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FGF and genes encoding transcription factors in early limb specification

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Abstract

SnR, twist and *Fgf10* are expressed in presumptive limb territories of early chick embryos. When FGF-2/FGF-8 beads are implanted in chick flank, an ectopic limb develops and *SnR* is irreversibly activated as early as 1 h. Ectopic *Fgf10* and *twist* expression are activated much later at 17 and 20 h, respectively. FGF-10 can also induce *SnR*, but much later, and in this case activation occurs simultaneously with that of *twist* and *Fgf10* via the *Fgf8*- expressing ridge. *Tbx-4* and *Tbx-5* are expressed in leg and wing forming regions, respectively, in a similar pattern to *SnR* and *twist*. FGF-2 leads to ectopic expression of *Tbx-4* and *Tbx-5* as rapidly as ectopic expression of *SnR*, but the patterns of ectopic transcripts suggest that induction of *SnR* and *Tbx* gene expression occur via different pathways.

Keywords: Limb development; *SnR*; *twist*; *fgf10*; *Tbx-5*; *Tbx-4*; Chick embryos; FGF-2; FGF-10

1. Introduction

The development of two pairs of limbs is fundamental to the tetrapod body plan. Embryological manipulations in chick embryos show that the lateral plate mesoderm becomes determined to form limbs in precise locations along the main body axis long before there are any visible buds. When mesoderm from limb forming regions, as early as stage 11, is transplanted to the flank, ectopic limbs develop (Pinot, 1970; Kieny, 1971). There is growing evidence that FGF signals are important in specifying limb formation but the molecules that are involved in the immediate response are unknown. Here we report that a Snail family member is involved in specification of lateral plate mesoderm to form a limb in chick embryos.

Vertebrate limbs can be initiated by application of FGF. Several different FGF's can induce limbs, including FGF1, FGF2, FGF4, FGF8 and FGF10. When beads soaked in FGF are applied to the flank of early chick embryos, ectopic limbs develop (Cohn et al., 1995; Ohuchi et al., 1995; Crossley et al., 1996) and in mouse chimeras containing FGF4-expressing cells, small outgrowths develop from the flank (Abud et al., 1996).

An important question is whether FGF signals initiate normal limb development. Removal of FGF function in mice, by targeted mutations of FGF receptors, leads to an embryo which lacks limb buds (Xu et al., 1998). *Fgf8* is expressed in intermediate mesoderm adjacent to the limb-forming lateral plate mesoderm in chick embryos (Crossley et al., 1996) and there are several lines of evidence suggesting that signalling by medial tissues, and in particular intermediate mesoderm, is necessary for limb development, although this is somewhat controversial (Geduspan and Solursh, 1992; Fernandez-Teran et al., 1997). Another member of the FGF family, *Fgf10*, has been found to be expressed in presumptive limb forming regions of chick embryos (Ohuchi et al., 1997). Mice in which *Fgf10* is functionally inactivated, have severe limb truncations (Sekine et al., 1999) or lack limbs completely (Min et al., 1998).

The ability to reprogramme chick flank with FGF provides an opportunity to dissect out the steps that are

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necessary for limb formation. A discrete bud in the flank region is visible about 24 h after FGF application (Cohn et al., 1995) and *Fgf10* is induced within 17 h (Ohuchi et al., 1997). The ectopic bud becomes equipped with an apical ridge that mediates outgrowth and a polarizing region that patterns the limb (Cohn et al., 1995). *Fgf8* gene expression, which provides one of the ridge signals, can be detected in the ectoderm 14 h after FGF application (Crossley et al., 1996) and *Shh*, which characterizes polarizing region signalling, is expressed in mesoderm at 24 h (Cohn et al., 1995). So far, one of the earliest known events in respecification of mesenchymal flank cells to form a limb, is reprogramming of expression of *Hox 9* paralogs, 9 h after FGF application (Cohn et al., 1997). It has been suggested that the pattern of *Hox* gene expression in lateral plate mesoderm could determine the position in which limbs are formed along the main body axis.

SnR, a homologue of the gene encoding the *Drosophila* zinc finger transcription factor Snail, which has been shown to be involved in specification of left/right asymmetry early in development (Isaac et al., 1997), is expressed in chick limb buds (Sefton et al., 1998). In *Drosophila,* Snail is involved in mesoderm formation (Simpson, 1983; Grau et al., 1984) and, later on, *snail* and a related zinc finger gene, *escargot,* are expressed in, and are required for correct development of wing imaginal discs (Fuse et al., 1996). In addition to *SnR*, another gene in this family, *slug,* is expressed in chick limb buds (Buxton et al., 1997; Ros et al., 1997; Sefton et al., 1998).

In *Drosophila*, Snail interacts with other transcription factors. A bHLH transcription factor, *twist*, cooperates with *snail* in mesoderm formation in early embryos (Ip et al., 1992a). *Twist* is expressed in imaginal discs (Emori and Saigo, 1993), but there is no evidence that it cooperates with *snail* in this region. However, in vertebrates, *twist* has been implicated in limb bud development (Chen and Behringer, 1995; Bushdid et al., 1998; Kanegae et al., 1998). Recently, much interest has been aroused by *Tbx-4* and *Tbx-5*, which are expressed early in leg and wing respectively. Respecification of flank to limb results in an ectopic bud which expresses *Tbx* genes (Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998), and misexpression of *Tbx-4* in the wing, where *Tbx-5* is normally found, can lead to a wing containing leg elements (Logan and Tabin, 1999; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999).

Here, we compare expression of the transcription factors, *SnR, Twist, Tbx-4* and *Tbx-5* with that of *Fgf10*, now thought to play an essential role in limb development (Ohuchi et al., 1997; Min et al., 1998; Sekine et al., 1999), in normal and FGF treated chick embryos. All these genes are expressed in presumptive limb regions of chick embryos at the right time to be involved in limb bud induction and are initiated rapidly in the flank in response to FGF. The most rapidly induced of these genes, *SnR*, is examined in more detail.

