Diversification of the vertebrate limb: sequencing the events

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Abstract

Naturalists leading up to the early 20th century were captivated by the diversity of limb form and function and described its development in a variety of species. The advent of discoveries in genetics followed by molecular biology led to focused efforts in few 'model' species, namely mouse and chicken, to understand conserved mechanisms of limb axis specification and development of the musculoskeletal system. 'Non-traditional' species largely fell by the wayside until their recent resurgence into the spotlight with advances in next-generation sequencing technologies (NGS). In this review, we focus on how the use of NGS has provided insights into the development, loss, and diversification of amniote limbs. Coupled with advances in chromatin interrogation techniques and functional tests *in vivo*, NGS is opening possibilities to understand the genetic mechanisms that govern the remarkable radiation of vertebrate limb form and function.

Introduction

Limbs of different shapes and proportions enabled amniotes to invade a variety of ecological niches. The diversification of limb form allowed kangaroos and jerboas to hop, humans to walk upright, and bats and birds to fly. Limb loss and reduction, on the other hand, has allowed squamates to slither beneath the ground or under water. For decades, chicken and mouse limbs have served as a crucial experimental paradigm to uncover conserved developmental programs that control pattern formation in all three axes, cellular differentiation, and proximal-distal growth of the amniote limb [1,2]. However, modifications to these conserved developmental programs that could explain divergent limb forms in nature have remained less understood until recently. Rapid advances in next-generation sequencing

(NGS)[3] and comparative genomic approaches[4,5], including reduced costs, are now bridging this gap to enable comparative assessments of gene expression and chromatin architecture in the developing limbs of a variety of species[6–17]. We focus on a few of these examples to illustrate the promise that NGS holds to advance our current understanding of the evolutionary diversification of the amniote limb.

Shared developmental origins of genitalia and limbs during amniote evolution

The limbs and genital tubercle, the precursor to the external genitalia/phallus, are among the most apparent outgrowths in the developing mouse embryo (Figure 1a). The genital tubercle and cloaca, a signaling center that controls genital tubercle development and that is retained as a separately functional structure in many other species, lie posterior to the limb buds in the caudal regional of the embryo [7]. Despite their non-overlapping embryonic locations, there is a functional overlap of key transcription factors and signaling pathways that determine the pattern of both the genital tubercle and hindlimbs [18,19]. Collectively, these observations have hinted that the evolutionary origin of external genitalia in terrestrial vertebrates may be somehow developmentally linked to limb bud pattern and outgrowth.

In contrast to the single medial phallus in mouse, the external male genitalia in squamates are a pair of outgrowths, called the hemi-penis. Also, unlike mouse, the developing squamate hemi-penis and cloaca lie in close proximity to the embryonic limb field (Figure 1b). In fact, the squamate hemi-penis emerges from the hindlimb-bud (anole lizards) or its remnants (pythons and house snakes), giving further credence to the hypothesis that developing limbs and external genitalia shared a common developmental origin early in the evolution of amniotes [7].

To confirm and gain deeper understanding of the shared developmental trajectory of these appendages, Tschopp and colleagues also applied comparative transcriptome analysis of the early- and late-developing limb buds and external genitalia (hemi-penis or genital tubercle) of anole and mouse embryos [7]. They discovered that a great degree of molecular architecture is shared between hindlimb buds and external genitalia during early development in anole, and in fact these structures computationally cluster together in anole but not in mouse. Most of

the similarity lies in transcription factors and signaling molecules, highlighting molecular programs at play in the shared developmental origin of limbs and external genitalia. The authors suggest that the developmental divergence of the squamate hemi-penis and the genital tubercle in mammals may have followed the posterior shift in embryonic position of the cloaca in mammals (Figure 1a, b), though vestiges of the ancestral similarity to limbs remain.

How did the molecular similarities between amniote appendages arise, and how have they been retained? Are there pleiotropic *cis*-regulatory elements that drive transcription of genes in both external genitalia and limb buds, or is all similarity due to the additive effects of elements with activity that is specific to each appendage? To answer this question, Infante and colleagues performed ChIP-Seq to detect the H3K27ac histone modification that marks active enhancers and promoters and discovered more than 9,600 putative regulatory elements that are active in mouse embryonic genital tubercle and/or limb buds [8]. Though a majority (~84%) of these elements showed appendage-specific acetylation patterns, a smaller proportion (~16%) were indeed acetylated in both of these structures.

