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Analysis Of The Expression Of Selected Suppressors Of Cytokine Signaling, Socs1 And Socs3, Genes In Response To Limb Amputation In Xenopus Laevis

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ANALYSIS OF THE EXPRESSION OF SELECTED SUPPRESSORS OF CYTOKINE SIGNALING, SOCS1 AND SOCS3, GENES IN RESPONSE TO LIMB AMPUTATION IN *XENOPUS LAEVIS*

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Doctor of Philosophy

By

Lubna Fawzi Abu-Niaaj

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Analysis of the Expression of Selected Suppressors of Cytokine Signaling, SOCS1 and SOCS3, Genes in Response to Limb Amputation in *Xenopus Laevis*

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ABSTRACT

Identification of genes that are possibly stimulators or inhibitors in limb regeneration in vertebrates is important in understanding the regeneration mechanism and the cause of regeneration failure in non-regenerative structures. It is proposed that the development of the immune system of adult anurans modifies the capacity of limb regeneration. This modulation is mainly due to the participation of variety of immune cells in the response to inflammation caused by amputation. The influence of cytokines and their modulators including **S**uppressors **o**f **C**ytokine **S**ignaling (SOCS) proteins is critical in determining the nature of tissue response to injury and the degree of tolerance of new cells forming regenerating structures. The South African clawed frog, *Xenopus laevis,* has a complete limb regenerative capacity at early developmental stages that is gradually decreased in the adult as the immune system matures. This study reports a successful isolation of a full-length *X. laevis* SOCS1 cDNA for the first time, and a full-length SOCS3 cDNA. An analysis of the temporal expression indicated an expression for both genes at the stage when the formation of a limb bud starts. The comparison of the expression of SOCS1 and SOCS3 in regenerative blastemas and non-regenerative pseudoblastemas showed an induction of both genes within one-day post amputation with a higher expression of SOCS3. The expression was maintained though five days with a higher expression in non-regenerative pseudoblastemas. Using whole mount *in situ* hybridization, it was determined that the induced expression was in mesodermal cells.

Data of amputated limb cultures postulated that the induced expression is a local response. Beryllium, which is known to repress regenerative capacity, showed a suppressed expression of the patterning gene, Shh, in correlation with an induction of SOCS1 and SOCS3 in beryllium-treated blastema.

In conclusion, this study addresses the role of SOCS1 and SOCS3 in the limb regeneration.

DEDICATION

To my homeland

To the soul of my father; without his support, advice, love and encouragement, my life has not been successful.

To my mother; without her support and love, my life has no value.

To My sisters, Fadwa and Muna.

To my brothers, Rami, Nimer and Imad.

To my uncles, Ziad Halawa and Mufeed Halawa; without their support, this work would not been completed.

To my husband, Osama Al-Halawat for love and support.

To my two wonderful children, Yasmeen and Mohammad, for their love and enjoyment.

To the memory of my grandfathers and grandmothers and to everyone I love.

Above all, I give my gratitude and love to God, the creator, and the source of all life, whose knowledge and infinite wisdom is above all and encompasses all.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

"If there were no regeneration, there could be no life. If everything regenerated, there could be no death."

Richard J. Goss (1969)

Regeneration is a fascinating biological mechanism because it takes place in all living organisms; however, the capacity of regeneration varies among species, tissues and with the level of biological organization within the organism. Regeneration is a term that refers to diverse healing activities including wound healing and tissue repair to maintain or restore the original architecture of a tissue or an organ. Understanding the process of regeneration is significant in cell biology and development because it holds the key for directing the molecular events for forming a new tissue to replace a damaged one. These events include *in vitro* and *in vivo* stimulation of tissue regeneration from cells in biodegradable scaffolds and from healthy residual tissue, respectively. Induction of regeneration *in situ* in non-regenerative systems is the future goal for developing the regenerative medicine. Characterization of genes that enhance or suppress regeneration using different animal models is significant in understanding the molecular signals for initiation of regeneration. Application of this knowledge into human medical research enables therapeutic approaches to cure diseases and injuries caused by regeneration failure such as in heart damage, spinal cord injuries and loss of appendages.

Limb Regeneration in Amphibians

Amphibians (anurans and urodeles) are useful models for studying regeneration due to their remarkable capacity to regenerate diverse structures including limbs, tails, jaws, and portion of their spinal cord and forebrain (Crawford, 2001; Straube & Tanaka, 2006; Stoick-Cooper, Moon, & Weidinger, 2007). Frogs and toads, which belong to the order Anura, have powerful abilities for limb regeneration at early larval stages. This ability gradually decreases when the animal undergoes metamorphosis (Dent, 1962; Muneoka, Holler-Disnmore & Bryant, 1986; Straube & Tanaka, 2006; Suzuki et al., 2006; Stoick-Cooper et al., 2007; Yokoyama, 2008).

Animal Model: *Xenopus laevis*

The South African clawed frog, *Xenopus laevis,* who belongs to order Anura, has a full capacity for limb regeneration during early development that is completely absent at later stages of life (Fig. 1). Limb regeneration is complete at early larval stages (st48-53; Nieuwkoop & Faber, 1967), when the formation of a small, round, limb bud starts with the first appearance of digit primordium. The capacity for limb regeneration is reduced when the animal undergoes metamorphosis; until the regenerative ability diminishes by later development (st57–59) when the tail starts to disappear (Dent, 1962; Wolfe, Nye, & Cameron, 2000). Therefore, stages 48-53 are considered as regeneration competent, stages 54-56 are regeneration limited, and stages 57-59 are regeneration non-competent stages.

Studies have shown unsuccessful regeneration of anuran limb that have lost the regenerative capacity when grafted to a host at the limb competent stage. On the other hand, grafting a limb capable to regenerate to a host that has lost its own regenerative

capacity, maintained the regenerative capacity (Carlson, 2007 p.280) Therefore, failure of regeneration in non-competent stages was suggested to be due to an absence ability to re-activate key signaling pathways required for limb development (Christen & Slack, 1997; Cannata, Bagni, Bernardini, Christen, & Filoni, 2001; Matsuda, Yokoyama, Endo, Tamura, & Ide, 2001; Gardiner, Endo, & Bryant, 2002; Beck, Christine, Barker, & Slack, 2006; Tazaki et al., 2005; Yokoyama, 2008).

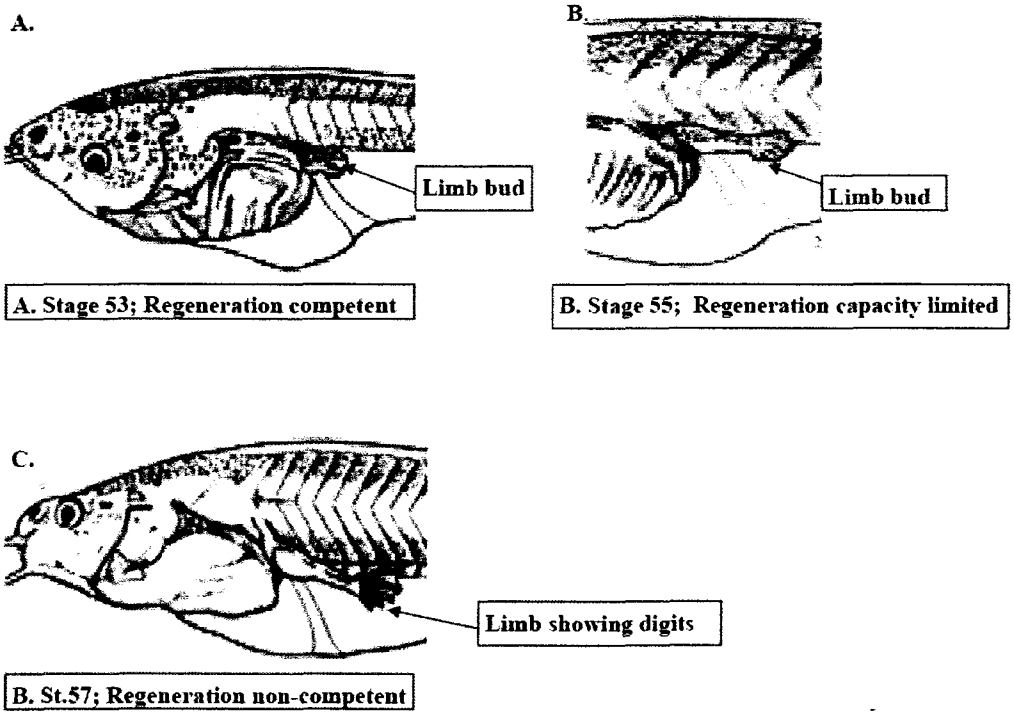


Figure 1. Schematic representation of the morphology of *Xenopus* hindlimb at selected developmental stages according to Nieuwkoop and Faber (1967).

During regeneration competent stages, *Xenopus* completely replaces all the lost structures of hindlimbs with maintenance of proper patterning and functioning (Fig. 2). The regenerating limb, called "blastema", will increase in size until it resembles the intact counterpart (Crawford, 2001). Amputation at progressively later larval stages (st54-56)

results in a limited regeneration where a small limb usually forms with less muscle and fewer digits. Amputation of a hindlimb during the regeneration non-competent stages, or as froglets, result in an outgrowth of a cartilaginous non-segmented "spike" covered by skin and lacking muscle (Dent, 1962; Muneoka et al., 1986; Goss & Holt, 1992; Endo, Bryant, & Gardiner, 2004; Satoh, Ide, & Tamura, 2005; Suzuki et al., 2006). The deficient regenerating limb is called a "pseudoblastema."

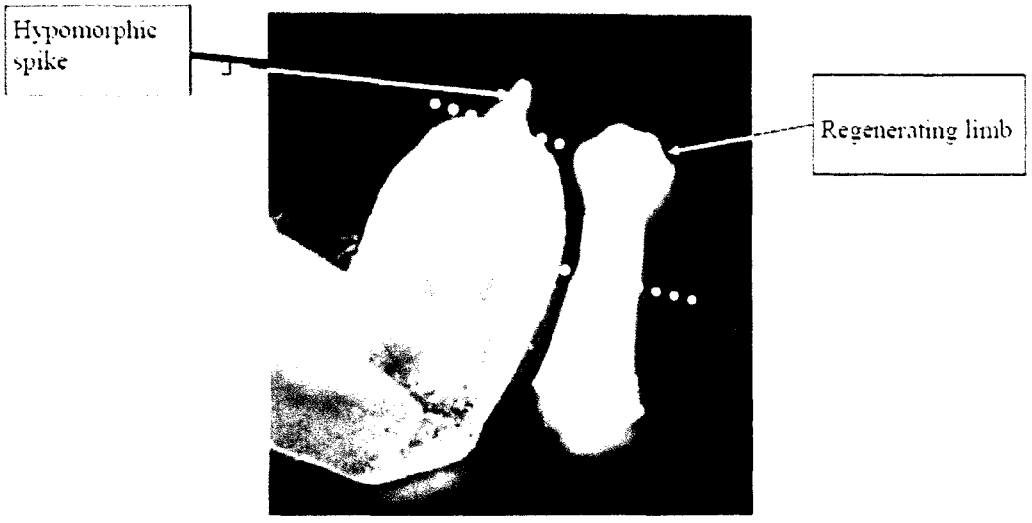


Figure 2. Regeneration at 21 days after amputation illustrating a non-complete vs. a complete regeneration at different larval stages in *Xenopus laevis*. Dotted lines represent plane of amputation. Left: regeneration non-competent limb at stage 59; Right: regeneration competent limb at stage 53. (Harty et al., 2003)

Using *Xenopus* is ideal in developmental studies for various reasons (Deuchar, 1972). The main advantage is the stage specificity for limb regeneration that allows the study of complete vs. incomplete limb regeneration in the same organism.This interesting phenomenon can be used to discover, by differential gene screening, the stimulatory and

the inhibitory genes involved in regeneration. Another advantage of using *Xenopus* is the ease of embryo manipulation in molecular studies mainly due to the external fertilization and the large size of oocytes that allow micro-surgery. One additional consideration is the female responsiveness around the year to the commercial hormone injection to induce ovulation compared to the limited breeding seasons of other amphibians (Deuchar, 1972; Kay & Peng, 1991; Tinsley & Kobel, 1996).

The most compelling reason for using *Xenopus* in developmental biology is the large number of genes identified in *X. laevis.* This allows for a diversity of *Xenopus* cloned genes in the public databases since enormous genes were identified in *X. laevis* (King et al., 2003; Tazaki et al., 2005; Grow, Neff, Mescher, & King, 2006; Pearl, Barker, Day, & Beck, 2008). Recently, completion of the genome project in *X. tropicalis* (Blitz, Andelfinger, & Horb, 2006) and application of transgenic technology on *X. laevis* and *X. tropicalis*, has served as useful tools for efficient manipulation of gene expression in this species (Slack, Beck, Gargioli & Christen, 2004; Beck et al., 2006; Yokoyama, Ogino, Stoick-Cooper, Grainger, & Moon, 2007; Pearl et al., 2008).

Genetic Studies on Limb Regeneration

In amphibians, several genes and signaling molecules identified in the embryonic tail and limb buds have been similarly detected in the regenerating structures (Matsuda et al., 2001). Limb regeneration studies on *Xenopus* reported many genes, previously identified in early limb development, that also have possible roles in the mechanism of limb regeneration (Stark, Gates, Brockers, & Ferretti, 1998). A global analysis of gene expression of *X. laevis* hindlimbs identified many genes that were differentially expressed among regeneration competent vs. non-competent stages (King et al., 2003; Nye,

Cameron, Chemoff, & Stocum, 2003; Katogi et al., 2004; Grow et al., 2006; Suzuki et al., 2006; Pearl et al., 2008).

Using subtractive hybridization to study genes expressed in regeneration competent (st53) vs. non-competent (st59) stages in *X. laevis* hindlimbs led to the classification of genes into three distinctive categories (King et al., 2003). One category includes genes expressed in both blastemas as well as in pseudoblastemas. The other two categories represent genes at higher levels either only in blastema or only in pseudo-blastema. The expression pattern of selected genes in each category indicated their roles as cell-signaling centers within the limb buds and/or within blastemas, suggesting their roles in directing the positional specification of developing and regenerating limbs (Fig. 3).

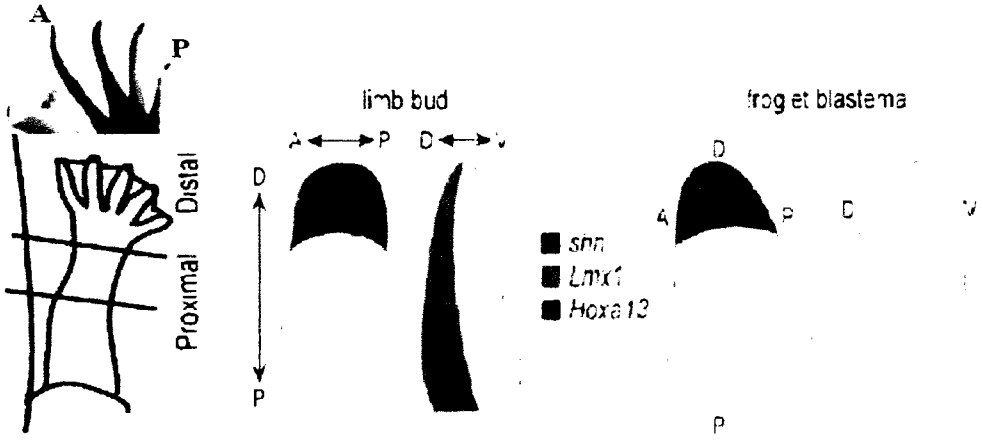


Figure 3. Schematic representation showing the positional identity of a limb and the gene expression related to patterning in tadpole limb bud and froglet blastema. A-P: Anterior–Posterior; D-V: Dordal-Ventral; P-D: Proximal-Distal. *Shh* (blue) is expressed in the mesenchymal cells; *Lmx1* (orange). *Hoxa 13* (purple). Only *Hoxa 13* is expressed in both, tadpole limb bud and froglet blastema. (Suzuki et al., 2006)

Mechanism of Limb Regeneration

Limb regeneration in amphibians represents epimorphosis (Goss & Holt, 1992; Agata, Saito, & Nakajima, 2007), where regeneration occurs via formation of the blastema. The blastema is a cone-like structure resulting from proliferation of dedifferentiated cells from many tissues proximal to the wound epithelium (Stark et al., 1998). These cells continue to proliferate, differentiate and re-pattern to form a regenerating limb (Hay & Fischman, 1961; Stark et al., 1998; Chen, Lin, & Slack, 2006; Morrison, Loof, He, & Simon, 2006; Stoick-Cooper et al., 2007).

The mechanism occurs in three roughly distinguishable phases that are: wound healing, blastema formation, and re-patterning (Chalkley, 1954; Hay & Fischman, 1961; Endo, Bryant, & Gardiner, 2004; Campbell & Crews, 2007). The wound healing is initiated via migration of cells, without dividing, from the edges of an amputated limb to cover the wound surface within hours after amputation, thus forming the wound epidermis or the apical epidermal cap (AEC) (Thornton, 1957; Tassava & Olsen, 1982; Call & Tsonis, 2005). The formation of wound epidermis is critical for successful regeneration of anurans hindlimbs. The AEC is the thickened epithelial tissue formed underneath the wound epithelium and it lacks the basement membrane as well as the dermis (Neufeld, 1996). The wound epithelium consists of up to fifteen layers, whereas the normal epidermis consists of three to four layers (Singer & Salpeter, 1961). The basal columnar cells of AEC have a regenerative capacity because they are essential in blastema growth and maintenance through signals to the underlying mesenchymal cells (Christensen, & Tassava, 2000; Wolfe, Crimmins, Cameron, & Henry, 2000). The contact of the wound epidermal cells with the underlying mesenchymal cells is necessary

for successful regeneration. It is proposed that regenerative capacity is controlled by mesoderm and not by the epidermis based on a study using a preparation of *Xenopus* recombinant limb buds that were composed of regenerative epidermis and non-regenerative mesenchyme and vice versa (Yokoyama et al., 2000). Regarding patterning, studies showed that regeneration failed if wound epidermis was only composed of cells characterized by a single positional identity that is genetically directed (Carlson, 2007; Campbell & Crews, 2008). This suggests that the proper patterning requires different positional identities of cells forming the wound epidermis (Fig. 3).

Major Initiative Signals Involved in Limb Regeneration

Numerous factors influence the capacity of limb regeneration in amphibians. Many signaling pathways, mainly induced in epidermal cells, fibroblasts, and nerves, are collectively vital for successful limb regeneration. Selected pathways are briefly discussed.

Epidermal cells are reported to enable outgrowth of limbs and control patterning (Gardiner et al., 2002). Once the epidermal cells migrate across the fibrin clot at the amputation site, they begin to synthesize their own extracellular matrix proteins, including laminin and collagens (type IV and type XII) (Del Rio-Tsonis, Washabaugh & Tsonis, 1992; Wei, Yang, Klatt, & Tassava, 1995). Regulation of collagen turnover and fiber formation occurs via selected degradation proteins known to play a role in tissue remodeling as collagenases and matrix metalloproteinases (MMPs) (Beck, Christen, Barker, & Slack, 2006; Benoît, 2007).

The proposed role of MMPs in cell dedifferentiation and formation of wound epidermis was based on studies indicating an increased expression of MMPs in tadpoles

that were regenerating limbs soon after amputation (Miyazaki, Uchiyama, Imokawa & Yoshizato, 1996; Call & Tsonis, 2005; Mescher, & Neff, 2006; Yokoyama, 2008). In addition, a failure in limb regeneration upon inhibition of MMPs activity was reported in newts, which have a regenerative capacity throughout their lives. The onset of expression of different MMPs varied in regenerating limbs occurring either detected early in wound epidermis of regenerating limbs as for MMP3/10b (Kato et al., 2003), or detected in the wound epithelium as soon as healing is completed as for MMP9 (Yang, Gardiner, Carlson, Nugas, & Bryant 1999).

Other factors proposed to play a significant role in vertebrate limb regeneration include members of the fibroblast growth factor (Fgf) family (Martin 1998; Capdevila & Izpisua Belmonte, 2001; Tickle & Munsterberg, 2001). Along with Wnt/β-catenin signaling, Fgf were reported to be required for progenitor cell proliferation and specification in amphibians, thus allowing maturation of wound epidermal cells (Mescher, 1996; Kawakami et al., 2006; Stoick-Cooper et al., 2007; Yokoyama et al., 2007; Yokoyama, 2008). At least five of the 22 known *Fgf* genes are expressed in the developing limb and affect other genes directly or indirectly. These are *Fgf-2, Fgf-4, Fgf-8, Fgf-9, and Fgf-10* (Han, An, & Kim, 2001). Receptors of *Fgf-1 and Fgf-2* were also reported to play a role in development (Martin, 1998). Some *Fgfs* are expressed at early stages of limb development as well as in regenerating limbs.

For example, expression of *Fgf-8* was reported in *Xenopus* hindlimbs at st48, when the limb bud is forming in the distal ectodermal tip as a broad stripe along the dorsoventral boundary (Han et al., 2001). Of note, studies showed no expression of *Fgf-8* and *Fgf-10* in pseudoblastemas. Additionally, *Fgf-8* was reported to be localized in the

basal layer of the wound epidermis and *Fgf-10* was reported in the distal mesodermal cells of the developing limb bud (Martin, 1998; Yokoyama et al., 2000).

The expression level of *Fgf-8* and *Fgf-10* was low in an intact limb (Christensen, Weinstein, & Tassava, 2002), however, there was evidence that *Fgf-8* and *Fgf-10* are involved in improving regeneration when application of *Fgf-10* soaked beads stimulated regeneration in *Xenopus* non-regenerative stumps with the maintenance of a proper patterning (Yokoyama, Ide, & Tamura, 2001). This manipulation caused reactivation of several other genes such as sonic hedgehog (*Shh*), *Msx-1* in addition to *Fgf-10* itself. Because ability of regeneration depends on the proliferation of mesenchymal tissue, and not on the epidermal tissue, the effect of *Fgf-10* was suggested to be a positive feedback mechanism on *Fgf-8* (Yokoyama et al., 2000; Yokoyama et al., 2001; Yokoyama, 2008).

The Wnt/β-catenin signaling is another important pathway reported in improving limb regeneration. A loss-of-function study indicated that inhibition of Wnt/β-catenin signaling had a negative effect on limb regeneration, which was reversed by injection of β-catenin. This inhibition caused a failure in the formation of wound epidermis. Similar to the role of *Fgfs*, the Wnt/β-catenin signaling is required for progenitor cell proliferation and patterning, thus allowing maturation of wound epidermal cells (Kawakami et al., 2006; Stoick-Cooper et al., 2007; Yokoyama et al., 2007; Yokoyama, 2008). Data indicated that inhibition of Wnt/β-catenin signaling affected *Fgf-8,* suggesting that Wnt/β-catenin signaling is required along with *Fgfs* for blastema formation (Han et al., 2001; Yokoyama et al., 2007; Yokoyama, 2008). A long-term *in vitro* study using chicken limbs had indicated the involvement of Wnt signaling in the

maturation and hypertrophy programs of cartilage development (Daumer, Tufan, & Tuan, 2004).

