Sim2 prevents entry into the myogenic program by repressing MyoD transcription during limb embryonic myogenesis

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SUMMARY

The basic helix-loop-helix transcription factor MyoD is a central actor that triggers the skeletal myogenic program. Cell-autonomous and non-cell-autonomous regulatory pathways must tightly control MyoD expression to ensure correct initiation of the muscle program at different places in the embryo and at different developmental times. In the present study, we have addressed the involvement of Sim2 (single-minded 2) in limb embryonic myogenesis. Sim2 is a bHLH-PAS transcription factor that inhibits transcription by active repression and displays enhanced expression in ventral limb muscle masses during chick and mouse embryonic myogenesis. We have demonstrated that Sim2 is expressed in muscle progenitors that have not entered the myogenic program, in different experimental conditions. MyoD expression is transiently upregulated in limb muscle masses of Sim2−/− mice. Conversely, Sim2 gain-of-function experiments in chick and Xenopus embryos showed that Sim2 represses MyoD expression. In addition, we show that Sim2 represses the activity of the mouse MyoD promoter in primary myoblasts and is recruited to the MyoD core enhancer in embryonic mouse limbs. Sim2 expression is non-autonomously and negatively regulated by the dorsalising factor Lmx1b. We propose that Sim2 represses MyoD transcription in limb muscle masses, through Sim2 recruitment to the MyoD core enhancer, in order to prevent premature entry into the myogenic program. This MyoD repression is predominant in ventral limb regions and is likely to contribute to the differential increase of the global mass of ventral muscles versus dorsal muscles.

KEY WORDS: Sim2, MyoD (Myod1), Embryonic myogenesis, Limb, Chick, Mouse

INTRODUCTION

The basic helix-loop-helix (bHLH) transcription factor MyoD (also known as Myod1) is central to triggering the skeletal myogenic program during the successive waves of embryonic, foetal, perinatal and adult myogenesis. A key issue is to understand the regulatory networks that drive MyoD expression at precise places and times in the embryo.

Embryonic myogenesis leads to the formation of the first multinucleated muscle fibres from embryonic muscle progenitors. The main role of this first wave of myogenesis is to initiate skeletal muscle differentiation at the appropriate locations in the embryo, while the second wave of foetal myogenesis uses embryonic fibres as a scaffold for muscle growth. Consequently, it is essential to understand the intrinsic and extrinsic pathways that specify the entry into the embryonic myogenic program at different places in the embryo. In addition, the embryonic myogenic program must be tightly regulated to retain a muscle progenitor pool for the following waves of myogenesis. Embryonic muscle progenitors rely on the activation of the skeletal muscle differentiation program based on the myogenic regulatory factors (MRFs). The MRFs includes four bHLH transcription factors: Myf5, MyoD, Mrf4 and myogenin. Myf5, Mrf4 and MyoD constitute the core regulatory network for the myogenic program. In their absence, myoblasts are lacking and skeletal muscles do not form (Kassar-Duchossoy et al., 2004). Conversely, they are sufficient for skeletal muscle differentiation in ectopic contexts in vitro and in vivo (Weintraub et al., 1991; Delfini and Duprez, 2004). The epistatic relationships between Myf5, Mrf4, MyoD and other transcription factors modulating their expression differ according to muscle groups in different anatomical locations in the embryos (Bismuth and Relaix, 2010).

Limb embryonic myogenesis involves delamination and migration of muscle progenitor cells from the hypaxial dermomyotome (Duprez, 2002). The transcription factors Lbx1, Pax3 and Six1/4 are all involved in muscle progenitor migration into the limb buds (Bismuth and Relaix, 2010). In addition, Pax3 and Six1/4 positively regulate Myf5 expression in the limbs by direct binding to different regulatory regions of the Myf5 promoter (Bajard et al., 2006; Giordani et al., 2007). The initiation of MyoD expression in limb skeletal muscles is also directly and positively regulated by the paired-related homeodomain transcription factor Pits2, independently of Myf5 (L’Honore et al., 2010). The decision to enter the myogenic program or to remain undifferentiated must be tightly regulated in order to maintain the pool of muscle progenitors during development. The identified transcription factors that act autonomously in limb myogenic cells have been shown to modulate positively Myf5 or MyoD expression (Bajard et al., 2006; Giordani et al., 2007; L’Honore et al., 2010). However, to date, no intrinsic repressor activity has been described to regulate the entry into the embryonic muscle program in limbs.

