

Distribution of insulin-like growth factor peptides in the developing chick embryo

J. R. RALPHS¹, L. WYLIE and D. J. HILL*

Department of Anatomy and Developmental Biology, University College and Middlesex School of Medicine, Windeyer Building, Cleveland Street, London W1P 6DP, UK

¹ Present address (and correspondence): Department of Anatomy, University of Wales College of Cardiff, PO Box 900, Cardiff CF1 3YF, UK

* Lawson Research Institute, St Joseph's Health Centre, 268 Grosvenor Street, London, Ontario N6A 4V2, Canada.

Summary

Growth factors are likely to be of major significance in developmental biology. Here, the distribution of insulin-like growth factor (IGF) peptides is described during development of the chick embryo. IGF was immunolocalised using a polyclonal antibody to human IGF I detected with a modified Vectastain ABC procedure. Under the conditions used, the antibody binds strongly to IGF I and weakly to IGF II; thus the distribution of IGF peptide, rather than the individual factors, is described.

Muscle, peripheral nerve and the notochord were labelled whenever present. Muscle label was associated with the myotubes and neural labelling with neurons; Schwann cells were unlabelled. IGF distribution changed during differentiation of connective tissues. Regions of mesenchyme destined to form cartilage labelled weakly or not at all, and cartilage condensations were unlabelled. In the limb, chondrocytes became

labelled once cartilage rudiments had formed; however, in later development, label was absent in zones of rounded and flattened chondrocytes and appeared strongly at the onset of hypertrophy. Early osteogenic mesenchyme was also unlabelled, although later bone cells were strongly stained. In the neural tube, label was associated with differentiating neuroblasts and cell bodies and with axons, especially in the developing dorsolateral tracts.

These results show a possible correlation between IGF label and cell division in early mesenchyme; cartilage condensations, which have reduced mitotic indices, do not label. In other tissues, notably muscle and nerve but also later connective tissues, label is associated with differentiating, rather than dividing, cells.

Key words: IGF peptides, immunocytochemistry, chick embryo, development.

Introduction

The functions of growth factors in embryos are of major interest in developmental biology. Whilst there is a considerable body of information dealing with the effects of growth factors on cultured cells, relatively little is known of their actual roles in embryos. Some factors have been shown to have novel roles in vertebrate development, for example transforming growth factor- β (TGF- β) and fibroblast growth factor (FGF) appear to act as signals to specify mesoderm in early amphibian embryos (Kimelman and Kirschner, 1987; Slack *et al.* 1987; Weeks and Melton, 1987); however, in most cases the precise function of growth factors remains obscure. An important step in elucidating the functions of growth factors in embryos is the identification of factors present and the investigation of their distribution and relationship with developmental processes. This approach has recently been taken, with

descriptions of distributions of TGF- β in mouse embryos (Heine *et al.* 1987), insulin-like growth factor (IGF) I and II in human foetuses (Han *et al.* 1987a) and the identification of FGF-like activity in the limb buds of chick embryos (Seed and Hauschka, 1988). Here, we describe the distribution of IGF peptides in the chick embryo, as revealed by immunocytochemistry.

IGFs have significant effects on proliferation and differentiation of embryonic cells *in vitro* (Zapf *et al.* 1984; Froesch *et al.* 1985). Important roles for these factors in embryos are suggested by the presence of specific receptors from early in development (Bassas *et al.* 1985, 1987, 1988; Bhaumic and Bala, 1987; Smith *et al.* 1987), and by the ability of explants of embryonic tissues to synthesise IGF *in vitro* (D'Ercole *et al.* 1980). Recently, mRNAs coding for IGF II have been detected by *in situ* hybridisation in rat embryos over a wide developmental period (Beck *et al.* 1987; Styliano-poulou *et al.* 1988). In addition, Engstrom *et al.* (1987)

detected IGF mRNA in chick limb buds, a result confirmed in our laboratory in a preliminary investigation of chick limb mRNA for messages coding for growth factors (Taylor pers. comm.). The distributions of IGF peptides has been mapped in human fetuses (Han *et al.* 1987b); however, at these stages all major organ systems are already established. There are no studies of IGF peptide distribution prior to or during patterning and differentiation.

As a first step in analysing roles of IGFs in developing chick embryos, we have mapped the distribution of IGF peptides using a polyclonal antibody raised to human IGF I (Morrel *et al.* 1989). We find that at early stages IGFs can be detected in the mesenchyme of the limb, face and body. The distribution is not even; in regions where cartilage will differentiate, cells are unlabelled. IGF peptides appear in chondrocytes as they mature, and in the late cartilage rudiment of the limb are distributed according to the stage of differentiation of the chondrocytes. IGF is also present in developing bone, muscle and nervous tissues. The results are discussed in the light of recent reports describing the distribution of mRNA coding for IGF peptides in rat and human embryos (Beck *et al.* 1987; Han *et al.* 1987b; Stylianopoulou *et al.* 1988).

Materials and methods

Fertilised eggs from Ross white chickens were incubated at 38°C in a humidified incubator. Eggs were windowed on the second day of incubation and the embryos staged according to Hamburger and Hamilton (1951). They were then reincubated until they reached the desired stages. Embryos were used from stage 20 to stage 36 (approx. 3.5–10 days).

Preparation of tissue

Embryos were removed from the egg and washed in phosphate-buffered saline (PBS). Embryos were dissected into pieces. At stages 20–28 both the head and the trunk region at the level of the wing bud were cut away and fixed; at later stages, limbs and specific facial regions were dissected from the embryos. The fixation procedure used was that of Han *et al.* (1987a). In addition, mouse embryos of age 13 days *post coitum* were dissected from the uterus and treated in the same way as stage 20–28 chick embryos. Specimens were fixed for 16–24 h at 4°C in 2% paraformaldehyde/2% glutaraldehyde in PBS. After washing in several changes of PBS over a 48 h period, they were dehydrated in a methanol series, cleared in histoclear and embedded in paraffin. Sections were cut at 10 µm, mounted on 'Histostik'-coated slides (Histolab, Hemel Hempstead, UK) and baked at 45°C for 48 h. Some chick embryos were prepared for cryosectioning. These were immersed in OCT compound (BDH, Poole, UK) and frozen in liquid nitrogen. Cryosections were cut at 10 µm and stored at –20°C until use. Sections of the head were cut in the frontal plane, whereas those of the body were cut transversely at wing level.

Immunohistochemistry

Three antibodies were tested for immunohistochemical reactivity with chick tissues. These were (1) polyclonal rabbit anti-human IGF I (kindly provided by Dr D. Morrell, Institute of Child Health, London, UK); (2) monoclonal anti-human IGF

I (kindly provided by Drs A. J. D'Ercole and J. J. Van Wyk, University of North Carolina, USA); and (3) rabbit anti-rat IGF II (kindly provided by Dr S. P. Nissley, NIH, Bethesda, USA). In each case, the labelling procedure was the same, and used a modified Vectastain ABC technique (Vector Laboratories) with appropriate biotinylated second antibody. Sections were dewaxed, rehydrated and stored in PBS. They were then treated with 0.012% trypsin (Gibco, Paisley, Scotland) in PBS for 5 min at room temperature and washed in three changes of PBS. The sections were then incubated in antibody (1), (2) or (3) at 4°C for 48 h. Various dilutions of the antibodies were used, from 1:500 to 1:2000 in PBS containing 0.1% bovine serum albumin and 0.05% sodium azide. After thorough washing, sections were incubated in 1:500 dilution of biotinylated second antibody for 2 h at room temperature. After further washes, the sections were incubated in Vectastain avidin/peroxidase complex for 2 h. The localisation was completed by incubation in 0.05% diaminobenzidine hydrochloride/0.05% hydrogen peroxide in Tris buffer, pH 7.4 for 5–7 min. Sections were counterstained with Meyer's haemalum, dehydrated, cleared and mounted in DPX.

