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Bmp Signaling at the Tips of Skeletal Muscles Regulates the Number of Fetal Muscle Progenitors and Satellite Cells during Development

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SUMMARY

Muscle progenitors, labeled by the transcription factor Pax7, are responsible for muscle growth during development. The signals that regulate the muscle progenitor number during myogenesis are unknown. We show, through in vivo analysis, that Bmp signaling is involved in regulating fetal skeletal muscle growth. Ectopic activation of Bmp signaling in chick limbs increases the number of fetal muscle progenitors and fibers, while blocking Bmp signaling reduces their numbers, ultimately leading to small muscles. The Bmp effect that we observed during fetal myogenesis is diametrically opposed to that previously observed during embryonic myogenesis and that deduced from in vitro work. We also show that Bmp signaling regulates the number of satellite cells during development. Finally, we demonstrate that Bmp signaling is active in a subpopulation of fetal progenitors and satellite cells at the extremities of muscles. Overall, our results show that Bmp signaling plays differential roles in embryonic and fetal myogenesis.

INTRODUCTION

Skeletal muscle development, growth, and regeneration rely on muscle stem cells. An important goal is to understand the source and the nature of the signals regulating these muscle stem cells during myogenesis.

During vertebrate development, the successive phases of primary (embryonic), secondary (fetal), and postnatal (adult) myogenesis leading to the formation of skeletal muscles involve different muscle progenitor populations. Primary myogenesis is the formation of the first multinucleated muscle fibers from embryonic progenitors, which differentiate by fusing with each other. This will establish the scaffold of muscles. This phase is usually considered to take until Embryonic Day (E) 6 in the chick (Stockdale, 1992) and E14.5 in the mouse (Biressi et al., 2007a). Secondary myogenesis depends upon fusion of fetal progenitors, which give rise to secondary fibers. Secondary myogenesis is important for the growth and maturation (fiber type) of muscles during embryonic development. During the perinatal period, there is considerable growth of muscle, mediated by adult muscle progenitors—the satellite cells. During all adult life, satellite cells reside around the muscle fibers in a quiescent state, and are solicited for muscle homeostasis, hypertrophy, and regeneration. Embryonic, fetal, and adult progenitors differ in their in vitro characteristics (Biressi et al., 2007a; Stockdale, 1992). In vivo, they will generate primary, secondary, and adult fibers, respectively, which differ in their morphology, myosin heavy chain isoforms, and muscle genes that they express (Biressi et al., 2007a; Stockdale, 1992). Over time, these muscle progenitors have distinct genetic requirements (Hutcheson et al., 2009; Lepper et al., 2009). However, despite this heterogeneity over time, a pool of resident progenitors is maintained in developing muscles during embryonic development and postnatal growth. The paired-box transcription factors, *Pax3* and *Pax7*, define this progenitor cell population during all the stages of skeletal muscle formation (Hutcheson et al., 2009; Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Schienda et al., 2006). In the absence of both *Pax3* and *Pax7*, muscle development is arrested (Relaix et al., 2005). *Pax3* defines the embryonic myoblast population, and is required for its formation, while *Pax7* labels fetal and adult myoblasts, and is required for their formation (Hutcheson et al., 2009; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). However, *Pax3* and *Pax7* function is dispensable for adult muscle regeneration (Lepper et al., 2009).

The signals regulating the pool of muscle progenitors during embryonic, fetal, and perinatal myogenesis have not been clearly identified. Classical signaling pathways, such as the Notch and Wnt pathways, were thought to be involved in this process. Notch signaling is involved in the maintenance of embryonic and fetal muscle progenitors and in the generation of satellite cells during mouse embryogenesis (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). Components of the canonical and noncanonical Wnt pathways have been implicated in the proliferation of embryonic muscle progenitors in chick somites (Galli et al., 2004), in the maintenance of fetal muscle progenitors in mouse limbs (Hutcheson et al., 2009), and in the expansion of satellite cells (Le Grand et al., 2009; Otto et al., 2008; Perez-Ruiz et al., 2008). However, modification of Wnt signaling has provided conflicting results concerning muscle differentiation, with various manipulations of Wnt signaling reported to both inhibit and promote myogenic differentiation in vitro (Gavard et al., 2004; Goichberg et al., 2001; Kim et al., 2008; Perez-Ruiz et al., 2008) and in vivo (Anakwe et al., 2003; Hutcheson et al., 2009).

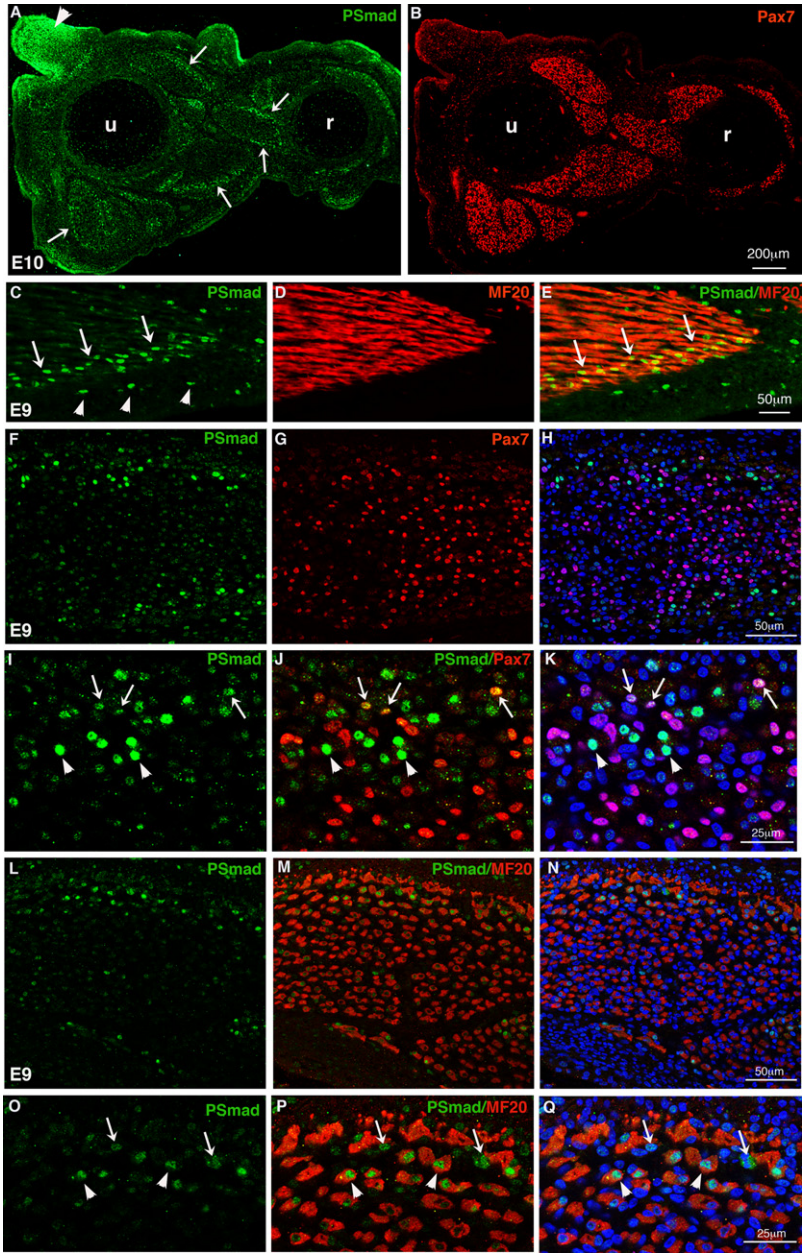

Figure 1. Bmp Signaling Is Active at the Tendon/Muscle Interface

(A and B) Transverse E10 limb sections were simultaneously incubated with the PSmad antibody showing active Bmp signaling (green) and the Pax7 antibody (red) revealing the muscles. Arrows point to PSmad expression at the muscle borders, and the arrowhead shows active Bmp signaling in a feather bud.

