The dissociation of the Fgf-feedback loop controls the limbless state of the neck

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Summary

In tetrapods, limbs develop at two specific positions along the anteroposterior axis of the embryo, whereas other regions of the embryo, most prominently the neck and the flank, are limbless. However, the flank can generate an ectopic limb when the Fgf-feedback loop crucial for the initiation of limb budding is activated. Thus, despite its limblessness, the flank is a limb-competent area.

Using the chick embryo as model, we investigated whether the neck, as the flank, has the competence to form a limb, and what mechanism may regulate its limblessness. We show that forelimb lateral mesoderm plus ectoderm grafted into the neck can continue limb development, suggesting that the neck does not actively inhibit this process. However, neck tissues themselves do not support or take part in limb formation. Hence, the neck is limb-incompetent. This is due to the dismantling of Fgf signalling at distinct points of the MAPK signalling cascade in the neck lateral mesoderm and ectoderm.

Key words: Limblessness, Limb development, Neck, Flank, Fgf signalling, Chick embryo

Development

Introduction

In today's tetrapods, the limbs reside at defined positions along the anteroposterior axis of the body, invariant for each species (Burke et al., 1995). These positions, the neck-thorax interface for the forelimb and the lumbosacral transition for the hindlimb, are commonly determined on the base of the regionalized vertebral column. However, limbs evolved from lobed fins before the regional diversification of vertebrae (Clack, 2002; Jarvik, 1980). In particular, ribs were still present on the neck vertebrae of basal crown-group tetrapods such as *Pholiderpeton scutigerum* (Clack, 2002). Thus, the regionalization of the vertebral column is not a sufficient explanation for the establishment of limb-bearing versus limbless regions of the body.

Interestingly, the neck as a limb-free area between head and forelimbs is a recent acquisition of tetrapods, fully developed only in amniotes (Clack, 2002). Initially, in the ancestors of tetrapods (and still in extant fishes), the shoulder girdle to which the forelimbs attach was an integral part of the breathing apparatus. It provided the rear wall of the gill chamber and was held in place via a series of bones that attached the shoulder girdle to the skull. These bones were lost in stem-group tetrapods such as *Acanthostega gunnari* Jarvik (Clack, 2002). Next, the function of the shoulder girdle in supporting the gills ceased when breathing with lungs and through the skin became more prominent. An indicator for this event is the absence of the postbranchial lamina on the cleithrum, as seen in *Pholiderpeton* (Clack, 2002). However, the shoulder girdle remained close to the head, possibly because the branchiomeric muscles that insert here and previously worked the gills were still required to facilitate breathing via air gulping. This mode of breathing prevailed in modern amphibians (Clack, 2002). Amniotes then, by developing a closed rib cage and intercostal and abdominal muscles to alter its volume, found the means to more efficiently ventilate their lungs. At this time, the shoulder girdle was 'set free'. It relocated to a more posterior position, yet maintaining the original muscular scaffold (Clack, 2002; Matsuoka et al., 2005). The consequence of this evolution was the establishment of a neck as a limbless area between shoulder girdle and head, which is of moderate length in mammals, but considerably longer in birds (Clack, 2002).

To unravel the events that may have established the limbless state of the neck during amniote evolution, we have to investigate the control of limbness versus limblessness, beginning with known amniote models. Possibly the best understood model for amniote limb development is the chick embryo (Capdevila and Izpisua-Belmonte, 2001). The prospective limb fields are specified in the lateral mesoderm around stage HH10-11 (stages according to Hamburger and Hamilton, 1992). Limb induction, i.e. the initiation of limb budding, occurs between stages HH13 and 15 next to somites 15-20 (forelimb) and somites 25-30 (hindlimb). The specification of the limb fields is known to involve the localized expression of Tbx5 (forelimb) or Tbx4 (hindlimb) and the localized signalling of Wnt2b (forelimb) (Ng et al., 2002; Takeuchi et al., 2003) but is otherwise poorly understood. Nevertheless, these factors establish Fgf10 expression in the lateral mesoderm of the limb fields, which signals to the overlying surface ectoderm to induce the differentiation of the

apical ectodermal ridge (AER) at the interface between prospective dorsal and ventral territories. Moreover, Fgf10, via Wnt3a as a mediator, triggers expression of Fgf8 in the AER, which signals back to the mesoderm to maintain the expression of Fgf10 (Kawakami et al., 2001; Kengaku et al., 1998; Ohuchi et al., 1997). Furthermore, the Fgf molecules promote cell proliferation and hence outgrowth. Thus, the Fgf feedback loop is crucial to initiate limb budding, simultaneously determining the proximodistal axis of the limb. Once established, the AER participates in the installation of the zone of polarizing activity (ZPA) at the posterior margin of the limb that controls anteroposterior patterning and acts back onto the AER in a further feedback loop. Finally, signalling from the ectodermal jacket to the underlying mesoderm establishes the dorsoventral axis of the limb, crucial for the correct patterning of bone, the development of extensor and flexor muscles and their innervation.

As limb development depends on locally acting regulatory cascades, and forelimbs were 'moved' from the head to a more posterior position during tetrapod evolution, it has been speculated that the set of regulatory cascades for limb formation has simply been relocated (Capdevila and Izpisua-Belmonte, 2001). However, while limbs were secondarily lost, for example in snakes (Cohn and Tickle, 1999), in no species has the forelimb ever returned to its previous layout, with the shoulder girdle joined to the head (Clack, 2002). Moreover, the anteroposterior dimension of the limb is closely regulated (Capdevila and Izpisua-Belmonte, 2001). Thus in addition to relocating the positive signals for limb development, it is conceivable that a mechanism was installed during evolution that both prevented the return of the forelimb to the original position and that limited the anterior extent of the limb: there may be a specific mechanism in the neck that ensures its limbless state.

Besides the neck, the flank is another prominent limbless area. However, embryological studies in the chick have demonstrated that the flank supports an ectopic limb grafted into this region (Hamburger, 1938; Capdevila and Izpisua-Belmonte, 2001). Moreover, using exogenous Fgf10 to mimic the mesodermal signalling or Fgf8 to substitute for the ectodermal signal, the Fgf feedback loop can be kick-started, and the flank mesoderm plus ectoderm will generate a limb (Yonnei-Tamura et al., 1999). Using this paradigm, we investigated the state of limblessness in the neck.

First, grafting forelimb-derived lateral mesoderm plus ectoderm, we investigated whether the neck environment may actively suppress limb development. We found that the neck permits limb development. Second, as the ectopic limb buds were poorly integrated into the neck, we investigated whether neck lateral mesoderm and ectoderm may contribute to the ectopic limb buds. However, this was not the case. Third, as the flank tissues participate in limb development in response to Fgf signalling (Crossley et al., 1996; Yonnei-Tamura et al., 1999), we investigated the responsiveness of neck ectoderm and lateral mesoderm to Fgf10 and Fgf8. We found that in the neck neither the Fgf feedback loop nor bud outgrowth could be achieved. Fourth, we investigated whether Fgf signalling was incapacitated in the neck due to the loss of receptor. However, Fgfr2, the crucial receptor to transduce Fgf10 and Fgf8 signalling in limb development (Revest et al., 2001; Xu et al., 1998), was expressed. Finally, as Fgf signalling in limb development employs the MAPK signal transduction system (Corson et al., 2003; Schlessinger et al., 2000), we investigated whether this system was operational in the neck. We found that in the neck lateral mesoderm, the MAPK signalling travels as far as the phosphorylation of the kinases ERK1 and ERK2. However, this point is never reached in the ectoderm. Thus, our study shows that limblessness in the neck is controlled by the dismantling of the Fgf feedback loop through the interruption of MAPK signalling at distinct points in the neck lateral mesoderm and ectoderm.

Materials and methods

Chick embryos

Fertilized hens' eggs (Winter Farm, Royston, UK) were incubated at 38.5°C in a humidified incubator and staged according to Hamburger and Hamilton (1992).

Protein-loaded beads

Heparin beads (Sigma) were washed in PBS and loaded overnight at 4°C with Fgf8 or Fgf10 (R&D) at 500 μg/ml (limb induction assays) or at 1 mg/ml, 250 μg/ml or 50 μg/ml (assays for MAPK signalling) in PBS/1% BSA. Before transplantation the beads were rinsed in PBS and kept on ice.

