Int. J. Dev. Biol. 41: 747-750 (1997)



*Short Contribution*

# Local origin of cells in FGF-4 - induced outgrowth of amputated chick wing bud stumps

KONSTADINA KOSTAKOPOULOU[1]*, NEIL VARGESSON[1], JONATHAN D.W. CLARKE[1], PAUL M. BRICKELL[2] and CHERYLL TICKLE[1]

[1]*Department of Anatomy and Developmental Biology, University College London, and* [2]*Molecular Haematology Unit, Institute of Child Health, University College London, London, United Kingdom*

**ABSTRACT Urodele amphibians are the only vertebrates that can regenerate amputated limbs, even as adults. However, we have previously shown that amputated chick wing bud stumps can be induced to «regenerate» and to form a complete set of correctly-patterned skeletal elements, following implantation of beads soaked in fibroblast growth factor-4 (FGF-4). We have now performed DiI injection experiments to determine which cells contribute to FGF-4-induced chick wing bud «regenerates». We show that the FGF-4-induced outgrowth of the regenerating wing bud stump is comprised of mesenchyme cells that originate from a region within 200 μm of the FGF-4 bead, and that cells proximal to the bead move distally.**

KEY WORDS: *limb bud regeneration, fibroblast growth factor-4, DiI injection*

Adult urodele amphibians have the remarkable capacity to regenerate their limbs. One important question is why other vertebrates do not have this capacity. It has recently been found that treating stumps of amputated chick embryo wing buds with fibroblast growth factors (FGFs) can induce them to «regenerate» and form a full set of skeletal elements (Taylor *et al.*, 1994; Kostakopoulou *et al.*, 1996). This «regeneration» appears to depend upon the ability of FGF to re-establish the signaling network that acts during normal development to produce limb pattern, implying that regeneration of adult urodele limbs may also involve the reiteration of processes that occur during limb development. It is not clear, however, to what extent the regeneration of embryonic chick limbs, induced by FGFs, parallels the regeneration of adult urodele limbs.

A key feature in regeneration is the formation of a mass of undifferentiated mesenchyme cells, which then undergo proliferation and patterning to replace missing structures. In adult urodeles, this mass of cells is known as the blastema and appears to arise as a result of de-differentiation of cells in the immediate vicinity of the cut surface (reviewed by Ferretti and Brockes, 1991). In the present study, we have mapped the precise origin of the cells that contribute to the FGF-4-induced outgrowth of amputated chick wing bud stumps, by labeling small populations of wing bud mesenchymal cells with the fluorescent lipophylic dye, DiI.

Amputation at 300-400 μm from the tip of wing buds at stage 23-24 leads to truncation at the level of ulna and radius. Such stumps can be rescued by posterior application of FGF-4, so that complete ulna, radius and digits develop. Injections of DiI were made at a number of different locations in wing bud stumps with FGF-4- or PBS-soaked control beads stapled posteriorly, as illustrated in Fig. 1. The stage of the embryo (23-24), the amputation level (300 μm from the tip), the time of injection (2 h after bead implantation) and the volume of DiI injected were the same in each case.

To check the position and number of cells initially labeled, embryos were fixed immediately after DiI injection 0-50 μm proximal to the bead. Labeled cells formed a discrete population proximal to the bead (Fig. 2A). To reveal the spatial and temporal pattern of cell contribution to the regenerating wing buds, embryos were fixed 48 h after DiI injection. In control amputated wing buds, in which a PBS-soaked bead had been implanted and DiI injected 0-50 μm proximal to the bead (n=5), labeled cells remained as a discrete population behind the bead 48 h later (Fig. 2B). A similar result was obtained when DiI was injected at each of the other positions (Fig.1) in amputated wing buds implanted with PBS-soaked beads (data not shown, n=5 for each injection site). In all of these control specimens, there was a relatively small number of labeled cells scattered outside the main region of labeling (Fig. 2B). Similar scattered cells were also seen in all experimental specimens. The identity of these cells is unknown, but they could be macrophages that have moved away from the site of DiI injection (Vargesson *et al.*, 1997).

