Cranial muscle defects of Pitx2 mutants result from specification defects in the first branchial arch

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Pitx2 expression is observed during all states of the myogenic progression in embryonic muscle anlagen and persists in adult muscle. Pitx2 mutant mice form all but a few muscle anlagen. Loss or degeneration in muscle anlagen could generally be attributed to the loss of a muscle attachment site induced by some other aspect of the Pitx2 phenotype. Muscles derived from the first branchial arch were absent, whereas muscles derived from the second branchial arch were merely distorted in Pitx2 mutants at midgestation. Pitx2 was expressed well before, and was required for, initiation of the myogenic progression in the first, but not second, branchial arch mesoderm. Pitx2 was also required for expression of premyoblast specification markers Tbx1, Tcf21, and Msc in the first, but not second, branchial arch. First, but not second, arch mesoderm of Pitx2 mutants failed to enlarge after embryonic day 9.5, well before the onset of the myogenic progression. Thus, Pitx2 contributes to specification of first, but not second, arch mesoderm. The jaw of Pitx2 mutants was vestigial by midgestation, but significant size reductions were observed as early as embryonic day 10.5. The diminutive first branchial arch of mutants could not be explained by loss of mesoderm alone, suggesting that Pitx2 contributes to the earliest specification of jaw itself.

homeobox gene | muscle development

Craniofacial skeletal muscles include four groups: the branchial, extraocular, laryngoglossal, and axial (1). Vertebrate craniofacial muscles originate from preotic somitic, unsegmented cranial paraxial, and prechordal mesoderm (2). Axial muscles derive from the preotic somites and move the head with respect to the body. Laryngoglossal muscles arise from preotic somites and branchial arch (BA) mesoderm and move the larynx and tongue. Extraocular muscles are derived from prechordal and first BA mesoderm and move the eye. Other BA-derived muscles are associated with jaw, hyoid cartilage, and caudal BA derivatives (3–5). The first BA gives rise to mandibular adductors, intermandibular muscles, suprahyoid muscles, and at least two extraocular muscles. The second BA gives rise to mandibular depressors, stapedial muscle, and facial expression muscles (2).

Trunk muscles are derived from a relatively uniform source, the somites, whereas head muscles are of diverse origin. Despite these varied origins, the classic myogenic progression seems to be quite similar for most, if not all, muscles. In both trunk and head, early stages of the myogenic progression can be followed by observing the expression of the myogenic regulatory factors (MRFs). Proliferative myoblasts, which have undergone initial myogenic commitment, are marked by the expression Myf5 or MyoD. Later myogenic differentiation is marked by myogenin. Myogenin expression marks the stage of the myogenic progression when cells pull out of the cell cycle and terminally differentiate into contractile cells. More mature stages can be followed by proteins specific to the contractile apparatus.

Although myogenic progression is similar in all developing muscle groups, it seems that the specification of cells just before the myoblast differs significantly between head and trunk, (6, 7). The transcription factors that mark progenitor cells just before the expression of MyoD or Myf5 differ greatly in different parts of the embryo. In limb level somites, Lbx1 and Pax3 mark the premyoblast cells that will enter the limb, diaphragm, or intrinsic tongue (8). Somites that produce body wall muscles are marked by Pax3 but not Lbx1. Both Lbx1 and Pax3 are required for limb muscle formation (9, 10). Pax3 is required for activating the myogenic progression in this developmental field. However, these two factors are not required for head muscle formation and do not seem to be expressed in the premyoblast mesoderm that gives rise to the head musculature.

In contrast, Tbx1 is expressed in the premyoblast mesoderm in the first and second BA and is required for the development of some head muscles (11). Tbx1 is required for activating the myogenic progression in this developmental field but is expressed only after the onset of myogenetic commitment in the trunk. Similarly, expression of at least one of the basic helix–loop–helix repressors Tcf21 (capsulin) or Msc (MyoR) is required for activation of Myf5 in the premyoblast BA mesoderm that gives rise to facial muscles (12). Double mutant mice lack first BA-derived muscle groups, such as the temporalis, masseter, and pterygoids. It seems that different premyoblastic regions of the embryo require different combinations of transcription factors to activate either MyoD or Myf5, and thereby initiate the myogenic progression. The myogenic progression can be viewed as a plug-in module that can be accessed by cells with various specifications. Specifications are defined by combinatorial codes of expressed transcription factors. Indeed, different elements control Myf5 expression in trunk and head muscle (13, 14) consistent with the view that different combinations of transcription factors activate this plug-in to the myogenic progression.

Pitx2 is a bicoid–related homeobox gene that is specifically expressed in all MyoD$^+$, Myf5$^+$, and myogenin$^+$ cells of embryonic muscle anlagen. Pitx2 therefore marks the myogenic progression more completely than any of the MRFs alone and provides the most comprehensive marker of muscle anlagen to date. Pitx2 labels virtually all muscle anlagen throughout embryogenesis and muscles in adults. Regions surrounding the anlagen generally lack Pitx2 (15). However, unlike the MRFs, Pitx2 also has expression domains outside of the muscle lineage where it plays critical roles in development. Ablation of all three Pitx2 isoforms (Pitx2abc$^{-/-}$) (16–19) causes lethality in mouse at embryonic day (E) 10.5–E14.5 with axial malformations, open body wall, laterality and heart defects, and arrest of organ development.

