**Pitx2 promotes development of splanchnic mesoderm-derived branchiomeric muscle**

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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 hypomorph embryos had defective branchiomeric muscle. Lineage tracing with a Pitx2Cre allele indicated that Pitx2 mutant descendants moved into the first branchial arch. However, markers of both undifferentiated core mesoderm and specified branchiomeric muscle were absent. Moreover, lineage tracing with a Myf5Cre 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

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**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). Myf4 (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 progenitor populations, with distinct developmental histories, in branchiomeric muscle development is unknown.

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 (Dielh 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 Pitx2flox, Pitx2mut and Pitx2hypo alleles have been described. Briefly, the Pitx2mut allele contains LoxP sites flanking Pitx2 exon 5 and has been shown to be a true conditional null allele (Gage et al., 1999). The Pitx2hypo allele is a 4 kb deletion that removes Pitx2 exons 5 and 6 and the intervening intron (Lu et al., 1999). The Pitx2hypo allele is a weak hypomorphic allele, previously called Pitx2Δab, 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% H2O2 in methanol for 10 minutes at room temperature. The primary antibody used was mouse anti-chicken polyclonal antibody (from Developmental Studies Hybridoma Bank). This was followed by incubation with horse anti-mouse IgG (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 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 MgCl2, in 0.1 mol/l Na2HPO4 pH 7.3) for 20-30 minutes. Following three washes with the rinse buffer (0.1% sodium deoxycholate, 0.2% NP40, 2 mmol/l MgCl2, in 0.1 mol/l NaH2PO4 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). 35S-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 37.5°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 were fixed for 1 hour in 4% paraformaldehyde, washed with PBS and incubated with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature. Sections were washed with PBS and treated with nucleiase for 15 minutes at room temperature. Sections were then incubated with 0.5% Triton X-100 in PBS for 5 minutes. Following three washes with PBS, sections were incubated with 200 μg/ml RNase A for 1 hour at 4°C. Sections were then incubated with a 1:50 dilution of fluorescein isothiocyanate- labeled avidin (Vector Labs) for 1 hour at room temperature. Sections were then washed with PBS and incubated with 10 μg/ml propidium iodide (Sigma) for 15 minutes at room temperature. Sections were then washed with PBS and mounted with Fluoromount-G (Southern Biotechnology Associates, Inc. Birmingham, Alabama).

Student’s t-test
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 Pitx2null 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 Pitx2null;Pitx2hypo 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 Pitx2null mutant embryos, indicating that myoblasts failed to be specified in the first branchial arch of Pitx2null 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 Pitx2null 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 Pitx2null 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 Pitx2null mutant embryos. To study this question in more detail, we performed lineage tracing with a Myf5Cre 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
performing lineage tracing in mouse embryos (Soriano, 1999). Myf5 expression is activated in the branchial arches at approximately 9.25 dpc. In Pitx2\textsuperscript{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\textsuperscript{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\textsuperscript{null} mutants, Myf5\textsuperscript{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\textsuperscript{cre}-marked cells had started to form the masseter muscle mass spanning the maxilla and mandible. In the Pitx2\textsuperscript{null} embryos, a small group of Myf5\textsuperscript{cre}-marked cells was apparent, indicating that a reduced number of cells in the mandibular process activated Myf5 expression (Fig. 3E,F). Taken together, these findings indicate that specified branchiomeric myoblasts are drastically reduced in Pitx2\textsuperscript{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.

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

We performed a lineage-tracing experiment with the Wnt1\textsuperscript{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\textsuperscript{null} mutant embryo, there was a reduction in the number of mesoderm cells; however, core mesoderm was present in the Pitx2\textsuperscript{null} mutant (Fig. 4A,B).

![Fig. 3. Specified, Myf5-positive myoblasts are drastically reduced in the Pitx2\textsuperscript{null} mutant branchial arch.](image)

(A,B) Lineage tracing with the Myf5\textsuperscript{cre} allele indicates that the Myf5 lineage is drastically reduced in the first branchial arch of 9.5 dpc Pitx2\textsuperscript{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\textsuperscript{null} branchial arch. LacZ-positive cells are detectable dorsal to the branchial arch in both control and Pitx2\textsuperscript{null} embryos (arrow in D). Also the Myf5 lineage that contributes to extraocular muscle, was 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\textsuperscript{null} mutant embryos.

