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REGULAR ARTICLE

Avian pelvis originates from lateral plate mesoderm and its development requires signals from both ectoderm and paraxial mesoderm

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Abstract The pelvic girdle is composed of three skeletal elements: ilium, pubis, and ischium. In comparison with other parts of the postcranial skeleton, its development is not well known to date. To elucidate the embryonic origin of the avian pelvic girdle and the signaling centers that control its development, we have performed extirpation and quail-to-chick grafting experiments. The results reveal that the entire pelvic girdle originates from the somatopleure at somite levels 26 to 35. No somitic cell contribution to skeletal elements of the pelvis has been detected. Removal of the surface ectoderm covering the lateral plate mesoderm has revealed that ectodermal signals control the development of the pelvic girdle, especially the formation of the pubis and ischium. The impaired development of the ischium and pubis correlates with the downregulation of *Pax1* and *Alx4*, two transcription factors that control the normal development of the ischium and pubis. Although of somatopleural origin, the development of the ilium depends on somitic signals. Insertion of a barrier between somites and somatopleure disrupts the expression of *Emx2* and prevents normal development of the ilium but does not affect the expression of *Pax1* or *Alx4* and the development of the pubis and ischium. Thus, the development of the ilium, but not of the pubis and ischium, depends on somitic and ectodermal signals.

Keywords Pelvic girdle · Somites · Somatopleure · *Emx2* · *Pax1* · *Alx4* · Chick (White Leghorn, *Gallus gallus domesticus*) · Japanese quail (*Coturnix japonicus*)

Introduction

The vertebrate limb bud is a well-established model in developmental biology. Nevertheless, the development of the proximal elements of the appendicular skeleton, i.e., the limb girdles is not well understood. Some data are available concerning the developmental (Chevallier 1977; Huang et al. 2000; Matsuoka et al. 2005) and evolutionary (Burke 1991a,b; Vickaryous and Hall 2006) origin of the shoulder girdle and the genes controlling its development (Selleri et al. 2001; Pröls et al. 2004; Kuijper et al. 2005). However, our knowledge of the development of the pelvic girdle, especially the ilium, remains controversial (Chevallier 1977; Malashichev et al. 2005).

The avian pelvic girdle consists of three bones, viz. the ilium, pubis, and ischium, whose structure and morphological development have been previously described (Mehnert 1887; Johnson 1893; Baumel et al. 1979; Malashichev et al. 2005). In the avian embryo, the ilium is the first visible skeletal element of the pelvic girdle and can be detected as early as HH-stage 26 (Hamburger and Hamilton 1951) as a local mesenchymal condensation at the position of the prospective

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acetabulum. It later chondrifies and expands rapidly in a caudal and then in a cranial direction. At HH-stage 36, the ilium stretches along 12 synsacral vertebrae (Malashichev et al. 2005). Its dorsal pre- and postacetabular wings (*Alae prae-* and *postacetabulares ilii*) form the ilio-synsacral joint, connecting the appendicular with the axial skeleton. The pubis and ischium develop subsequently from individual mesenchymal condensations and form the ventral part of the pelvic girdle. Interestingly, the entire pelvic girdle chondrifies relatively late during embryogenesis after the initiation of chondrification in the more distal elements of the limb skeleton.

The classical view is that limb girdles originate entirely from the lateral plate mesoderm (Bardeen and Lewis 1901; Osburn 1907). This view has been challenged by the finding that at least the scapular blade of the chick originates from the somites and not from the somatopleure (Chevallier 1977; Huang et al. 2000; Wang et al. 2005). Chevallier (1977) has suggested that the pelvic girdle is entirely of somatopleural origin but has not shown this explicitly. Evidence is now accumulating that the scapular blade and the ilium are subjected to control mechanisms that differ from those of the rest of the girdle elements and/or are derived from somitic material (Malashichev et al. 2005). Two knockout mice, *Emx2*$^{-/-}$ (Pellegrini et al. 2001) and *Fgf10*$^{-/-}$ mice (Sekine et al. 1999), with ablated scapular blade and ilium or preserved clavicula, anterior scapula blade and rudimentary ilium, respectively, suggest that proximal bones in both shoulder and pelvic girdles share a common developmental mechanism. In view of the somitic origin of the scapula (Chevallier 1977; Huang et al. 2000) and the proximo-distal homology of the scapula and ilium in terms of comparative anatomy (Drennan 1927), we might assume that the iliacal wings also originate from the somites. One aim of this study has been to investigate the contribution of the somites to the development of the ilium.

We have, furthermore, investigated the signaling centers essential for pelvic girdle development. In a previous report, we have shown that the ectoderm overlying the lateral plate mesoderm is necessary for the normal development of the pelvic girdle (Malashichev et al. 2005). Signaling at stages prior to limb bud formation (HH-stages 16 and earlier; Hamburger and Hamilton 1951) is indispensable for the formation of the ilium, ischium, and pubis. Ectoderm ablation experiments have revealed a differential regulation of the three pelvic elements by the ectoderm with the pubis being most prone to defects after such a treatment. In contrast, the postacetabular wing of the ilium still develops, at least partially, even after full ectoderm ablation at an early stage (HH-stage 12), suggesting that signals from other sources also control its development and can maintain its growth and differentiation (Malashichev et al. 2005). Therefore, we have aimed to find the source(s) of such signals.

