Experimental myocardial infarction triggers canonical Wnt signaling and endothelial-to-mesenchymal transition

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SUMMARY

Despite available therapies, myocardial infarction (MI) remains a leading cause of death worldwide. Better understanding of the molecular and cellular mechanisms that regulate cardiac repair should help to improve the clinical outcome of MI patients. Using the reporter mouse line TOPGAL, we show that canonical (β-catenin-dependent) Wnt signaling is induced 4 days after experimental MI in subepicardial endothelial cells and perivascular smooth muscle actin (SMA)-positive (SMA+) cells. At 1 week after ischemic injury, a large number of canonical-Wnt-positive cells accumulated in the infarct area during granulation tissue formation. Coincidently with canonical Wnt activation, endothelial-to-mesenchymal transition (EndMT) was also triggered after MI. Using cell lineage tracing, we show that a significant portion of the canonical-Wnt-marked SMA+ mesenchymal cells is derived from endothelial cells. Canonical Wnt signaling induces mesenchymal characteristics in cultured endothelial cells, suggesting a direct role in EndMT. In conclusion, our study demonstrates that canonical Wnt activation and EndMT are molecular and cellular responses to MI and that canonical Wnt signaling activity is a characteristic property of EndMT-derived mesenchymal cells that take part in cardiac tissue repair after MI. These findings could lead to new strategies to improve the course of cardiac repair by temporal and cell-type-specific manipulation of canonical Wnt signaling.

INTRODUCTION

Acute cardiac ischemic injury [also known as myocardial infarction (MI)] afflicts about 1.5 million people in the USA every year and is a leading cause of mortality, accounting for one in every four 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 can kill cardiomyocytes within minutes, but the extent of the injury depends on the location and duration of the blood flow obstruction. The widespread cell death causes an immediate and massive inflammatory response that gradually clears out the injury site, leaving behind sparse tissue with enlarged capillaries. After cellular debris is removed from the injury area, the gap fills with granulation tissue. This process starts a few days after the initial ischemic assault and involves activation and proliferation of endothelial cells and infiltration of myofibroblasts (Frangogiannis, 2008). Angiogenesis leads to the formation of new vessels in an attempt to restore blood supply, whereas myofibroblasts deposit collagen and other extracellular matrix proteins. A week after infarction, the granulation tissue starts to mesh into a dense scar. The extensive local and systemic response to preserve ventricular integrity, coupled to the low innate regenerative capacity of the heart, causes permanent loss of cardiac tissue, leading to ventricular remodeling and heart failure.

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. Better understanding of the molecular and cellular mechanisms that control the intrinsic cardiac repair processes could lead to optimal management of angiogenesis and scar formation and prevent or delay the onset of heart failure in MI patients. A potentially important regulatory mechanism of cardiac repair is the canonical Wnt/β-catenin pathway. A number of studies have shown that several Wnt factors, along with intracellular mediators of canonical signaling such as disheveled and β-catenin, are induced after experimental MI in various animal models of cardiac injury (Blankenstein et al., 2000; Barondon et al., 2003; Barondon et al., 2005; Chen et al., 2004; Kobayashi et al., 2009). 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, leads to improved cardiac function, reduced infarct size and less cardiac rupture after MI in mice, suggesting that blocking Wnt signaling is cardioprotective (Barondon et al., 2003; Mirotsou et al., 2007; Alfaro et al., 2008). Paradoxically, inactivation of sFRP2 also leads to better 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, causes a reduction in myocardial infarct size in a rat MI model, suggesting that canonical Wnt signaling improves cardiac wound healing (Hahn et al., 2006).

These apparently conflicting results might be due to variations between animal models, differential regulation of the canonical Wnt pathway and downstream gene targets by specific modulators, or involvement of sFRPs in processes that are independent of their typical Wnt signaling roles, such as procollagen cleavage (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). Finally, the cell specificity and timing of canonical Wnt pathway activation or suppression probably play a cell-type- and phase-dependent role in the MI response, comparable to the described stage-specific roles of the Wnt pathway in heart development (Aisagbonhi and Hatzopoulos, 2011).

Therefore, to establish whether the induction of various pathway mediators after MI results in a net activation of canonical Wnt signaling, we investigated temporal, spatial and cell-type-specific changes in canonical Wnt/β-catenin signaling after permanent occlusion of the left coronary artery. To this end, we used the TOPGAL reporter transgenic mouse line, which carries the lacZ gene (encoding β-galactosidase) under the control of three tandem β-catenin-responsive consensus TCF/LEF-binding motifs upstream of a minimal fos promoter, as an unbiased readout system of canonical Wnt activity at different stages post-MI (DasGupta and Fuchs, 1999).

