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Identity and fate of *Tbx4*-expressing cells reveal developmental cell fate decisions in the allantois, limb, and external genitalia

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Abstract

T-box gene *Tbx4* is critical for the formation of the umbilicus and the initiation of the hindlimb. Previous studies show broad expression in the allantois, hindlimb, lung and proctodeum. We have examined the expression of *Tbx4* in detail and used a *Tbx4-Cre* line to trace the fates of *Tbx4*-expressing cells. *Tbx4* expression and lineage reveal that various distinct appendages, such as the allantois, hindlimb, and external genitalia, all arise from a single mesenchymal expression domain. Additionally, although *Tbx4* is associated primarily with the hindlimb, we find two forelimb expression domains. Most notably, we find that, despite the requirement for *Tbx4* in allantoic vasculogenesis, the presumptive endothelial cells of the allantois do not express *Tbx4* and lineage tracing reveals that the umbilical vasculature never expresses *Tbx4*. These results imply that endothelial lineages are segregated prior to the onset of vasculogenesis, and demonstrate a role for the peri-vascular tissue in vasculogenesis.

Keywords

Tbx4; allantois; limb; hindlimb; lung; genital tubercle; mouse; development

INTRODUCTION

T-box genes code for a family of DNA-binding transcription factors known to play critical roles in the formation of numerous embryonic structures and maintenance of certain adult tissues (Showell et al., 2004; Naiche et al., 2005; King et al., 2006; Wardle and Papaioannou, 2008). Mutations of T-box genes in vertebrate organisms result in dramatic developmental defects including peri-implantation lethality, embryonic axis truncation, and debilitating heart and skeletal defects. T-box gene mutations are also known to be causative for a number of human developmental syndromes, including Holt-Oram, ulnar-mammary, and DiGeorge syndromes (Naiche and Papaioannou, 2007b).

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T-box gene *Tbx4* is primarily known for its role in hindlimb development. *Tbx4* was originally described as one of the few genes expressed specifically in hindlimbs and not forelimbs (Chapman et al., 1996; Gibson-Brown et al., 1996) and has been shown to be required for early stages of hindlimb development and for the development of hindlimb skeletal elements, such as the femur (Naiche and Papaioannou, 2003; Naiche and Papaioannou, 2007c). The hindlimb specificity of *Tbx4* has been observed throughout the gnathostomes, including mouse, chick, opossum, zebrafish, and shark (Gibson-Brown et al., 1996; Gibson-Brown et al., 1998a; Tamura et al., 1999; Ruvinsky et al., 2000; Tanaka et al., 2002; Horton et al., 2008; Keyte and Smith, 2010). Direct evidence for hindlimb-specific expression has not been shown in humans, but human mutations in *TBX4* cause small patella syndrome, which results in abnormalities of the pelvis, knee, and foot (Bongers et al., 2004). Hindlimb specific *Tbx4* expression may have evolved concomitantly with the evolution of pelvic fins in fish, which is the origin of the posterior limb pair (Horton et al., 2008).

Whether the hindlimb specificity of *Tbx4* confers hindlimb, or leg-like, identity and morphology to the hindlimb is still controversial. Studies in chick have indicated that ectopic *Tbx4* can induce limbs with leg-like characteristics (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999), while loss-of-function studies in the mouse showed no change in hindlimb identity or morphology (Naiche and Papaioannou, 2007c). Gene-swap experiments with the closely related forelimb-specific gene, *Tbx5*, in the mouse forelimb have shown that *Tbx4* can replace *Tbx5* in driving limb outgrowth and *Pitx1* is the actual determinant of hindlimb morphology (Minguillon et al., 2005). Conversely, similar experiments in the hindlimb have shown that only *Tbx4* can support normal morphology of the hindlimb, and that *Tbx4* and *Tbx5* have different transcriptional activities (Ouimette et al., 2010).

In addition to the hindlimb, *Tbx4* is expressed in the allantois (Chapman et al., 1996), an outgrowth from the posterior of the embryo which is the precursor to the umbilical cord and the vascular portions of the fetal placenta (Inman and Downs, 2007). The allantois is one of only three regions known to undergo vasculogenesis, the de novo differentiation of endothelial cells and formation of blood vessels out of undifferentiated mesenchyme, as opposed to the more common angiogenesis, which entails sprouting and extension of existing blood vessels (Baron and Fraser, 2005). *Tbx4* null embryos show severe defects in growth of the allantois and in vasculogenesis, resulting in the death of such embryos due to their failure to establish an umbilical connection (Naiche and Papaioannou, 2003).

