Wnt/β-catenin signaling has an essential role in the initiation of limb regeneration

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Abstract

Anuran (frog) tadpoles and urodeles (newts and salamanders) are the only vertebrates capable of fully regenerating amputated limbs. During the early stages of regeneration these amphibians form a “blastema”, a group of mesenchymal progenitor cells that specifically directs the regrowth of the limb. We report that wnt-3a is expressed in the apical epithelium of regenerating Xenopus laevis limb buds, at the appropriate time and place to play a role during blastema formation. To test whether Wnt/β-catenin signaling is required for limb regeneration, we created transgenic X. laevis tadpoles that express Dickkopf-1 (Dkk1), a specific inhibitor of Wnt/β-catenin signaling, under the control of a heat-shock promoter. Heat-shock immediately before limb amputation or during early blastema formation blocked limb regeneration but did not affect the development of contralateral, un-amputated limb buds. When the transgenic tadpoles were heat-shocked following the formation of a blastema, however, they retained the ability to regenerate partial hindlimb structures. Furthermore, heat-shock induced Dkk1 blocked fgf-8 but not fgf-10 expression in the blastema. We conclude that Wnt/β-catenin signaling has an essential role during the early stages of limb regeneration, but is not absolutely required after blastema formation.

Keywords: Xenopus laevis; Limb; Regeneration; Wnt/β-catenin signaling; Transgenic; fgf-8, fgf-10

Introduction

All tetrapod limbs are thought to have evolved from paired fins of fish, to employ conserved mechanisms of development that originated with fins, and generally to have a common skeletal pattern (Tamura et al., 2001). Intriguingly, the regenerative responses of limbs after amputation are quite different between species. Animals such as mammals, birds, and lizards cannot restore lost limbs but instead merely undergo a wound healing response. In contrast, urodele amphibians such as newts and salamanders can regenerate their amputated limbs, while anuran amphibians are intermediate between urodele amphibians and other vertebrates in terms of their regenerative capacity. Xenopus laevis can completely regenerate developing hindlimb buds prior to the onset of metamorphosis, but the regenerative capacity declines gradually as metamorphosis proceeds (Dent, 1962; Muneoka et al., 1986).

In both urodele and anuran amphibians, limb regeneration progresses through a characteristic series of steps, beginning with wound healing, followed by formation of the blastema, and finally a redevelopment phase (Bryant et al., 2002; Gardiner et al., 2002; Han et al., 2005). Although the redevelopment stage of limb regeneration is thought to be equivalent to limb development, the early steps that result in the genesis of the blastema are critical in determining whether an amputated limb can successfully regenerate or whether it will undergo wound healing without regeneration. Considering the highly conserved mechanisms of limb development and conserved limb skeletal pattern among tetrapods, it is possible that elucidation of critical factor(s) important for blastema formation in regenerating limbs would provide insights into the mechanisms of limb regeneration.
amphibian limbs will contribute to development and improvement of tissue and organ replacement therapies (Stocum, 1997; Brockes and Kumar, 2005).

Based on the known roles for Wnt/β-catenin signaling during limb development (reviewed by Yang, 2003), we hypothesized that this signaling pathway might play an essential role in limb regeneration. Specifically, Wnt/β-catenin signaling is involved in the initiation of chick limb development and zebrafish pectoral fin formation, by inducing fgf-10 expression in the presumptive limb and fin region, respectively (Kawakami et al., 2001; Ng et al., 2002). In chick and mouse embryos, Wnt/β-catenin signaling also has an essential role in the formation of a specialized ectodermal structure, the apical ectodermal ridge (AER) in the limb buds, through induction of fgf-8 expression (Kengaku et al., 1998; Barrow et al., 2003; Soshnikova et al., 2003). The feedback loop between FGF-10 and FGF-8 is well known to be crucial for the outgrowth of the developing limb buds of chick (Ohuchi et al., 1997; Xu et al., 1998). Similarly, several recent studies indicate that both fgf-10 and fgf-8 are expressed in Xenopus and axolotl limb blastemas suggesting a crucial role in limb regeneration as well (Christen and Slack, 1997; Christensen et al., 2001, 2002; Endo et al., 2000; Han et al., 2001; Suzuki et al., 2005; Yokoyama et al., 2000, 2001).

