INVESTIGATING MOLECULAR AND CELLULAR RESPONSES TO MYOCARDIAL INFARCTION: CANONICAL WNT ACTIVATION AND ENDOTHELIAL-TO-MESENCHYMAL TRANSITION

By

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To my wonderfully supportive family; my mom, Rebecca Okpere, my dad, Charles Aisagbonhi, my uncle and aunt, Anthony and Veronica Okpere, and my aunties Anthonia Aisagbonhi Obeto and Nina Okpere Akhimiemona

and

To my inspiring teachers from kindergarten through medical/graduate school

Specially dedicated to the memory of my high school science teacher, Mr. Makunjuola

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APC: adenomatous polyposis coli

BIO: 6-bromoindirubin-3’-oxime

β–gal: beta-galactosidase

dsh: disheveled

End: Endothelial

EndMT: Endothelial-to-mesenchymal transition

FACS: Fluorescence-Activated Cell Sorting

FS: Fractional Shortening

GSK-3β: Glycogen synthase kinase-3β

IF: Immunofluorescence

IHC: Immunohistochemistry

JNK: Janus Kinase

LAD: Left Anterior Descending artery

LEF: Lymphoid enhancing factor

MI: Myocardial Infarction

NFAT: Nuclear Factor of Activated T cells

PCR: Polymerase chain reaction

Sca: Stem cell antigen

SCL: Stem cell leukemia

S.E.M: Standard Error Measurement

sFRP: secreted frizzled-related protein

SMA: Smooth muscle actin

TCF: T-cell factor

X-gal: 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

CHAPTER I

INTRODUCTION

Acute myocardial infarction (MI), also known as a heart attack, afflicts about 1.5 million people in the United States every year and is a leading cause of mortality accounting for 1 in every 4 deaths. MI usually results from occlusion of a coronary artery after atherosclerotic plaque rupture and thrombosis (Antman and Braunwald, 2001). The ensuing ischemia in the cardiac tissue downstream from the blocked blood vessels kills cardiomyocytes within minutes. The widespread cell death triggers an immediate and massive inflammatory response that gradually clears out cellular debris. Inflammation is followed by granulation tissue formation which involves the activation and proliferation of endothelial cells and infiltration of myofibroblasts (Frangogiannis, 2008). Endothelial cell proliferation and migration from existing capillary beds, also known as angiogenesis, leads to the formation of new vessels in an attempt to restore blood supply, whereas myofibroblasts deposit collagen and other extracellular matrix proteins, in order to fill in the wound and prevent infarct spread. The granulation tissue eventually matures into a dense scar (Boudoulas and Hatzopoulos, 2009; Fig. 1.1).

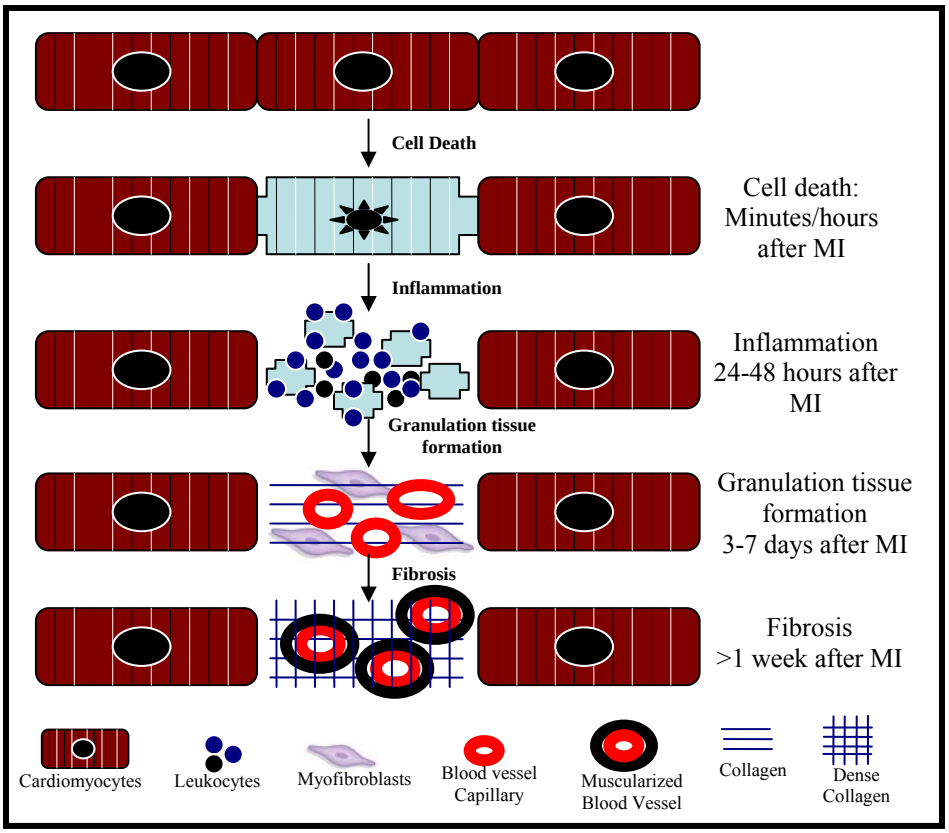


Figure 1.1: **The Myocardial Infarction Response**

Although the original reaction to ischemic assault and cell death is necessary to heal the wound and stabilize the ventricular wall, excessive scar formation acts as a barrier to proper electromechanical coupling between relatively healthy regions of the heart, thus compromising efficient and synchronous contraction. As a result, death from MI not only occurs acutely, but also due to chronic complications like heart failure (Canto et al., 2000).

Current treatment alternatives for patients who suffer a myocardial infarction are limited to revascularization to prevent further myocyte death and medical therapy to preserve residual heart function and slow down ventricular remodelling and heart failure. In the

last decade, there have been a number of reports in experimental models and human patients indicating that stem cells injected directly into the myocardium or delivered to the coronary circulation can improve cardiac function (Miyahara et al., 2006; reviewed by Boudoulas and Hatzopoulos, 2009; Schoenhard and Hatzopoulos, 2010). However, there seems to be limited long-term engraftment and minimal differentiation of transplanted stem cells into mature cardiovascular tissue (Suzuki et al., 2004; Reinecke et al., 2002; Kupatt et al., 2005a).

Instead, the current evidence suggests that donor cells exert a favorable paracrine effect on the injured myocardium, preventing apoptosis and promoting healing (Heil et al., 2004; Kupatt et al., 2005b; Gnecchi et al., 2008; Uemura et al., 2006). Some of these beneficial effects have been attributed to specific products of transplanted progenitor cells such as thymosin β4, which promotes wound healing, or the Wnt antagonist sFRP2 (secreted frizzled-related protein 2), which protects cardiomyocytes from hypoxia-induced apoptosis (Kupatt et al., 2005b; Gnecchi et al., 2005, Mirotsou et al., 2007; Hinkel et al., 2008; Alfaro et al., 2008; reviewed by Joggerst and Hatzopoulos, 2009). These studies suggest that it is possible to improve cardiac wound healing using various biological factors. Therefore, understanding the cellular and molecular regulatory mechanisms of innate repair may lead to new ways to improve cardiac function after MI, independently or in combination with stem cell therapy.

A potentially important regulatory mechanism for cardiac repair is the canonical wnt/β–catenin signaling pathway because not only has the pathway been implicated in

the cardioprotective paracrine effect of post-infarction stem cell transplantation (Kupatt et al., 2005b; Mirotsou et al., 2006) but also, a number of studies have shown that endogenous wnt pathway mediators are induced after MI (Blankensteijn et al., 2000; Barandon et al., 2003; Chen et al., 2004; Kobayashi et al., 2009). This dissertation therefore focuses on the canonical wnt signaling pathway in attempts to better understand the molecular mechanisms controlling the intrinsic cardiac repair processes.

The Wnt signaling pathway

Signal transduction in the canonical wnt/β–catenin signaling pathway often begins with the binding of a wnt ligand to its corresponding frizzled receptor and LRP-5/6 coreceptor. This interaction, which leads to activation of disheveled (dsh), can be blocked by secreted frizzled-related proteins (sFRPs) and Dickkopf (dkks) proteins. Activated dsh engages a multi-protein degradation complex containing axin, glycogen synthase kinase-3β (GSK-3β) and adenomatous polypopsis coli (APC). GSK-3β in the multi-protein complex normally phosphorylates β–catenin to cause its degradation by the proteasome, but upon binding of dsh, GSK-3β is inhibited, resulting in beta-catenin stabilization. Stabilized β–catenin accumulates in the nucleus where it binds members of the T cell factor (TCF) and lymphoid enhancing factor (LEF) families to cause transcription of target genes. In the non-canonical wnt signaling pathway, binding of wnt ligands to frizzled receptors trigger β–catenin-independent signaling events such as nuclear factor of activated T-cell (NFAT) and Janus Kinase (JNK) activation (Huelsken and Behrens 2002, Nelson and Nusse 2004, Moon et al 2004, Clevers 2006; Fig. 1.2).

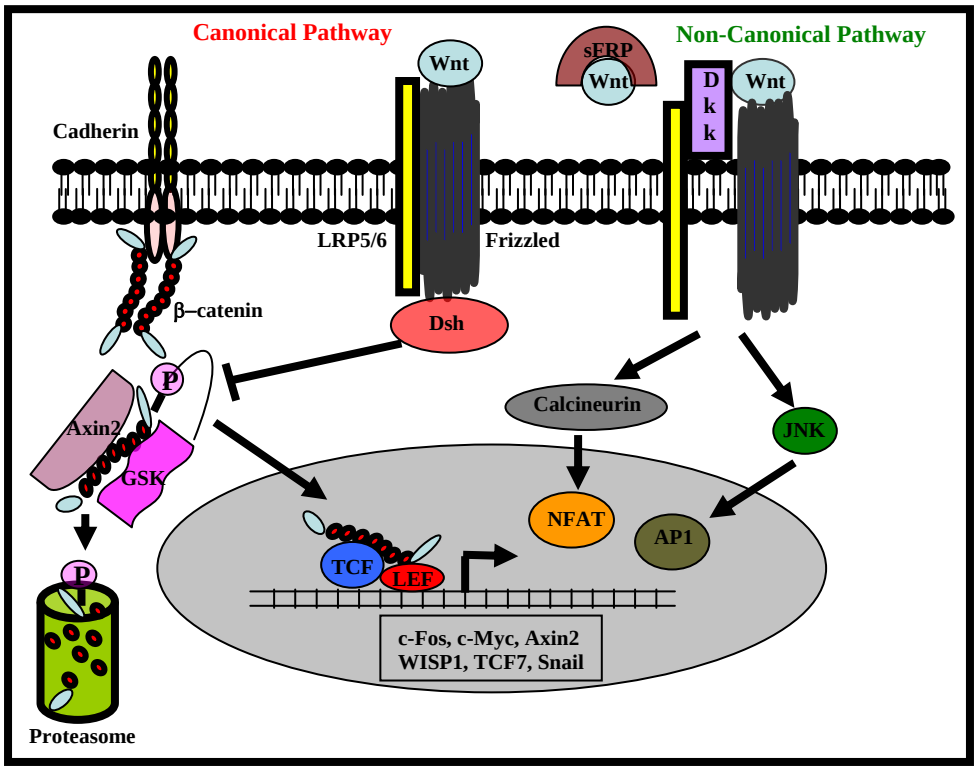


Figure 1.2: **The Wnt Signaling Pathway**

The Wnt signaling pathway in myocardial infarction

Multiple intermediates of the wnt signaling pathway including wnt proteins (Barandon et al., 2003), dsh (Chen, 2004), sFRPs (Barandon et al 2003, Kobayashi et al 2009) and β–catenin (Blankensteijn 2000), have been shown to be induced after MI; which suggests that the wnt signaling pathway may potentially be an MI therapeutic target**.** However, reports about the consequences of wnt pathway manipulation on cardiac recovery after MI have been somewhat contradictory. For example, overexpression of secreted Frizzled Related Proteins sFRP1 and sFRP2, two antagonists of wnt signaling, resulted in improved cardiac function, reduced infarct size and less cardiac rupture after MI in mice, suggesting that blocking wnt signaling is cardioprotective (Barandon et al., 2003;

Mirotsou et al., 2007; Alfaro et al., 2008). Paradoxically, inactivation of sFRP2 was also shown to improve recovery in a mouse MI model (Kobayashi et al., 2009). Consistent with this last outcome, adenovirus-mediated transfer of constitutively active β–catenin, the primary canonical wnt pathway activator, in a rat MI model, resulted in a reduction in myocardial infarct size and improved wound healing, suggesting that activating canonical wnt signaling helps cardiac recovery (Hahn et al., 2006).

These apparently conflicting results may be due to variations between animal models, differential regulation of canonical vs. non-canonical wnt pathway and downstream gene targets by specific modulators, or involvement of wnt intermediates in processes independent of their typical wnt signaling roles. For instance, sFRPs have been shown to be involved in collagen processing (Kobayashi et al., 2009). It is also possible that gain and loss of function approaches differentially trigger competing molecules such as the retinoic acid receptor, which has been shown to prevent stabilized β–catenin from interacting with TCF/LEF factors and activating transcription of target genes (Easwaran et al., 1999; Xiao et al., 2003). Finally, the cell-specificity and timing of wnt pathway activation or suppression likely plays a cell type- and phase-dependent role in the myocardial infarction response, comparable to its described stage-specific roles in heart development (Ueno et al., 2007; reviewed by Aisagbonhi and Hatzopoulos, 2010).

Therefore, to clearly establish whether the canonical wnt/β–catenin signaling pathway should be targeted for MI therapies, it is important to know if the induction of various

wnt pathway mediators after MI results in a net activation of canonical wnt signaling, and in what phase of the MI response canonical wnt induction occurs.

Specific Aims

This dissertation project was aimed at investigating the role the canonical wnt signaling pathway plays in the myocardial infarction response. To this end, we embarked on a detailed analysis of canonical wnt/β–catenin signaling activity in the healthy adult mouse heart and in the adult mouse heart at defined phases of the inherent myocardial infarction response process. We reasoned that answering questions about whether the canonical wnt pathway is induced after MI, at which stage of the infarction response induction occurs and in which cell types, will give clues into the post-MI function of canonical wnt signaling. Such an analysis will help determine whether the canonical wnt pathway should be therapeutically modulated post-MI and at what wound healing stage such interventions should occur.

CHAPTER II

THE CANONICAL WNT SIGNALING PATHWAY IS ACTIVATED AFTER MYOCARDIAL INFARCTION

Introduction

Wnt pathway intermediates have been shown to be increased after myocardial infarction in rat (Hahn et al., 2006), mouse (Blankensteijn et al., 2000; Barandon et al., 2003; Chen et al., 2004; Kobayashi et al., 2008) and even human (Castrillón et al., 2009). However, the simultaneous induction of wnt ligands known to activate canonical and non-canonical branches of wnt signaling, as well as the increase in the expression levels of canonical wnt pathway antagonists such as sFRP2, make it difficult to predict whether canonical wnt activity is induced overall, if activity is confined in specialized areas, or associated with specific cardiac tissue repair processes. To distinguish between these possibilities, the global response of wnt pathway mediators to myocardial infarction was first evaluated. Then, the transgenic mouse line, TOPGAL, which carries the β-galactosidase reporter under the control of a canonical wnt pathway activated promoter that consists of three β–catenin responsive LEF/TCF-binding sites upstream of a minimal *c-fos* gene promoter (DasGupta and Fuchs, 1999), was used to definitively analyze the cardiac regions and cell populations that engage in canonical wnt signaling at baseline, in the healthy adult mouse heart, and after MI.

Results

Expression of Wnt pathway mediators is induced after experimental myocardial infarction

To evaluate the response of wnt pathway components to myocardial ischemic injury, and validate published reports, experimental myocardial infarction was induced by occluding the Left Anterior Descending (LAD) coronary arteries of C57BL/6J mice. Sham-operated mice that underwent the same surgical procedure without arterial ligation served as controls.

Analysis for the expression of the entire set of nineteen wnt ligands, as well as three of Dickkopf (Dkk) family of canonical wnt signaling mediators, using conventional and real-time quantitative RT-PCR analysis, revealed that a number of Wnt ligands are expressed in normal heart including Wnt-2, Wnt-5a, Wnt-5b, Wnt-7b, Wnt-9a and Wnt-11 (Fig. 2.1). Following experimental myocardial infarction, there is a strong upregulation in the transcript levels of both wnt ligands and members of the Dkk family (Fig. 2.1). Specifically, by quantitative analysis we observed an induction of Wnt-2 (11-fold), Wnt-4 (18-fold), Wnt-10b, (6-fold), Wnt-11 (3-fold), Dkk-1 (11-fold) and Dkk-2 (6-fold) (Fig. 2.2).

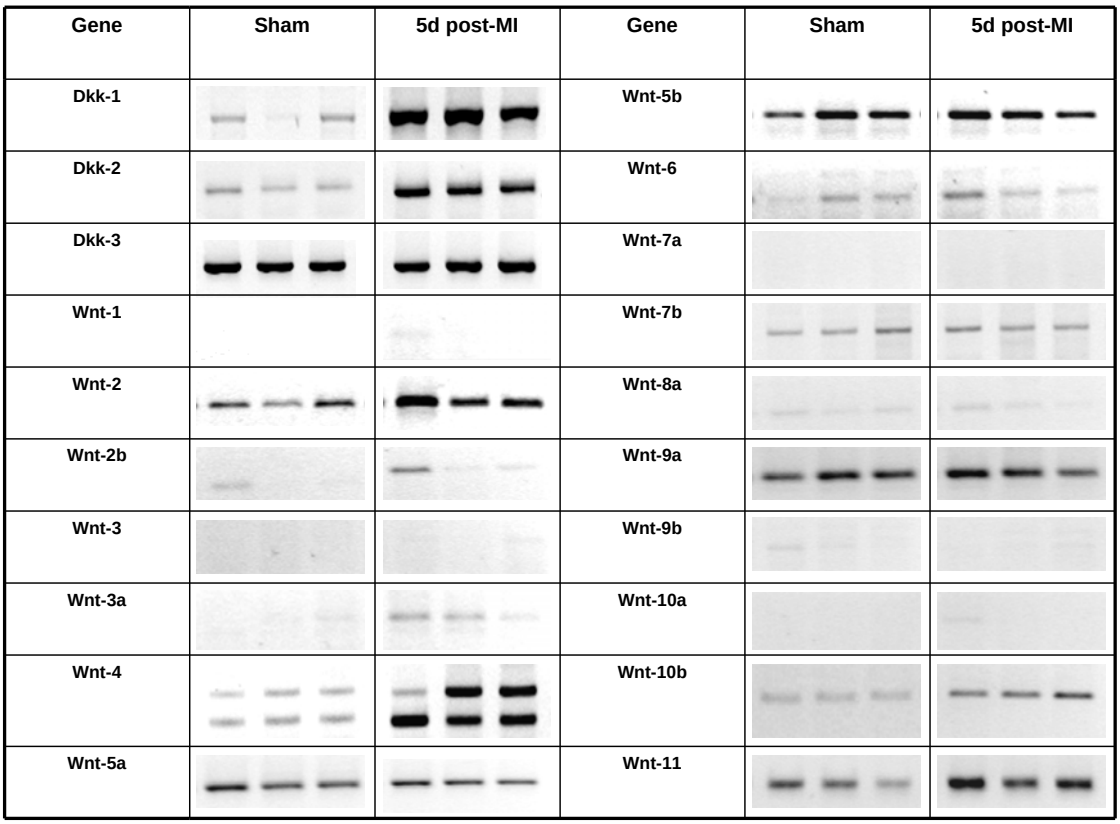


Figure 2.1: **Wnt pathway intermediates are increased after myocardial infarction.** RT-PCR was performed on RNA isolated from C57BL/6 hearts five days after LAD occlusion or sham surgeries

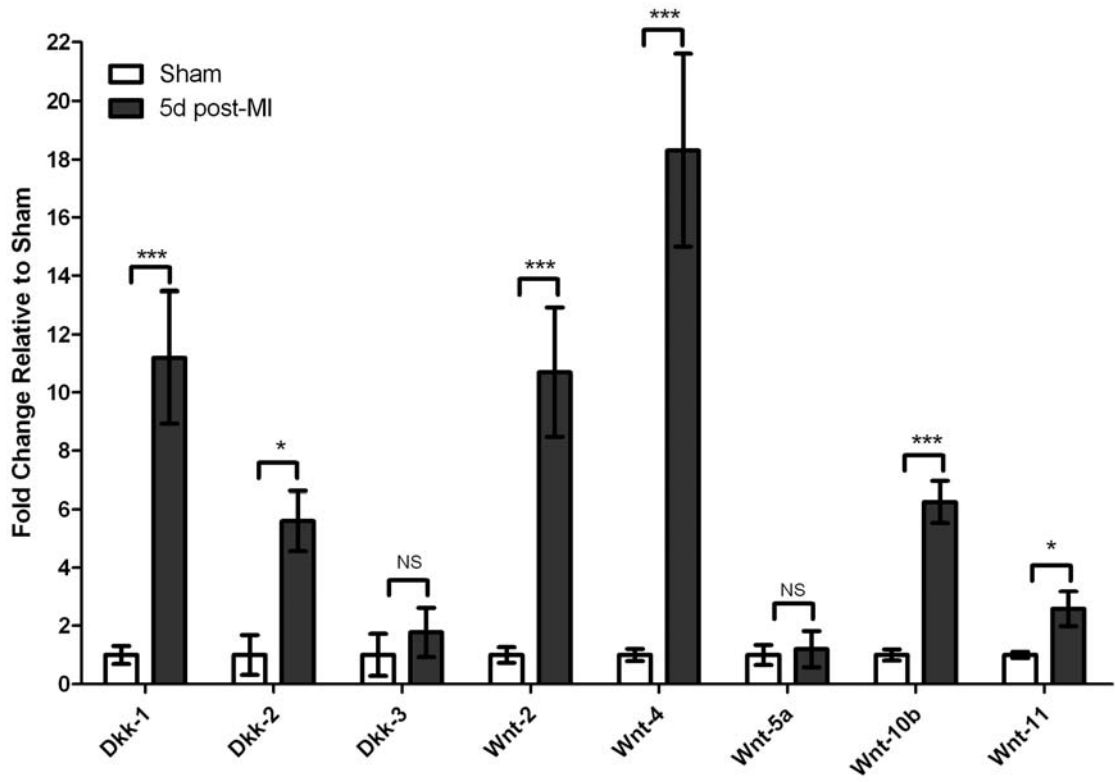


Figure 2.2: **Quantitative PCR analysis for the expression of Wnt pathway intermediates.** Quantitative real-time RT-PCR analysis for the expression of wnt pathway mediators was performed on mRNA isolated from the hearts of C57BL/6 mice five days after being subjected to either permanent LAD occlusion or sham surgeries. *P<0.05, **P<0.01, ***P<0.001. Sham N=6; MI N=6

Immunohistological analysis for changes in β–catenin expression revealed an increase in β–catenin staining at the cardiomyocyte intercalated disc 24 hours after MI (Fig 2.3), followed by an increase in cytoplasmic and nuclear β–catenin 7 days after MI (Fig 2.4).