2. Results

2.1. Comparison of genes expressed early in limb development

We compared expression patterns of the transcription factors *SnR, Twist, Tbx-4* and *Tbx-5* with that of *Fgf10* in lateral plate mesoderm of early chick embryos. At stage 14 (Hamburger and Hamilton, 1951), *SnR* is expressed in the posterior lateral plate (Fig. 1A) in a pattern very similar to that of the leg specific gene *Tbx-4* (Fig. 1J). These cells expressing *SnR* and *Tbx-4* will later contribute to the leg (M. Tanaka, personal communication). At stage 15 *SnR* expression appears in presumptive wing (Fig. 1B), opposite somites 16–20, in a pattern similar to that of the wing specific gene *Tbx-5* (Fig. 1K). *Fgf10* is expressed as early as *SnR* but at stage 14 is found in future wing and posterior lateral plate (Fig. 1D). At stage 15, *Fgf10* expression remains in the presumptive wing and posterior lateral plate, although posterior expression is much weaker (Fig. 1E). At stage 16, *SnR* and *Fgf10* are expressed in a similar pattern in both future wing and leg (compare Fig. 1C and 1F).

Even though *twist* has been implicated in vertebrate limb development (Chen and Behringer, 1995; Bushdid et al., 1998; Kanegae et al., 1998) no detailed expression pattern for this gene has been described so far. Therefore we compared *twist* expression in early chick limb development with that of the other genes described above. By stage 14, *twist* expression is located in presumptive wing and posterior lateral plate mesoderm (Fig. 1G) in a pattern similar to that of *Fgf10* (Fig. 1D) and, by stage 15, *twist* expression in both presumptive wing and leg also looks remarkably similar to that of *SnR* at this stage (compare Fig. 1B and 1H). Later, anterior lateral plate expression of *twist* sweeps posteriorly until it extends throughout the inter-limb region to meet presumptive leg expression by stage 16/17 (Fig. 1I). In contrast *SnR* and *Fgf10* transcripts are never detected in the flank region.

2.2. Comparison of induction of gene expression by application of FGF-2

In order to explore the possible significance of *SnR, Twist, Tbx-4* and *Tbx-5* in limb development, we implanted FGF-2 coated beads at different positions in the flank of stage 12–15 chick embryos. At 0.5 h ($n = 9$) and 0.75 h ($n = 9$) after FGF application, no ectopic *SnR* expression was detected in the flank, but at 1 h ($n = 22$), strong ectopic expression of *SnR* was seen (Fig. 2A). In the majority of cases ($n = 20/22$), ectopic *SnR* expression in the flank was located in a semi-circular patch lateral to the bead; that is, extending anteriorly, posteriorly and laterally in relation to the main body axes of the embryo, regardless of anterior-posterior positioning of the bead. In one case, ectopic expression was located only anterior to the bead, and in one case, only posterior. Expression was never seen medial to the

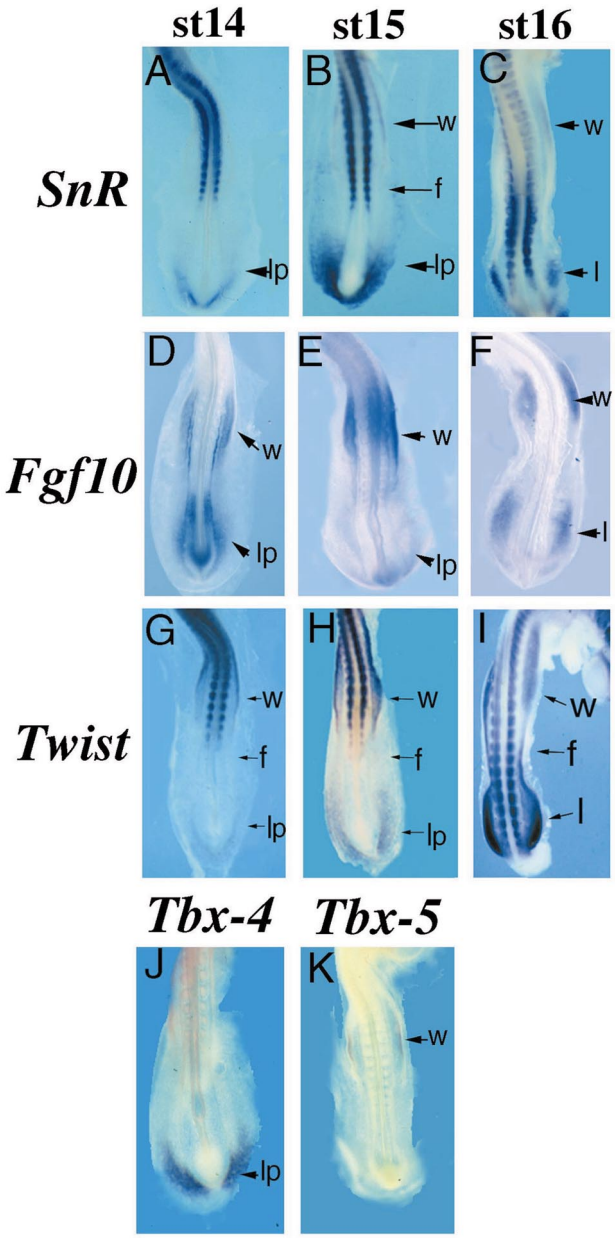

Fig. 1. Gene expression in limb regions of normal chick embryos as detected by whole-mount in situ hybridisation. Embryos orientated with anterior at top. (A) Expression of *SnR* in posterior lateral plate mesoderm (lp) at stage 14 is located in cells that will contribute to leg. Expression is absent from presumptive wing and flank region. (B) Expression of *SnR* in lateral plate mesoderm at stage 15 is located in presumptive wing (w) and becomes more extensive in posterior lateral plate (lp). No transcripts are detected in the flank (f). (C) At stage 16, expression of *SnR* is now present in wing (w) and leg (l) regions. (D) At stage 14, *Fgf10* expression is located in the presumptive wing region (w) in addition to the posterior lateral plate (lp). (E) At stage 15, *Fgf10* expression remains in presumptive wing (w) but has switched off in the posterior lateral plate (lp). (F) At stage 16 Fgf10 is now present in wing (w) and leg (l). (G) At stage 14, *twist* is expressed in wing (w) and leg (l) forming regions, but absent from flank (f). (H) At stage 15, *twist* is expressed in presumptive wing (w) and leg region (l). (I) At stage 16, *twist* expression has now spread to flank lateral plate mesoderm (f) in addition to wing (w) and leg (l). (J) At stage 14, *Tbx-4* is expressed in the posterior lateral plate (lp). (K) At stage 15, *Tbx–5* is expressed in presumptive wing (w).

bead. The distance of the ectopic *SnR* patch from the bead varied in that sometimes ectopic expression was immediately adjacent to the bead and sometimes several cell diameters away. We could find no defined relationship between location of the *SnR* domain and position of bead implantation or stage of embryo or duration of FGF treatment. Neither was distance of ectopic *SnR* expression from the bead reduced when beads soaked in a lower concentration of FGF (0.25 mg/ml) were implanted ($n = 12$; data not shown).

