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Developmental mechanisms of vertebrate limb evolution

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Abstract. Over the past few years, our understanding of the evolution of limbs has been improved by important new discoveries in the fossil record. Additionally, rapid progress has been made in identifying the molecular basis of vertebrate limb development. It is now possible to integrate these two areas of research in order to identify the molecular developmental mechanisms underlying the evolution of paired appendages in vertebrates. After the origin of paired appendages, several vertebrate lineages reduced or eliminated fins and limbs and returned to the limbless condition. Examples include eels, caecilians, snakes, slow worms and several marine mammals. Analyses of fossil and extant vertebrates show that evolution of limblessness frequently occurred together with elongation of the trunk and loss of clear morphological boundaries in the vertebral column. This may be suggestive of a common developmental mechanism linking these two processes. We have addressed this question by analysing python embryonic development at tissue, cellular and molecular levels, and we have identified a developmental mechanism which may account for evolution of limb loss in these animals.

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Skeletal morphology has two histories; one evolutionary and one developmental. These histories are intimately linked, but the details of this relationship have not been understood until very recently. This new focus owes largely to a number of factors coming together at approximately the same time, including (a) extremely rapid progress in the area of limb developmental genetics, (b) new palaeontological discoveries catalysing an interest in mechanisms of morphological development, and (c) a resurgence of interest in the relationship between embryonic development and evolution (a field now commonly referred to as 'evo-devo'). This integration of developmental and evolutionary biology has been bolstered by quite significant communication, including collaborative research, between developmental biologists and palaeontologists (e.g. Coates & Cohn 1998, Shubin et al 1997, Smith et al 1994).

One area which has seen considerable progress in recent years is the limb skeleton. Thanks to a rich fossil record, the evolutionary history of vertebrate limbs has become much clearer over the past decade. For example, work on Devonian amphibian fossils, such as *Acanthostega gunnari*, has changed our view of the fin-to-limb transition by raising strong evidence that polydactyly, rather than pentadactyly, is the primitive condition for tetrapod limbs (Coates & Clack 1990). Developmental genetics of limb development has contributed a mechanistic perspective to the study of limb evolution. Among these genetic studies, none has generated more discussion about developmental pathways of limb evolution than the work on *Hox* genes. The *Hox* complex is an evolutionarily ancient family of transcription factors which play fundamental roles in patterning the bodies of animal embryos (for review see Akam 1998). These homeobox-containing genes are arranged in clusters along the chromosome, and are best known for their roles as 'selector genes' which confer identity to cells (Rijli et al 1998). Quantitative and qualitative differences in *Hox* gene expression can, for example, determine whether a group of cells will form an antenna or a leg in flies, or a thoracic or cervical vertebra in vertebrates. *Hox* genes play very important roles in limb development. During early stages of development they are involved in specifying the position at which limbs will bud along the trunk (Cohn et al 1997, Rancourt et al 1995) and, at later stages of limb development, they govern cell identity, proliferation, adhesion and growth (Davis et al 1995, Duboule 1995, Yokouchi et al 1995). *Hox* gene expression is highly dynamic, and spatiotemporal changes in *Hox* expression domains correspond to the progression of limb development (Davis et al 1995). The discovery that late phases of *Hox* gene expression in the limbs control formation of digits (Dollé et al 1993) set the scene for some very exciting work in comparative developmental biology that led to the first suggestion of a specific molecular mechanism for a major transition in vertebrate limb evolution. Duboule and colleagues set-out to test the hypothesis that the late phase of *Hox* gene expression in the distal aspect of the limb bud was involved in specification of digits during the fin-to-limb transition. Their comparative analysis of *Hox* gene expression in mice and zebrafish revealed an intriguing difference in the dynamics of *Hox* expression in fins and limbs; zebrafish fins, which lack digits, also lack the late phase of distal *Hox* gene expression (Sordino et al 1995). This work gave rise to a model which suggested that changes in *cis*-regulation of *Hox* gene expression in distal fin/limb buds may have been a key step in the evolution of digits (reviewed in Zákány & Duboule 1999). This interesting study is an example of how one can relate the evolutionary and developmental histories of the skeleton to one another through an experimental approach.

Vertebrate limbs have diversified into an impressive range of anatomical patterns. In many cases, such as birds, salamanders, horses and sloths, these

changes have involved reductions in the number of digits. More extreme examples of limb reduction include animals which have dispensed with limbs altogether, such as snakes. While snakes are probably the most widely known case of secondary limb loss, limblessness has evolved on many independent occasions in different vertebrate classes. Although the extent of limb reduction and the order in which limbs have been lost varies in different vertebrate lineages, evolution of limblessness frequently occurs together with elongation of the trunk and loss of clear axial regionalization of the vertebral column. This could suggest a common developmental basis of limb loss and homogenization of the axial skeleton. These anatomical changes, like those discussed above, have their bases in embryonic development, when the body plan is laid out. We took an experimental approach to try to identify the molecular mechanisms which may have generated the snake body plan.