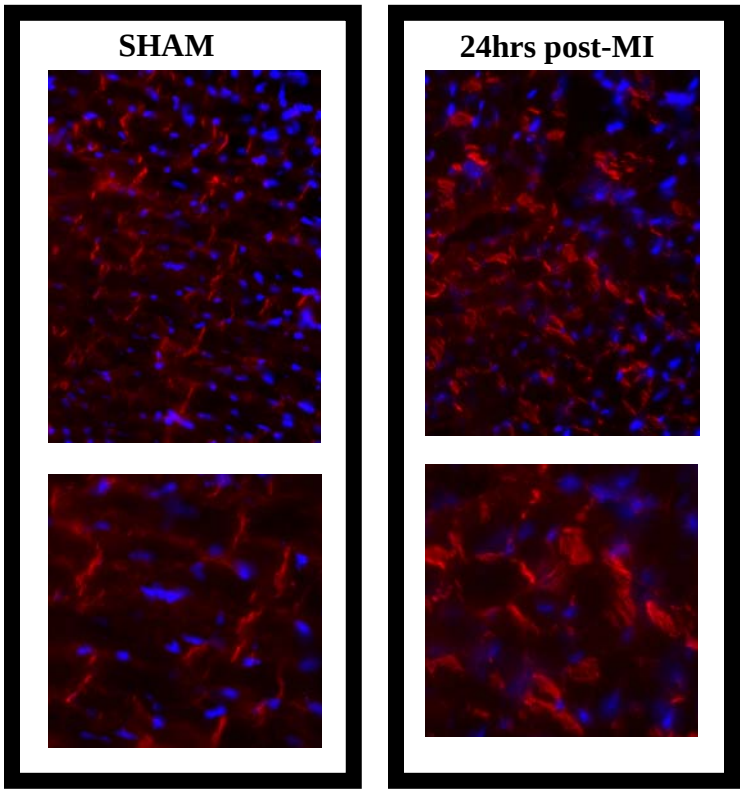


Figure 2.3: **Increased β–catenin immunoreactivity at the cardiomyocyte intercalated disc 24 hours after experimental MI.** C57BL/6 mice were subject to LAD occlusion or sham surgeries. Hearts were isolated 24 hours after surgery and stained for β–catenin

The increase in wnt pathway intermediates, such as β–catenin, after MI do not necessarily mean that canonical wnt signaling is activated, as molecules like the retinoic acid receptor can prevent stabilized β–catenin from interacting with TCF/LEF factors and activating transcription of target genes. Therefore, to more directly assess canonical wnt signaling activity, we turned to the TOPGAL transgenic canonical wnt reporter mouse line to evaluate canonical wnt activity in the healthy adult mouse heart and after MI.

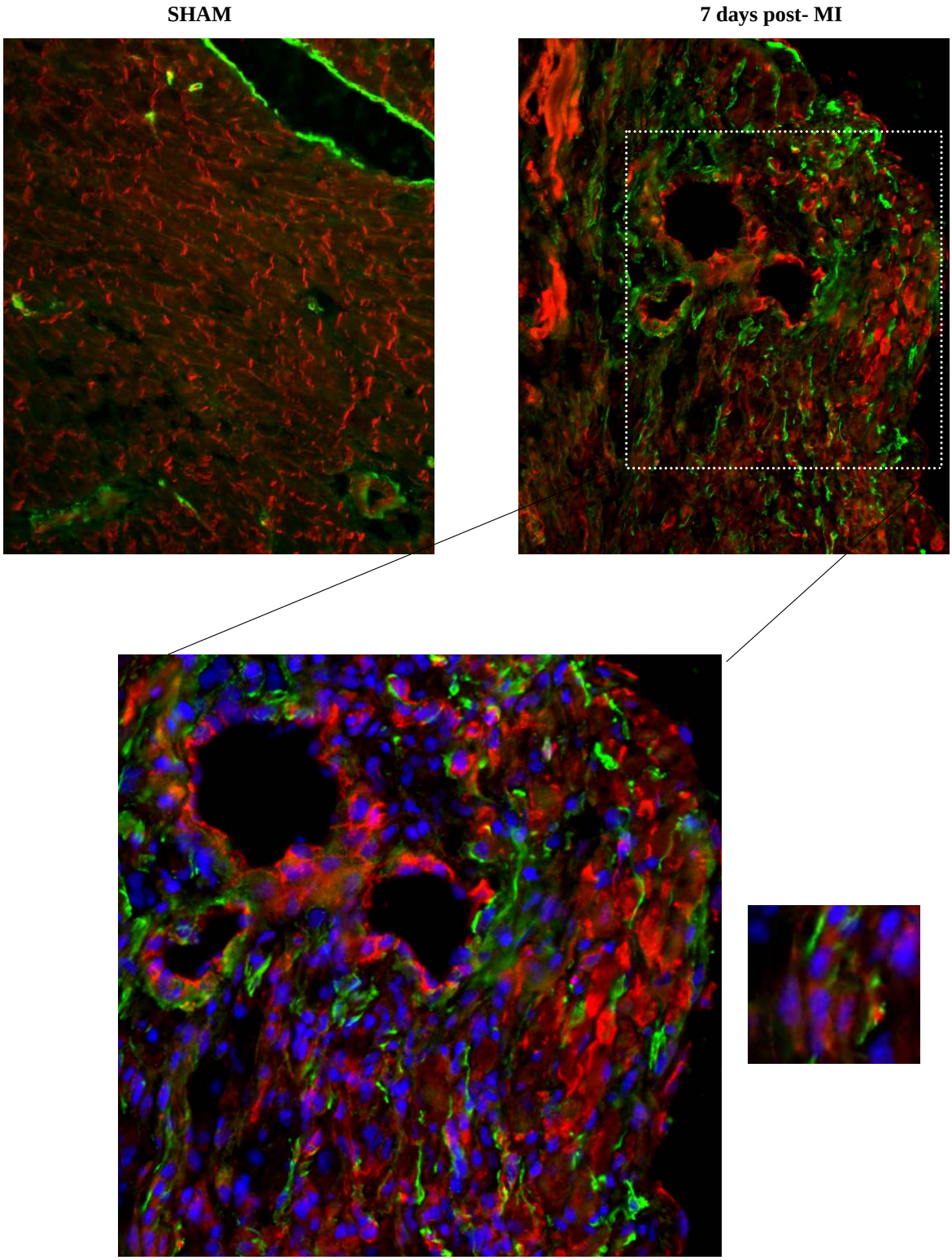


Figure 2.4: **Increased cytoplasmic and nuclear β–catenin 7 days after MI**. C57BL/6 mice were subject to LAD occlusion or sham surgeries. Hearts were isolated 7 days after surgery and stained for the canonical wnt activator, β–catenin (red) and smooth muscle actin (green). Notice that in the sham heart, smooth muscle actin-positive cells are contained within blood vessels but after MI, there are migratory smooth muscle actin-positive cells (myofibroblasts) that have β–catenin in their nuclei.

Canonical wnt signaling is active in the vasculature and valves of the adult mouse heart

To appreciate changes in canonical wnt signaling after ischemic injury following arterial ligation, canonical wnt/β–catenin signaling in the normal adult heart was first analyzed. To this end, hearts of TOPGAL mice were isolated and subjected to whole mount staining for β-galactosidase activity and serial histological analysis (Fig. 2.5). The results show that in the normal heart, canonical wnt signaling activity is present primarily in large blood vessels at the base of the heart and the valves (Fig. 2.5 A,B). Histological sections revealed that β–catenin signaling is active in the media and intima of the aorta and pulmonary vessels, and in the valve mesenchyme. Furthermore, there is activity in coronary vessels and subepicardial microvasculature (Fig. 2.5 C).

The staining patterns suggest that canonical wnt activity marks subpopulations of both endothelial and smooth muscle cells in the heart. To confirm this finding, histological sections stained with antibodies against the endothelial-specific marker CD31 (PECAM-1) and smooth muscle actin (SMA) were analyzed (Fig. 2.6 A,B). The results confirmed expression in subsets of smooth muscle and endothelial cells. Taken together, the results suggest that during cardiac homeostasis, canonical wnt activity is confined in select subpopulations of endothelial and smooth muscle cells as well as in valve mesenchymal cells.

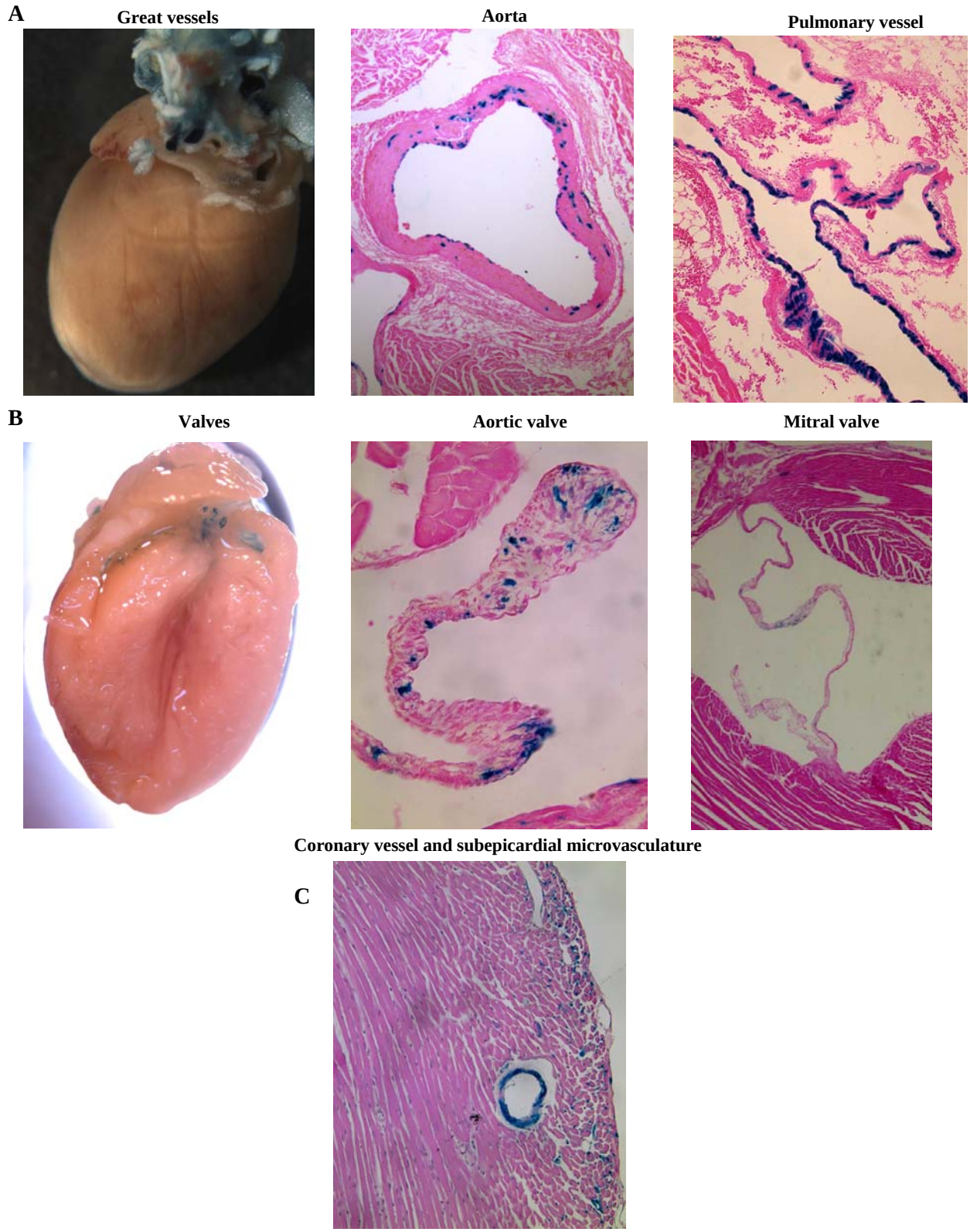


Fig. 2.5**: Canonical wnt signaling activity in the healthy adult mouse heart.** Whole mount and histological analyses of canonical wnt activity in the adult heart using the TOPGAL mouse line. (A) X-gal-stained whole mount heart shows canonical wnt activity in the great vessels at the base of the heart. Tissue sections of this area show canonical wnt activity in the media and intima of aortic and pulmonary vessels. (B) Whole mount X-gal staining of partially dissected heart to show cells with canonical wnt activity in cardiac valves (left). Histological sections (right panels) reveal cells positive for canonical wnt activity in the mesenchyme of outflow tract (aortic), and atrioventricular (mitral) valves. (C) Histological section shows canonical wnt activity in coronary arteries and subepicardial microvasculature (right).

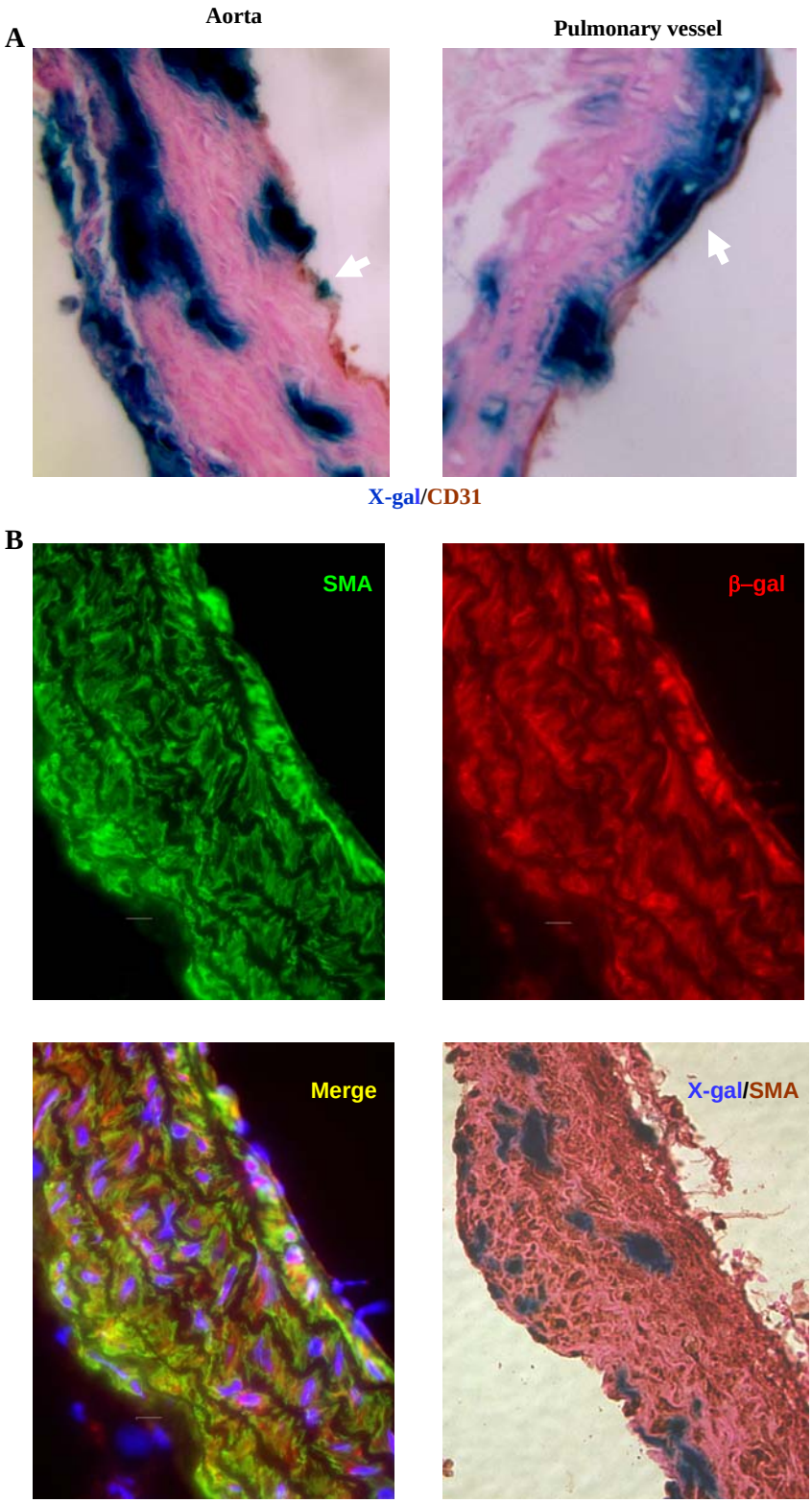


Figure 2.6: **Canonical wnt signaling is active in endothelial and smooth muscle cells.** (A) CD31 antibody stain (brown) on X-gal-stained section from great vessels of TOPGAL mouse shows canonical wnt activity in vascular endothelial cells (white arrow). (B) IF images of tissue sections stained with antibodies recognizing a-SMA in vascular smooth muscle cells (green) and β–galactosidase (red). Merged images show colocalization of the two antigens in cells of the intima and media areas of the aorta. Bottom right, SMA antibody stain (brown) on X-gal-stained aorta section indicates canonical wnt activity (blue) in perivascular SMA+ cells.

Canonical wnt signaling is activated in the infarct and peri-infarct areas during granulation tissue formation

To test whether the pattern of canonical wnt pathway is altered in response to myocardial infarction, TOPGAL mice were subjected to LAD occlusion or sham surgeries, hearts were isolated at defined time points after arterial ligation, i.e. at 24 hours post-MI during the cell death and inflammation phase, 4-7 days during granulation tissue formation and 3 weeks when scar tissue had matured, and then stained for β-galactosidase activity (Fig. 2.7).

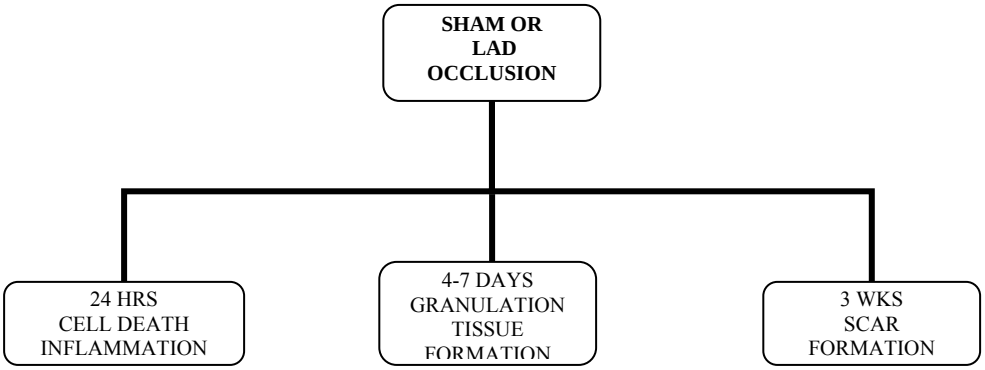


Figure 2.7: **Experimental Scheme: The post-MI timepoints at which TOPGAL mouse hearts were isolated and stained for β–galactosidase activity**.

The results show that canonical wnt signaling is not activated as part of the early infarction response as the β–galactosidase activity in TOPGAL mouse hearts isolated within the first 24 hours after coronary artery ligation is practically indistinguishable from sham-operated controls (Fig. 2.8). In contrast, canonical wnt positive cells appear scattered throughout the myocardium 4 days after LAD ligation. By 7 days post-MI, canonical wnt activity is primarily localized to large numbers of cells within the infarct and peri-infarct areas. This activity is transient and disappears by the time of scar maturation, 3 weeks post-infarction.

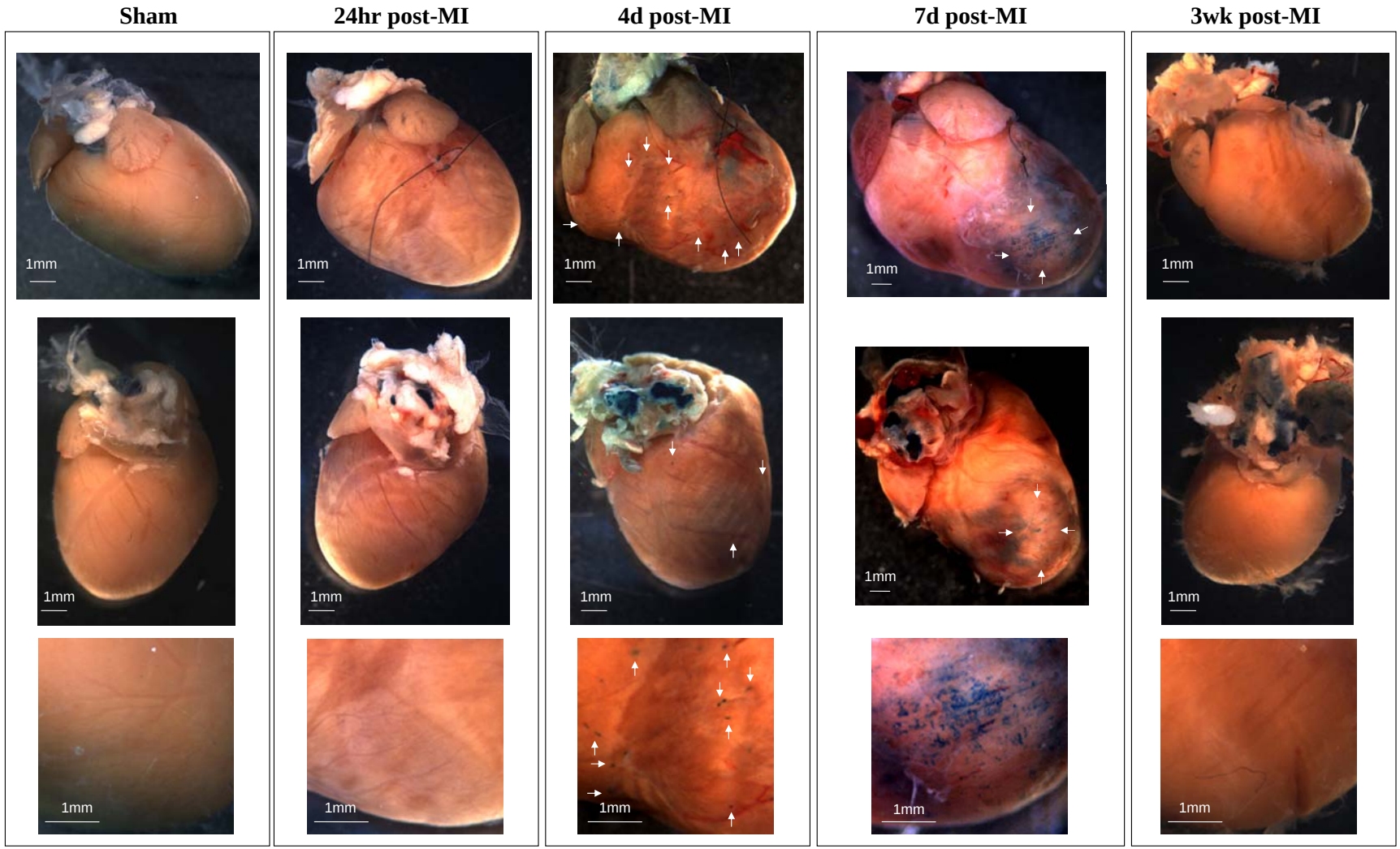


Figure 2.8: **The canonical wnt signaling pathway is activated during the granulation tissue formation stage of the MI response**. Experimental model of MI was induced by subjecting TOPGAL mice to LAD occlusion or sham surgeries. Hearts were isolated at defined stages of the MI response process and stained with X-gal to assess canonical wnt activity. Panel 1: Whole mount X-gal-stained sham heart shows gross canonical wnt activity is limited to the great vessels. Panel 2: Whole mount X-gal-stained heart 24hrs after LAD occlusion shows induction of the canonical wnt signaling pathway does not occur in the early stages of the myocardial infarction response. Panel 3: Whole mount X-gal-stained heart demonstrates novel activation of the canonical wnt signaling pathway throughout the myocardium 4 days after LAD occlusion. Panel 4: Whole mount X-gal-stained heart shows that induced canonical wnt activity becomes limited to the peri-infarct area 7 days after LAD occlusion. Panel 5: Whole mount X-gal-stained heart shows that infarction-induced canonical wnt activity is down-regulated by 3 weeks. Top images show anterior views, middle images show posterior views and bottom images represent higher magnifications of areas distal to LAD occlusion.