In this article, we examine the muscle anlagen of Pitx2 mutant embryos to determine what function is associated with the near universal expression of Pitx2 in muscle anlagen. Surprisingly, Pitx2 null mutants form all but a few muscle anlagen. Many muscle anlagen are distorted, and these distortions are generally associated with the malformation of a body part onto which the muscle

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Abbreviations: BA, branchial arch; E*n*, embryonic day *n*; MRF, myogenic regulatory factor.

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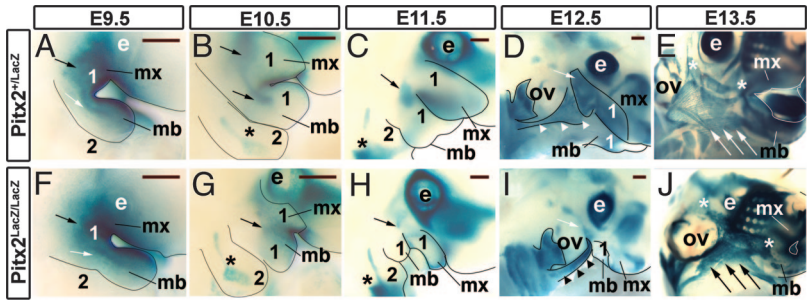

Fig. 1. Loss of head muscle in Pitx2 mutants. Whole-mount X-Gal staining was performed to trace and compare the head muscle anlagen in $Pitx2^{+/LacZ}$ (*A–E*) and $Pitx2^{LacZ/LacZ}$ (*F–J*) mouse embryos. Branchial arch structures are outlined. (*A* and *F*) At E9.5, Pitx2 was expressed in the first BA. No significant anatomical change was observed in the mutants. (*B* and *G*) At E10.5, Pitx2(β-Gal) was detected in both first and second BA (*B*, arrows and asterisk). In the Pitx2 mutant, the size of first BA was slightly smaller but the X-Gal-positive area was largely reduced (*G*, arrow). (*G*, asterisk) No significant changes in the second BA size or Pitx2 expression were observed. (*C* and *H*) At E11.5, Pitx2 mutants were characterized by hypocellular first BA. (*H*, asterisk) No significant changes in the second BA were observed. (*H*, arrow) Only a residual presumptive muscle anlage was found in first BA of the mutant. (*D* and *I*) At E12.5, first BA-derived muscles were absent (arrow), and the second BA-derived muscles were deformed in the mutant (arrowheads). (*E* and *J*) At E13.5, severe deformity and complexity of muscle anlagen was observed in the mutant. The maxillary and mandibular muscles were not properly formed (asterisks), and the second BA-derived muscles were significantly deformed in the mutant (arrows). e, eye; mb, mandibular component; mx, maxillary component; ov, otic vesicle; 1, first BA; 2, second BA.

attaches. Loss of muscle anlagen was observed only in the eye, jaw, and body wall. Much of the periocular and jaw musculature is derived from the first BA. The first BA of mutants was reduced at E10.5 and vestigial by midgestation, indicating that muscle loss was due to the loss of this structure. Pitx2 was expressed in the mesodermal cores of all BA at E10.5 when the first myoblasts were detected. However, it was expressed before the onset of the myogenic progression only in the first BA. Pitx2 was required for initiation of the myogenic progression in the first but not the other BA. Furthermore, Pitx2 was required for the expression of the premyoblast specification markers Tbx1, Tcf21, and Msc in the first BA, but not in the second BA. Thus, Pitx2 is required to set up the premyoblast specification in the first BA. It is also required for proper development of teeth, which derive from the Pitx2 expressing surface ectoderm that covers the early mesodermal core of the first BA (18). Pitx2 therefore seems to be involved in specifying the first BA itself, before the specification of muscle anlagen from the mesodermal core or teeth from the overlying ectoderm.

Results

Loss of First Branchial Arch and Deformation of Second Branchial Arch Muscle Anlagen. The morphology of muscle anlagen in Pitx2 mutant embryos was examined in detail to discover defects in muscle formation. Whole-mount X-Gal staining of Pitx2LacZ mice provided a convenient means to compare muscle anlagen at many stages of development. Mutant ($Pitx2^{LacZ/LacZ}$) and heterozygotes ($Pitx2^{+/LacZ}$) embryos were initially compared at E13.5. Anlagen for the deep back musculature showed no apparent defects. Muscles associated with the body wall, which fails to form in Pitx2 mutants, were deformed or absent (data not shown). Limb muscle anlagen also showed apparent morphological defects. The distortion of limb muscle anlagen was greater in those limbs that showed larger overall malformation because of failed body wall closure. Thus, the left hindlimb, which projected dorsally and caudally from the body of mutants, showed the greatest distortion in anlagen shapes. In contrast, the right forelimb, which was situated quite normally with respect to the body, showed no significant distortions of anlagen shapes. Although many limb muscle anlagen were distorted in mutants, no loss of anlagen was apparent (data not shown). Muscle anlagen in the head and neck appeared grossly distorted, particularly in the region between the eye, otic vesicle, and jaw. The digastic, masseter, platysma, and temporalis branchiomeric muscles were significantly smaller in mutants [Fig. 1 *E* and *J* and supporting information (SI) Table 1]. It was difficult to associate the defects in head muscle anlagen with defects in body wall closure.

