Published in final edited form as:
Cell Stem Cell. 2009 June 5; 4(6): 535–547. doi:10.1016/j.stem.2009.03.013.

Wnt7a Activates the Planar Cell Polarity Pathway to Drive the Symmetric Expansion of Satellite Stem Cells

Fabien Le Grand[1,2,3], **Andrew E. Jones**[1,2], **Vanessa Seale**[1], **Anthony Scimè**[1,2], and **Michael A. Rudnicki**[1,2,4]

[1] Sprott Center For Stem Cell Research, Ottawa Health Research Institute, Regenerative Medicine Program, 501 Smyth Road, Ottawa, ON, Canada, K1H 8L6

[2] University of Ottawa, Department of Medicine, 451 Smyth Road, Ottawa, ON, Canada, K1H 8M5

Summary

Satellite cells in skeletal muscle are a heterogeneous population of stem cells and committed progenitors. We found that quiescent satellite stem cells expressed the Wnt-receptor Fzd7, and that its candidate ligand Wnt7a was upregulated during regeneration. Notably, Wnt7a markedly stimulated the symmetric expansion of satellite stem cells but did not affect the growth or differentiation of myoblasts. Silencing of Fzd7 abrogated Wnt7a binding and stimulation of stem cell expansion. Wnt7a signaling induced the polarized distribution of the planar cell polarity effector Vangl2. Silencing of Vangl2 inhibited Wnt7a action on satellite stem cell expansion. Wnt7a overexpression enhanced muscle regeneration and increased both the number of satellite cells and the proportion of satellite stem cells. Muscle lacking Wnt7a exhibited a marked decrease in satellite cell number following regeneration. Therefore, Wnt7a signaling through the planar cell polarity pathway controls the homeostatic level of satellite stem cells and hence regulates the regenerative potential of muscle.

Introduction

Satellite cells in adult skeletal muscle are located in small depressions between the sarcolemma of their host myofibers and the basal lamina. Upon damage, such as physical trauma, repeated exercise, or in disease, satellite cells become activated, proliferate and give rise to a population of myogenic precursors cells (myoblasts) expressing the myogenic regulatory factors (MRF) MyoD and Myf5. In the course of the regeneration process, myoblasts undergo multiple rounds of division before committing to terminal differentiation, fusing with the host fibers or generating new myofibers to reconstruct damaged tissue (Charge and Rudnicki, 2004).

During skeletal muscle regeneration, the satellite cell population is maintained by a stem cell subpopulation, thus allowing tissue homeostasis and multiple rounds of regeneration during the lifespan of an individual (Kuang et al., 2008). Transplantation experiments of either intact myofibers with their associated satellite cells (Collins et al., 2005), or FACS-sorted satellite cells (Kuang et al., 2007; Montarras et al., 2005), or individual cells (Sacco et al., 2008), demonstrated that a subpopulation of quiescent satellite cells are capable of both extensive

4 Correspondence should be addressed to M.A.R., Tel: (613) 739-6740, Fax: (613) 739-6294, mrudnicki@ohri.ca.
[3]Present Address: Institut Cochin, INSERM U567, Université Paris Descartes, Paris, France

contribution to muscle regeneration and self-renewal, by giving rise to new satellite cells within the transplanted host muscle.

Recent findings from our laboratory using Cre/LoxP lineage-tracing identified a subpopulation of satellite cells which have never expressed Myf5 and function as a stem cell reservoir (Kuang et al., 2007). Satellite stem cells ($Pax7^+/Myf5^-$) represent about 10% of the adult satellite cell pool, and give rise to daughter satellite myogenic cells ($Pax7^+/Myf5^+$) through asymmetric apical-basal cell divisions. Transplantation of both $Myf5^-$ and $Myf5^+$ FACS-sorted satellite cells demonstrated that satellite stem cells are capable of repopulating the adult satellite cell niche as well as self-renewal (Kuang et al., 2007). However, our knowledge of the molecular networks regulating satellite stem cell fate decisions has remained unclear.

The paired-box transcription factor Pax7 plays a central regulatory role in satellite cell function and survival (Kuang et al., 2006; Seale et al., 2000). The satellite cell population in Pax7-deficient mice is progressively lost, and the residual cells in the satellite niche are unable to sustain efficient skeletal muscle regeneration (Kuang et al., 2006; Oustanina et al., 2004). Recent work has revealed that Pax7 recruits the Ash2L-Wdr5-MML2 histone methyltransferase complex to target genes such as *Myf5* leading to Histone 3 K4 trimethylation and subsequent gene activation (McKinnell et al., 2008). However, the signaling pathways and molecular mechanisms that regulate the activity of Pax7 in satellite stem cells are undefined.

Wnt signaling plays a key role in regulating developmental programs through embryonic development, and in regulating stem cell function in adult tissues (Clevers, 2006). Wnts have been demonstrated to be necessary for embryonic myogenic induction in the paraxial mesoderm (Borello et al., 2006; Chen et al., 2005; Tajbakhsh et al., 1998), as well in the control of differentiation during muscle fiber development (Anakwe et al., 2003). Recently, the Wnt planar cell polarity (PCP) pathway has been implicated in regulating the orientation of myocyte growth in the developing myotome (Gros et al., 2009). In the adult, Wnt signaling is necessary for the myogenic commitment of adult stem cells in muscle tissue following acute damage (Polesskaya et al., 2003; Torrente et al., 2004). Other studies suggest that the canonical Wnt/β-catenin signaling regulates myogenic differentiation through activation and recruitment of reserve myoblasts (Rochat et al., 2004). In addition, the Wnt/β-catenin signaling in satellite cells within adult muscle appears to control myogenic lineage progression by limiting Notch signaling and thus promoting differentiation (Brack et al., 2008).

In this study, we undertook a molecular characterization of satellite stem cells using a subtractive hybridization approach to identify uniquely expressed genes. We found that the Wnt receptor Fzd7 was markedly upregulated in quiescent satellite stem cells suggesting a role for non-canonical Wnt signaling. Investigation of this hypothesis revealed that Wnt7a is expressed during muscle regeneration and acts through its receptor Fzd7 and Vangl2, a component of the planar cell polarity (PCP) pathway, to induce symmetric satellite stem cell expansion and dramatically enhance muscle regeneration. Together these results reveal a novel role for the PCP pathway in regulating the homeostatic maintenance of the stem cell compartment during adult skeletal muscle regeneration.

Results

Frizzled7 is Highly Expressed in Quiescent Satellite Stem Cells

Satellite cells are a heterogeneous population composed of stem cells and committed progenitors. All satellite cells express Pax7 and markers such as CXCR4, however, a subset of about 10% have never expressed Myf5 during their developmental history (Figure 1A). As previously demonstrated, $Pax7^+/Myf5^-$ satellite cells represent a stem cell population within the satellite cell niche (Kuang et al., 2007).

Towards performing gene expression analysis of quiescent satellite stem cells, we first improved our previously described methodology for satellite cell isolation by fluorescence activated cell sorting (FACS) as described in the Methods. FACS-purified cells ($CD34^+$, α7-$Integrin^+$, $CD31^-$, $CD45^-$, $CD11b^-$, $Sca1^-$) (Figure S1A), were >95% satellite cells as determined by Pax7 and Syndecan4 expression (Figure S1B), and exhibited robust growth and differentiation potential *in vitro* (Figure S1C).

FACS-purified satellite cells were further separated on the basis of Myf5-conditional YFP fluorescence (Figure 1B). *In vitro* cultured YFP^- satellite cells gave rise to proliferating cells expressing Pax7, but not YFP or Myf5 protein, when maintained at low density (Figure S2), thus validating that YFP^- cells do not and have not expressed Myf5. Real-Time PCR analysis of freshly sorted cells confirmed Pax7 expression (Figure 1C), as well as several other satellite cell markers such as cMet, Syndecan4, Caveolin1 and α7-Integrin (not shown) in isolated YFP^+ and YFP^- satellite cells. In addition, YFP and Myf5 transcripts were detected in YFP^+ satellite cells while virtually no YFP and Myf5 expression (not significantly different from RT^- controls) were detected in YFP^- satellite cells (Figure 1C).

To gain insight into the molecular mechanisms responsible for regulating satellite stem cell function, suppressive subtractive hybridization (SSH) of cDNAs (Diatchenko et al., 1997) was employed to identify genes expressed specifically in quiescent $Pax7^+/Myf5^-$ satellite stem cells. Notably, one of the differentially expressed clones encoded a fragment from within the Frizzled7 (Fzd7) mRNA. Fzd7 is a G-protein-coupled transmembrane Wnt receptor that belongs to a protein family encoded by multiple genes (Egger-Adam and Katanaev, 2008). Real-time PCR analysis confirmed that Fzd7 transcripts were abundantly expressed in YFP^- satellite cells and only marginally detected in YFP^+ satellite cells (Figure 1C).

To confirm the differential expression suggested by Real-Time PCR, we examined Fzd7 protein expression on myofibers fixed immediately following isolation from *extensor digitorum longus* (EDL) muscles. Immunohistological analysis revealed that 12 ± 3 % of $Pax7^+$ satellite cells expressed readily detectable levels of Fzd7 (n=3 mice, > 150 cells/mouse). Analysis of myofibers isolated from *Myf5-Cre/ROSA26-YFP* EDL muscles demonstrated that Fzd7 was specifically upregulated in satellite stem cells ($Pax7^+/Myf5^-$) that do not contain detectable levels of YFP (Figure 1D).

However, culture of fibers in suspension for 2 days resulted in upregulation of Fzd7 in virtually all satellite cells (99%, n=3 mice, ≥100 cells per mouse) (Figure S3). Furthermore, examination of regenerating myofibers from EDL muscle following cardiotoxin (CTX) induced damage of the *tibialis anterior* (TA) muscle (Kuang et al., 2007), revealed Fzd7 expression on doublets of $Pax7^+/Myf5^-$ and $Pax7^+/Myf5^+$ cells (Figure 1E).

Taken together, these results demonstrate that in resting muscle, the Wnt receptor Fzd7 is specifically expressed in quiescent satellite stem cells. However, Fzd7 is also upregulated in proliferating satellite cells and myoblasts.

Wnt Expression During Muscle Regeneration

Coexpression of Fzd7 and Wnt7a during embryonic myogenesis suggests that Wnt7a is a candidate ligand for Fzd7 (Cossu and Borello, 1999). Moreover, Wnt7a has been implicated as a major regulator of embryonic and adult myogenesis (Chen et al., 2005; Polesskaya et al., 2003; Tajbakhsh et al., 1998). We therefore employed Real-Time PCR-Array analysis of freeze-injured TA muscle to document Wnt expression during regenerative myogenesis. We chose freeze injury of muscle because of the significantly reduced inflammatory response relative to other methods such as cardiotoxin (CTX) injection. Changes in gene expression were analyzed at 3 days post-injury, during the acute phase of regeneration, where most of the

Pax7$^+$ cells are proliferating, and at 6 days post-injury, when satellite cells have returned to a quiescent sub-laminar position (Figure 2A).

