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HOXA5 protein expression and genetic fate mapping show lineage restriction in the developing musculoskeletal system

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ABSTRACT HOX proteins act during development to regulate musculoskeletal morphology. HOXA5 patterns skeletal structures surrounding the cervical-thoracic transition including the vertebrae, ribs, sternum and forelimb girdle. However, the tissue types in which it acts to pattern the skeleton, and the ultimate fates of embryonic cells that activate *Hoxa5* expression are unknown. A detailed characterization of HOXA5 expression by immunofluorescence was combined with Cre/LoxP genetic lineage tracing to map the fate of *Hoxa5* expressing cells in axial musculoskeletal tissues and in their precursors, the somites and lateral plate mesoderm. HOXA5 protein expression is dynamic and spatially restricted in derivatives of both the lateral plate mesoderm and somites, including a subset of the lateral sclerotome, suggesting a local role in regulating early skeletal patterning. HOXA5 expression persists from somite stages through late development in differentiating skeletal and connective tissues, pointing to a continuous and direct role in skeletal patterning. In contrast, HOXA5 expression is excluded from the skeletal muscle and muscle satellite cell lineages. Furthermore, the descendants of *Hoxa5*-expressing cells, even after HOXA5 expression has extinguished, never contribute to these lineages. Together, these findings suggest cell autonomous roles for HOXA5 in skeletal development, as well as non-cell autonomous functions in muscle through expression in surrounding connective tissues. They also support the notion that different *Hox* genes display diverse tissue specificities and locations to achieve their patterning activity.

KEY WORDS: *musculoskeletal development, somite, sclerotome, dermomyotome, lateral plate mesoderm, genetic lineage tracing*

Introduction

Formation of the musculoskeletal system, including cartilage, bones, muscles, tendons and ligaments, must be highly coordinated, allowing functional integration of the resulting structures. Failures of musculoskeletal development result in birth defects and post-natal musculoskeletal diseases. Each musculoskeletal element has a particular morphology associated with its position in the body. The HOX family of transcription factors are central regulators of positional identity. The role of the Hox code in vertebrates was first defined through loss-of-function phenotypic analysis, mainly in the skeleton (Kmita and Duboule, 2003, Mallo *et al.*, 2010). Loss-of-function mutations lead to patterning defects including homeotic transformations, in which structures develop with morphologies inconsistent with their position. However, the mechanisms of HOX-mediated patterning, including the tissue types in which they act and the cellular processes that they regulate, are the subjects of ongoing investigation.

Accumulating evidence shows that HOX proteins function at many stages to pattern the musculoskeleton, and roles from gastrulation through differentiation are likely to be important for this process. Some HOX proteins act very early, during gastrulation and body axis elongation (Casaca *et al.*, 2016, Denans *et al.*,

Abbreviations used in this paper: BAT, brown adipose tissue; DIC, differential interference contrast; DML, dorso-medial lip of the myotome; DV, dorso-ventral; LPM, lateral plate mesoderm; MCT, muscle connective tissue; VLL, ventro-lateral lip of the myotome.

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2015, Iimura and Pourquie, 2006, Wong *et al.*, 2015, Young *et al.*, 2009), and to specify segmental identities in pre-somitic mesoderm (Carapuco *et al.*, 2005, Nowicki and Burke, 2000, Nowicki *et al.*, 2003). HOX proteins control differentiation and morphogenesis of numerous tissues pre- and postnatally (Rezsohazy *et al.*, 2015). During skeletal development, HOX proteins are now understood to affect diverse processes with impacts on skeletal morphology. These include cell recruitment to pre-cartilagenous condensations (Aubin *et al.*, 2002b, Goff and Tabin, 1997, Rijli *et al.*, 1993), modification of cell proliferation rates (Goff and Tabin, 1997, Yueh *et al.*, 1998), and regulation of cartilage differentiation and ossification (Boulet and Capecchi, 2004, Dolle *et al.*, 1993, Goff and Tabin, 1997, Kanzler *et al.*, 1998, Massip *et al.*, 2007, Yokouchi *et al.*, 1995, Yueh *et al.*, 1998). *Hox* expression also affects adhesion properties of condensing mesenchyme (Stadler *et al.*, 2001, Yokouchi *et al.*, 1995), which can influence size and shape of precartilagenous condensations. HOX proteins can regulate perichondrium patterning and formation of cortical vs. trabecular bone (Villavicencio-Lorini *et al.*, 2010). Finally, HOX proteins govern growth plate proliferation and maintenance, affecting both fetal and postnatal bone development (Kjosness *et al.*, 2014, Pineault *et al.*, 2015).

Recent evidence also indicates that HOX proteins vary in the tissue-types within which they act to pattern the skeleton. For example, HOXB6 activity in muscle precursors is sufficient to direct rib formation, showing that its role in skeletal patterning is not cell autonomous(Vinagre *et al.*, 2010). Similarly, *Hoxa11* regulates limb skeletal pattern but its expression is limited to tendon, muscle connective tissue (MCT), and to outer perichondrium, which mediates attachment of tendon and ligament to bone. *Hoxa11* is not expressed in muscle, chondrocytes or in the inner perichondrium, the latter being the source of bone osteoblasts (Swinehart *et al.*, 2013). For most *Hox* genes, however, the tissue types in which they act to perform their patterning functions remain unknown.

In mice, *Hoxa5* non-redundantly patterns musculoskeletal elements spanning the cervical-thoracic transition. The null skeletal phenotype includes homeotic transformations from the third cervical to the second thoracic vertebrae (C3-T2), rib malformations and fusions, and defects in sternal morphology and sternebra number (Jeannotte *et al.*, 1993), (Aubin *et al.*, 2002b, Aubin *et al.*, 1998, Jeannotte *et al.*, 2016, Tabaries *et al.*, 2007). Pectoral girdle defects include loss of the acromion of the scapula, or its replacement by a ligament (Aubin *et al.*, 2002b).

Within somites, which give rise to much of the axial skeleton, *Hoxa5* mRNA is expressed in the lateral part of the sclerotome, which is the mesenchymal, cartilage-forming compartment (Chen *et al.*, 2013, Mansfield and Abzhanov, 2010). This restricted expression is important for function because *Hoxa5* misexpression throughout the somite leads to cartilage defects (Chen *et al.*, 2013). *Hoxa5* negatively regulates the chondrocyte specification gene *Sox9* in somites and acromion (Aubin *et al.*, 2002b, Chen *et al.*, 2013). It also positively regulates the formation of *Pax1*-positive acromion condensations, indicating at least two functions in acromion skeletogenesis (Aubin *et al.*, 2002b). However, it is unknown whether either gene is a direct transcriptional target of the HOXA5 protein. In developing organs including lung, thyroid, gut, mammary gland and ovary, *Hoxa5* expression is also restricted to mesenchyme, where it regulates interactions between mesenchyme and epithelia (Jeannotte *et al.*, 2016). Although the phenotypic consequences of *Hoxa5* disruption have been well characterized, little is known about the mechanisms through which HOXA5 directs musculoskeletal patterning: the cell types in which it is required, the cellular behaviors it regulates, and ultimately its transcriptional targets.

Here, we applied a combination of protein expression analyses by immunostaining and genetic lineage tracing with Cre/loxP. We thus identified musculoskeletal lineages with a history of *Hoxa5* expression, as a means to spatially and temporally localize its action. We found that HOXA5 protein shows spatial localization to a subset of somitic cells within segments, similar to the RNA profile, and tissue specificity in later stages. Further, expression persists in axial skeletal elements and muscle connective tissue throughout differentiation, suggesting a direct role for HOXA5 from somite patterning to differentiation of somite derivatives. Further, we found that cells with a history of expressing *Hoxa5* show tissue-type restriction: they contribute abundantly to skeletal tissues (chondrocytes, osteoblasts and perichondrium), tendon and ligament, muscle connective tissue, and dermal and brown adipose tissues. However, *Hoxa5* is never expressed in muscle or muscle satellite cells. Further, no cell with a history of *Hoxa5* expression contributes to either muscle or muscle satellite lineages.

Results

HOXA5 protein expression in musculoskeletal structures and in their precursors in somites and lateral plate mesoderm

To better understand the role of HOXA5 protein in directing musculoskeletal development, we undertook a detailed characterization of its expression in musculoskeletal structures and in their precursors: the somites and the lateral plate mesoderm (LPM). We focused on segments spanning C6-T1, which are located within the antero-posterior (AP) expression domain of HOXA5. As a positive control, we confirmed expression in regions where it was previously described. HOXA5 was detected in the phrenic motor column (PMC) of the neural tube from embryonic day (E) 9.5 to E11.5 (Fig. 1 A-C;(Dasen *et al.*, 2005, Joksimovic *et al.*, 2005)). HOXA5 protein was also present in the foregut at E9.5-10.5 (Fig. 1 A-B; (Aubin *et al.*, 2002a)) and in tracheal mesenchyme at E12.5 (Fig. 2B;(Landry-Truchon *et al.*, 2017)). Specificity of the antibody was further demonstrated by absence of signal in *Hoxa5*$^{-/-}$ embryos (Supp. Fig. 1).

