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Pitx2 promotes development of splanchnic mesoderm-derived branchiomeric muscle

Feiyan Dong[1], Xiaoxia Sun[1], Wei Liu[1], Di Ai[1], Elizabetha Klysik[1], Mei-Fang Lu[1], Julia Hadley[2], Laurent Antoni[2], Li Chen[1,3], Antonio Baldini[1], Pip Francis-West[2] and James F. Martin[1,*]

Recent experiments, showing that both cranial paraxial and splanchnic mesoderm contribute to branchiomeric muscle and cardiac outflow tract (OFT) myocardium, revealed unexpected complexity in development of these muscle groups. The *Pitx2* homeobox gene functions in both cranial paraxial mesoderm, to regulate eye muscle, and in splanchnic mesoderm to regulate OFT development. Here, we investigated Pitx2 in branchiomeric muscle. *Pitx2* was expressed in branchial arch core mesoderm and both *Pitx2* null and *Pitx2* hypomorphic embryos had defective branchiomeric muscle. Lineage tracing with a *Pitx2*^{cre} allele indicated that *Pitx2* mutant descendents moved into the first branchial arch. However, markers of both undifferentiated core mesoderm and specified branchiomeric muscle were absent. Moreover, lineage tracing with a *Myf5*^{cre} allele indicated that branchiomeric muscle specification and differentiation were defective in *Pitx2* mutants. Conditional inactivation in mice and manipulation of *Pitx2* expression in chick mandible cultures revealed an autonomous function in expansion and survival of branchial arch mesoderm.

KEY WORDS: Homeobox, Branchiomeric muscle, Mouse, Chick

INTRODUCTION

Craniofacial muscle, comprised of branchiomeric (branchial arch muscle) and extraocular muscle, has distinct origins and developmental regulatory mechanisms from that of the trunk muscle. For example, Wnt signaling has been shown to promote trunk skeletal muscle differentiation, while inhibiting craniofacial muscle development (Tzahor et al., 2003). Moreover, investigation of transcriptional regulation of *Myf5*, one of the four muscle regulatory factors (MRFs), revealed separable elements controlling *Myf5* expression in trunk and craniofacial muscle (Carvajal et al., 2001; Hadchouel et al., 2003). *Mrf4* (*Myf6* – Mouse Genome Informatics), another MRF, appears to be dispensable for head muscle development but is crucial in trunk muscle (Kassar-Duchossoy et al., 2004). The paired domain factors, Pax3 and Pax7, have important functions in trunk muscle development but are not expressed in head muscle (Tajbakhsh et al., 1997).

Classically, two sources have been shown to contribute to branchiomeric and ocular muscle, cranial paraxial mesoderm (CPM) and the prechordal plate mesoderm (Noden and Francis-West, 2006; Chai and Maxson, 2006). Recent work has revealed overlap in the progenitors that contribute to branchiomeric and cardiac muscle. For example, lineage tracing in mouse embryos revealed the existence of the second cardiac lineage, derived from splanchnic mesoderm, that contributes to both cardiac and branchiomeric muscle (Buckingham et al., 2005). Fate-mapping studies in mouse and chick embryos revealed that CPM, in addition to branchiomeric muscle, also contributes to the cardiac outflow tract (OFT) (Tirosh-Finkel et al., 2006; Trainor et al., 1994). The significance of separate precursor populations, with distinct developmental histories, in branchiomeric muscle development is unknown.

[1]Institute of Biosciences and Technology, Texas A&M System Health Science Center, 2121 Holcombe Blvd, Houston, TX 77030, USA. [2]Department of Craniofacial Development, King's College, London Guy's Tower, London SE1 9RT, UK. [3]Program in Cardiovascular Sciences, Department of Medicine, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA.

*Author for correspondence (e-mail: jmartin@ibt.tamhsc.edu)

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Heterotopic grafting experiments in mouse embryos revealed substantial plasticity in the CPM, as transplanted CPM was competent to assume the characteristics of the recipient site (Trainor et al., 1994; von Scheven et al., 2006a). This observation is consistent with the demonstration that environmental cues are crucial for the normal diversification of CPM. *Bmp4* was shown to promote cardiac differentiation and inhibit skeletal muscle differentiation (Tirosh-Finkel et al., 2006). Similarly, Fgf8 was shown to promote branchiomeric muscle development while inhibiting extraocular muscle (EOM) development (von Scheven et al., 2006a). These findings indicate that signaling from surrounding tissues determines the fate of progenitor cells within the CPM.

Less is known about the cell-autonomous mechanisms regulating branchiomeric muscle development. *Tbx1* has been shown to be required for branchiomeric muscle and cardiac OFT development. In the OFT, *Tbx1* regulates proliferation of progenitor cells by regulating expression of Fgf ligands (Vitelli et al., 2002; Xu et al., 2004). A similar mechanism may underlie Tbx1-mediated regulation of branchiomeric muscle development (Kelly et al., 2004). Capsulin and MyoR (Tcf21 and Msc, respectively – Mouse Genome Informatics), two basic helix-loop-helix (bHLH) transcription factors that mark undifferentiated progenitor cells, are necessary for branchiomeric muscle development (Lu et al., 2002; von Scheven et al., 2006b). Mice that are double mutant for *MyoR* and capsulin lack a subset of first branchial arch-derived muscles, such as the temporalis, masseter and pterygoids. *MyoR* and capsulin probably function as survival factors in differentiating head muscle, although there may also be a migration defect in *MyoR;* capsulin mutants.

Pitx2 is a paired-related homeobox gene mutated in Rieger syndrome type I, an autosomal dominant, haploinsufficient disorder that includes tooth anomalies, anterior segment eye defects and facial dysmorphologies as cardinal features (Diehl et al., 2006; Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999; Semina et al., 1996). *Pitx2* also plays an essential role in the late aspects of left right asymmetry (LRA) and cardiac OFT development (Ai et al., 2006; Kioussi et al., 2002). Recent work has shown that the *Pitx2* OFT phenotype can be traced to a defect in

cardiac cells derived from the second cardiac lineage (Ai et al., 2006). In this work, we investigated the role of *Pitx2* in branchiomeric muscle.

Our data uncover an evolutionarily conserved role for *Pitx2* in growth and survival of branchiomeric muscle progenitors. Both conditional ablation of *Pitx2* in mouse embryos and manipulation of Pitx2 dose in chick embryo primary cultures reveal an autonomous *Pitx2* function in branchiomeric muscle precursors. Our findings also show that *MyoR* fails to be expressed in *Pitx2* mutants, indicating a defect in undifferentiated muscle progenitors. Expression of *Tbx1* is preserved in *Pitx2* mutant embryos suggesting that the *Pitx2* and *Tbx1*-mediated genetic pathways in branchiomeric muscle are distinct. Taken together, our data reveal a crucial role for *Pitx2* in branchiomeric muscle development and reveal a branching of genetic pathways upstream of the MRFs in branchiomeric muscle.

