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Hox Genes, Digit Identities and the Theropod/Bird Transition

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Contents

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ABSTRACT Vargas and Fallon (2005) propose that *Hox* gene expression patterns indicate that the most anterior digit in bird wings is homologous to digit 1 rather than to digit 2 in other amniotes. This interpretation is based on the presence of *Hoxd13* expression in combination with the absence of *Hoxd12* expression in the second digit condensation from which this digit develops (the first condensation is transiently present). This is a pattern that is similar to that in the developing digit 1 of the chicken foot and the mouse hand and foot. They have tested this new hypothesis by analysing *Hoxd12* and *Hoxd13* expression patterns in two polydactylous chicken mutants, Silkie and *talpid*^{2}. They conclude that the data supports the notion that the most anterior remaining digit of the bird wing is homologous to digit 1 in other amniotes either in a standard phylogenetic sense, or alternatively in a (limited) developmental sense in agreement with the Frameshift Hypothesis of Wagner and Gautier (‘1997, i.e. that the developmental pathway is homologous to the one that leads to a digit 1 identity in other amniotes, albeit that it occurs in the second instead of the first digit condensation). We argue that the *Hoxd12* and *Hoxd13* expression patterns found for these and other limb mutants do not allow distinguishing between the hypothesis of Vargas and Fallon (2005) and the alternative one, i.e. the most anterior digit in bird wings is homologous to digit 2 in other amniotes, in a phylogenetic, or developmental sense. Therefore, at the moment the data on limb mutants does not present a challenge to the hypothesis, based on other developmental data (Holmgren ’55, Hinchliffe ´84, Burke and Feduccia ´97, Kundrát et al. 2002, Larsson and Wagner 2002, Feduccia and Nowicki 2002), that the digits of bird wings are homologous to digits 2,3,4 in amniotes. We recommend further testing of the hypothesis by comparing *Hoxd* expression patterns in different taxa.

INTRODUCTION

Vargas and Fallon (2005) propose a new significance to the expression of *Hoxd12* and *Hoxd13* gene expression patterns in the developing digits of forelimbs and hindlimbs of amniotes. They propose that the combined expression pattern of these genes provides a reliable marker either of the condensation homologous (in a phylogenetic sense, with mice as the reference species; see our definition of the essential concepts in the appendix) to that from which the most anterior digit develops in pentadactyl amniotes (i.e. condensation 1), or of the condensation from which a digit develops with a digit 1 identity, i.e., a digit with the same adult identity (again to be interpreted in a phylogenetic sense) as that of the most anterior digit in pentadactyl amniotes, regardless of the condensation from which the digit develops. In practice we follow Tabin ('92) and Litingtung et al. (2002) in that the identity of a digit is inferred from the number of its phalanges, its size and its morphology, i.e., the size, shape and structure of the adult digit*). The second proposal by Vargas and Fallon (2005) provides a problem, because of the evolutionary diversity of digit 1 identities among amniotes, which is manifested in a large variation of shapes, sizes and phalanx numbers (e.g. on the one hand the forelimbs of ichthyosaurs and plesiosaurs with.a multitude of phalanges and on the other hand the limbs of many species such as cats with only one or no phalanx). The proposal may, however, be evaluated in taxa in which there has been relatively little evolutionary change in forelimbs and/or hindlimbs.

