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A proximo-distal gradient of FGF-like activity in the embryonic chick limb bud

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Abstract. In a microassay for anchorage-independent growth in soft agar, NR6 cells form colonies in a dose-dependent manner in the presence of fibroblast growth factor (FGF). Using this assay system, the ability of thin sequential slices of embryonic chick limb bud to promote colony formation was investigated. A functional gradient of colony-promoting ability along the proximo-distal axis of the developing chick limb bud (stages 22–26) was observed. The highest number of colonies was observed in the presence of the most distal slices, and colony number decreased progressively at proximal levels. This gradient was specifically eliminated by the addition of anti-FGF antibody to the assay, indicating that it was caused by a functional gradient of an FGF-like molecule. Limbs of stages 21–26 were assayed: before this time limb buds are too small to slice in the proximo-distal axis in the required manner. The FGF-like gradient was observed at stages 22 to 26.
Key words. Fibroblast growth factor (FGF); embryonic; limb; chick; gradient.

Abbreviations. AER = apical ectodermal ridge, FCS = fetal calf serum, EGF = epidermal growth factor, FGF = fibroblast growth factor, bFGF = basic fibroblast growth factor, PBS = phosphate buffered saline, TGF = transforming growth factor.

There are a number of pieces of evidence which implicate fibroblast growth factors (FGFs) as controlling factors in limb distalization. mRNAs for at least three FGFs have been found in varying locations within the limb bud. FGF-2 is located throughout the bud, in both ectoderm and mesoderm [1]. It has already been shown by immunostaining of the chick limb bud that FGF-2 is present in the limb ectoderm and sub-epithelial mesoderm from stages 16–25 [1]. FGF-4 transcripts are found in the posterior apical ectodermal ridge (AER), while FGF-8 is found throughout the AER [2]. FGF-8 transcripts have been found to be present in the ectoderm of the limb bud territories prior to the onset of limb bud growth in both chick and mouse embryos [3]. Purified FGF-8 protein has been shown to rescue limb bud outgrowth in mouse limbs lacking an AER, but has failed to maintain the expression of sonic hedgehog gene (*shh*) [3]. In chick embryos it has been shown that FGF-8 can replace the AER to support *shh* expression and outgrowth and patterning of the limb bud [4]. The effect of the AER in chick limbs can also be substituted by application of FGF-4 [5, 6]. FGF-4 also maintains the signal for polarizing activity produced by the posterior mesenchyme in the normal limb [5]. In vitro, FGF-4 has been shown to stimulate the proliferation of late limb mesenchymal cells [7].

Even though FGF-4 mRNA is expressed in the AER and FGF-4 stimulates limb mesenchyme cells to divide [8], this still does not prove that the growth factor is present in functional form in the limb extracellular compartment, since the molecule must be translated, exported and often extensively modified before becoming active [9].

The aim of this investigation is therefore to ascertain if a functional gradient of exported, processed and active FGF exists along the proximal/distal axis of the developing chick limb, using a sensitive microassay. This employs NR6 cells, which are derived from NRK cells, and which lack functional EGF and TGF-α receptors. They respond to the presence of members of the FGF family in a dose-dependent manner by showing transformed behaviour in soft agar anchorage-independence assays. No other single growth factors are known to have this effect [9].

Methods and materials

Cell culture. NR6 cells were cultured in alpha-Eagles medium containing 10% fetal calf serum (FCS) and passaged at 3–4 day intervals. Equal volumes of 0.6% agar and double strength medium, containing 10^5 NR6 cells/ml, were mixed gently by inversion. The 0.6% agar was boiled for 15 minutes and then transferred to a 40 °C water bath for 30 minutes. The medium supplements were such that at final concentration the agar/medium mixture contained 10% FCS, 50 IU/ml penicillin, 50 mg/ml streptomycin and 20 mM HEPES buffer. The mixture was dispensed in 0.1 ml volumes to each of the 60 centre wells of a sterile 96-well plate. The outer wells were filled with phosphate buffered saline (PBS) to prevent drying out. The microwell plates were