(C–E) Longitudinal E9 limb sections were simultaneously incubated with the PSmad antibody showing active Bmp signaling (green) and the MF20 antibody (red) revealing the muscles. Arrows point to the PSmad$^+$ cells in muscles, and arrowheads indicate to the PSmad$^+$ cells in tendon regions.

(F–Q) Forelimbs of E9 chick embryos were cut transversely and incubated with either PSmad and Pax7 antibodies (F–K) or PSmad and MF20 antibodies (L–Q).

(I–K) are higher magnifications of (F–H). Arrows point to the Pax7$^+$ cells displaying active Bmp signaling, while arrowheads indicate PSmad$^+$ cells not expressing Pax7.

(L–N) Lower magnifications of (O)–(Q).

(O–Q) Arrowheads indicate the PSmad expression in MF20$^+$ fibers at the borders of muscles, while arrows indicate PSmad$^+$ cells outside MF20$^+$ fibers.

(H, K, N, and Q) The merged pictures of (F) and (G), (I) and (J), (L) and (M), and (O) and (P), respectively, combined with Hoechst labeling. r, radius; u, ulna.

Bmps (bone morphogenetic proteins) constitute a subgroup of the transforming growth factor (TGF)-β super family, the members of which act through heteromeric complex of serine/threonine kinase receptors. Smad1, Smad5, and Smad8 (referred as Smad1/5/8) are the specific intracellular transducers of Bmp ligands. Upon Bmp stimulation, Smad1/5/8 are phosphorylated by Bmp-activated receptors, associate with Smad4, and translocate into the nucleus to regulate gene expression (Massague, 2008; Nohe et al., 2004). Bmps are usually considered potent inhibitors of embryonic, fetal, and adult muscle differentiation (Amthor et al., 1998; Biressi et al., 2007b; Dahlqvist et al., 2003; Frank et al., 2006; Tzahor et al., 2003), although application of low levels of Bmps upregulates *Pax3* expression during primary myogenesis in early chick limbs and somites (Amthor et al., 1998, 1999). The in vivo role of Bmp signaling on fetal muscle growth and satellite cell formation during development has never been addressed.

In the present study, we show that Bmp gain-of-function experiments increased the number of fetal muscle progenitors and satellite cells. The increase in fetal progenitor number was accompanied by an increase in the number of fetal muscle fibers. Conversely, blocking of Bmp signaling led to the opposite phenotype (i.e., a global diminution in the number of fetal progenitors and of satellite cells, ultimately leading to small muscles). We have identified a subpopulation of Pax7$^+$ cells responding to Bmp signaling at the extremities of the muscles close to the tendons, which produced Bmp4. We conclude that active Bmp signaling at the tips of muscles regulates the correct number of fetal progenitors and satellite cells during myogenesis.

RESULTS

Active Bmp Signaling Is Increased at the Muscle-Tendon Interface

In order to define the cells in which Bmp signaling was active, we used an antibody that detected phosphorylated Smad1/5/8 (referred as PSmad), reflecting active Bmp signaling pathway (Faure et al., 2002). In chick limbs, this PSmad antibody labeled the ectodermal buds (Figure 1A, arrowhead), consistent with the presence of *Bmp4* transcripts in this tissue (see Figure S1A [arrowhead] available online). In E10 muscles, Bmp activity

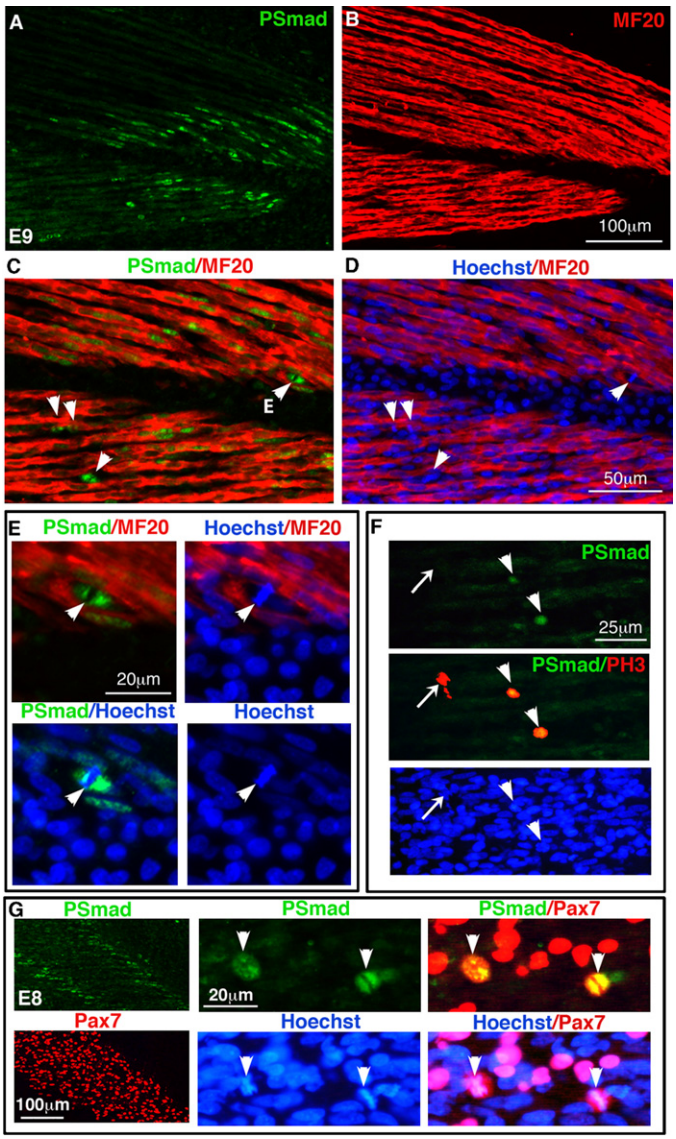

Figure 2. Bmp Signaling and Cell Division

(A and B) Forelimbs of E9 chick embryos were cut longitudinally and then incubated with the PSmad and MF20 antibodies.

(C and D) is a larger view of the muscle extremities displaying intense Bmp activity. Arrowheads in (C) and (D) point to PSmad$^+$ cells displaying a metaphasic state, indicating a mitotic figure.

(E) A higher magnification of a PSmad$^+$ cell displaying a metaphasic state.

(F) View of the muscle tips from longitudinal sections showing PH3$^+$ cells that are PSmad positive (arrowheads) or PSmad negative (arrow).

(G) Forelimbs of E8 chick embryos were cut longitudinally and then incubated with the PSmad and Pax7 antibodies. High magnification of the muscle tips shows PSmad$^+$ and Pax7$^+$ cells displaying mitotic figures. Arrowheads indicate two PSmad$^+$ and Pax7$^+$ cells being in a metaphasic state.

displayed a restricted location (Figures 1A and 1B, arrows), close to the *Bmp4* expression in tendons (Figures S1A and S1B). Longitudinal sections along the muscle axis confirmed the preferential location of Bmp-responsive cells at the muscle extremities (Figures 1C–1E, arrows), close to the tendons, where Bmp4 is produced (Figures S1C and S1D). In order to define which muscle cell types were responsive to Bmp signaling, we compared PSmad location at the tips of muscles with that of Pax7, expressed in fetal muscle progenitors and with that of myosin heavy chains, exclusively expressed in multinucleated muscle fibers. We clearly observed PSmad in Pax7$^+$ cells (Figures 1F–1K, arrows), showing that a subset of Pax7-positive cells located near the muscle extremities was responsive to Bmp signaling. We also observed PSmad inside the MF20$^+$ fibers, close to the muscle extremities (Figures 1L–1Q, arrowheads). We conclude that Bmp signaling is active at the muscle-tendon interface, and that a subpopulation of fetal muscle progenitors close to the muscle extremities is responsive to Bmp signaling.