Regulation of patterning of regenerative *Xenopus* hindlimbs occurs due to many genes including those expressing Wnts proteins, bone morphogenetic proteins (BMP) and Shh protein (Fig. 3). The expression pattern of some Wnt genes suggested a role of Wnt signaling in proximodistal and dorsoventral patterning, as well as in the maintenance of limb outgrowth (Yang, Topol, Lee, & Wu, 2003). The BMP family plays a significant role in setting up the dorsoventral polarity and determining the digit growth during blastema formation (Beck et al., 2006; Benoît, 2007; Yokoyama, 2008). The DNA labeling showed significant inhibition of proliferation of blastema cells by induction of Noggin, which is a BMP inhibitor. The study suggested that BMP is required, directly or indirectly, as a mitogenic factor for regenerating limbs (Beck et al., 2006).

Furthermore, studies suggest the importance of Shh in regulating the anterio-posterior patterning by determining the number and identity of digits (Fig. 3) (Stark et al., 1998; Suzuki et al., 2006; Yakushiji et al., 2007). The knockout studies indicated that Shh itself is not essential for the initiation of the anterio-posterior patterning (Te Welscher, Fernandez-Teran, Ros, & Zeller, 2002). Yakushiji and colleagues (2007) reported that lack of Shh expression in pseudoblastemas of *Xenopus* froglets was due to a high methylation of the region containing the *Shh* enhancer. The study suggested a correlation between the level of methylation of the enhancer and the spike formation in amputated limbs at later developmental stages.

Other genes, as *Hox* and *Lmx-1*, were detected in the dorsal mesenchymal cells indicating their role in dorsal-ventral identity and patterning of regenerating limbs

(Mescher, 1996; Martin, 1998; Torok, Gardiner, Shubin, & Bryant, 1998; Matsuda et al., 2001; Suzuki et al., 2006) (Fig. 3).

Nerve Influence on Limb Regeneration

The influence of innervation on limb and tail regeneration in *X. laevis* has been reported (Singer, 1952; Mullen, Bryant, Torok, Blumberg, & Gardiner, 1996; Carlson, 2007; Taniguchi Sugiura, Tazaki, Watanabe, & Mochii, 2008). It was suggested that denervation, before or simultaneously with limb amputation caused an inhibition of cell proliferation and blastema growth, but it did not affect the formation of wound epidermis and cell dedifferentiation. This inhibition was proposed to be due to loss of protein synthesis in proliferating cells (Singer 1952; Shepherd, Downing, & Miyan, 2005). Addition of extracts containing nerve-derived soluble factors induced protein synthesis in the proliferating cells of denervated limbs leading to blastema growth (Singer 1952; Singer, Maier, & McNutt, 1976).

The failure in limb regeneration due to denervation was suggested to be dependent on many criteria such as the time of denervation, the site of amputation (distal or proximal), and the developmental stages at which amputation was performed (Kurabuchi & Inoue, 1983; Filoni & Paglialunga, 1990). For example, it was reported that severing nerves at the trunk before or simultaneously with limb amputation allowed the initial phases of wound healing and cell dedifferentiation. However, the proliferative capacity of cells was inhibited by denervation causing failure of regeneration (Crawford, 2001). The study of Kurabuchi and Inoue (1983) showed a complete failure of limb regeneration in *X. laevis* when denervation was performed immediately after amputation. The study showed a reduced protein synthesis and mitotic activity of regenerating limb.

In addition, the study reported that failure in regeneration was fastest when denervation occurred during the early days post amputation (20-40 days) suggesting that this regression was mainly due to cartilage degeneration (Kurabuchi & Inoue, 1983).

The study of Filoni and Paglialunga (1990) reported a success in regeneration when denervation occurred after blastema formation, thus suggesting that an influence of intrinsic factors on regenerative ability other than innervation during development. It also suggests that neutrotrophic factors are survival factors for proliferating cells during limb regeneration (Mescher, 1996; Bryant, Endo, &Gardiner, 2002; Yokoyama, 2008). Among the reported neutrotrophic factors are: Fgf-2, glial growth factor (Ggf) and transferrin, the iron transport protein in blood. Studies indicated that implantation of soaked beads with *Fgf-2* and Ggf in denervated blastema had induced limb regeneration. In addition, removal of transferring reported to cause an inhibition of blastema growth through inhibition of cell proliferation, and this inhibition was reversed upon the addition of transferrin *in vitro* (Mescher, 1996; Mescher & Neff, 2006).

Regeneration and Immunology

Loss of regeneration in anurans limbs coincides with major changes in the immune system, including development of the adaptive immune system and appearance of new cell types and signaling centers. Data of the comparative and developmental immunology suggest that development of the adaptive immune system influences the wound microenvironment and consequently affects signaling and patterning of mesenchymal cells, therefore a complete restoration of the damaged tissue cannot be achieved (Harty, Neff, King, & Mescher, 2003). The changes in regenerative capacity in amphibians including *Xenopus*, were correlated with changes in the immune system as

the animal matures (Harty, Neff, King, & Mescher, 2003; Mescher & Neff, 2005; Mak & Saunders (2006); Mescher & Neff, 2006). It was indicated that the immature immune system allows for fetal wound healing by tolerating the proliferation of cells of blastemas forming a new limb. On the other hand in the mature immune system, the appearance of new cell types and signaling centers favors rapid healing over regeneration leading to fibrosis and scarring of wounds. This inverse relationship between the capacity of limb regeneration and development of the adaptive immunity in amphibians is based on two facts (Harty et al., 2003; Mescher & Neff, 2005; Mescher & Neff, 2006). First, the adaptive immune system in urodeles, which have a regenerative capacity for limbs throughout their lives, is less efficient than adult anurans. Second, tadpoles' limbs regenerate completely but froglets limbs do not (Flajnik, Miller, & Du Pasquier, 2003; Toumerfier et al., 1998; Cohen, 1971; Mescher & Neff, 2006).

Studies indicated that the rejection of skin allografts by *X. larvae* is slower than by adults, and that allogeneic skin grafts were tolerated much longer during the metamorphic period than similar grafts made after metamorphosis (Izutsu & Yoshizato, 1993; Rollins-Smith, & Cohen, 1996). This difference in tolerance is mainly because of the difference in the efficiency of the immune system between larvae and adults.

To accommodate the larval-adult transition, general and local mechanisms are involved to avoid self-destruction by autoimmune mechanisms. The general mechanism involves a variation in antibodies, a significant turnover of lymphocytes, and secretion of significant amounts of immunosuppressants, mainly corticosteroids (Mescher & Neff, 2006). The study of Izutsu and Yoshizato (1993) showed an acute graft rejection in adult *Xenopus* even though the skin graft was excised from the same donor at larval stages,

cryopreserved and then grafted back to the same animal after metamorphosis.
In contrast, comparing "semi-xenogeneic" skin grafts at st53–54 (from *X.laevis –X. borealis* hybrids) yielded different results (Ono &Tochinai, 1995). In this study, an active induction of tolerance was developed to the skin grafts. The self/nonself tissue rejection was due to antibodies produced by adult *Xenopus*, which have greater diversity and binding affinity as well as more cytotoxicity for tumor cells than antibodies produced by larvae. In addition, the significant lymphocyte turnover in dermis and epidermis during metamorphosis caused incompatibility between larval and adult tissues in the same animal (Du Pasquier, Schwager, & Flajnik, 1989; Mescher & Neff, 2006). The tolerance was induced when these lymphocytes were isolated and injected intraperitoneally in a new host (Ono & Tochinai, 1995), however, this tolerance was not developed when thymectomy was performed on animals before injection (Cohen, 1971).

The local accommodation mechanisms in *X. laevis* larval-adult transition require the involvement of a variety of cells that promote immune tolerance in both epidermis and in the wound epithelia of regenerating limbs. These cells include Langerhans cells, dendritic cells, and other antigen presenting cells (Du Pasquier & Flajnik, 1990; Mescher & Neff, 2006). Complement factors are other modulators in the larval-adult accommodation and studies suggested their role in successful regeneration in amphibians, especially in proliferation and establishment of blastema (Kimura et al., 2003; Grow et al., 2006).

Cytokines and Limb regeneration

Cytokines are glycoproteins produced by a large number of cells. They are known for their roles in enormous biological processes including development, inflammation,

wound healing, apoptosis, hematopoiesis, differentiation and the function of myeloid and lymphoid cells (Yoshimura, Naka, & kubo, 2007; Krebs & Hilton, 2001). Cytokines and regulators of cytokines are believed to play a major role in determining limb regenerative capacity due to their role in resolving the inflammatory response that is usually triggered by amputation. Cytokines act by binding to specific membrane receptors, which lack an internal kinase activity. The receptors are associated with tyrosine kinases called Janus kinase (JAK) proteins. The second messengers, often tyrosine kinases, convey cytokine-induced signals into nucleus to alter cellular gene expression. Regulation of the induced signaling pathways is required to avoid the damaging consequences of excessive secretion of cytokines due to inflammation.

Cytokine binding induces receptor aggregation, which causes an activation of JAKs via cross-phosphorylation. The activated JAKs phosphorylate multiple tyrosine residues in the cytoplasmic domain of the receptor, forming docking sites for other signaling proteins containing Src-homology2 (SH2) or phosphotyrosine binding domains. Thus, cytokine stimulation initiates binding of many proteins including the signal transducers and activators of transcription (STAT).

Binding of STAT proteins via their SH2 to the tyrosine-phosphorylated receptors causes STAT phosphorylation on specific C-terminal tyrosine residues by JAKs, leading to their dissociation from the receptor to form homo- or heterodimers through their SH2 domains. The formed STAT dimers immediately enter the nucleus and then bind specific DNA sequences in the promoter regions of target genes for inducing their transcription (Krebs & Hilton, 2001). Among the induced genes are SOCS (Fig. 4).

Cytokine signaling is mainly regulated by negative feedback mechanisms. These mechanisms may either cause inhibition to JAKs in a direct or indirect way, or may cause ubiquitination and subsequent degradation of neighboring proteins by recruiting the proteasomal machinery to signaling complexes (Krebs & Hilton, 2001; Yoshimura et al., 2007). To date, three families of proteins are involved in the inhibitory process: tyrosine phosphatases, protein inhibitors of activated STATs, and SOCS proteins (Fig. 4).

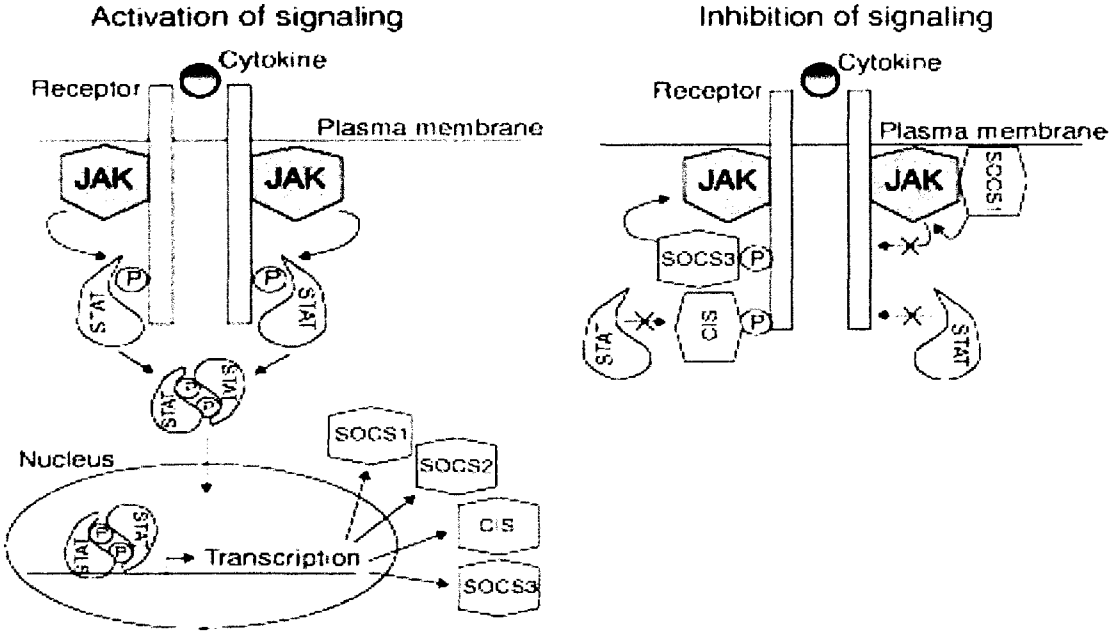


Figure 4 Proposed molecular regulatory mechanism of SOCS for cytokines

SOCS Family

SOCS are intracellular proteins known to be involved in various biological activities including development. To date, the SOCS family has eight proteins that have structural and functional homology. These are SOCS1 through SOCS7 and a cytokine-inducible SH2-domain-containing protein (CISs; also known as CISHs) (Fig. 5) (Krebs &

Hilton, 2001; Dalpke, Heeg, Bartz, & Baetz 2008). Presently, there are three nomenclature systems for SOCS proteins/genes although the SOCS nomenclature has become the most widely used. The primary amino acid sequence has shown that selected pairs of SOCS family are more similar to each other such as SOCS1 and SOCS3, SOCS2 and CIS, SOCS4 and SOCS5, and SOCS6 and SOCS7 (Krebs & Hilton, 2001; Yoshimura et al., 2007).

Many stimulators including cytokines induce SOCS proteins. Other inducers are cell stimulatory factors, growth hormones, lipopolysaccharide, statins, cAMP, and isoproterenol(Krebs & Hilton, 2001; Kubo, Hanada, & Yoshimura, 2003; Ilangumaran, Ramanathan, & Rottapel, 2004; Yoshimura et al., 2007; Dalpke et al., 2008).

Discovery of SOCS

In 1997, three independent groups using different experimental approaches identified *Socs1*. Using a functional screen for inhibitors of cytokine signaling, Starr and colleagues cloned and named a suppressor of cytokine signaling gene as SOCS1. Naka et al. identified SOCS1 as a protein recognized by a monoclonal antibody directed against the STAT3 SH2 domain, and referred to SOCS1 as STAT-induced STAT-inhibitor. The third group also identified SOCS1 as a protein that bound to the catalytic domain of JAK2 in a yeast two-hybrid screen and therefore called it a JAK-binding protein (Endo et al., 1997).

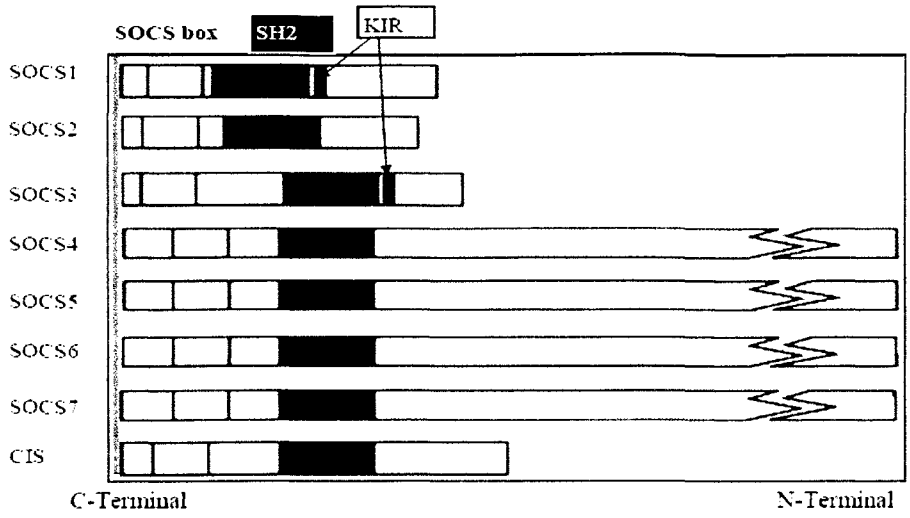


Figure 5. The general structures of SOCS proteins. The kinase inhibitory regions (KIR) of SOCS1 and SOCS3 are indicated in red. (Krebs and Hilton, 2001)

Structure of SOCS

All SOCS proteins have been shown to share three regions: a central SH2 domain, an amino-terminal domain of variable length and sequence ranging from 50-380 amino acids, and a carboxy-terminal with a conserved module of 40 amino-acid known as the SOCS box. The SOCS box has been found also in other proteins and is known to be involved in protein ubiquitination and proteasomal degradation (Kamura, Sato, Haque, & Li, 1998; Krebs & Hilton, 2001). The SOCS box Studies showed no recognizable motifs in the N-terminal region with the exception of SOCS7 that contained a recognized nuclear localization signal with multiple proline-rich regions (Krebs & Hilton 2001; Yoshimura et al., 2007). Only, SOCS1 and SOCS3 have a 12-residue kinase inhibitory region (KIR) located in the N-terminal of the SH2 region (Fig. 5). This domain was revealed to act as a pseudosubstrate to JAK enzymes inhibiting their catalytic activity Studies reported that

point mutations in KIR would completely shut down the suppressive effect of SOCS1 and SOCS3 on cytokine signaling (Kerbs & Hilton, 2001; Dalpke et al., 2008).

Molecular Mechanisms of SOCS

Via binding to a variety of signaling proteins *in vitro,* SOCS proteins were identified to play a role in a wide range of biological processes (Krebs & Hilton, 2001; Wormald & Hilton, 2007; Dalpke et al., 2008). An important function for SOCS proteins is their role as negative feedback regulators for cytokines. Because cytokines and other stimulatory signals are constantly present in the microenvironment of immune cells, SOCS proteins and other cytokine regulators are important for proper control of inflammation induced in response to amputation. This function is critical in determining the capacity for limb regeneration in an organism. Regulation of cytokine signaling via SOCS proteins can be through direct or indirect mechanism. For example, SOCS1 binds directly to the JAK causing an inhibition for cytokine activity in a direct mechanism. Other SOCS proteins, as SOCS3 and CIS, use an indirect inhibition such; however, each uses a different strategy. For example, SOCS3 binds to JAK-proximal sites on receptor blocking binding of cytokine, while CIS blocks the binding of STAT5 to the activated cytokine receptors (Krebs & Hilton, 2001). These pathways together culminate in the regulation of gene expression in the target nucleus, leading to cell differentiation, proliferation, survival, apoptosis or activation (Krebs & Hilton 2001). Expression of SOCS1 and SOCS3 is regulated by STAT as they both contain the KIR-domain. Both SOCS1 and SOCS3 employ different mechanisms for binding to JAK proteins. Studied showed that inhibition of JAK activity via SOCS1 is through a direct binding to the JAK catalytic domain through both KIR and SH2 domains, while SOCS3 binding to JAK

proteins is via proximal sites on the intracellular domain of activated cytokine receptors (Naka et al., 1997; Krebs & Hilton, 2001). SOCS1 was reported to interact directly with JAK1, JAK2, JAK3, and tyrosine kinase 2 (Naka et al., 1997). Additionally, other studies identified that SOCS1 has a higher affinity to JAK than SOCS3 (Krebs & Hilton, 2001; Yoshimura et al., 2007). Transcripts encoding SOCS1, SOCS2, SOCS3, and CIS are at low levels or undetected in cells; however, they are rapidly induced by many cytokines, both *in vitro* and *in vivo* (Wormald & Hilton, 2007).

In vitro systems where SOCS1 and SOCS3 were overexpressed, studies showed an inhibition of many cytokines. However, known roles of SOCS1 and SOCS3 are limited *in vivo*. Studies of knockout phenotypes and transgenic phenotypes for SOCS proteins are summarized in Table 1 (Dalpke et al., 2008).

Table 1

Summary of SOCS Knockout and Transgenic Phenotypes in Animals Suggesting the Possible Biological Roles of SOCS Proteins.

Gene	Knockout Phenotype	Transgenic Phenotype
SOCS1	• Neonatal lethality • Fatty liver degeneration • Lymphopenia • Accelerated apoptosis	• Disturbed T-lymphocyte development • Decreased number of γδ T-cells • Spontaneous T-cell activation
SOCS2	• Gigantism • Dysregulated growth hormone and IGF-1 signaling	• Gigantism • Dysregulated growth hormone
SOCS3	• Embryonic lethality • Placenta defects • Disturbed erythropoiesis • Altered IL-6 and LIF signal transduction	• Embryonic lethality • Increased Th2 differentiation • Altered IL-2 and NFAT signaling
SOCS5		• Disturbed IL-4 signaling • Disturbed Th2 differentiation
SOCS6	• Slightly decreased growth	
SOCS7	• 10% Smaller • 50% Lethality within 15 weeks postnatal due to hydrocephalus	
CIS	• No significant phenotype • 10% Smaller	• Reduced weight • Disturbed lactation • Altered IL-2 signaling

Effect of Beryllium on the Expression of SOCS1 and SOCS3

Beryllium toxicity was reported on different living tissues including liver, kidney, lymph nodes and skeleton. Direct contact with beryllium implicated in the development of skin cancer in individuals (CA: A Cancer Journal for Clinicians,1951). In amphibians, studies reported that immediate application of beryllium immediately to injured limbs blocked regeneration completely (Tsonis, 1990; Tsonis, English, & Mescher, 1991). A teratogenic effect of beryllium was also reported on early development (Witschi, 1970; ATSDR, 2002). In addition, beryllium was also found to have an inhibitory effect on gross differentiation of embryonic tissue and limb regeneration (Kimmerle, 1966; Witschi, 1970). To determine the precise mechanism for this inhibition, Witschi (1970) indicated that an intravenous injection of beryllium caused an *in vivo* inhibition for regenerating rat liver within 24 hours. This inhibitory effect was shown to be due to an inhibition of DNA synthesis via decreased activity of selected enzymes involved in DNA synthesis. Different immunological responses have been elicited by beryllium in different strains of guinea pigs. However, the precise inhibitory mechanism needs more investigation (Polák, Barnes, & Turk, 1968).

Significance

Limb regeneration decreases gradually in anuran amphibians as the animal progresses from early to late tadpole stages. There is evidence that changes in the tissue environment, particularly within the immune system, are responsible for the loss of the regenerative capacity. Suppression of the immune system in adult frog limbs might be one way to improve the regenerative capacity of limbs. Therefore, identification of immunomodulatory genes and determination of their expression pattern and functional

roles in limb regeneration are critical in improving the regenerative capacity in non-regenerative limbs. This knowledge may contribute to the medical therapies for conditions associated with failure of regeneration in humans.