For this study we focused on pythons, a primitive group of snakes which lack all traces of forelimbs, but have retained very small rudiments of the hindlimbs. When we began to analyse the skeletal anatomy of different python species, it became clear that the subdivisions of the vertebral column common to almost all tetrapods — cervical, thoracic, lumbar, sacral and caudal — were impossible to identify (Fig. 1A). Posterior to the atlas, all of the vertebrae looked similar down to the level of the hindlimb rudiment. The vertebral bodies showed very little regionalization, and each possessed a pair of true ribs, giving the appearance of a long series of thoracic vertebrae. Experimental data from a variety of organisms has demonstrated that vertebral identity is controlled by differential expression of *Hox* genes in paraxial mesoderm (from which vertebrae develop) along the primary body axis of the embryo. In order to determine whether development of vertebrae with thoracic morphology along most of the axial skeleton was associated with changes in *Hox* gene expression, we examined the distribution of three HOX proteins; HOXC6 and HOXC8 which, in other tetrapods, are restricted to thoracic somites, and HOXB5 which is expressed in all somites. In python embryos, we found that both thoracic markers, HOXC6 an HOXC8, were expressed over a broad domain extending from the first somite posteriorly to the level of the hindlimb buds, where a posterior boundary of expression was detected. This is in stark contrast to the general tetrapod condition, in which these genes are expressed in a domain restricted to the thorax (Fig. 1B). These gene expression patterns co-localize with the region of the python trunk that will form rib-bearing, or thoracic, vertebrae. The posterior boundary of expression lies at the posterior limit of the thoracic series, where there is an abrupt transition in vertebral identity from vertebrae with true ribs to vertebrae with short, forked fused ribs known as lymphapophyses (Fig. 1C). Thus, extension of thoracic identity along the python axial skeleton is associated with extension of *Hoxc6* and *Hoxc8* expression domains. Expansion of these domains in transgenic mice result in

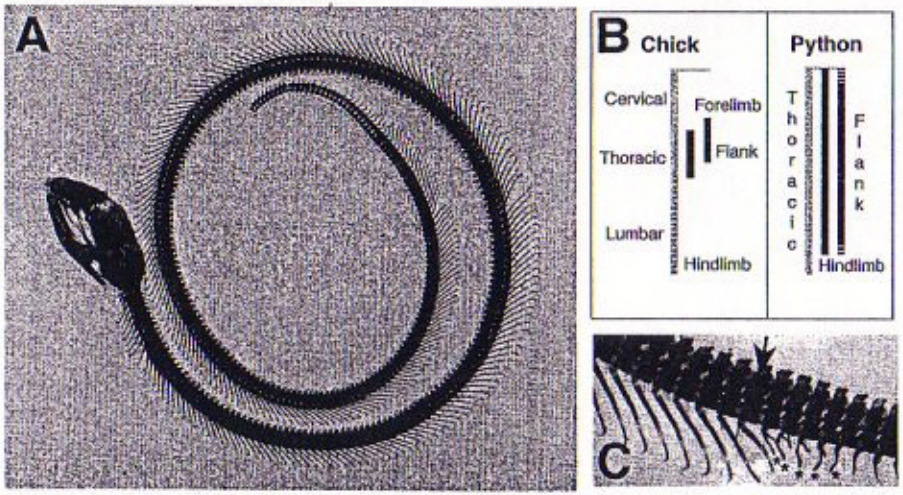


FIG. 1. Morphological and molecular regionalization of the python axial skeleton. Anterior to left in (A) and (C). (A, C) Alcian blue- and alizarin red-stained skeletal preparation of python embryo at 24 days of incubation. (A) Lateral view of complete skeleton. Note homogeneity of vertebrae, almost all of which bear ribs and have a thoracic appearance. (B) Schematic diagram comparing expression domains of HOXB5 (light grey), HOXC8 (black) and HOXC6 (dark grey) in chick and python embryos. Broken line at anterior and posterior extremes of red line indicates lack of certainty about precise limits of HOXC6 expression. Note that expansion of HOXC8 and HOXC6 domains in python correlates with expansion of thoracic identity in axial skeleton and flank identity in lateral plate mesoderm. (C) High magnification view of cloacal region of embryo shown in (A). Arrow indicates position of the hindlimb (removed) relative to axial skeleton. Hindlimb position corresponds to a transitional vertebra with intermediate morphology (arrow), separating vertebrae with large, movable ribs (left) from vertebrae with lymphapophyses in cloacal region (right, with asterisks).

expansion of ribs along the mouse axial skeleton (Jegalian & De Robertis 1992, Pollock et al 1995), and therefore, there may be a causal relationship in snakes. *Hoxb5* is also expressed over a broad anteroposterior domain, but this is true of all tetrapods examined. An interesting difference, however, is seen in lateral plate mesoderm, the tissue which will give rise to the limbs and body wall. In other tetrapods, *Hoxb5* is expressed in the proximal, anterior part of the forelimb, where it plays a role in determining forelimb position (Rancourt et al 1995). In python embryos, we were unable to detect this regionally specific pattern of expression; instead we saw widespread expression of *Hoxb5* throughout the lateral plate mesoderm. Loss of regionally specific expression in lateral plate mesoderm is associated with loss of forelimb specification, and in the context of the altered forelimb position seen in *Hoxb5* mutants, may underlie the failure of forelimb specification in python embryos.

