A muscle resident cell population promotes fibrosis in hindlimb skeletal muscles of mdx mice through the Wnt canonical pathway

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Trensz F, Haroun S, Cloutier A, Richter MV, Grenier G. A muscle resident cell population promotes fibrosis in hindlimb skeletal muscles of mdx mice through the Wnt canonical pathway. Am J Physiol Cell Physiol 299: C939–C947, 2010. First published September 1, 2010; doi:10.1152/ajpcell.00253.2010.—Previous work has pointed to a role for the Wnt canonical pathway in fibrosis formation in aged skeletal muscles. In the present study, we studied the dystrophic mdx mouse, which displays skeletal muscle fibrosis. Our results indicated that the muscle resident stromal cell (mrSC) population in the muscles of dystrophic mice is higher than in the muscles of age-matched wild-type mice. Wnt3α promoted the proliferation of and collagen expression by cultured mrSCs but arrested the growth of and collagen expression by cultured myoblasts. Injections of Wnt3α in the tibialis anterior muscles of adult wild-type mice significantly enhanced the mrSC population and collagen deposition compared with the contralateral muscles. Conversely, an injection of the Wnt antagonist Dickkopf protein (DKK1) into the skeletal muscles of mdx mice significantly reduced collagen deposition. These results suggested that the Wnt canonical pathway expands the population of mrSCs and stimulates their production of collagen as observed during aging and in various myopathies.

Wnt3α; β-catenin; collagen; myopathy; Sca1

Fibrosis is a general term used to describe the accumulation of collagen and other ECM components in soft tissues. It is also associated with muscular atrophy during the normal physiological process of aging and after muscular damage and immobilization (15, 24, 25, 27). Muscle fibrosis can lead to a decrease in muscle strength and elasticity, as well as reduced muscle fiber contractility (20, 27). These functional impairments contribute to the inability of patients to perform ordinary tasks of independent living such as grasping objects and walking. It can also significantly slow down muscle rehabilitation and compromise the success of myoblast transplantation and/or gene therapy strategies.

The Wnt canonical pathway may be a major actor in inducing skeletal muscle fibrosis during aging (4). Wnt signaling occurs through various signaling pathways that are activated by specific Wnt proteins (17). Unlike the planar cell polarity pathway, the main function of the Wnt canonical pathway is to carry out β-catenin. After Wnt induction, β-catenin is stabilized and translocated into the nucleus (17). In the nucleus, β-catenin and its binding partners T cell factor (TCF) and lymphoid enhancer-binding factor transactivate the expression of Wnt canonical target genes such as Axin2. An important characteristic of the Wnt canonical pathway is that it can be antagonized by several proteins, including the soluble Frizzled-related protein (sFRP), Wnt inhibitory factor (WIF1), and members of the Dickkopf (DKK) protein family (31).

In adults, myogenic stem cells are influenced by Wnt signaling, but the precise downstream effect is an object of intense debate. Le Grand et al. (29) showed that Wnt7a, acting through the planar cell polarity pathway, markedly stimulates the symmetric expansion of satellite stem cells but does not affect the growth or differentiation of myoblasts (29). The canonical Wnt protein Wnt3α has also been shown to induce the activation of satellite cells (37). On the other hand, Brack et al. (4) have suggested that Wnt promotes the differentiation of myogenic progenitor cells and canonical Wnt3α-induced muscle fibrosis in aging.

In addition to myogenic stem cells, skeletal muscles contain other mesenchymal progenitor cells. While different cell subpopulations may exist, these mesenchymal cells share certain common features. First, they can differentiate into various mesodermal lines, including adipocyte, endothelial, chondrocyte, and osteocyte lineages (3, 18, 26, 36, 44, 45). Second, they can be cultured from the adherent fractions of the Sca1-positive (Sca1+) cell population. Immunolabeling of skeletal muscles with Sca1 antibody has shown that this cell population is closely associated with vessel walls, which are also in close contact with myogenic progenitor cells (6, 18, 26). Finally, the muscle resident stromal cell (mrSC) population expands during muscle degeneration/regeneration and contributes to muscle regeneration (3, 18, 26).