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The single-minded 2 (Sim2) transcription factor has been previously shown to be expressed in a restricted pattern in embryonic myogenic cells during chick and mouse limb development (Coumailleau and Duprez, 2009). Sim2 expression is transiently enhanced in ventral limb muscle masses (Coumailleau and Duprez, 2009). Sim2 contains a bHLH domain, two PAS (Per-Arnt-Sim) domains and one HST (Hif1-α/SIM/TRH) domain. Although most bHLH-PAS proteins are transcriptional activators, Sim2 acts as a repressor of transcription in mammalian cells (Moffett et al., 1997; Moffett and Pelletier, 2000; Metz et al., 2006). The repressor activity has been localised to two independent repression domains in the C-terminal region of mouse Sim2 protein (Moffett et al., 1997; Moffett and Pelletier, 2000; Metz et al., 2006). The location of the SIM2 gene on the human chromosome 21, its expression in brain and the behaviour of transgenic mice with three copies of the Sim2 gene, have led to the suggestion that SIM2 is important in the mental retardation associated with Down’s syndrome (Ema et al., 1999; Chrast et al., 2000). A short splice variant of the SIM2 gene, SIM2s, which lacks one of the two repression domains of SIM2, has been alternatively identified as a tumour suppressor or activator depending on the tumour type (DeYoung et al., 2003; Aleman et al., 2005; Halvorsen et al., 2007; Kwak et al., 2007; Laffin et al., 2008). Despite the loss of one repression domain, SIM2s has been shown to repress directly target gene transcription in different cellular models (Kwak et al., 2007; Laffin et al., 2008; Woods et al., 2008; Farrall and Whitelaw, 2009; Wellberg et al., 2010). Sim2 mutant mice die perinatally owing to lung atelectasis and breathing failure, but display multiple other phenotypes such as rib, vertebral and craniofacial abnormalities (Goshu et al., 2002; Shambrott et al., 2002). The only muscle phenotype described in the absence of Sim2 activity in mouse mutants is a diaphragm hypoplasia at birth (Goshu et al., 2002).

In the present study, we investigated the involvement of Sim2 in limb embryonic myogenesis. We have shown that Sim2 is expressed in muscle progenitor cells that have not entered the myogenic program in normal or experimental conditions. Sim2 gain- and loss-of-function experiments in mouse, chick and Xenopus embryos show that Sim2 represses MyoD expression. In addition, Sim2 represses the activity of the mouse MyoD promoter in primary myoblasts and is recruited to the MyoD core enhancer in embryonic mouse limbs. Moreover, Sim2 is negatively and non-autonomously regulated by the dorsalising factor Lmx1b. Taken together, our results establish that Sim2 represses MyoD transcription in limb muscle masses, via the recruitment of Sim2 to the MyoD core enhancer, in order to prevent a premature entry into the myogenic program.

MATERIALS AND METHODS
Chick embryos and mouse lines
Fertilised chick eggs from commercial sources, JA 57 strain (Morizeau, Dangers) or White Leghorn (HAAS, Kaltenhouse) were incubated at 38°C. Embryos were staged according to somite number or Hamburger and Hamilton (HH) stages (Hamburger and Hamilton, 1992). MyoD+/- (Kablar et al., 1997), Myf5lacZ (Kassar-Duchossoy et al., 2004), Sim2+/- (Goshu et al., 2002) or Pax3RESm2lacZ (Relaix et al., 2004) mouse embryos were collected after natural overnight matings. For staging, fertilisation was considered to take place at midnight.

