Pitx2 defines alternate pathways acting through MyoD during limb and somitic myogenesis

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SUMMARY

The MyoD gene is part of the core regulatory network that governs skeletal myogenesis and acts as an essential determinant of the myogenic cell fate. Although generic regulatory networks converging on this gene have been described, the specific mechanisms leading to MyoD expression in muscles of different ontology remain misunderstood. We now show that the homeobox gene Pitx2 is required for initial activation of the MyoD gene in limb muscle precursors through direct binding of Pitx2 to the MyoD core enhancer. Whereas Myf5 and Mrf4 are dispensable for limb muscle progenitor fate, inactivation of Myf5 and Mrf4 in Pitx2 mutants results in a drastic decrease of limb MyoD expression. Thus, Pitx2 and Myf5 define parallel genetic pathways for limb myogenesis. We show a similar dependence on Pitx2 and Myf5(Mrf4) in myotome, where MyoD expression is initially activated by Myf5 and Mrf4. In their absence, MyoD expression is eventually rescued by a Pax3-dependent mechanism. We now provide evidence that Pitx2 contributes to the rescue of MyoD expression and that it acts downstream of Pax3. We thus propose that myogenic differentiation of somite-derived muscle cells relies on two parallel genetic pathways, with the Pitx2 pathway being of primary importance for limb myogenesis but the Myf5 and Mrf4 pathway predominating in myotome. Muscle-specific wiring of regulatory networks composed of similar transcription factors thus underlies development of distinct skeletal muscles.

KEY WORDS: Muscle, Differentiation, Regulatory networks, Mouse, Myod1

INTRODUCTION

Much that we have learned about muscle development has emphasized a general regulatory network driving myogenesis. The program for skeletal muscle development depends on a genetic network that is centred on a group of basic helix-loop-helix muscle regulatory factors (MRF) that control determination of myogenic progenitors and differentiation of myoblasts. The implication of this core genetic network in all skeletal muscles, together with the shared properties of MRFs, has led to a relatively simple view of muscle development. By contrast, different skeletal muscles clearly achieve different functions through intrinsic differences that are probably built into their developmental program. The molecular mechanisms that underlie the unique identity of different muscles remain elusive. We already have indications that two groups of body skeletal muscles have distinct mechanisms of formation although they both derive from somites. Indeed, most muscles of the trunk develop by growth, expansion and reorganization of the myotome (Cossu et al., 1996; Denetclaw et al., 1997; Tajbakhsh et al., 1996b), giving rise to back, intercostal and ventral body muscles (Christ et al., 1983; Christ and Brand-Saberi, 2002). By contrast, several muscle groups including limb, diaphragm, intrinsic tongue and pharynx muscles do not go through a myotomal intermediate. Instead, myogenic progenitor cells of the hypaxial dermomyotome undergo an epithelial-to-mesenchymal transition and migrate as single cells to their respective destinations (Bladt et al., 1995; Christ and Ordahl, 1995; Mackenzie et al., 1998; Noden, 1983; Ordahl and Williams, 1998). Finally, extraocular and branchiomeric muscles do not derive from somites, but rather from cranial mesoderm (Noden and Francis-West, 2006).

In spite of these developmental differences, all skeletal muscles rely on the regulatory network involving MRFs. Thus, Myf5 is expressed at the onset of myogenesis in the mouse embryo (Ott et al., 1991) when, together with Mrf4, it determines the myogenic cell fate (Kassar-Duchossoy et al., 2004). MyoD is subsequently expressed and can direct cells into the myogenic program in the absence of Myf5 and Mrf4 (Braun et al., 1992). Myf5, Mrf4 and MyoD thus constitute the core regulatory network for myogenic determination. In their absence, precursor myoblast cells are lacking and skeletal muscles do not form (Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993). By contrast, myogenin functions as an essential differentiation factor, as inactivation of its gene prevents formation of functional muscle fibres in vivo without affecting myoblast determination (Hasty et al., 1993; Nabeshima et al., 1993). Although this core network has been implicated in all skeletal myogenesis, various studies on hierarchical interactions between MRFs and with upstream transcription factors such as Pax3 suggested that “wiring” of the myogenesis network is different in trunk, limbs or head muscles (Bajard et al., 2006; Kablar et al., 1999; Kassar-Duchossoy et al., 2004; Sambasivan et al., 2009). In particular, different transcription factors were shown to modulate the core network in these muscles (Dastjerdi et al., 2007; Grifone et al., 2005; Grifone et al., 2007; Mankoo et al., 1999).

The Pitx2 gene has previously been shown to be expressed during embryonic myogenesis (Diehl et al., 2006; Dong et al., 2006; L’Honoré et al., 2007; Shih et al., 2007b) of different skeletal muscle types, including extraocular, branchiomeric, trunk and limb muscles. Whereas Pitx2 was suggested to regulate MRF transcription in extraocular and branchiomeric muscles (Diehl et al., 2006; Dong et al., 2006), it was also implicated in proliferation and survival of muscle progenitor cells in branchiomeric muscles.
(Dong et al., 2006; Shih et al., 2007a). During body muscle development, Pitx2 is expressed in muscle progenitor cells and early differentiating cells and its expression decreases at terminal differentiation (L’Honoré et al., 2007; Shih et al., 2007b). However, the precise role of Pitx2 in those muscles is unknown.

