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Sim1 and *Sim2* expression during chick and mouse limb development

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ABSTRACT The *Drosophila* Single minded *(Sim)* transcription factor is a master regulator of cell fate during midline development. The homolog mouse *Sim1* and *Sim2* genes are important for central nervous system development. Loss of *mSim1* activity leads to an absence of specific neuroendocrine lineages within the hypothalamus, while overexpression of *mSim2* leads to behavioural defects. We now provide evidence that vertebrate *Sim* genes might be important for limb muscle formation. We have examined by *in situ* hybridisation the expression of the *Sim1* and *Sim2* genes during limb development in chick and mouse embryos. The expression of both *Sim* genes is mainly associated with limb muscle formation. We found that each *Sim* gene has a similar temporal and spatial expression pattern in chick and mouse embryonic limbs, although with some differences for the *Sim2* gene between species. In chick or mouse embryonic limbs, *Sim1* and *Sim2* display non-overlapping expression domains, suggesting an involvement for Sim1 and Sim2 proteins at different steps of limb muscle formation. *Sim1* gene expression is associated with the early step of muscle progenitor cell migration in chick and mouse, while the *Sim2* gene is expressed just after the migration process. In addition, chick and mouse *Sim2* gene expression is enhanced in limb ventral muscle masses versus dorsal ventral muscle masses. Our results provide a basis for further functional analysis of the *Sim* genes in limb muscle formation.

KEY WORDS: *single-minded, chick, mouse, limb, muscle*

Introduction

The mammalian *Sim1* and *Sim2* genes encode proteins of the basic helix-loop-helix and Period-Arnt-Sim (bHLH-PAS) transcription factor family homologous to the *Drosophila* single minded (*Sim*) gene. The *Drosophila Sim* gene is key regulator of the development of the midline cells of the central nervous system (CNS) (Crews, 1998; Crews and Fan, 1999). Null mutations of murine *Sim1* and *Sim2* genes in mice have provided evidence that both genes are important for embryonic survival, since both *Sim1* and *Sim2* mutant mice die shortly after birth (Michaud *et al.,* 1998; Goshu *et al.,* 2002, Shamblott *et al.,* 2002). The disruption of the *Sim1* gene in mice has shown that *mSim1* is required for the development of two hypothalamic nuclei, the paraventricular nucleus (PVN) and supraoptic nucleus (Michaud *et al.,* 1998). In mouse, *Sim1* haploinsufficiency induces obesity by increasing food intake (Michaud *et al.,* 2001; Holder *et al.,* 2004). The absence of one *mSim1* copy leads to a hypocellular PVN, a region of the hypothalamus that controls food intake (Michaud *et al.,* 2001), but also affects adult PVN by altering the physiological pathways controlling food intake, indicating the involvement for *mSim1* both in PVN embryonic development and adult PVN physiology (Yang *et al.,* 2006). Interestingly, in humans, a decrease of *SIM1* also leads to obesity (Holder *et al.,* 2000). The human *SIM2* gene is located in the region of the human chromosome 21 known to be associated with the etiology of Down Syndrome phenotype and *mSim2* maps to the region of chromosome 16 syntenic to human chromosome 21 (Dahmane *et al.,* 1995; Chrast *et al.,* 1997). Transgenic mice with three copies of *mSim2* exhibited some of the Down Syndrome phenotype (Ema *et al.,* 1999; Chrast *et al.,* 2000). *Sim2* mutant mice die at birth due to lung atelectasis and breathing failure and display rib, vertebral and craniofacial abnormalities (Goshu *et al.,* 2002; Shamblott *et al.,* 2002). *Sim* homologs were also identified in chick, *Xenopus*

Abbreviations used in this paper: bHLH, basic helix-loop-helix; PAS, Period-Arnt-Sim; Sim, single minded transcription factor.

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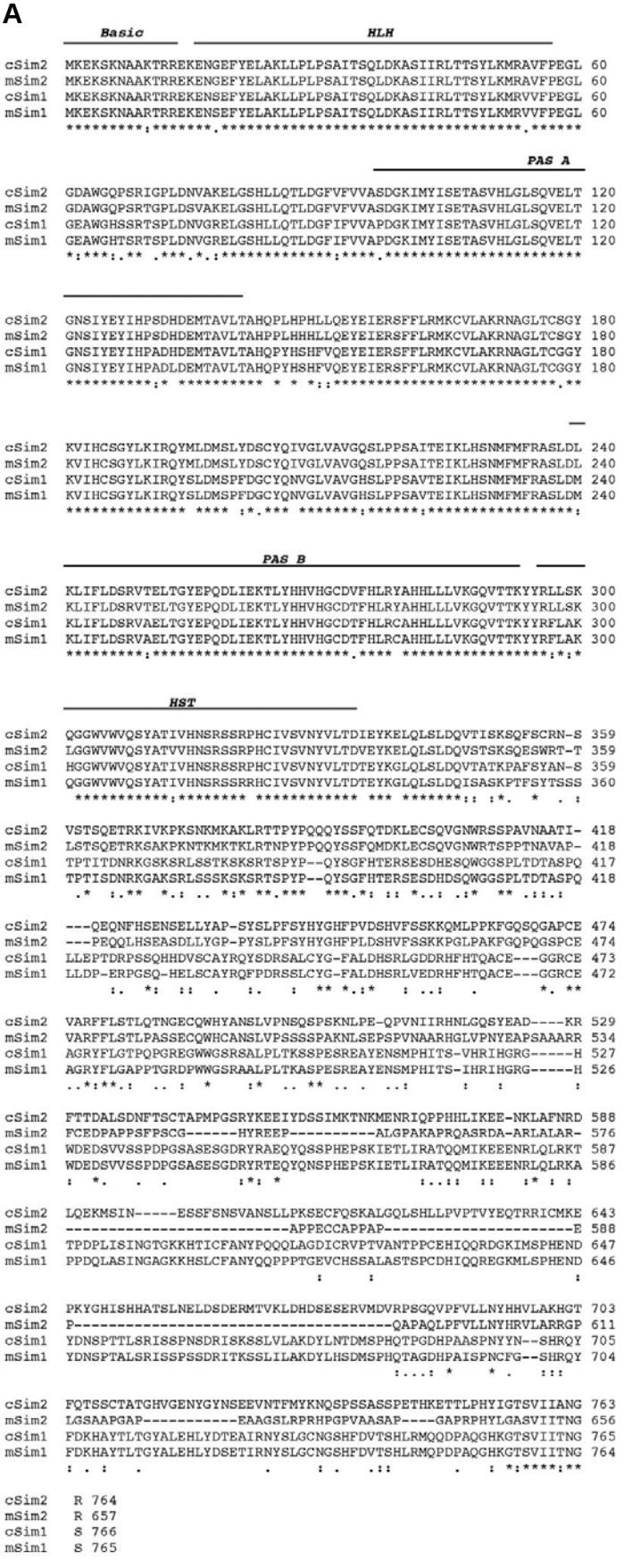
A



