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Establishing the pattern of the vertebrate limb

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ABSTRACT

The vertebrate limb continues to serve as an influential model of growth, morphogenesis and pattern formation. With this Review, we aim to give an up-to-date picture of how a population of undifferentiated cells develops into the complex pattern of the limb. Focussing largely on mouse and chick studies, we concentrate on the positioning of the limbs, the formation of the limb bud, the establishment of the principal limb axes, the specification of pattern, the integration of pattern formation with growth and the determination of digit number. We also discuss the important, but little understood, topic of how gene expression is interpreted into morphology.

KEY WORDS: Limb, Digits, Pattern formation, Growth, Signalling

Introduction

Limb buds form at reproducible antero-posterior positions on the flank of the embryo and are composed of a multipotent population of undifferentiated cells derived from the somatopleural layer of the lateral plate mesoderm that are ensheathed by an epithelial layer (Tickle, 2015). Limb bud mesoderm cells differentiate into cartilage, perichondrium, dermis, muscle connective tissues, ligaments and tendons, while the epithelium gives rise to the epidermis of the skin (Pearse et al., 2007). The spinal cord and somites also contribute cells that give rise to major tissue types, including the nerves and muscles, respectively.

In this Review, we cover the early stages of limb development, which are important because the axial positions at which the limb buds form relate to their identity. For example, the anterior region of the embryo forms the forelimbs, while the posterior region forms the hindlimbs. Another important aspect is that the limb fields are polarised (i.e. cells in different axial positions have different developmental potential) with respect to both the antero-posterior and dorso-ventral axes of the embryo, well before the limb buds form. Polarisation of the presumptive limb bud establishes signalling centres in the outgrowing bud, which specify the pattern of structures along each of its principal axes: antero-posterior (thumb to little finger) (Fig. 1A); proximo-distal (shoulder to fingertips) (Fig. 1B) and dorso-ventral (knuckle to palm) (Fig. 1C). Vertebrate limb anatomy along the proximo-distal axis comprises an invariant pattern of stylopod (i.e. humerus), zeugopod (i.e. radius/ulna) and autopod (wrist/digits) (Fig. 1D). However, along the antero-posterior axis digit number varies (i.e. three in the chick wing, four in the chick leg and five in mouse/human limbs) (Fig. 1D). Although it remains controversial, considerable effort has been invested into understanding how signals specify the pattern of limb structures along each of the primary axes. We will see for the proximo-distal axis that a coherent model is emerging from chick and mouse studies; however, there are differences for the antero-posterior axis, and the dorso-ventral axis has not been explored in as much detail. One of the areas that is least understood is how positional information (instructions received by cells to determine how they differentiate in respect to their position relative to other parts of the body) is interpreted into patterns of gene expression that determine anatomy, and we highlight recent attempts to gain insights into this problem. We focus on the molecular regulation of mouse and chick limb patterning where most progress is still being made, and the reader is directed towards other recent reviews that focus on clinical and evolutionary aspects (Pickering and Towers, 2014; Saxena et al., 2017).

Limb positioning

Vertebrate limbs form at distinct and reproducible locations along the main body axis. Forelimbs always form at the cervico-thoracic vertebrae boundary and hindlimbs at the lumbar-sacral boundary. The relative position at which these boundaries are found varies greatly between vertebrates (Table 1), and this has contributed greatly to the differences in the extent of body extension observed across evolution.

Classical fate-mapping and tissue transplantation experiments in the chick embryo have revealed that cells in distinct regions of the lateral plate mesoderm (LPM) are in position to form the limbs as early as the 2-somite stage (Chaube, 1959; Rosenquist, 1971). Candidates for specifying the position of the limbs include Hox (homeobox) family genes, which are expressed in gastrulating cells, and later along the antero-posterior axis of the LPM. Hox genes are expressed in the order in which they are found on the chromosome in the 3′-5′ direction – a process called spatial and temporal co-linearity (Box 1). Indeed, it has long been suspected that Hox proteins are important determinants of forelimb position, because the functional inactivation of Hoxb5 in the mouse repositions the forelimbs anteriorly (Rancourt et al., 1995). Recent evidence from the chick has suggested that the determination of forelimb position coincides with when Hox4-Hox9 paralogous group genes are first expressed in gastrulating cells (Moreau et al., 2019) (Fig. 2A). For example, the overexpression of a dominant-negative form of Hoxb4 in chick mesoderm cells causes an anterior shift of the forelimb (Moreau et al., 2019). By contrast, the overexpression of Hoxb4, together with a dominant-negative form of Hoxc9 in the interlimb at around the 20-somite stage, shifts the position of the wing bud posteriorly (Fig. 2B). This experiment was performed after the onset of endogenous Hox gene expression, therefore suggesting that earlier positional information can be re-specified. However, the overexpression of the dominant-negative version of Hoxc9 alone has no effect, indicating that both active repression and activation of target genes by Hox proteins is crucial for determining limb position. In a broader context, the timing of Hoxb4 expression correlates with the position of the forelimb in a range of avian
species, including the ostrich and zebra finch (Moreau et al., 2019). The expression of Hox genes is influenced by the distribution of retinoic acid (RA), which is higher in anterior regions of the elongating trunk compared with posterior regions (Langston and Gudas, 1994), and treatment of chick embryos with RA, or antagonists of RA signalling, shifts Hoxb4 expression posteriorly and anteriorly, respectively (Moreau et al., 2019).