Cytokines are involved in limb regeneration due to their role in resolving the inflammatory response triggered by limb amputation. SOCS proteins are integral to inflammatory responses, development and regeneration because they participate in the feedback inhibition of cytokines. No particular study, to my knowledge, was performed to determine the expression profile of any SOCS protein in response to amputation in *X. laevis*. In a microarray study performed to determine genes differentially expressed in regeneration competent vs. regeneration non-competent stages in *X. laevis*, SOCS3 was among the immune genes with a higher expression in regeneration non-competent stages. Studies indicate that the loss of regeneration is correlated to development of the immune system as the animal matures and the response to inflammation triggered by limb amputation changes. Because suppressors of cytokine signaling proteins are part of the immune system, the modification of their expression may contribute to improve regenerative capacity.

The primary goal of this three-part study involves the identification of two SOCS genes and determination of their expression patterns in response to limb amputation. The first part of the study isolated full-length cDNAs of SOCS1 and SOCS3 to allow functional studies in addition to *in vivo* localization of SOCS mRNA via synthesis of labeled-RNA probes used in whole mount *in situ* hybridization (WMISH). The comparison of the expression of SOCS1 and SOCS3 between regeneration competent and non-competent stages allowed for possible correlation between the development of the

immune system and the inflammatory response triggered by amputation. The second part of the study involved the determination of SOCS1 and SOCS3 expression in response to amputation in cultured blastemas at regenerative stage 53 for the assessment of the nature of the induction signal for expression if it was related to a local response or due to systemic signaling. The obtained data suggest that the response to amputation is a local response, which suggested a possible *in vitro* manipulation of limb regeneration. The third part of the study used limb cultures to determine the effect of beryllium as a hyper-inflammatory agent on the expression of SOCS1 and SOCS3, in addition to the expression of certain inflammatory and patterning genes (*IL-1β* and *Shh*, respectively).

Objectives

1. Isolation of a full-length SOCS1 cDNA from *Xenopus laevis.*
2. Determination of the temporal expression of SOCS1 in early developmental stages using reverse transcriptase -polymerase chain reaction (RT-PCR).
3. Isolation of a full-length SOCS3 cDNA from *Xenopus laevis.*
4. Determination of the temporal expression of SOCS3 in whole animals at early developmental stages using RT-PCR.
5. Determination of the temporal expression of SOCS1 and SOCS3 at both; regeneration competent stage53 and regeneration non-competent stage57 using RT-PCR.
6. Comparison of the spatial expression of SOCS1 at regeneration competent stage 53, regeneration limited stage55 and regeneration non-competent stage57, using whole mount *in situ* hybridization (WMISH).
7. Determination of the expression of SOCS1 and SOCS3 in cultured limbs in response to amputation to justify whether the induced expression in response to limb amputation as shown by RT-PCR was due to a local inflammatory effect or due to a systemic signaling.
8. Assessment of beryllium effect on the expression of SOCS1 and SOCS3 in response to limb amputation and the correlation to the expression of inflammatory and patterning genes as IL-1β and Shh, respectively.

MATERIALS AND METHODS

Preparation of solutions and media is included at the end of this chapter.

Animals and Surgery

Tadpoles of *Xenopus laevis* were obtained from Nasco (Fort Atkinson, Wisconsin, USA) or were raised from *in vitro* fertilized eggs in our laboratory. Developmental stages (Fig. 6) were described according to Nieuwkoop and Faber (1967). Hindlimbs at either regeneration competent (st53) or non-competent (st57) stages were amputated unilaterally or bilaterally at the mid-zeugopodia level. Tissues were collected 1mm proximal to the original level of amputation on 1, 3, 5, and 7 days post amputation (dPA). Total RNA was isolated using RNaqueous micro kit (Ambion, Inc.). Ethyl *p*-amino-benzoate (0.002% w/v) was used for all the surgical procedures. Anesthesia and surgical procedures were performed in accordance with U.S. government standards under the supervision of the institutional animal use committee.

In vitro Fertilization

To induce ovulation in a *Xenopus* female, an injection of human chorionic gonadotrophic hormone (hCGH) (Sigma) was given to each lateral side (10 units hCGH/ 0.15ml/side). Injections were given into the dorsal lymph sacs by inserting the needle just beneath the skin and between the dorsal lateral line stripes. The injected frogs were returned to their tanks, and left at room temperature overnight. Females were gently squeezed to collect eggs in Petri dishes containing artificial pond water, and eggs from

each female were treated individually.

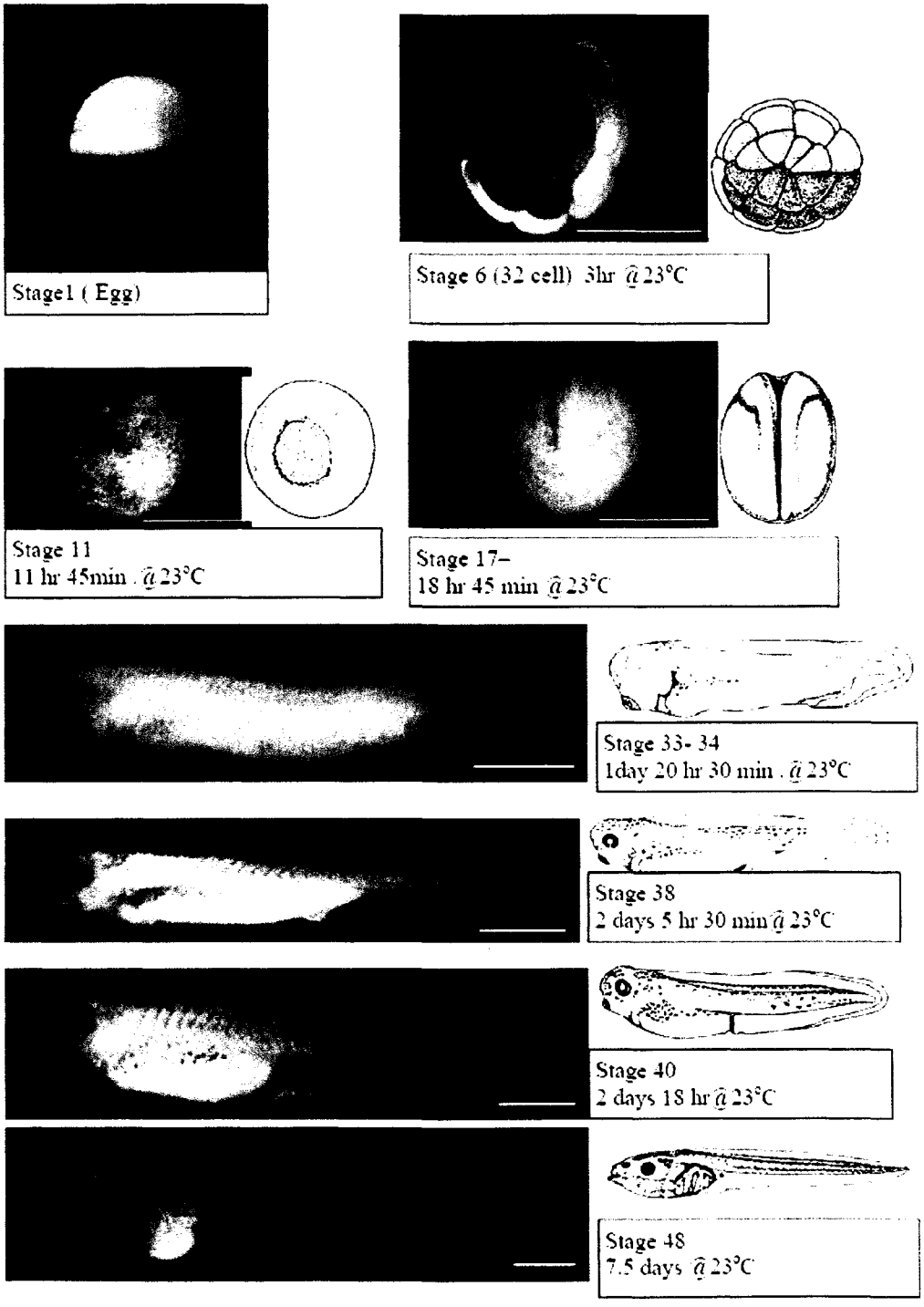


Figure 6. Diagram of anatomical characteristics of *X. laevis* at selected early developmental stages according to Nieuwkoop and Faber (1967).

For preparation of a sperm solution required for *in vitro* fertilization, a *Xenopus* male was euthanized via immersion in a solution containing an overdose of 0.02% (w/v) benzocaine (ethyl *p*-aminobenzoate) solution (Sigma). The male was dissected on ice to remove testes away from surrounding fat and tissues. Testes were placed individually in 1.5 ml Eppendorf tubes, each containing 1.0 ml of ice cold 0.1 X MMR buffer. Using a plastic pistil, testes minced finely on ice to release sperms. The viability and motility of sperms checked under the microscope before use for *in vitro* fertilization.

The process of *in vitro* fertilization begins with addition of 0.3ml of a previously prepared sperm solution was added to each Petri dish, and left for 2-3 min with gentle whirling of dishes to allow fertilization. Then, a sufficient volume of a freshly prepared 0.1X MMR buffer was added, and kept for an hour. The yolk rotation of eggs was checked periodically under the microscope for a successful fertilization. To loosen the surrounding jelly coats, eggs were transferred to clean Petri dishes containing 5-10 ml of 2% (w/v) L-cysteine HCl (Sigma), pH 7.9, and kept for almost 10 min with a gentle whirling. Eggs were washed three times in 10 ml of fresh 0.1X MMR buffers, each for 2-3 min to completely remove L-cystein. Then, the eggs were transferred to clean Petri dishes containing 0.1X MMR buffer containing gentamycin (5 mg/1ml) to avoid bacterial growth. The solution was changed twice over 8-9 hrs and the debris was removed regularly. When embryo cleavage reached stage 9, eggs from all dishes were combined and transferred to a tadpole-rearing tank containing artificial pond water until they hatched. Debris was removed repeatedly and tadpoles were fed four to five days after hatching, when the gastrointestinal tract was developed.

Polymerase Chain Reaction (PCR)

The PCR was performed for nucleic acid amplification using Platinum Taq DNA polymerase (Invitrogen/Life Technologies). Ornithine decarboxylase (ODC) was used as internal standard for mRNA expression (King, 1997). The volume of a PCR reaction was 25 µl and contained 1X hot master Taq buffer (Eppendorf), 2 m*M* $MgCl_2$, 10 pmol of each downstream and upstream primers, 200 µ*M* of all four deoxynucleotide 5′-triphosphates (dNTPs) mixture, 2–3 U Taq polymerase (Eppendorf) and 1µl of the desired RT-template (equivalent to 16.7ng of RNA input). The control reaction contained all components except the template. The volume for each reaction was adjusted to 25µl using DNase-free water. The amplification reaction was for 30 cycles; each cycle consisted of denaturation, annealing and extension. The PCR parameters were as follows: denaturation occurred at 94°C for 30 sec, except for the initial cycle, which was run for 5 min; annealing was set at 62°C for 30 sec, and extension occurred at 72°C for 30 sec. The PCR product was analyzed by agarose gel electrophoresis and photographed by ultraviolet transillumina-tion in the EpiChemi II Darkroom (UVP, California).

Reverse Transcriptase (RT) Reaction

Total RNA samples were extracted from whole embryos, blastemas and pseudoblastemas using the RNAqueous system (Ambion, Inc.) The RT-reaction was performed in 60 µl with 1µg of total RNA as template. An RT reaction equivalent to 16.7 ng of RNA was subjected to PCR. As templates for the PCR reaction, single strand DNAs were produced from total RNA obtained from the desired developmental stages by using SMART II cDNA synthesis (BD Biosciences Clontech, Inc.).

Quantitative PCR (qPCR)

The qPCR reactions were performed using the Mx3000P machine (Stratagene, La Jolla, CA) and SYBR Green chemistry for fluorescence detection (Bio-Rad, California). The 24-mer qPCR primers were high performance liquid chromatography purified (HPLC-purified). The optimal primer concentration was determined using a five-fold reciprocal dilution series for both primers starting with a primer concentration of 250 n*M* and ending with 50 n*M*. Each 25µl qPCR reaction was composed of 150nM of each primer and 2.8ng equivalents of RNA input of each RT reaction. All qPCR reactions were run for 40 cycles and were carried out in triplicate. Each RT reaction was equalized for RNA input by assessing the level of expression of the relatively invariant house-keeping gene ODC. Standard curves were generated for each primer pair using five-fold reciprocal dilution series ranging from 16.7ng to 0.27ng RNA equivalent of a stage that easily exhibits detectable expression of the gene of interest. Expression of each gene of interest was normalized to the level of ODC. Expression of each qPCR reaction of SOCS1 and SOCS3 was corrected for RNA input variation based on ODC level. A given qPCR value for both genes was then arbitrarily set to 1 (after normalization to ODC) and the remaining values were compared to that set value.

Primer Design

All primers were 24-mers with no more than a 50% G-C content. Primers were designed to be useful with an annealing temperature of 62°C and above (IDT Integrated DNA Technologies). The concentration of working solution of a primer used in PCR was 10 pmol/µl. Primers for qPCR had the same sequence as the end point PCR primers but were purified by HPLC (DNA Technologies) to ensure consistency of length.

SOCS1 primers were designed based on whole animal EST of *X. laevis* in the existing database. The accession number is BE026949. SOCS3 primers were designed based on existing data from a non-full length *X. laevis* cDNA with an accession number BC054214.

Primers SOCS 1

XSOCS1-U 5′-GTCGTTAATGCCCTGTGTTAGATC-3′

XSOCS1-D 5′-ATGCTGGGAAATTCAGTCTAGATG-3′

Primers SOCS3

XSOCS3-U 5′-CTTTAAGGGCCTTGGTTCTGGTTC-3′

XSOCS3-D 5′-GCGACGTCACCATTCATTGTCCAG-3′

Gel Electrophoresis

The agarose gel concentration was directly related to the size of fragments being analyzed. For determination of the size of PCR product, 2 % (w/v) agarose gels were used, while 1% (w/v) were used for analyzing products of plasmid digestion obtained by restriction endonucleases. Agarose low EEO powder (FisherBioTech.) was dissolved in 1X TAC buffer to obtain the desired concentration. The gel solution was slightly covered and heated in a microwave until it was completely melted. To facilitate DNA visualization by UV light, ethidium bromide (Sigma) in a final concentration of 0.5 μg/ml was added to gels with gentle whirling to avoid formation of air bubbles. The heated gel was left out on the counter until it cooled down to about 50°C. The cooled gel was poured into a casting rack with a 24-well comb which was sitting in an electrophoresis tank. After gel solidification, a sufficient volume of electrophoresis buffer, 1X TAC, was added to cover the gel, and then the comb was removed. To allow visualization of the

migrating DNA, the loading dye bromophenol blue juice was added to the PCR products (12% v/v) before sample loading them into the wells. The lid and power leads were placed on the apparatus and electrophoresis was applied at 130 volts and 400 mAmp for 45-50 min. The gel was visualized by ultraviolet transillumination. A standard 1kbp DNA ladder (Promega Inc.) was used to determine the desired DNA size. The control PCR reaction, which had no template, was run to the left of the DNA ladder to ensure cleanness of the control sample.

Growth of XL-1 Blue

The protocol for screening a cDNA library is summarized in (Fig. 7). It shows the requirement for initiating an overnight culture of the appropriate host strain of *Escherichia coli* (*E.coli*), XL-1 blue, for Lambda (λ) phage. Bacterial cells were incubated in about 3.0 ml of NZCYM media supplemented with 1.0 m*M* $MgSO_4$ and 0.3% maltose to ensure optimal phage infection and growth (Maniatis, Fritsch, & Sambrook, 1992; King 1997 p. 377). The culture was incubated at 37°C in the incubator shaker (C25 Incubator Shaker, New Brunswick Scientific) with continuous agitation. The standard volume of the overnight culture used for plating was 100µl /100 mm plate.

Screening of Recombinant DNA Library (cDNA)

Clone detection in a cDNA library was done using a PCR)-based selection method (King, 1997). To detect the presence of SOCS1 and SOCS3 clones, a screening was performed using libraries of various developmental stages of *X. laevis*. For each gene of interest, the cDNA library was tested using a 1 µl undiluted aliquot as a template using specific primers for the PCR assay. When a desired clone was detected, its amplification

using the designated cDNA library was performed (Fig. 7). The cDNA libraries used to isolate SOCS1 and SOCS3 clones were stage 53(st53) at three days post amputation (3dPA) cDNA and stage 11(st11) embryos, respectively. The cDNA libraries used in this study were generated previously by Dr. Michael W. King (Indiana University, Center for Regenerative Biology and Medicine) using total RNA isolated from the designated developmental stages and cloned into Uni-ZAP (Stratagene, Inc.), a bacteriophage Lambda (λ)-based vector.

Amplification of cDNA library

The primary screening started with plating ten 100 mm NZCYM plates using a selected dilution of the cDNA library. This required a previous initiation of an overnight culture of the appropriate host, XL-1 Blue of *E.coli*. One microliter (1μl) of the cDNA library was diluted in an appropriate volume of SM buffer to obtain the recommended plaque density for primary screening. To obtain the desired plaque density on plates have the final library concentration, an appropriate volume of this diluted aliquot was added to a 1000 μl aliquot of the overnight culture of XL1-Blue (100 μl culture/ 100mm plate). Prior to use, the NZCYM plates were warmed at 37°C for 20-30 to avoid immediate solidification of top agarose and to avoid formation of non-homogenous layer. The bacterial cells and phage mixture was incubated at 37°C for 10 min to allow initiation of the infection cycle of bacteria by phage. The mixture volume was divided into ten aliquots, equal to the number of plates used. To each aliquot of the culture mixture, 3 ml of 45°C melted NZCYM top agarose added by pouring on a previously warmed plate with gentle whirling to allow formation of a homogenous layer. The plates were left at room temperature to cool down and then incubated upside down at 37°C for a sufficient

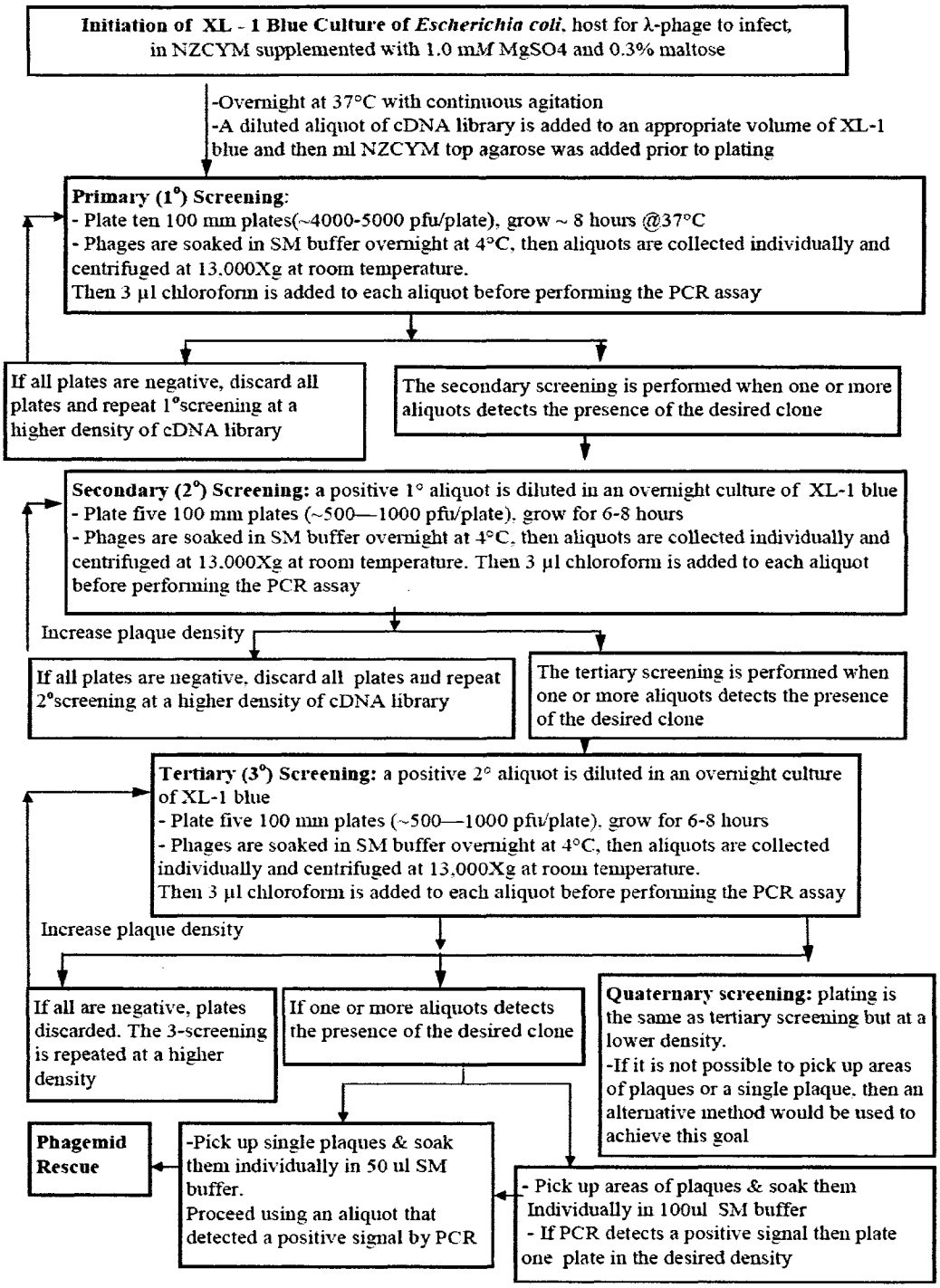


Figure 7 **General Protocol for the isolation of a clone from a cDNA library.**

time to form plaques at a density of 4000-5000 plaque forming unit (pfu)/plate. The incubation period was approximately 7-9 hours. The plates having the plaques were soaked in 3ml SM buffer overnight at 4°C to release phages containing clones. The aliquots were collected individually from each plate into 1.5 ml microcentrifuge tubes and centrifuged at 13,000 rpm for 10 min in an Eppendorf centrifuge (Centrifuge 5415 D) to remove bacterial debris and agar. Only 1ml of each aliquot was transferred to a new 1.5ml Eppendorf tube where 0.03% chloroform spectrograde (ACROS, New Jersey) was added to prevent bacterial growth and allow longer storage at 4°C. One microliter (1 µl) of each collected aliquot was used as a template for the PCR assay and the remainder was saved at 4°C for further possible use. All aliquots were amplified by PCR and the products were analyzed by gel electrophoresis. If one or more aliquots gave a positive signal for the desired clone, the next screening was performed (secondary, tertiary, and quaternary) using a similar procedure used for the primary screening. One modification for subsequent screenings was that five NZCYM plates were used for each round of screening. Each aliquot of interest was diluted in SM buffer and then added to 500 µl of an overnight culture of XL-1 Blue, a sufficient volume for five plates to obtain the desired plaque density on the plates.

