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Notes:

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Inflammatory myopathies (IM) are acquired diseases of skeletal muscle comprising dermatomyositis (DM), polymyositis (PM), and inclusion-body myositis (IBM). Immunosuppressive therapies, usually beneficial for DM and PM, are poorly effective in IBM. We report the isolation and characterization of mesoangioblasts, vessel-associated stem cells, from diagnostic muscle biopsies of IM. The number of cells isolated, proliferation rate and lifespan, markers expression, and ability to differentiate into smooth muscle do not differ among normal and IM mesoangioblasts. At variance with normal, DM and PM mesoangioblasts, cells isolated from IBM, fail to differentiate into skeletal myotubes. These data correlate with lack in connective tissue of IBM muscle of alkaline phosphatase (ALP)-positive cells, conversely dramatically increased in PM and DM. A myogenic inhibitory basic helix–loop–helix factor B3 is highly expressed in IBM mesoangioblasts. Indeed, silencing this gene or overexpressing MyoD rescues the myogenic defect of IBM mesoangioblasts, opening novel cell-based therapeutic strategies for this crippling disorder.

The idiopathic inflammatory myopathies (IM), characterized by mononuclear cells infiltration of skeletal muscle, are the largest group of acquired muscle diseases and encompass three major forms: dermatomyositis (DM), polymyositis (PM), and inclusion-body myositis (IBM) (1). Causes of DM, PM, and IBM are unknown, but an autoimmune pathogenesis is supported by marked up-regulation of cytokines and adhesion molecules, evidence of a T cell-mediated myocytotoxicity in PM and IBM and of a complement-mediated microangiopathy in DM (2). Current immunotherapies are usually effective in DM and PM patients, whereas IBM, the most frequent myopathy in elderly patients, responds poorly or not at all to immunosuppressive therapies and its course steadily progresses to severe disability. In IBM muscle, the presence of degenerative features, such as vacuolated fibers containing amyloid and amyloid-related proteins (3), reflects a complex pathogenesis involving misfolded and unfolded proteins and increased oxidative stress in the context of a cellular "aged" milieu acting in concert with chronic inflammation (4). Regeneration and repair of muscle fibers are fundamental processes accounting for rebuilding muscle integrity and gradual recovery of muscle strength in IM after suppression of mononuclear cells infiltration. Satellite cell-dependent regeneration occurs also in IBM muscle wherein multiple metabolic pathways normally involved in muscle development are activated (5, 6). However, in IBM, despite the activation of potentially repairing mechanisms, regeneration is inefficient.

Mesoangioblasts are vessel-associated stem cells, firstly isolated from dorsal aorta of mouse embryos (7), able to differentiate into a variety of mesoderm tissues including skeletal, cardiac and smooth muscle (8, 9). When delivered intraarterially, mesoangioblasts

restore to a significant extent muscle morphology and function in a mouse model of muscular dystrophy (10).

Because mesoangioblasts express numerous receptors for inflammatory cytokines, we assumed that the human counterpart of murine mesoangioblasts should be recruited in high numbers during muscle inflammation.

Here, we describe the isolation and functional characterization of pericyte-derived adult mesoangioblasts (herein simply called mesoangioblasts) from diagnostic muscle biopsies of IM patients and show that IBM mesoangioblasts fail to differentiate into skeletal muscle. This differentiation block can be corrected *in vitro* by transient expression of MyoD, making these cells potential attractive candidates for cellular therapy of this disabling disease.

Results

Mesoangioblasts Are Efficiently Isolated from IM Muscle Biopsies.

After 10–15 days of organ culture from biopsies of three normal controls, three DM, three PM, and six IBM, we isolated a population of cells morphologically different from satellite cells. Approximately $3\text{--}4 \times 10^4$ cells could be obtained from each biopsy. From the first passage on, cells were characterized by a triangular, adherent, refractive shape and by a floating/loosely adherent round component, particularly abundant in DM (Fig. 1A). Peculiar cell morphology, phenotypic characteristics, and differentiation potential indicated that our cells were human mesoangioblasts, as recently characterized (A. Dellavalle, M.S., R. Tonlorenzi, E.T., B. Sacchetti, L. Perani, B. G. Galvez, G. Messina, R.M., S. Li, G. Peretti, J. S. Chamberlain, W. E. Wright, Y. Torrente, S.F., P. Bianco, and G.C., unpublished data).

