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“Master of Science” (MSc)

Molecular factors required for maintenance of regeneration in the ectopic nerve-induced blastema on the anterior upper arm of the forelimb in *Ambystoma mexicanum*

presented by

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Statement of Academic Honesty

I hereby declare that this thesis is entirely the results of my own work except where indicated otherwise. I have only used the resources given in the list of reference

Dresden, 31.08.2012

Elena Shabanova _____

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Abstract

It is already known that deflection of the nerve to the lateral anterior wound results in formation of ectopic blastema in axolotl limb, indicating induction of regeneration process. However, the of blastema stops growing at the early stage and eventually regresses. According to Meinhardt model, intersection of segments with distinct positional identity (posterior, anterior/ventral, anterior/dorsal) is a compulsory requirement for the maintenance of regeneration, along with the wounding and nerve signals. Endo et al. confirmed it by showing that graft of the skin of posterior identity transplanted next to the nerve-supplied anterior wound is able to maintain regeneration and results in accessory limb (AL) outgrowth. However, particular factors, derived from the posterior skin, responsible for creating anterior-posterior (AP) discontinuity, and, thus maintenance of regeneration were not identified. In this research we have tested several molecules for their ability to stimulate AL growth from anterior nerve-induced blastema and demonstrated that Hedgehog pathway agonist (Hh-Ag) when delivered as a soluble drug to the animals induced formation of patterned, although abnormally, accessory limb in a concentration-dependent fashion. We have also tested Sonic Hedgehog (Shh) and dHAND for the same ability, by electroporating the plasmids, containing corresponding genes directly into the anterior nerve induced bump, or into mature upper arm with subsequent wounding and nerve deviation. Our results suggest that Shh is the factor responsible for the maintenance of regeneration and the limb outgrowth.

In parallel we tried to find out the exact mechanism of how Hh-Ag maintained regeneration. We propose that Hh-Ag achieves this in three ways: 1) by changing the identity of the cells from anterior to posterior and thus creating AP discontinuity 2) by maintaining the FGF expression in the wound epidermis, as in development Shh was shown to promote FGF expression 3) by combination of both 1 and 2.

Our preliminary data suggests that Hh-Ag could change the identity of the anterior cells to posterior. In agreement with this we show here that after treatment of amputated limbs with Hh-Ag the resulting mispatterned regenerates did not restore normal structure after second round of amputation. Although this result is not conclusive, it would suggest that Hh-Ag posteriorize the cells. Upon inhibition of Hh pathway spike-like structures formed instead of normal forelimbs. After repeated amputation and regeneration rounds some limbs did not

compensate fully and lack ulna bone and some posterior digits. This indicates that Shh signaling plays a role in determining posterior identity of the cells during regeneration.

To test if Hh-Ag induces AL formation by maintenance of FGF expression (2nd hypothesis) we add Hh-Ag to the posterior wounds, asking if AL forms. Unfortunately, the experiment didn't work and results are discussed.

List of abbreviations

AEC – apical ectodermal cap

AER – apical ectodermal ridge

AL – accessory limb

AP – anterior-posterior

DV –dorso-ventral

FGF – fibroblast growth factor

Hh-Ag – Hedgehog-pathway agonist

LA – lower arm

LW – lateral wound

LFL – left forelimb

RFL – right forelimb

UA – upper arm

UALWA – upper arm lateral wound anterior

NI-UALWA – nerve-induced upper arm lateral wound anterior

RA - retinoic acid

WE – wound epidermis

ZPA – zone of polarizing activity

1. Introduction

1.1 Axolotl regeneration ability and limb anatomy

Unlike other vertebrates *Ambystoma mexicanum* (axolotl) exhibits high regenerative ability in postembryonic life, allowing usage of them as a tool to uncover the mechanisms of regeneration. Axolotls are able to regenerate their appendages (fore- and hind limbs), tail and gills. This study is focused on forelimb regeneration. When a urodele limb is amputated, the remaining cells in the stump are able to proliferate, differentiate and arrange in proper order to eventually reconstruct the missing parts of the limb.

