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LETTERS

Collinear activation of *Hoxb* genes during gastrulation is linked to mesoderm cell ingression

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The vertebral column exhibits segmentation and regionalization along the antero-posterior axis. During embryogenesis, the rhythmic production of the precursors of the vertebrae, the somites, imposes a segmented aspect to the spine, whereas the spine's regional differentiation is controlled by *Hox* genes[1,2]. Here we show that in the paraxial mesoderm, *Hoxb* genes are first activated in a temporal collinear fashion in precursors located in the epiblast lateral to the primitive streak. Our data suggest that collinear activation of *Hoxb* genes regulates the flux of cells from the epiblast to the streak and thus directly controls the establishment of the genes' characteristic nested expression domains in the somites. This suggests that establishment of the spatial co-linearity in the embryo is directly controlled by the *Hox* genes themselves.

The precursors of the vertebrae are segmented embryonic structures called somites. They derive from the presomitic mesoderm (PSM) located bilaterally on both sides of the neural tube in the posterior part of the embryo. At the anterior extremity of the PSM, pairs of somites form in a rhythmic fashion, thus establishing the metameric pattern of the embryonic axis. Depending on their location along the antero-posterior (AP) axis, somites will differentiate according to specific morphogenetic programs, resulting in the establishment of distinct anatomical domains like the cervical, thoracic, lumbar and sacral regions. This regionalization of the vertebral column along the AP axis is largely controlled by a group of transcription factors called *Hox* genes. In mammals, these genes are organized in four clusters containing a total of 39 genes[2]. Within each cluster, the genes are arranged in a sequence that reflects their order of expression during development (temporal co-linearity[3]) and the position of the anterior boundary of their expression domains along the AP body axis (spatial co-linearity[4,5]). This nested distribution of *Hox* genes along the AP axis results in each vertebral precursor being endowed with a unique combinatorial expression of *Hox* genes that controls their AP identity[6,7]. In amniote embryos, activation of genes along the *Hox* clusters occurs sequentially in the primitive streak and the tail bud during axis elongation[8].

We analysed the activation schedule of *Hoxb* genes in the paraxial mesoderm, in early chick embryos. *Hoxb1*, *Hoxb2*, *Hoxb3*, *Hoxb4*, *Hoxb7*, *Hoxb8* and *Hoxb9* were first detected in scattered cells in the epiblast adjacent to the posterior two-thirds of the primitive streak (Fig. 1a, d, g and j; top and bottom panels, Supplementary Fig. 1 and

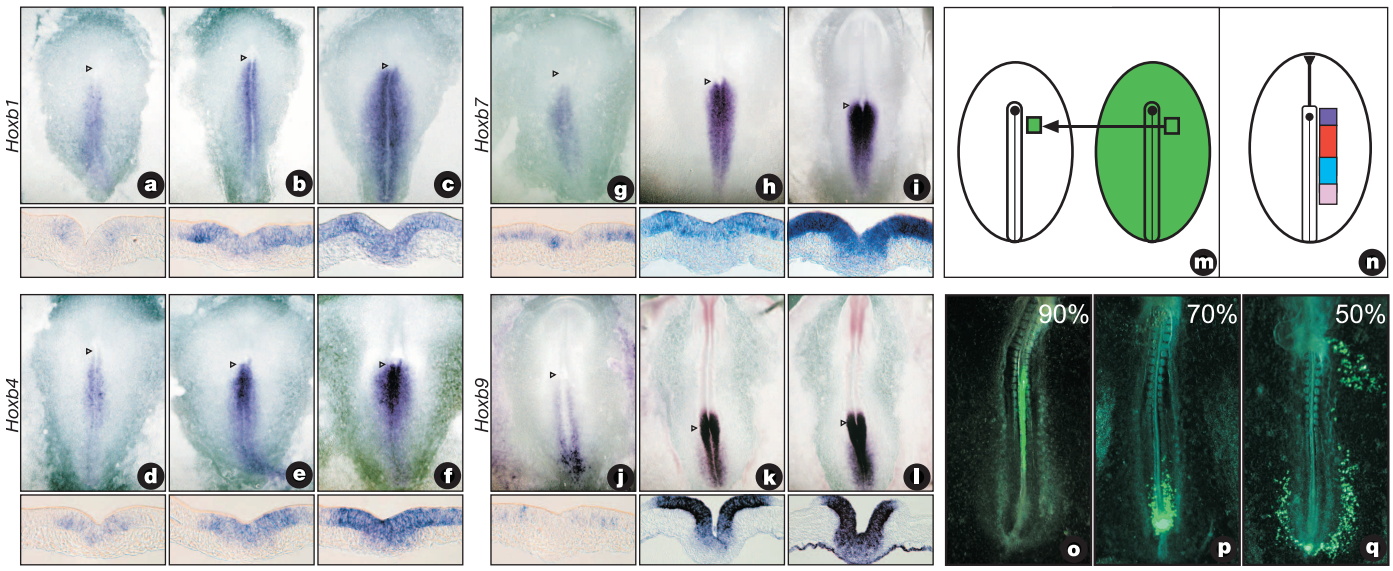

Figure 1 | *Hox* gene activation begins in the mesodermal territory of the epiblast. **a**–**l**, Top panels, whole-mount *in situ* hybridization showing the activation of *Hoxb1* (**a**, stage 3+HH; **b**, 4HH; **c**, 5HH), *Hoxb4* (**d**, 4HH; **e**, 5HH; **f**, 6HH), *Hoxb7* (**g**, 5HH; **h**, 6HH; **i**, 7HH) and *Hoxb9* (**j**, 7HH; **k**, 5-somite; **l**, 6-somite). Bottom panels, sections of embryos shown in top panels at the 70% level of the streak. Dorsal views, anterior to the top. Arrowhead marks the 90% streak level. **m**, Homotopic and homochronic grafting of fragments of EGFP-electroporated epiblast lateral to the primitive streak. **n**, Summary of the results of the epiblast fate mapping. Colours indicate the fate of the grafted tissue. Dark blue, neural; red, paraxial mesoderm; lighter blue, lateral plate mesoderm; pink, extraembryonic mesoderm. **o**–**q**, Embryos grafted as shown in **m** after 24 h of reincubation. A small fragment of labelled epiblast grafted adjacent to the 90% streak level mostly contributes to the neural tube (**o**); whereas, at the 70% level, cells are found in the paraxial mesoderm (**p**) and at the 50% level in the extraembryonic mesoderm and in the ectoderm (**q**). Ventral views, anterior to the top.

