CHAPTER

1

Development of vertebrate limbs: insights into pattern, evolution and dysmorphogenesis

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CONTENTS

Abstract

The vertebrate limb is a powerful model system for studying the cellular and molecular interactions that determine morphological pattern during embryonic development. Recent advances in our understanding of these interactions have shed new light on the molecular mechanisms of vertebrate limb development, evolution and congenital malformations. The transfer of information has, until recently, been largely one way, with developmental studies informing our understanding of the fossil record and clinical limb anomalies; however, evolutionary and clinical studies are now beginning to shed light onto one another and onto basic developmental processes. This chapter discusses recent

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advances in these fields and how they are interacting to improve our understanding of vertebrate limb biology.

INTRODUCTION

Paired limbs are one of the defining features of jawed vertebrates. Important morphological differences distinguish forelimbs and hindlimbs, although the basic skeletal pattern is shared, with a single proximal long bone (humerus in forelimb and femur in hindlimb)

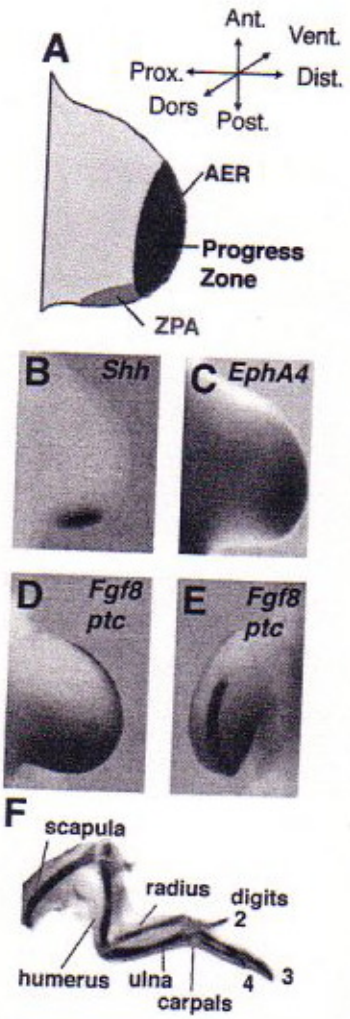

Figure 1 Signalling regions and gene expression patterns in the chick wing bud. (A) Schematic diagram of a stage 21 chick wing bud in lateral view, with axes indicated above. Major signalling regions are the apical ectodermal ridge (AER), the progress zone and the zone of polarising activity (ZPA). (B–E) Gene expression patterns as revealed by whole mount *in situ* hybridisation. (B) Expression of *Sonic hedgehog* in ZPA, (C) Expression of *EphA4* (formerly *Cek8*) in progress zone. (D and E) Double *in situ* hybridisation showing expression of *Fgf8* in apical ridge and *ptc* in posterior mesenchyme. (F) Whole mount skeletal preparation of 10-day chick wing, stained and cleared to show cartilage pattern.

articulating distally with a pair of long bones (ulna and radius in forelimb and tibia and fibula in hindlimb), followed by a series of carpals (forelimb) or tarsals (hindlimb) and digits (Fig. 1F). This complex, three-dimensional pattern of structures is polarised along three main axes; the proximodistal (shoulder to fingertips), anteroposterior (thumb to small finger) and dorsoventral (dorsum to palmer) (Fig. 1A). During embryonic development, the first visible sign of limbs is the appearance of paired buds from lateral plate mesoderm. These buds consist of undifferentiated mesenchyme cells covered by an ectodermal jacket. A spectacular process transforms this homogeneous population of cells into the highly ordered series of structures that makes up the mature limb. The cellular basis of this process has been the focus of experimental investigations for most of the twentieth century (reviewed in Harrison, 1969; Hinchliffe & Johnson, 1980), and the major signalling regions that specify pattern in the early limb bud have been identified. The molecular basis of these interactions has been the focus of considerable research, and specific genes have been linked to these cellular interactions. Molecular control of earlier events in limb development, such as specification of limb position and identity, and initiation of limb budding, has been a major area of investigation over the past few years, and a detailed understanding of the molecular genetics of limb development is becoming a reality.

SPECIFICATION AND INITIATION OF LIMBS

Paired limbs (and fins) are specified in lateral plate mesoderm at particular levels along the main body axis of jawed vertebrates. The lateral plate mesoderm is subdivided into splanchnic and somatic components, with the former giving rise to smooth muscle of the gut and the latter giving rise to forelimbs, hindlimbs and intervening flank regions (Fig. 2). Why limb budding is initiated at only two positions within lateral plate mesoderm of all tetrapods is a major unresolved question, although a hypothesis linking this evolutionarily conserved process to regionalisation of the gut has recently been proposed (see Coates & Cohn, 1998).

The molecular basis of limb initiation has come into focus within the past four years. In 1995, we reported that carrier beads loaded with fibroblast growth factor (FGF) and applied to the flank (or interlimb region) of chick embryos can induce development of complete additional limbs (Fig. 2A, E; Cohn *et al.*, 1995). This discovery, together with similar findings by Ohuchi *et al.* indicated that FGF alone is sufficient to activate the genetic pathway required for limb development (Cohn *et al.*, 1995; Ohuchi *et al.*, 1995). Subsequent work showed that *Fgf10* and *Fgf8* are expressed in lateral plate and intermediate mesoderm, respectively, prior to the onset of limb budding, and that *Fgf8* is later expressed in limb ectoderm as budding is initiated (Crossley *et al.*, 1996; Vogel *et al.*, 1996; Ohuchi *et al.*, 1997). *Fgf8* and *Fgf10* appear to interact, perhaps through *Fgf* receptor 2 (FGFR2), during limb bud initiation (Ohuchi *et al.*, 1997; Xu *et al.*, 1998). The earliest visible sign of ectopic limb formation after FGF application is a thickening in the flank, which is the result of increased cell number (Fig. 2B). The flank cells give rise to a limb bud (Fig. 2C), and an apical ridge subsequently forms in the ectoderm overlying the ectopic bud (Fig. 2D). The bud has its own signalling regions and develops autonomously to form a complete limb (Fig. 2E). Very brief exposure of flank cells to FGF (as little as 1 hour) is sufficient to activate the limb development cascade, suggesting that FGF may function as a master switch in limb induction (Cohn *et al.*, 1995; MJC and A. Isaac, unpublished). Although during normal development, flank cells do not contribute to limbs, FGF can respecify the same population

