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Growth of limb muscle is dependent on skeletal-derived Indian hedgehog

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During embryogenesis, muscle and bone develop in close temporal and spatial proximity. We show that Indian Hedgehog, a bone-derived signaling molecule, participates in growth of skeletal muscle. In $Ihh^{-/-}$ embryos, skeletal muscle development appears abnormal at embryonic day 14.5 and at later ages through embryonic day 20.5, dramatic losses of hindlimb muscle occur. To further examine the role of Ihh in myogenesis, we manipulated Ihh expression in the developing chick hindlimb. Reduction of Ihh in chicken embryo hindlimbs reduced skeletal muscle mass similar to that seen in *Ihh*$^{-/-}$ mouse embryos. The reduction in muscle mass appears to be a direct effect of Ihh since ectopic expression of Ihh by RCAS retroviral infection of chicken embryo hindlimbs restores muscle mass. These effects are independent of bone length, and occur when Shh is not expressed, suggesting Ihh acts directly on fetal myoblasts to regulate secondary myogenesis. Loss of muscle mass in Ihh null mouse embryos is accompanied by a dramatic increase in myoblast apoptosis by a loss of p21 protein. Our data suggest that Ihh promotes fetal myoblast survival during their differentiation into secondary myofibers by maintaining p21 protein levels.

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Introduction

Vertebrate limb skeletal muscle originates from muscle precursor cells that migrate into the developing limb bud from limb level somites (Brand-Saberi and Christ, 2000). Muscle formation is achieved by two waves of differentiation, primary and secondary myogenesis, mediated by embryonic and fetal myoblasts, respectively (Biressi et al., 2007a, 2007b; Duprez, 2002; Tajbakhsh, 2009). Early in limb development (chick embryonic day (ED)3 to ED7; mouse ED10.5 to ED12.5), embryonic myoblasts predominate. These cells: (i) require conditioned media for clonal growth, (ii) differentiate into small fibers with few nuclei in culture, and (iii) form primary muscle fibers *in vivo* (Biressi et al., 2007a, 2007b; Butler et al., 1982; Cossu et al., 1989; Rutz et al., 1982; Seed and Hauschka, 1984; Seed and Hauschka, 1988). Later in development (chick ED6 to ED13; mouse ED14.5 to ED20.5), fetal myoblasts predominate (Zappelli et al., 1996). These cells differ from embryonic myoblasts by: (i) no requirement of conditioned media for clonal growth, (ii) an ability to form large multinucleated myotubes in culture, (iii) the initial formation of small "secondary" myofibers adjacent to established primary myofibers *in vivo*, and (iv) in gene expression profiles (Biressi et al., 2007a, 2007b; Stockdale, 1992; Wigmore and Dunglison, 1998; Zappelli et al., 1996).

Understanding the roles of signaling molecules that regulate embryonic and fetal myoblasts *in vivo* has been possible, in part from retroviral manipulation of gene expression in the developing chick embryo (Bren-Mattison and Olwin, 2002; Edom-Vovard et al., 2001; Flanagan-Steet et al., 2000; Mitchell et al., 2002; Wang et al., 2010). Among the several growth factors shown to regulate muscle is Sonic hedgehog (Shh). Previously, we have shown that Shh regulates terminal differentiation of embryonic myoblasts located in the posterior of the limb during primary myogenesis (Bren-Mattison and Olwin, 2002). Shh mRNA is detectable by *in situ* hybridization only during primary myogenesis (Bren-Mattison and Olwin, 2002; Goodrich et al., 1996); however, Indian hedgehog (Ihh), another member of the hedgehog family, begins to be expressed by developing chondrocytes immediately preceding secondary myogenesis (Vortkamp et al., 1996). Ihh regulates long bone development beginning at ED5 in avian embryos and ED12.5 in mouse embryos (Bitgood and McMahon, 1995; Vortkamp et al., 1996). Previous work has suggested that organization of muscle tissue is influenced by skeletal elements (Lanser and Fallon, 1987; Robson et al., 1994; Yamamoto et al., 1998) and the close proximity of Ihh to the expanding musculature, make Ihh a candidate for a skeletal-derived signaling molecule capable of regulating secondary myogenesis.

Here, we present the evidence that bone-derived Ihh is required for secondary myogenesis. We show that loss of Ihh expression results in loss of the majority of skeletal muscle groups and loss of Ptch1 expression. Additionally, we show that down-regulation of Ihh expression in the developing chick hindlimb causes a loss of skeletal muscle tissue and phenocopies long bone defects observed in the *Ihh*$^{-/-}$ mouse. We propose that Ihh, produced by the developing bone anlagen, is critical for promoting the survival of differentiating fetal

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myoblasts forming secondary myofibers that contribute to the bulk of skeletal musculature.

Materials and methods

Embryos

Fertilized White Leghorn chick embryos were purchased, embryos grown, staged and injected as previously described (Flanagan-Steet et al., 2000). Ihh$^{+/-}$ mice were provided by Valerie Wallace (Ottawa Health Research Institute). *Ptch1*$^{+/nLacZ}$ (Ptch1tm1Mps/J) mice were purchased from The Jackson Laboratory. Embryos were harvested following timed pregnancies (the morning a vaginal plug was detected was considered day 0.5).

Viral constructs and production

RCAS(BP)A-Nkx-3.2 was provided by Andrew Lassar and RCAS (BP)A-Ihh was provided by Cliff Tabin. Primary chicken embryo fibroblasts were isolated from ED12 chick embryos grown, transfected and virus isolated as previously described (Flanagan-Steet et al., 2000).

Whole-mount immunohistochemistry

Fixation and preparation of tissue for whole-mount immunohistochemistry were as previously described (Bren-Mattison and Olwin, 2002). A monoclonal pan anti-myosin antibody, MF20 (Developmental Studies Hybridoma Bank (Bader et al., 1982)) was used with staining performed as described previously (Bren-Mattison and Olwin, 2002; Flanagan-Steet et al., 2000).

Section immunohistochemistry and immunofluorescence

Embryos were harvested in cold PBS, then fixed with 4% PFA at 4 °C for 12 h and washed with PBS before being incubated in 15% sucrose for 12 h, followed by incubation in 30% sucrose for 12 h. Embryos were then embedded in OCT Compound (Tissue-Tek) and sectioned at 8 μm using a cryostat (Leica). X-gal staining was performed by Premier Laboratory, LLC, Longmont, CO. For immunofluorescence, slides containing sections were washed with PBS to remove OCT, then blocked with 5% goat serum and 0.1% Triton X-100 for 1 h at room temperature. Sections were then incubated in primary antibody (anti-Desmin supernatant (Developmental Studies Hybridoma Bank (Ip, 1983); 1:2, anti-myosin heavy chain (MF20) 1:10 for 1 h at 37 °C, and washed three times for 30 min with blocking solution at room temperature. Sections were then incubated in appropriate secondary antibodies Alexa Fluor 594-conjugated (Molecular Probes; 1:500) and/or FITC-conjugated (Promega Corp.; 1:100) for 1 h at room temperature. Coverslips were mounted over sections using Vectashield fluorescent mount media containing 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories).