Tbx4 expression has also been noted in the lung (Chapman et al., 1996). Loss of *Tbx4* alone does not appear to alter lung development (Naiche and Papaioannou, 2003; Naiche and Papaioannou, 2007c), but *Tbx4* may play a role in lung bud branching in concert with *Tbx5*, which is co-expressed in this tissue (Cebra-Thomas et al., 2003; Sakiyama et al., 2003).

By examining the expression of *Tbx4* in greater detail and developmental range than previous studies, we have found several previously unreported aspects of this gene's expression. We find that tracing the lineage of *Tbx4* expressing cells reveals previously unrecognized developmental cell fate decisions, in particular the pre-patterning of vascular and perivascular fates in vasculogenic mesenchyme, continuity between multiple outgrowths of the posterior mesenchyme, and the origin of the pelvic girdle. We also describe the regions of recombination of the *Tbx4-Cre* allele, which will be of great use as a tool to conditionally excise genes throughout the limb, lung, and genital mesenchyme from their earliest inception, and to selectively ablate perivascular tissue in the allantois.

RESULTS AND DISCUSSION

***Tbx4* expression in hindlimb**

As previously reported, *Tbx4* expression in the lateral plate hindlimb mesenchyme initiates at embryonic day (E) 9.5, prior to hindlimb outgrowth (Fig. 1A). At E10.5-11.5, expression was observed throughout the extending hindlimb, with the proximal boundary of expression at the body wall (Fig. 1B-C). At later stages, *Tbx4* expression shifted distally but remained widespread in the hindlimb through E14.5 (Fig. 1D-F).

Lineage tracing of *Tbx4*-expressing cells in hindlimb

To explore the role of *Tbx4*-expressing cells in the limb, we examined the fate of cells that have expressed *Tbx4* using a *Tbx4-Cre* mouse line, where an internal ribosomal entry site (IRES) followed by the *Cre* recombinase gene has been inserted into the 3' UTR of the *Tbx4* gene (Luria et al., 2008). *Tbx4-Cre* homozygous mice are viable and fertile, confirming that *Tbx4* function is not disrupted, and *Tbx4-Cre* embryos do not show abnormal apoptosis in regions of *Cre* expression, as has been reported for some *Cre* lines (Naiche and Papaioannou, 2007a). *Tbx4-Cre* mice were mated to the *R26R-lacZ* and *R26R-YFP* *Cre* reporter lines (Soriano, 1999; Srinivas et al., 2001), which undergo excision of a transcriptional stop in *Cre*-expressing cells and irreversibly activate expression of the reporter, allowing us to trace the eventual distribution of all cells that ever express *Tbx4*, hereafter referred to as the *Tbx4*-lineage or *Tbx4*-lineage cells.

Both reporter lines produced similar results (Fig. 1G-H), but we noted that detection of the YFP fluorescence lagged behind the appearance of endogenous *Tbx4* expression by approximately half a day (Fig. 1I-J). No such delay was observed using the *lacZ* reporter, possibly due to the enzymatic amplification inherent in the assay, and we therefore conducted most of the following studies with *R26R-lacZ*.

Whole mount β -galactosidase staining of *Tbx4-Cre; R26R-lacZ* embryos showed apparently complete constitution of the hindlimb by *Tbx4*-lineage cells (Fig. 1G, K, L). Additionally, it was clear at postnatal day 1 (P1) that tissues associated with hindlimb mesenchyme but medial to the limb itself were also part of the *Tbx4*-lineage; the dermis showed β -galactosidase staining over most of the haunch, and the gluteal muscles that extend nearly to the spine were also strongly stained (Fig. 1L).

The limb is derived from two tissues sources: the lateral plate mesoderm of the hindlimb field gives rise to the cartilage, bone, perichondrium, tendons, ligaments, and connective tissue, whereas muscle and blood vessels arise from somitic cells that migrate into the limb (Chevallier et al., 1977). Sections through *Tbx4-Cre; R26R-lacZ* embryos showed that the *Cre* reporter is completely recombined in the hindlimb field mesenchyme as early as E9.5 (Fig. 1M). By E13.5, when the skeletal elements of the hindlimb can be discerned and the muscle primordia have entered the limb, all tissues of the hindlimb appear to be *Tbx4*-lineage cells (Fig. 1N), suggesting that much of the somite-derived tissue of the hindlimb also upregulates *Tbx4* at some point in its migration into the limb.