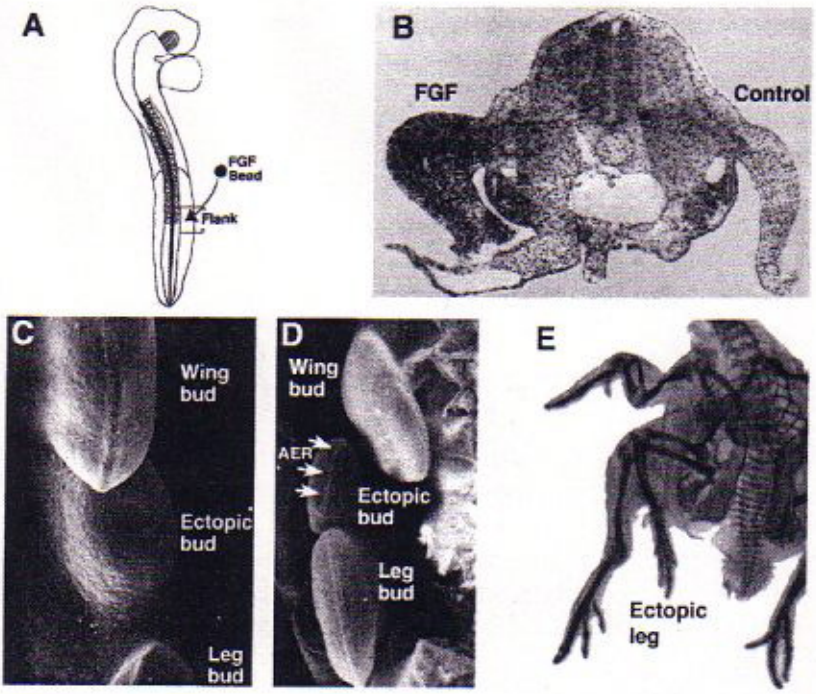

Figure 2 FGF induction of additional limb from the flank of chick embryo. (A) FGF-loaded bead is implanted in the prospective flank region on the right side of the embryo prior to limb budding, at stage 14. (B) Transverse section through embryo 24 hours after FGF application. A dramatic increase in cell number is observed on the FGF-treated side of the embryo compared with the untreated contralateral side. (C) Scanning electron micrograph of flank 36 h after FGF application. A discrete ectopic limb bud is visible between the wing and leg buds. The apical ridge is not yet visible. (D) At 48 h after FGF application the bud is well developed and capped by an apical ectodermal ridge (AER, arrows). (E) Complete ectopic leg at ten days of development, stained with alcian green and cleared.

of flank cells to give rise to forelimb or hindlimb, according to the anteroposterior position at which FGF is applied (Cohn *et al.*, 1997). Application of FGF to the anterior region of the flank respecifies flank cells to form a forelimb, and application to posterior flank respecifies them to form a hindlimb. Members of *Hox* paralog group 9, *Hoxb9*, *Hoxc9* and *Hoxd9*, are expressed in lateral plate mesoderm in regionally specific patterns related to limb specification and budding. For example, the anterior expression boundaries of *Hoxb9*, *Hoxc9* and *Hoxd9* overlap at the level of the forelimb, and the anterior limit of the hindlimb is positioned at the posterior boundary between high and low levels of *Hoxb9* expression (Cohn *et al.*, 1997). FGF-induced respecification of flank cells towards limb identity shifts the boundaries of *Hox9* expression in lateral plate mesoderm to reproduce a forelimb or hindlimb pattern of Hox expression in the flank (Cohn *et al.*, 1997). Direct evidence for the role of *Hox* genes in determining limb position comes from a loss of function mutation in the *Hoxb5* gene, which results in an anterior shift in the position of the forelimb (Rancourt *et al.*, 1995). Thus, it appears that specific combinations of *Hox* gene expression are involved in determining whether forelimbs, flank or hindlimbs develop at specific axial positions. It is noteworthy, however, that functional inactivation of *Hoxa9*, *Hoxb9* or *Hoxc9* individually results in axial skeletal defects, but limbs appear to develop

normally (Suemori *et al.*, 1995; Fromental-Ramain *et al.*, 1996; Chen & Capecchi, 1997) . In contrast, *Hoxd9* mutants have forelimb malformations, and these defects are more severe when both *Hoxa9* and *Hoxd9* are inactivated in the same animal (Fromental-Ramain *et al.*, 1996). This suggests that *Hoxd9* may compensate for *Hoxa9*, but *Hoxa9* cannot fully compensate for *Hoxd9*. Double mutants from *Hoxa9* and *Hoxb9* also have axial but not limb defects (Chen & Capecchi, 1997). If *Hox9* genes interact to position the limbs, then compound mutants should reveal this, and therefore it will be interesting to see whether loss of the full complement of *Hox9* genes has an effect on limb position. This approach may be complicated by the ability of *Hox* genes to interact with orthologous as well as paralogous genes, as several studies have demonstrated that inactivation of a single *Hox* gene can alter other *Hox* expression patterns (Suemori *et al.*, 1995; Fromental-Ramain *et al.*, 1996; Chen & Capecchi, 1997).

A key question arising from this work is whether *Hox* genes act as transcriptional activators of FGFs during limb specification. An interesting clue has come from work on melanoma cell lines, which has shown that *Hoxb7* directly activates transcription of *Fgf2* by binding to specific homeodomain binding sites in the *Fgf2* promoter region (Caré *et al.*, 1996). Although no such link has yet been demonstrated between the *Hox* genes and FGFs involved in limb initiation, it is indeed an attractive possibility that a similar interaction among these genes coordinates positioning of limbs and initiation of budding.

LIMB IDENTITY: FORELIMBS OR HINDLIMBS?

The discovery that FGF can induce both forelimbs and hindlimbs to form from the same population of flank cells has raised new questions about the molecular control of limb identity. What determines whether a limb bud will give rise to forelimb or hindlimb structures? In addition to the quantitative and qualitative differences in *Hox* gene expression in prospective forelimbs and hindlimbs, important new work has shown that another family of transcriptional regulators, the T-box (*Tbx*) genes, are also differentially expressed in forelimbs and hindlimbs of vertebrates (Gibson-Brown *et al.*, 1996, 1998; Isaac *et al.*, 1998; Logan *et al.*, 1998; Ohuchi *et al.*, 1998). *Tbx4* expression is restricted to the leg, and *Tbx5* is expressed in forelimb and flank (Gibson-Brown *et al.*, 1998; Isaac *et al.*, 1998; Logan *et al.*, 1998; Ohuchi *et al.*, 1998). Two other *Tbx* genes, *Tbx2* and *Tbx3*, are expressed in both forelimbs and hindlimbs (Gibson-Brown *et al.*, 1996). FGF induction of extra limbs from the flank alters the pattern of *Tbx4* and *Tbx5* expression in a pattern consistent with the identity of the ectopic limb (Gibson-Brown *et al.*, 1998; Isaac *et al.*, 1998; Logan *et al.*, 1998; Ohuchi *et al.*, 1998). When chick wing bud mesenchyme cells are transplanted under the apical ridge of the leg bud, and vice versa, the pattern of *Tbx* expression in the graft is stable, consistent with work which showed that the grafted cells retain their original identity (Isaac *et al.*, 1998). Together, these results suggest that *Tbx* plays a role in determining forelimb and hindlimb identity. Recent discoveries of *Tbx* mutations in human syndromes affecting the limbs are consistent with these genes playing an important role in pattern formation (see below), to have expression patterns restricted to one pair of limbs, such as the homeobox genes *Ptx1* and *Backfoot*, which are expressed in hindlimbs but not forelimbs (Shang *et al.*, 1997; Logan *et al.*, 1998). Studies of spontaneous mouse mutants, human limb malformations and mutagenesis screens have not yet revealed a complete reversal of limb identity, which suggests that control of limb identity may be more complex than single 'identity' genes. Limb mesenchyme cells may acquire forelimb or hindlimb

pattern by interpreting presence or absence of a gene product as well as differences in gene dosage or expression patterns.

