Wnt10b Deficiency Promotes Coexpression of Myogenic and Adipogenic Programs in Myoblasts

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Adult myoblasts retain plasticity in developmental potential and can be induced to undergo myogenic, adipogenic, or osteoblastogenic differentiation in vitro. In this report, we show that the balance between myogenic and adipogenic potential in myoblasts is controlled by Wnt signaling. Furthermore, this balance is altered during aging such that aspects of both differentiation programs are coexpressed in myoblasts due to decreased Wnt10b abundance. Mimicking Wnt signaling in aged myoblasts through inhibition of glycogen synthase kinase or through overexpression of Wnt10b resulted in inhibition of adipogenic gene expression and sustained or enhanced myogenic differentiation. On the other hand, myoblasts isolated from Wnt10b null mice showed increased adipogenic potential, likely contributing to excessive lipid accumulation in actively regenerating myofibers in vivo in Wnt10b^{−/−} mice. Whereas Wnt10b deficiency contributed to increased adipogenic potential in myoblasts, the augmented myogenic differentiation potential observed is likely the result of a compensatory increase in Wnt7b during differentiation of Wnt10b^{−/−} myoblasts. No such compensation was apparent in aged myoblasts and in fact, both Wnt5b and Wnt10b were down-regulated. Thus, alteration in Wnt signaling in myoblasts with age may contribute to impaired muscle regenerative capacity and to increased muscle adiposity, both characteristic of aged muscle.

INTRODUCTION

Adult skeletal muscle has remarkable regenerative capacity, largely mediated by satellite cells that reside between the sarcolemma and basal lamina of myofibers in postnatal muscle and remain throughout adult life (Bischoff, 1994). After muscle injury, satellite cells give rise to myoblasts that proliferate, migrate to the site of injury, and fuse into myofibers, thereby regenerating damaged or degenerating myofibers (Seale and Rudnicki, 2000). Deficits in the regenerative process occur as a function of age, some of which may be due to a decline in myoblast proliferative capacity (Schultz and Lipton, 1982; Dodson and Allen, 1987; Johnson and Allen,

1993; Renault *et al.*, 2000) or to loss of factors required for satellite cell activity (Barton-Davis *et al.*, 1998; Conboy *et al.*, 2003). Although these deficits in myoblast activity may contribute to loss of muscle mass with age, they do not account for other features of aged muscle, in particular, increased lipid content (Goodpaster *et al.*, 2001). We previously showed that compared with myoblasts isolated from adult mice (8 mo), those isolated from the muscle of aged mice (23 mo) showed increased expression of genes normally restricted to the adipocyte lineage (Taylor-Jones *et al.*, 2002). Although genes involved in lipid trafficking and storage were overexpressed, a fully differentiated adipocyte phenotype is not achieved (Guan *et al.*, 2002). Alteration of the expression and/or activity of peroxisome proliferator-activated receptor γ (PPAR γ) and CAAT/enhancer binding protein α (C/EBP α), both activators of adipocyte-specific genes (Tontonoz *et al.*, 1994; Mandrup and Lane, 1997; Hamm *et al.*, 2001), was apparent as a function of age in myoblasts, potentially contributing to the increased adipogenic potential observed (Taylor-Jones *et al.*, 2002). This is consistent with the observation that the muscle cell line C2C12 is converted to adipocytes by overexpression of PPAR γ and C/EBP α (Hu *et al.*, 1995; Rosen *et al.*, 2000). Similarly, treatment with thiazolidinediones, potent activators of PPAR γ , results in induction of genes involved in fatty acid uptake, storage, and metabolism (Teboul *et al.*, 1995; Grimaldi *et al.*, 1997).

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Abbreviations used: C/EBP α , CAAT/enhancer binding protein α ; DM, differentiation medium; FABP4, fatty acid binding protein 4; GM, growth medium; GSK, glycogen synthase kinase; IBMX, 3-isobutyl-1-methylxanthine; PPAR γ , peroxisome proliferator-activated receptor γ ; RT-PCR, reverse transcriptase-polymerase chain reaction; TCF, T cell factor.

Figure 1. Myoblasts and differentiated myotubes from aged mice express adipocyte genes. (A) RT-PCR analysis of RNA from myoblasts maintained in GM from adult (Ad) and aged (Ag) mice shows differences in the expression of the indicated genes involved in adipocyte differentiation. (B) Western blot analysis of PPAR γ shows age-related differences in protein abundance and phosphorylation. Filters were stained with Ponceau S to verify equivalent loads. (C) Immunocytochemical analysis of multinucleated myotubes after 72 h in DM shows FABP4 accumulates to high levels in aged (Ag) but not adult (Ad) differentiated myotubes.

