MAKING TRANSGENIC AXOLOTL FOR LINEAGE TRACING CONNECTIVE TISSUES DURING LIMB REGENERATION

MASTER'S THESIS

Technische Universität Dresden

Biotechnologisches Zentrum (BIOTEC)

Master Course "Molecular Bioengineering"

To achieve the academic degree

"Master of Science" (MSc)

by

Liubov Plyasunova

Completed at: CRTD/DFG-Center for Regenerative Therapies Dresden - Cluster of Excellence

Submitted on: October 1, 2012

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First Supervisor:

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Statement of Academical Honesty

I hereby declare that I have not used any auxiliary source for my Thesis work other than have been cited in my thesis.

Dresden, October 1, 2012

Liubov Plyasunova

ABSTRACT

Salamanders are known for their ability to reconstitute such complex structures as limb after amputation. Immediately after injury, the lineage-restricted pool of progenitor cells called blastema is formed. The major contributors to blastema formation are different subtypes of connective tissues [1]. However, their true potential to blastema formation is not examined so far. In order to study specific cell types and their participation during limb regeneration we generated tissue-specific Cre line. These animals will be useful for cell-lineage tracing experiments in association with the Cre/LoxP technology. These transgenic axolotl will be crossed with Lox-P transgenic axolotl to trace tissue during regeneration. Particularly, my project was focused on one candidate marker, Scleraxis, which specifically expressed in tendons and ligaments in mouse model. During this project, pScleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-Kan-FRT construct was made using recombineering method and transgenic animals were generated; specific expression presumably in fibroblasts was detected.

Accumulating studies report, that TGF-β signaling pathway is involved in scar formation during wound healing in mammals. However, recently TGF-β signaling was shown to be crucial for limb regeneration in axolotl. Due to these controversial data the second part of my project was focused on studying TGF-β pathways' role during limb regeneration. TGF-β and TGF-β RII genes were isolated from cDNA library, in-situ hybridization experiments were performed and it was observed that both ligand and receptor are ubiquitously expressed in all tissues of limb and blastema. Plasmid constructs, harboring TGF-β and TGF-β RII DN genes under control of CAGGS promoter were designed and validated by *in vitro* experiments. Constructs were also electroporated into blastema in order to overexpress genes TGFβ-1 and TGFβ RII DN. Transgenic animals expressing TGFβ-1 and TGFβ RII were generated for experiments utilizing Cre-loxP system to study cell fate and gene function during limb development and regeneration.

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OUTLINE:

ABBREVIATIONS

AEC	Apical ectodermal cap
BMP	Bone morphogenic proteins
ECM	Extracellular matrix
FAs	Focal adhesions
FN	Fibronectin
GDF	Growth and differentiation factor
LAP	Latency associated polypeptide
LTBP	Latent TGF-β binding protein
MAPK	Mitogen-activated protein kinase
MIS	Muellerian inhibiting substance
MMP	Matrix metalloproteinase
SBEs	Smad binding elements
Ssx	Scleraxis
TGFβ	Transforming growth factor beta β
TGFβ R	Tranforming growth factor β receptor
Tnmd	Tenomodulin
WE	Wound epidermis
α-SMA	α-smooth muscle actin

Introduction

Scleraxis: a potential marker of ligament and tendon

The salamander is an excellent vertebrate model to study regeneration, since it can reconstitute functional limb after amputation. Immediately after injury, the lineage-restricted pool of progenitor cells called the blastema is formed [1]. The major contributors to its formation are different subtypes of connective tissue such as bone, cartilage, tendon, ligament, fascia and dermis. However, their true potential in blastema formation is not examined due to the lack of markers.

Tendons and ligaments are soft connective tissues, made of proteoglycan matrix, fibroblasts and collagen fibers, oriented in parallel, providing motion and stability of joint in musculoskeletal system. Tendons connect muscle to bone, and ligaments connect bone to bone. The main function of tendons and ligaments is to transmit tensile load, the way to provide smooth movement of joints under normal circumstances and to restrain excessive joint displacements under high loads. The biochemical content, tensile properties and repair capabilities differ not only in tendons and ligaments, but also in different ligaments from the same joint [2].

There are many questions remain to answer, such as: what is the percentage contribution of tendons and ligaments to blastema formation, what is the trans-differentiation ability of tendon and ligament, does an undifferentiated progenitor for tendons and ligaments exist or do all cells have equal capability to participate in regeneration, what are the signals that promote blastema formation in these cells?

The one candidate marker, which we would like to test, is Scleraxis. Scleraxis (Scx) is a basic helix-loop-helix transcription factor (bHLH), which is expressed in progenitor cells and differentiated cells of tendon and ligament. Scleraxis was first identified in 1995 by Peter Cserjesi and co-workers using yeast-two-hybrid system as a bait protein. Scleraxis was defined

from 10.5 day old mouse embryo-derived library as a novel cell type specific bHLH protein, which dimerizes and binds to the E-box consensus sequence with E12 and activates transcription throughout its multimerized DNA binding site. The expression of Scleraxis was first found in mesenchymal precursors of cartilage and connective tissues. Based on its expression pattern, binding properties and transcriptional activity, it was suggested, that scleraxis may be involved in regulation of gene expression within chondrogenic lineages and dense connective tissue during embryogenesis [3]. It was reported, that scleraxis promotes fibroblast proliferation and collagen synthesis in tendons, and has an important role in somitic tendon progenitors development [4], and regulates several other characteristic genes, such as *collagen type I, decorin, fibromodulin, and tenomodulin (Tnmd).* The *Scx* knock-out mice displayed reduced development of force-transmitting and intermuscular tendons and suppressed expression of other genes, such as *Col1a1* (type I collagen) and *Tnmd* [5]. *Scleraxis* continues to be expressed in tendons and ligaments following development. *Scleraxis* expression was also detected in monolayers of cultured fibroblasts [6].

The expression of *Scx* was evaluated by Ronen Schweitzer ad co-workers in E18.5 mice, using *Scx*-GFP transgenic reporter mouse in which green fluorescent protein expression was driven by regulatory sequences of the *Scx* gene. The *Scx* gene consists of two exons. The exon1 includes the majority of the coding sequence. The *Scleraxis* gene is located within the fourth intron of a second gene called *Bop1*. *Bop1* is transcribed in a reverse manner. The nine blocks of highly conserved sequence were identified between 100 bp and 400 bp, and were suggested to play a regulatory function. Moreover, block 8 and 9 were assumed to represent repressor elements. The conserved noncoding domains were grouped near the *Scx* gene, mostly within the *Bop1* intron, that includes the *Scx* gene. Additional conserved noncoding domains were found downstream of the *Bop1* gene. The construct was designed without these two (8 and 9) blocks. *Scx*-GFP was subcloned such that the GFP-fused fragment was introduced into *Scxg12* genomic clone, with GFP replacing most but not all of the first exon. *Scx-GFP* transgenic reporter specifically labeled tendon and ligament progenitor cells and all differentiated cell types within these connective tissues [7].

We assume, that *Scx* may serve as a potential marker and permit cell lineage tracing also in axolotls, which would be helpful to address above-mentioned questions. In this experiment, to build the transgenic constructs, we will use a 12-kb genomic clone from the mouse *Scx* locus (*Scxg12*). The TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT construct will be cloned within this genomic fragment. The method we will use is Recombineering.

Recombineering

Recombineering is the revolutionary method, which relies on homologous recombination. It allows a wide range of modifications of DNA molecules at any chosen position with minimized risk of unwanted mutations. Construction of recombinant DNA molecules by conventional cloning methods is limited in size, due to the usage of restriction enzymes. In contrast, recombineering permits engineering of DNA molecules of any size, including large BACs. This is particular applicable in the following experiment and cannot be accomplished by conventional cloning methods, since large cassette of 5.5 kb has to be inserted in exact region. The λ-mediated recombination finds many applications in DNA engineering, such as, subcloning by gap repair, insertion and/or deletion of selectable fragments, oligonucletide-directed mutagenesis [8].

In λ-mediated recombination, target DNA molecules are precisely altered by homologous recombination in strain *E.coli* DH10B that expresses phage-derived proteins Redα/Redβ from λ phage where Redα - 5'- 3' is an exonuclease, and Redβ is a DNA annealing protein. Interaction between these proteins is required to catalyze the homologous recombination. Redαβγ recombineering is based on crossover step between a targeting construct that comprise homology arms and the target, which is any gene locus in plasmid vector or BAC. Homology arms can be chosen freely and any position in target DNA can be altered in a specific and faithful manner. A double-strand-break repair is initiated by Redα which degrades the DNA in a 5'-3' direction and creates a 3' ss DNA overhang. Then, Redβ binds and forms protein-nucleic acid filament, which further aligns with homologous DNA allowing single strand annealing or strand invasion. The 3' end becomes a primer for DNA replication [9].

To use the *E. coli* host for recombineering, the recombination proteins Red αβγ need to be expressed. The BAD αβγ operon can be present on the low copy plasmid, pSC101-BAD-αβγA or it can be integrated into the genome (*E. coli* GB08-red). In this experiment *E.coli* strain GB008 will be used, where BAD- αβγA operon is integrated into the genome. The, β, γ genes of the λ phage together with *recA* gene in a polycistronic operon are under the control of arabinose inducible BAD-promoter [10]. The pBAD is regulated by the product of *araC* gene. AraC is a transcriptional regulator that forms a complex with L-arabinose. After addition of L-arabinose, expression of Red operon, which is under the control of arabinose-inducible promoter pBAD, can be induced and allows recombineering of electroporated cassette [11].

Limb regeneration in axolotl vs wound healing in mammals

Axolotl (*Ambystoma mexicanum*) are best known for their ability to regenerate amputated body parts, such as limbs, lens, tail, spinal cord. Tissue regeneration is accomplished by total restoration of structure and function of the damaged limb or organ. The regeneration process consists of three important steps: wound healing, formation of blastema, and a phase of redevelopment of lost tissue. The first phase is very similar with mammalian wound healing, although mammals cannot regenerate amputated appendages and form scars after healing the wounds. On the contrary, salamanders never form scars [12].

In most mammals, a superficial injury of the skin will heal rapidly. However, a deep skin injury, which damage dermis, leads to formation of scar. Mammalian wound healing process is divided into three steps: 1) inflammation, 2) proliferation and 3) maturation [13].

Wound healing process in mammals

Haemostasis is the earliest process that happens and is achieved initially by the formation of a platelet plug, followed by a fibrin matrix, which becomes the scaffold for infiltrating cells. The inflammation phase starts immediately and lasts for 4-6 days and involves clot formation, entry of neutrophils and macrophages, and initiation of reepithelization [14]. Inflammation is mainly caused by the release of cytokines, such as epidermal growth factor, transforming growth factor-β, tumor necrosis factor-α, the interleukins α or β, by the adjacent

extracellular matrix (ECM) and immune cells. These cytokins influence cell response, ECM, and skin cells movement and proliferation and thus direct the outcome of wound healing [12].

During the proliferative phase new tissues are formed. This phase involves angiogenesis, collagen deposition, granulation tissue formation, epithelialization and wound contraction [15]. Keratinocytes migrate under the scab to reestablish the epidermal layer. In addition, some fibroblasts around the wounded area transform into myofibroblasts, characterized by expression of α-SMA (α-smooth muscle actin) and migrate in the wound. They facilitate wound closure by contraction [12]. The last step of wound healing is characterized by remodeling of wounded area. Most of the immune cells and myofibroblasts involved during first two phases are removed by apoptosis. Extracellular proteins such as matrix metalloproteinases (MMP) rearrange the collagen fibers in the scar tissue during next weeks and months [12].

Two of the most common types are hypertrophic and keloid scarring. Hypertroffic scars don't extend beyond the confines of original wound; however keloid scars are more aggressive continually growing and invading into normal surrounding tissue and rarely regress overtime. The major reason of abnormal wound healing and fibrotic disorders is differences in the regulation of the expression of ECM. The transcription and translation of collagen I and III, fibronectin, laminin, periostin and tenascin are increased in hypertrophic scars and further increased in keloid scars compared with normal tissue. However expression of hyaluronic acid (HA), dermatopontin and decorin is decreased in dermal scarring [16].

The main component of ECM is collagen, which forms a relaxed network of cross-linked long-chain fibers and gives the integrity to skin. There are two types of collagen which play important role in wound repair process- type I and III. All scarring is composed of the same collagen as the tissue it has replaced, but with differences in composition. The expression of collagen III is prominent during early proliferative phase of wound healing and then it's replaced by collagen I in the late proliferative and remodeling phases. Both types of scar experience excessive stiff collagen bundled growth instead of network and are less organized. The ratio of collagen I to collagen III in keloid scar is 17:1, and in hypertrophic scar it is 6:1 [16].

The scarring is created by fibroblast proliferation, a process that begins with a reaction to the clot. During the fibroproliferative phase of wound healing, fibroblasts form a new extracellular matrix, composed of granulation tissue, which replaces the fibrin clot. The ECM provides structural support and acts as a skeleton for cells, connective tissue, and other components to adhere to and grow. In response to injury, fibroblasts undergo a phenotype transition into myofibroblasts, which are characterized by the expression of α-SMA, increased contractile activity and ECM synthesizing activity [17]. These myofibroblasts establish a grip on the wound edges and contract themselves, making the wound smaller. Afterwards, when wound heals, myofibroblasts are removed by apoptosis. When the control mechanisms during wound healing are abnormal, the hypertrophic scar formation occurs [18].

Although, molecular mechanisms of fibroblast-to-myofibroblast transition are poorly understood, growth factors and especially TGF-β are reported to play important role in fibroblast-myofibroblast transdifferentiation. Inhibition of TGF-β could inhibit fibroblast-to-myofibroblast transition or development of organ fibrosis [17].

Diferentiated myofibroblasts share a number of similarities with smooth muscle cells including the expression of SMC-characteristic cadherins, such as R-, T-cadherin, and cadherin 6B. It is suggested that acquisition of specific cadherins may be induced by TGF-β [19]. During wound healing *in vivo* and after TGF-β induction *in vitro*, myofibroblast differentiation is accompanied by an increase of OB-cadherin expression and a decrease of N-cadherin expression. The α-SMA is incorporated into stress fibers and lead to the formation of specialized contacts with ECM that are called supermature focal adhesions (FAs) *in vitro* and fibronexus *in vivo*. FAs are absent in normal tissue fibroblasts that do not develop stress fibers. They couple the stress fibers of adjacent myofibroblasts to coordinate their contractile activity during connective tissue remodelling [19].

In mammalian embryos wound healing is scar-free until the half of the second trimester of gestation. Later in development and in adults wound healing is differently regulated and leads to the formation of scar [20].

Moreover, human and mouse neonates also maintain the ability to regenerate their digit tips [21]. Some experiments demonstrated that wounds in fetus have deficient amount of TGF-β in comparison with wounds in adult tissue. TGF-β1 and TGF-β2 are expressed within developing fetal dermis. No differential up-regulation in the healing wound was observed. However in adult wounds the expression of TGF-β1 and TGF-β2 was increased [22].

An addition of TGF-β to fetal wounds induced scar formation [23]. When human fetal skin was transplanted into rat subcutaneous zone, subsequent wounding resulted in tissue recover without scaring [23]. In adult wound low-dose subcutaneous injection of TGF-β resulted in fibrosis and accumulation of fibroblasts, macrophages and granulocytes [24].

