical and non-canonical pathways are activated. However, the presence of GSK3β suggests β-catenin expression could be regulated at the protein level at these time points.

One aspect of the non-canonical Wnt pathway that is consistently shown is its ability to abrogate β–catenin. There are a number of studies suggesting that this occurs in a non-GSK3β manner (Ishitani, Kishida *et al.* 2003; Topol, Jiang *et al.* 2003; Mikels and Nusse 2006). The non-canonical pathway may (Ishitani, Kishida *et al.* 2003) or may not (Topol, Jiang *et al.* 2003) signal through a G-protein, calcium influx or CamKIIα.

During osteogenesis using D3 ES cells, results show that Wnt5a and CamKIIα both express on day 6 (Figure 16). Furthermore, ectopic treatment of Wnt5a along with VD3 increases PKC gene expression (Figures 17) on day 6 thus supporting the findings by Ishitani *et al.* 2003. By day 8, IFC indicates that Wnt5a without VD3 could be re-routing β–catenin from the nucleus, where it is located on day 5, to the cell membrane (Figure 15). This result can be explained by the Wnt5a signalling pathway blocking β–catenin activity in the nucleus as suggested in Ishitani *et al.* 2003 and β-catenin being translocated to the cell membrane for its role in cell-cell adhesion. In conclusion, Wnt5a treatment of differentiating ES cells increases PKC mRNA and VD3 treated ES cells show CamKIIα and Wnt5a expression at similar timepoints, which support Ishitani *et al.* 2003. Wnt5a signalling seems to cause β-catenin to be translocated from the nucleus to the plasma membrane in a similar fashion as vitamin D_3.

ALP is a molecule known to be up-regulated in the presence of β-catenin (Bain, Muller *et al.* 2003) and vitamin D_3 (Ingram, Bonde et al. 1994). Although it may not be regulated directly by the transcriptional activity of β-catenin, ALP is up-regulated during proliferation (zur Nieden, Kempka *et al.* 2003). This thesis shows that by day 11, ALP and OPN are up-regulated in response to both vitamin D_3 and Wnt5a treatment over either treatment alone (Figures 12 and 13). This suggests that on day 11 during *in vitro* osteogenesis when Wnt5a is added to the media, ES cells are in the proliferative phase. Wnt5a could therefore be responsible for accelerating osteogenesis *in vitro*.

4.5 1α, 25-dihydroxyvitamin D$_3$ and Wnt5a

Endochondral bone formation is known to involve both vitamin D$_3$ and Wnt5a. The two

molecules appear to overlap in many areas of cell signalling and function. Vitamin D$_3$

activates PKC, mobilizes calcium, is responsible for cell survival *i.e* is anti-apoptotic,

inhibiting proliferation and enhancing differentiation (Krishnan, Cramer *et al.* 1995;

Gurlek, Pittelkow *et al.* 2002).   Wnt5a also activates PKC, increases intracellular

calcium, is anti-apoptotic, inhibits proliferation and enhances differentiation (Kuhl,

Sheldahl *et al.* 2000; Ishitani, Kishida *et al.* 2003; Almeida, Han *et al.* 2005). In addition,

the effect of these molecules on β–catenin is also similar, with each blocking β–catenin

from binding DNA. While vitamin D$_3$ and Wnt5a appear to have overlapping functions,

however, there are some differences. Vitamin D$_3$ does not increase OPN expression on

day 11 unless Wnt5a is added to the media, in which case, the effect on OPN expression

is synergistic over either Wnt5a or vitamin D$_3$ alone at this timepoint. This contradicts

the proposed role of vitamin D$_3$ in directly stimulating OPN expression (Rangaswami,

Bulbule *et al.* 2006). Vitamin D$_3$ was thought to increase OPN expression which in turn

inhibits mineral resorption, apatite crystal formation and apoptosis. In addition, *in vitro*

ES cell differentiation has shown OPN expression on day 8 and on day 18 (zur Nieden,

Kempka *et al.* 2003), which differs from what is shown when Wnt5a is added along with

VD3. These time-points correspond to the initial induction of osteogenesis and just prior

to mature osteoblast formation during cell proliferation (zur Nieden, Kempka *et al.*

2003). This is supported by the suggestion that OPN acts through the PI3K to inhibit

apoptosis like Wnt5a (Almeida, Han *et al.* 2005). This suggests that on day 11, it is

Wnt5a stimulation of OPN that causes the anti-apoptotic, pro-proliferative effect rather than vitamin D_3.