At 3 days post-injury we detected significant increases (as compared to contralateral leg, n=3 mice, $p<0.05$) in 31 transcripts including those for multiple Wnts (*Wnt-1, -2, -5b, -8b, -10a, -16a*), Frizzled receptors and sFRP inhibitors (Figure S4). Notably, at 6 days post-injury we detected a significant increase (n=3 mice, $p<0.05$) in the transcript levels for *Wnt7a* and *Wnt10a*, (Figure 2B). *Wnt3a* levels were below the limit of detection in our analyses at both 3 and 6 days of regeneration (Supplemental Table S1). Therefore, *Wnt7a* mRNA was markedly upregulated at the time when satellite stem cells replenish the resident satellite cell pool.

To confirm Wnt7a up-regulation during muscle regeneration in another muscle injury model, we performed immunohistochemical analysis of Wnt7a protein expression on cryosections of CTX-injured TA (fixed 4 days post-injury) and the contralateral resting TA. In undamaged muscle, Wnt7a was not expressed at detectable levels (Figure 2E, left). By contrast, Wnt7a was strongly upregulated in regenerating fibers (of smaller size than the intact fibers and containing Myogenin$^+$ nuclei), and was not expressed by CD144$^+$ endothelial cells (Figure 2E, right).

To determine whether Wnt7a is a ligand for Fzd7, cultured satellite cell-derived myoblasts were incubated with recombinant human Wnt7a protein for 30 minutes, washed, fixed and immunostained with anti-Wnt7a antibody. Cells incubated with BSA did not show membrane staining for Wnt7a protein. By contrast, cells incubated with Wnt7a protein exhibited immunostaining on the membrane (Figure 2C). Importantly, transfection of Fzd7 siRNA abrogated binding of Wnt7a (Figure 2C). Fzd7 silencing was effective, specific and did not significantly alter the other Frizzled transcripts expressed in myoblasts (Figure S6A). Taken together, these data provide compelling evidence that Fzd7 is the receptor for Wnt7a in myogenic cells.

Wnt7a has been described as either a canonical (Hirabayashi et al., 2004) or non-canonical Wnt (Kengaku et al., 1998), depending on cell-type and receptor context. To evaluate the possible function of Wnt7a as a canonical Wnt, we stimulated satellite cell-derived myoblasts with Wnt7a and Wnt3a proteins for 24 hours. Wnt3a activates the canonical Wnt pathway in myogenic cells (Brack et al., 2008), and in our experiment, Wnt3a stimulation resulted in increased expression of β-catenin/TCF target genes, for example a 5-fold and 50-fold increase of *Tcf7* and *Axin2* mRNAs respectively, (n=5, $p\leq0.001$). By contrast, Wnt7a stimulation did not result in any significant change in *Tcf7* and *Axin2* levels which were similar to BSA-treated samples (Figure 2D). In addition, Wnt3a but not Wnt7a stimulation robustly induced the stabilization and nuclear localization of activated-β-catenin (Figure S5), and Wnt3a but not Wnt7a robustly activated the β-catenin luciferase reporter TOP-Flash in transient transfection experiments (not shown).

Taken together, these results indicate that Wnt7a is markedly upregulated by newly formed myofibers during regenerative myogenesis, binds to the Fzd7 receptor at the surface of myogenic cells, and does not utilize the canonical Wnt-β-catenin signaling pathway.

Symmetry of Satellite Stem Cell Divisions is Regulated by Wnt7a-Frizzled7

The expression of Fzd7 specifically in quiescent satellite stem cells and the marked up regulation in Wnt7a during muscle regeneration suggested that Wnt7a-Fzd7 signaling is involved in regulating muscle stem cell function. In addition, Wnt7a had no effect on the growth or differentiation of cultured primary myoblasts *in vitro* (Figure S8). Therefore, to investigate the role of Wnt7a-Fzd7 signaling in satellite cells, we examined the ability of recombinant

Wnt7a to alter the ratio between asymmetric and symmetric cell divisions of satellite stem cells *in vitro*.

Myofibers were isolated from *Myf5-Cre/ROSA26-YFP* EDL muscle and cultured under non-adherent conditions. In our culture system, quiescent satellite cells become activated immediately following myofiber isolation. Satellite cells leave their niche, migrate across the basal lamina, and undergo their first cell division in a synchronous fashion. Thus, we visualized the outcome of the first division by fixing and staining the myofibers after 42hours of culture. Importantly, live imaging analysis confirms that satellite cells do not move on myofibers before dividing and that scored cell doublets are of clonal origin (Kuang et al., 2007).

Satellite stem cells (YFP^-) either underwent a symmetrical cell division to give rise to two YFP^- daughter cells, or an asymmetric cell division to give rise to one YFP^- stem cell and one YFP^+ committed precursor (Figure 3A). When stimulated with Wnt7a, we observed a dramatic increase in the proportion of symmetric cell divisions from 30% to 67% (n=3, n≥152 pairs, p=0.009). By contrast, Wnt3a treatment did not induce any significantly change (Figure 3B). Therefore, Wnt7a stimulated an increase in symmetric satellite stem cell divisions.

Our experimental analysis suggested that Wnt7a specifically binds the Fzd7 receptor (Figure 2C, 2D). Therefore, to determine whether the induction of symmetric stem cell divisions by Wnt7a required the presence of Fzd7, we performed Fzd7 knock-down experiments on isolated myofibers. Immunostaining of treated fibers demonstrated extensive silencing of Fzd7 expression after 42 hours (Figure 2C). Importantly, siRNA-induced knock-down of Fzd7 resulted in a complete abrogation of the ability of Wnt7a to induce symmetric satellite stem cell divisions (n=3, ≥123 pairs, p≤0.02). By contrast, scrambled siRNA treatment did not significantly affect this activity of Wnt7a (Figure 3D). Consistent with these results, the proportion of satellite stem cells ($Pax7^+/YFP^-$) after the second division (50 hours) was significantly increased by 13% after Wnt7a treatment (n=3, ≥1203 cells, p≤0.03) this resulting in an increase in the number of stem cells per fibers (Figures 3E, S6B). Similarly, Fzd7 silencing efficiently blocked the effect of Wnt7a stimulation. We observed that the total number of $Pax7^+$ cells per fiber remained constant between each condition, confirming that Wnt7a does not effect cell proliferation or differentiation (Figure S6C).

These results demonstrate that Wnt7a signals via Fzd7 to stimulate symmetric satellite stem cell division and thus drive the expansion of the satellite stem cell pool.

Role for the PCP Component Vangl2 in Satellite Stem Cell Self-Renewal

Our analysis indicated that Wnt7a does not activate the canonical Wnt/β-catenin signaling pathway (Figures 2D, S4), and that Wnt7a signals through the Fzd7 receptor to drive satellite stem cell symmetric divisions (Figures 2C, 3B, 3D). In *Xenopus laevis*, xFzd7 is considered a component of the planar cell polarity (PCP) signaling pathway involved in gastrulation (Goto et al., 2005). Therefore, we hypothesized that Wnt7a acts through Fzd7 to activate the PCP pathway and drive satellite stem cell expansion.

To investigate if Wnt7a activates the PCP pathway, we first analyzed the relative transcript levels of a set of core PCP components (Seifert and Mlodzik, 2007) in myogenic cells. Interestingly, myoblasts expressed significant levels of *Dvl-2* and *-3*, *Fzd-3* and *-7*, *Pk-1* and *-2* and *Vangl-1* and *-2*, and low levels of *Celsr2*. Other PCP component genes tested were called absent with *cut-off* values over 30 cycles (Figure 4A). In addition, cultured satellite stem cells (YFP^-) expressed significantly higher levels of all PCP components (n=3, p<0.05), with a marked upregulation of *Vangl2*, consistent with a role for PCP signaling in regulating satellite stem cell function.

Vangl2 is a crucial regulator of PCP and non-canonical Wnt-signaling in Drosophila and vertebrates (Torban et al., 2004). In cells with active PCP signaling, Vangl2 protein is distributed at the poles at either end of the axis of polarization and this distribution is lost in PCP mutants (Montcouquiol et al., 2006). Vangl2 protein was not detected in quiescent satellite cells on isolated myofibers, but was upregulated in activated satellite cells as they entered the cell cycle by 24 h in culture. After 48h, all $Pax7^+$ activated satellite cells were also positive for Vangl2 (100%, n=3 mice, ≥100 cells per mouse) (Figure 4B). We also confirmed the expression in satellite cells of Prickle1 and Celsr2 proteins that interact with Vangl2 *in vivo* (not shown).

Importantly, in presence of Wnt7a, a significant proportion of dividing doublets of satellite cells on cultured myofibers (29 ± 4%, n=3, ≥240 pairs, p≤0.006) exhibited polarized localization of Vangl2 on opposite poles of the daughter cells (Figure 4C, 4D). By contrast, following BSA (control) or Wnt3a-treatment, Vangl2 protein was uniformly dispersed in satellite cell doublets (90 ± 2% and 89 ± 2% respectively) (Figure 4C, 4D). Moreover, double staining with anti-Vangl2 and anti- α7-integrin antibodies revealed that Wnt7a appeared to induce enhanced membrane localization of Vangl2 and polarized distribution α7-integrin. This redistribution did not occur in untreated cells or in cells undergoing apical-basal cell divisions (Figure 4E). Taken together, these observations strongly support the assertion that Wnt7a induces a redistribution of the polarity effector Vangl2 and α7-integrin and the upregulated expression of α7-integrin at the poles of daughter cells allows them to remain adherent with the basal lamina and to remain in the stem cell niche.

To investigate the role of Vangl2 in satellite stem cell function, we performed siRNA silencing of Vangl2 on single *Myf5-Cre/ROSA26-YFP* myofibers stimulated with Wnt7a. Myofibers were first stained with Pax7 and Syndecan4 antibodies to allow visualization of the plane of satellite cell division relative to the fiber, and cell divisions scored as either planar or apical-basal (Figure 5A). At 42h after the first cell division, Wnt7a stimulation induced planar divisions and accordingly resulted in a 12% decrease in apical-basal cell divisions. By contrast, Vangl2 silencing increased the proportion of apical-basal cell divisions by 15%, (n=3, ≥154 pairs, p≤0.02) (Figure 5B). Myofibers from the same experiments were also stained with Pax7 and YFP antibodies, and the percentage of symmetric cell divisions scored. We observed a close inverse correlation between the proportions of apical-basal versus symmetric cell divisions. Wnt7a stimulation increased the proportion of symmetric cell divisions, whereas Vangl2 knockdown markedly impaired the ability of Wnt7a to stimulate symmetric cell divisions (n=3, ≥65 pairs, p≤0.02) (Figure 5C).