Whole-mount in situ hybridization

Embryos were harvested at stages specified in text and fixed in 4% PFA at 4 °C for 12 h. Whole-mount in situ hybridization was performed as previously described (Wilkinson and Nieto, 1993) with the following exception, older embryos (HH 26–27) were treated with 40 mg/ml Proteinase K for 15 min at room temperature. Digoxigenin (DIG) -labeled riboprobes were made as previously described (Wilkinson and Nieto, 1993). Plasmids used to generate cShh were provided by Dr. Cliff Tabin (Harvard University). Plasmids used to generate cMyoD-DIG labeled riboprobes for in situ hybridization were provided by Dr. Bruce Paterson (National Institutes of Health).

Skeletal muscle harvest, wet mass determination and measurement of bone length

Wild type and Ihh null embryos were harvested in cold PBS. For wet mass determinations, the left and right hindlimbs were removed, and weighed using an analytical balance. Following measurement of each whole limb, epidermis and connective tissue of the limb was removed and limb muscle was removed from the cartilage/bone elements using number five forceps and muscle mass was determined with an analytical balance. Bone length measurements were made using a transparent ruler and a stereoscope dissecting microscope. To extract proteins, the muscle tissue was removed as described above and placed in lysis buffer containing protease inhibitors (Pierce). The tissue was homogenized, vortexed several times, and incubated on ice for 1 h, centrifuged at 10,000 *g* for 15 min at 4 °C, aliquots flash frozen in liquid N_2 and stored at −70 °C. Protein concentrations were determined with the Pierce Commassie 200 Plus protein assay reagent using a bovine serum albumin standard curve as a reference. The embryo genotypes were readily detected by morphology and genotypes confirmed by PCR (St-Jacques et al., 1999).

Gel electrophoresis and Western Blotting

Protein extracts (20 μg) from wild type and Ihh null embryos were boiled in SDS-PAGE sample buffer and separated on 4–20% SDS-PAGE gradient gels for 2–3 h at 85 V in a Bio-Rad Mini-Protean II gel apparatus. Proteins were transferred to PVDF membrane (Bio-Rad) using a mini-gel transfer apparatus (Bio-Rad) at 30 V overnight at 4 °C. The membrane was placed in PBS containing 5% nonfat dry milk and 0.1% Triton X-100 (blocking solution) for 1 h. Primary antibodies for Patched (1:500, Research Diagnostics), p21 (1:100, Santa Cruz (H-164): sc756; may partially react with p27), and 〈-tubulin (1:1000, Novus Biologicals) were diluted in the same blocking solution, incubated with the membrane for 1 h at room temperature, and washed 3 times for 10 min each. Blots were then incubated in appropriate secondary antibodies anti-mouse-HRP (Promega, 1:5000) or anti-rabbit HRP (Promega, 1:5000) for 1 h at room temperature, washed once on blocking solution and 3 times in PBS for 15 min and developed according to the manufacturer's instructions for Chemiluminescence (Amersham, Inc.).

Quantitative RT-PCR

Hindlimb tissue was harvested from ED16.5 and ED18.5 *Ihh*$^{-/-}$ and wild type littermates. Total RNA was extracted and Reverse Transcription was performed using Superscript III. SYBR green detection of PCR amplicons was employed using an ABI QT-PCR machine. Corresponding level of MRF expression was normalized to 18S rRNA abundance levels from the same sample. Primer sequences for MyoD, Myf5, and Myogenin were previously described (Hannon et al., 1992). Primer sequences for Ptch1 were previously described (Liu et al., 2010). Primer sequences for 18S rRNA (FOR 5′GCCGCTAGAGGTGAAATTCTTG; REV 5′CTTTCGCTCTGGTCCGTCTT.

Apoptosis assay

Cryosections prepared and stained for MyHC using MF20 described above for wildtype and *Ihh*$^{-/-}$ ED16.5 embryos were processed per manufacturer's instructions to detect cell death using the Roche Cell Death Detection Kit and apoptotic cells visualized with TMR Red. Digital deconvolution microscopy and image acquisition were as described previously (Bren-Mattison and Olwin, 2002). Positive and negative controls were performed per manufacturer's instructions.

Primary cell culture and immunofluorescence

Primary chick myoblasts were cultured from chicken embryos, infected, BrdU labeled and assayed using established procedures as described previously (Bren-Mattison and Olwin, 2002). Primary mouse myoblasts were explanted from ED16.5 embryos by dissection of muscle tissue from hindlimbs digested for 30 min at 37 °C in 400 U/ml Type I Collagenase (Worthington). Digested tissue was washed with F12C (F12 media + 4 mM CaCl2) with 15% horse serum and 2% chick embryo extract, titerated with an 18 G needle, and filtered through a 40 μm filter. Explanted cells were plated onto a 10 cm tissue culture plate containing 10 ml of F12C, 15% horse serum and 2% chick embryo extract and incubated at 37 °C for 45 min. Floating cells were collected and either 1) directly dried onto gelatin coated coverslips or, 2) plated in 2 ml of media in 6-well tissue culture plates containing gelatin coated coverslips or, 3) harvested for RNA purification and quantitative PCR (see above). Coverslips were fixed with 4%PFA either immediately after drying onto coverslips or following overnight incubation at 37 °C. For immunofluorescence, cells were permeabilized with 0.5% Titron-X 100 in PBS for 5 min. Cells were blocked by incubation in 5% BSA for 1 h at room temperature, incubated with anti-MyoD antibodies (1:10, Vector Laboratories, clone 5.8A, VP-M669) or anti-Myogenin antibodies(neat, F5D mouse monoclonal), and anti-Ptch1 antibodies (1:100, Santa Cruz Biotechnology, sc-6149) overnight at 4 °C, then washed and incubated with either anti-mouse Alexa Fluor 555 or anti-goat Alexa Fluor 488, raised in donkey (1:500). As a negative control, coverslips were incubated with secondary antibodies alone. Images acquired with a Zeiss ABSO2 confocal microscope.

Results

Ihh is required for normal muscle development and Ptch1 receptor expression in muscle

Roles for Shh regulation of primary myogenesis have previously been reported (Bren-Mattison and Olwin, 2002; Duprez et al., 1998; Kruger et al., 2001). Based on these studies the observed effects of Shh overexpression on skeletal muscle occurring later in development (during secondary myogenesis) were presumed to be either indirect since Shh expression is not detectable following primary myogenesis, or from another hedgehog possibly involved in skeletal muscle development. Since Ihh expression in the bone anlagen begins during secondary myogenesis, we asked if Ihh null mice exhibited phenotypes consistent with a role for Ihh in developing muscle.