Pitx2 belongs to the Pitx gene family that includes three members in vertebrates. This family encodes paired-related homeodomain transcription factors that play major roles in early patterning and organogenesis. Pitx2 mutations have been associated with human Axenfeld-Rieger syndrome type I, an autosomal dominant disorder that includes dental hypoplasia, anterior segment eye defects, craniofacial dysmorphologies and heart defects as cardinal features (Gage et al., 1999; Kioussi et al., 2002; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999; Semina et al., 1996). Pitx2 is an essential effector for left-right asymmetry, and it is essential for pituitary and craniofacial development (Gage et al., 1999; Lin et al., 1999). The mouse Pitx2 gene produces three spliced isoforms (a, b and c) from two different promoters. Ablation of all three Pitx2 isoforms in the mouse embryo causes lethality at mid-gestation (E10.5-E13.5) with axial malformations, open body wall, heart malformations, laterality defects and arrest of multiple organ development.

In this report, we defined the role of Pitx2 in both limb and myotome muscle development. We show that Pitx2 is crucial for the onset of MyoD gene expression in limb muscle progenitors and that it acts on the MyoD core enhancer. This action is later compensated by a Myf5-dependent mechanism. By contrast, initiation of MyoD expression in myotome is not dependent on Pitx2 but we show that Pitx2 acts in a Pax3-dependent genetic pathway parallel to Myf5 and Mrf4 such that Pitx2 rescues MyoD-mediated myogenesis in their absence. We propose that the joint actions of Myf5, Mrf4 and Pitx2 are crucial for control of MyoD expression in muscles with different contributions of each gene depending on muscle ontology.

MATERIALS AND METHODS
Animal handling and genotyping
All animal procedures were approved and conducted in accordance with IRCM Animal Ethics Review Committee regulations. Pitx2−/−, Myf5−/−, Myf5lacZ and Splotch mice have been described previously (Gage et al., 1999; Tajbakhsh et al., 1996a; Underhill et al., 1995). Genotyping was carried out by PCR using DNA isolated from umbilical cord/amniotic sacs (protocols.asp) using digoxigenin-labelled riboprobes for detection of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA52242, USA); monoclonal anti-MyoD, 1:100 (Pharmingen, Mississauga, ON, Canada); and polyclonal anti-Ki67, 1:100 (LabVision/Neomarkers, Fremont, CA, USA). Secondary antibodies were coupled to biotin (anti-rabbit BA1000, or anti-mouse BA2000, Vector Labs, Burlington, ON, Canada) and used at dilution of 1:150, or coupled to a fluorochrome, Alexa 488 or 555 (Molecular Probes/Invitrogen, Carlsbad, CA, USA) and used at dilution of 1:250. Streptavidin was coupled to peroxidase HRP (NEL750, NEN Life Science, Bridgewater, NJ, USA) and used at 1:1000 or coupled to a fluorochrome, Alexa 488 or 555 (Molecular Probes/Invitrogen, Carlsbad, CA, USA) and used at 1:500. For immunohistochemistry, reactions were performed using diaminobenzidine (DAB, Sigma-Aldrich, St Louis, MO, USA) as peroxidase substrate (L’Honoré et al., 2007) and slides were counterstained with Methyl Green. For immunofluorescence, slides were mounted using Mowiol.

Plasmids and transfection assays
The MyoD CE and CE-mut luciferase reporter plasmids were constructed in pXP2 driven by the mouse MyoD proximal promoter (−285/+15 bp). The intact MyoD 258 bp CE was cloned upstream of this promoter and the CE-mut plasmids included mutations in either or both PRE as indicated in Fig. 4A. The following oligonucleotides were used for site directed mutagenesis of the PitxRE sites: PRE1fwd, GAGCAGCTGTG-TCCAAACGAGCCAGTTCTC; PRE1rev, GAGGACTCATGGGATCTTGCTGTCGAGCTGTCG; PRE2fwd, GTGAAATCCAGGTGAGTCAGTCTCAGTTCTC; and PRE2rev, GTGTTTGGAGTGCTACGGTTTTAACAGTGCA-GCAGCTCTGGGGAATTC. The reporter plasmids were sequenced to ascertain the expected sequences.