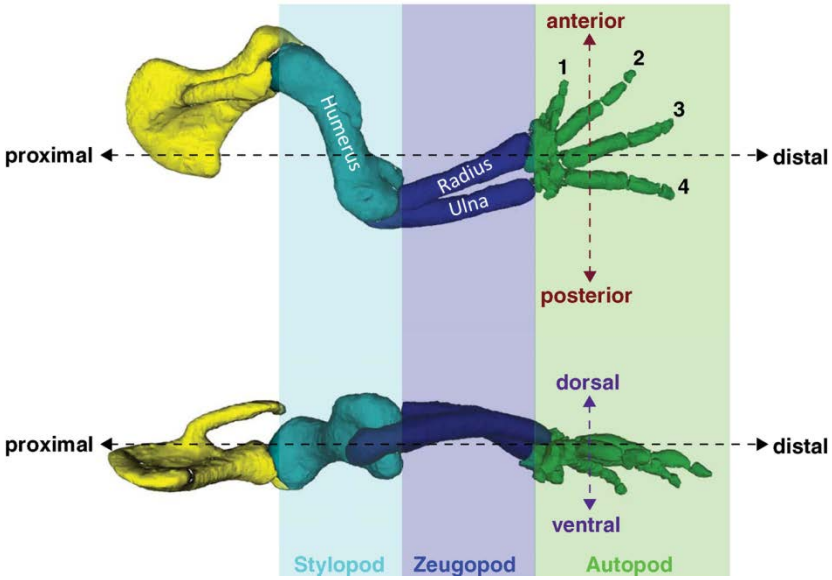


Figure 1. Forelimb anatomy. Adapted from Building limb morphology through integration of signaling modules, Veronique Duboc (2009)

Speaking of the limb, one often uses the 3-dimensional coordinate system related to the three main axes of the limb: ***anterior-posterior*** (AP) from thumb (1st digit) to pinky (4th digit), ***dorsal-ventral*** (DV) from palm to knuckles and ***proximal-distal*** (PD) from shoulder to fingers (Figure 1). In the anatomy of the axolotl forelimb three major patterns along the PD axis are distinguished: upper arm or *stylopod*, which corresponds to Humerus in the limb skeleton; lower arm or *zeugopod* corresponding to Radius and Ulna, and the most distal part, called hand or *autopod*, which consists of carpal bones, metacarpals and phalanges of four digits [1]. No matter at which level the limb will be amputated, it goes through all the same stages of regeneration.

1.2 Stages of regeneration.

The sequence of regeneration can be broken into a series of stages, progressing from amputation through blastema formation, growth and differentiation to the fully regenerated limb (Figure 2).

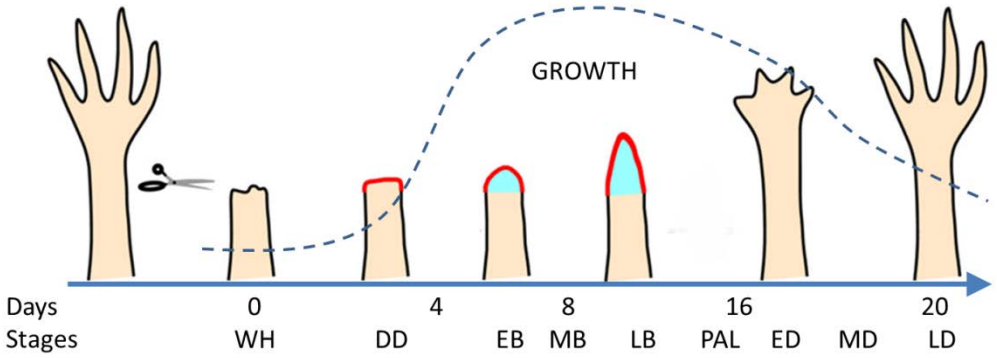


Figure 2. Urodele limb regeneration. The stages of regeneration are based on morphological criteria (Iten and Bryant 1973; Tank et al. 1976) and are referred to as WH (wound healing), DD (dedifferentiation), EB (early bud blastema), MB (mid bud blastema), LB (late bud blastema), PAL (palette), ED (early differentiation), MD (mid differentiation), LD (late differentiation). Figure was adapted from Whited and Tabin, 2009

Within hours after amputation, the epidermis at the periphery of the wound migrates to cover the wound surface. This wound epidermis (WE) thickens to form apical epidermal cap (AEC) of the regenerate. The blastema is formed by degradation of the extracellular matrix (ECM) local to the amputation surface, resulting in loss of contacts between ECM and integrin receptors of the cells, tissue disorganization (histolysis) and liberation of individual cells, followed by loss of phenotypic specialization and proliferation of the cells [2]. Within the first few days, the cells from the periphery begin to migrate under the WE, accumulate at the center of the stump and give rise to the blastema cells [3]. The process, by which blastema cells are generated, is classically referred to as *dedifferentiation*. The connective tissue, in particular dermis, makes a disproportionate contribution to the blastema [3]. However, muscle cells, chondrocytes, muscle fibroblasts and Schwann cells were also shown to give rise to the blastema cells [3], [5], [6]. Regardless of the specialization of their parent cells, blastema cells have the morphological appearance of limb bud mesenchymal cells. Matrix degradation ceases once the blastema is formed by extensive proliferation of the blastema cells. As the ECM is degraded, it is replaced by an embryonic limb bud-type ECM that helps to maintain the blastema cells in the undifferentiated state [7]. As the blastema increases in size it flattens along the dorsal-ventral axis (late bud stage). During the next Palette stage of regeneration formation of a flattened paddle is observed, which will eventually form the distal elements of the replacement limb [8]. Undifferentiated blastema cells stop dividing at the early

differentiation stage. Redifferentiation occurs and progresses as a wave, from the base of the blastema (proximal) towards its distal tip.