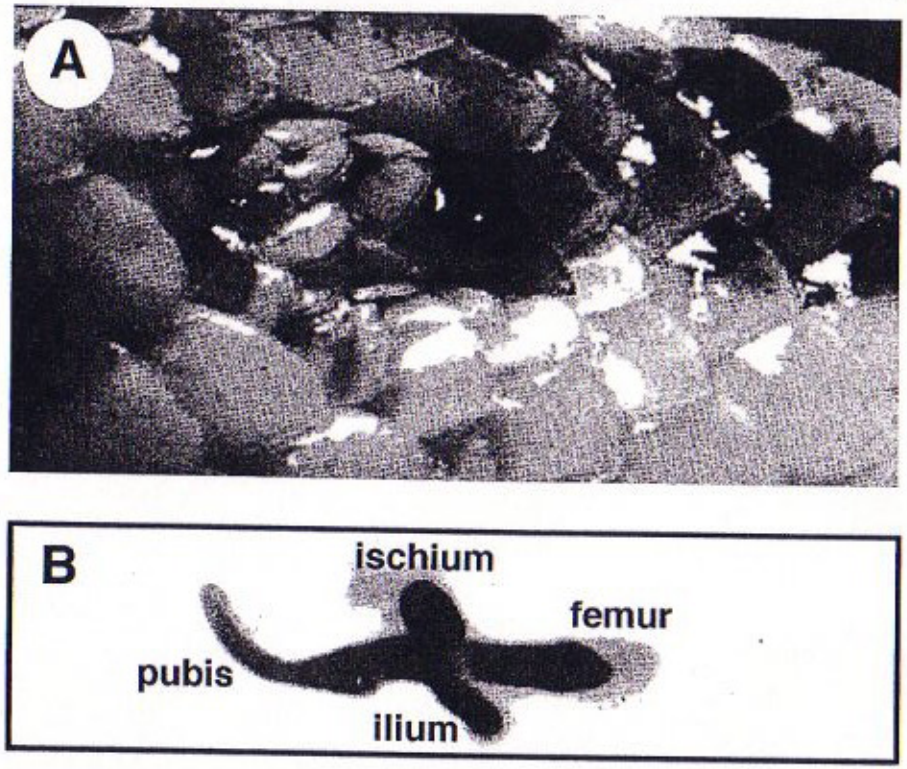


FIG. 2. Rudimentary hindlimbs of pythons. (A) Lateral view of left hindlimb protruding from the body wall of adult Python regius. (B) Skeletal preparation of hindlimb and associated pelvis dissected from Burmese python embryo at 14 days incubation. Femur and all three elements of pelvic girdle are present (pubis, ilium and ischium).

Pythons, unlike more derived snakes, have partially developed hindlimbs, known as spurs (Fig. 2A). The truncated limb skeleton consists of all three elements of the pelvic girdle, and a severely stunted femur (Fig. 2B). Early development of the hindlimb during embryogenesis appears to be normal, as a pair of well-formed limb buds emerge from lateral plate mesoderm on either side of the cloaca. Shortly after initiation of limb budding, bud outgrowth arrests. Outgrowth of the tetrapod limb skeleton is controlled by the apical ectodermal ridge (AER), a specialized epithelial ridge which runs along the distal edge of the limb bud (Cohn & Bright 1999). Surgical removal of this ridge from early limb buds of chick embryos results in the arrest of limb outgrowth and loss of distal skeletal structures. To determine whether hindlimb development arrests in python embryos due to a failure of apical ridge function, we analysed early limb buds for morphological and molecular evidence of an AER. Scanning electron

microscopy showed a relatively smooth ectodermal jacket covering the limb bud, in contrast to the chick limb bud in which the AER is clearly visible. Immunohistochemical analysis also failed to reveal expression of genes associated with AER function in the python limb bud ectoderm. These results suggested that hindlimb bud outgrowth arrests in python embryos because they lack an AER.

