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Beta-catenin-dependent Wnt signaling: a pathway in acute cutaneous wounding

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Abstract

Background—Acute wound healing is a dynamic process that results in the formation of scar tissue. The mechanisms of this process are not well understood; numerous signaling pathways are thought to play a major role. Here, we have identified β -catenin-dependent Wnt signaling as an early acute phase reactant in acute wound healing and scar formation.

Methods—We created 6 mm full thickness excisional cutaneous wounds on adult β -catenin-dependent Wnt signal (BAT-gal) reporter mice. The expression of canonical Wnt post-wounding was analyzed using X-gal staining and quantitative real-time polymerase chain reaction (qRT-PCR). Next, recombinant mouse Wnt3a (rmWnt3a) was injected subcutaneously to the wound edge, daily. The mice were sacrificed at stratified time points, up to 15 days post-injury. Histological analysis, qRT-PCR, and western blot were performed.

Results—Numerous individual Wnt ligands increased in expression after wounding, including Wnt3a, Wnt4, Wnt10a, and Wnt11. A specific pattern of Wnt activity was observed, localized to the hair follicle and epidermis. Mice injected with rmWnt3a exhibited faster wound closure, increased scar size, and greater expression of FGFR2 and Type I Collagen.

Conclusion—Our data suggest that β -catenin-dependent Wnt signaling expression increases shortly after cutaneous wounding and exogenous rmWnt3a accelerates re-epithelialization, wound matrix maturation, and scar formation. Future experiments will focus on the intersection of Wnt signaling and other known pro-fibrotic cytokines.

INTRODUCTION

Wound healing is a dynamic and synchronized process involving inflammation, cell proliferation and migration, extracellular matrix (ECM) deposition, and remodeling. Wound healing can be divided into four distinct but overlapping phases: The first phase is hemostasis, which includes vasoconstriction, platelet plug formation, and activation of the coagulation cascade. The second phase includes the inflammatory phase, which involves the

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localization, diapedesis, and degranulation of inflammatory cells. The third phase, or granulation phase, consists of 4 sub-phases including: a) fibroplasia, b) provisional matrix deposition, c) angiogenesis, and d) re-epithelialization. The fourth phase is the remodeling phase.¹

There have been a number of publications that have demonstrated that β -catenin may play an important role in wound healing.¹⁻⁶ The level of β -catenin is tightly regulated by a triad of proteins which consist of GSK3- β , Axin, and APC. These proteins phosphorylate the amino terminal Ser/Thr residues of β -catenin. When these residues are phosphorylated, β -catenin is targeted for ubiquitin-mediated proteosomal degradation. Wnt is a family of proteins that is a key component of embryological development but is also involved in all phases of the wound healing response.⁷ When Wnt binds to their receptor a family of trans-membrane proteins known as Frizzled and their co-receptors, LRP5 or LRP6, Dishevelled phosphorylates Axin, hence inhibiting the phosphorylation of β -catenin. β -catenin is allowed to accumulate in the cytosol and enter the nucleus, where the transcriptional repressor Groucho is displaced and β -catenin binds to the TCF/LEF transcription factor permitting gene transcription. This is referred to as the canonical Wnt pathway. One of the transcribed genes is Axin2 which accumulates in the nucleus and forms a new triad with the GSK3- β and APC which phosphorylates β -catenin and targets it for ubiquitination. Hence, Axin2 plays a major role in the negative feedback loop system of the canonical Wnt pathways; it can also be used as a surrogate marker for the activation of Wnt protein.