A number of marker genes are expressed in the mesenchyme of the proximal regions of limb buds. Because of their role in the development of proximal girdle elements, *Emx2*, *Pax1*, and *Alx4* are of particular interest. The homeobox-containing gene *Emx2* is expressed in the proximal mesenchyme of limb buds, the region that gives rise at least to the ilium (Pröls et al. 2004; Malashichev et al. 2005). This gene determines the proximal identity of limb buds during normal chick embryonic development (Pröls et al. 2004) and during regeneration in amphibians (Beauchemin et al. 1998). In *Emx2*$^{-/-}$ mutant mice, the most proximal elements of both shoulder and pelvic girdles, i.e., the scapula and ilium, are absent (Pellegrini et al. 2001). The paired-box containing gene *Pax1* is expressed in developing sclerotomes and in mesenchyme, which gives rise to the scapula in the shoulder and the pubis in the pelvic girdle (Timmons et al. 1994; Malashichev et al. 2005). Analysis of *Pax1*-deficient *undulated* mice has revealed an important role of *Pax1* in the development of the axial skeleton and shoulder girdle (Timmons et al. 1994; Wilm et al. 1998). An additional marker of the pubis anlage is the aristaless-related gene *Alx4*, which has a complementary function with another aristaless-related gene, *Cart1* (Kuijper et al. 2005). Targeted disruption of *Alx4* causes the non-formation of the proximal pubis, whereas *Alx4*$^{-/-}$/*Cart1*$^{-/-}$ double-knockout mice completely lack pubic cartilage and *Pax1* expression (Kuijper et al. 2005).

To elucidate molecular pathways involved in the control of pelvic girdle development, we have studied the expression patterns of *Emx2*, *Pax1*, and *Alx4* in normal chick embryos and in embryos after micromanipulations affecting the development of pelvic girdle skeletal elements. We discuss the possible regulatory pathways involved in the control of pelvic girdle development.

Materials and methods

Chick embryos

Fertilized eggs of White Leghorn domestic fowl (*Gallus gallus domesticus*) and Japanese quail (*Coturnix japonicus*) were incubated at 38°C and 80% humidity. The embryos were staged (Hamburger and Hamilton 1951) and used for microsurgery or grafting experiments as described below or processed for in situ hybridization. The general approach for microsurgery was performed as described by Ordahl and Christ (1998). All operations were performed on the right side of the embryo.

Extirpation of lumbosacral somites or somatopleure

A row of 4–6 somites (somite 25 and more caudal) opposite the hind limb bud was extirpated in embryos at HH-stages

17–18. The dermomyotomes were removed completely or up to 90%, whereas we never succeeded in completely removing the corresponding sclerotomes (80% in the center, 50% at the ends of the wound). To prevent regeneration of somites, wounds were filled with egg-shell crumbs. In the second experimental series, the somatopleure was completely removed at the level of presumptive or early hind limb bud (along somites 25–33) at HH-stages 12–17.

Quail-to-chick grafting experiments

Over 90 transplantation experiments were performed by grafting paraxial mesoderm of various stages (segmented and non-segmented) and somatopleure from quail to chick embryos of the same developmental stage. The somatopleural grafts included the overlying ectoderm. Single somite grafting was carried out as described by Ordahl and Christ (1998) on newly formed epithelial somites of stage III (Christ and Ordahl 1995) and involved somites 23–31. A series of somites or dermomyotomes were transplanted at HH-stages 17–19. At HH-stages 14–16, presomitic paraxial mesoderm spanning the length of six or more somites was also grafted from quail to chick. In addition to Nile blue staining, the cranial end of the mesodermal band was marked by one or two somites (stages I and II) for the proper orientation of the graft in the chick host. In the last series of transplantation experiments, somatopleure was grafted from quail to chick at HH-stages 15–17. Manipulated embryos were reincubated for 3–4 days until HH-stages 28–32 before fixation and subsequent histological or skeletal staining.

Histology and immunostaining

Embryos were sectioned and stained to visualize skeletal muscle and cartilage as described by Malashichev et al. (2005). Before being sectioned, embryos were fixed in Serra's fixative (Serra 1946), dehydrated in an alcohol series, incubated in Rotihistol, and embedded in paraffin. Quail cells in chimeric embryos were visualized with Feulgen staining (Schiff's reagent, Sigma) or were stained immunologically by using anti-quail antibody (Sigma). Serial sections (7 μm thick) were washed in a 0.3% solution of H_2O_2 in methanol to block endogenous peroxidases, preincubated in a 1% solution of bovine serum albumin in phosphatase buffer, and hybridized with primary polyclonal anti-α-desmin (Sigma) and monoclonal anti-quail antibodies (Sigma). After overnight incubation with secondary goat-anti-rabbit antibodies conjugated with horse-radish peroxidase (Sigma) and goat-anti-mouse antibodies conjugated with alkaline phosphatase (Sigma), goat-anti-rabbit antibodies were visualized by means of the diaminobenzidine reaction followed by visualization of goat-anti-mouse antibodies with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate in alkaline-phosphatase buffer and nuclear red counterstaining. As a result, muscle in sections stained brown to black, quail nuclei stained blue, and chick nuclei stained red. Some sections through quail-chick chimeras were additionally stained with instant Harris hematoxylin (Sigma) and a 0.01% aqueous solution of a mixture of Alcian blue and Alcian green for better visualization of quail and chick cartilage. The mixture of Alcian dyes stained sulfate and carboxyl groups blue and yellow, respectively, whereas acidic mucopolysaccharides were stained green (Burck 1988). We found that this mixture stained quail and chick cartilage differently, probably because of the different proportions of the stained components. Chick cartilage was stained azure and quail cartilage cyan.