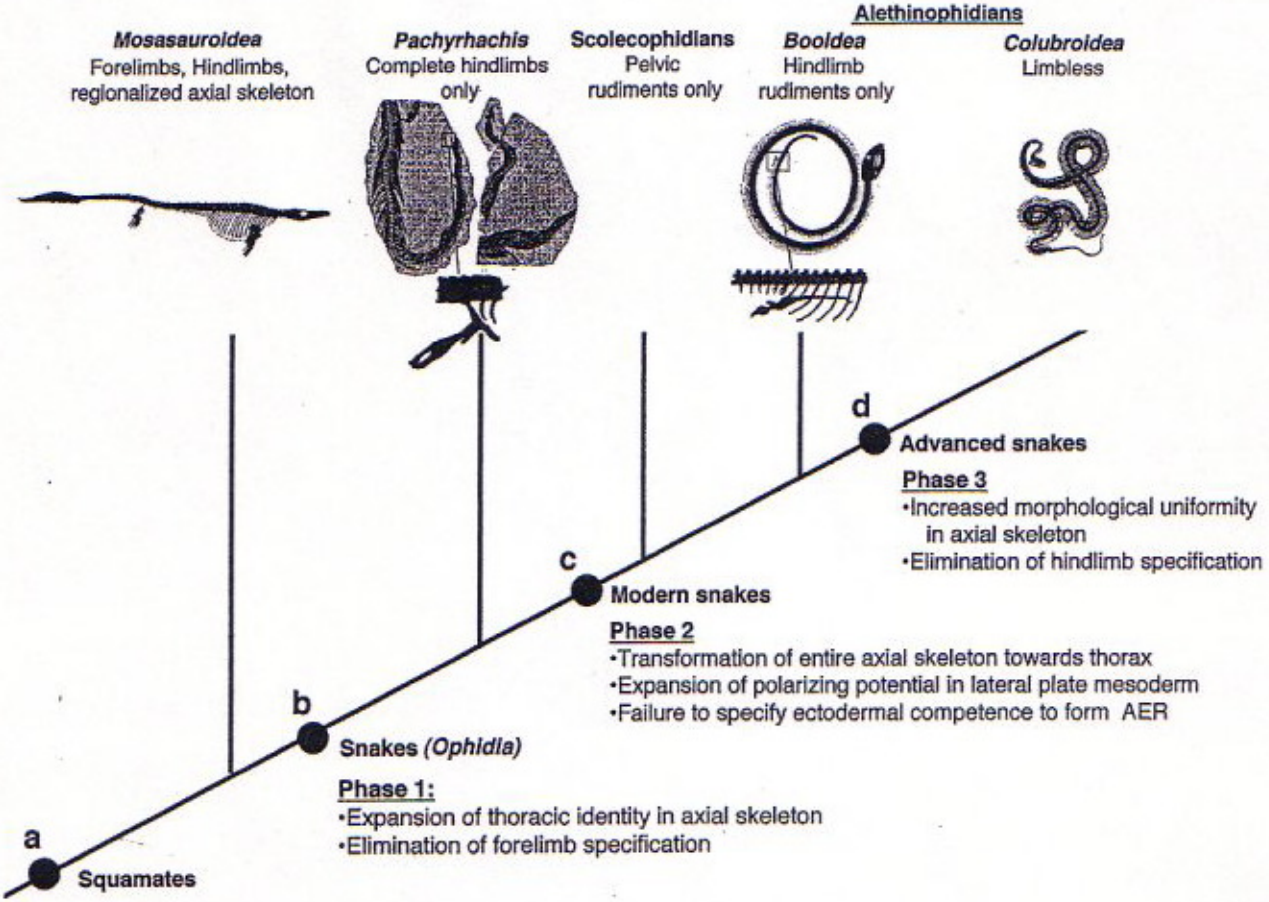
The AER maintains another signalling region in the limb bud, known as the zone of polarizing activity (ZPA) or polarizing region. The polarizing region is a specialized group of mesenchymal cells at the posterior margin of the limb bud which controls patterning of the limbs along the anterior to posterior (thumb to small finger) axis. These cells express a gene called Sonic hedgehog (*Shh*), which mediates the polarizing activity of the ZPA. Maintenance of *Shh* expression, and signalling activity of ZPA cells, depends on fibroblast growth factors secreted by the apical ectodermal ridge. We were interested in determining whether pythons had retained any evidence of a polarizing region from their limbed ancestry, and what effect the lack of an AER might have on these cells. To determine whether any cells in python hindlimb buds have molecular characteristics of ZPA cells, we examined the distribution of SHH protein in limb bud-stage python embryos. We were unable to detect any SHH in the hindlimb buds, although strong expression was seen in the notochord and in the floor plate of the neural tube. Thus, in the absence of an AER, the underlying mesenchymal cells fail to express *Shh*. We next tested whether these cells have the ability to polarize a limb bud. Mesenchymal cells were transplanted from the posterior and anterior margins of python hindlimb buds to the anterior margin of chick wing buds. Surprisingly, cells from both positions induced mild digit duplications in chick wings, indicating that they have retained polarizing potential even though they do not express *Shh*. When we assayed the transplanted posterior cells for *Shh* expression, we found that SHH could be detected in the python cells after they were grafted under a functional chick apical ridge. This indicated that python hindlimb cells have retained the potential to express *Shh* and polarize a limb in the presence of AER signals. Moreover, polarizing potential extends into the anterior part of the limb bud. This differs from the condition found in other vertebrate embryos, in which polarizing activity is always confined to the posterior margin of the limb bud. The anteroposterior extent of polarizing potential in mouse lateral plate mesoderm is related to the extent of *Hoxb8* expression, and, as such, expansion of this potential in python lateral plate mesoderm may be related to expansion of *Hox* gene expression domains.