The Wnt family of genes, encoding at least 19 lipid-modified signaling proteins (Willert *et al.*, 2003), has been implicated in regulating both adipogenic and myogenic differentiation through the canonical β -catenin and noncanonical pathways (for review, see Huelsken and Behrens, 2002). In the absence of Wnt signaling, β -catenin is phosphorylated by glycogen synthase kinase (GSK), leading to rapid degradation (Kikuchi, 2000). Wnt ligands bind to transmembrane receptors of the Frizzled family, leading to inactivation of GSK within the complex, thereby stabilizing β -catenin, allowing translocation to the nucleus where it regulates T-cell factor (TCF)/leukocyte enhancing factor-dependent gene transcription. In the developing embryo, Wnt family members have been shown to be essential for differentiation of epaxial muscles (for review, see Buckingham *et al.*, 2003). More recently, it has been shown that different Wnts have distinct effects during limb myogenic differentiation (Anakwe *et al.*, 2003). Moreover, Wnt5a, 5b, 7a, and 7b promote myogenic commitment in adult muscle progenitor cells (Polesskaya *et al.*, 2003). By contrast, Wnt signaling seems to inhibit adipogenic differentiation (Ross *et al.*, 2000; Bennett *et al.*, 2002; Moldes *et al.*, 2003; Zhou *et al.*, 2004). Blocking β -catenin activity in C2C12 cells through expression of dominant negative TCF resulted in inhibition of myogenic differentiation and activation of adipogenic differentiation (Ross *et al.*, 2000). In particular, Wnt10b has been demonstrated to inhibit adipogenic differentiation both in C2C12 myoblasts and preadipocytes (Ross *et al.*, 2000; Bennett *et al.*, 2002). Because Wnt10b abundance decreases in muscle as a function of age (Taylor-Jones *et al.*, 2002), the present study was designed to test the hypothesis that altered Wnt10b activity contributes to increased intramyocel-

lular lipid accumulation that accompanies aging. Results suggest that Wnt10b specifically controls adipogenic potential in myoblasts and regenerating muscle, whereas other Wnt family members influence myogenic potential.

MATERIALS AND METHODS

Animals

All studies were approved by the University Animal Care and Use Committee and overseen by the Division of Laboratory Animal Medicine (University Arkansas for Medical Sciences). For aging studies, DBA/2JNIA female mice were obtained from the NIA Aging Rodent Colony at Harlan (Indianapolis, IN) and were maintained on a normal chow diet until sacrifice. Generation and characterization of FVB Wnt10b $^{-/-}$ mice will be described in detail elsewhere (Lane and Leder, unpublished data). At 4 wk of age, female mice were randomly assigned to receive a low-fat diet (10% fat, D12450; Research Diets, New Brunswick, NJ) or a high-fat diet (45% fat, D12451; Research Diets). Wild-type and Wnt10b $^{-/-}$ mice were fed low- or high-fat diets until sacrifice.

Muscle Regeneration

To induce localized muscle injury, mice were anesthetized by isoflurane inhalation and an incision (~5 mm) made in the skin of the lower hindlimb. The skin was opened with forceps, and the exposed tibialis anterior muscle was subjected to mild damage by application of a 2-mm, dry ice-tempered metal rod for 5 s. The incision was closed, and the mice killed at indicated times after injury. For widespread muscle degeneration, 10 μ M cardiotoxin (snake venom from *Naja mossambica*; Sigma-Aldrich, St. Louis, MO) was injected directly into the tibialis anterior as described previously (Polesskaya *et al.*, 2003).

Tissue Culture

Myoblasts were isolated from the tibialis anterior of aged (23 mo, $n = 4$) and adult (8 mo, $n = 4$) DBA/2JNIA, and 9-mo-old FVB/C57 Wnt10b $^{+/+}$ ($n = 4$), and Wnt10b $^{-/-}$ ($n = 4$) mice as described previously (Rando and Blau, 1994). Pure populations (>98%) of each myoblast type were derived and maintained in growth medium (GM) containing 20% fetal calf serum (Taylor-Jones *et al.*, 2002). For aging experiments, comparable results were obtained from myoblasts derived from individual mice, so results shown were obtained from myoblasts pooled from the four animals in each group. Individual myoblast cultures were established and analyzed from the four wild-type and 4 Wnt10b $^{-/-}$ mice. For differentiation experiments, myoblast cultures were switched to differentiation medium (DM) containing 2% horse serum for 72 h with daily refeeding. To promote adipogenic differentiation, myoblasts were incubated in GM supplemented with a cocktail of 3-isobutyl-1-methyl-xanthine (IBMX) (115 μ g/ml; [Sigma-Aldrich]; 5×10^{-4} M dexamethasone (Sigma-Aldrich); and 0.25 U/ml insulin [Novolin, Clayton, NC]) as described previously (Taylor-Jones *et al.*, 2002) for 3 d. In some experiments, cells were treated with IBMX + LiCl (L9650; Sigma-Aldrich) at a final concentration of 25 mM, and all treatments were repeated once a day for 3 d. Myoblasts were infected with either pLNCX (empty vector), pLCNWnt7b (Shimizu *et al.*, 1997), or pLNCWnt10b as described previously (Ross *et al.*, 2000). Cultures were assessed for adipogenic differentiation by Oil Red O staining as described below.