In situ hybridisation to tissue sections and to whole-mount embryos
Chick and mouse embryos were fixed and processed for digoxigenin-labelled probe in situ hybridisation to whole mounts and to wax sections as previously described (Bonnet et al., 2010). For BrdU analyses, chick embryos were incubated with BrdU 1 hour before fixation. For double fluorescent in situ hybridisation, the two probes were tagged with digoxigenin-11-UTP or DNP-11-UTP, and were detected using Perkin Elmer Life Sciences TSA plus Cy3/Cy5 fluorescence dual detection system. For non-fluorescent double in situ hybridisation, the two probes were tagged with digoxigenin-11-UTP or fluorescein-11-UTP and detected with NBT/BCIP and INT/BCIP reagents, respectively, as already described (Delfini and Duprez, 2000). The antisense mRNA probes were used as previously described: chick (c) Fgf4, cPax3, cMyoD and cDelta1 (Delfini et al., 2000); mouse (m) Myf5, mMyoD, mMyog and GFP (Bonnet et al., 2010); Xenopus MyoD (Li, H. Y. et al., 2010); cLmx1b (Michaud et al., 1997); and cSim2 and mSim2 (Coumailleau and Duprez, 2009). The mSim2 probe used for endogenous mSim2 expression in mouse limbs recognises exon 11 and consequently does not recognise the splice variant mSim2s (Metz et al., 2006). The mSim2 probe used after chick electroporation experiments was specially designed not to crossreact with endogenous cSim2 expression, by PCR amplification using the Sim2N2 primers (see Table S1 in the supplementary material) from the from mSim2/Myc/pEFIRESpuro (Woods et al., 2008). For in situ hybridisation experiments of mouse embryos, sections of mutant and wild-type embryos were mounted on the same slide and consequently were treated exactly in the same conditions to facilitate comparison. Quantification of MyoD expression after situ hybridisation was performed by particle counting using the Image J software. For each in situ hybridisation experiment, at least three mutants and stage-matched wild-type mouse embryos from three different litters were used.

Production and grafting recombinant/RCAS-expressing cells
The Delta1/RCAS-expressing cells were prepared for grafting as previously described (Delfini et al., 2000). The left wings were used as internal controls and compared with the grafted wings at the same proximodistal level. Six chick embryos grafted with Delta1/RCAS-expressing cells were analysed by in situ hybridisation.

Neural tube and somite electroporation
Chick neural tube and somite electroporation experiments were performed as already described (Delfini and Duprez, 2004; Bonnet et al., 2010). Neural tubes were co-electroporated with mMyf5-pCAβ or mSim2-pCAβ in association with eGFP-pCAβ. Somites were co-electroporated with mSim2-pCAβ and eGFP-pCAβ. Control embryos were electroporated with eGFP-pCAβ alone. The mSim2-pCAβ plasmid was obtained by replacing the GFP EcoRI-Xhol fragment of eGFP-pCAβ by a mSim2 fragment amplified by PCR using the primers Sim2CS (supplementary material Table S1) from mSim2/Myc/pEFIRESpuro (Woods et al., 2008). Embryos were harvested 24 hours after electroporation. The expression of each gene was analysed on at least four different electroporated embryos in each condition.

RNA extraction and quantitative real-time PCR
Total RNAs were extracted from forelimbs of mutant and control mice using the RNeasy kit (Qiagen) and reverse-transcribed using the Superscript II Reverse Transcriptase kit (Invitrogen) according to manufacturer’s instructions. Quantitative real time PCR was performed using a Lightcycler 480 Sybergreen mix (Roche). Primer sequences are listed in supplementary material Table S1. For mRNA levels, gene changes were quantified using the ΔΔCt method. Quantitative data shown as histograms are expressed as means and S.E. Fold changes were assessed for statistical significance by Student’s t-test. Asterisks in figures indicate the different P values (**P<0.01; ***P<0.001). The experiment was performed with three independent samples for each genotype, and Q-PCR was carried out in triplicate for each gene.

Xenopus injections
Xenopus eggs were artificially fertilised with mixed testes. Synthetic capped mRNAs for mSim2 and β-galactosidase (β-gal) were obtained by in vitro transcription using SP6 RNA polymerase. Microinjection and β-gal staining using red-gal as a substrate were performed as previously described (Li, H. Y. et al., 2010).
Cell culture
Primary myoblasts were prepared from hindlimb muscles of 10-day-old chick embryos as described (Spitz et al., 1997; Giordani et al., 2007). Cells were transfected with 50 ng pTK-renilla, 100 ng cGFP-pCAβ, 500 ng mSim2-pCAβ or mMyoD-pCAβ or both and 850 ng of reporter plasmids, pCore-TATA-luciferase, pDPR-TATA-luciferase or TATA-luciferase according to the lipofectamine transfection kit (Invitrogen) protocol. The pDPR-TATA-luciferase was provided by Pascal Maire (Institut Cochin, Paris, France). The pCore-TATA-luciferase was obtained as followed: the core enhancer was amplified by RT-PCR using the primers MyoDcore (see supplementary material Table S1) from MyoD EnhancerMDE-1 (provided by Shahragim Tajbakhsh, Institut Pasteur, Paris, France) and was then inserted in the TATA-luciferase vector. Cells were harvested 3 days after transfection and luciferase activity was measured according to the dual-luciferase reporter assay system protocol (Promega). All transfection experiments were performed in triplicate and repeated five times.