Our results show that, in the normal adult mouse heart, canonical Wnt pathway activity is limited to a subset of vascular and perivascular cells, mainly around the large vessels at the base of the heart, and in valve mesenchyme. After experimental coronary artery ligation, there was a dramatic increase in canonical Wnt signaling activation starting at day 4 after MI and peaking during granulation tissue formation. Immunohistological analyses revealed that canonical Wnt activity is primarily confined to endothelial cells and α-smooth muscle actin (SMA)-expressing mesenchymal cells. To test whether these two canonical-Wnt-signaling-positive cell types are linked, we used a double transgenic line carrying the inducible Cre recombinase under the endothelial-specific enhancer of the stem cell leukemia (SCL) gene (Göthert et al., 2004) and the R26RstoplacZ locus (Soriano, 1999). Cell lineage tracing experiments showed that canonical-Wnt-activity-marked SMA+ cells, which congregate in the infarct and peri-infarct areas, are derived from endothelial cells, possibly through endothelial-to-mesenchymal transition (EndMT).

Consistent with this model, we found that activation of canonical Wnt signaling in cultured, mature endothelial cells induces morphological and molecular traits of the mesenchymal phenotype. Taken together, our results suggest that canonical Wnt signaling is involved in EndMT after ischemic injury and that this process might be a key contributor to new endothelial cells and myofibroblasts during granulation tissue formation. Thus, it seems that two important cardiac tissue repair processes after MI, i.e. neovascularization and scar formation, are more intrinsically linked than previously thought. These findings could lead to the development of novel ways to manipulate cardiac recovery after ischemic injury in order to promote angiogenesis and moderate scar tissue formation.

RESULTS
Expression of Wnt pathway mediators is induced after experimental MI
To evaluate the response of Wnt pathway components to myocardial ischemic injury, we permanently occluded the left anterior descending (LAD) coronary artery of C57BL/6J mice. Sham-operated mice, which underwent the same surgical procedure without arterial ligation, served as controls. We isolated mRNA from whole heart tissue 5 days after surgery and assessed the expression of the entire set of 19 Wnt ligands, as well as three members of the Dickkopf (Dkk) family of canonical Wnt signaling mediators, using conventional and real-time quantitative reverse-transcriptase (RT)-PCR analysis (Fig. 1).

We found 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. 1A). Following experimental MI, there is a strong upregulation in the transcript levels of both Wnt ligands and members of the Dkk family (Fig. 1A). Specifically, quantitative analysis showed 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. 1B). Two Wnt proteins, Wnt-8b and Wnt-16, did not produce detectable signals in normal heart and after MI (not shown).

These results suggest that significant changes in Wnt signaling take place in the heart following ischemic injury. However, the simultaneous induction of Wnt ligands known to activate canonical and non-canonical branches of Wnt signaling, e.g. Wnt7b and Wnt-11, respectively, as well the strong increase in the expression levels of canonical Wnt pathway antagonists such as Dkk-1, make it difficult to predict whether canonical Wnt activity is induced overall or is confined in specialized areas and cell types, or associated with specific cardiac tissue repair processes. To distinguish between these possibilities, we used the TOPGAL reporter mouse to determine changes in canonical Wnt signaling after ischemic injury.

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, we first analyzed canonical Wnt/β-catenin signaling in the normal adult heart. To this end, hearts of TOPGAL transgenic mice were isolated and subjected to whole-mount staining for β-galactosidase activity and serial histological analysis (Fig. 2). Our 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 in the valves (Fig. 2A,B). Histological sections revealed that β-catenin signaling is active in the media and intima of the aorta and pulmonary vessels as well as in coronary vessels and subepicardial microvasculature. In the valves, expression is mainly found in valve mesenchyme.

The staining patterns suggest that canonical Wnt activity marks subpopulations of both endothelial and smooth muscle cells in the heart. To confirm this finding, we analyzed histological sections...
stained with antibodies against the endothelial-specific marker CD31 (PECAM-1) and SMA (Fig. 2C,D). The results confirmed expression in subsets of smooth muscle and endothelial cells. Taken together, our results indicate that, during cardiac homeostasis, canonical Wnt activity is confined to select subpopulations of endothelial and smooth muscle cells, as well as to valve mesenchymal 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 MI, we occluded the LAD of TOPGAL mice or performed sham surgery, isolated hearts at defined time points after arterial ligation, and stained for β-galactosidase activity. In brief, we collected hearts 24 hours post-MI (during the cell death and inflammation phase), at 4-7 days post-MI (during granulation tissue formation) and at 3 weeks post-MI (when scar tissue has matured) (Fig. 3A).