During limb development in mice and chickens 5' group *Hox* genes play an important role in the anterio-posterior (A-P) patterning of the limbs, including the digits. Recently much progress has been made in understanding the determination of digit identity and several new models have been proposed (e.g. Sanz-Ezquerro and Tickle 2003, Suzuki et al. 2004, Harfe et al. 2004, Ahn and Joyner 2004, Zakany and Duboule 2004). The full picture of digit identity determination and in particular the role of *Hox* genes is, however, unclear. Unravelling the role of *Hox* genes in the A-P patterning of digits is difficult, because the signaling of these genes forms part of a complicated and dynamically changing feedback-loop that involves many other A-P patterning genes(e.g. Drossopoulou et al. 2000, Chiang et al. 2001, Te Welscher et al. 2002, Litingtung et al. 2002, Ross et al. 2003, Tickle 2004, Zakany et al. 2004, Selever et al. 2004, Harfe et al. 2004, Ahn and Joyner 2004, Zakany and Duboule 2004). The complexity of the interactions is further increased by the integration of A-P patterning with dorsoventral and proximodistal patterning (Tickle 2004) and by the high functional redundancy of *Hox* genes (Fig. 1, Goff and Tabin 1997, Davis and Capecchi 1996, Zakany and Duboule 1999, Kmita et al. 2002). Only if the *Hox* genes are the first genes to be expressed in a clear differentiated manner correlated with digit identity, can they tentatively be inferred to steer the local network leading to the digit form. As long as data of the required fine temporal resolution are lacking, the *Hox* expression pattern can only be viewed as no more than a useful correlate of digit identity.

Vargas and Fallon (2005) draw attention to interesting similarities of *Hoxd12* and *Hoxd13* expression patterns in the developing digits of forelimbs and hindlimbs of mice and chickens. *Hoxd13* is present in all developing digit condensations whereas *Hoxd12* has a more posterior boundary and is not expressed in the most anterior condensation of the remaining digits, i.e., the second digit condensation in the chicken wing (the first condensation is transiently present and disappears at an early stage, Prein 1914, Holmgren ´55, Kundrát et al. 2002, Larsson and Wagner 2002, Feduccia and Nowicki 2002) and the first digit condensation in the hand and foot of the mouse and the foot of the chicken. Vargas and Fallon now propose in their first scenario that in birds and mammals the absence of *Hoxd12* expression combined with the presence of *Hoxd13* expression in this condensation should not only be seen as a marker of the most anterior condensation of the remaining digits, but specifically of the condensation of digit 1 (in a phylogenetic homological sense), i.e., the local *Hox* expression pattern indicates the condensation of digit 1 in amniotes, regardless of the differences in identity of the adult digit 1 between taxa and between forelimbs and hindlimbs. They deduce from this that the second condensation in the developing bird would then be the condensation of digit 1 and the first condensation that of the prepollex. In this scenario bird wings would not have digits 2,3,4, as commonly assumed by developmental biologists (e.g. Burke and Feduccia '97, Hinchliffe'97, Wagner and Gauthier '99, Kundrát et al. 2002), but digits 1,2,3 (although 1,3,4 and 1,2,4 are also possible). This interpretation would remove the long-standing problem of how birds presumed to have digits 2,3,4 remaining at the end of evolution, can have descended from theropods that are generally assumed to have had digits 1,2,3 (e.g. Wagner and Gauthier '99). This problems stems from the difference between a homology interpretation of digits in birds that is based on a comparison of early amniote development, in particular of the branching pattern and the number of the digit condensations from which condensations 2,3,4 continue developmentally and one that is based on a comparison of the adult morphology of the digits of birds and theropods, where in theropods and birds adult digits 1,2,3 appear to have remained in the fossil record and digits 4 and 5 are reduced (Wagner and Gauthier '99). In an alternative scenario Vargas and Fallon (2005) propose that the mutant data also supports the Frameshift Hypothesis by Wagner and Gauthier ('99). This hypothesis states that the digits of bird wings have undergone homeotic identity shifts in their evolutionary past such that a digit with the adult identity of digit 1 develops in the condensation formerly of digit 2, a digit with the adult identity of digit 2 in the condensation formerly of digit 3 and a digit with the adult digit 3 identity in the condensation formerly of digit 4. This hypothesis was specifically proposed to solve the above-mentioned discrepancy between the homology interpretations based on developmental data of amniotes (2,3,4) and paleontological data of adult theropods and birds (1,2,3). For this alternative scenario, Vargas and Fallon (2005), thus, hypothesize that in amniotes the absence of *Hoxd12* expression combined with the presence of *Hoxd13* expression indicates in forelimbs and hindlimbs the condensation from which a digit develops with the identity of a digit 1, rather than that of digits 2, 3, 4 or 5, regardless of the position of the digit condensation. They propose, thus, that a specific *Hox* pattern indicates the condensation from which a digit 1 (in a phylogenetic homological sense, below referred to as a digit with identity 1) develops, regardless of both the anterio-posterior postion of the condensation and of the considerable shape differences of adult digits 1 between taxa and between forelimbs and hindlimbs.