Mdx mice are a widely used model of Duchenne muscular dystrophy because they lack the dystrophin protein, an essential component that links the extracellular matrix (ECM) to the cytoskeleton. The absence of dystrophin causes mechanical instability when fibers generate twitches and disrupts the myofiber membrane. This results in repeated cycles of fiber degeneration/regeneration, which are in turn associated with chronic inflammation and progressive fibrosis, especially in the diaphragm and, to a lesser extent, in the hindlimb muscles (10, 12, 13, 22, 50).

As fibrosis is linked to several pathological processes, it is important to address its origin. We hypothesized that the Wnt canonical pathway has a universal role in fibrosis formation other than just during the aging process. We tested this hypothesis by assessing the mrSC population, which expands during muscle degeneration/regeneration. Our results showed that the activation of the Wnt canonical pathway in skeletal muscles promotes the proliferation of mrSCs, which differentiate into collagen-secreting fibrotic cell types. In addition, the inhibition of the Wnt canonical pathway using a natural antagonist significantly reduced fibrosis formation in mdx mice. To our knowledge, the present study is the first to show that...
mrSCs are involved in fibrosis formation and thus provide a target cell population for developing therapies aimed at preventing fibrosis.

MATERIALS AND METHODS

Animals and immobilization procedures. Six- and 16-wk-old male C57Bl/6 mice (Charles River, Montreal, QC, Canada) and C57BL/10ScSn-Dmd/J (mdx mice) (The Jackson Laboratory, Bar Harbor, ME) were used. The animal experiments were approved by the Animal Ethics Committee of Université de Sherbrooke and were performed in accordance with Canadian Council on Animal Care guidelines (protocol no. 133-06B).

Serum isolation. Whole blood from anesthetized, age-matched mdx and wild-type mice was collected retro-orbitally. The blood samples were clotted at 37°C for 4 h. Serum was isolated by centrifugation (1,500 g for 10 min). The supernatant was collected and stored at −80°C until used.

Measurement of Wnt/β-catenin/TCF pathway activation. Super(8X) TOPFlash (200 ng; Addgene plasmid no. 12456) that reports TCF activity, or the mutated control Super(8X)/FOPFlash (200 ng; Addgene plasmid no. 12457) [from the laboratory of Randall Moon (48)], and pRL-CMV (20 ng; Promega, Madison, WI) were incubated with Fugene 6 (1 µl; Roche Mannheim, Germany) in serum-free DMEM (800 µl; Invitrogen, Burlington, ON, Canada) in 24-well plates containing HEK293 cells (ATCC, 100,000 cells per well) at 37°C. After 16 h, serum (10%) from mdx or wild-type mice was added to the cells. Wnt3a (100 ng/ml) was used as a positive control. Luciferase activities were measured 24 h later according to the manufacturer’s instructions by first removing the medium and then adding Dual-Glo assay solutions (Promega). The fold activation was normalized to Renilla luciferase. At least three independent data points were averaged for each treatment.

Cell and muscle fiber isolation. Muscle resident stromal cells were isolated and analyzed immediately or cultured as previously described (18, 43, 44). Briefly, gastrocnemius (Gas) muscles were minced and digested with collagenase I (1 mg/ml; Sigma-Aldrich, Oakville, ON, Canada) for 45 min at 37°C. The tissue slurry was washed with DMEM containing 10% fetal bovine serum (FBS; Hyclone, Thermo Scientific, Logan, UT) (DMEM-FBS) and poured through 100-µm nylon mesh. The supernatant was collected. Total protein in the supernatant was determined using Bradford’s method (Bio-Rad, Mississauga, ON, Canada). The homogenate was centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatant was collected. Total protein in the supernatant was determined using Bradford’s method (Bio-Rad, Mississauga, ON, Canada). The qPCR assays were performed using 50 ng of template DNA. The conditions for all the reactions were as follows: an initial denaturation step (neutral buffer, 10%; Sigma-Aldrich), and included in paraffin. Sections (5 µm) were stained with Masson’s trichrome (MT) stain, Bielbrich Scarlet, acid fuchsin, and aniline blue. Otherwise, TA and GAS muscles were immersed in successive baths of PBS containing increasing concentrations of sucrose (5, 15, and 30%). The TA were then embedded in OCT containing 30% sucrose (Tissue-Tek OCT, Torrance, CA), frozen in isopentane chilled in liquid nitrogen, and stored at −80°C until used. Sections (5 µm) were cut beginning at the midpoint (ink mark) using a cryostat (Leica CM1850, Richmond Hill, ON, Canada).

