THERAPEUTIC INHIBITION OF THE WNT SIGNALING PATHWAY TO PROMOTE REGENERATIVE REPAIR OF TISSUE INJURY

By

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Dissertation
Submitted to the Faculty of the
Graduate School of Vanderbilt University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
in

Pathology
May, 2016
Nashville, Tennessee

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For my loving mother,

and all that she dreamed of and dared to be…

ACKNOWLEDGEMENTS

Education is not preparation for life, it is life itself.
- John Dewey

There are many people who I have to thank for making this work possible. Dr. Pampee Young has been an amazing mentor, constantly encouraging me, stimulating me intellectually, and pushing me to do the best work I can. I cannot thank her enough for having faith in my abilities, and supporting my intellectual or other endeavors both in and outside the lab. The Young lab family deserves special thanks for helping me question, learn, grow and persevere as a researcher. Dr. Sarika Saraswati, who has been an integral part of this work, has not only helped push past the many research setbacks, but has also been an amazing friend, offering infinite support and patience during my moments of exasperation. I am deeply grateful to the many teachers along the way who instilled a deep curiosity and love for Science, specially Dr. Ron Rosen, who showed me the joys of leading a life full of curiosities and learning during my formative years as a college student.

Words cannot express my gratitude to my parents, who taught me by example, to value education as the greatest wealth; to my three siblings who have always set examples of independence and courage for me to follow; and to *Hajurbua* and *Ama* who showed what selfless love is. I am deeply grateful to my partner-in-crime, Bibek, who has been both my constant motivator, and a happy distraction from the all-too-common agonies of research over the last five years. I am so happy to have found you as my companion and look forward to our adventures together. This work was supported by Veterans Affairs Merit Award and Vanderbilt University Clinical and Translational Science Award Grant (UL1 RR024975-01 from NCRR/NIH) to P. Young, AHA Predoctoral Fellowship 3PRE16080004 to D. Bastakoty, and NIH NIGMS. R01GM081635 and R01GM103926 to E. Lee.

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- AEC II: Alveo-epithelial type II cells, 11
- AKI: acute kidney injury, 13
- ANOVA: Analysis of Variance, 51
- APC: adenomatous polyposis coli, 7
- BASCs: bronchio-alveolar stem cells, 12
- BCL-9: B cell lymphoma 9, 7
- BrdU: Bromodeoxyuridine, 15
- Brg1: Brahma-related gene 1, 7
- CBP: cyclic AMP response element binding protein, 14
- Ci: cubitus interruptus, *139*
- CK1α: Casein Kinase 1 alpha, 4
- CKD: Chronic kidney disease, 14
- Cox2: cyclooxygenase 2, *25*
- cTnI: cardiac Troponin I, 49
- Dkk1: Dickkopf-1, 14
- DMSO: dimethylsulfoxide, 98
- DVL: Disheveled, 7
- EF: Ejection Fraction, 45
- ESCs: Embryonic stem cells, *123*
- FDA: Food and Drug Administration, *27*
- FOXO: Forkhead box protein O, 4
- FS: Fractional Shortening, 45
- FSP-1: fibroblast-specific protein-1, 54
- Fzd4: Frizzled receptor 4, 13
- GSK3β: Glycogen synthase kinase 3 beta, 4
- HATs: histone acyltransferases, *123*
- IC_{50}: half-maximal inhibitory concentration, 37
- IFE: interfollicular epidermis, 15

- IGFBP3: insulin growth factor binding protein 3, *21*
- IPF: Idiopathic pulmonary fibrosis, 11
- LAD: left anterior descending artery, *136*
- LEF: Lymphoid enhancing factor, 4
- LRCs: label retaining cells, 15
- LRP: Lipoprotein receptor-related protein, 3
- LVIDd: Left ventricular internal dimensions at diastole, 45
- LVIDs: Left ventricular internal dimensions at systole, 45
- MI: myocardial infarct, 20
- mTOR: mammalian target of Rapamycin, 4
- MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 63
- NSAIDS: Non-steroidal anti-inflammatory agents, *25*
- PCNA: proliferating cell nuclear antigen, 54
- PD: pharmacodynamic, 37
- PK: pharmacokinetic, 37
- Pygo: Pygopus, 7
- Sca1: Stem cell antigen, 21
- sFRP: secreted Frizzled-related protein, *22*
- SV40: simian virus 40, 58
- TLE: Transducin-like enhancer protein, 4
- TUNEL: Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling, 63
- UUO: unilateral ureteral obstruction, 14
- VEGF: vascular-endothelial growth factor, *23*
- vWF: von Willebrand factor, 56
- αMHC: alpha myosin heavy chain, 21
- αSA: Alpha Sarcomeric Actin, 49
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CHAPTER 1

WNT/B-CATENIN PATHWAY IN TISSUE INJURY: ROLES IN PATHOLOGY AND THERAPEUTIC OPPORTUNITIES FOR REGENERATION

Introduction

The Wnt/β-catenin pathway is an evolutionarily conserved set of signals with critical roles in embryonic and neonatal development across species. In mammals, the pathway is quiescent in most organs. It is re-activated in response to injury, and is reported to play complex and contrasting roles in promoting regeneration and/or fibrosis. Here we review the current understanding of the role of the Wnt/β-catenin pathway in injury of various mammalian organs, and discuss the current advances in and potential of Wnt inhibitory therapeutics towards promoting tissue regeneration and reducing fibrosis.

Wnt pathway: diverse roles in development, regeneration and homeostasis

The Wnt signaling pathway is believed to have evolved with the first multicellular organisms (metazoa), and is considered an important mediator of the advent of multicellularity (Holstein, *Cold Spring Harb Perspect Biol* 2012). The pathway is important for embryonic and postnatal development in vertebrates and insects (Holstein, *Cold Spring Harb Perspect Biol* 2012). In invertebrates and the few vertebrate species that display regeneration, such as urodele amphibians, the pathway remains active in the adult organism, playing a role in stem cell maintenance and self-renewal for normal tissue homeostasis, and for regeneration following injury (Caubit et al., *Dev Dyn* 1997; Kawakami et al., *Genes Dev* 2006). In mammals and other non-regenerating vertebrates, the role of the pathway in the adult organism becomes more complex and context dependent. In organs, such as colon, that undergo constant turnover, and have a clearly defined, active pool of tissue-resident stem cells, the pathway remains active and

continues to regulate stem cell homeostasis (Fevr et al., *Mol Cell Biol* 2007). But in non-regenerating organs, such as heart in higher vertebrates, the pathway is quiescent and is often activated in response to injury (Oka et al., *Semin Cell Dev Biol* 2007; Aisagbonhi et al., *Dis Model Mech* 2011). The role this pathway then plays is incompletely understood and controversial, but increasing body of data suggest that rather than simply reviving the pool of quiescent stem cells and promoting regeneration in these injured organs, the pathway may additionally be involved in fibrotic processes often seen as part of the imperfect healing in these organs (Konigshoff et al., *PLoS One* 2008; Zelarayan et al., *Proc Natl Acad Sci USA* 2008; Zhang et al., *J Mol Cell Cardiol* 2009; Bergmann, *Circ Res* 2010; Poon et al., *PLoS One* 2012).

As a complex pathway with diverse effects on different cells and tissues, parsing out the exact role of reactivated Wnt pathway in response to injury has been difficult. The efforts at understanding the effects of Wnt pathway modulation in injury are further complicated by the limitations of classical genetics approaches, that rely on Wnt modulation through specific promoters, and therefore do not always target the complete wound milieu (Gat et al., *Cell* 1998; Baurand et al., *Circ Res* 2007), or carry unintended effects on the cells used for targeting (Ito et al., *Nature* 2007; Duan et al., *EMBO J* 2012). Recently however, with increasing focus on developing Wnt modulatory therapeutics aimed at Wnt-driven cancers, new studies that investigate the effects of modulating Wnt signals in regeneration are emerging (Chen et al., *Nat Chem Biol* 2009; Saraswati et al., *PLoS One* 2010; Bastakoty et al., *FASEB J* 2015). These studies are helping gain a better understanding of the role of the pathway in regeneration and more importantly, are pioneering the development of Wnt-modulatory therapeutics. In this chapter we will discuss the current understanding of the complicated role of the Wnt/β-catenin pathway in mammalian organ injury, focusing on the lung, kidney, skin and heart. We will also

outline the development of relevant Wnt modulators and deliberate on the challenges and promises of pharmacologic Wnt inhibition to promote regeneration.

Wnt/β-catenin signaling pathway in development: a historical perspective.

Wnt gene was first identified in 1982 as a mammalian oncogene *Int1 (Nusse et al., Cell 1982)*, a few years after identification of *Wingless (Sharma et al., Dev Biol 1976; Nusslein-Volhard et al., Nature 1980)* necessary for *Drosophila* wing development. With the finding that the two genes are homologous, the field of Wnt signaling was born (Nusse et al., *EMBO J* 2012). In the three-and-a-half decades since, the conserved roles of the Wnt family of genes in development and disease continue to be uncovered. The Wnt pathway as it is currently described consists of a family of 19 secreted glycoproteins (in mammals) (Cadigan et al., *Genes Dev* 1997), Frizzled family of transmembrane receptors (Bhanot et al., *Nature* 1996; Yang-Snyder et al., *Curr Biol* 1996), Lipoprotein receptor-related protein (LRP) family of co-receptors, and a number of other downstream components and effector proteins that fall under either the canonical or one of the two major 'non-canonical' arms of the Wnt signaling pathway. β-catenin, which was originally identified as *Armadillo (Peifer et al., Development 1991; Peifer et al., Dev Suppl 1993)* in *Drosophila* is an important effector protein that defines the canonical or β-catenin-dependent arm of the signaling pathway. This arm is involved in segment polarity, stem cell homeostasis, oncogenesis and tissue repair, which will be the focus of this chapter. The non-canonical arms that also signal through Frizzled, but in combination with a distinct set of co-receptors (such as tyrosine kinase receptors Ror and Ryk (Green et al., *Cold Spring Harb Perspect Biol* 2014)) control cell polarity and calcium signaling (Green et al., *Cold Spring Harb Perspect Biol* 2014). These non-canonical arms of the Wnt signaling pathway are also critical in

development and disease (Sugimura et al., *Birth Defects Res C Embryo Today* 2010), but are beyond the scope of our investigations.

The canonical Wnt pathway **(Fig 1)** is activated when a subset of the Wnt ligands bind their cognate Frizzled receptors and LRP5/6 co-receptors. This causes phosphorylation-dependent sequestration of Axin, a rate-limiting component of the cytoplasmic β-catenin degradation complex (Lee et al., *PLoS Biol* 2003). The β-catenin degradation complex includes the kinases Glycogen Synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α) that phosphorylate and target β-catenin for ubiquitin-mediated degradation (Marikawa et al., *Mech Dev* 1998; Amit et al., *Genes Dev* 2002; Price, *Genes Dev* 2006). It is hypothesized that with axin2 sequestration, GSK3β, and possibly other members of the β-catenin degradation complex are recruited to the membrane to form a 'signalosome', thereby preventing β-catenin degradation (Bilic et al., *Science* 2007). The stabilized β-catenin translocates to the nucleus by a yet-unknown mechanism (Nusse et al., *EMBO J* 2012). In the nucleus, β-catenin replaces Groucho/TLE (Transducin-like enhancer protein) in binding to TCF/LEF (T-cell factor/Lymphoid enhancing factor) transcription factors (Hanson et al., *Mol Cell* 2012), and in association with other transcriptional co-activators (Kramps et al., *Cell* 2002; Kim et al., *Biochem J* 2013), promotes transcription of its target genes. In addition to this classical cascade of signals, new studies describe other signaling arms of the canonical Wnt pathway. For example, during oxidative stress, β-catenin binds directly to, and promotes transcriptional activity of, Forkhead box protein (FOXO) family of transcription factors (Essers et al., *Science* 2005). Further, Inoki *et.al* showed in 2006 that Wnt pathway can also activate translation through mammalian target of Rapamycin (mTOR) signaling independent of β-catenin mediated transcription (Inoki et al., *Cell* 2006). In the Wnt producing cell, important players are involved in Wnt post-translational modification,

which is considered necessary for the ligand secretion and activity. Works from various groups have indicated that most Wnt ligands are N-glycosylated. This post-translational modification is not essential for Wnt secretion, but predominantly enhances palmitoylation—a lipid modification indispensable for Wnt ligand secretion and Frizzled receptor binding (Kadowaki et al., *Genes Dev* 1996; Mikels et al., *Oncogene* 2006; Komekado et al., *Genes Cells* 2007). The membrane-bound acyltransferase, Porcupine, essential for this modification in the endoplasmic reticulum is now being widely explored as a target for anti-Wnt therapeutics (Chen et al., *Nat Chem Biol* 2009). The transport of the lipidated Wnt proteins into secretory vesicles, for secretion of the ligand into the extracellular space, is carried out by the multipass transmembrane protein Wntless/Evi (Banziger et al., *Cell* 2006; Bartscherer et al., *Cell* 2006).

A gradient in the expression of Wnt genes controls axial patterning during embryonic development of organisms ranging from mammals to frogs, worms, birds and echinoderms (Petersen et al., *Cell* 2009). Perturbation of the pathway, or alteration of this gradient results in dramatic phenotypes caused by anteroposterior patterning defects (Petersen et al., *Cell* 2009). In later stages of embryonic development, Wnt modulators (both positive and negative) continue to be expressed and play crucial roles in development of organs. In heart development, for example, the Wnt pathway needs to be repressed during early stage of heart formation, but its re-activation later is critical for the development of the outflow tract and the valves (Hurlstone et al., *Nature* 2003). Similarly an initial repression and a later reactivation of Wnt signaling in specific cells is necessary during neural crest formation (Steventon et al., *Dev Biol* 2012). The strict temporal and spatial regulation of Wnt activity appears to be an important attribute of the pathway in its various roles in development and homeostasis/regeneration/fibrosis as will be discussed in the following sections.

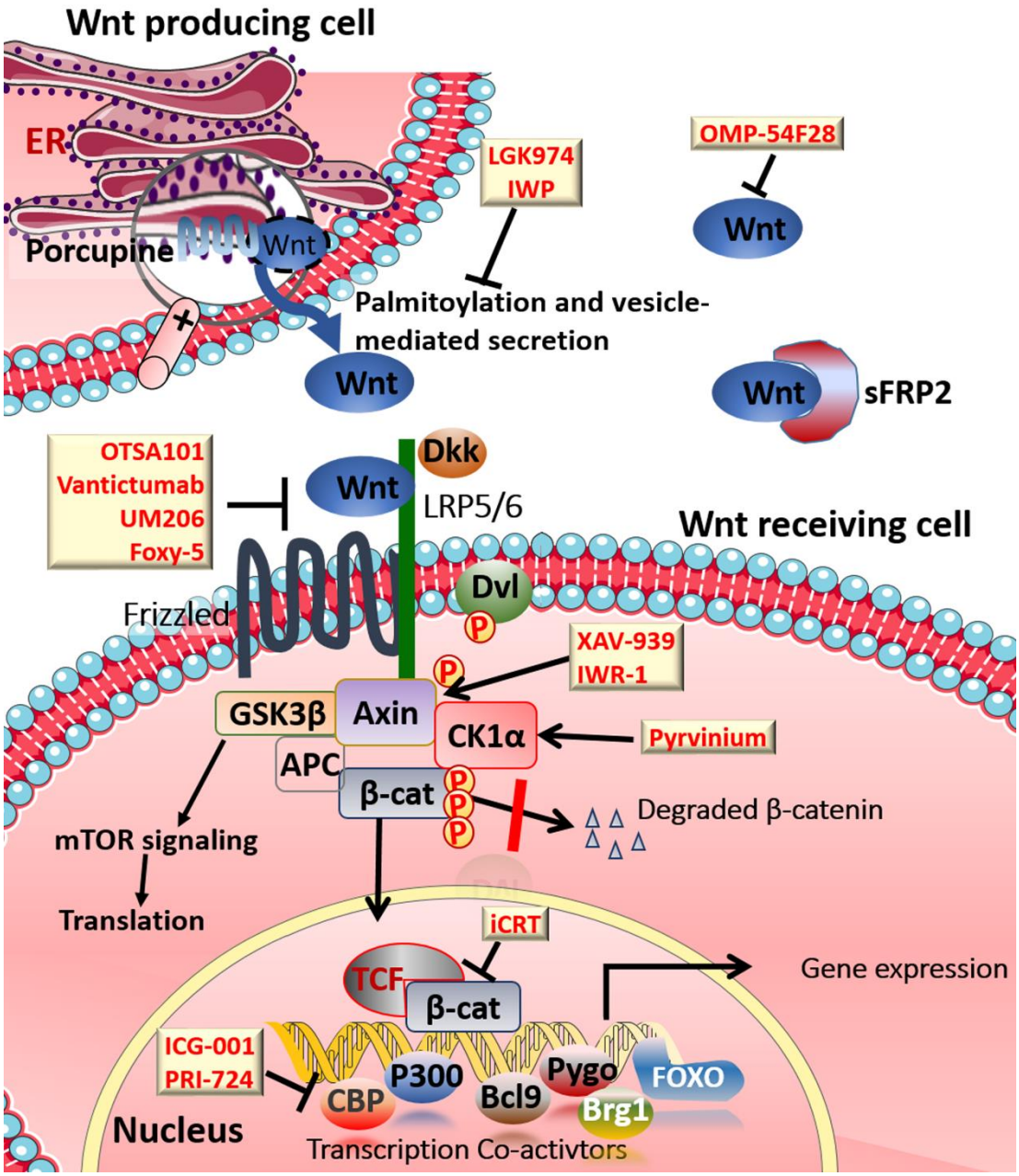

Figure 1: Simplified model of the canonical Wnt pathway and inhibitors.
The WNT–β-catenin pathway consists of secreted glycoproteins (Wnt ligands) and Frizzled family of transmembrane G-protein coupled receptors or LRP5/6 transmembrane receptors. In the Endoplasmic reticulum (ER) of the Wnt producing cells, the membrane bound O-acyltransferase, Porcupine, acrylates the Wnt protein, necessary for secretion of the Wnt ligand.

The secreted WNT ligand can be sequestered by secreted Frizzled-related protein (sFRP) family of secreted proteins that can sequester the WNT ligand and prevent binding to receptors. Dickkopf-related protein 1 (Dkk1), another secreted Wnt inhibitor prevents binding of WNT ligand to LRP5/6 receptors. In the absence of extracellular WNT glycoproteins, a destruction complex — including the proteins adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), casein kinase 1α (CK1α), and AXIN — phosphorylates β-catenin, targeting it for ubiquitylation and proteasomal degradation. When WNTs bind to the Frizzled and LRP5/6 (low-density lipoprotein receptor-related protein) co-receptors, Disheveled (DVL) helps phosphorylate and sequester Axin to the LRP5/6 receptor, causing disassociation of the β-catenin degradation complex. The stabilized β-catenin enters the nucleus, binds to T cell factor family of transcription factors (TCF) and activates transcription of target genes. Distinct co-activators of transcription such as cyclic AMP response element-binding protein (CBP), E1A-associated protein p300 Pygopus (Pygo), B cell lymphoma 9 (BCL-9), Brahma-related gene 1 (Brg1) etc. are involved in the transcription of distinct set of target genes. Independent of TCF, β-catenin can also associate with FOXO family of transcription factors to activate transcription of genes, primarily involved in aging. In the cytoplasm, inhibition of GSK3β activity by WNT ligand binding, can also simultaneously activate the mTOR complex 1 (mTORC1) signaling which results in mRNA translation into proteins. Inhibitors of the pathway and their respective targets are shown in red font on yellow tabs.

Wnt in regeneration and fibrosis: the good, the bad and the ugly

Wnt pathway in invertebrates and regenerating vertebrates

The canonical Wnt signaling pathway plays diverse roles during regeneration of different organisms. In bilaterally polarized invertebrates such as planarians the pathway is reactivated in response to injury and is necessary for appropriate head vs. tail polarity during regeneration, consistent with its role in patterning during development (Gurley et al., *Dev Biol* 2010). Wnt pathway perturbation does not prevent regeneration, but it promotes ectopic head formation in these organisms (Gurley et al., *Science* 2008), and even in lower invertebrates such as hydra (Broun et al., *Development* 2005). This observation mirrors the observation, first made by McMahon and Moon, that int-1 RNA injection in Xenopus embryos results in dual axis formation (McMahon et al., *Cell* 1989).

Few vertebrate species such as zebrafish and urodele amphibians display regenerative abilities as adults. The Wnt pathway is implicated in part of the regenerative process in these organisms. In both zebrafish fin regeneration and urodele amphibian limb regeneration, the blastema, which is an organized mass of tissue with a well-defined progenitor cell population, is thought to drive the mesenchymal regenerative process. The Wnt pathway is activated in a spatiotemporally regulated manner during blastema formation (Yokoyama et al., *Dev Biol* 2007) and is considered to play a role in orchestrating tissue organization and differentiation or proliferation of the blastemal cells (Caubit et al., *Dev Dyn* 1997; Poss et al., *Dev Dyn* 2000; Stoick-Cooper et al., *Development* 2007; Wehner et al., *Cell Rep* 2014).

Wnt pathway in non-regenerating vertebrates: stem cell homeostasis vs. fibrosis

In non-regenerating vertebrates, including mammals, the Wnt/β-catenin pathway activity is maintained in the adult organism only in specific organs/tissues with high cell turnover, such

as the hematopoietic compartment (Reya et al., *Nature* 2003), the intestinal epithelium (Fevr et al., *Mol Cell Biol* 2007; Krausova et al., *Cell Signal* 2014) and the epidermis (Lim et al., *Cold Spring Harb Perspect Biol* 2013). Wnt pathway activity is associated with stem cell self-renewal necessary for normal homeostasis in these organs. In the small intestine and colon, LGR5, a common Wnt target gene, is considered a marker of a subset stem cells (Barker et al., *Nature* 2007). Knockdown of TCF4 (canonical Wnt) signal in the small intestine depleted the crypt stem cell compartment needed for self-renewal of cells in the intestinal epithelium (Korinek et al., *Nat Genet* 1998). $LGR5^+$ cells are also considered to be the self-renewing progenitor cells in gastric epithelium (Barker et al., *Cell Stem Cell* 2010), mammary gland (Plaks et al., *Cell Rep* 2013) and epidermis (Haegebarth et al., *Am J Pathol* 2009; da Silva-Diz et al., *Oncogene* 2013). Accordingly, mutations in components of the Wnt pathway are implicated in epithelial cancers in these organs (Kinzler et al., *Cell* 1996; Liu et al., *Nat Genet* 2000), in which tumorigenesis is often driven by aberrantly proliferative 'cancer' stem cells (Morin et al., *Science* 1997; Oskarsson et al., *Nat Med* 2011).

In other mammalian tissues, however, the Wnt pathway is quiescent in the adult, and is re-activated in response to injury. These tissues possess either quiescent or differentiated 'facultative' stem cells (Kotton et al., *Nat Med* 2014) that can de-differentiate upon injury as in the lungs (Kotton et al., *Nat Med* 2014), or that may lack stem cells capable of orchestrating complete regeneration (e.g.: heart) **(Fig 2)**. The involvement of Wnt pathway in response to injury and its contributions to injury repair through regenerative (or more commonly fibrotic) processes are discussed in more detail in the following sections.

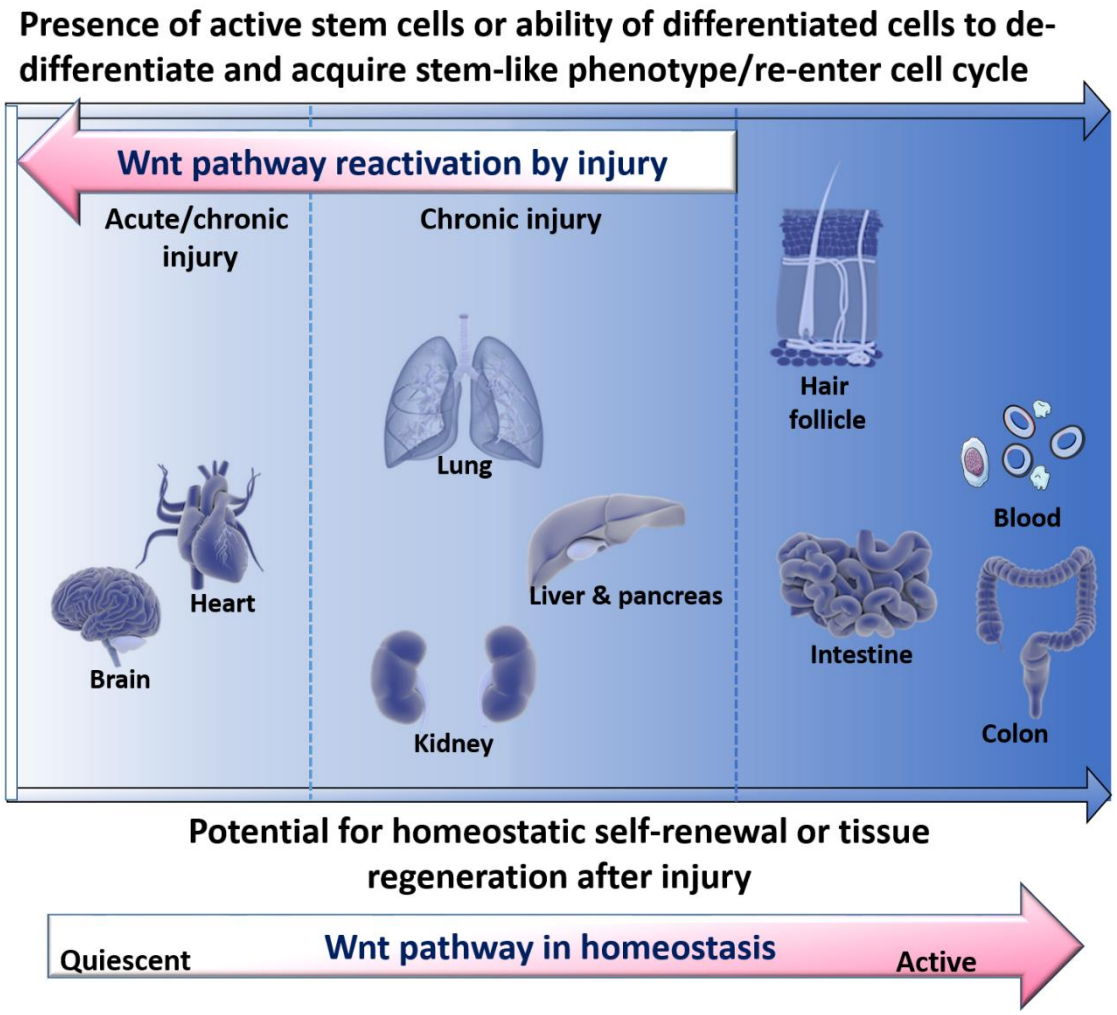

Figure 2: Regenerative capacity of mammalian tissues is correlated with the presence of stem/stem-like cells and Wnt pathway activity at homeostasis.
Tissues such as brain and heart that lie on the non-regenerative (left) end of the spectrum tend to lack an active stem cell population and are typically unable to jumpstart regenerative processes following injury, or they do not have effective regenerative processes in place, even if the regenerative signals can be initiated. Tissues in the middle range such as lung, kidney and pancreas undergo "facultative regeneration". The partly quiescent stem cells or differentiated cells capable of trans-differentiation in these tissues mediate regeneration following acute injury in some instances, but fail to do so in chronic insults. Tissues on the regenerative end of the spectrum (right side of the graph) such as the hematopoietic compartment, hair follicle and the intestine undergo constant homeostatic turnover, aided by an active population of tissue-resident stem cells. The Wnt pathway activity at baseline/homeostasis in these tissues correlates with their turnover/homeostatic regeneration potential. Wnt is involved in self-renewal/differentiation of stem/stem-like cells, although the necessity of the Wnt pathway in regeneration or its overall effect on regeneration is dependent on the tissue and the context. In response to injury in the non-regenerative organs (brain and heart), and to chronic injury in the tissues that undergo "facultative regeneration" (lung, kidney etc.), the Wnt pathway is activated and is considered part of the pro-fibrotic signal.

Lung

The lung is a complex organ, composed of multiple specialized epithelial and mesodermal-derived cells spatially arranged along an arborized architecture. The lung epithelium undergoes slow homeostatic turnover, replacing most of its cells from a progenitor pool (once every four months in rats (Blenkinsopp, *Exp Cell Res* 1967)). It is mostly quiescent except when activated by injury. The differentiated epithelial cells of the lung are capable of dedifferentiation, proliferation, and transdifferentiation into diverse cell lineages. These epithelial cells are renewed from a pool of stem/progenitor cells distributed along multiple niches in the proximal-distal epithelium. Mediated both by the regenerative epithelial cells, and also by the multiple progenitor cell subtypes, the process of regeneration in the lung is complex and spatially regulated (Giangreco et al., *Am J Pathol* 2002; Hong et al., *Am J Pathol* 2004; Beers et al., *J Clin Invest* 2011). Likewise, the involvement of the Wnt pathway in the process is complicated and incompletely understood. The Wnt pathway is activated in the lung in response to injury. Chilosi *et. al* reported increased nuclear localization of β-catenin and expression of Wnt target genes cyclinD1 and matrilysin in bronchiolar lesions, damaged alveoli and fibrotic foci of lung samples from patients with Idiopathic pulmonary fibrosis (IPF) (Chilosi et al., *Am J Pathol* 2003). Similar findings of Wnt pathway activation in the Alveo-epithelial type II cells (AEC II) in patients with IPF was reported by Koingshoff *et. al* (Konigshoff et al., *PLoS One* 2008). AEC II cells are considered the facultative progenitors of the distal lung, and are reported to replace the alveolar epithelium and lung parenchyma in bleomycin-induced and hyperoxic rodent lung injury models (Asselin-Labat et al., *Open Biol* 2012). Reports indicate a role for Wnt pathway in survival of AEC in bleomycin induced lung injury models, and increased AEC death upon selective depletion of β-catenin in AECs. In contrast, multiple reports have also shown

attenuation of bleomycin-induced pulmonary fibrosis by inhibition of the Wnt pathway through administration of small molecule Wnt inhibitors (Henderson et al., *Proc Natl Acad Sci U S A* 2010; Wang et al., *Am J Physiol Cell Physiol* 2014) although this may occur, in part, through effects on the mesenchyme rather than on the epithelial cells. Wnt pathway was activated in the bronchio-alveolar stem cells (BASCs) in response to naphthalene-based acute lung injury. Although Wnt activation induced proliferation of BASCs, inhibition of Wnt activity by GATA6 was deemed necessary for differentiation and proper regeneration of the damaged epithelium (Zhang et al., *Nat Genet* 2008). Likewise, Stripp and colleagues showed by Cre-mediated expression of mutant β-catenin in airway epithelial cells that Wnt pathway activity was not needed for repair of bronchiolar epithelium following naphthalene-induced injury (Zemke et al., *Am J Respir Cell Mol Biol* 2009).

Despite the presence of multiple resident regenerative cell lineages, effective regeneration following injury does not always occur in the lung, as evident with the increasing prevalence of fibrotic diseases of the lung (Ley et al., *Clin Epidemiol* 2013). Repeated injuries and persistent inflammatory insults, and in some cases, genetic predispositions compromise the regenerative potential of lung cells, leading to activation of fibrotic signals driving IPF, chronic obstructive pulmonary disease and other ailments (Kotton et al., *Nat Med* 2014). The Wnt pathway, is widely reported to be activated in response to injury, and is shown to promote proliferation of at least a subset of regenerative cell lineages, and yet, the regeneration of lung after injury appears insufficient to ward off fibrotic diseases. Moreover, the increasing reports citing Wnt pathway involvement in initiating pro-fibrotic signals by mesenchymal cells (Kim et al., *J Clin Invest* 2009) indicates a dual effect of Wnt activation resulting in regenerative

epithelial signals, but also activation of pro-fibrotic signaling which drives the pathology in chronic fibrotic pulmonary diseases.

Kidney

The kidney is capable of recovery from acute kidney injury (AKI) induced by ischemia or nephrotoxic drugs, commonly affecting the renal tubule. Although the cells contributing to the repair are still under investigation, a significant body of work suggests that proliferative and/or de-differentiated tubular epithelial cells are the major contributors to repair (Humphreys et al., *Cell Stem Cell* 2008; Berger et al., *Proc Natl Acad Sci U S A* 2014; Berger et al., *Semin Nephrol* 2014; Kusaba et al., *Proc Natl Acad Sci U S A* 2014). Despite this intrinsic healing potential, AKI results in significant morbidity and mortality, indicative of the inherent limitations to regeneration (Bonventre et al., *J Clin Invest* 2011; Berger et al., *Semin Nephrol* 2014).

The Wnt/β-catenin pathway is activated in response to AKI, as indicated by β-galactosidase expression in reporter mice expressing the protein in response to Wnt/β-catenin transcriptional activation or Wnt target gene axin2 expression (Lin et al., *Proc Natl Acad Sci U S A* 2010). In ischemia-reperfusion-induced kidney injury model, Duffield and colleagues showed that kidney macrophages secrete Wnt ligands following injury, and that the Frizzled receptor 4 (Fzd4)-expressing tubular epithelial cells are Wnt responsive. The disruption of Wnt/β-catenin signals in these cells by mutation of Fzd4 receptor or LRP5/LRP6 co-receptors reduced regeneration of tubular epithelium by increasing apoptosis of tubular epithelial cells, although the effect was modest even with depletion of both Fzd4 and LRP5/6 activity. Another study also reported aggravation of AKI through increase in epithelial cell apoptosis upon tubule-specific depletion of β-catenin (Zhou et al., *Kidney Int* 2012). However, an important caveat to this study

is that β-catenin depletion in epithelial cells can alter β-catenin/E-cadherin signals, affecting the survival and regenerative potential of these cells independent of Wnt pathway activity.

Chronic kidney disease (CKD) is characterized by progressive interstitial fibrosis, often leading to kidney failure. A recent study showed that sustained Wnt/β-catenin pathway activation after AKI is associated with progression to CKD, even though transient Wnt activity may aid epithelial repair. The authors also showed that partial blockade of Wnt/β-catenin pathway activity by pharmacologic inhibition of β-catenin binding to transcription co-factor (cyclic AMP response element binding protein) CBP could block progression to CKD (Xiao et al., *J Am Soc Nephrol* 2015). The effects of Wnt pathway on specific cells of the kidney have been implicated in CKD pathogenesis in multiple studies. For example, the pericytes, which are described as cells that differentiate into the fibrogenic myofibroblasts (Bonventre et al., *J Clin Invest* 2011; Kramann et al., *Semin Nephrol* 2014), express the myofibroblast marker alpha smooth muscle actin (αSMA) in response to Wnt signaling activation (DiRocco et al., *J Am Soc Nephrol* 2013). Forced upregulation of Wnt/β-catenin signals by stabilizing β-catenin in the interstitial cells was sufficient to induce increased myofibroblast differentiation, indicating a critical role for Wnt pathway in interstitial fibrosis in the kidney (DiRocco et al., *J Am Soc Nephrol* 2013). In unilateral ureteral obstruction (UUO), which is the most commonly used murine model of kidney fibrosis, Wnt ligand expression and β-catenin nuclear localization increased in both the kidney tubules and fibrous interstitial regions. He *et. al* reported that blockade of Wnt/β-catenin signaling by intravenous injection of a Dkk1-encoding plasmid vector reduced renal αSMA mRNA expression and interstitial fibrosis (He et al., *J Am Soc Nephrol* 2009). Wnt/β-catenin pathway activation was also reported to contribute to CKD progression by promoting podocyte dysfunction (Dai et al., *J Am Soc Nephrol* 2009). In another study, both hyper-activation and

ectopic down-regulation of Wnt/β-catenin signals led to podocyte dysfunction and compromised glomerular filtration (Kato et al., *J Biol Chem* 2011). Taken together, these studies suggest that in the kidney, as in the lung, transient Wnt/β-catenin pathway may induce regeneration by putative 'facultative progenitors'; however, upon prolonged injury, a hyperactivation of the Wnt pathway appears to initiate signals in multiple cell types leading to progressive fibrosis.

Skin

As a tissue that undergoes significant turnover and harbors multiple distinct stem and progenitor cells, and yet routinely heals with a scar, skin displays a unique dichotomy of regeneration and fibrosis upon injury. The involvement of Wnt pathway in skin injury response is similarly dichotomous, with Wnt activity playing a critical role in epidermal stem cell maintenance, hair follicle development and regeneration (Huelsken et al., *Cell* 2001; Blanpain et al., *Annu Rev Cell Dev Biol* 2006), but also in dermal, fibroblast mediated scarring (Cheon et al., *Proc Natl Acad Sci U S A* 2002; Cheon et al., *FASEB J* 2006).