At time points of 2, 3, 4, 5, 6, 7, 8, 9 and 15 h after FGF application ($n = 32$), strong ectopic *SnR* expression remained in a semi-circle around the bead, but in addition, at later time points (15 h), expression extended throughout the flank ($n = 8$, Fig. 2B). By 20–24 h after FGF application ($n = 18$) the future ectopic bud, detected as a slight thickening, was uniformly *SnR* positive and the non-thickened flank region, if present, was *SnR* negative (Fig. 2C). By 48 h ($n = 3$), the now obvious ectopic bud was *SnR* positive throughout (Fig. 2D).

Fgf10 expression was induced within 17 h after FGF-2 implantation into the flank (Fig. 2L; $n = 2$) in a similar pattern to *SnR* (cf Fig. 2C). Before this time point no *Fgf10* expression was detected ($n = 10$), and is thus not induced as rapidly as *SnR*.

Twist expression in the flank was not induced as rapidly as *SnR* when an FGF-2 bead was implanted. At time points between 1 and 15 h after application, FGF-2 did not alter *twist* expression even though these embryos would have gone on to develop ectopic limbs (Fig. 2F,G; $n = 32$). However, by 24 h after FGF-2 application, *twist* was expressed in the flank (Fig. 2H; $n = 15$) in a similar pattern to *SnR* (cf Figs. 2C and 3A).

Like *SnR*, *Tbx-5* and *Tbx-4* are ectopically expressed in the flank in rapid response to FGF-2 (after 1–1.5 h), thus, indicating that reprogrammed flank quickly makes the decision whether to become wing or leg. In contrast to *SnR*, which is induced within 1 h regardless of the position of the FGF-2 bead, rapid ectopic *Tbx-5* expression only occurs when the bead is placed in anterior flank (Fig. 2K; $n = 3$), and rapid ectopic *Tbx-4* expression is seen only when the bead is placed in posterior flank (Fig. 2J; $n = 2$). When FGF-2 beads are placed in mid-flank, no rapid change in either *Tbx-4* ($n = 4$) or *Tbx-5* ($n = 3$) is seen. A further difference between ectopic *SnR* and ectopic *Tbx* is that the ectopic *Tbx-4* and *Tbx-5* expression in the flank appears as an extension of the endogenous expression. This is unlike the early expression of *SnR*, which appears in a patch just lateral to the bead (Fig. 2A). Also, in the case of *Tbx-5,* expression in the wing region intensifies (Fig. 2K). Thus, initially only a small subset of cells in the flank will express both *SnR* and *Tbx* genes. Later, however, when *SnR* expression extends throughout the flank (Fig. 2B,C), the same cells will express both *SnR* and *Tbx-5* and/or *4*. These data together suggest that induction of expression of *SnR* and *Tbx* genes by FGF-2 takes place by different routes.