An unresolved question is what determines the position of the limbs with respect to the dorsoventral axis of the embryo. *Fgf8* expression in the prospective forelimb and hindlimb ectoderm is activated in the same dorsoventral plane, and subsequently, forelimb and hindlimb buds are initiated in register with one another (Crossley *et al.*, 1996). Application of FGF beads to the flank also induces ectopic limbs along this dorsoventral line, irrespective of whether the beads are placed dorsally or ventrally within the lateral plate (Crossley *et al.*, 1996; Altabef *et al.*, 1997). This suggests that limbs are positioned along a dorsoventral boundary in the lateral plate, consistent with the model of Meinhardt (1983). The molecular basis of limb specification along the dorsoventral axis is not yet understood, but the pace at which work in this area is moving suggests that the answer may come soon.

OUTGROWTH AND PATTERNING: GENERATING BONES FROM BUDS

Proximodistal axis I: apical ridge formation

After initiation of limb budding, limb buds continue to grow out under the influence of a specialised epithelial ridge at the apex of the bud, known as the apical ectodermal ridge (AER) (Fig. 1A). The apical ridge runs along the boundary between the dorsal and ventral limb ectoderm. The ridge is induced and maintained by a signal from underlying mesenchyme. The precise mesenchymal signal that induces apical ridge formation has not yet been determined, but the observation that this signal is not restricted to apical mesenchyme indicates that dorsoventral localisation of the ridge must be determined by the ectoderm (Carrington & Fallon, 1986). Recent work has identified several genes expressed in limb bud ectoderm that act to position the ridge at the apex of the limb ectoderm. For example, *Radical Fringe* (*r-Fng*) is expressed in the dorsal half of the limb ectoderm prior to ridge formation, and the ridge develops at the boundary of *r-Fng*-expressing and non-expressing cells (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997). The role of *r-Fng* in determining ridge position can be demonstrated by over-expression of the gene using a retroviral vector, which causes displacement of the ridge to the new expression boundary (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997).

Two members of the *Wnt* gene family, *Wnt3a* and *Wnt7a*, are also expressed in dorsal limb ectoderm (Parr *et al.*, 1993; Kengaku *et al.*, 1998). *Wnt3a* and *r-Fng* become restricted to the apical ridge later in development, and mis-expression of *Wnt3a* can induce ectopic expression of *r-Fng*. Ectopic expression of *Wnt3a* can, like *r-Fng*, displace the apical ridge and *Fgf8* expression into the ventral ectoderm (Kengaku *et al.*, 1998). WNT7A, in contrast, seems to be involved in specification of dorsovental pattern in the limb (see below), but is not involved in localisation of the apical ridge in chick embryos (Kengaku *et al.*, 1998), however WNT7A does appear to be required for ectopic ridge formation in *En1* mutant mice (Cygan *et al.*, 1997). WNT3A activates *Fgf8* expression through the β-*catentin/Lef1* pathway, whereas WNT7A signals through a separate, unknown pathway (Kengaku *et al.*, 1998). This important finding demonstrates that these two *Wnt* genes have evolved separate functions in limb development by utilising distinct signalling pathways.

Proximodistal axis II: apical ridge signalling

The apical ridge is the source of secreted signalling molecules that maintain the underlying mesenchyme in an undifferentiated, proliferative state. In a classic experiment, John W. Saunders Jr demonstrated that the apical ridge is required for proximodistal outgrowth of the limb by surgically removing it from early limb buds. Removal of the ridge causes limb development to arrest, resulting in loss of distal structures (Saunders, 1948). The severity of limb truncation depends on the stage at which the ridge is removed, with earlier removals resulting in more severe truncations (Summerbell, 1974). The activity of the apical ridge is mediated by FGF. Three members of the FGF family are expressed in the apical ridge; *Fgf4* is expressed posteriorly and *Fgf2* and *Fgf8* are expressed throughout the ridge (Fig. 1D, E; Niswander & Martin, 1992; Suzuki *et al.*, 1992; Savage *et al.*, 1993; Heikinheimo *et al.*, 1994; Crossley & Martin, 1995; Mahmood *et al.*, 1995; Savage & Fallon, 1995). Application of any one of these FGFs after ridge removal is sufficient to rescue outgrowth and patterning of the limb (Niswander *et al.*, 1993; Fallon *et al.*, 1994; Vogel *et al.*, 1996), indicating that FGF is a key outgrowth signal produced by the apical ridge.

Proximodistal axis III: the progress zone

Fibroblast growth factors from the apical ridge maintain two specialised regions of mesenchymal cells: the progress zone and the polarising region, or zone of polarising activity (ZPA; Vogel and Tickle, 1993). The progress zone is a narrow band of distal mesenchyme cells subjacent to the apical ridge, in which the proximodistal identity is specified in the limb (Fig. 1A). There is considerable experimental evidence to suggest that the period of time that cells spend in the progress zone determines their address along the proximodistal axis (Summerbell *et al.*, 1973). According to this idea, cells exiting the zone after a short period will acquire a proximal positional address to form, for example, a humerus, whereas cells remaining in the progress zone for longer periods acquire progressively more distal addresses, such that the last cells to leave will give rise to terminal phalanges in the digits. This model predicts that distal mesenchyme cells measure the length of time that they are in the presence of a specific factor or group of factors. This could be achieved by a counting mechanism; however, a more attractive possibility is that distal mesenchyme cells may employ a quantitative response to a factor that accumulates in response to ridge signals, perhaps by a mechanism similar to that which controls the timing of cell differentiation in other organ systems (Durand *et al.*, 1997). Several genes are now known to be expressed in the progress zone, including transcription factors such as *rel/NFkappaB* (Bushdid *et al.*, 1998; Kanegae *et al.*, 1998), the LIM-homeodomain gene *Lhx2* (Rodriguez-Esteban *et al.*, 1998), the homeobox genes *Msx1* and *Evx1* (Davidson *et al.*, 1991; Niswander & Martin, 1993), the signalling molecules *Wnt5a* and *Fgf10* (Parr *et al.*, 1993; Ohuchi *et al.*, 1997), an *Eph* receptor tyrosine kinase known as *EphA4* (Fig. 2C; Patel *et al.*, 1996), and the zinc finger gene *Slug* (Ros *et al.*, 1997). Transcription of most of these genes depends on FGF signalling from the apical ridge. The *Rel/NFkappaB* gene, a vertebrate homologue of the *Dorsal* gene in *Drosophila*, regulates expression of *Twist*, a helix–loop–helix transcription factor (Bushdid *et al.*, 1998; Kanegae *et al.*, 1998). If the *NFkappaB* pathway in vertebrates mirrors the *Dorsal* pathway in flies, then it may act through *Twist* to control expression of FGF receptors in the distal limb (Tickle, 1998), which would be a mechanism by which FGF could indirectly regulate its own receptor to control limb outgrowth.