CV-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% foetal bovine serum and penicillin/streptomycin antibiotics and maintained in a humidified incubator (37°C, 5% CO2). For transfection, 40,000 cells were plated in 12-well-plates. Cells were transfected by the calcium phosphate co-precipitation method using a total of 1.5 μg of DNA (100 ng of CE-wt or CE-mut reporter plasmid, 0-50 ng of RSV-Pitx2 or empty RSV-expression vector, 50 ng of CMV-β-galactosidase as internal control and 1.3 μg of carrier DNA). Cells were harvested 24 hours later using transfection lysis buffer [0.1 M Tris (pH 8.0), 0.5% NP-40, 1 mM DTT] and luciferase activity was assayed by injection of 100 μl of luciferase [0.5 mM in 100 μM Tris (pH 8.0)] to 100 μl of lysates using the Glomax luminometer (Promega, Madison, WI, USA). β-Gal activity was determined using the β-gal reporter gene Galacto-Star (TROPIX) assay system.

Chromatin immunoprecipitation
Chromatin immunoprecipitations were performed as described previously (Coulon et al., 2007) on either E12 hindlimbs, carefully dissected hindlimb muscles at E15 or E12 myotomes. Similar results were obtained using a Pitx2-specific antibody (L’Honoré et al., 2007) as well as an antibody that recognizes equally well the three Pitx factors. For ChIP analyses of the MyoD locus, the following primers were used in qPCR: CEfwd, CACAGTTAATCTCCAGAGTGC; CRErev, TGGATCTAGAGAAC; PRE1fwd, AGGTGCATGGTCTCTTGTG; DRRrev, CATTTCAGCTCCCTTGCTAGTCT; PRfwd, ACTCCTATGTTCTGCCGTGTC; and PRrev, ATAGCGGCATTCTGGGATCC. Enrichments were calculated relative to q-PCR analyses of a control sequence within the POMC gene promoter that is not expressed in muscles or limbs: POMCFwd, TGCCAGGTGAATCCAAGTATTGCG; and POMCrev, TGGTTTCAACAAGATACACTTCCCTCC. The β-actin and GAPDH loci were also used as negative control and yielded similar results.

RESULTS
Having previously shown (L’Honoré et al., 2007) that Pitx2 is expressed before MyoD in Pax3-positive myogenic progenitors that have entered limb buds (Fig. 1A-C), we wanted to assess Pitx2 expression in relation to the first expressed myogenic bHLH factor...
Myf5. Using Myf5\(^{\text{nlacZ}}\) heterozygous mice that carry a lacZ insertion into the Myf5 locus (Tajbakhsh and Buckingham, 1994), we did co-labelling by immunofluorescence for Pitx2 and \(\beta\)-galactosidase (Myf5) in E11.0 forelimb buds. Whereas all Myf5-expressing cells co-express Pitx2 (Fig. 1D-F), some Pitx2-positive Myf5-negative cells (13±1% (n=6) are also observed (Fig. 1F, arrowheads). As the expression of MyoD is more restricted at this developmental stage (Fig. 1G-J) (L'Honoré et al., 2007), these data show that Pitx2 expression precedes activation of the limb myogenic program (Fig. 1K).

MyoD expression is decreased in Pitx2\(^{-/-}\) limb buds

In order to assess a putative role of Pitx2 in the myogenic program, we crossed the Myf5\(^{\text{nlacZ}}\) indicator allele into the Pitx2-null background. Expression of \(\beta\)-galactosidase (Myf5) in Pitx2\(^{+/-}\) embryos is identical to Pitx2\(^{+/-}\) embryos (data not shown), and thus heterozygote embryos were used throughout as control. Mice heterozygous for Myf5\(^{\text{nlacZ}}\) did not show significant differences in \(\beta\)-galactosidase (Myf5) expression in E11.0 Pitx2\(^{+/-}\) forelimb (FL) and hindlimb (HL) buds compared with Pitx2\(^{+/-}\) embryos (Fig. 2A,B; Fig. S1A,B in the supplementary material). The early expression of Myf5 in limb progenitors thus appears to be largely independent of Pitx2. By contrast, the onset of hindlimb bud MyoD expression (Fig. 2C) is markedly decreased in Pitx2\(^{-/-}\) embryos (Fig. 2D). A similar decrease was observed in FL buds at E10.5 (data not shown) and decreased MyoD transcripts are still observed in E11.5 FL and E12.5 HL (Fig. 2F, compare with 2E; see Fig. S1C,D in the supplementary material). However, MyoD expression is largely recovered in E12.5 FL buds (Fig. 2G,H). As myogenin expression is dependent on MyoD during limb myogenesis (Sassoon et al., 1989; Wright et al., 1989), we assessed its expression by in situ hybridization in Pitx2\(^{-/-}\) embryos. We observed decreased myogenin transcripts, in agreement with their purported dependence on MyoD (Fig. 2J, compare with 2I). As shown for MyoD in E12.5 FL (Fig. 2G,H), similar patterns of MyoD and myogenin were observed in E13.0 FL and HL (see Fig. S1E,F in the supplementary material and data not shown), suggesting full recovery of their expression.

Delayed myogenic differentiation in Pitx2\(^{+/-}\) limb buds

The decreased MyoD expression in Pitx2 mutant limb buds may be due to altered progenitor number or delayed myogenic differentiation. In order to assess these possibilities, we analyzed myogenic precursors and their proliferation status by co-labelling for Pax3 and Ki67. The number of Pax3-positive cells is unaltered...
MyoD-positive cells in E13.5 HL buds revealed a complete recovery of the number of MyoD-expressing cells (Fig. 3M-O). These results clearly indicate that the absence of Pitx2 causes a delay in MyoD expression that is fully recovered by E13.5.