Interestingly, the severity of the injury, estimated by echocardiography-based measurements of fractional shortening and other cardiac functional parameters, directly correlates with post-MI day 7 levels of canonical wnt activity as assessed by β−galactosidase staining, suggesting a role for canonical wnt positive cells in the cardiac tissue repair process during granulation tissue formation (Fig. 2.9).

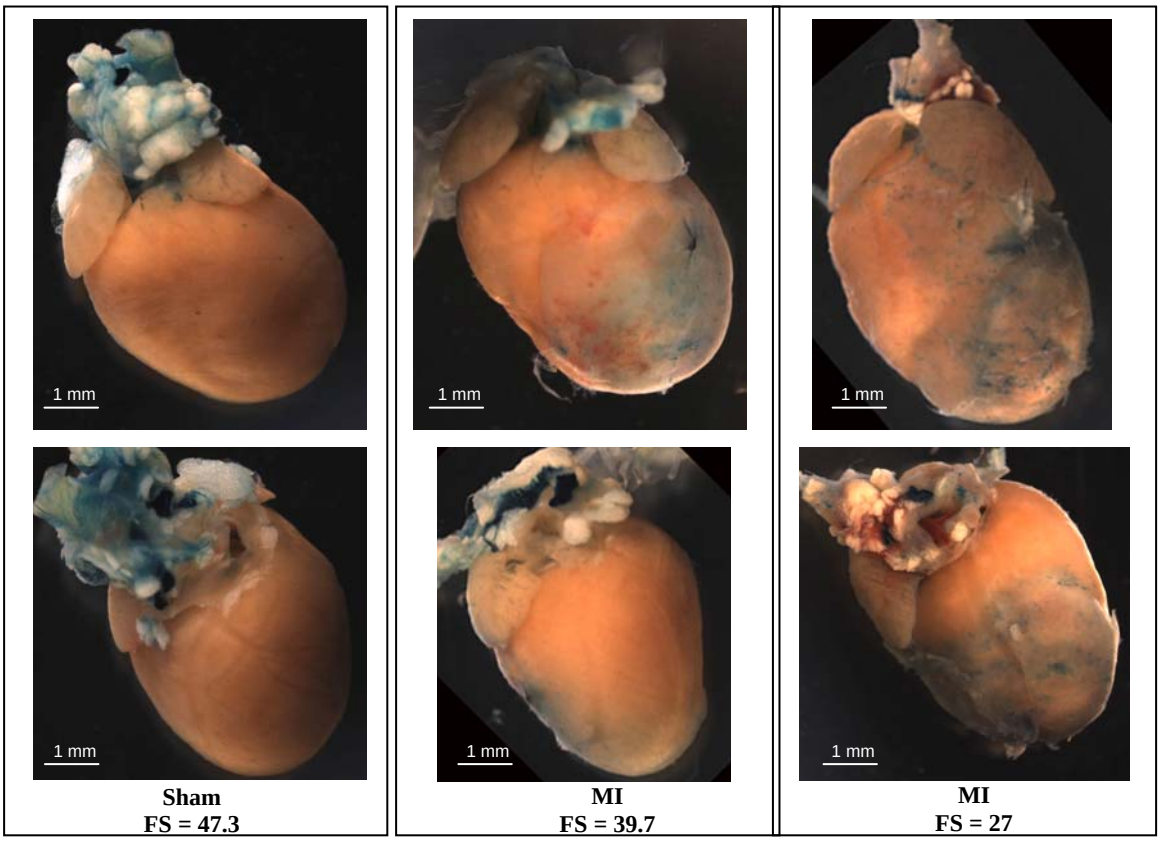


Figure 2.9: **Peri-infarct canonical wnt activity correlates to infarct severity.** Myocardial infarctions of different severities were performed in TOPGAL mice by varying the heights of LAD ligation sutures. 7 days after surgery, echocardiography was performed to evaluate cardiac function. Hearts were then isolated and X-gal stained to assess canonical wnt activity in relation to infarct severity.

Canonical wnt targets are induced during post-MI granulation tissue formation

As an independent read-out for canonical wnt pathway activation, real-time quantitative PCR analysis was performed to follow the expression of down-stream gene targets of the β–catenin signaling pathway at the defined MI response stages.

Consistent with the timing of β-galactosidase staining in TOPGAL mice post-MI, real-time quantitative PCR analysis shows a strong up-regulation of *bona-fide* β–catenin signaling gene targets such as *c-Fos, c-Myc, Wnt-10b, Wisp1*, *Tcf7 and Snail* seven days post-MI (Fig. 2.10). Among tested canonical wnt signaling targets, the early response genes, *c-Fos* and *c-Myc* show the strongest induction at 24 hours, probably through canonical wnt-independent pathways.

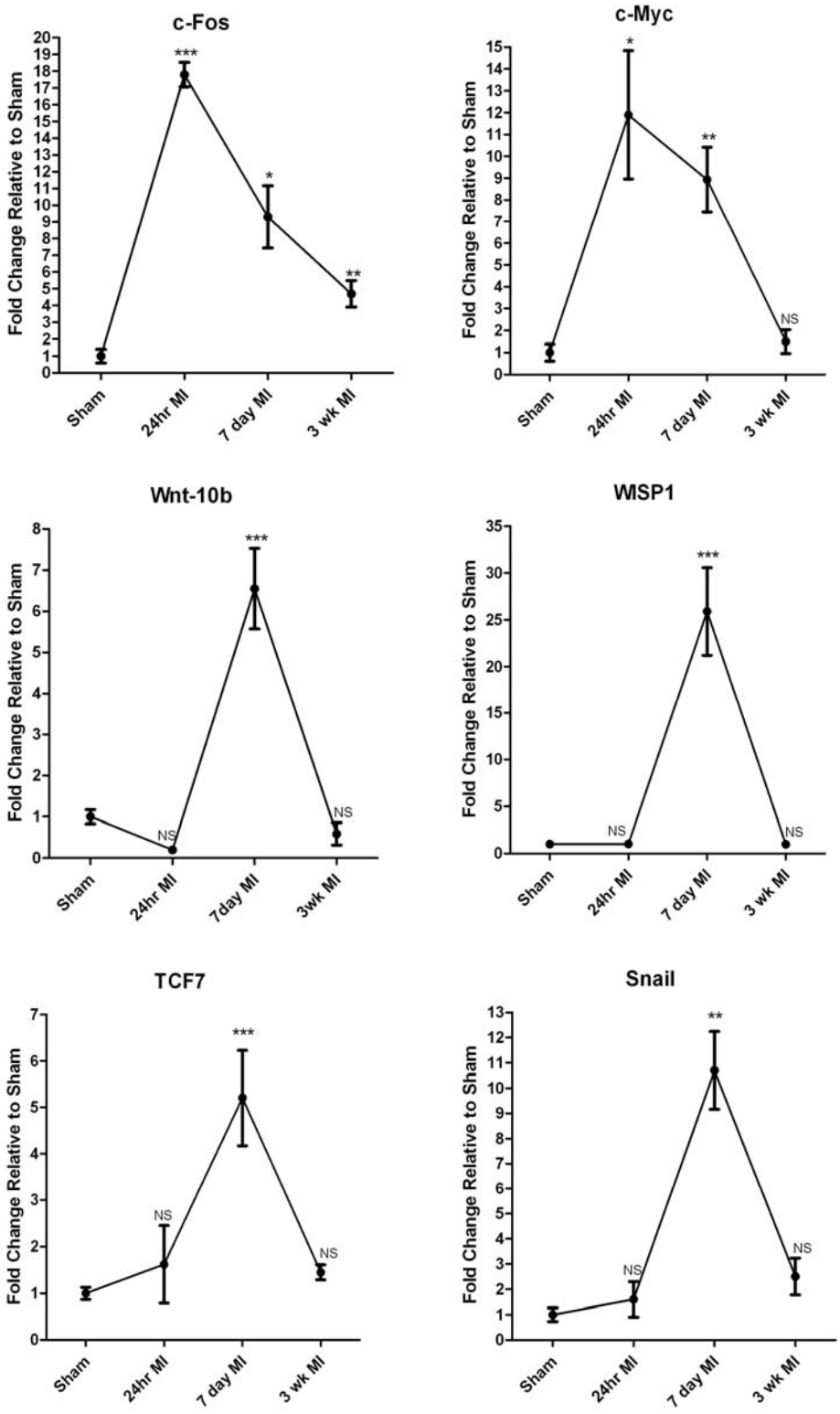


Figure 2.10: **Canonical wnt targets are induced after MI.** Real-time quatitative RT-PCR analysis for the expression of canonical wnt targets was performed on mRNA isolated from TOPGAL mouse hearts at defined stages of the myocardial infarction response process. *P<0.05, **P<0.01, ***P<0.001. Sham N=6; 24hr N=3; 7-day N=6; 3-week N=3.

Canonical wnt signaling is activated in endothelial and mesenchymal cells after MI

Granulation tissue formation is characterized by endothelial cell proliferation and migration to form new blood vessels and myofibroblast activation and migration to lay down collagen for subsequent scar formation. To identify the cell types marked by canonical wnt signaling activity during this stage, histological sections of TOPGAL mouse hearts 7 days after LAD occlusion or sham surgery were analyzed. The analyses show that surrounding the infarct area after MI, there is an increase in canonical wnt signaling active endothelial cells.

However, the intense staining observed in the whole-mount images at day 7 post-MI (Fig. 2.8) is mainly due to the *de novo* appearance of large numbers of subepicardial mesenchymal-like cells marked with canonical wnt signaling activity (Fig. 2.11 A and B). It should be noted that canonical wnt active-mesenchymal cells are not present in the subendocardium (Fig. 2.11 C).

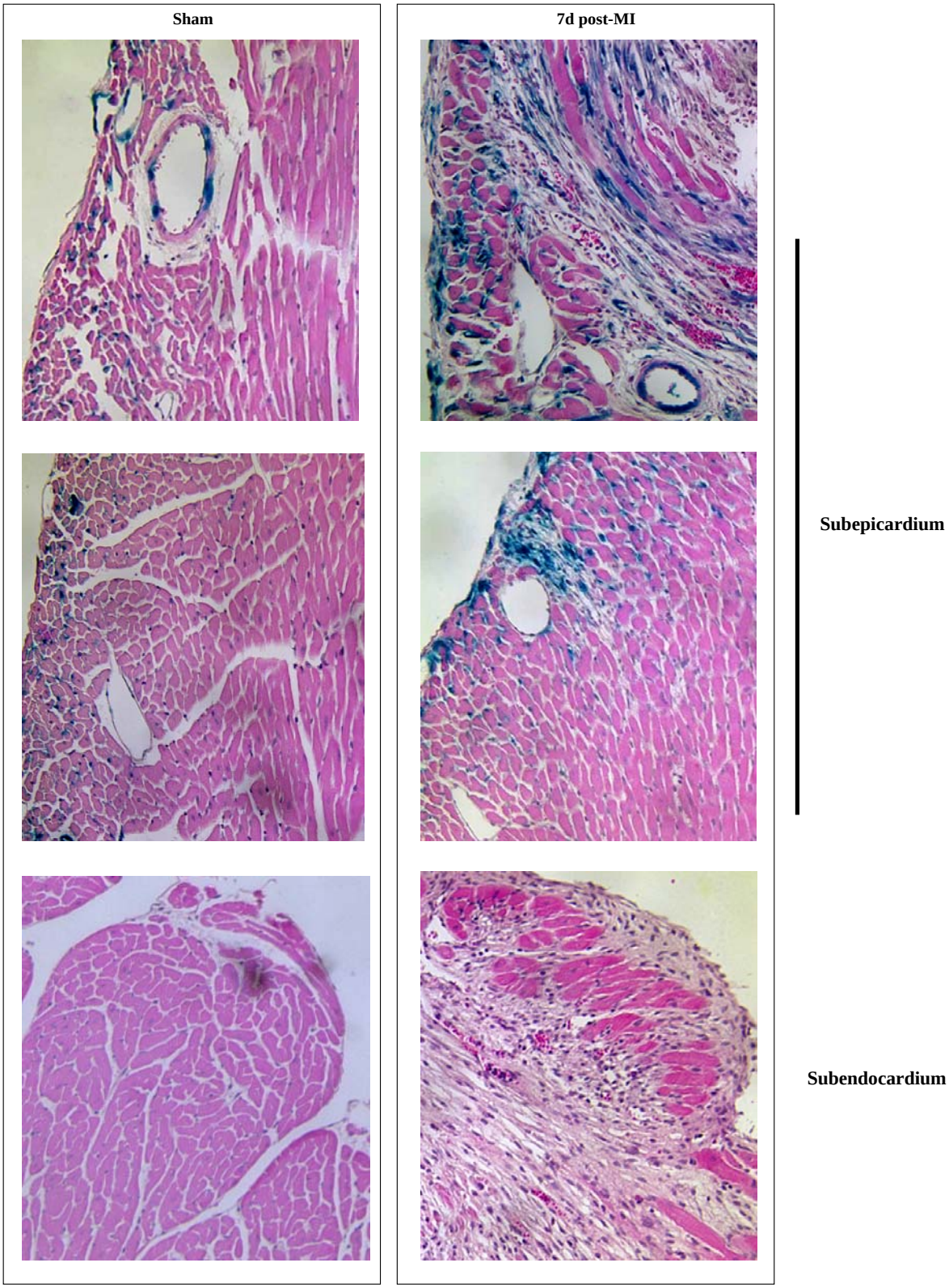


Figure 2.11: **Canonical wnt signaling is active in peri-infarct endothelial and mesenchymal cells after MI**. Histological analysis of canonical wnt activity was performed on TOPGAL mouse hearts 7 days after LAD occlusion or sham surgery. Tissue sections of X-gal-stained mouse hearts shows that in sham cardiac tissue, β–galactosidase/canonical wnt signaling activity is limited to the endothelium and pericytes of coronary vessels and in microvascular endothelial cells (left panel). After MI, in addition to coronary vasculature and microvasculature, canonical wnt signaling marks newly appearing subepicardial mesenchymal-like cells surrounding the infarct area and a subset of epicardial cells. In contrast, canonical wnt signaling active-cells are not present in subendocardial regions (bottom panel).

Immunohistochemical (IHC) and immunofluorescence (IF) analyses using antibodies recognizing the endothelial marker CD31 and the myofibroblast marker SMA confirmed that post-MI canonical wnt signaling is active in endothelial cells and SMA-expressing mesenchymal cells in the infarct and peri-infarct areas (Fig. 2.12 A,B).

To get a measure of the scale of the increase in canonical wnt activity, canonical wnt-positive endothelial and mesenchymal cells in the infarct and peri-infarct areas were counted and cell counts were compared to canonical wnt-active cell counts in the corresponding cardiac tissue sites of sham-operated mice. The results showed that the fraction of endothelial cells marked with β-galactosidase activity increases from about 30% in normal subepicardial heart tissue to 50% after MI. In parallel, the overall fraction of β-galactosidase positive SMA-expressing cells rises from almost undetectable levels to 12% of the entire subepicardial cell population. The quantitative cell count analysis also shows that canonical wnt-active SMA^+ mesenchymal cells represent about 40% of SMA^+ cells in the peri-infarct subepicardial area (Fig. 2.13 A). Interestingly, the number of SMA^+ cells in the infarcted myocardium, as measured by FACS analysis, correlates with the severity of injury as assessed by echocardiographic measurement of fractional shortening (Fig. 2.13 B).

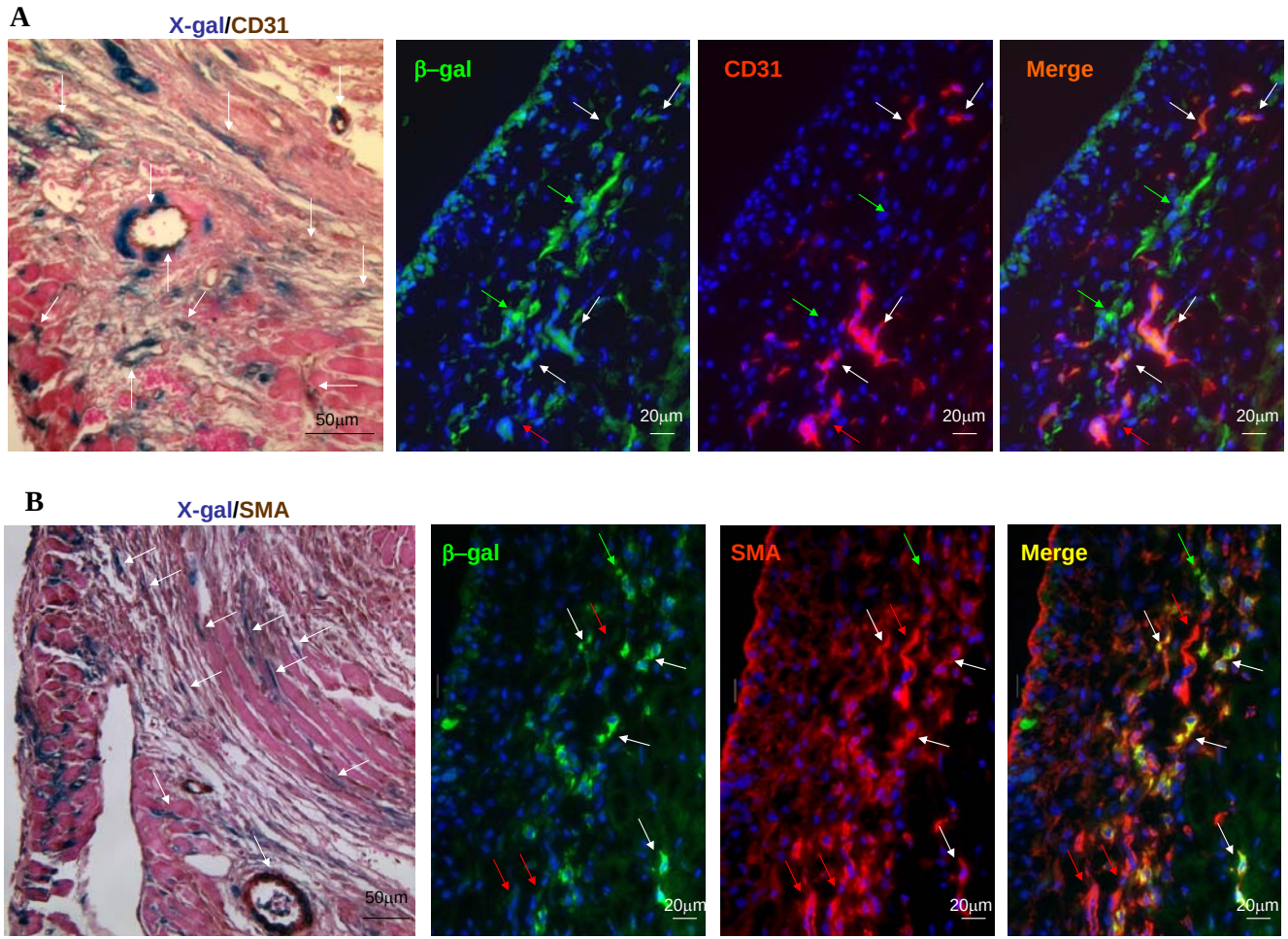


Figure 2.12: **Post-MI canonical wnt active cells express CD31 and Smooth muscle actin.** (A) IHC using the anti-CD31 antibody on X-gal stained cardiac tissue shows canonical wnt activity (blue) in endothelial cells (brown) in the peri-infarct area (white arrows). The three left panels display IF results obtained using the anti- β–galactosidase (green) and CD31 (red) antibodies and the corresponding merged image. Canonical wnt activity marks endothelial cells (white arrows). Examples of endothelial cells without canonical wnt activity (red arrows) and non-endothelial, canonical wnt-pathway active cells (green arrows) are indicated. (B) IHC analysis using the anti-α–SMA antibody on sections of X-gal stained cardiac tissue from TOPGAL mice shows canonical wnt activity (blue) in SMA+ cells (brown) in the peri-infarct area (white arrows). The three left panels display IF results obtained using the anti- β–galactosidase (green) and α–SMA (red) antibodies and the corresponding merged image. Canonical wnt activity marks SMA+ cells (white arrows). Green arrows point to SMA- cells with canonical wnt-pathway activity and red arrows to SMA+ cells lacking canonical wnt activity.