A recent study in mice has indicated that Oct4 indirectly controls forelimb position by repressing posterior 5’ Hox genes (Hox10-Hox13 paralogues), because the inactivation of Oct4 precociously activates the posterior programme of embryo development and results in posterior truncations (Fig. 2A,B), which, in less severe cases, can cause the hindlimb to form next to the forelimb (DeVeale et al., 2013). Conversely, when the duration of Oct4 expression is extended, more-posterior development of the embryo is delayed, which elongates the trunk (Aires et al., 2016). These effects are associated with corresponding changes in the timing of Hox gene expression along the antero-posterior axis of the embryo.

The position of presumptive hindlimb cells is determined later than forelimb cells (Tickle, 2015). Genetic studies have also implicated Hox genes in hindlimb positioning, because deletion of Hoxc8 in the mouse results in the posterior repositioning of the hindlimbs (van den Akker et al., 2001). A role for the TGFβ family member growth/differentiation factor 11 (Gdf11) in specifying the posterior body plan upstream of Hox genes has also been revealed (Fig. 2C). Thus, when Gdf11 expression is inactivated in the mouse, the trunk is extended (McPherron et al., 1999; Jurberg et al., 2013). Conversely, when Gdf11 is prematurely activated in the axial mesoderm, the trunk is shortened and the hindlimb forms next to the forelimb (Jurberg et al., 2013). Similarly, when the onset of Gdf11 expression is advanced or delayed in the posterior axial mesoderm of chick embryos at the 9-10-somite stage, the position of the hindlimb is shifted either anteriorly or posteriorly, respectively (Matsubara et al., 2017) (Fig. 2D). Furthermore, the timing of Gdf11 expression in the posterior axial mesoderm strongly correlates with the antero-posterior position at which the hindlimb develops in a range of species, including frogs, mice, chickens and snakes (Matsubara et al., 2017) (Table 1). Gdf11 signalling regulates the onset of Hox9-Hox13 expression in both posterior axial mesoderm, where it is expressed, and in the adjacent LPM at about the 10-somite stage of chick development (Matsubara et al., 2017). In

Table 1. Limb position in different vertebrates

| Species            | Cervico-thoracic vertebrae position | Lumbar-sacral vertebrae position |
|--------------------|------------------------------------|---------------------------------|
| African clawed frog| 2                                  | 10                              |
| Axolotl            | 3                                  | 17                              |
| Python             | 3                                  | 328                             |
| Mouse              | 8                                  | 23                              |
| Human              | 8                                  | 25                              |
| Chicken            | 15                                 | 17                              |
| Emu                | 18                                 | 36                              |
addition, in the mouse, Gdf11 is likely to repress the anterior programme by suppressing RA signalling via its regulation of the gene encoding the RA-catabolising enzyme Cyp26a1, which is then expressed in the prospective LPM (Fig. 2C) (Lee et al., 2010; Moreau et al., 2019). Therefore, Oct4 and Gdf11 play opposing roles in specifying the basic body plan (anterior versus posterior), upstream of the Hox genes that confer axial identity to these broad regions (Mallo, 2018) (Fig. 2). It remains unclear how Oct4 and Gdf11 influence the expression of Hox genes and whether this involves interaction with different enhancers, as is the case for Hoxd expression during later limb development (Box 1). Therefore, these studies reveal a hierarchical regulation of antero-posterior patterning of the embryo and the positioning of the limbs. In the first step, Oct4 and Gdf11 specify broad regions of the embryo as anterior and posterior, and then Hox genes act downstream to provide local identity (i.e. lumbar versus sacral) (Aires et al., 2016).

**Limb polarity**

Once cells are in position to form limbs along the antero-posterior axis of the embryo, they become polarised along two developmental axes. 180° tissue rotation experiments in the chick embryo have shown that the antero-posterior polarity and dorso-ventral polarity of the limb is determined in the LPM at pre-limb bud stages: at the 9- to 10- and 13-somite stages, respectively (Chaube, 1959; Michaud et al., 1997). The establishment of antero-posterior polarity results in the formation of the polarising region (or zone of polarising activity, ZPA) – a group of posterior limb bud mesoderm cells that express *Shh* (sonic hedgehog), which pattern the antero-posterior axis. A distant cis-regulatory sequence containing multiple enhancers called the ZPA regulatory sequence (ZRS) controls *Shh* expression (Box 2).