Tissue grafting

Host embryos were at HH10-14, as indicated in Table S1 in the supplementary material. Using flame-sharpened tungsten needles (Dietrich et al., 1997), a slit was made in the occipital, neck or flank ectoderm plus lateral mesoderm, next to the somites. Donor embryos were at HH14. They were pinned down in a Sylgard (Dow Corning) dish dorsal side up, a fragment of forelimb lateral plate mesoderm plus the covering ectoderm, or of forelimb lateral mesoderm only (square of two somites length) was excised with tungsten needles, aspirated with a serum-coated pipette, then released into the slit within the host and manoeuvred into place with tungsten needles. The embryos were incubated until they reached HH20-21.

Bead grafting

Host embryos were prepared as above, and protein-loaded beads were pipetted into the slit and manoeuvred into place with tungsten needles.

In-ovo electroporation followed by tissue grafting

The plasmid pCaβ-IRES-eGFP (Alvares et al., 2003) was injected into the neck or the flank coelom of HH12 or HH14-15 embryos, respectively, using a PV820 pneumatic picopump (WPI). The lateral mesoderm corresponding to 2-3 somite-lengths was electroporated with two 20 ms/18V rectangular pulses by an intracept TSS10 electroporator (Intracell) with a 0.1 mm flame-sharpened tungsten (negative electrode) wire placed under the embryo and a 0.5 mm platinum (positive electrode) wire placed on top. The site of electroporation was recorded, using the position of the neighbouring somites as reference. The electroporated embryos were then incubated for 2-3 hours, and the tissue grafting was carried out into the electroporated lateral plate mesoderm as described above.

In situ hybridization

Whole-mount in situ hybridization was carried out according to Mootoosamy and Dietrich (Mootoosamy and Dietrich, 2002). Probes and their expression pattern are detailed in: *Bmp2* (Francis et al., 1994), *En1* (Logan et al., 1992), *Fgf4* (Streit and Stern, 1999), *Fgf8* (Mahmood et al., 1995), *Fgf10* (Ohuchi et al., 1997), *Gremlin* (Capdevilla et al., 1999), *Lmx1* (Riddle at al, 1995), *Myf5* (Saitoh et al., 1993), *Shh* (Johnson et al., 1994), *Tbx5* (Isaac et al., 1998), *Wnt3a* (unpublished PCR product), *Wnt7a* (Dealy et al., 1993), *Fgfr2* (Patstone et al., 1993).

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Immunohistochemistry

RMO270 staining

Whole-mount tracing of the nervous system was carried out according to Guthrie and Lumsden (Guthrie and Lumsden, 1992), using the RMO270 antibody (Zymed), which recognizes the 155 kDa intermediate neurofilament subunit. Primary antibodies were detected using anti-mouse IgG conjugated with horseradish peroxidase (Dako). The detection was made using diaminobenzidine (DAB) staining.

Detection of MAPK signalling

To detect activated MAPK signalling, rapid fixation in 4% PFA was required, followed by dehydratation with MeOH and rehydratation. Embryos were incubated serially with intervening washes in anti-diphosphorylated ERK2 mouse IgG monoclonal antibody (1:250; # M8159, Sigma), Vectastain Biotinylated Goat anti-mouse IgG secondary antibody (1:200; Vector labs), and Vectastain ABC solution (Vectastain ABC Elite kit, Vector labs). The diphosphorylated ERK1 and 2 proteins were revealed by DAB staining.

Vibratome sectioning

Embryos were embedded in 20% gelatine, fixed in 4% paraformaldehyde at 4°C and 40-μm sections were cut transversely using a Pelco 1000 vibratome.

Photomicroscopy

After completion of the staining reactions, embryos were cleared in 80% glycerol/PBS and split midsagitally (except electroporated embryos). Embryos and the vibratome sections were photographed on a Zeiss Axioskop, using fluorescence or Normaski optics.

Results

A graft of limb mesoderm plus the overlying ectoderm in the neck gives rise to an ectopic limb bud

Neck and flank are limbless regions in every tetrapod. In the chick, however, when limb-derived lateral mesoderm plus its overlying ectoderm is grafted into the flank of a host embryo, the graft develops into an ectopic limb. This indicates that the flank is a permissive environment for limb development (Hamburger, 1938). To determine if the neck is also a permissive environment for limb development, the forelimb lateral mesoderm and covering ectoderm from stage HH13-14 embryos were grafted into the neck at the right side of HH10-14 embryos. The grafts were placed next to somites 6-14 (see Table S1 in the supplementary material). Embryos were harvested at HH20, the stage at which for control graftings in the flank, the ectopic limb buds were well developed (not shown). We observed that also in the neck, ectopic limb buds developed in the operated embryos, regardless of the position of the graft along the anteroposterior axis of the neck (Fig. 1).

To establish if the ectopic limb buds obtained in the neck developed properly, the expression of genes crucial for normal limb development was investigated. Fgf10 is a key factor for limb induction, outgrowth and proximodistal patterning (Ohuchi et al., 1997; Yonei-Tamura et al., 1999). It induces the differentiation of the AER and the expression of *Fgf8* in the AER through the expression of *Wnt3a* (Kawakami et al., 2001; Kengaku et al., 1998; Ohuchi et al., 1997). Fgf8, partially redundant with Fgf4 (Boulet et al.,