B

	Full length cSim1 Nterm cSim1 Cterm cSim1	Full length cSim2 Nterm cSim2 Cterm cSim2	Full length mSim1 Nterm mSim1 Cterm mSim1	Full length mSim2 Nterm mSim2 Cterm mSim2
Full length cSim1	100%	53%	89%	55%
Nterm cSim1		90%	98%	90%
Cterm cSim1		23%	82%	23%
Full length cSim2		100%	51%	77%
Nterm cSim2			90%	97%
Cterm cSim2			20%	56%
Full length mSim1			100%	57%
Nterm mSim1				89%
Cterm mSim1				24%
Full length mSim2				100%
Nterm mSim2				
Cterm mSim2				

and Zebrafish (Pourquié *et al.,* 1996; Coumailleau *et al.,* 2003, Eaton and Glasgow, 2006). *cSim1* is used as a maker of lateral somitic compartments, although its function in this region is not known (Pourquié *et al.,* 1996, Cheng *et al.,* 2004, Ahmed *et al.,* 2006). *xSim* has been identified and characterized but its function is not known (Coumailleau *et al.,* 2000, 2003). In zebrafish, zSim1 has been recently shown to be required for isotocin cell development, indicating an evolutionary conservation of neuroendocrine cell development between mammals and fish (Eaton and Glasgow, 2006). At a molecular level, the murine Sim1 and Sim2 proteins have been shown to form heterodimers with the Arnt (Arylhydrocarbon receptor nuclear translocator) and Arnt-2 proteins (Probst *et al.,* 1997; Moffet *et al.,* 1997; Woods and Whitelaw, 2002). *In vitro* biochemical analyses have shown that the murine Sim1 and Sim2 proteins have different transcriptional properties. mSim1 can strongly activate transcription via the transactivation domain of Arnt, while mSim2 inhibits transcription by active repression (Moffet *et al.,* 1997; Moffet and Pelletier, 2000).

Although most mammalian *Sim* studies focus on CNS, the endogenous expression of the mouse *Sim2* gene has frequently been observed to be enhanced in embryonic and adult skeletal muscle tissues (Fan *et al.,* 1996; Moffett *et al.,* 1996; Ema *et al.,* 1996). However, the *Sim2* transcript distributions related to the different steps of limb myogenesis have not been described. Limb skeletal muscle cells originate from somites. Cells from the ventro-lateral lips of the dermomyotomes of the limb somites will undergo an epithelial to mesenchyme transition, delaminate and then migrate to limb mesenchyme. Once they have reached the limb, somitic muscle cells aggregate into dorsal and ventral limb regions. The dorsal and ventral muscle progenitors will activate the skeletal muscle program upon the expression of the myogenic regulatory factors (MRFs), a family of four bHLH transcription factors, Myf5, MyoD, Myogenin and Mrf4. Formation of limb

Fig. 1. Comparison of chick and mouse Sim proteins. (A) *Comparison of the primary structures of chicken and mouse full length Sim proteins, cSim1, cSim2, mSim1 and mSim2. Identical amino acids (*) and conservative changes (: or.) are indicated according to Blast program. Dashes indicate deletions to maximize the sequence similarity. The classical domains of the bHLH/PAS transcription factors are indicated: basic, HLH, PAS-A, PAS-B and HST.* **(B)** *Table showing the percentage of homologies between the chick and mouse Sim proteins. The percentages for the N-terminal halves (Nterm) were calculated using the sequences from AAN°1 (M) to AAN°334 (D), including all the classical domains of bHLH/PAS proteins. The percentages for the Carboxyl-terminal regions (Cterm) were calculated using the sequences from the AAN° 335(I) just after the HST domain to the stop codon.*

skeletal muscles during vertebrate embryogenesis also involves other steps such as proliferation, growth arrest and skeletal muscle differentiation (reviewed in Duprez, 2002). Null mutations in mice have revealed hierarchical relationships and apparent functional overlap among the MRFs (reviewed in Buckingham, 2006). Besides the recognized master role of the MRFs in triggering myogenesis in vertebrates, there is emerging evidence that other transcription factors are important for myogenesis. In addition, distinct genetic hierarchies have been identified controlling the formation of each category of muscles, axial, limbs and head (Buckingham, 2006). However, the function of each of the components of the genetic network involved in limb myogenesis is not fully characterised.

In this paper, we investigated the expression pattern of the *Sim1* and *Sim2* genes during chick and mouse limb development. Due to the fact that the experiments of *in situ* hybridisation to wholemount embryos have some limitations, such as insufficient probe penetration, we focused our expression analysis using *in situ* hybridisation to tissue sections. Our results show that *Sim1* and *Sim2* gene expression are related to different steps of limb myogenesis during chick and mouse embryonic development.

