RAPID COMMUNICATION

Developmental Basis of Evolutionary Digit Loss in the Australian Lizard *Hemiergis*

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ABSTRACT

Loss of limb skeletal elements is a recurring theme in tetrapod evolution, but the developmental mechanisms underlying this phenomenon remain largely unknown. The Australian lizard genus *Hemiergis* offers an excellent model system to study limb reduction among closely related, naturally occurring populations with different numbers of digits. Evolutionary digit loss in *Hemiergis* does not result from simple truncation of a pentadactyl skeletal developmental program. Rather, the duration of embryonic expression of the patterning molecule Sonic hedgehog (SHH) is shortened in limbs with reduced numbers of digits, and is correlated with decreased cell proliferation in the posterior aspect of the limb. Moreover, this comparative analysis suggests an early role for SHH in specification of digit identity and later importance in maintaining cell proliferation and survival. Subtle changes in spatial or temporal regulation of SHH may alter proliferation and patterning of the developing limb, thereby effecting divergence in adult limb morphology among closely related species. In contrast, expression of MSX and Distal-less proteins were similar among embryos from different populations. *J. Exp. Zool. (Mol. Dev. Evol.)* 297B:48–56, 2003. © 2003 Wiley-Liss, Inc.
Correlations between morphological diversity and evolutionary changes in the expression of embryonic patterning molecules can yield important insights into the developmental mechanisms that underlie phylogenetic modifications in ontogeny. The gene Sonic hedgehog (Shh), which encodes a secreted intracellular signal expressed in the zone of polarizing activity (ZPA), is critical to normal outgrowth and patterning of the developing limb, including determination of the number and identity of digits (Litingtung et al., 2002; te Welscher et al., 2002). SHH expression is transient in limb development, declining just prior to or during digit condensation, and is codependent upon other molecules such as fibroblast growth factors (FGFs) in the neighboring apical ectodermal ridge (AER) (Laufer et al., '94; Niswander et al., '94; Zúñiga et al., '99). Breakdown of this positive feedback loop through targeted disruption of Shh expression (Chiang et al., '96) and removal of the ZPA (MacCabe et al., '73) has demonstrated that limb morphology can be altered by experimental manipulation, but such experiments reveal little about the role of this molecule in generating novel phenotypes in natural populations. A single study of SHH protein expression in pythons provided an important comparison between traditional model species and a limbless species (Cohn and Tickle, '99), but its results are not easily extrapolated to species with intermediate cases of limb reduction.

We examined the possible role of SHH in Hemiergis, which exhibits less severe, but more finely graded, limb reduction than that seen in snakes. We found a clear correlation between adult digit number and duration of SHH expression early in limb development. Moreover, truncated expression of SHH was correlated with reduced mesenchyme proliferation in the limb buds of embryos from populations with fewer digits.

Fig. 1. Limb skeletal development in Hemiergis. In all diagrams, distal is at the top and anterior is to the left. Following shared, early skeletal configurations (left), the developmental trajectories of H. initialis (A; 5/5), H. peronii (B; 4/4), H. peronii (C; 3/3), and H. quadrilineata (D; 2/2) autopodia diverge, culminating in different adult morphologies (right). The shared and intermediate stages depict forelimb configurations only, but hind limb data are virtually identical. Data from cleared and stained whole mounts and serial sections (Shapiro, 2002). Scale bars = 1 mm for adults.
MATERIALS AND METHODS

Collection, fixation, and staging of embryos

Embryos of *Hemiergis quadrilineata* (2/2), *H. peronii* (3/3 and 4/4), and *H. initialis* (5/5) were collected, harvested, and fixed as described (Shapiro, 2002). Embryos were precisely staged prior to immunochemistry using a staging table for *Lacerta vivipara* (Dufaure and Hubert, '61; Porter, '72). Key staging criteria included position (or presence) of the endolymphatic sacs, number of somites, number or presence of branchial slits, heart morphology, eye (and eyelid) morphology, and lower jaw morphology. Limb morphology was also considered, but limb size and shape often differed among the four morphs at otherwise similar embryonic stages (e.g., see Shapiro, 2002: figure 7, for a comparison of stage 33 embryos).