One such shared limb-genital tubercle enhancer, called HLEB, regulates expression of *Tbx4*, a T-box transcription factor that is necessary for the development of the hindlimb [20] and external genitalia in mice [8]. HLEB is conserved not only in the limbed mouse and anole but also in vestigial-limbed (pythons) and limbless snakes (king cobras). An assessment of squamate HLEB activity in transgenic mouse embryos showed that though anole HLEB has retained its activity in both appendages, snake HLEB sequences drive expression only in the mouse genital tubercle but not in the limb, suggesting appendage-specific degeneracy of this dual-activity enhancer (Figure 1a-c). A recent advance in the ability to access and edit the anole germ line genome [21], a major limitation in many amniote species, opens possibilities to dissect the *in vivo* function of the HLEB in the limb bud and hemi-penis in embryos that retained their ancestrally shared developmental origins.

Comparative genomic analysis between mouse and squamate genomes further revealed a surprising degree of conservation of experimentally validated mouse limb enhancers in limbed anoles as well as in three distinct snake species that have vestigial (pythons & boas) or no limbs (king cobras) [8]. Such conservation in snakes suggests selective pressure might have

preserved limb enhancer elements that also drive gene expression in other structures, including the external genitalia. In contrast, lack of selection to preserve limb-specific enhancers in snake genomes, starting when limb function was reduced or lost, might allow these to diverge more rapidly.

One such example of the extreme degeneracy of a limb-restricted *cis*-regulatory element in snakes is the 'ZPA regulatory sequence' (ZRS) enhancer, which initiates and sustains expression of the anteroposterior patterning gene *sonic hedgehog* in developing limb buds and is highly conserved among limbed tetrapods (Figure 1a-c). The ZRS of both vestigial-limbed [6] and limbless snakes [9] has degenerated to an extent that it drives aberrant, reduced, or no lacZ reporter expression in transgenic mouse limbs. Since the ZRS is essential for limb outgrowth in mice, replacement of the mouse ZRS with the most severely attenuated python or cobra ZRS causes severe limb truncation [9]. There is ample evidence for other such degenerate sequences throughout the snake genome; an alignment of 29 vertebrate genomes, including boa and python, identified 5,439 conserved non-coding elements (CNEs) that are highly and specifically divergent in snakes, which includes the ZRS. The nearest genes to these snake-divergent CNEs are enriched for genes with demonstrated importance during limb development, and many overlap experimentally validated limb enhancers [5]. The most-divergent snake CNEs among these provide excellent targets for further *in vivo* experiments that may provide evidence for the proximate and causative mechanism(s) of limb loss in the snake lineage.

Overall, NGS and comparative genomic approaches have allowed us to discover the shared developmental origins and evolutionary emergence of genitalia from limbs of amniotes. These studies have also revealed and partially explained the surprising conservation of limb enhancers in limbless species. Pleiotropic limb enhancers shared with genitalia and other structures as well as enhancers with activity limited to the limb may explain how constraint and degrees of selection each act to diversify vertebrate genomes and consequent development of the limbs.

Diversification of amniote limb form and proportion

The feathered wings and scaled feet of birds, elongated fingers in the bat wing, and disproportionately long feet of three-toed jerboas are compelling examples of diversity within the fore and hindlimbs of a species and between homologous limb elements of different species. Focusing on these examples, here we discuss how NGS, comparative genomics, and functional tests have revealed genetic signatures that generate such diversity of limb form.