4.5.1 Wnt5a and the Wnt signalling pathway

In the first four to five days, Wnt4 and Wnt8a have increased expression (Figure 16), suggesting their potential role in early mesoderm or mesenchymal condensation. Day 5 through day 10 is marked by the possibility of both canonical and non-canonical signalling, with Wnt3a and Wnt5a respectively, increasing expression (Figure 16). As Wnt3a increases β-catenin levels in the cytoplasm, I hypothesize that Wnt5a regulates the localization of β-catenin within the cell at this time. Wnt4 and Wnt3a increase mRNA expression on day 10 through day 22. Both are canonical Wnts involved in cell proliferation and are up-regulated during the time of cell proliferation during *in vitro* osteogenesis (zur Nieden, Kempka *et al.* 2003). Wnt11 increases expression starting on day 8 and extends to day 22 during VD3 induced osteogenesis. This time period, starts at the pre-osteoblast stage, based on ALP and OPN expression and extends to when ALP peaks the second time during osteogenesis on day 22 (zur Nieden, Kempka *et al.* 2003). Wnt11 is a non-canonical Wnt protein that has been suggested to be involved in osteogenesis both *in vivo* (Lako, Strachan *et al.* 1998) and *in vitro* (Boland, Buitrago *et al.* 2005). It also is known to act during *in vitro* chondrogenesis (Sekiya, Vuoristo *et al.* 2002) and signal through PKC and jun terminal kinase (JNK) (Eisenberg, Gourdie *et al.* 1997; Eisenberg and Eisenberg 1999; Pandur, Lasche *et al.* 2002). The last 8 days of osteogenesis do not include the expression of any Wnt proteins (Figure 16). This could mean that the proteins within the Wnt pathways needed for the final stages of osteogenesis are already activated. The time dependent expression of regulatory

molecules within the Wnt signalling pathways is key to how this pathway is involved in osteogenesis.

4.5.1.1 Wnt5a and GSK3β

Wnt5a mRNA expression overlaps with the mRNA expression of GSK3β (Figure 16) and both molecules are thought to independently regulate β-catenin (Ishitani, Kishida *et al.* 2003; Topol, Jiang *et al.* 2003).

GSK3β is a molecule involved in both canonical and non-canonical signalling. It phosphorylates β-catenin for degradation in the cytoplasm aiding the non-canonical pathway in regulating the location β-catenin within the cell. Lithium chloride is a common pharmaceutical used to inhibit GSK3β dependent phosphorylation and degradation of β-catenin in the cytoplasm (Phiel and Klein 2001). RT-PCR indicates that GSK3β is present from day 4 through day 10 (Figure 16) and the data from the microarray indicate mRNA expression day 4, 6, 8 and 30 (Appendix A: Figure 7). By inhibiting GSK3β with lithium chloride at these time-points, mineralization is increased. Wnt5a expression is increased as well at these time-points and this thesis shows that ectopic addition of Wnt5a increases calcification (Figure 7 and 8). In addition, this thesis suggests that Wnt5a is possibly translocating β-catenin from the nucleus to the plasma membrane (Figures 15). As the localization of β-catenin at the membrane could increase mineralization, it can be hypothesized that cell-cell contact must be crucial for the production of the ECM. Lithium chloride treatment spanning the period of proliferation and mineralization stages of osteogenesis (zur Nieden, Kempka et al. 2003), day 10 through day 20, decreases the amount of calcium laid down by the differentiating ES

cells (Figure 18). Therefore cytosolic β-catenin must be regulated during the proliferative phases of osteogenesis. Finally, lithium chloride treatment will decrease mineralization of cells if given in final 10 days of osteogenesis *in vitro* (Figure 18). In conclusion, the regulation of β-catenin by both Wnt5a and GSK3β is necessary for ES cells to go through the necessary stages of osteogenesis.