To analyze the role of Vangl2 in satellite cell proliferation and myogenic potential, we cultured fibers for 50 hours and assessed Vangl2 silencing by immunostaining (Figure 5D). At this point, Vangl2 knock-down continued to increase the rate of apical-basal cell divisions (n=5, ≥150 pairs, p=0.001) (Figure 5E), while depleting the population of satellite stem cells (n=3, ≥330 cells, p=0.03) (Figure 5F). This resulted in a marked diminution in the total number of satellite cells per fibers (n=5, ≥500 cells, p=0.001) (Figure 5G). At 3-days after knock-down of Vangl2 (Figure 5H), we observed a doubling in the number of cells expressing myogenin, an early marker for differentiation (n=4, ≥550 cells, $p=10^{-5}$) (Figure 5I) along with a loss of half the cells on fibers (n=4, ≥550 cells, p=0.001) (Figure 5J). Vangl2 silencing on satellite cell-derived myoblasts resulted in reduced levels of Pax7 and Myf5 transcripts, along with increased levels of Myogenin (n=4, p≤0.05) (Figure 5K). Together, these data suggest that Vangl2 is required for self-renewal of both satellite stem cells and the generation of transient-amplifying myoblasts.

These data demonstrate that Wnt7a signaling through Fzd7 requires Vangl2 to induce symmetric expansion of the satellite stem cell pool. Wnt7a also induces polarized distribution of Vangl2 protein at the opposite poles of cells undergoing a symmetric planar cell division.

Hence, Wnt7a utilizes the planar cell polarity pathway to control the orientation of satellite cell division and their fate within the niche.

Wnt7a Enhances Muscle Regeneration by Expanding the Stem Cell Pool

To investigate the role of the Wnt7a-Fzd7-Vangl2 pathway in muscle regeneration *in vivo*, we over-expressed Wnt7a by electroporation of a CMV-Wnt7a expression plasmid into TA muscles of 3-month old mice. Histological analysis of muscles electroporated with CMV-LacZ plasmid revealed that majority (>80%) of the myofibers expressed the β-galactosidase (Figure S7A) and that we did not detect any regeneration deficit following electroporation of control plasmid (Figure S7B). In addition, immunostaining revealed that myofibers electroporated with CMV-Wnt7a plasmid secreted readily detectable levels of Wnt7a protein (Figure S7C).

Notably, TA muscles electroporated with CMV-Wnt7a exhibited an 18 ± 4% ($p=0.009$, $n=8$) increase in mass after 3 weeks. Examination of serial sections of electroporated muscles revealed an increase in the overall size of the muscle as well as a significant increase in caliber size and numbers of fibers throughout the body of the muscle (Figure 6). By contrast, over expression of Wnt3a resulted in a larger increase in the number of myofibers but these exhibited a dramatic reduction in cross-sectional area, resulting in reduced regeneration efficiency (Figure 6). We did not observe an effect of Wnt7a over-expression on other cell types in muscle tissue. However, overexpression of Wnt3a resulted in abnormal matrix deposition suggesting an enhancement of proliferation of fibroblastic/smooth muscle progenitors resulting in increased fibrosis (Figure 6B). Taken together, our results indicate that over-expression of Wnt7a markedly enhances muscle regeneration, as evidenced by the presence of increased numbers of larger fibers and the significantly increased mass of muscle.

As previously noted, Wnt7a treatment did not alter the growth or differentiation of activated satellite cells or primary myoblasts *in vitro* (Figure S6C, S8A, S8C, S8D). In addition, Wnt7a did not induce the expression of MyoD or of Wnt/β-catenin target genes in differentiated myocytes (Figure S8B, S8E). However, our *in vitro* experiments established that Wnt7a-Fzd7-Vangl2 signaling stimulated the symmetrical expansion of satellite stem cells, which would then give rise to transient amplifying progenitors that undergo normal proliferation and differentiation. To assess whether Wnt7a similarly stimulates the expansion of satellite stem cells *in vivo*, we enumerated the numbers of satellite cells and satellite stem cells in regenerated muscle following electroporation of CMV-Wnt7a.

Over-expression of Wnt7a resulted in about a 2-fold increase in the number of $Pax7^+$ satellite cells per myofiber on sections at 3 weeks after electroporation ($p=0.03$, $n=4$). By contrast, over-expression of Wnt3a did not alter the number of satellite cells (Figure 7A, 7B). To enumerate the proportion of satellite stem cells, FACS-isolated satellite cells were isolated from *Myf5-Cre/ROSA26-YFP* TA muscle at 3 weeks following electroporation, cultured for 24 h, then immunostained for Pax7 and YFP (Figure 7C). Consistent with our observations that Wnt7a induces symmetrical satellite stem cell divisions *in vitro*, we observed that overexpression of Wnt7a in regenerating muscle in about a 20% increase ($n=5$, $p=0.0001$) in the proportion of $Pax7^+/YFP^-$ satellite stem cells (Figure 7C, 7D). Therefore, these data indicate that similarly Wnt7a acts on the satellite stem cell compartment *in vitro* and *in vivo*.

To investigate satellite stem cell function in the absence of Wnt7a, we analyzed the regeneration phenotype of 3 mo-old $Wnt7a^{-/-}$ null mice (Miller and Sassoon, 1998). Quantification of Pax7-expressing satellite cells on freshly isolated myofibers from EDL muscle demonstrated that $Wnt7a^{-/-}$ null mice exhibit about an 18% decrease in number of satellite cells per fiber ($p=0.03$, $n=4$) (Figure 7E). Three-weeks following a freeze-crush injury, the regenerated $Wnt7a^{-/-}$ TA muscles appeared grossly normal (Figure 7F). Importantly, examination of sections of regenerated $Wnt7a^{-/-}$ TA muscles revealed a significant 36% decrease in the numbers of

satellite cells (p=0.03, n=3) (Figure 7G). This result strongly supports the notion that Wnt7a plays an important role in regulating satellite stem cell function.

Overexpression of Wnt7a in muscle drives expansion of the satellite stem cell pool, and conversely, the satellite cell compartment becomes depleted in the absence of Wnt7a. Together, our data demonstrate a novel role for Wnt7a signaling via the PCP pathway to stimulate satellite stem cell symmetrical cell division to drive expansion. Therefore, Wnt7a regulates the homeostatic levels of the satellite stem cell compartment and thus regulate the efficiency of regenerative myogenesis in adult skeletal muscle.

Discussion

We undertook a gene expression analysis of satellite stem cells towards identifying signaling pathways that regulate their function. Satellite stem cells represent a small subpopulation of satellite cells that are capable of self-renewal and long-term reconstitution of the satellite cell niche following transplantation (Kuang et al., 2007). Our experiments identified the non-canonical Wnt-receptor Fzd7 as being specifically expressed in quiescent satellite cells (Figure 1). Wnt7a was examined as a candidate receptor for Fzd7 because of its expression and role during embryonic and adult myogenesis (Chen et al., 2005; Cossu and Borello, 1999; Polesskaya et al., 2003; Tajbakhsh et al., 1998). We found by Real-time PCR and immunohistochemistry that Wnt7a was markedly upregulated in newly formed myofibers during regenerative myogenesis (Figure 2B, 2E), and that the Fzd7 receptor is necessary for Wnt7a binding at the surface of myogenic cells (Figure 2C).

Satellite stem cells undergo apical-basal asymmetric cell divisions to give rise to committed myogenic cells that express Myf5, and to maintain their population through self-renewal. Alternatively, satellite stem cells can undergo planar symmetric cell divisions to drive expansion of their population (Kuang et al., 2007). Importantly, we found that recombinant Wnt7a protein dramatically stimulated the symmetric expansion of satellite stem cells and that this expansion required Fzd7 and Vangl2 (Figures 3 and 5), both components of the planar cell polarity (PCP) signaling pathway. Moreover, Wnt7a induced polarized localization of Vangl2 at opposite poles in pairs of dividing cells (Figure 4), in a manner consistent with Wnt7a activating PCP signaling. Over expression of Wnt7a during muscle regeneration resulted in an impressive enhancement of the regeneration process, generating more fibers of bigger caliber, independent of an effect on myoblast proliferation or differentiation (Figure 6). Importantly, Wnt7a over-expression resulted in a large expansion of the satellite stem cell population, and Wnt7a loss resulted in impaired maintenance of the satellite cell compartment (Figure 7). These results provide important new insights into the molecular regulation of satellite cell self-renewal, and for the first time implicate the Wnt non-canonical PCP pathway in the regulation of adult stem cell function.

The Wnt-PCP pathway plays a role in patterning by instituting polarity of cells within a tissue, such as with the organized orientation of epithelial cells in Drosophila (Zallen, 2007). In vertebrates, PCP signaling, and particularly its effecter Vangl2 (also known as Strabismus), is required for the polarization of stereociliary bundles in the cochlea (Montcouquiol et al., 2003), for convergent extension (CE) movements regulating gastrulation and neurulation (Torban et al., 2004), neural tube closure (Torban et al., 2008), and in regulating myocyte orientation in the developing myotome (Gros et al., 2009). During zebrafish neurulation, loss of Vangl2 abrogates polarization of neural keel cells by preventing re-intercalation of daughter cells into the neuroepithelium, resulting in ectopic neural progenitor accumulation (Ciruna et al., 2006).

We propose that the symmetric expansion of satellite stem cells results from a PCP-mediated orientation of the axis of stem cell division. Since PCP is a positional signaling relying on the redistribution of effectors proteins, polarization of PCP core molecules on opposite poles of the daughter cells allows both cells to maintain contact with the basal lamina and thus preserve their orientation relative to the niche (Figure 4 and S9). Notably, Wnt7a induced polarized distribution of Vangl2 and α7-integrin (Figure 4E). The upregulated and polarized localization of α7-integrin allows both daughter cells to remain attached to the basal lamina. By contrast, α7-integrin expression is reduced and evenly distributed in apical-basal oriented cell divisions. Daughter cells that are "pushed" towards the sarcolemma thus losing contact with the basal lamina, activate Myf5 transcription and commit to a progenitor state (Kuang et al., 2007). Therefore, these data suggest that the PCP pathway intersects with the mechanisms that control apical-basal cell divisions and commitment and function through a mechanism that promotes adhesion to the basal lamina.

Our experiments suggest that polarized distribution of Vangl2 protein at the poles of a couplet of daughter cells allows both cells to remain attached to the basal lamina, and therefore maintain a stem cell state, resulting in expansion of the stem cell population. Subsequent cell divisions will generate larger numbers of committed daughter cells through apical-basal asymmetric divisions that will undergo normal expansion and differentiation (Figure 6). We previously noted that the proportion of $Pax7^{+}/Myf5^{-}$ satellite stem cells increased from 10% to about 30% at 3 weeks following injury (Kuang et al., 2007), and that overexpression of Wnt7a further increased the level to 50% (Figure 7C, 7D). By contrast, satellite cell numbers decreased by 36% in Wnt7a-deficient muscle following injury and regeneration Figure 7G). These data strongly support the model through which Wnt7a regulates the homeostatic maintenance of the satellite stem cell pool by modulating the increase in satellite stem cell expansion during regenerative myogenesis and that basal levels of PCP signaling are insufficient to maintain the satellite cell pool at normal levels.

