ANATOMY AND DEVELOPMENT OF TENDONS IN VERTEBRATE LIMBS

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To my family

ABSTRACT

The gross and microscopical anatomy of developing tendons in chick limbs is described. Expression patterns of genes encoding *EphA4*, a tyrosine kinase receptor involved in direct cell-cell signalling, *Six-1*, a transcription factor, and *Follistatin*, a TGF-β antagonist, are documented in developing chick tendons. *EphA4* expression is compared with tenascin and collagen I expression. Follistatin applied ectopically to chick limbs inhibits tendon development suggesting a role for TGF-β signalling. Manipulations on chick limbs are carried out to examine co-ordination of tendon and cartilage development. Manipulations that invoke ectopic cartilage lead to ectopic tendons expressing both *Follistatin* and *EphA4* while manipulations that invoke cartilage truncation lead to loss of tendons and expression. Ectodermal signalling is known to control dorso-ventral limb pattern including tendons. *EphA4* is expressed in both dorsal and ventral tendons but the former are flattened while the latter round. In limbs of transgenic mice in which *Wnt-7a*, a dorsalising signal produced by dorsal ectoderm, had been functionally inactivated, the *EphA4* expression pattern is ventralised early in dorsal tendon development. In chickens in which Lmx-1, a transcription factor expressed in dorsal mesenchyme in response to Wnt-7a signalling, is ectopically expressed ventrally, no early changes in *EphA4* expression pattern in ventral tendons could be detected. However, later, established tendons in ventral regions come to resemble dorsal tendon and double nails form. Spatial and temporal expression patterns of *Wnt-7a* and *Lmx-1* were examined and compared with *EphA4* expression in tendons. These analyses suggest that dorso-ventral patterning and specification of tendons involves complex series of parallel interactions between tendon-forming cells and both ectoderm and mesenchyme. A polydactylous human foot with double nails is dissected and the toes identified by the tendons. The anatomy is interpreted from a developmental viewpoint.

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LIST OF ABBREVIATIONS

AER	Apical ridge
A-P	Antero-Posterior
BMP	Bone bone morphogenetic
BMPR	Bone bone morphogenetic receptor
CHAPS	3-((3-cholamidopropryl)-dimethyl-ammonio)-1-propane sulphonate
CST	Cortico-spinal tract
D-V	dorso-ventral
DiI	1,1 dioctadecyl 1-3,3,3',3'-tetrametllindo-carbocyanine perchloride
dpp	decapentaplegic
ECM	Extra cellular matrix
En	Engrailed
Eph	Erythropoietin producing human hepatocellular carcinoma cell line
Ephrin	Eph family receptor interacting proteins
Eya	Eyes absent
FGF	Fibroblast growth factor
MARP	Muscle Ankyrin Repeat Protein
Msx	Muscle segmentation homeobox class-homeobox gene
PBS	Phosphate buffered saline
PBT	Phosphate buffered saline + Triton X-100
P-D	Proximo-distal
PFA	Paraformaldehyde
RA	Retinoic acid
RAR	Retinoic acid receptor
Shh	Sonic hedgehog
TCA	Trichloracetic acid
TGF	Transforming growth factor
TSP	Thrombospondin
ZPA	zone of polarising activity

CHAPTER ONE

GENERAL INTRODUCTION

Chapter One: General Introduction

1.1 Development of the vertebrate limb

One of the most essential features of members of the animal kingdom is the ability to move. This requires co-ordinated development of components of the musculo-skeletal system of which tendons perform the role of transmitting the force generated by a muscle to the bone, thus providing locomotion. The chick embryo is an ideal model system to study the development of the limb (Tickle and Eichele 1994, Tickle 1999) as it is quite convenient to explore mechanisms of tissue outgrowth, pattern formation and identify molecules that mediate these processes and to carry out surgical manipulations in vivo.

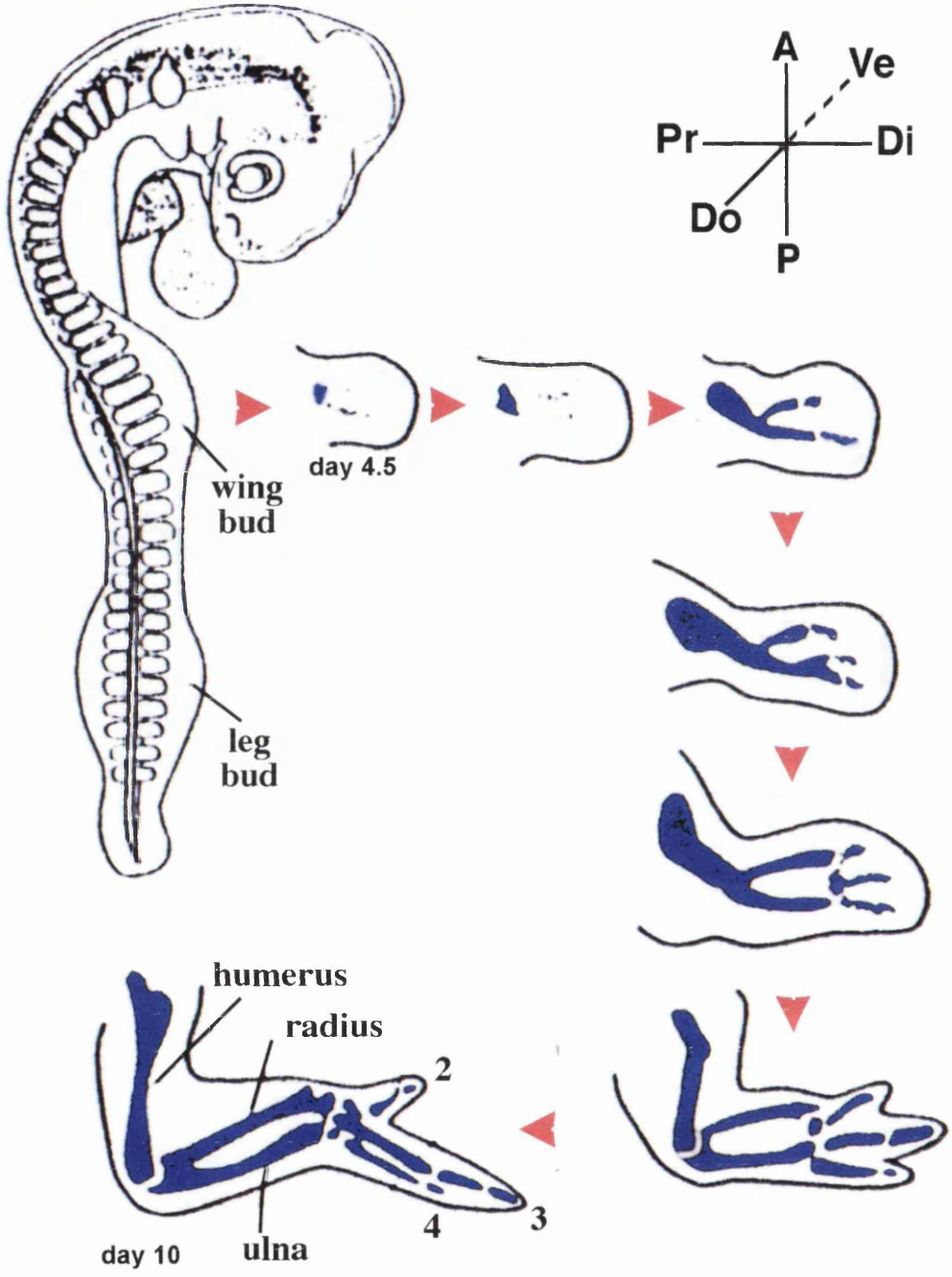
1.2 Outline of limb development

Overt limb development in the chick begins at stage 16 when condensation of somatopleural mesoderm occurs at precise locations along the anterior-posterior axis of the trunk. The wing develops opposite somites 15-20 and leg opposite somites 26-32 (Stages, Hamburger and Hamilton 1951) (Fig. 1.1). By stage 18, the limb bud is rimmed at the tip by a specialised epithelium, termed the apical ectodermal ridge (AER). The apical ridge runs antero-posteriorly and plays a crucial role in limb development (Saunders 1948; Toldt and Fallon 1984). Ectoderm overlying the paraxial, intermediate and lateral somatopleural mesoderm gives rise to the ectoderm overlying the limb bud (Altabef et al 1997; Michaud et al 1997). The outgrowth of the limb bud occurs due to decreased proliferation of tissue in the flank region (Searls and Janner 1971). At early stages, limb bud mesenchyme is undifferentiated and highly proliferative, but gradually there is a fall in mitosis (Hornbruch and Wolpert 1970; Searls 1973). Nearer the tip, cells keep dividing whereas proximally, cells differentiate into the various tissues that constitute the limb. Cells condensation precedes visible differentiation of tissues and proceeds from proximal to distal regions. By stage 36, cartilage elements have been laid down. Three limb segments can be distinguished, the stylopod made up of humerus (femur in leg), zeugopod containing radius and ulna (in leg, tibia and fibula) and the autopod. In the wing, the autopod consists of 3 digits numbered 2, 3 and 4, while the leg has 4 digits numbered 1, 2, 3 and 4 from anterior to posterior. At this stage, muscles and tendons are also well developed. The tip of the limb becomes a flattened plate in which

Figure 1.1 Diagram showing development of chick wing bud from day 3 (stage 17) to day 10 (stage 36)

Cells differentiating into cartilage to establish skeletal pattern are shown in blue within the limb bud. Digits at day 10 are numbered 2, 3 and 4 from anterior to posterior.
A, anterior; P, posterior; Pr, proximal; Di, distal; Do, dorsal; Ve, ventral. (Figure adapted from Tickle and Eichele 1994)

Fig. 1.1


A
Ve
Pr
Di
Do
P
wing bud
leg bud
day 4.5
humerus
radius
ulna
2
3
4
day 10

digit primordia develop. Initially, digits are joined by a web of tissue, which eventually disappears and the digits separate.

1.3 Initiation of limb development

The process of limb development begins even before an overt limb bud is seen. In chick embryos the entire region from future neck to future leg region (at stages 11-14) has the potential to form limbs. When mesonephros adjacent to the presumptive limb region was removed microsurgically or by laser ablation, limbs did not develop (Geduspan and Solursh 1992) suggesting that signals originating from the intermediate mesoderm have an inductive role in initiating limb bud development. In some limbless chick mutants, limb structures and mesonephros are lacking (Zwilling 1956) but in others (Prahalad et al 1979), kidney development is normal. Members of the Fibroblast Growth Factor (FGF) family, FGF-1, -2, -4 (Cohn et al 1995), -8 (Vogel et al 1996; Crossley et al 1996) and–10 (Ohuchi et al 1997), when applied to the presumptive flank of chick embryos, induce ectopic limb bud formation suggesting that FGFs initiate limb outgrowth. It was proposed that *Fgf-8* a member of the fibroblast growth factor (FGF) family functioned in initiating limb bud outgrowth by virtue of its spatial and temporal expression in the mesonephros at wing level (Crossley et al 1996; Vogel et al 1996). However *Fgf-8* is normally expressed in the intermediate mesoderm of the *limbless* mutant (Ros et al 1996) and removal of the mesonephric tissue expressing *Fgf-8* does not prevent limb bud outgrowth (Fernandes-Teran et al 1997). When interaction between Wolffian duct and intermediate mesoderm is blocked using barriers, mesonephric differentiation is prevented there is no *Fgf-8* expression is in the mesonephric tissue. Nevertheless limb buds form and limb development is normal suggesting that FGF-8 is not the factor responsible for limb bud initiation (Fernandes-Teran et al 1997). It is possible that *Fgf-8* acts via a relay mechanism inducing another signal in the lateral plate mesoderm such as *Fgf-10*. (Ohuchi et al 1997). *Fgf-10* knock out mice show that FGF-10 is required for limb development (Min et al 1998; Sekine et al 1999).

1.4 Signalling during limb bud development

During formation of the vertebrate limb, cells must be specified along three axes, proximo-distal, anterior-posterior and dorso-ventral (Fig.1.2b). These axes can be defined in relation to a hand, where, proximal refers to regions closer to the body wall,

distal to the tip of the fingers; thumb is at anterior, and little finger at posterior while dorsal refers to the back of the hand and ventral to the palm. A number of molecules involved in signalling have been identified and signalling systems along the three axes, interact with one another (Reviewed Tickle 1996, Tabin 1991; Johnson et al 1994; Johnson and Tabin 1997; Cohn and Tickle 1996).

1.4.1 Proximo-distal development of the limb

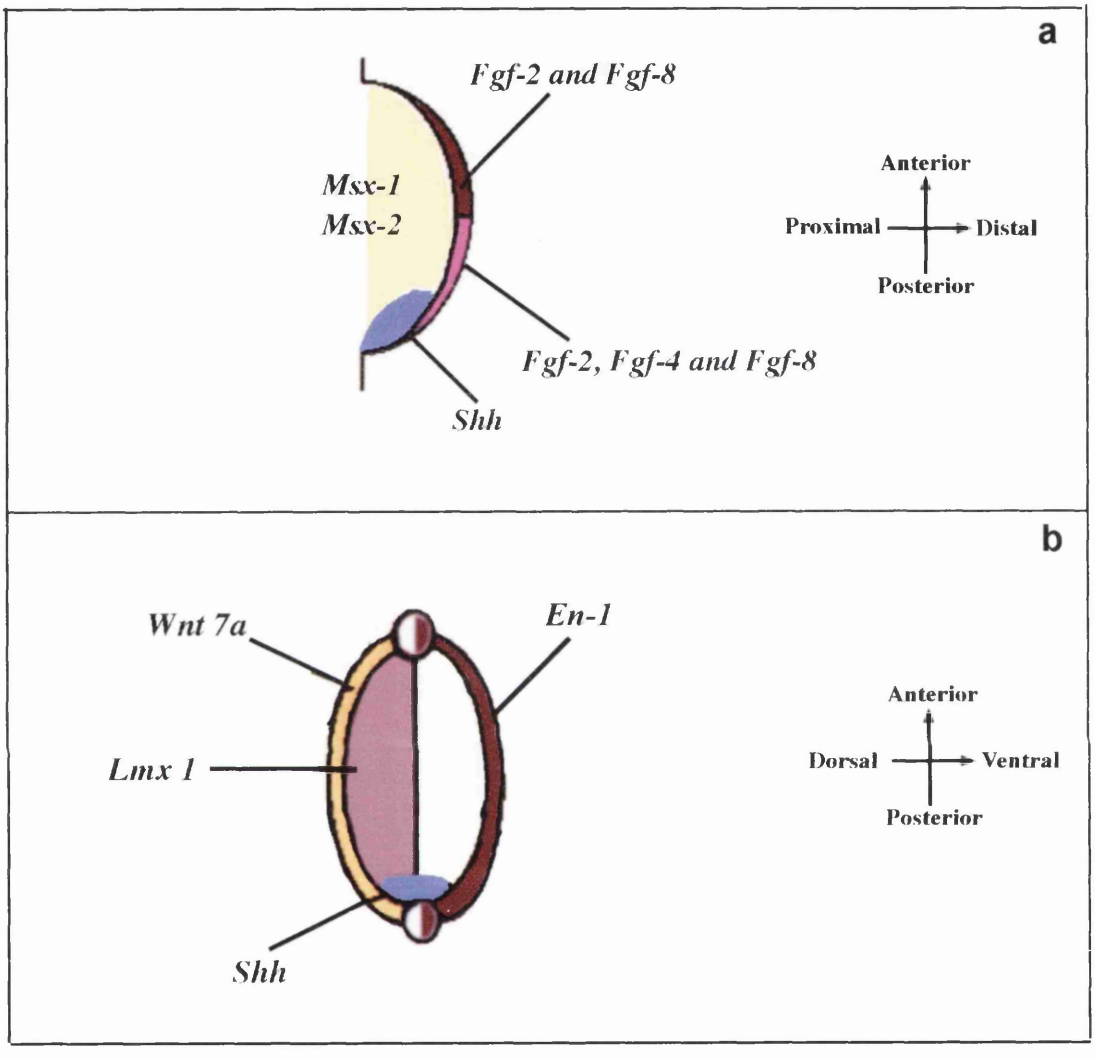
Signals that control limb bud outgrowth are produced by the apical ridge. The apical ridge thus mediates bud outgrowth and maintains a zone of undifferentiated mesenchyme cells, the so-called progress zone, at the tip of the limb bud. When the ridge is removed, the bud stops elongating and the limb that develops is truncated (Saunders 1948, 1972; Summerbell 1974b). The severity of truncation depends on the time of apical ridge removal. Skeletal structures develop along the proximo-distal (P-D) axis of the limb in sequence, with proximal structures forming first and distal structures last. Thus removal of ridge at early stages, results in a severely truncated limb, while, when the ridge is removed later, only distal-most skeletal elements such as digits are missing and proximal structures develop normally. Further, when an additional ridge is grafted to the surface of a limb bud, a second outgrowth is induced (Saunders et al 1976). However the ridge does not dictate which structure will develop from the mesenchymal cells at any particular stage, as when apical ridge is exchanged between limbs of different stages, the limb pattern develops according to the mesenchyme (Rubin and Saunders 1972). Similarly, tissue recombinations in which mesoderm and ectoderm of hind and forelimbs are interchanged show that mesoderm and not ectoderm determines the type of limb formed (Zwilling 1955) Recently it was shown that *Tbx* genes that encode transcription factors play a role in determining limb identity. *Tbx-5* and *Tbx-4* are expressed exclusively in wing and leg bud respectively (Isaac et al 1998; Logan et al 1998). *Pitx1* which is expressed in the posterior region of the early embryo can induce *Tbx-4* expression and when misexpressed in chick wing bud alters some features of the wing into leg-like features (Logan and Tabin 1999).

A number of genes that encode secreted molecules are expressed in the apical ridge (Fig. 1.2a). Among them are FGFs, *FGF receptor 1* (Peters et al 1992; Noji et al 1993), Bone Morphogenetic Proteins (BMP) *BMP-2* (Lyons et al 1990), *BMP-4* (Jones et al 1991), *Retinoic Acid Receptor ß* (Schofield et al 1992) and the vertebrate homologues of *Drosophila* genes *distalless* (*dlx*) and *engrailed* (Dolle et al 1992). A number of FGFs have been described and three of them are expressed in the apical ridge, *Fgf-2*, *Fgf-4*

Figure 1.2 Molecules involved in patterning the limb bud

a) Limb bud showing expression of *Fgf-2*, *Fgf-4* and *Fgf-8* in apical ectodermal ridge; *Shh* in posterior distal mesenchyme of the bud, *Msx-1* and *Msx-2* in distal mesenchyme.

b) Molecules that participate in dorso-ventral patterning in transverse section through a limb bud.*Wnt-7a* in dorsal ectoderm; *En-1* in ventral ectoderm and ventral apical ridge; *Lmx-1* in dorsal mesenchyme. *Wnt-7a* helps to maintain *Shh* expression.


a
Fgf-2 and Fgf-8
Msx-1
Msx-2
Fgf-2, Fgf-4 and Fgf-8
Shh
Anterior
Proximal
Distal
Posterior
b
Wnt 7a
En-1
Lmx 1
Shh
Anterior
Dorsal
Ventral
Posterior

(Niswander and Martin 1992; Suzuki et al 1992) and *Fgf-8* (Crossley and Martin 1995) and play a major role in signalling between AER and underlying mesoderm. *Fgf-2* and –*Fgf-8* are expressed along the entire length of the apical ridge while *Fgf-4* is expressed in the posterior half of the ridge (Niswander and Martin 1992; Suzuki et al 1992; Savage et al 1993; Dono and Zeller 1994). FGF-4 can maintain proliferation of cells in organ cultures of mouse limb stripped of their AER (Niswander and Martin 1993a). All 3 FGFs can substitute for the AER signal and rescue the limb when ridge is removed (Niswander et al 1993a; Fallon et al 1994; Crossley et al 1996; Vogel et al 1996). Bone Morphogenetic Proteins (BMPs), belonging to the transforming growth factor (TGF) superfamily are involved in bone formation and repair (reviewed Rosen and Thies 1992). Three of these, *Bmp-2*, *-4* and *–7* are expressed in the AER (Francis et al 1994) but their role in limb bud outgrowth is not clear. However, misexpression of *Bmp-2* in chick limb bud, leads to the ectopic activation of *Fgf-4* in the AER (Duprez et al 1996b). Another group of genes involved in proximo-distal patterning are members of the *Distal-less* (*Dlx*) family (Simeone et al 1994), *Dlx-1*, *-2* and *–5* expressed in the apical ridge (Ferrari et al 1995).

The progress zone is the undifferentiated limb mesenchyme at the tip of the limb bud where cells divide. The length of time spent by cells in this zone specifies which structures they will form. Cells that spend a short time in the progress zone form proximal structures, whereas cells that stay longer in the progress zone form distal structures (Summerbell et al 1973). Time spent in this zone may be measured by the number of cell divisions that a cell undergoes.

Mesenchymal cells in the progress zone express several genes encoding regulatory proteins. *Msx-1* and *Msx-2* are two related homeobox genes expressed in the progress zone (Hill et al 1989; Robert et al 1989) (Fig. 1.2a). *Msx-2* is strongly expressed in the ridge and *Msx-1* weakly. Expression of *Msx-1* in distal mesenchyme is regulated by the ridge as, when proximal non-expressing cells were transplanted distally they expressed *Msx-1* (Davidson et al 1991) and, when the apical ridge was removed, expression in cells in progress zone was lost (Ros et al 1992). Application of FGF-4 protein induces *Msx-1* (Kostakopoulou et al 1996). It is possible that *Msx* genes are responsible for maintaining the lability of cells in the progress zone. In this context, it is interesting that Song et al (1992) showed that when a myogenic cell line was transfected with *Msx-1*, differentiation was inhibited.

1.4.2 Patterning along the anterior-posterior axis

The discovery of the polarising region, a group of mesenchymal cells at the posterior margin of the limb bud (Saunders and Gasseling 1968), opened up avenues into the study of patterning along the anterior-posterior axis. When an additional polarising region is grafted at the anterior margin of the limb in contact with the apical ridge (Saunders and Gasseling 1968), additional digits develop. Instead of the normal digit pattern of 2 3 4, the wing develops a mirror image symmetry with digit pattern of 4 3 2 2 3 4. The extra elements are derived from the host tissue indicating that the polarising region is a signalling centre. When polarising region is grafted at the apex of the limb bud in contact with the apical ridge the digit pattern that develops is 2 3 4 4 3 4 from anterior to posterior. The polarising region can thus act on cells both anteriorly and posteriorly. It has been shown that the specific ectopic digit formed depends on the number of polarising region cells that are grafted indicating that the polarising region acts in a dose-dependent manner.

These data fit a model in which the polarising region is a source of a diffusible morphogen (Tickle et al 1975). A morphogen is a gradient-forming molecule that specifies in a concentration-dependent fashion the fate of cells. The postulated morphogen produced in the polarising region would diffuse anteriorly in a graded fashion, such that, cells at different positions along anterior-posterior axis will be exposed to different concentrations of morphogen. The different concentrations of the signal are interpreted by particular cells as a specific "positional value" and this allows them to differentiate into particular skeletal elements. Low levels of morphogen would specify digit 2 and high levels a digit 4 (Tickle et al 1975). This long-range signalling would have to occur over several diameters of cells.

Retinoic acid mimics the effect of polarising region grafts, can signal in a dose-dependent manner and is freely diffusible in the limb bud (Tickle et al 1982; 1995). In vivo, in the chick limb bud, retinol, which is the main dietary form of vitamin A, can enzymatically be converted into all-*trans*-retinoic acid (Thaller and Eichele 1990). A high level of endogenous retinoic acid is present at the posterior region of the limb bud (Thaller and Eichele 1987, Maden et al 1998). When applied exogenously, retinoic acid forms a concentration gradient across the anterior-posterior axis (Tickle et al 1985) and digits are duplicated. However controversy over the role of retinoic acid exists to this day. Retinoic acid can confer polarising activity to cells, since cells adjacent to the retinoic acid soaked bead acquire polarising activity (Wanek et al 1991; Noji et al 1991;

Tamura et al 1993) and expression of *RAR-β*, a retinoic acid receptor is induced (Noji et al 1991). On the other hand, polarising grafts do not confer polarising activity to neighbouring cells nor can they activate *RAR-β* expression, suggesting that retinoic acid may not be the polarising region morphogen. There is evidence however that retinoic acid may play a role in establishing the polarising region (Helms et al 1996; Stratford et al 1996).

Expression of the signalling molecule *Sonic hedgehog (Shh)* (Riddle et al 1993) co-localises with the polarising region in the chick limb bud (Fig. 1.2a). Grafting Sonic Hedgehog producing cells or protein at the anterior margin of the limb bud produces mirror image duplications similar to those induced by a polarising graft (Riddle et al 1993; Lopez-Martinez et al 1995; Yang et al 1997). Thus *Shh* functions as a regulator of anterior-posterior patterning (Niswander and Martin 1992; Niswander et al 1993; Laufer et al 1994; Haramis et al 1995). However it is not known whether Shh acts directly or through a relay mechanism. Immunohistochemistry suggests that Shh does not diffuse far from the cells in which it is synthesised (Marti et al 1995; Lopez-Martinez et al 1995) and experiments have suggested that Shh regulates anterior-posterior patterning via BMPs (Yang et al 1997). Expression of *BMP-2*, a member of the TGF-ß superfamily, overlaps with the expression of *Shh* in the mesoderm (Francis et al 1994; Francis-West et al 1995) and Shh induces ectopic *Bmp-2* expression in the anterior mesoderm (Laufer et al 1994; Niswander et al 1994). However BMP-2 alone cannot re-specify structures along the anterior-posterior (Francis et al 1994).

Fgf-4 expressed in the AER is important for creating a positive feedback loop with *Shh*. In the chick limb bud in vivo, when AER is removed, application of FGF-4 at posterior limb bud margin maintains polarising activity, which would have been lost due to removal of AER (Vogel and Tickle 1993). Thus signalling along each axis does not occur independently. FGF-2 also can maintain polarising activity (Anderson et al 1993) and promote growth along the P-D axis when AER is removed (Fallon et al 1994).

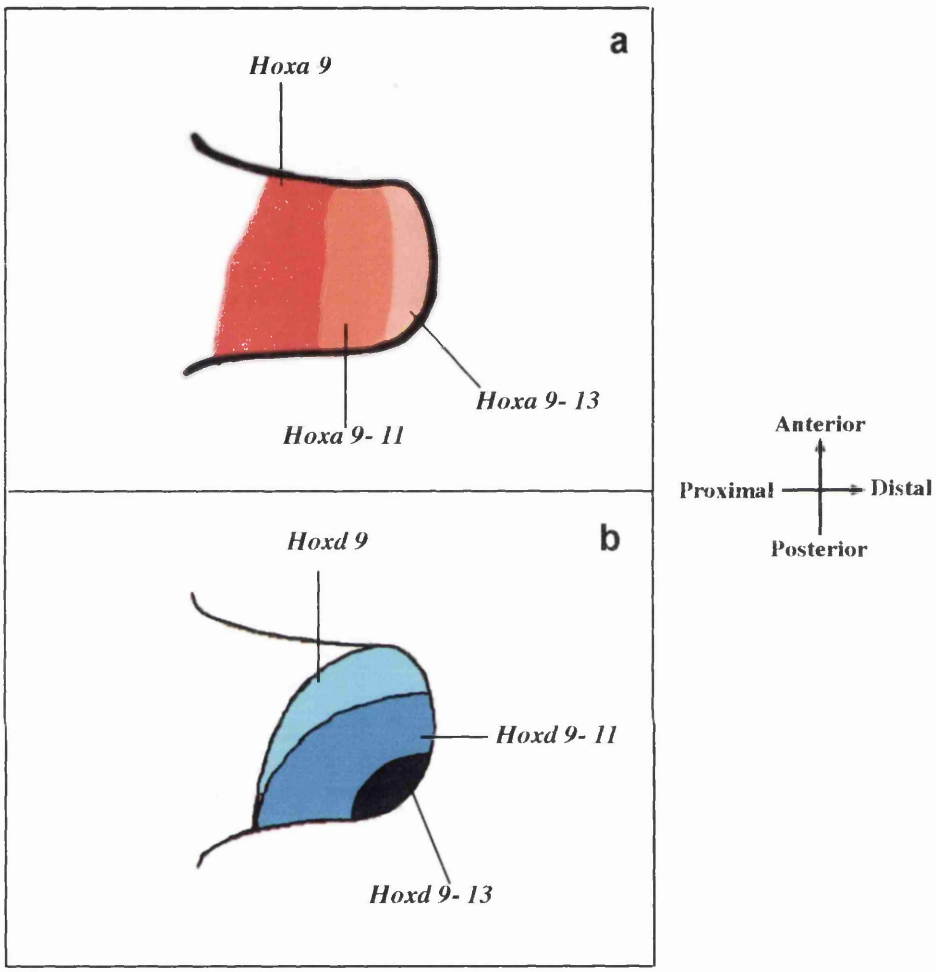
Hox genes

Grafts of polarising region, or application of retinoic acid or Shh at anterior margin of the limb bud induce ectopic expression of *Hoxd* genes (Ispizua-Belmonte et al 1991, 1992; Nohno et al 1991; Riddle et al 1993; Nelson et al 1996) prior to the appearance of ectopic digits. This suggests that anterior-posterior asymmetry maybe established by sequential activation of the *Hoxd* genes in the proliferating mesoderm.

Figure 1.3 Diagrammatic representation of expression pattern of *Hoxa* and *Hoxd* genes in developing limbs

a) *Hoxa*-9 to *-13* is expressed along the proximo-distal axis.

b) *Hoxd*-9 to -13 is expressed in nested domains along the anterior-posterior axis


a
Hoxa 9
Hoxa 9- 13
Hoxa 9- 11
b
Hoxd 9
Hoxd 9- 11
Hoxd 9- 13
Anterior
Proximal
Distal
Posterior

Hox genes encode homeodomain-containing transcription factors that exhibit a striking pattern of nested gene expression along the anterior-posterior and proximo-distal axes of the limb. *Hox* genes are organised into four clusters (*HoxA*, *HoxB*, *HoxC* and *HoxD*) and the organisation of these vertebrate genes is closely related to that of *Drosophila Bithorax* and *Antennapedia* homeotic complexes. The spatial and temporal expression of *Hox* genes correlates with their positional order in the genome such that genes located at the 3′ end of the cluster are expressed prior to those at the 5′ end and is called temporal colinearity. In addition the 3′ gene expression domains extend more anteriorly than the 5′ domain and this is called spatial colinearity (Fig. 1.3).

Hoxd and *Hoxa* are expressed along the anterior-posterior and proximo-distal axes of the limb bud and sequentially activated from the 3′ to 5′ during early limb bud development, leading to colinear and nested domains of expression (Izpisua-Belmonte and Duboule 1992; Yokouchi et al 1991). Initially *Hoxd-9* and *Hoxd-10* are expressed along the entire A-P length of both limb buds. Subsequently *Hoxd-11*, *Hoxd-12* and Hoxd-13 are activated sequentially at the posterior border of the limb bud (Nelson et al 1996) creating the nested pattern of *Hoxd* expression (Fig. 1.3b). The expression domain of *Hoxa-13* is also restricted to the distal region of the early limb bud while *Hoxa*-10, 11 are expressed more proximally (Yokouchi et al 1991; Haack and Gruss 1993) (Fig. 1.3a). *Hoxd* and *Hoxa* gene expression patterns subdivide the limb along the anterior-posterior and proximo-distal axes respectively.

Hox gene ablation experiments in mice showed that lack of a particular gene gives a phenotype with specific skeletal elements missing. In each case, the missing element derives from tissue that would normally express the ablated gene (Zakany et al 1997). However the severity of the phenotype is more pronounced in double or triple *Hox* mutants (Small and Potter 1993; Davis and Capecchi 1994, 1996; Favier et al 1995; Davis et al 1995). For example, by using loss-of-function alleles of various *Hox* genes, it has been shown that the size and number of digits is strictly linked to levels of the most posterior members *(11, 12* and *13*) of the *Hoxa* and *Hoxd* complexes. The combination of these studies suggests that *Hox* proteins pattern the vertebrate limb in a quantitative, dose-dependent manner, rather than through a *Hox* code in which different genes have different qualitative functions (Zakany et al 1997). Misexpression studies in chick also suggested that the spatial pattern of *Hoxd* gene expression was related to anterior-posterior axis development (Morgan et al 1992; Goff and Tabin 1997).

A mutation in *Hoxd-13* has been shown to be responsible for the human synpolydactyly condition. In this limb abnormality in humans, there is syndactyly of fingers 3 and 4 with either an extra digit or metacarpal in the hand, whereas, in the leg, there is syndactyly of toes with associated postaxial polydactyly (Muragaki et al 1996). Overall, digits are very short and Xrays reveal that the metacarpals/metatarsals are transformed into carpals/tarsals and there are extra carpal bones as well. *Hoxd*-$13^{-/-}$ mice (Dolle et al 1993) have some similarities, such as short and thick metacarpals. The appearance of extra digits suggests that *Hoxd*-13 may be required for patterning along both the anterior-posterior and the proximo-distal axes.

1.4.3 Patterning along dorso-ventral aixs

1.4.3.1 Role of ectoderm

Patterning of structures across the dorso-ventral axis of the limb is controlled by signals from the ectoderm. Evidence for this came initially from recombinant experiments in which an ectodermal jacket was rotated 180° such that dorsal ectoderm was in contact with the ventral mesenchyme and vice versa. The resulting limb had reversed dorso-ventral polarity (Pautou and Kieny 1973; MacCabe et al 1974; Geduspan and MacCabe 1987; 1989; Akita 1996). When an additional apical ridge is grafted to the dorsal or ventral ectoderm of the limb, outgrowths resulted which were bi-dorsal and bi-ventral respectively (Errick and Saunders 1976; Shellswell and Wolpert 1977). In the homozygous embryos of the chick mutant, *eudiplopodia*, ectopic AERs form on the dorsal surface resulting in extra digits with conical nails that lacked normal ventral curvature (Goetinck 1964). In homozygous *limbless* chick mutant (Prahlad et al 1979, Fallon et al 1983), limbs are initiated normally, but regress because they never form apical ridge. When an FGF bead was applied to the rudimentary bud of the mutant, the limb was rescued but bi-dorsal limbs developed (Noramly et al 1996; Ros et al 1996; Grieshammer et al 1996).

1.4.3.2 Models for dorso-ventral patterning

Different gradient models (Parr and McMahon 1995; Akita 1996, reviewed in Tickle 1995) similar to those put forward for anterior-posterior patterning have been suggested wherein long range signals from the ectoderm or apical ridge are responsible for dorso-ventral patterning of tissues in the limb. Serial short range signalling may also be involved (Tickle 1999). It is not clear whether dorso-ventral pattern depends on

distance from the ectoderm or on dose dependent signalling by the ectoderm. In fact, muscle and tendon primordia form just beneath the ectoderm (Murray and Wilson 1997; Ros et al 1995; Patel et al 1996) and, later come to be related to the cartilage elements deep in the mesoderm. This suggests that cells maybe told how to differentiate and then they get displaced to the correct position along the dorso-ventral axis (Tickle 1999).

1.4.3.3 Molecules involved in dorsal patterning

Recently, genes expressed in the ectoderm have been identified. *Wnt-7a*, a signalling molecule expressed in dorsal ectoderm (Parr et al 1993), induces *Lmx-1*, a LIM homeobox-containing gene, in the dorsal mesenchyme of the limb bud and both play a role in dorsal development (Parr and McMahon 1995; Riddle et al 1995; Vogel et al 1995). Mice lacking a functional *Wnt-7a* gene have ventralised limbs (Parr and McMahon 1995) while retrovirus-mediated ectopic expression of *Lmx-1* in ventral mesenchyme of chick limbs causes dorsalisation (Riddle et al 1995; Vogel et al 1995). *Lmx-1* appears to be a downstream target of *Wnt-7a* signalling, since ectopic expression of *Wnt-7a* in the limb bud induces *Lmx-1* expression in ventral mesenchyme but the converse is not true. Removal of dorsal ectoderm also results in loss of *Lmx-1* in the underlying mesenchyme. *En-1*, a homeobox-containing gene related to *Drosophila engrailed*, is expressed in ventral ectoderm. Mice homozygous for a null allele of *En-1* develop limbs that are dorsalised, thus indicating that *En-1* function is required for ventral development of the limb (Loomis et al 1996). The chick *limbless* mutant, develops bi-dorsal structures, when rescued with FGF, and *Wnt-7a* is expressed in the entire ectoderm of the rudimentary bud while *En-1* is absent (Ros et al 1996). The roles of *Wnt-7a* signalling and of *En-1* and *Lmx-1* expression in tendon development are examined in this thesis in chapter 5.

a) *Wnt* gene family

The *Wnt* gene family encodes a large and diverse group of signalling molecules (Nusse and Varmis 1992). There are more than 12 closely related *Wnt* genes in vertebrates (Nusse and Varmus 1992). These genes encode secreted proteins that are associated with cell surface and extracellular matrix and thus may act locally (Bradley and Brown 1990; Papkoff and Schryer 1990; van den Heuvel et al 1989; Jue et al 1992). Many members of the *Wnt* gene family are implicated in regulation of pattern formation and morphogenesis in vertebrate and invertebrate development (reviewed McMahon 1992; Nusse and Varmus 1992). The vertebrate *Wnt* gene family is related to the

Drosophila segment polarity gene *Wingless* (*wg*) (Rijsewijk et al 1987). In Drosophila, *Wingless* (*wg*) has a function in the organisation of wing pattern along the dorso-ventral axis. *Wnt-1* the homologue of *Wingless*, is not expressed in mouse limbs. However, ectopic Wnt-1 expression in the developing limb bud leads to disruption in normal limb development showing that *Wnt* signalling is important (Zakany and Duboule 1993).

Several *Wnt* genes are expressed in the developing limb bud. In 9.5 day mouse embryo, *Wnt-3*, *Wnt 4*, *Wnt-6* and *Wnt-7b* are expressed fairly uniformly through out the limb ectoderm. *Wnt-7a* is expressed in dorsal ectoderm and *Wnt-5a* in the ventral ectoderm and mesoderm (Parr et al 1993) and *Wnt 12* is expressed in the ridge from 10.5 dpc in mouse (Christiansen et al 1995). Recently it was shown that *Wnt-10b* was expressed in epithelium (Wang and Shackleford 1996) and mesoderm (Cygan et al 1997) in the mouse. *Wnt 11* is expressed in mesenchyme from the time the limb bud is visible (Christiansen et al 1995). *Wnt-5a* has also been shown to be expressed in chick limb buds in the apical ridge and in 3 quantitatively distinct domains of expression in the mesenchyme. In early limb bud (stage 19) *Wnt-5a* is expressed in mesoderm in an antero-posteriorly graded fashion with the highest expression biased posteriorly. Later (stage 22-25) there are domains along the proximo-distal axis of the limb mesoderm that may correspond to the regions that will give rise to the 3 distinct proximo-distal segments of the limb i.e. autopod, zeugopod and stylopod (Dealy et al 1993).

Wnt genes are also expressed in the nervous system. In the chick, *Wnt-7a* transcripts are present (stage 20) in lateral mesencephalon and the ventro-lateral regions of the myelencepalon flanking the floor plate (Dealy et al 1993). *Wnt-5a* is also present in the central nervous system of the chick in the ventral midline of the mesencephalon and myelencephalon. In mouse and frog embryos, expression of other members of the *Wnt* family have also been described in distinctly different sectors of the neural tube. *Wnt-1* and *Wnt-3a* are expressed in the roof plate (Wilkinson et al 1987; Roelink and Nusse 1991; Wolda et al 1993) and *Wnt-4* in the floor plate (McGrew et al 1992). The presence of these genes in the central nervous system suggests that distinct members of the *Wnt* family might be involved in specification of positional identities along the dorso-ventral axis of the spinal cord (Dealy et al 1993).

Members of the *Frizzled* gene family are involved in the response to *Wnt* signalling. It has been suggested that *Frizzled* a gene that encodes a cell-surface protein with an N-terminal cysteine-rich domain (CRD) and seven putative transmembrane segments. may be a receptor for *Wnt* proteins (Bhanot et al 1996;Yang-Snyder et al 1996; He et al 1997; Perrimon et al 1996). In parallel, a secreted frizzled-like protein Frzb-1, with a

conserved N-terminal CRD domain was identified (Hoang et al 1996) capable of binding to members of the *Wnt* family in vitro (Wang et al 1997; Leyns et al 1997). *mFrzb-1* in the mouse is seen at 9.5 dpc in ventral limb mesenchyme, in a pattern complementary to the expression of *Wnt-7a* in the dorsal ectoderm (Hoang et al 1996). Later, expression is in perichondrium surrounding condensing cartilage. Recent experiments indicate that *mFrzb-1* physically interacts with *Wnt-7a* in vitro (Lin and Luyten unpublished data- in Hoang et al 1998) and both are expressed in the same stage in vivo. The complementary expression pattern of *mFrzb-1* and *Wnt-7a* is similar to that seen with *Frzb-1* and *Wnt*-8 in Spemann's organiser (Wang et al 1997; Leyns et al 1997). This inverse pattern of *Frzb-1* and *Wnt-7a* expression may enforce a gradient of *Wnt-7a* activity across the dorso-ventral axis of the limb thereby excluding dorsalising signals from the ventral portion of the limb bud (Hoang et al 1998). It is also suggested that *Wnt-7a* may have an inhibitory effect on cartilage differentiation as when, chick micromass cultures were infected with retoviral vector carrying *Wnt-7a* cDNA, no cartilage nodules were detected (Rudnicki and Brown 1997).

b) LIM Homeobox genes

Members of several families of transcription factors are involved as regulators of embryonic pattern. LIM homeobox genes (German et al 1992) belong to one such family and encode proteins characterised by a pair of cysteine-histidine rich LIM domains and a homeodomain (reviewed Sanchez-Garcia and Rabbitts 1994; Dawid et al 1995). They appear to function as regulators of cell fate in different tissues and organisms (reviewed Curtiss and Heilig 1998). Expression of the *Drosophila apterous (ap)* gene, encoding a protein of the LIM-homeodomain family, is restricted to the dorsal compartment of the wing disc and is required for dorso-ventral compartmentalisation and for specification of dorsal cell fates (Cohen et al 1992; Blair 1995). In vertebrates *Lmx-1* (chick) and *Lmx-1b* (mouse), LIM homeoxbox genes are expressed in dorsal limb bud mesenchyme (Riddle et al 1995; Vogel et al 1995; Cygan et al 1997).

Lmx-1 transcripts are first detected in the chick limb mesenchyme at stage 15, at the same time of onset as *Wnt-7a*. At stage 17, expression is confined to the region of the limb mesoderm adjacent to the prospective dorsal ectodermal domain that expresses *Wnt-7a*. At stage 21, when *Wnt-7a* is expressed in dorsal limb ectoderm, *Lmx-1* is present in the dorsal mesenchymal cells with a sharp boundary at the border between dorsal and ventral mesenchyme and persists till stage 30 (the latest stage examined

Vogel et al 1995). *Lmx-1* is excluded from the cells in dorsal mesenchyme that had begun the process of chondrification (Riddle et al 1995). Thus onset and pattern of expression of *Lmx-1* in dorsal mesenchyme follows closely that of *Wnt-7a* in the dorsal ectoderm and defines a dorsal mesenchymal domain in the limb bud (Riddle et al 1995).

c) Ventral cues involved in dorso-ventral patterning

Engrailed-1 (*En-1*), a homeodomain-containing transcriptional regulator expressed in ventral limb ectoderm (Davis et al 1991; Gardner and Barald 1992) (Fig 1.2b), is critical for directing the development of ventral limb structures and appears to act, in part by suppressing *Wnt-7a* function in the ventral limb (Loomis et al 1996; Logan et al 1997). *En-1* is first expressed in ventral ectoderm of the chick embryonic trunk at stage 15 (just like *Wnt-7a* and *Lmx-1*) predominantly in the presumptive limb fields and by stage 16 is expressed throughout the ventral body wall. At stage 18, the anterior limit of expression is at anterior edge of wing bud at level of somite 15, but posteriorly expression continues under tail bud through the midline and merges with expression on contralateral side. In the limb bud, expression is uniform in the ventral ectoderm and extends into ventral half of apical ridge. Expression persists at least until stage 25, the latest stage examined (Logan et al 1997).

Phenotype of mice homozygous for *En-1* loss of function alleles, suggest that *En-1* regulates dorso-ventral patterning of the limb. *En-1* mutants also have extra digits. Digits in the mutants are flexed dorsally and have circumferential nails on digits 1,2,3 and 4. Hair follicles are present in dorsal integument but eccrine glands that normally are present on ventral side are absent. Mesodermal derivatives, such as tendons were poorly developed or absent, and sesamoids characteristic of ventral side were absent (Loomis et al 1996). *En-1* mutants not only have partial dorsal transformation of the ventral paw but also display a ventro-proximal expansion of the apical ridge suggesting that an additional regulatory relationship exists between dorso-ventral patterning of the limb and apical ridge formation. (Loomis et al 1996). In the chick *limbless* mutant, no apical ridge is formed, *Wnt-7a* is uniformly expressed throughout the limb ectoderm and *En-1* is lacking ventrally (Ros et al 1996; Grieshammer et al 1996).

Misexpression of *En-1* in dorsal ectoderm using a retrovirus, repressed endogenous expression of *Wnt-7a* in dorsal ectoderm and expression of *Lmx-1* in underlying mesoderm is decreased (Logan et al 1997). As infected embryos did not show complete ventralisation of dorsal mesoderm, it was suggested that other factors in addition to *En-1*, may be required to transform dorsal mesenchyme to a more ventral phenotype.

Alternately, dorsal mesoderm may not be capable of responding to ventralising signals. In dorso-ventral rotation of ectoderm experiments, bi-ventral phenotypes are rarely observed (MacCabe et al 1974; Geduspan and MacCabe 1987; 1989; Akita 1996). Akita (1996) found that ventral mesoderm was more readily respecified than dorsal mesoderm following rotation of ectoderm. In addition, recombinations made at earlier stages showed a more complete reversal in dorso-ventral patterning and distal regions were more affected than proximal ones. When *En-1* was ectopically expressed in dorsal ectoderm, similar results were found in that, distal structures were more affected than proximal ones (Logan et al 1997). The *Wnt-7a* mutant also shows incomplete ventralisation affecting primarily distal structures (Parr and McMahon 1995).

1.5 Differentiation of tissues in the limb

Chick-quail chimeras reveal that tendon and muscle have different embryonic origins. Mesenchymal cells in the limb bud are derived from two sources. The lateral plate mesoderm gives rise to tendons, cartilage and other connective tissue elements, whereas cells from the somites, migrate into the limb bud to give rise to the myogenic cells of the muscles (Chevalier et al 1977, Christ et al 1977, Jacob et al 1979. The migrating muscle cells become organised into pre-muscle masses, which then split to give rise to individual muscles (Schroeter and Tosney 1991). The early myogenic cells that migrate into the limb receive cues about which muscle they should form from the connective tissue, which is derived from the lateral plate mesoderm. When somites from leg level are transplanted to a wing level, the muscle cells that migrate into the wing form normal wing muscles (Butler et al (1988) indicating that limb environment controls patterning. Similarly, when the skeletal pattern of wing was duplicated, either by grafting polarising region or adding a bead soaked in retinoic acid, the muscle pattern was duplicated (Shellswell and Wolpert 1977; Robson et al 1994). Thus it appears that appropriate tissue arrangement depends on common connective tissue patterning, which may also involve expression of regulatory genes such as the *Hox* genes (Yamamoto et al 1998).

Even though a number of gene expression patterns have emerged, there is a large gap in our understanding between patterns of gene expression and the final differentiation of cells to form specialised tissue. BMPs (Bone morphogenetic proteins) that belong to the TGF-ß superfamily, for example, appear to be the signals that regulate cartilage differentiation. However it is not clear how gene expression at a particular site

can bring about particular cells to differentiate into cartilage and how the pattern of differentiation is controlled to give individual skeletal elements.

1.6 Tendons

In this thesis, the main tissue that will be considered is tendon. Tendons attach muscles to bones and transmit the pull generated by the muscle. The position at which a tendon inserts is of key importance to its function. As a rule, tendons in the digits, are attached immediately distal to the joint on which they principally act. Tendon consists of bundles of fine collagen filaments in which sparse cells are embedded. Individual bundles are wrapped in connective tissue. Where it is necessary to minimise friction against adjacent surfaces, tendons are enclosed in a tendon sheath. The inner part of the sheath closely invests the tendon and consists of circumferential collagen fibrils, while the outer is fixed closer to the bone. Between the two layers, a thin lubricant solution which includes hyaluronic acid reduces frictional resistance to the sliding tendon. Tearing of tendons is a common injury and the healing process involves cell infiltration and then laying down of fibrils. Cells involved in the healing process are not resident in the tendon, but come from connective tissue around tendon. Thus a torn tendon that has an intact sheath does not heal well (Potenza 1962; Peacock and Van Winkle 1970).

A number of structures associated with tendons control and facilitate their movements. For example, where flexor tendons pass over joints in the digits, they are held in place by retaining bands called fibrous sheaths. Tendons glide beneath them because they are surrounded by lubricating synovial membrane. In contrast, extensor tendons do not have fibrous or synovial sheaths.

Extracellular matrix (ECM) assembled in a complex structural arrangement is the main component of tendons and accounts for their biomechanical properties (Reviewed Birk and Zycband 1994). The composition of the matrix includes collagens, glycoproteins, proteoglycans and elastic matrix components (Greenlee and Ross 1967; de Carvalho et al 1994; Ros et al 1995). Collagen type I is the main component of ECM of tendons but other collagens such as type III, V, VI and XII are also present (Birk et al 1989; Fleischmajer et al 1991; Tsuzaki et al 1993). Tenascin and Fibronectin are also components of tendons (Swasdison and Mayne 1989; Chiquet and Famborough 1984; Hurle et al 1989).

The appearance of digital tendons is preceded by mesenchymal condensations that form at about the same time as that of cartilage anlage (Greenlee and Ross 1967). The

position of tendons in developing chick toes is marked by accumulation of sheets of tenascin-rich ECM beneath the ectodermal basement membrane (Hurle et al 1989; Ros et al 1991). Mesenchymal cells condense on these sheets in longitudinal rows that are pushed apart by the accumulation of ECM to form tendons. Procollagen, the precursor of collagen synthesis begins within the rough endoplasmic reticulum of fibroblasts and triple helix formation occurs within the Golgi apparatus, but procollagen is converted to collagen in the ECM (Butler et al 1978). Though further assembly of collagen occurs in the ECM, it is completed under cellular control (Birk and Zycband 1994).

1.6.1 Development of tendon

The morphological events of tendon formation in the avian wing and leg, have been described by Sullivan (1962) and Wortham (1948) respectively. Proximally, muscle is inserted into the skeleton almost directly, but distally it is attached to skeleton by tendon. During development, pretendinous mesenchymal blastemas undergo a proximo-distal differentiation process to join muscle to phalangeal elements of the digits. Tendon must migrate towards the nearest epiphysis to avoid being left behind as the bone grows (Frank et al 1988; Grant et al 1978; Dorfl et al 1980a, b) and long bones only grow in length at their epiphyseal plates. However phalanges being the most distal elements in the limb, are the last to be formed (Rowe and Fallon 1982). Hence there is some means of guiding the developing tendons from their origin to their distal phalangeal attachment.

As with other components of the limb, growth and differentiation of tendons progress in a proximo-distal sequence. In the chick embryo, three pairs of tendon primordia recognised by tenascin staining, develop in association with the 3 major joints of the leg bud (Kardon 1998) (Fig. 1.4). Between stage 24-27, the proximal tendon primordia appear at the junction of thigh and shank, the future knee joint. The second pair, the intermediate tendon primordia appear at shank-foot junction, the future intertarsal joint. Finally, the distal tendon primordia appear at the junction of metatarsus and phalanges and at interphalangeal joints. The proximal tendon primordia extend from the basement membrane of ectoderm to the surfaces of the cartilage whereas intermediate and distal tendon primordia develop subjacent to the ectoderm and are not in close proximity with the developing cartilages. Thigh, shank and foot muscle masses both on dorsal and ventral sides differentiate in between the three pairs of tendon primordia. Thigh myotubes differentiate proximal to the proximal tendon primordia; shank myotubes in between proximal tendon primordia and intermediate tendon

Figure 1.4 Relation of muscle and tendon development between stage 24 and 28

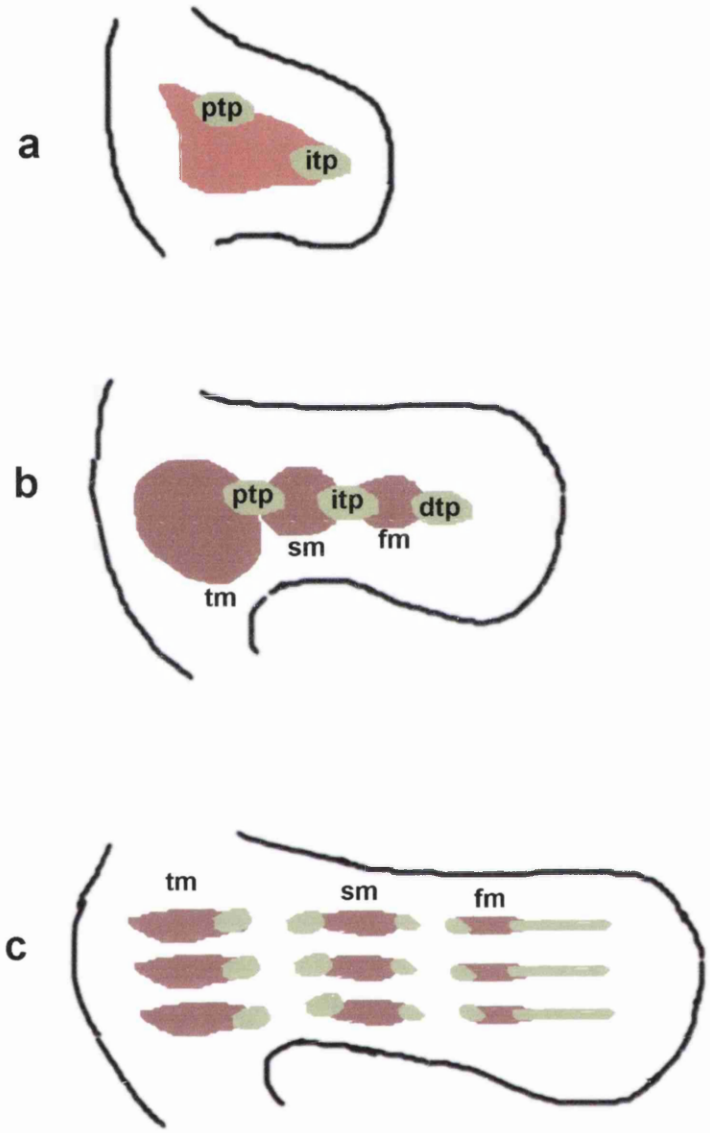
Brown, distribution of myogenic precursors/differentiating myotubes; green, tendon primordia; ptp, proximal tendon primordium; itp intermediate tendon primordium; dtp, distal tendon primordium; tm, thigh muscles; sm, shank muscles, fm, foot muscles.

a) Stage 25. Myogenic precursors in relation to proximal (ptp) and intermediate tendon primordia (itp).

b) Stage 26. Thigh muscle mass (tm) is proximal to proximal tendon primordia (ptp), shank muscle is between (ptp) and (itp) and foot muscles (fm) are between (itp) and (dtp).

c) Beyond stage 28. Tendon primordia form origin and insertion tendons for the muscles that lie on either side of it. (Figure adapted from Kardon 1998).

Fig. 1.4



primordia and foot myotubes differentiate between intermediate and distal tendon primordia (Fig. 1.4b). Tendon primordia become anatomically connected with the thigh, shank and foot muscles. Proximal tendon primordia will give rise to insertion tendons of thigh muscles and origin tendons for shank muscles; intermediate tendon primordia will form insertion tendons for shank muscles and origin tendons for foot muscles; distal tendon primordia will form insertion tendons for foot muscles (Fig. 1.4c) and some of the shank muscles that insert into the phalanges. Individual tendons form adjacent to their associated muscle. The distal parts of the long tendons of the foot are derived from the dorsal and ventral tissue extensions around metatarso-phalangeal and interphalangeal joints. These tissue extensions appear concurrently with the formation of their associated joints and capsules.

Distal tendon primordia subdivide into four tendinous blastemas associated with the four toes subjacent to ectoderm on dorsal and ventral sides of the developing cartilage (Hurle et al 1989; 1990; Ros et al 1995; Kardon 1998) and later these blastemas segregate into individual tendons. The long tendons of the foot form concomitantly with the establishment of the skeletal elements (Wortham 1948; Sullivan 1962; Hurle 1990).

1.6.1.1 Formation of digital tendons

In the autopod of the chick leg, tendons develop between day 6 and 9 of development (stages 29-35) along the dorsal and ventral surfaces of the developing digital rays and is described in 6 stages (Ros et al 1995). At stage I, (stage 29 of chick embryo development), the ectoderm-mesenchyme interface first gets thickened in the digital region and immediately thereafter at stage II, a mesenchymal lamina (Hurle et al 1989) of extracellular matrix appears running parallel to the basement membrane. The mesenchymal lamina is continuous both distally and laterally with the ectodermal basement membrane and mesenchymal cells are densely packed in the proximity of the lamina, but a tendon blastema is not yet recognisable. At stage III (day 6.5 to 7), an irregular cellular aggregation is recognisable in the centre of the mesenchymal lamina, but the outline is not well defined. At stage IV, the aggregate is clearly recognisable, but there is still no clear boundary between it and the surrounding mesenchyme and the lamina on either side of the cellular aggregate is still in continuity with the basement membrane (Fig. 3.19a, b). At stage V, (day 7-8) the cellular aggregate is clearly demarcated from the surrounding mesenchyme, remnants of the lamina are within the tendon blastema but the lamina has disintegrated on either side. At stage VI, (day 8) the tendon blastema is well defined and is surrounded by flattened cells, the epitenon. As

tendons develop in a proximo-distal sequence, they are at different stages of development along their length. For example at stage VI the distal tendons cells are just assembling around the mesenchymal lamina. From day 9-10, tendons undergo a stage of maturation along the entire length (Ros et al 1995).

1.6.1.2 How do tendons attach appropriately to the skeleton?

Dorso-ventral fibrils, rich in tenascin, collagen I and fibronectin, run dorsoventrally from the mesenchymal lamina towards the differentiating phalanges and it is suggested that this fibrillar scaffolding may represent a transitory structural system attaching the developing tendon blastema and ectoderm to the differentiating cartilage (Hurle et 1989b). Mechanical tension across the developing tendons may then select the definitive zones of skeletal attachment of each tendon. The perichondrium distal to the developing joints may have a major involvement in the patterning of the tendinous insertions. (Hurle et al 1990).

1.6.1.3 Muscle-tendon-cartilage relationship

Initial development of tendon occurs independent of its muscle. When tendons and muscles are prevented from interacting by grafting the forelimb region of the chick embryo onto the flank, the normal pattern of digital tendons appear, but later regress (Shellswell and Wolpert 1977; Kardon 1998). When muscle precursor cells are ablated prior to migration into limb, similar results are obtained (Kieny and Chevalier 1979). If the tip of the bud is inverted, tendons develop normally but join to the wrong muscles, ventral tendons to dorsal muscles and vice versa (Shellswell and Wolpert 1977). This shows that muscle is necessary for further development and maintenance of a tendon and that any muscle suffices, so long as its direction of pull matches that of tendon. This supports the idea (Miliare 1963) that tendon and muscle blastemas develop autonomously and join up later. However, others suggest that muscles and tendons develop in contact and in tandem (Sullivan 1962, Wortham 1948; Kardon 1998). Development of muscle and tendon and the muscle-tendon-bone insertion complex may involve separate mechanisms (Hurle et al 1990). Mature myotendinous junctions do not begin to form until stage 34 (Tidball and Lin 1989), but developing muscle and tendon may be closely associated as early as stage 25/26.

Studies on the temporal and spatial relationship between tendon and cartilage in the foot (Hurle et al 1989, 1990, Ros et al 1995) suggest that tendons develop separately

from their cartilage of origin and insertion sites and only attach relatively late. Kardon (1998) has suggested that tendon attaches to the distal phalanx by day 9 of development.

Muscle pattern is not autonomously patterned and pre-specified within the somite myogenic precursor cells (Chevalier et al 1977). It appears to be patterned by the lateral plate mesoderm of the limb bud but it is not clear whether the undifferentiated limb mesoderm, developing tendons or developing skeleton are the source of patterning information. During early limb development, myotubes differentiate within three particular regions of the limb in between 3 tendon primordia and are bounded by them, suggesting that tendon may be critical for basic division of muscle into thigh, shank and foot musculature. During normal development, the dorsal proximal tendon primordium defines the non-muscle region between thigh and shank muscle masses and when the dorsal proximal tendon primordium was removed, muscle invaded the area (Kardon 1998).

Conversely, the role of muscle in specification of tendon seems unlikely as it has been shown that tendons can develop in the absence of muscle (Brand et al 1985, Kieny and Chevalier 1979, Shellswell and Wolpert 1977). Recently it was shown that in the leg, the three pairs of tendon primordia develop autonomously in the absence of muscle in a normal temporal and spatial pattern however, the proximal and intermediate tendon primordia degenerate before segregation into individual tendons. In contrast, the distal tendon primordia, subdivide into individual tendons, make contact with tissue extensions at insertion sites and thereafter degenerate in the absence of muscle (Kardon 1998). Shellswell and Wolpert 1977 suggest that these structures develop autonomously but muscle-tendon patterning is ultimately coordinated by the mutual use of a three dimensional system of positional information present within the undifferentiated limb mesoderm.

1.6.1.4 Myotendinous junction

Tenascin staining shows that the mesenchymal lamina runs proximally through the sub-ectodermal mesenchyme and becomes continuous with a matrix on surface of the developing muscle masses. Tenascin positive fibrils penetrate the superficial muscle layer. In chick legs, elastin and fibrillin are found in the matrix region intercalated between distal tips of muscle masses and proximal end of developing tendons, prior to being detected in tendon at day 8-8.5. Important events occur around day 8, end to end musculo-tendinous junction is established (Ros et al 1995) and muscle bellies become individualised (Pautou et al 1982) and contractile (Watson and Bekoff 1990). It is at this

stage that the tendons that develop in muscleless limbs disintegrate (Kieny and Chevalier 1979). Hence these elastic matrixes may have a role to play in stability of developing tendons and onset of functional activity.

Muscle Ankyrin Repeat Protein (MARP) a nuclear protein, may play a crucial role in signalling from prospective tendon mesenchyme to forming muscle. MARP is expressed at the ends of primary myotubes and is accompanied by a complementary pattern of Thrombospomdin-4 (TSP4) an extracellular matrix protein in adjacent mesenchyme/prospective tendon mesenchyme (Baumeister et al 1997) which also expressed tenascin transcripts. Muscle is excluded in TSP-4 expressing regions. MARP and TSP-4 may be involved in myotendinous junctions as in vitro it was shown that myoblasts adhere to a peptide corresponding to the cell-binding carboxyl-terminal end of TSP-4 (Adams and Lawler 1994).

1.7 Molecules expressed in tendons

A number of molecules are now known to be expressed in developing tendons. *EphA4* a direct cell-cell signalling molecule is expressed in developing tendons . This will be described in chapter 4 of this thesis. In addition, other more long range signalling molecules have been found to be expressed in tendons and sets of related transcription factors that include the *Six* and *Eya* genes.

1.7.1 Eph receptors and ligands

The Eph receptor family is the largest of all known tyrosine kinase receptor families. Eph receptors are involved in transduction of extracellular signals across the cell membrane and play critical roles in cell growth, differentiation, survival and migration (van der Geer et al 1994). They are unique in mediating signalling only between neighbouring cells. Typically, they have an extracellular ligand-binding domain consisting of an IgG-like domain, a cysteine-rich region and two fibronectin Type III domains, a transmembrane region and an intracellular kinase domain. Eph receptors are classified into 2 subclasses either EphA or EphB according to their interaction with ligands (Eph Nomenclature Committee 1997). Ephrin-A ligands are membrane proteins anchored to the cytoplasmic membrane by a glycosylphosphatidylinositol (GPI) linkage and bind to EphA receptors. Ephrin-B ligands are anchored by a transmembrane domain and bind to EphB receptors.

However, with 14 receptors and eight ligands identified, the specificity with which these interact with one another is not quite clear. In vitro binding studies have suggested

that within the EphA/Ephrin-A and EphB/Ephrin-B subgroups, binding is promiscuous (Brambilla and Klein 1995; Brambilla et al 1995; Gale et al 1996a, b) (Table 1.1). It seems likely that specific ligands will activate different receptors to varying degrees (Monschau et al 1997). Complementary and overlapping domains of expression of receptors and ligands have been demonstrated in various tissues (Flenniken et al 1996; Gale et al 1996a, b).

1.7.1.1 Receptor signalling

Phosphorylation on tyrosine is critical for the recruitment of downstream-signalling molecules to interact with tyrosine kinase receptors (van der Geer et al 1994). As 14 tyrosine residues are conserved among Eph family receptors, signalling molecules interacting with one member may also interact with other members. However, there is considerable variation in sequences in the conserved tyrosine residues, indicating that each receptor may have specific interactions with down-stream signalling molecules (reviewed Zhou 1998).

