Antagonists of Wnt and BMP signaling promote the formation of vertebrate head muscle

Eldad Tzahor,[1,4] Hervé Kempf,[1,5] Roy C. Mootoosamy,[3,5] Andy C. Poon,[1] Arhat Abzhanov,[2] Clifford J. Tabin,[2] Susanne Dietrich,[3] and Andrew B. Lassar[1,6]

[1]Department of Biological Chemistry and Molecular Pharmacology and [2]Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA; [3]Department of Craniofacial Development, King's College London, Guy's Hospital, London Bridge, London SE1 9RT, UK

Recent studies have postulated that distinct regulatory cascades control myogenic differentiation in the head and the trunk. However, although the tissues and signaling molecules that induce skeletal myogenesis in the trunk have been identified, the source of the signals that trigger skeletal muscle formation in the head remain obscure. Here we show that although myogenesis in the trunk paraxial mesoderm is induced by Wnt signals from the dorsal neural tube, myogenesis in the cranial paraxial mesoderm is blocked by these same signals. In addition, BMP family members that are expressed in both the dorsal neural tube and surface ectoderm are also potent inhibitors of myogenesis in the cranial paraxial mesoderm. We provide evidence suggesting that skeletal myogenesis in the head is induced by the BMP inhibitors, Noggin and Gremlin, and the Wnt inhibitor, Frzb. These molecules are secreted by both cranial neural crest cells and by other tissues surrounding the cranial muscle anlagen. Our findings demonstrate that head muscle formation is locally repressed by Wnt and BMP signals and induced by antagonists of these signaling pathways secreted by adjacent tissues.

[*Keywords*: Skeletal myogenesis; cranial neural crest; head mesoderm; Wnt; BMP; Noggin; Gremlin; Frzb]

Received September 22, 2003; revised version accepted October 30, 2003.

Vertebrate locomotion crucially depends on trunk skeletal muscles, which all derive from the segmented paraxial mesoderm termed somites (for review, see Christ and Ordahl 1995). During the past decade, the tissues and signaling molecules that induce the formation of skeletal muscle from somites have been intensively studied. These studies have indicated that somitic myogenesis in the trunk is affected by signals emanating from the axial tissues, the surface ectoderm, and the lateral plate mesoderm. Wnt family members expressed in the dorsal neural tube work together with Sonic hedgehog (Shh) expressed in the notochord to activate myogenic bHLH gene expression (that is, *Myf*-5 and *MyoD*) in the epaxial component of the myotome (Munsterberg et al. 1995; Stern et al. 1995; Tajbakhsh et al. 1998; Borycki et al. 2000; Gustafsson et al. 2002). In addition, Wnt signals from the dorsal ectoderm have been demonstrated to up-regulate the expression of *MyoD* in the hypaxial component of the myotome (Tajbakhsh et al. 1998). Furthermore, BMP signals from the lateral plate have been shown to delay the activation of myogenic bHLH gene expression in the hypaxial muscle progenitors relative to those that form the epaxial musculature (Pourquié et al. 1996). In contrast to our understanding of how skeletal muscle is induced in the trunk, the tissues and signaling pathways that induce the formation of skeletal muscle in the head have not yet been elucidated.

In the vertebrate head, ~40 skeletal muscles are present, which, instead of serving for locomotion, rather move the eye, control the cranial openings, or facilitate food uptake and, in humans, speech (for review, see Wachtler and Jacob 1986). Although the hypobranchial muscles, the tongue muscles, and the muscles of the posterior branchial arches (BAs), develop from the somites, the remainder of the head muscles (that is, the "genuine" head muscles) develop from the paraxial and prechordal mesoderm located in the preotic levels of the head. These latter head muscles encompass the extraocular muscles and the muscles of the 1st, 2nd, and 3rd BAs. The BA muscle precursors stream from the cranial paraxial mesoderm into the neighboring BAs to fill their cores in concert with migratory cranial neural crest (CNC) cells, which surround the muscle anlagen,

[4]Present address: Department of Biological Regulation, The Weizmann Institute of Science, Rehovot, 76100, Israel.
[5]These authors contributed equally to this work.
[6]Corresponding author.
E-MAIL andrew_lassar@hms.harvard.edu; FAX (617) 738-0516.
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1154103.

thereby separating the myoblasts from the overlying surface ectoderm. The CNC cells give rise to most of the skeletal elements of the head and also serve as precursors for connective tissues and tendons (Noden 1988; Couly et al. 1993; Le Douarin et al. 1993).

It has long been suspected that the CNC cells may play a role in patterning the head musculature (Noden 1983a,b; Couly et al. 1992; Kontges and Lumsden 1996; Schilling and Kimmel 1997). Extirpation of CNC cells in amphibians apparently does not block the formation of cranial skeletal muscle but rather leads to defects in its patterning (Sadaghiani and Thiebaud 1987; Olsson et al. 2001). However, these results must be interpreted with caution, as neural crest cells are known to regenerate following extirpation (Scherson et al. 1993; Saldivar et al. 1997; Vaglia and Hall 1999). Therefore, the cranial muscles that formed in such operated embryos might have been induced by regenerating neural crest tissue. This view is supported by the phenotype of the zebrafish mutant *chinless* that lacks both cranial cartilage and skeletal muscle (Schilling et al. 1996). These authors showed that the *chinless* gene is required in a cell-autonomous fashion to promote the differentiation of cranial neural crest-derived cartilage, but in a non-cell-autonomous fashion to promote the formation of cranial skeletal muscles, consistent with a positive role for the CNC in head myogenesis (Schilling et al. 1996).

Although the genuine head muscles will finally exhibit the same tissue architecture as muscles in trunk, their development is remarkably distinct. The preotic head mesoderm lacks any overt sign of segmentation and never forms somites (for review, see Wachtler and Jacob 1986). In addition, components of the molecular clock that drive trunk mesoderm segmentation and hence somite formation are only transiently expressed in cranial mesoderm (Jouve et al. 2002), which soon after gastrulation merges to form a continuous strip of mesenchyme on either side of the cranial neural tube (for review, see Wachtler and Jacob 1986). Although the cranial mesoderm gastrulates from the primitive streak before the somitic mesoderm, myogenic differentiation is delayed in the head relative to the trunk. In the chick, somitic expression of the muscle regulatory gene *Myf-5* appears at stage 9–10, whereas in the BAs for instance, expression of this gene commences considerably later at stage 13–14, followed by the first sign of *MyoD* expression at stage 15, and the onset of *myosin heavy chain* (*MHC*) expression occurring not before stage 21 (Hacker and Guthrie 1998; Noden et al. 1999).

When cranial paraxial mesoderm was grafted adjacent to the axial tissues in the trunk, activation of the myogenic program in this ectopic position was inhibited, indicating that signals from the axial tissues and surface ectoderm that stimulate myogenesis in the somite are unable to elicit this effect in cranial paraxial mesoderm (Mootoosamy and Dietrich 2002). Consistent with the notion that different signals activate myogenesis in the head and trunk, distinct regulatory sequences have been found to drive *Myf-5* expression in these two regions of the embryo (Hadchouel et al. 2000; Carvajal et al. 2001). Furthermore, genetic studies have also indicated that formation of head and trunk muscle is regulated by distinctly different regulatory pathways. Whereas skeletal muscle formation in both regions of the embryo requires either *MyoD* or *Myf-5* (Rudnicki et al. 1993), mice lacking both *Myf-5* and *Pax-3* are completely devoid of trunk muscles yet retain normal head muscles (Tajbakhsh et al. 1997). In addition, mice lacking both *MyoR* and *Capsulin* display a deficit in jaw muscle formation, whereas the trunk musculature remains normal (Lu et al. 2002). In summary, embryological and genetic studies indicate that distinct regulatory circuits control the formation of head versus trunk skeletal muscles. In this work, we explore the signals that modulate the head-specific program of skeletal muscle formation.