MATERIALS AND METHODS

Mouse alleles used in this study

The *Pitx2*^{flox}, *Pitx2*^{null} and *Pitx2*^{hypo} alleles have been described. Briefly, the *Pitx2*^{flox} allele contains LoxP sites flanking *Pitx2* exon5 and has been shown to be a true conditional null allele (Gage et al., 1999). The *Pitx2*^{null} allele is a 4 kb deletion that removes *Pitx2* exons 5 and 6 and the intervening intron (Lu et al., 1999). The *Pitx2*^{hypo} allele is a weak hypomorphic allele, previously called *Pitx2* δ*ab*, that contains a deletion of the *Pitx2a* and *Pitx2b* isoforms and has reduced *Pitx2c* function (Liu et al., 2001). The *β-catenin* conditional null allele has been described (Brault et al., 2001).

Immunohistochemistry

Embryos were fixed, dehydrated and embedded in paraffin blocks and sectioned at 5 μm. The slides were deparaffinized and rehydrated according to standard protocols. Antigen retrieval was performed by heating the slides in a 95°C water bath for 30 minutes in 0.01 mol/l sodium citrate (pH 6.0) followed by slowly cooling down to room temperature. Sections were blocked in 3% H_2O_2 in methanol for 10 minutes at room temperature. The primary antibody used was mouse anti-chicken polyclonal antibody (from Developmental Studies Hybridoma Bank, The University of Iowa) diluted in 1:100 and incubated overnight. The Zymed Histostain-Plus kit was used according to the manufacturer's protocol.

Whole-mount *LacZ* staining and section

After dissection, the embryos were fixed in the fresh-made fixing buffer (0.2 glutaraldehyde, 2% formalin, 5 mmol/l EGTA, 2 mmol/l $MgCl_2$, in 0.1 mol/l Na_2HPO_4 pH 7.3) for 20-30 minutes. Following three washes with the rinse buffer (0.1% sodium deoxycholate, 0.2% NP40, 2 mmol/l $MgCl_2$, in 0.1 mol/l NaH_2PO_4 pH 7.3), the samples were stained with the staining buffer (1 mg/ml X-gal, 5 mmol/l potassium ferricyanide, 5 mmol/l potassium ferrocyanide, in rinse buffer) until the optimized results appeared. After removing the staining, the embryos were then rinsed with 1× PBS for 5 minutes. All the above procedures were performed at room temperature. The embryos were finally post-fixed with 10% formalin and could be stored in this buffer at 4°C. The *LacZ*-stained embryos were dehydrated in ethanol and isopropanol, embedded in paraffin blocks and sectioned at 10 μm.

Whole-mount and section in situ hybridization

Whole-mount in situ hybridization was performed as previously described (Lu et al., 1999). The mouse *Pitx2* probe was an exon6 fragment that hybridizes to all *Pitx2* isoforms. The myogenin, *MyoD*, *Tbx1* and *MyoR* probes have been previously described (Kelly et al., 2004). In situ hybridization to whole chick embryos was carried out as described by Francis-West et al. (Francis-West et al., 1995). ^{35}S-in situ hybridization to tissue sections was performed on 7 μm wax sections as described (Francis-West et al., 1994). The *Pitx2* probe is described by Yu et al. (Yu et al., 2001) and the chick MyoD clone by Lin et al. (Lin et al., 1989).

Chick embryology

Fertilized Ross White chicken eggs were supplied by Henry Steward & Co. Ltd (Lincolnshire, UK) and were incubated at 37±1°C. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Stage 20/21 mandibular primordia micromass cultures were prepared as described (Anakwe et al., 2003) and were plated in the presence of high titer RCASBP viruses encoding an activated version of Pitx2 or a dominant-negative Pitx2 construct (Yu et al., 2001). Micromasses were cultured for 3 days, fixed briefly in ice-cold methanol and immunostained with the pan-myosin antibody, A4.1025 (1 in 100), and A4.840 (1 in 50), which recognizes cells expressing the slow MyHC isoforms SM3 and SM1 (from the Developmental Studies Hybridoma Bank). This was followed by incubation with horse anti-mouse IgG (γ-specific) conjugated to FITC (Vector; 1:400) and donkey anti-mouse IgM (μ-specific) conjugated to Cy3 (Jackson; 1:800) for at least 1 hour at room temperature. Following three PBS washes for 5 minutes, cultures were mounted under coverslips with PBS:glycerol (1:9) with 0.1% phenylenediamine as an antifade reagent. Values shown are the mean and standard error of the mean of at least nine cultures from three independent experiments. The data was analysed using Student's *t*-test.

Histology and apoptosis

For histology, embryos were fixed overnight in Bouin's fixative or buffered formalin, dehydrated through graded ethanol and embedded in paraffin. Sections were cut at 7-10 μm and stained with H&E. For TUNEL, embryos