4.6 Future Studies

Although this thesis has made some progress in addressing some controversial issues regarding the role of Wnt5a in osteogenesis and its signalling pathway, there are a number of experiments left that will help to fully understand Wnt signalling and osteogenesis. This thesis addresses two major issues regarding Wnt5a and osteogenesis, a) is it involved in osteogenesis with regard to mineralization and gene expression and b) which downstream signalling pathway is activated by Wnt5a during *in vitro* osteogenesis. Wnt5a is a non-canonical Wnt signalling molecule and its characterization has been in its role during chondrogenesis *in vivo* or with regard to pluripotent cells already fated to the chondrocyte, osteoblast and adipocyte lineage (Westendorf, Kahler *et al.* 2004). This is the first study looking at the role of Wnt5a starting at the ES cell and inducing the cells to become osteoblasts. This study looks at calcification of ES cells, as calcium is the major mineral in the developing ECM of osteoblasts. However, the effect of Wnt5a on collagen production and incorporation into the ECM was not explored. Such studies would increase the understanding of how the ECM is laid down and the order of events that lead to complete mineralization *in vitro*. As this project looked at key osteoblast markers and not the entire osteoblast molecular pathway, a more in depth study into the expression and timing of FGF, parathyroid hormone related protein, bone sialoprotein and others

when cells are treated with Wnt5a would also increase knowledge regarding the stages and timing of endochondral bone formation *in vitro*. The stages and timing could then be compared to the known molecular stages *in vivo*. This comparison will be useful if *in vitro* production of osteoblasts is to be used as a cytotherapy, particularly with regard to knowing the stage of differentiation the ES cells can be inserted for tissue repair. As this thesis based most of its work on mRNA expression, western blot analysis depicting the change in protein expression throughout osteogenesis would useful, especially as some markers and molecules could be regulated at the protein level. Also, Wnt5a is not known to scavenge calcium from the external environment of the cell for mineralization of the extracellular matrix. As Wnt5a without vitamin D_3 can increase calcification of differentiating ES cells to similar levels as VD3 media, the role of Wnt5a specifically with regard to mineralization of the ECM should be studied in more detail. I would start by looking at the early production of the ECM specifically collagen production, through RNAi and collagen gain of function studies.

As the phenotype seen with β-catenin over-expression is the opposite of what is seen with Wnt5a over-expression, it has been suggested that these two pathways regulate each other during endochondral bone formation (Yang, Topol *et al.* 2003). The non-canonical Wnt pathway is characterized by the presence and activation by a Wnt protein that does not signal through β-catenin. Instead, the signalling pathway for Wnt5a is thought to signal through a G-protein followed by a calcium influx and activation of CamKIIα, and the MAPK cascade. Therefore this will result in NLK phosphorylating Tcf/Lef transcription factors, preventing the binding of β–catenin/Tcf/Lef complex to the

DNA. This thesis did not look at the involvement of a G-protein in the Wnt5a pathway during osteogenesis. However, the addition of pertussis toxin to the culture media would be effective in determining if Wnt5a signalling during osteogenesis involves a G-protein. Other controversies that could be studied involving the Wnt pathway is whether the inhibition of β-catenin from binding DNA in the nucleus is due entirely to the non-canonical pathway. In fact, Wnt 1, a canonical Wnt, has been shown to activate NLK in a calcium independent manner (Smit, Baas *et al.* 2004). The role of canonical Wnts on inhibiting β-catenin during osteogenesis could be explored.