**Pitx2 acts directly on the MyoD core enhancer**

We considered the possibility that Pitx2 may act directly on the MyoD gene. Appropriate spatiotemporal expression of MyoD is reproduced by a 24 kb fragment of 5′ regulatory sequences that contains two enhancers called core enhancer (CE) (Faerman et al., 1995; Goldhamer et al., 1992; Goldhamer et al., 1995) and distal regulatory region (DRR) (Asakura et al., 1995; Tapscott et al., 1992). Although DRR activity is restricted to differentiated cells and maintains MyoD expression at foetal stages and postnatally (Asakura et al., 1995; Chen et al., 2002; Hughes et al., 1993; L’Honoré et al., 2003), genetic studies have ascribed initiation of MyoD expression to the CE (Chen and Goldhamer, 2004; Kablar et al., 1999). The highly conserved 258 bp CE element is located about 20 kb upstream of the MyoD gene and it recapitulates the skeletal muscle pattern of MyoD expression during embryonic development (Faerman et al., 1995; Goldhamer et al., 1992; Goldhamer et al., 1995).

Interestingly, targeted deletion of the MyoD CE leads to a delay of MyoD expression in limbs (Chen and Goldhamer, 2004) that is similar to that observed in Pitx2−/− embryos (Figs 2 and 3), suggesting that the MyoD CE may be a direct target of Pitx2. To assess this hypothesis, we analyzed the CE sequence (Goldhamer et al., 1995) and this revealed two well conserved putative Pitx binding sites (Fig. 4A). The sites (TAATCT and TAAGCT) are conserved between mouse and human (see Fig. S3A in the supplementary material) and correspond to sequences previously shown to bind and be activated in response to Pitx1 and Pitx2 (Tremblay et al., 2000); furthermore, the sites are in opposite orientation relative to each other, as often observed in pituitary Pitx target genes (Tremblay et al., 1998). We used gel retardation assays to verify that Pitx2 binds these two sites (PRE1 and PRE2) in vitro (see Fig. S3B in the supplementary material). Similar binding was observed at each site and a probe containing both sites showed cooperative binding. The in vivo occupancy of the CE was ascertained directly by chromatin immunoprecipitation (ChIP) using HL buds from E12 and E15 embryos and anti-Pitx2 antibody. Significant recruitment of Pitx factors was observed both in E12 and E15 buds at the CE but not at the DRR (Fig. 4B). Interestingly, recruitment was also observed at about −600 bp in the promoter region (PR), in agreement with the presence of a putative Pitx binding site at −615 bp. This site was tested by gel retardation and shown to bind Pitx2 (see Fig. S3B in the supplementary material). Similar binding was observed at each site and a probe containing both sites showed cooperative binding. The in vivo occupancy of the CE was ascertained directly by chromatin immunoprecipitation (ChIP) using HL buds from E12 and E15 embryos and anti-Pitx2 antibody. Significant recruitment of Pitx factors was observed both in E12 and E15 buds at the CE but not at the DRR (Fig. 4B). Interestingly, recruitment was also observed at about −600 bp in the promoter region (PR), in agreement with the presence of a putative Pitx binding site at −615 bp. This site was tested by gel retardation and shown to bind Pitx2 (see Fig. S3B in the supplementary material). The CE may thus be a direct target of Pitx factor action and this possibility was tested directly in co-transfection experiments in CV1 cells. The reporters containing the CE inserted upstream of the proximal MyoD promoter exhibited dose-dependent activation in response to increasing amounts of Pitx2 and mutagenesis of the two putative Pitx-binding sites in CE prevented this activation (Fig. 4C). Linker-scanning mutagenesis of the CE provided an in-depth functional analysis of this element (Kucharczuk et al., 1999) but, surprisingly, it did not identify any essential sequence for MyoD limb expression. In particular, mutants LS-8 and LS-9 that respectively encompass the PRE1 and PRE2 did not affect limb activity. To assess the hypothesis that PRE1 and PRE2 may have redundant activities, we performed co-transfection experiments for Pitx2 activation of a wild-type or mutant CE reporter (Fig. 4D). Whereas mutagenesis of either site had a marginal effect, their combined mutation abrogated activation, which highlights their redundancy. These experiments support the
model that Pitx2 is directly recruited to the MyoD CE to activate MyoD expression in early development, notwithstanding a possible action through other Pitx sites. The model is further supported by equivalent delays of limb MyoD expression observed in Pitx2–/– (Fig. 2) and mice deleted of the MyoD CE (Chen and Goldhamer, 2004).