TGF-β knock-out mice showed the reduction in the amount of granulation tissue and an increased level of epithelialization [24]. Various studies *in vivo* and *in vitro* demonstrated that blocking TGF-β activity with antibody decreased scar formation. Treatment of wound with TGF-β 1,2, 3 antibodies at early time points resulted in delayed wound healing without reduction in scaring. Later when epithelialization is completed treatment with antibodies resulted in reduction of scaring [25].

Axolotl limb regeneration

Axolotl limb regeneration consists of two phases. First phase starts immediately after amputation and is referred as preparation phase. It is characterized by formation of wound epithelium over the wounded area, cellular differentiation and migration, which eventually lead to formation of blastema. During the second - redevelopment phase blastema cells start redifferentiation to form the lost part [26].

The preparation phase of regeneration in axolotl is very similar to wound healing in mammals. Both regeneration and wound healing are triggered by trauma, which is followed by the up-regulation of stress signals, inflammation and formation of blood clot. All these events occur within minutes and are followed by formation of wound epithelium. The formation of wound epidermis (WE) takes 2-6 hours in urodeles after amputation and 12-48 hours after wounding in mammals. This is a crucial step in regeneration and wound healing [27]. If the formation of WE is prevented, regeneration does not happen and wound healing is retarded.

Preparation phase is characterized by extensive remodeling of ECM through action of metalloproteinases and tissue inhibitors of metalloproteinases, as for wound healing in mammals. Inhibitors of MMP were reported to inhibit limb regeneration and led to formation of scar-like structures with collagen deposits on the stump of amputated area [26].

The wound epithelium thickens and forms an apical epithelial cap (AEC). The secretion of MMP and formation of AEC promote generation of blastema, likely through process of undifferentiation [15]. The blastema is the heterogeneous pool of progenitor cells restricted to their own identity. The mature limb consists of multiple tissues, including the epidermis, dermis, muscle, nerve, blood vessels and skeletal elements. These tissues potentially contribute to the blastema. Dermis was reported to be the most flexible tissue. Through resident stem cells or reversion of fibroblast phenotype, it can regenerate cartilage, tendon, but not muscle or Scwann cells,. Muscle make muscle, but not cartilage or epidermis. Spinal cord radial ganglia can form muscle or cartilage. Blastema cells form different tissues are localised in disinct subregions. For example, dermis is localazed in distal part of blastema [1].

Redevlopment phase involves growth of blastema, pattern formation and redifferentiation. Blastema continues to grow distally via cell proliferation untill the entire limb is regenerated. During regeneration interactions between the blastema and AEC are essential for elongation of limb regenerate. These interactions are likely to be mediated by several growth factors including TGF-β. Along with elongation, blastema directs re-differentiation and re-patterning to form a new limb [28].

The proteomic analysis of blastema formation in the amputated limbs of Axolotl revealed number of significant changes in protein expression related to cell signaling, transcription, metabolism, cell protection and cell cycle. The pathways regulated by transcription factors c-Myc and SP1 regulate 36.2% of the proteins expressed during axolotl limb regeneration [28].

TGF-β signaling is required for the formation of wound epidermis after amputation. TGF-β and Smad2, the component of TGF-β-mediated signaling pathway, are up-regulated during formation of wound epidermis and later in the blastema [28]. Fibroblasts invade granulation tissue and mediate wound contraction in mammals. In the axolotl they dedifferentiate

and provide one of the main sources of blastema cells. Possibly, during preparation phase TGF-β promotes detachment and migration of mesenchymal cells towards the blastema [27].

TGF-β 1 can lead to the expression of SP1 and fibronectin (FN), which is produced by blastema cells and AEC. FN then activates c-Myc via integrins and wnt signaling. Moreover, transcription factors such as Klf4, Oct4, Lin28 common for embryonic stem cells, induced pluripotent stem cells and blastema cells are also connected to c-Myc and SP1 [28].

Inhibition of TGF-β signaling with inhibitor of SMAD phosphorylation SB431542 immediately after amputation reduces FN expression and prevents establishment of wound epidermis and initiation of blastema formation [12, 28].

TGF-β signaling pathway

The TGF-β pathway controls diverse processes in most cells, including cell proliferation, recognition, differentiation, apoptosis, and specification of developmental fate during embryogenesis. Subsequent studies have shown that TGF-β causes fibrosis and promotes inflammation in multiple tissues including skin, liver, kidney, lung, colon, heart [24]. Increased TGF-β signaling is also associated with several diseases of skeletal muscle, including many of the muscular dystrophies [29]. TGF-β also plays an important role in cancer progression. In an early phase, TGF-β acts as a tumor suppressor, while later it participates in tumor progression [30]. Mutations in TGF-β family ligands are responsible for a number of human diseases including hereditary chondrodysplosia in humans and persistent mullerian duct syndrome [31]. Numerous studies over the last decades revealed a TGF-β signaling pathway, which is relatively simple and highly conserved among various species, including worms, flies and vertebrates [32].

The TGF-β is the cytokine family, which includes two subfamilies: TGF-β/activin/nodal subfamily and BMP (bone morphogenetic protein)/GDF (growth and differntiation factor)/MIS (Muellerian inhibiting substance). All of these growth factors have a cluster of conserved cystein residues that form a common cystein knot structure. The active form of TGF-β is a dimer stabilized by hydrophobic interactions which are strenghtened by disulfide bridges.

The TGF-β family is encoded by 33 genes encoding structurally related polypeptides that correspond to ligand precursors [33]. Mammalian TGF-β itself has at least three isoforms, including TGF-β 1, TGF-β 2, and TGF-β 3. TGF-β 1 contains 390 aminoacids and TGF-β 2 and 3 contain 412 aminoacids [34] . The three TGF-β isoforms share 70-80% aminoacid homology, and almost 100% conserved across species. They show similar functions *in vitro*. However, they are differentially expressed temporally and spatially during embryogenesis, wound repair, and carcinogenesis, suggesting distinct roles *in vivo* [35]. Specifically, TGF-β 1 is absent in normal dermis, whereas most dermal fibroblasts strongly stain for TGF-β 3, and TGF-β 2 is present only in SM cells. TGF-β 1 is detectable in granulation tissue only in the late stages of wound healing, whereas TGF-β 2 and 3 are present after 1 day of wounding and diminish with wound healing progression [36]. Embryonic wounds that heal without a scar have low levels of TGF-β1 andTGF-β2, low levels of platelet-derived growth factor and high levels of TGFβ-3 [37].

TGF-β is secreted in a latent form, in which it is complexed with two other polypeptides, latent TGF-β binding protein (LTBP) and latency associated polypeptide (LAP). The LAP region includes 20-30 aminoacids. Release of active TGF-β form from complex is accomplished by serum proteinase [34]. The mature TGF-β protein dimerizes and produces a 25 KDa active molecule [38]. TGF-β triggers signaling by binding and bringing together TGF-β receptor I and TGF-β receptor II (serine/threonine kinases) on the cell surface. The TGF-β receptor I phosphorylates TGF-β receptor II, which then distribute a signal through phosphorylation of Smad proteins [31, 39] (Fig.1).

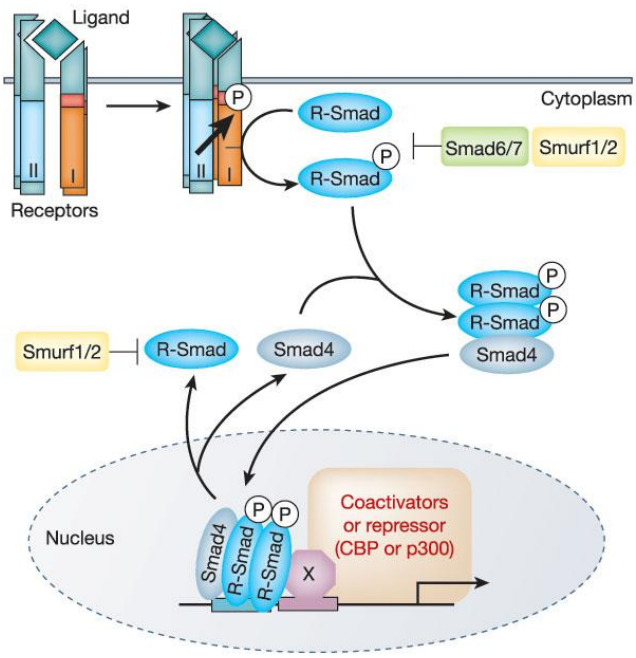

Figure 1. Tgf-β signaling pathway [39].

There are eight different Smad proteins divided into three groups: the receptor-regulated Smad (R-Smad), co-mediator (Co-Smad), and the inhibitory Smad (I-Smad). R-Smad are directly activated by TGF-β receptor I and form heteromeric complexes with Co-Smad, Smad4. The activated Smad-complexes are translocated into the nucleus and regulate transcription of target genes along with other nuclear cofactors. Smad6 and Smad7 (both I-Smads) inhibit TGF-β signaling by competing with R-Smad for receptor or co-Smad interaction and target TGF-β for degradation [40].

The access of TGF-β ligand to receptor is controlled by two groups of molecules. One group of proteins acts as ligand binding traps preventing its access to membrane receptors. For example, decorin and alpha-2-macroglobulin bind to free TGF-β. The other group includes membrane-anchored proteins acting as accessory receptors, or co-receptors. The proteoglycan β glycan (TGF-β III receptor) mediate TGF-β binding to type II receptor [31].

There are twelve members of serine/threonine kinase family in human genome involved in Tgf-β signaling pathway. Among them are 7 of type I and 5 of type II. Around 500 aminoacids comprise TGF-β receptor organized into N-terminal extracellular ligand binding domain, a transmembrane region and C-terminal serine/threonine kinase domain. Both type I and

type II receptors contain a characteristic SGSGSG sequence, named GS domain. Phosphorylation of GS domain by type II receptor activates type I receptor [31].

The activated type I receptor phosphorylates the C-terminal di-serine motif of Smad proteins. These receptor-activated Smads (R-Smads) exhibit specificity in their interaction with type I receptor. There are R-Smads Smad2 and Smad3, which recognize the L45 loop region of ALK4, ALK5 and ALK7 (type I receptors for TGF-β, activines and some GDFs). R-smads Smad1, Smad5, and Smad8 show specificity for ALK1, ALK2, ALK3 and ALK6 (type I receptors for BMPs, GDFs and AMH) [41]. The binding of the R-Smads to the TGF-β receptor complex is facilitated by a FYVE domain-containing adaptor protein called SARA [42]. C-terminal phosphorylation of the R-Smads leads to a conformational change of the C-terminal conserved domain, called Mad-homology 2 (MH2) domain. Once phosphorylated, the R-Smads dissociate from the receptor complex. Phosphorylated R-Smad oligomerize with itself or with Co-Smad (Smad4) and transported into nucleus [40].

Nuclear import of R-Smads does not actually require Smad4, although Smad4 cotranslocates with the R-Smads. Nuclear import of Smad1 and Smad3 is conferred by a lysine-rich nuclear localization sequence (NLS) in the MH1 domain that is conserved in all R-Smads. However, thenuclear translocation of Smad proteins can occur independently of importins, because Smad proteins can directly interact with nucleoporins. Nucleocytoplasmic shuttling of Smad2 requires its MH2 domain to interact with nucleoporins CAN/Nup214 and Nup153. In contrast to ligand-dependent import of R-Smads, Smad4 continuously shuttles between the nucleus and cytoplasm owing to the combined activities of a constitutively active NLS in the MH1 domain and a nuclear export signal (NES) in the linker region [39].

Smads bind with low affinity to DNA sequences named Smad-binding elements (SBEs) that include the minimal motif 5’-GTCT-3’ or its complementary 5’-AGAC-3’, to which primarily Smad3 binds, or G/C rich motif to which Smad4 and BMP-specific R-Smads bind. The N-terminal domain, or “Mad-homology 1” (MH1) domain is highly conserved in all R-Smads and Smad4, but not in Smad6. The linker region is divergent. The C-terminal domain MH2 is conserved in all Smads. MH1 domain is the DNA-binding module stabilized by bound zinc atom. The contact with DNA is established by beta-hairpin structure [43]. Binding of Smads to

DNA can be negatively regulated by phosphorylation of the MH1 domain of Smad2 or Smad3 by kinases, such as protein kinase or calcium-calmodulin kinase II and can be positively regulated by acetylation of the MH1 domain of Smad2 and Smad3 by acetyl-transferases such as p300 CBP and P/CAF [43].

The MH2 domain is the protein-interacting modulus in signal transduction. The MH2 domain of both R-Smads and Smad4 interacts with the transcriptional coactivators CBP, p300, ARC105, and Smif [44]. The C-terminal motif Ser-X-Ser is conserved in all R-Smads that is phosphorylated by activated receptor. A set of contiguous hydrophobic patches on the surface of MH2 domain mediates interactions with cytoplasmic retention proteins, components of nuclear pore complex and with DNA-binding cofactors. A region overlapping the linker and MH2 regions mediates interaction with transcriptional activators and repressors [43].

TGF-β receptor signaling is negatively regulated by the Smad7 inhibitory Smad (I-Smad). Complexes of Smad7 and the Smurf2 E3 ligase compete with SARA for binding to the TGF- β receptor and promote the ubiquitination and degradation of the TGF- β receptor complex [43].

Many genes are activated in response to GF-β ligands, whereas others are repressed. TGF-β inhibits cell-cycle progression by affecting the transcription of cell cycle regulators. Among them c-Myc and Id family members are down-regulated. Smad 3 represses the c-Myc transcription in association with transcription factors E2F4 and E2F5, and the co-repressor p107. In Id downregulation TGF- β -activated Smad 3 directly induces ATF3 expression, and ATF3 and Smad then form complex that represses the Id promoter. TGF-β also inhibits myoblast, osteoblast, adipocyte differentiation through functional repression of key transcription factors, which drive the differentiation pathways. Smad3 represses transcription by Runx2/CBFA1 in osteoblast differentiation, MyoD and other myogenic basic helix-loop-helix transcription factors in myoblasts, and CCAAT/enhancer-binding proteins in adipocyte differentiation [39].

Becides Smad-mediated transcription, TGF-β signaling is linked to other signaling cascades, including MAPK. Some of these pathways regulate Smad activation, others induce responses unrelated to transcription. TGF-β can activate the Erk, JNK and p38 MAPK kinase

pathways [39, 45] TGF-β-induced activation of the Erk and JNK pathways can result in Smad phosphorylation and regulate Smad activation. Also, TGF-β-induced activation of Ras/Erk MAPK signalling can induce *TGF-β 1* expression, thereby amplifying the TGF- β response and inducing secondary TGF- β responses [39, 46]. The dual ability of TGF- β to activate Smads and MAPK signalling has a role in TGF- β -induced epithelial-to-mesenchymal transdifferentiation, which depends in part on the Erk and/or p38 MAPK pathways. Depending on the cell line, TGF-β can rapidly activate Rho-like GTPases, including RhoA, Rac and Cdc42. Rac and Cdc42 regulate JNK and p38 MAPK pathway activation, presumably by directly interacting with MAPKKKs upstream of JNK and p38 MAPK, whereas Rho, Rac and Cdc42 affect the cytoskeletal organization [39, 47]. Activation of Rac1, RhoA and p38 MAPK, an effector of Cdc42, is required for rapid membrane ruffling and lamellipodia formation in response to TGF-β. Activation of RhoA and its effector kinase $p160^{ROCK}$, as well as Cdc24, p38 MAPK and Smad signalling have been implicated in TGF-β induced stress-fiber formation and epithelial-to-mesenchymal transdifferentiation [48]. Selective inhibition of the MAPK p38 prevents the Tgf beta-induced transdifferentiation of human fibroblasts to myofibroblasts [45]. Tgf beta inhibits cell cycle progression through up-regulation of gene expression of the p21 cell cycle inhibitor. Smad3 and Smad4 functionally cooperate with Sp1 to activate p21 promoter [30].