While most birds have feathered wings and scales on their feet, feathers cover the feet of many species, such as grouse and snowy owls. In domestic pigeons, breeders have favored a feather-footed phenotype so much that some breeds exhibit extreme 'muffs' of feathers as long as those of the wing that would appear to have no selective advantage in natural populations. However, the genetic mechanisms that transform aspects of hindlimb identity to more forelimb-like may similarly underlie both artificially and naturally selected foot feathering. Quantitative trait locus (QTL) analysis and comparative ChIPSeq analysis of scale- and feather-footed pigeon breeds, powered by whole genome assemblies, revealed mutations upstream of *Pitx1* and *Tbx5*, genes that associate with feathered feet (Figure 2b). These *cis*- regulatory mutations in developing feathered feet contribute to reducing the expression of the hindlimb transcription regulator *Pitx1* and to misexpressing *Tbx5* in the hindlimb, a transcription factor ordinarily restricted to and necessary for forelimb development [10, Figure 2a]. Comparative transcriptomic analysis of developing limbs of these breeds identified *Pitx1*- and *Tbx5*-dependent transcriptional targets and revealed partial co-option of a forelimb-specific genetic program that could explain how molecular shifts in limb identity from hindlimb to forelimb drove the development of feathers on the feet [11]. In addition to explaining the molecular mechanism underlying a domesticated trait in pigeons that has fascinated breeders for centuries, these findings provide insight into putative genetic targets of natural selection in diverse bird species.

The only mammals capable of bird-like powered flight are the bats, though they achieve this feat using wings with an entirely different structure than birds. Bat wings with elongated digits and broad interdigital membranes and unwebbed feet with shorter toes also present yet another case of contrasts in the fore- versus hindlimb. Transcriptomic analysis comparing embryonic bat fore- and hindlimbs identified differential expression of known limb patterning

and developmental pathways [13,14]. Of particular note is the enrichment of long non-coding RNAs (lncRNAs) and lower expression of ribosomal proteins in the developing wing [13]. Perhaps most interestingly, comparative genomics identified 'bat accelerated regions' of rapid evolution within H3K27ac peaks, and several of these are located near genes implicated in limb development [13,22]. These transcriptomic and *cis*-regulatory changes provide promising candidates to design experiments that will test their precise molecular function *in vivo,* perhaps using mice as an experimental system, and identify causative mechanisms that drove extreme forelimb modifications during bat evolution and development.

Diversification is often initiated in the embryo, as exemplified above, but is further amplified in neonatal and juvenile animals by postnatal growth differences. For example, differential postnatal growth of metatarsals has resulted in drastically elongated feet that enable bipedality in the three-toed jerboas [23]. With exception of species like pigeons with phenotype and genotype variation in breeds that is tractable using genetic approaches, most species of interest in the evolution of limb development are distantly related to a 'reference species', namely mice, for which we know the most about the genetics of limb development. The phylogenetic position of the jerboa, with the most extremely divergent hindlimb architecture among the nearest relatives of *Mus musculus*, therefore provides an extraordinary opportunity for direct comparisons between species to identify mechanisms that diversified skeletal proportion.

Though not a typical application of differential RNA-seq analysis, the relatively close phylogenetic relationship of jerboas and mice allowed us to identify 10% of the transcriptome associated with the disproportionate postnatal growth acceleration of jerboa metatarsals, which could explain the evolution of bipedality. Among many expression differences that likely belie a complex polygenic mechanism [24], we observed expression of *Shox2* in jerboa metatarsal growth cartilages [unpublished]. The *Shox2* transcription factor is restricted to the proximal skeleton of all other vertebrate species that have been analyzed [25], and its misexpression in the distal limb is sufficient to elongate mouse metatarsals [unpublished]. Similarly, comparing differential chromatin accessibility between species by ATAC-Seq analysis revealed a chromatin region upstream of *Shox2* in jerboa that might harbor *cis*-regulatory changes that modularize the gain of *Shox2* expression in jerboa metatarsals (Figure 3).

Identification of such differentially accessible regions associated with differentially expressed genes throughout jerboa and mouse genomes can help us define genomic regions of interest for functional manipulation in mice to identify mechanisms of differential skeletal growth.

Future of limb development with next-generation sequencing

The few examples we highlight in this review, and others we briefly reference, demonstrate how NGS techniques have facilitated the unbiased discovery of transcriptomic changes, differential enhancer activity, and sequence evolution associated with limb diversification across 'non-traditional' amniote species. Our ability to sequence, assemble, annotate, and compare genomes has seen unprecedented advances [3,4] since the first animal genome was sequenced just over two decades ago [26]. With large-scale efforts to make thousands of vertebrate genomes publicly available [27], opportunities are rapidly emerging to discover and functionally test the consequences of genome evolution with respect to limb diversification. Rather than 'stamp collecting', these efforts may begin to reveal a set of principals by which evolution has reshaped species.