The ontogeny of head muscle anlagen was therefore compared in mutant and heterozygote embryos between E9.5 and E13.5 to determine how the apparent anlagen defects observed at E13.5 arise (Fig. 1). The severe deformity and complexity of the anlagen defects made it difficult to identify corresponding anlagen in mutants and heterozygotes at E13.5 (Fig. 1 *E* and *J*). At E12.5, the pattern of X-Gal staining was simpler and allowed equivalent anlagen staining to be traced in the areas posterior and anterior to the jaw. However, the mandibular and maxillary components of the jaw were vestigial in mutants, and only a few residual blue spots were observed in these regions, indicating that most jaw-associated muscle anlagen were absent. A fan-shaped anlage originating ventral to the otic vesicle and inserting on the ventral aspect of the mandible seemed to be the anlage for a mandibular depressor, which derives from the second BA (11). This anlage was present but is no longer fan-shaped in mutants (Fig. 1 *D* and *I*). One crescent-shaped anlage just anterior to the eye seemed to extend toward the dorsal aspect of the jaw. This anlage was significantly shorter in mutants.

At E11.5, it was still possible to identify the mandibular and maxillary components of the developing jaw, or first BA, in mutants. However, both components were much smaller than normal. In contrast, the size of the second, or hyoid, arch showed no significant reduction (Fig. 1 *C* and *H*, asterisk). Pitx2 was normally expressed in a broad domain in the posterior half of the maxillary component and in a smaller more dorsal domain between the maxillary and mandibular components. These expression domains were not, or were, vestigial in mutants (Fig. 1 *C* and *H*, arrow). Instead, the maxillary component showed ectopic expression that resembled second arch expression. The Pitx2 expression domains in the second BA showed no significant defects in mutants.

At E10.5, the first BA of mutants was only slightly smaller, but striking differences in the X-Gal stain were still observed (Fig. 1 *B* and *G*). Some of the X-Gal stain at E10.5 is likely to correspond to muscle anlagen. However, the broad diffuse staining observed at E9.5 (Fig. 1 *A* and *F*) was due to Pitx2 expression in surface ectoderm. Ectoderm expression has also been reported at E10.5 in this region. The loss or malformation of jaw associated muscle anlagen was obvious at later stages when the jaw was vestigial. The first BA was still present at earlier stages but showed striking changes in the pattern of Pitx2 expression, suggesting that muscle anlagen were defective before loss of the structure. In contrast, hyoid arch-associated muscle anlagen, which showed deformities only at later stages, were not absent. No significant changes in the second BA size or Pitx2 expression were observed at earlier stages. Taken together, these results suggest that second BA anlagen were formed and became distorted, whereas first BA muscle anlagen were not properly formed in Pitx2 mutants.

Pitx2 Is Required for Initiation of Myogenic Progression in the First but Not Second Branchial Arch. The expression of Pitx2 in nonmyogenic tissues of the first BA at the earliest stages suggests that the whole-mount X-Gal analysis may not show a true picture of muscle anlagen in the developing jaw. No clusters of myogenin$^+$ cells that lacked Pitx2(β-gal) expression were observed in serial sections of

