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 (bHLH) 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 dorsalisning 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 embryo. In addition, the embryonic myogenic program must be tightly regulated 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.

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; Shamblott 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 dorsalisising 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), Myf5\(^{galac}Z\) (Kazaras-Duchcossey et al., 2004), Sim2\(^{−/−}\) (Goshu et al., 2002) or Pax3\(^{Shelke}\) (Relaxi 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 in 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 Deltau/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 Deltau/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-pCA-B or mSim2-pCA-B in association with eGFP-pCA-B. Somites were co-electroporated with mSim2-pCA-B and eGFP-pCA-B. Control embryos were electroporated with eGFP-pCA-B alone. The mSim2-pCA-B plasmid was obtained by replacing the GFP EcoRI-Xhol fragment of eGFP-pCA-B 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. Total RNAs were extracted from forelimbs of mice of the same conditions to facilitate comparison. Quantification of gene expression after situs 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.

**Xenopus injections**

Xenopus eggs were artificially fertilised with minced testes. Synthetic capped mRNAs for mSim2 and β-galacosidase (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, pDRR-TATA-luciferase or TATA-luciferase according to the lipofectamion transfection kit (Invitrogen) protocol. The pDRR-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 EnhancerpmDE-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 (brown). Arrowheads indicate the cSim2+ cells in situ hybridisation. BrdU was detected using a monoclonal antibody (Roche) after in situ hybridisation. Regression experiments were performed in triplicate and repeated five times.

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 (Counailleau 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 myogenic cells not expressing MyoD (Lagha et al., 2008). In chick limb, Fgfr4 transcripts were observed in replicating muscle progenitors from HH20 and were excluded from muscle fibres (Marcelle et al., 1995; Edom-Voillard et al., 2001; Bonnet et al., 2010). In order to correlate Sim2 expression with that of muscle progenitor markers, we performed double Sim2 in situ hybridisation with cFgfr4 and cPax3 probes to chick limb sections. We observed a co-localisation of cSim2 transcripts with that of cFgfr4 (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

Fig. 1. Sim2 is expressed in chick limb muscle cells not expressing MyoD. (A–F) Transverse sections of HH22 chick embryos at forelimb level were co-hybridised with cSim2 (green) and cFgfr4 (red) fluorescent probes (A–C) or with cSim2 (digoxigenin, purple) and cPax3 (fluorescein, orange) probes (D–F). (A–D) Arrowheads indicate ventral muscle masses; arrows show dorsal muscle masses. (G–H) Limb sections of HH22 chick embryos, incubated with BrdU 1 hour before fixation, were hybridised with cSim2 (E) and cPax3 (F) digoxigenin-labelled probes (blue) and then incubated with the BrdU antibody (brown). Arrowheads indicate the cSim2+ and cPax3+ cells in ventral muscle masses. (I–N) Transverse sections of HH26 chick forelimbs were labelled by double in situ hybridisation with cMyoD (green) and cSim2 (red) fluorescent probes. (I, K, M) In ventral muscle masses, arrowheads indicate the cSim2 expression domains not overlapping with that of cMyoD, whereas arrows indicate the overlapping cSim2 and cMyoD expression domains. (J, L, N) High magnifications of ventral muscle masses show that in overlapping expression domains, cSim2 mRNA and cMyoD mRNA are not expressed in the same cells.
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, 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 \textit{cSim2} 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, \textit{cDelta1} expression revealed the extent of the virus spread (Fig. 2B). In these conditions, \textit{cSim2} expression was not affected, nor that of \textit{cFgfr4}, whereas \textit{cMyoD} 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.

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 m\textit{Myog} 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, m\textit{Sim2} 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 m\textit{Sim2} 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}, s\textit{Sim2}, 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+/− mice compared with control mice (D,E), indicating a delay of muscle differentiation in Myf5−/−. On adjacent sections, mSim2 expression is increased in dorsal and ventral muscle masses 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 increased in the absence of MyoD activity.
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 increase in limbs of E11.5 Sim2−/− versus Sim2+/+. Quantification of mMyoD mRNAs in muscle masses in Sim2−/− versus Sim2+/+ indicates an increase of 27% in dorsal muscle masses and of 38% in ventral muscle masses in hindlimbs, and an increase of 36% in dorsal muscle masses and of 41% in ventral muscle masses in forelimbs (supplementary material Fig. S3). However, the increase of mMyoD expression was transient, because at E14.5, Sim2−/− mutant mice displayed no obvious difference in mMyoD expression in forelimbs and hindlimbs (data not shown). We conclude that MyoD expression is transiently upregulated in the absence of Sim2 activity in fore- and hindlimbs during embryonic myogenesis, suggesting a transcriptional repressor activity for mSim2 towards MyoD expression.

**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 Sim2 overexpression on cMyoD expression. Interlimb somites were electroporated in HH16 chick embryos (27 somite stage) with mSim2 and GFP expression vectors or with a GFP 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, compared with the uninjected sides (M, arrows and brackets).