Chromatin immunoprecipitation
ChIP assays were performed as already described (Havis et al., 2006). Fifty limbs from E11.5 mouse embryos were homogenised using a mechanical disruption device (Lysing Matrix A, Fast Prep MP1, 30 sec). 10 μg of the rabbit polyclonal Sim2 antibody (Millipore), or 8 μg of the acetylated histone H4 (ACH4) antibody (Upstate Biotechnology) as a positive control, were used to immunoprecipitate 25 μg of sonicated chromatin. ChIP products were analysed by PCR. Three pairs of primers were used to amplify fragments associated with the core enhancer, the DRR and the PRR, respectively (supplementary material Table S1). The ChIP experiments were performed three times.

Immunohistochemistry
Progenitor and differentiated muscle cells were detected with the monoclonal antibodies Pax7 and MF20, respectively. The Pax7 and MF20 antibodies developed by investigators A. Kawakami and D. A. Fischman, respectively, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa. GFP protein was detected using a monoclonal antibody (Amersham) after in situ hybridisation. BrdU was detected using a monoclonal antibody (Roche) after in situ hybridisation. 

RESULTS

Sim2 is expressed in chick limb myogenic cells not expressing MyoD
The onset of Sim2 expression in chick limbs was observed at HH20 after the migration process but before the onset of MyoD expression (Couvainaleau and Duprez, 2009). Pax3 is the main marker of embryonic muscle progenitors (Relaix et al., 2004). The fibroblast growth factor receptor 4, Fgfr4 lies genetically downstream of Pax3 and is the main marker of muscle progenitors from HH20 and were excluded from muscle fibres (Relaix et al., 2004). In order to correlate Sim2 expression with that of muscle progenitor markers, we performed double Sim2 in situ hybridisation with cFgf4 and cPax3 probes to chick limb sections. We observed a colocalisation of cSim2 transcripts with that of cFgf4 (Fig. 1A-C) and cPax3 (Fig. 1D-F) in ventral limb muscle masses at HH22. Moreover, we identified cSim2-positive cells that have incorporated BrdU as cPax3-positive cells in limb muscle masses (Fig. 1G,H, arrowheads), suggesting that Sim2 was associated with a proliferative state. Once limb myogenic cells have entered the differentiation program assayed by MyoD expression, muscle masses contain a heterogeneous population of myogenic cells, which are not at the same stage of muscle differentiation. In order to define the cellular expression of Sim2 versus that of MyoD, we performed double in situ hybridisation in chick forelimbs. At HH26, cMyoD mRNAs were expressed in myoblasts in dorsal and ventral muscle masses, whereas cSim2 transcripts were observed mainly in ventral muscle masses. In ventral muscle masses, cSim2 and cMyoD transcripts were not
observed in the same cells (Fig. 1I-N). These results indicate that c\textit{Sim2} and c\textit{MyoD} expression is mutually exclusive in chick limb ventral muscle masses. 

\textit{Sim2} is expressed in embryonic muscle progenitors that have been experimentally or genetically prevented from fully entering the myogenic program