At E9.5, HOXA5 protein expression was not yet detected in the dermomyotome or sclerotome of the somites, but it was present in LPM (Fig. 1A), which will give rise to the forelimb and body wall. At E10.5, HOXA5 protein was abundant in both somites and LPM, with spatial restriction within segments similar to that previously described for *Hoxa5* mRNA (Mansfield and Abzhanov, 2010): HOXA5 accumulated in the lateral and dorsal regions of sclerotome but was largely absent from the central sclerotome (Fig. 1 B-C). It was also observed in few cells of the dermomyotome (Fig. 1 B-C). Outside of the forelimb region, HOXA5 expression was found in the LPM along the body wall similar to E9.5 (not shown), and in the proximal forelimb bud (Fig. 1B). In the limb, HOXA5 showed a graded pattern from proximal to distal (Fig. 1B). By E11.5, sclerotome expression was further spatially restricted to a subset of cells when compared to E10.5. HOXA5 was detected in the dorsal and lateral-most sclerotome only, and was most abundant in a cap of sclerotome cells surrounding the ventro-lateral lip of the myotome (VLL; Fig. 1 D-E). At E11.5, HOXA5 expression was also seen in the central dermomyotome (Fig. 1 D-E), and in its derivative, the

dorsal dermis (Fig. 1). Expression in LPM was reduced compared to earlier stages, but still apparent (Fig. 1D).

By E12.5, musculoskeletal tissues including cartilage, muscle, dermis and connective tissue, have been specified and are beginning to differentiate. HOXA5 expression persisted in a subset of these differentiating tissues. In the skeleton, it was present in vertebral bodies, neural arches, and T1-T2 rib condensations (Fig. 2 A-E). Counterstaining with TENASCIN, which transiently marks condensing cartilage mesenchyme and later becomes refined to the perichondrium, showed significant overlap with HOXA5 (Fig. 2 B-C). HOXA5 expression was observed in round, proliferating chondrocytes and in all layers of the perichondrium of all of skeletal structures (Fig. 2 F-I). Although HOXA5 expression was present in all somite-derived skeletal elements in the C3-T2 area, it was stronger in lateral skeletal structures (neural arches, ribs) compared with the central skeletal structures (vertebral bodies), which was most evident when observed in low-power view (Fig. 2 A-B).

HOXA5 protein was not detected in the sternal bands (Fig. 2 B-C); in contrast to the above skeletal structures, these are derived from LPM. The sternum is also developmentally delayed compared to the vertebrae and ribs: at E12.5, sternal bands have not yet fully fused at the midline and they strongly expressed TENASCIN in condensing mesenchyme.

HOXA5 expression was also examined in non-skeletal tissues. Expression was high in dorsal (primaxial) mesenchyme surrounding the axial skeleton (Fig. 2A). This region gives rise mainly to muscle and muscle connective tissue (MCT). A lateral border of HOXA5 expression appeared to track the somitic frontier, which separates primaxial, or entirely somite-derived tissues, from abaxial tissues, in which somite derivatives have migrated into LPM and include contributions from both mesoderm populations (Nowicki *et al.*, 2003). This is notable because it has implications for the location of HOXA5 patterning activity. We therefore co-labeled embryos using the Prx1-Cre deleter mouse line, which marks LPM and thus demarcates the primaxial and abaxial regions (Durland *et al.*, 2008). Fig. 2 J-K showed differentiating muscles and MCT on either side of the frontier; indeed, HOXA5 expressing cells were abundant in the primaxial region, and although there were HOXA5 low-expressing cells in abaxial tissues, there was a clear boundary in expression between the two regions. Fig. 2 H-I showed a T1 rib highly expressing HOXA5 in chondrocytes and growing into the ventral body wall composed of abaxial tissue.

Interestingly, despite abundant HOXA5 expression in these regions of muscle formation, HOXA5 expression was never observed in skeletal muscle cells themselves (Fig. 2L). Instead, HOXA5 expression was abundant in TCF4-positive MCT cells (Fig. 2M).

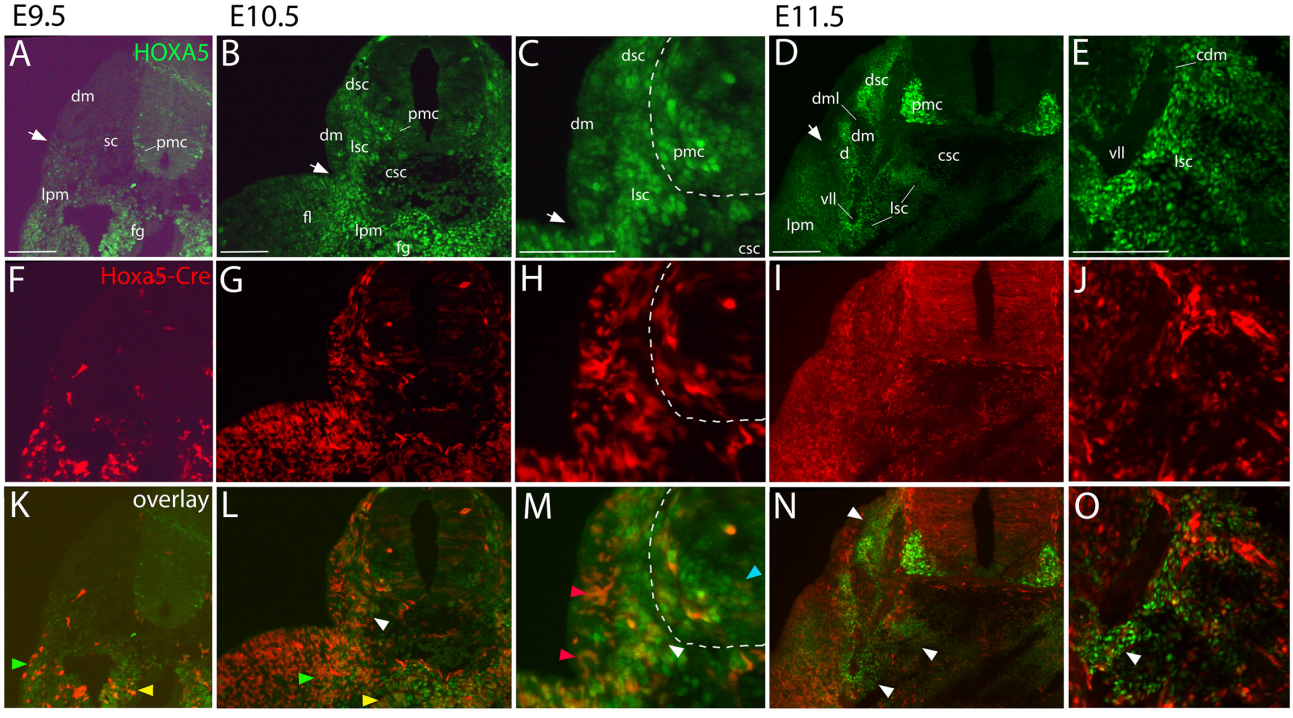

Fig. 1. HOXA5 protein expression in somites and lateral plate mesoderm (LPM), and comparison to Hoxa5-Cre reporter expression. *HOXA5 immunofluorescence is shown in green* **(A-E)**, *while Hoxa5-Cre reporter staining is in red* **(F-J)**. *Panels* **(K-O)** *show the overlay.* **(A-E)** *HOXA5 expression is dynamic and spatially restricted in the somites and LPM from E9.5 to E11.5.* **(F-K)** *Hoxa5-Cre reporter expression follows that of HOXA5 protein at E9.5 and E10.5. By E11.5, it is much broader than HOXA5 protein expression. Expression overlap between HOXA5 protein and Hoxa5-Cre reporter is indicated by the following arrows on panels* **(K-O)**: *LPM and forelimb bud (green arrowheads); foregut (yellow arrowheads); lateral and dorsal sclerotome (white arrowheads) including the cap of cells surrounding the VLL; dermomyotome (red arrowheads), phrenic motor column (blue arrowhead). The boundary between somites and LPM, the incipient somitic frontier, is marked by an arrow in (A-D). The neural tube is outlined by a dotted line in the section shown in (D,H,M). All sections are transverse, dorsal is up, and lateral is to the left. Abbreviations: cdm, central dermomyotome; csc, central sclerotome; d, dermis; dm, dermomyotome; dml; dorso-medial lip of the dermomyotome; dsc, dorsal sclerotome; fg, foregut; fl, forelimb; lpm, lateral plate mesoderm; lsc, lateral sclerotome; pmc, phrenic motor column; sc, sclerotome; vll, ventro-lateral lip of the dermomyotome. Hoxa5-Cre reporter line was either tdTOMATO (E9.5-E10.5) or nYFP (E11.5). Scale bars, 20 μm (A-O).*

Thus, the high expression described above in primaxial compared to abaxial muscles was in fact due to differential expression in the MCT of these two muscle types. With the exception of muscle, HOXA5 was expressed in other dermomyotome derivatives including dermal cells marked by Dermo1-Cre (Fig. 2N) and brown adipose tissue (BAT), which we first detected when BAT becomes morphologically defined at E13.5 (not shown).