RNA Isolation and Gene Expression

RNA was isolated using the RNAqueous extraction kit (Ambion, Austin, TX). Purity and concentration were determined using the Spectramax 384 Plus (Molecular Devices, Sunnyvale, CA). Gene expression was assayed by semi-quantitative, gel-based reverse transcriptase-polymerase chain reaction (RT-PCR) and by real-time RT-PCR. Semiquantitative RT-PCR was performed as described previously (Taylor-Jones *et al.*, 2002). Briefly, Advantage RT-for-PCR and Advantage cDNA PCR kits (BD Biosciences Clontech, Palo Alto, CA) kits were used with glyceraldehyde-3-phosphate dehydrogenase as the positive control for sample normalization. Reactions were carried out using an Applied Biosystems (Foster City, CA) GeneAMP PCR System 2400. Cycle number and annealing temperatures were empirically determined for each set of primers to ensure that amplification reactions were specific and within the linear range. PCR products were resolved on 2% agarose gels by combining UltraPure agarose and FMC (Rockland, ME) NuSieve GTG agarose 1:1 and visualized with ethidium bromide on the ChemiImager Imaging System 5500 (Alpha Innotech, San Leandro, CA). The figure shown is representative, and reactions were repeated a minimum of three times.

Real-time quantitative RT-PCR was performed using the protocols, chemistries, and the amplification and detection systems of Applied Biosystems. For each sample, cDNA was synthesized from 2 μ g of total RNA by using components from the Taqman reverse transcription reagents (Applied Biosystems). The reaction volume of 100 μ l also contained $1 \times$ reverse transcriptase buffer, 5.5 mM MgCl₂, 0.5 mM dNTPs, 2.5 mM random hexamers, 40 U

Figure 2. Overexpression of Wnt10b results in decreased adipogenic gene expression in aged myoblasts with no effect on myogenic differentiation. (A) Real-time RT-PCR analysis of Wnt10b, C/EBPα, PPARγ, and FABP4 gene expression in aged myoblasts overexpressing Wnt10b by retroviral-mediated gene transfer compared with empty vector-infected cells after exposure to DM for 24 h. Data are expressed as mean ± SE. Asterisk (*) indicates significant difference from empty vector-infected cells, $p \leq 0.05$. (B) Western blot analysis of cytosolic β-catenin in aged myoblasts overexpressing Wnt10b compared with empty vector-infected cells and to uninfected aged (Ag) and adult (Ad) myoblasts after exposure to DM for 24 h. A constitutively expressed protein visualized with Ponceau S is shown to verify equivalent loads. (C) Myoblasts overexpressing Wnt10b accumulate less lipid than empty vector-infected myoblasts, as demonstrated by Oil Red O (ORO) staining (top). Both cell populations differentiate efficiently to form myotubes that accumulate myosin heavy chain (MyHC; bottom) 72 h after exposure to DM.

of RNase inhibitor, and 375 U of Multi-scribe reverse transcriptase. The primers were allowed to anneal for 10 min at 25°C before the reaction proceeded for 1 h at 37°C followed by 5 min at 95°C. Samples were aliquoted and stored at -80°C. Taqman primer/probes were selected using Applied Biosystems Assays on Demand: Mm00442104_m1, Mm00445880_m1, Mm00440387_m1, Mm00446194_m1, Mm00437337_m1, Mm00437347_m1, Mm00437357_m1, Hs99999901_s1, Mm00514283_s1, and Mm00440945_m1. The latter primers to PPARγ amplified both PPARγ-1 and -2 mRNAs. PCR reactions were assembled so that only the addition of cDNA template and primers were required. Control reactions were run lacking template to check for reagent contamination. To optimize assay efficiency, PCR standard curves were produced using a pool containing each sample cDNA. Data points were generated using fourfold serial dilutions of cDNA. Eight microliters of the diluted cDNAs was added to 25 μl of final reaction volume. Gene expression was compared in individual samples by using 16 ng (1 ng of 18S) of RNA equivalents of cDNA and the standard curve method described in Applied Biosystems User Bulletin No. 2. Template amounts were adjusted for instances in which expression levels did not fall within the range of the standard curve. The reactions were performed in triplicate by using the ABI Prism 7700 sequence detection system and the instrument's universal cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, and then 60°C for 1 min. RNA abundance for each gene of interest is expressed as a ratio normalized to RNA abundance of 18S in the same sample.