The pathway that Wnt5a uses during osteogenesis and in other tissues appears to be dependent on the receptor to which it binds (Nishioka, Dennis *et al.* 2005; Mikels and Nusse 2006). By binding to Fzd4 and the co-receptor LRP5, not LRP6, Wnt5a can activate the canonical pathway (Mikels and Nusse 2006). However, when Wnt5a binds to the orphan tyrosine kinase, Ror2 which processes an extracellular cysteine rich Wnt binding domain (CRD), it will activate Jun kinase and inhibit the canonical signalling. Ror2 and Wnt5a have overlapping expression patterns and the knockout phenotype is similar for both molecules supporting their interaction. It has been suggested that Ror2 acts through Siah2 as well to inhibit β–catenin signalling (Mikels and Nusse 2006). The expression of the Fzds present during osteogenesis and the ability of Wnt5a to bind them should be studied, as this will determine which pathway Wnt5a signals through during osteogenesis *in vitro*.

A time course of the expression of all 10 Fzds as well as each component of the non-canonical and canonical pathway, through RT-PCR for VD3 and Wnt5a + VD3 treated cultures would give a greater understanding of the expression of the Wnt

pathways during in vitro osteogenesis. In addition, western blot analysis of β-catenin probing specifically for changes in cytoplasmic, nuclear or plasma membrane located protein would determine if, in fact, vitamin D_3 and Wnt5a are translocating β-catenin out of the nucleus to the plasma membrane. Also, the effect of Wnt5a was only studied at least one day after treatment; the immediate effect of Wnt5a on signalling during osteogenesis should be studied. This would be effectively studied by IFC.

4.7 Overall Conclusions

In conclusion, Wnt5a increases mineralization in differentiating ES cells. The role it plays appears to overlap partially with the role of vitamin D_3, as far as the expression of key osteoblast specific proteins early in osteogenesis and both have the ability to induce mineralized osteoblasts. In addition, it works with vitamin D_3 to localize β-catenin to the cell membrane earlier than either treatment alone (Figures 15 and 20). This project shows for the first time that Wnt5a increases mineralization of osteoblasts during endochondral bone formation. Its role in osteogenesis could involve rerouting β-catenin to the cell membrane indirectly by inhibiting the activity of nuclear β-catenin in order to increase cell-cell adhesion (Figure 20). The data from this thesis can be used toward the creation of cytotherapies for patients suffering from bone and cartilage degenerative diseases.
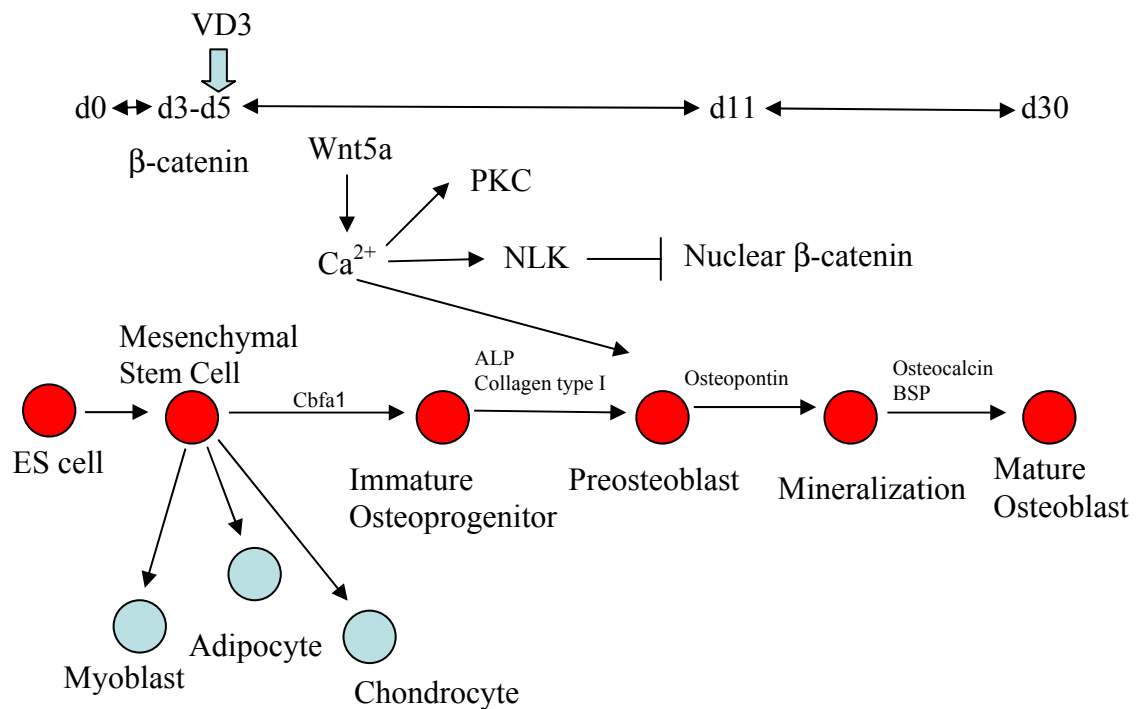
Figure 20: Proposed model of Wnt5a's role in osteogenesis. Wnt5a may play a role in regulating the localization of β-catenin during osteogenesis by inhibiting β-catenin activity within the cell nucleus. This figure is a cartoon depiction of the 30 day differentiation process. This figure shows that Wnt5a increases expression just after VD3 is added to the media. Also, it shows the time in which Wnt5a and VD3 synergistically act to increase osteopontin and alkaline phosphatase expression. It is possible that Wnt5a could activate the signalling pathway that causes NLK to inhibit nuclear β-catenin activity during osteogenesis.