Interestingly, during embryonic development, TGF-β signaling induces expression of the transcription factor Scleraxis, which promotes fibroblast proliferation and collagen synthesis in tendons. TGF-β RII knockout (KO) embryos have a marked reduction in scleraxis expression and a subsequent disruption in the formation of limb tendons [49]. Experiments with mice demonstrated, that TGF-β decreased muscle fiber size and dramatically reduced maximum isometric force production. TGF-β also induced Scleraxis expression in muscle fibroblasts, and increased procollagen Iα2 and atrogin-1 levels in muscles[50].

It is suggested, that apart from Smads there are other signaling pathways involved in regulation of cell functions by TGF beta. Wnt-signaling pathway is reported to regulate the effects of TGF-β/Smad signaling. It was demonstrated, that the expression of beta-catenin protein was up-regulated by TGF-β mainly at protein level, which in turn inhibits the TGF-β1 induced fibroblasts-to-myofibroblasts transition. B-catenin accumulation inhibited the

contractility of myofibroblasts induced by TGF-β, which was associated with down-regulation of α-SMA expression in the fibroblasts [17].

Members of mitogen-activated protein kinase (MAPK) signaling cascades are also activated by TGF-β. P38 direct activation is based on TAK and MMk3/6 activation without involvement of Smad proteins. An indirect activation relies on expression of stress-inducible protein GADDF45-β. Mechanical tension and integrin signaling are prerequisites for the acquisition and maintenance of the myofibroblast's phenotype. *In vitro* experiments showed that selective inhibition of the MAPK p38 prevents the TGF-β-induced transdifferentiation of human tenon fibroblasts to myofibrobalsts [45].

TGF-β and its link to cytoskeleton

The members of TGF-β family enfluence the cytoskeleton organization. By targetting actin cytoskeleton TGF-β changes the overall cellular architecture, which affect cell proliferation and differentiation and facilitates cellular motility [51].

Upon TGF-β ligand binding, TGF-β R II phosphorylates Par6 which recruits the ubiquitine ligase Smurf, which in turn leads to Rho A degradation [52]. RhoA is a small GTP-ase, which positively regulates F-actin and tight junction assembly, and promotes apico-basal polarity and junction stability. As the result, TGF-β signaling leads to local disassembly of actin microfilaments, associated with the assembly of the junctions [41].

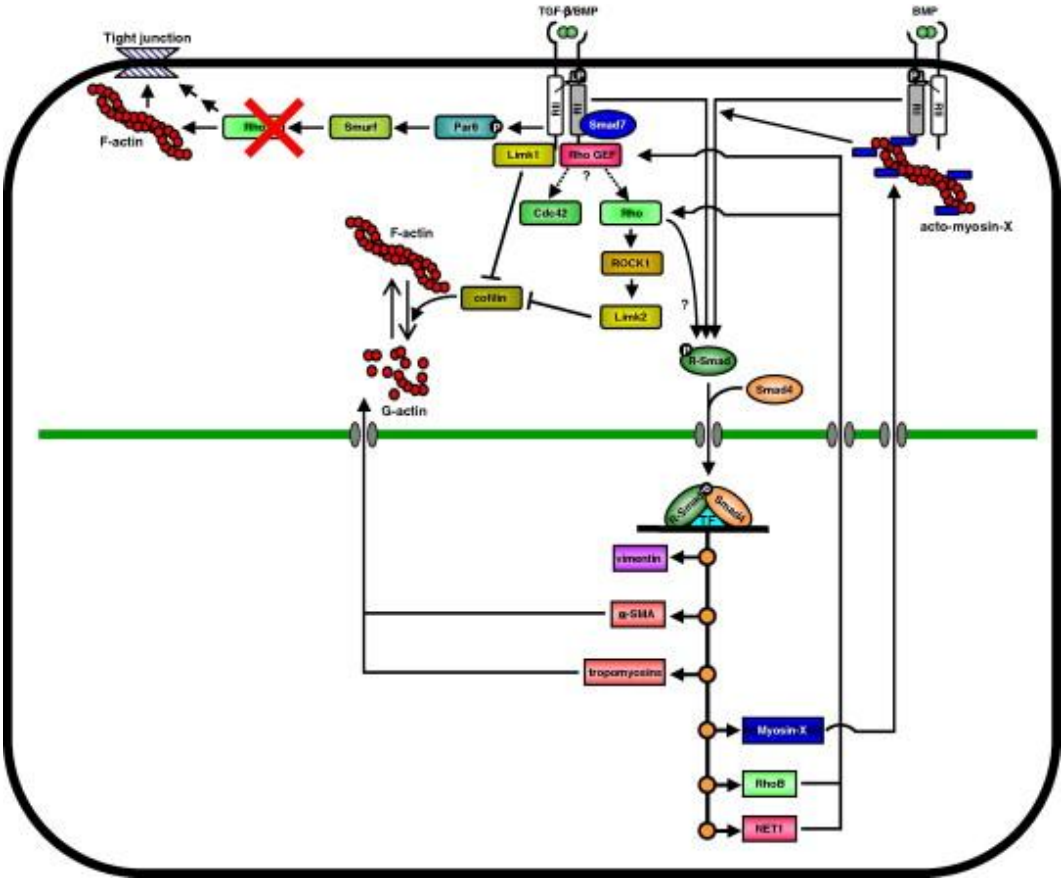

Figure 2. TGF-β/BMP signaling to the cytoskeleton.

Adherens junctions are mediated by interactions of the extracellular domains of E-cadherin. Intracellular domains link the protein with actin cytoskeleton via α- and β-catenins. TGF-β induces MMP-s mediated E-cadherin disruption, which causes nuclear translocation of β-catenin, the transcriptional induction of Slug, and the repression of E-cadherin transcription in tubular epithelial cells [53]. Signals from TGF-β or BMPs activate small GTPases of the Rho-family (RhoA or Cdcd42) and positively regulate the assembly of new actin filaments [41]. BMP RII binds and activates LimkI that phosphorylates and inhibits cofilin, an actine depolymerizing factor, favoring globular actin accumulation. BMP or TGF-β type I receptors activate small GTPases such as Cdc42 and RhoA and downstream p38 MAPK, as well as RhoB by uknown mechanism which invoves Smad7 and receptor bound Rho-GEF. Rho activates ROCKI kinase, which activates Limk2 which inhibits cofilin and positively affect actin polymerization in fibroblasts and epithelial cells (Fig.2) [54]. In addition to cytoskeletal remodeling, RhoA is

involved in E-cadherin clustering during adherens junction formation while Rac1 and Cdc42 GTPases control interactions of adherens junctions and actin filaments [55].

RhoGTPases also promote Smad signaling which regulates expression of variuos genes that regulate cytoskeleton. Synthesis of vimentin leads to a new intermediate filament cytokeleton. Synthesis of α-SMA and tropomyosin contributes to the assembly of new acto-myosin networks that promote cell motility. Smad3/smad4 complex in cooperation with Rho family GEF NET1 accumulates and promotes actine polymerization and also induces tropomyosin expression that regulates assembly of contractile apparartus [41].

The role of TGF-β signaling is appeared to be complex and contraversial. Various studies have shown that transforming growth factor-beta plays an important role in diverse processes in most cells, during embryogenesis, wound healing, tumor progression, fibrotic diseases, and others [56] [40]. In mammals TGF-β is a key mediator of wound healing process and scar formation. However, the axolotl can regenerate its limb after amputation without any residual scar. Recent experiments implicated TGF-β signaling in the initiation and control of regeneration process in axolotl. The study of similarities and differences between these processes of regeneration and wound healing would help us to understand how regeneration is achieved in urodeles but not in mammals [26].

In connection with these observations, we would like to study TGF-β functioning during limb regeneration in axolotl and its role in stress-fibers formation by overexpressing TGF-β as well as TGF-β R II DN (Dominant Negative), which blocks TGF-β signaling pathway. Plasmid constructs pCAGGS-AmTGFb-T2A-Cherry and pCAGGS-TGFR-DN-T2A-Cherry were designed, where the promoter CAGGS drives the expression of TGF-β or TGF-β R II DN genes. The constructs were further injected and electroporated into mature limbs. After limbs amputation and blastema formation, immunohistochemistry experiments will be performed. The role of TGF-β in stress-fibers formation will be analyzed. We have also generated germline transgenic animals expressing TGF-β and TGF-β RII DN. These animals will be used further to study cell fate during limb regeneration.

Materials and Methods

Screening Tgf-β and Tgf-β R II genes from cDNA library

Tgf-β and Tgf-β RII genes were screened from custom manufactured long insert cDNA library derived from axolotl tissues. We used PCR method to identify particular clones.
Axolotl cDNA long insert library was divided into 14 super-pools, each of them comprised of 48 pools. Each pool from positive super-pool was screen for TGF-β and TGF-β R II sequences by PCR.

PCR conditions

PCR mix:

Polymerase Red Taq and buffer mix	5 µl
Primer Forward	0.2 µl
Primer Reverse	0.2 µl
Water	Fill up to 10 µl

PCR Program:

Initial melting	95 degree	5 min	
Melting	95 degree	30 sec	29 times
Annealing	56 degree	30 sec	
Amplification	72 degree	40 sec	
Final extension	72 degree	10 min	

Halt 4 degree

Once identified positive super-pool particular clone was screened from the set of pools. Bacteria from positive well were plated on plates with ampicillin and colonies were screened for desired plasmid by colony-PCR.

For colony PCR we used following reaction mix:

Polymerase Taq: Pfu 0.5 µl

Buffer 2 µl

dNTP 0.8 µl

Primer Forward 0.5 µl

Primer Reverse 0.5 µl

Water Fill up to 20 µl

Primers were designed relying on precise sequence information of target genes (Tgf-β and Tgf-β R II)

For Tgf-β screen from cDNA library primers used:

Primer Forward (Pr. 276): ACCAGCCAGAAACCTACGAGACAC,

Primer Reverse (Pr.277): TCCCCTCCTTCACACTGATACAGC.

For Tgf-β R II screen:

Primer Forward (Pr. 298): GCTCCAACATCCTGGTGAAGAG

Primer Reverse (Pr.299): GAATGACTCCACGGTTCTCCAAG

Positive clones were identified and isolated. Sequencing data confirmed that clones contained sequences corresponding to axolotl TGF-β 1 and TGF-β RII.

Cloning

1) Pm-84, pCAGGS-Lp-GFP-Lp-Control-T2A-Cherry

In order to generate, control vector for transgenesis, Mlu1 digested and AnP phosphatase treated PM-75 (pCAGGS-Lp-GFP-Lp-T2A-Cherry) was incubated with boiled and annealed primer pair of PR-331 and PR-332.

Primer Forward (Pr. 331): CGCGTACGCCACCATGA

Primer Reverse (Pr. 332): CGCGTCATGGTGGCGTA

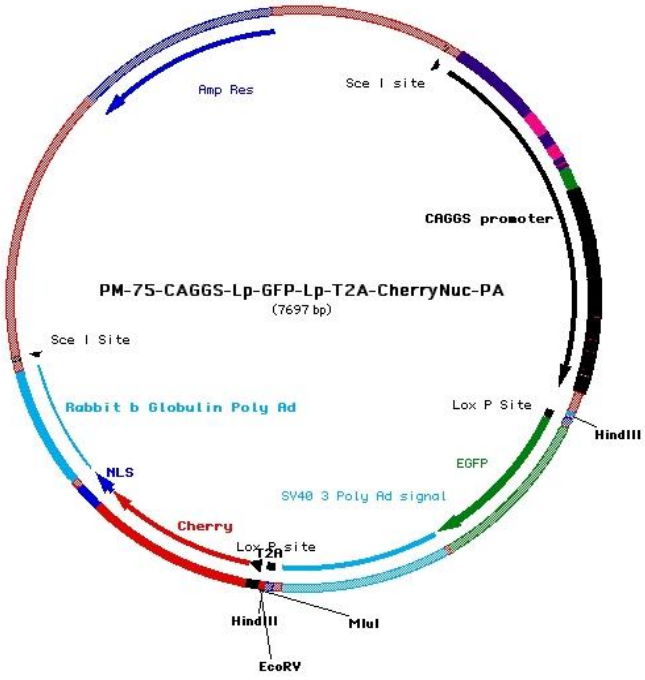

Figure 3 Map of the Pm-75 plasmid vector.

Restriction digest

For restriction digest following reaction mix was prepared:

DNA	5 µg
Enzyme buffer Neb4	8 µl
Mlu-1	2 µl
Water	fill up to 80 µl

Reaction mix was incubated for 1 hour at 37 °C, and then column purified using PCR Purification Kit (Qiagen). Plasmid DNA was eluted into 20 µl water.

Primers were boiled at 95 °C for 1 min and cooled down at room temperature. For reaction mix 3µl each primer and 4µl water were used.

Ligation

For ligation of insert into plasmid vector we used:

Column-purified plasmid Pm-75	3 µl
T4 DNA ligase buffer	1 µl
T4 DNA ligase	0.5 µl
Insert	6 µl

The reaction mix was incubated at room temperature for 1 hour and then preceded for transformation.

Transformation

For transformation we used chemically competent DHSα cells.

5 µl of ligation mix was mixed and incubated with 50 µl DHSα cells on ice for 30 min. Then cells were heat-shocked by changing the temperature to 42 °C for 90 sec and then

immediately were placed on ice for 2 min. Afterwards, cells were mixed with 400 µl of L.B. medium and incubated at 37 °C for 1 hour. To plate cells we used agar plates with ampicillin and kept them over night at 37 °C.

Positive clone were screen based on ampicillin resistance and colony PCR.

For colony-PCR reaction each selected colony was mixed up with reaction mix:

Enzyme Taq Pfu 0.5 µl

Enzyme Buffer 0.5 µl

dNTP 0.8 µl

Primer Forward 0.5 µl

Primer Reverse 0.5 µl

Water fill up 20 µl

PCR Program:

Initial melting	95 degree	5 min	
Melting	95 degree	30 sec	29 times
Annealing	56 degree	30 sec	
Amplification	72 degree	40 sec	
Final extension	72 degree	10 min	
Halt	4 degree		

PCR reaction mix was run on 1.5% agarose gel at 150 V for 40 min. Positive clones were identified by release of 200 bp DNA fragment. DNA was isolated using plasmid maxi preparation kit (Qiagen), and sequence of generated plasmid vector was confirmed by sequencing.