Ectoderm removal and barrier insertion

Ectoderm was removed over the somatopleure as described by Malashichev et al. (2005) and replaced with gold foil. the impermeable barrier (gold foil) was inserted between the somitic and somatic mesoderm at the level of somites (or non-segmented paraxial mesoderm) 24–32 at HH-stages 14–16, i.e., prior to limb bud formation (Fig. 2a). We first made a longitudinal cut with a tungsten needle, cutting off the somatopleure from the paraxial mesoderm. A rectangular piece of gold foil was then inserted vertically into the obtained gap; after the albumin in the egg was lowered, the edges of the gap came together and fixed the foil in the vertical position.

Whole-mount cartilage visualization

To visualize the cartilaginous skeleton, embryos ranging from day 7 to day 10 were fixed in 100% alcohol for at least 24 h and stained in a 0.015% solution of Alcian blue dissolved in 80 ml absolute ethanol and 20 ml glacial acetic acid. After being stained for 4–6 days, the embryos were cleared in 0.5–2% KOH and a series of KOH-glycerol solutions of increasing composition of glycerol for up to 1 week.

Whole-mount in situ hybridization

The cDNA clone of the chick transcription factor *Alx4* (ChEST699f4) was purchased from ARK Genomics (Roslin Institute, UK). After amplification and verification of the sequence by sequence analysis, the plasmid was restricted with *Sac*II to generate the antisense riboprobe by means of T3 polymerase. The sense control riboprobe was generated after restriction of the plasmid with *Xho*I by using T7 polymerase. *Emx2* riboprobe was the gift of A.

Lumsden (Bell et al. 2001) and *Pax1* was kindly provided by Martin Goulding. The sense and antisense riboprobes were labeled with a digoxigenin RNA labeling kit as recommended (Boehringer, Mannheim, Germany). Normal and experimental embryos were fixed overnight at 4°C in 4% paraformaldehyde prepared in diethyl-pyrocarbonate-treated phosphate buffer with Triton X-100 (PBT). Embryos were then washed twice in PBT, dehydrated in methanol, and stored at −20°C. In situ hybridization was performed as previously described (Nieto et al. 1996).

Results

Paraxial mesodermal cells do not contribute to avian pelvic girdle

In order to investigate the possible contribution of the paraxial mesoderm to the skeleton of the avian pelvic girdle, four to six lumbosacral somites were extirpated at HH-stages 17–19 (Hamburger and Hamilton 1951). Eight embryos analyzed at HH-stages 31–32 showed no substantial defects in the ilium, whereas the neural arches of adjacent vertebrae were absent or severely damaged in two or more segments (Fig. 1a–c). In the youngest embryo of this series (early HH-stage 17), the preacetabular wing of the ilium was shortened (not shown).

In the shoulder girdle, the scapular blade is formed by cells from the dermomyotome (Huang et al. 2000). In order to investigate whether somitic cells contribute to the formation of the pelvic girdle, we have performed homotopic quail-to-chick transplantation experiments. After transplantations of single somites, rows of somites, or rows of 4–6 lumbosacral dermomyotomes from quail to chick embryos at HH-stages 17–19, no contribution of quail cells to the pelvic girdle could be observed (n=13; Fig. 1d–f). In three cases, they formed small spherical or rod-like ectopic quail cartilages between the chick ilium and neural arches of the lumbosacral vertebrae (not shown). These cartilages were arranged in a clear segmental order (when more than one) and were always separate from the ilium.

In order to study the possible contribution of paraxial mesoderm cells to the pelvic girdle in earlier stages (HH-stages 14–17), homotopic transplantations of somites or presomitic mesoderm (somite numbers ranging from 23 to 29) were performed at various axial levels (somite 24 to prospective somite 32). In all 19 chimeras, no quail cells participated in the formation of the pelvic girdle (Fig. 1g–j). Seven chimeric embryos had a shortened preacetabular wing of the ilium.

Removal of the somatopleure at the level of the prospective hind limb bud at HH-stages 15–17 always (n=15) resulted in the complete absence of the distal limb skeleton, whereas the pelvic girdle was either absent or partially rescued possibly because of regulation of the tissue (Fig. 1k,l).