1.3 Limb development vs. limb regeneration.

In recent years, analysis of gene expression has confirmed that patterns seen in the later (blastema) stages of regeneration are comparable to those observed during similar stages of limb development. However, this is not the case during the early preblastema stages. In hindsight, these findings are perhaps to be expected since no stage in development corresponds to the preblastema stage of regeneration, in which cells of the mature tissue of the stump give rise to the limb bud–like cells of the blastema.

Therefore, when compared to the embryonic limb development successful limb regeneration could be seen as a two-step process, consisting of regeneration-unique preparation stage and redevelopment stage, which is quite similar to the developmental limb formation (Figure 3).

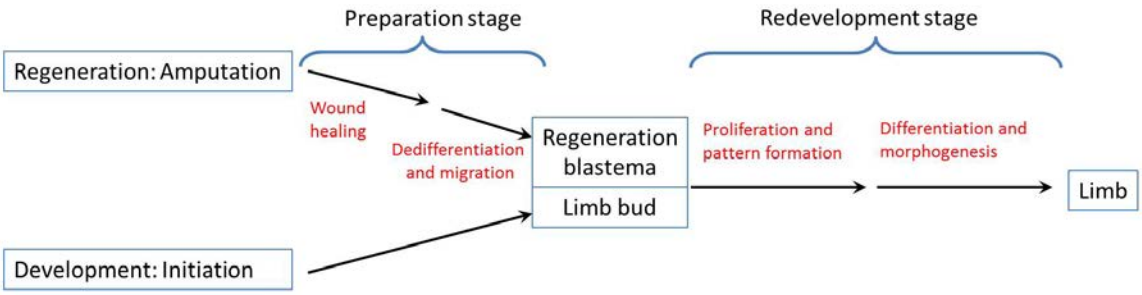


Figure 3. Diagram showing converging pathways for development and regeneration of urodele limb. (Based on Gardiner and colleagues [2002].) Figure was adapted from "Principles of regenerative biology", Bruce M. Carlson, 2007.

During the first preparation stage in the tissue underlying the zone of skin injury rapid expression of certains genes was detected [9], [10], [11]. Expression of these genes, as well as the wound healing itself, does not occur if the amputation surface is covered by full-thickness skin, which inhibits regeneration [12]. This points out that the presence of skin injury and formation of WE are vital for the initiation of regeneration. Innervation is required for the next step of regeneration, namely, dedifferentiation and proliferation (discussed in more detail in Chapter "1.4.2 Neural factors"). In contrast, for initiation of limb development neither skin injury nor neural signals are required. During the period of late dedifferentiation and early blastema formation, the regenerative process makes the transition into a phase, in which both the morphology and the underlying controls of the regenerative process begin to closely resemble those of the developing embryonic limb. Blastema itself is equivalent to the limb

bud, as it was shown in number of tissue-grafting experiments [13], [14]. Differences and similarities of the embryonic development of the limb and postembryonic limb regeneration are broadly reviewed by Nacu & Tanaka in 2011 [15]. They summarize that patterning mechanisms seem to be similar in both processes. This similarity allows the researchers to refer to the urodele limb development and known facts about it when study regeneration phenomena.

The exact mechanism of the outgrowth and patterning of the embryonic limb bud is still under debate. Over the past years, with the accumulation of experimental data a number of models have been put forward in attempts to explain molecular events taking place during limb patterning. However, several major points are no longer in doubt and consistent with all models proposed. It is now ubiquitously considered that limb patterning during embryonic development is influenced by the three interdependent signaling centers, also called organizers, and their interactions (Figure 4).

