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Developmental Expression Pattern

A comparative analysis of *Meox1* and *Meox2* in the developing somites and limbs of the chick embryo

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ABSTRACT We have examined the expression pattern of the avian *Meox1* homeobox gene during early development and up to late limb bud stages. Its expression pattern indicates that it is involved in somite specification and differentiation. The domains of expression are similar but different to those of *Meox2*. *Meox1* is expressed from stage 6 in the pre-somitic mesoderm and as development proceeds, in the tail bud, the dermomyotome of the rostral somites and in the dermomyotome and sclerotome of the caudal somites, the lateral rectus muscle, truncus arteriosus of the heart and the limb buds. Unlike *Meox1, Meox2* is not expressed in the pre-somitic mesoderm, but is expressed first in somites formed from stage 11 onwards. In the developing limb, both genes are expressed in the dorsal and ventral limb mesoderm in adjacent domains with a small region of overlap. In the limb bud, *Meox1* is co-expressed with *Meox2* but neither *Meox* gene is co-expressed with *MyoD*. These expression patterns suggest that these two genes have overlapping and distinct functions in development.

KEY WORDS: *chick, somitogenesis, limb bud, sclerotome, dermomyotome*

Meox1 and *Meox2,* formerly known as *Mox1* and *Mox2,* are closely related homeobox genes with mesoderm and mesenchyme specific expression during mouse embryonic development (Candia *et al.,* 1992). Mice homozygous for a null mutation of *Meox2* have defects in limb muscle differentiation resulting in an overall reduction in muscle mass and absence of specific muscles (Mankoo *et al.,* 1999). *Meox1* mutant mice exhibit mild defects in sclerotome-derived vertebral and rib bones (Mankoo *et al.,* unpublished). Compound mutant embryos (*Meox1*$^{-/-}$;*Meox2*$^{-/-}$) displayed a dramatic phenotype associated with disrupted somite development; the axial skeleton failed to develop and most skeletal muscles were absent or reduced in size (Mankoo *et al.,* 2003). These studies demonstrated that *Meox 2* is required for limb muscle development and both *Meox1* and *Meox2* have a critical concerted function during somite morphogenesis (Mankoo *et al.,* 2003). To understand the function of these two transcription factors during chick embryogenesis requires a detailed knowledge of their expression at relevant stages of development.

In chick, *Meox2* expression during development has been previously described (Rallis *et al.,* 2001). Here, we describe for the first time in chick the distribution of *Meox1* and compare it to *Meox2*. *Meox1* has both unique and overlapping expression domains with *Meox2* in chick and similar but not identical expression patterns to *Meox1* in mouse.

A chicken *Meox1* cDNA clone, ChEST805f3, was obtained from the BBSRC Chicken EST Project (Hubbard *et al.,* 2005). The nucleotide sequence homology of this clone showed 86% identity to human *Meox1* and 86% to murine *Meox1*.

Meox1 expression first appears in the chick embryo at the time of formation of the headfold (stage 6, Hamburger and Hamilton, 1951) in the pre-somitic mesoderm (Fig. 1A). Expression in this domain continues through stage 7 (Fig. 1B) and at stage 8 is in the pre-somitic mesoderm and the first somites (Fig. 1C). A section through the embryo in Fig. 1B confirms that expression of *Meox1* is in the pre-somitic mesoderm (psm, Fig. 1E, red arrows) and not in the ectoderm and endoderm. In the initial epithelial stage, the entire somite expresses *Meox1.* In mouse, like chick, *Meox1* expression begins during the gastrulation stage (E7.0-7.5) in the posterior mesoderm (Candia *et al.,* 1992). At stage 10, *Meox1* expression is located in the developing somites and the most anterior pre-somitic mesoderm. There is strong expression in the posterior somites but the signal rapidly diminishes in the more rostral somites (Fig. 1D). By stage 14 chick *Meox1* expression is much stronger in the pre-somitic mesoderm and the most caudal somites suggesting a role for this gene in somite formation and differentiation (Fig. 1F, black arrow). The most anterior somites also show higher expression levels and correspond to cervical somites (Fig. 1F, CS, black band). The thoracic somites initially

