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Recruitment of 5′ Hoxa genes in the allantois is essential for proper extra-embryonic function in placental mammals

Martina Scotti and Marie Kmita*

SUMMARY

The Hox gene family is well known for its functions in establishing morphological diversity along the anterior-posterior axis of developing embryos. In mammals, one of these genes, *Hoxa13*, is crucial for embryonic survival, as its function is required for the proper expansion of the fetal vasculature in the placenta. Thus, it appears that the developmental strategy specific to placental mammals is linked, at least in part, to the recruitment of *Hoxa13* function in developing extra-embryonic tissues. Yet, the mechanism underlying this extra-embryonic recruitment is unknown. Here, we provide evidence that this functional novelty is not exclusive to *Hoxa13* but is shared with its neighboring *Hoxa11* and *Hoxa10* genes. We show that the extra-embryonic function of these three Hoxa genes stems from their specific expression in the allantois, an extra-embryonic hallmark of amniote vertebrates. Interestingly, *Hoxa10-13* expression in the allantois is conserved in chick embryos, which are non-placental amniotes, suggesting that the extra-embryonic recruitment of *Hoxa10*, *Hoxa11* and *Hoxa13* most likely arose in amniotes, i.e. prior to the emergence of placental mammals. Finally, using a series of targeted recombination and transgenic assays, we provide evidence that the regulatory mechanism underlying Hoxa expression in the allantois is extremely complex and relies on several cis-regulatory sequences.

KEY WORDS: Hox genes, Endothelial cells, Enhancer, Placenta, Transcriptional regulation, Vascular progenitors, Mouse, Chick

INTRODUCTION

The Hox gene family is well known for its major role, conserved throughout the animal kingdom, in the establishment of the body architecture during embryogenesis (Kmita and Duboule, 2003; Krumlauf, 1994; Young and Deschamps, 2009). In addition to this ancestral function, Hox genes have been recruited in the course of evolution to achieve a variety of different functions, including the morphogenesis of evolutionarily novel structures (Pearson et al., 2005). The genome of most vertebrates contains 39 Hox genes physically grouped into four clusters referred to as the *HoxA*, *HoxB*, *HoxC* and *HoxD* clusters. Individual inactivation of the various Hox genes has revealed that *Hoxa13* is the only member of this gene family that is required for embryonic survival (Fromental-Ramain et al., 1996; Shaut et al., 2008; Stadler et al., 2001). Accordingly, mutants carrying deletion of the *HoxB*, *HoxC* or *HoxD* cluster are viable, at least until birth (Medina-Martinez et al., 2000; Spitz et al., 2001; Suemori and Noguchi, 2000). The lethality of *Hoxa13*$^{-/-}$ embryos is due to impaired expansion of the fetal vasculature in the placental labyrinth, which precludes adequate exchanges between maternal and fetal blood to ensure embryonic survival (Shaut et al., 2008). Thus, at least in mice, the function of *Hoxa13* is not restricted to the embryo proper. Importantly, it also suggests that the function of *Hoxa13* might have played a crucial role in the emergence of the developmental strategy that characterizes placental mammals. In this study we have addressed two key questions relevant to this role: how has *Hoxa13* been recruited in the extra-embryonic compartment and is this recruitment restricted to placental vertebrates?

We present evidence that *Hoxa10* and *Hoxa11*, the closest neighboring genes to *Hoxa13*, also contribute to the proper formation of the labyrinthine vasculature, indicating that extra-embryonic recruitment is not restricted to *Hoxa13*. We show that the extra-embryonic function of these 5′ Hoxa genes is linked to their expression in the allantois, a mesoderm derivative of the posterior primitive streak and hallmark of amniote embryos (Downs, 2009). Interestingly, we found that 5′ Hoxa genes are also expressed in the allantois of a non-placental amniote, suggesting that the extra-embryonic recruitment of 5′ Hoxa genes predates the emergence of placental vertebrates. Finally, our work reveals a specific transcriptional control underlying 5′ Hoxa extra-embryonic expression, and we propose that the emergence of the reproductive strategy of placental species was tightly linked to the evolution of Hoxa gene regulation.

MATERIALS AND METHODS

Mouse strains

HoxAflox, *Hoxa13null*, *Rosa26R*, *mT/mG*, *mox2Cre* and *CMV:Cre* lines were previously described (Dupe et al., 1997; Fromental-Ramain et al., 1996; Kmita et al., 2005; Muzumdar et al., 2007; Soriano, 1999; Tallquist and Soriano, 2000). The *HoxAdel* line was generated by crossing *HoxAflox* mice with *CMV:Cre* partners. The TAMERE approach (Herault et al., 1998) was used to generate *HoxAdelneo* and *del(5′)* mutants (M.K. and D. Duboule, unpublished). *HoxAdelneo* was obtained from meiotic recombination of the *HoxAflox* allele, and *del(5′)* from meiotic recombination between the *evx1flox* (gift of D. Goldman and G. Martin) and *HoxAflox* alleles. In the *Hoxa13Cre* allele, *Hoxa13* first exon is replaced by the *Cre:IRES:Venus* cassette (M.S. and M.K., unpublished). The *IR50* transgene was generated using the recombineering technique (Copeland et al., 2001). Transgenes *a* to *l* carry the chicken β-globin minimal promoter and a LacZΔCpG NLS reporter. *H19* insulators are located at both extremities of the transgenes. All transgenic embryos were generated by pronuclear injection.

Laboratory of Genetics and Development, Institut de Recherches Cliniques de Montréal (IRCM), Université de Montréal, 110 avenue des Pins Ouest, Montréal, Québec, H2W1R7 Canada.

*Author for correspondence (marie.kmita@ircm.qc.ca)

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In situ hybridization, immunohistochemistry and X-gal staining

Whole-mount in situ hybridizations were carried out using standard procedures (Kondo et al., 1998; Nieto et al., 1996). Chicken probes are as previously described (Burke et al., 1995). Mouse *Hoxa1* and *Hoxa13* probes are as described (Dupe et al., 1997; Warot et al., 1997). Probe templates for *Hoxa2*, *Hoxa3*, *Hoxa4*, *Hoxa5*, *Hoxa7*, *Hoxa9*, *Hoxa10* and *Hoxa11* were provided by J. Deschamps, C. Fromental-Ramain and B. Tarchini. The *hygromycin* probe was generated using the 600 bp *Eco*RI-*Hin*cII bacterial gene.