Myf5 cooperates with Pitx2 for limb MyoD expression

The delay in MyoD expression observed in Pitx2–/– limb buds thus appears to be due to the failure to activate the MyoD core enhancer. This delay is not compensated for by the related Pitx3 as the double Pitx2–/–;Pitx3–/– embryos show similar myogenin expression patterns as the Pitx2–/– embryos (see Fig. S4 in the supplementary material). As Myf5 is activated independently of Pitx2 during early myogenesis, we then tested the possibility that Myf5 may contribute to MyoD recovery in Pitx2–/– limbs. As previously reported (Tajbakhsh et al., 1997), MyoD expression is not affected in limb buds of Myf5nLacZ/nLacZ mutant embryos indicating that Myf5 and Mrf4 (which is inactivated in cis in this mutant) are dispensable for non-myotomal MyoD expression (Fig. 5B, compare with 5A,E). However, inactivation of Myf5(Mrf4) in the Pitx2–/– background resulted in almost complete loss of MyoD expression at E12.5 compared with Pitx2–/–;Myf5nLacZ/+ embryos (Fig. 5D,H, compare with 5C,G). As for the Pitx2 knockout (Fig. 2A-D), the double Pitx2 and Myf5(Mrf4) loss-of-function did not appear to
Fig. 6. Pitx2 is sufficient for hypaxial myotome expression of MyoD in absence of Myf5 and Mrf4. (A-D) Dorsal views of interlimb myotomes from Pitx2<sup>+/+</sup>;Myf5<sup>fl<ins>_lacZ</ins></sup>/+ (A), Pitx2<sup>+/+</sup>;Myf5<sup>fl<ins>_lacZ</ins></sup>/fl<ins>_lacZ</ins> (B), Pitx2<sup>−/−</sup>;Myf5<sup>fl<ins>_lacZ</ins></sup>/+ (C) and Pitx2<sup>−/−</sup>;Myf5<sup>fl<ins>_lacZ</ins></sup>/fl<ins>_lacZ</ins> (D) embryos at E12.5 stained by whole-mount in situ hybridization for MyoD transcripts. The black arrows indicate hypaxial myotome. See also Fig. S4A,B in the supplementary material. (E-H) Staining by immunofluorescence for MyoD (red, E,G) or β-galactosidase (Myf5, green, F,H) on transverse sections of thoracic somites from E11.5 Pitx2<sup>+/+</sup>;Myf5<sup>fl<ins>_lacZ</ins></sup>/+ (E,F) and Pitx2<sup>−/−</sup>;Myf5<sup>fl<ins>_lacZ</ins></sup>/fl<ins>_lacZ</ins> (G,H) embryos. The white arrow indicates hypaxial myotome and the asterisk indicates non-specific staining of red blood cells. See also Fig. S6G,H in the supplementary material for myogenin expression. (I) Chromatin immunoprecipitation (ChIP) analysis for the presence of Pitx2 at the indicated MyoD regulatory sequences were performed on chromatin isolated from dissected myotome of wild-type E12 embryos. The histogram shows enrichments (±s.e.m., n=3) obtained at CE, DRR and PR relative to the POMC promoter used as reference.

Pitx2 in myotome expression of MyoD

We have previously shown Pitx2 expression in myogenic cells of the myotome (L'Honoré et al., 2007). Myotome expression of MyoD (Fig. 2F and data not shown) and of myogenin (Fig. 2J) is not delayed in Pitx2<sup>−/−</sup> embryos, in contrast to muscle cells. We wanted to test the hypothesis that Pitx2 and Myf5/Mrf4 may also cooperate for MyoD expression in myotome. In Myf5<sup>fl<ins>_lacZ</ins></sup>/fl<ins>_lacZ</ins> mutant embryos where both Myf5 and Mrf4 are absent, MyoD expression in myotome is delayed by ~2 days. Recovery of MyoD expression, which is dependent on Pax3, appears by E11.5 and is complete by E12.5 (Tajbakhsh et al., 1997). Investigation of E12.5 Pitx2<sup>−/−</sup>;Myf5<sup>fl<ins>_lacZ</ins></sup>/fl<ins>_lacZ</ins> embryos that are deficient in Pitx2, Myf5 and Mrf4 showed an almost complete loss of MyoD myotome expression (Fig. 6D) compared with Myf5 (Fig. 6B) and Pitx2 (Fig. 6C) mutants (also see Fig. S6A-D in the supplementary material). The remaining weak expression of MyoD in these double mutants was ascertained by immunohistochemistry on E11.5 transverse thoracic sections. Staining for MyoD and myogenin revealed a small patch of positive cells in the most epaxial part of the myotome in Pitx2<sup>−/−</sup>;Myf5<sup>fl<ins>_lacZ</ins></sup>/fl<ins>_lacZ</ins> embryos (Fig. 6G), compare with 6E; see Fig. S6G,H in the supplementary material). However, the loss of Pitx2 did not affect the appearance of myotome cells as revealed by β-galactosidase staining (Fig. 6F,H; see Fig. S6E-H in the supplementary material). Pitx2<sup>−/−</sup> embryos are characterized by severe trunk distortion (see Fig. S6C,D in the supplementary material). It is noteworthy that this distortion had no effect on MyoD expression in myotome of Pitx2 mutants, clearly not supporting the idea of non cell-autonomous effects in the myotome of Pitx2<sup>−/−</sup>;Myf5<sup>fl<ins>_lacZ</ins></sup>/fl<ins>_lacZ</ins> embryos. In view of the many apparent targets of Pitx2 recruitment/action at the MyoD locus revealed by ChIP in limb buds (Fig. 4B), we also ascertained the presence of Pitx2 at the same sites in E12 myotome. The experiments clearly indicated recruitment of Pitx2 at the CE, DRR and ~600 bp regions of the MyoD gene (Fig. 6I). These data thus suggest multiple targets for Pitx2 action on the MyoD gene and, in particular, support the involvement of the DRR for myotome MyoD expression (Chen et al., 2002).