A sequence of control incubations were performed using antibody (1). The controls were as follows: omission of primary antibody, omission of second antibody and substitution of non-immune rabbit serum for primary antibody. The primary antibody was in addition preincubated overnight at 4°C with IGF peptide preparations prior to use in a series of blocking experiments. Preincubations were: 2–400 ng ml⁻¹ human IGF I, 2–400 ng ml⁻¹ rat IGF II and 1 µg ml⁻¹ partially pure chick IGF (kindly provided by C. Goddard, Poultry Research Centre, Roslyn, Edinburgh; fraction from gel chromatography likely to contain both putative chick IGF 1 and 2).

Cryosections were labelled with an affinity-purified rabbit polyclonal antibody to chick type II collagen (kindly provided by Dr C. Archer, Institute of Orthopaedics, London, UK) using standard procedures for indirect immunofluorescence after preincubation of sections with a mixture of hyaluronidase (*Streptomyces hyalurolyticus*: 1.45 units ml⁻¹; Sigma, Poole, UK) and chondroitinase ABC (*Proteus vulgaris*: 0.25 units ml⁻¹; Sigma, Poole, UK) for 30 min at 37°C. Specimens were examined under epifluorescent illumination using a Zeiss Photomicroscope III microscope.

Results

Antibody specificity

No positive staining was obtained with antibody (2). Slight positive staining was obtained with antibody (3) at 1:500 dilution, but not at 1:1000 or 1:2000. Strong positive staining was obtained with antibody (1) at 1:1000 and 1:2000 dilutions. The specificity of antibody 1, a polyclonal rabbit anti-human IGF I, was investigated using a series of controls and by preincubating antibody (1) solutions with various concentrations of purified IGF I and II in label blocking studies. Control incubations, involving either omission of primary antibody, omission of secondary antibody or substitution of non-immune rabbit serum, were negative. In blocking studies, preincubation of antibody with 2–20 ng ml⁻¹ human IGF I did not block IGF staining but 100 ng ml⁻¹ blocked completely. Preincubation with 2–200 ng ml⁻¹

rat IGF II did not block whereas 400 ng ml^{-1} blocked slightly. Staining was completely blocked with partially pure chick IGF (a fraction containing both putative chick IGF I and II) at a concentration of $1 \mu\text{g ml}^{-1}$. Previous work using radioimmunoassay has demonstrated that antibody (1) has negligible binding to insulin (0.1% of binding to IGF I; Morrell pers. comm). In addition, Armstrong *et al.* (1989) have shown that in adult chicken serum the antibody binds only to IGF I and does not bind to IGF binding proteins. Our competition controls demonstrate strong binding to chicken IGF I, and weak binding to IGF II. These results indicate that whilst the antibody binds more strongly to IGF I than to IGF II, we cannot use it to distinguish between the two peptides (see also Han *et al.* 1987a). Therefore we describe below the distribution of IGF peptides, rather than the individual growth factors. Attempts were made to immunoblot the antiserum against chick embryo preparations run on 15% SDS-polyacrylamide gels under reducing and non-reducing conditions; however, no bands were detected. This does at least demonstrate the absence of non-specific binding of the antiserum to chick proteins. In addition, the distributions of label were similar in mouse embryos (see later), which indicates similarity of binding of the antiserum to mammalian and avian IGF peptides under conditions of immunocytochemistry.

Distribution of IGF peptide during development of the limb, face and body

The distribution of immunocytochemical label was investigated in the limb buds and facial primordia of embryos of stages 20–28. The laying down of the vertebral column occurs in a cranio-caudal sequence and so labelling in the body region was investigated in sections taken consistently at the level of the wing at stages 20, 24 and 28. Some specimens of stage 36 were also studied, as was a mouse embryo of 13 days gestation. Sample test and control specimens are shown in Fig. 1; the distribution of IGF peptides is described in detail below, in developmental sequence.

Stage 20–24

At stages 20 and 24 immunolabel was present in connective, myogenic and neural tissues. In limb mesenchyme at stage 20, label was strongest at the periphery, with the core region being weakly labelled (Fig. 2). This pattern was more pronounced at stage 24 (Fig. 3) where the proximal core region, corresponding to the position of the cartilage condensation, was unlabelled (Fig. 4A). Labelling intensity increased peripherally and distally away from the condensation and was denser than that seen at stage 20. A region of high labelling intensity extended from the limb mesenchyme into the ventral body wall, beneath the ectoderm. Immunolabel in the peripheral mesenchyme was clearly associated both with cells and extracellular matrix (Fig. 4B). The overlying ectoderm contained labelled and unlabelled cells. In the body, there was little or no labelling of sclerotomally derived mesenchyme surrounding the notochord and extending dorsally between the neural

tube and myotome; mesenchyme lateral to the myotome was labelled, however (Fig. 5). Cells of the notochord itself were labelled (Fig. 6). Craniofacial mesenchyme also contained immunolabel, at moderate density (not shown).

Strong labelling was present in skeletal muscle of the myotomes in association with the myotubes (see Fig. 5), the heart muscle (Fig. 7) and, at stage 24, the developing smooth muscle of the wall of the aorta (Fig. 8). Labelling in the myotome was far stronger than the occasional non-specific label observed in controls of stage 20 embryos. In the heart, the developing endocardial cushions were unlabelled, in clear contrast to the muscle. In spinal nerves, strong immunolabel was associated with axons, Schwann cells being unlabelled (Fig. 9). The neural tube was largely unlabelled, with the exception of a very thin layer at its periphery at stage 24.

Stage 28

At stage 28, the cartilages of the limb are clearly defined with dense matrices containing type II collagen (see Von der Mark *et al.* 1976a,b; Dessau *et al.* 1980; results confirmed here). Chondrocytes within the rudiments contained IGF peptide; however, the cartilage rudiments were enclosed by a region of unlabelled mesenchyme (Fig. 10), which included the perichondrium. Mesenchyme surrounding this was strongly labelled. In the developing vertebral column, unlabelled cells were present in a region concentric to the heavily labelled notochord (Fig. 11). This region contained abundant type II collagen (not shown; see also von der Mark *et al.* 1976b). Most cells within this region did not label for IGF peptide; however, some strongly labelled cells were encountered immediately adjacent to the notochord sheath. The surrounding mesenchymal cells were moderately labelled, with the exception of those extending dorsolaterally around the neural tube to form the vertebral arch, which were unlabelled. Mesenchyme of the developing face was moderately labelled, with unlabelled regions corresponding to early phases of development of Meckel's cartilage and the prenasal cartilage (Fig. 12); in the latter case, a region of low labelling intensity occupied a large proportion of the frontonasal mass. This region corresponded to a region containing diffuse label for type II collagen which forms part of the early chondrocranium and prenasal cartilage (not shown).

Developing skeletal, heart and smooth muscle was strongly labelled. As at stage 24, the endocardial cushions, now with developing valves, were unlabelled (Fig. 13). In the neural tube, sections taken at wing level (Fig. 14) showed the neuroepithelial region to be weakly labelled, the mantle zone to contain more label and the marginal zone also to be labelled. Within the mantle zone, densely labelled cells were present ventrolaterally where the ventral lateral motor columns will form. In the marginal zone, labelling was particularly strong in the dorsolateral tracts. The dorsal root ganglia also contained foci of immunolabel. Peripheral nerves were densely labelled wherever observed.