Primers used for clone-confirmation:

Primer Forward (Pr. 331): CGCGTACGCCACCATGA

Primer Reverse (Pr. 222): GGAGCCCTCCATGTGCACCTTGAA

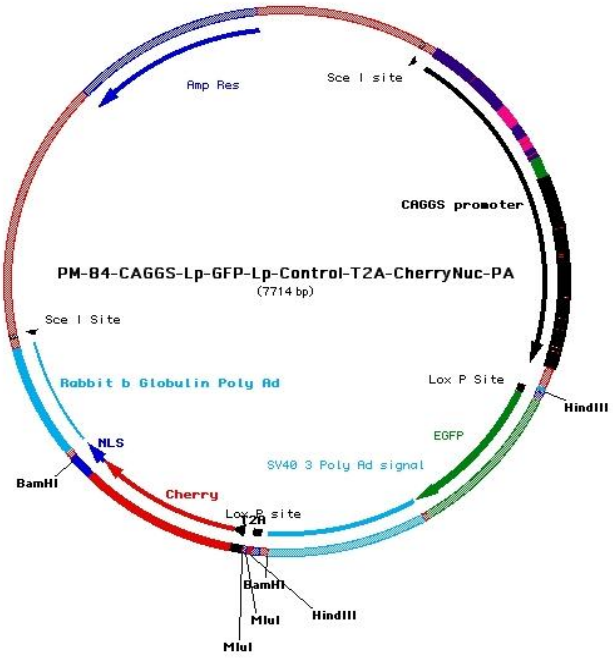

Figure 4 Map of the Pm-84, pCAGGS-Lp-GFP-Lp-Control-T2A-Cherry.

2) Pm-90, pCAGGS-Control-T2A-Cherry.

The pCAGGS-Lp-GFP-Lp-Control-T2A-Cherry construct was digested with Hind III enzyme. The same volumes of reaction components were used as for Pm-75 digest with Mlu-1 (described above). This reaction was performed to release Lp-GFP-Lp fragment from the plasmid vector. The reaction mix was run on agarose gel 0.8 % for 40 min at 150 V. The band corresponding to the vector sequence without Lp-GFP-Lp fragment (6 Kb) was excised from gel and purified using gel purification kit (Qiagen). The gel-purified band was eluted into 20 µl water. The purified plasmid DNA was processed for self-ligation reaction:

T4 ligase 0.5 µl

T4 ligse buffer 1 µl

Gel eluted band 3 µl

Water fill up 10 µl

The reaction mix was kept at room temperature for 1 hour and then transformed into chemically competent DHSα cells (the protocol is described above). DNA was isolated and confirmed by sequencing. This construct was used for electroporation into blastema of WT axolotl as the control vector.

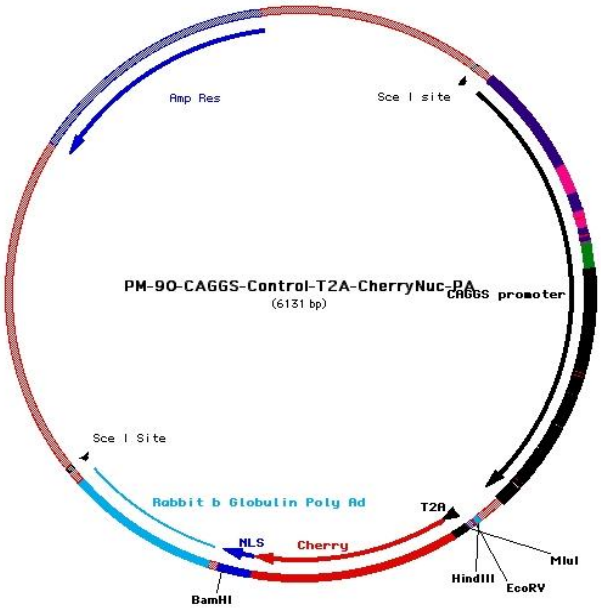

Figure 5 Map of the Pm-90, pCAGGS-Control-T2A-Cherry.

3) Pm-88. pCAGGS-Lp-GFP-Lp-Am-TGFb-T2A-Cherry.

This vector was designed to study gene of function of TGFb1 during limb regeneration using transgenesis.

pExpress1-AmTGFb1, which was screened from cDNA plasmid library was used as a template to amplify AmTGFb1 ORF.

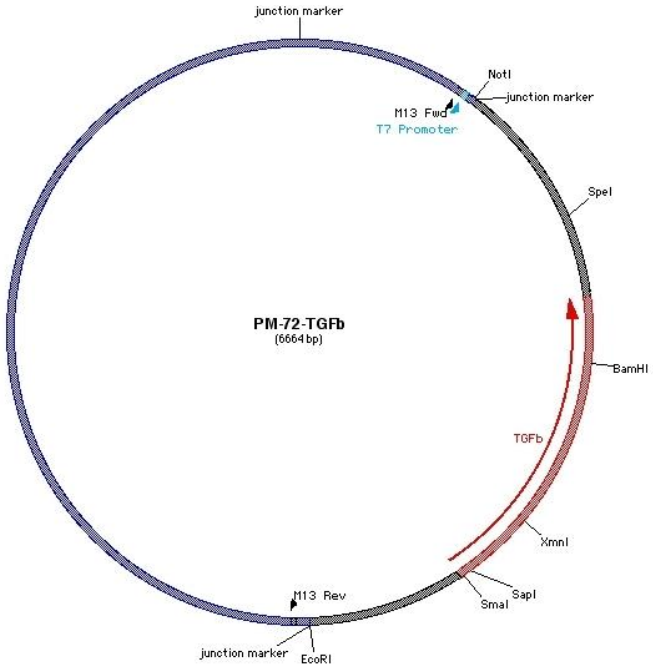

Figure 6 Map of the Pm-72(pExpress1-AmTGFb1) plasmid vector, encoding Tgf-β 1 gene.

Primers used for PCR reaction:

Primer Forward (Pr.276):
TCCGACGCGTACGCCACCATGGAGATCCCCCGGGCGCTG

Primer Reverse (Pr. 277):

GCCCTCACGCGTGCTGCATTTGCAGGTCTTCAC

The PCR reaction mix:

Template plasmid vector	1 µl
Primer Forward	1 µl
Primer Reverse	1 µl

dNTP	2 µl
10x HiFi Buffer	5 µl
$MgCl_2$	3 µl
Enzyme Hi Fi	5 µl
Water	fill up 50 µl

PCR conditions were used the same as for *Taq Pfu* enzyme, except that the polymerization was done at 68^0 C. Reaction mix was run on gel 0.8 % for 40 min at 140 V and gel-purified.

Pm-75 was used as a backbone-vector. It was digested with Mlu-1 resctriction. Then, 5' phosphates from plasmid vector were removed by Anp phosphatase to prevent self-ligation of the vector (the protocol is described above).

Gel-purified PCR product was subcloned into Pm-75 vector after digestion with Mlu1 (protocol for ligation and transformation are described above). The positive clone was identified by colony-PCR and confirmed by sequencing.

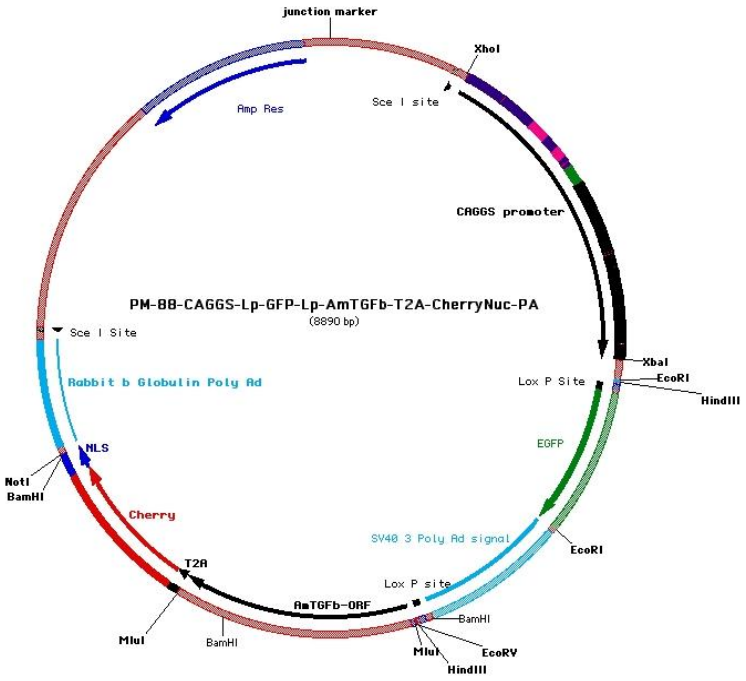

Figure 7 Map of the Pm-88, pCAGGS-Lp-GFP-Lp-Am-TGFb-T2A-Cherry.

4) Pm-95. pCAGGS-Am-TGFb-T2A-Cherry

This plasmid vector was designed to overexpress TGF-β 1 gene and study effect of the gene on α-SMA formation during axolotl limb regeneration using electroporation.

pCAGGS-Lp-GFP-Lp-Am-TGFb-T2A-Cherry was digested with Hind III enzyme (protocol for restriction digest is described above) to remove Lp-GFP-Lp fragment from plasmid vector. The gel band corresponding to vector sequence without Lp-GFP-Lp fragment was purified and self-ligated (see protocol above). After transformation and colony-PCR the positive clone was identified and confirmed by sequencing (described above).

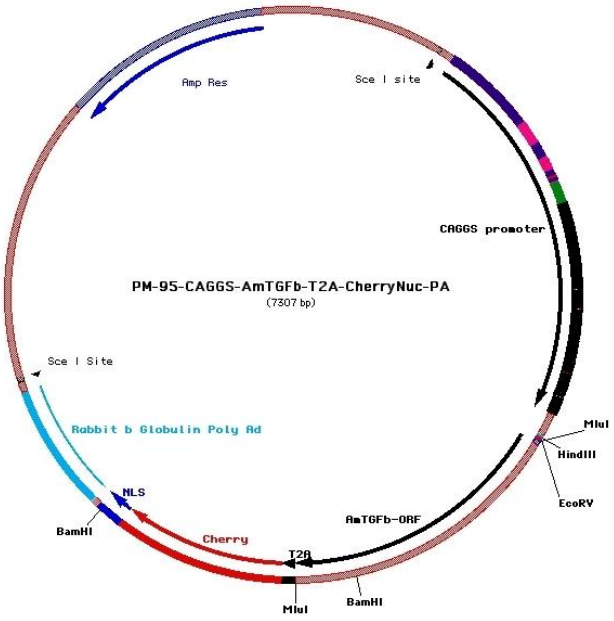

Figure 8 Map of the Pm-95, pCAGGS-Am-TGFb-T2A-Cherry.

5) Pm-94, pCAGGS-MmTGFbR2-DN-T2A-Cherry.

This construct was designed along with *pCAGGS-Lp-GFP-Lp-AmTGFb-DN-T2A-Cherry* for transgenesis and experiments utilizing Cre-LoxP system to study gene function. Plasmid vector Pm-75 was digested with Hind-III and ER-V enzymes and HindIII digested PCR product of MmTGFR-DN was cloned into it. For PCR, MmTGFR-DN construct obtained from Dr. Lalage wakefield (NCI, NIH, USA) was used as a template. The PCR mix and PCR conditions used were same as for PCR with enzyme HiFi. Transformation and clone confirmation procedures were used the same as described above.

To amplify the *TGFbR2-DN* region we used primers:

Primer Forward (Pr. 321): ACTTGTCATCGTCATCCTTATA

Primer Reverse (Pr. 322): ACCGGCAAGACGCGGAAGCTC

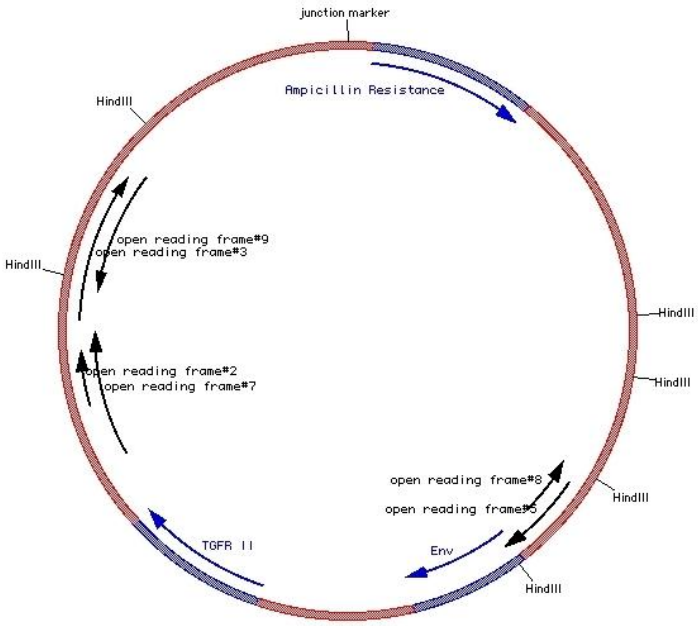

Figure 9 Map of the Pm-76 (TGFR-DN construct from Dr. Lalage Wakefield).

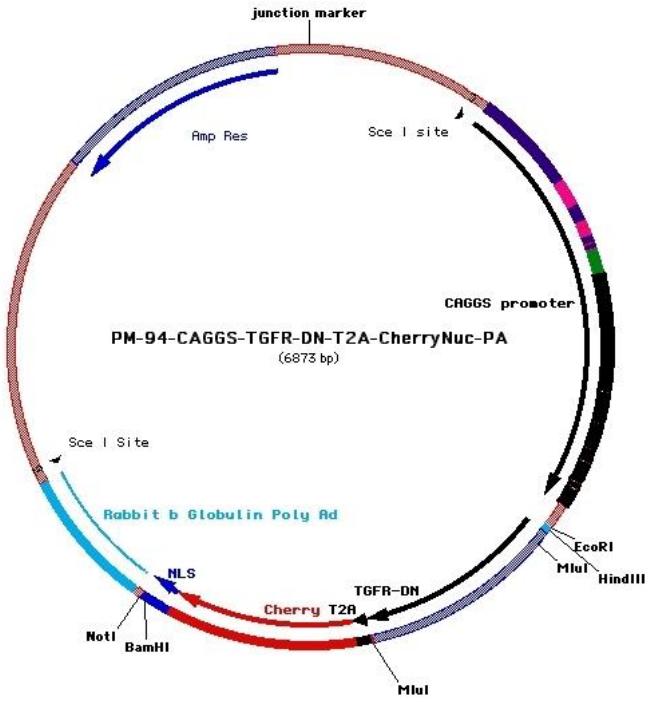

Figure 10 Map of the Pm-94, pCAGGS-MmTGFbR2-DN-T2A-Cherry.

6) Pm-97. pCAGGS-Lp-GFP-Lp-MmTGFbR2-DN-T2A-Cherry.

To make this construct, *pCAGGS-AmTGFb-DN-T2A-Cherry* was digested with Hind-III enzyme and AnP phosphatase treated. Lp-GFP-Lp (1.4 kb) was released from PM-75 using Hind-III and was ligated into above backbone. Ligated mix was transformed and colony PCR was done to identify positive clones (should release 500 bp).