As Pax3 was involved in recovery of MyoD expression in myotome of Myf5/Mrf4 mutants (Kassar-Duchossoy et al., 2004; Tajbakhsh et al., 1997), we investigated a putative role of Pax3 in myotome expression of Pitx2. We first excluded the possibility that Pax3 might act directly on PREs using gel retardation (see Fig. S3B in the supplementary material). The Pax3 mutant Splotch mouse exhibits disorganized dermomyotome and it has slightly delayed myogenic differentiation. Analysis of these mutants at E11.5, when myogenesis resumes (as evidenced by myogenin expression, Fig. 7B,D), revealed a complete deficit of Pitx2 expression (Fig. 7A,C); this deficit is restricted to the myotome and is not observed in neighbouring mesenchyme, indicating that Pitx2 is downstream of Pax3 during myotome myogenesis. If myotome expression of Pitx2 is downstream of Pax3, inactivation of both genes should have no greater effect than either mutation: this was indeed observed in myotome (Fig. 7E-G). These results indicate that Pitx2 is a likely intermediate downstream of Pax3 for MyoD recovery in Myf5/Mrf4 mutant myotomes. Interestingly, we observed a similar relationship between Pax3 and Pitx2 at shoulder girdle level. MyoD expression in a muscle opposite the forelimb is truncated in Pax3<sup>−/−</sup> embryos (Fig. 7H,I). This truncation is also observed in Pitx2<sup>−/−</sup> embryos (Fig. 7K,M) and the absence of both Pax3 and Pitx2 did not aggravate this truncation (Fig. 7H-J). As previously described, this truncation is not observed in Myf5<sup>fl<ins>_lacZ</ins></sup>/fl<ins>_lacZ</ins> mutants (Fig. 7K,L) (Tajbakhsh et al., 1997), but we now provide evidence that MyoD expression in this muscle is also dependent on both Pitx2 and Myf5/Mrf4 pathways, as the double mutant is almost completely devoid of MyoD (Fig. 7K-N). These data are consistent with Pax3 regulation of Pitx2 expression in both myotome and shoulder girdle, and with a primary role of the Pax3/Pitx2 pathway in the shoulder girdle.

**DISCUSSION**

Many studies have supported the idea that one core regulatory MRF is sufficient to initiate myogenesis in skeletal muscles of various origins. Whereas Myf5 and Mrf4 are primarily involved in...
initial determination of the myogenic program, it appears that MyoD is the point of convergence for regulatory pathways that drive myogenic differentiation. Recent work has identified Pitx2 as an upstream regulator for MRF expression in extraocular muscles (Diehl et al., 2006) and Pitx2 was also shown to cooperate with Tbx1 for activation of the muscle fate in the pharyngeal arch (Dong et al., 2006; Shih et al., 2007a). The present work identifies a crucial role for Pitx2 in control of somite-derived myogenesis, placing this gene either downstream of Pax3 in myotome and in a parallel pathway to Myf5 and Mrf4 in limbs where Pitx2 directly controls onset of MyoD expression. Taken collectively, these data support models that include Pitx2-dependent pathways in all skeletal muscles (Fig. 8A).

The present work documented a delay of limb bud MyoD expression in Pitx2−/− embryos. This delay is similar to the 1- to 2-day delay of MyoD activation in limb buds of MyoD CE-deleted mice. Accordingly, we show Pitx2 binding to the MyoD CE in embryonic limb buds by ChIP and binding site-dependent transcriptional activation by Pitx2. Thus, the MyoD core enhancer is the likely target for Pitx2 control of MyoD expression during limb myogenesis. CE-deleted mutant embryos are characterized by delayed MyoD expression not only in limbs but also in muscle precursors of the first branchial arch (Chen and Goldhamer, 2004). During branchiomeric development, Pitx2 is expressed both in surface ectoderm and in the mesodermal core of the first arch, and interestingly conditional inactivation of Pitx2 in pharyngeal mesoderm results in severe reduction of MyoD and myogenin expression (Dong et al., 2006). The CE may thus also constitute a cis target for Pitx2-dependent MyoD expression during branchiomeric myogenesis. In both Pitx2+/− and CEloxP/loxP mutants, the functional consequence of delayed MyoD expression was a delay in muscle differentiation as revealed by myogenin expression. This defect is reminiscent (somewhat less pronounced) of delayed limb and branchial arch differentiation observed in MyoD−/− embryos (Kablar et al., 1997). Collectively, these data suggest similar regulatory networks for Pitx2-dependent MyoD activation in MyoD-dependent lineages of the head and limb.