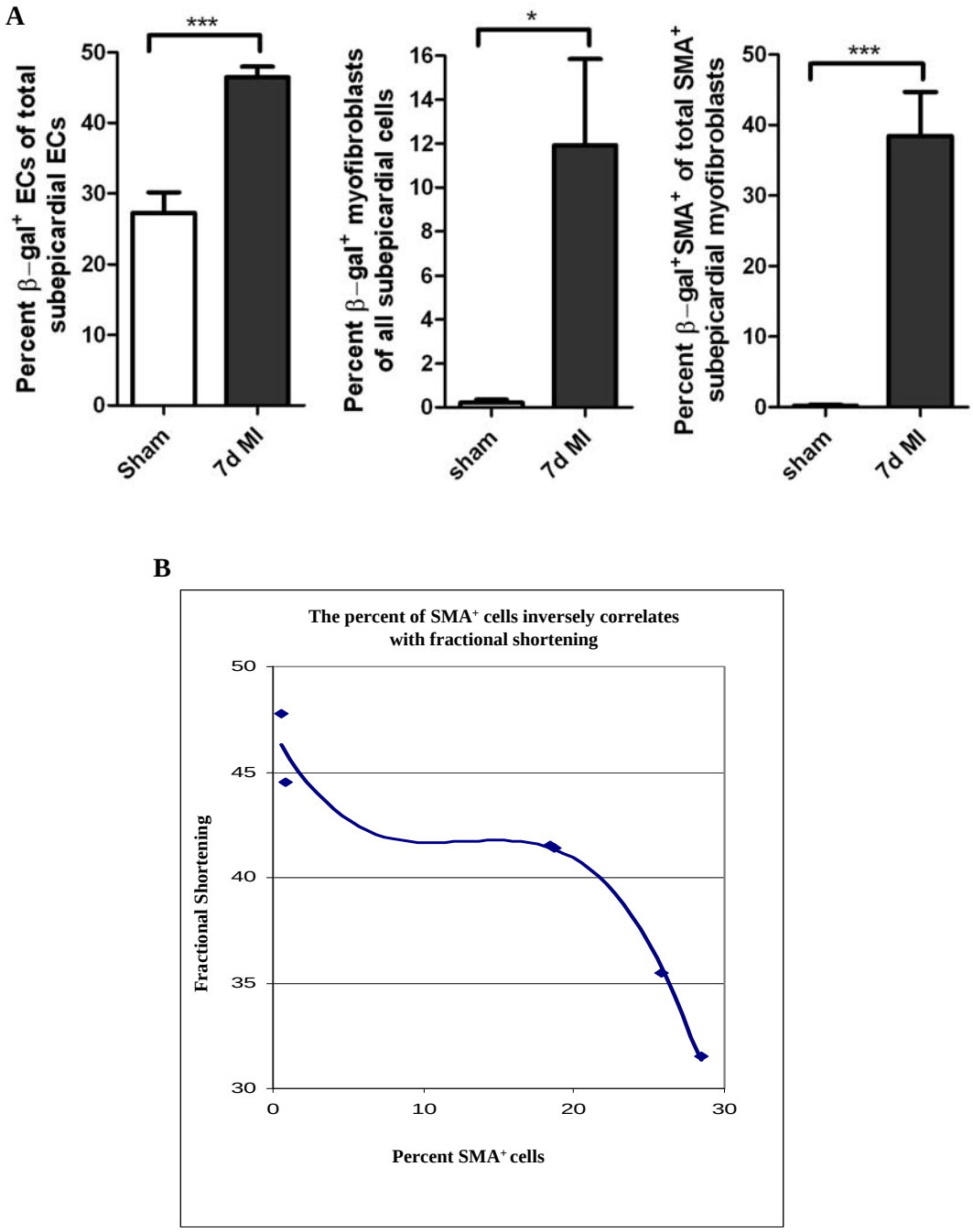


Figure 2.13: **Significant increase in canonical wnt-active cells after MI.** (A) Canonical wnt-activity-marked endothelial cells were quantified by counting the number of double X-gal-stained and CD31-positive cells per visual field. Canonical wnt-active SMA+ cells were quantified by counting the number of SMA-expressing X-gal-positive cells as a percentage of the total cell population in a given visual field, or as a percentage of the total SMA+ cell population. **P*<0.05, ***P*<0.01, ****P*<0.001, NS: not significant. Control sham *N*=5; MI samples *N*=5. (B) Percentage of SMA^+ cells correlates to infarct severity. Echos were performed on mice 7 days after LAD occlusion or sham surgeries. Hearts were then isolated and subjected to FACS analysis with anti-SMA antibody.

Discussion

The canonical wnt signaling pathway may be an important MI therapeutic target because transplanted stem/progenitor cells which yield beneficial paracrine effects after cardiac injury have been shown to express wnt pathway mediators (Kupatt et al., 2005b; Mirotsou et al., 2006; Alfaro et al., 2008). In addition, endogenous wnt pathway intermediates are increased after myocardial infarction (Blankensteijn et al., 2000; Barandon et al., 2003; Chen et al., 2004; Kobayashi et al., 2009). However, because controversies exist as to the effect wnt pathway manipulation has on post-infarction cardiac wound healing and remodeling, we sought to study post-MI canonical wnt signaling activity in more detail.

Our results show that in the normal, uninjured mouse heart, there is active canonical wnt signaling in subpopulations of endothelial cells and pericytes mainly around the aorta and pulmonary vessels, in coronary arteries and subepicardial microvasculature. Moreover, interstitial cells marked with canonical wnt activity are present within the connective tissue of the valve leaflets. After MI, there is an increase in canonical wnt active-cells that begins at 4 days and is followed by a massive accumulation of canonical wnt-positive cells in the infarct and peri-infarct areas 7 days post-MI. Therefore, it appears that canonical wnt signaling does not play a significant role in the original stages of ischemic injury during cardiomyocyte death and inflammation. Instead, its activity peaks in late reparative processes, during granulation tissue formation, where the number of canonical wnt positive cells directly correlates with the severity of the original ischemic injury.

Consistent with the whole mount and histological staining for β-galactosidase/active β-catenin, the expression of known canonical wnt signaling targets also shows maximum levels during the same time – with the exception of *c-Fos* and *c-Myc* which are induced shortly after infarction. These results are in agreement with a recent study, which showed that the activity of the *Axin2* promoter, a canonical wnt signaling target, also reaches its height 1-2 weeks after experimental MI (Oerlemans et al., 2010).

Taken together, the results show that the canonical wnt signaling pathway is induced during post-MI granulation tissue formation, a period characterized by proliferation and migration of endothelial cells and myofibroblasts. Consistent with this, the induced canonical wnt signaling activity is observed in these cell types. The question of what process or processes canonical wnt pathway activation mediates in endothelial cells and myofibroblasts is addressed in subsequent chapters.

Methods

Mice

The TOPGAL mice express β–galactosidase under the control of β–catenin-responsive consensus LEF/TCF-binding motifs upstream of a minimal *c-fos* promoter promoter (DasGupta and Fuchs 1999). All procedures were carried out with the approval of the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center. The TOPGAL mouse line was purchased from Jackson Laboratories.

Experimental myocardial infarction

Mice underwent open chest surgery under anesthesia. During surgery, a 10−0 nylon suture was placed through the myocardium into the anterolateral Left Ventricular wall around the left anterior descending artery and the vessel was permanently ligated. After surgery, the chest was closed and the animals were allowed to recover. At defined time points after surgery, i.e., 1 day, 4 days, 1 week and 3 weeks, mice were euthanized and whole hearts were isolated for whole-mount X-Gal staining and histological analysis, or used to obtain RNA for gene expression studies. Sham-operated animals underwent similar procedures without coronary artery ligation. Surgeries were performed in the Vanderbilt mouse Cardiovascular Pathophysiology & Complications Core.

Echocardiography

Two-dimensional M Mode echocardiography was performed on conscious hand held-restrained mice using a 15-6L probe. Analysis was performed on HP Sonos 5500.

β-galactosidase activity whole tissue staining assay

Whole hearts were isolated into cold 1X phosphate-buffered saline (PBS) and then fixed for 30 minutes at 4^{o}C in 1X PBS solution containing 1% formaldehyde, 0.2% glutaraldehyde and 0.02% NP40. After fixation, hearts were washed twice with 1X PBS for 20 minutes each, and then placed overnight at 30^{o}C in X-gal staining solution (1mg/mL X-gal, 5mM potassium ferro- and ferricyanate and 2mM magnesium chloride in 1X PBS). Whole mount hearts were photographed and then stored in 1X PBS at 4^{o}C

until embedded in paraffin and cut in 5 μm sections. Sections were deparaffinized, counter-stained with eosin, dehydrated and mounted.

Immunofluorescence (IF) and immunohistochemistry (IHC)

For IF experiments, freshly isolated hearts were embedded in Optimal Cutting Temperature compound (OCT) and cut in 5 μm sections. Before antibody staining, slides were thawed at room temperature, immersed in 4% para-formaldehyde and fixed for 5 minutes on ice. Slides were washed three times in 1X PBS for 5 minutes each wash. Sections were blocked in a 1X PBS solution of 1% bovine serum albumin and 0.05% saponin for 1 hour at room temperature. Sections were then incubated with primary antibodies overnight at 4^{o}C. Afterwards, slides were washed three times in 1X PBS for 5 minutes each, incubated with secondary antibodies for 1 hour at room temperature, washed in 1X PBS three times for 5 minutes each, and mounted with Vectashield fluorescent mounting medium (Vector Laboratories).

For IHC stains, 5 μm sections of LacZ-stained paraffin-embedded hearts were deparaffinized through histoclear and graded alcohols as per standard protocol. Endogenous peroxidase activity was quenched by immersing slides in 0.3% peroxidase dissolved in blocking solution for 5 minutes at room temperature. Sections were then blocked and stained with primary antibodies as described above for IF. Afterwards, sections were washed three times for 5 minutes each in 1X PBS and then incubated with ImmPress Universal reagent (Vector laboratories) when mouse or rabbit primary antibodies were used, or with Anti-Rat Ig Horse Rabbit Peroxidase detection kit (BD Pharmingen) when rat primary antibodies were used. Kits were used according to

manufacturers' instructions. Slides were counter-stained, dehydrated and mounted with Permount histological mounting medium (Fisher). Histological services were perfomrd by the Vanderbilt Histology Core.

Antibodies used for histological analysis and their various dilutions in blocking solution were: Rabbit IgG fraction against β−galactosidase (Cappel Pharmaceuticals; 1:2,000 dilution), monoclonal Mouse anti-β−catenin (Sigma; 1:800 dilution) monoclonal Mouse anti-smooth muscle alpha actin (Sigma; 1:800 dilution) and Rat anti-mouse CD31/PECAM1 (BD Pharmingen; 1:100 dilution).

Secondary antibodies used for IF were: Goat anti-Mouse Cy3-conjugated, Donkey anti-Rabbit Cy3, Goat anti-Rat Cy3, Goat anti-Mouse Alexa488 and Goat anti-Rabbit Alexa488. Cy3-conjugated antibodies were obtained from Jackson Immunoresearch and Alexa488-conjugated antibodies from Invitrogen. All secondary antibodies were used in a 1:200 dilution. To visualize and quantify cells in IF experiments, cardiac tissue sections were stained with the fluorescent dye DAPI (4',6-diamidino-2-phenylindole) (1:5,000 dilution; Invitrogen) to mark cellular nuclei.

FACS analysis

Suspensions of cardiac cells depleted of myocytes were prepared as follows. Murine hearts were washed to remove blood and aseptically isolated after incision at the base of the aorta. The atria were entirely removed. The heart's ventricles were minced and digested with 10 mg/ml collagenase II (Worthington), 2.4 U/ml dispase II (Roche

Diagnostics), DNase IV (Sigma) in 2.5 mM $CaCI_2$ at 37°C for 20-25 minutes and then passed through a cell strainer. The myocyte-depleted cell suspension was centrifuged at 1,500 x *g* for 5 minutes and resuspended in 1X PBS containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA.

To prevent non-specific binding, cells were incubated with FcR Blocking Reagent (Miltenyi Biotec Inc). For intracellular staining to detect α-smooth muscle actin (α-SMA), cells were fixed and permeabilized using 4% paraformaldehyde and 0.1% saponin in 1X PBS. Aliquots of cells (10^5 cells in 100 μL buffer) were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-α-SMA (Sigma) for 30 minutes at 4°C. Next, cells were washed once with 0.1% saponin and resuspended in 1X PBS containing 0.5% BSA and 2 mM EDTA. Data acquisition was performed on a FACScalibur flow cytometer (BD Immunocytometry Systems) in the Vanderbilt Flow Cytometry Core and the data were analyzed with the WinList 5.0 software. Antigen negative background binding was defined by the fluorescent intensity of isotype controls.

Quantitative RT-PCR analysis

Total RNA was isolated from mouse hearts using the Trizol reagents (Invitrogen) following the manufacturers' instructions. To reverse-transcribe RNA into cDNA, 3 μg of RNA was mixed with 100 ng $oligo(dT)_{15}$ and incubated for 5 minutes at 65°C. 1 mM dNTPs, 60 mM KCl, 15 mM Tris-Cl pH 8.4, 3 mM $MgCl_2$, 0.3% Tween 20, 10 mM β-mercaptoethanol, 10 U RNasin (Promega), and 100 U Mo-MLVRT (Invitrogen) were added, and the mix was incubated for 55 minutes at 37°C. Enzyme was inactivated by

incubating at 95°C for 5 minutes. In order to perform PCR, 20 ng cDNA was incubated with Taq DNA polymerase (Promega) and respective primers at 0.25 μM concentration for 35 cycles (1 minute at 95°C, 1 minute 60-65°C, and 1 minute 72°C). *β-actin* or *Gapdh* were used as internal controls. Quantitative PCR was performed using the iQ SYBR Green Supermix kit (BioRad) on an iCycler (BioRad). Relative gene expression levels were quantified using the $2^{(-DDCt)}$ formula (Livak and Schmittgen, 2001). Primer sequences have been included in Table 1.

Statistical analysis

The GraphPad Prism software was used to handle data. Results are reported as mean $\pm$ S.E.M. Student's *t*-test was used for comparing two groups. **P*<0.05, ***P*<0.01, ****P*<0.001.

CHAPTER III

CANONICAL WNT PATHWAY ACTIVATION DURING POST-MI GRANULATION TISSUE FORMATION COINCIDES WITH ENDOTHELIAL-TO-MESENCHYMAL TRANSITION

Introduction

The concurrent activation of canonical wnt activity in endothelial cells and SMA^+ mesenchymal cells raised the possibility that the latter are derived from activated endothelium. Consistent with this notion, endothelial-to-mesenchymal transition (EndMT) has been linked to angiogenesis, where tip cells acquire a mesenchymal phenotype during vessel branching (Gerhardt et al., 2003). EndMT has also been linked to myofibroblast activation and subsequent cardiac fibrosis after aortic banding (Zeisberg et al., 2007). We reasoned that since the granulation tissue formation phase of the MI response is characterized by neovascularization and myofibroblast activation, EndMT may play a role in this phase of the MI response. Furthermore, because we observed that the canonical wnt signaling pathway is activated in endothelial and mesenchymal cells during post-MI granulation tissue formation, we reasoned that canonical wnt signaling may mediate EndMT after MI.

Results

Endothelial-to-mesenchymal transition at homeostasis in the vasculature and valves of the adult mouse heart

In order to evaluate de novo EndMT after ischemic injury following arterial ligation, EndMT in the normal, uninjured adult heart was first analyzed. To this end, the endothelial-SCL-Cre-ER^T mouse line, in which the 5' endothelial-specific enhancer of the stem cell leukemia (SCL) locus drives tamoxifen-inducible Cre-ER^T (Göthert et al., 2004) was crossed to the R26RstoplacZ mouse line (Soriano, 1999). The SCL gene is expressed in both haematopoietic and endothelial cells (Schlaeger et al., 2005; Gottgens et al., 2002; Sanchez et al., 1999). However, the 5' enhancer has been shown to be exclusively active in endothelial cells thus allowing specific and permanent genetic labeling of endothelial cells and their progeny (Sinclair et al., 1999; Gothert et al., 2004).

Adult progeny of the double transgenic line SCL-Cre-ER^T/R26RstoplacZ, named End-SCL-LacZ mice, were injected with tamoxifen to induce Cre recombinase and labeling of endothelial cells. The results show that in the normal healthy adult mouse heart, most of the LacZ labeling is observed in vascular endothelium. Interestingly, there are some LacZ-expressing cells in the media of the great vessels and in the valve mesenchyme, suggesting that endothelial-to-mesenchymal transition occurs basally in the great vessels and valves (Fig 3.1 A). It should be noted that within the myocardium, LacZ labeling is limited to coronary and microvascular endothelial cells; LacZ-expressing peri-vascular or other mesenchymal-like cell are not detected in the healthy myocardium (Fig 3.1 B).

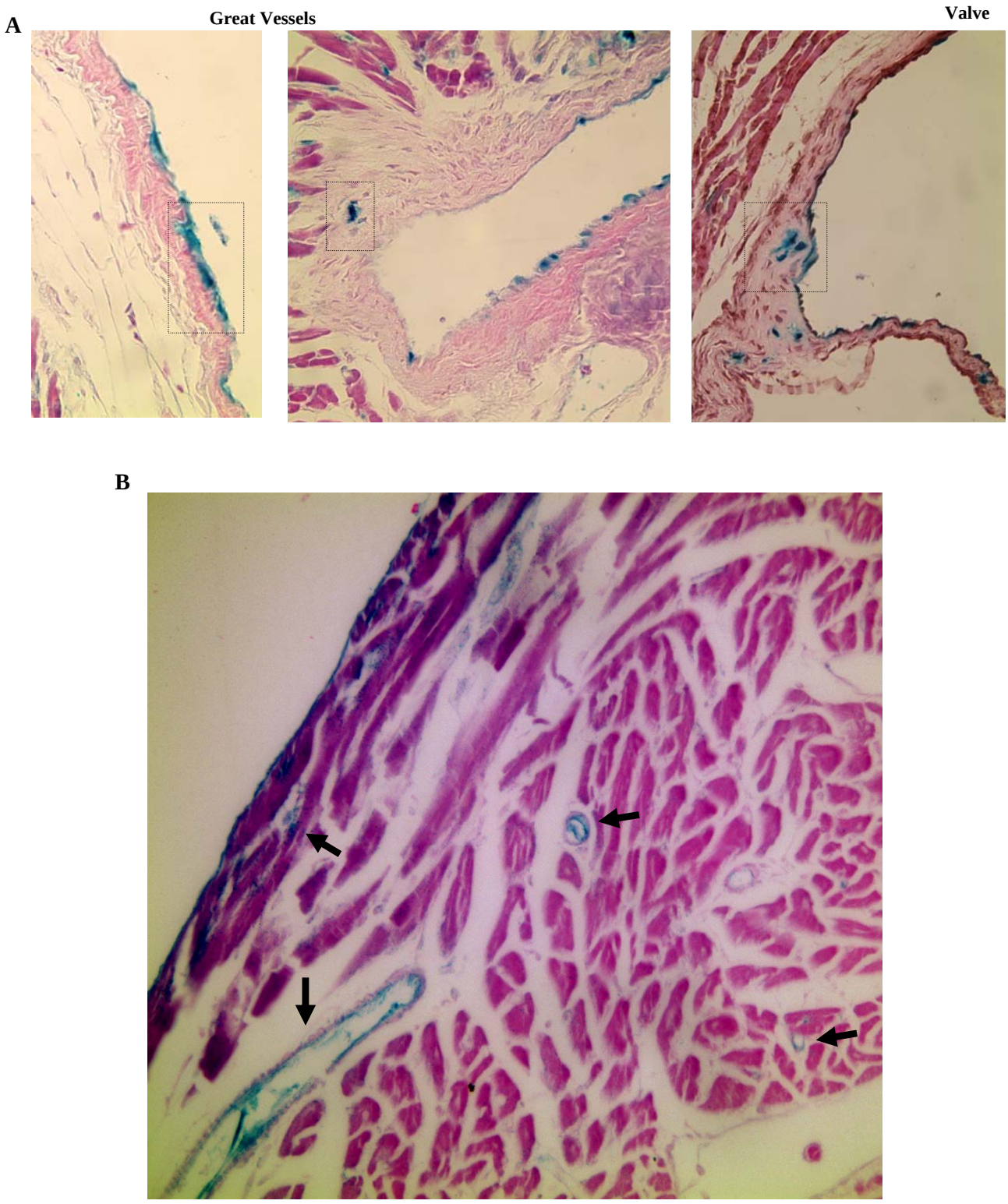


Figure 3.1: **EndMT at homeostasis in great vessels and valves at the base of the heart**. Hearts were isolated from tamoxifen-treated end-SCL-LacZ mice. A. X-gal stain lineage tracing reveals the presence of endothelial-derived mesenchymal cells in great vessels and valves at the base of the heart. B. Section through left ventricle shows that there is no mesenchymal labeling within the myocardium; only endothelial cells are labeled

Cells co-expressing CD31 and Smooth muscle actin are increased during post-MI granulation tissue formation

One hallmark feature of EndMT is the appearance of cells dually positive for endothelial and mesenchymal gene markers such as CD31 and SMA (Paruchuri et al., 2006; Sales et al., 2006; Goumans et al., 2008). To examine whether $CD31^{+}/SMA^{+}$ cells appear after experimental MI, cardiac tissue sections were analyzed by immunostaining. The results show a significant number of dually positive $CD31^{+}/SMA^{+}$ cells suggesting that EndMT takes place post-MI (Fig. 3.2 A).

To verify and quantify these results, cardiac tissues from mice that had undergone LAD occlusion or sham surgery were dissociated to single cell preparations 7 days after surgery, stained with antibodies against CD31 and SMA and analyzed by FACS. As shown in Fig. 3.2 B, there is a significant increase in $CD31^{low}SMA^{low}$ double positive cells 7 days post-MI. Specifically, $CD31^{+}SMA^{+}$ cells rise from less than 1% in normal cardiac tissue to almost 25% of the total cell number (Fig. 3.2 C).