The greatest limitations now are the ability to rear animals in captivity, or collect embryonic and juvenile samples from wild populations, and the ability to access and manipulate the germ line genome of amniotes. Overcoming these challenges in even a few representative species will unlock opportunities for targeted *in vivo* experiments to help us comprehend how variations in gene expression that alter conserved developmental programs caused the diversification of vertebrate limb form and function. Are some genes more 'evolvable' than others? Are some genes controlled by more modular *cis*-regulatory elements than others so that pleiotropic functions are under non-pleiotropic control? What are the phenotypic consequences of sequence changes, individually and collectively? Together with genetic studies that focused largely on conserved coding sequence functions in traditional model species in the intervening decades before the resurgence of evo-devo, studies in non-traditional species will give comprehensive insight to understand not only development from egg to adult but also the evolutionary explosion of countless adult forms.

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- ● of special interest
- ●● of outstanding interest

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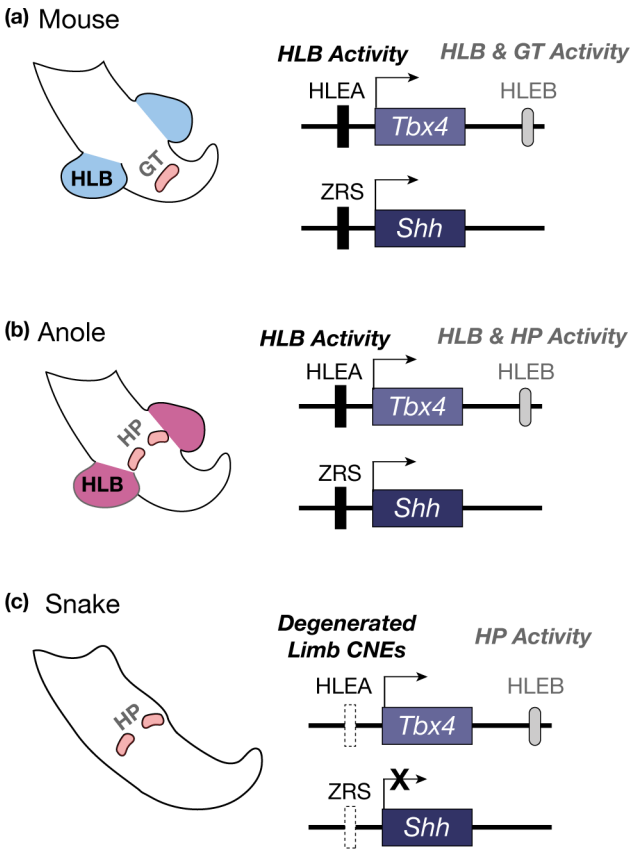

Figure 1. Development of hindlimb buds and external genitalia in **(a)** mouse, **(b)** anole lizard, and **(c)** snake. HLB, hind limb bud. GT, genital tubercle. HP, hemi-penis. In each species, *cis*-regulatory control of *Tbx4* by the hindlimb element A (HLEA) and dual activity hindlimb element B (HLEB) and control of *sonic hedgehog* by the ZPA regulatory sequence (ZPA) are depicted. Hindlimb activity of all three elements has degenerated in snakes.

