

## CO-EXPRESSION PATTERN ANALYSIS OF *Fgf4*, *Fgf8* AND *Shh* GENE EXPRESSION AT DIVERSE SIGNALLING CENTERS DURING MOUSE DEVELOPMENT

David BUENO<sup>1</sup> and John K. HEATH<sup>2</sup>

<sup>1</sup> Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, 08071 Barcelona, Catalonia (Spain).

<sup>2</sup> School of Biochemistry, The University of Birmingham, Edgbaston, B15 2TT Birmingham, UK.

The *Fgf* gene family constitutes a family of structurally related ligands that act to promote the growth and differentiation of many mesoderm and ectoderm cells by binding to specific receptors. Nine members of the *Fgf* gene family (*Fgf1* - *Fgf9*) have been described in mammals to date (reviewed by Wilkie et al., 1995). Many *Fgfs* as well as their receptors (Fibroblasts Growth Factor Receptors, *Fgfrs*) exhibit distinct patterns of expression during embryogenesis, which suggests that they could play distinct roles in mammalian development. Moreover, different experimental approaches have provided evidence that FGF signal transduction pathways play key roles in the regulation of growth and patterning in the vertebrate embryo. Experimental manipulation of chick limb buds (Niswander et al., 1994; Cohn et al., 1995) have also provided evidence of inductive interactions within members of the FGF family and other molecules involved in cellular interactions as Sonic Hedgehog (SHH). It has been suggested that a local source of FGF at the appropriate A-P axis level in the flank could initiate the formation of a limb bud, maintaining cell proliferation and leading to activation of *Shh* in cells with polarizing potential. Then, *Shh* could initiate *Fgf4* expression in the posterior half of the AER, and the expression of both genes may then be coordinately regulated by a positive feedback loop (Laufer et al., 1994). A candidate for the establishment of the limb field and initial limb outgrowth is *Fgf8* (MacArthur et al., 1995).

The aim of the present study is to examine the relationship between the expression patterns of *Shh*, *Fgf4* and *Fgf8* in a number of organising centers during mouse development as they are not only simultaneously localized in limb buds but also in other known signalling centers, such as the floor plate and the node. We have used a double in situ hybridization technique (Bueno et al., 1996) that permits the expression of two genes relative to one another and their co-expression (if any) to be precisely determined, allowing us to assess the roles of these molecules in a variety of signalling centers. Expression sites of these genes have been described previously (*Shh* - Echelard et al. 1993; *Fgf4* - Niswander and Martin 1992, Drucker and Goldfarb 1993; *Fgf8* - Crossley and Martin 1995).

Double in situ hybridization indicates that *Fgf4*, *Fgf8* and *Shh* are expressed in adjacent cells at the posterior boundary of the node, similar to their expression in the limb bud (see Fig. 1A for summary). In the node region, at 7.75 days post coitum (dpc), *Fgf4* and *Fgf8* expression domains (within the primitive streak) and *Shh* expression domain (in the midline mesoderm) are adjacent in the posterior margin of the node. The contact area comprises only a few cells and, under the resolution provided by the technique used, we have never seen any area expressing the three transcripts simultaneously. At that stage, *Fgf8* expression domain within the primitive streak is detected in more posterior regions than *Fgf4*. In the limb bud, at 10.5 dpc, *Fgf4* and *Fgf8* expression domains (within the apical ectodermal ridge, AER) and *Shh* expression domain (in posterior mesenchyma cells) come into close contact. Areas expressing the three transcripts simultaneously have not been detected. At that stage, *Fgf8* expression domain within the AER is detected in more anterior cells than *Fgf4*. Thus, the co-expression pattern of these three transcripts is similar in both organizing centres, but in an inverted manner (what is expressed anteriorly in the node region is expressed posteriorly in the limb bud). The analogies observed in the relative expression patterns of *Fgf4*, *Fgf8* and *Shh* between the developing limb and the node suggest that they could be acting and interacting in the node in a manner similar to their action in the limb.

The temporal order of expression of these genes differs between the node and the limb (see Fig. 1B for summary). In both structures, *Fgf8* transcripts are detected earliest, suggesting that *Fgf8* may be involved in a similar inductive pathway in the initiation and control of embryo outgrowth in the A-P axis (Crossley and Martin, 1995). However, the relative order of expression of *Shh* and *Fgf4* differs. Following *Fgf8* expression, the first transcript to be detected in the limb is *Shh*, whereas in the node region it is *Fgf4*. This difference in the hierarchy of expression indicates that, although all three genes are expressed in a similar combination in both structures, the way(s) they are activated could be different. Given that several FGFRs can bind both FGFs with a similar specificity it is possible that FGF4 and FGF8 could be functionally interchangeable in some inductive processes (MacArthur et al., 1995). Differences in the relative timing of expression of *Fgf4* and *Shh* may not, therefore, be highly significant since *Fgf8* expression precedes that of *Fgf4* and *Shh* in both structures. On the other hand, the interaction of these molecules with other different molecules also has to be considered.