Wnt has been implicated in wound healing and the transition from fetal scarless to adult scar-forming repair.^{6,7} When a topical application of liposomal Wnt3a was used to treat a non-healing wound, endogenous Wnt signaling was enhanced and led to better skin wound healing.⁸ Another study has shown that Wnt inhibition via topical application of small molecule Wnt inhibitors can reduce fibrosis and promote regenerative cutaneous wound repair.⁹ Beyond wound repair, Wnt has also been implicated in hair follicle regeneration.^{10,11} Secreted factors of the Wnt and fibroblast growth factor (FGF) signaling pathways can stimulate hair follicle regeneration after wounding. Recent findings suggest that hair follicles, in turn, can change the fate of myofibroblasts (a known cellular player in scar formation) into adipocytes through a signaling pathway involving bone morphogenetic protein (BMP).¹² The implications for these findings are huge: Wnt, FGF, and BMP treatment could present a biphasic strategy to decrease scar formation and promote skin regeneration.¹³

In our previous communication, we showed that recombinant mouse Wnt3a (rmWnt3a) up-regulated the transcription of low molecular weight hyaluronan in fibroblasts.¹⁴ These hyaluronan are subsequently digested to oligo-hyaluronan, which have been postulated in many studies to have angiogenic as well as pro-fibrotic effects.¹⁵ We also showed that rmWnt3a up-regulated the transcription of TGF- β 1 in fibroblasts *in vitro* in a dose-dependent manner.¹⁴ TGF- β 1 is known to be pivotal in wound healing in diverse aspects, such as tubulogenesis and neovascularization, as well as increasing the rate of re-epithelialization, in part by enhancing epithelial cell migration and proliferation.^{16,17} We also showed that rmWnt3a and recombinant TGF- β 1 seemed to have similar effects on postnatal dermal fibroblasts, including an increase in proliferation and an increased

expression of Type I Collagen.¹⁴ In this study, we demonstrated that canonical Wnt is one of the earlier proteins that gets translated post cutaneous wounding. We hypothesized that canonical Wnts may be a positive acute phase reactant similar to TGF- β 1 and may play a role in acute cutaneous wound healing.

To further advance our hypothesis we conducted the following experiments: First, we mimicked the early wound environment by placing dermal fibroblasts in hypoxia and assayed Wnt expression. Next, we evaluated Wnt expression post-injury in full thickness wounds of wild type CD-1 mice and β -catenin-dependent Wnt signal (BAT-gal) reporter mice. Finally, we injected rmWnt3a to the edges of full thickness cutaneous wounds and evaluated rate of wound closure, re-epithelization, and scar formation *in vivo*.

MATERIALS AND METHODS

Animals and Materials

Wild type CD-1 mice and BAT-gal mice were purchased from Charles Rivers Laboratories (Wilmington, MA). Animal procedures were conducted in accordance with university-approved protocols according to NIH guidelines. The RNeasy kit was from Qiagen (Valencia, CA). All other reagents for quantitative real-time polymerase chain reaction (qRT-PCR) were purchased from Applied Biosystems (Foster City, CA). rmWnt3a was from R&D Systems (Minneapolis, MN).

Cutaneous Wounding

For cutaneous injuries, the O-ring technique was performed on 21 day old mice as previously described.¹⁸ rmWnt3a (60 uL of a 100 ng/mL solution) or vehicle control (PBS) was injected daily to the wound edge (15 uL subcutaneously to four quadrants [rostral, caudal, left, right] of each wound) using a fine gauge needle (n=4 mice per group or 8 wounds per group). Tissues were harvested 0-15 days post-injury for analysis of histology and gene and protein expression.

Histology

Tissues were formalin fixed and paraffin embedded per standard protocol and sectioned at a thickness of 8 μ m. Routine stains included Hematoxylin and Eosin (H&E), Trichrome, and Picrosirius red. X-gal staining was performed in BAT-gal reporter mice to assess Wnt activity. Slides were fixed in 0.5% glutaraldehyde solution for 24 hrs, washed for 2 hrs in X-gal washing solution, and stained with X-gal solution (1%) for 24 hrs. Wnt3a *in situ* hybridization was performed as previously described.¹⁹

Histomorphometric and Morphometric Analysis

Rate of wound closure was assessed by gross morphometric measurements. A Nikon D40 camera was used for digital photographs, which were taken on postoperative day (POD) 0-15 (n=9 per group). The wound diameters (in mm) and areas (in mm²) were calculated from wound perimeter tracings by using photographic analysis with ImageJ software by a single blinded reviewer and confirmed by a second independent investigator. Wound areas

were interpreted as 1.00 on day 0 of wounding; wound areas on subsequent days were expressed as a fraction of the day 0 value.