We next turned our attention to the apical ridge to investigate the basis of failed ridge formation. In the chicken *limbless* mutant, limb outgrowth fails because apical ridge formation fails. It is also known that dorsoventral polarity is lost in limb ectoderm of these mutants, which is significant because dorsoventrally polarized expression of genes such as *Radical Fringe* and *Wnt3a* is needed for

normal ridge formation (Kengaku et al 1998, Laufer et al 1997, Zeller & Duboule 1997). When we examined dorsoventral gene expression in python hindlimb buds, we found that both *Engrailed* (an ventral ectodermal marker) and *Wnt7a* (a dorsal mesenchymal marker) were expressed in their normal positions. These results demonstrate that the mechanism underlying limb truncation in pythons differs from that which affects *limbless* mutants.

These experiments allowed us to eliminate a number of possibilities for the basis of hindlimb truncation, but the nature of the mechanism underlying failure of ridge formation remained unclear. During normal tetrapod limb development, the ridge is induced in apical ectoderm by a signal from underlying mesenchymal cells. The ectoderm responds to that signal by activating expression of genes such as *Fgf4* and organizing itself into a pseudo-stratified, columnar epithelium. Failure of AER formation could be due to a deficiency in the inductive signal or in the response to such a signal. To determine whether python limb mesenchyme is competent to produce a ridge-inducing signal, we transplanted python limb bud mesoderm under the non-ridge ectoderm of a chick wing and then monitored expression of *Fgf8* (a marker for apical ridge cells). We found that python cells were able to extend the domain of *Fgf8* expression into ectoderm overlying the graft, indicating that a functional ridge-inducing signal was produced by the python limb mesenchyme. This suggests that the deficient tissue in python hindlimbs could be the ectoderm, although this hypothesis will require further testing by recombining python limb bud ectoderm with chick limb bud mesoderm.

Our findings uncover a remarkable amount of the limb development program intact in pythons, which is surprising given that digits were probably last present in snakes during the Cretaceous. This retention of signalling potential and molecular polarity of python limb buds suggests that limb outgrowth could be rescued if an apical ridge could be restored. Because fibroblast growth factors (FGFs) mediate the signalling activity of the AER, we grafted FGF-loaded carrier beads to the distal margin of python limb buds. Although technical difficulties associated with *in ovo* operations prevented us from maintaining the embryos beyond 24 h after surgery, we did observe a dramatic increase in the proximodistal outgrowth of FGF-treated limb buds within the first day, suggesting that FGF can sustain python limb development beyond the normal stage of arrest. This is somewhat similar to the observations of Raynaud et al (1995), who demonstrated that outgrowth of slow worm hindlimb buds in culture can be stimulated by addition of FGF to the culture media. Whether replacement of a single growth factor will be sufficient to fully restore limb development in pythons is unclear at present, but the ability of FGFs to catalyse complete limb development in the flank (inter-limb) region of avian embryos suggests that autonomous limb development can be initiated by a single molecular switch.


Alethinophidians
Mosasauroidea
Forelimbs, Hindlimbs, regionalized axial skeleton
Pachyrhachis
Complete hindlimbs only
Scolecophidians
Pelvic rudiments only
Booidea
Hindlimb rudiments only
Colubroidea
Limbless
d
Advanced snakes
Phase 3
•Increased morphological uniformity in axial skeleton
•Elimination of hindlimb specification
c
Modern snakes
Phase 2
•Transformation of entire axial skeleton towards thorax
•Expansion of polarizing potential in lateral plate mesoderm
•Failure to specify ectodermal competence to form AER
b
Snakes (Ophidia)
Phase 1:
•Expansion of thoracic identity in axial skeleton
•Elimination of forelimb specification
a
Squamates