Immunohistochemistry was carried out on 10-12 μm cryosections according to standard procedures or on whole-mount specimens as previously described (Gregoire and Kmita, 2008). Antibodies against CD31 (BD Biosciences, 1:100) and β-galactosidase (Cappel, 1:1000) were used. The *mT/mG* Cre reporter allele expresses GFP at the cell membrane and thus direct GFP fluorescence was used for colocalization with CD31, which is also expressed at the cell membrane. X-gal staining of embryos and placentas was carried out as described by Downs and Harmann (Downs and Harmann, 1997) and for older specimens according to Zakany et al. (Zakany et al., 1988). Immunostaining on sections was imaged using a Zeiss LSM710 confocal microscope. For all analyses of placenta sections, we used only sections that encompassed the junction with the allantois/umbilical cord to ensure accurate comparison of the various placenta specimens. For each genotype and stage, analyses were performed on a minimum of three placentas.

RESULTS

Hoxa10 and *Hoxa11* together with *Hoxa13* contribute to the development of the labyrinthine vasculature

Inactivation of individual Hox genes in mice has revealed that *Hoxa13* is the only member required for embryonic survival (Fromental-Ramain et al., 1996; Shaut et al., 2008). Unexpectedly, whereas live *Hoxa13*$^{-/-}$ embryos can be recovered at embryonic day (E) 14.5 (Fromental-Ramain et al., 1996; Shaut et al., 2008), we found that embryos homozygous for the deletion of the entire *HoxA* cluster (referred to as *HoxAdel/del* hereafter) do not survive later than E12. As mid-gestation lethality is typically related to cardiovascular and/or placental defects (Copp, 1995) and mortality of *Hoxa13*$^{-/-}$ embryos is associated with placental dysfunction (Shaut et al., 2008), we hypothesized that the early lethality of *HoxAdel/del* mutants is the consequence of an exacerbated placental defect as compared with the single *Hoxa13* inactivation. Consistent with this assumption, abnormal placental morphology and marked reduction of the endothelium within the labyrinth are observed in all E10.5 *HoxAdel/del* placentas analyzed (Fig. 1), whereas *Hoxa13*$^{-/-}$ placenta remains undistinguishable from wild-type specimens until E11.5 (Shaut et al., 2008). Previous studies identified the requirement of *Hoxa10* and *Hoxa11* for the proper function of the uterus (Benson et al., 1996; Gendron et al., 1997; Satokata et al., 1995), raising the possibility that the more severe phenotype of *HoxAdel/del* placenta could be due to a combination of loss of *Hoxa13* function in the labyrinth and reduced *HoxA* dosage in the mother's uterus. However, epiblast-specific conditional inactivation of the *HoxA* cluster, using *HoxAflox* mice (Kmita et al., 2005) and the *mox2Cre* deleter strain (Tallquist and Soriano, 2000), resulted in the same placental phenotype as *HoxAdel/del* mutants (not shown), indicating that this phenotype is due to the loss of Hoxa genes in epiblast derivatives.

The vasculature of the labyrinth originates from the allantois (Inman and Downs, 2007; Rossant and Cross, 2001), a mesoderm derivative of the posterior primitive streak (Downs et al., 2004; Kinder et al., 1999; Lawson, 1999). Allantoic vascularization occurs de novo similarly to the embryo and yolk sac vascularization (Downs et al., 1998; Drake and Fleming, 2000).

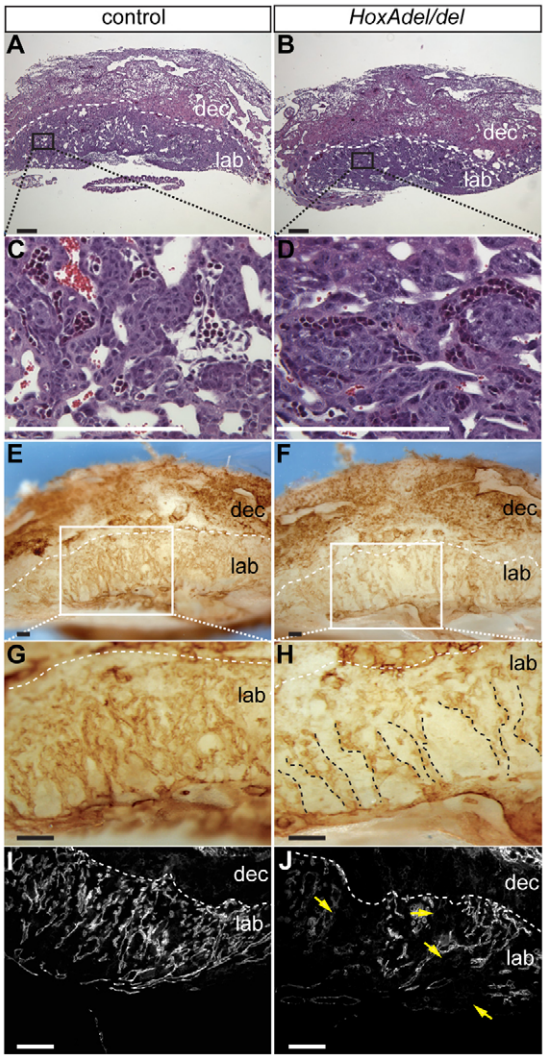

Fig. 1. Deletion of the *HoxA* cluster leads to impaired vasculature in the placental labyrinth. (**A-D**) Histology of wild-type (A) and *HoxAdel/del* mutant (B) mouse placentas at E10.5 as revealed by Hematoxylin and Eosin staining of paraffin sections. At high magnification, the mutant labyrinth (D) appears more compact than wild-type (C), which is likely to be a consequence of reduced fetal vasculature. (**E-H**) Whole-mount CD31 immunostaining of the vascular endothelium of wild-type (E) and mutant (F) hemi-placentas at E10.5. The boxed regions of E,F are magnified in G,H. In the wild-type placenta, the vasculature expands into the entire labyrinth (E,G). In the mutant (F,H), large labyrinth regions are deprived of vasculature and characterized by the absence of brown staining (H, black dashed lines delimit vasculature-deprived regions within the labyrinth). (**I**,**J**) Immunohistochemical analysis of CD31 expression on cryosections of wild-type (I) and mutant (J) placentas at E10.5 (*n*=11). Arrows point to the large regions deprived of endothelial cells in the mutant labyrinth. In all panels, white dashed lines mark the boundary between the labyrinthine region (lab) and the decidua (dec). Scale bars: 200 μm.