Results

Cranial skeletal muscles develop adjacent to tissues expressing Wnt, BMP, and Shh

In searching for possible regulators of head musculature, we initially focused on signals that are known to affect trunk myogenesis; namely, Wnts, Shh, and BMPs. To evaluate if these signals might affect the formation of head muscle, we first analyzed their expression in the head region relative to that of *MyoD* and *Myf-5* (Fig. 1). *MyoD* (Fig. 1A,A′,A′′) and *Myf-5* (Fig. 1B,B′,B′′) were detected in the core of the 1st BA as well as in some extraocular muscles by whole-mount in situ hybridization at stages 18–20. Similar analyses indicated that *BMP-4* (Fig. 1C,C′) and *BMP-7* (Fig. 1D,D′) are expressed in both the dorsal neural tube and, in a regionalized fashion, in the ectoderm lining the first two BAs. Both *Wnt-1* and *Wnt-3a*, which are known to signal via the canonical Wnt pathway, are expressed in the dorsal neural tube lying adjacent to the cranial paraxial mesoderm. In addition, *Wnt-13* (Wnt-2b) is strongly expressed both in the ectoderm of the 1st and 2nd BAs and in the dorsal neural tube (Fig. 1E,E′). Expression of *Shh* was detected in both the endoderm underlying the BAs as well as in the posterior ectoderm of the 2nd BA (Fig. 1F,F′). Thus, the cranial muscle anlagen are surrounded by tissues secreting BMP, Wnt, and Hedgehog family members, raising the possibility that such signaling molecules might act to modulate the formation of skeletal muscle in the head.

An in vitro explant culture system to study cranial myogenesis

To discern how BMP, Wnt, or Shh signals might affect skeletal muscle formation in the head, we established an in vitro explant culture system from the preotic head mesoderm of the chick embryo (Fig. 2A). At stage 10, the prechordal and the nonsomitic paraxial head mesoderm form a merged strip of mesenchyme on either side of the neural tube that will give rise to both the extraocular muscles and the muscles of BAs 1–3. We therefore dissected explants of this tissue in conjunction with both

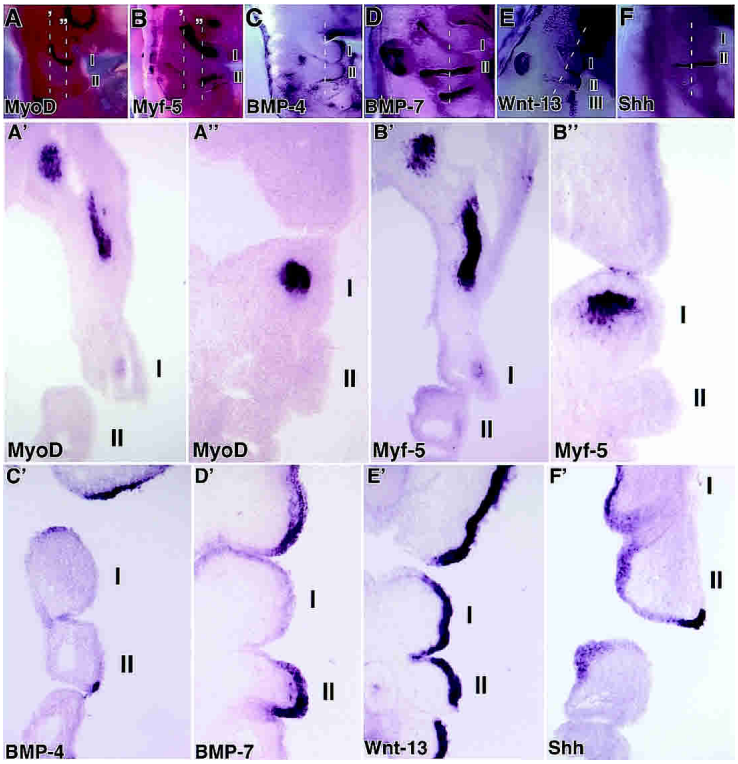

Figure 1. Expression of *BMP-4*, *BMP-7*, *Wnt-13*, and *Shh* relative to *MyoD* and *Myf-5* in the branchial arches. (*A–F*). Whole-mount in situ hybridization of stage 17–20 chick embryos for the indicated genes shown in the branchial arch (BA) region (lateral views, dorsal to the *left*, anterior to the *top*). (*A′–F′*) Frontal sections at levels designated by dashed lines in embryos depicted in *A–F* for the indicated genes in the BA region. (I and II marks the 1st and 2nd BAs, respectively).

the ectoderm and endoderm (termed CPMEE for cranial paraxial mesoderm, endoderm, ectoderm; situated between the dotted lines displayed in Fig. 2B). In addition, CPMEE explants contain migratory neural crest cells (see below). These explants were embedded in collagen, cultured in vitro for differing lengths of time, and harvested for RT-PCR analysis to evaluate gene expression. To assay skeletal myogenesis, we followed the expression of both the myogenic regulators, *MyoD* and *myogenin*, and the differentiation marker, *myosin heavy chain* (*MHC*). *MyoD* expression was first detected after 2 d of culture (Fig. 2C, lane 3), followed by the expression of *myogenin* and *MHC* at day three (Fig. 2C, lane 4); all three skeletal muscle markers continued to be robustly expressed after 4 d of culture (Fig. 2D, lanes 1,3,5,7,9, 11,13). Although *MyoD* expression in the cranial mesoderm in vitro was delayed relative to that observed in vivo, its expression in cranial mesoderm explant cultures preceded that of *myogenin* and *MHC*, similar to the course of myogenic differentiation documented in this tissue in vivo.

Inhibition of cranial myogenesis in vitro and in vivo by BMP, Wnt, and Shh signals

We next tested the effect of exogenous BMP, Wnt, and Shh on the progress of myogenic differentiation in stage 10 CPMEE cultures. Application of BMP-4 to these explants blocked skeletal myogenesis (Fig. 2D, lane 2; similar results were observed in 20/20 explants), reminiscent of the repressive effects of BMP-4 on myogenesis in the trunk. Surprisingly, infection of stage 10 explants with a retrovirus encoding *Wnt-3a* also blocked myogenic differentiation in these cultures (Fig. 2D, lane 4; similar results were observed in 14/18 explants), suggesting that Wnt signals, which promote trunk myogenesis, may act to repress skeletal myogenesis in the head. Because several Wnt family members, including *Wnt-3a*, are known to signal via the "canonical" Wnt pathway by stabilizing β-catenin levels in the cell, we evaluated whether expression of a stabilized form of β-catenin would also block cranial myogenesis in explant culture. Indeed, expression of stabilized β-*catenin* prevented skeletal myogenesis of CPMEE explants in vitro, similar to the effects of *Wnt-3a* (Fig. 2D, lane 6; similar results were observed in 6/9 explants). Likewise, forced expression of *Wnt-13* (*Wnt-2b*), which also signals via the canonical Wnt pathway, and is broadly expressed in BA ectoderm (Fig. 1E,E′), blocked myogenesis in these explants (Fig.2D, lane 8; similar results were observed in 6/8 explants). Thus, as opposed to trunk paraxial mesoderm, where Wnt signals have been found to promote skeletal myogenesis, the canonical Wnt signaling pathway blocks skeletal muscle differentiation in cranial paraxial mesoderm.

We also investigated if Shh signals, which in combination with Wnt-1/Wnt-3a induce myogenic differentiation in the trunk, would affect skeletal muscle formation in explants of the cranial mesoderm. We found that administration of Shh blocked skeletal myogenesis in stage 10 CPMEE explants (Fig. 2D, lane 10; similar results were observed in 10/14 explants). Likewise, the combi-