They have tested this hypothesis by analysing *Hoxd12* and *Hoxd13* expression patterns in two polydactylous chicken mutants, Silkie and *talpid*^{2}.

ANALYSIS OF CHICKEN AND MICE MUTANTS

Does data on polydactylous chickens support the hypothesis?

In Silkie chickens the most common variant has feet with an extra digit with three phalanges anterior to digit 1. The extra digit is morphologically very similar to digits 2,3 and 4 of the ordinary foot. By most authors the Silkie polydactyly is interpreted as a partial mirror image duplication of the foot with a mirror image digit 2 anterior to digit 1 (i.e. digits with identities 2,1,2,3,4, Grönberg 1894, Braus ‘08, Gabriel ‘48, Landauer ‘48). They base this on the range of digit variations displayed by both homozygous Silkie chickens and hybrids with normally-toed breeds. Indeed, the *Hoxd12* and *Hoxd13* patterns in Silkie are mirrored in the duplicated digit 2 condensation, i.e. both *Hoxd12* and *Hoxd13* are expressed. In experimentally induced full and partial mirror image duplications of chick wings *Hoxd11*, *Hoxd12* and *Hoxd13* patterns are similarly mirrored during development (Izpisúa Belmonte et al. ’91 and Nohno et al. ‘91). If indeed the polydactyly in the Silkie mutant should be interpreted as a partial mirror image duplication, the *Hoxd12* and *Hoxd13* patterns in the developing extra digit does not seem to provide support for Vargas and Fallon’s hypothesis, because the mirror image duplication is most likely induced upstream of the *Hox* gene expression at embryonic day 8 (e.g. by ectopic anterior *Shh* expression), allowing the possibility that digit identity is induced by the same upstraem stimuli, at least in part independent of the *Hox* expression. It is, thus, necessary to investigate first whether or not the Silkie polydactyly should be seen as a mirror image duplication and what induces the mirror duplication, before the Silkie data can be adduced as an argument in favour of Vargas and Fallon’s hypothesis.

In chicken *talpid*^{2} mutants the limbs have many digits that have lost wild-type morphological identity and that are fused (Fig. 2, Goetinck and Abbott ´64, MacCabe and Abbott ´74, Dvorak and Fallon ´91, Caruccio et al.´99). The A-P polarity is disturbed and weakened, which is assumed to be causally implicated in the loss of wild-type identity. In the foot the digits mostly consist of three phalanges, although some phalanges (and some digits) disappear during development due to apoptosis. Vargas and Fallon assume that the first digit has become posteriorized in *talpid*^{2} mutant feet, because in all digits the number of phalanges is three as in wildtype posterior digit. *Hoxd12* and *Hoxd13* are expressed in all digits on day 7 and this is in agreement with Vargas and Fallon's hypothesis, if indeed the assumption holds true that the first digit has assumed the identity of more posterior digits. We feel that the number of phalanges is not sufficient to conclude this, given the loss of wildtype identity of all digits, including the most anterior digit. The assumption of a posteriorization of the identity of the first digit is not supported by the majority of anatomical characters that determine the identity of a digit (Fig. 2). In this respect it is important to note that in chick wings, the number of phalanges has been reduced in all adult digits compared to *Archaeopteryx*. Nonetheless, the evolutionary reduction that took place in the second adult digit of birds from three phalanges to two has never been considered sufficient reason to conclude that the identity of digit has been changed into that of the first digit, because of the differences between the other anatomical parameters. In addition, the *Hoxd12* and *Hoxd13* expression patterns also do not support this, because in the chicken wing both *Hoxd12* and *Hoxd13* are expressed in the condensation of the second and third remaining digits. Hence, in chicken limbs the combination of *Hoxd12* and *Hoxd13* expression is associated with the development of digits with one, two and three phalanges.

