Watch-ing out for chick limb development

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Synopsis Time control is a crucial issue during embryonic development. Nevertheless, little is known about how embryonic cells measure time. Until recently, the only molecular clock known to operate during vertebrate embryonic development was the somitogenesis clock, exclusively functioning in coordinating the precise timing of each new pair of somites formed from the presomitic mesoderm. We have recently evidenced that a similar molecular clock also underlies the timing at which autopod chondrogenic precursors are laid down to form a skeletal limb element. In addition, we herein suggest that the molecular clock is not the only parallelism that can be established between somitogenesis and limb-bud development. In an evolutionary perspective, we support the previously proposed idea that the molecular mechanisms involved in the segmentation of the body axis may have been partially reused in the mesoderm of the lateral plate, thereby allowing the emergence of paired appendages.

Introduction

Growth and patterning of the vertebrate tetrapod limb has been a subject of intensive investigation for about three decades (Niswander 2003). Limb buds form at specific positions along the axis of the body of an embryo. At presumptive limb levels, cells of the lateral plate mesoderm (LPM) proliferate under the epidermal tissue, initiating the formation of growing buds (Searls and Janners 1971; Chevallier et al. 1977; Christ et al. 1977). Later on, this mesenchyme gives rise to skeletogenic precursors, among other cell types, while muscle precursor cells invade the limb upon delamination from the lateral edges of the nearby somites (Buckingham et al. 2003). Three orthogonal axes describe the anatomy of the limb: proximal–distal (p–d), from the shoulder to the fingertips; anterior–posterior (a–p), from the thumb to the little finger; and the dorsal–ventral (d–v), from the back of the hand to the palm. The limb skeleton is formed by a distinct number of bones with a characteristic size and shape that are laid down along the p–d axis—humerus (stylopod), ulna and radius (zeugopod), metacarpals, and phalanges (autopod)—as the limb bud grows. P–d outgrowth is controlled by cells of the apical ectodermal ridge (AER), a specialized ectodermal structure that runs along the length of the distal tip of the limb bud and that expresses proteins belonging to the fibroblast growth factor (FGF) family of secreted proteins (reviewed by Martin 1998). The intracellular responses to FGFs are mediated by the MAPK signal-transduction cascade (Javerzat et al. 2002). In 2003, Kawakami and colleagues found *MAPK phosphatase 3* (*mkp3*), a gene encoding a negative regulator of the MAPK/ERK pathway (Groom et al. 1996; Mourey et al. 1996; Muda et al. 1996; Smith et al. 1997) to be a downstream factor of the FGF8 signalling pathway during limb bud development.

Among the three cardinal axes of the limb, the mechanisms that lead to specification of cell fate along the p–d axis are the least understood. Dudley and co-workers proposed that populations of cells giving rise to the different p–d skeletal structures of the limb are specified very early during limb-bud development—"early specification model." Later, already specified populations of cells differentiate sequentially giving rise first to the more proximal structures and forming the distal structures last (Dudley et al. 2002). Another paradigm to explain limb development along this axis, largely unmodified since its conception 30 years ago, is the "progress zone model" (Summerbell et al. 1973). According to this model, the time cells spend in the distal-most mesenchyme, defined as the progress zone (PZ), specifies their fate along the p–d axis. Cells in the PZ would change their positional value over time, this being fixed when they leave this zone

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(Summerbell et al. 1973). The cells that leave the PZ earlier give rise to the more proximal skeletal elements of the wing (humerus) and those leaving later give rise to the more distal ones (phalanges). This model suggests that a clock-like mechanism could be operating, measuring the time that these cells spend in the PZ.

Palmeirim et al. (1997) demonstrated the existence of a molecular clock underlying chick somitogenesis by showing that presomitic mesodermal (PSM) cells undergo several cycles of *hairy1* gene expression, with a 90 min periodicity, corresponding to the time required to form one somite (Palmeirim et al. 1997). This oscillatory expression provided the first molecular evidence for a developmental clock linked to somitogenesis, in which both the segment number and the time of formation are extraordinarily constant and species-specific. This molecular clock operates in all vertebrate groups that have been studied and an increasing number of genes belonging to the FGF, Notch, and Wnt signaling pathways were found to have a cyclic behavior in the PSM cells (reviewed by Andrade et al. 2005; Freitas et al. 2005; Dequéant et al. 2006; Stern et al. 2006).