Distribution in late development of cartilage and bone

At stage 36, the rudiments of the humerus, radius and ulna are well developed with regions of rounded, flattened and hypertrophic chondrocytes. Occasional cells were labelled in the rounded and flattened cell zones, but label appeared abruptly in all cells at the onset of hypertrophy (Fig. 15). Labelling was not seen in cartilage matrix. Osteocytes and osteoblasts were strongly labelled, in subperiosteal bone of the limb rudiments and in membrane bones of the upper jaw (Fig. 16A,B). Bone matrix was unlabelled.

Distribution in the mouse embryo

The distribution of IGF peptide in the 13 day mouse embryo was essentially the same as that observed in chick embryos. Label was present in non-chondrogenic connective tissues, skeletal heart and smooth muscle and peripheral and central nervous system. Label was absent in most chondrocytes and in the endocardial cushions.

Discussion*Patterns of IGF peptide distribution during early connective tissue differentiation*

Insulin-like growth factor peptides can be detected in mesenchyme of the developing limb, the early primordia of the face and the spinal column. Prechondrogenic cells contain little or no IGF peptide, whereas undifferentiated and non-chondrogenic cells do label. For example, in the proximal part of the limb, unlabelled areas are present that correlate with regions of mesenchyme undergoing chondrogenesis, as shown by the onset of type II collagen synthesis (Von der Mark *et al.* 1976a,b; Dessau *et al.* 1980). In contrast at the tip of the limb, where cells are still undifferentiated, IGFs are present. In the spine and face, regions of reduced labelling with IGF peptide also develop. In both sites, it is striking that equivalent sections show a similar correlation of absence of IGF peptides and appearance of type II collagen.

Later connective tissue differentiation is associated with changes in cellular labelling for IGF peptides

Although at the initial stages of chondrogenesis (stage 24 in the limb) the differentiated cartilage cells are unlabelled, by stage 28 (approx. 1 day later) the limb chondrocytes contain IGF peptide. Since IGFs promote synthesis of cartilage matrix (see reviews, Froesch *et al.* 1985., Zapf *et al.* 1984) it may be that the peptides are involved in early growth and morphogenesis of the cartilage rudiments. The long bone of the limb at stage 36 provides a suitable structure for determining how IGF peptide distribution is correlated with later stages of chondrocyte differentiation. In the established rudiment, the rounded cells proliferate. This generates cells that then undergo a series of differentiative steps involving becoming flattened and then hypertrophic. At this late stage, IGF peptide distribution does not correlate quantitatively with matrix synthesis, as the

Fig. 1. Distribution of IGF peptide in stage 20 embryos, transverse sections at level of wing buds.

(A) Immunolabelled with 1:2000 dilution anti-IGF serum and (B) with same dilution of non-immune rabbit serum. Faint endogenous peroxidase activity is present in the myotome; otherwise, no labelling is observed in controls. Arrow on (A) indicates myotome; see Fig. 5. Bar represents 100 μ m.

Fig. 2. Distribution of IGF peptide in wing bud of stage 20 embryo; vertical longitudinal section. Mesenchyme in the proximal core region is weakly labelled; label increases distally and peripherally. Bar represents 50 μ m.

Fig. 3. Distribution of IGF peptide in wing bud of stage 24 embryo. Distribution is similar to that at stage 20. The cartilage condensation (c) does not label; label increases peripherally and distally (see Fig. 4). Peripheral label extends into the ventral body wall (arrow). Bar represents 300 μ m.

Fig. 4. High-power view of mesenchyme from limb of stage 24 embryo. (A) mesenchyme of the cartilage condensation; (B) peripheral mesenchyme. Cells of the cartilage condensation do not label, whereas those of peripheral mesenchyme are labelled. Extracellular matrix is also labelled in the peripheral region. Most cells of the ectoderm appear unlabelled. Bars represent 20 μ m.

Fig. 5. (A) Transverse section through brachial somite of stage 24 embryo. The myotome (m) is strongly labelled. Sclerotomally derived mesenchyme (s) central to myotome is weakly labelled; mesenchyme lateral to the myotome is more strongly labelled. A similar area is indicated on Fig. 1A. Bar represents 20 μ m. (B) High power view of myotome; strong label is associated with myotubes (m). Bar represents 10 μ m.

Fig. 6. High-power view of part of the notochord of stage 24 embryo. Labelled cells are present within the notochord (arrow). The sclerotomal cells surrounding the notochord are unlabelled. See Fig. 11 for lower power view of notochord. Bar represents 5 μ m.