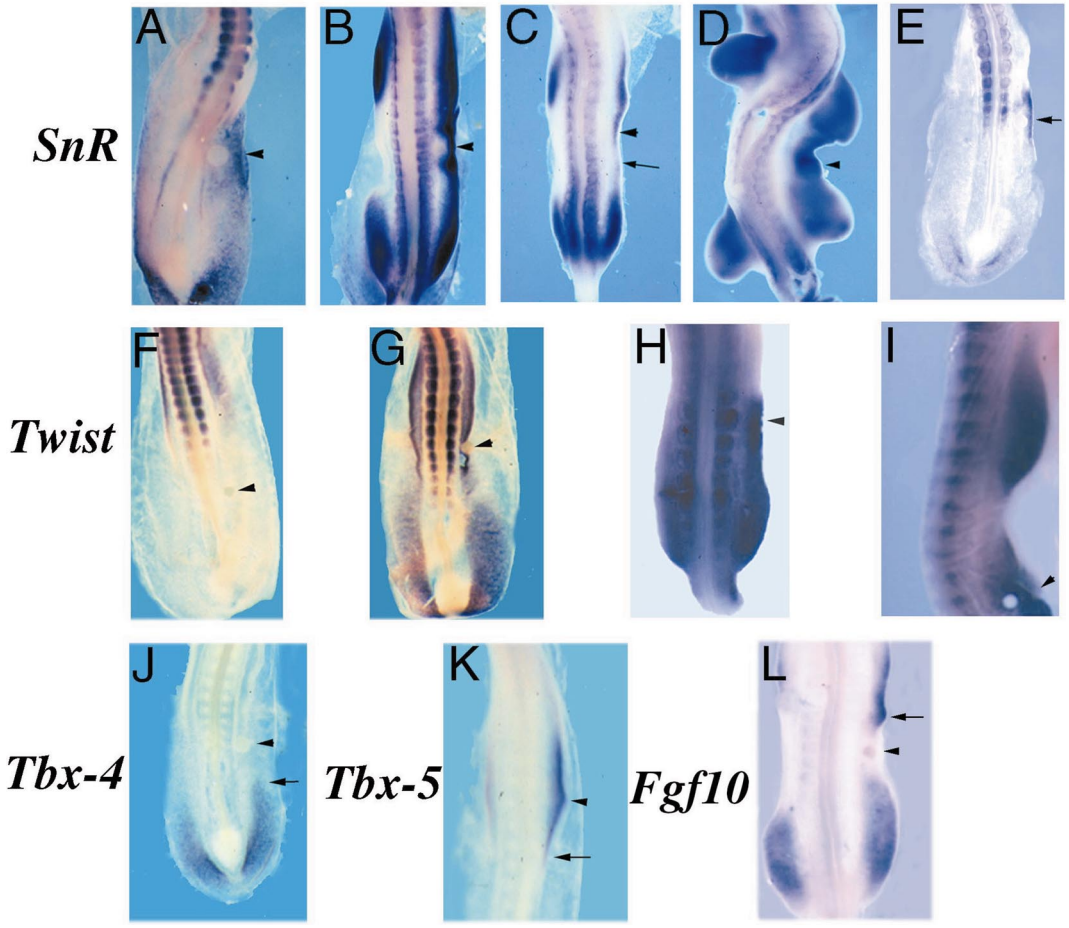

Fig. 2. Gene expression in limb regions of FGF-treated chick embryos as detected by whole-mount in-situ hybridisation. Embryos orientated with anterior at top. (A) Expression of *SnR* 1 h after bead implantation of FGF-2 opposite somite 23 of a stage 15 embryo. Transcripts (arrow head) found anterior, lateral and posterior to bead, but not medially. (B) Expression of *SnR* 15 h after bead implantation opposite somite 23 of a stage 16 embryo. Transcripts found throughout the flank and anterior and posterior to bead (arrow head). (C) Expression of *SnR* 24 h after bead implantation opposite somite 21 of a stage 14 embryo. Transcripts found in the forming ectopic bud (arrow head), but are absent from the flank (arrow). (D) Expression of *SnR* 48 h after bead implantation opposite somite 24 of a stage 14 embryo. Transcripts found throughout the ectopic bud (arrowhead). (E) Expression of *SnR* 1 h after bead implantation of FGF-8 opposite somite 23 of a stage 14 embryo. Transcripts (arrow head) found anterior, lateral and posterior to bead, but not medially. Note similarity with (A) and Fig. 3C. (F) Expression of *twist* 9 h after FGF-bead implantation into a stage 13 embryo. No transcripts found near bead (arrowhead). (G) Expression of *twist* 15 h after FGF bead implantation into a stage 14 embryo. *Twist* expression, now located in the flank of normal embryos, is unaltered (arrowhead). (H) Expression of *twist* 24 h after FGF-bead implantation opposite somite 24 of a stage 14 embryo. Transcripts found in region that will form ectopic bud (arrow head). (I) Expression of *twist* 20 h after FGF-8 bead implantation opposite somite 25 of a stage 14 embryo. Transcripts found in region that will form ectopic bud (arrow head). (J) Expression of *Tbx-4* (arrow)1 h after FGF-2-bead implantation (arrowhead) opposite somite 25 of a stage 15 embryo. Expression has expanded anteriorly as compared with contralateral side (arrow). Note expression is 'creeping' towards the bead rather than being located adjacent to the bead (cf *SnR* expression in A). (K) Expression of *Tbx-5* 1.5 h after FGF-2-bead implantation opposite somite 21 at stage 15. Expression has expanded posteriorly (arrow) and has also intensified (arrowhead). Note the bead has fallen out of this embryo. (L) Expression of *Fgf10* 17 h after FGF-2-bead implantation opposite somite 23 of a stage 14 embryo. Transcripts found in the forming ectopic bud (arrow), but are absent from the flank (arrowhead).

2.3. FGF-8 application also leads to rapid activation of SnR

In order to check the possible significance of a role for early induction of *SnR* by FGF-2, we applied FGF-8 to the flank of stage 13–15 chick embryos and looked for ectopic *SnR* induction after 1 h. In 5/5 embryos ectopic *SnR* expression was induced (Fig. 2E) in a pattern remarkably similar to that seen after FGF-2 induction (compare with Figs. 2A and 3C). Timing of *twist* induction by FGF-8 is also identical to that caused by FGF-2. At 4 h after FGF-8 application no change in *twist* expression is detected ($n = 6$, data not shown), however, by 20 h after FGF-8 application, strong *twist* expression is detected throughout the ectopic thickening (Fig 2I; $n = 5$). We confirm the results of Ohuchi et al. (1997) that *Fgf10* expression is induced within 17 h of FGF-8 application (data not shown).

2.4. FGF-2 induction of SnR is irreversible and not an immediate early response

Previous experiments have shown that application of an FGF-2 bead for just 1 h is sufficient to irreversibly repro-

gramme flank to form limb (Cohn et al., 1995). To determine whether FGF-2 induction of *SnR* in the flank is irreversible, we surgically removed FGF-2 beads 1 h after implantation, and incubated the embryos for a further 19 h. In 4/4 embryos examined, *SnR* expression was maintained (Fig. 3A), showing that irreversible activation of *SnR* in the flank correlates with reprogramming of flank to wing.

The rapidity of the appearance of *SnR* transcripts in response to FGF-2 suggests that *SnR* may be an early immediate response to FGF signalling. To assess this possibility, we compared the extent of the *SnR* domain induced by an FGF-2 bead and the distance over which FGF-2 released from the bead travelled. We implanted beads soaked in DIG-labelled FGF-2 and examined distribution of labelled protein and of *SnR* transcripts. After 1.5 h, labelled FGF-2 protein could be detected only immediately adjacent to the bead (see also Storey et al., 1998), while *SnR* transcripts were seen in cells much further away (Fig. 3B). This suggests that FGF-2 released from beads does not directly activate *SnR* expression but instead results in production of a signal which is rapidly propagated. However, it is possible that FGF-2 protein is present further away from the bead but in such low levels as to be undetectable.

We also tested whether FGF-2 induction of *SnR* is protein synthesis dependent by treating embryos with cycloheximide. Control embryos at stage 13/14 incubated with FGF-2 beads in New culture had strong ectopic *SnR* expression after 1 h (Fig. 3C; $n = 9$). Identically treated embryos incubated in the presence of cycloheximide had no obvious ectopic *SnR* expression around the bead (Fig. 3D; $n = 21$). This result is consistent with the idea that expression of *SnR* is not a direct response to FGF signalling. It should be noted, however, that in cycloheximide treated embryos, *SnR* expression was generally enhanced throughout the flank on both treated and untreated sides. Cycloheximide treatment of cultured chick embryos also led to increased expression of the *snail*-related chick gene, *slug*, while causing no change in *Tbx-4* and *Fgf10* expression patterns (not shown).

