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A Temporal Switch from Notch to Wnt Signaling in Muscle Stem Cells Is Necessary for Normal Adult Myogenesis

Andrew S. Brack,[1,4] Irina M. Conboy,[1,3,4] Michael J. Conboy,[1] Jeanne Shen,[1] and Thomas A. Rando[1,2,*]
[1]Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, CA, 94305-5235 USA
[2]GRECC and Neurology Service, VA Palo Alto Health Care System, Palo Alto, CA 94304, USA
[3]Present address: Department of Bioengineering, University of California Berkeley, Evans 479, Berkeley, CA 94720-1762, USA.
[4]These authors contributed equally to this work.
*Correspondence: rando@stanford.edu
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SUMMARY

The temporal switch from progenitor cell proliferation to differentiation is essential for effective adult tissue repair. We previously reported the critical role of Notch signaling in the proliferative expansion of myogenic progenitors in mammalian postnatal myogenesis. We now show that the onset of differentiation is due to a transition from Notch signaling to Wnt signaling in myogenic progenitors and is associated with an increased expression of Wnt in the tissue and an increased responsiveness of progenitors to Wnt. Crosstalk between these two pathways occurs via GSK3β, which is maintained in an active form by Notch but is inhibited by Wnt in the canonical Wnt signaling cascade. These results demonstrate that the temporal balance between Notch and Wnt signaling orchestrates the precise progression of muscle precursor cells along the myogenic lineage pathway, through stages of proliferative expansion and then differentiation, during postnatal myogenesis.

INTRODUCTION

Muscle regeneration in response to injury requires a careful orchestration of the activation and proliferation of muscle progenitor cells and their subsequent differentiation into multinucleated myotubes to form new muscle tissue. We have previously shown that activation of the Notch signaling pathway is essential for the early phases of this process, controlling activation of resident muscle stem cells (satellite cells) and promoting the proliferation of an intermediate progenitor cell population characterized by expression of high levels of Pax3 (Conboy and Rando, 2002; Boutet et al., 2007). However, little is known about the signaling pathways that control the transition from proliferation to differentiation in this population. Wnt signaling has been shown to be necessary for muscle formation during embryogenesis (Cossu and Borello, 1999) and has also been shown to have an essential role in myogenic differentiation (Anakwe et al., 2003; Petropoulos and Skerjanc, 2002) and myogenic stem cell fate in the adult (Brack et al., 2007).

Although it has been suggested that Wnt may indirectly affect muscle regeneration by inducing a subpopulation of $CD45^+$ cells to adopt myogenic potential and, thus, potentially participate in myogenesis (Polesskaya et al., 2003), the physiologic role of $CD45^+$ cells in muscle regeneration is uncertain (Sherwood et al., 2004; Zammit et al., 2002). We sought to determine if Wnt signaling may play an important role in postnatal myogenesis, akin to that in development, by acting directly on the bona fide muscle stem cells and their progeny to control myogenic lineage progression and therefore muscle regeneration.

In the current study, we show that Wnts act directly on myogenic progenitors, and the activation of this signaling cascade results in the transition of progenitors from the proliferative phase to the differentiation phase during postnatal myogenesis. Furthermore, we show that the increase in Wnt signaling reflects a critical transition from high Notch activation to high Wnt activation and that this molecular switch is essential for effective muscle regeneration. Importantly, the balance between these pathways is reflected by the state of activation of GSK3β, which is maintained in the active state by Notch signaling and inactivated by Wnt signaling. Thus, GSK3β is a pivotal determinant of cell fate in muscle stem cell progeny, integrating inputs from the Notch and Wnt signaling pathways.

RESULTS

Wnt Signaling Is Increased in Myogenic Progenitors Progressively during Lineage Progression and Muscle Regeneration

To investigate if active Wnt signaling was present in regenerating muscle, we analyzed a downstream target of Wnt signaling, Axin2 (Jho et al., 2002), in uninjured and regenerating muscle (see Figure S1A available with this article online). Axin2 was upregulated in regenerating muscle. In order to assess the potential role of canonical Wnt signaling in muscle progenitor cells during postnatal myogenesis, we used the TOPGAL reporter mouse (DasGupta and Fuchs, 1999). There was no detectable evidence of Wnt signaling in uninjured muscle, but there was a striking increase in Wnt signaling in many mononucleated cells within regenerating muscle at 2 and 5 days after injury (Figure 1A). By costaining for the myogenic lineage marker MyoD and for β-galactosidase as an indication of active Wnt signaling, we were able to confirm, both in vivo (Figure 1A, insert) and in vitro

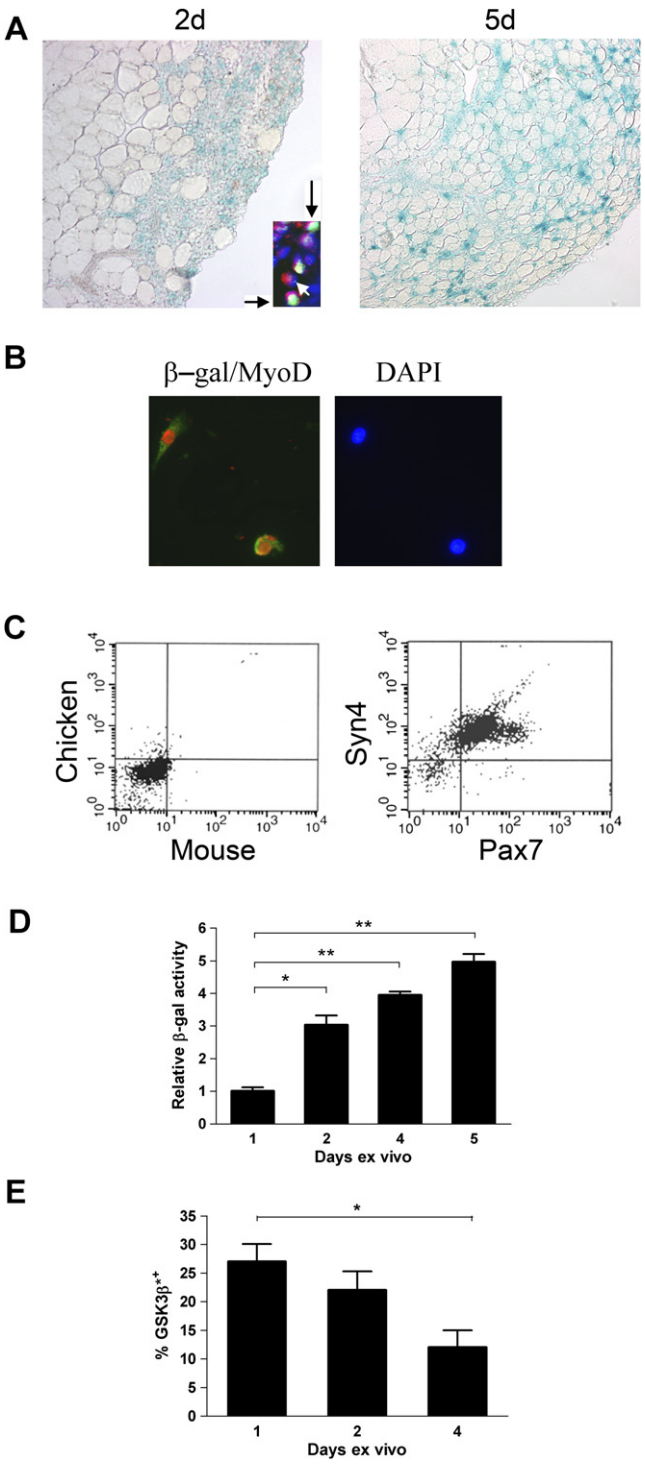

Figure 1. Active Wnt Signaling Increases Progressively during Myogenic Lineage Progression and Muscle Regeneration