Results

Comparison between chick and mouse Sim proteins

To search for chick homologs of the *mouse Sim* genes, we analyzed the recently sequenced genome of chick *Gallus Gallus* (International Chicken Genome Sequencing Consortium, 2004). Our search analysis in the database concluded that the chick genome only contains two genes that are highly related to the *mouse* and *Drosophila Sim* genes, *cSim1* (accession number XP_419817) and *cSim2* (accession number XP_416724). No other *Sim* sequence could be found from our blast search in the chicken genome. The chick full length cDNA sequences for the *Sim1* and *Sim2* genes encode a 766-amino acid protein (predicted molecular mass of 86 kDa) and a 764-amino acid protein (predicted molecular mass of 86.4 kDa), respectively (Fig. 1A). The comparison of the deduced amino acid sequences of Sim proteins between chick and mouse showed that each chick Sim protein has a strong homology with its mouse counterpart: chick and mouse Sim1, 89%, chick and mouse Sim2, 77%, (Fig. 1B). The Sim homologies between species are higher that the homologies observed between the Sim1 and Sim2 within the same species (Fig. 1B). The three classical domains of bHLH/PAS transcriptions factors (basic, Helix-Loop-Helix and PAS domains) are highly conserved between chick and mouse. The bHLH domains of the cSim1 and cSim2 proteins are 100% identical with that of the mSim1 and mSim2, respectively. In addition, bHLH domains between Sim1 and Sim2 proteins share 94% identical amino acids both in chick and mouse. Strong similarities in the PAS domains were also apparent between murine and chick sequences, since PAS domains of the Sim proteins share 98% (Sim1) and 97% (Sim2) identical amino acids. These high homologies within these domains, which are included in the N-term region of the Sim proteins (Fig. 1B), strongly suggest that Sim1 and Sim2 proteins have similar DNA binding and dimerisation properties across species, but also between them. The carboxy-terminal regions of the predicted amino acid sequences, which contain the trans-regulation domains, showed relatively high conservation of sequences between chick and mouse Sims (Fig. 1B). However, there is no significant amino acid identity of the carboxyl-terminal halves between the two chick proteins (23%) and between the two mouse proteins (24%), (Fig. 1B). The low identity of the carboxyl-terminal sequences between Sim1 and

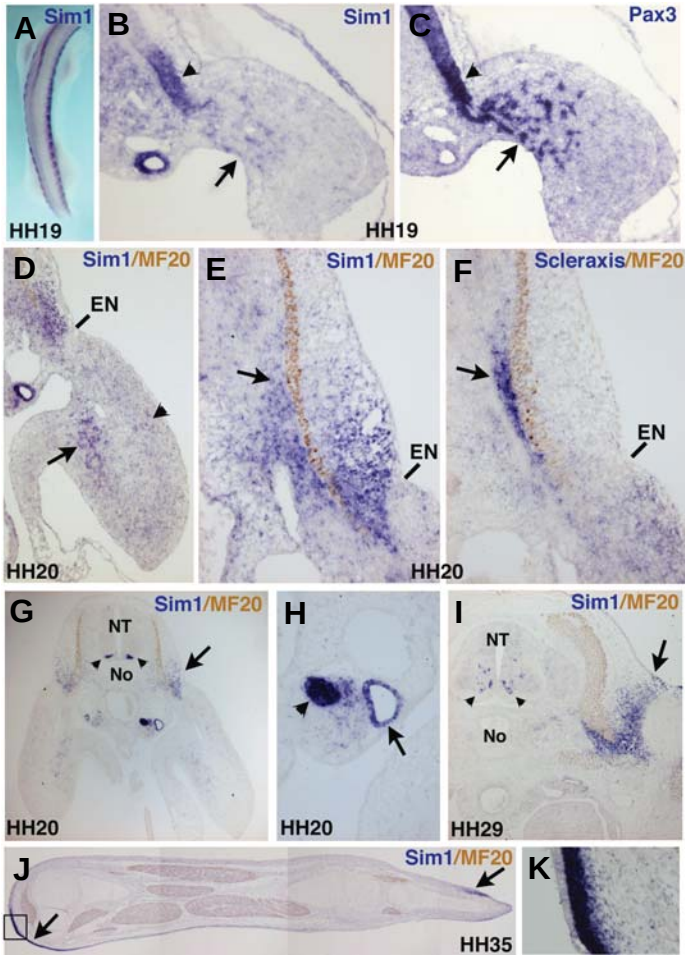


Fig. 2. *cSim1* expression during chick limb development. (A) In situ *hybridisation to HH19 embryo with the* cSim1 *probe.* **(B,C)** *Consecutive transverse sections of HH19 embryos at the forelimb level hybridized with the* cSim1 *(B) and* Pax3 *(C) probes. Arrows point to* cSim1*- and* Pax3*-migrating cells. Arrowheads show the lateral parts of the dermomyotomes expressing the* cSim1 *and* Pax3 *genes. Transverse sections of HH20 (D-H) and HH29 (I) embryos at the forelimb levels were hybridised with the* cSim1 *(D,E,G-I) or* Scleraxis *(F) probes and then incubated with the MF20 antibody (brown) that recognises myosins.* **(D)** *The arrow points to the* cSim1*-expressing cells in the ventral muscle mass, while the arrowhead shows the faint* cSim1 *expression in the dorsal muscle regions.* **(E,F)** *Arrows point to the regions expressing the* cSim1 *and* Scleraxis *genes.* **(G,I)** *The arrows point to the lateral somitic regions expressing* cSim1 *of HH20 (G) and HH29 (I) embryos. The two arrowheads point to the* cSim1 *expression in the ventral parts of the neural tube of HH20 and HH29 embryos.* **(H)** *Arrowhead points to the mesonephros and the arrow to the Wolffian duct.* **(J)** *Longitudinal sections of forelimbs from HH35 embryos. (J) Arrows point the* cSim1 *expression sites.* **(K)** *Higher magnification of the inset drawn in (J) shows that* cSim1 *expression is restricted to the dermis and does not cover the ectoderm. For all the sections (B-K) dorsal is to the top. NT, neural tube; No, notochord; EN, ectodermal notch.*