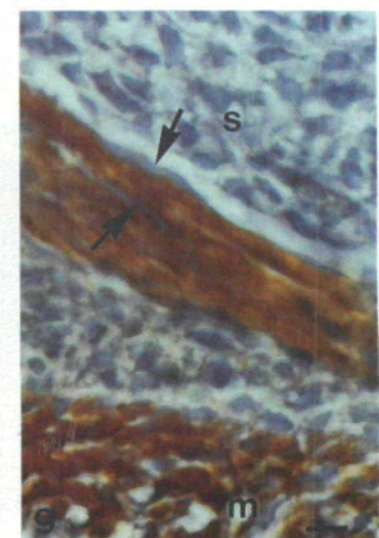
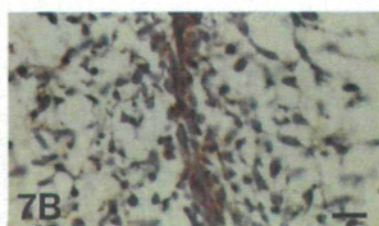
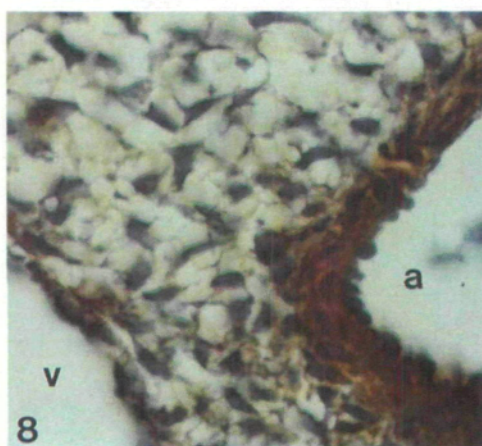
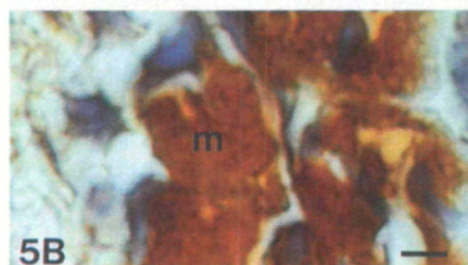
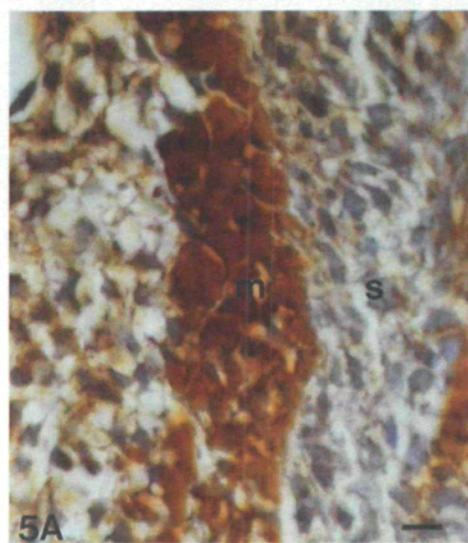
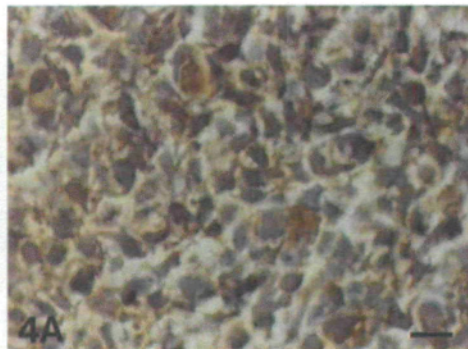
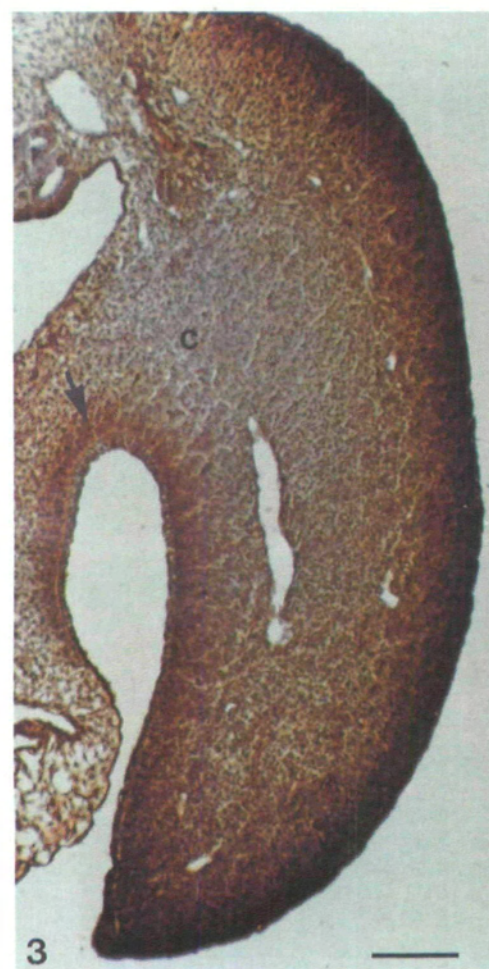
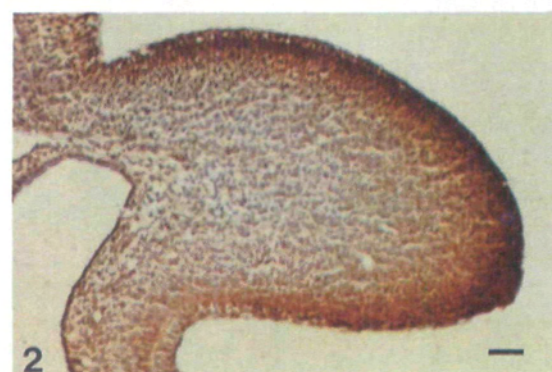
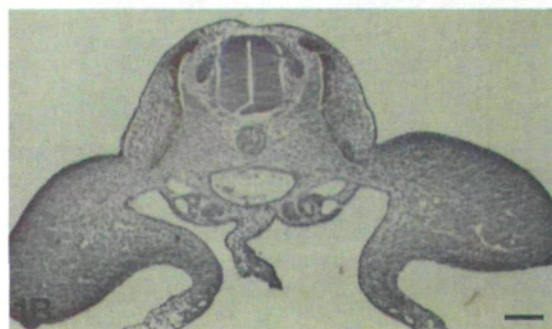
Fig. 7. (A) Heart of stage 20 embryo. The heart muscle is strongly labelled; cells of the developing endocardial cushions are unlabelled. Bar represents 200 μ m. (B) Higher power view of developing endocardial cushions: cells are unlabelled. Bar represents 20 μ m.

Fig. 8. Transverse section of part of dorsal aorta of stage 24 embryo. The developing smooth muscle layer surrounding the aorta (a) is labelled; there is no label around the posterior cardinal vein (v). Bar represents 20 μ m.

Fig. 9. Spinal nerve of stage 24 embryo in section taken at brachial level. Strong labelling is present in neurons; Schwann cells (arrows) are unlabelled. The figure also shows strongly labelled myotome (m) and unlabelled sclerotomally derived mesenchyme of the vertebral arch (s). Bar represents 20 μ m.

region of maximum synthesis of cartilage matrix is the flattened cell zone (Stocum *et al.* 1977), which does not contain IGF peptide. However, there is a correlation with qualitative changes. At the onset of hypertrophy when cells regain IGF label, an entirely new gene product, type X collagen, is synthesised (Schmidt and Linsenmeyer 1985a,b).

The cartilage model of the long bone serves as a template for bone deposition. This starts as a thin shell of bone, which is deposited in the mid-region beneath



