Positive and Negative Regulation of Muscle Cell Identity by Members of the *hedgehog* and *TGF-β* Gene Families

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Abstract. We have examined whether the development of embryonic muscle fiber type is regulated by competing influences between Hedgehog and TGF-β signals, as previously shown for development of neuronal cell identity in the neural tube. We found that ectopic expression of Hedgehogs or inhibition of protein kinase A in zebrafish embryos induces slow muscle precursors throughout the somite but muscle pioneer cells only in the middle of the somite. Ectopic expression in the notochord of Dorsalin-1, a member of the TGF-β superfamily, inhibits the formation of muscle pioneer cells, demonstrating that TGF-β signals can antagonize the induction of muscle pioneer cells by Hedgehog. We propose that a Hedgehog signal first induces the formation of slow muscle precursor cells, and subsequent Hedgehog and TGF-β signals exert competing positive and negative influences on the development of muscle pioneer cells.

During vertebrate embryogenesis, the paraxial mesoderm gives rise to somites, which are paired blocks of mesoderm that lie adjacent to the notochord and neural tube. As somites mature, they become subdivided, with cells in different regions of the somite developing into different cell types, sclerotome, myotome, and dermatome. The differentiation of the somite into specific cell types is under the influence of inductive signals from surrounding tissues, such as notochord, neural tube, and the surface ectoderm (for review, see Hauschka, 1994; Christ and Ordahl, 1995).

A variety of extracellular signaling molecules, including members of *hedgehog* (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994), *Wnt* (Munsterberg et al., 1995), and *TGF-β* (Pourquié et al., 1996) gene families, have been implicated in patterning the somite. Ventral midline tissues express Sonic hedgehog, which plays a critical role in sclerotome and myotome induction (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994). Wnts, which are expressed in the neural tube, act in combination with Hedgehog to induce myogenesis in vitro (Munsterberg et al., 1995). Lateral plate mesoderm in chick embryos expresses BMP4, a *TGF-β* gene family member that is a candidate for inducing the differentiation of the lateral myogenic precursors in the somite, which give rise to the muscles of the limbs and body wall (Pourquié et al., 1996). This effect of BMP4 is opposed by an unknown diffusible factor expressed in the neural tube (Pourquié et al., 1996).

Vertebrate skeletal muscle contains muscle fibers of several types, which can be broadly classified as slow or fast fibers on the basis of differences in contraction speeds, metabolic activities, and motoneuron innervation. The earliest developing embryonic muscle fibers have intrinsic fiber type properties (Butler et al., 1982; Thornell et al., 1984; Crow and Stockdale, 1986; Harris et al., 1989; Fredette and Landmesser, 1991*a*,*b*; Hughes et al., 1993; Devoto et al., 1996*b*). Transplantation experiments and in vitro clonal analyses have demonstrated that these early myoblasts are committed to form particular fiber types (Miller and Stockdale, 1986*a*,*b*; Van Swearingen and Lance-Jones, 1995). However, the factors that regulate the embryonic development of myogenic precursor cell identity are still unknown.

We have examined the potential roles of members of the *hedgehog* and *TGF-β* gene families in the development of different muscle fiber types in zebrafish. We provide evidence that slow muscle cells are induced by Hedgehogs, and that this induction is likely due to respecification of fast muscle precursor cells into slow muscle cells. We also show that ectopic expression of Hedgehogs induces supernumerary muscle pioneer cells. This induction of muscle pioneers is repressed by ectopic expression in the notochord of Dorsalin-1, a BMP4-related protein. Our data suggest that members of the *hedgehog* and *TGF-β* gene families play opposing roles in patterning the developing somite.

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Materials and Methods

Animals

Embryos were staged by hours (h) after fertilization at 28.5°C (Kimmel et al., 1995; available at World Wide Web address http://zfish.uoregon.edu). Chorions were removed with watchmaker's forceps, and embryos were maintained in Ringer's solution (Westerfield, 1995). Older embryos were anesthetized in a 0.6 mM solution of tricaine (Sigma Chemical Co., St. Louis, MO) to inhibit movement during observation.

Plasmid Constructions

pCS-twhh-β-gal. To link the *tiggy-winkle hedgehog* (*twhh*)[1] promoter to the gene encoding nuclear β-galactosidase (*nβ-gal*), plasmid pGEM-7Z-*twhh*5.5 containing a 5.5-kb genomic fragment of the *twhh* gene (Ekker et al., 1995*b*) was digested with SacI and BamHI and then deleted at the 3′ end of the promoter to position −49 with respect to the ATG translation start codon by EXO-III nuclease using an Erase-a-Base kit (Promega Corp., Madison, WI). The resulting plasmid, pGEM-7Z-*twhh*5.2, containing the 5.2-kb *twhh* promoter, was partially digested with BstXI and EcoRI to release the DNA insert. The EcoRI site at the 5′ end of the insert was then blunted by Klenow DNA polymerase and subcloned into the pBluescript-SK BstXI site that had been partially blunted by T4 DNA polymerase. The resulting plasmid, pBluescript-SK-*twhh*5.2, was digested with SacI and then blunted by T4 DNA polymerase. This linearized plasmid, pBluescript-SK-*twhh*5.2, was subsequently digested with SalI. The DNA insert containing the 5.2-kb *twhh* promoter sequence was purified and cloned into plasmid pCS-*nβ-gal* (a gift from D. Turner, R. Rupp, J. Lee, and H. Weintraub, Fred Hutchinson Cancer Research Center, Seattle, WA) at the SalI and HindIII sites, the latter site having been blunted by Klenow DNA polymerase. The resulting plasmid, pCS-*twhh-β-gal*, contains the 5.2-kb *twhh* promoter and the nuclear *β-gal* reporter gene.

pCS-twhh-β-gal-vec. To make the *twhh* promoter into a convenient expression vector for expressing heterologous cDNAs, the BamHI site upstream of the *twhh* promoter in the plasmid pCS-*twhh-β-gal* was deleted. The resulting plasmid, which retains the BamHI site between the promoter and the *β-gal*, was isolated and designated pCS-*twhh-β-gal*-vec. Genes of interest can be cloned into the BamHI and XhoI sites of this vector by replacing the *β-gal* sequence.

pCS-twhh-bGFP. The reporter gene, "bright" green fluorescent protein (bGFP) with a serine 65 to threonine mutation (Heim et al., 1995), was cloned into vector pCS-*twhh-β-gal*-vec BamHI/XhoI sites by blunt end ligation. The resulting plasmid was named pCS-*twhh-bGFP.*

pCS-twhh-dsl-1^{myc}. *dorsalin-1* cDNA was amplified from 9-d chick embryos by reverse transcriptase PCR using primers based on the published chick *dsl-1* DNA sequence (Basler et al., 1993). The sequences for the 5′ and 3′ PCR primers were 5′ CTCTGTCTGTAAAGATTCAAC 3′ and 5′ GTACAGTTTCACAGACAGCAG 3′, respectively. The PCR product was subcloned into the pCR-II vector (Invitrogen, San Diego, CA). The c-myc–tagged derivative (*dsl-1*^{myc}) was constructed as previously described (Basler et al., 1993), with all subcloning steps carried out in the pCR-II vector. To place *dsl-1*^{myc} after the *twhh* promoter, the DNA insert of *dsl-1*^{myc} was first subcloned into the EcoRI site of expression vector pCS^{2+} (a gift from D. Turner, R. Rupp, J. Lee, and H. Weintraub), which has a cytomegalovirus promoter and a polyadenylation site. The resulting plasmids were named pCS^{2+}-*dsl-1*^{myc}. To link *dsl-1*^{myc} to the *twhh* promoter, the *dsl-1*^{myc} insert was released from pCS^{2+}-*dsl-1*^{myc} by BamHI and XhoI digestion and then subcloned into pCS-*twhh-β-gal*-vec BamHI and XhoI sites by replacing the *β-gal* sequence. The final construct, pCS-*twhh-dsl-1*^{myc}, contains the 5.2-kb *twhh* promoter and *dsl-1*^{myc}.

pT7TSshh, pT7TStwhh, pT7TS-X-shhfs, pCS2^{+}dnPKA-GFP, and pSP64T-PKA*. Plasmid pT7TS*shh* and pT7TS*twhh* contain the zebrafish *shh* and *twhh* cDNAs, respectively (Ekker et al., 1995*b*); plasmid pT7TS-X-*shhfs* contains the *Xenopus shh* with a single base pair insertion, resulting in a frame shift in amino acid position No. 39 (Ekker et al., 1995*a*). Plasmid pCS2^{+}*dnPKA-GFP* contains the dominant negative form of PKA regulatory subunit (Ungar and Moon, 1996). Plasmid pSP64T-*PKA** contains the constitutively active PKA catalytic subunit (Hammerschmidt et al., 1996*a*).