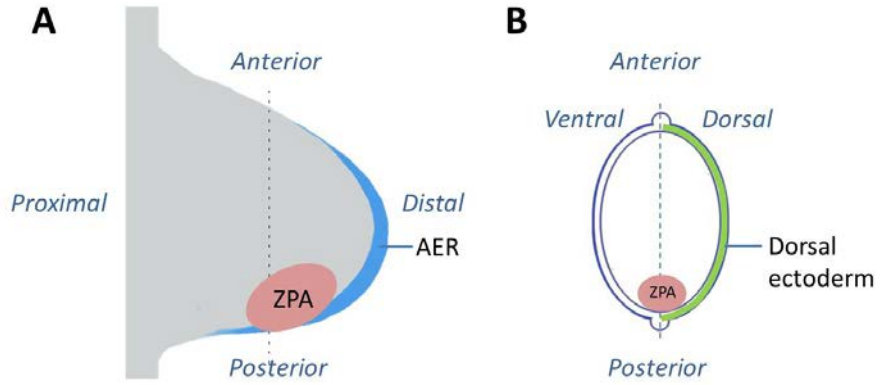


Figure 4 Limb bud (**A**) and its cross section (proximal view) (**B**). Patterning of the limb is orchestrated by interactions between three organizers: the apical ectodermal ridge (AER, *blue*), the zone of polarizing activity (ZPA, *red*), and the nonridge dorsal and ventral ectoderm (*white* and *green*, respectively). (Figure was adapted from C. Owen Lovejoy et al. 2003).

The proximo-distal growth and differentiation of the limb bud are made possible by a series of interactions between the apical ectodermal ridge (AER), which is similar to the AEC at the tip of regeneration blastema, and the limb bud mesenchyme directly beneath it. The Zone of Polarizing activity (ZPA) at the posterior edge of the mesenchyme controls AP patterning, and the dorsal ectoderm controls DV patterning [16], [17]. These signaling centers produce a variety of transcription factors and morphogens - diffusible molecules that are secreted by the cells with so-called 'organizer' properties. Similar signaling centers are

Morphogens - diffusible substances that are secreted by the cells with 'organizer' properties, spread from the source forming a concentration gradient and govern patterning.

involved in patterning during amphibian limb regeneration, and it appears to be regulated by many of the same transcription factors as will be discussed below.

1.3.1 Signaling networks involved in limb patterning

Genetic and embryological studies have identified many of the key signals controlling different temporal stages of limb development, such as the WNT, HOX, BMP, Hedgehog and FGF proteins. In this chapter it will be briefly discussed what is known about transcriptional targets of these signals, how these key signals interact with each other in development, and how they relate to the limb regeneration.

It is evident that limb patterning along PD, AP and DV axes occurs almost simultaneously and closely interrelated with each other. However, for simplicity, patterning is very often described separately for each axis.

Proximal-Distal patterning. Initiation of limb development starts when mesenchyme cells enter the limb field of the embryo. These cells secret fibroblast growth factor 10 (FGF-10), which induces the overlying ectoderm to form the AER [18], [19]. FGF-10 in the mesenchyme induces expression of FGF-8 in the AER, which in turn maintains FGF-10 expression in the mesenchyme, thus establishing the positive feedback loop intended to keep underlying AER cells in the proliferating state [20], [21], [22], [23], [24]. According to the *two signal gradient model* PD growth is the result of two opposing morphogen gradients, created along the PD axis in the early limb development: FGF secreted by AER comes from the distal end of the limb bud and responsible for determination of the distal structures, whereas another morphogen, retinoic acid (RA), is secreted in the flank of the animal and induces the proximal specification of limb bud mesenchyme [25],[26] (Figure 4).

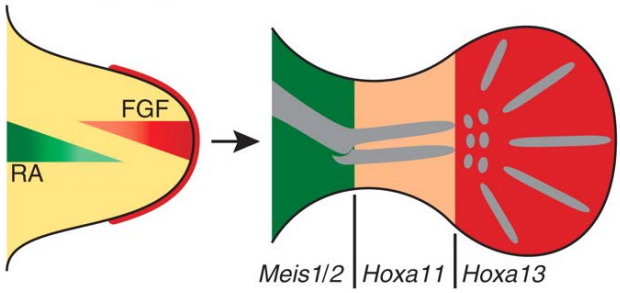


Figure 4. Two signal gradient model of PD patterning during limb development. Adapted from Zeller 2009

Beside its own morphogenetic influence, RA targets *Meis1* and *Meis2* and induces their expression in the proximal stylopod territory. *Meis* genes involved in determination of more proximal structures during development. Overexpression of either Meis1 or Meis2 leads

to inhibition or truncation of distal limb compartments [27],[28].In addition, ectopic distal *Meis1* expression inhibits the progressive distalization, resulting in limbs with proximally shifted identities along the PD axis [28].

AER-FGFs in turn lead to the activation of Homeobox genes *HoxA11* and *HoxA13*, which is indicative of the specification of more distal fates that will give rise to the zeugopod and autopod as limb bud outgrowth progresses. A few other *Hox* genes are also activated during PD patterning.

