Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 304 (2007) 675–686
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Transgenic *Xenopus* with *prx1* limb enhancer reveals crucial contribution of MEK/ERK and PI3K/AKT pathways in blastema formation during limb regeneration

Makoto Suzuki, Akira Satoh, Hiroyuki Ide, Koji Tamura *

Department of Developmental Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University, Aobayama Aoba-ku, Sendai 980-8578, Japan

Received for publication 25 November 2006; revised 9 January 2007; accepted 9 January 2007
Available online 17 January 2007

Abstract

Understanding the mechanisms that control amphibian limb regeneration should allow us to decipher the critical differences between amphibians and humans, which have the limited ability of organ regeneration. However, many issues at the cellular and molecular levels still remain unresolved. We have generated a transgenic *Xenopus laevis* line that expresses green fluorescent protein (GFP) under the control of mouse *prx1* limb enhancer, which directs reporter gene expression in limb mesenchyme in mice, and found that GFP accumulated in blastemal mesenchymal cells of the transgenic froglets after limb amputation. Thus, this transgenic line should provide a new approach to gain insights into the cellular dynamics and signaling pathways involved in limb blastema formation. We have also developed a culture system for forelimb explants of froglets and found that treatment with inhibitors of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) kinase 1/2 (MEK1/2) and phosphatidylinositol 3-kinase (PI3K) repressed GFP expression. These effects were partially reversible, and down-regulation of GFP was associated with inhibition of cell-cycle progression and induction of ectopic apoptosis. In addition, we found that ERK1/2 and AKT, downstream mediators of MEK1/2 and PI3K pathways, were activated in amputated forelimb stumps. These results demonstrate that MEK/ERK and PI3K/AKT pathways regulate limb blastema formation in the *X. laevis* froglet.

Keywords: *Xenopus*; Limb regeneration; Blastema; Dedifferentiation; Transgenesis; *prx1*

Introduction

Regeneration of vertebrate limbs, which is observed in amphibians, is a complex process involving large-scale tissue remodeling and activation of a number of signaling pathways. In urodeles such as newts and salamanders, epidermal cells around the stump rapidly migrate and form a dermis-free epithelial structure, wound epidermis (WE), within 1 day after limb amputation. Under the WE, mesodermal cells of mature tissues locally change their characteristics to proliferative and undifferentiated mesenchymal cells (blastemal cells) in a process referred to as "dedifferentiation." Consequently, the blastema, a growing cone-shaped mass of blastemal cells, is formed and regenerates a new limb structure. One of the most critical issues is how blastemal cells are generated in the early phase of limb regeneration, especially at the cellular and molecular levels (Brockes and Kumar, 2005; Gardiner et al., 2002; Suzuki et al., 2006). Various classical attempts to reveal the mechanisms controlling epimorphic limb regeneration in urodeles have suggested that injury, nerves and WE are prerequisite for proper blastema formation (Tassava and Mescher, 1975). Denervation of amputated stumps or prevention of WE formation disrupts proliferation of blastemal cells for blastema outgrowth (Maden, 1978; Mescher, 1976; Mescher and Tassava, 1975), suggesting that factors from nerves and WE are critical for the maintenance of cell cycle progression of mesodermal cells, which is one of the critical factors for success of epimorphic limb regeneration.

For the first step for dedifferentiation, quiescent differentiated cells in intact limbs are stimulated in response to limb amputation and reenter into the cell cycle, and these events have been shown to occur independently of nerves and the WE (Endo et al., 2004; Maden, 1978; Mescher, 1976; Mescher and Tassava, 1975;

* Corresponding author. Fax: +81 22 795 3489.
E-mail address: tam@biology.tohoku.ac.jp (K. Tamura).