Measurements of scar size were performed using Adobe Photoshop CS2. Representative slides per specimen were stained with Mallory's trichrome stain in which collagen fibrils stain blue. The total number of blue pixels was calculated using the magic wand tool (tolerance = 60, n=4 per group).

Primer Design and qRT-PCR

The gene specificities of the primers were tested by Blast in GeneBank and the PCR product from one primer pair showed a band of appropriate size by running on a 2% agarose gel. Primers were used as described in Table 1. Gene expression was assayed after cutaneous injury by qRT-PCR. Briefly, a meticulous dissection of the wound was performed accompanied by a minimal rim of uninjured skin. Groups were pooled, snap frozen in liquid nitrogen, and homogenized by sonication. Total RNA was isolated from each cell sample using RNeasy kit per the manufacturer's protocol. 1 ug RNA was reverse transcribed using TaqMAN® Reverse Transcription Reagents. qRT-PCR was performed in a 384-well plate with Power Sybr green detection on a 7900HT Sequence Detection System. GAPDH was used as internal control gene for data calculation. All detection was performed in triplicate wells.

Western Blot

Nuclear extraction was performed using the NE-PER kit pierce (Rockford, Illinois). Protein detection was performed with primary antibodies against β -catenin, TGF- β 1 (1:200 dilution), and β -actin (1:5000 dilution; Lab Vision, Fremont, CA) in 5% milk/Tris-buffered saline-T overnight at 4°C (n=3 per group). Blots were then incubated with the corresponding horseradish peroxidase-linked secondary antibodies (1:10,000 dilution; BD Pharmingen, San Jose, CA) for 1 hr at room temperature. Blots were developed with ECL detection reagent (Amersham, United Kingdom) and exposed for 1 to 10 mins using Biomax-MS film (Eastman Kodak, Rochester, NY).

Cell Isolation, Culture, and Assays

Postnatal dermal fibroblasts were isolated as previously described.¹⁴ Cells were cultured in growth medium, consisting of Dulbecco's Modified Eagle Medium (GIBCO Life Technologies, Carlsbad, CA), 10% FBS (Omega Scientific, Tarzana, CA) and 1% penicillin/streptomycin (GIBCO Life Technologies). Cells were kept at 37°C in a humid incubator with 5% CO₂. Passage 2-4 cells were used for all experiments.

Cellular proliferation was assessed by bromodeoxyuridine (BrdU) incorporation and cell counting assays, as previously described.^{14,20,21} Cell counting was performed in 12-well plates (5,000 cells/well) using 10% serum conditions; cells were counted by hemocytometer (n=3 per oxygen tension). BrdU assays were performed according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Briefly, cells were seeded in 96-well plates (1,000 cells/well) using 2% serum conditions (n=6 per oxygen tension). After 48 hrs, BrdU incorporation assays were performed.

Gene expression was assayed in cells after exposure to various oxygen tensions, as previously described.^{21,22} Briefly, cells were seeded at a density of 50,000 cells per well in 12-well plates. After attachment, cells were transferred to 21% or 1% oxygen tension in low serum conditions (2% FBS); gene expression was evaluated 48 hrs thereafter using the technique described above.

Statistical Analyses

Mean and standard deviation were calculated from numerical data, as presented in the text, figures, and figure legends. In graphs, columns represent means whereas error bars represent one standard deviation. Statistical analysis was performed using the ANOVA two-factor with replication when more than two factors were compared. In supplement, the Welch's two-tailed *t*-test was used when standard deviations between groups were unequal. Due to the nature of some experiments with limited sample size, * *p* 0.01 was considered to be significant.

RESULTS

Wnt Expression in Fibroblasts is Driven by Hypoxia

Dermal fibroblast proliferation in hypoxia (1% oxygen) was evaluated as compared to atmospheric conditions (21% oxygen). Using both cell counting and BrdU incorporation, dermal fibroblasts proliferated significantly faster in hypoxia (Fig 1A-B; **p*<0.01). Axin2 expression (as a marker of canonical Wnt signaling) was observed to significantly increase in hypoxia, as was Wnt4 expression (Fig 1C; **p*<0.01). Thus, hypoxia-induced fibroblast proliferation and a concomitant up-regulation of canonical Wnt signaling.