Following the initiation of vasculogenesis, the distal tip of the allantois fuses to the chorion and subsequent expansion of the distal allantoic vascular plexus within the chorionic plate gives rise to the fetal vasculature of the labyrinth (Inman and Downs, 2007; Rossant and Cross, 2001). To establish which Hoxa genes are involved in the development of the labyrinthine vasculature, we analyzed the

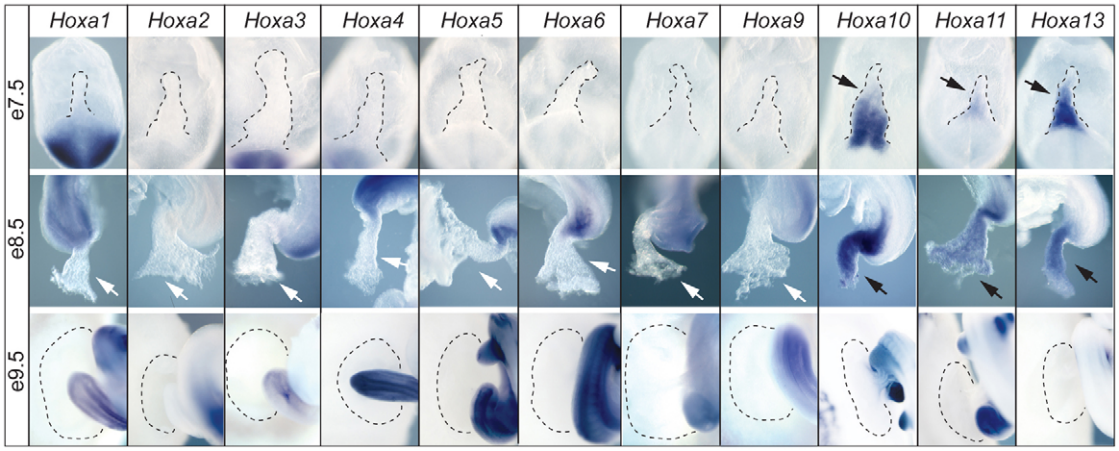

Fig. 2. *Hoxa10*, *Hoxa11* and *Hoxa13* are the only members of the *HoxA* cluster expressed in the allantois. Wild-type expression patterns of Hoxa genes as revealed by whole-mount in situ hybridization on E7.5 (top), E8.5 (middle) and E9.5 (bottom) mouse conceptuses. At E7.5 and E8.5, only the most 5′ genes (*Hoxa10*, *Hoxa11* and *Hoxa13*) are expressed in the allantois (black arrows). Note that these genes are expressed prior to genes located at more 3′ positions. At E8.5, all Hoxa genes are transcriptionally activated, but none of the group 1 to 9 Hoxa genes is expressed in the allantois (white arrows). By contrast, 5′ Hoxa expression in the allantois is barely, if at all, detectable in E9.5 allantois and there is no evidence for Hoxa expression in the labyrinth (bottom panel, dashed ovals).

expression of all Hoxa genes starting at allantoic bud stage (E7.5). As shown in Fig. 2, *Hoxa13* is expressed in the allantois together with its closest neighbors, *Hoxa10* and *Hoxa11*, both prior to and after chorio-allantoic fusion. Unexpectedly, this co-expression is transient and by E9.5 the extra-embryonic expression of *Hoxa10* and *Hoxa11* is only detected in the maternal part (decidua) and not in the labyrinth (Fig. 2 and supplementary material Fig. S1).

5′ Hoxa genes are expressed in progenitors of the labyrinthine vasculature

The early and transient co-expression of 5′ Hoxa genes suggests that the precocious vascular defect in *HoxAdel/del* placenta, when compared with the single *Hoxa13* loss of function, is due to the combined 5′ Hoxa inactivation in the allantois and/or nascent chorio-allantoic interface. However, at the stage of chorio-allantoic fusion, there is no apparent reduction of the endothelial cell population in *HoxAdel/del* allantois (supplementary material Fig. S2), thereby excluding impaired endothelial differentiation and/or expansion in the allantois as a cause for the labyrinthine phenotype. In turn, this result raises the possibility that 5′ Hoxa expression actually occurs in progenitor cells of the labyrinthine vasculature, but its functional outcome is only detectable at later stages of labyrinth development. In an attempt to clarify this issue, we investigated the fate of the allantoic cells expressing these genes. We used a mouse line driving expression of the Cre recombinase in all cells in which *Hoxa13* is normally expressed such that, in the presence of a *Cre* reporter transgene, *Hoxa13*-expressing cells and their descendants permanently express the reporter transgene. As genetic fate mapping is a three-step process (activation of *Cre* transcription, recombination of the reporter transgene and synthesis of the reporter protein), we first established the delay that exists between *Cre* transcriptional activation (i.e. *Hoxa13* activation) and the actual expression of the reporter protein. We found that the reporter protein is detectable 20-24 hours after the initial *Cre* transcription (not shown). To verify that the *Hoxa13Cre* allele is functional in all cells that normally express *Hoxa13*, we first looked at Cre reporter expression in developing limbs, where *Hoxa13* has been extensively studied and where, as in the allantois, its transcriptional activation occurs in mesenchymal cells. One day after *Hoxa13* transcriptional activation, Cre reporter expression is found in all mesenchymal cells of the distal limb buds (supplementary material Fig. S3A,B), providing evidence that our *Hoxa13Cre* allele is an efficient tool for tracing the fate of *Hoxa13*-expressing cells.

In the allantois, reporter expression is first detected at E8.25 (Fig. 3B), consistent with the delay between *Cre* transcriptional activation and Cre-mediated recombination, such that reporter expression at E8.25-8.5 highlights the fate of the first *Hoxa13*-expressing cells and their progeny (referred to as Hoxa13lin$^+$ cells hereafter). Interestingly, whereas *Hoxa13* is predominantly expressed in the proximal domain of the allantois at E7.5 (Fig. 3A), at E8.25-8.5 a large proportion of Hoxa13lin$^+$ cells are located at the chorio-allantoic interface/nascent labyrinth and only a few Hoxa13lin$^+$ cells are found in the proximal allantois (Fig. 3B). This early fate map indicates that a significant subset of cells in which *Hoxa13* is initially activated contributes to the formation of the labyrinth. Surprisingly, at E8.5, virtually none of these cells is of endothelial identity, as revealed by co-immunostaining for the endothelial marker platelet endothelial cell adhesion molecule 1 (Pecam1, also known as CD31; Fig. 3C-E). However, the proportion of Hoxa13lin$^+$ cells expressing CD31 (Hoxa13lin$^+$/CD31$^+$) increases progressively during embryogenesis (Fig. 4) and, at late gestation, all Hoxa13lin$^+$ cells are part of the fetal vasculature in the labyrinth, forming the labyrinthine endothelium as well as vascular smooth muscles that surround larger blood vessels at the base of the labyrinth (Fig. 4; data not shown). Consistent with the pool of Hoxa13lin$^-$/CD31$^+$ cells in the nascent labyrinth and undetectable *Hoxa13* expression beyond E9, the endothelium in the mature labyrinth is formed of both Hoxa13lin$^+$ and Hoxa13lin$^-$ cells (supplementary material Fig. S3C-E). In marked contrast, the endothelium of the mature umbilical cord is completely deprived of Hoxa13lin$^+$ cells, which are found exclusively adjacent to the endothelium and forming vascular smooth muscles (Fig. 4J-L). Together, these results show that endothelial differentiation of Hoxa13lin$^+$ cells takes place exclusively in the labyrinth and suggest that the ultimate fate of this cell population is influenced by extrinsic factors. However, *Hoxa13* appears