The stem/stem-like cells and their respective proliferative progenitors (often referred to as transient amplifying cells, TACs) in the skin are mostly distributed in niches in the basal layer of the interfollicular epidermis (IFE), and the bulge of the hair follicles (Ito et al., *Nat Med* 2005; Fuchs, *J Cell Biol* 2008). The IFE consists of partially differentiated progenitors that undergo asymmetric division giving rise to TACs and ultimately to basal epidermal cells that contribute to epidermal stratification during development and homeostatic turnover (Ro et al., *EMBO Rep* 2004; Lechler et al., *Nature* 2005). The bulge stem cells, originally identified as slow cycling "label retaining cells" (LRCs) based on their longer retention of Bromodeoxyuridine (BrdU) label (Cotsarelis et al., *Cell* 1990), are considered multipotent stem cells that can differentiate

either to form cells of the hair follicle or give rise to the progenitors of the IFE upon injury (Ito et al., *Nat Med* 2005; Blanpain et al., *Annu Rev Cell Dev Biol* 2006; Levy et al., *FASEB J* 2007). The Wnt pathway plays an integral role in the homeostasis and multipotency of both of these stem cell subtypes in the skin. Aside from being necessary for hair follicle development during embryogenesis (Dasgupta et al., *Dev* 1999; Alonso et al., *Genes Dev* 2003), Wnt-Lef1 transcription activation in bulge stem cells is specifically associated with initiation of hair cycle in the adult skin (Dasgupta et al., *Dev* 1999). In partially committed progenitor cells such as the IFE stem cells (Gat et al., *Cell* 1998), or differentiated cells of the dermal papillae (Kishimoto et al., *Genes Dev* 2000), Wnt pathway activation is reported to revert these cells to a multipotent stem-like phenotype whereby they acquire an ability to form de novo hair follicles. Consistent with this observation, focal Wnt activation in IFE stem cells is reported to drive self-renewal and proliferation to promote wound closure after injury (Lim et al., *Science* 2013), and de novo hair follicle formation from IFE stem cells likely by enhancing reversal to a multipotent phenotype (Ito et al., *Nature* 2007). Hence, there is clear evidence that Wnt/β-catenin/TCF/LEF signals are important for stem cell maintenance, hair follicle formation and turnover of epithelium during homeostasis and/or in response to injury. Although the role of the Wnt pathway in promoting wound resurfacing by the epidermis and its role in hair follicle regeneration has been widely studied, none of the studies have assessed the effect on dermis, which is the region that undergoes scarring. Indeed, in human wounds, and in most rodent models, the limiting factor in regenerative wound repair is not re-epithelialization, but scar resolution in the dermis. Since the initiating signal for hair follicle morphogenesis during development originates in the dermis ("first dermal message (Yang et al., *J Dermatol Sci* 2010) "), and has not been completely decoded, the lack of hair follicle regeneration in most scarred wounds may be explained by the

scarred non-regenerative healing in the dermis. Dermal papillae cells are known to induce hair follicle differentiation of epithelial cells during development and in hair reconstitution assays, which indicates a need for dermal regeneration for new hair follicles to form after repair of full thickness skin wound (Yang et al., *J Dermatol Sci* 2010).

Studies of skin wound healing that examine scar formation in the dermis demonstrate that Wnt pathway activation plays a pro-fibrotic role in this compartment (Cheon et al., *Proc Natl Acad Sci U S A* 2002; Cheon et al., *FASEB J* 2006; Akhmetshina et al., *Nat Commun* 2012; Amini-Nik et al., *J Clin Invest* 2014). Alman and colleagues have shown that β-catenin transcriptional activity, which is upregulated in mouse dermis in response to injury (Cheon et al., *Proc Natl Acad Sci U S A* 2002), caused hyper-proliferation of fibroblasts and increased wound size (Cheon et al., *FASEB J* 2006) and fibromatosis in rodent excisional wound model (Cheon et al., *Proc Natl Acad Sci U S A* 2002). Conversely, β-catenin destabilization by adenoviral cre-mediated deletion of β-catenin exon 3 reduced wound size and reversed the fibromatosis phenotype (Cheon et al., *FASEB J* 2006). Similarly, prolonged activation of Wnt/β-catenin signaling has been observed in human hyperplastic wounds (Cheon et al., *Lab Invest* 2005). Skin biopsies from patients with systemic sclerosis also reveal altered expressions of Wnt pathway components resulting in an activation of Wnt signaling (Lemaire et al., *J Invest Dermatol* 2010; Wei et al., *Arthritis Rheum* 2011).

Hence, the notion that Wnt pathway promotes regeneration in the skin may apply to hair follicle regeneration in conditions such as alopecia, or epidermal abrasion. However, for the regeneration of full-thickness dermal wound, or burn wounds, the role of Wnt signaling needs to be re-examined with a focus on dermal scarring. While Wnt activity is clearly needed for re-epithelialization, Wnt inhibition specifically in the dermis may be important to reduce scar. For

example, our studies using small molecule inhibitors of the Wnt pathway, that caused a more appreciable reduction in β-catenin/TCF driven transcription specifically in the dermis, promoted regenerative wound repair with less scarring (Bastakoty et al., *FASEB J* 2015). This finding aligns with the report by Alman's group that adenoviral Cre mediated conditional depletion of β-catenin, which inhibits the Wnt/β-catenin signaling throughout the wound, and is not limited by use of an epithelial or hair follicle-specific promoter, successfully drives reduction in fibrosis and wound size (Cheon et al., *FASEB J* 2006). Although the same authors have reported that complete ablation of Wnt pathway in the dermal macrophages prevented wound closure by impeding granulation tissue formation (Amini-Nik et al., *J Clin Invest* 2014), the study lends credence to the idea that fine tuning of Wnt inhibition in the dermis may be key to promoting scar-less wound healing in the skin. Development of effective pharmacological Wnt inhibitors and dosing strategies that result in an appropriately calibrated Wnt signaling reduction specifically targeted to the dermis may lead to new and effective wound healing therapeutics.

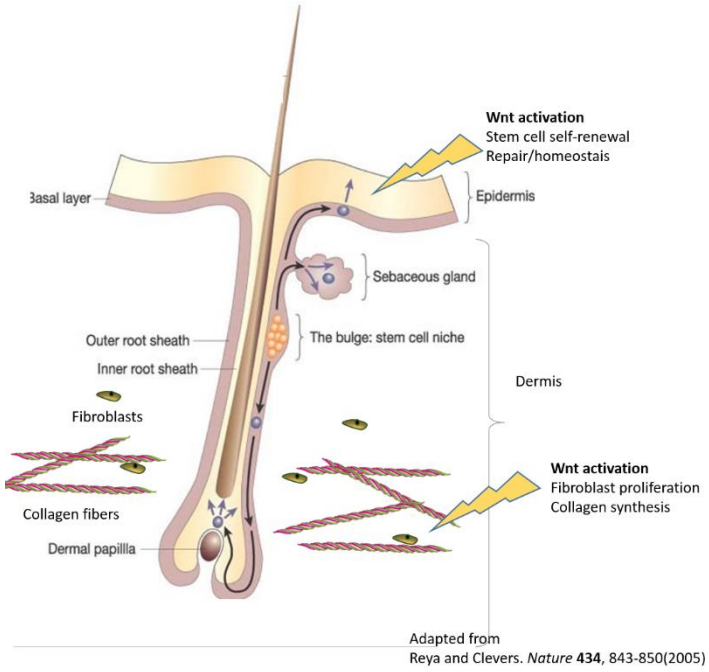

Figure 3: Differential response to Wnt pathway in the epidermal and the dermal compartments of skin.
In the epidermal compartment, which also consists of the hair follicles, stem/stem-like cells (indicated as blue spherical structures) are distributed in the basal layer of epidermis or the hair follicle bulge. These stem cells proliferate and differentiate into more specialized cells of the stratified squamous epithelium or the hair follicle during homeostasis. This process is aided by an active Wnt pathway. In response to injury these stem cells contribute to epidermal cells that cover the wound, also aided by the Wnt pathway activity. In the dermal compartment, the fibroblasts (yellowish elongated structure) constitute the majority of the cells. In response to Wnt activation as a result of injury, these cells proliferate and secrete collagen matrix, which forms granulation tissue, but also results in scarring. The known stem-like cells in the dermal compartment may reside in the dermal papillae, and they are reported to contribute towards hair follicle formation in development and in hair-reconstitution assays. However, there is no evidence that they have any contribution towards regeneration in the dermis. Therefore, the dermal response to Wnt activation is primarily fibrosis, whereas the epidermal (and hair follicle) response to Wnt activation is regeneration.

Heart

The heart was historically considered a non-regenerative organ devoid of a functional stem cell population and unable to replace dying cardiomyocytes through mitosis. However, in the last few decades, both of these views are being challenged by evidence of cardiomyocyte replenishment during the adult lifetime of humans (Beltrami et al., *N Engl J Med* 2001; Bergmann et al., *Science* 2009), and more significantly by reports of multiple cardiac-resident stem/progenitor cells in the adult heart (Beltrami et al., *Cell* 2003; Oh et al., *Proc Natl Acad Sci U S A* 2003; Messina et al., *Circ Res* 2004; Laugwitz et al., *Nature* 2005; Yoon et al., *Exp Mol Med* 2007) with the potential to regenerate cardiomyocytes following injury. Some of these studies have led to clinical trials in which autologous cardiac stem cells were infused into the coronary artery of myocardial infarct (MI) patients after *ex vivo* culture and expansion (Yacoub et al., *Glob Cardiol Sci Pract* 2013). The positive outcomes from these studies have been modest at best, mirroring the initial enthusiasm-followed-by-disinterest that cell therapy with bone-marrow derived ($CD34^+$) mononuclear cells had garnered a few years prior (de Jong et al., *Circ Cardiovasc Interv* 2014). Given the limited success of these expensive therapeutic approaches, the interest in understanding signals that can augment regenerative healing with or without the participation of endogenous cardiac stem cells has persisted. As with its involvement in injury response in other tissues, the Wnt/β-catenin pathway has been reported by many studies to be involved in both pro-reparative and pro-fibrotic response to cardiac ischemic injury (Zelarayan et al., *Proc Natl Acad Sci USA* 2008; Oikonomopoulos et al., *Circ Res* 2011; Duan et al., *EMBO J* 2012).

The Wnt/β-catenin pathway is activated in multiple cells of the heart starting 72 hours post-injury (Oerlemans et al., *Basic Res Cardiol* 2010; Aisagbonhi et al., *Dis Model Mech* 2011).

Consistent with its role in specification of cardiac progenitors during heart development (Gessert et al., *Circ Res* 2010), re-activation of the Wnt pathway after injury is reported to affect cardiac progenitors in multiple ways. For example, Bergmann and colleagues have shown that conditional depletion of β-catenin in alpha myosin heavy chain (αMHC)-expressing cells of the heart post-infarct promotes infarct recovery by increasing cardiomyogenic differentiation of $\alpha MHC^{+}Sca1^{+}$ (cells partially committed to cardiomyocyte lineage) cardiac progenitors. Stabilization of β-catenin using the same promoter reduced cardiomyogenic differentiation (indicated by GATA4 co-expression) of the partially committed progenitors (Zelarayan et al., *Proc Natl Acad Sci USA* 2008). In more recent studies, the group have used knock-down of *Kruppel-like 15* transcription factor, a negative regulator of β-catenin/TCF transcriptional activity, to show that increased Wnt/β-catenin signaling pushed $Sca1^{+}$ progenitors towards an endothelial as opposed to a cardiomyogenic lineage commitment in the adult mouse heart (Noack et al., *EMBO Mol Med* 2012). Exogenous administration of secreted Frizzled-related protein 2 (sFRP2), a secreted Wnt inhibitor, also promoted cardiomyogenic differentiation of $Sca1^{+}$ progenitors in the infarcted heart through inhibition of canonical Wnt pathway and concurrent activation of non-canonical Wnt signals (Schmeckpeper et al., *J Mol Cell Cardiol* 2015). In a study focusing on cardiac side population cells that were identified based on their ability to efflux Hoechst 3342 dye (Pfister et al., *Circ Res* 2005), Wnt pathway activation by injecting recombinant Wnt3a in the peri-infarct region depleted the endogenous pool of cardiac progenitors and worsened cardiac remodeling post-infarct (Oikonomopoulos et al., *Circ Res* 2011). *In vitro,* Wnt3a exerted an anti-proliferative effect on these side-population progenitors through insulin growth factor binding protein 3 (IGFBP3) signaling (Oikonomopoulos et al., *Circ Res* 2011).

Independent of stem/progenitor cells, the Wnt pathway is reported to affect infarct repair through many other cells in the heart. Wnt inhibition by sFRP2, for example, is credited with promoting survival of cardiomyocytes (Mirotsou et al., *Proc Natl Acad Sci USA* 2007; Zhang et al., *J Mol Cell Cardiol* 2009). Conversely, injection of recombinant Wnt3a in the infarct zone increased cardiomyocyte apoptosis (Oikonomopoulos et al., *Circ Res* 2011). Both of these studies point to a direct effect of the Wnt pathway on cardiomyocyte apoptosis, which is an important aspect of infarct pathology. However, other works have demonstrated an opposite pro-survival effect of the Wnt pathway in cardiomyocytes by adenovirus-mediated gene transfer of non-phosphorylatable, constitutively active β-catenin in the infarct zone (Hahn et al., *J Biol Chem* 2006).

The Wnt pathway and its modulation also affect cardiac fibroblasts and their activity post-ischemic injury. Overexpression or exogenous addition of sFRP1 and sFRP2 in the infarcted heart are reported to promote healing by modulating matrix synthesis and remodeling, at least in part, through Wnt pathway inhibition; however, Wnt independent roles of the protein are also considered important for this effect (Barandon et al., *Circulation* 2003; He et al., *Proc Natl Acad Sci U S A* 2010). Other reports suggest that a pro-fibrotic effect of Wnt pathway activation actually promotes adaptive cardiac remodeling post-infarct. In a study by Hyo-Soo Kim and colleagues, adenoviral gene transfer of stabilized β-catenin was used to activate the Wnt pathway post-infarct; the authors reported enhanced fibroblast proliferation and differentiation of fibroblasts into myofibroblasts, which surprisingly resulted in a slight improvement in cardiac function, and a reduction in infarct size and adverse remodeling (Hahn et al., *J Biol Chem* 2006). More recently, Duan *et.al* showed that Wnt1/β-catenin signaling is activated in the epicardium following infarct, and leads to differentiation of epicardial cells into fibroblasts through the

process of epithelial-mesenchymal transition (EMT) (Duan et al., *EMBO J* 2012). Disruption of this signal in either the epicardium or in fibroblasts using inducible genetic models increased cardiac dysfunction and ventricular dilatation following ischemia-reperfusion injury (Duan et al., *EMBO J* 2012). Other studies have shown that Wnt inhibition by sFRP2 overexpression increases the potency of exogenous bone-marrow derived mesenchymal stem cells in promoting cardiac repair post-infarct (Alfaro et al., *Proc Natl Acad Sci USA* 2008; Alfaro et al., *J Biol Chem* 2010). Wnt signals also affect angiogenesis during infarct repair. Laermans *et. al* showed that blocking Wnt/Frizzled signaling by a peptide mimic of Wnt3a/5a improved neovascularization (Laeremans et al., *Circulation* 2011), while another study reported a positive role for the Wnt pathway in mediating neovascularization in the infarcted heart through vascular-endothelial growth factor (VEGF) and Ang1 signals (Paik et al., *Circ Res* 2015). The role of Wnt pathway in infarct pathology and repair is complex and multi-faceted, and this complicates efforts at understanding effects of Wnt modulation using genetic models, that are limited by the promoters or cells types used for targeted mutations.

Other tissues

Other tissues illustrate the complicated, and more commonly, negative role of Wnt pathway in injury and repair. Skeletal muscle, a partially regenerating tissue uses satellite cells with a remarkable ability to facilitate regeneration of injured muscle. An initial inhibition of the Wnt pathway followed by a brief activation is necessary for complete regeneration of skeletal muscle (Brack et al., *Cell Stem Cell* 2008). In keeping with the theme of Wnt being a pro-fibrotic factor, a sustained increase in activity of the canonical Wnt pathway in the myogenic progenitors is associated with increased conversion of satellite cells from myogenic to fibrogenic lineages in aging mice (Brack et al., *Science* 2007). In the liver, another tissue with facultative progenitors

that successfully regenerate the tissue after injury, fibrosis can occur as a result of chronic injury from drug or alcohol toxicity and persistent viral infection. Wnt pathway activity is implicated in promoting fibrosis through myofibroblast differentiation of liver progenitor cells, known as hepatic stellate cells (Myung et al., *FEBS Lett* 2007; Miao et al., *Biochimie* 2013). In contrast, Wnt is ascribed the role of mediating homeostasis and regeneration through proliferation of both the hepatocytes (Gougelet et al., *Int J Hepatol* 2012; Wang et al., *Nature* 2015), and the more quiescent liver progenitors (Yang et al., *Cancer Res* 2008). Hence, in many mammalian tissues, regardless of their intrinsic regenerative and self-renewal potential, or lack thereof, the recurring pattern appears to be a positive, albeit complex and context- or cell subtype-dependent, role for the Wnt pathway in stem cell homeostasis/proliferation/differentiation, and a concurrent pro-fibrotic effect from longer term Wnt activation (Akhmetshina et al., *Nat Commun* 2012). In the face of this dichotomy, pharmacologic agents that allow targeting of the overall Wnt pathway (as opposed to specific ligands or receptors targeted by genetic models), and fine tuning of the level of Wnt inhibition in a spatio-temporally restricted manner may be crucial to achieve a therapeutically relevant wound healing outcome.

Wnt therapeutics: small molecules for big gains in regeneration

The involvement of Wnt pathway in fibrotic diseases, and even more commonly, cancers (Kinzler et al., *Cell* 1996; Morin et al., *Science* 1997; Liu et al., *Nat Genet* 2000; Oskarsson et al., *Nat Med* 2011), has engendered widespread interest in developing Wnt inhibitory therapeutics. However, progress has been slow because of the enormous complexity of the pathway, and the wide-ranging effects it has on homeostasis of multiple tissues. A number of FDA-approved drugs that inhibit the Wnt pathway are in use in the clinic, although Wnt inhibition may not be recognized as their primary mode of action. Non-steroidal anti-

inflammatory agents (NSAIDS) and cyclooxygenase 2 (Cox2) inhibitor (celecoxib) can inhibit Wnt/β-catenin mediated transcription (Dihlmann et al., *Oncogene* 2001), and reduce polyp formation in patients and in mouse models of colorectal cancer (Giardiello et al., *N Engl J Med* 1993; Tuynman et al., *Cancer Res* 2008). More recently, high-throughput screening using Wnt-activated luciferase reporters has led to identification of novel compounds or repurposing opportunities of known drugs as Wnt inhibitors **(Figure 1 and listed in Table1)**.

Table 1. Wnt pathway inhibitors and progress in the clinical and pre-clinical pipeline

Drug class	Compound	Molecular target	Drug developmental stage	Reference/Company
Small molecule	Pyrvinium	Activates CKIα	FDA-approved anti-helminth	(Thorne et al., *Nat Chem Biol* 2010)
	Niclosamide	Inhibits Frizzled endocytosis	FDA-approved anti-helminth	(Chen et al., *Biochemistry* 2009)
	XAV-939	Inhibits tankyrase/stabilizes Axin	Preclinical	(Huang et al., *Nature* 2009)
	IWR-1	Stabilizes Axin	Preclinical	(Chen et al., *Nat Chem Biol* 2009)
	iCRT	Prevents β-catenin binding to TCF	Preclinical	(Gonsalves et al., *Proc Natl Acad Sci U S A* 2011)
	ICG-001	Prevents β-catenin binding to CBP	Preclinical	(Emami et al., *Proc Natl Acad Sci U S A* 2004)
	PRI-724	Prevents β-catenin binding to CBP	Phase I for advanced solid tumors *clinicaltrials.gov: NCT01302405*	Prism Pharma
	IWP	Inhibits Porcupine activity	Preclinical	(Chen et al., *Nat Chem Biol* 2009)
	LGK-974	Inhibits Porcupine activity	Phase I for Wnt-driven malignancies *clinicaltrials.gov: NCT01351103*	Novartis
Biologics	OTSA101 mAb	Frizzled-10	Phase I for synovial sarcoma *clinicaltrials.gov: NCT01469975*	OncoTherapy Science
	Vantictumab	Frizzled	Phase I for solid tumors *clinicaltrials.gov: NCT01345201, NCT01957007, NCT01973309, NCT02005315*	OncoMed Pharmaceuticals
	OMP-54F28	Wnt scavenger	Phase I for solid tumors *clinicaltrials.org: NCT01608867, NCT02050178, NCT02092363, NCT02069145*	OncoMed Pharmaceuticals
	UM206 (Wnt3a/5a peptide mimetic)	Blocks Frizzled	Preclinical	(Laeremans et al., *Circulation* 2011; Uitterdijk et al., *Lab Invest* 2016)

Pyrvinium, a Food and Drug Administration (FDA)-approved anti-helminth was identified as a potent Wnt inhibitor in a Wnt/TCF-responsive luciferase screen (Thorne et al., *Nat Chem Biol* 2010). The compound potentiates CK1α, enabling degradation of β-catenin (Thorne et al., *Nat Chem Biol* 2010), and it has been shown to improve infarct repair (Saraswati et al., *PLoS One* 2010), and promote regenerative healing of cutaneous injury (Bastakoty et al., *FASEB J* 2015). However, in cardiac studies, intra-cardiac injection of the drug resulted in significant mortality of the animals (Saraswati et al., *PLoS One* 2010), curbing the potential of this drug as a therapeutic agent, unless it can be reformulated to counter the systemic toxicity. Another anti-helminth, niclosamide, was also identified by a similar approach as an inhibitor of the Wnt pathway. Niclosamide acts by preventing Frizzled endocytosis upon ligand binding (Chen et al., *Biochemistry* 2009). Both Pyrvinium and Niclosamide, although FDA-approved, have the limitation of being developed originally as orally administered compounds targeting the gastrointestinal tract, and hence present a need for thorough examination of their systemic bioavailability and safety.

Another small molecule Wnt inhibitor that targets the β-catenin degradation complex by stabilizing axin2, a rate-limiting component of the β-catenin degradation complex, was identified by Feng Cong and colleagues in a Wnt-responsive luciferase screen. XAV-939 stabilizes axin by inhibiting tankyrases (Huang et al., *Nature* 2009), and has been reported to suppress growth of Wnt-driven cancer cells derived from colorectal cancer (Waaler et al., *Cancer Res* 2012) and neuroblastoma (Tian et al., *J Exp Clin Cancer Res* 2013). In our study of a cutaneous injury model, topical treatment of skin injury with XAV-939 showed remarkable improvement in regenerative wound repair with reduction in fibrosis, and regeneration of auricular cartilage (Bastakoty et al., *FASEB J* 2015).

Since components of the β-catenin degradation complex, particularly APC, are mutated in a number of Wnt-driven cancers, some investigators have focused on inhibiting β-catenin responsive transcription (CRT) directly in the nucleus by targeting β-catenin binding to TCF or other transcription co-factors in the nucleus (Gonsalves et al., *Proc Natl Acad Sci U S A* 2011). Besides circumventing the limitations of dysregulation of other Wnt pathway components, this approach counters the effects of altering β-catenin protein levels, and hence interfering with E-cadherin/β-catenin signals. ICG-001, which inhibits β-catenin/TCF mediated transcriptional activity by binding to transcription co-factor CBP (CREB Binding Protein), was identified by Emami *et.al (Emami et al., Proc Natl Acad Sci U S A 2004).* Although the drug successfully reduced proliferation of colorectal carcinoma cells, improved heart function in rat myocardial infarct model (Sasaki et al., *PLoS One* 2013), reversed pulmonary fibrosis (Henderson et al., *Proc Natl Acad Sci U S A* 2010), attenuated renal fibrosis (Hao et al., *J Am Soc Nephrol* 2011), and inhibited Wnt-driven cholangiocarcinoma (Boulter et al., *J Clin Invest* 2015), concerns about the off-target effect of preventing CBP binding to its many other transcription factor partners persist among researchers (Vo et al., *J Biol Chem* 2001; Gonsalves et al., *Proc Natl Acad Sci U S A* 2011). A second generation CBP/β-catenin inhibitor PRI-724 with improved plasma half-life entered Phase I dose escalation study for advanced solid tumors in 2011, and showed no safety issues, but the study was terminated for unclear reasons *(clinicaltrials.gov: NCT01302405).* Meanwhile, other CRT inhibitors targeting β-catenin binding to its transcription co-factors have also been investigated. Shivdasani and colleagues developed a cell free β-catenin/TCF binding-based high-throughput screen, and identified small molecules that inhibit β-catenin-TCF binding (Lepourcelet et al., *Cancer Cell* 2004). Unfortunately, since the screen was performed in a cell-free system, the small molecules showed off-target inhibition of β-catenin-APC binding when

tested in cell lines. Similarly, Gonsalves *et.al* identified specific inhibitors of β-catenin-driven transcription using RNAi based chemical genetic screen of small molecules (Gonsalves et al., *Proc Natl Acad Sci U S A* 2011). The lead compounds identified in the screen inhibited Wnt/β-catenin reporter activity and reduced proliferation in myeloma cells (Narayanan et al., *Anticancer Res* 2012).

While these CRT inhibitors have moved towards the clinic with slow, halting progression, the recent identification of the acyltransferase porcupine, that is needed for Wnt ligand secretion as a druggable Wnt pathway target has garnered much enthusiasm. Consequently, new small molecule Porcupine inhibitors have been described by multiple groups (Chen et al., *Nat Chem Biol* 2009; Liu et al., *Proc Natl Acad Sci U S A* 2013; Proffitt et al., *Cancer Res* 2013; Kabiri et al., *Blood* 2015). One of the porcupine inhibitors identified in those screens, LGK974, is currently in Phase I dose escalation studies for malignancies driven by Wnt ligands *(clinicaltrials.gov: NCT01351103)*. In work currently in preparation for publication, we reported a significant improvement in cardiac recovery post infarct by treatment with an analog of LGK974 (Bastakoty, *Manuscript in preparation* 2016).

Although small molecules constitute the bulk of the drugs being developed to target the Wnt pathway, biologics such as blocking antibodies and Wnt peptide mimetics have also been developed, and some are being tested in clinical trials (Blagodatski et al., *Mol Cell Ther* 2014). Antibodies against Wnt5a (Hanaki et al., *Mol Cancer Ther* 2012) and Wnt1 (He et al., *Oncogene* 2005) have been described to be effective against Wnt-driven gastric and colorectal cancer in rodent models. However, none of the anti-Wnt antibodies have reached clinical studies yet. On the other hand, antibodies against Frizzled receptors are making considerable progress in the clinical or pre-clinical pipelines. OTSA101, a chimeric humanized monoclonal antibody against

Frizzled-10, developed by OncoTherapy Science, has reached Phase I clinical trial for synovial sarcoma, in which Frizzled-10 is uniquely expressed (Blagodatski et al., *Mol Cell Ther* 2014) *(clinicaltrials.gov: NCT01469975)*. Another anti-Frizzled monoclonal antibody, Vantictumab, developed by Oncomed Pharmaceuticals has completed Phase I trials for solid tumors *(clinicaltrials.gov: NCT01345201)*, and is in ongoing Phase I studies for lung cancer *(ClinicalTrials.gov: NCT01957007)*, metastatic breast cancer *(clinicaltrials.gov: NCT01973309)*, and stage IV pancreatic cancer *(clinicaltrials.gov: NCT02005315)* as a combinatorial therapy with cytotoxic chemotherapeutic agents. A different biologic by Oncomed Pharma, OMP-54F28, a Wnt scavenger formed by fusing the Wnt binding region of Frizzled-8 to Fc region of IgG (DeAlmeida et al., *Cancer Res* 2007), is also in Phase I dose escalation studies for a number of different solid tumors either as solo agent or in combination with cytotoxic chemotherapeutics *(clinicaltrials.org: NCT01608867, NCT02050178, NCT02092363, NCT02069145).*

Wnt mimetic peptides are another class of biologics that are being developed for targeting the Wnt pathway, and they have made considerable progress in clinical or pre-clinical studies (Blagodatski et al., *Mol Cell Ther* 2014). UM206, a Wnt3a/5a peptide mimic that blocks Wnt/Frizzled signaling, prevented infarct expansion and development of heart failure in both mouse (Laeremans et al., *Circulation* 2011) and swine (Uitterdijk et al., *Lab Invest* 2016) models.

These interventions to target the Wnt pathway in the clinic have enormous potential in promoting tissue regeneration, although many of them have been originally developed as cancer therapeutics. In addition to the development of pharmacologic agents, design of new tools or repurposing of existing ones for targeting these therapeutics to the tissue of interest, and fine tuning their delivery would be paramount to their success as therapeutics. Since Wnt pathway

activity is critical in homeostasis of certain tissues, and the role of Wnt activation or inhibition is often biphasic and context or cell type dependent in most tissues, the optimization of delivery, dosage and targets is critical for safety of patients. The need for carefully assessing safety and off-target effects of Wnt antagonists has been exemplified in the case of the Frizzled-10 mAb Vantictumab, and the Wnt scavenger OMP-54F28 (Grover, *Reuters Life* 2014). The Phase I clinical trials for these drugs, developed by Oncomed Therapeutics, were put on temporary hold by the FDA because of mild-to-moderate bone-related side effects on patients. Although the studies have resumed after revision of study protocols to mitigate side-effects (*BioSpace Life Sciences News+Jobs* 2014), this experience reiterates the need to optimize delivery and dosage so as to minimize the systemic exposure to the drugs, and to ward off collateral effects on tissues or cells that require Wnt activity for homeostasis. In cutaneous injury, for example, controlled-release scaffolds placed in the dermis may allow targeting of the drug specifically to the dermis, where fibrogenesis occurs, without undesired effects on the epidermis or hair follicles. Similarly, in the heart, use of implantable stents to release the drug directly into the infarct region, and hence reduce the dose and systemic exposure to the drug, may aid in achieving optimal Wnt inhibition while minimizing the off-target effects associated with systemic delivery.

Conclusion

The Wnt pathway, with its contradictory roles in regeneration/stem-cell maintenance and fibrosis presents a conundrum for therapeutic targeting. After more than three decades of intense investigation, understanding the complete role of the Wnt pathway in homeostasis and disease is still a work-in-progress, and no Wnt therapeutics have succeeded in reaching patients (Nusse et al., *EMBO J* 2012; Kahn, *Nat Rev Drug Discov* 2014). This can mostly be attributed to the increasing complexity of the pathway, and its wide ranging, disparate roles in various tissues.

These aspects make targeting more complicated until more knowledge of the pathway is gained. Based on the lessons learned from the genetic studies, whereby the effects seem to vary depending on the specific part of the Wnt signaling cascade that is targeted (Baurand et al., *Circ Res* 2007; Paik et al., *Circ Res* 2015), innovative interdisciplinary approaches such as systems biology approaches and computational modeling may be able to provide additional insights into the outcomes of targeting specific members of the pathway. Additionally, future studies that elucidate co-activators of β-catenin transcription involved in activating transcription of specific Wnt/β-catenin target genes in a given context may allow development of targeted inhibitors with potentially fewer off-target effects (Teo et al., *Proc Natl Acad Sci U S A* 2005; Miyabayashi et al., *Proc Natl Acad Sci U S A* 2007).

Despite the challenges in safely and effectively targeting the Wnt/β-catenin pathway for regeneration, given its wide-ranging effects in multiple tissues and pathologies, any concrete progress in the area is likely to result in big gains in reaching the patient. Our constantly improving understanding of the intricacies of the Wnt pathway, and availability of increasingly more sophisticated tools to target it in the tissue of interest present unprecedented opportunities for effective Wnt inhibitory therapeutics that can significantly improve regenerative healing of multiple types of tissue injuries.

CHAPTER 2

INHIBITION OF WNT/BETA-CATENIN PATHWAY PROMOTES REGENERATIVE CARDIAC REPAIR FOLLOWING MYOCARDIAL INFARCT

Introduction

The Wnt/β-catenin pathway is temporarily activated in the heart following myocardial infarction. Despite data from genetic models indicating both positive and negative role for the Wnt pathway, the overall effect of therapeutic inhibition of Wnt pathway on post injury outcome are unknown. We sought to investigate whether temporary pharmacologic inhibition of the Wnt pathway post-infarct can mitigate cardiac dysfunction and fibrosis using a newly available, small molecule, GNF6231, which averts Wnt pathway activation by inhibiting activity of the O-acyltransferase porcupine, and hence blocking Wnt secretion. Short-term treatment with GNF-6231 significantly reduced the decline in cardiac function, prevented adverse cardiac remodeling, and reduced infarct size. Histologically, Wnt inhibition augmented proliferation of interstitial cells, particularly in the distal myocardium, inhibited apoptosis of cardiomyocytes, and reduced myofibroblast proliferation in the peri-infarct region. In vitro studies showed that Wnt inhibition increased proliferation of Sca1+ cardiac progenitors, improved survival of cardiomyocytes, and inhibited collagen I synthesis by cardiac myofibroblasts. These data point to a promising role for Wnt inhibitory therapeutics as a new class of drugs to drive post MI repair and prevent progression to heart failure.

Myocardial infarct pathology and disease progression

Cardiovascular disease is the leading cause of death in the United States, and coronary heart disease, which leads to ischemic myocardial injury, the most common cardiovascular

disease with a toll of 370,000 deaths each year in the US (Mozaffarian et al., *Circulation* 2015). Cardiomyocyte apoptosis and fibrosis initiated by an infarct result in continuing adverse remodeling of the myocardium, which leads to eventual heart failure (Sutton et al., *Circ* 2000; Mill et al., *Braz J Med Biol Res* 2011). Current therapies for MI focus mostly on disease management and prevention of MI recurrence. Hence, there is a need to develop effective therapeutics that addresses the core pathophysiology: particularly the ongoing cardiomyocyte death and fibrosis that occur in the aftermath of an infarct.

Wnt/β-catenin pathway in myocardial infarct repair

The Wnt/β-catenin pathway is activated in various cardiac cells in response to ischemic injury (Oerlemans et al., *Basic Res Cardiol* 2010; Aisagbonhi et al., *Dis Model Mech* 2011), and has been studied by many groups in the context of myocardial infarct repair. In genetic model of Wnt pathway modulation through β-catenin stabilization or depletion in cardiomyocytes, Wnt activation leads to adverse remodeling after ischemic injury, whereas Wnt inhibition improves function (Baurand et al., *Circ Res* 2007; Zelarayan et al., *Proc Natl Acad Sci USA* 2008). Studies using overexpression or exogenous administration of secreted Wnt inhibitors (secreted Frizzled-related protein 1 and 2; sFRP1, and sFRP2 (Barandon et al., *Circulation* 2003; He et al., *Proc Natl Acad Sci U S A* 2010)) have shown that Wnt inhibition stimulates recovery of cardiac function and reduces scarring after MI through effects on cardiomyocyte survival (10), matrix deposition (Barandon et al., *Circulation* 2003; He et al., *Proc Natl Acad Sci U S A* 2010) and immune infiltration (Barandon et al., *Circulation* 2003). Our work has also shown that overexpression of sFRP2 in mesenchymal stem cells (MSCs) transplanted into infarcted heart improves cardiac repair. Moreover, pharmacologic inhibition of

the Wnt pathway also, prevented adverse cardiac remodeling, induced revascularization in the infarcted heart (Saraswati et al., *PLoS One* 2010; Murakoshi et al., *PLoS One* 2013) and reduced scarring, possibly by reducing fibroblast survival (Murakoshi et al., *PLoS One* 2013).

However, there are conflicting reports suggesting that Wnt activation can also enhance post-MI cardiac repair. Paik et.al showed that gain-of-function of a canonical Wnt ligand, Wnt 10b, in cardiomyocytes can orchestrate recovery by augmenting neovascularization (Paik et al., *Circ Res* 2015). Interruption of Wnt 1/β-catenin signals specifically in the epicardium through β-catenin deletion was reported to impede adaptive pro-fibrotic response (Woulfe et al., *Circ Res* 2010; Duan et al., *EMBO J* 2012), whereas post-infarct injection of adenoviral vector with constitutively active β-catenin reduced infarct size by boosting cardiomyocyte survival, and granulation tissue formation by myofibroblasts (16). Some of the discrepant observations may be explained by ligand (Woulfe et al., *Circ Res* 2010) and cell-type dependent (Hahn et al., *J Biol Chem* 2006) effects of Wnt signaling on healing. Moreover, the genetic models that use cell-specific promoters to modulate Wnt signaling have caveats such as incomplete targeting of the microenvironment, inability to transiently inhibit the signal, and unintended physiological effects on the cell types targeted (for example, complete β-catenin mutation in epicardial cells (Duan et al., *EMBO J* 2012) may lead to deficiencies in the developing heart as reported in previous studies (von Gise et al., *Dev Biol* 2011) complicating the investigation of its effect on the infarcted heart). Hence the overall effect of the pathway may be different based on the time-point and dosage of Wnt signal—a short or low Wnt activity being needed for initiation of healing and granulation tissue formation (Duan et al., *EMBO J* 2012), and a long term Wnt activation leading to fibrosis, hypertrophy (Woulfe et al., *Circ Res* 2010) and subsequent disease progression. Given these factors outlined above, and given that the majority of studies, including our own

work (Bastakoty et al., *FASEB J* 2015), suggesting a pro-reparative role for post-injury Wnt inhibition in multiple injury models (Cheon et al., *Proc Natl Acad Sci U S A* 2002; Kim et al., *Tohoku J Exp Med* 2011), we sought to investigate the effect of short-term therapeutic Wnt inhibition on cardiac recovery following MI in a mouse model of permanent ligation of the left ventricle descending coronary artery.