For confirmation by colony-PCR following primers were used:

Primer Forward (Pr. 250): CGCCGTCCAGCTCGACCA

Primer Reverse (Pr. 151): ATGGTCCTGCTGGAGTTCGT

Recombineering

In this experiment we used plasmid Pm-52 as a template, harboring TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT cassette, which will be further used for insertion into target vector. (Fig.9)

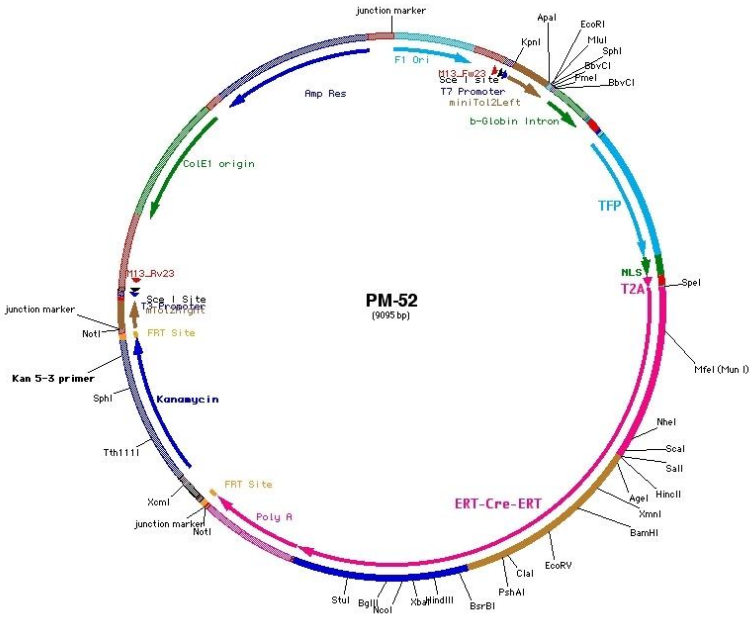

Figure 11 Pm-52, template plasmid, harboring TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT cassette.

Pm-56 – is the target vector with *Scx* genomic area of 12 kb (*Scxg12*). (Fig.10)

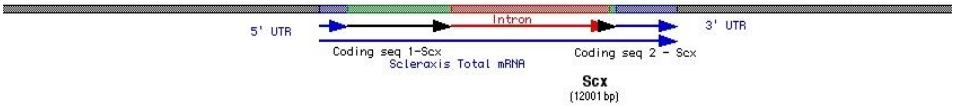

Figure 12 Pm-56, target vector with Scx genomic area of 12 kb.

Outline

1. Transformation of Pm-56 *(Scleraxis)* into GB008 cells (GB008 *E.coli* with integrated BAD-αβγA operon into the genome).

2. Introduction of homology arms to TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT cassette by PCR. Pm-52 plasmid is used as a template.

3. Induction of *E.coli* GB008, containing Scleraxis plasmid (Pm-56) with 0.2% arabinose, to induce the expression of recombineering proteins α,β,γ

4. electroporation of the PCR product into GB008 *E.coli*, containing Scleraxis plasmid (Pm-56). The cells successfully introduced TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT cassette can be identified based on antibiotic selection for kanamycin

Transformation of the Pm-56 into E.coli GB008

The plasmid Pm-56 (Sclarexis) was transformed by electroporation into E.coli

Cells were first made electrocompetent by washing them with cold water. Then, electroporation of Pm-56 was done at 1800 V pulse. Afterwards, cells were recovered at 37°C for 1 h and plated on LB agar plates containing Ampicillin. Plates were incubated at 37°C overnight. Positive colonies were inoculated in 4 ml L.B. medium containing Amp, and incubated at 37°C for 2h. Afterwards, cells were divided into 2 different tubes. One of them was induced with 20 µl of 10% L-arabinose, giving a final concentration of 0.2% so that it triggers the expression of proteins necessary for recombineering. Another was left as a negative control. After incubation at 37 °C, cells were preceded for electroporation.

Introduction of homology arms to TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT *cassette by PCR*

The oligos used as primers in PCR were designed to have 50 bp homologous to Pm 56 and 22 bp that are homologous to the template cassette, which will prime the template with 50 bp homology regions.

Sclarexis Fwd Primer (Primer, 278)

GGCGGCATGAGCAGCGCACGGTGGAGCTGACGCCGCGCCCCCTGCCCGGCCC**CGCCACCATGGTGAGCAAGGGC**

Sclarexis Rev Primer (Primer, 279)

CATCGGGGCCCCTCGGTGCTCCCAGCCCTGCTCACCAACTTTCTCTGGTT**GCCGCGGCAGATCGTCAGTCAG**

Table 1 PCR mix.

PCR mix	
Buffer 10x	5 µl
dNTP	5 µl
MgCl2	3 µl
Primer Fwd (PR-278)	1 µl
Primer Rev (PR-279)	1 µl
Template	1 µl
Platinum Tag HiFi	1.5 µl
dH2O	32.5 µl
Total	50 µl

Step	Temperature	Time	Go to cycle	Number of cycle
1	95°C	3’	-	-
2	95°C	20’’		
3	58°C	30’’	2	11
4	68°C	5’30’’		
5	95°C	20’’		
6	58°C	30’’	5	14
7	68°C	5’30’’*10’’/cycle		
8	68°C	10’		-
9	4°C	Halt	-	-

The product of 5.5 kb was detected on 0.8% agarose gel. After gel purification it was used further for recombineering.

Transformation of PCR product into GB008 cells containing Pm-56

300 ng of linear vector fragment with homology arms (PCR product, 66 ng/ µl) was transformed into GB008 cells containing Pm 56 by electroporation. Cells were then recovered at 37°C for 1 h and plated on LB agar plates containing ampicilin and kanamycin.

In Situ Hybridization

Sequences of axolotl TGFb1 and TGFbR2 were identified by blasting (tblastx) specific *Mus musculus* sequence against axolotl cDNA assembly. Using Macvector software, screening primers were designed against 5' seq of these genes and their cDNA clones were screened out from long-insert library, which were later verified by sequencing. These plasmids, encoding TGF beta 1 and TGF beta RII sequences were linearized with appropriate restriction enzymes (here, BamH I and Sph I respectively) in order to make a double stranded cut at its 5' and column purified using QIAquick PCR Purification Kit. Using T7 promoter, which was present at the 3' of these gene, dig-labeled anti-sense transcripts were prepared as probes for *in situ* hybridization. The reaction mix was prepared as follow:

- 10x transcription buffer	2 µl
- DIG-NTP mix	2 µl
- Linearized DNA	x µl (1µg)
- 0.1M DTT	2 µl
- RNase inhibitor	1 µl
- RNA polymerase (T7)	2 µl
- H2O (RNase free)	Fill up to 20 µl

For probes synthesis, above reaction mixture with T7 RNA polymerase was incubated at 37 °C for 2.5 hours, later RNA probes were column purified using RNAesy kit (Qiagen) and their quality was checked by agarose gel electrophoresis. The yield was estimated by nanodrop and was found in the range of 150 ng/µl. 10 µM thick slide-mounted paraffin sections of mature

limbs and blastema at different time points (1 day, 3 day, 5 day, 8 day) were washed with xylol and then subsequently rehydrated with 100% ethanol, 75 % EtOH, 50 % EtOH, 25 % EtOH, and PBS by incubating 10 mins. For hybridization, DIG-labeled RNA probes (10 µl) were applied in hybridization buffer (50% formamid, 10% dextran, 5xSSC, 0.1% Tween, 1mg/ml yeast RNA, 100 µg/µl heparin, 1x Denhardt's solution, 0.1% CHAPS, 5mM EDTA) and incubated at 70°C over night. Afterwards, slides were washed 3 times for 1 hour at 70°C in 50% formamid, 5x SSC, 0.1% Tween, twice in 50% formamid, 2x SSC, 0.1% Tween, and then 2 times for 5 minutes and 1 time for 20 minutes in MAB (100mM Maleic acid pH 7.5, 150 mM NaCl, 0.1% Tween) at room temperature. Following which, sections were incubated for 1 hour in blocking solution (1% Blocking reagent (Roche#110 961 76 001) in MAB) and incubated with anti-DIG antibody (Roche #110 932 74 910), diluted 1:5000 in blocking solution and kept at 4°C over night. Then, slides were washed 5 times for 10 minutes with MAB and 2 times for 10 minutes with AP buffer (100mM TRIS pH 9.5, 50 mM $MgCl_2$, 100mM NaCl, 0.1% Tween). For detection, sections were covered with BM Purple and developed at room temperature. The reaction was stopped in cold PBS/1mM EDTA. Images were taken using an inverted motorized Zeiss Axio Observer microscope.

Immunocytochemistry

For cell culture experiments HEK293 (human embryonic kidney) cells were used. Cells were put into 8-wells chambers (0.8 cm^3/well) mounted on glass slides at density $80*10^3$ cells per well. Cells were grown and maintained in MEM (minimum essential media) at 37 °C in a cell incubator over night. Cell culture was 60% confluent for transfection.

For 400 ml MEM solution:

Eagle's MEM	252 ml
FCS	40 ml
Penicillin/streptomecin	4 ml
Insulin (1 mg/ml)	4 ml of 1 mg/ml

Glutamin	4 ml
DdH_2O	100 ml

Cells were transfected with following plasmid constructs: Pm-94 (pCAGGS-MmTGFbR2-DN-T2A-Cherry), Pm-95 (pCAGGS-AmTGFb-T2A-Cherry), Pm-88 (pCAGGS-Lp-GFP-Lp-AmTGFb-T2A-Cherry), Pm-97 (pCAGGS-Lp-GFP-Lp-MmTGFbR2-DN-T2A-Cherry), Pm-38(expresses CAGGS promoter).

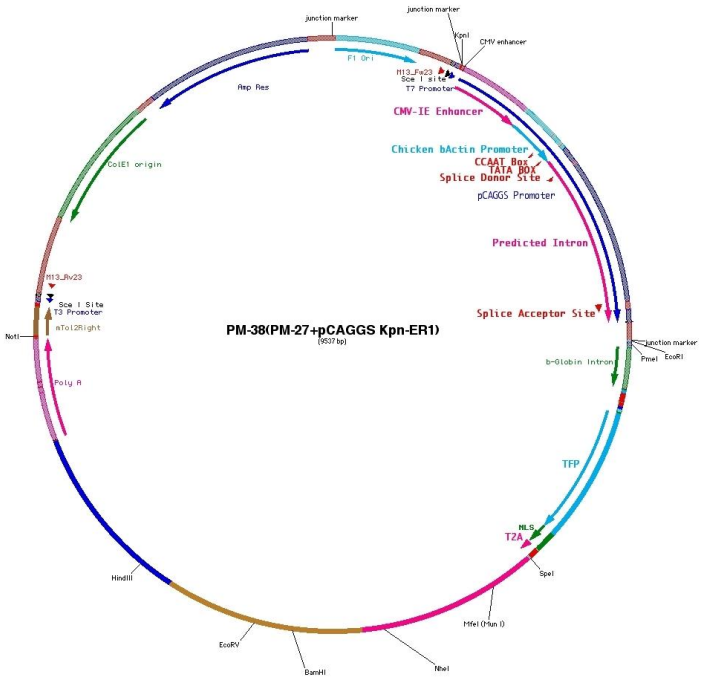

Figure 13 Map of the Pm-38 vector plasmid.

For transfection in one well, 1µl Lipofectamine 2000 (Invitrogen) and 1 µl DNA (1µg/µ) were diluted in 25µl media separately. After 5 mins, both solutions were mixed together and incubated at room temperature for 20 min. The transfection mix was mixed with media and added into cell culture.

Next day, cells transfected with Pm-88 together with Pm-38, as well cell transfected with Pm-97 with Pm-38 were tamoxifen-induced. Tamoxifen treatment was also done for cells transfected with Pm-97 or Pm-88 only as the controls. 1mM Tamoxifen in DMSO was used to treat cells at 1:1000 dilutions.

For Immunocytochemical staining cultures were fixed in 4% paraformaldehyde in TBS for 10 min at room temperature. After washing with TBS, cells were incubated for 30 minutes in 0.1% Triton-X-100 to permebilize cell membrane. They were then incubated for 1 hour in the primary antibodies, washed with TBS three times and incubated for 1 hour in secondary antibodies (Alexafluorophores, Invitrogen) with DAPI. Finally, cells were again washed with TBS three times and mounted with coverslip.

Following antibodies were used: TGFβ RII (D-2) mouse monoclonal antibody, raised against aminoacids 1-567 of TGFβ RII of human origin (1:50, sc-17799 Santa Cruz Biotechnology), Actin, smooth muscle Ab-1 (1:200, Clone 1A4; Thermo scientific, MS-113-P0), TGF beta 1 rabbit polyclonal (1:50, sc-146, Santa Cruz Biotechnology), mouse Cherry (1:400, 700-A10-10-3), anti RFP (1:400, 600-401-379, Rockland).

Gene Overexpression by electroporation

For limb electroporation constructs pCAGGS-AmTGFb-T2A-Cherry, pCAGGS-MmTGFbR2-DN-T2A-Cherry, pCAGGS-Control-T2A-Cherry and pScleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-Kan-FRT were used. Highly concentrated DNA (4 μg/μl) was prepared using Qiagen Plasmid Kit. DNA was eluted into water. For microinjection a pressure injector PV 830 Pneumatic Pico Pump (World Precise Instrumentation) mounted alongside of an Olympus Stereo SZX10 dissecting microscope were used. Needles were back-filled with DNA mixed with few crystals of Fast Green dye. Plasmids have been introduced inside limbs until DNA spread over the whole limb. All animals were injected in a similar manner: right limbs of WT animals were injected with a construct harboring Tgf beta or Tgf beta-dn, left limbs were injected with control. The pScleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-Kan-FRT construct was injected and electroporated into both limbs of Lp-GFP-Lp-Cherry animals. Shortly after microinjection limbs were placed between electrodes, and 9 pulses of 50V with duration of 50 ms were applied. Three days later the success of transfection was analyzed using fluorescence microscope Olympus SZX16.

Animal care and handling

The axolotl (*Ambystoma mexicanum*) larvae were kept separately in cups with tap water at room temperature. For the experiments animals of approximately 2 to 2.5 cm length (from snout to cloaca) were used. The animals were fed with artemia (brine shrimp). In experiments we used mainly white mutant (d/d) axolotl. To electroporate pScleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-Kan-FRT and observe tissue specific expression we used Lp-GFP-LP-Cherry transgenic axolotl. For all experiments the axolotl were anaesthetized with 0.01% Benzocaine.

Transgenesis

In all injection experiments single-cell state embryos were used. Collected embryos were rinsed with 70% Ethanol and cleaned with tap water thoroughly. Embryos were dejellied with fine forceps and kept in 1x MMR solution with penicillin and streptomycin. The 10x MMR solution was prepared as follow: 5M NaCl, 1M KCl, 1M $MgCl_2$, 1M $CaCl_2$, 0.5M EDTA, 1M HEPES.

The solution was adjusted up to 7.8 pH with 10M KOH and autoclaved. Before injection dejellied embryos were transferred to 1XMMR/20% Ficoll solution with penicillin and streptomycin. Injections were performed using a pressure injector Picospritzer II (Parker Instrumentation) mounted alongside of an Olympus Stereo SZX12 dissecting microscope. Needles were back-filled with DNA +ISceI Enzyme (0.05 µl DNA 1µg/µl, 0.2 µl IScel enzyme, 0.1 µl IScel buffer in 10µl reaction mix). Enzyme and buffer were obtained from New England Biolabs, USA. 5 nl of solution was injected into each of one-cell stage embryos. After injection embryos were kept in 20% Ficoll/1X MMR solution for 2 h to prevent yolk leakage and then were transferred to 5% Ficoll/0.1X MMR/pen-strep and left in this solution overnight. Finally, embryos were transferred to 0.1X MMR/pen-strep, until the hatching stage, when animals were kept in aerated tap water at room temperature.