Endogenous Wnt Expression After Cutaneous Injury

Next, the temporospatial expression of Wnt signaling post-wounding was examined using BAT-gal reporter mice, as previously described.¹⁴ At 24 hrs after injury, there was strong Wnt signaling in the epidermis, hair bulge, and dermis, whereas in intact skin the signal was faint and restricted to the basement membrane and hair follicles (Fig 2 A-C). PCNA staining on adjacent sections found that X-gal positive staining coincided with cells that were also actively dividing (Fig 2D). Finally, numerous individual Wnt ligands were elevated at 24 hrs post-wounding in adult mouse skin, including >14-fold increase in *Wnt3a* (n=3) (Fig 2E; **p*<0.01). These findings were corroborated by performing *Wnt3a in situ* hybridization on wounded skin at POD 3 and 5 (See Figure, Supplemental Digital Content 1, *Wnt3a in situ* hybridization shows increased expression after wounding. *Wnt3a in situ* hybridization was performed on POD 3 and 5. *Wnt3a* mRNA is increased at POD 3 (AS=anti-sense, S=sense; n=3), INSERT HYPER LINK). *Wnt3a* mRNA gene expression was increased at POD 3.

Recombinant Wnt3a Speeds Wound Closure and Increases TGF-β1 Expression

Next, 6 mm full thickness skin wounds were created, which were splinted by a rubber ring to prevent wound contraction. An increased rate of wound closure was observed in mice treated with rmWnt3a, most prominently after POD 5 (Fig 3A-B; **p*<0.01). Nuclear β-catenin was assayed by western blot (Fig 3C). Increased nuclear accumulation of β-catenin was observed in the rmWnt3a-treated group. TGF-β1 also increased after rmWnt3a injection (Fig 3C).

Recombinant Wnt3a Increases Re-epithelization, Scar Formation, and FGFR2 Expression

By routine H&E, a clear difference in the extent and thickness of re-epithelization was noted (Fig 4). Control wounds had less re-epithelialization (Fig 4A, left) than rmWnt3a-treated wounds (Fig 4B, left). In comparison to control, rmWnt3a-treated wounds were noted to be hypercellular at POD 5. In addition, picrosirius red staining demonstrated a greater abundance of collagen fibrils in rmWnt3a-treated wounds (Fig 4A-B, right). In effect, rmWnt3a treatment results in a larger scar size in a short period of time after wounding. Further attempts were made to quantify scar size between control and rmWnt3a-treated wounds (Fig 5). Scar size was quantified by assessing mean pixel number in serial sections stained with Mallory's Trichrome (Fig 5A). At both 5 and 10 days postoperatively, a significantly greater scar was observed. Collagen expression was also evaluated by qRT-PCR: again, a significant increase in collagen transcript abundance was observed after rmWnt3a treatment (Fig 5B). Finally, FGFR2, a gene marker of epithelial cell migration and proliferation, was assayed by qRT-PCR. Results showed >2-fold increase in FGFR2 among rmWnt3a injected wounds in comparison to control on POD 3 (See Figure, Supplemental Digital Content 2, which shows Recombinant Wnt3a increases FGFR2 expression. FGFR2 expression in control-treated (white) as compared to rmWnt3a-treated wounds (grey) on POD 3. A significant increase in gene expression >2-fold was observed (n=3; *p<0.01), [INSERT HYPER LINK](#)).