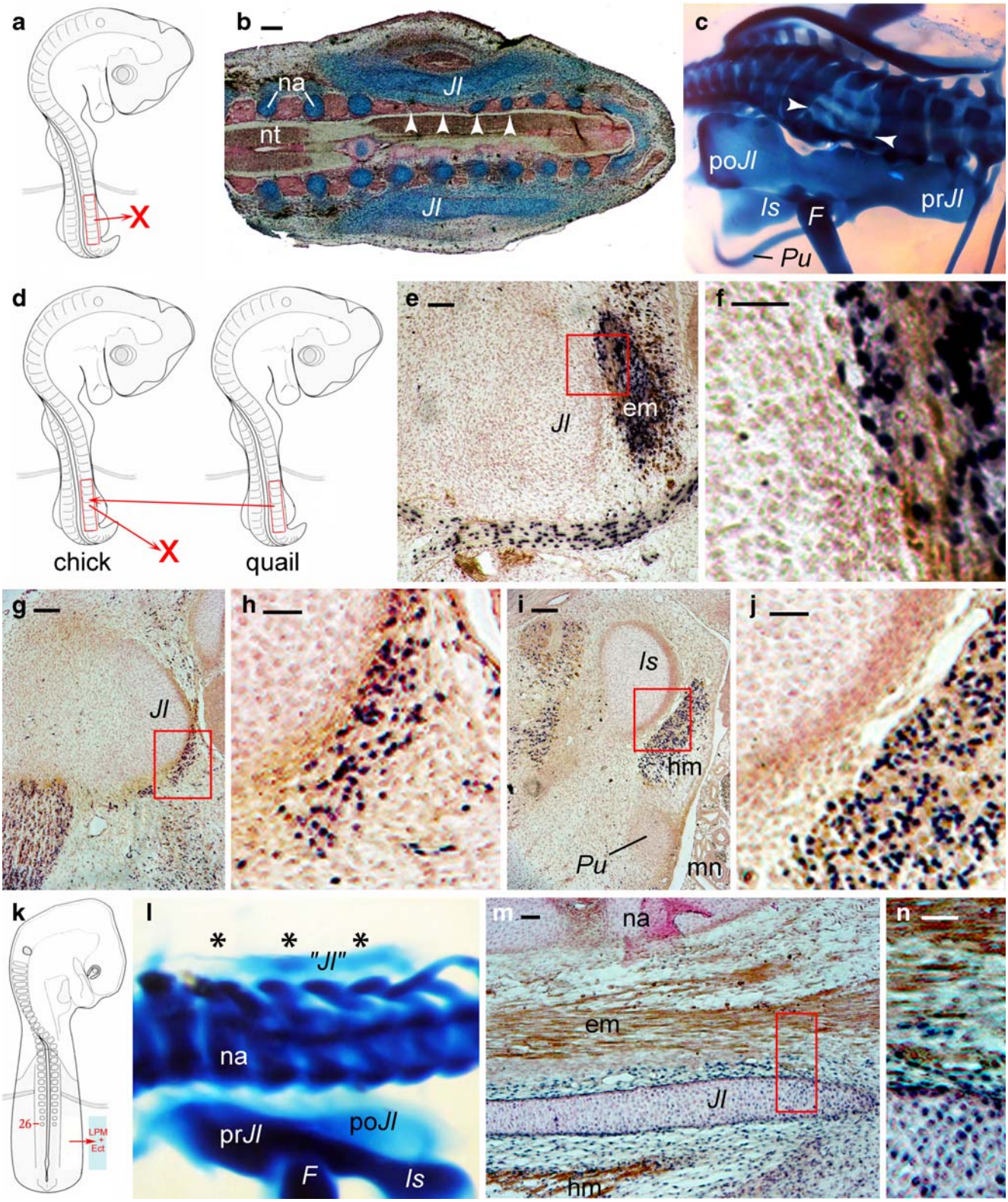
We also grafted somatopleure at the hind limb bud level from quail to chick (n=4). These grafts gave rise to all skeletal elements of the pelvic girdle, including the whole ilium, which consisted exclusively of quail cells (Fig. 1m,n). In two cases, the caudal or cranial tip of the ilium consisted of chick cells. This was because the transplant was shorter than the ilium anlage or had shifted slightly cranially or caudally. As the axial levels of all operations were known precisely, we could determine that the somatopleure at the level of somites 26 to 35 gave rise to the ilium anlage.

These experiments clearly show that the paraxial mesoderm does not contribute to any part of the avian pelvic girdle, and that the somatopleure is the only source of cells that gives rise to the pelvic bones.

Signaling from paraxial structures is indispensable for ilium formation

Although our transplantation experiments indicated that all cartilaginous elements of the pelvic girdle were derived