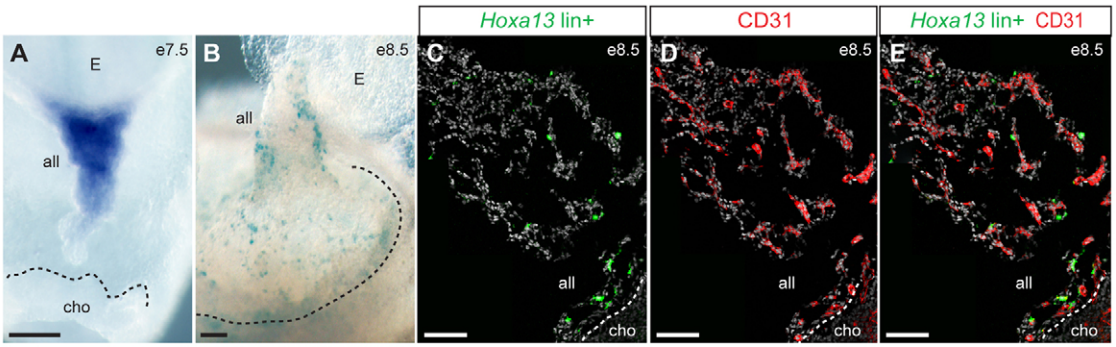

Fig. 3. Initial expression of *Hoxa13* does not occur in endothelial cells of the allantoic vasculature. (**A**,**B**) Whole-mount X-gal staining of *Hoxa13Cre/+;Rosa26R/+* mouse conceptus at E8.5 (B) reveals the fate of cells that have expressed *Hoxa13* at E7.5 (A). Note the significant proportion of Hoxa13lin$^+$ cells at the chorio-allantoic interface. (**C-E**) Immunostaining on allantois cryosections showing that most Hoxa13lin$^+$ cells (green) do not express the endothelial cell marker CD31 (red) at E8.5. The *mT/mG Cre* reporter allele expresses GFP at the cell membrane and was used for colocalization with CD31, which is also expressed at the cell membrane. Nuclei are labeled with DAPI (gray). Dashed lines highlight the limit between the allantois (all) and chorionic plate (cho). E, embryo. Scale bars: 100 μm.

dispensable for endothelial differentiation as the fate map of *Hoxa13*-expressing cells in the absence of Hoxa13 protein shows that the Hoxa13lin$^+$ cell population is reduced but remains capable of differentiating into endothelial cells (supplementary material Fig. S4).

Expression of 5′ Hoxa genes in the allantois is required for embryonic survival

Our fate map and in situ data suggest that 5′ Hoxa function in the proper expansion of the labyrinthine endothelium is associated with their expression in endothelial cell progenitors initially located in the allantois. As a consequence, gene inactivation after E8.5 should have little or no effect on the development of the labyrinthine vasculature. To identify the temporal requirement of 5′ Hoxa function, we took advantage of the spatial and temporal specificity of the *Hoxa13Cre* allele. Since *Hoxa13* coding sequence is disrupted in the *Hoxa13Cre* allele, we generated *Hoxa13Cre/HoxAflox* mutants in which *Hoxa13* inactivation occurs in all cells that normally express *Hoxa13* but with the 20- to 24-hour delay inherent to the Cre-mediated recombination. We found that *Hoxa13Cre/HoxAflox* mutants are fully viable and, accordingly, the vasculature of *Hoxa13Cre/HoxAflox* labyrinth is undistinguishable from that of wild-type specimens (Fig. 5A-C). This conditional inactivation has a distinct effect on limb development, during which *Hoxa13* expression is detectable over several days. Indeed, *Hoxa13Cre/HoxAflox* mice exhibit limb defects (Fig. 5D,E) that are reminiscent of the phenotype associated with complete *Hoxa13* inactivation (Perez et al., 2010), thereby demonstrating the efficiency of *Hoxa13Cre*-mediated inactivation of the *HoxAflox* allele. Together, these results provide evidence that transient *Hoxa13* expression in the allantois is sufficient to ensure proper vasculature development in *Hoxa13Cre/HoxAflox* labyrinth and survival of the embryo. Thus, expression of *Hoxa13* in the allantois up to the chorio-allantoic fusion stage is key for proper function of the placental labyrinth.

Extra-embryonic recruitment of 5′ Hoxa genes is specific to the allantois and is not restricted to placental mammals

The placental phenotype of both *Hoxa13*$^{-/-}$ (Shaut et al., 2008) and *HoxAdel/del* mutants (this study) provides evidence that 5′ Hoxa genes play a key role in the proper formation of the labyrinthine vasculature. By contrast, the vasculature in mutant and wild-type yolk sacs is indistinguishable (supplementary material Fig. S5A-D). Accordingly, analysis of the *Hoxa13Cre/+; Rosa26R* conceptus shows that Hoxa13lin$^+$ cells do not contribute to the formation of the yolk sac (supplementary material Fig. S5E), indicating that the extra-embryonic recruitment of Hoxa genes is specific to the allantois and its derivatives. Since the allantois is an extra-embryonic hallmark of amniote vertebrates, the recruitment of 5′ Hoxa genes in this tissue could have arisen prior to the emergence of placental species. To test this possibility, we investigated Hoxa expression in chick embryos. In this non-placental amniote, 5′ Hoxa genes are also specifically expressed in the allantois (Fig. 6A), indicating that extra-embryonic recruitment of 5′ Hoxa genes is not restricted to placental species.

Previous studies revealed that the allantois is a mesoderm derivative of the posterior primitive streak that buds and extends into the exocoelom (Downs et al., 2004; Kinder et al., 1999; Lawson, 1999). Knowing that vertebrate Hox genes are activated in epiblast cells prior to ingression through the primitive streak (Iimura and Pourquie, 2006), the possibility exists that the extra-embryonic expression of 5′ Hoxa genes is a mere collateral effect of the emergence of the allantois, i.e. the activation of 5′ Hoxa genes in the epiblast prior to formation of the epiblast-derived ‘appendage’ into the exocoelom. However, at early stages, Hoxa13lin$^+$ cells are located exclusively in the extra-embryonic compartment (Fig. 4A), indicating that the initial activation of 5′ Hoxa genes occurs in epiblast-derived cells only once these cells are already engaged in the extra-embryonic fate. This specificity suggests that the activation of 5′ Hoxa genes in the allantois is most likely independent of the mechanism underlying initial Hox activation in the embryo proper.