However, at P1 it is clear that the *Tbx4* lineage does not form the entirety of the hindlimb; there is a clear proximo-distal gradient of *Tbx4*-lineage tissue (Fig. 1O). In the hindlimb autopod, all tissues other than the epidermis appeared to be composed of *Tbx4*-lineage cells (Fig. 1P-Q). In the stylopod and zeugopod, the lateral plate-derived tissues, particularly bone, tendon, and perichondrium, appear to be completely composed of *Tbx4*-lineage cells (Fig. 1R-S), while the major muscles of the leg show more modest *Tbx4*-lineage contribution (Fig. 1 O, R, T). The low contribution of the *Tbx4* lineage to proximal muscle

reflects the case of *Tbx5*, which is expressed only at low levels in migrating myocytes (Hasson et al., 2010).

Interestingly, even though *Tbx4* expression is not observed within the body wall after E9.5, all of the elements of the pelvic girdle and its surrounding perichondrium, which lie deep within the body, are *Tbx4*-lineage tissues (Fig. 1 U-W). This data supports previous studies in chicks suggesting that the pelvis derives from lateral plate mesoderm of the hindlimb field (Malashichev et al., 2008). However, our data cannot rule out somitic contribution to the pelvic girdle, since we observe that some somite-derived tissue becomes part of the *Tbx4* lineage. Indeed, the hindlimb muscles that attach to the body core (i.e., the gluteal and groin muscles) contain significant *Tbx4* lineage contribution (Fig. 1L). This would suggest that the progenitors of the proximal hindlimb muscles migrate laterally into the hindlimb, upregulate *Tbx4*, and then reverse their migration to form muscles within the body wall, in a process similar to that which has been demonstrated for the muscles of the urogenital region (Valasek et al., 2005).

The early and complete recombination afforded by *Tbx4-Cre* in the hindlimb demonstrates that this is an excellent tool for limb-specific ablation of genes, including those involved in the very earliest stages of limb development and pelvic girdle formation.

***Tbx4* expression and lineage in forelimb and sternum**

Despite the predominantly hindlimb-specific expression of *Tbx4*, the earliest descriptions of *Tbx4* expression noted faint forelimb expression at E9.5 (Gibson-Brown et al., 1996). Similarly, we observe a small domain in the proximal core of the forelimb from E10.5-11.5 (Fig. 2A-B) that expresses *Tbx4* at lower levels than in the hindlimb (compare forelimbs in Fig. 2A-D with corresponding hindlimbs in Fig. 1B-E, which originated from the same embryos). This proximal forelimb expression disappears by E12.5. A novel domain of *Tbx4* expression in the forelimb autopod appears faintly at E12.5 and becomes more intense by E13.5, disappearing gradually thereafter (Fig. 2C-D).

When we examined the *Tbx4* lineage in the forelimb at E11.5-13.5, we observed the appearance of scattered lineage-marked cells primarily near the prospective elbow, corresponding to the proximal core expression domain noted above (Fig. 2E). At later stages, in addition to cells near the elbow, dense accumulations of *Tbx4*-lineage cells were observed in the ventral hand, corresponding to the autopod expression domain observed at E13.5 (Fig. 2F-H).

Sections of E13.5 embryos showed that, while there were a few *Tbx4*-lineage cells in the stylopod near the shoulder, the bulk of the early forelimb *Tbx4* lineage was concentrated in the bone and nearby tissues of the elbow joint (Fig. 2I-J). By P1, sections of the corresponding region show that these cells contribute mostly to the tendons around the elbow and to the periphery of the bone (Fig. 2K). In the autopod, *Tbx4*-lineage cells are likewise concentrated in the tendons and bone periphery, but scattered cells also appear in the muscle and epidermis (Fig. 2L-N).

At E13.5, *Tbx4*-lineage cells were also scattered throughout the ventral body wall and by P1 this lineage domain resolved into the cartilage of the anterior portion of the sternum and the xyphoid process (Fig. 2O-P). This region probably corresponds to the previously reported *Tbx4* expression in the thoracic body wall (Chapman et al., 1996).

The developmental significance of forelimb expression of *Tbx4* is unclear. Embryos with partial or complete loss of *Tbx4* function show no obvious defects in overall forelimb size or skeletal formation, but detailed analysis of forelimb tendons, where most *Tbx4* expression

occurs, was not performed (Naiche and Papaioannou, 2007c; Menke et al., 2008). It has been suggested that forelimb expression of *Tbx4* may impose features of hindlimb morphology in the forelimb (Wang et al., 2010). In bats, *Tbx4* is expressed in the first digit of the forelimb, which is the only digit that retains a more hindlimb-like morphology and does not elongate to form part of the wing (Wang et al., 2010). However, in mouse there is no obvious correlation between the regions of forelimb *Tbx4* expression and hindlimb-like morphology; on the contrary, the carpal bones and tendons, where *Tbx4* expression is concentrated, are some of the most divergent structures between limb pairs.