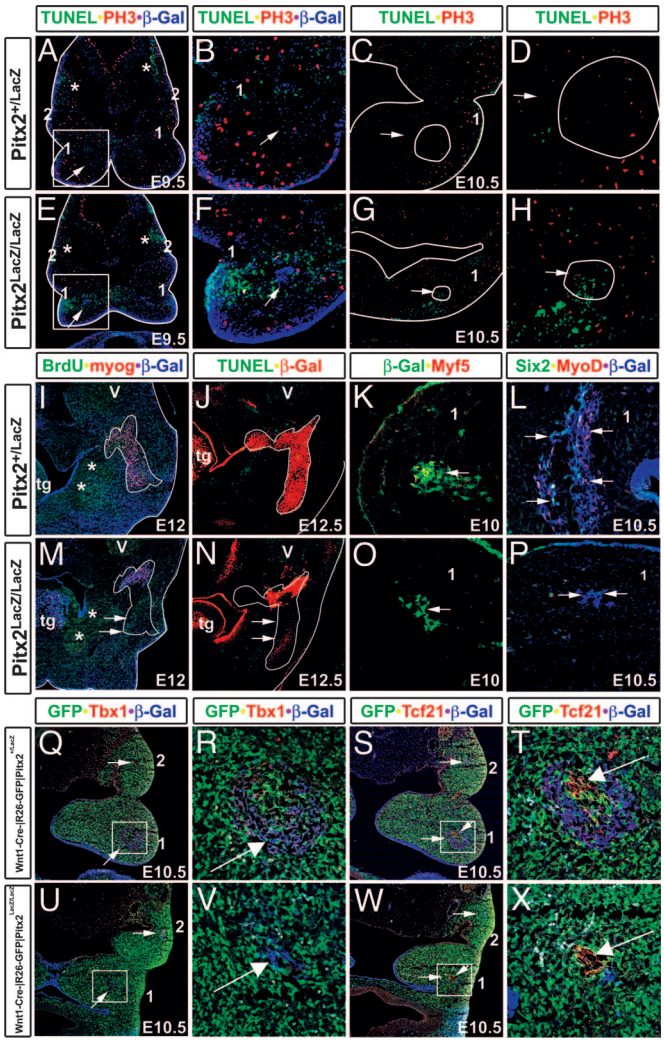

Fig. 2. Pitx2 specifies first BA myoblasts. (*A*, *B*, *E*, and *F*) TUNEL/PH3/β-Gal(Pitx2) triple labeling immunohistochemistry on transverse head sections of E9.5 $Pitx2^{+/LacZ}$ (*A* and *B*) and $Pitx2^{LacZ/LacZ}$ (*E* and *F*). (*C*, *D*, *G*, and *H*) TUNEL/PH3 double labeling on frontal head sections of E10.5 $Pitx2^{+/LacZ}$ (*C* and *D*) and $Pitx2^{LacZ/LacZ}$ (*G* and *H*). (*E–H*) Significant programmed cell death increase was observed in the mutant mice at E9.5 and E10.5. (*B*, *F*, *D*, and *H*) High-magnification images. (*I–P*) BrdU/myogenin/β-Gal(Pitx2) triple labeling immunohistochemistry on frontal head sections of E12 $Pitx2^{+/LacZ}$ (*I*) and $Pitx2^{LacZ/LacZ}$ (*M*). Muscle anlagen were outlined by Pitx2(β-Gal)$^+$ territory. No significant difference in BrdU labeling was observed in this territory. Myogenin was expressed only in a residual Pitx2(β-gal)$^+$ territory in the mutant, indicating a massive muscle reduction in the jaws (*M*, arrow). (*J* and *N*) TUNEL/Pitx2(β-Gal) double labeling immunohistochemistry on frontal head sections of E12.5 $Pitx2^{+/LacZ}$ (*J*) and $Pitx2^{LacZ/LacZ}$ (*N*). No significant change in TUNEL signal was observed within the outlined Pitx2(β-Gal)$^+$ territory in the heterozygote and mutant mice. (*K* and *O*) Myf5/β-Gal(Pitx2) double labeling immunohistochemistry on transverse head sections of E10 $Pitx2^{+/LacZ}$ (*K*) and $Pitx2^{LacZ/LacZ}$ (*O*). In the heterozygote, Myf5 was colocalized with a Pitx2(β-gal)$^+$ cell subpopulation in the first BA muscle anlagen (*K*, arrow). (*O*, arrow) Expression of Myf5 was not observed in the residual Pitx2(β-gal)$^+$ territory in the mutant. (*L* and *P*) Six2/MyoD/β-gal(Pitx2) triple labeling immunohistochemistry on sagittal head sections of E10.5 $Pitx2^{+/LacZ}$ (*L*) and $Pitx2^{LacZ/LacZ}$ (*P*). (*Q–X*) EGFP/Tbx1/Pitx2(β-Gal) (*Q*, *R*, *U*, and *V*) and EGFP/Tcf21/β-gal(Pitx2) (*S*, *T*, *W*, and *X*) triple labeling immunohistochemistry on transverse head sections of E10.5 Wnt1Cre R26EGFP $Pitx2^{+/LacZ}$ (*Q–T*) and Wnt1Cre R26EGFP $Pitx2^{LacZ/LacZ}$ (*U–X*). Tbx1$^+$ cells were colocalized with the Pitx2(β-Gal)$^+$ cells in the mesodermal cores of the first and second BA in the heterozygote mice (*R*, arrows). These cells were surrounded with the GFP$^+$ neural crest cells. Expression of Tbx1 was barely detectable in the mesodermal core of first BA in the mutant mice (*U* and *V*). Tcf21$^+$/Pitx2(β-Gal)$^+$ cells were detected in the mesoderm core of the first BA in the heterozygote mouse (*S* and *T*). This cell population was not observed in the mutant mouse (*W* and *X*). Expression of Tcf21 was also observed in the ectoderm-derived component of the first BA, which was located inside the core (*S* and *W*, arrow). (*R*, *T*, *V*, and *X*) Higher-magnification images of outlined area. 1, first BA; 2, second BA; V, trigeminal ganglion; tg, tongue.

the head (data not shown). Thus, Pitx2 still marked the muscle anlagen of the jaw. However, immunohistochemical studies at E12.5 revealed that the first BA contained many Pitx2$^+$ cells outside the myogenin$^+$ territories (Fig. 2*I*). These Pitx2$^+$/myogenin$^-$ cells may be either surface ectoderm or neural crest derivatives. Pitx2 expression in neural crest derivatives has also been observed in other regions of the head (20–23). Pitx2 expression was generally not observed in nonmyogenic territories surrounding anlagen in other regions of the body (17) Severe losses in myogenin$^+$/Pitx2$^+$ territories were observed in the region adjacent to the tongue that represents the vestigial jaw (Fig. 2 *I* and *M*). Thus, muscle anlagen of the first BA were severely reduced or absent in Pitx2 mutants.

The severe reduction in first BA size was associated with the loss of first BA muscle anlagen. Arch size reduction could result from decreased proliferation or increased cell death in either the muscle anlagen, the nonmyogenic components of the arch, or both. If the loss of first BA muscle anlagen was due to reduced cell proliferation or increased apoptosis, then one should observe decreased BrdU incorporation and PH3$^+$ cells or increased TUNEL in the relevant myogenin$^+$/Pitx2$^+$ territory. Section across the jaw at E9.5 (Fig. 2 *A*, *B*, *E*, and *F*) and E10.5 (Fig. 2 *C*, *D*, *G*, and *H*) showed increased TUNEL staining in the mutants (Fig. 2 *E* and *H*). No change in the PH3 staining was detected. Matched sections across the jaw at E12.5 show myogenin$^+$/Pitx2$^+$ territories that represent muscle anlagen in the tongue and the surrounding jaw. No significant difference in BrdU labeling was observed in these territories. In contrast, the regions outside these territories showed a slightly higher density of BrdU labeling in heterozygotes (Fig. 2 *I* and *M*, asterisk). TUNEL staining showed no significant differences inside or outside of these territories (Fig. 2 *J* and *N*). Thus, the reduction in first BA size in Pitx2 mutants was likely caused by increased cell death inside and outside of the muscle anlagen at E9.5-E10.5. The reduction in size of myogenin$^+$ muscle anlagen did not seem to be proportional to the overall size reduction. The loss of muscle cannot alone account for the deformation of jaw. The shape of the myogenin$^+$ muscle anlagen was also very different in mutants and heterozygotes. Taken together, these observations indicate that the jaw was incorrectly patterned and all components were smaller. The loss of Pitx2 resulted increased cell death and in the loss of nearly all myogenin$^+$ cells, or muscle anlagen, in the first BA.

