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Wnt Signaling in the Mouse Heart:
A Role for Wnt 11 in Cardiogenesis

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University of Ottawa
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Ph.D. Thesis Abstract

Specification and early patterning of the vertebrate heart is dependent on both canonical and non-canonical wingless (Wnt) signal pathways, however, the impact of each Wnt pathway on the later stages of myocardial development and differentiation remains contentious. Here, we sought to define the respective roles of canonical and non-canonical Wnt signaling during mammalian cardiogenesis, specifically cardiomyocyte differentiation and maturation. The cellular components of both canonical and non-canonical Wnt pathways were present throughout murine cardiac development, yet canonical Wnt signaling had a restricted region of activity in the mouse heart, primarily in the non-myocardial regions including the outflow tract and semi-lunar valves. Conversely, elevated non-canonical Wnt11 signaling was associated with expansion of the myocardium and *de novo* induction of cardiomyocyte differentiation. The pro-myogenic effect of Wnt11 resulted in a direct inhibition of canonical Wnt/β-catenin activity in Wnt11-treated progenitor cells suggesting that non-canonical signaling enhanced cardiogenesis through suppression of canonical Wnt pathways. Wnt11 stimulation suppressed canonical Wnt signaling by destabilizing full length β-catenin, an effect that could be reversed by inhibiting caspase 3/8 activity. Stimulation of progenitor cells with Wnt11 promoted the activation of caspase 3/8 and inhibition of either protease resulted in inhibition of cardiomyocyte differentiation. Taken together, our results suggest that non-canonical Wnt11 signaling promotes cardiogenesis, in part, by caspase-mediated suppression of canonical Wnt/β-catenin activity.

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I also want to acknowledge all the laboratory members (past and present) I have worked with over the years that have helped me with any questions I have had and with finding any reagents that I needed. I have learned a tremendous amount from them and, in turn, I hope they have benefited in some way from working with me.

Once again, I want to express my appreciation to everyone for making these past few years interesting and unique.

ABSTRACT

Specification and early patterning of the vertebrate heart is dependent on both canonical and non-canonical wingless (Wnt) signal pathways, however, the impact of each Wnt pathway on the later stages of myocardial development and differentiation remains contentious. Here, we sought to define the respective roles of canonical and non-canonical Wnt signaling during mammalian cardiogenesis, specifically cardiomyocyte differentiation and maturation. The cellular components of both canonical and non-canonical Wnt pathways were present throughout murine cardiac development, yet canonical Wnt signaling had a restricted region of activity in the mouse heart, primarily in the non-myocardial regions including the outflow tract and semi-lunar valves. Conversely, elevated non-canonical Wnt11 signaling was associated with expansion of the myocardium and *de novo* induction of cardiomyocyte differentiation. The pro-myogenic effect of Wnt11 resulted in a direct inhibition of canonical Wnt/β-catenin activity in Wnt11-treated progenitor cells suggesting that non-canonical signaling enhanced cardiogenesis through suppression of canonical Wnt pathways. Wnt11 stimulation suppressed canonical Wnt signaling by destabilizing full length β-catenin, an effect that could be reversed by inhibiting caspase 3/8 activity. Stimulation of progenitor cells with Wnt11 promoted the activation of caspase 3/8 and inhibition of either protease resulted in inhibition of cardiomyocyte differentiation. Taken together, our results suggest that non-canonical Wnt11 signaling promotes cardiogenesis, in part, by caspase-mediated suppression of canonical Wnt/β-catenin activity.

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LIST OF ABBREVIATIONS

Wnt.....Wingless-iNTegration

Fzd.....Frizzled receptor

Dvl.....Dishevelled

Daam.....Dishevelled associated activator of morphogenesis

APC.....Adenomatosis Polyposis Coli

GSK3β.....Glycogen synthase kinase 3β

TCF.....T-Cell Factor

Myoc.....Myocardium

OFT.....Outflow tract

cNCC.....Cardiac Neural Crest Cells

PrH.....Primitive heart sac

PrA.....Primitive atrium

PrV.....Primitive ventricle

PV.....Pulmonary valve

AV.....Aortic valve

Ant-Pos.....Anterior-posterior axis

Cr-Ca.....Cranial-caudal axis

L-R.....Left-right axis

RVOT.....Right Ventricle Outflow Tract

RC.....Rib cage

PKC.....Protein Kinase C

Wnt11-cm.....Wnt11 conditioned medium

PNGase F.....Peptidyl N glycosidase F

DMSO.....Dimethyl sulfoxide

shRNA.....short hairpin RNA

Tgfβ2.....Transforming growth factor β2

cTnT.....cardiac-specific Troponin T

DSHB.....Developmental Studies Hybridoma Bank

MF20.....antibody from DSHB directed against myosin heavy chain

α-MHC.....alpha myosin heavy chain

RT-PCR.....reverse transcriptase PCR

GAPDH.....glyceraldehyde-3-phosphate dehydrogenase

Hsp68.....heat shock protein 68 promoter element

LacZ.....β-galactosidase protein (encoded by lacZ gene)

TCF-lacZ (+/+).....T-Cell Factor-lacZ homozygous reporter mice

SuperTOPflash.....Super (TCF Optimal Promoter) flash luciferase plasmid

TUNEL.....Terminal deoxynucleotidyl transferase dUTP nick end labeling

DAPI.....4',6-diamidino-2-phenylindole

STS.....Staurosporine

hESC.....human embryonic stem cells

GPCR.....G-Protein Coupled Receptor

MEF2C.....Myocyte Enhancer Factor 2C

IP-Ag.....Immunoprecipitation-silver stain procedure

H+E.....Hematoxylin and Eosin stain

LC-MS/MS.....Liquid chromatography-mass spectrometry

Caspase.....Cysteinyl aspartate protease

DEVD.....Caspase 3 recognition site (Asp-Glu-Val-Asp) tetrapeptide

IETD.....Caspase 8 recognition site (Ile-Glu-Thr-Asp) tetrapeptide

amc.....amino-methylcoumarin (reagent for fluorogenic substrates)

fmk.....fluoromethyl ketone (reagent for irreversible caspase inhibition)

Casp-inh.....Caspase inhibitor (DEVD-fmk or IETD-fmk)

Chapter 1 - INTRODUCTION

Mammalian heart development

The heart is among the first functional organs to appear in the mammalian embryo and is critical to sustaining life at even the earliest developmental stages (Zaffran and Frasch 2002). Formation of the heart is a complex process that can be subdivided into 4 stages: i) specification of cardiac progenitor cells, ii) formation of the linear heart tube, iii) cardiac looping, and iv) septation and cardiac valve formation to form the multi-chambered and mature beating heart (Harvey 2002; Srivastava 2006); see Figure 1. The first stage (Cardiac crescent stage) is identified by the presence of a population of cardiac progenitor cells in the cranio-lateral region of the embryo shaped as a crescent. The second stage (Linear heart tube stage) is initiated when cardiac progenitors migrate ventrally towards the midline of the embryo and fuse to form a transient structure called the linear heart tube. Blood enters the inflow region (primitive atria) of the linear heart tube at the caudal end of the embryo, whereas its outflow tract region is located cranially. The linear heart tube then undergoes a complex set of morphological movements (Cardiac looping stage), in which the tubular heart initiates a rightward spiral loop. During cardiac looping, the inflow region of the heart is forced dorsally and cranially so that the common atria now rest above the developing ventricles. Finally, the heart assumes its final structure where septation of the ventricles, atria and atrioventricular valves results in the mature four-chambered heart. Furthermore, and notably after cardiac looping, cardiac neural crest cells migrate into the outflow tract from the neural folds to help septate the outflow tract and pattern the bilaterally symmetric aortic arches (III, IV, and VI) to contribute to formation of the mature heart. Septation of the outflow tract

forms the pulmonary and aortic arteries which pump deoxygenated blood to the lungs and oxygenated blood to the rest of the organism, respectively. Aortic arches III, IV and VI eventually give rise to arteries that supply the head, neck, and arms with oxygenated blood.

The structure of the mature heart illustrates the fact that it is comprised of two separate but anatomically fused pumping circuits: the pulmonary circuit (pumping de-oxygenated blood to the lungs) and the systemic circuit (pumping oxygenated blood to the organism). The right atrium receives deoxygenated blood from tissues and organs of the body and passes it through the tricuspid valve to the right ventricle, which pumps it through the pulmonary artery to the lungs. Oxygenated blood from the lungs is returned to the left atrium of the heart through the pulmonary veins, and is passed to the left ventricle through the bicuspid valve. Finally, from the left ventricle, blood is pumped through the aorta sending oxygenated blood to nourish tissues and organs of the whole organism.

Induction and Differentiation of Cardiac Progenitor Cells

During gastrulation, cardiac progenitor cells move through the primitive streak (Garcia-Martinez and Schoenwolf 1993; Kinder et al. 2001), and migrate towards the cranio-lateral parts of the embryo to form the cardiac crescent, which begins to express cardiac transcription factors (Harvey 2002). The earliest molecular markers for cardiac

Figure 1

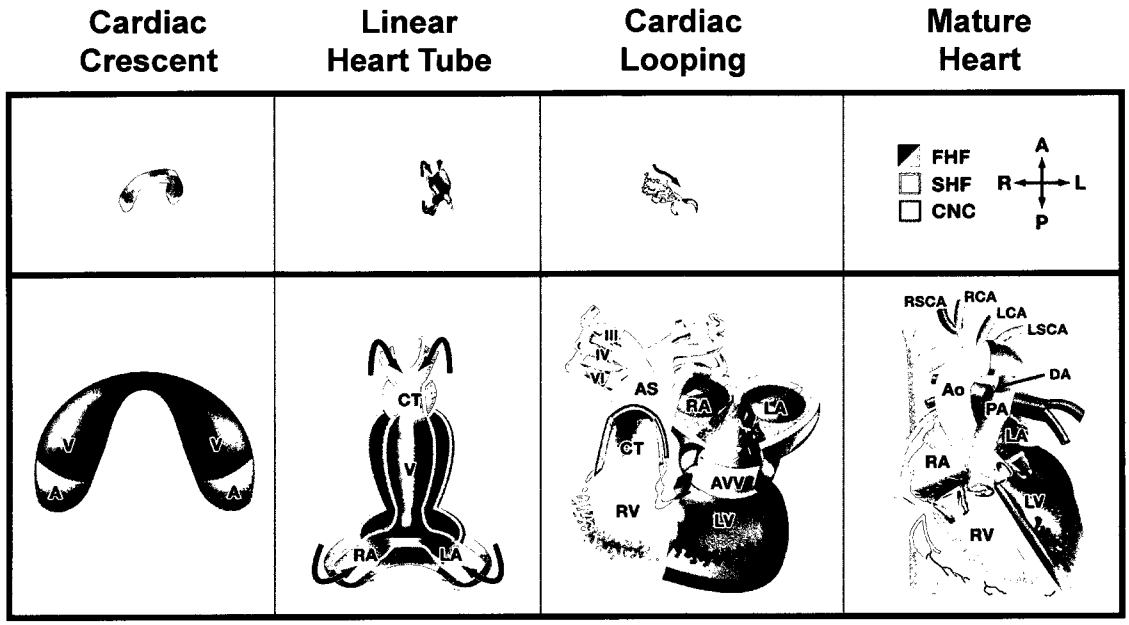

Cardiac Crescent
Linear Heart Tube
Cardiac Looping
Mature Heart
FHF
SHF
CNC
A
R
L
P
V
A
CT
RA
LA
III
IV
VI
AS
AVVs
RV
LV
RSCA
RCA
LCA
LSCA
DA
Ao
PA

Figure 1. Stages of mammalian cardiac development. Oblique views of whole embryos (upper panels) and corresponding frontal views of cardiac precursors (lower panels) are shown. (Cardiac Crescent stage) Heart progenitors are recognizable as a crescent-shaped tissue located at the cranio-lateral parts of the embryo. First Heart Field (FHF) cells contribute to progenitors in the crescent region (forming eventual left ventricle) whereas Second Heart Field (SHF) cells form the eventual right ventricle, outflow tract and atria. These cells then migrate towards and fuse along the midline of the embryo to form a transient structure called the linear heart tube (Linear Heart Tube stage). The linear heart tube then undergoes a complex set of movements (Cardiac Looping stage) where the tubular heart adopts a spiral shape via a rightward loop of the primitive linear heart tube, resulting in the atria positioned on top of the ventricles. In addition, cardiac neural crest (CNC) cells migrate into the outflow tract (arrow) from the neural folds to septate the outflow tract and pattern the symmetric aortic arches (III, IV, and VI). (Maturation stage) Finally, the heart undergoes a process of maturation where septation of the ventricles, atria, and atrioventricular valves (AVV) results in the mature four-chambered heart. V, ventricle; A, atrium; LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium; CT, conotruncus region (RV and outflow tract); AS, aortic sac; Ao, aorta; PA, pulmonary artery; RSCA, right subclavian artery; LSCA, left subclavian artery; RCA, right carotid artery; LCA, left carotid artery; DA, ductus arteriosus. (Figure adapted from Srivastava et al. 2006).

progenitor cells, Mesp1 and Mesp2 (Mesoderm Posterior 1 and 2), are expressed transiently in newly formed mesoderm at the primitive streak stage (Kitajima et al. 2000). This is required for the movement of cells towards the anterior region of the embryo where descendants of these cells colonize the whole myocardium, hence marking the myocardial cell lineages. There is evidence for plasticity of early mesodermal cells in the primitive streak, for example, mouse epiblast cells (the embryonic cell layer that generates endoderm and mesoderm by migration of cells through the primitive streak) will differentiate into cardiomyocytes if grafted into the heart-forming region of post-gastrulation embryos, whereas cells from the cardiac crescent, when introduced into the epiblast, will re-gastrulate and contribute to multiple cell lineages, including the heart (Harvey 2002).

Commitment of cardiac progenitors to the cardiac lineages is highly influenced by the dynamic spatio-temporal movements of gastrulation. In mammals, the cardiac progenitors lie adjacent to progenitors of the head in a region called the cardiac progenitor zone or cardiac crescent mesoderm (see Figure 2A and B). Head development requires potent inhibition of BMP, Wnt and Nodal signaling, and this is achieved by specific inhibitors that are secreted from inducing tissues including the overlying neural plate and the axial mesendoderm (Niehrs et al. 2001; Perea-Gomez et al. 2001). By contrast, cardiac cell lineages are induced at the lateral edges of the cardiac progenitor zone, where Wnt signaling is still inhibited but BMP signaling is robustly maintained (Marvin et al. 2001; Schneider and Mercola 2001). The endoderm that is in direct contact with the cardiac mesoderm has potent heart-inducing properties (Nascone and

Mercola 1995). In fact, members of the BMP family are highly expressed in the endoderm, as well as in the adjacent ectoderm (Schultheiss et al. 1997; Solloway and Robertson 1999; Yamagishi et al. 2001). In addition, genetic and biochemical analyses in many organisms have demonstrated a key role for BMP signaling in specification and maintenance of the myocardial lineage (Zhang and Bradley 1996; Schultheiss et al. 1997; Andree et al. 1998; Schlange et al. 2000; Shi et al. 2000). Furthermore, BMP receptor-regulated transcription factors of the Smad family seem to directly activate genes that encode cardiac transcription factors including Nkx2-5 (Schwartz and Olson 1999; Sparrow et al. 2000; Liberatore et al. 2002; Lien et al. 2002). Endodermal expression of Fgf8 is also induced by BMPs, where expression of both BMP and Fgf8 are required to induce cardiogenesis (Alsan and Schultheiss 2002). Wnt inhibitors of the secreted Frizzled-related proteins and Dickkopf families are also expressed in the endoderm that is adjacent to cardiac mesoderm (Marvin et al. 2001; Schneider and Mercola 2001). Surprisingly, cardiac differentiation can be activated by exposing the posterior non-cardiogenic mesoderm of chicken and *Xenopus* embryos with both BMP and Wnt inhibitors (Marvin et al. 2001; Schneider and Mercola 2001; Tzahor and Lassar 2001); and, notably in *Xenopus*, these conditions can lead to formation of ectopic-beating cardiomyogenic tissue (Schneider and Mercola 2001).

The cardiac crescent is not only regulated by positive signals (BMPs, Fgf8, and Wnt antagonists), but also by negative signals emanating from Wnts in the overlying neural tube and from BMP inhibitors expressed in axial tissues (Figure 2B). In response to these positive and negative inductive cues, the cardiac crescent activates several

cardiac transcription factors including: Gata4/5/6, Nkx2-5 (NK2 transcription factor-related, locus 5), Mef2c (Myocyte enhancer factor 2c), Hand1/Hand2 (Heart and neural crest derivatives expressed transcript 1 or 2), and Tbx5/Tbx20 (T-box 5 or 20) (Srivastava et al. 1995; Harvey 1996; Bruneau et al. 1999; Meins et al. 2000; Molkentin 2000), thus establishing a positive transcriptional network for cardiac differentiation. In turn, this transcription factor program engages a specific cardiomyocyte gene expression program including: Nkx2-5, Mlc2-v (Myosin light chain 2-ventricle), and cTnT (cardiac Troponin T).

Recent studies have revealed that the cardiogenic mesoderm actually consists of two distinct cell lineage populations or heart fields that contribute to the developing heart (Buckingham et al. 2005). The primary or first heart field (FHF) is derived from cells in the cardiac crescent mesoderm located in the ventral and cranio-lateral region of the embryo. FHF cells contribute to formation of the left ventricle and express cardiac markers such as Nkx2-5, Hand1, and Tbx5. The second heart field (SHF) is marked by the Islet1 (Isl1) transcription factor and is derived from cells dorsal and medial to the cardiac crescent. Expression of Isl1 is diminished as progenitor cells express markers of cardiac differentiation, but Isl1 is necessary for SHF-derived cells to populate the heart. SHF contributes progenitor cells to the right ventricle, outflow tract, and atria, while expressing the markers Isl1, Fgf8, Mef2c, Hand2, and Tbx20. In support of these observations, mutant mice of FHF derivatives (Nkx2-5, Hand1, Tbx5) display defects in left ventricular development (Lyons et al. 1995; Firulli et al. 1998; Srivastava 1999; Tanaka et al. 1999; Bruneau et al. 2001; Takeuchi et al. 2003) whereas perturbations in

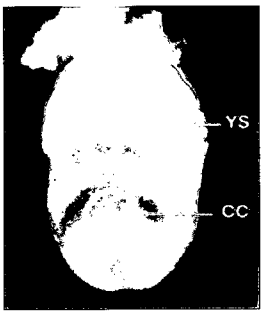
the SHF lineage components (Isl1, Fgf8, Mef2c, Hand2, Tbx20) primarily affect the developing outflow tract and right ventricle (Srivastava et al. 1995; Lin et al. 1997; Srivastava et al. 1997; Abu-Issa et al. 2002; Cai et al. 2003; Dodou et al. 2004; Stennard et al. 2005; Takeuchi et al. 2005).

Cardiac progenitor cell populations in the mouse heart

Three populations of embryonic cardiac progenitor cells have been identified in the mouse heart including: the cardiogenic mesoderm (described above), the cardiac neural crest and the proepicardial organ (see Figure 2C and D) (Laugwitz et al. 2008). Each of these represents a spatio-temporally distinct pool of cardiac progenitors that gives rise to the diverse muscle and non-muscle cell types found in the mouse heart. First, the cardiogenic mesoderm (E7.5), gives rise to progenitors that differentiate into endocardial cells and atrial/ventricular cardiomyocytes that form the bulk mass of the myocardium. Second, the cardiac neural crest cells, which migrate into the heart at a later stage (E10.5), give rise to the vascular smooth muscle of the outflow tract great vessels, ductus arteriosis, and the cardiac autonomic nervous system. Third, the proepicardium, contributes to the coronary vasculature and interstitial fibroblasts.

Figure 2A, 2B

A



Nkx2-5/Cre-lacZ
Cardiac Crescent
(E7.5)

B

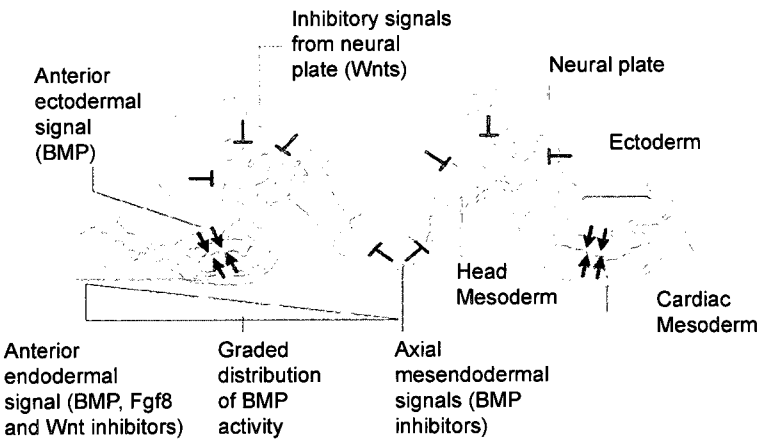
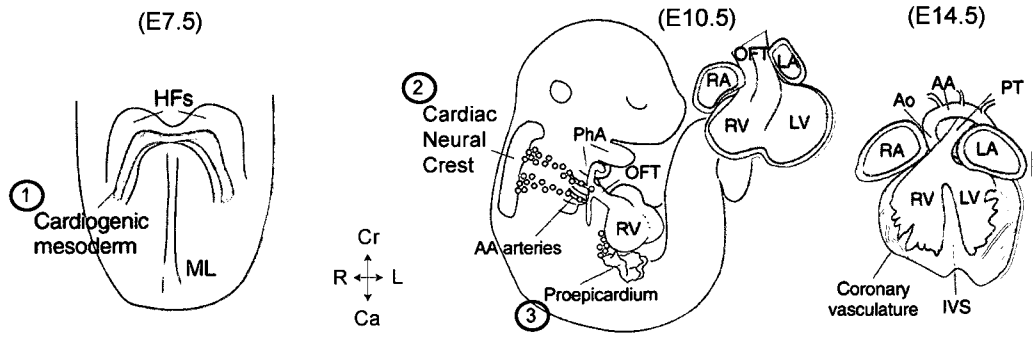


Figure 2C, 2D

C



D

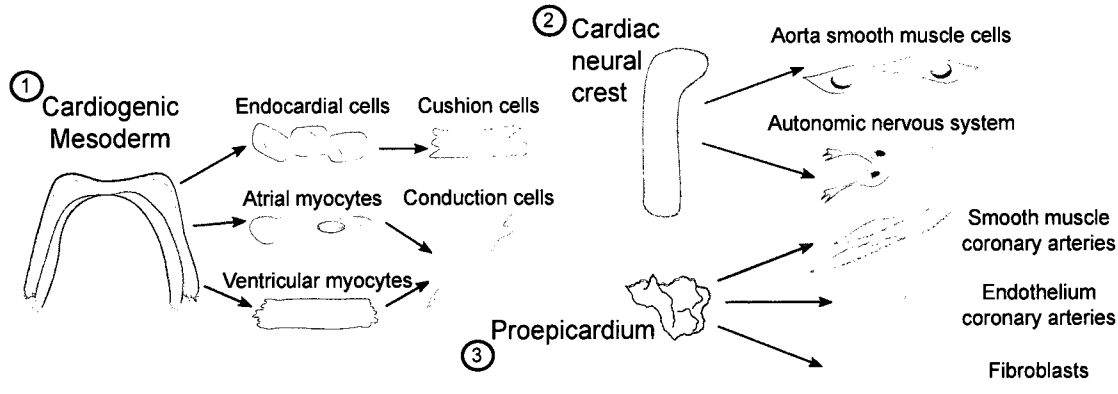


Figure 2. Positive and negative signals that shape the cardiac progenitor zone (A and B), and the origin and lineage relationship of cardiac cell types (C and D). (A) The cardiac crescent (CC), corresponding to the location of the heart progenitors, is shown here in an E7.5 mouse embryo (ventral view), and is highlighted by β-gal staining. The embryo was produced by crossing a cardiac-specific Nkx2-5 driven Cre recombinase mouse with a mouse that carries the Cre-dependent LacZ transgenic reporter. Part of the extra-embryonic region (yolk sac, YS) is also shown. (B) Positive and negative signals shaping the cardiac progenitor zone. A transverse section of an E7.5 mouse embryo illustrating the structure of the cardiac progenitor zone. Cardiac mesoderm is highlighted by β-gal staining. Positive and negative interactions are shown by arrows and bars, respectively. See text for details. BMP, bone morphogenetic protein; Fgf8, fibroblast growth factor 8. (C) Contribution of three populations of embryonic cardiac progenitor cells: cardiogenic mesoderm (red), cardiac neural crest (purple), and pro-epicardial organ (yellow) to different heart compartments during cardiac morphogenesis in the mouse. Progenitors of the cardiogenic mesoderm (left panel) are first recognizable under the head folds (HFs) of the embryo at E7.5, then migrate and fuse along the midline (ML) of the embryo to form the linear heat tube and ultimately the mature heart at E14.5. At E10.5, the cardiac neural crest cells (right panel) migrate towards and contribute to the formation of the outflow tract. At the same time, the proepicaridial organ precursors (right panel) contact the surface of the developing heart, giving rise to the epicardial mantle (yellow), and contributing to the coronary vasculature and epicardium. (D) Lineage diversification of the embryonic cardiac progenitor populations gives rise to many cardiac cell types. See text for details. Cr-Ca, Cranial-Caudal axis; L-R, Left-Right axis; A, atrium; V,

ventricle; OFT, outflow tract; AA, aortic arch; Ao, aorta; PT, pulmonary trunk; IVS, interventricular septum. (Figure 2A adapted from Stanley et al. 2002; Figure 2B adapted from Harvey 2002; Figure 2C and 2D adapted from Laugwitz et al. 2008).