Western Analysis

Fractionation of cytosolic and membrane-bound proteins for β-catenin analysis was performed as described by Young *et al.* (1998). Samples (30–35 μg of protein) were resolved on 10% SDS-PAGE Criterion, precast gels (#345-0009; Bio-Rad, Hercules, CA) and blotted as described previously (Kiyokawa *et al.*, 1994). Filters were stained with Ponceau S (P7170; Sigma-Aldrich) to verify equivalent loads. Primary antibody against β-catenin (610154; BD Transduction Laboratories, Lexington, KY) was diluted 1:1000. Total cellular extracts (50 μg of protein) were resolved for Western analysis with a PPARγ antibody (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:167. A horseradish peroxidase-conjugated, goat anti-rabbit secondary antibody (Pierce Chemical, Rockford, IL) was added at a dilution of 1:100,000. Incubation times and washes were as described previously (Taylor-Jones *et al.*, 2002). Protein bands

were detected with the ChemiGlow West chemiluminescent substrate kit (CGW-8000; Alpha Innotech) per the user's manual and visualized on the ChemiImager 5500 Imaging System (Alpha Innotech).

Immunohistochemistry and Histochemistry

Tibialis anterior muscles were isolated from wild-type and Wnt10b^{-/-} mice (n = 8 each) fed either a high-fat or low-fat diet (n = 4 each). From each animal, one tibialis anterior served as a control with the contralateral side subjected to freeze or cardiotoxin injury. Muscles were collected 7 or 28 d postinjury and flash frozen in liquid nitrogen. Serial cryostat sections (7 μm) were stained with hematoxylin and eosin or with Oil Red O as described previously (Taylor-Jones *et al.*, 2002). For immunohistochemistry, sections were fixed in 2% paraformaldehyde for 30 min, blocked with 0.6% H₂O₂ for 30 min to block endogenous peroxidase activity, and incubated for 1 h at room temperature with an antibody recognizing FABP4 (provided by D. A. Bernlohr, University of Minnesota, St. Paul, MN), myosin heavy chain (A4.1025, provided by H. M. Blau, Stanford University, Stanford, CA), or myogenin (Dupont-Versteegden *et al.*, 1998). For myogenin detection, all incubations contained 0.1% Igepal (CA-630; Sigma-Aldrich) for permeabilization. Incubation with biotin-conjugated secondary antibodies (Zymed Laboratories, South San Francisco, CA) was followed by streptavidin peroxidase (Zymed Laboratories) and the DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA). Slides were rinsed in distilled water, dehydrated through alcohols/xylene, and coverslipped. For cells in culture, an alkaline phosphatase-conjugated secondary antibody was used (BD Biosciences Pharmingen, San Diego, CA) and the alkaline phosphatase substrate kit (Vector Laboratories). Sections were visualized with a Nikon Eclipse E600 microscope by using Nikon Plan Fluor 20×/0.50 or 40×/0.75 objectives and photographed with a Photometrics CoolSnapES camera at room temperature.

Statistical Analysis

Experiments were conducted a minimum of three times on separate cell isolates with the different regimens. Western and PCR analysis were performed at least three times from each batch of treated cells, with individual samples run in either duplicate or triplicate. The variances for the groups

Figure 3. Mimicking Wnt signaling with LiCl results in decreased adipogenic and increased myogenic potential. (A) Western blot analysis of cytosolic β -catenin in aged control myoblasts (C), NaCl-treated 4 h a day for 3 d, LiCl-treated 1 h a day for 3 d, LiCl-treated 4 h a day for 3 d, or LiCl-treated 24 h a day for 3 d. PC is the β -catenin-positive control included with the antibody. All cells were cultured in GM + IBMX. A constitutively expressed protein visualized with Ponceau S is shown to verify equivalent loads. (B) Real-time RT-PCR quantification of myogenin (MyoG), FABP4, C/EBP α , and PPAR γ mRNAs in untreated cells compared with cells treated chronically with LiCl (24 h LiCl from A). Data are expressed as mean $\pm$ SE. Asterisk (*) indicates significant difference from untreated, $p \leq 0.05$.

were determined to be unequal using Bartlett's test. In light of this, a Welch's *t* test was used to compare the groups. The significance level was 0.05.

RESULTS

We reported previously that adipogenic potential increases as a function of age in isolated mouse myoblasts (Taylor-Jones *et al.*, 2002). Analysis of gene expression in myoblasts from aged (24 mo) compared with adult (8 mo) mice demonstrated that genes normally associated with adipocyte differentiation are overexpressed, including C/EBP α and fatty acid binding protein 4 (FABP4, previously known as ap2) (Figure 1A). Although no difference in PPAR γ 2 mRNA abundance was apparent, PPAR γ 2 phosphorylation decreased with age (Figure 1B), suggesting increased activity. On the other hand, Wnt10b gene expression, which inhibits adipogenic differentiation (Ross *et al.*, 2000; Bennett *et al.*, 2002), was down-regulated with age (Figure 1A), potentially contributing to increased adipogenic potential. The relative abundance of myogenic and adipogenic gene products in aged myoblasts suggested that there was coexpression of these differentiation programs. Immunohistochemistry demonstrated that FABP4 was highly expressed in fully differentiated myotubes derived from aged mice, but not in myotubes from young adult mice (Figure 1C), confirming that both differentiation programs operate within individual cells with age. Furthermore, clonal analysis demonstrated that of the 26 clones analyzed, all underwent myogenic differentiation in low serum-containing myogenic DM and when exposed to a cocktail that promotes adipocyte differentiation in preadipocytes (IBMX), 25 of 26 were induced to express adipogenic markers, including C/EBP α , FABP4, and lipoprotein lipase as well as to accumulate lipid visualized by Oil Red O staining (our unpublished data).