The desired density of plaques was 1000-500 pfu/plate in a secondary screening, 100- 500 pfu/plate in a tertiary screening, and 50 pfu/ plate in a quaternary screening. Culturing and screening continued until a single plaque containing the desired cDNA was successfully isolated, which usually occurred by quaternary screening. Otherwise, a different method would be used which will be described later for SOCS1. In case the clone of interest was not detected in all aliquots obtained from a particular screening, an

increased concentration of the previous positive aliquot would be used to perform a second round of the same screening.

For isolation of SOCS1 cDNA, a single plaque representing a phage containing the clone could not be picked up with multiple repeats for the quaternary screenings. Consequently the radioactive gene-screening method was performed (Fig. 8).

For isolation of SOCS3 cDNA, the PCR assay indicated the presence of a phage containing the clone after the quaternary screening. Therefore, this screening was repeated using the same tertiary aliquot that had given positive quaternary plates and at the same density. However, the formed plaques were not soaked in SM buffer but plates were cooled at 4°C in order to pick up individual plaques. The technique for picking up single plaques will be discussed in the following section.

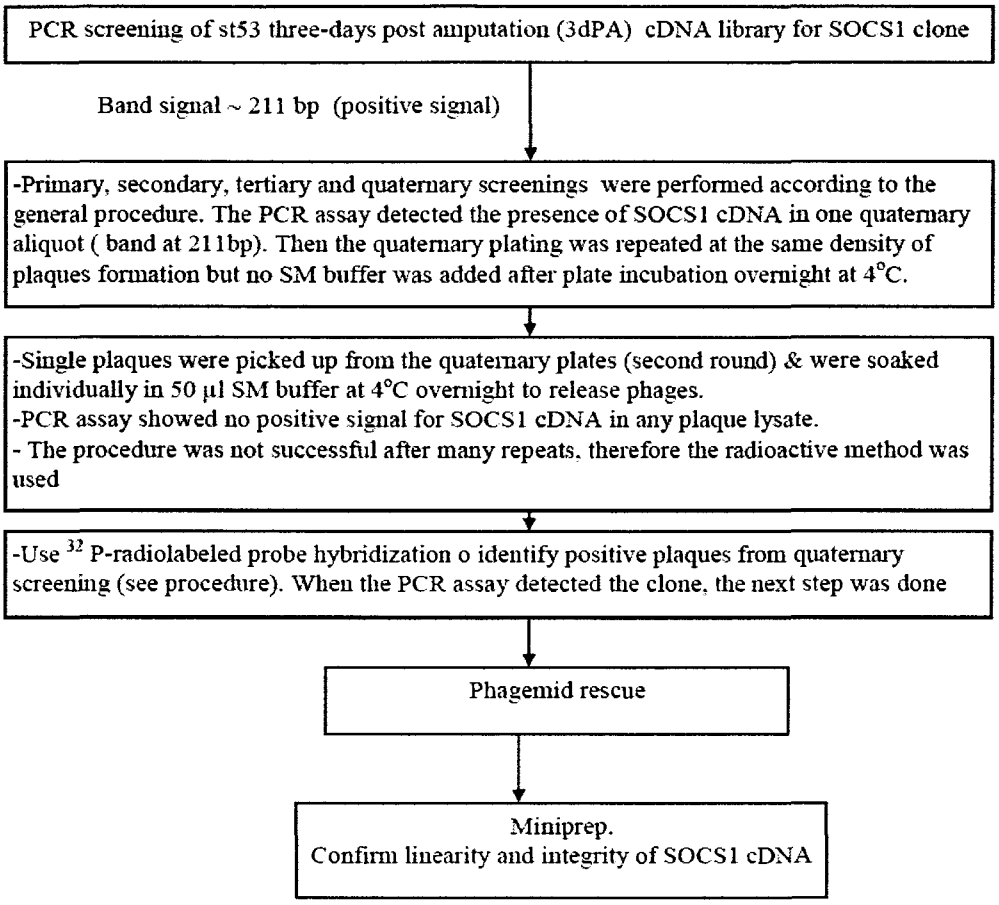


Figure 8. Summary for SOCS1 isolation in *X. laevis* using st53 (3dPA) cDNA library.

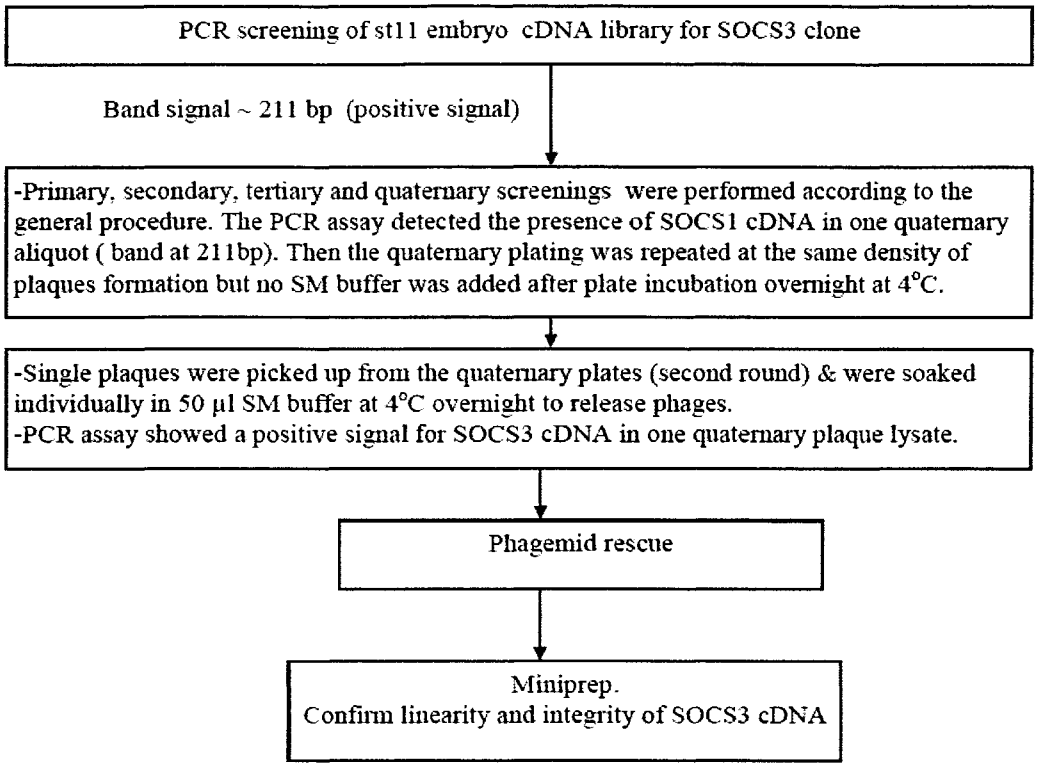


Figure 9. Summary for SOCS3 isolation in *X. laevis* using st11 cDNA library.

Single Plaque Screening (Phage Stock)

For SOC3, sterile Pasteur pipettes were used to pick up individual plaques off plates obtained by a second round of a quaternary screening. Each plaque was then eluted in 50 µl of SM buffer and kept at 4°C overnight to release phages. These single plaque aliquots were used as templates for the PCR assay using 1 µl of the aliquot. Analysis of PCR products by gel electrophoresis and visualization under UV light detected the presence of SOCS3 clone in one aliquot. The single plaque aliquot containing SOCS3 cDNA was the Lambda (λ) phage stock, and ideally it would contain $>1\times10^5$ phage particles. This phage stock was used for later isolation of plasmid and excision of SOCS3 cDNA (Fig. 9).

***In vivo* Excision of Phagemid from Lambda UniZAP Vector (Phagemid Rescue)**

The phagmid was isolated from the λ-UniZAP vector to allow later excision of SOCS3 cDNA. The protocol per manufacturer (Stratagene) started with inoculation of individual cultures of XL-1 Blue and SOLR. The XL-1 Blue *E.coli* bacteria, the appropriate host for λ-phage, was inoculated into 5 ml of NZCYM media supplemented with 1.0 m*M* $MgSO_4$ (Sigma) and 0.3% maltose (Sigma), while SOLR *E.coli* bacteria was inoculated into 5ml of LB broth. Both cultures were grown overnight at 37°C in the incubator shaker (C25 Incubator Shaker, New Brunswick Scientific). A 40 µl aliquot of a fresh overnight XL-1 Blue culture was diluted in 1 ml NZCYM media with supplements and incubated at 37°C for 1 hr to allow proliferation of bacterial cells. Only 200 µl of this diluted culture was mixed with 5 µl of λ-phage stock plus 1 µl of ExAssist helper phage (titer $>1 \times 10^6$ pfu/ml) (Stratagene) and incubated at 37°C for 15 min with agitation to allow phage attachment to bacterial cells. To this mixture, 3 ml of LB media were added and the entire solution was incubated for another 2.5hr. One and a half milliliters (1.5 ml) of the culture mixture was transferred to an Eppendorf microcentrifuge tube, and centrifuged at 4000 xg for 5min to remove cell debris. The supernatant was decanted into a new microcentrifuge tube and heated at 70°C for 15 min to kill bacterial cells. To pellet the cell debris, the mixture was centrifuged at 8000 xg for 5min, and the supernatant transferred to another microcentrifuge tube. This stock was the rescued phagemid because it was an excised pBluescript (PSK-) phagemids packaged as double-stranded DNA into phage protein particles. Only 1ml of the supernatant was transferred to a new microcentrifuge tube, which would be stored at 4°C for up to 1-2 months.

For a successful isolation of the cloned SOCS3 cDNA from the excised phagemid, SOLR cells were infected with the excised phagemid and plated on LB-amp plates.The use of SOLR allowed only the excised pBluescript phagemid to replicate because it excluded the possibility of co-infection from the ExAssist helper phage present in the phagemid aliquot due to a genetic mutation. The use of ampicillin plates allowed only the growth of infected cells with pBluescript double-stranded phagemid containing the DNA insert. To 100 µl freshly grown overnight SOLR culture, 5 µl of phagemid stock was added and incubated at 37°C for 15 min. Using two LB-ampicillin agar plates, 10 µl of the culture mixture were poured on one plate, while the remaining 95 µl of the mixture was poured onto the second plate. Both plates were streaked well and incubated overnight at 37°C. Only colonies with the pSK-plasmid had the plasmid containing the cDNA insert grown on LB-amp plates. These colonies were required for the miniprep procedure.

cDNA Recovery from Plasmid (Miniprep.)

The miniprep protocol was prepared per QIAGEN manufacturer's instructions. A QIAprep 8 miniprep kit used for high-copy DNA purification was used. Sterile toothpicks were used to transfer 5-6 colonies into individual culture tubes containing 3 ml of LB-amp broth. These tubes were kept overnight at 37°C in the incubator shaker. Each culture was remained treated individually to ensure uniformity of colonies. From each culture, only 1.5 ml was transferred into a microcentrifuge tube while the remaining volume was stored at 4°C for possible later use. Each culture suspension was then centrifuged at 13,000 xg for 5min to allow cell precipitation. The supernatant was aspirated and cell pellet was dissolved well in a minimal amount of SM buffer and then subjected to the miniprep procedure.

The protocol started with lysis of bacterial cells under alkaline condition. The lysate was subsequently neutralized, adjusted to high-salt binding conditions, and purified on a QIAprep silica membrane. The isolated plasmid was absorbed on a silica membrane, eluted and stored at -20°C for later use.

After completing the miniprep preparation, supernatants were transferred to clean microcentrifuge tubes for PCR assay. One negative control was used to ensure cleanness of the reactions and two positive controls were used to ensure the size of the plasmid containing the desired insert. The two positive controls used were a previously positive plaque aliquot and the phage stock. The size of PCR product was analyzed by electrophoresis and visualized by UV light. When one or more miniprep samples showed the proper size of SOCS3 cDNA, the bacterial glycerol stock was prepared using the remaining bacterial culture that was previously stored at 4°C.

The miniprep procedure was also done for SOCS1 using an aliquot of a single plaque that was obtained from the radioactive gene-screening after it was detected to represent a single phage containing the SOCS1 insert.

Radioactive Gene-Screening Method (DNA Radioactive Probe)

The PCR-based selection method was not successful for the isolation of SOCS1 cDNA, therefore in an attempt to isolate a single plaque containing the SOCS1 cDNA from st53 at 3dPA cDNA library, an alternative method was used (Fig. 10). This method utilizes X-ray film images of five plates obtained through a second round of tertiary screening with no addition of SM buffer after plaques formation. The method depends on lifting phage particles from plates onto 85mm nylon filters. These filters were then hybridized with a previously prepared complementary ^{32}P-labeled DNA probe as

summarized later. The original plates used for filter lifting were then stored at 4°C for later possible use. The filter lifts were considered as replicas of the plates. Therefore, these filters were laid onto X-ray film to produce an identical image of the plates. When the X-ray film detected possible single plaques containing SOCS1 cDNA on plates, these plaques were picked up using sterile Pasteur pipettes and soaked in 50 µl of SM buffer overnight to release phages. The PCR assay was performed on plaque aliquots using SOCS1 primers to detect the presence of the desired clone.

The summary for detection of SOCS1 clone by hybridization is summarized below:

I. **Filter lifting**: Transferring and fixing a portion of the amplified cDNAs of st53 at 3dPA cDNA library including SOCS1 cDNA from plates onto nylon 85 mm filters.
II. **Probe Hybridization**: Hybridizing the transferred SOCS1 cDNA onto the filters with a complementary ^{32}P-labeled radioactive labeled probe.
III. **Autoradiography:** Localizing the radioactively labeled cDNA via exposing filters to X-ray film at -80°C overnight. The film was developed in the dark and dried off to observe dark spotting that usually represents plaques possibly containing the gene of interest. The film aligned with plates via marks to identify representing plaques.
IV. **Plaque Pick-up:** Plaques representing the phages containing the SOCS1 cDNA were picked up and soaked in SM buffer overnight. The aliquots were amplified by PCR assay and checked for SOCS1 cDNA by electrophoresis and UV light.

Filter Lifting

To isolate SOCS1 clone from the cDNA at st53 at 3dPA, five 100 mm NZCYM plates were obtained by a second round of quaternary screening. After formation of plaques at a density of 50 pfu/plate, no SM buffer was added to the plates. In order to

avoid agarose detachment during filter lifting, the plates were then kept at 4°C.
A replica for each plate was created onto an 85 mm nylon filter (Millipore Co., Bedford). The procedure of filter lifting was done at room temperature using forceps and wearing gloves. Three non-symmetrical ink marks were positioned on the edges of each filter to specify orientation. With the marks facing up, filters were laid with gentle pressing onto the plates and left for 5 min to allow plaque transferring. Formation of wrinkles and bubbles was avoided to obtain identical images of plates. During this time, aligned marks on filters were placed on the back of the plate to ensure proper orientation upon matching with filters after hybridization. Filters were gently lifted off of the plates and laid plaque side up individually onto a sheet of plastic wrap containing small volumes of a denaturing solution (1.5 *M* NaCl, 0.5 *M* NaOH) for 5 min. This allowed denaturation of the cDNA on the filters. Excess solution was dried off the filters using paper towels. Filters, with plaque side up were then placed on another sheet of a plastic wrap with a neutralization solution (1.5 *M* NaCl, 0.5 *M* Tris-HCl; pH 7.5) for 5 min. Filters were dried off completely on paper towels and were placed into the UV chamber (GS Gene Linker, BioRad) at 150 mJ for 2 min to allow cDNA immobilization and cross-linking. Filters were stored between two pieces of blotting paper in a closed dry place until hybridization started.

Preparation of ^{32}P-Labeled DNA Probe

The ^{32}P-labeled probe was prepared using a kit called Prime-a-Gene Labeling System (Promega). This kit uses a mixture of standard random hexadeoxyribonucleotides. The principle is based on a method developed by Feinberg and Vogelstein (1983a & b) whereby DNA synthesis is primed *in vitro* from a linear double-stranded DNA

template and incorporates more than 60% of the labeled hexamers.

The procedure for preparation of the DNA template used is described in a later section (see PCR product section). The concentration of this template was 25 ng/ml. Initially, the linear double-stranded DNA template was denatured at 95°C-100°C for 2min and then was chilled immediately on ice to prevent annealing of DNA strands. Preparation of the as per manufacturer's protocol and the radioactivity of the synthesized probe was checked to standards by the Geiger counter. The probe was used immediately in hybridization or stored at -20 ° C for later use.

Probe Hybridization

Prior to starting, the genomic hybridization buffer was warmed to 65°C to ensure complete dissolving of ingredients. The prepared filters with the immobilized cDNA were rolled, with plaque side facing inside, and were placed into cylindrical hybridization glass bottles. Then for prehybridization, a sufficient volume of the prewarmed genomic hybridization buffer was added to the hybridization bottle. The bottle was kept at 65°C with continuous rotation for at least 4 hrs. in a hybridization oven (HYBAID, Midwest Scientific, St. Louis). The buffer was then discarded and two solutions were added to the bottle, 5 ml of warm genomic hybridization buffer and a previously prepared ^{32}P-radio-labelled probe resulting in a probe concentration of 10^6-10^7 cpm/ml buffer.

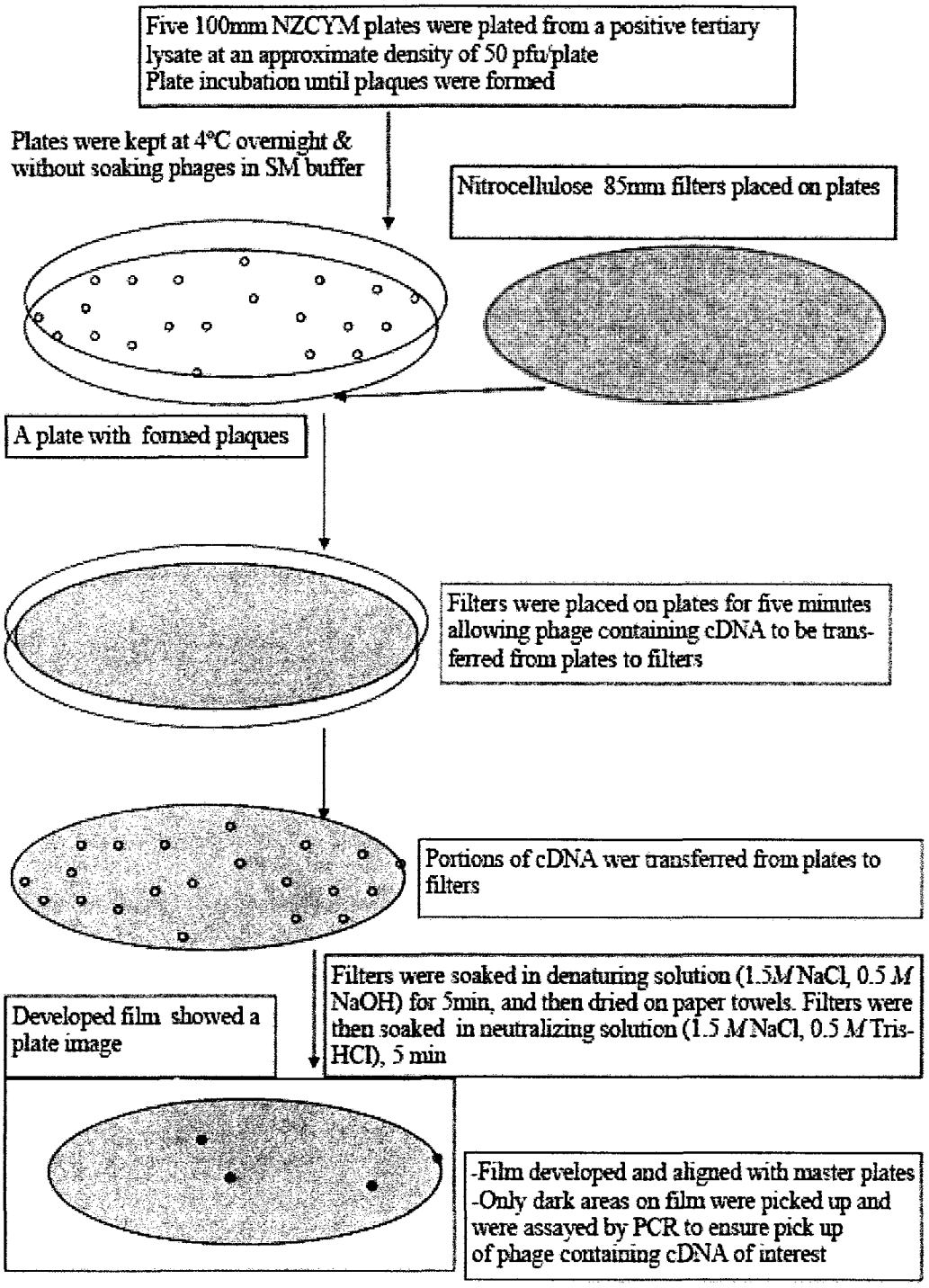


Figure 10. Procedure for filter lifting which was required for the radioactive screening method.

Prior to the probe use, it was boiled for 5 min and then snap-chilled on ice for 2-4 min. The hybridization was performed at 65°C for almost 18 hrs with continuous rotation in the hybridization oven to ensure uniform probe distribution. The probe was then discarded according to the safety standards and filters were washed with a minimal volume of washing buffer (0.2 X SSC / 0.1% SDS; see solutions). Filters were transferred to a Pyrex dish containing 500 ml of washing buffer in a water bath that was set at 65°C in the hot shaker (Bellco Glass, NJ). To ensure removal of unhybridized probe, washing was done twice at 45 min each time using 500 ml of washing buffer with gentle shaking. Filters were then slightly dried off with paper towels and wrapped with a sheet of a plastic wrap with edges folded to avoid fluid leaking onto X-ray film. In the dark, the wrapped filters with plaque facing up were laid onto an X-ray film cassette and the film was laid on top of filters. The cassette was tightly locked and kept overnight at -80° C. The next day, the film was developed and dried out to visualize dark spots. After film development, a few dark spots were observed indicating the possible presence of single phages containing SOCS1 cDNA. To allow proper pick up of plaques represented by the dark spots, marks on filters and plates were aligned. Using sterile Pasteur pipettes, plaques were picked up individually, and each plaque was transferred to an Eppendorf tube containing 50 µl of SM buffer. The aliquots were kept at 4°C overnight to allow the release of phages from the agarose and subsequent cDNA diffusion. The PCR assay was performed on all samples, and products were analyzed by gel electrophoresis and visualized by UV light. One aliquot was found to contain the SOCS1 cDNA. This aliquot was used for phagemid excision and DNA recovery.

Preparation of a Linearized Double-Stranded DNA containing SOCS1 cDNA (PCR Product)

For a preparation of the complementary ^{32}P-labeled DNA probe, an amplified sample containing a linearized double-stranded plasmid containing SOCS1 cDNA was required. Three PCR reactions were performed using a positive lysate obtained from tertiary screening as a template. Electrophoresis using 2% agarose gel was performed on the PCR products in three adjacent wells in addition to a positive control. The gel was visualized under UV light to assess the size of the product. The bands were excised from the gel with a razor blade under UV light. The bands were combined in one Ultrafree-DA Millipore column (Amicon). The column was placed in a 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for 10 min to elute the SOCS1 cDNA from the gel. The eluted solution was labeled as PCR product of SOCS1 cDNA and stored at -20°C until use.

