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Analysis of the expression and function of Wnt-5a and Wnt-5b in developing and regenerating axolotl (*Ambystoma mexicanum*) limbs

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Urodele amphibians are unique adult vertebrates because they are able to regenerate body parts after amputation. Studies of urodele limb regeneration, the key model system for vertebrate regeneration, have led to an understanding of the origin of blastema cells and the importance of positional interactions between blastema cells in the control of growth and pattern formation. Progress is now being made in the identification of the signaling pathways that regulate dedifferentiation, blastema morphogenesis, growth and pattern formation. Members of the Wnt family of secreted proteins are expressed in developing and regenerating limbs, and have the potential to control growth, pattern formation and differentiation. We have studied the expression of two non-canonical Wnt genes, *Wnt-5a* and *Wnt-5b*. We report that they are expressed in equivalent patterns during limb development and limb regeneration in the axolotl (*Ambystoma mexicanum*), and during limb development in other tetrapods, implying conservation of function. Our analysis of the effects of ectopic *Wnt-5a* expression is consistent with the hypothesis that canonical Wnt signaling functions during the early stages of regeneration to control the dedifferentiation of stump cells giving rise to the regeneration-competent cells of the blastema.

Key words: axolotl, dedifferentiation, limb, regeneration, Wnt.

Introduction

Urodele amphibians are unique among adult vertebrates in their remarkable ability to regenerate lost or damaged body parts, including their limbs. These animals provide the opportunity to understand the molecular basis of regeneration in a model system where regeneration occurs naturally, in order to guide therapeutic advances toward stimulating regeneration in higher mammals, including humans. One basic insight from such studies is that a critical step in regeneration involves the transition from the mature stump to the blastema that forms the new limb structures. The events that occur during this transition are collectively referred to as 'dedifferentiation' (see Gardiner 2005). Several signaling pathways have been implicated in the regulation of the cellular processes associated with dedifferentiation, and the key to inducing regeneration in humans lies in identifying the critical pathways and understanding how to manipulate them to induce a regenerative response. Wnt signaling pathways are among those that are likely to function in the control of limb regeneration (see Kawakami *et al*. 2006; Yokoyama *et al*. 2007).

Wnts are secreted proteins that control diverse developmental processes including limb development and regeneration (Dealy *et al*. 1993; Parr *et al*. 1993; Parr & McMahon 1995; Riddle *et al*. 1995; Kengaku *et al*. 1998; Kawakami *et al*. 1999; Yamaguchi *et al*. 1999; Barrow *et al*. 2003; Yang 2003; Kawakami *et al*. 2006; Stoick-Cooper *et al*. 2007; Yokoyama *et al*. 2007). Among the Wnts that are expressed during vertebrate development, *Wnt-3a*, *Wnt-5a*, *Wnt-5b* and *Wnt-7a* are expressed in early limb buds, and have been implicated in regulating growth and pattern formation of the proximodistal and dorsoventral axes

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(Gavin *et al*. 1990; Dealy *et al*. 1993; Parr & McMahon 1995; Riddle *et al*. 1995; Kengaku *et al*. 1998; Yang 2003). Functional analyses of *Wnt-5a* and *Wnt-5b* have demonstrated an important role in controlling growth and differentiation of the developing limb skeleton (Kawakami *et al*. 1999; Hartmann & Tabin 2000; Church *et al*. 2002). In the present study, we have investigated the temporal and spatial expression patterns of the axolotl (*Ambystoma mexicanum*) orthologues of *Wnt-5a* and *Wnt-5b* during limb development and regeneration. We have also used a vaccinia-based vector to ectopically express *Wnt-5a* in different regions of the regenerating limb blastema, and at different times during blastema formation and growth. The results from these functional studies are consistent with evidence of a role for canonical Wnt signaling during the critical events of dedifferentiation (Kawakami *et al*. 2006). Thus, Wnt signaling pathways are important targets for future therapies to induce regeneration in human limbs.

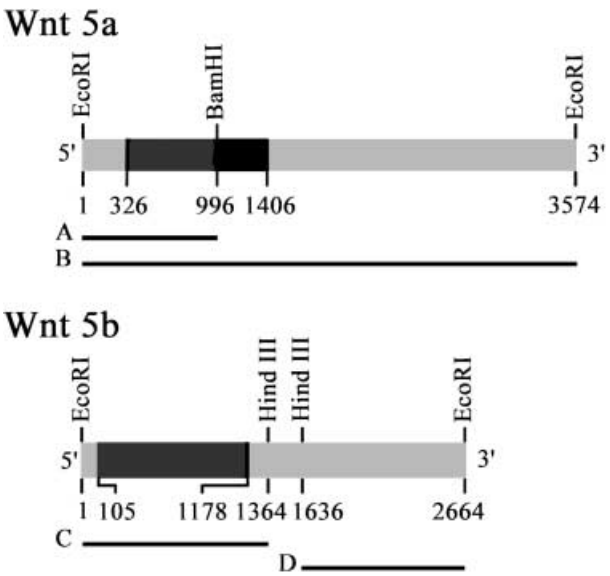

Fig. 1. Restriction map of axolotl *Wnt-5a* and *Wnt-5b* cDNAs. Regions indicated by B and D were used for Northern hybridization analyses for *Wnt-5a* and *Wnt-5b*, respectively. Regions indicated by A and D were used to generate antisense RNA probes for whole mount *in situ* hybridization analyses of *Wnt-5a* and *Wnt-5b*, respectively. Black boxed areas indicate the open reading frame (ORF) for each transcript.