Bmp Signaling and Cell Proliferation

We next analyzed the proliferating state of the cells displaying active Bmp signaling. Analysis of longitudinal sections along the muscle axis highlighted PSmad$^+$ cells outside the muscle fibers (at the tips of muscle), which were displaying mitotic figures (Figures 2A–2E, arrowheads), indicating that these PSmad$^+$ cells were dividing. Consistently, PSmad$^+$ cells at the muscle extremities were positive for the proliferating marker, phospho histone H3 (PH3) (Figure 2F). Finally, these dividing PSmad$^+$ cells at the tips of muscles were also Pax7$^+$ (Figure 2G). Since analysis was performed on sections, it was impossible to obtain significant data about whether the Pax7$^+$ cells were proliferating more at the tips of muscle than in other muscle regions. We conclude that the muscle progenitors displaying active Bmp signaling pathway at the tips of muscles proliferate.

Activation of Bmp Signaling Increases the Number of Fetal Muscle Progenitors and Fibers

In order to define the role of Bmp signaling in fetal muscle growth, we modified Bmp signaling with the replicative RCAS virus in chick embryonic limbs (Duprez et al., 1996a). In order to avoid the Bmp effect on cartilage (Duprez et al., 1996a, 1996b), grafts were performed in the dorsal aspects of E5 wings. By performing such grafts, the virus progressively spread to infect the dorsal mesenchymal tissues, including muscles, tendons, vessels, and connective tissues, but spared the cartilage elements and the ventral limb regions until E9 (Figures S2A–S2D). These types of grafts specifically targeted secondary myogenesis, as opposed to primary myogenesis. Grafts of control cells did not modify limb muscle formation (Figures S2E–S2M). Ectopic Bmp4 activity in dorsal limb regions altered fetal muscle formation (Figure 3). PSmad expression was clearly increased in the *mBmp4*-infected regions of experimental limbs compared to the normal restricted expression of PSmad at the borders of muscles in control limbs (Figures 3A–3C). Following Bmp activation, 90% of the Pax7$^+$ cells expressed PSmad, while only 15% of the Pax7$^+$ cells were PSmad$^+$ in normal conditions (Figures 3D–3J). This demonstrated that Pax7$^+$ cells were directly responding to the ectopic activation of Bmp signaling following Bmp4 overexpression. Following Bmp signaling activation, the number of Pax7$^+$ cells displayed a two-fold increase compared to control muscles (Figures 3D, 3G, and 3K–3M). Consistently, the number of

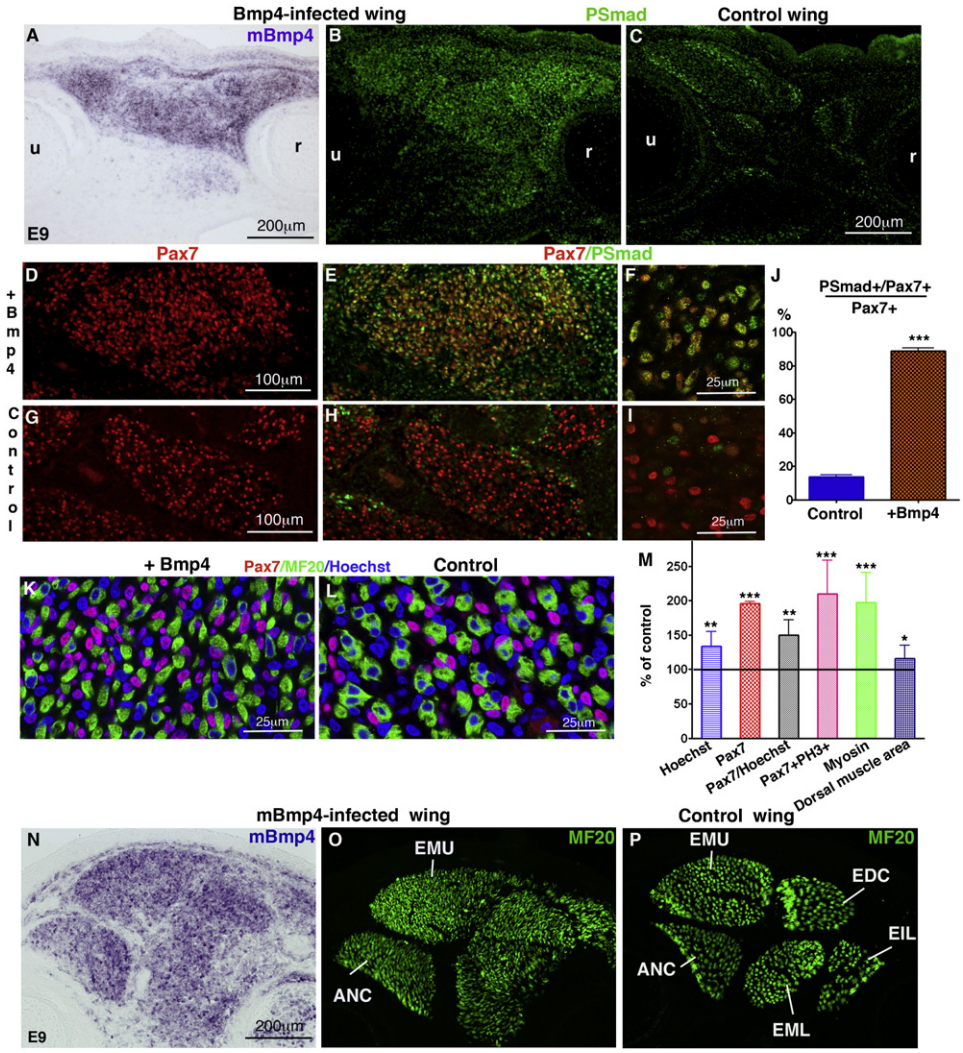

Figure 3. Ectopic Bmp Signaling Increases the Number of Fetal Muscle Progenitors and Fibers

Bmp4-grafted (A, B, D–F, K, N, and O) and controlateral (C, G, H, I, L, and P) forelimbs from E9 chick embryos were cut transversely and analyzed for activated Smad1/5/8 and muscle markers by immunohistochemistry. (A–C) Adjacent sections of experimental and control wings were hybridized with *mBmp4* probe (A) and incubated with the PSmad antibody (B and C). Ectopic activation of Bmp signaling increased the number of $Pax7^+$ cells in dorsal limb regions (D versus G). Following Bmp4 exposure, 90% of $Pax7^+$ cells were $PSmad^+$ (E, F, and J), while 15% of $Pax7^+$ cells were $PSmad^+$ in normal conditions (H–J). The number of $MF20^+$ fibers following Bmp4 treatment was also increased (K–M). Histograms are expressed as means and standard error of the mean (SEM). Analysis of the dorsal muscle areas indicated a global increase of 15% of dorsal muscle surface area in Bmp4-treated muscles compared with control muscles (M–P). Asterisks in the histograms indicate the different p values: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. ANC, anconeus; EDC, extensor digitorum communis; EIL, extensor incidis longus; EML, extensor medius longus; EMU, extensor metacarpi ulnans; r, radius; u, ulna. Dorsal is at the top.