In the mouse, the products of Hox genes specify the antero-posterior polarity of the developing forelimb (Fig. 3A), just as they specify antero-posterior position along the main body axis.

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**Box 1. Hox gene regulation**

Hox genes encode a subfamily of homeobox transcription factors, each containing a conserved DNA-binding domain known as a homeodomain, which consists of a helix-turn-helix motif. Hox genes are closely localised within their respective chromosomes and are arranged into four main clusters: Hoxa-Hoxd. Hox gene transcription displays colinearity, whereby the order in which the genes are expressed along the antero-posterior axis of the body and proximo-distal axis of the limb, relates to their order along the chromosome. Two topologically associated domains (TADs) – regions of chromatin with specific 3D structures – flank the Hox gene cluster at its 3′ and 5′ ends: telomeric (T-DOM) and centrosomal (C-DOM), respectively. The switch from T-DOM to C-DOM regulation drives the transition from 3′ Hoxd8-Hoxd10 expression during early proximal patterning to 5′ Hoxd11-Hoxd13 expression during late distal patterning (Andrey et al., 2013). Studies using Hox13 mutants have revealed that Hoxa13 (later cooperatively with Hoxd13) represses the T-DOM regulatory region by recruiting polycomb repressor complex proteins and this maintains the activity of the 5′ C-DOM regulatory region (Beccari et al., 2016; Rodríguez-Carballo et al., 2019). In addition, Hoxa13 and Hoxd13 drive the expression of an antisense Hoxa11 transcript, which confines Hoxa11 to more-proximal regions (Kherdjemil et al., 2016).
Deletion of all Hox9 paralogous group genes in the mouse embryo results in the loss of posterior polarity, and the failure to establish Shh expression in the polarising region via an intermediate transcription factor, heart- and neural crest derivatives-expressed protein 2 (Hand2) (Xu and Wellik, 2011). Conversely, deletion of all Hox5 paralogous genes results in the loss of anterior polarity, and Shh expression becomes detectable at the anterior margin of the limb bud (Xu et al., 2013). Hox5 proteins regulate expression of the gene encoding the promyelocytic leukaemia zinc-finger protein (Plzf) transcription factor, which represses Shh expression and the formation of an anterior polarising region (Xu et al., 2013). Sal1 is involved in the anterior region of Gli3 that encodes the major transcriptional effector of Shh signalling. Gli3-dependent transcription in the anterior part of the limb is inhibited by Gata4 and Gata6 transcription factors that promote its repressor function (Hayashi et al., 2016). Gata4 and Gata6 also contribute to the direct suppression of Shh in the anterior part of the limb (Kozhemyakina et al., 2014). Antero-posterior polarity is maintained because Hand2, which is expressed in the posterior part of the limb bud and regulates Shh expression directly, also mutually inhibits Gli3 expression posteriorly (te Welscher et al., 2002a). In addition, RA signalling is involved in specifying antero-posterior polarity by its regulation of anterior Hox5-Hox9 paralogous genes and posterior Hand2 expression in presumptive forelimb regions (Fig. 3A).

The antero-posterior polarity of the presumptive hindlimb is also specified at early stages and does not appear to involve Hox genes, but instead involves regionalised transcription factors (Fig. 3B). For example, islet 1 (Is1) is indirectly involved in the initiation of Shh expression in the posterior part of the limb via its induction of posterior Hand2 expression (Itou et al., 2012), while Sal1, and members of the Iroquois transcription factor family (Irx3 and Irx5), stimulate Gli3 expression anteriorly (Akiyama et al., 2015; Li et al., 2014). In addition, as in the forelimb, Gata6 represses Shh expression in the anterior part of the hindlimb (Kozhemyakina et al., 2014) (Fig. 3B).
The establishment of dorso-ventral polarity in the ectoderm of the trunk of the embryo involves bone morphogenetic protein (Bmp) signalling from the mesoderm, which is graded in the chick due to the action of its inhibitor, noggin (Nog), produced by the somites (Pizette et al., 2001). The cells that coalesce at the boundary between dorsal and ventral ectoderm of the trunk will become the apical ectodermal ridge: a thickening of the epithelium at the distal tip of the limb that is essential for and supports outgrowth along the proximo-distal axis (Fig. 1B) (Altabe et al., 1997; see Tickle, 2015 for additional detail). The dorso-ventrality of the ectoderm will be transferred to underlying limb mesoderm cells once they begin to form buds (see the section ‘Dorso-ventral specification’). Thus, polarisation triggers outgrowth of the limb away from the body wall and allows signalling centres to be established at early limb bud stages.