We next injected DiI into a series of sites at the posterior margin of amputated wing buds that were treated with FGF-4. When DiI

*Abbreviations used in this paper:* DiI, 1,1 dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchloride; FGF, fibroblast growth factor; PBS, phosphate-buffered saline.

***Address for reprints:** Department of Anatomy and Developmental Biology, Medawar Building, University College London, Gower Street, London WC1E 6BT, United Kingdom; FAX: 44-171-813-2813. e-mail: uegakko@ucl.ac.uk

0214-6282/97/$05.00

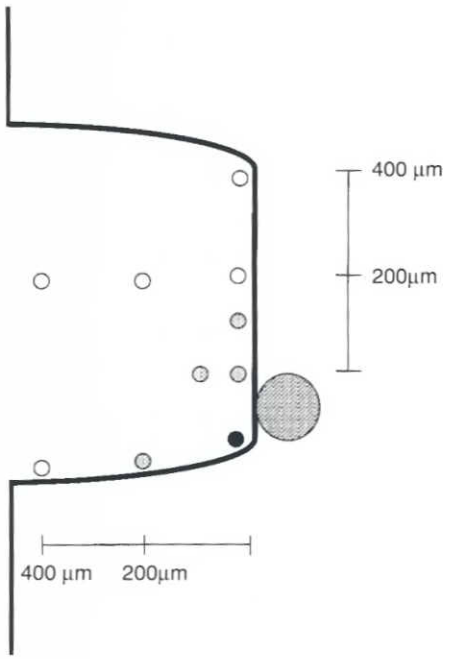


**Fig. 1. Positions of DiI injections in limb buds after amputation and application of FGF-4 bead (large shaded circle) to the posterior of the stump.** *Black circle, site at which essentially all labelled cells were found in the FGF-4-induced outgrowth; stippled circles, sites at which only a small number of labelled cells were found in the FGF-4-induced outgrowth; clear circles, sites at which no labelled cells were found in the FGF-4-induced outgrowth. The scales indicate distances from the FGF-4 bead, along the distal-proximal (right to left) and posterior-anterior (bottom to top) axes of the bud.*

was injected at the posterior margin of the bud, 0-50 μm proximal to an FGF-4 bead (Fig. 1, black circle), a stream of DiI-labeled cells was distributed evenly throughout the mesenchyme distal to the bead, right to the tip of the outgrowth (n=7; Fig. 2C). When DiI was injected at the posterior margin of the bud, 200 μm proximal to the FGF-4 bead, a long stream of labeled cells was found in proximal parts of the stump and, in addition, a few scattered labeled cells were found in the new outgrowth (n=6; Fig. 2D). However, when DiI was injected at the posterior margin of the bud, 400 μm proximal to the FGF-4 bead, no labeled cells were found in the outgrowth, with most cells remaining at the site of injection (n=5; Fig. 2E).

DiI was then injected into a series of sites at the plane of amputation in wing buds treated with FGF-4. When DiI was injected 0-50 μm anterior to the FGF-4 bead, most of the labeled cells remained at the injection site, but a small number of labeled cells were found in the outgrowth (n=5; Fig. 2F). A similar result was found when DiI was injected 100 μm anterior to the FGF-4 bead at the plane of amputation (n=5; data not shown) or 100 μm proximal to the anterior edge of the FGF-4 bead (n=3; data not shown). However, when DiI was injected 200 μm anterior to the FGF-4 bead, at the plane of amputation, no labeled cells were found in the outgrowth, and virtually all cells were distributed as a round patch in the future ulna/radius region of the wing (n=7; Fig. 2G). Similarly, when DiI was injected 400 μm from the FGF-4 bead, at the plane of amputation, labeled cells did not contribute to the outgrowth but rather remained as a round patch in the anterior of the wing, with a few scattered cells being found more centrally (n=5; Fig. 2H).

Finally, we injected DiI into central regions of the bud, where differentiation is already beginning to occur at stage 23-24. At injection sites 200 μm (n=7) or 400 μm (n=5) from the amputation plane, labeled cells did not contribute to the outgrowth but rather remained as a round patch in the region of the future elbow or humerus, respectively.