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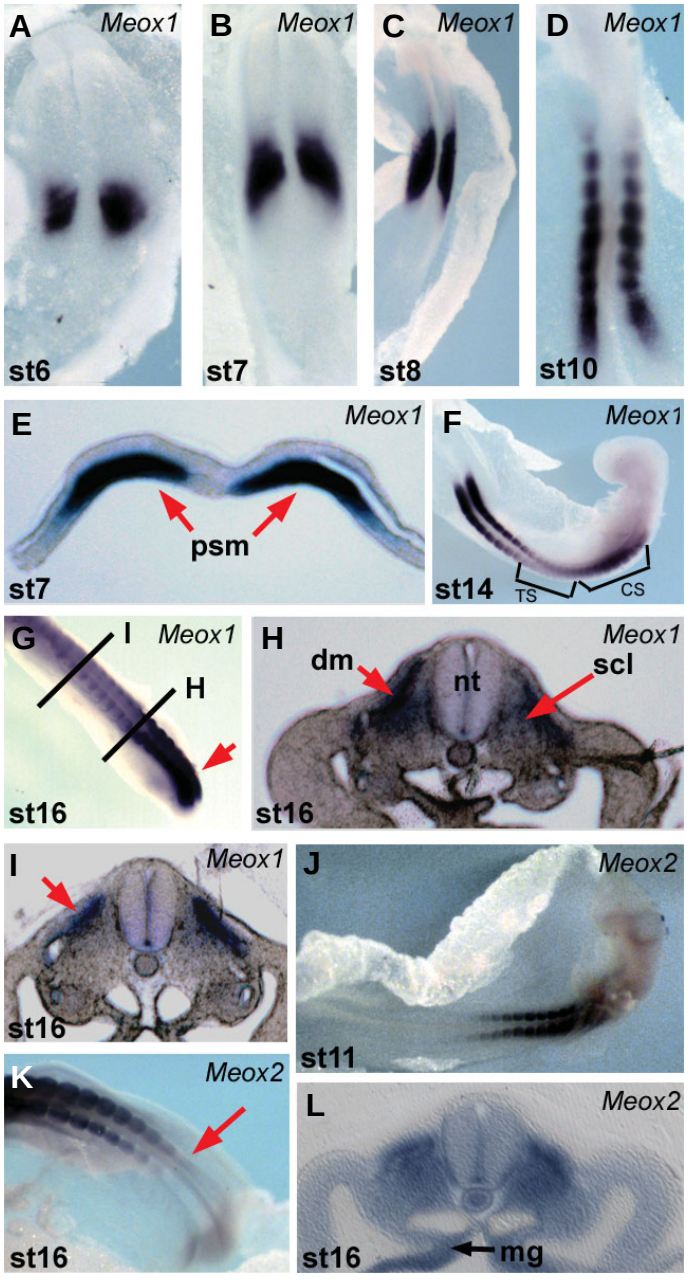
have high *Meox1* expression, but as they mature this drops to a much lower level (Fig. 1F, TS, black band). Murine *Meox1* expression at neurulation stages (E8.0-9.5) is described in intermediate and lateral plate mesoderm (Candia *et al.,* 1992). In chick, in contrast, *Meox1* was not detected in intermediate and lateral plate mesoderm (Figs. 1H and 1I).

As previously described by Rallis and colleagues (Rallis *et al.,* 2001), the onset of *Meox2* expression (Fig. 1J) is at stage 11 in all cervical somites, far later than that of *Meox1.* This is in contrast with murine *Meox2* which is expressed from E8.0 onwards in all newly formed somites. At stage 16, unlike *Meox1, Meox2* expression is not detected in the pre-somitic mesoderm and is first seen in newly formed epithelial somites (compare Figs. 1G, red arrow with 1K, red arrow). Furthermore, *Meox2* expression is maintained at a high level in the somites unlike *Meox1* which decreases in mature somites. A transverse, vibratome section through the caudal somites of the stage 16 embryo in figure 1G (plane of section shown by black line, H) shows that *Meox1* expression is in both the dermomyotome and sclerotome (dm and scl, red arrows, Fig. 1H). The dermomyotome gives rise to muscle and dermis and the sclerotome to cartilage and vertebrae. Figure 1I shows a more rostral, transverse section through the embryo in figure 1G (plane of section shown by black line I) and reveals that *Meox1* expression is restricted to the dermomyotome (red arrow). At E9.5 the murine *Meox1* signal is detected in the dermomyotome and the sclerotome (Candia *et al.,* 1992). Chick *Meox2* is detected in the whole somite as shown by a transverse section through a stage 16 chick embryo (Fig. 1L). There is also expression in the developing mid-gut (Fig. 1L, mg, black arrow).



Expression in stages 17-25 emphasises differences between Meox1 *and* Meox2

In chick, there are very few changes in the expression of *Meox1* from stage 16 to 20. At stage 17 *Meox1* begins expression in the lateral rectus muscle of the eye and at stage 20, (Fig. 2A), the muscle precursors of the first pharyngeal arch. Its somitic expression is restricted to the posterior halves, strong expression continues in the tailbud and in the lateral rectus muscle, clearly visible in a region lateral to the eye. (Fig. 2A). A close-up of the head of the stage 20 embryo in Fig. 2A shows *Meox1* expression in the lateral rectus muscle (Fig. 2J, red arrow) and the first pharyngeal arch (Fig. 2J, black arrow). A close-up of the somitic domain confirms that *Meox1* expression is restricted to the posterior halves of the somites (Fig. 2L, red arrow). At stage 22, chick *Meox1* expression is first detected in the fore limb buds (Fig. 2B, red arrow) and continues in the eye region (Fig. 2K) At stage 25, expression continues in the eye domain, somites, tail bud and the limb buds signal is stronger (Fig. 2C, red arrows). Extended