The role of *HoxA* and *HoxD* genes in PD patterning and growth promotion during early limb development in mammals was shown in numerous knockout experiments, where their partial or full combined inactivation leads to severe truncations [29], [30]. In particular, mice forelimbs that lack all *HoxA* and *HoxD* functions are arrested early in their developmental patterning [29]. When *HoxD13* and *HoxA13* are both functionally inactivated in mouse, digit development is severely impaired [31], while when *HoxD11* and *HoxA11* are knocked out, it is the lower arm/shank that is affected [30]. The limb phenotypes of knockouts of *Hox10* and *Hox9* paralogs suggest that these genes are involved in the patterning of the upper arm [32], [33].

Homeobox – DNA sequence, encoding protein domain (**homeodomain)**, which can bind DNA. Homeobox genes encode transcription factors that typically switch on cascades of other genes during morphogenesis.

In regenerating limb apical ectodermal cap (AEC), which is an analogue of the AER, is formed right after wound healing. FGF-8 expression was noted in the basal layer of the AEC and the underlying thin layer of mesenchymal tissue during blastema formation [34]. RA as well was found in regenerating axolotl limbs [35]. Mercader et al (2000) showed proximalizing effect of RA on distal blastemas through upregulation of MEIS [36] (Figure 6, A).

Expression of *Meis-1* and *-2* referentially associated with the stylopodial region of the regeneration blastema after amputation through the upper arm, whereas *Meis* knockdown inhibits RA proximalization of limb blastemas. *Meis* genes are thus crucial targets of RA proximalizing activity on blastema cells [37]. However, conclusive evidence that RA plays a role in proximal patterning during limb regeneration has not been shown.

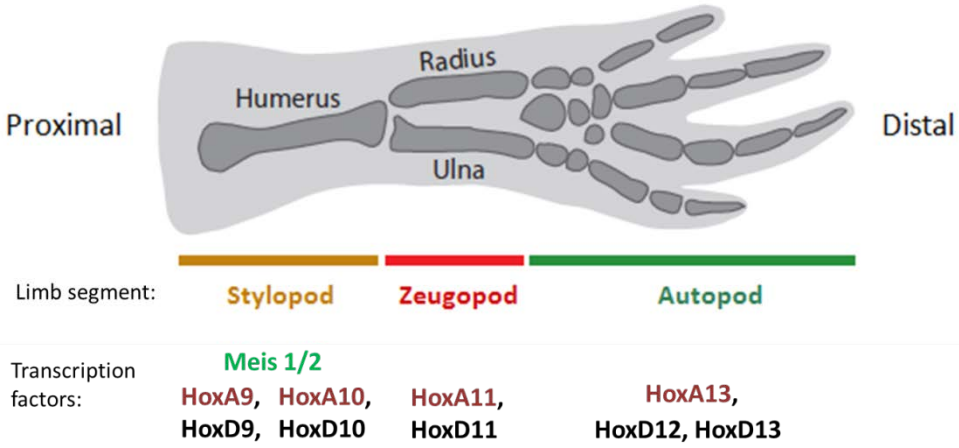


Figure 5. Spatial pattern of Homeobox genes activation during specification of limb segments along proximo-distal axis of the forelimb. Figure adapted from Tanaka & Nacu review.

The final spatial *HoxA* and *HoxD* expression pattern is the same as in developing limbs of all vertebrates, including urodeles, although during limb regeneration, 5' *HoxA* genes are expressed synchronously in stump cells regardless of the level of amputation along the proximal-distal limb axis [39]. In contrast, during limb development *HoxA* expression shows conserved spatial and temporal patterns. The more 5' genes (*Hoxa9–13*) are expressed progressively later, and in progressively more distally restricted regions of the limb bud, whereas the more 3' genes are expressed earlier, and in an expression domain that extends further proximally [38], [39].

Anterior-Posterior patterning. Mesenchymal differences responsible for the anterior-posterior polarity of the proximal part of the limb are set up very early prior to limb bud formation (reviewed by Panman and Zeller 2003) [40]. The asymmetry is generated by the transcription factors GLI3 and dHAND which mutually antagonize each other's expression. Expression of the gene encoding the transcription factor dHAND (*(**h**eart **a**nd **n**eural crest **d**erivatives),* also called HAND2, is initialized throughout limb-forming regions. Later, expression becomes localized to the posterior region of the buds by GLI3. Thus, dHAND expression is one of the earliest factors involved in shaping the limb and stays active during the whole process. [41].

Anterior GLI3 expression not only restricts dHAND expression to the posterior limb but also represses the transcription of the genes at the centromeric end of the *HoxD* cluster (the 5' *HoxD* genes — *Hoxd10, d11,d12 and d13*) [42], [43]. This anterior repression is a critical event as *dHand* and the 5' *HoxD* genes have each been shown to be necessary and sufficient for the transcriptional activation of *Sonic hedgehog* (*Shh),* which is expressed in a

portion of the limb known as the zone of polarizing activity(ZPA) [44], [45] [46] [47]. *Shh* expression from the ZPA regulates the number and identity of the digits formed in the limb, the ultimate readout of anterior–posterior patterning [48], [49], [50], [51], [52].