We observed a dramatic loss of skeletal muscle tissue during secondary myogenesis in Ihh null limbs as compared to wild type limbs (Fig. 1). At ED14.5, near completion of primary myogenesis, *Ihh*$^{-/-}$ muscle is morphologically distinct from wild type littermates with mutant muscle groups localized to the limb periphery rather than around the developing bone (Fig. 1A). However, it appears that the amount of differentiated muscle is similar in both wild type and *Ihh*$^{-/-}$ ED14.5 embryos (Fig. 1A). By ED16.5, *Ihh*$^{-/-}$ embryo hindlimbs have lost most of their differentiated muscle compared to wild type littermate hindlimbs (Fig. 1B). Older mutant embryo hindlimbs from ED18.5 (Fig. 1C) through ED20.5 (data not shown), consistently possess less muscle tissue than wild type control hindlimbs. Unfortunately, we were unable to examine embryos beyond ED20.5 because *Ihh*$^{-/-}$ animals are not viable.

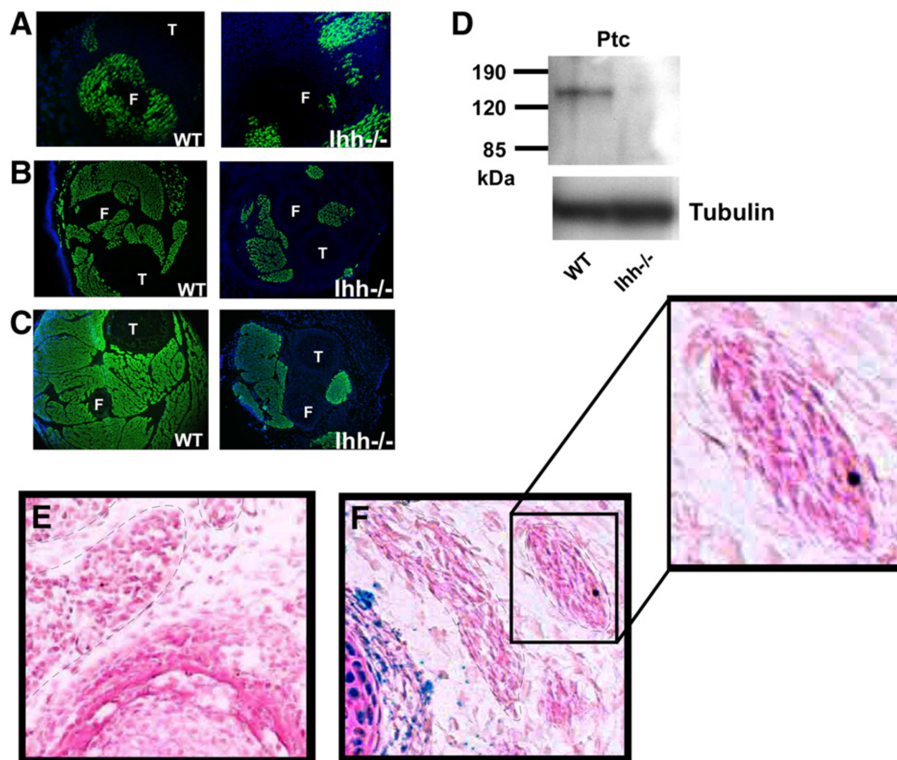

Fig. 1. Ptch1 is expressed in muscle tissue and *Ihh*$^{-/-}$ embryos lose hindlimb skeletal muscle. Wild type and Ihh$^{-/-}$ littermates were harvested at (A) ED14.5, (B) ED16.5, and (C) ED18.5. Hindlimb cross-sections (8 μm) were stained with a pan MyHC antibody to detect all differentiated skeletal muscle. F and T mark the fibula and tibia, respectively. (D) Lysates of skeletal muscle tissue from ED16.5 Ihh null and wild type littermates were western blotted for the presence of Ptch1 protein and alpha tubulin was used as a loading control. (E and F) Hindlimb sections were assessed for Ptch1 expression in muscle tissue. (E) Hindlimbs from ED16.5 wild type mice were sectioned and stained with nuclear fast red and X-gal. (F) Hindlimbs from ED16.5 *Ptch1*$^{+/nLacZ}$ heterozygous littermates were sectioned and stained with nuclear fast red and x-gal to detect beta-galactosidase expression. Inset is enlarged in order to show X-gal+ (blue) muscle tissue (the black spot is an artifact). Dashed lines outline developing muscle tissue (E) and muscle tissue that is β-galactosidase-positive (F).

Hedgehogs are capable of both short range and long range signaling (Gritli-Linde et al., 2001; Gualeni et al., 2010; Koziel et al., 2004; Lewis et al., 2001; Zeng et al., 2001) and signal via the Patched 1 receptor (Lum and Beachy, 2004). Since Ptch1 provides a sensitive readout of hedgehog activity (Goodrich et al., 1996), we predicted that if muscle receives direct Ihh signaling, Ptch1 would be expressed in developing skeletal muscle. To test this, skeletal muscle tissue was harvested from ED16.5 wild type and *Ihh*$^{-/-}$ hindlimbs. Western blots were probed for Ptch1; and as expected, Ptch1 is present in wild type but not *Ihh*$^{-/-}$ hindlimb muscle on ED16.5 (Fig. 1D). To further support that Ptch1 is expressed in muscle, we examined at ED16.5 β-galactosidase activity in heterozygous *Ptch1*$^{+/nLacZ}$ embryos, in which β-galactosidase expression is under the control of the Ptch1 regulatory elements. Sections from control (wild type) and *Ptch1*$^{+/nLacZ}$ embryos were stained with nuclear fast red and X-Gal to detect β-galactosidase activity. Cross sections through the lower hindlimb clearly show no β-gal activity in wild type control embryos (Fig. 1E); whereas in *Ptch1*$^{+/nLacZ}$ embryos, β-gal activity was observed (Fig. 1F). Magnified images from cross sections revealed that the majority of β-galactosidase activity is present in the perichondrium, as expected (Fig. 1F). However, distinct loci of β-galactoside activity were seen in developing skeletal muscle (Fig. 1F). Ptch1 message is present in primary myoblasts as cells explanted from ED16.5 muscle enriched for myoblasts by pre-plating and subjected to quantitative PCR are positive for Ptch1 message (Supplemental 1A). Myoblasts, immunoreactive for the myogenic markers, MyoD (Supplemental Fig. 1B) and Myogenin (Supplemental Fig. 1C) were also immunoreactive for Ptch1 (Supplemental Fig. 1 B–D), demonstrating that Ptch1 protein is present in myoblasts. At ED16.5 there is no detectable expression of either Shh or desert hedgehog in the developing limb and thus Ptch1 expression is likely due to Ihh signaling (Colnot et al., 2005; Vortkamp et al., 1996).

Ihh regulates fetal myoblasts undergoing secondary myogenesis

The most severe muscle phenotype in the Ihh$^{-/-}$ mouse was observed at ED16.5, which coincides temporally with high levels of expression of Ihh in cartilage (Bitgood and McMahon, 1995). At this developmental age, skeletal muscle is undergoing secondary myogenesis, where the bulk of the musculature is forming by fusion of fetal myoblasts (Biressi et al., 2007a, 2007b). Secondary myofibers first appear as smaller diameter myofibers that use larger primary myofibers as scaffolds (Duxson and Usson, 1989) (Fig. 2A; arrowheads) In Ihh null mice, smaller secondary myofibers were not readily discernible (Fig. 2B) and when quantified, the average myofiber size in *Ihh*$^{-/-}$ mice was significantly larger than in wild type mice (Fig. 2C; p-value$<$0.01; n$=$3). These data support a role for Ihh in regulating secondary myogenesis.