Notch signalling regulates the maintenance of embryonic muscle progenitors and inhibits muscle differentiation (Delfini et al., 2000; Schuster-Gossler et al., 2007; Vasyutina et al., 2007; Bonnet et al., 2010; Rios et al., 2011). In chick embryonic limbs, Delta1-activated Notch led to a downregulation of expression of the muscle differentiation genes (\textit{MyoD}, myogenin, desmin, \textit{Mef2c}) and myosins, whereas the expression of muscle progenitor markers (\textit{Pax3}, \textit{Six1} and \textit{Fgfr4}) was not affected (Delfini et al., 2000; Bonnet et al., 2010). In order to determine whether \textit{Sim2} expression was affected by Notch signalling, we analysed c\textit{Sim2} expression after \textit{Delta1} overexpression in chick limbs. Delta1/RCAS-expressing cells were grafted into the lateral plates of HH17 embryos at limb level (Fig. 2A). Seventy-two hours later, c\textit{Delta1} expression revealed the extent of the virus spread (Fig. 2B). In these conditions, c\textit{Sim2} expression was not affected, nor that of c\textit{Fgfr4}, whereas c\textit{MyoD} expression was downregulated in ventral and dorsal muscle masses (Fig. 2C-H). We conclude that \textit{Sim2} expression is not regulated by Notch and that \textit{Sim2} regulation by Notch signalling is similar to that seen in muscle progenitor markers.

\textit{Sim2} expression was increased in forelimbs of \textit{MyoD}\textsuperscript{–/–} mutant mice

We next analysed \textit{Sim2} expression in MRF mutant mice, in which limb embryonic myogenesis is delayed. Although being crucial for muscle formation, \textit{Myf5}, \textit{MyoD} or \textit{Mrf4} single mutant mice displayed no dramatic muscle phenotype, because of compensatory function between these genes (Kassar-Duchossoy et al., 2004; Bismuth and Relaix, 2010). However, \textit{MyoD}\textsuperscript{–/–} mutant mice exhibited an early delay of limb muscle differentiation by about 2.5 days (Kablar et al., 1997). This delay was assayed by the absence of \textit{mMyog} expression in limbs of E12 \textit{MyoD}\textsuperscript{–/–} mutant compared with its normal expression in wild-type limbs (Fig. 3A,B). When muscle differentiation is arrested, \textit{mSim2} expression domain was extended in ventral and dorsal muscle masses compared with its restricted expression in control limbs (Fig. 3C,D, arrows). The increase of \textit{mSim2} expression was confirmed by q-RT-PCR analyses in forelimbs of mutant and wild-type mice (Fig. 3E). Transcripts for the short splice variant of \textit{Sim2}, \textit{sSim2}, were hardly present in embryonic wild-type and \textit{MyoD}\textsuperscript{–/–} limbs (Fig. 3E). The relative levels of \textit{Myf5}
mRNAs also increased in the absence of MyoD activity in forelimbs (Fig. 3E). In order to determine whether Sim2 expression was dependent on Myf5, we analysed Sim2 expression in Myf5 gain- and loss-of-function experiments. Overexpression of mMyf5 in chick neural tubes leads to ectopic myogenesis (Delfini and Duprez, 2004). In these experiments, mMyf5 does not activate cSim2 expression, whereas activating cMyoD expression (Fig. 4A-C). Conversely, in the absence of Myf5 (Mrf4) activity, mSim2 expression was observed in E11.5 forelimbs (data not shown) and hindlimbs (Fig. 4F,G). We also noticed an increase of mSim2 expression in hindlimbs of Myf5−/− hindlimbs (G, arrows), compared with mSim2 in control hindlimbs (F, arrows). a, anterior; p, posterior; D, dorsal; V, ventral.

**MyoD expression is transiently upregulated in limbs of Sim2−/− mutant mice**

The inverse correlation between Sim2 expression and muscle differentiation during normal and experimental conditions and the repressor activity of Sim2 in vitro (Moffett et al., 1997; Moffett and Pelletier, 2000; Metz et al., 2006) led us to hypothesise that Sim2 could negatively regulate MyoD expression during limb embryonic myogenesis. We therefore analysed mMyoD expression in limb muscle masses of Sim2−/− mutant mice during embryonic myogenesis. In the absence of Sim2 activity, we observed an upregulation of mMyoD expression in dorsal and ventral muscle masses of forelimbs and hindlimbs (Fig. 5). In forelimbs, the mMyoD upregulation was more pronounced in posterior regions of muscle masses (Fig. 5A-F, arrows), where endogenous mSim2
expression is enhanced (Fig. 3C; supplementary material Fig. S1). In hindlimbs, the mMyoD expression domain was extended in ventral regions of in Sim2+/− compared with control limbs (Fig. 5G,H arrows; supplementary material Fig. S3), consistent with the predominant endogenous mSim2 expression in ventral muscle masses (supplementary material Fig. S2). Global quantification by q-RT-PCR analyses confirmed the MyoD expression in the absence of Sim2–/– in the presence of −/– versus Sim2+ M, arrows and brackets).