Although mutations in many of the cardiac transcription factors such as Gata4/5/6 and Nkx2-5 cause severe cardiac defects, none of these factors are exclusively required for the specification of cardiogenic mesoderm. Cardiac specification relies on a more intricate interaction between these cardiac transcription factors and several families of secreted signaling molecules involved in cardiogenesis including: BMPs, FGFs, and the Wnt protein family (Cohen et al. 2008).

The Wnt protein family

One class of extracellular growth factors commonly associated with mediating these complex morphological changes during cardiogenesis is the Wnt protein family. The term 'Wnt' is derived from the names of two orthologous genes: the *Drosophila* cell-polarity gene *Wingless*, and the mouse proto-oncogene *Int-1*, now known as Wnt1 (Giles et al. 2003). The first Wnt gene, mouse Wnt1, was discovered in 1982 as being a proto-oncogene activated by the integration of the mouse mammary tumour virus (MMTV) into the Wnt1 promoter region in mammary gland tumours (Nusse and Varmus 1982). Wnts are highly-conserved secreted glycoproteins involved in a variety of biological processes including cell growth and proliferation, cell polarity and migration, apoptosis, and differentiation (Cadigan and Nusse 1997; Wodarz and Nusse 1998; Cohen et al. 2008).

Nineteen Wnt genes have been identified in mammals that share a 27% to 83% amino acid sequence identity (Miller 2002). Wnt proteins range in size from 39 kDa (Wnt7A) to 46 kDa (Wnt10A) and they all share characteristic features typical of secreted growth factors including: i) an N-terminal signal peptide targeting Wnts to the secretory pathway, ii) 23 to 24 cysteine residues, the spacing of which is highly conserved, and important for proper protein folding, iii) sites for post-translational modifications including N-linked glycosylation, a requisite for fully mature and active Wnt protein.

Canonical and non-canonical Wnt pathways

The Wnt proteins are typically classified in two broad categories based on their respective signaling properties: the Wnt/β-catenin (canonical) pathway and the β-catenin-independent (non-canonical) pathway (see Figure 3). β-catenin is a multi-functional protein that plays at least two roles. It interacts with cadherins to mediate cell adhesion (McCrea and Gumbiner 1991) and it is a central downstream component and mediator of the canonical Wnt pathway (Cadigan and Nusse 1997). The well-characterized canonical Wnt/β-catenin pathway regulates β-catenin stability (Figure 3A). In the absence of Wnts, β-catenin is targeted for ubiquitination and proteosomal degradation by a cytoplasmic β-catenin degradation complex consisting of the scaffolding molecules APC and Axin, and the serine/threonine kinase GSK3β. Canonical signaling is triggered by Wnts binding transmembrane Frizzled (Fzd) receptors, leading to de-stabilization of the cytosolic

degradation complex and, hence, stabilization of β-catenin. Although Fzd receptors seem to be sufficient for Wnt binding, studies in *D. Melanogaster* and mice suggest that the LRP5/6 Wnt co-receptors are essential for the full Wnt signaling activation (Tamai et al. 2000; Wehrli et al. 2000; Angers and Moon 2009); and see Figure 3A. Binding of Wnts to Fzds and to their co-receptors LRP5/6 (Low density lipoprotein receptor-related protein 5/6) induces the formation of a Wnt-Fzd-LRP5/6 complex which then recruits the cytoplasmic Disheveled protein to the plasma membrane. Subsequently, the Axin-GSK3β proteins are recruited and LRP5/6 becomes phosphorylated at the proline-rich motifs (Pro-Pro-Ser-Pro) present on its cytoplasmic tail by GSK3β. In turn, axin binds with high affinity to the phosphorylated form of LRP5/6. It is this Axin recruitment which inhibits formation of the β-catenin degradation complex, leading to β-catenin stabilization. Stabilized β-catenin then translocates into the nucleus to interact with the High Mobility Group (HMG)-box family member T-cell transcription factor (TCF) thereby regulating expression of target genes (Giles et al. 2003).

In contrast, the non-canonical Wnt pathways (Figure 3B) are generally β-catenin-independent and mediate their respective activity through activation of calcium-sensitive enzymes such as Protein Kinase C (PKC), calcium/calmodulin-dependent kinase (CaMK) and calcineurin (CaCN). This pathway also activates Rho family GTPases and jun N-terminal kinase (JNK) (Pandur et al. 2002b).

Figure 3

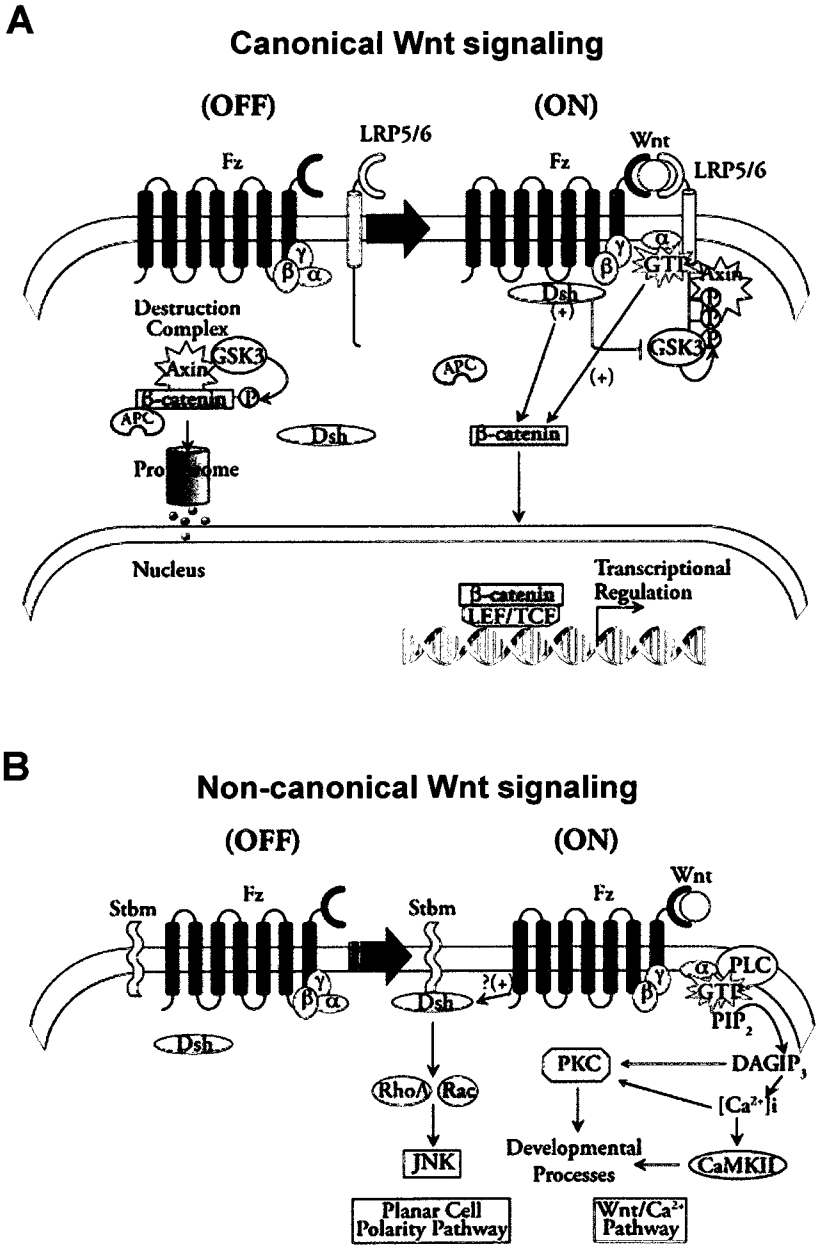

A
Canonical Wnt signaling
(OFF)
(ON)
LRP5/6
Fz
Wnt
Destruction Complex
GSK3
Axin
β-catenin
APC
Dsh
Nucleus
Transcriptional Regulation
LEF/TCF
B
Non-canonical Wnt signaling
Stbm
PLC
PIP2
DAGIP3
PKC
[Ca2+]i
RhoA
Rac
JNK
Developmental Processes
CaMKII
Planar Cell Polarity Pathway
Wnt/Ca2+ Pathway

Figure 3. Illustrative diagrams of the Canonical and Non-canonical Wnt signaling pathways. (A) Canonical Wnt signaling: (OFF) In the absence of Wnt ligand binding to the Frizzled (Fz) and LRP5/6 coreceptors, cytoplasmic Dsh remains inactive, and β-catenin is thereby targeted for ubiquitination and proteosomal degradation by the β-catenin degradation complex (consisting of APC, Axin, and GSK3β). (ON) Upon Wnt ligand binding to the Fz-LRP5/6 receptor complex, Dsh is activated resulting in sequestration (and inactivation) of GSK3β from the β-catenin degradation complex, thereby enabling stabilization and nuclear accumulation of β-catenin where it complexes with TCF/LEF transcription factor. (B) Non-canonical Wnt signaling; shown as two distinct pathways: the Wnt/Ca2+ pathway and Planar Cell Polarity (PCP) pathway. Wnt/Ca2+ signaling activates calcium-sensitive enzymes like PKC/CaMKII via activation of Fz receptors causing heterotrimeric G-protein-dependent Ca2+ release. PCP signaling is initiated by Rho/Rac-mediated activation of JNK through Dsh. LRP5/6, LDL receptor-related protein isoforms 5/6; Dsh, disheveled; APC, adenomatosis polyposis coli; GSK3β, glycogen synthase kinase 3β; TCF/LEF, T-cell factor/lymphoid enhancer factor. (Figure adapted from Flaherty et al. 2008).

Multiple roles of canonical Wnt signaling in cardiogenesis

Preparing for gastrulation. One of the earliest stages in tissue development of vertebrates is the process of gastrulation, which is marked by formation of the primitive streak and subsequent development of the embryonic germ layers, including the anterolateral mesoderm (cardiogenic) layer which is the primary source of cells that contributes to the developing heart (Tam et al. 2006; Abu-Issa and Kirby 2007; Tam and Loebel 2007). Interestingly, the canonical Wnt/β-catenin pathway was found to play a critical role in this very early stage of development. For example, β-catenin knockout (KO) mice are embryonic lethal due to defective primitive streak formation and, hence, lack of subsequent mesoderm formation (Huelsken et al. 2000; Morkel et al. 2003). In addition, mutant mice of other components of the canonical Wnt pathway (Wnt3 KO, and LRP5/6 double KO) displayed similar phenotypes (Liu et al. 1999; Kelly et al. 2004). These observations indicate that canonical Wnt signaling plays a significant role in initiating gastrulation and subsequent mesoderm formation, thus setting the stage prior to the onset of cardiac specification at the cardiac crescent stage of heart development (Figure 1).

Early cardiac specification. Early specification of cardiac tissue is regulated by a balance of activators and inhibitors of canonical Wnt/β-catenin signaling (Figure 2B). In chicken and *Xenopus* models, canonical Wnt activators (Wnt3A, Wnt8) were found to inhibit the expression of early cardiac genes, including Nkx2-5 and Gata4 (Marvin et al. 2001; Schneider and Mercola 2001). Furthermore, canonical Wnts (Wnt1, Wnt3A)

which are expressed in the neural plate and neural tube also inhibit cardiac specification in the non-cardiogenic posterior mesoderm. In contrast, secreted Wnt antagonists are expressed in the underlying endoderm, such as Crescent - which competes with Frizzleds for Wnt binding, and Dickkopf (Dkk1) - which inhibits canonical Wnt signaling by promoting endocytosis of LRP5/6 co-receptors (Schneider and Mercola 2001). Expression of Crescent and Dkk1 in the non-cardiogenic posterior mesoderm was found to induce cardiac gene expression accompanied by the presence of beating cardiomyocyte clusters (Marvin et al. 2001). These studies suggest that canonical Wnt signaling normally represses cardiac cell fate specification in the anterolateral/cardiogenic mesoderm.

More recent studies in mouse ESCs have shown that canonical Wnt signaling likely plays a biphasic role in cardiac specification. Expression of canonical Wnt ligands are transiently increased in differentiating ESCs prior to expression of cardiac genes such as Nkx2-5 and Gata4 (Naito et al. 2006; Ueno et al. 2007). Interestingly, blocking canonical Wnt signaling during the early stages of differentiation inhibits the expression of early cardiac genes and the appearance of beating cardiomyocyte clusters, whereas activating canonical Wnt signaling during later stages of ESC differentiation inhibited cardiac differentiation (Naito et al. 2006). Similar effects were observed in zebrafish embryos where stimulation of canonical Wnt signaling promoted cardiogenesis prior to gastrulation (i.e. early cardiac differentiation) and inhibited cardiogenesis after gastrulation, as tested using heat-shock inducible (hs)Wnt8 and hsDkk1 transgenic zebrafish embryos as activators and inhibitors of canonical Wnt signaling, respectively

(Ueno et al. 2007). In line with these observations, specific endodermal knockout of β-catenin leads to multiple ectopic heart formation in mice (Lickert et al. 2002), suggesting that the role of canonical Wnt signaling is partly due to an early cell autonomous effect emanating from the endoderm. Further evidence for the early role of canonical Wnt signaling during cardiac differentiation comes from studies of Wnt2A. Wnt2A has not only been shown to activate the canonical Wnt signaling pathway (Monkley et al. 1996), but also, Wnt2A-deficient ESCs displayed enhanced hematopoietic differentiation at the expense of both cardiac and endothelial differentiation (Wang et al. 2007). Collectively, these data show that canonical Wnt/β-catenin signaling plays a biphasic role in cardiac induction, positively influencing early expression of cardiac genes and then inhibiting the later stages of cardiogenesis.

Specification of secondary heart field progenitors. Canonical Wnt/β-catenin signaling also appears to regulate the specification and proliferation of Isl1+ cardiac progenitor cells which mark the secondary heart field (SHF) and its derivatives, including the right ventricle (RV), outflow tract (OFT) and atria (mentioned earlier). SHF-specific deletion of β-catenin using Isl1-cre mice causes a significant decrease in the levels of Isl1 expression and the numbers of cells that express Isl1 (Cohen et al. 2007; Lin et al. 2007a). Interestingly, β-catenin was found to directly bind to and regulate the Isl1 promoter (Lin et al. 2007a). Furthermore, myocardial-specific deletion of β-catenin in the heart using an Nkx2-5-cre mouse line causes a reduction in the overall numbers of Isl1+ cells without affecting the levels of Isl1 expression, and this is accompanied by a significant reduction in the ventricular size of the heart, particularly the RV (Kwon et al.

2007). This effect seems to be the result of a dramatic reduction in the proliferation of Isl1+ cells in the OFT, preventing the expansion of the Isl1 progenitors. This evidence suggests that canonical Wnt/β-catenin signaling regulates both the specification and proliferation of the Isl1+ cardiac progenitors of the SHF.

Role in cardiac neural crest cells. In addition to the direct effects observed in the myocardium, Wnt signaling has been implicated in the development of the cardiac neural crest cells (cNCC). The cNCCs delaminate from the neural tube and migrate along the aortic arches to help populate and eventually divide the OFT by contributing to the OFT cushion mesenchyme (Jiang et al. 2000). Two canonical Wnt genes (Wnt1, Wnt3A) are expressed in the dorsal region of the neural tube from which cNCCs are derived and have been associated with cNCC specification. Moreover, a neural crest-specific Wnt1-Cre lacZ reporter mouse strain has been generated to specifically monitor the effects on NCC migration (Jiang et al. 2000). Interestingly, mutations in a cytoplasmic adaptor molecule of the canonical Wnt pathway, Disheveled 2 (Dvl2), cause OFT defects that are similar to those caused by depletion of cNCCs, including double outlet right ventricle (DORV) and persistent truncus arteriosus (PTA) (Hamblet et al. 2002). In agreement with these observations, deletion of β-catenin from cNCCs results in OFT defects similar to those seen in Dvl2 mutant mice, in addition to a dramatic decrease in cNCC proliferation (Kioussi et al. 2002).

Role in the adult myocardium. There is very little evidence that active canonical Wnt/β-catenin signaling occurs in the adult myocardium. This is deduced from

studies that have used several common lacZ reporter mouse strains: TOPGAL, BATGAL, and TCF-lacZ which contain transgenic reporter cassettes expressing β-galactosidase (β-gal) placed downstream of TCF DNA-binding sites (DasGupta and Fuchs 1999; Maretto et al. 2003; Mohamed et al. 2004). Intriguingly, none of these reporters show high levels of canonical Wnt signaling activity in the myocardium, despite the expression of Wnt8A, a strong activator of the canonical Wnt pathway (DasGupta and Fuchs 1999; Maretto et al. 2003; Cohen et al. 2007; Lin et al. 2007a). As a result, studies have focused on Wnt-independent activation of β-catenin in the adult myocardium, specifically the role of β-catenin stability and GSK3β in postnatal myocardial growth (Hardt and Sadoshima 2002; Masuelli et al. 2003; Tseng et al. 2006). Stabilized β-catenin in isolated cardiomyocytes, or *in vivo*, results in increased cardiomyocyte growth with or without hypertrophic stimuli (Haq et al. 2003; Tseng et al. 2006). These studies implicate the PKB/Akt pathway where Akt phosphorylates and, in turn, inhibits GSK3β, thus rendering a more stabilized β-catenin. Furthermore, postnatal deletion of β-catenin was found to attenuate cardiac hypertrophy following pathological stress (Chen et al. 2006). These observations suggest that canonical Wnt/β-catenin signaling activity is required for normal and stress-induced cardiac hypertrophy.

Role in vasculature development. There is *in vivo* evidence that canonical Wnt/β-catenin signaling plays a role during endothelial cell development and formation of the vasculature. Studies have shown that endothelial cell (EC)-specific deletion of β-catenin results in impaired endocardial cushion formation, defective cardiac valve development, decreased canonical Wnt activity in the endocardial cushions, and defective

integrity of EC cell adhesion, ultimately leading to embryonic lethality (Cattelino et al. 2003; Liebner et al. 2004). Moreover, targeted disruption of the canonical Wnt2A gene results in defective formation of the placental vasculature (Monkley et al. 1996). Interestingly, loss of Frizzled 5 (Fzd5) also results in defective angiogenesis in the placenta leading to embryonic lethality in mice (Ishikawa et al. 2001). The similar vasculature phenotypes observed in the Wnt2A and Fzd5 mutant mice suggest that canonical Wnt signaling through the Wnt2A-Fzd5 ligand-receptor pair may play a role in regulating EC development.

Lastly, canonical Wnt signaling was found to regulate cell proliferation of vascular smooth muscle cells (VSMCs). Studies have shown that inhibiting canonical Wnt signaling results in decreased proliferation of VSMCs via decreasing cyclin D1 expression and increasing p21 (cyclin-dependent kinase inhibitor) expression (Quasnichka et al. 2006). Moreover, studies have shown that canonical Wnt signaling is activated following injury to the carotid artery (Wang et al. 2002). It is thus reasonable to suggest that active canonical Wnt signaling may be required to stimulate cell proliferation of VSMCs and, hence, promote regeneration of tissue after injury.

Roles of non-canonical Wnt signaling during cardiogenesis

Non-canonical Wnt11 features and functions. The prototypical non-canonical Wnt (Wnt11) was initially isolated from an embryonic day 14.5 whole mouse embryo cDNA (Adamson et al. 1994), and its human homologue has also been isolated, cloned and characterized (Lako et al. 1998; Kirikoshi et al. 2001). The Wnt11 gene was mapped to chromosome 7 and 11 in mice and humans, respectively. Wnt11 encodes a 354 amino acid polypeptide (39 kDa), wherein both human and mouse Wnt11 share a 97% amino acid identity. Wnt11 is expressed in many tissues including the heart, lungs, kidney, liver, pancreas, skeletal muscle, bones, adrenal cortex, and urogenital tract; however, it is not expressed in the neuroepithelium of the central nervous system.

Wnt11 has also been shown to play a role in a variety of biological processes. For example, in zebrafish, the silberblick (slb) gene encodes Wnt11 and has been implicated in the phenotype of the naturally occurring zebrafish slb-mutant. In the absence of Wnt11, abnormal convergent extension (CE) of axial tissues results in the classic cyclopia (single eye) phenotype observed in slb-mutant zebrafish, suggesting that Wnt11 is required for normal migration of cells during CE in zebrafish (Heisenberg et al. 2000; Ulrich et al. 2003; Ulrich et al. 2005). Additionally, Wnt11 was shown to be involved in mouse kidney development where the GDNF(glial-cell derived neurotrophic factor)/Ret kinase pathway targets Wnt11 signaling in a positive feedback loop to coordinate ureteric branching that is required for proper kidney development (Majumdar et al. 2003). Furthermore, Wnt11 was recently shown to act as a directional cue to properly organize

the elongation of early skeletal muscle fibers in mouse (Gros et al. 2009). Finally, numerous studies have pointed to an important role for Wnt11 in cancer. For instance, Wnt11 was found to promote the transformation of not only C57MG mammary gland epithelial cells but also of IEC6 intestinal epithelial cells (Christiansen et al. 1996; Ouko et al. 2004). Moreover, Wnt11 mRNA was found to be significantly up-regulated in a number of cancerous cell lines including: MKN45 (gastric cancer cell line), SKG-IIIa (cervical cancer cell line), and MCF-7 (breast cancer cell line) (Kirikoshi et al. 2001; Lin et al. 2007b). In addition to the many biological roles described above, Wnt11 has also been shown to be an integral player during cardiogenesis.

Wnt11 signaling required for cardiac specification. Studies have shown Wnt11 to be expressed in the cardiac crescent region (Christiansen et al. 1996; Eisenberg and Eisenberg 1999; Pandur et al. 2002a; Garriock et al. 2005; Cohen et al. 2008) suggesting that it likely plays an important role during the early stages of cardiac specification. The earliest lines of evidence associating Wnt11 with cardiogenesis came from studies showing that blocking Wnt11 signaling in the anterior mesoderm of *Xenopus* embryos blocks expression of the cardiac genes Nkx2-5, Gata4, and Tbx5, whereas over-expressing Wnt11 in the posterior (non-cardiogenic) mesoderm in *Xenopus* and chicken embryos was able to induce ectopic cardiac gene expression that was accompanied by the appearance of beating cardiomyocytes (Eisenberg and Eisenberg 1999; Schneider and Mercola 2001). In *Xenopus*, Wnt11 was found to enhance cardiogenesis in animal cap explants without inducing the expression of mesoderm markers, indicating that the effect of Wnt11 on cardiac specification was direct and not

fortuitously due to increased mesoderm induction (Pandur et al. 2002a). Interestingly, this study also demonstrates that the cardiogenic effect of Wnt11 was dependent upon JNK and PKC signaling. These data show that non-canonical Wnt11 promotes cardiac specification in the early embryo.

