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Temporal requirement for bone morphogenetic proteins in regeneration of the tail and limb of *Xenopus* tadpoles

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Abstract

Bone morphogenetic protein (BMP) signalling is necessary for both the development of the tail bud and for tail regeneration in *Xenopus laevis* tadpoles. Using a stable transgenic line in which expression of the soluble BMP inhibitor noggin is under the control of the temperature inducible *hsp70* promoter, we have investigated the timing of the requirement for BMP signalling during tail regeneration. If noggin expression is induced followed by partial amputation of the tail, then wound closure and the formation of the neural ampulla occur normally but outgrowth of the regeneration bud is inhibited. Furthermore, we show that BMP signalling is also necessary for limb bud regeneration, which occurs in *Xenopus* tadpoles prior to differentiation. When noggin expression is induced, limb bud regeneration fails at an early stage and a stump is formed. The situation appears similar to the tail, with formation of the limb bud blastema occurring but renewed outgrowth inhibited. The transcriptional repressor *Msx1*, a direct target of BMP signalling with known roles in vertebrate appendage regeneration, fails to be re-expressed in both tail and limb in the presence of noggin. DNA labelling studies show that proliferation in the notochord and spinal cord of the tail, and of the blastema in the limb bud, is significantly inhibited by noggin induction, suggesting that in the context of these regenerating appendages BMP is mainly required, directly or indirectly, as a mitogenic factor.

Keywords: *Xenopus* tadpole; Tail; Limb; Regeneration; BMP; Noggin; Msx1

1. Introduction

Bone morphogenetic proteins (BMPs) were first discovered for their ability to convert muscle tissue into bone and cartilage (Ulrist, 1965). Since their discovery, BMPs have been implicated in numerous roles during embryogenesis and organogenesis, regulating processes as diverse and fundamental as regional specification and cell proliferation, differentiation, survival and death (Hogan, 1996). There are now over 20 known members of the BMP family including the growth and differentiation factors (GDFs), forming a subset of the larger transforming growth factor β (TGFβ) superfamily, and found across the animal kingdom from cnidarians to humans. BMPs and GDFs bind to their receptors as dimers, and are controlled by a range of external inhibitors including noggin, chordin and gremlin (Hsu et al., 1998; Piccolo et al., 1996; Zimmerman et al., 1996). Noggin has been shown specifically to bind to BMP2 and -4 and, at lower affinity, also to BMP7. It antagonizes the action of these BMPs directly by blocking the interface of the binding epitopes for the type I and II receptor (Groppe et al., 2002). Our own lab has shown that BMP signalling is intimately involved in both the development of the *Xenopus* tailbud and in the regeneration of the tadpole tail (Beck et al., 2003; Beck and Slack, 1999). During tail development, BMPs have two roles, which can be dissected experimentally by manipulating their genetic pathways. Development of the tail neural tube requires BMP-dependent activation of Notch signalling at the

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dorsal/ventral boundary of the tail tip. BMP may facilitate this by ensuring that Notch ligands and accessory factors such as lunatic fringe become appropriately expressed. BMP also appears to have a direct role, independent of Notch, in the formation of muscle tissue in the tail (Beck and Slack, 1999, 2002).

Amphibians have long been used in the study of regenerative processes, as they have an ability to regenerate limbs, tails, jaws and the lens of the eye. This ability is considered by many authors to be ancestral and to have been lost in other vertebrates, including humans (Sanchez Alvarado, 2000). While regeneration shows many parallels with ontogeny, there are some different processes involved. Regeneration-competent wound healing is generally rapid and involves covering the wound surface with a specialised epidermis that lacks an underlying dermis and basement membrane(Neufeld et al., 1996; Tschumi, 1957). Following wound healing, the differentiated cells of the stump must dedifferentiate and reenter the cell cycle, or else reserve stem cell populations must be mobilised and recruited to the wound site. Gene expression analyses in *Xenopus* have shown that subsequent regeneration involves the re-activation of various developmental signalling pathways (Beck et al., 2003; Cannata et al., 2001; Christen et al., 2003; Christen and Slack, 1997; Matsuda et al., 2001; Sugiura et al., 2004; Tazaki et al., 2005; Yokoyama et al., 2000). Despite many years of study, rather few gene function studies have been reported, with most focusing on the members of the fibroblast growth factor family (FGFs) (Bosco et al., 1997; Yokoyama et al., 2001). In order to study the role of BMPs in regeneration we previously constructed an inducible transgene where the *noggin* coding sequence is under the control of the *Xenopus hsp70* promoter (Beck et al., 2003). Tadpoles carrying this transgene develop normally until subjected to a brief heat shock, after which noggin expression is induced strongly in all cells. Using this and other similar transgenes in founder tadpoles, we demonstrated that BMP signalling is sufficient and necessary for tail regeneration (Beck et al., 2003).