Sim2 overexpression inhibits MyoD expression in chick and Xenopus embryos

Mouse Sim2 loss-of-function experiments suggested that Sim2 represses MyoD expression during embryonic myogenesis. We therefore analysed the consequences of Sim2 gain-of-function for MyoD expression, in chicken and Xenopus embryos. We used somite electroporation in chick embryos (Wang et al., 2011) to assess the effect of mSim2 overexpression on cMyoD expression. Interlimb somites were electroporated in HH16 chick embryos (27 somite stage) with mSim2 and GFP expression vectors or with a control expression vector only, as control. The embryos electroporated with mSim2 displayed a loss of cMyoD expression in the electroporated regions (Fig. 6A-E, arrows), whereas normal cMyoD expression is observed in control embryos injected with β-gal only (L, arrows). At tail bud stages, mSim2 overexpression also leads to a loss of cMyoD expression in the most anterior somites on the injected sides.

In order to confirm that Sim2 acts as a repressor of MyoD expression, we overexpressed mSim2 in Xenopus embryos. Xenopus (x) MyoD zygotic expression occurs at mid-blastula transition and is maintained during mesoderm induction (Rupp and Weintraub, 1991). mSim2 mRNAs (500 pg) were injected in the dorsal-lateral equatorial region of eight-cell stage Xenopus embryos. β-gal mRNAs were either co-injected with mSim2 mRNAs to label the targeted regions (Fig. 6K,M) or were injected alone as a control (Fig. 6L). At early gastrula stage, mesodermal cells overexpressing mSim2 displayed a loss of cMyoD expression (Fig. 6K, arrows), compared with control electroporated somites (F-J). (K-M) Eight-cell stage Xenopus embryos were injected bilaterally (K,L) or unilaterally (M) with mSim2 and β-gal mRNAs (K,M) or with β-gal mRNAs as control (L) and were processed for whole-mount in situ hybridisation to cMyoD expression. At early gastrula stages, cMyoD expression is inhibited by mSim2 overexpression in β-gal-positive regions (K, arrows), whereas normal cMyoD expression is observed in control embryos injected with β-gal only (L, arrows). At tail bud stages, mSim2 overexpression also leads to a loss of cMyoD expression in the most anterior somites on the injected sides.

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with control embryos, in which β-gal overexpression did not affect xMyoD expression (Fig. 6L, arrows). At tail bud stage, we also observed a loss of xMyoD expression in the most anterior somites, where mSim2 was overexpressed, compared with the uninjected sides (Fig. 6M). We conclude that mSim2 overexpression represses xMyoD expression in Xenopus embryos.

Collectively, these Sim2 gain-of-function experiments show that mouse Sim2 represses MyoD expression in chicken and Xenopus embryos.

Sim2 represses MyoD transcription via Sim2 recruitment to the core enhancer of the mouse MyoD promoter

Sim2 expression, regulation and loss- and gain-of-function experiments indicated that Sim2 represses MyoD transcription. We next determined whether Sim2 could repress MyoD expression via the regulatory elements of the mouse MyoD promoter. We took advantage of the characterisation of two muscle-specific enhancers in mammals, the core enhancer and the distal regulatory region (DRR) located respectively –20 kb and –5 kb upstream from the MyoD transcription start site (Goldhamer et al., 1992; Tapscott et al., 1992; Asakura et al., 1995; Goldhamer et al., 1995). Mouse mutagenesis experiments have shown that the core enhancer regulates the initiation of MyoD in limb buds during embryonic development (Kablar et al., 1999; Chen et al., 2001; Chen and Goldhamer, 2004), whereas the DRR is associated with the onset of skeletal muscle differentiation (Asakura et al., 1995; Chen et al., 2001) and is essential for normal MyoD expression in adult muscles (Chen et al., 2002). The core and DRR regulatory elements were cloned upstream the luciferase reporter gene and transfected into primary myoblasts with mSim2 and mMyoD expression vectors. mSim2 was able to significantly repress the reporter gene expression via the core (Fig. 7A) and DRR (Fig. 7B) elements but not via a minimal promoter (Fig. 7C). We also observed that mMyoD activates its own transcription via the core element and that this activation was not significantly prevented by mSim2 (Fig. 7A). We conclude that mSim2 represses the transcriptional activity of both the core and DRR elements of the MyoD promoter in vitro.