On the basis of the above results, our current view is that loss of limbs and axial regionalization in snakes may stem from changes in the regulation of *Hox* gene expression along the primary body axis. Both limb position and axial skeletal identity are regulated by these factors, and as such, they are good candidates for coordinating changes to the axial and appendicular skeletons. This does not necessarily imply that *Hox* gene expression in paraxial and lateral plate mesoderm are co-regulated by the same *cis*-acting elements; this linkage may occur at the level of secondary or tertiary signalling between paraxial and lateral plate mesoderm, or via *trans*-acting factors which operate on global *Hox* expression. Our model suggests that the major morphological transitions in snake evolution can be accounted for by several phases of expansion of *Hox* gene expression domains along the anteroposterior axis of the trunk (Fig. 3). While some of these hypotheses concerning fossil taxa are not directly testable by an experimental approach, we can test predictions at the top and bottom of the tree by expanding our comparative analysis of development. For example, experiments are currently underway to test the hypothesis that more derived snakes, which lack limbs

FIG. 3. Developmental model for the evolution of snakes. Tree shows evolutionary relationships among the following: *Colubroidea* (advanced snakes) which lack both forelimbs and hindlimbs and have a large number of nearly-identical vertebrae; *Booidea* (including pythons and boas) which lack forelimbs, but have rudimentary hindlimbs and a large number of morphologically uniform vertebrae with few or equivocal regional differences; Scolecophidians, which have pelvic rudiments and a large number of morphologically uniform vertebrae; the primitive snake *Pachyrhachis problematicus*, which lacks forelimbs, but has complete (or nearly-complete) hindlimbs and a large number of similar vertebrae which nonetheless have identifiable regional differences; and mosasaurs, which have a morphologically regionalized axial skeleton and complete, normally polarized forelimbs and hindlimbs. According to this model, progressive expansion of Hox gene expression domains can account for loss of forelimbs, hindlimbs and regional identity in the axial skeleton. Additionally, the increase in vertebral number would have required continuous production of mesoderm for axial elongation, and this could have been achieved by sustained growth of the tail bud and movement of mesoderm through the primitive streak (Wilson & Beddington 1997). Node 'a' indicates origin of squamates. (b) *Hox* expansion initiated prior to the divergence of the *Pachyrhachis* lineage could have lead to reduction of regional differentiation in the axial skeleton and elimination of forelimb specification, with hindlimb development remaining unaffected. (c) Continued expansion of *Hox* domains after the divergence of the *Pachyrhachis* lineage could have lead to transformation the entire axial skeleton (anterior to the tail) towards thoracic identity and to reduction of hindlimb development by eliminating ectodermal competence to form an apical ridge and expanding polarizing potential (competence to express *Shh*). This condition is retained in scolecophidians and in modern pythons, which together with boas comprise the *Booidea*. (d) Further homogenization of *Hox* gene expression domains is predicted to have lead to the origin of advanced snakes/*colubroidea*. (Phylogenetic relationships among these taxa based on Caldwell & Lee 1997; Figures modified from Caldwell & Lee 1997, Carroll 1988, Gasc 1976.) Reproduced with permission from Cohn & Tickle (1999).

completely, will show less regionalization of *Hox* expression domains than do pythons.

How broadly applicable are the principles that we have discovered in python embryos? While it is tempting to speculate that secondary limb loss in other lineages may have been driven by the same developmental mechanisms, such speculation is avoidable when these hypotheses can be tested with relative ease in the laboratory. Analyses similar to the one we have described above can be performed using phylogenetically relevant taxa to determine whether independent evolution of limblessness in different vertebrate lineages may stem from similar developmental mechanisms.

Acknowledgements

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DISCUSSION

Kronenberg: What abnormalities are you expecting to find (or have you found) in the *Hox* gene pattern in the python mesenchyme that might correlate with the expansion of the thoracic vertebrae?

M. Cohn: We know that the somites, which form the vertebrae, are regionalized by nested domains of *Hox* gene expression. In transgenic mouse experiments in which the *Hoxc6* and *Hoxc8* genes are mis-expressed and expanded anteroposteriorally, there is an expansion of rib-bearing vertebrae beyond the normal thorax. This might mimic, to some degree, what has happened in snakes. The lateral plate mesoderm, which forms the limbs and body wall, is also regionalized by *Hox* gene expression. This is something that Cheryll Tickle and I showed a couple of years ago (Cohn et al 1997). Molecular regionalization of the lateral plate is an important step in determining the position at which limbs develop relative to the main body axis. The same principle of regionalization by differential *Hox* gene expression is operating in the axial skeleton and in the lateral plate

mesoderm. I'm arguing that, in snakes, expression of some *Hox* genes has been homogenized in both of these tissues. This might account for expansion of thorax in the axial skeleton, and expansion of flank — the limbless part of the body wall — in the lateral plate. The latter would posteriorize the positional identity of cells that would otherwise form forelimbs.

Kronenberg: That's the disappearance of the anterior limb. But it looked as if the hindlimb ended up in the right place, so why don't you get a normal hindlimb *Hox* pattern?