Characterization of the *Tbx4* locus has identified an enhancer element which drives *Tbx4* expression in the forelimb, but this element is poorly conserved outside of the mammalian class and is dispensable for viability and forelimb morphology in mouse (Menke et al., 2008). Collectively, these data suggest that the forelimb expression of *Tbx4* is not evolutionarily conserved and may have little developmental relevance, but this domain should be noted by anyone using the *Tbx4-Cre* allele for hindlimb-specific gene recombination.

The *Tbx4* lineage in tissues derived from posterior ventral mesenchyme

Expression of *Tbx4* can also be seen in the developing external genitalia throughout development (Fig. 3A-F). *Tbx4* expression in this region initiates at E9.5 in the mesenchyme surrounding the cloacal slit, in the region that will form the genital folds and the genital tubercle (Fig. 3A-B). Interestingly, at this early time point, *Tbx4* expression exists as a single contiguous expression domain in the ventral mesenchyme that extends laterally to the hindlimbs, posteriorly to the genital folds, and anteriorly to the allantois. During later development, *Tbx4* expression in each of these three regions becomes more distal and becomes separate expression domains (Fig 3C-F).

Lineage tracing of *Tbx4* reflects this early continuity: at E13.5 sections through the posterior of the embryo show a broad swath of *Tbx4*-lineage tissue that encompasses the mesenchyme of the hindlimb, genital tubercle, and urogenital sinus (Fig. 3G-I). In neonates, much of the urogenital system is derived partially or wholly from the *Tbx4* lineage (Fig. 3J). The mesenchyme (but not the epithelium) of the bladder, which arises from the urogenital sinus, is entirely composed of *Tbx4*-lineage cells (Fig. 3K). The urachus, which helps hold the bladder in place and is the post-natal remnant of the allantois, is also wholly composed of *Tbx4*-lineage cells (Fig. 3L), while the uterus, cervix, and hindgut mesenchyme show partial contribution of *Tbx4*-lineage tissues (Fig. 1N, W, Fig. 3G, J, M). All mesenchyme of the male and female external genitalia, except for the proximal ventral border, is comprised of *Tbx4*-lineage cells, including the prepuce, glans, and erectile tissues (Fig. 3N-O). Also in the posterior embryo, we note *Tbx4* lineage contribution at low levels in both the ovary and the testes (Fig. 3P-Q).

Tbx4 expression and lineage in heart and lung

Expression of *Tbx4* can be seen in the lung by early E10.5, when the first bifurcation of the lung bud is observed (Fig. 4A). Uniform expression is seen throughout the mesenchyme of the lung at all stages examined (Fig. 4A-C). Lineage tracing confirms the expression data; the entire lung mesenchyme is positive for the *Tbx4* lineage reporter (Fig. 4D). At E13.5, the rostral-most extent of the *Tbx4* lineage is seen in scattered cells around the trachea at the level of the laryngeal aditus (Fig. 4E). Caudal to the pharyngeal opening, the mesenchyme of the trachea and branching lung is entirely composed of *Tbx4*-lineage cells, while the epithelium shows no reporter expression (Fig. 4F-G). Similar results were observed at P1 (Fig. 4H). Interestingly, the pulmonary vein and artery are also composed of *Tbx4*-lineage cells, suggesting that these vessels arise from the surrounding lung mesenchyme (Fig. 4H).

Conflicting data exist regarding the expression of *Tbx4* in the heart. One study reports expression in the atria (Chapman et al., 1996), another reports no expression in the atria but weak expression in the outflow tract and right ventricle (Krause et al., 2004), while yet another found no expression of *Tbx4* in the heart (Gibson-Brown et al., 1998b). In E12.5-P1 mice, we found no *Tbx4* expression or *Tbx4*-lineage cells in any region of the heart (Fig. 4B-D, G, H), suggesting that *Tbx4* is not expressed at physiological levels at any stage of development in this organ.

***Tbx4* expression in allantois**

Previous work has shown *Tbx4* expression in the allantois, the primordium of the umbilicus (Inman and Downs, 2007), at E7.5, but little is known about the localization or temporal limits of its expression (Chapman et al., 1996). Whole mount *in situ* hybridization of wildtype embryos shows that faint *Tbx4* expression initiates in the extraembryonic mesoderm as early as the late streak stage (Downs and Davies, 1993), before the morphological allantoic bud can be seen in unstained embryos (Fig. 5A, A'), and strong expression can be observed throughout the allantois by the late bud stage (Fig. 5B). When examined by whole mount *in situ* hybridization, *Tbx4* expression encompasses the entire allantois through all stages of its growth and attachment and fusion to the chorion (bud stage-E8.5, Fig. 5C-F). After remodeling of the allantois into the umbilical cord, *Tbx4* expression is observed in the umbilical vessels and the fetal placenta until E11.5, after which *Tbx4* expression is faint or absent in the umbilicus and placenta (Fig. 5G-I and data not shown).