1.7.1.2 Ligand signalling

The ligand-receptor interaction may generate bi-directional signalling because the Ephrin ligands are unique in that they can act as receptors themselves and transmit signal into ligand-expressing cells. Intracellular domains of the Ephrin-B subclass ligands are highly conserved with the C-terminal amino acids virtually identical, suggesting that this region may play a key role in ligand signalling. Biochemical analysis shows that ligands are phosphorylated on tyrosine located in the C-terminal region upon receptor binding (Holland et al 1996; Bruckner et al 1997). However ligands do not possess intrinsic kinase activity and kinases such as those of the Src family may associate with ligands(Holland et al 1996). PDGF stimulation induces Ephrin-B1 phosphorylation suggesting that the ligand may be a direct target of the Platelet –Derived Growth Factor (PDGF) receptor (Bruckner et al 1997). Moreover, in vivo, it is also a substrate for the Platelet –Derived Growth Factor (PDGF) receptor which suggests cross talk between Ephrin-B1 signalling and signalling cascades activated by tyrosine kinases.

1.7.1.3 Eph signalling during development of Nervous System

Eph signalling has been implicated in several patterning events in the nervous system which include guidance of nerve cell migration, axon guidance and hindbrain

Table 1.1 Interaction between Eph ligands and receptors

Receptors	Ligands that interact
	GPI-Linked
EphA1	**Ephrin-A1**
EphA2	**Ephrin-A1,** -A2, **-A4**, **-A5**
EphA3	**Ephrin-A2, -A3, -A4, -A5**
EphA5	**Ephrin-A1, -A2, -A3 –A4, -A5**
EphA6	**Ephrin-A2, -A3, -A4, -A5**
EphA7	**Ephrin-A1, -A2, -A3 –A4, -A5**
EphA8	**Ephrin-A1, -A2, -A3 –A4, -A5**
EphA4	**Ephrin-A1**, -A2, -A3 –**A4, -A5**
	Transmembrane
EphA4	Ephrin-B1 **-B2**, **-B3**
EphB1	**Ephrin-B1, -B2**
EphB2	**Ephrin-B1,** -B2
EphB3	**Ephrin-B1, -B2** -B3
EphB4	**Ephrin-B2**
???	**Ephrin-B3**
EphB5	**???**
?EphB6	

Ligands in bold bind with high affinity whereas others bind with weak affinity

segmentation. A good example of a patterning event which involves Eph signalling is establishing the retinotectal map.

Retinotectal mapping is highly organised, such that, temporal axons terminate in anterior tectum and nasal axons map to posterior tectum (Bonhoeffer and Huf 1985) probably, by repulsive cues in tectum. Eph family ligands show a graded distribution from anterior to posterior in the developing chick and mouse tectum (Monschau et al 1997; Cheng et al 1995; Nakamoto et al 1996) suggesting a role for Eph signalling in establishing appropriate connections in the retinotectal system. In the chick, Ephrin-A5 and Ephrin-A2 ligands interact with EphA4 expressed throughout the retina and with EphA3 expressed in a nasal to temporal gradient (Cheng et al 1995). The graded distribution of ligands in tectum and Eph receptors in retina, indicate that Eph signalling is responsible for setting up the retinotectal map.

Another example of Eph-Ephrin signalling is seen in establishing connectivity is seen in preoptic region. Normally *EphB2* is expressed in preoptic area, ventral to the anterior commissure and *Ephrin-B1*, a ligand for EphB2 is expressed in the commisural axons. In *EphB2* receptor-null mice, commissural axons invade the preoptic area aberrantly (Henkemeyer et al 1996). This suggests that the activation of the ligand by receptor binding generates a repulsive signal that prevents axons from growing into receptor-rich areas Henkemeyer et al 1996; Holland et al 1996; Bruckner et al 1997; Wang and Anderson et al 1997).

In the central nervous system of mice, *EphA4* is highly expressed within the intermediate and ventral regions of the spinal cord where axons of corticospinal tract (CST) do not terminate (Dottori et al 1998). In the mouse at E18.5, *Ephrin-B3* is expressed in motor neurons in sensorimotor cortex, thereby suggesting that it is expressed in the motor neurons of CST during development (Dottori et al 1998). *EphA4* is thus expressed in structures surrounding the CST where it acts as a signal for CST axons bearing ephrin ligands to be guided appropriately (Dottori et al 1998). In *EphA4* null mutants (Dottori et al 1998) there are a reduced number of corticospinal tract (CST) axons in the lower spinal cord and medulla and the mutant mice have loss of coordination of limb movement with resultant hopping gait.

Eph receptors and ligands may regulate neural connectivity in the peripheral nervous system (Donoghue et al 1996) such that neurons derived from specific rostrocaudal levels of the neuraxis selectively project to the corresponding muscles at the same level (Wigston and Sanes 1982; Laskowski and Sanes 1987). *Ephrin-A5* is expressed at higher levels in rostral than caudal muscles and in vitro inhibits the growth

of neurites from caudally derived neurons more effectively than rostrally derived ones (Donoghue et al 1996). In rat and chick embryos, neural crest cells expressing an Eph receptor are excluded from migrating through the caudal half of the somite, due to repulsive activity of Ephrin-B ligand (Wang and Anderson 1997; Krull et al 1997).

EphA4 binding ligands act as guidance signals for axon outgrowth into chick limb bud. *EphA4* is expressed in motor neurons of spinal cord (Ohta et al 1996) at E4 just before axonal growth into the limb bud and expression peaks at E5-E6 and ceases at E14 after neuron-muscle connection has been established. *EphA4* is expressed predominantly in motor neurons in brachial and lumbar regions innervating limb muscle, but is not present in thoracic motor neurons that innervate body muscle. *Ephrin-A5* and *Ephrin-A2* are expressed strongly in the dorsal-anterior and weakly in ventral–anterior areas of leg buds but not in anterior and posterior-medial parts of the limb bud. It has been suggested that these two ligands by repulsion function to keep motor neuron axons expressing *EphA4* from invading inappropriate areas of the limb (Ohta et al 1997).

Bi-directional repulsion by the Eph family ligands and receptors is particularly suited for the role in boundary formation between two cell populations. Ligands and receptors are expressed in mutually exclusive and complementary fashion in many different regions during embryogenesis of the developing nervous system (Gale et al 1996; Wang and Anderson 1997). It as been suggested that the two different types of cells do not mix because both ligand and the receptor generate repulsive signals.

A good example of Eph-Ephrin expression in discrete regions of embryos is seen in hind-brain rhombomeres. *EphA4* is expressed at first in presumptive rhombomeres (r) r3 and then in r3 and r5 in mouse embryos. *EphA2* is expressed only in r4. *EphB2* and *–B3* are expressed initially in r2 and r3 but later restricted to r3 and r5. Transmembrane ligand, *Ephrin-B2* is expressed in r1, r2, r4 and r6 (Bergemann et al 1995) and *Ephrin-B3* is expressed in r2, r4 and r6 (Gale et al 1996a). As *EphA2* and *EphA4* are expressed in presumptive rhombomeres prior to morphological segmentation, which occurs at E9 in mouse embryos (Becker et al 1994), they may have a role in defining cell identity of rhombomeres. Another possibility is that *EphA4* may be responsible for boundary formation between rhombomeres. This idea is supported by overexpression of the dominant negative truncated *EphA4* receptor in zebrafish embryo. The resultant mutant embryos were found to have disruption in the rhombomere boundaries (Xu et al 1995). The boundary was also lost in the chick when *EphA4* expression was reduced by application of retinoic acid (Nittenberg et al 1997) but no mixing of cells occurred.

EphA4 has also been suggested to contribute to cell fate restrictions in developing forebrain of zebrafish where it is normally expressed in the region of the forebrain fated to become diencephalon and not in the region fated to become eye tissue (Xu et al 1996). Inhibition of EphA4 using a dominant negative construct resulted in the formation of retina instead of ventral diencephalon (Xu et al 1996). Cell-labelling with lipophilic dye DiI showed that cells of diencephalic origin were present in the eye. These results are consistent with a proposal that EphA4 may be involved in cell association and/or adhesion since eye and diencephalic tissue development involves extensive morphogenetic movements (Xu et al 1996).

1.7.2 TGF-ß family members involved in cartilage and tendon development

Several members of the Tgf-ß family (one of the sub-groups of the TGF-ß superfamily), which are highly homologous, show specific patterns of expression in prechondrogenic condensations. These include *Tgf-ß2* in mouse (Millan et al 1991) and *Tgf-ß3* in chick (Roark and Greer 1994) limb buds and in high density mesenchymal cell cultures (TGF-ß1-TGF-ß3-Leonard et al 1991; Roark and Greer 1994). These growth factors are chondrogenic when added to limb mesenchymal cell cultures (Kulyk et al 1989; Schofield and Wolpert 1990; Leonard et al 1991).

Tgfß2 is expressed in relation to developing digital rays in chick limb buds from stage 26, at first, dorsal and ventral to the digital condensation and by stage 28-29 in developing tendon, interphalangeal joint regions and in the distal tip of the growing cartilage (Merino et al 1998). Implantation of TGF-ß beads in the interdigital region lead to induction of *Tgfß2* gene. *Tgfß3* is expressed only in the developing tendons (Merino et al 1998). In contrast, some members of the BMP family of growth factors. *Bmp-2*, *Bmp-4* (Lyons et al 1990; Francis et al 1994) and *Bmp-7* (Luo et al 1995) are expressed in interdigital spaces of the developing autopod prior to establishment of cell death. TGF-ß1 or TGF-ß2 protein, implanted in the interdigital mesenchyme inhibits cell death and promotes interdigital cartilage indicating that TGF-ßs may be the signal required for cartilage condensation. BMPs implanted in interdigital region accelerate cell death or digit bifurcation and ectopic cell death when implanted at the digit tip.

Bone Morphogenetic Proteins (BMPs) are members of the Transforming Growth Factor-β (TGF-β) superfamily of secreted factors and were originally identified by their ability to induce ectopic endochondral bone in rats (Wozney et al 1988). Within the developing autopod, *Bmp-2*, *Bmp-4* and *Bmp-7* are also expressed later in the perichondrium of the forming digits (Lyons et al 1990; Jones et al 1991; Wozney et al

1993; Macias et al 1997). *Bmp-6* is expressed in prehypertrophic and hypertrophic chondrocytes (Wozney et al 1993; Vortkamp et al 1996). Besides expression in interdigital region and perichondrium, *Bmp-7* is also expressed in developing tendons, but the expression in perichondrium is interrupted at level of joints (Macias et al 1997). Expression of *Bmp-4* in chick tendons will be described in chapter 4.

Cells in the autopod can have 2 different fates, chondrogenesis or apoptic cell death, depending on whether they are incorporated into the digital rays or into the interdigital region. In skeletal development, BMPs may act on uncommitted multipotent stem cells to promote their entry into the chondrogenic rather than the myogenic or adipogenic pathway (Yamaguchi et al 1991; Gimble et al 1995). Alternately, they may stimulate cells committed to the chondrogenic lineages and promote programmed cell death or transdifferentiation of cells committed to other lineages (Katagiri et al 1994; Duprez et al 1996a). Thus TGF-ßs and BMPs may control the alternating pattern of digit and interdigital regions in the developing autopod.

BMP signalling involves two types of transmembrane serine/threonine kinases called Type I and Type II receptors (Massague 1996). Ligand binding to both receptors results in the phosporylation of the Type I receptor by the Type II receptor. The type I receptor then transduces the signal by phosphorylating the intracellular targets including members of the Smad family (Massague et al 1997). In vertebrates two Type I Bone Morphogenetic Protein Receptors (BMPR-1A and BMPR-1B) have been identified (Zou et al 1997). It was proposed, based on expression patterns that *BMPR-1a* receptors are involved in interdigital death while *BMPR-1b* receptors participate in chondrogenesis (Kawakami et al 1996; Enomoto-Iwamoto et al 1998). However when BMP signalling was blocked by retrovirus-induced overexpression of dominant negative constructs of either type I receptors, both produced similar effects, defective digit growth, loss of phalanges and inhibition of interdigital cell death (Zou and Niswander 1996; Yokouchi et al 1996). Misexpression with either constitutively active *BMPR-1a* or *BMPR-1b* promoted chondrogenesis but *BMPR-1b* also increased cell death in the interdigital zone (Zou et al 1997) indicating that the latter receptor had a dual role.

A number of extracellular proteins including Follistatin, Noggin and Chordin probably bind BMPs in vivo and thus limit their availability and domain of influence. Follistatin can interact directly with BMP-4 in vitro (Fainsood et al 1997) and BMP-7 (Nakamura et al 1990; Yamashita et al 1995). Noggin counteracts the effect of BMP-4 and it is likely that it binds BMP-4 protein directly blocking its biological activity thus

restricting the region over which it can act in vivo (Harland 1994; Re'em-Kalma et al 1995). In *Xenopus*, it has been shown that Chordin counteracts the ventralising effect of BMP-4 (Holley et al 1995). Expression of *Follistatin* in developing tendons will be described in chapter 4 of this thesis.

Application of BMPs to cartilage-forming regions in chick limbs induces expression of the BMP antagonist *Noggin*, thereby setting up a negative feedback loop, which may regulate the size and shape of the developing cartilage (Merino et al 1998). *Noggin* is normally expressed throughout the condensing digital rays from stage 29-30. Later it is concentrated in prehypertrophic cartilage of diaphyseal region and in mesenchyme of differentiating joints.

1.7.3 Transcription factors in tendon

1.7.3.1 *Six* genes

Two members of a highly diverged family of murine homeo-box containing genes, *Six 1* and *Six 2* were identified (Oliver et al 1995) as homologs of the Drosophila gene, *sine oculis* (Cheyette et al 1994). *Six 1* and *Six 2* are expressed in a complementary fashion in developing mouse limb tendons. *Six 1* is stronger in extensor blastemas while *Six 2* is stronger in ventral flexor tendon blastemas. Later expression of both genes is associated with the cartilage condensations. As *Six 1* is found to be present in myoblast precursors as well as in mesenchymal cells that give rise to connective tissues, it is suggested that these transcriptional factors may be involved in patterning connective tissue and muscle, perhaps by conferring similar positional value to cells migrating into the limb bud (Oliver et al 1995). Expression of *Six 1* in chick tendons will be described in chapter 4.

1.7.3.2 *Eya* genes

More recently *Eya* genes that encode a nuclear protein have been implicated in development of tendons. *Eya1* and *Eya2*, two mouse homologs of *Drosophila eyes absent* (*eya*) genes were found to be present in connective tissue precursor cells and later in developing tendons (Xu et al 1997a). *Eya1* expression is largely restricted to flexor tendons, while *Eya2* to extensor tendons and ligaments of phalangeal elements of the limb. These observations suggest that they may be involved in connective tissue patterning. However haploinsufficiency for human *EYA1*, found to be responsible for branchio-oto-renal syndrome (Abdelhak et al 1997b), does not involve connective

tissue. *Eya* genes are also expressed in somites, myoblast precursors (Xu et al 1997b) and in other skeletal muscles and connective tissue (Xu et al 1997a). Hence it is suggested that *Eya1* and *Eya2* may be involved in patterning connective tissue-derivatives, tendon and ligament in limbs, and may ensure that tendon and muscle precursor cells expressing the genes migrate to appropriate places (Xu et al 1997b).

A common observation about expression of *Eya* genes and *Six 1* in developing mouse tendons is that the 2 genes in each group have a predominance in either flexor or extensor tendons. *Eya1* is expressed a day earlier (day 10) than *Six 2* in the limb bud and is expressed ventrally in condensing mesenchyme cells whereas *Six 2* is expressed in dorsal and ventral developing tendons though more strongly ventrally. Both *Six* genes and *Eya* genes are associated with developing phalanges and it is suggested that *Six* genes are associated with ligaments around developing joints.

1.8 Work described in this thesis

The aim of this thesis is to understand how patterning of tendons is specified in vertebrate limb development. As the basis for the experimental work of the thesis, the gross and microscopical anatomy of the developing tendons in chick limbs is described. The expression of *EphA4*, a tyrosine kinase receptor, which is a direct cell-cell signalling molecule is documented and compared with expression of tenascin and procollagen I, other molecules known to be associated with tendons. Expression of *Six 1*, a transcription factor, associated with tendon is also described. *Follistatin* an antagonist that inhibits signalling of TGF-β superfamily members is also expressed during tendon development. The effects of applying Follistatin to the limb are explored. In addition manipulations are carried out to explore whether tendon patterning is correlated with cartilage patterning and expression of *EphA4* monitored. To examine roles of dorso-ventrally expressed genes in tendon patterning, expression of *Wnt-7a* and *Lmx-1* is compared in relation to expression of *EphA4* in developing chick tendons. Expression of *EphA4* in tendons is used to study dorso-ventral patterning of tendons in the *Wnt-7a* knockout mice. *Lmx-1* is misexpressed in ventral limb mesenchyme of the chick limb bud and expression of *EphA4* and tendon patterning is studied. Lastly, a human foot with polydactyly and alterations in dorso-ventral pattern is dissected and its morphology interpreted in the light of studies in developmental biology.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

Chapter Two: General Materials and Methods

2.1 Embryos for manipulations or analytical study

Fertile White Leghorn chicken eggs were obtained from Poyndon farm, Waltham Cross, Hertfordshire, U.K. Eggs were incubated at 38 ± 1°C and windowed on day 2 or 3 of incubation. Eggs were briefly swabbed with tissue soaked 70% alcohol and rotated 90° to ensure that the embryo was not stuck on the inside of the shell. The blunt end of the egg was pierced with forceps, followed by piercing of the upper surface. Tape was placed over this hole and a "window" was cut from the shell using scissors, thus exposing the embryo. Embryos were staged according to the method of Hamburger and Hamilton (1951) and put back into the incubator and used for manipulation or fixing depending on the stage required. For manipulation, the embryo was exposed by removing the vitelline and amniotic membranes around the leg region.

2.2 Preparation of fixatives

a) Formol saline: 1g sodium dihydrogen orthophosphate anhydrous, 1.625g disodium hydrogen phosphate anhydrous, 8.5g sodium chloride, 75ml 40% formaldehyde, make up to 1 litre with distilled water. Adjust pH to 7 with 1M NaOH.

b) 4% Paraformaldeyde: Sterile 10x phosphate buffered saline (PBS, Gibco) was diluted 1:10 with sterile distilled water. 4% (w/v) paraformaldehyde powder (Sigma) was issolved in PBS by heating gently. Aliquots of 4% paraformaldehyde (PFA) were stored at –20°C.

2.3 Fixing embryos

2.3.1 For paraffin wax sections

Embryos were dissected in 1x phosphate buffer (PBS) then fixed in formol saline, dehydrated through graded series of alcohol (50%, 70%, 85%, 95% and absolute), cleared in toluene, embedded in paraffin wax, sectioned at about 8 μm thickness.

2.3.2 For in situ hybridisation or antibody staining

Embryos were dissected in cold 1x PBS, fixed in 4% paraformaldehyde (PFA) and then processed for whole mount in situ hybridisation or antibody staining.

2.4 Staining with Mallory's trichrome

Serial wax sections taken on slides, were immersed carefully in different solutions as follows:

Histoclear for 5 minutes; rehydrated in 90% absolute alcohol 3minutes; 70% alcohol 3minutes; 50 % alcohol 3 minutes; distilled water 3 minutes; acid fuschin 10 seconds; wash in distilled water 10 seconds; phosphomolybdic acid 60 seconds; wash in distilled water 10 seconds; Mallory's stain 75 seconds; wash in distilled water 10seconds; dehydrate by passing through 90% alcohol, and twice in absolute alcohol for 10 seconds each, finally in histoclear and mounted with DPX mounting medium.

2.5 Alcian green staining for cartilage

Embryos were fixed in 5% trichloracetic acid (TCA), overnight at room temperature. Subsequently they were stained with 0.1% (w/v) alcian green in acid alcohol (70% (v/v) ethanol, 1% HCl), for 4 hours to overnight. Embryos were then washed in acid alcohol for 3 hours to overnight and then washed several times in absolute ethanol for 3 hours minimum. Finally, the embryos were cleared and stored in methyl salicylate.

2.6 Whole mount in situ hybridisation

2.6.1 Preparation of Linear DNA from plasmid DNA

As Linear DNA is required to produce probes, plasmid (circular) DNA is cut with restriction enzymes to produce linear DNA.

Cutting the plasmid: For a total digestion volume of 200µl, 20µl of enzyme buffer (10%total volume), 10µl restriction enzyme and 20µg plasmid DNA is made up to 200µl with sterile water. Mix, centrifuge briefly and incubate for 2 hours at 37°C. To ensure the plasmid DNA has been linearised, run on a 1% agarose gel (melt 1% low melting point agarose (Sigma) in 1x Tris acetate with EDTA (TAE). Add 0.002% 10mg/ml Ethidium Bromide. Load 1-2µl cut or uncut DNA with 1µl loading buffer. Run against a DNA ladder to estimate the size of the cut DNA The cut DNA will appear as a single band, the uncut DNA as a streak of bands. Once cut, precipitate by adding an equal volume (200µl) of phenol:choloroform. Vortex and centrifuge at 14,000rpm for 5 minutes. Remove the top layer (100µl) and add an equal volume of phenol:chloroform, vortex and centrifuge as before. Remove the top layer (100µl). To this add, 1 / 4 volume

10M ammonium acetate and 2.5x volume of ethanol. Freeze at –20°C for 30 minutes to precipitate. Centrifuge at 14,000rpm for 10 minutes to produce a pellet, then carefully tip the supernatant off. To clean, re-suspend in 70% ethanol, centrifuge and remove supernatant. Air dry and re-suspend in 20µl of water.

2.6.2 Synthesis of riboprobe for whole mount in situ hybridisation

Riboprobes are synthesised by linearising plasmid DNA using appropriate restriction enzymes. Typically, 1µg of plasmid DNA was digested with 1 unit of enzyme, for at least 1 hour at 37°C, in a total volume greater than 10 times the volume of enzyme used. The method of linearisation of each plasmid will be listed separately in relevant chapter. For all steps of in situ protocol, sterile RNase free water was produced by treating distilled water with 0.05% diethylpyrocarbonate (DEPC) for several hours before autoclaving.

Transcription reaction is set up by mixing the following at room temperature in a sterile eppendorf. 10µl of sterile distilled water; 4µl of 5x transcription buffer (Boehringer);2µl of 0.1M DTT (Dithiothretol); 2µl of DIG 10x nucleotides mix (Digoxigenin labelled nucleotides, Boehringer); 1.5µl of Linearised plasmid (1µg/µl); 0.5µl of RNase inhibitor; 1.5µl of RNA polymerase (T3 or T7, Boehringer).
Incubate in a water bath at 37°C for 2-3 hours. For precipitation add the following ice cold: 50µl water, 1µl glycogen (20µg/µl, from mussels, Boehringer), 25µl ammonium acetate and 200µl ethanol. Mix place on dry ice for 30 minutes or overnight at 70°C. Centrifuge at 14,000rpm for 20 minutes at 4°C. Remove supernatant, wash pellet with 70% ethanol, centrifuge for 5 minutes. Remove supernatant and air dry. Re-suspend in 50µl sterile water and aliquot. The reaction was monitored by loading 1µl of the reaction mixture on a TAE 1% agarose gel before precipitation and after the final re-suspension and visualising the riboprobe after brief electrophoresis. Preparation of individual probe will be described in appropriate chapter.

2.6.3 Preparation of embryos for hybridisation

Fix embryos in 4% PFA for 2-24 hours depending on stage of embryo. Wash in PBT (PBS + 0.1% Triton 100). (If embryos are to be stored, dehydrate in ascending series of methanol (Analar grade) in PBT –25%, 50%, 2x 100% and store for up to 2months at 20°C. Rehydrate in descending series of methanol in PBT (50%, 25%, 2x PBT). Digest embryos with 20µg/ml proteinase K (Sigma) in PBT. Embryos of stage

20-24 were digested for 20 minutes, stages 25-30 for 25 minutes and an extra minute for every stage thereafter. Wash in PBT 5 minutes. Refix in 4% PFA, 25% gluteraldehyde (Sigma, tissue culture grade), 20 minutes. Wash in PBT 5 minutes. Incubate in preheated hybridisation mix (see below) at 60°C overnight.

Hybridisation mix is as follows:

25ml- formamide (Fluka)

3,25ml- 20xSSC (3M NaCl, 0.3M NaCitrate, pH to 4.5 with citric acid)

0.5ml- 0.5M EDTA pH8

250µl- 20mg/ml Yeast tRNA (Bakers yeast, Boehringer)

100µl- Triton 100

2.5ml- 10% CHAPS (Boehringer)

100µl- 50mg/ml heparin (Sigma)

1gm blocking powder (Boehringer)

Make up to 50ml with sterile water. Store at –20°C.

2.6.4 Hybridisation, post hybridisation washes and visualising the signal

Wash embryos in pre-warmed hybridisation mix. Incubate in pre-warmed hybridisation mix with 1µl/ml labelled probe overnight at 60°C. Wash twice in 2xSSC, 0.1% CHAPS, 20 minutes at 60°C. Wash twice in 0.2% SSC, 0.1% CHAPS, 20 minutes at 60°C. Wash in KTBT (50mM Tris pH7.5, 150mM NaCl, 10mM KCl, 1% Triton), 5minutes at room temperature. Incubate in 50% lamb serum (Gibco) in KTBT for 4 hours at room temperature. Meanwhile, prepare 50% lamb serum in KTBT plus 1/1000 anti-DIG antibody (Boehringer) and rock on ice for 4 hours. Incubate in this antibody solution at 4°C overnight.

Next day wash with KTBT at room temperature for hour 5-6 times and then overnight at 4 °C. Wash in Alkaline phosphate buffer, NTMT (1ml-5M NaCl, 2.5ml 2M Tris-HCl pH9.5, 1.25 ml-2M $MgCl_2$, 500µl-Tween-20 (Sigma), make up to 50ml with sterile water), 10 minutes at room temperature. Prepare colour reagent as follows and keep in dark: NTMT + 4.5µl NBT (4-Nitrobluetetrazlium)/ml + 3.5µl BCIP (5-Bromo-4-chloro-3-indolyl-phospate)/ml. Incubate embryos in colour reagent, in dark, until purple stain appears to sufficient intensity. Wash for 1 hour, x2 in NTMT at room temperature. Refix in 4% PFA.

2.7 Preparation of Template DNA

Plasmids are generally prepared from bacterial cultures grown in the presence of a selective agent such as an antibiotic.

2.7.1 Bacterial Transformation

1) Dissolve LB Agar – 7.5 gms (Difco Lab) in 500ml of distilled water and autoclave. To this add 500µl of 100mgm/ml ampicillin (this ensures that only ampicillin resistant cells grow). Pour into plates maintaining sterility. Allow to set, cool and air dry.

2) Meanwhile to 5µl of competent cells (Strain JM109-Promega) in an eppendorf, add a 10-20ng cDNA and keep on ice for 1 ½ hour (this allows the plasmid DNA to be incorporated into cells).

3) Glucose/ LB broth (Luria Broth base (Millars LB base for molecular biology) 25 gms in 1000ml of distilled water, autoclave to sterilise and cool) Take 100µl of 200mM glucose made up in LB broth and make up to 1ml. Add this 1ml of the Glucose/LB broth to the eppendorf containing cDNA and cells after bringing to room temperature. Shake in an incubator at 37°C for 1 hour.

4) Then take 100µl of cells from eppendorf and drop on to the agar plates prepared in (1) above, using spreaders evenly spread on the plate. Place plate upside down in incubator at 37°C overnight. (Can make higher concentration of cells for plating) This allows colonies to grow overnight.

5) Next morning, select and mark single plaques in the centre of plate that are not clumped together (to be used for mini culture).

5) Add 1µl of 100mgm/ml ampicillin to 3ml of LB broth in a tube. Using a tip, carefully remove a selected plaque from the plate (by gently touching the plaque with the tip) and add to the tube containing LB broth/ampicillin. Keep the cap of the tube loose and shake overnight at 160rpm at 37°C. (other plaques can be treated in the same way in case this one fails). If there is good growth, the tube will be murky.

2.7.2 Mini (Zippy) Preparation of Plasmid DNA

1) Take 1.5 ml from the tube above into a 2ml round bottomed eppendorf and make up to 2ml with distilled water (Store the remaining 1.5ml in fridge until results are obtained).

2) Spin at 13,000rpm for 10 minutes at room temperature, remove supernatant and discard. Re-suspend in 0.7ml of Zippy buffer (to make 100ml add 8gm glucose, 0.5ml Triton 100, 10ml 0.5M EDTA, 5ml 1M Tris pH 8).

3) Add 50µl of 10mgm/ml Lysozyme (Sigma) made up in Zippy buffer

4) Meanwhile boil water in a beaker and place the eppendorf in boiling water for 45 seconds (using a polystyrene holder such that the bottom of eppendorf is in the boiling water).

5) Spin at 13,000rpm for 15 minutes. Remove gelatinous muck that stands on top, with a wooden tooth pick. Then add equal volume of Isopropanol (BDH Analar) and spin immediately at 15,000rpm for 15 minutes, remove supernatant and add 75% ethanol to wash pellet, spin for 15,000 for 15 minutes, remove supernatant and re-suspend pellet in 20µl of 1x TAE buffer.

6) To 450ml of LB broth, add 450µl of ampicillin. Take 80ml in a conical flask and tip the contents of the tube in 1) above into it. Place a loose lid or foil over the mouth and put into shaker at 37°C overnight and allow to grow to saturation.

Plasmid DNA purification was done using Qiagen Plasmid Maxi Prep Kit

2.8 Frozen sectioning of embryos

Limbs used for wholemount in situ hybridisation were re-fixed overnight in 4% PFA (w/v) overnight and then washed in ice cold PBS. Embryos were then placed in increasing concentrations of sucrose in PBS up to 30% (w/v). Embryos were then processed in OCT compound (Tissue Tek) for 1 hour to allow infiltration and then slowly frozen in liquid nitrogen and stored at –70°C prior to sectioning. Sections of 10-15µm thickness were prepared and mounted with glycerol before analysis under the microscope.

CHAPTER THREE

MACROSCOPIC AND MICROSCOPIC PATTERN OF TENDONS IN CHICK LIMBS

Chapter Three: Macroscopic and Microscopic Pattern of Tendons in Chick Limbs

3.1 Introduction

In order to gain detailed first hand knowledge of how tendon pattern in the autopod is established during chick limb development, I first dissected the adult wing and leg of a chick and then went on to section embryonic limbs to study the histological pattern. Sullivan (1962) described the muscles and tendons in the wing of adult domestic fowl and all of these are present and identifiable at stage 36. As this thesis was addressing tendons in the autopod it was decided to confine all work to the digital plate. Sullivan (1962) also showed details of embryonic tendons between stages 25 to 38 using drawings reconstructed from sections. Shellswell and Wolpert (1977) also described the wing at stage 36 using diagrams and sections. Nomenclature used in this work is according to Sullivan (1962).

The chick foot is quite complex because of the number of tendons approaching the foot and also because of the number of toes and the varying number of phalanges in each toe. Although tendons do not complete their insertions to the bone until the eleventh day according to Wortham (1948), this study did not go beyond embryonic day 10.

The muscles and tendons in the foot are divided into intrinsic and extrinsic groups (Raikow 1985) (Table 3.1). The intrinsic group lies entirely within the foot, arising from the tarsometatarsus and inserting on the proximal phalanges. The extrinsic group lies in the crus, arising from the femur, tibiotarsus or fibula and their long tendons run distally around the intertarsal joint and down the tarsometatarsus to their phalangeal insertions (Fig. 3.1a). The basic movements of the toes are flexion and extension. Flexor tendons (Fig. 3.1a, b and c) lie on the plantar aspect and are responsible for grasping movement, while extensors are on the dorsal aspect and are responsible for releasing the grip. More strength is required for grasping than for letting go. Hence the flexor system contains larger extrinsic muscles whereas the extensors are mostly intrinsic and smaller. The only extrinsic extensor is the extensor digitorum longus (Fig. 3.1b) and the only intrinsic flexor is the flexor hallucis brevis (Fig. 3.1d). The intrinsic tendons (Table 3.1) never reach the terminal phalanx.

An important feature of late tendon development is the formation of tendon sheath. Many problems about the sheath are still not answered e.g. the origin of the tendon

Figure 3.1 Diagrammatic representation of the tendons in the chick foot

Fdl, flexor digitorum longus; Fpd, flexor perforans digiti; Fp pd, flexor perforans et perforatus digiti; Fhl, flexor hallucis longus; Fhb, flexor hallucis brevis; Edl extensor digitorum longus; Ehl, extensor hallucis longus.

a) Pattern of divisions and insertions of (extrinsic) long tendons to digits 2, 3 and 4.
b) Extrinsic tendons qualified by the term "longus" go to the terminal phalanges of all digits for final insertion.
c) Extrinsic flexor tendons that do not insert terminal phalanges but go to more proximal ones of digit 2, 3 and 4.
d) Intrinsic tendons that go to digit 1. (modified after Raikow (1985)

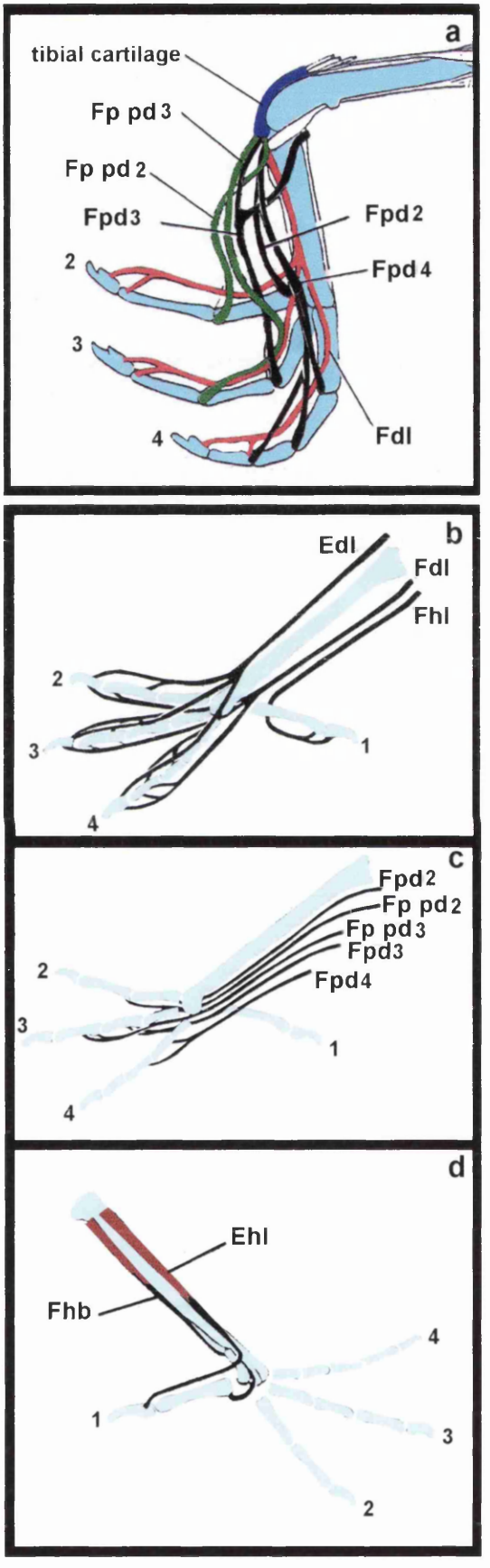

a
tibial cartilage
Fp pd 3
Fp pd 2
Fpd3
Fpd2
Fpd4
2
3
4
Fdl
b
Edl
Fdl
Fhl
2
3
4
1
c
Fpd2
Fp pd2
Fp pd3
Fpd3
Fpd4
2
3
4
1
d
Ehl
Fhb
4
1
3
2

Table 3.1 Tendons in the chick foot (Raikow 1985)

Extensors	Intrinsic	**Extrinsic**
Digit 1	Extensor hallucis longus	
Digit 2	Adductor digiti 2 Abductor digiti 2	Extensor digitorum longus
Digit 3	Extensor proprius digiti 3 Extensor brevis digti 3	Extensor digitorum longus
Digit 4	Extensor brevis digiti 4 Extensor proprius digit 4 Adductor digiti 4 Abductor digiti 4	Extensor digitorum longus
Flexors		
Digit 1	Flexor hallucis brevis	Flexor hallucis longus
Digit 2		Flexor perforatus digiti 2 Flexor perforans et perforatus digit 2 Flexor digitorum longus
Digit 3		Flexor perforatus digiti 3 Flexor perforans et perforatus digit 3 Flexor digitorum longus
Digit 4		Flexor perforatus digiti 4 Flexor digitorum longus

All the extrinsic "longus" tendons insert on the terminal phalanx and have multiple insertions

sheath, how a synovial space forms and whether cell death plays a role. The chick flexor tendon sheath is similar in anatomy, size and function to that in the human hand (Lindsay and Thomson 1960) and hence is a good model for studying development and healing of tendons.

In the human hand, superficial transversely oriented fibres form fibrous flexor sheaths around flexor tendons and form part of a pulley system. The fibrous flexor sheath (Fig. 3.2) is a specialised part of the palmar fascia and consists of arcuate fibres that arch over the tendon and are attached to the side of the phalanges (Fig. 3.2b). Fibres arranged in a cruciate fashion overlie joint regions. The fibrous sheath has a retinacular role in preventing tendons springing away from the underlying bone. To perform this function however, the fibrous flexor sheath must have considerable strength and a system of lubrication to prevent functional resistance to movement of tendons within it. Each finger thus has an osseo-fibrous tunnel (Fig. 3.2) made up of the fibrous flexor sheath and the phalanges and is lined with a double layer of synovial membrane (Fig. 3.2b). The outer parietal synovial layer lines the fibrous flexor sheath, is adherent to it and is reflected on to the surface of the tendon forming the inner visceral layer or epitenon (Fig. 3.2b) that closely invests the tendon. Folds of synovial membrane called vincula (Fig. 3.2b) contain a loose plexus of fascial fibres, carry blood vessels (Edwards 1946; Brockis 1953; Chaplin 1973; Verdan 1972) to the tendons at defined points.

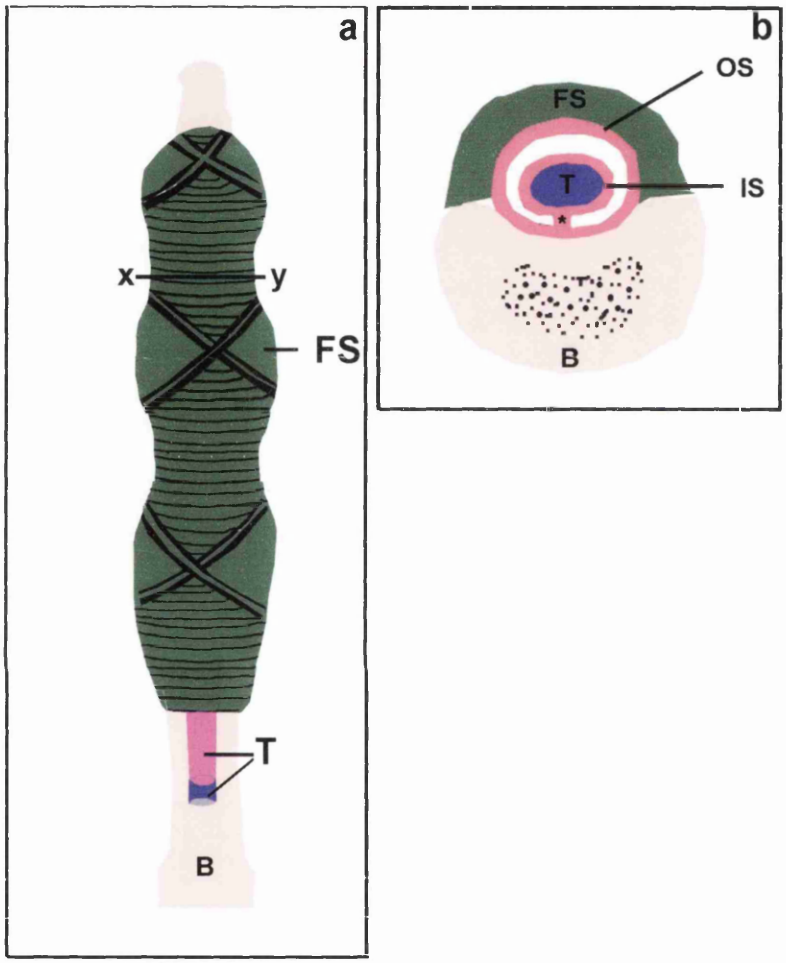
Tendon sheath integrity provides an enclosed nutrient environment and prevents external adhesion when tendons heal after injury (Tonkin 1991). The synovial lining of the flexor sheath has regenerative capability (Potenza 1962; Eiken, Lundborg and Rank 1975) in tendon healing in man. Migration of epitenon cells into lacerated chicken flexor tendons in vivo was noted (Lindsay and Thomas 1960, Garner et al 1988) and the same was also observed in canine flexor tendons (Gelberman et al 1990). Paralysis of chick embryo affects formation of synovial cavity, even though the pulley is formed and tendon is normal (Beckham et al 1977). The formation of joint cavity is similarly affected (Drachman and Skoloff 1966). This suggests that movement may be required for the formation of tendon sheath. Textbooks of adult anatomy describe that "tendons are invaginated into the sheath" in order to account for how a 2 layered covering is formed (Bannister et al 1995). However it is not clear if this happens during development. I examined formation of tendon sheath of flexor digitorum profundus in chick wing.

Figure 3.2 Diagrammatic representation of tendon sheath and its components in human finger

a) Gross appearance of ventral view of a finger showing fibrous sheath (FS) which is attached to the sides of the skeleton (B) in the finger and encloses flexor tendons (T). Over the joints the sheath is thinner and has fibres arranged obliquely.
Line x-y is equivalent to the plane of the transverse section shown in (b).

b) Tranverse section equivalent to plane x-y in (a). Fibrous sheath (FS) on ventral side is attached to sides of the phalanx (B) enclosing the flexor tendon (T) in an osseo-fibrous tunnel. The inner (visceral) layer of synovial membrane (IS) is adherent to the tendon (T) and is continuous with the outer (parietal) layer (OS) of synovial membrane which is adherent to the fibrous sheath; asterisk, vinculum.

Fig. 3.2



3.2 Materials and methods

Fresh adult chick wing and leg were purchased at the local market. Skin was removed and the long tendons in the digits were carefully dissected and exposed on both dorsal and ventral sides and then photographed.

Embryos were fixed as described in section 2.3a and stained with Mallory's trichrome as per section 2.4. Alcian green staining for cartilage was performed as described in section 2.5 using embryos at day 10.

3.3 Results

3.3.1 Pattern of tendons in adult wing

3.3.1.1 Extensor tendons

On the dorsal surface (Fig. 3.3a), extensor digitorum communis (Edc) (Fig. 3.3a) is a muscle that arises in the proximal humerus (not indicated), extends over the entire forearm, and then becomes tendinous just before the metacarpus (not indicated). Over the metacarpus, Edc tendon lies (Fig. 3.3a, black arrowhead) superficial to the tendon of extensor medius longus (Eml) (Fig. 3.3a) and then runs anterior to Eml and separates into 2 slips (Fig. 3.3a). The anterior slip is short and attaches to the base of digit 2, the posterior slip is longer and runs distally over the dorsal aspect of metacarpal 3 to attach to the base of the proximal phalanx of digit 3, posterior to the extensor medius longus (Eml) tendon. Before its distal attachment, it passes (Fig. 3.3a arrow) beneath the tendon of extensor medius longus (Eml) (Fig. 3.3a). The latter (Eml) (Fig. 3.3a) has its proximal attachment to the radius (not shown). It lies between ulna and radius and is overlapped by muscle Edc (not shown) and becomes tendinous just before the metacarpus where it lies anterior to extensor digitorum communis (not indicated). The Eml tendon extends over the dorsal aspect of metacarpal 3 (Fig. 3.3a) and muscle extensor medius brevis (not shown) and then runs along the anterior margin of digit 3 (Fig. 3.3a, white arrowheads) to get attached to its distal phalanx on its anterior aspect. In its course, it is peculiarly related to the tendon of extensor digitorum communis (Edc) which crosses it at 2 points, once superficial (Fig. 3.3a, black arrowhead) and once deep (Fig. 3.3a, arrow). The changing anterior-posterior relationship between these two tendons is shown in (Table 3.2 and Fig. 3.4).

Figure 3.3 Digital tendons in adult chick wing

Digits are numbered 2, 3, and 4 from anterior to posterior.

a) Dorsal view showing extensor digitorum communis (edc) tendon lying superficial to extensor medius longus (eml) (black arrowhead) before dividing into 2 slips for digit 2 (edc2) and digit 3 (edc3); slip to digit 3 (edc3) crosses (arrow) deep to eml from anterior to posterior before its insertion; extensor medius longus (eml) coursing to its insertion nearer the anterior edge (white arrowheads) of digit 3; interrosseus dorsalis (id) and interrosseus palmaris (ip), note that the latter is on a more ventral plane.

b) Ventral view showing flexor digitorum profundus (fdp) tendon dividing into 2 slips, one coursing to digit 2 (fdp2), the other to digit 3 (fdp3), flexor digitorum superficialis (fds) seen over digit 3 fusing (arrowhead) with fdp and running distally for insertion.

c) Dorsal view of the wing showing planes 1, 2, 3, 4 and 5. Equivalent to histological transverse sections taken from 10 day embryoinc wing are shown in Fig 3.5.

Fig. 3.3

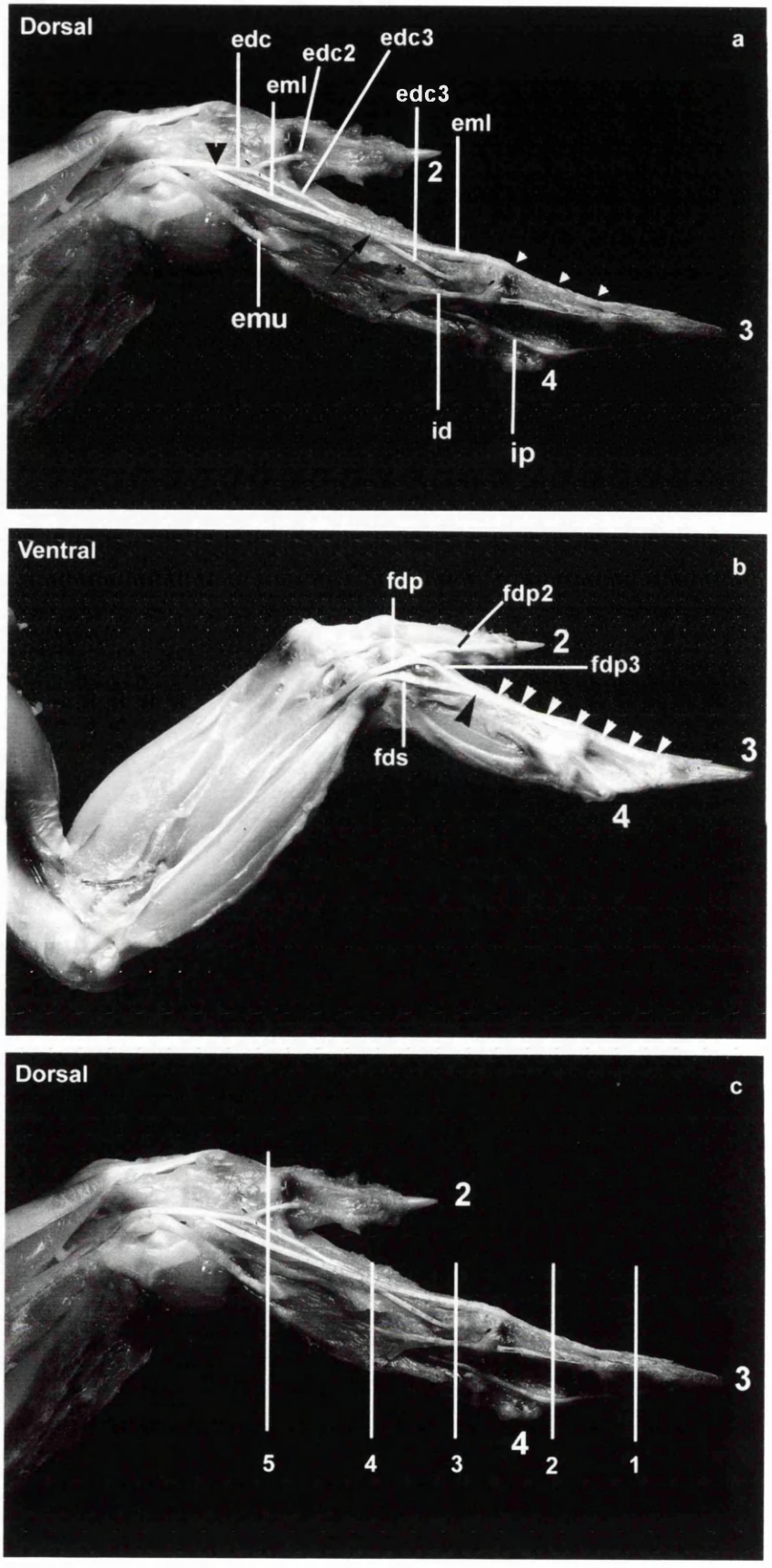

Dorsal
a
edc
edc2
edc3
eml
edc3
eml
2
emu
3
4
id
ip
Ventral
b
fdp
fdp2
2
fdp3
fds
3
4
Dorsal
c
2
3
4
5
4
3
2
1

Table 3.2 Tendons in chick wing showing their dorso-ventral and antero-posterior position along the proximo-distal axis

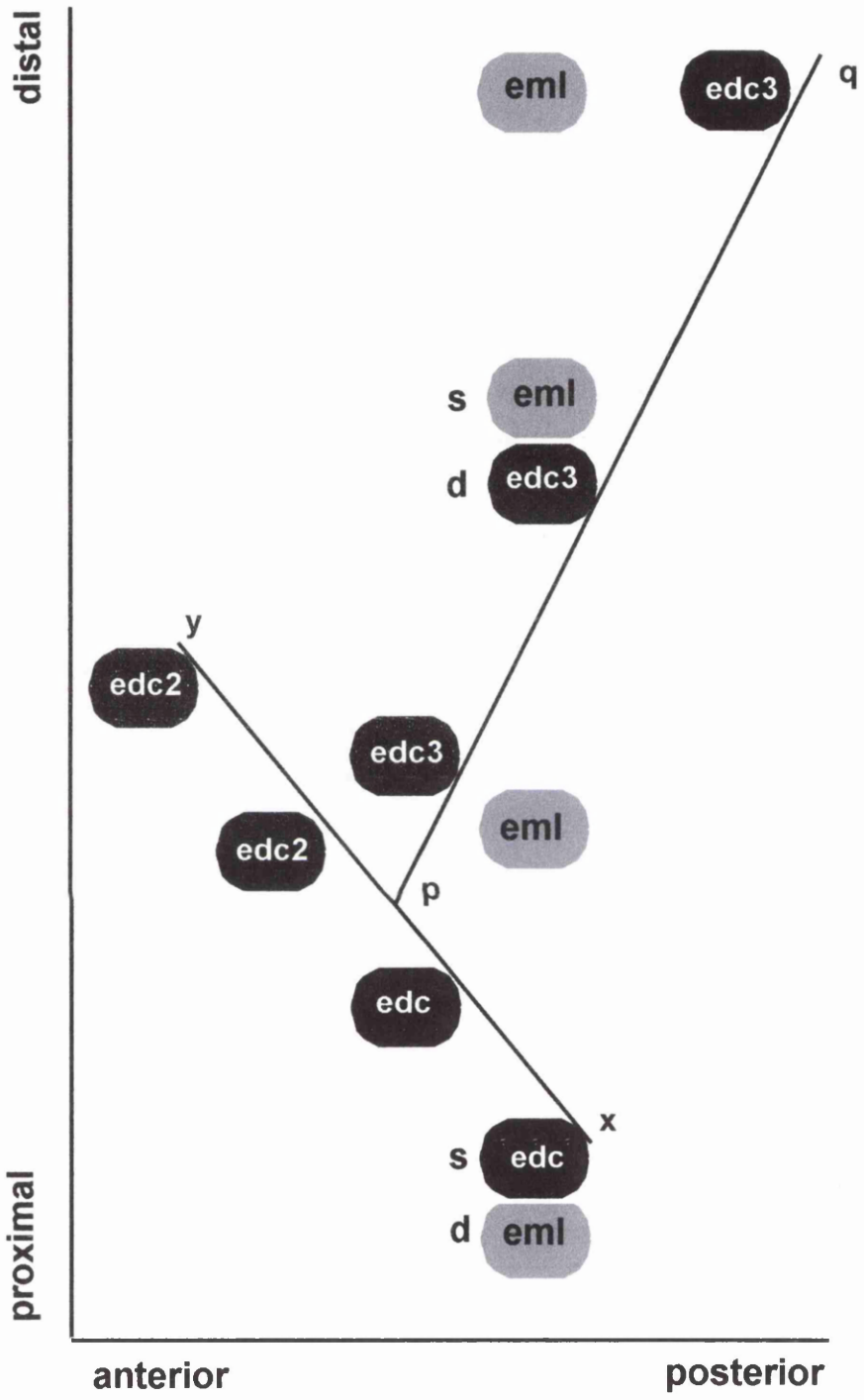
		Anterior					Posterior
		Digit 2	**A**	**Digit 3**		**P**	**Digit 4**
Distal	**Dorsal**		eml				
	Ventral		fdp				
	Dorsal		eml		id	ip	
	Ventral		fdp				
	Dorsal		eml	edc-3	id	ip	
	Ventral		fdp fds				fdq
	Dorsal		eml edc-3		id (m)		
	Ventral		fdp	fds	(m) ip		fdq
	Dorsal	edc-2	edc-3	eml			emu
	Ventral	fdp	fdp	fds			fdq
	Dorsal	edc	eml				emu
	Ventral		fdp		fds		
	Dorsal		edc eml				emu
Prox	Ventral		fdp			fds	

eml, extensor medius longus
edc, extensor digitorum communis
emu, extensor metacarpi ulnaris
id, interosseus dorsalis
ip, interosseus palmaris
fdp, flexor digitorum profundus
fds, flexor digitorum superficialis
fdq, flexor digitorum quarti
m, muscle
A, anterior; P, posterior; Prox, proximal
Interosseus dorsalis, interosseus palmaris and flexor digitorum quarti are intrinsic and the latter does not have a long tendon.
Extensor metacarpi ulnaris has a very short tendinous course in the digit plate.

Figure 3.4 Relationship of extensor digitorum communis (edc) to tendon of extensor medius longus (eml) along the anterior-posterior, proximo-distal and dorso-ventral axes in chick wing

s, superficial; d, deep; edc, extensor igitorum communis; eml, extensor meius longus. Proximally, edc is superficial to eml, then moves to a position anterior to eml and divides in two for digit 2 (edc2) and digit 3 (edc3). Branch to digit 3 (edc3) crosses deep to eml to reach its insertion posterior to eml. The line x-y seems to indicate that edc maybe a tendon for digit 2. If it was a tendon that developed originally for digit 3 then line p-q should have been a more vertical line going directly from x to q. The peculiar course taken by tendon edc3 seems to indicate that it is a secondary development.

Fig. 3.4



edc, extensor digitorum communis(before branching
edc2, extensor digitorum communis for digit 2
edc3, extensor digitorum communis for digit 3
eml, extensor medius longus
s, superficial
d, deep

3.3.1.2 Flexor tendons

On the ventral aspect (Fig. 3.3b), the flexor digitorum profundus (Fdp) muscle arises from the ulna (not indicated) and becomes tendinous distally. At the base of the metacarpus, the tendon splits into two slips, an anterior slip (Fig. 3.3b) which attaches to the base of the distal phalanx of digit 2 and a posterior slip (Fig. 3.3b). The posterior slip reaches the base of the distal phalanx of digit 3 coursing nearer the anterior edge of digit 3 and related to muscle abductor medius (Am) (not shown). Over the metacarpus the flexor digitorum superficialis (Fds) fuses (Fig. 3.3b arrowhead) with Fdp and thus gets inserted into the base of the distal phalanx of digit 3. Proximally, Flexor digitorum superficialis (Fds) muscle seems to be part of flexor carpi ulnaris (not indicated), as described by earlier work (Sullivan 1962).

3.3.1.3 Interrosseus tendons

The interrosseus muscles and tendons lie in between digits 3 and 4 (Fig. 3.3a). Interrosseus dorsalis (id) muscle has a bipennate origin (Fig. 3.3a asterisks) from the adjacent sides of metacarpal 3 and 4 and its tendon runs towards digit 3 (Fig. 3.3a) to be attached to the base of the distal phalanx on its dorsal aspect. Interrosseus palmaris (Ip) muscle (Fig. 3.3a) takes origin from the adjacent sides of metacarpal 3 and 4 ventral to id. Its tendon runs towards the distal phalanx of digit 3 passing dorsal (Fig. 3.3c) to the fused distal ends of metacarpal 3 and 4 thus though Ip arises ventrally it courses dorsally to reach its insertion.

3.3.2 Tendon pattern in 10-day embryonic wing reflects that seen in the adult.

This study was done by analysing histological sections of a wing at stage 36 after 10 days of incubation. Five cross section pictures (Fig. 3.5) are shown at varying positions along digit plate with reference to the adult wing (Fig. 3.3c).

3.3.2.1 Extensor tendons

At the base of the digit plate extensor medius longus (Eml) tendon develops on the dorsal aspect of digit 3 superficial to muscle extensor medius brevis (Emb) and anterior to the insertion of extensor metacarpi ulnaris (Emu) (Fig. 3.5e). More distally, Eml occupies a dorsal position (Fig. 3.5d), and still more distally, is antero-dorsal in relation to digit 3 (Fig. 3.5a, b and c) even though proximally (Fig. 3.5e) it appears at a more posterior position (also compare with Fig. 3.3c plane 5 with 3).

Extensor digitorum communis tendon is superficial to Eml (not shown) just before the metacarpus region, then on the metacarpus it is found anterior to extensor medius

Figure 3.5 Light microscope appearance of tendons in a ten day (stage 36) embryonic wing

Sections pass through 5 different planes shown in Fig 3.3c, anterior to left and dorsal to top.

a) Section passing through plane 1. Dorsally extensor medius longus (eml) and ventrally flexor digitorum profundus (fdp) tendons are seen flanking cartilage of digit 3.

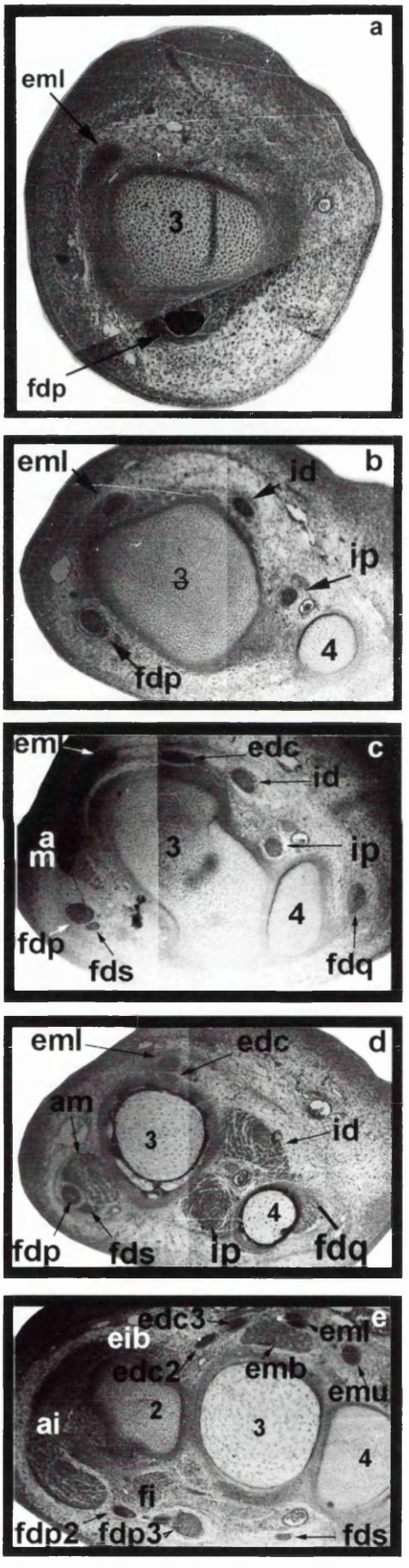
b) Section passing through plane 2 showing eml tendon and interrosseus dorsalis (id) dorsal to digit 3, interrosseus palmaris (ip) lies between digits 3 and 4. Ventrally flexor digitorum profundus (fdp) tendon is seen. Notice that eml and fdp are nearer the anterior of the cartilage.

c) Section passing through plane 3. Eml and id are in the same relationship to the cartilage of digit 3, as seen in (b) except that a new tendon extensor digitorum communis (edc) is seen between the two, having crossed from anterior to posterior; interrossseus palmaris (ip) is seen dorsally in between the cartilage of 3 and 4; flexor digitorum quarti (fdq) is posterior to digit 4. Ventrally, flexor digitorum superficialis (fds) is a small tendon lying close to flexor digitorum profundus (fdp) with which it fuses distally. Abductor medius (am) muscle is seen on the anterior edge of digit 3.

d) Section passing through plane 4. Interrosseus dorsalis (id) and palmaris (ip) are now muscular and lying in interosseus space between digit 3 and 4 from which they take origin; flexor digiti quarti (fdq) muscle is closely related to cartilage 4. Tendon of extensor digitorum communis (edc) is deep to extensor medius longus (eml) where it is crosses from anterior to posterior; abductor medius is seen deep to flexor digitorum profundus (fdp) and flexor digitorum superficialis (fds).

e) Section passing through plane 5. Flexor digitorum profundus split into two slips for digits 2 (fdp2) and 3 (fdp3): similarly extensor digitorum communis is also split in two for digits 2 (edc2) and 3 (edc3). Proximal muscles associated with digit 2 flexor indicis (fi) abductor indicis (ai) and extensor indicis brevis (eib) are seen, including tendon of extensor metacarpi ulnaris (emu) nearing its insertion.

Fig. 3.5


a
eml
3
fdp
b
eml
id
ip
3
4
fdp
c
eml
edc
id
am
3
ip
4
fdp
fds
fdq
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edc
am
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ip
fdq
e
edc3
eml
eib
edc2
emb
emu
ai
2
3
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fi
fdp2
fdp3
fds

longus (not shown) and more distally (Fig. 3.5e) it branches into two small tendons one for digit 2 and the other for digit 3. Still more distally (Fig. 3.5d), it is deep to extensor medius longus and this can be correlated with the adult anatomy where extensor digitorum communis crosses from anterior to posterior deep to extensor medius longus (Fig. 3.3a and c plane 4). Further distal (Fig. 3.5c) just before the base of proximal phalanx of digit 3 which is its insertion point, it is found in a posterior position lying in between the developing tendons of Eml and interosseus dorsalis. This changing antero-posterior relationship between extensor medius longus, extensor digitorum communis and other tendons is summarised in Table 3.2 and Fig. 3.4).

3.3.2.2 Flexor tendons

Proximally, flexor digitorum profundus (Fdp) muscle is found anterior to flexor digitorum superficialis (Fds) and ventral to digit 3 (not shown). Distally, Fdp tendon splits into 2 slips for digits 2 and 3, the latter slip in line with anterior margin of metacarpal 3 (Fig. 3.5e). The tendon of Fds lies posterior to Fdp and is in line with posterior edge of metacarpal 3 (Fig. 3.5e). This seems to fit with the muscle arrangement. Distally Fds tendon is very close to Fdp and both are now in line with anterior edge of digit 3 (Fig. 3.5d, c). Even more distad, Fds tendon fuses with Fdp (Fig. 3.5b) and thus there is only one tendon, on the ventral side of digit 3, (Fig. 3.5a). Thus it can be seen that Fds tendon is posterior proximally (Fig. 3.5e) whereas distally it is found more anterior (Fig. 3.5c) (Table 3.2).

3.3.2.3 Interosseus tendons

Interosseus muscles, dorsalis and palmaris develop in the space between digit 3 and 4 on dorsal and ventral sides respectively (Fig. 3.5d). Distally, both their tendons develop on a dorsal plane, interosseus dorsalis postero-dorsal to digit 3 and interosseus palmaris dorsal to an articulation between digit 3 and 4 (Fig. 3.5c). Further distal, the two tendons are found dorsally (Fig. 3.5b). This fits the picture seen in adult anatomy where interosseus palmaris tendon can be seen on the dorsal side of the adult wing, though proximally it can be seen emerging from under the interosseus dorsalis (Fig. 3.3c, 3.5c) and coursing towards digit 3 (Fig. 3.3a) for attachment.

3.3.3 Gross and histological study of tendons in chick toes

I studied the gross anatomy of the avian foot in an attempt to unravel the complexity of the bundle of tendons in the foot. The skeleton comprises of the femur, the tibiotarsus and a short fibula in the leg (crus) and the tarsometatarsus with the

phalanges in the foot. Digits in the leg are numbered 1, 2, 3 and 4 from anterior to posterior The number of phalanges in each digit vary, digit 1 has 2; digit 2 has 3; digit 3 has 4 and digit 4 has 5 phalanges (Fig. 3.6a).

3.3.3.1 Extensors

On the extensor surface there is only one long tendon (Fig. 3.6b, 3.8a) extensor digitorum longus, supplying toes 2, 3 and 4 and extensor hallucis longus goes to toe 1 (Table 3.1).

3.3.3.2 Flexors

Digit 1 and 4 have 2 flexor tendons, digits 2 and 3 have 3 flexor tendons. As digit 3 is longer, I dissected out the tendons in that digit. To expose the flexor tendons (Fig. 3.6c) a tough fibrous sheath similar to that seen in the human had to be cut. The inner surface of this sheath was smooth and shiny indicating that it was lined by synovial membrane. Flexor digitorum longus is on a deep plane very close to the bone and runs to the terminal phalanx for insertion. The other 2 tendons are more superficial but terminate more proximally; flexor perforans inserts on the sides of the proximal phalanx, whereas flexor perforans perforatus inserts on an intermediate one. Thus to allow the passage of Fdl superficial tendons split into 2 slips (Fig. 3.7). Because tendons split to attach to their insertions and to allow the deep tendon to pass through, there are varying number of tendons at different levels (Fig. 3.6c). In Fig. 3.8b, which is a cross section passing through plane 1 (Fig. 3.6d) there are 3 tendons, flexor digitorum longus is the deepest and there are 2 other tendons superficial to it. The arrangement of these 3 tendons, has a cabbage leaf appearance, meaning that one layer (Fig. 3.8b tendon outlined in black) partially overlaps the other (tendon outlined in white). In Fig. 3.8c which is a more proximal section through plane 2 (Fig. 3.6c) there are 2 tendons, a deep (Fdl) and a superficial Fp et pd just before the latter splits to allow the deep tendon to pass through.