If Ihh plays a role in secondary myogenesis, and fetal myoblasts are lacking or impaired in Ihh null mice, we predict that myoblasts explanted from ED20.5 Ihh null mice would not exhibit characteristics

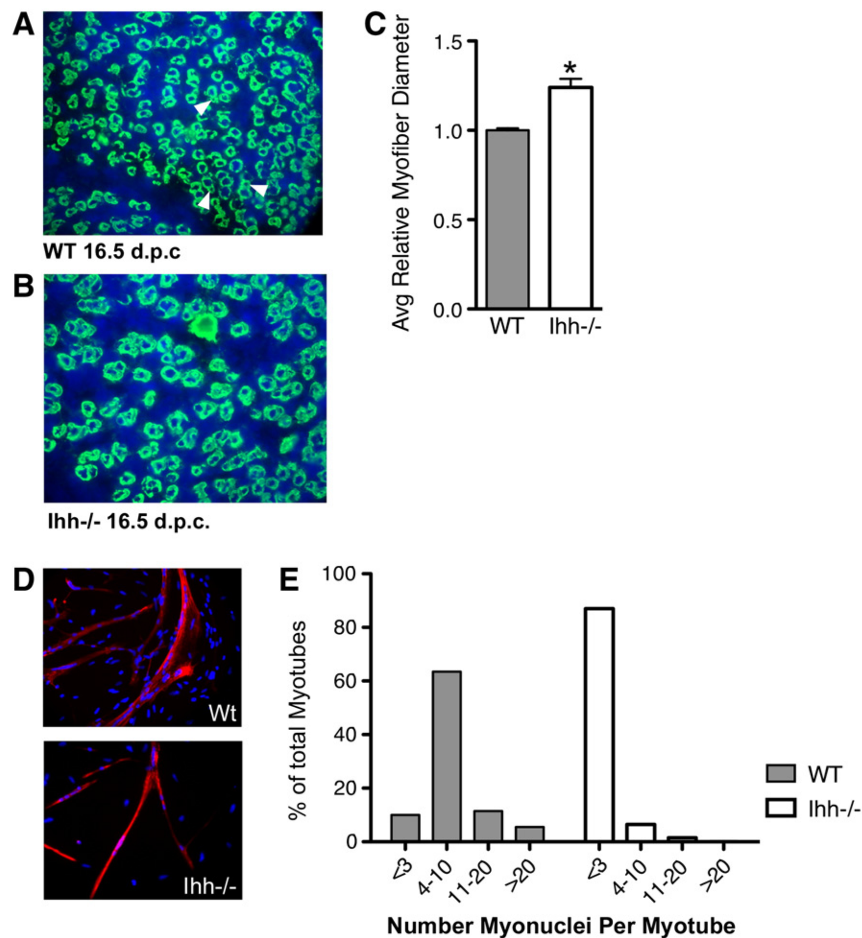

Fig. 2. Ihh regulates secondary myogenesis. Cross sections at the mid-thigh level of hindlimbs from (A) ED16.5 wild type and (B) *Ihh*$^{-/-}$ mice stained for MyHC and nuclei reveal larger diameter fibers in Ihh null sections. (A) Arrowheads identify smaller secondary myofibers in sections from wild type limbs. (C) The fiber diameters from sections similar to those shown in (A and B) derived from 3 embryos of each genotype were quantified and the average fiber diameter ± SEM (standard error of the mean) was plotted (*p ≤ 0.01). Myoblasts were explanted from ED20.5 wild type and *Ihh*$^{-/-}$ hindlimb muscle, plated at equal density, cultured for 3 days and assayed for their ability to form myotubes. (D) Myotubes and nuclei were visualized by using pan MyHC in red and DAPI, respectively. (E) The number of myonuclei per myotube was quantified and plotted as a function of the percent of the total number of myotubes.

of fetal myoblasts. At this developmental age in wild type muscle, no embryonic myoblasts are present and all myoblasts are expected to fuse into large multinucleated myotubes (Biressi et al., 2007a, 2007b; White et al., 1975). Myoblasts explanted from both ED20.5 wild type and ED20.5 *Ihh*$^{-/-}$ hindlimbs were able to differentiate in tissue culture (Fig. 2D); however, myoblasts explanted from Ihh$^{-/-}$ hindlimbs formed small myotubes containing 3 or fewer nuclei (Fig. 2E). These data indicate that defects in the Ihh null myoblasts are cell-autonomous and not due to extrinsic factors in the developing limb. The formation of small myotubes in culture (Vivarelli et al., 1988; White et al., 1975) and the larger diameter of Ihh null myofibers in vivo (Biressi et al., 2007b; Condon et al., 1990; Kelly and Zacks, 1969; Wigmore and Dunglison, 1998), are all consistent with the hypothesis that secondary myogenesis is altered in *Ihh*$^{-/-}$ embryos.

Manipulation of Ihh expression using retroviral mediated gene transduction in chick

A complete loss of Ihh severely affects long bone development (St-Jacques et al., 1999) likely influencing the developing musculature. To test whether Ihh signaling directly affects skeletal muscle development, we either augmented or reduced Ihh levels in the developing chicken embryo (Petropoulos and Hughes, 1991). In an attempt to decouple long bone development and myogenesis, we aimed to reduce rather than eliminate Ihh expression. Identification of an appropriate shRNA that would reduce rather than eliminate Ihh and that could be rescued *in vivo* proved technically difficult. Therefore, we opted to reduce Ihh expression by using retroviral-mediated gene expression and infecting the developing bone region with RCAS(BP)A-Nkx3.2 (Fig. 3A).

Nkx3.2 and its mouse homolog Bapx1 belong to a family of homeodomain transcription factors related to Drosophila bagpipe (McMahon, 2000; Tribioli and Lufkin, 1999) which is expressed in prechondrogenic cells that will eventually give rise to bone as well as the muscle sheath and surrounding mesenchyme tendons (Church et al., 2005). Overexpression of Nkx3.2 reduces Ihh expression by preventing the maturation of chondrocytes (Provot et al., 2006). When injected into the central region of the developing hindlimb on ED3, RCAS(BP)A-Nkx3.2 reduced Ihh expression levels on ED12 (Fig. 3B). Injection of RCAS(BP)A-Nkx3.2 throughout the entire limb severely reduced Ihh expression sometimes to undetectable levels (data not shown), similar to that previously observed (Provot et al., 2006).