2.5. FGF-10 does not induce rapid expression of SnR

To explore the relationship between *SnR*, *Fgf10* and *twist* expression, we applied FGF-10 protein to the flank of stage 12–13 chick embryos. Unlike with FGF-2 or FGF-8, *SnR* expression was not induced by 1 h after FGF-10 application ($n = 3$; data not shown) or at various time points between 1 and 17 h ($n = 11$, data not shown), but was expressed by 24 h (Fig. 3E; $n = 12$). *Fgf10* (Fig. 3F; $n = 7$) and *twist* (Fig. 3G; $n = 3$) are also expressed by 24 h after FGF-10 application. In the case of *twist*, ectopic expression in the thickening (arrowhead) is stronger than in the rest of the flank (arrow), although expression is seen throughout the flank due to weaker endogenous expression present here (arrow).Unlike with FGF-2 and FGF-8 application, which lead to induction of *SnR*, *Fgf10* and *twist* lateral to the bead, FGF-10 application leads to ectopic expression of *SnR*,

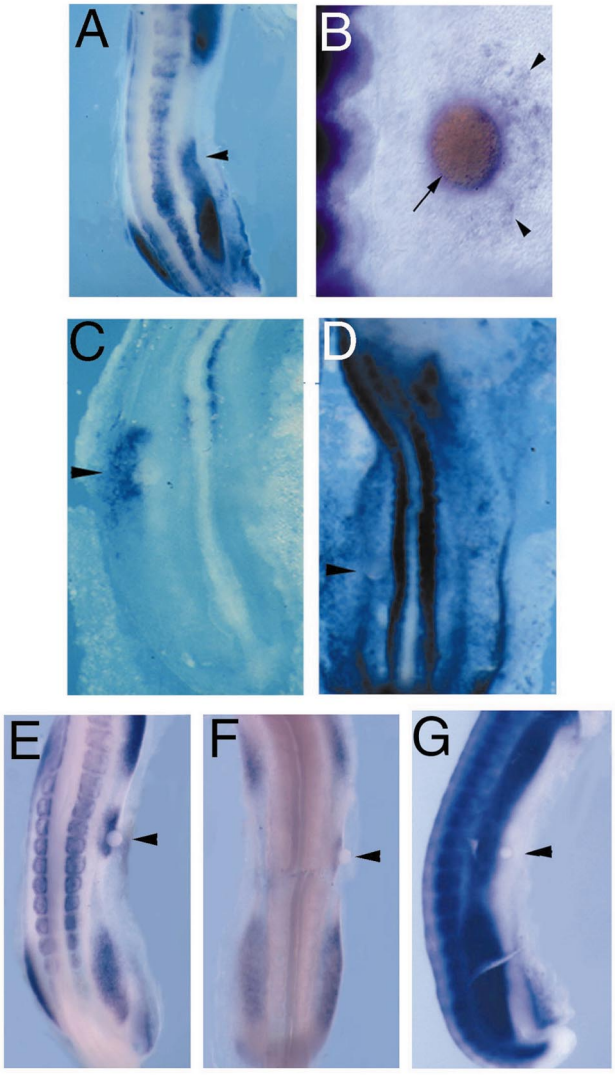

Fig. 3. Gene expression in FGF-treated chick embryos. (A) FGF-2 bead implanted opposite somite 25 of a stage 15 embryo and removed 1 h after implantation. *SnR* expression at 19 h after bead removal. Transcripts found in the forming ectopic bud (arrow head). (B) Double labelling of *SnR* transcripts (blue) and FGF protein (red) 1.5 h after bead implantation. Labelled FGF protein can only be detected immediately adjacent to bead (arrow), whereas *SnR* transcripts are found in a punctate pattern in cells much further away (arrow heads). (C,D) Expression of *SnR* 1 h after FGF-2 bead implantation to embryos in Ring culture with or without cycloheximide. (C) Control embryo cultured in the absence of cycloheximide. Transcripts found near FGF-2 bead (arrow head). (D) Embryo cultured with cycloheximide. No obvious transcripts detected near FGF-2 bead (arrow head). Note that *SnR* expression is enhanced throughout the whole embryo including the flank on both sides. (E–G) FGF-10 beads implanted into the flanks of chick embryos in ovo. (E) *SnR* is expressed adjacent to the bead (arrowhead) 24 h after FGF-10-bead implantation opposite somite 23 of a stage 13 embryo. (F) *FGF-10* is expressed adjacent to the bead (arrowhead) 24 h after FGF-10-bead implantation opposite somite 23 of a stage 13 embryo. (G) *Twist* is expressed adjacent to the bead (arrowhead) 24 h after FGF-10 bead implatation opposite somite 22 of a stage 13 embryo. Note that the non-thickened flank region is expressing edogenous *twist*, but at a lower level.

Fgf10 and *twist* medial to the bead. In the case of FGF-2 and FGF-8, the mesoderm begins to bud before any thickening of the ectoderm into a ridge is observed. In contrast, FGF-10 application leads to a thickening in the ectoderm followed by budding of the underlying mesoderm (Yonei-Tamura et al., 1999; and our observations). Double in-situs show that *SnR, Fgf10, twist* and *Fgf8* are induced at approximately the same time following FGF-10 application (20 h in our hands; data not shown), and it has therefore been impossible to dissect out the exact order of appearance of these genes following this treatment.

3. Discussion

We have shown that a number of genes are expressed at the right time and place to be early mediators of limb induction, and that expression of *SnR*, in particular, is activated very rapidly when FGF-2 and FGF-8 beads are implanted in the flank to induce ectopic limbs. *SnR* expression is irreversibly induced by a brief exposure to FGF-2, but this does not appear to be an early immediate response. *Fgf10* appears to be expressed in limb forming regions just prior to *SnR*, and has been shown to be required for limb formation (Ohuchi et al., 1997; Min et al., 1998; Sekine et al., 1999). Induction of *SnR* by FGF-10 in the ectopic limb bud is slow suggesting that FGF-10 signalling does not directly influence *SnR*. *Twist* expression overlaps with that of *SnR* in lateral plate mesoderm, but is induced much more slowly in response to FGF-2. Expression of the limb specific genes, *Tbx-4* and *Tbx-5*, also overlaps with that of *SnR*, and their expression can be induced in the flank as rapidly. The difference in the initial patterns of ectopic expression of *SnR* and *Tbx* genes suggests, however, that they are induced by different mechanisms.