HOXA5 expression persisted through late embryogenesis in skeletal structures, in connective tissues including TCF4-positive MCT, and in BAT. At E13.5, HOXA5 protein was abundant in the ribs and vertebral bodies of anterior thoracic segments. Within skeletal elements, expression was robust in the chondrocytes and perichondrium (Fig. 2 U-V), and in RUNX2-positive osteoblasts known to be derived from perichondrium (Fig. 2R). HOXA5 expression was also detected in sternum mesenchyme at this stage (Fig. 2O). At E14.5, a similar expression pattern was observed in

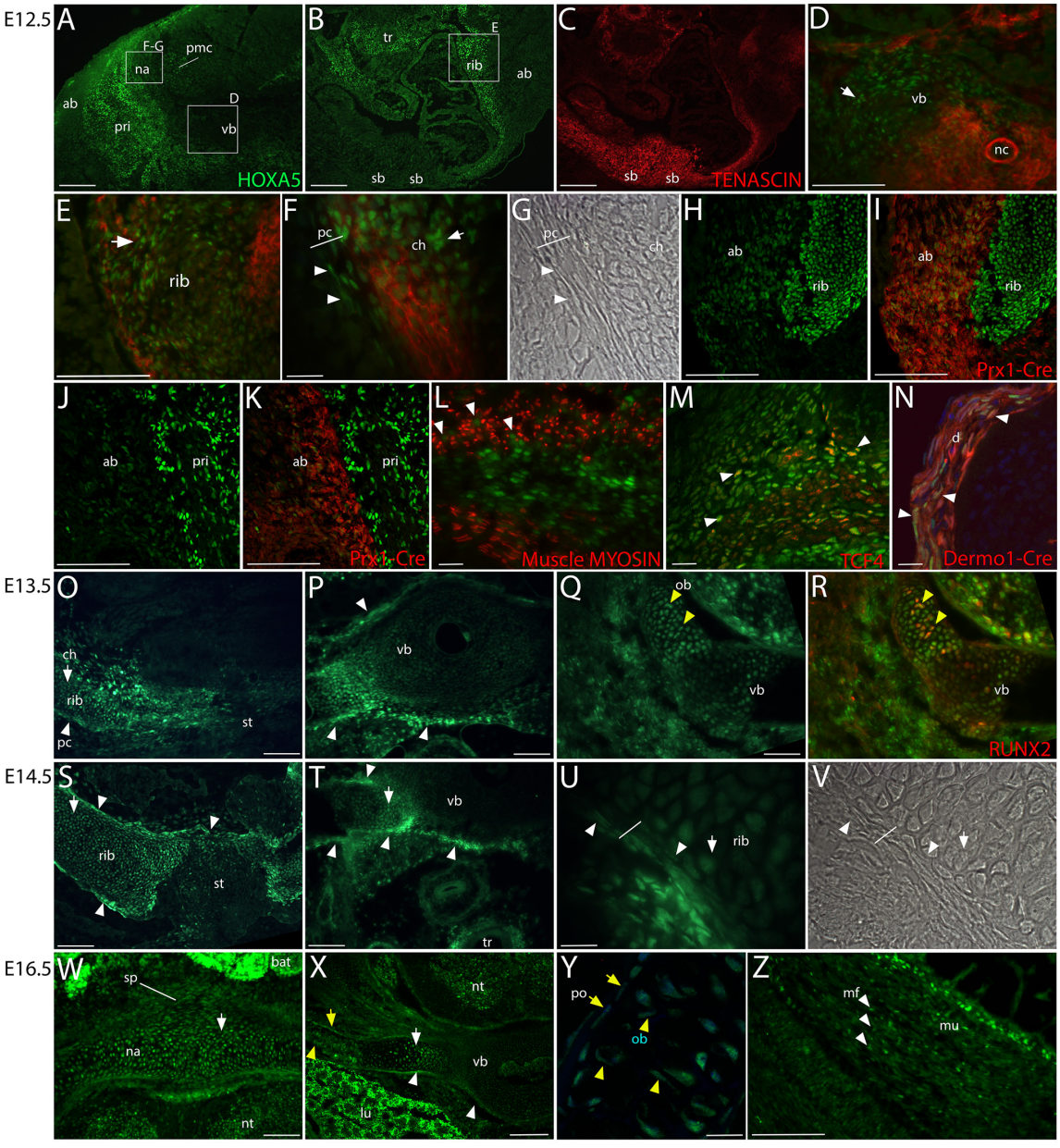

Fig. 2. HOXA5 protein expression in somite and lateral plate mesoderm (LPM) derivatives from E12.5 to E16.5. *HOXA5 immunofluorescence is shown in green and other markers including the Prx1-Cre reporter are in red.* **(A-B)** *Overview of the dorsal (A) and ventral (B) sides of an E12.5 embryo showing HOXA5 expression in somite-derived structures. Cartilage condensations in (B) are revealed by TENASCIN staining in* **(C)**. *Boxed regions are magnified in (D-G).* **(D-F)** *HOXA5 expression in TENASCIN-positive condensing cartilage is detected in proliferating chondrocytes (arrows), and in the perichondrium (arrowheads) of the vertebral body, rib and neural arch.* **(G)** *DIC optics confirm perichondrial cells by morphology.* **(H-K)** *Co-labeling with HOXA5 and tdTOMATO reporter in* $Tg^{Prx1Cre+}$; $Rosa26^{tdTomato+}$ *embryo (H-I) show HOXA5 expression in rib chondrocytes and abaxial muscle region into which the rib migrates. (J-K) show the somitic frontier between abaxial (Prx1-Cre-positive) and primaxial muscle (Prx1-Cre negative) regions.* **(L)** *Muscle MYOSIN staining reveals absence of HOXA5 expression in skeletal muscle cells. Instead, HOXA5-expressing cells are interspersed within the muscle (arrowheads).* **(M)** *HOXA5-expressing cells co-express TCF4, suggesting they are fated to become muscle connective tissue.* **(N)** *HOXA5 is expressed in dermis, labeled with Dermo1-Cre. At E13.5* **(O-R)**, *E14.5* **(S-V)**, *and E16.5* **(W-Y)**, *HOXA5 expression persists in cartilage elements including vertebral bodies, neural arches and ribs, and further is detected in the developing sternum by E13.5 and spinous process by E16.5. Expression in perichondrium is marked with white arrowheads, in chondrocytes with white arrows, in periosteium with yellow arrowheads, and in osteocytes with yellow arrows.* **(P)** *HOXA5 continues to be expressed in chondrocytes and perichondrium, and by E13.5 also becomes localized in the layer of cells surrounding the perichondrium (arrowheads).* **(Z)** *Expression of HOXA5 in muscle-embedded fibroblasts persists at E16.5 (arrowheads). All sections are transverse, dorsal is up. Cre reporters are tdTOMATO except for (H-K), which are nYFP. ab, abaxial; bat, brown adipose tissue; ch, chondrocytes; d, dermis; lu, lung; mf; muscle fibroblast; na, neural arch; nc, notochord; ob, osteoblast; pc, perichondrium; po, periosteium; pmc, phrenic motor column; pri, primaxial; sb, sternal band; st, sternum; tr, trachea; vb, vertebral body. Scale bars: 20 μm (A-E, W-X), 2 μm (F-G, L-N, U-V,Y), 10 μm (H-K), 50 μm (X).*

the vertebrae, ribs and sternum (Fig. 2 S-T). Although HOXA5 protein continued to be detected throughout skeletal elements, expression levels appeared to diminish in chondrocytes and inner perichondrium but remained high in mesenchymal cells surrounding the perichondrium (Fig. 2 U-V). Expression at E16.5 showed a spatial distribution like that at E14.5. HOXA5 immunostaining was also seen in the dorsal neural arch and spinous process (Fig. 2W), which form cartilagenous condensations between E14.5 and E16.5. While most axial skeletal structures are ossifying by E16.5, HOXA5 expression continued to be present in both osteoblasts and periosteium, the latter being the source of osteoblasts (Fig. 2 X,Y). Continuous HOXA5 was also observed in muscle-embedded fibroblasts (Fig. 2Z), in dermis (not shown) and in BAT (Fig. 2W).

Hoxa5 *lineage labeling recapitulated HOXA5 expression in early somites*

Because HOXA5 expression is dynamic, we could not determine the ultimate fate of HOXA5 expressing cells using immunostaining. We therefore used Cre/LoxP genetic lineage labeling to permanently mark all embryonic cells with a history of *Hoxa5* expression and thus track their fates. The previously described *Hoxa5-Cre* transgene includes the *Hoxa5* mesodermal enhancer that drives *Hoxa5* expression in somitic and lateral plate mesoderm, as well as in the gastrointestinal and respiratory tracts, and closely matches *Hoxa5* mRNA expression (Berube-Simard and Jeannotte, 2014, Larochelle *et al*., 1999). When used to conditionally delete *Hoxa5* gene function, it fully recapitulates the null skeletal phenotype, indicating that all aspects of HOXA5 expression involved in skeletal patterning are reproduced (Berube-Simard and Jeannotte, 2014).