How is FGF transferred from the apical ridge and integrated into the progress zone? FGF4 and FGF8 are known to be secreted from the cell, and FGF2 may be released by cell damage or cell death (McNeil, 1993), which is known to occur in the ridge. Transfer of FGF from the apical ridge cells to the FGF receptors (FGFR) on the underlying mesenchyme cells may be facilitated by CD44, a cell surface proteoglycan, which is co-expressed with FGF8 in the apical ridge (Sherman *et al.*, 1998). Blocking CD44 activity using specific antibodies interferes with presentation of FGF8 and FGF4 to the adjacent mesenchyme cells and inhibits outgrowth of the treated limb (Sherman *et al.*, 1998). CD44 on one cell may act to present FGF on the same cell to its receptor or to heparan sulphate proteoglycans in the limb bud mesenchyme (Sherman *et al.*, 1998).

Dorsoventral axis

It should be apparent from the above discussion of apical ridge localisation that considerable interplay exists between the proximodistal and dorsoventral axes of the limb bud. In addition to specifying ridge position, dorsoventrally restricted gene expression patterns establish the dorsoventral pattern of the limb. Initial dorsoventral polarity of the prospective limb mesenchyme may be determined by planar signalling from adjacent cell populations; the somites provide a dorsalising factor and the lateral somatopleure (superficial layer of the lateral plate) provides a ventralising signal (Michaud *et al.*, 1997). The prospective limb mesenchyme then signals to the overlying ectoderm to specify ectodermal dorsoventral polarity (Geduspan & MacCabe, 1989). After dorsal and ventral identities are established in the overlying ectoderm, the ectoderm signals back to the mesenchyme to determine the final pattern of the limb (MacCabe *et al.*, 1974). Once this transfer of command has taken place, 180° rotation of the limb bud ectoderm along the dorsoventral axis results in respecification of dorsoventral pattern in the distal limb bud mesenchyme (MacCabe *et al.*, 1974).

Dorsal pattern of the limb appears to be controlled in part by the *Wnt7a* gene, which is expressed in the dorsal ectoderm (Parr & McMahon, 1995). Loss-of-function mutation of the mouse *Wnt7a* gene results in ventralisation of the distal dorsal aspect of the limb (Parr & McMahon, 1995). WNT7A induces expression of *Lmx1* in the dorsal limb mesenchyme (Riddle *et al.*, 1995; Vogel *et al.*, 1995) and inactivating the *Lmx1b* gene results in partial loss of dorsal structures (Chen *et al.*, 1998). Ectopic expression of either *Wnt7a* or *Lmx1* in chick limbs is sufficient to induce development of dorsal features on the ventral aspect of the limb (Riddle *et al.*, 1995; Vogel *et al.*, 1995). In the ventral half of the limb ectoderm, the homeobox gene *Engrailed1* (*En1*) is expressed. Loss of *En1* causes the ventral aspect of the limb to be dorsalized (Loomis *et al.*, 1996). Loss of *En1* expression allows *Wnt7a* and *Lmx1* expression to spread into the ventral aspect of the limb, where they induce a dorsal fate. Interestingly, loss of *Wnt7a* does not alter *En1* expression, demonstrating that the default fate of limb cells is to have a ventral identity and this is prevented dorsally by WNT7A (Parr & McMahon, 1995). Acquisition of dorsal fate in the ventral limb of *En1* mutants is therefore achieved by ectopic *Wnt7a* and *Lmx1* expression (Cygan *et al.*, 1997). EN1 normally prevents dorsalisation in the ventral limb by repressing expression of *Wnt7a* in the ectoderm (Logan *et al.*, 1997), and as a result, *Lmx1* expression is confined to the dorsal limb mesenchyme.

Another feature of *En1* mutants is the expansion of the apical ridge into the ventral ectoderm (Loomis *et al.*, 1996), pointing to a role for *En1* in restriction of the apical ridge to the apex of the bud. Overexpression of *En1* leads to elimination of the apical ridge, or

displaces it into the dorsal ectoderm (Logan *et al.*, 1997). The boundary of *En1* expression may determine the ventral limit of the apical ridge by defining the boundary of *r-Fng* expression at the apex of the limb ectoderm.

Anteroposterior axis

The anteroposterior axis is controlled by the polarising region or zone of polarising activity (ZPA), a specialised mesenchymal signalling region located at the posterior margin of the limb (Fig. 1A). In the chick wing, which contains only three digits, digit 2 is the most anterior, followed by digit 3 in the middle and digit 4 posteriorly. Transplantation of an additional polarising region to the anterior margin of the limb bud results in a mirror-image duplication of the digits, such that the anterior-to-posterior pattern of digits is 4–3–2–2–3–4, rather than the normal 2–3–4 pattern (Saunders & Gasseling, 1968). This experiment demonstrates that the polarising region is the source of a signal that bestows a posterior identity on limb mesenchyme cells, with cells closest to the polarising region acquiring the most posterior fate. Cells in the polarising region express the *Sonic hedgehog* (*Shh*) gene, which codes for a secreted signalling molecule (Fig. 1B) (Riddle *et al.*, 1993). Application of SHH protein or Shh-expressing cells to the anterior margin of the limb can mimic the effect of a polarising region graft by inducing a mirror-image pattern of digits. Retinoic acid is enriched in the posterior region of the limb (Thaller & Eichele, 1987; Maden *et al.*, 1998) and application of retinoic acid to the anterior margin of the limb bud can also induce mirror-image duplication of the digits (Tickle *et al.*, 1982). Application of retinoic acid activates the *Shh* pathway in the limb (Riddle *et al.*, 1993), and both retinoic acid and SHH can act in a dose-dependent and time-dependent manner, with higher doses and longer exposure periods inducing more posterior fates (Tickle *et al.*, 1985; Yang *et al.*, 1997). Retinoic acid appears to be required for *Shh* expression, as application of retinoid antagonists to the posterior aspect of the limb results in loss of *Shh* expression (Stratford *et al.*, 1996). *Hoxb8* is expressed in lateral plate mesoderm with an anterior expression boundary located in the posterior region of the forelimb bud in chick and mouse embryos (Charite *et al.*, 1994; Lu *et al.*, 1997; Stratford *et al.*, 1997). Retinoic acid application to the anterior limb induces a direct, rapid induction of *Hoxb8* anteriorly (Lu *et al.*, 1997). Transgenic experiments have revealed that anterior extension of the *Hoxb8* expression boundary results in an ectopic zone of *Shh* expression, which leads to polydactyly in the forelimbs (Charite *et al.*, 1994). Thus, *Hoxb8* expression in lateral plate mesoderm along the main body axis appears to specify the position of the polarising region within the limb. Retinoic acid appears to lie upstream of *Hoxb8* expression, which lies upstream of *Shh* expression, in the polarising region pathway.