**Limb bud initiation**

Following the establishment of limb field polarity, the genes encoding the T-box transcription factors Tbx4 and Tbx5 are activated in the LPM. In the mouse, Tbx4 and Tbx5 are essential for fibroblast growth factor 10 (Fgf10)-dependent limb initiation (Agarwal et al., 2003; Ng et al., 2002), which depends on a reciprocal feedback loop operating between the mesoderm (Fgf10) and apical ectodermal ridge (Fgf8) (Fig. 4). Both Fgf8 and Fgf10 are essential for mouse limb development (Sekine et al., 1999; Mariani et al., 2008; Min et al., 1998; Ohuchi et al., 1997; Xu et al., 1998), and the implantation of an Fgf-soaked bead into the interlimb of a chick embryo is sufficient to induce limb outgrowth (Cohn et al., 1995). In addition, Wnt signalling and genes encoding the S6p and Sp8 transcription factors are important intermediates involved in establishing the Fgf10-Fgf8 loop at early stages of mouse limb development (Barrow et al., 2003; Haro et al., 2014; Kawakami et al., 2001).

The mechanism that controls the onset of Tbx4 and Tbx5 expression in the LPM – corresponding to the 19- to 20-somite stage of chick development (Saito et al., 2002) – differs between forelimbs and hindlimbs (reviewed by Sheeba and Logan, 2017). Transgenic mouse studies have implicated Hox4 and Hox5 paralogues, Wnt/β-catenin signalling and RA signalling in regulating forelimb Tbx5 expression via specific regulatory elements located in Tbx5 intron 2 (Minguillon et al., 2012; Nishimoto et al., 2014, 2015) (Fig. 4A). However, deletion of these elements by CRISPR/Cas9 gene editing in the mouse does not abolish Tbx5 expression (Cunningham et al., 2018). By contrast, mouse studies have suggested that RA signalling indirectly regulates Tbx5 by repressing Fgf8 in axial tissues of the main body (Cunningham et al., 2013) (Fig. 4A). Additional work is required to resolve these findings, but they suggest that complex and redundant mechanisms control Tbx5 expression.

As with the specification of antero-posterior polarity, hindlimb initiation involves a distinct developmental programme that is controlled by regionally restricted transcription factors (Pitx1 and Isl1), which co-operatively regulate Tbx4 expression in the LPM of the mouse (Duboc and Logan, 2011; Kawakami et al., 2011) (Fig. 4B). Isl1 acts downstream of Gdf11 signalling (Jurberg et al., 2013), and also activates the Wnt signalling pathway, which is essential for hindlimb development (Kawakami et al., 2011) (Fig. 4B). Evidence that Hox proteins regulate Pitx1 expression comes from capture Hi-C (chromatin conformation capture that determines the number of interactions between genomic loci in defined 3D space) approaches in the mouse, which show that Hoxc9-Hoxc11 interact with a enhancer (Pen) (Kragestein et al., 2018) (Fig. 4B). Although RA has been proposed to act as a co-factor with Tbx4 to initiate Fgf10 expression and hindlimb outgrowth in the chick (Nishimoto et al., 2015), the genetic or pharmacological removal of RA signalling in mouse and chick embryos, respectively, prevents the initiation of the forelimb, but not the hindlimb bud (Niederreither et al., 2002; Stratford et al., 1996).

Therefore, although we are gaining detailed information of how limb bud initiation is controlled at the molecular level, there are still gaps in our understanding about the underlying differences between forelimbs and hindlimbs.

**Limb pattern specification**

**Proximo-distal specification**

How is the pattern of tissues specified along the proximo-distal axis of the limb? The ‘progress zone model’ was influenced by Saunders’ apical ectodermal ridge removal experiments, which...
A later model proposes that proximal and distal signalling gradients co-operatively specify proximo-distal positional values. This ‘two-signal model’ is based on the observation that Fgf8s from the apical ectodermal ridge antagonise RA signalling (using Meis1 and Meis2 in the proximal part of the limb bud as a read-out of RA signalling) from the flank of the embryo (Mercader et al., 1999, 2000). Further studies in the chick have confirmed RA as a signal capable of specifying proximal fate (Cooper et al., 2011; Rosello-Diez et al., 2011). Retinoic acid is also likely to coordinate the outgrowth of the limb with proximo-distal patterning, because it needs to be cleared from the early chick wing bud by a combination of active degradation and displacement by growth to allow the programme of 5’ Hoxa/d11-13 gene expression to be activated (Rosello-Diez et al., 2014). Recent evidence from the mouse obtained by the conditional inactivation of Meis1 and Meis2 has been presented in support of the two-signal model (Delgado et al., 2020). The absence of Meis function results in the loss or severe reduction of proximal structures in both forelimbs and hindlimbs, which have normal digit development. The authors explain these results in terms of an instructive model in which the Fgf to RA ratio is interpreted into a gradient of Meis1 and Meis2 abundance that specifies proximo-distal positional values: high Meis1 and Meis2 would specify proximal positional values, low Meis1 and Meis2, intermediate positional values and absent Meis1 and Meis2, distal positional values (Fig. 5) (Delgado et al., 2020). The diminishing levels of RA, Meis1 and Meis2 would allow the progressive activation of Hoxa11 to Hoxa13. Therefore, proximal structures are lost in Meis1 and Meis2 mutants because of the precarious activation of the 5’-most Hox genes. However, Hoxa11 and then Hoxa13 are still progressively activated in the absence of Meis function (Delgado et al., 2020), which suggests that a timing mechanism underlies this transition (Fig. 5). Indeed, in the chick wing, manipulations of RA and Fgf signalling fail to advance the timing of Hoxa13 expression (Vargesson et al., 2001; Rosello-Diez et al., 2014) (Fig. 5).