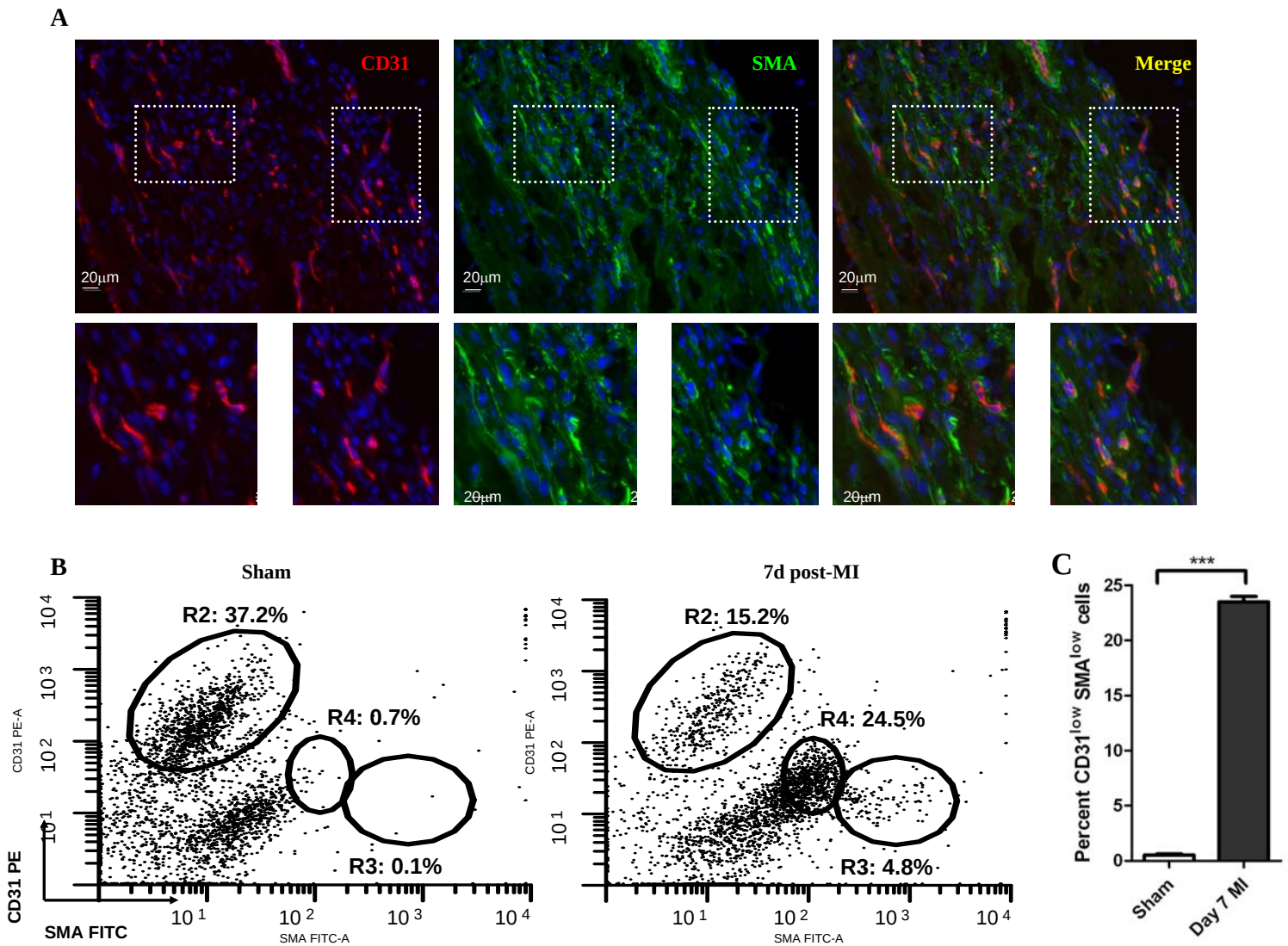


Figure 3.2: **Increase in cells co-expressing CD31 and SMA during post-MI granulation tissue formation.** (A) Immunohistological analysis with CD31 (red) and smooth muscle actin (green) antibodies, performed on sections from mouse hearts isolated 7 days after MI, shows the presence of cells co-expressing CD31 and smooth muscle actin (merge). (B) FACS analysis with CD31 and smooth actin antibodies was performed on peri-infarct tissue isolated 7 days after MI or sham surgeries. There is a marked increase in the CD31lowSMAlow population of cells on post-MI day 7. (C). Graph of average from FACS analysis. Sham N = 3; MI N = 3. *P<0.05, **P<0.01, ***P<0.001.

EndMT-associated genes are induced during granulation tissue formation after MI

Other genes that have been linked to mesenchymal transition include *Snail* (Medici et al., 2006; Kokudo et al., 2008), *Fsp1* (Zeisberg and Neilson 2009), *Vimentin* (Milsom et al., 2008), *MMP2* (Song et al., 2000; Duong and Erickson 2004), *Tgfβ1* (Wang et al., 2005; Deissler et al., 2006; Tavares et al., 2006; Zeisberg et al., 2007; Goumans et al., 2008) and *Col1A1* (Zeisberg et al., 2007; Hashimoto et al., 2010). Quantitative real-time RT-PCR analysis for the expression of EndMT-related genes at different time points after MI revealed that the peak in the expression of genes such as *Snail*, *Fsp1*, *Tgfβ1*, *Vimentin* and *Col1a1* corresponds to the appearance of $CD31^{+}/SMA^{+}$ cells on day 7 post-MI (Fig. 3.3).

Taken together, the results suggest that EndMT takes place after ischemic injury, and reaches its highest point during granulation tissue formation.

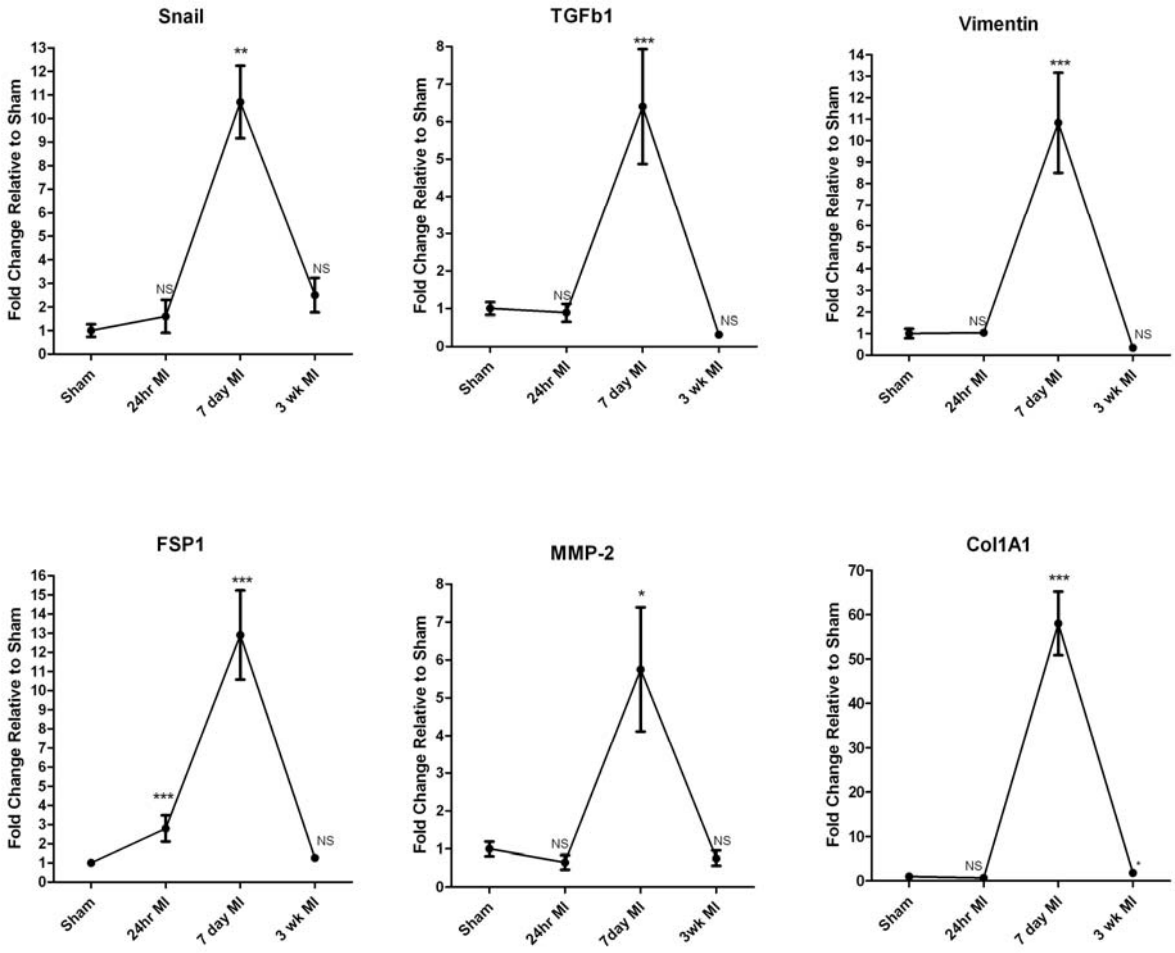


Figure 3.3: **EndMT-associated genes are induced during granulation tissue formation**. Real-time quatitative RT-PCR analysis for the expression of EndMT-associated genes was performed on mRNA isolated from TOPGAL mouse hearts at defined stages of the myocardial infarction response process.

Post-MI myofibroblasts are endothelial-derived

To confirm the endothelial origin of peri-infarct mesenchymal cells, End-SCL-LacZ mice were injected with tamoxifen to induce Cre recombinase and labeling of endothelial cells. One week after the last tamoxifen dose, the mice underwent LAD or sham surgery, and histological analyses were performed seven days after surgery (Fig 3.4 A). The analyses showed that in sham-operated mice, ventricular β-galactosidase expression is limited to endothelial cells. In contrast, the cardiac tissues of mice subjected to LAD occlusion have both increased LacZ-positive endothelial cells and a novel population of β-galactosidase-expressing mesenchymal cells (Fig. 3.4 B). Anti-SMA staining confirmed the presence of double positive β-galactosidase/SMA cells in peri-vascular and subepicardial peri-infarct regions (Fig. 3.4 C).

Quantification of histological sections showed that β-galactosidase^{+}/SMA^{+} cells represent 35-40% of the subepicardial SMA^{+} cell population (Fig. 3.4 D). Because tamoxifen induction labels only a fraction of resident endothelial cells, it is likely that this number is an underestimation of the endothelium-derived SMA^{+} cells.

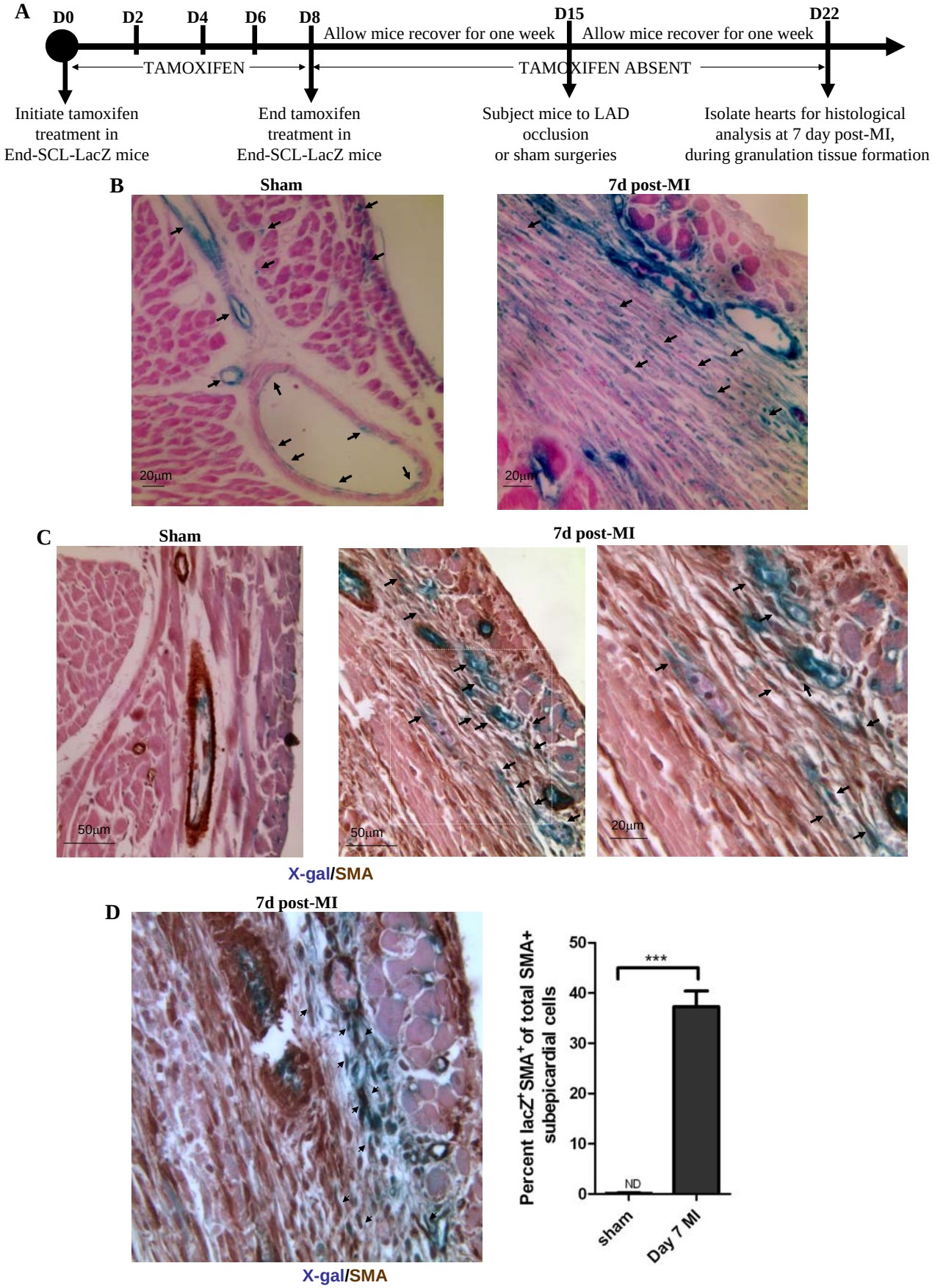


Figure 3.4: **EndMT is induced during granulation tissue formation.** Tamoxifen-treated End-SCL-LacZ mice were subject to LAD occlusion or sham surgeries. A. Experimental scheme. Lineage tracing reveals increased endothelial labeling after MI and the presence of endothelial-derived mesenchymal cells in the day 7 MI, but not in the sham, myocardium (B and C). C and D. Immunohistochemical stain with smooth muscle actin antibody was performed on heart sections from End-SCL-LacZ mice isolated 7 days after coronary ligation. Endothelial-derived myofibroblasts are observed in the peri-infarct subepicardial region.

Canonical wnt signaling is active in endothelial-derived mesenchymal cells

By lineage tracing with the End-SCL-LacZ mouse line, we observe basal EndMT in the great vessels and valves of the healthy adult mouse heart. These areas, the great vessels and valves, are the areas in which we observe the highest levels of canonical wnt signaling activity in the healthy adult mouse heart. This pattern of canonical wnt signaling activation in areas undergoing EndMT suggests a link between canonical wnt signaling and EndMT in the adult heart. This link is further supported by the finding that after MI, peak canonical wnt pathway signaling coincides with the time of highest EndMT. Therefore, we investigated whether canonical wnt signaling is active in EndMT-derived mesenchymal cells after MI.

To this end, cardiac tissue sections of TOPGAL mice 7 days post-MI were stained with antibodies to β–galactosidase and the mesenchymal transition-inducing gene, *Snail*. The results show that Snail is expressed in cells marked by canonical wnt signaling activity (Fig. 3.5). Furthermore, analysis of cardiac tissue sections of tamoxifen-treated End-SCL-LacZ mice obtained 7 days after LAD or sham surgery shows nuclear β–catenin in β-galactosidase stained mesenchymal cells of endothelial origin (Fig. 3.6).

Taken together, the results show that canonical wnt signaling is active in cells undergoing EndMT.

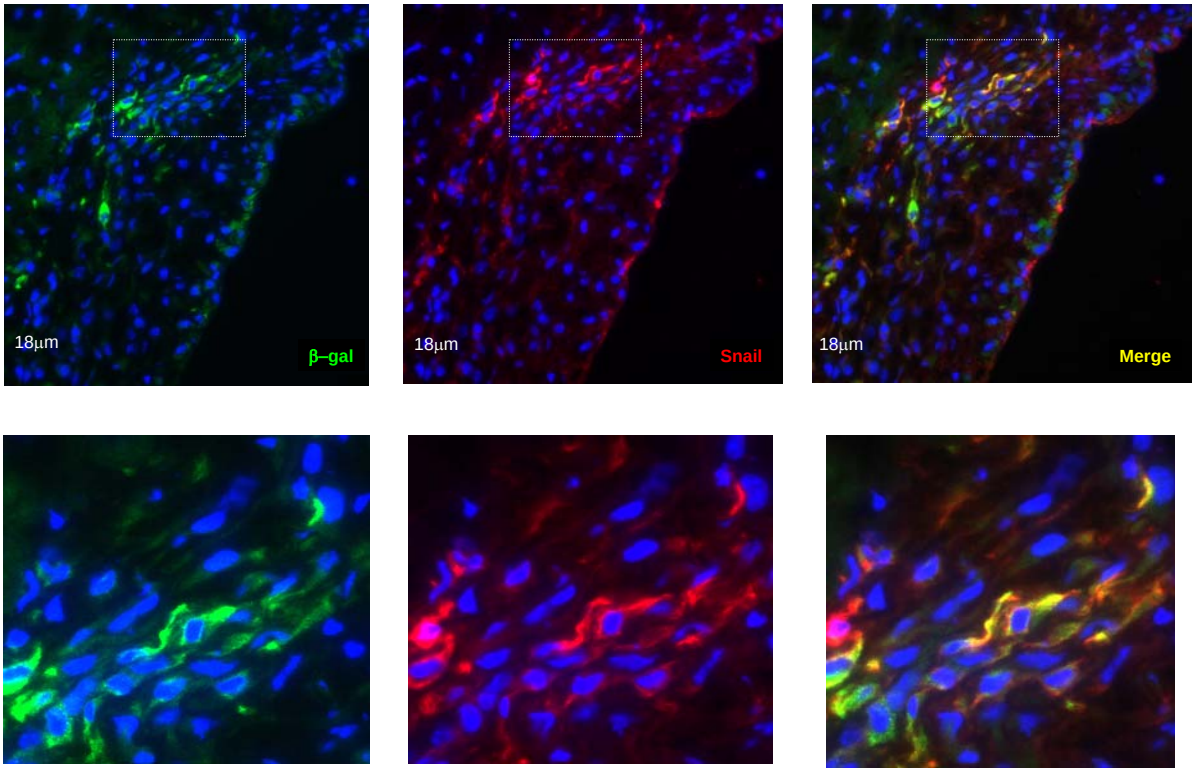


Figure 3.5: **Cells in which canonical wnt signaling is activated express the EndMT-associated gene, *Snail*.** IF analysis on sections from TOPGAL mouse hearts 7 days after experimental MI using the anti- β–galactosidase (green) and Snail (red) antibodies.

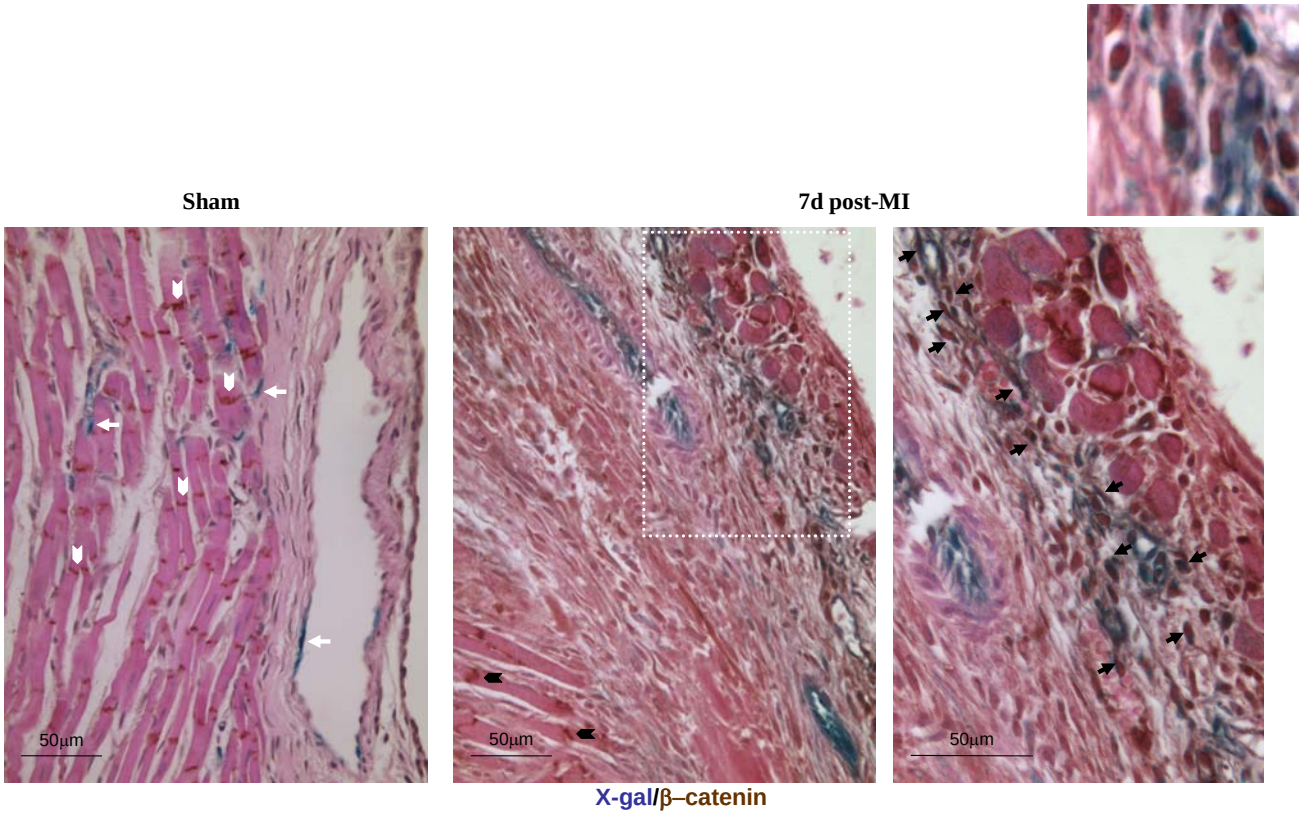


Figure 3.6: **Canonical wnt signaling is active in EndMT-derived mesenchymal cells**. IHC analysis using the anti-β–catenin antibody on sections of X-gal-stained cardiac tissue of tamoxifen-treated End-SCL-Cre/Rosa-LacZ mice 7 days post-MI. Left panel: sham control shows β–catenin accumulation in the intercalated disks of cardiomyocytes (white arrowheads) and a subset of endothelial cells (white arrows). Middle and right panels: 7 days post-MI, nuclear β–catenin is also present in mesenchymal cells (black arrows). Right panel depicts a greater magnification of area boxed in the middle image.

Discussion

EndMT has been extensively studied during heart development. EndMT occurs during aortic and pulmonary vessel development (Arciniegas et al., 2005), as well as during valve development (Yamagushi et al 1999, Sugi et al 2004, Lincoln et al 2004, reviewed by Armstrong and Bischoff 2004). A role for canonical wnt signaling in cardiac cushion EndMT has been demonstrated by studies showing canonical wnt activation in developing mouse cardiac cushions (Gitler et al 2003), studies showing disruption of valve formation upon interruption of canonical wnt signaling in zebrafish (Hurlstone et al 2003), and studies showing the requirement of endothelial β–catenin for proper mouse cushion development (Liebner et al 2004). Our results indicate that canonical wnt signaling and EndMT persist in the adult mouse valves, and also persist in the adult great vessels, where the endothelium serves as a source for peri-vascular smooth muscle cells.