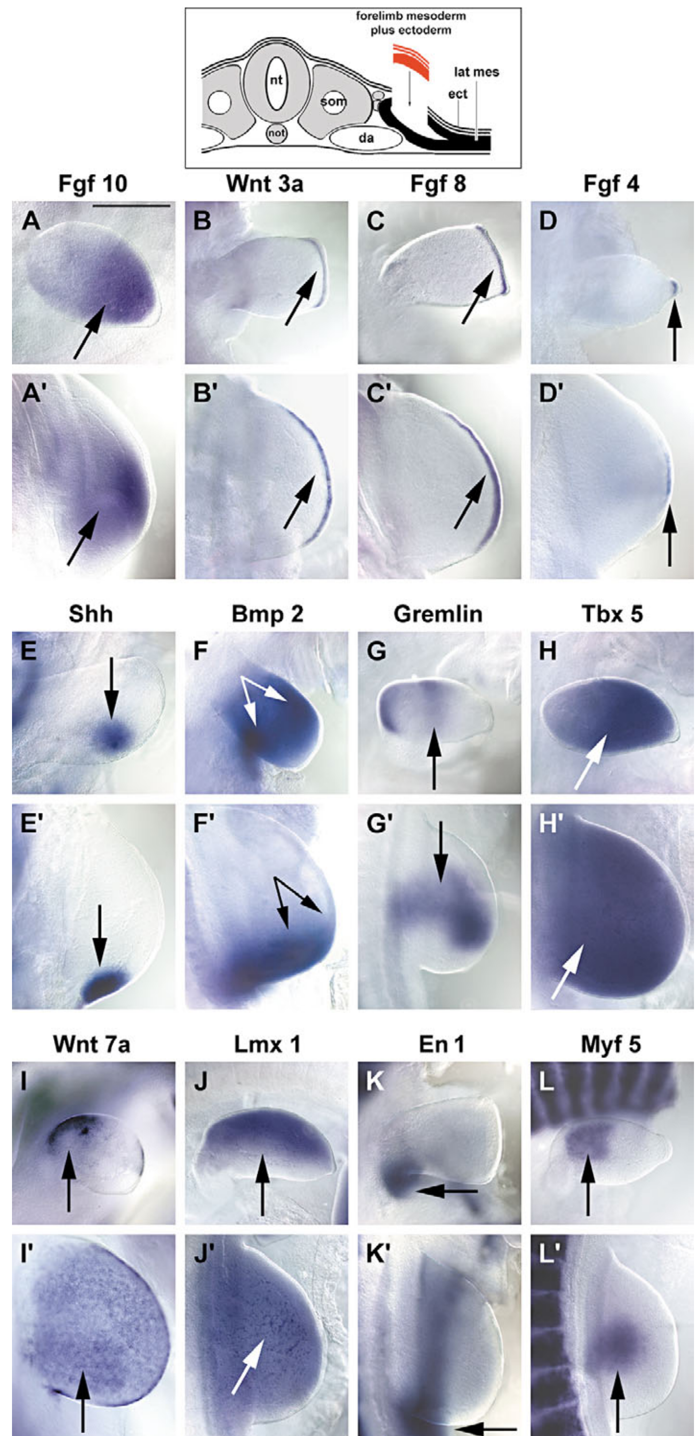

Fig. 1. A graft of forelimb lateral mesoderm plus its covering ectoderm in the neck develops into an ectopic limb bud. A scheme of operation is shown on top of the figure. (A-L) Dorsal views (A-I, K-L) or lateral view (J) of the ectopic limb buds observed in the neck. (A′-L′) Dorsal views onto the right forelimb buds of the embryos with the ectopic limb buds shown in A-L. Expression of *Fgf10* (A,A′), *Wnt3a* (B,B′), *Fgf8* (C,C′), *Fgf4* (D,D′), *Shh* (E,E′), *Bmp2* (F,F′), *Gremlin* (G,G′), *Tbx5* (H,H′), *Wnt7a* (I,I′), *Lmx1* (J,J′), *En1* (K,K′) and *Myf5* (L,L′) mRNAs are shown in dark blue; arrows. In all the pictures (except J), anterior is to the top and distal is to the right. In J, dorsal is to the top and distal is to the right. Scale bar: 500 μm.

2004), then signals back to the limb mesenchyme to maintain Fgf10 (Ohuchi et al., 1997). A mesodermal-ectodermal Fgf feedback loop is thus established. We found that *Fgf10* was expressed in the mesenchyme of the ectopic buds in the neck (*n*=14/15, Fig. 1A,A′). An AER was differentiated at the distal tip of the buds, expressing *Wnt3a* (*n*=17/17, Fig. 1B,B′), *Fgf8* (*n*=6/6, Fig. 1C,C′) and *Fgf4* (*n*=8/9, Fig. 1D,D′).

Fgf4 expression in the AER is established upon Shh signalling from the ZPA, which is situated in the posterior mesenchyme of the bud and organizes the anteroposterior patterning of the limb (Tickle et al., 1975). Fgf4 then acts back onto the ZPA to maintain Shh (Laufer et al., 1994; Niswander et al., 1994). The establishment of this Shh-Fgf loop requires the expression of the Bmp antagonists Gremlin and Noggin in the bud mesenchyme. These prevent Bmp2, 4 and 7 in the bud mesenchyme and AER from downregulating *Fgf4* and disorganizing the AER (Capdevilla et al., 1999; Kawakami et al., 1996; Pizette and Niswander, 1999; Zuniga et al., 1999). We found that in the ectopic buds in the neck, *Shh* was expressed in the ZPA (*n*=6/13, Fig. 1E,E′), and *Bmp2* (*n*=20/24, Fig. 1F,F′) and *Gremlin* (*n*=15/17, Fig. 1G,G′) were expressed in the mesenchyme.

Tbx5 and *Tbx4* expression distinguishes between forelimb and the hindlimb fields, respectively (Gibson-Brown et al., 1996; Logan et al., 1998), but both genes have common roles in the initiation and maintenance of limb outgrowth (Minguillon et al., 2005). *Tbx5* was expressed in the ectopic buds (*n*=7/7, Fig. 1H,H′).

Dorsal and ventral limb territories are demarcated by the expression of *Wnt7a* in the dorsal ectoderm, which induces *Lmx1* in the dorsal mesoderm and is antagonized by *En1* in the ventral ectoderm (Davis and Joyner, 1988; Dealy et al., 1993; Loomis et al., 1996; Parr et al., 1993; Vogel et al., 1995). In the ectopic limb buds in the neck, *Wnt7a* (*n*=3/5, Fig. 1I,I′) and *Lmx1* (*n*=15/15, Fig. 1J,J′) were correctly expressed. *En1* was also expressed, more weakly in the ventral ectoderm and strongly in the ventral base of the ectopic buds (*n*=6/6, Fig. 1K,K′).

When a limb bud develops, the mesodermal cells eventually differentiate into the bones, tendons and connective tissues (Capdevilla and Izpisua-Belmonte, 2001). Limb skeletal muscles, however, are made from precursors that emigrate from the somites (Buckingham et al., 2003). One of the markers for muscle cells is *Myf5* (Saitoh et al., 1993). We found that *Myf5* was expressed in half of the neck ectopic buds (*n*=5/10, Fig. 1L,L′).

During the development of the chick embryo, motor axons extend from the spinal cord towards the limb buds in order to innervate them (Jacob et al., 2001). When limb lateral mesoderm is grafted into the flank, the resulting ectopic limb is properly innervated (Hamburger, 1939). Using the RMO270 antibody to trace the developing nervous system, we investigated whether the ectopic limb buds in the neck also received innervation. We observed that whereas the forelimb and hindlimb buds, as well as ectopic buds in the flank, were always properly innervated (Fig. 2B,C), the ectopic limb buds in the neck were not (Fig. 2A). However, in two cases, where the graft had been inserted at the occipital-cervical interface, the hypoglossal nerve had formed a side branch and innervated the limb (not shown).

The limb mesenchyme, i.e. the lateral mesoderm-derived tissue plus immigrated myoblasts, produce chemoattractants for the growth cones of the incoming axons (Jacob et al., 2001). The fact that half of the ectopic limbs in the neck recruited muscle precursors but only a few were innervated suggests that failure of innervation was caused by the limb mesenchyme independent of the presence of muscle. To confirm this, limb buds were generated in the mid-cervical region, and double-stained for *Myf5* and RMO270. We found that, indeed, the ectopic limbs were not innervated, even in the presence of *Myf5* (*n*=2/2, Fig. 2D).

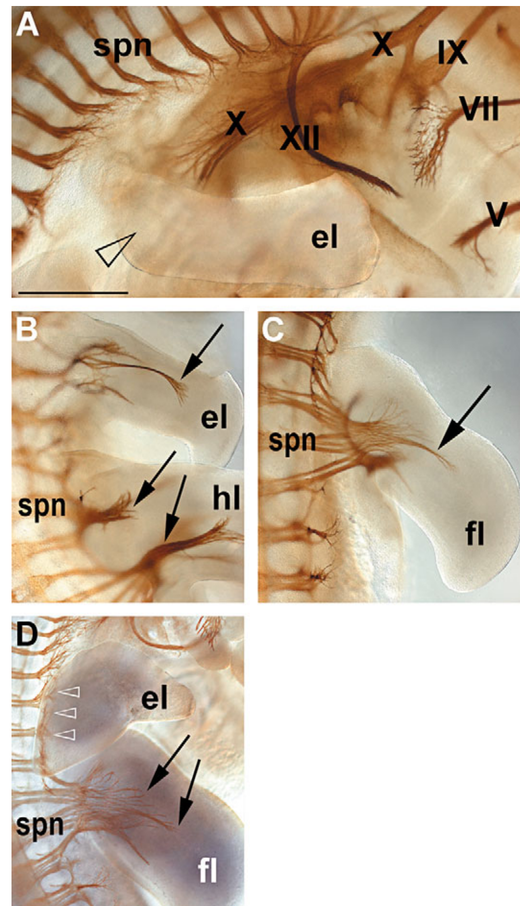

Fig. 2. The ectopic limb buds obtained in the neck are not innervated. Dorsal views of an ectopic limb bud obtained in the neck (A,D) or in the flank (B) and of a hindlimb bud (B) and a forelimb bud (C,D) of an embryo stage HH 25. The axons are stained in brown. (D) The embryo is double stained for Myf5 to reveal the developing muscles. Note that the axons extend into the forelimb and hindlimb buds and into the ectopic limb bud that develops in the flank (arrows in B,C,D) but not in the ectopic limb bud that develops in the neck (arrowheads in A,D). el, ectopic limb bud; fl, forelimb; hl, hindlimb; IX, glossopharyngeal nerve; spn, spinal nerves; V, trigeminal nerve; VII, facial nerve; X, vagal nerve; XII, hypoglossal nerve. Scale bar: 500 μm.