Fig. 1. Expression patterns of *Meox1* (A-I) and *Meox2* (J-L) in stage 6-16 chick embryos. (A) *Expression of* Meox1 *begins at stage 6 in the pre-somitic mesoderm. Expression continues in this region through stages 7 and 8* **(B-C)** *until stage 10 when it becomes localised in the developing somites* **(D)**. **(E)** *A transverse vibratome section through a stage 7 embryo confirms that* Meox1 *expression is in the pre-somitic mesoderm (psm, red arrows).* **(F)** *At stage 14,* Meox1 *expression continues in the somites with strong expression in the most caudal somites and the as yet undifferentiated pre-somitic mesoderm (black arrowhead). The cervical somites exhibit strong* Meox1 *expression, (CS) and the thoracic somites (TS) initially have strong expression, decreasing as the somites mature.* **(G)** *At stage 16,* Meox1 *is still expressed in the somites and strong expression continues in the tail bud (red arrow).* **(H)** *Sections through the caudal somites of the embryo in (G) (marked by black line - H), show* Meox1 *expression throughout the somite in the dermomyotome (dm, red arrow) and the sclerotome (scl, red arrow).* **(I)** *Rostral transverse sections through the stage 16 embryo in G (black line – I), reveal that* Meox1 *expression in the rostral somites is restricted to the dermomyotome (red arrow).* **(J)** Meox2 *expression begins at stage 11 in the developing somites.* **(K)** *At stage 16,* Meox2 *expression continues in the somites but not in the pre-somitic mesoderm [compare (G) and (K) red arrows].* **(L)** *A transverse section through the somites of the embryo in (K) shows* Meox2 *expression in the dermomyotome and the sclerotome and the developing mid-gut (mg, black arrow). Abbreviations: cs, cervical somites; dm, dermomyotome; mg, mid-gut; nt, neural tube; psm, pre-somitic mesoderm; scl, sclerotome; st, stage; ts, thoracic somites.*

staining revealed additional sites of expression (Fig. 2D) namely the oesophagus (o), truncus arteriosus of the heart (ta) and the dorsal somites (black arrow). As in chick, the mouse *Meox1* signal at E11.5 is also detected lateral to the eye (Fig. 2G, upper red arrow). At E11.5 murine *Meox1* expression is similar to chick expression in the somites with stronger expression in the caudal somites and expression begins in the limb bud (Fig. 2G) and the pharyngeal arches (Fig. 2G, lower red arrow). At later stages, murine *Meox1* expression has also been reported to be localised to regions of the developing heart (truncus arteriosus) (Candia *et al.,* 1992).

From stages 16 to 20, the chick *Meox2* signal, like *Meox1*, does not dramatically change. At stage 20 *Meox2* expression is found in the posterior halves of the somites, similar to *Meox1* (Fig. 2E). In limb buds, *Meox2* expression is first observed in stage 21 chick embryos (this study and Rallis *et al.,* 2001). A lateral view of a stage 25 chick embryo shows strong *Meox2* expression in the developing limb buds(Fig. 2F, red arrows) and expression continues in the somites (Fig. 2F). A new domain of expression begins at this stage, the 2nd pharyngeal arch (Fig. 2F, white arrow). In chick, unlike *Meox1,* there is no *Meox2* expression detected in the heart. Stage 25 chick embryos were hybridised with *Pax3* for comparison with *Meox1* and *Meox2* expression in the somites. Figure 2H shows *Pax3* expression in the limbs at stage 25 and is expressed in the epithelial dermomyotome with strong expression in the ventral domain of the somites. *Scleraxis* expression at stage 25 in the chick is in the limb buds and the intersomitic mesenchyme (Fig. 2I).

Expression in stage 22 and 25 hind limb buds

Avian *Pax3* (Goulding *et al.,* 1994); *Scleraxis* (Schweitzer *et al.,* 2001); *MyoD* and *Myf5* (Pownall and Emerson, 1992) expression patterns have been previously described. Here they are used for comparison of *Meox1* and *Meox2* expression in hind limbs of stage 22 and stage 25 chick embryos. Figures 3A-F upper panel, shows the hind limbs of stage 22 embryos where the myogenic pathway has been activated and prolif-