1. *Abbreviations used in this paper*: β-gal, β-galactosidase; bGFP, bright green fluorescence protein; PKA, protein kinase A; *twhh*, *tiggy-winkle hedgehog.*

In Vitro mRNA Synthesis

shh and *twhh* RNAs were transcribed from DNA plasmid T7TS*shh* or T7TS*twhh* as described (Ekker et al., 1995*b*). Capped mRNAs were transcribed from linearized DNA template with a T7 RNA polymerase in vitro transcription kit (mMESSAGE mMACHINE T7, Ambion, Inc., Austin, TX) according to the manufacturer's instructions.

β-Gal Labeling

β-Gal labeling was carried out with minor modification of published procedures (Westerfield et al., 1992). Embryos were fixed for 30 min at room temperature with rotation, with or without removing the chorion, in 4% paraformaldehyde, 0.2% glutaraldehyde, 4% sucrose, 0.15 mM $CaCl_2$, and 1× PBS. To visualize β-gal activity, embryos were rinsed twice for 15 min with PBS containing 0.1% Triton X-100 and then incubated in reaction solution containing 0.04% x-gal (bromo-4-chloro-indoxyl-β-D-galactoside), 1 mM $MgCl_2$, 3.3 mM $K_4[Fe_3(CN)_6]$, and 3.3 mM $K_3[Fe_2(CN)_6]$ at room temperature for 1–2 h. The reaction was stopped by replacing the substrate solution with PBS.

Microinjection

For DNA microinjection, linearized DNA was dissolved in distilled H_2O to a final concentration of 50 μg/ml. For mRNA injection, mRNA was dissolved in distilled H_2O to a final concentration of 100 μg/ml. A final concentration of 0.1% phenol red was added to the DNA or RNA solution to facilitate visualization during microinjection. Approximately 2 nl of DNA or RNA solution was microinjected into the cytoplasm of zebrafish embryos at the one- or two-cell stage.

Antibody Labeling

For antibody labeling, embryos were fixed in 4% paraformaldehyde in 1× PBS for 2 h at room temperature. Embryos were washed twice for 5 min in 1× PBS and once for 5 min in water. Embryos were then soaked in cold acetone for 10 min at −20°C. Embryos were washed once with water for 5 min, and twice with 1× PBS for 5 min each, and once with BDP (0.1% bovine serum albumin, 1% dimethylsulfoxide, 1× PBS) for 5 min. For labeling using monoclonal antibodies, the embryos were incubated with avidin-blocking reagent (Vector Laboratories, Burlingame, CA) and 10% goat serum in BDP for 30 min at room temperature. The embryos were then rinsed twice for 5 min in BDP and subsequently incubated with 1:5 diluted antiengrailed monoclonal antibody 4D9 (Patel et al., 1989) and/or 1:500 diluted anti–c-myc monoclonal antibody (Oncogene Science, Cambridge, MA) together with biotin-blocking reagent (Vector Laboratories) overnight at 4°C with shaking. The embryos were then washed three times for 30 min with BDP, followed by incubation with 1:500 diluted biotin-labeled secondary antibody (Vector Laboratories) in BDP for 1 h at 37°C. Embryos were then washed three times for 30 min with BDP and incubated with 1:1 diluted ABC solution (avidin biotin complex) for 30 min at room temperature. Embryos were washed three times for 30 min in BDP and then presoaked in DAB solution (0.05% diaminobezidine, 1% DMSO, 1× PBS) for 10 min at room temperature. The DAB soaking solution was then replaced by DAB staining solution containing 0.003% of H_2O_2 in DAB soaking solution. The staining was monitored and stopped by washing twice for 10 min with BDP. Embryos were photographed in PBS.

Immunofluorescent labeling of sections with the F59 and 4D9 monoclonal antibodies was done as previously described (Crow and Stockdale, 1986; Devoto et al., 1996*b*).

Results

Zebrafish Muscle Fiber Type Development

Three distinct types of embryonic muscle fibers can be identified in zebrafish based on position, gene expression, and pattern of immunoreactivity with several monoclonal antibodies. Their development is summarized in Fig. 1. Slow muscle precursors, known as adaxial cells, develop adjacent to the notochord and then migrate radially through the somite to become a monolayer of muscle cells on the surface of the myotome (Devoto et al., 1996*b*). A

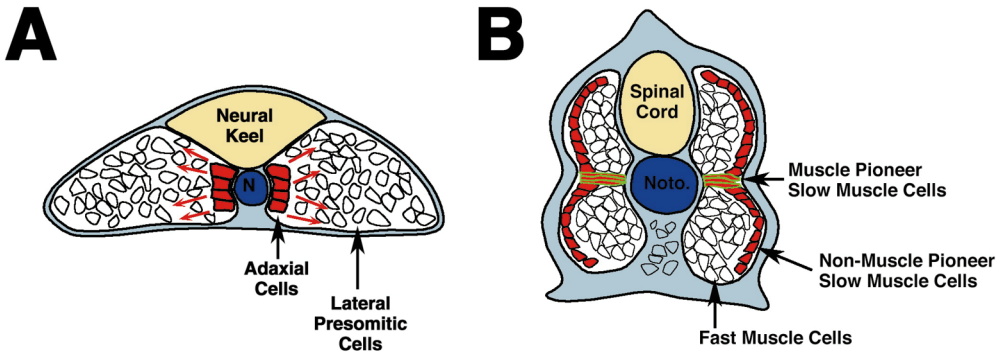

Figure 1. Development of zebrafish muscle fiber types. (*A*) Schematic transverse section through the segmental plate. Adaxial cells (*red*) are in the segmental plate adjacent to the notochord (*N*) and are precursors to both muscle pioneer slow muscle cells and non–muscle pioneer slow muscle cells. Lateral presomitic cells (*white*), which include precursors to fast muscle cells, are lateral to the adaxial cells. Arrows indicate that adaxial cells will migrate past the lateral presomitic cells after somite formation. (*B*) Schematic transverse section through the trunk of a 24 h embryo. The non–muscle pioneer slow muscle cells (*red with black outline*) have migrated to the surface of the myotome, while the muscle pioneer slow muscle cells (*red with green outline*) have become flattened cells that contact both the notochord and the lateral surface of the myotome. The fast muscle cells (*white*) are now deep in the myotome.

subset of the slow muscle precursors, located at the future horizontal myoseptum, remain in contact with the notochord and become flattened cells that extend from the notochord to the lateral surface of the myotome (Waterman, 1969; van Raamsdonk et al., 1974; Devoto et al., 1996*b*). These cells, called muscle pioneers (Felsenfeld et al., 1991), are the only slow muscle precursors to express the *engrailed1* and *engrailed2* genes at high levels (Hatta et al., 1991; Ekker et al., 1992). Fast muscle precursors, in contrast, develop from lateral presomitic cells and remain deep within the myotome.