$Pax7^+$ cells expressing PH3 displayed a two-fold activation (Figure 3M). This increase in the number of $Pax7^+$ cells was clearly accompanied by an increase in the number of MF20-positive cells (Figures 3K and 3L). The *Bmp4*-treated muscles displayed smaller $MF20^+$ fibers compared with the control fibers (Figures 3K–3M), consistent with the notion that Bmp4 increases the number of fetal muscle fibers, which are smaller than embryonic fibers (Biressi et al., 2007a; Stockdale, 1992). Moreover, the global area of dorsal muscles, visualized with the MF20 antibody, was slightly but significantly increased following Bmp4 activation compared with the control limbs (Figures 3M–3P).

Since, upon Bmp signal, the expression of the Bmp receptor, BmpR-IA was induced (Figures S1E–S1H), we asked whether a constitutively active form of BmpR-IA (caBmpRIA) could mediate the Bmp4 effect on muscle progenitors. Ectopic expression of caBmpRIA (Zou et al., 1997) increased the number of $Pax7^+$ progenitors and fibers in dorsal infected areas compared with control limbs (Figure S3). The similar effect on fetal muscle progenitors and fibers observed with Bmp4 and caBmpRIA indicates that Bmp4 is acting via BmpR-IA to regulate secondary myogenesis.

Secondary myogenesis is marked by fiber type differentiation (Duprez et al., 1999; Kardon et al., 2002). We compared slow muscle differentiation with active Bmp signaling in normal and experimental conditions. We did not observe any obvious correlation between active Bmp signaling and slow muscle

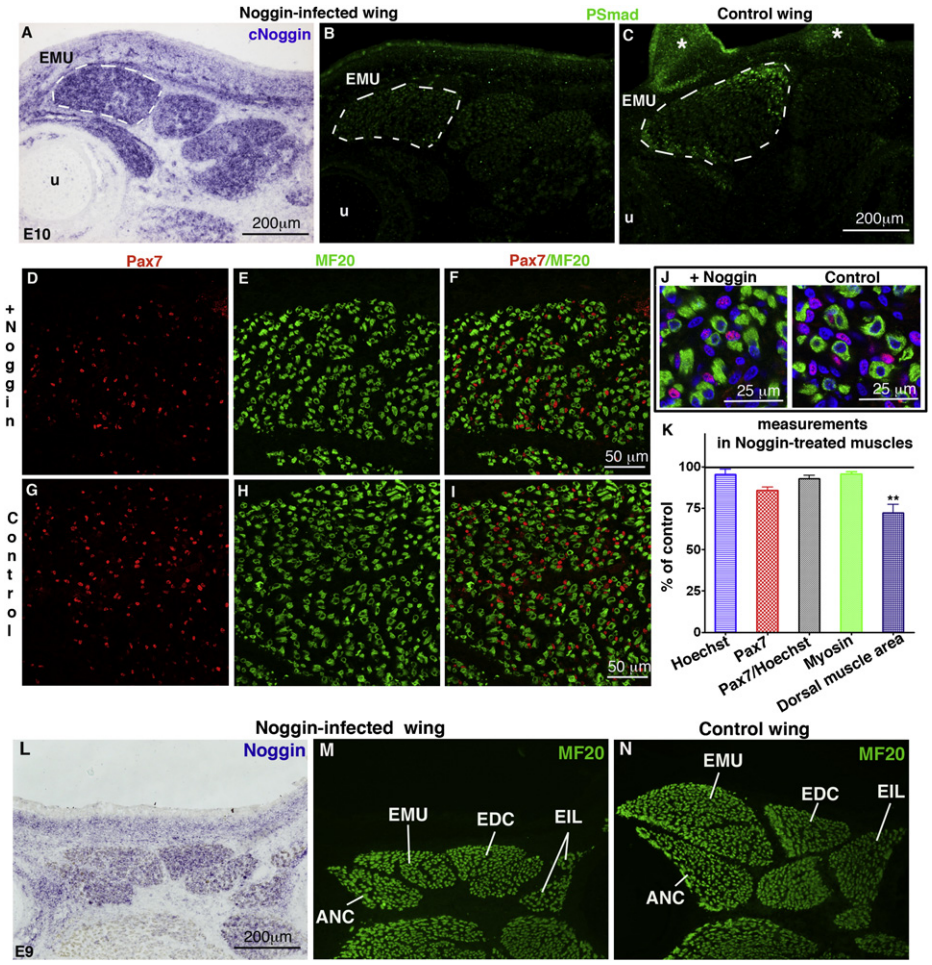

Figure 4. Blocking Bmp Signaling Diminishes the Number of Fetal Muscle Progenitors and Fibers

Noggin-grafted (A, B, D–F, J, L, and M) and controlateral (C, G, H, I, J, and N) forelimbs of E9/E10 chick embryos were cut transversely and analyzed for activated-Smad1/5/8 and muscle markers. (A and B) Adjacent sections of experimental right wings were hybridized with chick *Noggin* probe (A) and incubated with the PSmad antibody (B) in order to observe the inhibition of Bmp signaling in dorsal limb muscles expressing *Noggin*. (A–C) Following Noggin overexpression, Bmp activity is clearly downregulated in a dorsal and posterior muscle, the EMU, compared to normal condition. The noggin-infected muscles appear smaller compared to the control muscles (B, C, K, L–N), while the density of the $Pax7^+$ cells appears only slightly modified (D–K). Asterisks in (C) indicate the feather buds. Dorsal is to the top. Histograms are expressed as means and standard error of the mean (SEM). $^{**}p < 0.01$.

differentiation in normal conditions or after ectopic Bmp activation (Figures S4), suggesting that Bmp signaling does not promote slow differentiation.

From all the Bmp gain-of-function experiments, we conclude that Bmp signaling is sufficient to increase the number of fetal muscle progenitors and fibers.

Noggin-Mediated Blocking of Bmp Signaling Reduced the Global Number of Fetal Muscle Progenitors and Fibers

In order to analyze the consequences of the absence of Bmp signaling for limb fetal muscle formation, we misexpressed Noggin, known to be a potent inhibitor of Bmp signaling (Groppe et al., 2002; Pizette and Niswander, 2000), with the RCAS virus. Blocking of Bmp signaling following Noggin overexpression led to a disappearance of PSmad expression in Noggin-infected regions compared with the restricted expression of PSmad in control limbs (Figures 4A–4C), showing that Noggin-treated muscles were not able to respond to any Bmp signal. Consistent with the Bmp involvement in feather formation (Lin et al., 2006), the E10 Noggin-infected limbs displayed a total absence of feather buds compared with control limbs (Figures 4A–4C). The inhibition of Bmp signaling by Noggin led to small muscles compared with the control limbs (Figures 4B, 4C, 4M, and 4N). Conversely to the positive Bmp effect on $Pax7^+$ cells, we observed a slight diminution in the number of $Pax7^+$ progenitors compared with the control muscles (Figures 4D–4I). Normalized to area, this diminution was modest (Figure 4K). There was no change in the density of differentiated cells, and no obvious change in fiber morphology in E10 Noggin-treated muscles (Figures 4J and 4K). Combined with the clear global diminution of muscle size (Figures 4K–4N), we conclude that blocking Bmp signal in embryonic muscles led to a global diminution in the number of fetal muscle progenitors and fibers during secondary myogenesis.

Bmp Signaling and Tendon Markers

Muscle and tendon interactions occur during fetal myogenesis (Edom-Vovard and Duprez, 2004). Since *Bmp4* transcripts were located in tendon cells (Figures S1A–S1D), and active Bmp signaling was also observed in tendon regions in addition to muscles extremities (Figure 1C, arrowheads), we analyzed the consequences of Bmp misexpression for tendon markers. The expression of the tendon marker, *Scleraxis*, was not modified 2 days after activation or blocking of Bmp signaling, while the increase and diminution of muscle differentiation started to be observed in E7 Bmp- and Noggin-treated limbs, respectively (Figures S5A and S5B). At 4 days after grafting, ectopic activation of Bmp signaling did not modify the tendon pattern visualized with *Scleraxis* and *collagen1* expression, despite the increase of muscle mass (Figures S5C and S5D). Nevertheless, we observed a diminution of the tendon marker expression in Noggin-treated limbs (Figures S5E and S5F). However, we interpreted this diminution as being the consequence of the muscle loss, since muscles are necessary for late tendon formation (Edom-Vovard et al., 2002; Kardon, 1998). We conclude that the Bmp effect on muscle is not a consequence of a tendon defect.