Pharmacologic inhibition of the Wnt/β-catenin pathway following myocardial infarct

The paucity of safe and effective Wnt inhibitors suited for clinical use has delayed efforts to assess therapeutic Wnt pathway inhibition in cardiac injury (Anastas et al., *Nat Rev Cancer* 2013; Kahn, *Nat Rev Drug Discov* 2014). Recently, with the new class of Wnt ligand secretion inhibitors—an analog of which, has advanced to clinical studies (Novartis, *Clinicaltrials.gov* 2014), we are able to investigate the potential of systemic Wnt inhibition for post-MI cardiac injury repair. Our study shows that short-term pharmacologic Wnt inhibition following experimental MI augments cardiac repair, characterized by improvement in functional and remodeling parameters, and reduction in collagenous scar. Our data suggests that this occurs through effects on multiple facets of cardiac pathology including, proliferative response in interstitial (possibly, progenitor) cells in the heart, through reduction in cardiomyocyte apoptosis, and through mitigation of myofibroblast proliferation and collagen synthesis.

Results

GNF-6231, a porcupine inhibitor, downregulates Wnt target gene expression and is bioavailable in vivo

In order to investigate the physiological and cellular effects of Wnt inhibition on post-MI cardiac regeneration, we utilized GNF-6231, a small molecule inhibitor of the acyltransferase,

Porcupine. Porcupine is the enzyme responsible for the post-translational palmitoylation of Wnt proteins that is required for both Wnt secretion as well as binding of Wnts to their receptors (Liu et al., *Proc Natl Acad Sci U S A* 2013) (**Fig 4A**). An analog of the compound is currently in Phase I clinical trials as Wnt inhibitory therapeutics for cancer (Novartis, *Clinicaltrials.gov* 2014). GNF-6231 inhibits porcupine enzymatic activity with a cellular IC_{50} of 0.8 nM, and does not show cytotoxicity up to 20 μM (Cheng, *To be submitted* 2016). In Wnt3a overexpressing cells, it potently inhibits Wnt pathway activation as indicated by Axin2 mRNA levels (**Fig 4B**). In vivo, the pharmacokinetic (PK) and pharmacodynamic (PD) relationship of GNF-6231 was investigated following a single 5 mg/kg intravenous administration to C57BL/6J mice. GNF-6231 showed high plasma level, and free plasma concentrations (mouse plasma protein binding of GNF-6231 is 88%) above its in vitro Porcupine IC_{50} for at least 16 h (**Fig 4C)**. Plasma half-life of GNF-6231 was estimated to be 2.3 hours (**Fig 4C**). The expression of the Wnt target gene Axin2 was measured in liver tissues. Inhibition of Axin2 expression occurred upto 24 hours post intravenous injection (**Fig 4D**). Since GNF-6231 inhibits the enzymatic activity of Porcupine, impeding Wnt secretion, an expected time delay was observed between peak GNF-6231 plasma concentration (5 min) and the maximum PD response as measured by Axin2 inhibition similar, to that described by Liu at el (Liu et al., *Proc Natl Acad Sci U S A* 2013) (**Fig 4D**).

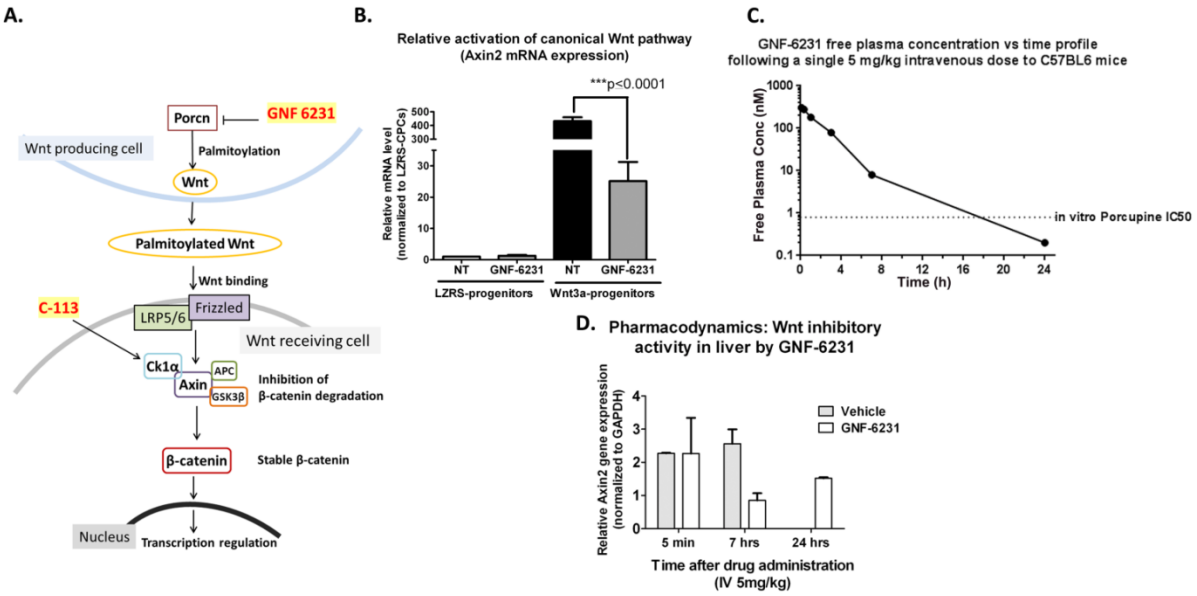

Figure 4: GNF-6231 inhibits canonical Wnt pathway activity in vitro.
(A) Schematic of the Wnt pathway and point of action of Wnt inhibitors, GNF-6231 and C-113. **(B)** Fold change in Axin2 gene expression in Wnt3a overexpressing cardiac cells showing GNF6231 treatment reduced Wnt target gene expression (N=3 per group; ***P≤0.0001; Repeated measures ANOVA with Bonferroni correction for multiple comparisons). **(C)** IV free plasma level of GNF-6231 after a single intravenous injection of 5 mg/kg. The plasma half-life of the drug was approximately 2.3 hours; GNF-6231 free plasma concentrations were above in vitro Porcupine IC_{50} for >12 h. **(D)** qRT-PCR showed inhibition of Axin2 gene expression in liver at different time points following a single 5 mg/kg intravenous treatment with GNF-6231 (N=2 per timepoint). Bars represent Mean±SD.

Systemic administration of GNF-62321 does not cause toxicity to Wnt-dependent tissues in vivo.

Since the canonical Wnt pathway is constitutively active in certain tissues such as colon and skin, we assessed the potential toxicity of inhibiting Wnt signaling in these tissues. With 6 daily consecutive treatments of 5mg/kg GNF-6231 intravenously (dosage and regimen used in our studies; described in **Fig 7A**), there was no effect on histology of colon (**Fig 5A**), or β-catenin expression and localization as detected by immunostaining (**Fig 5B**), signifying no GI tract toxicity of the drug. Likewise, no effect on skin histology (**Fig 5C**) was observed in GNF-6231 treated animals compared to vehicle-treated controls.

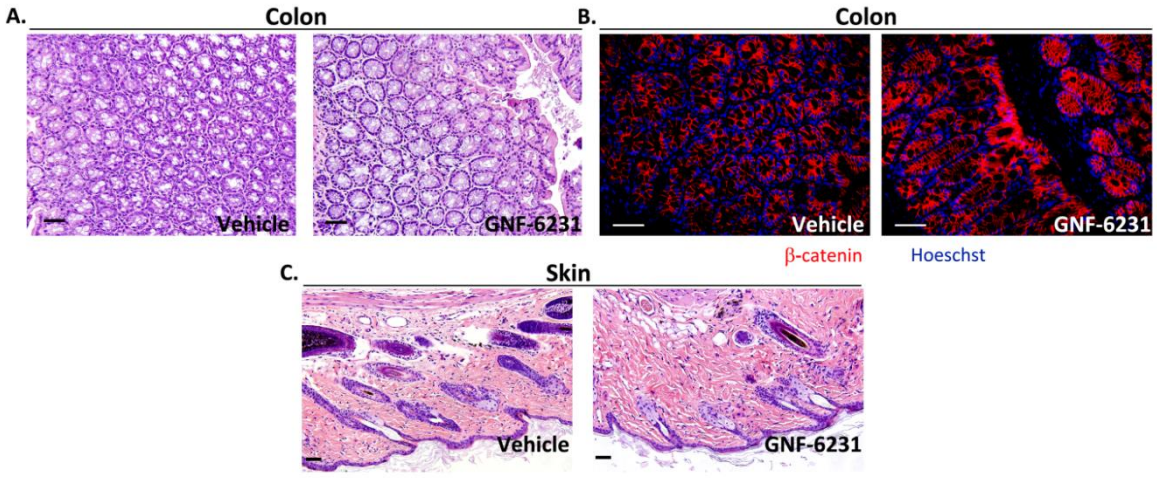

Figure 5: GNF-6231 causes no detectable toxicity in Wnt-dependent tissues.
Representative **(A)** H&E stained sections, and **(B)** β-catenin immunostained sections of the colon depicted that GNF-6231 treatment had no effect on colon morphology and β-catenin protein level and localization in the tissue. **(C)** Representative H&E stained sections of skin from GNF-6231 or vehicle-treated animals. Scale bars in A and B equal 50µm.

The Casein Kinase Iα activator C-113 inhibits Wnt target gene expression in cells treated with recombinant Wnt3a

To confirm our findings with GNF-6231 in vitro, we used a small molecule Casein Kinase1-alpha (CK1α) activator VU-WS113 (Thorne et al., *Nat Chem Biol* 2010), referred to in this chapter as C-113. It targets the β-catenin degradation complex (18, 26), and hence inhibits the Wnt pathway by a mechanism of action that is distinct from GNF-6231 (**Fig 4A**). Quantitative real-time PCR for Wnt target gene, Axin2 showed that C-113 inhibits Wnt pathway activation induced by treatment with recombinant WNT3A (**Fig 6**). Since C-113 targets the Wnt pathway downstream of Wnt ligand secretion, it allowed us to investigate the effect of Wnt inhibition without the need to overexpress *Wnt3a*.

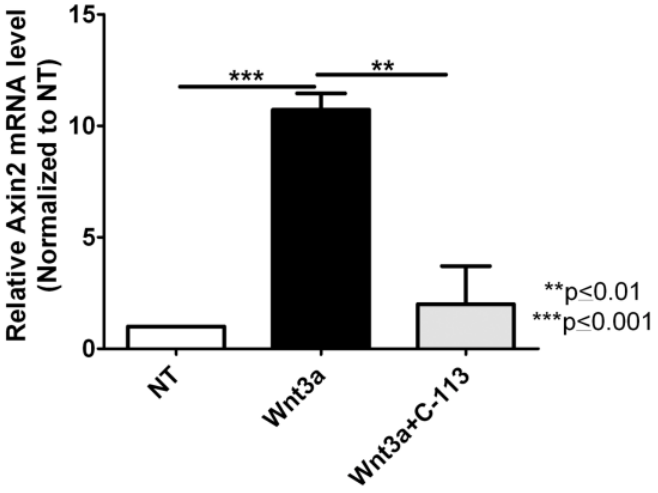

Figure 6: C-113 inhibits Wnt target gene expression.
Graph showing relative Axin2 mRNA expression detected by qRT-PCR in Sca1+ progenitors treated with recombinant WNT3A or with recombinant WNT3A and Wnt inhibitor C-113. Bars represent mean±SD. N=3; **P≤0.01 and *P≤0.001; Kruskal-Wallis test with Dunns correction for multiple comparisons.

Treatment with GNF-6231 inhibits post-MI Wnt/β-catenin pathway activation in the infarcted heart

Previous studies have shown that the canonical Wnt pathway is activated in the infarcted heart starting around 72 hours post-experimental MI (Oerlemans et al., *Basic Res Cardiol* 2010; Aisagbonhi et al., *Dis Model Mech* 2011). Wnt pathway activation is reported to peak between 7 to 14 days post-injury, after which it begins to recede to baseline levels (Aisagbonhi et al., *Dis Model Mech* 2011). To avert this early, transient post-injury Wnt activation, we treated mice (C57Bl6 and Wnt reporter, TOPGAL mice (Aisagbonhi et al., *Dis Model Mech* 2011); age≥12 weeks) with intravenous injection of 5mg/kg GNF-6231 or vehicle every 24 hours through day 6 after injury (**Fig 7A**). Immunostaining for β-catenin (in C57Bl/6J), and for β-galactosidase (in TOPGAL mice) showed that GNF-6231 treatment reduced nuclear and cytoplasmic β-catenin levels (**Fig 7B and B'**), and total β-galactosidase protein levels (**Fig 7C**) in the peri-infarct region compared to vehicle treatment.

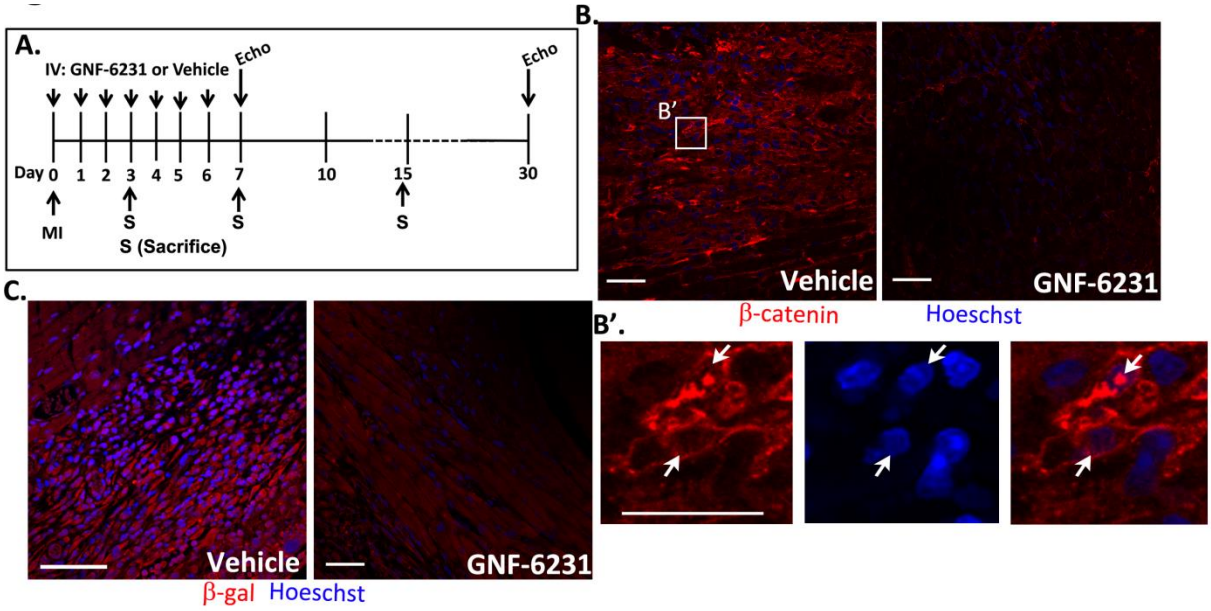

Figure 7: Porcupine inhibitor treatment inhibits Wnt pathway activity in the infarcted heart.
(A) Schematic summarizing animal study timelines. Mice were treated with daily intravenous injection of 5mg/kg drug or vehicle following MI and continued through day 6. For cardiac recovery studies, mice underwent echocardiography at day 7 and 30. For histology, a separate cohort of mice was sacrificed on day 3, 7 and 15. **(B)** β-catenin immunostaining of peri-infarct region of ventricles at day 7 showed reduction in β-catenin levels with GNF6231 treatment. **(B')** High magnification image of vehicle-treated tissue showed nuclear localization of β-catenin signifying Wnt pathway activation. **(C)** β-galactosidase immunostaining in ventricle sections from Wnt reporter, TOPGAL mice demonstrated inhibition in Wnt activity at day 7 post infarct with GNF6231 treatment. Scale bars equal 50μm.

GNF-6231 improves post-MI recovery/repair.

In order to determine the physiological effect of temporary post-MI Wnt inhibition, cardiac function and remodeling were assessed at day 7 and day 30 post-MI using echocardiography (**Table 2**). Left ventricular internal dimensions at diastole and systole (LVIDd and LVIDs respectively) were used as measures of cardiac remodeling. At day 30 post-MI, the GNF-6231 treated hearts had lower LVIDd and LVIDs compared to vehicle-treated mice (LVIDd: 3.83±0.45mm vs. 4.32±0.68mm, p=0.0377; LVIDs: 2.35±0.38mm vs. 2.84±0.64mm, p=0.0446; **Table 2**). To account for variations between mice within the experimental groups, we calculated percent change in each of the parameters from day 7 to day 30 for each individual mouse. The percent change in both of the parameters of ventricular remodeling (ΔLVIDd% and ΔLVIDs %) were significantly lower in GNF-6231 treated vs. vehicle-treated hearts (ΔLVIDd%: -2.287±12.36 vs. 16.109±8.53, p=0.0024; ΔLVIDs %: -3.011±12.65 vs. 17.198±8.91, p=0.0015; **Fig 8A and B, Table 2**), indicating that Wnt inhibition prevented adverse ventricular remodeling. Ejection Fraction (EF) and Fractional Shortening (FS) were measures of cardiac function. At day 30 post-MI, GNF-6231 treated mice had higher EF (0.75±0.05 vs. 0.71±0.06, p=0.0421), and higher FS (38.71±4.13% vs. 34.89±4.86%, p=0.0325; **Table 2**) compared to vehicle-treated controls. The percent change from day 7 to 30 in both parameters of cardiac function (ΔEF% and ΔFS%) were on average, significantly higher for each mouse in GNF-6231 treated group compared to vehicle-treated group (ΔEF%: 0.83±1.25 in GNF-6231 treated vs. -1.723±2.36 in vehicle-treated, p=0.0138; and ΔFS%: 1.4±2.312 in GNF-6231 treated vs. -1.713±3.59 in vehicle-treated, p=0.0265; **Fig 8C**, **Table 2**), suggesting that Wnt inhibition prevented worsening of cardiac function in the injured heart.

The percent infarct area, as determined by blinded histomorphometry of Masson's trichrome stained left-ventricular sections (**Fig 8E and F**) by a pathologist at day 30, was significantly lower in GNF-6231 treated hearts compared to vehicle control (9.07±3.99% in GNF-6231 treated vs. 17.18±4.97% in vehicle-treated; p=0.0152), indicative of a reduction in myocardial scarring with Wnt inhibition. Hence, GNF-6231 augmented overall cardiac repair and recovery following LV infarct.

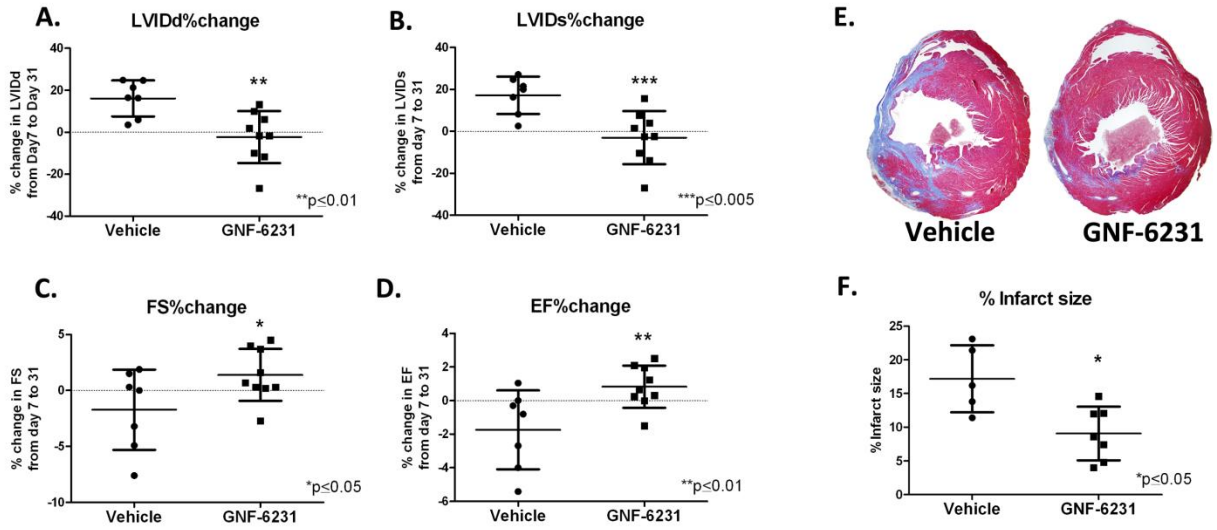

Figure 8: Porcupine inhibition improves cardiac function and reduces adverse remodeling after MI.

(A and B) Left ventricular remodeling was measured as % change in **(A)** LVIDd and **(B)** LVIDs. LV function was measured as % change in **(C)** FS and **(D)** EF. Data showed no increase in left ventricular diameter **(A and B)**, and improved cardiac function **(C and D)** with GNF6231 treatment compared to vehicle. **(E)** Masson's trichrome stained representative sections of the left ventricle at day 30 depicted more collagen stained (blue) area in vehicle-treated LV compared to GNF6231-treated. **(F)** Quantification of infarct size. Each data point on graphs represents individual mouse; **P≤0.05, **P≤0.01 or ***P≤0.005*; Unpaired t-test.

Table 2. GNF-6231 treatment promotes cardiac recovery post-MI

Echo parameters		VEHICLE	GNF-6231	p values
LVIDd (mm)	DAY 7	3.74 ± 0.60	3.95 ± 0.42	ns; p=0.3229
	DAY 30	4.32 ± 0.68	3.83 ± 0.45	*p=0.0377
LVIDs (mm)	DAY 7	2.43 ± 0.55	2.45 ± 0.39	ns; p=0.7378
	DAY 30	2.84 ± 0.64	2.35 ± 0.38	*p=0.0446
FS %	DAY 7	35.48 ± 4.65	38.24 ± 4.69	ns; p=0.174
	DAY 30	34.89 ± 4.86	38.71 ± 4.13	*p=0.0325
EF	DAY 7	0.72 ± 0.06	0.75 ± 0.06	ns; p=0.149
	DAY 30	0.71 ± 0.06	0.75 ± 0.05	*p=0.0421
Δ LVIDd %		16.109±8.53	-2.287±12.36	**p=0.0024
Δ LVIDs %		17.198±8.91	-3.011±12.65	**p=0.0015
Δ FS %		-1.713±3.59	1.4±2.312	*p=0.0265
Δ EF %		-1.723±2.36	0.83±1.25	**p=0.0138
N		7	9	

The top eight rows represent mean±SD values for each parameter and treatment at day 7 and 30. The mean±SD percent difference between day7 and day 30 for each mouse (Δ) are listed in the bottom four rows. Statistical difference between parameters in each two columns was determined by unpaired t-test.

Wnt inhibition causes proliferation of interstitial cells in the infarcted heart

Based on previous studies reporting an anti-proliferative effect of the Wnt pathway in models of skeletal muscle (Brack et al., *Cell Stem Cell* 2008) and cardiac injury (Oikonomopoulos et al., *Circ Res* 2011), we asked whether the reparative effects of GNF-6231 treatment was mediated in part by proliferation of specific cardiac cells. Immunostaining for proliferation markers Ki67 and phospho-Histone-H3 (pHisH3) showed that in the peri-infarct region (defined in **Fig 9A**), there was a remarkable increase in $pHisH3^+$cells at day 3 in both GNF-6231 and vehicle-treated hearts (**Fig 9D)**. At day 7, the proliferative response was significantly reduced, but there were 2.3 fold more $pHisH3^+$ cells in the peri-infarct region of GNF-6231 treated hearts, although the difference was not statistically significant (**Fig 9D)**. By day 15, the proliferative response had largely subsided in both treatment groups. In the distal myocardium however, GNF-6231 treated hearts had significantly more $pHisH3^+$ cells compared to vehicle-treated at both day 3 and day 7 (5.67-fold higher; ***$p \leq 0.001$, and 2.65-fold higher *$p \leq 0.05$ than control respectively; **Fig9 B-D**).

Co-immunostaining for cardiomyocyte marker Alpha Sarcomeric Actin (αSA) with pHisH3 indicated that most of the cells that were proliferating in the distal myocardium (higher in proportion in GNF-6231 treated ventricles) were interstitial cells in both treatment and control groups (**Fig 9E**). This was verified by co-staining for cardiac Troponin I (cTnI) and Ki67; a representative high magnification confocal microscopy image is shown in **Fig 9F**. We observed rare cardiomyocytes with nuclear pHisH3 staining in both GNF-6231 treated and control animals (a representative example is shown in **Fig 9G**), but it wasn't clear whether these were only undergoing karyokinesis or were truly dividing cardiomyocytes. Similarly, *in vitro* BrdU (Bromodeoxyuridine) incorporation assay with HL-1 cardiomyocyte cell line showed that

recombinant WNT3A and/or Wnt inhibitor, C-113 had no effect on cardiomyocyte proliferation (**Fig 9H**). Since the proliferating cardiomyocytes were so rare, we focused our investigations on identifying the interstitial proliferative cells.

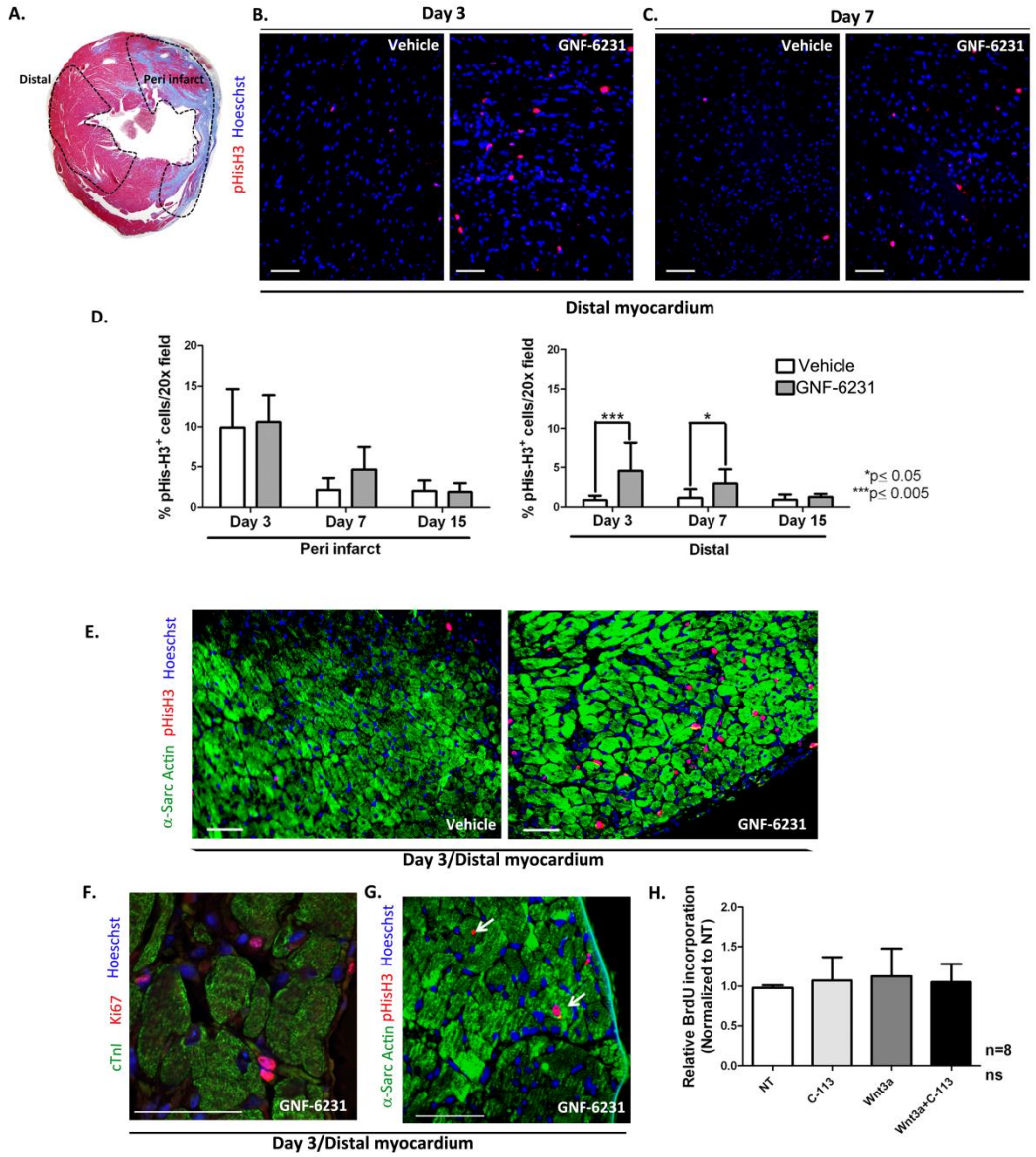

Figure 9: Wnt inhibition promotes proliferation of interstitial cells in the infarcted heart. **(A)** H&E stained cross-section of the heart demarcating peri-infarct and distal regions of the left ventricle as defined in the study. Representative pHisH3 stained sections of the ventricles at **(B)** day 3 and **(C)** day 7 showing more proliferative cells in the distal myocardium of GNF-6231 treated hearts. **(D)** Quantification of percent pHisH3+ cells. Bars represent mean±SD; $N \geq 3$ mice per group; $*P \leq 0.05$, $***P \leq 0.005$; One-Way ANOVA with Bonferroni Correction for multiple comparisons. Representative sections of the distal myocardium at day 3 post-MI **(E)** co-stained with αSarcomeric Actin and pHisH3, and **(F)** high magnification confocal microscopy image of ventricle co-stained with cTnI and Ki67, demonstrating that the majority of proliferative cells in the GNF6231-treated tissue localized to the interstitium of myofibers. **(G)** αSarcomeric Actin/pHisH3 co-stained LV depicting the rare proliferating cardiomyocytes (white arrows). **(H)** Relative proliferation measured by BrdU incorporation by HL-1 cardiomyocytes treated with recombinant Wnt3a or Wnt3a and C-113, showing that Wnt pathway modulation did not affect their proliferation. $N=8$ replicates.

Wnt inhibition selectively reduces proliferation of myofibroblasts in the peri-infarct region, and augments proliferation of αSMA negative interstitial cells in the distal myocardium

In the infarcted heart, Alpha Smooth Muscle Actin (αSMA) positive myofibroblasts are the major matrix producing cells responsible for granulation tissue formation and fibrosis (Hahn et al., *J Biol Chem* 2006). Hence, we performed co-immunostaining for αSMA and Ki67 (**Fig 10A**). Not unexpectedly, proliferating myofibroblasts (αSMA and Ki67 double positive cells) were present in both the GNF-6231 and vehicle-treated tissue, since some level of pro-fibrotic signaling is necessary to initiate granulation tissue formation and prevent infarct rupture (Duan et al., *EMBO J* 2012). Interestingly, in the peri-infarct region, the proportion of proliferating myofibroblasts ($Ki67^{+}\alpha SMA^{+}$ cells; upper dark portions of the bar in **Fig 10B**) was significantly lower in the GNF-6231 treated hearts (2.21 fold lower in GNF-6231 treated; **p=0.0013) at day 3 (**Fig 10B**). However, the proportion of αSMA negative proliferating cells ($Ki67^{+}\alpha SMA^{-}$, lower white portions of the bar in **Fig 10B**) in the peri-infarct region was higher in the GNF-6231 treated hearts than in vehicle-treated hearts at day 3 and day 7 post infarct (2.7-fold; p=*0.0135 and 2.2 fold; #p=0.0587 respectively; **Fig 10B**). Likewise, in the distal myocardium, the proportion of αSMA negative proliferating cells ($Ki67^{+}\alpha SMA^{-}$ cells) was 3.3-fold higher in the GNF-6231 treated hearts than the vehicle-treated hearts at day 3 (*p=0.012) post-infarct (**Fig 10C**).

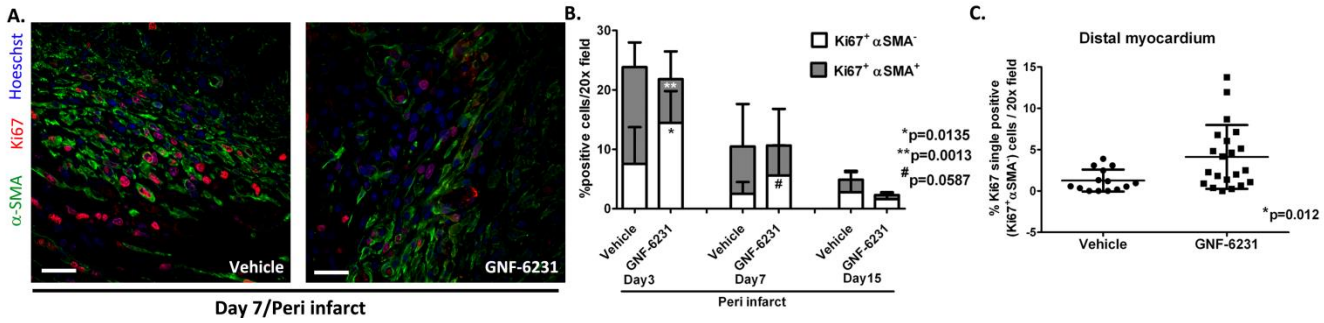

Figure 10: Wnt inhibition reduces proliferation of interstitial αSMA positive cells in the infarcted heart, and augments proliferation of αSMA negative interstitial cells in the distal myocardium.

(A) Proliferating myofibroblasts were identified by αSMA/Ki67 co-staining as depicted in the representative section from the peri-infarct region at day 7. **(B)** Quantification of αSMA/Ki67 co-stained cells revealed that the percentage of proliferating myofibroblasts (grey shaded portion of the bars) was significantly lower in GNF6231-treated peri-infarct tissue than control at day 3 (**$P=0.0013$) and lower ($^{\#}P=0.0587$) at day 7. In contrast, the percentage of proliferating non-myofibroblasts (αSMA^- cells; lower white portion of the graphs) was significantly higher (*$P=0.0135$) in GNF-6231 treated ventricles compared to control at day 7. Bars represent mean±SD. $N\geq3$ mice per group and $N\geq3$ sections per mouse were imaged. *P-values* for individual comparisons between each two groups of data were calculated using Mann-Whitney test. Scale bars equal 50µm. **(C)** Quantification of $Ki67^+\alpha SMA^-$ cells in the distal myocardium showing higher proportion of proliferating αSMA negative cells in GNF6231-treated hearts. $N\geq14$;*$P=0.0120$; Unpaired t-test.

Wnt inhibition does not affect proliferation of αSMA negative fibroblast populations in the infarcted heart

Co-immunostaining of proliferating cell nuclear antigen (PCNA) or Ki67 with markers of other fibroblast cell populations, fibroblast-specific protein-1 (FSP-1; **Fig 11 A-B**), Periostin (**Fig 11C**) and Vimentin (Zeisberg et al., *Circ Res* 2010) (**Fig 11D**) showed no effect of GNF-6231 treatment on proliferation of these cells. Based on these observations, we posit that GNF-6231 treatment selectively reduces myofibroblast proliferation in the infarcted hearts, while promoting proliferation of other interstitial cells that do not include FSP-1^{+}, Periostin^{+} or Vimentin^{+} fibroblasts.

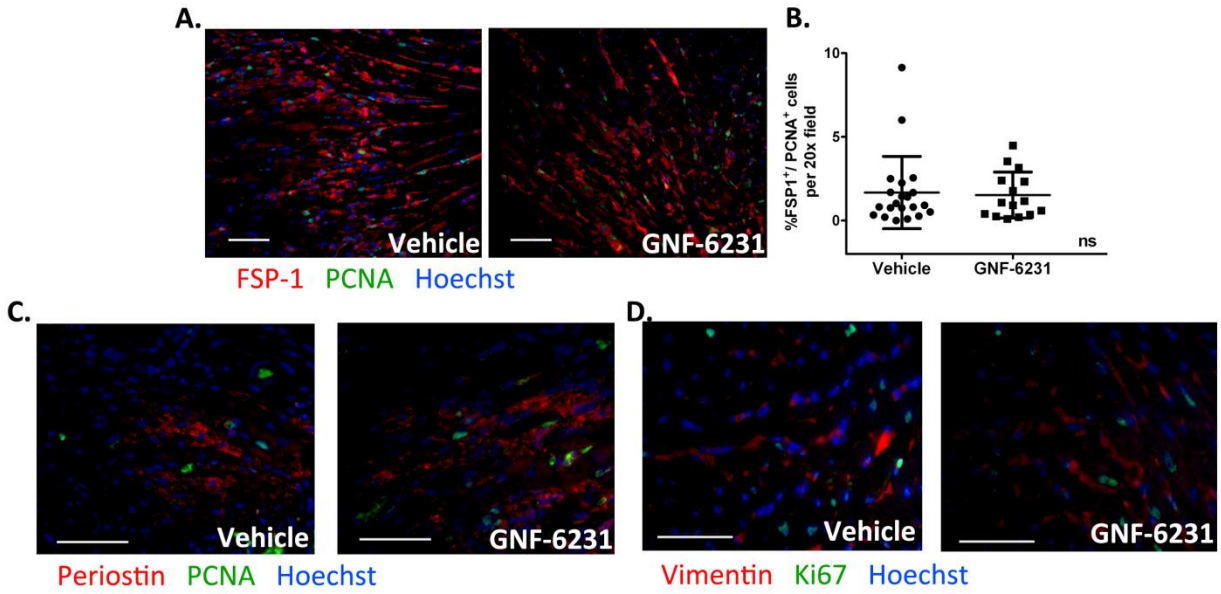

Figure 11: Wnt inhibition does not affect proliferation of other fibroblast subtypes. **(A)** Co-immunostaining for PCNA and fibroblasts marker FSP-1; quantification is presented in **(B)** showing no difference in percent double positive ($FSP\text{-}1^{+}PCNA^{+}$) cells between GNF-6231 and vehicle treated hearts. *N≥15;* Mann-Whitney test. Representative co-immunostaining for **(C)** PCNA and Periostin, and **(D)** Ki67 and Vimentin; no significant difference in percent double stained cells between GNF-6231 and vehicle-treated sections were observed for both Periostin and Vimentin. Scale bars in B, D and E equal 50µm.