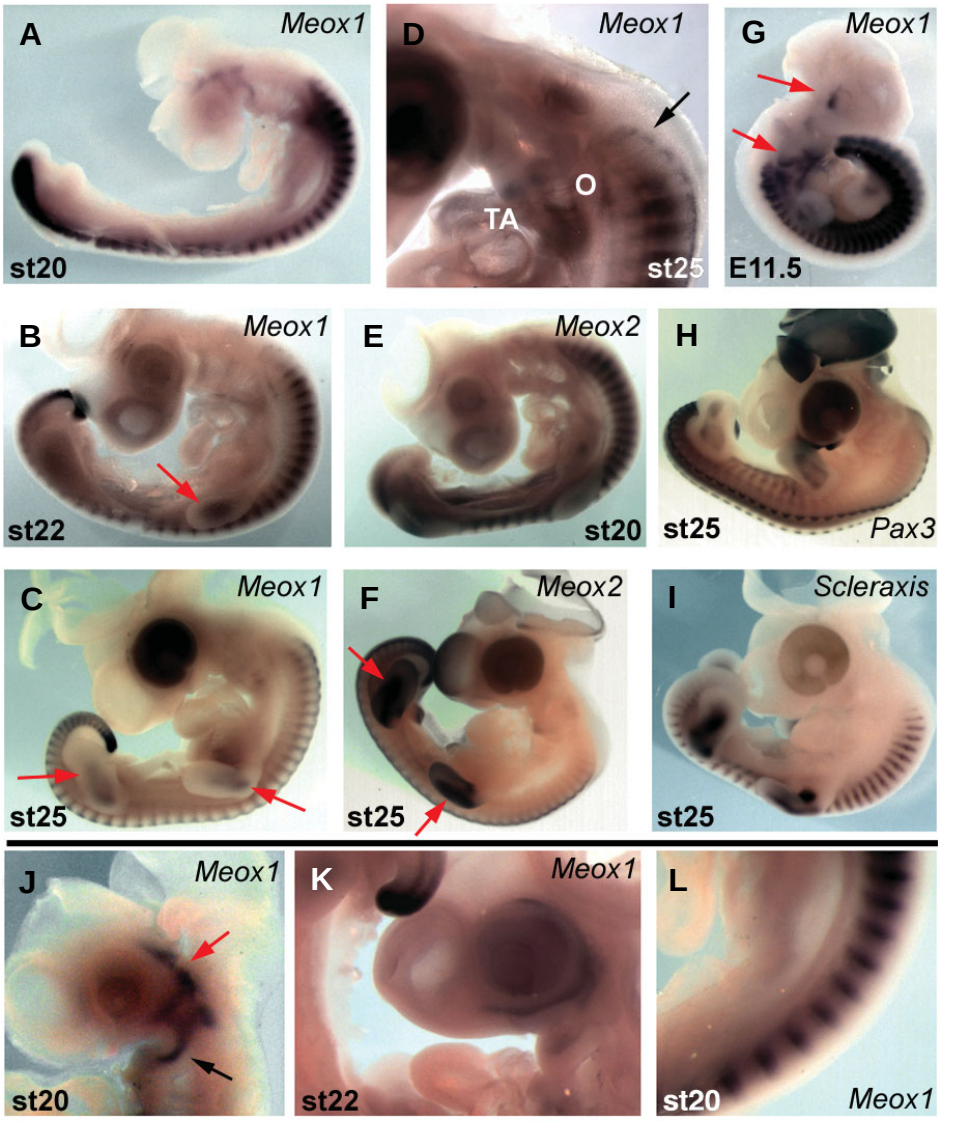


Fig. 2. Expression patterns of *Meox1* (A-D, J-L), *Meox2* (E-F), *Pax3* (H) and *Scleraxis* (I) in stage 20-25 chick embryos and *Meox1* in an E11.5 mouse embryo (G). (A) *A lateral view of* Meox1 *in a stage 20 embryo showing expression in the tail bud, posterior halves of somites, the lateral rectus muscle and the muscle of the first pharyngeal arch.* **(B)** *At stage 22 the same expression domains are present as in* **(A)** *and expression now begins in the limb buds, red arrow.* **(C)** *At stage 25, expression in the wing and leg buds is more pronounced, red arrows.* **(D)** *A close-up of a stage 25 embryo reveals* Meox1 *expression in the dorsal region of the most anterior somites (black arrow), the 4th and 5th pharyngeal arches, the truncus arteriosus of the heart (ta) and oesophagus (o). [N.B. the staining around the otic vesicle in* **(D)** *is non-specific].* **(E)** *At stage 20,* Meox2 *expression is in the posterior halves of the somites.* **(F)** Meox2 *is strongly expressed in the limb buds by stage 25 (red arrows) and the 2nd pharyngeal arch (white arrow).* **(G)** Meox1 *expression in an E11.5 mouse embryo shows expression in the somites, the pharyngeal arches (lower red arrow) and a region lateral to the eye (upper red arrow).* **(H)** Pax3 *expression is detected in the limbs at stage 25 and in the epithelial dermomyotome of the somites, with stronger expression in the ventral domain.* **(I)** Scleraxis *expression at stage 25 is in the intersomitic mesenchyme and limb buds.* **(J)** *A close-up of the embryo in (A), showing* Meox1 *expression in the lateral rectus muscle of the eye, red arrow and the first pharyngeal arch (black arrow).* **(K)** *A close-up of a stage 22 embryo where the eye domain expression continues.* **(L)** *A stage 20 embryo showing* Meox1 *expression in the posterior halves of the somites. Abbreviations: o, oesophagus; ta, truncus arteriosus.*