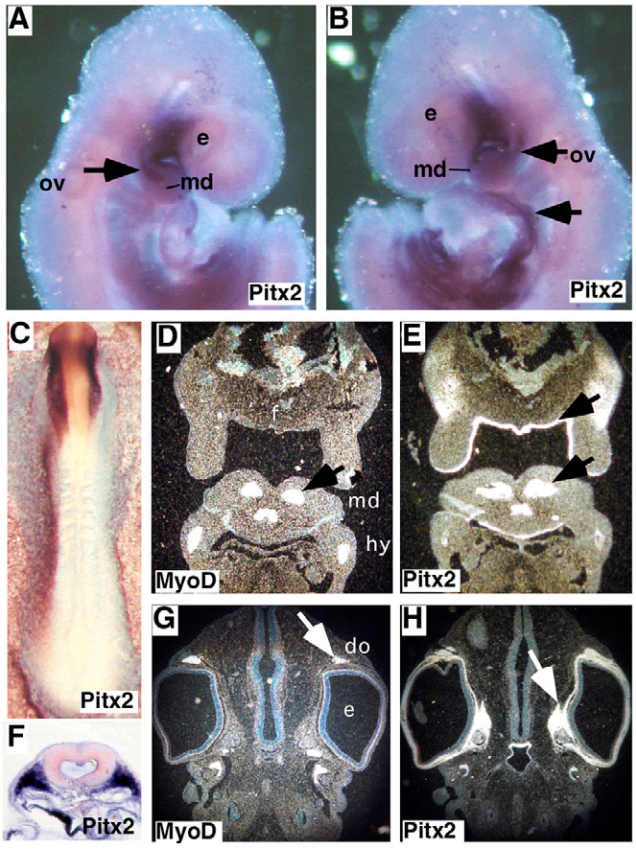

Fig. 1. *Pitx2* is required for survival of branchiomeric muscle precursors. (**A**,**B**) Right and left views of 9.5 dpc mouse embryos after whole-mount in situ hybridization with a *Pitx2* probe (arrows denote hybridization signal). (**C-H**) Expression in chick embryos of *Pitx2* (C,E,F,H) and *MyoD* (D,G) analysed by whole-mount in situ hybridization (C,F) and radioactive in situ hybridization to tissue sections (D,E,G,H) of stage 10 (C,F), 24 (D,E) and 26 (G,H) chick embryos. C is a dorsal view and D,E,G,H are frontal sections through the facial primordial. F is a transverse vibratome section through the head of the embryo in C. Arrows in D,G indicate developing muscles, in E the ectodermal and mesodermal expression of *Pitx2*, in H the ectomesenchymal *Pitx2* expression. do, dorsal oblique muscle; e, eye; f, frontonasal mass; hy, hyoid arch; md, mandibular primordia.

were first stained for *LacZ* using the whole-mount protocol, then embedded in paraffin and sectioned. TUNEL staining was performed according to the manufacturer's protocol (Serologicals Corporation).

RESULTS

Pitx2 expression in branchial arch mesoderm is conserved between mouse and chick

We evaluated *Pitx2* expression in mouse and chick embryos. At 8.5 days post coitum (dpc) in the mouse, *Pitx2* was expressed in oral ectoderm and head mesoderm (Mitsiadis et al., 1998; Mucchielli et al., 1997). At 9.5 dpc, *Pitx2* was expressed in mesoderm-derived, core cells of the first branchial arch (Fig. 1A,B) (Mucchielli et al., 1997). In chick embryos, *Pitx2* was expressed in head mesoderm (Fig. 1C,F) and in the core mesoderm of the branchial arch (Fig. 1D,E). More dorsal sections indicated that *Pitx2* was also expressed in periocular mesenchyme and extraocular muscles (Fig. 1G,H). As in the mouse, *Pitx2* was also expressed in oral ectoderm (Fig. 1E). *Pitx2* expression colocalized with the muscle marker *MyoD* in branchial arch mesoderm and extraocular muscle (Fig. 1D,E,G,H). Taken together, these findings suggest that the function of Pitx2 in branchiomeric muscle is probably conserved between mouse and chick.

Pitx2 function is required for *MyoR* but not *Tbx1* expression in first branchial arch muscle precursors

We next looked at markers of muscle development in *Pitx2*^{null} and *Pitx2* hypomorphic mutant embryos. myogenin encodes one of the four bHLH MRFs and is required for muscle development (Hasty et al., 1993). Moreover, myogenin is a late muscle marker that is important for muscle differentiation. In *Pitx2* control embryos, myogenin was highly expressed in the branchial arch core cells, while in *Pitx2* hypomorphic embryos, myogenin was greatly reduced (Fig. 2A,B). Importantly, in *Pitx2*$^{null-/-}$ embryos myogenin was absent, indicating that muscle precursors in the branchial arch core fail to activate myogenin, an essential regulator of muscle differentiation (Fig. 2C). MyoD is another MRF and is a marker of committed myoblasts. Similar to myogenin, we found that *MyoD* expression was absent in the *Pitx2*^{null} mutant embryos, indicating that myoblasts failed to be specified in the first branchial arch of *Pitx2*^{null} mutants (Fig. 2D,E). It is also possible that defects in myogenin and *MyoD* expression result from a loss of progenitor cells (see below).

Previous studies have identified a requirement for the bHLH transcription factors MyoR and capsulin in development of first branchial arch muscle (Lu et al., 2002). In addition, *MyoR* marks undifferentiated muscle precursor cells (von Scheven et al., 2006b). Moreover, in the *MyoR;* capsulin double-mutant embryos, elevated apoptosis was detected in the core branchial arch cells. In *Pitx2*^{null} mutant embryos, *MyoR* failed to be expressed in the core cells of the first branchial arch (Fig. 2F,G). Previous work has also established that the T-box transcription factor *Tbx1* is expressed in core cells of the branchial arches and is required for first branchial arch muscle development, although *MyoR* continues to be expressed in the *Tbx1* mutants (Kelly et al., 2004). In *Pitx2*^{null} mutants, *Tbx1* was still expressed in the first branchial arch, although in a reduced expression domain (Fig. 2H,I). In addition, expression of *Pitx2* in Tbx1 null mutants was also unaffected (Fig. 2J,K). Taken together, these findings indicate a defect in undifferentiated muscle progenitors in *Pitx2* mutant embryos.

Committed myoblasts are absent in the *Pitx2* null mutant first branchial arch

The expression analysis suggested that specification of myoblasts was defective in *Pitx2*^{null} mutant embryos. To study this question in more detail, we performed lineage tracing with a *Myf5*^{cre} allele that marks cells that have activated expression of *Myf5* in committed myoblasts (Tallquist and Soriano, 2003). This is a very sensitive method for following the developmental progression of *Myf5*-expressing descendents. Induction of recombination at the *Rosa 26* reporter locus by cre recombinase is heritable and irreversible and so is a reliable method for