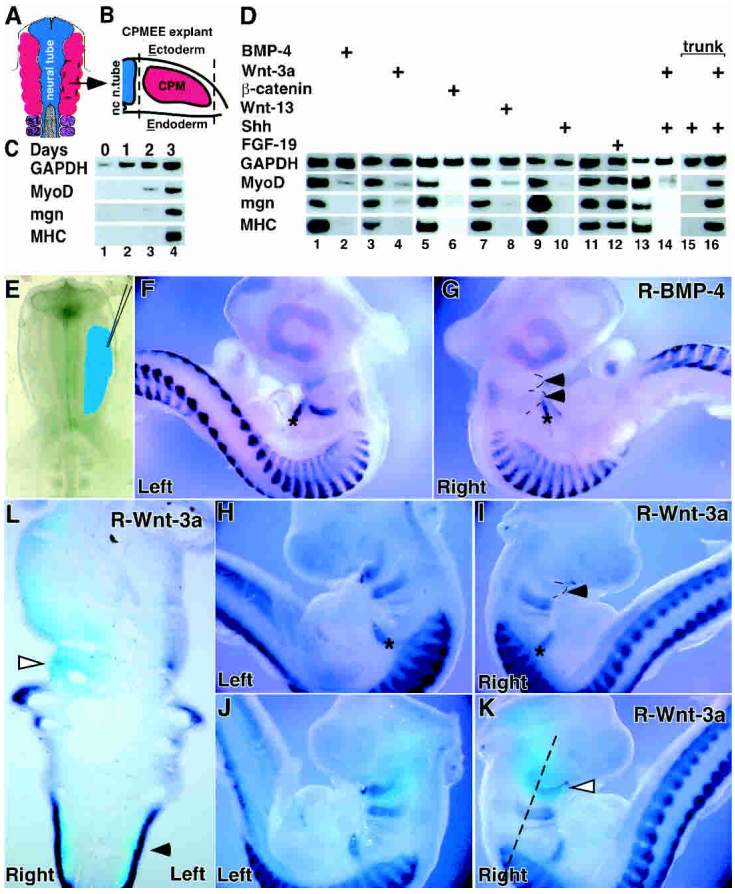

Figure 2. Ectopic BMP, Wnt, and Hedgehog signals can block myogenesis in the cranial paraxial mesoderm. (*A*) Diagram of a stage 10 chick embryo, showing the location of the cranial paraxial mesoderm (red) adjacent to the neural tube (blue) and anterior to the first pair of somites (purple). (*B*) Scheme of the germ layers included in the dissected tissue, showing the cranial paraxial mesoderm covered dorsally by the ectoderm and ventrally by the endoderm, termed CPMEE. (*C*) Skeletal myogenesis in CPMEE explants cultured in vitro. CPMEE explants were dissected and cultured in vitro for the indicated number of days prior to harvest. RT-PCR analysis was performed to monitor expression of the indicated genes. (*D*) The effect of various signaling molecules on myogenesis in cranial and trunk paraxial mesoderm. Explants of either CPMEE (lanes *1–14*) or somites I–III (lanes *15,16*) isolated from stage 10 chick embryos were cultured in vitro. Explants were cultured in medium containing either BMP-4 (lane *2*), Shh (lanes *10,14, 15,16*), or FGF-19 (lane *12*), or infected with a nondefective retrovirus (RCAS) encoding either *GFP* (lanes *3,5,7,13,15*), *Wnt-3a* (lanes *4,14,16*), a stabilized form of β-*catenin* (lane *6*), or *Wnt-13* (lane *8*). A representative experiment is shown; however, similar results were obtained in at least four independent experiments. (*E*) Cranial paraxial mesoderm of stage 9 embryos targeted for infection by RCAS viruses (blue) in *F–L*. (*F,G*) Effect of *RCAS-BMP-4* infection of the cranial paraxial mesoderm. *MyoD* expression in either the control side (*left*) or the *RCAS-BMP-4*-infected side (*right*), respectively. The asterisk marks developing tongue muscles that migrate from occipital somites, closed arrowheads identify *BMP-4*-infected branchial arches. (*H,I*) Effect of *RCAS-Wnt-3a* infection of the cranial paraxial mesoderm. *Myf-5* expression in either the control side (*left*) or the *RCAS-Wnt-3a*-infected side (*right*), respectively. The asterisk marks developing tongue muscles that migrate from occipital somites. The closed arrowhead identifies *Wnt-3a*-infected branchial arch. (*J,K*) Secondary in situ hybridization to detect ectopic viral gene expression (light blue; open arrowhead). Light blue staining in the branchial arch region of *J* is from staining on the contra-lateral *RCAS-Wnt-3a*-infected right side of the embryo; see *L*. (*L*) A frontal section of the embryo depicted in *J* and *K* (at the level labeled with a dashed line) documents viral expression (light blue) in both the right side of the branchial arches (which lack *Myf-5* expression; open arrowhead) as well as the somitic mesoderm (which retains *Myf-5* expression; closed arrowhead).

nation of Shh and Wnt-3a repressed skeletal muscle differentiation in these cultures (Fig. 2D, lane 14; similar results were observed in 7/8 explants), whereas this same combination of signals induced myogenesis in explants of somitic mesoderm (Fig. 2D, lane 16; similar results were observed in 4/4 explants). It should be noted that not all signaling molecules expressed in the head blocked myogenic differentiation of head mesoderm in vitro, as FGF-19, which is expressed in a dynamic fashion within the three germ layers during facial development did not interfere with skeletal myogenesis in the CPMEE cultures (Fig. 2D, lane 12; similar results were observed in 5/5 explants). We conclude that Wnt and Shh signals, which promote skeletal myogenesis in the trunk, paradoxically block this differentiation program in cranial paraxial mesoderm, and that BMP signals block skeletal myogenesis in both head and trunk muscle precursors.

To test if either BMP or Wnt signals would similarly block skeletal myogenesis in head mesoderm in vivo, we infected the right side of the cranial paraxial mesoderm in stage 9 chick embryos *in ovo* with a retrovirus encoding either *BMP-4* or *Wnt-3a* (Fig. 2E–L). Eggs were sealed and incubated for an additional 2 d. In situ hybridization for *MyoD* (Fig. 2F,G) or *Myf-5* (Fig. 2H–L) revealed a loss of the BA musculature on the right side of embryos infected with these retroviruses (Fig. 2G,I), and normal expression of these same markers in the BAs on the control left side of the embryos (Fig. 2F,H). To detect viral spreading, we performed in situ hybridization for the viral transcripts (shown in light blue; Fig. 2J,K,L). This procedure revealed that *Wnt-3a*-infected BA tissue failed to express *Myf-5*, whereas infected somitic tissue (which was infected by virus that was spread bilaterally underneath the dorsal ectoderm) robustly expressed *Myf-5* (Fig. 2L). In addition, implantation of beads laden with Shh into the cranial region blocked the formation of nearby skeletal muscles in vivo (S. Dietrich, unpubl.). Thus, Wnt, BMP, and Shh signals are potent inhibitors of cranial skeletal muscle formation both in vitro and in vivo.

Wnt signals from the dorsal neural tube block cranial myogenesis in vitro

Our findings indicate that the formation of genuine head muscles is blocked by BMP, Wnt, and Shh signals. Given that a prominent site of expression for these signaling molecules is the neural tube and that cranial muscle, and in particular the BA musculature, develops at sites distant from the neural tube, we reasoned that BMP, Wnt, and/or Shh secreted by the neural tube might act to prevent premature myogenic differentiation of the mesoderm prior to its migration away from the axial tissues. To investigate if the axial tissues secrete inhibitors of cranial skeletal muscle differentiation, we cultured stage 10 CPMEE explants in either the absence or presence of the adjacent neural tube (from the hindbrain region) and underlying notochord. When cultured in the absence of the neural tube/notochord, stage 10 CPMEE explants underwent robust myogenic differentiation (Fig. 3A, lane 1; similar results were observed in 22/24 explants). In contrast, when cultured in the presence of the axial tissues, the explants failed to show significant levels of muscle differentiation. (Fig. 3A, lane 2; similar results were observed in 14/15 explants). Thus, signals from the neural tube/notochord block cranial myogenesis in vitro, indicating that muscle differentiation in the head, in contrast to the trunk, may be actively inhibited in the vicinity of the axial tissues. Next, we investigated if the inhibitory signal(s) that blocks skeletal myogenesis in cranial paraxial mesoderm explants resides in either the dorsal or ventral region of the axial tissues. Activation of the skeletal muscle differentiation program was efficiently blocked in stage 10 explants cultured in the presence of only the dorsal half of the neural tube (Fig. 3A, lane 4; similar results were observed in 8/8 explants). In contrast, myogenesis took place in similar explants cultured with only the ventral half of the neural tube and notochord (Fig. 3A, lane 6; similar results were observed in 5/5 explants). In other words, dorsal neural tube ablation restores cranial myogenesis in CPMEE explants. Thus, skeletal myogenesis in the cranial paraxial mesoderm is strongly inhibited by a signal(s) emanating from the dorsal neural tube, and is consistent with the expression of *Wnt-1*, *Wnt-3a* , and BMP family members in this region of the neural tube. Because skeletal muscle differentiation in CPMEE explants took place in the presence of the ventral midline tissues (that is, the notochord and floor plate) in vitro, it suggests that physiological levels of Shh expressed by these tissues do not block myogenesis in the cranial paraxial mesoderm.