In the chick PSM, posterior FGF8 and anterior retinoic acid activities form two opposing gradients (Dubrulle et al. 2001; Diez del Corral et al. 2003). The confrontation of these gradients gives rise to the so-called determination front that moves posteriorly as the embryonic axis elongates (Dubrulle et al. 2001). When the determination front is experimentally shifted anteriorly by placing an FGF8-coated bead in the mid-PSM, smaller somites form. Conversely, FGF8 inhibition at the same level induces the formation of larger somites (Dubrulle et al. 2001). Thus, FGF8 maintains posterior PSM cells in an immature state, negatively regulating differentiation in chick PSM.

In summary, in the somitogenesis system, a molecular clock operates in the PSM and regulates the periodicity of the formation of the segmented elements (the somites) and a maturation wavefront controls the size of the elements that are formed. An interesting question thus arises: are the same molecular mechanisms used in other tissues to control the formation of different segmented structures?

Hairy2 is cyclically expressed in the precursor cells of the autopod limb bones

Hirata et al. (2002) showed that periodic expression of *Hes1* can be triggered in a variety of cultured cell lines in response to a serum shock, suggesting that the genes of the segmentation clock might play a role in other cell types and biological processes. *In vivo*, however, such oscillations of gene expression have only been reported in presomitic cells. Recently, we described the dynamic expression of *hairy2* (the chick *hes1* homologue) in the distal part of stage HH20-28 forelimb mesenchyme, in an area of undifferentiated chondrogenic precursor cells (Fig. 1). By performing *in ovo* microsurgery, we demonstrated that *hairy2* expression is cyclic in phalanx chondrogenic precursors with a 6 h periodicity (Pascoal, Carvalho et al. 2007; Fig. 2). In contrast, *hairy2* expression is never detected in the central core of the limb where the already-specified cartilaginous elements are localized, suggesting that cartilaginous precursor cells stop performing *hairy2* expression cycles when they differentiate (Pascoal, Carvalho et al. 2007).

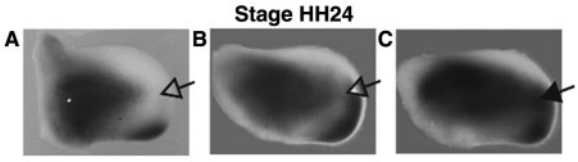

Fig. 1 *Hairy2* dynamic expression pattern in distal limb mesenchyme of a chick wing at stage HH24. *hairy2* dynamic expression pattern of a limb-bud stage HH24. At the level of the distal limb mesenchyme one can observe diverse expression patterns of *hairy2*: (**A**) a clear negative *hairy2* expression pattern, (**B**) an intermediate *hairy2* expression pattern, and (**C**) a strongly positive *hairy2* expression pattern. (A) Hollow arrows point to the absence of *hairy2* expression in the distal limb mesenchyme. (B) Partially filled arrows indicate an intermediate *hairy2* expression pattern. (C) Filled arrows point to the strong *hairy2* expression in the distal limb mesenchyme. All pictures show a dorsal view. Adapted from Pascoal, Carvalho et al., 2007.

We demonstrated that *hairy2* is cyclically expressed in the distal mesenchyme of the limb with a 6 h periodicity (Pascoal, Carvalho et al. 2007). Interestingly, the 6 h time-period is a multiple of the 1.5 h period reported for *hairy2* in somite precursor cells. This is strikingly in accordance with the ''clock and trail model'' proposed by Kerszberg and Wolpert (2000), which concentrates on the somitogenesis clock and in which period-doubling oscillations can arise from an oligomerization mechanism between transcription factors that act either by activating or inhibiting genes, including themselves. These authors argue for the existence of cyclic gene expression with a period different from 1.5 h, but multiples of this value, and these cycles could specify hemi-somites