3.3.4 Embryonic development of tendons in the wing

At stage 25, there are no signs of tendon, although two muscle masses, dorsal and ventral, can be made out (Fig. 3.9a) with conventional histology. A mesenchymal lamina stained by silver has been reported to be present even at stage 25 (Hurle et al 1989) paralleling closely the location of the distal tip of myogenic mass (Rong et al 1987). At stage 27, at both proximal (not shown) and distal (Fig. 3.9b) levels, there is no tendon or mesenchymal lamina formed though cartilage is present. By stage 28, there is

Figure 3.6 Tendon pattern in adult chick leg

a) Alcian green staining at day 10 shows that digits 1, 2, 3 and 4 have phalanges 2, 3, 4 and 5 respectively.
b) Dorsal surface, arrows indicate extensor digitorum longus tendon to digits 2 and 3.
c) Plantar surface, showing many flexor tendons (arrows).
d) Flexor tendons in digit 3, black and white arrowhead, flexor perforans digiti; black arrowhead, flexor perforans et perforatus digiti; white arrowhead, flexor digitorum longus, black double arrowhead indicates the vinculum. Lines 1 and 2 are equivalent to the planes through which cross-sections are shown in Fig. 3.8b and c.

Fig. 3.6

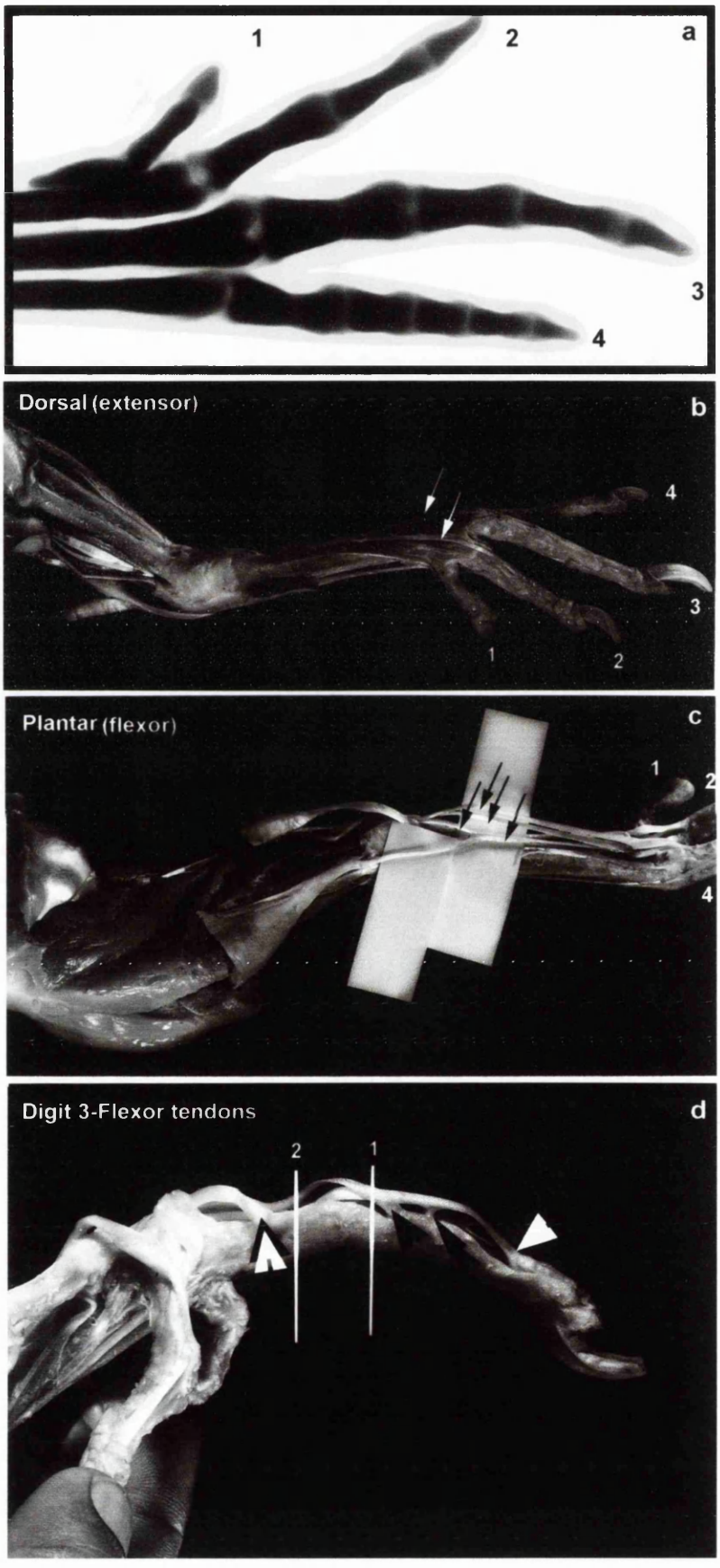

1
2
a
3
4
Dorsal (extensor)
b
4
3
1
2
Plantar (flexor)
c
1
2
4
Digit 3-Flexor tendons
d
2
1

Figure 3.7 Diagrammatic representation of tendons in digit 3 of the chick leg

Pink elements numbered 1, 2, 3 and 4 are the phalanges in digit 3. Three tendons are present in this toe, each tendon splits in 2 slips before it reaches its insertion at the sides of the phalanx. The most superficial tendon, flexor perforans digiti (FPD) inserts to 2nd phalanx; flexor perforans et perforatus digiti inserts into 3rd phalanx; and the deepest tendon flexor digitorum longus (FDL) inserts into the terminal phalanx. Different planes along the P-D axis of the digit would have different number of tendons in transverse section. For example, at the point where tendon FPD is labelled, a transverse section would show 3 tendons and at the point where FPD divides just before its insertion, a transverse section would show 4 tendons.

Fig. 3.7

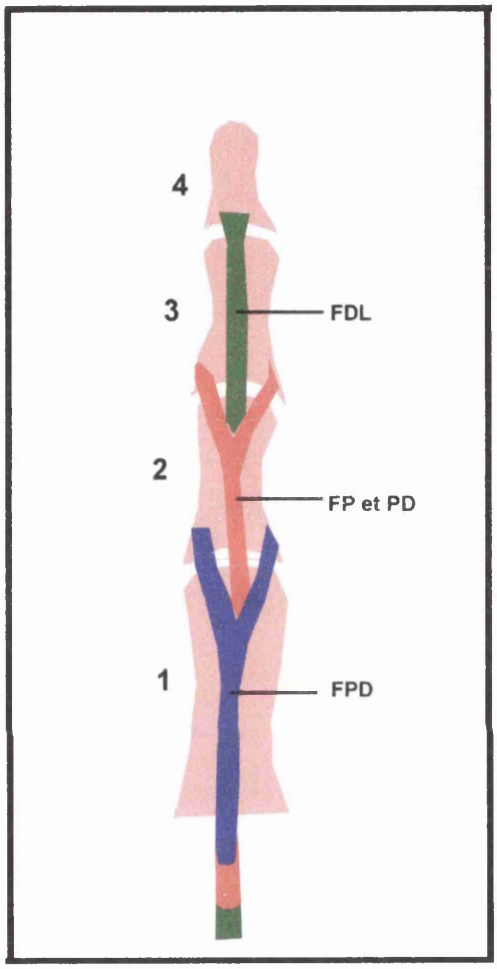


Figure 3.8 Sections showing tendons in digit 3 of the chick leg at stage 36

Sections in (b) and (c) are equivalent to cross-sections passing through planes 1 and 2 of Fig. 3.6c.

a) Tendon of extensor digitorum longus (arrow).

b) Deep flexor digitorum longus (fdl) tendon, overlain by the splitting (dotted outline) flexor perforans et perforatus digiti (fp et pd) tendons; asterisks indicate fibrous flexor sheath.

c) Flexor digitorum longus tendon is deep (D) and flexor perforans et perforatus digiti is superficial (S).

Fig. 3.8

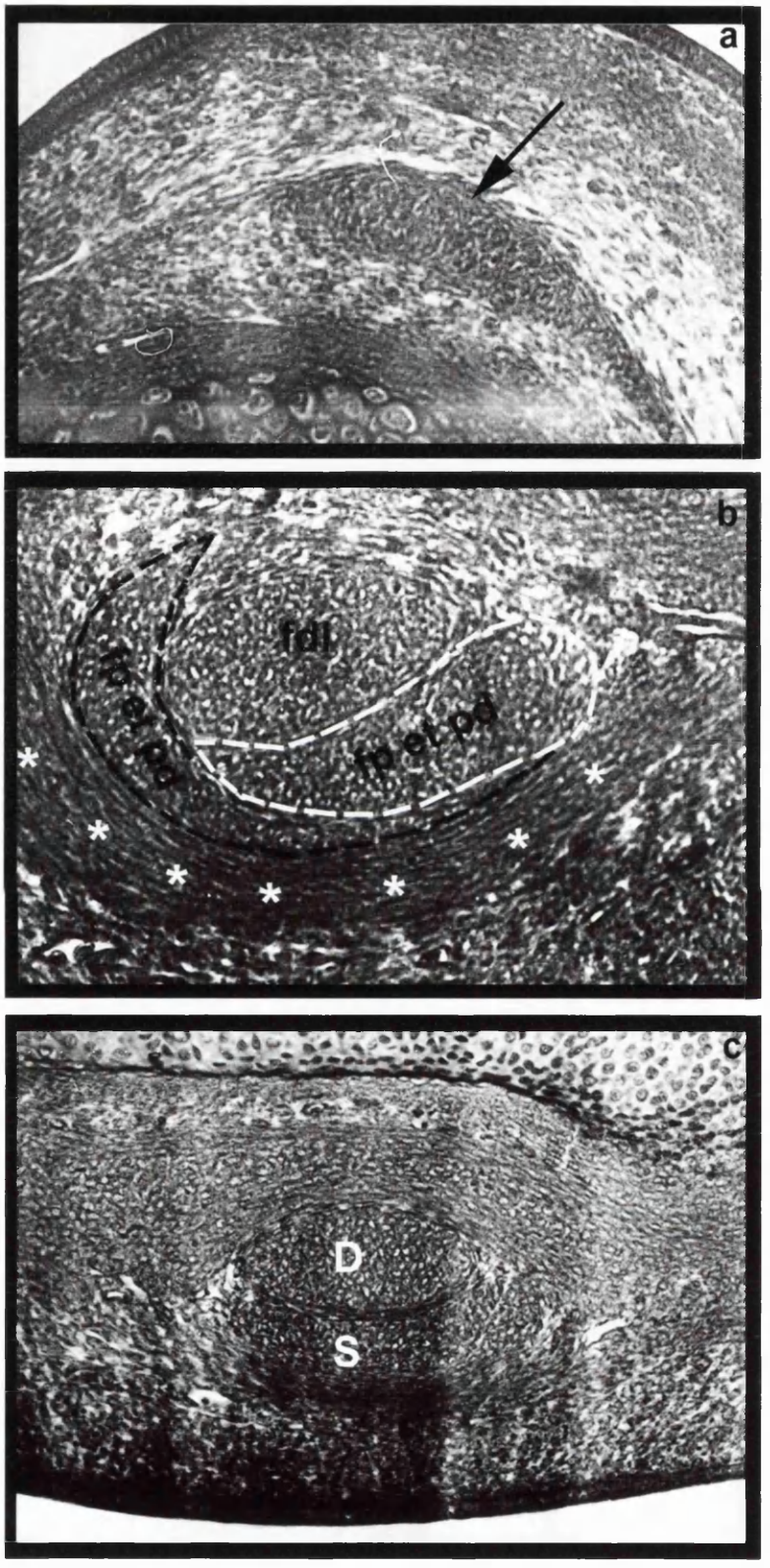


Figure 3.9 Transverse sections of the chick wing bud at stages 25 to 31/32

Dorsal to top; anterior to left.

a) Proximal section at stage 25, dorsal (arrow) and ventral (arrowhead) muscle mass seen close to ectoderm.

b) Distal section at stage 27, no muscle or tendon has developed in the distal digit plate.

c) Section at stage 28 with 2 dorsal muscle masses (arrows) and a ventral muscle mass (arrowhead) but no tendon developing.

d) Section at stage 29 showing mesenchymal lamina (white arrows) away from dorsal ectoderm (arrows) and continuous with basement membrane (arrowhead).

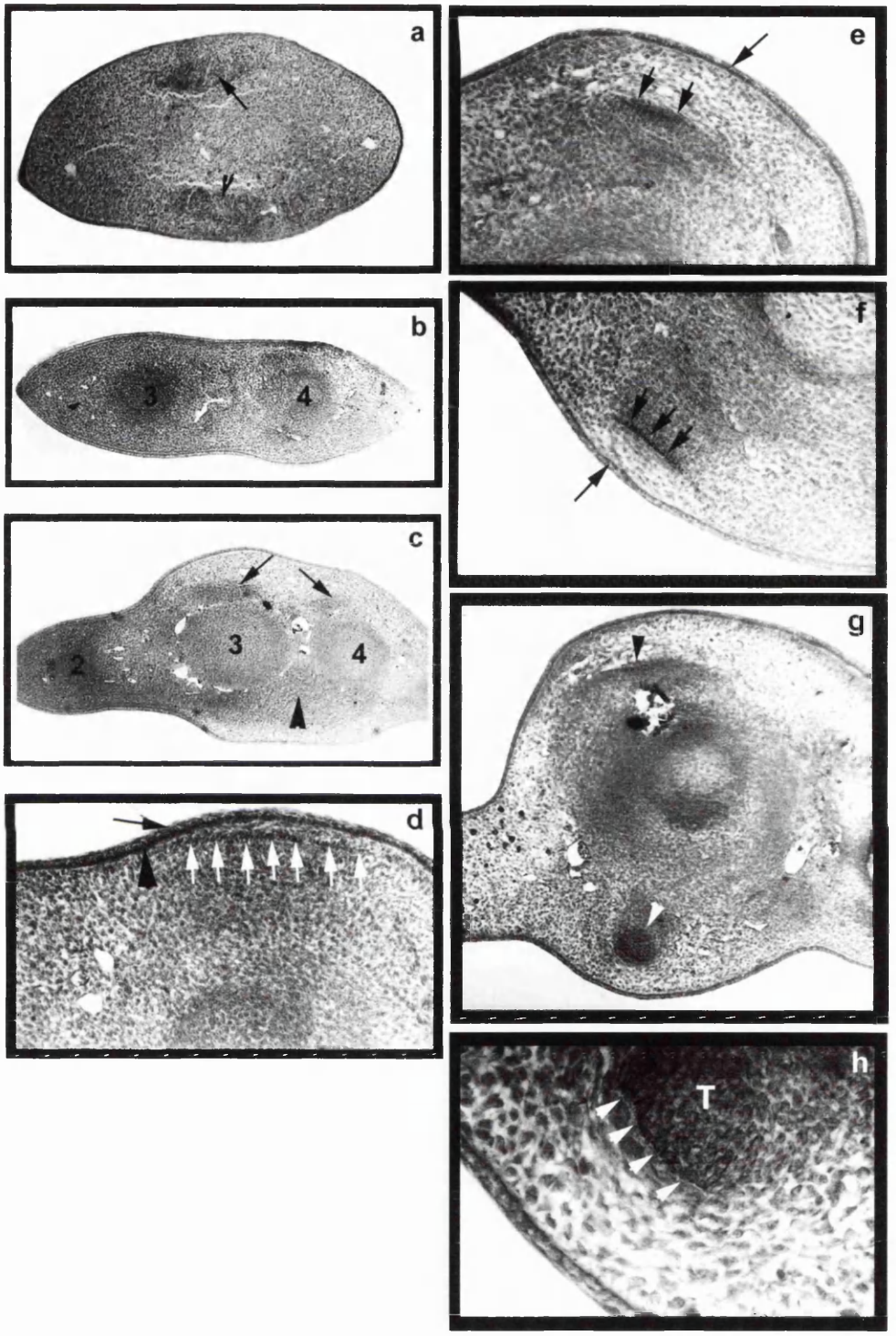
e) Mesenchymal lamina and condensing tendon cells (small arrows) have moved away from dorsal ectoderm (big arrow) at stage 30.

f) Ventral mesenchymal lamina and condensing tendon cells (small arrows) in same section shown in (e).

g) Developing dorsal tendon (black arrowhead) and ventral (white arrowhead) at stage 31/32.

h) High power magnification of the ventral mesenchymal lamina and developing tendon seen in (g). Arrows indicate the mesenchymal lamina with (T) tendon cells seen on its inner aspect.

Fig. 3.9



still no development of the tendon or mesenchymal lamina at distal (not shown) or proximal levels (Fig. 3.9c). However 2 dorsal muscle masses (arrows) and a huge ventral muscle mass (arrowhead) can be seen quite deep away from the ectoderm.

At stage 29, the mesenchymal lamina, a layer just under the ectoderm, continuous with the basement membrane on either side (Fig. 3.9d) is seen with conventional histology. As development proceeds, cells condense on the inner face of the mesenchymal lamina to form tendon blastemas. The lamina-tendon cell complex (Fig. 3.9e dorsal and 3.8f ventral) then slowly gets displaced deeper into the mesenchyme away from the ectoderm and the mesenchymal lamina on either side of each tendon disappears. As the developing tendon is becoming displaced (Fig. 3.9g. 3.9h), the mesenchymal lamina can be seen within the condensing tendon cells. Even at stage 34, when tendon approaches its insertion to cartilage, the mesenchymal lamina is within the developing tendon (Fig. 3.10a and b). Hurle et al (1989) had seen the mesenchymal lamina up to stage 33. When one looks proximally, at stage 34, a second tendon has started to develop and appears as a new aggregation of tendon cells forming in association with the already formed tendon (Fig. 3.10c, d).

3.3.5 Formation of tendon sheath

The sheath around the tendon of flexor digitorum profundus in the chick wing appears to form at stage 34. Multiple layers of flattened cells become arranged circumferentially around the developing tendon (Fig. 3.11a). Later, a space starts to develop in between these cells (Fig. 3.11b) in such a way that the cells that were compact initially, now become loosely arranged where the space forms. As the space widens, cells are pushed and or displaced away from the tendon but at least a single layer of cells remain very closely related to the tendon. During this process, there are also some cells scattered within the space (Fig. 3.11c). Most of the cells within the space are flattened (Fig. 3.11c) but some have a rounded profile (Fig. 3.12a). The space does not form around the complete periphery of the tendon. On one side, flattened cells may persist (Fig. 3.12a) to form the vinculum. When the flexor digitorum profundus tendon splits into 2 separate slips, for digit 2 and for digit 3 (Fig. 3.12b), it appears that the developing sheath i.e. the cells that surround the main tendon is dragged along with the slip that is splitting (Fig. 3.12b). When the two split tendons are far apart, the cells seem to be organizing once again to surround each tendon individually (Fig. 3.12c). When 2 tendons do the opposite and come closer together to fuse before the insertion, cells not only surround each individual tendon but also enclose both tendons together

Figure 3.10 Sections of chick wing at stage 34

a) Tendon cells (arrow) surrounding mesenchymal lamina (arrowheads) lies very close to the cartilage.

b) As tendon approaches its insertion (arrow) to cartilage, mesenchymal lamina (arrowheads) is still present.

c) and (d) flexor digitorum superficialis (arrow). Tendon sheath formation with cells arranged circularly (arrowhead) around circumference of flexor digitorum profundus tendon (t). Space forming between the tendon (t) and the circumferentially arranged cells is also seen.

Fig. 3.10

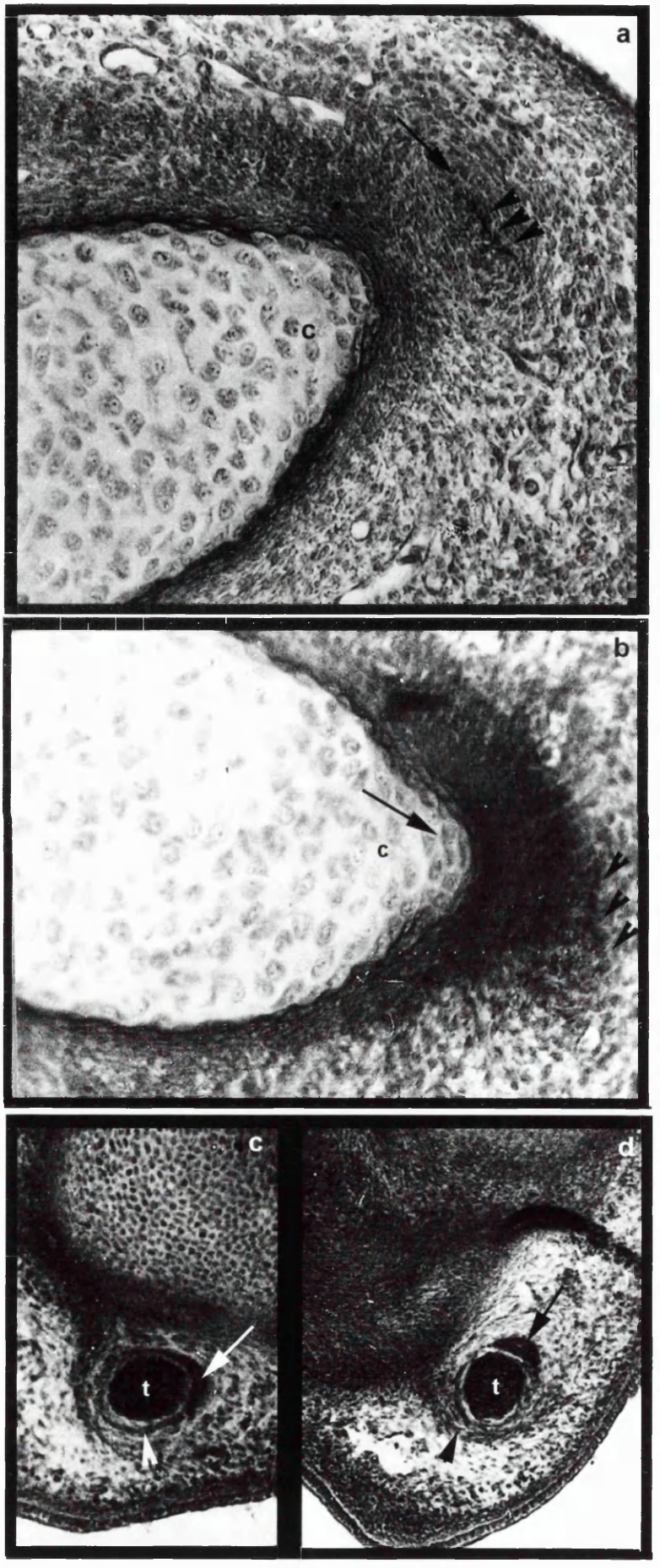


Figure 3.11 Light microscope analysis of cross sections of the formation of tendon sheath in the chick wing

(t), tendon.

a) Cells arranged circumferentially around tendon (arrows).

b) Cells (arrow) surrounding the tendon, space (arrowhead) around tendon.

c) White arrow shows cells further away from tendon that form the outer parietal layer of synovial sheath; white arrowhead shows cells that are close to tendon and form inner visceral layer of synovial sheath ; black arrow shows flattened cells scattered in the space.

Fig. 3.11

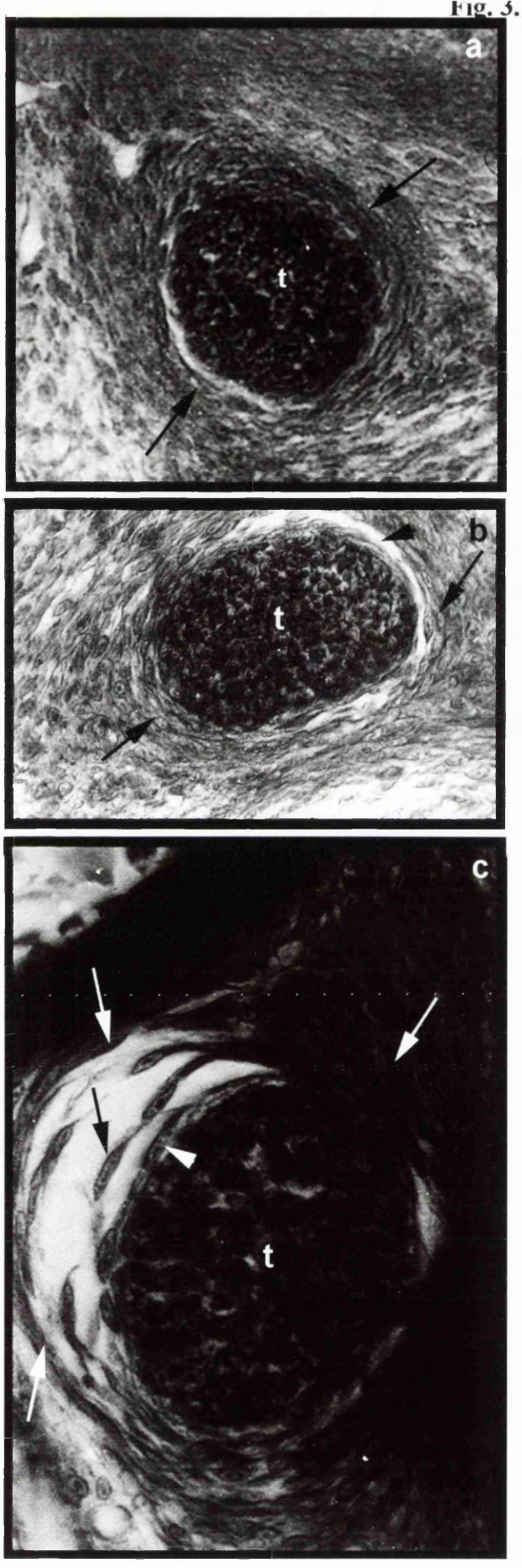


Figure 3.12 Tendon sheath in association with splitting and fusion of tendons in chick wing

Sections a-d are from proximal to distal.

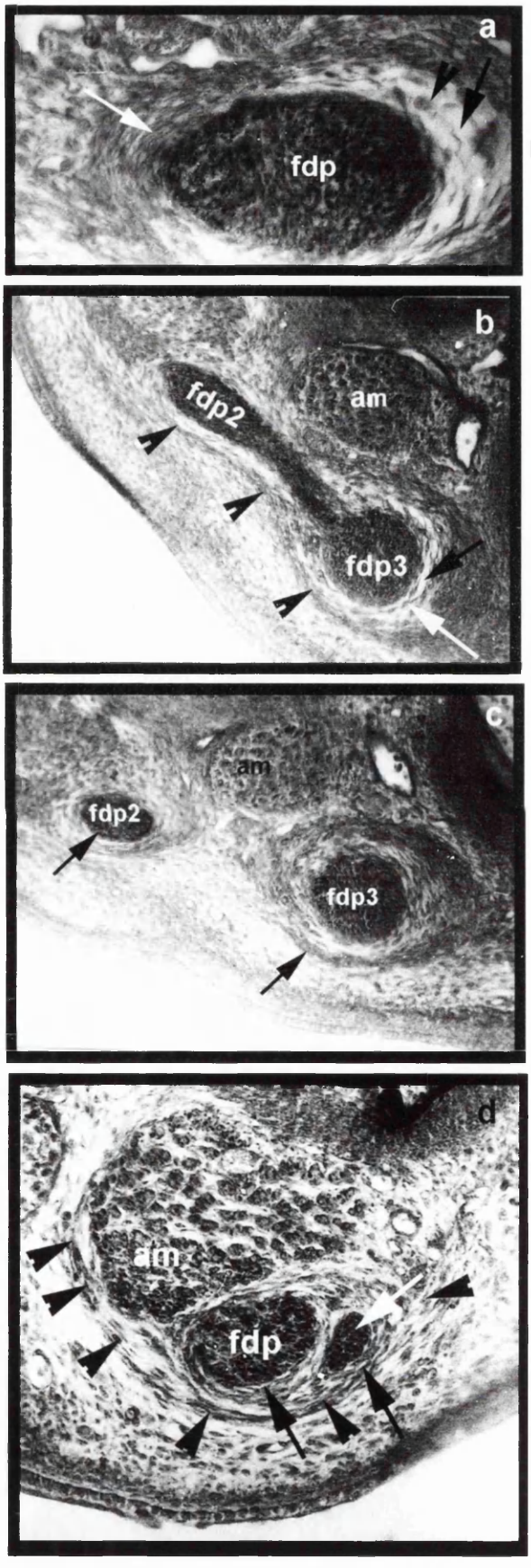
a) Flattened cells (white arrow) associated closely with tendon; space (black arrow) surrounding tendon; rounded cell (black arrowhead) in the space.

b) Flexor digitorum profundus splitting for digits 2 (fdp2) and 3 (fdp3); space (black arrow); cells (white arrow) in the space; cells (arrowheads) that surround both slips of tendon.

c) Flexor digitorum profundus tendons (fdp2) and (fdp3) for digit 2 and 3 respectively; cells organising around individual tendons (arrows).

d) Tendon of flexor digitorum profundus (fdp) for digit 3, flexor digitorum superficialis tendon (white arrow), cells (black arrows) enclosing individual tendons; cells (arrowheads) surrounding both tendons and the underlying abductor medius muscle (am).

Fig. 3.12



(Fig. 3.12d). When a muscle is lying deep to the tendon it appears that cells enclose both the tendon and the muscle (Fig. 3.12d).

3.4 Discussion

3.4.1 Development of individual digital tendons

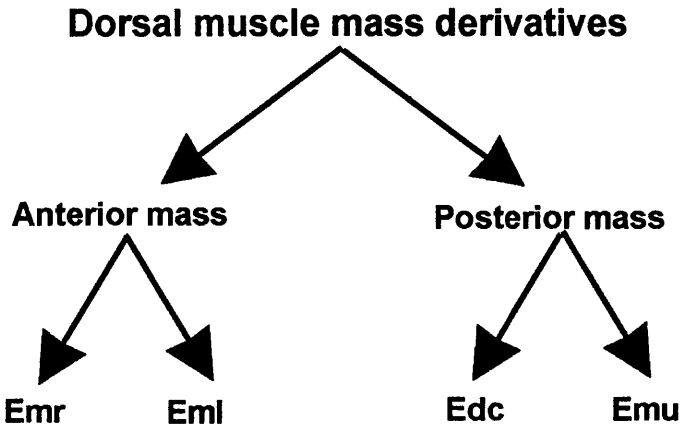
During early development of the wing, the initial tendon blastema has to divide into separate tendon blastemas along the anterior-posterior axis, for each of the different digits. Some tendons lie in line with their muscle and run from proximal to distal without gross change in anterior-posterior or dorso-ventral direction connecting its muscle to its insertion point on the same surface (Fdp, Eml, Id). Other tendons run distally for attachment but show relative changes in anterior-posterior position. These tendons connect a muscle derived from posterior muscle mass to an anterior position (Fig. 3.13). For example, Edc muscle is derived from the posterior muscle mass and its tendon shows a change in its anterior-posterior course (Fig. 3.4). Edc in its proximal course (Fig. 3.4 line x-y) is directed towards digit 2, but then distally gives off a branch for digit 3 instead. The branch for digit 3 now courses posteriorly (Fig. 3.4line p-q) to insert posterior to Eml. From the course taken by the Edc tendon originally, it appears that it may have been formed morphologically and functionally in relation to digit 2 and that the branch for digit 3 is a secondary attachment. If Edc was formed in relation to digit 3 then line p-q in Fig. 3.4 should be more parallel to the P-D axis and instead extend from point x to q in Fig. 3. Hence I suggest that Edc originally forms in relation to digit 2. Changes in tendon direction could be related to their action on the digits. Sullivan (1962) has described that Edc of digit 3 after passing beneath Eml, near its insertion is located in a groove at distal end of metacarpus 3, and the groove turns anteriad, such that Edc approaches its insertion from a posterior direction. Thus the pull of the muscle is transmitted such that it helps to effect a supinating movement of tip of digit 3 (Sullivan 1962).

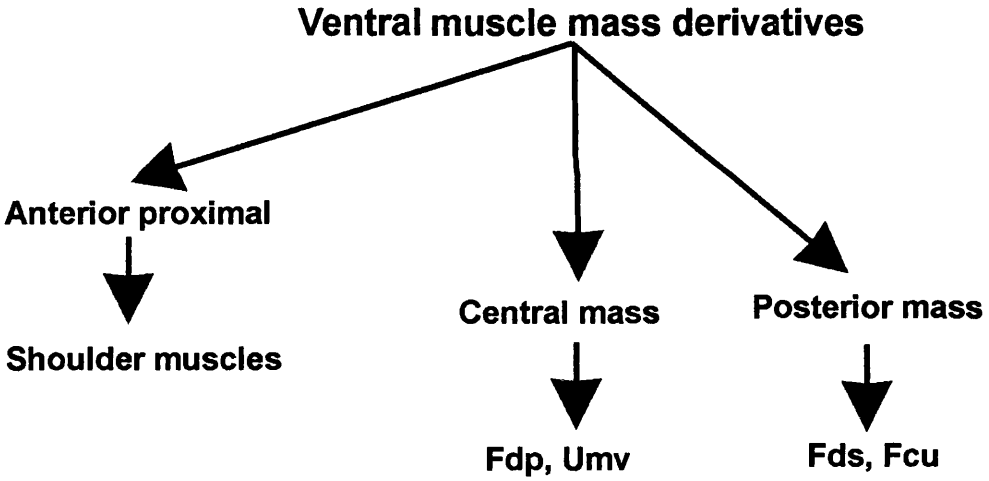
Another example in which tendons change relative positions is seen in Fds. Fds muscle is derived from the ventral posterior muscle mass along with flexor carpi ulnaris (FCU) at stage 30-31 (Robson et al 1994) (Fig. 3.13). Proximally, Fds tendon lies in a posterior position (Fig. 3.5e) in relation to the position of its muscle but distally in a more anterior position (Fig. 3.5b and d) in relation to its cartilaginous insertion. To explain the course of Fds (and Edc) one would have to predict that the proximal segment and the distal segment develop independently and then make precise secondary contact.

Figure 3.13 Muscles derived from the dorsal and ventral muscle masses.

(Figure adapted after Robson et al 1994)

Fig. 3.13


Dorsal muscle mass derivatives
Anterior mass
Posterior mass
Emr
Eml
Edc
Emu


Ventral muscle mass derivatives
Anterior proximal
Shoulder muscles
Central mass
Fdp, Umv
Posterior mass
Fds, Fcu

The tendon of Fds fuses with Fdp tendon before its insertion into the base of the distal phalanx of digit 3 (Sullivan 1962) (Fig. 3.5b, c and d). However Fdp muscle is derived from the centre of ventral muscle mass while Fds muscle is derived from the posterior of the ventral mass. Hence the posterior part of the Fds tendon probably develops independently, whereas the anterior part which is in close relation to Fdp tendon may have developed by delineating from the Fdp tendon during development. It has been suggested for leg flexor tendons that the superficial tendon flexor perforans separates from the deeper flexor digitorum longus which differentiates first (Hurle et al 1989).

Tendon of Edc also has a changing dorso-ventral relationship with the tendon of Eml. Proximally, it is superficial (dorsal) to tendon of Eml but, as it courses posteriorly, it crosses Eml again but this time is deep (ventral) to Eml. As there is this superficial-deep relation between Edc and Eml, the blastema may also divide dorso-ventrally. This is not hard to perceive but it is difficult to understand how, at a proximal point, dorso-ventral splitting occurs such that Eml is formed deep but at a distal point the same tendon is formed superficially.

A third class of tendons are those which develop on the dorsal side but make contact proximally with muscles on the opposite side i.e. ventral, thereby attaching ventral muscle to cartilage on the dorsal side. There is only one such tendon which I have noticed in this work i.e. interosseus palmaris. Tendon of interosseus palmaris (Ip) has a dorsal course and inserts on side of the phalanx. It is probably derived from the dorsal distal tendon primordium (Kardon 1998), but its muscle arises ventral to the interosseus dorsalis muscle and has been described as being derived from the ventral muscle mass (Sullivan 1962). Certainly it is possible for dorsally and ventrally derived tendons/muscles to fuse. When the distal tip of a limb bud is inverted dorso-ventrally, the ventral tendons of the graft join with nearby dorsal tendons in the stump (Shellswell and Wolpert 1977). Another example of this phenomenon in the literature is flexor digiti quarti (Fdq), an intrinsic muscle of digit 4 in the wing derived from ventral muscle mass, which makes secondary contact proximally with muscle ulnimetacarpalis dorsalis (umd), a muscle derived from dorsal muscle mass (Sullivan 1962).

3.4.2 Role of mesenchymal lamina in tendon-cartilage attachment

It has been suggested that the mesenchymal lamina is linked to the pre-chondrogenic elements by dorso-ventral fibrils (Hurle et al 1989). Here, the mesenchymal lamina is seen within the tendon at stage 34 as the tendon lies very close

to its insertion to cartilage and this certainly indicates that it may have a role in distal connection between tendon and cartilage.

3.4.3 Tendon sheath

My observations confirm the general features of the description given by previous work (Greenlee and Ross 1967). It certainly does appear that the cells that remain around the tendon as the synovial cavity develops maybe destined to become the inner visceral layer of synovium of the tendon sheath. Cells that are not in close proximity to the tendon may form the fibrous flexor sheath and the innermost of these, may become the outer parietal layer of the synovial lining, which is adherent to the fibrous flexor sheath. The scattered cells in the forming synovial cavity are probably those that are getting displaced away from the tendon. However, the question still arises as to how a space is formed in the first place.

Hyaluronic acid is a good candidate to be involved in formation of synovial space in tendon sheath. In chick limb, hyaluronic acid has been associated with the development of joint cavity (Archer et al 1994). Hyaluronic acid is a constituent of extracellular matrix found between the mesenchyme cells of the limb bud. When cartilage condensation begins, hyaluronic acid is concentrated in the periphery and is relatively absent in the core of the limb bud (Singley and Solursh 1981). At stage 37, hyaluronic acid is preferentially located at the interzone and during the ensuing cavitation, at stage 41, is localised to the newly formed joint cavity, separating articular surfaces and in developing synovium of the chick metatarsophalangeal joint.

Hyaluronic acid has been shown to be necessary for maintenance of the cell free space normally visible under the neural groove between neural ectoderm and the endoderm (Fisher and Solursh 1977). Hyaluronic acid is also important in forming space in the developing corneal stroma (Toole and Telstrad 1971).

Observations made in this study do not offer any support to the idea that tendons are invaginated in the synovial sheath put forward in textbooks (Bannister et al 1995). Instead it appears that cells surrounding the tendon become the synovial sheath as suggested by Greenlee and Ros (1967).

CHAPTER FOUR

MOLECULAR SIGNALLING IN TENDON DEVELOPMENT

Chapter Four: Molecular Signalling in Tendon Development

4.1 Introduction

In this chapter it is shown that *EphA4*, a cell-cell signalling molecule is expressed in a dynamic manner during tendon development (see also Patel et al 1996). The *EphA4* gene, was first described in mice. Transcripts were found in the hindbrain, restricted to rhombomeres 3 and 5 in a 9.5-day old mouse embryo, and hence the gene was called segmentally expressed kinase (Sek) (Gilardi-Hebenstreit et al 1992). The chick homologue (*Cek-8*) was isolated by Sajjadi and Pasquale (1993), the human (*Hek8*) by Fox et al (1995) and the Xenopus (*Paggliaccio*) by Winning and Sargent (1994). In this thesis the new nomenclature according to Eph nomenclature committee (1997) is adopted. It has been suggested that the function of *EphA4* may be conserved, since expression patterns in different species are very similar (Nieto et al 1992; Fox et al 1995; Ohta et al 1996; Patel et al 1996; Winning and Sargent 1994). During embryogenesis, prominent expression is found in presumptive somites, hindbrain, otocyst, limb buds and lateral motor column in brachial and lumbar segments of the spinal cord. Later in development, high levels are also seen in tendons (see later, this thesis) and in the ventricular zone of the developing brain. In the adult, expression is maintained in several tissues including brain, kidney, heart, lung, skeletal muscle, testis, and ovary (Sajjadi and Pasquale 1993; Mori et al 1995). The diverse spatial and temporal expression of *EphA4* suggests that it may be involved in multiple developmental processes (reviewed in Zhou 1998). *EphA4* interacts strongly with GPI linked ligands *Ephrin-A1, -A4* and *–A5*. It also interacts weakly with *Ephrin-A2* and *–A3*, and with *Ephrin-B1, B2 and -B3* transmembrane ligands and is the only EphA receptor that is known to interact with transmembrane ligands (Table 1.1).

Six 1 and *Six 2,* two novel murine homeobox-containing genes have been reported to be expressed in a complementary fashion during development of tendons in mouse embryos (Oliver et al 1995). *Six 1* is expressed strongly in dorsal tendons whereas *Six 2* is strong in ventral tendons. Here I document the pattern of *Six 1* expression in developing chick tendons and also show that a signalling molecule *Bmp-4,* and its natural antagonist *Follistatin,* are expressed in tendon. BMPs belong to a subset of the TGF-ß superfamily of growth factors which also includes TGF- ß subset. BMPs exert their biological functions by interactions with cell surface receptors of which there are two types. BMP signalling through the *BMP-R1* is thought to lead to chondrogenesis (Zou and Niswander 1996; Yokouchi et al 1996; Merino et al 1998).

Follistatin, encodes a protein that antagonises the action of some members of the TGFß super-family, including BMP-2, BMP-4 and Activin, but not for example TGFß1 (reviewed by Patel 1998). Follistatin can neutralise BMP function by preventing BMP binding to its cognate BMP receptors. Follistatin can regulate the long range signalling activity of TGFß super-family proteins, while Eph-mediated signal transduction requires cell-to-cell contact. *BMP-7* (Macias et al 1997) and *Tgfß3* genes have been reported to be expressed in developing tendons whereas *Tgfß2* is expressed in developing tendon and cartilage (Merino et al 1998).

Tendon cells, like cartilage cells, originate from lateral plate mesoderm. The mechanism by which mesenchymal cells derived from the same source later differentiate into cartilage, tendon and other connective tissue elements with specialised function is not known. It is well established that muscle and tendon can develop autonomously (Kieny and Chevalier 1979, Christ et al 1979) but the relationship between tendon and cartilage development is not so clear. For example, when a digit is truncated by removal of ridge from the tip of digit, dorsal and ventral tendons fuse around the distal tip of the truncated digit (Hurle et al 1990) indicating that tendon can develop independent of cartilage. However when ectopic cartilage is induced in interdigital mesenchyme, it was accompanied by the development of tendon (Ganan et al 1996). Here we look at relationship between tendon development and skeletal development, and expression of *EphA4* and *Follistatin* in developing tendons.

In order to test the relationship of development of tendon and cartilage, a number of manipulations that either induce ectopic cartilage in the digital plate or truncate digit cartilage were performed. Apical ectodermal ridge removal from the distal tip of digits in the chick leg leads to truncation (Saunders 1948, Summerbell 1974, Shellswell and Wolpert 1977, Rowe and Fallon 1982). Application of FGF-2 or FGF-4 to the autopod of chick leg buds prevents chondrogenesis and results in digit truncation (Merino et al 1998). TGF-ß1 application to inter-digital tissue (Ganan et al 1996) or removal of ridge from interdigital region induces cartilage. By monitoring expression of *EphA4* and *Follistatin*, we explored how these manipulations affected tendon formation. Further, it was also tested if TGFß signalling was capable of initiating development of tendon as well as skeletal elements.

4.2 Materials and Methods

Experimental manipulations, sectioning, and photography analysis work done in this chapter were done by me. *In situ* hybridisation was performed by K. Patel and me as detailed below.

4.2.1 Antibody staining

Bring cryosections to room temperature and encircle sections with grease pen. Wash in 1x PBS for 5 minutes. Add full strength primary antibody (procollagen type I – (M-38 -McDonald et al 1986) or tenascin (M1-B4 Chiquet and Fambrough et al 1984 - Hybridoma bank) and incubate at room temperature for one hour. Then wash three times in 1x PBS, for 5 minutes each time. Add secondary antibody (antimouse IgG FITC conjugate Sigma) at 1:50 dilution and incubate in the dark at room temperature for 45 minutes. Wash three times in 1x PBS for 5 minutes each and mount in DABCO/glycerol (50% glycerol in 1x PBS wit 100mgm/ml of DABCO).

4.2.2 In situ hybridisation

In situ hybridisation whole mounts shown in Plate 4.1 were done by me with probe prepared by K. Patel. Preparation of probes and situ hybridisation of whole mounts of *EphA4*, *Follistatin* and *Bmp-4* in Fig. 4.3, 4.11-4.15, was performed by K. Patel. *Six 1* whole mounts were processed by G.Oliver.

4.2.3 Preparation and application of beads

Heparin acrylic beads of size 100-150μm in diameter are selected using an eyepiece graticule and stage micrometer, washed in PBS and then soaked in 1μl of either TGFß1 (R and D Systems) at 10μg/ml, or human recombinant Follistatin at 1mg/ml, (a kind gift from The National Hormone and Pituitary Repository, Maryland, USA), or FGF-4 (a kind gift from John Heath) at 0.7mg/ml or BMP-4 (Genetics Institute, Cambridge, Massachusetts) at 100μg/ml for at least 1 hour in a humidified chamber. Control beads were soaked in PBS only. Beads were applied either at the tip of digit 3 or at the 3rd inter-digit at stages 28-29 except where otherwise stated.

4.2.4 Removal of apical ectodermal ridge

Ridge from the tip of digit 3 or from the 3rd inter-digit was removed using a fine tungsten needle at stage 27-29.

4.2.5 Alcian green staining

Staining for cartilage was done according to section 2.4.

4.3 Results

4.3.1 Characterisation of expression pattern of *EphA4* in chick leg tendons

4.3.1.1 Early expression pattern of *EphA4* in foot plate

EphA4 is first detected in the posterior part of the developing wing bud at stage17. Subsequently weak expression extends throughout and by stage 22/23, expression is confined to the distal tip of the wing bud (not shown). Here expression in relation to the leg bud is studied. As the limb bud grows out further, expression still remains at the distal tip. The posterior boundary of expression is well defined but anteriorly, expression extends more proximally and narrows down and is less intense and well defined (Fig. 4.1a). Sections reveal that *EphA4* is expressed in mesenchymal cells but not in ectoderm (Fig. 4.2a).

In the leg at stage 25, there is also a patch of expression proximally (Fig. 4.1a) which, on sectioning, was found to be related to developing cartilage (Fig.. 4.2b). This may be the proximal tendon primordium which appears at this time in the thigh-shank region (the future knee) described by Kardon (1998) (Fig. 4.1a arrow). At stage 26, expression in proximal tendon primordium is weaker but still present and a new area of expression is now seen between it and expression in the digital plate (Fig. 4.1b asterisk). This coincides with the intermediate tendon primordium described by Kardon (1998) which appears in the shank-foot junction (future intertarsal joint). However now, there is reduced expression in the distal anterior parts of the digital plate, but a patch of strong expression remains at the posterior distal extremity (Fig. 4.1b, 14c).

At stage 27, the posterior distal expression remains but a new broad band of expression is present at the base of the digital plate extending straight across the entire anterior-posterior axis (Fig. 4.1c). This seems to be the distal tendon primordium which appears at the future metatarso-phalangeal joint (Kardon 1998). Expression is very much reduced at the apex of the bud (Fig. 4.1c). At stage 28, the posterior distal patch at the tip of the bud is still present but now expression in the distal tendon primordium is in the shape of an arc from which a band of expression extends into the digital plate (Fig. 4.1d). This band of expression is seen both dorsal and ventral to the developing digital cartilage. On the dorsal side, the arc of expression is not well defined (not shown). Sections reveal that, distally, expression is in mesenchyme subjacent to the ectoderm (Fig. 4.2d) in the region of developing tendons. Proximally, expression is in a continuous layer deep to the ventral ectoderm (Fig. 4.2 e) whereas dorsal expression coincides with tendons that have moved away from ectoderm. It can also be made out that *EphA4* expression in dorsal tendon is a thinner layer when compared to that on the

ventral side. By stage 29, three bands are seen dorsally and ventrally in the digital plate in relation to the developing digits (Fig. 4.1e, f). Expression is seen in sub-ectodermal mesenchyme (Fig. 4.2f, g). On the ventral surface, the bands diverge from an arc of expression at the base of the digital plate (Fig. 4.1f). However, dorsally, there is no clear arc but faint expression is present in base of digit plate (Fig. 4.1e) and from this bands of expression extend into digital plate in relation to the developing digits. Proximal and distal sections show that the bands are condensations of expressing cells in sub-ectodermal mesenchyme. However it must be noted that both at distal and proximal levels, *EphA4* expressing cells form a thin layer on the dorsal side and a thicker layer on the ventral side (Fig. 4.2f, g).

4.3.1.2 Description of tendon development in relation to *EphA4*

From stage 27 onwards, expression of *EphA4* is related to development of tendons, based on description of tendon morphogenesis by others (Hurle et al 1989, 1990, Ros et al 1995). Up to and including stage 29 inclusive, I was not able to make out the mesenchymal lamina in association with *EphA4* expression. This may be because the expression of *EphA4* under the ectoderm is so very strong. As development progresses, from stage 27 onwards, the broad domain of *EphA4* expression under the ectoderm (Fig. 4.2d) gradually condenses. By stage 31, expression condenses ventrally into a tight knot of cells having a round/oval cross section, whereas the dorsal condensation is flattened or crescentic (Fig. 4.2j). From stage 29 onwards, narrow strips of expression related to the digital elements extend proximo-distally (Fig. 4.3a, b) and become broadened out distally (Fig. 4.3a, b white arowheads) to end just short of the digit tip. These strips are developing tendons. However, between the broadened ends of the strips of expression and the tips of the digits, there is diffuse weak *EphA4* expression (Fig. 4.3a, b arrows). At stage 31, the narrow strips become broad nearer the tip of the digit (Fig. 4.3b). At about the middle of each digit, there is a new increased area of expression on either side of the strips of expression in the tendon itself (Fig. 4.3a, b black arrowheads). This is not seen at stage 30.

Sections at stage 31 reveal that the strip of *EphA4* expression distally is right beneath the ectoderm, dorsal and ventral to the cartilage (Fig. 4.2h), but, proximally, the tendon is seen away from the ectoderm (Fig. 4.2i and j). The difference in shape of dorsal and ventral tendons can be made out in cross sections (Fig. 4.2i). At proximal and distal sites, the layer of *EphA4* expression in sub-ectoderm and deeper mesoderm on dorsal side is distinctly thinner when compared to the ventral side (Fig. 4.2h, i, and j). Proximally as the ventral tendons round up, a thin layer of *EphA4* expressing cells is

found at the superficial aspect of the rounded tendon (Fig. 4.2j and k). This represents an early stage in formation of the superficial tendons.

4.3.1.3 Relation of *EphA4* to the mesenchymal lamina

From the above description of sections of the leg at stage 31, expression of *EphA4* during progressive development of the tendon is documented. *EphA4* expressing cells are displaced from a superficial to a deep level and, as tendons condense, they form differently shaped dorsal and ventral tendons. In a similar way by studying a series of sections from distal to proximal, the association of *EphA4* expressing cells with the mesenchymal lamina can be traced. However, the mesenchymal lamina is not visible on the ventral side because *EphA4* expression is very strong and does not become weaker until later stages. Distally, *EphA4* is expressed immediately under the ectoderm (Fig. 4.4a). At the edges of the *EphA4* expressing domain, underneath the ectoderm, the basement membrane is visible (Fig. 4.4a arrowheads) whereas centrally, the basement membrane is not visible between the *EphA4* domain and ectoderm (Fig. 4.4a arrows). In a more proximal section, the mesenchymal lamina is very clear within the domain of *EphA4* expression in the tendon which has just begun to peel away from the basement membrane (Fig. 4.4b white arrowheads) and it is continuous (Fig. 4.4b arrow) with the basement membrane (Fig. 4.4b arrowhead). Cells that express *EphA4* are also seen between the mesenchymal lamina and the ectoderm. Still more proximally (Fig. 4.4c), cells expressing *EphA4* have been displaced deeper into the mesenchyme along with the mesenchymal lamina (Fig. 4.4c arrows, see also inset). The mesenchymal lamina now appears to have lost (Fig. 4.4c arrowhead) its connection with the basement membrane. Furthermore, the basement membrane between tendon and ectoderm has now been completely re-established (compare Fig. 4.4c with Fig. 4.4a).

4.3.1.4 Expression of *EphA4* in later stages (32-36)

At stage 32, tendon expression has further condensed (Fig. 4.3c) and expression that was present on either side of the mid-region of the tendon in stage 31 is now seen at additional distal sites along the digit (Fig. 4.3c black arrowheads). These sites of *EphA4* expression are at the level of interphalangeal joints (Fig. 4.5a, b) and appear to coincide with ventral tissue extensions that contribute to the distal parts of long tendons of the foot muscles (Kardon 1998**).** At these sites of *EphA4* expression, the tendon is slightly enlarged (Fig. 4.3c white arrowheads). The posterior distal patch of *EphA4* expression in the footplate has now disappeared but there is still faint expression at each digit tip. On the dorsal side, expression in the tendons in considerably reduced and there are no enlarged areas (not shown).

Figure 4.1 Expression of EphA4 chick legs at stage 25 to 29

Lines indicate planes through which sections are shown in Fig. 4.2

a) Stage 25 dorsal view. Expression is mostly at distal tip of bud (arrowhead); proximally there is a patch of expression (arrow) in the proximal tendon primordium.

b) Stage 26 dorsal view. Expression at the distal limb bud with high expression at posterior (arrowhead); weak expression persists at the proximal tendon primordium (arrow); a new area of weak expression in intermediate tendon primordium (asterisk).

c) Stage 27 dorsal view. A band of expression extends from anterior to posterior at the base of digit plate, the distal tendon primordium (white arrowhead); posteriorly, there is strong expression nearer tip (black arrowhead) which seems to have some continuity with the distal tendon primordium; intermediate tendon primordium (asterisk).

d) Stage 28 ventral view. 3 bands of expression extend into digit plate (arrows); expression in distal tendon primordium is in the shape of an arc (white arrowhead); posterior distal expression (black arrowhead); intermediate tendon primordium (asterisk).

e) Stage 29 dorsal view. 3 individual bands of expression on digit plate (arrows) separated proximally by an area of low expression (white arrowhead), the distal tendon primordium; posterior distal expression (black arrowhead). Note the discontinuity between middle band and the expression in distal tendon primordium (white arrowhead).

f) Stage 29 ventral view. 3 bands of expression extending further into digit plate (arrows) and proximally continuous with the arc of expression, the distal tendon primordium (white arrowhead); posterior distal expression (black arrowhead).

Fig. 4.1

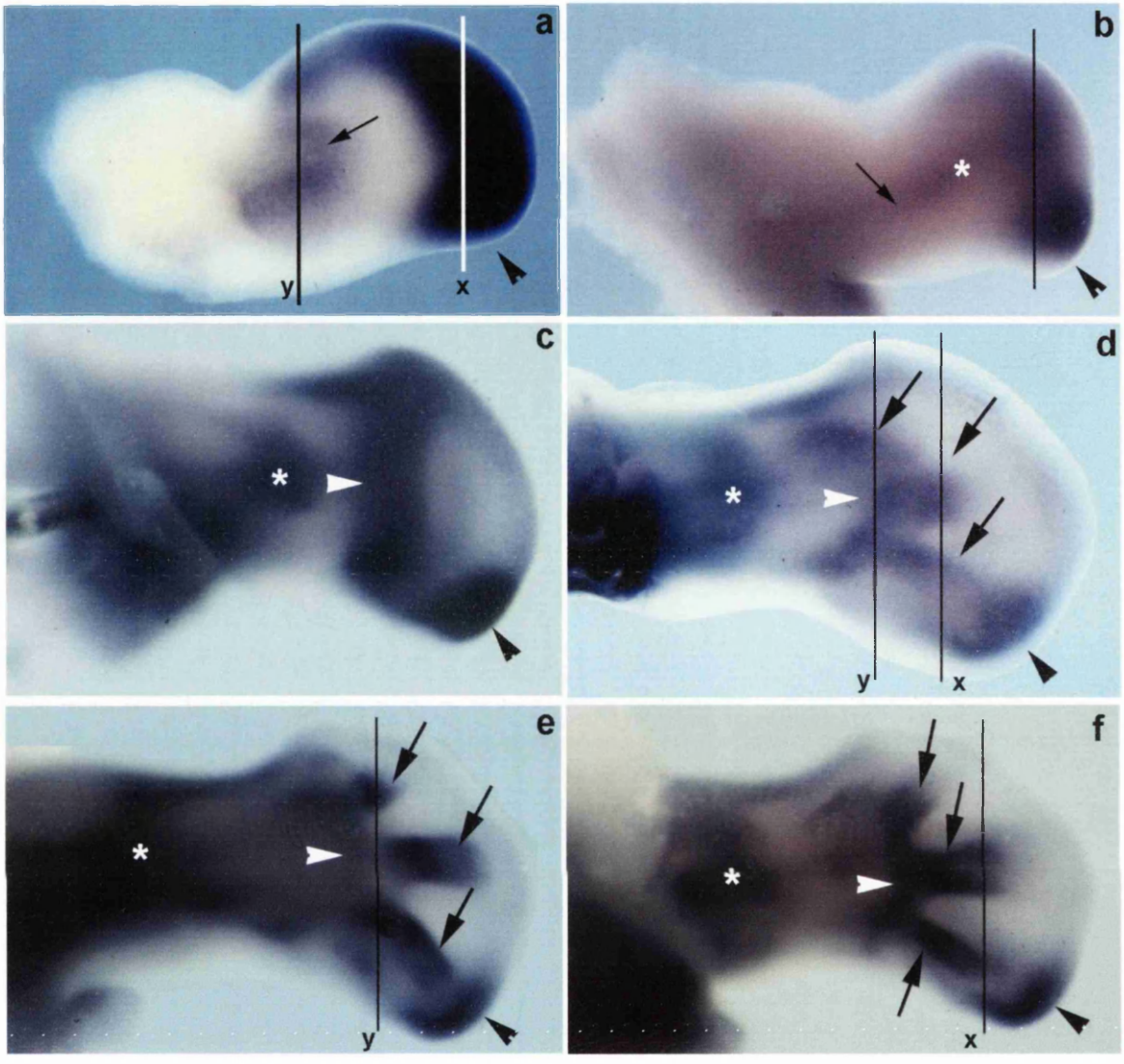


Figure 4.2 Transverse sections showing *EphA4* in developing tendons

a) –g) are sections of whole mounts shown in Fig. 4.1. h) –k) are sections of whole mount shown in Fig. 4.3a and b. Anterior to left, dorsal to top.

a) Section at distal tip of stage 25 through plane x of Fig. 4.1a, showing *EphA4* expression is in the mesoderm and stronger posteriorly.

b) Section through plane y of Fig. 4.1a. *EphA4* expression in proximal tendon primordium of limb bud (white arrowheads), dorsal and ventral areas of expression close to ectoderm (arrows); anterior expression (black arrowhead).

c) Section of stage 26 leg through plane indicated in Fig. 4.1b. Posterior expression (arrow) is a thick layer; anterior expression (arrowheads) is fainter and separated into dorsal and ventral domains.

d) Section of stag 28 leg through plane x of Fig. 4.1d. *EphA4* expression in sub-ectodermal mesenchyme dorsal (arrowheads) and ventral (arrows) to the developing cartilage.

e) Section of stage 28 leg through plane y of Fig. 4.1d. Dorsal expression (arrowheads) has moved away from ectoderm, ventral expression is a continuous layer (arrow).

f) Section of stage 29 leg through plane x of Fig. 4.1e and f. *EphA4* expression in dorsal (arrowheads) and ventral (arrow) sub ectodermal mesenchyme.

g) Section of stage 29 leg through plane y of Fig. 4.1e and f. Dorsal (arrows) and ventral (arrowheads) expression in sub ectodermal mesenchyme.

h) Section through stage 31 leg. Dorsal (arrowhead) and ventral (arrow) *EphA4* expression just under the ectoderm.

i) Section through stage 31 leg more proximal to that in h. Both dorsal and ventral tendon expression have moved a little deeper. Dorsal (arrowhead) is flat and ventral (arrow) has become more rounded.

j) Section through stage 31 proximal to that in i. Dorsal (arrowheads) and ventral (arrows) tendons have moved deeper into mesoderm.

k) Magnification of the ventral tendons of digit 3 and 4 in (j). Ventral tendon (t) of digit 3 has a thin layer of expressing cells (double arrowhead) superficial to it that appears to have separated from (t). This may be compared with the tendon (t) in digit 4 where there is a broad area of expression (arrowhead) which appears to be in continuity with the tendon (t).

Fig. 4.2

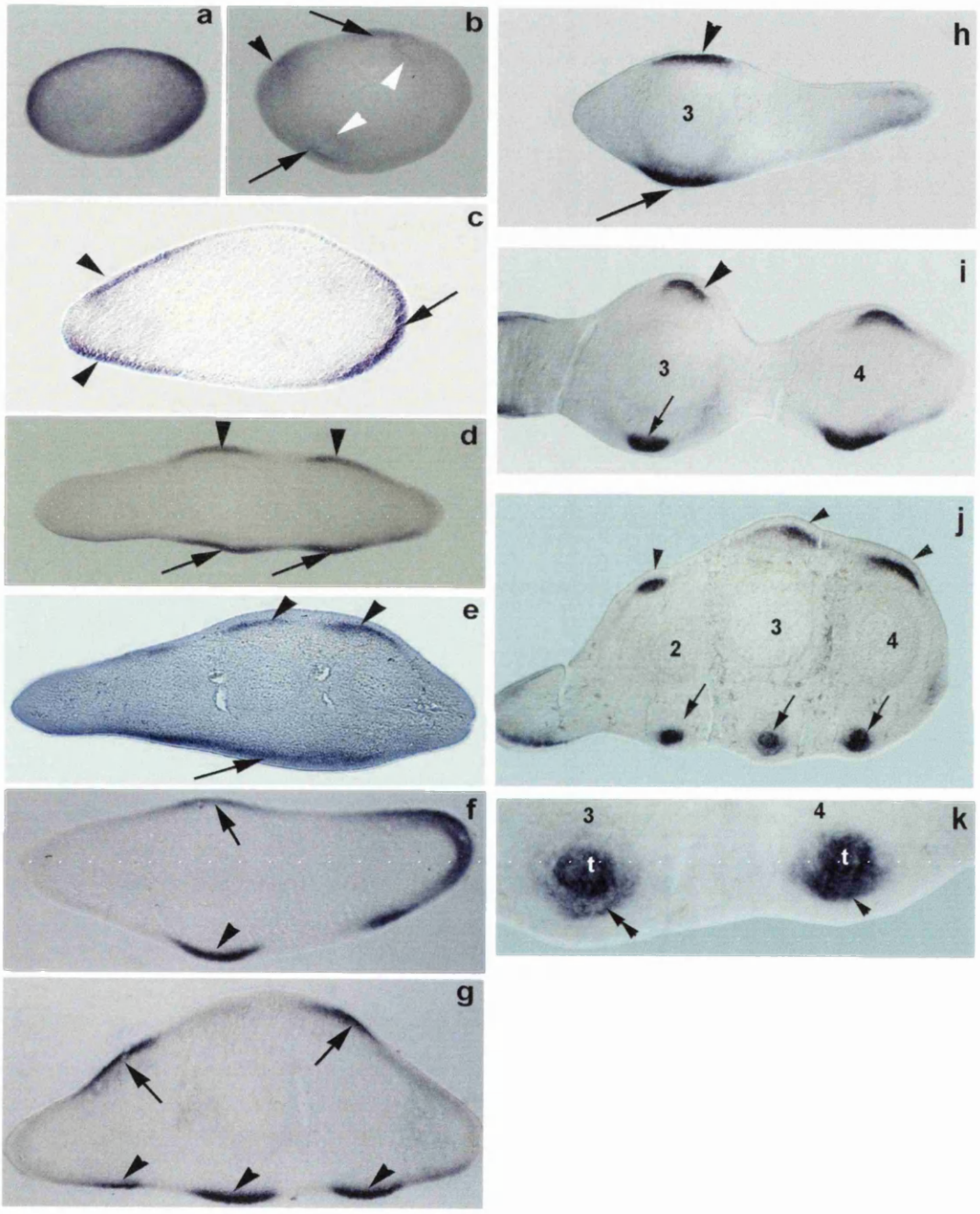


Figure 4.3 Expression of *EphA4* in stages 31 to 34

a) Dorsal and **(b)** ventral view of stage 31 leg. Bands of expression extending along digits become broad distally (white arrowheads). Tips of the digits show weak expression (arrows). Expression seen flanking the tendon (black arrowheads), but separate from it represents the first dorsal and ventral tissue extensions that will contribute to formation of foot tendons.

c) Ventral view of stage 32 leg. Bands of *EphA4* expression extending over the digits show intermittent thickened areas (white arrowheads); Patches of expression in tissue extensions at level of joints (black arrowheads) have increased in number.

d) Ventral view stage 33/34 with areas of digit 2 and 4 in rectangles that are enlarged in (e) and (f) respectively.

e) Digit 2 of (d). *EphA4* expression in tissue extensions (arrows) that are now in contact with the expression in tendon; tendon splitting at level of proximal tissue extension (white arrowhead); distal end of tendon splitting tendon (black arrowheads). Asterisk is expression at the tip of the digit not in continuity with the tendon.

f) Digit 4 of (d). Big arrowhead indicates the distal tip of tendon; small arrowheads mark the splitting tendon at level of most proximal patch (arrows) of expression flanking the tendon. The patches of expression in tissue extensions (arrows) are now further away from tendons; asterisk is expression at the tip of the digit.

g) and (h) end-on view of the tip of digits 2 and 4 respectively shown in (d). Black arrowhead is the distal end of ventral tendon, white asterisk is the expression at the ventral tip; white arrowhead is the expression at dorsal tip enclosing a non-expressing area (black asterisk).

i) End-on view of the tip of digit 4 at stage 36. The dorsal and ventral expression at the tip of the digit seen in (h) has now fused and formed a horseshoe shaped domain.