Wnt inhibition does not affect proliferation of vWF positive endothelial cells in the infarcted heart

We next sought to determine the identity of the proliferating interstitial cells that were higher in number in the GNF-6231 treated hearts. To assess whether these proliferative interstitial cells were endothelial cells lining the coronary vasculature, co-immunostaining for von Willebrand factor (vWF) and PCNA was performed. Although a small percentage of proliferating endothelial cells were observed in both GNF-6231 and vehicle treated hearts, there was no significant difference in the double positive cells between the drug and vehicle treated groups (**Fig 12 A-B**).

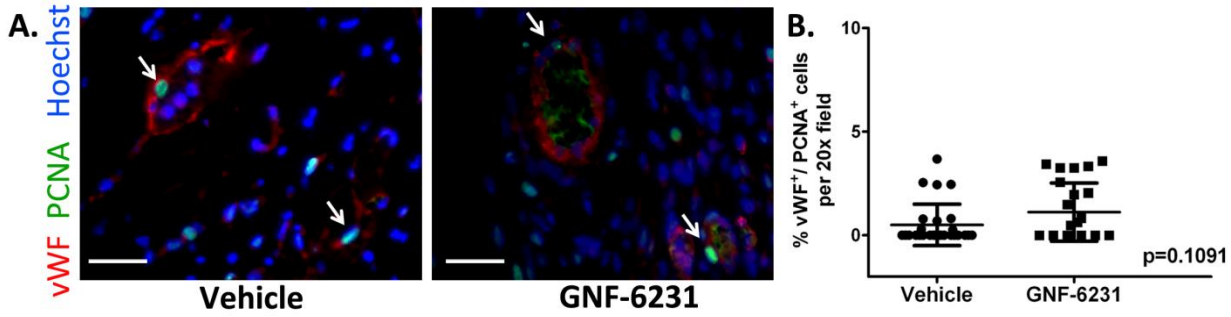

Figure 12: GNF-6231 treatment does not affect proliferation of vWF^{+} endothelial cells in the infracted heart.
(A) Representative vWF (red) and PCNA (green) immunostained sections of LV treated with GNF-6231 or vehicle. Sections were counterstained with Hoechst (blue); white arrows mark double stained cells. Scale bars equal 50µm. **(B)** Quantification of vWF and PCNA double positive cells showed no significant difference in percent double positive cells between vehicle and GNF-6231-treated hearts. *N≥20* per group; *P=0.1091;* Mann-Whitney test.

Investigating the proliferative cells in the distal myocardium responsive to Wnt inhibition

We and others have shown that Wnt inhibition causes proliferation of adult stem/progenitor cells—bone-marrow-derived MSCs (Alfaro et al., *Proc Natl Acad Sci USA* 2008) and cardiac tissue resident-side population progenitors (Oikonomopoulos et al., *Circ Res* 2011). Additionally, some of the proliferating interstitial cells in the immunostained sections of distal myocardium were spherical with large nuclei, and were localized in what appeared similar to 'stem cell niche' for tissue-resident progenitor cells described in the literature (Kimura et al., *Cardiovasc Diagn Ther* 2012) (**Fig 9F**). These proliferative αSMA negative interstitial cells were significantly higher in proportion at the distal myocardium in GNF-6231 treated tissue compared to vehicle treated myocardium as discussed in the previous section (**Fig 10C**). We hypothesized that post-injury treatment with GNF-6231 induced proliferation of a cardiac progenitor population. We used $Sca1^+$ progenitors isolated from murine hearts for this purpose.

$Sca1^+CD31^-CD45^-CD117^-$ cells isolated from murine hearts can express markers of different cardiac cell lineages when cultured in differentiation media

We assessed the effect of Wnt inhibition on $Sca1^+CD31^-CD45^-CD117^-$ cells isolated from murine heart homogenates by Fluorescent Activated Cell Sorting (FACS; **Fig 13A**). Mice expressing thermolabile simian virus SV40 T antigen (H-2Kb-tsA58 transgenic or Immorto mice) were used for this purpose (Ryzhov et al., *J Pharmacol Exp Ther* 2012). The conditionally immortalized cells isolated from these mice, under non-permissive conditions do not express the T-antigen and behave as primary cells (Ryzhov et al., *J Pharmacol Exp Ther* 2012). These cells were tested for multipotency based on their ability to differentiate into all three major cell types in the heart: cardiomyocytes, fibroblasts and endothelial cells (**Fig 13B and C**).

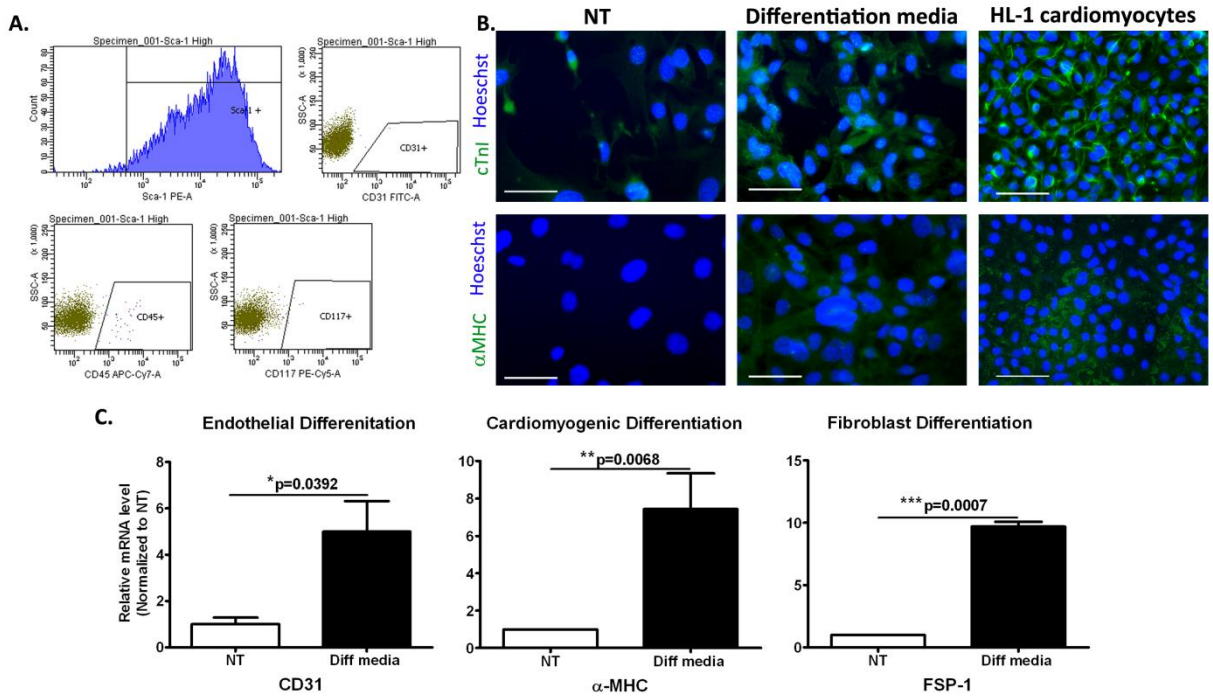

Figure 13: Conditionally immortalized mouse heart-derived Sca1+ cells are negative for CD31, CD45 and c-kit expression, and can upregulate myocyte, endothelial and stromal markers in specific culture conditions.
(A) FACS of $Sca1^{+}CD31^{-}CD45^{-}CD117^{-}$ cardiac progenitor cells. **(B)** Images showing immunostaining for αMHC and cTnI of $Sca1^{+}$ cells (untreated, left panels), cultured in cardiomyocyte differentiation media (middle panels) or HL-1 cardiomyocytes as control (right panel) demonstrated that conditionally immortalized murine $Sca1^{+}$ cells retain capacity to express cardiomyocyte markers in culture. Scale bars equal 50µm. **(C)** Relative gene expression of CD31 (left chart), αMHC (middle chart) and FSP-1 (right chart) in untreated $Sca1^{+}$ cells and following culture in endothelial cell-, cardiomyocyte-, and fibroblast-specific differentiation media, respectively to demonstrate their multilineage potential. *N≥3* replicates per group; **P=0.0392, **P=0.0068,* and ****P0.0007;* Mann-Whitney test.

Wnt inhibition increases proliferation of cardiac-derived Sca1$^+$ progenitor cells

Sca1$^+$ cells stably overexpressing *Wnt3a* were generated in order to assess the proliferative response to Wnt activation and subsequent inhibition with GNF-6231 or C-113. *Wnt3a* overexpression reduced proliferation compared to vector only (LZRS) control, as measured by BrdU incorporation (**Fig 14A and B**). Wnt inhibition by either GNF-6231 (**Fig 14A**) or by C-113 (**Fig 14B**) rescued the anti-proliferative effect of *Wnt3a* overexpression. Similar effects were observed in Sca1$^+$ cells stably expressing *Axin2*, a Wnt pathway negative regulator (data not shown). We were unable to confirm the effects Wnt pathway on proliferation of Sca1$^+$ cells *in-situ* because of technical challenges in identifying *Sca1* or *c-kit* expressing progenitors in the heart by immunostaining. Interestingly, GATA4 transcription factor, which is expressed by early differentiating cardiomyocytes (Schmeckpeper et al., *J Mol Cell Cardiol* 2015), was localized to the nucleus in more cardiomyocytes in the infarct border zone of GNF-6231 treated hearts compared to control (**Fig 15 C-D**). Taken together, these observations suggest that the proliferating cells in the GNF-6231 treated myocardium may include myogenic progenitors.

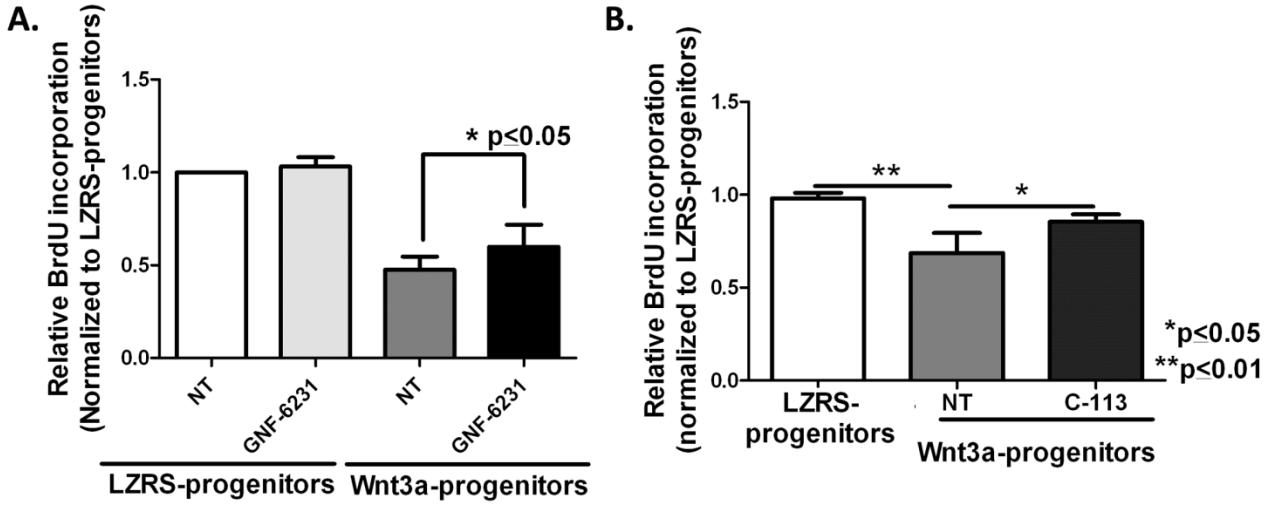

Figure 14: Wnt inhibition increases proliferation of Sca1$^+$progenitor cells isolated from murine hearts.

(A and B) Relative BrdU incorporation by Sca1$^+$ progenitor cells stably expressing LZRS (empty vector) or *Wnt3a*-LZRS revealed that proliferation was reduced by *Wnt3a* overexpression and this effect was reversed by **(A)** GNF6231 treatment and **(B)** C-113 treatment. Data are presented as Mean±SD. (A) *N=5* and (B) *N=3* replicates; *$P \leq 0.05$* and **$P \leq 0.01$*; Kruskal-Wallis test with Dunns correction for multiple comparisons.

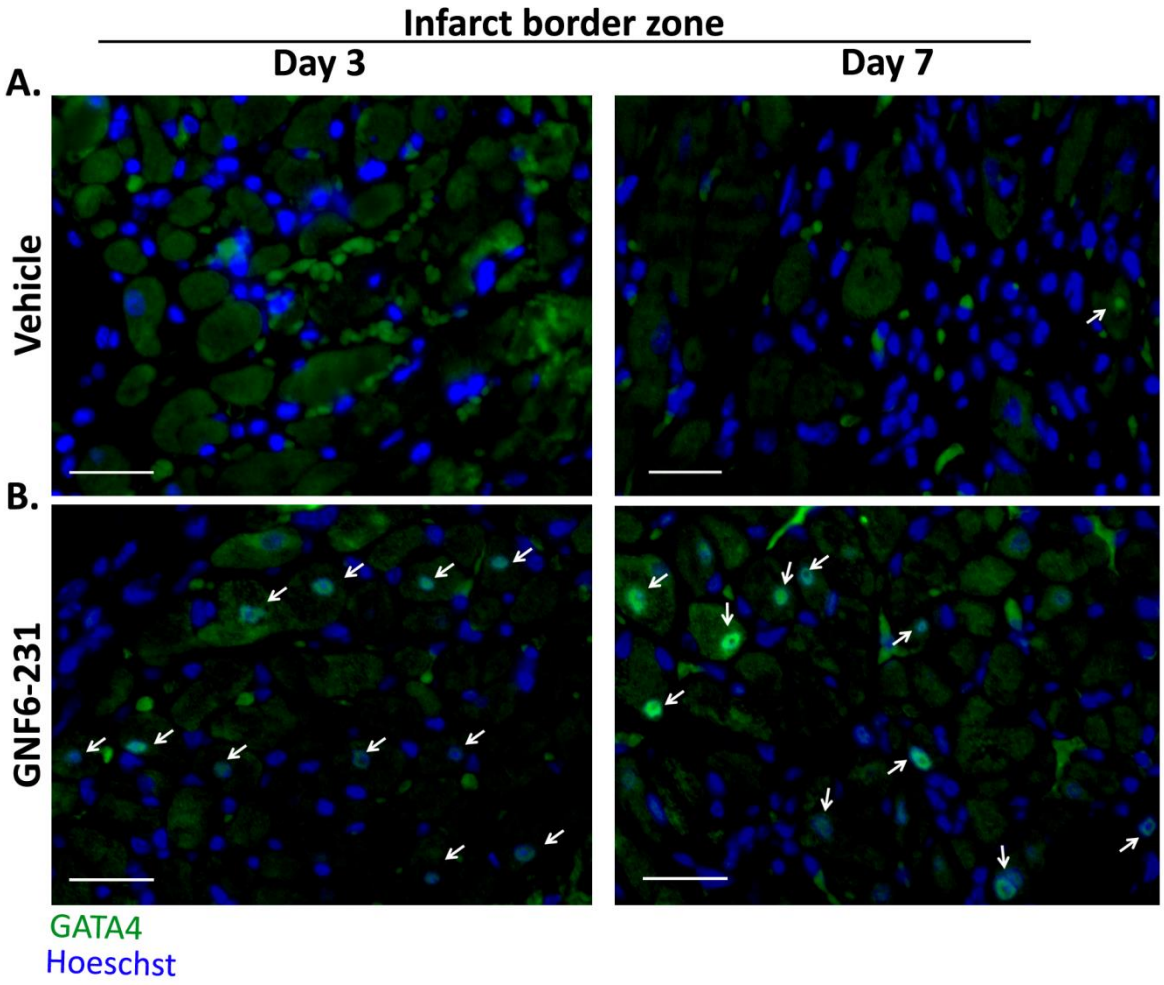

Figure 15: Wnt inhibition stimulates new cardiomyocyte formation as indicated by GATA4^{+} myonuclei.
Representative GATA4 immunostained sections of infarct border zone at day 3 (left panels) and day 7 (right panels) post-MI of **(A)** vehicle-treated hearts and **(B)** GNF-6231 treated hearts. White arrows point to GATA4 stained nuclei. Scale bars equal 50µm.

Wnt inhibition enhances survival of cardiomyocytes

We next asked whether the preservation of myocardial function and smaller infarct size in GNF-6231 treated animals could be accounted for, at least in part, by an effect on myocyte death or survival. Previous studies have shown that Wnt inhibition by sFRP2 improves cardiomyocyte survival in the MI model (Mirotsou et al., *Proc Natl Acad Sci USA* 2007) and in culture, specifically by binding to Wnt3a and blocking its pro-apoptotic signals (Zhang et al., *J Mol Cell Cardiol* 2009). Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) was performed to detect cell death *in situ*. Co-immunostaining for TUNEL and the cardiomyocyte marker cTnI showed that in the infarct border zone, there were significantly lower percent $TUNEL^+$ (apoptotic or necrotic) cardiomyocytes in the GNF-6231 treated hearts compared to vehicle-treated (2.03 fold lower in GNF-6231 treated ventricles; *p=0.022; **Fig 16A-C**). For further verification of the positive effect of Wnt inhibition on cardiomyocyte cell survival in vitro, cell survival and cell death assays were performed on isolated cardiomyocytes. In HL-1 cardiomyocyte cell line, assessment of cell viability by metabolic activity resulting in reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide—MTT— to insoluble formazan (MTT assay), showed that Wnt pathway activation by recombinant WNT3A treatment significantly reduced survival of these cells (by 16.4% over control; *p≤0.05; **Fig 16D**), which could be rescued by treatment with Wnt inhibitor, C-113 (18.78% increase over WNT3A treatment; *p≤0.05; **Fig 16D**). Since BrdU incorporation assay had indicated no effect on proliferation of these cells by Wnt pathway modulation (**Fig 9H**), we concluded that the effect on cell viability was exclusively due to reduction in cell death.

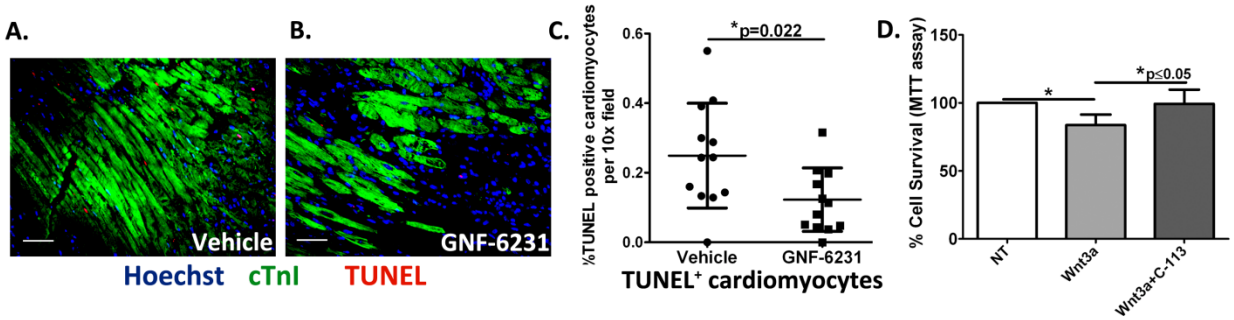

Figure 16: Wnt inhibition reduces cardiomyocyte cell death *in vivo* and enhances their survival *in vitro*

(A and B) Representative sections co-stained with cTnI and TUNEL of infarct border zone of **(A)** vehicle-treated hearts, and **(B)** GNF-6231 treated hearts. **(C)** Quantification of percent TUNEL positive cardiomyocytes revealed significantly fewer apoptotic cardiomyocytes in GNF-6231 treated hearts. *N=5* mice per group, **P=0.022,* Unpaired t-test. **(D)** Percent cell survival of HL-1 rat cardiomyocytes as measured by metabolic uptake (MTT) assay showed significant reduction in survival with recombinant Wnt3a treatment, which was rescued by addition of C-113. Bars represent mean±SD; *N=3* replicates; **P≤0.05;* Kruskal-Wallis test with Dunns correction for multiple comparisons.

To further confirm these findings, we used human iPSC-derived cardiomyocytes (iCell® Cardiomyocytes[2]; Cellular Dynamics International, Madison, WI). This highly pure population of cardiomyocytes expresses cardiomyocyte markers, cTnI (**Fig 17A**), and Sarcomeric Alpha Actinin (Ref (Zhang et al., *Circ Res* 2009) and manufacturer's datasheet), and beats in culture. TUNEL assay with these cells showed that in the presence of oxidative stress induced by 250μM H_2O_2, treatment with recombinant WNT3A significantly increased cell death (percent $TUNEL^+$ cells 1.8 fold higher over control; $^{**}p \leq 0.01$), whereas Wnt inhibitor treatment reduced cell death (by 3.6 fold over WNT3A treatment; $^{***}p \leq 0.001$; **Fig 17B-C**), further suggesting that enhanced myocyte survival and reduced myocyte death contributed to the observed pro-reparative effect of post-injury Wnt inhibition.

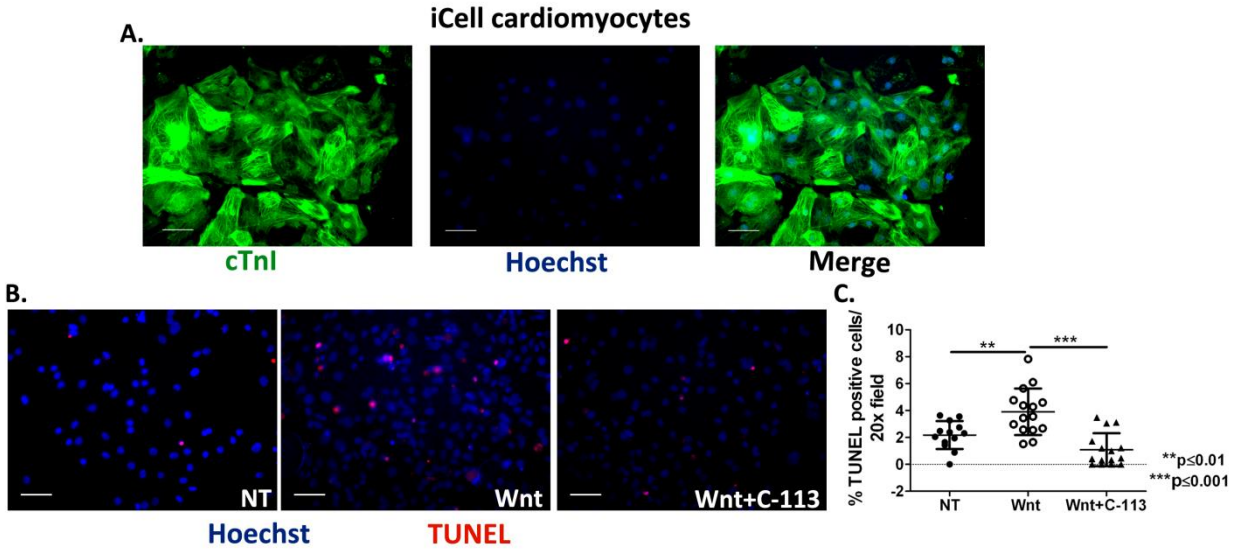

Figure 17: Wnt pathway activation increases cell death of human iPSC-derived cardiomyocytes, and Wnt inhibition reverses the effect.
(A) Representative images of human iPSC-derived iCell[2] Cardiomyocytes immunostained with the cardiomyocyte marker, cTnI (green). Sections were counterstained with Hoechst (blue). Scale bars equal 50µm. **(B)** Representative images of human iPSC-derived iCell cardiomyocytes treated with recombinant WNT3a or WNT3a and C-113 in presence of 250µM H2O2 showing that under stress, treatment with recombinant WNT3A increased cell death, which was rescued by Wnt inhibition with C-113. **(C)** Quantification of %TUNEL positive iCell cardiomyocytes per 20x field. *N≥12* per group; ***P≤0.01* and ****P≤0.001*; One-Way ANOVA with Bonferroni correction for multiple comparisons. Scale bars in A-B and E represent 50µm.

Wnt inhibition reduces collagen synthesis by cardiac myofibroblasts

As discussed in the preceding sections, GNF-6231 treatment reduced myofibroblast proliferation compared to vehicle-treatment (**Fig 10B**). Since myofibroblasts are the major matrix synthesizing cells responsible for scar formation, we tested whether Wnt inhibition also affected type I collagen synthesis by cardiac myofibroblasts. Primary αSMA^{+} myofibroblasts (**Fig 18A**) were generated from adult mouse hearts as previously described (Kong et al., *Am J Physiol Heart Circ Physiol* 2013). Treatment of these cells with Wnt inhibitor for 48 hours reduced Collagen1α1 gene expression (by 39.16% over control; *p=0.0251) as determined by qRT-PCR (**Fig 18B**). These data suggest that Wnt inhibition reduced pro-fibrotic effects in the infarcted heart by modulating both the proliferation and the matrix synthesis activity of cardiac myofibroblasts.

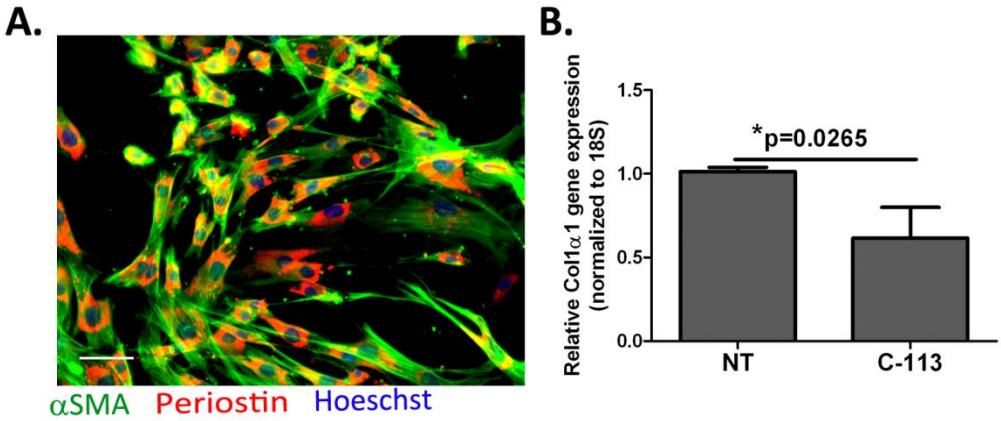

Figure 18: Wnt inhibition reduces collagen synthesis activity of αSMA positive myofibroblasts *in vitro*.
(A) Primary myofibroblasts in culture confirmed by αSMA (green) and Periostin (red) staining; scale bar equals 50µm. **(B)** Relative *Col1α1* gene expression in primary cardiac myofibroblasts with and without Wnt inhibitor treatment revealed significant reduction in *Col1α1* gene expression in response to Wnt inhibitor treatment. Bars represent mean±SD. *N=4*; **P=0.0265*; Mann-Whitney test.

Discussion

Several studies have reported that canonical Wnt signaling is temporally increased after MI. In this study we showed that temporary systemic inhibition of the Wnt/β-catenin for several days post-MI prevented this post-MI tissue Wnt activation. Furthermore, therapeutic Wnt inhibition following infarct alleviated adverse cardiac remodeling, improved ventricular function, and reduced infarct size. Temporary Wnt inhibition post-infarct increased cell proliferation and cardiomyocyte survival, and reduced myofibroblast proliferation and their matrix synthesis activity in the heart.

Early after infarct, Wnt inhibition caused an increase in proliferation of interstitial cells, particularly in the distal myocardium. The cardiac cells that showed a proliferative response to GNF-6231 treatment mostly excluded cardiomyocytes, endothelial cells and various stromal populations, including αSMA^+ myofibroblasts, and $Vimentin^+/Periostin^+/FSP1^+$ fibroblasts. Interestingly, we discovered that Wnt pathway activation downregulated proliferation of isolated $Sca1^+CD31^-CD45^-CD117^-$ cardiac progenitor cells, which are one of the tissue resident stem cells reported to reside in the interstitial niche. Wnt inhibition by treatment with two mechanistically distinct Wnt inhibitors, or via overexpression of Axin2 reversed the anti-proliferative effect of Wnt activation in these cells. This is in agreement with published reports of anti-proliferative effects of recombinant WNT3A on side population progenitors, *in vitro* and *in vivo* in the infarcted heart (Oikonomopoulos et al., *Circ Res* 2011). Our own work, and work by others in cardiac injury and other injury models (Brack et al., *Science* 2007; Alfaro et al., *Proc Natl Acad Sci USA* 2008; Saraswati et al., *PLoS One* 2010) report the Wnt pathway as a negative regulator of cell proliferation, particularly of stem/progenitor cells. Although these data may appear incongruous with reports of Wnt being necessary for stem cell homeostasis and self-

renewal in other adult organs (Huelsken et al., *Cell* 2001; Fevr et al., *Mol Cell Biol* 2007; Plaks et al., *Cell Rep* 2013), and during development (Kelly et al., *Cell Stem Cell* 2011; Merrill, *Cold Spring Harb Perspect Biol* 2012), our data support a model in which Wnt exerts multi-phasic, context dependent effects on stem cells. For example, during heart development (Hurlstone et al., *Nature* 2003), just as in skeletal muscle regeneration (Brack et al., *Cell Stem Cell* 2008), a temporal regulation of Wnt contributes to a balance between stem cell proliferation and differentiation. Also, in the now well-optimized and commercially used methods of cardiomyocyte differentiation from iPSCs, a biphasic regulation of Wnt activity is sought in order to achieve optimal cardiomyocyte generation (Lian et al., *Proc Natl Acad Sci U S A* 2012; Minami et al., *Cell Rep* 2012). The observed expansion of $GATA4^{+}$ (i.e. newly differentiating) cardiomyocytes in the infarct border zone provided *in vivo* support of the role of Wnt inhibition in enhancing neomyogenesis.

Our data also suggest an anti-fibrotic effect of Wnt inhibition following MI. We found that Wnt inhibition reduced the number of proliferating myofibroblasts *in vivo*, and also downregulated collagen I expression in cultured cardiac myofibroblasts. These results are not surprising against the backdrop of numerous studies reporting that Wnt activation is a driver of fibrosis in heart (Bergmann, *Circ Res* 2010) and many other forms of tissue injury (Brack et al., *Science* 2007; He et al., *J Am Soc Nephrol* 2009; Kim et al., *Tohoku J Exp Med* 2011; Akhmetshina et al., *Nat Commun* 2012).

In addition to effects on cell proliferation and myofibroblast activity, Wnt inhibition also reduced cardiomyocyte cell death, which is the major cause of the subsequent progression to heart failure (Ruiz-Meana et al., *Rev Esp Cardiol* 2009). This observation aligns with published reports of pro-apoptotic effects of Wnt (Oikonomopoulos et al., *Circ Res* 2011), and pro-survival

effects of Wnt inhibition (Mirotsou et al., *Proc Natl Acad Sci USA* 2007; Zhang et al., *J Mol Cell Cardiol* 2009) on cardiomyocytes.

In this study, we focused exclusively on the effects of porcupine inhibition through the β-catenin-dependent arm of the Wnt pathway, based on the significant body of literature suggesting a critical and complicated role—both maladaptive (Baurand et al., *Circ Res* 2007; Zelarayan et al., *Proc Natl Acad Sci USA* 2008; Oikonomopoulos et al., *Circ Res* 2011), and in some cases pro-reparative (Hahn et al., *J Biol Chem* 2006; Duan et al., *EMBO J* 2012; Paik et al., *Circ Res* 2015)—for this signaling cascade in infarct pathology. However, there are reports implicating both the Ca^{2+}/CAM Kinase and the JNK arms of the non-canonical Wnt signaling pathways (Wang et al., *J Biol Chem* 1998; Dawson et al., *J Physiol* 2013; Schmeckpeper et al., *J Mol Cell Cardiol* 2015) in infarct pathology and repair. Hence, future studies investigating the effect of inhibition of Wnt ligand secretion on the non-canonical arms of the Wnt signaling pathways may provide a more complete picture of the mechanisms mediating cardiac recovery by porcupine inhibitor treatment.

That said, our data demonstrating the potential of short term Wnt inhibition in counteracting the key drivers of post-infarct LV dysfunction and eventual failure—cardiomyocyte death and fibrosis—are clinically significant since the current standard-of-care for myocardial infarct focus mainly on thrombolytic and palliative interventions, and do not address the ongoing disease progression driven by the initial infarct. Moreover, in the context of complicated (Hahn et al., *J Biol Chem* 2006; Alfaro et al., *Proc Natl Acad Sci USA* 2008; He et al., *Proc Natl Acad Sci U S A* 2010; Aisagbonhi et al., *Dis Model Mech* 2011; Paik et al., *Circ Res* 2015) and multifaceted roles of the Wnt pathway in infarct repair in the existing literature, our data may speak to the potential of temporally regulated scalable pharmacologic Wnt

inhibition in reconciling the discordant observations based on genetic models (Baurand et al., *Circ Res* 2007; Zelarayan et al., *Proc Natl Acad Sci USA* 2008; Duan et al., *EMBO J* 2012) of Wnt modulation. With additional details on the mechanism-of-action and safety data emerging with continuing studies, and ongoing clinical trials (Novartis, *Clinicaltrials.gov* 2014; Kabiri et al., *Blood* 2015), GNF-6231 and the new class of porcupine inhibitors hold significant potential as effective therapeutics for cardiac regeneration.

Materials and Methods

Antibodies

The following antibodies were used: β-catenin (1:200; BD Pharmingen, 610153); β-galactosidase (1:100; AbCam, Ab616); Alpha Smooth Muscle Actin (α-SMA) (1:1000; Sigma A2547); Ki67 (1:400; AbCam, Ab15580); phospho-Histone-H3 (1:200; Millipore, 06-570); cTnI (1:1000; AbCam, Ab6556); Alpha Sarcomeric Actin (1:200; Sigma, A2172); GATA4 (1:200; Santa Cruz, SC25310); Periostin (1:100; Santa Cruz, SC67233); PCNA (1:100; Santa Cruz Biotech, SC-56); FSP1 (1:100; Millipore, 07-2274); Vimentin (1:200; Sigma, V2258); vWF (1:200; Takara, M116) .

Wnt modulators

The small molecule Porcupine inhibitor GNF6231 was a generous gift from the Genomic Institute of the Novartis Research Foundation. Small molecule Wnt inhibitors (CK1α activator) VU-WS113 (C-113) was a generous gifts from Dr. Ethan Lee, Department of Cell and Developmental Biology, Vanderbilt University (Thorne et al., *Nat Chem Biol* 2010). XAV-939 (Huang et al., *Nature* 2009), a small molecule stabilizer of Axin2 was purchased from Selleck Chemicals (S1180; Houston, TX). Recombinant mouse Wnt3a was purchased from AbCam (ab81484) or from the Vanderbilt Antibody and Protein Resource (VAPR).

Cell lines

HL1 cell line, a kind gift from Dr. William C. Claycomb (Louisiana State University Medical Center, New Orleans, LA), were cultured on gelatin/fibronectin (25μg fibronectin in 2 ml of 0.02% gelatin in water)-coated plates (fibronectin and gelatin from Sigma-Aldrich). The HL1 cell line was maintained at 37°C in Claycomb medium (SAFC Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (SAFC Biosciences), 100μM norepinephrine

(Sigma) in 30mM ascorbic acid (Sigma), 2mM L-Glutamine (Sigma), penicillin, and streptomycin (Life Technologies, Grand Island, NY).

iCell Plus Cardiomyocytes, which are primarily ventricular cardiomyocytes derived from human induced pluripotent stem (iPS) cells, were purchased from Cellular Dynamics International and maintained in 0.1% gelatin coated plastic plates in manufacturer's proprietary maintenance medium.

Primary mouse cardiac fibroblasts were isolated from hearts of C57Bl/6 mice that were at least 12 weeks old following previously described protocol (Kong et al., *Am J Physiol Heart Circ Physiol* 2013). Briefly, heart tissue was minced and placed into Kreba-Henseleit (Sigma; K3753a) buffer with 2.9mM $CaCl_2$ and 24mM $NaHCO_3$ containing a cocktail of 0.25mg/mL Liberase Blendzyme 3 (Roche Applied Science), 20U/mL DNase I (Sigma Aldrich), 10mmol/L HEPES (Invitrogen) and 0.1% sodium azide in HBSS, and shaken at 37ºC for 20 min. Cells collected after digestion were passed through 40µm nylon mesh and centrifuged (15min, 200g, 4ºC). Finally, cells were reconstituted with DMEM-F12 medium containing 10% FBS and 1% Penicillin/Streptomycin and seeded onto plastic plates (Corning) for separation of fibroblasts by selective adhesion for 4 hours at 37º.