Materials and methods

Animal procedures

Experiments were carried out on albino or white axolotls (*Ambystoma mexicanum*) that were spawned either at University of California, Irvine or at the Axolotl Colony, Indiana University (now the *Ambystoma* Genetic Stock Center, University of Kentucky, Lexington). Larvae were maintained at 20–22°C in 40% Holtfreter's solution. Animals measuring 5–6 cm snout to tail tip were used to generate blastemas for whole-mount *in situ* hybridization. Animals were anesthetized in a 0.1% solution of MS222 (Sigma, St. Louis, MO, USA) prior to amputation to induce regeneration, and for virus injection. Limbs were amputated either through proximal (mid-humerus or femur) or through distal (mid-radius/ulna or tibia/fibula) levels. For RNA isolation, blastemas were generated on animals measuring 10–15 cm snout to tail tip. The designation of regeneration stages was based on Tank *et al*. (1976), and the stages of limb bud development were identified based on the larval stages for *Ambystoma punctatum* described by Harrison (1969). Samples for skeletal preparations were fixed overnight in Bouin's fixative, and then processed as in Bryant & Iten (1974). X-gal staining was carried out according to published protocols (Miller 1972; Roy *et al*. 2000).

RNA isolation and northern hybridization

RNA isolation and northern hybridization were carried out as described in Gardiner *et al*. (1995). The amount of RNA loaded (10 μg per lane) was quantified spectrophotometrically, and normalized relative to the 18 s and 28 s ribosomal RNA bands visualized by either UV shadowing or ethidium bromide staining of the gels. Filters were probed with different fragments of the *Wnt-5a* and *Wnt-5b* clones, which yielded the same results (Fig. 1). The results reported below are based on the expression detected by the 3574 bp sequence (probe B) for *Wnt-5a*, and the 1028 bp 3′ non-coding sequence (probe D) for *Wnt-5b* (Fig. 1).

Whole-mount in situ hybridization

The procedure for whole-mount *in situ* hybridization was as described previously (Gardiner *et al*. 1995) with the following modifications. Conditions for proteinase K treatment were adjusted for each tissue: embryos, 10 μg/mL at 4°C for 30 min followed by 10–30 min at 25°C; limb blastemas, 30 μg/mL at 4°C for 30 min followed by 15–30 min at 37°C. The *Wnt-5a* digoxigenin probe (probe A, Fig. 1) was a 996 bp *Eco*RI/*Bam*HI fragment containing 5′ untranslated region (UTR) and part of the coding region (326–996 bp). The *Wnt-5b* probe (probe C, Fig. 1) was a 1364 bp *Eco*RI/Hind-III fragment containing 5′ UTR and part of the coding region (105–1178 bp) along with 186 bp of 3′ non-coding region. Both probes were hybridized to the tissues and were washed posthybridization at 67°C.

A specifically hybridized probe was localized by an Alkaline Phosphatase (AP)-conjugated antidigoxigenin secondary antibody reacted with BM purple (Roche Diagnostics Corp., Indianapolis, IN, USA), after which, samples were postfixed in neutral buffered formalin, dehydrated in methanol and photographed. After whole-mount *in situ* hybridization, some samples were rehydrated in phosphate-buffered saline (PBS), frozen in Optimal Cutting Temperature (OCT) compound, and cryosectioned at a thickness of 30–40 μm.

Generation and microinjection of Wnt-5a vaccinia virus

To analyze the function of *Wnt-5a* during axolotl limb regeneration, we cloned the full-length cDNA coding sequence of *Wnt-5a* into the vaccinia viral vector as detailed in Roy *et al*. (2000). In addition to the coding sequence, a c-myc epitope tag (10 amino acid sequence, EQKLISEEDL) was added to the 3′ end of the transgene. The *Wnt-5a* cDNA was amplified using a *Wnt-5a* specific 5′ primer (5′-CCACCATGGCCAC-CAC*GCACCTG*-3′) and a Wnt-5a/c-myc specific-3′ primer (5′-*CAGGTCCTCTTCGCTA* ATCAGCTTTTGTTC-CTTGCACACAAACTGGTCCAC-3′). The c-myc tagged sequences were then subcloned into a shuttle vector and packaged into viral particles as detailed previously (Roy *et al*. 2000).

For microinjection of the viral transgene, we used either albino or white axolotls in order to allow for the detection of the X-gal reaction product in the control injections. Multiple injections, resulting in a total injection volume of 1–2 μL of either control β-gal or experimental *Wnt-5a* virus (10^9 pfu/mL) were made into the mesenchymal compartment of blastemas at progressively later stages of regeneration. Regenerating limbs were injected at the stages of early dedifferentiation, early bud, medium bud, and late bud. In most experiments, injections were made into the central region of the blastema; however, in some experiments injections were made into either the dedifferentiating stump tissues at the base of the blastema or into the distal region of the blastema. We previously determined that the transgene (β-gal) is expressed over a large area of the blastema surrounding the injection site for a period of about 1 week (Roy *et al*. 2000).

Results

Analysis of Wnt-5a and Wnt-5b expression by northern hybridization

The axolotl orthologues of *Wnt-5a* and *Wnt-5b* were cloned several years ago by Busse & Seguin 1992). The axolotl *Wnt-5a* clone consisted of 3574 bp encoding an open reading frame (ORF) of 359 amino acids, and the axolotl *Wnt-5b* clone consisted of 2664 bp encoding an ORF of 357 amino acids. We subcloned both the genes into appropriate vectors in order to generate probes for northern hybridization and *in situ* hybridization analysis (Fig. 1).