In *Xenopus* tadpoles, the limb, unlike the tail, loses the ability to regenerate as it differentiates (Overton, 1963). However, prior to differentiation, complete limbs can regenerate from a small remnant of the original limb bud mesoderm (Christen and Slack, 1998; Dent, 1962). This depends on the formation of an apical epidermal cap (AEC) which directs formation of a mesenchymal blastema of proliferating *Msx1*-expressing cells that grows and differentiates to form the missing distal limb structures. This capacity to recover from such a severe loss of tissue is unique to amphibians. By contrast, in the chick embryo removal of the tip region, or even just the apical ectodermal ridge, causes permanent distal defects (Summerbell, 1974). The capacity of the *Xenopus* limb bud to regenerate depends on the mesenchymal tissues. It is linked to timing of *FGF10* expression and limited by the onset of skeletal differentiation (Wolfe et al., 2000; Yokoyama et al., 2000). As in the case of tail regeneration, limb bud regeneration seems to depend on the reactivation of developmental genes and pathways (Christen et al., 2003; Christen and Slack, 1997, 1998). BMP signalling has been shown to be required for both dorsal-ventral and proximodistal axis patterning in the chick limb, and can direct the formation of ectopic AERs (Pizette et al., 2001). While proximodistal patterning appears to use the same mechanisms in *Xenopus*, the dorsal ventral axis may be set up differently (Christen and Slack, 1998).

Msx transcription factors, direct targets of BMP signalling, are intimately associated with regeneration in several systems. The key role of Msx1 appears to be in the inhibition of differentiation. *Msx1* is expressed during regeneration in urodele limbs (Koshiba et al., 1998), anuran tails and limb buds (Beck et al., 2003; Yokoyama et al., 2000), FGF-induced chick limb buds (Kostakopoulou et al., 1996), fish fins (Murciano et al., 2002), and mouse digit tips (Han et al., 2003; Reginelli et al., 1995). In urodeles and mammals, Msx1 can induce differentiated, multinucleate myofibers to cellularise, forming actively dividing mononucleate cells *in vitro* (Kumar et al., 2004; Odelberg et al., 2000). Correct regulation of *Msx1* expression by BMPs and their inhibitors may therefore be critical for regeneration of appendages.

Here we use a transgenic line of *Xenopus laevis hsp70-noggin* tadpoles to advance understanding of the role of BMP in regeneration. A genetic line provides a higher degree of reproducibility than the founder tadpoles used previously. The tadpoles of a line are available in unlimited numbers and all have the same transgene, wheras every individual founder transgenic represents a different insertion site and copy number. Using this line, we show that the requirement for BMP signalling in tail regeneration is relatively late: at 24–48 hours after amputation. It is required for the re-expression of *Msx1* and for cell proliferation, particularly in the notochord and spinal cord. In addition we use this line to show, for the first time, that BMP signalling is necessary for limb bud regeneration, specifically for the growth of the blastema.

2. Results

2.1. Normal expression of noggin and BMP in tails and limbs

BMP2 and *-4* are expressed ventrally during tail bud initiation (Fainsod et al., 1994), with *noggin* restricted to the organiser region, which later becomes the chordoneural hinge (Gont et al., 1993; Smith and Harland, 1992). However, during outgrowth of the tail, expression of these *BMPs* becomes restricted to the fin (Fig. 1A) and *noggin* is expressed in a small region of posterior dorsal neural tube previously named the dorsal roof (Beck and Slack, 1998) no longer being seen in the chordoneural hinge (Fig. 1B). During regeneration of the tail, *BMP2* and *noggin* are re-expressed in equivalent domains, i.e. respectively the edge of the forming fin and the tip of the spinal cord (Fig. 1C and D). A previous study of localisation of