Proliferation assay

Cell proliferation was assessed by 5-bromo-2'-deoxyuridine (BrdU) cell proliferation assay (Calbiochem, Gibbstown, NJ). Briefly, CPCs, HL-1 cells, or primary fibroblasts were seeded on 96-well plates (gelatin/fibronectin-coated for HL-1 cells), with recombinant Wnt3a where indicated. Following attachment onto plates, Wnt modulators were added and cultured for 24 hours. BrdU for 16 hours, and BrdU incorporation was assessed by enzyme-linked immunosorbent assay (ELISA) and read at a dual wavelength of 450/595 nm using the

SOFTMax Version 2.35 software (Molecular Devices, LLC, Sunnyvale, CA) following manufacturer's recommendations.

Cell viability assay

HL-1 cardiomyocytes or CPCs were serum starved overnight in 2% FBS, and seeded onto (gelatin/fibronectin-coated for HL-1) 96-well plate at a density of 10^4 cells/well. Following attachment of cells over 24 hours, Wnt modulators were added and the cells incubated for 48 hours. Cell viability was assessed by incubating with 0.5 mg/mL of 3-[4,5-dimethylthiazol-2-yl] 2,5,-diphenyltetrazolium bromide (MTT) (Sigma) in PBS for 4 hours at 37°C, and measuring MTT reduction into formazan by viable cells. Formazan crystals were dissolved in 75µL/well DMSO 10 minutes at 37 °C. Photometric measurement was carried out at 540 nm. Percentage viability was calculated by as follows: % Cell survival =($OD_{Control}$-$OD_{Sample)}$ x 100%/ $OD_{Control}$. Non-treatment cells were used as control. Results represent three independent experiments each performed in triplicate.

Animals

All procedures were carried out in accordance with Vanderbilt Institutional Animal Care and Use Committee. C57Bl/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained by PPY. TOPGAL (Aisagbonhi et al., *Dis Model Mech* 2011) mice were a generous gift from Dr. Antonis Hatzopoulos (Department of Cell and Developmental Biology, Vanderbilt University).

Surgical LAD ligation (myocardial infarction) model, drug treatment, Echocardiography and infarct size calculations

C57Bl6J mice (at least 3 months of age) were anesthetized under sodium pentothal (50 mg/kg) and endotracheal intubation was performed under direct laryngoscopy. Mice were ventilated with a small animal respirator (tidal volume = 1.0 ml, rate = 110 breaths/min). With

the use of a surgical microscope, a left thoracotomy was performed. The fourth intercostal space was entered using scissors and blunt dissection. A 7-0 silk suture was placed through the myocardium into anterolateral LV wall and the left anterior descending artery was ligated. The heart was monitored with continuous EKG throughout the procedure to ensure successful infarction. The chest was closed in layers with 6-0 silk (6-0 nylon to close the skin) and the animal was gradually weaned from the respirator to avoid complicating pneumothorax.

Starting from within 6 hours after surgery through day 6 post-infarct, mice were treated every 24 hours with intravenous (tail-vein) injection of 5mg/kg (100uL volume) GNF-6231 or vehicle (3% D-α-tocopheryl polyethylene glycol succinate or Vitamin E in 20%Polyethylene glycol).

For cardiac recovery study, cardiac dimensions were obtained from 2-D guided M-mode images (100 frames/sec) and were read blinded using short axis and a parasternal long-axis views All measurements were done on unsedated mice at day 7 and day 30 post MI. Measurements were averaged over 3 consecutive beats from the LV posterior wall (LVPW) the interventricular septum (IVS) and LV internal diameter (LVID). After day 30 Echocardiography, hearts were excised, immersion-fixed in and paraffin-embedded to obtain serial sections in order to measure the infarct size (% area of tissue stained blue for collagen in H&E stained sections).

Separately, for longitudinal studies, hearts were excised at 3, 7, and 15 days post MI, and processed to obtain paraffin sections for immunostaining studies.

Histology and Morphometry

Hearts were fixed in 10% buffered formalin for 24 hours, embedded in paraffin and sectioned into 5 microns-thick transverse sections. H&E and Masson's Trichrome staining was

performed by the Vanderbilt Translational Pathology Shared Resource. Olympus DP71 microscope camera (Olympus America, Center Valley, PA) was used for imaging H&E and Masson's Trichrome stained sections.

For immunofluorescence staining, slides were deparaffinized and hydrated through xylene and ethanol steps. Heat-mediated antigen retrieval was performed by boiling in citrate buffer (pH 6). Cells seeded onto coverslips were fixed for 1 hour at room temperature in 1% paraformaldehyde, permeabilized with 0.1% Triton-X in 0.1% sodium citrate for 2 min on ice, and washed several times with PBS. Following blocking with 10% goat serum in 1% BSA solution for 1 hour at room temperature, sections were incubated with primary antibody at 4^{o}C overnight, and Alexa Fluor 488 or Cy3 conjugated secondary antibodies at room temperature for 1 hour. The slides were then counterstained with Hoechst 33342 (H21492 Invitrogen, Carlsbad, CA) and mounted with Slowfade Gold (S36936 Life Technologies, Grand Island, NY). For TUNEL staining, a 1:10 mix of enzyme: label diluted 5 fold with TUNEL dilution buffer (InSitu Cell Death Detection Kit TMR Red, Roche 12 156 792 910) was added to samples along with the secondary antibody and incubated for 60 minutes at 37^{o}C. Samples were then counter stained with Hoechst and mounted as usual. Images were taken at 10x, 20x or 40x magnification using Axio Imager2 microscope (Carl Zeiss, Thornwood, NY) and CoolSNAP HQ CCD camera (Photometrics, AZ), and quantified using ImageJ. For confocal microscopy, LSM510 (Zeiss) microscope was used to capture 1 µm optical slices. All images are presented with scale bars that equal 50µm.

PK/PD study

Single dose PK/PD profile of GNF-6231 was investigated in C57Bl/6 mice following a 5 mg/kg intravenous bolus injection. GNF-6231 was formulated at 1.5 mg/mL using a solution

containing 20% PEG300 and 3% vitamin E. At specific time points following intravenous administration, plasma samples were taken and plasma concentrations of GNF-6231 were quantified by Liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis. Briefly, aliquots of plasma samples were added internal standard and acetonitrile/methanol (3/1), samples were vortexed and centrifuged at 4,000 rpm for 5 minutes at 4^{o}C to precipitate the plasma proteins. Supernatant was transferred to a clean 96-well plate, and diluted with distilled water. The extracted samples were injected (10 µL) onto a Zorbax SB-C8 analytical column (2.1 x 30 mm, 3.5 µm, Agilent Technologies Inc., Palo Alto, CA, USA). Mobile phases consisted of 0.05% formic acid in water (solvent A) and 0.05% formic acid in acetonitrile (solvent B), and a gradient elution method at a flow rate of 700 µL/min was used for compound elution and separation. Mass spectral analyses were carried out using atmospheric pressure chemical ionization (APCI) in the positive ion mode, with multiple reaction monitoring (MRM) of GNF-6231 (449.2>221.0). The lower limit of quantitation (LLOQ) in plasma was 1.0 ng/mL. Pharmacokinetic parameters were calculated by non-compartmental regression analysis using an in house fitting program.

PD study was performed as described previously (Liu et al., *Proc Natl Acad Sci U S A* 2013). Briefly, total RNA was isolated using the Qiagen RNeasy kit; TaqMan analyses were performed using Axin2 and Gapdh probes (Applied Biosystems) according to the manufacturer's instructions. mRNA expression levels for the target gene, Axin2, was normalized to Gapdh mRNA levels and data were analyzed using SDS 2.0 software (Applied Biosystems) to calculate relative RNA quantities.

RNA isolation and qRT-PCR

RNA was isolated from cells were using Trizol Reagent (Invitrogen, 15596026) following manufacturer's protocol. First strand DNA synthesis was performed with 1 μg RNA using iScript cDNA synthesis kit (Bio-Rad 170-8890). Quantitative Real-time PCR was performed in triplicate for each sample with iCycler (BioRad) and fluorescence detection (SsoFast EvaGreen; 172-5200; BioRad). Each reaction was normalized against 18S. Primer sequences are as shown in **Table 3**.

Table 3. List of q-RT-PCR primers I

Gene	Forward	Reverse
Axin2	GGACAGTAGCGTAGATGGAG	CGGAAAATGAGGTAGAGACA
Col1α1	GCCAGATGGGTCCCCGAGGT	GGGGGTCCAGCAGCACCAAC
CD31	GTGAAGGTGCATGGCGTATC	CACAAAGTTCTCGTTGGAGGT
αMHC	CCACTGTGGTGCCTCGTTC	GCGTCCGTCATTCTGTCACTC
FSP1	CGGTTACCATGGCAAGACCC	TGTGCGAAGCCAGAGTAAG
18S	CGCCGCTAGAGGTGAAATTCT	GAACCTCCGACTTTCGTTCCT

Statistical analysis

The statistical significance between experimental and control groups were determined by One-way ANOVA with Bonferroni correction for multiple comparisons when multiple groups were compared. The D'Augustino and Pearson omnibus or the Shapiro-Wilk tests were used to determine whether the data sets were normally distributed. For data sets that were not normally distributed or had $N<7$, the Kruskal-Wallis H-test was used instead of One-way ANOVA. For comparison between two groups of data, unpaired t-test was used for normally distributed data-sets, and Mann-Whitney test was used for data that were not normally distributed. GraphPad

Prism (San Diego, CA) software was used for all statistical analyses. $p<0.05$ was considered statistically significant in two-tailed hypothesis tests.

CHAPTER 3

INHIBITION OF WNT/BETA-CATENIN PATHWAY PROMOTES REGENERATIVE REPAIR OF CUTANEOUS AND CARTILAGE INJURY

Introduction

Wound healing in mammals is a fibrotic process. The mechanisms driving fibrotic (as opposed to regenerative) repair are poorly understood. Herein we report that therapeutic Wnt inhibition with topical application of small molecule Wnt inhibitors can reduce fibrosis and promote regenerative cutaneous wound repair.

In the naturally stented model of ear punch injury, we found that Wnt/β-catenin pathway is activated most notably in the dermis of the wound bed early (day 2) after injury and subsides to baseline levels by day 10. Topical application of either of two mechanistically distinct small molecule Wnt pathway inhibitors (a tankyrase inhibitor, XAV-939, and the FDA-approved casein kinase activator, pyrvinium) in C57Bl/6J mice resulted in significantly increased rates of wound closure (72.3±14.7% with XAV-939; and 52.1±20.9% with pyrvinium) compared to contralateral controls (38.1±23.0% and 40.4±16.7% respectively). Histologically, Wnt inhibition reduced fibrosis as measured by α-smooth muscle actin positive myofibroblasts and Col1α1 synthesis. Wnt inhibition also restored skin architecture including adnexal structures in ear wounds, and dermo-epidermal junction with rete pegs in excisional wounds. Additionally, in ear punch injury Wnt inhibitor treatment enabled regeneration of auricular cartilage.

Our study shows that pharmacologic Wnt inhibition holds therapeutic utility for regenerative repair of cutaneous wounds.

Epidemiology and pathology of cutaneous wounds

Acute wounds related to trauma, surgery or burns are a major medical problem, driving approximately 11 million emergency room visits every year in the United States (Centers for Disease Control and Prevention, 2011). Repair of these wounds leads to persistent scarring causing long term pain, discomfort or disability. Since mammalian skin does not possess an ability to regenerate following cutaneous injury, wounds often heal with a residual fibrotic scar devoid of adnexal structures (Atala et al., *MRS Bull* 2010). Therapeutic interventions to effectively direct wound healing towards regenerative rather than the default fibrotic mode are lacking.

Regenerative repair of cutaneous wounds has been observed in some "healer" strains or species of mice such as MRL/MpJ (Clark et al., *Clin Immunol Immunopathol* 1998; Gourevitch et al., *Dev Dyn* 2003; Seifert et al., *Nature* 2012) or the African spiny mice, *Acomys (Seifert et al., Nature 2012)* . There is poor understanding of the mechanisms driving this enhanced regeneration, although certain signals such as p21 signaling (Bedelbaeva et al., *Proc Natl Acad Sci U S A* 2010) or processes such as blastema formation (Bedelbaeva et al., *Proc Natl Acad Sci U S A* 2010; Seifert et al., *Nature* 2012) or inflammation (Gourevitch et al., *Adv Wound Care (New Rochelle)* 2014), have been attributed to enhanced regeneration.

Wnt/β-catenin pathway in cutaneous wounds

Our goal in this study was to identify signaling pathways that are mediators of regeneration, and that can be modulated therapeutically to achieve regenerative repair with minimal scarring. Based on previous studies from our group (Alfaro et al., *Proc Natl Acad Sci USA* 2008; Saraswati et al., *PLoS One* 2010) and others (Brack et al., *Dev Biol* 2009; He et al., *J*

Am Soc Nephrol 2009; He et al., *Proc Natl Acad Sci U S A* 2010; Kim et al., *Tohoku J Exp Med* 2011) of the role of Wnt/β-catenin pathway in multiple adult injury models, we focused our attention on the Wnt signaling pathway. We have shown that that the mesenchymal stem cells derived from the super healer MRL/MpJ mice have elevated expression of the secreted Wnt inhibitor sFRP2 and that overexpression of sFRP2 in MSCs derived from C57Bl6 mice enhances their regenerative potential in cell therapy for cutaneous and cardiac injury (Alfaro et al., *Proc Natl Acad Sci USA* 2008). Subsequent studies using small molecule Wnt inhibitor recapitulated these findings in both injury models (Saraswati et al., *PLoS One* 2010; Saraswati et al., *Wound Repair Regen* 2012). There is extensive literature suggesting a role for the Wnt/β-catenin pathway in promoting fibrosis in multiple injury models (Cheon et al., *Proc Natl Acad Sci U S A* 2002; He et al., *J Am Soc Nephrol* 2009; Kim et al., *Tohoku J Exp Med* 2011) including cutaneous injury (Russell et al., *J Invest Dermatol* 2010; Akhmetshina et al., *Nat Commun* 2012). Mutations in humans resulting in activation of the Wnt/β-catenin pathway causes fibromatoses that arise from over-proliferation of fibroblasts (Alman et al., *Am J Pathol* 1997; Tejpar et al., *Oncogene* 1999).

However, in cutaneous injury, Wnt pathway activity is linked with regeneration (Whyte et al., *PLoS One* 2013), particularly regeneration of hair follicles (Lowry et al., *Genes Dev* 2005; Ito et al., *Nature* 2007; Gay et al., *Nat Med* 2013). Most of these studies are based on genetic models of Wnt pathway driven by epidermal or hair follicle-specific promoters (Andl et al., *Dev Cell* 2002; Choi et al., *Cell Stem Cell* 2013), and present a folliculocentric story. Since dermal signals are important components of the wound healing response (Werner et al., *Proc Natl Acad Sci U S A* 1992), these genetic models may not provide a complete picture of the effect Wnt signaling in cutaneous wound healing. Indeed, the studies that have employed conditional β-

catenin stabilization spanning both the dermis and the epidermis have reported that Wnt/β-catenin signals promote fibrosis and increase in wound size (Cheon et al., *Proc Natl Acad Sci U S A* 2002; Cheon et al., *FASEB J* 2006). We sought to reconcile these contrasting observations regarding the role of dermal or epidermal Wnt/β-catenin signals in the context of wound therapy using small molecule Wnt antagonists.

Skin injury models used for the study

We utilized two distinct wound models for our study—full-thickness excisional wound on the backs of C57Bl6 mice, and the through-and-through ear punch injury model. The full-thickness injury model, which is a widely used model of cutaneous wound in mice was used for investigating the effect of Wnt inhibitor treatment on regenerative vs. scarred repair. However, this model presents limitations of extensive wound contraction and rapid closure observed in mice, but not recapitulated in human wounds. Hence, we utilized the naturally stented model of ear punch injury to minimize the effect of wound contraction, and additionally, to allow more accurate quantification of wound closure, and investigation of regeneration of complex sub-dermal structures such as cartilage.

Results

Wnt/β-catenin pathway is activated temporally in the injury region after ear punch

Wnt pathway activation is reported in full thickness excisional wounds (Cheon et al., *Proc Natl Acad Sci U S A* 2002). We tested Wnt pathway activity in through-by-through wounds made with a 2mm biopsy punch in the central cartilaginous region of mouse ear pinnae. We used Wnt pathway RT-PCR array (SA Biosciences, Valencia, CA) to test the expression of Wnt/β-catenin pathway related genes in the wound bed **(Fig 19)** after injury, and found that the pathway is activated in the wound tissue between day 2- 5 after injury in C57Bl/6 mice **(Fig 20A)**.

Quantitative real-time PCR validation for Wnt ligand (Wnt3a), Wnt/β-catenin direct target genes (Dkk1, CyclinD1 and cMyc) and secreted Wnt inhibitor (sFRP2) corroborated the PCR array data **(Fig 20B)**. In contrast, the super-healer MRL mice, that are reported to completely close full thickness ear punch wounds (Clark et al., *Clin Immunol Immunopathol* 1998), showed a significant decrease in expression of Wnt pathway agonists and target genes at day 2 after injury compared to uninjured control as indicated by RT-PCR array **(Fig 20A)** and qRT-PCR **(Fig 20B)**. This was in agreement with our previous report that increased expression of the secreted Wnt inhibitor sFRP2 contributes to the enhanced reparative potential of mesenchymal stem cells derived from MRL mice (Alfaro et al., *Proc Natl Acad Sci USA* 2008). To our knowledge, a link between low Wnt pathway activity and regenerative repair in the MRL mice has not been reported (Heydemann, *Front Biol (Beijing)* 2012).

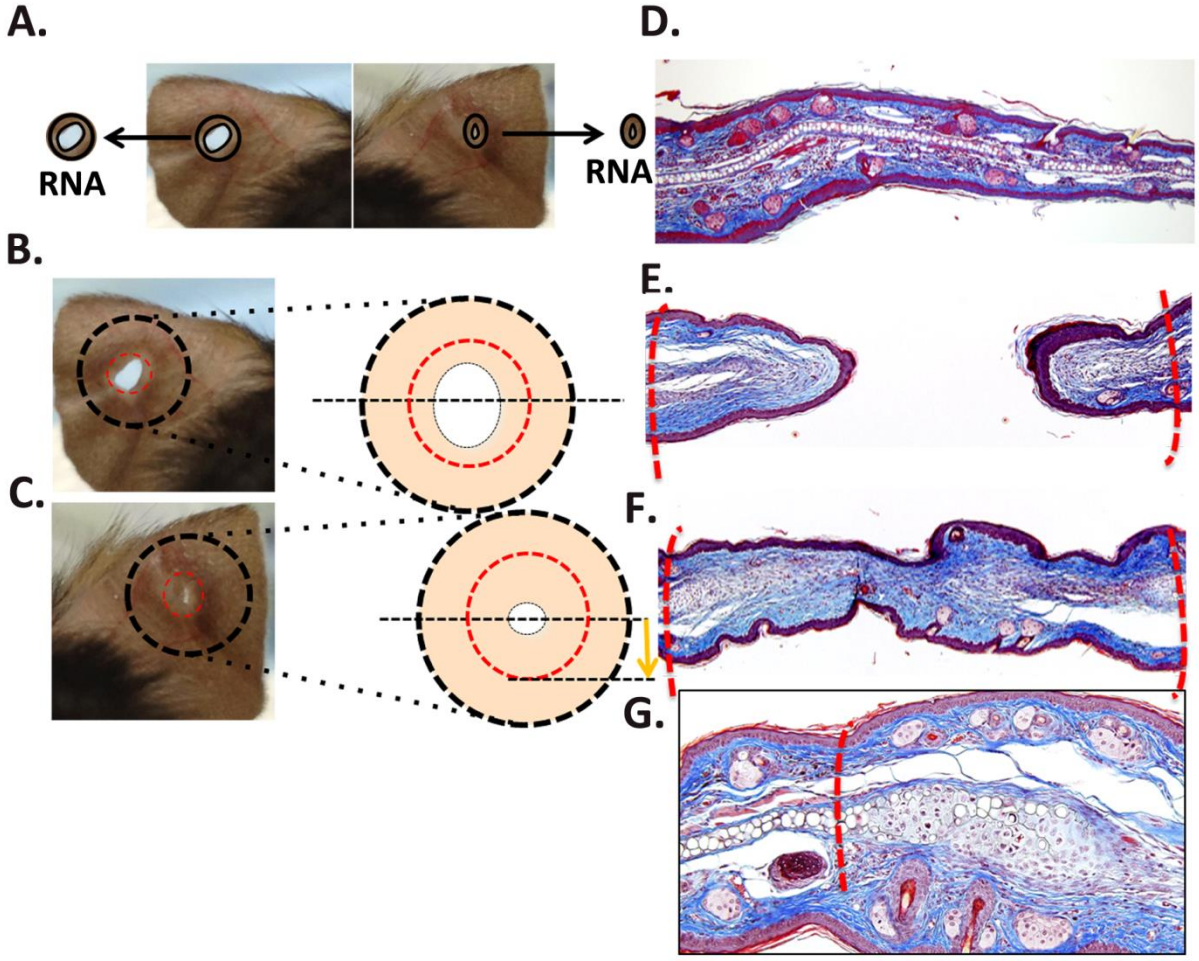

Figure 19: Ear punch wound model schematic.
(A) Representative images of ear punch wounds at 30 days post-injury demarcating the tissue region harvested for RNA isolation. **(B-C)** Schematic showing tissure region sectioned for histology and **(E-F)** histological image of sectioned tissue resulting from tissue with (B) less closure of wound vs. (C) more closure of wound. Red dashed lines in B-C and E-G represent wound margin. Area indicated by the yellow arrow in (C) shows region sectioned for histology within the wound bed, beyond the margin (red dashed line). **(D)** shows uninjured tissue for comparison, and **(G)** shows higher magnification image clearly indicating the leading edge of the existing cartilage demarcated as the wound margin (red dashed line).

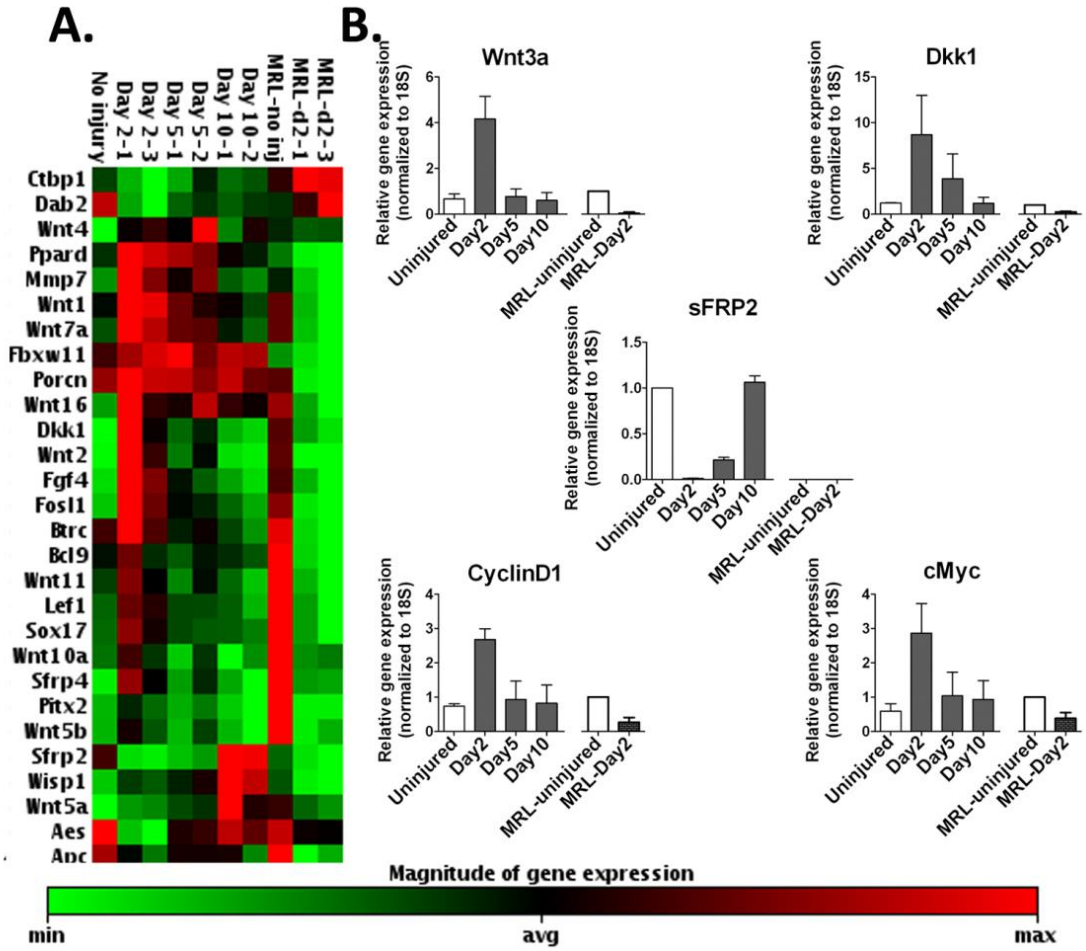

Figure 20: Wnt signaling is activated in cutaneous wounds after injury.
(A) Heat map generated from gene expression array of Wnt pathway-associated genes. **(B)** qRT-PCR validation of change in expression of Wnt agonists (Wnt3a), and Wnt direct transcriptional targets (Dkk1, CyclinD1 and c-Myc), and Wnt inhibitor (sFRP2); MRL-uninjured: *N=1;* all other groups: $N \geq 3$ per group.

Wnt/β-catenin pathway activation in response to wounding occurs primarily in the dermis

To investigate the nature of Wnt activation observed after injury, we performed immunostaining in the wound bed tissue (schematic in **Fig 19B-G**) for β-catenin in C57Bl/6 mice, and for β-galactosidase protein in the Wnt reporter TOPGAL mice. We found detectable Wnt pathway activation compared to uninjured control, specifically in the wound dermis, with both β-catenin **(Fig 21A-B**; nuclear localization highlighted in **Fig 21D** and **Fig 22E and F**) and β-galactosidase immunostaining **(Fig 21F, G** and **K and Fig 22A-B)**. High magnification imaging of β-galactosidase-stained sections indicated that perichondrocytes flanking the cartilage near the wound margin, and elongated fibroblast-like cells were among the Wnt-responsive dermal cells early after injury **(Fig 22D)**. The baseline Wnt activity in the epidermis did not appear to change significantly in response to injury as detectable by immunofluorescence for β-catenin **(Fig 21A-B)** and β-galactosidase **(Fig 22A-B)**. Consistently, daily topical treatment with distinct Wnt inhibitors **(Fig 21E)** caused a reduction in staining intensity of β-catenin **(Fig 21 B-C)** and total β-galactosidase (in TOPGAL mice) **(Fig 21 G-M** and **Fig 22B-C)** proteins most notably in the dermis.

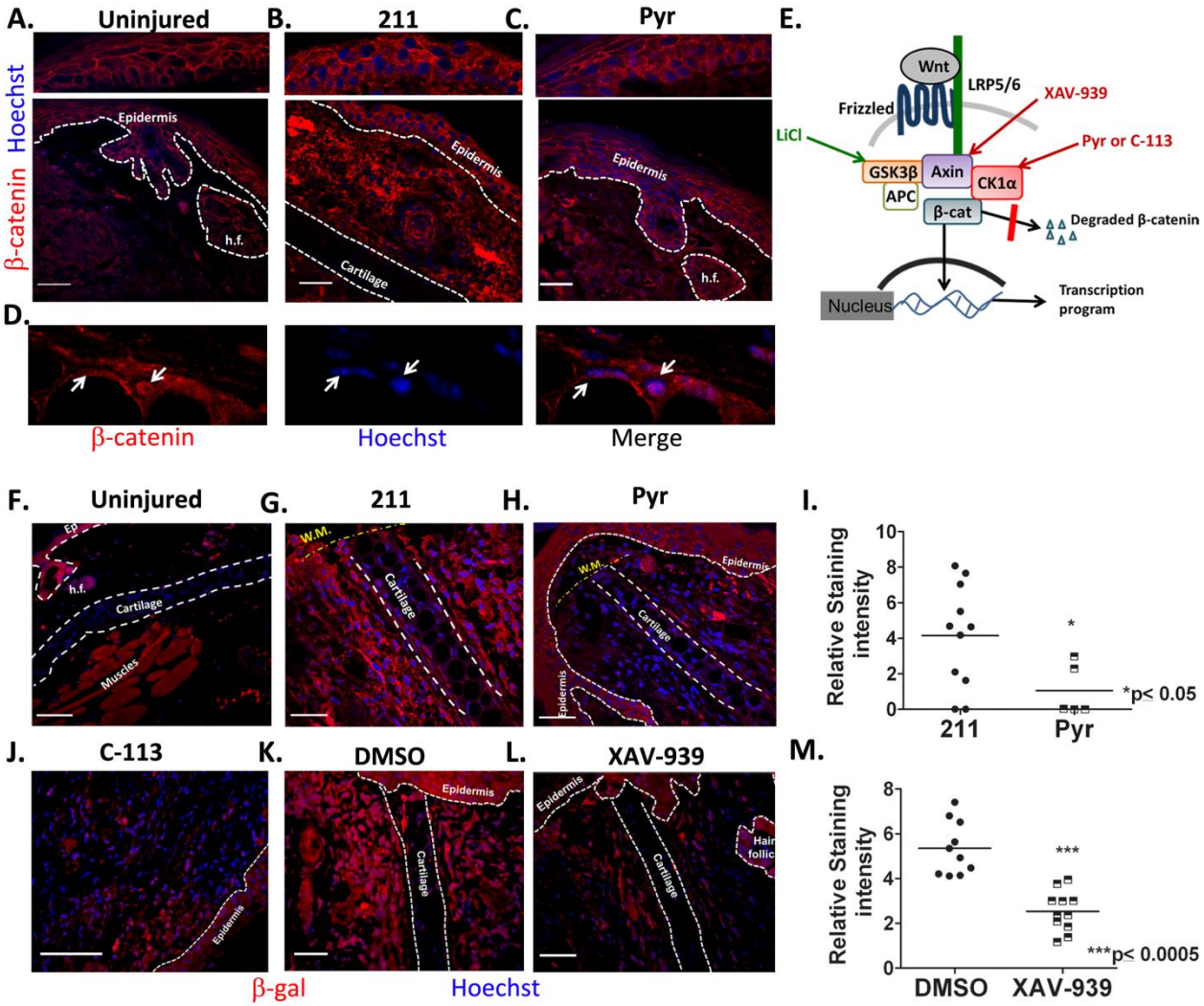

Figure 21: Wnt pathway activation and inhibition occurs specifically in the dermis of wound bed.

(A) Immunostaining for β-catenin (red) on **(A)** uninjured, **(B)** 211-treated, and **(C)** pyrvinium-treated tissue sections showing Wnt activation in the dermis (B) compared to uninjured control (A), and inhibition by pyrvinium treatment (C). Top panels represent relatively undetectable change in epidermal β-catenin levels in response to injury and to treatment. **(D)** High magnification image of injured dermal tissue highlighting nuclear localization of β-catenin (white arrows). **(E)** Schematic of the Wnt/β-catenin pathway showing target molecules activated or stabilized by Wnt agonist (LiCl) or antagonists (Pyr, C-113 and XAV-939) used in the study. **(F-L)** β-galactosidase immunostaining in TOPGAL mice that express LacZ gene under the Wnt-responsive TCF promoter showing dermal (and not epidermal) Wnt activation in **(G)** 211, and **(K)** DMSO-treated sections compared to **(F)** uninjured. Treatment with Wnt inhibitors **(H)** pyrvinium, **(J)** C-113 and **(L)** XAV-939 reduced dermal β-galactosidase staining intensity; nuclei are counterstained blue with Hoechst. **(I** and **M)** Quantification of β-gal protein expression, measured as integrated pixel density in 20x images by ImageJ. *N≥3* mice per group. Scale bars equal 50µm.

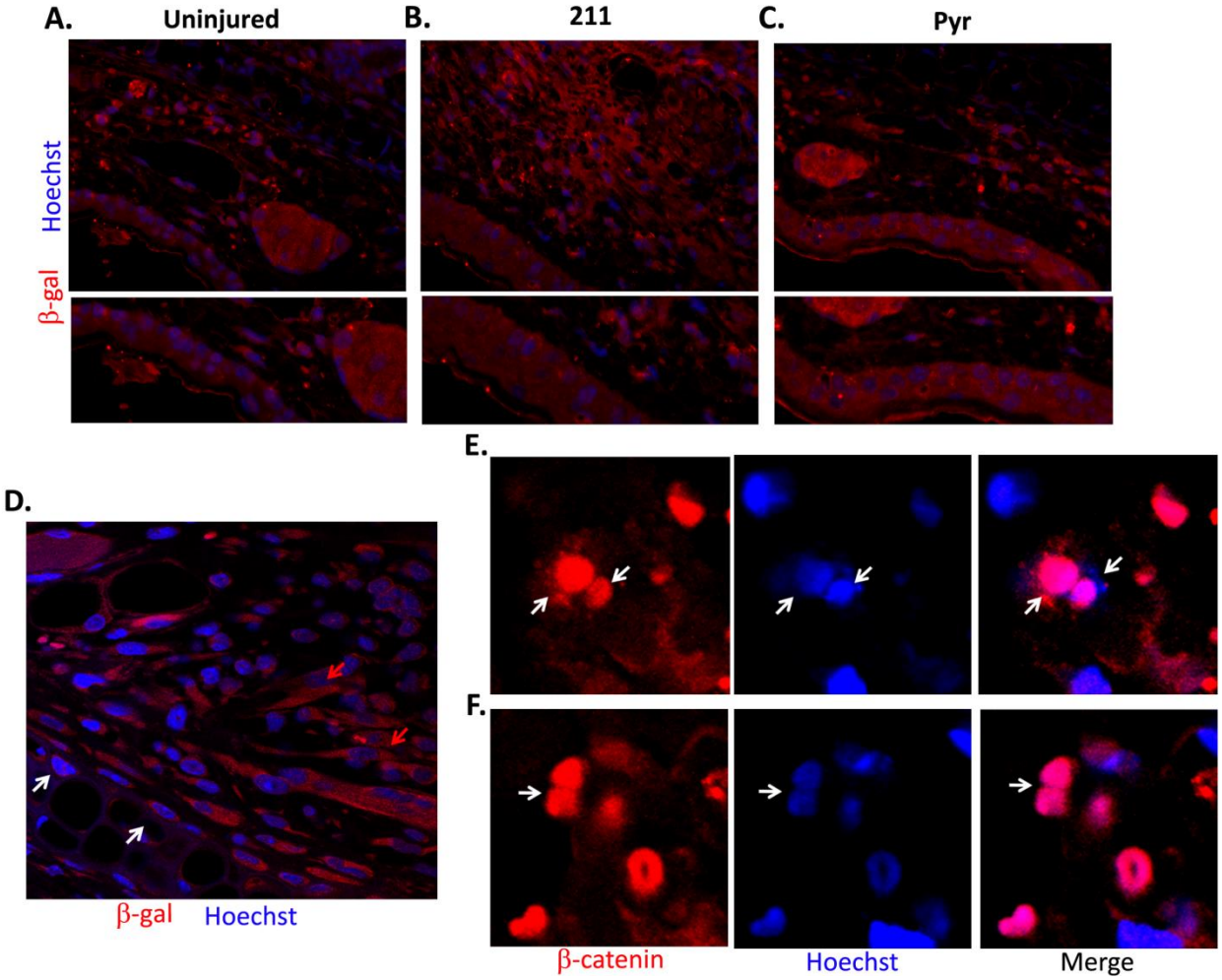

Figure 22: Wnt activation upon injury, as detected by β-galactosidase immunostaining is localized most notably to cells in the dermis, and is inhibited by Pyrvinium treatment.
(A) Immunostaining for β-galactosidase (red) on **(A)** uninjured, **(B)** 211 control-treated, and **(C)** pyrvinium-treated tissue sections from TOPGAL mice showing Wnt activation in the dermis in response to injury (B) compared to uninjured control (A), and inhibition by pyrvinium treatment(C). Bottom panels highlight the relatively undetectable change in epidermal β-galactosidase levels in response to injury and to treatment. **(D)** High-magnification confocal microscopy images highlight β-galactosidase staining in perichondrocytes (indicated by white arrows), and elongated fibroblast-like cells of the dermis (red arrows) in the wound bed. **(E** and **F)** High-magnification images of β-catenin stained sections showing nuclear localization of β-catenin in wound bed tissue at day 2 post-injury. Nuclei are counterstained with Hoechst.