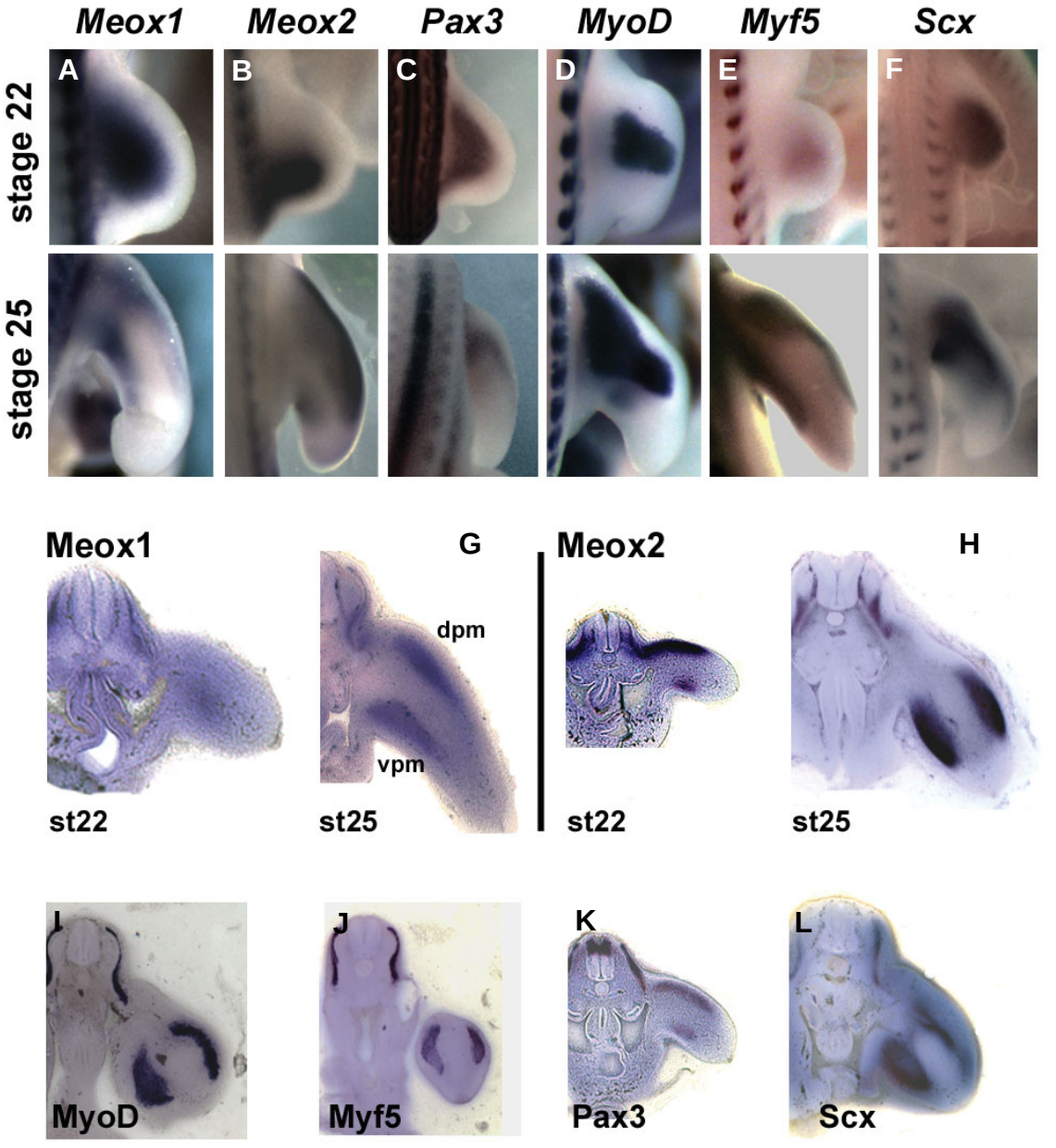


Fig. 3. *Meox1* and *Meox2* expression in stage 22 and 25 chick hind limb buds compared with *Pax3, MyoD, Myf5* and Scleraxis at equivalent stages. (A-F) *Dorsal views of hind limb buds of stage 22 (upper panel) and stage 25 (lower panel) chick embryos stained for expression of* Meox1 **(A)**, Meox2 **(B)**, Pax3 **(C)**, MyoD **(D)**, Myf5 **(E)** *and* Scleraxis (Scx), **(F)**. **(G)** *Transverse sections through the stage 22 hind limb in (A) (upper panel) shows* Meox1 *expression in the dermomyotome and ventral to the central core of the limb and at stage 25 embryo* Meox1 *expression continues in the dermomyotome and in the same region as the dorsal pre-muscle mass (dpm) and the ventral pre-muscle mass (vpm).* **(H)** *Transverse section of a stage 22 chick embryo stained for the expression of* Meox2. *Note the* Meox2- *positive muscle precursor cells migrating from the dermomyotome into the limb bud. At stage 25* Meox2 *expression is located in a region of the limb corresponding to the dorsal and ventral pre-muscle masses. The expression of the* Meox1 *(G) and* Meox2 *(H) genes at stage 25 is similar to other myogenic markers at this stage such as* MyoD **(I)**, Myf5 **(J)** *and* Pax3 **(K)**. **(L)** *This is in contrast to the expression of* Scleraxis *at stage 25 in the somites -* Scleraxis *is expressed in the syndetome - and the limb buds. Abbreviations: dpm, dorsal pre-muscle mass; vpm, ventral pre-muscle mass.*

erating cells expressing myogenic markers *Pax3* (Fig. 3C) *MyoD* (Fig. 3D), *Myf5* (Fig. 3E) and the tendon marker, *Scleraxis* (Scx, fig. 3F) can be seen. *Meox1* and *Meox2* positive cells are also detected in stage 22 limb buds (Figs 3A and 3B respectively).

In figures 3A-B, lower panel, *Meox1* and *Meox2* transcripts are detected in stage 25 chick hind limbs and at this stage *Pax3, MyoD, Myf5* and *Scleraxis* are also detected in hind limbs (Figs. C-F, lower panel).

Transverse sections were cut from stage 22 and stage 25 hind limbs to determine where *Meox1* and *Meox2* are expressed. *Meox1* is expressed in the dermomyotome at stage 22 and transcripts can be detected in the proximal domain, ventral to the

central core of the hind limb but sectioning at the limb level did not show evidence of staining in limb muscle progenitors migrating from the dermomyotome (Fig. 3G, left side). At stage 25, expression is still detected in the dermomyotome and there is now strong expression in the dorsal and ventral limb mesoderm, apparently overlapping with the dorsal and ventral pre-muscle masses (dpm, vpm, Fig. 3G, right side). At stage 