Despite the apparently homogenous expression seen in whole mount preparations, sectioning reveals that *Tbx4* is expressed only in a subset of the cells of the allantois (Fig. 5J-M). At all stages examined, *Tbx4* is expressed in all cells of the mesothelial (outer) layer of the allantois (Fig. 5J-L and data not shown). In pre-fusion allantoises, *Tbx4* is expressed in most of the allantois, but a few cells that do not express *Tbx4* (*Tbx4*-negative cells) are seen scattered throughout the allantois with no obvious pattern (data not shown). In 8-10 somite allantoises, clear organization can be seen in the *Tbx4*-positive and negative cells (Fig. 5J-L and Supp. Fig. 1). At the base of the allantois, where cells have recently emerged from the primitive streak (Downs and Harman, 1997; Kinder et al., 1999), most cells are *Tbx4* positive and only a few scattered *Tbx4*-negative cells are observed (Fig. 5J). In the middle of the allantois, the field of *Tbx4*-positive cells is interspersed with *Tbx4*-negative cells, which appear in small clusters frequently located immediately subjacent to the mesothelium (Fig. 5K). At the tip of the allantois, which contains cells that have been in the allantois for the longest period, the *Tbx4* negative cells are clearly localized to large clusters subjacent to the mesothelium and to the central umbilical vessel (Fig. 5L). After remodeling of the allantois, *Tbx4* expression surrounds both umbilical vessels, but the endothelium is *Tbx4*-negative (Fig. 5M).

The observation that the endothelium of E9.5 umbilical vessels does not express *Tbx4* led us to investigate whether the *Tbx4*-negative cells in the allantois might also be developing endothelial cells. In 2-4 somite embryos, *Pecam* protein, which marks developing endothelial cells, is found in small clumps of cells scattered throughout the mesoderm of the allantois (Downs et al., 1998), but excluded from the mesothelium (data not shown). By 8-10 somites, the base of the allantois contains scattered *Pecam*-positive cells, while the distal allantois shows *Pecam* staining in cell clusters subjacent to the mesothelium and in the central vessel (Fig. 5N-P). *Pecam* also marks circulating erythrocytes, which can be observed in the lumen of some sections of the central vessel (Fig. 5P, Supp. Fig. 1C). This pattern appears to be complementary to the pattern of *Tbx4* expression (Fig. 5Q), suggesting that *Tbx4* and *Pecam* expression is mutually exclusive. Unfortunately, double labeling was not possible due to the lack of a suitable *Tbx4* antibody. Additionally, it could not be

determined from expression data alone whether cells in the allantois mesenchyme merely downregulate *Tbx4* as they differentiate into endothelium, or if potential endothelial cells never express *Tbx4*, so we examined the lineage fates of *Tbx4* expressing tissues.

Fate of the *Tbx4* lineage in the allantois

As expected, the *Tbx4* lineage comprised most of the allantois and umbilicus at E8.5-13.5, as well as tracing the vasculature and other allantois-derived tissues of the fetal placenta (Fig. 6A-D). In sections of early stage allantoises, the majority of the allantois was derived from the *Tbx4* lineage, although scattered cells could be seen which were not part of the *Tbx4*-lineage and whose identity could not be clearly determined (Fig. 6E). No *Tbx4* lineage was observed in other extraembryonic tissues, such as the yolk sac, amnion, or unfused portions of the chorion (data not shown).

After remodeling of the allantois into the umbilical cord it became clear that, while most of the umbilicus is part of the *Tbx4* lineage, the endothelial lining of the umbilical arteries and veins show no contribution of *Tbx4*-lineage tissues (Fig. 6F). Within the fetal placenta, this situation is more variable, with both vessels composed of *Tbx4*-lineage cells and vessels with no *Tbx4*-lineage (Fig. 6G). We examined whether the tissue that was negative for the *Tbx4* lineage trace was endothelium by double labeling with antibodies against β -galactosidase and Pecam (Fig. 6H-M), and thus confirmed that the endothelium of the umbilicus and parts of the placenta are derived from cells that are not part of the *Tbx4* lineage.