To investigate if Wnt signals from the neural tube block skeletal myogenesis in explants containing cranial mesoderm, we investigated whether administration of the Wnt antagonist Frzb-IgG (Tzahor and Lassar 2001) to explants of cranial paraxial mesoderm cultured in the presence of the dorsal neural tube would restore skeletal myogenesis in such cultures. Indeed, although coculture of stage 10 CPMEE with the dorsal neural tube completely blocked the expression of *MyoD* (Fig. 3B, lane 1), addition of Frzb-IgG negated the inhibitory effects of the dorsal neural tube and induced the expression of *MyoD* in 40% of such treated cultures (Fig. 3B, lane 2; similar results were observed in 4/10 explants). These findings indicate that Wnt signals from the dorsal neural tube repress skeletal muscle formation in cocultured cranial paraxial mesoderm, and suggest that such signals may serve to block premature cranial myogenesis in the vicinity of the neural tube.

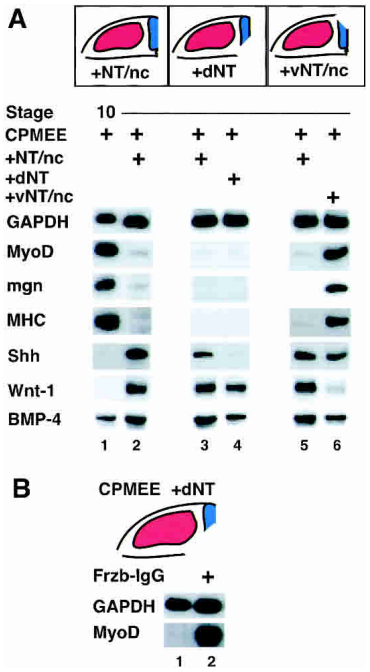

Figure 3. Inhibition of myogenesis in the cranial paraxial mesoderm by signals from the dorsal neural tube. (*A*) Diagrams of transverse sections through explants show the cranial paraxial mesoderm (red) in relation to the adjacent axial tissues (blue). RT-PCR analysis of gene expression in explants that had been isolated from stage 10 chick embryos and cultured for 4 d in either the absence (lane *1*) or the presence of the adjacent neural tube and notochord (NT/nc; lanes *2*,*3*,*5*), the dorsal neural tube (dNT; lane *4*) or the ventral neural tube and notochord (vNT/nc; lane *6*). Skeletal myogenesis was observed in 1/15 explants cocultured with NT/nc (lanes *2*,*3*,*5*), in 0/8 explants cocultured with dNT (lane *4*), and in 5/5 explants cocultured with vNT/nc (lane *6*). (*B*) Administration of Frzb-IgG can induce *MyoD* expression in CPMEE explants cultured in the presence of the dNT. CPMEE plus the adjacent dorsal neural tube were isolated from stage 10 chick embryos and were cultured in either the absence (lane *1*) or presence (lane *2*) of Frzb-IgG. *MyoD* induction was observed in 40% of such explants (n = 10).

Expression profile of BMP and Wnt antagonists in the head

In the chick embryo, BMP and Wnt signals are secreted by both the dorsal neural tube from neurulation stages onward and are also present in the surrounding tissues when muscle precursors enter the BAs. We therefore speculated that a mechanism must be in place to antagonize both BMP and Wnt signals in the BAs to promote

myogenic differentiation in these structures. We began to explore this possibility by investigating the expression patterns of BMP and Wnt antagonists at the sites of the developing cranial muscle anlagen. We found that *Noggin* is expressed in migrating CNC cells at stage 11 just prior to the onset of myogenic differentiation (Fig. 4A). This expression was retained by tissues surrounding the eye but lost by the CNC cells that had migrated into the BAs at stage 16 (Fig. 4B). At this stage, however, expression of a different BMP inhibitor, *Gremlin*, commenced in the BAs region (Fig. 4C,D) in the subectodermal layer of the CNC-derived BA mesenchyme and in the pharyngeal endoderm in stage 21 embryos (Fig. 4E,F).

At stage 11, expression of the Wnt antagonist *Frzb* overlapped with that of *Noggin* in the migrating CNC cells (Fig. 4G). Later, *Frzb* is expressed by the CNC-derived mesenchyme of the BAs, and displays a wider distribution than the BMP antagonists in this tissue (Fig. 4H–J). Similar to *Gremlin*, however, the expression of *Frzb* was not limited to CNC-derived tissues, but included the ectoderm covering the distal arches (Fig. 4I). To more closely investigate the spatial relationship of the cranial muscle anlagen and *Frzb* expressing mesenchyme, we performed in situ hybridization of adjacent sections at stage 19. This analysis revealed a striking complementary expression pattern for *Frzb* and *Myf-5*. The crest-derived mesenchyme of such embryos expressed *Frzb* (Fig. 4J), surrounding a mesodermal core expressing *Myf-5* (Fig. 4K). Thus, at the time of myogenic differentiation, tissues surrounding the myogenic mesoderm, in particular CNC cells, provide the means to antagonize both BMP and Wnt signals and thereby may act to promote myogenic differentiation. We therefore analyzed the role that CNC cells and BMP or Wnt inhibitors might play during cranial muscle formation in vitro and in vivo.

CNC cells can induce cranial myogenesis in vitro

CNC cells start to leave the neural folds prior to neural tube closure at stage 8+, and by stage 10, most of the CNC cells have left the neural folds and reside between the cranial mesoderm and ectoderm. We therefore reasoned that the myogenic differentiation observed within stage 10 CPMEE explants may be stimulated by signals secreted by CNC cells present within these explants. Consequently, we investigated whether explants taken from stage 8 embryos prior to CNC migration would undergo myogenic differentiation. Explants dissected at stage 10 and cultured for 3 d in vitro robustly expressed *MyoD*, *myogenin*, and *MHC* (Fig. 5A, lane 6; similar results were obtained in 38/44 explants). In striking contrast, explants dissected at stage 8 failed to differentiate into skeletal muscle after 3 d in culture (Fig. 5A, lane 3; similar results were observed in 22/26 explants). Although both stage 8 and stage 10 CPMEE explants expressed *Wnt-13* and *BMP-4* (Fig. 5A) in the ectodermal layer (see following), only stage 10 explants were populated by CNC cells, as evidenced by the expression of several neural crest markers including *Pax-3*, *Slug*, *Id2*, and *Noelin* (Fig. 5B, lane 2). Notably, *Frzb*, *Noggin*, and *Gremlin*, which are expressed by CNC cells, were detectable in stage 10 but not stage 8 CPMEE explants (Fig. 5B, lane 2). Thus, activation of skeletal myogenesis in explants of cranial paraxial mesoderm indeed correlated with the presence of *Frzb*/*Noggin*/*Gremlin* expressing CNC cells within these explants.

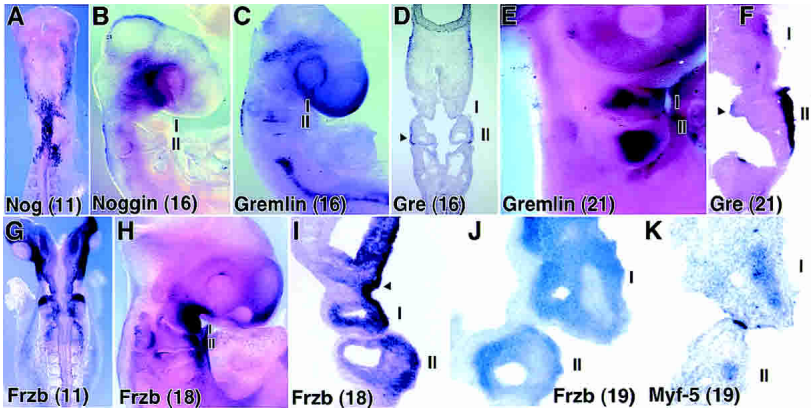

Figure 4. The BMP antagonists *Gremlin* and *Noggin* and the Wnt antagonist *Frzb* are expressed by both the cranial neural crest and other facial tissues. Gene expression analysis of the BMP antagonists *Noggin* and *Gremlin* (*A–F*) and the Wnt antagonist *Frzb* (*G–J*). (*A*,*B*) *Noggin* expression. (*A*) Whole-mount in situ hybridization of *Noggin*, showing its expression in migrating CNC cells at stage 11 (dorsal view, anterior to the *top*). (*B*) At stage 16, *Noggin* expression is reduced in the BA region (lateral view, dorsal to the *right*, anterior to the *top*). (*C–F*) *Gremlin* expression. (*C*) Lateral view and (*D*) frontal section (anterior to the *top*) at stage 16. *Gremlin* is expressed in a subset of CNC cells beneath the surface ectoderm that migrate to the 2nd BA at stage 16. (*E*) Lateral view and (*F*) frontal section (pharynx to the *left*, surface of the embryo to the *right*) at stage 21. Note enhanced expression of *Gremlin* in the subectodermal mesenchyme and in addition, the pharyngeal endoderm (*F*, arrowhead). (*G–K*) *Frzb* expression. *Frzb* is expressed in migrating cranial neural crest cells at stage 11 (*G*, dorsal view) and in the cranial neural crest-derived mesenchyme of the BAs at stage 18 (*H*, lateral view). (*I*) Frontal section of the embryo in *H* (orientation as in *F*). In addition to its expression in the BA mesenchyme, *Frzb* is also expressed in the surface ectoderm (arrowhead). (*J*,*K*) Adjacent frontal sections through the BAs of a stage 19 chick embryo stained for *Frzb* (*J*) and *Myf-5* (*K*), pharynx toward the *top left* corner. Note the complementary expression patterns of *Frzb* in neural crest-derived mesenchyme and *Myf-5* in paraxial mesoderm-derived cells located in the core of the BA.