Cells were kept in culture up to 25 population doublings (PD) (25 for control and DM, 19 for IBM, and 20 for PM) with a proliferation rate comparable for DM, IBM, and PM and independent from patients' age. Doubling time from all biopsies was 33.5 ± 2.38 h (Fig. 1B). At both early and late passages, cells kept a diploid karyotype (data not shown). There were no differences in the number of cells isolated from freshly dissected or fresh-frozen muscles at both early

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The authors declare no conflict of interest.

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Abbreviations: ALP, alkaline phosphatase; IM, idiopathic inflammatory myopathies; IBM, inclusion-body myositis; DM, dermatomyositis; PM, polymyositis; SDMC, satellite-derived myogenic cells; *mdx*, mouse muscular dystrophy; bHLH, basic helix–loop–helix; BHLHB3, bHLH domain containing class B3 transcription factor.

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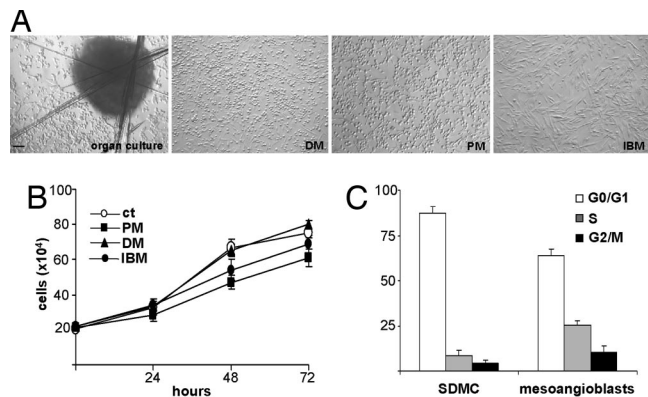


Fig. 1. Cell morphology, growth curve, and cell cycle. (A) From the organ culture on, refractive triangular, adherent, and round loosely adherent/floating cells were observed. (Scale bar: 40 μ m.) (B) Cell growth was assessed after 24 h, 48 h, and 72 h. Viable cells were judged by trypan blue exclusion. Results are expressed as absolute counts. Bars represent mean \pm SD of triplicate samples of one representative experiment of three. (C) Cell cycle distribution of proliferating normal human SDMC and mesoangioblasts from three controls, three DM, three PM, and six IBM (run in duplicate) after 24 h of culture was assessed by propidium iodide and FACS. For each sample, percentage of cells in G₀/G₁, S, or G₂/M phases of cell cycle is indicated. One representative experiment of three is shown.

and late passages, neither phenotypic characteristics of the 12 IM studied were significantly different after 8 and 18 PD (Fig. 2A and Fig. 7, which is published as supporting information on the PNAS web site). Because we isolated on average of $3\text{--}4 \times 10^4$ cells from a single biopsy, the estimated final number of cells after 25 PD is $50\text{--}120 \times 10^{10}$, and the real number that could be obtained before the appearance of senescent cells in significant proportion is between 10 and 20×10^9 cells. This number would be suitable for intraarterial delivery to adult patients, based on a per kg comparison with the mouse model used before (10).

Clonogenic Potential, Cell Cycle, and Phenotypic Characteristics Do Not Differ Among IM Mesoangioblasts. We dissociated mesoangioblasts to single cell suspension and cloned them by limiting dilution: clones appeared in 9.75 ± 3.9 , 8.87 ± 3.1 , and 10.5 ± 4.0 wells for DM, PM, and IBM, respectively, all with the same double morphology of the original cells. By replating the clones at clonal density they were able to give rise to new clones.