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data not shown). Double *in situ* hybridization with *Hoxb* genes and *Sox2*, which labels the neural plate, indicates that the genes are initially activated at a distance from the neural territory (Supplementary Fig. 1 and data not shown). The whole *Hoxb* cluster is activated in a collinear fashion in the epiblast in less than 12 hours between stages 4HH and 6HH (see Methods for stage nomenclature). Expression of the *Hoxb* genes subsequently appears to spread anteriorly and posteriorly to the entire lateral epiblast (Fig. 1 b, e, h and k; top and bottom), reaching the level of the anterior primitive streak (arrowhead in Fig. 1b, e, h, k; top) and the posterior neural plate (Fig. 1b, e, h, k; top). Expression was then seen in cells ingressing into the primitive streak (Fig. 1c, f, i, l; top and bottom) and was maintained in the mesodermal derivatives produced by these expressing cells (not shown). Similar kinetics of *Hox* activation have been observed in the frog and mouse embryos, suggesting that this expression sequence is conserved across vertebrates[9,10].

Because the fate of the epiblast adjacent to the primitive streak after the beginning of node regression is poorly characterized[11–13], we performed a series of homotopic grafts of enhanced green fluorescent protein (EGFP)-labelled fragments of different levels of the epiblast adjacent to the primitive streak between stages 4HH and 7HH (Fig. 1m). The anteriormost epiblast, which lies adjacent to the node and the anterior primitive streak, essentially gave rise to neural and ectodermal derivatives (Fig. 1n, o, $n = 14$). We found that the epiblast region lateral to the primitive streak at the 90% to 60% levels essentially contained paraxial mesoderm precursors (Fig. 1n, p, $n = 25$). Posterior to the 60% level of the streak, the epiblast derivatives were essentially found in the lateral plate or extra-embryonic mesoderm (Fig. 1n, q, $n = 11$). These observations indicate that ingression of somite precursor cells from the lateral epiblast continues long after the beginning of primitive streak regression. In the paraxial mesoderm, therefore, activation of the *Hoxb* cluster is first initiated in the somite precursors in the epiblast in a collinear fashion before their ingression into the primitive streak.

The activation kinetics of *Hox* genes in paraxial mesoderm precursors strikingly parallels the sequence of ingression of the epiblast into the primitive streak. To test if *Hox* genes control the ingression of epiblast cells into the streak, we grafted small fragments of the anterior streak overexpressing either *Hoxb1*, *Hoxb4*, *Hoxb7* or *Hoxb9* with a ZsGreen reporter into stage-matched embryos. We then scored the position of labelled descendants of grafted cells (Supplementary Table 1). In stage 5HH embryos grafted with an 80% primitive streak fragment overexpressing *Hoxb7* or *Hoxb9* constructs, labelled cells were found to extend more posteriorly than in embryos grafted with fragments overexpressing the *Hoxb1*, *Hoxb4* or a control construct (compare Fig. 2a–c with d, e). We then monitored the posterior distribution of labelled cells in grafted embryos incubated for a further 24-hour period (Fig. 2f–j). The *Hoxb1* and *Hoxb4* grafts did not contribute to the posteriormost end of the embryo, whereas overexpression of more posterior *Hox* genes resulted in labelled cells in the tail bud (compare Fig. 2g, h with i, j). Remarkably, the final distribution along the AP axis of the labelled descendants of the grafts overexpressing *Hoxb1* to *Hoxb9* was strictly collinear (Fig. 2b–e, g–j).

In order to directly compare the effect of different *Hox* genes in the same embryos, we orthotopically grafted at the same streak level two half-primitive streak domains coming from stage-matched embryos overexpressing either *Hoxb1*, *Hoxb4*, *Hoxb7*, *Hoxb9* or control constructs with ZsGreen or DsRed (Fig. 2k, l). We first compared the fate of grafts overexpressing *Hoxb4* labelled with DsRed with grafts overexpressing *Hoxb9* labelled with ZsGreen (Fig. 2l–p). After a short incubation of 6 hours, *Hoxb4* cells were already found in the PSM (Fig. 2m, red), while the *Hoxb9*-expressing cells were still located at the level of the primitive streak (Fig. 2m, green). Analysis of transverse sections demonstrated that cells overexpressing *Hoxb9* were still located in the epiblast layer and that most retained a characteristic epithelial columnar shape (Fig. 2p, green); in contrast, a large fraction of cells expressing *Hoxb4* had lost their epithelial characteristics and were located in the primitive streak and the ingressed mesoderm (Fig. 2p, red). When embryos were analysed after a 16-hour incubation period, the red-labelled cells were always found to extend several somites anterior to the green-labelled cells (Fig. 2n, o, $n = 6$). When the embryos were incubated an additional 24 hours, only the green-labelled cells were found to extend to the posterior tip of the embryo (Fig. 2o). Similar results were observed with double grafts comparing *Hoxb1*-overexpressing cells labelled with DsRed, and *Hoxb7*-overexpressing cells labelled with ZsGreen (data not shown).