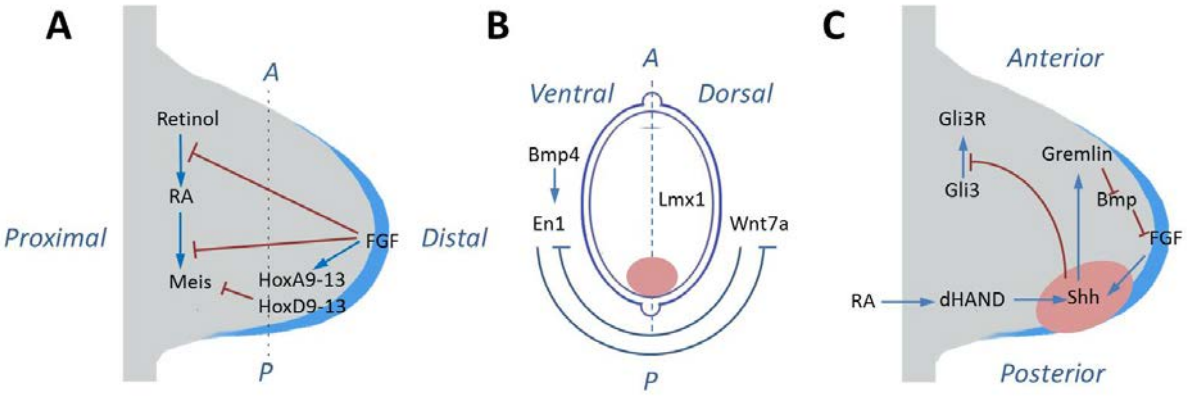


Figure 6. Some oft he key genes for limb bud implicated in patterning. (A) Signaling cascade for limb outgrowth and proximo-distal patterning. (B) Signaling cascades for limb specification along dorsal-ventral axis. (C) Signaling cascade for establishing anterior-posterior pattern of limb bud. Figure adapted from "Fins into Limbs" edited by Brian K. Hall

Expression of posterior dHAND together with homeobox D13 (HOXD13) promotes expression of the cell-cell signaling molecule sonic hedgehog homolog (Shh) [45]. Shh produced by the polarizing region is also involved in the control of antero-posterior limb bud patterning [47] (Figure 6, C). The relatively late expression of the Shh gene (at medium bud) suggests that it is not essential for AP patterning of the most proximal segments of the limb, but rather is involved in specification of digit number and their patterning, and probably patterning of the lower arm. This indeed was proven in a number of experiments. When Shh-expressing cells or beads soaked in Shh are applied to the anterior margin of chick wing buds concentration-dependent changes in digit pattern are induced [49], [52]. Genetic inactivation of *Shh* disrupts establishment of the AP limb axis as the zeugopod is reduced to one anterior bone, the radius, and the autopod is lost with exception of one rudimentary digit of likely anterior character [53], [54].

When dHand is misexpressed at the anterior margin of either chick or mouse limb buds, ectopic Shh expression is induced, resulting in formation of additional digits [41], [44]. In mice lacking *dHAND shh* is not expressed in the limb buds and the limbs are severely truncated [1]. Deletion of *hand2* in the limb bud results in lack of posterior structures similar to sonic hedgehog deletion during mouse limb development [45].The *shh* gene appears to indirectly activate FGF protein coming from the newly formed apical ectodermal ridge (AER). In turn, FGF signaling by the posterior AER maintains the SHH signaling polarizing region [55]. Thus the SHH/FGF feedback loop is established in the posterior mesenchyme

through localized activation of the bone morphogenetic protein (BMP) antagonist GREMLIN, which relays the SHH signal from responding mesenchymal cells to FGFs expressed by the posterior AER [56], [57].

Transcriptional repressor GLI3 restricts *dHAND* expression to posterior mesenchyme prior to activation of SHH signaling in mouse limb buds. dHAND, in turn, excludes anterior genes such as*Gli3* and *Alx4* from posterior mesenchyme. Furthermore, genetic interaction of GLI3 and dHAND directs establishment of the SHH/FGF signaling feedback loop by restricting the BMP antagonist GREMLIN posteriorly. These interactions polarize the nascent limb bud mesenchyme prior to SHH signaling [43].

The mechanism of anterior-posterior patterning during limb regeneration is very similar to the developing limb. Shh is also expressed in the ZPA on the posterior margin of medium bud stages of regenerating newt, axolotl, and Xenopus tadpole limbs, and moves distally with outgrowth, becomes restricted to the postero-distal region as digits begin to condense, then ceases. Expression of Shh in Xenopus developing limb buds and regenerating blastemas was proved by whole-mount in situ hybridization [58]. When transfected into the anterior cells of axolotl blastemas, Shh evokes digit duplications [59].