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.01.019

Suzuki et al., 2005). Careful examinations of temporal and spatial patterns of the reentry of mesodermal cells into the cell cycle during blastema formation have shown that the initial cell cycle takes place in the stumps slightly apart from the amputation plane (Chalkley, 1954; Hay and Fischman, 1961). The cycling cells in the proximal region move distally and gradually accumulate under the WE. Therefore, initiation of limb regeneration occurs by unknown signaling pathways in the mature tissues rather than by signaling molecules elicited by transected nerves and the WE. However, what signaling pathways stimulate the cell cycle progression and motility of progenitors of blastemal cells leading to blastema formation is poorly understood.

In order to further explore the mechanism controlling blastema formation, the cellular and molecular framework of limb blastema formation has been addressed by using the *Xenopus laevis* froglet as a model system. An amputated limb of a *Xenopus* froglet has been shown to form a blastema through a molecular mechanism closely related to that in urodeles (Endo et al., 2000; Suzuki et al., 2005, 2006), whereas it gives rise to an incomplete regenerate because of the deficient ability for pattern formation and tissue differentiation (Endo et al., 1997, 2000; Matsuda et al., 2001; Satoh et al., 2005a,b; Yokoyama et al., 1998). In a series of studies, we found that *prx1* is expressed in blastemal cells in early blastemas of *Xenopus* froglet forelimbs (Suzuki et al., 2005), suggesting that up-regulation of *prx1* is another reliable marker of early blastemal cells and their progenitors. *Prx1*, which encodes a paired-type homeobox-containing transcription factor related to aristaless, is one of the earliest genes that are expressed in mesenchyme of the prospective limb-forming region in mouse and chick embryos (Kuratani et al., 1994; Leussink et al., 1995; Meijlink et al., 1999; Nohno et al., 1993). It has also been shown that a 2.4-kb genomic sequence upstream of mouse *prx1* contains an enhancer element that directs expression of reporter genes in limb bud mesenchyme in mice (Martin and Olson, 2000). These findings suggest that the mouse *prx1* limb enhancer also directs expression of reporter genes in *Xenopus* limb blastemal cells and provide a new approach for visualizing early blastemal cells and for addressing the signaling mechanism controlling blastema formation.

In this study, we established a transgenic *Xenopus* line expressing GFP under the control of the mouse *prx1* limb enhancer and examined features of GFP-positive cells in the process of blastema formation. Furthermore, by developing an organ culture system of the froglet forelimb, we found that MEK/ERK and PI3K/AKT pathways are involved in a variety of cellular processes, including upstream regulation of *prx1* expression in blastemal cells. Our results suggest the usefulness of the newly established transgenic *Xenopus* to study vertebrate limb regeneration and the importance of MEK/ERK and PI3K/AKT pathways during organ regeneration in vertebrates.

Materials and methods

Construction and transgenesis

A 2.4-kb genomic sequence upstream of mouse *prx1* has a 530-bp core region (CR) that contains two conserved non-coding regions, CR1 and CR2, and deletion of these sequences results in loss of the limb-specific activity of the transgene (Martin and Olson, 2000). We confirmed that a genomic sequence upstream of frog (*Xenopus tropicalis*, closely related species to *X. laevis*) *prx1* has a homologous region to CR1 (Supplementary Fig. 1A, see Discussion). To generate the plasmid *Mprx1-GFP*, a 2.4-kb genomic sequence upstream of mouse *prx1* (Martin and Olson, 2000) was amplified from the C57BL/6J mouse genome using KOD plus DNA polymerase (TOYOBO) with primers 5′-(*Sal*I)-GTCGACTTGCTACAGGTTTCTAGAACAATG-3′ and 5′-(*Hin*dIII)-AAGCTTAATAGGAGCCTGTAATTACGTG-3′. The PCR product was cloned into pCR-Blunt (Invitrogen) and sequenced. Then this 2.4-kb sequence excised using *Sal*I and *Hin*dIII was subcloned into the *Sal*I and *Hin*dIII sites of pCSGFP3 (Bronchain et al., 1999). For transgenesis, *Mprx1-GFP* was linearized with *Ssp*I and cleaned by Wizard SV gel and PCR clean-up system (Promega). Transgenesis was performed as previously described (Kroll and Amaya, 1996) with minor modifications.