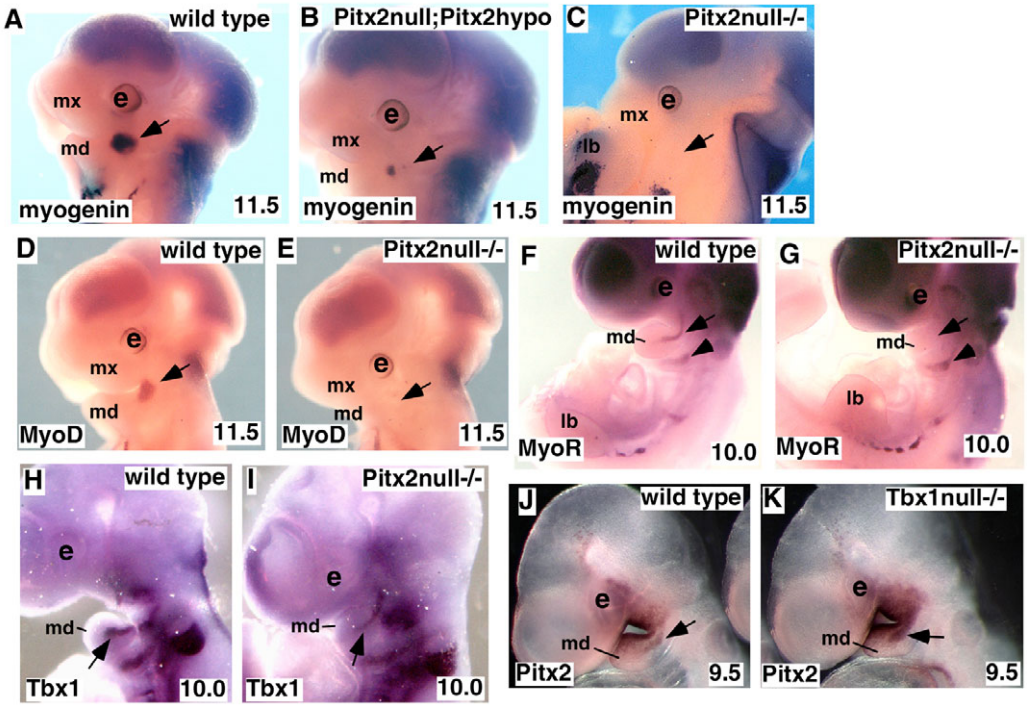

Fig. 2. Defective specification of first branchial arch undifferentiated core mesoderm in *Pitx2* mutants. (**A-C**) Myogenin and (**D**,**E**) *MyoD* fail to be expressed normally in *Pitx2* mutant embryos. (**F-I**). *MyoR*, a marker of undifferentiated core mesoderm, fails to be expressed in the *Pitx2*^{null} mutant embryos while another marker of undifferentiated progenitors. (**J**,**K**) *Pitx2* expression in control and *Tbx1*^{null} mutant embryos. Arrows denote first branchial arch hybridization signal or absence of signal. Arrowheads in F,G denote signal in the second branchial arch.

performing lineage tracing in mouse embryos (Soriano, 1999). *Myf5* expression is activated in the branchial arches at approximately 9.25 dpc. In *Pitx2*^{null} mutants, the number of *LacZ*-marked *Myf5* descendents was drastically reduced in the first branchial arch, consistent with the expression data indicating that myoblast specification in core cells of the first branchial arch was defective (Fig. 3A,B).

At 10.5 dpc, *Myf5*^{cre} marked cells were present both within the branchial arch and dorsal to the first branchial arch in control embryos. The dorsal cells probably represent myoblasts that are migrating from the cranial paraxial mesoderm (Tirosh-Finkel et al., 2006; Trainor et al., 1994). In *Pitx2*^{null} mutants, *Myf5*^{cre}-marked cells were excluded from the first branchial arch but were still present in the dorsally located migrating myoblasts (Fig. 3C,D). By 11.5 dpc, *Myf5*^{cre}-marked cells had started to form the masseter muscle mass spanning the maxilla and mandible. In the *Pitx2*^{null} embryos, a small group of *Myf5*^{cre}-marked cells was apparent, indicating that a reduced number of cells in the mandibular process activated *Myf5* expression (Fig. 3E,F). It is also notable that *Myf5*^{cre}-marked cells, contributing to extraocular muscles, were present but failed to localize properly around the developing eye (Fig. 3D,F). Taken together, these findings indicate that specified branchiomeric myoblasts are drastically reduced in *Pitx2*^{null} mutant embryos. We interpret these data to indicate a defect in muscle specification, although it should be noted that defective migration or a developmental delay may also contribute to the phenotype.

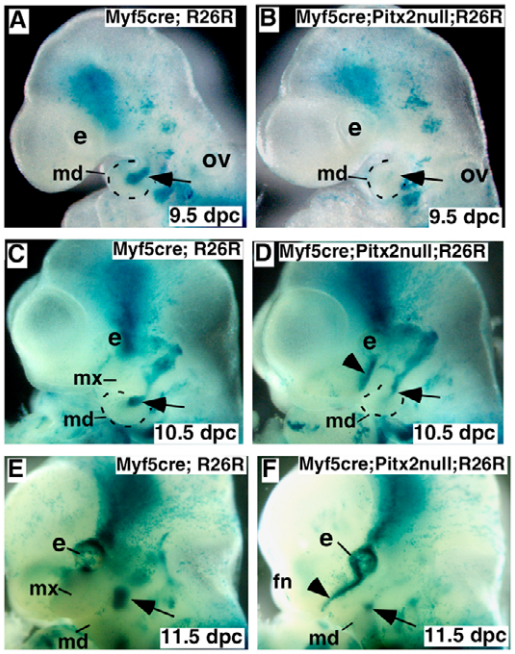

Fig. 3. Specified, *Myf5*-positive myoblasts are drastically reduced in the *Pitx2*^{null} mutant branchial arch. (**A**,**B**) Lineage tracing with the *Myf5*^{cre} allele indicates that the *Myf5* lineage is drastically reduced in the first branchial arch of 9.5 dpc *Pitx2*^{null} mutant embryos (arrows). (**C**,**D**) By 10.5 dpc, the *Myf5* lineage has expanded in the wild-type first branchial arch (arrow in C) but is still greatly reduced in the *Pitx2*^{null} branchial arch. *LacZ*-positive cells are detectable dorsal to the branchial arch in both control and *Pitx2*^{null} embryos (arrow in D). Also the *Myf5* lineage that contributes to extraocular muscle is mislocalized in *Pitx2*^{null} embryos (arrowhead in D). (**E**,**F**) In the 11.5 dpc embryo, a small number of *Myf5*^{cre}-labeled cells can be detected, indicating that a few specified myoblasts are present in the *Pitx2*^{null} mutant branchial arch (arrow in E,F). Abnormal localization of the EOM precursors is still observed in the *Pitx2*^{null} embryo (arrowhead in F). e, eye; fn, frontonasal mass; md, mandible; mx, maxilla; ov, otic vesicle.

Pitx2 descendents move into the first branchial arch but fail to form mature muscle

We performed a lineage-tracing experiment with the *Wnt1*^{cre} transgenic line that directs cre activity in the neural crest that surrounds, and therefore outlines, the mesoderm in the branchial arch (Chai et al., 2000). In control embryos, *LacZ*-negative mesoderm-derived cells were outlined by blue, neural crest derivatives (Fig. 4A). In the *Pitx2*^{null} mutant embryo, there was a reduction in the number of mesoderm cells; however, core mesoderm was present in the *Pitx2*^{null} mutant (Fig. 4A,B).