Neck mesoderm does not participate in limb bud formation

Ectopic buds in the flank are known to recruit host cells, which change their fate to become limb cells and contribute to the bud (Dhouailly and Kieny, 1972). Thus, the bud is made from the

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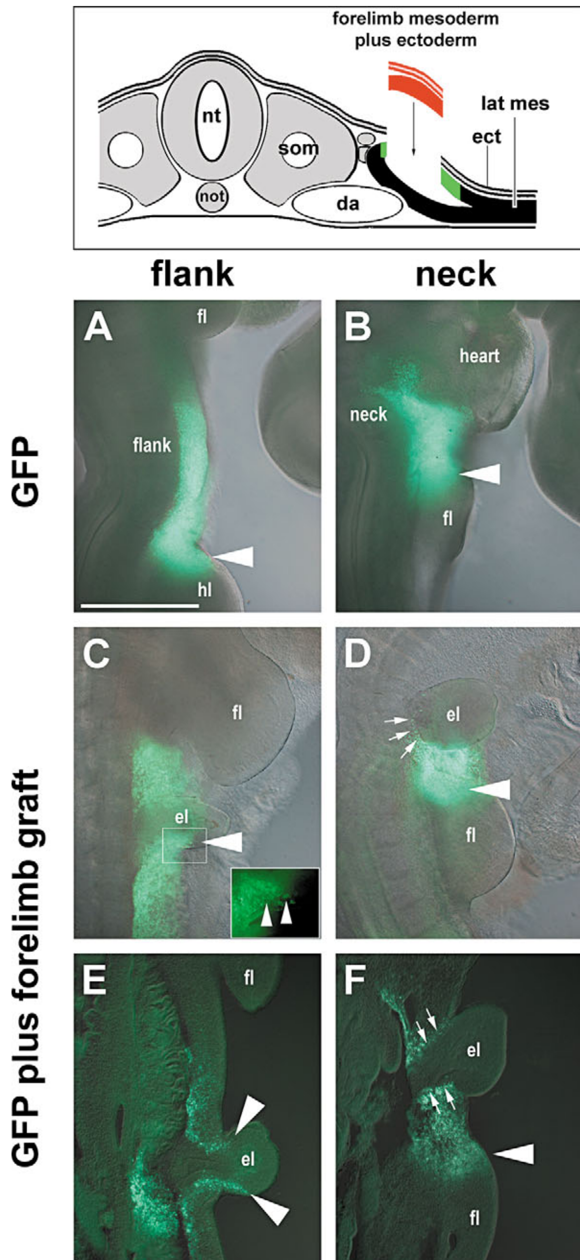

Fig. 3. The neck mesoderm is not recruited to contribute to the bud. A scheme of operation is shown on top of the figure. (A,B) Lateral views of the flank (A) and the neck (B) lateral plate mesoderm of chick embryos at HH20, expressing GFP upon electroporation with the pCaβ-IRES-GFP construct at stage HH14 and 12, respectively. A part of the limb lateral plate mesoderm was also electroporated, leading to GFP expression in the anterior part of the hindlimb or the forelimb (arrowheads). (C,D) Lateral views of the ectopic limb buds obtained in the flank (C) or in the neck (D) of stage HH20 embryos after grafting of limb lateral plate mesoderm plus the overlying ectoderm into the areas electroporated with the pCaβ-IRES-eGFP plasmid. Fluorescent flank mesodermal cells are present in the ectopic bud (C, arrowheads, enlarged area in window). In the neck, fluorescent cells are found around the base of the ectopic bud (D, arrows) but never inside. As the anterior limb field was co-electroporated, fluorescent cells are also found in the forelimb (D, arrowhead). (E,F) Frontal sections of the buds shown in C,D, respectively. In the flank, fluorescent cells are present in the flank lateral mesoderm and in the mesoderm of the ectopic bud (E, arrowheads). In the neck, fluorescent cells are present in the neck mesoderm around the base of the bud (F, arrows). The co-electroporated limb mesoderm also contains fluorescent cells (F, arrowhead). Significantly, no fluorescent cells contributed to the ectopic bud in the neck. Scale bar: 1000 μm in A-D; 500 μm in E,F.

grafted tissues but also from flank cells, which allows it to be well integrated into the flank. Many of the ectopic limb buds that develop in the neck had an abnormal pear-like shape (Fig. 1H,J,L), and their base was generally very thin and fragile in comparison with the wide base of a forelimb bud (Fig. 1H′,J′,L′, for instance), or the base of ectopic limb buds in the flank (Fig. 4I). This suggests that in the neck the buds were not properly integrated, possibly due to a failure of recruitment of host cells.

To investigate whether or not neck lateral mesoderm can be recruited by the graft to contribute to the bud, we carried out cell-tracing experiments. In a first step, the neck lateral mesoderm at the right side of HH12 host embryos was labelled by electroporating the pCaβ-IRES-eGFP plasmid (Alvares et al., 2003) into this tissue. In a second step, forelimb lateral mesoderm and overlying ectoderm from HH13-14 donor embryos was grafted into the electroporated neck of the hosts. A control experiment was carried out in the flank (Fig. 3).

Fig. 3A,B shows the distribution of GFP in the flank and the neck tissues of control embryos that were electroporated but not grafted. The electroporated tissues developed normally. Notably, when the flank lateral mesoderm was electroporated and then received a graft, fluorescent cells were found inside the bud (*n*=6/6, Fig. 3C,E). The cells resided along the margins of the limb, indicating that they did not contribute to the limb via random cell mixing. Rather, they were incorporated into the limb in response to signals from the graft. By contrast, the ectopic buds that developed in the neck never contained fluorescent cells. The fluorescent mesodermal cells were all around the base of the bud but never inside. Moreover, they formed a sharp boundary with the mesoderm of the graft (*n*=6/6, Fig. 3D,F). Thus, the neck lateral mesoderm did not contribute to the ectopic buds. Consequently, the bud remained poorly integrated into the neck.

Neck ectoderm cannot differentiate into an AER

It has been shown that when the limb lateral plate mesoderm without its covering ectoderm is grafted into the flank of a chick embryo, the host ectoderm will cover the graft (Hamburger, 1938). Subsequently, the graft signals to the host ectoderm to induce an AER from a subset of ectodermal cells, while the remaining ectoderm will express the dorsal and ventral markers of the ectodermal jacket. Once the AER is in place, this bud grows and differentiates normally.

To investigate whether the neck ectoderm, like the flank ectoderm, is able to contribute to a limb bud, we grafted forelimb lateral mesoderm alone into the neck. For this, forelimb lateral mesoderm from stage HH13-14 chick embryos, i.e. already expressing *Fgf10*, was separated from the ectoderm and inserted into the neck at the right side of HH10-14 embryos, and in the flank of HH13-14 embryos as a control (see Table S2 in the supplementary material). Embryos were harvested at HH20 as before. Whereas an ectopic limb with a differentiated AER developed in the flank as previously

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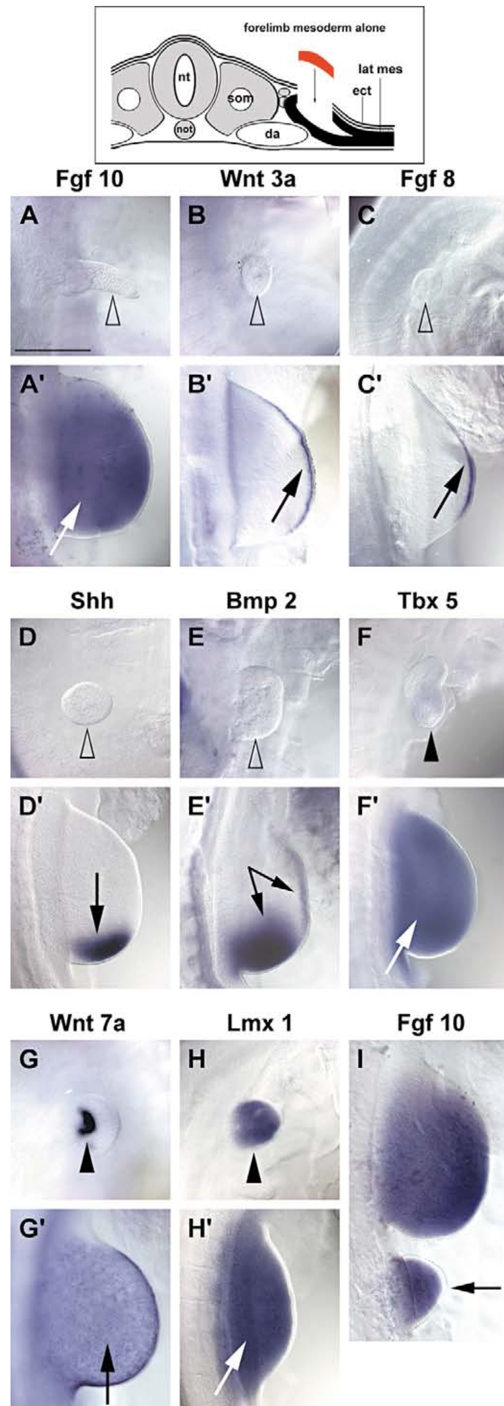

