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Gremlin1 induces anterior–posterior limb bifurcations in developing *Xenopus* limbs but does not enhance limb regeneration

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ABSTRACT

Gremlin1 (*grem1*) has been previously identified as being significantly up-regulated during regeneration of *Xenopus laevis* limbs. Grem1 is an antagonist of bone morphogenetic proteins (BMPs) with a known role in limb development in amniotes. It forms part of a self-regulating feedback loop linking epithelial (FGF) and mesenchymal (shh) signalling centres, thereby controlling outgrowth, anterior posterior and proximal distal patterning. Spatiotemporal regulation of the same genes in developing and regenerating *Xenopus* limb buds supports conservation of this mechanism. Using a heat shock inducible *grem1* (*G*) transgene to created temperature regulated stable lines, we have shown that despite being upregulated in regeneration, *grem1* overexpression does not enhance regeneration of tadpole hindlimbs. However, both the regenerating and contralateral, developing limb of *G* transgenics developed skeletal defects, suggesting that overexpressing *grem1* negatively affects limb patterning. When *grem1* expression was targeted earlier in limb bud development, we saw dramatic bifurcations of the limbs resulting in duplication of anterior posterior (AP) pattern, forming a phenotypic continuum ranging from duplications arising at the level of the femoral head to digit bifurcations, but never involving the pelvis. Intriguingly, the original limbs have AP pattern inversion due to de-restricted Shh signalling. We discuss a possible role for Grem1 regulation of limb BMPs in regulation of branching pattern in the limbs.

1. Introduction

Gremlin was first identified as a bone morphogenetic protein (BMP)-specific antagonist and dorsalising factor in *Xenopus laevis* (now termed Gremlin1 and hereafter referred to as Grem1 Hsu et al., 1998). The protein product acts as a glycosylated homodimer, and contains a cysteine knot motif (Wordinger et al., 2008) in common with other extracellular BMP antagonists, such as Noggin, Chordin, Follistatin, Cerberus, DAN, and PRDC or Gremlin2 (reviewed in Brazil et al., 2015). Grem1 has a well-established role as a BMP antagonist in development of two vertebrate organs, the kidney and the limb (Khokha et al., 2003; Michos et al., 2004), and is known to bind with high affinity to BMPs 2, 4 and 7 (Eimon and Harland, 1999; Hsu et al., 1998). The balance between BMP antagonists and BMPs themselves is critical not only for development but also has a role in cancer, skeletal homeostasis and fibrosis of the kidney as well as other organs (for review, see Brazil et al., 2015).

The role of Grem1 as a BMP antagonist in limb development was identified soon after its discovery (Capdevila et al., 1999; Merino et al., 1999). Tetrapod limbs develop autonomously from limb buds, which in turn develop from perpendicular outgrowths of lateral plate mesoderm (LPM). The early vertebrate limb bud is comprised of mesenchymal cells surrounded by ectoderm, and its subsequent growth and development requires reciprocal signalling between these two cell types (for review see Butterfield et al., 2010; Zeller et al., 2009). Normal developmental patterning of the vertebrate limb is regulated by two main signalling centres, the apical ectodermal ridge (AER), a morphological boundary between the dorsal and ventral ectodermal surfaces of the limb bud, and the zone of polarising activity (ZPA), which resides in the posterior/distal mesenchyme. Repression of BMPs by Grem1 links these two centres forming part of a self-potentiating feedback loop (Zuniga et al., 1999; Capdevila et al., 1999; Khokha et al., 2003; Michos et al., 2004). The AER expresses several members of the fibroblast growth factor (FGF) signalling growth factor family, principally *fgf8*, and directs distal growth of the limb bud. Removing the AER in amniote embryos results in a truncated limb (Summerbell, 1974), since embryos cannot recover from the developmental loss of this signalling centre, nor can they regenerate limbs. Expression of the Shh morphogen in the ZPA directs AP pattern of the distal half of the limb bud, comprising the future zeugopod and autopod (Chiang et al., 2001). Reciprocal expression of *shh* and *grem1* in the remaining mesenchyme, along with AER *fgf8*, form a feedback loop that maintains function of the AER (Khokha et al., 2003; Niswander, 2002; Scherz et al., 2004). The loop is

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terminated by a negative feedback mechanism triggered by threshold levels of FGFs in the AER, after which expression of *fgf8*, *shh* and *grem1* itself, decline (Verheyden and Sun, 2008). Recently, conditional *grem1* overexpression in the mouse limb bud mesenchyme was shown to result in polydactyly, suggesting that restriction of *grem1* is important in establishing digit number (Norrie et al., 2014).

In comparison to amniotes, we know relatively little about how limbs are formed in amphibians. Limbs develop late in this group, with some exceptions, and the source of the limb bud cells is different, as they migrate from the coelomic epithelium (Tschumi, 1957). In amniotes, where the limb develops early in embryogenesis, limb buds are pre-programmed with AP information from the LPM. Since most frog limbs develop much later and are made up of cells that migrate from the coelomic epithelium (Tschumi, 1957), it is not clear to what extent this pre-pattern is present in amphibian limb buds, if any. In axolotls, which can regenerate their limbs throughout life, there is neither a morphological AER in developing limbs nor is expression of *fgf8* restricted to the ectoderm or dorsal/ventral boundary (Han et al., 2001). In the anuran amphibian *X. laevis* however, there is both a morphological ridge (Tarin and Sturdee, 1971) and a localised expression of *fgf8* indicating a functional AER (Christen and Slack, 1997). This localised expression of *fgf8*, but not the morphological ridge, is re-established in young, regeneration competent *Xenopus* limb buds (Christen and Slack, 1998). *Fgf8* transcripts are localised in the basal columnar cell layer of the multi layered apical epidermal cap (AEC) in both *Xenopus* limb bud (Wang and Beck, 2014) and axolotl limb regeneration (Han et al., 2001). *Shh* is expressed, similarly to amniotes, in the ZPA of developing limbs, and this is re-established during regeneration (Christen and Slack, 1998; Endo et al., 1997). This would seem to indicate that the Shh–Fgf loop is conserved in amphibians and that it functions in both limb development and regeneration, however, the role of Grem1 in regulating this loop in amphibians has not yet been addressed.