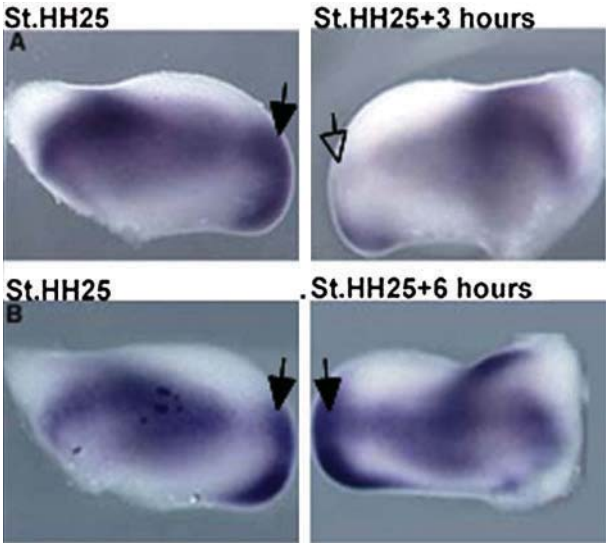

Fig. 2 The molecular clock in limb bud autopod precursor cells shows a periodicity of 6 h. Comparison of the *hairy2* expression pattern between the two wings of the same embryo in which the right wing was surgically removed and fixed immediately, while the left wing was reincubated (*in ovo*) for different time periods. Both wings were hybridized simultaneously ensuring the same conditions. (**A**) The *hairy2* expression pattern in the left wing is distinct from the one in the right wing that has been incubated for 3 h. (**B**) The *hairy2* expression pattern is similar in left wing and right wing both of which have been incubated for 6 h. Hollow and filled arrows point to the absence or the presence of *hairy2* expression in the distal limb mesenchyme. All pictures show a dorsal view. Adapted from Pascoal, Carvalho et al., 2007.

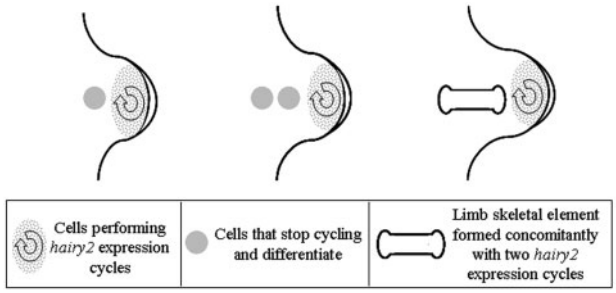

Fig. 3 Proposed model for the correlation between the periodicity of *hairy2* cycles and the formation of skeletal elements. The undifferentiated cells localized at the tip of the limb perform cycles of *hairy2* expression since early stages of limb-bud development (stage 20HH). As the limb bud grows, cells leave the undifferentiated zone, stop cycling, and differentiate. Each cycle of *hairy2* in autopod limb chondrogenic cells lasts 6 h and an autopod skeletal element takes 12 h to be formed. This means that two cycles of *hairy2* expression occur during the laying down of a new autopod skeletal element.

(period of 45 min), somites (1.5 h), or even pairs of somites (3 h) (Kerszberg and Wolpert, 2000).

During somitogenesis, each cycle of expression of a molecular clock gene underlies the formation of a new pair of somites and both events take 1.5 h. In the limb, the formation time of skeletal elements was unknown.

By measuring the time between the appearances of two consecutive gdf5 (joint marker) stripes of expression corresponding to the second and third joint of digit 3, we found that the formation time of the second phalanx is 12 h (Pascoal, Carvalho et al. 2007). Previous work suggested that each of the seven limb bone primordials take about the same length of time to be laid down (Wolpert et al. 1975). If this is the case, the formation of the entire set of seven limb elements would take 3.5 days, which is in accordance with the experimentally-determined time required to form a chick forelimb (3–4 days; Hamburger and Hamilton 1951).

During somite formation, each PSM cell undergoes many molecular clock gene cycles before escaping the influence of FGF8 and becoming committed to incorporate a somite. Similarly, we propose that autopod chondrogenic precursor cells go through several cycles of *hairy2* expression until they incorporate a cartilaginous primordial of an autopod bone and that two *hairy2* cycles represent the time period required to form an autopod skeletal element (Pascoal, Carvalho et al. 2007; Fig. 3). Hence, this study unveils the existence of a limb molecular clock operating during autopod p–d outgrowth and patterning, with a specific time period that can be correlated with the time required to form an autopod skeletal element.