For Sirius red staining, sections were fixed in 4% paraformaldehyde (PFA) for 10 min, rinsed, air dried, and stained for 1 h in 0.1% (wt/vol) Sirius red (Sigma-Aldrich) dissolved in saturated aqueous picric acid (Sigma-Aldrich). The sections were then rinsed extensively with tap water and mounted in aqueous mounting medium. Micrographs from muscles were captured using a ×10 objective, and whole muscle was reconstituted by merging the images using Photoshop. Collagen deposition around fibers (endomysium) was quantified by randomly selecting images from 10 fields. Regions containing collagen deposition foci were excluded. Images were processed using Image Pro software (Media Cybernetics, Silver Spring, MD). Pixel density related to staining intensity was then graphed.

For immunofluorescence, frozen sections (7 µm) or isolated fibers were fixed in 2% PFA (10 min at RT), blocked, and permeabilized in PBS containing 10% goat serum, 1% BSA, and 0.2% Triton X-100. The sections or fibers were then incubated with FITC-conjugated rat monoclonal anti-Scal (1:400; BD Bioscience, Mississauga, ON, Canada), mouse anti-Pax7 (1:2; DSHB, Sigma-Aldrich) primary antibodies. After being rinsed several times in PBS-Tween, the sections or fibers were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1,000) or Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:1,000) secondary antibody (Invitrogen). As a control, primary antibodies were omitted. Cell nuclei were labeled with DAPI reagent (Sigma-Aldrich). Indirect immunofluorescence was examined without counterstaining using an Axioskop 2 phase-contrast/epifluorescence microscope (Carl Zeiss, Thornwood, NY). Photomicrographs were processed using Image Pro software (Media Cybernetics).

Western blot analysis. Frozen TA muscles were crushed and homogenized in RIPA buffer (0.5% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) containing Complete protease inhibitor cocktail (Roche Molecular Biochemical, Laval, QC, Canada). The homogenate was centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatant was collected. Total protein in the supernatant was determined using Bradford’s method (Bio-Rad, Mississauga, ON, Canada). The protein extracts (30 µg) were separated on 7.5% polyacrylamide gels and transferred to PVDF membranes (Millipore, Bedford, MA). Blotted membranes were incubated overnight at 4°C in PBS-T (3.5 mM Na2HPO4, 17.4 mM NaH2PO4, 3.5 mM KCl, 137 mM NaCl, 0.1% Tween-20) with anti-β-catenin (1:500; BD Bioscience) or anti-GAPDH (1:1,000; Santa Cruz) antibody. The membranes were washed twice with PBS-T and were then incubated for 1 h at RT with the appropriate peroxidase-conjugated secondary antibody. After being extensively washed with PBS-T, the membranes were incubated with ECL Plus reagent (GE Healthcare, Baie d’Urfé, QC, Canada) according to the manufacturer’s instructions, and the immunostained bands were revealed on BioMax ML films (GE Healthcare). The membranes were scanned and the bands were quantified by densitometry using ImageJ software (NIH, Bethesda, MD) (1).