Constructs used for injection were: Pm-88 (pCAGGS-Lp-GFP-Lp-AmTGFb-T2A-CherryNucPA), Pm-97 (pCAGGS-Lp-GFP-Lp-TGFR-DN-T2A-CherryNucPA), and recombined Pm-100 (pScleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-Kan-FRT).

Results

Recombineering of Scx

Recombineering allows a wide range of DNA modifications, including deletions, insertions, and substitutions, by means of homologous recombination so that any position on target molecule can be specifically changed.

The purpose of this experiment was to insert TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT cassette from template plasmid (Pm-52) (Fig.9) into target vector (Pm-56) with *Scx* genomic area of 12 kb (*Scxg12*) (Fig.10).

In order to do that, Pm-56 *(Scleraxis)* was first electroporated into GB008 cells (GB008 *E.coli* with integrated BAD-αβγA operon into the genome). In parallel, 50 bp homology arms were introduced into TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT cassette by PCR. Pm-52 was used as a template. PM-56 electroporated GB-008 *E. coli* were grown to 0.6 OD and then induced by L-arabinose for the expression of α, β, γ recombineering proteins. PCR product was electroporated in arabinose-induced cells. The colonies, which successfully introduced TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT cassette, were identified based on antibiotic selection for kanamycin and confirmed by colony PCR for estimated product of 450 bp (Fig.12).

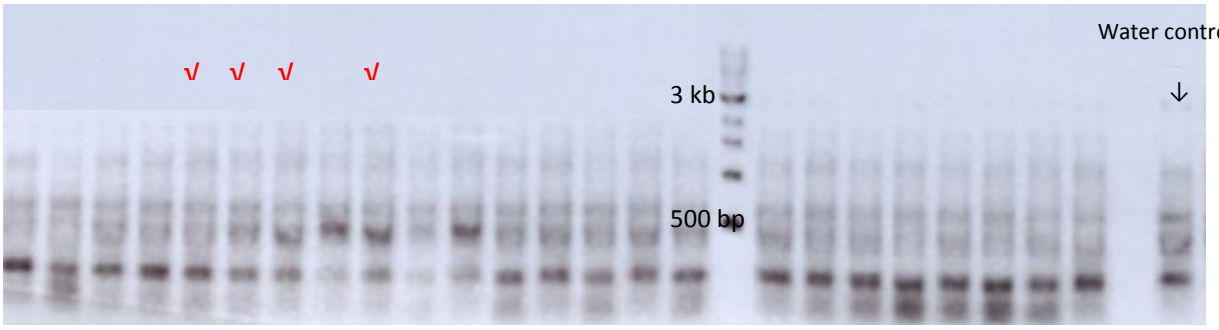

Figure 14 Clone confirmation by colony-PCR.

The positive clones were selected; DNA was isolated and confirmed by Sanger sequencing (Fig.12)

This construct (Fig.13) was used afterwards and was proved to work for Transgenesis and Immunohistochemistry experiments.

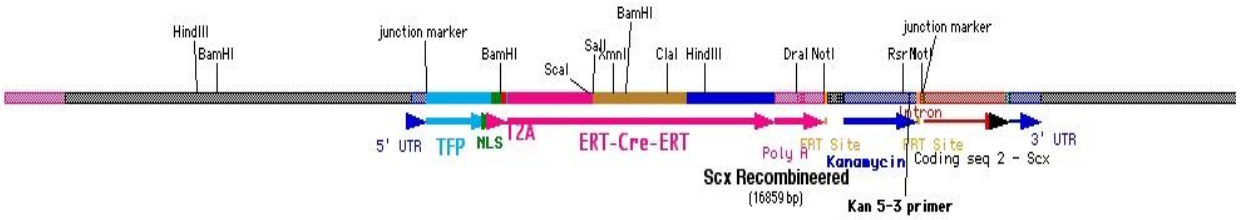

Figure 15 Recombineered ScxG12.

Screening of TGF-β and TGF-β R II from cDNA library

We screened TGF-β 1 and TGF-β RII genes from custom manufactured long insert cDNA library using PCR method. By increasing the abundance of a particular sequence, PCR method allows easy identification of a particular clone in portion of library.

The original library was divided into set of pools of decreased complexity. Each super pool and pool can be screened for particular sequence this way. A super pool, which contains the desired clone is subdivided into smaller pools, each was screened using the same PCR conditions. The positive TGF -β clones are expected to amplify band of 270 bp whereas clones positive for TGF-β RII gave rise to 190 bp (Fig.14). By screening cDNA library we isolated clones with sequence corresponding to axolotl TGF-β 1 and TGF-β R II and confirmed by sequencing. These clones were used afterwards for IHC and cloning experiments.

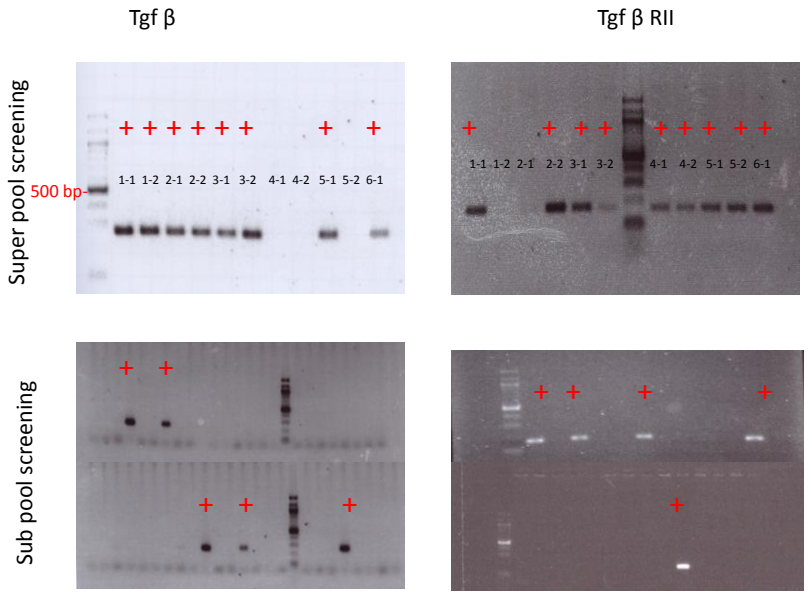

Figure 16 Super pool/pool screening of Tgf-β and Tgf-β RII from cDNA library.

In situ hybridization

To analyze the expression pattern of TGF-β and TGF-β RII during limb regeneration we examined sections taken from blastema at 3 day after amputation and mature limb (Fig.15).

In situ analysis suggest that TGF-β 1 is expressed in a variety of cell types. Transcripts are found in epidermis, connective tissues, muscles and blastema. The expression pattern of TGFβ RII correlated well with the expression of TGF-β 1. It supports the previous observations fo mammalian models, that nearly every cell in the body including epithelial, endothelial, hematopoetic, neuronal, and connective tissues produces TGF-β and its receptor. This phenomenon is known as autocrine signaling [57].

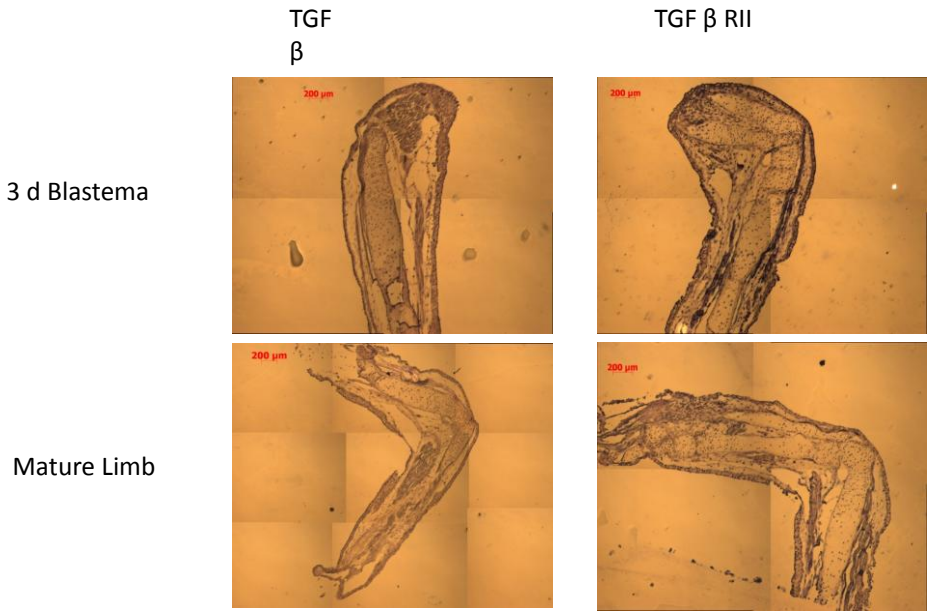

Figure 17 In Situ Hybridization of axolotl limb and blastema at 3d for Tgf-β and Tgf-β RII RNA.

Gene overexpression by electroporation

After cloning the genes TGF-β and TGF-β RII DN we wanted to analyze their characteristic during axolotl limb regeneration by introducing directly into blastema. The goal of the experiments was to observe the phenotypic changes regarding fibroblast-to-myofibroblast differenetiation and α-SMA production associated with changing the expression of TGF-β gene. For this purpose we overexpressed TGF-β gene and mutant form of TGF-β RII (TGF-β R II DN), which is a truncated TGF-β RII, lacking the serine-threonine kinase domain for downstream signaling.

Electroporation is an effective method to introduce DNA into mammalian cells. In this technique electric field is applied to disrupt plasma membrane stability transiently, creating pores in cell membrane through which DNA is driven due to its negative charge [58]. Using this method we transfected plasmids pCAGGS-AmTGFb-T2A-Cherry, and pCAGG-TGFR-DN-

T2A-Cherry into axolotl mature limbs. After three days recovery limbs were amputated and lower arm blastema formed. Cherry expression of all constructs was observed in blastema, confirming successful plasmid transfection.

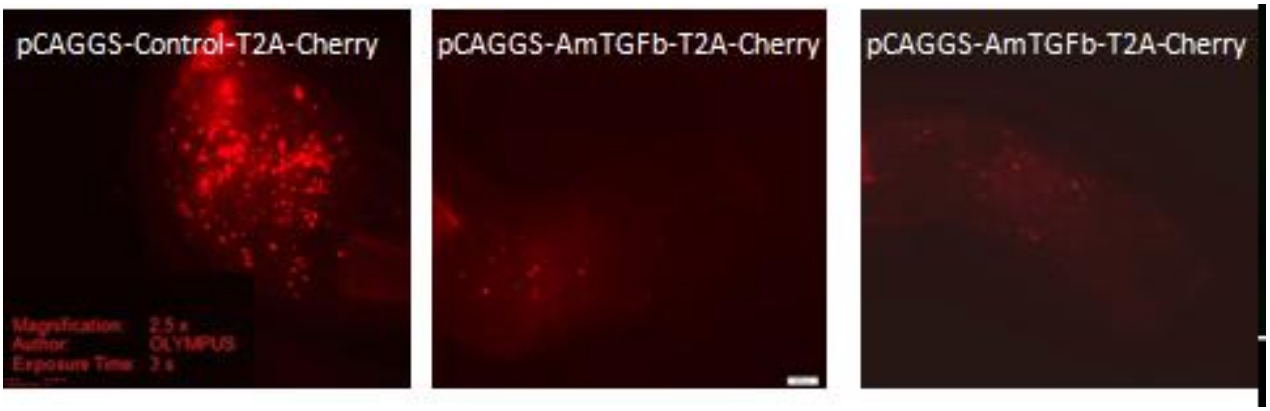

Figure 18 Analyis of gene over-expression in 14 d blastema after electroporation of pCAGGS-Control-T2A-Cherry, pCAGGS-AmTgf-β-T2A-Cherry, pCAGG-Tgf-βR-DN-T2A-Cherry.

We have also successfully electroporated pScleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-Kan-FRT into mature limbs of *Lp-GFP-Lp-Cherry* transgenic axolotl. This would help us to identify tissue specific expression by means subsequent IHC experiments. Upon tamoxifen induction conversion from *GFP*-to *Cherry* expression was detected. However, conditions for electroporation need to be modified.

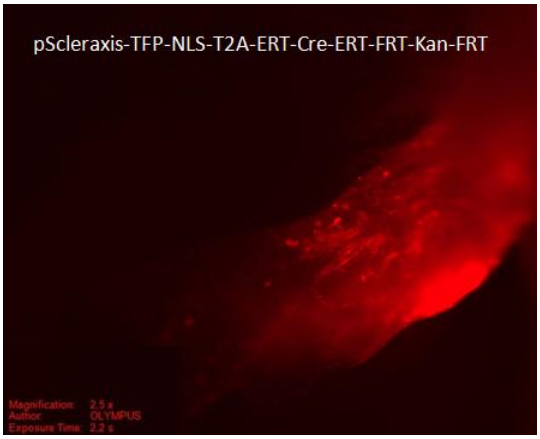

Figure 19 Electroporation of pScleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-Kan-FRT into mature limbs of Lp-GFP-Lp-Cherry transgenic axolotl.

Immunocytochemistry.

In order to validate constructs and Cre-loxP system, we resorted to *in vitro* experiments. In one experiment, electroporation compatible constructs Pm-94 (pCAGGS-MmTGF-βRII-DN-T2A-Cherry) and Pm-95 (pCAGGS-AmTGFβ-T2A-Cherry) were transfected in HEK 293 cells and checked for the expression of TGF-β1 or TGF-βRII-DN by their specific antibodies. Results suggest that *Cherry* expressing cells were co-stained with TGF-β1 or TGFβ-R2 antibody respectively (Fig. 18 and 19).

In second experiment, Cre-loxP system was validated using co-transfection of PM-38 (CAGGS-TFP-NLS-T2A-ERt-Cre-ERt) with either PM-88 (pCAGGS-Lp-GFP-Lp-AmTGF-β1-T2A-Cherry) or PM-97 (pCAGGS-Lp-GFP-Lp-MmTGF-βRII-DN-T2A-Cherry). Co-transfected cells were either treated with DMSO control or 1nM tamoxifen for the ability of inducible Cre enzyme's activity. Results suggest that both PM-88 and PM-97 successfully converted and turned on *Cherry* fluorescent Proteins (Fig. 20 and 21).