(A) Cross-sections of muscle from TOPGAL reporter mice after focal injury. X-gal reactivity (blue) was observed in mononucleated cells in injured areas at 2 days (left panel) and 5 days (right panel) of regeneration. (Insert, sections stained for MyoD [green], β-galactosidase [red], and DAPI [blue]. MyoD$^+$/ β-gal$^+$ cells [arrows] were observed in regenerating areas, demonstrating myogenic cells signaling through the canonical Wnt pathway. Nonmyogenic cells that were responsive to Wnt [MyoD$^-$/ β-gal$^+$; arrowhead] were also present.)

(B) Wnt signaling in myogenic progenitors. Myogenic progenitors isolated from regenerating muscle (3 days after injury) of TOPGAL mice were stained for β-gal (red), MyoD (green), and DAPI (blue). Confirming results in vivo (insert, [A]), myogenic cells were actively signaling through the Wnt cascade (MyoD$^+$/ β-gal$^+$ cells).

(C) Mononucleated cells freshly isolated from bulk myofiber explants cultured for 1 day were analyzed for Syndecan-4 (Syn4) and Pax7 expression by FACS. Isotype controls are shown in the left panel. In these representative FACS plots, 92% of cells were positive for both Syn4 and Pax7, demonstrating a very high proportion of myogenic cells.

(D) Increased Wnt signaling during myogenic lineage progression. Myofibers from TOPGAL mice were maintained in proliferation medium for 1–5 days. Purified myogenic progenitor cells were stripped off the fibers (see Figure 1B) and analyzed for β-gal activity. The levels of β-gal activity are presented relative to the levels at day 1, demonstrating increased Wnt signaling during lineage progression in myogenic progenitors. Error bars, SEM. (*$p < 0.05$, **$p < 0.01$)

(E) Cells stripped off myofibers after 1–4 days in culture were analyzed for Syndecan-4 and GSK3β^{pY216} expression by FACS. GSK3β^{pY216} is the active form of GSK3β (GSK3β*) that declines when the canonical Wnt signaling cascade is activated. Data are presented as the percentage Syndecan-4$^+$ cells that are GSK3β** (*$p < 0.05$).

(Figure 1B), that most myogenic progenitors in the regenerating muscle were signaling through the canonical Wnt pathway. To further investigate if myogenic progenitors signal via Wnt during postnatal myogenesis, we isolated mononucleated cells from myofiber explants. Approximately 92% of mononucleated cells from fiber explants were positive for both Pax7 and Syndecan-4 (sensitive and, in combination, specific markers of the myogenic lineage) as determined by FACS analysis (Figure 1C), allowing for direct assessment of Wnt signaling in this population. The levels of Wnt signaling increased in the satellite cell progeny progressively with time in culture (Figure 1D and Figure S1B).

We also analyzed the state of activation of GSK3β, a component of the canonical Wnt signaling cascade, as an indication of active Wnt signaling in myogenic progenitors. Dephosphorylation of GSK3β at tyrosine 216 is necessary for GSK3β to phosphorylate β-catenin and thereby promoting for canonical Wnt signaling (Hagen et al., 2002; Yuan et al., 1999). FACS analysis demonstrated that the fraction of myogenic cells with detectable phosphorylated GSK3β at tyrosine 216 (GSK3β^{pY216}) was relatively high (indicative of low Wnt signaling) 1 day after injury (Figure 1E), a time when Notch signaling is high (Conboy and Rando, 2002). There was a progressive decline in the levels of GSK3β^{pY216} from day 1 to day 4 (Figure 1E), consistent with increased Wnt signaling seen by reporter gene expression in the TOPGAL mice.

To test for the pattern of expression of canonical Wnts and their receptors in muscle, we performed real-time qRT-PCR on isolated myofiber explants with associated satellite cells after 1 or 4 days in culture. We observed an increase in canonical Wnts after 4 days in culture, the largest increase being in Wnt 3A (Figure S1C). The Wnt receptors, Frizzled-1 and Frizzled-2, also increased, as did Axin2. Therefore, ligand, receptor, and a downstream target were all induced in preparations consisting of only myofibers and activated myogenic progenitors. When the myogenic progenitors were dissociated from the myofibers during this time course and analyzed for ligand (Wnt3A) and receptor (Frizzled-1) expression, an increase only in Frizzled-1 was detected (Figure S1D). These data demonstrate that there is an

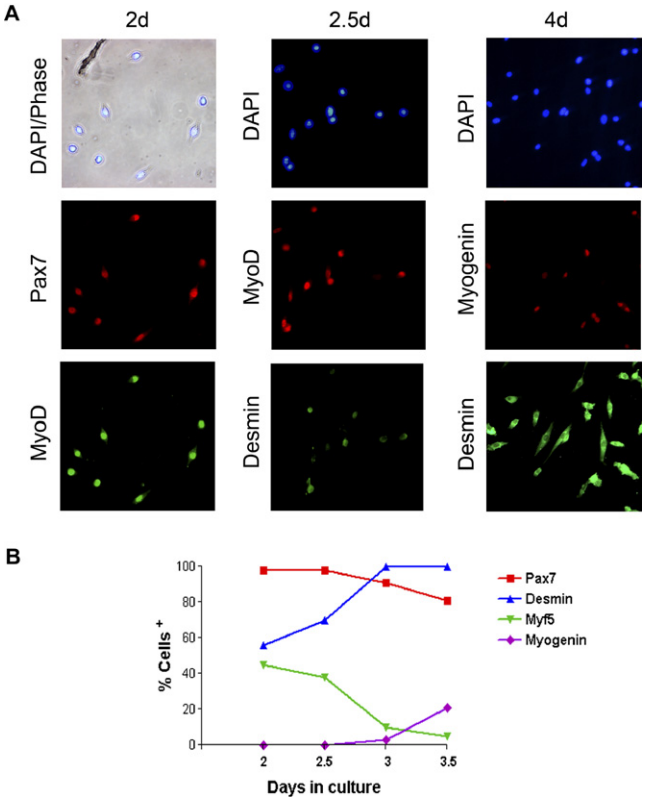

Figure 2. Expression of Myogenic Markers during Lineage Progression

(A) Single fibers were cultured and satellite cell progeny were immunostained with a panel of antibodies (Pax7, MyoD, Desmin, and Myogenin) at different times in culture. Nuclei were visualized with DAPI (blue).

(B) The percentages of cells expressing indicated proteins as determined in (A) were quantified after various times in culture.

increased Wnt responsiveness of myogenic progenitors coincident with an increased expression of Wnt in the tissue during the process of muscle regeneration.