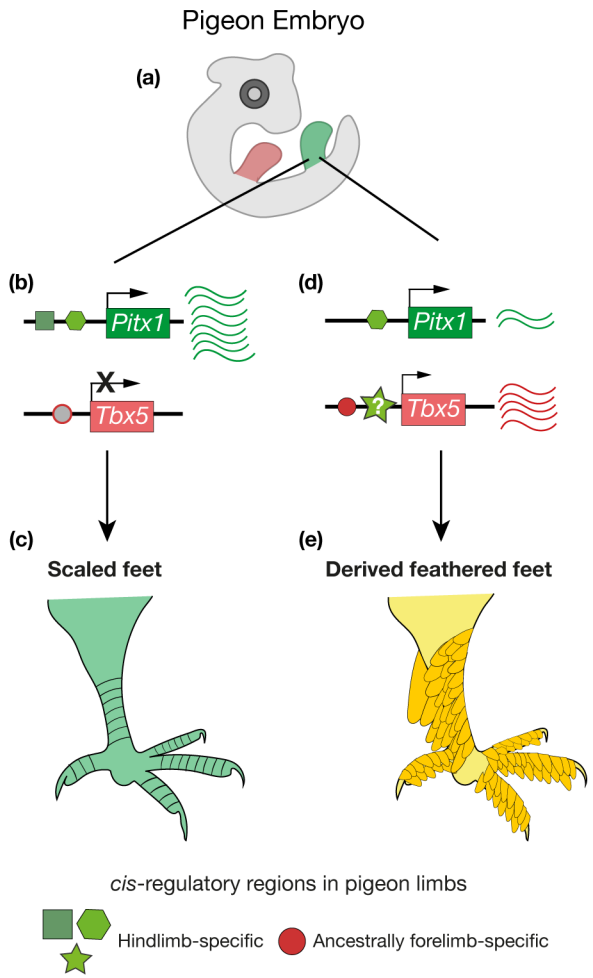

Figure 2. In the developing hindlimbs (green) of the **(a)** scaled feet pigeon breeds, hindlimb-specific *cis*-regulatory regions drive robust *Pitx1* expression. Forelimb-specific *Tbx5* *cis*-regulatory regions are inactive (grey circle) in the hindlimbs, resulting in no *Tbx5* expression. **(b)** In feather-footed breeds, a deletion upstream of *Pitx1* is associated with reduced hindlimb *Pitx1* expression, and *cis*-regulatory mutations near *Tbx5* are associated with its misexpression in the hindlimbs. These *cis*-regulatory mutations could lead to the reactivation of forelimb-specific *Tbx5* enhancers (conceptually represented using a red circle) in the hindlimbs and/or a gain of novel hindlimb-specific enhancers (green star).

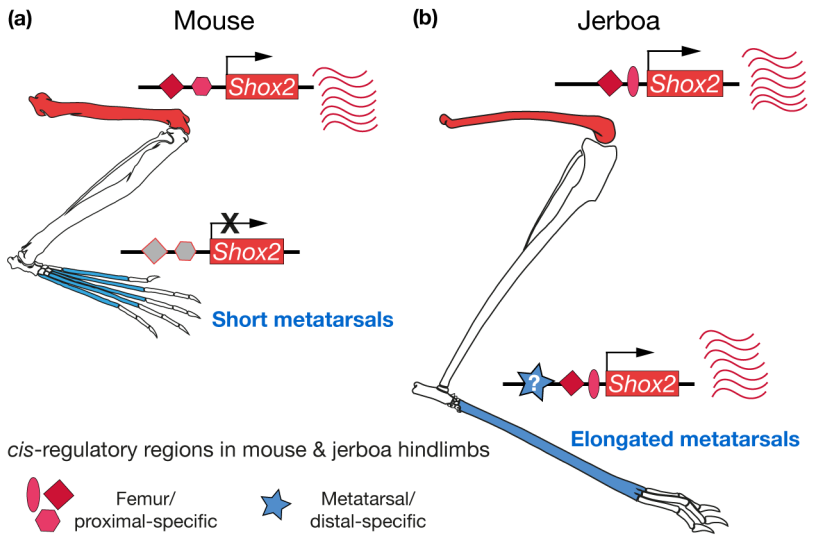

Figure 3. Hindlimbs of **(a)** mouse and **(b)** jerboa with femurs (red) and metatarsals (blue) highlighted. Proximally active (red) *cis*-regulatory regions drive *Shox2* expression in mouse and jerboa femurs. These regions are inactive (grey) in the distal mouse limb, and no *Shox2* expression is detected in mouse metatarsals. Jerboa metatarsals express *Shox2*, which may be explained by the activation of *cis*-regulatory sequences typically restricted to the proximal limb (red).