22, *Meox2* positive myoblasts are migrating from the dermomyotome into the dorsal and ventral hind limb (Fig. 3H, left side). At stage 25, *Meox2* transcripts are observed in the dermomyotome and in the same region as the dorsal and ventral pre-muscle masses (Fig. 3H, right side). Transverse sections of stage 25 hind limbs were *in situ* hybridised with myogenic markers *MyoD* (Fig. 3I), *Myf5* (Fig. 3J) and *Pax3* (Fig. 3K). These show that *Meox1* and *Meox2* transcripts appear to localise to the same domains as these myogenic marker genes, namely the dermomyotome and the dorsal and ventral pre-muscle masses. The domains of *Scleraxis* expression are slightly different at stage 25 (Fig. 3L). In the somite it is located to the syndetome and the limb, in cells beginning to coalesce into tendon precursors.

In the developing mouse embryo limb buds at E9.75 and E10.75, *Meox2* is expressed in the somite derived myoblasts that coalesce to form the dorsal and ventral pre-muscle masses; *Meox1* mRNA was not detected in the limb bud at these stages (Mankoo *et al.,* 1999). We detected low levels of the *Meox1* signal at E11.5 in the limb buds in the dorsal and ventral mesenchyme (data not shown).

To determine the identity of the cells in the dorsal and ventral pre-muscle mass regions that express *Meox1* and *Meox2*, two-colour fluorescence labelled *in situ* hybridisation was performed on transverse cryosections of stage 25 chick limbs. Figure 4 A-O shows confocal images at high magnification (20X) in the same region as the dorsal pre-muscle mass (see Fig. 3G, dpm). The domain of *Meox1* (green) expressing cells slightly overlaps with but shows no co-expression with the *Pax3* (red) positive myogenic precursor cells (Figs. 4A-C). Double labelling for *Meox1* (green) and the committed myogenic precursor marker gene *MyoD* (red) show expression in the same domain but not in the same cells (Figs. 4D-F). *Meox2* (green) and *Pax3* (red) expression overlap with a proportion of cells co-expressing both genes (Figs. 4G-I). *Meox2* (green) and *MyoD* (red) have adjacent, non-overlapping expression domains in the distal limb with *Meox2* in the peripheral limb mesoderm (Figs. 4J-L) whereas in the proximal limb domain, the two expression domains overlap but there is no co-expression (see Fig. 4L, inset). *Meox2* (Fig. 4M, green) and *Meox1* (Fig. 4N, red) have abutting domains of expression with regions of overlap but *Meox2* is expressed in a more peripheral, broader domain in the dorsal and ventral limb mesenchyme than *Meox1* and there is a narrow region of overlap where both genes are co-expressed (Fig. 4O, white line).