We first confirmed that expression of the *Hoxa5-Cre* reporter gene closely matched endogenous HOXA5 expression in early-stage somites and LPM. RFP and HOXA5 proteins were visualized in 14.5kb *Hoxa5-Cre*$^{+}$*; Rosa26-tdtomato*$^{+}$ embryos. Cre activity was detectable at E9.5, in a pattern overlapping with HOXA5 (Fig. 1 A,F,K), which at this stage consisted mainly of foregut and LPM cells; somitic expression was not yet observed. By E10.5, when HOXA5 protein expression initiated and showed maximum extent of spatial expression in somites, the *Hoxa5-Cre*$^{+}$*; Rosa26-tdTomato*$^{+}$ specimens showed an overlapping pattern in the lateral and dorsal regions of the sclerotome and in dermomyotome, although with fewer cells labelled with the reporter compared to direct HOXA5 detection (Fig. 1 B-C, G-H, L-M). This might in part be explained by a temporal lag in reporter activation, However, it also appeared that recombination did not occur with 100% efficiency, such that only a subset of HOXA5 expressing cells activated the reporter. Importantly, the fact that reporter activity was confined to HOXA5 expressing cells indicated that it lacks ectopic activity. Reporter labeling also overlapped extensively with HOXA5 expressing cells in the foregut and proximal limb. However, Hoxa5-Cre/ROSA26-tdTomato labeling was abundant throughout the forelimb, and extended distal to the region of high HOXA5 protein expression (Fig. 1 B,G,L). This widespread labeling in the limb was consistent with two observations: first, at early stages HOXA5 was expressed in the forelimb field LPM, which would produce extensive labeling throughout forelimb mesenchyme. In addition, low levels of HOXA5 expression were observed distally in the limb buds (not shown), which may be sufficient to drive recombination there.

By E11.5, Hoxa5-Cre-labelled cells were more abundant and more broadly distributed than HOXA5 expressing cells (Fig. 1 D-E, I-J, N-O). This was consistent with the dynamic HOXA5 expression pattern, which was spatially constricted at E11.5 compared to E10.5, indicating that some cells extinguished HOXA5 expression between these stages. This was also coherent with the proliferation and migration of the descendants of HOXA5-expressing cells as somites mature.

Overall, labelling with the Hoxa5-Cre/Rosa26-tdTomato reporter system recapitulated the pattern of HOXA5 protein expression in mesoderm, with a near-complete overlap at E9.5-E10.5, although some spottiness in labeling indicated imperfect recombination efficiency. By E11.5, when the spatial domain of HOXA5 expression constricted, tdTomato labeling revealed that many *Hoxa5*-descendant cells no longer expressed HOXA5 protein.

Finally, although Hoxa5-Cre labelled neurons in the spinal cord (Fig. 1 F-I), the neuronal spatial pattern of HOXA5 expression was not fully recapitulated. The Cre transgene contains the *Hoxa5* brachial spinal cord (BSC) enhancer and was previously shown to reproduce the spatial pattern of *Hoxa5* mRNA in the CNS, including an anterior boundary in the caudal hindbrain, and transcript distribution along the DV extent of the spinal cord (Berube-Simard and Jeannotte, 2014, Gaunt *et al*., 1990, Joksimovic *et al*., 2005, Larochelle *et al*., 1999). In contrast to the mRNA, however, HOXA5 protein is only detected in the ventral spinal cord at E9.5-E12.5, where it accumulates in the PMC (Dasen *et al*., 2005, Joksimovic *et al*., 2005). From E13.5-E18.5, HOXA5 expression expands dorsally and is broadly expressed along the DV axis of the spinal cord (Joksimovic *et al*., 2005). Hoxa5-Cre-labelled neurons were found distributed along the extent of the DV axis in the cervical and brachial spinal cord from E10.5 onward. Further, the concentration of HOXA5 expressing cells within the PMC was not recapitulated by the reporter (compare PMC neurons labelled in Fig. 1 A-D to Fig. 1 F-I). This broader expression of Cre in the CNS may reflect a lack of endogenous translational control required to restrict HOXA5 expression to the motor neurons. Alternatively, it is possible that low levels of HOXA5, below the level of IHC detection, are in fact expressed in dorsal spinal cord earlier than E13.5.

Contribution of the **Hoxa5** *genetic lineage to somitic compartments and fates*

To assess the tissue-type contribution of *Hoxa5* descendant cells in the mesoderm, we compared *Hoxa5* descendants to markers of somite compartments. At E10.5, most of the sclerotome, which gives rise to axial cartilage and tendon, is marked by the early chondrocyte regulator SOX9. The *Hoxa5* lineage substantially overlapped with SOX9 (Fig. 3A), confirming *Hoxa5* descendant presence in the sclerotome. Dermomyotome gives rise to skeletal muscle, dorsal dermis, and, at the axial level of *Hoxa5* expression, also to brown adipose tissue. We examined expression of two dermomyotome markers, PAX7 and PAX3. At E10.5, many *Hoxa5* descendant cells in the dermomyotome expressed PAX7, but none were observed to express PAX3; the latter is a marker for the muscle lineage (Fig. 3 B-C). It was previously shown by inducible lineage tracing that PAX7-positive cells at E10.5 give rise to numerous dermomyotome derivatives, while by E11.5, PAX7-positive cells are primarily skeletal muscle precursors (Lepper and Fan, 2010). Interestingly, we found no *Hoxa5* descendants expressing PAX7 at E11.5 (Fig. 3D), despite the broad distribution of descendants at this stage. Also at E11.5, somitic *Hoxa5* descendants did not express PAX3 (not shown), or muscle actin (Fig. 3E). Moreover,

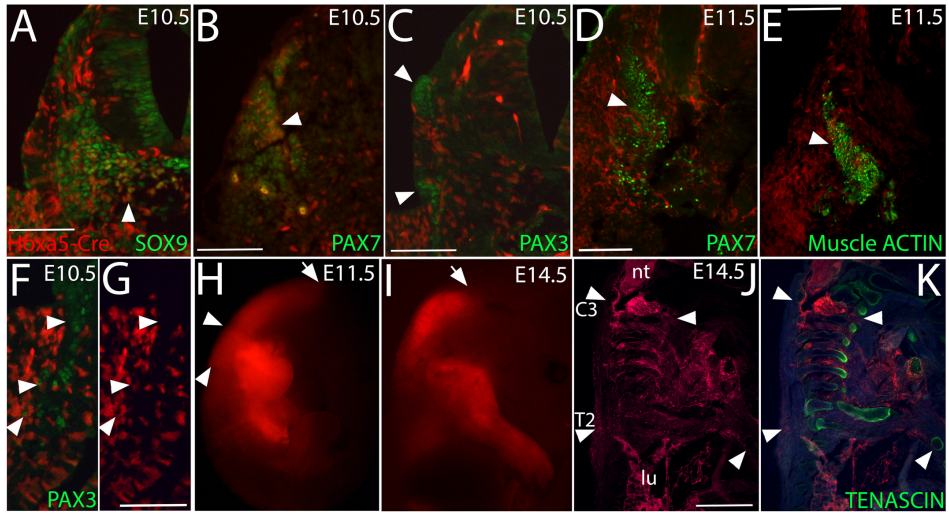

Fig. 3. Distribution of *Hoxa5* descendants in compartmentalized somites and differentiating tissues. Hoxa5 *descendants are labelled in red following Hoxa5-Cre activity and stained with cell markers by immunofluorescence. At E10.5,* Hoxa5 *descendants co-label with sclerotome marker SOX9* **(A)** *(arrowhead) and dermomyotome marker PAX7* **(B)** *(arrowhead) but not with PAX3, a marker of the muscle lineage. PAX3 muscle progenitors are detected in the dorso-medial and ventro-lateral myotome lips* **(C)** *(arrowheads).* **(D)** *At E11.5,* Hoxa5 *descendants no longer co-label with PAX7 (arrowhead), which at this stage marks the muscle lineage almost exclusively.* **(E)** Hoxa5 *descendants also fail to co-label with muscle ACTIN.* **(F-G)** *There is also no contribution of* Hoxa5 *descendants to PAX3-positive muscle progenitors that migrate ventrally from the somites, and will give rise to forelimb and diaphragm musculature. Arrowheads show that PAX3 positive cells are not labelled with the* Hoxa5 *lineage label. The PAX3 channel is included (F) and excluded (G) to highlight absence of Hoxa5 descendants.* **(H)** *In E11.5 whole-mount embryo, somitic reporter expression is largely confined to the future C3-T2 region (between arrowheads).* **(I)** *Three days later,* Hoxa5 *descendants are broadly distributed along the DV and medio-lateral axes, and clear borders of somitic expression are not obvious in whole-mount. Arrows in (H,I) indicate the rostral border of expression in the neural tube, which is similar at these two stages.* **(J,K)** *In an E14.5 sagittal section,* Hoxa5 *descendants are broadly distributed along the dorsal ventral and medio-lateral axes within the domain from C3-T2 (arrowheads mark the dorsal and ventral sides of C3 and T2). Vertebrae and ribs are visualized by TENASCIN staining in perichondrium. Sections are transverse in (A-G) (dorsal is up) and sagittal in (J-K) (dorsal is to the left, anterior is up). Hoxa5-Cre activity is visualized with tdTOMATO reporter (A, C-K) or nYFP reporter (B). lu, lung; nt, neural tube. Scalebars: 20 μm (A-E), 5 μm (F-G), 50 μm (J-K).*

Hoxa5 descendants did not contribute to PAX3-positive cells that had migrated ventrally out of the somites at E10.5 (Fig. 3 F-G) or E11.5 (not shown). This population of migrating PAX3-positive cells includes precursors of forelimb and diaphgram musculature. Thus, *Hoxa5* descendant cells appeared to be completely excluded from the skeletal muscle lineage.