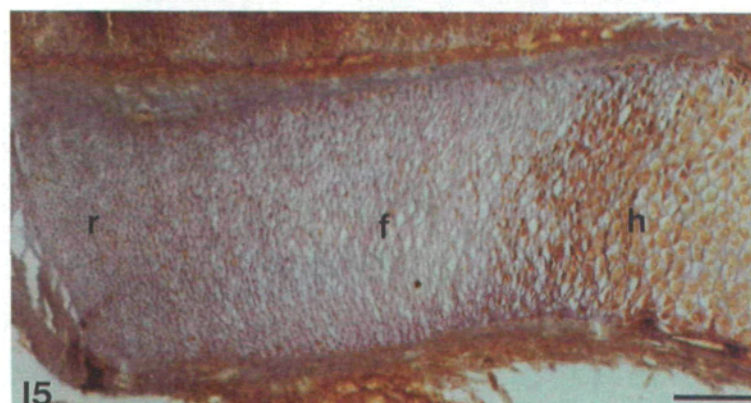
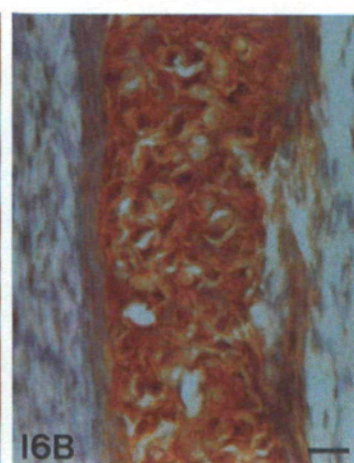
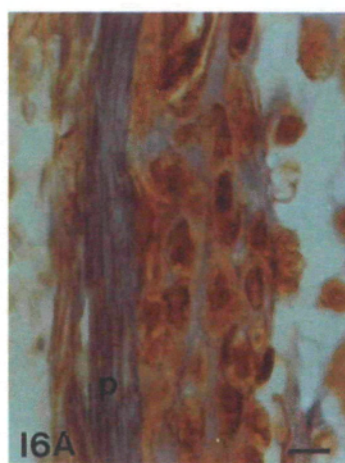
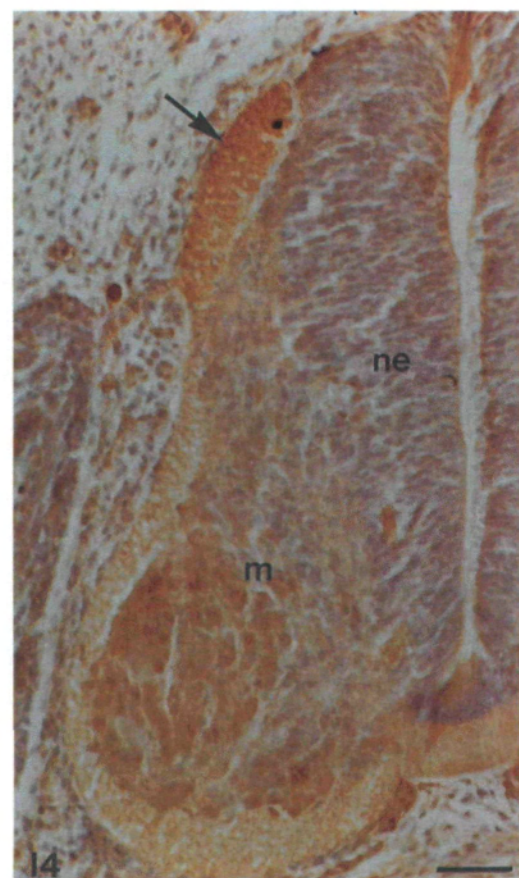
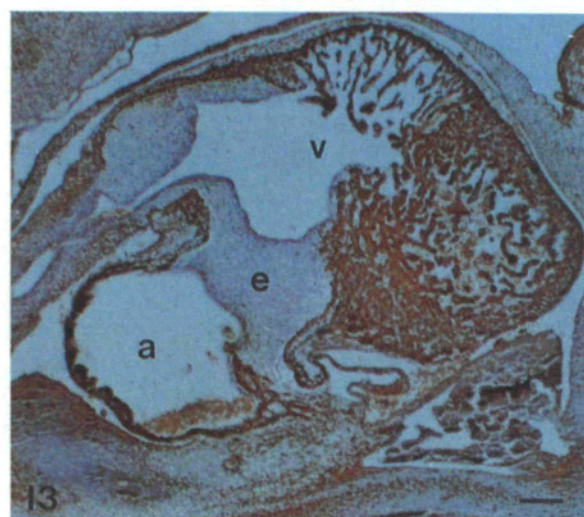
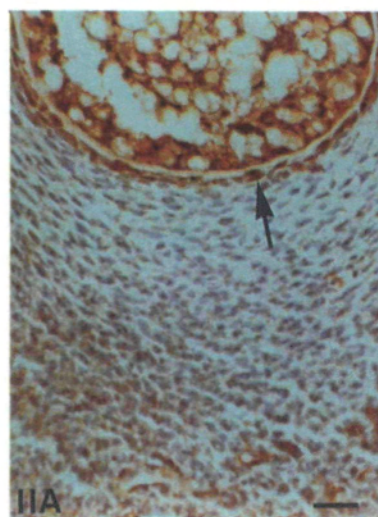
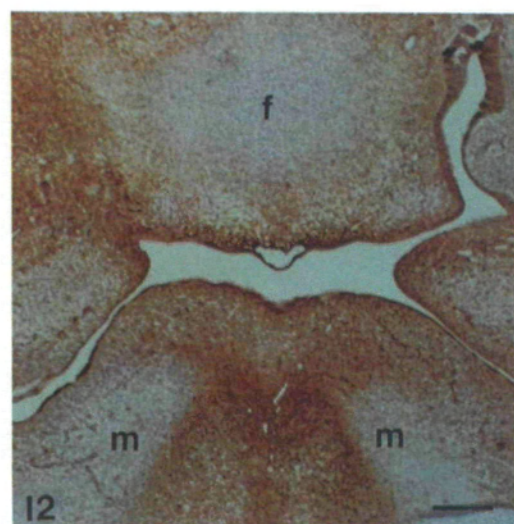
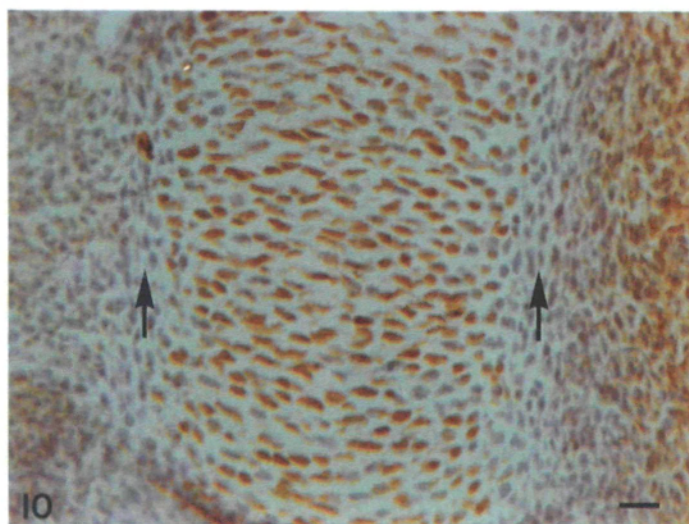


Fig. 10. Longitudinal section of the cartilage rudiment of the humerus of stage 28 embryo. Most chondrocytes are labelled; cells of the perichondrium are unlabelled (arrows). Bar represents 20 μm .

Fig. 11. (A,B) Notochord and developing vertebra of stage 28 embryo. Cells of the developing vertebra are unlabelled, except for cells immediately adjacent to the notochord sheath (arrows). (A) Bar represents 50 μm . (B) Bar represents 10 μm .

Fig. 12. Frontal section of the face of a stage 28 embryo. Regions of unlabelled mesenchyme are present in the mandible, corresponding to the developing Meckel's cartilages (m), and in the central portion of the frontonasal mass (f). Bar represents 200 μm .

Fig. 13. Heart of stage 28 embryo. The ventricular (v) and atrial (a) muscle labels strongly; the endocardial cushions (e) remain unlabelled. Bar represents 50 μm .

Fig. 14. Neural tube of stage 28 embryo. Cells of the neuroepithelium (ne) are unlabelled; label is present in the mantle zone (m), particularly ventrolaterally in association with cell bodies. The marginal zone is also labelled, especially in the developing dorsolateral tract (arrow). Bar represents 50 μm .

Fig. 15. Longitudinal section of cartilage rudiment of the humerus of stage 36 embryo. Most cells in the rounded (r) and flattened (f) cell zones are unlabelled; All cells are labelled in the hypertrophic cell zone (h). Bar represents 50 μm .

Fig. 16. Sections of bone from stage 36 embryo.

(A) Perichondrial bone from mid-diaphysis of the humerus; osteoblasts and osteocytes are strongly labelled. The periosteum (p) and bone matrix are unlabelled.

(B) Membrane bone from the upper beak; bone cells are strongly labelled. Bars represents 20 μm .

the periosteum. The bone cells arise from the periosteum. Like mature chondrocytes, osteoblasts in subperiosteal bone are strongly positive for IGF, although the periosteum itself is unlabelled. It is interesting that the region of mesenchyme from which these cells arise is the region that encloses the cartilage rudiments and is unlabelled for IGF peptides at stage 28; these regions contain type I collagen (von der Mark 1976a,b) and correspond to the position of the perichondrium. Later, at stage 36, the periosteum remains unlabelled. These observations could be analogous to early cartilage differentiation. Regions that give rise to bone cells are unlabelled for IGF; subsequent stages of differentiation regain labelling ability. In contrast to long bones, bone in the face arises in a different manner, being formed *de novo* from mesenchyme as membrane bone without the involvement of cartilage. Despite their different origin, these bone cells are also strongly labelled.

IGF peptides are present in differentiating muscle and nerve

Throughout the period studied, IGF peptides co-localise with differentiating muscle and nerve. According to Holtzer *et al.* (1957), muscle-specific myosin is first elaborated in the brachial somites at stage 15. Differentiated muscle is therefore present in the body wall at all stages investigated here, with IGF peptide being present in the myotubes. Our recent studies of myogenesis

in the face (Ralphs *et al.* 1989) have shown that at stage 24 no muscle blocks are present in the facial primordia. At this stage, no discernible pattern of IGF peptide distribution is present; mesenchyme is diffusely stained throughout. In the mandible at stage 28, muscle-specific myosins and IGF peptide are present in differentiated muscle blocks. In addition to skeletal muscle, smooth and heart muscle contained IGF peptide. Using a similar antibody recognising IGF peptides, Han *et al.* (1987a) also observed peptide in skeletal and heart muscle in human foetuses.