This similarity contrasts with Myf5/Mrf4 regulation of MyoD. Indeed, while MyoD expression is delayed in myotome of Myf5nlacZ/nlacZ mutant embryos, it is unaffected in limb buds and branchial arch-derived myogenic cells (Kablar et al., 1997; Tajbakhsh et al., 1997). Consistent with this, CE activity is not affected in Myf5nlacZ/nlacZ limb buds nor in branchial arch, suggesting that Myf5 is dispensable for both CE activity and early MyoD expression in these lineages (Chen and Goldhamer, 2004).

Fig. 7. Cooperation between Pax3/Pitx2 and Myf5/Mrf4 pathways for MyoD expression in myotome and shoulder girdle muscle. (A-D) Immunohistochemical analyses of Pitx2 and myogenin expression on transverse sections of thoracic somites from E11.5 Pax3Sp+/+ and Pax3Sp+/− embryos. The black arrows indicate unaffected Pitx2 expression in mesenchyme, whereas dotted lines indicate myotomes. (E-G) Whole-mount in situ hybridization for MyoD performed on E12.0 (E-I) and E12.5 (K-N) embryos of the indicated genotypes. (E-G) Bracket indicates width of myotomes. (H-N) Forelimb buds are outlined with black dotted lines, whereas white dotted lines indicate shoulder girdle muscle; furthermore, the extent of this shoulder muscle is indicated by a white bar. Second branchial arch muscles affected by Pitx2 mutation (Shih et al., 2007a) are outlined with green line. Asterisk indicates first branchial arch that is absent in Pitx2 mutant (Dong et al., 2006). White arrows in K-N indicate presence (K-M) or absence (N) of shoulder muscle. OV, otic vesicle; FL, forelimb bud.

Fig. 8. MyoD gene regulation. (A) Gene networks controlling MyoD expression in limb, myotome, extra-ocular and pharyngeal arch muscles. Pitx2 has a crucial role in initiation of MyoD expression in limb buds, but not in myotome, where Myf5 and Mrf4 play a predominant role. Nonetheless, Pitx2, Myf5 and Mrf4 are jointly contributing to MyoD expression in both limb and myotome, as revealed by their compound mutants. Pax3 is required for Pitx2 expression in myotome. In head (extra-ocular and 1st pharyngeal arch) muscles that do not express Pax3, Pitx2 acts upstream of Myf5 and Mrf4 (rather than in parallel), and in association with Tbx1 in arches (Sambasivan et al., 2009). (B) Differential recruitment of Pitx2 to different regions of the MyoD gene depending on muscle ontology, in support of functional redundancy models (Frankel et al., 2010).
The present work nonetheless supports a role of Myf5(Mrf4) in limb MyoD expression and positioned these two genes in a parallel pathway relative to Pitx2 (Fig. 8A).

MyoD regulation has been studied extensively in myotomal lineages and supported models in which Myf5, Mrf4, as well as Pax3, operate upstream of MyoD (Tajbakhsh and Cossu, 1997). In Myf5<sup>lacZ</sup>/Myf5<sup>lacZ</sup> mutant embryos, MyoD expression is delayed by ~2 days in myotome (Kablar et al., 1997; Tajbakhsh et al., 1997) and this delay appeared to be due to developmental arrest of myogenic progenitors along the dermomyotome (Tajbakhsh et al., 1996b). Using allelic series of Myf5 mutants that differentially affect Mrf4 expression together with Mrf4 mutants, it has been shown that Myf5<sup>lacZ</sup>/Myf5<sup>lacZ</sup> and Myf5<sup>Mrf4</sup>/Mrf4 mutants exhibit the same delayed MyoD expression, supporting the conclusion that both Mrf4 and Myf5 act upstream of MyoD in myotomes (Kassar-Duchossoy et al., 2004). This dependence on Myf5 and Mrf4 was transient and the recovery of MyoD expression operates through a Pax3-dependent mechanism, although this may be mediated through a secondary factor (Tajbakhsh et al., 1997). We complemented this model by positioning Pitx2 under control of Pax3 (Fig. 8A). As the double mutant for Pitx2 and Myf5(Mrf4) is deficient in myotome expression of MyoD, Pitx2 is a likely intermediate for Pax3 control of MyoD. Thus, Pitx2 defines a complementary pathway for activation of the myogenic pathway through MyoD.