The product was cloned into the pCS2+ vector multicloning site. In order to linearize the vector using the proper restriction endonucleases, Xho1 and Hind III, a reaction was initiated with a solution containing 1.0µl of each enzyme (final conc. 1X), 5.0µl SOCS1 cDNA, and 3.0µl of 10X buffer (final conc. 1X). The volume was adjusted to 30 µl using DNase-free water and the reaction was kept at 37°C for 1.5hr. The size and integrity of the linearized vector was confirmed by 1% gel electrophoresis. This aliquot had the linearized double-stranded pCS2+ vector with the cloned SOCS1 cDNA and it was used as a template in preparation of the ^{32}P-labeled DNA probe.

Glycerol Stock

A bacterial glycerol stock was maintained in the laboratory for long-term storage for every construct. After confirming the presence of SOCS1 and SOCS3 in the miniprep

culture, this positive miniprep culture was used for preparation of 32% glycerol stocks for both cDNAs, SOCS1 and SOCS3. This glycerol concentration was obtained by mixing 940 µl of 80% glycerol and 560 µl of the positive miniprep culture with a vigorous vortex to ensure homogenization. The glycerol stock was kept at -80°C for later use.

Preparation for Whole Mount *in situ* Hybridization (WMISH)

WMISH requires preparation of blastemas and pseudoblastemas at the proper stages. In addition, this procedure requires preparation of digoxigenin (DIG)–labeled RNA probes of the gene of interest (Mescher et al., 2007). A summary for the procedure is in Figure 11.

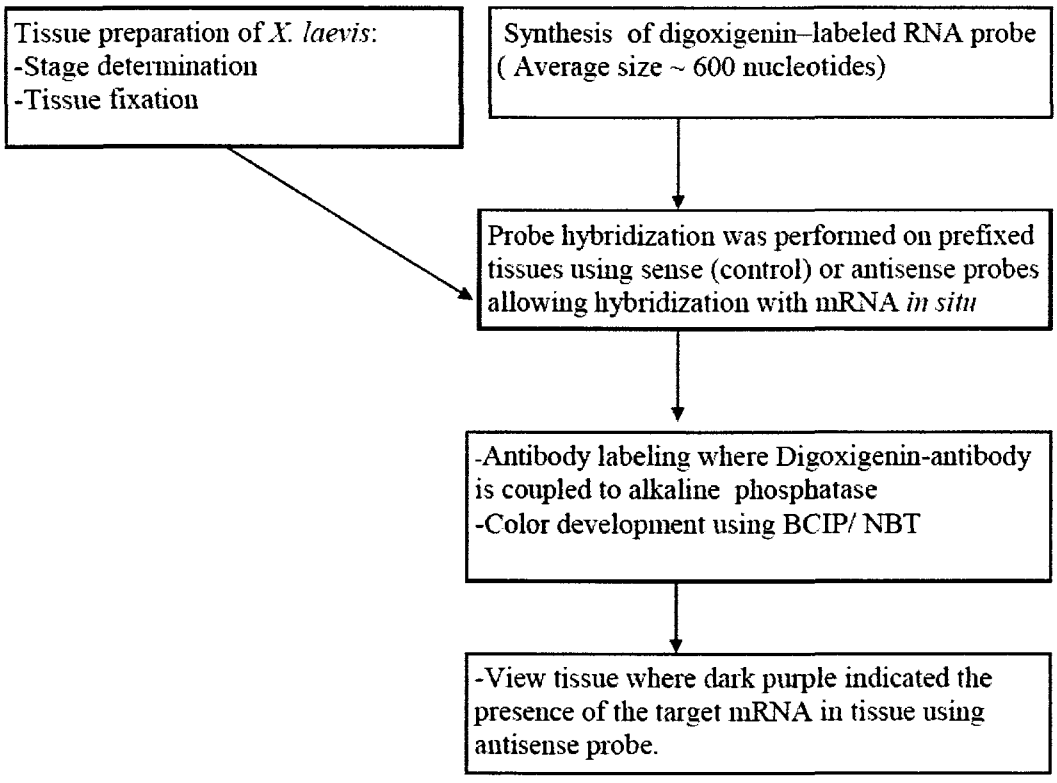


Figure 11. Protocol for whole mount *in situ* hybridization (WMISH).

For tissue preparation, stages of *Xenopus laevis* larvae at regeneration competent (st53), limited (st55) and non-competent (st57) stages were determined. Larvae were fixed overnight at room temperature in MEMFA [1X MEM buffer (pH 7.4) /4% formaldehyde (Sigma)]. Then, larvae were stored in 100% methanol (Merck, Germany) at -20° C until use. Tails were removed and larvae were dissected midsagittally to provide experimental and control tissues of the same larva.

For the preparation of DIG–labeled RNA probes, 5µg of pSK-plasmid was linearized with EcoR1 in a 50-µl reaction total volume at 37°C for 1.5 hr. The product was purified using an enzyme remover cartridge (Millipure AZ, Amicon). Plasmid integrity and purity were checked by analyzing 5µl of the linearized plasmid via electrophoresis using 1% agarose gel. Promega Riboprobe system buffers and nucleotides, and Roche DIG- UTP were used to prepare the DIG-probe.

Two transcription reactions were initiated, a sense probe as a control and an antisense probe which was the complementary strand to the transcribed SOCS1 mRNA in cells. Each reaction had a total volume of 50µl containing 5µl (1 µg/ µl) of the linearized plasmid (pSK) with the cloned SOCS1 cDNA, 3µl of RNase–free water, 1µl Rnasin (Promega), 10 µl 5x transcription buffer, 10 µl of 100 m*M* DTT, 5µl of 10 m*M* each of the nucleotides (rATP, rGTP, rCTP), 3.2 µl (10mM) rUTP, 1.8 µl DIG-UTP and 1.5 µl RNA polymerase. The polymerase used was either T7 polymerase (Promega) (for the antisense probe) transcription or SP6 (Invitrogen) (used for the sense probe transcription). Both reactions were incubated for two hours at 37°C and then each reaction was divided into two aliquots, one of which was saved for a possible use later. To the second aliquot of each reaction, 2µl of RQ1 Dnase was added and incubated for 15 min at 37°C to get

rid of the untranscribed DNA template. Then, to stop the transcription reaction, a 25 µl of 7.5*M* ammonium acetate (Sigma) was added to the reaction and then followed by the addition of 150 µl of cold 100% ethanol (-20°C). Immediately thereafter, the solution was centrifuged at 14,000rpm for 30 min at 4°C to precipitate the transcribed RNA. The supernatant was removed carefully to avoid pellet disturbance. The pellet was representing the DIG-labeled RNA probe. This pellet was washed carefully with 700 µl of 70% cold ethanol (-20°C) and centrifuged at 13,200rpm for 2min at room temperature. The supernatant was poured out without excessive dryness to allow complete dissolving of the pellet in 80 µl of RNase-free water. The optical density was determined using Eppendorf's Biophotometer. For *in situ* hybridization, the probe was base-hydrolyzed into 600 base-pair fragments immediately before use. The following formula was used to determine the desired length of the probe:

$$\text{Hydrolysis time in minutes} = \frac{\text{(starting probe size – desired size)}}{0.11\ \text{(start size x desired size)}}$$

The hydrolysis was done by adding 20 µl of a basic solution composed of an equal volume of 0.4 *M* sodium bicarbonate and 0.6*M* sodium carbonate to the RNA probe with a quick vortex followed by a centrifugation for few seconds. Then, the reaction was heated at 60°C for a calculated time sufficient, from the above formula, to generate 600bp fragments. The hydrolysis was terminated by the addition of 50 µl of lithium chloride (from Ambion mMessage mMachine kit) with well vortex. The reaction was then placed at -20°C for at least 30 min. before it was centrifuged at 14,000rpm at 4 °C for 30 min. The supernatant was removed carefully and discarded, while the pellet was washed, without vortex, using 700 µl of cold (-20°C) 70% ethanol. The pellet, the DIG probe, was centrifuged at 13,000rpm for 2 min. The supernatant was removed carefully and

discarded, while the pellet was dried well using the speed vacuum. Then, the probe was resuspended in 10-20 µl of RNase-free water and the optical density was read. The concentration of the probe was at least 1 µg RNA to be used in WMISH.

Whole Mount *in situ* Hybridization (WMISH)

The three-day procedure was done at room temperature, unless otherwise noted.

Day 1: Hybridization

RNase-free conditions and reagents, were required on the first day of the procedure. The previously fixed tissues were rehydrated into a final concentration of 1X PTw (1X PBS/0.1% w/v Tween 20) (Sigma) using decreasing concentrations of methanol in 1X PTw in a step gradient solution. After 15 min, tissues were treated for 2 min with Proteinase K (Sigma) at 10µg/ml in 1X PTw at room temperature, and then tissues were washed in 1X PTw twice for 15 min each. The limb tissues were refixed in 4% paraformaldehyde (Sigma) in 1X PTw for 20 min and then washed with 1X PTw several times for 5 min each. The 1X PTw was replaced with the hybridization buffer for 3-7 hr at 70°C. The concentration of a stock DIG-labeled RNA probe was determined via spectrophotometer. Immediately prior to use, the RNA probes were heated at 95°C for 3 min, and then diluted with 2 ml of a preheated hybridization buffer to obtain a final probe concentration of 1µg/ml. The concentration for the sense and anti-sense probes was the same. Tissues were placed in small vials for hybridization and either sense or antisense probes were added. The hybridization was done at 70°C for 12- 15 hr.with gentle rotation in a water bath.

Day 2: Blocking Reaction

The RNA probe was replaced with a pre-warmed hybridization buffer and was kept at the hybridization temperature (70°C) for 10 min under constant rotation. Tissues were washed in 2X SSC several times for 20 min each at 37°C, then washed twice with 0.2X SSC for 30 min each at 70°C. A stepwise change for tissues from the hybridization buffer to 0.1 *M* maleic acid buffer (Sigma) was done at room temperature. Then the reaction was blocked at room temperature for 3-4 hrs using 2% blocking buffer (Roche Diagnostics), which was prepared as per manufacturer's instructions. The blocking buffer was then replaced with an antibody solution (alkaline phosphatase conjugated anti-digoxigenin antibody) (Roche Diagnostics) previously diluted to 1:2000 using blocking solution (2 µl antibody/1.0 ml of fresh 2% blocking buffer). The blocking reaction was conducted overnight at 4°C with a continuous agitation. No primary antibody was added to the control samples that were treated with the sense probe.

Day 3: Color Development

The principle of color development was dependent on the formation of an insoluble black-purple precipitate. The formation of this precipitation was the result of an alkaline phosphatase reaction with a combination mixture of BCIP and NBT [5-bromo-4-chloro-3-indolyl-phosphate (Roche Diagnostics) and 4-nitro blue tetrazolium chloride (Roche Diagnostics)]. The intensity of the developed color was monitored by observation.

Tissues were washed several times for 1 hr each in 0.1 *M* maleic acid buffer at room temperature, then washed with gradually decreased concentrations of maleic acid in 1X PTw. After a final wash in 1X PTw, tissues were placed in TTLMN buffer for 1 hr at

room temperature. The color reaction through alkaline phosphatase was performed in the dark per manufacturer's protocol. This protocol was done by the addition of a solution containing 7.0 µl of BCIP and 9.0 µl of NBT in TTLMN to tissues on ice. Color development in tissues was monitored in the light every 15 min and the reaction was stopped upon achieving the desired color intensity by washing tissues several times in fresh cold TTLMN buffer at room temperature for 2 hrs. Tissues were then dehydrated stepwise with solutions with gradually increasing concentration of methanol until 100% methanol was reached. To remove excess staining, tissues were treated with 10-15% hydrogen peroxide in 70% methanol for about 2-5 hrs. Tissues were then placed in 100% methanol for 1-2 hrs with continuous agitation and then re-fixed in MEMFA. The tissues were washed multiple times with methanol and photographed digitally using a bright-field Nikon stereoscope.

Cultured Limbs

For each round of experimentation, five larvae of *X. laevis* at a regeneration competent stage (st53) were bathed overnight in a sterile 10% Steinberg's/antibiotic solution to avoid infection upon amputation. The larvae were anesthetized in benzocaine solution and then transferred to the amputation solution to perform the amputation while larvae were submerged in the solution. The sequences of the cuts are described as follows (Fig. 12). First, the tip of the limb was cut and labeled as the paddle, while the remaining part of the limb was amputated from the trunk, and it was cut into two pieces; the piece closer to the trunk was labeled as the proximal while the other piece was labeled as a distal (the portion containing the blastema) (Fig. 12). The pieces were then subjected to RNA collection immediately (0dPA) or each group was cultured in 800 µl of culture

medium for either 1dPA, 3dPA, 5dPA, or 7dPA. At the day of RNA collection, each group of pieces (paddle, distal, proximal) was placed in 300 µl of LB. The RT-PCR assay was performed on all samples to determine the induced expression of SOCS1 and SOCS3 in response to amputation in the collected pieces of limbs.

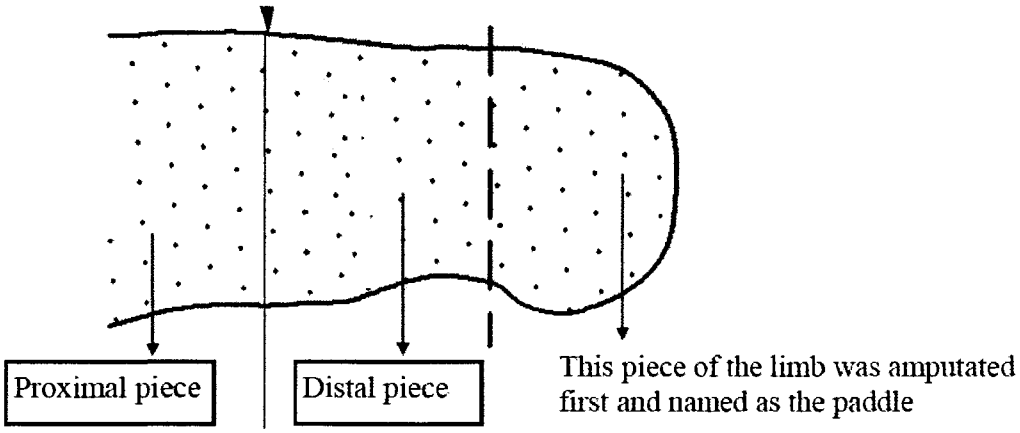


Figure 12. Diagram for limb amputation into three pieces for limb cultures. The paddle was cut first, then the remaining piece of the limb as cut into distal and proximal pieces. PA: post amputation. Dotted line indicates the first amputation plane while the arrow and solid indicates the second amputation plane.

Effect of Beryllium on the Expression of SOCS1 and SOCS3

For beryllium treatment, an initial insult to the limb was made by making a partial snip (Fig. 13). The limbs used as controls were not dipped in beryllium. Immediately after making this cut, while the limb was still attached to the trunk, it was dipped in beryllium solution (10 m*M* $BeSO_4$ in 0.67X PBS). Then, two cuts were made: a partial cut at the amputation plane was completed and the tip of the limb was discarded. The remaining portion of the limb was amputated from the trunk (Fig.13). The limbs were

cultured in LB media for either 1, 3, 5, or 7dPA and then subjected to RNA collection. The 0dPA samples were subjected to RNA collection immediately after the second cut was made. The RT-PCR assay was performed on all samples (control and beryllium- treated) to determine the expression profile of SOCS1, SOCS3, Shh and IL-1β.

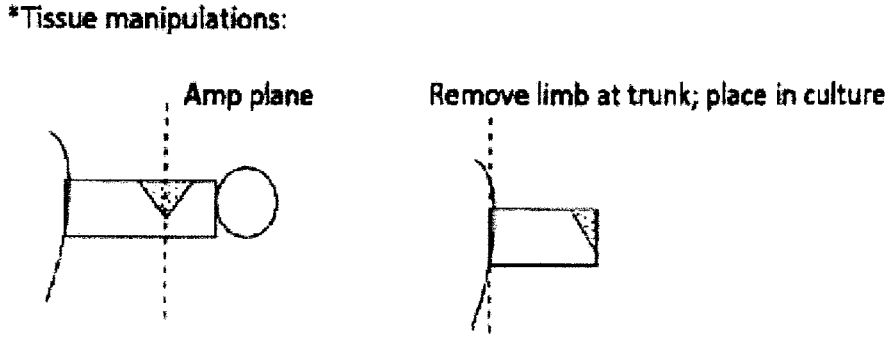


Figure 13. Schematic representation showing limb manipulation for beryllium treatment Dotted lines indicate amputation plane. Amp represents amputation.

SOLUTIONS AND MEDIA

Artificial Pond Water

It consisted of an equal volume of stock solution A and stock solution B, to which a water conditioner called NovAqua plus (Kordon) was added. For a 44 gallon barrel, 680ml of stock A, 680ml of stock B and 25ml Novaqua was needed.

For preparation of 2 Liters Stock A, 175g NaCl and 35g $CaCl_2$ were dissolved completely in distilled water, 200ml of 30% KI was added and the volume was adjusted to 2 liters by distilled water. For stock B, 5g $NaHCO_3$ were dissolved in a final volume of 2 L distilled water.

10X MMR buffer

To prepare a 500ml total volume of the buffer, the following components were dissolved in almost 450 ml of reverse osmosis (RO) water: 1 *M* NaCl (29.22), 18 *M* KCl (0.67g), 20 m*M* $CaCl_2$ (1.47g), 10 m*M* $MgCl_2$ (0.48), and 1 *M* HEPES (25ml) (Fischer, enzyme grade). The pH was adjusted to 7.6 using 10N NaOH and the volume then was completed to 500 ml using RO water.

For preparation of 0.1X solution, 10 ml of 10X stock MMR solution was diluted using RO water to 1 L and the pH adjusted to 7.6.

For preparation of 0.1X solution containing gentamycin, 5 μg/ml gentamycin was added to the previously prepared 0.1X MMR buffer after adjusting the pH to 7.6.

L-Cystein (2%)

In about 90 ml of RO water, 2gm of L-cystein was dissolved well and the pH of the solution was adjusted to 7.9 using 10 *M* NaOH. Then, the volume was completed to 100 ml using RO water.

NZCYM Media for Plates

In 500ml final volume of tap water, 11g NZCYM (Fischer Scientific) and 7.5g bactoagar (Fischer Scientific) were dissolved and autoclaved for 15 min. After cooling to approximately 45°C, the media was poured on 100mm plates (~25ml/plate). The plates were left on the counter to cool down before using or they were stored at 4°C.

NZCYM Top Agarose

In 100ml final volume of tap water, (2.2% w/v) NZCYM powder (Fischer Scientific) and (0.75% w/v) agarose low EEO powder (FisherBioTech.) were dissolved and autoclaved for 15min. Upon use, the media's temperature had to be around 45°C to

avoid killing of the host bacterial cells. If solidified, the medium was previously warmed in a water bath and heated in the microwave to completely dissolve the agarose. The agarose was left on the counter to cool down to 45°C before use.

NZCYM Broth

In 100ml final volume of tap water, (2.2% w/v) NZCYM powder (Fischer Scientific) was dissolved and autoclaved for 15min. After cooling down, this media was supplemented with a previously autoclaved 1m*M* $MgSO_4$ (Sigma-Aldrich) and a sterilized 0.3% maltose.

Maltose(0.3%)

In 100ml final volume of previously autoclaved water, 3g of maltose were dissolved well. The sterilization of the maltose solution was done using 0.22mm bacterial filters.

SM Buffer

In a final volume of 500ml tap water, 2.9 g sodium chloride, 1.0g $MgSO_4$.7H2O (0.008M) and 25ml 1*M* Tris-Cl buffer (pH 7.5) were dissolved. The media was autoclaved for 15 min and cooled to room temperature before use.

20X TAC Buffer

For the preparation of 2 liters, 0.8 *M* (193.8 g) Tris (Sigma), 2m*M* (1.52g) EDTA (Sigma), and 100 *mM* (16.4g) sodium acetate (Sigma) were dissolved in a minimal volume of distilled water. The pH was then adjusted to 8.3 using glacial acetic acid and the volume was completed to 2L using distilled water. For the preparation of a 1X TAC, the 20X solution was diluted using RO-distilled water for dilution.

Blue Juice Dye

Equal volumes of 20X TAC and glycerol (5 ml each) were combined and mixed well. One drop of each, bromophenol blue and xylene cyanol, were added to the solution with well mixing.

LB–Ampicillin Agar

In a final volume of 1L` of double distilled water, 7.5g BactoTryptone, 2.5g yeast extract, 5g Bactotrypton, and 1.0g NaCl were dissolved in reversed osmosis distilled water (RO-water). The media was autoclaved for 15 min and cooled to 55°C before adding ampicillin (100 µg/ml). The agar was poured into Petri dishes and cooled completely before use.

20X SSC: (3 *M* NaCl, 0.3 *M* sodium citrate)

The following solutes were dissolved in most 800 ml of RO-water: 175.3 g NaCl, 88.2 g sodium citrate. The pH was then adjusted to 7.0 using concentrated HCl. The volume was completed to 1L using RO-water.

LB Broth

In a final volume of 100ml tap water, 1.0g BactoTryptone, 0.5g Bacto-yeast extract and 1.0g NaCl were dissolved. The media was autoclaved for 15 min and cooled to room temperature before use.

Genomic Hybridization Buffer

The following solutes were dissolved in a sufficient amount of warm RO- water to ensure complete dissolving: 5x using 125 ml 20X SSPE (see preparation later), 100µg/ml boiled 10 mg/ml salmon sperm DNA (Sigma), 0.2% using 10ml of 10% Sodium Dodecyl Sulfate (SDS) (Life Technologies), 0.5 g polyvinylpyrrolidone (PVP)

(Sigma), 0.5 g Ficoll, 0.5 g BSA (Bovine Serum Albumin (BSA) (Sigma).

The volume was then adjusted to 500ml using RO- water.

20X SSPE

The following solutes were dissolved in a minimal volume (~800 ml) of RO-water: 175.3 g of NaCl, 27.6 g of NaH_2PO_4.H20, 7.4 g of EDTA. The pH was adjusted to 7.4 using 10 N NaOH, then the volume was completed to 1L using RO-water.

Washing Post-Hybridization Buffer (0.2 x SSC/0.1% SDS)

The components were dissolved RO-water: 0.2X Sodium chloride sodium citrate (SSC) (Sigma), 10 ml of 20X SSC 0.1% (w/v), 0.1% (w/v) Sodium Lauryl Sulfate (SDS) (Fischer)] and 10 ml of 10% SDS. The volume was then adjusted to 1L and mixed well. The buffer was freshly prepared fresh prior to use.