Cyclic *hairy2* expression and the acquisition of temporal information

The major difference between the ''progress zone model'' (Summerbell et al. 1973) and the ''early specification model'' (Dudley et al. 2002) is the timing at which p–d specification takes place. The progress zone model proposes sequential p–d specification, while the early specification model suggests that all cell fates are already specified within the early limb bud and that progressive formation of p–d segments results from a temporally coordinated expansion of the progenitor populations in each domain (Dudley et al. 2002). In light of the new data, we propose that cells in the proliferative region of the limb can record the number of *hairy2* oscillations that they perform and thus acquire a temporal value. This temporal value could be (1) translated in positional value, in agreement with the progress zone model or (2) it could establish the expansion time for the cell populations

Table 1 Experimental data on the putative role of Notch signalling pathway in both mouse and chick forelimb p–d development

Mouse				
Gene	**Expression in distal limb mesenchyme**	**Expression in the AER**	**Limb phenotype in KO or misexpression studies**	**References**
			Mouse	
Notch1	–	+	Hyperplastic AER; Syndactyly	Francis et al. 2005 (Fig. 1A) Shawber et al. 1996 (Fig. 1F)
Notch2	+	*	Unknown; KO mouse die at E11.5	Shawber et al. 1996; Hamada et al. 1999
Delta1	–	–	Muscle hypotrophy; Inhibition of both muscle and chondrocyte differentiation	Schuster-Gossler et al. 2007; Watanabe et al. 2003
Serrate1/Jagged1	+	–	Unknown; KO mouse die at E11.5	McGlinn et al. 2005 (Fig. 1G) Francis et al. 2005 (Fig. 1B) Shawber et al. 1996 (Fig. 2E) Xue et al. 1999
Serrate2/Jagged2	–	+	Hyperplastic AER syndactyly	Sidow et al. 1997 Jiang et al. 1998 Francis et al. 2005 (Fig. 1C) Shawber et al. 1996 (Fig. 2D) Pan et al. 2005
Presenilin	+	*	Clinched forelimb digits, truncated digits, some missing distal phalanxes	Pan et al. 2005
Hes1	+	+	*	McGlinn et al. 2005 (Fig. 1P) Jouve et al. 2000; Ishibashi et al. 1995
Hey1	+	*	*	McGlinn et al. 2005 (Fig. 1S) Fischer et al. 2004
			Chick	
notch1	–	+	*	Myat et al. 1996; Hayashi et al. 1996 (Fig. 6A)
notch2	+	–	*	Hayashi et al. 1996
delta1	–	–	Inhibition of both muscle and chondrocyte differentiation	Delfini et al. 2000; Crowe et al. 1999
serrate1	+	–	*	Myat et al. 1996 (Fig. 5B–E) Hayashi et al. 1996 (Fig. 6C) Vargesson et al. 1998
serrate2	–	+	*	Hayashi et al. 1996 (Fig. 6B) Delfini et al. 2000; Vargesson et al. 1998
hairy1	+	–	Reduction of limb skeletal elements size	Vasiliauskas et al. 2003 Our unpublished observation
hairy2	+	+	*	Pascoal, Carvalho et al. 2007

*No data available in the literature.

already specified according to the early specification model. Thus, the proposal that *hairy2* expression provides temporal information is compatible with either model (Pascoal, Carvalho et al. 2007).

Notch signaling pathway in p–d limb outgrowth

Studies in both chick and mouse embryos provide data compatible with a role for the Notch signaling pathway in the development of the distal mesenchyme of the forelimb (Table 1). Limb phenotypes of mouse embryos lacking *Notch1* or *Serrate2/Jagged2* (expressed in both chick and mouse AER) in the AER implicate Notch signaling in the development of the AER and in interdigital apoptosis, possibly precluding a role for these particular genes in the molecular clock machinery of the limb bud (chick: Hayashi et al. 1996; Myat et al. 1996; mouse: Shawber et al. 1996; Sidow et al. 1997; Jiang et al. 1998; Francis et al. 2005; Pan et al., 2005). In contrast, *Notch2*, and its ligand *Serrate1/Jagged1*, are coexpressed in the distal mesenchyme of the limb, rather than in the AER, both in chicks