To determine whether Wnt10b controls gene expression in myoblasts with age, its expression was manipulated and the effect on differentiation potential was determined (Figure 2). Retroviral-mediated gene transfer of Wnt10b into aged myoblasts resulted in approximately threefold overexpression,

quantified by real-time RT-PCR (Figure 2A). This increase was associated with increased abundance of cytosolic β -catenin to levels comparable with those observed in adult myoblasts (Figure 2B). Furthermore, Wnt10b overexpression led to inhibition of adipogenic gene expression, evidenced by decreased C/EBP α , PPAR γ , and FABP4 mRNA abundance (Figure 2A), as well as decreased Oil Red O staining compared with empty vector-infected cells (Figure 2C). Myogenic differentiation was not significantly affected by Wnt10b overexpression, and fully differentiated myotubes expressing myosin heavy chain were apparent irrespective of Wnt10b abundance (Figure 2C). Treating aged myoblasts with LiCl, which inhibits activity of GSK, thereby stabilizing β -catenin, mimicked some aspects of Wnt10b overexpression. Western blot analysis demonstrated that daily treatment with LiCl for 4 h each day for 3 d (Figure 3A) resulted in increased cytosolic β -catenin, which was even more stabilized if cells were exposed to LiCl chronically for 3 d (Figure 3A, 24-h LiCl). Quantification of FABP4, C/EBP α , and PPAR γ mRNA by real-time RT-PCR after chronic treatment with LiCl demonstrated that LiCl treatment decreased adipogenic gene expression (Figure 3B). LiCl treatment had the opposite effect on myogenic gene expression, augmenting expression of genes such as myogenin that promote myogenic differentiation (Figure 3B). Thus, although the canonical Wnt signaling pathway seems to promote myogenic differentiation and inhibit adipogenic differentiation in primary myoblasts, Wnt10b seems specific to the latter.

Because Wnt10b clearly inhibits adipogenic gene expression in myoblasts, we hypothesized that knocking out its expression in primary myoblasts would cause them to express an "aged" phenotype, with increased expression of markers of adipogenic differentiation. To test this hypothesis, myoblasts were isolated from mice at 9 mo of age with a deletion of the Wnt10b open reading frame (Lane and Leder, unpublished data). The most striking feature of the Wnt10b $^{-/-}$ myoblasts was that myogenic differentiation was significantly accelerated compared with myoblasts ob-

Figure 4. Wnt10b^{-/-} myoblasts demonstrate accelerated myogenic differentiation and augmented adipogenic gene expression. (A) Myoblasts isolated from 9 mo Wnt10b^{-/-} or wild-type (Wnt10b^{+/+}) mice cultured 24 h in DM were incubated with an antibody to myosin heavy chain. Wnt10b^{-/-} myoblasts form massive, fully differentiated myotubes by this stage. (B) Real-time RT-PCR analysis of MyoD, FABP4, C/EBPα, and PPARγ gene expression after 24 h in DM. Data are expressed as mean ± SE. Asterisks (*) indicates significant difference from wild type, $p \leq 0.05$. (C) Real-time RT-PCR analysis of FABP4 gene expression after 72 h in DM. Data are expressed as mean ± SE. Asterisks (*) indicates significant difference from wild type, $p \leq 0.05$.

tained from wild-type mice (Figure 4). Within 24 h of exposure to DM massive fusion of myoblasts into large myotubes expressing myosin heavy chain was observed from knock-out mice (Figure 4A), whereas wild-type myoblasts were just initiating myogenic differentiation (Figure 4A). Increased fusion in Wnt10b^{-/-} myoblasts was apparently not due to increased follistatin production (Iezzi *et al.*, 2004), because follistatin mRNA was expressed at slightly higher levels in wild-type compared with Wnt10b^{-/-} myoblasts (our unpublished data). The MyoD gene was overexpressed in Wnt10b^{-/-} myoblasts 24 h after induction of differentiation (Figure 4B), which may account for the increased fusion potential (Brennan *et al.*, 1990). Transcripts encoding the adipogenic transcription factors C/EBPα and PPARγ also were overexpressed in null compared with wild-type myoblasts at this early stage of differentiation. By 72 h in DM, no difference in transcription factor gene expression was apparent between Wnt10b^{-/-} and wild-type myotubes (our unpublished data), however, at this stage, downstream genes, such as FABP4, were differentially expressed (Figure 4C).