In the fly, it has been demonstrated that in parallel to the frizzled-induced PCP signaling, Strabismus collaborates with Flamingo to mediate planar cell orientation. A secondary PCP mechanism is regulated by the protocadherins Fat and Dachsous independently of the core effectors Frizzled and Strabismus (Zallen, 2007). In our *in vitro* single myofiber system, the basal rate of symmetric divisions was not perturbed by the loss of Fzd7. By contrast, silencing of Vangl2 not only abrogated Wnt7a stimulation of symmetric expansion but also reduced the basal state of symmetric stem cell division. Therefore, it is likely that other PCP mechanisms, dependent on Vangl2, or on the protocadherins, mediate basal PCP signaling independent of Wnt binding.

It is possible that basal levels of PCP signaling are activated through other effectors. One such candidate is Syndecan4 (Syn4), a cell surface heparan sulfate proteoglycan expressed by quiescent satellite cells and their activated progeny (Cornelison et al., 2001). Satellite cells in mice deficient for *Syn4* are compromised in activation, proliferation, and differentiation *in vitro*, and they fail to reconstitute damaged muscle *in vivo* (Cornelison et al., 2004). Importantly, during Xenopus embryogenesis, xSyn4 interacts directly with xFzd7 and xDsh to activate non-canonical Wnt-signaling to regulate convergent and extension (CE) movements (Munoz et al., 2006). In addition, Syn4 and non-canonical Wnt signaling regulates the directional migration of neural crest cells (Matthews et al., 2008). Therefore, Syn4 and Fzd7 appear to cooperate to activate non-canonical Wnt signaling through the PCP pathway. Consistent with this hypothesis, we found that Syn4 expression was severely perturbed in satellite cells following Vangl2 silencing on myofibers (not shown). Thus, the inability of satellite cells from $Syn4^{-/-}$ mice to regenerate muscle may be a consequence of a defect in PCP signaling.

Canonical Wnt-signaling plays a well-documented role in regulating myogenic growth and differentiation. Recently, the temporal balance between proliferation and differentiation of satellite cells in adult muscle was demonstrated to be regulated by cross-talk between Notch and Wnt/β-catenin signaling pathways (Brack et al., 2008). Activation of the Wnt/β-catenin signaling pathway inhibits Notch-mediated maintenance of the undifferentiated state and thus facilitates differentiation. Our experiments indicate that activation of Wnt/β-catenin signaling using Wnt3a did not interfere with satellite stem cell choice between commitment and symmetric expansion (Figures 3B, 4D). Nevertheless, over-expression of Wnt3a *in vivo* appeared to impair regeneration possibly by promoting premature differentiation and the formation of myofibers of reduced size (Figure 6). Indeed, Wnt3a stimulation of satellite cells on single myofibers drove their differentiation as evidenced by significant increase in the number of $Pax7^{-}/MyoD^{+}$ cells (not shown). However, we were unable to detect Wnt3a expression in undamaged or regenerating skeletal muscle by Real-Time PCR. Potentially other upregulated Wnts, such as Wnt10a (Figure 2B, S4), may function to activate the Wnt/β-catenin signaling pathway in myogenic cells. Furthermore, we observed a large up-regulation of the Wnt-inhibitors sFRPs during the early stages of the regenerative process. Perhaps this represents a physiological feedback system that inhibits canonical Wnt signaling, allowing the proliferation of myogenic progenitors. Thus, as hypothesized by Brack et al., (2008), inhibition of Wnt/β-catenin signaling would act to promote muscle regeneration.

In Xenopus embryos, the Vangl2 homolog Strabismus inhibits Wnt/β-catenin activated transcription by competing for Disheveled (Park and Moon, 2002). Thus, PCP signaling may also act to keep satellite stem cells in an uncommitted state by antagonizing canonical Wnt/β-catenin signaling. In Drosophila eye development, Frizzled (Fz)/PCP signaling induces cell-fate specification of the R3/R4 photoreceptors through regulation of Notch activation in R4 (del Alamo and Mlodzik, 2006). This raises the possibility that cross-talk between Frizzled/PCP and Notch pathways, as well as Wnt/β-catenin pathways, act to coordinate satellite stem cell choice between self-renewal/commitment versus expansion.

Molecular characterization of satellite stem cells is providing important insights into the molecular mechanisms regulating their function. Our identification of a role for the Wnt7a/Fzd7/Vangl2 signal transduction cascade reveals an unanticipated role for the PCP pathway in regulating the symmetric expansion of satellite stem cells. This finding represents a significant advance in our understanding of satellite cell biology and muscle regeneration. Future experiments will investigate both the utility of modulating the PCP pathway to augment muscle regeneration towards ameliorating the loss of muscle function in neuromuscular disease.

Experimental Procedures

Mice and Animal Care

Adult (8-12 weeks of age) *Myf5-Cre/ROSA26-YFP* mice were obtained by crossing the knock-in *Myf5-Cre* (Tallquist et al., 2000) heterozygous mice with *ROSA26-YFP* (*Srinivas et al., 2001*) homozygous reporter mice. *ROSA26-YFP* mice were used as wild type controls. Wnt7a-null mice and their littermates controls were obtained by crossing heterozygous $Wnt7a^{+/-}$ mice. All mice were maintained inside a barrier facility and experiments were performed following the University of Ottawa regulations for animal care and handling.

Cell Sorting

Mononucleated muscle derived cells were isolated from hind-limb muscles and staining was performed as previously described (Ishibashi et al., 2005; Kuang et al., 2007). Cells were separated on a MoFlo cytometer (DakoCytomation), equipped with 3 lasers. Dead cells and

debris were excluded by Hoescht staining, and by gating on forward and side scatters profiles (Figure S1).

Myofiber Isolation, Culture and Immunohistochemistry

Single myofibers were isolated from the EDL muscles as previously described (Charge et al., 2002). Isolated myofibers were cultured in suspension in 6-well plates coated with horse serum to prevent fiber attachment (Kuang et al., 2006). Fibers were incubated in plating medium consisting of 15% FBS (Hyclone) and 1% chick embryo extract (CEE, Accurate Chemicals) in DMEM containing 2% L-glutamine, 4,5% Glucose and 110 mg/ml Sodium Pyruvate. For myoblast culture, satellite cells were sorted and plated on collagen-coated dishes in Ham's F10 medium supplemented with 20% FBS and 5ng/ml of basic FGF (Invitrogen). For Wnt stimulation, recombinant Wnt7a or Wnt3a proteins were added in the plating medium (25ng/ml, R&D Systems). For *in vivo* activation of satellite cells, regeneration was induced by CTX injection in the TA muscle, and four days later, individual myofibers were isolated from the neighboring EDL muscle. Immunochemical labeling of cryosections, myofibers and cells were performed at previously reported (Kuang et al., 2006). The primary antibodies used are listed in Supplemental Table S2.

siRNA Knock-Down

For EDL myofibers, transfections were carried at 4hours and 24hours post-dissection in plating medium using Lipofectamine 2000 reagent (Invitrogen) as per manufacturer's instructions. Fibers were re-fed in fresh media on the next mornings and fixed after 42hours to 72hours of culture. For Satellite cell-derived myoblasts, cells were re-fed 3 hours prior to transfection and transfections were carried in growth medium. Cells were washed and re-fed with Ham's Complete media 6 hours following transfections. RNA was harvested 48 hours following transfection. siRNA duplexes were from Ambion siFzd7 (ID s66314), siVangl2 (ID s96802) and used at the final concentration of 10nM each. Transfection efficiency was monitored using Cy3-labeled siRNA. Knock-down efficiency was assessed by real-time PCR (Figures S6A and S5K).

Real-Time PCR

RNA was isolated using the RNEasy kits (Qiagen) and subjected to on column DNase digestion as per manufacturers instructions. cDNA synthesis was performed using the Superscript III reverse transcriptase and random hexamer primers (Invitrogen). Real-Time PCR was carried out as previously described (Ishibashi et al., 2005). Transcript levels were normalized to GAPDH transcript levels. Relative fold change in expression was calculated using the $\Delta\Delta$CT method (CT values < 30). For relative transcript quantification, each cDNA sample was run on a 5-point standard curve as to assure a PCR efficiency of $\geq$ 95%. Wnt Signaling Pathway PCR Arrays were purchased from Superarray Bioscience Corporation (PAMM-043) and analysis was performed as per manufacturer' instructions. See Table S3 for primer sequences.

Statistical Analysis

A minimum of 3 and up to 5 replicates was done for experiments presented. Data are presented as standard error of the mean. Results were assessed for statistical significance using Student's T Test (Microsoft Excel) and differences were considered statistically significant at the $p<0.05$ level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Shihuan Kuang, Feodor Price, Iain McKinnell, Mark Gillespie and Jeffrey Dilworth for helpful discussions throughout the study, and Caroline Vergette and Paul Oleynick for cell sorting. We thank Drs. P. Soriano for the *Myf5-Cre* mice, F. Costantini for the *ROSA-YFP* mice, Alysia vandenBerg and David Sassoon for the *Wnt7a*$^{-/-}$ mice. M.A.R. holds the Canada Research Chair in Molecular Genetics and is an International Research Scholar of the Howard Hughes Medical Institute. This work was supported by grants to M.A.R. from the Canadian Institutes of Health Research, Muscular Dystrophy Association, the National Institutes of Health, the Howard Hughes Medical Institute, the Canadian Stem Cell Network, and the Canada Research Chair Program. The authors declare no conflict of interest.

References

Anakwe K, Robson L, Hadley J, Buxton P, Church V, Allen S, Hartmann C, Harfe B, Nohno T, Brown AM, et al. Wnt signalling regulates myogenic differentiation in the developing avian wing. Development 2003;130:3503–3514. [PubMed: 12810597]

Bae GU, Gaio U, Yang YJ, Lee HJ, Kang JS, Krauss RS. Regulation of myoblast motility and fusion by the CXCR4-associated sialomucin, CD164. J Biol Chem 2008;283:8301–8309. [PubMed: 18227060]

Borello U, Berarducci B, Murphy P, Bajard L, Buffa V, Piccolo S, Buckingham M, Cossu G. The Wnt/beta-catenin pathway regulates Gli-mediated Myf5 expression during somitogenesis. Development 2006;133:3723–3732. [PubMed: 16936075]

Brack AS, Conboy IM, Conboy MJ, Shen J, Rando TA. A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. Cell Stem Cell 2008;2:50–59. [PubMed: 18371421]

Cerletti M, Jurga S, Witczak CA, Hirshman MF, Shadrach JL, Goodyear LJ, Wagers AJ. Highly efficient, functional engraftment of skeletal muscle stem cells in dystrophic muscles. Cell 2008;134:37–47. [PubMed: 18614009]

Charge SB, Brack AS, Hughes SM. Aging-related satellite cell differentiation defect occurs prematurely after Ski-induced muscle hypertrophy. Am J Physiol Cell Physiol 2002;283:C1228–1241. [PubMed: 12225986]

Charge SB, Rudnicki MA. Cellular and molecular regulation of muscle regeneration. Physiol Rev 2004;84:209–238. [PubMed: 14715915]