Wnt Signaling Promotes Myogenic Lineage Progression and Premature Muscle Differentiation

Mononucleated cells isolated from single fiber cultures yield a very pure myogenic population of cells (Rosenblatt et al., 1995; Zammit et al., 2002). Nearly 100% of cells expressed both Pax7 and MyoD 2 days after isolation and Desmin 3 to 4 days after isolation (Figures 2A and 2B). Over time in culture, there was a progressive increase in the fraction of cells expressing Myogenin and Desmin and a decline in the fraction of cells expressing Myf5 (Figures 2A and 2B). This expression pattern was used to determine the state of differentiation in the myogenic progenitors when Wnt signaling was manipulated.

We used recombinant Wnt3A protein and a GSK3 inhibitor to activate canonical Wnt signaling. In response to both treatments, we observed an increase in Wnt reporter activity in myogenic progenitors (Figure S2A). Furthermore, treatment of the cells with Wnt3A resulted in an increase in the percentage of myoblasts expressing active β-catenin (Figure S2B) and nuclear localization of β-catenin, which was not observed in proliferating myoblasts under control conditions (Figure S2C).

We analyzed the effects of activation of the Wnt signaling pathway on myogenic lineage progression. Myogenic progenitors were isolated from regenerating muscle 2 days after injury. Relatively few of the myogenic cells expressed Desmin (~10%), as determined by FACS analysis (Figure 3A). During a further 2 days in culture, the fraction of myogenic cells expressing Desmin increased substantially (to ~45%). Strikingly, in cells treated with Wnt3A, there was a much greater increase in the percentage of myogenic progenitors expressing Desmin (to ~75%).

Single fibers were isolated from uninjured muscle, activated for 2 days, and either maintained overnight in control medium or treated overnight with either exogenous Wnt3A or a GSK3 inhibitor to activate the Wnt pathway. Progenitor cells were then analyzed for the state of myogenic differentiation (see Figures 2A and 2B). Activation of Wnt signaling accelerated myogenic lineage progression in progenitors activated ex vivo. After treatment with Wnt3A or the GSK3 inhibitor, a larger percentage of cells expressed high levels of Desmin (Figure 3B and Figure S3A). There was also a lower percentage of cells expressing Myf5 (Figure S3B), concomitant with a decrease in proliferation (Figure S3C). Thus, Wnt signaling is not only upregulated during myogenic lineage progression, but can promote myogenic commitment and lineage progression in satellite cell progeny.

In order to confirm that these studies in vitro reflected the behavior of myogenic progenitors in vivo, Wnt3A was injected into tibialis anterior muscles after freeze injury during the early proliferative phase of myogenic progenitors (2 days after injury). The muscles were analyzed 2 and 3 days later to determine the effect of Wnt3A on myogenic lineage progression and early myogenic differentiation. On day 4 after injury, myotubes in Wnt3A-treated muscles were larger than in control muscles at this time point (Figure 3C), and there was a greater number of nascent myotubes compared to control muscles (Figure 3D), effects that could be mimicked by injection of a GSK3 inhibitor (data not shown). These findings reflect a premature induction of progenitor cell differentiation in vivo, are consistent with the effects observed in vitro, and confirm that Wnt signaling facilitates lineage progression and leads to accelerated differentiation during postnatal myogenesis in vivo. However, this promotion of differentiation did not result in an enhancement of muscle regeneration. Between 4 and 5 days of regeneration, there was an increase in the number of nascent fibers formed in control muscles, whereas in Wnt-treated muscles, there was almost no increase in fiber number (Figure 3D), an effect of the premature differentiation and thus depletion of myogenic progenitors. The end result was that there were large areas of injured muscle devoid of new myofiber formation in Wnt-treated muscles (Figure 3E). Therefore, the enhancement of differentiation by exogenous Wnt3A during the early, proliferative phase was ultimately detrimental to effective tissue regeneration.

Inhibition of Endogenous Wnt Signaling Prevents Myogenic Lineage Progression

As a test of the role of endogenous Wnt signaling in satellite cell activation and lineage progression, we added the soluble Wnt inhibitor, sFRP3, to single fiber cultures at two different times during myogenic lineage progression. Wnt was inhibited either

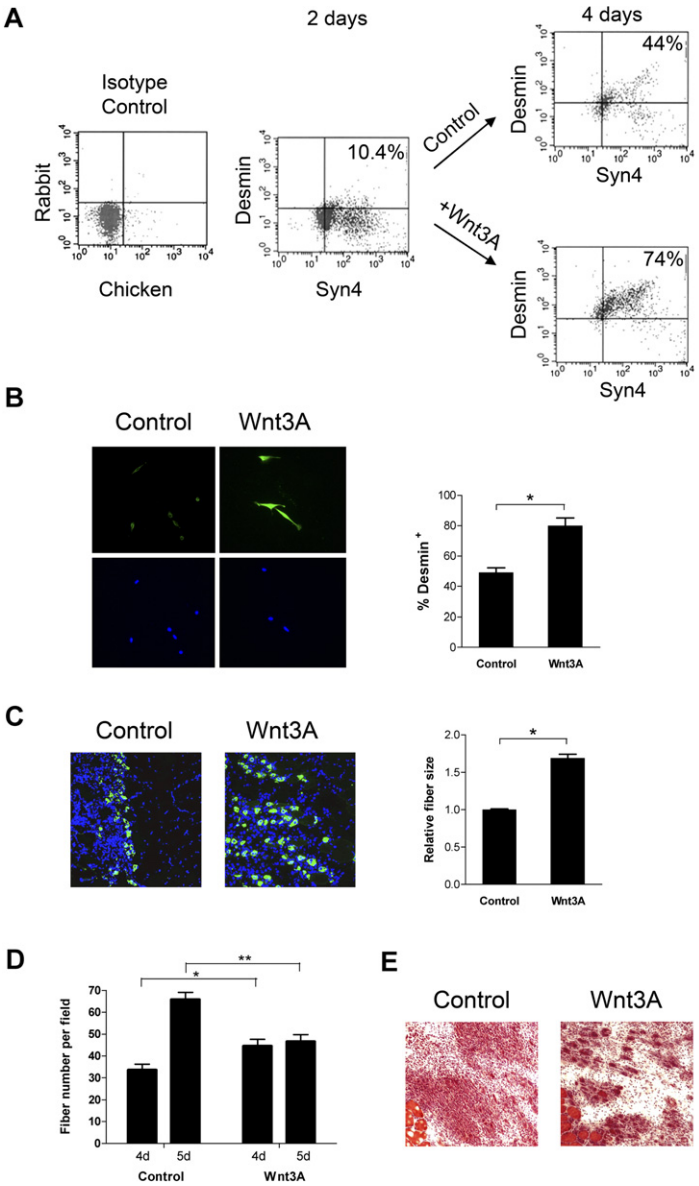

Figure 3. Exogenous Wnt Promotes Myogenic Lineage Progression and Differentiation

(A) Mononucleated cells freshly isolated from a regenerating muscle (2 days after injury) were fixed for analysis or treated with Wnt (100 ng/ml) or control (0.1% BSA) in proliferating conditions for 2 days. Cells were analyzed for Syndecan-4 (Syn4) and Desmin expression by FACS. Isotype controls are shown in the left panel. Representative FACS plots are shown.