Using northern hybridization analysis, we detected four *Wnt-5a* transcripts (hash marks in Fig. 2A) that were 5.0 kb, 4.2 kb, 3.0 kb and 1.5 kb in length. Expression of all four *Wnt-5a* transcripts was detected in both developing limb buds and regenerating limb blastemas (Fig. 2A), but none were detected in mature limbs (data not shown). *Wnt-5a* was expressed in both proximal and distal blastemas, beginning at dedifferentiation stages (LDD, late dedifferentiation) and continuing through the early digit stage, when re-differentiation had already begun (Fig. 2A). We manually separated the apical epidermal cap (AEC; Fig. 2A, MB-Ep) from the underlying blastema cells (Fig. 2A, MB-Mes) of medium bud blastemas, and observed that *Wnt-5a* was expressed at higher levels in the mesenchymal cells as compared with the AEC keratinocytes.

We detected a single 3.0 kb transcript of *Wnt-5b* that was expressed in both developing limb buds and regenerating limb blastemas (Fig. 2B). We did not detect *Wnt-5b* transcripts in mature limb tissues (data not shown). We could detect *Wnt-5b* expression in the mesenchyme of medium bud blastemas (Fig. 2C, MB-Mes), but not in the AEC (MB-Ep). *Wnt-5b* appears to be expressed at higher levels in proximal blastemas as compared with distal blastemas (Fig. 2D).

Analysis of Wnt-5a and Wnt-5b expression by in situ hybridization

During forelimb development, *Wnt-5a* expression was detected as early as stage H36 when the limb bud first grew distally from the flank tissues (Fig. 3A). Expression appeared stronger in the apical ectoderm and weaker in the distal mesenchyme. From the earliest stages it was detected, *Wnt-5a* expression was restricted to the distal regions of the limb bud (Fig. 3A–C). A similar spatial and temporal pattern of expression was observed in developing hindlimbs (data not shown).

Wnt-5b expression was also detected at early stages of forelimb bud outgrowth (Fig. 3D, stage H37), and was expressed predominantly in the mesenchyme until the end of development (Fig. 3E; H41, digit stage). *Wnt-5b* was also expressed in developing hind limbs (data not shown). *Wnt-5b*

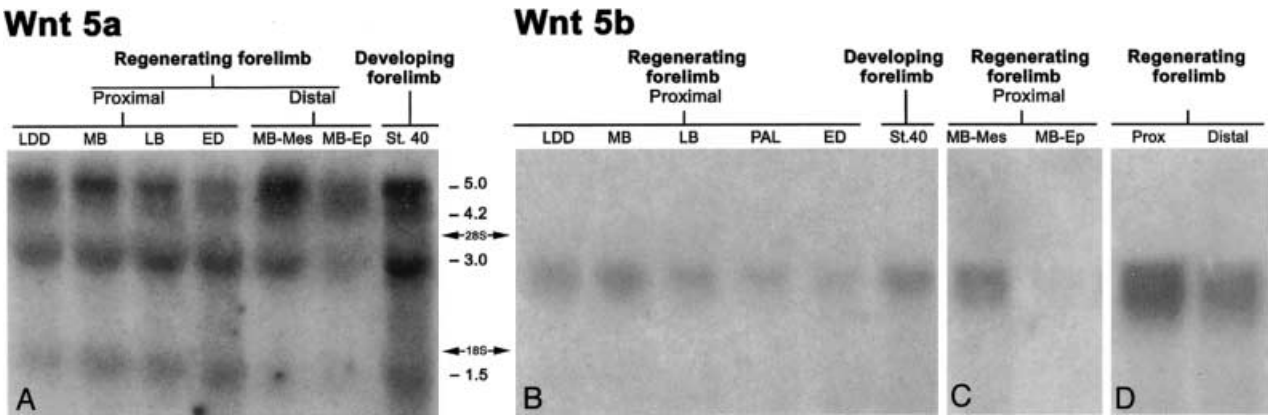

Fig. 2. Northern hybridization blots indicating the expression of *Wnt-5a* and *Wnt-5b* transcripts in developing and regenerating axolotl limbs. (A) Expression of *Wnt-5a* at progressively more advanced stages of regeneration from a proximal amputation (ED, early digits; LB, late bud; LDD, late dedifferentiation; MB, medium bud), in the mesenchyme of a distal medium bud blastema (MB-Mes), in the epidermis of a distal medium bud blastema (MB-Ep), and at stage 40 developing forelimbs. The positions of the four *Wnt-5a* transcripts (5 kb, 4.2 kb, 3 kb and 1.5 kb) are indicated by hash marks. (B) Expression of *Wnt-5b* at progressively more advanced stages of regeneration from a proximal amputation (abbreviations as in A, with the addition of a sample from the palette (Pal) stage of regeneration), and at stage 40 developing forelimbs. (C) Expression of *Wnt-5b* in the mesenchyme of a proximal medium bud blastema (MB-Mes) and in the epidermis of a proximal medium bud blastema (MB-Ep). (D) Expression of *Wnt-5b* in proximal compared to distal medium bud blastemas from a regenerating forelimb. The position of the single *Wnt-5b* transcript (3 kb) is indicated by a hash mark. The sizes of the transcripts were determined relative to the mobility of axolotl 28S rRNA and 18S rRNA as indicated between panels A and B. 10 μg of total RNA were loaded for each lane.