Chen AE, Ginty DD, Fan CM. Protein kinase A signalling via CREB controls myogenesis induced by Wnt proteins. Nature 2005;433:317–322. [PubMed: 15568017]

Ciruna B, Jenny A, Lee D, Mlodzik M, Schier AF. Planar cell polarity signalling couples cell division and morphogenesis during neurulation. Nature 2006;439:220–224. [PubMed: 16407953]

Clevers H. Wnt/beta-catenin signaling in development and disease. Cell 2006;127:469–480. [PubMed: 17081971]

Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, Morgan JE. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 2005;122:289–301. [PubMed: 16051152]

Cornelison DD, Filla MS, Stanley HM, Rapraeger AC, Olwin BB. Syndecan-3 and syndecan-4 specifically mark skeletal muscle satellite cells and are implicated in satellite cell maintenance and muscle regeneration. Dev Biol 2001;239:79–94. [PubMed: 11784020]

Cornelison DD, Wilcox-Adelman SA, Goetinck PF, Rauvala H, Rapraeger AC, Olwin BB. Essential and separable roles for Syndecan-3 and Syndecan-4 in skeletal muscle development and regeneration. Genes Dev 2004;18:2231–2236. [PubMed: 15371336]

Cossu G, Borello U. Wnt signaling and the activation of myogenesis in mammals. Embo J 1999;18:6867–6872. [PubMed: 10601008]

del Alamo D, Mlodzik M. Frizzled/PCP-dependent asymmetric neuralized expression determines R3/R4 fates in the Drosophila eye. Dev Cell 2006;11:887–894. [PubMed: 17141162]

Diatchenko L, Lau Y, Campbell A, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov E, et al. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A 1997;93:6025–6030. [PubMed: 8650213]

Egger-Adam D, Katanaev VL. Trimeric G protein-dependent signaling by Frizzled receptors in animal development. Front Biosci 2008;13:4740–4755. [PubMed: 18508542]

Goto T, Davidson L, Asashima M, Keller R. Planar cell polarity genes regulate polarized extracellular matrix deposition during frog gastrulation. Curr Biol 2005;15:787–793. [PubMed: 15854914]

Gros J, Serralbo O, Marcelle C. WNT11 acts as a directional cue to organize the elongation of early muscle fibres. Nature 2009;457:589–593. [PubMed: 18987628]

Hirabayashi Y, Itoh Y, Tabata H, Nakajima K, Akiyama T, Masuyama N, Gotoh Y. The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells. Development 2004;131:2791–2801. [PubMed: 15142975]

Ishibashi J, Perry RL, Asakura A, Rudnicki MA. MyoD induces myogenic differentiation through cooperation of its NH2- and COOH-terminal regions. J Cell Biol 2005;171:471–482. [PubMed: 16275751]

Kengaku M, Capdevila J, Rodriguez-Esteban C, De La Pena J, Johnson RL, Belmonte JC, Tabin CJ. Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. Science 1998;280:1274–1277. [PubMed: 9596583]

Kuang S, Charge SB, Seale P, Huh M, Rudnicki MA. Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. J Cell Biol 2006;172:103–113. [PubMed: 16391000]

Kuang S, Gillespie MA, Rudnicki MA. Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell 2008;2:22–31. [PubMed: 18371418]

Kuang S, Kuroda K, Le Grand F, Rudnicki MA. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell 2007;129:999–1010. [PubMed: 17540178]

Matthews HK, Marchant L, Carmona-Fontaine C, Kuriyama S, Larraín J, Holt MR, Parsons M, Mayor R. Directional migration of neural crest cells in vivo is regulated by Syndecan-4/Rac1 and non-canonical Wnt signaling/RhoA. Development 2008;135:1771–1780. [PubMed: 18403410]

McKinnell IW, Ishibashi J, Le Grand F, Punch VG, Addicks GC, Greenblatt JF, Dilworth FJ, Rudnicki MA. Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex. Nat Cell Biol 2008;10:77–84. [PubMed: 18066051]

Miller C, Sassoon DA. Wnt-7a maintains appropriate uterine patterning during the development of the mouse female reproductive tract. Development 1998;125:3201–3211. [PubMed: 9671592]

Montarras D, Morgan J, Collins C, Relaix F, Zaffran S, Cumano A, Partridge T, Buckingham M. Direct isolation of satellite cells for skeletal muscle regeneration. Science 2005;309:2064–2067. [PubMed: 16141372]

Montcouquiol M, Rachel RA, Lanford PJ, Copeland NG, Jenkins NA, Kelley MW. Identification of Vangl2 and Scrb1 as planar polarity genes in mammals. Nature 2003;423:173–177. [PubMed: 12724779]

Montcouquiol M, Sans N, Huss D, Kach J, Dickman JD, Forge A, Rachel RA, Copeland NG, Jenkins NA, Bogani D, et al. Asymmetric localization of Vangl2 and Fz3 indicate novel mechanisms for planar cell polarity in mammals. J Neurosci 2006;26:5265–5275. [PubMed: 16687519]

Munoz R, Moreno M, Oliva C, Orbenes C, Larrain J. Syndecan-4 regulates non-canonical Wnt signalling and is essential for convergent and extension movements in Xenopus embryos. Nat Cell Biol 2006;8:492–500. [PubMed: 16604063]

Oustanina S, Hause G, Braun T. Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. The EMBO journal 2004;23:3430–3439. [PubMed: 15282552]

Park M, Moon RT. The planar cell-polarity gene stbm regulates cell behaviour and cell fate in vertebrate embryos. Nat Cell Biol 2002;4:20–25. [PubMed: 11780127]

Polesskaya A, Seale P, Rudnicki MA. Wnt signaling induces the myogenic specification of resident CD45 + adult stem cells during muscle regeneration. Cell 2003;113:841–852. [PubMed: 12837243]

Rochat A, Fernandez A, Vandromme M, Moles JP, Bouschet T, Carnac G, Lamb NJ. Insulin and wnt1 pathways cooperate to induce reserve cell activation in differentiation and myotube hypertrophy. Mol Biol Cell 2004;15:4544–4555. [PubMed: 15282335]

Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM. Self-renewal and expansion of single transplanted muscle stem cells. Nature 2008;456:502–506. [PubMed: 18806774]

Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Pax7 is required for the specification of myogenic satellite cells. Cell 2000;102:777–786. [PubMed: 11030621]

Seifert JR, Mlodzik M. Frizzled/PCP signalling: a conserved mechanism regulating cell polarity and directed motility. Nat Rev Genet 2007;8:126–138. [PubMed: 17230199]

Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol 2001;1:4. [PubMed: 11299042]

Tajbakhsh S, Borello U, Vivarelli E, Kelly R, Papkoff J, Duprez D, Buckingham M, Cossu G. Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. Development 1998;125:4155–4162. [PubMed: 9753670]

Tallquist MD, Weismann KE, Hellstrom M, Soriano P. Early myotome specification regulates PDGFA expression and axial skeleton development. Development 2000;127:5059–5070. [PubMed: 11060232]

Torban E, Kor C, Gros P. Van Gogh-like2 (Strabismus) and its role in planar cell polarity and convergent extension in vertebrates. Trends Genet 2004;20:570–577. [PubMed: 15475117]

Torban E, Patenaude AM, Leclerc S, Rakowiecki S, Gauthier S, Andelfinger G, Epstein DJ, Gros P. Genetic interaction between members of the Vangl family causes neural tube defects in mice. Proc Natl Acad Sci U S A 2008;105:3449–3454. [PubMed: 18296642]

Torrente Y, Belicchi M, Sampaolesi M, Pisati F, Meregalli M, D'Antona G, Tonlorenzi R, Porretti L, Gavina M, Mamchaoui K, et al. Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. J Clin Invest 2004;114:182–195. [PubMed: 15254585]

Zallen JA. Planar polarity and tissue morphogenesis. Cell 2007;129:1051–1063. [PubMed: 17574020]

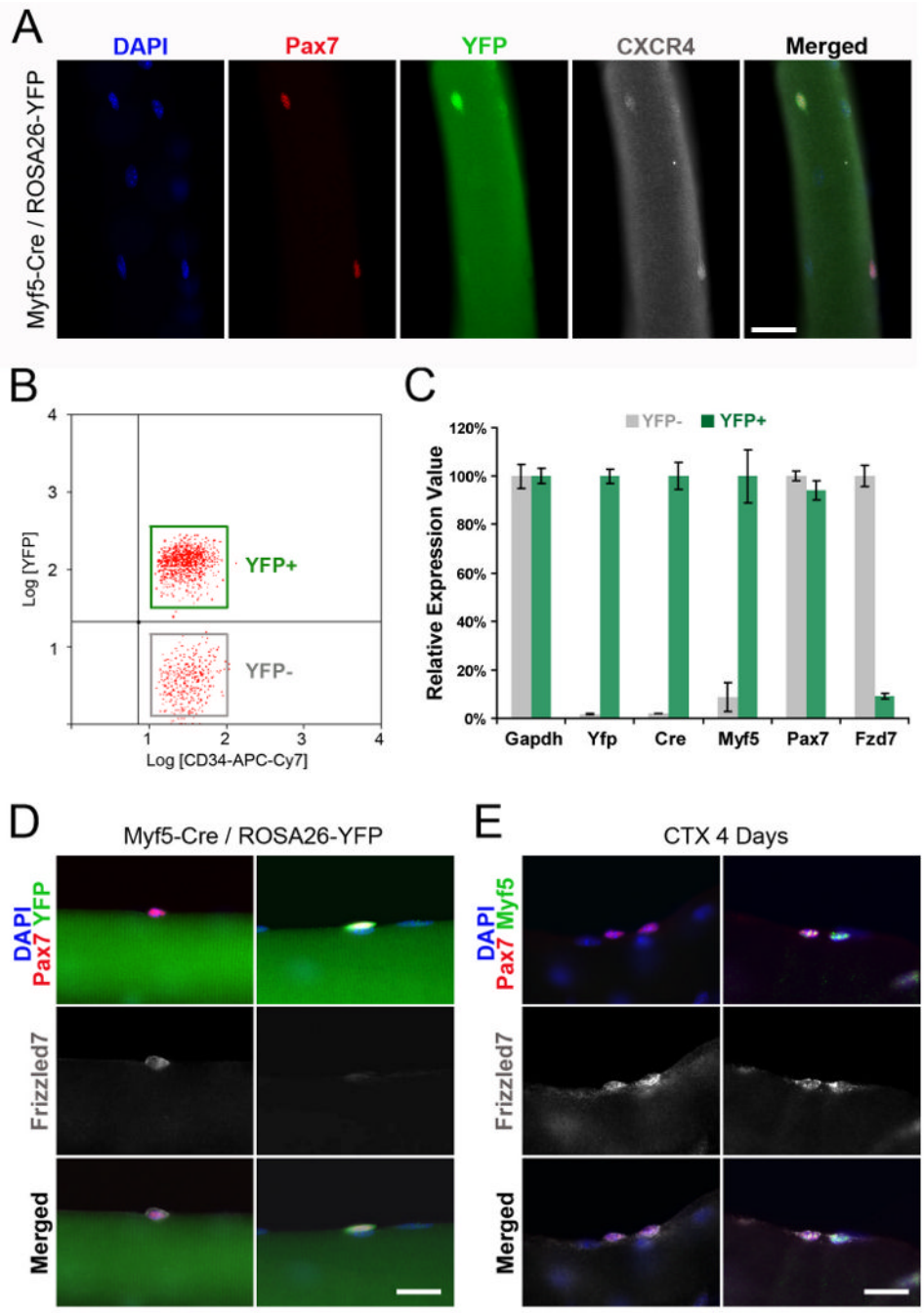

Figure 1. Satellite Stem Cells Express the Wnt receptor Frizzled7

(A) Single myofibers isolated from *Myf5-Cre/ROSA26-YFP* mice. 90% of $Pax7^+$ cells expressed YFP, and 10% of $Pax7^+$ cells were YFP^-. Satellite cells uniformly expressed the stem cell marker CXCR4.