Contribution of the Hoxa5 *genetic lineage to differentiating musculoskeletal tissues*

We also examined *Hoxa5* descendant cells in older embryos. In E11.5 whole-mount embryos, Hoxa5-Cre activity was confined to somites in the C3-T2 region (Fig. 3H), which corresponds to the segmental boundaries of HOXA5 protein expression (Coulombe *et al.*, 2010). Reporter expression was also present in the neural tube extending rostral to the somite boundary, and in the LPM extending to the abdominal region and throughout the forelimb. By E14.5, *Hoxa5* descendant cells were broadly disbributed along the dorsal-ventral and medial-lateral axes of C3-T2 segments, as shown in whole mount specimen (Fig. 3I) and sagittal sections (Fig. 3 J-K). Descendants were found within skeletal elements, marked by co-staining with TENASCIN, which detects the perichondrium at this stage (Fig. 3K), and also in non-skeletal tissues. Note that the sagittal section cut through the neural tube rostral to C2, revealing labeling there, but caudal to this saggital cut was through the neural arches of the vertebrae, which are located lateral to the neural tube.

Sclerotome derivatives

Consistent with HOXA5 expression in sclerotome, *Hoxa5* descendants were abundant in all skeletal elements in the C3-T2 region: vertebral bodies, neural arches, ribs, scapula and sternum (Fig. 4 A-O, white arrows). All these elements showed morphological phenotypes in *Hoxa5* null mutants (Jeannotte *et al.*, 1993). *Hoxa5* descendants were also present in all skeletal elements of the forelimb (Fig. 4C), consistent with HOXA5 expression in the LPM forelimb field (Fig. 1A) and in the proximal region of the forelimb bud (Fig. 1B).

The identity of *Hoxa5* descendants within skeletal elements was defined by their morphology under brightfield differential interference contrast (DIC) optics (Fig. 4 D,F,H,J,L,N,P) and by co-labelling with markers, such as SOX9 for chondrocytes (Fig. 4R) and COL1A1 for osteoblasts (Fig. 4S). *Hoxa5* descendants were abundant in the perichondrium of all skeletal elements (Fig. 4 A-O), a likely explanation for the high labeling in osteoblasts, which arise from perichondrium. High magnification revealed that they contributed to all layers of the perichondrium, including the outer perichondrium, which produces tendon-cartilage attachments, and the inner perichondrium, which generates osteoblasts (Fig. 4 P,Q). Interestingly, these skeletal elements have origins either in somites (neural arches, vertebral bodies, rib chondrocytes) or in LPM (sternum, T1 perichondrium, most of the scapula), and derivatives of both were found to contain *Hoxa5* descendant cells. Thus, *Hoxa5* expressing cells from both tissue types contributed to the skeletal lineage, despite differences in the timing of HOXA5 expression in those tissues. We note that RFP signal appeared brighter in osteoblasts than in chondrocytes, making the marked chondrocytes more difficult to visualize at low power, especially in the limb (Figs. 1 A-C); however extensive chondrocyte labeling was present (Fig. 1C, inset) and more apparent in higher magnification views in both the axial skeleton and limbs (Fig. 1 D-S and not shown).

A subcompartment of the sclerotome, the syndetome, gives rise to the axial connective tissue, such as tendon and ligament (Brent *et al.*, 2003). We observed that *Hoxa5* descendants also contributed to these tissues as shown by co-labelling with COL1A1 (Fig. 5C) or TENASCIN (Fig. 5 B,D,E,F). As for skeletal tissue, tendons of both LPM and somitic origins were labelled as shown

for the scapula (Fig. 5 B,C), vertebrae (Fig. 5D), ribs (Fig. 5E) and even the nuchal ligament (Fig. 5F), which connects the occipital bone of the skull to the cervical vertebrae, and which, in contrast to most connective tissues, is neural crest-derived (Matsuoka *et al.*, 2005). Given that HOXA5 expression has not been reported in neural crest or its derivatives (Joksimovic *et al.*, 2005), we confirmed that this reflected endogenous expression. Indeed, HOXA5 protein can be detected in the nuchal ligament at E16.5, where it overlaps with Cre reporter expression (Fig. S2).

Dermomyotome derivatives: dermis, adipose tissue and skeletal muscle

Hoxa5 descendants were abundant in dermomyotome derivatives, which included the dermis (Fig. 5A), where *Hoxa5* descendant cells co-labeled with the dermatocyte marker PDGFRα (Fig. 5G). HOXA5 expression continued at this stage in dermatocytes as well (not shown). Based on morphology and co-expression with PPARγ, *Hoxa5* descendants also contributed to brown adipocytes and stromal fibroblasts within the BAT (Fig. 5A,H,I;(Mueller *et al.*, 2002)). HOXA5 protein continued to be stongly expressed in BAT (Fig. 5J; Fig. 2W). *Hoxa5* descendant cells were never observed in differentiated skeletal muscles (Fig. 5 K-M) consistent with the absence of HOXA5 protein expression in muscle (Fig. 2). Moreover, these data further extended to show exclusion from muscle of any cells with an HOXA5 expression history, even after expression has been extinguished.

Finally, *Hoxa5* descendant cells were found as fibroblasts distributed throughout skeletal muscles (Fig. 5 A, K-M), consistent with our observation of HOXA5 expression in TCF4-positive cells at E12.5. These lineage-labelled cells were found in both abaxial and primaxial muscles, including scapular muscles (Fig. 5K), trapezius (Fig. 5L), and vertebral muscles (Fig. 5M). In all cases, descendants had a fibroblast-like morphology and did not co-label with muscle actin.

Muscle interstitial fibroblasts

We further characterized the identity of these *Hoxa5* descendant cells found throughout the skeletal muscle. One possibility was that