(B) Exogenous Wnt3A (100 ng/ml) or control solution was added overnight to single fiber cultures 2 days after isolation, and cells were analyzed for Desmin expression (quantitative analysis shown in the right panel; mean ± SEM) (*$p < 0.05$).

(C) Cross-sections of muscles after 4 days of regeneration treated with either Wnt3A (10 μl of 100 ng/ml) or control solution (10 μl of 0.1% BSA) 1.5 to 2 days after injury. Left, sections stained for embryonic myosin heavy chain (eMHC, green) to identify newly formed myotubes. Right, quantitative analysis of fiber size in control and Wnt3A-treated muscles. Fiber size in regenerating area is relative to fiber size in uninjured area and normalized to control muscles (mean ± SEM) (*$p < 0.05$).

(D) Quantitative analyses of nascent fiber number normalized to cross-sectional area of regenerating tissue in control and Wnt3A-treated muscles analyzed after 4 and 5 days regeneration (mean ± SEM) (*$p < 0.05$; **$p < 0.001$).

(E) Muscles were injected as in (C) and analyzed 5 days after injury. Muscle cryosections were stained with H&E.

early (day 2), when satellite cell progeny are rapidly expanding, or later (day 3.5), when most progeny have progressed to further commitment and differentiation (Zammit et al., 2004). When sFRP3 was added early, no effect on cell fate was observed compared to controls (Figure 4A). By contrast, inhibition of Wnt produced a phenotype when added late, resulting in cultures with a less differentiated phenotype. Therefore, endogenous Wnt signaling acts directly on myogenic progenitors to promote the progression from early, proliferating progenitors to more differentiated progenitors.

We wanted to determine whether this temporal pattern of Wnt activity in satellite cell progeny was observed in vivo during muscle regeneration. Wnt was inhibited by an intramuscular sFRP3 injection either during the proliferative phase (day 2) or the differentiation phase (day 3.5). There was no observable phenotype if Wnt was inhibited during the proliferative phase. By contrast, when the inhibitor was injected later, both the number and size of nascent myotubes was decreased (Figures 4B and 4C). These data confirm that Wnt, acting directly on myogenic progenitors, is essential for normal muscle regeneration and that there is a critical temporal switch from early Notch signaling to later Wnt signaling.

Because of the possibility that changes in endogenous Wnt signaling could affect regeneration by acting on other cells besides satellite cell progeny, we tested for Wnt signaling in inflammatory cells that infiltrate areas of muscle damage. Specifically, we tested for evidence of active Wnt signaling in $CD45^+$ cells, since it has been shown that a subpopulation of $CD45^+$ cells may respond to exogenous Wnt to adopt a myogenic fate (Polesskaya et al., 2003). We found no evidence of active Wnt signaling in the bulk population of $CD45^+$ cells in resting or injured muscle, all of which were lineage$^+$ immune cells and showed no evidence of a proliferative response to Wnt (Figure S4). These data suggest that there is no significant contribution of $CD45^+$ cells to the population of progenitor cells that actively regenerate muscle and that the effects of Wnt signaling described here are not a reflection of any subpopulation of $CD45^+$ cells.

The Notch Pathway Feeds into the Wnt Cascade via GSK3β in Adult Myogenic Progenitors

To further characterize this temporal switch between Notch and Wnt signaling, we used a soluble Jagged fusion protein (Jagged1-Fc) to inhibit Notch signaling and a γ-secretase inhibitor, which has been shown to block Notch signaling in different cell types, including myogenic progenitors (Dahlqvist et al., 2003). Inhibition of Notch signaling promoted myogenic lineage progression both in vitro and in vivo (Figures 5A and 5B), thus confirming previous results that Notch signaling maintains an early lineage fate (Conboy and Rando, 2002; Conboy et al., 2003). However,

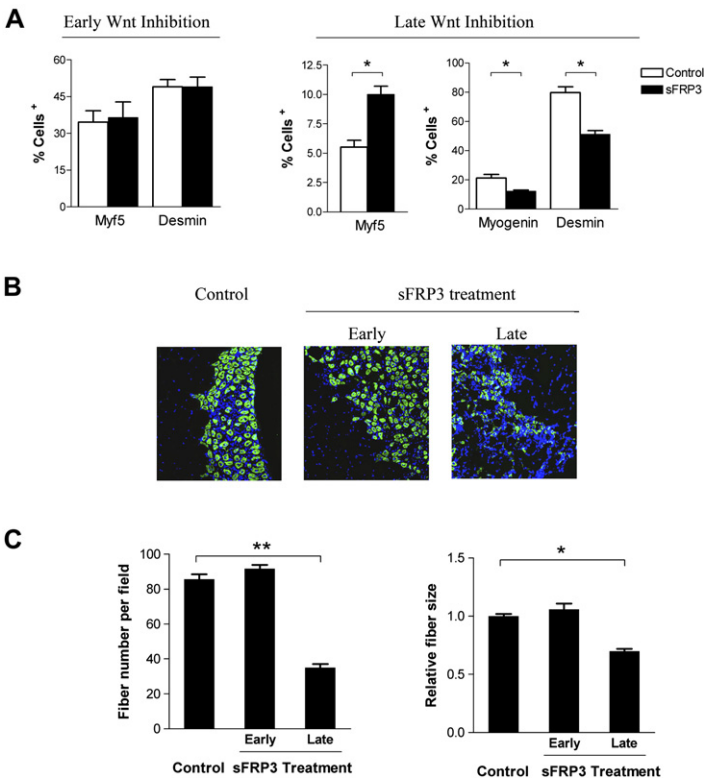

Figure 4. Temporal Regulation of Wnt Signaling Is Necessary for Lineage Progression and Effective Muscle Regeneration

(A) sFRP3 (30 ng/ml) was added overnight to single fiber cultures to inhibit Wnt either 2 days ("early") or 3.5 days ("late") after isolation. The fraction of cells expressing an early myogenic lineage marker (Myf5) or late myogenic lineage markers (Desmin and Myogenin) were quantified (mean ± SEM). Histograms showing the state of myogenic lineage progression, depending upon the timing of inhibition of Wnt signaling, are shown (*$p < 0.05$).

(B) Injured muscle was injected with sFRP3 (10 μl of 30 ng/ml) or 0.1% BSA (10 μl) either 2 days ("Early") or 3.5 days ("Late") after injury. Muscles were analyzed after 5 days of regeneration by immunostaining for eMHC (green) and DAPI (blue).

(C) Muscles treated as in (B) were analyzed by determining the number of $eMHC^+$ fibers in a normalized field from the regenerating area (left histogram) and fiber size relative to control (right histogram; data expressed as mean ± SEM) in H&E-stained cryosections (*$p < 0.05$, **$p < 0.001$).

when the Notch inhibitor was injected during the differentiation phase in vivo (Figure 5B), the effects were negligible, indicating a functional decline in Notch signaling at a time when Wnt signaling is increasing. This suggests a functional antagonism between these two pathways.

In order to test for crosstalk between the Notch and Wnt signaling pathways in myogenic progenitors, we first examined readouts of Wnt signaling in cells in which Notch signaling was altered. When Notch signaling was inhibited during the early proliferative phase, we observed a premature increase in Wnt signaling (Figure 6A), comparable to that seen in response to exogenous Wnt3A or GSK3 inhibition (Figure S2A), suggesting that active Notch might suppress Wnt signaling during this phase. Indeed, direct activation of Notch using an activating antibody in Wnt3A-treated myoblasts led to fewer cells expressing detectable nuclear-localized β-catenin (Figure 6B).