3.1. SnR induction is an early event in limb specification

The early expression of *SnR* in limb forming regions and in response to FGF suggests that it may be important in vertebrate limb determination. *Snail* together with another zinc finger gene related to *snail*, *escargot,* is expressed in *Drosophila* imaginal discs and a role in determination of the fly wing has been proposed (Fuse et al., 1996). When we prevented protein synthesis in chick embryos, *SnR* appears to be upregulated throughout the embryo, including the flank. This raises the possibility that repression of *SnR* by a labile protein is important in confining *SnR* to limb forming regions. There is evidence from other systems that expression of *snail* genes sets up boundaries during body plan formation by repression and/or by being repressed themselves. For example, in *Drosophila Snail* sets up a mesoderm/neurectoderm boundary (Ip et al., 1992b), in *Ciona Snail* sets up a muscle/non-muscle boundary (Fujiwara et al., 1998) and in early chick embryos *SnR* is involved in setting up left-right differences (Isaac et al., 1997; Patel et al., 1999).

3.2. Relationship between expression of SnR and Fgf10

We can probably exclude the possibility that FGF-10 activates *SnR*. Even though *Fgf10* transcripts appear to become localized to presumptive limb regions just prior to *SnR*, we have shown that *SnR* is not induced rapidly in the flank by FGF-10. Furthermore, FGF-10 appears to be most closely related to keratinocyte growth factor (FGF7) and probably, like FGF7, acts via epithelial FGF receptors (Orr-Urtreger et al., 1993; Noji et al., 1993; Ornitz et al., 1996) and thus would not be expected to induce directly expression of genes in the mesoderm. Grafts of FGF-10 expressing cells have been shown to induce *Fgf10* expression in flank mesoderm but this appears to be via ridge formation and induction of *Fgf8* in the ectoderm (Ohuchi et al., 1997; Yonei-Tamura et al., 1999). Our data suggest that FGF-10 induces *SnR* expression in the same way, via the ridge.

We find that *SnR* is induced irreversibly in the flank by a short exposure to FGF-2 and FGF-8. *Fgf10* is induced later. This suggests that *SnR* may in fact be upstream of *Fgf10*. Application of an epithelially derived FGF such FGF-8 to the flank could be considered to mimic the apical ridge, or alternatively, the FGFs proposed to come from the mesonephros (Crossley et al., 1996). However, if the former were true, and our proposal that FGF-10 acts via the ridge is correct, then we would have expected that FGF-8 would induce expression of *SnR* and *Fgf10* at more or less the same time. It should be borne in mind that reprogramming the flank to form limb may not only involve the same cascade of genes as those required for normal limb specification but also inhibition of genes that normally repress limb formation in the flank. Another possibility is that an FGF antagonist such as *sprouty* could be rapidly induced by FGF application to the flank which prevents the early induction of *Fgf10* and *twist*, but not *SnR* (Minowada et al., 1999).

3.3. Relationship between SnR and Twist

In *Drosophila, snail* and *twist* are both required for mesoderm formation (Ip et al., 1992a). Our results show that both *SnR* and *twist* are co-expressed at early stages in chick limb development. When chick flank is reprogrammed to form a limb, *SnR* is induced much more rapidly than *twist,* therefore, *twist* expression is not necessary for activation of *SnR* in the flank. Expression of *DFR1*, an FGF receptor in *Drosophila* mesodermal primordia, requires *snail* and *twist* (Shishido et al., 1993). There could be a parallel here. Expression of an FGF receptor in the mesenchyme of vertebrate limbs could require *snail* and *twist,* and expression of this FGF receptor in the mesenchyme could enable the mesenchyme to respond, later, to FGF8 produced by the apical ectodermal ridge.

3.4. Tbx genes in early limb specification

The response of *Tbx-4* and *Tbx-5* gene expression to FGF

signalling is very rapid. There is now evidence that these genes are involved in determining limb type, with *Tbx-5* being involved in wing, and *Tbx-4* in leg determination (Logan and Tabin, 1999; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). However, initially only a subset of ectopic *SnR* expressing cells overlap with ectopic *Tbx* expressing cells in FGF treated flanks. This suggests that changes in *SnR* and *Tbx* gene expression induced by FGF involve different pathways. The initial expression of *Tbx-4* and *Tbx-5* is likely to be connected to patterning along the head-tail axis, which involves Hox genes (Cohn et al., 1997), while *SnR* appears to be linked to setting up a new outgrowth (Fig. 4).

4. Materials and methods

4.1. Whole-mount in situ hybridization

Whole-mount in situ hybridization's of chick embryos were performed as previously described (Wilkinson and Nieto, 1993). The RNA probes were as follows: *SnR*, *twist* (GenBank database accession number AF093816), *Tbx-4* and *Tbx-5* (Juan-Carlos Izpisua-Belmonté) *fgf-8* (Gail Martin, University of California at San Francisco, CA, USA); RCAS p27gag (Cliff Tabin, Harvard, USA). *Fgf10* was isolated as in Ohuchi et al., 1997.

4.2. FGF bead implantations into embryos in ovo

Heparin acrylic beads (Sigma H-5263; with a diameter of 125 μm) were washed in PBS for 2 h, and then incubated in FGF-2 or FGF-8 (1 mg/ml, R&D systems), or FGF-10 (John Heath, Birmingham University, UK) for at least 1 h at room temperature. A bead was inserted under the ectoderm in the lateral plate mesoderm of the flank of stage 12–15 chick embryos as described (Cohn et al., 1995; Ohuchi et al., 1997). The embryos were then incubated further at 38°C until they reached the desired stage. For the double labelling to show distribution of applied FGF-2 protein and of *SnR* transcripts, digoxygenin-labelled FGF-2 at a concentration of 0.25 mg/ml (John Heath, Birmingham University, UK) was used and in situ hybridization was performed essentially as described above. A fluorescein-labelled riboprobe for *SnR* was detected with anti-fluorescein AP antibody using NBT-BCIP. Embryos were incubated in 0.1 M acid glycine pH 2.2 to stop the colour reaction. Digoxigenin-labelled FGF-2 protein was subsequently detected with anti-digoxigenin AP antibody using magenta phosphate. This generated blue colour for *SnR* transcripts and pink colour for FGF-2 protein.