Numerous studies have also demonstrated that non-canonical Wnt11 promotes cardiac differentiation *in vitro* in a wide variety of adult cells. In fact, Wnt11-conditioned medium has been able to enhance cardiomyocyte differentiation of not only murine bone marrow-derived multipotent adult stem cells (mBM-MASCs) but also of unfractionated bone marrow mononuclear cells (BMMNCs) (Belema Bedada et al. 2005; Flaherty et al. 2008). Of important note, although Wnt11 could promote the expression of cardiac genes in these studies, the resulting cardiomyocytes showed characteristics of the fetal cardiac lineage, suggesting that other factors are likely to interact with Wnt11 in promoting complete cardiac differentiation. In agreement with these studies, Wnt11 was found to enhance cardiomyocyte differentiation of adult circulating endothelial progenitor cells (EPCs) only upon co-culture with neonatal rat ventricular cardiomyocytes (Koyanagi et al. 2005). Therefore, it is reasonable to ascertain that although Wnt11 alone can activate cardiac differentiation, additional signaling from other cardiogenic factors is likely required to produce a mature cardiomyocyte phenotype.

Non-canonical Wnt signaling coupled to inhibition of canonical Wnt/β-catenin signaling. One of the first lines of evidence to demonstrate that the non-canonical Wnt pathway can actively antagonize or suppress canonical Wnt/β-catenin

activity came from investigations into *Xenopus* body axis duplication. When over-expressed in *Xenopus* embryos, the canonical Wnts (e.g. Xwnt1, 3A, 8) were able to promote secondary axis formation (i.e. duplication of the embryonic axis) whereas non-canonical Wnts (e.g. Xwnt4, 5A, 11) were not able to induce this phenotype (Du et al. 1995). More importantly, co-expressing various non-canonical Wnt components along with canonical Wnts was able to inhibit the body axis duplication phenotype induced by the canonical Wnts (Torres et al. 1996), indicating that non-canonical Wnts can actively suppress signaling through the canonical Wnt pathway.

Recently, evidence has emerged suggesting that non-canonical Wnt signaling might suppress the canonical Wnt pathway through activation of a mitogen-activated protein kinase (MAPK)-related gene called Nemo-Like Kinase (NLK). NLK phosphorylates the TCF transcription factor, thereby inhibiting the DNA binding ability of the β-catenin/TCF complexes, thus suppressing the canonical Wnt pathway (Ishitani et al. 1999; Ishitani et al. 2003). Specifically, it has been shown that Wnt5A-Fzd2 interaction activates the non-canonical Wnt/Calcium pathway which, in turn, activates NLK to antagonize the canonical Wnt/β-catenin pathway (Ishitani et al. 2003). Most of the effects of non-canonical Wnt signaling have been ascribed to activation through Fzd receptors, however, recent evidence has raised the possibility that non-canonical Wnt signaling might be transduced by non-Fzd receptors, leading to suppression of the canonical Wnt/β-catenin pathway (reviewed in (Angers and Moon 2009)). Indeed, it has been demonstrated that non-canonical Wnt5A signaling is able to strongly suppress Wnt3A-induced activation of the canonical Wnt/β-catenin pathway through the Receptor

tyrosine kinase Orphan Receptor 2, ROR2; although the exact mechanism of action remains unknown (Mikels and Nusse 2006).

Finally, studies have shown that both phosphorylated and non-phosphorylated forms of β-catenin may be targeted for proteasomal degradation via the APC/Siah2 E3-ubiquitin ligase complex (Liu et al. 2001) and, as a consequence, suppresses the canonical Wnt/β-catenin pathway. Interestingly, non-canonical Wnt5A signaling was found to inhibit the canonical Wnt/β-catenin pathway during chondrocyte differentiation in embryonic mouse limb buds by promoting β-catenin degradation via APC/Siah2 (Topol et al. 2003).

Suppression of canonical Wnt/β-catenin signaling during cardiomyocyte differentiation. Although canonical Wnt/β-catenin signaling was reported to be essential for cardiomyocyte differentiation in P19 cells (Nakamura et al. 2003), the over-riding unanimity in the literature favours the idea that canonical Wnt/β-catenin signaling must be suppressed or antagonized for cardiac specification and differentiation to occur (Schneider and Mercola 2001; Eisenberg and Eisenberg 2006; Eisenberg and Eisenberg 2007; Ueno et al. 2007). Analyses of conditional β-catenin loss- and gain-of-function mice have indicated that canonical Wnt/β-catenin signaling is required to maintain and expand cardiac precursor cells, while suppression of this pathway must take place to allow cardiac cell differentiation (Grigoryan et al. 2008). Similarly, blockade of the canonical Wnt/β-catenin pathway in embryonic stem cells (ESCs) and pluripotent P19 cells appears to be a prerequisite for cardiomyocyte differentiation (David et al. 2008;

Zhu et al. 2008). Taken together, these studies suggest that cardiomyocyte differentiation may be driven by non-canonical Wnt signaling coupled to suppression of the canonical Wnt/β-catenin pathway. There is evidence suggesting that non-canonical Wnt signaling actively inhibits the canonical Wnt/β-catenin pathway by enhancing β-catenin degradation through an as yet undefined mechanism (Topol et al. 2003; Nemeth et al. 2007; Vijayaragavan et al. 2009). A plausible mechanism by which canonical Wnt/β-catenin signaling might be actively suppressed is through the concerted action of caspase proteases.

Caspase signaling

Interestingly, caspase proteases have been shown to target and cleave β-catenin, leading to a loss of canonical Wnt signal activity (Van de Craen et al. 1999; Steinhusen et al. 2000). Caspases (Cysteinyl aspartate proteases) play a central role in the initiation and execution of programmed cell death/apoptosis by proteolytically cleaving proteins vital for cell survival at specific tetrapeptide caspase recognition motifs (Fischer et al. 2003). Although the tetrapeptide motifs targeted by caspases are not absolute, caspases invariably cleave their substrates after the most downstream aspartic acid (Asp, D) residue. It is well accepted that caspases participate in two distinct pathways of cell death, termed the extrinsic and intrinsic apoptotic pathways (Budihardjo et al. 1999; MacFarlane and Williams 2004; Siegel 2006); see Figure 4A. The extrinsic pathway is activated by engagement of cell surface death receptors of the tumour necrosis factor

(TNF) receptor superfamily with their specific ligands. Death receptors (DR) include Fas/APO1/CD95, TNF-R1, and the TRAIL (TNF-Related Apoptosis Inducing Ligand) receptors TRAIL-R1/DR4, and TAIL-R2/DR5. The ligands for these death receptors are structurally related and belong to the TNF gene superfamily including: FasL/CD95L ligand binding to Fas, TNF binding to TNF-R1, and TRAIL binding to DR4 and DR5. Binding of ligand to death receptor induces formation of a trimerized receptor-ligand complex called the DISC (Death-Inducing Signaling Complex). FADD (Fas-Associating protein with Death Domain) is reported to be the universal cytoplasmic adaptor used by death receptors to recruit and activate the initiator caspase 8.

The intrinsic pathway is activated by stress-inducing stimuli such as DNA damage, toxic chemicals, and growth factor deprivation, resulting in perturbation of the mitochondria and subsequent release of proteins, such as cytochrome *c*, from the mitochondrial intermembrane space. The release of cytochrome *c* from the mitochondria is mediated by Bcl-2 family members, where anti-apoptotic (Bcl-2) and pro-apoptotic (tBid, Bax) members inhibit or promote the release of cytochrome *c*, respectively. Once released, cytochrome *c* binds to its cytosolic cofactors, Apaf-1 (Apoptotic Protease-Activating Factor-1) and procaspase 9, to form the Apoptosome complex, leading to activation of initiator caspase 9. The activated initiator caspases 8 and 9 then activate the effector caspases 3, 6 and 7, which are responsible for executing the cleavage of vital cellular substrates that triggers apoptosis.

Caspase activity is typically associated with apoptosis/programmed cell death, yet a growing body of evidence suggests that this protease family mediates cell fate decisions that are distinct from apoptosis/cell death (Fernando and Megeney 2007; Abdul-Ghani and Megeney 2008); see Figure 4B. For example, transient caspase 3 activation is required for the differentiation of a multitude of cell types including skeletal myoblasts, neural precursor cells, spermatids, hematopoetic and embryonic stem cells (Fernando et al. 2002; Arama et al. 2003; Fernando et al. 2005; Kanuka et al. 2005; Fujita et al. 2008; Janzen et al. 2008). It is proposed that precursor cells gauge the intensity and timing of caspase signals in mediating cell fate decisions, such that rapid and excessive caspase activation leads to cell death/apoptosis, whereas slow and transient caspase activation leads to cell differentiation.

Interplay of Caspase and Wnt signals

Interaction of caspase and Wnt signaling pathways has been studied in various cell processes and model systems. For example, apoptosis-induced cleavage of β-catenin, the central component of the Wnt signaling pathway, by caspases (3 and 8) has been observed in a variety of human and *Drosophila* epithelial cell lines (Steinhusen et al. 2000; Kessler and Muller 2009; Senthivinayagam et al. 2009). In addition, cerebral ischemia induced by occlusion of the carotid arteries (i.e. induced stroke) in rats resulted in degradation of β-catenin that was caspase 3 dependent, and interestingly this effect was reversible upon treating rats with caspase 3 specific inhibitors (Zhang et al. 2008).

Blockade of canonical Wnt signals (via small molecule inhibitors that specifically disrupt the β-catenin/TCF interaction), resulted in the activation of caspases 3 and 7 (via an as yet unknown mechanism) and were found to effectively induce apoptosis in certain carcinoma cell lines (Minke et al. 2009). By contrast, some components of the Wnt pathway are known to regulate caspase expression in a more direct manner. For instance, over-expression of APC (a component of the β-catenin degradation complex, also see Figure 3A) directly increases the expression and subsequent activation of caspases (3, 7, and 9), providing a mechanism to regulate the apoptotic activity in certain colon cancer cells (Chen et al. 2003).

Interestingly, caspase signals also regulate neural precursor (sensory organ precursor, SOP) cell differentiation in *Drosophila* (Kanuka et al. 2005). Activation of the *Drosophila* caspase Dronc (a homologue of mammalian Caspase 9, see Figure 4A) was found to target and activate the *Drosophila* Shaggy kinase (a homologue of mammalian GSK3β, see Figure 3A), and the activated kinase then antagonizes wingless (Wnt) signals allowing for the formation of SOP cells (Kanuka et al. 2005). These observations indicate that caspases may target and regulate activation of the Wnt pathway and, presumably, the resulting Wnt/caspase signal can lead to either cell death/apoptosis or to cell specialization/differentiation.

Figure 4

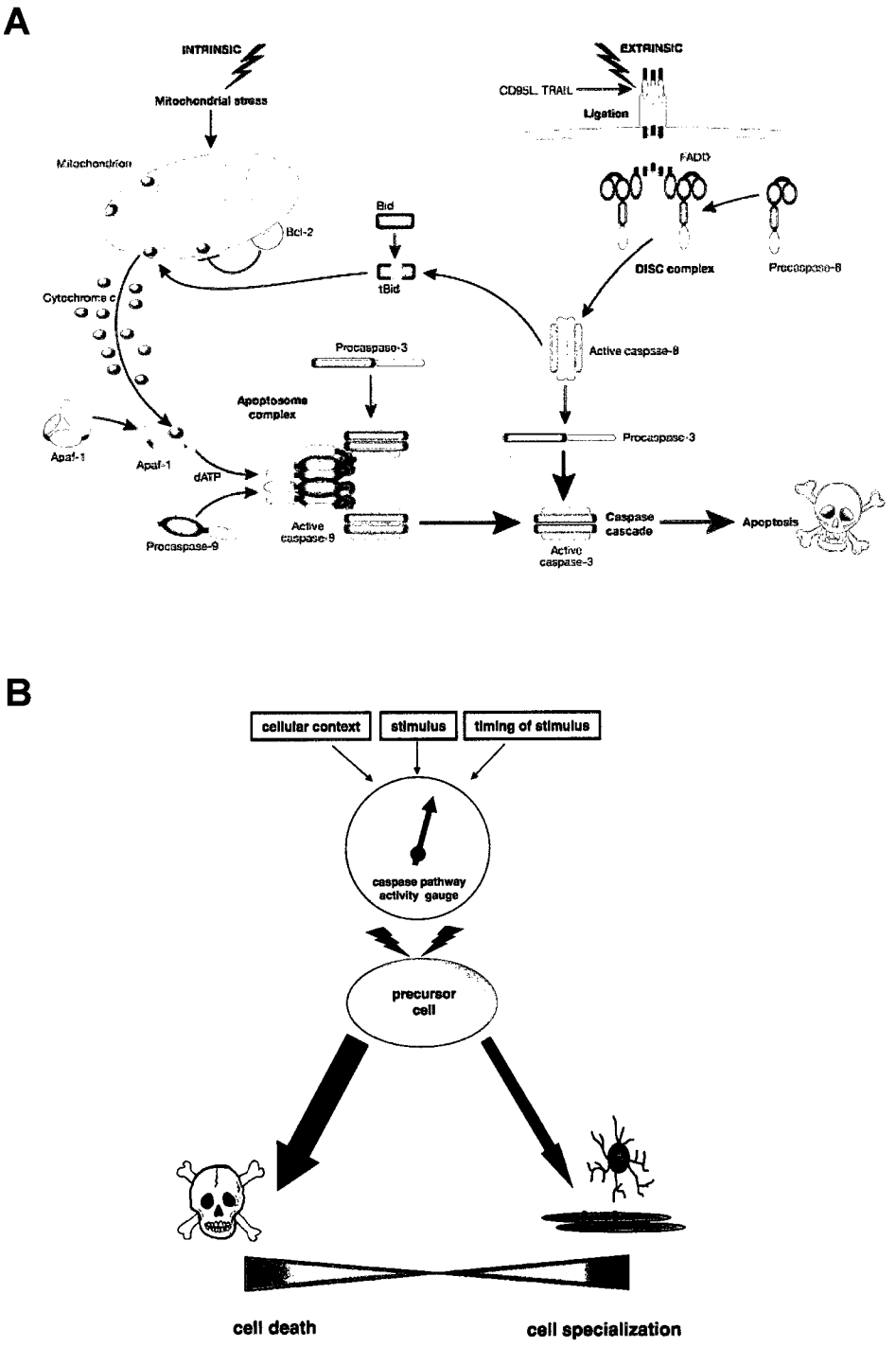

A
INTRINSIC
Mitochondrial stress
Mitochondrion
Bcl-2
Bid
tBid
Cytochrome c
Apaf-1
Apaf-1
dATP
Procaspase-9
Procaspase-3
Apoptosome complex
Active caspase-9
EXTRINSIC
CD95L, TRAIL
Ligation
FADD
DISC complex
Procaspase-8
Active caspase-8
Procaspase-3
Caspase cascade
Active caspase-3
Apoptosis
B
cellular context
stimulus
timing of stimulus
caspase pathway activity gauge
precursor cell
cell death
cell specialization

Figure 4. Caspase signaling mediates programmed cell death/apoptosis (A) and cell specialization/differentiation (B). (A) Illustrated are the extrinsic and intrinsic pathways to apoptotic caspase activation. The extrinsic pathway is activated via cell surface death receptors, where, triggering of death receptors (CD95/Fas, TRAIL-R1/R2) of the TNF receptor superfamily by ligands (CD95L/FasL, TRAIL) forms a trimerized receptor-ligand DISC complex which recruits the universal adaptor molecule FADD, resulting in rapid activation of the initiator caspase 8. The intrinsic pathway is activated by stimuli such as DNA damage and toxic chemicals, resulting in mitochondrial stress-induced release of cytochrome *c*. Release of cytochrome *c* is regulated by Bcl-2 family members, where anti-apoptotic (Bcl-2) and pro-apoptotic (tBid, Bax) members inhibit or promote cytochrome *c* release, respectively. Cytochrome *c* then binds to its cofactors (Apaf-1 and procaspase 9) to form the Apoptosome complex, leading to activation of initiator caspase 9. Activated initiator caspases 8 and 9 then activate effector caspases 3, 6 and 7, which then execute the cleavage of vital cellular substrates triggering apoptosis. TNF, Tumour Necrosis Factor; TRAIL, TNF-Related Apoptosis Inducing Ligand; DISC, Death-Inducing Signaling Complex; FADD, Fas-Associating protein with Death Domain; Bcl-2, B-cell lymphoma-2; BID, Bcl-2-homology domain 3(BH3)-domain-containing protein BID (BH3-interacting domain death agonist); tBID, truncated BID; Apaf-1, Apoptotic protease-activating factor-1. (B) Cell fate regulation of the caspase pathway. A model illustrating that a precursor cell integrates multiple caspase signals in a spatio-temporal manner to influence cell fate. During a specific stage of development (cellular context), a precursor cell gauges the intensity and timing of the caspase stimulus which, subsequently, influences whether the cellular response will be cell death or cell

specialization/differentiation. Rapid and excessive caspase activation (wide arrow) leads to cell death, whereas slow and transient caspase activation (narrow arrow) is a hallmark of the cell specialization/differentiation process. (Figure 4A adapted from MacFarlane et al. 2004; Figure 4B adapted from Fernando et al. 2007).

Summary of findings

Here, we sought to define the respective roles of canonical and non-canonical Wnt signaling during mammalian cardiogenesis, specifically cardiomyocyte differentiation and maturation. The intracellular components of both canonical and non-canonical Wnt pathways were present throughout murine cardiac development, yet canonical Wnt signaling had a restricted region of activity in the mouse heart, primarily in the non-myocardial regions including the outflow tract and semi-lunar valves. Conversely, elevated non-canonical Wnt signaling was associated with expansion of the myocardium and *de novo* induction of cardiomyocyte differentiation. The pro-myogenic effect of Wnt11 resulted in a direct inhibition of canonical Wnt/β-catenin activity in Wnt11-treated progenitor cells suggesting that non-canonical signaling enhanced cardiogenesis through suppression of canonical Wnt pathways. Wnt11 stimulation suppressed canonical Wnt signaling by destabilizing full length β-catenin, an effect that could be reversed by inhibiting caspase 3/8 activity. Stimulation of progenitor cells with Wnt11 promoted the activation of caspase 3/8 and inhibition of either protease resulted in inhibition of cardiomyocyte differentiation. In summary, our results suggest that non-canonical Wnt11 signaling promotes cardiogenesis, in part, by caspase-mediated suppression of canonical Wnt/β-catenin activity.

OBJECTIVES and HYPOTHESIS

The objectives of this study are to: 1) Undertake an expression analysis of components of the Wnt signaling pathway during embryonic and post-natal heart development, 2) Assess the extent of canonical versus non-canonical Wnt signaling in the heart using the TCF-lacZ (+/+) reporter mouse strain, and 3) Investigate the mechanisms of non-canonical Wnt11 during cardiac differentiation. A combinatorial approach of molecular cloning, *in vitro* cell culture techniques, as well as the use of *in vivo* and transgenic approaches was used to investigate Wnt signaling pathways in the heart and the role of Wnt11 during cardiac differentiation. We hypothesize that non-canonical Wnt11 signaling promotes cardiac differentiation, in part, through caspase-mediated suppression of the canonical Wnt/β-catenin pathway.

Chapter 2 - MATERIALS and METHODS

Media, cell culture, differentiation

P19 cells were maintained in Minimum Essential Medium alpha, MEMα, growth media (Invitrogen) supplemented with 10% FBS and 50 μg/ml gentamicin (Sigma). Undifferentiated cells were induced to undergo cardiac differentiation with 0.8% DMSO and aggregation. Briefly, cells were plated onto non-adherent bacterial petri dishes in the presence of growth media containing 0.8% DMSO and allowed to aggregate for 4 days, during which time the media was replenished every 24 hours. Aggregates were then transferred onto adherent tissue culture dishes in the absence of DMSO to allow for subsequent cardiac differentiation. Media was replaced every 48 hours for the duration of the differentiation timecourse. Wnt11 or control conditioned media was generated using Ad-293 cells. Briefly, a Wnt11-mycHis adenovirus (AdV) generated in our lab, or a control AdV, was used to infect Ad-293 cells maintained in MEMα growth media. After 2 days, conditioned media was harvested and spun at 200 × g for 10 min. to remove cell debris, then filtered at 0.1 μm (Nalgene) and aliquots stored at -80°C.

Cell lines, immunoblot analyses

Plasmids encoding Wnt11 shRNA targeting the 3' end (shRNA#1; clone V2LMM-53302) and middle (shRNA#2; clone V2LMM-54666) of the mouse Wnt11 transcript, and control shRNA (clone RHS4346) were purchased from Open Biosystems. shRNA knockdown cell lines were generated by transfecting 1×10^6 P19 cells with 5 μg

of respective DNA plasmid using FuGENE 6 (Roche) for 4 hours. The following day, stable cells were selected in media containing 2 μg/ml puromycin (Sigma) over a 2 week period. Cell lysates were obtained at specific timepoints throughout the P19 differentiation timecourse. Briefly, cells were washed in PBS and harvested by centrifugation at 1,500 × g for 7 min. Cells were resuspended in lysis buffer supplemented with protease inhibitors (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Tx-100, 1 mM EGTA, 1.5 mM $MgCl_2$, 20 mM NaF, 10 mM sodium pyrophosphate, 0.3 mM PMSF, 2.5 mM sodium orthovanadate) and incubated at 4°C for 1 hour. Crude lysate was centrifuged at 17,000 × g for 10 min. Equal amounts (20 μg) of protein were separated by SDS-PAGE and transferred to nitrocellulose. After blocking with TBST-milk (10 mM Tris pH 7.4, 150 mM NaCl, 0.05% Tween-20, 5% non-fat powdered milk), membranes were incubated with primary antibody overnight at 4°C followed by incubation with Horseradish Peroxidase (HRP)-conjugated secondary antibody for 1 hour. Protein expression was detected using the ECL detection kit (GE Healthcare). Primary antibodies used: mouse anti-myc (Invitrogen), mouse anti-cardiac Troponin T (clone 1C11; Abcam), goat anti-frizzled 4 (clone C-18; Santa Cruz), mouse anti-myosin heavy chain (clone MF20; Developmental Studies Hybridoma Bank-DSHB), mouse anti-β-tubulin (clone E7; DSHB), mouse anti-β-catenin stabilized (clone 7A7; Alexis Biochemicals), rabbit anti-β-catenin (N-terminus; Abcam), rabbit anti-(pan)phospho-PKC-βII Ser660 (Cell Signaling). Secondary antibodies used: HRP-conjugated goat anti-mouse/rabbit (BioRad) or donkey anti-goat (Santa Cruz).

RT-PCR expression analysis

Whole mouse hearts (ventricles and atria) were isolated from wild type CD-1 mice (Charles River Laboratories) at specified timepoints throughout embryonic and post-natal cardiac development. Total RNA was extracted using TRIzol and first strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Upon isolating total RNA, the TURBO DNA-*free*™ DNase digestion kit (Ambion) was used to remove any traces of contaminating genomic DNA from RNA preparations according to manufacturer's instructions. First strand cDNA was synthesized from 1 μg total RNA using SuperScript II (Invitrogen), and complementary RNA was then digested with RNase H (Roche). Twenty-five ng of cDNA product was used for subsequent PCR analysis using Wnt pathway-specific primers (see Supplementary Data (SD) Table 1 for details). cDNA obtained from whole embryos (E9.5/E14.5) and adult tissues (8 week brain/testes) were used as positive control samples when required. Qualitative RT-PCR expression analysis results are representative of two sets of hearts independently isolated at each timepoint analysed (SD Table 1 and SD Figure 1A). Quantitative PCR (qPCR) was performed on the Wnt11 gene (SD Figure 1B) using the RT^2 Real-Time SYBR Green PCR Master Mix reagent (SuperArray Bioscience) and samples were run on the Rotor-Gene 6000 real-time instrument detection system (Corbett Life Science).