To ensure that ectopic Nkx3.2 effects were mediated via reductions in Ihh, we first asked if Shh was affected. Over expression of Nkx3.2 in the hindlimb of ED3 embryos did not significantly alter or reduce Shh expression (Supplemental Fig. 2A). This finding agrees with previous studies examining the role of Bapx1; in Bapx1$^{-/-}$ mice transcript levels of either Shh or Gli2, a downstream component of Shh-mediated signaling, are not significantly altered (Tribioli and Lufkin, 1999). Since Shh has been shown to regulate MyoD expression, and to further rule out the possibility that Nkx3.2 affects Shh expression, we assessed whether Nkx3.2 overexpression altered MyoD during primary myogenesis. Two days after RCAS(BP)A-Nkx3.2 injection into ED3 hindlimbs, we examined the distribution of MyoD mRNA. In situ analysis revealed that ectopic expression of Nkx3.2 and its subsequent inhibition of Ihh did not significantly alter MyoD expression levels during primary (early) myogenesis (Supplemental Fig. 2B). These results, together with our previous finding that forced expression of Nkx3.2 does not appear to alter Shh distribution or

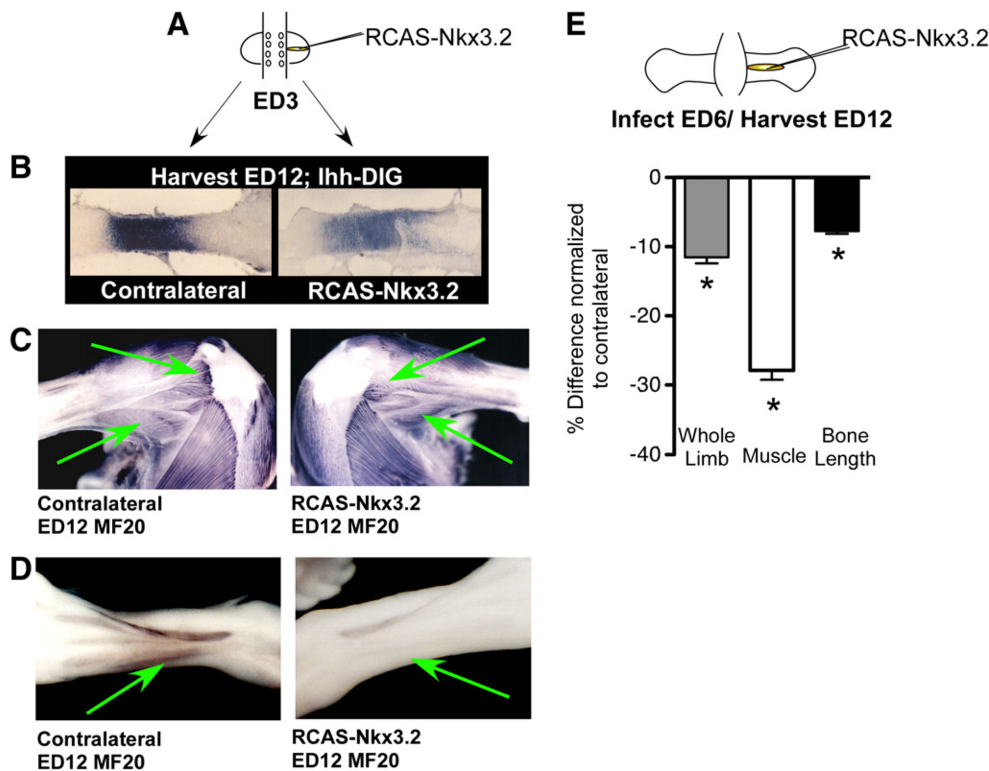

Fig. 3. Reduction of *Ihh* expression results in decreased skeletal muscle mass and long bone length. (A) Chick hindlimbs were injected with RCAS(BP)A-Nkx3.2 at ED3 in the mid-region of the hindlimb. (B) On ED12, the limbs were sectioned and an anti-sense Ihh-DIG probe was used to detect Ihh expression following low-dose viral injection. (C and D) Chick hindlimbs were injected with RCAS(BP)A-Nkx3.2 at ED3 and harvested at ED12. (C) Whole-mount immunohistochemistry for MyHC shows decreases in individual muscle sizes and a decrease in girth across the lower limb compared to the contralateral uninfected limb. (D) Extensor muscles (arrows) are not detectable by MyHC staining in Nkx 3.2 infected lower hindlimbs. (E) Forced expression of Nkx3.2 significantly reduces whole limb weight, wet muscle weight and bone length. Chick hindlimbs were injected with RCAS(BP)A-Nkx3.2 on ED6 and harvested on ED12. Both the injected limb and the contralateral control limb were removed and weighed (see Materials and methods). Average percent difference normalized to the contralateral control ± SEM were plotted (*$p \leq 0.01$; $n = 9$).

levels of expression, suggest that Nkx3.2 down-regulates Ihh expression without affecting Shh expression or primary myogenesis.

Reduction of Ihh expression significantly reduces muscle mass and bone length

RCAS(BP)A-Nkx3.2 injection of ED3 chick embryos into the mid-region of the hindlimb reduced the size of the musculature at ED12 (Fig. 3C). Effects on distal muscles appeared more prominent than on proximal muscles since extensors in the lower hindlimb were difficult to identify (Fig. 3D). We quantified whole limb and muscle mass of embryos injected on ED3 and found significant decreases when infected with RCAS(BP)A-Nkx3.2 compared to uninfected contralateral controls (Supplemental Fig. 3A), while injection of a control virus had no detectable effect (Bren-Mattison and Olwin, 2002). To confirm that reduction of Ihh expression reduces muscle in the absence of Shh, we injected chick hindlimbs with RCAS(BP)A-Nkx3.2 at a time point (ED6; Fig. 3E) when endogenous Shh expression was undetectable and quantified whole limb and muscle masses. Injection of RCAS(BP)A-Nkx3.2 into hindlimbs at ED6 significantly reduced whole limb mass by 12% and muscle mass by 28% (Fig. 3E). Consistent with regulation of long bone development by Ihh in mice (St-Jacques et al., 1999), we observed that RCAS(BP)A-Nkx3.2 injection into ED3 (Supplemental Fig. 3B) and ED6 hindlimbs (Fig. 3E) shortened long bones by 8%. Therefore, we were unable to definitively conclude that the loss of muscle following RCAS(BP)A-Nkx3.2 injection was not an indirect effect of bone length reduction.

Overexpression of Ihh Increases Muscle Mass without Affecting Bone Length

Ectopic expression of Nkx3.2 reduces Ihh mRNA levels suggesting that overexpression of Ihh should increase muscle mass; however, we wanted to target developing muscle without affecting long bone length. By injecting RCAS(BP)A-Ihh into the flanks of ED6 developing hindlimbs, we were able to decouple muscle mass regulation and bone length (Fig. 4A). We observed significant increases of 23% in whole limb and 27% in muscle masses independent of long bone length (Fig. 4A).