1X MEM buffer

The buffer has the following components: 1*M* MOPS, 20 m*M* EGTA and 10 m*M* $MgSO_4$. The volume was then adjusted to 1L using RO- water. The pH was 7.4

TTLMN Buffer

The buffer has the following components: 100 m*M* Tris–HCl, pH 9.5, 0.1% Tween 20, 2 m*M* Levamisole, 50 m*M* MgCl2, and 100 m*M* NaCl.

10X Steinberg's Stock Solution

The following components were dissolved completely in Sigma water: 0.6 *M* NaCl (35 g), 6.7 *M* KCl (0.5 g), 3.4 *M* Ca $(NO_3)_2$. 4 $H2OCl_2$ (0.8 g), 8.3 *M* $MgSO_4.7H_2O$ (2.05 g) and 46 m*M* Trizma Base (5.6g). The pH was adjusted to 7.4-7.6 then, the volume was completed to 1L. The solution was autoclaved and stored at and then the solution was stored in the refrigerator.

For preparation of the bathing solution, where larvae were kept overnight before culturing, 2ml 10X Steinberg's solution, 1ml antibiotic (Sigma) solution and 97ml Sigma water were mixed well together.

Culture Solution

For the preparation of 33 ml solution, 500 µl antibiotic solution was added to 33 ml of L-15 (Sigma) working solution. The volume was almost completed to 50 ml using Sigma water and the pH was adjusted to 7.4. Then, the volume was adjusted to 50ml.

For preparation of L-15 stock solution, an envelope of L-15 (Leibovitz medium with L-glutamine/GIBCO H-13) was dissolved well in 1 L Sigma water. The working solution (67%) of L-15 was prepared by mixing 2 parts of the stock solution of L-15 with one part of Sigma water.

Amputation solution

The following components were mixed well together: 33ml Benzocain stock solution, 2 ml Antibiotic solution and 165 ml Sigma water.

For the preparation of benzocain stock solution, 0.015g was dissolved completely in a minimal amount of absolute alcohol (~2ml). Complete the volume to 50 ml by slow addition of Sigma water.

The antibiotic solution was prepared by dissolving a lyophilized vial of antibiotic/antimycotic (Sigma) in 20 ml Sigma water using a sterile syringe and needle.

RESULTS

This study focuses on two selected genes in *Xenopus laevis* that are members of the suppressors of cytokine signaling (SOCS) family. These genes are SOCS1 and SOCS3.

Isolation of Full –Length cDNAs for SOSC1 and SOCS3

Full-length cDNAs were isolated for both SOCS1 and SOCS3 from cDNA libraries of stage 53 (st53) at three days post amputation (3dPA) and stage 11 (st11) embryos, respectively, using the PCR-based selection method and the appropriate primers.

The nucleotide sequencing showed isolation of a full-length SOCS1 cDNA of 633 nucleotides (Fig. 14) that encodes a 211-amino acid protein. This is the first isolation of SOCS1 cDNA from *X. laevis* that is deposited in the database with the accession number FJ696649. A comparison between the predicted protein for the isolated SOCS1 cDNA to a previously identified SOCS1 protein homologue in *X. tropicalis* showed that the proteins share 97% amino acid similarity, with a difference in three conservative substitutions and three non-conservative substitutions as shown in Figure 14. Sequencing of SOCS3 cDNA showed isolation of a full-length SOCS3 cDNA of 597 nucleotides (Fig. 16) that encodes a 199-amino acid protein. The predicted SOCS3 protein in this study demonstrated a 99 % identity to a previously identified SOCS3 protein in *X.*

tropicalis. The difference between the protein homologues is in two conservative substitutions as shown in Figure 17.

ATGGTAGCACACAGTAAGGTGGAAGCAGATAATGCAGTTGCAGATAGAAGA
TCTCCGCACCTGGAAGCTTCAGTCTCTGATCGTTCACAAATCCAACCTGCC
AGAGCACTCCATCCCAGCCCAGGACGTGCCACTCCTGCTTTGCTGCTCAGT
GATACCCACTTCCGCACCTTCCGCTCACACTCGGATTTCACTGTCATCACC
AAGACCAGTAGCATGCTGGATACTTGCGGCTTCTACTGGGGACCTATGGAT
GTCAACGTGGCTCACGACAAGCTCAAATCGGAGCCCATAGGTACCTTCCTC
ATCAGGGACAGTAAACAAAAGAACTGTTTCTTTGCAATCAGTGTTAAAACA
GCCAGGGAAACTGTCAGCATCCGGATTAAGTTTCATGCTGGTAAATTCAGC
CTAGATGGCAGCAAAGAGTTGTTCAGTTGCCTCTTTCAGCTGGTGGAACAT
TACATGACTTCTCCGAAGAAGATGCTGGTTTCTCCTTTAAGGAAAGTCAGG
TTACGACCTTTGCAAGAACTCTGCCGAAAAAGCATCTTAGCGACTTTTGGG
AGGCAGAACCTGGACAGTATCCCCTTGAACAGGGTGTTAAAGGACTACCTA
AAGTCTTTCCCGTTTCAGATC**TAA**CGCAGGGCATTAACGACCCGGGACACA
AGAACTAAACGTGTTACAAACGCCGCCTCCGTGGATTATTTTTTAAACCTT
ATTTAACTGTGGTCAAGTTTATTTTTGTAGCAATTTAACTGTATTTTGGGA
TGAAACTTGAACACTTGACTTTTAATGTTTACATGAAAATATTGTTTGGCA
CAAAGTAACAAGGTGAATANCCCCTTGAAATTNGTGTCTGCTGNGCAGANC
ACTATTTTATTNCATTTTAAGTAATTAACTTTAATAAACTTTATTATGAAA
TATTCNGNAAAAAAAACCAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 14. Nucleotide sequences of a full-length *Xenopus laevis* SOCS1 cDNA which is composed of 633 nucleotides. The underlined bold ATG represents a start codon, while the underlined bold TAA represents the stop codon.

The poly A nucleotides represent the 5′ end. "N" denotes uncertain sequence.

```
XtSOCS1     MVANSKVEADNAVADRRSQHLEASVSDRSQIQPARALHPSPGRATPALLLSDTHFRTFRS
XlSOCS1     MVAHSKVEADNAVADRRSPHLEASVSDRSQIQPARALHPSPGRATPALLLSDTHFRTFRS
            ***:**************;:****************************************

XtSOCS1     HSDFTIITKTSSMLDTCGFYWGPMDVNVAHDKLKSEPVGTFLIRDSRQKNCFFAISVKTA
XlSOCS1     HSDFTVITKTSSMLDTCGFYWGPMDVNVAHDKLKSEPIGTFLIRDSKQKNCFFAISVKTA
            *****:**********************************:********:**************

XtSOCS1     RETVSIRIKFYAGKFSLDGSKESFSCLFQLVEHYMISPKKMLVSPLRKVRLRPLQELCRK
XlSOCS1     RETVSIRIKFHAGKFSLDGSKELFSCLFQLVEHYMTSPKKMLVSPLRKVRLRPLQELCRK
            **********:***********;************;************************

XtSOCS1     SILTTFGRQNLDSIPLNRVLKDYLKSFPFQI
XlSOCS1     SILATFGRQNLDSIPLNRVLKDYLKSFPFQI
            ***:****************************
```

Figure 15 *Xenopus laevis* SOCS1 protein consists of 211 amino acids encoded by a full-length SOCS1 cDNA. *X. laevis* (xl) SOCS1 protein sequence is aligned with a previously identified *X. tropicalis* (xt) protein. Asterisks (*) indicate identity, colons (:) indicate conservative substitutions, and semi-colons (;) represent non-conservative constitutions.

GTC**ATG**GTAACGCAGAGCAAGTTCCCGAGCATGAGCCGGGCATTGGACTCT
AGTCTACGGCTTAAGACCTTCTCTTCTTGCAGCGAGTACAACCTTGTGTTG
ACTGCAGTGCGCAAACTCCAAGAGAGTGGTTTTTACTGGAGCACCGTAACA
GGGAGCCAGGCAAACCTACTATTGAGCACTGAACCTGCGGGAACTTTTCTG
ATCCGAGACAGCTCCGACCGGCGGCACTTCTTCACCCTCAGCGTCAAAACT
GAGTCTGGCACCAAGAACCTACGCATCCAGTGTGAGCCTTGTGGATTCTCC
TTGCAGACTGAACCACGCAGTGCCCAGCCAGTACCACGTTTTGACTGTGTC
TTAAAGCTGCTGTGCCATTATATGCCAACCAAGGACAGTAGCAGTGGCTCC
AAACGGGCATATTACATTTATTCTGGAGGGGAGCGTGTCCCCTTGCTTCTG
TCACGGCCACTATCAACCAATGTGTCTTCTCTACAGCACCTATGTAGAAAG
GCCGTGAATGGTACCTTAGAGGGGGGTGAAGGGCAAGAGCAGCTTCCATTG
CCCATAAAAGACTTTCTGCAGGAATATGACTCCCCAGTG**TAG**ACAA

Figure 16 Nucleotide sequences of a full-length *Xenopus laevis* SOCS3 cDNA is composed of 597 nucleotides. The underlined bold ATG represents a start codon, while the underlined bold TAG represents the stop codon.

```
XtSOCS3     MVTQSKFPSMSRALDSSLRLKTFSSRSEYNLVLTAVRKLQESGFYWSTVTGSQANLLLST
XlSOCS3     MVTQSKFPSMSRALDSSLRLKTFSSCSEYNLVLTAVRKLQESGFYWSTVTGSQANLLLST
            ************************************************************

XtSOCS3     EPAGTFLIRDSSDRRHFFTLSVKTESGTKNLRIQCEPCGFSLQTDPRSAQPVPRFDCVLK
XlSOCS3     EPAGTFLIRDSSDRRHFFTLSVKTESGTKNLRIQCEPCGFSLQTEPRSAQPVPRFDCVLK
            ************************************************************

XtSOCS3     LLCHYMPAKDSGSGSKRAYYIYSGGERVPLLLSRPLSTSVSSLQHLCRKAVNGTIEGGEG
XlSOCS3     LLCHYMPTKDSSSGSKRAYYIYSGGERVPLLLSRPLSTNVSSLQHLCRKAVNGTLEGGEG
            *******:****************************:**********************

XtSOCS3     REQLPLPIKDFLQEYDSPV
XlSOCS3     QEQLPLPIKDFLQEYDSPV
            ********************
```

Figure 17 *Xenopus laevis* SOCS1 protein consists of 199 amino acids encoded by a full-length SOCS3 cDNA isolated clone. *Xenopus laevis* (xl) SOCS3 protein sequence is aligned with a previously identified *X. tropicalis* (xt) protein. Asterisks (*) indicate identity, and colons (:) indicate a conservative substitution.

Expression of SOCS1 and SOCS3 mRNA During Early Developmental Stages

RT-PCR analysis using specific primers was performed to determine the expression patterns of SOCS1 and SOCS3 in a whole *X. laevis* during early developmental stages. The maternal expression was determined in egg (E) and stage 6 (st6), while the zygotic expression was determined in selected developmental stages including st11, st17, st34, st40, and st48 (Fig. 6).

There was a high level of expression for SOCS1 mRNA in the maternal stages (E and st6). The maternal SOCS1 mRNA was degraded prior to the onset of zygotic transcription and this decrease was constant through st40. However, SOCS1 expression was again increased by st48, the stage at which the limb bud formation is initiated (Fig. 18). There was no expression for SOCS3 mRNA in maternal stages (egg and st6). The expression from the zygotic genome was significantly elevated at st11, but barely detectable by the late neurula (st17). The expression of SOCS3 was again increased at st34 and this reactivation was sustained throughout st38, st40 and st48. Of note, this pattern is an indicative of a biphasic mode to the control of zygotic SOCS3 expression. Additionally, the expression of SOCS3 protein was significantly up-regulated by stage 48, when limb bud formation starts (Fig. 18). Consequently, at the stage of bud formation, both genes demonstrated an increase pattern of expression.

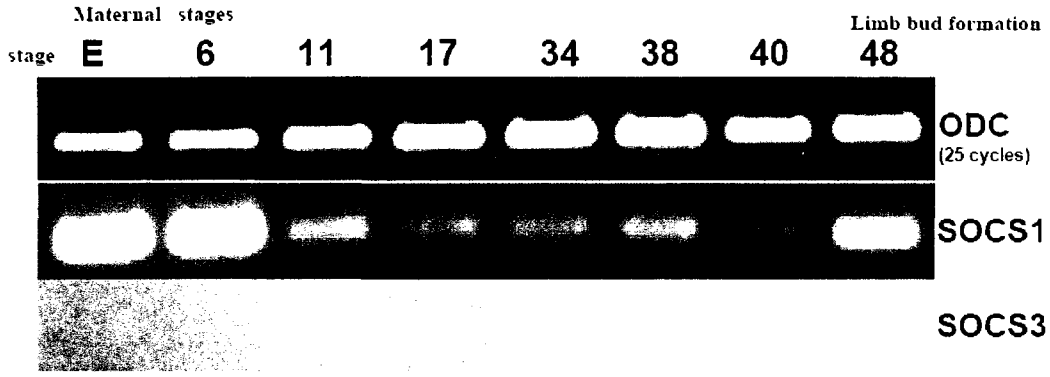


Figure 18 RT-PCR assay to determine the temporal expression of SOCS1 and SOCS3 mRNA during early developmental stages. Numbers on the top represent developmental stages according to Nieuwkoop and Faber (1967). Ornithine decarboxylase (ODC) used as an internal normalizer.

Comparison of SOCS1 and SOCS3 Induction in Response to Limb Amputation at Regeneration Competent (st53) vs. Regeneration Non-Competent (st57) Stages

RT-PCR analysis using specific primers was performed to determine the expression patterns of SOCS1 and SOCS3 in regenerative blastemas at st53 and non-regenerative pseudoblastemas at st57 (Fig. 19 A).

At st53, the expression of SOCS1 was induced at 1dPA and this induction was sustained at 3dPA but then decreased by 5dPA. For SOCS3, the expression was highly induced at 1dPA in response to amputation followed by a decrease at 3dPA and 5dPA.

At st57, the expression of SOCS1 and SOCS3 was induced in response to amputation by 1dPA and this increase was maintained until 5dPA.

In summary, the RT-PCR data analysis showed that SOCS1 and SOCS3 were induced in response to limb amputation in regenerative and non-regenerative stages at 1dPA. Both SOCS1 and SOCS3 transcripts at 5dPA were notably lower at st53 compared to st57 where the expression was barely detectable. In addition, the expression of SOCS3 was higher than the expression of SOCS1 especially at 1dPA st53 (Fig. 19).

Table 2

The expression of SOCS1 and SOCS3 expression as indicated by the RT-PCR at the regeneration competent (st53) blastemas vs. the non-competent (st57) pseudoblastemas immediately post amputation (0dPA) and at 1, 3, and 5 days post amputation.

STAGE / GENE	Regeneration Competent (st53)				Regeneration Non-Competent (st57)			
	0 dPA	1dPA	3 dPA	5 dPA	0 dPA	1 dPA	3 dPA	5dPA
Socs1	Minimal expression (< +)	++	++	<+	Minimal expression (< +)	+	+	+
Socs3	Minimal Expression (<+)	++++	++	<+	Minimal expression (< +)	++	++	+

The qPCR assay was performed to determine the level of induced expression of SOCS1 and SOCS3 at st53 and st57. The degree of induction was quantified in values compared to a given qPCR value which was set relative to ODC and then arbitrary set to 1 (after normalization to ODC).

At st53 at 1dPA the expression of SOCS1 was induced 6.1-fold while the expressionof SOCS3 was induced 11.5-fold. It is clear that the induction of SOCS3 was nearly two times more than SOCS1. For SOCS1, there was a minimal increase in values from 1dPA to 3dPA but by 5dPA the value was again decreased to levels similar to 0dPA. For SOCS3, the induction at 1dPA gradually decreased at 3dPA and 5dPA. The value of induction at 5dPA was nearly equivalent to 0dPA.

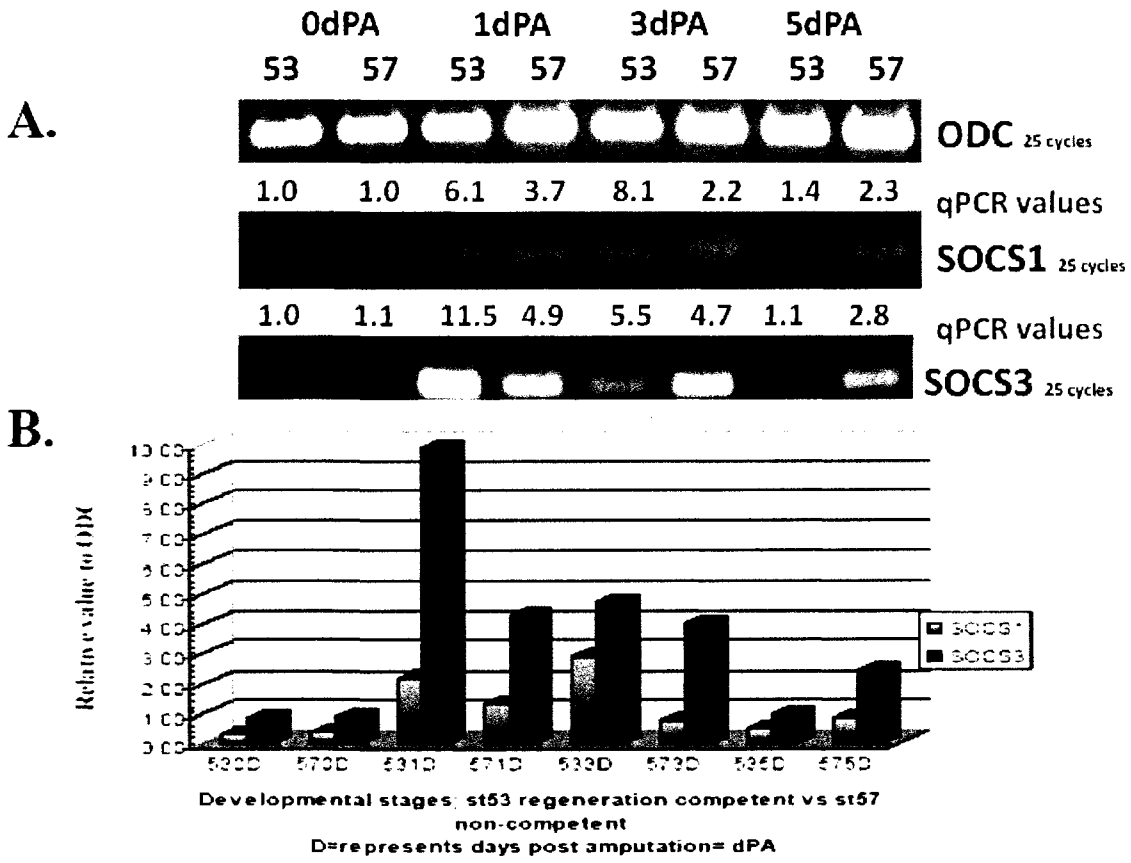


Figure 19. (A) RT-PCR and ***(B)*** qPCR assays comparing the expression of SOCS1 and SOCS3 in response to amputation in regeneration competent (st53) blastemas and noncompetent (st57) pseudoblastemas at zero-day post amputation (0dPA), 1dPA, 3dPA, and 5dPA. D represents days post amputation=dPA. Ornithine decarboxylase (ODC) was used as an internal normalizer.

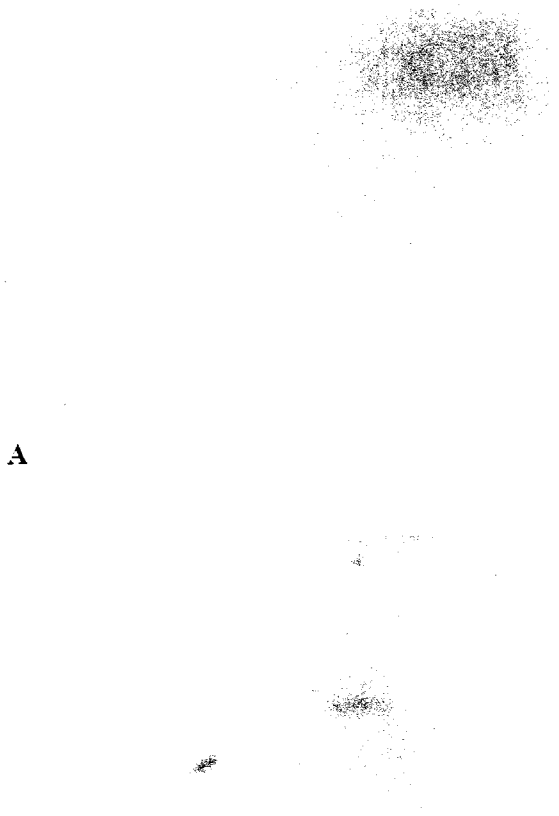
For st57, there was an induction of SOCS1 expression at 1dPA by 3.7-folds. The induction level was reduced at 3dPA to 2.2 and sustained to 5dPA (2.3). For SOCS3, there was an increased induction at 1dPA by 4.9-fold. The level was almost maintained at 3dPA (4.7) but reduced at 5dPA to 2.8.

In summary, the RT-PCR and qPCR data were consistent. There was an induction for SOCS1 and SOCS3 expression in response to limb amputation at st53 and st57 by 1dPA. Both genes were expressed at 1dPA and also remained expressed at 5dPA at the regeneration non- competent stage, however, for the expression at the regeneration competent stage, expression of SOCS1 and SOCS3 was comparable to values at 0dPA.

Whole Mount *In Situ* Hybridization (WMISH) for SOCS1

The spatial expression of SOCS1 mRNA *in situ* in *X. laevis* hindlimbs in response to limb amputation was determined using WMISH experiments performed on regeneration competent (st53) blastemas and non-competent (st57) pseusodoblastemas at 1dPA, 3dPA and 5dPA. In addition, WMISH was performed on intact limbs from both stages.