Ectopic Expression of hedgehogs Induces Extra Slow Muscle Cells

Previous studies have shown that ectopic expression of Hedgehog induces MyoD and supernumerary muscle pioneer cells, suggesting that it may play an important role in muscle development in zebrafish (Currie and Ingham, 1996; Hammerschmidt et al., 1996*a*; Weinberg et al., 1996). To examine directly whether *hedgehog* genes influence the development of muscle fiber type identity, we expressed zebrafish *sonic hedgehog* or *tiggy-winkle hedgehog* ectopically by injection of RNA into cleavage stage embryos. We then examined the developing embryos for induction of slow muscle cells using several monoclonal antibodies that recognize the entire population of slow muscle cells, including the muscle pioneers. F59 recognizes myosin heavy chain in fish (Miller et al., 1989); in zebrafish it specifically labels slow muscle fibers during the first day of development, and then later it also weakly labels fast muscle fibers (Devoto et al., 1996*b*). We also used zn5 (Trevarrow et al., 1990) and S58 (Crow and Stockdale, 1986) antibodies that also label slow but never label fast muscle fibers in zebrafish (Devoto et al., 1996*b*). We found that both Sonic hedgehog and Tiggy-winkle hedgehog induced the development of many extra slow muscle cells. Specifically, as in uninjected embryos, only one layer of slow muscle cells was present in the superficial layer of the somite in control embryos injected with frame-shifted *sonic hedgehog* RNA (Fig. 2 *A*), whereas in embryos injected with *sonic hedgehog* (Fig. 2 *B*) or *tiggy-winkle hedgehog* (Fig. 2 *C*) RNA, almost all cells in the somite differentiated into slow muscle. These ectopic slow muscle cells were also labeled by the S58 and zn5 antibodies, indicating that these cells had fully differentiated as slow muscle fibers (data not shown). Presumably, these extra slow muscle cells are formed at the expense of fast muscle because they occupy the locations where fast muscle cells normally form, and because nearly all the muscle cells in the somite exhibited these slow muscle properties. Both Sonic hedgehog (Fig. 2, *E* and *H*) and Tiggy-winkle hedgehog (Fig. 2, *F* and *I*) also induced extra muscle pioneer cells, as determined by labeling with the anti–engrailed monoclonal antibody, 4D9. In control embryos injected with frame-shifted *sonic hedgehog*, two to six muscle pioneer cells were normally present in each somite (Fig. 2, *D* and *G*) as in uninjected embryos, whereas Sonic hedgehog induced an average of 20 muscle pioneer cells per somite (88%, $n = 87$; Fig. 2, *E* and *H*), and Tiggy-winkle hedgehog induced an average of 10 muscle pioneer cells per somite (75%, $n = 105$; Fig. 2, *F* and *I*).

Hedgehog Signaling Is Required for Slow Muscle Development

Protein kinase A (PKA) is an integral part of the Hedgehog signaling pathway (for review see Perrimon, 1995). PKA constitutively represses Hedgehog target genes, and Hedgehog acts to relieve this repression. Thus, expression of a dominant negative isoform of PKA mimics Hedgehog signaling in both *Drosophila* (Jiang and Struhl, 1995; Li et al., 1995; Pan and Rubin, 1995) and in vertebrates (Fan et al., 1995; Hammerschmidt et al., 1996*a*; Ungar and Moon, 1996). Our results (Fig. 2) suggested that Hedgehog is sufficient to trigger slow muscle development. To test whether Hedgehog signaling is required for slow muscle development, we ectopically expressed the constitutively active PKA isoform (Orellana and McKnight, 1992). Compared with control embryos (Fig. 3 *A*), slow muscle cells labeled with F59 antibody appeared to be absent in embryos injected with RNA encoding the constitutively active isoform of PKA (Fig. 3 *B*). Frequently, injected RNAs are localized to one region of the embryo (Hammerschmidt et al., 1996*a*). Consistent with this, transverse sections through control (Fig. 3 *C*) and active PKA-injected embryos demonstrated a local loss of slow muscle cells in the active PKA injected embryos (Fig. 3 *D*). Together with

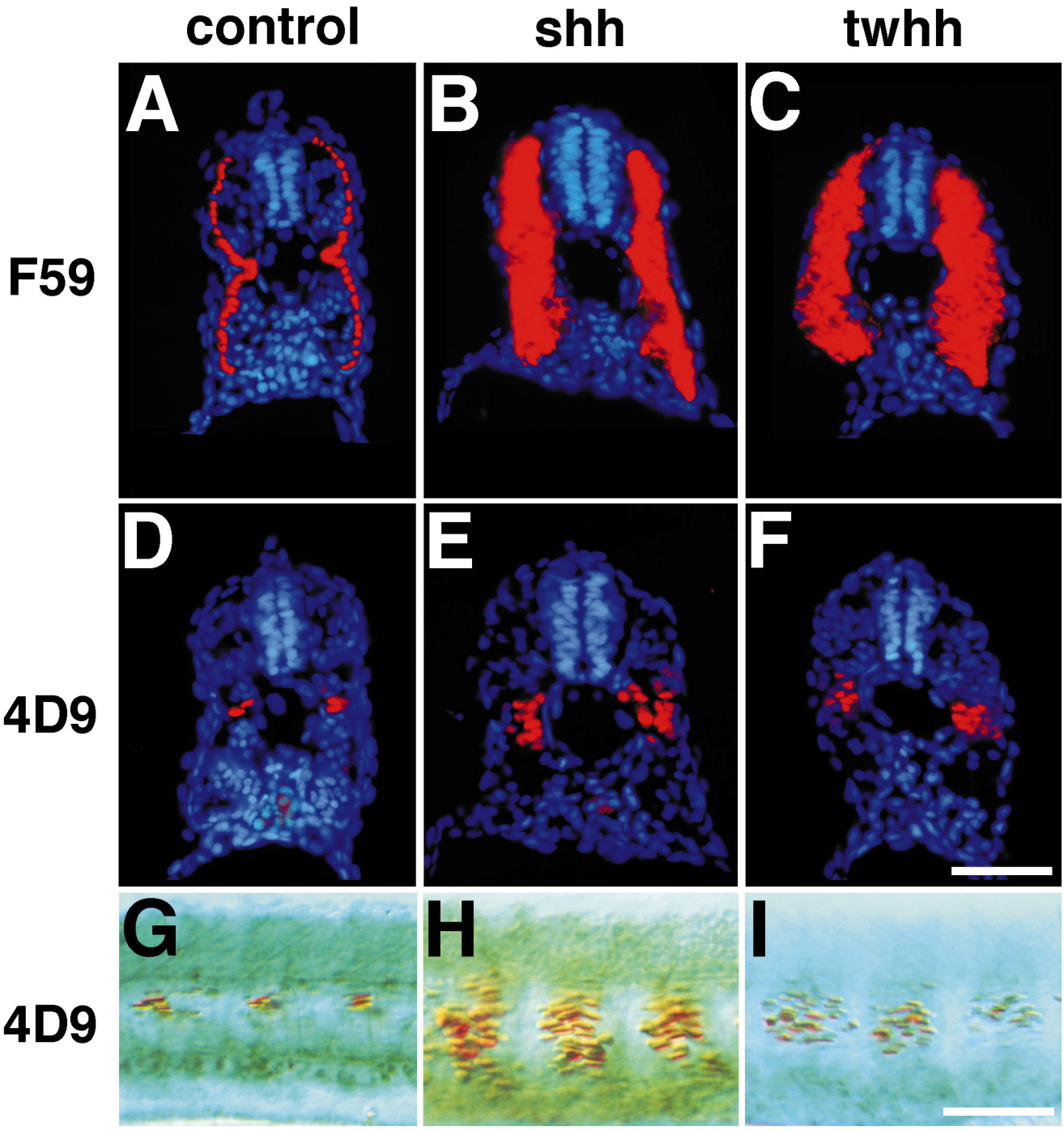

Figure 2. Induction of slow muscle cells by zebrafish Sonic hedgehog (Shh) and Tiggy-winkle hedgehog (Twhh). (*A*, *B*, and *C*) Sections (dorsal to the top) showing fluorescence localization of slow muscle cells labeled with F59, an anti–myosin heavy chain antibody, in embryos injected with frame shifted sonic hedgehog (Shhfs) (*A*), Shh (*B*), or Twhh (*C*). (*D*, *E*, and *F*) Sections (dorsal to the top) showing fluorescence localization of muscle pioneer cells labeled with 4D9, an anti-engrailed antibody, in embryos injected with Shhfs (*D*), Shh (*E*), or Twhh (*F*). (*G*, *H*, and *I*) Whole-mount Nomarski images showing muscle pioneer cells labeled with the 4D9 antibody in embryos injected with Shhfs (*G*), Shh (*H*), or Twhh (*I*). Embryos in *G*, *H*, and *I* are oriented in side views, with anterior to the left and dorsal to the top. Bars, 50 μm.

the Hedgehog ectopic expression data (Fig. 2), this result suggests that Hedgehog signaling is required for the development of all slow muscle cells, including muscle pioneer cells (Hammerschmidt et al., 1996*a*, and data not shown).