Our results show that canonical Wnt signaling is not activated during the early post-MI response, because the X-gal reactivity in TOPGAL mouse hearts that were isolated within the first 24 hours after coronary artery ligation was practically indistinguishable from sham-operated controls (Fig. 3B). By contrast, canonical-Wnt-positive cells were scattered throughout the myocardium 4 days after LAD ligation. By 7 days post-MI, canonical Wnt activity was primarily localized to large numbers of cells within the infarct and peri-infarct areas. This activation was transient and disappeared by 3 weeks post-infarction.
Consistent with the timing of β-galactosidase staining in TOPGAL mice post-MI, real-time quantitative PCR analysis of RNA samples collected at equivalent time points showed strong upregulation of bona-fide β-catenin signaling gene targets, such as Wisp1, Tcf7 and Myc, at 7 days post-MI (Fig. 3C). Among the tested canonical Wnt signaling targets, Myc also showed strong induction at 24 hours, probably through pathways that are independent of canonical Wnt signaling.

**Fig. 2. Canonical Wnt signaling activity in the normal 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 (center left image), pulmonary (center right), and coronary arteries and subepicardial microvasculature (right). (B) Whole-mount X-gal staining of partially dissected heart to show cells with canonical Wnt activity in cardiac valves (left). Histological sections (two right-most panels) reveal cells positive for canonical Wnt activity in the connective tissue of outflow tract (aortic) and atrioventricular (mitral) valves. (C) Anti-CD31 antibody stain (brown) on an X-gal-stained section from TOPGAL mouse aorta demonstrates canonical Wnt activity in vascular endothelial cells (arrow). (D) Anti-SMA antibody binding (brown) on an X-gal-stained aorta section indicates canonical Wnt activity (blue) in perivascular SMA+ cells. The three right panels are IF images of tissue sections stained with antibodies recognizing SMA in vascular smooth muscle cells (green) and β-galactosidase (red). Merged images (far right) show colocalization of the two antigens in cells of the intima and media areas of the aorta. DAPI was used for counterstaining of cellular nuclei (blue).
Canonical Wnt signaling is activated in endothelial cells and SMA+ cells after MI

Granulation tissue formation is characterized by endothelial cell proliferation and sprouting 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, we analyzed histological sections of TOPGAL mouse hearts 7 days after LAD occlusion or sham surgery. We found that, surrounding the infarct area after MI, there is an increase in endothelial cells in which there is active canonical Wnt signaling. However, the intense staining observed in the whole-mount images at day 7 post-MI (Fig. 3) is mainly due to the de novo appearance of large numbers of subepicardial mesenchymal-like cells marked with canonical Wnt.
signaling activity (Fig. 4A). There is also canonical Wnt signaling activation in a subset of epicardial cells.

Immunohistochemical (IHC) and immunofluorescence (IF) analyses using antibodies recognizing the endothelial marker CD31 and the myofibroblast marker SMA confirmed that canonical Wnt signaling was active in endothelial cells and SMA-expressing cells in the infarct and peri-infarct areas 7 days post-MI (Fig. 4B,C). Macrophages in the peri-infarct area were also marked by canonical
Fig. 4. Canonical Wnt activity marks endothelial cells and myofibroblasts after MI. Histological analysis of canonical Wnt activity was performed on TOPGAL mouse hearts 7 days after LAD occlusion or sham surgery. (A) Tissue sections of X-gal-stained mouse hearts show 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 (middle two panels). By contrast, cells with active canonical Wnt signaling are not present in subendocardial regions (right panel). (B) Immunohistochemical analyses of sections from TOPGAL mouse cardiac tissue. 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 right 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. DAPI was used for counterstaining of cellular nuclei (blue). (C) IHC analysis using the anti-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 right panels display IF results obtained using the anti-β-galactosidase (green) and -SMA (red) antibodies, and the corresponding merged image. Blue is nuclear DAPI counterstain. 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. A portion of SMA+ cells with canonical Wnt pathway activity are macrophages (supplementary material Fig. S1). (D) Canonical-Wnt-activity-marked endothelial cells were quantified by counting the number of double X-gal/CD31-stained and single 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<0.001. Control sham, n=5; MI samples, n=5.