Recent experiments in the chick support a ‘signal-time model’ in which signals specify proximal limb segments, as discussed (i.e. humerus), and then intrinsic timing specifies distal segments (i.e. wrist/digits) (Saiz-Lopez et al., 2015) (Fig. 5). When distal mesoderm from an early chick wing bud (Hoxa11 positive/ Hoxa13 negative) was grafted beneath the apical ectodermal ridge of a host wing bud that was 24 h older (Hoxa13 positive), the grafted cells maintained their intrinsic timing of cell proliferation and Hoxa13 and Hoxd13 expression, which marks the specification of distal positional values (Saiz-Lopez et al., 2015). Therefore, it appears that signals control the transition from proximal to intermediate specification (Hoxa10 and Hoxd10 to Hoxa11 and Hoxd11) and that timing controls the transition from intermediate to distal specification (Hoxa11 and Hoxd11 to Hoxa13 and Hoxd13) (Fig. 5). It remains unclear when this switch occurs, and one possibility is that a low level of RA signalling, Meis1 and Meis2 activity is required for the autonomous timer to start once Hoxa11 has been activated (Fig. 5).

**Dorso-ventral specification**

How the pattern of tissues along the dorso-ventral axis of the limb bud is specified has not been investigated in as much detail as the other axes. Tissue rotation experiments in the chick have shown that ectodermal signals specify the dorso-ventral polarity of the underlying mesoderm within the first 24 h of limb outgrowth (MacCabe et al., 1974; Akita, 1996). Further work has identified Wnt7a as a dorsal signal (Parr and McMahon, 1995) and Bmps as
ventral signals (Pizette et al., 2001). Accordingly, limbs of mice lacking Wnt7a function are ventralised (Parr and McMahon, 1995); those lacking the Bmp target gene engrailed 1 are dorsalised (Loomis et al., 1996), and the overexpression of the Wnt7a target gene Lmx1b dorsalises the chick limb (Riddle et al., 1995; Vogel et al., 1995). Akita has proposed a model in which high concentrations of a dorsal signal would specify dorsal tissues and low concentrations would specify ventral tissues (Akita, 1996). However, it is unclear how far ectodermal signals spread into the underlying mesoderm and whether they act through secondary signals. One observation is that, although the early limb consists of multipotent mesoderm cells that have the capacity to populate any of the segments along the proximo-distal axis, they are lineage restricted into dorsal and ventral compartments (Pearse et al., 2007; Arques et al., 2007). Therefore, cells in these compartments could respond differently to signals from other organisms, such as the polarising region or apical ectodermal ridge, and this could be a way by which limb anatomy could be refined. Furthermore, Wnt7a signalling, which emanates from the dorsal ectoderm of the limb, regulates Shh expression, thus showing how dorso-ventral and antero-posterior patterning are integrated (Parr and McMahon, 1995).

**Antero-posterior specification**

Several types of tissue-grafting experiments performed in the chick embryo have resulted in a positional information model of antero-posterior specification, based on graded signalling by the polarising region (reviewed by Tickle and Towers, 2017). The polarising region was discovered in experiments in which grafts of posterior chick wing mesoderm were made to the anterior margin of the wing bud of a host embryo. This resulted in the normal digit pattern (1, 2 and 3) being symmetrically duplicated (i.e. 3, 2, 1, 2 and 3) (Saunders and Gasseling, 1968). Lewis Wolpert interpreted the results of these experiments in terms of positional information, with the polarising region producing a signal, which specifies positional values that encode the different digit identities in a concentration-dependent manner (Tickle et al., 1975; reviewed by Vargesson, 2020) (Fig. 6A). As we have discussed, transcripts of Shh are restricted to the polarising region (Riddle et al., 1993), and its encoded protein fulfils the criteria required for a polarising region signal to specify antero-posterior positional values (reviewed by Tickle and Towers, 2017). However, here we consider recent evidence from both chick and mouse systems, which indicate that Shh might specify digit identity via secondary signals.

Digits do not form in the absence of Shh function in both knockout mice and naturally occurring chicken mutants (oligozoegodactyly), apart from a single dysmorphic digit 1 in their hindlimbs (Chiang et al., 2001; Ros et al., 2003). Timed experiments, in which Shh signalling has been either pharmacologically blocked in the chick wing (Towers et al., 2008, 2011) or genetically removed in the mouse limb (Zhu et al., 2008, 2020 preprint), both show that digit identities are specified during early stages of limb outgrowth (Fig. 6A,B). Lineage-tracing experiments have revealed that chick wing bud cells are sequentially ‘promoted’ through anterior to posterior positional values every 4 h by progressively higher concentrations of Shh signalling (Yang et al., 1997; Towers et al., 2011). Thus, by 4 h, Shh signalling specifies ‘digit 1’ positional values, by 8 h ‘digit 2’ positional values and by 12 h ‘digit 3’ positional values.