Bmp Signaling Regulates the Number of Satellite Cells during Muscle Development

We next analyzed whether the modification in the number of muscle progenitors following Bmp misexpression subsequently altered satellite cell formation. Satellite cells are identified by their location between the muscle fiber and the basal lamina (Mauro, 1961). During late fetal stages, $Pax7^+$ progenitor cells adopt a satellite cell position. Satellite cells are observed in E18 chick (Feldman and Stockdale, 1992; Hartley et al., 1992) and in E18.5 mouse (Relaix et al., 2005) embryos. During late fetal stages, it has been estimated that the majority of $Pax7^+$ cells are satellite cells (Hartley et al., 1992; Relaix et al., 2005). At E18, the global size of chick Bmp4-treated muscles was difficult to analyze, since the *Bmp4* virus had spread to the cartilage elements and increased cartilage formation at the expense of muscle, a phenotype previously reported (Duprez et al., 1996a, 1996b). However, in the remaining muscles, Bmp4 exposure increased the number of satellite cells by a factor three compared with control limbs (Figures 5A–5F). The number and the size of muscle fibers were difficult to quantify, since the residual Bmp4-treated muscles were rarely transverse on transverse limb sections. However, in the few muscles cut transversely, we observed a slight increase in the fiber number, and the fiber size was unchanged (Figures 5C, 5D, and 5F). Conversely, analysis of E18 limbs after Noggin exposure showed a marked diminution of muscle size (Figures 5K and 5L). Cell measurements showed a decrease of 25% in the number of $Pax7^+$ cells per unit area in Noggin-treated muscles compared with control muscles (Figures 5G–5L). The global diminution of muscle size combined with the diminution of $Pax7^+$ cell density in Noggin-treated muscles showed that blocking of Bmp signaling decreased the number of satellite cells. This decrease in satellite cell number was accompanied by a diminution in the number of muscle fibers in noggin-treated muscles compared with the control muscles (Figures 5I–5L). Finally, we observed an increase in the fiber size in noggin-treated muscles compared with control limbs (Figures 5I′, 5J′, and 5L). Interestingly, we observed active Bmp signaling in $Pax7^+$ cells at the tips of E18 muscles (Figures S6), indicating that this population of satellite cells, close to the muscle extremities, were responsive to a Bmp signal, similarly to the situation during early fetal myogenesis (E8).

We conclude that Bmp signaling is important for satellite cell formation in chick embryonic limbs.

Activated Satellite Cells in Mouse Adult Muscles Displayed Active Bmp Signaling

In E18 chick embryo, Bmp signaling was observed in a subpopulation of satellite cells close to the muscle tips (Figures S6). In order to determine which population of satellite cells displayed active Bmp signaling in postnatal muscles, we first used the mouse single myofiber system, which allows a clear visualization of adult satellite cells within their niche in vitro (Zammit, 2008; Kuang et al., 2007). In adult muscles, *Pax7* is expressed by quiescent, activated, and self-renewing satellite cells, but not by differentiated myocytes, nor by myonuclei (Collins et al., 2005; Shefer et al., 2006). PSmad expression was not observed in quiescent satellite cells in freshly isolated myofibers (T0 of culture, Figure 6A), indicating that satellite cells do not respond to Bmp signaling in resting conditions. However, PSmad was observed in all dividing clusters of $Pax7^+$ satellite cells along the isolated fibers after 3 days of culture (Figure 6B), showing that activated satellite cells displayed active Bmp signaling. Satellite cell heterogeneity is also illustrated with the orientation of cell division, which has been shown to determine the fate of the satellite cells (Kuang et al., 2007). $Pax7^+$ cells undergoing apical-basal division mainly divide asymmetrically, leading to the differentiation of one daughter cell, while $Pax7^+$ cells dividing in a planar orientation give rise to two self-renewing daughters via symmetric expansion (Le Grand et al., 2009). In chick embryonic muscles, we noticed that the PSmad-dividing cells close to the tips of muscles often (but not exclusively) displayed a division plane parallel to the axis of the fiber (Figures 2C and 2E; Figures S7). Since this observation was impossible to quantify in muscle tissues, we analyzed PSmad location during the first set of division of $Pax7^+$ cells along the isolated fiber. We observed that $Pax7^+$ cells undergoing planar and apical-basal divisions both expressed PSmad (Figures 6C and 6D). However, analysis of the regionalization of the clusters of dividing cells along the fibers showed that there were more clusters near the tips compared to the center of the fibers (Figures 6E and 6F). This observation indicated heterogeneity of the distribution of activated satellite cells along the isolated fibers. We conclude that Bmp signaling is active in all dividing $Pax7^+$ cells in isolated fibers, the only regionalization being the preferential location of the $Pax7^+$ dividing clusters close to the tips of the isolated fibers versus the middle.

Following muscle injury, $Pax7^+$ satellite cells are solicited and massively activated to regenerate the injured muscles (Zammit, 2008). We used the muscle regeneration system to determine whether active Bmp signaling was observed in activated $Pax7^+$ cells in vivo. We analyzed tibialis anterior (TA) muscles 4 days after cardiotoxin injection during the proliferative phase of satellite cells. In adult TA muscles, very few $Pax7^+$ cells were observed. These quiescent $Pax7^+$ cells were not $PSmad^+$. PSmad displayed a punctual expression in a subset of myonuclei