Fig. 4.3

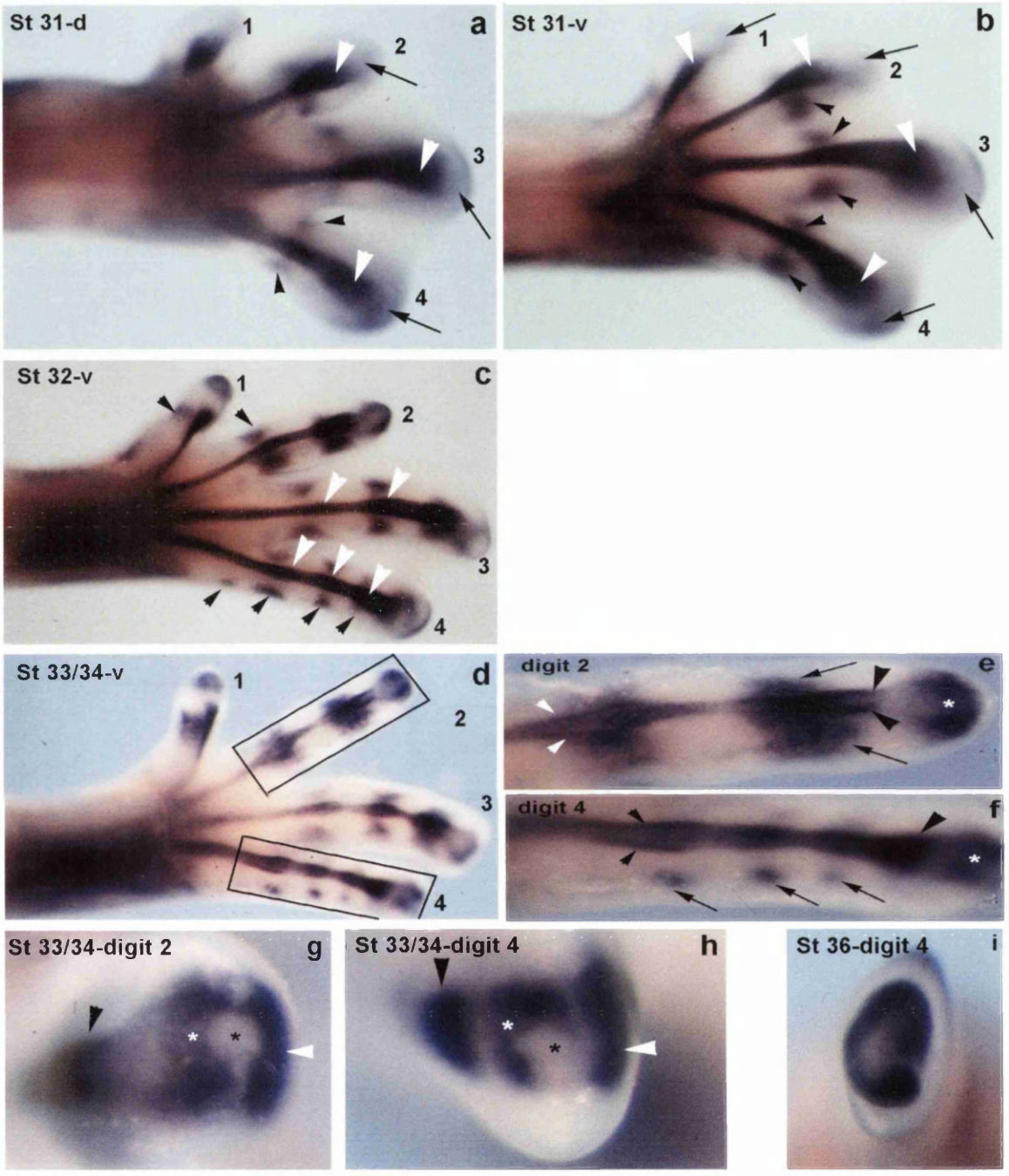

St 31-d
a
1
2
3
4
St 31-v
b
1
2
3
4
St 32-v
c
1
2
3
4
St 33/34-v
d
1
2
3
4
digit 2
e
digit 4
f
St 33/34-digit 2
g
St 33/34-digit 4
h
St 36-digit 4
i

Figure 4.4 Relation of *EphA4* expression to mesenchymal lamina

Sections through stage 31 leg showing the dorsal tendon blastema in 3 sections from distal to proximal.

(a) Basement membrane (arrowheads); expression in dorsal tendon blastema (arrows) where basement membrane cannot be made out.

(b) Mesenchymal lamina (white arrows) within the area of *EphA4* expression in dorsal tendon blastema and continuous (black arrow) with the basement membrane (arrowheads).

(c) Mesenchymal lamina (arrows) seems to have a beaded appearance. The lamina at the sides (black arrowhead) of the tendon has disappeared. Tendon expression and lamina have moved deep into mesoderm. Inset is enlarged area of mesenchymal lamina (white arrowheads).

Fig. 4.4

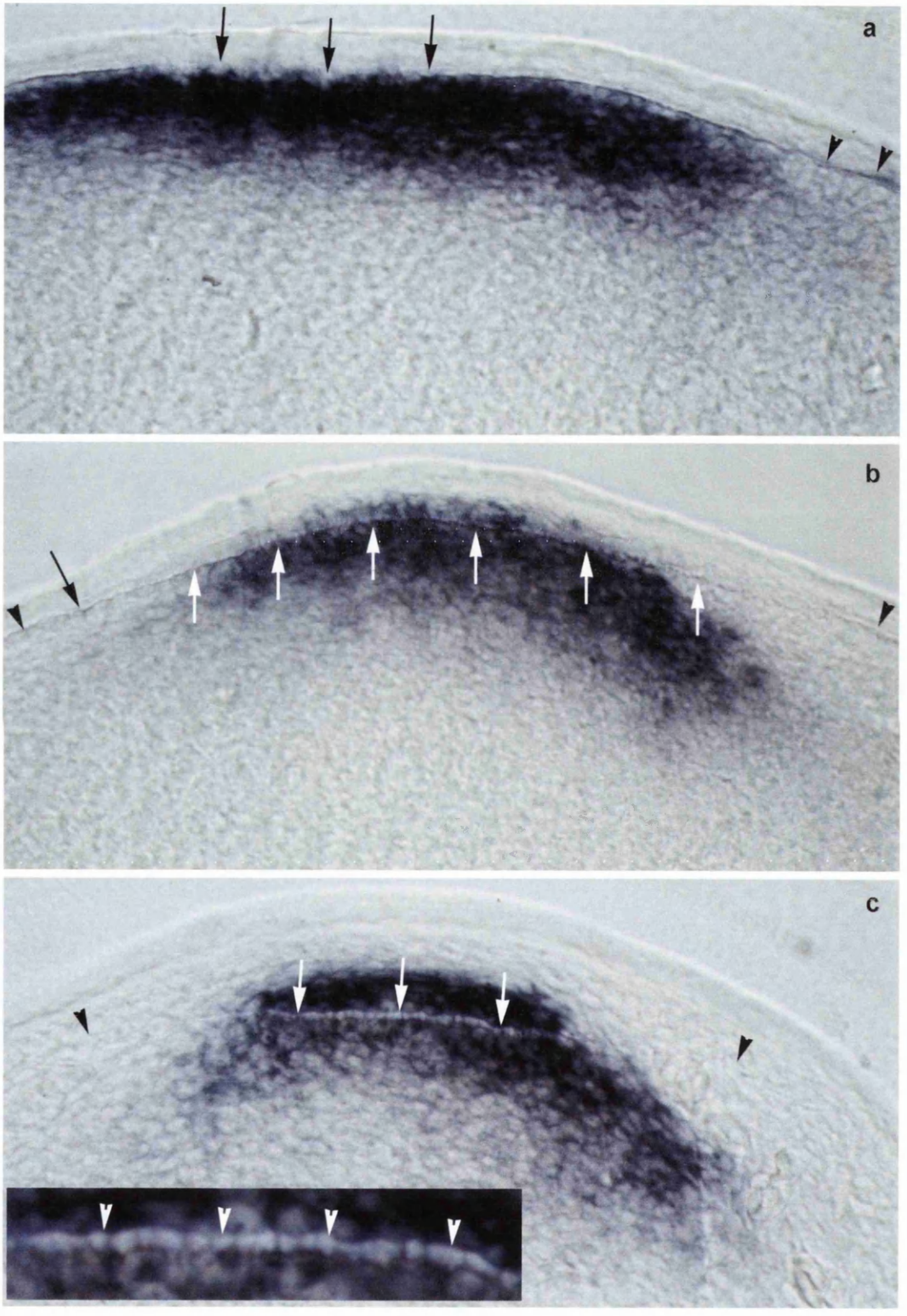


Figure 4.5 Expression of *EphA4* in chick legs at late stages

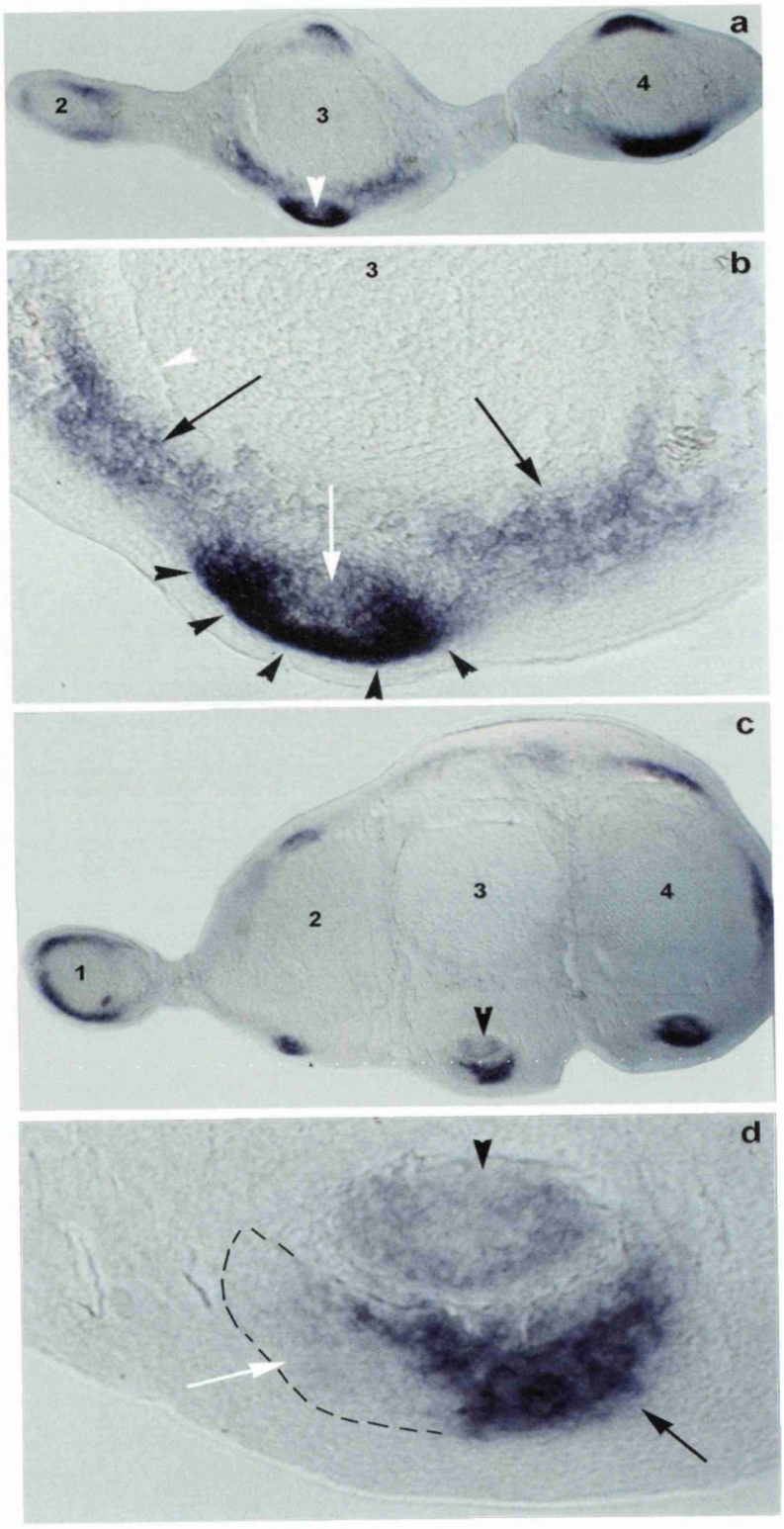
a) Distal section through stage 32 leg. *EphA4* expression (arrowhead) in ventral tendon of digit is reduced in the centre.

b) Magnification of ventral tendon in (a). Reduced *EphA4* expression in the central region (white arrow) and strong expression in the superficial area (black arrowheads). Black arrows indicate expression in tissue extension away from cartilage (white arrowhead).

c) Section through proximal region of stage 33/34. Two separate tendons are seen in digit 3, a deeper tendon in which expression is very faint and a superficial one in which expression is strong (arrow).

d) Magnification of ventral tendon of digit 3 in (c). Deep tendon, (arrowhead); superficial tendon (arrow), on one side expression is strong whereas on the other side (outlined with dotted line) expression is weak.

Fig. 4.3



By stage 34 (Fig. 4.3d), there is reduced expression of *EphA4* in proximal regions of tendons in digits 1, 2 and 3 but a striking feature is the beaded appearance seen clearly in the ventral tendon of digit 4 (Fig. 4.3d and f). Distally, tendons do not reach the tips of the digits and split some distance away (Fig. 4.3e, f-black arrowhead). *EphA4* expression that came on at stage 31 in the tissue extensions appears to have condensed in posterior digits and is more clearly associated with interphalangeal joint region. Expression of *EphA4* in tissue extensions of digit 2 (Fig. 4.3d, e), remains as two thick bands across the anterior-posterior axis (Fig. 4.3c) of the digit and on both dorsal and ventral sides. A closer look at expression in digit 2 shows that just proximal to expression in tissue extensions (Fig. 4.3e arrows), the tendon splits and splays out thus making the tendon look enlarged (Fig. 4.3e white arrowheads). The beaded appearance of the tendons that begins to appear at around stage 32 is due to an enlargement of the tendon area at intermittent intervals. On looking closer at expression of *EphA4* in ventral tendon of digit 2 and 4, it can be seen that, at least proximally, expression is split and 3 indistinct strips (Fig. 4.3e, f-arrowheads) can be made out. In digit 3, expression in tissue extensions has moved away from the tendon region (Fig. 4.3d) and in digit 4 it has receded still further away from tendon (Fig. 4.3d, f-arrows). From this dynamic pattern of *EphA4* expression in the tissue extensions, it appears that these make contact with the main tendon and appear as bands that extend across the anterior-posterior axis initially (Fig. 4.3e). Once this contact is established, expression persists only at the site of initial formation of the tissue extensions, which are the actual sites of tendon insertion. Expression in the intervening tissue is reduced (Fig. 4.3f). Thus there is a gap between expression in the tendon and expression in the sites of tendon insertions. Digit 2 has two sites of expression, digit 3, three sites and digit 4 four sites of expression in tissue extensions (Fig. 4.3d). As these patches are related to interphalangeal joints, they also give an indication of the number of phalanges that are formed. For example, in digit 3 there are 3 patches and 4 phalanges are formed in this toe. At the extreme tip of digit 2 and 4, there is a patch of expression (Fig. 4.3e, f white asterisk) which is not in continuity with the tendon. A view of the digit tip shows that this distal patch of expression along with a similar patch of expression dorsally (Fig. 4.3g, h white arrowhead) encloses a non-expressing area at the tip (Fig. 4.3g, h black asterisk). By stage 36, *EphA4* expression is almost completely lost except at the tip of the digits (Fig. 4.3i) where an annular domain of expression is present. Faint expression in the interphalangeal joint regions also remains (not shown).

4.3.2 Comparison of expression of *EphA4* with Tenascin and Collagen I in tendons

Sections immunolabelled with antibodies that recognize tenascin and procollagen I were compared with matching sections of whole mount limbs in which *EphA4* mRNA transcripts were labelled.

At stage 25, *EphA4* is expressed in the distal region of the limb bud in the mesoderm (Fig. 4.1a). In addition, *EphA4* is expressed proximally in the future thigh region which could be the site of tendinous attachment of thigh muscle to cartilage (Fig. 4.1a, 14b). Similarly tenascin labelling is present in the proximal limb dorsally and ventrally (Fig. 4.6a), but there is no labelling distally (not shown). Tenascin is first expressed in the digital plate at stage 28, when it is present as a continuous thin layer under the dorsal and ventral ectoderm (Fig. 4.6b). Proximally, tenascin is found in a thicker layer under the ectoderm (Fig. 4.6c). In comparison, at the same stage, *EphA4* expression distally is seen under the dorsal and ventral ectoderm as 2 distinct patches in relation to digits 3 and 4 (Fig. 4.2d) and not as a continuous layer. By stage 29, tenascin labels individual tendons dorsally and ventrally which have now been displaced a little away from ectoderm (Fig. 4.6d). This now mirrors expression of *EphA4* (Fig. 4.2g).

Sections through a stage 31 leg show the progress of tendon development in terms of tenascin expression when studied from distal to proximal (Fig. 4.7) and this is similar to expression of EphA4 (Fig. 4.4). Initially, tenascin is expressed in the sub-ectodermal mesenchyme, dorsal (Fig. 4.7a) and ventral (Fig. 4.7a') to the digital cartilage elements in association with the mesenchymal lamina (Fig. 4.7a, a') which is continuous with the basement membrane. A little proximally, the labelled region and the mesenchymal lamina is a little way away from the ectoderm (Fig. 4.7b, b') and the lamina is losing its continuity with the basement membrane. Still more proximally, the tendon is deeper in the mesenchyme (Fig. 4.7c, c') and the ventral tendon is rounded. At later stages (up to 34) tenascin can be seen in other flexor tendons (Fig. 4.6e) and has some similarity to *EphA4* expression (Fig. 4.5c, and d). However at stage 36, tendons are still labelled with tenascin antibody (Fig. 4.6f), whereas *EphA4* expression by now has decreased and persists only at the tip of the digit (Fig. 4.3i) and persists weakly in the area of interphalangeal joint.

Procollagen I is not detected in tendon cells up to stage 28 (Fig. 4.8a), and the antibody only appears to label the ectoderm. At stage 29, procollagen appears weakly in dorsal and ventral tendons (Fig. 4.8b). At later stages, labelling is strong in all leg tendons (Fig. 4.8c, d). At stage 36, it is very similar to that seen with tenascin (Fig. 4.6f)

Figure 4.6 Tenascin in early and late tendon development in the leg

a) Stage 25. Tenascin staining is seen only in proximal part of the leg in the proximal tendon primordium.
b) Stage 28. Tenascin is present as a sheet under the dorsal (arrowhead) and ventral (arrow) ectoderm.
c) More proximally, at stage 28, there is a thick layer of tenascin under the ventral ectoderm only.
d) Stage 29. Tenascin is present in the dorsal (arrowhead) and ventral (arrow) tendon blastema.
e) Stage 34. Difference in contour of dorsal (arrowhead) and ventral (arrow) tendons can be made out. In digit 3 there are 3 distinct tendons, flexor perforans digiti is attached to cartilage and is fused across the ventral surface of cartilage , the middle, flexor digitorum longus and the superficial, flexor perforans et perforatus digiti.
f) Digit 3, has flexor digitorum longus (fdl) the rounded deep tendon; superficial to it is the flexor perforans et perforatus digiti (fp et pd); two slips of flexor perforans digiti are inserted at the cartilage region and are also fused across the cartilage lying deep to flexor digitorum longus.

Fig. 4.6

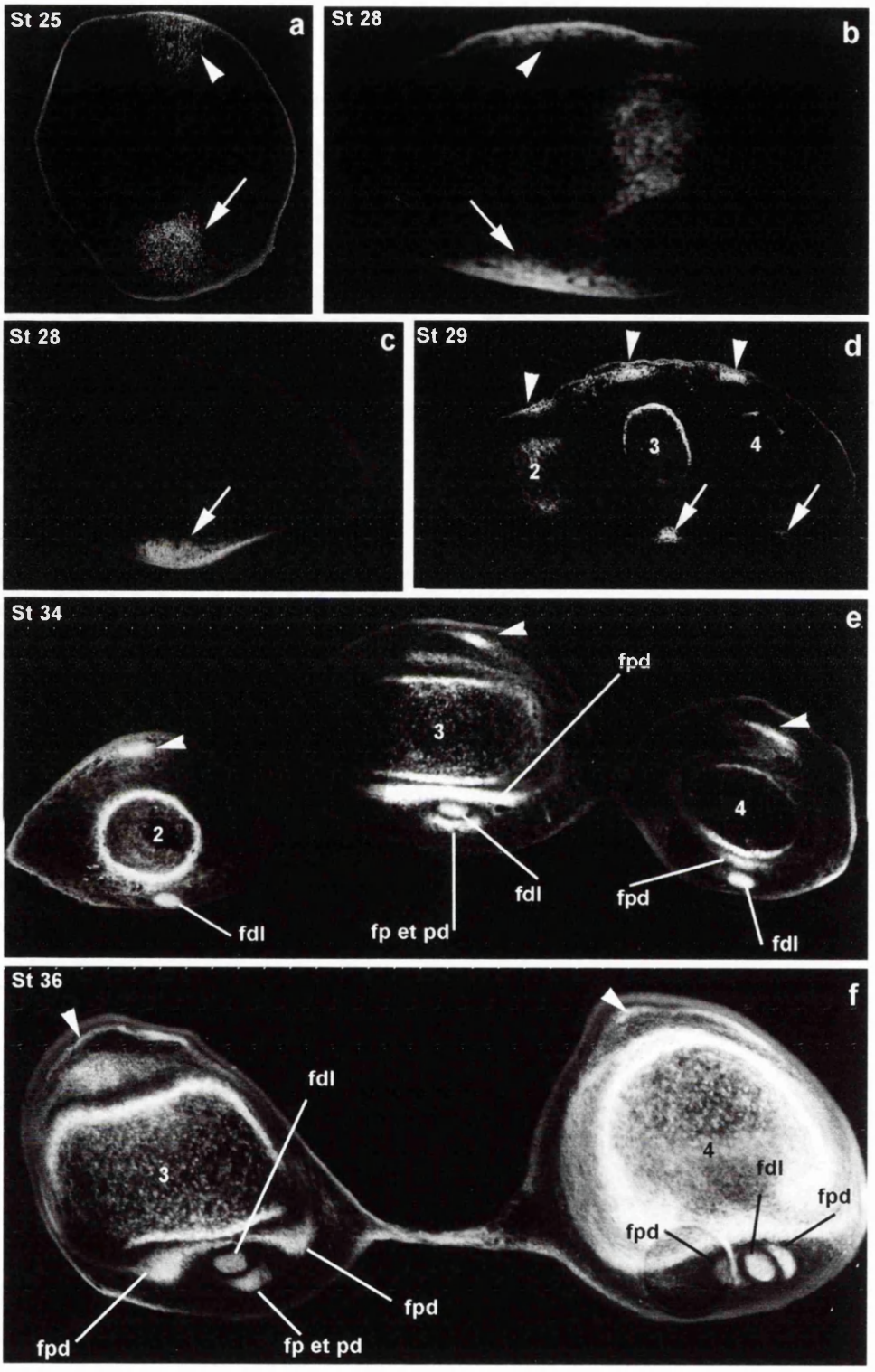

St 25
a
St 28
b
St 28
c
St 29
d
2
3
4
St 34
e
fpd
3
2
4
fdl
fp et pd
fdl
fpd
fdl
St 36
f
fdl
3
4
fdl
fpd
fpd
fpd
fpd
fp et pd

Figure 4.7 Tenascin is present in the tendon blastema and mesenchymal lamina of stage 31 chick legs

Sections are from distal to proximal. Dorsal mesenchymal lamina (white arrowheads); ventral mesenchymal lamina (white arrows).

a) Tenascin labelling in mesenchymal lamina (arrowheads) is present within the dorsal tendon blastema and is continuous with the basement membrane (white arrows). Note that the basement membrane is not distinct dorsal to the mesenchymal lamina.

a') Ventral part of the section shown in a). Tenascin labelling in mesenchymal lamina (arrows) in the ventral tendon blastema.

b) Section proximal to (a). Tenascin labelling in mesenchymal (arrowheads) and dorsal tendon blastema. The lamina and tendon have moved deeper into the mesenchyme and the basement membrane dorsal to the lamina has re-organised and is visible.

b') Ventral part of the section shown in b). Tenascin labelling in ventral tendon shows the rounded contour in cross section with part of the mesenchymal lamina within it (arrows).

c) Tenascin labelling in lamina (arrowheads) is almost completely discontinuous with the basement membrane but still within the tendon.

c') Ventral part of the section shown in c). Tenascin labelling seen in the rounded ventral tendon which is now deeper in the mesenchyme and some remnant of mesenchymal lamina (arrow) persists.

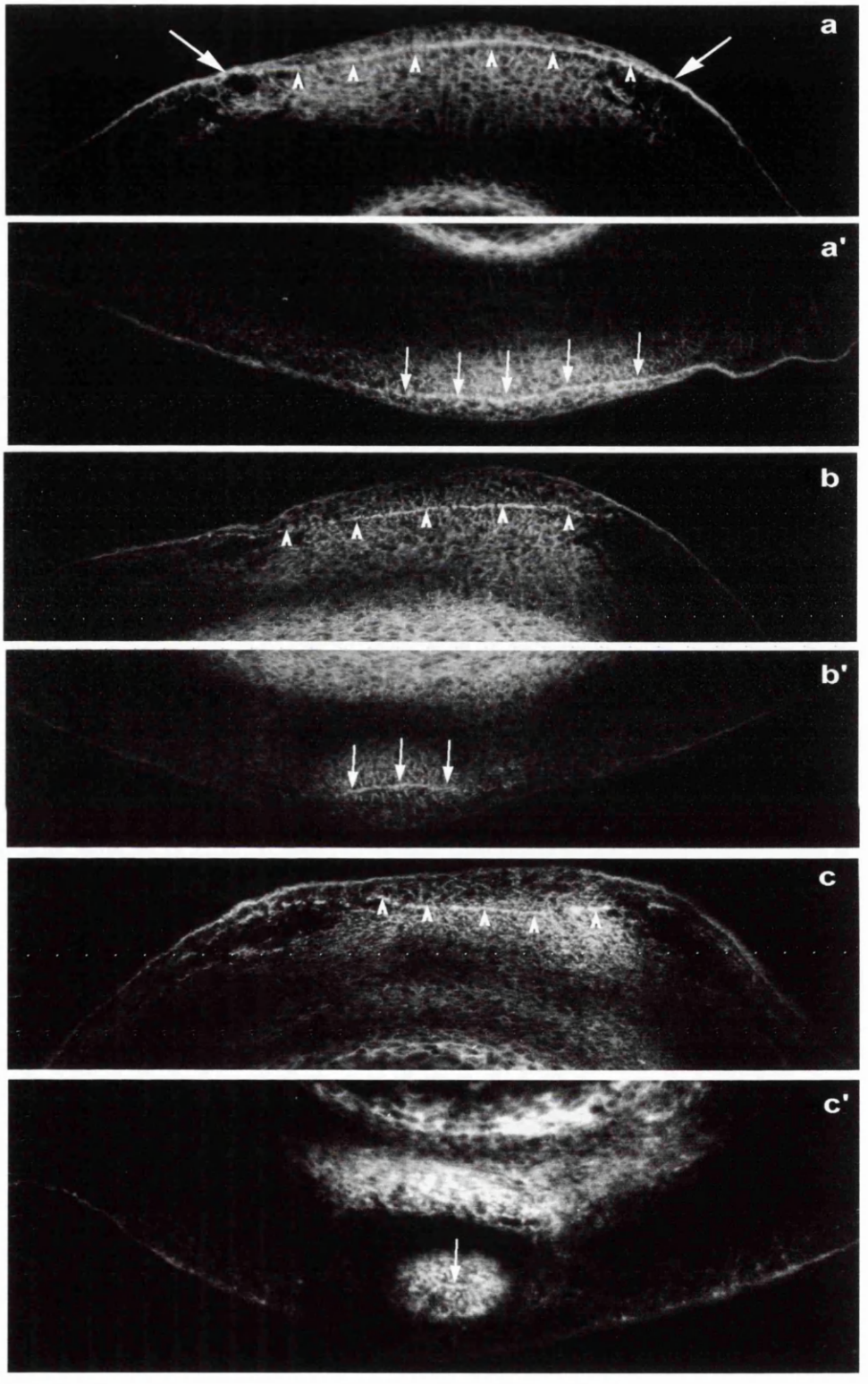

a
a'
b
b'
c
c'

Figure 4.8 Procollagen I is present at late stages in chick leg tendons

Dorsal tendons (white arrowheads); ventral tendons (white arrows).

a) Stage 28. Procollagen I appears to be present in the ectoderm (arrow).
b) Stage 29. Procollagen I is present only in proximal region of the leg in dorsal (arrowhead) and ventral (arrow) tendons.
c) Stage 32. Flattened dorsal (arrowhead) and the rounded ventral (arrow) tendons can be made out.
d) Stage 34. Some appearance of procollagen I detectable in 2 ventral tendons (arrow) in digit 3; dorsal tendons (arrowheads).
e) 3 different tendons associated with digits 3 can be made out labelled with procollagen I similar to those seen with tenascin (Fig. 4.6f), fdl, flexor digitorum longus; fpd flexor perforans digiti; fp et pd, flexor perforans et perforatus digiti.

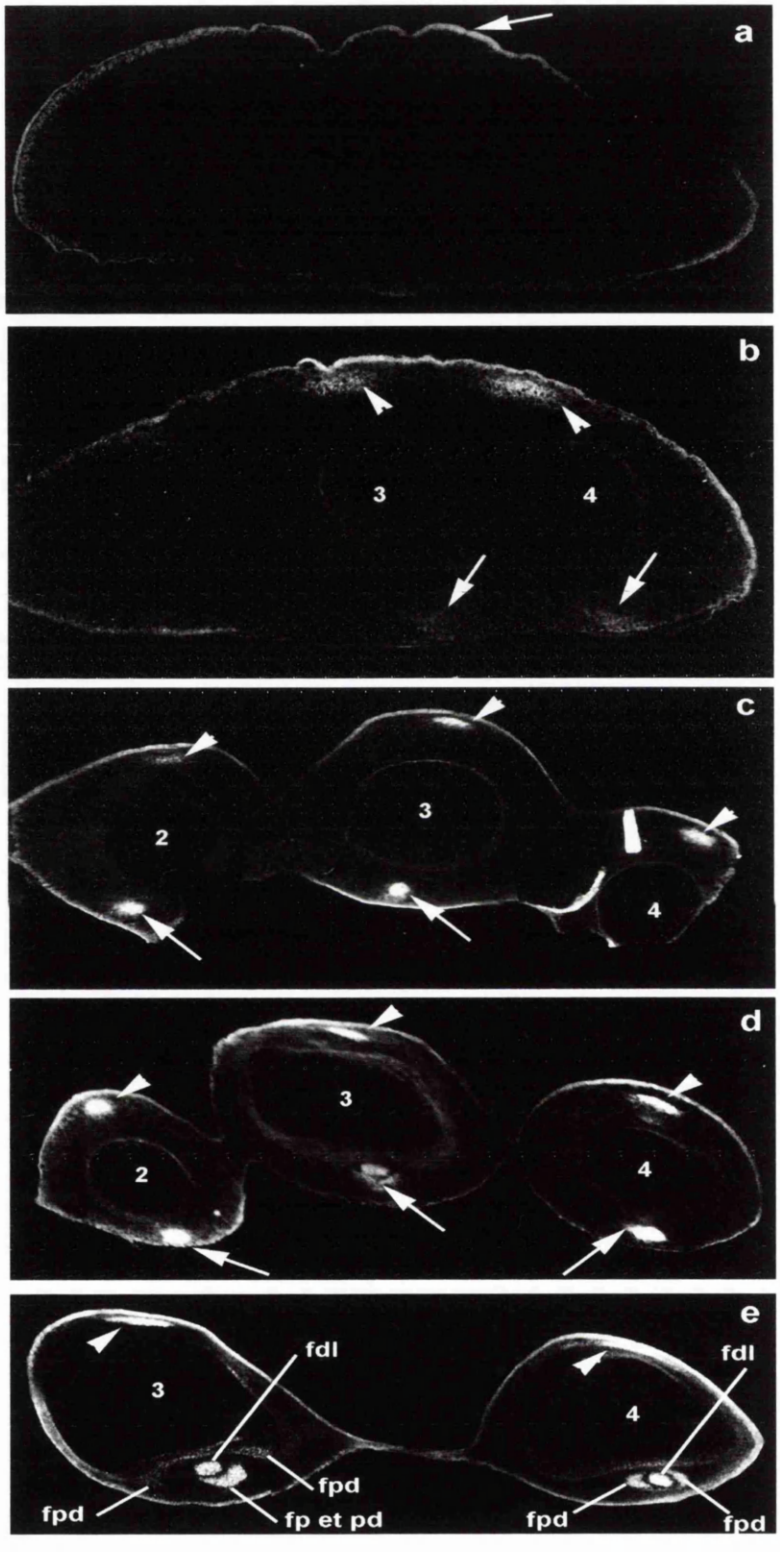

Procollagen I
a
b
3
4
c
2
3
4
d
2
3
4
e
3
fdl
fpd
fp et pd
fpd
4
fdl
fpd
fpd

Table 4.1 Comparison of expression of EphA4 with tenascin and procollagenI

Stage	Site	EphA4	Tenascin	Procollagen I
25	**distal** mes	+	-	-
	prox mes	+	+	-
28	**distal** sub-ect mes	individual dorsal tend individual ventral tend	continuous layer continuous layer	-
	proximal sub-ect mes	individual dorsal tend continuous layer	continuous layer continuous layer	-
29-30		individual tendons	individual tendons	tendons
31-32		tendons, tissue extensions	tendons mesl lamina	tendons
33-36		tendon tissue extensions tendon insertion sites	tendons tissue extensions	tendons tissue extensions

mes, mesenchyme

mesl, mesenchymal lamina

sub-ect, sub-ectoderm

and is present in all 3 tendons in digit 3 (Fig. 4.8e). Thus procollagen I appears rather late in tendons and is not expressed in early stages like tensacin and *EphA4* (Table 4.1).

4.3.3 Expression of *Six 1* in chick limb buds

At stage 25 (Fig. 4.9a), *Six 1* expression along the posterior half of the wing bud distally is similar to that seen in the mouse forelimb at 11.5dpc. Proximally, *Six 1* expression extends a little anteriorly, though the anterior edge and the anterior distal end of the bud do not express the gene (Fig. 4.9a). Expression in the distal posterior mesenchyme extends all across the dorso-ventral axis with highest concentration of transcripts dorsally (Fig. 4.10a). Proximally, posterior mesenchymal expression is reduced but expression is also present anteriorly in the dorsal and ventral mesenchyme (Fig. 4.10b). Still more proximally, the anterior edges of the dorsal and ventral expression domains have moved away from the ectoderm (Fig. 4.10c). By stage 28, posterior expression is strong but proximally there is reduced expression when compared to that seen in stage 25. Distal anterior and central parts of the digital plate have no expression (Fig. 4.9b). Posterior expression in the mesenchyme still extends across the dorso-ventral axis with dorsal dominance but no transcripts are seen in the developing cartilage (Fig. 4.10d).

In the wing at stage 31 (Fig. 4.9c), there is a layer of strong *Six 1* expression in dorsal mesenchyme subjacent to the dorsal ectoderm (Fig. 4.10e) with a small patch ventrally. Proximally, this layer (Fig. 4.10f) extends more anteriorly on the ventral side. Distally, a thin line of expression extends between the posterior of the cartilage of digit 4 to the expressing layer subjacent to the dorsal ectoderm (Fig. 4.10e). A small arc of *Six 1* expression, on a deeper plane, is related to tendon EML of digit 3. Postero-ventral to cartilage of digit 4, expression is related to the developing muscle and tendon of flexor digitorum quarti (Fig. 4.10e). An area of *Six 1* expression similar to that described in mouse by Oliver et al (1995) is seen closely flanking dorsal and anterior sides of the cartilage of digit 4 (Fig. 4.10e) and more proximally (Fig. 4.10f) the posterior side of the cartilage of digit 2. There are no tendons that develop at this level dorsal to digit 4 and hence this dorsal area of expression must be associated with other connective tissue. The above features indicate that, like the mouse, *Six 1* expression proceeds from posterior to anterior (Oliver et al 1995). In proximal regions, *Six 1* expression is prominent in muscle masses dorsal and ventral to digit 3, with higher levels of expression nearer the ectoderm (Fig. 4.10f).

In the chick leg at stage 31 (Fig. 4.9d), very weak *Six 1* expression is detected in ventral mesenchyme. Dorsal expression is present proximally at the base of the digital

Figure 4.9 Expression of Six 1 in chick limb buds

Anterior to top.

a) Stage 25-wing bud. Dorsal view, posterior expression extends to the tip (black arrowhead); anteriorly in proximal region there is some expression (black arrow), but does not extend to extreme anterior edge (white arrowhead); distally (white arrow) a major part of the anterior region does not express any Six 1 transcripts.
b) Stage 28-wing bud. Proximally there is reduced *Six 1* expression although strong expression persists at the posterior edge of the bud (arrowhead), no expression anteriorly (arrow).
c) Stage 31 wing. Strong expression on dorsal aspect (white arrowhead) some expression close to digit region (arrow).
d) Dorsal view of the leg at stage 31. Distally, no expression but proximally expression is related to metacarpal region (arrows). Posterior edge of limb has strong expression (arrowhead).
e) Stage 33 wing. *Six 1* expression is present in dorsal (white arrowhead), ventral (black arrowhead) and inter-phalangeal joint region (arrows).
f) Stage 35 leg. Weak expression in inter-phalangeal joint regions (arrowheads) and 2 streaks of expression on the proximal part of digits 2 and 3 (arrows) which also extend proximally.

Fig. 4.9

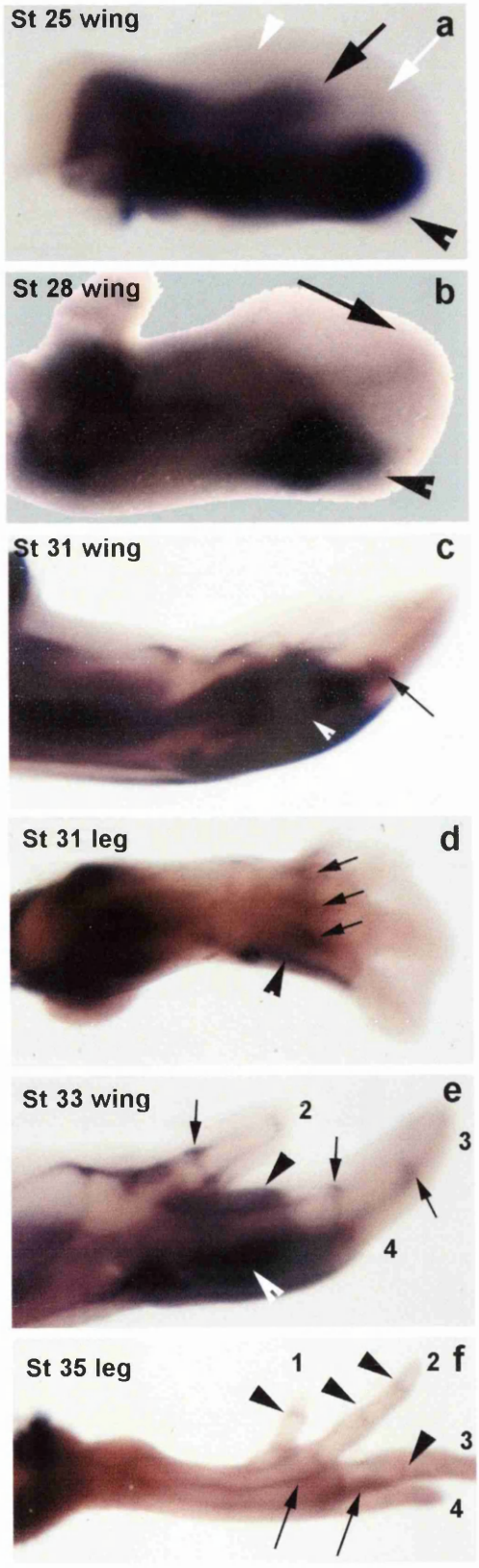

St 25 wing
a
St 28 wing
b
St 31 wing
c
St 31 leg
d
St 33 wing
e
2
3
4
St 35 leg
1
2 f
3
4

Figure 4.10 Sections showing localisation of *Six 1*

Transverse sections of in situs shown in Fig. 4.9.

Dorsal to top and anterior to left. a)-c) are sections of stage 25 wing.

a) *Six 1* expression is seen posteriorly with a higher distribution of transcripts dorsally (arrow).

b) Expression dorsally (white arrow) and ventrally (arrowhead) are separated from the posterior expression (black arrow); no expression centrally.

c) Anterior edge of expression is away from the ectoderm both on dorsal (white arrow) and ventral (arrowhead) sides, posterior expression (black arrow) is weak.

d) Stage 28. Distally expression is present through out the dorso-ventral axis. Dorsal expression (white arrow) is stronger than ventral (black arrow). Expression is excluded from cartilage.

e) Stage 31 wing. Distal section shows dorsal expression very close (arrow) to cartilage of digit 4; extensor medius longus tendon, (black arrowhead); muscle flexor digiti quarti (double arrowhead); a thick layer of strong expression just under dorsal ectoderm (big black arrowhead).

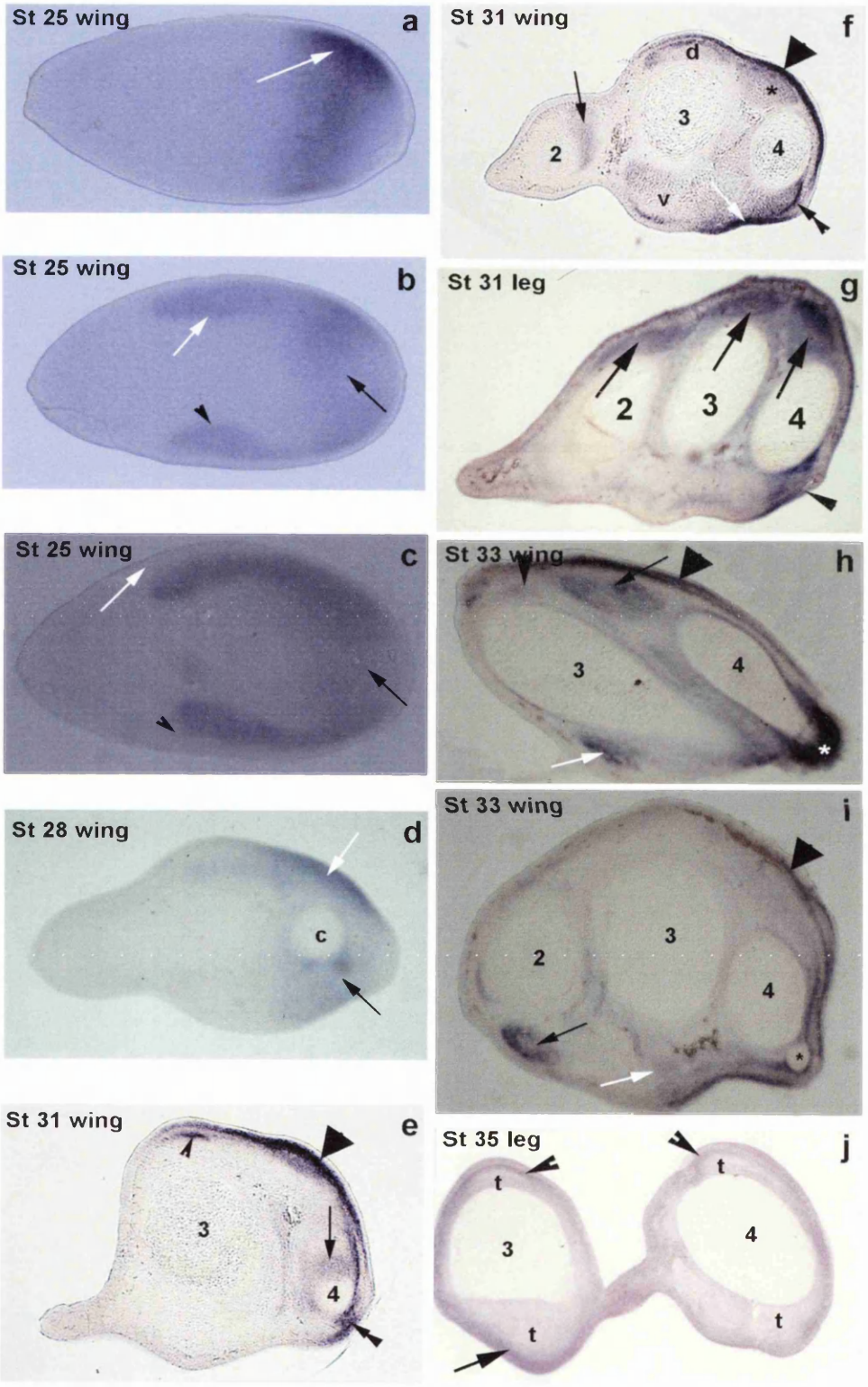
f) Proximal level of stage 31 wing. *Six 1* transcripts in muscles extensor medius muscle brevis (d), abductor medius (v), flexor digiti quarti (double arrowhead) and interosseus dorsalis (asterisk). Expression posterior to cartilage of digit 2 (arrow); thick strong expression under dorsal ectoderm over digits 3 and 4 (big black arrowhead) and under ectoderm ventral to digit 4 (white arrow).

g) Proximal section of stage 31 leg. *Six 1* expression in dorsal tendons (arrows), abductor muscle of digit 4 (arrowhead). No expression seen ventral to digits 2 and 3.

h) Distal section of stage 33 wing. Expression in interosseus dorsalis (arrow) is well made out; faint expression is seen in dorsal (arrowhead) and ventral (white arrow) tendons of digit 3 and in flexor digiti quarti tendon (asterisk). Expression subjacent to dorsal ectoderm is still present (big black arrowhead).

i) Proximal section of stage 33 wing. Expression in flexor tendon (black arrow) of digit 2 is prominent, weak expression subjacent to ectoderm (big black arrowhead) is seen dorsally, posteriorly and ventrally. Very weak expression is present ventral to digit 3 (white arrow).

j) Stage 35 leg. Dorsal expression (arrowhead) is seen outlining the dorsal surface of tendon; ventrally and on sides (arrow) there is diffuse expression between tendon and ectoderm.


St 25 wing
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St 25 wing
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d
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St 31 leg
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St 33 wing
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St 33 wing
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St 35 leg
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plate with linear streaks of stronger expression. These streaks correspond to *Six 1* expression in proximal regions of dorsal tendons. In the mouse, expression is seen even in distal parts of tendons. Expression is also present at the postero-ventral side of digit 4 in the chick leg abductor muscle (Fig. 4.10g).

Expression in muscle persists at stage 33 in the chick wing and is present in interosseus dorsalis, abductor medius of digit 3, abductor of digit 4 (Fig. 4.10h) and, proximally, in flexor indicis (Fig. 4.10i). In the leg, at stage 35 (Fig. 4.9f), weak expression is present in interphalangeal joint regions and there are 2 streaks of expression along the digits, which mark tissue surrounding the tendons on both the dorsal and ventral sides (Fig. 4.10j). Tendons themselves do not appear to express *Six 1* at this stage.

4.3.4 Expression of *Bmp-4* and *Follistatin* during chick tendon development

Although the expression of *EphA4* has already been described in section 4.3.1 it is included in this section for comparison. At stage 29, *Bmp-4* transcripts are present in the interdigital region (Fig. 4.11d and 4.12d). This is particularly striking in the 3rd inter-digital region (between digits 3 and 4) where a triangular region of expression with the base placed distally (Fig. 4.11d) was localised. Additional domains are seen in more proximal regions overlapping the developing digits. At stage 31, expression between digits increases next to the digits but is weaker centrally in the 3rd inter-digital region. Faint stripes of expression overlie the digits especially in proximal regions (Fig. 4.11e and 4.12e). By stage 33, there is even stronger expression at the edges of each digit. Inter-digital tissue not expressing *Bmp-4* is found centrally and extended from proximal to distal in the interdigit (Fig. 4.11f and 4.12f). Weak stripes of expression detected at stage 31 overlying the digits have faded and could only be detected at the base of each digit. At these proximal levels, sections show *Bmp-4* transcripts around the tendon (Fig. 4.12f).

Follistatin is first detected in the limb bud as a proximo-distal wave of expression from stage 23 onwards, in a sub-set of proximal muscles and surrounding mesenchyme. By stage 26, there is faint expression in the digital plate (Amthor et al 1996). At stage 29, there are bands of *Follistatin* expression dorsally (Fig. 4.11g) and ventrally (not shown) over the digits. Transcripts are distributed in sub-ectodermal mesoderm overlying digital cartilages (Fig. 4.12g). By stage 31, there is higher expression over the distal tips of the digits (Fig. 4.11h) and proximally, on the ventral aspect only, there are 2 faint streaks over each of the digits. Sections reveal that the faint streaks of expression represent a thin rim of transcripts at the periphery of each tendon (Fig. 4.12h).

Figure 4.11 Comparison of expression of *EphA4, BMP-4* and *Follistatin* in chick legs

EphA4 (a-c); BMP-4 (d-f); Follistatin (g-i)

All samples viewed from ventral aspect.

a) Stage 29 leg. Broad bands of EphA4 expression (arrows) overlie the developing digits.

b) Stage 31 leg. *EphA4* expression at the base of the foot plate (white arrowhead). Distally bands of expression associated with digits fan out (arrow). Note also expression at level of inter-phalangeal joints (black arrowheads).

c) Stage 33 leg. Slight weakening of *EphA4* expression compared to stage 31 in tendon regions which are now narrower. Expression at the inter-phalangeal joints which flanks stripes of expression in tendons (arrowheads).

d) Stage 29 leg. Strong expression of *BMP-4* transcripts in the 3rd interdigital region (arrowhead); some transcripts are also present over the digits (arrow).

e) Stage 31 leg. Strong *BMP-4* expression is evident in the proximal inter-digit (arrowhead) contrasting with lower expression in central inter-digit (asterisk). Faint thin stripes of expression (black arrow) are diverging from a common point of expression (white arrowhead).

f) Stage 33 leg. *BMP-4* expression in the interdigit (arrowhead). Expression over the digit is present (arrow) only proximally.

g) Stage 29 dorsal view showing broad bands of Follistatin expression extending over the digits (arrows).

h) Stage 31 leg Strong *Follistatin* expression at digit tips (arrowhead) and two streaks extending over digits (arrows).

i) Stage 33 leg. *Follistatin* expression at digit tips is faint, while streaks over the digits (arrow) are more distinct and expression at inter-phalangeal region (arrowhead) becomes evident.

Fig. 4.11

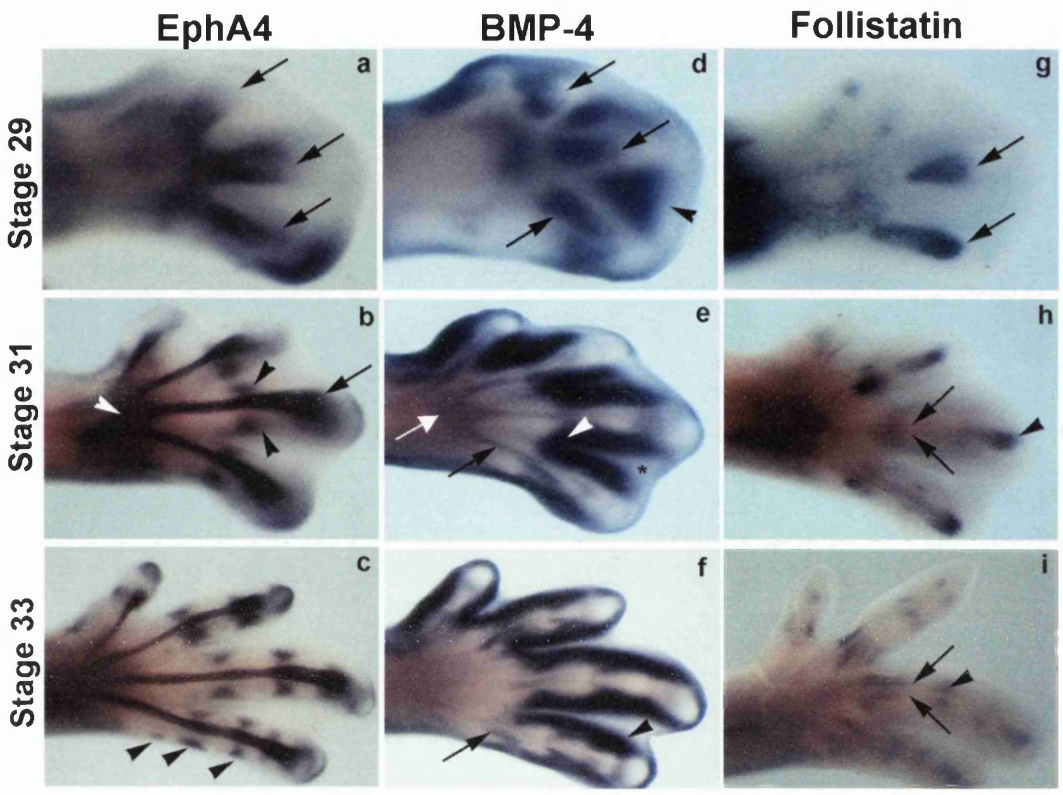

EphA4
BMP-4
Follistatin
Stage 29
Stage 31
Stage 33
a
b
c
d
e
f
g
h
i
*

Figure 4.12 Expression of *EphA4*, *BMP-4* and *Follistatin* in tendons

EphA4 (a-c); BMP-4 (d-f); Follistatin (g-i)

Sections are from limbs shown in Fig. 4.11. Dorsal to top.

a) *EphA4* transcripts in stage 29 leg detected in both dorsal and ventral subectodermal mesenchyme (arrows).

b) Stage 31 leg. Ventral expression domain of *EphA4* (arrow) is displaced away from the ectoderm.

c) Stage 33 leg. Reduction of expression of *EphA4* in ventral tendon (arrow) which is also more condensed.

d) Stage 29 leg. *BMP-4* expression in inter-digital region (arrow) and some expression over digit region (arrowhead).

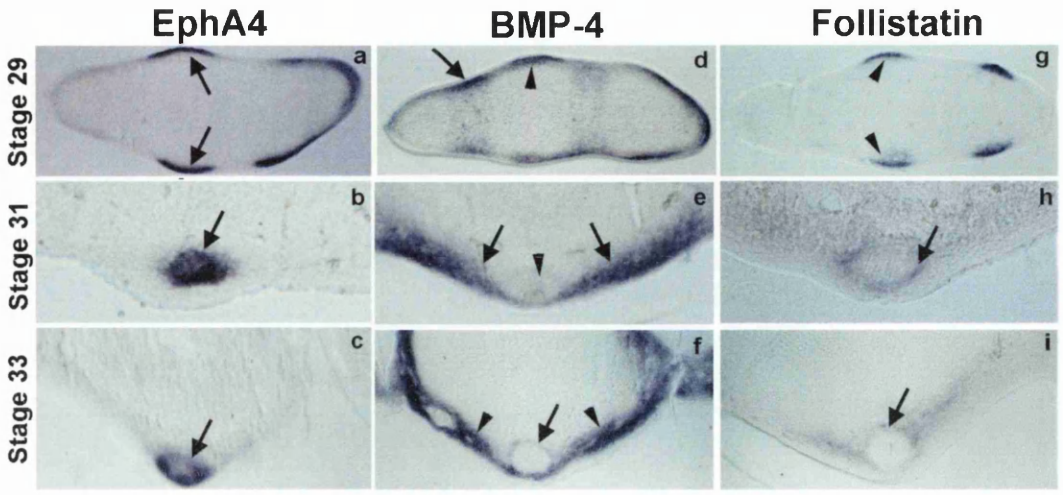
e) Stage 31 leg. Strong *BMP-4* expression in inter-digital region (arrow) and faint expression over the digit region (arrowhead).

f) Stage 33 leg. *BMP-4* expression detected around tendon (arrow) and in interdigital region (arrowhead).

g) Stage 29 leg. Expression of *Follistatin* over the digits just subjacent to the ectoderm (arrows).

h) Stage 31 leg. *Follistatin* expression detectable around the periphery of the ventral tendon (arrow).

i) Stage 33 leg. Expression is detectable around periphery of ventral tendon (arrow).


EphA4
BMP-4
Follistatin
Stage 29
Stage 31
Stage 33
a
b
c
d
e
f
g
h
i

Comparison of all three genes at stage 31, shows that *Follistatin* expression is the most distally situated while *EphA4* expression is located slightly more proximally. *EphA4* expression is found in the body of the tendon, whereas the tendon-associated expression of *Follistatin* and of *Bmp-4* is only found in the periphery. At stage 33, the strong distal *Follistatin* expression seen at the tip of digits decreases (Fig. 4.11i) but faint expression persists in tendon periphery (Fig. 4.11i, 4.12i). Reduction of *Follistatin* expression at the digit tips, coincides with the disappearance of the ridge. Areas of weak expression of *Follistatin* are present at the level of the inter-phalangeal joints similar to *EphA4* expression (Fig. 4.11c).

4.3.5 Relationship between cartilage and tendon development

4.3.5.1 TGF-ß1 induces molecules associated with early tendon development

Previous work has shown that application of TGF-ß1 or TGF-ß2 leads to formation of cartilaginous elements and also associated tendons in inter-digital regions (Ganan et al 1996). We therefore investigated whether TGF-ß1 induces molecules expressed in early tendon development.

Inter-digital mesenchyme at stage 29 expresses *Bmp-4*. However application of TGF-ß1 at this stage (prior to cell death in interdigital area) leads to reduced *Bmp-4* expression in this region (compare Fig. 4.13a treated with TGF-ß and 4.13b control limb). This result suggests that a decrease in *Bmp-4* expression is an early event associated with formation of ectopic cartilaginous elements. We also investigated whether *Follistatin* and *EphA4*, two genes associated with developing tendon, were ectopically expressed following TGF-ß1 application. We found that introduction of a bead soaked in TGF-ß1 to the inter-digit at stage 29, led to ectopic expression of *Follistatin* (Fig. 4.13c) and of *EphA4* (Fig. 4.13D) after 48 hours. These results show that the induction of cartilage by TGF-ß in the inter-digital region is accompanied by expression of early tendon-associated molecules.

4.3.5.2 Ectopic Follistatin results in loss of tendon-associated markers

We examined the consequences of disrupting the normal activity of TGF-ß super-family mediated signalling using the antagonistic activity of Follistatin in both digit and inter-digit regions. Application of Follistatin to the 3rd interdigit led to the loss of 2 phalangeal elements in digit 3 and loss of 3 phalangeal elements in digit 4 (Fig. 4.14a and 4.14b). At 48 hours after the same procedure, the broad distal *EphA4* expressing regions on both the dorsal and ventral aspects were lost (Fig. 4.14c and 4.14d). Thus a stunted tendon is produced (Fig. 4.17a). Furthermore, following an identical procedure,

Figure 4.14 Application of Follistatin leads to the loss of cartilage and tendons

Follistatin (1mg/ml) was applied to inter-digit (a-d) or digital sites (e-h) at stage 28/29. Anterior to top. Asterisk marks bead.

a) Cartilage organisation, 4 days after operation shown by Alcian green staining reveals loss of phalangeal elements in digits 3 and 4 following Follistatin applicationin inter-digital region; compare with control left leg (b).

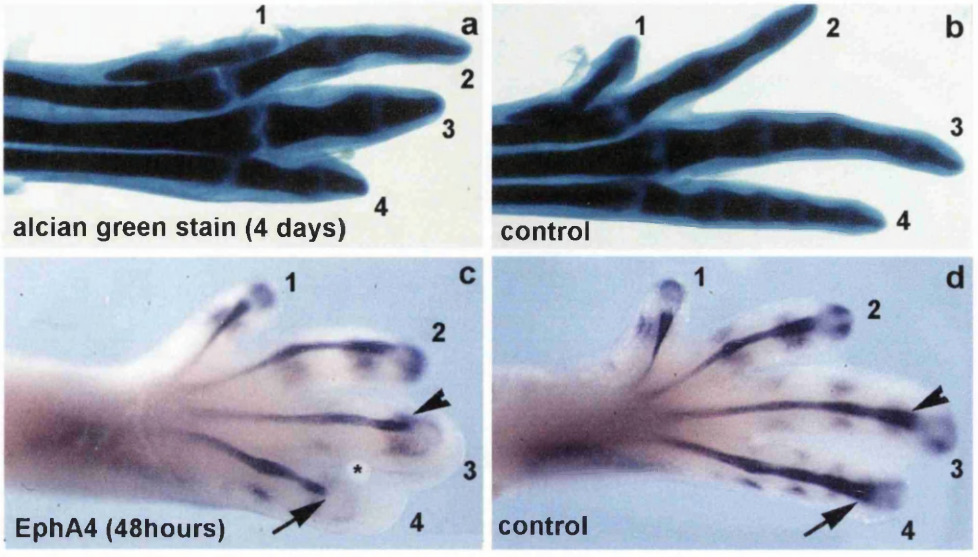
c) Follistatin application to inter-digit sites (aster) causes reduction in *EphA4* expression at distal sites 48h after operation. Some decrease in expression also seen in digit 3 (arrowhead). Note characteristic broad expression domain at the distal tip is missing in digit 4 (arrow) compare with control left leg (d).

e) Application of beads soaked in Follistatin to digit site for 48h leads to loss of distal *EphA4* expression (arrow). Note that webbing continues to develop in treated limb (arrowhead).

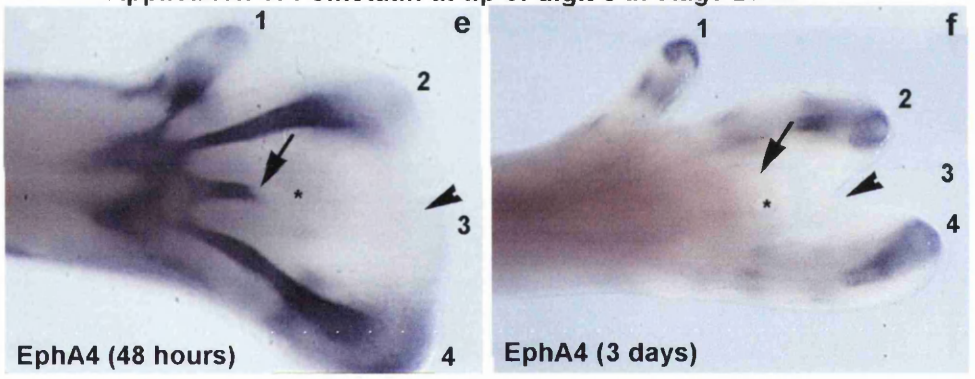
f) After 3 days, *EphA4* expression even further reduced (arrow). Inter-digital webbing now begins to recede (arrowhead).

g) Application of Follistatin to proximal tendon sites for 48h leads to reduced *EphA4* expression in proximal sites (arrow) but distal expression (arrowhead) appears similar to control leg (h).

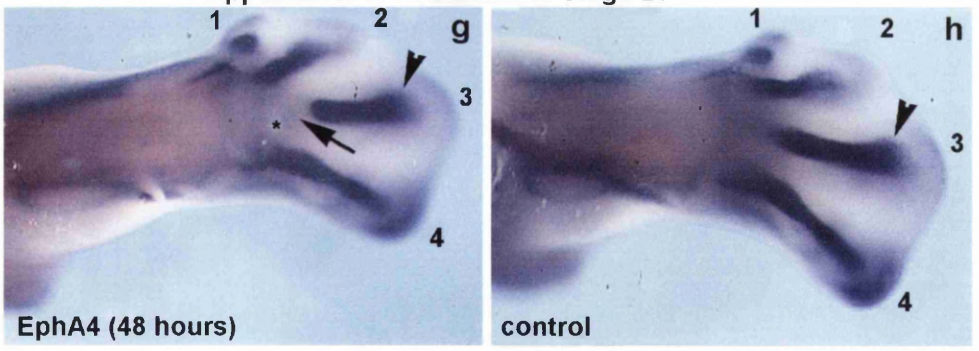
Application of Follistatin in interdigital region at stage 28/29



Application of Follistatin at tip of digit 3 at stage 28/29



Application of Follistatin at stage 26



the tendon associated expression of *Follistatin* is also lost in distal digital regions (data not shown).

When a Follistatin bead is implanted directly at tip of digit 3 instead of in the interdigital region, phalanges were lost only in digit 3 (data not shown). The same manipulation resulted in truncation of the broad band of *EphA4* expression normally associated with the digit tip (Fig. 4.14e) but normal expression is present in the neighbouring digits. Although digit 3 and associated tendon is truncated, a web-like tissue extended between digits 2 and 4 and subsequently regressed (Fig. 4.14f). These results show that application of Follistatin to either digital or inter-digital regions leads to loss of both distal cartilage and tendon.

We examined the effect of Follistatin on proximal cartilage and tendon by applying the protein at earlier stages. Application at stage 26 resulted in loss of proximal *EphA4* expression in tendon regions but distal expression of the gene is normal (Fig. 4h and 4g). Cartilage, although present in the region where *EphA4* is lost, has not developed normally but is smaller. These manipulations resulted in the loss of dorsal *EphA4* expression only. These results show that distal tendon development can occur without the need for proximal tendon.