We then grafted orthotopically at an anterior position along the primitive streak a half-primitive streak fragment from a *Hoxb9-DsRed* overexpressing donor and on the same side but slightly more posteriorly, a half-primitive streak from a *Hoxb1-ZsGreen* donor (Fig. 2q, r, $n = 4$). After a 4-hour reincubation period, a large number of descendants of the *Hoxb1*-expressing cells had already

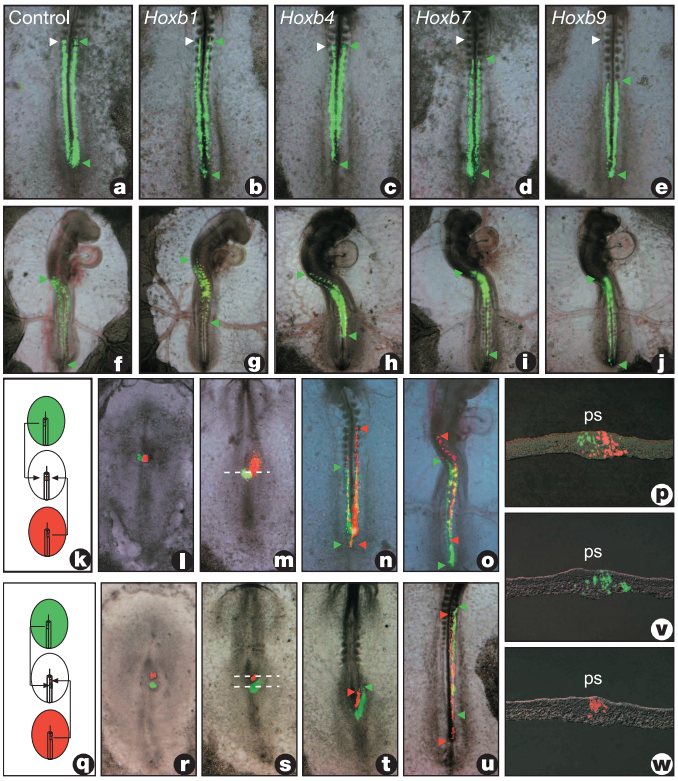

Figure 2 | *Hox* genes control the timing of ingression of epiblast cells into the primitive streak. **a–j**, Homotopic and homochronic grafts of fragments of the 80% level of the primitive streak electroporated with pCIZ control vector (**a**, **f**), or *Hoxb1* (**b**, **g**), *Hoxb4* (**c**, **h**), *Hoxb7* (**d**, **i**), *Hoxb9* (**e**, **j**) expression vectors driving internal ribosome entry site 2 *(IRES2)-ZsGreen* expression following a reincubation period of 16 h (**a–e**) or 40 h (**f–j**). White arrowheads mark the fifth somite level. **k**, Homotopic and homochronic double graft of fragments of the 80% level of the primitive streak from embryos electroporated with *Hoxb4-IRES2-DsRed* and *Hoxb9-IRES2-ZsGreen*, respectively. The grafted embryo is shown just before reincubation (**l**), and 6 h (**m**), 16 h (**n**) and 40 h (**o**) after reincubation. **p**, Section of an embryo grafted as in **k** incubated for 6 h at the level indicated in **m** (white hatched line). **q**, Homotopic and homochronic double graft of fragments of the 80% level of the primitive streak from an embryo electroporated with *Hoxb9-IRES2-DsRed* and of the 70% primitive streak level from an embryo electroporated with *Hoxb1-IRES2-ZsGreen*, respectively. The grafted embryo is shown just before reincubation (**r**), and 4 h (**s**, **v**, **w**), 8 h (**t**) and 28 h (**u**) after reincubation. Hatched lines in **s** indicate the level of transverse sections shown respectively in **v** and **w**. Green and red arrowheads mark the anterior and posterior extension of the descendants of the labelled-grafted cells by each reporter along the AP axis. **a–j**, **l–o**, **r–u**, Ventral views, anterior to the top. ps, primitive streak.

ingressed into the mesoderm (Fig. 2s, v, green); in contrast, most of the *Hoxb9*-expressing cells were still found in the epiblast layer of the primitive streak (Fig. 2s, w, red). After 8 hours, some *Hoxb1*-overepressing cells were found anterior to the *Hoxb9*-overexpressing cells (Fig. 2t). After a 28-hour reincubation period, the *Hoxb1*-overexpressing cells that had been grafted posteriorly were found to extend more anteriorly than the *Hoxb9*-overexpressing cells (Fig. 2u). In similar experiments in which only control red and green reporters were electroporated, cells grafted anteriorly in the streak were always found to lie more anterior in the somitic series (data not shown, Supplementary Table 1). These experiments indicate that overexpressing more posterior *Hox* genes in epiblast cells can delay the timing at which cells ingress from the epiblast into the primitive streak and the nascent mesoderm. This alters the fate of paraxial mesoderm cells along the AP axis, leading the cells to become more posterior than they normally would.

We then developed a procedure to successively electroporate two different constructs labelled with two distinct colours in the anterior primitive streak of chick embryos. This protocol results in essentially distinct cells being transfected with each construct. When we successively electroporated *Hoxb9-ZsGreen* and *Hoxb1-DsRed*, or *Hoxb9-ZsGreen* and a *DsRed*-expressing vector in the primitive streak, the descendants of the cells overexpressing *Hoxb9* were located in a more posterior region than the cells overexpressing *Hoxb1* or the control *DsRed* vector (compare the position of green and red arrowheads in Fig. 3a and data not shown). In all the combinations of *Hox* genes analysed, the descendants of the cells expressing the more 5′ gene were always located in a more posterior domain (Supplementary Table 2). Analysis of sections shows that ingression of the cells overexpressing *Hoxb9* (Fig. 3b, green) was delayed compared to cells overexpressing *Hoxb1* (Fig. 3b, red). This effect on cell ingression requires a functional homeodomain, since deleting the helix 3 of the *Hoxb9* homeodomain completely suppressed the delaying effect (Fig. 3c, red) compared with the full length *Hoxb9* overexpression (Fig. 3c, green).