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Appendix A

|	day 6	day 8	day 16	day 25	day 30
MMP13	-3.23	-4.68	53.14	4.09	1.51
vitamin D-24-hydroxylase	1.48	2.11	60.95	34.2	45.7
cartilage link protein	2.42	2.38	1.18	-6.08	2.31
parathyroid hormone receptor	1.96	1.19	-1.66	1.84	2.95

Table 1: The n-fold change in expression of four genes known to be involved in endochondral bone formation. The signal was normalized to the signal of ES cells that are spontaneously differentiating on each day of differentiation. The n-fold change in expression is shown. Decreasing expression is indicated with a "-". The largest n-fold increase for each gene is colored red. This table has been modified from the work of Dr. N.I. zur Nieden
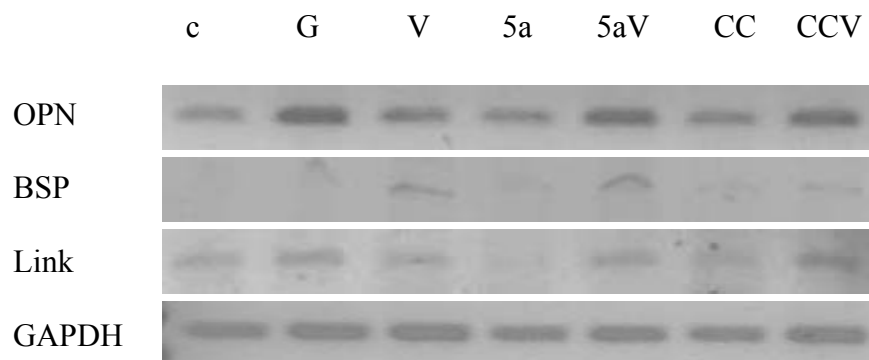
Figure 1: RT-PCR osteoblast and chondrocyte markers on day 11. The relative expression of gene markers for pre-osteoblast and mature osteoblast and chondrocyte stages of differentiation were deteremined compared against Gapdh. Osteopontin (OPN) increases expression on day 11 when EBs are treated with GPAA (G), Wnt5a+VD3 (5aV) and CC+VD3 (CCV). Bone Sialoprotein (BSP) increases expression only when EBs are treated with VD3 (V) or Wnt5a+VD3 (5aV). Link Protein (Link) expression is not as elevated when treated with Wnt5a (5a) or CC. c: ctrl, G: GPAA, V: VD3, 5a: Wnt5a, 5aV: Wnt5a+VD3, CC: CC, CCV: CC+VD3 (Table 1).