Maintenance of *Shh* expression and polarising activity in the limb also requires FGF4 from the apical ectodermal ridge (Laufer *et al.*, 1994). SHH, in turn, feeds back to maintain *Fgf4* expression in the apical ridge. This positive feedback loop between FGF4 in the apical ridge and SHH in the polarising region coordinates proximodistal outgrowth and anteroposterior patterning. Inactivation of *Shh* in mice results in proximodistal truncation of the limbs, confirming its role in maintaining the proximodistal outgrowth machinery (Chiang *et al.*, 1996). WNT7A from the dorsal ectoderm is also involved in maintaining *Shh* expression in limb bud mesenchyme (Yang & Niswander, 1995), although it appears that this is indirect (Cygan *et al.*, 1997). Thus, multiple molecular interactions link the anteroposterior, proximodistal and dorsoventral axes to generate the integrated system required for limb bud outgrowth and patterning.

How does SHH activate the polarising region pathway in the limb? Although SHH is a secreted protein, and can generate dose- and time-dependent effects, it does not appear to act over a long range. Instead, SHH remains tethered to the cell surface. Post-translational processing of SHH results in cleavage of the protein and addition of cholesterol to the N-terminal peptide. Attachment of lipophilic cholesterol results in binding of the N-terminal portion of the protein to the surface of the cell, thereby preventing its diffusion throughout the limb (Porter *et al.*, 1996; Yang *et al.*, 1997). This is consistent with the observation that SHH protein is confined to the region of *Shh* transcription in the polarising region (Marti *et al.*, 1995). The long-range effects of Shh must, therefore, be mediated by secondary signals in the limb, such as the bone morphogenetic proteins (BMPs). SHH induces transcription of *Bmp2*, by repression of Patched (Ptc) (Marigo *et al.*, 1996b). Patched is a transmembrane receptor that is expressed in regions of hedgehog gene expression (Fig. 1D, E; Marigo *et al.*, 1996a,b; for a detailed review of the hedgehog signalling pathway, see Hammerschmidt *et al.*, 1997). Other members of the hedgehog gene family can also act through Patched receptors (two *Patched* genes have been discovered in mice, and both appear to be co-expressed with *Sonic hedgehog*; Motoyama *et al.*, 1998). *Indian hedgehog* (*Ihh*) acts through *ptc* in the formation of cartilage, and is expressed later than *Shh* during limb development (Vortkamp *et al.*, 1996). Nonetheless, *Ihh*-expressing cells grafted to the anterior margin of the early limb bud can mimic the effect of SHH, ectopically activating the polarising region pathway and leading to digit duplications in the limb (Vortkamp *et al.*, 1996). Recent work on the *Doublefoot* mouse mutation has attributed the severe digit duplications in the mutants to ectopic IHH signalling, which activates both *Ptc1* and *Ptc2* anteriorly in the limb buds (Yang *et al.*, 1998).

Bmp2 is expressed in a pattern that broadly overlaps the *Shh* domain in the limb bud (Riddle *et al.*, 1993; Francis *et al.*, 1994), and the ability of *Bmp2*-expressing cells to induce mild digit duplications suggests that it could, at least in part, mediate *Shh* signalling in the limb (Duprez *et al.*, 1996). The inability of BMP2 on its own to induce a complete duplication of the digits could reflect a requirement for BMP heterodimerisation, which seems to increase potency of BMP signalling activity (Hazama *et al.*, 1995). *Bmp2*-expressing cells are capable of activating *Fgf4* anteriorly in the apical ridge, which, together with the observation that *Bmp2*, *Bmp4* and *Bmp7* are expressed in limb mesenchyme and ectoderm, suggests that BMPs could play a role in the feedback loop between limb bud mesenchyme and the apical ridge (Francis-West *et al.*, 1995; Duprez *et al.*, 1996).

HOX GENES IN LIMB DEVELOPMENT

The signalling molecules described above confer positional identity onto cells in the limb, and set in motion the regionalised programmes of differentiation which generate the limb pattern. *Hox* genes are key components in the interpretation of positional information during development (reviewed in Gellon & McGinnis, 1998). These transcription factors are organised in four gene clusters, known as *Hoxa–d*, in most jawed vertebrates, although additional clusters have been found in bony fish (Prince *et al.*, 1998). The multiple *Hox* clusters of vertebrates have arisen by duplication from an ancestral cluster during chordate evolution (reviewed in Aparicio, 1998; Holland, 1998), and these gene duplications have provided new genetic raw material for co-option into new developmental processes. In the limbs, genes located at the 5′ end of the *Hoxa* and *Hoxd* clusters are expressed in dynamic

patterns from the outset of budding (Dolle *et al.*, 1989; Yokouchi *et al.*, 1991; Nelson *et al.*, 1996). *Hoxd9–13* are expressed in nested domains centred around the polarising region in the posterior distal aspect of the bud. This pattern appears to be regulated by SHH from the polarising region, together with FGF from the apical ridge. Ectopic activation of the polarising region pathway by anterior application of polarising region cells, retinoic acid, SHH, or *Bmp2*-expressing cells under the apical ridge induces a mirror image pattern of *Hox* expression in the limb, foreshadowing the mirror-image pattern of digit duplication (Izpisúa-Belmonte *et al.*, 1991; Riddle *et al.*, 1993; Duprez *et al.*, 1996). The pattern of *Hox* gene expression changes considerably during the course of limb development, and the dynamic pattern is broadly divisible into three phases; in phase 1, the *Hoxd* domains are spread across the distal limb, in phase 2 these domains become centred on the posterior distal limb and in phase 3 the posteriorly restricted domains spread anteriorly in the distal limb. *Hoxa* gene expression is also dynamic, with *Hoxa13* expression spreading into the anterior part of the distal limb during phase three (Nelson *et al.*, 1996). This third phase of *Hox* expression correlates with specification of the digits (Nelson *et al.*, 1996), which has interesting implications for our understanding of the origin of digits during the fin to limb transition in tetrapod evolution (discussed below).

Determining the function of *Hox* gene expression during limb patterning has been no easy task, but thanks to the highly detailed approach several groups have taken to study *Hox* gene regulation and the interactions of different *Hox* genes during development, a considerable body of information is now available (Dolle *et al.*, 1993; Davis *et al.*, 1995; Mortlock *et al.*, 1996; van der Hoeven *et al.*, 1996b; Zákány *et al.*, 1997a). Two important ideas have shaped our understanding of *Hox* gene regulation. Temporal and spatial colinearity refer to the manner in which *Hox* genes are expressed, with the former referring to the sequential manner of *Hox* gene expression, with 3′ genes being expressed before their 5′ neighbours, and the latter referring to the spatial distribution of these transcripts in the embryo, with 5′ genes being expressed at more posterior positions than 3′ genes (Duboule, 1994). Collinearity can break down, such as the case during the late phase of *Hox* expression in limb development, and during amphibian limb regeneration (Gardiner *et al.*, 1995; Nelson *et al.*, 1996). Spatial and temporal collinearity are controlled by regulatory elements acting at three levels; some enhancers operate on a per-gene basis, other elements are shared between different *Hox* genes; and other 'higher order' control elements can act on the entire complex (Gérard *et al.*, 1996; van der Hoeven *et al.*, 1996b; Zákány *et al.*, 1997b). The precise timing of gene expression is as important as 'on' and 'off' decisions, and subtle alterations to the timing of gene expression (heterochronic changes) can induce severe morphological changes in the animal (Gérard *et al.*, 1997). Precise regulation of *Hox* gene dosage is also important, as variation in the dose of *Hox* gene products can cause severe patterning defects (Horan *et al.*, 1995; Zákány *et al.*, 1997a) The discovery that such control elements are shared among distantly related vertebrates indicates that they are phylogenetically ancient (Beckers *et al.*, 1996). Evolutionary conservation of *Hox* regulatory machinery has led to the idea that evolution of morphological changes in vertebrates may have been driven by very slight changes to the timing of *Hox* gene activation (Gérard *et al.*, 1997, see below). Moreover, the discovery of tissue- and region-specific *Hox* enhancers (Whiting *et al.*, 1991; Beckers *et al.*, 1996) suggests that such changes can be confined to highly specific locations of the embryo to allow regionalised rather than wholesale modifications of the body.