Quantitative PCR. Total RNA was extracted from flash-frozen TA muscles using TRIzol (Invitrogen). RNA (1 µg) was reverse-transcribed using Reverse Transcriptase Superscript II (Invitrogen). The qPCR assays were performed using 50 ng of template cDNA. The conditions for all the reactions were as follows: an initial 5-min denaturation step at 95°C followed by forty 40-s cycles at 95°C,
Table 1. Primer sets used for quantitative PCR

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<th>Genes</th>
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<th>Reverse</th>
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<td>Axin2</td>
<td>GGGGAAAAACACAGCTTACA</td>
<td>TCGACTGCGTCGCTTCTTT</td>
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<tr>
<td>Col1a1</td>
<td>GCCCGAGAAGCTCTTGGATCG</td>
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<td>HPRT</td>
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56°C, and 72°C. The qPCR assays were performed using a Rotorgene 6000 (Corbett Robotics, Australia) and IQSYBR Green Supermix (Bio-Rad). Results were calculated using the 2^ΔΔCT relative quantification method normalized to HPRT1. The primer sets are listed in Table 1.

Intramuscular injections. To modulate Wnt signaling in the skeletal muscles, serial intramuscular injections of recombinant Wnt3a and DKK1 were performed every 4 days using a tuberculin syringe. To study the effect of Wnt pathway activation, three doses of Wnt3A (250 ng/25 μl) were injected into the TA of wild-type mice. Eight doses of DKK1 (300 ng/25 μl) were injected into the TA and GAS muscles of 6-wk-old mdx mice to inactivate the Wnt pathway. The muscles were harvested 7 days after the last injection. As a control, saline (25 μl) was injected into the contralateral muscles.

Statistics. All data are expressed as means ± SE. Paired t-tests were used to assess the statistical significance between treated muscles and untreated contralateral muscles. Unpaired t-tests were used to compare two groups of mice or two different time points. P < 0.05 was considered to be statistically significant. Statistical values were obtained using GraphPad Prism 5.0 software.

RESULTS

The population of Sca1-positive resident cells is higher in fibrotic mdx muscles. The assessment of fibrosis in dystrophic muscles using paraffin Masson’s trichrome-stained cross-sections of Gas muscles revealed an obvious accumulation of fibrotic tissue (blue) in the interstitial space between the myofibers (Fig. 1A). The accumulation of collagen was more pronounced in the diaphragm of mdx mice than in wild-type mice (see supplemental Fig. S1 online at the AJPCellPhysiology website). Using quantitative PCR (qPCR), we assessed the gene expression of collagen I and III, the main types of collagen expressed in fibrotic tissue. As expected, both the levels of collagen I and III were significantly higher in mdx muscles than in control muscles (Fig. 1B). As mrSCs may be involved in ECM deposition, we determined whether this cell population was higher in mdx muscles. mrSCs express Sca1 and are mainly localized in the interstitial spaces between myofibers and around blood vessels. The immunostaining of Sca1 in muscle sections from mdx and wild-type mice revealed that this cell population appeared to be higher in muscles from mdx mice than in muscles from wild-type mice (Fig. 1C). Flow cytometry analyses showed that the mrSC subpopulation of Sca1-containing cells that were also negative for endothelial marker CD31 and blood lineage markers (Sca1^+CD31Lin^- or S^+CL^-) were significantly higher in dystrophic muscles than in wild-type muscles (Fig. 1D). These findings suggested that mdx muscles are highly dystrophic and possess significantly higher levels of a subpopulation of mrSCs expressing the Sca1^+ marker.

Wnt3a stimulates mrSC proliferation and collagen expression in vitro. We assessed the level of Wnt pathway activation in mdx muscles since the canonical pathway stimulates fibrosis during the aging process. To test for Wnt canonical pathway activation, we performed luciferase assays using 8xTOPFlash and control 8xFOPFlash reporter constructs transfected into HEK293 cells in the presence of serum (Fig. 2A).

We observed an 1.8-fold increase in TCF activity in the presence of serum from mdx mice than in the presence of serum from wild-type mice. This activity was specific to the Sca1^+ marker.