Analysis of other chick limb mutants

Analysis of other chicken and mouse limb mutants provides evidence that a digit 1 identity (including a phalanx number of two) can apparently develop in the presence of *Hoxd12* expression and, in addition, that the identity of digits 2,3,4 (including a phalanx number of three) can develop in the absence of *Hoxd12* expression. Therefore, in limb mutants in general there does not appear to be a very tight correlation between the *Hoxd12* and *Hoxd13* gene expression and digit identity (including phalanx number). Suzuki et al. (2004) show, for example, that misexpression of *Tbx2* leads to an anterior expansion of *Hoxd12* expression without a posteriorization of the identity of digit 1 (mutants have feet with digits with identities 1,3,4,4 and 1,2,4,4). Selever et al. (2004) show that in mutant limbs in which *BmP* expression is inactivated (*Prx1*^{cre}*;BmP4n/f* mutants) complete and partial posteriorizations of the identity digit 1 occur in the absence of a change in the expression pattern of *Hoxd12* and *Hoxd13* gene expression.

Similarly, two studies show that over-expression of *Hoxd11* leads to partial or complete posteriorization of the identity of digit 1, presumably in the absence of any changes in *Hoxd12* and *Hoxd13* expression (Morgan et al. ‘92, Goff and Tabin ‘97). Finally, Davis and Capecchi (‘96) show that in mutants in which *Hoxd12* expression is entirely absent (*Hoxd12*$^{-/-}$), there is virtually no change in the identity of the digits (Fig. 1). The results emphasize the functional redundancy of posterior *Hoxd* genes in the limb. This redundancy also follows from the fact that phenotypic effects in the digits are again very limited when *Hoxd13* is absent and *Hoxd12* expression is expanded anteriorly similarly to the normal expression of *Hoxd13* (as a result of the deletion of the *Hoxd13* locus, Kmita et al. 2002). This again limits the reliability of the *Hoxd12* and *Hoxd13* patterns as sole indicators of digit identity in limb mutants.

The analyses of these and other mutant limbs show that *Hoxd12* and *Hoxd13* play an important role in the patterning of the A-P identity of the digits. However, they do not allow the conclusion that the presence of *Hoxd13* expression together with the absence of *Hoxd12* in mice and chicken mutant digit condensations provides molecular evidence for the development of a digit 1 identity, to the exclusion of a more posterior digit identity.

FIVE INITIAL CONDENSATIONS IN THE BIRD WING

Prepollex in bird wings?

The ossifying digits in the bird wings develop, as in the feet, in the middle three of the initial five digital mesenchymal condensations (e.g. Prein 1914, Holmgren ´55, Kundrát et al. 2002, Larsson and Wagner 2002, Feduccia and Nowicki 2002). Vargas and Fallon (2005) suggest that the most anterior condensation, which is only transiently present, may represent the prepollex rather than the digit 1 condensation as generally assumed. This is highly improbable, because this would imply that digit condensations are present of the prepollex (digit 0) and digits 1,2, 3 and 4 and not of digit 5. The general consensus is that the prepollex was lost much earlier than digit 5 in the ancestral lineage of birds and reptiles (e.g. Romer ‘55, Wagner and Gauthier ‘99). Moreover evoutionary digit reduction tends to be extremely slow and gradual (Lande '78, Galis et al. 2002). Together these two facts suggest that there should still be a condensation present of the most recently lost digit 5 rather than one of the prepollex. The interpretation of a prepollex condensation is also not in agreement with the similarity of the branching pattern of the digit condensations in the chicken foot and wing and in other amniote limbs that also indicate that the remaining digits in the bird wings develop from digit condensations 2,3 and 4 (Fig. 3, Hinchliffe ´84, Burke and Feduccia ´97, Kundrát et al. 2002). This supports the notion that bird digits are digits 2,3,4 in a phylogenetic homological sense.