We next asked whether GSK3β, which is inactive in the presence of Wnt signaling and targets β-catenin for degradation, was regulated by Notch signaling. We used antibodies that recognize GSK3β when phosphorylated at serine 9 ($GSK3\beta^{pS9}$) or at tyrosine 216 ($GSK3\beta^{pY216}$); these are readouts of inactive and active GSK3β, respectively (Wang et al., 1994). Inhibition of Notch resulted in a decline in $GSK3\beta^{pY216}$ comparable to that seen in response to Wnt3A (Figure 6C). Conversely, $GSK3\beta^{pS9}$ staining was increased when Notch was inhibited (Figure 6D), suggesting that both inhibition of Notch and activation of Wnt signaling appear to inactivate GSK3β. Furthermore, direct activation of Notch led to a significant increase in $GSK3\beta^{pY216}$ compared to controls as detected by FACS analysis (Figure S5). When Notch was activated in cells treated with Wnt 3A, the Wnt-induced inactivation of GSK3β was partially inhibited (Figure 6E). Therefore, there is clearly crosstalk between the Notch and Wnt cascade reflected by the divergent regulation of GSK3β activation.

A Temporal Balance between Notch and Wnt Signaling Is Necessary for Effective Muscle Repair In Vivo

To investigate the balance between the Notch and Wnt pathways in vivo, we activated these pathways individually or together during the phase of progenitor cell proliferation. Activation of the Wnt pathway promoted differentiation, albeit prematurely, reflected by the decreased fiber number and larger fiber size (Figures 6F and 6G). Conversely when Notch was activated, there was a marked inhibition of myogenesis both in terms of number and size of nascent fibers, consistent with the role of Notch in inhibition of lineage progression (Figure 5). Strikingly, this block of myogenesis by enhanced Notch signaling could be rescued by activation of the Wnt pathway via GSK3β inhibition. These data further illustrate the fine balance between Notch and Wnt signaling that is necessary for effective regeneration with GSK3β as a key nodal point at which these two pathways interact.

DISCUSSION

The results presented here reveal molecular regulatory pathways involved in cell fate determination during postnatal myogenesis, and provide new insights into functional and molecular interactions between Notch and Wnt signaling pathways. We have previously shown that Notch activity is necessary during early activation and proliferation of satellite cells (Conboy et al., 2003). The results of the current study demonstrate that Wnt signaling is activated in myogenic progenitors later during lineage progression, leading to the generation of fusion-competent myoblasts. Moreover, the effects of exogenous activators and inhibitors of Wnt signaling on adult myogenesis strongly suggest that the Wnt pathway is a key regulator of myogenic commitment and terminal differentiation, consistent with findings during avian wing development (Anakwe et al., 2003).

The upregulation of Axin2 suggests that Wnts are present and actively signaling in regenerating muscle. Previous reports have

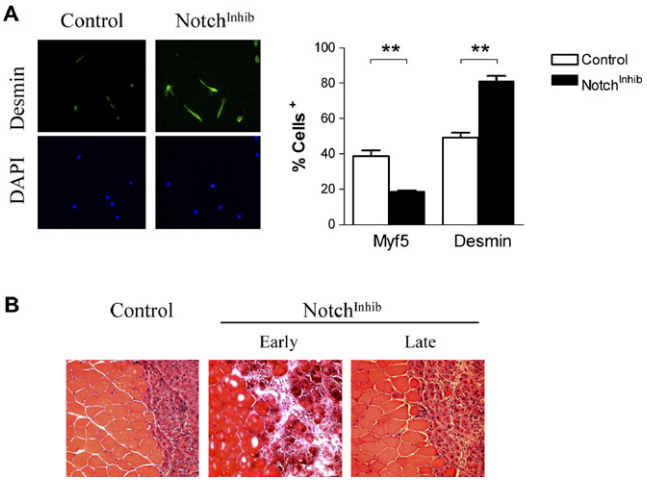

Figure 5. Inhibition of Notch Signaling Early during Satellite Cell Activation Promotes Myogenic Lineage Progression

(A) After 2 days in culture, myogenic cells from single fiber cultures were treated overnight with a γ-secretase inhibitor (L-685,458) (2 μM) to inhibit Notch signaling. Cells were immunostained for Myf5 and Desmin. Left, representative image of Desmin staining; right, quantitative analysis (mean ± SEM) (*$p < 0.05$, **$p < 0.01$).

(B) Injured muscle was injected with either a control solution (0.1% BSA [10 μl]) or a solution containing Jagged1-Fc (10 μl, 100 μg/ml) either 2 days ("early") or 3.5 days ("late") after injury. Muscles were analyzed after 5 days of regeneration by H&E staining of cryosections. Early inhibition caused premature differentiation, resulting in fewer but larger myotubes.

suggested some Wnts are expressed in regenerating muscle and that the muscle fibers are the source of Wnt (Polesskaya et al., 2003). A quantitative analysis of Wnt transcripts in regenerating muscle demonstrated that Wnts, such as Wnt5A, are expressed in uninjured muscle but appear to decrease during the first 24 hr after injury, a time when the muscle tissue is degenerating, then are restored as the muscle is healed (Zhao and Hoffman, 2004). We observed an increase in transcript levels of Wnt3A in myofibers isolated and placed in culture for 4 days. The current data are consistent with expression of Wnts being low during the early phase of regeneration, perhaps even reflecting a decrease from resting levels consistent with previous results (Zhao and Hoffman, 2004), when progenitor expansion is occurring and then increase in injured muscle to promote myogenic lineage progression. However, as important as the level of ligand expression is the presence of their cognate receptors. We have demonstrated that, during lineage progression, myogenic progenitors express both receptors and downstream targets of Wnt, arguing strongly that myogenic progenitors are signaling through canonical Wnt signaling.

During muscle repair, the progenitor cells responsible for the regenerative response are derived almost solely from the satellite cell population (Zammit et al., 2002; Collins et al., 2005). It has been reported that repeated daily injections of exogenous Wnt promoted the myogenic conversion of $CD45^+$ cells (Polesskaya et al., 2003), but the physiological significance of these rare cells in the process of muscle regeneration in vivo is unknown. With daily injections of Wnt inhibitors, fewer desmin-expressing cells were isolated from the regenerating muscle. This is consistent with our data and our conclusion that inhibition of Wnt signaling prevents myogenic lineage progression, but is likely due to the effects of the inhibitors on myogenic progenitors rather than on $CD45^+$ cells present in the regenerating tissue. In fact, our studies show that daily injections of Wnt actually interfere with, rather than enhance, muscle regeneration because of the negative effects on early phases of myogenesis. Furthermore, our studies of resting and injured muscle suggest that any $CD45^+$ cells, responding to Wnt and adopting myogenic properties, are likely to make only a very minor contribution, at most, to muscle regeneration.