Fig. 1 Extirpation and transplantation experiments (*Ect* ectoderm, *em* epaxial muscles, *F* femur, *hm* hypaxial muscles, *Is* ischium, *Jl* ilium, *LPM* lateral plate mesoderm, *mn* mesonephros, *na* neural arch, *nt* neural tube, *poJl* postacetabular ilium, *prJl* preacetabular ilium, *Pu* pubis, *26* somite 26). **a** Representation of sacral somite extirpation. **b** Frontal section of an operated embryo; cranial is *left*. Immunostaining against α-desmin visualizes skeletal muscles (*dark brown to black*). Alcian blue staining reveals cartilage (*blue*). Nuclear red counterstaining. *Bar* 250 μm. **c** Whole-mount Alcian-blue-stained skeleton of a day 10 embryo. The neural arches correspondent to the extirpated somites are missing, smaller in diameter (*white arrowheads*), or fused, whereas the iliac cartilage is normal on both the operated and control sides of the embryo. The modest damage to the dorsal edge of the ilium might be attributable to changes in the shape of the vertebral column or to slight injuries of the adjacent lateral plate mesoderm during the operation procedure. Note the lack of substantial gaps in the ilium cartilage. **d** Representation of transplantation of a row of somites or dermomyotomes from quail to chick embryo. **e** After somite or dermomyotome transplantation (transverse section), quail antigens were detected in the nuclei (*blue*, densely organized in groups) of epaxial and hypaxial muscle, tendons, and dermis and sometimes in myelinated sheaths of spinal nerves, but never in the cartilaginous elements of the pelvic girdle. *Bar* 100 μm. **f** Enlargement of *boxed area* in **e**. *Bar* 50 μm. **g–j** Transverse sections of the pelvic region of an embryo after transplantation of quail presomitic mesoderm (HH-stages 14–16). All pelvic skeletal elements, viz., ilium (**g**) and ischium and pubis (**i**) are devoid of quail cells. **h**, **j** Enlargements of *boxed areas* in **g**, **i**, respectively. *Bars* 100 μm (**g**, **i**), 50 μm (**h**, **j**). **k** Representation of extirpation of somatopleure. **l** In most cases, this resulted in the complete absence of the pelvic girdle on the operated side. Absence of the ilium is indicated by *"Jl"* and *asterisks*. Whole-mount preparation stained with Alcian blue. **m**, **n** Somatopleure grafting between quail and chick embryo of the same age as shown in **k**. **m** Frontal section; cranial is *right*. Quail cells mark the whole ilium, the connective tissue outside of it, and a part of the hypaxial muscles, whereas epaxial muscles and the axial skeleton are formed by chick cells. *Bar* 100 μm. **n** Enlargement of *boxed area* in **m**. *Bar* 50 μm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from the somatopleure, some embryos showed a slight or substantial underdevelopment of the iliac wings. We, therefore, investigated whether there was a temporal window of signals from the paraxial mesoderm indispensable for normal development of the pelvic girdle. An impermeable barrier (gold foil) was inserted between the somatopleure and the paraxial structures at hind limb level to prevent possible signaling from the paraxial mesoderm to lateral plate mesoderm. The experiments were performed at HH-stages 14–17. In this experimental series, the ilium was affected in all cases (n=14). The cranial wing of the ilium, the *ala preacetabularis ilii*, was either missing or was substantially shortened. The ilium was, thus, formed from only of a part of the postacetabular wing, which had developed to different extents (Fig. 2). The pubis and ischium developed normally. We concluded that signals from the paraxial mesoderm from HH-stage 14 onwards were required for normal development of the ilium but were not required for the development of the pubis and ischium.

Signals from ectoderm and paraxial mesoderm control expression of marker genes

To investigate signals involved in the development of the avian pelvic girdle, the expression of the marker genes *Pax1*, *Emx2*, and *Alx4* was investigated after surgical manipulation, i.e., removal of the ectoderm overlying the somatopleure (Malashichev et al. 2005) and barrier implantation between the somatopleure and paraxial mesoderm, at HH-stages 12–17.

The expression patterns of *Pax1* and *Emx2* in the limb regions of chick embryos have been described previously (Huang et al. 2000; Pröls et al. 2004; Malashichev et al. 2005). The *Alx4* expression pattern (Fig. 3a) in chick hind limb buds corresponds to the expression pattern of *Pax1*, but the onset of *Alx4* expression slightly precedes that of *Pax1*.

Removal of the ectoderm over the somatopleure at the hind limb bud level results in complete downregulation of both *Alx4* (Fig. 3d) and *Pax1* (Fig. 3e). However, *Emx2* expression is unaffected (Fig. 3f). We conclude that ectodermal signals control (directly or indirectly) the expression of both *Alx4* and *Pax1* but not of *Emx2*.

On the contrary, barrier implantation between somitic and lateral plate mesoderm at the hind limb bud level did not affect *Alx4* (Fig. 3g) or *Pax1* (Fig. 3h) expression, whereas *Emx2* expression was downregulated in the prospective pelvic region (Fig. 3i). The expression along the dorsal side of the hind limb bud was drastically downregulated at the operated side. The caudal patch of intensive *Emx2* expression was substantially reduced in size. These effects were seen only if the operation had been performed at HH-stage 16 or earlier. Complete down-