Transcriptional control of 5′ Hoxa genes in the allantois involves an enhancer-sharing mechanism

To gain insights into the mechanism underlying the recruitment of 5′ Hoxa genes in the allantois, we investigated whether it is linked to particular features of 5′ Hoxa promoters or is associated with an enhancer-sharing mechanism. We first investigated the expression of the transgene located at the 5′ end of the *HoxA* cluster in *HoxAflox* embryos. This transgene, which is located 3.5 kb from *Hoxa13*, contains the housekeeping phosphoglycerate kinase 1 (*Pgk1*; *PGK*) promoter, previously shown to respond to enhancer activity spanning the transgene insertion site (Herault et al., 1999).

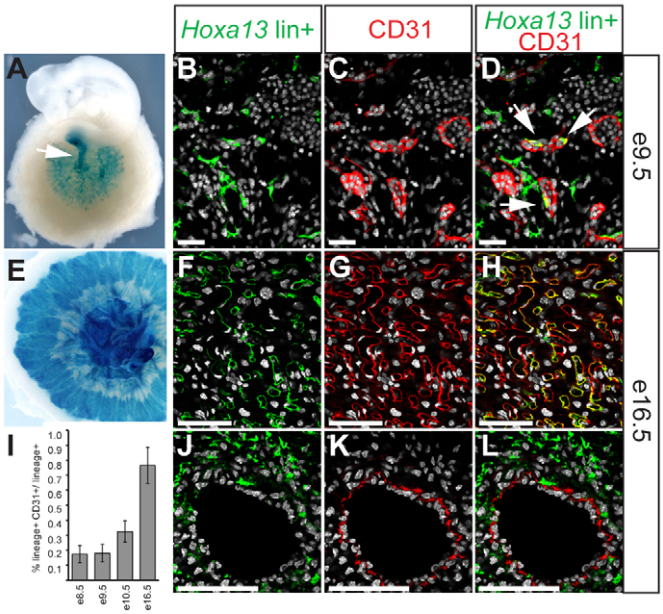

Fig. 4. Hoxa13lin$^+$ cells become progressively endothelial only in the labyrinth. (**A**) β-galactosidase activity reveals that Hoxa13lin$^+$ cells are restricted to the allantois and placental labyrinth. (**B-D**) Co-immunostaining for Hoxa13lin$^+$ cells and endothelial cells at E9.5. (**E**) Whole-mount X-gal staining of E16.5 mouse placenta. (**F-H**,**J-L**) Immunostaining showing both Hoxa13lin$^+$ and endothelial cells in mature placental labyrinth (F-H) and umbilical cord (J-L) at E16.5. *mT/mG Cre* reporter was used to mark Hoxa13lin$^+$ cells such that both the reporter protein and CD31 signals are targeted to the cell membrane, allowing unambiguous detection of protein colocalization (D, arrows). Nuclei are labeled with DAPI (gray). (**I**) Percentage of Hoxa13lin$^+$ signal colocalized with CD31$^+$ signal at distinct stages of labyrinthine development. Error bars indicate s.d. Scale bars: 30 μm.

When randomly inserted or targeted at the 5′ end of the *HoxD* cluster, this promoter has no detectable activity in the allantois (Kmita et al., 2000). By contrast, when targeted to the 5′ end of the *HoxA* cluster it becomes robustly expressed in the allantois (Fig. 6C), revealing the existence of an 'allantois' enhancer with activity that is shared between neighboring genes. Interestingly, this locus-specific expression persists in the absence of the *HoxA* cluster (Fig. 6D), suggesting that the enhancer is located outside the *HoxA* cluster. Yet, *Evx1*, the closest 5′ Hoxa neighboring gene outside the *HoxA* cluster, is not expressed in the allantois (Fig. 6E), raising the possibility that the 'allantois' enhancer is located within the *Hoxa13-Evx1* intergenic region but in the vicinity of *Hoxa13*. To test this hypothesis, we first generated transgenic mice carrying this 50 kb region linked to the *lacZ* reporter gene (*IR50* in Fig. 6F). Out of five independent lines, one failed to express the reporter but the four other lines showed *lacZ* expression in the allantois as well as the chorio-allantoic interface at E8.5 (Fig. 6F). Interestingly, at E9, the transgene is not expressed in the labyrinth and becomes downregulated in the allantois (Fig. 6F; data not shown), which is reminiscent of the 5′ Hoxa expression pattern. Together, these results show that the *Hoxa13-Evx1* intergenic region contains a regulatory element that is capable of activating gene expression in the allantois. To test whether this element is necessary and sufficient to drive the expression of 5′ Hoxa genes in the allantois, we analyzed the impact of deleting the endogenous *Hoxa13-Evx1* intergenic region. Unexpectedly, expression of 5′ Hoxa genes and of the *PGK* transgene remain detectable in the allantois of homozygous embryos carrying this deletion (*Del 5′*, Fig. 7), indicating the existence of additional regulatory element(s) underlying 5′ Hoxa expression in the allantois. Accordingly, *Del 5′* homozygous embryos survive until birth.

The presence of a transcriptional enhancer in the *Hoxa13-Evx1* intergenic region raises the possibility that the recruitment of 5′ Hoxa genes in the allantois originates from the appearance of an evolutionarily novel transcriptional regulatory element. Alternatively, this element might have already been functional in another tissue prior to the emergence of amniotes, and the presence of appropriate transcription factors in the allantois resulted in its functional co-option therein. Analysis of our *IR50* transgenic lines shows that the *Hoxa13-Evx1* intergenic region also triggers reporter gene expression in the tail bud and developing limbs (supplementary material Fig. S6, top), two domains where 5′ Hoxa genes are expressed. In an attempt to assess whether these expression domains rely on distinct or shared regulatory elements, we subdivided the 50 kb intergenic region into smaller DNA fragments, each one linked to the *lacZ* reporter gene driven by the β-globin minimal promoter (referred to as *β-lacZ*). To avoid variations in transgene expression due to position effects, each transgene was flanked with the *H19* insulator sequence. We generated 12 distinct transgenes (named *a* to *l* in supplementary material Fig. S6) and for each we analyzed at least five transgenic embryos at E8.5 and at least three at E12.5 (supplementary material Table S1). At E12.5, four of these transgenes trigger *lacZ* expression (supplementary material Fig. S6, transgenes *c*, *f*, *g* and *l*). Three of them show staining in limbs (supplementary material Fig. S6, transgenes *c*, *f*, *g*) that partially recapitulates the *IR50* expression pattern. We next analyzed these transgenes at E8.5 and did not detect any β-Gal staining, except for embryos carrying transgene *l*, in which staining is observed in the midbrain (not shown). These results suggest that regulatory elements capable of triggering gene expression in limbs are not functional in the allantois. We then tested expression of the other eight transgenes at E8.5, but, strikingly, none of them shows expression in the allantois or tail bud. Consistent with the lack of tail bud expression at E8.5, none of the E12.5 transgenic embryos expresses the *lacZ* reporter in the developing tail (supplementary material Fig. S6). Together, these results show that, whereas the entire *Hoxa13-evx1* intergenic region results in reporter expression in the allantois, tail bud and developing limbs, subdomains of this DNA fragment are only able to trigger reporter expression in limb buds when assayed individually.