The distinct in vitro differentiation potential of myoblasts from Wnt10b^{-/-} mice suggested that the muscle phenotype in vivo also would be altered. Analysis of cryostat sections demonstrated no difference in muscle size or morphology between wild-type Wnt10b^{+/+} (Figure 5A) and Wnt10b^{-/-} null mice (Figure 5C). To examine muscle regenerative capacity, both freeze injury and cardiotoxin injection were used to induce either localized (freeze) or wide-

spread (cardiotoxin) muscle fiber degeneration and satellite cell activation in the tibialis anterior. Muscle regeneration in wild-type muscle was typical (Figure 5B), characterized by satellite cell proliferation and fusion to regenerate muscle fibers, identified by their small size and central nuclei. Muscle regeneration also proceeded efficiently in Wnt10b^{-/-} mice (Figure 5D); however, areas of regeneration accumulated large amounts of lipid as detected by Oil Red O staining, whereas adjacent nonregenerating areas showed no increased lipid content. Higher magnification analysis of Wnt10b^{-/-} mice (Figure 6) showed that lipid accumulation in regenerating muscle was heterogeneous with some regenerating fibers demonstrating no accumulation, some showing small lipid inclusions (Figure 6A), and others becoming completely filled with lipid (Figure 6, A and B). Although lipid clearly accumulated in regenerating muscle fibers, small cells surrounding the fibers also were Oil Red O positive (Figure 6A). The location and frequency of these cells suggested they were activated satellite cells or myoblasts, verified by nuclear accumulation of myogenin (Dupont-Versteegden *et al.*, 1999) (Figure 7A). Myogenin was overexpressed in regenerating muscle from Wnt10b^{-/-} mice (Figure 7A), compared with wild type (Figure 7E), confirming in vitro findings that myogenic potential was increased in the absence of Wnt10b. Myogenic cells in Wnt10b^{-/-} mice (Figure 7B) but not Wnt10b^{+/+} mice (Figure 7F) coexpressed FABP4. The nearby lipid-filled fibers in Wnt10b^{-/-} mice (Figure 7C) did not survive fixation with alcohol and manifested as void volumes upon

Figure 5. In vivo analysis of Wnt10b^{-/-} and wild-type (Wnt10b^{+/+}) muscle from 9-mo-old mice fed a high-fat diet. Lipid accumulation visualized by Oil Red O staining on 7- μ m cryostat sections was assessed in Wnt10b^{+/+} (A and B) and Wnt10b^{-/-} (C and D) tibialis anterior muscle. No obvious difference in muscle was apparent in uninjured muscle (A and C). One week after freeze injury, lipid accumulation was apparent in Wnt10b^{-/-} muscle (D) compared with wild type (B), restricted to regenerating areas. No significant lipid accumulation was apparent in regenerating muscle from Wnt10b^{-/-} mice fed a low-fat diet (our unpublished data). One small area of lipid accumulation in wild-type muscle is indicated by the arrow in B. Bar, 100 μ m.

hematoxylin and eosin staining (Figure 7D), and immunohistochemical analysis (Figure 7, A and B), whereas muscle integrity was maintained through processing in Wnt10b^{+/+} mice (Figure 7, E–H). Thus, myogenic and adipogenic gene programs are simultaneously expressed in regenerating muscle from Wnt10b^{-/-} mice. This may have functional and metabolic consequences as the adiposity in muscle is apparent even 1 mo after injury in Wnt10b^{-/-} mice (Figure 8). It should be noted that the increased adipogenic potential of regenerating muscle in vivo is only observed in Wnt10b^{-/-} mice fed a high-fat diet. Muscle regeneration in Wnt10b^{-/-} mice maintained on a low-fat diet (our unpublished data) was indistinguishable from that observed in wild-type mice (Figure 5B), suggesting that a stimulus to accumulate lipid also is required.

The observations that Wnt10b^{-/-} muscle and isolated myoblasts undergo myogenic differentiation more efficiently than those from wild-type animals, even in the face of increased adipogenic gene expression, suggest two possibili-

ties: 1) Wnt10b normally dampens myogenic differentiation, or 2) other Wnt family member(s) that are more myogenic may be compensating for Wnt10b deficiency. That overexpression of Wnt10b in myoblasts inhibits adipogenic potential with no obvious affect on myogenic differentiation (Figure 2) argues against the first possibility. Real-time RT-PCR analysis demonstrated that the Wnt7b gene is overexpressed in Wnt10b^{-/-} compared with wild-type myoblasts during the first 24 h of differentiation (Table 1). This suggests that the myogenic potential of these cells is increased, because Wnt7b gene expression has been shown to increase rapidly in regenerating muscle and to induce myogenic commitment in resident muscle stem cells (Polesskaya *et al.*, 2003). No such compensation is apparent in aged myoblasts, and in fact, both Wnt5b and Wnt10b are down-regulated as a function of age (Table 1), resulting in decreased cytosolic β -catenin with age (Figure 2B).