The different roles of *Hox* genes have been proposed to reflect quantitative rather than qualitative variations of expression of these genes[14]. To evaluate the impact of quantitative variations of the same gene on the ingression process, we compared the effect of two *Hoxb9* constructs driven either by the strong CAGGS promoter (β-actin promoter and CMV enhancer) and by the weak thymidine kinase promoter (HSV-TK)[15]. Whereas a bright green signal was detected when *pCAGGS-Hoxb9-ZsGreen* was overexpressed in the chick PSM, very weak or no fluorescent signal was detected from the *pTK-Hoxb9-ZsGreen*, indicating that the two promoters drive very different expression levels in this tissue (data not shown). In order to follow the cells overexpressing the *pTK-Hoxb9-ZsGreen* in the embryo, we mixed the plasmid with a *pCAGGS-EGFP* vector. This co-electroporation procedure resulted in the vast majority of cells co-expressing the two vectors. We then electroporated cells sequentially with *pCAGGS-Hoxb9-DsRed* and with *pTK-Hoxb9-ZsGreen* mixed with *pCAGGS-EGFP* as described above. The green and red cells were found to extend to the same anterior level (Fig. 3d), indicating that the low level of *Hoxb9* expression driven from the *TK* promoter results in the same phenotype as the high level of *Hoxb9* expression from the CAGGS promoter. Similar observations were made with *Hoxb7* or when other weaker promoters like SV40 were compared to the CAGGS promoter[16] (Supplementary Table 2). This indicates that the dose of *Hoxb* gene expression has no significant influence on the final position of the cell along the AP axis.

The results described above also suggest that the 5′ *Hox* genes are functionally dominant over the 3′ *Hox* genes, consistent with the posterior prevalence of *Hox* genes[17]. To test this directly, we compared the distribution of labelled descendants from 80% level streak grafts overexpressing either *Hoxb4* or *Hoxb9* alone or co-expressing the two genes (Supplementary Table 1). Cells co-expressing *Hoxb4* and *Hoxb9* in somites extend to a similar anterior level to cells expressing *Hoxb9* alone, which lie several somites posterior when compared to cells expressing only *Hoxb4* (compare Fig. 3e–g). A similar effect was observed when co-expressing *Hoxb1* and *Hoxb7* (data not shown), indicating that posterior genes are able to suppress the effect of more anterior *Hox* genes.

These results imply that heterochronic grafts of primitive streak fragments, which normally express different combinations of *Hox*

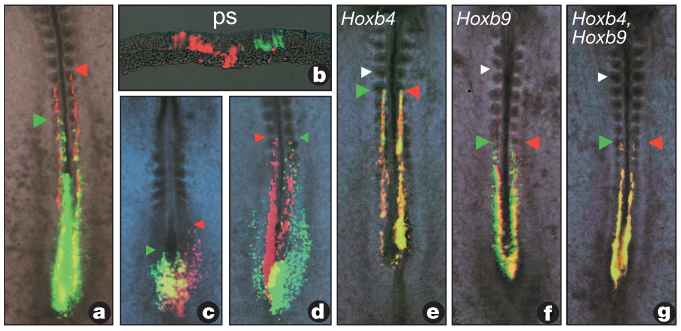

Figure 3 | Posterior prevalence of *Hoxb* genes. **a**, Embryo successively electroporated at the anterior primitive streak level at stage 5HH with *Hoxb9-ZsGreen* and *Hoxb1-DsRed*, 16 h after reincubation. **b**, Transverse section at the same level as the embryo shown in Fig. 2m (hatched line) of an embryo electroporated sequentially with *Hoxb1-IRES2-DsRed* and *Hoxb9-IRES2-ZsGreen* fixed 4 h after reincubation. **c**, Embryo successively electroporated with C-terminal-deleted-*Hoxb9-DsRed* and *Hoxb9-ZsGreen* at 6HH, 5 h after reincubation. **d**, Embryo successively electroporated with *Hoxb9* driven by the CAGGS promoter (in red) and *Hoxb9* driven by the TK promoter (in green) at 5HH, shown 16 h after reincubation. **e**–**g**, Co-electroporation of the anterior primitive streak region at 5HH with two constructs; **e**, *Hoxb4-DsRed* and *ZsGreen* vector, **f**, *DsRed* vector and *Hoxb9-ZsGreen*, and **g**, *Hoxb4-DsRed* and *Hoxb9-ZsGreen*. Green and red arrowheads mark the anterior extension of the descendants of the labelled grafted cells by each reporter along the AP axis. **a**, **c**–**g**, Ventral views, anterior to the top. ps, primitive streak.

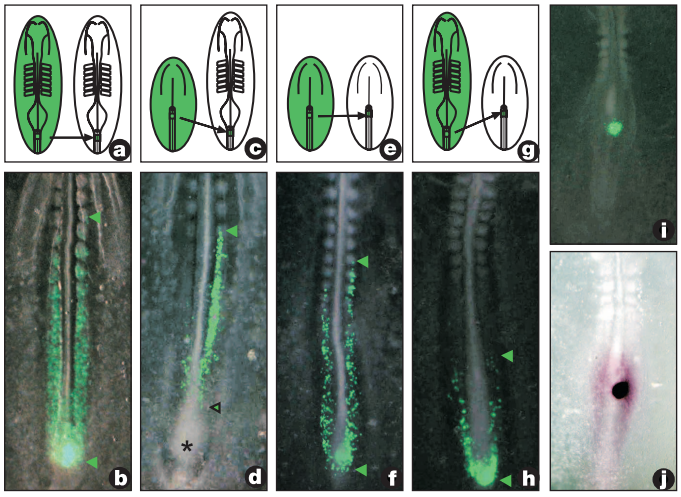

Figure 4 | Heterochronic grafts of the primitive streak. **a**, Homotopic and homochronic 80% level primitive streak graft from an EGFP-labelled 6-somite stage embryo. **b**, 20 h after incubation. **c**, Homotopic and heterochronic 80% level primitive streak graft from a stage 6HH EGFP-labelled donor to a 6-somite stage host. **d**, 20 h after incubation. Tail bud is marked by an asterisk. **e**, Homotopic and homochronic 80% level primitive streak graft from an EGFP-labelled 6HH embryo. **f**, 20 h after incubation. **g**, Homotopic and heterochronic 80% level primitive streak graft from a 6-somite stage EGFP-labelled donor to a 6HH host. **h**, 20 h after incubation. **i**, 6 h after incubation. **j**, *Hoxb9 in situ* hybridization of the embryo shown in **i**. Ventral views, anterior to the top. Green arrowheads mark the anterior and posterior extension of the descendants of the labelled-grafted cells along the AP axis.