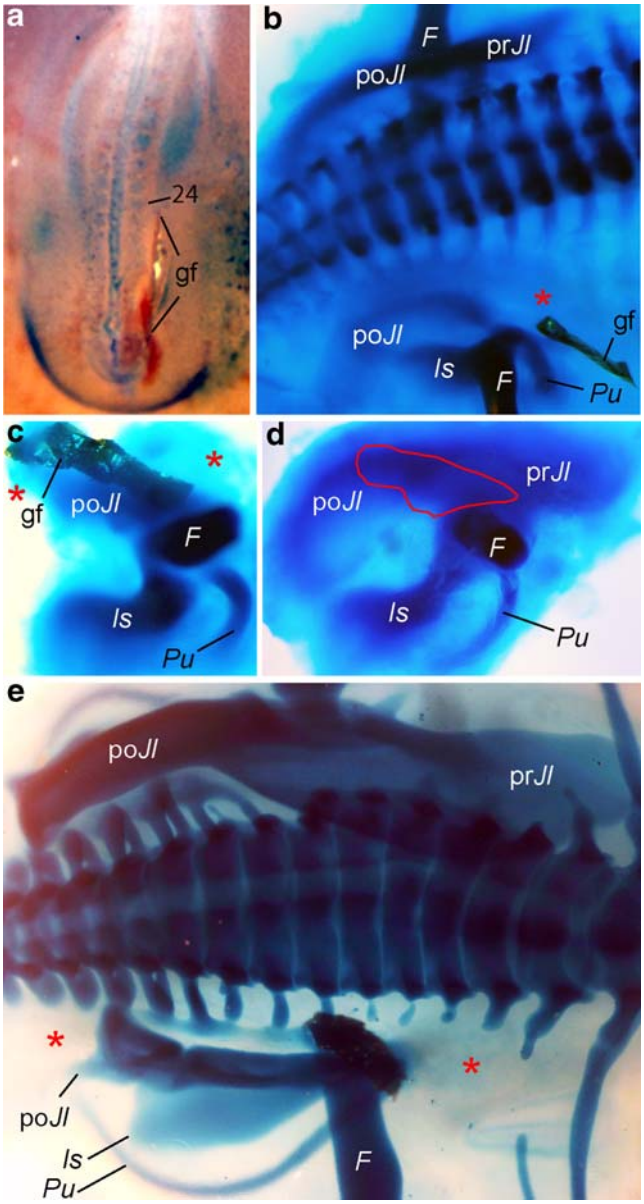

Fig. 2 Effects of insertion of an impermeable barrier (gold foil) between the anterior sacral somites and the somatopleure (abbreviations as in the legend to Fig. 1). **a** Embryo immediately after operation (*gf* position of insertion of gold foil, *24* 24th somite). ×20. **b** Whole-mount Alcian-blue-stained preparation of an operated embryo viewed from above (head is *right*). ×5. **c**, **d** Pelvic girdle from the operated and control sides, respectively, of another embryo with the same orientation and magnification. Note the lack (*red asterisks*) of the preacetabular (*prJl*) and most of the postacetabular (*poJl*) wings of the ilium. To enable estimation of the developmental deficits after barrier implantation, a *red contour line* that delineates the misshaped ilium in **c** is projected onto the regular normal shape of the ilium in **d**. ×10. **e** Another embryo, but reincubated after barrier implantation until day 10. At this stage, the formation of the cartilaginous pelvic girdle is completed. Note the lack or underdevelopment of iliac wings (*red asterisks*) indicating the lack of regeneration of the missing parts in late development. ×10