We next analysed the in vivo recruitment of Sim2 to the core enhancer of the MyoD promoter in vivo. We performed chromatin immunoprecipitation (ChIP) assays on E11.5 mouse limbs to analyse the Sim2 occupancy to the core, the DRR or the Proximal Regulatory Region (PRR) (Fig. 7D). We observed that mSim2 was recruited to the core of the mMyoD promoter and not to the DRR nor the PRR (Fig. 7E). The acetylation of Histone4 (ACH4) in the core and the PRR testifies to an open state of the chromatin of these regions at this stage (Fig. 7E). Interestingly, the DRR was not acetylated in E11.5 mouse limbs (Fig. 7E), reflecting an absence of DRR activity in E11.5 limbs, which is consistent with transgenic analyses (Asakura et al., 1995; Chen et al., 2001; Chen et al., 2002). These results established that mSim2 was recruited to the core element of the MyoD promoter in vivo in E11.5 limbs. We conclude that Sim2 represses MyoD expression via the core element in vivo during limb embryonic myogenesis.
Sim2 and limb myogenesis

Enhanced Sim2 expression in ventral muscle masses is correlated with a greater global muscle size in ventral regions compared to that of dorsal limb regions

Our results indicate that Sim2 prevents entry into the myogenic program by repressing MyoD transcription. We next asked why this delay of myogenic progression would be predominant in ventral limb muscle masses, where Sim2 expression is enhanced. In chick forelimbs, at the onset of embryonic myogenesis, the global mass of muscle progenitors, visualised with Pax3 transcripts (Fig. 8A,C) or with Pax7-positive cells (Fig. 8E), appeared to be equivalent in dorsal and ventral regions. We did not observe any obvious delay in myosin expression in dorsal versus ventral masses (Fig. 8F, no expression in muscle masses at HH22; Fig. 8G expression in both masses at HH23), indicating that muscle differentiation occurs simultaneously in both muscle masses in chick forelimbs. However, the global mass of forming muscles (including muscle progenitors and differentiated cells) is increased in ventral regions at the end of embryonic myogenesis (Fig. 8H,I). During foetal myogenesis, when forelimb muscles are individualised, the global mass of ventral muscles is also greater than that of dorsal muscles (Duprez et al., 1999). We observed a similar situation in chick hindlimbs, where the ventral mouse limb regions also display a greater muscle size, in forelimbs and hindlimbs (see supplementary material Figs S6, S7).}

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Fig. 10. MyoD regulation during limb embryonic myogenesis. (A) Gene network involved in the initiation of MyoD expression in limbs during embryonic myogenesis. Pax3, Myf5 and Pitx2 cell-autonomously activate the expression of Myf5 and MyoD, whereas Sim2 cell-autonomously represses MyoD expression. (B) Sim2 recruitment to the core element of MyoD promoter in E11.5 mouse limbs. (C) Sim2 expression is enhanced in ventral limb muscle progenitors. Lmx1b expressed in dorsal limb regions and excluded from myogenic cells is involved in the non-cell-autonomous repression of Sim2 expression in dorsal limb regions.

We conclude that the delay in the myogenic progression by Sim2 is enhanced in restricted areas of limb muscle masses, where the muscle size is increased at later stages. This delay is predominant in ventral limb muscle masses and correlates with the greater size of ventral musculature compared with dorsal musculature.

**mSim2 expression is negatively and non-autonomously regulated by the dorsalising limb signal Lmx1b**

As Sim2 expression was enhanced in ventral limb muscle masses during embryonic myogenesis, we next analysed whether the Sim2 ventral expression could be linked to dorsoventral limb patterning. The LIM-homeodomain transcription factor Lmx1b is known to be necessary and sufficient for specifying dorsal fates in limbs (Riddle et al., 1995; Vogel et al., 1995; Chen et al., 1998). In chick limbs, cLmx1b is expressed in dorsal limb regions, whereas cSim2 expression is increased in ventral muscle masses (Fig. 9A-C). In the absence of Lmx1b activity, mouse limbs displayed a biventral limb pattern, mainly in distal regions (Chen et al., 1998; Li, Y. et al., 2010). In the absence of Lmx1b activity, mSim2 expression was increased in dorsal fore- and hindlimb regions, compared with wild-type limbs (Fig. 9D-I). As Lmx1b is not expressed in myogenic cells (Li, Y. et al., 2010), we conclude that Lmx1b in dorsal connective tissue cells non-autonomously represses mSim2 expression in dorsal limb muscle cells.

