Insulin-like growth factor axis during embryonic development*

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The insulin-like growth factor (IGF) axis has been studied extensively in the developing vertebrate embryo. Knockout experiments have demonstrated that both IGF-I and -II are required for normal development in the mouse embryo, and mRNA and protein expression patterns for both growth factors, together with those for the type I IGF receptor and the six IGF-binding proteins, have been analysed in embryos from different species. Although the unique temporal and spatial expression patterns of these genes indicates important roles for the IGF axis during organ and whole animal development, the variation and complexity of expression makes these roles difficult to unravel. However, one possible mechanism unifying the IGF system in development is programmed cell death (apoptosis), which has been shown to be important in sculpting embryonic tissues, and, in particular, the developing limb bud. In addition, the very early onset of expression of various IGF family members in chicken embryos further emphasizes the fundamental importance of this system in development. This article reviews the work that has been carried out in this area in the context of current understanding of the IGF system.

The biological actions of the insulin-like growth factor (IGF) axis are well defined, and include profound effects on the growth and differentiation of most cell types (Hwa *et al.*, 1999). The established components of the IGF system include IGFs (IGF-I and IGF-II), type I and type II IGF receptors, a family of six secreted IGF-binding proteins (IGFBPs), and IGFBP proteases (Fig. 1). IGF-I and IGF-II are very similar small peptides of approximately 7 kDa and similar to insulin in structure. IGFs are expressed ubiquitously and are important mitogens affecting cell growth and metabolism. In addition to endocrine effects exerted by circulating IGFs, locally produced IGFs exert paracrine, as well as autocrine, effects on cell proliferation (Jones and Clemmons, 1995). The IGFs interact with specific cell surface receptors, designated type I and type II IGF receptors, and can also interact with the insulin receptor. The mitogenic effects of IGFs are mediated mainly through interactions with the type I IGF receptor, which has tyrosine kinase activity. The type II IGF receptor is structurally distinct, binds primarily IGF-II, but also serves as a receptor for mannose-6-phosphate-containing ligands (Nissley and Lopaczynski, 1991). The role(s) of the type II receptor in mediating IGF action is less well defined (Kornfeld, 1992).

In biological fluids, IGFs are normally bound by

members of a family of secreted IGFBPs of approximately 30 kDa. There are, to date, six well characterized mammalian IGFBPs, designated IGFBP-1 to -6. Since the affinity constants of the IGFBPs are 2–50-fold greater for binding IGFs than that of the IGF type I receptor, they can modulate IGF action by preventing the insulin-like effects of IGFs, controlling the half-life of IGFs in blood, acting as carrier proteins for IGFs and determining the distribution of IGFs between tissues and extracellular fluids (Hwa *et al.*, 1999). The affinity of IGFBPs for IGFs is controlled by phosphorylation, glycosylation and specific proteolysis (Clemmons, 1998). In the prostate, thyroid and mammary glands, the expression of a particular binding protein, IGFBP-5, coincides with a stage during tissue remodelling known as involution, when many cells are removed by programmed cell death (Guenette and Tenniswood, 1994; Phillips *et al.*, 1994; Tonner *et al.*, 1997). It has been proposed that IGFBP-5 triggers the apoptotic process through attenuation of IGF-I, which is necessary for cell survival. This attenuation may occur by sequestration of IGFs bound to IGFBP-5 in the extracellular matrix (ECM).

There is increasing evidence that IGFBPs not only regulate IGF bioavailability, but that they also have their own receptors that mediate IGF-independent actions. Cell surface receptors for IGFBP-1 (Jones *et al.*, 1993), IGFBP-2 (Rauschnabel *et al.*, 1999), IGFBP-3 (Oh *et al.*, 1993a) and IGFBP-5 (Andress, 1995, 1998) have been described, although, to date, none of these proteins have been cloned. Direct IGF-independent effects of IGFBPs include modulation of bone cell proliferation (Mohan *et al.*, 1995) and growth arrest of breast and prostate cancer cells (Oh *et*

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*This article is based on a presentation given at the British Society of Animal Science symposium 'Early Regulation of Mammalian Development' held in Aberdeen in September 2000.