This analysis shows that the limb bud «regenerate» is formed from cells in the immediate vicinity of the FGF-4 bead. The region within which cells can contribute to the outgrowth extends approximately 200 μm proximally along the posterior margin of the bud, and approximately 100 μm anteriorly along the amputation plane. Cells proximal to the bead, within this region, can move distally. Cells in other regions of the stump do not enter the outgrowth. In regenerating adult amphibian limbs, localized irradiation experiments and descriptive studies show that the blastema is also local in origin and that there is no migration of cells from the rest of the body (reviewed by Wallace, 1981). Thus, regeneration is not affected by irradiation of tissue behind the amputation surface, as long as the tissue immediately below the surface is not irradiated. Furthermore, DNA synthesis, as measured by incorporation of tritiated thymidine, is restricted to the region immediately beneath the amputation plane.

The finding that participation in outgrowth is restricted to cells close to the FGF-4 bead could reflect an inherent inability of more distant cells to participate in forming new structures. However, this possibility can be discounted, since although only cells immediately adjacent to the bead contributed to outgrowth of a wing bud stump amputated 300 μm from the tip of the bud, our previous work has shown that FGF-4 can induce outgrowth of wing bud stumps amputated up to 500 μm from the tip. It is therefore likely that the local recruitment of cells in FGF-4-treated wing bud stumps is due to the limited range of influence of the FGF signal. Similarly, in regenerating amphibian limbs, amputation at any position along the proximo-distal axis leads to blastema formation, but only local cells contribute to the blastema at any given level.

We have shown previously that cells in the FGF-4-induced outgrowth of amputated wing bud stumps express *Msx-1* (Kostakopoulou *et al.*, 1996). During limb development, *Msx-1* is expressed in undifferentiated mesenchyme cells at the tip of the limb bud. It has been suggested that *Msx-1* functions to maintain cell lability and responsiveness to positional cues, and that *Msx-1* is switched off as cells become specified (reviewed by Davidson, 1995). Since we have now shown that the outgrowth arises from cells immediately adjacent to the implanted FGF-4 bead, which do not express *Msx-1* initially, it is clear that outgrowth involves activation of *Msx-1* expression, probably in cells that had previously switched off *Msx-1* expression. Indeed, while FGF-4 beads placed in posterior mesenchyme can reactivate expression of both *Msx-1* and *Sonic hedgehog*, FGF-4 beads placed in central mesenchyme cannot (Kostakopoulou, 1996). Whilst our results therefore indicate that FGF-4-induced regeneration involves the re-programming of gene expression in mesenchymal cells adjacent to the bead, there is no reason to believe that fully differentiated cells within the wing bud stump, such as chondrocytes or myocytes, de-differentiate and contribute to the outgrowth. This contrasts with amphibian limb regeneration, where de-differentiation of tissues to contribute cells to the blastema is well-established (Steen, 1968; Namenwirth, 1974; Maden and Wallace, 1975; Casimir *et al.*, 1988; Lo *et al.*, 1993).