EndMT is emerging as a source for pro-fibrotic fibroblasts and myofibroblasts in a wide range of diseases. A number of mouse lineage tracing studies reveal that EndMT contributes to fibrosis in models of chronic renal (Zeisberg et al 2008), pulmonary (Hashimoto et al 2009), and heart (Zeisberg et al 2007; Widyantoro et al 2010) disease. Our lineage tracing study with the End-SCL-LacZ mouse line implicates the mature endothelium as a major source of myofibroblasts after MI.

The possibility that the End-SCL locus becomes activated so that Cre expression and lacZ labeling occurs in post-infarction myofibroblasts independently of endothelial origin, is very small because tamoxifen injections end fourteen days before hearts are

isolated and sections analyzed (fig 3.4 A); such an occurrence will suggest that Cre activation occurs independently of tamoxifen, which is unlikely as we and others (Sinclair et al., 1999; Gothert et al., 2004) have not observed this.

The argument can be raised that activation of the End-SCL locus, Cre expression and LacZ labeling occurs in circulating endothelial progenitors rather than mature endothelial cells, implying that our observed post-infarction LacZ-expressing myofibroblasts are bone marrow- rather than mature endothelial-derived. This argument is refuted by FACS analyses data on cells isolated from the bone marrow of End-SCL-EYFP mice which showed that the End-SCL locus is not active in the adult mouse bone marrow; EYFP-expressing cells were not detected in the bone marrow (Gothert et al., 2004).

Our study is therefore the first report to implicate EndMT as a major player in cardiac tissue repair after acute ischemic injury since our results show to that EndMT occurs after acute myocardial infarction, during granulation tissue formation.

In addition to the generation of fibrous scar-forming myofibroblasts, the granulation tissue formation stage of the MI response is characterized by new blood vessel formation, or neovascularization. Our data showing the occurrence of EndMT during granulation tissue formation suggest EndMT also contributes to neovascularization. A role for EndMT in new blood vessel formation is supported by Gerhardt and colleagues' study which showed that during vessel branching, endothelial cells acquire a mesenchymal phenotype (Gerhardt et al 2003). Tumor studies showing attenuation of angiogenesis

upon inhibition of EndMT-associated genes further support the link between EndMT and angiogenesis (Singh et al 2008; Lahat et al 2010).

All together, the data presented thus far show that (i). Canonical wnt signaling is activated during post-MI granulation tissue formation (ii). Canonical wnt activation during granulation tissue formation coincides with EndMT and (iii). Canonical wnt signaling is active in EndMT-derived cells. These results suggest, but do not directly show, that canonical wnt signaling mediates post-MI EndMT. The next chapter presents experiments that test whether activation of canonical wnt signaling in isolated endothelial cells is sufficient to elicit mesenchymal transition.

Methods

Mice

The SCL-Cre-ER^T mouse carries a fragment of the 5' endothelial-specific enhancer of the stem cell leukemia (SCL) gene locus driving the tamoxifen-inducible Cre-ER^T gene (Göthert et al., 2004). The endothelial-SCL-Cre-ER^T mice were crossed to R26RstoplacZ mice (Soriano, 1999) giving rise to End-SCL-LacZ mice. Cre recombinase was induced by treating adult end-SCL-LacZ mice with 2mg tamoxifen (Sigma) every other day for ten days. Surgical procedures on End-SCL-LacZ mice were carried out one week after the last dose of tamoxifen treatment. All procedures were carried out with the approval of the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center. The R26RstoplacZ mouse line was purchased from Jackson Laboratories. The endothelial-SCL-Cre-ER^T mouse line was a kind gift of Drs. Begley and Göthert.

Experimental myocardial infarction

Myocardial infarction was induced in mice following the protocol outlined in the previous chapter.

β–galactosidase activity staining

Whole hearts from End-SCL-LacZ mice were isolated at stated time points and stained with X-gal to assess β−galactosidase according to the previously outlined protocol.

Immunofluorescence (IF) and immunohistochemistry (IHC)

IF and IHC analysis were performed as previously outlined. Mouse anti-β–catenin antibody (Sigma) was used in a 1:800 dilution. Mouse anti-Snail antibody (Chemicon) was used in a 1:600 dilution.

FACS analysis

Experiments were performed as previously described. Single-cell suspensions (10^6 cells/ml) were labeled using phycoerythrin-conjugated anti-mouse CD31 (clone 390, eBioscience, Inc) and peridinin-chlorophyll-protein-conjugated anti-CD45 (clone 30-F11, eBioscience, Inc). For intracellular staining to detect α-smooth muscle actin (α-SMA), cells were fixed and aliquots of cells (10^5 cells in 100 μL buffer) were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-α-SMA (Sigma).

Quantitative RT-PCR analysis

Total RNA was isolated from mouse hearts using the Trizol reagents (Invitrogen) following the manufacturers' instructions. Reverse transcription of RNA into cDNA and quantitative PCR analysis were performed as previously described. Forward and reverse primer sequences are listed in Table 1.

Statistical analysis

The GraphPad Prism software was used to handle data. Results are reported as mean $\pm$ S.E.M. Student's *t*-test was used for comparing two groups. $*P<0.05$, $**P<0.01$, $***P<0.001$.

CHAPTER IV

CANONICAL WNT SIGNLAING TRIGGERS ENDOTHELIAL-TO-MESENCHYMAL TRANSITION *in vitro*

Introduction

Canonical wnt signaling has been shown to be important for EndMT during embryonic vessel and valve development (Monkley et al., 1996; Hurlstone et al., 2003). Specifically, endothelial β–catenin is required for proper cardiac cushion development via EndMT (Liebner et al., 2004). Our observations that in the healthy adult mouse heart, EndMT occurs at homeostasis in regions of high basal canonical wnt activity i.e. the great vessels and valves, that post-MI canonical wnt activation coincides with EndMT and that the canonical wnt signaling pathway is active in cells that express snail, a protein that has been linked to mesenchymal transitions, suggest that canonical wnt signaling may mediate adult endothelial EndMT.

To explore this possibility, we turned to the bovine aortic endothelial cell (BAEC) culture system, a pure population of mature endothelial cells routinely used in experiments to study endothelial cell behavior.

Results

Canonical wnt signaling triggers mesenchymal transition in cultured mature bovine endothelial cells

To determine if activation of canonical wnt signaling is sufficient to induce mesenchymal transition in a pure population of mature endothelial cells, we turned to the bovine aortic endothelial cell (BAEC) culture system. BAECs were treated with BIO or DMSO. We observed that the BIO-treated BAECs undergo dramatic morphological changes within 24 hours acquiring a distinct mesenchymal appearance, whereas vehicle (i.e., DMSO)-treated cells retain the characteristic cobblestone morphology of aortic endothelial cells (Fig. 4.1 A). To confirm canonical wnt activation in BIO-treated cells, immunocytology with β–catenin antibody was performed. We observed β–catenin localization to junctions of DMSO-treated cells and the cytoplasm and nuclei of BIO-treated cells (Fig. 4.1 B), suggestive of canonical wnt activation upon BIO-treatment.

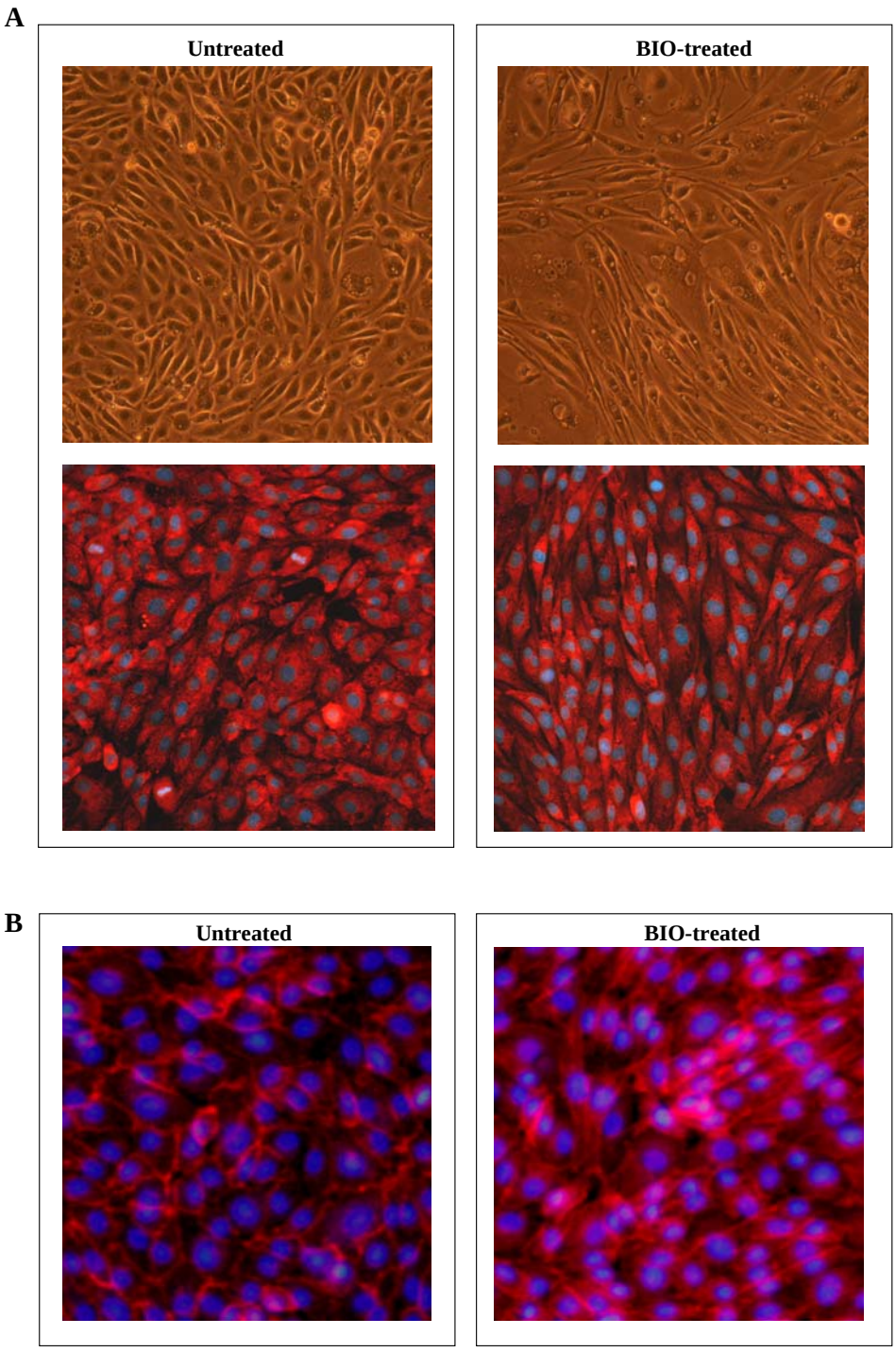


Figure 4.1: **The canonical wnt signaling pathway activator, BIO, induces mesenchymal phenotype in BAECs.** Mature bovine aortic endothelial cells (BAECs) were cultured with BIO or with DMSO as a control. BIO-treatment induced a mesenchymal phenotype within 24hrs of treatment. (A) Bright-field and fluorescent images. (B) IF stain with β–catenin (red) and dapi (blue).

To analyze the molecular phenotype of BIO-treated BAECs, mRNA from BIO- and vehicle-exposed controls were isolated 24 hours and 3 days after treatment. The response of a number of canonical wnt signaling targets was then analyzed by real-time quantitative RT-PCR using gene-specific primers.

Our results show that exposure to BIO results in a 50-150-fold induction of canonical wnt target genes such as *Tcf7* and *Axin2* within 24 hours and that high levels are sustained for the 3-day culture period (Fig. 4.2). BIO treatment also results in a significant decrease in the endothelial-cell-specific gene CD31 at both the 24 hours and 3 days time points. Interestingly, though the Snail-related gene Slug is moderately up-regulated within 24 hours of exposure to BIO, other EndMT-associated genes, such as SMA and Col1A1, are not induced until day 3 of BIO treatment, suggesting a gradual transition to the mesenchymal phenotype (Fig. 4.2).

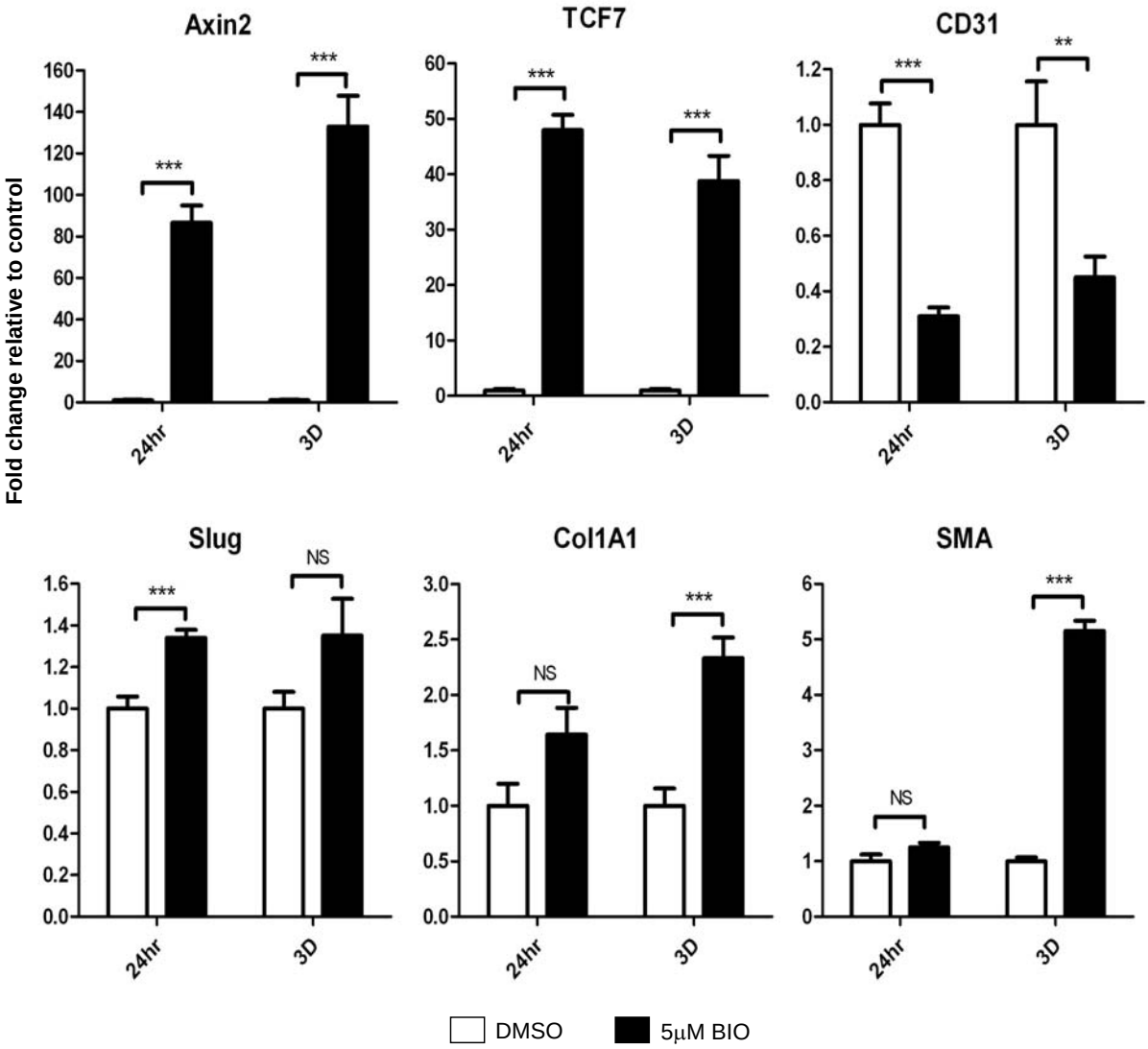


Figure 4.2: **Canonical wnt activation down-regulates endothelial and induces mesenchymal gene expression in BAECs.** Real-time quantitative RT-PCR analysis. BIO-induces the expression of canonical Wnt signaling gene targets (*Axin2*, *TCF7*) and EndMT-associated genes (*Slug*, *SMA*, *Col1A1*), whereas it leads to the downregulation of the endothelial-specific gene *CD31*. Induction of canonical wnt-pathway target genes (*Axin2*, *TCF7*) and down-regulation of the endothelial gene (*CD31*) occurs within 24 hours of BIO treatment, whereas induction of *SMA* and *Col1A1* is not observed until after 3 days of exposure to BIO.

Inhibition of canonical wnt signaling attenuates BIO-induced EndMT

In order to confirm that BIO-stimulated molecular alterations were specific to activation of the canonical wnt signaling pathway, cells were treated with the canonical wnt pathway inhibitor, pyrvinium (kindly provided by Drs. Curtis Thorne and Ethan Lee). As shown in Fig. 4.3, pyrvinium attenuates BIO effects on BAECs. Specifically, the molecular analyses indicate that blocking canonical wnt signaling attenuates the induction of *Axin2* and *Sma* and partially restores *CD31* expression.

Altogether, the *in vitro* experiments described above indicate that activation of the canonical wnt signaling pathway in mature endothelial cells induces morphological and molecular changes that are consistent with an initial suppression of endothelial, followed by an acquisition of mesenchymal characteristics.

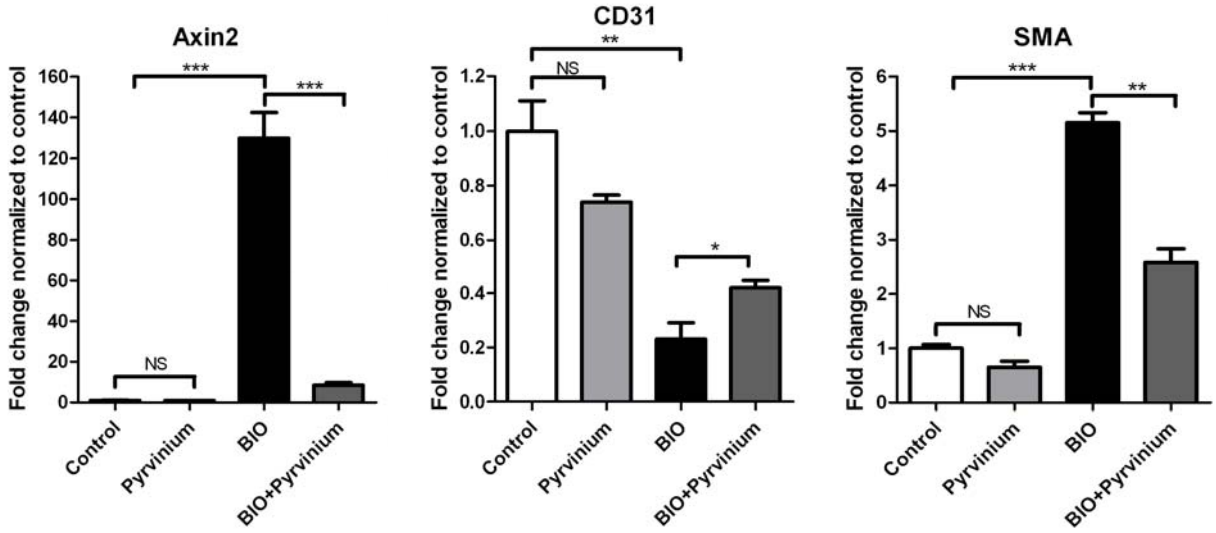


Figure 4.3: **Inhibition of canonical wnt signaling attenuates BIO-induced EndMT.** BAECs were cultured for 3 days with BIO in the presence of the canonical wnt inhibitor, pyrvinium.

Discussion

The link between canonical wnt signaling and EndMT during heart development has been extensively studied, and validated in zebrafish (Hurlstone et al., 2003), chick (Arciniegas et al., 2005) and mouse (Liebner et al., 2004) models. Our *in vivo* studies showing that at baseline in the healthy adult mouse heart, EndMT is ongoing in areas engaged in canonical wnt signaling, that after MI, peak canonical wnt signaling activation coincides with *de novo* EndMT and that canonical wnt signaling is active in EndMT-derived cells, suggest that canonical wnt signaling mediates EndMT not only during development, but also in the adult heart at homeostasis and after injury.

In this chapter, for the first time to our knowledge, we addressed the role of canonical wnt signaling in controlling EndMT in the mature endothelium. We used a pure culture of adult endothelial cells to show that activation of canonical wnt signaling is sufficient to induce the adult endothelium to undergo a mesenchymal transition.

All together, our *in vivo* observations coupled with our experiments *in vitro* provide evidence that EndMT occurs in the adult cardiac endothelium at baseline, is greatly up-regulated after injury, and is mediated by canonical wnt signaling.

These observations that canonical wnt signaling induces the mature endothelium to undergo mesenchymal transition not only demonstrate the importance of the canonical wnt signaling pathway but also highlight the endothelial cell as a dynamic cell type that

not only reproduces itself in response to injury, but also transforms into another cell type to contribute even more to the wound healing process. These data thereby suggest that the endothelial cell can be characterized as a cardiac stem cell. This idea is probed in more detail in the next chapter.

Methods

Cell culture

Bovine aortic endothelial cell culture

Bovine Aortic Endothelial Cells (BAECs) were used between sixth and eighth passages. Cells were cultured in standard high glucose DMEM medium containing 10% FBS, 1% Pen-Strep and 1% L-glutamine. Cells were treated with GSK-3 inhibitor IX, BIO (CalBiochem; 5 μM), Pyrvinium (kindly provided by Drs. Curtis Thorne and Ethan Lee; 10nM) or the equivalent volume of vehicle solution (DMSO, Sigma).

Immunocytology

Bovine aortic endothelial cells were platted in glass chambers pre-coated with 1% gelatin. Upon reaching confluence, cells were treated with 5 μM BIO or DMSO and cultured for 24 hours. Cells were fixed for 5 minutes with 2% para-formaldehyde on ice, and subjected to the procedures described for immunofluorescence on tissue sections.

Quantitative RT-PCR analysis

Total RNA was isolated BAECs using the RNase easy minikit (Qiagen) following the manufacturers' instructions.

Reverse transcription of RNA into cDNA and quantitative PCR analysis were performed as previously described. Forward and reverse primer sequences are listed in Table 1.

CHAPTER V

ENDOTHELIAL CELLS DISPLAY STEM CELL CHARACTERISTICS

Introduction

It has been since established that adult endothelial cells proliferate in response to myocardial ischemia (reviewed by Hudlicka et al., 1992). Our lineage tracing experiments using the End-SCL-LacZ mice in which tamoxifen injection and endothelial labeling is induced in adult mice show that mature adult endothelial cells are able to transform into mesenchymal cells after myocardial infarction *in vivo*. Furthermore, our experiments in isolated adult bovine aortic endothelial cells show that canonical wnt signaling is sufficient to induce mesenchymal transition in mature endothelial cells. Our data therefore add mesenchymal transformation to the repertoire of endothelial cells in their response to acute myocardial ischemic injury.