The cell cycle distribution was similar for all mesoangioblasts of 12 IM in three separate experiments (each one conducted in duplicate) (G₀/G₁, $65.5 \pm 6.4\%$; S, $23.7 \pm 4.3\%$; G₂/M, $10.8 \pm 2.9\%$) regardless of the IM type. The pattern of distribution was significantly different ($P \leq 0.01$) from that observed in control proliferating satellite-derived myogenic cells (SDMC) (G₀/G₁, $87.58 \pm 3.6\%$; S, $8.43 \pm 3.0\%$; G₂/M, $4 \pm 1.8\%$) (Fig. 1C). Results were always consistent throughout all experiments. Cells from all IM were strongly positive for CD44 and CD13, positive for CD49b, homogeneously negative for CD34, CD133, CD45 by FACS (Fig. 2A), consistently with what observed in normal human mesoangioblasts (A. Dellavalle, M.S., R. Tonlorenzi, E.T., B. Sacchetti, L. Perani, B. G. Galvez, G. Messina, R.M., S. Li, G. Peretti, J. S. Chamberlain, W. E. Wright, Y. Torrente, S.F., P. Bianco, and G.C., unpublished data). By immunocytochemistry and Western blot, all of the cells were positive for vimentin, weakly positive for α -SMA and desmin, and did not express glial fibrillar acidic protein (GFAP), nestin, β III-tubulin, and MyoD (data not shown). Alkaline phosphatase (ALP) staining was positive in all IM mesoangioblasts, with the highest levels observed in PM and only a weak labeling in IBM (Fig. 2B). Together, these markers identify human adult mesoangioblasts as the *in vitro* progeny of pericytes.

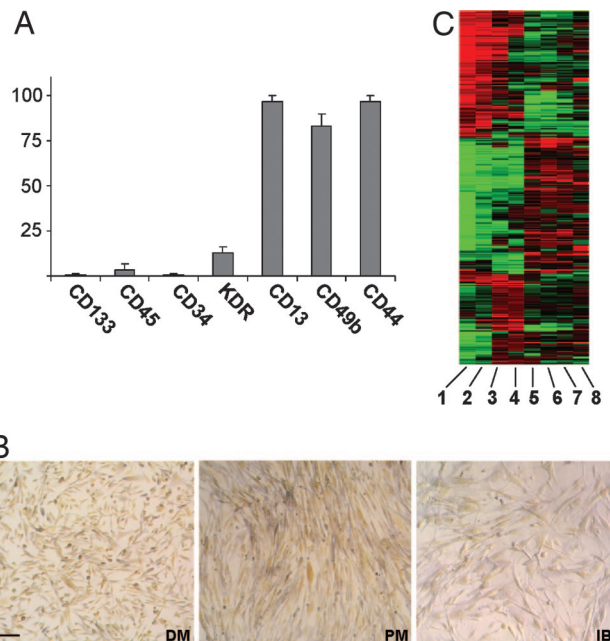


Fig. 2. FACS, immunophenotyping, ALP histochemistry, and gene expression profiling of IM mesoangioblasts. (A) More than 90% of cells from all samples were strongly positive for CD44 and CD13 with high percentage of cells CD49b-positive. None of the markers positive in murine mesoangioblasts were significantly expressed. Bars represent the mean \pm SD of 36 samples from the 12 patients with IM (3 DM, 3 PM, and 6 IBM) (each performed in triplicate). (B) IM mesoangioblasts *in vitro* are all ALP-positive. After simultaneous staining in the same culture conditions, more intensely labeled cells can be observed in PM and to a lesser extent in DM, whereas IBM mesoangioblasts are only weakly positive. (Scale bar: 20 μ m.) (C) Clustering results show two main classes: mesoangioblasts from normal controls (lanes 5–8), mesoangioblasts from DM (lanes 1 and 2), and IBM (lanes 3 and 4). Clustering procedure pairs together DM and IBM replicates.

The Ability to Differentiate into Smooth Muscle Cells (SMCs) and in Osteoblasts Are Similar Among All IM Mesoangioblasts. Murine mesoangioblasts differentiate into mature SMCs upon TGF β treatment (11, 12). Therefore, we exposed mesoangioblasts from all patients to TGF β . Approximately 80% of cells from all biopsies differentiated into strongly positive α -SMA-positive SMC, with no significant difference between the various IM (Fig. 8 which is published as supporting information on the PNAS web site).

Similarly to murine mesoangioblasts (8), human cells responded to BMP2 with a rather low percentage ($\approx 5\%$) differentiating into strongly ALP-positive osteoblast-like cells expressing osteocalcin and osteopontin (data not shown). In contrast, both control and IM mesoangioblasts failed to differentiate into neurons or glia when grown in neural stem cell differentiation media (data not shown).

Genome-Wide Gene Expression in IM Mesoangioblasts. Proliferating mesoangioblasts from normal and IM muscle were analyzed for gene expression by Affimetrix gene array. As expected, gene expression profile was similar in all samples with only few genes differentially expressed. Clustering results are shown in Fig. 2C. Two main classes were defined: the first included mesoangioblasts from normal muscle, whereas the second consisted of mesoangioblasts from DM and IBM. Interestingly, the clustering procedure paired together DM and IBM replicates.