Detection of TCF-lacZ activity

TCF-lacZ (+/+) reporter mice were provided by D. Dufort (McGill University, Montreal). Whole hearts or embryos were dissected and washed in PBS (pH 7.4) followed by tissue fixation in 4% paraformaldehyde (PFA) at 4°C for 3 hours. After fixation, tissues were prepared for either whole mount or frozen section β-gal staining. For whole mount β-gal staining: tissues were washed 3 times (10 min. each) in β-gal wash buffer (0.1 M sodium phosphate pH 7.4, 2 mM $MgCl_2$, 0.01% Na-deoxycholate, 0.02% NP-40) followed by overnight incubation at 37°C with β-gal staining buffer (0.1% X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.1 M sodium phosphate pH 7.4, 2 mM $MgCl_2$, 0.01% Na-deoxycholate, 0.02% NP-40). Next day, tissues were rinsed 3 times (5 min. each) in PBS followed by overnight post-fixation at 4°C in 2% PFA/0.1% glutaraldehyde dissolved in PBS. Whole mounts were preserved for long term storage in 70% ethanol. For frozen sections: tissues were washed 3 times (10 min. each) in PBS followed by overnight incubation at 4°C with cryoprotection equilibration buffer (25% sucrose in PBS). Next day, tissues were embedded in Shandon Cryomatrix (Thermo Scientific) and frozen with liquid nitrogen-isopentane. Frozen sections (10 μm) were prepared onto microscope slides and equilibrated to room temperature. Tissue sections were fixed in 0.2% glutaraldehyde dissolved in PBS (10 min.) followed by 3 washes in PBS (5 min. each), and tissues were stained overnight at 37°C in β-gal staining buffer (above). Next day, tissue sections were rinsed in PBS and counterstained with 1% alcoholic Eosin Y (Sigma).

SuperTOPflash luciferase assay

P19 cells were seeded onto 6 well plates (2.5×10^5 cells per well) and, the next day, cells were transfected using Lipofectamine 2000 and serum-free Opti-MEM for 4 hours. Transfections contained: 40 ng of SuperTOPflash or FOPflash plasmids, 4 ng of pRP2044 β-gal plasmid used as an internal transfection control, plus 400 ng of effector plasmid containing the gene of interest. Non-canonical Wnt signaling was activated using a pcDNA3.1/Wnt11-mycHis plasmid whereas β-catenin and Wnt3A plasmids were used to activate the canonical Wnt signaling pathway. Empty pcDNA3.1/mycHis vector was added to all transfections to normalize samples for total plasmid DNA content. Next day, luciferase activity (canonical Wnt activation) was measured using the Luciferase Assay System according to manufacturer's instructions (Promega). Luciferase values were normalized for transfection efficiency using β-gal and for total protein amount used per sample. To examine effects of caspase inhibition, P19 cells were transfected with β-catenin and/or Wnt11 plasmids onto 6 well plates (as above) and were incubated overnight with 10 μM caspase 3 or 8 inhibitors (DEVD-fmk or IETD-fmk) prior to SuperTOPflash luciferase measurements.

Caspase activity assay, caspase inhibition

Caspase activity measured by incubating cell lysates with caspase-specific fluorogenic substrates: DEVD-amc and IETD-amc for caspase 3 and 8, respectively

(BIOMOL International). Briefly, cells were lysed in caspase lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EGTA) and incubated at 4°C for 1 hour. Cell debris was pre-cleared by centrifugation at 17,000 × g for 10 min. Fifty μg of total protein was incubated with 0.1 mM fluorogenic caspase substrate in caspase activity buffer (25 mM HEPES-NaOH pH 7.5, 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 10 mM DTT) for 2 hours at room temperature. Resulting fluorescence intensity (caspase activity) was measured at 460 nm. To test for caspase activation by Wnt11 and Fzd4, 5 μg of Wnt11 and/or Fzd4 plasmids were transfected into P19 cells (2.0×10^6 cells per 10 cm dish) prior to measuring caspase 3 and 8 activity the following day. Control P19 cells were transfected with empty plasmid. For the apoptosis-induced positive control, P19 cells were incubated with 1 μM staurosporine (STS) in growth media for 5 hours prior to measuring caspase activation. To examine the effects of caspase inhibition, cells or tissues were incubated overnight with 10 μM caspase 3 or 8 specific irreversible peptide inhibitors: DEVD-fmk and IETD-fmk, respectively (BioVision Inc.) or with DMSO as control prior to subsequent analyses. To assess the levels of stabilized β-catenin upon exposure to Wnt11 and caspase inhibition, P19 cells transfected with 1 μg of the Wnt11 plasmid (onto 6 well plates seeded with 2.5×10^5 cells per well) were incubated overnight with 10 μM caspase 3 or 8 inhibitors (DEVD-fmk or IETD-fmk) or with DMSO (control) prior to immunoblot with anti-stabilized β-catenin. To measure the effects of inhibiting caspase activity *in vivo*, intact E12.5 embryos or hearts of TCF-lacZ (+/+) mice were incubated overnight at 37°C in DMEM growth media containing 10 μM caspase 3 or 8 inhibitors, or containing DMSO (control), prior to β-gal staining. Heart sections were counterstained with 1% alcoholic

Eosin Y (Sigma). To examine the effects of caspase inhibition during P19 differentiation, cells were induced to differentiate in the presence of DMSO (control) or 10 μM caspase 3 or 8 inhbitors (DEVD-fmk or IETD-fmk) prior to cell staining with MF20 Ab (at days 0, 5, 7) or prior to immunoblot with anti-cardiac Troponin T (at day 7). Caspase-derived fragments or mutations of β-catenin were amplified from a mouse β-catenin cDNA (Accession# NM_007614) and sub-cloned into the EcoRV site of the pcDNA3.1/mycHis(-)B plasmid (Invitrogen). The mycHis-tagged β-catenin mutants were over-expressed in P19 cells and subsequent analysis by SuperTOPflash assay, or by immunoblot (at day 7 of P19 differentiation), was conducted.

TUNEL assay

Percentage of apoptotic cell death/apoptosis during P19 cardiac differentiation was measured by TUNEL+ cell staining using the ApopTag®-Red apoptosis detection kit according to manufacturer's instructions (Chemicon). Briefly, cells were washed twice in PBS (5 min. each) followed by fixation in 1% paraformaldehyde in PBS at room temperature. After 15 min., cells were washed twice in PBS (5 min. each) followed by incubation in equilibration buffer (proprietary buffer containing potassium cacodylate) for 3 min. Buffer was removed and cells were incubated with working strength Terminal deoxynucleotidyl Transferase (TdT) enzyme reaction buffer at 37°C for 1 hour. TdT enzyme reaction buffer contained digoxigenin (DIG)-labeled nucleotides and unlabeled nucleotides in an optimized ratio, equilibration buffer, and the TdT enzyme to catalyze

addition of nucleotides to 3'-OH ends of double or single-stranded DNA. Reaction was stopped in stop/wash buffer for 10 min. at room temperature. Cells were then incubated with anti-DIG-Rhodamine (affinity purified sheep polyclonal antibody) at 37°C for 45 min. Finally, cells were washed three times in PBS (5 min. each), counterstained with DAPI (Sigma), and mounted onto microscope slides for subsequent analysis of apoptotic (TUNEL+) cells.

Nickel bead pulldown, PNGase F treatment, Fzd4 inhibition

Wnt11-mycHis conditioned media (described above) was incubated overnight at 4°C with Nickel-NTA agarose beads (Qiagen) in binding buffer (50 mM Na-phosphate, 150 mM NaCl, 10 mM imidazole; pH 8.0). Next day, beads were washed (50 mM Na-phosphate, 300 mM NaCl, 20 mM imidazole) followed by elution of mycHis-tagged Wnt11 (50 mM Na-phosphate, 300 mM NaCl, 250 mM imidazole). Eluate was deglycosylated with 1000 units of Peptidyl N-glycosidase F (PNGase F, New England BioLabs) at 37°C for 1 hour and subject to immunoblot with anti-myc. For the Wnt11-Fzd4 binding assay, 2.0×10^6 P19 cells were seeded onto 10 cm dishes and transfected with 5 μg of pcDNA3.1/Wnt11-mycHis plasmid. P19 cell lysates over-expressing Wnt11-mycHis were subject to nickel bead pulldown followed by immunoblot with endogenous anti-Fzd4 antibody. One μg/ml of Fzd4 Ab was added to P19 growth media (2 hours) to inhibit the Fzd4 receptor in cells transfected with Wnt11-mycHis prior to immunoblot with the non-canonical Wnt signaling marker anti-(pan)phospho PKC.

Fzd4-Ab blocking peptide (3 μg/ml, Santa Cruz) was used to neutralize the Fzd4-Ab. P19 growth media supplemented with 1 μg/ml Fzd4-Ab or Normal Goat Serum (control) was replenished every 24 hours during the 7 day differentiation timecourse prior to immunoblot with anti-cardiac Troponin T.

Immunocytochemistry and Histology

Fluorescent cell staining of P19 cells subject to various conditions during the cardiac differentiation timecourse was conducted on 25 mm glass coverslips. Briefly, cells were washed in PBS followed by fixation in 4% PFA for 30 min. Cells were permeabilized in blocking buffer (1% BSA, 2% normal goat serum, 0.4% Tx-100 in PBS) for 15 min. To detect cardiac Troponin T (cTnT), cells were incubated for 2 hours at room temperature with mouse anti-cTnT (Abcam) and then with Rhodamine-labeled donkey anti-mouse secondary antibody (Chemicon) for 1 hour. To detect myosin heavy chain, MF20 hybridoma antibody was used (DSHB) followed by the FITC-labeled donkey anti-mouse secondary antibody (Chemicon). Nuclei were counterstained with 4',6-diamidino-2-phenylindole, DAPI (Sigma) and cells were mounted onto microscope slides with DakoCytomation fluorescent mounting media (DakoCytomation Inc.). Digital fluorescent images were obtained using the Axioplan 2 Imaging System (Carl Zeiss MicroImaging Inc.). For histology of wild type and transgenic mouse tissues, hearts were paraffin embedded and sectioned (10 μm) followed by Hematoxylin and

Eosin (H+E) staining performed by the Department of Pathology and Laboratory Medicine (University of Ottawa, Ottawa).

Immunoprecipitation-Silver (IP-Ag) stain

E15.5 whole mouse heart tissue was isolated from TCF-lacZ(+/+) mice (CD-1 background) and immediately frozen in liquid nitrogen. Frozen tissue was homogenized by mortar and pestle and by dounce homogenization, followed by tissue lysis in SDS-containing lysis buffer (0.2% SDS, 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Tx-100, 1 mM EGTA, 1.5 mM $MgCl_2$, 20 mM NaF, 10 mM sodium pyrophosphate, 0.3 mM PMSF, 2.5 mM sodium orthovanadate) at 4°C for 1 hour. Crude lysate was centrifuged at 17,000 × g for 10 min. to remove cell debris. Five hundred μg of total protein was incubated overnight at 4°C with 2 μg of N-terminal directed β-catenin antibody (Abcam) for IP or with 2 μg of Normal Rabbit Serum (Jackson Immunoresearch Labs) for mock IP. Next day, IP samples were incubated with Protein G Sepharose beads (GE Healthcare) for 1 hour to pulldown β-catenin immunocomplexes, followed by washing with lysis buffer.

Samples were separated on a 10% polyacrylamide gel by SDS-PAGE, and the gel was subject to standard Ag-stain procedure according to manufacturer's instructions (GE Healthcare). Briefly, gel was placed in fixative solution (50% ethanol, 5% acetic acid in water) overnight at room temperature. Gel was washed once in 50% ethanol (10 min.)

followed by two washes (10 min. each) in water. Gel was then placed in sensitizer solution (0.02% sodium thiosulphate in water) for 5 min. followed by two washes (5 min. each) in water. Gel was exposed to silver nitrate staining solution (0.1% in water) for 45 min. followed by a brief rinse in water for 1 min. Gel was then placed in developer solution (0.04% formalin in 2% sodium carbonate in water) for 5 min. and the reaction was stopped by adding stop solution (5% acetic acid in water). Finally, the gel was placed in 1% acetic acid for long term storage and subsequent extraction of specific gel bands. Protein identification of the resulting Ag-stained gel bands were analysed by LC-MS/MS mass spectrometry at the Ottawa Hospital Research Institute Proteomics Facility (Ottawa, Canada).

Transgenic Wnt11-mycHis mouse production, and generation of cardiac-specific reporter construct

All studies involving mice were approved by the University of Ottawa Animal Care Committee. Wnt11 was amplified by PCR using cDNA obtained from an adult (8 week) mouse heart and the following primers: Wnt11-5'Xho1 (5'-GAG CTC GAG ATG AGG GCG CGG CCG CAG-3') and Wnt11-3'HindIII (5'-GGA AGC TTAG CTT GCA GAC GTA GCG CTC-3'). Wnt11 was cloned into the pcDNA3.1/mycHis(-)B vector (Invitrogen) in-frame with the mycHis tag. Wnt11-mycHis fragment was then PCR amplified for subsequent sub-cloning into the cardiac-specific α-myosin heavy chain (α-MHC) expression cassette plasmid provided by J. Gulick (University of Cincinnati,

Cincinnati, (Gulick et al. 1991)); also see Figure 7A. The vector backbone was excised from the plasmid using SacII enzyme and the resulting linearized Wnt11-mycHis expression cassette (~ 7.3 kb) was used to generate the transgenic mouse strain using a standard pronuclear microinjection method (Palmiter et al. 1982). Briefly, 10 pl of 2 ng/μl linearized transgenic expression cassette was microinjected into the male pronucleus of one-cell stage fertilized embryos derived from B6C3F1 mice (Charles River Laboratories). After overnight incubation, those embryos that had survived and cleaved to the two-cell stage were transferred into the oviducts of CD1 pseudopregnant foster mothers that were plugged on the day of transfer. Transgenic mice were identified by PCR genotyping. Briefly, 0.5 cm tails were digested overnight at 55°C in 350 μg/ml Proteinase K (Sigma) dissolved in genomic DNA buffer (10 mM Tris pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS). The next day, genomic DNA was extracted with phenol/chloroform/isoamyl alcohol and resuspended in 100 μl water. One μl of DNA was used as a template for each PCR reaction using the following primers which amplified a region spanning part of the Wnt11-mycHis sequence as well as the human growth hormone polyA (hGH polyA) sequence: Wnt11-forward (5'-GTG TAT CTA CAG AGC TCC-3') and hGH polyA-reverse (5'-CAA GGC TGG TGG GCA CTG GAG TGG C-3'). Thirty cycles of PCR were performed using the following conditions: 95°C for 30 sec, 55°C for 50 sec, and 72°C for 50 sec.

One mouse line was generated from a total of 4 pronuclear microinjections using the above mentioned cardiac-specific Wnt11 expression cassette. The analysis and comparison of multiple Wnt11 transgenic lines was hindered by the inability to generate

a stable mouse line. Variables that could cause this situation to occur include: i) the inability of the Wnt11 expression cassette to stably integrate into the mouse genome, ii) stable over-expression of Wnt11 in the heart could cause a detrimental phenotype in the majority of mice, where only those mice that compensate for this go on to survive. Evidence in support of this latter possibility comes from investigations into Wnt11 knockout mice, which display an embryonic lethal phenotype (Zhou et al. 2007), suggesting that disrupting the balance of Wnt11 expression by knockdown, or over-expression in our case, could result in lethality.

Materials and reagents acquired

To generate adenovirus, Wnt11-mycHis was sub-cloned into the pShuttle-IRES-hrGFP2 plasmid purchased from Stratagene. Shuttle vectors were recombined with pAdEasy-1 according to the manufacturer's instructions using the AdEasy XL Adenoviral Vector System (Stratagene). The Super8XTOPflash firefly luciferase reporter plasmid (driven by 8 tandem copies of the TCF binding site and the herpes simplex virus minimal thymidine kinase promoter) and the Super8XFOPflash plasmid (negative control containing mutations in the corresponding TCF binding sites) were provided by R. Moon (University of Washington, Seattle; (Kaykas et al. 2004)). The pRP2044 β-galactosidase plasmid (driven by the human elongation factor 1α promoter) was provided by R. Parks (Ottawa Hospital Research Institute, Ottawa). We also thank S. Millar for providing us with the Frizzled PCR primer sets (University of Pennsylvania,

Philadelphia; (Reddy et al. 2004)) and S. Heller for providing us with Wnt9B, Wnt16 and Wif1 primer sets (Hagedorn Research Institute, Denmark; (Heller et al. 2002)).

Statistical analysis

Results, expressed as mean ± SD, were compared by ANOVA followed by student's t-test, with $P<0.05$ considered significant.

Chapter 3 - RESULTS

We initially analysed the cardiogenic effect of the non-canonical Wnt family member Wnt11 using the P19 murine embryonic carcinoma cell line (Figure 5). The pluripotent P19 cell line has been used extensively as a model system to dissect the regulatory mechanisms involved in commitment and differentiation of the cardiomyocyte lineage. DMSO treatment leads to a robust induction of cardiomyocytes, an event that recapitulates all the major hallmarks of cardiogenesis *in vivo* while providing an accessible system for studying biochemical and genetic control mechanisms (McBurney 1993; Skerjanc 1999; van der Heyden and Defize 2003). Firstly, media over-expressing mycHis-tagged Wnt11 protein was generated and use of N-peptidyl glycosidase F (PNGase F) confirmed the presence of functionally mature glycosylated Wnt11 protein (Figure 6A). Subsequently, the engineered Wnt11 was found to promote cardiac differentiation in the absence of DMSO (Figure 6B), demonstrating that Wnt11 acts as a potent pro-cardiac differentiation factor. Next, we assessed the effects of Wnt11 on P19 cell cardiac differentiation using shRNA-mediated knockdown. There was significant knockdown of Wnt11 expression in both shRNA cell lines generated and the expression of Tgfβ2, a known downstream target of Wnt11 (Zhou et al. 2007), was also significantly decreased (Figure 6C) indicating inhibition of Wnt11 signaling. A differentiation timecourse showed that expression of the cardiac-specific marker Troponin T (cTnT) was significantly decreased in both Wnt11 shRNA knockdown cell lines when compared to control DMSO-induced P19 cells (Figure 6D) supporting the hypothesis that Wnt11 plays a role in mediating cardiac differentiation.

Subsequently, we sought to determine the Frizzled (Fzd) receptor utilized by Wnt11 during cardiomyocyte differentiation. Interestingly, Fzd4 expression is significantly increased during DMSO-induced cardiac differentiation in P19 cells (Peng et al. 2002). To test the role of Fzd4, P19 cells were incubated with growth media containing Fzd4 antibody to inhibit signaling through the receptor. Fzd4 inhibition blocked non-canonical Wnt11 PKC activation and this effect was reversed by the addition of Fzd4 blocking peptide (Figure 6E). Furthermore, inhibiting Fzd4 also decreased P19 cardiac differentiation as noted by a significant decrease in levels of the cardiac-specific differentiation marker Troponin T (Figure 6F). Finally, we noted that a mycHis-tagged Wnt11 co-immunoprecipitated with endogenous Fzd4 (Figure 6G), indicating that Wnt11 may be interacting directly with Fzd4 to mediate cardiac differentiation.

Next, we analysed the *in vivo* effects of over-expressing Wnt11 using a cardiac-specific (α-MHC) murine transgenic model (Figure 7A, see Materials and Methods for details). Transgenic expression of Wnt11-mycHis resulted in the development of an overt cardiac hypertrophy with a significant increase in the heart to body mass ratio in comparison to non-transgenic litter mates (Figure 7B). Moreover, we noted that Wnt11 transgenic animals displayed a hypertrophy phenotype that appeared to be free of many pathologic markers typical of adult onset hypertrophy such as fibrosis and calcium deposition (Figure 7C and D). Consistent with a role as a modulator of myocardial development rather than hypertrophy per se, we also noted that the ventricle region of Wnt11 transgenic hearts contained an increased number of cardiomyocytes compared to

Figure 5

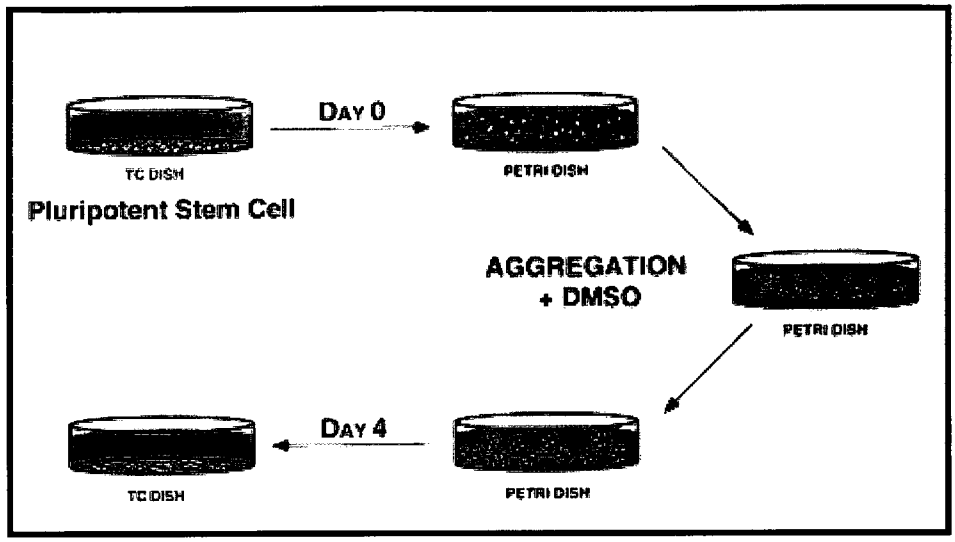
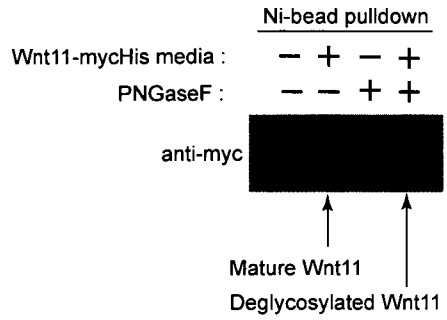

DAY 0
TC DISH
PETRI DISH
Pluripotent Stem Cell
AGGREGATION
+ DMSO
PETRI DISH
DAY 4
TC DISH
PETRI DISH

Figure 5. Procedure for cardiac differentiation of pluripotent P19 cells. Undifferentiated cells induced to undergo cardiac differentiation with 0.8% DMSO and aggregation (shaded area). At day 0, P19 cells are plated onto non-adherent bacterial petri dishes in the presence of MEMα growth media containing 0.8% DMSO and allowed to aggregate for 4 days. Aggregates are then transferred onto adherent tissue culture dishes in the absence of DMSO to allow for subsequent cardiac differentiation. Cardiac muscle appears by day 5 (Figure adapted from Skerjanc et al. 1999).