**DISCUSSION**

In this manuscript, we have demonstrated for the first time the functional involvement of the bHLH-PAS transcription factor Sim2 in limb embryonic myogenesis. Only few intrinsic transcription factors (Six1/4, Pax3 and Pitx2) have been identified as directly regulating the expression of the MRFs during limb embryonic myogenesis (Bajard et al., 2006; Giordani et al., 2007; L’Honoré et al., 2010). With the present work, we identified Sim2 as a new regulator of limb embryonic myogenesis. In contrast to the already identified MRF regulators, which have been shown to promote myogenesis, Sim2 acts as a negative regulator of myogenesis by repressing MyoD transcription in limb muscle progenitors (Fig. 10A). Sim2 does not seem to be downstream of Myf5, as Sim2 expression is observed in limbs of Myf5 mouse mutants and is not activated upon overexpression of Myf5 in chick embryos. Cell-autonomous regulators of Sim2 expression in muscle progenitors remain to be characterised. We have shown that Sim2 is recruited to the core element of the MyoD promoter in vivo in mouse limbs at E11.5 and represses MyoD transcription via this element in vitro (Fig. 10B). The specific Sim2 recruitment to the core element of the MyoD promoter in vivo is consistent with the idea that Sim2 negatively regulates the initiation of MyoD expression in limbs, as the core enhancer is essential for the timely initiation of MyoD expression in limb buds (Kablar et al., 1999; Chen and Goldhamer, 2004). We have identified Sim2 as a new and negative regulator of the MyoD core enhancer activity. The transient upregulation of MyoD expression in limbs of Sim2 mutant mice is consistent with the 2-day delays of limb MyoD expression in mice lacking the MyoD core enhancer (Chen and Goldhamer, 2004). Pitx2 has recently been shown to directly bind to and positively regulate the activity of the MyoD core enhancer in mouse limbs at E12 (L’Honoré et al., 2010). Further studies are required to analyse putative interactions between Pitx2 and Sim2 in regulating the core element activity during limb embryonic myogenesis.

Sim2 expression is enhanced in ventral limb muscle progenitors during chick and mouse embryonic myogenesis (Coumailleau and Duprez, 2009). One obvious difference between dorsal and ventral limb muscles is the greater global volume of ventral muscles compared with dorsal muscles at the autopod and zeugopod levels of chick and mouse limbs (Fig. 8; see supplementary material Figs S5-S7). Our hypothesis is that the presence of Sim2 in muscle progenitors will prevent them from prematurely entering the myogenic program. The enhanced expression of Sim2 in ventral progenitors will lead to a progressive increase of the muscle progenitor pool in ventral limb regions. The Sim2 repressor activity on muscle differentiation provides a mechanism that allows differential muscle growth between dorsal and ventral limb regions. In chick and mouse hindlimbs, the mass of muscle progenitors is smaller in ventral regions than that in dorsal regions (supplementary material Figs S5, S6), indicating that the delay of myogenic progression by Sim2 must be capable of overcoming this initial difference, in addition to increasing the pool of ventral muscle progenitors. The Sim2 repressor activity in muscle progenitors is obviously not the only mechanism involved in the differential muscle growth between dorsal and ventral limb regions, because Sim2 mutant mice display a normal limb muscle pattern at E14.5. Embryological experiments and genetic analyses have shown that the position, shape and size of limb muscles are driven by signals provided by non-myogenic limb cells (Kieny, 1982; Chen et al., 1998; Kardon, 1998; Duprez et al., 1999; Duprez,
In conclusion, these results identify Sim2 as a new intrinsic regulator of limb embryonic myogenesis by negatively regulating MyoD expression through the recruitment of Sim2 to the MyoD core enhancer. This prevention of entry into the embryonic myogenic program is predominant in ventral limb regions and is likely to contribute to the differential increase of the global mass of ventral muscles versus dorsal muscles.

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