A summary of the analysis is shown in Table 1, which is published as supporting information on the PNAS web site. In particular, mesoangioblasts from controls and IM (DM and IBM) did not express myogenic factors such as MyoD, or Pax3, Pax7, MEF2C, or MEF2D. As expected for mesoderm cells, mesoangioblasts did not

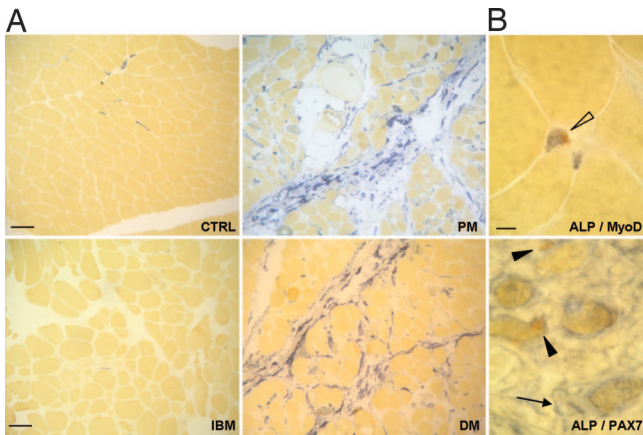


Fig. 4. ALP histochemistry in IM muscle biopsies. (A) In normal muscle only small arteries show ALP-positive staining. Intense ALP staining is present in PM and DM biopsies in perimysial and endomysial connective tissue, particularly strong in round cells associated to vessels' walls. IM biopsies contain also a variable number of small-sized regenerating muscle fibers with slight punctuate ALP positivity. IBM muscle shows no ALP connective tissue staining. (Scale bar: 40 μm .) (B) Immunohistochemistry for MyoD or Pax7 with peroxidase-antiperoxidase (PAP) on the same unfixed frozen sections of biopsies from PM, DM, IBM, and normal muscle from which mesoangioblasts were isolated. Representative fields of a PM biopsy showing a round vessel-associated cell (open arrowhead) strongly ALP-positive containing a MyoD-positive nucleus. Several PAX7-positive cells (filled arrowheads), identified as activated satellite cells, are associated with muscle fibers showing regenerative aspects with slight ALP positivity, whereas the roundish ALP-positive cells in the interstitium are PAX7-negative (arrow). CTRL, control. (Scale bar: 10 μm .)

expressing pericytes-derived cells seems correlated with the specific defect in myogenic commitment of this cell population in IBM.

Transient Expression of MyoD Rescues the Myogenic Defect of IBM Mesoangioblasts. IBM mesoangioblasts did not give rise to terminally differentiated myotubes and did not constitutively express MyoD mRNA, and MyoD could not be induced by our differentiating conditions (Fig. 3 B and C); therefore, we transduced IBM mesoangioblasts with an adenoviral vector expressing full-length murine *MyoD*. Expression of *MyoD* caused myogenic differentiation in $\approx 60\%$ (fusion index 0.6 ± 0.02) of cells, as defined by activation of muscle-specific genes and myotubes formation (Fig. 5A).

Myogenic potential of IM mesoangioblasts *in vivo* was tested by intramuscular transplantation in irradiated *scid/mdx* immunodeficient mice (Table 2, which is published as supporting information on the PNAS web site). After two consecutive injections, the tibialis anterior (TA) of mice treated with mesoangioblasts from DM displayed, in the injected areas, numerous muscle fibers containing human nuclei and expressing human dystrophin (average percentage of positive fibers was $60 \pm 15\%$ and $57 \pm 10\%$ in two animals transplanted with DM-derived mesoangioblasts, respectively; Fig. 5B Right). In contrast, none of such fibers could be detected in the TA of two mice injected with "wild-type" IBM mesoangioblasts. These muscles showed the presence of isolated (1–2%) human lamin A/C-positive nuclei in the interstitium and within some muscle fibers, but dystrophin expression was negligible (Fig. 5B Left). However, after transplantation of adenoMyoD-transduced IBM mesoangioblasts, the TA of the two mice treated showed, in the injected region, the presence of large areas reconstituted with dystrophin-lamin A/C-positive fibers ($33 \pm 10\%$ and $29 \pm 8\%$, respectively) (Fig. 5B Middle). Controls are shown in Fig. 9, which is published as supporting information on the PNAS web site).