Fig. 4. Neck ectoderm does not differentiate into an AER. A scheme of operation is shown on top of the figure. (A-H) Dorsal views onto the outgrowths obtained in the neck after grafting of forelimb-derived lateral mesoderm without ectoderm (arrowhead in A-H) and onto the right forelimbs (A′-H′) of the operated embryos shown in A-H. (I) Dorsal view of the ectopic bud developing from grafted forelimb mesoderm in the flank, and of the forelimb of the operated embryo. Expression of *Fgf10* (A,A′,I), *Wnt3a* (B,B′), *Fgf8* (C,C′), *Shh* (D,D′), *Bmp2* (E,E′), *Tbx5* (F,F′), *Wnt7a* (G,G′) and *Lmx1* (H,H′) mRNAs are shown in dark blue. The arrows in A′-H′ and I show the wild-type pattern of these genes in limb buds. In all the pictures, anterior is to the top and distal is to the right. Scale bar: 500 μm.

published (Fig. 4I) (Hamburger, 1938), only a small outgrowth was obtained in the neck (Fig. 4A-H). No AER could be seen in these outgrowths, and the lack of *Wnt3a* (*n*=10/10, Fig. 4B,B′) and *Fgf8* (*n*=5/5, Fig. 4C,C′) expression confirmed that no AER was differentiated.

Due to the failure of AER formation, we suspected that the AER-dependent signalling systems might be absent. Indeed, we found that *Fgf10* expression was lost from the bud mesoderm (*n*=7/7, Fig. 4A,A′). *Shh* (*n*=8/8, Fig. 4D,D′) and *Bmp2* (*n*=10/10, Fig. 4E,E′) were not expressed either. Moreover, *Tbx5* was expressed in only two of five outgrowths, and the expression was barely detectable (Fig. 4F,F′).

The establishment of dorsoventral limb polarity is to some extent independent of the processes regulating limb outgrowth (Capdevila and Izpisua-Belmonte, 2001). Thus, markers for the ectodermal jacket of the limb may be expressed in the ectoderm overlying the graft. Significantly, *Wnt7a* (*n*=8/8, Fig. 4G,G′) was expressed in the host-derived ectoderm and *Lmx1* in the donor-derived mesoderm (*n*=9/11, Fig. 4H,H′). However, the markers did not show a wild-type pattern, as *Wnt7a* was either expressed in a central dot (Fig. 4G) or as a crescent (not shown), and *Lmx1* was present throughout the outgrowth (Fig. 4H). Thus, despite the presence of *Wnt7a* and *Lmx1* expression, the outgrowths lacked a proper dorsoventral polarity.

The neck ectoderm does not respond to Fgf10 signalling

We have shown that neck lateral mesoderm cannot be recruited into an ectopic limb bud, and neck ectoderm cannot participate in limb development as it does not form an AER. In normal limb development and during the development of an ectopic limb in the flank, the recruitment of mesodermal and ectodermal cells and the subsequent development of a bud depend on reciprocal Fgf signalling between the lateral mesoderm and the overlying ectoderm (Ohuchi et al., 1997). We thus investigated whether the neck lateral mesoderm and ectoderm can respond to Fgf signals, supplying exogenous sources of Fgf.

The first step in the establishment of the Fgf feedback loop is the activation of *Wnt3a* and *Fgf8* in the AER, in response to Fgf10 from the lateral mesoderm (Kawakami et al., 2001; Kengaku et al., 1998; Ohuchi et al., 1997). This is followed by reciprocal Fgf8 signalling to the mesoderm, which stabilizes *Fgf10* expression. To investigate if the neck ectoderm is able to respond to Fgf10 and then signals back to the lateral mesoderm, beads soaked in Fgf10 protein were grafted into the neck lateral mesoderm at the right side of HH11-14 chick embryos at different positions along the anteroposterior axis (see Table S3 in the supplementary material). As a positive control, Fgf10 beads were placed into the flank of HH13-15 embryos, as Fgf10 has been shown to trigger the development of an ectopic limb (Yonei-Tamura et al., 1999). Indeed, Fgf10 beads induced an ectopic bud in the flank, the AER of which expressed both *Wnt3a* (*n*=2/2, Fig. 5D) and *Fgf8* (*n*=2/2, Fig. 5G), and consequently, the mesoderm of which expressed *Fgf10* (*n*=2/2, Fig. 5A). Thus, the beads released a sufficient amount of Fgf10 protein to establish the Fgf10-Fgf8 regulatory loop and to induce the development of a limb in the flank. By contrast, in the neck neither *Wnt3a* (*n*=17/17, Fig. 5E) nor *Fgf8* (*n*=6/6, Fig. 5H) were expressed above the Fgf10 beads and, as a result, *Fgf10* was not expressed in the neighbouring neck mesenchyme (*n*=11/11, Fig. 5B).

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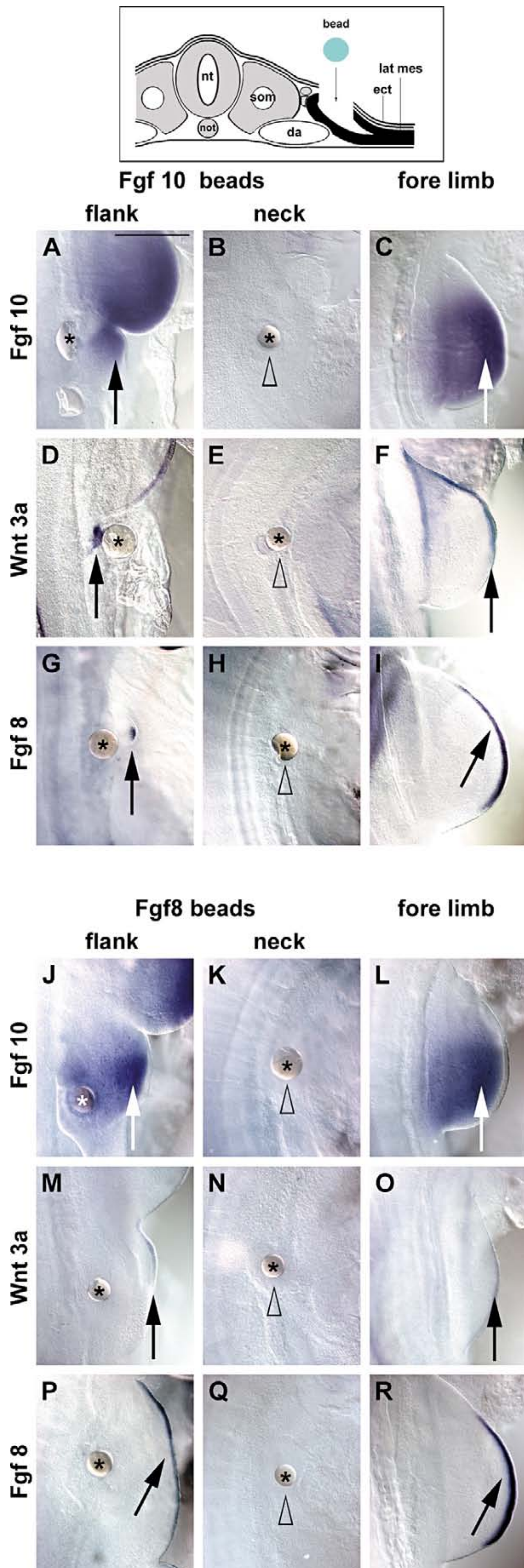

Fig. 5. Neck ectoderm and mesoderm do not respond to Fgf signals. A scheme of operation is shown on top of the figure. Fgf10 beads (A-I) or Fgf8 beads (J-R) were implanted into the flank (A,D,G,J,M,P) or the neck (B,E,H,K,N,Q) of host embryos. Expression of *Fgf10* (A-C,J-L), *Wnt3a* (D-F,M-O) and *Fgf8* (G-I,P-R) is shown in dark blue; arrows. Marker gene expression in the forelimb bud of the operated embryos is shown as a control (C,F,I,L,O,R). Note that in the flank, the beads triggered the development of an ectopic limb with normal marker gene expression. In the neck, neither limb budding nor *Fgf10*, *Wnt3a* or *Fgf8* expression was observed (arrowhead in B,E,H,K,N,Q). Scale bar: 500 μm.