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We observed an 1.8-fold increase in TCF activity in the presence of serum from mdx mice than in the presence of serum from wild-type mice. This activity was specific to the Wnt canonical pathway since the activity was not observed...
Fig. 2. The activation of the Wnt canonical pathway stimulates mrSC proliferation and collagen expression in vitro. A: relative luciferase activity of the Wnt/β-catenin/T cell factor (TCF) pathway reporter constructs 8xTOPFlash and its mutated control 8xFOP-Flash in HEK293 cells treated with recombinant Wnt3a, mdx, or wild-type (WT) control sera for 24 h. Sera was pooled from six mice. Four independent experiments were performed. B: Western blot of β-catenin in TA protein extracts from WT and mdx mice. GAPDH was used as loading control. Graph represents the relative expression of β-catenin using densitometry data from four muscle preparations. C: graph of the percentage of proliferating mrSCs in the presence of 0, 50, or 100 ng/ml of Wnt3a (n = 6). D: Western blot of β-catenin expression from mrSCs treated with Wnt3a (100 ng/ml) alone or with DKK1 (250 ng/ml). GAPDH was used a loading control. Graph showing the relative expression of β-catenin using densitometry data from four cell preparations. E: graph of the expression of the Axin2 and collagen I genes in mrSCs that were untreated (CTL), treated with Wnt3a (100 ng/ml) alone, or treated with both Wnt3a (100 ng/ml) and DKK1 (250 ng/ml) as measured by qPCR (n = 6).

with the control construct. We also assessed the expression of β-catenin, the principal downstream effector of the canonical Wnt signaling pathway. Western blot analyses revealed that β-catenin levels were 1.5-fold higher in dystrophic muscles than in control muscles (Fig. 2B), suggesting that the activation of the Wnt/β-catenin/TCF pathway was associated with a higher amount of β-catenin in fibrotic dystrophic muscles than in wild-type mouse muscle. We next assessed the effect of Wnt canonical pathway activation on mrSCs containing Sc11+ cells in vitro, mrSCs were isolated, cultured, and challenged with recombinant Wnt3a protein, a widely used Wnt family member known to specifically activate the Wnt canonical pathway. Cells treated with 50 and 100 ng/ml of Wnt3a displayed a significant 1.26- and 1.37-fold increase in proliferation, respectively, compared with untreated cells (Fig. 2C). Western blot analyses confirmed that the canonical pathway had been activated in cells treated with 100 ng/ml of Wnt3a since they exhibited an accumulation of β-catenin that was mitigated by the addition of 250 ng/ml of the canonical antagonist DKK1 (Fig. 2D). This was confirmed by qPCR, which revealed an increase in the expression of Axin2, a target gene of the canonical pathway. Axin2 levels increased 12-fold following an incubation with 100 ng/ml of Wnt3a. This was blocked by a treatment with 250 ng/ml of DKK1 (Fig. 2E). We next looked at whether treating mrSCs with Wnt3a would promote the upregulation of collagen, an ECM component whose dysregulation is a hallmark of fibrosis. A qPCR analysis of the gene coding for collagen I, a principal fibrillar collagen of skeletal muscles, revealed a significant increase in collagen expression after the Wnt3a treatment. Collagen I expression was down-regulated to its basal level in the presence of DKK1 (Fig. 2E). These results suggested that the Wnt canonical pathway activation is highly prevalent in dystrophic muscles can influence the proliferation of mrSCs and their potential to secrete collagen, a hallmark of fibrosis.