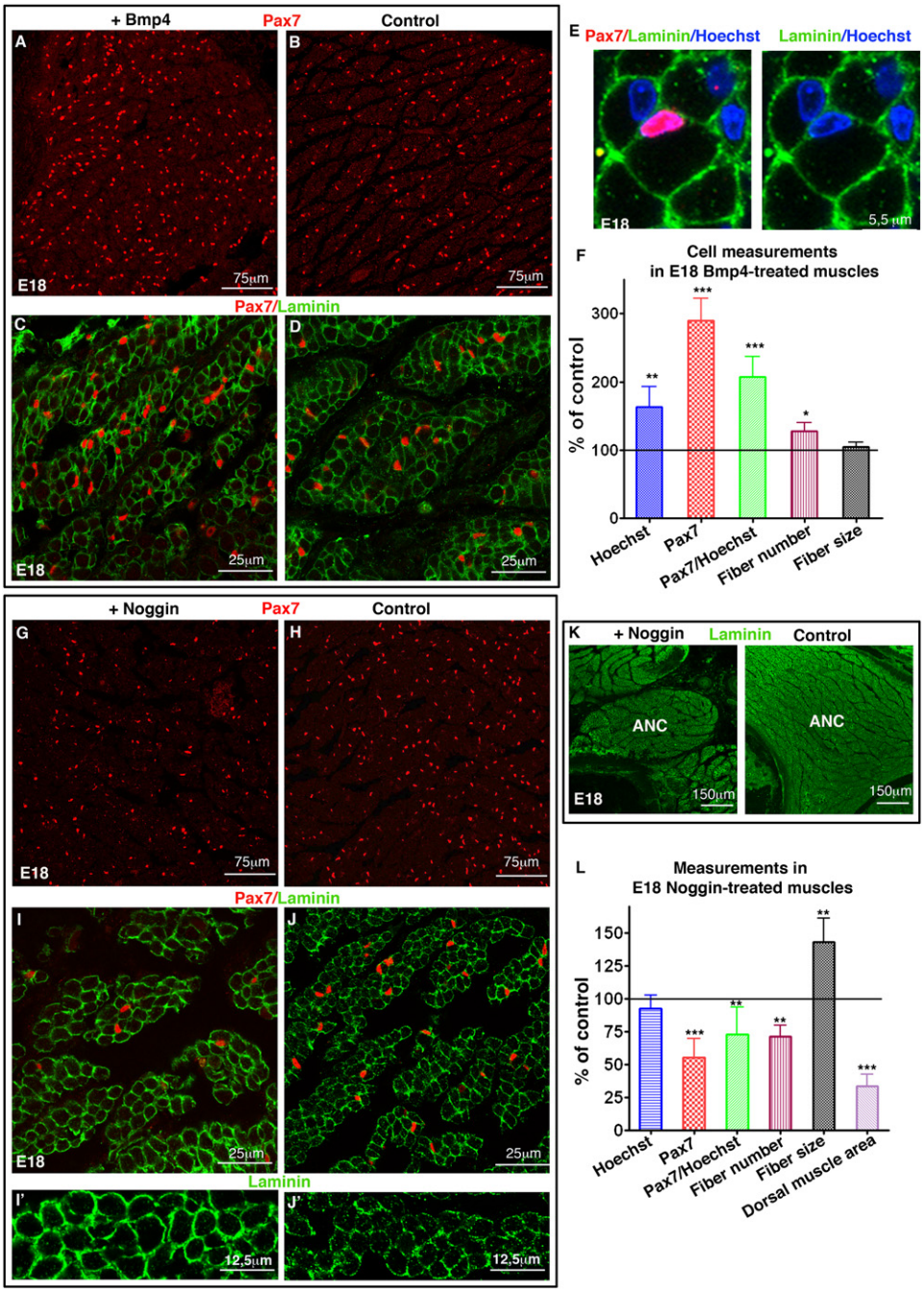

Figure 5. Misexpression of Bmp Signaling Affects the Number of Satellite Cells in the Embryo

Bmp4-grafted (A and C) and controlateral (B and D) forelimbs of E18 chick embryos were cut transversely and analyzed for Pax7 and Laminin markers by immunohistochemistry. Exposure to Bmp4 increases the number of satellite cells compared with control muscles by a factor of three (A–D and F). (E) Pax7 and Laminin labeling showing the location of the $Pax7^+$ cells beneath the basement membrane in E18 chick limb muscles. Noggin-grafted (G and I) and controlateral (H and J) forelimbs of E18 chick embryos were cut transversely and analyzed for Pax7 and Laminin markers by immunohistochemistry. Noggin-treated muscles were dramatically reduced compared with control muscles (K and L). Blocking of Bmp signaling diminished the number of satellite cells compared with control muscles by a factor of 1.5 (L). Following Noggin overexpression, the number of muscle fibers was diminished, and the Noggin-treated fibers were hypertrophic (I′, J′, and L). Histograms are expressed as means and standard error of the mean (SEM). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

(Figure 6G). In regenerating conditions, the number of $Pax7^+$ cells increased dramatically, as did the number of $PSmad^+$ cells (Figure 6H). Most of the $Pax7^+$ cells were also $PSmad^+$ (Figure 6H), showing that activated satellite cells displayed active Bmp signaling during the muscle regeneration process. In regenerating muscle, PSmad expression was also induced in myonuclei and in muscle connective tissue cells (Figure 6H and data not shown).

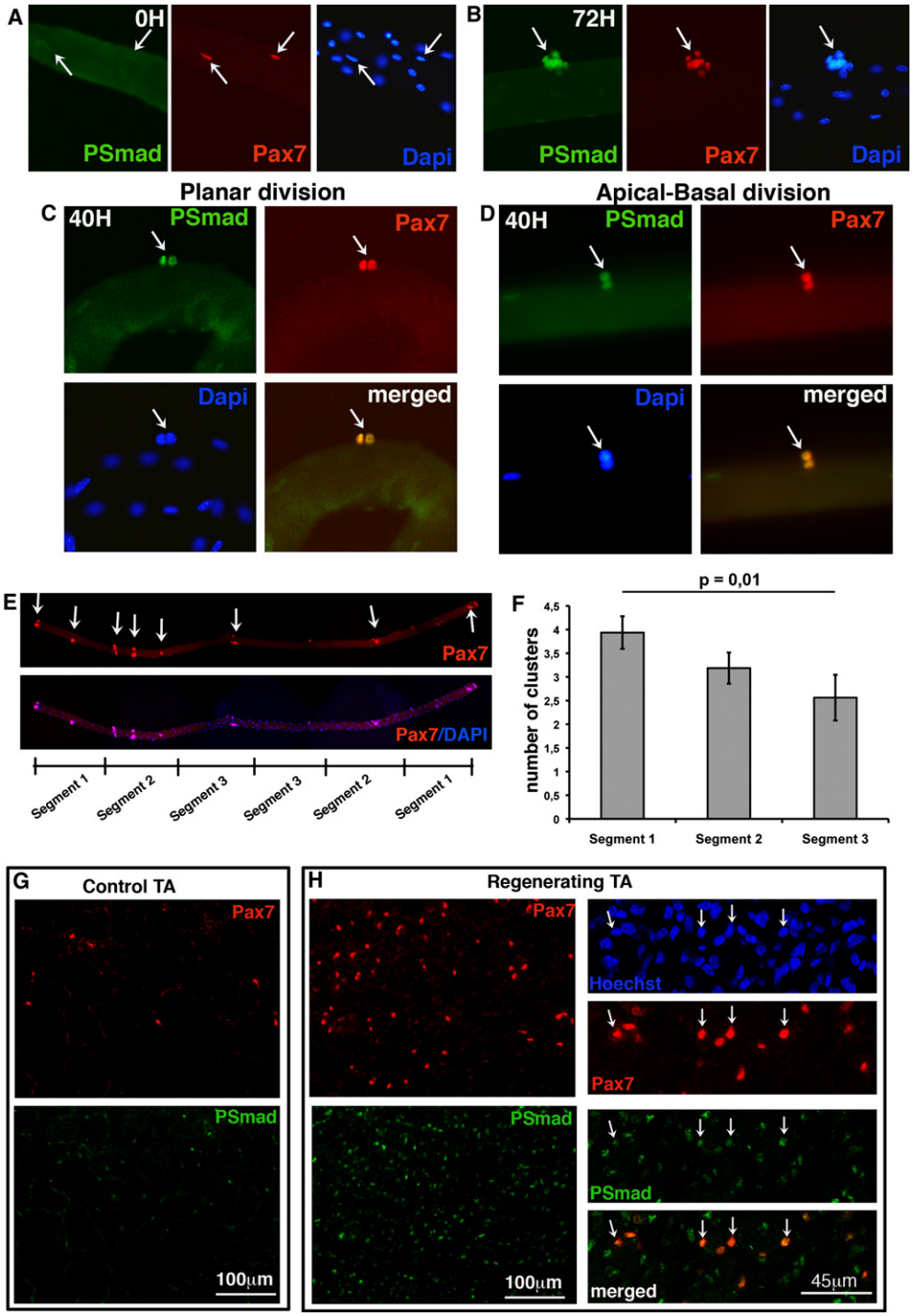

Figure 6. Proliferating Satellite Cells Displayed Active Bmp Signaling in Adult Mouse Muscle

(A–D) PSmad expression at 0 hr (A), 72 hr (B), and 40 hr (C and D) after isolation of single myofibers from EDL of 2-month-old mice. Quiescent Pax7$^+$ cells were not expressing PSmad (A), while activated Pax7$^+$ cells displayed active Bmp signaling, assayed by the expression of PSmad (B). During the first division of satellite cells, Pax7$^+$ cells undergoing planar (C) or apical-basal (D) -orientated division displayed active Bmp signaling (arrows in C and D).