The neck mesoderm does not respond to Fgf signalling

The second step in the establishment of the Fgf feedback loop is the maintenance of *Fgf10* expression in the mesoderm by Fgf8, or when ectopic limbs are induced in the flank, the de-novo activation of *Fgf10* expression in the mesoderm. Hence we tested if the neck mesoderm was able to respond to Fgf8. Beads soaked in Fgf8 protein were grafted into the neck lateral mesoderm at the right side of HH10-14 chick embryos at different positions along the anteroposterior axis (see Table S4 in the supplementary material). As a positive control, Fgf8 beads were inserted into the flank of HH13-15 embryos. The beads grafted into the flank induced ectopic limbs, which expressed *Fgf10* (*n*=6/6, Fig. 5J), *Wnt3a* (*n*=4/4, Fig. 5M) and *Fgf8* (*n*=4/4, Fig. 5P). In the neck, however, *Fgf10* (*n*=24/24, Fig. 5K) was not expressed in the mesoderm around the Fgf8 beads and, as a result, neither *Wnt3a* (*n*=9/9, Fig. 5N) nor *Fgf8* (*n*=7/7, Fig. 5Q) was expressed in the neck ectoderm.

During limb development, limb mesodermal cells underneath the AER stay mitotically active, allowing outgrowth of the bud. The best candidates to induce the division of these cells are Fgf molecules (Ohuchi et al., 1997). However, when Fgf beads were grafted into the neck mesoderm, no outgrowth was seen around the beads (*n*=98/98, Fig. 5B,E,H,K,N,Q).

Expression of Fgf receptor 2 in the neck

The non-response of the neck tissues to Fgf8 and Fgf10 suggests that the molecular network that transduces Fgf signals is missing or incomplete. We thus investigated the presence of Fgf receptor 2 (Fgfr2), as its IIIb isoform is exclusive to limb ectoderm and perceives the Fgf10 signal, while the IIIc isoform is exclusive to lateral mesoderm and perceives Fgf8 (Miki et al., 1992; Ornitz et al., 1996; Orr-Urtreger et al., 1993; Revest et al., 2001; Xu et al., 1998). Using a probe detecting the transcripts for all Fgfr2 isoforms, we simultaneously assayed for the presence of Fgfr2IIIb in the neck ectoderm and Fgfr2IIIc in the neck lateral mesoderm. We investigated the expression of this receptor at stage HH14, i.e. at the stage of limb induction, when Fgf10 is specifically expressed in the presumptive limb lateral plate mesoderm. We found that *Fgfr2* was expressed both in the neck mesoderm and ectoderm at this stage (Fig. 6).

Dissociation of the Fgf signalling pathway in the neck

It has been shown that during limb development, Fgf receptors signal through the MAPK pathway, which involves the di-phosphorylation of the kinases ERK1 and 2 (dpERK) (Corson

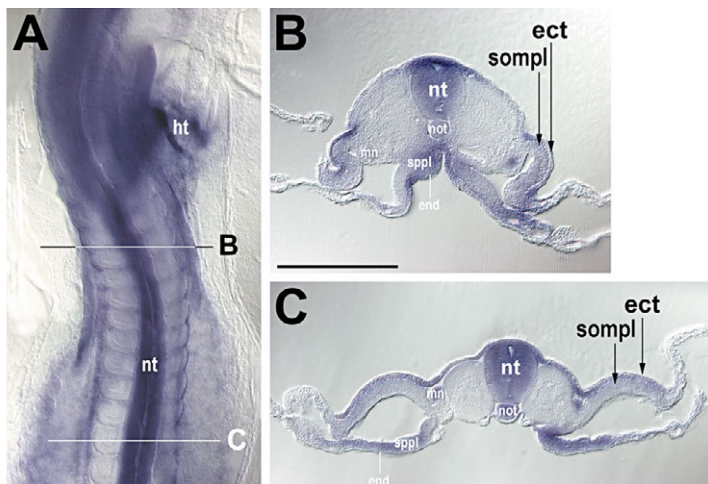

Fig. 6. Fgfr2 expression pattern. Dorsal view (A) and cross-sections of the cervical (B) and presumptive limb (C) region of wild-type chick embryos at HH14. Note that Fgfr2 is expressed in the neural tube, notochord, mesonephros and, most importantly, lateral mesoderm (somatopleure and splanchnopleure) and in the surface ectoderm. ect, surface ectoderm; end, endoderm; ht, heart; mn, mesonephros; not, notochord; nt, neural tube; sompl, somatopleure; sppl, splanchnopleure. Scale bar: 500 μm.

et al., 2003). To investigate if the MAPK pathway can be activated in the neck in response to Fgf8 or Fgf10, we thus assayed for the presence of dpERK. For this, beads soaked in Fgf10 protein or Fgf8 protein were grafted into the neck lateral mesoderm of HH11-14 chick embryos and in the flank of HH13-15 embryos as a positive control. As we noticed that Fgf8 beads induced a bigger ectopic limb in the flank than Fgf10 at a concentration of 500 μg/ml (compare Fig. 5A,D,G and J,M,P), we increased Fgf10 concentration to 1 mg/ml, and in some cases two beads were grafted. Conversely, we decreased Fgf8 concentrations to 250 and 50 μg/ml. We then obtained ectopic buds in the flank of equivalent sizes with both factors, which shows that they have equivalent properties at these concentrations (Fig. 7, compare A,C,E).

Using an antibody directed against dp-ERK (Corson et al., 2003), we found that in the flank, Fgf10 beads induced an ectopic limb bud displaying dp-ERK (Fig. 7A). However, dp-ERK was not detected in the neck (*n*=3/3, Fig. 7B). Fgf8 beads induced an ectopic limb bud in the flank, which also harboured dp-ERK (Fig. 7C,E). Notably, dp-ERK staining was also present in the neck mesoderm around the beads (Fgf8 at 50 μg/ml, *n*=3/3; Fgf8 at 250 μg/ml, *n*=5/5; Fig. 7D,F,G). No dp-