Considering the essential roles of both pathways in the earliest regenerative steps, it is reasonable to hypothesize that Wnt/β-catenin signaling may serve to control in the initiation of limb regeneration by regulating downstream β-catenin signaling (Glinka et al., 1998–2000; Han et al., 2001; Suzuki et al., 2005; Yokoyama et al., 2000, 2001).

Functional analysis of genes and signaling pathways that might participate in regeneration has been hindered by the difficulty of manipulating gene function in postembryonic amphibians. However, the recent development of a transgenic system in Xenopus enables us to manipulate regeneration in anuran amphibians. To test the functional importance of Wnt signaling in regeneration we engineered X. laevis that were transgenic for heat-shock-inducible Dickkopf-1 (Dkk1), a secreted inhibitor of Wnt/β-catenin signaling (Glinka et al., 1998; Mao et al., 2001). By inducing this transgene at different time points during limb regeneration, we provide data establishing that Wnt/β-catenin signaling is required for limb regeneration.

Materials and methods

Animal husbandry

X. laevis were obtained from Nasco (Fort Atkinson, WI, USA). Tadpoles were kept in dechlorinated tap water containing 59 g Instant Ocean Sea Salt (Aquarium System, INC., Mentor, OH, USA)/l at 21–23 °C, staged according to Nieuwkoop and Faber (1994), and fed with spirulina (Salt Creek, Inc.). At stage 58, the feeding was stopped until metamorphosis was completed.

DNA constructs and in situ hybridization

mmGFP5 (Siemering et al., 1996) was fused to the C-terminus of zebrafish Dkk-1 (GenBank Accession No. AB023488). The Dkk1GFP5 fusion was then cloned downstream of the CMV promoter of the vector pC52+ (CMV–Dkk1GFP5; Fig 1A). For the negative control, a plasmid in which only mmGFP5 is expressed under control of the CMV promoter was prepared (CMV–GFP5; Fig. 1A). For preparation of transgenic tadpoles, the Dkk1GFP5 was cloned downstream of the Xenopus hsp70 promoter (Wheeler et al., 2000; Hsp70–Dkk1GFP5; Fig. 2B).

Preparation of Dig-labeled wnt-3a (Wolda et al., 1993), fgf-8 (Yokoyama et al., 1998), fgf-10 (Yokoyama et al., 2000), Lmx-1 (Matsuda et al., 2001), Hoxa-13 (Endo et al., 2000) and mnx-2 (unpublished) probes and in situ hybridization were performed as described previously (Endo et al., 1997). For making serial cryosections, specimens were fixed in MEMFA, dehydrated with 30% sucrose/PBS, embedded in OCT compound (Sakura), and serially sectioned at a 12 μm thickness. Transcripts were detected by in situ hybridization on frozen sections using procedures described by Yoshida et al. (1996) with slight modifications.

Luciferase assays

A total of 25 pg Super8× TOPFlash DNA (Veeman et al., 2003) together with 4 pg pKlu-N1 (h) DNA (renilla reniform as the luciferase internal control; BioSignal Packard) was injected into two dorsal cells of four-cell-stage embryos. Three replicate samples each of four embryos were frozen for each group at late gastrula (st. 12.5) and luciferase assays were performed using the Promega luciferase assay system according to Tao et al. (2005) with slight modifications.

Transgenis in X. laevis

Transgenic X. laevis embryos were generated by the REMI technique as previously described (Offield et al., 2000). To minimize potential leakiness of the transgene under the hsp70 promoter, embryos were reared at 16 °C in 0.1× MMR (Wheeler et al., 2000) until tadpoles started swimming and feeding, then reared in 21–23 °C.