(Hayashi et al. 1996; Myat et al. 1996) and in mice (Shawber et al. 1996; Francis et al. 2005; McGlinn et al. 2005). Unfortunately, both *Notch2* and *Serrate1*/*Jagged1*-null mouse mutants die *in utero* by E11.5, preventing determination of the function of these genes in limb mesenchyme (Hamada et al. 1999; Xue et al. 1999). Notch downstream targets *hairy1 and hairy2* in the chick (Vasiliauskas et al. 2003; Pascoal, Carvalho et al. 2007 and our unpublished data) and *Hes1, Hey1* in the mouse (McGlinn et al. 2005) are coexpressed with *Notch2 and Serrate1/Jagged1* in the distal mesenchyme of the limb, indicating an activation and a putative function of this signaling pathway. In agreement, studies of the misexpression of *hairy1* in the chick implicate Notch pathway in the regulation of overall limb size (Vasiliauskas et al. 2003). Since no skeletal analysis has been performed for the *Hes1- and Hey1*-null mouse mutant (Ishibashi et al. 1995; Fischer et al. 2004), we do not know the role these genes play in skeletal limb-bud development. Moreover, while evidence has been provided that Notch can function nonautonomously across germ layers (Baker and Schubiger 1996), it is possible that different combinations of these receptors/ligands may mediate an interaction between the AER and the mesenchyme. Interestingly, overactivation of Notch signaling in the limb ectoderm produced a dramatic effect on limb skeletal development, involving extensive hind limb truncations, including complete or partial loss of zeugopods and autopods (Pan et al. 2005). Finally, loss of both presenilins from the mesenchyme of the mouse limb gives rise to mutant embryos with clenched forelimb digits, truncated digits, and digits with some distal phalanges missing, leading the authors to propose that a deficiency of presenilin in mesenchyme could affect endochondral skeletal differentiation (Pan et al. 2005).

In conclusion, data both from the chick and the mouse suggest a possible role of the Notch signaling pathway in the distal mesenchyme of the limb, possibly through the receptor/ligand duo *Notch2/Serrate1*, as has been previously proposed (Hayashi et al. 1996; Shawber et al. 1996; Pan et al. 2005). Our recent report of the cyclic expression of *hairy2*, a transcription factor downstream of Notch, allowing the identification of a molecular clock operating in the distal mesenchyme of the limb, reinforces this possibility (Pascoal, Carvalho et al. 2007). To further evaluate the role of the Notch signaling pathway in the distal mesenchyme of the limb, it is crucial to produce KO mice for *Notch 2* and *Jagged1/Serrate1* conditional for the distal limb mesenchyme and to perform further studies of misexpression of these and other genes in the chick embryo.

Is there a wavefront in the limb bud?

In 2001, Dubrulle et al. (2001) demonstrated a gradient of *fgf8* transcripts starting from the embryo tail bud and fading in the direction of the uppermost part of the PSM. This graded distribution of *fgf8* mRNA was shown to result from progressive mRNA decay, rather than from different transcriptional levels (Dubrulle and Pourquié 2004). Recently, we have shown that *mkp3*, a downstream effector of FGF8 (Kawakami et al. 2003), has a graded distribution of its transcripts in the mesenchyme of the limb bud, beginning in the most distal part of the limb (underneath the AER) and fading out in the direction of the more proximal part of the limb (Pascoal, Andrade et al. 2007). To further characterize this gradient, an intronic probe for *mkp3* was constructed and the distribution of *mkp3* nascent transcripts observed. The cells recognized by the intronic probe were restricted to the more distal part of the limb, mimicking the effect obtained with the intronic *fgf8* probe in the PSM (Dubrulle and Pourquié 2004). This result demonstrates that the graded distribution of *mkp3* in distal limb mesenchyme is a result of mRNA decay (Pascoal, Andrade et al. 2007).

These results led us to propose that the graded *mkp3* expression pattern in the chick limb bud might result from RNA decay as follows: (1) the FGF8 protein produced in the AER induces *mkp3* expression at the distal part of the limb; (2) as the limb bud grows the cells progressively move away from the FGF8 source; (3) once they are distant from the AER, the transcription of the *mkp3* is no longer induced and the mRNA molecules still present in these cells decay over time, giving rise to a spatial gradient of mRNA expression in the limb bud (Pascoal, Andrade et al. 2007).

The gradient of *mkp3* mRNA observed in chick wing bud corresponds to a gradient of Fgf8 activity, equivalent to that present in the chick presomitic mesoderm. Future work should clarify whether a wavefront is also involved in the establishment of the size of the limb bones.