Figure 6A, 6B

A



B

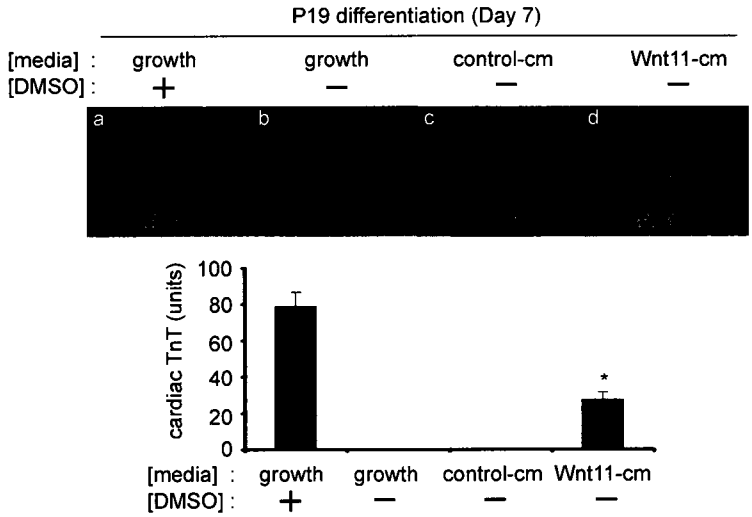
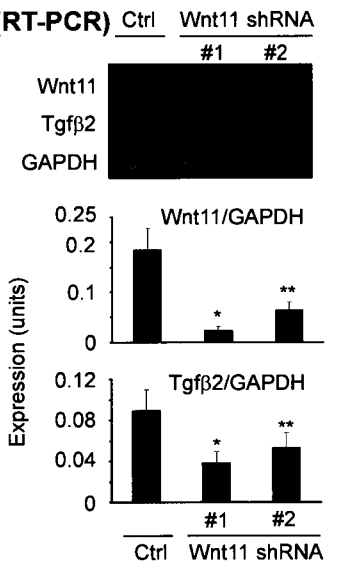


Figure 6C, 6D

C



D

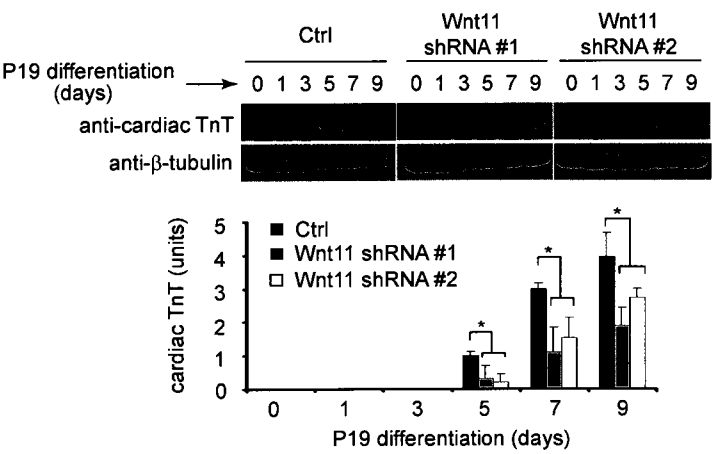
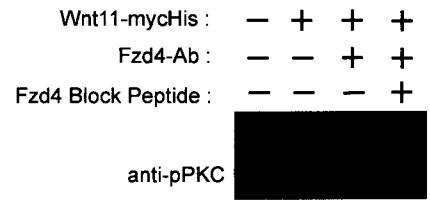
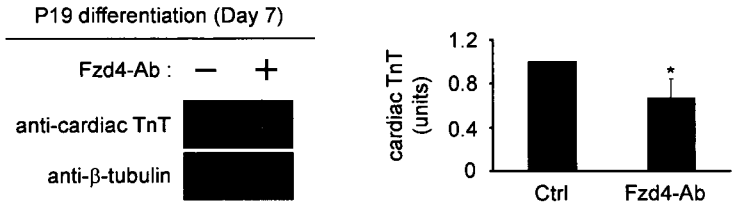


Figure 6E, 6F, 6G

E



F



G

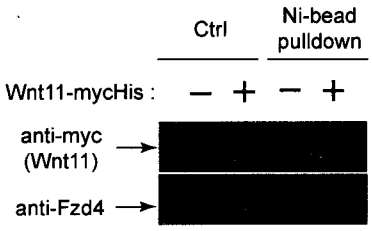


Figure 6. Non-canonical Wnt11 signaling promotes cardiac differentiation of P19 cells. (A) Nickel bead pulldown of Wnt11-mycHis from conditioned media. Incubation of Wnt11-mycHis with 1000 units of PNGaseF at 37°C for 1 hour confirmed expression of glycosylated, functionally mature Wnt11-mycHis protein upon immunoblot with anti-myc. (B) DMSO-induced P19 cardiac differentiation conducted in the presence of MEMα growth media or conditioned media (control-cm or Wnt11-cm). Wnt11 promoted cardiac differentiation at day 7. Cardiac TnT (cTnT) antibody used for cell staining (panels a to d) or for immunoblot (graph). (C) Wnt11 shRNA knockdown cells have a significant decrease in Wnt11 expression (*, 87% and **, 65%) and in Tgfβ2 (downstream of Wnt11 signaling pathway) expression (*, 57% and **, 40%) when compared to control shRNA cells (n=3; * and **, $P<0.05$). (D) Wnt11 shRNA knockdown cells inhibit cardiac differentiation throughout the 9 day timecourse (30-52% at day 9) versus control shRNA cells (n=3; *, $P<0.05$) as assessed by immunoblot with anti-cardiac TnT. (E) Fzd4 antibody (1 μg/ml in media) inhibits non-canonical Wnt11 signaling in P19 cells transfected with Wnt11-mycHis plasmid (measured by PKC activation) and this effect is reversed with Fzd4-Ab blocking peptide (3 μg/ml in media). (F) Inhibiting Fzd4 (1 μg/ml Fzd4-Ab in media) decreases cardiac differentiation (33%) when compared to control cells incubated with 1 μg/ml normal goat serum at 7 days of differentiation (n=5; *, $P<0.05$). (G) Direct interaction between Wnt11-mycHis and endogenous Fzd4. P19 cells transfected with Wnt11-mycHis plasmid were subject to nickel bead pulldown followed by immunoblot for endogenous Fzd4.

Figure 7

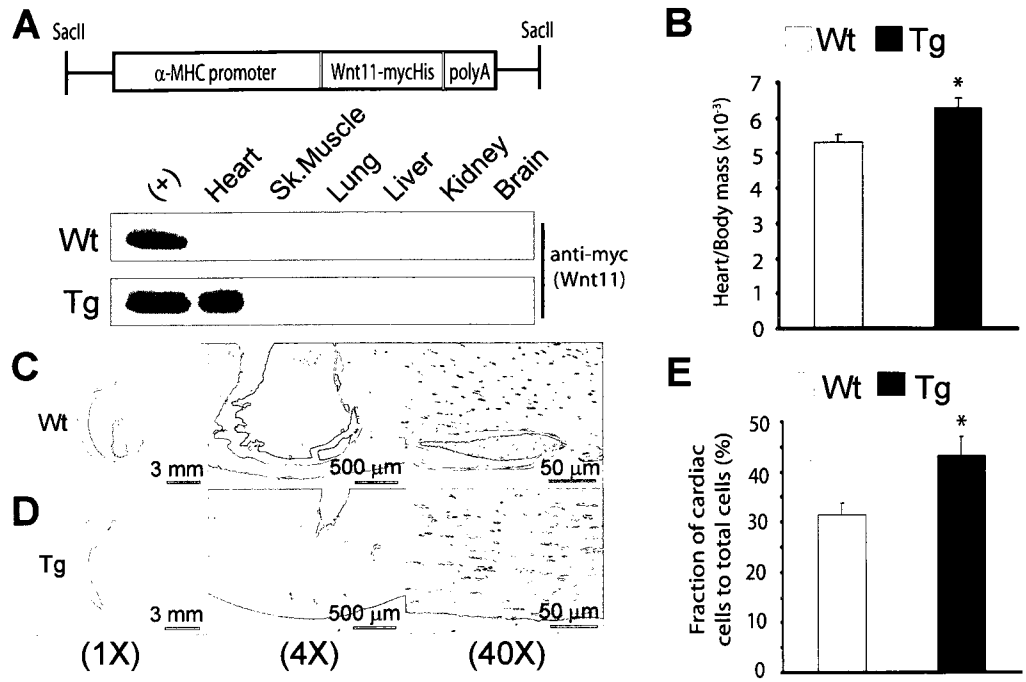

A
SacII
SacII
α-MHC promoter
Wnt11-mycHis
polyA
(+)
Heart
Sk.Muscle
Lung
Liver
Kidney
Brain
Wt
Tg
anti-myc
(Wnt11)
B
Wt
Tg
Heart/Body mass (x10⁻³)
C
Wt
3 mm
500 μm
50 μm
D
Tg
3 mm
500 μm
50 μm
(1X)
(4X)
(40X)
E
Wt
Tg
Fraction of cardiac cells to total cells (%)

Figure 7. Cardiac-specific transgenic over-expression of Wnt11-mycHis promotes hypertrophy and myocardial development. Analyses were conducted on 9 week old mice. (A) Tissue expression analysis of Wnt11-mycHis transgenic (Tg) mice versus Wild type (Wt) mice. Wnt11-mycHis transgene detected with anti-myc. H9c2 cells over-expressing recombinant Wnt11-mycHis via adenoviral infection were used as (+) control. (B) A significant increase (18%) in heart to body mass ratio of Tg vs. Wt mice was observed (n=3; *, $P<0.01$ vs. Wt). (C and D) Wt and Tg whole heart sections stained with H+E. Left, centre, and right panels correspond to 1X, 4X, and 40X magnifications of apical region, respectively. (E) An increase in the fraction of cardiac cells to total number of cells was observed (38%) in H+E sections taken from 3 Tg vs. 3 Wt hearts (n=3; *, $P<0.01$ vs. Wt). The relative cell counts were obtained from at least 10 fields of view in the apical region of each heart section analysed at 40X magnification.

age-matched non-transgenic animals (Figure 7C to E). Together, these data suggest that non-canonical based signals (as mediated by Wnt11) act as an inductive cue for cardiomyocyte development.

Although our observations indicated a positive role for non-canonical Wnt activity in cardiomyocyte differentiation, the role of canonical Wnt signaling in this context remained to be analysed. As an initial step in defining the role of canonical Wnt signaling, we conducted a comprehensive *in vivo* expression analysis of Wnt signaling factors (Table 1, also see Supplementary Data (SD) Table 1 and SD Figure 1). Whole mouse hearts were isolated at various timepoints throughout cardiac development including: embryonic day E14.5, E18.5, post-natal day (P0), 1 week, 2 and 4 weeks. These timepoints were selected as they uniformly represent later stages of cardiogenesis, typically associated with cardiomyocyte differentiation and maturation and not specification for the cardiac lineage. The analysis revealed that virtually all components of the Wnt pathway were detected throughout cardiac development including secreted factors such as Wnt antagonists of the Sfrp family (Secreted Frizzled-related proteins) and the plasma membrane Wnt receptors, namely Frizzleds. Moreover, intracellular components such as the canonical-specific β-catenin, the non-canonical-specific Daam1 (Dishevelled-associated activator of morphogenesis), and the bi-functional molecule Dvl1 (Dishevelled) were detected at all timepoints analysed. Wnt expression was not consistent during cardiogenesis, as Wnt genes were either differentially expressed or were not detected over the timecourse examined. Furthermore, both canonical and non-canonical Wnts were detected in the heart. Interestingly, the non-canonical Wnt11 (and

Table 1

Wnt pathway components		Throughout	Embryonic	Biphasic	Not detected
Secreted factors	Wnt	2, 4, 5A, 5B, 9B, 10B, 16	7B	2B, 11	1, 3, 3A, 6, 7A, 8A, 8B, 9A, 10A
	Sfrp	1-4			
	Wif	1			
Membrane receptors	Fzd	1-9	10		
Intracellular components		β-catenin Daam1 Dvl1			

Table 1. Expression analysis summary of Wnt pathway components during embryonic and post-natal mouse cardiac development. Whole hearts were analysed at the following timepoints: embryonic day E14.5, E18.5, birth, 1 week, 2 and 4 weeks. Results were grouped into 4 categories: i) Genes detected throughout cardiac development, ii) embryonic genes (i.e. genes detected prior to birth), iii) biphasic genes (i.e. embryonic genes whose expression decreases at or shortly after birth, then re-appears at 4 weeks), iv) genes not detected. See SD Table 1 and SD Figure 1 for details of primers and conditions used for RT-PCR.

Wnt2B) were found to have biphasic expression patterns (Table 1, SD Figure 1). These observations suggest that both canonical and non-canonical Wnt pathway components play a role during cardiogenesis.

To investigate the extent to which canonical versus non-canonical signaling impacted cardiogenesis, we examined canonical Wnt signal activity using the TCF-lacZ reporter mouse strain. Homozygous TCF-lacZ (+/+) transgenic mice were generated by linking 6 copies of the TCF-binding site together with a minimal hsp68 promoter element upstream of a lacZ reporter gene (Mohamed et al. 2004). The extent of canonical Wnt/β-catenin signaling during cardiac development was analysed by examining the β-galactosidase (β-gal) activity pattern of whole mount embryos or hearts as well as of frozen heart sections derived from various timepoints (Figure 8A, panels a-l). During early cardiogenesis at E8.5 and E9.5 (the looping heart tube stage), embryos revealed a striking absence of β-gal stain, with no detectable signal in the primitive heart sac at E8.5 (Figure 8A, panel c) or within the primitive atria and ventricles at E9.5 (Figure 8A, panel d). The outflow tract, OFT (Figure 8A, panels c-d) displayed intense β-gal staining suggesting that canonical Wnt signaling is active in this region. At later stages of cardiac development (E18.5, birth, 1 week of age), the OFT-derived pulmonary and aortic semi-lunar valves were clearly defined by a strong β-gal stain whereas the myocardium (ventricles and atria) remained largely devoid of β-gal activity (Figure 8A, panels e-h).

To eliminate the possibility that the absence of β-gal staining in the myocardium was due to impermeability of the β-gal staining reagent, heart sections were also analysed

(Figure 8A, panels i-l). In agreement with our whole mount observations, the cardiac muscle region at E14.5 was predominantly β-gal negative whereas the right ventricle outflow tract and the pulmonary semi-lunar valves were intensely β-gal positive (Figure 8A, panel j). For comparative purposes, a whole E14.5 heart with lungs attached clearly showed the contrasting β-gal staining pattern of the lungs and heart (Figure 8A, panel i). This is further supported by heart sections at birth (Figure 8A, panel k) where the cardiac muscle region was devoid of β-gal, and the OFT (enlarged in Figure 8A, panel l) was lined with numerous β-gal positive structures. The absence of canonical Wnt signaling in the myocardium proper strongly suggests that this branch of Wnt activity may be more prominent in regulating the formation of the OFT and the OFT-derived semi-lunar valves rather than heart muscle per se.

Given the observation that canonical Wnt/β-catenin pathway was inactive in the developing myocardium (Figure 8A), it was reasonable to hypothesize that Wnt11 may elicit a pro-myocardial effect by antagonizing the canonical Wnt/β-catenin pathway. To test this hypothesis directly, we examined whether Wnt11 administration modified TCF-dependent gene expression *in vitro*, as measured by the luciferase SuperTOPflash reporter assay (Kaykas et al. 2004) (Figure 8B). Stimulation with the canonical-specific Wnt pathway components β-catenin and Wnt3A resulted in a greater than 20 fold increase in TCF-dependent activation compared to its corresponding SuperFOPflash control. Interestingly, co-stimulation with Wnt11 and the canonical signaling components (β-catenin/Wnt3A) resulted in a substantial inhibition (57% decrease, $P<0.01$) of canonical Wnt activation (Figure 8B). Next, we investigated the involvement

of caspase proteases in mediating the Wnt11 suppression of canonical signaling. As noted above, caspase proteases are known to induce differentiation in a number of cell lineages, and caspase 3 has also been reported to target β-catenin during apoptosis. In addition, caspase 8 null mice display severe disruption in the development of the myocardium (Varfolomeev et al. 1998). These results suggest that an extrinsic-mediated apoptotic signal (caspase 8) may trigger caspase 3 activity to promote cardiomyocyte differentiation. To assess whether the suppressive effect of Wnt11 was caspase-dependent, we treated canonical β-catenin-activated cells with caspase-specific peptide inhibitors during Wnt11 stimulation. We observed that treatment with either caspase 3 or 8 inhibitors led to a significant restoration of β-catenin activity in the presence of Wnt11 (Figure 8C). Furthermore, inhibition of caspase 3 or 8 led to accumulation of stabilized β-catenin protein, even in the presence of Wnt11 expression (Figure 8D). These data suggest that Wnt11 inhibits canonical signaling, in part, through a caspase-mediated suppression of the canonical Wnt pathway.

To further confirm a role for caspase 3/8 in the suppression of canonical Wnt/β-catenin activity, we examined the effect of inhibiting caspase activity *in vivo* while monitoring the impact (if any) on TCF reporter activity. Intact embryos or isolated hearts of TCF-lacZ (+/+) mice incubated with caspase 3 or 8 inhibitors displayed a dramatic increase in β-gal staining compared to age-matched DMSO control treated tissue (Figure 9, panels b-d and f-h, arrowheads). Sectioning of treated embryos or isolated hearts revealed that the β-gal stained region was primarily localized in the epicardial region of the early myocardium (Figure 9, panels j-l, arrowheads). During cardiac development,

Figure 8A

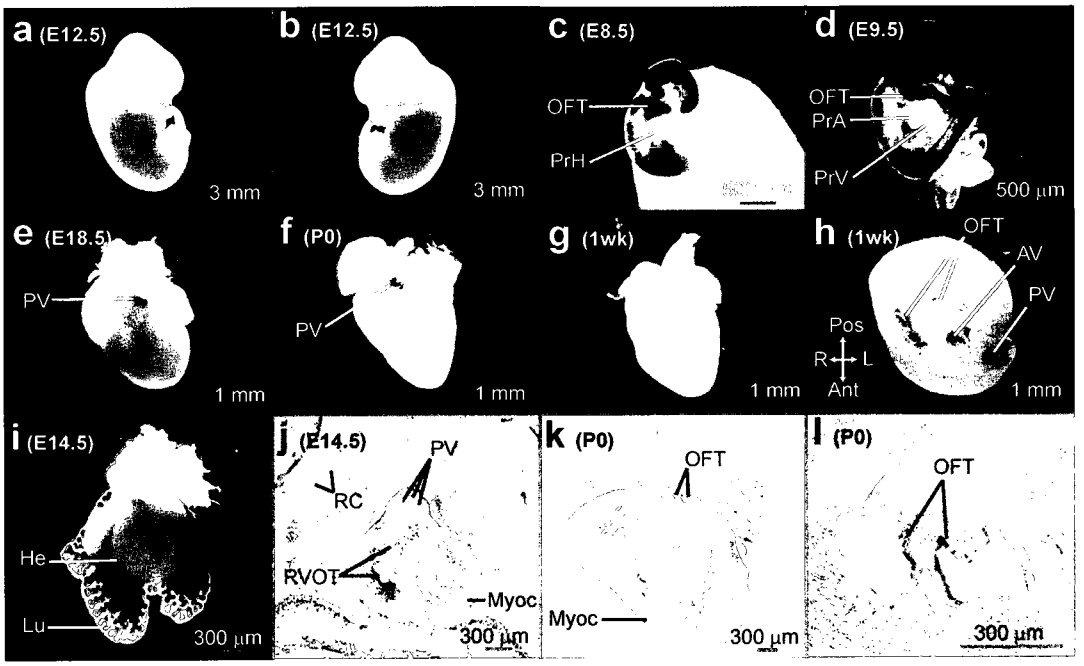
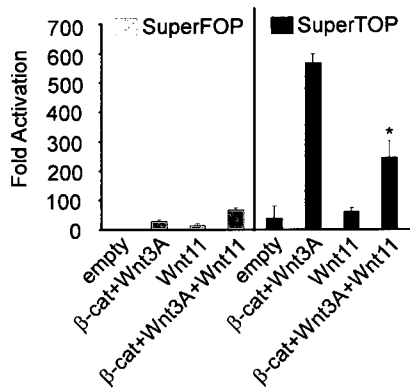
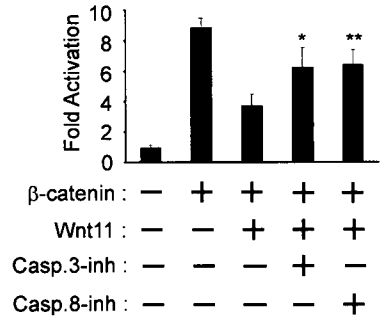

a (E12.5)
3 mm
b (E12.5)
3 mm
c (E8.5)
OFT
PrH
d (E9.5)
OFT
PrA
PrV
500 μm
e (E18.5)
PV
1 mm
f (P0)
PV
1 mm
g (1wk)
1 mm
h (1wk)
OFT
AV
PV
Pos
R
L
Ant
1 mm
i (E14.5)
He
Lu
300 μm
j (E14.5)
PV
RC
RVOT
Myoc
300 μm
k (P0)
OFT
Myoc
300 μm
l (P0)
OFT
300 μm

Figure 8B, 8C, 8D

B



C



D

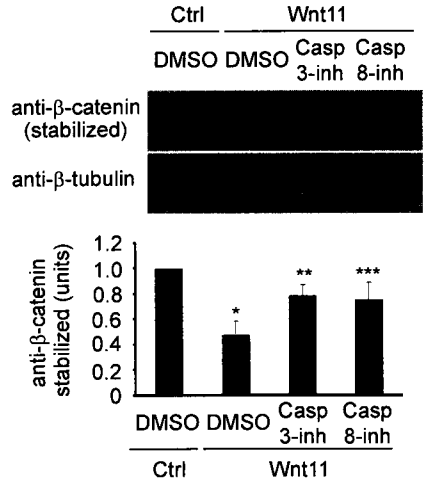


Figure 8. Limited canonical Wnt/β-catenin signaling in the myocardium (A); and non-canonical Wnt11 suppresses canonical Wnt activity in a caspase-dependent manner in P19 cells (B to D). (A) Whole embryos/hearts (panels a-i) and heart sections (panels j-l) from TCF-lacZ (+/+) mice during different stages of cardiac development. (panels a-b) Negative control unstained embryos. (panels c-d) E8.5 and E9.5 embryos showing the primitive heart sac and looping heart tube, respectively. (panels e-g) E18.5 heart, post-natal day 0 heart and 1 week old heart, respectively. Top portion of heart (panel g) dissected away to reveal β-gal positive structures (coronal view, panel h). (panels j-l) Heart sections showing β-gal positive structures in the OFT region and β-gal deficiency in the myocardial layers. (panel i) Whole E14.5 heart and lungs attached for comparative purposes. (panel j) E14.5 whole embryo section showing heart contained within thoracic cavity. (panel k) Post-natal day 0 whole heart section showing the OFT (magnified in panel l). β-gal sections counterstained with Eosin Y. OFT, outflow tract; PrH, primitive heart sac; PrA, primitive atrium; PrV, primitive ventricle; PV, pulmonary valve; AV, aortic valve; Ant-Pos, anterior-posterior; L-R, left-right; He, heart; Lu, lungs; RVOT, right ventricle outflow tract; RC, rib cage; Myoc, myocardium. (B) SuperTOPflash assay. Cells were transfected with plasmids encoding either canonical Wnt components (Wnt3A and β-catenin) or with the non-canonical Wnt11. Transfections contained: 40 ng of SuperTOPflash or FOPflash plasmid, 4 ng of β-gal plasmid as an internal transfection control, plus 400 ng of effector plasmid containing the gene of interest. Co-stimulation of both canonical and non-canonical Wnt pathways resulted in dramatic suppression (57%) of canonical Wnt pathway activation (n=3; *, $P<0.05$ vs. canonical Wnt stimulation). Luciferase values were normalized for transfection efficiency using β-gal

and for total protein amount used per sample. (C) Wnt11-mediated suppression of SuperTOPflash β-catenin activity in (B) was partially alleviated by incubating P19 cells with 10 μM caspase 3 or 8 inhibitors (28% and 31% alleviation, respectively) (n=4; * and **, $P<0.05$ vs. Wnt11-transfected P19 cells). (D) Levels of stabilized β-catenin were actively suppressed (*, 53%) in P19 cells transfected with Wnt11 plasmid, while treatment with 10 μM caspase 3 or 8 inhibitors reversed this effect (**, 28% and ***, 24% increase, respectively) relative to Wnt11-only transfected P19 cells (n=3; $P<0.05$). Control cells were treated with DMSO.