genes, should result in predictable outcomes. When a streak fragment originating from a younger host was grafted homotopically into the primitive streak of an older host, labelled cells reached a similar anterior level to homochronic control grafts (Supplementary Table 3; compare Fig. 4a, b and c, d), but no cells were observed in the tail bud (compare Fig. 4b and d). In contrast, when a primitive streak fragment from an older embryo was grafted into a younger host, labelled cells were found to extend from the tail bud to a level located more posteriorly by up to 6 somites than the level of grafted cells in stage-matched controls (Supplementary Table 3; compare Fig. 4e, f and g, h). In grafts from an older primitive streak, no emigration of cells from the grafted tissue was observed following a 6-hour reincubation period (Fig. 4i), consistent with the strong expression of posterior *Hox* genes like *Hoxb9* which was retained in the grafted cells (Fig. 4j). These findings are consistent with earlier observations in the mouse showing that heterochronic grafts of older tail bud into earlier mouse embryos result in grafted cells being located more posteriorly along the AP axis than expected[18]. Even though there might be more differences between an old and a younger graft from the same streak level than the mere expression of *Hox* genes, these observations support the idea that *Hox* genes expressed in the grafted territories at these stages control the ingression of somite derivatives from the graft.

Our data therefore suggest that at a defined time point, ingression of epiblast cells is controlled by the most 5′ *Hox* genes expressed by these cells. When the next more 5′ paralogous *Hox* gene becomes activated in a subpopulation of these epiblast cells, these cells will acquire slightly different migratory properties, and their ingression will be slightly delayed. Expression of this *Hox* gene rapidly spreads to the surrounding cells that now exhibit similar migratory properties until expression of the next 5′ *Hox* gene begins in scattered epiblast cells, altering their migratory properties once again. Overall, this process would ensure that ingressing cells expressing *Hox* genes from consecutive paralogous groups will sort out from each other along the AP axis. This ordered ingression of epiblast cells in the streak probably constitutes an initial step for the establishment of the nested *Hox* expression domains of the mesoderm (that is, the spatial co-linearity).

METHODS

Embryos and nomenclature. Fertilized chick eggs were obtained from commercial sources. Eggs were incubated at 38 °C in a humidified incubator, and embryos were staged according to Hamburger and Hamilton (HH)[19] and by counting somites.

RNA *in situ* hybridization and probes. Whole mount RNA *in situ* hybridizations were carried out as described[20]. Chicken *Hoxb1*, *Hoxb2*, *Hoxb3*, *Hoxb4*, *Hoxb5*, *Hoxb6*, *Hoxb7*, *Hoxb8* and *Hoxb9* probes have been described[21]. Two-colour *in situ* hybridization is described in Supplementary Information. Some embryos were embedded for cryosection and sliced at 14 μm.

Electroporation and fate mapping of the epiblast. Embryos ranging from stage 3HH to stage 7HH were prepared for EC culture[22]. *In vitro* electroporations were carried out as described in Supplementary Information. Small fragments of electroporated epiblast with unlabelled underlying mesoderm and endoderm were excised and grafted into the same position of stage-matched unlabelled host embryos[23]. After reincubation, the position of the fluorescent cells in the embryos was scored.

Hox expression constructs. Hox expression vectors were generated in expression vectors driven by different promoters including *pCIZ* and *pCIRX*, which are originally derived from the *pCAGGS* expression vector[24].

More detailed experimental procedures are available in Supplementary Information.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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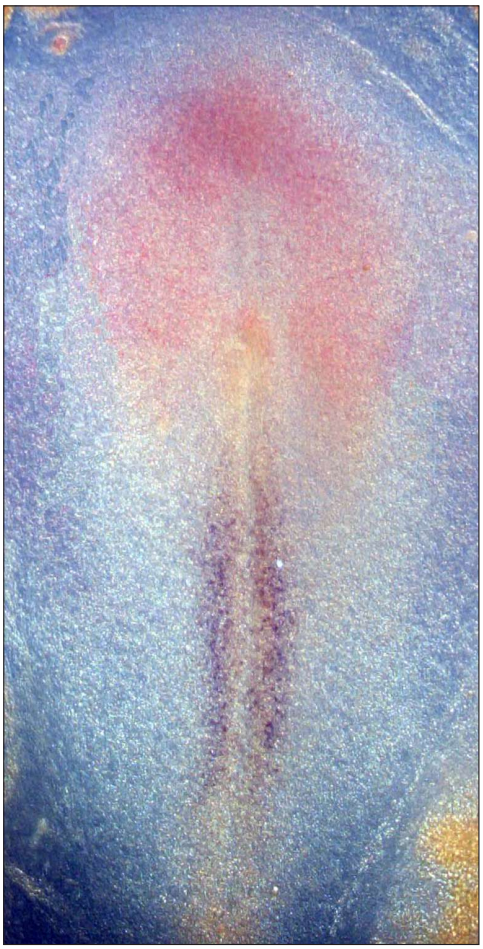

Iimura et al, supplementary Figure 1

Onset of Hoxb7 expression (blue) is in the epiblast adjacent to the primitive streak and this Hox domain is at a distance from the neural territory marked by Sox2 (red).