Loss of myogenin$^+$ muscle anlagen in the first BA may have resulted from a failure to specify a population of myoblasts in this region. Myf5 and MyoD label the onset of myogenic progression, and their expression in the first BA begins at E10 and E10.5, respectively (24) (25). Immunohistochemical analyses of the first BA at E10 and E10.5 demonstrate that heterozygotes produce Myf5$^+$ and MyoD$^+$ cells in the core of the first BA (Fig. 2 *K* and *L*) where X-Gal staining was observed (Fig. 1*B*). Both MyoD and Myf5 label subpopulations of the Pitx2$^+$ cell cluster at the mesodermal cores of both the first and second BA, consistent with previous studies (15). Pitx2 staining was also observed in the overlying ectoderm of the first but not second BA (Fig. 2 *I* and *K*). At E10, the Pitx2(β-Gal)$^+$ first BA core was already much smaller in mutants and Myf5$^+$ cells were not detected in it (Fig. 2 *L* and *P*). A few MyoD$^+$ cells were observed in the first BA core of mutants at E10.5, but their total number and the fraction of Pitx2(β-Gal)$^+$ cells that was MyoD$^+$ was dramatically reduced (Fig. 2 *L* and *P*). Drastic reduction of MyoD expression was also observed by whole-mount RNA *in situ* (Fig. 3 *E* and *J*). In contrast, the Pitx2$^+$ core of the second BA of mutants was normal in size and no defects in MyoD or Myf5 expression were observed in them (data not shown).

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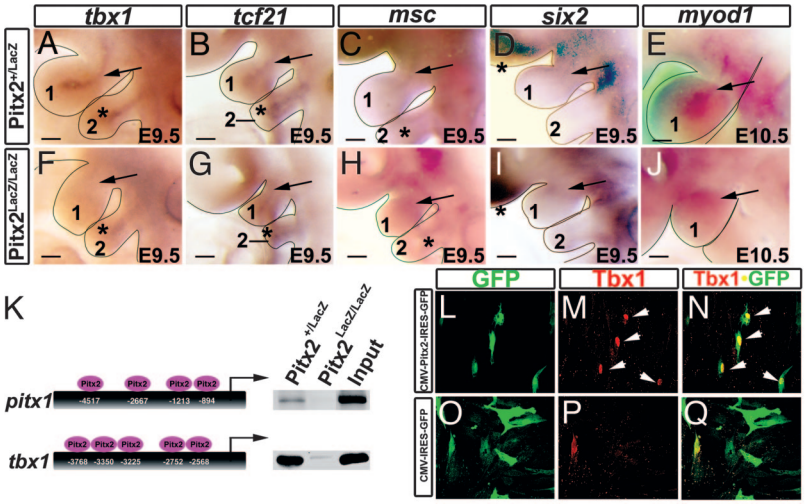

Fig. 3. Pitx2 regulated transcription factors in first BA. (*A–J*) RNA whole-mount *in situ* hybridization for tbx1 (*A* and *F*), tcf21 (*B* and *G*), msc (*C* and *H*), six2 (*D* and *I*), and myod1 (*E* and *J*) in $Pitx2^{+/LacZ}$ (*A–E*) and $Pitx2^{LacZ/LacZ}$ (*F–J*) E9.5 or E10.5 mice. The expression of tbx1 RNA was not observed in the first BA in the Pitx2 mutants (*F*, arrow), but no significant change was observed in the second BA (*F*, asterisk). The expression of tcf21 RNA was reduced in the first BA (*G*, arrow), but no significant change was observed in the second BA (*G*, asterisk). The expression of msc (*C* and *H*) and six2 (*E* and *J*) RNA was not observed in the first BA of the Pitx2 mutants. (*K*) *In vivo* ChIP assays from first and second BA chromatin extracts of E12 heterozygote mice indicated the presence of Pitx2a on the Pitx1 and Tbx1 promoters. The chromatin extracts from $Pitx2^{LacZ/LacZ}$ mice were used as a negative control. (*L–Q*) Overexpression of Pitx2a-IRES-EGFP under the control of CMV resulted in activation of Tbx1 expression (*L–N*, arrows). Nuclear staining of Tbx1 was not observed in C2C12 myoblasts transfected with an empty vector (*O–Q*). 1, first BA; 2, second BA.

These observations indicate that Pitx2 lies genetically upstream of the myoblast markers Myf5 and MyoD in the first, but not second, BA and that it plays a role in initiating myogenic progression only in the first BA.

Pitx2 Regulates Transcription Factors That Specify First Branchial Arch Myoblasts. Pitx2 expression precedes and is required for the expression of both Myf5 and MyoD in the mesodermal core of the first BA. Several other transcription factors have similar properties. Tcf21(capsulin) and Msc(MyoR) seem to encode a somewhat redundant pair of basic helix–loop–helix transcription factors, for which at least one needs to be present to form first BA associated mastication muscles(masseter, pterygoid, and temporalis) (12). Loss of both genes results in absence of Myf5 and severe reduction of MyoD in the first BA. Tbx1 is required for Myf5 and MyoD expression in the first and second BA and its loss leads to defects in first and second arch associated muscles (11). Six2 is specifically expressed in the first BA core at E9 and in the second BA core at slightly later stages (26). Functional analyses for this gene in the BA regions have not been reported. The expression of these four markers was examined in Pitx2 mutants.