EVOLUTION OF TETRAPOD LIMBS

Paired lateral appendages, fins and limbs, are unique to jawed vertebrates and their immediate ancestry (reviewed in Coates & Cohn, 1998). Tetrapod limbs evolved from paired fins of a fish-like ancestor during the Devonian, approximately 360 million years ago (Coates & Clack, 1990). The key breakthrough in the fin to limb transition was elaboration of the distal limb skeleton to give rise to endoskeletal digits. The earliest evidence of tetrapod limbs complete with digits is found in Devonian specimens such as Acanthostega, Ichthiostega and Tulerpeton. These limbs display the basic skeletal arrangement of modern tetrapods, with discrete endoskeletal digits. An important difference, however, is the number of digits on each limb, which is greater than the highly conserved tetrapod pattern of five digits (Coates & Clack, 1990). These discoveries overturned previous ideas that the ancestral pattern for tetrapod limb is pentadactyly (e.g. Jarvic, 1980).

Comparative molecular studies of teleost fin and tetrapod limb development have uncovered striking conservation of the genetic control of pattern formation (Sordino *et al.*, 1995; Reifers *et al.*, 1998; Vandersea *et al.*, 1998). Although little is known about the molecular basis of fin formation in lobe-finned fishes or sharks, phylogenetically the most relevant taxa in the context of limb evolution, the teleost–tetrapod comparative studies strongly suggest that the earliest tetrapod limbs were patterned by the same primitive genetic network. Indeed, the genetic toolbox used in fin and limb development is far older than the earliest chordates, as invertebrate appendages from antennae to limbs to genitals are patterned by the same genetic circuit (for a review see Shubin *et al.*, 1997). Among the genes shown to be expressed in both teleost fins and tetrapod limbs are *Shh*, *Ptc1*, *Bmp4*, *Fgf8*, *Distal-less* (*Dlx*), FGFRs, *Bmp2*, AbdB-related *Hox*a and *Hoxd* (early phases of expression), *Hoxc6*, *Msx*, *En1* and *Sal1* (*spalt*) (Molven *et al.*, 1990; Hatta *et al.*, 1991; Krauss *et al.*, 1993; Akimenko *et al.*, 1994, 1995; Sordino *et al.*, 1995; Thisse *et al.*, 1995; Concordet *et al.*, 1996; van der Hoeven *et al.*, 1996a; Chin *et al.*, 1997; Koster *et al.*, 1997; Laforest *et al.*, 1998; Reifers *et al.*, 1998). These similarities between fins and limbs extend beyond simple presence or absence of gene expression, as their precise spatial relationships and the cellular interactions in early fin development bear striking resemblance to those found early in tetrapod limb development.

If fish fin and tetrapod limb development involve the same pattern-forming genes, how can such extreme morphological differences be achieved? The most striking difference between fin and limb endoskeletons is found distally. Although both fins and limbs contain girdles and proximal bones (radials in fins and long bones in limbs) with clear anteroposterior and dorsoventral pattern, only tetrapod limbs have endoskeletal digits. The distal fin rays, or lepidotrichia, of bony fish are entirely dermal. It appears that patterning of the proximal elements is achieved by the same mechanisms in fish and tetrapods, but important differences in gene expression patterns occur later, when the distal elements are laid down. For example, *Shh* expression in the posterior bud mesenchyme controls anteroposterior pattern of the tetrapod limb all the way to the digits, whereas in zebrafish, *Shh* expression in the fin buds diminishes after the radials are laid-down, prior to ray formation (Laforest *et al.*, 1998). *Shh* is then re-expressed in each fin ray where it may play a role in scleroblast differentiation or matrix production (Laforest *et al.*, 1998). The early loss of *Shh* expression in the posterior fin bud is associated with cessation of *Hoxa* and *Hoxd* expression. In contrast to the triphasic pattern of *Hox* expression seen in tetrapod limbs, in zebrafish fin buds these genes undergo only the early phases of expression (Sordino *et al.*, 1995). The significance of this difference is that the third phase of *Hox* expression in tetrapod limbs, when

expression domains move anteriorly across the distal aspect of the bud, is associated with digit development (Nelson *et al.*, 1996). These observations led Duboule and co-workers to suggest that this tetrapod specialisation may have evolved together with the autopod (wrist/ankle and digits; Sordino *et al.*, 1995). Transgenic analyses in mice have identified an enhancer element in the vicinity of *Hoxd13* that is responsible for its distal limb expression (van der Hoeven *et al.*, 1996b; Zákány & Duboule, 1996; Herault *et al.*, 1998). If distal expression of *Hoxd10–13* is controlled as a unit, under the influence of a single *cis*-acting regulatory element, then evolution of digits in the earliest tetrapods could have resulted from a surprisingly simple genetic innovation (van der Hoeven *et al.*, 1996b). Another fascinating component of this work has linked development of limbs with external genitalia, providing an attractive developmental scenario by which tetrapod locomotion and internal fertilisation could have co-evolved (Kondo *et al.*, 1997). Both the genital bud and the digits are appendages with posterior or distal identity, in that they develop at the terminus of the trunk and limbs, respectively. At a molecular level, 5′ (posterior) members of the *Hoxa* and *Hoxd* clusters are expressed in the genital tubercle (which gives rise to the penis and clitoris) and distal limbs of mice. Compound loss of function mutations in *Hoxa13* and *Hoxd13* result in complete loss of digits and external genitalia (Kondo *et al.*, 1997). Thus, formation of both organs requires posterior *Hox* gene expression, which may mediate cell proliferation and outgrowth. Moreover, expression of *Hoxd* genes in limbs and genitals is controlled by a single enhancer (van der Hoeven *et al.*, 1996b; Herault *et al.*, 1998). This raises the intriguing possibility that the origin of digits and external genital organs during tetrapod evolution may have resulted from the appearance of a single transcriptional regulator. Such a genetic innovation could have freed early tetrapods from an aquatic environment by providing the anatomical hardware necessary for terrestrial locomotion and internal fertilisation (Kondo *et al.*, 1997). This evolutionary linkage between limbs and genitals at a genetic level provides a contextual explanation for syndromes in which development of limbs and genitals is perturbed, as in hand–foot–genital syndrome (discussed below). Perhaps the most striking congruence between this molecular scenario and the fossil record comes from the finding that morphogenesis of the digits and penis are sensitive to changes in *Hox* gene dosage. Recent work has shown that a quantitative decrease in the dose of *Hoxd11–13*, reduces the length of the penian bone and digits, as well as the number of digits (Zákány *et al.*, 1997a). This progressive reduction in digit number takes an interesting turn, however, in that the transition from five digits to complete lack of digits involves a step in which limbs are polydactylous. Considering this finding in light of the polydactylous nature of the earliest tetrapods (Coates & Clack, 1990), Zákány *et al.* suggested that, during tetrapod evolution, successive activation of *Hox* gene expression in the distal limb may have taken the limb from complete lack of digits to the pentadactyl pattern via a polydactylous phase.