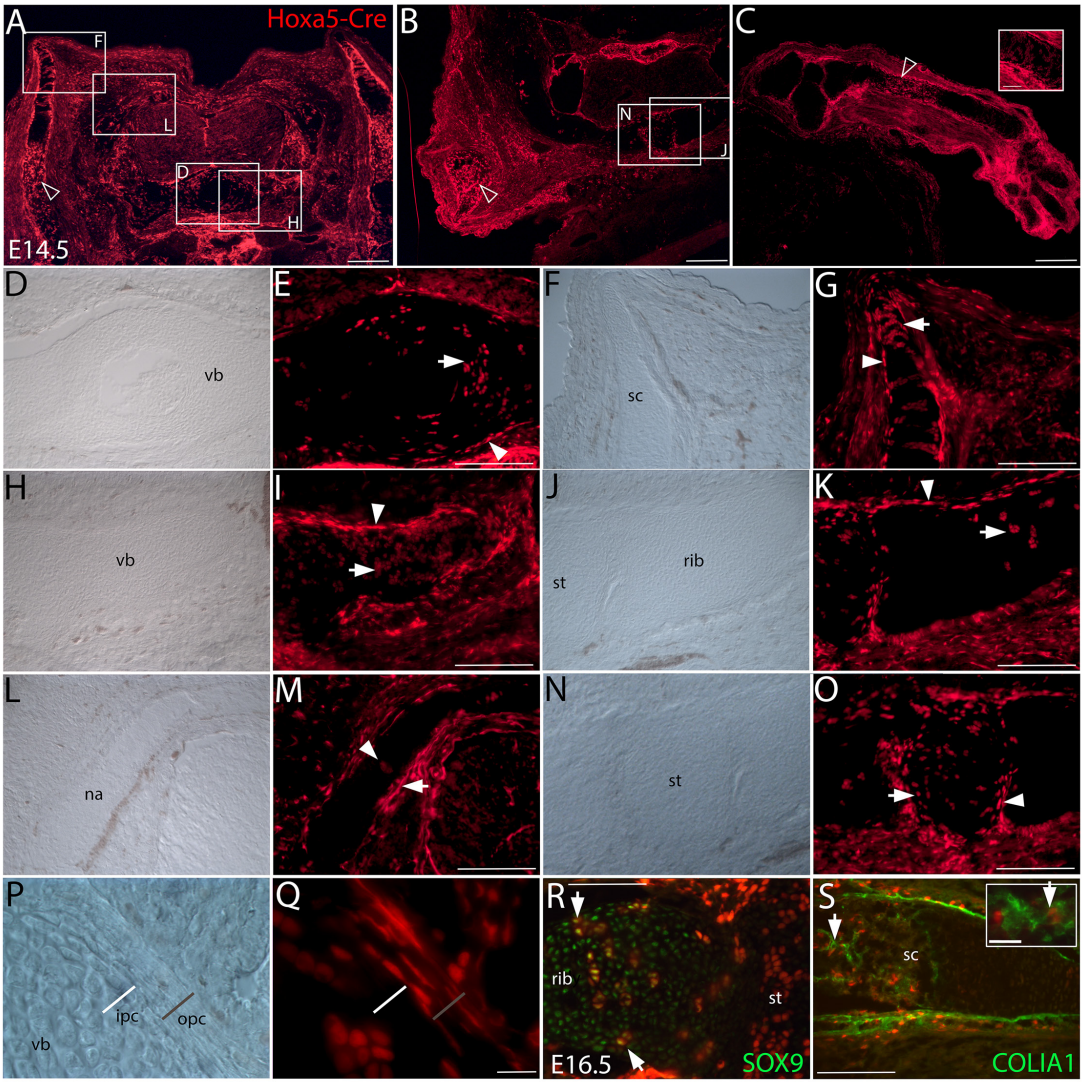

Fig. 4. Broad distribution of *Hoxa5* descendants in skeletal elements at E14.5 (A-Q) and E16.5 (R-S). Hoxa5 *descendants are labelled in red following Hoxa5-Cre activity and counterstained with cell markers by immunofluorescence. (A-C) Low power views of the dorsal body axis* **(A)**, *ventral body wall* **(B)**, *and forelimb* **(C)**. *Dorsal is up (A-B), or to the left (C). Boxes of closeup views shown in (D-O) are indicated.* **(D,F,H,J,L,N,P)** *DIC reveals skeletal cells by their morphology, and corresponding fluorescence panels* **(E,G,I,K,M,O)** *show* Hoxa5 *descendants. They contribute to cartilage (arrows) and perichondrium (arrowheads) of: vertebral body (D-E, H-I), scapula (F-G), rib (J-K), neural arch (L-M), sternum (N-O). Labeled osteoblasts show brighter signal than cartilage and are evident even at low power magnification, indicated by hollow arrowheads in the scapula (A), humerus (B) and ulna (C).* **(P-Q)** *High magnification image of vertebral body perichondrium. Within perichondrium,* Hoxa5 *descendant cells contribute to all layers.* **(R-S)** Hoxa5 *descendants also co-label with chondrocyte marker SOX9 (R; arrows) and with osteoblast marker COL1A1 (S; arrows). Inset in panel S shows closer view of* Hoxa5 *descendants embedded in the COL1A1-containing extracellular matrix. All sections are transverse. ipc, inner perichondrium; na, neural arch; opc, outer perichondrium; st, sternum; vb, vertebral body. Scalebars: 50 μm (A-C), 20 μm (E-O,R,S), 10 μm (inset in C), 5 μm (inset in S) and 2 μm (P-Q).*

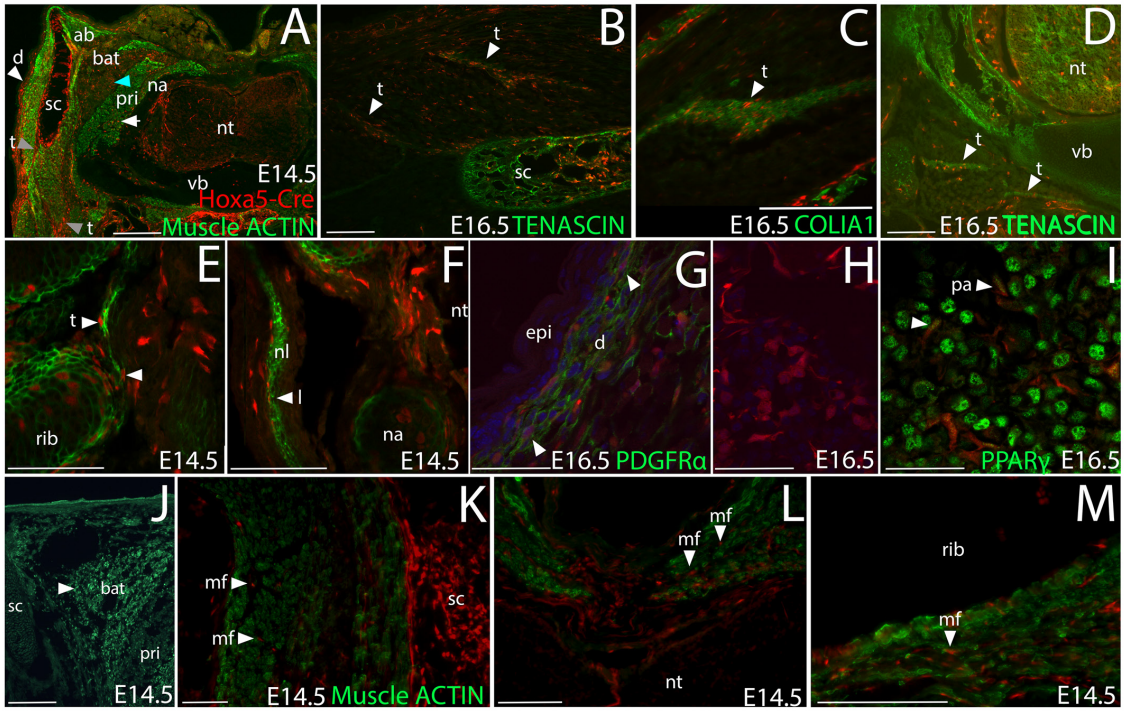

Fig. 5. Distribution of *Hoxa5* descendants in connective tissue, dermis, brown adipose tissue, but not skeletal muscle at 14.5-E16.5. Hoxa5 *descendants are labelled in red following Hoxa5-Cre activity and counterstained with cell markers by immunofluorescence.* **(A)** *Low power view shows that* Hoxa5 *descendants contribute to tendons (grey arrowheads), dermis (white arrowhead), brown adipose tissue (blue arrowhead), and fibroblasts within abaxial and primaxial skeletal muscle (white arrow).* **(B-F)** Hoxa5 *descendants contribute to tendon and ligament, which co-labeled with Col1A1 and TENASCIN. These include tendons of the subscapularis (B-C), vertebral body (D), rib (E) and nuchal ligament (F).* **(G)** Hoxa5 *descendants also contribute to dermatocytes, which are co-labelled with PDGFRα.* **(H-I)** Hoxa5 *descendants were found in brown adipose tissue including adipocytes and pre-adipocytes, which can be recognized by round shape and/or expression of PPARγ.* Hoxa5 *descendants were also detected in stromal fibroblasts of brown fat (arrows) and in cells that appeared to be pre-adipocytes based on their expression of PPARγ and fibroblast-like morphology (I).* **(J)** *HOXA5 protein continues to be expressed in BAT cells at E14.5 (arrowheads).* **(K-M)** Hoxa5 *descendants are found in fibroblasts throughout skeletal muscle (arrowheads) but do not co-label with muscle actin. Sections are transverse except for panels (E,F), which are sagittal. Abbreviations: a, adipocyte; ab, abaxial region; bat, brown adipose tissue; d, dermis; epi, epidermis; f, stromal fibroblast of brown adipose tissue; l, ligamentocyte; mf, muscle-embedded fibroblast; na, neural arch; nl, nuchal ligament; nt, neural tube; pa, preadipocyte; pri, primaxial sc, scapula; t, tenocyte; vb, vertebral body. Scalebars: 50 μm (A), 20 μm (B-D, J-M), 10 μm (E-F) and 5 μm (G-I).*

they correspond to muscle satellite cells, a stem cell population required in embryonic and adult muscle for development, homeostasis and regeneration. Muscle satellite cells can be distinguished by their position inside the basal lamina of muscle bundles, and by their expression of PAX7. LAMININ-co-staining revealed that *Hoxa5* descendants resided outside of muscle bundle laminae (Fig. 6A). Moreover, they never co-expressed PAX7 (Fig. 6B), supporting the notion that the HOXA5 lineage makes no contribution to muscle satellite cells. We also assessed whether these cells were MCT. In fact, several different muscle interstitial fibroblast types have been identified in recent years, with varied roles in muscle maintenance and/or regeneration (Nassari *et al.*, 2017). Most of the muscle-embedded *Hoxa5* descendant cells were found to co-express the fibroblast marker PDGFRα (Fig. 6C), which labels most but not all muscle fibroblast cell types. A subset of these also expressed TCF4 (Fig. 6D), revealing their identity as MCT and