(B) Gated satellite cells ($\alpha7\text{-Integrin}^+$, $CD34^+$, $CD45^-$, $CD31^-$, $CD11b^-$, $Sca1^-$) extracted from resting limb skeletal muscle were separated on the basis of *Myf5-Cre* activated YFP fluorescence.

(C) Real-time PCR analysis of sorted cells showing the absence of *Myf5* and *YFP* transcripts as well as the expression of *Fzd7* transcripts in YFP^- sorted cells (n=3).

(D) Fzd7 was expressed specifically in quiescent $Pax7^+/YFP^-$ satellite stem cells (left) but not in $Pax7^+/YFP^+$ satellite myogenic cells (right) in freshly isolated *Myf5-Cre/ROSA26-YFP* myofibers

(E) Proliferating satellite cells and myogenic precursor cells express Fzd7. Regenerating EDL myofibers were isolated 4 days after TA muscle injury. Both $Pax7^+/Myf5^-$ (left) and $Pax7^+/Myf5^+$ (right) dividing satellite cells expressed Fzd7.

Bars are 10 μm. Errors bars represent SEM.

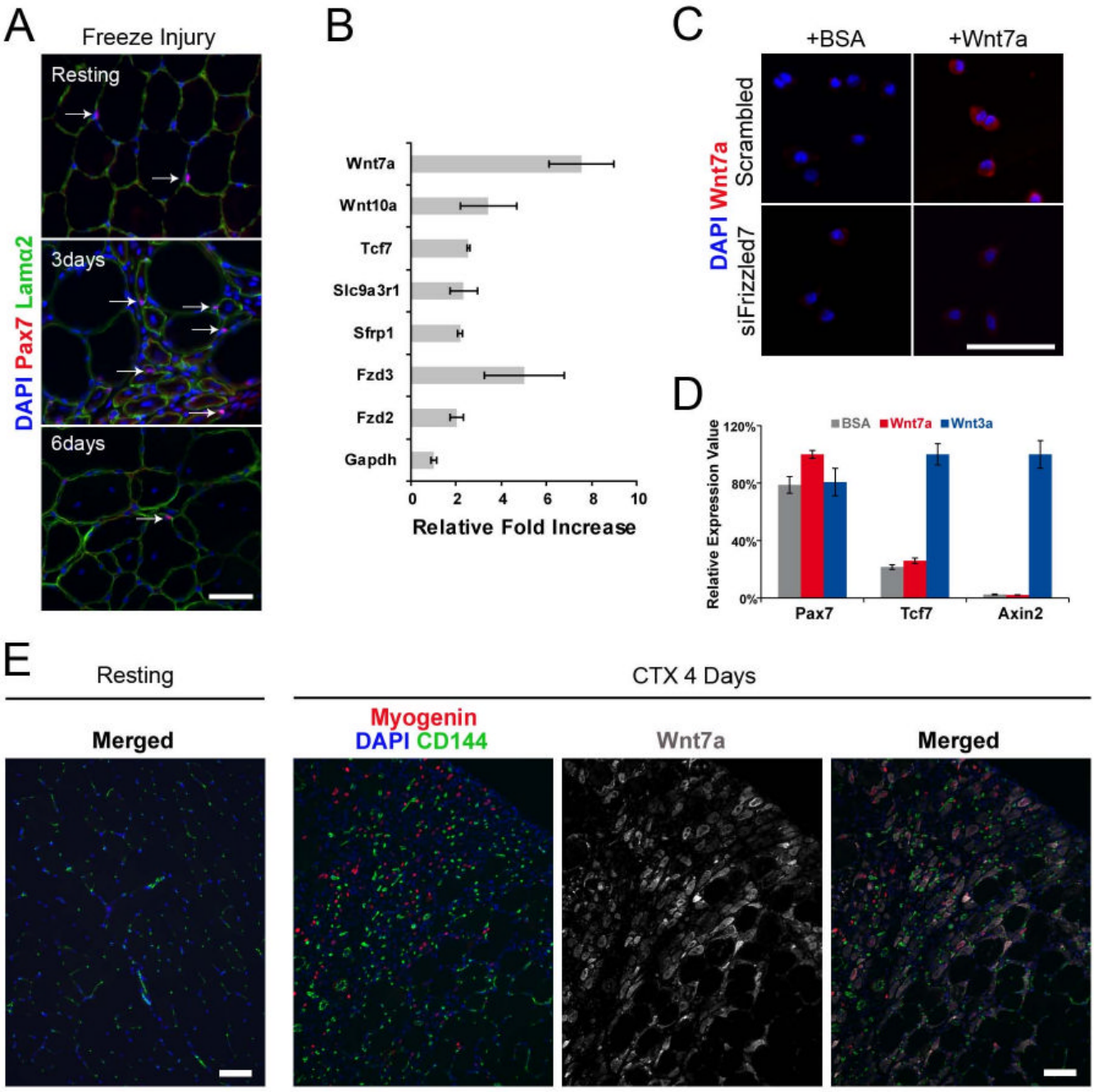

Figure 2. Wnt7a is Highly Upregulated During Muscle Regeneration

(A) Cryosections of resting (top) and freeze-injured TA muscles analyzed at 3 (middle) and 6 (bottom) days following injury. The basal lamina of myofibers is revealed by Laminin α2 chain staining and satellite cell nuclei were visualized by Pax7 staining.

(B) Real-time PCR-array analysis of regenerating TA muscle 6-days following freeze-injury, revealed upregulation of *Wnt7a* mRNA at the time that satellite cells return to quiescence (n=3).

(C) Recombinant Wnt7a protein binds Frizzled7 at the surface of myogenic cells, and this binding is abolished after knock-down of Frizzled7.

(D) Wnt3a but not Wnt7a activates β-catenin/TCF target genes. Real-time PCR analysis of cultured myogenic cells after stimulation with BSA (control), and recombinant Wnt proteins. Only Wnt3a induced the transcription of the β-catenin/TCF target genes *Tcf7* and *Axin2* (n=5). (E) Wnt7a protein is expressed by regenerating myofibers, and not by vascular endothelial cells. Cryosections of 4-days cardiotoxin-induced regenerating (left) and resting contralateral (right) TA muscles. Sections were examined for the expression of Myogenin (differentiating myogenic cells), CD144 (endothelial cells) and Wnt7a proteins.
Bars are 25 μm. Errors bars represent SEM.

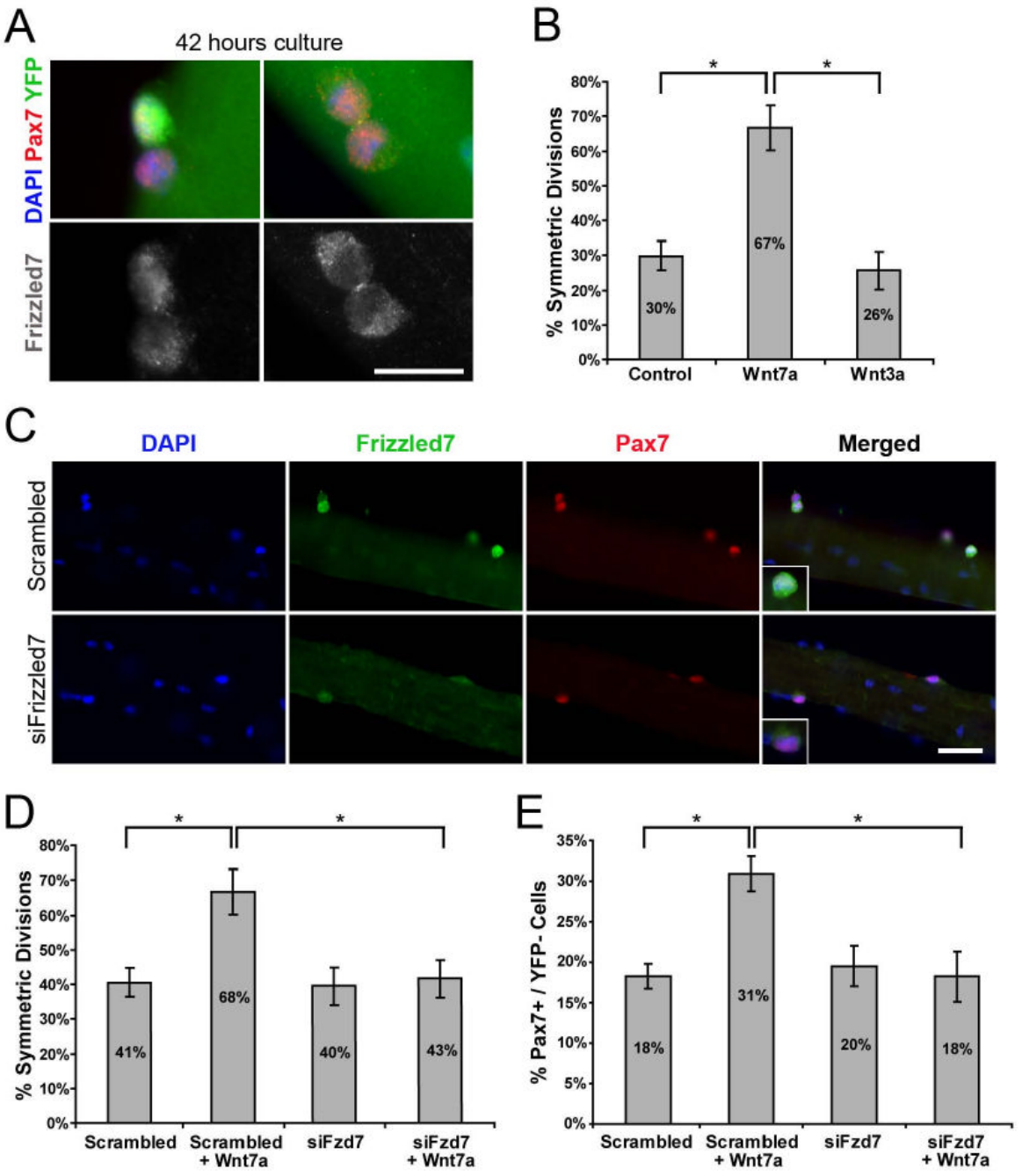

Figure 3. Wnt7a-Frizzled7 Signaling Drives Satellite Stem Cell Expansion

(A) First division of $Pax7^{+}/YFP^{-}$ satellite stem cells, 42 hours after isolation of EDL single myofibers from *Myf5-Cre/ROSA26-YFP* mice, cultured in floating conditions. Satellite stem cells either give rise to one YFP^{-} stem cell and one YFP^{+} committed cell, via asymmetric cell division (left), or alternatively give rise to two YFP^{-} daughter cells by symmetric cell division (right).