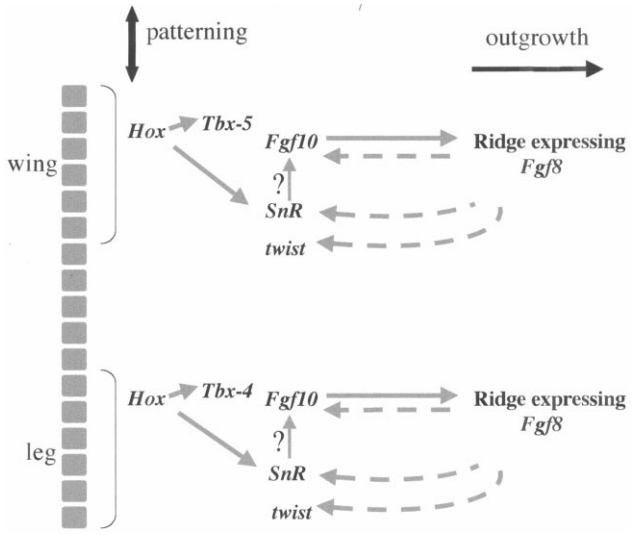

Fig. 4. Possible relationships between expression of different genes regulated by FGF signalling. *Tbx* genes, together with *Hox* genes, are concerned with patterning along the anterior-posterior axis and link limb position and limb type. FGF-10 induces *Fgf8* in the overlying ectoderm which maintains *Fgf10* expression in underlying limb mesenchyme. FGF-8 also maintains *SnR* and *twist* in the underlying limb mesenchyme. This feedback loop is essential for limb outgrowth. Solid lines indicate interactions that appear to be linearly related while dotted lines represent interactions that may not be linear and operate via expression of some of the other genes in the scheme.

4.3. Cycloheximide treatment

Chick embryos were set up in ring culture at HH stage 5/6 as previously described (Isaac et al., 1997), and incubated until they reached stage 13/14, the stage at which application of FGF-2 is carried out in ovo. FGF-2 coated beads were implanted into the lateral plate of the flank region. Control embryos were cultured with the standard 90% albumin/10% tissue culture medium (MEM) for 1 h at 38°C, whereas the experimental embryos were cultured with cycloheximide at a final concentration of 100 μg/ml (in albumin/tissue culture medium) for an equivalent time period. Embryos treated with cycloheximide were viable as indicated by beating hearts.

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References

Abud, H.E., Skinner, J.A., McDonald, F.J., Bedford, M.T., Lonai, P., Heath, J.K., 1996. Ectopic expression of fgf-4 in chimeric mouse embryos induces the expression of early markers of limb development. Dev. Genet. 19, 51–65.

Bushdid, P.B., Brantley, D.M., Yull, F.E., Blaeuer, G.L., Hoffman, L.H., Niswander, L., Kerr, L.D., 1998. Inhibition of NF-kappaB activity results in disruption of the apical ectodermal ridge and aberrant limb morphogenesis. Nature 392, 615–618.

Buxton, P.G., Kostakopoulou, K., Brickell, P., Thorogood, P., Ferretti, P., 1997. Expression of the transcription factor slug correlates with growth

of the limb bud and is regulated by FGF-4 and retinoic acid. Int. J. Dev. Biol. 41, 559–568.

Chen, Z-F., Behringer, R.R., 1995. Twist is required in head mesenchyme for cranial neural tube morphogenesis. Genes Dev. 9, 686–699.

Cohn, M.J., Izpisúa-Belmonté, J.-C., Abud, H., Heath, J.K., Tickle, C., 1995. Fibroblast growth factors induce additional limb development from the flank of chick embryos. Cell 80, 739–746.

Cohn, M.J., Patel, K., Krumlauf, R., Wilkinson, D.G., Clarke, J.D.W., Tickle, C., 1997. Hox9 genes and vertebrate limb specification. Nature 387, 97–101.

Crossley, P.H., Minowada, G., MacArthur, C.A., Martin, G.R., 1996. Roles for FGF8 in the induction, and maintenance of chick limb development. Cell 84, 127–136.

Emori, Y., Saigo, K., 1993. Distinct expression of two Drosophila homologues of fibroblast growth factor receptors in imaginal discs. FEBS Lett. 332, 111–114.

Fernandez-Teran, M., Piedra, M.E., Simandl, B.K., Fallon, J.F., Ros, M.A., 1997. Limb initiation and development is normal in the absence of the mesonephros. Dev. Biol. 189, 246–255.

Fujiwara, S., Corbo, J.C., Levine, M., 1998. The Snail repressor establishes a muscle/notochord boundary in the Ciona embryo. Development 125, 2511–2520.

Fuse, N., Hirose, S., Hayashi, S., 1996. Determination of wing cell fate by the escargot and snail genes in Drosophila. Development 122, 1059–1067.

Geduspan, J.S., Solursh, M., 1992. A growth-promoting influence from the mesonephros during limb outgrowth. Dev. Biol. 151, 242–250.

Gibson-Brown, J.J., Agulnik, S.I., Silver, L.M., Niswander, L., Papaioannou, V., 1998. Involvement of T-box genes Tbx2-Tbx5 in vertebrate limb specification and development. Development 125, 2499–2509.

Grau, Y., Carteret, C., Simpson, P., 1984. Mutations and chromosomal rearrangements affecting the expression of snail, a gene involved in embryonic patterning in Drosophila Melanogaster. Genetics 108, 347–360.

Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in development of the chick embryo. J. Morphol. 88, 49–92.

Ip, Y.T., Park, R.E., Kosman, D., Yazdanbakhsh, K., Levine, M., 1992a. Dorsal-twist interactions establish snail expression in the presumptive mesoderm of the Drosophila embryo. Gen. Dev. 6, 1518–1530.

Ip, Y.T., Park, R.E., Kosman, D., Bier, E., Levine, M., 1992b. The dorsal gradient morphogen regulates stripes of rhomboid expression in the presumptive neuroectoderm of the Drosophila embryo. Gen. Dev. 6, 1728–1739.