If the reduction in muscle mass after RCAS(BP)A-Nkx3.2 infection was due to loss of Ihh expression, then ectopic Ihh expression should rescue the muscle mass. Thus, we attempted to rescue RCAS(BP)A-Nkx3.2 infected hindlimbs by overexpressing Ihh. Since Ihh is produced in condensing chondrocytes, both limbs were first infected in the medial region of the limb with RCAS(BP)A-Nkx3.2 at ED3, in order to allow viral replication, infection, and reduction of Ihh (Fig. 4B). Subsequently, RCAS(BP)A-Ihh was injected 24 h later into the anterior and posterior regions of one limb to target the developing muscle (Fig. 4B). Both Nkx3.2 and Ihh viruses used were RCAS-A subtypes and could not simultaneously infect the same cells (Hughes et al., 1997), therefore this injection scheme establishes a boundary where neighboring cells ectopically express either Nkx3.2 or Ihh. Comparison of co-infected limbs with contralateral limbs injected only with Nkx3.2 demonstrated that ectopic Ihh expression effectively rescued Nkx3.2-induced muscle loss (Fig. 4C and D). Whole mounts of hindlimbs at ED10 stained for pan skeletal muscle MyHC demonstrate that the majority of muscles appear intact and that the hindlimb girth

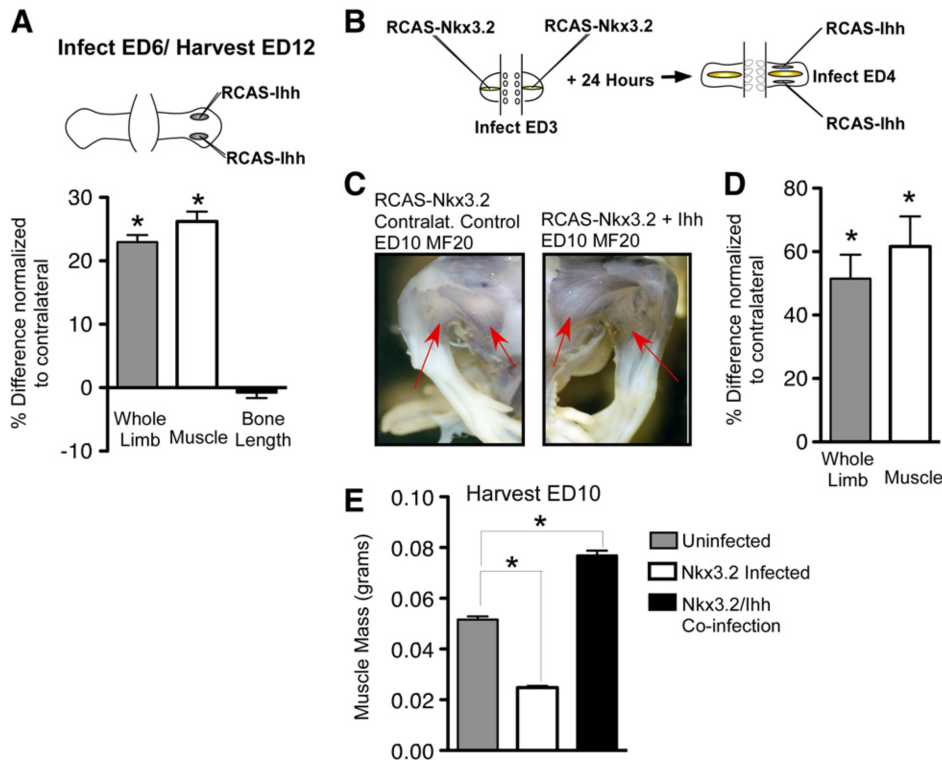

Fig. 4. Ectopic Ihh expression increases skeletal muscle mass and rescues Nkx3.2-mediated loss of skeletal muscle mass. (A) Hindlimbs were injected with RCAS(BP)A-Ihh on ED6 and harvested on ED12. Ectopic expression of Ihh increased average whole limb mass and muscle mass without affecting long bone length (for whole limb and muscle mass * is $p \leq 0.01$; $n = 9 \pm$ SEM). (B) Schematic of co-injection procedure used for rescue of RCAS(BP)A-Nkx3.2 infected limbs with RCAS(BP)A-Ihh. At ED3, both the left and right chick hindlimbs were injected in the mid-region with Nkx3.2 expressing virus. After 24 h, an Ihh virus was injected both anteriorly and posteriorly to the site of Nkx3.2 injection in one hindlimb and embryos were maintained until ED10. (C) Whole-mount immunohistochemistry for MyHC of Nkx3.2 and Nkx3.2/Ihh infected hindlimbs. RCAS-Nkx3.2 infection decreased individual muscle size; however, subsequent infection with RCAS-Ihh rescued limb girth and individual muscle sizes (arrows). (D) RCAS-Ihh rescued whole limb and muscle mass loss in Nkx3.2 infected limbs. Average percent difference of Nkx3.2/Ihh co-infected whole limb and wet muscle weight normalized to Nkx3.2 infected contralateral limb were plotted (*$p \leq 0.01$; $n = 5 \pm$ SEM). (E) Chick hindlimbs were injected with RCAS(BP)A-Nkx3.2 on ED3 and harvested on ED10. Average muscle mass from uninfected contralateral control, RCAS-Nkx3.2 injected, and Nkx3.2/Ihh co-infected limbs (as in D) at ED10 were plotted (uninfected vs. Nkx3.2 single infection; for ED10 harvest whole limb * is $p \leq 0.05$; for muscle * is $p \leq 0.01$; $n = 3 \pm$ SEM).

has been restored (Fig. 4C). When quantified, Nkx3.2/Ihh co-infection resulted in significant increases in whole limb mass of 58% and muscle mass of 65% when compared to the singly infected Nkx3.2 contralateral limb (Fig. 4D). In order to further assess the ability of RCAS-Ihh to rescue loss of muscle mediated by forced Nkx3.2 expression, we compared increases in whole limb and muscle masses of Nkx3.2/Ihh co-infected limbs to an uninfected control. Consistent with the prior data (Fig. 3), RCAS-Nkx3.2 injection on ED3 with harvest on ED10 significantly decreased whole limb (Supplemental Fig. 4) and muscle masses (Fig. 4E). Conversely, Nkx3.2/Ihh co-infection significantly increased whole limb (Supplemental Fig. 4) and muscle masses when compared to an uninfected ED10 limb (Fig. 4E). Co-infection of an empty control virus with Nkx3.2 did not rescue the loss in muscle mass, further demonstrating that the rescue of muscle mass is specific to Ihh (data not shown).

Ihh promotes myoblast cell survival

Our data support the hypothesis that Ihh regulates long bone development concomitantly with secondary myogenesis. Ihh regulation of secondary myogenesis could be mediated via several mechanisms including promotion of fetal myoblast proliferation, enhanced survival, or promotion of terminal differentiation. Alternatively, Ihh null muscle may be deficient in muscle regulatory factor expression effectively blocking progression through myogenesis. To better understand the mechanisms involved, we quantified expression of Myf5, MyoD, and