(E) The distribution of activated satellite cells was analyzed along the axis of the mouse isolated fibers. After 72 hr in culture, all Pax7$^+$ cells were activated and formed clusters of activated satellite cells, all of which were PSmad$^+$ (data not shown). Counts were performed in different regions along the isolated myofibers.

(F) The segments closest to the tips displayed significantly more clusters of activated satellite cells compared with other segments.

(G and H) Pax7 (red) and PSmad (green) expression on transverse sections of control TA muscle (G) and of regenerating TA muscle (H) 4 days after cardiotoxin injury in mouse hindlimbs. In H, high magnifications show the same picture, with Hoechst, Pax7, PSmad, and a superposition of the Pax7 and PSmad labeling pattern (merged). Arrows point to examples of double Pax7$^+$/PSmad$^+$ cells in regenerating conditions. Histograms are expressed as means and standard error of the mean (SEM). The p value is 0.01.

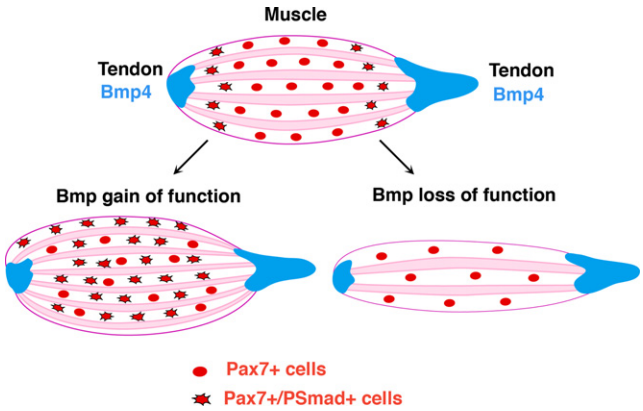

Figure 7. Schematic Representation of Bmp Function during Muscle Growth

In normal conditions, a Bmp signal produced by the tendons acts on a subpopulation of Pax7$^+$ cells located at the tips of muscle. Activation of Bmp signaling in these muscle progenitors will activate their proliferation and induce their differentiation. This will allow longitudinal muscle growth. Bmp gain-of-function experiments lead to an increase in the number of muscle progenitors and fibers, while Bmp loss-of-function experiments lead to a decrease in the number of muscle progenitors and fibers.

From these experiments, we conclude that active Bmp signaling was detected in activated/dividing Pax7$^+$ cells in adult isolated fibers and during adult regenerative myogenesis.

DISCUSSION

We have demonstrated that Bmp signaling is crucial for establishing the correct number of fetal muscle progenitors and fibers and that of satellite cells during development. In addition, we have shown that the fetal muscle progenitors and satellite cells, which are responsive to Bmp signal, are preferentially located at the tips of the muscles close to tendons, where a Bmp signal is produced.

Bmp Signaling Regulates the Number of Pax7$^+$ Fetal Progenitors Not at the Expense of Muscle Differentiation

We have demonstrated that Bmp signaling regulates the number of fetal muscle progenitors during chick limb myogenesis. Wnt and Notch signaling pathways have also been shown to be involved in regulating the number of fetal muscle progenitors in mouse embryos (Hutcheson et al., 2009; Schuster-Gossler et al., 2007; Vasyutina et al., 2007). Inhibition of Wnt or Notch signaling in the embryo has been shown to decrease the number of Pax7$^+$ cells, although the reduction appears more dramatic in the absence of Notch activity than after the blocking of β-catenin. The blockade of Notch signaling resulted in an uncontrolled myogenic differentiation of embryonic muscle progenitors, leading to a progressive depletion of the myogenic progenitor pool and to the ultimate phenotype of very small muscles (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). We believe that Bmp signaling acts on fetal muscle progenitors by a different mechanism to that of Notch, since we did not observe any obvious increase of muscle differentiation following Noggin overexpression, nor any transient inhibition of muscle differentiation following Bmp4 overexpression at early stages after grafting (see MF20 labeling in Figures S5A and S5B). Our in vivo experiments argue for a role of Bmp signaling regulating the number of fetal muscle progenitors, not at the expense of muscle differentiation. The exact relationships between Bmp signaling and the Notch and Wnt signaling pathways remain to be determined in the context of maintenance of the fetal muscle progenitor pool.

An increase of proliferation of human fetal skeletal muscle progenitors after Bmp4 exposure in vitro has already been observed (Frank et al., 2006). However, in contrast to our in vivo results, this effect was followed by an inhibition of skeletal muscle differentiation. One explanation would be that, in vitro, muscle progenitors have a limited choice of fate—either proliferate or differentiate, one being at the expense of the other. We favor the idea that Bmp signaling does not act on the balance between proliferation and differentiation, but activates the proliferation of a subpopulation of Pax7$^+$ progenitors, not at the expense of skeletal muscle differentiation.

Bmp Signaling and Skeletal Muscle Differentiation

Previous in vitro and in vivo studies have led to the conclusion that Bmp is a potent repressor of skeletal differentiation in all muscle progenitors (Amthor et al., 1998; Biressi et al., 2007b; Dahlqvist et al., 2003; Frank et al., 2006; Tzahor et al., 2003). In contrast to these studies, we clearly show that overexpression of Bmp signaling led to an increase in the number of fetal muscle fibers, while blocking of Bmp signaling led to small muscles. Although this is likely to be the consequence of the Bmp effect in regulating the number of muscle progenitors, we do not exclude an additional and direct role of Bmp signaling in the fetal skeletal muscle differentiation process. Consistent with this idea is the presence of PSmad in nuclei of differentiated muscle fibers at the tips of the muscles, close to the tendons (Figures 1E and 1L–1Q). One hypothesis is that Bmp signal, in addition to increasing the proliferation of a subpopulation of Pax7$^+$ cells, also drives these cells to fuse to form myotubes. The involvement of Bmp signaling on muscle differentiation requires more attention.

Bmp Signaling and Satellite Cells

We have shown that Bmp signaling influences the generation of satellite cells during development. Experimental chick work and genetic labeling in mice have demonstrated a continuous lineage between embryonic, fetal, and then satellite cells during development (Gros et al., 2005; Hutcheson et al., 2009; Relaix et al., 2005). Consequently, a modification in the number of fetal muscle progenitors is logically expected to have a consequence for satellite cell formation. However, the reduction of muscle progenitor numbers observed in distal limb muscles of *Lbx1* mutant mice was not correlated with a prevention of satellite cell formation in these affected muscles (Vasyutina et al., 2007). Conversely, the increase in the number of Pax7$^+$ cells during fetal myogenesis in the *myostatin* mutant mice (Manceau et al., 2008) was not followed by an increase in satellite cell number in this mutant (Amthor et al., 2009). The following lead us to believe that the increase in the satellite cell number upon Bmp treatment is not only a consequence of the effect on fetal progenitor number, but also results from an action of Bmp signaling on satellite cell formation: the absence of obvious

correlation between the number of fetal progenitors, and that of satellite cells; the exacerbation over time of the muscle phenotypes following Bmp gain- and loss-of-function; and the similar location of Bmp-responsive cells at the tips of the muscles during fetal myogenesis (E10) and satellite cell formation (E18). Finally, the absence of active Bmp signaling in quiescent adult satellite cells and the presence of active Bmp signaling in proliferating satellite cells in adult myofibers and in regenerating muscles strongly suggests that Bmp signaling is important for activated satellite cells in the adult. However, its precise role in adult muscle myogenesis remains to be determined.