The vessels of the allantois/umbilicus are created via a process called vasculogenesis, which differs from angiogenesis, in which new blood vessels form by sprouting from preexisting vessels (Baron and Fraser, 2005). In vasculogenesis, a previously unpatterned mesenchyme forms blood vessels via the *de novo* differentiation of individual endothelial cells from the vasculogenic mesenchyme, which then coalesce into tubules and eventually mature vessels. Vasculogenesis primarily occurs in the allantois, yolk sac, and the aorta-gonad-mesonephros (AGM) region of the embryo, and the vasculogenic endothelium is the tissue that later becomes capable of producing all of the hematopoietic stem cells (HSCs) of the body (Baron and Fraser, 2005).

Our results indicate that the scattered *Tbx4*-negative cells observed in the expression data are, in fact, the prospective endothelial cells, and that unlike the bulk of the allantois mesenchyme, these cells do not express *Tbx4* at any point in their development. This implies that the vasculogenic mesenchyme of the allantois is not naive; in fact, it contains a compartment of cells which do not at any point express the same genes as the surrounding mesenchyme and which are solely capable of responding to vasculogenic signals.

It is not clear whether this pre patterning is a feature unique to the allantois. The allantois is unlike other vasculogenic regions in that it can initiate vasculogenesis independent of Hedgehog signaling (Astorga and Carlsson, 2007), and the allantois-derived endothelium of the umbilicus and placenta may produce HSCs earlier and more abundantly than other tissues (Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Zeigler et al., 2006; Yokomizo and Dzierzak, 2010). However, it is equally possible that other vasculogenic tissues possess the same compartments, the discovery of which awaits a similar fortuitous lineage analysis.

Interestingly, our observations also imply that the severe defect in vasculogenesis in the *Tbx4* null mutant embryos is due to non-cell autonomous defects, since the endothelium does not express *Tbx4* at any point. This illustrates an important role for the surrounding mesenchyme in vasculogenesis, and identifies the allantois as a potential model system for investigating these interactions.

While not previously emphasized, expression of *Tbx4* in a single ventral domain that encompasses the allantois, hindlimb, and proctodeum appears to be an evolutionarily conserved feature and can be observed in possum, chick, and in the ancestral chordate *tbx4/5* gene of amphioxus (Gibson-Brown et al., 1998a; Horton et al., 2008; Keyte and Smith, 2010). These data might suggest that the *Tbx4*-positive mesenchyme has a particular facility for generating appendages, and/or that appendages in this region might share similar characteristics. It has long been known that the limb and genital tubercle show a number of regulatory similarities, including requirements for hedgehog and fibroblast growth factor (FGF) signaling for their growth and patterning, supporting this speculation (Kondo et al., 1997; Minelli, 2002; Yamada et al., 2006). Conversely, the allantois does not require hedgehog signaling for its growth or vascular patterning (Astorga and Carlsson, 2007), and while our recent work has shown that loss of FGF signaling impairs allantois growth (see supplemental material in Naiche et al., 2011), it is currently unclear whether the growth defect is secondary to defects in the primitive streak. Current evidence suggests that the allantois is actually not an appendage, but rather may be an extension of the primitive streak (Downs et al., 2009). Further study will elucidate whether all of the outgrowths of the *Tbx4* mesenchyme share common regulation.

EXPERIMENTAL PROCEDURES

In situ hybridization

Timed pregnancies from ICR females mated to ICR males were harvested at indicated developmental stages. For whole-mount lung staining, the lungs, still attached to the heart, were dissected out of fresh embryos and fixed separately. All samples were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4° for 24-48 hours, then dehydrated stepwise into methanol. Whole mount in situ hybridizations were performed according to established protocols (Nagy et al., 2003).

Lineage tracing

Timed matings were set up between heterozygous *Tbx4-Cre* (Luria et al., 2008) males and *R26R-lacZ* (Soriano, 1999) or *R26R-YFP* (Srinivas et al., 2001) homozygous females. For whole mount β -galactosidase staining, embryos were fixed briefly in 4% PFA (30-120 minutes) at 4° and transferred to X-gal stain at 37° overnight. For sections, E9.5 embryos were stained in whole mount and then paraffin embedded for sectioning; older embryos were frozen without fixation in OTC media (Tissue-Tek 4583), cryosectioned at 20 μ m, fixed briefly in PFA, and transferred to X-gal stain overnight. Counterstain was either neutral red or nuclear fast red. Controls for background staining were conducted on Cre-negative littermates in all experiments, but no background was observed except in the post-natal gut (Fig. 3J-M and data not shown).