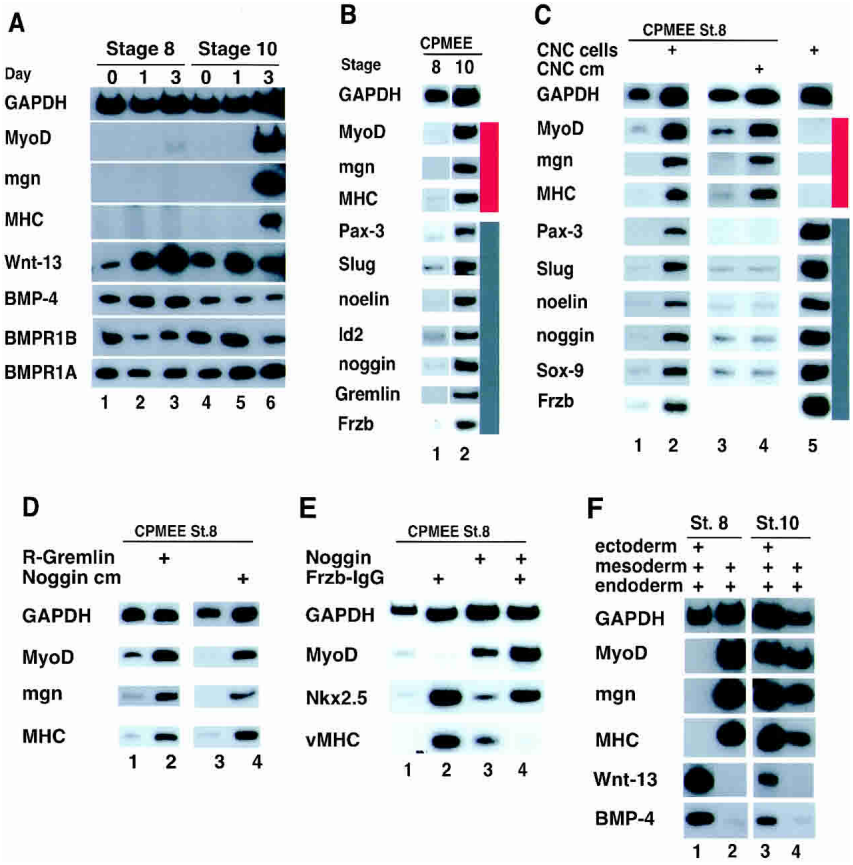

Figure 5. Activation of myogenesis in explants of cranial paraxial mesoderm by cranial neural crest cells, and by antagonists of BMP and Wnt signaling. (*A*) Myogenesis occurs in stage 10 but not stage 8 CPMEE explants. CPMEE explants dissected from stage 8 or 10 embryos were cultured for the indicated period of time. Transcript levels of the indicated genes were monitored by RT-PCR analysis. (*B*) Myogenesis in CPMEE explants correlates with the presence of the cranial neural crest. RT-PCR analysis of gene expression in CPMEE explants that were isolated from either stage 8 (lane *1*) or stage 10 (lane *2*) chick embryos and cultured in vitro for 4 d. (*C*) CNC cells or CNC-conditioned medium can induce myogenesis in stage 8 CPMEE explants. Stage 8 explants were cultured for 4 d either in the absence (lanes *1*,*3*) or in the presence of either CNC cells (CNC cells; lane *2*), or medium conditioned by CNC cells (CNC cm, lane *4*). In addition, CNC cells were cultured alone (lane *5*). Skeletal myogenesis was observed in 12% of explants dissected from stage 8 chick embryos that were cultured alone (lanes *1*,*3*; $n = 8$); in 75% of stage 8 explants that were cultured in the presence of isolated CNC cells (lane *2*; $n = 8$); and in 67% of stage 8 explants that were cultured in the presence of conditioned medium harvested from neural crest cells (lane *4*; $n = 6$). Isolated CNC cells cultured alone expressed all neural crest markers but failed to express skeletal muscle differentiation markers (lane *5*). In *B* and *C*, the skeletal muscle differentiation markers are identified by a red bar on the right; the neural crest markers are identified by a grey bar. (*D*) Administration of BMP antagonists can elicit myogenesis in stage 8 CPMEE explants. Stage 8 CPMEE explants were infected with a retrovirus encoding either *GFP* (lane *1*) or *Gremlin* (*R-Gremlin*, lane *2*; myogenesis was observed in 8/10 such explants) and cultured for 4 d. Similar explants were treated with medium conditioned by either control cells (lane *3*) or Noggin-expressing cells (*Noggin*, lane *4*; myogenesis was observed in 14/20 such explants). (*E*) Noggin and Frzb synergistically activate *MyoD* expression in stage 8 CPMEE explants. Stage 8 CPMEE explants were treated with either soluble Frzb-IgG (lane *2*), medium conditioned by Noggin-expressing cells (lane *3*), or both Frzb-IgG and Noggin-containing medium (lane *4*). The transcript levels of *Nkx2.5* and *ventricular Myosin Heavy Chain (vMHC)* were evaluated to assay for cardiogenesis. (*F*) Ectodermal signals suppress myogenesis in stage 8 CPMEE explants. Stage 8 or stage 10 cranial paraxial mesendoderm explants were cultured for 4 d in either the presence or absence of ectoderm as indicated. Similar results were obtained in multiple independent experiments.

To directly test if CNC cells express an activator of the cranial skeletal muscle differentiation program, we cocultured stage 8 explants with either purified CNC cells or medium conditioned by CNC cells (Fig. 5C). As discussed earlier, skeletal myogenesis occurred in only about 12% of CPMEE explants dissected from stage 8 chick embryos that were cultured alone (Fig. 5C, lanes 1,3; similar results were observed in 7/8 explants). In contrast, when cocultured with either CNC cells (Fig. 5C, lane 2; similar results were observed in 6/8 explants) or with medium conditioned by CNC cells (Fig. 5C, lane 4; similar results were observed in 4/6 explants), robust skeletal myogenesis was observed in stage 8 explants. Control cultures containing only CNC cells expressed the various neural crest markers, yet failed to express any skeletal muscle differentiation markers (Fig. 5C, lane 5; similar results were observed in 5/5 explants). Thus, CNC cells secrete a factor(s) that can promote skeletal myogenesis of cranial paraxial mesodermal cells in vitro.

BMP and Wnt antagonists expressed by the CNC cells mimic the muscle-inducing activity of this tissue in vitro

We found that the BMP antagonists *Noggin* and *Gremlin* and the Wnt antagonist *Frzb* are all expressed by cranial neural crest cells in vivo and in vitro. We therefore speculated that these secreted factors may act to stimulate cranial myogenesis by blocking the repressive effects of BMP and Wnt signals on this process. To evaluate if BMP antagonists might promote skeletal myogenesis in explant culture, we first cultured stage 8 explants with either Gremlin or Noggin (Fig. 5D). Infection of stage 8 explants with an avian retrovirus programmed to express Gremlin (Fig. 5D, lane 2; similar results were

observed in 8/10 explants) or culture of similar explants in Noggin-conditioned medium (Fig. 5D, lane 4; similar results were observed in 14/20 explants) led to activation of the skeletal muscle differentiation program, suggesting that these molecules can indeed trigger myogenic differentiation of the cranial mesoderm. In contrast, administration of the Wnt antagonist Frzb-IgG to these same explants failed to induce skeletal muscle differentiation markers, but rather induced cardiogenesis as monitored by the expression of *Nkx2.5* and *ventricular MHC* (Fig. 5E, lane 2; similar results were observed in 6/9 explants) as previously reported (Tzahor and Lassar 2001). However, administration of Noggin and Frzb-IgG in combination (Fig. 5E, lane 4; similar results were observed in 5/6 explants) augmented the expression of *MyoD* relative to that induced by Noggin alone (Fig. 5E, lane 3), and repressed the extent of cardiogenesis relative to that induced by FrzB-IgG alone (Fig. 5E, lane 2). These results suggest that the combinatorial action of BMP and Wnt antagonists secreted by the cranial neural crest may work to promote the differentiation of cranial mesodermal cells into skeletal rather than cardiac myoblasts.