For heat-shocking, tadpoles were placed in water warmed to 34 °C for 30 min as described by Beck et al. (2003). At 3 to 4 h after heat-shocking, tadpoles were examined under a fluorescent dissecting microscope and classified as GFP positive (hsDkk1GFP) or GFP negative (wild-type). Tadpoles with mosaic expression patterns of GFP, or that did not show GFP fluorescence 3 to 4 h after heat-shocking but showed weak GFP the next day were excluded from the experiment.

Tadpole surgery

Tadpoles were anesthetized in 1:5000 ethyl-3-aminobenzoate (Sigma–Aldrich) dissolved in Holtfreter’s solution. Left hindlimb buds were amputated at the presumptive knee level (according to the outside view and a fate map by Tschumi, 1957) with an ophthalmologic scalpel. After metamorphosis was completed, the cartilage pattern of amputated limbs was examined under a dissecting microscope to evaluate limb regeneration. If necessary, the limbs were stained with Alcian blue as described previously (Yokoyama et al., 2000). For in situ hybridization on sections of transgenic F0 tadpoles, both left and right hindlimb buds were amputated at the presumptive knee level.

Results and discussion

Heat-shock inducible inhibition of Wnt/β-catenin signaling in X. laevis

Our primary goal was to test the hypothesis that Wnt signaling is required for limb regeneration. To address this question we created transgenic Xenopus tadpoles that allowed us to inducibly inhibit endogenous Wnt/β-catenin signaling by overexpression of Dickkopf-1. Since a heat-shock-inducible
A transgenic line for GFP-tagged Dickkopf-1 (hsDkk1GFP) can efficiently inhibit Wnt/β-catenin signaling in zebrafish (Stoick-Cooper et al., 2007), we used the same Dkk1GFP clone in Xenopus. After confirming that this fusion protein inhibits Wnt/β-catenin signaling in Xenopus embryos (Fig. 1), we cloned it downstream of the Xenopus hsp70 promoter (Fig. 2B). This Hsp70-Dkk1GFP (hsDkk1GFP) construct was then used to generate transgenic F0 animals.

As reported by Wheeler et al. (2000), no transgene expression under control by the hsp70 promoter was detected in transgenic animals during embryonic stages when embryos were kept at 16 °C (data not shown), and under these conditions the embryos developed normally. Once embryos reached tadpole stages, leakiness of the transgene was not observed even at higher temperatures (21–23 °C). Suggesting that basal expression of the transgene was very low prior to heat-shock, we observed no fluorescence of Dkk1GFP in these transgenic tadpoles at rearing temperatures (21–23 °C; Fig. 2B inset). Establishing that the transgene was indeed induced by heat-shock, ubiquitous expression of Dkk1GFP was induced in F0 tadpoles 3 to 4 h following a 30-min heat-shock at 34 °C (Fig. 2B; 24% of total F0 tadpoles). Because of the random insertion of transgenes into Xenopus genomes by the REMI transgenic procedure (Kroll and Amaya, 1996), some F0 tadpoles did not express the transgene hence they were used as matched sibling negative controls (wild-type). The fluorescence of Dkk1GFP reaches a peak the day after heat-shock and persists for several days in transgenic F0 tadpoles (data not shown). Ambiguous tadpoles that did not show GFP fluorescence 3 to 4 h after heat-shock but showed weak GFP the following day were excluded from the experiment.

Wnt/β-catenin signaling is required for the early stages of limb regeneration

We used stage 52 hindlimb buds as they consistently regenerate complete hindlimbs after amputation at the presumptive