M. Cohn: There is a posterior boundary of *Hoxc8* expression precisely at the level of the hindlimb bud. Hindlimb position is specified and budding is initiated. Our data suggest that we're seeing a downstream effect in the hindlimb, in which formation of the apical ridge is affected. There is no evidence that ridge formation is related to *Hox* gene expression, even in the model systems, but I think that this is an interesting possibility.

Kronenberg: Why would whales be different? Is there anything you know yet about whales that explains the evolution of its thorax and limbs?

M. Cohn: Eocene whales like *Basilosaurus* challenge our idea that limb reduction and axial regionalization have to be wrapped-up in the same developmental package. They have elongated and homogenized the posterior part of the axial skeleton, yet they make hindlimbs which are complete all the way to the toes.

Wilkins: In principle there can be independent selection for retention of the limbs. One just needs genetic changes that uncouple the initiation of the bud development from these earlier signals that they used to be linked to. I don't think that this pre-historic whale disproves your idea.

Ornitz: In light of that, have you looked to see whether FGF10 is expressed in lateral plate mesoderm in the python? Is FGF receptor 2 expressed in ectoderm in the ridge?

M. Cohn: No, but I like the FGF receptor 2 idea. This could explain failure of the interaction between limb bud mesenchyme and the overlying ectoderm, which should normally result in formation of an AER.

Morriss-Kay: It would be good to look at this, because in the early mouse limb bud, there is no morphological AER — in this sense it resembles python more than chick

Have you looked at *Patched* (*Ptc*) expression in the limb? Although you have shown there is no *Shh* there, the limb clearly has the potential to turn *Shh* on. The limb also has a much broader region of polarizing activity, which in a minor way (since it doesn't form any digits) is reminiscent of the *Doublefoot* mutant, in which the whole limb mesenchyme acts as a polarizing region (Hayes et al 1998). Is there any *Ptc* expression in the python limb bud?

M. Cohn: I haven't looked at *Ptc* or BMPs in the python.

Ornitz: Have you looked in lateral plate mesoderm for *Tbx4* or *Tbx5* expression?

M. Cohn: No. It seems likely that *Tbx4* must be expressed, since the hindlimb field is specified. This is an interesting question with respect to the forelimbs: Is a forelimb field specified in python embryos? From the *Hox* pattern that we see, I would predict that there is not, but we should certainly look at *Tbx* genes.

Beresford: Is the assumption that if you carried out an experiment in which you grafted a chick AER onto the python mesenchyme, you would get a limb? That is, is the assumption that there is nothing intrinsically wrong with that mesenchyme, and that nothing has been lost over evolutionary time?

M. Cohn: This is an experiment we have tried for many years. The more we learn about python hindlimb bud mesenchyme, the more reason we have to believe that we can rescue limb outgrowth with an AER signal. I have been treating python embryos with FGF, which is the key ridge factor, by grafting FGF-loaded beads to the hindlimb bud. So far, what I have seen is that in the first 24 h, proximo-distal outgrowth is increased by about 30%. FGF can, therefore, sustain outgrowth over a longer period. However, we ran into technical problems doing these experiments *in ovo* because of the soft nature of snake eggs; they tend to collapse after being opened and there are problems with infection. The next step is to start doing this experiment in explant cultures, which I plan to start next season.

Newman: The fact that the python mesenchyme retains the ability to make *Shh* suggests two things. First is that *Shh* might be induced by FGFs in other regions of the embryo and, second, there probably isn't a limb-specific promoter for *Shh* induction.

M. Cohn: That is an interesting point. If *Shh* and FGF were always acting in a feedback loop in the embryo, then there would be strong selection to maintain this circuit. While they are often co-expressed in the embryo, this is not always the case. I would say that it does seem likely that *Shh* expression in the limb is controlled by some FGF-responsive mechanism, but we don't yet know whether there is a limb-specific hedgehog regulatory element.

Blair: Is there a theoretical basis for the uncoupling of the limb formation from the axial skeleton?

M. Cohn: There is an experimental basis for it. The morphological evidence suggests that the position of the forelimb in vertebrates tends to co-localize with the junction between thoracic and cervical vertebrae, but during development they can be uncoupled experimentally. One of our findings when we were looking at *Hox* regulation in lateral plate mesoderm was that FGF can reprogramme the *Hox* code and induce ectopic limb formation in the lateral plate without altering *Hox* expression or identity in the axial skeleton.

Blair: What I find confusing is that the *HoxC8* boundary still exists in the snake, but the hind limb rudiments are completely dissociated from the axial skeleton.

M. Cohn: The disarticulation of the pelvic girdle from the column could be an epiphenomenon that happens late, during differentiation or growth, and not

during specification of pattern. These tissues are patterned by *Hox* domains that lie adjacent to one another.

Mundlos: Could you not explain this simply by the loss of *Shh* expression? If Sonic is lost in the mouse, there is also truncation of limbs, and in the hindlimb there is exactly the same situation as you have seen in the python: there is a femur and nothing else. Perhaps loss of *Hox* expression leads to the regional turn off of *Shh* in the digit.