DISCUSSION

On the basis of *Hoxd12* and *Hoxd13* expression patterns the data on limb mutants unfortunately does not allow distinguishing between the hypothesis that the most anterior digit in the wing of birds is homologous to digit 1 in other amniotes and the alternative that it is homologous to digit 2, either in a phylogenetic or a developmental sense. More particularly, the disturbed nature of the digital patterning in most discussed mutants makes a proper evaluation of Vargas and Fallon’s hypothesis difficult. A more straightforward test of the hypothesis would be to investigate *Hoxd12* and *Hoxd13* gene expression patterns in amniote species other than chickens and mice. In particular it would be useful to examine species from taxa that have independently lost digit 1 in forelimbs or hindlimbs during evolution (e.g. ostriches, dogs and many reptilian species). Furthermore, it would be useful to also include analysis of *Hox* gene expression patterns (including *Hoxd11*) at earlier stages, when more of the digit identity has not yet been specified (e.g. the number of phalanges, Hartmann and Tabin 2001, see also Dudley et al. 2002 and Zakany and Duboule 2004 for early specification), even though it has been shown that at later stages part of the specification has not yet irreversibly happened (Dahn and Fallon 2000). Unfortunately the late *Hox* activity cannot anymore be causally implicated in the initial specification of the digit identity, although it is of course possible that it is correlated with it.

As at the moment the data on mutant limbs does not present a challenge to the hypothesis that is based on developmental data, that the digits of bird wings are homologous to digits 2,3,4 in other amniotes, this appears to leave the problem of how birds with digits 2,3,4 in their forelimbs can have descended from theropods that are generally assumed to have digits 1,2,3. However, the apparent conflict between homology based on developmental and paleontological data does not mean that we should seriously question the descent of birds from theropods. In an earlier paper (Galis et al. 2003) we have discussed two scenarios that reconcile a theropod descent of birds with the digit identities that have been found, a) the above-mentioned frameshift hypothesis by Wagner and Gauthier ('99) and, b) the hypothesis that the theropod ancestors of birds may have had hands with digits 2,3,4, rather than 1,2,3.

The Frameshift Hypothesis has several problems. The homeotic identity shifts cannot be achieved by straightforward identity shifts in four consecutive digits alone. The frameshift also requires: a) the reversal of the evolutionary reduction of digit 4 into a fully functional digit (a polydactylous change) and b) the induction of digit reduction in digit 1 (an oligodactylous change). Both polydactylous and oligodactylous changes are highly constrained as single mutational steps and have never been documented in amniotes at a species level, despite their common occurrence within species (i.e. high intraspecific and no interspecific variation, Lande, Galis et al. 2001, 2002). In addition, a homeotic shift of digits 1,2,3 into digits 2,3,4 in theropods without further anatomical changes does not appear to lead to an adaptive advantage. Therefore, it appears probable that not one, but several highly constrained mutational changes are necessary to achieve the proposed frame-shift, without as yet indications for a selective advantage that would favour these changes.

On the other hand, the scenario that theropod ancestors of birds may have had hands with digits 2,3,4 is also problematic. Analysis of the fossil data strongly suggests the reduction of digits 4 and 5 in pentadactyl theropods. However, the reduction of digit 4 is not absolutely certain, and, given the gaps in the Jurassic fossil record of theropods, a bilateral reduction of digits 1 and 5 cannot be excluded (Galis et al. 2003). Given the plausibility of a descent of birds from theropods and given the arguably low mechanistic plausibility of the Frameshift Hypothesis we think that the hypothesis that theropod ancestors of birds had hands with digits 2,3,4 should be explored more thoroughly. Larsson and Wagner (2003) in their reply to Galis et al. (2003) disagree with this reasoning and suggest that the inferred low mechanistic plausibility of the frameshift hypothesis is less relevant as it may be inaccurate due to the incompleteness of our scientific knowledge. Our opinion is that considerations about the plausibility of evolutionary transitions on morphogenetic or selective grounds should be an essential ingredient of any attempt to reconstruct such transitions in a phylogenetic context. In this context it may be noted that the Frameshift hypothesis was itself also put forward by Wagner and Gauthier ('99) to make the evolutionary transition from theropod hands with digits 1,2,3 to bird wings with digits 2,3,4 mechanistically plausible. Not only that, consideration of the plausibility of evolutionary transitions is actually a routine procedure (though generally unacknowledged as such) in the construction of cladograms when choices are made between the multiple loss or multiple gain of complex traits.