siRNA Inhibition of BHLHB3 Expression Restores a Normal Myogenesis in IBM Mesoangioblasts. IBM mesoangioblasts had increased mRNA levels of BHLHB3 (Fig. 10, which is published as supporting

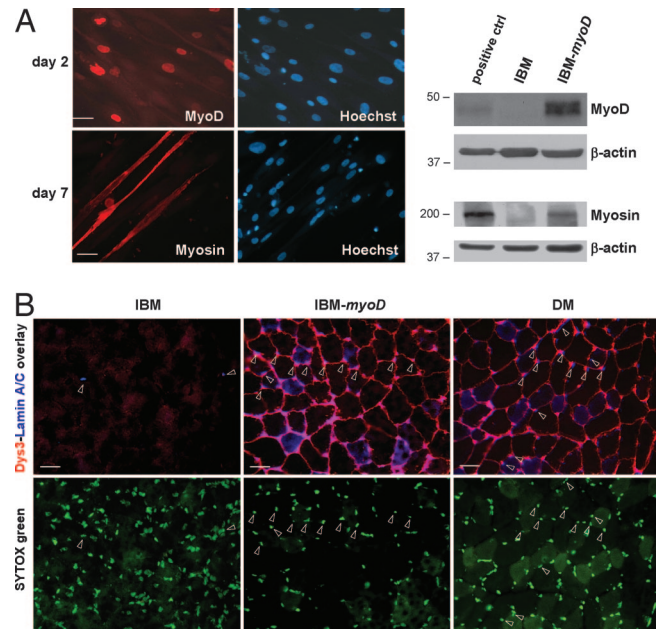


Fig. 5. MyoD transduction and induction of differentiation in IBM mesoangioblasts. (A) As shown by immunofluorescence and Western blot, AdenoMyoD-transduced IBM mesoangioblasts after 7 days in differentiation medium, fused into multinucleated myosin-positive myotubes. Approximately 90% of cells were MyoD-positive. (Scale bar: 10 μm .) Efficiency of transduction was evaluated 48 h after the infection by Western blot showing a marked up-regulation of MyoD. β -actin was used as loading control. A representative experiment of three, each performed in duplicate, is shown. (B) Double immunohistochemistry for human dystrophin and lamin A/C. *In vivo* myogenicity of IM mesoangioblasts was tested by intramuscular transplantation in *scid/mdx* mice. After two consecutive transplants, the TA of mice injected with IBM mesoangioblasts showed the presence of occasional human nuclei (blue with AMCA-labeled human lamin A/C, arrowheads) within muscle fibers and in the interstitium, but dystrophin expression was undetectable (Left). On the contrary, after IBM adenoMyoD-infected mesoangioblasts transplantation (Middle), large areas of injected muscle were reconstituted with fibers containing human lamin A/C-positive myonuclei (blue, arrowheads) and expressing dystrophin along the sarcolemma (red with Texas red-labeled specific antibody). When DM mesoangioblasts were used (Right), the majority of muscle fibers in the injected areas expressed dystrophin and contained human nuclei (positive for lamin A/C, arrowheads). (Scale bars: 10 μm .) SYTOX green staining on the same sections was used to identify both human and murine nuclei.

information on the PNAS web site), a group E subfamily member of bHLH factors, which negatively regulates myogenesis by modulating the transcriptional activity of MyoD (17). Therefore, we examined the effect of siRNA-mediated suppression of BHLHB3 in mesoangioblasts from three IBM patients. Interestingly, siRNA-transfected cells were able to differentiate, giving rise to multinucleated myosin-positive myotubes after 7 days in differentiation medium (Fig. 6). To verify the siRNA specificity, we used a second siRNA for BHLHB3 and found that also this siRNA had a similar ability to rescue myogenesis (data not shown), unlike nonsilencing control siRNA that was ineffective.