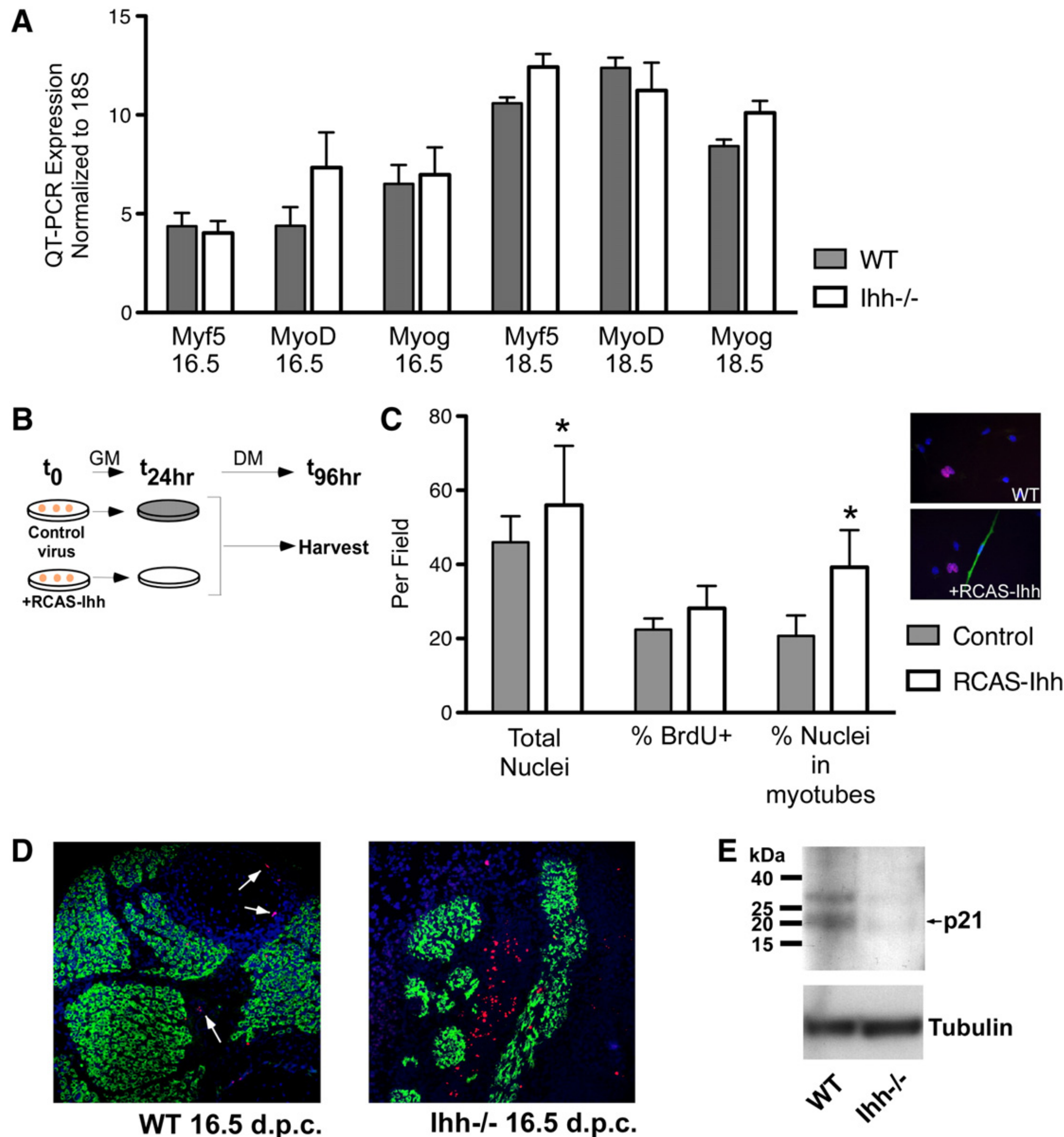

Fig. 5. Ihh-mediated signaling promotes myoblast survival. (A) Quantitative PCR analysis was performed on cDNA from wild type littermates and Ihh null hindlimbs harvested on ED16.5 or ED18.5. Muscle regulatory factor (MRF) expression levels were normalized to 18S rRNA and plotted as the average ± SEM. (n ≥ 3 embryos for each timepoint) (B) Schematic of experiment: wild type chick myoblasts explanted at ED12 were either infected with RCAS(BP)A-Ihh or RCAN control virus. After 24 h in culture, cells were switched to differentiation medium and maintained for an additional 72 h. Prior to fixation, cells were pulsed with BrdU for 1 h and then stained with antibodies to BrdU and MyHC to detect proliferating and differentiated cells, respectively. (C) The data were quantified and the average number of total nuclei, % BrdU+, and % of nuclei in myotubes ± SEMs were plotted per field (30 fields scored per condition; 3 independent experiments; for total nuclei $p \leq 0.05$; for % of nuclei in myotubes $p \leq 0.01$). Inset micrographs were stained with anti-BrdU in red, anti-MyHC in green and DAPI. (D) Hindlimb cross sections obtained from ED16.5 wild type and $Ihh^{-/-}$ mouse embryos were fixed, and subjected to TUNEL (red) to detect apoptotic cells, and stained for MyHC to detect differentiated muscle (green). Arrows in wild type sections indicate the small number of TUNEL positive cells. (E) Muscle tissue protein extracts were obtained from ED16.5 wild type and $Ihh^{-/-}$ hindlimb muscle, separated by SDS-PAGE and Western blotted. p21 protein was detected in wild type but reduced in Ihh null extracts. Alpha-tubulin was used as a loading control.

Myogenin at ED16.5 and ED18.5 in Ihh null embryos. Surprisingly, neither Myf5, MyoD nor Myogenin is deficient in Ihh null muscle when compared to wild type littermates (Fig. 5A).

Since loss of Ihh expression does not alter myogenic transcription factor expression, we further explored the mechanisms responsible for the effects of exogenous Ihh by examining fetal myoblast proliferation and differentiation. If Ihh acts as a classic mitogen, such as EGF, or a repressor of myogenesis similar to the FGFs or Shh (Bren-Mattison and Olwin, 2002; Clegg et al., 1987; Rheinwald and Green, 1977), then exogenous Ihh should either promote proliferation or inhibit myogenesis in fetal myoblasts under conditions that would normally promote cell cycle exit and differentiation. Chick fetal myoblasts were explanted, infected with RCAS(BP)A-Ihh or RCAN control virus in growth medium for 24 h and then switched to differentiation medium for an additional 48 h (Fig. 5B). Prior to fixation, the cells were pulsed for 1 h with BrdU. The cultures were then scored for the total number of nuclei per field, percentage of BrdU+ cells and percent nuclei in myotubes positive for MyHC. We observed no significant difference in the percentage of BrdU+ cells (Fig. 5C); therefore, we conclude that Ihh does not act as a fetal myoblast mitogen. Interestingly, infection with RCAS-Ihh promoted differentiation without a corresponding decrease in the total number of nuclei per field, which would be expected if Ihh promoted cell cycle exit (Fig. 5C). Since Hedgehogs are known to function as survival factors in other developing systems (Ahlgren and Bronner-Fraser, 1999; Varjosalo and Taipale, 2008), these data are consistent with Ihh promoting survival of fetal myoblasts undergoing differentiation. It is well established that myoblasts undergoing differentiation exhibit higher rates of apoptosis and that survival factors enhance muscle differentiation (Stewart and Rotwein, 1996; Wang and Walsh, 1996).