Wnt activity (supplementary material Fig. S1). To measure the increase in canonical-Wnt-signaling-positive cells, we counted CD31+ and SMA+ cells marked with β-galactosidase activity in the infarct and peri-infarct areas and compared the measurements with cell counts in the corresponding cardiac tissue sites of sham-operated mice. The results show that the fraction of endothelial cells marked with β-galactosidase activity increased from about 30% in normal subepicardial heart tissue to 50% after MI. In parallel, the overall fraction of β-galactosidase-positive SMA-expressing cells increased from almost undetectable levels to 12% of the entire subepicardial cell population. Finally, β-galactosidase (canonical Wnt pathway)-marked SMA+ cells represented about 40% of SMA+ cells in this area (Fig. 4D).

EndMT is induced post-MI

The concurrent activation of canonical Wnt activity in endothelial cells and SMA+ cells raised the possibility that the latter are derived from activated endothelium. Consistent with this notion, EndMT has been linked to tissue repair in various organs during angiogenesis and vessel branching, when tip cells acquire a mesenchymal phenotype (Gerhardt et al., 2003). EndMT has also been implicated in myofibroblast production and cardiac fibrosis after aortic banding (Zeisberg et al., 2007).

Hallmark features of EndMT include the appearance of cells that are double-positive for endothelial and smooth muscle gene markers such as CD31 and SMA (Paruchuri et al., 2006; Goumans et al., 2008). To examine whether CD31+/SMA+ double-positive cells emerge after experimental MI, we analyzed cardiac tissue sections by IF. We found a large number of double-positive CD31+/SMA+ cells, suggesting that EndMT takes place post-MI (Fig. 5A).

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. 5B and supplementary material Fig. S2, there was a significant increase in double-positive cells expressing both CD31 and SMA at 7 days post-MI. Specifically, the percentage of CD31+/SMA+ cells rose from less than 1% in normal cardiac tissue to almost 25% of the total number of isolated cells (Fig. 5C).

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 (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. 5D).

To test whether induction of proteins regulating mesenchymal transition, such as Snail, takes place in canonical-Wnt-positive cells, we stained cardiac tissue sections of TOPGAL mice 7 days post-MI. The results show that Snail is expressed in cells marked by canonical Wnt signaling activity (Fig. 5E). Taken together, our results suggest that EndMT takes place after ischemic injury and reaches its highest point during granulation tissue formation. Moreover, canonical Wnt activity marks cells undergoing EndMT.

Endothelial origin of post-MI myofibroblasts marked with canonical Wnt activity

To confirm the endothelial origin of peri-infarct mesenchymal cells, we crossed the endothelial-SCL-Cre-ER2 mouse line, in which the 5′ endothelial-specific enhancer of the SCL locus drives tamoxifen-inducible Cre-ER2 (Göthert et al., 2004), to the R26RstoplacZ mouse line (Soriano, 1999). The SCL gene is expressed in both hematopoietic and endothelial cells (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; Göthert et al., 2004).

We injected the adult progeny of the double transgenic line SCL-Cre-ER2/R26RstoplacZ, named end-SCL-lacZ mice, with tamoxifen to induce Cre recombinase and labeling of endothelial cells. At 7 days after the last tamoxifen dose, the mice underwent LAD or sham surgery. Histological analyses 7 days after surgery showed that, in sham-operated mice, β-galactosidase expression was limited to endothelial cells and to a few mesenchymal cells around the aorta and in the valves (Fig. 6A; supplementary material Fig. S3). Conversely, the cardiac tissues of mice subjected to LAD occlusion
had both increased β-galactosidase-positive endothelial cells and a novel population of β-galactosidase-expressing mesenchymal cells (Fig. 6A). Anti-SMA staining confirmed the presence of double-positive β-galactosidase/SMA cells in perivascular and subepicardial peri-infarct regions (Fig. 6B).

Quantification of histological sections showed that β-galactosidase+/SMA+ cells represent 35-40% of the subepicardial SMA+ cell population (Fig. 6B). 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.

Because peak canonical Wnt pathway signaling coincides with the time of highest EndMT activity, we investigated whether EndMT-derived mesenchymal cells are marked by canonical Wnt activity. In this experiment, we analyzed cardiac tissue sections of tamoxifen-treated end-SCL-lacZ mice 7 days after LAD or sham surgery with antibodies recognizing β-catenin. The results show nuclear β-catenin in β-galactosidase-stained mesenchymal cells of endothelial origin, indicating that EndMT-derived cells are marked by canonical Wnt activity (Fig. 6C).