Antibody staining

For antibody staining, embryos were dehydrated in 30% sucrose in PBS overnight at 4°, then embedded in OTC media and cryosectioned at 8-10 μ m. Immunohistochemistry was performed using standard protocols and anti-Pecam (BD Pharmingen # 550274) and anti- β -galactosidase (MP Biomedicals # 55976).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Tbx4 expression and lineage in the hindlimb. **A-F:** Whole mount *in situ* hybridization of *Tbx4* expression in the hindlimb at indicated stages. hlf = hindlimb field, a = allantois, hl = hindlimb. **G-H:** Comparison of *Tbx4-Cre* lineage observed using the *R26R-lacZ* reporter (G) or the *R26R-YFP* reporter (H). lu = lung, fl = forelimb. **I-J:** Comparison of the appearance of *Tbx4* transcript with the *R26R-YFP* lineage reporter. At E10.0, *Tbx4* transcript reaches both the anterior and posterior borders of the hindlimb, indicated with red lines (I). At this stage, YFP fluorescence appears in a more limited domain and appears similar to the *Tbx4* expression domain at E9.5, suggesting a half a day delay in appearance of this marker (J). **K-L:** Whole mount β -galactosidase staining marking *Tbx4* lineage in the hindlimb at indicated stages. de = the medial extent of *Tbx4* lineage in the dermis, mu = medial extent of *Tbx4* lineage in the muscle. **M-W:** Sectioned embryos at indicated stages with *lacZ* staining marking the *Tbx4* lineage. Distal is oriented to the right in all panels except M, where dorsal is toward the top, and O, where distal is down. **M:** Transverse section showing both hindlimb fields, with the cloaca (cl) at the midline. **N:** Transverse section of an E13.5 hindlimb, where the cartilage condensations can be identified. Insert is detail of the distal tip of the hindlimb, showing uniformly stained mesenchyme and unstained epithelium. pe = pelvis, fe = femur, ti = tibia, au = autopod, hg = hindgut mesenchyme. **O:** Sagittal section through the entire hindlimb and pelvic region. il = ilium, fi = fibula, ph = phalanges. **P-Q:** Sagittal (P) and transverse (Q) sections through the hindlimb foot. Insert is a detail of the skin, showing that epidermal derivatives such as hair follicles are not *lacZ*-positive. **R:** Sagittal section of the proximal hindlimb. **S-T:** Frontal sections through the femur near the knee (S) and the quadriceps immediately proximal to it (T). quad = quadriceps, der = dermis. **U-W:** Transverse sections through the pelvic girdle and its attachment to the vertebral pedicles of the axial skeleton. il = ilium, vp = vertebral pedicles, pu = pubis, ps = pubic symphysis, ur = urethra, ce = cervix, hg = hind gut.

Fig. 2.

Tbx4 in the forelimb and sternum. **A-B:** *Tbx4* expression in core mesenchyme (c) of the forelimb. **C-D:** *Tbx4* expression in the forelimb autopod (au). **E-H:** Whole mount β-galactosidase staining marking *Tbx4* lineage in the forelimb. **E-F:** Side view of forelimb at indicated stages. elb = elbow, sh = shoulder. **G:** Ventral view of forelimb autopod. tn = tendon. **H:** Dissected tendon from panel G. mu = muscle. **I-N:** β-galactosidase-stained transverse sections of *Tbx4* lineage marker. hu = humerus, sb = shoulder blade, tn = tendon, u = ulna, sk = skin. **I-J:** Shoulder and elbow at E13.5. **K-N:** Elbow, wrist, carpals, and digit tip (respectively) at P1. **O:** Sagittal section of the anterior tip of the sternum (st). **P:** Sagittal section through the posterior extension of the sternum, the xyphoid process (xp).

Fig. 3.

Tbx4 expression and lineage in developing urogenital organs. **A-F:** *Tbx4* expression in the posterior ventral mesenchyme at indicated stages. View is ventral in A-E, anterior in F. Tails have been removed for clarity. **A-B:** Expression is contiguous between the allantois/umbilicus, hindlimbs, and nascent proctodeum. a = allantois, gf = genital folds, cl = cloacal slit, hlf = hindlimb field, umb = umbilicus, hl = hindlimb. **C:** Expression becomes more distal, and a region with no *Tbx4* expression (arrowhead) can be seen between the genital tubercle (gt) and the hindlimb. **D-F:** Progressively more distal expression continues in the developing genital tubercle through late gestation. ug = urethral groove. **G-Q:** β-galactosidase stained sections of *Tbx4* lineage trace embryos at indicated stages. Panels G-N and P are transverse sections, O and Q are sagittal. Ventral is oriented toward the top of the panel in G-H, ventral is toward the right in I-Q. **G-I:** At mid-gestation, the *Tbx4* lineage encompasses much of the ventral posterior mesenchyme, but not epithelial tissues. hl = hindlimb, up = urethral plate, gt = genital tubercle, ugs = urogenital sinus, uge = urogenital epithelium, hg = hindgut, ua = umbilical arteries, ep = surface epithelium, umb = umbilicus, mg = midgut. **J-M.** *Tbx4* lineage in posterior organs. Midgut staining appears in negative controls and is probably due to intestinal flora, not the *lacZ* reporter. mg = midgut, bl = bladder, ut = uterus, epi = bladder epithelium, blm = bladder mesenchyme, urac = urachus. **N-O:** *Tbx4* lineage in external genitalia. ure = urethra, pr = prepuce, gc = glans clitoridis, gp = glans penis. **P-Q:** Scattered *Tbx4*-lineage cells in male and female gonads. ovi = oviduct, ova = ovary, et = efferent tubules, te = testis.