Animals and experimental manipulations

Maintenance and surgical operations of *X. laevis* were performed as previously described (Suzuki et al., 2005). For organ culture, *Xenopus* froglets were anesthetized, sterilized for 5 seconds with 70% ethanol and washed twice with PBS. Forelimbs were dissected at the distal and proximal zeugopodium to exclude epiphyses with microdissecting scissors, and skin was removed using fine forceps. After three washes with PBS, up to 6 explants were placed in a 35-mm Petri dish (Falcon #1008) with 2 ml of modified L-15 medium (70% L-15, 10% heat-inactivated FBS, 1% ITS supplement (GIBCO, #41400-045) and penicillin/streptomycin) and cultured at 25 °C. For chemical treatments, U0126 (Calbiochem) and LY294002 (Sigma) were added to the culture medium from 20 mM stock solutions in DMSO and exchanged every other day. DMSO was added to the experimental medium and control medium to a final concentration of 0.5%.

Histological staining and BrdU labeling

Preparation of a digoxigenin-labeled RNA probe for *X. laevis prx1* (Takahashi et al., 1998) and section in situ hybridization was performed as described previously (Suzuki et al., 2005). Whole-mount in situ hybridization was performed as described previously (Endo et al., 2000) with minor modifications. For bromodeoxyuridine (BrdU) labeling, 20 μl of 10 mM BrdU (Sigma) was injected intraperitoneally into froglets or 1 mM BrdU was added to the culture medium to a final concentration of 10 μM. Specimens were fixed 1 h after BrdU treatments.

For immunostaining, we used the protocol described by Kamal Sharma (http://sharmalab.bsd.uchicago.edu/research.html). Briefly, specimens were fixed for 2 h at 4 °C in 4% paraformaldehyde/PBS, decalcified overnight at 4 °C with 8% EDTA/PBS and embedded in O.C.T. compound. Sections were cut at a thickness of 8 μm and washed with wash buffer (PBS plus 0.02% Tween-20). For BrdU staining, sections were incubated in 100 U/ml DNase (TaKaRa, #2210A) and 1 mM $MgCl_2$ in PBS for 30 min at 37 °C. Then sections were incubated with primary antibodies diluted with blocking buffer (PBS plus 0.02% Tween-20, 0.1% Triton X-100 and 1% heat-inactivated goat serum) overnight at 4 °C. The primary antibodies used were mouse anti-myosin heavy chain (DSHB, MF20) at 1:500, rat anti-GFP (Nacalai Tesque, GF090R) at 1:1000, mouse anti-BrdU (DSHB, G3G4) at 1:500, rabbit anti-phospho-Histone H3 (Upstate, #06-570) at 1:1000, mouse anti-MSX1/2 (DSHB, 4G1) at 1:1000, rabbit anti-active caspase-3 (BD Pharmingen, C92-605) at 1:400 and mouse anti-diphospho-ERK1/2 (Sigma, MAPK-YT) at 1:500. After several washes with wash buffer, sections were incubated with secondary antibodies diluted with blocking buffer for 2 h at room temperature. The secondary antibodies used at 1:500 were anti-mouse FITC (Chemicon), anti-mouse Alexa594 (Molecular Probes), anti-rabbit Alexa594 (Molecular Probes) and anti-rat Alexa488 (Molecular Probes). Sections were counterstained with DAPI to detect nuclei, washed several times with wash buffer, and mounted in Vectorshield (Vector Laboratories).

For immunostaining with rabbit anti-phospho-(Ser/Thr) AKT substrate (CST, #9611), which recognizes one of the conserved motifs phosphorylated by activated AKT (R/K)X(R/K)XX(T*/S*), sections were washed with distilled water, boiled in 10 mM sodium citrate buffer (pH 6.0) using a microwave oven and then washed three times with TBST (TBS plus 0.1% Tween-20). After