Whole-mount immunohistochemistry

Limbs typically were removed from embryos before beginning the immunohistochemistry protocol, but whole embryos (stage 32 and younger) were stained occasionally. A modified version of the Vectastain Elite ABC peroxidase antibody kit (Vector Laboratories, Burlingame, CA) protocol was used for all whole-mount procedures. Tissues first were incubated for 2 hr in 6% hydrogen peroxide in 75% methanol to quench endogenous peroxidases. Following rehydration to PBT (PBS with 0.3–0.5% Triton X–100), specimens were incubated in a serum cocktail (95% calf serum, 5% DMSO) containing primary antibodies. SHH antibodies (Marti et al., '95) were diluted 1:50, MSX–1 and DLX antibodies (supernatant, Hybridoma Bank, University of Iowa) 1:10, and MSX–1 and DLX antibodies (Panganiban et al., '95) 1:100. Tissues were incubated overnight (MSX and DLX) or over two nights (SHH) at 4°C, then washed thoroughly in PBT and incubated overnight at 4°C in serum cocktail containing a biotinylated secondary antibody diluted 1:1000. Following three 10-min PBST washes, tissues were incubated in an avidin-peroxidase conjugate solution (Vector “A” and “B” reagents diluted 1:1000 each) for 1 hr at room temperature. Tissues were then washed in PBT, followed by signal development in a diaminobenzine substrate solution (Vector Elite DAB kit) according to manufacturer’s instructions.

BrdU immunohistochemistry

Following harvest from gravid females, in ovo embryos were incubated for 1–2 hr in bromodeoxyuridine (BrdU; approximately 0.25 mg/mL) in PBS or 0.8% saline. For immunohistochemical detection of BrdU, individual limbs were excised and then embedded in Paraplast according to standard procedures. Tissues were sectioned at 10 μm, rehydrated through an ethanol series, incubated for 30 min in 0.3% hydrogen peroxide to quench endogenous peroxidases, rinsed twice in H₂O, and then incubated for 1 hr in 2 N HCl to denature DNA. Following two rinses in PBS, sections were incubated for 5 min in 0.1% proteinase K to permeabilize cells and optimize antigen accessibility. Sections were blocked for 30 min with 3% horse serum and incubated overnight at 4°C with a BrdU antibody (Hybridoma Bank G3G4 clone, supernatant diluted 1:200 to 1:400 in 3% horse serum). Vector ABC Elite Universal peroxidase kit and Vector Elite DAB kit were used for secondary antibody conjugation and signal detection, respectively, according to manufacturer’s instructions.

RESULTS AND DISCUSSION

*Hemiergis quadrilineata* (2/2), *H. peronii* (3/3 and 4/4), and *H. initialis* (5/5) differ in their duration of SHH expression in the limbs. At stage 30, SHH immunoreactivity is observed in the posterior mesenchyme of the fore- and hind limbs of all four morphs (Fig. 2). By stage 31, however, forelimb expression in *H. quadrilineata* (2/2) is no longer detected, and hind limb expression is restricted to the posterodistal mesenchyme (Fig. 2G) or absent entirely (not shown). In the other morphs, expression at this stage is maintained along the full posterior edge of both the fore- and hind limb autopodia.

During stage 32, SHH is also downregulated in the hind limb of *H. quadrilineata* (2/2; Fig. 2I) and the forelimb of *H. peronii* (3/3; Fig. 2L). Expression also becomes restricted to the posterodistal mesenchyme (Fig. 2G) or absent entirely (not shown). In the other morphs, expression at this stage is maintained along the full posterior edge of both the fore- and hind limb autopodia.