To test the hypothesis that Wnt7b contributes to increased myogenic potential, Wnt7b was overexpressed in aged myoblasts by retroviral-mediated gene transfer (Figure 9). Whereas Wnt7b expression had no significant affect on the adipogenic genes FABP4 (Figure 9), C/EBP α , or PPAR γ (our unpublished data), myogenic gene expression was dramatically increased within 24 h of exposure to DM. Thus, the compensatory increase in Wnt7b in Wnt10b^{-/-} myoblasts leads to more robust coexpression of myogenic and adipogenic gene programs than in aged myoblasts, where low levels of Wnt10b may be permissive for expression of adipogenic genes with no augmentation of myogenic gene expression.

Figure 6. Lipid accumulation in regenerating Wnt10b^{-/-} muscle is heterogeneous. (A) Higher magnification analysis of sections shown in Figure 5 shows large Oil Red O-positive lipid droplets in regenerating fibers (arrows with tails). Small cells surrounding the fibers also accumulate lipid (arrows without tails). (B) In some cases, regenerating fibers, identified by central nucleation (arrows), become completely filled with lipid. Bars, 50 μ m (A) and 100 μ m (B).

DISCUSSION

The loss in muscle and bone mass with age is accompanied by an increase in fat mass (Pahor and Kritchevsky, 1998). Thus, aging in muscle is characterized not only by a decrease in the total number of muscle fibers and a reduced fiber cross-sectional area but also by decreased muscle density

Figure 7. Myogenic and adipogenic programs are coexpressed in regenerating *Wnt10b*^{-/-} muscle. Serial cryostat sections of muscle from *Wnt10b*^{-/-} (A–D) or wild-type (*Wnt10b*^{+/+}; E–H) mice 1 wk after freeze injury were processed for myogenin immunohistochemistry (MyoG; A and E), FABP4 immunohistochemistry (B and F), Oil Red O staining (ORO; C and G), or hematoxylin and eosin staining (H&E; D and H). Bar, 100 μ m.

associated with increased intra- and intermuscular fat (Porter *et al.*, 1995; Goodpaster *et al.*, 2001). These changes in body composition and tissue remodeling contribute significantly to the decline in muscle strength and function. In fact, the increase in lipid content within muscle fibers that accompanies aging may account for the observation that older subjects are weaker than one would predict based on muscle mass alone (Kallman *et al.*, 1990). Although mechanisms underlying increased muscle adiposity during aging are relatively undefined, a substantial literature exists examining lipid content within skeletal muscle and its link to insulin resistance and type 2 diabetes (for review, see Kelley and Goodpaster, 2001; McGarry, 2002; Unger, 2003). Defects in the pathways for fatty acid oxidation leading to diminished use of fatty acids and increased esterification and storage of lipid within skeletal muscle have been strongly implicated in the pathogenesis of insulin resistance (Bays *et al.*, 2004). Changes in fatty acid oxidation with age have been less well studied; however, availability of free fatty acids increases with age, likely contributing to increased lipid content of muscle through increased storage. Our results demonstrate that adipocyte-specific regulatory genes as well as genes involved in fatty acid trafficking are overexpressed in myoblasts from aged mice, potentially exacerbating muscle lipid accumulation with age. Other muscle degenerative processes such as Duchenne muscular dystrophy (Cullen and

Mastaglia, 1980) and denervation (Bacou *et al.*, 1982; Dulor *et al.*, 1998) also involve increased intramyocellular lipid accumulation. Thus, we hypothesize that changes in gene expression due to altered Wnt signaling in myogenic progenitors may contribute to muscle adiposity during aging and in other pathological conditions.

Wnts have been shown previously to initiate myogenesis during development (Munsterberg *et al.*, 1995; Stern *et al.*, 1995; Hoppler *et al.*, 1996; Ikeya and Takada, 1998; Tajbakhsh *et al.*, 1998; Borello *et al.*, 1999; Cossu and Borello, 1999; Ridgeway *et al.*, 2000) and to inhibit adipogenic differentiation (Ross *et al.*, 2000; Bennett *et al.*, 2002). Our results demonstrate that Wnt signaling promotes myogenic and inhibits adipogenic differentiation simultaneously within primary adult myoblasts. Analysis of *Wnt10b* null mice suggests that *Wnt10b* normally represses the aberrant expression of genes involved in lipid storage in adult muscle, but this function is only apparent during muscle regeneration. Thus, upon satellite cell activation leading to expression of the myogenic differentiation program, adipogenic genes were activated in *Wnt10b*^{-/-} myoblasts, leading to excessive lipid accumulation within regenerating myofibers. However, lipid in regenerating fibers was diffuse and thus may not be properly associated with lipid droplet-associated proteins for efficient utilization. In aged muscle, satellite cell acti-