Because BMP signals repress skeletal myogenesis in stage 8 CPMEE explants, and BMPs are prominently expressed in the facial ectoderm, we examined whether removal of the ectoderm from stage 8 cranial paraxial mesendoderm (CPME) explants would allow skeletal muscle differentiation to occur in this tissue, which lacks a cranial neural crest component. Indeed, culturing stage 8 CPME explants in the absence of ectoderm led to a robust induction of skeletal muscle differentiation, which correlated with the absence of both *Wnt-13* and *BMP-4* in the explants (Fig. 5F, cf. lanes 1 and 2). In contrast, skeletal muscle differentiation in stage 10 CPMEE versus CPME explants was unaffected by the removal of the ectoderm (Fig. 5F, lanes 3,4). Thus, in addition to signals from the dorsal neural tube, inhibitory signals from the ectoderm block muscle differentiation in stage 8 cranial paraxial mesoderm.

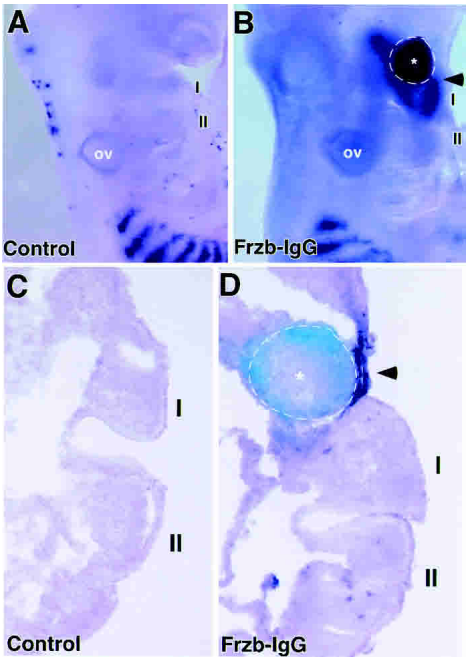

Figure 6. Ectopic application of the Wnt antagonist Frzb induces *MyoD* expression in vivo. (*A*) Lateral view of the BA region of a control embryo and (*B*) an embryo in which a pellet of 293 cells, programmed to express Frzb-IgG, was inserted into the cranial paraxial mesoderm on the right side at stage 9. After 2 d of reincubation, the embryo was harvested and *MyoD* expression was analyzed by whole-mount in situ hybridization. Following in situ hybridization, the embryos were immunostained with an antibody against IgG to identify the location of the Frzb-IgG-expressing cells (indicated by dotted line). The BA containing the Frzb-IgG cells shows robust levels of *MyoD* expression (*B*). (*C*,*D*) Frontal sections through the BAs of the embryos displayed in *A* and *B*. Arrowhead in *D* points to location of ectopic *MyoD* expression in the BA mesenchyme located adjacent to Frzb-IgG cells (indicated by dotted line). For controls, we used staged-matched embryos that were mock-operated in the same manner and incubated for the same time period (*A*,*C*). Other controls included implantation of pellets of either 293 cells, 293-IgG cells, COS7 cells, or CHO cells into the cranial paraxial mesoderm on the right side of a stage 9 chick embryo, which in all cases failed to induce *MyoD* expression in the BAs (data not shown).

Ectopic expression of a Wnt antagonist promotes cranial myogenesis in vivo

Because *Frzb*-expressing cells surround a core of *MyoD*/*Myf-5*-positive cells in the BAs (Fig. 4J,K), and this inhibitor counteracts Wnt signals from the dorsal neural tube that repress skeletal muscle differentiation of CPMEE explants in vitro (Fig. 3B), we speculated that inhibition of Wnt signaling by *Frzb* may promote skeletal muscle differentiation in the BAs in vivo. To explore this possibility, we applied Frzb-IgG to the cranial paraxial mesoderm in vivo, reasoning that increased levels of Frzb might, in conjunction with endogenous BMP inhibitors expressed in the embryo, act to accelerate the expression of skeletal muscle differentiation markers in vivo. Indeed, implantation of pellets of 293 cells, engineered to express the Frzb-IgG fusion protein, into stage 9 cranial paraxial mesoderm induced premature expression of *MyoD* in the vicinity of the Frzb-IgG cell pellet in the BA region (Fig. 6B,D). Notably, the induction of *MyoD* by Frzb-IgG cells was restricted to only a few cell layers adjacent to the cell pellet, consistent with the localized expression of FrzB-IgG to tissue immediately adjacent to the Frzb-IgG expressing cells (Fig. 6D). In the BAs of either stage-matched unoperated embryos or of embryos implanted with IgG control cells, *MyoD* expression was not detected (Fig. 6A,C; data not shown). These findings are consistent with our in vitro results, indicating that Wnt signals secreted by the neural tube repress cranial myogenesis, and suggest that myogenic differentiation in the head is stimulated by Wnt antagonists.

Redundant sources of BMP and Wnt inhibitors may activate cranial myogenesis in vivo

As discussed above, we have found that BMP and Wnt signals can block cranial myogenesis, whereas BMP and Wnt antagonists promote this process. A prominent site of expression for these BMP and Wnt inhibitors in the head are the CNC cells, which in vitro also promote skeletal myogenesis. To investigate whether CNC cells are required for skeletal myogenesis in vivo, we bilaterally ablated the dorsal half of the neural plate from the diencephalic levels to the level of rhombomere 5/6 at stage 8, prior to the onset of CNC cell emigration (Fig. 7A). The embryos were reincubated until stage 18–19 and analyzed for the presence of *Myf-5* (Fig. 7C,E, blue staining, showing control and operated embryos, respectively, $n = 7$) or *MyoD* (data not shown, $n = 2$) by whole-mount in situ hybridization. We monitored whether residual CNC cells were present in the BA region by analyzing the expression of the neural crest cell markers *Dlx5* (data not shown, $n = 3$) or *Frzb* (Fig. 7B,D, blue staining, showing control and operated embryos, respectively; Fig. 7E, showing an operated embryo; red staining, $n = 4$). The neural crest-ablated embryos, although exhibiting malformed, size-reduced, and often developmentally retarded heads, all developed BAs. In striking contrast to stage-matched control embryos (Fig. 7B,C), the BAs in CNC-ablated embryos lacked neural crest-associated expression of *Dlx5* (data not shown) and displayed a significant decrease in *Frzb* expression (Fig. 7, cf. B, D and E), suggesting that a significant fraction of neural crest-derived mesenchyme was absent from these structures. Interestingly, however, faint expression of *Frzb* throughout the BAs and stronger expression in both the ectoderm overlying the trigeminal ganglion and the distal ectoderm of the BAs persisted in such manipulated embryos (Fig. 7D,E, arrows), suggesting that *Frzb* may be expressed by either residual CNC and/or nonneural crest-derived tissues in such operated embryos. Accordingly, *Myf-5* (Fig. 7E; blue staining) or *MyoD* (data not shown) expression was still detectable in both the BA muscle anlagen and in the anlagen of the extraocular muscles. These results indicate that induction of myogenesis in the BAs can occur following extirpation of