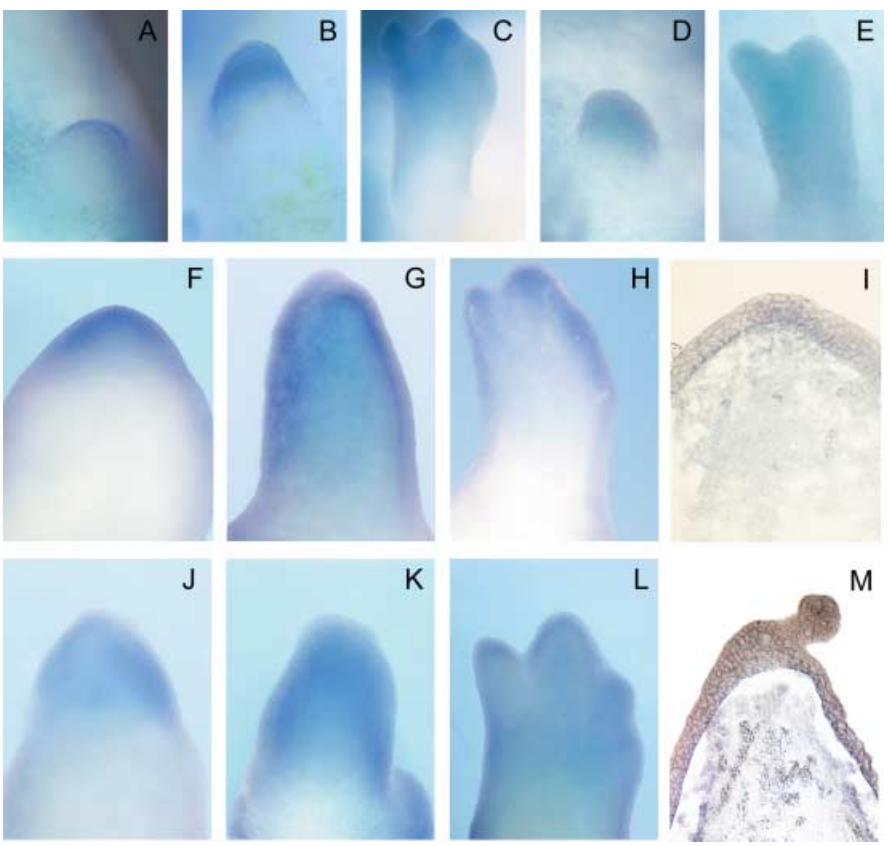

Fig. 3. Whole mount *in situ* hybridization analysis of *Wnt-5a* and *Wnt-5b* in developing and regenerating axolotl limbs. (A–C) Expression of *Wnt-5a* in developing forelimb at stages H36 (A), H38/39 (B) and H43(C). (D–E) Expression of *Wnt-5b* in developing forelimb at stages H37 (D) and H41 (E). (F–H) Expression of *Wnt-5a* in regenerating forelimbs at medium bud (F), palette (G), and early digit (H) stages after a distal amputation. (J–L) Expression of *Wnt-5b* in regenerating forelimbs at medium bud (J), palette (K), and early digit (L) stages after a distal amputation. (I, M) Cryosections of medium bud blastemas after *in situ* hybridization illustrating expression of *Wnt-5a* in the apical epidermis and mesenchyme (I) and expression of *Wnt-5b* in the apical mesenchyme (M). Specific reaction product appears blue, whereas, brown regions are non-specific discoloration resulting from tissue processing. All limbs are oriented with anterior to the left.

Table 1. Induction of hypomorphic regenerated limbs in response to injection of either control (Lac-Z) or experimental (*Wnt-5a*) vaccinia virus into progressively later stages of blastemas

Stage	Construct	Number of limbs	Normal	Hypomorph	Percentage hypomorph
Preblastema	Lac-Z	12	12	0	0
	Wnt-5A	32	6	26	81
Early bud blastema	Lac-Z	11	10	1	9
	Wnt-5A	22	12	10	45
Late bud blastema	Lac-Z	0	0	0	n/a
	Wnt-5A	9	8	1	11

expression was less distally restricted than *Wnt-5a* expression, and thus *Wnt-5b* was expressed at more proximal levels at each stage of limb bud development (compare Fig. 3A–C with D–E).

During limb regeneration, *Wnt-5a* was expressed in the distal region of the regenerating limbs. Expression was first detected 3 days after amputation, at which time stump cells were undergoing dedifferentiation, but was prior to formation of the blastema (data not shown). After the blastema had formed, *Wnt-5a* expression was restricted to the more distal regions (Fig. 3F) through to the late stages of regeneration (Fig. 3G,H), as was observed in developing limb buds. *Wnt-5a* expression in both the distal epidermis (AEC) and distal mesenchyme was confirmed in sections made after whole-mount *in situ* hybridization (Fig. 3I).

In contrast to *Wnt-5a*, we could not detect expression of *Wnt-5b* until a blastema had formed (Fig. 3J–L). Since we could detect *Wnt-5b* expression on Northern blots at the later stages of dedifferentiation, our inability to visualize expression at preblastema stages with whole-mount *in situ* hybridization presumably reflected differences in the levels of sensitivity of these two techniques. *Wnt-5b* expression appeared to be restricted to the mesenchymal compartment of the blastema, an observation consistent with the result from northern analysis (Fig. 2C) and further confirmed in sections made after whole-mount *in situ* hybridization (Fig. 3M).

Ectopic expression of Wnt-5a inhibited expression of Msx-2 and stump cell dedifferentiation in regenerating limbs

Ectopic expression of *Wnt-5a* in dedifferentiating stump cells and early blastema cells induced a hypomorphic phenotype at high frequency. We induced ectopic *Wnt-5a* expression by injecting recombinant vaccinia virus expressing axolotl *Wnt-5a* into regenerating limb tissues during dedifferentiation (prior to blastema formation), and during the early and late bud stages of blastema growth. Control injections of vaccinia virus expressing the lacZ gene resulted in normal regenerates in more than 95% of the limbs (Fig. 4A; Table 1, $n = 22$ of 23 regenerates). In contrast, nearly 60% of the *Wnt-5a* injected limbs exhibited the hypomorphic phenotype (Fig. 4B–F; Tables 1, $n = 37$ of 63 regenerates). The extent of inhibition of regeneration ranged from relatively mild, with shortened digits (Fig. 4B) to essentially a complete failure of regeneration (Fig. 4F). Both the frequency of hypomorphic regenerates, and the severity of the phenotype were greater when regenerating limbs were injected at earlier rather than later stages (Table 1).