Canonical Wnt signaling triggers mesenchymal transformation in cultured endothelial cells
Canonical Wnt signaling has been shown to be important for EndMT during embryonic valve development (Liebner et al., 2004), but whether Wnt signaling plays a role in mesenchymal transition of adult endothelial cells has not been addressed. To investigate this...
possibility, we treated bovine aortic endothelial cells (BAECs) with the compound BIO, which blocks GSK-3β phosphorylation of β-catenin, leading to stabilization and intracellular accumulation of β-catenin and thus activating canonical Wnt pathway target genes. BIO-treated BAECs underwent dramatic morphological changes within 24 hours of treatment, acquiring a distinct mesenchymal appearance, whereas vehicle (i.e. DMSO)-treated cells retained the characteristic cobblestone morphology of aortic endothelial cells (Fig. 7A). To examine the molecular phenotype of BIO-treated BAECs, we isolated mRNA from BIO- and vehicle-exposed controls 24 hours and 3 days after BIO treatment. We then analyzed the response of a number of canonical Wnt signaling targets by real-time quantitative RT-PCR using gene-specific primers.

Our results show that exposure to BIO results in a 50- to 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. 7B). By contrast, BIO treatment results in a significant decrease in expression of the endothelial-cell-specific gene CD31 at both the 24-hour and 3-day time points. Interestingly, although expression of the Snail-related gene Slug is moderately upregulated within 24 hours of exposure to BIO, other EndMT-associated genes, such as those encoding SMA and Col1A1, are not induced until day 3 of BIO treatment, suggesting a gradual transition to the mesenchymal phenotype (Fig. 7B). Altogether, the in vitro experiments described above indicate that activation of the canonical Wnt pathway in mature endothelial cells induces morphological and molecular changes that lead to suppression of the endothelial and induction of the mesenchymal phenotype followed by acquisition of smooth muscle and myofibroblast markers.
Fig. 7. Activation of canonical Wnt signaling causes mesenchymal transition in cultured endothelial cells. The effects of canonical Wnt signaling activation were investigated in adult BAECs and the mouse brain bEnd.3 endothelial cell line by treatment with the canonical Wnt signaling activator BIO. (A) BIO induces a mesenchymal phenotype in BAECs within 24 hours of treatment. (B) Real-time quantitative RT-PCR analysis using RNA samples from BIO-treated BAECs. 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 downregulation of 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. BIO treatment leads to downregulation of CD31 and upregulation of SMA, consistent with the molecular data obtained in BAECs. (D) IF analysis of bEnd.3 cells treated for 48 hours with BIO (+) or its inactive analog MeBIO (–) and stained with antibodies recognizing CD31 (green) and SMA (red). BIO treatment leads to nuclear accumulation of β-catenin, a hallmark of canonical Wnt signaling activation. DAPI stain (blue) marks cellular nuclei. All BIO treatment time points (1, 4 or 24 hours) showed nuclear β-catenin (arrowheads). The images are from the 4-hour exposure. (E) Luciferase analysis using protein extracts isolated from bEnd.3 cells transiently transfected with Super TOPFlash and pRL-TK Renilla Luciferase Reporter for normalization. bEnd.3 cells were treated with BIO, MeBIO or vehicle (DMSO) for 6 hours. BIO addition increases luciferase activity, indicating induction of the canonical Wnt pathway.
To test whether EndMT is a widespread response to activation of canonical Wnt signaling, we investigated the effects of BIO on the mouse endothelial line bEnd.3. As shown in Fig. 7C, BIO treatment led to downregulation of CD31 and upregulation of SMA, similarly to the effects on BAECs. Furthermore, BIO treatment led to nuclear translocation of β-catenin and to upregulation of the canonical Wnt signaling responsive promoter, further indicating that activation of the canonical Wnt pathway leads to induction of mesenchymal and suppression of endothelial markers (Fig. 7D,E).

Altogether, the in vitro data, the histological analyses and cell-lineage tracing experiments in mice are in support of a model postulating that activation of canonical Wnt signaling in endothelial cells after ischemic injury leads to EndMT and to generation of bipotential mesenchymal cells. These cells might then take part in both angiogenesis and fibrosis, giving rise to new blood vessels and scar tissue, respectively (Fig. 8).