Peripheral nerve is strongly labelled with the antibody, in association with axons; Schwann cells were unlabelled. It may be significant that culture studies have shown that IGF promotes neurite outgrowth (Bothwell, 1982; Recio-Pinto and Ishii, 1984) and is associated with nerve regeneration (Hansson *et al.* 1986). The neural tube of stage 28 embryos shows clear demarcations in its labelling pattern. In the inner neuroepithelium, where cells are dividing, there is little IGF peptide. In contrast in the middle layer of the neural tube, the mantle zone, where neuroblasts are differentiating into nerve cells (Tapscott *et al.* 1981), IGF peptide is present. The outer layer of the neural tube, which consists mainly of axons, labels in similar manner to peripheral nerves. Since at this stage glial cells have not differentiated in the neural tube (Tapscott *et al.* 1981) the observed labelling is probably associated with axons.

Cellular localisation of IGF peptide

Immunohistochemical localisation of IGF detects sites of accumulation of the peptides. Our results show that IGF peptides could be associated with extracellular matrix as well as with cells. Fibroblast growth factor can bind to heparan sulphate proteoglycan (Gospodarowicz and Cheng, 1986; Vlodavsky *et al.* 1987). Such associations have not been reported for IGF peptides, but could be significant here; changes in extracellular matrix composition at the onset of chondrogenesis could be an explanation for the absence of IGF peptides at this stage. The observed distribution of IGF peptides is likely to represent sites of action since specific IGF receptors have been localised to numerous embryonic tissues. IGF receptors have been identified in early chick and mouse embryos (Bassas *et al.* 1987, 1988; Smith *et al.* 1987) and have been detected in early limb buds (Bhaumic and Bala, 1987; Bassas *et al.* 1988), although precise localisations have not been performed. Receptors have been identified on chondrocytes (Foley *et al.* 1982; Sessions *et al.* 1987), muscle cells (Meuli and Froesch, 1976; Poggi *et al.* 1979; Pfeifle *et al.* 1982) and in neural tissue, including whole brain, (Bassas *et al.* 1985) and in cultures of neuronal, glial and neuroblastoma cells (Balloti *et al.* 1987; Shemer *et al.* 1987; Ota *et al.* 1988).

Han *et al.* (1987a,b) have demonstrated that in human foetuses the distribution of IGF peptide does not reflect the pattern of IGF synthesis. Whilst mRNA cross-hybridising with a cDNA probe for human IGF II was detected in undifferentiated mesenchyme of the

chick embryo limb and head (Engstrom *et al.* 1987), no study of cellular distribution of IGF mRNA has been undertaken in chick. However, *in situ* hybridisation patterns for IGF II mRNA have been described in the rat embryo (Beck *et al.* 1987; Stylianopoulou *et al.* 1988). It is of interest to compare the peptide distributions in chick embryos with mRNA distribution in rodent embryos. There is some justification for this, as we find similar distributions of IGF peptides in mouse and chick embryos. Three relationships emerge between the distributions. Firstly some tissues, for example, muscle and at some stages cartilage, both synthesise and accumulate IGFs; secondly, there are tissues that synthesise and do not accumulate IGFs, for example early cartilage condensations and late rounded and flattened cell cartilage zones; and finally some tissues do not synthesise IGFs but accumulate them, for example hypertrophic cartilage and neural tissues. These relationships are consistent with the factors having both autocrine and paracrine functions, although it should be noted that discrepancies between sites of IGF synthesis and localisation may also be explained by the cellular distribution of IGF-binding protein (Hill *et al.* 1989). It would be fascinating to know the precise source of IGFs accumulated by particular tissues of a given species, but whilst this question has been addressed in relatively late human foetal tissues (Han *et al.* 1987a,b) the juxtaposition of synthesising and accumulating tissues has not been studied in early embryos.

Role of IGF peptides in development

The precise role of IGF peptides in developing embryos is unclear. Cell proliferation, differentiation and patterning are key elements in the development of organised structures. Since IGF peptides have significant effects on proliferation and differentiation of cells in culture (Zapf *et al.* 1984; Froesch *et al.* 1985), they may also be of importance in these processes during embryonic development. One possibility is that the role of the IGFs is to promote cell proliferation. In the early limb, there is some evidence for this; where cartilage condensations form, cells do not stain for IGF peptides. In these regions, the mitotic index is significantly reduced when compared to the rest of the limb (see Ede, 1983). In other tissues, and in later connective tissues, IGF peptides are associated with differentiating rather than dividing cells. Examples of this are in muscle and the neural tube. *In vitro* evidence also suggests a role in cell differentiation in these tissues, as IGF promotes differentiation of myoblasts (Ewton and Florini, 1981; Schmid *et al.* 1983) and neuronal and glial cells (Bothwell, 1982; McMorris *et al.* 1986; Recio-Pinto *et al.* 1984) in culture. In connective tissues, the position is more complex. During cartilage differentiation, IGF is undetectable at early stages of chondrogenesis, but reappears and then becomes redistributed at later stages of differentiation. *In vitro*, IGF has been shown to enhance the production of differentiated cell products by chondrocytes, bone cells and fibroblasts (Froesch *et al.* 1976; Kato *et al.* 1980; Canalis, 1985;

Schmid *et al.* 1985). However, their distribution *in vivo* would suggest a more subtle role, possibly relating to particular stages of differentiation.

There is some indication that IGFs could be involved in signalling between tissues. It has been shown that the notochord is a significant promoter of growth and differentiation of neuroblasts in the neural tube and may play a role in establishing neural pathways (Van Straaten *et al.* 1985, 1989). The presence of IGF peptide in the notochord and its accumulation in the neural tube as development proceeds suggests that IGFs could have some role in the notochord-neural tube interaction.

Comparison of the distribution of IGF peptides with other factors

In assessing the biological roles of IGFs during development it is important to compare the peptide distribution with that of other growth factors. Recently, Heine *et al.* (1987) described the distribution of transforming growth factor- β during the development of the mouse embryo. The distribution overlaps to some extent with that of IGF peptide in the chick embryo. TGF- β , like IGF, was associated with mesenchyme and its derived tissues, connective tissue, cartilage and bone. The factors were also present in the central nervous system. The temporal aspects of the two distributions differ during connective tissue differentiation; TGF- β is present in early cartilage where IGF is absent. This was the case in limb, vertebral and head development. Later, the distributions are more similar, in that TGF- β is present in hypertrophic cartilage. It is of interest that, when comparing IGF mRNA distribution in this region with TGF- β peptide distribution, Stylianopoulou *et al.* (1987) suggested that the two factors are distributed differently; however, when the two peptides are compared, they are similar. There are differences in the precise localisation within differentiated connective tissues; TGF- β appears to be associated with the matrix of cartilage and bone, whereas IGF, when present, always appears cellular. In some structures, the distributions were completely opposite. This was well illustrated in developing muscle, where myotubes label for IGF peptides, and the associated connective tissues with TGF- β . In the heart, TGF- β is localised solely to the connective tissue of the developing valves, derived from the endocardial cushions. We find that IGF peptide is absent from the whole of the endocardial cushions, but is present in the heart musculature. The distribution of IGF has similarities with that of FGF, which is also found in striated muscle fibres and is absent from endocardial cushions (Joseph-Silverstein *et al.* 1989). Clearly there must be a complex interrelationship between growth factors in developing structures.

