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. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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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 FGFs 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 (Guduspan 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
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).

Tbx-4 and Tbx-5 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.

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.
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 implantation opposite somite 22 of a stage 13 embryo. Note that the non-thickened flank region is expressing endogenous twist, but at a lower level.](image-url)
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-situ 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. Grafs 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, digoxigenin-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-fluorescin 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.

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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