Ectopic Expression of Dorsalin-1 in the Notochord Inhibits Muscle Pioneer Development

Interestingly, we observed that the ectopic muscle pioneer cells induced by Hedgehogs appeared only in the region of

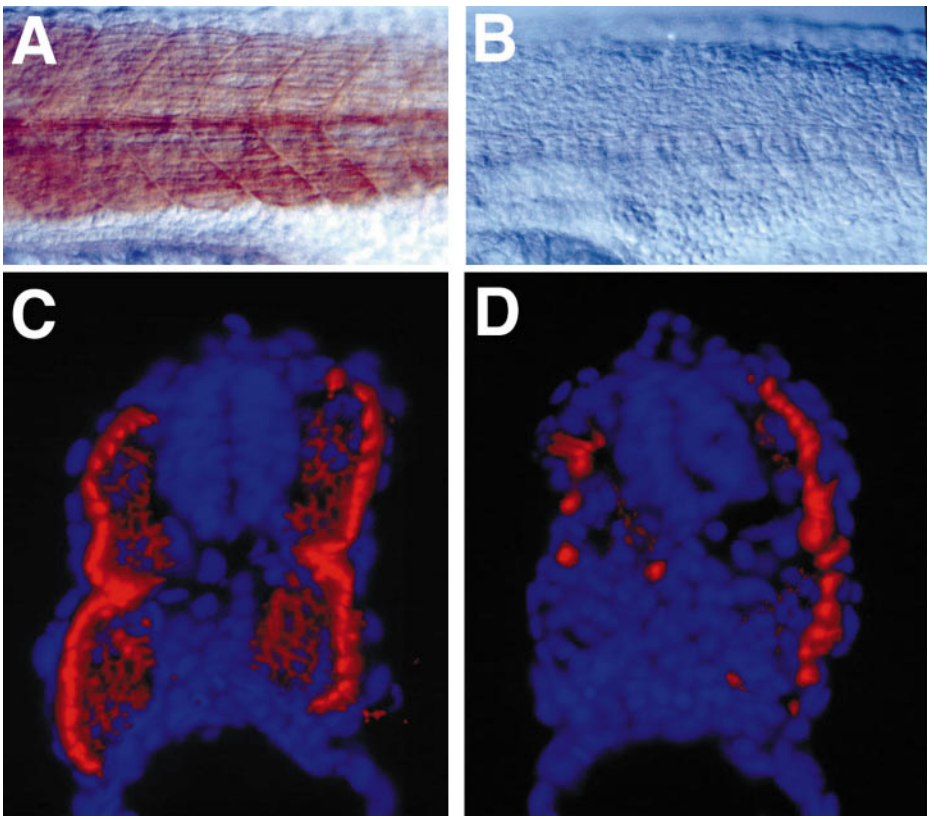

Figure 3. Inhibition of slow muscle cells by a constitutively active isoform of PKA. (*A*) Whole-mount Nomarski images showing slow muscle cells labeled with the F59 antibody in a representative control embryo. (*B*) Whole-mount Nomarski images showing slow muscle cells labeled with the F59 antibody in a representative embryo injected with a constitutively active form of PKA. (*C*) Section (dorsal to the top) showing localization of slow muscle cells labeled with F59 in a control embryo. (*D*) Sections (dorsal to the top) showing local loss of slow muscle cells in an embryo injected with a constitutively active form of PKA.

the somite nearest the notochord; ectopic muscle pioneers were absent in the dorsal or ventral thirds of the somite. Because Hedgehogs were active in the induction of non–muscle pioneer slow muscle cells in these regions, this suggested that inhibitory signals from tissues near these regions may antagonize the activity of Hedgehogs.

Competition between BMP4 expressed in the dorsal neural tube and Sonic hedgehog expressed in the ventral neural tube has been shown to play an important role in dorsoventral patterning of the spinal cord (Basler et al., 1993; Liem et al., 1995). Somite patterning may also be regulated by competing positive and negative signals, in-

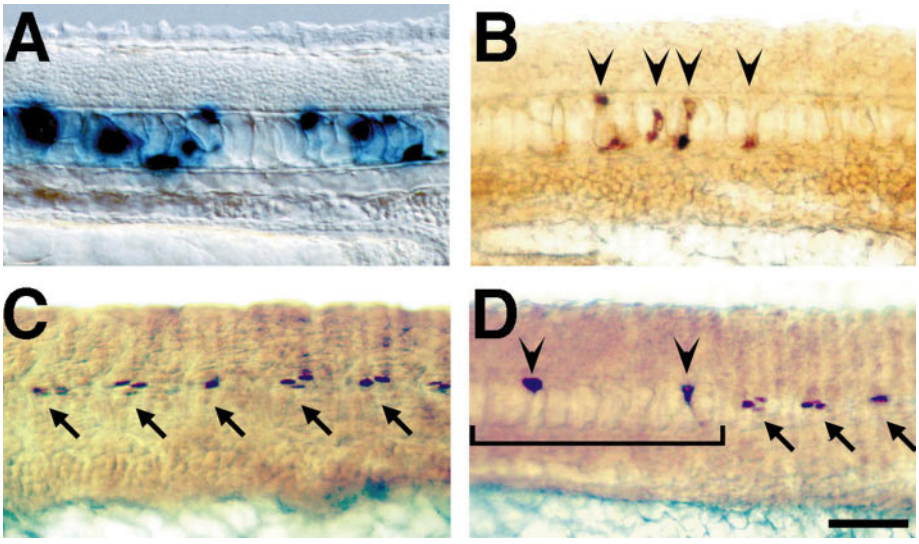

Figure 4. Dorsalin-1 blocks the development of muscle pioneer cells. (*A*) β-gal expression in embryos injected with *twhh-β-gal* was monitored by enzyme activity and Nomarski microscopy at early pharyngula stage (~24 h). At this stage, the expression of β-galactosidase was notochord specific in >90% (n = 120) of the injected embryos that expressed the construct. We first detected β-galactosidase expression in the early segmentation stage (~12 h), specifically in notochord cells of injected embryos (84%, n = 57, data not shown). (*B*) Immunolocalization with anti–c-myc antibody in embryos injected with the DNA construct *twhh-dsl-1*^{myc}. The *twhh* promoter drives expression of dsl-1^{myc} in notochord cells (*arrowheads*). (*C* and *D*) Double labeling with anti–c-myc antibody and anti-engrailed antibody, 4D9, in embryos injected with either the DNA construct *twhh-bGFP* (*C*) or *twhh-dsl-1*^{myc} (*D*). The bracket in *D* marks the region affected by Dorsalin-1. The dsl-1^{myc} expressing notochord cells in *D* are indicated by the arrowheads. Muscle pioneer cells in *C* and *D* are indicated by arrows. Cells in only some regions of the embryos expressed the transgenes (*B* and *D*, *arrowheads*), consistent with the mosaic expression of other injected DNAs (Westerfield et al., 1992). In 93% (n = 54) of the embryos lacking muscle pioneer cells in some of their somites, nearby notochord cells expressed Dorsalin-1. Embryos are oriented in side views, with anterior to the left and dorsal to the top. Bar, 50 μm.

cluding BMP4 (Fan and Tessier-Lavigne, 1994; Pourquié et al., 1996). To learn whether a BMP4-like protein can affect the development of muscle cell identity in zebrafish, we tested whether ectopic expression of Dorsalin-1, a BMP4-like factor, would inhibit the formation of muscle pioneer cells in surrounding somites. We used the chick Dorsalin-1 in this study for several reasons. First, the dorsal neural tube (Basler et al., 1993), a tissue known to play a role in somite patterning (Lassar and Munsterberg, 1996; Pourquié, et al., 1996), expresses Dorsalin-1. Second, Dorsalin-1 can antagonize Hedgehog signaling in the dorsoventral patterning of the neural tube (Basler et al., 1993; Liem et al., 1995). Third, at the time we initiated this study, no gene encoding BMP or a BMP-like protein expressed in the neural tube had been isolated from zebrafish. More recently, a BMP-like gene named *radar* was reported in zebrafish; however, this clone contains only the partial coding region (Rissi et al., 1995).