Both scenarios, thus, currently lack convincing support. Hopefully new paleontological data may provide either a plausible adaptive scenario for the frameshift, or support for a bilateral reduction in the hands of the theropod ancestors of birds (digits 2,3,4), or for yet another hypothesis. In addition, further testing of Vargas and Fallon’s hypothesis in amniote species with independently reduced numbers of digits will provide new information on the molecular basis of evolutionary digit reduction, and hence, on the transition of theropod digits to bird digits. For the moment, the enigma persists.

*) Position is also a form criterion, but the anterio-posterior position of the digit is excluded here.

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Figure legends

Fig. 1. Dorsal view of the forelimbs of a wildtype type mouse and of three mutant mouse strains produced by gene targetting (*Hoxd11*$^{-/-}$, *Hoxd12*$^{-/-}$ and *Hoxd13*$^{-/-}$). Loss of *Hoxd11* or *Hoxd12* in the mutants hardly affects the phenotype of the digits, suggesting functional redundancy among posterior *Hox* genes. The phenotype of the limb of *Hoxd13*$^{-/-}$ mice is more disturbed (From Davis and Capecchi 1996).

Fig. 2. From left to right, *talpid*^{2} wing, wildtype wing, *talpid*^{2} leg, wildtype wing. *Talpid*^{2} limbs have supernumerary digits that have lost wildtype morphological identity (From Goettinck and Abbott, 1964).

Fig. 3. Comparison of the wing (top row) and foot (bottom row) development in the chicken, dorsal views. Digit 4 is labelled in each specimen. Note the strong resemblance of the patterns. In the foot the transient condensation of digit 5 can be seen (From Burke and Feduccia 1997).

Appendix

Concepts

There are four concepts that we have to deal with, condensation number and identity and digit number and identity. Digit refers to the adult, condensation to the earliest recognisable shape in the embryo. Numbers are observationally determined ordinal numbers counted anterioposteriorly. Identities are determined by phylogenetic homology with adult mice as a reference. Condensations in mice derive their identity from the corresponding adult.

Two features, be they condensations or adult digits, in a parent and offspring are called homologous if they are closely similar. Two features in arbitrary organisms are called homologous if they can be linked through a phylogenetic chain of parent-offspring pairs. In the case of condensations similarity is determined by the combination of the four form parameters (position, size, shape and structure). In the case of digits similarity is based on adult form parameters only. (The implicit assumption is thus that over the generations the disappearance of condensations and the change of adult shapes occurs only gradually.)

Disappearance of the prepollex leads to a shift of digit nummer relative to digit identity. A shift of digit identity relative to that of the corresponding condensation is called a frameshift.

Fig. 1

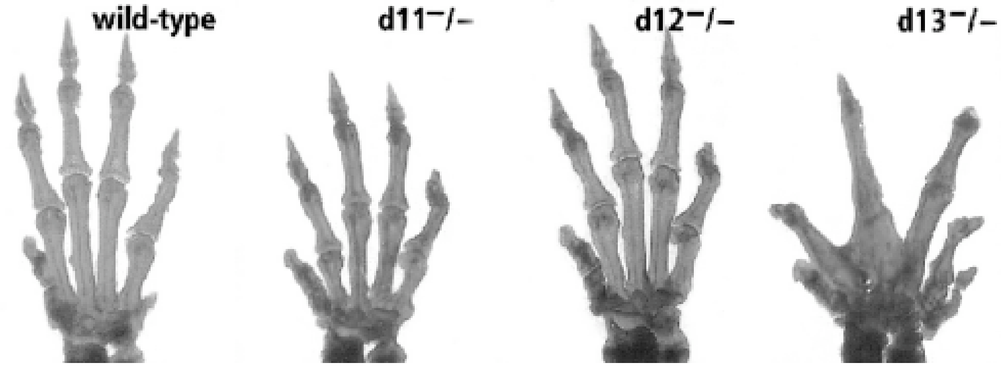

Fig. 2

Fig. 3