The relative expression patterns of *Shh*, *Fgf4* and *Fgf8* have also been examined in the floor plate and in its surroundings (the neural plate). Our results show that the relative distribution of these three transcripts in the neural plate region is somewhat different than that described for the developing limb and the node region. At 8.0-8.5 dpc, *Shh* expression domain (within the notochord) and *Fgf8* and *Fgf4* expression domains (within the neuroepithelium of the primitive streak region) come into close contact with adjacent cells in the anterior area of the primitive streak, at the end of the notochord and in the condensation of tissue at its caudal end. In the cephalic region of the embryo, however, no contact was detected between *Fgf8* and *Shh* expression domains, which are non adjacent. On the other hand, *Fgf4* expression domain in the cephalic region at that stage (within the neuroectoderm along the neural folds) and *Shh* expression domain (in the notochord and CNS ventral midline) come into close contact in adjacent cells. Again, we have not seen any cells at any of these regions simultaneously expressing *Fgf4*/*Fgf8* and *Shh*. Thus, although some molecular mechanisms seem to be conserved by different organising centers, there are differences in their relative spatial and temporal expression patterns. This suggests that the contribution of these molecules to the establishment, maintenance and/or promotion of processes associated with these centers may be somewhat different.

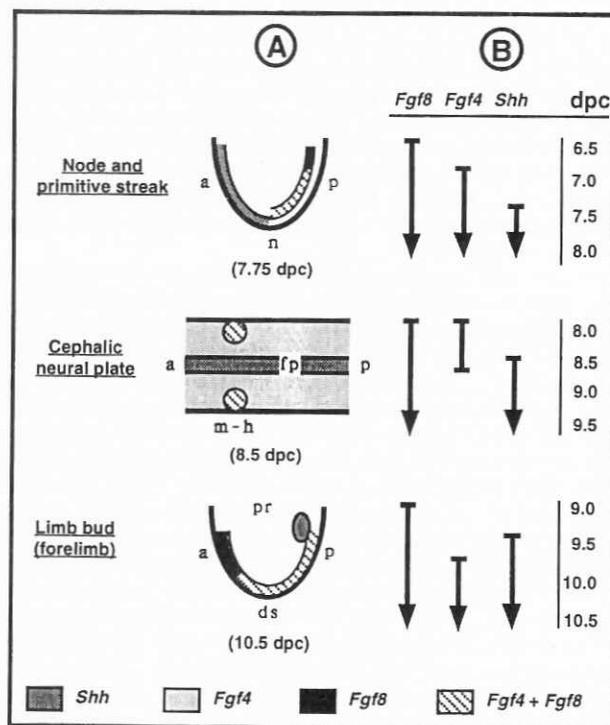
We have also analyzed the expression relationships of *Fgf8* and *Shh* in the developing brain at 9.5 dpc (*Fgf4* transcripts are not detected in the developing brain later than 8.5 dpc). *Fgf8* transcripts are detected in discrete sites in the developing brain. One of its expression domains in the neuroectoderm of the brain, the ventral midline of the hypothalamus at around the infundibular region, is very close to the expression domain of *Shh* in the ventral midline. *Shh* is detected in the ventral midline from the spinal chord to the

diencephalon where, in contrast to all other CNS regions, *Shh* is not detected at the ventral midline but in two ventrolateral strips. These two ventrolateral strips merge again in the floor of the forebrain at its rostral limit. Detection of *Fgf8* in the hypothalamus ventral midline is restricted to the area between these two *Shh* ventrolateral strips. We have not detected any cell expressing both transcripts together. No signalling centers for patterning the forebrain have yet been identified. As *Fgf8* is detected in the isthmus as well as in other known signalling centers, Crossley and Martin (1995) suggest that the sites of *Fgf8* expression in the developing forebrain may identify possible signalling centers responsible for patterning of the forebrain. Moreover, Crossley et al. (1996) have recently identified FGF8 as an important signalling molecule for midbrain development. It has also been suggested that *Shh*, which is detected within the ventral midline, is responsible for patterning the ventral forebrain (reviewed by Lumsden and Graham, 1995). We have shown that *Shh* expressing cells are adjacent to *Fgf8* expressing cells in the ventral midline and not in the other *Fgf8* expression sites in the developing brain. It is tempting to speculate that *Fgf8* and *Shh* could play important roles in the area where they are adjacently expressed, defining a signalling center for forebrain patterning.