Sim2 proteins is consistent with distinct transcriptional activities observed in cultured systems (Moffet *et al.,* 1997; Moffet and Pelletier, 2000).

cSim1 *gene expression during chick limb bud development*

In situ hybridisation to whole mount HH19 embryos showed that *cSim1* transcripts were located in the lateral parts of somites, all along the antero-posterior axis, as already described (Fig. 2A, Pourquié *et al.,* 1996). At the limb level, *in situ* hybridisation to sections showed enhanced *cSim1* expression in the lateral parts of the dermomyotomes, visualised by *Pax3* expression (Fig. 2B,C, arrowheads). *cSim1* transcripts were also observed in a subset of migrating muscle precursors, visualised by *Pax3* expression in forelimbs (Fig. 2B,C, arrows). *cSim1* transcripts appeared to be located in a ventral subpopulation of *Pax3*-positive migrating cells. At stage HH20*, cSim1* expression was still observed in ventral muscle cells, while a faint expression was consistently observed in dorsal muscle limb regions (Figure 2D, arrow and arrowhead). Similar *cSim1* expression (lateral parts of dermomyotomes and migrating cells) was also observed in HH21 hindlimbs (data not shown). From stage HH23 onwards, *cSim1* expression was no longer detected in the limb muscle cells (data not shown). In addition to *cSim1* expression in lateral dermomyotomes, *cSim1* transcripts were also observed in lateral regions of sclerotome and in dermomyotome derivatives such as the dermatome and myotome at various stages of development (Fig. 2B,D,E,G,I), in line with previous studies (Cheng *et al.,* 2004). At the limb level, at HH20, the ventral/lateral boundary of the dermal *cSim1* expression domain corresponds exactly to the ectodermal notch (Fig. 2D,E,G), which is a thickening of the ectoderm demarcating the somite- and lateral plate-derived dermis (Christ *et al.,* 1983). Comparison of *cSim1* expression with that of the tendon marker *Scleraxis* did not highlight any obvious correlation, although the *cSim1* expression domain did encompass the *Scleraxis* domain (Fig. 2E,F, arrows). In addition, *cSim1* transcripts were never detected in differentiated skeletal muscle cells, visualised by sarcomeric myosin expression (Fig. 2I and data not shown). At the axial level, the *cSim1* gene displayed the known sites of expression: the ventral regions of the neural tube (Fig. 2G,I, arrowheads), the mesonephros and the Wolffian duct (Fig. 2H). In HH35 limbs, only discrete sub-regions of the limb dermis displayed *cSim1* expression (Fig. 2J,K, arrows). In summary, the *cSim1* expression in the lateral parts of the dermomyotomes of the limb somites and the faint and transient *cSim1* expression in limb migrating cells suggest a role for the cSim1 protein in early steps of migration of muscle progenitor cells into the limb buds.

cSim2 *expression during chick limb development*

In contrast to *cSim1*, the *cSim2* gene was not expressed during the migration step of the muscle precursors into the chick limbs (data not shown). The first limb *cSim2* expression was observed at stage HH20 in the forelimbs (data not shown) and at stage HH21 in the hindlimbs (Fig. 3A). *cSim2* transcripts were not observed in limb somites; the dermomyotomes were visualised with *Pax3* expression (Fig. 3A,B, arrowheads). *cSim2* transcripts were specifically observed in the ventral muscle masses; the dorsal and ventral muscle masses were also visualised by *Pax3* expression (Fig. 3A,B). In the ventral muscle mass, the *cSim2* and *Pax3* expression domains were similar (Fig. 3A,B, arrows). At stage HH24, *cSim2* transcripts are still exclusively observed in the ventral muscle cells of the limbs, in a domain larger than that of *MyoD* (Fig. 3C,D). At stage HH29, c*Sim2* expression was still observed in the limb ventral muscle masses and not in the dorsal muscle masses, both masses were visualized with myosin expression (Fig. 3E). Detailed examination of the ventral muscle mass at HH29 (Fig. 3G) and on a ventral muscle at HH35 (Fig. 3H) shows that *cSim2* transcripts were not observed in MF20-positive cells, indicating that *cSim2* is not expressed by differentiated muscle cells. At stage