Fig. 1. Dkk1GFP suppresses the activity of the β-catenin responsive reporter SuperTOPFlash in frog embryos. (A) Schematic representation of the injected constructs. Designs of constructs are described in Materials and methods. (B) SuperTOPFlash reporter activation after injection of 250 pg CMV–GFP5 was compared to the activation occurring after injection of 250 pg CMV–Dkk1GFP5 with or without co-injection of 250 pg Xenopus CMV–Wnt3a DNA. Dkk1GFP5 suppressed both the endogenous activity of SuperTOPFlash (left two lanes) and the Wnt-3a-induced activation of SuperTOPFlash (right two lanes) in embryos. Firefly luciferase activity of the SuperTOPFlash reporter was normalized to renilla luciferase control activity. Error bars indicate the standard deviation from the mean (n=3).
Fig. 2. Wnt/β-catenin signaling is required for *Xenopus* limb regeneration. (A) Experimental scheme. Hindlimb buds of F0 tadpoles were amputated at the presumptive knee level (amp: represented as blue square). One heat-shock (hs: represented as red circle) was applied to tadpoles at 3 to 4 h prior to amputation (yellow line), 3 dpa (days post amputation; green line) or 5 dpa (blue line). (B) Map of the heat-shock inducible Dkk1GFP transgene. Details are described in Materials and methods. Expression of Dkk1GFP was induced in a transgenic tadpole carrying this transgene within 3 to 4 h after heat-shock (left panel, bright field; right panel, GFP). No GFP expression was detected in the same tadpole before heat-shock (inset). (C) Live limb buds were photographed when tadpoles were heat-shocked (st. 52–53; a–d). The same amputated limb buds were photographed again when regenerated limbs became obvious in controls (st. 57; e–h). A wild-type tadpole heat-shocked prior to amputation regenerated the amputated limb bud completely (a and e). While the hsDkk1GFP tadpoles heat-shocked prior to amputation (b and f) or at 3 dpa (c and g) failed to regenerate, hsDkk1GFP tadpoles heat-shocked at 5 dpa regenerated incomplete hindlimbs (d and h). Note that un-amputated right limb buds developed normally (black arrows). Arrowheads show the presumptive knee level (amputation level). Scale bars: 500 µm.
knee level (Dent, 1962; Yokoyama et al., 2000). We heat-shocked F0 tadpoles at stage 52 and then amputated their left hindlimb buds 3 to 4 h after heat-shock (Fig. 2A; yellow line). While 69% of wild-type F0 tadpoles regenerated hindlimbs completely (Figs. 2C-a, e and 3A), none of the hsDkk1GFP F0 tadpoles showed complete regeneration and only 18% showed partial regeneration (Figs. 2C-b, f and 3B, see Fig. 3 for n values). Interestingly, un-amputated right limb buds of the hsDkk1GFP tadpoles developed normally after heat-shock (see black arrows in Fig. 2C). Therefore, Wnt/β-catenin signaling is required for limb regeneration but not for limb development at this stage. Furthermore, the normal development of the matched right limb bud controls excludes the possibility that the Dkk1GFP transgene has nonspecific inhibitory effects on limb outgrowth.

To test for the requirement of Wnt/β-catenin signaling during subsequent phases of regeneration, left hindlimb buds of stage 52 F0 tadpoles were amputated at the presumptive knee level and heat-shocked following amputation, once at 3 dpa (days post amputation; Fig 2A; green line) or once at 5 dpa (Fig. 2A; blue line). At 3 dpa, the blastema is small, the reorganizing mesenchymal cells are in the process of accumulating and the overlying apical epithelium already appears thickened (see sections, Figs. 4C, F). When the F0 tadpoles were heat-shocked at 3 dpa, some regeneration response occurred in only 17% of the hsDkk1GFP tadpoles, compared with 84% in wild-type controls (Figs. 2C-c, g and 3A, B). Heat-shock induction of Dkk1GFP during apical epithelial thickening and early blastema formation reveals the requirement for Wnt signaling for regeneration at this stage.

By 5 dpa, a cone-shaped blastema is formed. When heat-shocked at 5 dpa, 64% of the hsDkk1GFP tadpoles regenerated at least partially, compared with 89% in wild-type controls (the hsDkk1GFP tadpoles regenerated two digits at best; Figs. 2C-d, h and 3A, B). This result indicates that Wnt/β-catenin signaling is important, but not absolutely required for limb regeneration at

Fig. 3. Percentage of wild-type and hsDkk1GFP tadpoles displaying varying degrees of regenerative responses after heat-shock as described in Fig. 1. (A) wild-type tadpoles. (B) hsDkk1GFP tadpoles. Regenerative capacity was evaluated by the number of regenerated digits.
this time-point. It is important to note that heat-shock itself at 5 dpa may have a slightly negative effect on regeneration. While about 70% of wild-type tadpoles heat-shocked before amputation or at 3 dpa regenerated completely (with 4 to 5 digits), only 40% of wild-type tadpoles heat-shocked at 5 dpa regenerated fully (Fig. 3A).