In initial experiments, we found that injection of Dorsalin-1 mRNA had a severe ventralizing effect during gastrulation, similar to that caused by injection of BMP4 mRNA (Hammerschmidt, et al., 1996*b*). Thus, to assess potential later effects on somite patterning, we expressed Dorsalin-1 in the notochord after gastrulation. Additionally, expression of Dorsalin-1 in the notochord localized the protein to the region of the somites, where we anticipated the lowest activity of the putative inhibitor of Hedgehog signaling. To express a potential inhibitor specifically in this region of the somites, we put *dorsalin-1* under the control of a promoter from the *tiggy-winkle hedgehog* gene. The floor plate normally expresses Tiggy-winkle hedgehog. Paradoxically, we found that 5.2 kb of the 5′-flanking sequence from the *tiggy-winkle hedgehog* gene leads to expression of heterologous proteins, including β-galactosidase, specifically in the notochord (Fig. 4 *A*; a further characterization of this promoter is in progress). Thus, we used this promoter fragment to express Dorsalin-1 in the notochord.

Embryos injected with the *dorsalin-1* DNA construct (*twhh-dsl-1*^{myc}) developed with apparently normal anteroposterior and dorsoventral axes. As expected, the *tiggy-winkle hedgehog* promoter drove expression of Dorsalin-1 specifically in notochord cells (Fig. 4*B*, *arrowheads*), consistent with the expression pattern of the *β-gal* reporter gene under control of the same promoter (Fig. 4*A*). To analyze whether Dorsalin-1 has an inhibitory effect on the development of muscle pioneer cells, we examined embryos injected with the *twhh-dsl-1*^{myc} for differentiation of muscle pioneer cells labeled with the 4D9 antibody. As shown by the bracket in Fig. 4 *D*, muscle pioneer cells (*arrows*) were absent in the somites adjacent to notochord cells expressing the *twhh-dsl-1*^{myc} construct. In contrast, muscle pioneer cells developed normally in embryos injected with the control construct, *twhh-bGFP* (Fig. 4 *C*, *arrows*). A single Dorsalin-1–expressing cell in the notochord was able to inhibit the formation of muscle pioneer cells in the flanking two to four somites. Usually there were more somites affected rostral than caudal to the Dorsalin-1–expressing notochord cell (data not shown). This is probably because notochord cells shift caudally relative to the somites, from about 12 h to at least 48 h (Devoto, S.H., and M. Westerfield, in preparation). This correlation between Dorsalin-1 expression in the notochord and the absence of muscle pioneer cells in adjacent somites indicates that the differentiation of muscle pioneer cells can be blocked by a BMP-like signal, establishing a BMP-like molecule as a viable candidate for an inhibitory signal that prevents muscle pioneer differentiation in the dorsal and ventral regions of the somite.

Notochord Expression of Dorsalin-1 Fails to Block the Development of Non–muscle Pioneer Slow Muscle Cells

Muscle pioneers are derived from a subset of slow muscle precursor cells, whereas most of the precursor cells develop into non–muscle pioneer slow muscle cells (Devoto et al., 1996*b*). To learn whether ectopic expression of Dorsalin-1 in the notochord inhibits the development of muscle pioneer cells specifically or whether non–muscle pioneer slow muscle cells are also affected, we injected embryos with *twhh-dsl-1*^{myc} DNA and labeled with the F59 antibody, which recognizes all of the slow muscle cells (Fig. 1; Devoto et al., 1996*b*). As shown by the bracket in Fig. 5 *A*, there was a gap in F59 labeling in the middle of some of the somites in embryos injected with *twhh-dsl-1*^{myc}. Transverse sections through unaffected regions (Fig. 5 *B*) and affected regions (Fig. 5 *C*) demonstrated that this gap in labeling is a result of the absence of the muscle pioneer population of slow muscle cells, which are normally located adjacent to the notochord (Fig. 5 *B*, *arrows*). In contrast, the dorsal and ventral populations of slow muscle cells (the non–muscle pioneer slow muscle cells; Fig. 5, *B* and *C*, *arrowheads*) are apparently unaffected by Dorsalin-1. These data demonstrate that notochord expression of Dorsalin-1 specifically interferes with the development of muscle pioneer cell identity and does not affect the development of the non–muscle pioneer slow muscle cells from adaxial cells.

Expression of Dorsalin-1 in Notochord Blocks Muscle Pioneer Cell Induction by Hedgehogs

These results demonstrate that Hedgehogs can induce slow muscle cells, including both muscle pioneers and non–muscle pioneer slow muscle cells, and that Dorsalin-1 can specifically inhibit the development of muscle pioneer cells. Dorsalin-1 could act by inhibiting the expression of *hedgehog* genes in the notochord, or by antagonizing Hedgehog protein activity. If Dorsalin-1 represses expression of *hedgehog* genes, then overexpression of Hedgehog should overcome the inhibitory effect of Dorsalin-1 on muscle pioneer formation. We tested this prediction by coinjecting Hedgehog RNAs with *twhh-dsl-1*^{myc} DNA and analyzing the injected embryos by double labeling with anti-myc antibody (labeling myc-tagged Dorsalin-1) and with the 4D9 antibody (labeling muscle pioneer cells). Compared to embryos injected with control RNA (Fig. 6 *A*), the expression of Dorsalin-1 in the notochord (Fig. 6 *C*, *arrowhead*) inhibited development of muscle pioneers in adjacent somites (Fig. 6 *C*, *bracket*), regardless of whether embryos were coinjected with RNA encoding Tiggy-winkle hedgehog (100%, $n = 65$; not shown) or Sonic hedgehog (100%, $n = 43$). In contrast, induction of muscle pioneers by Hedgehogs was unaffected in embryos coinjected with Sonic hedgehog RNA and the control DNA construct *twhh-bGFP* (Fig. 6 *B*). These data suggest

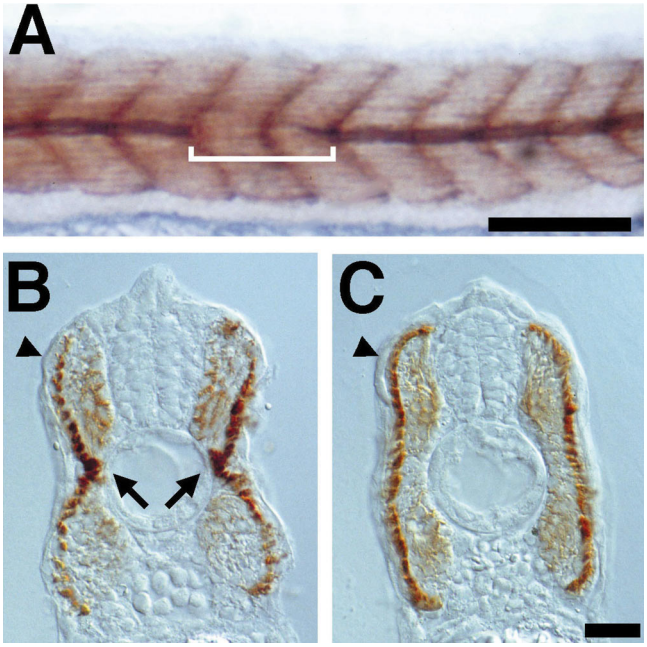

Figure 5. Dorsalin-1 specifically affects the development of muscle pioneer cells but not the non–muscle pioneer slow muscle cells. (*A*) Whole-mount staining of embryo injected with DNA construct *twhh-dsl-1*^{myc} using F59 to monitor slow muscle cells. The bracket marks the region affected by Dorsalin-1. (*B*) Section through an unaffected region with both muscle pioneer cells (*arrows*) and non–muscle pioneer slow muscle cells (*arrowhead*) labeled with F59. (*C*) Section through an affected region with normal non–muscle pioneer slow muscle cells labeled with F59 antibody (*arrowhead*) but no muscle pioneer cells. The orientation is anterior to the left (*A*) and dorsal to the top (*A–C*). Bars: (*A*) 100 μm; (*B* and *C*) 50 μm.