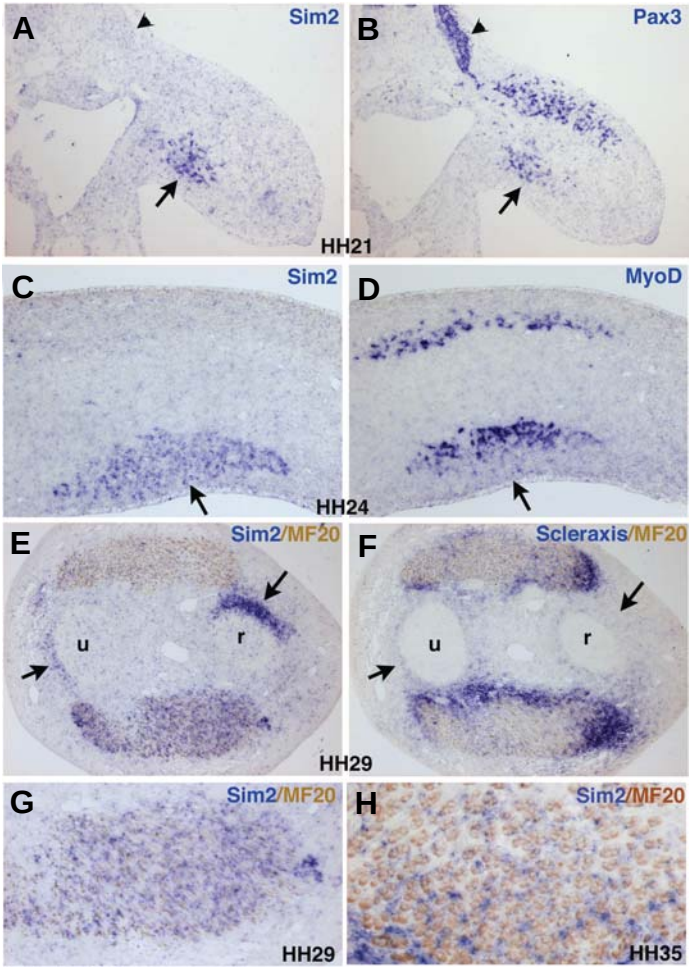


Fig. 3. *cSim2* expression during chick limb development. (A,B) *Consecutive transverse sections at the hindlimb level from HH21 embryos were hybridised with the* cSim2 *(A) and* Pax3 *(B) probes.* **(C,D)** *Consecutive transverse sections at the forelimb level from HH24 embryos were hybridized with the* cSim2 *(C) and* MyoD *(D) probes.* **(E,F)** *Consecutive transverse sections of forelimbs from HH29 embryos were hybridized with the* cSim2 *(E) and* Scleraxis *(F) probes and then incubated with the MF20 antibody (brown). Arrows indicate non-myogenic* cSim2 *expression surrounding the cartilage elements.* **(G)** *High magnification of the ventral muscle mass of (E).* **(H)** *Transverse sections of a ventral muscle from HH35 forelimbs hybridized with the* cSim2 *probe and then incubated with the MF20 antibody (brown) shows that* cSim2 *transcripts are located outside the MF20-positive cells. (A-D) For transverse sections of embryos (leading to longitudinal limb sections), dorsal is to the top and proximal to the left. (E-G) For transverse limb sections, posterior is to the left and dorsal to the top. r, radius, u, ulna.*

HH29, the *cSim2* gene was expressed around the cartilage elements (Fig. 3E, arrows). Comparison with *Scleraxis* expression on adjacent sections showed that these *cSim2* expression domains were *Scleraxis*-negative (Fig. 3E,F, arrows). At stage HH35, when the final muscle pattern is organised, *cSim2* transcripts were still observed in ventral, individualised muscles (Fig. 4A). No *cSim2* expression was observed in dorsal muscles, with the exception of one dorsal and posterior muscle, the Anconeus (Fig. 4A,C). This *cSim2* expression in the Anconeus muscle must be late since there was no obvious sign of dorsal *cSim2* expression at earlier stages. c*Sim2* transcripts were also expressed in some tendons, which appeared to be more dorsal (Fig. 4A,C, arrowheads), while most of the ventral tendons did not display any *cSim2* expression (Fig. 4A,E, arrows). The tendons were visualised with *Scleraxis* expression (Fig. 4B,D,F). In summary, *cSim2* is a specific marker of chick limb ventral muscle masses.

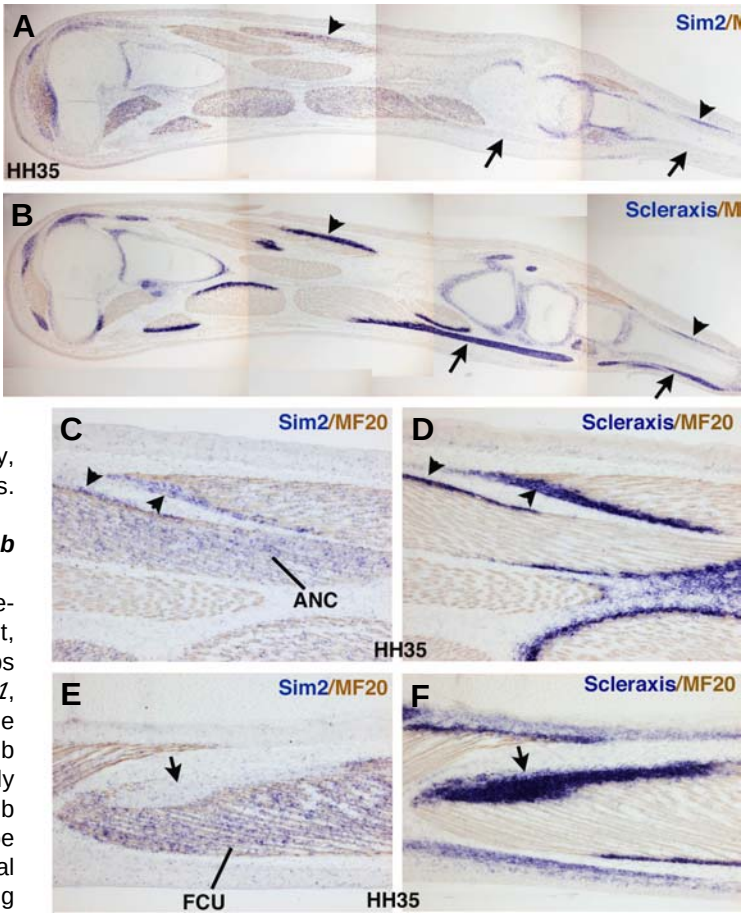


Fig. 4. *cSim2* expression in chick HH35 forelimbs. *Consecutive longitudinal sections of HH35 forelimbs were hybridized with the* cSim2 **(A,C,E)** *and* Scleraxis **(B,D,F)** *probes and then incubated with the MF20 antibody (brown).* **(A,B)** *These longitudinal sections are in anterior regions. (A,B) Arrows show the ventral tendons expressing* Scleraxis *and no* Sim2. *Arrowheads point to dorsal tendons expressing* cSim2 and Scleraxis. **(C,D)** *Focus on dorsal and posterior forelimb muscles of HH35 embryos showing the unique dorsal muscle expressing* cSim2, *the ANC (Anconeus). Arrowheads in (C) point to the tendons expressing the* cSim2 *(C) and* Scleraxis *(D) genes.* **(E,F)** *The ventral and posterior muscle, FCU (Flexor carpi ulnaris) expresses the* cSim2 *genes; however, the associated tendon does not (arrows).*