CONGENITAL LIMB ANOMALIES: LINKING MALFORMATIONS TO MOLECULES

Progress in the molecular genetics of limb development has started to shed light on the genetic basis of naturally occurring limb malformations. The aetiology of limb defects is complex, and includes mutations, environmental factors, chromosomal abnormalities and intrauterine accidents such as amniotic bands, which can amputate the limb by constriction (Ferretti & Tickle, 1997). This discussion will be restricted to malformations resulting

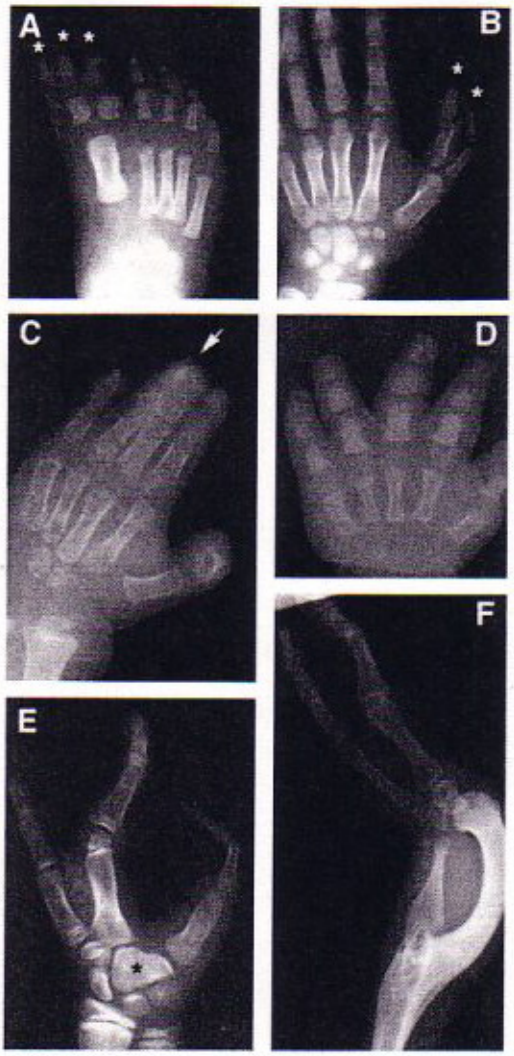

Figure 3 Congenital malformations of human limbs. (A) Polydactyly in the foot. Triplication of the first toe (white asterisks) results in the presence of seven digits. Note increased breadth of first metatarsal. (B) Polydactyly in the hand involving duplication of the thumb (white asterisks). (C) Syndactyly in hand of an individual with Apert syndrome. Arrow indicates distal bony fusion of digits. (D) Hand of child with achondroplasia. Note that metacarpal and phalangeal epiphyses have already fused although carpals remain largely unossified (compare with unfused bones in B and E). (E) Ecrodactyly in hand. Two digits are completely absent and hamate and capitate are fused (black asterisk). (F) Forelimb with severe ecrodactyly, dysplastic, hemimelic long bones and absence of elbow joint. Note shortness of ulna and radius relative to metacarpals.

from mutations in developmental control genes. Malformation of the limbs occurs frequently, and the spectrum of such defects is large (Figs. 3 and 4). Limb abnormalities are broadly divisible into three categories, reduction defects, duplication defects and dysplasias (Larsen, 1997). Some mutations can result in a combination of defects, and thus it is worth outlining the major types of defect within each category before considering these compound limb malformations.

Figure 4 Naturally occurring polydactyly in one of Hemingway's cats. Front paws of the cat show additional digits (arrows) on the anterior side of the limbs. The cat is a descendant of the original population living at Ernest Hemingway's house in Key West, Florida.

Reduction defects. The most extreme form of reduction defect is amelia, in which the entire limb is absent. Overall limb length may be shortened due to partial absence of the limb skeleton, meromelia, or stunting the development of long bones, termed hemimelia (Fig. 3F). Digital length can also be truncated both by shortening of the phalanges in brachydactyly, or by deletion of digits, as is the case in both ecrodactyly, when one or more of the digits is absent (Fig. 3E, F), and adactyly, the complete absence of digits on a limb.

Duplication defects. Duplication of proximal elements in the limb is extremely rare, and even experimental manipulations of the embryo rarely result in extra proximal elements (Wolpert & Hornbruch, 1987). In contrast, duplication of the digits, or polydactyly, is quite a common duplication defect (Fig. 3A, B; Fig. 4). Polydactyly is generally classified as either preaxial, when extra digits develop anteriorly (on the radial or tibial side of the limb; Fig. 3A, B), or postaxial, when extra digits develop posteriorly (on the ulnar or fibular side of the limb). Additional digits may be fully formed, complete with functional tendons and nerves, or poorly formed, appearing as only a skin tag in the mildest cases. Additional digits usually develop with respect to the anteroposterior polarity of the bud, such that formation of an ectopic polarising region anteriorly results in a mirror-image pattern of extra digits, with the most posterior digits, digit 5, appearing at the anterior and posterior margins of the hand or foot.

Dysplasia defects. This class of defects may be thought of as defects in the cellular differentiation programme, as opposed to defects in the specification of limb pattern as in polydactyly, although affected limbs may exhibit both classes of defects. Dysplasias often result in reduction defects such as *hemimelia* and *brachydactyly*, which are caused by deficient cell proliferation (*hypoplasia* or *aplasia*) during bone growth (Fig. 3D). Other dysplasias include *syndactyly*, in which digits are joined by soft tissue between the digits failing to break down, resulting in webbed digits, and *synostosis*, in which the bones themselves are fused (Fig. 3C). Clinically, syndactyly is subdivided into types I–V, with the five subclasses displaying varying degrees of soft tissue and bony fusions (Bergsma, 1979). For example, in type II syndactyly (synpolydactyly), the hand exhibits fusion of digits 3 and 4 together with duplication of the fourth finger and fifth toe, whereas in type V syndactyly, metacarpals and metatarsals are also fused (Bergsma, 1979). Soft tissue webbing is the result of a failure of programmed cell death between the digits, which normally functions to separate the digits of the hand plate after the entire skeleton has been laid down. Dysplasias may result in skeletal size or shape changes, or in deficiency defects by premature cessation of the bone growth programme.