Fig. 1. Components of the insulin-like growth factor (IGF) axis. IGF-binding protein 3 (IGFBP-3) is the main circulating binding protein and is transported in serum complexed with a glycoprotein. The binding protein and glycoprotein together make up a 150 kDa complex. IGFBPs are secreted proteins and all six have been shown to have autocrine effects in a variety of different cell types. Cell surface receptors have been identified for IGFBP-1, -2, -3 and -5, and several have been shown to bind to the extracellular matrix.

al., 1993a,b, 1995; Rajah *et al.*, 1997). However, owing to the diverse range of properties imparted by the IGFs and IGFBPs, their role during organ and whole animal development has been difficult to unravel.

The IGF axis in development

The mRNA and protein expression patterns of the various components of the IGF axis have been studied extensively in the developing embryo using *in situ* hybridization techniques and immunohistochemistry.

Mammalian development

For many years it was considered that there was a change from IGF-II to IGF-I during fetal to adult growth, as IGF-II is expressed more widely in the embryo (Bhaumick and Bala,

1987). The IGF-II gene is also a member of a small family of genes that have been shown to be subject to genomic imprinting (Reik *et al.*, 1996; Moore *et al.*, 1997). An imprinted gene is expressed primarily from one specific parental allele and such genes have been shown to exert important effects, primarily on fetal development. The use of homologous recombination technology proved conclusively that IGF-II was required for normal embryonic development, as IGF-II null mice (requiring only the disruption of the paternal allele due to parental imprinting) were 60% smaller than their wild-type littermates (DeChiara *et al.*, 1990, 1991; Fig. 2). These growth-deficient animals were otherwise apparently normal and fertile, demonstrating that IGF-II protein is not essential for development and survival. The phenotype of the null mutant mice was surprising and difficult to reconcile with the fact that IGF-II had been shown to have a specific expression pattern in

Fig. 2. Insulin-like growth factor I (IGF-I) and IGF-II knockouts cause growth deficiency during embryonic development in mice. (a) Chimaeric mice containing a targeted disruption of one IGF-II allele (DeChiara *et al.*, 1990, 1991). Germline transmission of the inactivated gene from male chimaeras yields heterozygous mutants ($wt/-$, grey mouse) that are 60% the size of wild-type (wt , white mouse) littermates. In contrast, when the disrupted gene is transmitted maternally, the heterozygous offspring are phenotypically normal, indicating that the maternal IGF-II allele is silenced by genetic imprinting. (b) Results of crossing chimaeras containing a disrupted IGF-I allele (Powell-Braxton *et al.*, 1993). Heterozygous offspring (grey mouse) are 10–20% smaller than wild-type littermates (white mouse), whereas homozygous mutants (black mouse) are < 60% the bodyweight of wild-type mice. In addition, > 95% of the homozygous mutant pups die perinatally (red cross).

many different tissues throughout the embryo (Wood *et al.*, 1990; Pintar *et al.*, 1991; Streck *et al.*, 1992). However, later experiments showed that mice with both copies of the IGF-I gene knocked out were not only reduced in size to a similar extent, but also displayed severe muscle dystrophy and most (> 95%) of these mice died at birth (Powell-Braxton *et al.*, 1993; Fig. 2). These findings clearly demonstrated that IGF-I is essential for correct embryonic development in mice. The reduction in size of both types of IGF knockout mice indicates a role for IGFs as cell survival factors. In support of this contention, studies *in vitro* with cells derived from null mutants for IGF-II (Lamm and Christofori, 1998) and the type I IGF receptor (Cui *et al.*, 1997) demonstrated an increased number of apoptotic cells, whereas animals transgenic for IGF-I (Neuenschwander *et al.*, 1996; Leri *et al.*, 1999), IGF-II (Petrik *et al.*, 1999) and the type I IGF receptor (Steller *et al.*, 1996) demonstrated a reduced number of apoptotic cells.

The expression of IGFBPs during embryogenesis has been the subject of investigation in several laboratories. Earlier studies were confined to the mRNA expression patterns of IGFBP-2 in rat embryos and how these compared with the

expression of IGF-II (Orlowski *et al.*, 1990; Wood *et al.*, 1990). These studies showed that during the mid-gestational period of rat development, IGF-II and IGFBP-2 expression patterns often overlapped. In general, it appeared that IGFBP-2 expression was either in populations of rapidly dividing cells or in regions that direct the growth and differentiation of neighbouring cells and tissues, indicating that IGFBP-2 may have important roles during development of numerous fetal tissues either by modulating IGF action or by acting independently of the IGFs. However, interaction between IGFBP-2 and IGF-II does not appear to be an essential method for directing autocrine or paracrine growth since the complete genetic ablation of IGF-II (DeChiara *et al.*, 1990, 1991) did not lead to severe deficits near tissues expressing IGFBP-2.