Proliferating in response to injury, otherwise known as cell self-renewal, and transformation into another cell type are characteristics of stem cells. Because the endothelium displays both characteristics, we probed whether endothelial cells display other stem cell characteristics.

Results

Endothelial cells proliferate at homeostasis in the healthy adult mouse heart and after myocardial infarction

In addition to proliferation in response to injury, stem cells, by definition, proliferate during homeostasis for self-maintenance. To examine whether endothelial cells proliferate during homeostasis and after myocardial injury, immunohistological analysis with the endothelial-specific marker, CD31, and the pan-proliferation marker, Ki67, was performed on sections from mouse hearts subject to LAD occlusion or sham surgeries.

The results show that endothelial cells are the dominant proliferating cardiac cell type at homeostasis (Fig. 5.1 A). Endothelial cells also proliferate after ischemic heart injury (Fig. 5.1 B). Interestingly, though little to no proliferation of other cardiac cell types is detected in the sham (Fig. 5.1 A; α–actinin and SMA stains), after MI, proliferation of SMA^+ cells and few cardiomyocytes can be detected (Fig. 5.1 B).

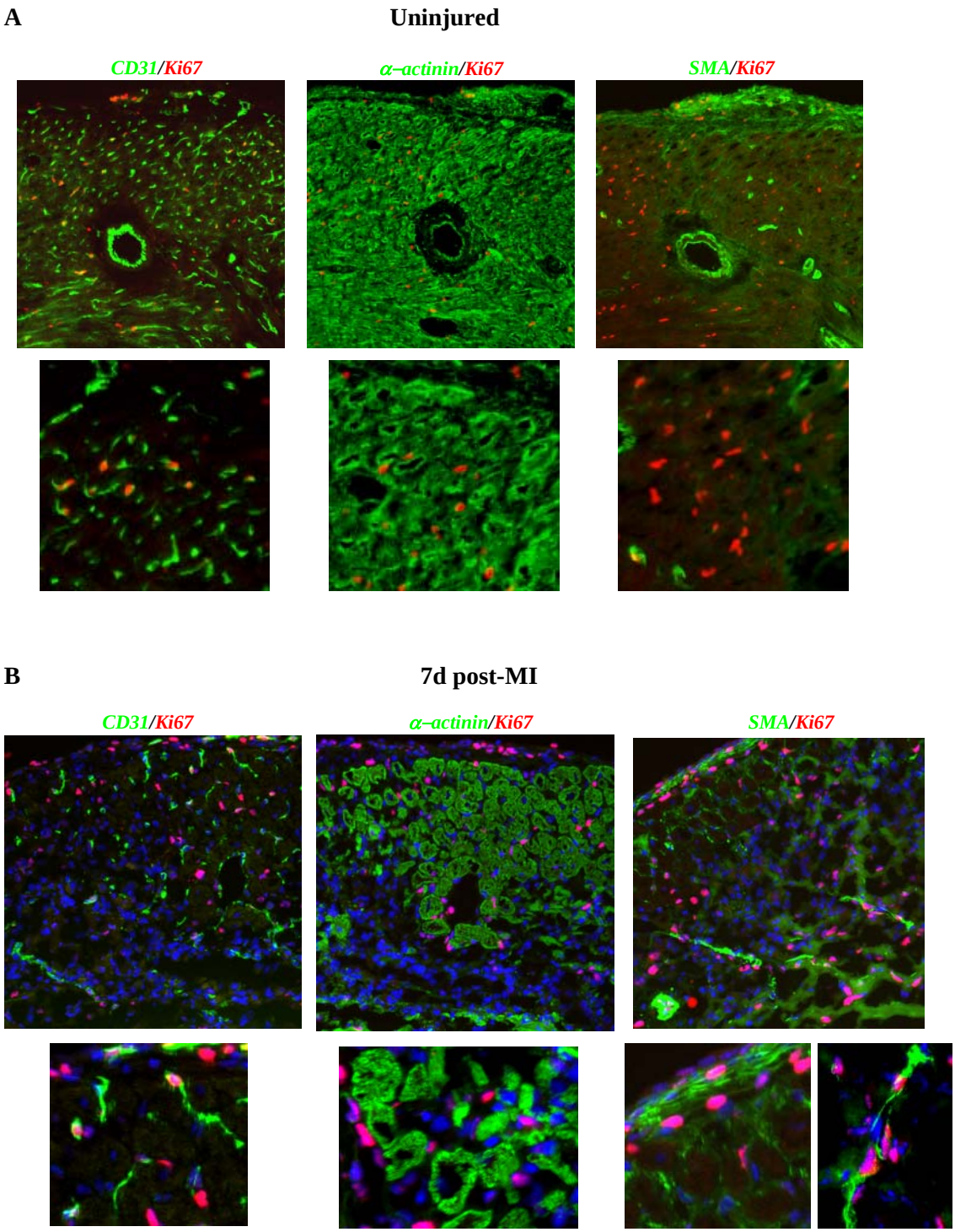


Figure 5.1: **Endothelial cells proliferate at homeostasis and after MI**. Immunofluorescence stain was performed with endothelial marker, CD31/PECAM (green; left panel), the cardiomyocyte marker, α–actinin (green; middle panel), the myofibroblast marker, SMA (green; right panel) and proliferation marker, Ki67 (red; all panels). A. Endothelial cells account for the majority of the proliferating cells at homeostasis. B. After MI, in addition to endothelial cells, high level of proliferation is observed in SMA^+ myofibroblasts (right panel). Proliferation in a few α–$actinin^+$ cardiomyocytes is also observed (middle panel)

Endothelial cells express cardiac stem cell markers

Lineage tracing experiments in mice and differentiating cultured embryonic stem cells have identified a number of genes to be associated with cardiac stem/progenitor cells. These genes include *Nkx2.5* (Stanley et al., 2002), *Isl-1* (Moretti et al., 2006) and *Stem cell antigen (Sca)-1* (Wang et al., 2006). To examine whether endothelial cells express these genes, cardiac tissue sections were subject to immunohistological analysis with antibodies against endothelial cells (CD31) and cardiac stem/progenitor markers, Nkx2.5 and Isl-1. The results show that endothelial cells express Nkx2.5 and Isl-1 as cells dually positive for CD31 and Isl-1 could be detected in the infarcted myocardium (Fig. 5.2 A; left panel). Also, by triple-labeling techniques, cells expressing CD31, Nkx2.5 and Isl-1 were detected within the myocardium (Fig. 5.2 A; right panel). FACS analysis on single cell suspensions isolated from mouse hearts revealed that the majority of cardiac endothelial cells express Sca-1 (Fig. 5.2 B).

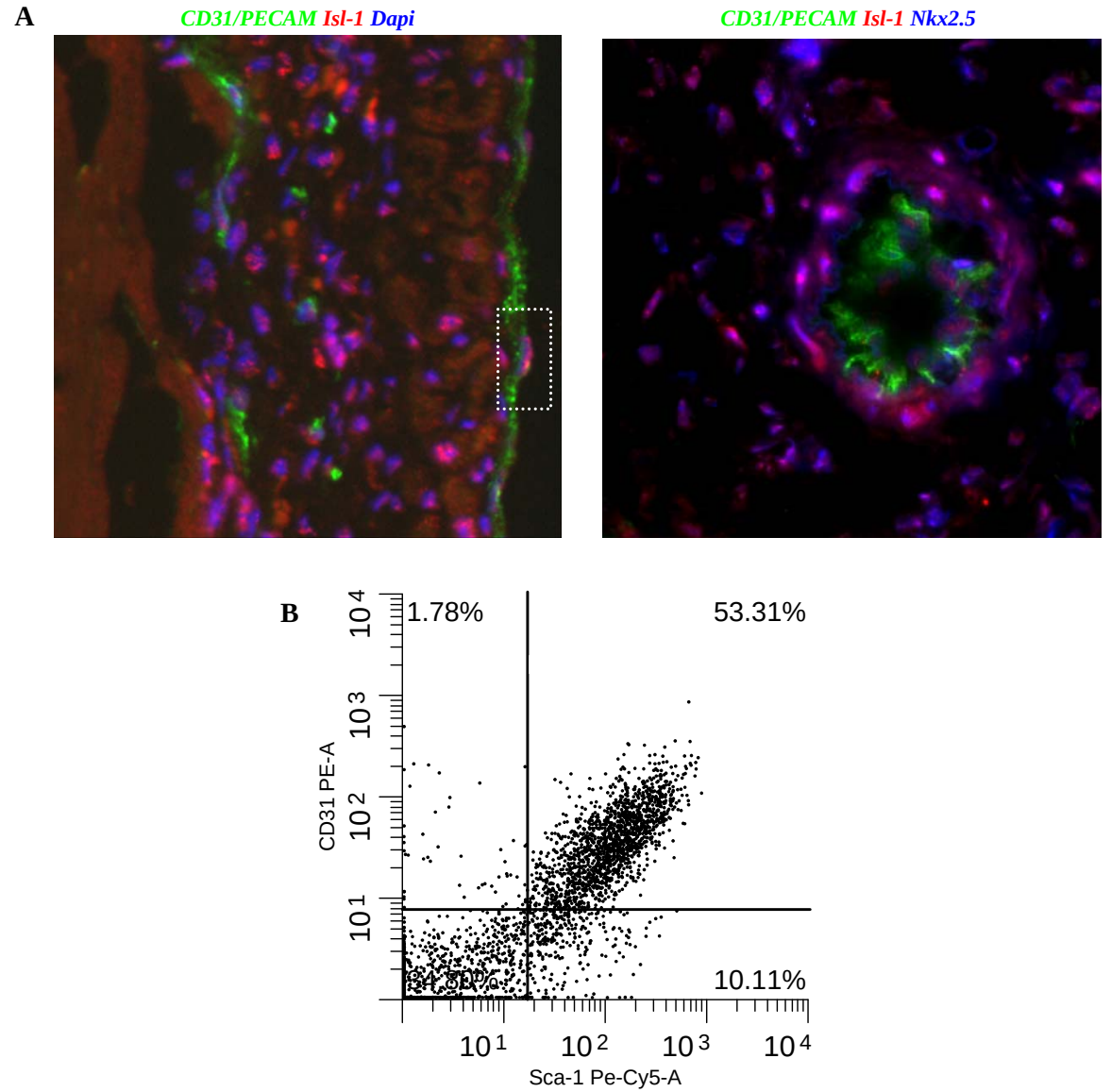


Figure 5.2: **Endothelial cells express cardiac stem cell-associated genes.** A IF stain with antibodies to cardiac stem cell-associated genes Nkx2.5 (blue in right panel) and Isl-1 (red) shows stem cell-associated gene expression in endothelial cells (green). B. FACS analysis was performed on single suspensions isolated from mouse hearts. Approximately 50% of all non-cardiomyocyte cardiac cells are endothelial cells that express stem cell antigen (Sca)-1; almost all endothelial cells express Sca-1.

Discussion

Our data showing that mature endothelial cells are able to transform into myofibroblasts for cardiac repair in vivo and transition into spindle-shaped mesenchymal cells *in vitro* suggested that endothelial cells may have other stem cell characteristics. Consistent with this, in this chapter, we showed that endothelial cells proliferate at homeostasis in the healthy adult mouse heart as well as after injury and that endothelial cells express proteins that have been linked to cardiac stem/progenitor cells, specifically, Nkx2.5, Isl-1 and Sca-1.

These findings that endothelial cells display stem cell characteristics are in line with findings that other epithelial cells including cardiac epicardial cells display stem cell behavior and aid in cardiac wound healing after injury (Limana et al., 2007). Consistent with the notion that the endothelium/epithelium is an important stem cell source that undergoes mesenchymal transition to generate critical repair cells for wound healing, during development, epithelial-to-mesenchymal transition (EMT) serves as the source for almost all specialized cell types – EMT generates mesodermal cells from the primitive ectoderm (Nakaya and Sheng, 2008) and neural crest cells from the neuroepithelium (Sauka-Spengler and Bronner-Fraser, 2008) – also, later in development, distinct sets of cardiac progenitors that give rise to blood vessels and valves arise from epicardial EMT and endothelial EndMT (Winter and Gittenberger-de Groot, 2007; Armstrong and Bischoff, 2004). Adult repair mechanisms may therefore share a great deal with normal developmental processes that shape the cardiac tissue during embryogenesis

All together, our data support the notion that the endothelium is a cardiac stem cell and that canonical wnt signaling regulates its behavior. Interestingly, canonical wnt signaling has been shown to be an important regulatory mechanism in a number of cardiac stem/progenitor cell studies, including Isl-1-expressing cardiac stem cells (Lin et al., 2007; Qyang et al., 2007), Mesp-1-expressing cardiac stem cells (Klaus et al., 2007) and mesenchymal stem cells (Mirotsou et al., 2007; Alfaro et al., 2008). Based on our findings that endothelial cells express a number of these genes, it will be interesting to know if these endogenous cardiac stem cells – i.e. endothelial cells and endothelial-derived mesenchymal cells, Sca-1-expressing cells, Isl-1-expressing cells, Nkx2.5-expressing cells, etc – are all really one and the same population of cells transitioning from one form to another, or distinct cardiac stem cell populations with unique functions.

Methods

Mice

All procedures were carried out with the approval of the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center.

Experimental myocardial infarction

The previously described protocol was followed

Immunohistology

The previously described protocol was followed. The primary antibodies used and their various dilutions were: Nkx2.5 (Santa Cruz; 1:50 dilution), Isl-1 (Abcam; 1:100 dilution),

CD31/PECAM (BD Pharmingen; 1:100 dilution), Smooth muscle actin (Sigma; 1:800 dilution), Cardiac α–actinin (Sigma; 1:1,000 dilution) and Ki67 (Abcam; 1:500 dilution).

FACS analysis

Performed as previously described. Stem cell antigen (Sca)-1 was purchased from ebioscience and used at a 1:100 dilution

CHAPTER VI

ATTEMPTS AT CANONICAL WNT PATHWAY MANIPULATION *in vivo* WERE UNSUCCESSFUL

Introduction

The data presented so far show that the canonical wnt signaling pathway is activated during the granulation tissue formation phase of the myocardial infarction response, that canonical wnt activation coincides with post-infarction endothelial-to-mesenchymal transition, and that canonical wnt signaling is sufficient to induce a mesenchymal transition in the mature vascular endothelium. Our data show that the endothelium is a versatile cell type that serves as a source of both new blood vessels and scar-forming myofibroblasts.

Our studies suggest that canonical wnt signaling, by regulating endothelial cell behaviour and mediating EndMT during post-MI granulation tissue formation, contributes to both new blood vessel formation and fibrosis. Our studies also suggest that post-MI cardiac repair may be improved by temporal and cell type-specific manipulation of canonical wnt signaling.

In initial attempts to test these hypotheses, TOPGAL mice were treated with recombinant protein and small molecule inhibitors of the canonical wnt pathway in order to manipulate post-MI canonical wnt activity and subsequent canonical wnt-mediated processes.

Results

Dkk-1, at a dose of 500 μg/Kg of body weight, did not affect MI-induced canonical wnt activation, EndMT or cardiac function decline

In order to assess the effect of canonical wnt pathway manipulation on post-infarction EndMT, angiogenesis and fibrosis, TOPGAL mice were treated with the canonical wnt inhibitor, Dkk-1 (500μg/Kg of body weight), or with vehicle (PBS), 3, 4, and 5 days after being subjected to LAD occlusion or sham surgeries. This dose of Dkk-1 has previously been reported to inhibit canonical wnt signaling activity *in vivo* (Aicher et al., 2008).

Our results show that 500μg/Kg of body weight of Dkk-1 was insufficient to block myocardial infarction-induced canonical wnt activation, as shown by the lack of statistically significant differences between vehicle and Dkk-1 treated mice in the post-MI expression of canonical wnt targets (Fig. 6.1 A). Consistent with its lack of effect on *in vivo* canonical wnt signaling, the given dose of Dkk-1 did not affect infarction-induced EndMT as assayed by RT-PCR for the expression of EndMT-associated genes and FACS analysis for the presence of cells doubly positive for CD31 and SMA (Fig. 6.1 B). Cardiac function, as assessed by echocardiography, did not vary significantly between vehicle and Dkk-1-treated animals (Fig. 6.1 C).

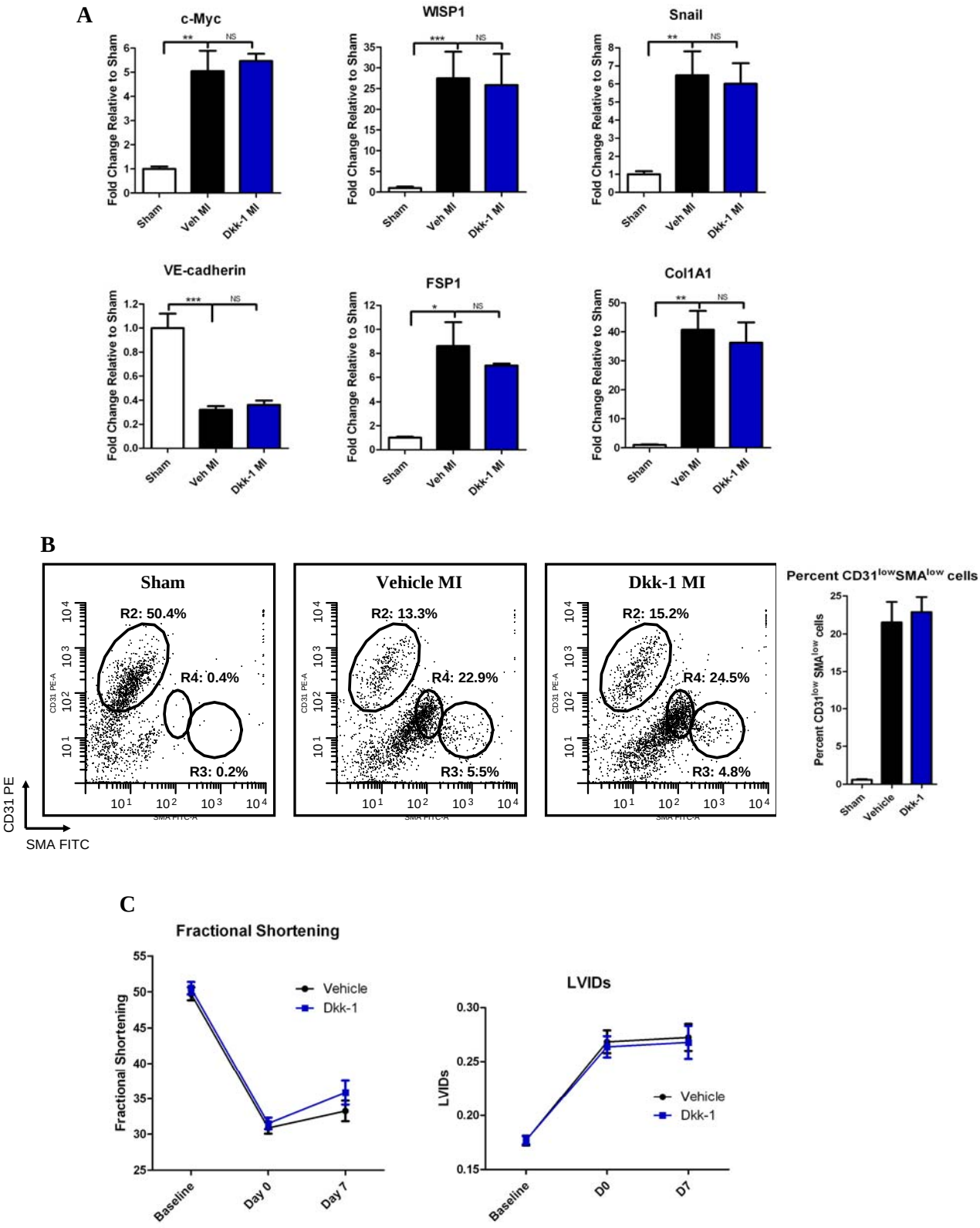
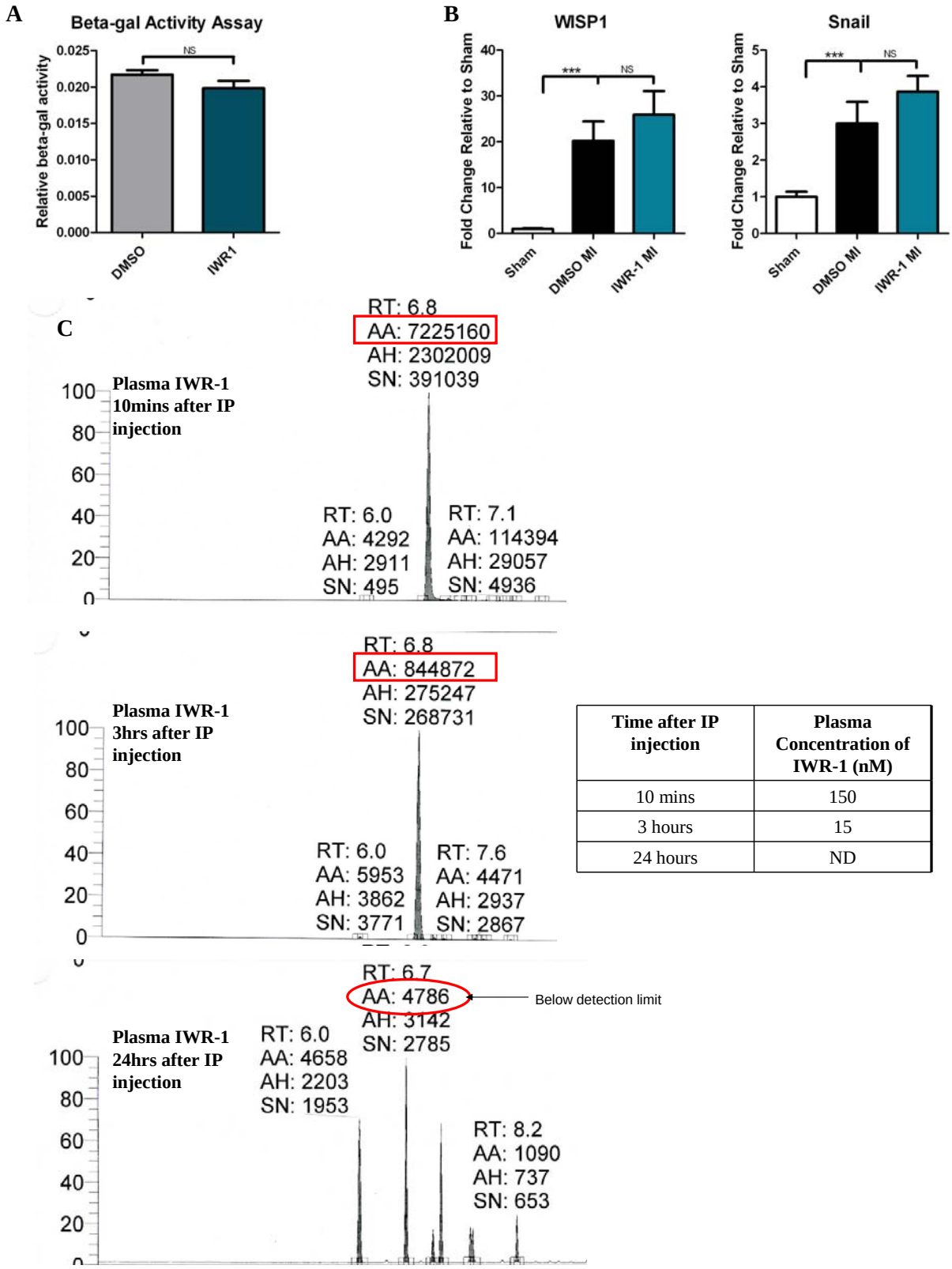


Figure 6.1: **500mg/Kg BW of Dkk-1 did not affect MI-induced canonical wnt activation, EndMT or cardiac function decline.** Mice were subject to LAD occlusion or sham surgeries and treated with vehicle or Dkk.1. A. Effect on expression of canonical wnt targets and EndMT-related genes was tested by real-time quantitative RT-PCR. B. Effect on generation of cells co-expressing CD31 and SMA was tested by FACS analysis. C. Effect on cardiac function was tested by echocardiography.