![Fig. 4. Fate mapping Pitx2 descendents in Pitx2 mutant embryos.](image)

(A,B) Lineage tracing with the Wnt1\textsuperscript{cre} transgenic line that outlines core mesoderm of the first branchial arch. (C,D) Lineage tracing with the Pitx2\textsuperscript{cre} allele showing Pitx2 lineage contributes to the core mesoderm in both control (arrow in C) and Pitx2\textsuperscript{null} embryos (arrow in D). (E,F) Lineage tracing with the Pitx2\textsuperscript{cre} allele in control (E) and Pitx2 hypomorphic embryos (F) at 16.5 dpc. 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\textsuperscript{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\textsuperscript{Cre} allele to mark Pitx2 descendents (Liu et al., 2002). This strategy also marks cells that are fated to express Pitx2. In control Pitx2\textsuperscript{Cre}\textsubscript{R26R} embryos, we found that Pitx2 descendents contributed to the core mesoderm of the first branchial arch (Fig. 4C). In Pitx2\textsuperscript{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\textsuperscript{null} homozygous mutant embryos are lethal at 14.0 dpc, we used a weak hypomorphic allele of Pitx2, referred to as the Pitx2\textsuperscript{bypo} 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\textsuperscript{null} embryos, LacZ-marked Pitx2 mutant descendents were TUNEL-positive, indicating that Pitx2\textsuperscript{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 branchiomerically 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 branchiomerically 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 branchiomerically 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 branchiomerically muscle.

We used the Mef2c AHF cre to trace the splanchnic mesoderm lineage in Pitx2\textsuperscript{null} mutant embryos. At 9.5 dpc, Mef2c AHF descendents were drastically reduced in the Pitx2\textsuperscript{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\textsuperscript{null} mutant embryos (Fig. 5E,F). At 11.5 dpc, Mef2c AHF descendents were no longer detectable in the Pitx2\textsuperscript{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 branchiomerically muscle in Pitx2\textsuperscript{null} mutant embryos.

Pitx2 autonomously promotes muscle expansion

Because Pitx2 regulates Fg8 and Bmp4-signaling pathways in branchial arch morphogenesis, we wanted to investigate the cell-autonomous role of Pitx2 in branchiomerically 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 late 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).

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.
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 Mesp1Cre knock-in and Pitx2floxc 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 Mesp1Cre allele directs cre activity broadly in mesoderm before muscle differentiation (Saga et al., 1999; Zhang et al., 2006).

Lineage tracing with the Mesp1Cre 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 (Mesp1Cre; Pitx2flox/null (f/n)) there was a deficiency in masseter development (Fig. 7B). Investigation of myogenin expression in the Mesp1Cre; Pitx2f/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-
specific myosin antibody of control and Mespi1mouse; Pitx2tm1b embryos also indicated that branchiomioceric muscle was severely defective in Pitx2 mutants (Fig. 7I-L).

We next used the Mef2cAHEm1, 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 AHE lineagewas 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 AHE 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 AHE 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 Mespi1mouse 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 Pitx2null 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-termimus of Pitx2a (Kioussi et al., 2002). In the heart, Pitx2 regulates proliferation of cardiomycocytes of the OFT (Ai et al., 2006). In extracardiac 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 Pitx2null 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 Pitx2null mutant branchial arch core mesoderm consistent with the notion that Tbx1 is an upstream regulator of Pitx2. However, Pitx2 was still expressed in Tbx1null mutants, indicating that a simple epistatic relationship is unlikely. In addition, by contrast to Pitx2null 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 (Lamote 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 branchiomyogenic 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 mutant embryos, 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 branchiomyogenic muscle**

Similar to that described for the cardiac OFT, multiple lineages with distinct developmental histories contribute to branchiomyogenic muscle. In the heart, the primary heart field contributes to the linear heart tube, while the second lineage is sequestered and moves into the cardiac pouches to form the cardiac cushions (Buckingham et al., 2005). In branchiomeric muscle, the addition of elements derived from the splanchnic mesoderm is required to investigate this idea. Further experiments will be required to understand the contribution of this lineage to craniofacial muscle development.

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