DISCUSSION

Current treatment alternatives for patients who suffer MI are limited to revascularization to prevent further myocyte death and medical therapy to preserve residual heart function and slow down ventricular remodeling 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 (Boudouhas 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 (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; Gneccchi et al., 2005; 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; Mirotsou et al., 2007; Hinkel et al., 2008; Alfaro et al., 2008; Joggerst and Hatzopoulos, 2009; Hinkel et al., 2010). 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 could lead to new ways to improve cardiac function after MI, independently or in combination with stem cell therapy.

Here, we focused on the Wnt signaling pathway because expression of a number of its individual components, including inhibitors such as sFRPs and activators such as β-catenin, is induced after MI. Moreover, canonical Wnt signaling has been implicated in angiogenesis (Hu et al., 2009; Zhang et al., 2010), fibrosis (Brack et al., 2007) and cardiomyocytic differentiation (Kwon et al., 2007; Lin et al., 2007; Qyang et al., 2007; Cohen et al., 2008).

Our results show that, in the normal, uninjured mouse heart, there is active canonical Wnt signaling in subpopulations of endothelial cells and pericytes; these subpopulations are located mainly around the aorta and pulmonary vessels, in coronary arteries and in subepicardial microvasculature. Moreover, interstitial cells marked with canonical Wnt activity are present within the connective tissue of the valve leaflets. Beginning 4 days after MI, there is an increase in canonical-Wnt-activity-marked cells around the heart, largely in peri-vascular areas. This original appearance is followed by a massive accumulation of canonical-Wnt-positive cells in the infarct and peri-infarct areas 7 days post-MI. Therefore, it seems 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. In support of this idea, our data show that canonical Wnt activity after MI is confined to smooth muscle and endothelial cells, as well as to infiltrating macrophages in the peri-infarct area.

Consistent with the whole-mount and histological staining for β-galactosidase and active β-catenin, the expression of analyzed canonical Wnt signaling targets also shows maximum levels at the same time, i.e. at 7 days post MI, with the exception of Myc, which is 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).
The proximity of endothelial and perivascular smooth muscle cells marked with canonical Wnt pathway activity in the adult heart and the initial induction around blood vessels prior to accumulation of canonical-Wnt-marked endothelial and SMA+ cells around the injury site raises the possibility that the two cell types share a common origin in vascular endothelium. Supporting this hypothesis, the cell lineage tracing analysis described here shows that at least half of the subepicardial SMA+ cells within granulation tissue are of endothelial origin. However, a caveat of cell lineage tracing experiments is the potential existence of unrecognized promoter activity in ectopic domains that might drive Cre recombinase transcription in non-endothelial cells. Although thorough analyses previously ruled out 5’ SCL enhancer activity in hematopoietic lineages or other cell types (Göthert et al., 2004), we cannot fully exclude that at least some of the ß-galactosidase-marked cells are derived from other intra- or extra-cardiac sources.

Our data also show that endothelial-derived SMA+ cells are marked by canonical Wnt activity, and canonical Wnt activation is sufficient to induce EndMT in vitro. During normal cardiac homeostasis, this course might replenish perivascular smooth muscle cells and valve interstitial mesenchyme. However, after ischemic injury, EndMT might become a source of endothelial cells and myofibroblasts for neovascularization and scar formation, respectively. Therefore, next to epicardial cells, which are also activated after injury (Limana et al., 2007), endothelial cells might be an additional crucial source of repair cells after infarction. These results suggest that cells from different origins respond to injury and contribute to cardiac tissue repair. However, it remains unclear whether they perform separate functions or whether this occurrence simply reflects a response to a catastrophic event that mobilizes all available resources to accelerate the repair process. EndMT has been extensively studied during heart development. In the embryo, EndMT occurs during valve development (Armstrong and Bischoff, 2004). Canonical Wnt signaling is crucial for cardiac cushion EndMT; several studies show disruption of valve formation upon disruption of canonical Wnt signaling in zebrafish and mouse (Hurlstone et al., 2003; Liebner et al., 2004). Our results indicate that canonical Wnt signaling and EndMT persist in the adult mouse valves, but also in a number of vascular beds, and that this trend is greatly accelerated during cardiac repair. Therefore, adult repair mechanisms might share a great deal with normal developmental processes that shape the cardiac tissue during embryogenesis.