Activation of the Wnt canonical pathway reduces proliferation but has no effect on collagen expression in myogenic progenitor cells. To determine whether the myogenic progenitor cell population might also be involved in fibrosis formation, we assessed the effect of the Wnt canonical pathway activation on primary myogenic precursors in vitro. Using fiber cultures, we analyzed the population of Pax7-positive (Pax7+) satellite cells, which are the main adult myogenic stem cells, and their progenies. The addition of Wnt3a to fibers in culture reduced the expansion of the satellite cells in the early stage of activation/proliferation compared with untreated fibers 4 days postisolation (Fig. 3A). Unlike mrSCs, the treatment of the primary myogenic precursors with increasing concentrations of Wnt3a caused a robust inhibition of cell proliferation (compare Fig. 3B with Fig. 2C). Moreover, a qPCR analysis showed that, while myogenic precursors treated with Wnt3a were activated as shown by a significant 20-fold upregulation of Axin2, there was no change in collagen I levels (Fig. 3C). These results suggested that myogenic cells react differently than mrSCs to
The myogenic progenitor Pax7

We investigated whether the activation of

We then quantified the expression of the genes coding for collagen I and III, which are involved in fibrosis. There was a significant upregulation of these two genes in Wnt3a-injected muscles (Fig. 4D). Histological examinations of Wnt3a-injected TA cross-sections stained with Sirius red, a dye specific for collagen, revealed that Wnt3a enhanced the deposition of collagen (Fig. 4E), with a moderate but consistent increase in Wnt3a-injected TA muscles compared with control saline-injected muscles (Fig. 4F). These results showed that the enhanced mrSC proliferation and collagen deposition caused by Wnt3a could also be observed in vivo.

Inhibition with the canonical Wnt antagonist DKK1 reduces collagen synthesis and β-catenin expression in mdx muscles. Since Wnt increased the levels of fibrosis formation markers, we assessed the effect of inhibiting the Wnt canonical pathway with DKK1 on controlling fibrosis in vivo. We performed eight injections of DKK1 (one every 1 days) in one TA muscle of mdx mice and saline in the contralateral muscle. As expected, Western blot analyses showed that the DKK1 injections induced a significant decrease in β-catenin protein expression levels in DKK1-injected mdx muscles compared with the contralateral control muscles (Fig. 5A). This decrease was accompanied by a significant decrease in collagen I expression as shown by qPCR (Fig. 5B). We next assessed the overall level of fibrosis in Gas mdx dystrophic muscles injected with Wnt3a. We used transversal Sirius red-stained sections to quantify the levels of collagen and observed a slight but constant decrease in the collagen deposition in the fibers of DKK1-injected Gas muscles compared with saline-injected contralateral muscles (Figs. 5C and S2). Taken together with our other results, these findings suggested that collagen deposition in fibrotic tissues results from the activation of the Wnt canonical pathway, which stimulates collagen production by Sca1+ cells, a subset of mrSCs.

### DISCUSSION

Muscle fibrosis is a common feature of numerous muscular traumatic states and diseases such as compartment syndrome and muscular dystrophies (10, 14). It is also associated with skeletal muscle atrophy caused by disuse and denervation, and with the physiological process of aging (15). Depending on the extent of the matrix deposition, muscular fibrosis may lead to an impairment of tissue functions (23, 47). While little is known about the cellular origins and signaling pathway(s) that trigger fibrosis, this clinically relevant problem is currently under intensive study (2, 4, 19). To gain more insight into fibrosis formation, we chose a mouse model of Duchene muscular dystrophy (mdx) in which progressive fibrosis occurs consistently due to chronic muscle degeneration/regeneration cycles (39, 49). We provide evidence that Wnt canonical pathway stimulation of muscle resident stromal cells (mrSCs) induces fibrosis in dystrophic skeletal muscles.

We showed that the Wnt canonical activation increases in the serum of mdx mice compared with wild-type mice and is