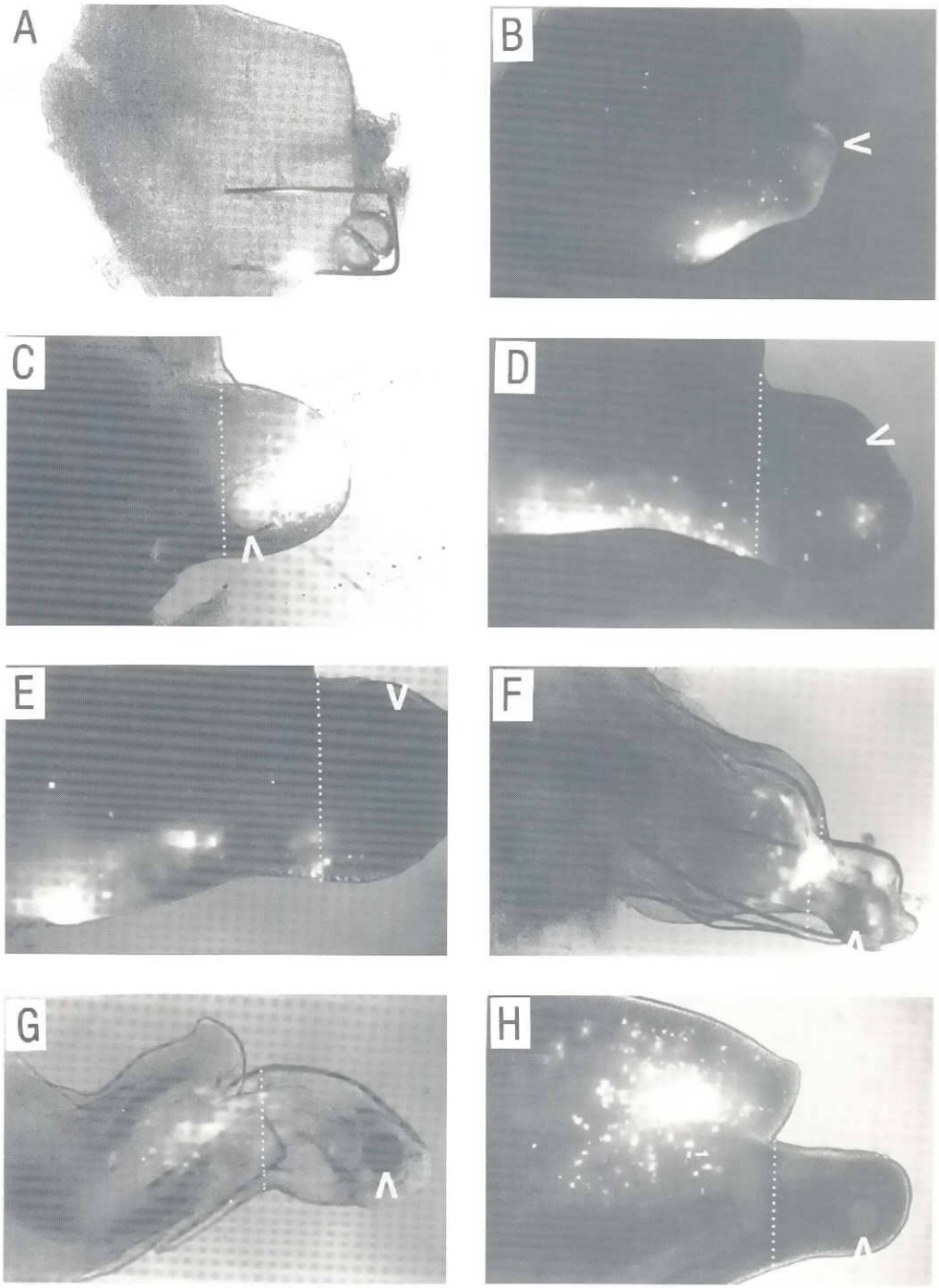


**Fig. 2. Origin of cells contributing to regenerating chick wing buds after amputation 300 μm from the tip of the bud at stage 23-24, application of FGF-4 and injection of DiI. (A)** *Embryo fixed immediately after FGF-4 application and DiI injection.* **(B)** *Embryo fixed 48 h after application of a PBS-soaked bead and DiI injection 0-50 μm proximal to the bead, at the posterior margin of the bud.* **(C-H)** *Embryos fixed 48 h after FGF-4 application and DiI injection 0-50 μm proximal to the bead, at the posterior margin of the bud* **(C)**; *200 μm proximal to the bead, at the posterior margin of the bud* **(D)**; *400 μm proximal to the bead, at the posterior margin of the bud* **(E)**; *0-50 μm anterior to the bead, along the plane of amputation* **(F)**; *200 μm anterior to the bead, along the plane of amputation* **(G)**; *400 μm anterior to the bead, along the plane of amputation* **(H)**. *Regions distal to the dotted lines constitute the outgrowth stimulated by the FGF-4 bead after 48 h. FGF-4 beads are indicated by arrow heads. Photographs were taken under bright-field (A) or dark-field (B-H) illumination.*