**Wnt-3a is a candidate for regulating Wnt/β-catenin signaling in limb regeneration**

Considering the inhibitory mechanism by which Dkk1 acts on Wnt/β-catenin signaling (Mao et al., 2001, 2002), a Wnt ligand that activates the β-catenin pathway should be expressed in regenerating limb buds during the period when heat-shock induced Dkk1GFP blocks regeneration. Among several Wnt ligands shown to activate β-catenin signaling (wnt-2b, wnt-3a, wnt-8, wnt-8b and wnt-10a), RT–PCR analysis showed that only wnt-3a was expressed in both regenerating limb buds during Dkk1GFP-sensitive regenerating window as well as in developing limb buds (data not shown). In chick embryo, wnt-3a is expressed in epithelial cell layers during the formation of the apical ectodermal ridge (AER), a specialized epithelial structure essential for the outgrowth and patterning of amniote limbs, and induces fgf-8 expression in β-catenin dependent manner (Kengaku et al., 1998). We examined the expression of wnt-3a and fgf-8 by in situ hybridization and found that both are expressed in the distal region of uncut stage 52 limb buds (Figs. 4A, D). Importantly, both genes were also expressed in the blastema of regenerating limbs (Figs. 4B, E). In situ hybridization on sectioned Xenopus regenerating limb buds further shows that wnt-3a and fgf-8 are specifically expressed in the apical epithelium of the blastema at 3 dpa (Figs. 4C, F).

These data suggest that wnt-3a is a candidate for mediating the function(s) of Wnt/β-catenin signaling during limb regeneration. In the initial process of amphibian limb regeneration, the amputated plane is rapidly covered with migrating epithelial cell layer that forms a specialized epithelial structure referred to as wound epithelium (Stocum, 1995; Han et al., 2005). As the regeneration process progresses, this epithelial cell layer thickens and forms an apical epithelial cap (AEC), a structure that is morphologically and functionally similar to the AER in amniote limb buds (Muneoka and Sassoon, 1992). The localization of transcript to the apical epithelium suggests that Wnt-3a and subsequent activation of Wnt/β-catenin signaling may function in the formation of the so-called “AEC” during limb regeneration.

To obtain more mechanistic insights into the roles of Wnt/β-catenin signaling in limb regeneration, we examined the expression of fgf-8 and fgf-10 following the induction of Dkk1GFP expression. F0 wild-type and hsDkk1GFP tadpoles were heat-shocked at 3 dpa or 5 dpa, and were fixed shortly (8 h) after heat-shock to address the effect of Dkk1GFP on fgf-8 and fgf-10 expression (Fig. 5A). When tadpoles were heat-shocked at 5 dpa, fgf-8 expression was suppressed in the blastema of hsDkk1GFP tadpoles (Fig. 5B-b; n=6/6), while in all wild-type tadpoles the expression of fgf-8 remained unchanged, localized to the inner layer of the apical epithelium of the blastema (Fig. 5B-a; n=6/6). Similarly, fgf-8 expression was also suppressed in the hsDkk1GFP tadpoles heat-shocked at 3 dpa (n=4/6; data not shown) while all wild-type tadpoles expressed fgf-8 (n=6/6; data not shown).