Wnt inhibition, and not off-target effects promotes closure of ear punch wound

In order to investigate the effect of Wnt pathway on wound healing, we treated ear punch wounds topically with small molecule Wnt inhibitors every day after injury for 30 days. To minimize "off-target" effects, we used well-characterized (Huang et al., *Nature* 2009; Thorne et al., *Nat Chem Biol* 2010) small molecules that are structurally distinct, and that inhibit the Wnt/β-catenin pathway through distinct mechanisms-of-action **(Fig 21E)**. XAV-939 is a commercially available tankyrase inhibitor, which stabilizes Axin (Huang et al., *Nature* 2009). Pyrvinium (Pyr) and its structural analog, VU-WS113 (C-113) activate casein kinase1α with different potencies (Thorne et al., *Nat Chem Biol* 2010). Despite having two distinct structures and modes of action (tankyrase inhibition vs. casein kinase1 activation), all three Wnt inhibitors promoted wound closure, reduced fibrosis and enhanced regeneration (as will be discussed in subsequent sections) indicating that their effect was a result of Wnt pathway inhibition and not off-target effects.

At day 30 post-injury, ear punch wounds treated with Wnt inhibitors displayed a significantly greater degree of closure (XAV-939: 72.3±14.7%; Pyr: 52.1±20.9%; and C-113: 63.7±8.1%) than their respective contralateral controls (DMSO: 38.1±23.0%; and VU-WS211 (referred to as 211 hereafter), an inactive analog of Pyr: 40.4±16.7%) **(Fig 23A-C)**. Conversely, treatment with lithium chloride (LiCl), which activates the Wnt pathway (Hedgepeth et al., *Dev Biol* 1997), inhibited wound closure compared to vehicle (PBS) treated wounds (LiCl: 13.3±6.3%; PBS: 25.1±13.8%; **Fig 23D)**. We did not see enhanced wound closure when the treatment was started 7 days after injury, or alternatively, started at day 0 and continued only until day 7 after injury indicating a need for treatment during the full 30-day period **(Fig 24)**.

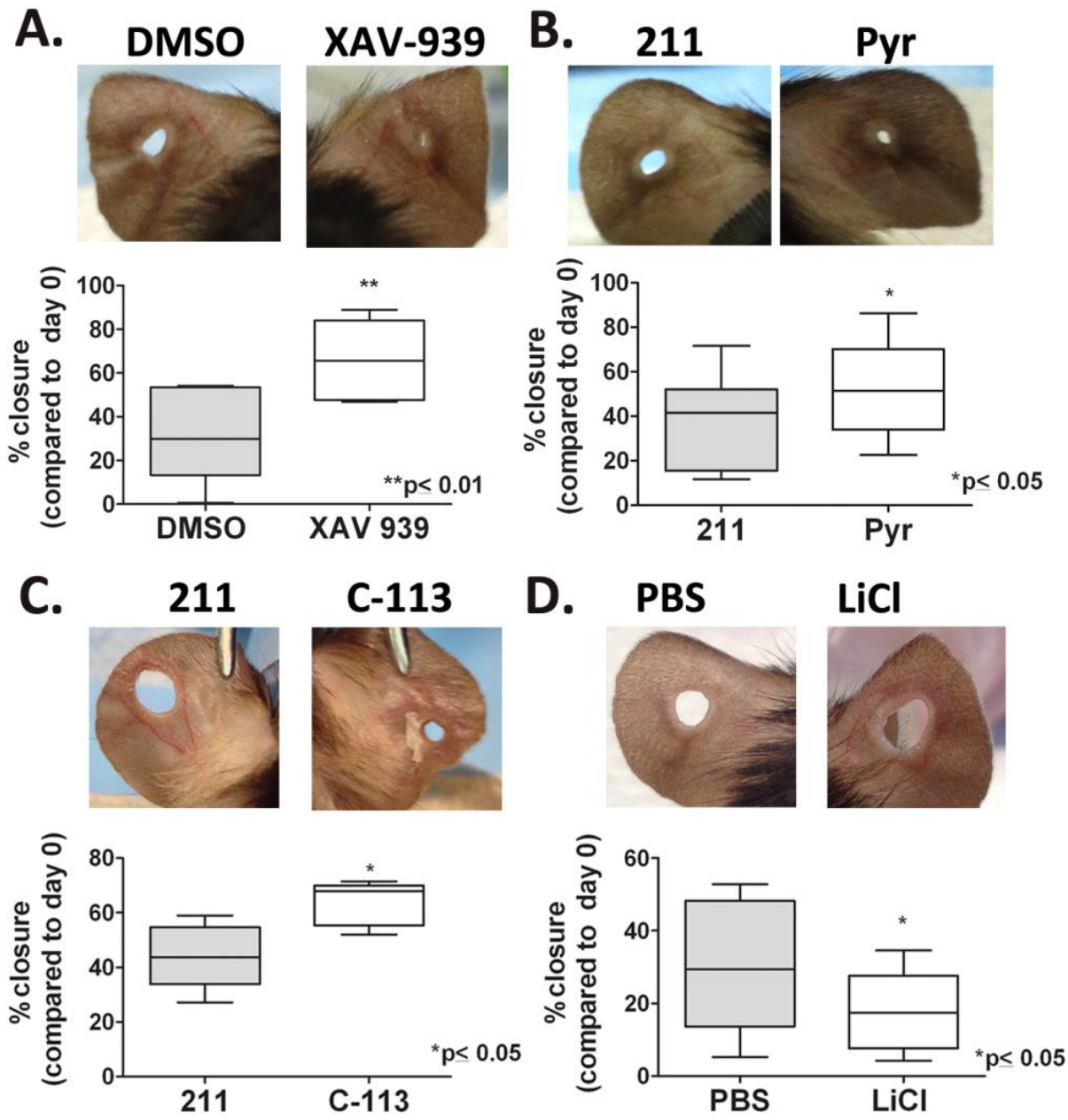

Figure 23: Wnt inhibitor treatment promotes closure of ear punch wounds.
Images of 2 mm ear punch holes treated topically for 30 days with **(A)** small molecule Wnt inhibitor XAV-939 (right panel) or contralateral control treated with vehicle only (DMSO; left panel); **(B)** small molecule Wnt inhibitor Pyr (right panel) or a non-functional analog, compound 211 (left panel); **(C)** small molecule Wnt inhibitor, analog of Pyr (C-113; right panel) or 211 (left panel); and **(D)** Wnt activator LiCl (right panel) or vehicle (PBS; left panel). The histograms underneath the panels represent quantification of percent closure of ear punches at day 30. Bars represent mean±SD; *p≤0.05 and **p≤0.01 were calculated using paired t-test with (A) *N=10*; (B) *N=6* per group; (C and D) *N=5* per group.

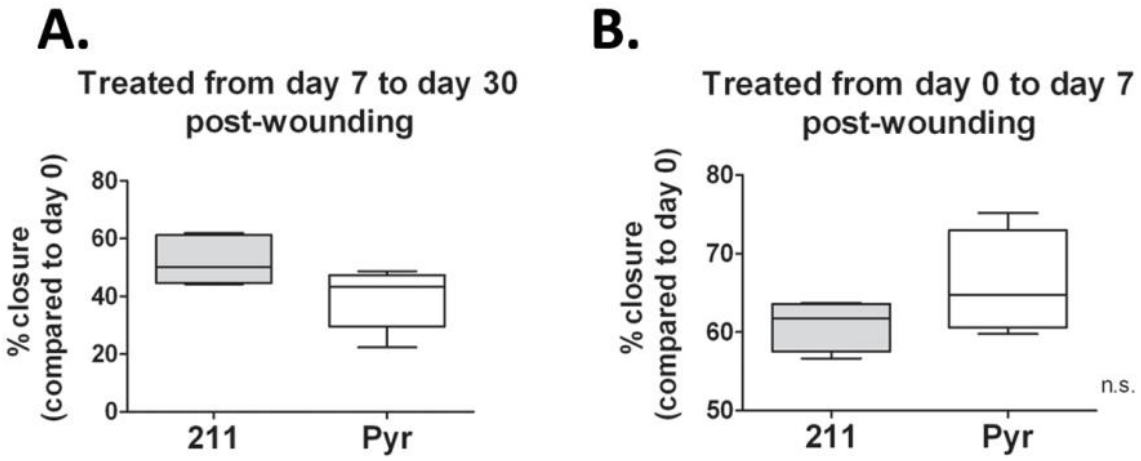

Figure 24: Wnt inhibitor treatment is necessary immediately after wounding and throughout the healing period for effective regenerative repair.
Quantification of closure at day 30 of ear punch wounds treated starting **(A)** day 7 until day 30, or **(B)** day 0 until day 7 after wounding with Pyr or 211 (contralateral control). p=0.0313 (A) and p=0.1250 (B) were calculated by paired t-test using *N=5* (A) and *N=4* (B).

Wnt/β-catenin pathway inhibition promotes regenerative repair and reduces fibrosis

An essential aspect of regenerative skin repair is controlling fibrosis and recapitulating the properties of normal skin. We investigated the effect of Wnt inhibitor treatment on this aspect of repair using both full-thickness excisional wound model on backs of mice, and ear punch injury model.

In 1cm by 1cm full thickness wounds on the back of C57Bl6 mice, daily topical treatment with pyrvinium promoted resolution of scar and restoration of reticular collagen lattice closer in arrangement to normal skin compared to 211 control as detected by Gomori's trichrome blue staining **(Fig 25 A-B)** and by picrosirius red staining observed under circularly polarized light **(Fig 25 C-D)**. Dermo-epidermal junction with rete pegs and dermal papillae, indicative of a more regenerative healing (D. T. Nguyen, *Biomaterials for treating skin loss* 2009), were also observed in pyrvinium treated skin, and not in 211-treated skin **(Fig 25 E-F)**.

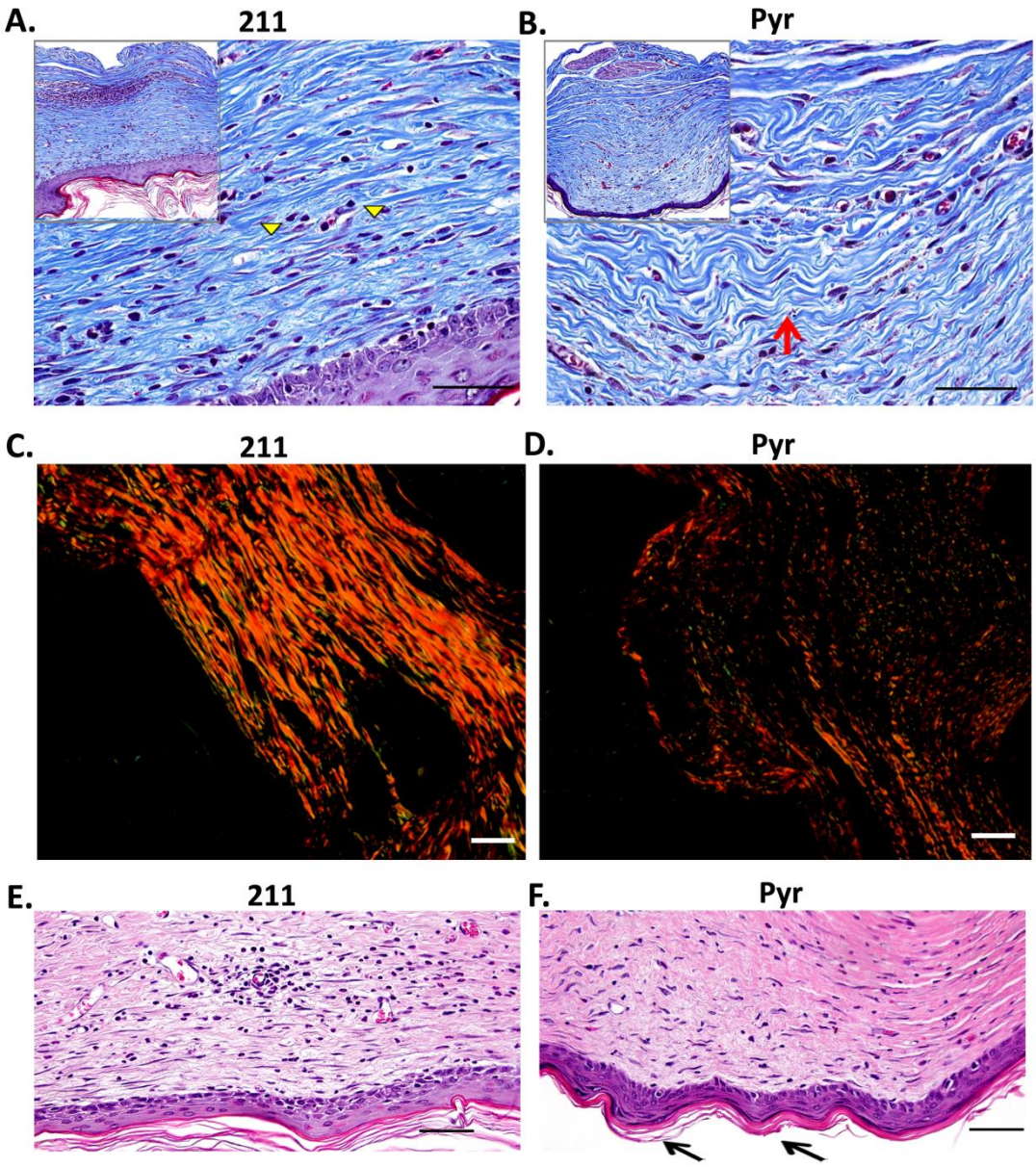

Figure 25: Wnt inhibition promotes scar resolution and rete ridges formation in healing skin.

(A-B) Gomori trichrome stained sections of skin from full-thickness excisional wound on backs of mice showing more linear (scar-like) extracellular matrix in **(A)** 211-treated sections (marked by yellow arrowheads), and more reticular collagen and organized extracellular matrix in **(B)** pyrvinium-treated sections (marked by red arrow). **(C-D)** Picrosirius red stained sections imaged with circularly polarized light showing difference in fiber thickness and alignment between 211-treated (C) and Pyr-treated (D) tissue. **(E-F)** H&E stained sections show increased rete ridge formation (black arrow) in **(F)** pyrvinium-treated compared to **(E)** 211-treated sections. Insets in A and B show low magnification images for orientation. Scale bars equal 50µm.

Histological analysis of ear punch wound also indicated more regenerative repair with Wnt inhibitor treatment than control **(Fig 26A-B)**. At day 30, contralateral control (DMSO or 211-treated) wounds were composed of immature granulation tissue or disorganized extracellular matrix, characteristic of scar tissue **(Fig 26A-B top panels** and **Fig 27A**). In contrast, wounds treated with Wnt inhibitors showed evidence of improved healing, with organized (more reticular) extracellular matrix and areas of regenerated tissue that closely resembled normal, uninjured cutaneous tissue (**Fig 26A-B bottom panels**, **Fig 27A**, and quantification of variance in collagen fiber alignment: **Fig 26E**). As expected, Wnt activation by LiCl treatment did not reduce scarring at day 30 **(Fig 27B)**.

In order to understand the cellular and molecular basis of this phenotype, we examined the major drivers of fibrosis. We found that Wnt inhibitor treatment resulted in reduced presence of alpha smooth muscle actin (α-SMA) positive myofibroblasts in the healing wound **(Fig 26C-D)**, which corresponded with lower mRNA expression of α-SMA **(Fig 26F),** Col1α1 and Col3 **(Fig 26G)** in Wnt inhibitor-treated tissue. Since inflammation at early stages of repair is considered an important determinant of subsequent fibrosis (Stramer et al., *J Invest Dermatol* 2007), we tested the effect of Wnt inhibition on inflammation. Immunohistochemistry with F4/80 showed no difference in macrophage content in the wound at 48 hours and 4 days post wounding **(Fig 28A-C)** in Pyr and 211-treated ears. Quantitative real-time PCR for IL-6 gene expression at day 2 post-injury confirmed this finding **(Fig 28D)**, indicating that the anti-fibrotic effect Wnt inhibition is likely not mediated by a reduction in inflammation.

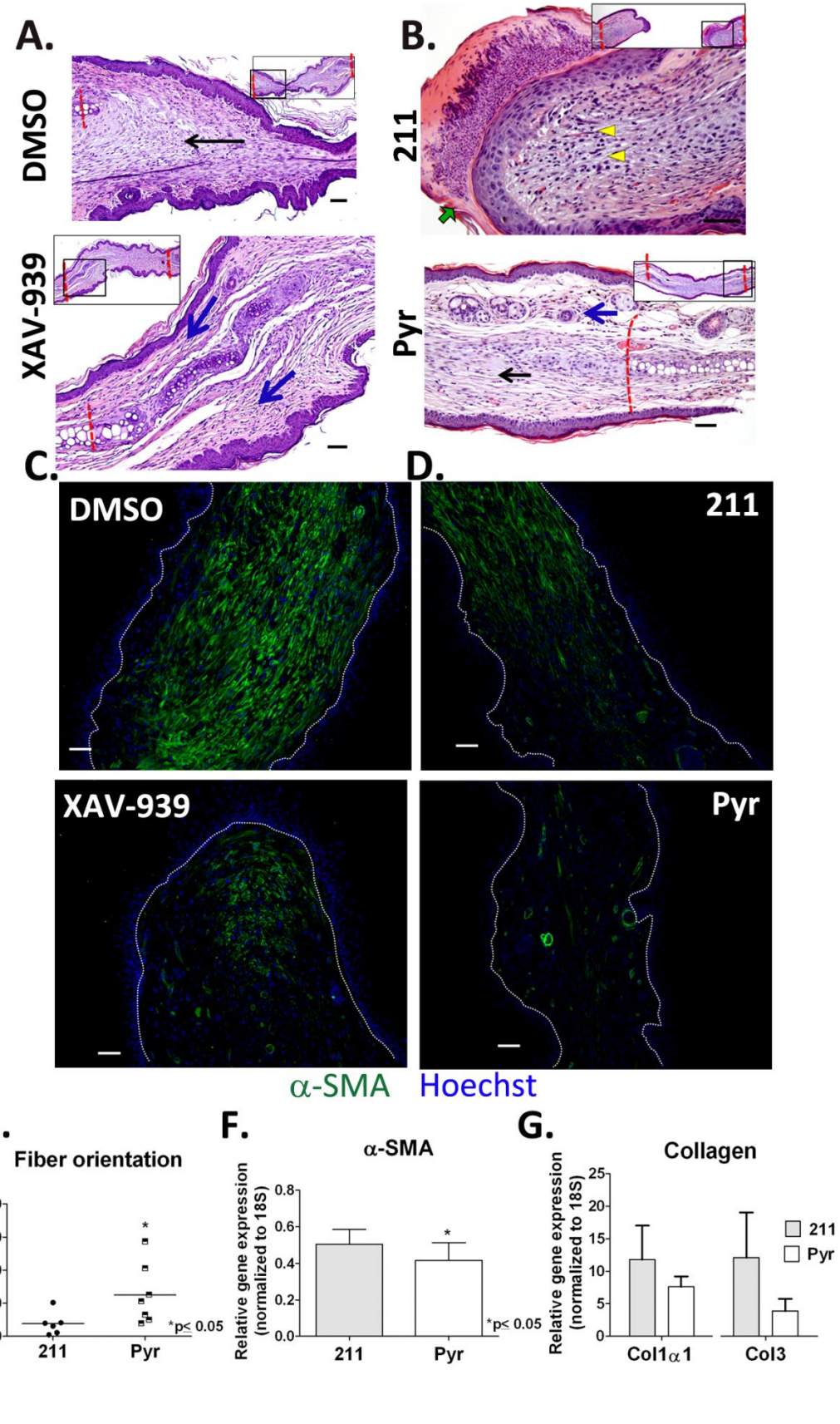
A.
DMSO
B.
211
XAV-939
Pyr
C.
D.
DMSO
211
XAV-939
Pyr
α-SMA
Hoechst
E.
Fiber orientation
Relative Variance
40
30
20
10
0
*p≤ 0.05
211
Pyr
F.
α-SMA
Relative gene expression (normalized to 18S)
0.8
0.6
0.4
0.2
0.0
*p≤ 0.05
211
Pyr
G.
Collagen
Relative gene expression (normalized to 18S)
25
20
15
10
5
0
211
Pyr
Col1α1
Col3

Figure 26: Wnt inhibitor treatment promotes regenerative repair of ear punch wounds.
Representative images of **(A)** XAV-939 or DMSO-treated tissue, and **(B)** Pyr or 211- treated tissue showing difference in regenerative repair. XAV-939 and Pyr-treated sections show scar resolution (black arrow in B) and regenerated tissue that closely resembles healthy skin (blue arrows in A and B). The 211-treated section shows organizing granulation tissue with higher cellularity (yellow arrowheads) and scale crust (green arrow) at day 30. DMSO-treated tissue shows an abundance of scar (black arrow) without regenerating tissue. Insets show lower magnification images of the tissue for orientation (black box indicates magnified regions). **(C-D)** Immunostaining for α-SMA on **(C)** DMSO and XAV-939; and **(D)** 211 and Pyr-treated tissue at day 20 post-wounding showing fewer α-SMA$^+$ myofibroblasts (green fluorescence) in Wnt inhibitor-treated tissue (C and D: lower panels). Nuclei are counterstained with Hoechst; white dashed lines demarcate dermis-epidermis border; scale bars equal 50μm. **(E)** Directional variance of collagen fiber alignment calculated from 20x images of Trichrome blue-stained sections. Relative mRNA expression of **(F)** α-SMA (*N=5* per group; paired t-test), and **(G)** Col1α1 and Col3 in wound bed tissue (*N=3* per group). Scale bars equal 50μm.

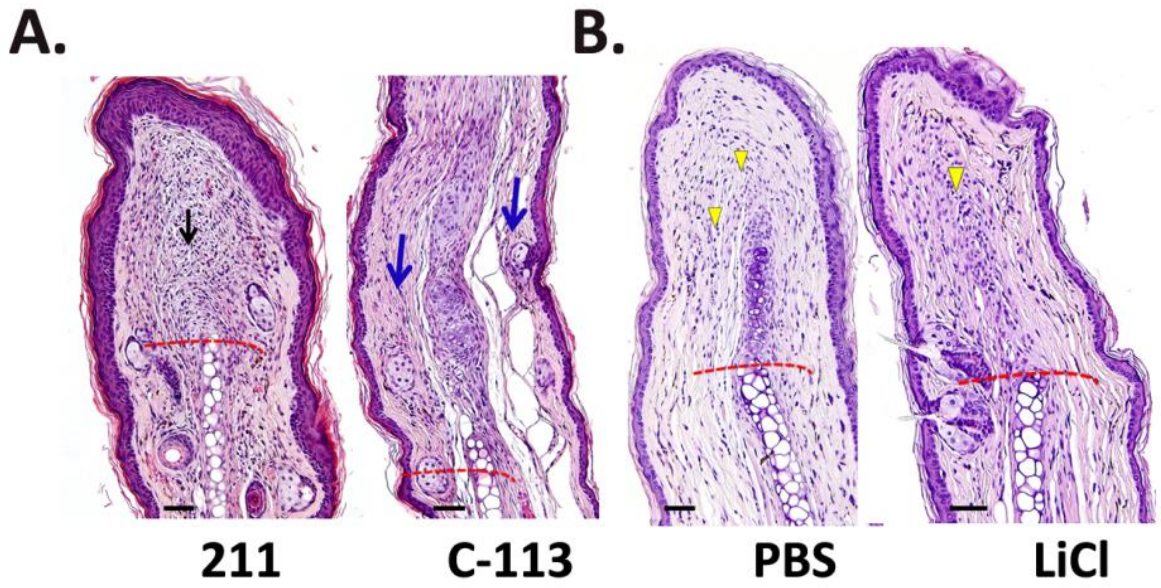

Figure 27: Activation of Wnt pathway reduces regenerative wound repair.
Images of H&E stained sections of wound at day 30 treated with **(A)** C-113 (right panel), and 211 (non-functional analog of C-113; left panel); and **(B)** LiCl (right panel) and vehicle (PBS; left panel. Images show immature scar tissue (black arrow) in 211-treated wounds (A) and immature granulation tissue (yellow arrowheads) in PBS or LiCl-treated wounds (B). C-113 treated section (A) shows regenerated tissue that closely resembles healthy skin (blue arrow).

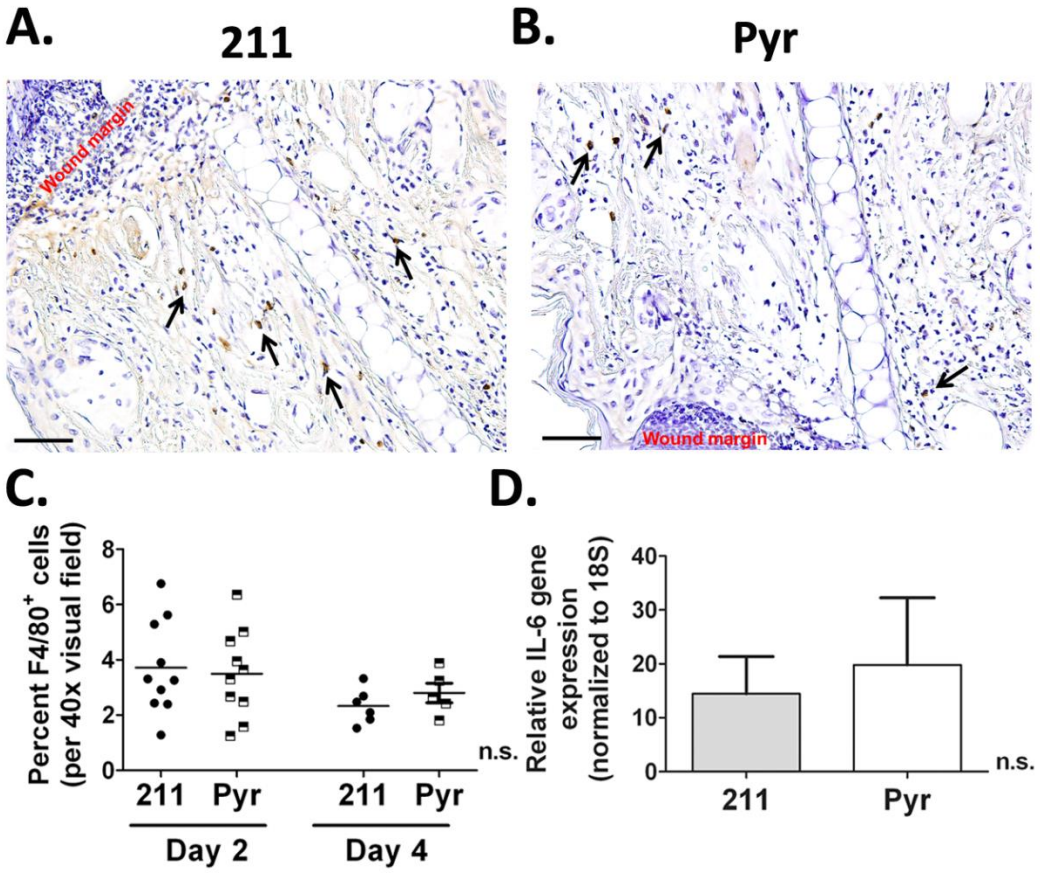

Figure 28: The regenerative repair induced by Wnt inhibition is not mediated through modulation of inflammation.

(A and **B)** Immunohistochemistry staining for F4/80 shows no difference in macrophage recruitment in the wound early after injury between **(A)** 211 and **(B)** Pyr-treated tissue, quantified in **C**. Arrows point to representative F4/80^{+} foci; scale bars equal 50µm. Sections from *N=4* mice per group were used. **(D)** Relative IL-6 mRNA expression in wound bed tissue. p=0.1826 was calculated by paired t-test using *N=5* per group.

Wnt inhibition promotes cartilage regeneration in wounded ears

An important component of regenerative repair in the ear punch injury model is cartilage regeneration, which is essential for complete restoration of tissue function at this site. Only a few "super-healer" strains or species of mice are capable of cartilage regeneration after ear punch injury (Heber-Katz et al., *Phil Trans R Soc Lond* 2004; Seifert et al., *Nature* 2012). The mechanism driving this regeneration is unknown in these models. We noted that Wnt inhibition resulted in increased chondrogenesis extending from the uninjured cartilage at the edge of the wound compared to control wounds **(Fig 29A-B)**. Toluidine blue **(Fig 29C-D and F)** and Safranin-O **(Fig 29E)** staining for chondroid matrix proteoglycans also showed that Wnt inhibition promotes the formation of cartilaginous matrix in the healing punch wound. Furthermore, immunostaining for type II and X collagen showed greater deposition in the leading edge of existing cartilage in ears treated with Wnt inhibitors compared to contralateral controls **(Fig 29G-I)**. The transcription factor Sox9 is essential for chondrocyte differentiation (Akiyama et al., *Genes Dev* 2002). We observed significantly greater numbers of Sox9-positive nuclei extending from the uninjured cartilage at the wound margin in tissue treated with Wnt inhibitors compared to their respective controls **(Fig 29J-L)**, suggesting that Wnt inhibition promotes chondrogenesis in this model of cutaneous injury.

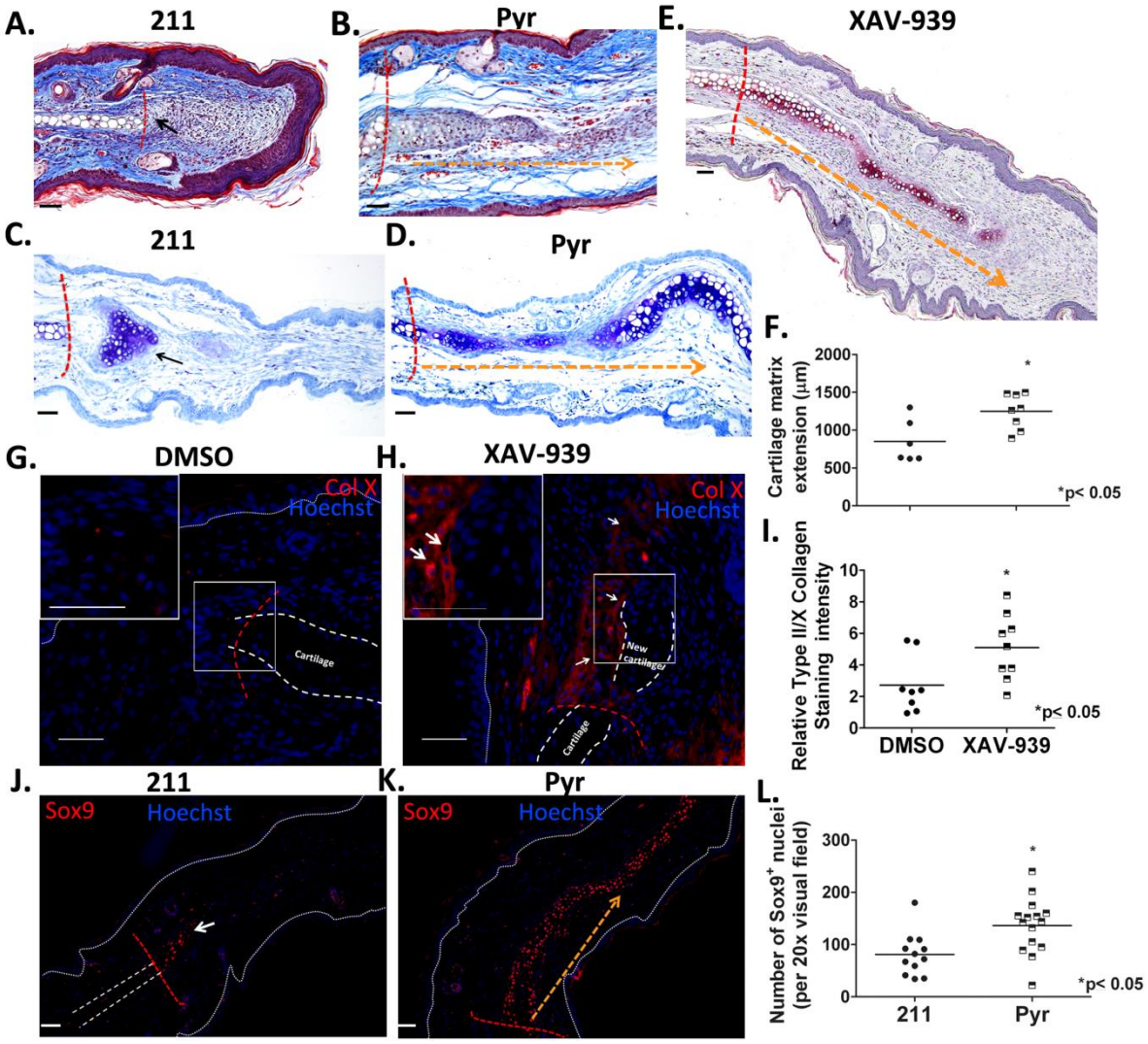

Figure 29: Topical treatment with Wnt inhibitors stimulates cartilage regeneration.
(A–B) Trichrome and **(C-D)** Toluidine blue-stained sections of tissues treated with 211 or Pyr. 211 treated sections **(A** and **C)** show very limited extension of cartilage matrix (black arrow). In contrast, Pyr-treated tissue **(B** and **D)** show extension of new cartilage matrix (indicated by orange dashed arrow) beyond wound margin (red dashed lines). Toluidine blue staining of new cartilage matrix is quantified in **F**. **(E)** Safranin-O staining of proteoglycan deposition (indicated by orange dashed arrow) beyond wound margin (red dashed line) in XAV-939-treated tissue. **(G-H)** ColX stained sections show increased ColX deposition at the leading edge of injured cartilage in **(H)** XAV-939-treated tissue than **(G)** DMSO-treated tissue. **(I)** Quantification of ColX and ColII immunostaining. **(J-K)** $Sox9^+$ chondrocytes (red fluorescence) extending from the leading edge of existing cartilage (red dashed line) indicates more chondrogenesis in **(K)** Pyr-treated wound compared to **(J)** 211-treated tissue. Dotted white lines mark epidermal layer, and dashed white lines in G marks existing cartilage. Nuclei are counterstained blue with Hoechst. **(L)** Quantification of $Sox9^+$ nuclei. (F) $N \geq 5$; (I) $N \geq 8$; and (L) $N \geq 4$ mice per group. Scale bars equal 50μm.

Topical treatment with Wnt inhibitors does not impede hair follicle neogenesis

Our analysis of the dermal and sub-dermal layers indicated a more regenerative and less fibrotic healing by treatment with Wnt inhibitors. However, given the large body of literature suggesting an essential role for epidermal Wnt signaling in hair follicle neogenesis in injured skin (Lowry et al., *Genes Dev* 2005; Ito et al., *Nature* 2007; Gay et al., *Nat Med* 2013), we were surprised to note that topical Wnt inhibitor treatment of ear punch wounds did not inhibit hair follicle regrowth in regenerating wound tissue. In contrast, XAV-939 **(Fig 30A)**, C-113 **(Fig 30B)** or Pyr **(Fig 30C and D)** treatment resulted in a significantly increased number of hair follicles in the regenerated tissue. Immunostaining for the hair follicle bulge stem cell marker cytokeratin 15 (Liu et al., *J Invest Dermatol* 2003) **(Fig 30E)**, and the epithelial placode marker cytokeratin 17 (McGowan et al., *J Invest Dermatol* 2000; Ito et al., *Nature* 2007) **(Fig 30F)** confirmed the presence of mature and nascent hair follicles. To further confirm that these follicles were nascent follicles arising from regenerated tissue, we stained for $Sox9^+$ epithelial placode cells, which mark the beginning of hair follicle formation (Vidal et al., *Curr Biol* 2005). More $Sox9^+$ placode cell clusters were detected in Wnt inhibitor treated wounds compared to respective controls **(Fig 30G-I)**. Although studies using epidermis-specific promoters to modulate Wnt signals (Lowry et al., *Genes Dev* 2005; Ito et al., *Nature* 2007; Gay et al., *Nat Med* 2013) have showed that epidermal Wnt activation is an important requirement for hair follicle neogenesis, it is possible that in our model, dermal Wnt inhibitory signals promote regeneration without disrupting follicle regeneration mediated by Wnt in the epidermis. This is also evident by our immunofluorescence data **(Fig 21B-C** and **Fig 22B-C)** where we did not observe a detectable reduction in Wnt signals in the epidermis as measured by β-catenin and β-galactosidase immunofluorescence intensity by treatment with our Wnt inhibitors.