4.3.5.3 Effects of ridge removal on *EphA4* and *Follistatin* expression in tendons

Apical ridge removal from the digit tip has been shown previously to result in the loss of distal cartilage elements followed by the fusion of dorsal and ventral tendons (Hurle et al 1990). We therefore examined the effects of removing the apical ridge from both digit and interdigit regions on expression of *EphA4* and *Follistatin*. Apical ridge removal from digit 3, at stage 28, resulted in the loss of *Follistatin* at the distal tip of digit 3 after 48h (Fig. 4.15a and b) and the normal broad end of the *EphA4* expression domain is also missing. However *EphA4* expressing cells extend around the truncated digit tip (compare Fig. 4.15c and d).

Ectopic application of FGFs at the tips of digits also leads to the inhibition of cartilage formation (Ganan et al 1996). Therefore we examined the effect of FGF-4 application on tendon markers. After FGF-4 application to the distal tip of digit 3, expression of both *Follistatin* (Fig. 4.15g and h) and *EphA4* is reduced (data not shown). Proximal expression however is not affected in either case. This change is seen as early as 24 hours after FGF4 application. Therefore FGFs inhibit not only formation of cartilage but also expression of tendon-associated markers. Inter-digital apical ridge removal has previously been shown to result in ectopic cartilage after 24 hours (Hurle and Ganan 1986). Similarly, we found that after interdigit ridge removal, *EphA4* is

Figure 4.15 Effect of Apical Ectodermal Ridge removal and application of either BMP-4 or FGF-4 on the expression of *Follistatin* and *EphA4*

Removal of the apical ridge from digit 3 (marked by asterisk) at stage 27/28 leads to reduction of *Follistatin* expression (arrow) after 48h.
Anterior at top.

a) Apical ridge removal from tip of digit 3 (asterisk) results in loss of *Follistatin* expression at the tip (arrow).

b) Normal expression of *Follistatin* in control left leg.

c) Apical ridges removal at the tip of digit 3. Broad tip of *EphA4* expression is missing (arrow) after 48h. Dorsal and ventral expression of *EphA4* is continuous (arrowhead).

d) Normal expression of *EphA4* does not extend to the distal tip of the digits (arrow) in control left leg.

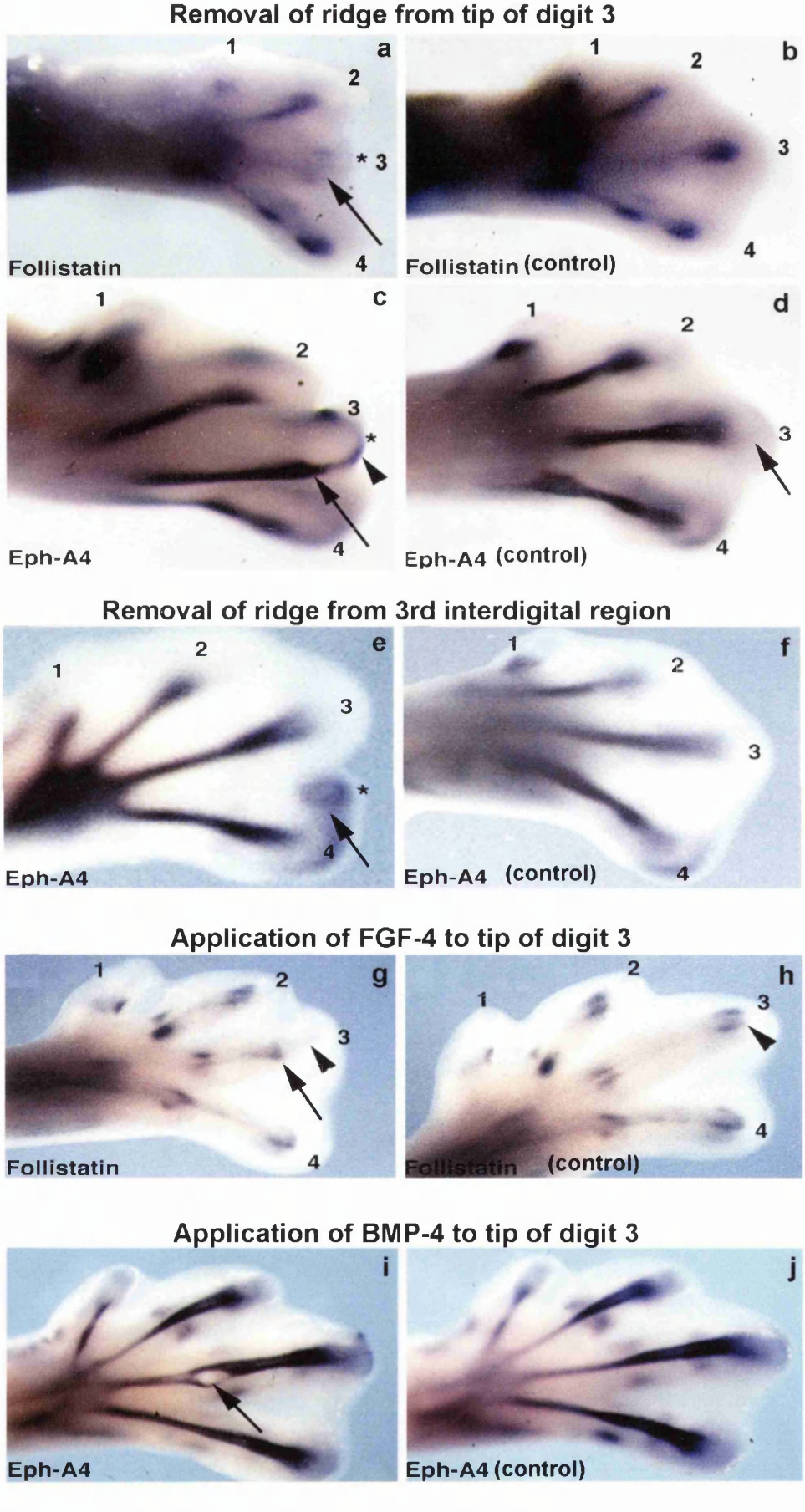
e) Inter-digital ridge removal between digits 3 and 4 (region marked by asterisk) results in ectopic expression of *EphA4* after 24h (arrow) ventral view.

f) Shows dorsal expression of *EphA4* in control left leg.

g) Application of FGF-4 to distal tip of digit 3 (arrowhead) leads to the loss of distal *Follistatin* expression after 48h. *Follistatin* expression ends at more proximal site (arrow).

h) Shows distal expression of *Follistatin* in control limb (arrowhead).

i) Application of BMP-4 for 48h to proximal region of the 3rd digit leads to a split in *EphA4* expression near the BMP-4 bead but *EphA4* expression is normal distally (compare with (j) control left limb).


Removal of ridge from tip of digit 3
a
1
2
* 3
4
Follistatin
b
1
2
3
4
Follistatin (control)
c
1
2
3
*
4
Eph-A4
d
1
2
3
4
Eph-A4 (control)
Removal of ridge from 3rd interdigital region
e
1
2
3
*
4
Eph-A4
f
1
2
3
4
Eph-A4 (control)
Application of FGF-4 to tip of digit 3
g
1
2
3
4
Follistatin
h
1
2
3
4
Follistatin (control)
Application of BMP-4 to tip of digit 3
i
Eph-A4
j
Eph-A4 (control)

induced in a tubular domain in the interdigital region (compare Fig. 4.15e and f). FGF-4 induced a similar effect on *Follistatin* expression. These results show that removal of inter-digital apical ridge leads not only to the formation of cartilage but also leads to the induction of the tendon associated markers *EphA4* and *Follistatin* within 24hours.

In contrast to the reduction in cartilage produced by FGF-2 or FGF-4, application of BMP-2 or BMP-4 to digital regions leads to the enlargement and bifurcation of cartilage elements (Merino et al 1998). Therefore we determined the effect of BMP-4 on *EphA4* expression. We applied beads soaked in 100ug/ml BMP-4 to the digital region of stage 28 embryos and found that, after 24h, the expression of *EphA4* is split. After 48hours, the bead and the split *EphA4* expression domain have been displaced more proximally (Fig. 4.15i) but both distal and proximal to the site of bead expression of *EphA4* appeared normal (compare with Fig. 4.15j). BMP-4 induced a similar effect on expression of *Follistatin* (data not shown).

4.4 Discussion

4.4.1 *EphA4* is expressed in relation to tendon development

EphA4 is expressed in chick limb from stage 17 up to stage 26 in proliferating mesenchymal cells at the distal end of the bud overlapping the progress zone. Later, from stage 27 onwards, expression in a layer subjacent to ectoderm becomes associated with developing tendon primordia. Expression persists in tendons up to stage 34. Thus, when tendons reach their correct position, expression goes off. From stage 31 onwards, expression also appears in tissue extensions and this remains up to stage 36. This expression pattern suggests that *EphA4* is expressed in proliferating and non-proliferating tendon cells as it has been shown that from stage 30/31 tendon cells fail to incorporate BrDu (Ros et al 1995).

The very early expression of *EphA4* in the subectodermal mesenchyme at stage 28 in regions where tendon blastema will form later suggests that *EphA4* has a role in tendon cell aggregation around the mesenchymal lamina. By stage 30/31, mesenchymal cell aggregation around the mesenchymal lamina is clearly made out (Ros et al 1995). At these stages *EphA4* expression becomes more condensed and is not seen as broad bands but as narrower strips overlying the digital cartilage. *EphA4* is not only expressed in tendons during the process of cell condensation but also as tendon moves from a site subjacent to ectoderm to a site deeper in the mesenchyme.

EphA4 is expressed in both dorsal and ventral tendons during development. Dorsal expression is thin and wide whereas ventral expression is thicker and narrower. From

the study of *EphA4* expression in tendons it appears that initially as *EphA4* expressing tendon forming cells assemble under the ectoderm they do so with a dorso-ventral differential pattern. Dorsally they assemble as a thin mass of cells in the subectodermal mesenchyme, while ventrally, cells assemble as a thicker layer and this difference can be made out even at early stages. Later the dorsal tendon cell mass moves away from ectoderm but remains as a thin layer of *EphA4* expressing cells, whereas the ventral tendon becomes a rounded mass of *EphA4* expressing cells.

EphA4 is expressed throughout all the dynamic events during development of deep and superficial tendons. Toes have more than one flexor tendon, for example, there are 3 flexor tendons in digit 3 (as described in chapter 3). According to Ros et al (1995), the superficial tendon flexor perforatus digiti appears as a peripheral condensation in relation to the deep tendon, flexor digitorum longus at stage 33 in proximal regions. The expression pattern of *EphA4* is consistent with the idea that superficial tendon delaminates from the deep tendon already formed. Superficial tendon may also form by assembling of new tendon cells. If this was the mechanism by which superficial tendon forms, then one might expect to see a mesenchymal lamina at the site of assembling of superficial tendon, and this is not seen in this work. Once the superficial tendon is formed, expression of *EphA4* is reduced in deep tendon. I could not follow the expression pattern of *EphA4* in development of the 3rd and most superficial tendon.

As superficial tendons reach their insertion points, they divide into two slips that attach to the sides of the phalanx, and allow the deeper tendon to run further and insert at a more distal site (Wortham 1948). *EphA4* is expressed during splitting of superficial tendon into 2 slips that attach to cartilage and allow the deep tendon to run distally. Once the 2 slips are formed, *EphA4* expression reduces in the anterior slip and then in posterior slip. It is not clear why this happens first in the anterior slip.

The comparative study between expression of *EphA4* with tenascin and procollagen I, indicates that *EphA4* is the earliest marker during tendon development (Table 4.1). However *EphA4* is detected by transcripts rather than protein.

4.4.2 EphA4 is expressed at tendon insertion sites to cartilage elements

The autopodial tendons of the foot are derived from two components, the distal tendon primordium and the dorsal and ventral tissue extensions around the metatarso-phalangeal and interphalangeal joints (Kardon 1998). From stage 31 onwards, in addition to expression in tendons, patches of *EphA4* expressing cells are present at the level of the digital joints. These patches become continuous with expression in tendons and probably represent the tissue extensions, which fuse with the tendon to form the

terminal most part of the tendon at attachment points with skeleton. According to Kardon (1998), tissue extensions which she recognised by tenascin, grow proximally to make contact with tendons extending from distal tendon primordium. There is no evidence for proximal extension of tissue extensions in this work. Tendons move from a superficial to a deep site, and it appears that the actual tendon splays out, splits into 2 slips which come into contact with the tissue extensions (Fig. 4.3e). Once connection between tendon and the tissue extension is made, *EphA4* expression is reduced in the connecting tissue and expression remains only at the attachment sites to cartilage up to stage 36 and not in the connecting tissue.

Elastin-positive fibrils that anchor tendon blastema to the epiphyses of developing phalanges are produced by tendon cells at stage 33/34 (Ros et al 1995). It is interesting to note that *EphA4* is expressed at insertion points at the same time as elastin positive fibrils.

4.4.3 Tendons are fully formed by stage 35

The expression patterns of *EphA4* mark interphalangeal joint regions and show how many phalanges have formed. At stage 34, it can be seen that all phalanges are formed as the number of expression sites in tissue extensions coincide with the total number of interphalangeal joints. For example, digit 2 in the leg has 3 phalanges, and there are 2 patches of *EphA4* expression in tissue extensions indicating 3 phalanges have formed (Fig. 4.3d). As expression in the tendon extends beyond the 2nd tissue extension which is at the joint between 2nd and distal phalanx, it suggests that tendon makes its distal attachment at around this stage. According to Kardon (1998), insertion of tendons into the distal most phalanges is complete around stage 35. This then suggests that thereafter tendon grows in length concomitant with elongation of cartilage and this may depend on its attachment to cartilage.

4.4.4 Potential role of *EphA4* during tendon development

EphA4 null mutant mice have loss of coordination of limb movement and a hopping gait with reduced number of corticospinal tract (CST) axons in the lower spinal cord and medulla (Dottori et al 1998). However defects in the limb or tendon were not reported and it would be very interesting to know if development of tendon is affected.

EphA4 provides tendon cells with the ability to respond to short-range signals presented by neighbouring cells. A number of potential ligands could interact with *EphA4* expressed in developing tendons. In mouse limbs, *Ephrin-A2, Ephrin-A4 and Ephrin-A5* ligands are expressed in the early distal limb mesenchyme and later *Ephrin-A2*, and *Ephrin-A5* are expressed in mesenchyme of interdigital regions and then around

edges of cartilage at day 12-13 when tendon region expresses *EphA4* (Flenniken et al 1996, Gale et al 1996b, this thesis, chapter 5). Therefore one possibility is that tendon cells expressing *EphA4* interact with neighbouring cells (non-tendon-forming mesenchyme) at the boundary of expression domains.

Interaction at the boundary of condensing cells in tendons could control formation of the tendon sheath (Patel et al 1996). Here and in mouse tendons (Chapter 5), *EphA4* expression is stronger around periphery of tendons. Around stage 34 when the sheath begins to form *EphA4* expression has already started to be reduced in tendons but the presence of *EphA4* expression at periphery of ventral tendons may prevent premature sheath formation. Another function of boundaries in development is to keep cell populations in their proper places. When proximal tendon primordium expressing tenascin was removed in chick limb bud, muscle cells invaded the area (Kardon 1998). Since this primordium also expresses *EphA4* (this thesis), this suggests that *EphA4* could be involved in limiting migration of myogenic precursors into the tendon regions.

Cells from different regions of limb bud sort out from each other when mixed in vitro (Ide et al 1994). When GPI-anchored membrane-bound proteins (*Ephrin-A)* were removed from chick limb bud cells in culture, segregation was inhibited. A neutralising antibody to *EphA4*, prevented clustering of cells (Wada et al 1998). Hence it was suggested that, when cells derived from different regions of the limb bud are mixed (Ide et al 1994), receptor-ligand interaction occurs and the two types of cells migrate away from each other (Wada et al 1998) probably by repulsion. *EphA4* receptor and its ligands may have some analogous function in tendon development.

A primary event following *EphA4* receptor activation could be a change in the shape of the cell. Recent studies have shown that activation of *Eph* receptors leads to direct interactions with regulators of GTPases including ras-GAP and indirectly with regulators of small GTPases via *Nck* (Holland et al 1997). These GTPases have been shown to be key molecules in regulating organisation of the membrane-actin cytoskeleton as well as the formation of focal contacts between cells (reviewed by Hall 1998). Cell shape changes do occur when the loosely arranged distal sub-ectodermal mesenchyme re-organises itself, using the mesenchymal lamina as a scaffold, into tendons.

It has also been suggested that *EphA4* may modulate cell adhesion, possibly by regulating cadherin function (Winning et al 1996) and this could be relevant to its role in tendon formation. This suggestion came from experiments which showed that, when activated chimeric EphA4 (Pag) receptor was injected into fertilised frog eggs,

extensive disruption of cell adhesion occurred and embryonic blastomeres disaggregated. The pathway through which *EphA4* achieves this embryonic dissociation is not known. However, it was rescued by co-injection of dominant-negative form of the chimeric receptor and also by C-cadherin (Winning et al 1996). Cadherins act as adhesion molecules at cell-cell adherens junctions, which are required for cell adhesion in early frog embryos (Muller et al 1994). In the rat, there are intercellular contacts through focal junctional sites located at the ends of relatively slender processes in between tendon cells arranged circumferentially around tendon, as well in developing tendon proper (Greenlee and Ross 1967). Hence another possiblity is that *EphA4* expression in developing tendons has a role in regulating cell adhesion. It obviously would be interesting to look at cell adhesion molecules during tendon development.

4.4.5 *Six 1* is expressed in proximal regions of developing tendons

An important observation about the expression pattern of *Six 1* in both chick wing and leg bud is that, except for expression in the interphalangeal joint region, *Six 1* is not expressed in the distal part of the digital plate from stage 28 up to stage 35, the latest stage studied. In the leg, tendons going to phalanges for insertion are derived from the distal tendon primordium with contributions from tissue extensions that differentiate from around interphalangeal joints (Kardon 1998). Thus if *Six 1* is associated with tendon development in the chick, then the spatial distribution of transcripts suggests that it is not involved in development of distal parts of long autopodial tendons. *Six 1* is expressed in proximal regions and seems to be associated with distal tendon primordium and muscle. *Six 1* is however expressed in interphalangeal regions where it could be involved either in formation of ligament around the joint or in differentiation of tissue extensions that form the most distal part of tendons; that is, near their insertion points.

In some ways, the pattern of *Six 1* expression in chick resembles the murine expression, but, in others, it does not. In the mouse, transcripts are initially found in connective tissue-precursor cells and later become differentially localised to dorsal extensor tendons of the digits. In the chick wing, expression is present in undifferentiated limb mesenchyme and later is localised in muscle and tendon both dorsally and ventrally. However in the chick foot, expression is prominent in dorsal tendons but not in ventral tendons.

The line of *Six 1* expression extending between the strong expression subjacent to the dorsal ectoderm and the posterior side of digit 4 may be related to the connective tissue bundles that anchor the roots of some of the flight feathers to the posterior bones

in the adult (King and McLleland 1975). On the other hand, it could also be associated with expansor secondariorum muscles that insert on roots of secondary flight feathers (King and McLleland 1975). As there is not much information available, it is hard to relate the expression of *Six 1* temporally and spatially to these structures. Another possibility is that the strong layer of expression subjacent to the ectoderm is associated with development of feather buds.

Six 1 expression is seen flanking cartilage in both chick and mouse. This expression is not seen throughout the cartilage but is seen only at joint regions and hence is associated with ligaments. In the mouse, Oliver et al (1995) described expression flanking the cartilage on anterior and posterior sides. However, in the chick, *Six 1* expression is very clear on dorsal side as well. Since *Six 1* expression is stronger posteriorly and as expression proceeds from posterior to anterior in the chick limb just as it does in the mouse, this could be an indication that the polarising region may have some control over its expression. Secondly as *Six 1* is stronger on the dorsal side than it is on the ventral, signals from dorsal ectoderm like *Wnt-7a* may regulate its expression.

4.4.6 Expression of *Follistatin* is associated with tendons

Follistatin is localised in the developing chick leg bud at sites associated with tendon formation. However the expression profile is quite different from that of *EphA4* in tendon regions. *EphA4* is expressed in the core while *Follistatin* is expressed at the periphery and this may be significant in the context of regulating the development of tendon. *Bmp-4* is expressed in the inter-digital region of limb buds and later also at the periphery of proximal regions of tendon. *Follistatin* is the most distally expressed of the three genes (*Follistatin, EphA4, Bmp-4*).

A number of genes encoding transcription factors have recently been found to be expressed in tendons. *EphA4* and *Follistatin* follow a similar spatial pattern; both genes are initially expressed in a broad sub-ectodermal layer, dorsal and ventral to developing cartilage. Subsequently these cells condense and the cell condensations are displaced more centrally. Ventral expression of both genes precedes dorsal expression. These expression profiles are very similar to those described by Oliver et al (1995) for the transcription factor *Six-2*. Recent work by Xu et al (1997a) has shown that *Eya2* and *Eya1*, two related transcriptional regulators are also expressed in tendons. Moreover, *Eya2* is expressed in dorsal extensor tendons while *Eya1* is expressed in the ventral flexor tendons. Similar to the expression patterns of *Follistatin* and *EphA4*, expression of the ventral marker *Eya1* precedes the expression of *Eya2* in dorsal tendon.

From the expression profile of *Follistatin*, we propose that the protein encoded by this gene also plays a role in tendon development. We have shown that *Follistatin* is expressed around the tendon and suggest that it may modulate signalling of TGF-ß super family members involved in tendon development. Although we showed here that *Bmp-4* is expressed in proximal tendons and application of BMP-4 results in tendon splitting, other TGF-ß family members are much more likely to act specifically to signal tendon development. *Gdf-5*, *Gdf-6* and *Gdf-7*, a group of genes very closely related to the BMPs have recently been shown to be expressed specifically in tendon and ligament forming regions during mouse embryogenesis (Wolfman et al 1997). Furthermore GDF-5, GDF-6 and GDF-7 have tendon inducing activity but not cartilage inducing activity, in contrast to BMP-2, which can induce cartilage but not tendon (Wolfman et al 1997). These results raise the possibility that Follistatin is regulating the activity of GDFs during tendon development (Fig. 4.16). Indeed there is evidence that BMP antagonists can directly bind GDF-6 and inhibit its activity in Xenopus embryos (Chang and Hemmati-Brivanlou pers comm).

4.4.7 Co-ordination of skeletal and tendon development

The results reported here, show that development of ectopic cartilage is accompanied by induction of expression of tendon-associated molecules. For example, application of TGF-ß in interdigital region and removal of apical ridge lead to ectopic expression of *EphA4* and *Follistatin*. Conversely, cartilage truncation is associated with tendon truncation and loss of expression of *EphA4* and *Follistatin*. Thus when limb pattern is manipulated, the effects on cartilage and tendon, on the whole, appear to be simultaneous. The only exception is when Follistatin is applied to an early limb bud and in this case tendon development is more markedly affected than cartilage development.

There are several possible explanations for this co-ordinated development. One possibility is that one tissue controls the development of the other. Another possibility is that co-ordinated development of skeleton and tendon is regulated by some common signalling mechanism. TGF-ß has been shown to be expressed in both cartilage and tendon (Merino et al 1998) and could act as a common signalling molecule (Fig. 4.16). *Gdf5* a member of the BMP family when implanted subcutaneously or intramuscularly in the rat, induces ectopic tendon (Wolfman et al 1997). In chick, GDF-5 implantation in the interdigital region did not produce ectopic tendon. However, when implanted at the tip of the growing digit, tendons were duplicated in the treated digit and the expression domain of *Tgf-ß2* and *Tgf-ß3* was enlarged and bifurcated ((Merino et al 1999). We therefore suggest that there could be parallel pathways in both tendon and

Figure 4.16 Diagram showing signals proposed to operate in cartilage and tendon development

Development of both cartilage and tendon is initiated by TGF-ß and then separate pathways lead to cartilage and tendon.

Fig. 4.16

Signals involved in tendon development

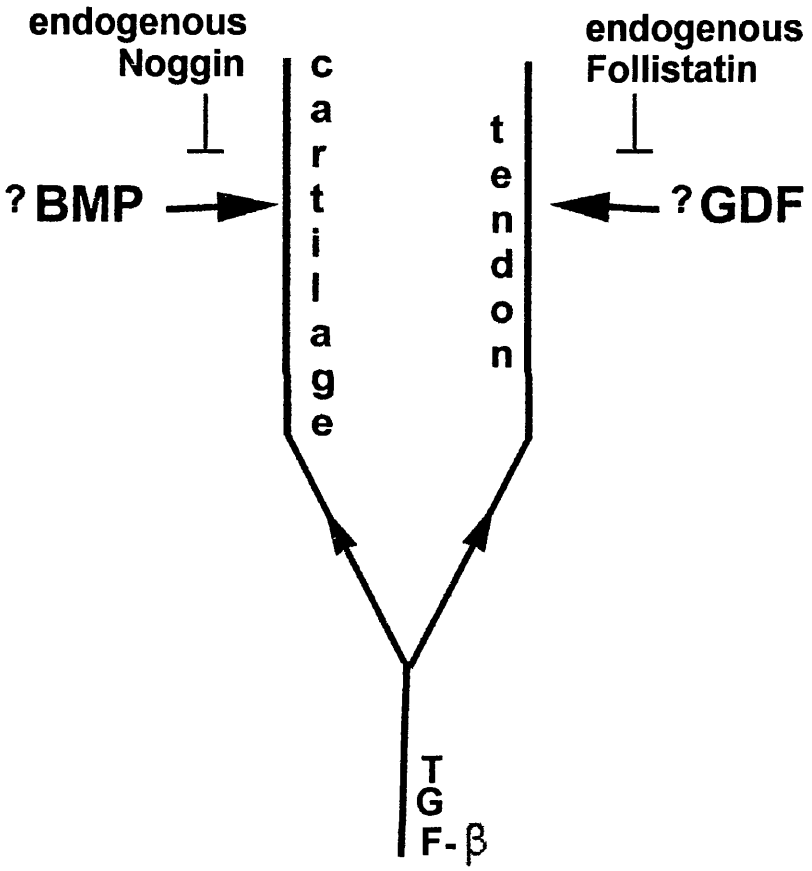


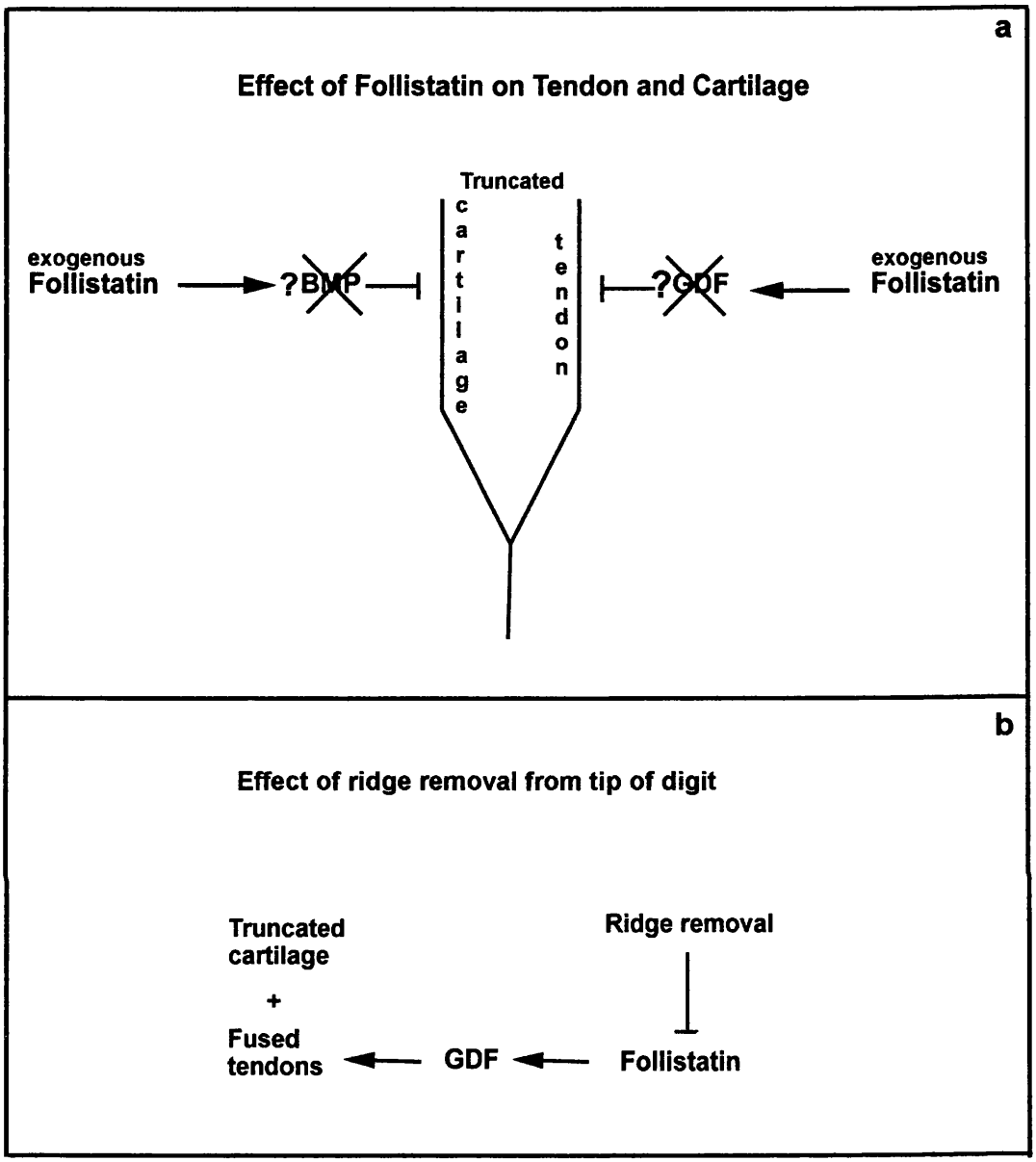
Figure 4.17

a) Diagram showing how Follistatin could affect both tendon and cartilage development

Application of Follistatin to either digit tip or inter-digital region leads to cartilage and tendon truncation by antagonising members of TGF-ß superfamily.

b) Diagram showing how ridge removal could promote tendon development

When ridge is removed from the tip of digit, *Follistatin* expression is reduced, and this could allow GDF to promote tendon development, leading to fusion of dorsal and ventral tendons around the tip of the truncated digit.


a
Effect of Follistatin on Tendon and Cartilage
Truncated
cartilage
tendon
exogenous
Follistatin
?BMP
?GDF
exogenous
Follistatin
b
Effect of ridge removal from tip of digit
Truncated
cartilage
+
Fused
tendons
GDF
Follistatin
Ridge removal

cartilage development involving TGF-ß, BMP-like molecules and their antagonists (Fig. 4.16). In the cartilage, according to Merino et al (1998) TGFß2 induces the expression of a BMPR1 receptor, which is activated by BMPs, and Noggin antagonises the action of BMPs. We have shown that TGF-ßs can induce tendon and furthermore that *Follistatin* is expressed in the tendon. Thus we hypothesise that TGFß-2 (based on its expression pattern) could induce GDF receptors in the tendons which are activated by GDFs and Follistatin could antagonise the action of GDFs (Fig. 4.17a). Apical ridge removal leads to loss of distal *Follistatin* expression. According to our hypothesis, outlined above, this would lead to greater GDF activity, since the antagonist Follistatin is no longer present. This enhanced activity of GDF could explain how dorsal and ventral tendons grow and join up around a truncated digit (Fig. 4.17b).

4.4.8 Does *Follistatin* have a role in joint formation?

In this work *Follistatin* is found to be expressed in inter-phalangeal joint regions just as *EphA4*. Hence both *EphA4* and *Follistatin* may have separate roles in development of the tendon extensions. These tendon insertions derived from the cartilage insertion sites contribute to the distal most part of tendons that form from the distal tendon primordium (Kardon 1998). Expression of *Follistatin* at the inter-phalangeal joint region may play a role in maintaining the joint. Recently, gene encoding the human *Follistatin related protein* (*FRP*) was cloned from synovium of rheumatoid arthritis patients. FRP is a secreted protein containing some similarity to amino acid sequence in *Follistatin*. It is a novel auto-antigen present in systemic rheumatic diseases (Tanaka et al 1998) for example rheumatoid arthritis. *FRP* gene expression was higher in synovial tissue resected from rheumatoid arthritis than that of osteoarthritis (control) patients. Rheumatoid arthritis is characterised by inflamed synovium, tender joints and thickened and nodular tendons (Collier et al 1995). It is suggested that auto-antibodies to FRP reduces activity of FRP and promotes tissue inflammation in rheumatoid arthritis patients.

Gdf5, just as in mouse, is expressed in joint-forming regions of developing chick digits (Francis-West et al 1996; Merino et al 1999). Implanting GDF-5 bead either at the digit tip or in interdigital region, results in inhibition of joint formation, and overexpression lead to increased length and width of cartilage. Hence it was suggested that GDF-5 is not involved in differentiation of synovial tissue but in controlling growth and differentiation of cartilage at the epiphyses of the phalanges in the joint region (Merino et al 1999; Francis-West et al 1996). In the light of these suggestions and as *Follistatin* is expressed in joint regions and is an antagonist of TGF-ß family members,

it seems likely that *Follistatin* may have a role in joint formation or formation of synovial tissue in relation to joints.

CHAPTER FIVE

PATTERNING OF TENDONS IN DORSO-VENTRAL AXIS OF VERTEBRATE LIMBS

Chapter Five: Patterning of Tendons in Dorso-ventral axis of Vertebrate Limbs

5.1 Introduction

Patterning along the dorso-ventral axis is controlled by signals from the ectoderm. Signalling from dorsal ectoderm is mediated by a member of the *Wnt* family of secreted proteins. *Wnt-7a* (Parr and McMahon 1995) is expressed uniformly throughout the dorsal ectoderm during initial stages of limb bud outgrowth in mouse (Gavin et al 1990; Parr et al 1993) and chick (Dealy et al 1993). In mice, *Wnt-7a* is expressed in the presumptive forelimb region at 8.75 days, extending from the level of the last few somites into the ectoderm overlying the pre-somite mesoderm (Par and McMahon 1995) and expression at the level of hindlimb comes on later at day 9.25. In chick, *Wnt-7a* is uniformly expressed in the dorsal ectoderm of the trunk at stage 15 prior to limb bud outgrowth (Riddle et al 1995, Vogel et al 1995). *Wnt-7a* is required for the acquisition of dorsal limb pattern (Parr and McMahon 1995). When *Wnt-7a* is functionally inactivated in transgenic mice, homozygous mutant mice show dorsal to ventral transformations of cell fate in the developing limbs. In wild type mice, ventral skin of the digits is devoid of fur, lacks pigmentation, has transverse striations and dermal footpads at the base and tip of the digits. Ventral tendons attach to ventrally placed sesamoid bones. Dorsal skin is pigmented, covered with fur and there are no dorsal footpads or sesamoid bones (Parr and McMahon 1995). *Wnt-7a* transgenic mice have pads on the dorsal aspect of the paws at day 15.5, which express *Pax-9* (a transcription factor normally expressed in ventral pads). Dorsal skin has less fur, ectopic pigmentation and striations. Nails grow at the dorsal-ventral interface of digits but many were truncated (Table 5.1). Ectopic footpads and dorsal striations are present in digits 2 and 3 and rarely in digits 4 and 5 (Table 5.2) (Cygan et al 1997). Mesodermally-derived structures such as tendons also have altered dorso-ventral patterning. Dorsal tendons are transformed into ventral-looking tendons and sesamoid bones are present on the dorsal side (Table 5.1). These ventralising features of the dorsal limb are more severe distally. Similar observations that distal limb is more severely affected than proximal limb, were made in chick experiments in which ectoderm was rotated (Geduspan and MacCabe 1987, 1989; Akita 1996).

Some *Wnt-7a* mutant mice also lack posterior digits indicating that *Wnt-7a* may be required for normal anterior-posterior patterning. Indeed, *Wnt-7a* is required for

Table 5.1 Characteristics of Wild type and mutant mice

Type	Flexure of digits	Skin	Nails	Tendons	Sesamoids
Wild type[1]					
Ventral	ventral	devoid of hair		normal	normal
		trans striations			
		eccrine glands[2]			
		no pigmentation			
		dermal footpads at base and distal tips of digits			
Dorsal		covered with hair	normal	normal	
		no striations			
		pigmentation +			
		no footpads			
Wnt-7a mutant[1]					
Ventral	straight	devoid of hair	truncated	normal	normal
		trans striations			
		no pigmentation	pigmented		
		dermal footpads at base and distal tips of digits	thickening overgrow the nails		
Dorsal		loss of hair		altered	ectopic +
		striations +			
		pigmentation on			
		ectopic footpads			
En-1mutant[2,3]					
	dorsal[2]	ectopic hair follicles[2]			loss[2]
		eccrine gland absent in proximal pads[3]	conical/cyl nails in		
		highly pigmented pads that develop into nails later[2]	digits 1-4 supplanting ventral pads[2]	absent/ poorly developed[3]	
		distal most pads absent[2]			
Wnt-7a/En-1[3]		D-loss of hair follicles	conical overgrown by tissue		ectopic
		ventral pads pigment become nails later			
		dorsal pads with pigmentation			
		V-less eccrine glands become nails later			

[1]Parr and McMahon 1995; [2]Loomis et al 1996; [3]Loomis et al 1998

Table 5.2 Dorso-ventral alterations in ectoderm of mouse mutants
(Cygan et al '97)

Digit	*Wnt-7a*$^{-/-}$		*Wnt-7a*/En-1	double mutant
	D- footpads	D- striations	D- footpads	V- ectopic hair
1				+
2	+	+	+ distal tip	+
3	+	+	+ distal tip	+
4	rarely	occasionally posterior half		+
5	rarely	occasionally posterior half		+

D, dorsal; V, ventral
Note:
Footpads and striations are normally found ventrally and hair is normally found dorsally.

expression of *Shh* and maintenance of polarising region (Yang and Niswander 1995; Parr and McMahon 1995). Recently, a spontaneous mouse mutation, Post-axial hemimelia (*px*), was reported as being a mutation in the gene encoding the *Wnt-7a* signalling molecule (Parr et al 1998). This mutant shows limb deformities with ectopic dorsal footpads and sesamoid bones, accompanied frequently by loss of posterior bones.

Wnt-7a induces expression of the LIM homeobox-containing gene, *Lmx-1* (Riddle et al 1995, Vogel et al 1995). In chick, both onset and pattern of *Lmx-1* expression in dorsal limb bud mesenchyme closely follows that of *Wnt-7a* in ectoderm. Removal of dorsal ectoderm leads to a corresponding loss of *Lmx-1* expression in dorsal mesenchyme especially distally, which can be rescued in vitro when dorsal limb mesenchyme deprived of ectoderm is grown on *Wnt-7a* expressing cells (Table 5.3). In addition *Wnt-7a* is sufficient to induce ectopic *Lmx-1* expression in ventral mesenchyme and leads to dorsalisation of ventral mesoderm.

Lmx-1 can be ectopically expressed in ventral limb mesenchyme of chick limb buds by injecting retroviral vector carrying the *Lmx-1* gene into the presumptive limb field at stage 8-10 (Riddle et al 1995) or at stage 12 (Vogel et al 1995). Infected chick legs at 11 days (Riddle et al 1995), and 14 days of incubation (Vogel et al 1995) have hyperextended toes and bi-dorsal characteristics. This is most evident in the tarsal region where ventral muscles and tendons resemble closely the pattern of muscles and tendons usually found dorsally. At the distal tips of the toes, the number of ventral tendons is reduced and tendon sheath is absent. (Riddle et al 1995). In the foot, ventral muscles are lost especially in digits 1, 3 and 4 and extensor hallucis longus muscle characteristic of dorsal side is present on ventral side. In infected wings, abductor medius muscle of metatarsus 3 on the ventral side is missing. Misexpression of *Lmx-1* also produces alterations in dorso-ventral pattern of ectodermally derived structures. Supernumerary feathers develop on the ventral side of wing and ectopic feathers in the leg. Some toes in infected legs show bi-dorsal scale patterns (Vogel et al 1995). For example when legs are examined after 14 days of incubation, sometimes digits 1, 3 and 4, sometimes digits 3 and 4 have bi-dorsal scale patterns whereas the ventral integument in other toes were unchanged. As *Wnt-7a* and *En-1* (a homeobox containing gene expressed in ventral ectoderm) expression in infected legs is restricted to dorsal and ventral ectoderm respectively as normal, it was suggested that ventral to dorsal alteration in ectodermal cell fate is due to mesenchymal changes in gene expression and ectopic *Lmx-1* expression in ventral mesenchyme (Vogel et al 1995).

Table 5.3 Regulation of *Lmx-1* in chick embryo
(Riddle et al 1995; Vogel et al 1995)

	Expression of	**Wild type**	**Mis express Wnt-7a @ st 8-12**	**Ectoderm removed in vivo at stage 22**	**Ectoderm removed st 20-21 in vitro culture for 36hrs**	**Ectoderm removed grown on Wnt-7a expg cells**	**AER removed at stage 21**
st 15	**Wnt-7a**	presu limb ect	limb ect and *flank ect				
upto st 23[r]		Dorsal ect					
	Lmx-1			barely detected[r] (after 24 hrs)			normal (after 48 hrs)
st 15		presu limb mes			#persists prox Dm, absent dist Dm ~absent Vm[r]	present Dm[r] present dist Vm	
st 17-30		Dm excluded from cart forming region					
st 22-24			Ve [v] distal Ve [r] *not in flank				

st, stage; dist, distal; prox, proximal; expg, expressing; D, dorsal; V, ventral;
ect/e, ectoderm, De, dorsal ectoderm ; Ve, ventral ectoderm;
mes/m, mesenchyme; Dm, dorsal mesenchyme; Vm, ventral mesenchyme
cart, cartilage.
presu, presumptive;

*Indicates that at stage 14-15 mesenchyme has acquired competence to respond to Wnt-7a.

# Indicates that dorsal mesenchyme has acquired autonomy of Lmx-*1* expression after a significant period of expression to ectoderm signals.

~ Indicates that ventral ectoderm does not repress Lmx-*1* expression.
[r] Riddle et al 1995
[v] Vogel et al 1995

The stability of *Lmx-1* expression in proximal versus distal mesenchyme cells in limb bud differs. When chick limbs denuded of ectoderm at stage 20-21 are cultured for 36 hours, the distal mesenchymal cells do not retain *Lmx-1* expression, but proximal mesenchyme retains expression (Riddle et al 1995). However when Vogel et al (1995) removed dorsal ectoderm in vivo at stage 22 limbs *Lmx-1* is barely detected after 48 hours. When ectoderm-denuded chick limbs are cultured on *Wnt-7a* expressing cells, *Lmx-1* is present in both proximal and distal dorsal mesenchyme. *Lmx-1* is also expressed in ventral mesenchymal cells distally but not proximally (Riddle et al 1995) indicating that more mature ventral mesenchymal cells have lost the competence to respond to the *Wnt-7a* signal. This difference might explain why, when *Lmx-1* was misexpressed in ventral mesenchyme, proximal regions did not show any obvious alterations. In classical embryological experiments, recombinations of ectoderm and mesoderm in which ectoderm is rotated at stage 22, show no alteration in proximal ventral regions. This might be due to the fact that proximo-ventral cells are already committed to a ventral cell fate (MacCabe et al 1974). Alternatively, other factors (like En-1 and its unknown downstream targets) also may play a predominant role in mediating dorso-ventral patterning in proximal limb (Riddle et al 1995).

In order to test whether the normal role of *Lmx-1* is to control dorsal pattern in the limb, *Lmx-1* function was inhibited in chick embryos. Chick embryos were infected with retrovirus encoding a chimaeric fusion construct between the repressor domain of Engrailed and the homeodomain of *Lmx-1*. The chimaeric protein when in excess would compete for and repress genes activated by *Lmx-1*. In such chimaeric infected limbs, in which *Lmx-1* activity is competitively inhibited, the overall result was a lack of dorsal structures and occasionally additional ventral structures on the dorsal side. Infected wings had reduced feather density on the dorsal side, the limb was generally straighter and distal tip of digit 2 was missing. In the legs, the large scales/scuta typical of the dorsal side were missing and instead tuberculae-like structures typical of ventral side were present. There was also reduction in claws and digits were more cylindrical than in normal chick legs. Ventral muscles and tendons were normal but dorsal muscles were smaller or absent especially that of digit 4 (interosseus dorsalis) and digit 3 (extensor medius brevis). Sometimes flexor indicis, a ventral muscle of digit 2, was present on the dorsal side. In these infected limbs, the expression pattern of distribution of transcripts of the three genes, *Wnt-7a* in dorsal ectoderm, *Lmx-1* in dorsal mesoderm and En-1 in ventral ectoderm known to be involved in dorso-ventral patterning was normal. These

experiments indicate that *Lmx-1* is necessary to define dorsal cell fate since inhibition results in limbs lacking dorsal-specific structures (Rodriguez-Esteban et al 1998).

The phenotype in mice resulting from targeted disruption of *Lmx-1b*, the mammalian homolog of *Lmx-1* in chicken, also shows that *Lmx-1b* is essential for specification of dorsal limb fate at both zeugopodial and autopodial level (Chen et al 1998). Homozygous mutant limbs lack dorsal hair follicles and nails are absent or hypoplastic. Footpads but not eccrine glands were duplicated dorsally. Ventral flexure seen in wild type digits was absent. Sections revealed mirror-symmetry of mesodermal derivatives, ventral tendons and ventral sesamoid bones were duplicated dorsally. At level of the metatarsus, ventral muscles were also duplicated. The dorsal-to-ventral re-structuring of limb extends from the tip to at least to the level of distal zeugopod. In the forelimb, distal ulna was absent, and patella was lost in the leg. Hence it was suggested that *Lmx-1b* might be required for an early step in cartilage morphogenesis. However there was no loss of posterior digits as seen in the *Wnt-7a* mutant. These features are similar to that seen in the dominantly inherited skeletal malformation Nail Patella Syndrome (NPS) seen in humans (Dreyer et al 1998). *Lmx-1b* maps to the NPS locus, and patients affected by Nail Patella Syndrome have hypoplastic nails, hypoplastic/absent patella and joint abnormalities with contractures (Jones 1997). As there were skeletal abnormalities in the mouse *Lmx-1b*-/- and in the human NPS patients it was suggested that *Lmx-1b* may have similar a role in mouse and human skeletal morphogenesis and in patterning of nails (Chen et al 1998; Dreyer et al 1998). The human phenotype is found with a heterozygous deficiency and resembles a milder variant of the homozygous phenotype in mice, whereas the heterozygous mice are phenotypically normal. This was suggested to be due to a difference in dosage sensitivity for this gene in the two species or a dominant-negative effect in the human condition.

Engrailed-1 (*En-1*), a homeobox-containing gene, is present throughout the ventral limb ectoderm in early stages in mouse (Davis et al 1991) and chick (Davis et al 1991; Gardner and Barald 1992). Mutational analysis has shown that *En-1* is involved in patterning along the dorso-ventral axis (Wurst et al 1994; Loomis et al 1996). Loss of *En-1* function in mice results in transformation of ventral paw structures to a more dorsal phenotype and, in the homozygous mouse mutant (Loomis et al 1996) *Wnt-7a* is expressed ventrally. In both *Wnt-7a* and *En-1* loss of function mutants, tendons showed morphological dorso-ventral transformation.

The general aim of this chapter is to understand how dorso-ventral patterning is accomplished with particular reference to tendons. As tendon pattern in the digits of the chick leg show clear dorso-ventral differences and as *EphA4* is expressed in tendons during early development, I examined in detail the relationship between expression of dorso-ventral patterning molecules, *Wnt-7a* and *Lmx-1* and expression of *EphA4* in developing tendons. Furthermore *EphA4* expression highlights the differences in shape of dorsal and ventral tendons; dorsal tendons are disc-like while ventral tendons are round. As tendon development starts around stage 29, I have concentrated on studying later stages of the chick leg bud in detail. *Wnt-7a* mutant mice in which, *Wnt-7a* is functionally inactivated, have double ventral pattern of tendons. Hence I analysed the expression pattern of *EphA4* in those limbs to understand how ectodermal signalling is translated into dorso-ventral patterning of tendons. It is not clear whether *Wnt-7a* acts directly on developing tendons or indirectly via *Lmx-1*. Therefore I misexpressed *Lmx-1* in ventral mesenchyme of chick limb buds to study the role of *Lmx-1* in dorso-ventral patterning of tendons.

5.2 Materials and Methods

5.2.1 Infection with virus

RCAS-*Lmx-1* virus at titre of $10^{6/7}$ (kind gift from M. Ensini) was injected at several sites into the presumptive hind limb mesoderm at stage 10-11 using a micropippette and General Valve Picospritzer II. For easy visualisation of the virus during injection, fast green (0.2%) was added to the virus stock before injection. Embryos were incubated and then fixed in 4% PFA at different periods after injection for whole mount RNA in situ hybridisation with anti-sense *EphA4* probe. After the whole mounts were photographed, they were cryosectioned to study the pattern of expression of *EphA4* in dorsal and ventral tendons.

5.2.2 Staining with antibody to *Lmx-1*

Embryos are fixed and cryosectioned as per section 2.2b and 2.8 respectively. Bring cryosections to room temperature. Wash in 1x PBS for 5 minutes. Add 1:40 dilution monoclonal antibody 50.5A5 (gift from M. Ensini) to which is added 1% heat inactivated goat serum, 0.1% triton and 1x PBS and keep at –4°C overnight. Wash a couple of times in 1x PBS. Add secondary antibody at 1:100 dilution (antimouse IgG FITC conjugate Sigma) to which is added added 1% heat inactivated goat serum, 0.1%

triton and 1x PBS and incubate in dark for one hour at room temperature. Wash in 1x PBS and mount using DABCO/glycerol. Photographed with flourescence microscope.

5.2.3 In situ hybridisation

Chick *EphA4* cDNA plasmid, was linearised with Not1, and transcribed with T3 as in section 2.5.2. Mouse *EphA4* and whole mounts shown in Fig. 5.1d-g, were performed by K.Patel. *Wnt-7a* plasmid DNA was linearised with HindIII, *Lmx-1* with Not1 and transcribed with T3 and T7 RNA polymerase respectively as described in section 2.3.2.

5.2.4 Histology

Limbs harvested at stage 36 and later were fixed in Formol saline as per section 2.2, embedded in paraffin wax, sectioned and stained with Mallory's trichrome as per section 2.3.

5.3 Results

5.3.1 Expression profile of Wnt-7a and Lmx-1 in relation to tendon development in chick limbs

5.3.1.1 Expression of *Wnt-7a* in late stages of limb development

In early chick limb development at stage 20, *Wnt-7a* expression is almost homogenous in the dorsal ectoderm but transcripts are not present in the apical ridge (Fig. 5.1a, 5.2a). Between stage 22-25, expression becomes patchy with a reduction in transcripts in proximal dorsal ectoderm but higher expression distally with highest expression anteriorly (Fig. 5.1b). By stage 27, there is very little expression proximally while, in distal dorsal ectoderm there is strong expression. In the region, where the posterior cartilage ray is developing, there seems to be some reduction with strong expression along the anterior and posterior edges of the developing digit (Fig. 5.1c). Sections reveal that distally the *Wnt-7a* expressing epithelium is thickened (5. 5.2b) whereas proximally, the *Wnt-7a* expressing epithelium is thinner (Fig. 5.2c and d). Nearer the ridge both anteriorly and posteriorly, *Wnt-7a* expressing epithelium is thicker still (Fig. 5.2b). At the junction of ridge and non-ridge ectoderm, the latter is very thick (Fig. 5.2b, c and inset). This thickening is not seen at the junction of the dorsal ectoderm and ridge at the distal tip at stage 20 (Fig. 5.2a). In proximal regions of limb buds from stage 27 embryos, it also seems as if the expression is in the region of the basement membrane underlying the ridge (Fig. 5.2c and inset). At stage 29, there is

Figure 5.1. Expression pattern of *Wnt-7a* in chick leg buds

All are dorsal view except a), which is tilted.

a) Stage 20. *Wnt-7a* is expressed strongly on dorsal surface (d), no expression in apical ridge (r), ventral side (v).

b) Stage 22. Distal limb bud ectoderm has more expression than proximal with highest levels of *Wnt-7a* transcripts at the anterior edge (arrowhead) of the bud.

c) Stage 27. Patch of *Wnt-7a* expression proximally (arrow), expression also strong distally. In region of developing posterior digit (black arrowhead), there is reduced expression; but anterior (white arrowhead) and posterior to cartilage forming region, expression is higher.

d) Stage 29. Proximally less *Wnt-7a* expression (white arrow); strong expression distally over interdigital region (black arrowhead); weaker over the digit (white arrowhead) and still weaker distal to the digits (black arrow).

e) Stage 30. Proximally low *Wnt-7a* expression (white arrow); distally strong expression over interdigital (black arrows) and regions flanking the developing cartilage. Low expression distal to digit (black arrowhead) and over distal interdigit (white arrowhead).

f) Stage 31. *Wnt-7a* expression over proximal regions of leg is weak (arrowhead) and almost absent over the digits.

g) Stage 34. Proximally there is some *Wnt-7a* expression (arrowhead).

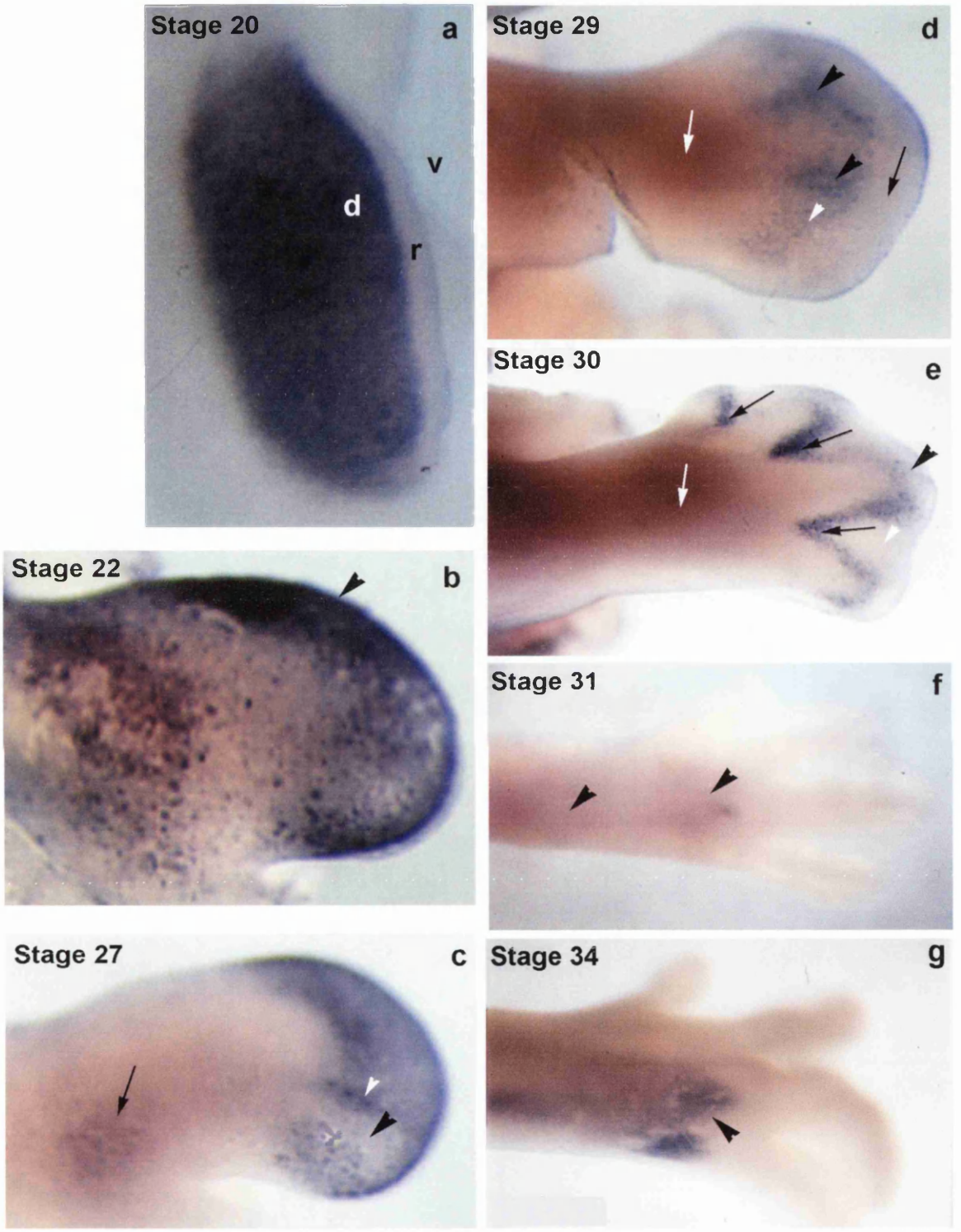

Wnt- 7a
Stage 20
a
v
d
r
Stage 29
d
Stage 30
e
Stage 22
b
Stage 31
f
Stage 27
c
Stage 34
g

Figure 5.2. Sections showing localisation of Wnt 7a transcripts in dorsal ectoderm of chick leg buds.

a) Stage 20 Longitudinal section; b)-d) are transverse sections of stage 27 leg from distal to proximal; e)-h) are transverse sections of stage 29 leg from distal to proximal. Dorsal to top and anterior to left.

a) *Wnt-7a* expression in dorsal ectoderm, (arrow); no expression in AER (arrowhead).

b) Apical ridge, (arrowhead); arrows, very thick layer of expression in dorsal ectoderm, anteriorly and posteriorly (arrows).

b′) and b″) are magnified anterior and posterior ridge regions of b); arrowhead indicates thickest region of expression.

c) Section proximal to that in b). Apical ridge, (arrowheads); expression in dorsal ectoderm (arrows).

c′) and c″) are magnified anterior and posterior ridge region with arrowhead indicating expression in dorsal ectoderm along basement membrane of apical ridge.

d) Section proximal to c). Expression is weak in the proximal part of limb bud (arrowheads). It appears that ridge is not present at this level.

e)-h) Sections from distal to proximal showing stronger expression distally and weaker expression proximally. Proximally expression in interdigit region (arrow) is thicker and stronger as seen in h) and enlarged in h′). Arrowhead indicates digital region.

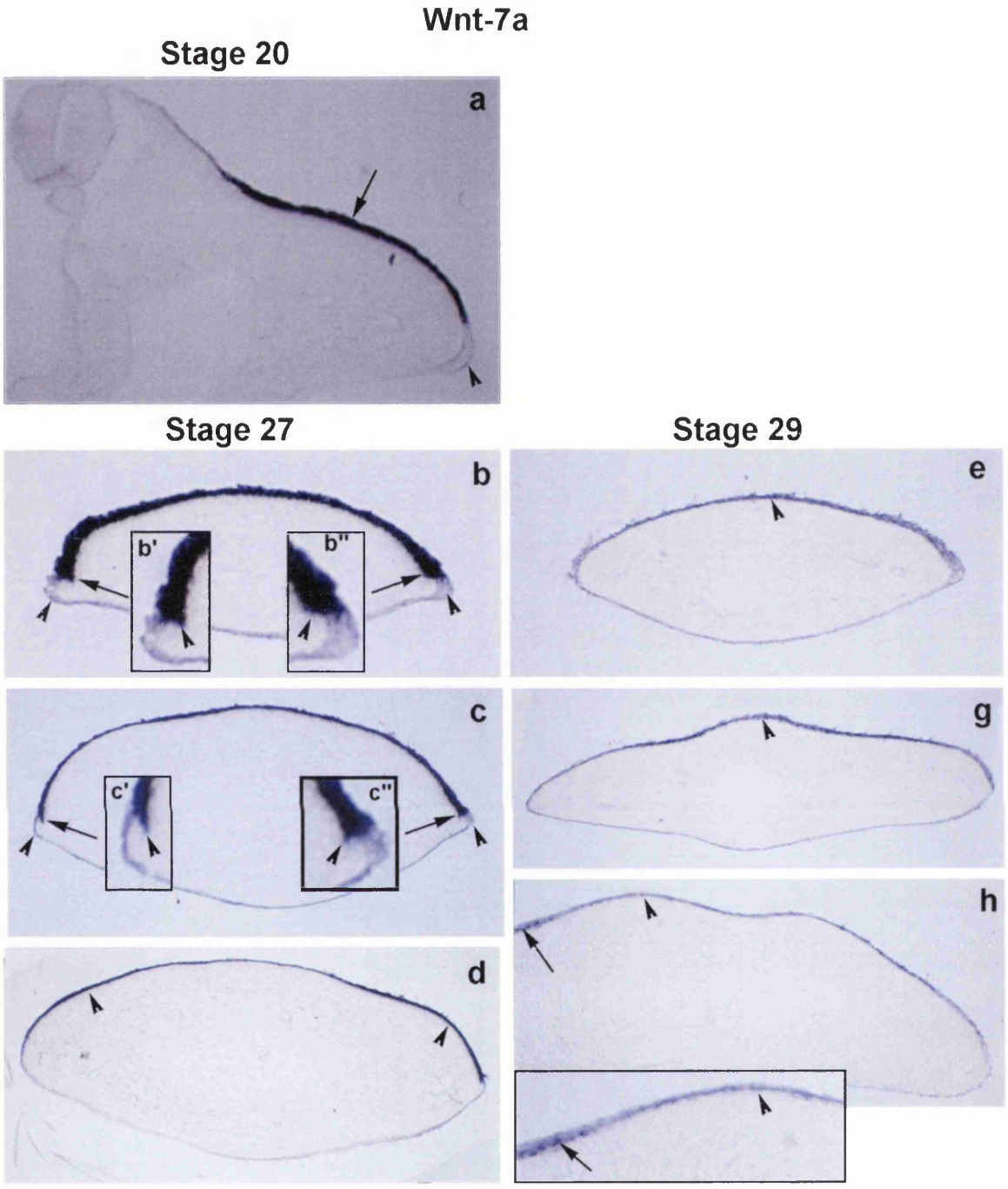

Wnt-7a
Stage 20
a
Stage 27
Stage 29
b
b'
b''
e
c
c'
c''
g
d
h

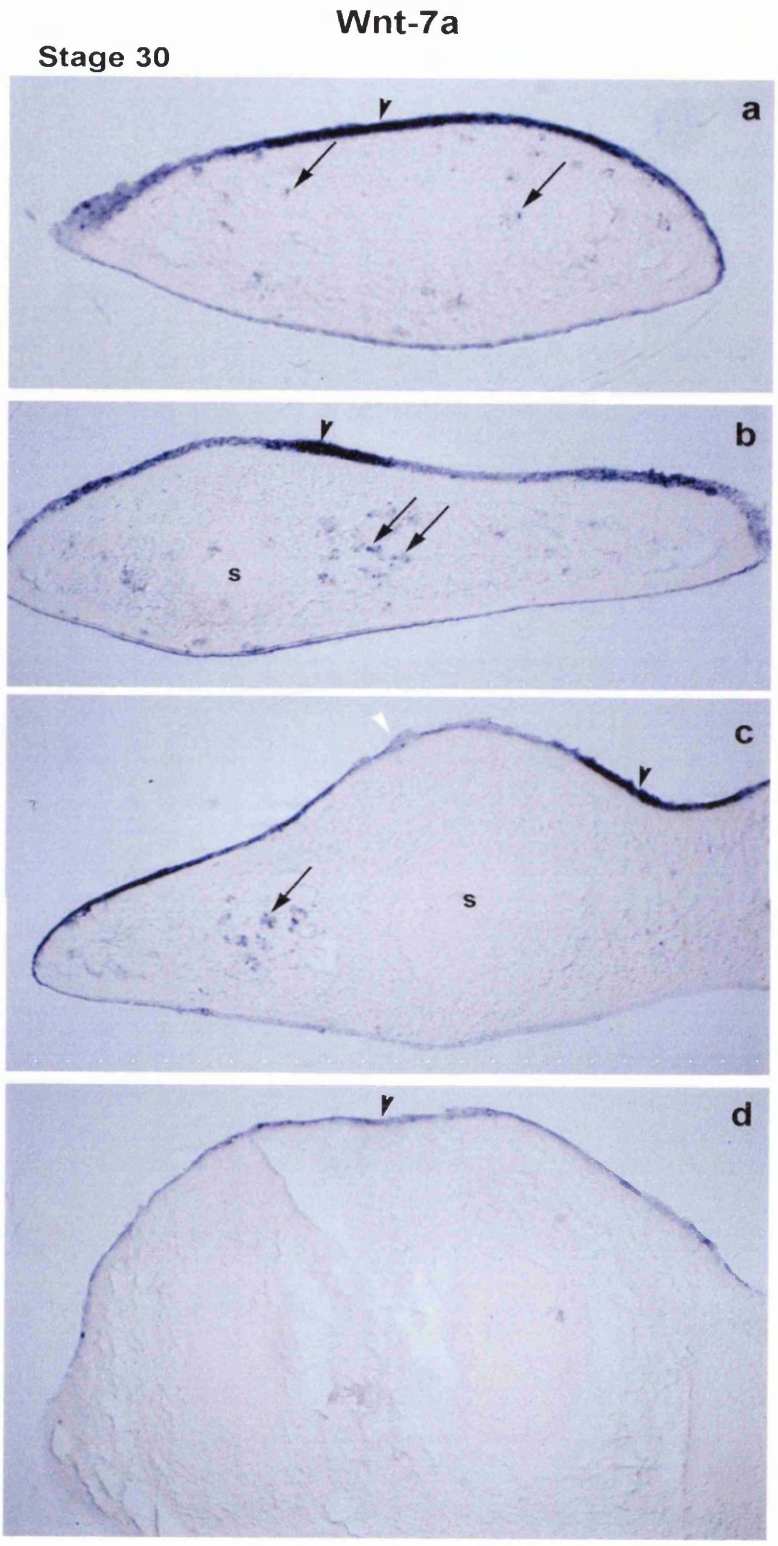
Figure 5.3 Expression pattern of Wnt-7a in transverse sections of chick leg at stage 30

a) Thick layer of expression in dorsal ectoderm, (arrowhead); sparsely scattered cells expressing *Wnt-7a* in the mesenchyme (arrow).

b) Section proximal to a). Stronger area of *Wnt-7a* expression in ectoderm nearer interdigit region, (arrowhead); digital cartilage (s); expressing cells in the mesenchyme (arrows).

c) Section proximal to b). Strong *Wnt-7a* expression in ectoderm overlying interdigit region, (black arrowhead); weak expression in ectoderm (white arrowhead) overlying digital cartilage (s); expression in mesenchyme (arrow).

d) Section proximal to c). Weak *Wnt-7a* expression in ectoderm (arrowhead). No expression in mesenchyme.