(B) Wnt7a but not Wnt3a stimulation markedly increased the proportion of symmetric cell divisions resulting in satellite stem cell expansion (n=3, *p=0.009).

(C) Activated satellite cells on cultured myofibers at 42 h after isolation, do not express Fzd7 (bottom) after knock-down of Fzd7 with siRNA, as compared to cells in non-silencing conditions (top).
(D) The Wnt7a-induced increase in the rate of symmetric satellite stem cell divisions was abrogated following silencing of Fzd7 on myofibers after 42 h of culture (n=3, *p<0.02).
(E) The increase in symmetric satellite stem cell numbers induced by Wnt7a was blocked by silencing of Fzd7 on myofibers after 52 h of culture (n=3, *p<0.03).
Bars are 10 μm. Errors bars represent SEM.

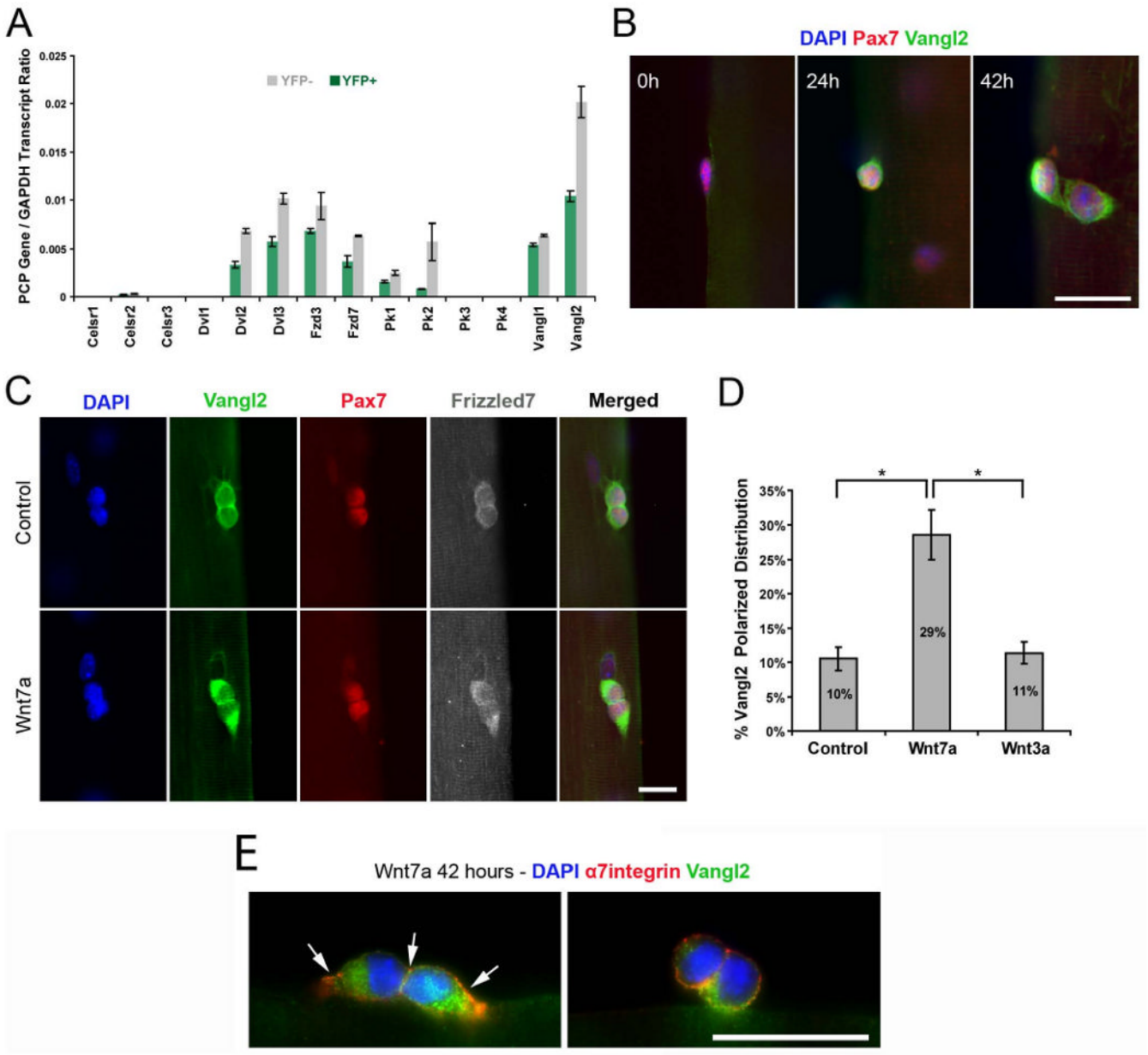

Figure 4. PCP Components are Expressed by Myogenic Cells

(A) Quantitative real-time PCR analysis indicated expression of PCP core component transcripts by YFP^+ and YFP^- satellite cell-derived myoblasts (n=3).

(B) Immunostaining indicated that Vangl2 is upregulated during activation of $Pax7^+$ satellite cells by 24 h on cultured myofibers.

(C) Wnt7a induces polarized Vangl2 cellular localization on opposite poles of dividing $Pax7^+$ satellite cells on cultured myofibers. EDL myofibers were cultured in control medium or medium supplemented with Wnt7a and fixed 42 hours after isolation.

(D) Effects of Wnt treatment on Vangl2 polarization during initial division. Wnt7a signaling, but not Wnt3a, induces polarized localization of Vangl2 and Fzd7 during satellite cell division (n=3, *p=0.006).

(E) Wnt7a-treated myofibers were immunolocalized for Vangl2 and the membrane marker α7-Integrin. Vangl2 is polarized and co-localize to the membrane in planar-dividing satellite

cells (arrows). Note the polarized and upregulated expression of α7-integrin, which facilitates adhesion to the basal lamina of both daughter cells.
Bars are 10 μm. Errors bars represent SEM.

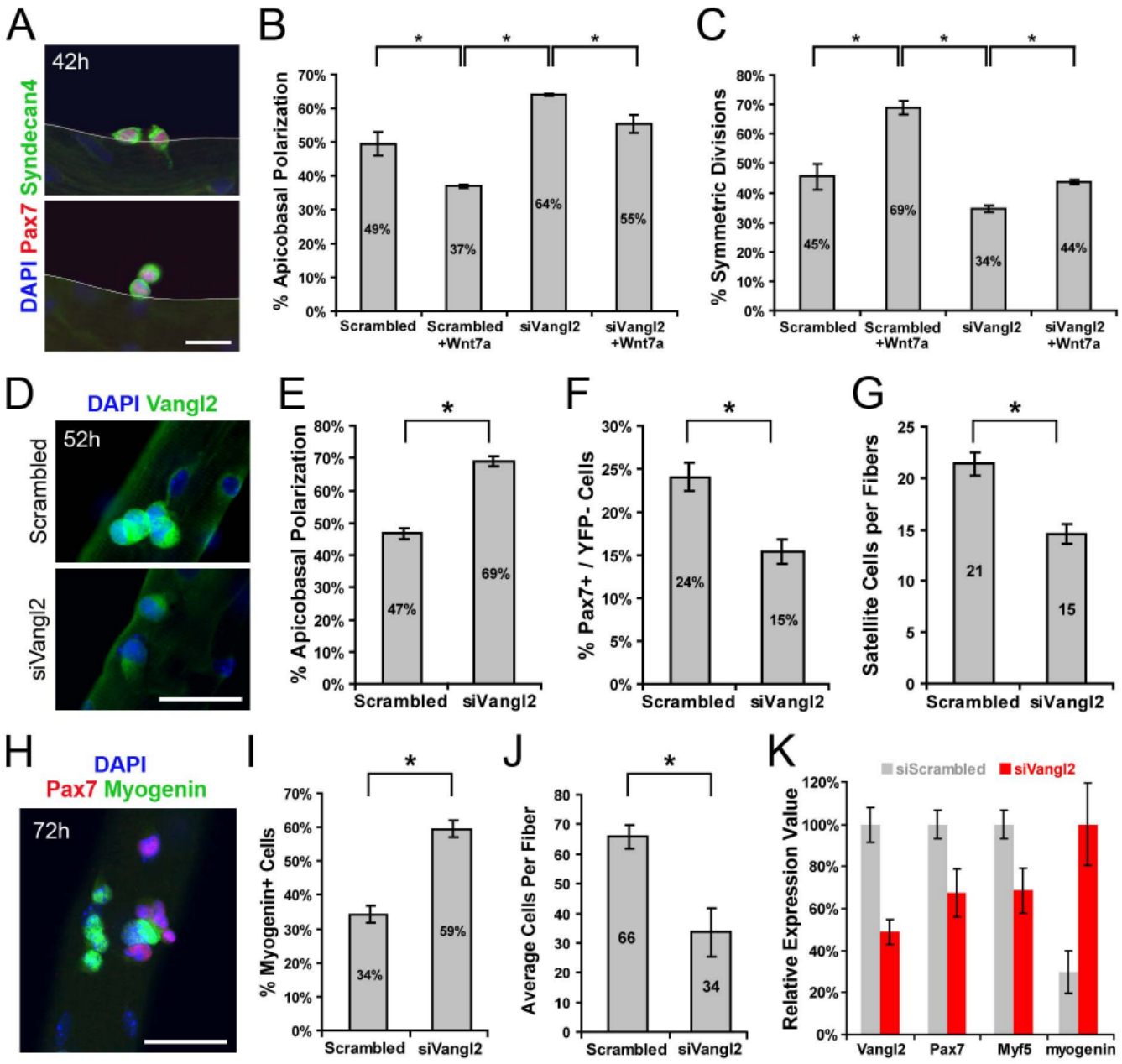

Figure 5. Vangl2 is Required for Symmetric Expansion of Satellite Stem Cells

EDL single myofibers from *Myf5-Cre/ROSA26-YFP* mice were cultured in floating conditions and subjected to either non-silencing or Vangl2 siRNA transfection.

(A) Orientation of $Pax7^{+}$/$Syndecan4^{+}$ satellite cell first cell division at 42 hours. Divisions were scored either as planar (top) or apical-basal (bottom). Note, in myofiber culture, satellite cells translocate to the outside surface of the basal lamina and apical-basal cell divisions are directed into the media.