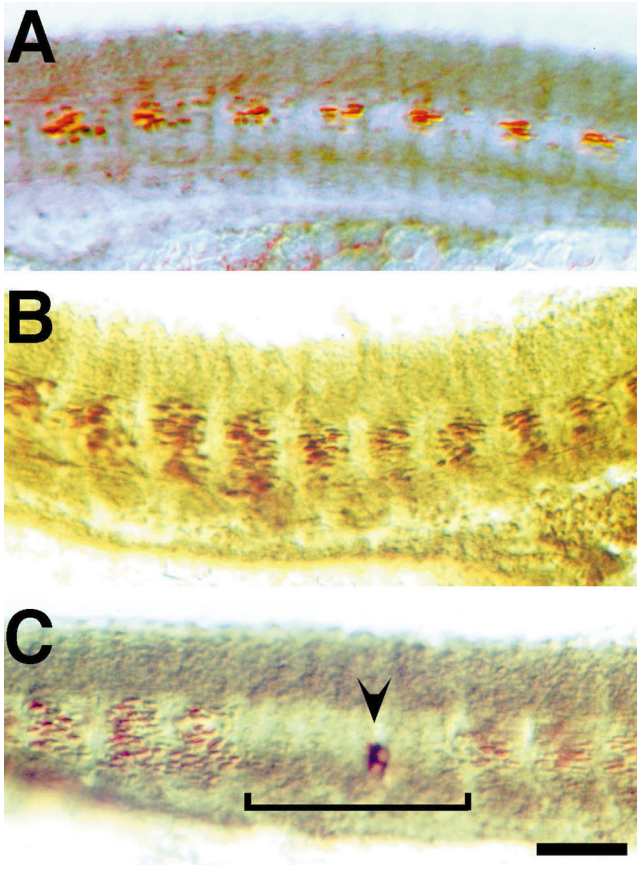

Figure 6. Ectopic Dorsalin-1 represses muscle pioneer cell induction by Hedgehogs. Embryos were double labeled with anti-myc antibody to monitor expression of dsl-1^{myc} and with the 4D9 antibody to monitor muscle pioneer cells. (*A*) Embryos injected with Shhfs RNA as control. (*B*) Embryos coinjected with *twhh-bGFP* DNA and zebrafish Shh RNA show induction of extra muscle pioneer cells. (*C*) Embryos coinjected with *twhh-dsl-1*^{myc} DNA and zebrafish Shh RNA also show induction of extra muscle pioneer cells, but not in the region (*bracket*) near the notochord cell expressing Dorsalin-1 (*arrowhead*). Embryos are oriented in side views, with anterior to the left and dorsal to the top. Bar, 50 μm.

that Dorsalin-1 blocks the differentiation of slow muscle precursor cells into muscle pioneer cells by antagonizing the activity of Hedgehogs rather than by simply inhibiting their expression.

Dorsalin-1 Likely Acts Downstream of PKA in Muscle Pioneer Development

As discussed above, Hedgehog signaling is mediated by inhibition of PKA activity (for review see Perrimon, 1995). PKA constitutively represses Hedgehog target genes, and inhibition of PKA by Hedgehog alleviates this repression. To test whether blocking PKA activity induces slow muscle cells, we injected RNA encoding a dominant negative mutant form of the PKA regulatory subunit (dnPKA; Ungar and Moon, 1996) or frame-shifted Sonic hedgehog as an injection control and then examined the somites by antibody labeling for muscle pioneer cells and for other slow muscle cells. dnPKA induced the development of extra slow muscle cells (94%, $n = 102$), including both the muscle pioneer (Fig. 7, *E* and *H*) and non–muscle pioneer slow muscle cells (Fig. 7 *B*). In contrast, injection of frame-shifted *sonic hedgehog* as a control had no effect on the development of slow muscle cells (Fig. 7, *A*, *D*, and *G*).

To learn whether Dorsalin-1 can antagonize the ability of dnPKA to induce both muscle pioneer cells and non–muscle pioneer slow muscle cells, we coinjected dnPKA RNA and *twhh-dsl-1*^{myc} DNA into zebrafish embryos and labeled serial sections or whole-mount embryos with the 4D9 antibody (to detect muscle pioneer cells) or with the F59 antibody (to detect all slow muscle cells). We found that Dorsalin-1 inhibited muscle pioneer induction by dnPKA (100%, $n = 84$; Fig. 7 *F* compared to *E*, and Fig. 7 *I* compared to *H*; *arrowhead* in *I* indicates Dorsalin-1–expressing cell). This result suggests that the inhibitory effect of Dorsalin-1 on muscle pioneers is downstream of PKA activity. In contrast, induction of non–muscle pioneer slow muscle cells by dnPKA was apparently unaffected by Dorsalin-1 (Fig. 7 *C* compared to *B*). In many dnPKA/*twhh-dsl-1*^{myc} coinjected embryos, the entire somite was transformed into slow muscle cells, even in regions where there were no muscle pioneers because of the action of Dorsalin-1. This is consistent with the results obtained in embryos injected with the *twhh-dsl-1*^{myc} DNA construct alone (Fig. 5). Dorsalin-1 apparently affected only the muscle pi-