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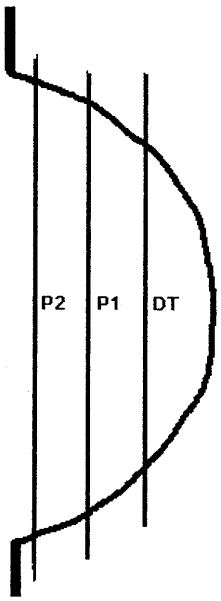

Figure 1. 200 μm transverse slices of the chick limb bud were made by a sharp tungsten needle against a 200 μm grid. DT: distal tip, P1: first proximal slice, P2: second proximal slice.

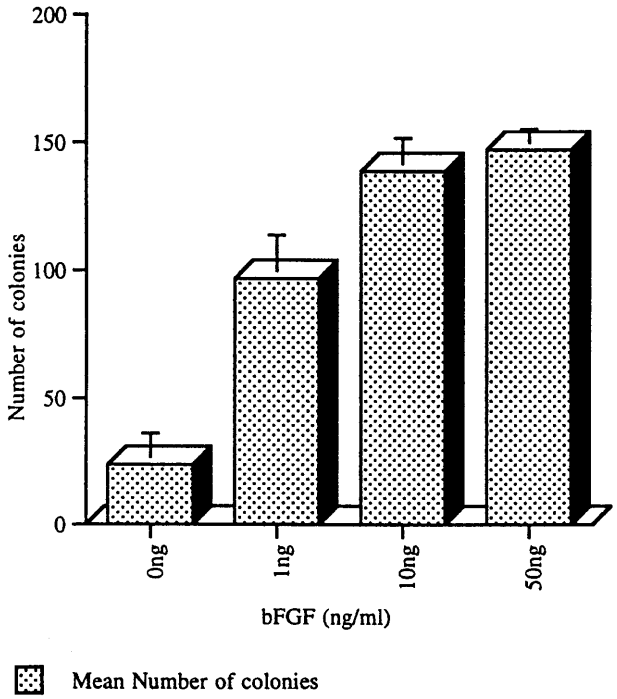

Figure 2. Dose-dependent transformation of NR6 cell line by bFGF. The transforming ability of bFGF appears to plateau at 10 ng/ml. Transformation was measured as the ability to form colonies of four or more cells. The number of these colonies was counted in each well, which at the start of the assay contained on average 500 cells. Error bars represent standard deviations.

cooled at 4 °C for 4 minutes to ensure that the agar mixture had solidified prior to incubation.

Preparation of embryonic material. Fertilized hen eggs were incubated blunt end up in a humidified environment at 38 °C for approximately 3–5 days, depending on the stage required. Entry was made via the air space, and embryos were removed to dishes of alpha-Eagles medium for cleaning (removing remaining attached membranes, cranial region) and examination. Each embryo was staged (stages 16–26) according to Hamburger and Hamilton, 1951 [10]. Using electronically sharpened tungsten needles, 200 μm slices were made through embryonic chick limbs from distal to proximal, transverse to the long axis of the limb, at a range of stages. This was achieved by placing a grid of 200 μm underneath the limb prior to cutting to ensure accuracy. The flank slices were cut using the same method. Two slices at each level and stage were added to the culture cells.

Scoring the assays. Once the tissue samples had been added to the wells of the assay, the plates were placed in a humidified incubator at 37 °C and 5% CO_2 for 7 days before being fixed by the addition of a drop of formal saline to each well. Colonies were examined unstained using an inverted microscope and only colonies containing four or more cells were scored. The chick limb cells cannot invade the semi-solid medium.

Extracted bovine pituitary FGF-2 (Sigma) was added to wells of the soft agar assay in varying concentrations as a positive dose-response control. In experiments to investigate the ability of anti-FGF antibodies (rabbit polyclonal, Sigma F3393) to inhibit colony-forming ability of limb bud explants, anti-FGF antibody was added to the wells in either 1:800 or 1:400 dilutions (alpha-Eagles medium was used to make up the dilutions). Blank solutions of equivalent volume but containing no additives were added to control wells.