Dorso-ventral patterning. Investigations of gene expression associated with DV axial polarity have so far been carried out only in regenerating Xenopus limbs. The dorsal–ventral axis is established by unknown signals emanating from the somites that pattern the prelimb mesenchyme [60]. The regulation of dorso– ventral patterning is then transmitted onto the nascent limb ectoderm. The ectoderm establishes dorsal–ventral polarity through the expression of *En1* and *Wnt7a* in the ventral and dorsal ectoderm, respectively. *En1* functions to restrict *Wnt7a* to the dorsal ectoderm (Figure 6, B). Loss of *En1* results in *Wnt7a* expression in the dorsal and ventral ectoderm and the loss of ventral structures such as footpads [61], [62]. *Wnt7a* promotes dorsal cell fate by positively regulating *Lmx1b* (mouse) or *Lmx1* (chicken) expression in the sub-ectodermal mesenchyme [63], [64], [65], [66]. Loss of either *Wnt7a* or *Lmx1b* results in the ventralization of the dorsal limb such that pawpads are now present on the dorsal limb surface [63], [64], [67].

Lmx-1 is expressed in the dorsal mesenchyme of tadpole regeneration blastemas formed after amputation through the zeugopodium at stages 51-53, but is not expressed in blastemas of stage 55 limbs, which regenerate poorly [68]. These results suggest that self organization of DV polarity in the limb regeneration blastema is specified by the effect of the wound epidermis on the expression of *Lmx-1* up through stage 52 or 53; after that the polarity

is fixed. These results indicate that the major players for patterning the limb during development are reestablished during amphibian limb regeneration. By performing gain- and loss-of-function experiments of specific members of the Wnt-pathway during appendage regeneration, It was demonstrated that this pathway is not only necessary for regeneration to occur, but it is also able to promote regeneration in axolotl, *Xenopus*, and zebrafish [69].

1.3.2 Positional signaling and cell memory

Interestingly the amputated forelimb is replaced during regeneration by a forelimb and not by a hind limb or a tail. Moreover, if the limb was amputated at the upper arm level, it reconstructs missing parts of stylopod, the whole zeugopod and hand, whereas only the hand will be rebuilt in case of amputation through the wrist. This accurate reformation of original structures was termed "*epimorphic*" regeneration and over decades raised a question of how the cells know, what is missing and how they restore exactly missing structures.

About a century ago, Driesch (1908) inferred from some of his experiments on sea urchin embryos that, in some manner, cells are able to recognize their relative positions within the embryo (cited in Wolpert 1971). Later Wolpert formulated a more rigorous and testable theory of positional information in development [70]. In essence, Wolpert's theory stresses that, within a developing organism or a field within the organism, the cells are able not only to recognize their positions, but also to respond to this information by differentiating into structures appropriate for their positions. From positional information the cells are assigned positional values. Epimorphic systems appear to have maintained an active system of positional information even when development is finished, which has been called *positional memory* [71]. According to this concept, the cells in a system capable of regeneration retain a memory of their originally assigned position even if they are moved to some other location within the regeneration territory. Positional memory, which can be viewed as the long-term persistence of positional values, is the basis for intercalation when two dissimilar regions are brought together experimentally.

The series of experiments, involving spatial rearrangement of stump tissue or regeneration blastemas showed that cells remember their positional identity during the process of regeneration.