Figure 8. Lipid accumulation persists in Wnt10b^{-/-} muscle 1 mo after injury. Oil Red O staining of Wnt10b^{-/-} and Wnt10b^{+/+} muscle 1 mo after cardiotoxin injection to induce widespread degeneration indicates that lipid accumulation persists specifically within muscle fibers from Wnt10b^{-/-} mice. Bar, 100 μ m.

vation and regeneration are ongoing due to increased susceptibility to damage and denervation (Brooks and Faulkner, 1994), suggesting that loss of Wnt10b that normally occurs during aging may contribute to increased muscle adiposity with age. This increase in adipogenic potential is likely mediated by C/EBP α and PPAR γ .

Other Wnt family members, in particular Wnt7b, seem to contribute to enhanced myogenic potential in Wnt10b^{-/-} myoblasts and when overexpressed in aged myoblasts. Using gain- and loss-of-function studies, it

Table 1. Wnt mRNA expression in wild-type, Wnt10b^{-/-}, and aged myoblasts

	Wnt 5a	Wnt 5b	Wnt 7b	Wnt 10b	FABP4
24 h in DM					
Wild type	5.1	5.3	9.0	nd	7.4
Wnt 10b ^{-/-}	4.2	2.9	21.9 ^a	nd	8.4
72 h in DM					
Wild type	6.0	14.9	14.0	nd	9.3
Wnt 10b ^{-/-}	6.4	12.8	17.0	nd	31.9 ^b
Adult	19.2	23.0	14.0	19.5	2.2
Aged	16.7	8.4 ^c	9.9	6.5 ^d	19.2 ^e

nd, not determined (Wnts 1, 3a, and 7a were below the level of detection).

^{a,b} Wnt10b^{-/-} significantly different from wild type, $p < 0.05$.

^{c,d,e} Aged significantly different from adult, $p < 0.05$.

Figure 9. Overexpression of Wnt7b results in increased myogenic differentiation with no significant effect on adipogenic differentiation. (A) Real-time RT-PCR analysis of Wnt7b, myogenin (MyoG), MyoD, and FABP4 gene expression in aged myoblasts overexpressing Wnt7b by retroviral-mediated gene transfer compared with empty vector-infected cells after exposure to DM for 24 h. Data are expressed as mean $\pm$ SE. Asterisk (*) indicates significant difference from empty vector-infected cells, $p \leq 0.05$.

was recently shown that different Wnt family members have distinct effects on chick limb muscle development, controlling the number of terminally differentiated cells and fiber type distribution (Anakwe *et al.*, 2003). Because both Wnt10b and Wnt7b seem to increase cytosolic β -catenin, the mechanism underlying the specificity in response is currently unknown, but it is consistent with the observation that stabilization of β -catenin promoted myogenic differentiation and inhibited adipogenic potential in aged myoblasts. Clearly, the regulation of differentiation potential in myoblasts by β -catenin is complex (Goichberg *et al.*, 2001; Martin *et al.*, 2002), and we cannot rule out the possibility that Wnt10b and Wnt7b also differentially affect noncanonical signaling pathways (Nelson and Nusse, 2004). This is supported by the observation that the abundance of cytosolic β -catenin was not consistently different between myoblasts from wild-type and null mice (our unpublished data). The compensatory increase in other Wnt family members in response to decreased Wnt10b did not occur in aged myoblasts, suggesting that alterations in Wnt signaling may contribute to both decreased regenerative potential and increased adiposity in muscle as a function of age.

That myoblasts can be induced to undergo adipogenic differentiation has been demonstrated in several different experimental paradigms *in vitro*. Enhancing PPAR γ activity by treatment with thiazolidinediones, synthetic ligands for PPAR γ , has been reported to convert C2C12 cells to adipocytes (Teboul *et al.*, 1995; Grimaldi *et al.*, 1997). Thiazolidinediones alone did not enhance adipogenic potential in primary mouse myoblasts (Taylor-Jones *et al.*, 2002), suggesting that additional events may be required, such as inhibition of the p38 pathway (Yeow *et al.*, 2001), or decrease in Wnt10b abundance that occurs in myoblasts with age. Thus, in aged mice and high-fat-fed Wnt null mice, abundant free fatty acid derivatives may act as natural ligands for PPAR γ enhancing the adipogenic potential of myoblasts *in vivo*. Moreover, aged muscle is a more oxidized environment with ongoing dener-

vation, both of which have been shown to augment adipogenesis in isolated myoblasts and muscle (Dulor *et al.*, 1998; Csete *et al.*, 2001). Together, these results suggest that during aging, altered Wnt signaling may combine with the effects of elevated circulating oxidized free fatty acids and ongoing denervation/regeneration to lead to the expression of genes that promote lipid storage in muscle.

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