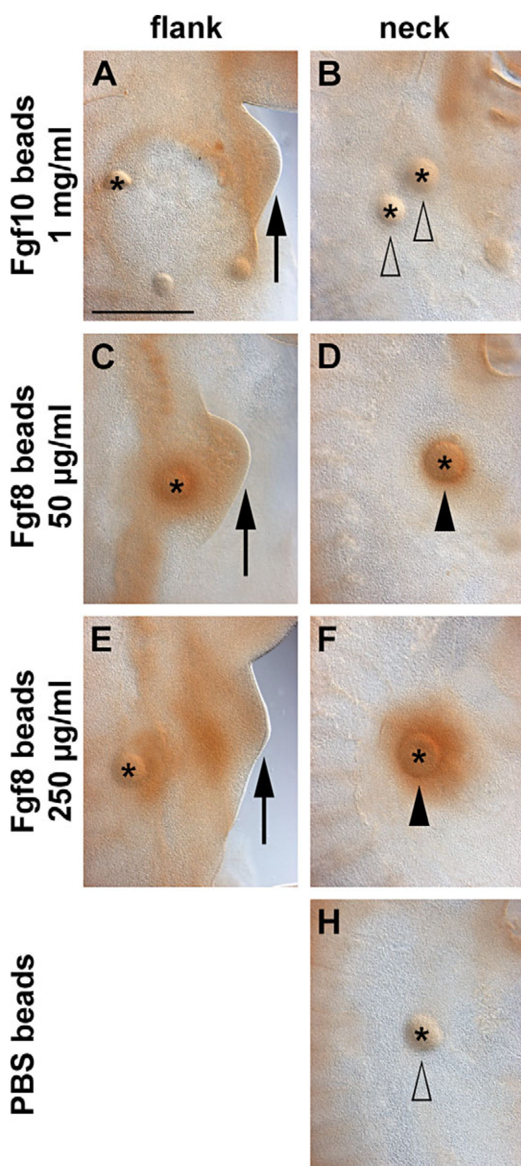

Fig. 7. The MAPK signalling pathway is activated in the neck after implantation of Fgf8 beads but not after implantation of Fgf10 beads. Dorsolateral views (A-F,H) and cross-section (G) of the grafted areas of HH20-21 chick embryos. Fgf10 beads at 1 mg/ml (A,B), Fgf8 beads at 50 (C,D) or 250 μg/ml (E,F,G) and PBS beads (H) were implanted into the flank (A,C,E) or neck (B,D,F,G) of host embryos. Expression of dp-ERK1 and 2 is shown in dark brown. Fgf10 and Fgf8 beads implanted in the flank induce the phosphorylation of ERK1 and ERK 2 (A,C,E, black arrow) and the formation of an ectopic bud. Fgf10 beads implanted in the neck induce neither the phosphorylation of ERK1 and ERK 2 (B, empty arrowhead) nor the formation of a bud. Fgf8 beads implanted in the neck induce the phosphorylation of ERK1 and ERK 2 (D,F,G; black arrowhead) but not the formation of an ectopic bud. Note that dp-ERKs are only present in the mesoderm around the bead (G, black arrowhead). ccv, common cardinal vein; d, dermatome; da, dorsal aorta; drg, dorsal root ganglion; in, intestine; lat mes, lateral mesoderm; m, myotome; not, notochord; nt, neural tube; scl, sclerotome; spn, spinal nerve. Scale bar: 500 μm.

Table 1. Summary of results

Grafted material	Grafted site: Flank	Grafted site: Neck
Forelimb lateral mesoderm plus ectoderm	– Graft forms limb – Graft recruits hosts lateral mesoderm into the limb	– Graft forms limb – Graft does not recruit host cells
Forelimb lateral mesoderm alone	– Graft signals to overlying host ectoderm and neighbouring host mesoderm and, together, they form a limb	– Graft forms small outgrowth, no limb – Graft triggers expression of dorsal limb ectoderm markers – Graft does not trigger the Fgf-feedback loop required for limb bud initiation
Fgf10 bead	– Triggers MAPK signalling cascade in overlying ectoderm and induces AER/Fgf8 expression – Stimulates growth in mesoderm Result: establishment of Fgf-feedback loop, ectopic limb	– MAPK cascade not activated – No AER/Fgf8 expression – No Fgf-feedback loop – No ectopic limb
Fgf8 bead	– Triggers MAPK signalling in underlying mesoderm and stimulates Fgf10 expression Result: establishment of Fgf-feedback loop, ectopic limb	– Triggers MAPK cascade to the point of ERK1,2 phosphorylation – No Fgf-feedback loop – No ectopic limb

ERK was detected in the neck around PBS-soaked beads (n=6/6, Fig. 7H).

Discussion

Limbs develop at the dorsoventral interface of the body at characteristic axial positions. In extant tetrapods, they are separated from the head by the neck and separated from each other by the flank. A host of studies has established that limb development depends on positive signals acting locally. However, the flank can also participate in limb development when the Fgf feedback loop that initiates limb budding is activated. This has led to the view that limbless areas are in principle limb-competent (Capdevila and Izpisua-Belmonte, 2001).

The limbless neck is a recent acquisition of tetrapods, fully developed only in amniotes (Clack, 2002). It evolved as a result of the separation of the shoulder girdle from the skull. Given its history, and given that limbs never returned to their original position with the shoulder immediately posterior to the skull, we wondered whether a specific mechanism was installed in the neck to ensure its limbless state.

Our study shows that limb development is not actively suppressed in the neck. However, both neck lateral mesoderm and ectoderm are incompetent to participate in limb development, as they are unable to install the Fgf feedback loop. This is due to the dismantling of MAPK signal transduction cascades at distinct points in the lateral mesoderm and ectoderm. Our results are summarized in Table 1.

Limb formation is not actively suppressed in the neck

It is known that a graft of limb lateral mesoderm plus overlying ectoderm develops into a limb when placed into the flank, indicating that the flank is a permissive environment for limb development (Hamburger, 1938). We showed that the same holds true for grafts in the neck: morphologically defined buds developed that expressed all the key genes involved in limb development. The presence of *Fgf10*, *Wnt3a* and *Fgf8* indicated that a functional AER and the Fgf feedback loop between AER and limb mesoderm had been established. Moreover, the expression of *Shh* and *Fgf4* reflected the establishment of the ZPA and the Shh-Fgf4 feedback loop. Furthermore, the correct dorsoventral patterning was demonstrated by the expression of *Wnt7a*, *Lmx1* and *En1*. Thus, the continuation of limb development from these grafts was not suppressed in the neck. Consequently, the neck tissues, as the flank tissues, do not harbour signalling molecules that actively shut down limb development.

Neck tissues are unable to participate in limb development

When limb-derived lateral mesoderm plus ectoderm are grafted into the flank, the grafted tissues provide the bulk of the ectopic bud (Hamburger, 1938). However, the ectopic buds also recruit surrounding cells to participate in limb development (Dhouailly and Kieny, 1972) (our results). This leads to the formation of a broad limb base, ensures the integration of the limb into the host tissues and the transmission of the limb-derived signals that organize innervation and attraction of muscle progenitors (Buckingham et al., 2003; Jacob et al., 2001). In the neck, by contrast, the ectopic buds soon showed deficiencies such as an irregular morphology and a fragile base. Moreover, only half of the buds attracted muscle precursors, and even fewer received innervation, indicating that the molecular tools required to efficiently relay signals to the somites and neural tube were absent or non-functional. This suggests that neck tissues may not be able to actively participate in limb development.

To systematically address whether neck tissues may or may not contribute to limbs, we first labelled the lateral mesoderm with a fluorescent marker, followed by the insertion of forelimb mesoderm plus ectoderm. Our control experiments confirmed that flank lateral mesoderm becomes incorporated into the ectopic limb. However, this was never the case in the neck. Moreover, the host cells formed a sharp boundary with the mesoderm of the graft, indicating that they are unable to take an active part in limb development.

Next we performed an ectoderm recruitment assay. It has been shown that when lateral mesoderm of the forelimb is grafted into the flank alone, it recruits the host ectoderm to form the ectodermal jacket and AER of the limb, to participate in the Fgf and Shh-Fgf4 feedback loops, and to support the development of a normal limb. In the neck, by contrast, the

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grafted mesoderm formed a small outgrowth, possibly because in the donor it was loaded with Fgf10 protein. Markers for the establishment of the AER and the Fgf feedback loop failed, and consequently, the Shh-Fgf4 feedback loop also failed, leading to the absence of defined proximodistal and anteroposterior axes. Thus, the neck ectoderm was also not limb-competent.

Notably, the forelimb lateral mesoderm when grafted into the neck induced *Wnt7a* expression in the overlying host ectoderm. As a consequence, *Lmx1* was strongly expressed in the grafted limb mesenchyme. This indicates that the neck ectoderm is not completely deaf to signals from the limb. However, both *Wnt7a* and *Lmx1* showed aberrant expression patterns, indicating that the dorsoventral axis was also incorrect.

In the neck, the Fgf10-Fgf8 feedback loop fails

When the forelimb-derived lateral mesoderm was grafted into the neck, neither Fgf10 in the surrounding mesoderm nor Fgf8 in the overlying ectoderm was induced. This implies that neck tissues are not able to establish the mesodermal-ectodermal Fgf feedback loop, which is crucial for the initiation of limb budding. In order to confirm this finding, we directly tested the response of neck tissues to ectopically applied Fgf. Significantly, in our control experiments Fgf10 and Fgf8 readily triggered limb development in the flank, as previously reported (Crossley et al., 1996; Yonei-Tamura et al., 1999). However, the formation of an outgrowth was never observed in the neck. Moreover, the expression of markers normally induced by these signalling molecules was lacking, both in the mesoderm and in the ectoderm. Thus, neck lateral mesoderm and ectoderm are deaf to Fgf signalling and consequently, reciprocal Fgf signalling fails. We conclude that limb development in the neck is prevented at its earliest step, namely the initiation of the bud.