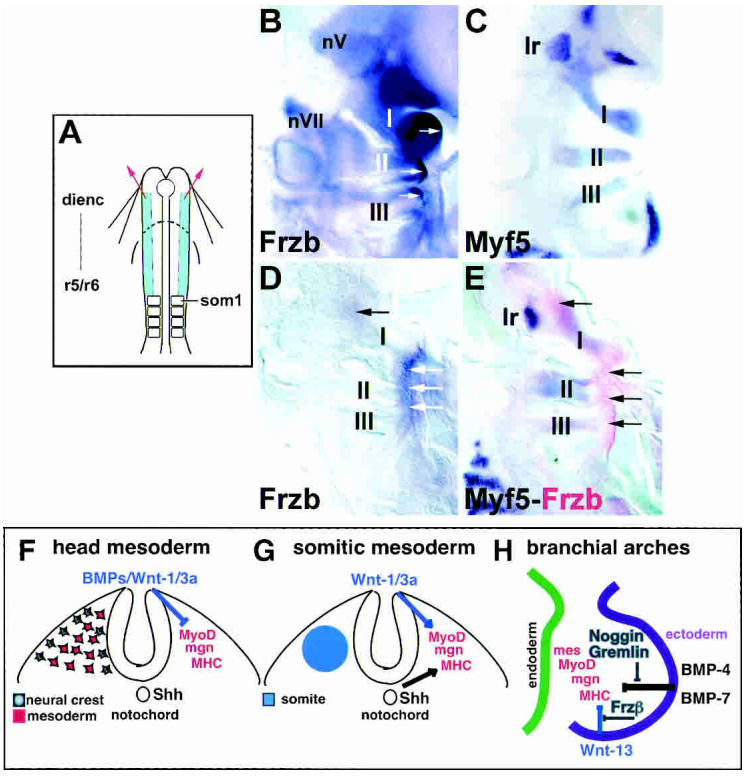

Figure 7. Myogenic bHLH genes are expressed in the branchial arches after ablation of the cranial neural crest; a model for cranial skeletal muscle induction. (*A*) Scheme of in ovo neural crest cell ablation. At stage 8, the dorsal half of the neural plate from diencephalic levels to rhombomere 5/6, anterior to the first somite (som1), was excised bilaterally as indicated. (*B–E*) Lateral views of the BA region of either control (*B*,*C*) or operated (*D*,*E*) embryos at stage 18–19 (dorsal is to the *left*, anterior to the *top*). (*B*) Control embryo stained for *Frzb*. Note the widespread expression of *Frzb*, including the trigeminal (nV) and facial (nVII) ganglia, the neural crest cells in the BA mesenchyme, and the ectoderm covering the trigeminal and the distal region of the BAs (arrows). (*C*) Expression of *Myf-5* in a control embryo. The anlagen of the muscles in BA I–III and of the lateral rectus muscle (lr) are indicated. (*D*) Neural crest ablated embryo stained for *Frzb*. Although neural crest cell-associated expression of *Frzb* is significantly diminished in the head region, *Frzb* is still present in the ectoderm overlying the trigeminal ganglia and the distal ectoderm of the BAs (arrows). (*E*) Neural crest ablated embryo stained for *Myf-5* in blue and *Frzb* in red. Note that *Myf-5* expression is still detectable both in the lateral rectus and in the BAs. In this embryo, low levels of *Frzb* are detectable throughout the BAs and higher levels are found in the ectoderm overlying the trigeminal ganglia and the distal ectoderm of the BAs (arrows). (dienc) diencephalon; (r5/6) rhombomeres 5 and 6. (*F–H*) Head muscle formation is cued by a combination of negative and positive signals from surrounding tissues. (*F*) Cranial neural crest cells (CNC, shown as grey) migrate away from the dorsal neural tube and into the region of the cranial paraxial mesoderm (red). While lying adjacent to the neural tube, muscle formation in the cranial paraxial mesoderm is blocked by BMP and Wnt signals from the dorsal neural tube (*G*) In the trunk, Wnt signals from the dorsal neural tube in combination with Shh signals from the notochord and floor plate promote epaxial myogenesis in the somites. (*H*) At sites of head muscle formation such as the BAs, CNC cells, which have migrated from the dorsal neural tube, surround a core of muscle precursors derived from the cranial paraxial mesoderm. BMP-4, BMP-7, and Wnt-13 signals from the BA ectoderm repress skeletal muscle differentiation in the mesodermal core. The BMP antagonists *Noggin* and *Gremlin* work together with the Wnt antagonist *Frzb*, which is expressed by both CNC cells and other BA tissues, to override BMP and Wnt inhibition and thereby promote skeletal myogenesis.

CNC tissue. However, because *Frzb* was still expressed at a low but detectable level in the BA region following ablation of the CNC, it is possible that expression of this molecule by either residual CNC cells or by other tissues in the head may compensate for the absence of the majority of the CNC to induce head muscle formation.

Discussion

Myogenesis in cranial and trunk paraxial mesoderm is controlled by distinct regulatory circuits

In this work, we demonstrate that the signals that induce myogenesis in the cranial versus the trunk paraxial mesoderm are notably distinct. Although signals from the dorsal neural tube promote myogenesis in the trunk, such signals block myogenesis of the cranial paraxial mesoderm in vitro. Indeed, expression of Wnt family members expressed in either the dorsal neural tube (*Wnt-3a*) or surface ectoderm (*Wnt-13*), or forced expression of stabilized β-*catenin*, which stimulates the canonical Wnt-signaling pathway, all blocked myogenesis in cranial paraxial mesoderm explants, and in the case of Wnt-3a, blocked myogenesis in the BA region in vivo. In striking contrast, Wnt family members expressed in either the dorsal neural tube or in surface ectoderm overlying the somites have been documented to promote skeletal myogenesis in this tissue (Takada et al. 1994; Munsterberg et al. 1995; Stern et al. 1995; Capdevila et al. 1998; Ikeya and Takada 1998; Tajbakhsh et al. 1998). Wnt-1 and Wnt-3a, which cooperate with Shh to stimulate myogenesis in the trunk and suppress myogenic differentiation of cranial mesoderm, are known to signal via the canonical Wnt signaling pathway by stabilizing intracellular β-catenin levels. Because overexpression of a stabilized form of β-catenin can mimic the effects of Wnts-1, -3a, and -13 on cranial muscle formation, it seems most likely that the effects of these Wnts are mediated by the canonical Wnt signaling pathway. Whereas in the trunk activation of this pathway triggers myogenic differentiation, in the head myogenic differentiation is blocked. It will be interesting to determine if the opposite effects of increased β-catenin levels on myogenesis in the head and trunk are both mediated by induction of TCF/LEF1 transcriptional activity. In contrast to the differential effects of Wnt signaling on cranial versus trunk paraxial mesoderm, BMP signals were found to repress myogenesis in either cranial (this report) or trunk (Pourquié et al. 1996; Hirsinger et al. 1997; McMahon et al. 1998; Reshef et al. 1998; Amthor et al. 1999) paraxial mesoderm. Consistent with the inhibitory affects of BMPs and Wnt signals on cranial muscle formation, we have found that induction of skeletal muscle in stage 8 cranial paraxial mesodermal explants requires the presence of BMP inhibitors such as Noggin and Gremlin, and is augmented by Wnt inhibitors such as Frzb. Interestingly, both BMP and Wnt signaling antagonists are secreted by both CNC cells and other tissues surrounding the cranial muscle anlagen.

Both Wnt and BMP signals may act to restrict head muscle formation in the vicinity of the neural tube

To test whether the levels of BMP and Wnt signaling present in the embryo have the same effect on myogenic differentiation as purified factors, we cultured the cranial paraxial mesoderm with either the neural tube/notochord complex, the dorsal neural tube, or the ventral neural tube plus notochord. We found that both the neural tube/notochord complex or the dorsal neural tube alone blocked myogenic differentiation of cranial paraxial mesoderm similar to overexpression of either BMP or Wnt molecules in this tissue. The repressive effect of the dorsal neural tube on cranial myogenesis could be partially reversed (in 40% of explants) by administration of the Wnt antagonist Frzb-IgG. These findings suggest that Wnt signals from the dorsal neural tube may act to inhibit or delay cranial myogenic differentiation in the embryo. However, although application of ectopic Shh was also able to block myogenesis of cranial mesoderm both in vitro and in vivo, coculture of this tissue with the ventral neural tube/notochord complex, which expresses presumably lower physiological levels of Shh, did not apparently interfere with this process. In this latter experiment, our assay did not distinguish whether myogenesis was selectively inhibited in cranial paraxial mesodermal cells that lay close to the ventral midline structures as opposed to mesodermal cells that had migrated away from the floor plate and notochord. However, because extirpation of both the notochord and floor plate of the neural tube in the cranial region of stage 10 chick embryos failed to affect head muscle formation in vivo (S. Dietrich, unpubl.), it seems most likely that physiological levels of Shh present in the notochord and floor plate of the neural tube may not be involved in the control of cranial muscle formation.