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., 2010) and heart (Zeisberg et al., 2007; Widyanarto et al., 2010) disease. Ours is the first report to implicate EndMT as a major player in cardiac tissue repair after acute ischemic injury. Previous studies have also shown that EndMT contributes to neovascularization, because endothelial cells acquire a mesenchymal phenotype during vessel branching (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). Interestingly, canonical Wnt signaling has also been implicated in fibrosis, providing a molecular link between fibrosis and angiogenesis (Bowley et al., 2007). Such a dynamic role might explain why studies manipulating the Wnt pathway in experimental MI models occasionally yield contradictory results.

In summary, our work shows an intricate cellular (EndMT) and molecular (canonical Wnt signaling) connection between two crucial repair mechanisms with apparently opposing effects: beneficial neovascularization and detrimental fibrosis. This intrinsic link might open the opportunity to improve the course of cardiac repair post-MI by temporal and cell-type-specific manipulation of canonical Wnt signaling.

**METHODS**

**Mice**

TOPGAL mice express ß-galactosidase under the control of ß-catenin-responsive consensus TCF/LEF-binding motifs upstream of a minimal fos promoter (DasGupta and Fuchs, 1999). The SCL-Cre-ERT mouse carries a fragment of the 5’ endothelial-specific enhancer of the stem cell leukemia (SCL) gene locus driving the tamoxifen-inducible Cre-ERT gene (Göthert et al., 2004). The endothelial-SCL-Cre-ERT 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 2 mg tamoxifen (Sigma) every other day for 10 days. Surgical procedures on end-SCL-lacZ mice were carried out 1 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 TOPGAL and R26RstoplacZ mouse lines were purchased from Jackson Laboratories. The endothelial-SCL-Cre-ERT mouse line was a kind gift of Glen Begley and Joachim Göthert (Telethon Institute for Child Health Research, West Perth, Australia).

**Experimental MI**

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 and Complications Core.

**ß-galactosidase activity in whole tissue staining assay**

Whole hearts were isolated into cold 1× phosphate-buffered saline (PBS) and then fixed for 30 minutes at 4°C in 1× PBS solution containing 1% formaldehde, 0.2% glutaraldehyde and 0.02% NP40. After fixation, hearts were washed twice with 1× PBS for 20 minutes each, and then placed overnight at 30°C in X-gal staining solution (1 mg/ml X-gal, 5 mM potassium ferro- and ferricyanate, and 2 mM magnesium chloride in 1× PBS). Whole-mount hearts were photographed and then stored in 1× PBS at 4°C until embedded in paraffin and cut in 5 μm sections. Sections were deparaffinized, counter-stained with eosin, dehydrated and mounted.
Immunofluorescence and immunohistochemistry
For IF experiments on cardiac tissue sections, 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% paraformaldehyde and fixed for 5 minutes on ice. Slides were washed three times in 1× PBS for 5 minutes each wash. Sections were blocked in a 1× PBS solution of 1% bovine serum albumin (BSA) and 0.05% saponin for 1 hour at room temperature. Sections were then incubated with primary antibodies overnight at 4°C. Afterwards, slides were washed three times in 1× PBS for 5 minutes each, incubated with secondary antibodies for 1 hour at room temperature, washed in 1× PBS three times for 5 minutes each, and mounted with VECTASHIELD fluorocite mounting medium (Vector Laboratories).

For IHC stains, 5-μm sections of β-galactosidase-stained paraffin-embedded hearts were deparaffinized through Histo-Clear and graded alcohols as per standard protocol. Endogenous peroxidase activity was quenched by immersing slides in 3% hydrogen peroxide 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 1× 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 counterstained, dehydrated and mounted with Permount histological mounting medium (Fisher). Histological services were performed 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; 1:2000 dilution), monoclonal mouse anti-SMA (Sigma; 1:800), monoclonal mouse anti-β-catenin (Sigma; 1:800), monoclonal mouse anti-Snail (Chemicon; 1:600), rat anti-mouse CD31/PECAM1 (BD Pharmingen; 1:100) and rat anti-mouse F4/80 (Abcam; 1:100). Secondary antibodies used for IF were: goat anti-mouse Cy3-conjugated, donkey anti-rabbit Cy3, goat anti-rat Cy3, goat anti-mouse Alexa-Fluor-488 and goat anti-rabbit Alexa-Fluor-488. Cy3-conjugated antibodies were obtained from Jackson ImmunoResearch and Alexa-Fluor-488-conjugated antibodies from Invitrogen. 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 (1:5000 dilution; Invitrogen) to mark cellular nuclei.