Subsequently, the mRNA expression patterns of the other five IGFBPs were established (Cerro *et al.*, 1993; Schuller *et al.*, 1993; Green *et al.*, 1994): for example, the expression of IGFBP-5 in a rat embryo at day 11.5 is shown (Fig. 3). All of the IGFBPs are expressed at least as early as day 14 in rat embryos, and each IGFBP has a unique pattern of expression, indicating that the members of this family have

Fig. 3. Whole mount *in situ* hybridization of an 11.5 day rat embryo showing the expression pattern of insulin-like growth factor binding protein 5 (IGFBP-5) mRNA. Dark blue colour corresponds to sites of message expression, and the arrow indicates the expression of IGFBP-5 in the apical ectodermal ridge of the developing forelimb. The full-length cDNA for rat IGFBP-5, (964 bp), in pGEM®-7zf (Promega), was kindly provided by S. Guenette, John Wayne Cancer Institute, Los Angeles, CA. Conditions for *in situ* hybridization and processing of embryos were according to Nieto *et al.* (1996).

distinct and tightly regulated functions in the development of specific tissues. However, IGFBP-2 and -5 display complementary expression patterns that often overlap or occur in adjacent cell populations (Cerro *et al.*, 1993; Schuller *et al.*, 1993; Green *et al.*, 1994). This finding may be indicative of complementary developmental roles for these two binding proteins in specific tissues. Recent immunohistochemical studies using antisera raised against IGFBP-1 to -6 enabled the comparison between IGFBP protein and mRNA localization in mouse embryos (van Kleffens *et al.*, 1999) and demonstrated that differences between mRNA and protein localization were extensive for IGFBP-3, -5 and -6, indicating that these IGFBPs are secreted and transported. In contrast, there was strong overlap of mRNA and peptide for IGFBP-2 and -4, which may indicate an autocrine or paracrine function for these binding proteins (IGFBP-1 mRNA and protein were confined to the liver). In addition, there is significant overlap of IGF axis mRNA expression patterns and regions of apoptotic activity, as measured by Nile blue staining, in both the head-neck

region and limb bud (see below) of mouse embryos (van Kleffens *et al.*, 1998). This finding is consistent with the function of IGFs as potent cell survival signals and of IGFBPs as regulators of this function.

Avian development

In later stages of development in chicken embryos, IGF peptides have been localized immunohistochemically using a polyclonal antibody to human IGF-I, which recognizes both IGF-I and -II (Ralphs *et al.*, 1990). Specific spatiotemporal patterns of expression are similar to those observed in mouse embryos. The cDNA for chicken IGF type I receptor has been cloned and used to generate an anti-sense probe for *in situ* hybridization, which demonstrated considerable overlap of receptor mRNA expression and the location of the IGF peptides (Holzenberger *et al.*, 1996). In addition, the cDNAs for both chicken IGFBP-5 (Allander *et al.*, 1997) and IGFBP-2 (Schoen *et al.*, 1995a) have also been cloned. *In situ* hybridization experiments using the chicken IGFBP-2 cDNA as an antisense probe have outlined the expression of IGFBP-2 mRNA in the embryonic eye, brain, branchial arches, somites and limb buds of chicken embryos 3.5 days after mating (Schoen *et al.*, 1995b). The expression patterns are similar to those observed for IGFBP-2 in mouse and rat embryos, indicating that this IGFBP is likely to perform similar functions in both avian and mammalian development.

Linkage of IGFBP and Hox genes

Each of the human IGFBP genes are localized to the same chromosomal regions as a specific homeobox (*Hox*) gene family (Allander *et al.*, 1995); for example, IGFBP-2 and -5 are linked to the *Hox D* gene family on human chromosome 2 in opposite transcriptional orientations. The most likely mechanism to explain this linkage is that IGFBP and *Hox* genes have co-evolved from a single ancestral genetic locus to multiple loci via the process of chromosomal duplication and translocation (Fig. 4). Whether this tight linkage between the two gene families has any functional significance is unknown, but it is interesting to note that vertebrate *Hox* genes are well known for their important functions during embryonic development (Krumlauf, 1994).