Tbx1 was expressed in most, if not all, $Pitx2^{+}(\beta\text{-Gal})^{+}$ cells in the mesodermal cores of the first and second BA of heterozygotes at E10.5 (Fig. 2 *Q* and *R*). Similarly, virtually all $Tbx1^{+}$ cells were $Pitx2^{+}$ $(\beta\text{-Gal})^{+}$. Pitx2 and Tbx1 therefore label identical cell populations in these two regions. This cell population was severely reduced in size in the first BA of mutants. Furthermore, the level of Tbx1 expression in the residual population, which could still be identified by X-Gal staining, was only barely detectable at high gain. In contrast, the core $Tbx1^{+}/Pitx2(\beta\text{-Gal})^{+}$ cell population showed no significant difference in size or Tbx1 expression in the second BA (Fig. 2 *U* and *V*). The size of the $Pitx2^{+}$ core populations was more similar in the first BA of heterozygotes and mutants at E9.5 (Fig. 2 *A* and *E*). Whole-mount RNA *in situ* analyses indicated that Tbx1 RNA was expressed in stripes of similar intensity in the first and second BA (Fig. 3*A*). In mutants, the stripe representing the mesodermal core of the first BA was not detected when the stripe representing the mesodermal core of the second BA was clearly visible (Fig. 3*F*).

Tcf21 was coexpressed with $Pitx2(\beta\text{-Gal})^{+}$ cells of the BA cores in a manner similar to Tbx1, indicating that Tcf21 and Pitx2 also label the same mesodermal cells. One important difference was noted. Tcf21 also labels the neural crest-derived cells that reside inside the mesoderm core (Fig. 2 *S* and *W*). $Pitx2(\beta\text{-Gal})^{+}/Tcf21^{+}$ cells were not detected in the residual $\beta\text{-Gal}^{+}$ core of the first BA of mutants (Fig. 2 *W* and *X*). However, a group of $Pitx2(\beta\text{-Gal})^{-}/Tcf21^{+}$ cells that expressed the neural crest lineage tracer were detected. No significant changes in Tcf21 were observed in the second BA core of mutants. Whole-mount RNA *in situ* analyses at E9.5 indicated that Tcf21 RNA was expressed in stripes of similar intensity in the first and second BA (Fig. 3*B*). In mutants, Tcf21 was still expressed in the first and second BA however the expression pattern in the first BA became thinner (Fig. 3 *B* and *G*, arrow), consistent with the maintenance of Tcf21 in the neural crest-derived population at the center of the mesodermal core.

Msc and Six2 RNAs were also expressed in a central stripe in the first BA at E9.5 (Fig. 3 *C* and *D*). A weak central stripe was observed for Msc, but not for Six2, in the second BA. Expression of Msc and Six2 RNA were not observed in the first BA core of mutants (Fig. 3 *C* and *H*). The weak Msc expression was not significantly altered in the second BA. Functional Pitx2 was cell autonomously required for proper Tbx1, Tcf21, Msc, and Six2 expression in the premyoblast precursors of first, but not second, BA. Pitx2 acted genetically upstream of all four transcription factors in the first BA. Three of these factors have been invoked in the specification or commitment of first BA myoblasts.

Pitx2 Specifies Premyoblast Mesoderm in First Branchial Arch. Immunohistochemical detection of the mesodermal core of the first BA currently requires expression of either Tbx1, Tcf21, or Pitx2(β-Gal) in the core. The results above indicate that loss of Pitx2 results in severe reduction or loss of Tbx1 and Tcf21 expression. They also show that the β-Gal-labeled area, referred to as the residual mesodermal core, was severely reduced in mutants. However, it is possible that Pitx2 was also required for its own expression in a positive feedback loop and that the reduced number of $\beta\text{-Gal}^{+}$ cells in mutants reflected a loss of Pitx2(β-Gal) expression rather than a loss of the mesodermal core itself. A Pitx2 independent means to detect the mesodermal core was needed to test this hypothesis.

In the developing BA, neural crest cells fill the space between the surface ectoderm and the enclosed mesoderm at early stages. Neural crest cells generate bone, cartilage, and neuronal cells, but not muscle cells, in the developing jaws. The Wnt1-Cre|Rosa-EGFP system indelibly labels the neural crest lineage from the time it is created (27). Examination of embryos bearing this tracing system revealed GFP^{-} holes in the BA. Double labeling with GFP and Tbx1, Tcf21, or Pitx2(β-Gal) showed that these holes were filled by the mesodermal cores (Fig. 2 *J* and *L*). If the loss of Pitx2 function merely resulted in down-regulation of Pitx2, then one would expect to see a $Pitx2(\beta\text{-Gal})^{-}$ hole defined by the neural crest lineage tracing system. This $Pitx2(\beta\text{-Gal})^{-}$ hole was not observed. The simplest interpretation of the data are therefore that the mesodermal cores of Pitx2 mutants are severely reduced between E9.5 and E10.5. Myogenic progression normally begins at E10 to E10.5.