DISCUSSION

The embryonic lethality resulting from impaired vascular development in the labyrinth of the *Hoxa13* mutant revealed that, in mice and possibly other vertebrate species, the function of Hox genes is not restricted to the embryo proper. This discovery raises the question of the evolutionary history underlying the extra-embryonic recruitment of *Hoxa13*. In this study, we used a combination of targeted genomic rearrangements, transgenesis and genetic fate mapping to gain insights into the transcriptional regulation underlying *Hoxa13* function in the placental labyrinth. The expression data, genetic fate mapping and conditional gene inactivation results presented here further reveal that the primary extra-embryonic function of *Hoxa13* relies on its expression in a subset of cells forming the allantois, well before defects in the labyrinthine vasculature are detectable in the *Hoxa13*$^{-/-}$ mutant. Interestingly, Cdx gene function in labyrinth development also relies on their expression in endothelial progenitors in the allantois (van

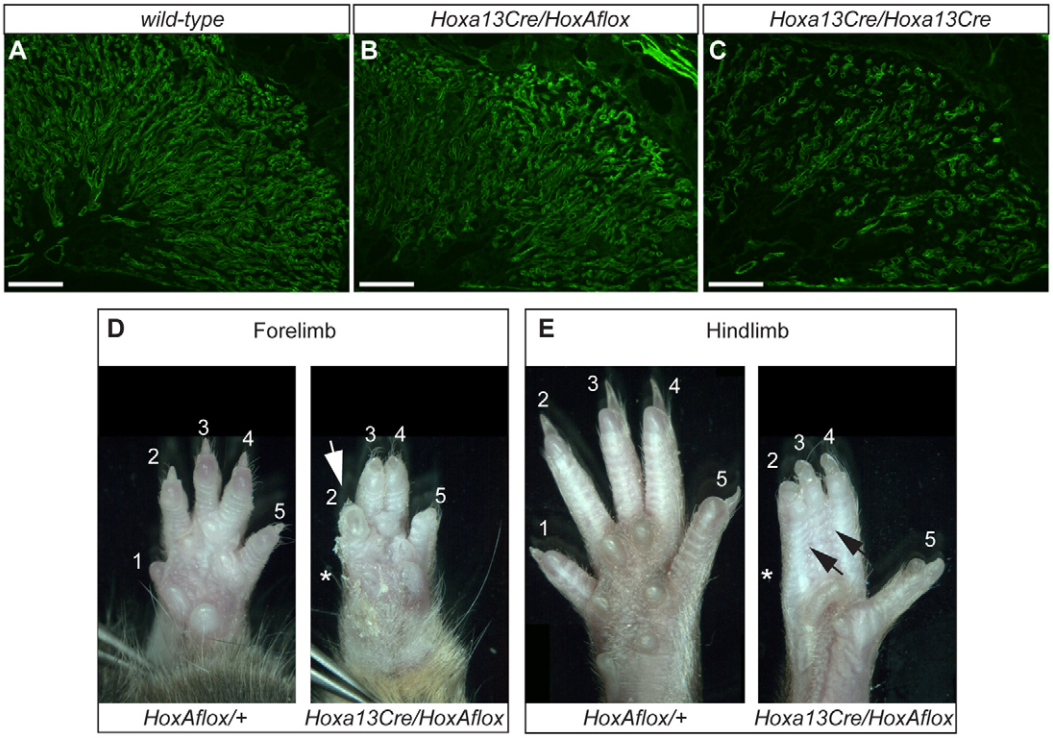

Fig. 5. Delay in the induction of *Hoxa13* inactivation is sufficient to ensure proper development of the labyrinth and survival of the embryo. (**A-C**) CD31 immunostaining on placenta cryosections at E14.5. *Hoxa13Cre/HoxAflox* labyrinthine vasculature (B) is comparable to that of wild type (A) and *Hoxa13Cre/Hoxa13Cre* mutant (C). (**D**,**E**) Forelimbs (D) and hindlimbs (E) of control (left, *HoxAflox/+*) and mutant (right, *Hoxa13Cre/HoxAflox*) mice at 6 months of age. Mutant limbs show a fully penetrant phenotype associated with the loss of *Hoxa13*, such as lack of digit 1 (asterisk), shortening and malformation of the other digits in the forelimb (white arrow) and fusion of digits 2, 3 and 4 in the hindlimb (black arrows). Scale bars: 200 μm.

Nes et al., 2006; Young et al., 2009) and reduced Cdx gene dosage results in a phenotype similar to that of the *HoxAdel/del* labyrinth. Such similarity between Cdx and Hox mutants is consistent with the role of Cdx proteins as regulators of Hox genes, as illustrated for some Hox genes during anterior-posterior patterning of the axial skeleton (reviewed by Young and Deschamps, 2009), and suggests that the role of Cdx genes in proper labyrinth formation is mediated, at least in part, by Hox genes.

Although the allantois contains progenitor cells of both labyrinthine and umbilical cord endothelium, those expressing *Hoxa13* do not contribute to the umbilical cord endothelium. This specificity could be explained by a non-cell-autonomous effect, whereby signaling from trophoblast cells would be required for endothelial differentiation of *Hoxa13*-expressing cells and their descendants. Consistent with this hypothesis, evidence has been obtained that cross-talk between trophoblast and allantois cells plays a key role in the development of the fetal vasculature in the labyrinth (Rossant and Cross, 2001). Of note, recent analysis of the fate map of *Tbx4*-expressing cells provided evidence for a key role of perivascular cells during vasculogenesis in the allantois (Naiche et al., 2011). However, in contrast to *Tbx4* (Naiche and Papaioannou, 2003), *Hoxa13* is dispensable for endothelial differentiation. Instead, our fate map shows a reduced Hoxa13lin^{+} cell population in *Hoxa13*$^{-/-}$ labyrinth, consistent with decreased expansion of the endothelial network.