Since ectopic *Wnt-5a* expression had the maximum effect during the earlier stages of regeneration when stump cells were dedifferentiating, and a much reduced effect at later stages when the blastema had formed and dedifferentiation had ceased (Tank 1977), we reasoned that dedifferentiating stump cells were the target of *Wnt-5a* misexpression. To test this hypothesis, we injected vaccinia-*Wnt-5a* into either the middle or distal regions of a medium bud blastema to transduce blastema cells that had already dedifferentiated, or at the junction between the stump and base of a medium bud blastema to transduce stump cells undergoing dedifferentiation prior to becoming blastema cells. Control injections resulted in normal regenerates in 90% of the limbs, regardless of the site of injection (Table 2). Similarly, vaccinia-*Wnt-5a* injections into central and distal blastema cells resulted in the regeneration of limbs with a normal phenotype (Tables 2, $n = 4$ of four limbs). In contrast, nearly all of the regenerating limbs that were injected at the junction between the stump and the base of the blastema ($n = 6$ of seven limbs) were inhibited, resulting in the hypomorphic phenotype (Table 2).

Since ectopic *Wnt-5a* expression appeared to inhibit dedifferentiation rather than blastema growth and pattern formation, we tested the effect of ectopic *Wnt-5a* on the expression of *Msx-2* which has been hypothesized to function in the regulation dedifferentiation during urodele limb regeneration, as does

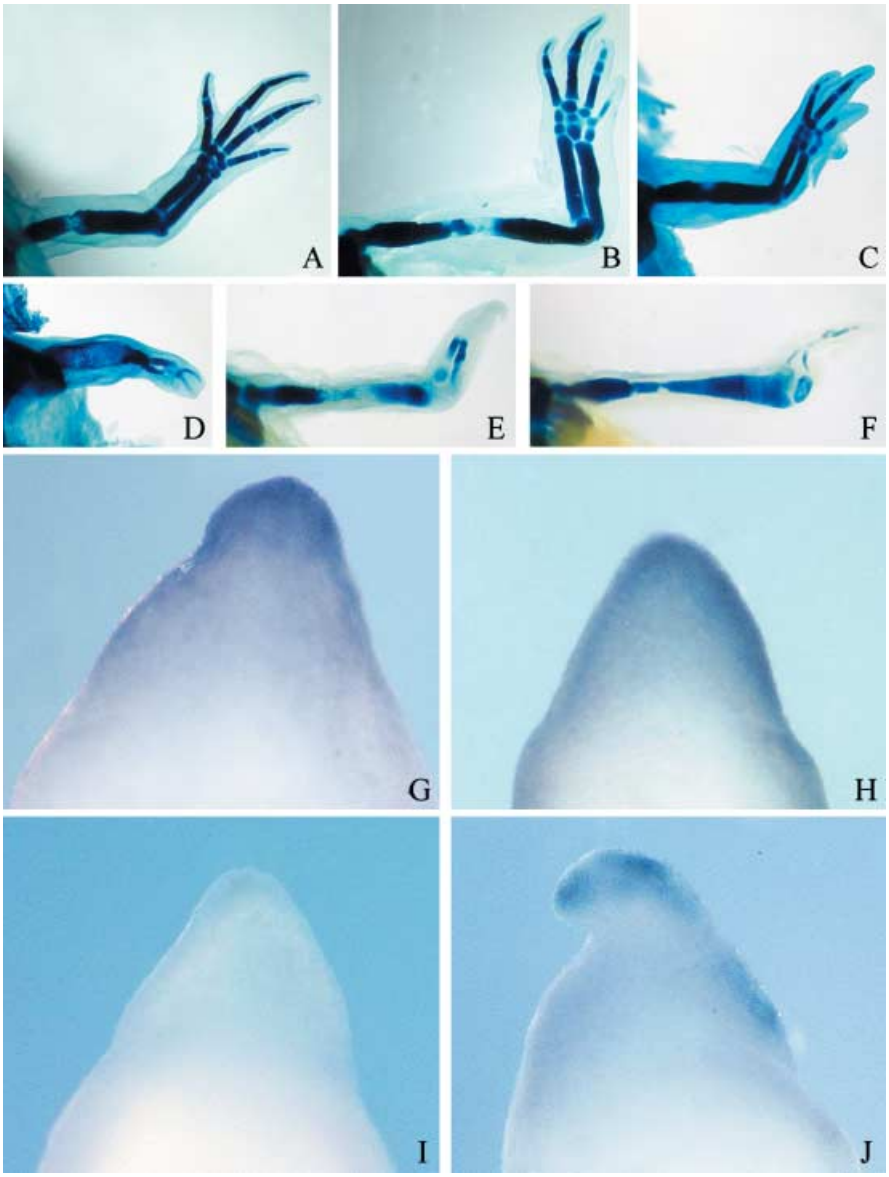

Fig. 4. Whole-mount, Victoria-blue stained skeletal preparations and *Msx-2* expression in regenerating blastemas injected with control or *Wnt-5a* vaccinia virus. (A) Skeletal preparation showing the normal limb pattern in control (Lac-Z vaccinia virus) injected limbs and (B–F) progressively more hypomorphic experimental (*Wnt-5a* vaccinia virus) limbs. (G) *Msx-2* expression in a control uninjected blastema, and (H) in a control (Lac-Z vaccinia virus) injected limb showing a normal pattern of *Msx-2* expression. (I) Lack of *Msx-2* expression, and (J) disrupted *Msx-2* expression in experimental (*Wnt-5a* vaccinia virus) injected limbs that result in the hypomorphic phenotypes illustrated in (B–F). Limbs and blastemas are oriented with anterior to the upper-left (A–F) or left (G–J).