The clock in an evolutionary perspective

The acquisition of paired appendages was a main evolutionary advance for locomotion in vertebrates. The tetrapod forelimb bud is considered the vertebrate homologue of the zebrafish pectoral fin bud, since they share structural organization and

repertoires of gene expression (Grandel and Schulte-Merker 1998), suggesting that the paired appendages are conserved among species. Thus, the chick limb-bud molecular clock could also be operative in the limbs/fins of other species.

The first vertebrate fossils that were discovered lack paired fins, but show brawny median fins (Coates 1994; Zhang and Hou 2004), implying that mechanisms of fin development were first gathered in the midline (Freitas et al. 2006). Freitas and co-workers (2006) studied this possibility and showed that the development of the median fin in sharks involves the same genetic programs that operate in paired appendages. By using molecular markers for different cell types, they showed that median fins arise predominantly from somitic mesoderm, whereas paired appendages develop from the mesoderm of the lateral plate. These results suggest that the molecular mechanisms used for fin development originated in the somitic mesoderm of the first vertebrates and the origin of paired appendages was associated with relocation of these mechanisms to the lateral plate mesoderm (Freitas et al. 2006). Combining these results with the discovery of a molecular clock in the chick limb bud, we consider the molecular clock to be a constituent of the molecular program of the somitic mesoderm that was reused, thereby allowing the emergence of the paired appendages from the mesoderm of the lateral plate.

The forelimb of whales and dolphins presents hyperphalangy (supernumerary finger bones). One of the developmental bases proposed for this terminal addition of extra phalanges is that the limb outgrowth controlled by the AER may be prolonged by a shift in timing or heterochrony (Richardson and Oelschläger 2002). Richardson and Oelschläger (2002) showed that in the spotted dolphin *Stenella attenuate*, hyperphalangy was evident on digits II and III. Histological analysis has shown that the apical ectoderm was thickened into a cap that persists on digits II and III at stages when it had disappeared from other digits (Richardson and Oelschläger 2002). This implies that the cells at the tip of the limb are maintained in an undifferentiated state longer than are those in the other digits. This could indicate that the molecular clock genes would undergo expression cycles more times than in other digits. This could be a complementary explanation for the supernumerary phalanges formed in these two particular digits of the dolphin.

Final remarks

The studies reported here allow us to propose a certain degree of parallelism between somitogenesis and limb bud development. Both tissues comprise a zone in which cells are maintained in an undifferentiated state (posterior PSM and distal limb mesenchyme). In addition, in the chick PSM, anterior retinoic acid and posterior FGF8 activities form two opposing gradients (Diez del Corral et al. 2003) and the same happens during limb bud development in which retinoic acid synthesis and signaling is restricted to the proximal limb by FGF activity (Mercander et al. 2000). Moreover, in both limb and PSM, a molecular clock operates, regulating the periodicity of structure formation. Finally, we propose the possibility that a wavefront could be present in the distal limb mesenchyme in a way reminiscent of the one in the PSM. Our data provides further support for the previously proposed idea that the molecular program used for a–p axis segmentation of the vertebrate body might be partially reused in the outgrowth of vertebrate limbs.

The discovery of the limb molecular clock unveiled a new mechanism in limb bud development. Taken together, the studies reviewed here have opened a completely novel line of investigation into limb development that could be further extrapolated to several developmental processes. Much work, however, must be performed before the machinery of this clock becomes completely understood.

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References

Andrade RP, Pascoal S, Palmeirim I. 2005. Thinking clockwise. Brain Res Brain Res Rev 49:114–9.

Baker R, Schubiger G. 1996. Autonomous and nonautonomous Notch functions for embryonic muscle and epidermis development in *Drosophila*. Development 122:617–26.

Buckingham M, Bajard L, Chang T, Daubas P, Hadchouel J, Meilhac S, Montarras D, Rocancourt D, Relaix F. 2003. The formation of skeletal muscle: from somite to limb. J Anat. 202:59–68.

Chevallier A, Kieny M, Mauger A. 1977. Limb-somite relationship: origin of the limb musculature. J Embryol Exp Morphol 41:245–58.

Christ B, Jacob HJ, Jacob M. 1977. Experimental analysis of the origin of the wing musculature in avian embryos. Anat Embryol 150:171–86.