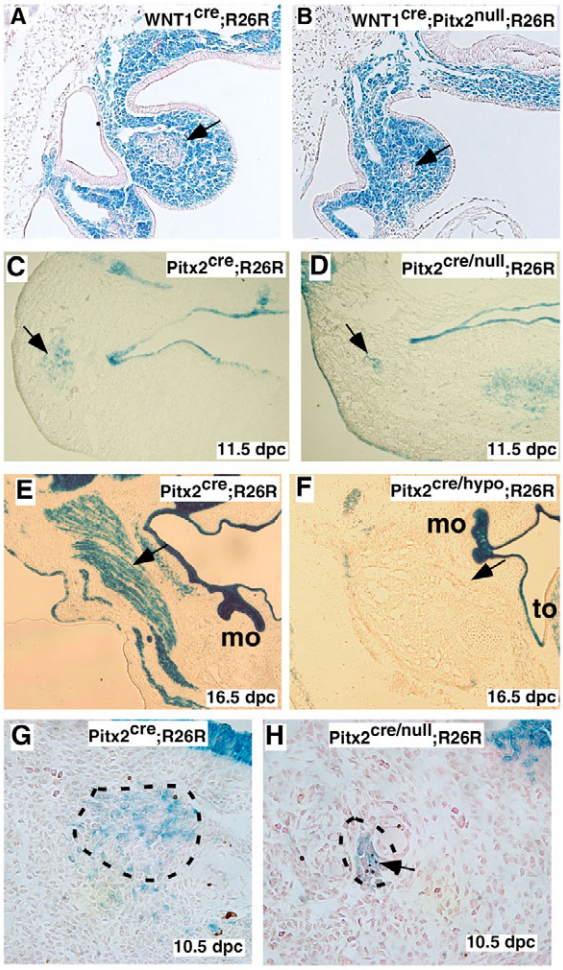

Fig. 4. Fate mapping *Pitx2* descendants in *Pitx2* mutant embryos. (**A**,**B**) Lineage tracing with the *Wnt1*^{cre} transgenic line that outlines core mesoderm of the first branchial arch. (**C**,**D**) Lineage tracing with the *Pitx2*^{cre} allele showing Pitx2 lineage contributes to the core mesoderm in both control (arrow in C) and *Pitx2*^{null} embryos (arrow in D). (**E**,**F**) Lineage tracing with the *Pitx2*^{cre} allele in control (E) and *Pitx2* hypomorphic embryos (F) at 16.5 doc. Pitx2 descendents contribute to branchiomeric muscle in the control, but in the mutant branchiomeric muscle is absent (arrow). (**G**,**H**) Lineage tracing and TUNEL double-labeling in control and *Pitx2*^{null} mutant embryos. The *LacZ*-positive cells are *Pitx2* descendents that show upregulated TUNEL-positive cells in the mutant (arrow). mo, molar tooth; to, tongue.

To establish more firmly that *Pitx2* descendents were present in the first branchial arch of *Pitx2* mutant embryos, we used the $Pitx2^{cre}$ allele to mark *Pitx2* descendents (Liu et al., 2002). This strategy also marks cells that are fated to express *Pitx2*. In control $Pitx2^{creneo}$;*R26R* embryos, we found that *Pitx2* descendents contributed to the core mesoderm of the first branchial arch (Fig. 4C). In $Pitx2^{null}$ embryos, *Pitx2* descendents were still present in the first branchial arch core, although in reduced numbers (Fig. 4D).

We next examined embryos at 16.5 dpc to evaluate if first branchial arch muscle was defective in *Pitx2* mutants. Because $Pitx2^{null}$ homozygous mutant embryos are lethal at 14.0 dpc, we used a weak hypomorphic allele of *Pitx2*, referred to as the $Pitx2^{hypo}$ allele for this experiment (see Materials and methods for allele description). In control embryos, *LacZ*-marked *Pitx2* descendents were observed in the masseter muscle, as well as oral and dental epithelium (Fig. 4E). In the *Pitx2* hypomorphic mutant, both *LacZ*-marked *Pitx2* descendents and first branchial arch muscle were absent (Fig. 4F).

To investigate the possibility that in *Pitx2* mutants, the core mesoderm cells of the branchial arch underwent apoptosis, we performed TUNEL analysis on embryos in which the *Pitx2* lineage was *LacZ*-marked.

In agreement with previous observations, control core mesoderm had little cell death (Fig. 4G) (Lu et al., 2002). By contrast, in $Pitx2^{null}$ embryos, *LacZ*-marked *Pitx2* mutant descendents were TUNEL-positive, indicating that $Pitx2^{null}$ cells were undergoing cell death (Fig. 4G,H). Together these data indicate that *Pitx2* is required for development of the first branchial arch muscle. Moreover, *Pitx2* mutant descendents are present in the branchial arch at 11.5 dpc, but they undergo apoptosis and are gone by 16.5 dpc.

Pitx2 is required for splanchnic mesoderm to contribute to branchiomeric muscle

Previous work revealed that a *Mef2c* enhancer element specifically directed *LacZ* expression in splanchnic mesoderm that contributed to the cardiac OFT but was not expressed in branchiomeric muscle (Dodou et al., 2004). Subsequent experiments using this *Mef2c* enhancer to direct cre activity indicated that descendents of the *Mef2c*-expressing splanchnic mesoderm contributed to branchiomeric muscle (Fig. 5A,B) (Verzi et al., 2005). Thus, the *Mef2c AHF cre* provides a valuable reagent to dissect the role of splanchnic mesoderm in branchiomeric muscle.

We used the *Mef2c AHF cre* to trace the splanchnic mesoderm lineage in $Pitx2^{null}$ mutant embryos. At 9.5 dpc, *Mef2c AHF* descendents were drastically reduced in the $Pitx2^{null}$ mutant embryos (Fig. 5C,D). One day later, at 10.5 dpc, a few *LacZ*-positive cells were visible in the first branchial arch of $Pitx2^{null}$ mutant embryos (Fig. 5E,F). At 11.5 dpc, *Mef2c AHF* descendents were no longer detectable in the $Pitx2^{null}$ mutant embryos. At this late stage, we noted an abnormal dispersion of *Mef2c AHF cre* descendents in the caudal branchial arches (Fig. 5G,H). The significance of this is unclear and is currently under investigation. Together, these data reveal a defect in development of the splanchnic mesoderm component of branchiomeric muscle in $Pitx2^{null}$ mutant embryos.