Evidence that Shh may not operate as a graded morphogen in the specification of antero-posterior positional values has been obtained by genetic lineage-tracing experiments in mouse forelimbs and hindlimbs, revealing that the two most-posterior digits (4 and 5 out of digits 1-5) are derived from the polarising region itself (Fig. 6B) (Harfe et al., 2004). Unexpectedly, the specification of these digit identities is independent of the concentration of Shh signalling, but is instead considered to depend on the length of time that cells are directly exposed to short-range Shh signalling (Harfe et al., 2004). GFP-expressing tissue transplantation experiments in the chick wing have shown that the polarising region does not contribute to the digit skeleton (Towers et al., 2011), consistent with a concentration gradient mechanism of long-range signalling for specifying the positional values that encode digit 1, 2 and 3 identities (Fig. 6A). In the chick leg, positional values that encode digit 1, 2 and 3 identities are specified by Shh signalling in the same manner as the equivalent digits of the wing (Towers et al., 2011). However, the chick leg has a fourth digit that arises from the cells of
In the mouse limb, the use of a *Gil1* reporter transgene showed that only polarising region cells directly receive Shh signalling during the 2-3 h it is required for specification (Zhu et al., 2020 preprint). Therefore, it is suggested that digits 1 to 3 (and possibly 4 and 5) are specified by secondary relay signals emanating from the polarising region (Zhu et al., 2008, 2020 preprint) (Fig. 6B). It is unclear whether Bmps are involved in the specification of antero-posterior positional values in the mouse limb, because the genetic removal of Bmp2, Bmp4 and Bmp7 function does not appear to cause overt transformations of digit identity (Bandyopadhyay et al., 2006). However, it has been noted that digits 2 to 5 of the mouse limb have very similar anatomies in terms of phalange number and proportion (Delgado and Torres, 2016). This observation could suggest that they developed from cells that were specified with ‘anterior’ positional values at a very early stage (Tickle and Towers, 2017; Towers, 2018). Distinct anatomical identities could arise from the subtle interpretation of these positional values, which could then be elaborated by differential growth of the limb bud at later stages (see the section ‘Digit number determination’). By contrast, the further promotion of positional values that encode definitive posterior digit identities in chick limbs could involve Bmps, and therefore explain the longer period of anterior to posterior positional value specification compared with the mouse (Fig. 6A,B).

In summary, the emerging view is that Shh signalling may not act directly to specify digit identities in chick and mouse limbs. Further work is required to understand how this is achieved, but, at least for the chick, Bmps are likely to function as secondary signals in the specification of antero-posterior positional values.

**Limb growth**

In order to understand many of the processes discussed so far, we need to consider the important contribution of growth. Early studies determined that proliferation is maintained in prospective chick wing cells and reduced in the adjacent interlimb flank (Sears and Janniers, 1971). However, it is unclear whether this alone can explain how the limb bud forms. Evidence from the chick has indicated that Tbx5 is involved in an epithelial-to-mesenchymal transition, in which cells from the coelomic lining of the somatopleure are recruited into the forelimb-forming field (Gros and Tabin, 2014). This influx of cells could supplement those derived from the LPM and influence localised budding from the body wall. Indeed, early work has shown that presumptive chick wing bud mesoderm is less cohesive than interlimb mesoderm (Heintzelman et al., 1978), and this could facilitate outgrowth from the flank of the embryo.
Contemporary live-imaging analyses of early mouse and chick limb buds has revealed that cells align themselves in the direction of outgrowth, while cells in more dorsal and ventral positions become oriented towards the overlying limb ectoderm (Gros et al., 2010; Wyngaarden et al., 2010). In addition, Wnt5a and Fgf signalling are required for limb elongation along the proximo-distal axis. An instructive gradient of Wnt5a is implicated in establishing planar cell polarity in the limb by promoting directional cell migration and/or directional cell division, while Fgf4 and Fgf8 signalling from the apical ectodermal ridge orients this process to promote a distal growth trajectory (Gao et al., 2018). However, the contribution that active cell migration and/or cell division play in directional outgrowth remains unclear. These findings help to explain earlier experiments in the chick wing bud, in which dye-labelled cells radiated towards an ectopic source of Fgf protein (Li and Muneoka, 1999). Computational modelling approaches in the mouse limb also predict the crucial requirement for directional cell division in 2-3 h period during which it is required for digit identity specification results in apoptosis of distal mesoderm cells and the failure to form digits (Zhu et al., 2020 preprint), it suggests there is a specific role for this process in the promotion of antero-posterior positional values in chick limbs (Fig. 6A) (Towers et al., 2008).