In addition to its role in limb development in amniotes, *grem1* has also been identified as one of the most up-regulated genes in 1 to 5 day regenerating *X. laevis* limb buds in two independent studies (Grow et al., 2006; Pearl et al., 2008). We have previously demonstrated a role for BMP signalling in limb regeneration in *X. laevis* by overexpressing *noggin* in a temporally controlled manner using a heat shock inducible promoter system (Beck et al., 2006). In these experiments, ectopic Noggin was able to completely ablate regeneration of early, regeneration competent limb buds, but also showed defects such as oligodactyly and brachydactyly in the contralateral, non-operated limb (Jones et al., 2013). Ectopic expression of noggin in chicken limb buds using the RCAS virus system had been previously shown to produce a similar range of defects, in the most extreme cases resulting in formation of only a single digit like structure from the limb bud (Capdevila and Johnson, 1998). Conversely, in younger larval *Xenopus*, induction of *noggin* at stages 49–50, when limb buds have become autonomous, resulted in ectopic limb formation (Christen et al., 2012; Jones et al., 2013). However, since endogenous *noggin* is not expressed in the limb mesenchyme early (Beck et al., 2006) and mouse knockouts develop with fairly minor skeletal defects (Brunet et al., 1998) we decided to investigate the role of Grem1, which has a known role in early limb patterning.

2. Materials and methods

2.1. Culture of X. laevis tadpoles

X. laevis embryos were generated as described previously (Beck et al., 2006) and cultured in 0.1 × MMR without antibiotics until stage 48, when they were transferred to 10-litre tanks and were fed daily with a slurry of spirulina powder and salmon starter food. About 20% of the 0.1 × MMR water was exchanged for carbon filtered tap water each day. Once feeding (stage 48) was established, tadpoles were transferred into a Marine Biotech XR1 aquarium at a density of approximately 25 tadpoles per litre with slow automatic water recirculation. They were staged according to Nieuwkoop and Faber (1967).

All experiments involving animals were subjected to New Zealand's welfare standards for vertebrates and were reviewed by the University of Otago Animal Ethics Committee. The Animal Ethics Committee approved all experiments under protocols AEC 56/09 and 56/12.

2.2. In situ hybridization

Whole-mount in situ hybridization of tadpole limbs was performed as previously described in Harland (1991) with modifications for limbs as described in McEwan et al. (2011). The *Xenopus gremlin1* (*grem1*) probe has been previously described (Pearl et al., 2008) as have the probes used for *shh*, *sox9* (Jones et al., 2013) and *fgf8b* (Wang and Beck, 2014). *Lmx1b* (based on the sequence of Haldin et al., 2003) probe was made using forward primer ACCCATTTCAGACCGGTTCC and reverse primer GGGTCTGTGCTGTAGCTGTT to amplify 807 bp of reverse transcribed RNA from stage 48 to 55 *X. laevis limb* buds. This partial sequence was cloned into pCR4-TOPO (Life Tech) and verified by sequencing. *NotI* restriction and T3 RNA polymerase transcription was used to generate an antisense, Digoxigenin-labelled RNA probe. mRNA was detected with NBT/BCIP staining to yield a dark purple precipitate.

2.3. Transgenic Xenopus lines

Fourteen transgenic founders were produced with confirmed insertion of the G transgene (*Hsp70-grem1-γ-crystallin-RFP*) and raised to adulthood. Transgenic animals were made by sperm nuclear injection using the method described in (Kroll and Amaya, 1996) with modification as in (Beck et al., 2003). The G6 transgenic F1 or F2 tadpoles used for the experiments in this study, were produced from outcrossing the G6 male founder frog, or one of his sons, containing a single insertion of the *Hsp70-grem1-γ-crystallin-RFP* transgene (Fig. 2). Transgenic animals were identified by the presence of RFP in the lens of the eye from around stage 45.

2.4. Hindlimb regeneration

All hindlimb amputations were at future knee level on the right limb. The amputations were performed using Vannas iridectomy scissors while the tadpoles were anaesthetised in 1/4000 (w/v) tricaine (MS222, Sigma). Heat shocks were used to regulate transgenic activity through immersion of tadpoles in water adjusted to 34 °C for 30 min followed by return to normal aquarium temperature at 25 °C. This heat shock regime was applied five times: once 2 h before limb amputation, and then daily for four days after amputation. Regeneration was scored at stage 58 as present if any identifiable limb skeletal structures formed, ranging from a single toe or spike to a fully formed limb. Non-regeneration was defined as a stump, where the wound had simply healed over with full thickness skin.

2.5. Heat shock activation of grem1 during limb development

Tadpoles were grown to the appropriate limb stage (48, 49, 50, 51 and 52) at ambient aquarium temperature (25 °C) before initiating a series of daily heat shocks of 34 °C for 30 min, by transferring to warmed aquarium water in 500 ml conical flasks in a water bath set to 34 °C, using a fine net. After the 30 min, tadpoles were returned to their original tanks. Daily heat shocks were applied for 5 days after which animals were maintained until they reached stage 58, when forelimbs become visible. Wild type tadpoles were also heat shocked as controls but showed no phenotypes, indicating that the phenotypes in G6 tadpoles were the result of the transgene.