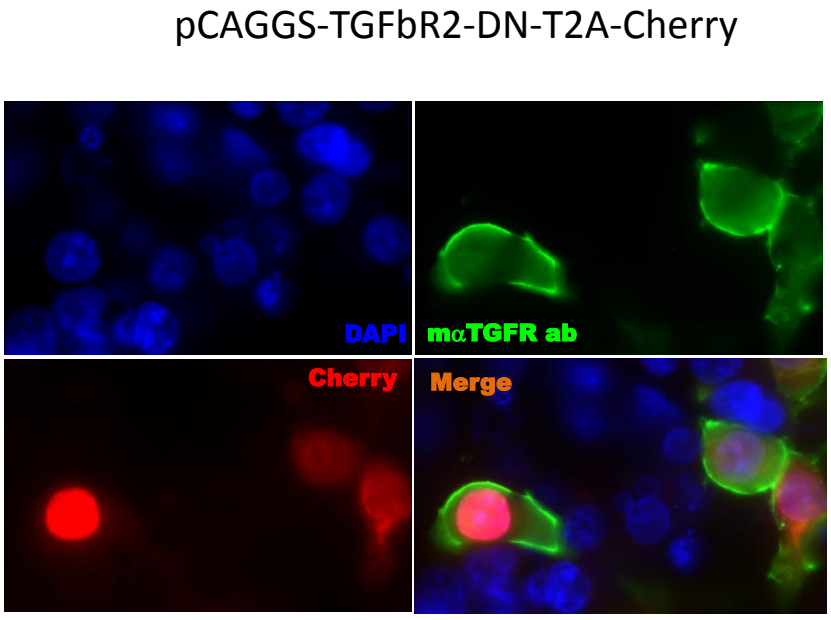

Figure 20 HEK293 cells transfected with pCAGGS-MmTGFbR2-DN-T2A-Cherry and stained with Tgf-β RII and Cherry antibodies.

pCAGGS-TGFb1-T2A-Cherry

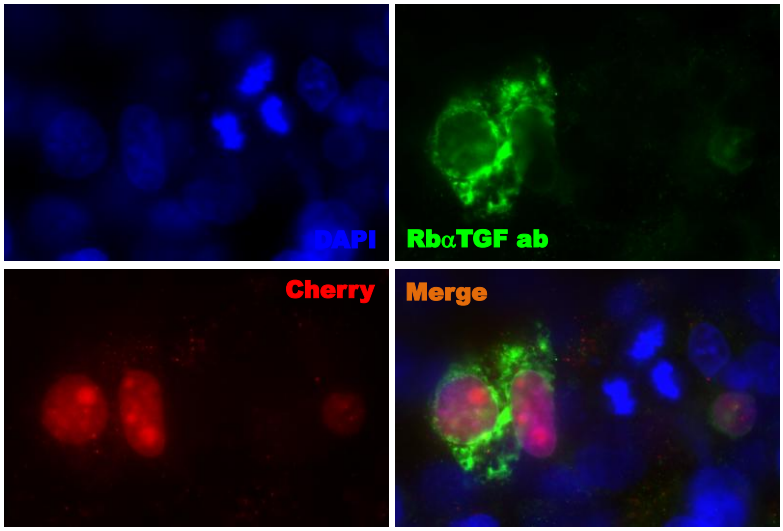

Figure 21 HEK293 cells transfected with pCAGGS-AmTGF-β-T2A-Cherry and stained with Tgf-β and Cherry antibodies.

Co-transfection of CAGGS-Cre and pCAGGS-Lp-GFP-Lp-TGFb-T2A-Cherry (No Tamoxifen)

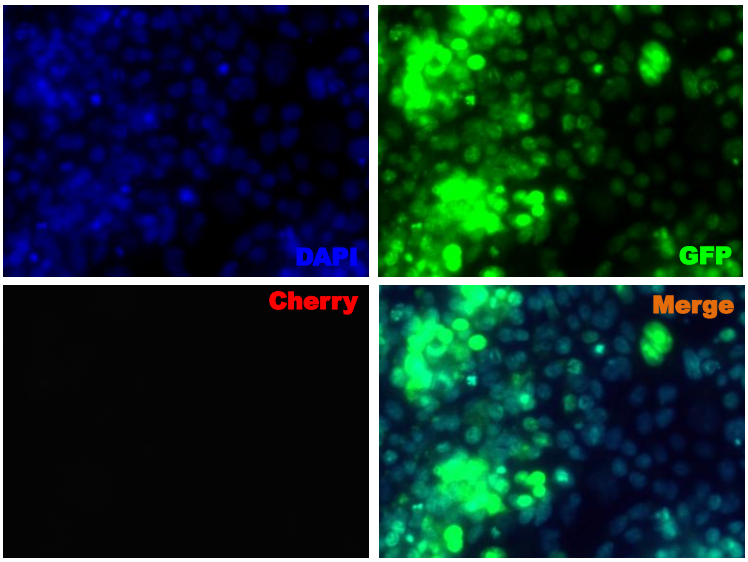

Figure 22 Cells co-transfected with pCAGGS-TFP-NLS-T2A-ERt-Cre-ERt and pCAGGS-Lp-GFP-Lp-TGFb-T2A-Cherry (No Tamoxifen).

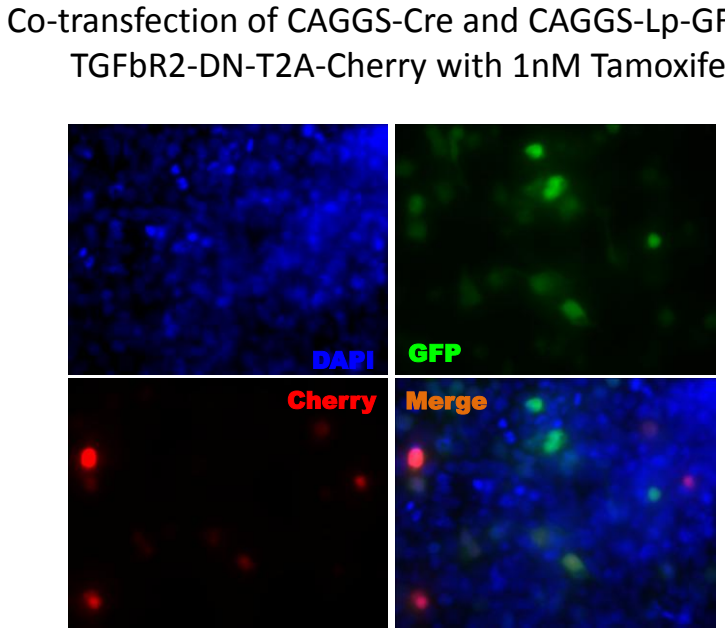

Figure 23 Cells co-transfected with pCAGGS-TFP-NLS-T2A-ERt-Cre-ERt and pCAGGS-Lp-GFP-Lp-TGFβRII-DN-T2A-Cherry. Conversion from GFP-to Cherry expression was observed upon tamoxifen induction.

Making transgenic axolotl

The production of transgenic animals is widely used in animal systems and represents a powerful tool to study regeneration. It allows for long-term cell lineage tracing and gene expression analysis.

To make transgenic animals we used technique described by Elly M. Tanaka and co-workers [59]. This technique was reported to be efficient in production of relatively large number of animals with least mosaicism in F0 animals. We co-injected plasmids with *I-Sce I* meganuclease into single-cell stage embryos and generated animals that display expression of the constructs. We injected following plasmids: pCAGGS-Lp-GFP-Lp-AmTGFb-T2A-Cherry, and pCAGGS-Lp-GFP-Lp-MmTGFR2-DN-T2A-Cherry, harboring CAGGS promoter which drives

the expression of Tgf β and Tgf β RII DN into single-cell stage embryos. CAGGS promoter was reported to drive very strong expression which persisted beyond 6 months [59].

The recombined construct Scleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT was also injected. Out of 280 embryos, injected with Scleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT, only 5 embryos yielded weak expression of TFP, and one embryo showed relatively strong and uniform expression of fluorescent protein (Fig.23). Such animals expressed the reporter gene widely in connective tissues including dermis and blood cells, but the intensity of expression decreased over time. We believe that this could be a specific expression for fibroblasts. However, further experiments are needed to identify and confirm the specificity of expression.

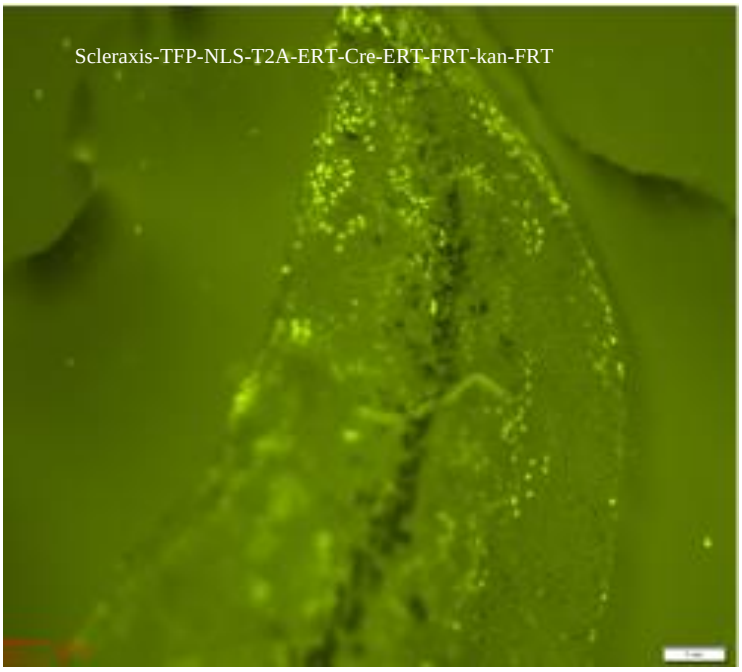

Figure 24 pScleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT transgenic axolotl.

The efficiency of injection of pCAGGS-Lp-GFP-Lp-AmTGFb-T2A-Cherry and pCAGGS-Lp-GFP-Lp-MmTGFbR-DN-T2A-Cherry was higher in comparison with Scleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT and resulted in 5% each classified as ‘‘strong’’. Few animals among them gave rise to “very strong” signal. They appear to express *GFP*

uniformly throughout their body, and yet for one month a very strong expression remained (Fig.24)

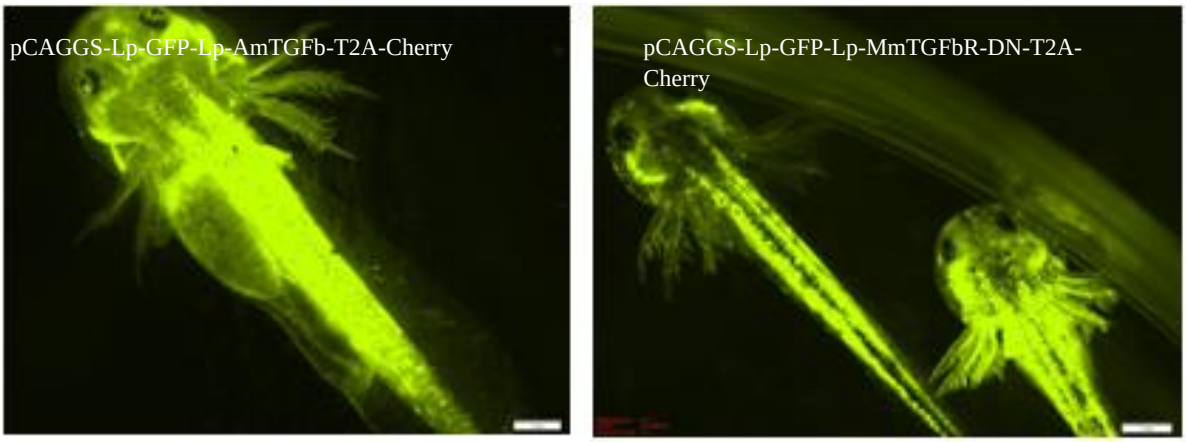

Figure 25 Transgenic axolotl, expressing pCAGGS-Lp-GFP-Lp-Am-Tgfβ-T2A-Cherry and pCAGGS-Lp-GFP-Lp-Mm-Tgf-βR-DN-T2A-Cherry.

All these transgenic animals, expressing pCAGGS-Lp-GFP-Lp-AmTGFb-T2A-Cherry, pCAGG-Lp-GFP-Lp-TGFR-DN-T2A-Cherry, and pScleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT will be used further to conduct experiments utilizing Cre-lox P system to study cell fate and gene function during limb development and regeneration.

Discussion

Based on recent studies that have identified different markers for subtypes of connective tissues, we focused on one molecular marker – scleraxis. Previously it was demonstrated for mouse model, that scleraxis is specifically expressed in ligaments and tendons. Using recombineering method we incorporated TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT cassette into 12 kb Scleraxis genomic sequence. The following construct pScleraxis-TFP-NLS-T2A-ERT-Cre-ERT-FRT-kan-FRT was injected into single-cell stage embryos in order to produce germ-line tissue-specific Cre-line. Founders showed expression of the reporter gene widely in connective tissues including dermis and blood cells. We believe that this could be a specific expression for fibroblasts at this stage. However, further experiments, including IHC, will be done to identify and confirm the specificity of expression. Our lab has also developed loxP reporter line in axolotl, which can be crossed with tissue specific tamoxifen inducible Cre line to lineage trace individual subtype of connective tissues in the course of regeneration.

In mammals Tgf -β is a key mediator of wound healing process and scar formation. However, the axolotl can regenerate its limb after amputation without any residual scar. Recent experiments implicated Tgf-β signaling in the initiation and control of regeneration process in axolotl, but why do axolotl never develop myofibroblast and never form scar?

We assume there are three parameters which need to be considered in order to spread light onto this problem:

1) Duration of TGF-β mediated signaling pathway

Presumably, the longer duration of TGF-β functioning during wound healing in mammals may be the reason of excessive collagen I production and as the result scar production.

2) Intensity of the signal. The other possible reasons may be related to intensity or amount of signal.

3) Molecular changes in TGF-β signaling pathway in regenerative and mammalian models.

Particularly, accumulating studies have shown controversial results in effect of Smad3 protein, which is a direct transducer of signal in Tgf-β pathway during fibroblast-myofibroblast differentiation.

Various studies suggest that R-Smads are key mediators in TGF-β-induced fibrosis and EMT. Experiments using Smad3 knockout mice, exhibited reduced matrix deposition. TGF-β downregulates intercellular junctions in a Smad-dependent manner by inducing the expression of the members of the Slug/Snail family [60]. These proteins are strong suppressors of E-cadherin, occludin and claudin-1. During fibrogenesis, Smad3 induces the expression of several collagen isoforms and regulates the expression of various matrix metalloproteinases and tissue type inhibitor of metalloproteases.

On the contrary, other studies provide evidence that Smad3 can act as a negative regulator of SMA expression and the activation of the myogenic program in the epithelium. In human mesangial and tubular cells, TGF-β caused robust downregulation of Smad3 (but not Smad2) protein, enhanced Smad3 ubiquitination and suppressed Smad3 mRNA transcription [61].

András Masszi and András Kapus suggested that Smad3 is a timekeeper and context-dependent modulator of EMyT [62]. Early on, active Smad3 contributes to the downregulation of epithelial markers and plays a major role in the induction of several mesenchymal genes. This mesenchymal phase is likely an important preparatory period for MF development. On the other hand, Smad3 postpones the myogenic program and commitment toward the MF phenotype in the injured epithelium. However, upon continued stimulation, Smad3 is degraded, which triggers the myogenic program. Thereby, Smad3 acts as an important switch or timekeeper that contributes to major fate-determining decisions during fibroblast-to-myofibroblast transition.

It is logical to assume, that Smad2/3 may be differently regulated in regenerative model (axolotl) and in mammalian model at different time points.

During this project we designed following plasmid constructs pCAGGS-AmTGFb-T2A-Cherry and pCAGGS-TGFR-DN-T2A-Cherry; their workability was validated by in vitro

experiments. In order to study TGF- β1 function during limb regeneration we overexpressed gene by electroporation the construct pCAGGS-AmTGFb-T2A-Cherry into axolotl blastema. pCAGGS-TGFR-DN-T2A-Cherry has also been electroporated in order to block TGF-β-mediated signaling pathway. In the sequel, immunohistochemistry experiments will be performed to analyze the role of TGF-β in stress-fibers formation.