Another role for Shh signalling in the control of proliferation has been uncovered in the chick wing. Grafting experiments showed that the duration of Shh expression and proliferation in the chick wing polarising region are controlled by an autoregulatory intrinsic timing mechanism, which is triggered by the depletion of RA signalling from the trunk of the embryo (Chinnaiya et al., 2014; Pickering et al., 2019) (Fig. 6A). Thus, during digit identity specification stages in the chick wing, Shh signalling stimulates G1-to S-phase entry via cyclin D2 (Ccdn2) and this could adjust the number of Shh-expressing polarising region cells. However, following digit identity specification, Shh signalling inhibits G1-to S-phase entry via the Bmp2-mediated regulation of the D cyclin-dependent kinase inhibitor p27kip1, which prevents the polarising region from producing at least one additional posterior digit (Pickering et al., 2019) (Fig. 6A). The fate of most chick wing polarising region cells is to undergo apoptosis, which is also controlled by Shh signalling (Sanz-Ezquerro and Tickle, 2000), thus further showing how morphogenetic processes are tightly regulated in the posterior part of the chick wing to restrict digit number.

It had been initially suggested that polarising region signalling, as well as specifying antero-posterior positional values that encode digit identity, could also determine digit number (Wolpert, 1969). However, ‘recombinant limb’ experiments, in which chick limb bud mesoderm cells are disaggregated and reaggregated, before being transferred into an epithelial jacket and grafted to a host embryo, have shown the astonishing ability of cells to self-organise into a rudimentary pattern of digits (Zwilling, 1964; Pautou, 1973). The grafting of a polarising region into a recombinant limb gives the digits distinctive morphological characteristics (Elisa Piedra et al., 2000), thus showing that the processes of digit specification (positional information) and digit number determination (self-organisation) are separable. In addition, many mouse mutants with de-repressed Shh signalling, such as the Shh/Gli3 double mutant, produce multiple digits of very similar anatomy (Litingtung et al., 2002; te Welscher et al., 2002b). These findings are consistent with digit number being determined by the width of the hand-plate, which provides boundary conditions for a ‘Turing-type’ self-organising system based on reaction-diffusion (Wilby and Ede, 1975; Newman and Frisch, 1979). From experimental evidence in the mouse, a model has been formulated that integrates the known roles of Bmp ligands as activators of digit formation and Wnt ligands as inhibitors, to converge on an early chondrogenic marker, Sox9, thereby producing a repeated pattern of digits and interdigits (Raspopovic et al., 2014). 5’ Hoxa and Hoxd proteins are implicated in the control of digit spacing, and hence digit number, by determining the wavelength of reaction-diffusion of Wnt and Bmp ligands (Sheth et al., 2012). However, it is unclear how this is achieved. Digit formation has also been modelled on the ability of mesoderm cells to sort themselves using their differential surface adhesion properties (Oster et al., 1983), which can occur in the absence of Sox9 in vitro (Barna and Niswander, 2007). Indeed, studies in the chick have implicated galectin proteins, which bind cell-surface carbohydrates, in facilitating self-organisation by adhesion (Bhat et al., 2011). Therefore, the interplay between reaction-diffusion and cell adhesion in digit number determination needs to be resolved.

Shh signalling controls 5’ Hoxd gene expression (Capellini et al., 2006; Lettice et al., 2017), and this could provide a mechanism that integrates digit identity specification and digit number.
Conclusions

We have presented a current view of how the vertebrate limb is patterned. This knowledge is crucial to our understanding of how a myriad of genetic disorders affect human limb development, and to the ultimate goal of designing regenerative therapies to enable the replacement of missing limb structures (Cox et al., 2019). However, many challenges remain and we will outline three. First, although we have discussed signalling molecules, the dynamics underlying gradient formation and their range of action remain unclear, which is complicated by the fact that they can be transported by different mechanisms, such as diffusion, or by filopodia in the case of Shh (Sanz-Ezquero and Tickle, 2003). Taken together, a common theme emerges in which the interpretation of positional information depends on the subtle regulation of the same genes involved in processes such as connective tissue/cartilage development.

Interpretation of gene expression into limb anatomy

A major gap in our understanding of limb development is how gene expression is translated into anatomy. The best candidates we have are Pitx1 as a hindlimb determinant and Lmx1b as a dorsal determinant: the mis-expression of Pitx1 in the mouse forelimb results in the acquisition of morphologies that are characteristic of the hindlimb (Minguillon et al., 2005); and, as mentioned previously, the constitutive overexpression of Lmx1b dorsalises the chick limb (Riddle et al., 1995; Vogel et al., 1995). So how do these transcription factors determine anatomy?