Pitx2 autonomously promotes muscle expansion

Because *Pitx2* regulates *Fgf8* and *Bmp4*-signaling pathways in branchial arch morphogenesis, we wanted to investigate the cell-autonomous role of *Pitx2* in branchiomeric muscle (Lu et al., 1999). We turned to the chick embryo system because of its utility as an experimental system. Furthermore, *Pitx2* expression is highly conserved between mice and chicks (Fig. 1) and *Pitx2* is expressed in the early chick cranial mesoderm before any onset of myogenesis (Fig. 1C,F). Then at later stages of development, *Pitx2* transcripts are found in all the cranial muscles derived from the unsegmented cranial mesoderm – i.e. those found in the mandibular and hyoid arch and the extraocular muscles (Fig. 1D,E,G,H).

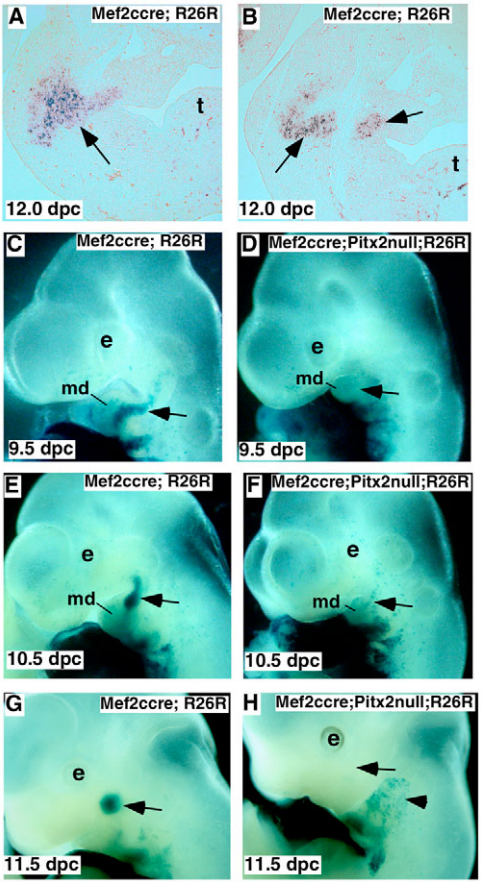

Fig. 5. Splanchnic mesoderm-derived *Mef2cAHFcre*-marked muscle precursors are reduced in the $Pitx2^{null}$ mutant branchial arch. (**A**,**B**) Lineage tracing with the *Mef2c AHF cre* transgenic driver and the R26R allele indicated that the *Mef2c*-marked splanchnic mesoderm lineage contributes to first branchial arch branchiomeric muscle (denoted by arrows). (**C-H**) Lineage tracing with the *Mef2c AHF cre* in the control and $Pitx2^{null}$ mutant embryos indicated a deficiency in the splanchnic mesoderm component of the first branchial arch muscle precursors (denoted by arrows) in $Pitx2^{null}$ mutant embryos at 9.5 dpc (C,D), 10.5 dpc (E,F) and 11.5 dpc (E,F). It is notable that in the 11.5 dpc *Pitx2* mutant embryo, *Mef2c AHF cre*-marked cells are found diffusely scattered in the caudal branchial arches (arrowhead). e, eye; md, mandibular process; mx, maxillary process.

Muscle precursor cells, including the surrounding ectomesenchyme but not the ectoderm, were isolated from the developing mandible and infected with a retrovirus expressing *Pitx2a*. This resulted in a statistically significant elevation in the number of myosin-positive cells when compared with control cells (Fig. 6A,B). We next used a retrovirus expressing a dominant-negative form of *Pitx2a* to decrease Pitx2 activity in muscle precursors. Primary cultures with reduced Pitx2a activity had lower numbers of myosin-positive cells, which was a statistically significant difference from the control (Fig. 6A,B). We also assessed whether Pitx2a had a differential effect on slow versus fast myocyte differentiation by immunostaining with a slow MyHC antibody

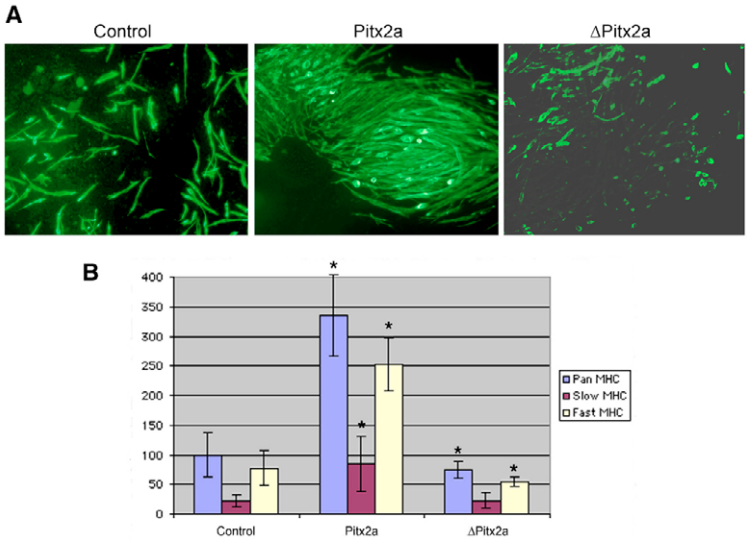

Fig. 6. Elevated and reduced Pitx2 activity in chick primary myoblasts. (**A**) Fluorescent images of mandibular micromass cultures, which have been infected with a control, Pitx2a or ΔPitx2a RCASBP retrovirus showing myocytes that have been visualized with the pan MyHc antibody A4.1025. (**B**) Bar chart quantifying these effects and also showing the effect on slow (red bar) and fast (yellow bar) myocyte development. *P<0.05.

(Hughes and Blau, 1992). This showed that loss and gain of *Pitx2a* function affected the development of both slow and fast myocytes (Fig. 6B). These data indicate that *Pitx2a* is necessary and sufficient for myocyte development in the context of craniofacial mesenchyme.

Conditional inactivation revealed a cell-autonomous function for *Pitx2* in branchiomeric muscle precursors

We used the *Mesp1*^{cre} knock-in and *Pitx2*^{flox} alleles to inactivate *Pitx2* specifically in mesoderm-derived cells. As *Pitx2* is expressed in oral ectoderm in the first branchial arch, *Pitx2* inactivation in mesoderm will leave the *Pitx2* ectoderm expression domain intact while removing *Pitx2* from branchiomeric muscle precursors. The *Mesp1*^{cre} allele directs cre activity broadly in mesoderm before muscle differentiation (Saga et al., 1999; Zhang et al., 2006).