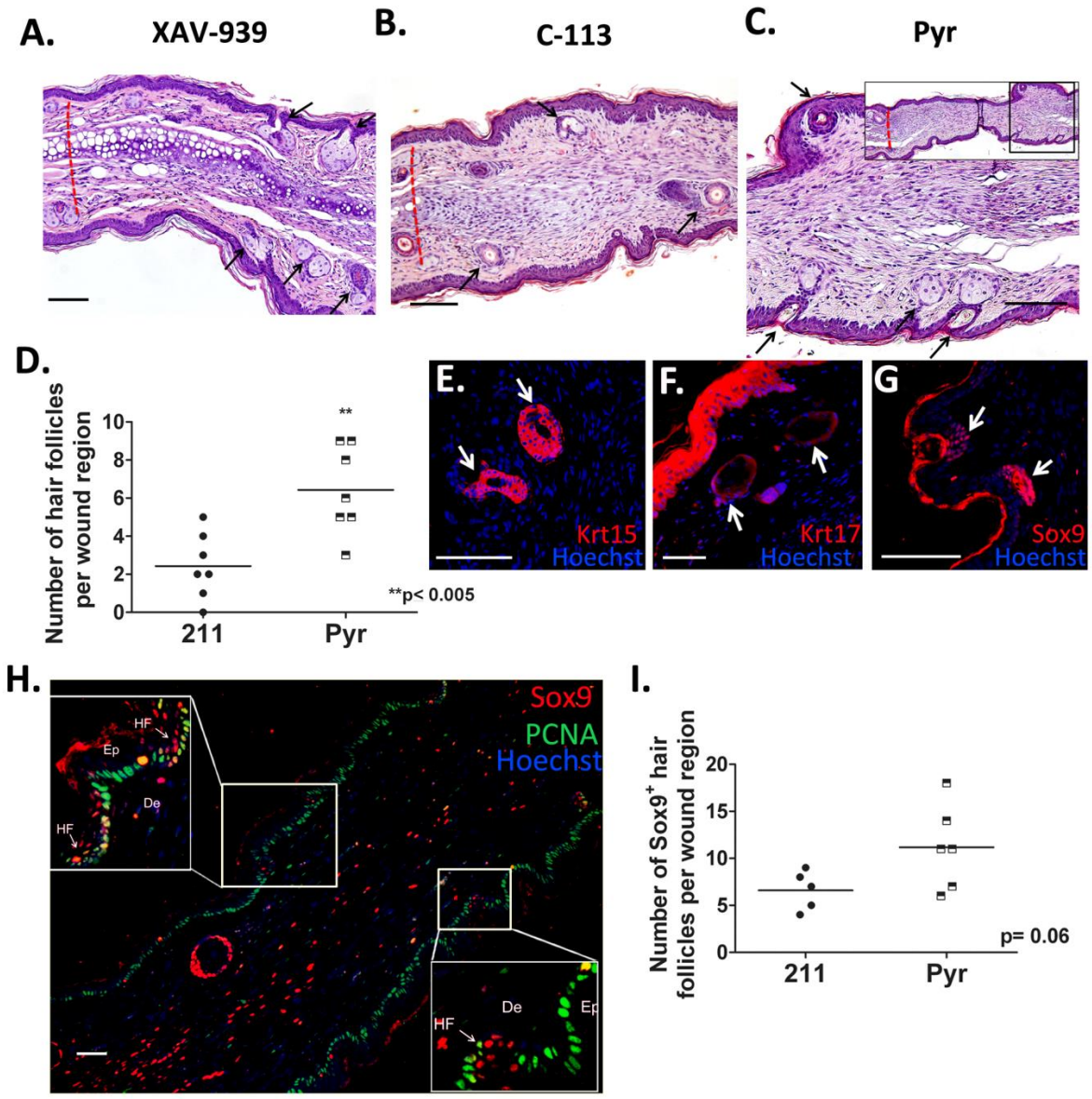

Figure 30: Wnt inhibition does not affect hair follicle neogenesis in newly regenerated tissue.

H&E stained sections of ear wound treated with **(A)** XAV-939, **(B)** C-113 or **(C)** Pyr showed newly regenerating hair follicles (black arrows). Dashed red lines represent wound margin; **(D)** Graphical representation of number of new hair follicles arising in the regenerated tissue with 211 or Pyr treatment. **(E)** anti-cytokertain-15 (red), **(F)** anti-cytokeratin-17(red), and **(G)** anti-Sox9 (red)-stained sections of XAV-939-treated tissue highlights new hair follicles arising in the regenerated tissue **(H)** Sox9 (red) stained section of Pyr-treated tissue showing positive staining of placode cells (magnified in the insets) in very early stages of hair follicle formation; PCNA (green) marks proliferating basal cells of epidermis; nuclei in E-H are counterstained blue with Hoechst. HF- hair follicle; Ep- Epidermis; D- Dermis. **(I)** Number of early hair placodes stained with Sox9. (D and I) *N≥5* per group. Scale bars equal 50µm

Discussion

In this study, we demonstrated a cutaneous wound healing response characterized by reduced fibrosis, and regeneration of auricular cartilage and skin adnexa in response to inhibition of Wnt/β-catenin signaling. Given the conflicting literature supporting the need for epidermal Wnt activation in hair follicle regeneration (Lowry et al., *Genes Dev* 2005; Ito et al., *Nature* 2007; Gay et al., *Nat Med* 2013), and dermal Wnt activity in promoting fibrosis (Russell et al., *J Invest Dermatol* 2010; Akhmetshina et al., *Nat Commun* 2012), our study highlights the potential of therapeutic Wnt inhibition in balancing both the aspects of wound repair.

Topical treatment with three Wnt inhibitors with two distinct mechanisms-of-action reduced β-catenin and β-galactosidase (in TOPGAL mice) protein levels most notably in the dermis. Unlike the epidermis, which exhibited high, continuous baseline Wnt activation, Wnt activity in the dermis was more dynamic-- relatively quiescent in uninjured skin and activated shortly after injury, similar to previously published reports in skin injury (Cheon et al., *Proc Natl Acad Sci U S A* 2002). This dynamic aspect of dermal Wnt activity may make it more susceptible to therapeutic inhibition. Similar phenomenon has been reported by de la Roche *et. al*, where transiently Wnt-stimulated cells responded robustly to tankyrase inhibition XAV-939, while long term over-stimulation with Wnt rendered the cells resistant to the Wnt inhibitory effect of the drug (de la Roche et al., *Cancer Res* 2014).

In the dermis, we noted that Wnt inhibition promoted granulation tissue resolution, promoting deposition of more organized extracellular matrix with reticular fiber alignment, in both the excisional wound model and ear punch injury model. In the excisional wound, Wnt inhibition promoted formation of rete-pegs and dermal papillae indicating better restoration of dermal-epidermal junction. Wnt inhibitor treatment in ear injury also reduced myofibroblast

number and modestly reduced gene expression of α-smooth muscle actin and collagen1 and 3—all major indicators of fibrosis (Wynn et al., *Nat Med* 2012). IL-6 gene expression and macrophage immunohistochemistry showed no significant difference in immune activation between Wnt inhibitor and vehicle-treated tissue, suggesting that the effect of Wnt inhibition on fibrosis was not mediated by modulating inflammation. Our findings are supported by other studies demonstrating the role of Wnt/β-catenin signals in promoting fibrosis in various wound models (He et al., *J Am Soc Nephrol* 2009; Kim et al., *Tohoku J Exp Med* 2011; Wei et al., *Arthritis Rheum* 2011; Akhmetshina et al., *Nat Commun* 2012). Within the wound milieu, Wnt pathway activity is associated with proliferative and migratory phenotype of fibroblasts (Cheon et al., *Proc Natl Acad Sci U S A* 2002), as well as with fibroblast differentiation into myofibroblasts (Akhmetshina et al., *Nat Commun* 2012), which can potentially contribute to fibrosis.

The role of Wnt pathway in folliculogenesis has been extensively studied. During mammalian development, the Wnt pathway is necessary for hair follicle initiation (Andl et al., *Dev Cell* 2002; Tsai et al., *Dev Biol* 2014), and stabilization of β-catenin in the epidermis leads to ectopic *de novo* hair morphogenesis (Gat et al., *Cell* 1998). Previous studies indicate that Wnt pathway activity is necessary for initiation of new hair follicle formation in the wounded epidermis (Lowry et al., *Genes Dev* 2005; Ito et al., *Nature* 2007; Gay et al., *Nat Med* 2013). However, most of these studies focus on folliculogenesis, and do not report an overall regenerative repair of cutaneous wound. Hence our results may be explained by the possibility that the molecular signals driving folliculogenesis in a regenerating wound may be distinct from the signals (such as Wnt) transiently activated specifically in the epidermis during the initial phase of hair follicle neogenesis. Indeed hair follicle maintenance in normal skin is thought to be

driven by ‘short-range’ Wnt activation signals, that are surrounded and blunted by ‘long-range’ Wnt inhibition signals (Lim et al., *Cold Spring Harb Perspect Biol* 2013).

The Wnt inhibitors allowed us to target the dermis, which may also explain our results that are in direct contrast to the studies of follicle neogenesis based mostly on epithelial or follicle-specific promoter-driven models of Wnt modulation (Lowry et al., *Genes Dev* 2005; Ito et al., *Nature* 2007; Gay et al., *Nat Med* 2013). In agreement with our data, previous studies in which β-catenin was stabilized both in the dermis and the epidermis (Cheon et al., *Proc Natl Acad Sci U S A* 2002; Cheon et al., *FASEB J* 2006), showed that Wnt activation promoted fibrosis, and increase in wound size. Given the strong evidence that Wnt inhibits regenerative repair in lung (Kim et al., *Tohoku J Exp Med* 2011), kidney (He et al., *J Am Soc Nephrol* 2009), heart (Bergmann, *Circ Res* 2010), and even skin (Beyer et al., *Ann Rheum Dis* 2013), it is important to examine folliculogenesis in the context of regenerative skin repair. Our findings may also be attributed to the fact that pharmacological Wnt inhibition can be titrated to achieve optimal therapeutic outcome, whereas genetic models of complete Wnt inhibition (e.g. through β-catenin inactivating mutation) are more likely to have a deleterious effect given the importance of the pathway in skin homeostasis. Indeed, we saw reduction in cytoplasmic β-catenin staining in the dermis, but did not notice an effect on β-catenin levels or localization in the epidermis of Wnt inhibitor treated tissues compared to controls. Moreover, our data suggest a negative role of Wnt signaling in the regeneration of cartilage. Although Wnt signaling has not been implicated directly in the degradation of auricular cartilage, the Wnt/β-catenin pathway is known to play an inhibitory role in cartilage development (Rudnicki et al., *Dev Biol* 1997), and promote hypertrophic chondrocyte maturation during endochondral ossification by suppressing chondrogenic gene expression program (Day et al., *Dev Cell* 2005). Wnt signaling is also

thought to promote cartilage degradation in osteoarthritis and rheumatoid arthritis by promoting chondrocyte de-differentiation and degradation of the cartilage extracellular matrix (Hwang et al., *J Biol Chem* 2004). Finally, β-catenin-mediated transcription has been shown to downregulate Sox9, an important mediator of chondrocyte differentiation from mesenchymal cells (Day et al., *Dev Cell* 2005).

Cartilage regeneration may also offer an explanation for our observation that treatment throughout the 30-day period was required for optimal ear punch closure, although a spike in Wnt activity is observed early after injury. Since treatment for two weeks was sufficient to induce a reduction in scarring in the excisional wound model, it is likely that in the ear punch injury model, the need for 30-day treatment is tied closely to the requirement for cartilage regeneration to enable wound closure in this model. Although with RT-PCR, we see Wnt pathway activity reduce to baseline levels by day 10, it is possible that Wnt pathway remains active in the leading edge of the injured cartilage—undetectable by RT-PCR of the whole tissue—and that quenching of the pathway with inhibitors promotes regeneration of the cartilage, driving closure of the wound. Studies that explore the role of Wnt inhibition on cartilage regeneration in this and other injury models may help test this hypothesis.

The Wnt pathway has been ascribed roles in regulating stem-ness and differentiation of various stem/progenitor cell types. Work by our group and others have shown a role for Wnt inhibition in maintaining stem-ness and inhibiting differentiation of mesenchymal stem cells (MSCs) (Gaur et al., *J Biol Chem* 2005; Otto et al., *Crit Rev Biochem Mol Biol*

2005; Alfaro et al., *Proc Natl Acad Sci USA* 2008). In the heart, the Wnt pathway inhibits proliferation of cardiac progenitor cells *in vivo (Oikonomopoulos et al., Circ Res 2011),*

and in skeletal muscle Wnt signaling is associated with differentiation of myogenic progenitors (Brack et al., *Dev Biol* 2009). Studies in cutaneous injury suggest similar roles for Wnt/β-catenin pathway activity—specifically, promoting differentiation of epithelial stem cells to follicle lineages during the hair cycle (Dasgupta et al., *Dev* 1999; Huelsken et al., *Cell* 2001). Consistent with this, we have observed in excisional wound model, and using engineered skin substitute *in vitro*, that Wnt inhibition slows keratinization and stratification of wound epidermis, possibly by inhibiting differentiation and promoting a proliferative phenotype in keratinocytes (data not shown), also in agreement with published report (Zhu et al., *PLoS Genet* 2014). It is known that quiescent stem cells localized in the hair follicle bulge directly contribute to wound repair (Ito et al., *Nat Med* 2005). Hence, Wnt inhibition may promote regenerative repair through its effect on epithelial stem cells. Further studies are necessary to confirm this hypothesis.

The clinical significance of topical application of an agent that effectively promotes regenerative cutaneous repair and inhibits scarring is immense. Scar-free wound healing can significantly improve the quality of life of thousands of burn and trauma survivors. We found regeneration of auricular cartilage with Wnt inhibitor treatment. Further studies may also reveal a role for Wnt inhibition in promoting regeneration of damaged articular cartilage in conditions such as degenerative joint disease in humans. Moreover, given the availability of pyrvinium, an FDA-approved drug that can be effectively repurposed for cutaneous Wnt inhibition, and the ongoing therapeutic development of Wnt inhibitors for other indications such as cancer (*clinicaltrials.gov*) (Novartis, *Clinicaltrials.gov* 2014), our findings have immediate and broad clinical impact.

Materials and Methods

Antibodies

β-catenin (1:200; BD Pharmingen, 610153); β-galactosidase (1:100; AbCam, Ab616); Cytokeratin (Krt)15 (1:100; AbCam, Ab52816); Krt17 (1:1000; AbCam, Ab53707); Sox9 (1:1000 Millipore, AB5535); Proliferating cell nuclear antigen (PCNA) (1:100; Santa Cruz, SC-56); Alpha Smooth Muscle Actin (α-SMA) (1:1000; Sigma A2547); Collagen type IIC1 (1:100; DSHB, Iowa); Collagen Type X (1:100; DSHB, Iowa).

Wnt modulators

The small molecule Wnt inhibitors (CK1α activators) pyrvinium (Pyr) and VU-WS113 (C-113), as well as the non-functional analog of the two drugs, VU-WS211 (211), were generous gifts from Dr. Ethan Lee, Department of Cell and Developmental Biology, Vanderbilt University (Thorne et al., *Nat Chem Biol* 2010). XAV-939 (Huang et al., *Nature* 2009), a small molecule stabilizer of axin2 was purchased from Selleck Chemicals (S1180; Houston, TX). LiCl (203637 Sigma, St. Louis, MO) at 100mM in PBS was used for Wnt activation.

Animals

All procedures were carried out in accordance with Vanderbilt Institutional Animal Care and Use Committee. C57Bl6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained by PPY. TOPGAL (Aisagbonhi et al., *Dis Model Mech* 2011) mice were a generous gift from Dr. Antonis Hatzopoulos (Department of Cell and Developmental Biology, Vanderbilt University).

Mouse ear punch model

C57Bl6 mice (at least 3 months of age) were anesthetized with 3-5% Isofluorine in O_2 administered using Table Top Laboratory Animal Anesthesia System (VetEquip Incorporated, Pleasanton, CA). A 2 mm biopsy punch wound was made in the center of the cartilaginous region of each ear using a disposable biopsy punch (Acuderm Inc., Fort Lauderdale, FL) as described previously (Rai et al., *Arthritis Rheum* 2012). The ears were treated topically with 5µL/ear of drug (1 µM Pyr, 10 µM C-113, 5 µM XAV-939 or 100mM LiCl) or control solution (1 µM 211, DMSO or PBS, respectively) every day. The ears were imaged at day 30 using Nikon Coolpix 8700 digital camera. For quantification of wound closure, ears were excised after the mice were sacrificed, placed on microscope slides (Denville Scientific, South Plainfield, NJ) and imaged using Nikon Coolpix 8700 affixed to Micromaster Inverted microscope (Fisher Scientific, Waltham, MA). Wound closure in the images was quantified by measuring the surface are of the hole using ImageJ (NIH) and calculating percent difference compared to original ear punch area. For longitudinal study of Wnt activation, mice were sacrificed 2, 3, 4, 5 or 10 days after injury. The ears were either paraffin embedded for histology or used for RNA isolation as described in the following sections.

Dermal full-thickness injury model

C57Bl6 mice (at least 3 months of age) were anesthetized with 3-5% Isoflurane in O_2 administered using Table Top Laboratory Animal Anesthesia System (VetEquip Incorporated, Pleasanton, CA). The backs of mice were shaved and disinfected by applying betadine followed by 70% ethanol wipe three times. 1cm by 1cm excision was made on the back of the mice using scalpel and skin was removed by using surgical scissors to separate the skin from the underlying muscle. The wound was covered with tegaderm after topical application of 1µM pyrvinium or

10μM 211 (control). The treatments were continued daily for two weeks following injury. At day 30, the healed tissue was excised and embedded in paraffin for histology.

Histology and Morphometry

Excised ears or skin were fixed for 24 hours in 10% buffered formalin, cut longitudinally across the injury and embedded in paraffin blocks. 5 micron slides were stained with H&E and Trichrome blue by the Vanderbilt Translational Pathology Shared Resource and imaged using an Olympus DP71 microscope camera (Olympus America, Center Valley, PA). Number of regenerated hair follicles originating beyond the wound margin (defined as the leading edge of healthy pre-existing auricular cartilage) was quantified using 20X images of H&E stained slides. For picrosirius red staining, nuclei were stained with Weigert's hematoxylin, followed by picrosirius red stain overnight. Excess stain was washed off in acidified water; slides were then dehydrated and mounted in resinous medium. Toluidine blue staining was performed as previously described (Rai et al., *Arthritis Rheum* 2012). Briefly, deparaffinized slides were covered in 0.5% Toluidine blue solution prepared in 0.1 M Na $CH_3CO_2^-$ buffer (pH 2.5) for 2 hours at room temperature. The slides were then rinsed in water, subjected to two quick dips each in 95% ethanol and xylene, and mounted with Permount (Fisher Scientific, Waltham, MA). For Safranin-O and Fast Green staining, deparaffinized and hydrated slides were stained with Wiegert's iron hematoxylin working solution for 10 minutes, washed in running tap water, stained with fast green for 5 minutes, rinsed for 15 seconds with 1% acetic acid solution, stained with 0.1% Safranin-O solution for 5 minutes, dehydrated and cleared with 95% and 100% ethanol followed by xylene. Images of sections were taken at 20-40X magnification using an Olympus DP71 microscope camera. Cartilage regeneration was quantified using ImageJ to measure the distance from the wound margin to the leading edge of newly laid down cartilage

matrix (stained purple with Toluidine blue). Collagen fiber directional variance (as an indicator of scarring) was quantified using OrientationJ plugin for ImageJ as described previously (Fonck et al., *Stroke* 2009; Rezakhaniha et al., *Biomech Model Mechanobiol* 2012). Briefly, the fiber alignment directional variance was calculated from the local angle distribution in Trichrome blue stained 20x images at each pixel using coherency-weighted alignment.

For immunofluorescence staining, slides were deparaffinized and hydrated through xylene and ethanol steps. Heat-mediated antigen retrieval was performed by boiling in citrate buffer (pH 6). Following appropriate blocking, primary (overnight) and secondary antibody incubation steps, the slides were counterstained with Hoechst 33342 (H21492 Invitrogen, Carlsbad, CA) and mounted with Slowfade Gold (S36936 Life Technologies, Grand Island, NY). Images were taken at 10x, 20x or 40x magnification using Axio Imager2 microscope (Carl Zeiss, Thornwood, NY) and CoolSNAP HQ CCD camera (Photometrics, AZ), and quantified using ImageJ. All images are presented with scale bars that equal 50µm.

RNA isolation and Wnt PCR Array

Two holes per ear were made in cartilaginous region of mouse ears using 2 mm biopsy punch. At day 2, 5 or 10 after injury, 1 mm of tissue surrounding the holes was excised and RNA was isolated using RNeasy Mini Kit (Qiagen). First strand DNA synthesis was performed with 1 µg RNA using iScript cDNA synthesis kit (Bio-Rad 170-8890). Gene expression of 84 Wnt pathway genes was determined using the Mouse WNT Signaling Pathway RT² Profiler PCR Array (PAMM-043Z; SABioscience, Valencia, CA). To verify the expression of select genes, quantitative Real-time PCR was performed in triplicate for each sample with iCycler (BioRad)

and fluorescence detection (SsoFast EvaGreen; 172-5200; BioRad). Each reaction was normalized against 18S. Primer sequences are as shown in Table 4.

Table 4. List of q-RT-PCR primers II

Gene	Forward	Reverse
cMyc	ATCGTCGTGGCTGTCGGGGT	TGCCCGCGATCAGCTCTCCT
CyclinD1	CTGGTGTTTGGAAGTAGGAA	CTGGTGTTTGGAAGTAGGAA
Dkk1	CGAAGTTGAGGTTCCGCAGTCC	CGAAGTTGGGTTCCGCAGTCC
MMP-7	AGGAAGCTGGAGATGTGAGC	TCTGCATTTCCTTGAGGTTG
sFRP2	ATGGAAACCCTTTGTAAAAATGACT	TCTTGCTCTTTGTCTCCAGGATGAT
Wnt3a	GCACCACCGTCAGCAACAGC	CAGGAGCGTGTCACTGCGAAAG
Col1a1	GCCAGATGGGTCCCCGAGGT	GGGGGTCCAGCAGCACCAAC
Col 3	GAAAAAACCCTGCTCGGAATT	GGATCAACCCAGTATTCTCCACTCT
α-SMA	CAGGCATGGATGGCATCAATCAC	ACTCTAGCTGTGAAGTCAGTGTCG
IL-6	CCGGAGAGGAGACTTCACAG	TCCACGATTTCCCAGAGAAC

Statistical analysis

The statistical significance between experimental and control groups were determined by Student's t-test (unpaired analysis unless otherwise noted in the figure legends), using GraphPad Prism (San Diego, CA). $p<0.05$ was considered statistically significant in two-tailed hypothesis tests.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

Conclusion and significance

Regenerative repair of mammalian tissue injury is an important frontier in medical research. As the aging population grows in the developed world, the need for therapeutics that can enable effective tissue regeneration in various organs including heart, kidney, lungs and liver increases. The Wnt pathway has been linked to injuries in these organs for many years, and despite concerted efforts from multiple groups, the complicated role of the Wnt pathway in repair of acute or chronic injury in these organs hasn't been completely understood. The survey of evidence from the literature (Chapter I) suggests that the role of Wnt pathway in mammalian tissue injury may depend significantly on the resident stem/progenitor cells, and the intrinsic homeostatic self-renewal capacity of the tissue. The Wnt pathway, while active and integral during development, is quiescent in most adult mammalian tissues except the few that are constantly undergoing turnover as part of their normal homeostasis (e.g. hair follicles, intestine, epidermis of the skin, etc.) (Reya et al., *Nature* 2003; Krausova et al., *Cell Signal* 2014). In other organs with limited or virtually no ability to regenerate in response to injury (depending on the activity and presence of stem-like cells in these tissues), the Wnt pathway is quiescent and re-activated in response to injury (Kotton et al., *Nat Med* 2014). In the partially regenerative organs such as lungs, kidney and liver, the Wnt pathway activity is associated with promoting regeneration through stem cell self-renewal or proliferation in response to acute injury (Zhang et al., *Nat Genet* 2008; Henderson et al., *Proc Natl Acad Sci U S A* 2010; Lin et al., *Proc Natl Acad Sci U S A* 2010; Gougelet et al., *Int J Hepatol* 2012; Zhou et al., *Kidney Int* 2012). However, overwhelming evidence has suggested a pro-fibrotic role for Wnt signaling, particularly as a

response to repeated or chronic injury (Akhmetshina et al., *Nat Commun* 2012; DiRocco et al., *J Am Soc Nephrol* 2013). Hence, development of therapeutic Wnt inhibitors that can block the fibrotic signals activated by the Wnt pathway may push tissue repair towards a regenerative rather than the fibrotic mode, which is the default in many mammalian organ injuries (Atala et al., *MRS Bull* 2010). Our studies in this dissertation have focused on understanding the potential of therapeutic Wnt inhibition in enabling regenerative injury repair, particularly in the context two injury models: myocardial infarct, and full thickness dermal injury.

In Chapter 2, we described our work investigating the effect of pharmacologic Wnt pathway inhibition following myocardial infarct. We reported that pharmacologic inhibition of the Wnt pathway using a new class of small molecules that block Wnt ligand secretion improved cardiac function post-infarct, reduced adverse cardiac remodeling, and reduced infarct size. We found that Wnt inhibition increased proliferation of interstitial cells in the heart, a subset of which were possibly myogenic progenitors. Wnt inhibition also reduced cardiomyocyte apoptosis in the infarcted heart, reduced proliferation of αSMA^+ myofibroblasts in the infarcted heart, and reduced their collagen expression *in vitro*. Hence we showed that temporary Wnt inhibition post infarct improved cardiac recovery by addressing the three major mediators of infarct pathology and repair: cardiac progenitor cell proliferation, cardiomyocyte death, and cardiac myofibroblast proliferation and matrix synthesis activity. These findings are particularly significant because the current standard-of-care for myocardial infarct is limited to pain and disease management and prevention of future infarct events. Given no current therapeutics address the ongoing myocyte death and increasing fibrosis that occur post infarct, leading to hypertrophic cardiomyopathy and subsequent heart failure in many cases, our findings that

therapeutic Wnt inhibition can target these core pathologies that ensue post-infarct is of immense clinical significance.

In Chapter 3, we discussed the role of Wnt pathway inhibition by small molecule inhibitors that target two distinct components of the Wnt/β-catenin pathway in promoting regenerative repair of cutaneous wound. Current understanding of the role of Wnt/β-catenin pathway on skin wound healing is informed mostly by the studies focused on epidermis or hair follicle regeneration by stem/progenitor cells residing in the basal layer of the epidermis or the various niches in or around hair follicles (Gat et al., *Cell* 1998; Ito et al., *Nature* 2007). These studies suggest that Wnt pathway activation is associated with stem cell proliferation/self-renewal and activation in response to injury. However, what is missing in these studies that have largely relied on models of Wnt pathway modulation using epidermis or hair follicle-specific promoters (Ito et al., *Nature* 2007), is a complete picture of overall wound healing including mitigation of fibrosis in the dermis. Consistently, studies that examine scarring or scar resolution in the dermis more closely, indicate a pro-fibrotic role for Wnt/β-catenin pathway activation in the dermis (Cheon et al., *Lab Invest* 2005). To counteract the limitation of genetic models, we used mechanistically distinct Wnt inhibitors in full thickness injury model in mouse dorsum and ear pinnae. We found that daily topical treatment of Wnt inhibitors promoted closure of ear wounds, reduced scarring, promoted cartilage regeneration and hair follicle neogenesis. Our findings indicate that despite the need for Wnt in hair follicle and epidermal homeostasis and replenishment, targeted Wnt inhibition can significantly reduce scarring in the dermis, and likely promote subsequent regenerative signals that can improve regeneration of skin adnexa and cartilage. Indeed, our immunostaining data **(Fig 21 and 22)** showed that the activation of Wnt signal in response to injury, and the inhibition by topical treatment with Wnt inhibitors was more

appreciable in the dermis, whereas, in the epidermis we did not observe a very notable reduction in nuclear β-catenin or β-gal staining in response to topical Wnt inhibitor treatment. These observations align with previously published studies by de la Roche *et. al,* reporting a robust response to Wnt inhibition by XAV-939 observed in transiently Wnt-stimulated cells, whereas resistance to Wnt inhibition in cells that were exposed to long term over-stimulation with Wnt ligand (de la Roche et al., *Cancer Res* 2014). Considering, scarred healing of skin wounds affects millions of patients and incurs annual health care cost of more than 12 billion dollars (Sen et al., *Wound Repair Regen* 2009), a topical agent with relatively lower safety barrier, which can effectively reduce dermal scarring can have a significant clinical impact.

In unpublished work (presented in Appendix I) systemic delivery of the CK1α activator C-113 in mice post-MI did not result in improved cardiac recovery, although C-113 was bioavailable, able to inhibit the Wnt pathway *in vivo,* and did not result in cardiac toxicity in uninjured mice. Moreover, C-113 induced a more dramatic proliferative effect on cardiac cells than GNF-6231. Our preliminary experiments indicated that the negative effect of C-113 on cardiac recovery may be explained by the collateral inhibition of Hedgehog pathway by CK1α activation (Li et al., *Cancer Res* 2014), which, according to data by Ornitz and colleagues, is necessary for coronary vasculature maintenance in the heart (Lavine et al., *J Clin Invest* 2008). However, since we saw no decline in cardiac function upon C-113 administration in uninjured mice, Hedgehog pathway inhibition may not completely account for our observations. An alternative explanation for the difference in healing observed with Wnt inhibition by GNF-6231 vs. C-113 may be a crosstalk between Wnt and another pathway such as TGFβ occurring at the cell membrane (with receptors) in the cytoplasm, and hence inhibited by GNF-6231 and not by C-113, which acts further downstream than Wnt secretion. Work by Duffield's group in the

UUO model of kidney fibrosis showed that Wnt pathway inhibition by Dkk-1, but not by XAV-939 inhibited the TGFβ-mediated effect on myofibroblast activity post-injury (Ren et al., *Proc Natl Acad Sci U S A* 2013), supporting our thinking that C-113 may not elicit the anti-fibrotic effect of GNF-6231. Both GNF-6231 and Dkk-1 (by targeting LRP6) (Bryja et al., *Mol Biol Cell* 2009) (Tahinci et al., *Development* 2007; Cselenyi et al., *Proc Natl Acad Sci U S A* 2008) can modulate both the canonical and non-canonical arms of the Wnt pathway, while C-113 and XAV-939 selectively target β-catenin stabilization (canonical arm of the Wnt pathway). This pathway selectivity may also be partially responsible for the observed differences in healing. The role of the non-canonical Wnt pathway in injury repair has previously been described (Le Grand et al., *Cell Stem Cell* 2009; von Maltzahn et al., *Nat Cell Biol* 2012; Schmeckpeper et al., *J Mol Cell Cardiol* 2015). More detailed studies will be needed to pinpoint the differences in effects of GNF-6231 vs. C-113. Taken together, these observations provide an impetus for investigating the detailed effects of drugs that target further downstream components of the pathway (e.g. CRT or β-catenin responsive transcription discussed in Chapter 1). Examples of this approach are the small molecule ICG-001 and the second generation PRI-724 currently in clinical trials (Miyabayashi et al., *Proc Natl Acad Sci U S A* 2007; Kahn, *Nat Rev Drug Discov* 2014). These reports highlighted the advantage of selective targeting of specific transcription co-factors that associate with β-catenin, particularly when a narrow and well-known set of target genes are responsible for the phenotype whose attenuation is sought. This approach, although challenging in terms of drug development with a protein binding screen rather than reporter activity based-screen, has a clear benefit of avoiding unintended, off-target effects.

Overall, the work in this thesis project highlights the potential of therapeutic Wnt inhibition in promoting regenerative tissue repair in two very different models of tissue injury

(outlined in **Fig 31)**. With complex and contradicting roles of the Wnt pathway reported by multiple studies in tissue regeneration, our study, adds to the increasing body of data (Henderson et al., *Proc Natl Acad Sci U S A* 2010; Hao et al., *J Am Soc Nephrol* 2011; Laeremans et al., *Circulation* 2011; Beyer et al., *Ann Rheum Dis* 2013; Sasaki et al., *PLoS One* 2013; Uitterdijk et al., *Lab Invest* 2016) that provide precedent for advancing Wnt inhibitory therapeutics to reduce fibrosis and promote regeneration.

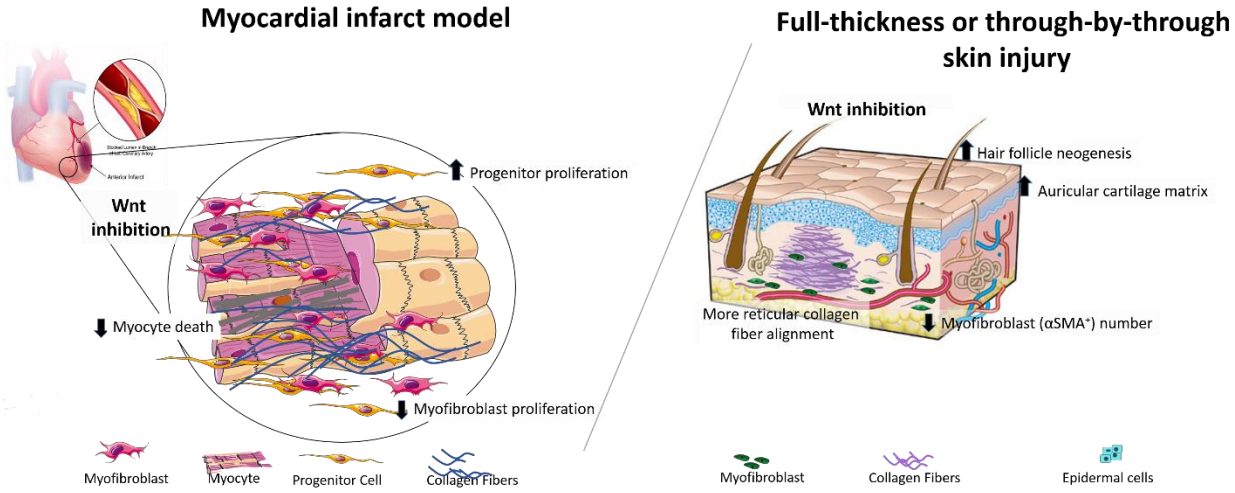

Figure 31: Model summarizing the data outlined in this dissertation
Left panel shows that in the myocardial infarct model, Wnt inhibition promotes increase of myocyte proliferation, reduces cardiomyocyte death, lowers myofibroblast proliferation and by extension, collagen matrix secretion. Right panel represents the reduction in fibrosis (reduced αSMA^{+} myofibroblast staining, and more reticular arrangement of collagen fibers in the wound bed), increase in hair follicle neogenesis and increase in synthesis of new cartilage matrix in the ear injury model.

Limitations and Future outlook

Our work elucidates the potential of pharmacologic Wnt inhibition in regenerative tissue repair, and uncovers some of the cellular and physiological basis for the regenerative repair in cardiac and cutaneous injury models. However, there are many unanswered questions, and hence many avenues this work could progress into.

Molecular mechanisms of Wnt inhibition-mediated regeneration: the proliferating cardiac cells

The primary lingering question in both cardiac and cutaneous regeneration by Wnt inhibition is the molecular mechanisms. In the heart, we have uncovered the cell types affected and the type of effect incurred by Wnt inhibition (e.g. increased proliferation of Sca1 progenitors *in vitro*, reduced cell death of cardiomyocytes post-infarct, reduced proliferation of myofibroblasts and reduced Col1α1 expression by isolated cardiac myofibroblasts *in vitro*). However, the Wnt target genes driving these wide-ranging cellular phenotypes are yet to be determined. To begin to understand the Wnt target genes involved, we plan to utilize the CyclinB1-GFP mouse (Klochendler et al., *Dev Cell* 2012) in which proliferating cells express GFP. By Flow sorting for the GFP^+ cells, we can not only identify the types and relative proportions of cells that are proliferating in response to Wnt inhibition, but also identify the changes in gene expression programs in proliferating cells by RNA sequencing.

Fibroblast populations as drivers of cardiac fibrosis

Cardiac fibrosis and fibroblast biology are another important aspects of cardiac repair, with direct link to the Wnt pathway activity (Chen et al., *Biochim Biophys Acta* 2013). With interest in identifying the specific populations of fibroblasts that are activated post-infarct and their respective contributions to repair, we are pursuing two distinct post-injury fibroblast

populations expressing either FSP1 or αSMA. Our goal is to identify the molecular signals emanating from these distinct cell populations in response to injury and to Wnt inhibition.

Molecular mediators of cardiac fibrosis: crosstalk with the TGFβ pathway

At a molecular level, an interesting avenue of exploration is the specific set of signals that act downstream of, or in conjugation with, the Wnt pathway leading to fibrosis. Work by multiple groups in the context of disparate injury models have shown that Wnt pathway activation leads to increased Collagen1 expression, fibroblast proliferation or fibrosis (Alman et al., *Am J Pathol* 1997; Kim et al., *Tohoku J Exp Med* 2011; Beyer et al., *Ann Rheum Dis* 2013). Crosstalk with the TGFβ pathway has been implicated in at least a subset of these studies (Chen et al., *Arterioscler Thromb Vasc Biol* 2011; Akhmetshina et al., *Nat Commun* 2012), but only few, mostly underdeveloped, hypotheses have been advanced to explain how the Wnt/β-catenin and TGFβ crosstalk might be occurring, have been advanced. For example, Akhmetshina *et.al.* reported that the Wnt-mediated increase in fibrosis likely occurs indirectly through new intermediate gene products rather than through direct effect on Col1A1 or αSMA gene expression. The authors proposed this based on absence of TCF binding to the Col1A1 or Acta2 promoters, and based on the observation that Wnt1-mediated increase in Col1α1 mRNA was inhibited by cycloheximide treatment (Akhmetshina et al., *Nat Commun* 2012). Work by Ren *et.al* demonstrated that in a kidney fibrosis model, the Wnt/β-catenin pathway activity is likely contributing to fibrosis by interaction between LRP6 receptor and TGFβ receptor I (TGFβRI) at the cell membrane, since Dkk-1, which binds to LRP6, inhibited the Wnt-driven fibrogenic differentiation of pericytes, and their migration, but XAV-939, which acts in the cytoplasm, did not have an effect (Ren et al., *Proc Natl Acad Sci U S A* 2013). The authors also demonstrated that LRP6 and TGFβR1 co-immunoprecipitate indicating a co-receptor interaction that occurs at

the cell membrane. GNF-6231 but not C-113 elicited a positive cardiac healing outcome reinforcing this hypothesis. However, we observed that C-113 inhibited Col1A1 gene expression in myofibroblasts *in vitro* (**Chapter 2, Fig 18B)**, whereas XAV-939 did not: a result that may be better explained by an independent role for axin 2 or tankyrases (targets of XAV-939 activity) in mediating fibrosis. To complicate things further, direct interaction of TGFβ-responsive transcription factors, Smads, together with β-catenin and TCF in the promoter region of Wnt/TGFβ co-operative target genes have also been reported (Hu et al., *Development* 2005; Labbe et al., *Cancer Res* 2007). Attisano and colleagues showed that in the APC mutant *Min* mice, colorectal adenomas ectopically expressed the cooperative Wnt/TGFβ target gene, *Inhba,* or Activin A, in epithelial cells, whereas in normal colon, Activin A expression would be localized to stromal cells (Labbe et al., *Cancer Res* 2007), demonstrating the importance of cellular context in these interactions. Moreover, a crosstalk with the non-canonical arms of the Wnt pathway can also partly account for the differences in healing observed by both Ren *et. al (Ren et al., Proc Natl Acad Sci U S A 2013),* and in our studies of C-113 vs. GNF-6231. As can be gleaned from this discussion, the mechanism by which Wnt pathway promotes fibrosis, and crosstalk with TGFβ signaling pathway is far from clear, and a difficult, yet important signaling network to understand.