Supplementary Table 1

Electroporation and Graft

Electroporated	Grafted at		Distribution of Grafted Cells		End of Incubation
Constructs	Stage, HH	Streak Level	Rostral-Most Somite	Caudal-Most Somite	Last-Formed Somite
Vector	St5	80%	5	21	30
Vector	St5	80%	5	28	28
Vector	St5	80%	5	22	28
Vector	St5	80%	5	19	27
Vector	St5	80%	6	25	28
Vector	St5	80%	6	23	28
HoxB1	St5	80%	5	23	24
HoxB1	St5	80%	4	20	26
HoxB1	St5	80%	6	23	28
HoxB1	St5	80%	5	23	28
HoxB4	St5	80%	5	PSM	25
HoxB4	St5	80%	5	PSM	26
HoxB4	St5	80%	5	25	28
HoxB4	St5	80%	5	27	27
HoxB4	St5	80%	6	26	28
HoxB4	St5	80%	6	21	28
HoxB4	St5	80%	5	24	28
HoxB4	St5	80%	5	23	28
HoxB7	St5	80%	7	PSM	25
HoxB7	St5	80%	7	TB	24
HoxB7	St5	80%	8	TB	25
HoxB7	St5	80%	7	PSM	25
HoxB7	St5	80%	9	TB	24
HoxB7	St5	80%	8	TB	26
HoxB7	St5	80%	8	TB	27
HoxB7	St5	80%	7	TB	27
HoxB7	St5	80%	7	TB	28
HoxB7	St5	80%	8	TB	30
HoxB9	St5	80%	9	TB	28
HoxB9	St5	80%	9	TB	30
HoxB9	St5	80%	9	TB	26
HoxB9	St5	80%	10	TB	28
HoxB9	St5	80%	11	PSM	27
HoxB9	St5	80%	9	TB	28
HoxB9	St5	80%	9	TB	29
HoxB9	St5	80%	9	TB	29
HoxB9	St5	80%	8	TB	28
HoxB9	St5	80%	9	TB	26
HoxB9	St5	80%	9	TB	28
HoxB9	St5	80%	10	TB	28
HoxB9	St5	80%	9	TB	28

Supplementary Table 1 (cont'd)

Electroporated Constructs	Grafted at Stage, HH	Streak Level	Distribution of Grafted Cells		End of Incubation
			Rostral-Most Somite	Caudal-Most Somite	Last-Formed Somite
Vector	St6	80%	9	PSM	30
Vector	St6	80%	8	PSM	30
HoxB1	St6	80%	9	21	25
HoxB1	St6	80%	9	25	28
HoxB1	St6	80%	9	25	27
HoxB1	St6	80%	9	24	26
HoxB1	St6	80%	9	19	30
HoxB1	St6	80%	8	26	27
HoxB4	St6	80%	8	28	28
HoxB4	St6	80%	7	28	28
HoxB4	St6	80%	7	22	30
HoxB7	St6	80%	11	TB	27
HoxB9	St6	80%	11	TB	28
HoxB9	St6	80%	11	TB	28

Co-Electroporation & Graft

Electroporated Constructs	Grafted at Stage, HH	Streak Level	Distribution of Grafted Cells		End of Incubation
			Rostral-Most Somite	Caudal-Most Somite	Last-Formed Somite
B1+B7	St5	80%	8	PSM	26
B1+B7	St5	80%	8	PSM	27
B4+Vector	St5	80%	6	23	26
B4+Vector	St5	80%	6	24	28
B4+Vector	St5	80%	6	23	28
B4+Vector	St6	80%	7	22	27
B4+B9	St5	80%	9	TB	30
B4+B9	St5	80%	10	TB	28
B4+B9	St5	80%	9	TB	28
B4Rx-B9Zs	St6	80%	12	TB	28
B9+Vector	St5	80%	8	TB	26
B9+Vector	St5	80%	9	TB	28
B9+Vector	St5	80%	10	TB	28
B9+Vector	St5	80%	9	TB	28
B9+Vector	St5	80%	11	TB	28
B9+Vector	St5	80%	11	TB	30

Supplementary Table 1 (cont'd)

Double Grafts Experiments

Electroporated Constructs	Grafted at Stage, HH	Streak Level	Distribution of Grafted Cells		End of Incubation
			Rostral-Most Somite	Caudal-Most Somite	Last-Formed Somite
B1-DsRed-Exp.	St5	80%	5	23	24
B7-ZsGreen		80%	9	TB	
B1-DsRed-Exp.	St5	80%	4	20	26
B7-ZsGreen		80%	8	TB	
B1-DsRed-Exp.	St6	80%	8	26	27
B7-ZsGreen		80%	11	TB	
B4-DsRed-Exp.	St5	80%	5	24	28
B9-ZsGreen		80%	10	TB	
B4-DsRed-Exp.	St5	80%	5	23	28
B9-ZsGreen		80%	9	TB	
B4-DsRed-Exp.	St6	80%	8	28	28
B9-ZsGreen		80%	12	TB	
B1-DsRed-Exp.	St5	80%	PSM	Streak	HH St7
B7-ZsGreen		80%	Streak/Epiblast	Streak	
B1-DsRed-Exp.	St6	80%	PSM	Streak	3
B7-ZsGreen		80%	Streak/Epiblast	Streak	
B1-DsRed-Exp.	St5	80%	PSM	Streak	HH St7
B9-ZsGreen		80%	Streak/Epiblast	Streak	
B1-DsRed-Exp.	St6	80%	PSM	Streak	3
B9-ZsGreen		80%	Streak/Epiblast	Streak	
EGFP	St5	80%	6	Streak	16
DsRed		70%	9	Streak	
DsRed	St5	80%	7	Streak	17
EGFP		70%	10	Streak	
B9-DsRed-Exp.	St5	80%	10	Streak	15
B1-ZsGreen		70%	10	PSM	
B9-DsRed-Exp.	St5	80%	12	Streak	17
B1-ZsGreen		70%	10	PSM	
B9-DsRed-Exp.	St5	80%	11	Streak	17
B1-ZsGreen		70%	10	PSM	
B9-DsRed-Exp.	St5	80%	Streak/Epiblast	Streak	HH St7
B1-ZsGreen		70%	PSM	Streak	
B9-DsRed-Exp.	St5	80%	Streak/Epiblast	Streak	3
B1-ZsGreen		70%	PSM	Streak	
B9-DsRed-Exp.	St5	80%	Streak/Epiblast	Streak	3
B1-ZsGreen		70%	PSM	Streak	