Fig. 4.

Tbx4 expression and lineage in lung and heart. **A-C:** *Tbx4* expression in the lung at indicated stages. lb = lung bud, h = heart, li = liver, t = trachea. **D:** Whole mount β -galactosidase staining to mark the *Tbx4* lineage. **E-H:** Sectioned embryos stained for β -galactosidase to mark the *Tbx4* lineage. Section plane is transverse, ventral is down. **E:** Section through the larynx at the axial level where it is open to the pharynx (laryngeal aditus). la = laryngeal aditus, t = trachea, es = esophagus. **F:** Section immediately below the separation of the trachea from the pharynx. **G:** Section through the anterior lungs and heart at E13.5. le = lung epithelium, lm = lung mesenchyme, a = atria, v = ventricle. **H:** Section through the anterior lungs and heart at P1. b = bronchi, pv = pulmonary vessel.

Fig. 5.

Expression of *Tbx4* in the allantois. **A-I:** Whole mount in situ hybridization with *Tbx4* at stages indicated. Posterior is to the right in all panels except C', which is a posterior view of the embryo in C. Red arrowheads = allantois, LS = late streak stage, LB = late bud stage, LHF = late head fold stage, umb = umbilical vessels, fp = fetal placenta, e = embryo. **A':** Detail indicated by dashed lines in panel A. White circle indicates a region of brown shadowing of the proamniotic fold, which is distinct from the blue hybridization signal in the allantois. **J-L:** Sections from different levels (illustrated in F) of a 10-somite stage allantois from an embryo hybridized with *Tbx4*. am = amnion, all = allantois, m = mesothelium, cl = clusters, cv = central blood vessel of the allantois. **M:** Section of an umbilical cord hybridized with *Tbx4*. en = endothelium. **N-P:** Corresponding sections of an 8-somite stage allantois stained with anti-Pecam antibody. Fine black line indicates division between allantois and amnion. **Q.** Schematic of reciprocal *Tbx4* and *Pecam* expression in the distal allantois.

Fig. 6. *Tbx4* lineage in the allantois and umbilicus. **A-D:** Whole mount fluorescence (A) or lacZ staining (B-D) shows where *Tbx4-cre* has excised *R26R-YFP* (A) or *R26R-lacZ* (B-G). In C-D, the placenta was bisected near the umbilicus to expose a cross section. The allantois/umbilicus (red arrowheads) and fetal placenta (fp) show expression of both Cre reporters. Epc = ectoplacental cone, e = embryo, mp = maternal placenta. **E-G:** Sectioned embryos stained with β-galactosidase to mark the *Tbx4* lineage. **E:** At E9.5, the *Tbx4* lineage marks most of the allantois, including cells invading the chorion (ch). **F:** At E12.5, the umbilicus shows reporter expression throughout the mesenchyme, but the endothelial lining (green arrowhead) of the lumen (lu) is negative for lacZ. Open arrowhead indicates region where the plane of section has grazed the vessel wall and separated the endothelium from the underlying mesenchyme, allowing clear visualization of the unstained endothelium. **G:** In the placenta, some vessels are part of the *Tbx4* lineage (green arrowheads) while others are not (black arrowheads). **H-M:** Double antibody staining against β-galactosidase protein (red) and Pecam (green), Hoechst stain (blue) marking nuclei. **H-J:** In the umbilical vessels, the endothelial lining (green arrowhead) does not co-localize with the lineage reporter. Open arrowhead marks grazing section of vessel wall, as in panel F. **K-M:** In the placenta, endothelial cells from the *Tbx4* lineage (white arrowheads) and not from the *Tbx4* lineage (green arrowheads) are both observed.