| ctrl | GPAA | VD3 | Wnt5a | Wnt5a+VD3 | CC | CC+VD3 |

Figure 2: Sample pictures of EBs under phase contrast on day 30 of differentiation. Pictures of EBs as visualized using phase contrast microscopy (2x magnification). See Chapter 2: Table 1 for contents of media. The extracellular matrix of mineralized osteoblasts appears black, therefore the Wnt5a+VD3 treated EB has increased osteoblast differentiation compared to other treatments.
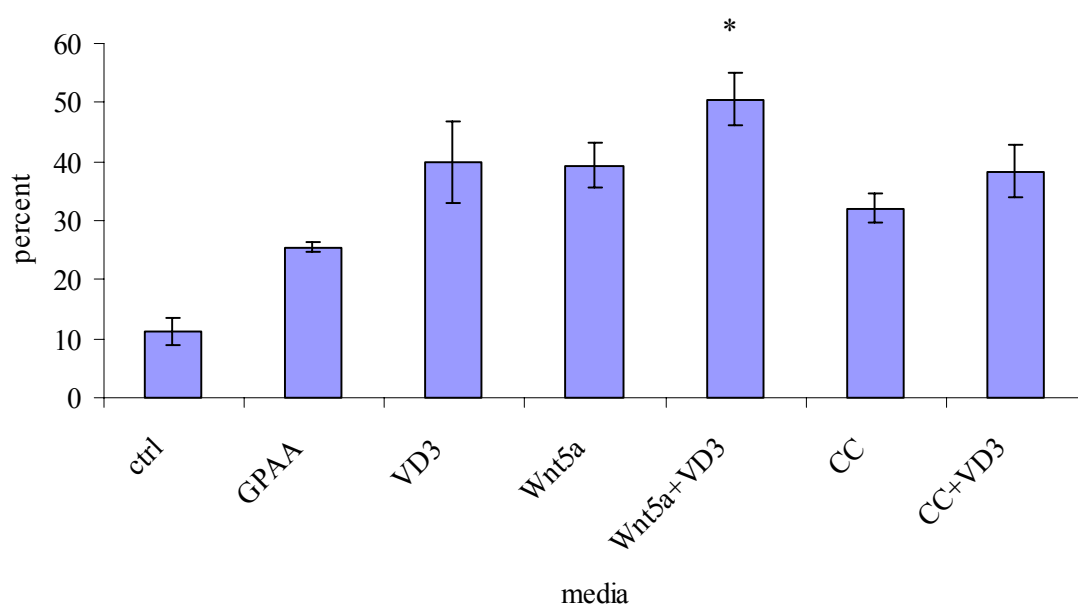
Figure 3: **Mineralization of EBs analyzed through morphometric analysis.** Pictures of EBs taken at 2x magnification under phase contrast microscopy were analyzed using Image J to determine the mean average number of pixels per picture that were black. See Chapter 2: Table 1 for contents of media. Values are averages +/- SD. * indicates significance over VD3 calculated using a student's t -test where $P < 0.005$, $n = 3$
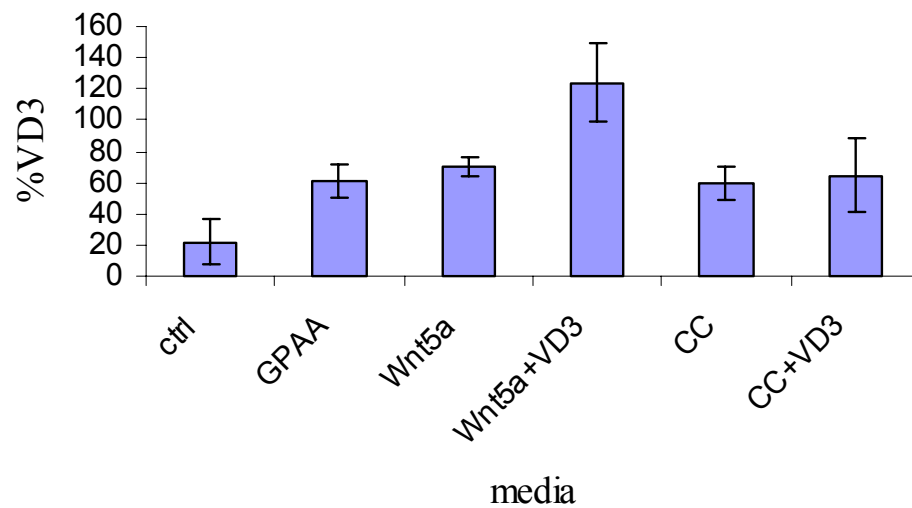
Figure 4: Calcium deposition of EBs treated with different media. Calcium deposition shown as average percentage of calcium that is deposited by EBs treated with VD3 media (%VD3). Therefore, the amount of mineralization deposited when EBs are treated with VD3 is set at 100%. Wnt5a + VD3 treated EBs show a slight increase in mineralization over other treatments but not significantly over VD3. Those given Wnt5a conditioned media or control conditioned media were treated from day 5 through day 10. Values are averages +/- SD. Significance over VD3 calculated using a student's t –test: n = 3.