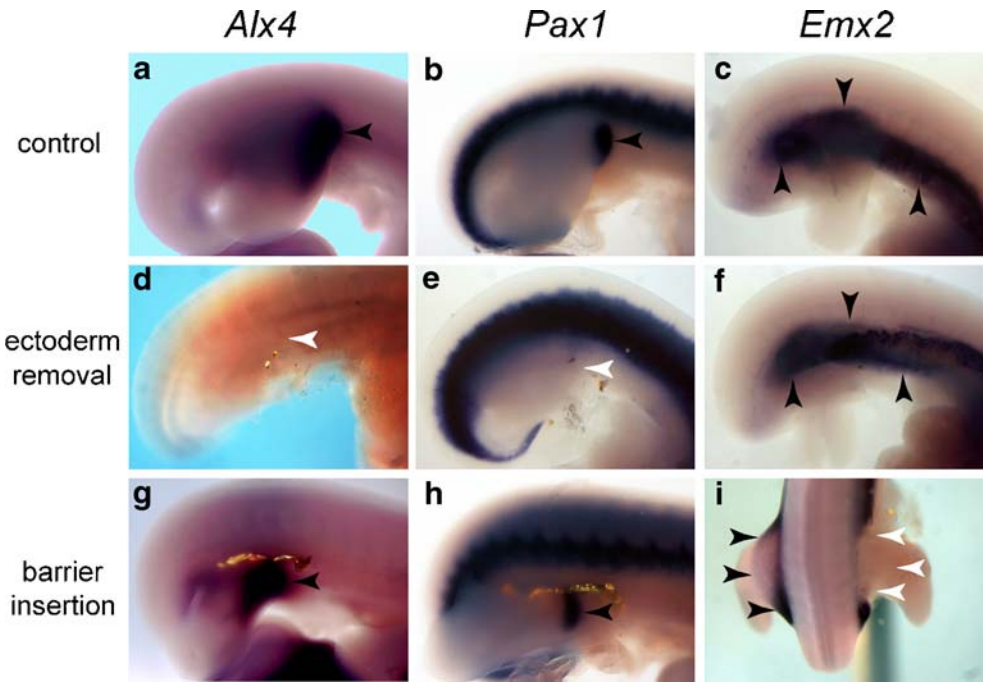

Fig. 3 Marker gene expression in normal embryos (**a**–**c**) and after two different operations, viz., ablation of the ectoderm over the lateral plate mesoderm (**d**–**f**, gold foil has been removed) and barrier implantation between paraxial mesoderm and somatopleure (**g**–**i**) at hind limb bud level (*black arrowheads* pattern of normal expression, *white arrowheads* sites at which normal expression is absent). *Alx4* is normally expressed in the anterior proximal region of the hind limb bud. It is downregulated after ectoderm ablation (**d**), whereas its expression is unaffected after barrier implantation (**g**). Normal expression of *Pax1* in the proximal hind limb bud (**b**) is completely abolished after ectoderm ablation (**e**) but preserved after barrier implantation (**h**). *Emx2* is normally expressed in the caudal area of the hind limb bud and in its most proximal dorsal part adjacent to the somites and in the intermediate mesoderm (**c**). No downregulation of *Emx2* is observed after ectoderm ablation (**f**), but its expression is significantly disturbed after barrier implantation (**i**). In the latter experiment, *Emx2* expression is fully preserved only in the intermediate mesoderm, whereas it is completely absent or substantially reduced in the proximal hind limb bud, especially opposite to the gold foil. The patch of expression caudal to the hind limb bud is also diminished in size, thus making the staining of the embryo highly asymmetric. The gold foil has been removed for better pattern visualization. ×10

regulation of *Emx2* at the hind limb bud level (especially caudal to the limb bud) was not possible, because this would have required an extremely long barrier, extending along at least 10 somites. Our attempts to perform such an operation disrupted the caudal bud and caused severe abnormalities of the embryo (not shown). *Emx2* expression in the intermediate mesoderm was always normal, indicating that signals from the paraxial mesoderm were important for maintaining the normal expression of *Emx2* in the lateral plate mesoderm at the level of the hind limb bud.

Discussion

Pelvic girdle derives from somatopleure

The embryonic origin of the avian ilium has been investigated by using extirpation and quail-to-chick grafting experiments. Extirpation of a series of lumbosacral somites causes severe damage of the vertebral column, but in most cases, not of the ilium and never of any other part of the pelvic girdle, suggesting that somites are probably not the source of cells required for pelvic girdle formation. The quail to chick somite transplantation experiments at HH-stages 17–19 have clearly revealed that somitic cells do not participate in the formation of the pelvic girdle. Only small pieces of quail-derived cartilage between the vertebral column and the pelvic girdle have been observed. Their morphology and segmental organization have led us to interpret these cartilaginous elements as being ectopic neural arches.

Extirpation of the somatopleure at somitic levels 23 to 35 leads to the complete absence or severe truncation of the pelvic girdle indicating that the pelvic girdle originates from the somatopleure. This finding is further supported by the results achieved by direct tracing of cell lineages after the grafting of somatopleure from quail to chick. These data show that all the elements of the pelvic girdle originate from the lateral plate mesoderm. We have further determined that the ilium recruits somatopleural cells from the somitic levels 26 to 35, indicating that the border of the presumptive pelvic girdle territory extends more caudally than has previously been presumed (Chevallier 1977).

Development of pelvic girdle depends on signals from the paraxial mesoderm

The extirpation and transplantation experiments performed in this study have revealed that somites never contribute cells nor influence the development of the pelvic girdle when embryos are operated at HH-stage 17 or later. However, similar operations at earlier stages result in the ablation of the ilium. The observed phenotypes resemble those reported by Kieny et al. (1972) following their substitution of the paraxial mesoderm of the prospective synsacral level with tissue of other axial levels or with non-mesodermal tissue. Accordingly, we presume that signals from the somites or non-segmented paraxial mesoderm at stages earlier than HH-stage 17 are important for the formation of the pelvic girdle and, in particular, for the formation of the ilium. This hypothesis has been confirmed by our barrier implantation experiments (Fig. 2). Insertion of an impermeable barrier (gold foil) between the paraxial and the lateral plate mesoderm at HH-stages 14 to 17, indeed, inhibits the development of the ilium, especially its cranial part, the *ala preacetabularis ilii*.

Several diverse operations result in a shortened or absent preacetabular wing of the ilium: (1) barrier implantation between the somites and somatopleure (this study); (2) removal of the ectoderm over somatopleure before hind limb bud formation (Malashichev et al. 2005); (3) early limb bud amputation (Spurling 1923; Malashichev et al. 2005); (4) embryo paralysis with bungarotoxin (Laing 1982). This is probably attributable to the late developmental onset of the preacetabular wing, which occurs after the development of the postacetabular ilium and the rest of the girdle (Malashichev et al. 2005). This late, cranially directed growth of the ilium cartilage also corresponds to its evolutionary novelty (Romer 1956; Malashichev 2006).