(B) Wnt7a induces a significant decrease in the proportion of apical-basal cell divisions after 42h of culture supporting its function in stimulating stem cell expansion. Knock down of Vangl2 inhibits the ability of Wnt7a to stimulate planar cell divisions (n=3, *p<0.02).

(C) The Wnt7a-induced increase in symmetric satellite stem cell divisions was abrogated following silencing of Vangl2 on myofibers after 42 h of culture (n=3, *p<0.02).

(D) Activated satellite cells on myofibers knocked-down for Vangl2 after 52 h of culture do not express Vangl2 (bottom) as compared to cells in non-silencing conditions (top).
(E) Knock-down of Vangl2 increased the rate of apical-basal divisions (n=5, *p=0.001).
(F) Knock-down of Vangl2 decreased the proportion of $Pax7^{+}/YFP^{-}$ stem cells (n=3, *p=0.03).
(G) Knock-down of Vangl2 decreased the number of cells per fibers (n=5, *p=0.001).
(H and I) Silencing of Vangl2 increased the proportion of differentiating $Myogenin^{+}/Pax7^{-}$ cells myofibers after 3 days of culture (n=4, * $p=10^{-5}$).
(J) Silencing of Vangl2 depleted the satellite cells pool (n=4, *p=0.001).
(K) Vangl2 silencing promotes myogenic differentiation as revealed by Real-time PCR analysis of gene expression in satellite cell-derived myoblasts (n=4).
Bars are 10 μm. Errors bars represent SEM.

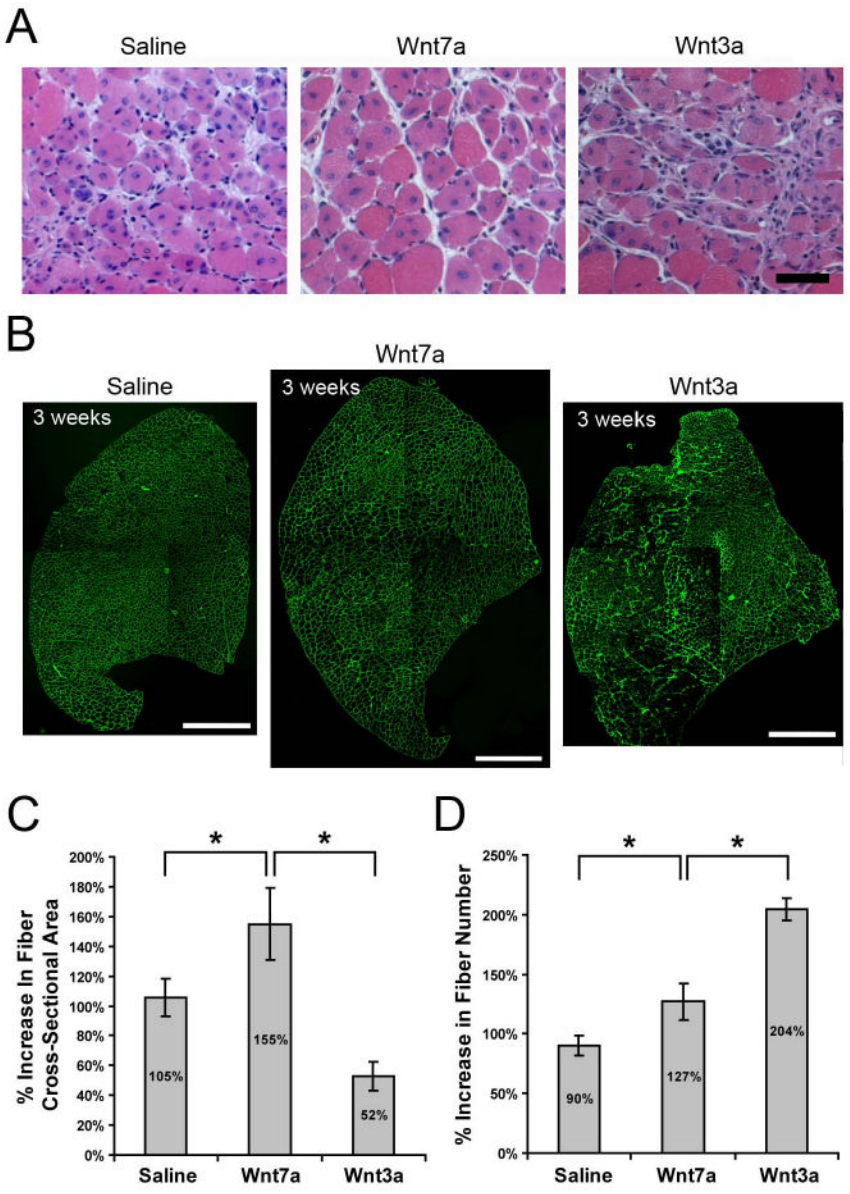

Figure 6. Ectopic Wnt7a Enhances Muscle Regeneration

(A) Representative histology of regenerated TA muscles of 3-month old mice, 8 days following electrotransfer-induced injury. Regenerated myofibers show centrally-located nuclei. Bar is 25 μm.

(B) Representative cryosections of TA muscles 3 weeks following electroporation with CMV-Wnt7a plasmid exhibit accelerated regeneration as evidenced by increased mass, and number and caliber of fibers. Electroporation with CMV-Wnt3a resulted in malformed muscle with abnormal accumulation of matrix. The basal lamina of myofibers was detected by Laminin α2 chain immunostaining. Bars are 200 μm.

(C) Quantification of muscle fiber caliber in TA muscles electroporated with either saline or a Wnt7a / Wnt3a expression plasmids, as compared to contralateral leg, 3 weeks after electroporation (n=4, *p≤0.008). Wnt7a and Wnt3a have divergent effects on myofiber caliber.

(D) Quantification of muscle fiber number in TA muscles electroporated with either saline or a Wnt7a / Wnt3a expression plasmids, as compared to contralateral leg, 3 weeks after electroporation (n=4, *$p \leq 0.03$).
Errors bars represent SEM.

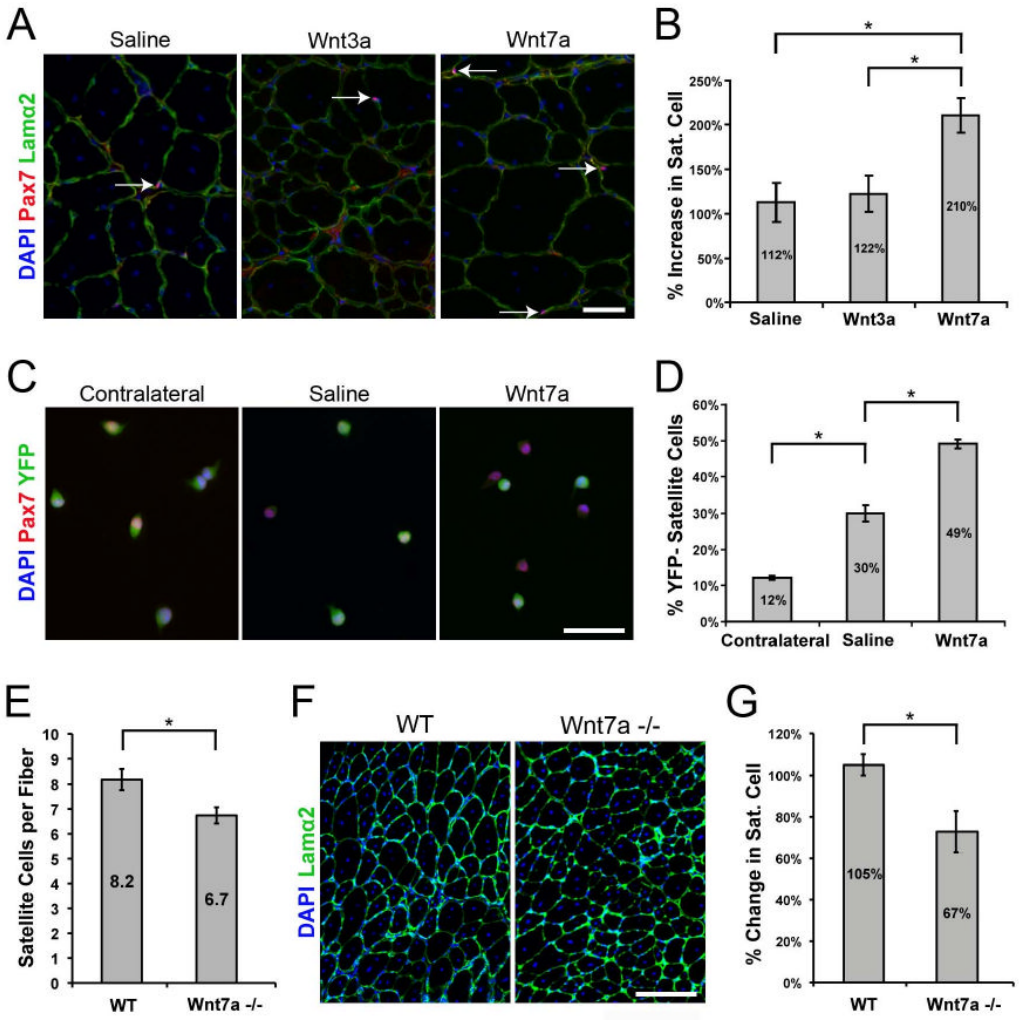

Figure 7. Wnt7a Drives Satellite Stem Cell Expansion

(A) TA muscles of 3-month old mice were electroporated with either saline or a Wnt7a / Wnt3a expression plasmid, and dissected after 3 weeks. Sublaminar $Pax7^{+}$ satellite cells were scored on cryosections of electroporated muscles. Note the increased numbers of $Pax7^{+}$ satellite cells following electroporation with CMV-Wnt7a plasmid. Bar is 25 μm

(B) The satellite cell population was increased by two-fold following electroporation of CMV-Wnt7a plasmid (n=4, *p≤0.03), as compared to Saline- or Wnt3a- electroporated samples.

(C) Satellite cells were FACS-sorted from electroporated *Myf5-Cre/ROSA26-YFP* TA muscles, 3 weeks after electroporation, and plated in culture for 24 hours, fixed and stained for Pax7 and YFP. Bar is 10 μm.

(D) The proportion of $Pax7^{+}/YFP^{-}$ satellite stem cells was significantly increased following overexpression of Wnt7a in electroporated TA muscles (n=5, *p≤0.0001).

(E) $Wnt7a^{-/-}$ myofibers showed a reduced population of Pax7+ satellite cells on myofibers were isolated from EDL muscle. (n=4, *p=0.03).

(F) Cryosections of freeze-injured TA muscles of 3-month old $Wnt7a^{-/-}$ null mice and their littermate controls analyzed at 3 weeks following injury. No significant difference in terms of structure or cross-sectional area was observed in the regenerated muscle. Bar is 20 μm. (n=3).

(G) Decreased numbers of satellite cells were observed in regenerated Wnt7a $^{-/-}$ TA muscles normalized to the number of myofibers in cross-sectional area and to the contralateral leg. (n=3, *p=0.03).

Errors bars represent SEM.