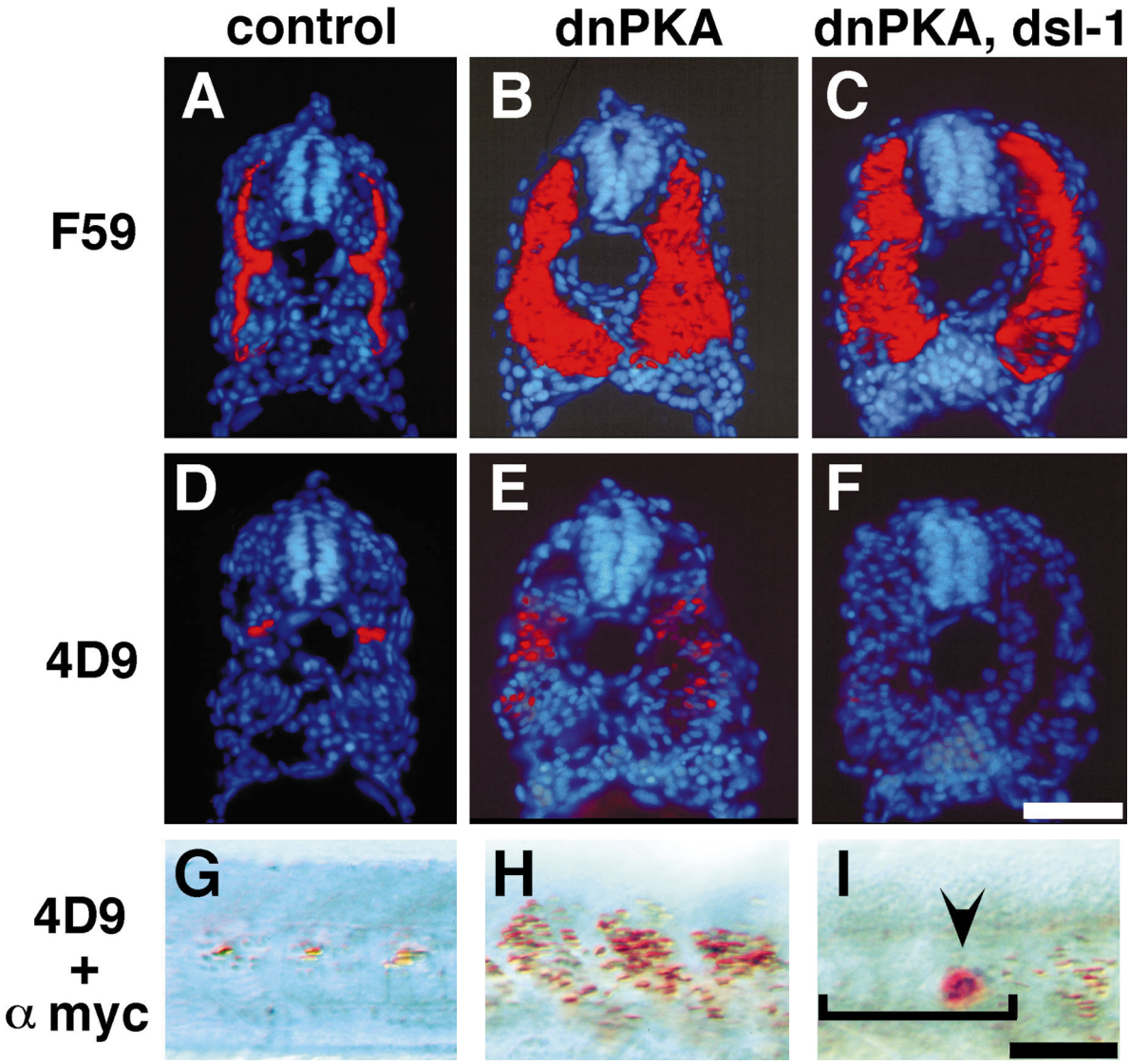

Figure 7. dnPKA induces slow muscle cells. (*A–C*) Sections showing slow muscle cells labeled with F59 in embryos injected with Shhfs (*A*), dnPKA (*B*), or dnPKA/*twhh-dsl-1*^{myc} (*C*). Extra slow muscle cells are induced in both *B* and *C*. (*D–F*) Sections showing muscle pioneer cells labeled with 4D9 in embryos injected with Shhfs (*D*), dnPKA (*E*), or dnPKA/*twhh-dsl-1*^{myc} (*F*). Muscle pioneer cells are induced by dnPKA (*E*) but are repressed by dsl-1 (*F*). (*G–I*) Whole-mount labeling with anti–*c-myc* and 4D9 antibodies in embryos injected with Shhfs (*G*), dnPKA (*H*), or dnPKA/*twhh-dsl-1*^{myc} (*I*). The bracket in *I* marks the region affected by Dorsalin-1. The dsl-1^{myc} expressing cell in *I* is indicated by the arrowhead. The orientation is dorsal to the top (*A–I*) and anterior to the left (*G–I*). Bar, 50 μm.

oneer population of slow muscle cells. These data confirm that the inhibitory effect of Dorsalin-1 on formation of slow muscle cells is specific for muscle pioneer cells. Together, these data suggest that Hedgehog signaling induces slow muscle cells and that BMP-like signaling is involved in the specification of distinct slow muscle cell identities.

Discussion

We have investigated the mechanisms regulating the induction and differentiation of slow muscle fibers in zebrafish. Our results suggest that Hedgehog signals are involved in the initial induction of slow muscle precursor cells, whereas the subsequent differentiation of these precursors into distinct types of embryonic slow muscle cells may involve an inhibitory TGF-β signal. This proposed inhibitory signal antagonizes the Hedgehog activity in dorsal and ventral regions of the somite. Our data suggest that opposing actions of *hedgehog* and *TGF-β* gene family members may regulate the differentiation of specific slow muscle fiber cell types in the zebrafish somite.

Induction of Slow Muscle by Hedgehogs

We have shown that ectopic expression of members of the *hedgehog* gene family during early zebrafish development induces extra slow muscle cells, suggesting that Hedgehog signaling participates in the establishment of slow muscle cell identity. This is further supported by our observation that inhibition of PKA, likely to occur during Hedgehog signaling, mimics the activity of Hedgehog in slow muscle

induction and that constitutive activation of PKA blocks the development of slow muscle cells. Several observations support the hypothesis that one or more Hedgehogs are the endogenous factors that induce the formation of slow muscle precursors during normal development. First, slow muscle precursors develop adjacent to the notochord, becoming apparent after notochord precursor cells begin to express *hedgehog* genes (Krauss et al., 1993; Roelink et al., 1994; Currie and Ingham, 1996; Devoto et al., 1996*b*). Second, all slow muscle precursors strongly express the *patched* gene, which is induced by Hedgehog signaling (Concordet et al., 1996), suggesting that they receive and respond to Hedgehog. Third, there is a loss of slow muscle cells in mutants in which Hedgehog signaling is reduced (Talbot et al., 1995; Devoto et al., 1996*a*; Weinberg et al., 1996). Together with the results reported here, these observations provide compelling evidence that Hedgehogs induce slow muscle cells.

Muscle Pioneer Induction

We found that ectopic expression of either Sonic hedgehog or Tiggy-winkle hedgehog induced ectopic muscle pioneer cells. Our data differ from a previous report that ectopic expression of Sonic hedgehog was unable to induce muscle pioneers, unless another member of the Hedgehog family, Echidna hedgehog, was coexpressed (Currie and Ingham, 1996). The reason for this discrepancy is unclear, although the two studies used different plasmids that generate RNAs with different untranslated regions. It is possible that these differences affected the stability or translation of the RNA. Our results are consistent with those reported by Hammerschmidt et al. (1996*a*), who found that ectopic expression of either mouse Sonic hedgehog or Indian hedgehog induced extra muscle pioneer cells in zebrafish embryos.

We propose that early signaling by Hedgehogs is sufficient to trigger the development of slow muscle identity, but that muscle pioneer development requires additional later exposure to Hedgehogs (Fig. 8 *A*). This hypothesis is supported by the following observations. First, slow muscle precursors are distinct from the other presomitic cells before muscle pioneers become distinct from the other slow muscle precursors. Second, injection of Hedgehog RNA was consistently more effective at inducing non–muscle pioneer slow muscle cells than muscle pioneer cells. Third, hedgehog RNA injection induces muscle pioneers more effectively in anterior somites than in posterior somites of the embryo (data not shown). If the injected hedgehog RNA is degraded over time, then there would consistently be more ectopic hedgehog early in development (in anterior somites) than there would be later in development (in posterior somites). Finally, in several mutants (*no tail*, *floating head*), Hedgehog expression becomes progressively reduced, relative to wild-type embryos, as development proceeds. In these mutants, muscle pioneers are missing, whereas other slow muscle cells develop relatively normally, especially in the earlier developing anterior somites (Devoto et al., 1996*a*).

A BMP-like Inhibitory Signal Opposing the Action of Hedgehogs on Muscle Pioneer Cells

During normal zebrafish embryogenesis, slow muscle precursor cells that move dorsally and ventrally in the somite develop into non–muscle pioneer slow muscle cells. Although ectopic expression of Hedgehogs and dnPKA induces ectopic non–muscle pioneer slow muscle cells throughout the somite, neither induces ectopic muscle pioneers in the dorsal or ventral thirds of the somite. We showed that expression in the notochord of *dorsalin-1*, a *BMP4*-like gene normally expressed in the neural tube (Basler et al., 1993), can inhibit the development of muscle pioneer cells. This suggests that an inhibitory signal such

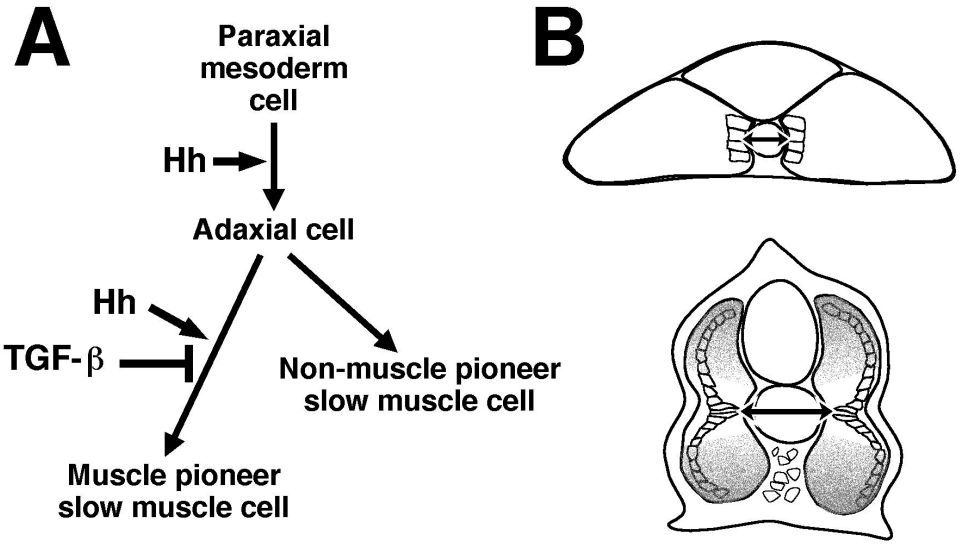

Figure 8. Opposing actions of Hedgehog and BMP4-like proteins regulate slow muscle cell identity. (*A*) At the tail bud stage, paraxial mesoderm cells are induced by Hedgehog secreted from notochord precursor cells to become adaxial cells, the slow muscle precursors. A subset of the adaxial cells located adjacent to the notochord are induced to become muscle pioneer cells by continued Hedgehog signal from the notochord cells and floor plate cells. In contrast, other slow muscle precursors migrate to the lateral surface of the myotome and differentiate into non–muscle pioneer slow muscle cells. BMP4-like inhibitory signal antagonizes the hedgehog signals in dorsal and ventral regions of the somite and blocks the induction of muscle pioneer cells in these regions. (*B*) Schematic illustration of this model in cross section at the tailbud stage (*upper embryo*, *arrow* denotes Hedgehog signal), and at the somitogenesis stage (*lower embryo*, *arrow* denotes Hedgehog signal and stippling denotes the BMP4-like signal). The distribution of muscle pioneers is determined by the distribution of these competing signals.

as Dorsalin-1 might normally prevent the development of muscle pioneers in the dorsal and ventral portions of the somite (Fig. 8 *B*). In addition to inhibitory BMP-like signals originating from the neural tube, other BMP-like factors expressed ventral to the notochord (Rissi et al., 1995) and elsewhere (Liem et al., 1995; Pourquié et al., 1996) are candidates for antagonizing Hedgehog signaling.