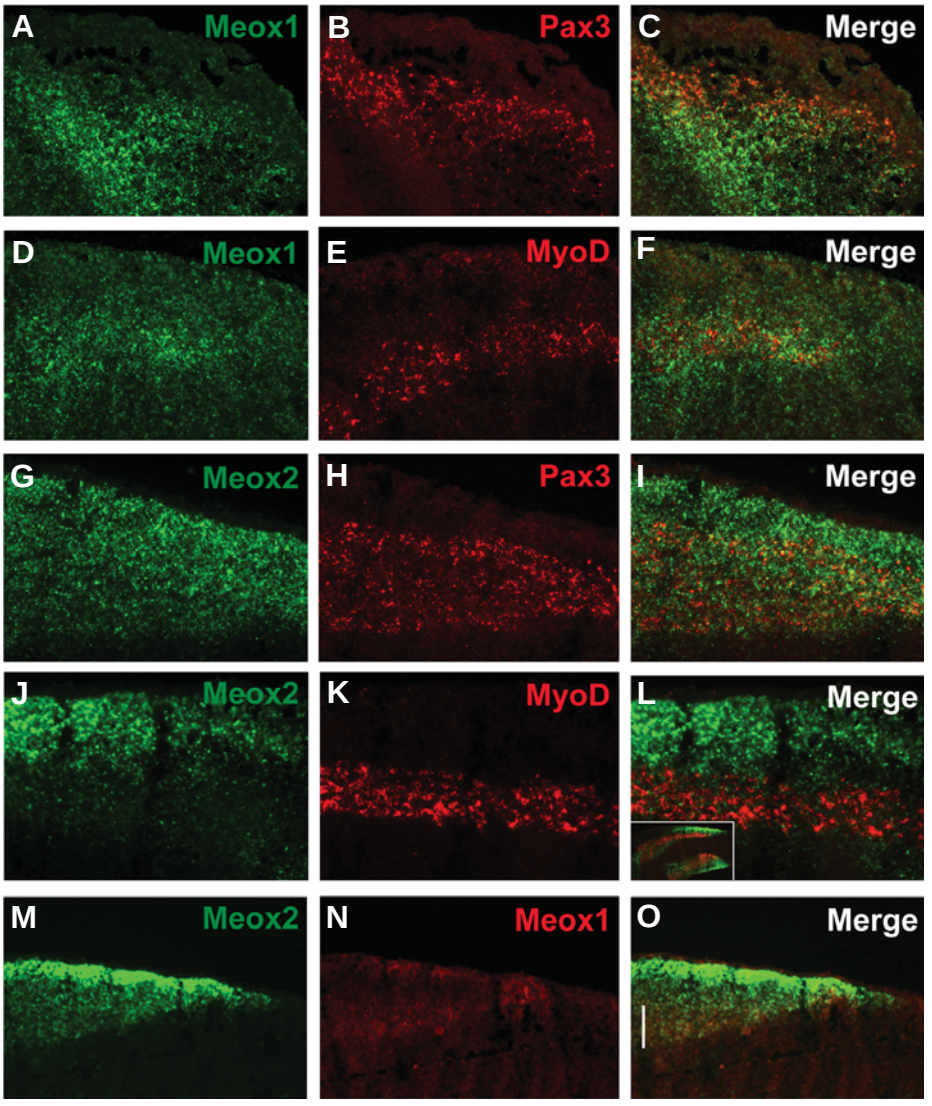


Fig. 4. Two-colour fluorescent *in situ* hybridisations with *Meox1* and *Pax3* (A-C), *Meox1* and *MyoD* (D-F), *Meox2* and *Pax3* (G-I), *Meox2* and *MyoD* (J-L) and *Meox2* and *Meox1* (M-O) on transverse sections of stage 25 chick hind limbs. *Expression domains of* Meox1 *(green)* **(A)** *and* Pax3 *(red)* **(B)** *slightly overlap but are not co-expressed* **(C)**. Meox1 *(green)* **(D)** *and* MyoD *(red)* **(E)** *positive cells overlap but there is no co-expression* **(F)**. Meox2 *(green)* **(G)** *and* Pax3 *(red)* **(H)** *overlap, a few cells show co-expression* **(I)**. Meox2 *(green)* **(J)** *and* MyoD *(red)* **(K)** *are not expressed in the same cells* **(L)**. Meox2 *(green)* **(M)** *and* Meox1 *(red)* **(N)** *exhibit a narrow band of co-expression (depicted by a white line in* **(O)***), suggesting separate roles for these two genes during chick limb development.*

Table 1 compares the similarities and differences between the expression domains of *Meox1* and *Meox2* in chick and mouse at equivalent developmental stages.