For IF on bEnd.3 cells, cells were cultured in DMEM (Gibco) containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate and 1× antibiotic antimycotic mix (Sigma) on Nunc Lab-Tek chambered slides. Cells were treated with 5 μM GSK-3β inhibitor BIO IX (CalBiochem; Cat#361550), or 5 μM of its inactive analog methyl-BIO (MeBIO; CalBiochem; Cat#361556) for 1, 4, 24 or 48 hours. Cells were washed with 1× PBS and fixed in 4% paraformaldehyde for 15 minutes at room temperature. Cells were washed three times with 1× PBS for 5 minutes each, permeabilized with 0.2% Triton X-100 in 1× PBS for 30 minutes at room temperature and then incubated with primary antibodies overnight at 4°C. The next day, cells were washed three times with 1× PBS for 5 minutes each, incubated with secondary antibodies for 1 hour at room temperature and washed three times with 1× PBS for 5 minutes each. Cellular nuclei were marked with the fluorescent dye DAPI (1:5000 dilution). Primary antibodies used were: monoclonal mouse anti-SMA (1:800), rat anti-mouse CD31 (1:100) and monoclonal mouse anti-β-catenin (Sigma; 1:800). As secondary antibodies, we used goat anti-mouse Cy3-conjugated (1:600) and donkey anti-rat Alexa-Fluor-488 (1:400).

FACS analysis
Suspensions of cardiac cells depleted of myocytes were prepared as follows. Murine hearts were washed to remove blood and asetically isolated after incision at the base of the aorta. The atria were entirely removed. The ventricles of the heart 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 CaCl2 at 37°C for 20-25 minutes and then passed through a cell strainer. The myocyte-depleted cell suspension was centrifuged at 1500 g for 5 minutes and resuspended in 1× PBS containing 0.5% BSA and 2 mM EDTA.

To prevent non-specific binding, cells were incubated with FcR Blocking Reagent (Miltenyi Biotec). Then single-cell suspensions (106 cells/ml) were labeled using phycoerythrin-conjugated anti-mouse CD31 (clone 390; eBioscience) and peridinin-chlorophyll-protein-conjugated anti-CD45 (clone 30-F11; eBioscience). Cells were incubated for 20 minutes at 4°C, washed and resuspended in PBS/BSA/EDTA buffer.

For intracellular staining to detect SMA, cells were fixed and permeabilized using 4% paraformaldehyde and 0.1% saponin in 1× PBS. Aliquots of cells (105 cells in 100 μl buffer) were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-SMA (Sigma) for 30 minutes at 4°C. Next, cells were washed once with 0.1% saponin and resuspended in 1× PBS containing 0.5% BSA and 2 mM EDTA. Data acquisition was performed on a FACSalibur 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.

Cell culture and luciferase assays
BAECs were used between the sixth and eighth passages. Cells were cultured in standard high-glucose DMEM containing 10% FBS, 1% Pen-Strep and 1% L-glutamine. Cells were treated with 1 μM GSK-3 inhibitor IX (BIO), or the equivalent volume of vehicle solution (DMSO, Sigma).

bEnd.3 mouse endothelial cells were seeded in 12-well plates (5×104 cells/well), grown for 48 hours and then transfected with 0.48 μg of Super TOPFlash Plasmid (Addgene; plasmid 12456), encoding Firefly luciferase gene and 0.02 μg of pRL-TK Renilla luciferase reporter vector (Promega), overnight using FuGENE6 (Roche Molecular Biochemicals) as transfection agent and following the manufacturer’s specifications. The next day, cells were stimulated with 1 μM BIO or 1 μM MeBIO for 6 hours. DMSO treatment served as a control. Luciferase activity was measured using the Dual-Luciferase Reporter Assay from Promega. Firefly luciferase activity was normalized to Renilla luciferase activity and data reported as fold induction over basal (DMSO, vehicle).
Conventional and quantitative RT-PCR analysis

Total RNA was isolated from mouse hearts using the Trizol reagents (Invitrogen) and from BAECs using the RNase easy mini kit (Qiagen) 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 MgCl2, 0.3% Tween 20, 10 μM β-mercaptoethanol, 10 U RNasin (Promega) and 100 U Mo-MLV RT (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. Next, 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 supplementary material Table S1.

Statistical analysis

We used the GraphPad Prism software to handle data. Results are reported as mean ± s.e.m. Student’s t-test was used for comparing two groups.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

O.A. and A.K.H. conceived and designed the experiments. O.A., M.R., S.R. and N.A. performed the experiments. O.A., S.R., I.F. and A.K.H. analyzed the data. O.A. and A.K.H. wrote the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.006510/-/DC1

REFERENCES


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