Wnt-7a
Stage 30
a
b
s
c
s
d

much less *Wnt-7a* expression especially proximally (Fig. 5.1d, 5.2h) with relatively higher expression distally (Fig. 5.2e) and in the interdigital regions (Fig. 5.2g, h, h').

At stage 30, higher levels of *Wnt-7a* transcripts are present in the digital plate and lower levels of expression proximally (Fig. 5.1e, 5.3d). Expression in the digital plate is weak over the cartilage rays except over the tip of the growing digit (Fig. 5.1e, 5.3a). Expression is stronger in the interdigital region, and appears to flank the sides of the digits (Fig. 5.1e). However sections reveal that there appear to be cells expressing *Wnt-7a* deep within the mesoderm in the interdigital region (Fig. 5.1e, 5.3a-c). Proximal to the interdigital region *Wnt-7a* is expressed only in dorsal ectoderm (Fig. 5.3d) and no expressing cells are seen in the mesoderm. In stage 31-34 limbs, there is weak expression in proximal region of the limb (Fig. 5.1f, g).

This analysis shows that *Wnt-7a* is expressed in dorsal ectoderm in early limb bud, but as limb grows out expression is reduced proximally. Between stages 28-30, when digits develop, less expression is seen in dorsal ectoderm overlying the digit and more on the sides. Surprisingly, some expression is also seen in the interdigital mesenchyme.

5.3.1.2 Spatial and temporal relation of expression of *Lmx1* to tendon development

When *Lmx-1* is misexpressed in ventral mesenchyme, there are ventral to dorsal transformations (Riddle et al 1995; Vogel et al 1995). Hence I compared the expression of *Lmx1* in mesenchyme with that of *EphA4*, a marker of early tendon development (Patel et al 1996) to try and understand how expression pattern of *Lmx-1* could be related to dorsal tendon development.

Spatial and temporal expression pattern of *Lmx1* is dynamic at least in relation to developing tendon. Initially, at stage 27-29, *Lmx-1* is expressed throughout the dorsal mesoderm and encompasses cells that will form tendon. At stage 27 (Fig. 5.4f), *Lmx1* appears to be expressed more or less throughout the dorsal limb bud, with a slight reduction near the tip and anterior margin of the digital plate (Fig. 5.4f). Sections show that *Lmx-1* transcripts are distributed in distal bud mesenchyme under the dorsal ectoderm (Fig. 5.5c). However, proximally, expression is reduced and surrounds an area in which muscle appears to be present (Fig. 5.5d). Anteriorly, posteriorly and dorsally expression of *Lmx1* overlaps that of *EphA4* (Fig. 5.5a,b).

At stage 28, *Lmx1* is reduced proximally but, over the digital plate, there is strong expression except at the anterior edge and tip (Fig. 5.4g). Weak expression is also present in the interdigital region (Fig. 5.4g asterisk). Proximal to the digital plate, there is expression along anterior and posterior margins of the but in between this area there

Figure 5.4. Comparison of expression pattern of *Lmx1* with that of *EphA4* in whole mounts of chick legs at different stages in development

Limbs used here are taken from the same embryo and probed with either *Lmx-1* or *EphA4*.

Whole mounts are viewed from dorsal side. Planes indicate the level at which sections are shown in subsequent figures with numbers referring to each figure.

a) Strong expression of *EphA4* in digit plate at stage 27.
b) Expression of *EphA4* in developing dorsal tendons over digits 2, 3 and 4 at stage 28.
c) Expression of *EphA4* in dorsal tendons of digits 1, 2, 3 and 4 at stage 30
d) Expression of *EphA4* in dorsal tendons and in inter-phalangeal joint regions at stage 33.
e) Expression of *EphA4* is weak in inter phalangeal joint region at stage 36.
f) Strong expression of *Lmx1* in a chick leg at stage 27 throughout the dorsal aspect of the limb except anteriorly (arrow) and at the tip (arrowhead) of limb where expression is weak.
g) Strong expression of *Lmx1* over the digit plate at stage 28/29 except at anterior edge (arrow), tip (arrowhead), and interdigital region (asterisk). Proximally strong expression at anterior (white arrowhead) and posterior (white arrow) edges; a longitudinal band of expression separates 2 areas of weak expression (m) where cartilage of tibia and fibula are developing.
h) *Lmx-1* expression over digit plate and proximal leg at stage 30 is now less strong. Reduced expression over central regions of digits3 and 4 but flanked by strong expression; no expression at distal interdigit but weak expression in proximal interdigital region (asterisk).
i) Expression has become weaker over central region of the digits, but expression still flanks the sides of the digits. Tips of the digits (arrow) do not express *Lmx-1*.
j) Expression of *Lmx1* is reduced in stage 36 leg.

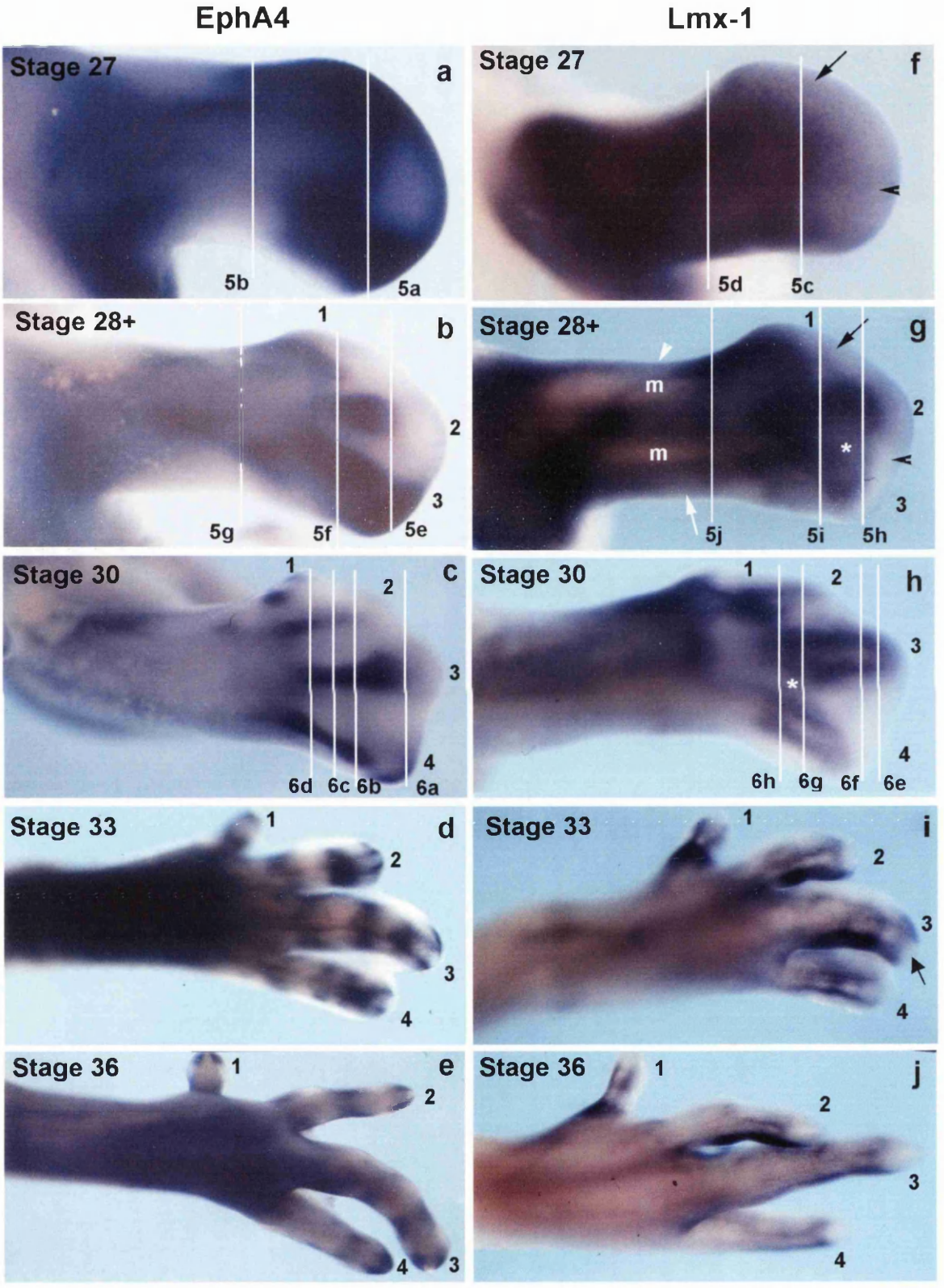

EphA4
Lmx-1
Stage 27
a
5b
5a
Stage 27
f
5d
5c
Stage 28+
b
1
2
3
5g
5f
5e
Stage 28+
g
1
m
m
2
*
3
5j
5i
5h
Stage 30
c
1
2
3
4
6d
6c
6b
6a
Stage 30
h
1
2
3
*
4
6h
6g
6f
6e
Stage 33
d
1
2
3
4
Stage 33
i
1
2
3
4
Stage 36
e
1
2
4
3
Stage 36
j
1
2
3
4

Figure 5.5. Comparison of expression of *EphA4* and *Lmx1* in tendons of developing chick legs

Sections are taken from limbs shown in Fig 5.4 a) and f) for stage 27, b) and g) for stage 28. Dorsal to top and anterior to left.

a)-d) are transverse sections of stage 27 legs; e)-j) are transverse sections of stage 28 legs.

a), b) and e)-g) show expression of *EphA4*; c), d) and h)-j) show expression of *Lmx1*.

a) Section through plane x. *EphA4* expression at anterior (arrowhead); expression at posterior (arrow); weak dorsal and ventral expression is also seen.

b) Section proximal to a). *EphA4* expression at anterior edge of limb (arrowhead); area of muscle with low expression (asterisk) and higher expression surrounding it.

c) Expression of *Lmx1* in dorsal mesenchyme (arrowhead) overlaps the area of expression of *EphA4* (compare with Fig 5a)

d) Expression of *Lmx-1* in the dorsal mesenchyme excludes and surrounds the dorsal muscle area (asterisk) and overlaps the expression domain of EphA4.

e) Faint expression of *EphA4* (arrowhead) in dorsal mesenchyme, but strong posteriorly.

f) *EphA4* expression in dorsal tendon blastemas (arrowheads) with strong expression posteriorly.

g) *EphA4* expression at anterior edge (arrowhead) of base of digit plate and posteriorly.

h) Expression of *Lmx-1* in dorsal mesenchyme (arrowhead), stronger nearer the ectoderm than it is deeper and overlaps *EphA4* expression domain seen in (e).

i) *Lmx-1* expression in dorsal mesenchyme (arrowhead), stronger near ectoderm and weaker deeper in mesenchyme and overlaps area of *EphA4* expression in developing tendon as seen in (f), and non tendon areas.

j) Proximal to the digital plate. *Lmx-1* expression is reduced in the anterior muscle mass (asterisk) and posterior muscle mass (arrow) but stronger expression in surrounding mesenchyme. There is overlap of the EphA4 expression domain seen in (g).

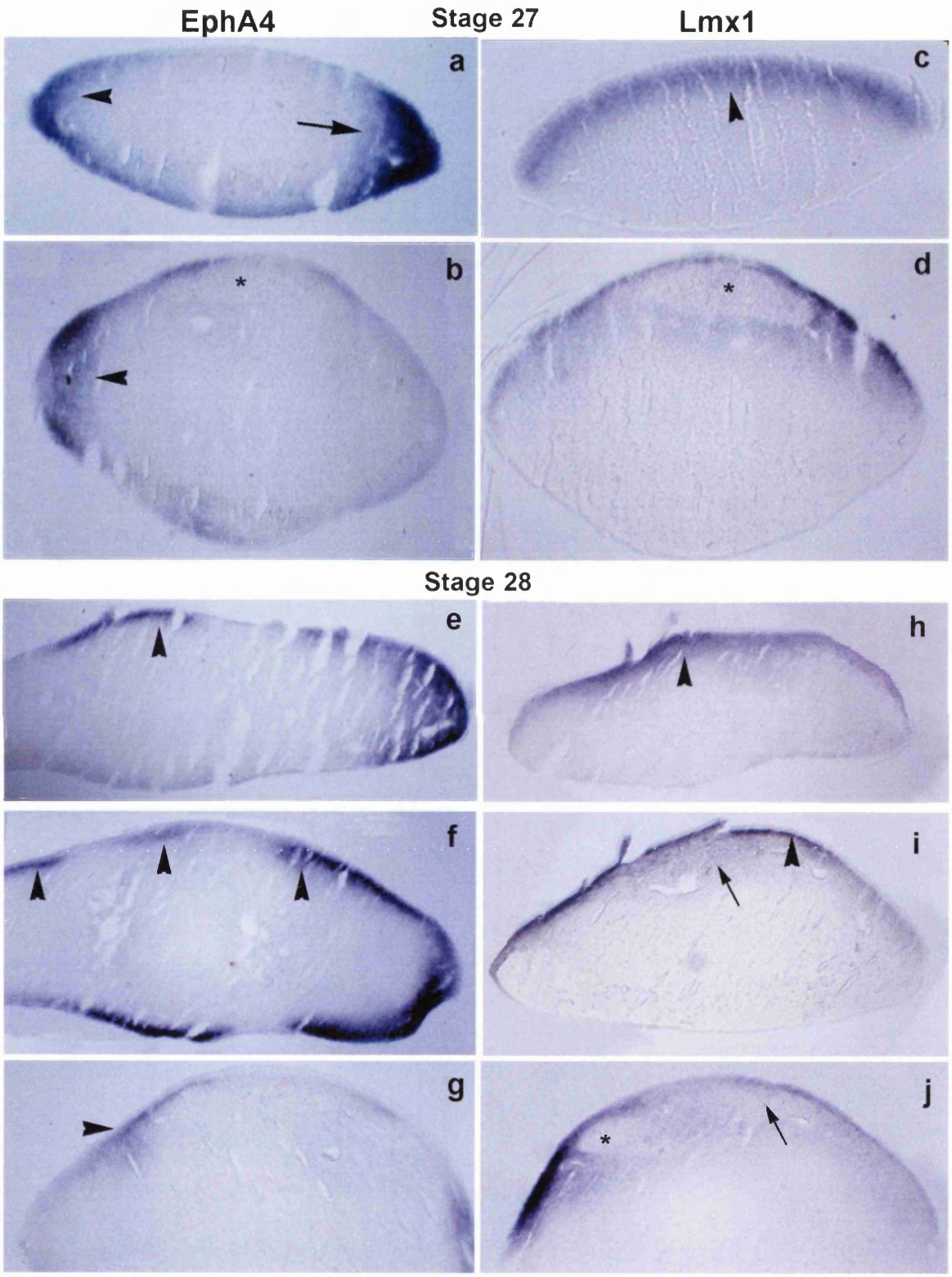

EphA4
Stage 27
Lmx1
a
c
b
d
Stage 28
e
h
f
i
g
j

Figure 5.6 Expression of *Lmx1* in relation to that of *EphA4* in late tendon development in chick legs

Sections are taken through limbs shown in Fig 5.4 c and h in the planes indicated.

a)-d) show expression of *EphA4*, e)-h) show expression of *Lmx1*.

a) Expression of *EphA4* in developing dorsal tendon of digit 3, subjacent to ectoderm.

a') magnified picture of expression of EphA4 (arrowhead) in dorsal tendon subjacent to ectoderm

b) Expression in dorsal tendon (arrowhead) which has moved away from ectoderm.

c) Expression of *EphA4* in dorsal tendon (arrowhead) has moved further from ectoderm.

c') magnified picture of expression of *EphA4* (black arrowhead) in tendon that has moved away from ectoderm (white arrowhead).

d) Expression of *EphA4* in tendon (arrowhead) proximal to that seen in c); white arrowhead, ectoderm.

e) Expression of *Lmx1* is reduced in area of forming tendon subjacent to ectoderm (arrowhead), but a thin layer of weak *Lmx-1* expression is present immediately deep to ectoderm. Expression on the sides of the developing tendon (arrows) is strong and is sloping down the sides of the digit.

e') magnified image of *Lmx1* expression in tendon area (arrowhead); expression on sides tendon (arrows).

f) Expression of *Lmx1* is strong in mesenchyme (arrow) between developing tendon and ectoderm where *EphA4* is weakly expressed. Tendon (arrowhead) itself does not express *Lmx-1*.

g) g') and h) indicate that as tendon (black arrowhead) which does express *Lmx-1* moves away from ectoderm, expression of *Lmx1* (black arrow) becomes more stronger in the mesenchyme between the tendon and the ectoderm; expression of *Lmx1* over digits 4 and 2 (white arrows).

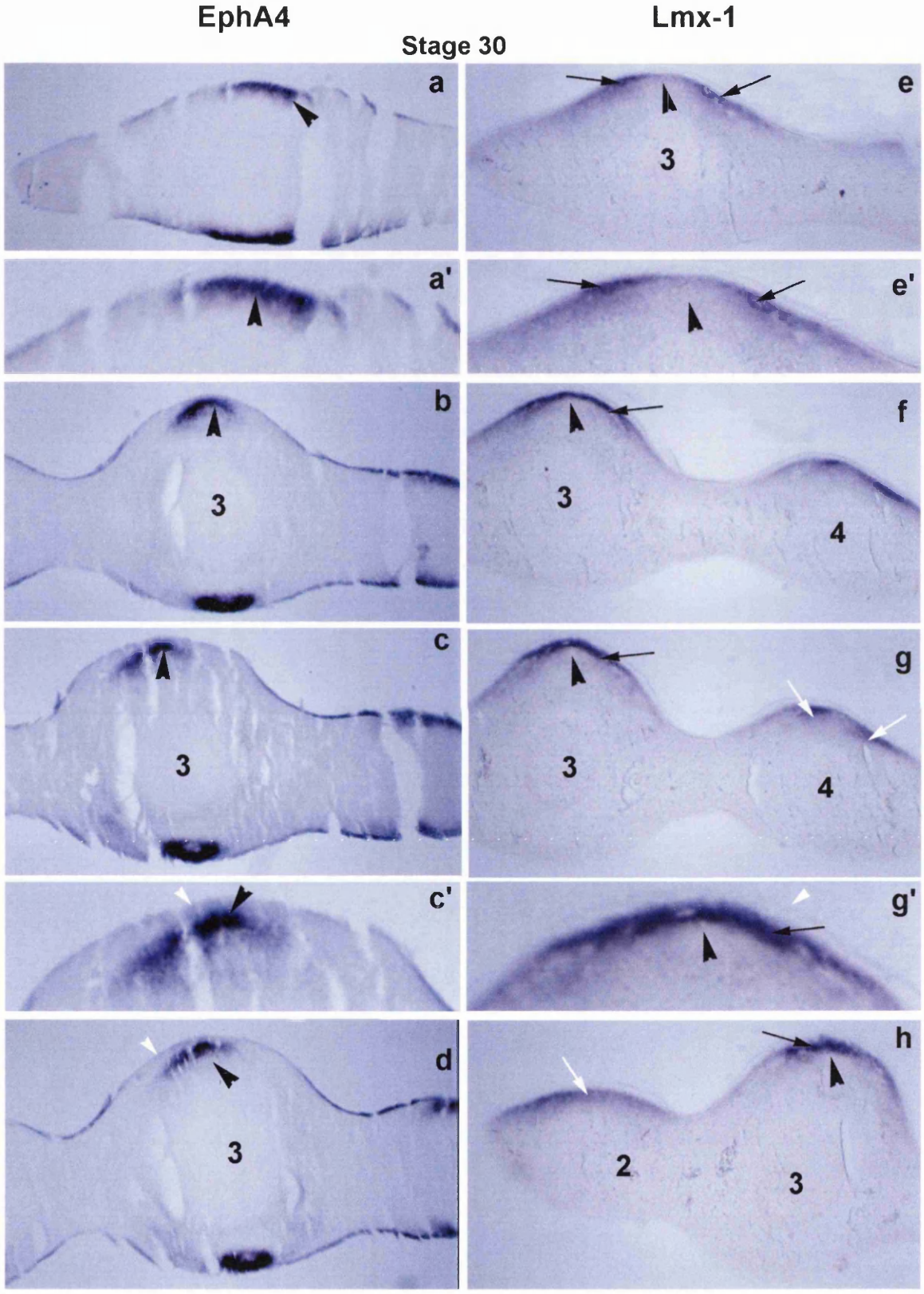
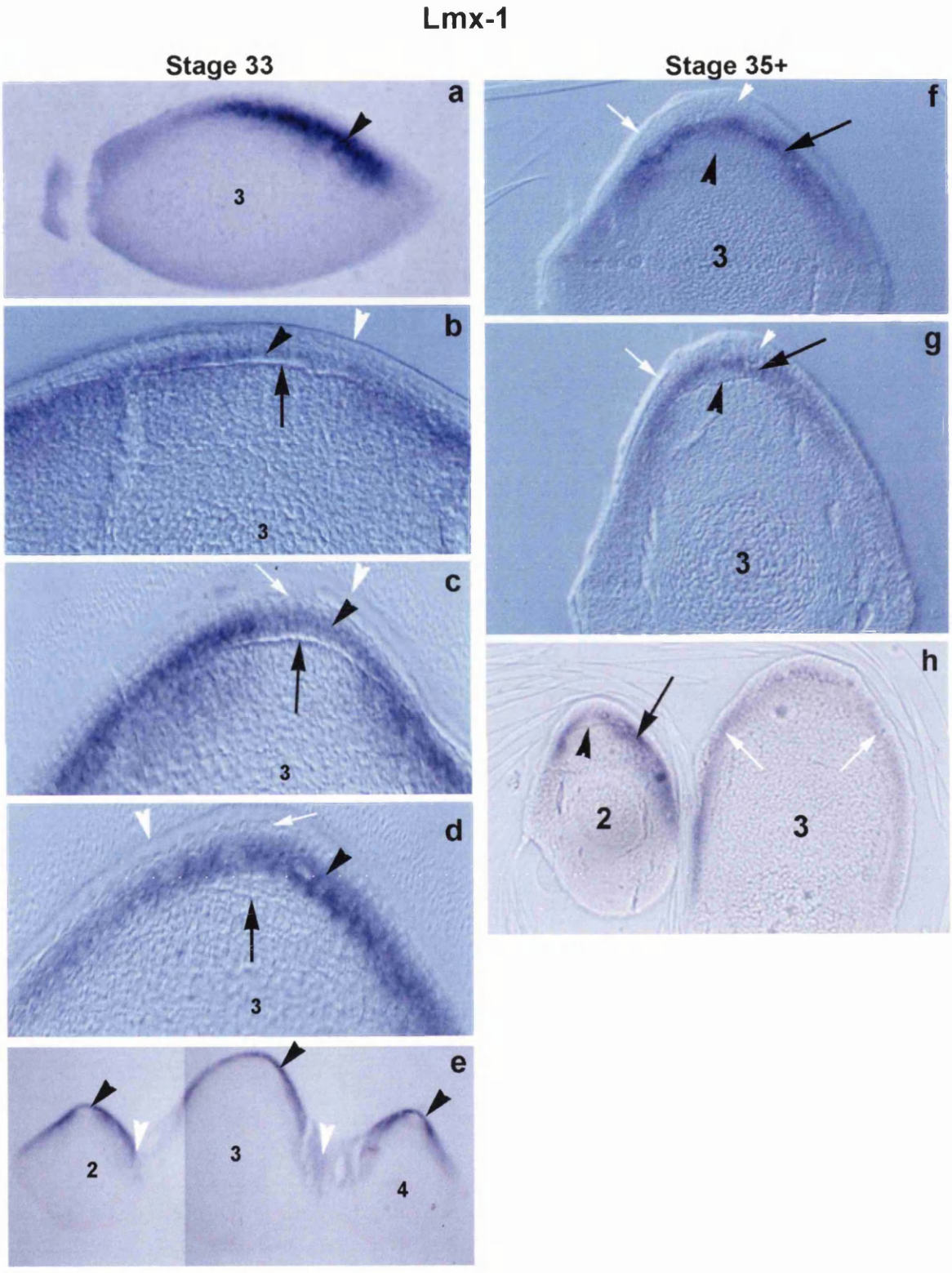

EphA4
Lmx-1
Stage 30
a
e
3
a'
e'
b
f
3
4
c
g
3
4
c'
g'
d
h
3
2
3

Figure 5.7. Expression of *Lmx1* at stage 33-35 in relation to tendon development in chick leg

a-e are from stage 33; f-h are from stage 35+

Expression in mesenchyme (arrowhead).

a) Strong expression (arrowhead) near tip of digit
b) When tendon/mesenchymal lamina (arrow) is close to ectoderm (white arrowhead) there is less expression in tendon cells; a thin layer of expression ((black arrowhead) between tendon and ectoderm
c) Mesenchymal lamina (black arrow); expression in mesenchyme (black arrowhead) between mesenchymal lamina and ectoderm (white arrowhead); some mesenchymal cells that are close to ectoderm have less expression (white arrow).
d) Mesenchymal lamina (black arrow); thick layer of expression (black arrowhead) in between mesenchymal lamina and ectoderm (white arrowhead); Notice that dorsal most mesenchymal cells (white arrow) do not express *Lmx-1*.
e) Proximal section. Expression in dorsal mesenchyme (black arrowheads); expression in interdigit region (white arrowheads).
f) Tendon (black arrowhead); expression of *Lmx1* (black arrow); area of mesenchyme not expressing *Lmx1* (white arrowhead) just under the ectoderm (white arrow).
g) Mesenchymal lamina (black arrowhead), the edges of which are within cells that express *Lmx-1* (black arrow); ectoderm (white arrow); dorsal mesenchymal cells close to ectoderm (white arrowhead) do not express *Lmx-1*.
h) Proximally *Lmx-1* expression remains on the sides of digit 3 (white arrowheads) while expression in digit 2 (arrow) is also between mesenchymal lamina (arrowhead) and the ectoderm.


Lmx-1
Stage 33
Stage 35+
a
3
b
3
c
3
d
3
e
2
3
4
f
3
g
3
h
2
3

is a long band of expression separating two areas of low expression. At this stage, *EphA4* is expressed along the digit rays (Fig. 5.4b) and is within the domain of *Lmx-1* expression. Sections confirm that distal expression of *Lmx-1* in the dorsal mesoderm overlaps the expression domain of *EphA4* at all levels in the digital plate even in regions where tendon is differentiating in the sub-ectodermal mesenchyme (compare Fig. 5.5h, i, j with Fig. 5.5e, f, g). Proximal expression of *Lmx-1* (Fig. 5.5j) is very weak (Fig. 5.5j arrow) or even absent (Fig. 5.5j aster) where muscle is differentiating, with some overlap with EphA4 expression in non-muscle region (Fig. 5.5g arrowhead).

From stage 30 onwards, there is low expression of *Lmx-1* in the tendon as it lies subjacent to the ectoderm. But when tendon and mesenchymal lamina have moved deeper into the mesoderm, expression is either weak or absent in tendon but strong in the mesoderm intervening between the developing tendon and the ectoderm. In whole mounts, expression of *Lmx-1* appears to outline the digits with lower expression in the interdigital region (Fig. 5.4h). Sections confirm that expression is reduced over the dorsal surface of the digital cartilage (Fig. 5.6e) in the region where *EphA4* is expressed in tendon (compare Fig. 5.6a, a'with Fig. 5.6e,e'). However there is higher expression on either side of tendon underneath the ectoderm (Fig. 5.6e, e'arrows) as it lies subjacent to the ectoderm (Fig. 5.6a arrowhead). More proximally, where tendons have moved away from ectoderm (Fig. 5.6b, c), *Lmx1* is expressed in the mesoderm intervening between the *EphA4* expressing dorsal tendon and the ectoderm. *Lmx-1* is expressed along the slope of the dorsal surface of the digits diminishing towards the interdigital region (Fig. 5.6f, g, h). There maybe some *Lmx-1* expression in the dorsal-most cells of the developing tendon and perhaps a low-grade expression of *Lmx1* throughout tendon. It appears as though domains of *EphA4* and *Lmx-1* are separate but may overlap at the edges. This suggests that at least some cells co-express both genes. Nevertheless, it appears that *Lmx-1* expression is reduced as tendon starts to develop.

At stage 33/34, expression of *Lmx-1* has become further reduced in the midline of the dorsal surface of digits (Fig. 5.4i). The tip of the digit has a thick layer of *Lmx-1* expressing cells in the mesoderm which maybe associated with the developing nail bed (Fig. 5.7a). *Lmx-1* is expressed distally in tendon cells both on outer and inner faces of the mesenchymal lamina, which is separating from the basement membrane (Fig. 5.7b). Where the mesenchymal lamina and associated tendon has moved deeper into the mesoderm (Fig. 5.7c, d), a thicker layer of *Lmx-1* expression intervenes between ectoderm and mesenchymal lamina although it appears that some of the mesenchymal cells that are in close contact with the basement membrane are not expressing *Lmx-1*

(Fig. 5.7c, d). Interdigital mesenchymal cells express of *Lmx-1* at low levels (Fig. 5.7e). At stage 35/36 expression of *Lmx-1* is much reduced(Fig. 5.7j). *Lmx1* is now not expressed in cells that are just under the ectoderm but expression seems to be hugging the dorsal surface of the developing tendon which has moved away from ectoderm (Fig. 5.7f, g). More proximally, *Lmx-1* is still expressed on the sides of the separating digits (Fig. 5.7h).

This analysis shows that at first *Lmx-1* is expressed in dorsal mesenchymal cells and in cells that will form tendon in the sub-ectodermal mesenchyme. Later as developing tendons start to move away from sub-ectodemal sites, expression of *Lmx-1* becomes reduced and is then lost in the tendon. However *Lmx-1* expression is present in cells that surround the tendon dorsally and on the sides.

5.3.2 Expression of *EphA4* in tendons of wild type and *Wnt-7a* mutant mouse

Parr and McMahon (1995) showed that tendons were morphologically bi-ventral in mice at day 15.5 in which Wnt-7a is functionally inactivated. However details of differences along the entire length of the digital tendon was not reported. Here we use EphA4 to examine tendon development in limbs of Wnt-7a mutant mice.

5.3.2.1 Expression of *EphA4* in wild type mouse limbs at day 14.5

In wild type mouse limbs at 14.5 dpc, distribution of *EphA4* transcripts shows clear differences in ventral and dorsal tendons. Ventrally, there are distinct strips of expression associated with the digits -these are the ventral tendons- while dorsally there is a broad area of expression over the digits. This pattern of expression resembles that seen in the chick leg tendons (refer earlier in chapter 3). This differential expression of *EphA4* serves as a good marker to detect alteration in dorso-ventral pattern of tendons.

Wild type forelimb

Distinctive strips of cells expressing *EphA4* are seen ventrally in digits 2, 3 and 4 (Fig. 5.8a). Expression is stronger at the sides of the strips than in the centres.
Expression becomes weaker very proximally and the strips converge on a patch of *EphA4* expression at the base of the digital plate. Distal expression at the tips (Fig. 5.8a) of the strips, which are wider here compared with proximally, seems to have some continuity with expression at the digit tips (Fig.5.8a). A curved band of expression is seen along the anterior margin of digits 2, 3 and 4 (Fig. 5.8a) which seems to be slightly segregated from the tendon.

Expression of *EphA4* in dorsal tendons is different from that seen in ventral tendons. Strong expression is present over a broad area extending over the whole width of the digit (Fig. 5.8b) with 2 linear streaks of expression proximally along anterior and posterior edges of the digits (Fig. 5.8b). At the base of the digital plate, a separate patch of weak expression is present (Fig. 5.8b black/white arrow). In digits 1 and 5, the strips of expression are not clearly demarcated. Nevertheless, strong expression associated with these digits seems to be diverging from the patch of expression (Fig. 5.8a b/w arrowhead) at the base of the digital plate.

The tips of the digits are constricted and expression of *EphA4* at the digit tips is weak (Fig. 5.8a, b). Sections reveal that *EphA4* transcripts are more abundant ventrally (Fig. 5.9a and c) especially over the convex boundary of the tendon nearer the ectoderm. By this stage of development, the ventral tendon is well away from the ectoderm (Fig. 5.9c) and the intervening tissue does not express *EphA4*. Dorsally, *EphA4* transcripts are distributed in the whole thickness of the mesoderm between ectoderm and periphery of the cartilage (Fig. 5.9b) and a slightly stronger area of expression (Fig. 5.9a) appears to be the dorsal tendon. Expression extends across the whole width of the dorsal side of the digit, except in interdigital region and seems to be associated with the development of tendon insertion sites at sides of cartilage elements.

Wild type hindlimb

The hindlimb (Fig. 5.8c, d) is less developed than the forelimb and webbing is still apparent. Strips of *EphA4* expression associated with ventral tendons are clearly visible ventrally (Fig. 5.8c) similar to those seen in the forelimb. Dorsally, no strips are seen and the patch of expression at the base of the digital plate (Fig. 5.8b b/w arrowhead) is very faint. In sections, strong *EphA4* expression is associated with ventral tendons which are lying between the cartilage and the ectoderm. Expression in tendons associated with digits 3 and 4 is stronger around the periphery (Fig. 5.9a) while expression is throughout the ventral tendon of digit 2 (Fig. 5.9a). Expression also flanks the cartilage (Fig. 5.9a).

5.3.2.2 Wnt7a mutants have bi-ventral pattern of tendons at 14.5 dpc

Mutant Forelimb

Dorsal expression of *EphA4* in the forelimb of the mutant (Fig. 5.8f) is very different from that seen in the wild type (Fig. 5.8b) and resembles instead the ventral pattern of expression. Whole mounts show that digits 2, 3 and 4 have strips of

Figure 5.8. Comparison of expression pattern of *EphA4* in wild type and *Wnt-7a* mutant mice at day 14.5 of development.

a) to d) are wild type, (e) to (h) are *Wnt-7a* knockout limbs.

Anterior at the top with digits numbered 1 to 5; b/w, black and white.

a) **Ventral view of wild type forelimb**. Strips of *EphA4* expression in ventral tendon (white arrow); strong expression distally in tendon (small white arrow); curved area of expression along anterior margin of digits (white arrowhead); expression at base of digit plate (b/w arrowhead) from which tendons are diverging out into the digits; tip of the digit which seems to be constricted has weaker expression (black arrowhead).

b) **Dorsal view of wild type forelimb**. *EphA4* expression over the entire width of the digit (white arrow); 2 linear streaks of expression along anterior and posterior edges of the digit (white arrowheads); strong expression along the posterior edge of digit 5 extends proximally (black arrow); b/w arrowhead, faint expression at the base of the digital plate.

c) **Ventral view of wild type hindlimb**. Tendon (white big arrow); distal part of tendon (small white arrow) has strong *EphA4* expression; expression along the anterior edge of the digit (white arrowhead); faint expression at the base of the digital plate (b/w arrowhead) from which tendons diverge; expression at the tip of digit (black arrowhead).

d) **Dorsal view of wild type hindlimb.** *EphA4* expression over the entire width of digit (white arrow); 2 streaks of expression along the anterior and posterior edges of the digit (white arrowheads); expression at digit tip (black arrowhead).

e) **Ventral view of knockout forelimb**. Strip of *EphA4* expression in tendon of digit 2 (white arrow) is also seen in digits 1, 3 and 4 and these tendons diverge from an area of expression at the base of the digital plate (big black arrowhead); strong expression in the distal region of the tendon (small white arrowhead); 2 streaks of expression flank the anterior and posterior margins of the digits (small black arrowheads); expression at digit tip (b/w arrowhead)which is not in continuity with the domain of expression proximal to it.

f) **Dorsal view of the knockout forelimb.** *EphA4* expression seen as a strip (white arrow) in digit 2 which is also seen in digit 3; the distal part of the tendon also has strong expression (white arrowhead); 2 streaks are seen along anterior and posterior edges of the digits (black arrowheads). These 3 features may be compared with the normal pattern in dorsal wild type (b) and with its ventral pattern in (e). Expression at the tip of digit (b/w arrowhead).

g) **Ventral view of hindlimb knockout.** *EphA4* expression in tendon (white arrow), and strong expression distally in tendon (white arrowhead) are similar to that seen in the wild type; streaks of expression along anterior and posterior margins (black arrowheads); and expression at the tip of digit (b/w arrowhead) are not seen in the wild type (c).

h) **Dorsal view of knockout hindlimb.** *EphA4* expression (White arrow) similar to a ventral tendon, with strong distal expression in distal tendon (white arrowhead); 2 streaks of expression at anterior and posterior edges (black arrowheads) and expression at the tip of the digit (b/w arrowhead) are features indicating a biventral pattern.

Expression of EphA4 at day 14.5

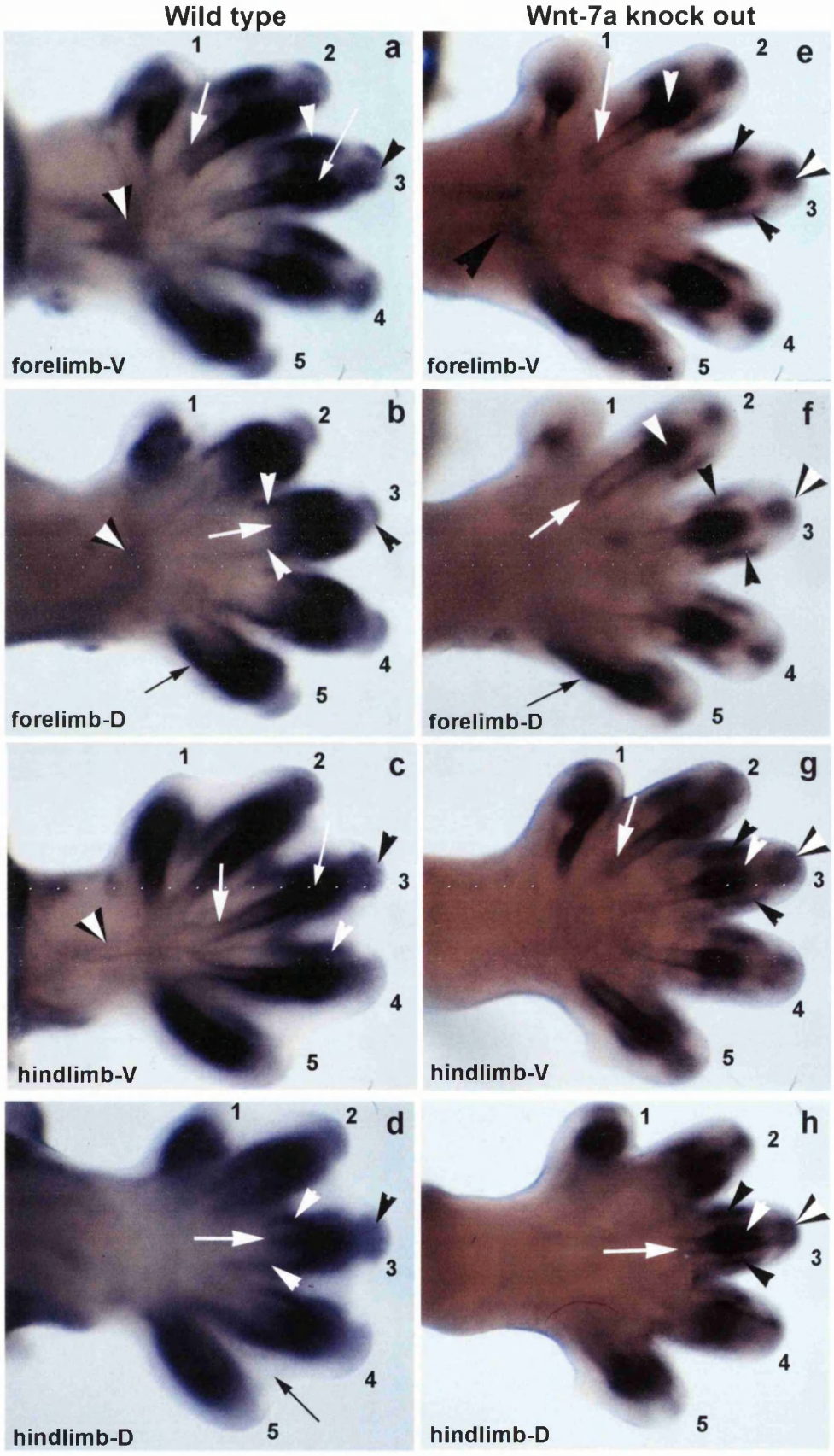


Figure 5.9. Expression of *EphA4* in transverse sections of the wild type and *Wnt-7a* knockout mouse forelimbs

Sections are from limbs shown in Fig 5.8. Dorsal is at top and anterior to left.

a) Wild type forelimb. *EphA4* expression dorsally (black arrowheads); expression along the dorsal and sides of the digit (white arrows); expression in ventral tendon (black arrow) which is some distance away from the ectoderm (white arrowhead).

b) High power magnification of dorsal aspect of digit 3 shown in (a). *EphA4* expression is seen in the whole thickness of mesoderm between edge of the cartilage (arrow) and the ectoderm, which does not express *EphA4* (arrowhead).

c) High power magnification of ventral expression in digit 3 shown in (a). Ventral tendon expression is different from that in dorsal having a convex distinct edge (black arrowheads). This edge lies at a distance from ectoderm (white arrow). Central core of tendon (black arrow) and area close to cartilage have less transcripts.

d) Knockout forelimb seen in Fig. 5.8 showing biventral pattern of *EphA4* expression in digit 3. Dorsal tendon (small black arrowhead) looks similar to the ventral tendon (black arrow). 2 arcs of expression (white arrows) flank cartilage on anterior and posterior sides. Ventral tendon and arcs of expression are away from the ectoderm (big black arrowhead). Note dorsal expression in digit 4 is different from that seen in digit 3 however the expression flanking the cartilage is somewhat similar to that in digit 3 (white arrows).

e) High power magnification of dorsal tendon in (d). Expression is more extensive in an anterior-posterior direction compared with that of the ventral tendon seen in (f). Dorsal tendon (small black arrowhead) is well away from the ectoderm (big black arrowhead) which shows expression. Core of the tendon (arrow) has fewer *EphA4* transcripts. Compare e) with b).

f) High power magnification of ventral tendon seen in (d). Edge of tendon (small black arrowhead) is away from ectoderm (big black arrowhead) which shows expression. The tendon core (arrow) has weaker expression.

g) Section more proximal to that in (d) showing that the digit 2 dorsal tendon (small black arrowhead) bears a reasonable resemblance to its ventral tendon (black arrow). Dorsal *EphA4* expression (big arrowhead) in digit 5 is not typical but mirror its ventral tendon.

Day 14.5 forelimb

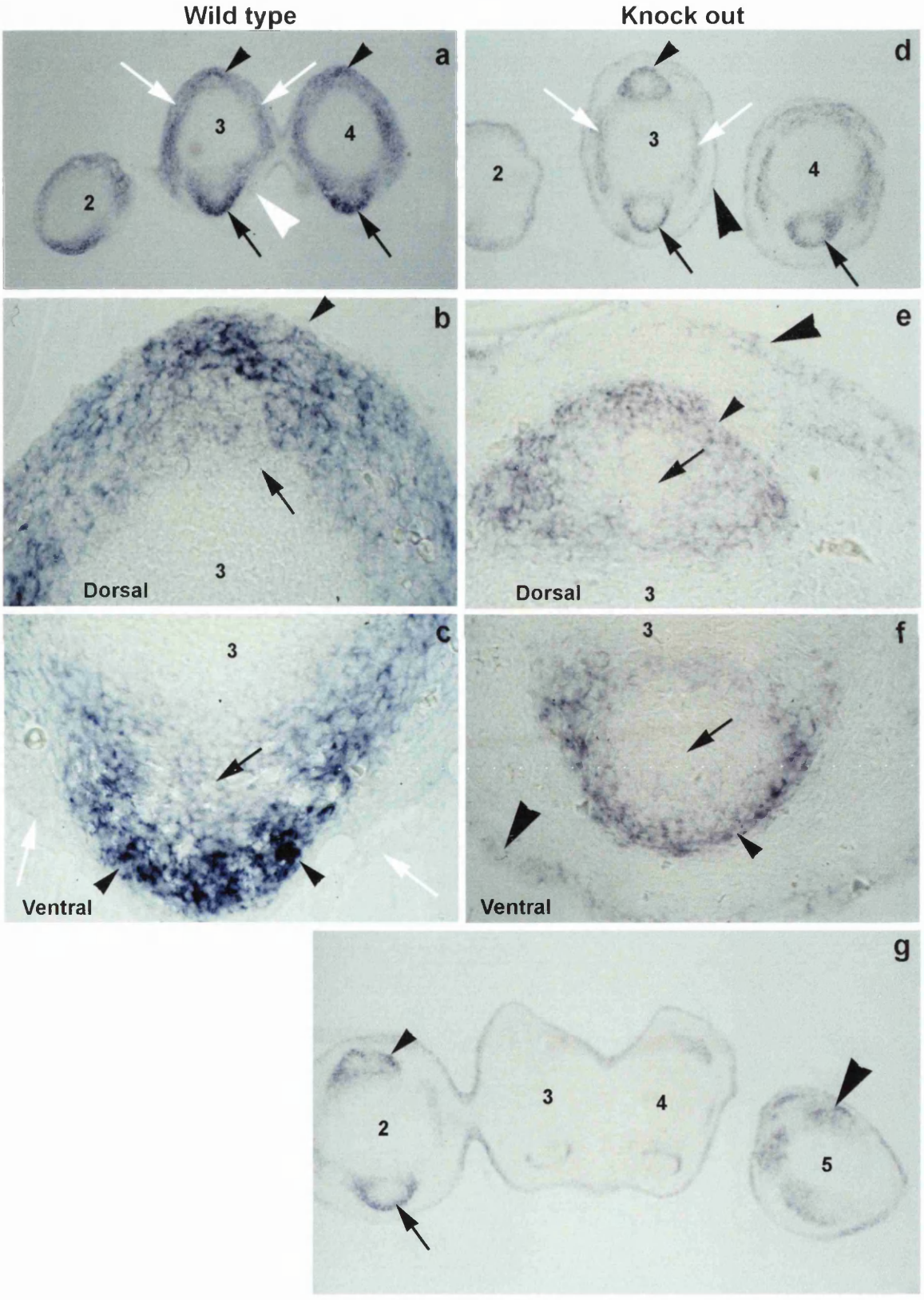


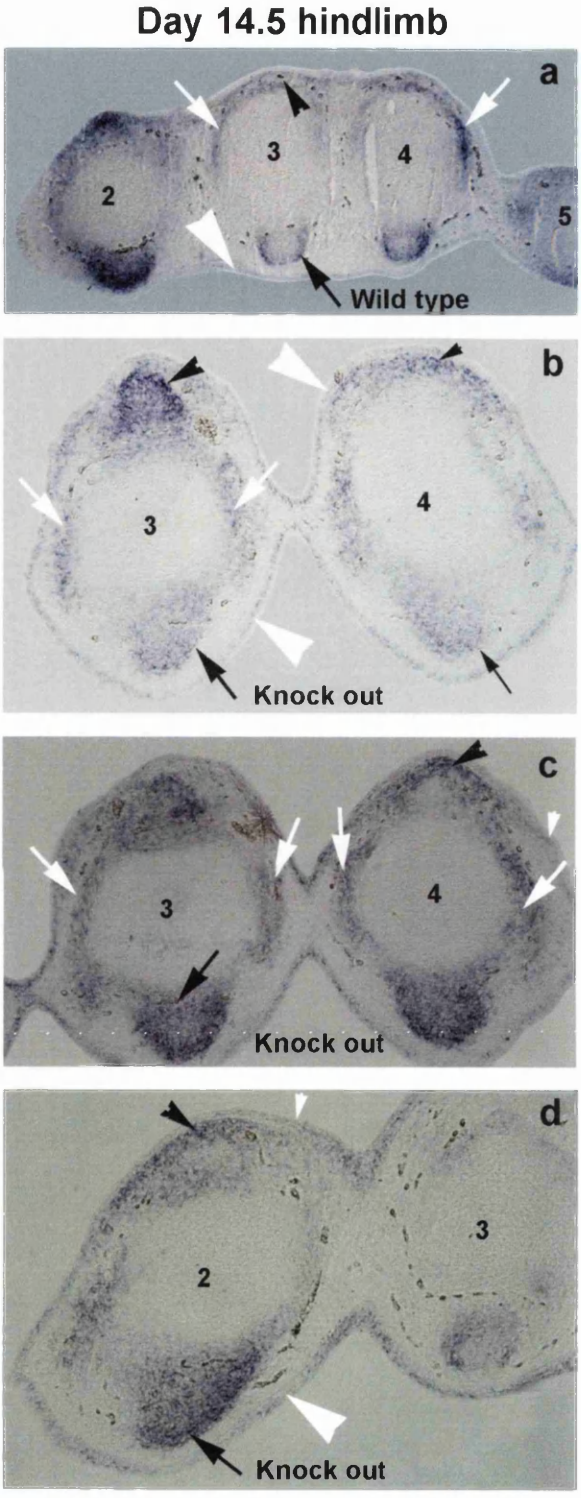
Figure 5.10. *EphA4* expression in transverse sections through wild type and *Wnt-7a* knockout mouse hindlimbs at 14.5 dpc

a) Proximal section through digits of a wild type limb showing dorsal (black arrowhead) and ventral (black arrow) tendons; ectoderm, (white arrowhead); *EphA4* expression flanking cartilage, (white arrow).

b) Section through hindlimb digits in the knockout. Rounded dorsal tendon expressing *EphA4*, (big black arrowhead) is similar to ventral tendon, (black arrow); also compare with dorsal tendon of digit 4, (small black arrowhead); expression flanking cartilage, (white arrow); ectoderm (white arrowhead).

c) Section proximal to (b). Dorsal tendon, (black arrowhead) of digit 4 does not look flat as in (b) but it is similar to ventral tendon (black arrow); expression flanking the cartilage, (white arrows); ectoderm, (small white arrowhead).

d) Section even more proximal to (c) shows tendons expressing *EphA4* in digit 2. Dorsal tendon, (black arrowhead) is atypical as it is rounded and displaced slightly away from the dorsal ectoderm (small white arrowhead) and resembles ventral tendon (black arrow) which is also displaced from the ventral ectoderm (big black arrowhead).


Day 14.5 hindlimb
a
2
3
4
5
Wild type
b
3
4
Knock out
c
3
4
Knock out
d
2
3
Knock out

expression on dorsal side (Fig. 5.8f) indicating a bi-ventral pattern of expression. Although digits 1 and 5 do not have strips of expression dorsally, expression is different from that seen in wild type (compare Fig. 5.8f with b). Sections clearly show that digits 2 (Fig. 5.9g), 3 (Fig. 5.9d), 4 (not illustrated) have a bi-ventral pattern of expression. For example, expression of *EphA4* in tendon on the dorsal side of digit 3 is not over a broad area but is a more circular domain, which mirrors that seen on the ventral side (Fig. 5.9d). Both dorsally (Fig. 5.9e) and ventrally (Fig. 5.9f), these tendons have a well-defined convex border nearer the ectoderm and a flattened border nearer the cartilage element. Both tendons are deep in the mesoderm at a considerable distance from the ectoderm (Fig. 5.9e and f). *EphA4* expression in both tendons is strong around the peripheral convex surface but weak in the centre (Fig. 5.9e and f). Digit 5 (Fig. 5.9g) also probably has bi-ventral pattern of expression as dorsal and ventral expression in the mutant looks similar. Thus in the knockout, expression of *EphA4* is the same in both sets of tendons.

In the wild type, *EphA4* expression dorsally is present in the whole thickness of mesoderm between cartilage and ectoderm and extends over the entire width of the digit (Fig. 5.9a, b), whereas expression in ventral tendon is well away from ectoderm (Fig. 5.9c). In the knockout, *EphA4* expression is not present in the whole thickness of mesoderm between cartilage and ectoderm but forms a thin layer on sides of the digit (Fig. 5.9d). This latter feature may be associated with a difference in tendon attachment to cartilage. It should be noted, however, that tendons seen on the dorsal side though transformed into a ventral pattern (Fig. 5.8f) do not diverge from a common point which is the case in ventral tendons (Fig. 5.8a, e) indicating that tendons may not be dorso-ventrally transformed proximally.

Mutant Hindlimb

Dorsal expression of *EphA4* associated with digit 3 (Fig. 5.8h) is similar to that seen on the ventral side (Fig. 5.8g) with a strip of expression proximally. Sections of the knockout hindlimb show that digits 3, 4 and 2 are bi-ventral (Table 5.4) (Fig. 5.10b, c and d). Distally, dorsal expression in digit 4 (Fig. 5.10b) looks just like a dorsal pattern. However, proximally (Fig. 5.10c) it resembles a ventral pattern more than it does a dorsal one. Hence, *EphA4* expression on the dorsal side in digit 4 has a dorsal pattern distally (Fig. 5.10b) and a ventral pattern proximally (Fig. 5.10c).

As noted in the mutant forelimb, even though the strips seem to diverge from the base of the digital plate ventrally, there is no expression in the base (Fig. 5.8g) akin to

that seen in the wild type (Fig. 5.8c). Expression of *EphA4* in tendons of digit 5 both proximally and distally has a normal dorso-ventral pattern (not shown).

From this analysis of mutant hind and forelimbs, it emerges that tendon pattern along the proximo-distal axis differs in different digits and same pattern is not seen through out the whole length of a digit (Table 5.4). Tendons in digit 3 of both forelimb and hindlimb are bi-ventral throughout, but, in digits 2 and 4, in both forelimb and hindlimb, tendons are bi-ventral proximally but have a distinct dorsal-ventral pattern distally (Table 5.4). Digit 5 is also bi-ventral only in proximal region in forelimb.

5.3.2.3 Early development of tendon in wild type and *Wnt-7a* mutant mouse limb

By day 14.5 of development, a bi-ventral tendon pattern can be clearly seen in mutant limbs. However, as the pattern differs in different digits, it is not clear how this arises during development. In order to address this, I compared tendon patterns in mutant and normal limbs at progressively earlier stages in development.

In whole mounts of 13.5 d.p.c mutant forelimb, the dorsal expression of *EphA4* (Fig. 5.11f) associated with digit 3 is similar to that on its ventral side (Fig. 5.11e) and to ventral expression of wild type (Fig. 5.11a). Thus digit 3 in the forelimb has a bi-ventral pattern even at day 13.5. Sections reveal that *EphA4* is expressed in the wild type as a thin layer close to ectoderm across the width of the dorsal surface of the digits excluding the interdigital region (Fig. 5.12a). Ventral expression distally is confined to a narrow oval domain close to ectoderm, and is associated with tendons in digits 3 and 4 which have already started to round up (Fig. 5.12a) but proximally expression is away from ectoderm (Fig. 5.12b). However in digit 2 and 5 it can be noticed that the layer of expression is thinner dorsally, when compared to the ventral expression which is thicker (Fig. 5.12b). In mutant forelimbs, expression of *EphA4* in digits 2, 3 and 4 (Fig. 5.12c) is bi-ventral. Dorsal expression in digits 3 and 4 is not present as layer across the entire width of the digit but in a more oval domain (Compare Fig. 5.12c with a) and is more akin to the ventral expression domain. However there are differences between expression domains seen in digits 3 and 4 and that seen in digit 2. In digits 3 and 4 the dorsal tendons have started to round up, but in digit 2 the dorsal expression is a thick layer in contrast to the wild type were expression is a thinner layer (compare Fig. 5.12c with b). The same appearance can be seen in digit 3 of the mutant hindlimb were the dorsal expression is thick (Fig. 5.12d) proximally but has a normal pattern distally (Table 5.4). None of the other dorsal tendons were altered.

Figure 5.11. *EphA4* expression in the wild type and *Wnt-7a* knockout mouse limb buds at 13.5 days of development

a) Ventral view of wild type forelimb showing *EphA4* expression in proximal tendon (big white arrow) and strong expression distally (small white arrow). *EphA4* expression at the anterior margin of digit (white arrowhead); expression at the tip of the digit (black arrowhead); expression at the base of the digital plate (b/w arrowhead).

b) Dorsal view of wild type forelimb. Broad *EphA4* expression across width of digit (big white arrow) and expression along sides proximally (white arrowheads). Distal tip of expression (small white arrowhead) is separate from the weak expression (black arrowhead) at the tip of digit

c) Ventral view of wild type hindlimb with *EphA4* expression in ventral tendon (white arrow) with distal stronger expression (small white arrow); expression in a linear streak at anterior edge of digit (white arrowhead); expression at tip (black arrowhead).

d) Dorsal view of wild type hindlimb with expression across digit (white arrow) and proximally at sides (white arrowheads); expression at tip of digit (black arrowhead) is separate from the expression at the tip of tendon (small white arrow).

e) Ventral view of knockout forelimb with expression similar to wild type forelimb. Expression in tendon proximally (big white arrow); expression at side of digit (arrowhead); expression at tip (black arrowhead).

f) Dorsal view of the knockout forelimb. Digit 3 has expression in tendon (big white arrow) similar to ventral expression in (e) with strong expression distally (small white arrow); expression at the tip of digit (black arrowhead). Generally other digits look very similar to (e).

g) Ventral view of the knockout hindlimb with typical ventral expression. *EphA4* expression in proximal tendon (white arrowhead) is strong distally (small white arrow); expression along side of digit (white arrowhead); expression at tip (black arrowhead).

h) Dorsal view of knockout hindlimb with some sign of a linear streak proximally in digit 3 (big white arrow); however the 2 streaks that are at the anterior and posterior edge of the digit (white arrowheads) look similar to the wild type limb in (d).

Expression of EphhA4 at day 13.5

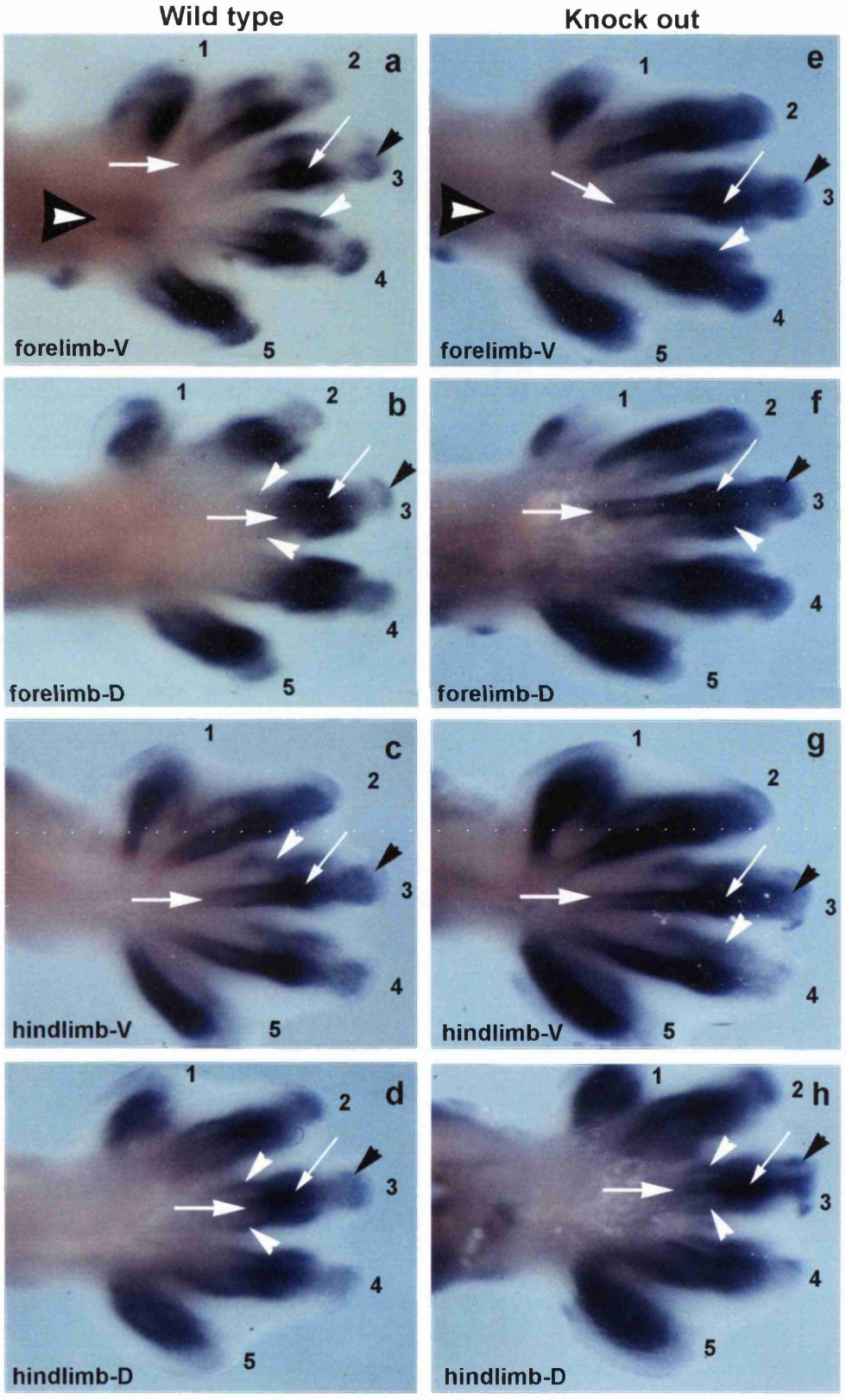


Figure 5.12. Expression of *EphA4* in transverse sections of wild type and *Wnt-7a* mouse mutant limb buds at 13.5 day of development

a) Forelimb wild type. *EphA4* expression dorsally (arrowhead); expression in ventral tendon (black arrow); ectoderm (white arrowhead).

b) Section more proximal to that in (a). dorsal tendon (arrowhead); ventral tendon (arrow). Note round ventral tendons, flat dorsal tendons.

c) Section through forelimb knockout. Expression in dorsal tendons look similar to ventral tendons seen in (a); arrows, expression in ventral tendons (Black arrowheads); ectoderm (white arrowhead); dorsal expression in digit 2 (asterisk) which is very strong when compared to that in (a) and (b). Note the similarity to the ventral.

d) Section through hindlimb knockout. Symmetrical expression of *EphA4* in dorsal (black arrowhead) and ventral tendons (black arrowhead) of digit 3. Compare with dorsal expression seen in (a) or (b); dorsal expression in digit 4 (white arrowhead).