DISCUSSION

Wnt proteins have important functions during development and regeneration.^{23,24} Expression of Wnt proteins in the developing skin, and their role in fetal versus postnatal wound healing has not been thoroughly investigated.^{6,25,26} In our previous communication, we evaluated scarless fetal and postnatal skin repair in a BAT-gal reporter mouse.¹⁴ β -catenin-dependent Wnt signaling was visible with X-gal staining in both fetal and postnatal mice. Wnt signaling increased dramatically during early postnatal skin repair (postnatal day 1). In contrast, Wnt signaling was unchanged during early scarless fetal cutaneous wound repair. Thus, enhanced Wnt signaling is associated with wounds that heal via a pro-fibrotic mechanism. High hyaluronic acid levels are associated with scarless wound healing,²⁷ and we found that Wnt significantly down-regulates hyaluronan synthase gene expression in postnatal fibroblasts.¹⁴ Wnt3a was observed to be a potent mitogen in cell culture, and induced Type I collagen expression as well as transcription of TGF- β 1 in cell culture. Moreover, these findings highly correlate with side-by-side experiments testing recombinant TGF- β 1 regulation of gene expression. Thus, rmWnt3a effected postnatal dermal fibroblasts in striking similarity to a known pro-fibrotic cytokine, and indeed we observed rmWnt3a to positively regulate TGF- β 1 transcription. These findings led us to conclude that β -catenin-dependent Wnt genes may be early and key regulators of wound healing and may play a role in scar formation; our present work is an extension of this line of investigation.

First, we added to our previous observations that postnatal wounding induced an up-regulation in Wnt expression and activity.^{5,14} Using a Wnt reporter mouse and qRT-PCR, we observed that cutaneous injury even to an adult mouse resulted in significantly increased Wnt expression circumscribing the wound within 24 hrs. Moreover, we observed that

numerous individual Wnt transcripts were up-regulated post-wounding, not limited as we previously observed to Wnt3a and Wnt4.¹⁴ A key regulator of gene expression in an early wound environment is hypoxia.²⁸ We hypothesized that hypoxia may be one of the triggering factors that induces Wnt expression after cutaneous wounding, hence its importance in wound healing. We tested this hypothesis by placing dermal fibroblast cell culture in a hypoxic environment (1% oxygen) to mimic the early wound environment. Indeed, within 48 hrs in this environment, fibroblasts showed a nearly 3-fold increase in Axin2, suggesting an increase in β -catenin-dependent Wnt signaling driven by hypoxia. Further studies are necessary to fully understand this relationship.

Our data thus far, as well as data from others in the literature, suggest a pro-fibrotic activity of β -catenin-dependent Wnt signaling.^{14,29-31} We directly tested this through the daily injection of recombinant Wnt3a to full thickness adult wounds. Our delivery of the lipophilic Wnt protein was successful, as it resulted in an increase in nuclear β -catenin by western blot. Moreover, an obvious phenotype resulted from Wnt3a injection, including faster wound closure, greater re-epithelization, and a larger scar matrix. These results corroborate the finding by Bastakoty *et al.* demonstrating that inhibition of Wnt leads to less scar formation. Although wounds would be expected to heal slower based on this prior study, the models are vastly different and conclusions cannot be drawn.⁹ Given these and other findings, manipulation of Wnt signaling may be a promising therapeutic modality to accelerate healing of chronic wounds or reduce scarring and promote regeneration. This lends further credence to the belief that wound healing has evolved to rapidly heal wounds at the cost of perfect regeneration.³²

There is mounting evidence that suggests increase in Wnt and TGF- β 1 transcript induce epithelial differentiation during wound healing.^{4,33} Furthermore, congruent results were obtained by Cheon *et al.* using conditional null or conditional stabilized alleles of β -catenin.¹ In their study, the size of the of the healed wounds were observed to correlate with the protein level of β -catenin. This high level of β -catenin was achieved by the injection of LiCl treatment, resulting in the concept that β -catenin signaling is central to wound healing and scarring. Important differences in experimental design exist between our study and those performed by Cheon *et al.*, most importantly, dysregulation of β -catenin expression and the use of LiCl. These two methods do not distinguish between the effect of Wnt ligands, but rather it is a disruption of the negative feedback loop system of the β -catenin-dependent Wnt signaling pathway. However, the physiological pathway involves the increase in translation of various individual Wnt proteins. Invariably, the difference in Wnt ligand expression levels in the skin during skin morphogenesis underlies their pluripotency, and no doubt serve different developmental purposes. Wounds were treated with rmWnt3a as representative of canonical Wnt protein, however the possibility that application of a different Wnt protein may have resulted in a substantially different outcome cannot be excluded. More research will focus on targeted up-regulation of other Wnt ligands known to be increased post-wounding.