## Experimental procedures

### *Limb bud amputation and FGF-4 application*

White Leghorn chicken eggs were incubated at 38°C until stage 24 (Hamburger and Hamilton, 1951). Eggs were windowed and membranes covering the embryo were slit and pulled back to expose the wing bud. Amputations were carried out by making an anterior-posterior incision through the limb bud with fine needles and removing the distal tissue with fine forceps. The level of amputation was measured using an eyepiece graticule. For FGF-4 application, heparin acrylic beads (H5263, Sigma), 200-250 μm in diameter, were soaked in 2μl of 700 μg/ml FGF-4 (a kind gift from John Heath) for 1 h at room temperature before application to the amputated mesenchyme of the limb. Control beads were soaked in PBS before application. Beads were kept in place with staples made of platinum wire (0.025 $mm^2$, Goodfellow Metals). Embryos were then fixed in either 5% (w/v) trichloroacetic acid or 4% (w/v) paraformaldehyde.

### *DiI injection*

Amputated stumps were injected with the lipophylic dye DiI (1,1 dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchloride, Molecular

Probes) at a concentration of 3 mg/ml in dimethyl formamide, using a pressure injector. The position of the injection site was determined using an eyepiece graticule. DiI is taken up by cells and passed on to their progeny, but is not passed to neighbouring cells and is not toxic (Honig and Hume, 1989; Vargesson *et al.*, 1997). Injected embryos were incubated for a further 48 h, fixed overnight in 4% (w/v) paraformaldehyde and examined by epifluorescence microscopy to visualize labeled cells.

*Acknowledgments*

*This work was supported by Action Research and the Astor Foundation. JC is supported by the Wellcome Trust. We thank John Heath for FGF-4 protein and Lewis Wolpert for helpful discussions.*

## References

CASIMIR, C.M., GATES, P.B., PATIENT, R.K. and BROCKES, J.P. (1988). Evidence for dedifferentiation and metaplasia in amphibian limb regeneration from inheritance of DNA methylation. *Development 104:* 657-668.

DAVIDSON, D.R. (1995). The function and evolution of Msx genes: pointers and paradoxes. *Trends Genet. 11:* 405-411.

FERRETTI, P. and BROCKES, J.P. (1991). Cell origin and identity in limb regeneration and development. *Glia 4:* 214-224.

HAMBURGER, V. and HAMILTON, H. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol. 88:* 49-92.

HONIG, M.G. and HUME, R.I. (1989). DiI and DiO: versatile fluorescent dyes for neuronal labelling and pathway tracing. *Trends Neurosci. 12:* 333-341.

KOSTAKOPOULOU, K. (1996). The Role of the Gene Msx-1 in Limb Development. PhD Thesis, University of London.

KOSTAKOPOULOU, K., VOGEL, A., BRICKELL, P.M. and TICKLE, C. (1996). Regeneration of wing bud stumps of chick embryos and reactivation of Msx-1 and Shh expression in response to FGF-4 and ridge signals. *Mech. Dev. 55:* 119-131.

LO, D.C., ALLEN, F. and BROCKES, J.P. (1993). Reversal of muscle differentiation during urodele limb regeneration. *Proc. Natl. Acad. Sci. USA 90:* 7230-7234.

MADEN, M. and WALLACE, H. (1975). The origin of limb regenerates from cartilage grafts. *Acta Embryol. Exp. 2:* 77-86.

NAMENWIRTH, M. (1974). The inheritance of cell differentiation during limb regeneration in the Axolotl. *Dev. Biol. 41:* 42-56.

STEEN, T.P. (1968). Stability of chondrocyte differentiation and contribution of muscle to cartilage during limb regeneration in the axolotl (Siredon mexicanum). *J. Exp. Zool. 167:* 49-77.

TAYLOR, G.P., ANDERSON, R., REGINELLI, A.D. and MUNEOKA, K. (1994). FGF-2 induces regeneration of the chick limb bud. *Dev. Biol. 163:* 282-284.

VARGESSON, N., CLARKE, J.D.W., VINCENT, K., COLES, C., WOLPERT, L. and TICKLE, C. (1997). Cell fate in the chick limb bud and relationship to gene expression. *Development 124:* 1909-1918.

WALLACE, H. (1981). *Vertebrate Limb Regeneration*, (Ed, Wiley), Chichester.

*Received: April 1997*
*Accepted for publication: July 1997*