Isaac, A., Sargent, G., Cooke, J., 1997. Control of vertebrate left-right asymmetry by a snail-related zinc finger gene. Science 275, 1301–1304.

Isaac, A., Rodriguez-Esteban, C., Ryan, A., Altabef, M., Tsuki, T., Patel, K., Tickle, C., Izpisúa-Belmonté, J.-C., 1998. Tbx genes and limb identity in chick embryo development. Development 125, 1867–1875.

Kanegae, Y., Tavares, A.T., Izpisa Belmonté, J.-C., Verma, I.M., 1998. Role of Rel/NF-kappaB transcription factors during the outgrowth of the vertebrate limb. Nature 392, 611–614.

Kieny, M., 1971. Les phase d'activité morphogène du mésoderme somatopleural pendent le développement précoce du membre chez l'embryon de poulet. Ann. Embryol. Morphogen. 4, 281–298.

Logan, M., Tabin, C.J., 1999. Role of Pitx1 upstream of Tbx4 in specification of hindlimb identity. Science 283, 1736–1739.

Logan, M., Simon, H.G., Tabin, C., 1998. Differential regulation of T-box and homeobox transcription factors suggests roles in controlling chick limb-type identity. Development 125, 2825–2835.

Min, H., Danilenko, D.M., Scully, S.A., Bolon, B., Ring, B.D., Tarpley, J.E., DeRose, M., Simonet, W.S., 1998. Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless. Genes Dev. 12, 3156–3161.

Minowada, G., Jarvis, L.A., Chi, C.L., Neubüser, A., Sun, X., Hacohen, N., Krasnow, M.A., Martin, G.R., 1999. Vertebrate Sprouty genes are induced by FGF signalling and can cause chondrodysplasia when overexpressed. Development 126, 4465–4475.

Noji, S., Koyama, E., Myokai, F., Nohno, T., Ohuchi, H., Nishikawa, K., Taniguchi, S., 1993. Differential expression of three chick FGF receptor genes, FGFR1, FGFR2 and FGFR3, in limb and feather development. In: Fallon, J.F., Goetinck, P.F., Kelly, R.O., Stocum, D.L. (Eds.). Limb Development and Regeneration, Wiley-Liss, Inc, New York, pp. 645–654.

Ohuchi, H., Nakagawa, T., Yamauchi, M., Ohata, Y., Yoshioka, H., Kuwana, T., Mima, T., Nohno, T., Noji, S., 1995. An additional limb can be induced from the flank of the chick embryo by FGF4. Biochem. Biophys. Res. Commun. 209, 806–809.

Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., Yoshioka, H., Kuwana, T., Nohno, T., Yamasaki, M., Itoh, N., Noji, S., 1997. The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. Development 124, 2235–2244.

Ohuchi, H., Takeuchi, J., Yoshiyasu, H., Ishimaru, Y., Ogura, K., Takahashi, N., Ogura, T., Noji, S., 1998. Correlation of wing-leg identity in ectopic FGF-induced chimeric limbs with the differential expression of chick Tbx5 and Tbx4. Development 125, 51–60.

Ornitz, D.M., Xu, J., Colvin, J.S., McEwen, D.G., MacArthur, C.A., Coulier, F., Gao, G., Goldfarb, M., 1996. Receptor specificity of the fibroblast growth factor family. J. Biol. Chem. 271, 15292–15297.

Orr-Urtreger, A., Bedford, M.T., Burakova, T., Arman, E., Zimmer, Y., Yayon, A., Givol, D., Lonai, P., 1993. Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR-2). Dev. Biol. 158, 475–486.

Patel, K., Isaac, A., Cooke, J., 1999. Nodal signalling and the roles of the transcription factors SnR and pitx2 in vertebrate left-right asymmetry. Curr. Biol. 9, 609–612.

Pinot, M., 1970. Le rôle du mésoderme somitique dans la morphogenèse précose des membres de l'embryon de Poulet. J. Embryol. Exp. Morph. 23, 109–151.

Rodriguez-Esteban, C., Tsukui, T., Yonei, S., Magallon, J., Tamura, K., Izpisua Belmonté, J.C., 1999. The T-box genes Tbx4 and Tbx5 regulate limb outgrowth and identity. Nature 398, 814–818.

Ros, M.A., Sefton, M., Nieto, A.M., 1997. Slug, a zinc finger gene previously implicated in early patterning of the mesoderm and neural crest, is also involved in chick limb development. Development 124, 1821–1829.

Sefton, M., Sanchez, S., Nieto, A.M., 1998. Conserved and divergent roles for members of the Snail family of transcription factors in the chick and mouse embryo. Development 125, 3111–3121.

Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N., Kato, S., 1999. Fgf10 is essential for limb and lung formation. Nat. Genet. 21, 138–141.

Shishido, E., Higashijima, S., Emori, Y., Saigo, K., 1993. Two FGF-receptor homologues of Drosophila: one is expressed in mesodermal primordium in early embryos. Development 117, 751–761.

Simpson, P., 1983. Maternal-zygotic gene interactions during formation of the dorsoventral pattern in Drosophila embryos. Genetics 105, 615–632.

Storey, K.G., Goriely, A., Sargent, C.M., Brown, J.M., Burns, H.D., Abud, H.M., Heath, J.K., 1998. Early posterior neural tissue is induced by FGF in the chick embryo. Development 125, 473–484.

Takeuchi, J.K., Koshiba-Takeuchi, K., Matsumoto, K., Vogel-Hopker, A., Naitoh-Matsuo, M., Ogura, K., Takahashi, N., Yasuda, K., Ogura, T., 1999. Tbx5 and Tbx4 genes determine the wing/leg identity of limb buds. Nature 398, 810–814.

Wilkinson, D.G., Nieto, A.M., 1993. Detection of messenger RNA by In situ hybridisation to tissue sections and whole-mounts. Methods Enzymol. 225, 361–373.

Xu, X., Weinstein, M., Li, C., Naski, M., Cohen, R.I., Ornitz, D.M., Leder, P., Deng, C., 1998. Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. Development 125, 753–765.

Yonei-Tamura, S., Endo, T., Yajima, H., Ohuchi, H., Ide, H., Tamura, K., 1999. FGF7 and FGF10 directly induce the apical ectodermal ridge in chick embryos. Dev. Biol. 211, 133–143.