Signals from the paraxial mesoderm control *Emx2* expression and ilium development

The homeobox-containing gene *Emx2* is expressed in the iliacal mesenchyme (Malashichev et al. 2005). The normal pattern of *Emx2* expression is asymmetric along the cranio-caudal axis of the hind limb bud, with a stronger expression in the posterior part. Barrier insertion between the somites and somatopleure lead to the downregulation of *Emx2* expression and to defects in ilium formation. Ablation of the ectoderm at the hind limb bud level at HH-stages 12–14 is followed by more defects in cranial, but not caudal, parts of the ilium (Malashichev et al. 2005), exactly corresponding to the levels of *Emx2* expression. The defects in the ilium corresponding to the downregulated levels of *Emx2* expression are in accordance with the function of *Emx2* acting as a proximalizing factor (Pröls et al. 2004). In addition, *Emx2* might serve as a survival factor for the *Emx2*-positive cell populations. Barrier insertion between the paraxial and the lateral plate mesoderm did not affect the expression of *Pax1* or *Alx4*, corresponding to the normal development of the pubis or ischium. Thus, according to our results and in conjunction with the $Emx2^{-/-}$ phenotype (Pellegrini et al. 2001), only the ilium is controlled by the paraxial mesoderm via *Emx2* expression.

Ectodermal signals control development of entire pelvic girdle

Whereas paraxial signals are essential for ilium formation, ectodermal signals are required for the normal development of all skeletal elements of the avian pelvic girdle, although the pubis and ischium seem to be more sensitive. Ectoderm removal results in the complete downregulation of the two marker genes investigated, *Pax1* and *Alx4*. This downregulation corresponds to the observed skeletal phenotypes, with no development of the pubis and ischium (Malashichev et al. 2005). In $Alx4^{-/-}$ mutant mice, the *Pax1* expression pattern has recently been shown to be unaffected (Kuijper et al. 2005) suggesting that *Alx4* and *Pax1* are members of independent signaling pathways. The expression of *Emx2* is never affected by ectoderm ablation (Pröls et al. 2004; Malashichev et al. 2005) indicating that it is not under the direct control of ectodermal signals. Ilium formation thus depends on signals from dual origins, viz., the paraxial mesoderm and the ectoderm overlying the somatopleure, whereas ischium and pubis are solely controlled by the ectoderm overlying the somatopleure.

Two regulatory genes, *Wnt10a* and *Wnt7a*, are expressed in the surface ectoderm and might control the development of the pelvic girdle skeleton. Both genes are expressed long before the initiation of limb budding. *Wnt10a* is involved in the formation of the apical ectodermal ridge in limb buds (Narita et al. 2005), but its particular role in the patterning of the skeleton is not clear. Mutations in *Wnt7a* have recently been shown as a primary cause of human syndromes (Woods et al. 2006), collectively known as Al-Awadi/Raas-Rothschild syndrome or Schinzel phocomelia (Olney et al. 2001; Kantaputra and Tanpaiboon 2005). In these congenital disorders, the pelvic girdle is either completely absent or severely malformed. These phenotypes largely resemble the skeletal malformations observed after ectoderm ablation in our experiments (Malashichev et al. 2005). Accordingly, *Wnt7a* seems to play a crucial role in the chondrogenesis of the pelvic girdle. Indeed, *Wnt7a* controls the dorso-ventral patterning in the vertebrate limb (Parr and McMahon 1995; Riddle et al. 1995; Kengaku et al. 1998). In adddition, *Wnt7a* signaling has been shown to induce *Sox9* degradation, and its downregulation by the BMP2/p38 MAPK pathway is a prerequisite for the initiation of chondrogenesis (Jin et al. 2006). The effect of *Wnt7a* on *Pax1* or *Alx4* expression remains to be elucidated.

Evolutionary aspects of the pelvic girdle

The avian pelvic girdle derives entirely from lateral mesoderm, whereas the scapula blade has been shown to originate from hypaxial dermomyotomes, and the scapula head from the lateral plate mesoderm, the scapula thus having two origins (Chevallier 1977; Huang et al. 2000; Wang et al. 2005). An interesting question arises as to whether this difference between shoulder and pelvic girdle development is maintained during evolution. The development of the scapula blade, at least, is not necessarily consistent during evolution. Although somite extirpation in turtles results in the ablation of the scapula blade, somite extirpations in axolotl do not affect shoulder girdle formation (Burke 1991a,b). This indicates that, in caudate amphibians, the scapula originates entirely from the lateral plate mesoderm, in contrast to the dual origin reported for higher vertebrates (amniotes). On the other hand, at least in some amphibians, the scapula originates from two mesenchymal anlagen (Baleeva 2001), indicating the evolutionary appearence of the dual origins as early as the level of amphibians.

Less is known about the origin of the pelvic girdle in vertebrates. In *Prx1*-Cre/Z/AP mice, in which the mesenchymal cells of the limb buds (but not the cells of the somites) have been marked by lacZ expression (Logan et al. 2002), all elements of the pelvic girdle stain blue (A. Burke, personal communication), suggesting that the murine pelvic girdle is not of somitic but also possibly of somatopleural origin. According to the data available, we can assume that, in amniotes (reptiles, birds, and mammals), the scapula has a dual origin (lateral plate mesoderm and somites), whereas the ilium has a single origin (lateral plate mesoderm).

To summarize, we have shown that the avian pelvic girdle originates entirely from the lateral plate mesoderm but requires morphogenetic signals from the paraxial mesoderm and the ectoderm overlying the somatopleure. Paraxial-mesoderm-derived signals control the expression of *Emx2* prior to limb bud formation and thus are specifically necessary for the normal development of the ilium. Ilium, pubis, and ischium also require signals from the ectoderm overlying the somatopleure.

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