A further possibility is that IGFs could be involved in the response of mesenchymal cells to the putative limb bud morphogen retinoic acid. Whilst we obtained no evidence of IGF distribution corresponding to the retinoic acid gradient present in limb buds (Thaller and Eichele, 1987) the distribution is strikingly similar to that of cellular retinoic acid binding protein (Maden *et al.* 1988), being present in peripheral limb mesenchyme

and absent from the cartilage condensation of the humerus. A connection between IGF and retinoid actions is not without precedent; embryonal carcinoma cells stimulated to differentiate *in vitro* produce IGF (Heath and Shi, 1986). Further investigation of the distribution of peptide growth factors in the context of their known biological effects *in vitro* are required before their roles in development can be elucidated.

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References

- ARMSTRONG, D. G., MCKAY, C. O., MORREL, D. J. AND GODDARD, C. (1989). Insulin-like growth factor I binding proteins in serum from the domestic fowl. *J. Endocrinol.* **130**, 373–8.
- BALLOTI, R., NIELSON, F. C., KOWALSKI, A., RICHARDSON, W. D., VAN OBERGHEEN, E. AND GAMMELTOFT, S. (1987). Insulin-like growth factor I in cultured rat astrocytes: expression of the gene and receptor tyrosine kinase. *EMBO J.* **6**, 3633–39.
- BASSAS, L., DEPABLO, F., LESNIAK, M. A. AND ROTH, J. (1985). Ontogeny of receptors for insulin-like peptides in chick embryo tissues: early dominance of IGF over insulin receptors in brain. *Endocrinology* **117**, 2321–9.
- BASSAS, L., DEPABLO, F., LESNIAK, M. A. AND ROTH, J. (1987). The insulin receptors of chick embryo show tissue-specific structural differences which parallel those of the insulin-like growth factor I receptors. *Endocrinology* **121**, 1468–76.
- BASSAS, L., LESNIAK, M. A., SERRANO, J., ROTH, J. AND DEPABLO, F. (1988). Developmental regulation of insulin and type I insulin-like growth factor receptors and absence of type II receptors in chicken embryo tissues. *Diabetes* **37**, 637–644.
- BECK, F., SAMANI, N. J., PENSCHOW, J. D., THORLEY, B., TREGEAR, G. W. AND COGHAN, J. P. (1987). Histochemical localisation of IGF-I and -II mRNA in the developing rat embryo. *Development* **101**, 175–184.
- BHAUMIC, B. AND BALA, R. M. (1987). Receptors for insulin-like growth factors I and II in the developing mouse limb bud. *Biochem. Biophys. Acta* **927**, 117–128.
- BOTHWELL, M. (1982). Insulin and MSA promote nerve growth factor-independent neurite formation by cultured chick dorsal root ganglionic sensory neurons. *J. Neurosci. Res.* **8**, 225–231.
- CANALIS, E. (1985). Effects of growth factors on bone cell replication and differentiation. *Clin. Orthop. Rel. Res.* **193**, 246–263.
- D'ERCOLE, A. J., APPLEWHITE, G. T. AND UNDERWOOD, L. E. (1980). Evidence that somatomedin is synthesised by multiple tissues in the fetus. *Dev. Biol.* **75**, 315–328.
- DESSAU, W. H., VON DER MARK, K., VON DER MARK, H. AND FISCHER, S. (1980). Changes in the pattern of collagens and fibronectin during limb bud chondrogenesis. *J. Embryol. exp. Morph.* **57**, 51–60.
- EDE, D. A. (1983). Cellular condensations and chondrogenesis. In *Cartilage, Volume 2: Development, Differentiation and growth* (ed. B. K. Hall) pp. 143–186. New York, London: Academic Press.
- ENGSTROM, W., BELL, K. M. AND SCHOFIELD, P. N. (1987). Expression of the insulin-like growth factor II gene in the developing chick limb. *Cell Biol. Int. Rep.* **11**, 415–421.
- EWTON, D. Z. AND FLORINI, J. R. (1981). Effects of the somatomedins and insulin on myoblast differentiation *in vitro*. *Dev. Biol.* **86**, 31–9.
- FOLEY, T. P., NISSLEY, S. P., STEVENS, R. L., KING, G. L., HASCALL, V. C., HUMBEL, R. E., SHORT, P. A. AND RECHLER, M. M. (1982). Demonstration of receptors for insulin and insulin-like growth factors on swarm rat chondrosarcoma chondrocytes. *J. Biol. Chem.* **257**, 663–9.
- FROESCH, E. R., SCHMID, C., SCHWANDER, J. AND ZAPF, J. (1985). Actions of insulin-like growth factors. *Ann. Rev. Physiol.* **47**, 443–67.
- FROESCH, E. R., ZAPF, J., AUDHYA, T. K., BEN-PORATH, E., SEGER, B. J. AND GIBSON, K. D. (1976). Nonsuppressible insulin-like activity and thyroid hormones: major pituitary dependant sulphation factors for chick embryo cartilage. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2904–8.
- GOSPODAROWICZ, D. AND CHENG, J. (1986). Heparin protects basic and acidic FGF from inactivation. *J. cell. Physiol.* **128**, 475–84.
- HAMBURGER, V. AND HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.
- HAN, V. K. M., D'ERCOLE, A. J. AND LUND, P. K. (1987b). Cellular localisation of somatomedin (insulin-like growth factor) messenger RNA in the human foetus. *Science* **236**, 193–197.
- HAN, V. K. M., HILL, D. J., STRAIN, A. J., TOWLE, A. C., LAUDER, J. M., UNDERWOOD, L. E. AND D'ERCOLE, A. J. (1987a). Identification of somatomedin/insulin-like growth factor immunoreactive cells in the human foetus. *Pediatr. Res.* **22**, 245–9.
- HANSSON, H. A., DAHLIN, L. B., DANIELSEN, N., FRYKLUND, L., NACHEMSON, A. K., POLLERYD, P., ROZELL, B., SKOTTNER, A., STEMME, S. AND LUNDBORG, G. (1986). Evidence indicating trophic importance of IGF-1 in regenerating peripheral nerves. *Acta Physiol. Scand.* **126**, 609–14.
- HEATH, J. K. AND SHI, W.-K. (1986). Developmentally regulated expression of insulin-like growth factors by differentiated murine teratocarcinomas and extraembryonic mesoderm. *J. Embryol. exp. Morph.* **95**, 193–212.
- HEINE, U. L., MUNOZ, E. F., FLANDERS, K. C., ELLINGSWORTH, L. R., LAM, H.-Y. P., THOMPSON, N. L., ROBERTS, A. B. AND SPORN, M. B. (1987). Role of transforming growth factor- β in the development of the mouse embryo. *J. Cell Biol.* **105**, 2861–2876.
- HILL, D. J., CLEMMONS, D. R., WILSON, S., HAN, V. K. M., STRAIN, A. J. AND MILNER, R. D. G. (1989). Immunological distribution of one form of insulin-like growth factor binding protein and IGF peptides in human foetal tissue. *J. molec. Endocrinol.* **2**, 31–38.
- HOLTZER, H., MARSHALL, J. M. AND FINCK, H. (1957). An analysis of myogenesis by the use of fluorescent antimyosin. *J. Biophysic. Biochem. Cytol.* **3**, 705–723.
- JOHNSON, G. D., DAVIDSON, R. S., MCNAMEE, K. C., RUSSEL, G., GOODWIN, D. AND HALBARROW, E. J. (1982). Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. *J. Immunol. Methods* **55**, 231–42.
- JOSEPH-SILVERSTEIN, J., CONSIGLI, S. A., LYSER, K. M. AND VER PAULT, C. (1989). Basic fibroblast growth factor in the chick embryo: immunolocalisation to striated muscle cells and their precursors. *J. Cell Biol.* **108**, 2459–66.
- KATO, Y., NASU, N., TAKASE, T., DAIKUHARA, Y. AND SUZUKI, F. (1980). A serum-free medium supplemented with multiplication stimulating activity (MSA) supports both proliferation and differentiation of chondrocytes in primary culture. *Expl Cell Res* **125**, 167–174.
- KIMELMAN, D. AND KIRSCHNER, M. (1987). Synergistic induction of mesoderm by FGF and TGF- β and the identification of an mRNA coding for FGF in the early amphibian embryo. *Cell* **51**, 869–77.
- MADEN, M., ONG, D. E., SUMMERBELL, D. AND CHYTIL, F. (1988). Spatial distribution of cellular protein binding to retinoic acid in the chick limb bud. *Nature* **335**, 733–5.
- McMORRIS, F. A., SMITH, T. M., DESALVO, S. AND FURLANETTO, R. W. (1986). Insulin-like growth factor I/somatomedin C: a potent inducer of oligodendrocyte development. *Proc. natn. Acad. Sci. U.S.A.* **83**, 822–6.
- MEULI, C. AND FROESCH, E. R. (1976). Binding of insulin and nonsuppressible insulin-like activity to isolated perfused heart muscle. Evidence for two separate binding sites. *Arch. Biochem. Biophys.* **177**, 31–8.
- MORRELL, D. J., DADI, H., MORE, J., TAYLOR, A. M., DABESTANI, A., BUCHANAN, C. R., HOLDER, A. T. AND PREECE, M. A. (1989). A monoclonal antibody to human Insulin-like growth factor I: characterisation, use in radioimmunoassay and effects on the biological activities of the growth factor. *J. molec. Endocrinol.* **2**, 201–206.
- OTA, A., WILSON, G. L., SPILBERG, O., PRUSS, R. AND LEROITH, D. (1988). Functional insulin-like growth factor I receptors are