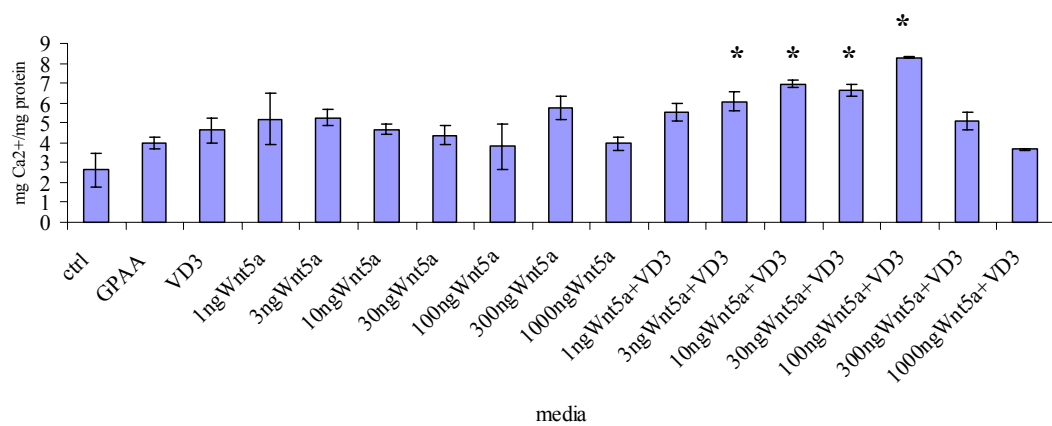
Figure 5: Calcium assay of transfected D3 cells at different concentrations of Wnt5a recombinant protein. D3 cells were stably transfected with Lef/Tcf vector. Wnt5a recombinant protein increased calcification of differentiating ES cells when given with VD3. The most calcification occurs at 100ng/ml of Wnt5a + VD3. 1000ng/ml was toxic to transfected D3 cells differentiating to osteoblasts. * indicates a significant increase in calcification over VD3 media where n=3 separate differentiations and P<0.05. Data is represented as a mean +/- SD. Differences were first analyzed by ANOVA to determine overall significant differences then by Tukey's posttest procedure.
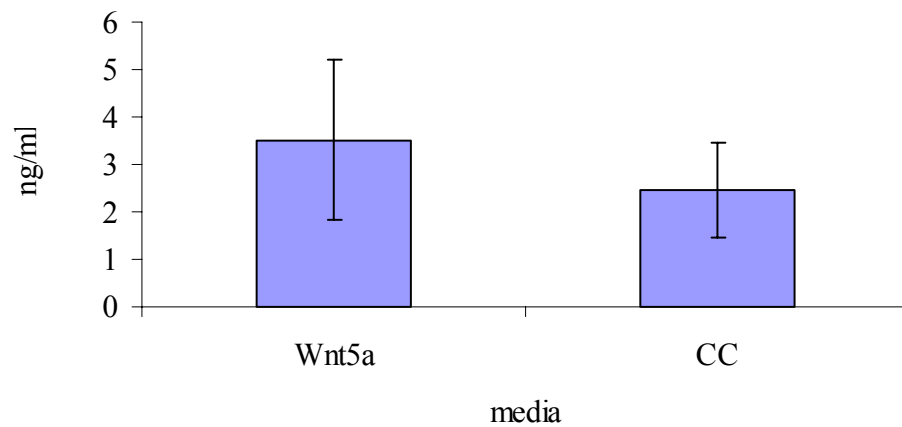
Figure 6: Average quantity of Wnt5a in conditioned media. ELISA analysis was used to determine quantity of Wnt5a. The mean average quantity of Wnt5a protein is increased slightly and insignificantly in conditioned media from Wnt5a transfected L-cells over CC - L-cells that are not transfected with the Wnt5a vector. The average amount of active Wnt5a in Wnt5a conditioned media was 3.52ng/ml while CC media contained 2.46ng/ml. Media was probed with goat anti-donkey Wnt5a primary antibody and anti-goat HRP secondary antibody.
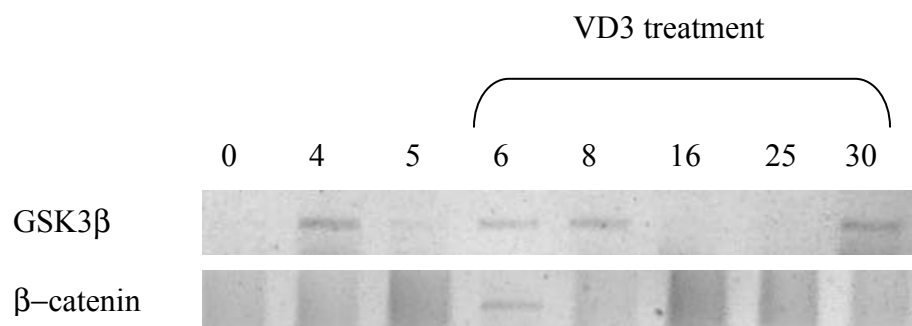
Figure 7: RT-PCR timecourse of Wnt signalling molecules. cDNA was made from total RNA collected for the microarray data. GSK3β increased expression early between day 4 and day 8 as well as day 30. β-catenin mRNA expression occurs day 6.