mSim1 *and* mSim2 *gene expression during mouse limb development*

In order to determine whether mouse *Sim* expression resembled to that of chick *Sim* expression during limb development, we examined *mSim1* and *mSim2* expression in fore- and hindlimbs of mouse embryos between E10 and E14.5. Similar to the *cSim1*, *mSim1* transcripts were observed in lateral parts of the dermomyotomes of the somites at the forelimb and hindlimb levels (Fig. 5A-D). *mSim1* expression was also detected faintly and transiently in a subset of somitic cells migrating to the limb buds; the migrating cells were visualised using the *Pax3* probe (Fig. 5A-D). As in chick, *mSim1* expression defined a lateral compartment of somites and of somitic-derived tissues, including lateral regions of sclerotome and dermomyotome and its derivatives, myotome and dermatome (Fig. 5 F,I and data not shown). At later stages, no subsequent *mSim1* expression was detected in fore- and hindlimbs (data not shown). In contrast to *mSim1,* but similarly to *cSim2*, *mSim2* was not expressed in limb somites or in early limb migrating cells (Fig. 5E). The first *mSim2* expression in limb muscle was observed in E10 forelimbs and E11 hindlimbs (data not shown). As soon as it was expressed, *mSim2* expression was clearly enhanced in ventral muscle progenitors compared to *Pax3* expression, which labels all muscle progenitors (Fig. 5 G,H,J,K). In E11.5 forelimbs, *mSim2* expression was enhanced in posterior regions of the muscle masses visualised by *MyoD* expression (Fig. 6A,C). In E11.5 hindlimbs, *mSim2* expression was mainly ventral and appeared to be complementary to that of *MyoD*, (Fig. 6E,G). At E12.5, *mSim2* expression was still enhanced in ventral muscle masses of fore- and hindlimbs (data not shown). At E14.5, *mSim2* expression was no longer observed in muscles but only around digit cartilage (data not shown). In order to confirm that *mSim2*-expressing cells in limbs were myogenic cells, we took advantage of the existence of *Pax3*-deficient mice, in which no myogenic cells are detected in the limbs (Relaix *et al.,* 2003). In the absence of muscle cells, muscle connective tissue and tendons initiate their development normally, showing the absence of muscle requirement for other limb tissue formation at early stages of development (Kardon, 1998; Kardon *et al.,* 2003, Bonnin *et al.,* 2005). Consequently, the absence of gene expression in early *Pax3* mutant limbs (before E12) strongly suggests that it is not an indirect consequence of the absence of muscle cells but reflects a normal expression restricted to myogenic cells. The absence of *mSim2* expression in distal fore and hindlimbs of *Pax3* mutant mice confirmed that *mSim2* expression was exclusively in myogenic cells (Fig. 6A-H). However, in proximal and posterior limb regions close to the body axis, we were able to observe *mSim2* expression domains, which were present in *Pax3* mutant limbs (Fig. 6I-L). These non-myogenic *mSim2* expression domains did not specifically correspond to *Scleraxis* expression domains (data not shown).

Discussion

In this paper we have addressed the precise tissue distribution of *Sim* transcripts during limb development in the chick and mouse embryos. We have established a link between the expres-