- expressed by neural-derived continuous cell lines. *Endocrinology* **122**, 145–152.
- PFEIFLE, B., DITSCHUMEIT, H. H. AND DITSCHUMEIT, H. (1982). Binding and biological actions of insulin-like growth factors on human arterial smooth muscle cells. *Horm. Metab. Res.* **14**, 409–14.
- POGGI, C., LE MARCHAND-BRUSTEL, Y., ZAPP, J., FROESCH, E. R. AND FREYCHET, P. (1979). Effects and binding of insulin-like growth factor I in the isolated soleus muscle of lean and obese mice: comparison with insulin. *Endocrinol.* **105**, 723–30.
- RALPHS, J. R., DHOOT, G. K. AND TICKLE, C. (1989). Differentiation of myogenic cells in micromass cultures of cells from chick facial primordia. *Devl Biol.* **131**, 189–196.
- RECIO-PINTO, E. AND ISHII, D. N. (1984). Effects of insulin, insulin-like growth factor II and nerve growth factor on neurite outgrowth in cultured human neuroblastoma cells. *Brain Res.* **302**, 323–4.
- SCHMID, C., STEINER, T. AND FROESCH, E. R. (1983). Preferential enhancement of myoblast differentiation by insulin-like growth factors (IGF I and IGF II) in primary cultures of chicken embryonic cells. *Febs. Lett.* **161**, 117–21.
- SCHMID, C., STEINER, T. AND FROESCH, E. R. (1986). Triiodothyronine increases the responsiveness of bone cells to parathyroid hormone. *Acta Endocrinol.* **111**, 213–6.
- SCHMIDT, T. M. AND LINSSENMEYER, T. F. (1985a). Immunohistochemical localisation of short chain cartilage collagen (type X) in avian tissues. *J. Cell Biol.* **100**, 598–605.
- SCHMIDT, T. M. AND LINSSENMEYER, T. F. (1985b). Developmental acquisition of type X collagen in the embryonic chick tibiotarsus. *Devl Biol.* **107**, 373–81.
- SEED, J., OLWIN, B. B. AND HAUSCHKA, S. D. (1988). Fibroblast growth factor levels in the whole embryo and limb bud during chick development. *Devl Biol.* **128**, 50–57.
- SESSIONS, C. M., EMLER, C. A. AND SCHALCH, D. S. (1987). Interaction of insulin-like growth factor II with rat chondrocytes: receptor binding, internalisation and degradation. *Endocrinol.* **120**, 2108–16.
- SHEMER, J., RAIZADA, M. K., MASTERS, B. A., OTA, A. AND LEROITH, D. (1987). Insulin-like growth factor I receptors in neuronal and glial cells. Characterisation and Biological effects in primary culture. *J. biol. Chem.* **262**, 7693–99.
- SLACK, J. M. W., DARLINGTON, B. G., HEATH, J. K. AND GODSAVE, S. F. (1987). Mesoderm induction in early *Xenopus* embryos by heparin-binding growth factors. *Nature, Lond.* **326**, 197–200.
- SMITH, E. P., SADLER, T. W. AND D'ERCOLE, A. J. (1987). Somatomedins/insulin like growth factors, their receptors and binding proteins are present during mouse embryogenesis. *Development* **101**, 73–82.
- STOCUM, D. L., DAVIES, R. M., LEGER, M. AND CONRAD, H. E. (1977). Development of the tibiotarsus in the chick embryo: biosynthetic activities of histologically distinct regions. *J. Embryol. exp. Morph.* **54**, 155–170.
- STYLIANOPOULOU, F., EFSTRATIADIS, A., HERBERT, J. AND PINTAR, J. (1988). Pattern of the insulin-like growth factor II gene expression during rat embryogenesis. *Development* **103**, 497–506.
- TAPSCOTT, S. J., BENNET, G. S., TOYAMA, Y., KLEINBART, F. AND HOLTZER, H. (1981). Intermediate filament proteins in the developing chick spinal cord. *Devl Biol.* **86**, 40–54.
- THALLER, C. AND EICHELE, G. (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. *Nature* **327**, 625–8.
- VAN STRAATEN, H. M. W., HEKKING, J. M. W., BEURGERS, J. P. W. M., TERWINDT-ROUWENHORST, E. AND DRUKKER, J. (1989). Effect of the notochord on proliferation and differentiation in the neural tube of the chick embryo. *Development* **107**, 793–803.
- VAN STRAATEN, H. M. W., THORS, F., WIERTZ-HOESSELS, L., HEKKING, J. AND DRUKKER, J. (1985). Effect of a notochordal implant on the early morphogenesis of the neural tube and neuroblast: histometrical and histological results. *Devl Biol.* **110**, 247–254.
- VLODAVSKY, I., SULLIVAN, R., SASSE, J., FOLKMAN, J. AND KLAGSBRUN, M. (1987). Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *Proc. natn. Acad. Sci. U.S.A.* **84**, 2292–96.
- VON DER MARK, H., VON DER MARK, K. AND GAY, S. (1976a). Study of differential collagen synthesis during development of the chick embryo by immunofluorescence. *Devl Biol.* **48**, 237–249.
- VON DER MARK, K., VON DER MARK, H. AND GAY, S. (1976b). Study of differential collagen synthesis during development of the chick embryo by immunofluorescence. II: localisation of type I and type II collagen during long bone development. *Devl Biol.* **53**, 153–170.
- WEEKS, D. L. AND MELTON, D. A. (1987). A maternal mRNA localised to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF- β . *Cell* **51**, 861–867.
- ZAPP, J., SCHMID, C. AND FROESCH, E. R. (1984). Biological and immunological properties of insulin-like growth factors. *Clin. Endocrinol. Metabol.* **13**, 3–30.

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