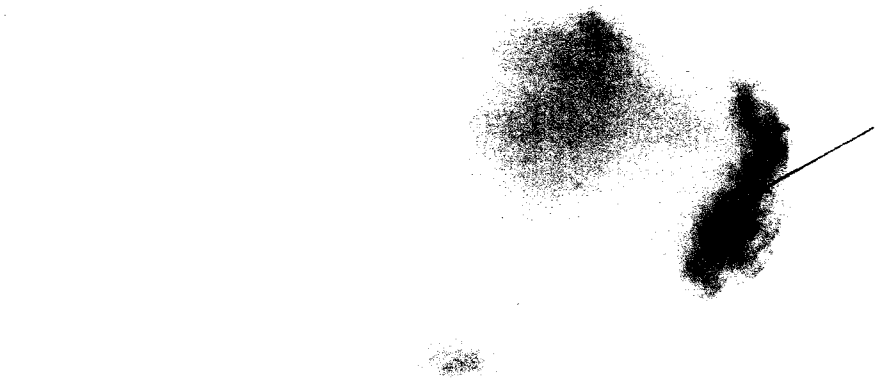
The WMISH showed no expression of SOCS1 in intact limbs using either the antisense or sense DIG-labeled RNA probes (Fig. 20). The WMISH assay showed induced expression of SOCS1 in response to amputation in st53 at 1dPA, 3dPA, and 5dPA (Fig. 21, 22, 23 and 24). These results were consistent with the data obtained from the RT-PCR and qPCR assays for expression of SOCS1 at st53. The induced expression of SOCS1 mRNA was detected in clusters of cells in the underlying mesenchymal cells (Fig. 22 and Fig. 23).



A

B

Figure 20. WMISH at the regeneration competent stage (st53) (side view)in intact limbs showing no difference in the expression of SOCS1 using the antisense (A) and sense (B) probes.



A.

B.

Figure 21 WMISH at the regeneration competent stage at one day post amputation (st53 1dPA) (side view) blastemas showing an expression of SOCS1 using antisense (A) compared to the sense (control) (B) probes. Arrow demarcates SOCS1 expression.

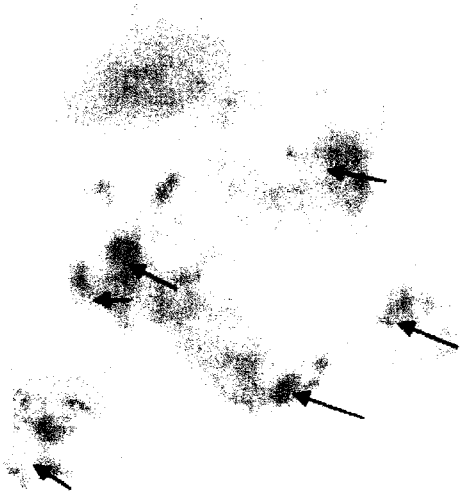
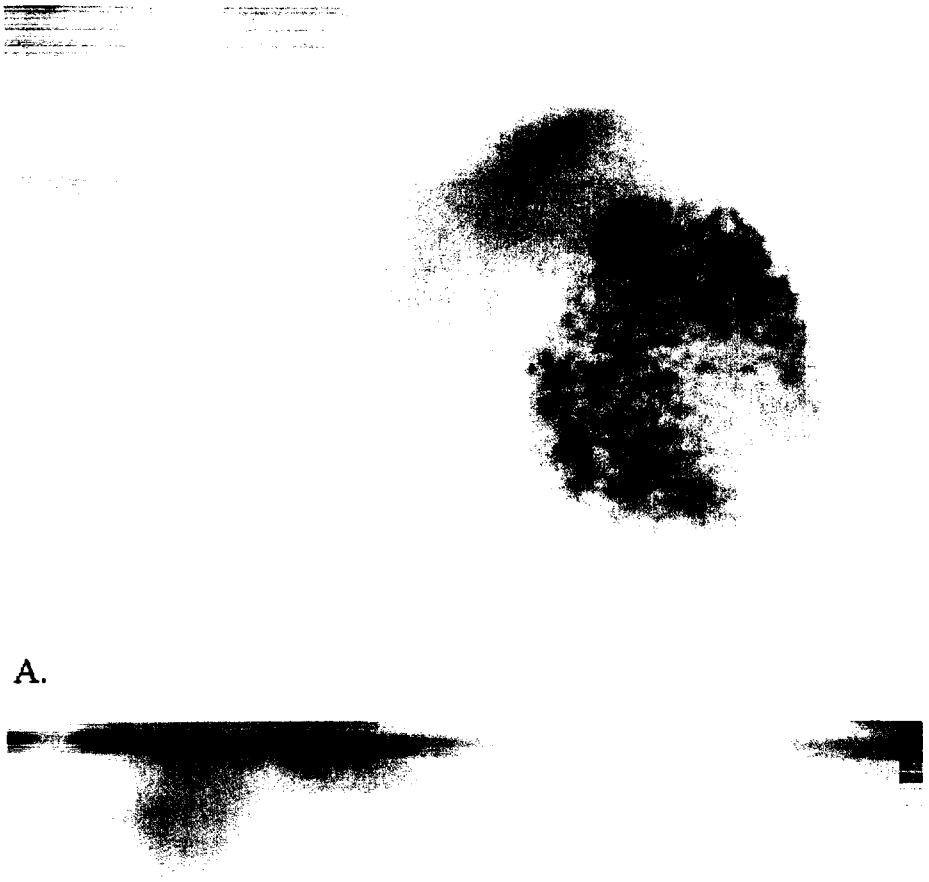


Figure 22. Antisense digoxigenin-RNA probe in blastemas of st53 at one day post amputation (end on view). Expression of SOCS1 was detected in underlying mesenchymal cells as indicated by the arrows.



A.

B.

Figure 23. WMISH at a regeneration competent stage at three days post amputation (st53 3dPA) (side view) blastemas showing expression of SOCS1 using digoxigenin-labeled RNA antisense probe (A) compared to the sense probe (control) (B).

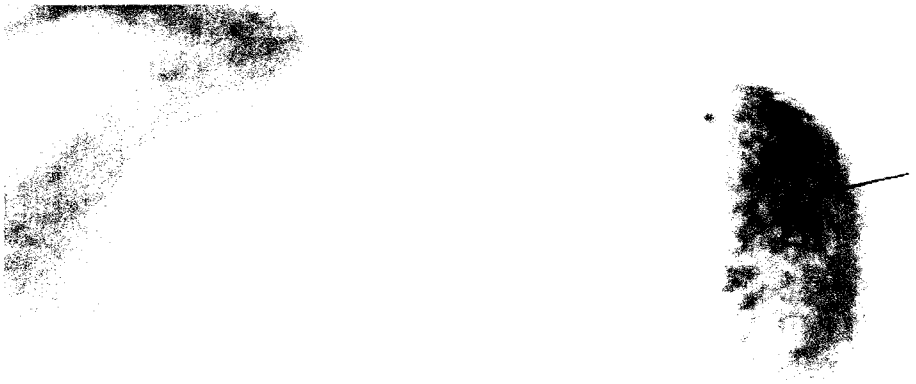


Figure 24. WMISH at the regeneration competent stage (st53) at five days post amputation (side view) using the antisense digoxigenin-labeled RNA probe. The expression of SOCS1 is indicated by the arrow.

Expression of SOCS1 at Stage 55 (Regeneration Limited Stage)

To determine if there was a difference in expression of SOCS1 in a regeneration limited stage (a transient stage between regeneration competent and non-competent stages), WMISH assay was performed on blastemas at st55 at 1dPA and 5dPA. The expression of SOCS1 mRNA was induced in response to limb amputation at st55 at 1dPA, however, there was no induction detected at 5dPA as shown in Figure 25.

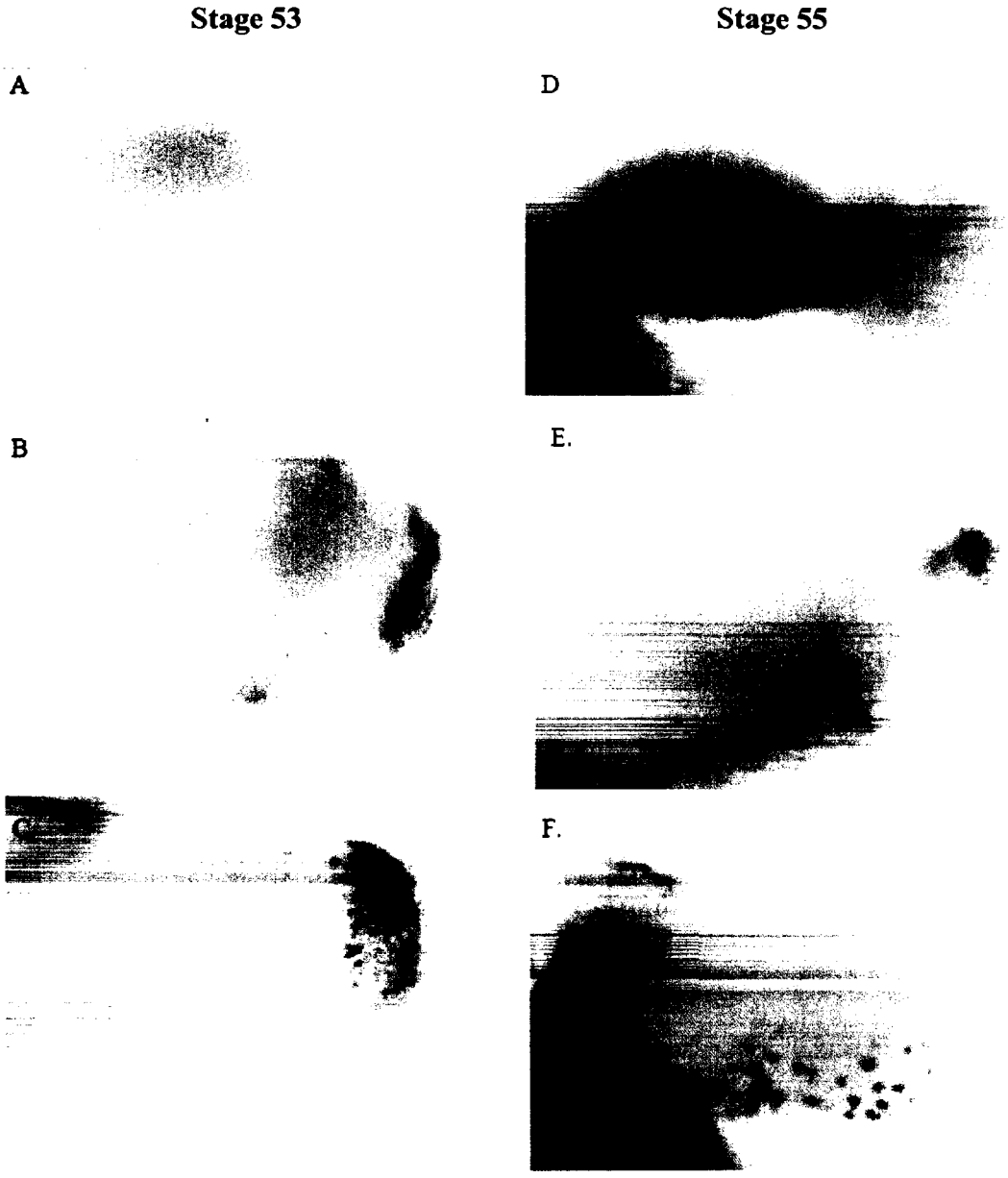


Figure 25. WMISH comparing the expression of SOCS1 in whole limbs (A and D) and in blastemas at certain days post amputation (dPA) at the regeneration competent stage (st53) vs. the regeneration limited stage(st55) at 1 dPA (B and E) and 5 dPA (C and F) using the sense probe.

Expression of SOCS1 at Stage 57 (Regeneration Non-Competent Stage)

The WMISH assay in a regeneration non-competent stage (st57) showed no expression of SOCS1 in an intact limb. In response to amputation, there was an induced expression of SOCS1 at 1dPA, 3dPA, and 5dPA (Fig. 26). This WMISH data were consistent with the RT-PCR and qPCR data (Fig. 19).

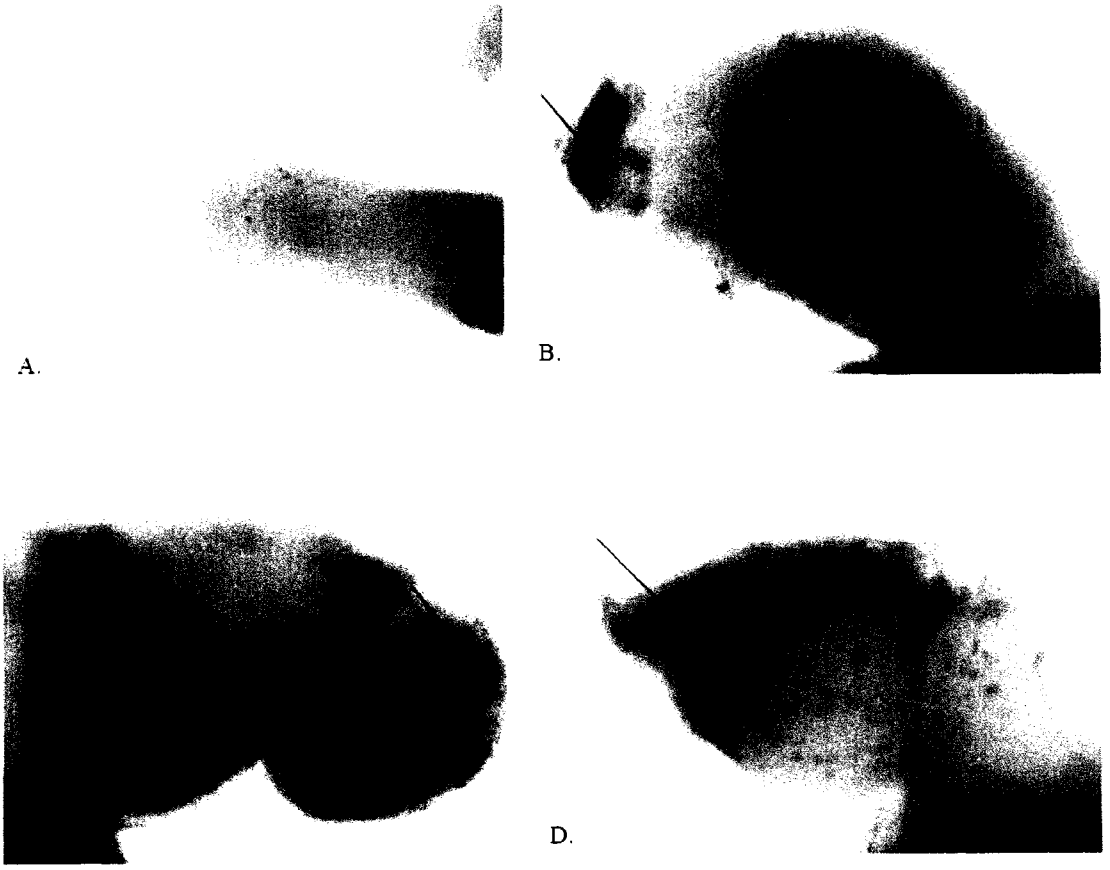


Figure 26. WMISH at the regeneration non-competent stage (st57) pseudoblastemas at certain days days post amputation (dPA). An induced expression of SOCS1 using the antisense probe at 1dPA (B), 3dPA (C), and 5dPA (D) compared to the intact limb (A).

Expression of SOCS1 and SOCS3 in Cultured Limbs

An RT-PCR assay was performed using RNA of amputated limb pieces cultured at 1PA, 3dPA, 5dPA, and 7dPA to determine the expression of SOCS1 and SOCS3. The samples collected immediately post amputation are referred to as 0dPA.

The RT-PCR assay indicated induced expression of SOCS1 and SOCS3 in all limb pieces (proximal, distal, and paddle) at the specific time periods studied (1dPA, 3dPA, 5dPA and 7dPA). The induction was observed to be higher in the distal and proximal pieces than that of the paddle especially for both SOCS1 and SOCS3 at 5 and 7 days.

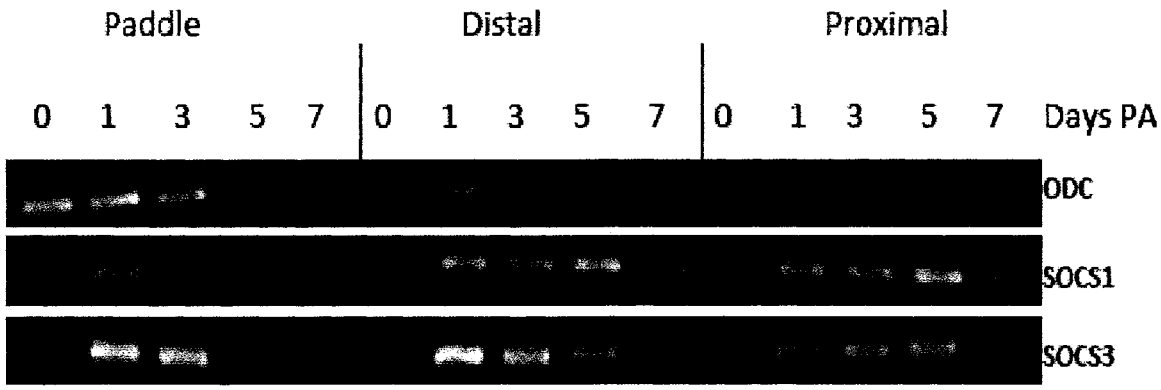


Figure 27. Expression of SOCS1 and SOCS3 genes in cultured limb pieces of *X. laevis* at the regeneration competent stage (st53). The amputated limb was cut into paddle, distal and proximal pieces (Fig. 13). Ornithine decarboxylase (ODC) was used as the internal normalizer.

Beryllium Effect on the Expression of SOCS1 and SOCS3

In response to amputation, there was a considerable induction of SOCS1 expression at 1dPA in the control and beryllium-treated blastemas; however, the induction was higher in beryllium-treated blastemas. The expression of SOCS1 was significantly high at 3dPA and 5dPA and was barely detectable at 7dPA. However, the expression of SOCS1 was still elevated in the control blastemas.

The induced expression of SOCS3 in beryllium-treated blastemas at 1dPA was higher than that of SOCS1. This expression gradually decreased at 3dPA and 5dPA followed by an increase at 7dPA. The induced expression of both genes in the control blastemas was less than the induced expression of beryllium-treated blastemas.

The expression of IL1-β, which is a proinflammatory factor, was induced at 1dPA in beryllium treated blastemas and this expression was maintained at 3dPA, 5dPA, and 7dPA. The control samples showed no expression of IL1-β.

On the other hand, the expression of the patterning gene Shh was induced at 3dPA in control blastemas followed by an increase at 5dPA and 7dPA. However, this gene was not expressed in beryllium-treated blastemas in response to amputation up to 7dPA.

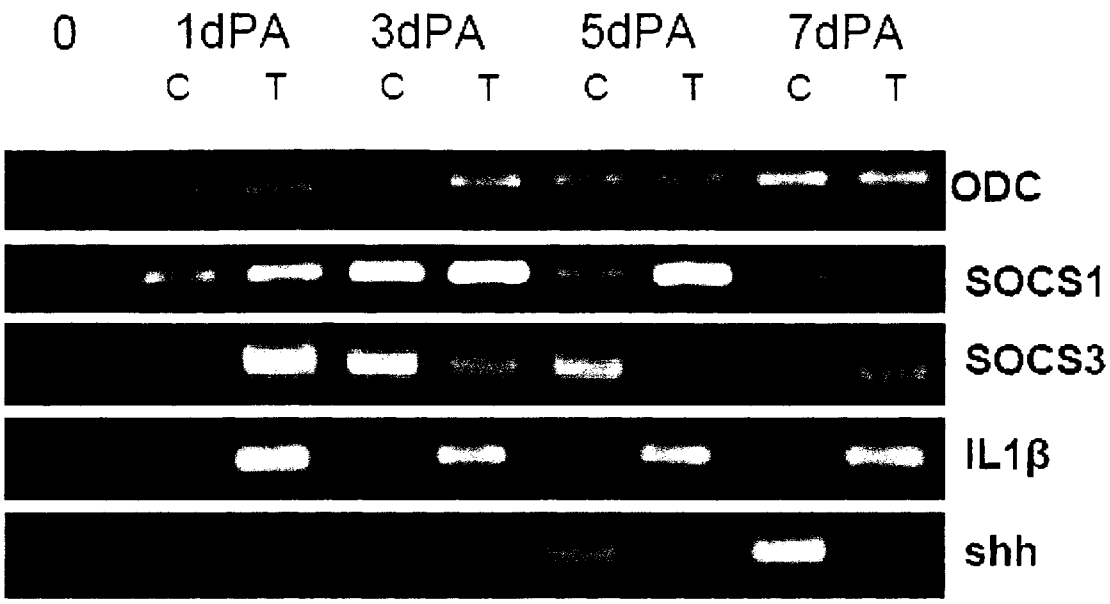


Figure 28. Effect of beryllium at a concentration of 10 m*M* on the expression of SOCS1, SOCS3, IL1-β and Shh in a regeneration competent stage (st53) at 0dPA, 1dPA, 3dPA, 5dPA and 7dPA. Control (C) samples were compared to the beryllium-treated (T) samples for each time period. The expression of ODC was used as the internal standard.

DISCUSSION

Isolation of SOCS1 & SOCS3 cDNA

The isolation of full-length cDNA clones for SOCS1 involved a PCR-based selection method and a radioactive method employing a cDNA library of stage 53 larvae at 3-days post amputation. The isolation of a full-length SOCS3 cDNA involved a PCR-based selection method using stage 11 embryos.

This study identified the first full-length nucleotide sequence of SOCS1 cDNA from *Xenopus laevis* (Fig. 14). The accession number FJ696649 is deposited in the NCBI database. In addition, this study identifies a full-length nucleotide sequence for SOCS3 cDNA (Fig. 16). This was not reported to the NCBI database because of an existing entry for *X. laevis* SOCS3 mRNA. Expressed sequence tags (ESTs) for SOCS1 and SOCS3 have been reported in the database with accession numbers BC054214 and BE026949, respectively.

The isolated clones of SOCS1 and SOCS3 are homologues to previously identified SOCS1 and SOCS3 of *X. tropicalis* as shown in the database. The highly conserved sequence of SOCS1 and SOCS3 among related species of the same genus most probably indicate a functional similarity.

Sequence alignments for proteins encoded by the isolated clones of SOCS1 and SOCS3 and previously isolated protein homologues from *X. tropicalis* showed extensive sequence similarity ranging from 97% to 99%, respectively (Fig. 15 and 17).

The comparison alignment data of SOCS3 proteins are consistent with previous results. Kuliyev et al. (2005) showed by sequence alignments an extensive sequence similarity between *X. laevis* and *X. tropicalis* SOCS3 proteins, and similarities with avian and mammalian SOCS3 proteins. Kuliyev's study (2005) indicated that the most conserved regions among SOCS were the Src-homology (SH2) domain and the SOCS box.

Temporal Expression of SOCS1 and SOCS3 During Early Development

Using RNA extracted from different developmental stages (Nieuwkoop & Faber 1967), the temporal expression profiles of SOCS1 and SOCS3 of *X. laevis* were examined by RT-PCR assay. The maternal gene expression was determined at egg (E) stage and 64-cell stage (stage 6; st6), while the zygotic expression was determined at st17, st34, st.40, and st48.