The timing of the regulation of the Wnt pathway is consistent with the hypothesis that low Wnt activity is essential during proliferative expansion of tissue-specific progenitor cells and that increased Wnt activity promotes the progression of those cells along the myogenic lineage (Figures 3A and 4A). Thus, a fine tuning of the temporal pattern of Wnt signaling activity might regulate the expansion versus fusion of myoblasts. Consistent with this prediction, exogenous Wnt added early during the proliferative phase of regeneration promoted premature differentiation; there were more nascent fibers and they were larger. This had a detrimental effect on muscle regeneration as areas of the injury area were devoid of nascent fibers (Figure 3E). Indeed, a high level of Wnt signaling in quiescent and early activated satellite cells during aging leads to progenitors losing their myogenic fate and acquiring a fibrogenic fate (Brack et al., 2007). Therefore, low Wnt signaling is required for the generation of adequate numbers of myogenic progenitors. Inhibition of Wnt at this early stage had negligible effects on the regenerative response (Figure 4), consistent with the relatively low level of Wnt signaling in young myogenic progenitors. By contrast, inhibition of Wnt after the proliferation phase resulted in a delay of lineage progression and terminal differentiation. This pattern was opposite to that of the Notch signaling pathway (Figure 5), where there was impaired regeneration in vivo when Notch was inhibited early but not late. Although levels of active Notch may continue to be elevated at these late time points, the increase in expression of Numb would inhibit active Notch signaling, consistent with the absence of any detectable effects of Notch inhibitors at these time points. Thus, cell-fate determination along the myogenic lineage is controlled by a precise balance between the (early) Notch and (later) Wnt signal transduction pathways. The mechanisms whereby Wnt promotes myogenic lineage progression and differentiation and the downstream targets of Wnts that are activated or repressed during this process remain to be determined. Interestingly, β-catenin can enhance the transcriptional activity of the myogenic regulatory factors and thereby induce myosin heavy-chain expression in P19 cells and promote differentiation in myogenic cells (Pan et al., 2005; Pan et al., 2006). The binding of β-catenin to myogenic determination genes is reminiscent of β-catenin's ability to bind to tissue-specific determination genes during development (Olson et al., 2006).

The temporal balance between early Notch and later Wnt signaling required for effective lineage progression and muscle regeneration is controlled in part by Notch signaling feeding into the Wnt cascade. We observed that Notch inhibition promoted active signaling via the canonical Wnt cascade (Figure 6A), whereas Notch activation inhibited the nuclear localization of β-catenin observed in the presence of Wnt signaling (Figure 6B). Our results indicate GSK3β as a focal point of the

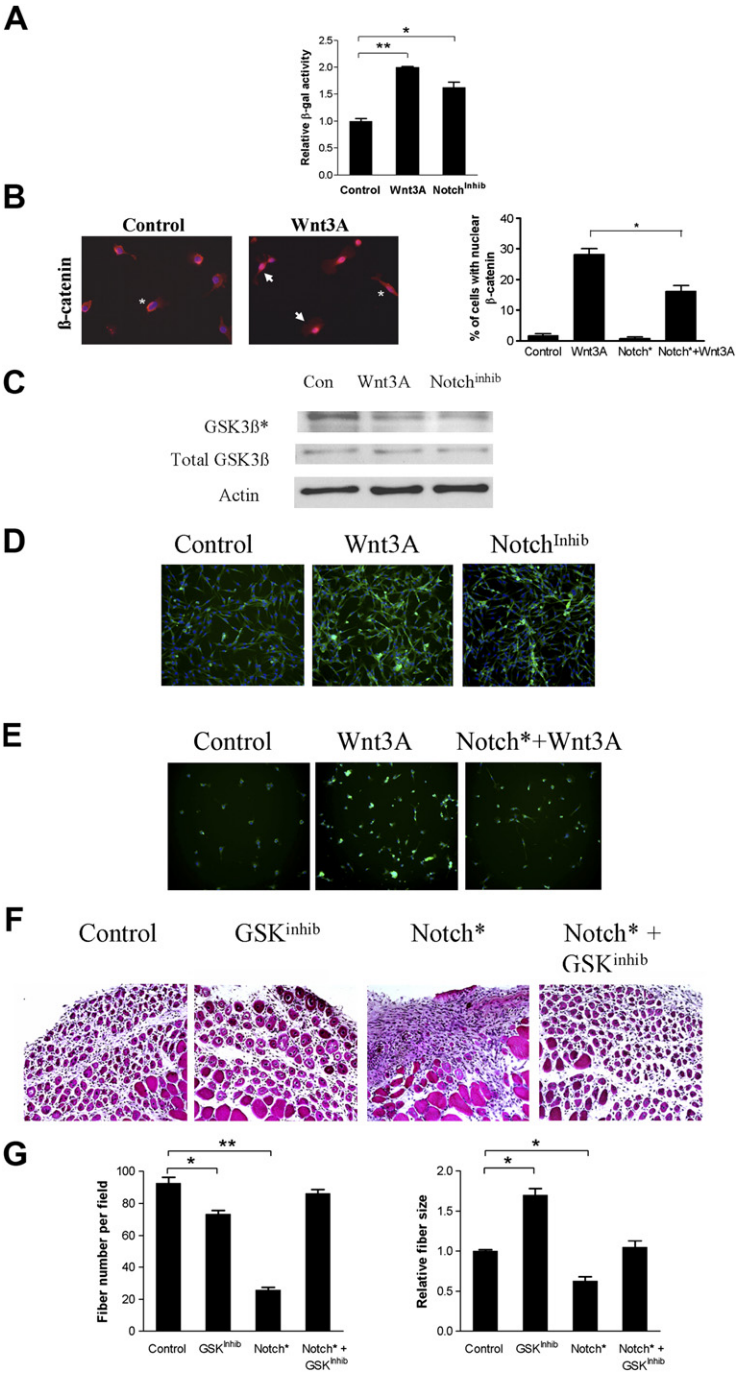

Figure 6. Crosstalk between Wnt and Notch Signaling Ensures the Correct Balance for Normal Muscle Regeneration

(A) Myofiber-associated progenitors were stripped off myofibers after 2 days in growth conditions. Cells were incubated in Wnt3A (100 ng/ml) or the γ-secretase inhibitor L-685,458 (5 ng/ml) overnight. β-gal activity was measured and normalized to total protein content, and the activity levels in the treated samples are presented relative to control levels (mean ± SEM) (*$p < 0.05$, **$p < 0.01$).

(B) Primary myoblasts were treated with an activating Notch antibody or control IgG for 1 hr and subsequently plated for 17 hr in media (5% horse serum in DMEM) containing Wnt (60 ng/ml) or BSA (0.2%) as a control. The cultures were then analyzed for nuclear localization of β-catenin (red) by immunohistochemistry. Representative images are shown on the left (arrows indicate β-catenin$^+$ nuclei; asterisk denotes β-catenin$^-$ nucleus). Quantitative analysis is shown on the right (mean ± SEM) (*$p < 0.05$).

(C) Western blotting was performed on extracts from progenitor cells to test for changes in the levels of active GSK3β ("GSK3β*", determined as the level of GSK3β $^{(pY216)}$) when the Wnt pathway was activated by exogenous Wnt3A or the Notch pathway was inhibited by the γ-secretase inhibitor, as in (A). Actin was used as a loading control. Blots from replicate experiments were quantified, after normalization to Actin, to determine to the ratios of GSK3β*/total GSK3β. The ratios were reduced to 41% ± 7% of control by Wnt activation and 51% ± 10% of control by Notch inhibition.