Table 2. Induction of hypomorphic regenerated limbs in response to injection of either control (Lac-Z) or experimental (*Wnt-5a*) vaccinia virus into blastemas (mid to distal regions) or into dedifferentiating stumps (junction between the distal stump and the base of the blastema)

	Construct	Number of limbs	Normal	Hypomorph	Percentage hypomorph
Stump	Lac-Z	6	5	1	17
	Wnt-5A	7	1	6	86
Blastema	Lac-Z	4	4	0	0
	Wnt-5A	4	4	0	0

Msx-1 during mouse digit regeneration (Simon *et al*. 1995; Carlson *et al*. 1998; Koshiba *et al*. 1998; Han *et al*. 2003; Satoh *et al*. 2007). We injected either vaccinia-*Wnt-5a* virus or vaccinia-LacZ virus into early bud blastemas, and then collected samples when the control blastemas reached the medium bud stage of regeneration. We analyzed expression of *Msx-2* by whole-mount *in situ* hybridization as described

in Carlson *et al*. (1998). In uninjected blastemas (Fig. 4G) and control-injected limbs (Fig. 4H), *Msx-2* was expressed at high levels in both the distal epidermis and blastema mesenchyme (Carlson *et al*. 1998; Satoh *et al*. 2007). In contrast, ectopic *Wnt-5a* expression either completely inhibited *Msx-2* expression (Fig. 4I), or induced irregular and incomplete expression (Fig. 4J).

Discussion

Wnts are secreted proteins that control diverse developmental processes including limb development and regeneration (see Kawakami *et al*. 2006; Stoick-Cooper *et al*. 2007; Yokoyama *et al*. 2007). In general, limb development and regeneration are positively regulated by Wnt ligands that signal through the canonical, β-catenin mediated pathway (see Poss *et al*. 2000; Yang 2003; Kawakami *et al*. 2006; Stoick-Cooper *et al*. 2007). In contrast, *Wnt-5a* and *Wnt-5b* signal through a non-canonical pathway, and function to antagonize β-catenin-mediated signaling (Topol *et al*. 2003; Stoick-Cooper *et al*. 2007). In the present study we report on the expression of two closely related Wnt genes, *Wnt-5a* and *Wnt-5b* during axolotl limb development and regeneration, and the function of *Wnt-5a* during dedifferentiation of stump tissues in regenerating limbs. Given the conserved function of molecular signaling pathways, and our discovery that ectopic *Wnt-5a* expression induces a hypomorphic phenotype in regenerating axolotl limbs that is remarkably similar to what is observed when canonical Wnt signaling is inhibited directly (Kawakami *et al*. 2006), we hypothesize that ectopic *Wnt-5a* expression also inhibits canonical Wnt signaling. As discussed below, this hypothesis is consistent with recent reports in the axolotl, Xenopus, and zebrafish (see Kawakami *et al*. 2006; Stoick-Cooper *et al*. 2007; Yokoyama *et al*. 2007).

The spatial and temporal patterns of *Wnt-5a* and *Wnt-5b* expression in developing axolotl limb buds are comparable to what has been reported in chick and mouse limb buds (Dealy *et al*. 1993; Parr *et al*. 1993; Hartman & Tabin 2000). Similarly, expression in blastema stages of regenerating limbs is high distally, and low or undetectable proximally. Thus it appears that the regulation of expression is conserved during development between vertebrate species, as well as between developing and regenerating limbs in the axolotl. The conserved spatial and temporal regulation of *Wnt-5a* and *Wnt-5b* expression implies that the function of these two genes is also conserved, and that the function of Wnt signaling in blastema growth and pattern formation during regeneration recapitulates the conserved function during development (see Gardiner 2005).

Our understanding of the function of *Wnt-5a* and *Wnt-5b* comes largely from studies of developing limb buds in which both genes signal through non-canonical pathways (see Topol *et al*. 2003), and function as antagonists of canonical Wnt signaling (Topol *et al*. 2003; Westfall *et al*. 2003). In mice that are mutant for *Wnt-5a* signaling, canonical Wnt signaling is upregulated distally in the domain in which *Wnt-5a* would normally be expressed. Consistent with the observation that canonical Wnt signaling inhibits chondrogenesis (Rudnicki & Brown 1997), ectopic canonical Wnt signaling distally in *Wnt-5a*$^{-/-}$ mouse embryos inhibits mesenchymal condensation and chondrocyte differentiation, resulting in the loss of digits (Topol *et al*. 2003). We hypothesize that *Wnt-5a (*as well as *Wnt –5b*) has a similar function in axolotl limb buds and blastemas in which it is expressed distally where it would inhibit canonical Wnt signaling, and thus allows for the differentiation of the distal limb structures.