Experiments were run to investigate the effects of a non-specific antibody (rabbit polyclonal anti-fd bacterophage, Sigma B7786) upon the colony-forming ability of NR6 cells . The non-specific antibody was added to the wells in either 1:800 or 1:400 dilution (alpha-Eagles medium was used to make up the dilutions).

Measuring the volume of the limb slice. It is important in principle to correct for the differing volumes of the slices, though in practice the shape of the limb means that volume increases from distal to proximal, thus acting against the spurious observation of a gradient with high distal values.

In order to measure the volume of the slices, a stage 23 limb was sectioned transversely to the proximo-distal axis at 5 mm intervals and then stained with haematoxylin and eosin. The sections were traced using an Olympus microscope with a drawing tube at 100× magnification and scanned into a Macintosh Performa 620 PowerPC. The areas of the scans were measured using NIH Image freeware, and the volume of each section was obtained. The volume of all the sections making up a 200 μm slice was summed to give the total volume of the slice.

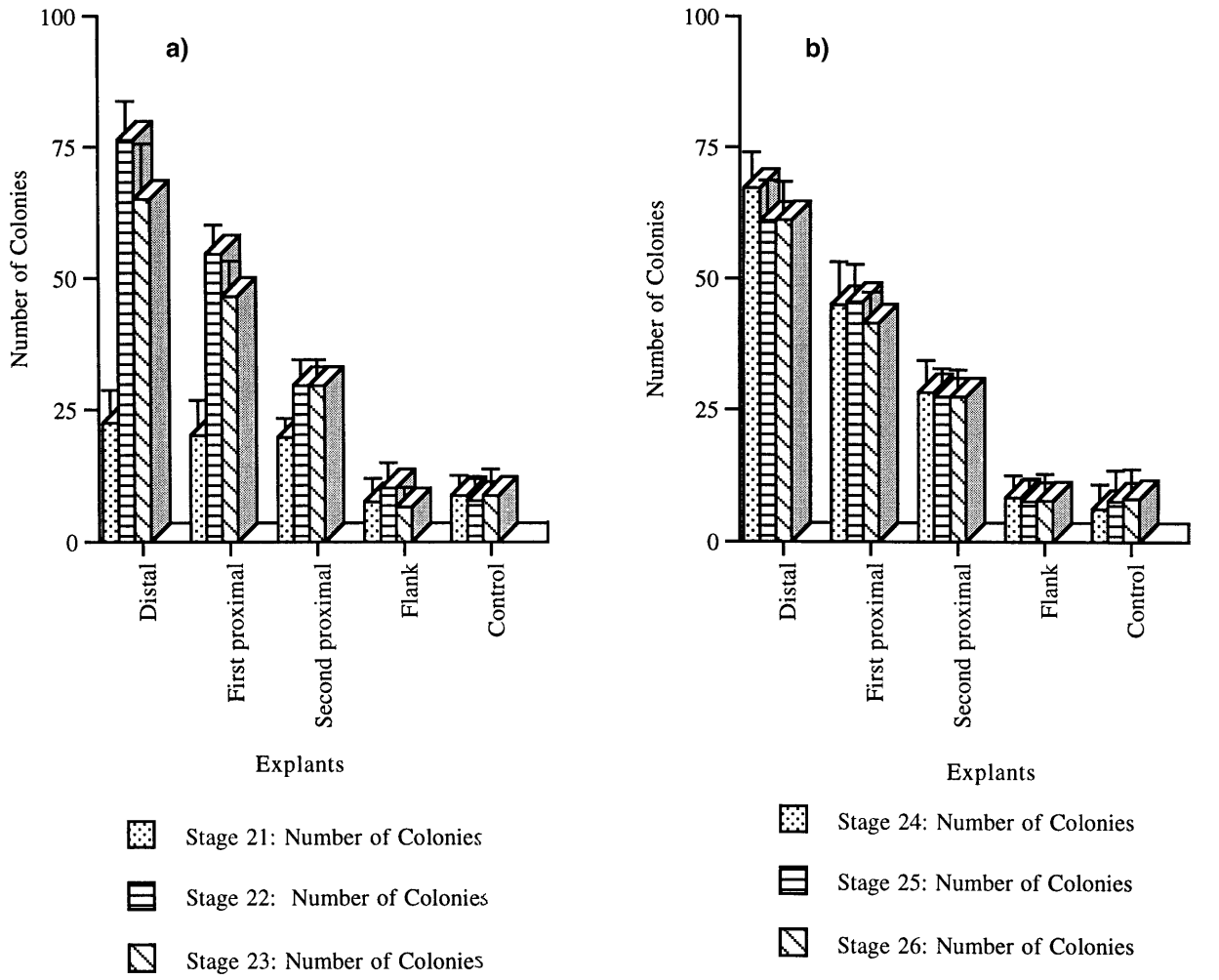

Figure 3(*a*, *b*). Transformation of the NR6 cell line by embryonic chick limb slices (200 μm) at stages 21–26, compared with controls in the absence of limb explants. By Tukey post-hoc test, there is a significant difference between wells containing limb slices with wells containing flank or no explants ($p < 0.001$).

Statistical analysis. Statistical analyses were carried out using Minitab. In all cases two-way analysis of variance (ANOVA) was performed to determine whether the experimental subgroups were from the same or different populations. If the assay categories were found not to belong to the same population, Tukey post-hoc analyses were carried out on the data sets to determine which data sets were significantly different from their neighbours.