PSM: presomitic mesoderm
TB: tail bud

Supplementary Table 2

Electroporated at			Distribution of Labelled Cells	End of Incubation
Stage, HH	Construct	Promotor	Rostral-Most Somite	Last-Formed Somite
St4	DsRed-Express		1	11
	ZsGreen		1	
St4	HoxB1-DsRedEx		PSM	HH St5
	HoxB9-ZsGreen		Streak/Epiblast	
St4+/5-	DsRed-Express	CAGGS	5	12
	ZsGreen	CAGGS	5	
St4+/5-	DsRed-Express	CAGGS	6	13
	B4-ZsGreen	CAGGS	6	
St4+/5-	DsRed-Express	CAGGS	3	13
	B9-ZsGreen	CAGGS	7	
St4+/5-	DsRed-Express	CAGGS	3	12
	B9-ZsGreen	CAGGS	7	
St4+/5-	B1-DsRed-Exp.	CAGGS	5	13
	B9-ZsGreen	CAGGS	8	
St4+/5-	B1-DsRed-Exp.	CAGGS	5	12
	B9-ZsGreen	CAGGS	8	
St4+/5-	B4-DsRed-Exp.	CAGGS	3	10
	B9-ZsGreen	CAGGS	6	
St4+/5-	B1-DsRed-Exp.	CAGGS	5	11
	B9-ZsGreen	CMV	9	
St4+/5-	B1-DsRed-Exp.	CAGGS	5	10
	B9-ZsGreen	CMV	9	
St4+/5-	B1-DsRed-Exp.	CAGGS	4	11
	B9-ZsGreen	SV40	7	
St4+/5-	B9-DsRed-Exp.	CAGGS	9	11
	B9-ZsGreen	SV40	9	
St4+/5-	B7-DsRed-Exp.	CAGGS	9	11
	B7-ZsGreen	TK	9	
St4+/5-	B9-DsRed-Exp.	CAGGS	9	10
	B9-ZsGreen	TK	10	
St4+/5-	B9-DsRed-Exp.	CAGGS	8	12
	B9-ZsGreen	TK	7	

Supplementary Table 2 (cont'd)

Electroporated at			Distribution of Labelled Cells	End of Incubation
Stage, HH	Construct	Promotor	Rostral-Most Somite	Last-Formed Somite
St5	DsRed-Express	CAGGS	6	14
	ZsGreen	CAGGS	7	
St5	DsRed-Express	CAGGS	6	13
	EGFP	CAGGS	6	
St5	DsRed-Express	CAGGS	7	14
	EGFP	CAGGS	7	
St5	DsRed-Express	CAGGS	7	14
	EGFP	CAGGS	7	
St5	DsRed-Express	CAGGS	7	13
	B4-ZsGreen	CAGGS	7	
St5	DsRed-Express	CAGGS	6	14
	B4-ZsGreen	CAGGS	7	
St5	DsRed-Express	CAGGS	7	13
	B9-ZsGreen	CAGGS	11	
St5	DsRed-Express	CAGGS	7	14
	B9-ZsGreen	CAGGS	12	
St5	B1-DsRed-Exp.	CAGGS	PSM	HH St7
	B9-ZsGreen	CAGGS	Streak/Epiblast	
St5	B1-DsRed-Exp.	CAGGS	PSM	HH St6
	B9-ZsGreen	CAGGS	Streak/Epiblast	
St5	B1-DsRed-Exp.	CAGGS	PSM	HH St7
	B9-ZsGreen	CAGGS	Streak/Epiblast	
St5	B1-DsRed-Exp.	CAGGS	8	10
	B9-ZsGreen	CMV	PSM	
St5	B7-DsRed-Exp.	CAGGS	8	11
	B7-ZsGreen	SV40	8	
St5	B7-DsRed-Exp.	CAGGS	9	12
	B7-ZsGreen	SV40	9	
St5	B1-DsRed-Exp.	CAGGS	6	12
	B9-ZsGreen	SV40	9	
St5	B9-DsRed-Exp.	CAGGS	10	12
	B9-ZsGreen	SV40	10	
St5	B9-DsRed-Exp.	CAGGS	13	13
	B9-ZsGreen	SV40	13	
St5	B9-DsRed-Exp.	CAGGS	9	12
	B9-ZsGreen	SV40	9	
St5	B7-DsRed-Exp.	CAGGS	9	12
	B7-ZsGreen	TK	9	
St5	B9-DsRed-Exp.	CAGGS	10	11
	B9-ZsGreen	TK	10	
St5	B9-DsRed-Exp.	CAGGS	10	11
	B9-ZsGreen	TK	10	
St5	B9-DsRed-Exp.	CAGGS	11	12
	B9-ZsGreen	TK	12	
St5	Deleted B9-DsRedEx	CAGGS	PSM	3
	B9-ZsGreen	CAGGS	Streak/Epiblast	
St5	Deleted B9-DsRedEx	CAGGS	PSM	3
	B9-ZsGreen	CAGGS	Streak/Epiblast	
St5	Deleted B9-DsRedEx	CAGGS	6	12
	B9-ZsGreen	CAGGS	12	
St5	Deleted B9-DsRedEx	CAGGS	7	13
	B9-ZsGreen	CAGGS	12	

Supplementary Table 2 (cont'd)

Electroporated at			Distribution of Labelled Cells	End of Incubation
Stage, HH	Construct	Promotor	Rostral-Most Somite	Last-Formed Somite
St6	DsRed-Express	CAGGS	10	14
	ZsGreen	CAGGS	9	
St6	DsRed-Express	CAGGS	9	14
	B4-ZsGreen	CAGGS	9	
St6	DsRed-Express	CAGGS	9	14
	B4-ZsGreen	CAGGS	9	
St6	DsRed-Express	CAGGS	10	14
	B9-ZsGreen	CAGGS	14	
St6	B1-DsRed-Exp.	CAGGS	PSM	HH St7
	B9-ZsGreen	CAGGS	Streak/Epiblast	
St6	B1-DsRed-Exp.	CAGGS	PSM	3
	B9-ZsGreen	CAGGS	Streak/Epiblast	
St6	B1-DsRed-Exp.	CAGGS	9	13
	B9-ZsGreen	SV40	13	
St6	B7-DsRed-Exp.	CAGGS	12	14
	B7-ZsGreen	TK	12	
St6	Deleted B9-DsRedEx	CAGGS	PSM	4
	B9-ZsGreen	CAGGS	Streak/Epiblast	
St6	Deleted B9-DsRedEx	CAGGS	PSM	4
	B9-ZsGreen	CAGGS	Streak/Epiblast	
St7	HoxB1-DsRed-Exp.	CAGGS	PSM	5
	HoxB9-ZsGreen	CAGGS	Streak/Epiblast	
St7	B7-DsRed-Exp.	CAGGS	15	16
	B7-ZsGreen	TK	16	
St7	HoxB1-DsRed-Exp.	CAGGS	15	20
	TK-B9-ZsGreen	TK	20	20
St7	HoxB9-DsRed-Exp.	CAGGS	18	15
	TK-B9-ZsGreen	TK	18	