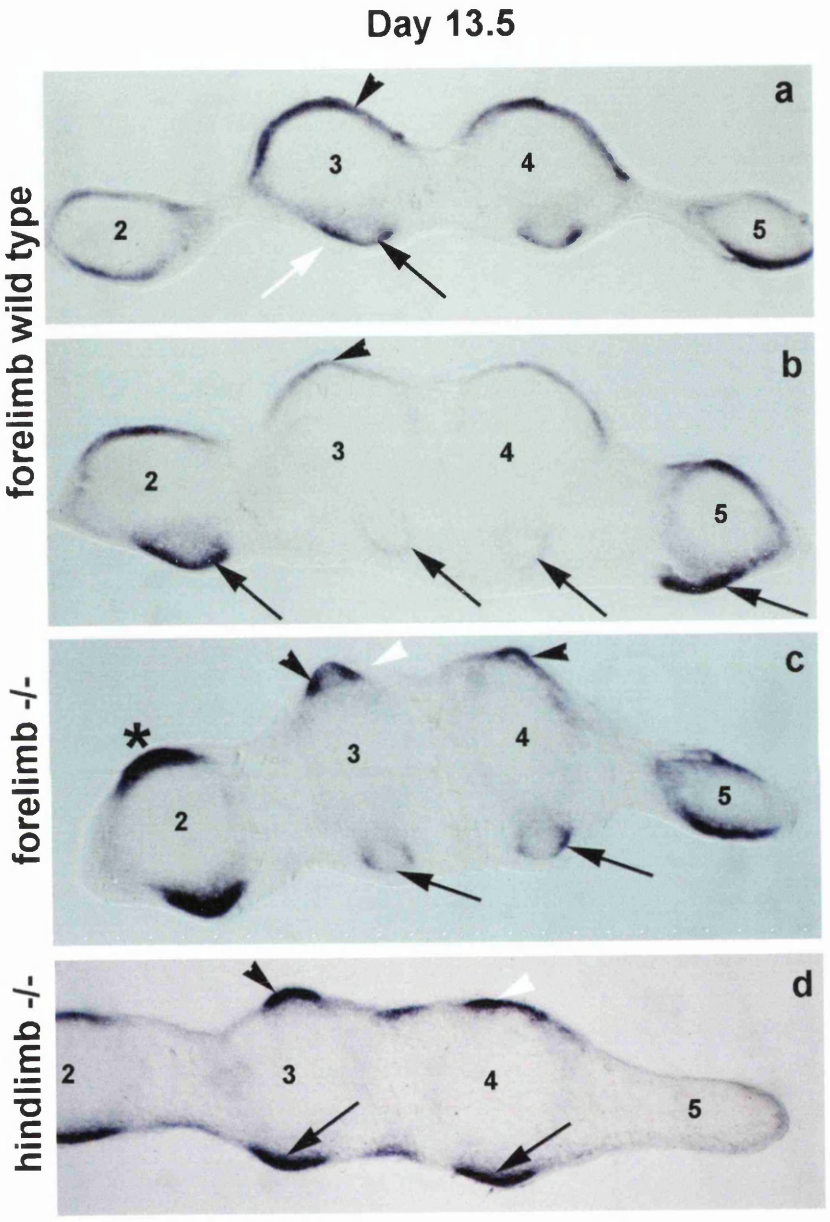

Day 13.5
forelimb wild type
forelimb -/-
hindlimb -/-
a
b
c
d
2
3
4
5
*

Table 5.4 Difference in tendon pattern along the proximo-distal axis in *Wnt-7a* mutant mice

Days	Digit	Limb	Proximal	Distal
14.5	2	fore	bi-ventral	dorso-ventral
		hind	bi-ventral	dorso-ventral
	3	fore	bi-ventral	bi-ventral
		hind	bi-ventral	bi-ventral
	4	fore	bi-ventral	dorso-ventral
		hind	bi-ventral	dorso-ventral
	5	fore	bi-ventral	dorso-ventral
		hind	dorso-ventral	dorso-ventral
13.5*	2	fore	bi-ventral	dorso-ventral
		hind	dorso-ventral	dorso-ventral
	3	fore	bi-ventral	bi-ventral
		hind	bi-ventral	dorso-ventral
	4	fore	bi-ventral	dorso-ventral
		hind	dorso-ventral	dorso-ventral

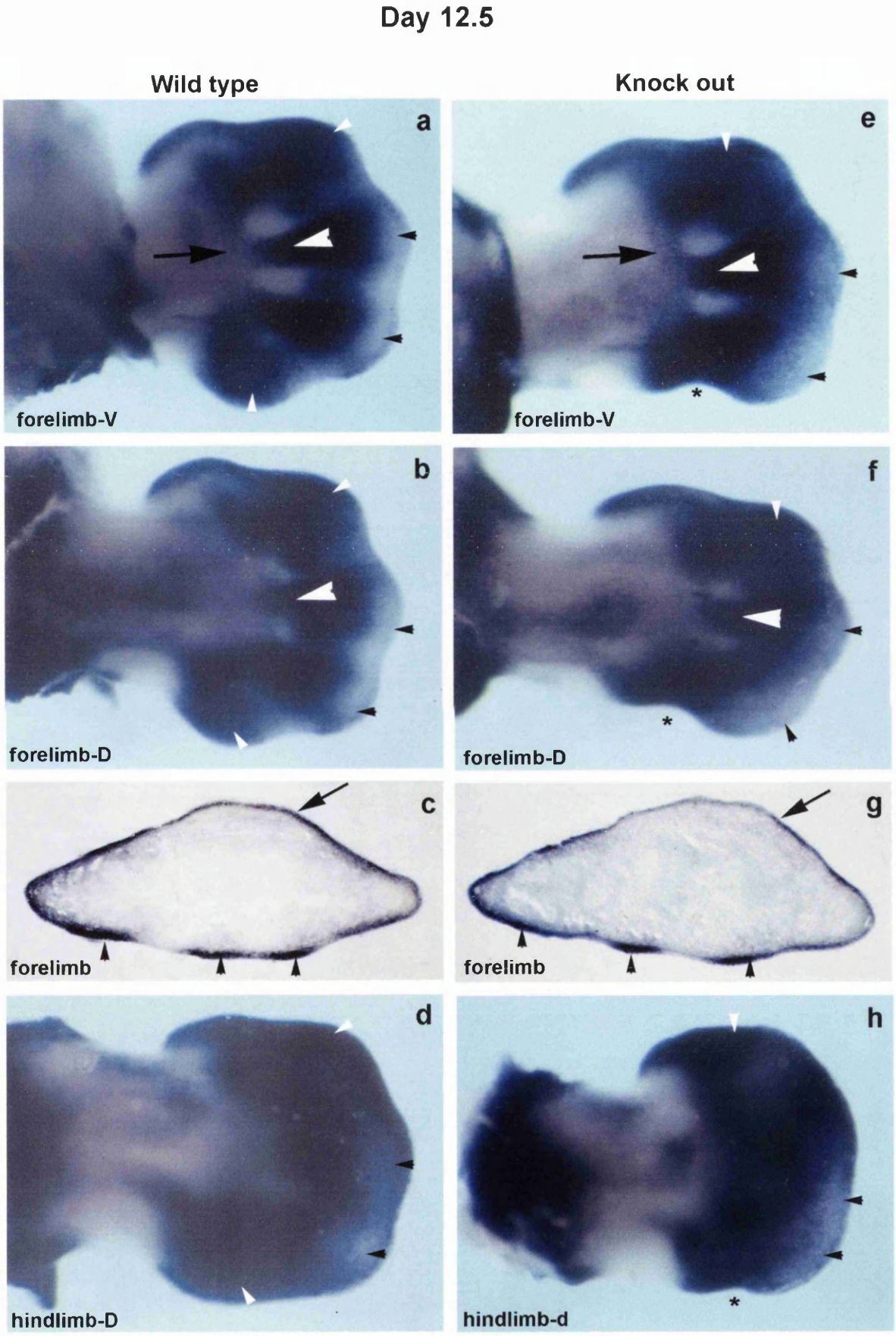
* Digits 1 and 5 were not distinct in sections and could not be scored.

Figure 5.13. Expression of *EphA4* in wild type and *Wnt-7a* mouse mutant limb buds at 12.5 day of development

White arrowhead indicates expression in early tendon primordia; black arrowheads indicate the distal tip of digit where expression is either low or absent; small white arrowhead is where expression extends to the tip of the digit plate; asterisk indicates the deficiency seen in the transgenic mice limbs.

a) Ventral view of wild type forelimb. *EphA4* expression in tendon of digit 3 (black arrowhead) which is also faintly demarcated in digits 2 and 4; expression at base of digit plate (black arrow) with which the tendon is in contact with proximally.
b) Dorsal view of the wild type forelimb shows *EphA4* expression in dorsal tendon (white arrowhead) of digit 3.
c) Transverse section of the wild type forelimb seen in a and b. *EphA4* expression in ventral tendon primordia (arrowheads)very close to the ectoderm; *EphA4* expression dorsally (arrow), tendons are not clearly demarcated.
d) Dorsal view of the wild type hindlimb showing diffuse expression over the whole digital plate except at the distal tips (black arrowheads). Expression is also seen at anterior and posterior edges (white arrowhead) of digit plate.
e) Ventral view of knockout forelimb shows *EphA4* expression in ventral tendon (big white arrowhead) which is in continuity with the expression at the base of the digit plate (black arrow).
f) Dorsal view of knockout forelimb with expression in dorsal tendon (white arrowhead).
g) Transverse section through the knockout forelimb shows ventral tendons (black arrowheads); and dorsal expression (arrow). This can be compared with (c) and looks very similar.
h) Dorsal view of knockout hindlimb. *EphA4* expression covers the digit plate and looks similar to wild type indlimb in d)..

Fig. 5.13


Day 12.5
Wild type
Knock out
a
e
forelimb-V
forelimb-V
b
f
forelimb-D
forelimb-D
c
g
forelimb
forelimb
d
h
hindlimb-D
hindlimb-d

At 12.5 dpc (Fig. 5.13), the dorso-ventral expression pattern of *EphA4* is normal in both forelimb and hindlimb of the mutants. 12.5 d.p.c mutant limbs appear to have a deficiency in the posterior digit region, which has been reported earlier (Parr and McMahon 1995). In this study, all the older limbs analysed had the correct number of digits.

Taken together, these observations show that changes in dorso-ventral patterning occur in the mutant between day 12.5 and 13.5.

5.3.3 Misexpression of *Lmx-1* in chick limb buds

A total of 83 embryos were infected, of these 10 were used for *Lmx-1* antibody staining, and 7 proved to have complete infection in dorsal and ventral mesenchyme at stage 24/25, more than 3 days after infection (Fig. 5.14g). Limbs examined at earlier stages were not completely infected. 55 were probed for *EphA4* between stages 25 to 34 and 15 were sectioned for histology between stage 33 to 36. Separately, 3 embryos were allowed to grow to 14 days.

5.3.3.1 Pattern of *EphA4* expression

In 5 out of 15 infected embryos between stage 33 and 36, some toes stood out straight rather than being curved or were hyperextended similar to earlier reports (Riddle et al 1995, Vogel et al 1995). In one limb, one of the digits was hyperextended to such an extent that it stood up almost vertically. The tips of toes (nail primordia) look normal, but scale pattern could not be made out on ventral side as these do not develop until beyond stage 36. However, none of these straight or hyperextended digits showed dorso-ventral alterations of tendon on histological examination. No alteration in pattern of *EphA4* expression could be detected in either whole mounts (Fig. 5.14a) or sections (Fig. 5.14b, c). Ventral tendon expression is seen as tight narrow bands diverging from a common point of expression near the base of the digital plate (Fig. 5.14a) and looks normal (compare Fig. 5.14d). Expression in sections also appears to be normal with rounded compact narrow tendons expressing *EphA4* on the ventral side and dorsal tendon expression in flat broader domains (compare Fig. 5.14b and c with e and f). However normally, expression of *EphA4* in dorsal tendons is weaker than expression in ventral tendons but in these infected limbs, expression on the dorsal side was is as strong as that on the ventral side (not shown). Thus, up to stage 36, day 10 of embryonic development, both by studying the expression of *EphA4* and, by histology no alteration in the dorso-ventral patterning was made out in infected limbs.

Figure 5.14. Expression pattern of *EphA4* in chick limb following viral misexpression of *Lmx-1*

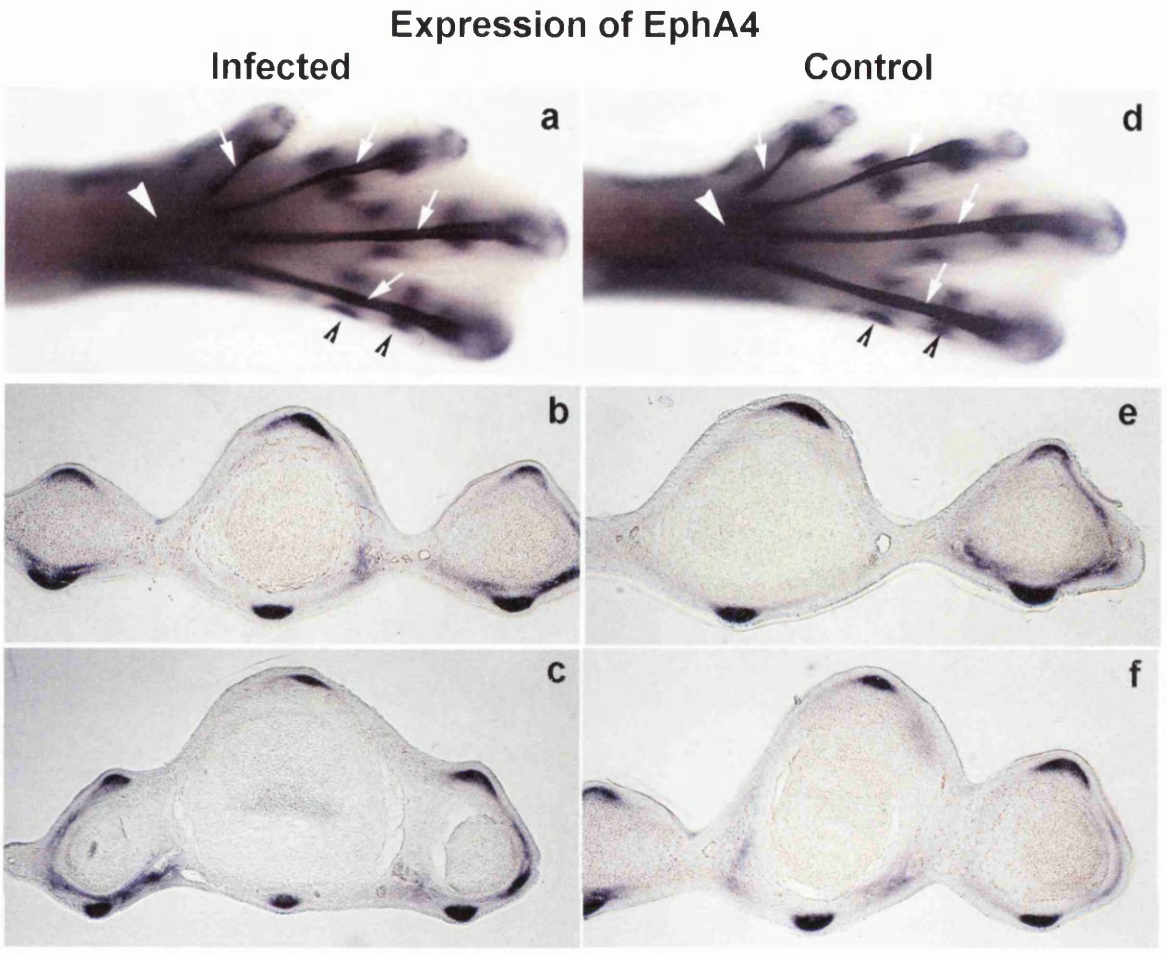
a)-c) limb that was infected with retrovirus carrying the *Lmx-1* gene d)-f) are controls.

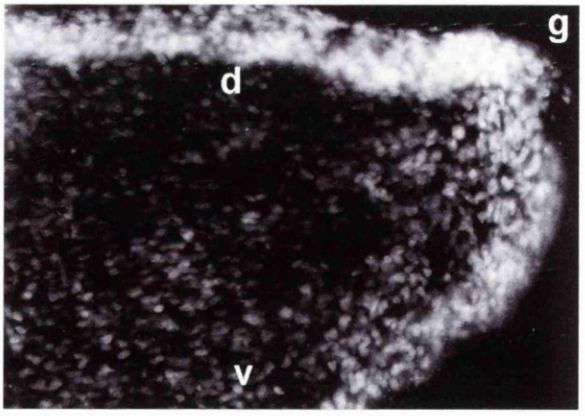
a) Whole mount ventral view of infected limb at 8 days showing expression pattern of *EphA4* similar to the control (d). Anterior to top.

b) and c) are distal and proximal sections of the infected limb showing flat dorsal tendons and rounder ventral tendons. Compare similar sections of uninfected left limb in e) and f).

d) Whole mount ventral view of a control limb showing expression of *EphA4* in ventral tendons.

g) Limb of embryo infected at stage 10, fixed at stage 24 and stained with Lmx antibody stain. Both dorsal (d) and ventral (v) cells are expressing *Lmx-1* protein.


Expression of EphA4
Infected
Control
a
d
b
e
c
f


Lmx 1 Antibody Stain
g
d
v

5.3.3.2 Dorso-ventral alterations after misexpression of *Lmx-1*

In previous studies, dorso-ventral patterning after mis-expression of *Lmx-1* was examined later. Hence 3 embryos were allowed to develop until 14 days. By this time EphA4 expression is switched off and therefore the limbs examined histologically using paraffin wax sections stained with Mallory's trichrome.

When limbs are allowed to grow to day 14 of incubation, in 2 out of 3 embryos the striking feature is that some of the toes have double claws (Fig. 5.15a) (Table 5.5) separated by a very small amount of soft tissue at the tip. Double nails appear shorter than the other normal nails in toes of the same foot. In both embryos the toes with double nails are not hyper-extended or straight, but instead curved ventrally (Fig. 5.15a). Only anterior digits have double nails (Table 5.5). Sections show details of nail formation in normal toes (Fig. 5.16i, j) and those with double nails (Fig. 5.16a-e arrowheads). These double nails are separate for a short distance from the tip (Fig. 5.16a) and fuse (Fig. 5.16 b-e) proximally. This forms a cylindrical structure that initially surrounds soft tissue (Fig. 5.16f) and more proximally encircles cartilage (Fig. 5.16g, h).

Another feature of 14 day infected embryos is an altered scale pattern. Normally, the ventral surface of toes has small scales called tuberculae (Fig. 5.15b arrowhead) and the dorsal has large broad scales called scuta (Fig. 5.15b arrow) (Table 5.6). In embryos examined at day 14, scales on the ventral surface are either normal shaped tuberculae (Fig. 5.15b arrowheads) or sometimes replaced by dorsal shaped large scuta (Fig. 5.15d) or the ventral surface was smooth with no evidence of any scales. Different toes on the same limb could show any of the above mentioned three features. In one instance, all three features are seen in the same toe, distally scuta (dorsal scales), intermediate region smooth and proximally tuberculae (ventral scales) (Fig. 5.15e) (Table 5.5). The double dorsal scale pattern is clearly evident in sections of the toes as well. Toes that had double claws did not have dorsal scales on the ventral surface (Table 5.5), they were atypical and smooth. Similarly toes that had double dorsal scale pattern did not have double claws.

Individual digits of these infected limbs at 14 days of development were sectioned and tendon pattern compared with the same toe of the contralateral side sectioned at approximately the same level. Only some toes in the same foot show alterations in tendon pattern. Digit 2 exhibits double dorsal tendon pattern in 2 of the 3 infected limbs. Toes 1, 3 and 4 have normal tendon pattern. Double dorsal tendons of the infected digit

Figure 5.15. Gross features in control and *Lmx-1* infected chick limbs

a) Limb infected with *Lmx-1* virus at stage 10 and fixed at day 14. Arrows indicate 2 nails on digit 2, note smooth ventral surface in digit 4.

b) Control left limb from same embryo as in a). Arrowheads indicate ventral scales; arrows, dorsal scales.

c) Dorsal view of an infected digit showing dorsal scales (arrows).

d) Ventral view of toe seen in c) showing dorsal type scales on ventral side (arrows).

e) Ventral view of another infected digit showing dorsal type scales distally (arrows); ventral type scales (arrowheads); and smooth area (asterisk), which has neither type of scale.

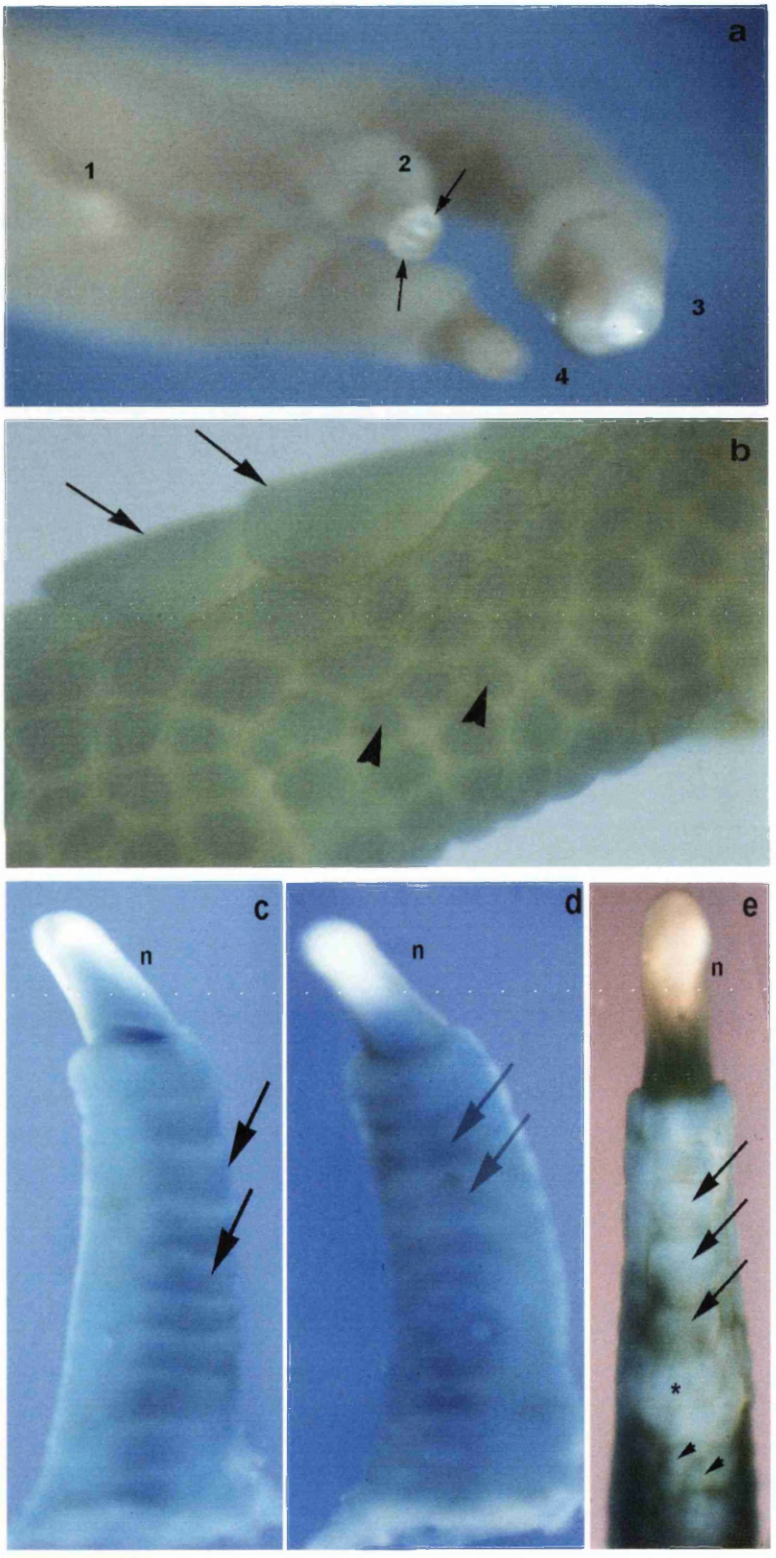

a
1
2
3
4
b
c
n
d
n
e
n
*

Figure 5.16. Transverse sections of nails in *Lmx-1* infected and control chick limbs

a)-e) shows sections of double nails found in digit 2 of infected limb from distal to proximal, shown in Fig 5.15a. Arrowhead indicates nail on dorsal side; arrow, nail on ventral side. Note that distally they are separate entities and fused proximally.

f)-h) sections of the nail proximally have fused and form a cylindrical structure around the phalanges (s).

i) and j) are sections of digit 2 of the control limb from the same embryo at approximately same level as f) and h) respectively.

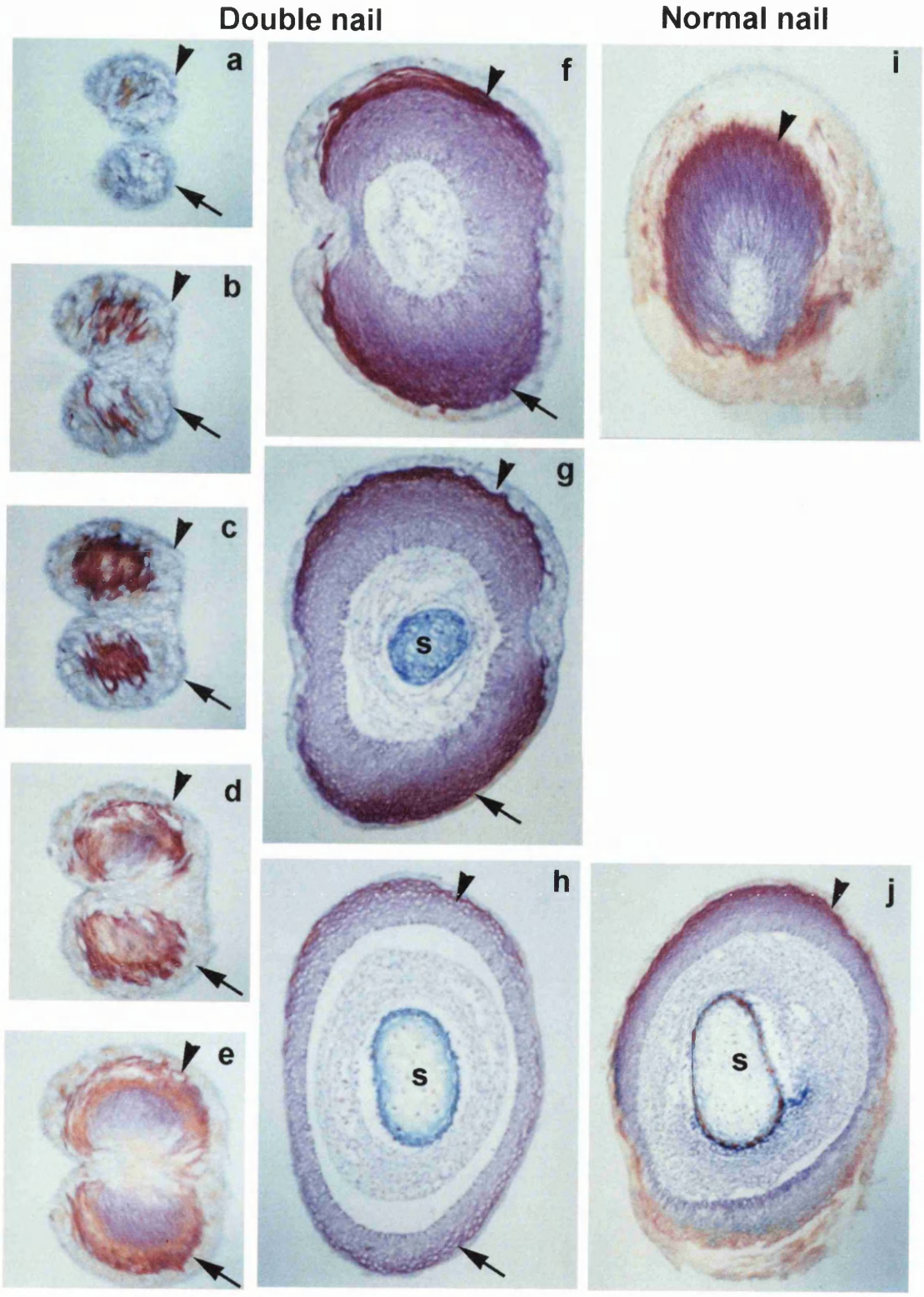

Double nail
Normal nail
a
b
c
d
e
f
g
h
i
j
s
s
s

Figure 5.17. Tendon pattern in a digit from an infected chick limb

Control and infected digits are from the same embryo infected at stage 10 and fixed at day 14.

a)-c) Control digit with a flat dorsal tendon (arrowhead) and a round ventral tendon enclosed by a sheath.

d) and e) are distal to proximal sections of infected digit in which dorsal (arrowhead) and ventral tendon (arrow) are flat and look similar.

f) and g) More proximal sections of infected digit. Ventral tendon (arrow) becomes less flat and instead takes on a round appearance though smaller than in the control c). Note the duplication of protuberance (white arrowhead) in f).

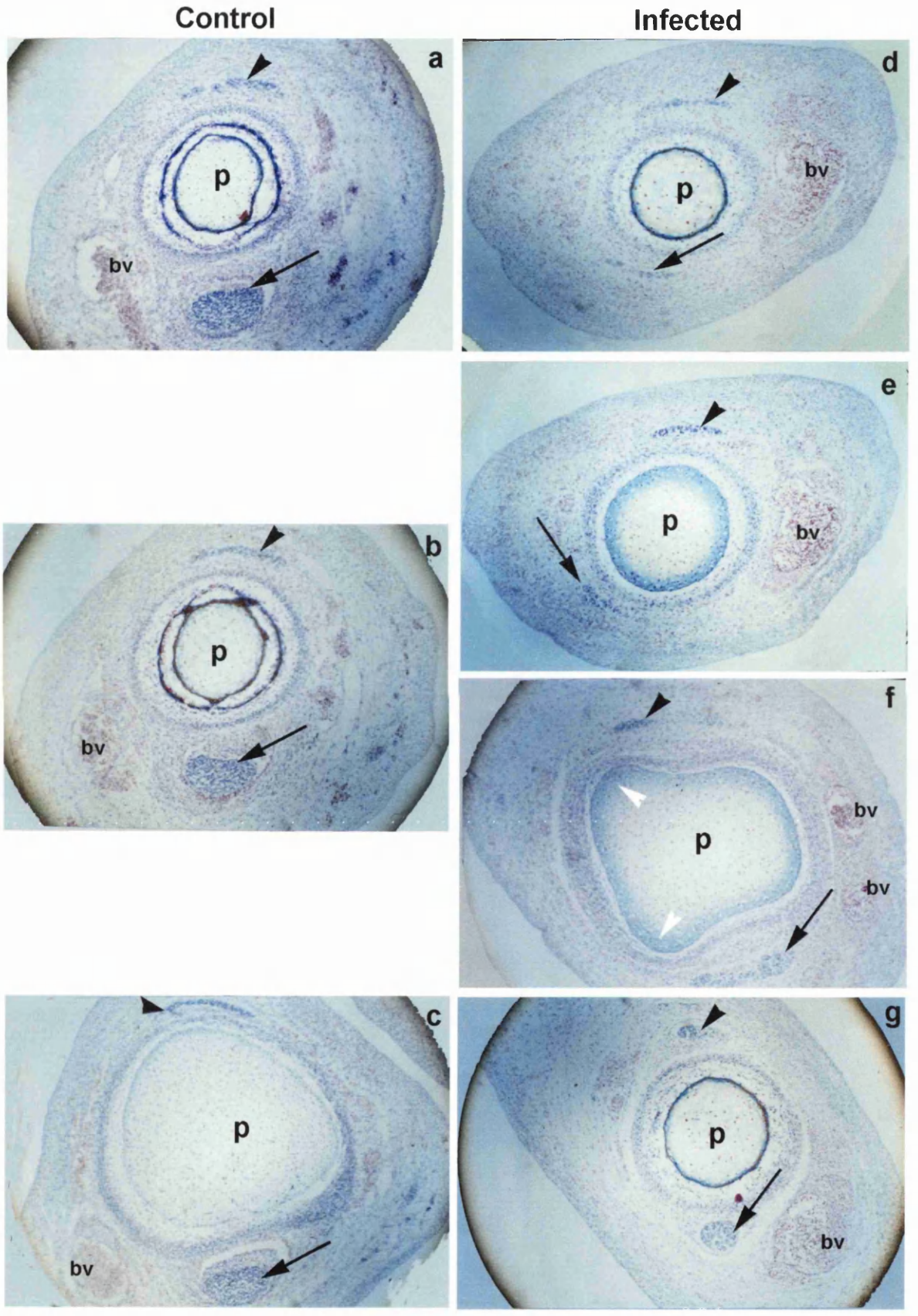

Control
Infected
a
b
c
d
e
f
g
p
bv

Table 5.5 Dorso-ventral alterations following misexpression of Lmx-1 in chick embryos

	Double nail	Ventral scale	Double dorsal tendon
Embryo 1			
Digit 1	+	Smooth	-
Digit 2	+	Smooth/ventral	+
Digit 3	-	Dorsal/Smooth/Ventral	-
Digit 4	-	Smooth	-
Embryo 2			
Digit 1	-	Smooth	-
Digit 2	+	Smooth	+
Digit 3	-	Dorsal	-
Digit 4	-	Smooth	-
Embryo 3			
Digit 1	-	Normal	-
Digit 2	-	Normal	-
Digit 3	-	Normal	-
Digit 4	-	Normal	-

Table 5.6 Misexpression of *Lmx-1* in chick embryos

Features	**Wild type**[2]	**Misexpression**[1,2]	**Misexpression in leg**[3]
Ectodermal			
Feather buds	D dense V less dense	V increased	
Scales	D broad scuta V small tuberculae	V-dorsal scales	V dorsal/atypical
Claws	curved ventral	dorsally curved/ straight	Double
Mesodermal			
Wing muscle		V muscle missing	
Leg muscle		V ,muscles missing replaced by D muscle	
Tendons		V ectopic dorsal tendon	V rounded tendon replaced by flat tendon distally
ectopic digits		+	+(one-not shown)

[1]Riddle et al 1995; [2]Vogel et al 1995; [3]This thesis

on both dorsal and ventral sides, had the same proximity to the phalangeal skeletal element (Fig. 5.17d-g). Distally there is no bulky flexor tendon on the ventral side (Fig. 5.17d-e) as seen in the control (Fig. 5.17a, b) but instead a flat tendon is seen (Fig. 5.17d) which becomes bulkier (Fig. 5.17f arrow) and then round (Fig. 5.17g arrow) nearer the proximal end of the digit. The rounded tendon appears to show signs of tendon sheath development but is much smaller than the control left one (Fig. 5.17c) which has a well developed tendon sheath. Of the two embryos that showed altered dorso-ventral patterning after *Lmx-1* mis-expression, only digits 1 and 2 had double nails. However in both embryos, only digit 2 had double dorsal tendon distally, associated with a smooth ventral ectoderm surface.

Cartilage elements in the infected digits are smaller in diameter and with a round contour (Fig. 5.17g) whereas a normal element has a flatter ventral side (Fig. 5.17c) at least nearer its ends. It has been reported earlier that cartilage in infected limbs had bi-dorsal pattern (Vogel et al 1995) an I observed a duplication of a ventral protuberance on the dorsal side (Fig. 5.17f).

5.4 Discussion

In chick leg buds, as digits and tendons develop, expression of *Wnt-7a* decreases over the digit region and is stronger over the slope of the digits and reduces after stage 30. Early expression of *Wnt-7a* in dorsal ectoderm is homogenous but between stage 22 to 27, expression becomes patchy and is reduced proximally. However strong expression was present as a thick layer in the ectoderm distally, hence *Wnt-7a* signal may have a proximo-distal gradient. *Lmx-1* expression in dorsal mesenchyme in chick leg bud overlaps areas of early tendon development and expression domains of *EphA4*. As the tendon moves away from ectoderm, *Lmx-1* expression becomes reduced in the tendon which still expresses *EphA4*. *Lmx-1* expression persists however in the mesenchyme surrounding the tendon on the sides and dorsally. At day 14.5 *Wnt-7a* mutants have a bi-ventral pattern of digits. Changes in dorso-ventral patterning in the *Wnt-7a* mutant occurs between day 12.5 to day 13.5. When *Lmx-1* is misexpressed in the ventral mesenchyme of chick leg bud, dorso-ventral alterations occur late in tendon development. Results also suggest a role for *Lmx-1* in nail formation and possibly in cartilage as well.

5.4.1 *Wnt-7a* signal may have a proximo-distal gradient

Expression of *Wnt-7a* in dorsal ectoderm becomes weaker proximally as limb grows out. The dorsal ectoderm over digital plate appears to be thicker distally than it is proximally. Histologically, however, this has not been reported before. This could affect *Wnt-7a* signalling along the proximo-distal axis. The signal from the dorsal ectoderm could be stronger distally over the progress zone. This could affect the amount of signal received by the mesenchyme at different distances from the ectoderm.

5.4.2 Relation of *Wnt-7a* to *Lmx-1*

Wnt-7a is expressed in the digit region only up to stage 30/31, whereas Lmx 1 is present in the mesoderm up to stage 35/36. This shows that *Lmx1* can be maintained at later stages in chick limb development without the *Wnt-7a* signal. Other work has also pointed to the ability of dorsal limb mesenchyme to maintain *Lmx-1* expression. When stage 20/21 chick limbs are grown in vitro for 36 hours without dorsal ectoderm, *Lmx1* expression is present in the proximal mesoderm but lost distally. It was therefore suggested that mesoderm cells require a certain period of exposure to the *Wnt-7a* signal (Riddle et al 1995) after which they can maintain *Lmx1* without the influence of *Wnt-7a* signalling (Table 5.3). However, in contrast, Vogel et (1995) found that *Lmx-1* expression is reduced throughout dorsal mesenchyme when ectoderm is removed (Vogel et al 1995).

Data in my work suggest that cells that lie dorsal-most in the dorsal mesoderm lose their *Lmx1* expression before deeper cells. and thus are most responsive to the absence of *Wnt-7a* signalling. These changes in *Wnt-7a* expression suggest that from stage 32 onwards, these dorsal mesenchymal cells may become more amenable for dorsal to ventral transformation in cell fate. However when En-1 was misexpressed, it did not lead to complete ventralisation of dorsal mesoderm. Hence it was suggested that dorsal mesoderm may not be capable of responding to ventralising signals (Logan et al 1997). However in only 4/38 infected embryos was *Lmx-1* expression completely abolished in dorsal mesoderm. In 23/38 infected embryos, much reduced *Lmx-1* expression persisted in more proximally located dorsal mesenchyme (Logan et al 1997).

5.4.3 Relation of *Wnt-7a* and *Lmx-1* expression to tendon development.

Wnt-7a is expressed in dorsal ectoderm during early tendon development. However there is less expression directly over the areas where tendons assemble under the ectoderm. *Lmx-1* is expressed in the mesenchyme subjacent to the ectoderm where

Table 5.7 Expression pattern of *Wnt-7a* and *Lmx-1* during tendon development in chick legs

Stage	Site	Tissue	*EphA4*	*Lmx-1*	*Wnt-7a*
27-29	Distal	Ectoderm	-	-	+
		Tendon subjacent to Ectoderm	+	+	
	Proximal	Ectoderm			+
		Tendon away from ectoderm	+	+	
		Mes between Ecto and tendon		+	
30-32	Distal	Ectoderm			+ *
		Tendon subjacent to Ectoderm	+	-/weak	
	Proximal	Ectoderm			-
		Tendon away from ectoderm	+	-	
		Mes between Ecto and tendon	-	+/strong	
33-36	Distal	Ectoderm			-
		Tendon subjacent to Ectoderm	+	+	
	Proximal	Ectoderm			-
		Tendon away from ectoderm	+	-	
		Mes between Ecto and tendon	-	+	

Ecto, ectoderm; Mes, mesenchyme; d, dorsal

* *Wnt-7a* is expressed only up to stage 30.

tendon will form, but later, as mesenchymal lamina and associated tendon move away from ectoderm, *Lmx-1* expression is reduced and is almost absent in differentiating tendon (Table 5.7). Thus the cells that form tendons appear to arise from *Lmx-1* expressing cells but, early in tendon development, *Lmx-1* expression is switched off in developing tendon itself but remains strong in the surrounding mesenchyme. A similar pattern of *Lmx-1* expression in relation to differentiated tissues is also seen in proximal limb level where high levels of expression are found in tissue that surrounds the dorsal developing muscle mass but expression is very low or absent within the muscle. It has also been reported that *Lmx-1* is not expressed in cells once they have begun the process of chondrification (Riddle et al 1995). Thus *Lmx-1* in early stages of limb bud development is present in the whole of the dorsal limb mesenchyme. Later it is excluded from condensing tendon, muscle and cartilage, thus it seems to follow a similar pattern of expression in each type of tissue. Its spatial and temporal presence around developing tissues suggests that it exerts its patterning effects via the connective tissue.

5.4.4 Dorso-ventral patterning of tendons in *Wnt-7a* null mutants

In the *Wnt-7a* mutant, at day 14.5 dpc, expression pattern of *EphA4* in dorsal tendons of digits 4 and 5 in forelimb is bi-ventral proximally but appears to show a normal dorso-ventral pattern distally (Fig. 5.18). According to Cygan et al (1997) digits 4 and 5 in these mutants do not express *Lmx-1b* (the murine homolog of *Lmx-1*) over the distal-most tip of the dorsal mesenchyme at this stage (Fig. 5.18) (Table 5.8), yet distally dorsal tendons have a dorsal pattern of *EphA4* expression. On the other hand, *Lmx-1b* is present proximally in digits 4 and 5 but in this region digits have ventral pattern of *EphA4* expression dorsally. This certainly seems contradictory. If *Lmx-1b* was involved in patterning dorsal tendon in the mouse, then digit 4 and 5 should have been bi-ventral distally and have normal polarity proximally because *Lmx-1b* is absent distally but present proximally. This suggests that expression of *Lmx-1b* is not directly related with dorso-ventral patterning of tendons. One possibility is that *Lmx-1b* does not dorsalise tendons but instead dorsalisation is brought about by some other factor. Similarly, *Lmx-1b* is never present in the entire dorsal mesenchyme of digit 2 at day 14.5 in the mutant (Cygan et al 1997) and yet I found that the *Wnt-7a* mutant in my study has bi-ventral tendon proximally and a normal dorso-ventral pattern distally. According to Cygan et al (1997) *Lmx-1b* expression is absent in the anterior half of digit 3 most often (but sometimes is completely absent). In this study, tendon pattern in digit 3 is bi-ventral both proximally and distally. Thus the expression pattern of *Lmx-1b* does

Figure 5.18 Comparison of *Lmx-1b* expression with dorso-ventral pattern of tendon in 14.5 day forelimb of *Wnt-7a* mutant

Expression pattern of *Lmx-1b* described by Cygan et al (1997) is compared and contrasted with dorso-ventral pattern of tendon at proximal and distal levels observed in *Wnt-7a* knockout by using *EphA4* as a marker.

Comparison of Lmx-1b expression with dorso-ventral pattern of tendon in 14.5 day forelimb of Wnt-7a mutant

A ⟷ P

	Lmx-1/Tend pattn	Lmx-1/Tend pattern	Predicted tend pattn
Digit 2			
distal	- dv	absent = dv	vv
proximal	- vv	absent = vv	vv
Digit 3			
distal	- + vv	absent/present = vv	
proximal	- + vv	absent/present = vv	
Digit 4			
distal	- dv	absent =dv	vv
proximal	+ vv	present = vv	dv
Digit 5			
distal	- dv	absent = dv	vv
proximal	+ vv	present = vv	dv

A, anterior, P, posterior, **dv, dorso-ventral; vv, bi-ventral;**
tend pattn, tendon pattern

▭ expression of Lmx-1b in dorsal mesenchyme of *Wnt-7a* null mutant (Cygan et al 1997)

▭ tendon - **seen in this thesis**

Table 5.8 Expression of Lmx-1 in wild type and mouse mutants
(Cygan et al '97)

Type	Upto 11.5 dpc	At 11.5 dpc	At 14.5 dpc
Wild type	Dorsal mesenchyme	Dorsal mesenchyme	
Wnt-7a -/-	Dorsal mesenchyme	D-Lost in distal anterior # and around lateral margin *	Digit 1 D-absent distal most,# sometimes totally absent Digit 2 always absent Digit 3 D-absent anterior half, sometimes totally absent Digit4 D-absent distal most * Digit 5 D-absent distal most *
En-1 -/-		Dorsal mesenchyme V-ectopic proximal and distal but patchy distal ventral, highest at anterior distal edge ~	Digit 1 D-normal expression V- ectopic expression Digit 2 D-absent distal tip, V-ectopic expression Digit 3 D-absent distal tip, V-ectopic expression Digit4 D-normal exp in mesench V-ectopic expression anterior half Digit 5
Double mutant		D-Lost in distal anterior and around lateral margin similar to that seen in Wnt-7a -/- but weaker V-no ectopic expression	Digit 1 absent throughout Digit 2 absent throughout Digit 3 absent throughout Digit 4 absent anterior half Digit 5

\# At 11.5 expression in Wnt 7a knockout is lost anterior distal and expression at 14.5 is lost at distal tip of digit 1.

* At 11.5 expression in Wnt 7a knockout is lost at lateral margin and so also at 14.5 expression is lost at distal tip of digit 4 and 5.

~ Note that at 11.5 ectopic ventral expression in En-1 knockout is highest at anterior distal, the same site where Lmx-1b is lost in Wnt-7a mutants.

not correspond precisely with the dorso-ventral pattern of tendons in the mutant. During development, the developing tendon is displaced deeper into the mesenchyme hence it may be out of range of ectodermal signals and instead maybe influenced by signals from the surrounding mesenchyme. Hence the difference between proximal and distal regions of tendons. The *Lmx-1b* mutant is said to have dorsal-ventral transformations from tip to distal zeugopod (Chen et al 1998). However sufficient details are not available to allow a comparison with my analysis of the *Wnt-7a* mutant.

5.4.5 *Wnt-7a* signalling and timing of dorso-ventral tendon specification

Expression patterns of EphA4 in tendons in both forelimb and hindlimb of 12.5, 13.5 and 14.5 day wild type embryos were compared with expression patterns in the mutants of the same age. Up to and including 12.5 day of development, expression of *EphA4* showed a differential dorso-ventral pattern of EphA4 expression.

At 13.5 dpc, tendons in digits 2, 3 and 4 of the mutant forelimb show a bi-ventral pattern of expression proximally (Table 5.4) and distally as well in digit 3. In the hindlimb, digit 3 has bi-ventral pattern of expression proximally but there is a differential pattern of *EphA4* expression associated with digit 3 distally and in all other digits. Thus early expression of *EphA4* is unchanged dorsally, and ventral tendons develop normally. At day 12.5 dorsal tendons as such do not seem to have developed, however the dorsal expression of *EphA4* does not resemble ventral expression. Within one day (day 13.5), tendon pattern in digit 3 is bi-ventral. However it should be noted that tendons of digit 2 and 4, even after a further 24 hours of development i.e. at day 14.5, do not become bi-ventral distally. The effects of absence of *Wnt-7a* signalling are manifested at day 13.5 as either a thicker layer or a more rounded shape dorsally. It should be noted that prior to 11.5 days, *Lmx-1b* expression in *Wnt-7a* mutant is normal and expressed in the whole of the dorsal mesenchyme (Cygan et al 1997) but at day 11.5, expression is reduced in the digit plate (Table 5.8). Therefore in the *Wnt-7a* mutant, *Lmx-1b* expression is reduced at the time tendons are forming.

Dorso-ventral alteration in tendon pattern in the *Wnt-7a* mutant occurs proximally first and then distally. This is in agreement with the idea that development of tendon proceeds from proximal to distal. Development of tendons in chick legs start first in digit 3 (the longest digit) and then in other digits (Hurle et al 1990). Chick leg tendons begin to develop at stage 29, and this is equivalent to day 13 in mouse embryo development (Table 5.9). Hence shorter digits may not have as yet established dorso-

Table 5.9 Mouse stages comparable to chick (Wanek et al 1989)

Mouse limb stage	Chick wing stage[1]	Approx gestation	mouse time[2]
		Forelimb	Hindlimb
1	18	E10	E10/11
2	19/20	E10/11	E10/11
3	20/21	E11	E11
4	22/23	E11	E12
5	24/25	E12	E12
6	25	E12	E12
7	26/27	E12	E13
8	28/29	E13	E13
9	30/31	E13	E14
10	32/33	E14	E15
11	34	E15	E15
12	35	E16	E16
13	36	E17-19	E17-19
14	37	PN1-4	PN1-4
15	38/39	PN5	PN5

[1] Hamburger and Hamilton 1951
[2] Cervical plug = E1

ventral patterning,or in the case of *Wnt-7a* knockout, dorso-ventral transformation and this may explain why *EphA4* expression in digits 2 and 4 is not bi-ventral. It would be interesting to see if, at later stages, the distal region of all digital tendons are transformed. Although Parr and McMahon (1995) have examined the *Wnt-7a* knockout at later stages not enough details have been reported to make a comparison between different digits along the entire proximo-distal axis.

5.4.6 Does *Wnt-7a* have a role in proximo-distal outgrowth?

Digits in the mutant embryonic forelimb become round and blunt at the tips in contrast to the constricted digit tips in the wild type. Nails in the new born *Wnt-7a* mutant were reported to be truncated (Parr and McMahon 1995). It was also noted that, in early mutant limb buds, there was some loss of *Fgf 4* expression in apical ridge and expression of *Shh* was reduced. The finding here that the digits are round and blunt is consistent with the idea that *Wnt-7a* has some control over the outgrowth of digits along the proximo-distal axis as well as dorso-ventral patterning. However it appears that this effect acts late in development as the digits at first appear to be normal in shape and length.

5.4.7 Dorso-ventral alteration of tendons occurs late after *Lmx-1* misexpression in chick

Up to 10 days of incubation, *Lmx-1* infected limbs did not show any visible alteration in dorso-ventral patterning of tendons. Expression of *EphA4* was normal and histological analysis also did not show any change in ventral tendons. Hence although one cannot discount the fact that there could have been insufficient infection, it is hard to believe that it could happen in 70 embryos. 70% of limb buds (7/10) examined for infection using *Lmx-1* antibody had complete infection in dorsal and ventral mesenchyme at stage 24/25. A total of 55 limbs were examined for EphA4 expression pattern at different periods after injection and 15 limbs were sectioned for histological study between stages 33-36. None of these limbs showed any dorso-ventral alteration. Normally, *Lmx-1* is expressed in dorsal mesenchyme of the limb bud from the beginning of bud outgrowth (Table 5.3) and in this thesis I have shown that it is present up to stage 36. Thus *Lmx-1* could potentially play a role in dorsalisation of structures late in limb development. Riddle et al (1995) reported dorso-ventral alterations at day 11 (stage 37) and Vogel et al (1995) at day 14 but it was not indicated whether any alterations occurred earlier. Thus it is possible that ventral tendon cannot respond to

ectopic *Lmx-1* during early development. In new born *Lmx-1*b null mutants there are alterations in dorso-ventral patterning but alterations if any, earlier in development was not described (Chen et al 1998). When *Lmx-1* is repressed in chicks, some dorsal muscles were either smaller or absent. However the report pertains to late stages (Rodriguez-Esteban et al 1998).

I found that structures that develop later in limb development, such as ectodermally-derived structures like scale and nail, that are seen only after stage 36 (10 days), can be re-patterned by ectopic *Lmx-1* in ventral mesenchyme. The ventral surfaces of infected digits in 2/3 embryos that have dorso-ventral alterations at day 14, show atypical scale patterns (Table 5.5). The skin was either smooth throughout or had dorsal scale pattern distally and ventral pattern proximally. During limb development, the earliest signs of scale on ventral side are ridges on ventral proximal pads at day 12 and at day 13 scales appear on proximal pads while distal pads are smooth. At day 14, the entire ventral skin is covered with small ventral scales. Hence ventral scale-forming cells are exposed to ventralising signals directly or indirectly from either ectoderm or mesoderm. In *Lmx-1* misexpressed limbs, they are exposed in addition to the mesenchyme-dorsalising factor *Lmx-1*. As complete infection occurs between day 4 – 4 ½ (stage 24/25) the dorsalising influence can occur only from this stage onwards. Here I found that at least 1 digit had ventral scales proximally plus dorsal scales distally on ventral side. Since digits develop in a proximo-distal sequence, it seems likely that the distal scale forming cells will have been exposed to dorsalising factor for longer and that this may be necessary to re-programme ventral cells. The smooth ectoderm in intermediate region of digit may represent an intermediate stage in dorsalisation. Normally by day 14 the entire ventral surface has ventral scales. Hence in *Lmx-1* infected limbs, the skin may have been patterned as ventral to begin with, then re-specified to form smooth surface in intermediate region of digit and finally dorsalised distally.

5.4.8 *Lmx-1* is required for nail formation

Primordia of claws in the chick are just visible in the wing at stage 36 (day 10). In chick, claws in the toes are seen as flattened ventrally curved structures at day 11. In this work double nails develop in toes when *Lmx-1* is misexpressed in chick embryos. Results here suggest that conical nails arise due to development of a ventral nail which later fuses with the dorsal nail in a proximo-distal direction.

Conical nails are reported in *Wnt-7a* and *Wnt-7a/En-1*$^{-/-}$ double mouse mutants and are also seen in chick *eudiplopodia.* Cylindrical nails have been reported in *En-1* mutants. Claws with a ventral convexity and a dorsal curvature have been reported before (Vogel et al 1995), however nails in this study were never dorsally curved. Double nails have not been reported in other work when *Lmx-1* was misexpresed in chick limbs (Riddle et al 1995, Vogel et al 1995). The double *Wnt-7a*/En-1$^{-/-}$ mutants in the post natal period also develop hard nail-like surfaces on ventral pads indicating that En-1 has a nail suppressing role in association with the pads (Loomis et al 1996, 1998) (Table 5.1). However the fact that a nail which is a dorsal structure develops on the ventral side when *Lmx-1* is misexpressed indicates that *Lmx-1* has altered the fate of ventral ectodermal cells probably by ectoderm-mesodermal interaction. Another possibility is that ectopic *Lmx-1* can produce this effect, only because En-1 is no longer expressed in ventral ectoderm at later stages. However it has been shown that *Lmx-1* misexpression does not alter the expression of *En-1* in ventral ectoderm (Vogel et al 1995).

Double nails seen here fuse in a proximo-distal direction which is evident as 2 separate entities at distal tips in contrast to fused proximal regions. This indicates that "conical", "cylindrical" nails and truncated nails that develop at the dorso-ventral interface reported in mouse and chick mutants are really double nails that fuse across the dorso-ventral axis to form one mass. In the *eudiplopodia* chicken mutant (Goetinck 1964) extra digits arise on dorsal surface of the limb and these have conical nails as well. These extra digits are covered with dorsal ectoderm on both sides and hence mesenchyme would receive dorsalising signals from the ectoderm on both sides, which could lead to two nails arising which then fuse to form conical nails.

Nail Patella Syndrome (NPS) (Dreyer et al 1998) is a dominantly inherited skeletal malformation seen in humans (Dreyer et al 1998) and *Lmx-1b* maps to the NPS locus and patients have hypoplastic nails. Similarly when *Lmx-1* is inhibited in chick embryos, claws were reduced (Rodriguez-Esteban et al 1998). Hence overall it appears that *Lmx-1* is necessary for nail formation.

5.4.9 Role of ectopic *Lmx-1* in dorso-ventral patterning of tendons

Formation of long tendons takes place concomitantly with the establishment of skeletal elements of the phalanges of the digits (Wortham 1948; Sullivan 1962; Hurle et al 1990). Histologically it was shown that tendons are formed from day 6 (stage 29) of development (Hurle et al 1990). According to Wortham (1948) tendons do not complete

their insertions to the bone until 11th day, when all skeletal elements are developed in cartilage. According to Kardon (1998) digital tendon makes connection with the distal phalanx by stage 35 and corroboration for this comes from work in this thesis, where expression pattern of *EphA4* at interphalangeal joint levels show that all phalanges are formed by stage 35 (chapter 3). Thus the whole length of the tendon is formed by stage 35 (day 9) and thereafter the tendon just has to grow in length in concert with growth of cartilage.

Chick limbs in which *Lmx-1* is misexpressed in ventral mesoderm did not show any histological or molecular alteration in dorso-ventral pattern of tendons up to day 10 of development (stage 36). Normally ventral tendon is round and dorsal tendon is flat in transverse section. The question then is, how the double dorsal tendon pattern seen at day 14 arises. It seems likely that distal region of ventral tendon was re-structured and changed from a rounded to a flattened shape between day 10 and 14. This suggests to me a continuing interplay between tendon and surrounding tissue during later stages of development.

5.4.10 Does *Lmx-1* have a role in cartilage pattern?

As a result of ectopic expression of *Lmx1* in the ventral mesenchyme it has been shown previously that either dorsal structures are present on ventral side or ventral structures are lost (Riddle et al 1995; Vogel et al 1995). Presence of dorsal structures on ventral side include dorsal type scales, increased number of feathers, extensor tendons (Riddle et al 1995; Vogel et al 1995; reported in this work) and nails (reported in this work). Cylindrical cartilage in cross-section has also been reported suggesting a bi-dorsal pattern because ventral surface of cartilage is normally flatter than the dorsal convex surface. Symmetrical cartilagenous protuberances has been reported (Vogel et al 1995) and also seen in this work. Although Vogel et al (1995) call it a bi-dorsal pattern, tubercle-like protuberances are normally present on the ventral side and are related to the attachment of ventral tendons. In limbs infected with *Lmx-1,* one would not expect a bi-ventral pattern i.e. appearance of a ventral protuberance on the dorsal side. A bi-ventral pattern is reported in the mouse *Wnt-7a* mutant, for example, sesamoid bones normally present on ventral side are present on the dorsal side also (Parr and McMahon 1995). Hence it is not clear how the ectopic presence of a dorsalising factor can produce an apparently bi-ventral cartilage pattern.

Competitive inhibition of *Lmx1* activity in chick inhibits dorsal cell fate (Rodriguez-Esteban et al 1998) and, overall, dorsal features are lost or reduced. These

include changes in epidermal differentiation such as reduced feather density, partial loss of dorsal scales with presence of ventral type scales on dorsal side, and reduction of claws. There is reduction in other distal tissues e.g. interosseus dorsalis and extensor medius brevis muscles are absent. In some cases, additional ventral structures such as ventral muscle flexor indicis are present on dorsal side. This suggests that absence or reduced *Lmx-1* alone can produce ventral structures or the expression of a ventrally restricted gene, now expands in the absence of *Lmx-1*. These *Lmx-1* reduced legs also showed an abnormal ventral curvature of the whole limb (Rodriguez-Esteban et al 1998) suggestive of defects in more proximal regions.

5.4.11 Role of *Wnt-7a* and *Lmx-1* in dorso-ventral patterning of digital tendons

Overall the comparative study of expression pattern of *Wnt-7a*, *Lmx-1* and *EphA4* in tendons shows that *Wnt-7a* is expressed in ectoderm overlying tendon forming mesenchyme up to stage 26. Thereafter as digits form expression is reduced in the midline of digits but persists away from the midline in ectoderm overlying the dorso-lateral sides of the developing digit. *Lmx-1* is expressed in tendon forming mesenchyme up to stage 29 and thereafter there is reduction. When tendon gets displaced deeper into mesenchyme, expression of *Lmx-1* becomes strong in mesenchyme that intervenes between tendon and ectoderm and persists upto stage 36. *EphA4* is expressed in developing tendon when it is subjacent to ectoderm and as it moves deep into mesenchyme (Fig. 5.19). This study hence suggests that *Wnt-7a* may have a role in dorso-ventral patterning of tendons in early stages whereas *Lmx-1* may have a role in early and late stages. In early stages *Lmx-1* may have direct dorsalising effect on tendon forming cells and later *Lmx-1* may have an indirect role on dorso-ventral patterning of tendon via the mesenchymal tissue that surrounds the tendon. Support for the latter role of *Lmx-1* in tendons, comes from the study of tendons in chick legs when *Lmx-1* is misexpressed in ventral mesenchyme (this thesis). Although the embryo is infected at stage 10 and is found to be completely infected at stage 24/25 (day 4-4 ½) there is no alteration in dorso-ventral pattern of tendon even at stage 36 (day 10). Altered shape of ventral tendon is seen at day 14 indicating a late role for *Lmx-1* in dorso-ventral patterning probably through an interaction between tendon and mesenchymal tissue surrounding tendon.

My detailed study of *EphA4* expression in tendons of the *Wnt-7a* mutant suggests that *Wnt-7a* has a very early role in specifying dorsal tendon (Fig. 5.20). In the absence of *Wnt-7a* there is an increase in the thickness of tendon-forming cell aggregation on the

Figure 5.19 Time course of expression of *Wnt-7a* and *Lmx-1* in relation to tendon formation in chick leg

Wnt-7a (red line) is expressed in dorsal ectoderm from stage 15. When digits and tendons start to form *Wnt-7a* (red dotted line) expression recedes from the ectoderm overlying developing tendons.

Lmx-1 (green line) is expressed in dorsal mesoderm from stage 15 and is expressed in sub-ectodermal mesenchyme in cells that form tendon as they develop under ectoderm. As tendons start to move away from ectoderm at stage 29, *Lmx-1* is expressed in connective tissue that is around the dorsal and sides of the developing tendon. This suggests that *Lmx-1* may have a direct role in patterning tendons in early stages and in later stages may do so indirectly through surrounding connective tissue.

EphA4 (blue dotted line) is expressed in limb bud mesenchyme from stage 17 and later at stage 27 becomes confined to tendon forming regions as it forms under the ectoderm and as it moves away from ectoderm (blue line).

Fig. 5.19

Time course of expression *Wnt-7a* and *Lmx-1* in relation to tendon formation in chick leg

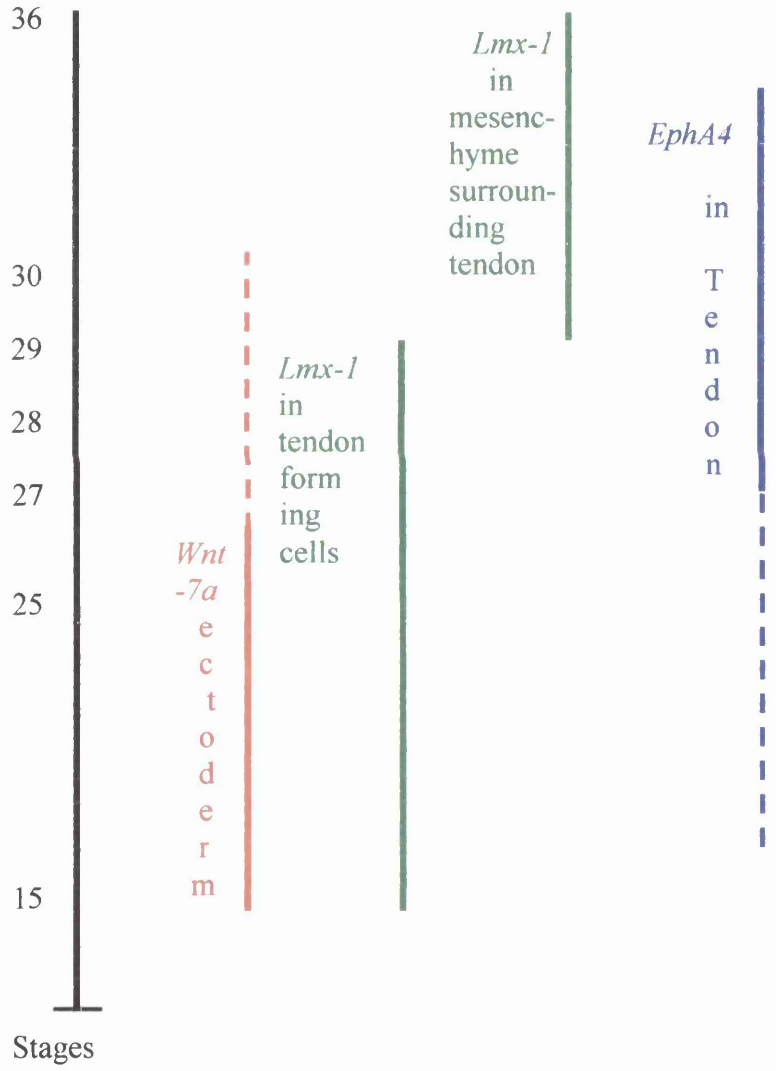


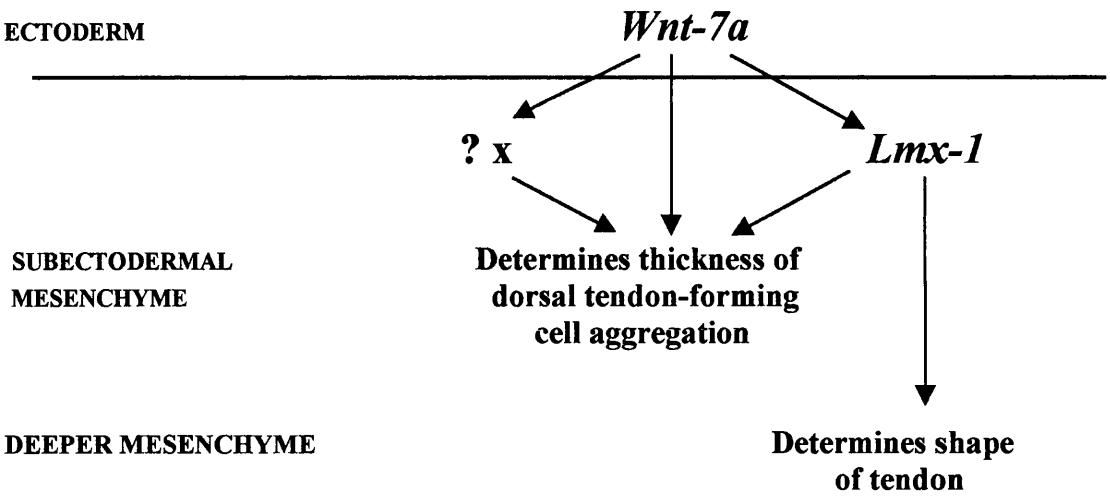
Figure 5.20 Diagram showing a model for how dorsal tendons are patterned by *Wnt-7a* and *Lmx-1*

The model is based on the analysis of expression patterns in chick leg and results obtained from misexpression of *Lmx-1* and analysis of tendon pattern in *Wnt-7a*$^{-/-}$ mutant.

Wnt-7a expressed in dorsal ectoderm induces *Lmx-1* in dorsal mesenchyme. Expression of *Lmx-1* coud affect sub ectodermal mesenchyme cell behaviour in a cell autonomous way at early stages, to determine the thickness of the tendon cell aggregation. Later, persistent expression of *Lmx-1* in deeper mesenchyme that surrounds dorsal tendon defines dorsal tendon shape. *Wnt-7a* may also act directly (or indirectly) independent of *Lmx-1* expression to determine the thickness of the tendon forming cell aggregation.

Fig. 5.20

Model for roles *Wnt-7a* and *Lmx-1* in specifying dorsal tendons



dorsal side which now therefore resembles that seen on the ventral side. There is additional evidence for a mechanism involving direct influence of *Wnt-7a* independent of *Lmx-1* from comparing my analysis of dorso-ventral pattern of tendons in *Wnt-7a* knockout and the expression of *Lmx-1b* described by Cygan et (1997). Thus it appears that the absence of *Wnt-7a* either directly or indirectly can influence dorsal tendon patterning through a pathway independent of *Lmx-1*.

CHAPTER SIX

A POLYDACTYLOUS HUMAN FOOT WITH "DOUBLE-DORSAL" TOES

Chapter Six: A Polydactylous Human Foot with"double-dorsal" toes

6.1 Introduction

Anomalies are often characterized by absence of structures or by the formation of additional structures, and usually originate in humans in the first trimester of pregnancy. Deformities can be genetically determined or can occur due to external influences. Common anomalies in the foot are excesses (e.g. polydactyly) or deficiencies (e.g. absent digits) or fusion (e.g. syndactyly). In many cases, the causes of these human malformations are not known. Experimental analysis of chick limb development over the last 50 years has considerably advanced the understanding of the general mechanisms that pattern limbs and much of this knowledge is believed to be relevant to human limb development. In addition, molecules that mediate interactions in developing limbs have been identified recently and this has allowed a direct link to be made to clinical genetics (see general introduction).