Regarding SOCS1; the maternal transcript of SOCS1 mRNA was highly expressed at E and st6. This transcript was degraded by zygotic st11 and remained at a relatively constant low level throughout the following developmental stages (st17 until st40) (Fig. 19). The expression of SOCS1 was induced again at st48.

The expression of any particular gene in maternal stages indicates that the encoded protein is produced during oogenesis and suggests a developmental role for such a gene during embryogenesis. The fact that SOCS1 was maternally expressed (E and st6) suggests that this gene may serve an important function during the development process of *X. laevis*. The detected low expression of SOCS1 from st17 until st40 might be due to two possibilities with no definite evidence for either. The first possibility is the decrease in SOCS1 expression during this period of development. The second possibility is the

zygotic gene silencing from expressing SOCS1 during this period; however, the low expression detected may be due to incomplete degradation of the maternal transcript that was extensively expressed earlier. The later induced expression of SOCS1 at st48 might be due to a role in limb development since the formation of limb bud starts at this stage.

Regarding SOCS3, there was no expression of SOCS3 maternal transcript (at E and st6) which starts to be induced from the zygotic genome. The expression of SOCS3 showed a biphasic pattern during zygotic development. There was an induced expression at early gastrulation (st11), followed by a decrease at late neurulation (st17) and then a re-induction of expression at st34 which was sustained almost at the same level until st48. A similar biphasic pattern of expression of SOCS3 during development was previously reported (Kuliyev et. al., 2005). This study of Kuliyev and colleagues (2005) indicated that SOCS3 was not present as a maternal transcript, while the zygotic expression began at late gastrula (st12) and remained at a relatively constant level throughout early development (st12 until st45) with the exception of st17 when the expression was barely detectable.

This biphasic pattern indicates that the induced expression of SOCS3 at st11 was subjected within hours to almost complete degradation to be barely detectable at st17. Later in the developmental stages there was a re-induction for SOCS3. As in the case with SOCS1, the induced expression the expression of SOCS3 at st48 might indicate a possible role in limb development because this stage is the onset of limb bud formation. Furthermore, it is possible that expression of SOCS1 and SOCS3 is highly induced at st48 because of the changes in regard the inflammatory response as the immune system is

becoming more developed as the animal matures, and these genes are involved in modulating the immune system.

Expression of SOCS1 and SOCS3 in Regeneration Competent (st53) vs. Non-Competent (st57) Stages Using RT-PCR and qPCR

Dent (1962) reported that amphibians have perfect limb regeneration early in the development (st51 through st53) that is decreased gradually during metamorphosis until it is completely lost at st57. Negative and positive roles of the immune system in restoration of tissue homeostasis and organ function after injury were reported in many species including *Xenopus* (Crawford, 2001). Harty and colleguaes (2003) hypothesized an inverse relationship between the ability for limb regeneration and the development of the immune system. Previous studies documented that the immature immune response in *Xenopus* larvae at st53 allows complete limb regeneration; however, development of the immune response by st57 causes a failure or incomplete limb regeneration (Harty et al., 2003; Mescher & Neff 2006). The efficiency of limb regeneration in amphibians was suggested to be modified by the immune response as new signals are evolved in the adaptive immune system as the animal matures. There is strong evidence that the immune response is critical to the quality of the tissue response to injury including successful limb regeneration. This is because of the importance of the tolerance of the immune system for the dedifferentiating cells of the blastema.

Cytokines are one of the most important players to resolve inflammation in a developed immune system. It is possible that the rapid mechanism to resolve inflammation induced in an amputated limb via excessive secretion of cytokines is the cause of the decreased efficiency of limb regeneration resulting in the incomplete

regeneration or formation of a cartilaginous tissue instead. Therefore, suppressors of cytokine signaling proteins (SOCS), which are feedback inhibitors for cytokines, may modify the limb regenerative capacity. An increasing number of studies have shown an induction of SOCS3 by proinflammatory cytokines such as *IL-6, IFN-γ*, and *TNF-α* (Campbell, 2001; Alexander, 2002). Therefore, SOCS3 is known as a potent regulator of cytokine signaling. This regulation was reported to be mediated via gp130, the common cytokine receptor subunit (Wang et al., 1998). In addition, the study of Campbell (2001) reported an induced expression of SOCS3 in response to partial hepatectomy and this induction as regulated by *IL-6.*

In an attempt to determine whether the expressions of SOCS1 and SOCS3 vary in regeneration competent vs. non-competent stages in response to amputation, the expression profiles of SOCS1 and SOCS3 were determined by RT-PCR comparing st53 to st57 immediately after amputation (zero day post amputation) and in response to amputation after 1 day, 3 days and 5 days.

The RT-PCR data showed that the expression of SOCS1 and SOCS3 was induced in response to limb amputation within one day post amputation (1dPA); however, both genes were more expressed at a regeneration competent stage (st53) than st57 where the innate immune system is dominant in tadpoles (Fig. 19). The data clearly showed that the induced expression of SOCS3 was significantly higher than that of SOCS1 at 1dPA and this difference was consistent at all time parameters. In addition, the data showed that by 5dPA, the expression of SOCS1 and SOCS3 at the regeneration non-competent stage (st57) was higher than at the competent stage and was higher than the expression at 0dPA

of both stages. Consistent with the results of this study, previous reports have indicated similar patterns of SOCS3 expression in response to tissue injury and limb amputation.

Many immunomodulatory genes involved in inflammation, including SOCS3, were identified in a microarray study to be differentially expressed either at a regenerative-competent or at non-competent stages in *X. laevis* (Grow et al., 2006). Using a subtractive hybridization method, Grow et al. showed that SOCS3 expression in intact limbs was significantly lower than in amputated tissues tested at certain times. In response to amputation, Grow at al. (2006) reported a rapid increase of SOCS3 expression at st53 at 1dPA followed by a decrease by 5dPA. However, the induction of SOCS3 was nearly unchanged in st57 tissue compared to st53 at 1dPA and this induction was maintained 3dPA and 5dPA. Using *in situ* hybridization, the study of Kuliyev et al., (2005) showed a similar result for the expression of SOCS3 in response to tissue injury. A rapid and transient increase in SOCS3 expression was detected in response to epithelial healing in *X. laevis* embryos. This induction was not blocked using mitogen-activated protein inhibitors, indicating a role of SOCS3 in attenuating the signaling pathways initiated by trauma and thus protect the responding cells to such signals.

Using other models of study, Katogi et al. (2004) showed that SOCS3 was expressed in regenerative Madaka fins at a higher level at 3dPA compared to 10dPA.

In addition, reports indicated an increase in SOCS3 expression in response to partial hepatectomy (Campbell, 2001) and also in the liver of rats that received a third degree skin burn (Ogle et al., 2000; Kong et al., 2002).

Compared to st53, the lower induced expression in response to amputation for SOCS1 and SOCS3 at st57 and the maintained expression of both genes to 5dPA might be an indication of prolonged inflammation at st57 and therefore be the cause for incomplete regeneration or failure of regeneration. This possibility is supported by previous documentions indicating that tadpoles at later developmental stages have more developed immune system. Therefore, the role of cytokines in resolving the triggered inflammation in response to amputation is considerable. Thus, SOCS proteins as feedback inhibitors for different cytokines, have a possible role in modulating the regenerative capacity and it is possible that both genes play a role in regeneration as in wound healing in *X. laevis*. Other histological studies showed that the changes in cellular activity, except the proliferative capacity of fibroblasts, are minimal in the limb stumps at st57 between 1dPA and 5dPA (Dent, 1962; Wolfe et al., 2004). This data might be an indication that the changes in the tissue composition is minimal at this period of time causing little or no change in the gene transcription due to the lack of or no regenerative capacity in limbs.

On the other hand, a study by Forrai et al. (2006) to determine the role of SOCS3 in embryonic stem cell signaling *in vivo* indicated that the fate and potency of murine embryonic stem (ES) cells was altered via the activation of cytokine signaling pathways. Forrai et al. determined that SOCS3 had a crucial role in regulating the self-renewal and pluripotency of the ES cells and that SOCS3 increased the cell potential for differentiation into the primitive endoderm lineage. The study indicated that in the murine ES cells, the absence of SOCS3 reduced self-renewal and promoted differentiation. This effect was due to the disruption of the STAT3 activation pathway

required for induction of SOCS3, which negatively modifies the ability of leukemia inhibitory factor (LIF). This LIF is necessary to maintain self-renewal of ES cells while constitutive expression of activated STAT3 prevents differentiation of ES cells after withdrawal of LIF. The induced expression of SOCS1 and SOCS3 in this study might also be due to the role of both genes in increasing the proliferative capacity of the dedifferentiated cells forming the blastema, thus favoring a successful regeneration. The maintained expression up to 5dPA in the non-regenerative stage most probably was due to increased inflammation and the demand for a higher expression of SOCS1 and SOCS3 to overcome the excessive inflammation to allow complete regeneration.

In summary, expression of SOCS1 and SOCS3 was induced in response to amputation in *X. laevis* possibly because of their potential roles in resolving inflammation at the site of amputation. The continued expression of SOCS1 and SOCS3 by 5dPA at the regeneration non-competent stage (st57) compared to the regeneration competent stage possibly contributes to the development of the immune system as the animal matures. The adaptive immune system is less tolerant to dedifferentiated cells recruited to the site of amputation to proliferate and form a regenerating limb. This is consistent with a previous study indicating the negative regulation of a chronic induced inflammation of st57 at 5dPA pseudoblastemas relative to st53 blastemas (Screiber et al., 2002). This postulation supports the hypothesis that the immune system and its signaling modulators influence the tissue response to injury and tolerance to the dedifferentiated cells, therefore affecting the capacity for limb regeneration.

Spatial Expression of SOCS1 Using WMISH at st53, st55 and st57

To determine the vicinity of induced expression for SOCS1 in regeneration competent blastemas (st53) vs. non-competent pseudoblastemas (st57) as indicated by RT-PCR and qPCR assays, whole mount *in situ* hybridization (WMISH) was done using DIG-labeled antisense riboprobes. The current study did not examine SOCS3 expression using the WMISH.

PCR and qPCR indicated that there was no significant expression for SOCS1 in intact regeneration competent blastemas (st53) and non-competent pseudoblastemas (st57). The WMISH results were consistent with those of RT-PCR and qPCR assays. The WMISH assay did not indicate the expression of SOCS1 in intact hindlimbs. However, an induced expression was shown in response to amputation at 1dPA at st53 and st57 which was sustained at 3dPA and 5dPA. At st53, the expression was almost sustained at 3dPA, but decreased by 5dPA to levels comparable to 0dPA. At st57, the induction was maintained by 5dPA to a higher level than at 0dPA. The induced expression of SOCS1 was observed in clusters of cells in the underlying mesoderm of the amputated limbs but not in the epidermis as shown in Figure 25.

SOCS proteins are negative regulators of adaptive and innate immune responses (Belardelli & Ferrantini, 2002; Qin et al., 2007). The inhibitory role of SOCS1 in inflammation is due to wide range of effects on different cell types as well as on different receptors (Yashimura et al., 2007; Wormald & Hilton, 2007; O´Shea & Murray, 2008). A previous study indicated an induction of SOCS expression in response to inflammation (Qin et. al., 2007). This induction was reported in many cells including T cells, microphages and dendritic cells.

The role of T cells in the adaptive immune system is essential in determining the response of tissue to injuries and thus the extent of fibrosis. It has been suggested that SOCS1 regulates T cell development. After these T cells are activated and able to produce IFN-γ, a cytokine with potent antimicrobial and antiviral properties, the SOCS1 inhibitory effect is directed to the responsive cells to modulate the damaging inflammatory effect of IFN-γ (Qin et. al., 2007; Yashimura et al., 2007; O´Shea & Murray, 2008). Using WMISH, this study compared SOCS1 expression at a stage where the capacity for regeneration was limited (st55) to the regeneration competent (st53) and the non-competent stages (st57). This comparison was done in an attempt to identify the correlation between the changes in a developing immune system, and the capacity for limb regeneration. It has been reported that the period between st53 and st59 is a transitional state between regeneration competence without scar formation and a regeneration incompetent state with scar formation (Yannas, Coly, & Wai, 1996). The study of Yannas and colleagues (1996) developed a schemata describing the regenerative capacity from early life stages of *X. laevis* (egg) through adulthood (frog). According to the schemata, early developmental stages possess complete capacity for regeneration and for wound healing without scar formation. In describing the influence of the immune system on the capacity for regeneration, the study (Yannas et al., 1996) cited an inverse relationship between the regenerative capacity and the development of the immune system. The schemata indicated that as the adaptive immune system is mature, there is no successful regeneration and wound healing occurs with a scar formation. Therefore, stage 55 was chosen in the current study for the determination of SOCS1 expression in the transitional period as described above.

The WMISH was used to compare SOCS1 expression in response to amputation for these three stages (st53, st55, and st57) at 1dPA and 5dPA in addition to the intact limbs. In intact limbs, the WMISH assay using st55 showed no expression of SOCS1, which was consistent with the result of the other two stages (st53 and st57). At st55, there was significant induction for SOCS1 expression in response to amputation at 1dPA (Fig. 27) similar to st53 and st57; however, the increased expression was concentrated at the distal area of the mesoderm at st55 at 3dPA. By 5dPA, surprisingly there was no detected expression of SOCS1 at st55 compared to st53 and st57 at 5dPA. It is unlikely that SOCS1 expression was undetectable at the normal stringency used in WMISH assay in this study (hybridization temperature is 70°C; Fig. 26D) because all other stages worked at this range of stringency. The possibility for this difference in gene expression is the transient status of the immune system from immaturity (innate) to the maturity (adaptive) at st55. Most likely, the immune system at st55 is maximally tolerant to inflammation leading to a minimal or no cytokine secretion. Therefore, it is suggested that SOCS1 was not expressed in view of the fact that there was no need for negative feedback for cytokine signaling.

In summary, WMISH data showed that the expression for SOCS1 was induced at 1dPA and was sustained through 5dPA in the regeneration competent blastemas in clusters of cells in the mesenchyme. Expression of SOCS1 most probably is a protective response to attenuate the inflammation triggered in the limb microenvironment. Therefore, This attenuation will allow for a successful regeneration. Because the identity of cells expressing SOCS1 was not determined, this gene induction in microphages or T-cells penetrating into the site of injury is possible. Determining cell type (s) expressing

SOCS1 is beneficial to determine the exact role of SOCS1 in regenerating limbs. Previous reports detected some genes involved in development, such as *Shh, Glia3* and *Hox,* were expressed in the mesodermal region (Te Weschler et al., 2002). It may be possible that that SOCS1 and SOCS3 affect such developmental genes, especially those involved in patterning.

Future studies are required to address the role of SOCS1 in limb regeneration by determining the specific targets for SOCS1 during inflammation, especially on T cells. Furthermore, the identity of cells expressing SOCS1 in the mesenchyme and their exact roles in limb regeneration has to be investigated.

Limb Cultures

The specific signaling that mediates the deterioration in the regenerative capacity in anurans are unknown but may be caused by local responses that are intrinsic to limb tissue rather than systemic or hormonal responses. This theory is based on regeneration studies where tissues of st53 larvae and postmorphic froglets were successfully grafted together (Sessions and Bryant 1988). In an attempt to detect whether the increased expression of SOCS1 and SOCS3 in response to amputation was due to a local effect or extrinsic factors, the expression of both genes was examined in limb cultures for regenerative blastemas at st53. Therefore, the RT-PCR assay was performed on the three regions of an amputated limb (paddle, distal, and proximal). The expression of SOCS1 and SOCS3 was determined in response to amputation at 1dPA, 3dPA and 5dPA in all pieces. There was an induced expression for both genes, however, the induction was less significant in the paddle region compared to the distal and proximal pieces. This data was consistent with that of the PCR assays performed on st53 blastemas at 0dPA, 1dPA,

3dPA and 5dPA (Fig. 20). This suggested that the response to amputation in *Xenopus* hindlimbs is definitely a local response because limbs were cut into pieces and isolated from the influence of the systemic signals on them (Fig. 28). This may have been due to the presence of different types of immune cells including neutrophils and macrophages that are involved in the response to inflammation triggered by amputation (Mescher et al., 2007; Qin et al., 2007).

Effect of Beryllium on the Expression of SOCS1, SOCS3, IL-β and Shh

In an attempt to confirm the effect of inflammation on the expression of SOCS1 and SOCS3 and to determine the possibility for an external manipulation for limb regeneration *in vivo*, the effect of beryllium, a hyperinflammatory agent, was examined on cultured regeneration competent blastemas (st53) at 0dPA, 1dPA, 3dPA, 5dPA and 7dPA (Fig. 29). In addition, the effect of inflammation on the patterning signaling was examined via determining the effect of beryllium on the expression of Shh, a gene involved in the posterior-anterior patterning of limbs in amphibians. As an indication for the induced inflammation by beryllium, the expression of IL-1β, an proinflammatory gene, was examined. Previous studies have reported the inhibitory effect of beryllium on limb regeneration (Polák et al., 1968; Tsonis, 1991); however, the exact inhibitory mechanism and factors involved in this inhibition were not determined. Studies performed to determine the initial mechanism of limb regeneration in the amphibian *Ambystoma mexicana* reported that immediate application of beryllium to the injured limbs caused a complete regeneration failure (Tsonis 1990; Tsonis et al., 1991). These studies suggested that beryllium inhibits production of inositol phosphates that are generated as an immediate response to amputation. The inhibitory role of beryllium on

limb regeneration is most likely due to its action as a phosphate analog, thereby blocking the phosphate transfer enzymes leading to a reduction in the production of inositol phosphates required for limb regeneration (Tsonis, et al., 1991).

In the current study, beryllium has caused an induced expression of IL-1β, a potent component of the IL-1, at 1dPA followed by a decrease expression by 3dPA that was maintained at 5dPA through 7dPA; however, no expression was detected in controls. This data confirmed that the inflammatory response caused by beryllium in blastemas compared to controls was triggered at 1dPA and sustained through 7dPA.

In addition, this study showed that beryllium-treated blastemas at st53 in a concentration of 10 m*M* prior to amputation caused an increased expression of SOCS1 and SOCS3 in response to amputation at 1dPA. The induced expression of SOCS1 at 1dPA was significantly higher in beryllium-treated blastemas compared to controls. For SOCS1, the induced expression in the treated samples was increased at 3dPA and maintained by 5dPA, however, by 7dPA, the expression was diminished. For SOCS3, there was an induced expression at 1dPA but this expression was gradually decreased at 3dPA and maintained through 5dPA in beryllium-treated blastemas, however, the expression of SOCS3 was slightly increased by 7dPA. Compared to SOCS1, the expression of SOCS3 was significantly higher at 1dPA.

The pattern for SOCS3 expression was almost biphasic where there was a significant increase in expression at 1dPA, a decline at 3dPA and 5dPA and then an increase at 7dPA. This biphasic pattern of expression was previously reported for IL-16, a proinflammatory cytokine that is known as a chemotactic factor for several cells that participate in the immune response to inflammation including T-lymphocytes, monocytes

and eosinophils (Wormald & Hilton, 2007). This might explain the pattern of biphasic SOCS3 expression in the present study. Wong and colleagues (2006) reported that IL-1 directly induces SOCS3 in macrophages. Therefore, regulating IL-1β expression via an endogenous increase in SOCS3 might be critical in attenuating the inflammation and thus in inducing limb regeneration. In addition, other studies suggested that exogenous delivery of SOCS3 has therapeutic benefits in the treatment of conditions associated with successive inflammatory responses such as in certain hematological malignancies and proliferative disorders (Wormald & Hilton, 2007).

On the other hand, the inhibitory effect of beryllium on the expression of Shh in the treated blastemas was suggested since treated samples did not show an expression for Shh gene compared to control samples. It has been reported that Shh was expressed in the posterior region of a developing hindlimb bud of a stage 53 tadpole but not in froglet pseudoblastemas (Te Welscher et al., 2002). Therefore, failure of regeneration in beryllium-treated blastemas at st53 (data not shown) was probably due to the inhibition of signaling pathways involved in limb patterning. Previous studies on Shh showed that expression of *X. laevis Shh* is restricted to regenerating structures; where the expression was limited to the posteriodistal area of a regenerative limb where digits develop. In addition, a reduced expression of Shh was reported in pseudoblastemas and none was detected in froglet pseudobalstemas (Endo et al., 1997; Matsuda et al., 2001; Yakushiji et al., 2007). This finding suggests the importance of Shh in regulating the anterio-posterior patterning by determining the digits number and identity.

As a result, this study suggests that the inflammation caused by beryllium caused an interruption to the patterning signaling of regenerative blastemas.

CONCLUSION

This study isolated full-length cDNAs of SOCS1 and SOCS3 in *Xenopus laevis* and found that both genes were expressed at early developmental stages. An induced expression of both genes was observed at the stage of limb bud formation (st48). The expression of SOCS1 and SOCS3 in response to limb amputation was determined in the regeneration competent stage (st53) and the regeneration non-competent stage (st57). For both stages, upon limb amputation, SOCS1 and SOCS3 were induced at one day post amputation with higher induction for SOCS3. Their expression decreased gradually at three days and five days post amputation. Of note, by 5dPA the expression was higher at st57 than st53. This data indicate that the sustained expression for SOCS1 and SOCS3 in non-regenerative limbs is due to their possible role in resolving inflammation triggered by amputation. Consequently sustained gene expression may be the cause for failure of regeneration.

Using whole mount *in situ* hybridization, the induced expression of both genes was localized in the mesedermal cells. This localization suggested that the expression of SOCS1 and SOCS3 occurs in proliferating regions of cells having a stem-like nature. The induced expression of SOCS1 and SOCS3 in cultured blastemas indicated that the response of both genes in response to limb amputation was due to a local signaling. Hence, external manipulation may be used to regulate the expression of these immunomodulatory genes, thereby promoting regeneration.

In studying the effect of induced inflammation by beryllium on regenerative blastemas at st53, the expression of SOCS1 and SOCS3 was higher in beryllium–treated blastemas than non-treated blastemas from 1dPA through 7dPA. In addition, there was a suppressed expression of the Shh gene, which plays a role in the patterning of regenerative limbs. This might indicate a correlation between a high inflammatory response and the suppression of the patterning signaling. This suppression may be an indicator of failure of regeneration due to the triggered excessive inflammation caused by beryllium.

In summary, SOCS1 and SOCS3 were induced in response to limb amputation with the maintenance of expression in non-regenerative blastemas where the immune system is more advanced. It is proposed that the development of the immune system of adult anurans modifies the capacity of limb regeneration. This hypothesis states that due to the participation of variety of immune cells in the response to inflammation caused by amputation, there is a modulation in regenerative capacity. Based on the study, the induced expression of SOCS1 and SOCS3 in response to limb amputation is regulated by the degree of the maturity of the immune system at various developmental stages. The induction of both genes in regenerating blastema at a higher level than pseudoblastemas is probably in the favor of inducing a successful regeneration. However, the exact role of SOCS1 and SOCS3 in limb regeneration must be characterized in further detail.

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