Limb bud development

The developing limb bud is an attractive model for studying tissue morphogenesis since a number of key developmental processes are understood at both the cellular and molecular level (Tickle, 1996; Cohn and Bright, 2000; outlined in Fig. 5). In vertebrates, limbs develop from paired buds that appear at appropriate points along the main antero-posterior body axis. At early stages, these buds consist of undifferentiated mesenchyme cells encased in ectoderm. At first, limb buds are small mounds (Figs 3 and 5), but these soon elongate. As the buds continue to grow out, they

Fig. 4. Co-evolution of the insulin-like growth factor (IGFBP) and Hox gene families. Numbers refer to human chromosomes.

become broader at the tip as digits begin to develop. Later, cells between the developing digits undergo apoptosis. A prominent feature of limb bud development is the reciprocal signalling between the apical ectodermal ridge (AER), which is a region of specialized pseudostratified epithelium covering the distal edge of the developing limb bud, and the underlying undifferentiated mesenchymal cell population, known as the progress zone. The AER is essential for outgrowth of the limb during development, when the mesodermal cells in the progress zone divide more rapidly. Experiments *in vitro* have confirmed that the AER has a mitogenic effect on limb mesoderm (Tickle, 1996). The polarizing region is an additional signalling region at the posterior margin of the limb mesenchyme, which controls the antero-posterior patterning of the limb bud (Cohn and Bright, 2000). Many experiments have addressed the role of the IGF system during limb development, and these are discussed below.

Expression of the IGF axis in the mammalian embryonic limb bud

The location of IGF-I and -II expression in the developing limb bud appears to be species dependent. For example, early in rat limb development, IGF-I and -II are expressed throughout the limb mesoderm, but later their expression becomes confined to the proximal part of the bud (Streck *et al.*, 1992). These results indicate that, at least during the early stages of rat limb outgrowth, IGF peptides are available in the region of rapidly proliferating undifferentiated mesoderm

at the distal tip of the limb, owing to production within or near that region. It is possible that locally produced IGF peptides are still present in this region during later stages as well, depending on the half-life of the proteins. However, only a proximal location of IGF mRNA in mouse limb buds has been detected (van Kleffens *et al.*, 1998) and an immunohistochemical study has shown that this location coincides with that of IGF peptides (van Kleffens *et al.*, 1999). This discrepancy between rat and mouse limb buds may reflect differences in embryonic stages or the techniques used for detection, and it is possible that, at some point, IGFs are also expressed in the mouse progress zone. In support of this contention, growth of undifferentiated mouse limb bud mesodermal cells has been shown to be stimulated by both IGF-I and IGF-II *in vitro* (Kaplowitz *et al.*, 1982).

The mouse and rat limb buds provide the most obvious example of the overlap of IGFBP-2 and -5 mRNA expression, with both binding proteins displaying strong expression along the whole length of the AER and weaker expression in a proximal region of the bud (Pintar *et al.*, 1991; Streck *et al.*, 1992; Wood *et al.*, 1992; Allan *et al.*, 1998; van Kleffens *et al.*, 1998; Fig. 3). In fact, IGFBP-2 and -5 are the only two of the six binding proteins expressed in the AER (van Kleffens *et al.*, 1998), and their expression at this site is consistent with them having a role in the directional outgrowth of limb mesenchyme. In addition to IGFBP-2 and -5, several members of the fibroblast growth factor (FGF) family, including FGF-2, -4 and -8 are expressed by the AER (Tickle, 1996). Other factors known to be involved in the apical-subridge mesoderm interaction are the TGF β family

Fig. 5. Development of forelimbs of mouse embryos at 11.5 and 16 days. The signalling regions in the 11.5 day bud are depicted in the expanded segment (circled with dotted line), including the apical ectodermal ridge (AER) and a region of mesenchymal cells responsible for antero–posterior patterning, known as the polarizing region. These signals operate in the undifferentiated mesenchyme immediately below the AER, known as the progress zone. Three main axes are indicated in the centre: pr–d, proximo–distal; do–v, dorso–ventral; a–p, antero–posterior.

members known as bone morphogenetic proteins (BMP)-2, -4 and -7, the homeobox containing transcription factors *Msx*-1 and -2, as well as some members of the retinoic acid family.