Summarising, chick *Meox1* and *Meox2* have similar but distinct spatial-temporal expression patterns during early devel-

TABLE 1

COMPARISON OF *MEOX1* AND *MEOX2* EXPRESSION PATTERNS IN CHICK AND MOUSE DURING EARLY EMBRYOGENESIS

	CHICK	MOUSE
	HH st. 6 (Headfold - pre-somitic)	**E7-7.25 (Headfold – pre-somitic)**
Meox1	Pre-somitic mesoderm	Pre-somitic mesoderm
Meox2	No expression detected at this stage	No expression detected at this stage
	HH st. 7-10 (1-10 somites)	**E7.5-8.5 (5-13 somites)**
Meox1	Developing somites, anterior pre-somitic mesoderm	Somitic, intermediate and lateral plate mesoderm, developing somites, anterior pre-somitic mesoderm
Meox2	No expression detected at this stage	Expression begins at E8.0 in the entire epithelial somite
	HH st. 11-16 (13-28 somites)	**E9-10.25 (14-30 somites)**
Meox1	Anterior pre-somitic mesoderm, dermomyotome in rostral somites, dermomyotome and sclerotome in caudal somites	Somitic, intermediate and lateral plate mesoderm, dermomyotome and sclerotome of somites
Meox2	Expression begins at stage 11 in all cervical somites, at stage 16 in dermomyotome and sclerotome, developing mid-gut	Expression is restricted to sclerotome of somites
	HH st. 17-22 (29-43 somites)	**E10.5-11.5 (35-45 somites)**
Meox1	Lateral rectus muscle, posterior halves of somites, limb buds, 1st pharyngeal arch	Eye muscle, limb buds, somites, pharyngeal arches, developing kidneys
Meox2	Posterior halves of somites, limb buds	Limb buds, somites, pharyngeal arches and derivatives
	HH st. 22-25	**E12.5**
Meox1	Eye domain, somites and dorsal somites, tail bud, limb buds, oesophagus, truncus arteriosus of heart	Eye domain, truncus arteriosus of heart, developing kidneys, pharyngeal arches and derivatives
Meox2	Limb buds, somites, 2nd pharyngeal arch	Limb buds, somites, pharyngeal arches and derivatives

opment and somitic differentiation. *Meox1* is expressed earlier and initially both are expressed over the whole epithelial somite. *Meox1* has strong expression in the pre-somitic mesoderm whereas *Meox2* transcripts are absent in this domain. In the newly formed somites, *Meox1* transcripts are detected throughout the somite in the sclerotome and dermomyotome. When the somites differentiate, *Meox1* expression is restricted to the dermomyotome. *Meox2* is expressed throughout the whole somite and its expression is maintained along the axis whereas *Meox1* expression is strongest in most caudal somites and is much reduced more anteriorly. Both genes appear conserved between chick and mouse. In the chick limb, two-colour fluorescent *in situ* hybridisation revealed that neither *Meox1* nor *Meox2* transcripts had overlap with *MyoD* expressing cells. This technique also revealed that chick *Meox1* and *Meox2,* although both being expressed in the same regions as the dorsal and ventral pre-muscle masses, show only a small domain of co-expression.

Meox1 and *Meox2* have overlapping domains of expression which suggest overlapping functions. However, within developing somites and the limb bud the non-overlap of expression indicates these genes have distinct and unique functions.

Experimental Procedures

Fertilized chicken eggs were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Full-length chick *Meox2* cDNA was cloned in pBluescript KS (+/-) (Stragene) and to generate digoxigenin (DIG) labelled (Roche) anti-sense probe, cut with SacI and transcribed with T3 RNA polymerase (Roche). *Scleraxis* anti-sense probe was generated from ChEST973H2 cDNA from the BBSRC Chicken EST Project, cloned into pBluescript KS II, cut with Not1 and transcribed with T3. *Meox1* antisense-probe was generated from ChEST805F3, cloned into pBluescript KS II, cut with NotI and transcribed with T3. *MyoD, Pax3* and *Myf5* anti-sense probes were a kind gift of Philippa Francis-West. Full-length mouse *Meox1* cDNA was cloned into pBluescript KS II, cut with PstI and transcribed with T3. Whole-mount *in situ* hybridisation was carried out using standard procedures.

Two-colour fluorescence *in situ* hybridisation was performed on fresh frozen sections as described by Tylzanowski *et al.* (2003). FITC-labelled probes were used instead of biotin-labelled probes and detected by anti-FITC-POD (Roche 1426346). Proteinase K digestion at 1μg/ml for 5-7 mins was used for tissue permeabilisation.

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