Results

The results demonstrate that:

(i) Increasing concentrations of FGF-2 give a dose-dependent response in terms of NR6 colony formation (fig. 2). ANOVA indicated that there were significant differences between samples ($p < 0.0001$). The Tukey pairwise comparison test (family error rate = 0.05) showed that there were significant differences between 0 ng/ml and 1 ng/ml, and between 1 ng/ml and 10 ng/ml FGF-2 treatments. No significant difference was observed between 10 ng/ml and 50 ng/ml.

(ii) Proximo-distal slices down the chick limb evoke a graded response in terms of NR6 colony formation, with the most distal tissue giving the highest number of colonies, at all stages tested from 22 to 26 (fig. 3a, b). At stage 21 there were significant difference between the samples by ANOVA ($F = 8.49$, $DF = 9, 40$, $p < 0.001$), but the Tukey's pairwise comparison showed that these differences existed between the limb slices and the flank and not between proximo-distal levels along the limb. At stages 22–26 there were significant differences between levels by ANOVA ($F = 126.50$, $DF = 9, 40$, $p < 0.001$) and Tukey pairwise comparison showed that these differences were significant between each sequential slice ($p \leq 0.001$).

(iii) This graded response was removed by addition of polyclonal anti-FGF-2 at all stages tested (fig. 4: stage 23 is shown as an example. An identical response was observed in all experiments). Anti-FGF was added to the wells at two different dilutions, 1:800 and 1:400. There was significant difference between the samples by ANOVA ($p < 0.0001$); Tukey's pairwise comparison

