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A Molecular Clock Operates During Chick Autopod Proximal-distal Outgrowth

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Temporal control can be considered the fourth dimension in embryonic development. The identification of the somitogenesis molecular clock provided new insight into how embryonic cells measure time. We provide the first evidence of a molecular clock operating during chick fore-limb autopod outgrowth and patterning, by showing that the expression of the somitogenesis clock component *hairy2* cycles in autopod chondrogenic precursor cells with a 6 h periodicity. We determined the length of time required to form an autopod skeletal limb element, and established a correlation between the latter and the periodicity of cyclic *hairy2* gene expression. We suggest that temporal control exerted by cyclic gene expression can be a widespread mechanism providing cellular temporal information during vertebrate embryonic development.

Keywords: *hairy2*; molecular clock; limb development; cycling genes; vertebrate embryo

Embryo limb development requires precise orchestration of cell proliferation and differentiation in time and space.[1] Limb skeletal elements emerge as cartilaginous primordia in a proximal-distal (p-d) sequence. Two models seek to explain cell fate specification along the p-d limb axis. Although fundamentally different, both models imply the existence of a limb bud distal zone where cells reside until they reach the time to differentiate, the progress zone (PZ) model,[2] or to expand, the early specification model.[3,4] However, how these cells measure time is not known.

Time control during embryo development is particularly evident during somitogenesis. Chick presomitic cells were shown to undergo several cycles of *hairy1* gene expression, providing evidence for the existence of a molecular clock underlying the rhythm of somitogenesis.[5] An increasing number of genes have been implicated in the molecular clock machinery;[6,7] namely, *hairy2*,

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Abbreviations used: p-d, proximal-distal; AER, apical ectodermal ridge.

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encoding a transcriptional repressor closely related to *hairy1*.[8] All studies regarding embryonic cyclic gene expression have focused on somitogenesis. Nevertheless, Hirata *et al*. showed that periodic expression of *Hes1* (mouse *hairy2* homologue) can be triggered in a variety of cultured cell lines.[9] This led us to postulate that a molecular clock could play a role in the temporal control of other embryonic structure formation.

We analyzed *hairy2* expression during forelimb development, from limb bud initiation to digit formation (Figure 1). A closer analysis of our results showed that, although forelimbs of the same embryo always presented the same *hairy2* expression pattern, forelimbs of different embryos at the same stage of development, showed variable *hairy2* expression patterns in the distal mesenchyme (up to 400 μm from the apical ectodermal ridge (AER)). Figure 2 shows representative patterns of *hairy2* expression in forelimb buds hybridized under precisely the same conditions. In some wings the distal domain under the AER was completely negative for *hairy2* expression (Figure 2(a), (d), (g), (j) and (m)), while in others *hairy2* was expressed strongly in this same region (Figure 2(c), (f), (i), (l) and (o)). Various intermediate patterns could also be observed (Figure 2(b), (e), (h), (k) and (n)). This variability in *hairy2* expression patterns is clear from stages HH20 to HH28. The region showing variable *hairy2* expression patterns is located between the limb central mesenchyme and the zone of polarizing activity at stages HH20–22 (Figure 2(p)), is displaced

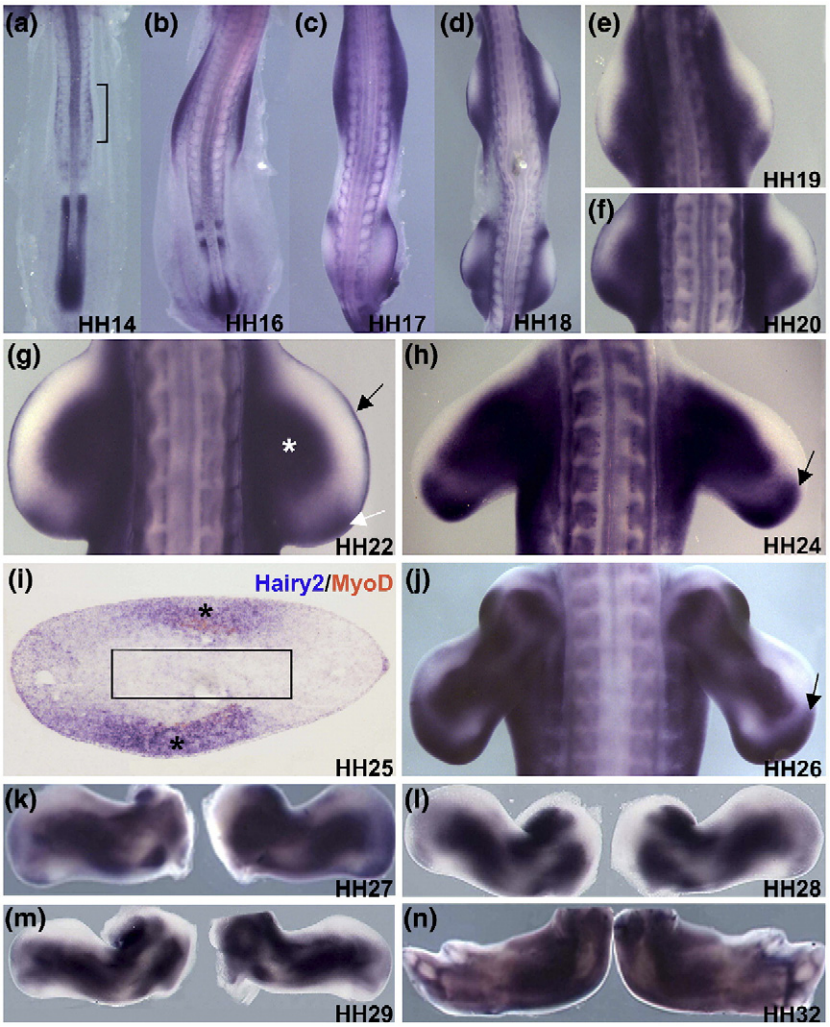

Figure 1. *hairy2* expression pattern during forelimb development. (a) *hairy2* transcripts are first detected at stage HH14 in the presumptive forelimb region (bracket). (b) and (c) At stages HH16 and HH17, *hairy2* transcripts are expressed uniformly within the early wing bud. From stage HH18 onwards, *hairy2* expression becomes localized to distinct expression domains: (1) *hairy2* transcripts are detected in the AER from its formation at stage HH18 until its progressive disappearance at stage HH32 ((d)–(n); black arrow in (g)); (2) from stages HH18 to HH28, *hairy2* is expressed in a posterior limb bud area including the zone of polarizing activity ((c)–(h) and (j)–(l); white arrow in (g)); (3) during all these stages, *hairy2* transcripts are present also in dorsal and ventral mesenchymal domains containing the limb muscles precursors cells as visualised by *MyoD* expression ((g) and (i) asterisks). *hairy2* expression was never found in the limb core, the region that contains the differentiating chondrogenic cells ((i) open rectangle); (4) *hairy2* expression is observed in the forming digits joints ((n)); and (5) in some embryos *hairy2* transcripts are found in the distal limb mesenchyme ((h) and (j) filled arrows), while others lack this expression domain (Figure 2). All pictures show a dorsal view.