Nemec and colleagues have used RNA-seq and ChiP-seq to identify Pitx1 targets in the mouse limb (Nemec et al., 2017). As appreciated in previous work, very few genes are expressed exclusively in forelimb or hindlimb buds (Cotney et al., 2012). Surprisingly, however, Pitx1 modulates the expression of genes that are active during both forelimb and hindlimb development, in particular, factors involved in chondrogenesis, including Sox9 (Nemec et al., 2017). In the search for additional candidates, a further study in the mouse has shown that Tbx4 interacts directly with the hindlimb-restricted Hoxc10 protein, and ChiP-seq analyses revealed that this complex activates many of the same genes as Tbx5 (Jain et al., 2018). Although this is unsurprising, it highlights the major challenge of understanding how the same genes could be involved in determining subtle anatomical variation.

Harro and colleagues have used a similar strategy involving ChiP-seq analysis to find Lmx1b targets in E12.5 mouse limb buds (Harro et al., 2017). Direct transcriptional targets include genes involved in various processes, most notably, in terms of tissue architecture: the extracellular matrix and bone development. Interestingly, one direct target of Lmx1b is the TGFβ family member, Gdf5 (growth differentiation factor 5), which is involved in joint formation, thus providing a link between gene expression and anatomy (Harro et al., 2017).

For proximo-distal patterning, Meis and 5' Hox proteins remain the best candidates for determining the anatomy of the main subdivisions of the limb. However, there is no evidence that the manipulation of these genes can cause the transformation of positional identity. Verified targets of 5' Hox proteins include genes involved in cell adhesion, such as those encoding ephrin receptors (Stadler et al., 2001; Salsi and Zappavigna, 2006), cadherins (Salsi et al., 2008) and genes involved in chondrogenesis, such as Bmp2 and Bmp7 (Knosp et al., 2004). Targets involved in cell adhesion are of particular interest because the stable memory of positional information is considered to reside in differential cell surface properties (Ide et al., 1994; Nardi and Stocum, 1984; Wada and Ide, 1994).

For translating antero-posterior positional information into digit identity, most studies have used genomic approaches in chick and mouse limbs to characterise the downstream response to Shh signalling, and have uncovered many of the same targets, such as Bmp2, Hoxd13, Tbx2, Tbx3 and Grem1 (Vokes et al., 2008; Bangs et al., 2010). Lewandowski and colleagues have undertaken detailed ChiP-sequencing and RNA-sequencing analyses of the posterior region of Shh/Gli3 mouse mutants (Lewandowski et al., 2015). However, several digits of similar anatomy form in the limbs of these mutants because of the de-repression of Shh signalling (Litingtung et al., 2002; te Welscher et al., 2002a), so it is unclear if they have distinct identities. Nonetheless, the results showed that Shh signalling controls gene expression, primarily by relieving repression by its main transcriptional effector, Gli3. In addition, three regional patterns of gene expression have been described in the limb bud (Lewandowski et al., 2015). In terms of the specification of digit identity, the most interesting region expresses the Hoxd13, Salt1 and Salt3 genes, which have previously been implicated in this process (reviewed by Tickle and Towers, 2017). So far, only the overexpression of Tbx2 and Tbx3 has been reported to change digit identity in the chick leg, albeit with low penetrance (Suzuki et al., 2004).

The evidence that Bmp signalling could act downstream of Shh signalling at early limb bud stages could make it worthwhile to determine if its downstream targets are involved in the specification of digit identity. This idea is lent support because the manipulation of Bmp signalling in the so-called phalanx-forming region (Suzuki et al., 2008) during chondrogenic stages can transform digit identity in the chick leg (Dahn and Fallon, 2000). Therefore, it is possible that Bmp signalling primes the activity of genes at early stages, which are expressed later in response to a second wave of Bmp signalling. In addition, Bmp signalling inhibits Fgf signalling by the apical ectodermal ridge – the duration of which determines the number of phalanges with a periodicity characteristic for each digit (Sanz-Ezquero and Tickle, 2003). Taken together, a common theme emerges in which the interpretation of positional information depends on the subtle regulation of the same genes involved in processes such as connective tissue/cartilage development.

determination. Indeed, the pharmacological inhibition of Shh signalling in the chick wing at a specific temporal window during anterio posterior positional value promotion can uncouple these two processes, and produce up to three morphologically similar digits [similar to digit 2 in terms of phalange number and proportion (Pickering and Towers, 2016)]. Recent research has provided a further mechanism for how Shh signalling and 5' Hox genes control digit number in the mouse limb. Digit 1 (thumb) development requires Hoxa13 to maintain Hoxd13 expression via inhibition of Gli3 (Bastida et al., 2020). This means that, in Hoxa13 mutant limbs, Gli3 represses Hoxd13 and this prevents thumb formation, emphasising once again the cross-repressive nature of Hox gene regulation (Bastida et al., 2020).
Furthermore, it is likely that such experimental data will need to be integrated with biophysical, computational and mathematical approaches to help understand how fine-scale anatomy is achieved. Finally, it is encouraging that, although this Review has largely concentrated on mouse and chick studies, attempts are being made to understand human limb development (Cotney et al., 2013), which is the ultimate goal of the field.

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Competing interests

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