Recent findings have provided evidence for the importance of Wnt signaling during regeneration in the axolotl, *Xenopus* and zebrafish. Inhibition or enhancement of canonical Wnt signaling either inhibited or enhanced a regenerative response, respectively (Kawakami *et al*. 2006; Stoick-Cooper *et al*. 2007; Yokoyama *et al*. 2007). Of particular relevance to our study is the demonstration that overexpression of *Wnt-5b* reduced expression of Wnt/β-catenin target genes and inhibited fin ray regeneration in the zebrafish (Stoick-Cooper *et al*. 2007). Similarly, inhibition of canonical Wnt signaling by ectopic expression of either Axin1 or Dkk1 (both inhibitors of the canonical Wnt pathway) during axolotl limb regeneration resulted in a symmetrical, hypomorphic phenotype that is comparable to what we have observed in response to ectopic *Wnt-5a* expression (Kawakami *et al*. 2006). An equivalent hypomorphic response was observed when Wnt signaling was inhibited in regenerating *Xenopus* limb buds (Kawakami *et al*. 2006; Yokoyama *et al*. 2007). Given that ectopic expression of *Wnt-5a* inhibited regeneration in axolotl limbs as it does in other systems, we hypothesize that the mechanism of inhibition involves the inhibition of canonical Wnt signaling, which is consistent with the previous data indicating that Wnt signaling has a critical functional role in limb regeneration.

In the present study, we provide data for a novel, specific function for Wnt signaling in the regulation of the earliest events of regeneration involving dedifferentiation and blastema morphogenesis. The highest frequency and severity of inhibition of

regeneration was observed when *Wnt-5a* was ectopically expressed during the early stages of regeneration, and at the junction between the stump and the base of blastema (dedifferentiating stump cells). In contrast, when *Wnt-5a* was ectopically expressed during later blastema stages, or in the distal cells of earlier blastema stages, regeneration was normal. Although cell proliferation is obviously inhibited in the hypomorphic regenerates, it is unlikely that *Wnt-5a* directly inhibits proliferation since central and distal blastema cells proliferate and regenerate normally whether or not they are transduced with the *Wnt-5a* transgene. Thus, we hypothesize that the observed inhibition of proliferation of cells at the stump-blastema junction is a secondary response associated with the inhibition of dedifferentiation, which would prevent the genesis of the population of proliferating blastema cells.

There are multiple, potential downstream targets if Wnt signaling functions early in the regeneration cascade (stump cell dedifferentiation), and there are multiple signaling pathways that are known to be regulated by Wnt signaling. Since canonical Wnt signaling can function upstream of Fibroblast Growth Factor (FGF) signaling (see Yokoyama *et al*. 2007), it may function through regulating the function of nerves and/or the wound epidermis, both of which are sources of FGF signaling and are required for limb regeneration (see Mullen *et al*. 1996). If Wnt-dependent FGF signaling is being targeted, it presumably functions during the early stages of regeneration associated with dedifferentiation and dermal cell migration (see Endo *et al*. 2004). Since ectopic *Wnt-5a* expression did not result in any evident phenotypic response in later stage blastemas, it is unlikely that FGF-mediated functioning of the apical epidermis during these later stages is disrupted.

Msx-mediated pathways are a second category of potential targets of early Wnt signaling. *Msx* expression is associated with repression of the differentiated phenotype and the induction of dedifferentiation in a number of developing and regenerating systems, including regenerating salamander limbs and mouse digits (see Han *et al*. 2003; Satoh *et al*. 2007). In the present study, we observed inhibition of *Msx-2* expression in association with the inhibition of regeneration by ectopic *Wnt-5a* expression. This finding provides evidence not only for *Msx* as a downstream target of Wnt signaling during limb regeneration, but also for the importance of *Msx* expression in dedifferentiation of stump cells leading to the formation of blastema cells. Given that *Wnt-5a* normally functions in the distal region of the blastema, the inhibitory effect of ectopic *Wnt-5a* expression likely disrupts the ability of proximal stump cells to interact with distal cells in order to stimulate intercalary growth and pattern formation leading to complete regeneration (see Gardiner & Bryant 1996; Agata *et al*. 2007).

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References

Agata, K., Saito, Y. & Nakajima, E. 2007. Unifying principles of regeneration I: Epimorphosis versus morphallaxis. *Dev. Growth Differ*. **49**, 73–78.

Barrow, J. R., Thomas, K. R., Boussadia- Zahui, O. *et al*. 2003. Ectodermal Wnt3/β-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. *Genes Dev*. **17**, 394–409.

Bryant, S. V. & Iten, L. E. 1974. The regulative ability of the limb regeneration blastema of *Notophthalmus viridescens*: Experiments *in situ*. *Roux's Arch. Dev. Biol*. **174**, 90–101.

Busse, U. & Seguin, C. 1992. Isolation of cDNAs for two closely related members of the axolotl Wnt family, Awnt-5A and Awnt-5B, and analysis of their expression during development. *Mech. Dev*. **40**, 63–72.

Carlson, M. R. J., Bryant, S. V. & Gardiner, D. M. 1998. Expression of Msx-2 during development, regeneration, and wound healing in axolotl limbs. *J. Exp. Zool*. **282**, 715–723.

Church, V., Nohno, T., Linker, C., Marcelle, C. & Francis-West, P. 2002. Wnt signaling during limb development. *J. Cell. Sci*. **115**, 4809–4818.

Dealy, C. N., Roth, A., Ferrari, D., Brown, A. M. & Kosher, R. A. 1993. Wnt-5a and Wnt-7a are expressed in the developing chick limb bud in a manner suggesting roles in pattern formation along the proximodistal and dorsovental axes. *Mech. Dev*. **43**, 175–186.

Endo, T., Bryant, S. V. & Gardiner, D. M. 2004. A stepwise model system for limb regeneration. *Dev. Biol*. **270**, 135–145.

Gardiner, D. M. 2005. Ontogenetic decline of regenerative ability and the stimulation of human regeneration. *Rejuvenation Res*. **8**, 141–153.