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**Fig. 3.** Activation of the Wnt canonical pathway reduces proliferation but has no effect on collagen expression in myogenic progenitor cells. A: graph showing the number of Pax7-positive (Pax7+) cells per muscle fiber cultured for 0 (CTL) or 4 days in the absence (CTL) or presence of Wnt3a (100 ng/ml). The myogenic progenitor Pax7+ cell population had expanded significantly after 4 days in the absence of Wnt3a compared with day 0 (n = 50). However, the expansion of the Pax7+ population was significantly inhibited in the presence of Wnt3a (n = 50). B: graph of the percentage of proliferating primary myoblasts cultured in the presence of increasing concentrations (10, 25, 50, 75, and 100 ng/ml) of Wnt3a (n = 6). C: graph showing the relative expressions of the Axin2 and collagen I genes in primary myoblasts that were untreated (CTL) or treated with Wnt3a (100 ng/ml) as measured by qPCR (n = 6). Note the absence of collagen modulation despite Wnt activation.
characterized by an increase in the stability of β-catenin and the expression of Axin2, a direct Wnt canonical gene target. These results suggested that the systemic environment of mdx mice can alter stem cell specification and function, in this case, muscle resident progenitor cells, and are in agreement with other studies showing that the systemic environment can modulate muscle stem cell activity (5, 16, 32, 40). Rando’s group used parasymbiosis of young and aged mice to elegantly show that a young systemic environment favors muscle regeneration (4, 7). The young environment was sufficient to rescue the regenerative potential of muscle stem cells that was believed to be lost with aging. Other investigators have also reported that the loss of myogenic stem cell activity with aging is due to an increase in Wnt canonical activation (4, 30). Interestingly, we recently suggested that the levels of Wnt canonical antagonists, notably sFRP2 and Wif1, are reduced during aging and that this might reflect the increase in Wnt canonical activation observed during the aging process (43).

Anti-Sca1 antibodies have made it possible to identify and enrich stromal cell populations from various tissues, including skeletal muscle (18, 26, 35). Sca1+ skeletal muscle stromal cells are located near blood vessels and are closely associated with satellite cells (6, 18, 26, 35). mrSCs are similar to other multipotent progenitor cells in muscles. One class of progenitor cells, the mesoangioblasts, which was identified by Cossu et al. (9, 38, 42), are located around blood vessels and also possess the Sca1 marker. Like mrSCs, mesoangioblasts can differentiate into many lineages. However, unlike mrSCs, mesoangioblasts can also differentiate into myogenic cells (8, 9, 18, 34, 42).

Fig. 4. Wnt3a stimulates Sca1-positive mrSC proliferation and collagen expression in vivo. A: Western blot of β-catenin of tibialis anterior (TA) muscles from the same mouse injected with three intramuscular doses of control saline solution (25 μl) in one TA and Wnt3a (250 ng) in the contralateral TA. GAPDH was used as a loading control. Graph represents the relative expression of β-catenin using densitometry (n = 5). B: graph of the percentages of Sca1-positive, CD31-negative, and lineage-negative (S-CL) cells in TA muscles treated as in A (n = 5). C: representative immunohistology of TA muscle sections treated as in A and immunolabeled for Sca1 (green). Sca1+ cells can be seen between fibers labeled for laminin (red). DAPI was used to stain nuclei (blue). D: graph showing the relative expressions of the collagen I and collagen III genes in TA muscles treated as in A and measured by qPCR (n = 5). E: representative Sirius red staining of TA muscles treated with Wnt3a. F: quantification of Sirius red staining in fibers from E comparing Wnt3a-injected and saline-injected contralateral TAs from three different mice.
In this work, we looked at whether mrSCs were affected in a chronic muscle degeneration model and whether they could account for the fibrosis induced by Wnt canonical signaling. Our results showed that, in the presence of Wnt3a, mrSCs proliferate significantly and secrete larger amounts of factors involved in fibrosis, thereby becoming profibrotic.

It has been reported that, during aging, satellite cells convert from a myogenic to a nonmyogenic phenotype, causing them to adopt a fibroblastic state (2, 4). To verify whether satellite cells might be involved in fibrosis formation, we cultured muscle fibers in the presence of Wnt3a. Our results showed that the Pax7+ satellite cell population derived from a single fiber culture does not proliferate in the presence of Wnt3a. Wnt3a also caused a dose-dependent arrest of the growth of cultured primary myoblasts but had no impact on collagen I expression. Taken together, our results indicated that Wnt3a stimulation of fibrosis formation is mainly induced by mrSCs rather than satellite cells. On the other hand, the stimulation of myoblasts by Wnt3a may promote the downregulation of Pax7+ cells (28), which could potentially favor their conversion from the myogenic to fibrogenic state. More work is clearly required to elucidate the contribution of myogenic cells to fibrosis.