If Ihh functions to enhance fetal myoblast survival, loss of muscle tissue in Ihh null mice could be due to increased cell death. TUNEL assays performed on ED16.5 wild type and $Ihh^{-/-}$ muscle tissue show virtually undetectable levels of TUNEL reactivity in wild type muscle (Fig. 5D). In contrast, a high level of TUNEL activity was observed in $Ihh^{-/-}$ muscle tissue (Fig. 5D). To further probe the mechanisms involved, we assayed for the presence of p21 in muscle tissue undergoing secondary myogenesis. Upregulation of p21 is critical for normal myoblast cell-cycle exit (Guo et al., 1995; Halevy et al., 1995; Wang and Walsh, 1996) and confers resistance to apoptosis in myoblasts committed to differentiate (Fujio et al., 1999; Lawlor and Rotwein, 2000; Ostrovsky and Bengal, 2003; Wang and Walsh, 1996). When probed for p21 protein, Western blots of extracts from ED16.5 $Ihh^{-/-}$ muscle tissue reveal a dramatic difference in the level of p21. While p21 is readily detectable in wild type skeletal muscle, it is virtually undetectable in Ihh null muscle tissue (Fig. 5E). Thus, Ihh signaling appears to promote survival of fetal myoblasts undergoing terminal differentiation during secondary myogenesis.

Discussion

Vertebrate limb musculature develops in close proximity and concurrently with bone. Bone can develop normally in the absence of muscle, since muscleless mouse mutants exhibit no obvious hindlimb skeletal defects (Nowlan et al., 2010). Since bone is necessary for insertion of skeletal muscle tendons and acts as a scaffold for muscle development (Marieb, 1998), it seems unlikely that muscle could develop appropriately in the absence of bone. Signals directing muscle patterning are normal in the absence of myoblasts (Kardon et al., 2003), implying that muscle development is influenced by the surrounding tissues. Here, we present evidence demonstrating that Ihh, a skeletal-derived factor, is involved in skeletal muscle development. We propose that Ihh is required for survival of the fetal myoblast population; and thus, Ihh appears to coordinate outgrowth of the skeleton with growth of the skeletal musculature.

Requirement of Ihh for secondary but not primary myogenesis

Loss of Shh dramatically affects skeletal muscle development during primary myogenesis (Bren-Mattison and Olwin, 2002; Duprez et al., 1998; Kruger et al., 2001); and inhibition of Shh affects muscle development as early as ED5 in the chicken embryo (Bren-Mattison and Olwin, 2002). Conversely, inhibition or loss of Ihh does not detectably alter muscle development until ED10–12 in the chicken and ED16.5 in mouse, indicating that Shh and Ihh act on temporally distinct myogenic cell populations. Consistent with this idea is the observation that secondary but not primary myogenesis appears affected in $Ihh^{-/-}$ mice and is supported by the following experimental observations: myofiber diameters in $Ihh^{-/-}$ cross sections are significantly larger than myofiber diameters of wild type mice; and smaller, secondary myofibers, readily visible in wild type sections, are rare or absent in sections from $Ihh^{-/-}$ embryos. Additionally, Ihh null myoblasts appear to have a cell autonomous defect as explanted myoblasts from $Ihh^{-/-}$ ED20.5 mouse embryos form myotubes possessing few nuclei.

Ihh regulates muscle mass independently from bone length

Ihh regulates long bone length through inhibition of the rate of chondrocyte differentiation (Vortkamp et al., 1996); and loss of Ihh results in severe shortening of long bones (St-Jacques et al., 1999). However, our analysis of the Ihh null mouse could not address whether loss of muscle was an indirect consequence of shorter limbs. Consequently, we used retroviruses to either augment or inhibit Ihh expression in the developing chicken hindlimb. To accomplish this, Nkx 3.2 was ectopically overexpressed in the developing limb bud. Although artificial, this allowed a reduction rather than elimination of Ihh to further test the requirement of Ihh in muscle development without the confounding effects on bone development. Thus, reduction of Ihh expression in chick hindlimb caused a 6% decrease in long bone length with a concurrent 30% decrease in muscle weight. The 6% decrease in bone length was much less severe than the 65% decrease in long bone length reported for the Ihh null mouse (St-Jacques et al., 1999) Ihh overexpression significantly increased muscle mass without affecting long bone length. These data and the observations that Ptch1 is expressed in developing muscle and in fetal myoblasts, suggest that Ihh regulates muscle mass independently of bone length and that long-range Ihh signaling is involved as the primary source of Ihh is the perichondrium (Ayers et al., 2010; Etheridge et al., 2010; Yan et al., 2010). Long-range Ihh signaling is dependent on heparan sulfate proteoglycans (Ayers et al., 2010; Yan et al., 2010), which are also involved in skeletal muscle development and regeneration (Casar et al., 2004; Cornelison et al., 2001; Cornelison et al., 2004; Olguin et al., 2003; Olguin and Brandan, 2001). These data and the observations that (i) Ptch1 is expressed in developing muscle, (ii) Ptch1 mRNA is in fetal myoblasts, (iii) Ptch1 protein is present in fetal proliferating myoblasts and committed myocytes, and (iv) fetal myoblasts are Ihh responsive support a direct role for Ihh signaling in skeletal muscle development.

Cellular signaling mediated by Ihh

Skeletal muscle development requires the coordinated effort of four muscle transcription factors where the severe loss of muscle in the $Ihh^{-/-}$ mice could be due to alterations in the timing of muscle transcription factor expression or in their expression levels. We observed normal levels and timing of Myf5, MyoD and Myogenin expression in Ihh null hindlimb muscle, indicating that myogenic specification and early stages of differentiation appear normal. Addition of Ihh to wild type fetal myoblast cultures significantly increases differentiated nuclei without a corresponding decrease in total nuclei, suggesting that Ihh enhances the survival of myoblasts committed to differentiate rather than promoting cell cycle exit and terminal differentiation. A similar role has been well

documented for p21, a cyclin dependent kinase inhibitor. Loss of p21 from skeletal muscle myoblasts increases apoptosis only in myoblasts committed to terminal differentiation, thereby reducing the number of differentiated nuclei (Wang and Walsh, 1996). These data support a model whereby Ihh is required for survival of fetal myoblasts undergoing terminal differentiation. Loss of Ihh may result in apoptosis of differentiating myoblasts which in turn would reduce the number myofibers in developing muscle tissue, resulting in catastrophic muscle loss. In agreement with this idea, we observe a dramatic increase in TUNEL staining of $Ihh^{-/-}$ hindlimb sections in regions where muscle is present in wild type littermates. Furthermore, our data support a direct role for Ihh in fetal myoblast survival since Ihh directly affects fetal myoblasts in culture, and Patched 1 protein, a direct readout of Ihh signaling, is present in wild type but not Ihh null muscle tissue. Loss of Ihh in myoblasts may induce apoptosis since the Patched 1 receptor in the absence of ligand can function as a dependence receptor (Thibert et al., 2003). In addition, the loss of Ihh appears to affect p21 expression in muscle tissue, enhancing apoptosis of differentiating cells which in turn results in reduction of differentiated muscle. In summary, our data provide evidence supporting a role for Ihh in regulating skeletal muscle development during secondary myogenesis where Ihh appears to maintain and promote the survival of fetal myoblasts undergoing myogenic differentiation.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.06.002.

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