The downregulation of *Tie2* (*Tek*), *Foxf1* and *Autotaxin* (*Enpp2*), which are *Hoxa13* target genes (McCabe and Innis, 2005; Shaut et al., 2008), was proposed to account for the reduced fetal vasculature in *Hoxa13*$^{-/-}$ labyrinth (Shaut et al., 2008). The function of *Autotaxin* and *Foxf1* is actually required in the allantois, where their inactivation prevents chorio-allantoic fusion and de novo vasculogenesis (Mahlapuu et al., 2001; van Meeteren et al., 2006). Our finding that cells forming the endothelium of the allantois/umbilical cord originate from cells in which *Hoxa13* is never expressed thus provides an explanation for proper formation of the endothelium in *Hoxa13*$^{-/-}$ allantois/umbilical cord. Nonetheless, this does not exclude the possibility that downregulation of *Autotaxin* and/or *Foxf1* in *Hoxa13*-expressing cells affects the development of the labyrinthine vasculature. Understanding the respective roles of *Autotaxin*, *Foxf1* and *Tie2* in the *Hoxa13*$^{-/-}$ labyrinth phenotype will require their conditional inactivation in *Hoxa13*-expressing cells.

Although endothelial cells in the allantois do not express *Hoxa13*, analysis of the *Hoxa13Cre/HoxAflox* mutant shows that the slight delay inherent to the Cre-mediated gene deletion is sufficient to ensure proper expansion of the fetal vasculature in the labyrinth and thus embryonic survival. This result suggests that *Hoxa13* expression in the allantois is crucial for subsequent development of the labyrinthine vasculature and is consistent with our in situ hybridization analysis showing that *Hoxa13*, as well as *Hoxa10* and *Hoxa11*, expression is only detectable until E9. The discrepancy between our expression data and that reported by Shaut et al. (Shaut et al., 2008) is likely to result from the difference in the experimental approach employed. Whereas we used whole-mount in situ hybridization to visualize *Hoxa13* transcripts, Shaut et al. analyzed the fluorescence of the *Hoxa13-*

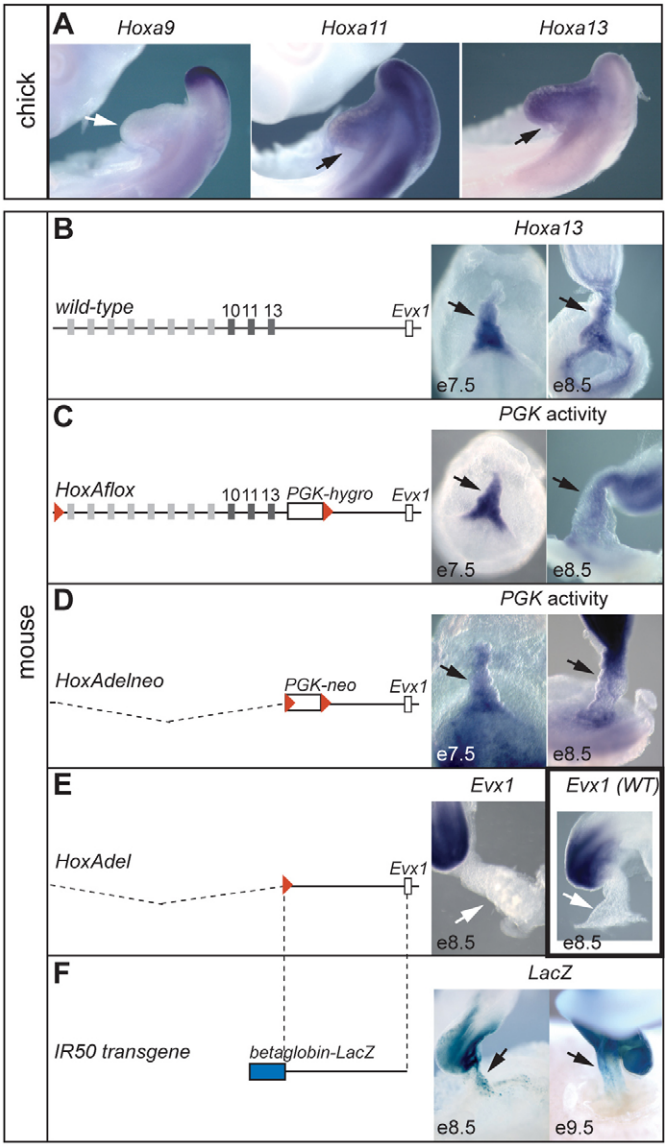

Fig. 6. Expression of 5′ Hoxa genes in chick allantois and evidence for a shared allantois enhancer in mice. (**A**) Whole-mount in situ hybridization on chick embryos at stage HH18. *Hoxa13* and *Hoxa11* are expressed in the allantois (black arrows), whereas *Hoxa9* is not (white arrow), illustrating that 5′ Hoxa expression in the allantois is not restricted to placental species. (**B-E**) Schematic representation of the wild-type *HoxA* cluster (B) and alleles carrying rearrangements or deletions within the *HoxA* cluster (C-E). For each allele, the expression pattern for 5′ Hoxa genes, *Evx1* or *PGK* transgenes is shown. (B) Wild-type expression of *Hoxa13*. (C) The *PGK* promoter is activated in the allantois when inserted at the 5′ end of the *HoxA* cluster (*HoxAflox* allele). (D) The *PGK* transgene remains expressed in the allantois even in the absence of the entire *HoxA* cluster (*HoxAdel*$^{neo+}$). (E) *Evx1* expression remains excluded from the allantois even when the *HoxA* cluster is deleted (*HoxAdel* allele). Wild-type expression of *Evx1* (black box). (**F**) The *IR50* transgene, which contains the 50 kb *Hoxa13-Evx1* intergenic region linked to the minimal promoter and *lacZ* reporter, is expressed in the allantois.