Figure 9

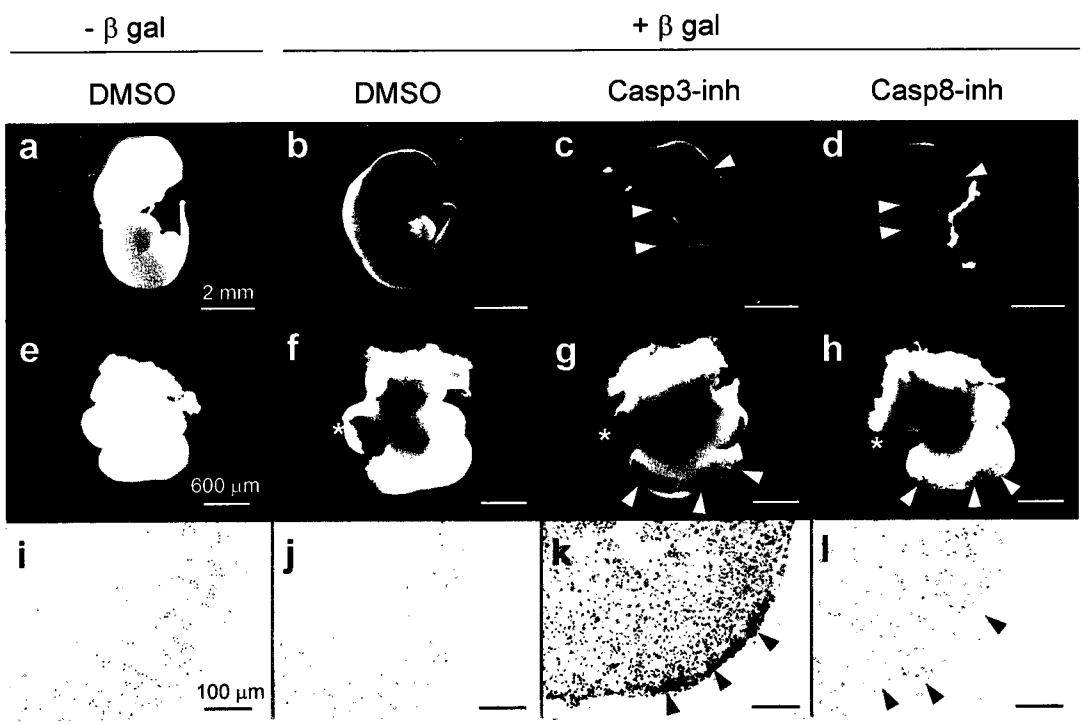

- β gal
+ β gal
DMSO
DMSO
Casp3-inh
Casp8-inh
a
b
c
d
2 mm
e
f
g
h
600 μm
i
j
k
l
100 μm

Figure 9. Caspase inhibition increases canonical Wnt pathway activation (β-gal expression) *in vivo*. E12.5 embryos/hearts of TCF-lacZ (+/+) mice were incubated overnight at 37°C in DMEM growth media containing DMSO (control) or 10 μM caspase 3 or 8 inhibitors and stained for β-gal. Non-stained negative controls show no fortuitous β-gal staining in whole tissue (panels a, e) or sectioned tissue (panel i). Increased β-gal staining (canonical Wnt activity) was observed compared to DMSO-treated control samples upon caspase 3 or 8 inhibition in embryos (compare panels c-d to b, arrowheads) or in the epicardial region of whole hearts (compare panels g-h to f, arrowheads). Heart sections, counterstained with Eosin Y, showed this increased β-gal staining to be localized primarily in the epicardial region (compare panels k-l to j, arrowheads). Note the non-specific β-gal staining (*) appearing in the right atrium (panels f-h).

the epicardium acts as the niche for committed cardiac progenitors, an area largely devoid of differentiated cardiomyocytes (Limana et al. 2007; Zhou et al. 2008). Our observations suggest that the loss of caspase activity in this region is synonymous with an expansion in canonical Wnt/β-catenin activity, a response that may delay the cardiac differentiation program.

Based on these observations, we examined whether the pro-differentiation effect of Wnt11 was mediated by caspase activity. Differentiating P19 cells displayed notable increases in both caspase 3 and 8 activity, peaking at around day 3 of differentiation (Figure 10A). Caspase 9 activity was negligible compared to caspase 8, implying that upregulation of caspase 3 during differentiation was a caspase 8/extrinsic cell death pathway response and was not derived from the caspase 9 intrinsic/mitochondrial mediated signal pathway. The elevated caspase activity observed during DMSO-induced P19 cardiac differentiation (Figure 10A) did not originate from apoptotic cell death as the proportion of TUNEL+ apoptotic cells was very low and did not increase during the differentiation time course (Figure 10B). P19 cells exposed to Wnt11 or Fzd4 were found to moderately stimulate caspase 3 and 8 activity, whereas co-stimulation with Wnt11/Fzd4 enhanced caspase 3 and 8 activity 2 fold in conditions that were not conducive to engaging differentiation (Figure 10C). This non-death activation of caspase 3 and 8 is considerably reduced compared to the level of caspase 3 and 8 activation that occurs during apoptosis (Figure 10C; caspase 3 and 8 activity: 670% and 620% increase, respectively). Finally, we analysed whether Wnt11 knockdown alone (via Wnt11 shRNA expression) would influence the caspase activity profile. P19 Wnt11 shRNA clones

Figure 10

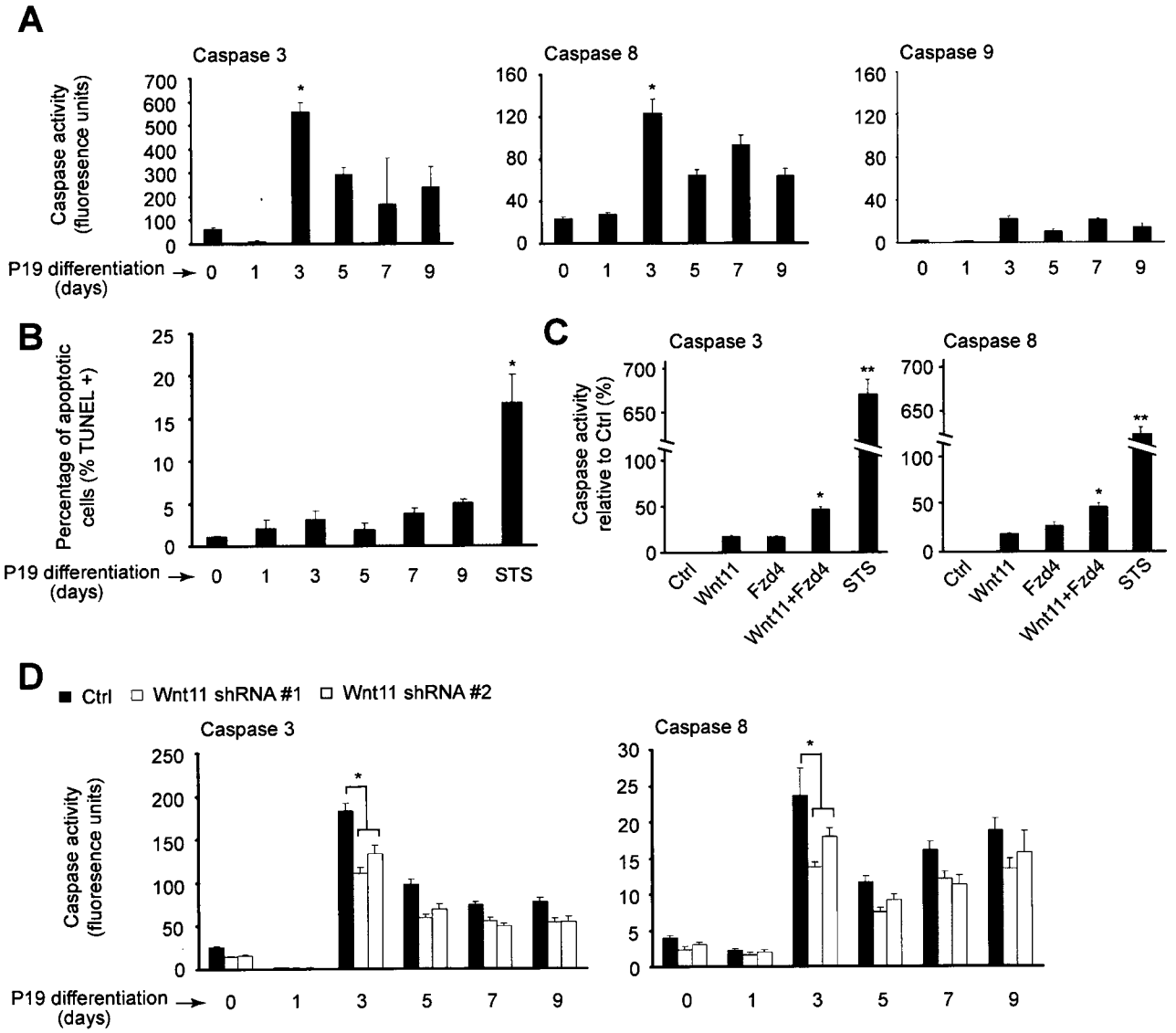

A
Caspase 3
Caspase 8
Caspase 9
Caspase activity (fluorescence units)
P19 differentiation (days)
B
Percentage of apoptotic cells (% TUNEL +)
STS
C
Caspase activity relative to Ctrl (%)
Ctrl
Wnt11
Fzd4
Wnt11+Fzd4
STS
D
Ctrl
Wnt11 shRNA #1
Wnt11 shRNA #2

Figure 10. Caspase activation during DMSO-induced P19 cardiac differentiation. (A) Activation of caspase 3 and 8 was observed during a 9 day differentiation timecourse showing peak activation at day 3. A 9 and 5 fold increase relative to day 0 was observed for caspase 3 and 8, respectively (n=4; *, $P<0.05$ vs. day 0). Caspase 9 activity was negligible compared to caspase 3 or 8. (B) Percentage of apoptotic (TUNEL+) cells was minimal (< 6%) at all timepoints analysed when compared to staurosporine (STS)-treated apoptotic cells (17%; n=3; *, $P<0.01$ vs. day 9). (C) P19 cells transfected with Wnt11 and Fzd4 plasmids moderately activate caspase 3 and 8, however, co-transfection with Wnt11/Fzd4 enhanced activity (45-50%) of both caspase 3 and 8 when compared to control P19 cells transfected with empty plasmid (n=4; *, $P<0.05$ vs. control). For the positive apoptotic control, P19 cells were incubated with 1 μM staurosporine (STS) in growth media for 5 hours prior to measuring caspase activation. STS-treated apoptotic cells resulted in excessive caspase 3 and 8 activity, 670% and 620%, respectively (n=3; **, $P<0.05$ vs. control). (D) Wnt11 shRNA cells exhibit decreased caspase 3 and 8 activation (27-39% and 24-42%, respectively) during P19 cardiac differentiation at day 3 (n=4; *, $P<0.05$ vs. control at day 3). Caspase activity measured by incubating cell lysates with caspase-specific fluorogenic substrates (DEVD-amc for caspase 3, IETD-amc for caspase 8) and resulting fluorescence intensity was measured at 460 nm.

displayed a moderate decrease in both caspase 3 and 8 activity during DMSO induced differentiation (Figure 10D), signifying that Wnt11 influences caspase-dependent signaling during cardiomyocyte maturation.

To directly test a pro-differentiation effect for the Wnt11/caspase signal conduit, we conducted the following experiments. First, P19 cells were induced to form cardiomyocytes in the presence or absence of specific caspase peptide inhibitors, then monitored for alterations in morphology and the expression of cardiac differentiation markers. At day 0 of differentiation, no cardiac muscle was observed (Figure 11A, panels a,e,i), whereas beginning at day 5, cuboidal-shaped myosin heavy chain positive cardiomyocytes begin to appear, increasing in frequency by day 7 (Figure 11A, panel d). However, caspase 3 or 8 inhibition led to a marked reduction in the formation of the typical cuboidal-shaped myosin heavy chain positive cardiomyocytes, with a dramatic increase in thin elongated cell types compared to control cultures (Figure 11A, panels d,h,l). To further assess the extent of cardiac differentiation, we examined levels of cardiac-specific cTnT at day 7. A significant decrease in the number of cTnT positive cells to the total number of cells (DAPI positive) was observed in the caspase 3 or 8 inhibited cells (31% and 42%, respectively) when compared to DMSO treated control cells (Figure 11B). In support of these observations, immunoblot analysis also showed a significant decrease in cTnT levels in the caspase 3 or 8 inhibited cells (35% and 47%, respectively) when compared to DMSO treated control cells (Figure 11C). Furthermore, incubating cells with Wnt11-mycHis-containing media during caspase 3 or 8 inhibition was not sufficient to rescue the defect in cardiomyocyte differentiation (Figure 11D).

Figure 11A

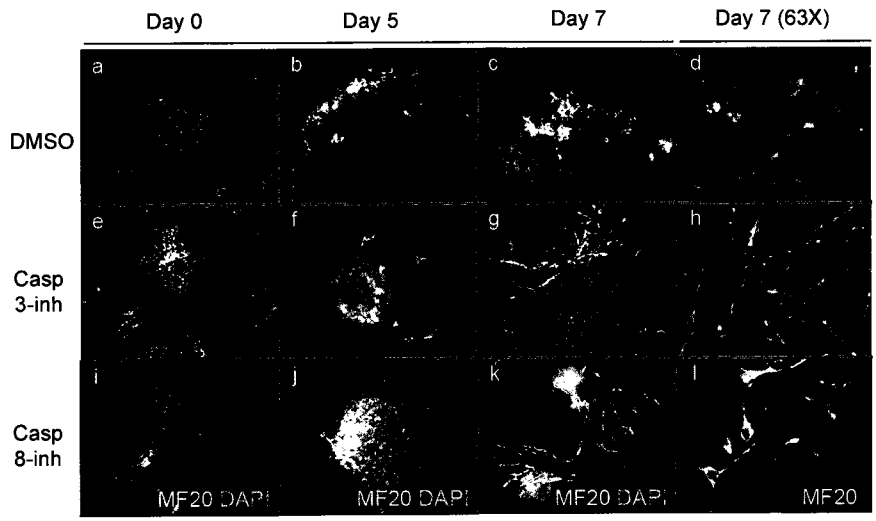
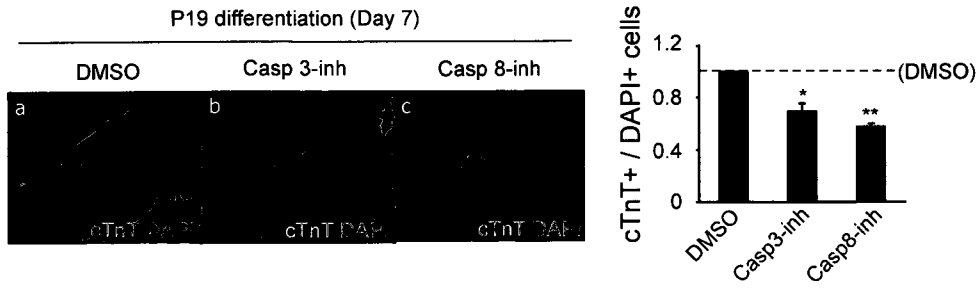
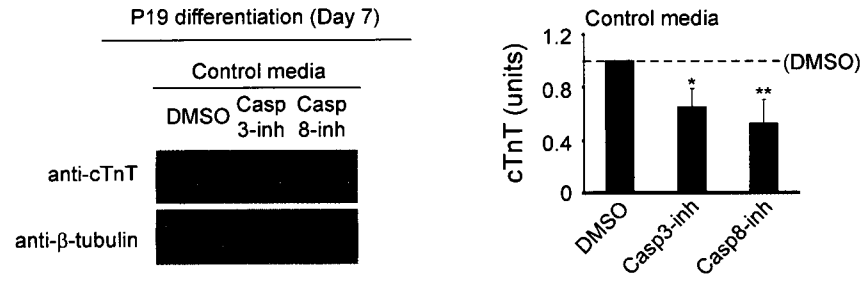

Day 0
Day 5
Day 7
Day 7 (63X)
DMSO
Casp 3-inh
Casp 8-inh
a
b
c
d
e
f
g
h
i
j
k
l
MF20 DAPI
MF20 DAPI
MF20 DAPI
MF20

Figure 11B, 11C, 11D

B



C



D

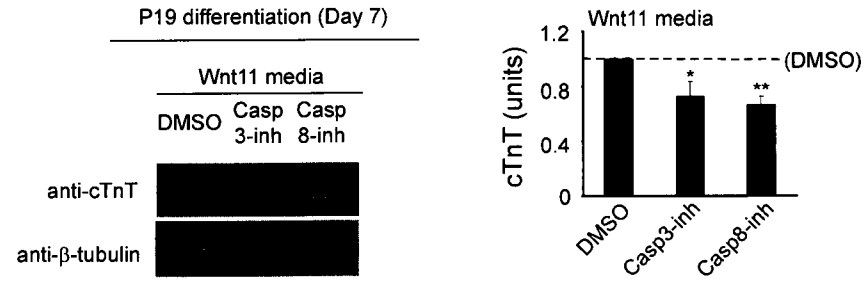


Figure 11. Caspase inhibition decreases P19 cardiac differentiation. (A) P19 cells were induced to differentiate in the presence of DMSO control (panels a-d) or 10 μM caspase 3 or 8 inhibitors (panels e-h and i-l, respectively). Cells were stained with MF20 antibody to detect myosin heavy chain (MHC) positive cardiomyocytes, wherein cardiac cells were identified morphologically as cuboidal-shaped cells. No cardiac muscle detected at day 0 (panels a,e,i), whereas at day 5, MHC-positive cardiomyocytes begin to appear (panels b,f,j), increasing in frequency by day 7 (panels c,g,k; also magnified in panels d,h,l). At day 7, a significant change in cell morphology was observed in caspase 3 or 8 inhibited samples (panels h and l, respectively) compared to control (panel d). Caspase inhibition gave rise to not only cuboidal-shaped cardiac cells, but also to thin elongated-shaped cells (compare panels h and l to panel d) indicating a marked reduction in cardiomyocyte formation. Images taken at 20X magnification except panels d, h and l (63X). (B) The relative number of cardiac Troponin T (cTnT) positive cells to the total number of cells (DAPI positive) shows a significant decrease in cardiac differentiation at day 7 in cells treated with 10 μM caspase 3 or 8 inhibitors (panels b,c) (31% and 42%, respectively) compared to DMSO treated control cells (panel a) (n=3, * and **, $P<0.05$). (C) Using control conditioned media, cells treated with 10 μM caspase 3 or 8 inhibitors also show a significant decrease in cardiac differentiation at day 7 (35% and 47%, respectively) compared to DMSO treated control P19 cells as assessed by cTnT immunoblot (n=5; * and **, $P<0.05$). (D) Incubating P19 cells with Wnt11 conditioned media was not able to reverse the effect seen in (C).

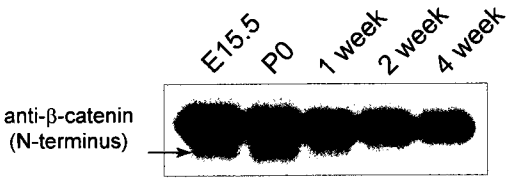
These data are consistent with the hypothesis that Wnt11 drives the cardiomyocyte differentiation program (in part) by activating a caspase 8/3 signal conduit, which in turn represses canonical Wnt signaling. To address this supposition directly, we sought to identify a substrate by which caspase 3/8 may limit canonical signaling activity. Interestingly, β-catenin, the central component of the canonical Wnt pathway, is a known substrate of effector caspases (3,6,7) during apoptosis (Van de Craen et al. 1999; Steinhusen et al. 2000). Accordingly, we noted that β-catenin immunoblots detected a faster migrating form that was evident only during times that coincided with *in vivo* cardiomyocyte differentiation (Figure 12A). LC-MS/MS of gel fragments that potentially corresponded to the mass of both the full length β-catenin and the faster migrating band identified both gel fragments as β-catenin (Figure 12B and C). In the faster migrating form of β-catenin, the LC-MS/MS identified peptides throughout the protein, however, there was no coverage in the region of β-catenin that corresponded to the C-terminal caspase 3 cleavage sites as described in Steinhusen et al. 2000 (see below). This suggested that the β-catenin species identified may have been subjected to a cleavage modification in the C-terminal region.

Accordingly, we generated and tested 1) β-catenin truncation mutants that corresponded to the known caspase cleavage sites at either the N- or C-termini (Steinhusen et al. 2000), and 2) β-catenin point mutants with a substitution of the preferred caspase 3 cleavage site from the DXXD motif to an alanine motif (Figure 13A, adapted from (Steinhusen et al. 2000)). Expression of β-catenin fragments was confirmed by immunoblot (Figure 13A). Transfection of P19 cultures with the ΔN and

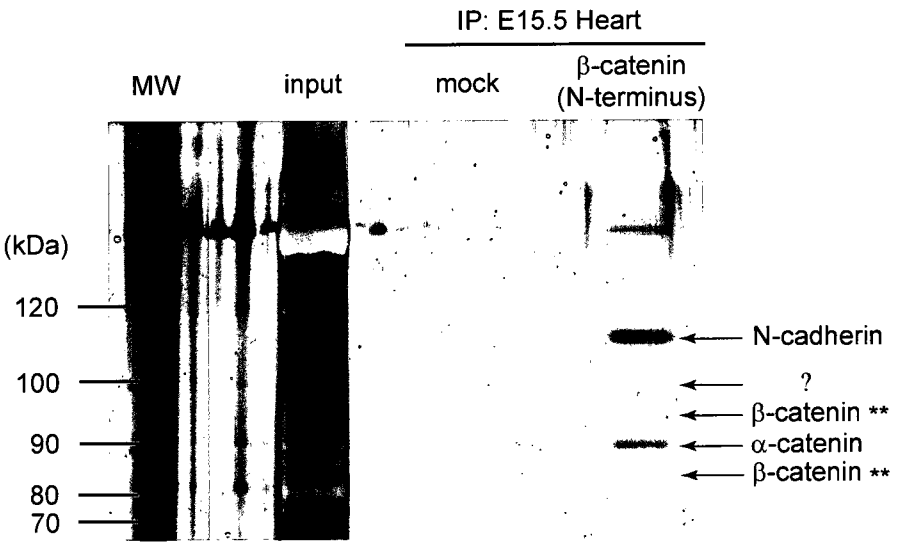
ΔC β-catenin fragments yielded a moderate reduction in canonical Wnt activation (as measured by SuperTOPflash assay) when compared to WT β-catenin, whereas the ΔNC fragment, lacking both N- and C-termini, had a dramatic decrease (71%, $P<0.01$) in canonical Wnt activation (Figure 13A). These results are consistent with those observed by Steinhusen et al. and suggest that caspase cleaved β-catenin acts as an effective repressive signal for canonical Wnt signaling. Alternatively, expression of the point mutations D115A and D751A, resulted in a minor but significant increase in canonical Wnt activation when compared to WT β-catenin (Figure 13A). Subsequently, we monitored the impact of the various β-catenin mutants on cardiomyocyte differentiation. Over-expression of β-catenin lacking the C-terminal end (ΔC and ΔNC) led to increased expression of cardiomyocyte differentiation markers (Figure 13B). Unexpectedly, expression of the individual β-catenin point mutants did not negatively impact expression of cardiomyocyte differentiation markers (Figure 13B). We surmise that expression of the individual β-catenin caspase cleavage site mutants do not possess sufficient dominant activity to enhance endogenous β-catenin signaling and thereby limit the cardiomyocyte differentiation program.