As the interval between the heat-shock and fixation was short (8 h), no significant morphological difference was observed among wild-type and hsDkk1GFP tadpoles. Our data show, then, that fgf-8 expression is dependent upon Wnt/β-catenin signaling during limb regeneration. In contrast to studies that show that fgf-10 is regulated by Wnt/β-catenin signaling in limb bud and fin formation (Kawakami et al., 2001; Ng et al., 2002; Agarwal et al., 2003), we observed that expression of fgf-10 was not directly affected by the Dkk1GFP
at neither 5 dpa nor 3 dpa (Figs. 5B-c, d and data not shown; n = 6/6, respectively). However, it is still possible that Dkk1GFP may indirectly inhibit fgf-10 expression through the suppression of fgf-8 in the blastema later than 8 h after heat-shock since FGF-10 and FGF-8 constitute a positive feedback loop essential for limb outgrowth in amniote embryo (Ohuchi et al., 1997; Xu et al., 1998). If this is also true during Xenopus limb regeneration, one possible explanation for the differences in the regeneration response among hsDkk1 tadpoles heat-shocked at 3 dpa and heat-shocked at 5 dpa could be that the feedback loop between FGF-10 and FGF-8 may be easily inhibited by Dkk1GFP through the suppression of fgf-8 during the blastema formation as expression levels of fgf-10 and fgf-8 are still low. However, once a cone-shape blastema is formed and strong expression of fgf-10 and fgf-8 is established, the feedback loop may be maintained even after the temporal suppression of fgf-8 by Dkk1GFP and result in partial limb regeneration.

Several reports strongly suggest that Wnt/β-catenin signaling controls the expression of fgf-8 in the developing limb buds of chick and mouse (Kengaku et al., 1998; Barrow et al., 2003; Soshnikova et al., 2003). Furthermore, in transgenic mice carrying a Wnt/β-catenin responsive reporter, the mice show reporter activity in the AER, in the fgf-8 expressing domain of limb buds. Moreover, defects in Wnt/β-catenin signaling caused the reduction of reporter activity as well as the absence of fgf-8 expression in the apical epithelium (Maretto et al., 2003). Based on these results, fgf-8 expression in the apical epithelium can be taken as an index of Wnt/β-catenin activity in limbs during morphogenesis. To exclude the possibility that the Dkk1GFP transgene suppressed not only Wnt/β-catenin signaling but non-specifically repressed other genes, in the present study we examined the expression of Lmx-1 (Figs. 5B-e, f; n = 4), Hoxa-13 (Figs. 5B-g, h; n = 6) and msx-2 (Figs. 5B-i, j; n = 6) and found that neither was altered by Dkk1GFP in the blastema. Based on these results, we concluded that the Dkk1GFP specifically blocked canonical Wnt/β-catenin signaling in blastema of tadpoles and resulted in the suppression of fgf-8 gene expression in the hsDkk1GFP tadpoles.

Although we still cannot exclude the possibility that there may be other Wnt ligands expressed that mediate Wnt/β-catenin signaling during limb regeneration, the wnt-3a expression domain clearly overlaps with that of fgf-8 in the blastema and furthermore, wnt-3a is known to induce fgf-8 expression during the AER formation process of limb bud in chick embryo (Kengaku et al., 1998). Therefore, it is likely that wnt-3a plays a role in the initiation of limb regeneration by inducing fgf-8 expression in a β-catenin-dependent manner.

Conclusions

Based on the critical roles of Wnt/β-catenin signaling in limb bud initiation during limb development and in stem cell
renewal in amniotes, we hypothesized that Wnt/β-catenin signaling plays an essential role in initiation of limb regeneration. To test this hypothesis, we created transgenic *X. laevis* tadpoles that express a Wnt/β-catenin antagonist, Dkk1, under the control of a heat-shock promoter and we used heat-shock at various time-points during limb regeneration to express Dkk1 and thus inhibit endogenous Wnt/β-catenin signaling. A single heat-shock, just prior to limb amputation or during early blastema formation, blocked limb regeneration with high efficiency. However, induction of Dkk1 by heat-shock after blastema formation allowed tadpoles to escape complete block of regeneration resulting in the production of incomplete limbs. Dkk1 inhibition of Wnt/β-catenin signaling during regeneration repressed fgf-8 but not fgf-10 in the regenerating blastema. These findings help to position Wnt signaling in the hierarchy of signaling events important during the early stages of limb regeneration. In conclusion, we demonstrate that Wnt/β-catenin signaling plays an essential role during the early phases of limb regeneration and is important, but not absolutely required, during the subsequent phases of limb regeneration in *Xenopus*.

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