M. Cohn: We did notice that the extent of hindlimb outgrowth in pythons is almost the same as the extent of outgrowth seen in the *Shh* knockout mice. This is an interesting point. Our observations that python mesenchymal cells are competent to express *Shh* in the presence of a functional AER, and that the ridge is normally absent from these limb buds, suggests that absence of *Shh* is due to absence of an AER.

Meikle: How long ago did the pythons loose their hind limbs? There is a limit to the amount of time which a gene can be silenced without undergoing some kind of mutational degradation.

Ornitz: But *Shh* is still used by pythons in other places. Perhaps there is a tissue-specific enhancer that is lost.

Kingsley: This is the part that isn't known: how modular are the controls that build these limbs? Once the limbs are gone you should lose selection to maintain those modules that are limb specific. If there are limb-specific regulatory elements that are no longer used in making a useful limb, one would expect to see secondary changes in these.

Wilkins: In the python the limbs are still useable to some extent, for certain functions. So in this case there would be some selection for retention of some of these regulatory elements.

M. Cohn: There is an evolutionary principle known as 'Dollo's Law', which says that evolution cannot reverse itself. More specifically, once a structure is lost from an animal, it can not re-evolve. This 'law' has to be re-examined in light of molecular developmental biology. If there is selection to maintain a developmental cassette like FGFSHH in another part of the embryo, then there should be no great difficulty in re-activating that cassette in the limb. But if development is truly modular and there is, for example, a transcriptional enhancer required for *Shh* expression in the limb, then I would agree that once limbs are lost, genetic drift could eventually make it difficult, if not impossible, for them to re-appear.

Wilkie: Do you know what underlies the changes in distribution of *Hox* gene expression? Is *Hox* gene organization altered, are there differences in particular *Hox* promoters, or is this all driven by another gene that's switching on *Hox* — which is itself relatively unchanged in gene organization ?

M. Cohn: We have no idea — we don't even know how many *Hox* genes these animals have. However, this is a key question. These developmental changes could

be associated with gene loss. Puffer fish, for example, have stripped-down their axial skeletons and lost a set of fins, and this is associated with loss of several *Hox* genes.

Hall: Now that you have done this quite prodigious study on a snake that retains hindlimb elements, it would be nice to do the whole thing all over again with a snake that completely loses hindlimb elements, or some of the legless lizards, because this might help you to get at the modularity and the connection to the *Hox* genes.

M. Cohn: We are, in fact, now looking at corn snakes and king snakes, both of which lack limbs entirely.

Hall: One of the reasons the limb buds regress in legless lizards is because the somitic cells move into the limb and inhibit cell death. Do you know anything about the somitic contributions coming into the python's limbs? There are clearly muscles there, so there must be a normal somitic contribution.

M. Cohn: I'm glad that you brought this up, because I was going to ask you about it! You are correct that there is muscle in these rudimentary limbs. One idea proposed by Raynaud several years ago is that limbless lizards have reduced limbs because of a quantitative deficiency in the number of somitic processes that invade and somehow 'stimulate' the lateral plate. Additionally, explant experiments in mouse suggested that a somitic contribution is needed for limb outgrowth. The data from chick somite-removal experiments, however, show that one can get perfectly good, but muscle-free, limbs without somites. In light of this, I wonder whether there may have been some misinterpretation of the mouse and legless lizard work. By removing or separating paraxial from lateral plate mesoderm, one might inadvertently remove the adjacent intermediate mesoderm. The intermediate mesoderm expresses Fgf8, and there are data which suggest that it is required for limb development. Somites, however, do not seem to be required for limb outgrowth. I have not looked for somitic processes in pythons primarily because I think that the somitic contribution to the limb bud is myogenic rather than stimulatory.

Hall: Jonathan Bard and I were chatting yesterday about the derivatives of the intermediate mesoderm, such as the mesonephros and the role that it plays in development.

There is a notion that the ability to be competent to make an AER is specified by the mesoderm. I had a feeling it must be known whether the initial ability to become AER is ectoderm specific or mesoderm specific in the chick.

Ornitz: In the mouse *Fgf10* knockout there is a very transient limb bud but the AER is not formed. *Fgf10* is a mesoderm-expressed gene.

Pizette: If you put FGF7 beads in the mesenchyme of the back, you can actually induce AER-specific gene expression in the overlying ectoderm (Yonei-Tamura et al 1999). This indicates that the competence to form an AER is in the mesenchyme.

Hall: Unless that back ectoderm is competent to respond, then what you probably mean is making it competent to respond.

Tickle: In those experiments, there's a difference in the competence of the ectoderm at the dorsal midline versus the competence of the ectoderm on the sides of the body. The competence dorsally extended much further anteriorly then it did on the lateral side of the body.

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