β-catenin transcription co-factors: determinants of cell-fate decisions

At the nuclear level, the histone acyltransferases (HATs) CBP and p300 that act as β-catenin transcription co-activators, have been described as important determinants of progenitor cell fate decisions by dictating transcription of separate sets of Wnt/β-catenin target genes in the context of ESCs, neuronal progenitors and other cells (Teo et al., *Proc Natl Acad Sci U S A* 2005; Miyabayashi et al., *Proc Natl Acad Sci U S A* 2007). The reported improvement in

regeneration with an inhibitor targeting specifically the CBP-mediated transcription in myocardial infarct, pulmonary fibrosis and renal fibrosis models increases enthusiasm for such targeted approach to inhibition of Wnt target gene transcription (Henderson et al., *Proc Natl Acad Sci U S A* 2010; Hao et al., *J Am Soc Nephrol* 2011; Sasaki et al., *PLoS One* 2013).

Proposed models and future studies

Taking into account all of these divergent ideas, a model (represented in **Fig 32**) that seems to be emerging is that in different cells within a tissue, one or more molecular mechanisms and pathway interactions initiated by the Wnt pathway may cause the phenotypes observed. For example, the CBP versus p300 interactions in the nucleus may account for proliferation of cardiac progenitors and myogenic differentiation ($GATA4^+$ nuclei) we observed in the heart. Since GNF-6231 does not selectively target one of these co-activators, the effect we observed may be explained by the relative abundance of CBP vs. p300 in progenitors at different stages of lineage commitment. Both differentiation and proliferation could be occurring in these progenitors at the same time, depending on where in the spectrum of stemness vs. lineage commitment each of the progenitor cells lies. Immunoblotting for relative abundance of these co-activators, and β-catenin binding affinity (by immunoprecipitation) for CBP vs. p300 in progenitors at different stages of differentiation, combined with Chromatin immunoprecipitation to assess abundance of these transcription co-factors at the promoters of activated Wnt target genes may give an idea of the validity of this hypothesis. In the context of ear injury, the lineage commitment of undifferentiated mesenchymal cells to chondrogenic or osteogenic progenitors is directly linked to the Wnt pathway (Day et al., *Dev Cell* 2005). Although we cannot assume that the same co-activators are involved in this fate decision, other chromatin modifying proteins that act as β-catenin co-activators may be involved. Recent reports of other β-catenin co-activators,

such as Bcl-9, that do not have HAT activity, but may act as β-catenin scaffolds to direct target gene transcription specific to cellular contexts may be implicated in cell specific effects of Wnt signals (Mosimann et al., *Nat Rev Mol Cell Biol* 2009). Overexpressed BCL-9 reportedly has a marked preference to augment reporter-based beta-catenin responses in lymphoid cells in comparison to fibroblasts—and hence its relative abundance could be a determinant of the effects in both models we used, since fibrosis is an important part of the pathology in these models.

Similarly, in both cardiac and dermal fibroblasts, we observed a reduction in collagen and/or αSMA gene expression with C-113. Although we did not directly examine these target genes in the infarcted heart in response to GNF-6231 treatment, the reduction in infarct size, and in proliferation of αSMA positive myofibroblasts with GNF-6231 suggests a possible combination of interactions between Wnt and TGFβ receptors at the cell surface, and between β-catenin, TCF and Smads in the nucleus as discussed in the preceding section. In unpublished work, we have found that sFRP2 inhibits Smad2/3 phosphorylation in fibroblasts, and that the inhibition is reversed by pre-treatment with heparin, indicating an interaction with TGFβ receptors at the cell membrane rather than an indirect effect through Wnt target gene transcription. To examine the role of these interactions in the phenotype observed in our studies, in future experiments, we could assess Wnt/TGFβ co-operative target gene transcription (Labbe et al., *Cancer Res* 2007) in cells with selective ablation of receptors or in response to treatment with inhibitors of these receptors. For example, in fibroblasts after LRP knock-down or treatment with LRP blocking antibodies (currently in development by our collaborator Dr. Ethan Lee), stabilization of β-catenin would be predicted to have no effect on Smad phosphorylation or Collagen expression if the cross-talk between the pathways occurred primarily at the cell

membrane/receptor level. Alternatively, if we express dominant negative TCF and/or destabilize β-catenin, treatment with Wnt ligand would be predicted to not increase TGFβ responsive target gene activation if the interaction between the two pathways is mediated primarily by TCF/β-catenin/Smad interaction at the nucleus. However, if we observe an increase in TGFβ target gene expression upon Wnt3a treatment in these cells, we could assume that Wnt-TGFβ pathway interaction can occur independent of their direct interaction at the nucleus, and certainly independent of β-catenin target gene expression (Summarized in **Fig 33)**.

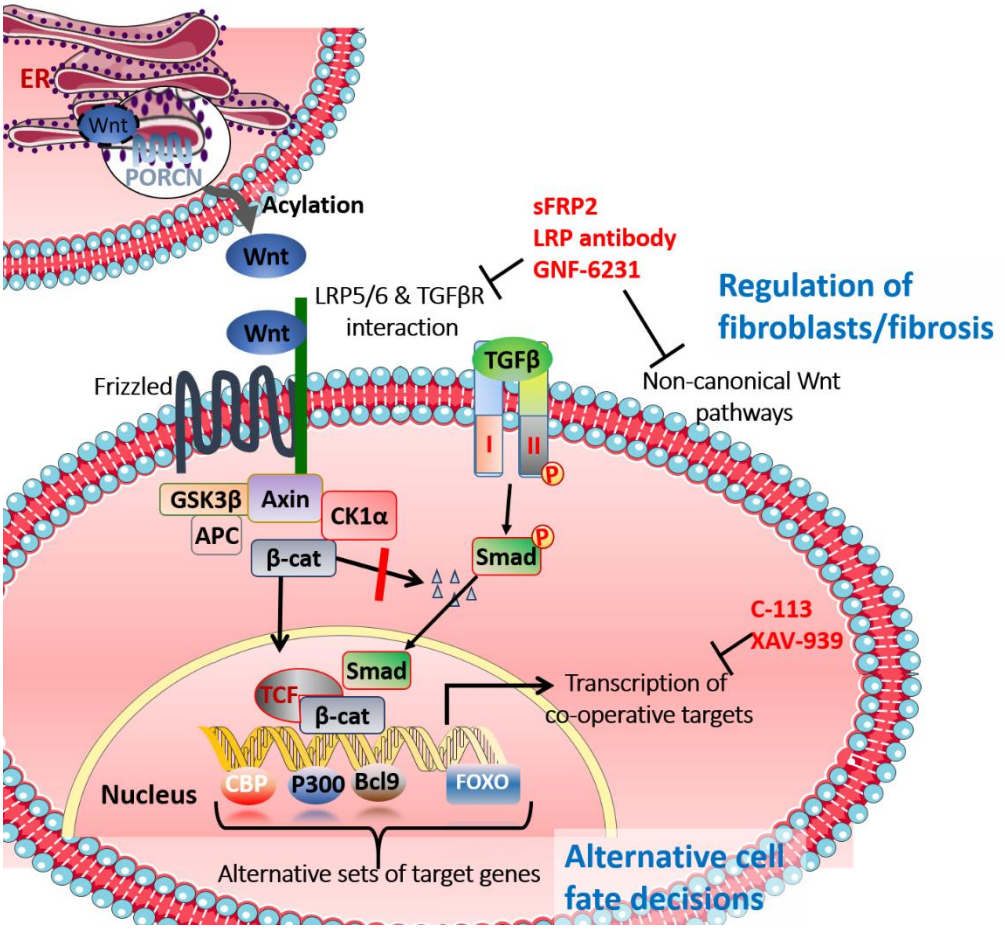

Figure 32: Model summarizing the possible molecular signaling and interactions driving phenotypes observed in our studies

At the cell membrane, LRP5/6 co-receptors may interact with TGFβ receptors. Inhibitors such as sFRP2, LRP antibody, Dkk1 or GNF-6231 would likely affect this interaction possibly resulting in reduced fibrosis. Most of these inhibitors would also inhibit the non-canonical arms of the Wnt pathway eliciting distinct effects. In the nucleus, Smad is reported to interact directly with β-catenin and TCF affecting transcription of Wnt/TGFβ co-operative targets. These target genes, as well as independent targets of β-catenin, but not Smad, transcriptional activity would likely be inhibited by C-113 or XAV-933. Also, CBP vs. p300 may direct cell fate decisions, proliferation etc of progenitors in the heart, or lineage specification of mesenchymal cells into cartilage in ear injury. Targeted inhibitors such as ICG-001 may specifically target this aspect of Wnt signaling.

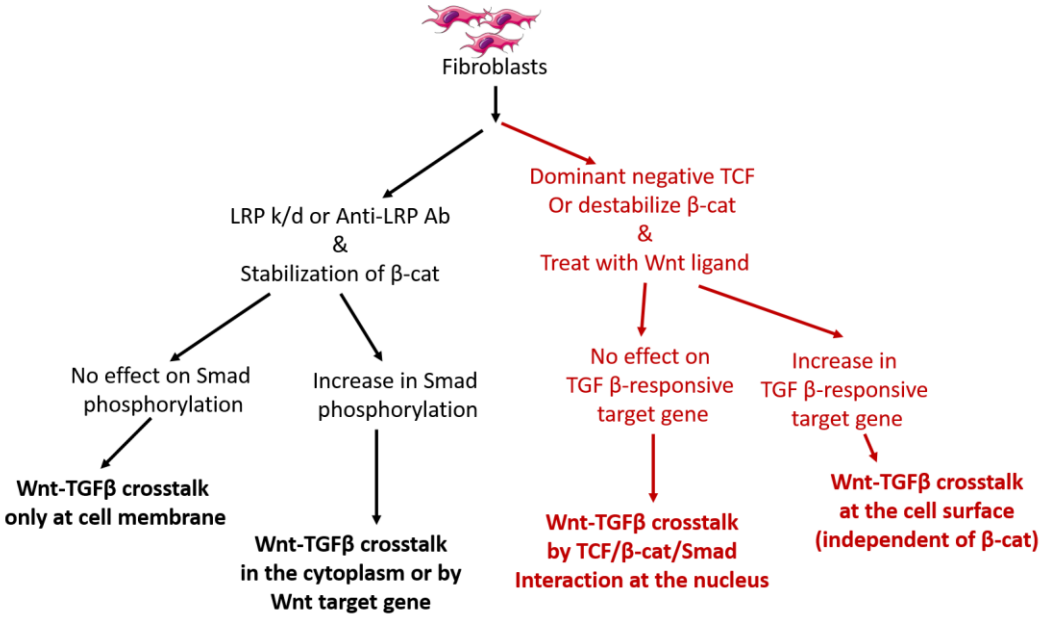

Figure 33: Decision tree summarizing predictions based on experiments to illustrate the crosstalk between Wnt/β-catenin and TGFβ signaling pathways.

Predictive models and systems biology approaches to future studies of Wnt pathway

Since one would expect significant number of feedback loops and cross-talks with other pathways occurring in parallel, these experiments, even if successful at yielding clearly interpretable results, may only provide a partial picture of the mechanisms at play in the tissue. To make inferences at the whole organism level, more outside-the-box approaches, such as computational modeling and predictive algorithms may have to be applied.

Some interesting work have been done with systems biology approaches. In 2003, Lee and Kirschner used a system of differential equations to predict the kinetics of axin and APC in the Wnt pathway in a cell-free system (Lee et al., *PLoS Biol* 2003). Unrelated to the Wnt pathway, the Cellular Potts model, used initially by Graner and Glazier for cell sorting simulation (Graner et al., *Phys Rev Lett* 1992) has been adopted by multiple investigators for making cell clustering predictions. Osborne recently adopted the model to describe colonic crypt cell shape, distribution and motility in colorectal cancer (Osborne, *Cancer Inform* 2015). In very exciting work, the Levin group has devised an *in silico* system that integrated literature on planarian regeneration to make accurate predictions of the complex signaling networks that would result in the dynamic regulation of patterning, growth and form during planarian regeneration (Lobo et al., *PLoS Comput Biol* 2015). This innovative work exemplifies the power of novel interdisciplinary approaches that combine knowledge gained from traditional molecular biology with constantly evolving machine learning capabilities. A complicated system of signaling such as the Wnt pathway, which varies widely among cells and microenvironments, would be the ideal avenue for trying to develop a predictive model. Although such sophisticated computational biology is beyond the expertise of this laboratory, it is very exciting to imagine that when these approaches are used in the context of Wnt signaling networks and continue to

improve, our abilities to make accurate predictions about the effect of Wnt pathway in disease and development could increase exponentially in the near future.

Wnt/β-catenin signaling in the dermis: a study of isolated compartments

These questions and conundrums regarding molecular mechanisms also apply to understanding fibrosis in the skin injury model. With the clear separation of the dermal and epidermal compartments unique to this model, and the differences in Wnt mediated signaling in these compartments, investigating Wnt driven signals separately in the dermis is likely to provide insights into the mechanisms of healing by Wnt inhibition. We have started to investigate this with RNA sequencing of dermis isolated from mouse dorsum following injury and topical treatment with Wnt inhibitor or vehicle. Our preliminary analysis of gene expression data obtained suggests that there is a clear difference between the gene expression programs turned on at different time points in Wnt inhibitor vs. vehicle treated tissue **(Fig 34)**. Further analysis of the data is likely to yield clearer picture of the relevant gene expression programs implicated. Additionally, the non-canonical arms of the Wnt pathway, with demonstrated roles in organogenesis and/or response to injury or homeostatic turnover in both heart and skin (Wang et al., *J Biol Chem* 1998; Widelitz, *Organogenesis* 2008; Schmeckpeper et al., *J Mol Cell Cardiol* 2015), would be important in the future to investigate in the context of our injury models.

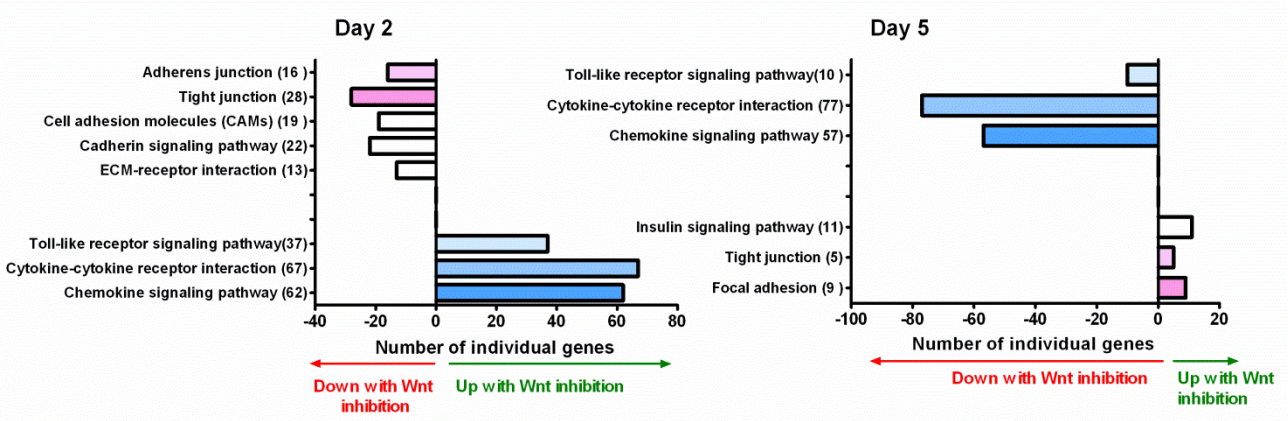

Figure 34: Wnt inhibitor treatment activates distinct set of gene expression programs in the dermis at day 2 and 5 post injury.
Gene ontology analysis of RNA sequencing data from dorsal dermis at day 2 and 5 post injury shows that inflammatory genes are upregulated at day 2 post-injury (left panel) in Wnt inhibitor-treated tissue compared to vehicle treated (blue bars), whereas cell-cell adhesion related genes are downregulated. At day 5 (right panel), the inflammation-related genes are down while the cell-to-cell interaction related genes are starting to go up in Wnt inhibitor-treated tissue.

Final remarks

The data that we have presented in this dissertation provide a starting point for future studies, some of which have been outlined above. These studies would be instrumental in advancing our understanding of the diverse role of the Wnt pathway in mammalian tissue repair and fibrosis. More excitingly, building upon this work could lead to development of Wnt inhibitory therapeutics for promoting regeneration. We are confident that with continuing data on safety and efficacy, and future preclinical studies with better injury models (e.g. swine), we can achieve success in developing a clinically feasible and safe Wnt inhibitor for tissue repair, and make considerable progress in driving tissue repair towards regenerative as opposed to fibrotic mode.

APPENDIX I

THE LIMITATIONS OF CASEIN KINASE I ALPHA ACTIVATION FOR SYSTEMIC WNT INHIBITION POST-INFARCT

Introduction

In Chapter 2, we discussed data suggesting an important role for pharmacologic inhibition of the Wnt pathway in promoting cardiac repair post-MI. Based on our data we posit that an important cellular mediator of cardiac recovery that we observed was post-infarct proliferation of myogenic progenitors driven by Wnt inhibition. This hypothesis is also based on previously published work from our group, which showed that therapeutic Wnt inhibition contributed to a proliferative response in the infarcted heart (Saraswati et al., *PLoS One* 2010). This appendix discusses unpublished work using C-113, which showed that CK1α activation and resulting Wnt inhibition had a proliferative effect on cardiac cells but also resulted in unexpected off-target effects.

Results

C-113 treatment enhanced proliferation of cardiac cells post-infarct in vivo and isolated Sca1$^+$ cells in vitro

Previously published work from our group had shown that a single intra-cardiac administration of Pyrvinium, an anti-helminth reported to inhibit Wnt/β-catenin signaling by CK1α potentiation (Thorne et al., *Nat Chem Biol* 2010), reduced adverse cardiac remodeling post-infarct (Saraswati et al., *PLoS One* 2010). **(Fig 1)**. Although, the study by Saraswati *et.al.* was limited by the toxicity of pyrvinium, the availability of C-113, a non-toxic analog of pyrvinium (Thorne et al., *Nat Chem Biol* 2010), enabled us to continue further studies to examine the mechanism by which pyrvinium, despite its toxicity, improved cardiac recovery with a single intra-cardiac injection.

Saraswati *et. al.* had shown that pyrvinium-treated hearts had higher numbers of Ki67 positive cells (Saraswati et al., *PLoS One* 2010). We asked whether these observations would be replicated with C-113, and whether we could achieve a superior healing outcome with the less toxic Wnt inhibitor C-113.We treated mice with intraperitoneal injection of 40mg/kg/day of C-113 or control (211, a non-functional analog of C-113) daily from day 1 through 4 following infarct. At day 7 post-infarct, harvested hearts were sectioned and stained for Ki67 **(Fig 35 A)**. We found that C-113 treatment significantly enhanced proliferation, as indicated by proportion of $Ki67^+$ nuclei per 10x section. To identify whether the proliferative cells were progenitors, we performed BrdU incorporation assay on isolated $Sca1^+$ cardiac progenitors (characterized in **Fig 13**), and found that treatment with recombinant Wnt3a reduced proliferation of $Sca1^+$ progenitors, and while C-113 reversed this effect **(Fig 35 B-C)**.

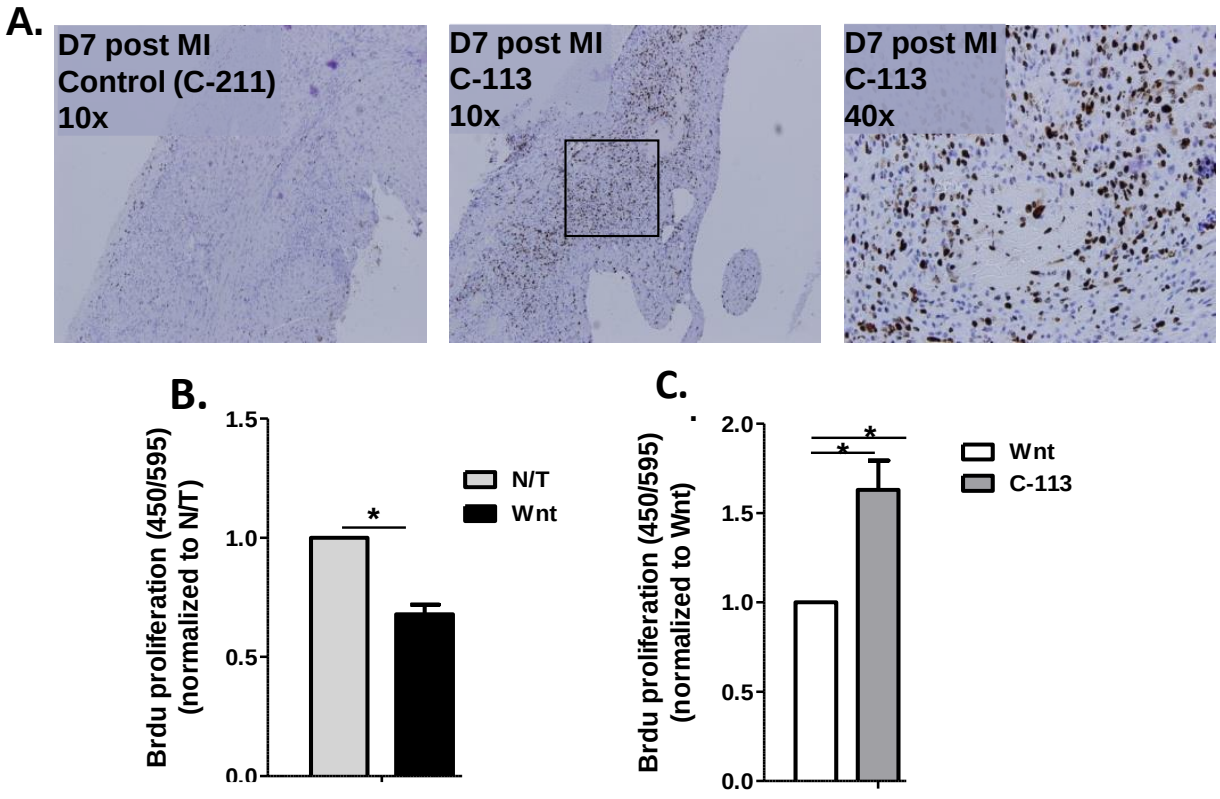

Figure 35: Treatment with C-113 induces cardiac cell proliferation in the infarcted heart, and of cardiac-derived Sca1$^+$ cells *in vitro*.

(A) Representative images of paraffin sections of peri-infarct region of ventricles stained with Anti-Ki67 antibody by immunohistochemistry from mice treated with 40mg/kg/day of C-113 or control (211) from day 1 through 4 post-MI. The right panel shows 40x magnification of the boxed area. Relative BrdU incorporation by Sca1$^+$ cardiac cells in response to **(B)** recombinant Wnt3a**,** and **(C)** Wnt3a and/or C-113 indicating that C-113 rescues the anti-proliferative effect of Wnt3a.

C-113 does not improve post-MI healing outcome, although Wnt inhibition is achieved with the dose, and no cardiac toxicity is observed in uninjured mice.

We built on our previously published findings with pyrvinium showing that post-MI Wnt inhibition prevents adverse cardiac remodeling (Saraswati et al., *PLoS One* 2010). Mice that received surgical LAD ligation were treated with 4 daily intraperitoneal injections of 40mg/kg C-113, based on our bioavailability study, which showed that the C-113 was bioavailable in most tissues at a dose of 40mg/kg **(Fig 36A)**. This dose produced significant inhibition of Wnt target gene expression **(Fig 36B)**. However, echocardiography at day 7 and 30 showed increases in adverse cardiac remodeling, indicated by increase in the Left ventricular dimension **(Fig 36C)**, and decline % fractional shortening from day 7 to 31 **(Fig 36D)** in C-113 treated mice compared to control. This effect could not be counteracted by modulating the treatment regimen to a single injection post-infarct instead of 4. **Fig 36C and D)**. The same dose and regimen of C-113 in uninjured mice did not cause a decline in cardiac function or an increase in adverse remodeling **(Table 5)**.

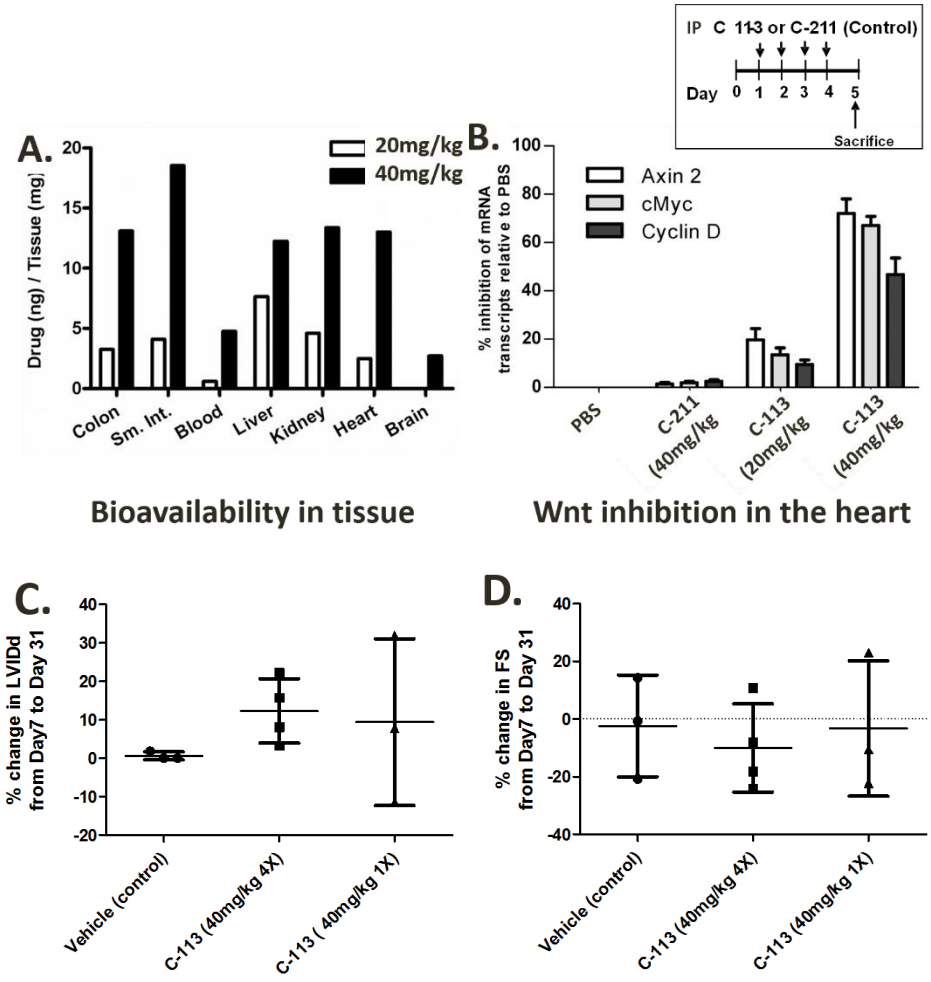

Figure 36: Treatment with C-113 does not improve recovery post-infarct, despite C-113 bioavailability and Wnt-inhibitory activity *in vivo*.

(A) Bioavailability study by LC-MS showed that the drug was bioavailable in most tissues except brain when administered at 20mg/kg and 40mg/kg. 40mg/kg was chosen as optimal dose to achieve Wnt inhibition in the heart. **(B)** qRT-PCR for Wnt target genes showing significant inhibition of Axin2, cMyc and CyclinD1 expression with 40mg/kg dosing of C-113. Inset shows experimental timeline for bioavailability and Wnt inhibitory activity studies. Left ventricular remodeling was measured as **(C)** % change in LVIDd, and LV function was measured as **(D)** % change in FS from day 7 to day 31 post-infarct. Data showed increase in left ventricular diameter (LVIDd) and decline or no improvement in cardiac function (%FS) upon treatment with 1 injection or 4 daily injections of 40mg/kg C-113 post-infarct.

Table 5. C-113 does not affect cardiac function and remodeling in uninjured mice

Echo parameters		C-113
LVIDd (mm)	DAY 7	3.645 ± 0.118
	DAY 30	3.575 ± 0.156
Δ LVIDd %		-1.832±5.69
LVIDs (mm)	DAY 7	2.093 ± 0.078
	DAY 30	2..005 ± 0.096
Δ LVIDs %		-4.077±5.989
FS %	DAY 7	42.600± 0.529
	DAY 30	43.750± 0.870
Δ FS %		2.714±2.550
EF	DAY 7	0.798 ± 0.004
	DAY 30	0.813 ± 0.008
Δ EF %		1.9±1.1
N		4

The mean±SD values for each parameter at day 7 and 30 are followed by the mean±SD percent difference between day 7 and day 30 for each mouse (Δ).

Collateral effects: C-113 treatment inhibits the Hedgehog pathway in vivo.

In order to understand the cause of the unexpected adverse healing outcome in infarcted heart by C-113 treatment, we searched the literature for other pathways that CK1α may interact with. Thorne *et. al* had previously shown that pyrvinium (and by extension, C-113) had no discernible effect on TGFα, BMP4, IL-4 and Notch pathways (Thorne et al., *Nat Chem Biol* 2010). However, published studies suggest that CK1α activation may result in inhibition of the Hedgehog signaling pathway by promoting partial degradation of cubitus interruptus (Ci), which is a Hedgehog-responsive transcription factor whose role in Hedgehog signaling pathway parallels that of β-catenin in the Wnt pathway (Price, *Genes Dev* 2006). In parallel, Ornitz and colleagues have demonstrated that Hedgehog pathway activity is necessary for maintenance of coronary vasculature in adult mice. Ablation of Hedgehog signaling leads to tissue hypoxia, cardiomyocyte cell death and subsequent lethality by loss of coronary blood vessels (Lavine et al., *J Clin Invest* 2008). We performed qRT-PCR for Hedgehog target genes Gli1 or Patched 1 in atria and cerebellum, which are tissues with high baseline Hedgehog activity in the adult mouse (Traiffort et al., *J Neurochem* 2010) **(Fig 37A-B)**, and in cardiac cells pre-treated with Smoothened Agonist (SAG), which activates Hedgehog signaling **(Fig 37C)**. We found that indeed, C-113 reduced Hedgehog target gene expression in these tissues and cells. Hence, the effect of C-113 on post-infarct cardiac recovery we had observed was, at least in part, mediated by CK1α inhibition of Hedgehog signaling. However, it is important to note that this may not completely explain our findings, because unlike mice with Hedgehog pathway ablation (by deletion of transmembrane receptor Smoothened) in the heart described by Ornitz and team (Lavine et al., *J Clin Invest* 2008), C-113 treatment did not result in cardiac pathology in uninjured mice **(Table 5)**.

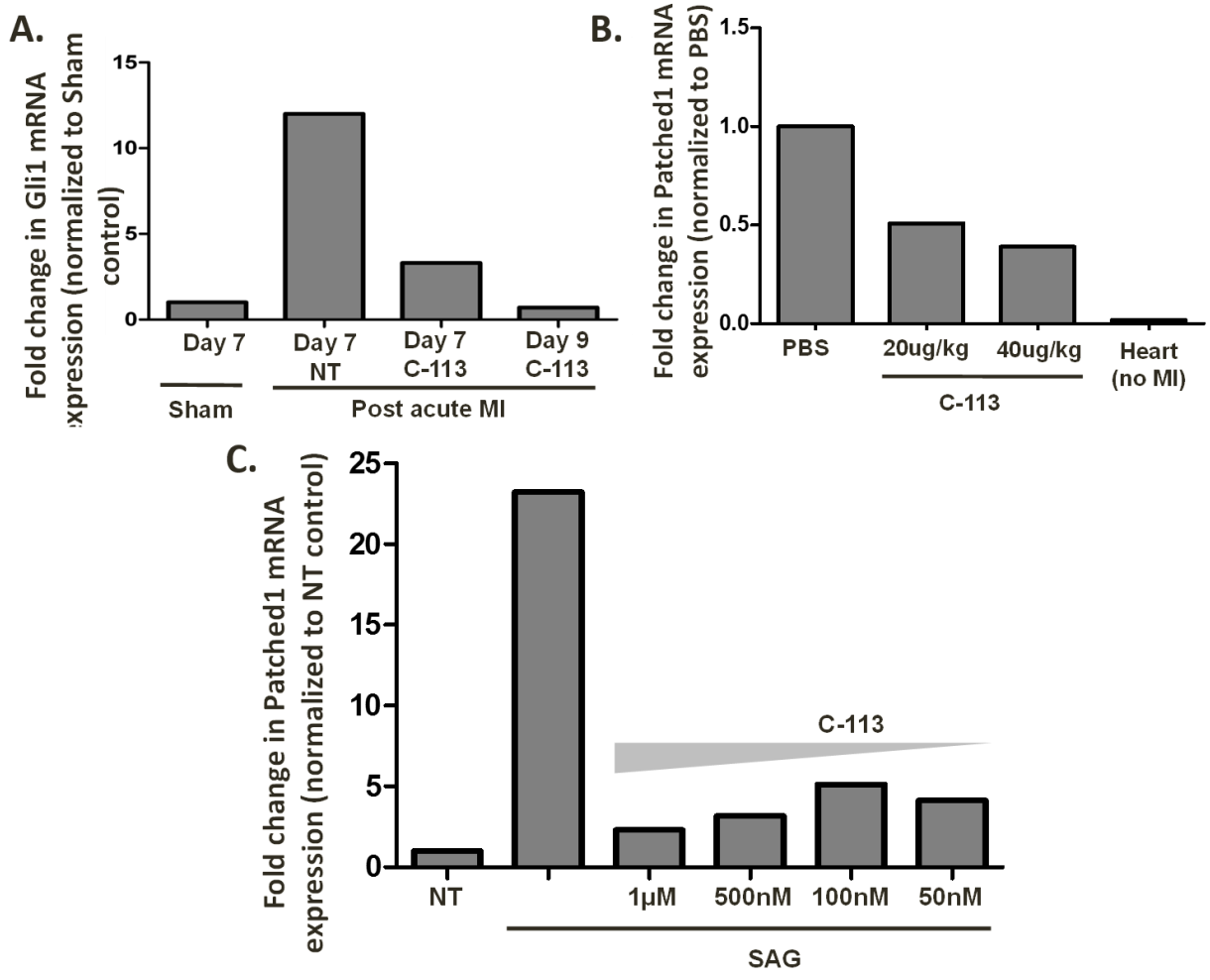

Figure 37: C-113 inhibits Hedgehog target gene expression in mouse atria and cerebellum, and in cardiac cells *in vitro*.

(A) Fold change in gene expression of Gli1 in mouse atria at day 7 and 9 post infarct with vehicle or C-113 treatment **(B)** qRT-PCR for Patched1 showing a dose-responsive reducetion in gene expression in the mouse cerebellum by C-113 treatment. **(C)** qRT-PCR for Patched1 in cardiac $Sca1^{+}$ cells pre-treated with Hedgehog agonist SAG showing a significant reduction in Patched1 gene expression with C-113 treatment at a range of concentrations.

Conclusion

These data although preliminary, point to an important need to carefully examine the collateral effects of Wnt inhibitors. Although the use of C-113 for cardiac regeneration is precluded by its effect on the Hedgehog pathway, it still has potential for improving regenerative healing of other tissues that do not require Hedgehog pathway activity for homeostasis or response to injury. Our study with skin wound model described in Chapter 3 showed that the CK1α activators pyrvinium and C-113 were both successful in promoting regenerative wound repair. This finding elucidates not only the potential of C-113 as a Wnt therapeutic, but also the benefit of topical or targeted drug delivery in tissues to counteract the issue of systemic toxicity or off-target effects on unintended tissues. Careful dose escalation is also important in achieving optimal healing outcomes while minimizing unintended effects and toxicity.

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