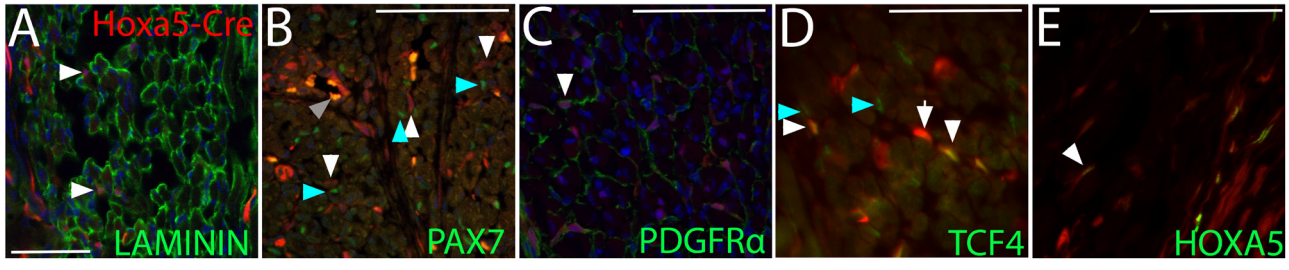

Fig. 6. Distribution of *Hoxa5* descendants within skeletal muscle bundles at E16.5. Hoxa5 *descendants are labelled in red following Hoxa5-Cre activity and counterstained with cell markers by immunofluorescence.* **(A)** Hoxa5 *descendants are localized outside the basal lamina of muscle fiber bundles (arrowheads).* **(B)** Hoxa5 *descendants (white arrowheads) do not co-label with PAX7 (blue arrowheads) indicating that they do not contribute to the muscle satellite cell lineage. Note that autofluorescent blood cells are found in this section (grey arrowhead).* **(C)** Hoxa5 *descendant cells do co-label with PDGFRα, a marker of fibroblasts in muscle (arrowhead).* **(D)** *Some* Hoxa5 *descendants co-label with TCF4, a marker for muscle connective tissue (arrowhead), while other do not (arrow). Conversely, some TCF4 cells do not express the* Hoxa5 *lineage label (blue arrowhead).* **(E)** *HOXA5 immunofluorescence (green) shows that many* Hoxa5 *descendant muscle fibroblasts continue to express HOXA5 protein (arrowhead). Sections are transverse through muscle bundles. Scalebars: 5 μm (A-E).*

consistent with HOXA5 expression in TCF4 positive mesenchyme earlier in development. However, some of these fibroblasts from the *Hoxa5* lineage did not express TCF4 (Fig. 6D), indicating that they corresponded to a different muscle fibroblast cell type. Finally, we observed that these *Hoxa5* descendants in muscle continued HOXA5 expression at later stages of development, at least up to E16.5 (Fig. 6E; Fig. 2Z).

Discussion

Dynamic HOXA5 expression profile in somites and lateral plate mesoderm

Hoxa5 is required to pattern somites that will give rise to the 3rd to the 9th vertebrae (C3-T2), and in the forelimb girdle (Aubin *et al*., 2002b, Jeannotte *et al*., 1993, Tabaries *et al*., 2007). HOXA5 immunostaining revealed a dynamic and spatially restricted expression pattern within these segments, suggesting relatively localized patterning roles (Figs. 1-2). Somitic expression begins in compartmentalized somites at E10.5 and accumulates mainly in the lateral and dorsal sclerotome. In late-stage somites (E11.5), HOXA5 sclerotome expression is further spatially restricted to the lateral-most cells, including a cap of mesenchyme surrounding the myotome VLL. This expression around the VLL reproduced that described for *Hoxa5* mRNA (Mansfield and Abzhanov, 2010). These cells are likely to be sclerotome-derived: they are not migrating muscle precursors delaminated from the dermomyotome because they do not co-express PAX3. As previously described, they also lack expression of the central sclerotome marker *Pax1* (Chen *et al*., 2013). This lateral Pax1-negative sclerotome contributes to distal ribs (Aoyama *et al*., 2005, Olivera-Martinez *et al*., 2000). It also plays a key role in rib formation: signaling between VLL and lateral sclerotome regulates specification and outgrowth of ribs and intercostal muscles into the ventral body wall (Huang *et al*., 2003, Vinagre *et al*., 2010). This interaction could be a potential target of HOX activity in determining whether a rib forms or not on a given vertebrae. Indeed, expression of *Hoxb6* in hypaxial myotome is sufficient to drive lumbar rib formation (Vinagre *et al*., 2010). *Hoxa5*, which is required for cervical rib repression and thoracic rib patterning (Jeannotte *et al*., 1993), may act on the same process, but within the sclerotome instead. Interestingly, *Hoxa5* mRNA and HOXA5 protein are also present in mesenchyme surrounding the dorso-medial lip of the myotome (DML, Fig. 1D and (Mansfield and Abzhanov, 2010)). The DML regulates dorsal migration of sclerotome to form vertebral spinous processes, similar to the role of the VLL in rib formation (Pickett *et al*., 2008).

HOXA5 is also expressed in the dermomyotome, which gives rise to skeletal muscle, dorsal dermis and brown adipose tissue. By E11.5, HOXA5 dermomyotome expression is largely restricted to the central region and to at least one of its derivatives, the dermal mesenchyme. HOXA5 is also expressed in LPM between E9.5-E10.5. From E11.5-E12.5, expression decreased in LPM-derived abaxial region. Interestingly, HOXA5 expression in differentiating sternum, an LPM-derived skeletal structure, was re-established as chondrogenesis proceeded.

The location of lineage labelled *Hoxa5* descendant cells closely matched those actively expressing HOXA5 at E9.5-10.5 (Fig. 1). While there was inefficiency in recombination such that not all of the HOXA5 expressing cells became labelled, importantly, mesodermal reporter activity was confined to HOXA5 expressing cells at these stages with no sign of ectopic activity. This, combined with a previous report that conditional deletion of *Hoxa5* using this Cre transgene recapitulates the *Hoxa5* loss-of-function phenotype indicates that it reflects the HOXA5 functional domain (Berube-Simard and Jeannotte, 2014). However, we cannot rule out the possibility that the Hoxa5-Cre transgene may be missing regulatory elements required to entirely recapitulate *Hoxa5* endogenous expression.

By E11.5, when protein expression constricted spatially in the sclerotome and LPM, *Hoxa5* descendant cells were found to be distributed much more broadly than expressers. This is not surprising given the spatially expansive expression at E10.5 and also extensive proliferation and migration that occurs at this time. This comparison further highlights the dynamic expression pattern of *Hoxa5*. Further, its spatial restriction is likely important for its function, as misexpression of *Hoxa5* throughout chick somites led to cartilage patterning defects (Chen *et al*., 2013).

Persistent HOXA5 expression in skeletal tissues suggests a direct and continuous role in skeletal morphogenesis and differentiation

HOXA5 expression persisted in differentiating skeletal structures from C3-T2 through late development. HOXA5 expression was detected in all skeletal structures (vertebral bodies, neural arches, spinous processes, ribs, sternum, scapula and humerus), although it was highest in the lateral regions of vertebral bodies and the T1-T2 ribs (Fig. 2 and humerus not shown). Further, expression was observed in all skeletal cell types including chondrocytes, osteoblasts, perichondrium and periosteum, as well as in mesenchyme surrounding the skeletal elements, and it was maintained up to at least E16.5. Cumulatively, the HOXA5 expression pattern suggests a direct role in skeletal patterning and differentiation.

Interesingly, while HOXA5 expression appears to be present continuously in vertebrae and ribs from somite stages onward, this was not true for the LPM-derived structures, where expression transiently decreased. For instance, HOXA5 was highly expressed in early LPM, but not in the LPM-derived sternal bands at E12.5. These sternal bands were extensively labelled in *Hoxa5-Cre^{+}; Rosa26-tdTomato^{+}* specimens (not shown), corresponding to the earlier phase of LPM expression. However, by E13.5, when sternal chondrogensis proceeded, HOXA5 expression reappeared in the structures.

Overall, the expression of HOXA5 in differentiating skeletal tissues is notable for its contrast with other *Hox* genes, many of which extinguished during early stages of chondrocyte differentiation, despite high expression in pre-cartilagenous condensations. For instance, *Hoxa11*, *Hoxa13* and *Hoxd13* are initially expressed in pre-cartilagenous mesenchyme, but not in SOX9 expressing, specified chondrocytes or in any later stage chondrocyotes or osteoblasts. Expression of these *Hox* genes is also excluded from the inner (cambial) perichondrium layer which gives rise to chondrocytes and later osteocytes (Stadler *et al*., 2001, Suzuki and Kuroiwa, 2002, Swinehart *et al*., 2013, Villavicencio-Lorini *et al*., 2010). Similar findings were reported for *Hoxa2* mRNA in the condensations of second branchial arch derivatives(Kanzler *et al*., 1998).

Taken together, these findings indicate that different HOX proteins have diverse tissue-specificity of expression and thus are likely to have diverse mechanisms in regulating skeletal patterning. The mechanisms through which *Hoxa5* regulates skeletal development

remain largely unknown. In the forming acromion, HOXA5 acts in at least two stages: first to positively regulate the acromial condensation, and second to negatively regulate *Sox9* expression within it (Aubin *et al.*, 2002b). Similarly, HOXA5 negatively regulates *Sox9* in chick somites (Chen *et al.*, 2013). However, the tissue-specificity or timing of these requirements are unknown.