Finally, we have shown that at none of the sites where *Shh* expression domain is adjacent to *Fgf8* and/or *Fgf4* expression domains, have we detected cells expressing *Shh* and *Fgf4* or *Fgf8* genes simultaneously (see Fig. 1A for summary). These results suggest the presence of some kind of boundary between *Shh* and *Fgf4*/*Fgf8* expression domains that does not allow cells expressing *Shh* transcripts to express *Fgf4*/*Fgf8* transcripts or viceversa. Martí et al. (1995), however, have reported the presence of SHH protein, using specific antisera, in areas where *Fgf8*/*Fgf4* should be expressed.

In conclusion, transcripts of the signalling molecules *Fgf4*, *Fgf8* and *Shh* are detected in adjacent areas at distinct sites and at different stages of mouse development. Most of these sites are known signalling centers. Knowing the spatial and temporal expression relationships of these genes in different signalling centers should provide a better understanding of how similar molecular mechanisms may serve to coordinate different developmental processes.

Figure 1. Summary of the spatial and temporal expression of *Fgf4*, *Fgf8* and *Shh* in the node and primitive streak, in the neural plate and in the developing limb bud. (A) Schematic representation of a 7.75 d.p.c. embryo, the neural plate at 8.5 d.p.c. and a developing forelimb bud at 10.5 d.p.c.. The expression domains of *Fgf4*, *Fgf8*, *Fgf4*/*Fgf8* and *Shh* are indicated; (B) Schematic representation of the temporal order of expression of *Fgf4*, *Fgf8* and *Shh* in the node and primitive streak, in the neural plate and in the developing forelimb. Vertical line, beginning/end of detection of gene expression; arrow, further detection of gene expression. Abbreviations: a, anterior; dpc, days post-coitum; ds, distal; fp, floor plate; m-h, midbrain-hindbrain junction; p, posterior; pr, proximal.



## Acknowledgements

We thank Drs. Judith Skinner and Helen Abud for invaluable help and discussions. D. B. also thanks Maria Tricas for invaluable help and discussion throughout the course of these studies. This work was supported by the Cancer Research Campaign. Part of this work has been performed under an award from the Comissionat per a la Recerca-CIRIT (Generalitat de Catalunya) to D.B.

## References

- Bueno, D., Skinner, J., Abud, H. and Heath, J. K. (1996) Double *in situ* hybridization on mouse embryos for detection of overlapping regions of gene expression. *Trends in Genet.* (In Press).
- Cohn, M. J., Izpisua-Belmonte, J. C., Abud, H., Heath, J. K. and Tickle, C. (1995) Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* 80: 739-746.
- Crossley, P. H. and Martin, G. R. (1995) The mouse *Fgf-8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* 121: 439-451.
- Crossley, P. H., Martinez, S. and Martin, G. R. (1996) Midbrain development induced by FGF8 in the chick embryo. *Nature* 380: 66-68.
- Drucker, B. J. and Goldfarb, M. (1993) Murine FGF4 gene expression is spatially restricted within embryonic skeletal muscle and other tissues. *Mech. Dev.* 40: 155-163.
- Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993) *Sonic hedgehog*, a member of a family of putative signalling molecules, is implicated in the regulation of CNS polarity. *Cell* 75: 1417-1430.
- Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A. and Tabin, C. (1994) *Sonic Hedgehog* and *Fgf-4* act through a signalling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* 79: 993-1003.
- Lumsden, A. and Graham, A. (1995) A forward role for hedgehog. *Curr. Biol* 5: 1347-1350.
- MacArthur, C. A., Lawshé, A., Xu, J., Santos-Ocampo, S., Heikinheimo, M., Chellajiah, A. T. and Ornitz, D. M. (1995) FGF-8 isoforms activate receptor splice forms that are expressed in mesenchymal regions of mouse development. *Development* 121: 3603-3613.
- Martí, E., Takada, R., Bumcrot, D. A., Sasahi, H. and McMahon, A. P. (1995) Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. *Development* 121: 2537-2547.
- Niswander, L. and Martin, G. R. (1992) *Fgf-4* expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* 114: 755-768.
- Niswander, L., Jeffrey, S., Martin, G. R. and Tickle, C. (1994) A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* 371: 609-612.
- Wilkie, A. O. M., Morriss-Kay, G. M., Jones, E. I. and Heath, J. K. (1995) Functions of fibroblast growth factors and their receptors. *Curr. Biol.* 5: 500-507