	C	G	V	5a	5aV	CC	CCV
CamkIIα							
GSK3β							
GAPDH							
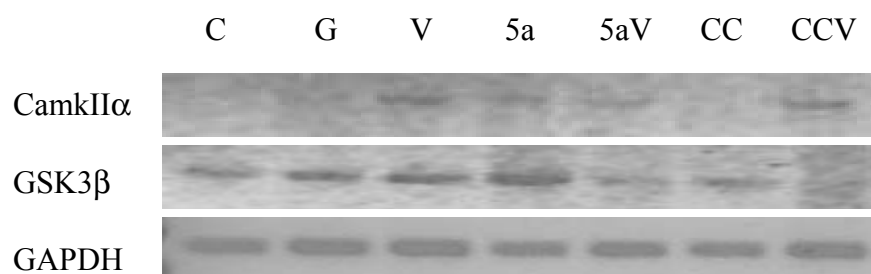
Figure 8:   RT-PCR showing the expression of three Wnt signalling genes on day 11 of differentiation given different media.   β-catenin did not change its mRNA expression on day 11.   CamKIIα was present when treated with VD3, or Wnt5a. GSK3β increased its expression slightly when given Wnt5a conditioned media and decreased expression when given Wnt5a +VD3, or CC+VD3. c: ctrl, G: GPAA, V: VD3, 5a: Wnt5a, 5aV: Wnt5a+VD3, CC: CC, CCV: CC+VD3 (Table 1).