**Fig. 6. mSim2 overexpression inhibits MyoD expression in chick and Xenopus embryos. (A-J)** Chick embryos were electroporated at interlimb regions with mSim2 and GFP expression vectors (A-E) or with GFP expression vector only (F-H). Transverse sections (A-C,F-H) or whole-mount embryos (D,E,J) were hybridised with the cMyoD probe and then incubated with GFP antibody to visualise the electroporated regions. (B,C,G,H) High magnifications of the boxed regions in A,F. mSim2 overexpression in somites leads to a loss of cMyoD expression in electroporated regions (A-E) compared with control electroporated with GFP only (F-H), in which electroporated cells express cMyoD. Arrows indicate the loss of cMyoD expression where GFP is expressed in mSim2-electroporated somites (A-E) and cMyoD expression in 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 xMyoD expression. At early gastrula stages, xMyoD expression is inhibited by mSim2 overexpression in β-gal-positive regions (K, arrows), whereas normal xMyoD 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 xMyoD expression in the most anterior somites on the injected sides, compared with the uninjected sides (M, arrows and brackets).
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 mouse MyoD promoter in E11.5 mouse limbs. (A-C) Primary myoblasts were co-transfected with GFP-, mMyoD- or mSim2-expression vectors and MyoD reporter constructs, in which the luciferase reporter gene was cloned downstream of two regulatory elements of the mMyoD promoter, the core (A) and the DRR (B) or a minimal promoter (C). mSim2 represses the luciferase activity of the core (A) and the DRR (B), but not that of the TATA promoter (C). Luciferase activity was measured in the same conditions for the experiments in A, B and C. The same arbitrary units (A.U.) therefore apply to all panels. (D) Schematic representation (not in scale) of the regulatory elements of the mouse MyoD promoter and of the position of the three amplified fragments used for ChIP experiments. (E) ChIP assays were performed from limbs of E11.5 mice with antibodies against Sim2 or Ach4, or without any antibody (no Ab) as a negative control. ChIP products were analysed by PCR to study the presence of mSim2 on regulatory regions of mouse MyoD promoter. We could detect the binding of mSim2 to the core element. PCR amplifications were performed on chromatin isolated before immunoprecipitation (Input) as positive control.

Fig. 7. mSim2 represses the transcriptional activity of MyoD promoter in vitro and is recruited to the core element in vivo in E11.5 mouse limbs. (A-C) Primary myoblasts were co-transfected with GFP-, mMyoD- or mSim2-expression vectors and MyoD reporter constructs, in which the luciferase reporter gene was cloned downstream of two regulatory elements of the mMyoD promoter, the core (A) and the DRR (B) or a minimal promoter (C). mSim2 represses the luciferase activity of the core (A) and the DRR (B), but not that of the TATA promoter (C). Luciferase activity was measured in the same conditions for the experiments in A, B and C. The same arbitrary units (A.U.) therefore apply to all panels. (D) Schematic representation (not in scale) of the regulatory elements of the mouse MyoD promoter and of the position of the three amplified fragments used for ChIP experiments. (E) ChIP assays were performed from limbs of E11.5 mice with antibodies against Sim2 or Ach4, or without any antibody (no Ab) as a negative control. ChIP products were analysed by PCR to study the presence of mSim2 on regulatory regions of mouse MyoD promoter. We could detect the binding of mSim2 to the core element. PCR amplifications were performed on chromatin isolated before immunoprecipitation (Input) as positive control.
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 global mass of ventral musculature is greater than that of dorsal musculature (see supplementary material Figs S6, S7). Interestingly, in mouse forelimbs, where mSim2 expression is also enhanced in posterior regions (Fig. 3C; supplementary material Fig. S1), the global muscle size is also greater in posterior regions compared with anterior regions (supplementary material Fig. S5).

Fig. 8. Differential increase of dorsal versus ventral muscle masses in chick forelimbs. In situ hybridisation to whole-mount (A,B) or to tissue sections (C,D) of forelimbs of HH22 embryos using the cPax3 (A,C) or cSim2 (B,D) probes. (A) Dorsal and ventral views of the same forelimbs hybridised with the cPax3 probe. (B) Ventral view of forelimbs hybridised with the cSim2 probe. (C,D) Transverse and adjacent sections of HH22 forelimbs were hybridised with the cPax3 (C) and cSim2 (D) probes. (E-I) Double immunohistochemistry using the Pax7 and MF20 antibodies on transverse sections of forelimbs at the level of the mid-forearm, at different stages of development: HH22 (E,F), HH23 (G) and HH29 (H,I). D, dorsal; u, ulna; r, radius; V, ventral.

Fig. 9. mSim2 expression is upregulated in dorsal regions of embryonic limbs deficient for Lmx1b activity. (A-C) In situ hybridisation experiments on transverse HH29 chick limb sections with the cLmx1b (A), cSim2 (B) and cMyoD (C) probes show that cLmx1b expression in dorsal limb regions is excluded from the cMyoD expression domain and from the cSim2 expression domain in ventral limb muscles. (D-I) E10.5 forelimbs (D,E) and E11.5 hindlimbs (F-I) from wild-type (D,F,H) and Lmx1b mutant mice (E,G,I) were hybridised with the mSim2 probe (D-I). mSim2 expression is upregulated in dorsal limb regions of Lmx1b-deficient limbs (E,G,I, arrows), compared with wild-type limbs (D,F,H). In all pictures, the dorsal and ventral limb regions are separated by a line. Arrows indicate the extension of the mSim2 expression domain in dorsal limb regions in Lmx1b mutants. a, anterior; p, posterior; D dorsal; V, ventral.
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 four 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'Honore 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 mutant 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'Honore 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 (Coumaillieau 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 (Kiery, 1982; Chen et al., 1998; Kardon, 1998; Duprez et al., 1999; Duprez,
Bonnet, A., Dai, F., Brand-Saberi, B. and Duprez, D. 2002). However, it is interesting to note that Sim2, although it is not the master gene for regulating the muscle size, is negatively regulated by the factor specifying the dorsal limb pattern, Lmx1b (Fig. 10C).

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 MyoD (Fig. 10C).

References
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