To determine the impact of chronic Wnt canonical stimulation on skeletal muscle, we performed serial intramuscular injections of Wnt3a in resting muscles of wild-type mice. Stimulation by Wnt3a was sufficient to induce significant muscle fibrosis as indicated by Sirius red collagen staining. We also showed that the proliferation of mrSCs increases in vivo, which supported our in vitro results. In addition, the increased expression of β-catenin and the upregulation of Axin2 transcripts showed that the Wnt canonical pathway is activated and potentiates fibrosis. Interestingly, Brack et al. (4) showed that a single injection of Wnt3a induces massive fibrosis in aged damaged muscle, suggesting an additive effect of the injected Wnt in the systemic environment. In agreement with their findings, we showed that fibrosis can occur in undamaged normal adult muscles that are chronically exposed to canonical Wnt, which indicates that Wnt3a has the potential to expand the mrSC population and alter its phenotype.

We also looked at whether the inhibition of the Wnt canonical pathway might prevent fibrosis. Intramuscular injections of the Wnt antagonist DKK1 in young mdx mice prevented significant fibrosis formation concomitant with the downregulation of collagen I, the main collagen type involved in fibrosis. This result could be of particular clinical interest for muscular dystrophies in which diaphragm muscles display functional impairment because of fibrosis (21). In addition, our findings indicated that the relationship between Wnt canonical pathway agonists/antagonists may play an important role in maintaining muscle homeostasis. Indeed, an increase in Wnt pathway activation due to a decrease in the expression of antagonists may be sufficient to cause a muscle regenerative deficit and fibrosis formation as previously observed in aged mice (43).

Taken together with previous findings, our study adds significantly to a model where local and systemic factors such as chronic inflammation due to, and/or associated with, muscle tissue degeneration/regeneration can lead to an enhanced activation of the Wnt canonical pathway either through direct stimulation or Wnt antagonist suppression (43). In the context of aging or muscular dystrophy, we showed that a Wnt agonist/antagonist imbalance favoring agonistic activity of local or

study characterized a nonsatellite cell resident progenitor cell (PIC) subpopulation that possesses strong myogenic potential (35). Because of their localization and because they share many characteristics such as being Sca1+, it is possible that these various cell types are related. mrSCs, PICs, and FAPs may thus originate from the expansion and commitment of a common progenitor, perhaps mesoangioblasts, which would explain their various levels of potencies. Interestingly, a pericyte subpopulation has been identified in human skeletal muscle that is related to mesoangioblasts (11, 46). It is thus possible that mrSCs may have a human counterpart that might derive from this pericyte subpopulation.
systemic origin can lead to the proliferation and activation of Sca1+ mrSCs, which is associated with a profibrotic phenotype that results in the production of collagens in affected muscles, leading to a decrease in muscle strength, elasticity, and contractile capacity (20, 27). During aging, this process may further be enhanced by Wnt activation of satellite cells and decreased activity of myogenic stem cells and their differentiation into profibrotic cell types (4), possibly due to a lack of counter-inhibition by natural antagonists (43). This further highlights the important role played by natural antagonists of the canonical Wnt pathway such as sFRP, Wif1, and the DKK family as a counterbalance during the Wnt-associated profibrotic process (43).

In summary, our results showed that the activation of the Wnt canonical pathway increases the population of mrSCs and alters their phenotype, leading to an increase in the synthesis of collagen and contributing to fibrosis formation. mrSCs may thus be an important choke point in the Wnt canonical pathway and thus a target for therapies to prevent fibrosis formation during ageing and various myopathies. More studies are needed to determine whether Wnt antagonist treatments can improve muscle function impaired by fibrosis in humans.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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