Coates MI. 1994. The origin of vertebrate limbs. Development (Suppl):169–80.

Crowe R, Zikherman J, Niswander L. 1999. Delta-1 negatively regulates the transition from prehypertrophic to hypertrophic chondrocytes during cartilage formation. Development 126:987–98.

Delfini MC, Hirsinger E, Pourquie O, Duprez D. 2000. Delta 1-activated notch inhibits muscle differentiation without affecting Myf5 and Pax3 expression in chick limb myogenesis. Development 127:5213–24.

Dequeant ML, Glynn E, Gaudenz K, Wahl M, Chen J, Mushegian A, Pourquie O. 2006. A complex oscillating network of signaling genes underlies the mouse segmentation clock. Science 314:1595–98.

Diez DC, Olivera-Martinez I, Goriely A, Gale E, Maden M, Storey K. 2003. Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron 40:65–79.

Dubrulle J, McGrew MJ, Pourquie O. 2001. FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. Cell 106:219–32.

Dubrulle J, Pourquie O. 2004. Fgf8 mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. Nature 427:419–22.

Dudley AT, Ros MA, Tabin CJ. 2002. A re-examination of proximodistal patterning during vertebrate limb development. Nature 418:539–44.

Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M. 2004. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. Genes Dev 18:901–11.

Francis JC, Radtke F, Logan MP. 2005. Notch1 signals through Jagged2 to regulate apoptosis in the apical ectodermal ridge of the developing limb bud. Dev Dyn 234:1006–15.

Freitas C, Rodrigues S, Saude L, Palmeirim I. 2005. Running after the clock. Int J Dev Biol 49:317–24.

Freitas R, Zhang G, Cohn MJ. 2006. Evidence that mechanisms of fin development evolved in the midline of early vertebrates. Nature 442:1033–37.

Grandel H, Schulte-Merker S. 1998. The development of the paired fins in the zebrafish (*Danio rerio*). Mech Dev 79:99–120.

Groom LA, Sneddon AA, Alessi DR, Dowd S, Keyse SM. 1996. Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. EMBO J 15:3621–32.

Hamada Y, Kadokawa Y, Okabe M, Ikawa M, Coleman JR, Tsujimoto Y. 1999. Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. Development 126:3415–24.

Hamburger V, Hamilton HL. 1951. A series of normal stages in the development of the chick embryo. Dev Dyn 195:231–72.

Hayashi H, Mochii M, Kodama R, Hamada Y, Mizuno N, Eguchi G, Tachi C. 1996. Isolation of a novel chick homolog of serrate and its coexpression with C-Notch-1 in chick development. Int J Dev Biol 40:1089–96.

Hirata H, Yoshiura S, Ohtsuka T, Bessho Y, Harada T, Yoshikawa K, Kageyama R. 2002. Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. Science 298:840–43.

Ishibashi M, Ang SL, Shiota K, Nakanishi S, Kageyama R, Guillemot F. 1995. Targeted disruption of mammalian hairy and enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev 9:3136–48.

Javerzat S, Auguste P, Bikfalvi A. 2002. The role of fibroblast growth factors in vascular development. Trends Mol Med 8:483–89.

Jiang R, Lan Y, Chapman HD, Shawber C, Norton CR, Serreze DV, Weinmaster G, Gridley T. 1998. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. Genes Dev 12:1046–57.

Jouve C, Palmeirim I, Henrique D, Beckers J, Gossler A, Ish-Horowicz D, Pourquie O. 2000. Notch signalling is required for cyclic expression of the hairy-like gene HES1 in the presomitic mesoderm. Development 127:1421–1429.

Kawakami Y, et al. 2003. MKP3 mediates the cellular response to FGF8 signalling in the vertebrate limb. Nat Cell Biol 5:513–9.

Kerszberg M, Wolpert L. 2000. A clock and trail model for somite formation, specialization and polarization. J Theor Biol 205:505–10.

Martin GR. 1998. The roles of FGFs in the early development of vertebrate limbs. Genes Dev 12:1571–86.

McGlinn E, et al. 2005. Pax9 and Jagged1 act downstream of Gli3 in vertebrate limb development. Mech Dev 122:1218–33.

Mercader N, Leonardo E, Piedra ME, Martinez AC, Ros MA, Torres M. 2000. Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. Development 127:3961–70.