After removal of the AER by apoptosis, the morphology of the digits has to be established. Apoptosis is an important mechanism in sculpting limb morphology and is responsible for removing inter-digital tissue (Milaire, 1992). In addition, programmed cell death may play a role in the positioning of muscle precursors in the limb (Amthor *et al.*, 1998). The BMPs play a major role in the specification of digital and interdigital regions of the limb and may act as apoptotic signals in the presence of *Msx* and growth factors (Ganan *et al.*, 1996). During this later stage in mouse limb bud development (13.5 days after mating), it was found that most of the IGF system co-localized with areas of apoptosis; IGFBP-2, -4 and -5 were found in the interdigital zone, whereas IGFBP-3 and IGF-I bordered this region (Allan *et al.*, 1998; van Kleffens *et al.*, 1998; Fig. 6 shows expression of IGFBP-5 in the interdigital zone of a mouse forelimb bud 13.5 days after mating). In addition to the limb bud, the IGF system co-localizes to apoptotic regions in the head–neck region of mouse embryos (van Kleffens *et al.*, 1999).

Fig. 6. Whole mount *in situ* hybridization of mouse embryo forelimb at 13.5 days showing the expression of insulin-like growth factor 5 (IGFBP-5) mRNA (dark blue) in the interdigital zones.

Expression of the IGF axis in the avian wing bud

Most of the cellular interactions involved in limb morphogenesis are believed to be common for all vertebrates. The later development of the chicken bud is analogous to that of the rat limb bud, in that IGF-I and its receptor are expressed below the AER (Geduspan *et al.*, 1992; Dealy and Kosher, 1995). Although IGF-I does not appear to be expressed in the chicken AER itself, IGF peptides have been detected in the AER by immunohistochemistry (Ralphs *et al.*, 1990), indicating that IGF-I expressed in the subridge mesoderm may be transmitted to the AER. As described above, the mouse and rat AER are sites of strong overlapping expression of both IGFBP-2 and -5.

Owing to the accessibility of the chicken embryo in the egg, it is feasible to carry out limb bud experiments that involve physical and molecular manipulation. Experiments of this kind have shed further light on the potential role of the IGF axis in limb development. Several lines of evidence point to an IGF-I autocrine regulatory loop in AER–subridge mesoderm interaction. First, both IGF-I and insulin are able to induce a thickened ridge-like structure in the distal anterior ectoderm, raising the possibility that IGF-I or insulin may be involved in the initial induction of AER formation in the limb bud (Dealy and Kosher, 1995). Second, exogenous IGF-I and insulin can promote the outgrowth of limb mesoderm in the absence of the AER (Dealy and Kosher, 1995). In addition, IGF-I expression in the subridge mesoderm is dependent on FGF expression in the AER, and the ability of these FGFs to

promote the outgrowth and proliferation of limb mesoderm is dependent on IGF-I activity (Dealy *et al.*, 1996). Other lines of evidence link the IGF axis to genes expressed in the AER. First, IGF-I appears to regulate the expression of the homeobox containing transcription factor *Msx-2* in the AER (Dealy and Kosher, 1996). Second, studies in bone cell cultures have demonstrated that BMP-2 can enhance IGF-I and -II synthesis and inhibit IGFBP-5 synthesis (Gabbitis and Canalis, 1995).

Alteration of IGF axis expression in Hypodactyl limb buds

Study of abnormal limb development in mutants has proved very valuable, as it has shed light on the patterning and differentiation processes in limb morphogenesis. During limb development, programmed cell death is essential for the modelling and fine tuning of limb shape (Milaire, 1992), and it is interesting that an alteration in the shape of limb buds is often correlated with changes in the pattern of cell death. The semi-dominant lethal mouse mutant *Hypodactyl* (*Hd*), which is caused by a deletion in the *Hoxa13* on chromosome 6, develops pointed limb buds that are unlike the paddle shape common to *wild-type* mice (Hummel, 1970; Mortlock *et al.*, 1996). The *Hoxa13* mutation may be a gain of function, as it actually leads to the expression of a mutant HOXA13 protein (Post *et al.*, 2000). Homozygous (*Hd/Hd*) pups have only a single 'digit' on both forelimbs and hindlimbs, and usually die *in utero*. While limb initiation in *Hd* embryos appears to be normal, the AER is more pronounced, persists for longer and does not appear to be closely associated with the underlying mesenchyme (Robertson *et al.*, 1996). This abnormal AER may result in a change in reciprocal signalling between ridge and mesenchyme, which could account for the massive increase in cell death in the progress zone observed in *Hypodactyl* (Robertson *et al.*, 1996). The concomitant reduction of both anterior and posterior mesenchyme in the distal part of the bud results in the distinctive pointed *Hd* limb bud. The expression patterns of some of the important limb bud signalling molecules, including sonic hedgehog (*Shh*), FGF-4, *Hoxd13* and *Hoxd11*, have been analysed in *Hd* limb buds (Robertson *et al.*, 1997), and it was found that the pattern of transcripts for genes in the mutant did not differ markedly.