(D) Primary myoblasts were incubated in Wnt3A (60 ng/ml), the γ-secretase inhibitor, or control solution for 17 hr. Cells were stained with a GSK3β^{S9} (green) antibody which reacts with the serine 9 phosphorylated form of GSK3β and is a readout of the inactive form of the enzyme. DAPI stains nuclei blue.

(E) Primary myoblasts were treated with control IgG or Notch activating antibody, followed by incubation in Wnt3A or control solution, and then analyzed by immunohistochemistry for GSK3β^{S9} (green). Wnt3A treatment increased levels of GSK3β^{S9}, and this was partially prevented by incubation with the Notch activating antibody. DAPI stains nuclei blue.

(F) Two and a half days after injury, muscles were injected with either 10 μl of 0.1% BSA ("Control"), the GSK3 inhibitor (0.35 ng/ml) to activate the Wnt pathway (GSK3^{inhib}), the activating Notch antibody (1:4 dilution) to activate the Notch pathway (Notch*), or both (Notch* + GSK3^{inhib}). Five days after injury, cryosections were stained with H&E to assess the effectiveness of muscle regeneration under these different conditions.

(G) Quantitation of results from experiments as described in (D) by determining the fiber number (left panel) and fiber size (right panel; normalized to control values) in regenerating muscles under the different conditions (mean ± SEM) (*$p < 0.05$, **$p < 0.001$).

crosstalk between Notch and Wnt pathways that is functionally significant for the cell-fate determination in adult tissue repair. Consistent with these findings, genetic screening of Notch gain-of-function alleles in *Drosophila* identified the GSK3β homolog, Shaggy, to be downstream of Notch signaling (Ruel et al., 1993). It was shown that active GSK3β can phosphorylate Notch-1 and -2 (Foltz et al., 2002; Espinosa et al., 2003), again suggesting that this may represent a point of direct interaction between Notch and Wnt signaling (Wesley, 1999; Axelrod et al., 1996). GSK3β is a regulator that is at the crossroads of multiple signaling pathways, including those initiated by calcium fluxes, G protein-coupled receptor activation, and Hedgehog signaling, and participates in such diverse cellular glycogen metabolism to cell-cycle regulation to tissue patterning (Doble and Woodgett, 2003). We show that the antagonistic nature of Notch and Wnt pathways is mediated by reciprocal effects on the activation status of GSK3β in myogenic progenitor cells (Figure 6B). GSK3β is inactivated by Wnt (Kim and Kimmel, 2000; Ding et al., 2000; Patel et al., 2004), although the precise mechanism remains elusive. We show that GSK3β is active in

undifferentiated progenitors (Figure 1E), at a time when Notch signaling is high (Conboy and Rando, 2002), and Wnts and their receptors are low in the tissue. Furthermore, when Notch signaling was directly activated, there was a corresponding increase in the activation of GSK3β represented by increased phosphorylation at Y216 (Figure S5). When Notch signaling was maintained in vivo (Figures 6C and 6D), which presumably maintained GSK3β activity high, the addition of a GSK3 inhibitor was sufficient to restore the balance and, more importantly, the normal regeneration process.

The promotion of myogenic lineage progression by exogenous Wnt was associated with a decrease in tyrosine phosphorylation at residue 216 of GSK3β, consistent with previous results showing the important role of the phosphorylation state of this residue for β-catenin stabilization and, thus, downstream Wnt signaling (Yuan et al., 1999; Hagen et al., 2002). We also observed an increase in serine phosphorylation at residue 9 of GSK3β after Wnt treatment, consistent with GSK3β being less active. Ding et al. reported that phosphorylation of GSK3β at serine 9 in CHO cells was not affected by a brief (10 min) treatment with Wnt-conditioned media (Ding et al., 2000). There are many technical differences between that study and our studies presented here to account for this difference. For example, the duration of exposure to Wnt could be important. We did not observe changes in GSK3β^{S9} even after 2 hr in Wnt3A, but changes were evident after approximately 7 hr. Activation of Notch signaling increased phosphorylation of tyrosine 216 of GSK3β whereas dephosphorylation of this residue was observed following treatment of the cells with a Notch inhibitor. Consistent with the reciprocal effects of Notch and Wnt on GSK3β activity, activation of Notch inhibited the increase of serine 9 phosphorylation due to Wnt3A treatment. The regulation of the phosphorylation status of GSK3β by Notch could involve the regulation of both kinases and phosphatases that act on GSK3β (Kim et al., 2002). However, we cannot exclude the possibility that there are other points of crosstalk between the Notch and Wnt pathways, upstream of GSK3β, that fine tune the balance between these two functionally antagonistic pathways in myogenic progenitors.

We present a model of the balance between Notch and Wnt signaling in satellite cell progeny, and the normal temporal switch that occurs during lineage progression (Figure S6). This balance serves to assure the production of the appropriate numbers of myogenic progenitors and to precisely regulate their cell fate during postnatal myogenesis. During initial activation and proliferation of satellite cells, Wnt signaling is low and the Notch pathway is activated. Low Wnt signaling is due to both low secretion of Wnt in the injured tissue, low expression of Wnt receptors on myogenic progenitors, and repression of Wnt signaling via Notch activation, reflecting the convergence of these two pathways on the regulation of GSK3β activity in these cells. Low Wnt signaling is necessary for myogenic progenitors to maintain their fate soon after activation (Brack et al., 2007). Once adequate numbers of progenitors have been generated, Wnt signaling increases in the progenitors, whereas Notch activity begins to decrease because of the upregulation of Numb (Conboy and Rando, 2002), consistent with the finding that ectopic Wnt3A leads to the increase in Numb levels in somite cultures from the chick (Holowacz et al., 2006). Due to the crosstalk between the Notch and Wnt cascade via GSK3β, the inhibition of Notch signaling would facilitate the activation of Wnt signaling further. The crosstalk between these two pathways via GSK3β is suitable for a rapid temporal switch to activate the Wnt pathway and repress the Notch pathway, thus enabling the rapid differentiation and fusion of myoblasts into nascent myotubes. These studies also highlight a role of Wnt to promote lineage progression rather than to maintain cells in a stem cell stage (Reya and Clevers, 2005). We have recently shown that Wnt can also cause myogenic progenitors to convert to a fibrogenic fate if Wnt signaling is activated during quiescence or early activation of adult myogenic stem cells (Brack et al., 2007). Therefore, apparently opposing actions of Wnt signaling may reflect differential effects depending on the cellular context. More generally, understanding the reciprocal roles of Notch and Wnt signaling during muscle regeneration is essential for therapeutic approaches that aim to regulate expansion versus differentiation of myogenic progenitor cells to enhance tissue repair.

EXPERIMENTAL PROCEDURES

Animals

Two- to four-month-old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). TOPGAL mice were kindly provided by Elaine Fuchs (Rockefeller University, NY). Animals were housed and handled in accordance with the guidelines of Veterinary Medical Unit of the VA Palo Alto Health Care System and the Administrative Panel on Laboratory Animal Care of Stanford University.