Figure 12A, 12B

A



B



LC-MS/MS Matching Peptides List: β-catenin (1 - 781)

(upper band)		(lower band)	
Start - End	Sequence	Start - End	Sequence
1 - 19	MATQADLMELDMAMEPDRK	1 - 19	MATQADLMELDMAMEPDRK
293 - 312	FLAITTDCLQILAYGNQESK	134 - 151	HAVVNLINYQDDAELATR
551 - 565	TSMGGTQQQFVEGVR	293 - 312	FLAITTDCLQILAYGNQESK
566 - 582	MEEIVEGCTGALHILAR	313 - 329	LIILASGGPQALVNIMR
613 - 625	VAAGVLCELAQDK	434 - 449	NKMMVCQVGGIEALVR
626 - 647	EAAEAIEAEGATAPLTELLHSR	497 - 508	LLHPPSHWPLIK
648 - 661	NEGVATYAAAVLFR	648 - 661	NEGVATYAAAVLFR
		685 - 710	TEPMAWNETADLGLDIGAQGEALGYR

Figure 12C

LC-MS/MS Matching Peptides to β-catenin (1 - 781)

(upper band)

MATQADLMELDMAMEPDRKAAVSHWQQQSYLDSGIHSGATTTAPSLSGKGNPEEEDVDTS -60
QVLYEWEQGFSQSFTQEQVADIDGQYAMTRAQRVRAAMFPETLDEGMQIPSTQFDAAHPT -120
NVQRLAEPSQMLKHAVVNLINYQDDAELATRAIPELTKLLNDEDQVVVNKAAVMVHQLSK -180
KEASRHAIMRSPQMVSAIVRTMQNTNDVETARCTAGTLHNLSHHREGLLAIFKSGGIPAL -240
VKMLGSPVDSVLFYAITTLHNLLLHQEGAKMAVRLAGGLQKMVALLNKTNVKFLAITTDC -300
LQILAYGNQESKLIILASGGPQALVNIMRTYTYEKLLWTTSRVLKVLSVCSSNKPAIVEA -360
GGMQALGLHLTDPSQRLVQNCLWTLRNLSDAATKQEGMEGLLGTLVQLLGSDDINVVTCA -420
AGILSNLTCNNYKNKMMVCQVGGIEALVRTVLRAGDREDITEPAICALRHLTSRHQEAEM -480
AQNAVRLHYGLPVVVKLLHPPSHWPLIKATVGLIRNLALCPANHAPLREQGAIPRLVQLL -540
VRAHQDTQRRTSMGGTQQQFVEGVRMEEIVEGCTGALHILARDVHNRIVIRGLNTIPLFV -600
QLLYSPIENIQRVAAGVLCELAQDKEAAEAIEAEGATAPLTELLHSRNEGVATYAAAVLF -660
RMSEDKPQDYKKRLSVELTSSLFRTEPMAWNETADLGLDIGAQGEALGYRQDDPSYRSFH -720
SGGYGQDALGMDPMMEHEMGGHHPGADYPVDGLPDLGHAQDLMDGLPPGDSNQLAWFDTD -780
L -781

(lower band)

MATQADLMELDMAMEPDRKAAVSHWQQQSYLDSGIHSGATTTAPSLSGKGNPEEEDVDTS -60
QVLYEWEQGFSQSFTQEQVADIDGQYAMTRAQRVRAAMFPETLDEGMQIPSTQFDAAHPT -120
NVQRLAEPSQMLKHAVVNLINYQDDAELATRAIPELTKLLNDEDQVVVNKAAVMVHQLSK -180
KEASRHAIMRSPQMVSAIVRTMQNTNDVETARCTAGTLHNLSHHREGLLAIFKSGGIPAL -240
VKMLGSPVDSVLFYAITTLHNLLLHQEGAKMAVRLAGGLQKMVALLNKTNVKFLAITTDC -300
LQILAYGNQESKLIILASGGPQALVNIMRTYTYEKLLWTTSRVLKVLSVCSSNKPAIVEA -360
GGMQALGLHLTDPSQRLVQNCLWTLRNLSDAATKQEGMEGLLGTLVQLLGSDDINVVTCA -420
AGILSNLTCNNYKNKMMVCQVGGIEALVRTVLRAGDREDITEPAICALRHLTSRHQEAEM -480
AQNAVRLHYGLPVVVKLLHPPSHWPLIKATVGLIRNLALCPANHAPLREQGAIPRLVQLL -540
VRAHQDTQRRTSMGGTQQQFVEGVRMEEIVEGCTGALHILARDVHNRIVIRGLNTIPLFV -600
QLLYSPIENIQRVAAGVLCELAQDKEAAEAIEAEGATAPLTELLHSRNEGVATYAAAVLF -660
RMSEDKPQDYKKRLSVELTSSLFRTEPMAWNETADLGLDIGAQGEALGYRQDDPSYRSFH -720
SGGYGQDALGMDPMMEHEMGGHHPGADYPVDGLPDLGHAQDLMDGLPPGDSNQLAWFDTD -780
L -781

Figure 12. Immunoblot and Immunoprecipitation-Silver (IP-Ag) stain of early stage E15.5 embryonic hearts reveals multiple β-catenin fragments. (A) Whole heart lysates (50 μg) obtained at different timepoints during cardiac development were analysed by immunoblot using an N-terminal directed β-catenin antibody. Appearance of a faster migrating band (arrow) under the full-length β-catenin band was observed during early stages of cardiac development (E15.5 and P0), and it gradually disappears during later stages of cardiac development. (B) Five hundred μg of whole E15.5 mouse heart lysate was subject to IP using the N-terminal directed β-catenin antibody (above), whereas Normal Rabbit Serum was used for mock IP. Fifty μg of whole E15.5 mouse heart lysate was used as input. Subsequent silver stain and LC-MS/MS analysis revealed the presence of the following peptides: N-cadherin, α-catenin, β-catenin and a faster migrating form of β-catenin (**). (C) Alignment of LC-MS/MS peptides with β-catenin protein sequence (red and green sequences). Caspase 3 cleavage sites, as described by Steinhusen et al. 2000, are shown at the N and C-termini (boxed areas). Refer to Materials and Methods for details of IP-Ag stain procedure.

Figure 13

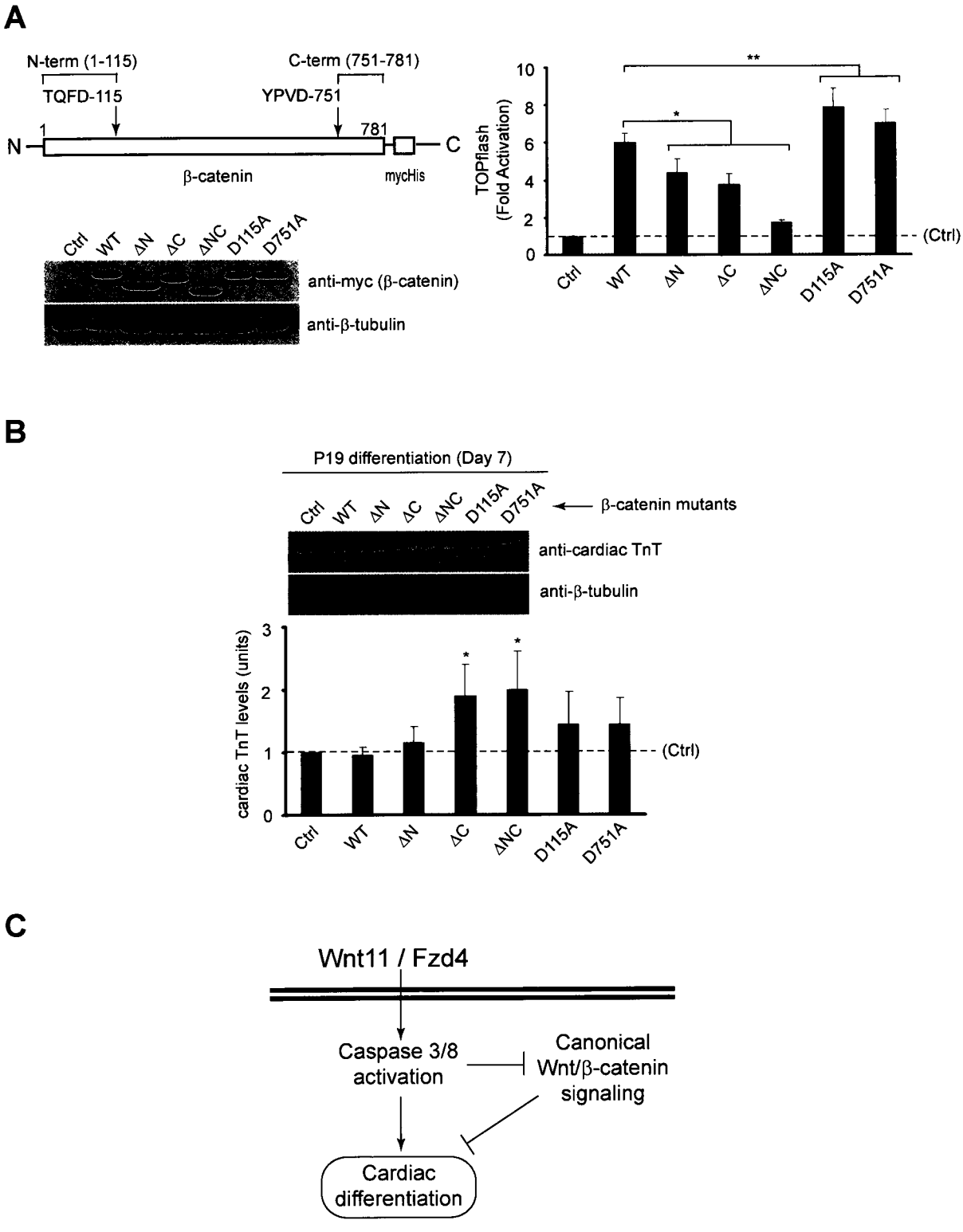

A
N-term (1-115)
TQFD-115
C-term (751-781)
YPVD-751
1
781
N
C
β-catenin
mycHis
Ctrl
WT
ΔN
ΔC
ΔNC
D115A
D751A
anti-myc (β-catenin)
anti-β-tubulin
TOPflash (Fold Activation)
(Ctrl)
B
P19 differentiation (Day 7)
β-catenin mutants
anti-cardiac TnT
anti-β-tubulin
cardiac TnT levels (units)
C
Wnt11 / Fzd4
Caspase 3/8 activation
Canonical Wnt/β-catenin signaling
Cardiac differentiation

Figure 13. Caspase-derived β-catenin mutants alter canonical Wnt pathway activation and subsequent cardiac differentiation. (A) mycHis-tagged β-catenin truncation and point mutants corresponding to known caspase 3 cleavage sites at either N- or C-termini were generated (Figure 13A, adapted from (Steinhusen et al. 2000)). Activation of the canonical Wnt/β-catenin pathway was analysed, via SuperTOPflash assay, in P19 cells transfected with caspase-derived β-catenin mutants. ΔN and ΔC β-catenin mutants yielded a moderate reduction in canonical Wnt activation compared to WT β-catenin (27% and 37%, respectively) whereas the ΔNC fragment had a dramatic suppression (71%) in canonical Wnt activation (n=4; *, $P<0.05$). Point mutants, D115A and D751A, resulted in increased canonical Wnt activation (30% and 17%, respectively) compared to WT β-catenin (n=4; *, $P<0.05$). (B) P19 cells over-expressing caspase-derived β-catenin mutants were examined for subsequent effects on cardiac differentiation. β-catenin lacking the C-terminal region (ΔC and ΔNC) increased cardiac differentiation (90% and 98%, respectively) after 7 days compared to WT β-catenin (n=4; *, $P<0.05$). (C) Proposed model of non-canonical Wnt11 mediating P19 cardiac differentiation.

Chapter 4 - DISCUSSION

The observations of this study demonstrate that non-canonical Wnts engage caspase dependent signal activity to promote cardiomyocyte differentiation. In turn, Wnt11 stimulated caspase activity targets and disables β-catenin thereby disrupting the canonical Wnt signaling pathway. Collectively, this study provides the novel insight that cardiomyocyte differentiation is dependent on the co-ordinated integration of two seemingly disparate signal pathways. Our observations indicate that Wnt11 initiates the pro-myogenic cascade through interaction with the Fzd4 receptor. We have shown that blocking Fzd4 signaling results in decreased cardiac differentiation, similar to the effect seen when knocking down expression of Wnt11. In addition, while Wnt11 and Fzd4 individually stimulate caspase activity, the combinatorial action of Wnt11 and Fzd4 leads to a dramatic increase in caspase function. In support of our observations, a study measuring the differential ability of Fzd homologs to activate known pro-myogenic signals showed that Fzd4 retained a high capacity for activating PKC (Sheldahl et al. 1999). Moreover, global gene expression profiling of human ESC derived cardiac cell clusters and DMSO-induced P19 cardiomyocytes revealed an upregulation of a number of Wnt ligands and Fzd receptors, including Wnt11 and Fzd4 (Peng et al. 2002; Cao et al. 2008).

We hypothesize that Fzd receptors provide a convenient means of transducing the caspase signal that is initiated by Wnt11 at the membrane surface. Frizzled receptors belong to the family of 7 transmembrane spanning G protein-coupled receptors (GPCRs) where subsequent activation of heterotrimeric G-proteins can initiate a multitude of intracellular signaling pathways including PKC activation, similar to that seen in non-

canonical Wnt11 signaling (Salazar et al. 2007; Schulte and Bryja 2007). It has also been demonstrated that activation of certain GPCRs results in several intracellular changes including caspase 3 activation (Adams et al. 2000; Revankar et al. 2004), and it has also been shown that GPCR signaling can suppress activation of the canonical Wnt/β-catenin pathway (Wang et al. 2009).

Alternatively, Fzd receptors may engage caspase activity by directly interacting with membrane components of the classic death receptor pathway. To review, we have noted that caspase 8 activity is upregulated in synchrony with caspase 3 and that inhibition of caspase 8 is as effective in blocking the differentiation program. These results indicate that the caspase 3 dependent signal conduit is initiated by the caspase 8 extrinsic cell death pathway. Caspase 8 can be engaged by a variety of ligand receptor based signals including the TNF receptor family, TNFR1 and TNFR2, the TRAIL receptor family including DR4, DR5, DR6, RANK-R, the FAS/APO1/CD95 complex and potentially the TRAIL related decoy receptors DCR1 and DCR2. These transmembrane receptor complexes interact with cognate cytoplasmic signal intermediates to activate the caspase proteases 8/10 and are usually referred to as DISC components (death inducing signaling complex) (Fernando and Megeney 2007). To date, there have been no reports demonstrating a direct physical link between a Fzd receptor and one of these DISC components. However, a recent study has shown that addition of TRAIL ligand can elicit a caspase associated cleavage of β-catenin during apoptosis, suggesting that DISC components are receptive to integration with Wnt signaling pathways (Senthivinayagam et al. 2009). Clearly, our observations support the

hypothesis that Fzd4 engages the extrinsic cell death pathway (at an as yet undefined point) to promote caspase-dependent differentiation signals.

Suppression of canonical Wnt/β-catenin signaling appears to be a common mechanism in the differentiation of a broad range of cell types including intestinal epithelial progenitor cells, chondrocytes, and adipocytes (Ross et al. 2000; Ryu et al. 2002; van de Wetering et al. 2002). The mechanism by which Wnt11 suppresses canonical signaling may be related to the ability of this factor to engage antagonistic signal pathways. For example, the nuclear β-catenin-interacting protein Chibby was found to not only block canonical Wnt activity by directly binding to the C-terminal region of β-catenin, but also to antagonize canonical Wnt signaling in facilitating cardiomyocyte differentiation of mouse ESCs (Takemaru et al. 2003; Singh et al. 2007). Irrespective of a role for Chibby, our observations are consistent with the probability that Wnt11 may enhance cardiogenesis by promoting the cleavage or de-stabilization of β-catenin itself (Figure 8D), leading to suppression of the canonical Wnt/β-catenin pathway (Figure 13A and B). This provides a parallel mechanism of action to that of Chibby whereby the C-terminus of β-catenin in this case is not bound by Chibby, but targeted by caspases leading to the differentiation of cardiomyocytes (see proposed model Figure 13C).

Recent investigations provide compelling *in vivo* evidence as to the importance of β-catenin stability/levels in the differentiation of cardiovascular progenitor cells (Qyang et al. 2007; Zelarayan et al. 2008). In one study, expression of de-stabilized β-catenin

was able to attenuate cardiac remodeling following induced-cardiac infarction in mice through enhanced cardiac precursor cell differentiation (Zelarayan et al. 2008). In a second study, canonical Wnt signaling, via the expression of stabilized β-catenin, actively inhibited differentiation of cardiovascular progenitor cells (Qyang et al. 2007). Furthermore, investigations of Wnt11 null mice have shown that the absence of Wnt11 results in the accumulation of full-length β-catenin, an event that is concurrent to a disruption in ventricular myocardial development (Nagy et al. 2009). A prevailing assumption with these studies is that the primary mode of regulating β-catenin content and function is achieved by the prototypical GSK-3β mediated phosphorylation event. Although we cannot rule out the influence of this signaling pathway, we have clearly shown that caspase directed alteration of β-catenin is an equally dramatic effector of the canonical Wnt/β-catenin pathway and hence cardiomyocyte differentiation. For example, β-catenin fragments lacking either the N- or C-terminus (ΔN or ΔC) have reduced potential for canonical Wnt pathway activation, whereas β-catenin lacking both N- and C-termini (ΔNC) yields a significant suppression of the canonical Wnt response (Figure 13A). Our results are similar to those observed by Steinhusen et al. (Steinhusen et al. 2000), although there was complete abrogation of canonical Wnt/β-catenin activity in the prior study. In addition, we noted that mutations at the N- or C-terminus (D115A and D751A, respectively) resulted in increased canonical Wnt pathway activation when compared to the wildtype β-catenin. Presumably, the caspase resistant β-catenin constructs render a more stable form of the protein which results in the hyperactivity of the canonical Wnt pathway. Interestingly, expression of the truncated β-catenin fragments (ΔC and ΔNC) led to an increase in cardiac differentiation, implying that

caspase mediated reduction of the full-length β-catenin is a direct stimulus for engaging cardiac differentiation.

In light of evidence suggesting that suppression of the canonical Wnt/β-catenin pathway (i.e. inhibiting β-catenin activity) promotes cardiomyocyte differentiation, the direct function engaged by β-catenin with respect to mediating cardiomyocyte differentiation is not well known. Possible roles that β-catenin may directly be involved in include: limiting self-renewal, and altering cytoskeletal dynamics.

Development of tissues depends on a delicate equilibrium between self-renewal, differentiation, and apoptosis/cell death. A core set of transcriptional regulators including Oct4, Sox2 and Nanog, appear to maintain the pluripotent state of ESCs by activating the expression of genes controlling self-renewal while repressing genes that drive differentiation (Chambers and Tomlinson 2009). Recent studies have shown that activation of caspase 3 promotes the differentiation of ESCs and hematopoietic stem cells (HSCs) indirectly, via limiting self-renewal of the stem cell populations, rather than by direct engagement of the differentiation program (Abdul-Ghani and Megeney 2008; Fujita et al. 2008; Janzen et al. 2008). These studies demonstrated that stem cells escape from the self-renewal state by caspase-mediated cleavage of one or more of these core transcription factors, allowing ESCs to rapidly exit the cell cycle and enter differentiation. By comparison, evidence demonstrates that β-catenin signaling also regulates the self-renewal potential of ESCs and neuronal progenitor cells (Zechner et al. 2003; Anton et al. 2007). Specifically, activation of β-catenin was found to prolong

expression of self-renewal genes (Oct4, Nanog) while delaying expression of differentiation genes (Nkx2-5, Mef2c, Tbx5, and α-MHC) in ESCs (Anton et al. 2007). Moreover, stabilized β-catenin was found to increase the neuronal progenitor population in mouse brain tissue (Zechner et al. 2003). These observations show that in the presence of stabilized β-catenin, progenitor cells exit the cell cycle less frequently, and instead, continue to proliferate. Therefore, inhibiting β-catenin activity would conceivably limit the self-renewal capacity of progenitor cells, thus favouring differentiation.

Interestingly, uncontrolled self-renewal due to elevated β-catenin activity has been detected in a number of cancerous tissues including colon cancer (Cadigan and Nusse 1997; Giles et al. 2003). The elevated β-catenin activity in such tumours is, in part, a result of uncontrolled activation of cyclin D1, a major regulator of cell cycle progression and self-renewal (Sherr 1996), and an important downstream target of the canonical β-catenin/TCF pathway (Shtutman et al. 1999; Tetsu and McCormick 1999). Furthermore, transcriptional activation of the cyclin D1 gene in such cells can be inhibited by enhancing the degradation of β-catenin (Shtutman et al. 1999). It is thus reasonable to speculate that, like the studies conducted by Fujita et al. 2008 and Janzen et al. 2008 (above), decreasing β-catenin activity would limit self-renewal (as mediated by cyclin D1), thus promoting differentiation.

Recent evidence suggests that limited self-renewal could be a direct result of the negative regulation of β-catenin transcriptional activity. The TIP60-p400 Histone Acetyl Transferase (HAT) chromatin remodeling complex has been shown to bind the C-

terminus of β-catenin (Sierra et al. 2006; Mosimann et al. 2009). Intriguingly, the TIP60-p400 complex is also required for maintaining the self-renewal potential and pluripotency of mouse ESCs by maintaining Nanog expression (Fazzio et al. 2008; Ho and Crabtree 2010). It is reasonable to suggest that caspase-directed cleavage of β-catenin at the C-terminus would disrupt the β-catenin/TIP60-p400 interaction, thus limiting self-renewal and inducing subsequent cardiac differentiation. Consistent with the role played by chromatin remodeling factors during cell differentiation, it has recently been shown that the cardiac-specific chromatin remodeling factor, BAF60c, was essential for directing the ectopic differentiation of mouse mesoderm into beating cardiomyocytes by binding to, and promoting the transcriptional activities of Gata4 and Tbx5 (Lickert et al. 2004; Takeuchi and Bruneau 2009; Ho and Crabtree 2010). One can ascertain that identifying other β-catenin-interacting components in the cardiac-specific chromatin remodeling complexes, and determining whether those components (if any) are also targeted by caspases, would shed further light onto the role played by chromatin regulation during cardiogenesis.

β-catenin is commonly associated with mediating TCF-dependent signaling events in the nucleus via the canonical Wnt pathway, however, β-catenin signaling can also directly regulate the cytoskeleton through transcription-independent mechanisms (Schlesinger et al. 1999; Ciani et al. 2004; Walston et al. 2004). Importantly, β-catenin is known to associate with components of the cytoskeleton including actin filaments and microtubules (Tao et al. 1996; Ligon et al. 2001; Huang et al. 2007) as well as with cadherin adhesion receptors that link to the cytoskeleton (Potter et al. 1999; Lien et al.

2006) to mediate biological processes such as: establishment of the plane of cell division, changes in cell shape and polarity, cell migration, and differentiation (Salinas 2007).

For example, it has been shown that β-catenin associates with microtubules and mediates mitotic spindle formation (Kaplan et al. 2004; Bahmanyar et al. 2008), to ensure that cells divide equally during mitosis. Precise orientation of the spindle apparatus establishes the plane of cell division and is an essential event during early development because this process influences the segregation of cell-fate determinants to daughter cells, and thus affects the fate of daughter cells (Salinas 2007). Many stem cells divide asymmetrically to generate two developmentally different daughter cells: one daughter cell maintaining stem cell characteristics (self-renewal) and the other daughter cell committed to differentiation. Indeed, recent evidence indicates that β-catenin activity mediates asymmetric divisions (Mizumoto and Sawa 2007; Fuentealba et al. 2008) and, therefore, may also directly influence the balance between self-renewal and differentiation.

Alternatively, the cytoskeleton undergoes drastic changes during differentiation from a progenitor cell in order to carry out the functions performed by the resulting differentiated/specialized cell. For example, cardiomyocytes are composed of an organized array of contractile units (sarcomeres) that are not found in the corresponding cardiomyocyte progenitor cells (Capetanaki et al. 2007). In this context, it is envisioned that decreasing β-catenin activity would destabilize the cytoskeleton, allowing for a

dynamic reorganization of cytoskeletal components necessary to form the sarcomeric units in a differentiated cardiomyocyte.

We have provided evidence that both canonical and non-canonical Wnt signaling components are expressed throughout the developing mouse heart, yet each Wnt pathway appears to be associated with unique aspects of myocardial development, i.e. canonical signal activity was restricted to the outflow tract (OFT) whereas non-canonical activity modified the differentiation capacity of cardiomyocytes.

Canonical Wnt/β-catenin signaling appears to be integral for promoting the formation of non-myocyte components within the heart itself. For example, the β-gal staining pattern observed in our TCF-lacZ reporter mouse hearts (Figure 8A) revealed striking similarities to the β-gal staining pattern observed in the neural crest-specific Wnt1-Cre lacZ reporter mice at early stages of development (Jiang et al. 2000). This area of intense β-gal positive cells likely represents the cardiac neural crest cell population which is known to contribute to a variety of tissue types including the formation and septation of the cardiac OFT (Creazzo et al. 1998). That is, the OFT region as well as the aortic and pulmonary vessel linings stain positive for β-gal. Recent evidence suggests that neural crest cells retain their multipotential characteristics and persist in the semi-lunar valves in late fetal and neonatal hearts (Nakamura et al. 2006) which is consistent with our β-gal staining seen at E18.5, P0 and 1 week (Figure 8A). Therefore, our data supports the hypothesis that canonical Wnt signaling plays a role in cardiac OFT formation, in part, via regulation of the neural crest cell population.

Collectively, our observations implicate the non-canonical Wnt11 as a potent pro-myogenic factor in mammalian heart development. Wnt11 expression has been detected in the cardiac crescent region (Kispert et al. 1996) which is consistent with the early expression of several cardiac transcription factors including Nkx2-5 (Lyons et al. 1995; Tanaka et al. 1999) and MEF2C (Lin et al. 1997), suggesting an early role in cardiac differentiation. Furthermore, other evidence substantiates the argument for cross-talk between the non-canonical Wnt11 pathway and other pro-cardiomyogenic transcription factors. A comparative genomic analysis of the Wnt11 gene has revealed Nkx2-5 binding sites within exon 1, which appear to be conserved across mammalian Wnt11 orthologs (Katoh and Katoh 2005). Assuming that Nkx2-5 binds exon 1 to regulate transcriptional activation of Wnt11, these data suggest an early feedback mechanism between Wnt11 and Nkx2-5 to moderate cardiomyocyte gene expression. In addition, it has recently been shown that Nkx2-5 also regulates the expression levels of full-length β-catenin in ventricular cardiomyocytes (Riazi et al. 2009) suggesting a common mode of action between Nkx2-5 and Wnt11.