Intraperitoneal injection of the small molecule canonical wnt inhibitor, IWR-1, did not affect canonical wnt signaling or EndMT *in vivo* likely because IWR-1 is rapidly cleared

The high cost of Dkk-1 made it unfeasible to escalate the dose of Dkk-1 given to mice. Instead, we sought small molecule alternatives to recombinant proteins for *in vivo* experimentation with canonical wnt inhibition. IWR-1 is a small molecule compound which inhibits canonical wnt signaling by stabilizing Axin2 and thus enhancing the degradation of β–catenin (Chen et al., 2009). TOPGAL mice were treated with 3mg/Kg of body weight of IWR-1, twice a day, for three days. On the third day, mice were sacrificed, livers isolated and assayed for β–galactosidase activity using an optical density measurement system. The results show that the given doses of IWR-1 did not significantly affect baseline β–galactosidase activity (Fig. 6.2 A). The effect of IWR1 treatment on MI-induced canonical wnt activation and EndMT was assessed by treating mice with vehicle (DMSO) or 3mg/Kg of body weight of IWR-1, 3, 4, and 5 days after being subjected to LAD occlusion or sham surgeries. The results show that the given IWR-1 drug regimen did not affect MI-induced up-regulation of the canonical wnt target, *WISP1* or EndMT-associated gene, *Snail* (Fig 6.2 B). To assay the pharmacokinetic properties of IWR-1, plasma was isolated from mice 10 minutes, 3 hours and 24 hours after intraperitoneal injection of 3mg/Kg of body weight of IWR-1. Isolated plasma was subjected to mass spectrometric analysis. The results show that IWR-1 is rapidly cleared with 50-70% of the initial dose being detected after 10 minutes, 5-10% after 3 hours and 0% after 24 hours (Fig. 6.2 C).



Time after IP injection	Plasma Concentration of IWR-1 (nM)
10 mins	150
3 hours	15
24 hours	ND

Figure 6.2: **IP injection of IWR-1 did not affect canonical wnt signaling or EndMT *in vivo* likely because IWR-1 is rapidly cleared**. A. TOPGAL mice were given five IP doses of DMSO or 3mg/Kg BW IWR-1 over the course of three days. Livers were isolated and assayed for beta-gal activity. B. Mice treated with DMSO or 3mg/Kg BW were subject to sham or LAD occlusion surgeries. Hearts were isolated and real-time quantitative PCR for the expression of canonical wnt targets and EndMT-associated genes was performed 7 days after surgery. C. IWR-1 clearance was measured by mass spectrometry on plasma isolated 10mins, 3hrs and 24hrs after IP injection of 3mg/Kg BW.

Discussion

Our *in vivo* and *in vitro* data from previous chapters suggest that canonical wnt signaling mediates EndMT during post-MI granulation tissue formation and by so doing, likely contributes to both new blood vessel formation and fibrosis. In this chapter, attempts to manipulate canonical wnt signaling *in vivo* and thereby test the hypothesis that canonical wnt signaling activation is necessary for post-MI EndMT and subsequent neovascularization and fibrosis, fell short. Our experiments treating mice with 500 μg/Kg of body weight of the endogenous canonical wnt inhibitor, Dkk-1, showed no effect on *in vivo* canonical wnt signaling activity and as a result, no effect on post-MI EndMT or other cardiac parameters. Our experiments with Dkk-1 suggest that a higher dose of the protein, or a different delivery method, may be necessary to attenuate myocardial infarction-induced canonical wnt activation and EndMT, and subsequently affect post-MI cardiac function.

The lack of success with Dkk-1 led us to seek alternatives to recombinant proteins for the post-infarction manipulation of canonical wnt signaling activity. We turned to the small molecule, IWR-1, which inhibits canonical wnt signaling activity by stabilizing Axin2 and promoting the degradation of β–catenin. The data show that treating mice with IWR-1 did not cause any measurable effect on canonical wnt signaling activity or EndMT. Mass spectrometric analysis of the pharmacokinetics of IWR-1 showed that the compound is rapidly cleared suggesting that if IWR-1 is to be used to attenuate canonical wnt signaling activity *in vivo*, continuous intravenous (IV) dosing may be necessary to

produce an effect on myocardial infarction-induced canonical wnt activation and endothelial-to-mesenchymal transition.

In summary, our initial attempts at manipulating *in vivo* canonical wnt signaling via intraperitoneal injections of recombinant protein or small molecule wnt pathway inhibitors proved unsuccessful. The question of whether post-infarction neovascularization and fibrosis are altered by manipulation of canonical wnt signaling thereby remains open.

Methods

Mice

All procedures were carried out with the approval of the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center.

Experimental myocardial infarction

The previously described protocol was followed

Echocardiography

Performed as previously described

FACS analysis

Performed as previously described.

Mouse plasma isolation

Mice were injected with 50 μl of 50 mg/ml heparin solution. Ten minutes after injection, mice were anesthetized and sacrificed. Blood was isolated from mouse hearts and spun for 7 minutes at 13,200rpm in order to obtain plasma, the top liquid layer.

Mass spectrometry

Isopropanol (500 μl) was added to mouse plasma (250 μl). The plasma solution was mixed by vortexing, and centrifuged at 18,000g for 5 minutes at 5^{o}C. The clear supernatant was filtered, dehydrated under nitrogen and reconstituted in 20% acetonitrile solution. Plasma solution was then injected into LC/MS for mass spectrometric analysis. Mass spectrometric analyses were performed at the Vanderbilt University Mass Spectrometry Research Center.

β-galactosidase activity optical density assay

Measurement of β−galactosidase activity was performed using high sensitivity β−galactosidase assay kit (Agilent) according to the manufacturer's protocol. Briefly, mouse livers were harvested and lysed in lysis buffer, containing Triton-X 100. Lysates were treated with chlorophenol red−β−D-galactopyranoside (CPRG), a substrate for β−galactosidase enzyme that turns from yellow-orange to red upon cleavage, and incubated at 37^{o}C till reaction mixture turned dark red. Optical density was read at 595 nm.

Quantitative RT-PCR analysis

Total RNA was isolated from mouse hearts using the Trizol reagents (Invitrogen) following the manufacturers' instructions. Reverse transcription of RNA into cDNA and quantitative PCR analysis were performed as previously described. Forward and reverse primer sequences are listed in Table 1.

Statistical analysis

The GraphPad Prism software was used to handle data. Results are reported as mean $\pm$ S.E.M. Student's *t*-test was used for comparing two groups.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Summary

Myocardial infarction is the leading cause of death worldwide. Current available MI therapies, including thrombolytics and percutaneous coronary interventions, are aimed at opening the blocked coronary artery and preventing further ischemic injury to the heart. However, no therapies currently exist to repair already damaged cardiac tissue and as a result, heart failure is often a complication of myocardial ischemic injury. A better understanding of the cellular and molecular events that occur after ischemic heart injury may lead to the identification of new therapeutic targets that may ultimately result in myocardial repair. The fact that canonical wnt pathway intermediates are induced after MI suggests that the canonical wnt pathway may be a novel MI therapeutic target However, the effect of wnt pathway manipulation on post-MI wound healing and recovery has been controversial, with several studies yielding contradictory reports. As these contradictions may be due to ambiguities surrounding the activation state of the canonical wnt pathway after MI, the issue of whether the post-MI increase in wnt pathway intermediates leads to canonical wnt pathway activation, at what phase of the injury response such activation occurs, and what processes are mediated by activated canonical wnt signaling, was the focus of this dissertation project.

The experiments presented here use the canonical wnt reporter, TOPGAL transgenic mouse line, and RT-PCR for the expression of canonical wnt targets, to show that the canonical wnt signaling pathway is induced after myocardial infarction. The results show that post-MI canonical wnt activation occurs during granulation tissue formation, and coincides with endothelial-to-mesenchymal transition (EndMT). The data show that canonical wnt signaling is active in endothelial-derived myofibroblasts generated during granulation tissue formation. Furthermore, the experiments show that activation of the canonical wnt signaling pathway is sufficient to induce EndMT in cultured endothelial cells.

Implications

These findings show that canonical wnt activation and endothelial-to-mesenchymal transition are myocardial infarction responses that occur during granulation tissue formation. The study suggests that the canonical wnt signaling pathway may mediate EndMT after MI. Our findings are in support of a model postulating that activation of canonical wnt signaling in endothelial cells after ischemic injury leads to EndMT and generation of bipotential mesenchymal cells. These migratory mesenchymal intermediate cells may then take part in neovascularization and myofibroblast generation, giving rise to new blood vessels and laying down collagen for subsequent scar formation (Fig. 7.1).

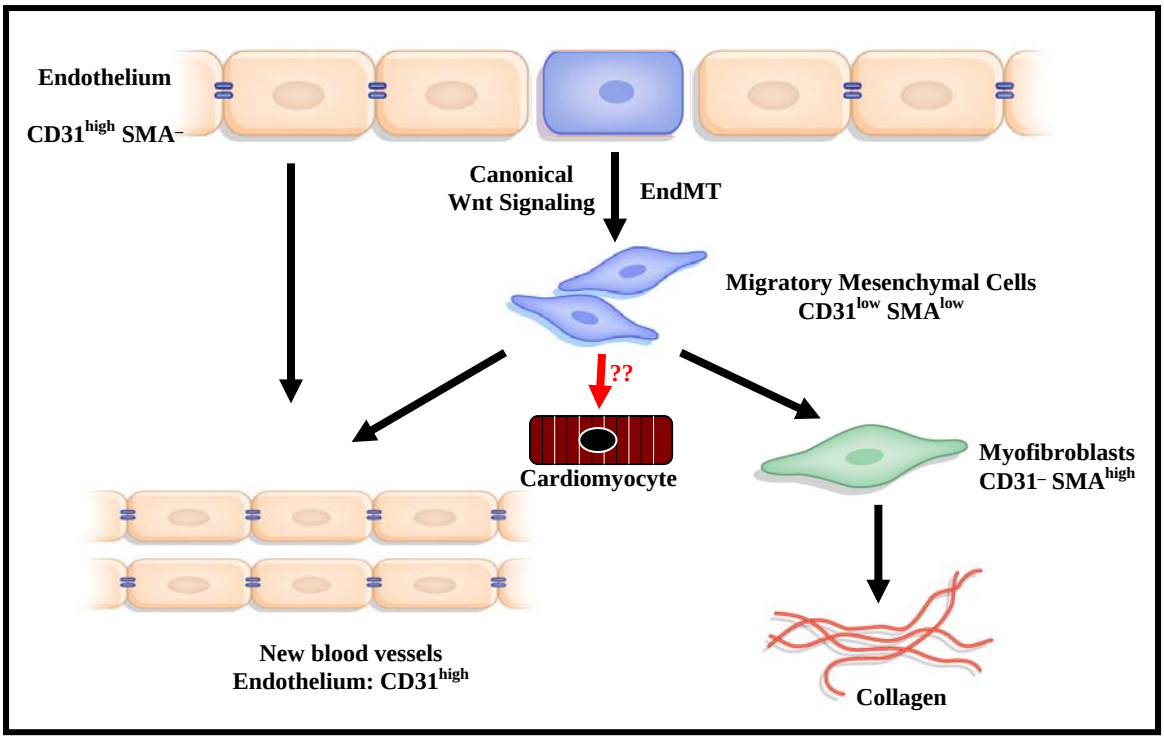


Figure 7.1: **Model: The endothelium serves as a cardiac stem cell source**. Our data show that endothelial cells are a versatile population of cells, capable of self-renewal and transformation into myofibroblasts when the need arises. Our data also show that the canonical wnt signaling pathway acts as a regulator of endothelial cell behavior; activation of canonical wnt signaling is able to induce EndMT *in vitro*. We propose a model whereby canonical wnt signaling initiates a mesenchymal transition in endothelial cells to yield a migratory intermediate cell population that gives rise to both new blood vessels and scar-forming myofibroblasts during post-MI granulation tissue formation. The expression of cardiac progenitor cell-associated genes in endothelial and wnt-active cells raises the possibility that the endothelium may also give rise to cardiomyocytes. Teasing out the signals that push migratory mesenchymal intermediate cells to form cardiomyocytes and new blood vessels, instead of myofibroblasts, and vice versa, will be important for the development of new MI therapies.

In support of our model, both endothelial-to-mesenchymal transition and canonical wnt signaling have been shown to contribute to angiogenesis and fibrosis in development and disease. EndMT has been shown to play a role in angiogenesis in the retina (Gerhardt et al., 2003), tumor angiogenesis (Potenta et al., 2008), and fibrosis in a number of chronic diseases (Zeisberg et al 2008; Hashimoto et al 2009; Zeisberg et al 2007; Widyantoro et al 2010)). Similarly, canonical wnt signaling has been shown to be important for proper vascular formation in the placenta (Monkely et al., 1996). The link between canonical

wnt signaling and angiogenesis is additionally supported by tumor studies which show that blocking wnt signaling inhibits angiogenesis (Hu et al 2009; Zhang et al 2010). Canonical wnt signaling has also been implicated in fibroproliferative diseases such as, fibromatoses, Dupuytren's contracture and hypetrophic skin keloid scars (Bowley et al., 2007), as well as in fibrosis during aging (Brack et al., 2007). Ours is the first to connect canonical wnt signaling to EndMT and subsequent angiogenesis and fibrosis in the healing myocardium.

Our study implicates the endothelium as a major source of repair cells during granulation tissue formation. Our findings show that the endothelium not only reproduces itself, but also transforms into another cell type, the mesenchymal myofibroblast. Defined this way, as a cell that reproduces itself and differentiates into other cell types, the endothelium can be considered as a stem cell. Consistent with this notion, endothelial cells proliferate at homeostasis in the healthy adult mouse heart and also after ischemic injury. Furthermore, endothelial cells express the cardiac stem cell-associated genes, *Nkx2.5*, *Isl-1* and *Stem cell antigen (Sca)-1*.

In summary, our work shows an intricate cellular (EndMT) and molecular (canonical wnt signaling) connection between two critical repair mechanisms with apparently opposing effects, beneficial neovascularization and detrimental fibrosis. Such a dynamic role may explain why studies manipulating the wnt pathway in experimental MI models occasionally yield contradictory results.

Limitations and Future directions

The data presented show that the canonical wnt signaling pathway is activated during the granulation tissue formation phase of the myocardial infarction response, that canonical wnt activation coincides with post-infarction endothelial-to-mesenchymal transition, and that canonical wnt signaling is sufficient to induce a mesenchymal transition in the mature vascular endothelium.

Our studies suggest that canonical wnt signaling mediates EndMT during granulation tissue formation after myocardial infarction, and by so doing, contributes to both new blood vessel formation and fibrosis. Our studies also suggest that post-MI cardiac repair may be improved by temporal and cell type-specific manipulation of canonical wnt signaling.

Attempts to manipulate canonical wnt signaling *in vivo* and test these hypotheses via intraperitoneal injections of recombinant protein or small molecule wnt pathway inhibitors proved unsuccessful. Therefore, the issue of whether post-MI cardiac repair can be improved by temporal and cell type-specific manipulation of canonical wnt signaling still needs to be studied. Other than treatment with recombinant protein or small molecule canonical wnt inhibitors, the use of conditional inducible cre strategies to attenuate canonical wnt signaling activity during the period of granulation tissue formation may also help address the issue.

Finally, based on the technologies at our disposal, our study was limited to elucidating the post-MI role of the canonical wnt/β–catenin branch of the wnt signaling pathway. However, wnt pathway intermediates, such as Wnt-4 and Wnt-11, which have been implicated in non-canonical wnt signaling (Du et al., 1995; Pandur et al., 2002; Maurus et al., 2005; Chang et al., 2007), are also induced post-MI. In addition, dkk-1, which has been implicated in preferentially activating the non-canonical wnt branch while inhibiting the canonical/β–catenin branch (Semënov et al., 2001; Chien et al., 2009), is up-regulated after MI. Therefore, elucidating the post-MI interactions between the canonical and non-canonical branches of the wnt signaling pathway may help further knowledge to improve the course of cardiac recovery.

Conclusions

In conclusion, this dissertation project sought to understand the role the canonical wnt signaling pathway plays in the myocardial infarction response. Our work showed that canonical wnt signaling is induced in the healing (granulation tissue formation) stages of myocardial infarction. We identified endothelial-to-mesenchymal transition as a cellular response to MI that occurs during granulation tissue formation, coincident with canonical wnt activation. Furthermore, we show that canonical wnt signaling is sufficient to induce endothelial-to-mesenchymal transition in the mature vasculature. Our findings suggest that the granulation tissue formation stage is a target for potential therapies to modulate canonical wnt signaling and EndMT. Our studies also suggest that canonical wnt and EndMT-modulating therapies may affect both beneficial neovascularization and detrimental fibrosis. Future studies elucidating the signals that cue endothelial-derived

mesenchymal cells to form new blood vessels rather than myofibroblasts and vice versa will be important for the generation of novel MI therapies.

Table 1. Sequence of primers used for PCR amplification

Mouse Primers

Gene	Forward primer	Reverse primer
Col1A1**	ATGGATTCCCGTTCGAGTACG	TCAGCTGGATAGCGACATCG
c-Myc	CAAATCCTGTACCTCGTCCGATTC	AGCTGGATAGTCCTTCCTTGTGGAG
Dkk-1	CAACTACCAGCCCTACCCTTGCG	CAGACGGAGCCTTCTTGTCCTTTG
Dkk-2	ATGAACCAAGGACTGGCTTTCGG	CGAGCACAACAAAACCCATCAATG
Dkk-3	GCCTCAGATGACGGTTTCAGGTGC	GCTGTGCTGCCTCTCCTTAGTTTCAAC
FSP1**	TCAGGCAAAGAGGGTGACAAG	AGGCAGCTCCCTGGTCAGT
Snail	GATGAGGACAGTGGCAAAAGCTC	AGAATGGCTTCTCACCAGTGTGG
TCF7	ATCCACCACAGGAGGAAAAAGAAAT	GTTAGGCTGCCTGTCTCTGAGATTC
TGFβ1	AGATTAAAATCAAGTGTGGAGCAAC	GTCCTTCCTAAAGTCAATGTACAGC
Vimentin	GGTACAAGTCCAAGTTTGCTGACCT	CATTGAGCAGATCTTGGTATTCACG
WISP1	CAGATGGCTGTGAATGCTGTAAGAT	GGATGCAACACCTATTGTCAGTACA
Wnt-1	AGTCCTGCACCTGCGACTAC	CCTCGGTTGCCGTAAAGGAC
Wnt-2	ACTTCAGGAAAACAGGCGACTATC	AATTTACACTCACACTTGGTCATCC
Wnt-2b	GACTACCTGAGGAGGCGATATG	CATCCACAGTGTTTCTGCACTC
Wnt-3	GGAGAAACGGAAGGAGAAATG	GAGAGACGTTAGTTGAGAAAGAAGC
Wnt-3a	GAGGGATCAGCTGCTATCTGTG	GTCATACTTGTCCTTGAGGAAATC
Wnt-4	AGCAGGTGTGGCCTTTGCAGTGAC	CGTTGTTGTGAAGATTCATGAGTGCC
Wnt-5a	GAGACAACATCGACTATGGCTACC	GTTGACCTGCACCAGCTTGC
Wnt-5b	CAGAGAGGGTGAGCTGTCCAC	CTGTCGTACTTCTCCTTCAAACG
Wnt-6	CAACTGGCTCTCCAGATGCT	AGAGCTTTGCCGTCGTTGGT
Wnt-7a	GAAGGAGCTCAAAGTGGGTATTC	CTTACACTCCAGCTTCATGTTCTC
Wnt-7b	AGTGGATCTTTTACGTGTTTCTCTG	CTGGTTGTAGTAGCCTTGCTTCTC
Wnt-8a	CTGCAGCGACAACGTGGAGT	GCTCTGTTGCCAGCCCTTAG
Wnt-8b	AGAATGTAAATACCAGTTTGCTTGG	CATTGTTGTGCAGATTCATGG
Wnt-9a	AAGTACAGCAGCAAGTTTGTCAAG	CCACCTCCTGACCCAGAAGC
Wnt-9b	CGCTGTACTTGTGACGACTCC	GTGTCATAGCGTAGCTTCAGC
Wnt-10a	AGAACGCTTCTCTAAGGACTTTCTG	AGTCGGGAGATTTCTCAAAGTAGAC
Wnt-10b	AGAAGTTCTCTCGGGATTTCTTG	CAAAGTAAACCAGCTCTCCAG
Wnt-11	GCTACCTGTCAGCCACGAAGGTG	CTGCCGTTGGAAGTCTTGTTGCAC

** *Reference: Zeisberg et al., 2007*

Table 1, continued

Bovine Primers

Gene	Forward primer	Reverse primer
Axin2	CGAGCTGGTGGTCACTTACTTCTTC	AGGGCTCAGTCGATCCTCTCC
CD31	GAATGTAGCAATTCATCAGGCCAAG	ACTAGCCCCTAAGCTAGGCCACTCT
Col1A1	CTCTGACTGGAAGAGCGGAGAATAC	CTGTTCTTGCAGTGGTAGGTGATGT
SMA	CAGGAAGGACCTCTATGCTAACAACG	GACAGACAAGAGAGCAGGGAGTGTC
Slug	TCCACCTCTGAGCGACAAGTAAAAG	TTACAAATCCCGTACAAGGCAACAC
TCF7	CCCTGACCTCTCTGGCTTCTACTCT	CAATGACTTTGGCTCTCATCTCCTT

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