Reagents

Antibodies to the following proteins (and sources) used were: Pax7 (DSHB); Myogenin, total β-catenin, GSK3β^{pY216}, and total GSK3β (PharMingen/Beckton-Dickinson, San Jose, CA); actin and desmin (Sigma, St. Louis, MO); activated Notch-1 (Abcam, Cambridge, MA); activated (nonphosphorylated) β-catenin and phospho-H3 (Upstate Biotechnology, Lake Placid, NY); and GSK3β^{pS9} (Cell Signaling, Danvers, MA); β-galactosidase (Covance, Denver, PA). The anti-Notch activating antibody used for in vivo injections was from Upstate Biotechnology (hybridoma clone 8G10). The chicken polyclonal Syndecan-4 antibody, eMHC antibody, and Myf5 antibody were generously provided by Brad Olwin (University of Colorado), Grace Pavlath (Emory University), and Dr. Steve Konieczny (Purdue University), respectively. Fluorophore secondary conjugates used for immunofluorescence detection were donkey-anti-chicken Alexa488, goat-anti-mouse APC, goat-anti-mouse Alexa546, goat anti-rabbit Alexa488, and donkey-anti-rat Alexa488 (Molecular Probes). Recombinant Jagged1-Fc, sFRP3, and Wnt3A proteins were from R&D Systems (Minneapolis, MN). Inhibitors of γ-secretase (L-685,458) and GSK3 (BIO) were from Calbiochem (San Diego, CA).

Single Fiber Cultures, Explant Cultures, and Satellite Cell Isolation

Single fiber cultures, explant cultures, and satellite cell isolations were performed as previously described were prepared as described previously (Chargé et al., 2002; Conboy and Rando, 2002). Details are presented in the Supplemental Data.

Muscle Injury and Modulation of Notch and Wnt Signaling in Regenerating Muscle

Injury to whole muscle was made by injection of barium chloride (50 μl, 1.2%) into 30 sites in the lower limb. Focal injuries to tibialis anterior muscles were made by applying a metal probe, 4 mm in diameter, that had been cooled on dry ice directly to the exposed muscle surface for 10 s. To modulate Wnt or Notch signaling, 10 μl of treatment (Wnt3A, sFRP3, GSK3 inhibitor, Jagged1-Fc) or control solution was introduced into the muscle surrounding the injury site by direct intramuscular injection at different times after the initial injury. To activate Notch signaling, 10 μl of activating anti-Notch antibody supernatant at a 1:4 dilution or an isotype-matched hamster IgG was injected as previously described (Conboy et al., 2003). The GSK3 inhibitor and

activating Notch antibody were mixed in an aliquot immediately before injecting into the injured muscle for experiments when they were to be added in combination. To activate the Notch pathway in vitro, primary myoblasts were incubated with activating anti-Notch antibody supernatant or isotype-matched hamster control antibody as described previously (Conboy et al., 2003). Cells were subsequently plated on ECM coated dishes in 5% HS/DMEM and cultured for 1 hr at 37°C, 5% CO_2 and then isolated and prepared for FACS analysis.

Histology and Immunofluorescence

Muscles were dissected and embedded for cryostat sectioning as previously described (Conboy and Rando, 2002). Immunofluorescence was performed on fixed cells (4% PFA, 10 min) after permeabilization with 0.2% Triton X-100 in PBS (PBT) for 10 min and block with 5% goat serum (GS) in PBT. Cells were incubated in primary antibodies overnight at 4°C at the following dilutions: Myf5 (1/200), Pax7 (1/3), Desmin (1/120), Myogenin (1/100), phospho-H3 (1/400), and BrdU (1/500). Cells were washed and blocked in 5% GS/PBS and then incubated with fluorophore-conjugated antibody (Alexa goat anti-mouse546 and goat anti-rabbit488 at 1/1500) and DAPI to visualize nuclei for 1 hr at room temperature.

Analysis of Fiber Size and Fiber Number in Injured Area

At different times after muscle injury, the cross-sectional area of every regenerating muscle fiber (denoted by central nucleation) was quantified in the mid-region of the injury using Axiovision AC software (Zeiss) and normalized to cross-sectional areas of adjacent noninjured muscle fibers in H&E stained sections. The numbers of fibers were counted in the regenerating area within a normalized size field of view (20× objective). A minimum of three sections per muscle was analyzed.

Fluorescent Activated Cell Sorting

Cells were fixed with 4% PFA. For detection of single antibodies, cells were permeabilized with 5% FBS in 0.1% Triton X-100. Fixed cells were then stained with primary antibodies or with isotype-matched control antibodies for 1 hr at room temperature followed by incubation with fluorochrome-labeled secondary antibodies (1/1000, goat anti-mouse Alexa 546, Molecular Probes) for 1 hr at room temperature. For double antibody labeling experiments, the primary antibodies were sequentially applied. Cells were blocked in 10% BlokHen (Aves Labs, Tigard, OR) for 30 min and stained with the Syndecan-4 antibody (1/500) for 1 hr. Cells were washed and blocked in 5% GS in 0.1% PBT for 15 min. Secondary antibody (1/1500; goat-anti-chick Alexa 488, Molecular probes) was applied for 1 hr. Cells were then washed and fixed in 4% PFA for 5 min. The second antibody (goat anti-mouse APC) was then applied using the same protocol as single antibody labeling. Cells were analyzed by FACScaliber (Beckton-Dickinson).

Western Blotting

For western analysis, cells were lysed in RIPA buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% NP-40) containing 90 μg/ml PMSF, 20 μg/ml aprotinin, and 20 μg/ml leupeptin (Sigma). Immunoblotting was performed as previously described (Conboy et al., 2003).

Detection of β-Galactosidase in TOPGAL mice

For histological analysis, muscle sections were fixed in 0.2% glutaraldehyde for 10 min, and stained with X-gal. For biochemical analysis, muscle was homogenized in 0.1% SDS in RIPA buffer and analyzed for total protein (BCA kit, Pierce) and β-gal activity (Galacto-light, Tropix).

Real Time RT-PCR

RNA was isolated from muscle using TRIZOL reagent (Invitrogen) and cDNA was synthesized using Superscript First Strand Synthesis System for RT-PCR (Invitrogen) according to manufacturer's instructions. Relative quantitation by real-time PCR was carried out using SYBR-green detection of PCR products in real time using the MyiQ single-color detection system (Biorad). In each experiment, GAPDH gene was amplified as the reference standard. Each real-time PCR reaction (25 μl) contained 2 μl of cDNA, 10 μl of 2X SYBR Green Master Mix (Applied Biosystems Inc. Foster City, CA), including Amplitaq polymerase (Perkin-Elmer), and primers (available on request) at a final concentration of 20 pM.

Gene expression was quantitated using an ABI SYBR green PCR detection system (Applied Biosystem). All reactions were performed using the following thermal cycler conditions: 95°C for 10 min followed by 45 cycles of a three step reaction, denaturation at 95°C for 30 s, annealing at 60°C for 2 min, and extension and data collection at 72°C for 30 s.

Statistical Analysis

A minimum of 3 and up to 5 replicates was done for experiments presented. Data are presented as means and standard errors of the mean. Comparisons between groups were done using Kruskall Wallis comparison and a Dunn's multiple comparison post hoc test. Differences were considered statistically significant at the $p < 0.05$ level.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/2/1/50/DC1/.

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