Bmp Signaling during Muscle Growth

We have observed that a subpopulation of fetal progenitors and satellite cells responding to Bmp signaling are preferentially located at the tips of muscles, close to the tendons in chick embryos. In addition, clusters of activated satellite cells are more concentrated at the extremities of isolated adult muscle fibers. This preferential location is fully consistent with previous observations that there is a greater concentration of $Pax7^+$ satellite cells at the ends of growing skeletal muscle fibers of post-hatch chicken (Allouh et al., 2008), and an increase of satellite cell proliferation close to the tendons (Tsujimura et al., 2006). Moreover, studies have highlighted that the ends or terminal tips of skeletal muscle fibers are the preferential sites for longitudinal growth. Nuclei are preferentially added to the ends of growing myotubes in rats during secondary myogenesis (Zhang and McLennan, 1995), and new sarcomeres tend to be added serially more at ends rather than in the middle of the muscle fibers of adult mice (Williams and Goldspink, 1971). We propose that a Bmp signal in tendons acts on a subset of $Pax7^+$ cells at the tips of the muscles to activate and maintain their proliferation. This Bmp signal will maintain a pool of $Pax7^+$cells that have the possibility of undergoing proliferation or differentiation. These $Pax7^+$ cells could either be incorporated at the extremities of the muscle fibers or proliferate. Active Bmp signaling at the muscle tips would be responsible for the progressive longitudinal growth of muscles during myogenesis (Figure 7).

EXPERIMENTAL PROCEDURES

Chick Embryos

Fertilized chick eggs from commercial sources (JA 57 strain; Institut de Sélection Animale, Lyon, Fraance; White Leghorn from HAAS, Strasbourg, France) were incubated at 37°C. Embryos were staged according to days in ovo.

Production and Grafting Recombinant/RCAS-Expressing Cells

The GFP/RCAS (Le Guen et al., 2009), Bmp4/RCAS (Duprez et al., 1996a), caBmpRIA/RCAS (Zou et al., 1997), and Noggin/RCAS (Pizette and Niswander, 2000) -expressing cells were prepared for grafting as previously described (Edom-Vovard et al., 2002). Pellets of approximately 50–100 μm in diameter were grafted into the dorsal regions of the right wings at E5. The embryos were fixed 2–14 days after grafting. At various times after grafting, embryos were harvested and processed for immunohistochemistry or in situ hybridization to tissue sections. The left wings were systematically used as internal controls. In total, 40 Bmp4-treated, 8 caBmpRIA-treated, and 30 Noggin-treated embryos were analyzed at E7 (n = 20), E9/E10 (n = 52), and E18 (n = 6).

In Situ Hybridization to Tissue Sections

Normal or manipulated wings were fixed in farnoy (60% ethanol, 30% formaldehyde, 40% and 10% acetic acid) and processed for in situ hybridization to wax tissue sections, as previously described (Tozer et al., 2007). The grafted right wings and the control left wings from the same manipulated embryos were precisely positioned in the same way for transverse sectioning. This allows an easy comparison between grafted and control wings at the same proximodistal level. The digoxigenin-labeled mRNA probes were used as previously described: chick and mouse *Bmp4* (Duprez et al., 1996a); Chick and human *BmpR-IA* (Zou et al., 1997); chick *Noggin* (Pizette and Niswander, 2000); chick *Scleraxis* (Edom-Vovard et al., 2002); and *Collagen 1* (Tozer et al., 2007).

Immunohistochemistry

For antibody staining, control and manipulated chick wings were fixed with paraformaldehyde 4% and then cut in 12 μm cryostat sections. Differentiated muscle cells were detected with the monoclonal antibody, MF20, recognizing sarcomeric myosin heavy chains (Developmental Hybridoma Bank). Two isoforms of the slow myosin heavy chain (SM2 and SM3) were detected with the monoclonal Na8 antibody (Developmental Hybridoma Bank). Fetal progenitors and satellite cells were detected with the monoclonal Pax7 antibody (1/100; Developmental Hybridoma Bank). Active Bmp signaling was detected with the polyclonal PSmad antibody recognizing the complex Bmp-activated receptor-phosphorylated Smad1/5/8 (1/100; Cell Signaling). Laminin was detected with a Laminin polyclonal antibody (Sigma). Proliferating cells were visualized with the PH3 antibody (Cell Signaling). The following secondary antibodies (Molecular Probes) were used: goat anti-mouse IgG coupled to Alexa Fluor 555; goat anti-mouse IgG1 coupled to Alexa Fluor 568; goat anti-mouse IgG2 coupled to Alexa Fluor 488; and goat anti-rabbit IgG coupled to Alexa Fluor 488 or 555. To label nuclei, sections were incubated for 15 min with Hoechst 33342 (Molecular Probes). For adult mouse muscle analysis, muscles were freshly frozen and cut in to 12 μm cryostat sections. Immediately after sectioning, slides were fixed in 4% PFA. Serial transverse sections of each TA muscle were simultaneously incubated with the monoclonal Pax7 (1/100) and polyclonal PSmad (1/100) antibodies, and then incubated for 15 min with Hoechst 33342 (Molecular Probes) to label nuclei. Stained sections were examined with a Leica SP5 confocal microscope or a Nikon Eclipse 80 microscope.

Muscle Measurements

At least three treated limbs and three associated control limbs were used for muscle area analysis and cell counting. A total of 8–10 sections from each limb were analyzed. All measurements and counting were performed with Image-Pro plus 6.0 software. For muscle area analysis, AOI (area of interest) function was used to draw a contour surrounding the muscles of dorsal areas on transverse sections in control and experimental limbs. After converting AOI to Object, pixel values were obtained. For cell counting ($Pax7^+$ cells, $Myosin^+$ fibers, $Hoechst^+$ nuclei, double $PSmad^+Pax7^+$ cells, and double $Pax7^+PH3^+$ cells), counts were performed manually and normalized with muscle area. Fiber size in E18 muscles was analyzed by laminin immunostaining, which delineated the fiber outline on transverse sections. The sizes of approximately 300 fibers were quantified in total (for each experimental case) and normalized to fiber numbers. Fiber numbers in E18 muscles were manually counted using laminin immunostaing and normalized to muscle area. Standard deviations were calculated with GraphPad Prism version 4.02 software. Quantitative data shown as histograms are expressed as means and SEM. Results were assessed for statistical significance by Student's t test (integrated in GraphPad Prism software), and differences were considered statistically significant at $p < 0.05$. Asterisks in the histograms indicate the different p values: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Isolated Fibers

Single myofibers were isolated from the EDL muscles of 2-month-old C57BL6 mice as previously described (Kuang et al., 2007). Myofibers were cultured in suspension in six well plates coated with horse serum to prevent fiber attachment. Fibers were incubated in plating medium consisting of 15% FBS and 1% chick embryo extract (CEE; Accurate Chemicals) in DMEM. At 0, 40, and 72 hr of culture, individual fibers were picked and fixed in 1% PFA, permeabilized with 0.2% Triton X-100 in PBS, and then processed for immunostaining. For quantification of satellite cell numbers, myofibers were prepared from 3 different animals, and at least 10 fibers were scored per sample.

Cardiotoxin Injury in Adult Mice

Regeneration studies were carried out on adult wild-type C57BL/6J mice. Animals were anesthetized with a mixture of Ketamine/Xylazine, and received a single injection of cardiotoxin (50 μl of 12 μM Latoxan) into the left TA muscles. TA of the right legs was used as control. TA muscles from the left (injected) and right (control) hindlimbs were collected 4 days after injury, and subsequently processed for immunohistochemistry analyses.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and is available with this article online at doi:10.1016/j.devcel.2010.02.008.

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