At stage 33, early digit condensations are visible externally in all morphs. SHH is no longer detected in *H. quadrilineata* (2/2; Fig. 2J) or in either morph of *H. peronii* (3/3 and 4/4; Fig. 2O,T, respectively), but small foci of staining persist distally in most (75%; n = 4) stage 33 *H. initialis* (5/5) hind limbs (Fig. 2Y).
Overall, SHH expression is downregulated earlier in morphs with fewer digits. Conversely, limbs of lizards with more digits are exposed to SHH for a longer period. Yet, despite changes in the relative timing of SHH expression among Hemiergis morphs, the dynamics of expression appear to be unchanged and similar to that seen in other tetrapods. As seen in both mouse and chicken...
embryos, Shh is expressed along the full posterior edge of the early limb bud. As the limb bud grows, Shh expression is progressively restricted distally to the posterior subapical mesenchyme, and it is downregulated as the digits begin to condense.

To assess the possible involvement of other gene products in digit loss in *Hemiergis*, we analyzed the protein expression patterns of two additional gene families, Msx and Distal-less (Dlx). In chicken embryos, Msx gene expression is associated with apopotic regions of the limb, and misexpression of Msx-2 induces ectopic apoptosis, leading to digit loss (Ferrari et al., '98). Dll/Dlx genes are expressed in the apical (distal) portion of developing appendages in most animals (Bendall and Abate-Shen, 2000). Dlx5 and Dlx6 are essential for proper autopod development in mammalian limbs, and these genes have been implicated in the human split-hand/split-foot malformation, which is characterized by a loss of digits (Merlo et al., 2002; Robledo et al., 2002). Moreover, DLX is absent from the limb buds of python embryos, which develop only rudimentary limbs (Cohn and Tickle, '99). Patterns of both MSX and DLX immunostaining in embryonic limbs of *Hemiergis* closely resemble those reported for chicken and mouse embryos at all stages of development analyzed, and no significant differences were observed among *Hemiergis* morphs (Figs. 3, 4). Thus, interpopulational variation in MSX or DLX distribution is unlikely to be directly responsible for selective digit loss in *Hemiergis*.

Experimentally reduced mesenchyme proliferation in developing lizard limbs can induce patterns of digit loss remarkably comparable to those observed in natural populations of *Hemiergis* (Raynaud, '90). Because SHH stimulates limb mesenchyme proliferation through FGF expression (Lauffer et al., '94; Niswander et al., '94; Ohuchi et al., '97), premature downregulation of SHH signaling may effect digit loss in *Hemiergis* by curtailing normal cell proliferation. To test this tissue limitation hypothesis, we analyzed cell proliferation in stage 32 *H. quadrilineata* (2/2) and *H. peronii* (3/3 and 4/4) embryos by monitoring BrdU immunoreactivity (Fig 3). BrdU is incorporated into the DNA of limb mesenchyme during DNA synthesis; hence, increased BrdU incorporation indicates increased DNA synthesis, a precursor to cell division. In *H. quadrilineata* (2/2), little BrdU staining was observed in the posterior limbs. This low level of proliferation is correlated with the absence of SHH expression, which is extinguished in the forelimb by stage 31 and in the hind limb by stage 32. In *H. peronii* (3/3) cell proliferation and SHH expression are also low in the posterior aspects of the hind limb relative to *H. peronii* (4/4), in which BrdU staining is more intense. These findings are significant because digit V, whose phalanges do not form in *H. quadrilineata* (M; 2/2) and digit V of *H. peronii* (N; 3/3), forms in the posterior autopod. The relatively low proliferation of posterior mesenchyme in these two morphs—correlated with curtailed SHH expression—may be responsible for the loss of digit V phalanges. Based on Alcian blue staining of