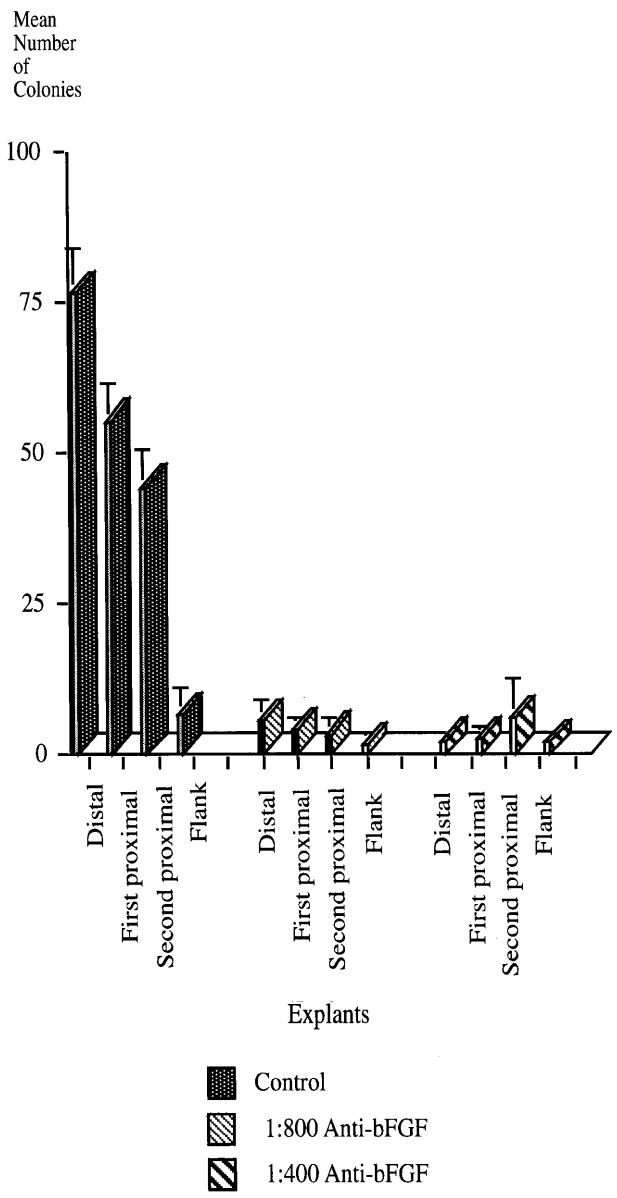

Figure 4. Transformation of NR6 cell line by embryonic chick limb slices (200 μm) at stage 23, in the presence of anti-FGF. Wells contained blank solution, 1:800 and 1:400 dilutes of anti-FGF. By Tukey post hoc test, there are significant differences between wells containing limbs only and wells containing 1:800 and 1:400 dilutions of anti-FGF ($p < 0.0001$).