Discussion

Our study demonstrates that human adult mesoangioblasts can be efficiently isolated from diagnostic muscle biopsies of patients with IM. Antigenic and molecular characterization of these cells indicated that mesoangioblasts represent a distinct type of mesoderm progenitor cells, different from mesenchymal stem cells (E.T., and S.F., unpublished observations). In all IM patients, these cells retain the same proliferation ability of cells isolated from normal muscle, and can be grown and expanded for as many as 25–30 passages, although not indefinitely. Here we have shown that exposure of DM

combined with regenerative cell therapy, given the inflammatory background of IBM muscle. Also rare cases of unresponsive PM and DM would be potentially treatable as well by targeting muscle groups essentials for motor and respiratory functions.

Methods

Patients. Diagnostic muscle biopsies were performed after informed consent at the Neurology Department of Catholic University. We used fresh and fresh-frozen muscles from three normal controls (one fresh, two frozen) (48–84 years of age, average 64 ± 18.33 years of age) and 12 patients with IM: 3 DM (2 fresh, 1 frozen), 3 PM (1 fresh, 2 frozen) (33–75 years of age, average 52.5 ± 9.5 years of age), and 6 sporadic IBM (3 fresh, 3 frozen) (56–75 years of age, average 67.4 ± 18.1 years of age). Diagnosis was based on clinical evaluation and laboratory studies. None of the patients received steroids or immunosuppressive therapy before biopsy. This research was approved by the ethical committee of our institution.

Cell and Organ Cultures. Fragments of intramuscular vessels and surrounding mesenchymal tissue were plated as described (8, 10). Details are provided in *Supporting Methods*, which is published as supporting information on the PNAS web site.

A fragment from the same muscle biopsy was also cultured to obtain primary muscle cultures from satellite cells by using the explantation reexplantation method (18).

Characterization of Human Mesoangioblasts from IM by FACS, Cell-Cycle Analysis, and Growth Curve. Cells (5×10^4) were incubated with FITC-, PE-, or APC-conjugated mAbs directed against AC133/1, CD34, VEGF-RII (KDR), CD45, CD49b, CD44, and CD13. Details are provided in *Supporting Methods*.

In Vitro Differentiation. Skeletal muscle differentiation. Mesoangioblasts were (i) cultured under standard differentiating conditions for SMC (17); (ii) cocultured with a 4-fold excess of C2C12 myoblasts; (iii) cultured in normal human SMC-conditioned medium and then exposed to differentiation medium. At each time point, cells were fixed or harvested for protein extraction. Differentiation assays were performed in all IM samples studied and repeated at least three times for each patient with consistent results. **Smooth muscle, osteoblasts, and neural differentiation.** Differentiation of mesoangioblasts into SMCs, osteoblasts, and neural cells was tested as described (8, 12, 25).

Immunostainings. Immunostainings were performed as described (5, 6). Details are available in *Supporting Methods*.

Double Immunohistochemistry-Histochemical ALP Staining. Immunocytochemistry for MyoD or Pax7 was performed with peroxidase-antiperoxidase followed by histochemical ALP staining on the same unfixed frozen sections of the biopsies used for mesoangioblast isolation.

Gene Expression Profiling and Data Analysis. Proliferating mesoangioblasts from normal and IM muscles were analyzed for gene expression by Affimetrix gene array (26, 27). Details are provided in *Supporting Methods*.

Western Blot Analysis and RT-PCR. Protein expression was analyzed by Western blot according to standard methods. Details on antibodies and primers are available in *Supporting Methods*. Primers and PCR conditions for BHLHB3 have been described (28).

Cell Transduction. Mesoangioblasts from three IBM patients were adenoMyoD-transduced (29), cultured for 24 h in growth medium, and then either shifted to differentiation medium for 7 days or injected *in vivo*. Details are available in *Supporting Methods*.

Intramuscular Transplantation of DM, IBM, and AdenoMyoD-Transduced IBM Mesoangioblasts into Irradiated *scid/mdx* Mice. Mesoangioblasts from DM, IBM, and IBM adenoMyoD-transduced were injected into the right or left TA of six mice (two per group). Details are available in *Supporting Methods*.

BHLHB3 siRNA. Predesigned siRNA directed against human BHLHB3 (Hs BHLHB3 1 and Hs BHLHB2 HP siRNA; Qiagen, Valencia, CA) were transfected into IBM mesoangioblasts. Details are available in *Supporting Methods*.

Statistical Analysis. All data were expressed as mean \pm SD. One-way ANOVA was used to compare differences between groups. Statistical significance was set at $P \leq 0.05$.

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