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**Fig. 3.** MSX-1+2 immunoreactivity in embryonic stages 32–34 *Hemiergis*. (A, E, I, M) Stage 32 embryos of *H. quadrilineata* (A; 2/2), *H. peronii* (E; 3/3), *H. peronii* (I; 4/4), and *H. initialis* (M; 5/5) in right lateral view showing combined MSX-1 and -2 (MSX-1+2) protein expression. Immunoreactivity is detected not only in the forelimbs (fl) and hind limbs (hl), but also in the head, including the maxillary region (mx). Other panels depict limbs of stages 32–34 *Hemiergis* embryos. In panels with two limbs, forelimbs are on the left and hind limbs are on the right; anterior is to the left and distal is up. All limbs are shown in dorsal view. (B, F, J, N) Stage 32 forelimbs. MSX-1+2 immunoreactivity is detected in the autopodial distal ectoderm and mesenchyme in all morphs. Staining is also visible along the anterior edge of the limb proximal to the autopod (arrowheads in B). (C, G, K, O) Stage 33 hind limbs. MSX-1+2 immunoreactivity remains in the distal ectoderm and mesenchyme; additional expression is visible between the early digit condensations (arrowheads in C). (D, H, L, P) Stage 34 limbs. MSX-1+2 is still detected distal to the digits at this stage, and interdigital expression is expanded relative to stage 33. The quantities and placement of digits are clearly visible, and MSX-1+2 immunoreactivity characterizes autopod tissues that have not, or will not, form skeletal structures. Note that staining is excluded from the highly reduced digits II and V of *H. quadrilineata* (M; 2/2) and digit V of *H. peronii* (N; 3/3). Digit numbers are indicated by roman numerals. Scale bars = 1 mm in (N–P) and apply to all panels in their respective columns.
whole-mount and serially-sectioned limbs, digit I condensations are never observed in the developing limbs of *H. quadrilineata* (2/2) or *H. peronii* (3/3 and 4/4) (Shapiro, 2002), contrary to a model of digit loss in which all five digit precursors form but some are later destroyed (Galís et al., 2001). (However, we have not used peanut agglutinin, which may detect cryptic mesenchymal condensations; Kundrát et al., 2002; Larsson and Wagner, 2002.) Instead, we speculate that the loss of anterior structures may result from increased anterior apoptosis of mesenchyme due to insufficient SHH signaling (Lewis et al., '99; Drossopoulou et al., 2000; Sanz-Ezquerro and Tickle, 2000), increased Gli3 signaling in the anterior autopod (Litingtung et al., 2002; te Welscher et al., 2002), or both.

The present study also provides insight into the role of SHH in digit specification. In the developing mouse limb, digits may be transformed to more anterior identities by greatly lowering the
Fig. 6. Model depicting dual intervals of SHH action in digit specification in *Hemiergis*. Intense, early expression of SHH (shaded area) is critical for specification of digit identity in the developing limb. (A) Sustained high levels of SHH promote proliferation of limb mesenchyme and may prevent anterior apoptosis, resulting in a full complement of digits. (B) When SHH expression is curtailed during the proliferative interval, anterior and posterior elements fail to form, but the remaining digits maintain their posterior identities (digits II–V). (C) Digit specification depends on reaching a threshold of SHH expression intensity during the identity interval. When levels fall below this threshold, digits are “anteriorized” and reduced in number (see Lewis et al., 2001).

quantity—but not the timing—of SHH signaling (Lewis et al., 2001). Low, sustained levels of SHH are not sufficient to specify posterior digits. *Hemiergis* provides a complementary, natural experiment regarding SHH function in digit specification, in which the level of SHH expression remains constant but its duration is abbreviated. The interval of intense SHH expression is truncated in *Hemiergis* morphs with fewer complete digits, but digit identity is unaffected (Shapiro, 2002). For example, metatarsal 5 of all morphs—including those with no phalanges on digit V—retains the “hooked” morphology characteristic of lepidosaurs (Estes and Pregill, ’88). Other digits retain their identities as well: metacarpal 3 is at least as long as metacarpal 4 (Estes and Pregill, ’88) and metatarsal 4 has the broadest proximal epiphysis in all morphs, despite differences in phalangeal counts. Thus, early expression of SHH in all morphs appears to be sufficient to specify digits II–V.