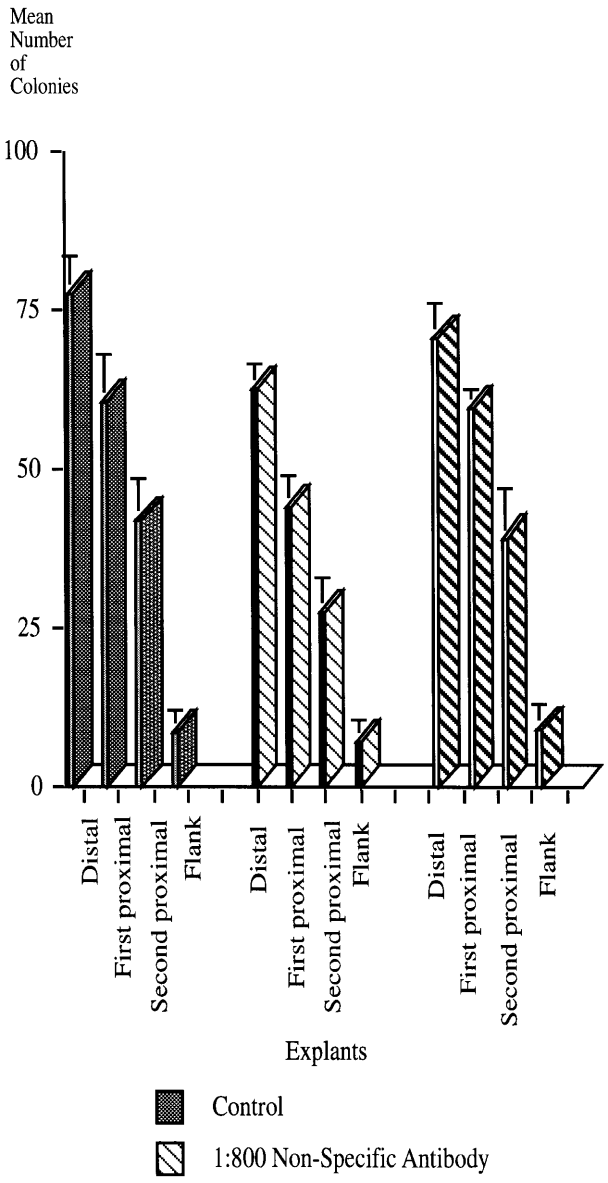

Figure 5. Transformation of the NR6 cell line by embryonic chick limb slices (200 μm) at stage 23 in the presence of blank solution, 1:800 and 1:400 dilutions of non-specific antibody. By Tukey post hoc test there is no significant difference between wells containing limbs only and wells containing limbs with 1:800 or 1:400 dilutions of non-specific antibody.

showed that these differences existed between the limb slices treated with anti-FGF and the positive controls. There was no significant difference between the colony-forming of the limb slices at 1:800 and 1:400 anti-FGF dilutions ($p < 0.0001$). At stages 21–26 there was significant difference by ANOVA ($F = 203.46$, $DF = 5, 24$, $p < 0.0001$) and Tukey's pairwise comparison showed that these differences were significant between the slices treated with anti-FGF and the slices treated with non-specific antibody. The non-specific antibody did not have any effect on the formation of colonies by limb explant slices (fig. 5).

(iv) In all experiments 200 μm transverse slices of limb buds were added to the assay wells. However, proximal limb bud slices have a greater diameter, and were therefore larger than distal slices. Volume measurements in a representative limb showed that the volume for the 200 μm distal tip slice was 0.04275 mm^3 while that for the first proximal slice was 0.07838 mm^3. Correcting for this change in size markedly enhances the gradient effect, and we believe this justifies neglecting it in the first instance.

Slight variations were observed from day to day in both experimental and control values. However, in general

the results were sufficiently similar to be directly compared without the necessity of converting to a common base line.

Previous experiments at stages 16–19 where the limb bud is 200 mm or less have failed to show a difference between whole limb buds and flank (McLachlan, unpublished observations).

Discussion

NR6 cells respond in a dose-dependent manner to the presence of FGF by forming colonies; they also respond to limb slices by forming colonies, with colony number increasing from proximal to distal. This colony-forming ability is removed by anti-FGF antibodies (cells respond to members of the FGF family), but not by non-specific antibodies. It therefore seems reasonable to conclude that the gradient effect is mediated by an FGF-like molecule. Identification of the FGF involved awaits the development of suitable specific antibodies.

FGF is regulated by being exported from cells, where it appears to be cell-associated with heparan sulfate-related proteoglycans [11]. Our observations are consistent with the hypothesis that a functional gradient of an active FGF-like molecule is secreted by the AER at stages 22–26. The results are consistent with the idea that a chemical gradient of growth factor may exist in vivo, acting over a range of approximately 600 μm. However, this is not the only possible explanation. FGF and FGF receptors are present in the underlying mesenchyme, and thus a functional gradient of FGF could be produced in the mesenchyme itself. This may therefore prove to be a suitable test system to distinguish between long range diffusible morphogens and cell-cell relays as a signalling system [12]. At stage 21 no significant difference between the distal tip and the zone immediately proximal to it was observed, and this may reflect an absence of a gradient before stage 22. Alternatively the microassay used may not have been sensitive enough to detect a gradient. In these experiments no distinction was made between the wing and the leg buds. However, in previous investigations it has been observed that whole leg buds induce slightly fewer colonies than wing buds [13]. No correlation was observed between the number of colonies formed and the distance from the explant, suggesting that the FGF-like molecule diffuses evenly throughout the agar.

In conclusion, these results are consistent with the theory that FGFs are locally produced by the AER and exert a graded influence over the underlying mesoderm, and extend it by demonstrating for the first time that there is indeed a functional gradient of FGF from distal to proximal in the limb, as required by the theory.

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