Regulation of MyoD expression is complex: although it can be recapitulated by the complementary activities of the CE and DRR, various transgenic studies have concluded that both enhancers are dispensable either for Myf5 regulation or Pax3-dependant expression of MyoD in myotomal lineages (Chen et al., 2002; Chen and Goldhamer, 2004; Kucharczuk et al., 1999). With the exception of E-boxes, the two enhancers lack cis-motifs known to regulate muscle genes during development. Taken together, these observations suggest a functional redundancy in cis-regulatory mechanisms controlling MyoD expression. Such redundancy of transcriptional control mechanisms appears to be a frequent feature of developmental regulatory pathways, as elegantly shown recently in Drosophila larvae (Frankel et al., 2010) and the action of Pitx2 on the MyoD gene is consistent with this model (Fig. 8B). Whereas the CE appears to be the predominant site of action for MyoD expression in limb buds, ChIP analyses also revealed Pitx2 recruitment in the ~600 bp PR region, but not at the DRR. This proximal site, which is close to documented sites for Pax3 and FoxO3 (Hu et al., 2008), may thus also contribute to expression. Furthermore, similar ChIP analyses in myotome revealed Pitx2 at the three MyoD regulatory regions (CE, DRR and PR). Pitx2 recruitment to the DRR was unexpected as no consensus Pitx-binding site is present in this enhancer. Pitx2 may be indirectly recruited to the DRR through its interaction with another transcription factor. Pitx2 may be a likely candidate as the DRR contains a SRF-binding CArG element previously shown to be required for MyoD expression in skeletal myoblasts (L’Honoré et al., 2003) and Pitx2 has been shown to interact with SRF and increase its association with DNA (Shang et al., 2008).

In Pitx2;Myf5(Mrf4) mutants, progenitor cells are still present at E11.5-E12.5 in both myotome and limb, as revealed by β-galactosidase staining but most of these cells do not express MyoD or myogenin. This situation is reminiscent but somewhat less pronounced than in Myf5;Mrf4;MyoD triple mutants, where in absence of all myogenic bHLH, somitic progenitors cells fail to commit to the muscle lineage. Those progenitors have been reported to assume nonmuscle fates in trunk and limbs, being first integrated in cartilage primordia, but then to undergo apoptosis. This death occurs several days after their birth (E13.5) (Kablar et al., 1999; Kablar et al., 2003) and may be linked to their failure to commit. It is, thus, tempting to speculate that a similar mechanism occurs in Pitx2;Myf5(Mrf4) mutants: analysis of those mutants at E12.5 revealed a slight increase of apoptosis in myotome and limb compared to control. Unfortunately, the early lethality (E13) of Pitx2;Myf5(Mrf4) mutants did not allow us to perform analyses at later stages.

As reported for Pax3;Mry5;Mrf4 mice (Kassar-Duchossoy et al., 2004; Tajbakhsh et al., 1997), we note an incomplete penetrance and residual MyoD and myogenin expression in Pitx2;Myf5(Mrf4) embryos, leading to sporadic myogenesis in the myotome (particularly in the most epaxial part) and in the limb (dorsal mass). This could be due to compensation by another factor. Likely candidates include: Pax7, which is expressed in precursors from E11.5 (Kassar-Duchossoy et al., 2005; Relax et al., 2005); Tbx1, which is initially expressed in dorsal masses of the limbs (Dastjerdi et al., 2007) and reminiscent of residual MyoD expression observed in Pitx2;Myf5(Mrf4) mutants; and, finally, Pitx3, which is expressed after Pitx2 during embryonic myogenesis (L’Honoré et al., 2007). Pitx3 does not compensate for loss of Pitx2 towards myogenin or MyoD expression in limbs (see Fig. S4 in the supplementary material), but we cannot exclude the possibility that it could exert its action in absence of both Pitx2 and Myf5. These seemingly Pitx2-independent myogenic cells may represent a subpopulation that is regulated by yet another alternate myogenic pathway.

The present work delineated differential requirements for Pitx2 in migratory versus non-migratory somite-derived myogenic cells. These body myogenic cells share a crucial dependence on Pax3 for their fate, in contrast to extra-ocular and branchial arch muscles (Sambasivan et al., 2009), which do not express Pax3. It is noteworthy that Pitx2 is complementary to Myf5 and Mrf4 in Pax3-dependent body muscles, whereas it is genetically upstream of the MRF core regulatory network in Pax3-independent head muscles. Thus, all skeletal muscles include Pitx2 in their genetic program. Different muscles have evolved the striking ability to co-opt selected elements of a core regulatory network together with a complementary genetic pathway in order to direct similar, yet distinct, muscle cell fates at different anatomical locations. Pitx transcription factors have been shown to interact physically and functionally with factors of multiple structural families, including POU-homeo factors (Szeto et al., 1996), bHLH factors (Poulin et al., 2000), Smads (Nudi et al., 2005), Tbox (Lamolet et al., 2001), Egr and nuclear receptors (Tremblay and Drouin, 1999), and LEF1 (Vadlamudi et al., 2005). In view of these multiple interactions, the implication of Pitx2 in the myogenic program provides numerous alternative mechanisms to modulate unique muscle identities in limb, trunk, pharyngeal or ocular muscles.

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Competition for MyoD function.

Supplementary material

References