Mourey RJ, Vega QC, Campbell JS, Wenderoth MP, Hauschka SD, Krebs EG, Dixon JE. 1996. A novel cytoplasmic dual specificity protein tyrosine phosphatase implicated in muscle and neuronal differentiation. J Biol Chem 271:3795–802.

Muda M, Boschert U, Dickinson R, Martinou JC, Martinou I, Camps M, Schlegel W, Arkinstall S. 1996. MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase. J Biol Chem 271:4319–26.

Myat A, Henrique D, Ish-Horowicz D, Lewis J. 1996. A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. Dev Biol 174:233–47.

Niswander L. 2003. Pattern formation: old models out on a limb. Nat Rev Genet 4:133–43.

Palmeirim I, Henrique D, Ish-Horowicz D, Pourquie O. 1997. Avian hairy gene expression identifies a molecular clock

linked to vertebrate segmentation and somitogenesis. Cell 91:639–48.

Pan Y, Liu Z, Shen J, Kopan R. 2005. Notch1 and 2 cooperate in limb ectoderm to receive an early Jagged2 signal regulating interdigital apoptosis. Dev Biol 286:472–82.

Pascoal S, Carvalho CR, et al. 2007. A molecular clock operates during chick autopod proximal-distal outgrowth. J Mol Biol 368:303–9.

Pascoal S, Andrade RP, et al. 2007. Progressive mRNA decay establishes an mkp3 expression gradient in the chick limb bud. Biochem Biophys Res Commun 352:153–57.

Richardson MK, Oelschlager HH. 2002. Time, pattern, and heterochrony: a study of hyperphalangy in the dolphin embryo flipper. Evol Dev 4:435–44.

Schuster-Gossler K, Cordes R, Gossler A. 2007. Premature myogenic differentiation and depletion of progenitor cells cause severe muscle hypotrophy in Delta1 mutants. Proc Natl Acad Sci USA 104:537–42.

Searls RL, Janners MY. 1971. The initiation of limb bud outgrowth in the embryonic chick. Dev Biol 24:198–213.

Shawber C, Boulter J, Lindsell CE, Weinmaster G. 1996. Jagged2: a serrate-like gene expressed during rat embryogenesis. Dev Biol 180:370–6.

Sidow A, Bulotsky MS, Kerrebrock AW, Bronson RT, Daly MJ, Reeve MP, Hawkins TL, Birren BW, Jaenisch R, Lander ES. 1997. Serrate2 is disrupted in the mouse limb-development mutant syndactylism. Nature 389:722–5.

Smith A, Price C, Cullen M, Muda M, King A, Ozanne B, Arkinstall S, Ashworth A. 1997. Chromosomal localization of three human dual specificity phosphatase genes (DUSP4, DUSP6, and DUSP7). Genomics 42:524–27.

Stern CD, Charité J, Deschamps J, Duboule D, Durston AJ, Kmita M, Nicolas JF, Palmeirim I, Smith JC, Wolpert L. 2006. Head-tail patterning of the vertebrate embryo: one, two or many unresolved problems? Int J Dev Biol 50:3–15.

Summerbell D, Lewis JH, Wolpert L. 1973. Positional information in chick limb morphogenesis. Nature 244:492–96.

Vargesson N, Patel K, Lewis J, Tickle C. 1998. Expression patterns of Notch1, Serrate1, Serrate2 and Delta1 in tissues of the developing chick limb. Mech Dev. 77:197–99.

Vasiliauskas D, Laufer E, Stern CD. 2003. A role for hairy1 in regulating chick limb bud growth. Dev Biol 262:94–106.

Watanabe N, et al. 2003. Suppression of differentiation and proliferation of early chondrogenic cells by Notch. J Bone Miner Metab 21:344–52.

Wolpert L, Lewis J, Summerbell D. 1975. Morphogenesis of the vertebrate limb. Ciba Found Symp 0:95–130.

Xue Y, Gao X, Lindsell CE, Norton CR, Chang B, Hicks C, Gendron-Maguire M, Rand EB, Weinmaster G, Gridley T. 1999. Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. Hum Mol Genet 8:723–30.

Zhang XG, Hou XG. 2004. Evidence for a single median fin-fold and tail in the lower Cambrian vertebrate, *Haikouichthys ercaicunensis*. J Evol Biol 17:1162–6.