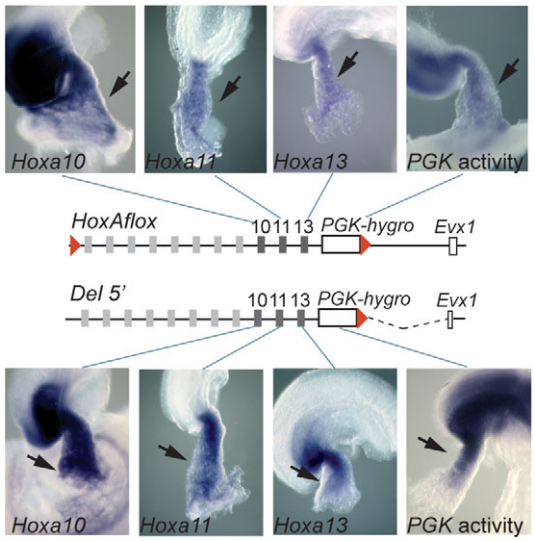

Fig. 7. Deletion of the *Hoxa13-Evx1* intergenic region does not prevent *Hoxa10*, *Hoxa11* and *Hoxa13* expression in the allantois. Whole-mount in situ hybridization for 5′ Hoxa genes or *PGK* transgenes in *HoxAflox/flox* (top) and *Del(5′)/Del(5′)* mouse embryos (bottom) at E8.5. These rearrangements in the vicinity of the *HoxA* cluster do not prevent 5′ Hoxa expression in the allantois, nor that of the *PGK* transgene. Note that deletion of the 50 kb *Hoxa13-Evx1* intergenic region (*Del 5′*) does not interfere with 5′ Hoxa expression in the tail bud. Arrows indicate to the allantois.

GFP allele, i.e. the protein produced by this targeted allele. Nevertheless, the proper labyrinth development in our conditional mutant, together with the genetic fate map of *Hoxa13*-expressing cells and the in situ data, indicate that the primary function of *Hoxa13* in the extra-embryonic compartment relies on its early expression in the allantois. As a consequence, implementation of the mechanism underlying *Hoxa13* transcriptional activation in the allantois was likely crucial for species requiring the function of a chorio-allantoic placenta to ensure embryonic survival. Our analysis also shows that *Hoxa10* and *Hoxa11* are co-expressed with *Hoxa13* in the allantois, indicating that extra-embryonic recruitment was not restricted to *Hoxa13*.

Analysis of several targeted rearrangements within and outside the *HoxA* cluster reveals that the mechanism underlying expression of these 5′ Hoxa genes in the allantois involves at least two transcriptional enhancers, one of which is located within the 50 kb *Hoxa13-Evx1* intergenic region. Surprisingly, subdivision of this intergenic region into smaller DNA fragments failed to recapitulate reporter gene expression in the allantois. A similar result was obtained for tail bud/trunk expression. By contrast, three of these overlapping transgenes were able to drive reporter expression in developing limbs, which recapitulates the limb enhancer activity of the entire 50 kb region (*IR50* transgene), thereby establishing that allantois and tail bud expression rely on cis-regulatory sequences distinct from those driving expression in limbs. Loss of reporter expression in the allantois and tail bud upon fragmentation of the *Hoxa13-Evx1* intergenic region raises the possibility that both expression patterns rely on the same regulatory sequences. In this view, the extra-embryonic recruitment of 5′ Hoxa genes could be the consequence of the functional co-option of the tail bud enhancer in the allantois, both tissues being epiblast derivatives. However, in contrast to the *IR50* transgene, 5′ Hoxa genes are expressed in the allantois but not in the tail bud, at least up to E8.5. Thus, if expression of the *IR50* transgene is driven by the same regulatory sequences in allantois and tail bud, absence of 5′ Hoxa expression in the tail bud implies the existence of a repression

mechanism that prevents activation of the 5′ Hoxa genes in this tissue, consistent with the recent finding that precocious expression of 5′ Hoxa genes in the tail bud is detrimental for the posterior elongation of mice embryos (Young et al., 2009). Nonetheless, the fact that allantois expression could not be triggered using fragments of the *Hoxa13-Evx1* intergenic region suggests that the integrity of this 50 kb region is required to drive reporter expression in the allantois. It is widely accepted that long-distance enhancer-promoter interaction involves chromatin looping. In this view, it is possible that both allantois and tail bud enhancers located in the *Hoxa13-Evx1* intergenic region require a defined three-dimensional chromatin organization to establish proper contacts with their target promoters. As a consequence, fractioning of the intergenic region would result in loss of proper chromatin organization, while the distance between the enhancer and the minimal promoter of the reporter might be too large to permit efficient transcriptional activation without chromatin looping. Consistent with this hypothesis, analysis of Hoxd gene regulation in developing limbs revealed that the underlying control is extremely complex and cannot be easily assessed by analysis of simple reporter transgenes (Tschopp and Duboule, 2011).

Although it remains to be established whether the recruitment of 5′ Hoxa function in the allantois was elicited by the co-option of tail bud enhancer(s) or the implementation of evolutionarily novel cis-regulatory sequences, the *Hoxa13*$^{-/-}$ and *HoxAdel/del* placental phenotypes suggest that 5′ Hoxa expression in the allantois is vital for the survival of mouse embryos and most probably for other placental species. Expression analysis in the allantois of chick embryos, which are non-placental amniotes, suggests that 5′ Hoxa extra-embryonic recruitment is likely to have occurred in amniotes, prior to the emergence of placental animals. It is thus likely that recruitment of 5′ Hoxa genes in the allantois has subsequently played a key role in the implementation of the developmental strategy that characterizes placental species. It will be of particular interest to investigate whether the regulatory mechanism controlling 5′ Hoxa expression in the allantois is conserved between placental and non-placental amniotes or whether it has evolved concomitantly with the emergence of placental species.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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Table S1. Summary of transgene analyses

Transgene (Tg)	Position		E8.5			E12.5		
	Start	End	Tg/total	Expressing/Tg (allantois)	Expressing/Tg (tail bud)	Tg/total	Expressing/Tg (limb)	Expressing/Tg (tail)
a	6:52214326	6:52215187	8/43	0/8	0/8	12/45	0/12	0/12
b	6:52215554	6:52216314	7/25	0/7	0/7	10/36	0/10	0/10
d	6:52216243	6:52220703	9/45	0/9	0/9	4/33	0/4	0/4
e	6:52220683	6:52221080	7/68	0/7	0/7	13/52	0/13	0/13
g	6:52226204	6:52226809	13/42	0/13	0/13	10/44	7/10	0/10
f	6:52225971	6:52231612	7/23	0/7	0/7	9/34	4/9	0/9
c	6:52215337	6:52227090	7/45	0/7	0/7	4/54	4/4	0/4
h	6:52231584	6:52240112	5/29	0/5	0/5	6/50	0/50	0/50
i	6:52240064	6:52243437	15/53	0/15	0/15	7/31	0/7	0/7
j	6:52241134	6:52251078	8/37	0/8	0/8	10/47	0/10	0/10
k	6:52250154	6:52258385	6/38	0/6	0/6	3/38	0/3	0/3
l	6:52258283	6:52264475	9/68	0/9	0/9	4/72	0/4	0/4