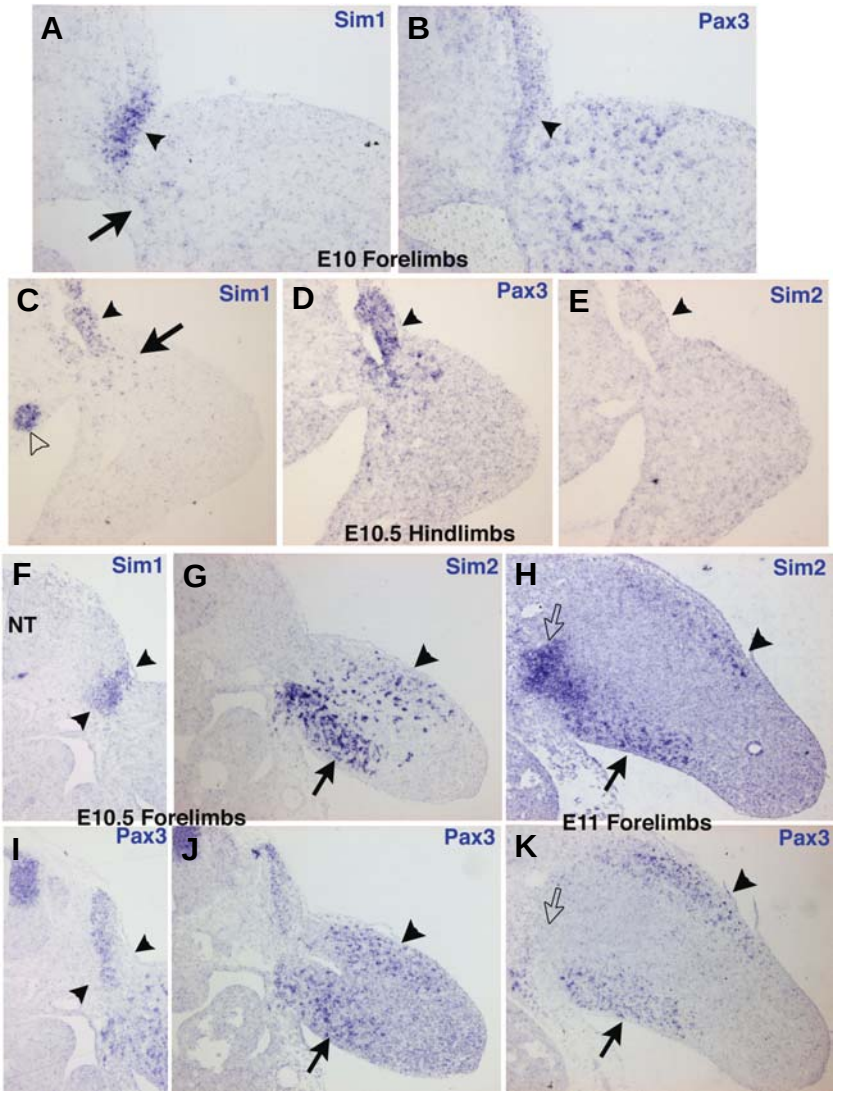


Fig. 5. *mSim1* and *mSim2* expression during mouse limb development. *Consecutive transverse sections at the forelimb level from E10 (A,B), E10.5 (F,G,I,J) and E11 (H,K) embryos or at the hindlimb level from E10.5 embryos (C-E) were hybridized with the* mSim1 **(A,C,F)**, mSim2 **(E,G,H)** *and* mPax3 **(B,D,I-K)** *probes. Arrowheads in (A-E) indicate the lateral regions of the dermomyotomes at the limb levels, expressing* mSim1 *(A,C),* Pax3 *(B,D) and no* mSim2 *(E). Arrows in (A,C) point to the faint* mSim1 *expression in a subset of the migrating muscle progenitor cells.* **(C)** *The open arrowhead shows the mesonephros expressing* mSim1. **(F,I)** *Arrowheads point to the lateral somitic* mSim1 *expression domain (F), which include the lateral* Pax3 *expression (I). Arrows in (G,H,J,K) show the ventral muscle masses expressing* mSim2 *(G,H) and* Pax3 *genes (J,K). Arrowheads in (G,H,J,K) point to the dorsal muscle masses visualised with* Pax3 *expression, which display faint* mSim2 *expression. The open arrows in (H,K) indicates a* mSim2*-positive domain (H), which is not* Pax3*-positive (K). NT, neural tube.*

sion of the *Sim1* and *Sim2* genes and different steps of limb muscle formation, in chick and mouse.

Limb Sim1 *expression in chick and mouse embryos*

Sim1 transcripts displayed a similar expression patterns in chick and mouse limbs. In mouse embryos, *mSim1* has previously been described as being restricted to the central dermomyotome at E10.5 at the interlimb region (Sporle, 2001). Our observation of *mSim1* expression in lateral limb dermomyotomes could reflect a difference of axial level, although it is consistent with previous observations that described *mSim1* expression in the lateral compartment of the dermomyotomes at the interlimb region (Ikeya and Takada, 1998). It is also consistent with the *cSim1* expression in chick embryos (Fig. 2, Pourquié *et al.,* 1996; Cheng *et al.,* 2004). The enhanced expression of *cSim1* and *mSim1* in lateral parts of the dermomyotomes of limb somites and the faint, transient expression in migrating somitic cells suggest an involvement in the migration step of limb muscle progenitors in chick and mouse embryos. However, no limb muscle phenotype has been described in the *Sim1* mutant mice. Interestingly, using a cell aggregation assay it has been shown that the *cSim1*-expressing cells in the lateral dermomyotomes do not mix with the medial Engrailed1-expressing cells, suggesting different properties of *Sim1*-expressing cells in the dermomyotomes versus the medial dermomyotomal cell population (Cheng *et al.,* 2004). *Sim1* is not exclusive to the lateral parts of chick and mouse dermomyotomes and is also expressed in lateral regions of the sclerotome, myotome and dermatome, defining a lateral somitic-derived region, for both species (Olivera-martinez *et al.,* 2000, 2002, Ben-Yair *et al.,* 2003)

Limb Sim2 *expression in chick and mouse embryos*

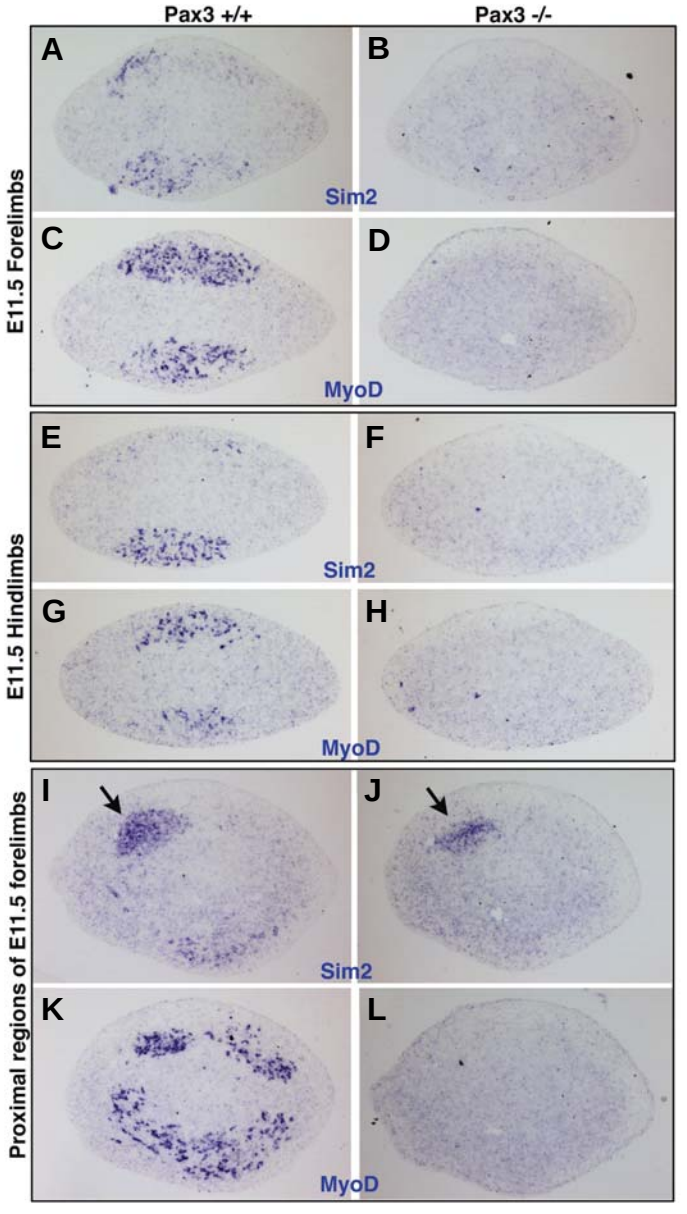
One major feature of the *Sim2* limb expression was its enhancement in ventral muscle cells (versus dorsal muscle cells). *cSim2* expression was almost exclusive to chick ventral muscle masses. In mouse, although *mSim2* expression was clearly enhanced in ventral muscle masses of fore- and hindlimbs, we could detect *mSim2* expression in the dorsal muscle masses, specifically in the forelimbs. This restricted/enhanced expression pattern in ventral limb muscles was observed until E9 chick limbs and until E12.5 mouse limbs. The transient *mSim2* expression in mouse limb muscle masses and the absence of *cSim2* expression in chick muscle fibres indicate that *Sim2* labels limb myoblast progenitors. To our knowledge, *Sim2* represents of the first example of a gene displaying this enhanced expression in ventral muscle masses, since all the known muscle genes (associated with any steps of myogenesis) are located in both dorsal and ventral muscle masses. The reason for this enhanced ventral expression is not clear and could reflect an involvement of the *Sim2* gene in patterning the ventral limb muscles. However, embryological data have established that the positional information for limb muscle patterning is not located in myogenic cells but within limb lateral plate-derived mesenchyme cells (reviewed in Duprez, 2002). The only muscle defect described in the *Sim2* mutant mice is a thinner diaphragm, contributing to the pulmonary atelectasis (Goshu *et al.,* 2002). Further