We were interested in what other genes might be operating downstream of *Hoxa13* that could lead to the phenotype observed in *Hypodactyl*. As described above, an increase in cell death is observed in *Hd* limb buds, indicating that factors involved in apoptosis during limb development may play a part in the manifestation of the *Hd* phenotype. The results of our studies using both *in situ* hybridization and bead implantation, and the detailed study of van Kleffens *et al.* (1998), indicate strongly that members of the IGF system are involved in the regulation of apoptosis during development of the limb bud. For this reason, we decided to determine whether there was any alteration in the expression patterns of

IGF-I, IGFBP-2 and -5 in heterozygous and homozygous *Hd* embryos using *in situ* hybridization. IGFBP-2 and -5 expression patterns have been found to be altered in the AER in *Hd* limb buds, and this is the first example of ridge markers that are affected in this mutant (Allan *et al.*, 2000). IGFBP-2 expression is completely downregulated in the AER as early as 11.5 days after mating, whereas the expression of the gene at more proximal sites was not affected. This would imply that IGFBP-2 is only downregulated in regions where there was mutated *Hoxa13* gene transcription. In contrast, IGFBP-5 was only downregulated in specific regions of the AER in *Hd* mutants. This alteration in expression indicates that IGFBP-2 and -5 are involved in the manifestation of the mutant phenotype displayed in *Hd* limb buds, and adds support to the contention that members of the IGF axis are involved in AER-subridge mesoderm interactions and programmed cell death during limb bud development. In addition, these findings indicate that IGFBP-2 and -5 lie somewhere downstream of *Hoxa13* in a signalling pathway. This conclusion is particularly intriguing when the genetic linkage between IGFBP and *Hox* genes is considered.

The IGF axis in early embryonic development

The chicken embryo model has the advantage of being accessible from the period of gastrulation through neurulation and organogenesis until hatching. Studies on the expression pattern of the IGF system in early chicken development have been confined to ligand-binding analysis of the IGF type I receptor in chick embryos during gastrulation and neurulation (Bassas *et al.*, 1985; Girbau *et al.*, 1989). An alternative explanation for these expression patterns of the labelled IGFs is that the probe is bound by IGFBPs that are expressed early in development, as the binding proteins have a much greater affinity than the receptor for IGFs (Hwa *et al.*, 1999). However, confirmation that the IGF type I receptor is also expressed at these developmental stages is provided by the findings that IGF-I-dependent tyrosine kinase activity was present in membrane preparations from whole day 2 embryos (Girbau *et al.*, 1989) and that receptor mRNA was detected by polymerase chain reaction (PCR) analysis in chicken blastoderms at day 0 (Scavo *et al.*, 1991). In addition, PCR analysis has demonstrated that IGF-I is expressed in chicken embryos during organogenesis (Serrano *et al.*, 1990). Expression of the IGF type I receptor in chicken blastoderm indicates a potential role for IGF signalling before neurulation or even gastrulation. Since the chicken embryo possesses neither blood circulation nor a liver at these early stages, conceptually, any effects of IGF-I mediated by its receptor are autocrine-paracrine effects.

Conclusions

Members of the IGF axis have unique temporal and spatial mRNA and protein expression patterns in embryos from different vertebrate species, indicating that these genes have distinct and tightly regulated functions in the development of specific tissues. These expression patterns often co-

localize with known areas of apoptosis, with the strongest evidence relating to the programmed cell death that occurs in the developing limb bud and, in particular, during the removal of interdigital tissue. These observations are consistent with the function of IGFs as potent cell survival signals and IGFBPs as regulators of this function. Further support for this hypothesis comes from recent work with the mouse mutant *Hypodactyly* (*Hd*), in which homozygous mutants displayed a large increase in cell death in the limb buds. Loss of expression of both IGFBP-2 and -5 has been observed in the AER of the mutant buds, indicating the involvement of these binding proteins in the manifestation of the mutant phenotype, possibly through their potential to influence apoptosis. Finally, several components of the IGF system are expressed at very early stages of in the chicken embryo, underlining the importance of this gene family in development.

The authors thank Derek Cooney for help with the preparation of this manuscript. This work was partly funded by the Scottish Executive Rural Affairs Department. K. Patel wishes to acknowledge the generous funding provided by the Wellcome Trust (Grant 046379).

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