Here we report the anatomy of a human polydactylous left foot. We try to give an identity to the nine toes, for which a study of the muscles, tendons and skeleton of the foot was undertaken by gross dissection and by magnetic resonance (MR) and X-ray imaging. A possible explanation for how the peculiarities seen in this foot may have arisen is given. The peculiarities consist of supernumerary toes and toes with "double-dorsal" features i.e. 2 extensor tendons, no flexor tendons and conical nails, instead of dorsally placed flat nails. These two features, supernumerary toes and "double-dorsal" toes suggest to us abnormalities in the antero-posterior (rostral-caudal) and dorso-ventral patterning of the limb respectively. For digit specification in the early leg bud, antero-posterior (rostral-caudal) refers to the "big toe-little toe" axis whereas for descriptions of the gross anatomy of the child's foot after rotation has occurred, we use medio-lateral to refer to this axis. While there have been many reports of polydactyly (Davidson 1918, Warkany 1971 Phelps and Grogan 1985, Hesselschwerdt and Heisel 1990, Wang et al 1996) including central polydactyly and a few reports of palmar nails (Al-Qattan et al 1997), there have been no previous reports of polydactyly associated with "double-dorsal" features. What is of particular interest is that "double-dorsal" and "double-ventral" paws recently been described in mice in which genes that are

expressed either ventrally or dorsally respectively, have been functionally inactivated (Loomis et al 1996, Parr and McMahon 1995).

6.2 Materials and methods

X-rays were taken in different views and then MRI (magnetic resonance image) scanning was done. The foot was dissected to study muscles and tendons. After having taken photographs, the skeleton was dissected and a diagram was made.

6.3 Results

6.3.1 Case history

A female infant was born in 1985 with a severely abnormal polydactylous left foot with 9 toes. Other deformities of the left lower limb included soft tissue hypertrophy of the left thigh and knee. Pre-operative x-ray examination showed the left tibia to be 5mm shorter than the right. There were no other morphological abnormalities and chromosome studies were normal. There was no known family history relevant to this abnormality, the pregnancy was uneventful and no known teratogens were taken. At the age of eleven months, the child underwent a through ankle amputation.

6.3.2 External appearance of the foot

The foot (Fig. 6.1a-dorsal view) has nine toes and is a peculiar shape. The toes were labeled A-I from medial to lateral (Fig. 6.1a,1b-end on view). The dorsal surface is sloping on two sides (Fig. 6.1a, b) and the plantar surfaces are apposed (Fig. 6.1b). As the dorsal surface has 2 slopes, for convenience in description, we will refer to the dorsal surface as a medial part which consists of toes "A" to "E" and a lateral part consisting of toes "E" to "I"(Fig. 6.1b). MR imaging show that the foot has a Y shape cross section (Fig. 6.2b, c) The stem of the Y contains the toes labeled "D" and "E" (Fig. 6.2b, c) and both are rather different in morphology from the rest of the toes, having conical nails with a round cross section situated at the tip of the toes (Fig. 6.1b). All the other toes have nails that are flat and situated on the dorsal aspect (Fig. 6.1b). **Toe D** is rather peculiar in that, the base of the metatarsal is next to the base of metatarsal of toe C (Fig. 6.2d, 2e) but the head is deviated laterally(Fig. 6.2c, 2e) and

so the metatarsal is obliquely placed leaving a gap between its distal end and that of toe C (Fig. 6.2e). Alhough the head of the metatarsal is more lateral, the actual toe is bent medially (Fig. 6.4b) **Toe E** is at the dorsal-most part of the dorsum (Fig. 6.1a,b, 2b, c).

6.3.3 Description of the muscles and tendons

Medial part of dorsal surface (Toes A-E)

There is a common thick tendon (Fig. 6.1c- Edl) on this aspect of the dorsum giving rise to extensor digitorum longus tendons for toes B, C, D and E. Toe A has its own extensor hallucis longus (Fig. 6.1d-ehl). Proximally and more laterally just deep to the extensor digitorum longus tendons, there is a muscle mass - the extensor digitorum brevis muscle (Fig. 6.1d-edb) giving rise to 3 tendons for toes A, B and C (Fig. 6.1d-arrowheads).

Toe A resembles a normal great toe (Fig. 6.1b) being rather broader than the other toes. It has a long extensor tendon going to the terminal phalanx- the extensor hallucis longus (Fig. 6.1d-ehl) and a smaller tendon deep to it, coming from the extensor digitorum brevis (Fig. 6.1d-arrowhead). A tendon is also attached to the base of the metatarsal- the tibialis anterior (Fig. 6.1d). **Toes B and C** resemble normal 2nd and 3rd toes respectively (Fig. 6.1b, c).Extensor digitorum longus tendons are attached to the distal phalanges of each toe and also smaller tendons coming from the extensor digitorum brevis muscle. Toe B has the insertions of 2 dorsal interossei (Fig. 6.3a), one on the medial and the other on its lateral side. Here we refer to the phalangeal insertions in our description. Dorsal interossei are bipennate at their origin from adjacent sides of metatarsals and insert into the proximal phalanx of the toe on which they act. Thus all metatarsals give origin to dorsal interrossei but not all toes receive an insertion (Fig. 6.3a). Toe C lacks such an attachment (Fig. 6.3a). **Toe D** receives a tendon from the common extensor digitorum longus tendon (Fig. 6.1c-Edl) on this aspect. It also receives another extensor tendon from the lateral part (Fig. 6.1c) of the dorsal aspect which does not go to the phalanx but is attached to the shaft of the metatarsal. There is no dorsal interosseous associated with this toe (Fig. 6.3a). **Toe E** has tendons from extensor digitorum longus on both the medial (Fig. 6.1c-Edl) and lateral parts (Fig. 6.1c-edl) of the dorsal surface running all the way to the distal phalanx. At the base of the metatarsal two tendons of peroneus brevis are attached side by side (Fig. 6.1c). No dorsal interosseous is present (Fig. 6.3a).

Figure 6.1 Gross anatomical features in the human polydactylous foot

a) Dorsal view shows nine toes labelled A-I from medial to lateral. This surface has 2 slopes, one medially (m-d) and another laterally (l-d). The lines kl, pq, rs and xy are the planes through which scans in Fig. 6.2 a, b, c and d were done.

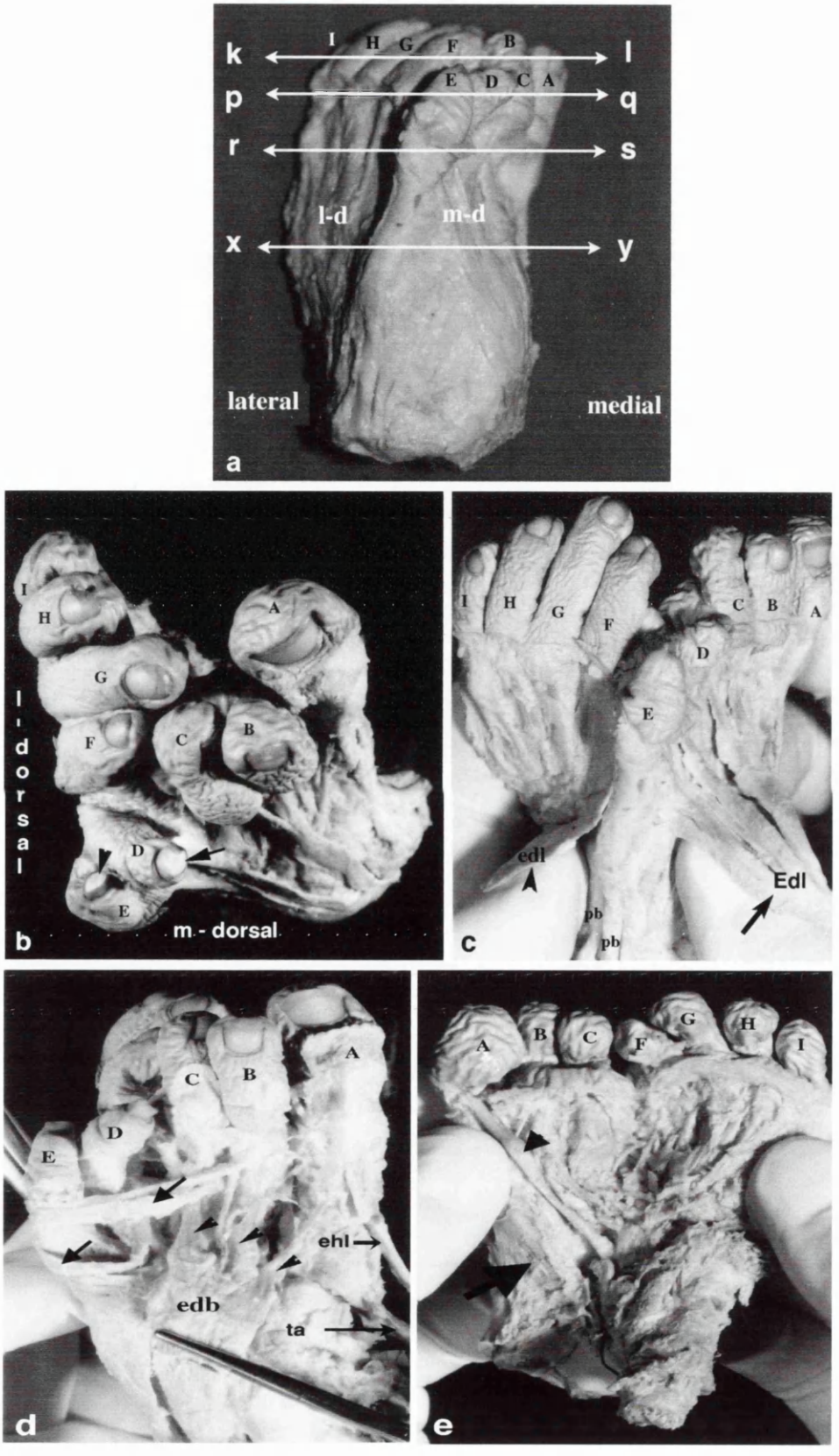
b) View from distal end. Toes are labelled A-I from medial to lateral. Toes are arranged in the shape of a Y with toes D and E in the stem of the Y. Toe E is at the summit of 2 sloping parts of the dorsal surface; the medial part from toe A to toe E (m-dorsal), the lateral part from toe E to toe I (l-dorsal). Plantar surfaces of the medial and lateral parts are almost facing each other. Arrow indicates the nail of toe D, cut to show its outline in cross section and arrowhead indicates that of toe E and these can be compared with the flat dorsal nails of toes A, B, C, F, G, H and I.

c) Dorsal view with toes labelled A-I from medial to lateral. Edl is the common tendon mass on the medial part of the dorsal surface which gives slips to toes B, C, D and E. edl is the common tendon mass on the lateral part dividing into tendinous slips for toes D, E, F, G, H and I. Toes D and E receive slips from both the medial (Edl) and lateral (edl) extensor digitorum longus tendon masses. Toe E receives 2 peroneus brevis tendons (pb).

d) Medial part of the dorsum with toes labeled A-E, ta, tibialis anterior; ehl, extensor hallucis longus; arrows, extensor digitorum longus tendons that are turned distally to show the extensor digitorum brevis muscle, edb with 3 slips (arrowheads) to toes A, B and C.

e) Plantar aspect of foot. Toes D and E cannot be seen. (arrowhead), flexor hallucis longus tendon; (arrow) short muscles of the big toe A.

Fig. 6.1


k
I H G F B
l
E D C A
p
q
r
s
l-d
m-d
x
y
lateral
medial
a
I
H
A
G
l-dorsal
F
C
B
D
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m - dorsal
I H G F C B A
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edl
Edl
pb
pb
c
A
C B
D
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ehl
edb
ta
d
A B C F G H I
e

Figure 6.2 Imaging and radiographic study of arrangement of skeletal elements

a-f, 3-dimensional MR scans; g, h, radiographs.

a) Through level k-l as seen in Fig. 6.1a. Toes D and E are not seen.

b) Through level p-q as seen in Fig. 6.1a. The Y shaped appearance of the foot in cross section can be made out with toes D and E forming the stem of the Y. Continuity of soft tissue between toes C and F (arrow) can be seen and toes D and E are dorsally situated with no plantar surface.

c) Through level r-s as seen in Fig. 6.1a. Arrow indicates a gap between the heads of metatarsal C and D due to the obliquity of metatarsal D.

d) Through level x-y as seen in Fig. 6.1a. Gap between toes C and D is less, as the base of the metatarsal D is situated normally.

e) Horizontal scan through the toes in the medial part of the foot, showing gap (asterisk) between the heads of metatarsal C and D and the proximity between the bases.

f) Horizontal scan through the medial part of the foot showing tarsal bones ***t*a**, talus; ***n***, navicular; ***cm***, medial cuneiform; ***ci***, intermediate cuneiform; ***cl*** lateral cuneiform. Talus articulates with the navicular. The 3 cuneiforms articulate proximally with the navicular, and distally with the metatarsals A, B and C. Note that the base of metatarsal B which was identified as digit 2 is more proximally situated than that of A and C.

g) X-Ray Dorsal-Plantar view of the foot. Proximal row of tarsals ***k***, ***l*** maybe equivalent to calcaneum in the normal foot and ***m*** the talus which articulates with navicular ***n***. In the distal row ***o***, ***p*** and ***q*** are smaller than the cuboid ***r***, lateral to it. ***s***, ***t*** and ***u*** are similar in shape and size to ***r***.

h) X-ray Plantar-Dorsal view. **k, l** and **m** are bones in the proximal row; **r, s, t** and **u** are in the distal row; C, D, E, F, G, H and I are the metatarsals . Note that the base of metacarpal F is at a more proximal level than G and thus appears to be digit 2.

Fig. 6.2

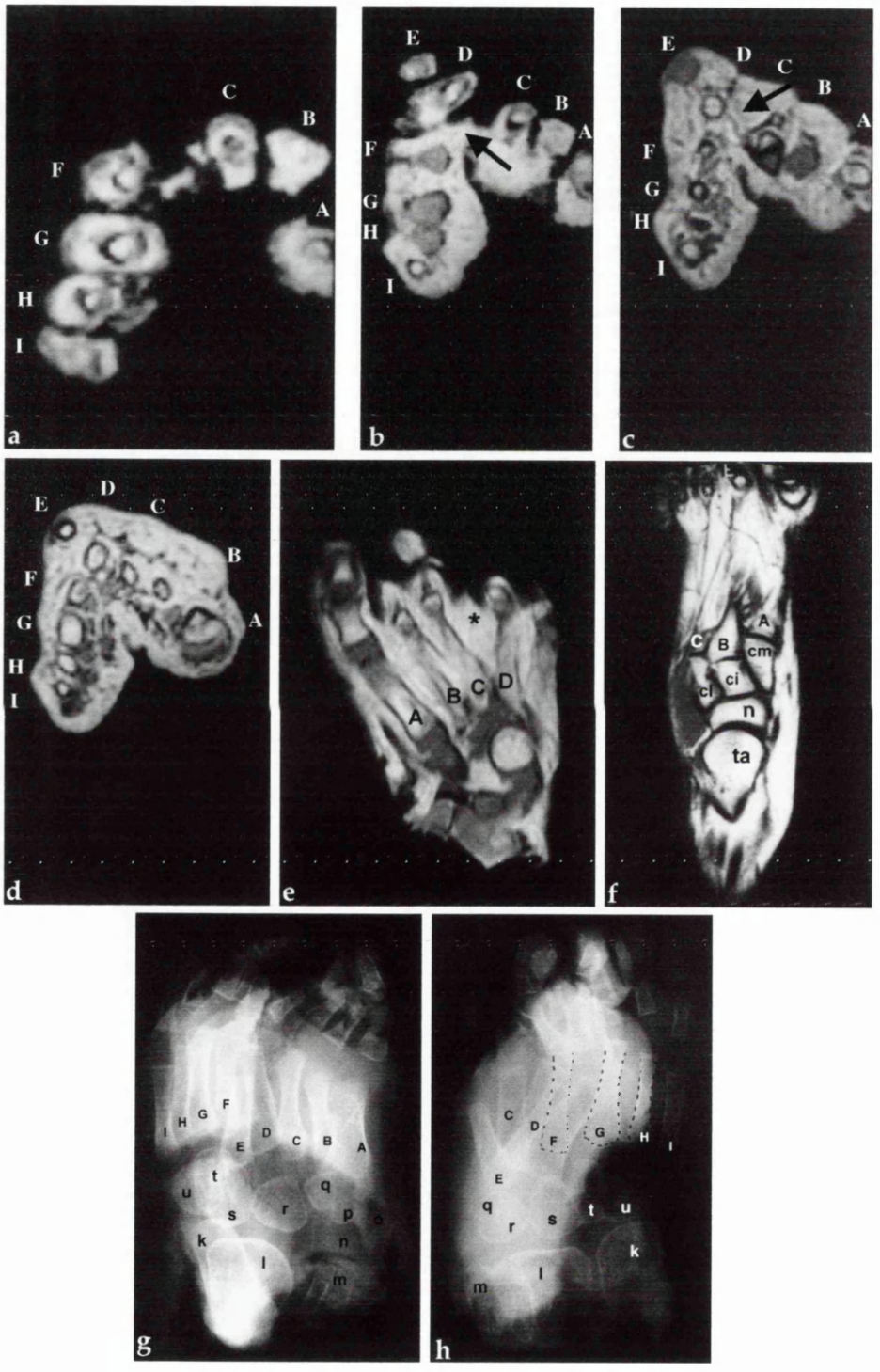
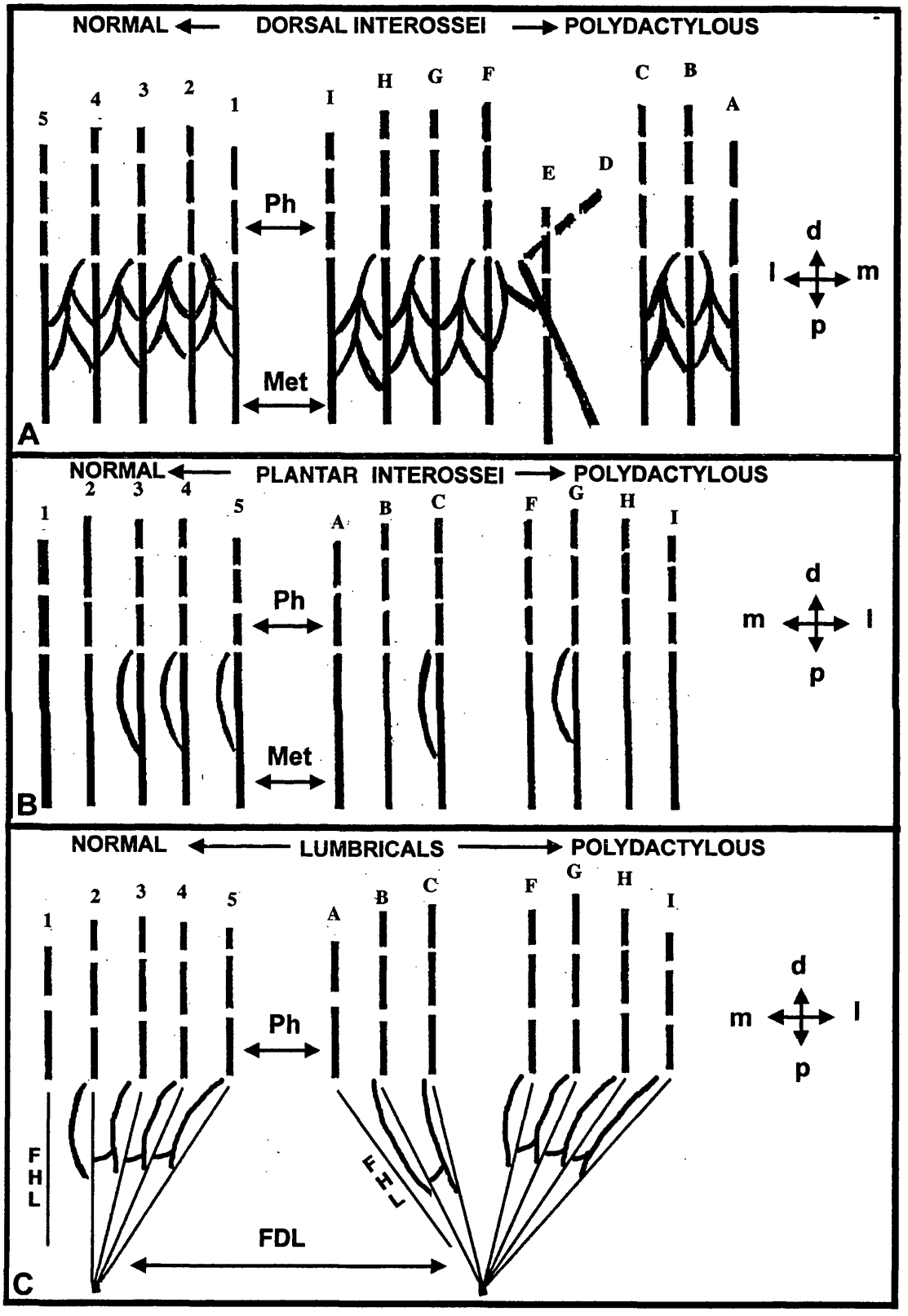


Figure 6.3 Line diagram representing the flexor tendons, interossei and lumbricals present in the normal foot and the polydactylous foot

Ph, phalanges; Met, metatarsals; FDL, flexor digitorum longus tendons; FHL, flexor hallucis longus tendon; m, medial; l, lateral; p, proximal; d, distal.

a) Attachment of dorsal interossei (curved lines) in the normal and the polydactylous foot. Note that toe B and F receive 2 dorsal interossei, thus identifying them as digit 2.
b) Attachment of plantar interossei (curved lines) in the normal and polydactylous foot.
c) Attachment of the lumbricals (curved lines) in the normal and the polydactylous foot. (modified after Chaurasia BD 1981)

Fig. 6.3


NORMAL
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NORMAL
LUMBRICALS
POLYDACTYLOUS
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Lateral part of the dorsal surface (Toes E-I)

Toes D, E, F, G, H and I all receive an extensor digitorum longus tendon from a common large tendon (Fig. 6.1c-edl) which splits into smaller ones distally and this is distinctly separate from the one on the medial part. Extensor digitorum brevis could not be made out on this lateral part of the dorsum. **Toe F** has the insertions of 2 dorsal interossei, one medial and one lateral and toes **G and H** have one each on the lateral aspect (Fig. 6.3a). **Toe I** has a tendon attached at the base of its metatarsal, reminiscent of peroneus brevis (not shown).

Plantar aspect

The cut end of an artery could be seen dividing into 3 branches but this was not dissected further. There are 2 main tendons proximally on this aspect, flexor hallucis longus(Fig. 6.1e-arrow-head) and flexor digitorum longus (Fig. 6.3c) that send a communicating slip to the hallucis tendon. The attachment of flexor accessorius muscle to flexor digitorum longus (not shown) can be made out.. There is plantar aponeurosis and flexor digitorum brevis muscle (not shown). A fibrous flexor sheath is present in toes A, B, C, F, G, H and I. Tibialis posterior is attached to the navicular (not shown).

Toe A has a muscle mass - the short muscles of the big toe (Fig. 6.1e-arrow) and a flexor hallucis longus (Fig. 6.1e) tendon going to the distal phalanx. Adductor hallucis (not shown) is present as a very thick sheet of muscle. **Toes B and C** each have a flexor digitorum longus (Fig. 6.3c) going to the distal phalanx. Both toes have a lumbrical on the medial side (Fig. 6.3c). **Toe C** has a plantar interosseous (Fig. 6.3b) on its medial aspect. **Toes D** and **E** do not have any flexor tendons. **Toes F, G, H and I** each have a long flexor tendon (Fig. 6.3c) and a lumbrical on the medial side(Fig. 6.3c). Tendons of flexor digitorum brevis could not be made out on any of these toes. **Toe G** has plantar interosseous on its medial side (Fig. 6.3b). **Toe I** has a muscle mass on its plantar aspect reminiscent of the short muscles of the little toe (not shown).

6.3.4 Skeleton

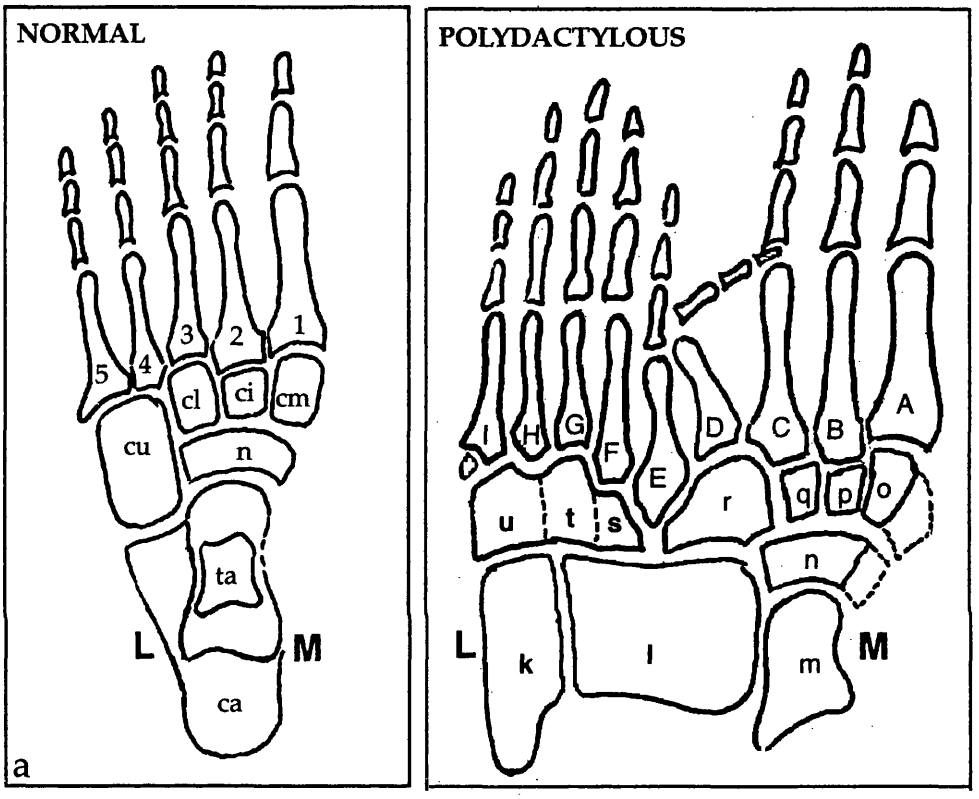
Gross dissection and study shows that all toes except toe A have 3 phalanges while toe A has 2 indicating that this was a digit 1. X-ray showed that there are 3 large tarsal bones k, l and m, in the proximal row (Fig. 6.2g), a navicular labelled 'n', in the intermediate row and 7 bones labeled o, p, q, r, s, t, and u in the distal row (Fig. 6.2g). Gross dissection showed that the 3 medial bones in the distal row are the 3 cuneiforms

Figure 6.4 Drawing of the dorsal view of the skeleton of foot

a) Normal foot. ca, calcaneum; ta, talus; n, navicular; cm, medial cuneiform; ci, intermediate cuneiform; cl, lateral cuneiform; cu, cuboid. The metatarsal bones are numbered 1-5 from medial, M to lateral, L.

b) Polydactylous foot. Proximally there are 3 bones **k, l** and **m. m** resembles the talus with a rounded distal end articulating with the navicular **n**. The 3 cuneiforms **o, p** and **q** articulate with the bases of metatarsals A, B and C. Note that the base of metatarsal B lies at a more proximal level than metatarsal A and C. The dotted parts of bones **n** and **o** are turned plantarwards. Bone **r** articulates with the base of metatarsal C, D and E. Bones **s t u** articulate with the bases of metatarsals E, F, G, H and I. The base of the metatarsal F is at a proximal level, and resembles toe B. Bones **o, p** and **q** resemble cuneiforms. Bone **r** though it appears to be of slightly different shape from a cuboid, articulates with metatarsal E which we have identified as a digit 5, and may be a cuboid. Bone **s, t, u** is one piece in gross dissection but are 3 separate bones as seen in the x-ray (Fig. 6.2g, h) where they resemble the bone **r,** in size and shape, and therefore seem to be cuboids.

Fig. 6.4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labeled, o, p and q, articulating with the base of the metatarsals of toe A, B and C (Fig. 6.4b). The base of metatarsal B lies at a more proximal level than Toe A or C (Fig. 6.4b). The bone 'r,' next to the lateral cuneiform 'q,' is the cuboid which articulates with the base of metatarsals C, D and E. The lateral 3 tarsal bones s, t and u, articulate with the base of the metatarsals E, F, G, H and I. It must be noted that the base of the metatarsal F (Fig. 6.2h, Fig. 6.4b) is lying distinctly, at a more proximal level than the base of metatarsals G, H and I, thus toe F resembles digit B (Fig. 6.2g) which was identified as digit 2. The 3 tarsal bones s, t and u, could be extra cuboids, as the X-ray (Fig. 6.2g) showed that they resemble the normal cuboid, r, which is next to the cuneiforms.

6.3.5 Digit Identity

Digit identity was assigned from the detailed examination of the muscles, tendons and skeleton of the foot.

Toe number	(lateral)	I	H	G	F	E	D	C	B	A (medial)
Digit identity	(lateral)	5	3/4	3/4	2	5	?	3/4	2	1 (medial)

It should be noted that toes D and E which are underlined are on a different plane from the rest of the toes (Fig. 6.2c, d). They are not seen when the foot is viewed from the plantar aspect (Fig. 6.1g).

6.3.6 Reasons for assigning digit identity

Toe A was assigned identity as **digit 1** by virtue of its size and morphological shape. It also has tibialis anterior attached at the base of the metatarsal. There are short muscles on its plantar side associated with the metatarsal. There are no dorsal or plantar interossei and lumbricals associated with it. It has only 2 phalanges.

Toe B was identified as **digit 2**. It has dorsal interossei on its medial and lateral sides and there is no plantar interosseous. Its articulation with the cuneiforms is at a more proximal level (Fig. 6.2g, Fig. 6.4b). **Toe C** could be **digit 3 or 4**. It has a plantar interosseous (Fig. 6.3b) and a lumbrical (Fig. 6.3c) on its medial side. **Toe D**. This toe has a long extensor tendon on the medial part of dorsum (Fig. 6.1c,-Edl) going to the distal phalanx and it also has an extensor on the lateral part of the dorsum (Fig. 6.1c - edl) which is attached to the metatarsal shaft, and this may be the reason why, it is bent almost 90° towards toe C (Fig. 6.4b). There are no tendons on its plantar aspect. No digit identity could be given to this toe.

Toe E receives two extensor tendons, one on the medial part of the dorsum (Fig. 6.1c- Edl) and one on the lateral part of the dorsum (Fig. 6.1C - edl) but there are no tendons on its plantar side. This toe however did have 2 peroneus brevis tendons (Fig. 6.1 C) and hence this toe was identified as **digit 5. Toe F** was identified as **digit 2** because it receives two dorsal interossei, one medial and one lateral (Fig. 6.3a). Its metatarsal articulates with the tarsal at a more proximal level than its neighbours (Fig. 6.2h, Fig. 6.4b). **Toes G and H** could not be given specific identity but because they have lumbricals and plantar interossei on the medial side and dorsal interossei on the lateral side, both could either be **digit 3 or digit 4. Toe I** was identified as **digit 5** because there is a tendon attached at the base of its metatarsal - the peroneus brevis and there is a short muscle mass on the plantar aspect of its metatarsal. There were no plantar or dorsal interossei. It might appear from the sequence of toes, that digit 2 (toe F) is next to a digit 5 (toe E) but this is not the case, as, toes D and E are pushed more dorsally (Fig. 6.2b)and digit 2 (toe F) is next to digit 3 (toe C)(Fig. 6.1e, 2a).

6.3.7 "Double-dorsal" toes

Figure 2b and c show that toes A, B, C, D and E are on the same plane, but at the same time it can also be noted that toes D and E are pushed more dorsally and toe F is next to toe C (Fig. 6.2b) on another plane. The nail in toes D and E are conical and placed at the tip of the toes (Fig. 6.1b) with a round cross section, whereas all the other toes have flat nails on the dorsal aspect (Fig. 6.1b). These two toes do not have any flexor tendons. Both toes receive two extensor digitorum longus tendons, one from the medial and the other from the lateral part of the dorsum. Toe E has two peroneus brevis tendons attached at the base of its metatarsal. Based on the above features we designated toes D and E as being "double-dorsal" with no characteristic plantar features. We did not note the type of skin surrounding toes D and E i.e. whether it is thin as is characteristic of dorsal skin or thick as is normally found ventrally.

6.4 Discussion

From the analysis just described we deduced that the toe sequence in the human polydactylous foot is 1, 2, 3/4, ?, 5, 2, 3/4, 3/4, 5 (from medial to lateral) and the **2 toes underlined** are "double-dorsal". We now wish to discuss how such abnormalities could have occurred.

6.4.1 Acquisition of extra digits

Additional digits could result from ectopic polarizing activity. The chick limb polarizing region is now known to express the gene *Sonic hedgehog Shh* (Riddle et al 1993). All vertebrate limb buds have a polarising region that governs anterior-posterior pattern. *Shh* is expressed in mammalian limb buds (Echelard et al 1993) and the human *Shh* gene has also been identified (Chang et al 1994). In several mouse polydactylous mutants (Masuya et al 1995, Chan et al 1995), an ectopic polarizing region expressing *Shh* has been detected at the anterior margin.

In chick embryos, when polarizing region is grafted from the posterior to the anterior margin of a leg bud, a duplicate pattern of toes 4 3 2 1 1 2 3 4 (reading anterior to posterior) results (Summerbell and Tickle 1977). When the additional polarizing region is grafted at the leg bud apex, signalling occurs in both anterior and posterior directions and digit patterns such as 1 2 3 4 4 3 4 can develop (Summerbell and Tickle 1977) (Fig. 6.5a). It should be noted that in these limbs, despite experimental manipulations, digits always develop in the same relative positions, so that digit 4 never forms next to digit 1.

In the human foot described here, if there had been an ectopic polarizing region anteriorly (rostrally i.e. near the position in which the big toe would normally form), mirror image digit patterns such as 54321/12345 or 5432 / 2345 would have been predicted. However this foot does not have a mirror image duplication. If there was an ectopic polarizing region at the apex of the limb bud, the digit sequence 1 2 3 4 5/4345 could result (Fig. 6.5b). This almost fits the pattern in the human foot, but digit F then should be a 4 instead of a digit 2 (Fig. 6.5c) because digit 2 in this position does not maintain correct digit order

Polyactylous mutants identified in mice include Alx-4, extra toes, hemimelic extra toes, Strong's luxoid, Rim-4. Many of these mutations show ectopic *Shh* expression (reviewed Niswander 1997) indicating that ectopic *Shh* results in polydactyly. Homozygous mice with targeted null mutation of *Alx-4* have preaxial polydactyly (Qu et al 1997; Hudson et al 1998). Recently it was shown *Strong's luxoid* (Chan et al 1995), a polydactylous mutant mouse, has a deletion of the homeobox region of Alx-4 protein and that there is a negative feedback loop between *Alx-4* and *Shh* in the limb (Takahashi et al 1998). Mutations in the human SHH gene and genes that encode its downstream signalling pathway may be responsible for Greig cephalopolysyndactyly

syndrome (GCPS) as loss of one copy of the human GLI-3 gene results in abnormalities similar to GCPS including preaxial and postaxial polysyndactyly. The polydactylous mouse mutant, *extra toes* (*xt*) contains a deletion in *Gli*-3 gene (Hui and Joyner 1993) and there is ectopic *Shh* expression (Buscher et al 1997). *Gli* genes encode proteins that are downstream in the *Shh* signalling pathway (Lee et al 1997). Recently it was shown that *Shh* expression was normal in the *doublefoot* (*dbf*) mutant, however there is ectopic expression of *ptc* , which is downstream in the *Shh* signalling pathway (Yang et al 1998). The most important feature in the *doublefoot* mutant is that digits that are duplicated are not anterior digits but posterior digits and this certainly is more akin to the human polyactylous foot described here. Recently it was shown that mice with mutations in *prx-1* and *prx-2*, closely related homeobox genes had preaxial polydactyly (Lu et al 1999) but *Shh* expression was normal.

Additional digits have been shown to result not only from ectopic polarizing activity but also from alterations in response to polarizing signals. Expression of genes in the 5' region of the *Hox-D* cluster from *Hoxd-9* to *Hoxd-13* can be regulated by polarizing region signalling (Izpisúa-Belmonte et al 1991). These genes are expressed in nested domains that overlap across the antero-posterior axis of early limb buds so that anterior (rostral) cells express just *Hoxd-9* and posterior(caudal) cells express *Hoxd-9* through *Hoxd-13* (Dollé et al 1989) (see chapter 1). When extra digits are formed there is induction of expression of more posterior *Hoxd* genes such as *Hoxd-11* and Hox*d-13* in anterior cells (Izpisúa-Belmonte et al 1991). In the chicken polydactylous *talpid* mutant, *Sonic hedgehog* is expressed at the posterior margin of the limb bud as normal (Francis-West et al 1995) but *Hoxd 13* is expressed uniformly across the limb bud (Izpisúa-Belmonte et al 1992) instead of being posteriorly restricted. There can be up to 10 digits in a talpid wing and they all have a uniform appearance with no distinct antero-posterior differences in morphology. Mutations in *Hoxd-13* have been found to be responsible for the human condition *Synpolydactyly*. In heterozygous individuals, this leads to the formation of a branched digit (Muragaki et al 1996). Targeted deletion of the *Hoxd* genes in mice phenocopies human synpolydactyly (Zákány and Duboule 1996). Additional digit primordia develop which later fuse with neighbouring original digit primordia to give branched digits. Since the digits in the foot described here are morphologically distinct and not branched, it seems unlikely that this polydactyly could be based on alterations in the response to polarizing signalling.

6.4.2 How to obtain "double-dorsal" digits

"Double-dorsal" toes can arise if toes develop from a limb bud covered on both sides by dorsal limb ectoderm and can be produced by grafting an extra apical ridge is on to the dorsal surface of a chick limb bud. As a result, a second outgrowth is induced from the dorsal surface of the chick wing bud covered on both sides with dorsal ectoderm and a "double-dorsal" pattern of tendons and muscles results (Saunders et al 1976, Shellswell and Wolpert 1977). In a chick mutant, *eudiplopodia,* there are accessory ridges on the dorsal aspect of the limb buds. Here also, extra digits are formed which have "double-dorsal" phenotype with conical nails (Goetinck 1964) reminiscent of those in this human foot. Recently, "double-dorsal" toes with conical nails have been seen in a transgenic mouse strain in which *engrailed-1*, a gene encoding a transcription factor is functionally inactivated (Loomis et al 1996; 1998). *Engrailed-1* is normally expressed in ventral ectoderm (Davis et al 1991, Gardner and Barald 1992) (Fig 1.3 chapter1). Another gene *Wnt-7a*, encoding a signalling molecule, is expressed in dorsal ectoderm (Dealy et al 1993, Parr et al 1993) (Fig 1.3 chapter 1) and activates expression of *Lmx1,* a transcription factor, in dorsal mesoderm (Riddle et al 1995, Vogel et al 1995). Ectopic expression of *Lmx1* in ventral mesoderm induces ectopic dorsal structures (Riddle et al 1995, Vogel et al 1995) and an additional nail is formed on the ventral side, which fuses across the dorso-ventral axis to form one mass (see chapter 5). The fusion of these 2 nails may result in the appearance of a conical nail. In Nail Patella Syndrome, a human dominant inherited skeletal malformation (Dreyer et al 1998), patients have hypoplastic or absent nails and the mammalian *Lmx-1*b maps to the NPS locus. Simlarly when *Lmx-1* was inhibited in chick limbs, there there was lack of dorsal structures, additional ventral structures and claws were reduced (Rodriguez-Esteban et al 1998). In mice with targeted disruption of *Lmx-1b*, dorsal structures are absent, ventral structures are duplicated and nails are hypoplastic. These observations suggest that *Lmx-1* may be involved in formation of nails.

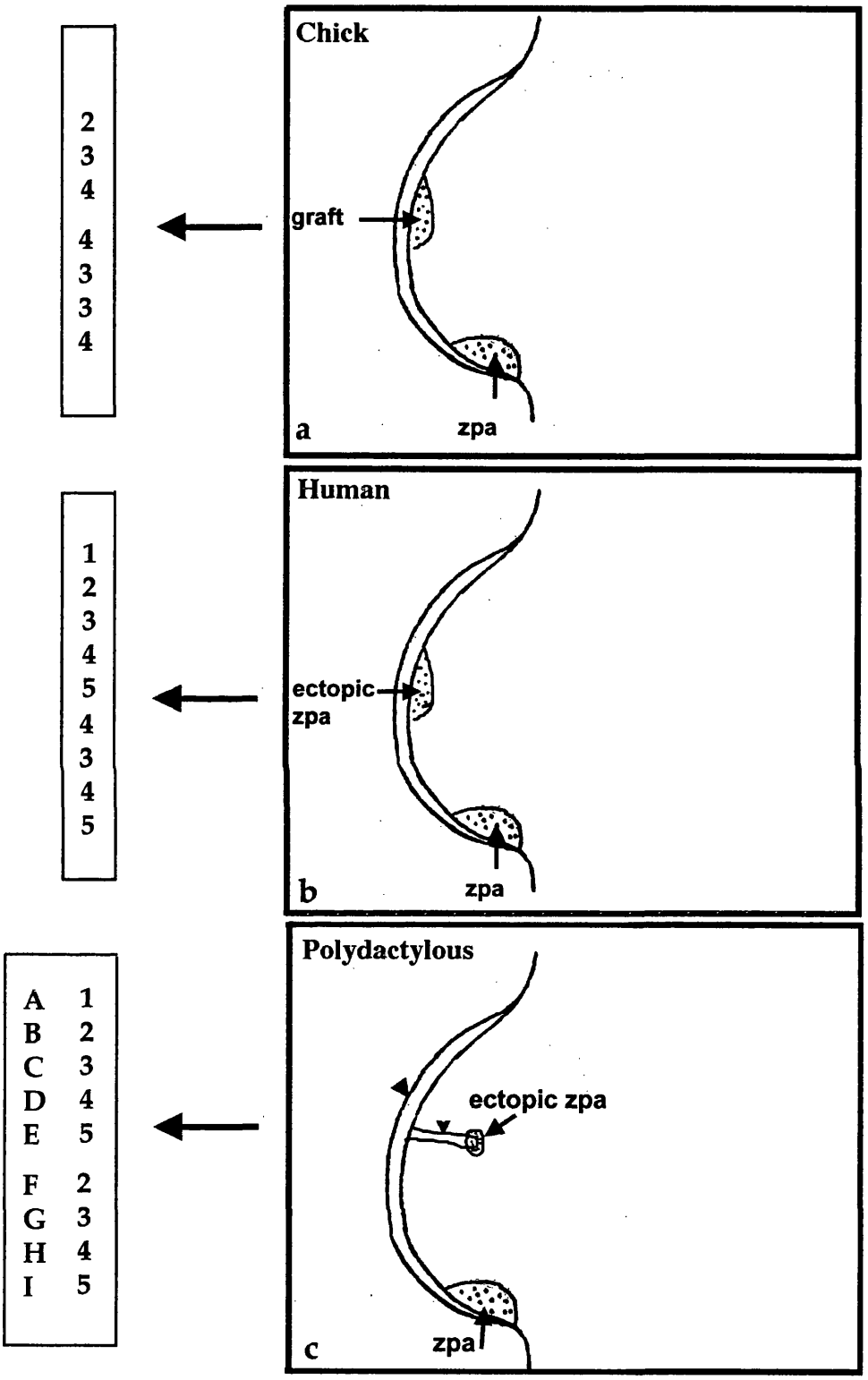
6.4.3 Polydactyly with "double-dorsal" toes

It is difficult to explain how one could obtain both additional digits and "double-dorsal" digits. This appears to require both changes in the distribution of polarizing activity and an ectopic ridge on the dorsal surface of the leg bud. It is still not clear however, why digit F, is a 2. Development of either a digit 3 or a digit 4 in this position

Figure 6.5 Possible ways of obtaining a human foot with polydactyly and "double dorsal" toes

a) Results that are obtained when a piece (graft) of polarizing region is grafted under the ridge at the apex of the chick limb bud giving a digit pattern of 234/4334. zpa, zone of polarizing activity. (Summerbell and Tickle 1977).
b) Predicted results that may be obtained if an ectopic zpa, zone of polarizing activity, were to occur at the apex of a human limb bud resulting in a 12345/4345 digit pattern.
c) Possible explanation of what could have happened in this human poldactylous foot. An accessory ridge (small arrowhead) on the dorsal aspect of the limb bud, perpendicular to the normal ridge (big arrowhead) associated with an ectopic zone of polarizing activity, and a normal zone of polarizing activity, zpa resulting in a 12345/2345 digit pattern.

Fig. 6.5


2
3
4
4
3
3
4
Chick
graft
zpa
a
1
2
3
4
5
4
3
4
5
Human
ectopic
zpa
zpa
b
A 1
B 2
C 3
D 4
E 5
F 2
G 3
H 4
I 5
Polydactylous
ectopic zpa
zpa
c

would maintain correct order. However it should be noted that digits D and E lie dorsally and digits A B C and F G H I lie in the same plane. In this case, F, digit 2, lies next to C, digit 3/4.

It seems unlikely that the basis of this polydactylous foot is an inherited gene mutation similar to that seen in the chick mutant *eudiplopodia*. Only the left foot was affected and the right was normal although there could be mosaicism. Another possibility is that the developing limb bud was damaged in some way and part of the apical ridge deflected on to the dorsal surface. In this case, we would have to postulate the development of an ectopic polarizing region in the middle of the bud associated with the deflected ridge (Fig. 6.5c). Current maps of polarizing activity suggest that cells with the potential to produce polarizing signals are restricted to the posterior margin of the limb bud (MacCabe et al 1973, Honig and Summerbell 1985, Hinchliffe and Sansom1985). It would therefore have to be assumed that cells with polarizing activity were displaced with the deflected ridge. Nevertheless it is still difficult to explain why digit F is a 2 rather than a 4, since a polarizing region placed apically can signal both anteriorly and posteriorly.

CHAPTER SEVEN

GENERAL DISCUSSION

Chapter Seven: General Discussion

7.1 Main findings

The main results of my thesis are summarised below. My analysis of the gross and microscopical anatomy of tendons in chapter 3, show that tendons can follow complicated paths in their course to insertion sites. Some course from a posterior position to an anterior one and vice versa, while others move from a ventral to a dorsal site for insertion. This suggests that proximal and distal parts of tendons develop independently and join up later. Before insertion, tendons may divide to go to separate digits for attachment or two tendons may fuse to attach to a common point.

EphA4, *Bmp-4*, *Follistatin* and *Six 1* are expressed in developing tendons (chapter 4). When Follistatin is implanted into the limb bud, tendon and cartilage development is inhibited. Other manipulations of the limb bud also indicate that tendon and cartilage respond together. The exception was when the apical ridge is removed from the digit tip, digit cartilage was truncated but tendons of the dorsal and ventral side grew and fused around the tip. *EphA4*, is expressed in tendons very early, even as they assemble under the ectoderm that overlies the digit forming regions and expression persists until they are displaced deeper in the mesenchyme and superficial tendons are formed.

Wnt-7a and *Lmx-1* are expressed in dorsal ectoderm and dorsal mesoderm respectively during early limb bud development. When tendons start to assemble subjacent to ectoderm, *Wnt-7a* is still expressed in dorsal ectoderm but, later after stage 30, when tendons get displaced, expression disappears. *Lmx-1* is expressed in tendon-forming cells as they lie under ectoderm, but later as tendon gets displaced *Lmx-1* is excluded from tendon, but expression is persists in mesenchyme that surrounds the tendon (chapter 5). The pattern of expression of *EphA4*, is not altered up to day 12.5 in the *Wnt-7a* knockout when tendon cell aggregation is visible under the ventral ectoderm as a thicker layer when compared to the dorsal. At day 13.5, *EphA4* expression in tendons is shows a bi-ventral pattern. Dorsal flattened expression is replaced by a thicker or rounded domain of expression, which mirrors that seen on ventral side. *Lmx-1* overexpression in chick limb bud reveals that *EphA4* expression in ventral tendon is not altered up to day 8 after which *EphA4* expression goes off. Later, at day 14, a bi-dorsal tendon pattern is seen histologically. Attachment of tendons can be used to identify digits and this was done in the human polydactylous foot. Some toes in the

polydactylous foot were "double-dorsal" and these were identified by the presence of conical nails, absence of flexor tendons and the presence of two extensor tendons.

7.2 Tendons course along different directions to reach insertion points

Some tendons like flexor digitorum profundus (Fdp) lie in line with their muscle and run to the distal attachment without an obvious change in direction. Other tendons like extensor digitorum communis (Edc) run to their distal attachment exhibiting a relative change in position along the anterior-posterior axis. Muscle interosseus palmaris (Ip) arises on the ventral side and is derived from the ventral muscle mass but its tendon courses dorsally to be attached to a dorsal site on digit 3. During development, genes are known to regulate dorsal and ventral pattern of structures. *Wnt-7a,* and downstream *Lmx-1*, is responsible for patterning dorsal ectoderm and mesenchyme-derived structures and *En-1* expressed in ventral ectoderm is responsible for ventral patterning. These molecules are expressed in the limb bud during early development and therefore a ventrally derived structure such as Id, has a tendon that is derived probably from the dorsal mesenchyme and thus patterned by *Lmx-1*. It has been shown experimentally, however, that dorsal and ventral tendons can fuse when digits are truncated (Hurle et al 1990; this thesis) or when digit tip is isolated and grafted back to the stump (Shellswell and Wolpert 1977). *Six 1 and Six 2* genes are restricted to dorsal and ventral tendons respectively (Oliver et al 1995) and *Eya1* and *Eya2* genes to ventral and dorsal tendons respectively (Xu et al 1997a). However, despite these molecular differences dorsal and ventral tendons can fuse. In contrast *EphA4* is expressed in both dorsal and ventral tendons and I have found that *EphA4* is expressed in the fused part of the tendon after digit truncation (chapter 4) and this may explain why tendons can fuse around the tip.

7.3 Distal and proximal parts of tendons develop independently

The study of the embryonic wing at day 10 suggests different components of Edc develop individually along the proximo-distal length as serial sections show that tendon is present at the different sites in the same embryo. For example 2 Edc tendons are seen proximally in association with digit 2 and 3. Distally Edc tendon for digit 3 is seen deep to tendon of extensor medius longus (Eml) and further distally it is seen posterior to Eml. This indicates that it is not a single tendon blastema that divides, but that, different segments develop independently and then fuse to give the adult anatomy. A question of

interest then would be how the different components of a tendon connect up. Kardon (1998) has described that autopodial tendons arise from distal tendon primordium and from tissue extensions that develop from around joint regions. *EphA4* is expressed in distal tendon primordium, digital tendon blastemas and in tissue extensions. Further it can be seen that initially *EphA4* is expressed along the entire length of the tendon and later expression appears in the tissue extensions first proximally and then further distally. At later stages, at the level of tissue extensions, expression in tendons is split and overlaps expression in tissue extensions suggesting that contact between the two may be established at that time. *Follistatin* is expressed in the region of interphalangeal joints and hence this spatial expression at the time of joint formation suggests that Follistatin may have a role in attachment of tendons to cartilage. Furthermore as *Gdf5* is expressed in joint regions (Francis-West et al 1996; Merino et al 1999), Follistatin by its antagonistic action may have a role in preventing tendon from attaching to joint capsule or ligaments.

7.4 Tendons split in two before distal attachment

As tendons reach their insertion, they split to attach to the sides of the phalanges and also allow a deeper tendon to run further. As there is more than one tendon in toes, the question arises as to what makes the superficial tendon split just before the level of interphalangeal joint and why the deep tendon does not split. Does the formation of joint itself have any relation to splitting of tendons and is there some molecule that attracts the split ends of the tendon? Both *EphA4* and *Follistatin* are expressed in the region of the interphalangeal joint (chapter 5) and *Gdf-5* is another molecule associated with interphalangeal joint. Furthermore, *Six* genes, *Eya* genes and *Tgf-ß2* are expressed at joint regions and it will be useful to know the role played by these genes in attachment of tendons.

What attracts the split ends of the tendon to the cartilage for attachment? Cells need positional information about whether they are in an appropriate relationship to the ECM and surrounding cells and this information is provided through cell adhesion receptors (Aplin et al 1998). Integrins are cell surface glycoproteins that act as receptors for ECM proteins. *Delilah* a novel basic Helix-Loop-Helix Protein is expressed only at attachment sites of epidermis for somatic muscles in *Drosophila* (Armand et al 1994). The sites of attachment are called tendon cells and serve as connectors of somatic muscles to the chitinous exoskeleton. Integrins are critical to the tendon cell-muscle

junction and other cell-cell junctions in the fly (Zusman et al 1990). Mutation in integrin leads to tendon cell-muscle separation in the mutant *lethal myospheroid* (Leptin et al 1989; MacKrell et al 1988). Position-specific (PS) integrins are present in *Drosophila* and show complementary expression at tissue interfaces including muscle attachment sites (Bogaert et al 1987). Hence it is possible that cell adhesion molecules could have a similar role in attachment of tendon to cartilage at the insertion points in the chick embryo. *Drosophila* PS integrin subunits share extensive amino acid sequence homology with vertebrate integrin subunits (Bogaert et al 1987). There is also considerable functional conservation between insect and vertebrate integrins.

7.5 Genes expressed in tendons

EphA4 is first expressed in mesenchyme of the chick wing bud at stage 17, but later, by stage 28, *EphA4* expression becomes associated with developing tendons and persists through all the dynamic processes of tendon development. *Follistatin* is first seen in chick limb bud at stage 23, later at stage 28/29 is expressed in the sub-ectodermal mesenchyme in region of developing tendon. Later at stage 31-33 *Follistatin* is expressed at the distal tip of tendon and in the periphery of tendons. *Bmp*-4 is present subjacent to ectoderm in tendon forming regions of chick limbs at the same time as *Follistatin* and later is present in the core of the tendon and still later in the periphery of tendon. There is no evidence to suggest that expression of *EphA4* lead to expression of *Follistatin* and *Bmp-4*. Expression of *Bmp-4* and *Follistatin* seems to come on at the same time in tendon. It would be interesting to study the expression pattern of both *Follistatin* and *Bmp-4* when *EphA4* is overexpressed or inhibited (using either a constitutively active or a dominant negative form of the receptor) and whether any new genes are expressed.

Six and *Eya* genes are expressed in developing tendons of vertebrate limbs. Both *Six* (*So*) and *Eya* (*eya*) homolog genes are required for development of eyes in *Drosophila* along with *eyeless* (*Pax6*) and both regulate common steps in eye development (Xu et al 1997b) including cell proliferation and patterning. In *eya* mutants, progenitor cells in the eye disc undergo programmed cell death anterior to the morphogenetic furrow and do not proceed to retinal differentiation. These transcription factors are conserved in vertebrates and it is very surprising these genes are expressed in tendons during vertebrate development. A study of vertebrate mutants may indicate the role that *Eya* genes play in tendon and eye development. However the human homolog

EYA gene underlies Branchio-oto-renal syndrome (Abdelhak et al 1997a; 1997b) and there is no report of the eye or tendon being affected.

7.6 Functions of molecules expressed in tendons

In muscle development, it has been proposed that *Follistatin* may regulate the presentation of TGF-ß–related proteins to myogenic cells and thus control migration, proliferation and differentiation of muscle cells (Amthor et al 1996). Overexpression of TGF-ß family members de-differentiates myoblasts or transdifferentiates them into cartilage (Katagiri et al 1994) or inhibits muscle altogether (Duprez et al 1996) and hence TGF-ß family members must be regulated. Further, in mice that lack *Follistatin* (Matzuk et al 1995) muscle mass is reduced supporting the idea that *Follistatin* has a role in muscle differentiation. The pattern of expression of *EphA4* in the *Follistatin* mutant could throw light on the role of *Follistatin* in tendon development.

Six and *Eya* genes that have been shown to be expressed in tendons of mice (Oliver et al 1995; Xu et al 1997a) and in chick (this thesis) are also known to regulate development of eyes in the fly. When *eya* genes are absent in retinal progenitor cells, they undergo programmed cell death leading to absent eyes or reduced eye phenotype (Bonini et al 1998). The relationship of these transcription factors to the expression of *Follistatin* and *Bmp-4* is not known, however absence of *eya* genes in the fly has been shown to lead to cell death and BMP-4 is known to mediate programmed cell death (Graham et al 1994; Ganan et al 1996).

The functions of EphA4 in tendon development are not yet known. The effects of expressing viral constructs of constitutively active or dominant negative forms of EphA4 in chick embryos could be informative. The *EphA4* knockout mouse as a "hopping" gait but it is not clear whether this is due solely to defect in nerves or whether there are changes in tendons. It maybe that there is functional redundancy as several Eph family members are expressed in the limb and probably in tendons as well and can substitute for *EphA4*. One could think of several possible roles for *EphA4* signalling. One possibility is that *EphA4* signalling is involved in changes of cell shape required for condensation of pre-tendon mesenchymal cells as they lie subjacent to ectoderm. *EphA4* could also modulate cell adhesion during tendon development, possibly by regulating cadherin function. Presence of *EphA4* expression at periphery of ventral tendons could prevent premature sheath formation. Yet another possibility is

that *EphA4* could be involved in limiting migration of myogenic precursors into the tendon regions.

7.7 Connection between dorso-ventral patterning and tendon development

Wnt-7a signalling induces *Lmx-1* expression in dorsal mesenchyme very early, even before overt limb development. As tendons start to form, they are right under ectoderm expressing *Wnt-7a* and cells from which they arise appear to express *Lmx-1* but later, expression of *Lmx-1* reduces very quickly. In *Wnt-7a*$^{-/-}$, in the absence of *Wnt-7a* signal, a thicker aggregation of tendon cells form on the dorsal side thus resembling ventral cell aggregation. Tendon cell aggregation under ectoderm is a very early event in tendon formation. When *Lmx-1* is overexpressed ventrally, tendon pattern is not perturbed until very late in development. This suggests that early events in tendon morphogenesis may be mediated directly by *Wnt-7a* signalling or via *Lmx-1* and later events may require mesenchyme-mesenchyme signalling involving *Lmx-1*.

The above hypothesis could be substantiated by studying tendon pattern in either *Lmx-1*b mutants with targeted deletion of the gene in mice (Chen et al 1998) or chicken embryos in which *Lmx-1* activity has been inhibited (Rodriguez-Esteban et al 1998). Similarly the study of the mouse mutant, Post-axial hemimelia (*px*), in which there is a spontaneous mutation in the gene encoding the *Wnt-7a* signalling molecule (Parr et al 1998) may throw light on the role of dorso-ventral patterning molecules on tendon patterning. Further, grafting pieces of dorsal tendon into the ventral side and ventral tendon into dorsal side and studying whether the shape of the grafted tendon is reorganised under the influence of mesenchymal signalling should be enlightening. It would also be interesting to study the expression pattern of *EphA4* in the *En-1* mutant.

7.8 Polydactylous foot

In chapter 6, the anatomy of a human polydactylous foot is described. This was very topical because at the time when analysis was being made, the only evidence that conical nails were associated with dorso-ventral patterning defects came from the description of the chick mutant *eudiplopodia.* Recently, there have been many instances of conical nails reported in *Wnt-7a* knockout (Loomis 1998) in En-1 knock out (Loomis et al 1996, 1998) and in the *Wnt-7a/En-1*$^{-/-}$ double mutant (Loomis et al 1998).

I used the anatomy of tendons to identify toes in the polydactylous foot. Tendons transmit the force of muscles on cartilage to enable particular movements. Therefore tendons are attached at particular sites and this attachment can be used to give identity to toes. For example, dorsal interossei muscles produce abduction of toes; hence their tendons must be attached appropriately to carry out that action. The second toe, digit 2, in man, has 2 dorsal interossei attached to medial and lateral sides (embryonic anterior and posterior sides) as abduction of that toe can be done in two directions. Whereas digits 3 and 4 have a dorsal interosseus attached on the lateral side as abduction moves those toes in that direction. Hence digit 3 and 4 cannot be differentiated from each other, but certainly can be differentiated from digit 1, 2 and 5. Digit 5 on the other hand has the attachment of tendon peronius brevis to the lateral side of the base of its metatarsal so that the tendon produces eversion of the foot.

7.9 Some outstanding questions to be answered in development of tendons

During tendon development, tendon cells first aggregate subjacent to the ectoderm and later get displaced deeper into mesenchyme. Tendons must get closer to attachment sites on cartilage but it is not known what makes tendons move from a superficial position to a deep one, and what tissue intervenes between the displaced tendon and the ectoderm. However it is known that no distinct dermis is recognisable in the trunk of chick embryo at day 5 of development (Sengal 1976) which indicates that dermis in limb bud may form later. Preliminary cell lineage analysis by injecting DiI into sub-ectodermal cells suggests that labelled sub-ectodermal cells fated to become tendons are displaced or move towards the cartilage elements. Displacement could occur due to development of the dermis which could extend progressively more distally, driving as it were a wedge between ectoderm and assembling tendon. Further this can be explored by making chick-quail chimaeras (Le Douarin 1973). Small cubes of mesenchyme along with the overlying ectoderm from the developing foot regions of chick embryos can be cut out and replaced with equivalent regions from quail embryos at the time that tendons are forming beneath the ectoderm. The quail cells can be followed by using quail-specific antibody (QCPN) and tendon cells by procollagen I antibody. Double labelling can establish whether quail cells contribute to the tendon and the origin of the cells that come to lie between tendons and ectoderm.

Many questions on development of tendon sheath are still not answered such as the origin of cells that form tendon sheath, how a synovial space forms and if cell death is involved in the formation of a synovial space. How synovial space around tendon is formed in the first place is a question that has not been addressed. Hyaluronic acid has been associated with the development of joint cavity (Archer et al 1994) and maintenance of extensive intercellular spaces in the chick primary mesenchyme (Fisher and Solursh 1977; Toole and Telstrad 1971). Histological study of tendon sheath formation in chapter 3 shows that cells are initially compactly arranged around tendons and then move away from the tendon and a space is formed. It has been postulated that hyaluronic acid inhibits extensive intercellular interactions thus cells separate (Toole 1972). These observations and the fact that synovial cells secrete hyaluronic acid suggest that hyaluronic acid maybe involved in formation of synovial space in tendon sheath.

Tendon cells have vascular supply (Edward 1946; Brockis 1953; Chaplin 1973; Verdan 1972), respiratory activity (Peacock 1959) and amino acid turnover within the collagen. Hence tendon must be regarded as one of living tissues of the body. However it is unique in lacking any intrinsic repair potential and is quite passive in the healing process whereas the sheath tissue is the active healing agent. Tendon healing experimentally has been shown to involve ingrowth of granulation tissue from the synovial sheath and tendon tissue has no power of regeneration. Potenza (1962) experimentally studied tendon healing and reported that a digital flexor tendon heals by cellular activity of the sheath and surrounding tissue. Hence a knowledge of the cell biology of tendon sheath is of prime importance in understanding tendon healing.

Mesenchymal cells in the region overlying digits, aggregate around a mesenchymal lamina that separates from the basement membrane of the ectoderm. Later these tendon cells condense to become compact strips and the mesenchymal lamina disappears (Hurle et al 1989; 1990). Kardon (1998) proposed that the autopodial tendons arise from several components, intermediate tendon primordium, distal tendon primordium and from tissue extensions, which connect up later. In this thesis, using histology and expression of *EphA4* it appears that formation of tendon around a mesenchymal lamina pertains to tendons as they lie over digit regions and that the different components that constitute autopodial tendons develop independently and fuse later. Further work here also indicates that superficial tendon may delineate from the deeper tendon that is formed first.

7.10 Future prospects

In Drosophila, during development of myotubes inductive interactions between muscle cells and their specific attachment cells are responsible for terminal differentiation of epidermal muscle attachment (EMA) cells to tendon cells. A number of genes are expressed in epidermal muscle attachment (EMA) cells. *Stripe*, an early growth response (EGR) –like putative transcription factor, is expressed specifically in EMA cells (Lee et al 1995) and is necessary for induction of cell-specific genes for example, *delilah*. Initially *delilah* is expressed in EMA cells independent of muscle but later there is elevation of levels of *delilah* in tendon cells, after muscle binding. *Stripe* may be responsible for initially triggering an attractive activity that guides myotubes to interact specifically with EMA cells. It has also been suggested that *delilah* maybe the key factor in terminal differentiation of tendon cells in *Drosophila* (Yarnitzky et al 1997). It would be interesting to determine if a vertebrate homolog of *delilah* exists and study its expression pattern because this may indicate whether a similar molecule participates in attachment of tendons to their insertion sites in vertebrates.

It has been reported that expression of *EphA7*, a tyrosine kinase receptor belonging to the Eph family, was down regulated in dorsal mesoderm of the chick limb when *Lmx-1* was competitively inhibited (Rodriguez-Esteban et al 1998). In some preliminary work that I have done, I removed leg bud ectoderm at stage 26/27 and found that *EphA4* expression was reduced. Hence, this suggests that *Wnt-7a* signalling may regulate *EphA4* directly or indirectly through *Lmx-1* and more work on this will have to be done to confirm relation of expression of *EphA4* to *Wnt-7a* signalling.

More detailed analysis of the pattern of tendon along the proximo-distal length in different stages of *Lmx-1b* and *En-1* mutants will have to be done to further elucidate the role of dorso-ventral patterning in tendon development. *En-1*, which is expressed in the ventral ectoderm is responsible for ventral patterning. However its expression in ventral ectoderm has been shown only upto stage 25 in chick embryos and it is not known whether it is expressed in later stages. A study of expression pattern of *En-1* in later stages may explain why when *Lmx-1* was misexpressed ventral tendon pattern remained normal up to stage 36. The question that needs to be answered is how *En-1* expression regulates ventral pattern. As *En-1* is a transcription factor and acts cell autonomously it presumably controls expression of a signalling molecule in ventral ectoderm, which then diffuses into underlying ventral mesenchyme to control patterning.

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