PSM: presomitic mesoderm

Supplementary Table 3

Host	Donnor	Grafted at	Dstribution of Grafted cells		End of Incubation
Stage, HH	Stage, HH	Streak Level	Rostral-Most Somite	Caudal-Most Somite	Last-Formed Somite
St4+	St4+	90%	2	Streak	10
St4+	St4+	90%	3	Streak	10
St4+	St7	90%	7	Streak	11
St4+	St7	70%	7	Streak	10
St4+	St7	70%	8	Streak	12
St4+	3ss	70%	8	Streak	10
St5	St5	70%	6	Streak	10
St5	St5	90%	5	Streak	10
St5	St7	70%	PSM	Streak	10
St5	St7	70%	PSM	Streak	10
St5	5ss	70%	PSM	Streak	10
St5	5ss	70%	PSM	Streak	12
St6	St6	90%	8	Streak	11
St6	St6	90%	8	Streak	13
St6	St6	90%	8	Streak	11
St6	St6	70%	9	Streak	12
St6	6ss	70%	PSM	Streak	12
St6	6ss	70%	PSM	Streak	14
St6	6ss	70%	PSM	Streak	14
St6	6ss	90%	PSM	Streak	15
5ss	5ss	80%	16	Streak	20
5ss	St4+	70%	15	PSM	15
5ss	St4+	80%	16	PSM	16
6ss	6ss	80%	18	TB	20
6ss	6ss	80%	18	TB	21
6ss	6ss	80%	18	TB	22
6ss	St4+	80%	18	PSM	18
6ss	St6	80%	19	PSM	22
6ss	St6	80%	18	PSM	22
8ss	8ss	80%	21	TB	23
8ss	St7	80%	21	PSM	23
8ss	St5	80%	22	PSM	22

ss: somite-stage
PSM: presomitic mesoderm
TB: tail bud

Supplementary methods

Two-color *in situ* hybridization: Chicken *Sox2* cRNA probe and *Hox* probes (*Hoxb1*, *Hoxb4*, *Hoxb7* and *Hoxb9*) were labeled, respectively, by fluorescein and DIG labeling Mix (Roche). *Sox2* probe and a *Hox* probe were mixed and hybridized with stage 4HH to stage 8HH chicken embryos at 70C° overnight. After washing the probes, the embryos were incubated with anti-DIG and anti-fluorescent alkaline phosphatase-conjugated antibodies (Roche) at 4C° overnight. The *Hox* and *Sox2* expression were successively detected by NBT-BCIP substrates (Promega) and Fastred substrate (SIGMA) for the alkaline phosphatase.

Electroporation and fate mapping of the epiblast: Embryos ranging from stage 3HH to stage 7HH were prepared for EC culture. A DNA solution containing the fluorescent protein expression vectors (*pCAGGS-EGFP*, or *pCAGGS-DsRed*)) (1.0-5.0 μg/μl) was microinjected in the space between the vitelline membrane and the epiblast lateral to the streak. *In vitro* electroporations were carried out with five successive square pulses of 8V for 50ms, keeping 4mm distance between anode and cathode using Petri dish type electrodes (CUY701P2, Nepa Gene, Japan) and a CUY21 electroporator (Nepa Gene, Japan). This procedure strictly labels the superficial epiblast layer. Electroporated embryos were cultured for 2-3 hours at 38ºC in a humidified incubator until EGFP fluorescence was observed in the electroporated embryo; then, EGFP-positive lateral epiblast fragments with unlabeled underlying mesoderm and endoderm, approximately 100μm x 100μm, were excised and grafted into the same position of stage-matched unlabeled host embryos. After reincubation, the position of the anterior and posterior-most fluorescent cells in the embryos was scored. Pictures were taken using a CCD camera (Progeress, Germany), bright field image, GFP filtered image and/or Rhodamine filtered image of each embryo were superimposed with Photoshop software. Frozen sections were obtained from the fixed cultured embryos.

Hox Expression Constructs: Plasmid constructs to express chicken Hox proteins were generated in expression vectors: *pCIZ (pCAGGS-IRES2-ZsGreen1), pCIRX (pCAGGS-IRES2-DsRed-Express), pCMV-IZ (pCMV-IRES2-ZsGreen1), pCMV-IRx (pCMV-IRES2-DsRed-Express), pSV40-IZ (pSV40-IRES2-ZsGreen1), pSV40-IRx (pSV40-IRES2-DsRed-Express), pIZ-TK (pTK-IRES2-ZsGreen1)* and *pTK-IRx (pTK-IRES2-DsRed-Express)*. *pCIZ* and *pCIRX* are derived from the *pCAGGS* expression vector, in which an *IRES2-ZsGreen1* (Clontech) cassette or an *IRES2-DsRed-Express* (Clontech) cassette, respectively, was inserted downstream of a multi-cloning site. *pCMV, pSV40* and *pTK* vectors were derived from *pRL-CMV, pRL-SV40* and *pRL-TK* (Promega) in which each *IRES2-reporter* cassette was respectively inserted after removal of R*luc* sequence. Full coding sequences of chicken *Hoxb1, Hoxb4, Hoxb7* and *Hoxb9* were amplified by PCR from chick embryo-derived cDNAs and then subcloned into these expression vectors. A deletion mutant of chick *Hoxb9* that lacks 28 amino acids from the C-terminal was constructed with introducing a stop codon at the position of 220 Asn just after the helix2 by PCR. These constructs (5.0μg/μl) were independently electroporated, and the fate of fluorescent protein-positive cells was analyzed. Empty vectors were electroporated in control experiments. After the electroporation as described above, a fragment of the 80% level of the donor primitive streak was grafted at the same level of the primitive streak of