Lineage tracing with the *Mesp1*^{cre} and *R26R* alleles revealed that the *Mesp1*-expressing lineage contributed efficiently to masseter muscle in the control (Fig. 7A). By contrast, in *Pitx2* conditional mutants (*Mesp1*^{cre}*; Pitx2*$^{flox/null\ (f/n)}$) there was a deficiency in masseter development (Fig. 7B). Investigation of myogenin expression in the *Mesp1*^{cre}*; Pitx2*$^{f/n}$ embryos also indicated that branchiomeric muscle was defective in *Pitx2* conditional mutants (Fig. 7C,D). Sections with H&E staining (Fig. 7E-H) and immunohistochemistry with a muscle-

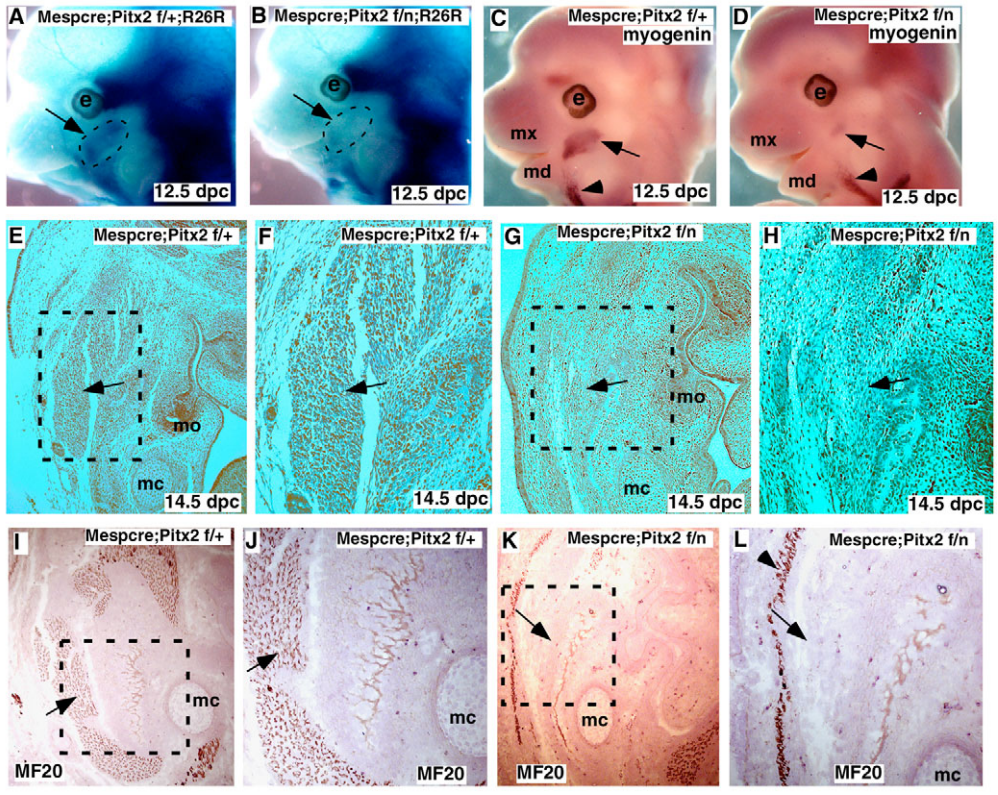

Fig. 7. Conditional ablation of *Pitx2* in mesoderm-derived cells reveals an autonomous role for *Pitx2* in branchiomeric muscle development. *Pitx2* deletion in mesoderm using the *Mesp1*^{cre} and *Pitx2*^{flox} alleles. (**A**,**B**) Lineage tracing indicates a reduction in the number of *LacZ*-marked *Pitx2* mutant descendents in the masseter muscle (outlined, arrow). (**C**,**D**) Myogenin expression (arrow) is greatly reduced in the *Pitx2* mutant compared with control. Sections through the masseter muscle of control (**E**,**F**) and *Mesp1*^{cre}*; Pitx2 n/f* mutant (**G**,**H**) indicate a muscle deficiency in the *Pitx2* conditional mutant (arrows). (I-L) Myosin immunostaining with the MF20 antibody in 14.5 control (**I**,**J**) and *Pitx2* conditional null mutant (**K**,**L**) to mark branchiomeric muscle in control and show deficiency in mutant (arrows). Note that in the mutant (L) there is residual subcutaneous muscle that shows myosin reactivity (arrowhead). e, eye; mc, Meckel's cartilage; md, mandible; mx, maxilla.

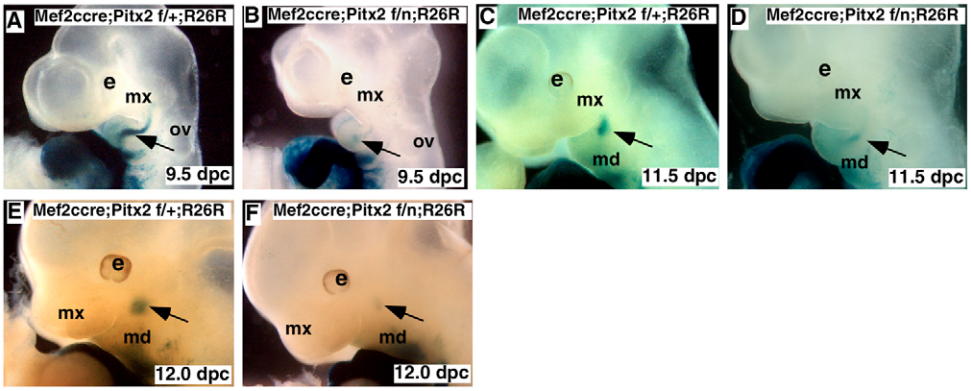

Fig. 8. Ablation of *Pitx2* in splanchnic mesoderm with *Mef2cAHFcre*$^{-}$ reveals an autonomous role for *Pitx2* in branchiomeric muscle development. (A-F) Lineage tracing with the *Mef2c AHF cre* transgenic driver (arrows) indicated that the *Mef2c*-marked splanchnic mesoderm lineage is reduced in the first branchial arch of *Mef2c AHF cre*; *Pitx2 n/f* conditional mutant embryos at 9.5 dpc (**A**,**B**), 11.5 dpc (**C**,**D**) and 12.0 dpc (**E**,**F**). e, eye; md, mandibular process; mx, maxillary process.

specific myosin antibody of control and *Mesp1*^{cre}*; Pitx2*$^{f/n}$ embryos also indicated that branchiomeric muscle was severely defective in *Pitx2* mutants (Fig. 7I-L).