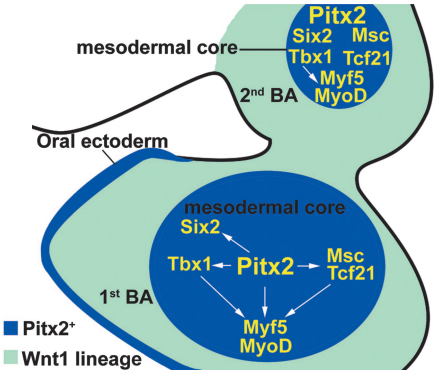

Fig. 4. Model of Pitx2 roles in the first BA muscle development. Pitx2 is expressed in the oral ectoderm and first and second BA. Pitx2 was expressed before the myogenic onset in the first BA mesodermal core and was required for the first BA muscle specification by modulating premyoblast specification markers, such as Tbx1, Tcf21, and Msc in early stages. These transcription factors are required for the Myf5 and MyoD activation in the first BA muscle precursors. Pitx2 may also directly control the expression of MRFs and regulate the differentiation of the muscle in later stages. Pitx2 was not required for the second BA specification.

Thus, Pitx2 seems to be required for specification premyoblast mesoderm in the first but not second BA.

Pitx2 Directly Interacts with Tbx1 Regulatory Elements. Chromatin immunoprecipitation using BA tissue and Tbx1 expression in myoblast cell cultures were used to test whether the regulation of Tbx1 by Pitx2 was due to a direct molecular interaction between Pitx2 protein and Tbx1 regulatory sequences. Five potential Pitx2 binding sites were identified in the 3 kb of genomic sequence that lie between the transcription initiation site of the Ensembl gene model for Tbx1 and the initiation site of a gene model for a divergent transcript of unknown function (Fig. 3*K*). This sequence has promoter activity in cell culture studies (11). Primers were designed to encompass these putative binding sites. Chromatin prepared from the BA of heterozygotes and mutants at E12.5 was sheared and immunoprecipitated with anti-Pitx2a antibodies. Tbx1 promoter fragments were amplified from heterozygote, but not from mutant precipitates (Fig. 3*K*). Pitx1 promoter fragments were also selectively amplified in heterozygous precipitates. Pitx1 is expressed in the first BA and is down-regulated in Pitx2 mutants (20) (28). These data indicate that Pitx2 occupies sequences upstream of Tbx1 in BA. A bicistronic expression vector containing the Pitx2a cDNA and IRES-GFP under the control of the CMV promoter was used to transiently overexpress Pitx2a in the C2C12 mouse myoblast cell line. Transfected cells that over-expressed Pitx2a were identified by GFP expression. These cells expressed high levels of Tbx1 that were not detected in cells transfected by the control plasmid, CMV-IRES-GFP (Fig. 3 *L* and *Q*). Taken together, the results are consistent with the idea that the Pitx2 transcription factor regulates Tbx1 by directly interacting with its promoter.

Discussion

Homeobox genes generally display discrete zones of expression and are thought to engage in different molecular mechanisms in each of the zones. Pitx2 is strongly expressed in muscle (15), neural crest (20–22), cardiac fields (23) (C.K., unpublished data) and brain cell lineages (29). Mutants of the Pitx2 homeobox gene have phenotypes in body parts corresponding to each of the gene's embryonic expression domains. However, the reported muscle phenotypes in Pitx2 mice have defied a consistent explanation. Whereas it is clear that virtually all muscle anlagen in embryos express Pitx2 in all stages of the myogenic progression (15), only a few muscles show an apparent phenotype. Most of these phenotypes are shape distortions. The most expedient explanation is that anlagen distortions are induced by the loss or distortion of one muscle insertion point. The loss of body wall and jaw in Pitx2 mutants leads to deformation but not loss of muscle anlagen that insert with only one end into these structures (latissimus dorsi, mandibular depressor). Muscle anlagen that are entirely associated with the missing structures are absent (body wall muscles, mandibular adductors, intermandibular). Some extraocular muscle precursors fail to condense in Pitx2 mutants and their expression of myogenic markers is aborted (17) (30). Some extraocular muscles and the mastication muscles that connect maxilla and mandible to other regions of the skull are derived from the first BA, which is vestigial in Pitx2 mutants by E13.5. In this article, we examine the loss of first BA muscles by tracking the earliest known muscle specification events and find that there is a drastic reduction of first BA mesoderm and in the expression of the premyogenic markers Tbx1, Tcf21, Msc, and Six2 that happens before the normal onset of MyoD and Myf5 expression. Thus, the muscle phenotypes in this region are most simply explained by the loss, or respecification, of the structure itself and have nothing to do with the any role of Pitx2 in MRF expressing cells (Fig. 4). It is likely that Pitx2 plays some as yet undiscovered roles in MRF expressing cells or in mature muscle, but we expect that this phenotype will be seen in all muscle groups of the body.