Finally, our data revealed a complete correlation between HOXA5 expression and the skeletal phenotypes reported in *Hoxa5* null mutants. Indeed, *Hoxa5* mutants show patterning defects in the skeletal structures derived from all the regions of expression described above, including the ribs and other ventral vertebral processes, the spinous processes, the sternum and the acromion of the scapula (Aubin *et al.*, 2002b, Jeannotte *et al.*, 1993, Tabaries *et al.*, 2007). Moreover, *Hoxa5* was also shown to play a redundant role in forelimb skeletal patterning (Xu *et al.*, 2013).

Hoxa5 *expressing cells and their descendants are excluded from muscle and muscle satellite cells, but contribute to muscle connective tissue*

The function of *Hoxa5* in soft tissue patterning of the axial musculoskeleton, including muscles, connective tissues, dermis and brown adipose tissue, has not yet been characterized. However, our expression and lineage tracing data indicate that HOXA5 expressing cells contribute abundantly to all these tissues with the notable exception of skeletal muscle, from which the *Hoxa5* lineage was excluded. HOXA5 was expressed in the dermomyotome (Fig. 1), which contains proliferative cells of the dermal, adipogenic, myogenic, and muscle satellite cell lineages (Christ *et al.*, 2007). At E10.5, it is co-expressed with PAX7, which marks all of these lineages at this stage. However, by E11.5 most cells except the muscle lineage have extinguished PAX7. At this stage, we no longer observed any overlap between PAX7 and HOXA5 expressing cells, consistent with the absence of coexpression with muscle markers at any stage. Hoxa5-Cre lineage labeling confirmed activation of *Hoxa5* expression in dermomyotome. Further, persistent HOXA5 expression was detected in the dermis, brown adipocytes and stromal fibroblasts of brown adipose tissue of late embryos (Fig. 5), and *Hoxa5* was previously reported to be expressed in pre-adipocytes (Wang *et al.*, 2014). Interestingly, BAT arises in the scapular region of the body axis, a region corresponding to the HOXA5 expression domain. BAT is a specialized derivative of the dermomyotome that is closely related to skeletal muscle and arises from *Myf5*-positive myotome via a lineage respecification (Seale *et al.*, 2008).

In contrast to the tissues above, HOXA5 expression was notably absent from the skeletal muscle lineage, both in the body axis and limbs (Figs. 2, 5), and in the diaphragm (Landry-Truchon *et al.*, 2017). A similar exclusion of *Hoxa11* expression from limb muscle was previously reported (Swinehart *et al.*, 2013). However, this is apparently not a common theme for HOX proteins. *Hoxb6* exerts skeletal patterning activity from within the *Pax3*-positive muscle progenitor lineage (Vinagre *et al.*, 2010), and both *Hoxa10* and *Hoxd9* were shown to function cell-autonomously within the myotome (Alvares *et al.*, 2003). Interestingly, these latter genes regulate expression of *Lbx1*, a marker for migratory myocytes in the VLL (Alvares *et al.*, 2003). As described above, *Hoxa5* expressing cells in the sclerotome accumulate directly adjacent to these *Lbx1* positive cells (Fig. 1;(Chen *et al.*, 2013). Further, the AP expression of HOXA5 from C3-T2 corresponds closely to the somites from which the forelimb, forelimb girdle and diaphragm muscle progenitors originate (C4-T2; (Babiuk *et al.*, 2003, Houzelstein *et al.*, 1999)).

Strikingly, HOXA5 expression is not only excluded from embryonic muscle, but lineage mapping shows that all descendants of *Hoxa5* expressing cells are similarly excluded from the muscle and PAX7-positive muscle satellite cell lineages. The potential functional relevance of this lineage restriction is unknown, but future studies can test whether it is important for development of muscle or other tissues.

Hoxa5 descendant cells are abundant among muscle-embedded fibroblasts including TCF4-positive muscle connective tissue cells. Further, expression of HOXA5 in muscle-embedded fibroblasts persists at least through late embryogenesis (Figs. 5, 6). MCT has emerged as a key regular of muscle patterning (Huang, 2017, Kardon *et al.*, 2003, Mathew *et al.*, 2011). While the developmental origin of MCT in the axial musculature is unknown, primaxial MCT is not labeled with the Prx1-Cre lineage marker (Durland *et al.*, 2008) indicating its origin is likely in the somites instead. In contrast, abaxial muscle MCT is marked by *Prx1-Cre*, and thus likely derives from LPM, like the limb MCT (Chevallier and Kieny, 1982, Chevallier *et al.*, 1977). HOXA5 expression, and *Hoxa5* descendant cells, are found within both types of MCT, but expression is substantially higher in primaxial regions (Fig. 2), probably reflecting higher overall HOXA5 expression in the somites compared to LPM at the axial levels we examined.

Hoxa5 descendants also contribute to TCF4 negative muscle-embedded fibroblasts (Fig. 6). Several fibroblast cell types distinct from the muscle satellite cell lineage have recently been described (Nassari *et al.*, 2017). These show variation in expression profiles, fate potential, and function in muscle regeneration, homeostasis, fibrogenesis and adipogenesis. Interestingly, *Hoxa5* is known to play a functional role in lung alveolar myofibroblasts (Mandeville *et al.*, 2006), which share a number of characteristics with muscle connective tissue fibroblasts (Barron *et al.*, 2016). Lung alveolar fibroblasts are required for alveolar septation, and HOXA5 activity is necessary for this process. *Hoxa5* loss of function mutants show aberrant expression of alveolar myofibroblast markers, including *PDGFRa*, and impaired migration of alveolar myofibroblasts out of lung interstitium into the developing alveolae (Abbott *et al.*, 2005, Landry-Truchon *et al.*, 2017, Mandeville *et al.*, 2006).

Altogether, the expression and lineage contribution of HOXA5 to numerous soft tissue lineages suggests that it plays patterning roles in them. It is clear that skeletal and soft-tissue patterning is co-regulated, although they can be uncoupled in some cases (Swinehart *et al.*, 2013). The above characterization of the sites of HOXA5 expression and the fate-mapping analysis of *Hoxa5* descendants can guide future functional studies that will define the sites of HOXA5 musculoskeletal patterning activity and in turn, identify potential transcriptional targets.

Material and Methods

Mouse strains and genotyping

The following mouse strains were used: *Tg(Hoxa5-cre)447BLjea* (Berube-Simard and Jeannotte, 2014); *B6.129X1-Twist2tm1.1(cre)Dor/J* (Sosic *et al.*, 2003); *B6.Cg-Tg(Prrx1-cre)1Cjt/J* (Logan *et al.*, 2002); *B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J* (Madisen *et al.*, 2010); *B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J* (Srinivas *et al.*, 2001); *Hoxa5tm1Rob* (Jeannotte *et al.*, 1993). In all crosses, males bearing a *Cre* allele were crossed to females homozygous for an RFP or nYFP Cre

reporter. *Hoxa5*$^{-/-}$ null embryos were generated by crossing heterozygous parents. Timed-pregnant females were sacrificed to collect embryos at the stages indicated. Genotyping on tail snips or embryonic yolk sac DNA was performed with primers for Cre, RFP, nYFP, *Hoxa5*$^{+}$ or *Hoxa5*$^{-}$ alleles.

All procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Columbia University IACUC. Genotyping primers (5'to 3') were as follows. Cre: forward GCGGTCTGGCAGTAAAAACTATC, reverse GTGAAACAGCATTGCTGTCACTT; tdTOMATO/RFP: forward CTGTTCCTGTACGGCATGG, reverse GGCATTAAAGCAGCGTATCC; YFP/GFP: forward GCACGACTTCTTCAAGTCCGCCATGCC, reverse GCGGATCTTGAAGTTCACCTTGATGCC; *Hoxa5*$^{+}$ allele: forward ACTGGGAGGGCAGTGCCCCCACTTAGGACA, reverse CTGCCGCGGCCATACTCATGCTTTTCAGCT; *Hoxa5*$^{-}$ allele forward ACTGGGAGGGCAGTGCCCCCACTTAGGACA, reverse GGCTACCTGCCCATTCGACCACCAAGCGAA.

Immunofluorescence

Immunostaining was performed as described (McGlinn *et al.*, 2018). Briefly, embryos were fixed two hours to overnight in 4% paraformaldehyde at 4° C and embedded in OCT. Sections of 6-20 μm were cut, dried at 37° C, and frozen at -80° C. After air drying and rinsing in PBS, sections were subjected to citrate-based antigen retrieval, 0.1% Triton-X, or were not permeabilized. Slides were blocked for 60 minutes in 5% Normal Donkey Serum/ 2% Boehringer Blocking Reagent/ PBT or 0.1% Triton-X/ PBS, and incubated in primary antibodies overnight at 4° C. Primary antibodies are listed in Supplementary Table 1. The following day, slides were washed in PBT, incubated in Cyanine Dye or Alexa Fluor conjugated secondary antibodies (Jackson Immunoresearch, diluted 1:400) for 2 hours at room temperature, washed in PBS, counterstained with DAPI, and mounted in Prolong Diamond.

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