The GATA family of cardiac transcription factors (GATA4/5/6), like Wnt11, are expressed in the early cardiac crescent tissue (Molkentin 2000; Patient and McGhee 2002), and also regulate cardiogenesis (Grepin et al. 1994; Grepin et al. 1995; Sebastiani et al. 1995; Grepin et al. 1997; Kuo et al. 1997; Molkentin et al. 1997; Holtzinger and Evans 2007; Peterkin et al. 2007). In addition to cross-talk with Nkx2-5 signaling mentioned above, the non-canonical Wnt11 pathway and GATA signaling were found to interact during cardiogenesis in the *Xenopus* model system (Afouda et al. 2008).

Moreover, Wnt11 was found to be a direct downstream target of GATA4/6 signaling, suggesting that the pro-cardiogenic effects of Wnt11 may be preceded by GATA4/6 function. Interestingly, activation of canonical Wnt/β-catenin signaling inhibited cardiogenesis via inhibition of GATA4/6 gene expression, whereas restoring GATA4/6 function counteracted the canonical Wnt/β-catenin-mediated inhibition and rescued cardiogenesis. As a result, this study shows that GATA4/6 transcription factors play a central role in integrating the canonical Wnt/β-catenin and non-canonical Wnt11 pathways during cardiogenesis.

Our observations support the contention that Wnt11/caspase signaling promotes cardiogenesis by antagonizing β-catenin activity/TCF dependent gene expression. Nevertheless, these observations do not preclude a role for additional Wnt11/caspase directed targeting events in the differentiation process. Indeed, it is reasonable to assume that a complex alteration in cell fate such as cardiomyocyte differentiation may be dependent on multiple signaling events. Caspases have been demonstrated to activate well known pro-differentiation MAPK signal cascades (MST1/MKK6/p38) in a non-apoptotic context, i.e. skeletal muscle and neural stem cell differentiation (Fernando et al. 2002; Fernando et al. 2005; Fernando and Megeney 2007). Downstream components of these same MAPK signal cascades are also known to phosphorylate and up-regulate the transcriptional activity of MEF2A/C, factors that are integral for differentiation in numerous cell lineages including cardiomyocytes (McKinsey et al. 2002). As such, it is reasonable to assume that Wnt11/caspase activity may promote cardiomyocyte

differentiation by kinase directed activation of MEF2 activity concurrent to targeted repression of canonical Wnt activity.

Interestingly, caspases may influence other signal pathways by targeting substrates other than those which suppress the canonical Wnt pathway (i.e. β-catenin). For example, the class II histone deacetylase, HDAC4, is not only a bona fide non-kinase caspase 3 substrate (Liu et al. 2004), but also regulates the entry of mesoderm cells into the cardiac muscle lineage (Karamboulas et al. 2006). These are interesting observations given that HDACs are known to interact with and inhibit the pro-differentiation activity of MEF2 proteins (McKinsey et al. 2000; Mejat et al. 2005). It is thus reasonable to suggest that targeting of HDACs by caspases may relieve the inhibition on MEF2, paving the way for differentiation to take place.

Wnt11/caspase signaling might also influence the engagement of the Protein Kinase C (PKC) pathway. With regards to cardiac differentiation in adult progenitor cells, several studies have shown that non-canonical Wnt11 induces cardiac gene expression in a PKC-dependent manner in a wide variety of cell types including: endothelial progenitor cells, bone marrow derived mesenchymal stem cells, and unfractionated bone marrow mononuclear cells (Belema Bedada et al. 2005; Koyanagi et al. 2005; Flaherty et al. 2008). Moreover, Wnt11-induced cardiac-specific expression was abolished by the use of PKC inhibitors in these prior studies. Interestingly, caspases are known to target several PKC isoforms for proteolytic cleavage and subsequent activation including: PKCδ, PKCε, PKCθ, and PKCζ (Emoto et al. 1995; Ghayur et al.

1996; Datta et al. 1997; Mizuno et al. 1997; Frutos et al. 1999; Smith et al. 2000; Hoppe et al. 2001). Recent studies of the non-canonical Wnt pathway, utilizing Wnt5A, have implicated the specific activation of PKCδ (and not PKCα or PKCε) in promoting cardiac gene expression in human circulating endothelial progenitor cells (Koyanagi et al. 2009). These observations bring forth the prospect that caspase activation of isoform-specific PKC substrates may not only mediate cardiomyocyte differentiation, but may also provide an additional mechanism by which PKC activation can integrate into the traditional non-canonical Wnt/Calcium pathway (Figure 3B).

Compelling evidence from a number of *in vivo* studies provides strong support for the role of caspases in cardiomyocyte differentiation. Targeted homozygous disruption of the mouse caspase 8 gene was found to be lethal *in utero*, and caspase 8 null embryos exhibited impaired heart muscle development caused by thinning of the myocardium (Varfolomeev et al. 1998). In support of these observations, downstream components of the caspase 8/extrinsic death receptor pathway (FADD null mice) and various caspase proteases (caspase 3 and 7 double null mice) also displayed defective myocardial phenotypes (Yeh et al. 1998; Lakhani et al. 2006). The primary interpretation of these studies was that caspase activity was required for normal developmental apoptosis and the absence of cell death resulted in cardiac malformations. However, in light of our observations, these studies suggest death receptor-mediated pathways may be an essential component in mediating cardiomyocyte differentiation.

The exact role of non-canonical Wnt11 is complicated by observations showing that not only is Wnt11 a target of the canonical Wnt pathway, but also that Wnt11 -/- mutant mice have defects in cardiac OFT formation in addition to interventricular septal defects (Zhou et al. 2007). This study highlighted a requirement for Wnt11 in the cross-talk between three major signaling pathways: the canonical Wnt pathway, the non-canonical Wnt pathway, and TGFβ signaling to achieve correct cardiac OFT formation and myocardial development. In addition, non-canonical Wnt11 signaling was found to be essential for migration of neural crest cells (NCCs) in *Xenopus*, which are known to contribute to cardiac OFT development (De Calisto et al. 2005). Furthermore, and again in apparent contrast to the generally accepted notion of the non-canonical role of Wnt11, maternally-derived *Xenopus* Wnt11 was shown to specifically activate the canonical Wnt/β-catenin pathway to regulate dorsal axis formation (Tao et al. 2005). Therefore, although we have shown that non-canonical Wnt11 signaling plays a role in cardiac differentiation, a more complex network of interactions between components of both the canonical and non-canonical Wnt pathways as well as with caspase signals likely underlie the intricate mechanisms that control cell differentiation throughout the heart. Nevertheless, our evidence leads us to hypothesize that Wnt11 mediates cross-talk between the non-canonical Wnt pathway and caspase signaling to regulate cardiomyocyte differentiation in the heart.

A variety of complementary approaches and analyses could be used with regards to future directions for this area of research. One can analyse the *in vivo* effects of the caspase-derived β-catenin fragments by generating corresponding cardiac-specific

transgenic mice that over-express these fragments and, one would anticipate that there would be increased cardiac differentiation in mice over-expressing the C-terminal truncated β-catenin. Whole hearts from these mice could also be used for protein complex isolation to determine whether the C-terminal truncated form of β-catenin forms a complex with different proteins when compared to complexes isolated from full-length β-catenin transgenics. Any differences in protein complex binding could give further clues as to the mechanism by which C-terminal truncated β-catenin promotes cardiac differentiation. If Wnt11 is acting through caspase 3/8 signaling *in vivo*, one could cross the Wnt11 transgenic mice with mice that have a significant knockdown in cardiac-specific caspase 3 or 8 expression. The resulting double mutant mice would be predicted to have decreased cardiac differentiation. Crossing these double mutant mice with the TCF-lacZ reporter mice should yield even more interesting results with regards to the effect on canonical Wnt/β-catenin signaling in the heart. It is expected that the decreased caspase activity acting on β-catenin in the heart would increase β-gal staining in the myocardium which, based on this study, has predominantly been localized only to the OFT. In addition to the H+E staining done on caspase 3 or 8 inhibited heart sections (as shown in Figure 9), one could perform immunofluorescent staining on the number of cTnT+/β-gal+ cells. If caspase inhibition decreases cardiac differentiation by increasing canonical Wnt/β-catenin signaling (i.e. β-gal staining), then it is anticipated there would be a decrease in the ratio of cTnT+/β-gal+ double positive cells in caspase inhibited hearts compared to control DMSO treated hearts. A combination of these approaches along with other similar *in vitro* and *in vivo* analyses would provide interesting results with regards to future directions for this area of research.

CONCLUSION

In summary, this study sheds further light on the respective roles of canonical versus non-canonical Wnt signaling during cardiac development. Although both traditional Wnt pathways are likely to mediate cardiogenesis, this study provides evidence that the non-canonical Wnt signaling pathway (as mediated by Wnt11) plays an important role during maturation of the mammalian heart by promoting cardiac differentiation and does so, in part, by caspase-mediated suppression of canonical Wnt/β-catenin activity.

Chapter 5 - APPENDIX

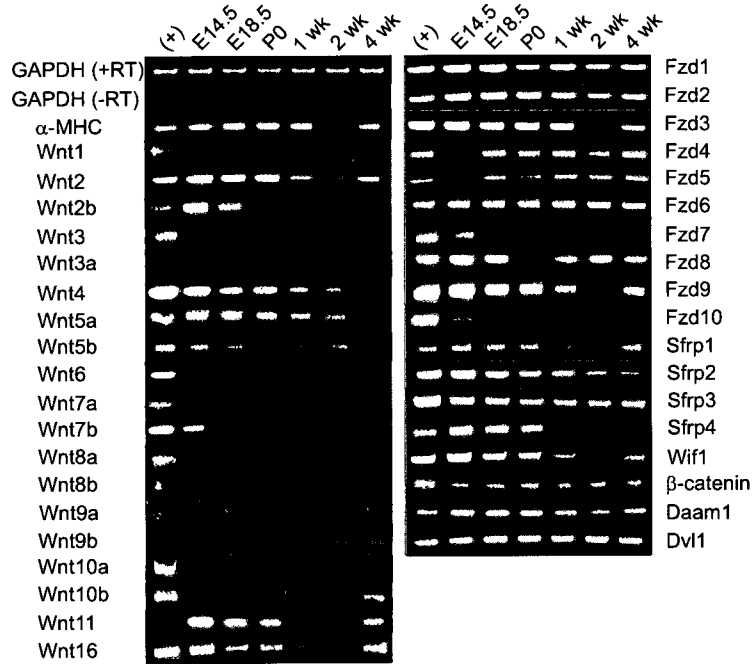
Supplementary Data - SD Table 1

Gene Name	Unigene #	Fwd/Rev Primer Binding Sites (bp)	Size (bp)
Wnt1 *‡	Mm.1123	SuperArray (Cat. No: PPM03491A)	125
Wnt2	Mm.33653	323-342/819-836	514
Wnt2B	Mm.10740	730-748/1048-1066	337
Wnt3 †	Mm.159091	484-503/782-801	318
Wnt3A †	Mm.1367	40-59/256-273	234
Wnt4	Mm.20355	77-97/403-421	345
Wnt5A	Mm.287544	628-647/833-852	225
Wnt5B	Mm.321818	604-623/809-828	225
Wnt6	Mm.268282	556-575/779-798	243
Wnt7A †	Mm.56964	412-431/700-719	308
Wnt7B	Mm.306946	271-290/512-531	261
Wnt8A *¶	Mm.558	SuperArray (Cat. No:PPM04771A)	196
Wnt8B *‡	Mm.88365	SuperArray (Cat. No:PPM05478A)	132
Wnt9A †§	Mm.218794	366-385/532-551	186
Wnt9B	Mm.215161	637-653/718-737	101
Wnt10A †§	Mm.5130	72-95/760-779	708
Wnt10B *‡	Mm.4709	SuperArray (Cat. No:PPM05463A)	100
Wnt11	Mm.22182	820-837/1048-1065	246
Wnt16	Mm.137403	761-780/979-998	238
Fzd1	Mm.246003	1522-1539/1887-1905	384
Fzd2	Mm.36416	542-561/800-822	281
Fzd3	Mm.243722	1967-1985/2356-2374	408
Fzd4	Mm.387968	1485-1502/1941-1958	474
Fzd5 *	Mm.150813	SuperArray (Cat. No:PPM05438A)	178
Fzd6	Mm.4769	1719-1735/2110-2126	408
Fzd7	Mm.297906	1574-1591/1959-1976	403
Fzd8 †‡	Mm.184289	1604-1624/1859-1879	276
Fzd9	Mm.6256	1323-1341/1743-1759	437
Fzd10	Mm.197628	1325-1344/1464-1483	159
Sfrp1	Mm.281691	300-319/467-486	187
Sfrp2	Mm.19155	93-116/356-374	282
Sfrp3 †‡	Mm.427436	367-389/551-576	210
Sfrp4 *¶	Mm.42095	SuperArray (Cat. No:PPM05165A)	173
Wif1	Mm.32831	1591-1610/1767-1786	196
β-catenin	Mm.291928	13-37/356-377	365
Daam1	Mm.87417	2233-2252/2392-2411	179
Dvl1	Mm.3400	510-529/716-735	226
α-MHC	Mm.290003	917-937/996-1016	100
Cripto1	Mm.5090	105-124/327-346	242
GAPDH	Mm.309092	141-164/766-789	649

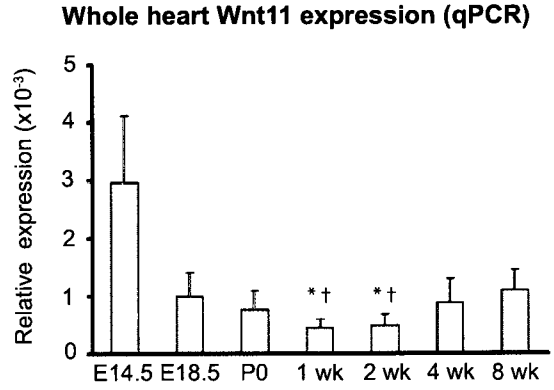
SD Table 1. Primers and conditions used for RT-PCR expression analysis during embryonic and post-natal cardiac development (also see SD Figure 1). Thirty cycles of PCR were performed using the following conditions: 95°C for 30 sec, 55°C for 50 sec, and 72°C for 50 sec. Reactions contained 2.25 mM $MgCl_2$ and E14.5 whole embryos were used as positive control tissues unless otherwise specified. (*) Proprietary primer sequences purchased from SuperArray Bioscience. (†) 1.25 mM $MgCl_2$ used in PCR reaction. (‡, §, ¶) Adult (8 week) testes, brain and E9.5 whole embryo used as positive control tissues, respectively. Fzd, Frizzled; Sfrp, Secreted Frizzled-related protein; Wif, Wnt inhibitory factor; Dvl, Dishevelled; Daam, Dishevelled associated activator of morphogenesis; Unigene numbers found at http://ncbi.nlm.nih.gov. We also thank S. Millar for providing us with Frizzled PCR primer sets (Reddy et al. 2004) and S. Heller for providing Wnt9B, Wnt16, and Wif1 primer sets (Heller et al. 2002).

Supplementary Data - SD Figure 1

A



B



SD Figure 1. RT-PCR expression analysis of Wnt signaling pathway components during embryonic and post-natal cardiac development. (A) Qualitative expression of Wnt pathway components during cardiac development (also see SD Table 1 for primers, PCR conditions, and positive control tissues used, and Table 1 for summary). (B) SYBR Green Quantitative PCR (qPCR) of Wnt11 gene in whole mouse heart from E14.5 to 8 weeks. Wnt11 mRNA expression expressed relative to *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH), used as a constitutive control (n=4; *, $P<0.05$ vs. E14.5; †, $P<0.05$ vs. 8 weeks). Note the biphasic expression pattern of Wnt11 in (A) and (B).

Rehabilitation of a Contract Killer: Caspase-3 Directs Stem Cell Differentiation

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Activation of caspase-3 is generally acknowledged as a penultimate step in apoptotic cell death pathways. Two studies in this issue of *Cell Stem Cell* (Fujita et al., 2008; Janzen et al., 2008) provide compelling data to demonstrate that caspase-3 is also a conserved inductive cue for stem cell differentiation.

Tissue development and maintenance are dependent on a complex interplay of stem cell self-renewal, differentiation, and apoptosis/programmed cell death. The phenotypic difference between renewal, differentiation, and death implies that each event is governed by a unique cohort of factors. In this issue of *Cell Stem Cell*, Fujita et al. (2008) and Janzen et al. (2008) report that the key apoptotic protease, caspase-3, mediates the differentiation of embryonic stem cells (ESCs) and hematopoietic stem cells (HSCs), respectively.

Based on the premise that stem cell differentiation corresponds with a loss of self-renewal capacity, Fujita et al. (2008) and Janzen et al. (2008) conducted a series of elegant studies to explore the role of caspase-3 as a probable gatekeeper of stem cell function. Using an inducible caspase-3-specific cleavage sensor, Fujita and colleagues observed that initiation of caspase activity coincided with early stages of ESC differentiation in otherwise healthy cells. Further, caspase-3 null ESCs displayed significant defects in in vitro assays of differentiation as well as an inability to form differentiated progeny when transplanted in vivo. The authors reasoned that caspase-3 mediated its effects through targeted cleavage of a pluripotent factor(s), demonstrating Nanog to be a priority target/substrate. Consistent with this hypothesis, expression of a caspase-3-resistant Nanog promoted ESC self-renewal while inhibiting differentiation. The results of Janzen et al. (2008) were equally persuasive in linking caspase-3 to the differentiation of HSC. Using a null mouse model, the authors established that loss of caspase-3 resulted in the accumulation of phenotypically defined long-term (LT) repopulating HSCs, with a corresponding reduction in circulating mature hematopoietic cells. In addition, no changes in the percent of apoptotic HSCs were observed. The observed differentiation defect was shown to be cell autonomous, as transplanted bone marrow from caspase-3 null animals replicated the aberrant hematopoietic lineage profiles in wild-type recipient mice. Interestingly, cytokine responsiveness and cytokine-mediated signals were elevated in caspase-3 null HSC, yet this alteration did not appear to be dependent on the enzymatic activity of the protease. Together, these findings provide compelling evidence that a protein with an established apoptotic role is also indispensable for the regulation of stem cell development and differentiation.

The discovery of a nondeath role for caspase-3 in stem cell self-renewal and differentiation appears to be counterintuitive. For example, stem cells and their immediate progeny need to remain free of factors that can cause lasting cell damage if they are to maintain pluripotency, genome integrity, and LT survival. Typically, the activation of the proteolytic enzyme caspase-3 acts as a convergent point for a number of death-signaling pathways. Once engaged, the effecter caspase enzymes, such as caspase-3, -6, and -7, cleave vital protein substrates, an event that precipitates many of the morphologic changes associated with cell death (Fischer et al., 2003). Given the wealth of observations that have positioned caspase-3 as the arbiter of the death signal, how is it then possible that this enzyme could do anything other than kill a stem cell?

The answer to this question may arise with a deeper appreciation of the mechanics that govern cell-fate decisions. Indeed, while there exists an obvious contradiction in the final outcomes of apoptosis and differentiation, there is also a remarkable degree of morphologic and biochemical symmetry between each process. Cells undergoing either apoptosis or differentiation display comparable cytoskeletal rearrangements, membrane fusion, and fission events as well as similar alterations in chromatin and nuclear architecture (Fernando and Megeney, 2007). This parallel is most evident in cells that engage a differentiation phenotype resembling attenuated cell death (i.e., are enucleated and short lived) such as red blood cells and epithelial derivatives. The differentiation program in these cell lineages has been shown to be dependent on a transient caspase-3 signal, unlike the sustained caspase-activation profile that triggers apoptosis (Ishizaki et al., 1998; Zermati et al., 2001). Similarly, transient activation of caspase-3 also mediates differentiation of longer-lived cell types such as skeletal muscle, osteoblasts, and neurons (Fernando et al., 2002; Miura et al., 2004; Fernando and Megeney, 2007). The observations from the current studies greatly extend this paradigm, suggesting that caspase-3-directed differentiation is a primordial feature of the cell-fate decision-making process (Figure 1A).

The primary interpretation of Fujita et al. (2008) and Janzen et al. (2008) is that caspase-3 promotes stem cell differentiation indirectly, by limiting self-renewal per se rather than directly engaging a differentiation program (Figure 1B). A corollary to

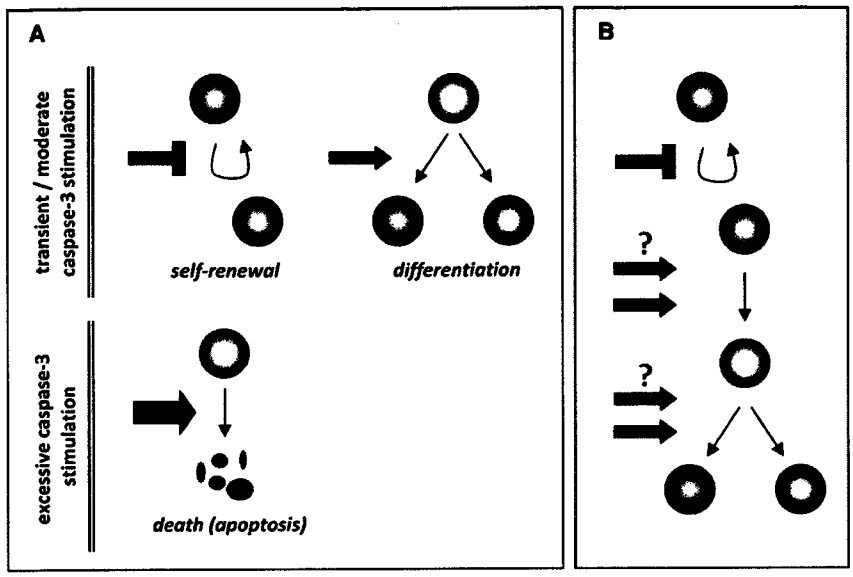

Figure 1. Caspase Regulation of Cell-Fate Decisions
(A) Caspase activation (green arrows) induces stem cell self-renewal, cell differentiation, or cell death (apoptosis). The specific outcome may depend on the extent and/or kinetics of caspase activation.
(B) Fujita et al. (2008) and Janzen et al. (2008) postulate that caspase-3 indirectly promotes stem cell differentiation by limiting self-renewal of the stem cell pool, hence favoring differentiation. Other, largely unidentified, factors (depicted with blue arrows) are also likely to participate. Alternatively, caspase-3 may influence differentiation more directly, by actively engaging factors that promote the gene expression and phenotype of a differentiated cell type, in addition to limiting self-renewal.

this hypothesis is that additional factors (as-of-yet undefined) promote the stem cell differentiation program following caspase blockade of self-renewal. Data from both studies demonstrate that caspase inhibition leads to a dramatic increase in the stem cell population, and Fujita and colleagues have shown that caspase-3 limits ESC pluripotency through direct cleavage of one protein alone, Nanog. An alternative interpretation of the current results may also be proposed. That is, caspase-3 may participate in stem cell differentiation in a more direct fashion rather than by simply limiting self-renewal. In this model, caspase-3 may simultaneously engage factors to promote the gene expression profile and resulting phenotypic changes that result in a specific differentiated cell type (Figure 1B). In potential support of this hypothesis, Janzen and colleagues have shown that loss of caspase-3 results in a dramatic reduction in early B lymphocyte maturation, a step far removed from the self-renewal process of an HSC. This multitask interpretation is also more consistent with previous observations that have demonstrated a direct role for caspase-3 in the terminal differentiation of numerous lineage-committed cell types (as noted above). A definitive position for either model will require a more complete mapping of potential caspase substrates.

Collectively, the work of Fujita et al. (2008) and Janzen et al. (2008) suggests that caspase-3 acts at multiple steps in the stem cell life cycle, affecting both self-renewal and differentiation. Yet, like all exciting observations, the solution to one mystery raises so many more questions. Paramount among these, how is caspase-3 activity restrained and guided to influence stem cell differentiation rather than cell death? The study of cell differentiation in other systems may provide the answer. *Drosophila* spermatid differentiation is dependent on the entire apoptotic signal cascade that culminates in activation of caspase homologs (Huh et al., 2004). However, caspase activity in this system is tightly scripted through ubiquitin-mediated degradation of caspase-inhibitory proteins (Arama et al., 2007). Such a mechanism ensures a level of caspase activity that is sufficient to drive spermatid differentiation yet not high enough to engage an apoptotic program. Whether this or other control mechanisms operate in various mammalian stem cells remains unknown.

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