Based on these observations, we propose a model in which thresholds of SHH concentration specify digit identity (Fig. 4). In this model, an early interval of high SHH expression is critical for digit specification as well as promoting proliferation, whereas relatively prolonged SHH expression may stimulate further proliferation in morphs with more complete autopodia. Consequently, morphs with persistent SHH expression have more digits, whereas truncated expression yields fewer, but properly identified, complete digits. This model differs from those proposed in earlier studies that suggest distinct early and late roles for SHH in determining digit quantity and identity, respectively (Drossopoulou et al., 2000). The action of SHH in determining digit quantity and identity in *Hemiergis* likely is intimately tied to the SHH-inhibitor role of GLI3, as has been demonstrated recently in mouse models of limb development (Aoto et al., 2002; Litingtung et al., 2002; te Welscher et al., 2002). Future studies of *Gli3* expression in *Hemiergis* will help elucidate the role of this gene in the evolution of limb reduction among natural populations of vertebrates. For example, it will be interesting to determine whether SHH is downregulated on its own, or in response to expanded expression of GLI3 or another inhibitory signal.

An important class of evolutionary changes in morphology involves alterations in developmental timing, or heterochronies (Alberch et al., ’79). Since skeletal elements within the limb form in a discrete sequence, hypotheses of developmental truncations have been put forward to explain evolutionary digit reductions (Essex, ’27; Müller, ’91). Such hypotheses posit that observed reductions in adult digit number are the result of truncation of the ancestral sequence of limb ontogeny. Detailed skeletal analyses of limb development in *Hemiergis*, however, reveal that digit loss does not result from truncation of a complete (five-digit) limb chondrogenesis sequence (Shapiro, 2002) (Fig. 1). Truncation of a putative ancestral chondrogenesis sequence in *Hemiergis* would produce incomplete digits, not fewer numbers of complete ones, and this was not seen. As predicted (but never tested) for other amniotes with similar reduction patterns (Shubin and Alberch, ’86; Greer, ’91), these observations in *Hemiergis* confirm that reduced adult limbs do not resemble intermediate morphologies of pentadactyl relatives (Shapiro and Carl, 2001; Shapiro, 2002). Therefore, at the level of the whole limb skeleton, limb reduction in *Hemiergis* cannot be explained by standard heterochronic methodology (Alberch, ’85). Alternate interpretations of heterochronies may be possible at other levels of analysis, such as segmentation of individual digits or bifurcations of the digital arch (see Müller, ’91; and Shapiro and Carl, 2001). At the level of SHH expression, however, our data are consistent with a hypothesis of paedomorphic heterochrony: the timing of SHH expression differs among the four
morphs of *Hemiergis*, but the biological role of SHH is likely conserved (Rice, '97). Our results are also consistent with Müller’s (‘91: p. 396) prediction that “very subtle changes” in the expression of important patterning molecules underlie the evolutionary transformation of the autopod.

A detailed knowledge of the evolution of developmental mechanisms can inform our understanding of the diversification of organisms. In this study, we show how duration of gene expression of a key signaling molecule is correlated with morphological variation among a group of closely related vertebrates. Molecular mechanisms of limb development are generally assumed to be highly conserved among tetrapods (but see (Christen and Slack, '98), yet actual tests of this assumption beyond the traditional chick, mouse, and frog models are rare (Cohn and Tickle, '99; Hanken et al., 2001). Reptiles comprise an extraordinarily diverse group of tetrapods with a multitude of limb morphologies, but they are the focus of few studies of limb development; this study is the first to investigate expression of patterning molecules during limb development in any lizard, and only the third in any reptile (Cohn and Tickle, '99; Loredo et al., 2001). Future studies comparing vertebrate development among natural populations will offer further opportunities to test hypotheses about evolutionary changes in development not easily addressed using traditional model systems.

ACKNOWLEDGMENTS

We are grateful to K. Aplin (Western Australian Museum) and M. Grounds (University of Western Australia) for laboratory space and logistic support; B. Maryan and R. Browne-Cooper (Western Australian Society of Amateur Herpetologists) for critical collecting assistance; E. Slonimsky for technical assistance; and members of the Hanken and Rosenthal laboratories for advice, protocols and helpful discussions. C. Tabin helped guide this project from its inception, and he, A. W. Crompton, F. A. Jenkins, Jr., D. Menke, R. Rountree, G. Wagner, and two anonymous reviewers provided useful comments on earlier drafts of the manuscript. A. P. McMahon kindly supplied the SHH antibody used in this study, and G. Panganiban and S. Carroll made available the DLX antibody to the Hanken lab.

LITERATURE CITED