work is necessary to determine the function of the *Sim2* gene in the ventral muscle masses in chick and mouse embryos. *cSim2* was also expressed in a subset of chick limb tendons, but we did not observe similar *mSim2* expression in mouse limbs, highlighting a difference between *Sim2* expression in chick and mouse limbs.

Sim1 *and* Sim2 *genes display distinct expression profiles during embryonic limb development*

Sim1 and *Sim2* expression domains did not overlap during chick and mouse limb myogenesis, suggesting an absence of functional redundancy in muscle formation. The only overlapping expression between the *Sim1* and *Sim2* genes was observed in HH20 chick forelimbs in ventral muscle masses. In addition to being expressed in different steps of muscle formation, the homologies between the *Sim1* and *Sim2* genes (in chick or mouse) are very low compared to those between *cSim1* and *mSim1* and between *cSim2* and *mSim2* (Fig. 1B). *Sim1* expression suggests an involvement in early steps of limb muscle formation (specification or/and migration of lateral muscle precursors), while *Sim2* expression indicates an involvement after the migration step. This is reminiscent of the *mSim1* and *mSim2* expression in CNS, where the murine *Sim1* and *Sim2* genes display different expression profiles that overlap in certain regions of the anterior hypothalamus (Fan *et al.,* 1996). *Sim* mutant analysis showed that *Sim1* acts upstream of *Sim2* and partially compensates for the loss of *Sim2* in PVN embryonic development (Michaud *et al.,* 1998, Goshu *et al.,* 2002, Goshu *et al.,* 2004). However, *mSim1* and *mSim2* act along compensatory pathways in mammillary body axonal development (Marion *et al.,* 2005).

In summary, the *Sim1* gene is expressed mainly in early limb muscle precursor cells and *Sim2* expression is enhanced in ventral limb myoblasts in chick and mouse embryonic limbs. *Sim* expression analysis provides a basis for analysing the function of the *Sim* genes within the gene network involved in limb muscle formation.



Materials and Methods

Chick and mouse embryos

Fertilized chick eggs from commercial sources (JA 57 strain, Intitut de Sélection Animale (ISA), Lyon, France) were incubated at 37°C. Embryos were staged according to Hamburger and Hamilton (1992). Embryos from wild type and Pax3$^{-/-}$ mutant mice were collected after natural overnight matings (Relaix *et al.,* 2003). For staging fertilization was considered to take place at 6 am.

In situ *hybridisation to tissue sections or to wholemount embryos*

Chick or mouse embryos were fixed overnight at 4% (v/v) formaldehyde and processed for *in situ* hybridisation to whole mounts and to paraffin sections as previously described (Delfini *et al.,* 2000). Antisense RNA probes were labelled with digoxigenin according to manufacturer's instructions (Roche Diagnostics). The probes were detected by an alkaline phosphatase-coupled antibody against digoxigenin using nitroblue tetrazolium/5-bromo-chloro-3-indolyl phosphate (NBT/BCIP) as the chromogenic susbstrate for alkaline phosphatase. Antisense digoxigenin-labelled RNA probes were prepared as described: chick *Pax3*, chick *MyoD*, and mouse *MyoD* (Delfini and Duprez, 2004; Tozer *et al.,* 2007); chick and mouse *Scleraxis* (Bonnin *et al.,* 2005); mouse *Pax3* (Relaix *et al.,* 2003); chick *Sim1* (Pourquié *et al.,* 1996) and *Sim2* (Caqueret *et al.,* 2005); mouse *Sim1* (Michaud *et al.,* 1998) and *Sim2* (Goshu *et al.,* 2002).

Immunohistochemistry

Differentiated muscle cells were detected on sections as previously described using a monoclonal antibody against sarcomeric myosin heavy chain, MF20 (Developmental Hybridoma Bank, University of Iowa, Iowa City). Immunohistochemistry were performed following the *in situ* hybridisation experiments.

Fig. 6. Limb *mSim2* expression in the absence of muscles. *Transverse sections of forelimbs (A-D, I-L) or hindlimbs (E-H) from E11.5 wild type embryos (A,C,E,G,I,K) or E11.5* Pax3 *mutant embryos (B,D,F,H,J,L) were hybridised with the* mSim2 **(A,B,E,F,I,J)** *or* MyoD **(C,D,G,H,K,L)** *probes. The sections hybridized with the* mSim2 *probe are adjacent of the sections hybridized with the* MyoD *probe respectively. Residual* mSim2 *expression in muscleless limbs of* Pax3 *mutant mice shows that non-myogenic expression of the* mSim2 *gene is located in the proximal and posterior limb region (arrows in I,J). For all the sections, dorsal is to the top and posterior to the left.*

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