Gardiner, D. M., Blumberg, B., Komine, Y. & Bryant, S. V. 1995. Regulation of HoxA expression in developing and regenerating axolotl limbs. *Development* **121**, 1731–1741.

Gardiner, D. M. & Bryant, S. V. 1996. Molecular mechanisms in the control of limb regeneration: the role of homeobox genes. *Int J Dev Biol* **40**, 797–805.

Gavin, B. J., McMahon, A. P. & McMahon, A. P. 1990. Expression of multiple novel Wnt-1/int-1 related genes during fetal and adult mouse development. *Genes Dev*. **4**, 2319–2332.

Han, M., Yang, X., Farrington, J. E. & Muneoka, K. 2003. Digit regeneration is regulated by Msx1 and BMP4 in fetal mice. *Development* **130**, 5123–5132.

Harrison, R. G. 1969. *Organization and Development of the Embryo*. Yale University Press, New Haven, CT.

Hartmann, C. & Tabin, C. 2000. Dual roles of Wnt signaling during chondrogenesis in chicken limb. *Development* **127**, 3141–3159.

Kawakami, Y., Rodriguez E. C., Raya, M. et al. 2006. Wnt/β-catenin signaling regulates vertebrate limb regeneration. Genes Dev. 20, 3232–3237.

Kawakami, Y., Wada, N., Nishimatsu, S. L., Ishikawa, T., Noji, S. & Nohno, T. 1999. Involvement of Wnt-5a in chondrogenic pattern formation in the chick limb bud. *Dev. Growth Differ*. **41**, 29–40.

Kengaku, M., Capedevilla, J., Rodriguez-Esteban, C. *et al*. 1998. Distinct WNT pathways regulating AER formation and dorso-ventral polarity in the chick limb bud. *Science* **280**, 1274–1277.

Koshiba, K., Kuroiwa, A., Yamamoto, H., Tamura, K. & Ide, H. 1998. Expression of *Msx* genes in regenerating and developing limbs of axolotl. *J. Exp. Zool*. **282**, 703–714.

Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mullen, L. M., Bryant, S. V., Torok, M. A., Blumberg, B. & Gardiner, D. M. 1996. Nerve dependency of regeneration: the role of *Distal-less* and FGF signaling in amphibian limb regeneration. *Development* **122**, 3487–3497.

Parr, B. A. & McMahon, A. P. 1995. Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. *Nature* **374**, 350–353.

Parr, B. A., Shea, M. J., Vassileva, G. & McMahon, A. P. 1993. Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* **119**, 247–261.

Poss, K. D., Shen, J. & Keating, M. T. 2000. Induction of Lef1 during zebrafish fin regeneration. *Dev. Dyn*. **219**, 282–286.

Riddle, R. D., Ensini, M., Nelson, C., Tsuchida, T., Jessell, T. M. & Tabin, C. 1995. Induction of LIM homeobox gene Lmx1 by WNT 7a establishes dorsovental pattern in vertebrate limb. *Cell* **83**, 631–640.

Roy, S., Gardiner, D. M. & Bryant, S. V. 2000. Vaccinia as a tool for functional analysis in regenerating limbs: ectopic expression of Shh. *Dev. Biol*. **218**, 199–205.

Rudnicki, J. A. & Brown, A. M. 1997. Inhibition of chondrogenesis by Wnt gene expression in vivo and in vitro. *Dev. Biol*. **185**, 104–118.

Satoh, A., Gardiner, D. M., Bryant, S. V. & Endo, T. 2007. Nerve-induced ectopic limb blastemas in the axolotl are equivalent to amputation-induced blastema. *Dev. Biol*. **312**, 231–244.

Simon, H. G., Nelson, C., Goff, D., Laufer, E., Morgan, B. A. & Tabin, C. 1995. Differential expression of myogenic regulatory genes and *Msx-1* during dedifferentiation and redifferentiation of regenerating amphibian limbs. *Dev. Dyn*. **202**, 1–12.

Stoick-Cooper, C., Weidinger, G., Riehle, K. J. *et al*. 2007. Distinct Wnt signaling pathways have opposing roles in appendage regeneration. *Development* **134**, 478–489.

Tank, P. W. 1977. The timing of morphogenetic events in the regenerating forelimb of the axolotl, *Ambystoma mexicanum*. *Dev. Biol*. **57**, 15–32.

Tank, P. W., Carlson, B. M. & Connelly, T. G. 1976. A staging system for forelimb regeneration in the axolotl, Ambystoma mexicanum. *J. Morph*. **150**, 117–128.

Topol, L., Jianng, X., Cho, H., Garrett-Beal, L., Carolan, P. J. & Yang, Y. 2003. Wnt 5a inhibits the canonical Wnt pathway by promoting GSK-3 independent beta catenin degradation. *J. Cell. Biol*. **162**, 899–908.

Westfall, T. A., Brimeyer, R., Twedt, J. *et al*. 2003. Wnt-5/pipetail functions in vertebrate axis formation as a negative regulator of Wnt/beta-catenin activity. *J. Cell Biol*. **162**, 889–898.

Yamaguchi, T. P., Bradley, A., McMahon, A. P. & Jones, S. 1999. A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development* **126**, 1211–1223.

Yang, Y. 2003. Wnts and wing: Wnt signaling in vertebrate limb development and musculoskeletal morphogenesis. *Birth Defects Res*. **69**, 305–317.

Yokoyama, H., Ogino, H., Stoick-Cooper, C. L., Grainger, R. M. & Moon, R. T. 2007. Wnt/b-catenin signaling has an essential role in the initiation of limb regeneration. *Dev. Biol*. **306**, 170–178.