CONCLUSION

β -catenin-dependent Wnt signaling expression increases after adult cutaneous wounding. These data suggest that exogenous rmWnt3a accelerates re-epithelialization, wound matrix maturation, and scar formation. Future experiments will focus on the intersection of Wnt signaling and other known pro-fibrotic cytokines and its potential clinical role in the treatment of chronic non-healing wounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Wnt expression in fibroblasts is driven by hypoxia

Postnatal dermal fibroblasts were cultured in normoxia (21% oxygen) or hypoxia (1% oxygen). (A-B) Proliferation was assessed by cell counting (A) and bromodeoxyuridine (BrdU) incorporation assays (B). (C) After 48 hrs in normoxic or hypoxic conditions, gene expression for Axin2 and Wnt4 by qRT-PCR. (n=3 for cell counting and gene assays; n=6 for BrdU incorporation; *p<0.01)

Figure 2. Endogenous Wnt expression post adult cutaneous injury

(A-B) X-gal staining in BAT-gal reporter mice, 24 hrs post-wounding. (C) Uninjured adult mouse skin for comparison. (D) PCNA staining on adjacent slide, demonstrating similar distribution of staining. (E) Wnt gene expression 24 hrs post-injury (red) in comparison to uninjured adult skin (grey), by qRT-PCR. (n=3; * p<0.01)

Figure 3. Recombinant Wnt3a speeds wound closure and increases TGF- β 1 expression
(A) Representative photographs of adult cutaneous wounds injected daily with a rmWnt3a suspension or control. Differential healing was observed beginning POD 5. (n=7) **(B)** Quantification of wound area in rmWnt3a or control wounds from 0-15 days. **(C)** Protein expression of nuclear β -catenin, cytoplasmic TGF- β 1, and β -actin at 0, 1, and, 5 days postoperatively, by Western blot. (n=3 for each time point)

Figure 4. Recombinant Wnt3a increases re-epithelization

(A) Routine H&E (left) and picrosirius red staining of control wound at POD 5. (B) H&E (left) and picrosirius red staining of rmWnt3a-treated wound at POD 5. A substantially thicker layer of re-epithelization was noted in Wnt-treated wounds on H&E. Greater picrosirius red staining, corresponding to increased collagen content was observed among rmWnt3a-treated groups. (n=3)

Figure 5. Recombinant Wnt3a increases scar formation

(A) Quantification of collagen pixels in Mallory's Trichrome stain per 5x field in control (blue) as compared to rmWnt3a-treated wounds (red) at POD 5 and 10. (n=3 for each group)
 (B) Relative Type I Collagen expression in control (blue) as compared to rmWnt3a-treated wounds (red) through POD 10, as assessed by qRT-PCR. (n=3 for all gene assays; *p<0.01)

Table 1

Polymerase Chain Reaction Genes and Primer Sequences

Gene Name	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')
<i>Axin2</i>	TGACTCTCCTTCCAGATCCCA	TGCCCACACTAGGCTGACA
<i>Type I Col-±</i>	AACCCGAGGTATGCTTGATCT	CCAGTTCTTCATTGCATTGC
<i>FGFR2</i>	GCCTCTCGAACAGTATTCTCCT	ACAGGGTTCATAAGGCATGGG
<i>Wnt3a</i>	GCTCAACGCCAACACAGTG	CGAAAACCTCGGCTGAAGATG
<i>Wnt4</i>	GCTGGCACTGTCCAAGACTC	CTCCCGTGTACCTCTCTCCA
<i>Wnt5a</i>	CAACTGGCAGGACTTTCTCAA	CATCTCCGATGCCGGAAC
<i>Wnt10a</i>	GCTCAACGCCAACACAGTG	CGAAAACCTCGGCTGAAGATG
<i>Wnt11</i>	GCTGGCACTGTCCAAGACTC	CTCCCGTGTACCTCTCTCCA
<i>GAPDH</i>	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA