Unwinding Limb Development

Preview article

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ONLINE SYNOPSIS

The molecular mechanisms underpinning vertebrate body plan evolution are beginning to be unravelled. In this issue of Cell, Kvon et al, spectacularly demonstrate how transplanting snake-specific genetic changes found uniquely in serpent enhancers leads to limb loss in mice.

MAIN TEXT

The genetic and developmental changes that have led to the huge diversity of forms among vertebrate limbs is a fascinating line of investigation, and snake limb loss has long been of particular interest (Cohn and Tickle, 1999). Anatomically, the vast majority of snake species have lost all trace of skeletal limb structures; these advanced snakes co-exist with a handful of basal snakes, such as pythons, that retain vestigial pelvic and femur structures. Fossil evidence attests to extinct evolutionary intermediates (Martill et al., 2015). Comparative sequence approaches have gradually elucidated the genetic and epigenetic innovations that underlie snake morphology. In this issue of *Cell,* Kvon et al., demonstrate how transplanting snake-specific limb enhancers into mice can recapitulate limb truncation.

The Zone of Polarizing Activity Regulatory Sequence (ZRS) is one of the best-characterized long-distance transcriptional enhancers in mammals. Present in most vertebrate genomes, the ZRS controls the expression of Sonic hedgehog (Shh) in developing limb buds. However, the ZRS sequence, as well as its transcriptional regulatory activity, have substantially diverged among snakes (Kvon et al., 2016; Leal and Cohn, 2016).

In heroic experiments, Kvon et al., replace the endogenous ZRS mouse sequence with orthologous sequences from limbed (human or coelacanth, a rare order of fish) or limbless (python and cobra) vertebrates (**Figure 1**). Despite up to 400 million years of divergence since their last common ancestor, the human and coelacanth enhancer sequences functionally recapitulate normal limb development in mice; in stark contrast, transgenic mice with snake ZRS enhancer sequences replacing normal murine sequences had severe limb truncations (Figure 1). Sequence analysis suggests that a small 17 bp snake-specific deletion within the ZRS disrupts an ETS transcription factor binding site (TFBS). Kvon and colleagues then perform rescue experiments by reintroducing this sequence into their transgenic mice, thus restoring normal limb development *in vivo*.

In complementary experiments now reported in *Current Biology*, (Leal and Cohn, 2016) dissect how sequence changes in limb-specific enhancers for *Shh* (notably including the same ZRS as above) as well as *Hoxd* (see also Guerreiro et al., 2016) contribute to limb loss in snakes. The authors combine transgenic reporter assays in python embryos and mice with *in vitro* biochemical approaches to analyse *Shh* signalling and ZRS enhancer activities. Using directed mutagenesis, Leal and colleagues show how three snake-specific deletions in the python ZRS sequence (one of which corresponds to the 17 bp deletion analysed by Kvon et al., 2016) have a potentially additive effect on ZRS activity. Leal and Cohn provide evidence that ZRS regulation by *Hoxd13* is also likely disrupted by snake-specific deletions. Despite pointing to different regulatory culprits (that is, ETS *vs* Hoxd13), both pioneering studies reveal how mutations in functional non-coding sequences—even when poorly conserved—can disrupt transcriptional networks required for organismal development.

The remarkable phenotypic results obtained by Kvon et al., 2016 crystalizes the extraordinary progress in combining comparative functional genomics and genome engineering. The rapidly accelerating success of this strategy has implications for exploring mechanisms of vertebrate evolution, in understanding human disease and diversity, and (most profoundly) on the future of re-engineering the vertebrate genome.

Firstly, in many different vertebrate lineages, limb morphology frequently evolves. Bat wings are essentially repurposed hands, and even among primates such, as the aye-aye, finger morphology has radically diverged. Limb atrophy, similar to that seen in snakes, is the simplest of these modifications, and has independently occurred in salamanders, lizards, and cetaceans. The analytical strategy outlined by Kvon and Leal (Kvon et al, 2016; Leal and Cohn, 2016) could rapidly accelerate our understanding of the constellation of enhancer changes that can and have led to limb reduction in our closer relatives. Many of the same enhancers disabled in snakes are likely to have also been *functionally* rewired in mammals. Striking limb morphological adaptations have evolved at least in part by tuning *Shh* or upstream *Fgf* signalling to truncate hind-limb development in whales and dolphins (Thewissen et al., 2006) and to massively remodel fore-limb development in bats (Eckalbar et al., 2016) **(Figure 1)**.

Secondly, these studies may provide further insight into how mutations in enhancers can lead to developmental disorders in humans (Anderson et al., 2012). Mutations in the same regulatory regions that control Shh tissue-specific expression have been linked to a range of congenital abnormalities, including limb malformations such as preaxial polydactyly. Moreover, human mutations associated with altered limb development include numerous single nucleotide polymorphisms in the ZRS itself, as well as duplications and deletions in other limb enhancers. These sequence changes often occur on evolutionarily conserved nucleotides and alter binding sequences of the ETS family of transcription factors.

Finally, Kvon et al. show how comparative functional genomics is increasingly a powerful tool to design genetic modifications in enhancers that have an accurately predictable outcome on the organismal phenotype. Most mammalian studies suggest that more elegant and functional anatomical restructuring, such as resurrecting limb development in snakes, will almost certainly require the simultaneous engineering of multiple enhancers (Cretekos et al., 2008; Leal and Cohn, 2016). Fortunately, there are ambitious efforts to map all enhancer and promoters across a diversity of mammals that will unlock species-specific functions in many tissues (Eckalbar et al., 2016; Villar et al., 2015).

The example set by Kvon and colleagues is an important waypoint towards consciously restructuring the vertebrate form by re-writing transcriptional enhancer sequences.

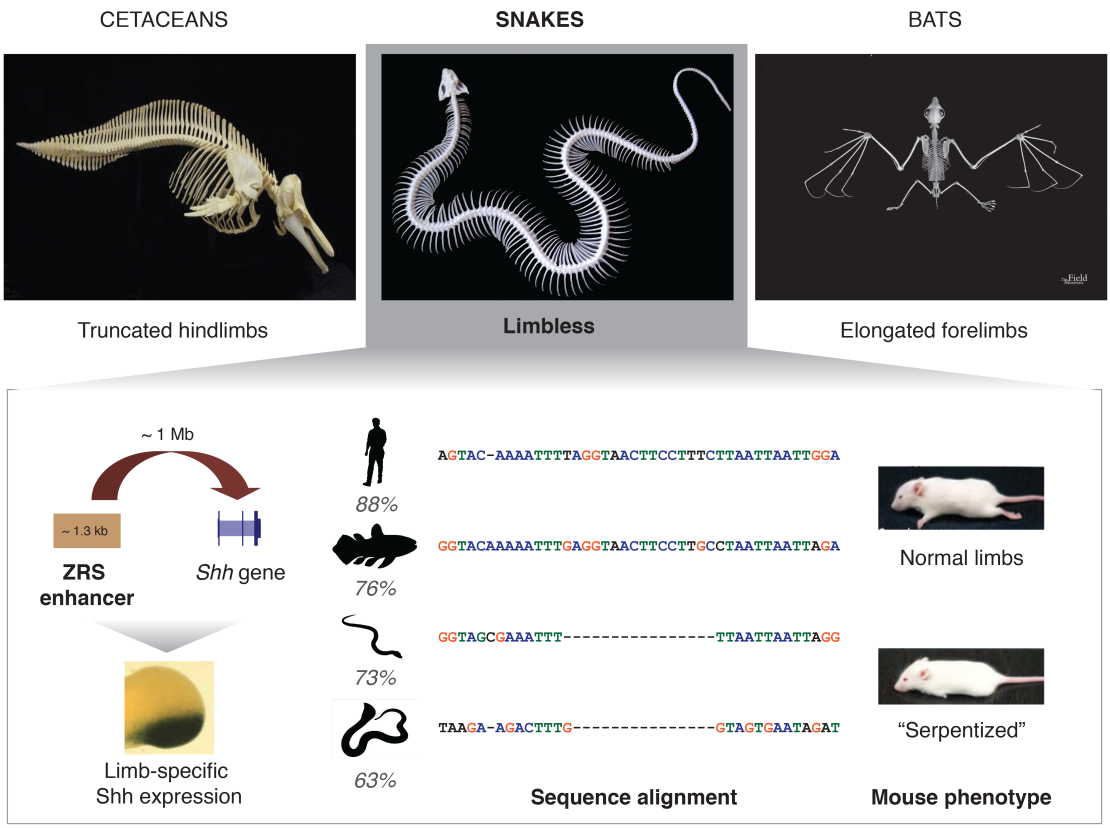

Figure 1: Most snakes have evolved complete limb loss (center). Similarly in mammals, whales and dolphins have lost their hind limbs, and conversely, bats have massively remodelled segments of their forelimbs. The ZRS is a well-characterised long-range enhancer directing limb-specific expression of *Sonic hedgehog*. Kvon et al. employ a combination of comparative genomics and genetic engineering to introduce snake-specific deletions in this enhancer element into the orthologous mouse genome, resulting in truncated limb development *in vivo*. The sequence alignment of human, coelacanth, python and cobra sequences corresponds to a 42 bp region of the ZRS containing a serpent-specific deletion. Numbers below each species silhouette indicate percentage nucleotide identity over the core ZRS (~0.8 kb), using the mouse enhancer as reference. ZRS: Zone of Polarizing Activity Regulatory Sequence. Shh: Sonic hedgehog.

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