Transgenic animals expressing TGFβ-1 and TGFβ RII were generated for subsequent experiments utilizing Cre-loxP system. These experiments will be focused on study cell fate and Tgf -β-1 function during limb development and regeneration.

References

1. Martin Kragl, D.K., Eugen Nacu, Shahryar Khattak, Malcolm Maden, Hans Henning Epperlein, Elly M. Tanaka, *Cells keep a memory of their tissue origin during axolotl limb regeneration.* Nature, 2009. **460**(7251): p. 60-67.
2. Savio L.-Y. Woo, R.E.D., Jennifer Zeminski, Steven D. Abramowitch, Serena S. Chan Saw, MS, and James A. Fenwick, *Injury and repair of ligaments and tendons.* Annu Rev Biomed Eng., 2000. **2**: p.83-118.
3. Cserjesi P, Brown D., Ligon KL, Lyons GE, Copeland NG, Gilbert DJ, Jenkins NA, Olson EN., *Scleraxis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis.* Development, 1995. **121**(4): p. 1099-1110.
4. Brent AE, S.R., Tabin CJ, *A somitic compartment of tendon progenitors.* Cell, 2003. **113**(2): p. 235–248.
5. Murchison ND, P.B., Conner DA, Keene DR, Olson EN, Tabin CJ, Schweitzer R., *Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons.* Development, 2007. **134**(14): p. 2697-2708.
6. Scott A, D.P., Abraham T, Fong G, Sampaio AV, Underhill TM., *Mechanical force modulates scleraxis expression in bioartificial tendons.* J Musculoskelet Neuronal Interact 2011. **11**(2): p. 124-132.
7. Pryce BA, B.A., Murchison ND, Tabin CJ, Schweitzer R, *Generation of transgenic tendon reporters, ScxGFP and ScxAP, using regulatory elements of thescleraxis gene.* Dev.Dyn 2007. **236**(6): p. 1677-1683.
8. Shyam K. Sharan, L.C.T., Sergey G. Kuznetsov, Donald L. Court, *Recombineering: A Homologous Recombination-Based Method of Genetic Engineering.* Nat Protoc, 2009. **4**(2): p. 206–223.
9. Alexander W Bird, A.E., Jun Fu, Jean-Karim Hériché, Marcello Maresca, Youming Zhang, Anthony A Hyman and A Francis Stewart, *High-efficiency counterselection recombineering for site-directed mutagenesis in bacterial artificial chromosomes.* Nature Methods 2012. **9**(1): p. 103-109.
10. Jun Fu, M.T., Konstantinos Anastassiadis, William Skarnes, A. Francis Stewart, *A Recombineering Pipeline to Make Conditional Targeting Constructs.* Methods in Enzymology, 2010. **477**: p. 125–144.
11. Copeland NG, J.N., Court DL., *Recombineering: a powerful new tool for mouse functional genomics.* Nature Reviews Genetics, 2001. **2**(10): p. 769-779.
12. Lévesque M, V.E., Roy S., *Skin Wound Healing in Axolotls:A Scarless Process.* J. Exp. Zool. (Mol. Dev. Evol.), 2010. **314**(8): p. 684-697.
13. Latha Satish and Sandeep Kathju, *Cellular and Molecular Characteristics of Scarless versus Fibrotic Wound Healing.* Dermatology Research and Practice, 2010: p. 1-11.
14. Gurtner GC, W.S., Barrandon Y, Longaker MT., *Wound repair and regeneration.* Nature, 2008. **453**(7193): p. 314-321.
15. Yokoyama H., *Initiation of limb regeneration: The critical steps for regenerative capacity.* Develop. Growth Differ., 2008. **50**(1): p. 13-22.
16. Sidgwick GP, B.A., *Extracellular matrix molecules implicated in hypertrophic and keloid scarring.* JEADV, 2012. **26**(2): p. 141-152.

17. Liu J, W.Y., Pan Q, Su Y, Zhang Z, Han J, Zhu X, Tang C, Hu D., *Wnt/b-catenin pathway forms a negative feedback loop during TGF-b1 inducedhuman normal skin fibroblast-to-myofibroblast transition.* Journal of Dermatological Science, 2012. **65**(1): p. 38–49.
18. Midwood, K.S., Williams LV, Schwarzbauer, J.E, *Tissue repair and the dynamics of the extracellular matrix.* The International Journal of Biochemistry and Cell Biology 2004. **36**(6): p. 1031-1037.
19. B. Hinz, P.P., J. Smith-Clerc, C. Chaponnier,and J.-J. Meister, *Myofibroblast Development Is Characterized by Specific Cell-Cell Adherens Junctions□.* Molecular Biology of the Cell, 2004. **15**(9): p. 4310-4320.
20. Adzick NS, L.H., *Cells, Matrix, Growth Factors, and the Surgeon. The Biolofy of Scarless Fetul Wound Repair.* Ann Surg., 1994. **220**(1): p. 10-18.
21. Manjong Han, X.Y., Lee Jangwoo, Christopher H. Allan, and Ken Muneoka, *Development and Regeneration of the Neonatal Digit Tip in Mice.* Dev. Biol, 2008. **315**(1): p. 125-135.
22. Rahul K. Nath, M.L., Herbert Markham, George A. Ksander, and Paul M. Weeks, *The expression of transforming growth factor type beta in fetal and rabbit rabbit skin wounds. Journal of Dermatological Science*, 1994. **29**(3): p. 416-429.
23. Chia Soo, F.-Y.H., Xinli Zhang,Yubert Wang,Steven R. Beanes, H. Peter Lorenz, Marc H. Hedrick, Richard J. Mackool, Ana Plaas, Soo-Jin Kim, Michael T. Longaker, Earl Freymiller, and Kang Ting, *Differential Expression of Fibromodulin, a Transforming Growth Factor-β Modulator, in Fetal Skin Development and Scarless Repair.* American Journal of Pathology, 2000. **157**(2): p. 423-433.
24. Abraham, A.L.a.D.J., *TGF-β signaling and the fibrotic response.* The FASEB Journal, 2004. **18**(7): p. 816-827.
25. Leonard Lu, M., Alexandrina S Saulis, MD, W Robert Liu, PhD, Nakshatra K Roy, Jerome D Chao, MD, Steven Ledbetter, PhD, Thomas A Mustoe,, *The Temporal Effects of Anti-TGF-1, 2, and 3 Monoclonal Antibody on Wound Healing and Hypertrophic Scar Formation. J Am Coll Surg*, 2005. **201**(3): p. 391-397.
26. Mathieu Lévesque, S.G., Kenneth Finnson, Sophie Desmeules, Éric Villiard, Mireille Pilote, Anie Philip, and Stéphane Roy, *Transforming Growth Factor: b Signaling Is Essential for Limb Regeneration in Axolotls.* PLoS ONE 2007. **2**(11): p. 1-14.
27. Ferris DR, S.A., Mandefro B, Cummings GM, Gardiner DM, Rugg EL., *Ex vivo generation of a functional and regenerative wound epithelium from axolotl (Ambystoma mexicanum) skin.* Dev Growth Differ., 2010. **52**(8): p. 715-724.
28. Deepali Jhamb, Nandini Rao, 6, Derek J Milner, Fengyu Song, Jo Ann Cameron, David L Stocum, Mathew J Palakal, *Network based transcription factor analysis of regenerating axolotl limbs.* BMC Bioinformatics, 2011.**12**: p. 1-12.
29. Chen Y-W, N.K., Bakay M, McIntyre O, Rawat R, Shi R, Hoffman EP, *Early onset of inflammation and later involvement of TGFbeta in Duchenne muscular dystrophy.* Neurology, 2005. **65**(6): p. 826-834.
30. Erin C. Connolly, Juliana Freimuthm and Rosemary J. Akhurst, *Complexities of TGF-β Targeted Cancer Therapy.* Int J Biol Sci., 2012. **8**(7): p. 964–978.
31. Joan Massagué1, S.W.B., Roger S Lo1, *TGFβ Signaling in Growth Control, Cancer, and Heritable Disorders.* Cell, 2000. **103**(2): p. 295–309.
32. Attisano L, Wrana JL, *Signal Transduction by the TGF-b Superfamily.* Science, 2002. **296**(5573): p. 1646-1647.
33. Sakaki-Yumoto M, Katsuno Y, Derynck R, *TGF-β family signaling in stem cells.* Biochim. Biophys. Acta, 2012: p. 1-17.
34. Khalil, N., *TGF-β: from latent to active.* Microbes and Infection, 1999. **1**(15): p. 1255-1263.

35. Leslie I. Gold1, Tommi Jussila2, Norbert E., Fusenig3, Frej Stenbäck2, *TGF-β isoforms are differentially expressed in increasing malignant grades of HaCaT keratinocytes, suggesting separate roles in skin carcinogenesis.* The Journal of Pathology, 2000. **190**(5): p. 579-588.
36. Neilson2, R.K.a.E.G., *Transforming Growth Factors b1, b2, and b3 and Their Receptors Are Differentially Regulated during Normal and Impaired Wound Healing.* The journal of biological chemistry, 1996. **271**(17): p. 10188-10193.
37. Ferguson MW, O'Kane S.O'Kane, *Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. Phil. Trans. R. Soc. Lond*, 2004. **359**(1445): p. 839-850.
38. Herpin A, Lelong C, *Transforming growth factor-β-related proteins: an ancestral and widespread superfamily of cytokines in metazoans.* Developmental and Comparative Immunology, 2004. **28**(5): p. 461–485.
39. Derynck R, Zhang YE, *Smad-dependent and Smad-independent pathways in TGF- family signalling.* Nature, 2003. **425**(6958): p. 577-584.
40. Shi Y, Massagué J, *Mechanisms of TGF-beta Signaling* Cell, 2003. **113**(6): p. 685-700.
41. Moustakas A, Heldin CH, *Dynamic control of TGF-b signaling and its links to the cytoskeleton.* FEBS Letters, 2008. **582**(14): p. 2051-2065.
42. Wu G, Ye-Guang Chen, Ozdamar B, Gyuricza CA, Chong PA, Wrana JL, Massagué J, Shi Y., *Structural basis of Smad2 recognition by the Smad anchor for receptor activation.* Science., 2000. **287**(5450): p. 92-97.
43. Joan Massagué , Joan Seoane, and David Wotton, *Smad transcription factors.* Genes Dev, 2005. **26**(19): p. 2783-2810.
44. Yoichi Kato1, R.H., Yu Katsuyama, Anders M. Näär and Xi He, *A component of the ARC/Mediator complex required for TGF/Nodal signalling.* nature, 2004. **418**: p. 641-646.
45. Tobias Meyer-ter-Vehn, S.G., Walter Sebald, Mathias Buttmann, *p38 Inhibitors Prevent TGF-beta-Induced Myofibroblast Transdifferentiatioin in Human Tenon Fibroblasts.* IOVS, 2006. **47**(4): p. 1500-1509.
46. Yu, L., Hebert, M. C. & Zhang, Y. E., *Yu, L., Hebert, M. C. & Zhang, Y. E. TGF- receptor-activated p38 MAP kinase mediates Smad-independent TGF- responses.* EMBO J., 2002. **21**(14): p. 3749–3759.
47. Bishop, A.L., Hall. A, *Rho GTPases and their effector proteins.* Biochem. J, 2000. **348**(Pt2): p. 241–255.
48. Edlund, S., Landström, M., Heldin, C. H. and Aspenström, P., *Transforming growth factor--induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA. .* Mol. Biol. Cell 2002.**13**(3): p. 902-914.
49. Pryce BA, Walson S.S., Murchison ND, Staverosky JA, Dünker N, Schweitzer R, *Recruitment and maintenance of tendon progenitors by TGF-beta signaling are essential for tendon formation.* Development, 2009. **136**(8): p. 1351–1361.
50. Christopher L. Mendias, Jonathan P. Gumucio, Max E. Davis, Caleb W. Bromley, Carol S. Davis, Susan V. Brooks, *Transforming growth factor-beta induces skeletal muscle atrophy and fibrosis through the induction of atrogin-1 and scleraxis.* Muscle Nerve, 2012. **45**(1): p. 55-59.
51. Moustakas, A., Heldin CH., *Signaling networksguiding epithelial-mesenchymal transitions during embryogenesis and cancer progression.* Cancer Sci., 2007. **98**(10): p. 1512-1520.
52. Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H.R.,Zhang, Y. and Wrana, J.L, *Regulation of the polarityprotein Par6 by TGFb receptors controls epithelial cell plasticity.* Science, 2005. **307**(5715): p. 1603-1609.
53. Guoping Zheng, James Guy Lyons, Thian Kui Tan, Yiping Wang, Tzu-Ting Hsu, Danqing Min, Lena Succar, Gopala K. Rangan, Min Hu, Beric R. Henderson, Stephen I. Alexander, and David C.H.

Harris, *Disruption of E-Cadherin by Matrix Metalloproteinase Directly Mediates Epithelial-Mesenchymal Transition Downstream of Transforming Growth Factor-β1 in Renal Tubular Epithelial Cells.* Am J Pathol., 2009. **175**(2): p. 580–591.

54. Vardouli, L., Moustakas, A. and Stournaras, C., *LIM-kinase2 and cofilin phosphorylation mediate actin cytoskeleton reorganization induced by transforming growth factor beta.* J. Biol.Chem., 2005. **280**(12): p. 11448–11457.
55. Takashi Watanabe, Kazuhide Sato, and Kozo Kaibuchi, *Cadherin-mediated Intercellular Adhesion and Signaling Cascades Involving Small GTPases.* Cold Spring Harb Perspect Bio., 2009. **1**(3): p. 1-13.
56. Gerard C. Blobe, William P. Schiemann, and Harvey F. Lodish, *Role of Transforming Growth Factor β in Human Disease.* N Engl J Med, 2000. **342**(18): p. 1350-1358.
57. Schmid P, Itin. P., Rufli T., *In situ analysis of transforming growth factor-beta s (TGF-beta 1, TGF-beta 2, TGF-beta 3), and TGF-beta type II receptor expression in malignant melanoma.* Carcinogenesis., 1995. **16**(7): p. 1499-503.
58. Krull, C.E., *A Primer on Using In Ovo Electroporation to Analyze Gene Function.* Developmental dynamics, 2004. **229**(3): p. 433-9.
59. Sobkow L., Epperlain HH., Herklotz S., Straube W., Tanaka EM., *A germline GFP transgenic axolotl and its use to track cell fate: Dual origin of the fin mesenchyme during development and the fate of blood cells during regeneration.* Developmental Biology, 2006. **290**: p. 386-397.
60. Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin CH, Moustakas A*., Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition.* J Cell Biol. 2006. 174(2): p. 175-83.
61. Anne-Christine Poncelet, H. William Schnaper, Ruoyun Tan, Youhua Liu, and Constance E. Runyan *Cell phenotype-specific down-regulation of Smad3 involves decreased gene activation as well as protein degradation.* J. Biol. Chem 2007: p. 1-8.
62. András Masszi, Pam Speight, Emmanuel Charbonney, Monika Lodyga, Hiroyasu Nakano, Katalin Szászi, and András Kapus Fate-determining mechanisms in epithelial–myofibroblast transition: major inhibitory role for Smad3 J. Cell Biol., 2010. **188**(3): p. 383-399.