We next used the *Mef2cAHF*^{cre}, which directs cre activity in the splanchnic mesoderm beginning at stages before branchiomeric muscle development (Dodou et al., 2004; Verzi et al., 2005), to conditionally ablate *Pitx2* in the splanchnic mesoderm component of branchiomeric muscle. We have recently shown that conditional deletion of *Pitx2* with *Mef2c AHFcre* resulted in severe cardiac defects (Ai et al., 2006). We used lineage tracing with R26R to follow the progression of branchiomeric muscle progenitors in the control and *Pitx2* conditional mutant embryos. At 9.5 dpc, the *Mef2c AHF* lineage was clearly marked in the core cells of the first branchial arch, while in the *Pitx2* conditional mutant there was a deficiency in *LacZ*-marked cells (Fig. 8A,B). Similarly, for 11.5 and 12.0 dpc embryos, we consistently found a deficiency in the *LacZ*-marked *Mef2c AHF* lineage in the *Pitx2* conditional mutants (Fig. 8C-F). It is notable that in all embryos examined, we found a small contribution of *LacZ*-marked cells in the *Pitx2* conditional mutant embryos. This observation suggests that the *Mef2c AHF cre* failed to completely inactivate *Pitx2* in branchiomeric muscle progenitors. It is also possible that there is a subpopulation of muscle precursors that are *Pitx2* independent. Nonetheless, taken together with the *Mesp1*^{cre} data, our findings indicate that *Pitx2* has an autonomous function in the development of branchiomeric muscle progenitors.

DISCUSSION

The findings presented here uncover a requirement for *Pitx2* in branchiomeric muscle development and provide insight into the genetic pathways controlling development of branchiomeric muscle. In *Pitx2*^{null} embryos, branchiomeric muscle precursors were initially present but failed to expand and activate the myogenic program. Moreover, lack of *MyoR* expression and elevated apoptosis indicated a defect in survival of undifferentiated muscle progenitor cells. Conditional *Pitx2* inactivation and overexpression and knockdown in chick primary cultures supported a direct role for *Pitx2* in branchiomeric muscle development. In addition, we showed that *Pitx2* has a function in the splanchnic mesoderm-derived component of branchiomeric muscle.

Pitx2 function in muscle

Pitx2 is expressed in multiple muscle types, including extraocular muscle, branchiomeric muscle, cardiac muscle and trunk skeletal muscle (Ai et al., 2006; Kitamura et al., 1999). The in vivo function of *Pitx2* in trunk skeletal muscle is poorly understood. Previous experiments investigating *Pitx2* in the C2C12 myoblast cell line, derived from satellite cells of the adult leg, uncovered a direct role for *Pitx2* in regulating myoblast proliferation through a mechanism mediated by the N-terminus of Pitx2a (Kioussi et al., 2002). In the heart, *Pitx2* regulates proliferation of cardiomyocytes of the OFT (Ai et al., 2006). In extraocular muscle, it has been suggested that Pitx2 may directly regulate MRF transcription (Diehl et al., 2006).

Our data indicate that, in branchiomeric muscle, *Pitx2* regulates undifferentiated precursor cells and probably controls expression of genes that are involved in muscle expansion and survival. Recent experiments revealed a role for *MyoR* and capsulin in the survival of a subset of first branchial muscle precursors (Lu et al., 2002). The *Pitx2*^{null} mutant branchiomeric muscle precursors fail to express *MyoR* and undergo apoptosis. It is notable that there is evidence in the pituitary that Pitx2 and the related factor Pitx1 promote cell survival by regulating expression of *Lhx3* (Charles et al., 2005; Zhao et al., 2006). In addition, the third member of the *Pitx* family, *Pitx3*, is required for postnatal survival of midbrain dopaminergic neurons (van den Munckhof et al., 2003). The requirement for *Pitx2* in undifferentiated precursor cells contrasts with the function of *Pitx2* in asymmetric organ morphogenesis. In left right organ morphogenesis, Pitx2 activity is needed in the organ primordium rather than in undifferentiated precursors (Ai et al., 2006; Shiratori et al., 2006). This may reflect a difference in tissues that only express *Pitx2c*.

Pitx2 and *Tbx1* in craniofacial muscle development

Tbx1 mutants have sporadic failure of craniofacial muscle development with loss of *Tlx1* and *Fgf10* expression (Kelly et al., 2004). Moreover, *Tbx1* has been suggested to directly activate *Pitx2* in the second cardiac lineage by binding to an element upstream of exon 6 (Nowotschin et al., 2006). *Tbx1* was still expressed in undifferentiated cells of the *Pitx2*^{null} mutant branchial arch core mesoderm consistent with the notion that *Tbx1* is an upstream regulator of *Pitx2*. However, *Pitx2* was still expressed in *Tbx1*^{null} mutants, indicating that a simple epistatic relationship is unlikely. In addition, by contrast to *Pitx2*^{null} mutant embryos, *Tbx1* mutants continue to express *MyoR* in the branchiomeric progenitors, further arguing against a linear, epistatic relationship (Kelly et al., 2004).

Alternatively, it may be that *Pitx2* and *Tbx1* regulate parallel pathways that may converge on common target genes. *Pitx* and *Tbx* genes have been shown to coordinately regulate gene expression in the pituitary. *Pitx1* and *Tpit* (*Tbx19*) bind to proximate but distinct recognition elements in the POMC promoter (Lamolet et al., 2001). In this system, *Pitx1* synergized with *Tbx19* but failed to

transcriptionally synergize with *Tbx1*, suggesting that cell-type-specific co-factors may be required for any potential synergism between *Pitx2* and *Tbx1* in branchiomeric muscle progenitors (Lamolet et al., 2001). It is notable that in the zebrafish mutant *van gogh*, which carries a mutant allele of *Tbx1*, muscle expression of endothelin 1 (*edn1*) is reduced (Piotrowski et al., 2003). In *Pitx2*^{null} mutants, *edn1* expression is reduced in the oral ectoderm, suggesting the possibility that the *Tbx1* and *Pitx2*-mediated pathway may converge on edn1 (Liu et al., 2003). Further experiments will be required to investigate this idea.

Splanchnic mesoderm contribution to branchiomeric muscle

Similar to that described for the cardiac OFT, multiple lineages with distinct developmental histories contribute to branchiomeric muscle. In the heart, the primary heart field contributes to the linear heart tube, while the second lineage is sequestered and moves into the OFT at a later stage. The later addition of the secondary heart field is required for proper OFT lengthening and morphogenesis (Buckingham et al., 2005). In branchiomeric muscle, the addition of cells from multiple lineages may play a similar role in controlling the size and pattern of craniofacial muscle.

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