Jaw Specification. By E11.5 and E12.5, the jaw reduction clearly demonstrated that the Pitx2 homeodomain protein was essential for the development of first BA structures besides the muscle lineage. Underdevelopment of first BA in Pitx2 mutant was not due solely to loss of mesoderm components. Indeed, tooth morphogenesis in Pitx2 mutants is also impaired at early stages and involves the first arch surface ectoderm, which also expresses Pitx2. The neural crest cell lineage is a also a major contributor to the jaw structures (27), but these cells enter the BA well after the onset of Pitx2 in the mesodermal core and surface ectoderm. At E10.5 and E11.5, when the mutant first BA is smaller but still apparent, the muscle anlagen of the first BA resemble those of the second BA, suggesting that the mutant first BA exhibits second BA characteristics. Pitx2 was expressed as early as E8.5 in two broad patches corresponding to somatopleure and first BA progenitors (15). These are the two regions of the body where muscle anlagen are lost rather than just distorted. We suspect that the anterior-posterior patterning events that occur during gastrulation may initiate these two early Pitx2 expression domains and that Pitx2 may contribute to the combinatorial transcription factor codes, or network kernels, that specify jaw and abdominal body wall very early in ontogeny. Because Pitx2 is a homeobox gene, we would predict that its loss will result in a respecification event, perhaps to a primordial, less elaborate arch that more closely resembles the early second BA.

The first BA-derived mandibular muscles developed late during evolution compared with the trunk muscles. These muscles operate in breathing movements and in capturing and manipulating food and are therefore under strong selective pressure that depends greatly on the habitat of the animal. The adductors mandibulae in amphibians and reptiles exhibit many forms and seem to have evolved specializations corresponding to feeding behavior. The temporalis, masseter, and pterygoid seem to be the corresponding muscle groups in mammals. The intermandibularis helps pump air to the lung in amphibians and is thought to correspond to the mylohyoid and anterior digastic in mammals. Grafting studies suggested that development of mastication muscles shared distinct and highly conserved genetic pathways from fish to mammals (31). Interestingly, in lamprey, a jawless fish with strong mastication muscles, the Pitx2 and Tbx1 orthologous genes are also expressed in first BA mesodermal core (32, 33).

Pitx2 and Tbx1 Interactions in Myogenesis. Pitx2 and Tbx1 are molecular partners in different developmental fields including

cranial, limb and heart muscle lineages. Recent studies indicated that Tbx1 and Pitx2 are in the same genetic pathway during cardiac development (34). Tbx1 is expressed in both first and second BA at E9.5 and when mutated leads to severe perturbation or absence of both first and second BA muscles (11). Tbx1 also maintains the number of myocytes in the head and limb (35). Interestingly, like Pitx2, expression of Tbx1 follows the onset of myogenic commitment in the limb muscle anlagen but precedes the speciation event in the first BA muscle precursors (15). Our data show that Pitx2 is not only required but is also sufficient to activate Tbx1. Therefore, it is possible that Pitx2 controls the number of muscle precursors through Tbx1 in the first BA mesodermal core (Fig. 4). Furthermore, our microarray and real-time PCR data from myoblasts indicated that Tbx1 was down-regulated in the limb muscle anlagen of Pitx2 mutant (H.P.S., unpublished data) and places Pitx2 upstream of Tbx1 in the skeletal muscle lineages.

Materials and Methods

Mouse Strains. Pitx2-LacZ knockin mice (18) on ICR (outbred stock from the Institute of Cancer Research) background were used.

X-Gal Staining, Immunohistochemistry, BrdU Labeling, and TUNEL Stains. For general immunohistochemical studies and X-Gal staining, mouse embryos were dissected free of membranes; then we proceeded as described (15). Specific antibodies against MyoD, myogenin, Myf5 (Santa Cruz Biotechnology, Santa Cruz, CA), β-galactosidase (Cappel, Aurora, OH), PH3 (Upstate, Lake Placid, NY), BrdU (Accurate Chemical Scientific Corporation, Westbury, NY), and EGFP (H.P.S., Oregon State University) were applied at empirically determined optimal titers. BrdU/PBS solution (50 μg/g of body weight) was injected i.p. 2 hr before killing. BrdU immunohistochemistry was performed in postfixed immunolabeled tissue followed by acid depurination and neutralization. TUNEL assay was also performed as recommended by the manufacturer (Dead End kit; Promega).

RNA Whole-Mount *in Situ* Hybridization. RNA *in situ* hybridization was performed according to standard procedures (26). Digoxigenin-labeled antisense RNA riboprobes were generated by in vitro transcription kit (Roche Molecular Biochemicals). AP-conjugated anti-DIG antibody was used to detect the hybridization signals (Roche Molecular Biochemicals).

Tissue Culture and Transient Transfection. C2C12 cells were grown on 10-cm dishes in DMEM (Cellgro) supplement with 10% FBS (HyClone), 5 mM glutamine, penicillin, and streptomycin. The C2C12 cells were then seeded onto poly(L-lysine) (0.1 mg/ml)-coated glass coverslips in 12-well plates. Cells at 50% confluence were transfected and then maintained in the medium without antibiotics according to the instructions of the manufacturer (Lipofectamine 2000; Invitrogen). After 24 hr, the transfected cells were examined by immunohistochemical staining.

***In Vivo* ChIP Assays.** BA were dissected and collected from E12.5 embryos. Dissected BA were mechanically triturated several times by using 1-ml tip on ice to dissociate the tissue. Dissociated cells were cross-linked with 1% formaldehyde/PBS solution for 10 min at 25°C, resuspended in lysis buffer, and then sonicated (average length of sheared fragments was ≈300–1,000 bp). Ten percent of the soluble chromatin complex was saved for positive control. Soluble chromatin complexes were diluted and then incubated with specific IgGs against Pitx2a (15, 20) overnight at 4°C. Chromatin/antibody complexes were pulled down by protein-A Sepharose beads and then eluted. Eluted immunoprecipitates were heated at 65°C for 6–18 hr to reverse the formaldehyde cross-linking. DNA fragments were purified with a QIAquick spin kit (Qiagen, Chatworth, CA).

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