In the trunk, the dorsal neural tube specifically stimulates the development of the epaxial muscle anlagen, which remain in the vicinity of the axial midline tissues to give rise to the deep muscles of the back (Burke and Nowicki 2003). In contrast, cranial skeletal muscle anlagen develop at a distance from the dorsal neural tube (Noden 1983a, 1986; Couly et al. 1992; for review, see Wachtler and Jacob 1986), in either the core of the BAs (branchiomeric muscles) or around the eye (extraocular muscles). It is therefore conceivable that both Wnt and BMP signals secreted by the dorsal neural tube block cranial muscle differentiation in the vicinity of the axial tissues. In addition, both BMP family members and Wnt-13 (Wnt2b), which are able to block muscle differentiation in cranial mesoderm explants, are secreted by the ectoderm overlying the BAs, suggesting that even at their final location, differentiation of cranial muscle precursors may be repressed by ectodermal signals. Indeed, in stage 8 CPMEE explants, we found that removal of *BMP-4*/*BMP-7*/*Wnt-13*-expressing ectoderm derepressed skeletal muscle differentiation in this tissue. In the limb, BMP molecules limit the rate of myogenic differentiation to prevent the exhaustion of the myogenic precursor pool (Amthor et al. 1999). Similarly, it has been shown

that Wnt-1 and Wnt-3a expressed in the dorsal neural tube act as mitogens in the developing spinal cord (Megason and McMahon 2002). It therefore is possible that in a similar fashion, BMP and Wnt molecules may control the balance between myogenic precursor proliferation and differentiation in the head.

Wnt and BMP inhibitors are inducers of cranial myogenesis

The repressive effect of BMP and Wnt signals on cranial myogenesis implies that these factors need to be antagonized in order to promote muscle formation in the head. In support of this idea, we found that the BMP inhibitors *Noggin* and *Gremlin*, as well as the Wnt inhibitor *Frzb*, are expressed in tissues surrounding the differentiating cranial myoblasts. In vitro, Noggin and Gremlin strongly supported myogenic differentiation of cranial mesoderm. It is formally possible that other BMP antagonists in addition to Gremlin and Noggin may also play a role in promoting cranial myogenesis. In contrast to the effects of BMP antagonists, administration of the Wnt antagonist Frzb to cranial mesoderm, stimulated cardiogenesis rather than skeletal muscle formation (Tzahor and Lassar 2001; this study), but when added in combination with Noggin could enhance skeletal myogenesis. Moreover, Frzb was able to neutralize the repressive function of the dorsal neural tube and restore myogenic differentiation in the explant culture system. In vivo implantation of Frzb-IgG-expressing cells in the cranial paraxial mesoderm similarly promoted the premature expression of *MyoD* in the BAs. These findings suggest that Frzb (and potentially other Wnt antagonists expressed in the head; i.e., *Sfrp-2* [Ladher et al. 2000]) may work in conjunction with endogenous BMP inhibitors to promote cranial myogenesis in the embryo.

Wnt and BMP inhibitors are expressed by both the cranial neural crest and other tissues in the head

Our expression analysis revealed that the CNC cells are a prominent source of BMP and Wnt inhibitors in the head. Moreover, the presence of CNC or CNC-conditioned medium triggered skeletal myogenesis of cranial mesoderm explants in vitro in the same fashion as did administration of either Noggin or Gremlin. The time course of skeletal myogenesis in these cultures correlated with the timing of expression of both these BMP inhibitors and the Wnt inhibitor *Frzb* by the CNC cells (data not shown), suggesting that secretion of these factors by CNC cells may act to promote cranial myogenesis in the head. This interpretation is in line with CNC transplantation experiments in chick and genetic studies in the zebrafish, suggesting a positive role for CNC cells in the patterning of the head musculature (Noden 1983a,b; Schilling et al. 1996; Schilling and Kimmel 1997). CNC ablation in the frog led to complex changes in muscle patterning, yet apparently not a loss of head muscles (Olsson et al. 2001). Similarly, myogenic marker genes were detected in the anlagen of the BAs as well as the extraocular muscles following CNC ablation in the chick (this study). Significantly, although the CNC-associated expression of marker genes including *Frzb* was significantly diminished in these embryos, a low level of *Frzb* expression throughout the BAs was often apparent, whereas other sites of *Frzb* expression such as in the ectoderm overlying the trigeminal ganglion and in the distal BA ectoderm remained unaffected. Thus, it is unclear whether a small number of residual CNC cells remaining in these operated embryos or other redundant sources of both BMP and Wnt antagonists secreted by adjacent tissues allowed cranial myogenesis to occur in these operated embryos. This issue can be more definitively resolved by analyzing head muscle formation in mice embryos engineered to lack either all or a subpopulation of their CNC cells.

Model for the regulation of skeletal muscle formation in the head

In summary (Fig. 7F–H), we have identified signals that may act to modulate cranial muscle formation. Our findings suggest that in the head, both BMPs and Wnts-1 and -3a, which are secreted by the dorsal neural tube, act as repressors of skeletal muscle formation, possibly to prevent myogenic differentiation of the cranial mesoderm in the vicinity of the neural tube (Fig. 7F). In stark contrast, these same Wnt molecules work in conjunction with Shh to stimulate (epaxial) myogenesis in the trunk (Fig. 7G). CNC cells, which surround the muscle anlagen in the head, secrete both BMP inhibitors (Noggin, Gremlin) and Wnt inhibitors (Frzb), which we have found to promote myogenic differentiation of the cranial mesoderm (Fig. 7H). However, because removal of a substantial amount of the CNC did not significantly negate the cranial expression of Myf-5, we surmise that other tissues in the head that similarly express either BMP and/or Wnt antagonists may serve in a redundant capacity to promote myogenic differentiation in the absence of CNC cells.

Materials and methods

In situ hybridization (ISH)

Whole-mount ISH was performed as described (Riddle et al. 1993) and double in situ staining according to Mootoosamy and Dietrich (2002). Chick DIG-labeled cRNA probes were generated from appropriate plasmids (available on request) encoding either *MyoD*, *Myf-5*, *BMP-4*, *BMP-7*, *Wnt-13*, *Shh*, *Gremlin*, *Noggin*, *Frzb*, or "Rsc" (probe for the RCAS virus).

Explant culture and analysis

Dissection of the cranial tissue explants was performed with tungsten needles in Tyrode solution using 1% agar dishes as a base. Techniques for CNC isolation and abation in vivo are available upon request. Explants were cultured in serum-free medium containing insulin, transferrin, and selenium and supplemented with 2% chick embryo extract (Life Technolo-

gies). Purified Shh-N protein was added to a final concentration of 200 ng/mL, and BMP4 protein to 60 ng/mL, as previously described (Murtaugh et al. 1999). Noggin or control-conditioned medium prepared from CHO B3 or CHO dhfr- cells, respectively (generously provided by Dr. Richard Harland, University of California, Berkeley), as described (Murtaugh et al. 1999), was used as a 75%:25% mixture of normal culture medium and conditioned medium. Individual explants were lysed and RNA was purified using a QIAGEN Rneasy minikit. RT-PCR was performed to evaluate gene expression in single explants as described in Munsterberg et al. (1995), and the PCR products amplified from the RNA of a single explant was separated by polyacrylamide gel electrophoresis. PCR primers used are available on request.

Retroviral infection

RCAS constructs for *GFP*, *Wnt-3a*, a stabilized form of β-*catenin*, *Wnt-13*, *Noggin* (gift of Randy Johnson, M.D. Anderson Cancer Center), and *Gremlin* (gift of J.-C. Belmonte, Salk Institute) were grown as described (Morgan and Fekete 1996).

Acknowledgments

This work was supported by grants to A.B.L. from the N.I.H. (GM054879) and the Muscular Dystrophy Association, to S.D. from the BBSRC, to C.J.T. from the NIH (PO1 DK56246), and to E.T. from the Muscular Dystrophy Association. E.T. was previously supported by an EMBO fellowship. Support for H.K. was provided by a fellowship from ARC and a long-term fellowship from the Human Frontier Science Program. H.K. is a recipient of a Fondation Bettencourt Schueller Award. R.C.M. is supported by a KCL Dental School fellowship. A.A. is supported by the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship.

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