Mutational analyses in mice have identified a large number of genes which can generate limb malformations from each of the above classes (for reviews see Ferretti & Tickle, 1997; Niswander, 1997). These advances have recently begun to yield results in humans, with naturally occurring human mutations being identified at the molecular level (Table 1). Because many of these genes have been studied for years in the laboratory, there is often a considerable amount known about the cell and molecular biology of these mutations by the time they are identified in humans. Perhaps the best example of this is *HOX* gene mutations. Type II syndactyly, or synpolydactyly, is caused by a mutation in the *HOXD13* gene. The mutation involves an expansion of the polyalanine stretch in the amino-terminal region of the peptide, which may interfere with DNA binding or interaction of *HOXD13* with other *Hox* proteins (Muragaki *et al*., 1996). A total loss of function mutation has been generated in mice (Dolle *et al*., 1993), and although this does not phenocopy human synpolydactyly, some aspects of the phenotype are shared. In particular, there appears to be a common defect in the length of the digits, consistent with the role of *Hox* genes in controlling growth and proliferation. Elimination of the *Hoxd11–13* gene products in mice results in a phenotype closely resembling human synpolydactyly, suggesting that the human condition could involve functional suppression of other *HOXD* genes (Zákány & Duboule, 1996). The human Hand–Foot–Genital mutation, and the mouse Hypodactyly mutation are both caused by mutations in *Hoxa13* (Mortlock *et al*., 1996; Mortlock & Innis, 1997). Hypodactyly mutants have more severe reduction defects distally, and cellular analysis has shown that this defect involves increased cell death in the distal limb and a cell-autonomous defect affecting mesenchymal cell behaviour and cartilage differentiation (Robertson *et al*., 1996). Members of the Hedgehog pathway have also been implicated in congenital malformations affecting the limbs. *Gli3* is a zinc finger gene which is negatively regulated by *Shh*. Mutations in the *Gli3* gene are found in humans with Greig cephalopolysyndactyly syndrome and Pallister–Hall syndrome, both of which involve polysyndactyly (Vortkamp *et al*., 1991; Kang *et al*., 1997; Wild *et al*., 1997). Mouse *extra-toes* mutants, which are also characterised by polysyndactyly, have deletion mutations within the *Gli3* gene (Hui & Joyner, 1993). Townes–Brockes syndrome is caused by a mutation in SALL1, a zinc finger gene homologous to the *spalt sal* genes of *Drosophila*, mouse, frog and fish, which may be positively regulated by hedgehog signalling (Koster *et al*., 1997; Kohlhase *et al*., 1998).

Although the function of SALL/sal in the SHH pathway is unclear, it is nonetheless intriguing that patients develop preaxial polydactyly in Townes–Brockes syndrome, given that the mutation is thought to result in loss of function (Kohlhase *et al.*, 1998). In Holt–Oram syndrome, which is caused by a mutation in *TBX5* (Basson *et al.*, 1997; Li *et al.*, 1997), limb defects are restricted to the forelimbs, which is consistent with observations that *Tbx5* is expressed in forelimb, but not hindlimb buds of chicks and mice (described above). Mutations in *Tbx3* cause ulnar mammary syndrome, which involves mild to severe reduction defects in the forelimbs (Bamshad *et al.*, 1997). Restriction of the limb phenotype to forelimbs is somewhat puzzling in light of the observation that, during development, *Tbx3* is expressed in a similar pattern in both forelimb and hindlimb buds (Gibson-Brown *et al.*, 1996, 1998; Isaac *et al.*, 1998). Dorsoventral patterning defects of the limbs are less common than anteroposterior and proximodistal defects. Absence of the patellae and hypoplasia of the nails in human nail–patella syndrome may be interpreted as precisely such a defect, as it is the dorsal limb structures that are affected. It is therefore satisfying that mutations in the *LMX1B* gene, known to be involved in specification of dorsal structures in chicks and mice, have now been identified as the cause of nail–patella syndrome in humans (Dreyer *et al.*, 1998; Vollrath *et al.*, 1998). The numerous limb dysplasia syndromes caused by mutations in FGF receptors (FGFRs) implicated these genes in later phases of limb development, during growth and differentiation (Table 1) (Wilkie *et al.*, 1995a,b). Premature closure of the cranial sutures and epiphyses of the limb are a common feature of most of these syndromes (Fig. 3D), which points to a key role of FGFRs in maintaining cell proliferation or inhibiting differentiation during skeletal growth. Similar growth defects were observed in the limbs of transgenic mice over-expressing FGF2 (Lightfoot *et al.*, 1997). The gap in our understanding of FGF function during these late events in limb development highlights the role of natural mutations in identifying future areas of investigation for developmental biology.

Another poorly understood area of limb development is the relationship between skeletal morphogenesis and epigenetic events such as mechanical loading. The potential of bone cells and their precursors to assess and respond to mechanical stresses to remodel the skeleton has long been known to skeletal biologists and orthopaedists (Lanyon, 1987). Remodelling occurs *in utero* and in adults as a response to extrinsic mechanical forces (McLeod *et al.*, 1998). Fracture repair in adults and joint formation during development are also influenced by the loading regime of the skeleton. Until recently, however, precisely how these extrinsic forces are translated into biochemical signals has been unknown. Recent work has shown that even prechondrogenic mesenchyme cells can respond to compressive loading by increasing cartilage matrix production, and this is mediated by activation of the *Sox9* pathway (Takahashi *et al.*, 1998). *Sox9* is expressed in developing limb buds (and numerous other tissues; Ng *et al.*, 1997), and directly activates transcription of type II collagen, which produces the major cartilage matrix protein (Bell *et al.*, 1997). Thus, SOX9 is involved initially in development of the skeleton and later in the remodelling response to mechanical loading. Similarly, *Indian Hedgehog* and *PTHrP*, which participate in a feedback loop that mediates the rate of endochondral ossification during skeletal development, are also expressed postnatally during bone growth and fracture repair (Vortkamp *et al.*, 1998). Although the role of this signalling network in these later events is unclear at present, it is tempting to speculate that its initial activation during development may be controlled by a hard-wired genetic programme and re-expression during bone repair may be catalysed by mechanical stimuli. Further work on the molecular bridge between mechanical loading and cell behaviour should help to integrate our understanding of pattern formation with skeletal biology, dysmorphogenesis and evolution.

CONCLUSIONS

Although developmental biology has provided enormous groundwork for clinical genetics by identifying candidate genes for congenital malformations and uncovering their signalling pathways and functions, it is now possible to reverse direction and use new clinical genetic discoveries to tackle developmental questions. Availability of newly discovered human mutations for generation of transgenic mice and transfection studies *in vivo* and *in vitro* should provide new insights into the potential for development and evolution. Similarly, the fossil record not only provides a phylogenetic context for interpretation of phenotypes and demonstrates the morphological potential of pattern formation, but also, and perhaps most importantly, sets questions for the future. Much of the morphological detail of vertebrate skeletons is due to load-induced remodelling, which allows continuous fine-tuning of the skeleton. In a phylogenetic context, this point is paramount, as distinguishing skeletal traits which arise as a consequence of genetic change from those which have arisen as a remodelling response to, say, locomotor pattern, will have dramatic consequences for our view of evolution. Understanding how gene expression, cell behaviour and environment interact to generate morphological pattern in the limb is the next frontier.

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