We have shown that when it is expressed in the notochord, Dorsalin-1 has a specific effect on muscle pioneer identity and does not affect the differentiation of non–muscle pioneer slow muscle fibers. Several studies have suggested that BMPs have a limited range of diffusion, raising the question of whether non–muscle pioneer cells are also exposed to Dorsalin-1 protein when it is expressed in the notochord. We think that the specificity of Dorsalin-1 action on muscle pioneer cells but not on the non–muscle pioneer cells due to a difference in the exposure to Dorsalin-1 is unlikely for several reasons. First, Dorsalin-1 was expressed in the notochord just before the migration of adaxial cells away from the notochord, and thus all slow muscle precursors would be exposed to Dorsalin-1 (data not shown). Second, in the case of dominant negative PKA and Dorsalin-1 coinjection, non–muscle pioneer slow muscle cells were induced in the region adjacent to the notochord cells expressing Dorsalin-1, whereas muscle pioneer cells were inhibited in this region (compare Fig. 7, *C* with *F*), suggesting that the lack of effect on non–muscle pioneer cells is unlikely the result of a limited range of Dorsalin-1 action.

It is likely that slow muscle identity is established earlier than muscle pioneer cell identity. Slow muscle precursors are morphologically and molecularly distinct at the end of gastrulation (Devoto, et al., 1996*b*; Weinberg, et al., 1996), whereas muscle pioneers are not identifiably separate from the other slow muscle precursors until the time of somite formation, when they express *engrailed* genes and develop their distinctive morphology (Felsenfeld et al., 1991; Hatta et al., 1991; Ekker et al., 1992). Expression of *dorsalin-1* from the *twhh* promoter begins before the appearance of muscle pioneer identity and before the migration of the slow muscle precursors away from the notochord (data not shown). Thus, all of the slow muscle cells are likely to be exposed to Dorsalin-1; this suggests that the development of non–muscle pioneer slow muscle precursors is unaffected by exposure to Dorsalin-1 at this time, perhaps because they are already committed to a slow muscle fate. We have not tested whether BMP-like signals acting earlier, during gastrulation, influence the development of non–muscle pioneer slow muscle cells.

twhh Promoter

In this study, we showed that the 5.2-kb *twhh* promoter could drive expression of heterologous cDNAs specifically in the notochord. This notochord specificity was unexpected considering that the endogenous *twhh* gene is exclusively expressed in the floor plate. It is possible that the 5.2-kb *twhh* promoter we isolated and used may lack a repressor sequence present in the *twhh* gene that inhibits the notochord expression of the *twhh* gene. Our results highlight the power of using tissue-specific promoters to direct expression of proteins to specific tissue types, at specific times, in the zebrafish embryo. This will be generally useful for analyzing later functions of genes that also have functions during gastrulation (see also Kroll and Amaya, 1996).

Model

Based on our results and studies by other laboratories, we propose that the differentiation of slow muscle cells in zebrafish is regulated by at least two signals, Hedgehogs and BMP-like proteins (Fig. 8). During early stages of development, Hedgehogs secreted from midline cells induce paraxial mesodermal cells to become adaxial cells, the precursors of slow muscle. We propose that this early exposure to Hedgehogs is sufficient to signal the development of non–muscle pioneer slow muscle cells; however, it is insufficient for the development of muscle pioneer cells. Differentiation of muscle pioneers requires prolonged exposure to the inductive Hedgehog signals, and minimal exposure to an inhibitory BMP-like signal. In the dorsal and ventral regions of the somite, an inhibitory BMP4-like signal blocks the response to Hedgehog, whereas in the middle region of the somite, this inhibitory BMP-like activity is absent or very low and consequently restricts the development of muscle pioneer cells to the middle region of the somite. The mechanism for setting up this low BMP-like activity in the middle region of the somite is unknown. One possibility is an uneven distribution of the BMPs and BMP-like protein within the somite. The dorsal and ventral regions are exposed to a high concentration of the BMP-like protein, while the middle region is exposed to a low concentration of BMP-like protein. Evidence supporting this hypothesis comes from studies of mediolateral patterning of the chick somite. In chick embryos, the lateral part of the somite is exposed to a high concentration of BMP4 expressed in the lateral plate mesoderm (Pourquié et al., 1996). BMP4 from this source acts as a diffusible lateralizing signal to specify the hypaxial muscle lineage (Pourquié et al., 1996). In zebrafish, several BMPs and BMP-like proteins have been shown to be expressed in tissues near the somite during segmentation stages. For example, a BMP-like gene, *radar*, is specifically expressed in the dorsal neural tube and hypochord cells (Rissi et al., 1995), and BMP2 and BMP4 are expressed primarily in the mesenchyme of dorsal and ventral fins (Nikaido et al., 1997). Therefore, it is likely that there is a gradient in the distribution of BMP and BMP-like proteins within the somite, with higher concentrations in the dorsal and ventral regions of the somite and lower concentrations in the middle region of the somite. Alternatively, the BMP inhibitory activity might be reduced in the middle region of the somite (around the notochord) by an opposing signal from the notochord that blocks the BMP-like activity in this region. Chordin (Piccolo et al., 1996), Noggin (Zimmerman et al., 1996), and Follistatin (Hemmati-Brivaniou et al., 1994) can each bind to and inactivate BMPs and other TGF-β family members, and in *Xenopus* these genes are also expressed in the notochord (Smith and Harland, 1992; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994). Thus, Chordin, Noggin, or Follistatin could repress the BMP-like activity in the notochord region, allowing the development of muscle pioneer cells. Further experiments are re-

quired to learn which mechanism is correct, and possibly both mechanisms are used to establish an uneven distribution of BMP-like inhibition of muscle pioneer development. This BMP4-like signal may also stimulate migration of adaxial cells toward the surface of the somite, where they are consequently exposed to a lower concentration of Hedgehogs. Regardless of the mechanism, this model predicts that these opposing signals determine slow muscle cell identities; adaxial cells that remain near the notochord express *engrailed* and develop into muscle pioneers, whereas the adaxial cells in the dorsal and ventral regions of the somite do not develop into muscle pioneers.

Our model is similar to that proposed for the dorsoventral patterning of the spinal cord (Liem et al., 1995; Ericson et al., 1996). Sonic hedgehog expressed by the notochord induces ventral cell types, such as floor plate and motorneurons, whereas BMP4 expressed in the dorsal neural tube induces dorsal cell types, such as neural crest, roof plate cells, and dorsal commissural neurons (Liem et al., 1995). The activity of Hedgehog and BMP4 are mutually antagonistic; Hedgehog inhibits the responses to BMP4, and BMP4 in turn inhibits the responses to Hedgehog (Liem et al., 1995). It has been suggested that patterning of the chick somite also involves the opposing actions of signals from surrounding tissues, including the neural tube and notochord (Fan and Tessier-Lavigne, 1994; Pourquié et al., 1996). These signals include Sonic hedgehog and BMP4 (Munsterberg et al., 1995; Pourquié et al., 1996). Our results suggest that in addition to regulating the broad subdivisions of the somite into sclerotome and dermamyotome, the opposing actions of *hedgehog* and *TGF-β* gene family members also regulate the development of embryonic muscle fiber-type identity.

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