Presence of Fgf receptors

As the neck tissues were unable to install the Fgf feedback loop, it was paramount to investigate at which point Fgf signalling was defective. It has been established that in limb development Fgf signals are perceived through the Fgf receptor 2, with the IIIb isoform binding Fgf10 and the IIIc isoform taking up Fgf8 (Revest et al., 2001; Xu et al., 1998). Moreover, expression studies showed that Fgfr2 IIIb is only expressed in the limb ectoderm (Revest et al., 2001), while IIIc is exclusive to the lateral mesoderm (Miki et al., 1992; Ornitz et al., 1996; Orr-Urtreger et al., 1993). Thus, using an *Fgfr2* probe detecting all isoforms, we were able to simultaneously assay for the presence of Fgfr2IIIc in the neck lateral mesoderm and Fgfr2IIIb in the ectoderm. We found that they were correctly expressed. Thus, if the transcripts are translated at sufficient levels, Fgf perception in the neck is possible.

Fgf signalling is interrupted at specific points within the MAPK signalling pathway

In the neck lateral mesoderm, Fgf10 is expressed temporarily at the time the future limb fields are established (Ohuchi et al., 1997). Moreover, our data showed persistent expression of at least *Fgfr2* mRNA, suggesting that the system is geared up for functional Fgf signalling. However, both Fgf10 signalling from the mesoderm to the ectoderm and Fgf8 signalling from the ectoderm to the mesoderm were unsuccessful. This suggests that Fgf signalling is interrupted within the signal transduction cascade.

Fgf signalling predominantly operates through the MAPK pathway (Corson et al., 2003; Schlessinger et al., 2000). This pathway encompasses a series of protein phosphorylation events and can be monitored using antibodies against the di-phosphorylated forms of the kinases ERK1 and ERK2 (Corson et al., 2003). Employing this approach, we show that in the neck Fgf8-loaded beads are able to trigger phophorylation of ERK1 and ERK2. This confirms that functional receptors are present in the neck to bind Fgf8. It furthermore indicates that Fgf8 signal transduction is interrupted downstream of ERK1 and ERK2. By contrast, Fgf10 did not trigger ERK phosphorylation, indicating that signalling is interrupted upstream. Thus, in the neck lateral mesoderm and ectoderm, Fgf signalling was dismantled separately and at distinct points of the signalling cascades.

Model: the neck is limb-incompetent due to the dissociation of Fgf signalling cascades at distinct points in the lateral mesoderm and ectoderm

Limb induction, i.e. the initiation of limb budding, depends on reciprocal Fgf signalling between lateral mesoderm and ectoderm (Fig. 8A). At stage HH14 in the chick, Fgf10 is expressed in the presumptive limb lateral plate mesoderm. Fgf10 binds to the Fgfr2 IIIb present on the surface of the limb

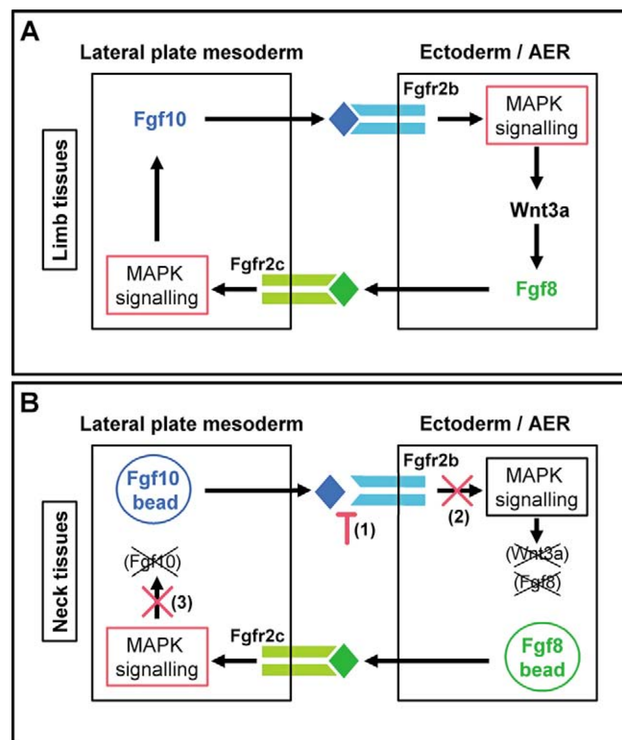

Fig. 8. Interruption of the Fgf feedback loop in the neck. (A) Schematic representation of the Fgf feedback loop that initiates limb budding for a normal limb and for an ectopic limb in the flank; details in the text. (B) In the neck, Fgf signalling is interrupted at distinct points in the neck lateral mesoderm and ectoderm, rendering this region limb-incompetent. Fgfr2b/2c, Fgfr2IIIb/IIIc.

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ectodermal cells. The MAPK signalling pathway is activated in the ectodermal cells, triggering expression of Wnt3a, which in turn induces expression of Fgf8 in the AER. Fgf8 binds to Fgfr2IIIc present on the surface of the mesodermal cells. MAPK signalling is activated and leads to the maintenance of the expression of Fgf10. In the flank, this signalling cascade is silent until exogenously applied Fgf10 or Fgf8 kick-starts the system. Thus, all components of the molecular network involved in Fgf signalling are present in the flank – the flank is a 'limb in waiting'.

The neck, by contrast, is limb-incompetent. This is not due to the presence of specific inhibitors of limb development. Rather, key components of the signalling cascades positively regulating limb development have been lost (Fig. 8B). When exogenous Fgf10 is supplied to substitute for the signal emerging from the lateral mesoderm, MAPK signalling does not proceed to the point of ERK1/2 phosphorylation. Thus, signalling is interrupted upstream in the cascade, Wnt3a and consequently Fgf8 are not expressed in the ectoderm, and no AER develops. This prevents the activation of Fgf10 and the development of a limb. Our expression analysis suggests that the receptor to bind Fgf10 is present, although this needs to be confirmed at the protein level. If the receptor is functional, then either molecules that operate outside the cells to facilitate Fgf10 binding to the receptor (Fig. 8B-1), or factors that operate inside the cell, between the receptor and the phosphorylation of ERK1/2 (Fig. 8B-2), are missing.

When an exogenous source of Fgf8 is provided in the neck to substitute for the signals derived from the AER, then dpERK1/2 are produced in the lateral mesoderm. This indicates that Fgfr2IIIc is present and that signal transduction through the MAPK pathway has commenced. However, signal transduction downstream of ERK1/2 is not completed, as Fgf10 is not upregulated in the neck mesoderm. This indicates that the molecular pathway between ERK1/2 phosphorylation and FGF10 activation is defective (Fig. 8B-3).

Outlook

Our study shows that, in the neck, Fgf signalling was interrupted at distinct points in the lateral mesoderm and ectoderm. This does not exclude the possibility that further factors involved in limb development have also been lost. For example, the regulators that act upstream of Fgf10, such as Tbx5/4 and Wnt2b, may be required to install the components for successful Fgf-MAPK signalling. These factors are absent from the neck. However, at least at mRNA levels, there is some expression of these markers in the neck at the time of limb field specification, as is the case for Fgf10 (Gibson-Brown et al., 1998; Kawakami et al., 2001; Ohuchi et al., 1997; Ohuchi et al., 1998). This suggests that yet further factors may be involved. Candidates are Hox/HOM genes, suspected to provide a 'Hox-code' for fore- and hindlimbs in the lateral mesoderm (Cohn et al., 1997), and amendment of their expression boundaries has been suggested as a cause for the loss of forelimbs in snakes (Cohn and Tickle, 1999). By contrast to the neck, the flank has all tools for limb development in store. Thus, to investigate which signalling cascades have to be reconstituted in the future to correct amelic conditions in humans, the neck is possibly the most appropriate test system.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/24/5553/DC1

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