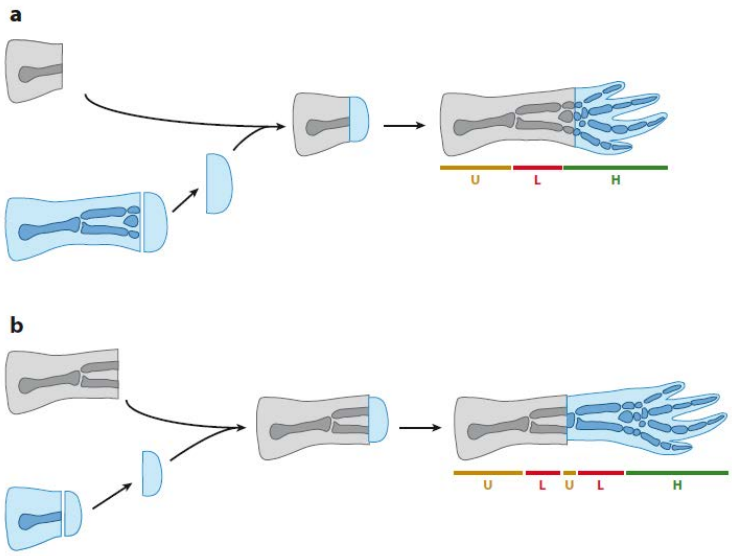


Figure 7. Effects of positional discontinuity along the proximal/distal (Px/Ds) axis. (a) A wrist blastema (blue) transplanted onto an upper arm stump (gray) results in normal regeneration. The blastema contributes to the formation of the hand elements (blue), whereas the intercalated lower arm comes from the stump. (b) An upper arm blastema (blue) transplanted onto a lower arm stump (gray) causes duplications along the Px/Ds axis. The blastema forms the structures it would have formed in situ, the distal part of upper arm, the lower arm and the hand (blue). Original experiments described in Maden (1980a) and Pescitelli & Stocum (1980). U, upper arm; L, lower arm, H, hand. Nacu & Tanaka review, 2011.

Thus, for example, distal blastema grafted to a more proximal stump results in normal regeneration, where blastema cells contribute only to the hand structure, and all intercalated segments originate from the stump (*intercalation phenomena)* (Figure 7, A). In the converse case, proximal blastema grafted to the lower arm stump behaves according to its origin, meaning that it develops all proximal and distal segments, over already existing distal segments in the stump (Figure 7, B) [72], [73]. These and other observations led to the conclusion, that cells in the limb interacted, thus determining behavior of each other. Interaction is mediated through signaling molecules that define positional identity of the blastema cells along the PD, DV, and AP axes of the limb, and is inherited from precursor cells in the stump. All in all, growth and pattern formation during regeneration was believed to be a consequence of short-range interactions between cells with different positional information.

An important tool for analyzing the nature of positional information came with the discovery that retinoic acid (RA) could change positional information in a precise concentration-dependent fashion. [35]. Mercader et al in 2000 showed that retinoic acid (RA) is an upstream activator of the proximal determinant genes Meis1 and Meis2 [36]. Meis act through the small cell surface protein Prod 1 (proximodistal 1), identified by da Silva (2002) [74]. Meis homeoproteins directly regulate Prod 1 during axolotl limb regeneration [75]. Prod 1 is expressed by blastemal cells at a 1.7-fold higher level in proximal than distal blastemas, and it is upregulated 15-fold by retinoic acid treatment.

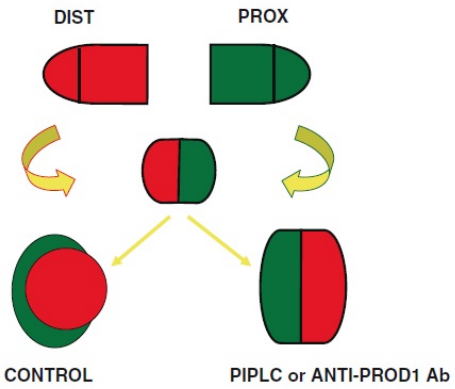


Figure 8. In vitro differential adhesion assay demonstrating that Prod1 (CD59) is one of the surface molecules that determines the level of adhesivity associated with positional identity in regenerating newt limbs. Distal (green) blastemas engulf proximal (red) blastemas in control cultures. Treatment of the blastemas with PIPLC, which cleaves Prod1 from the surface, or with an antibody to Prod1, abolishes the engulfment behavior. Picture was taken from "Regenerative biology and medicine" Stocum 2006

Surprisingly this same level of difference between proximal and distal is also present in the normal, unamputated limb, suggesting that the assessment of positional information is not a property only of dedifferentiated blastemal cells. When proximal and distal blastemas were confronted in culture and the proximal blastema engulfed the latter, then an antibody against the Prod 1 protein showed strong immunoreactivity to the proximal blastema and low immunoreactivity to the distal blastema (Figure 8). When phospholipase C, which removes Prod 1 molecules, was placed into the culture medium, then no engulfment took place. Most importantly, engulfment also failed to take place when either of two different Prod 1 antibodies was added to the medium. Overexpression of Prod1 causes distal blastema cells to translocate to a more proximal position when transplanted into proximal blastemas [76], suggesting that Prod1 plays a major role in the expression of the adhesivity gradient exhibited by blastemas from different PD levels. Summarizing these results one can argue, that the essence of proximal positional identity of the cells is in the expression of surface molecule Prod 1, which is regulated by Meis homeobox proteins, which are in turn upregulated in proximal regions by RA gradient. Thus the positional information is seem to be the result of two types of influences on the inner and outer level of the cell: a short cell-cell interactions through adhesive molecules and diffusive signaling molecules (morphogenes), creating a concentration gradient, which apparently can be sensed by the cells.

Although abundant experimental evidence exists for positional information, positional values, and positional memory, the molecular basis underlying the phenomenology represented by these concepts remains largely unknown.

Acquisition of dorso-ventral, anterior-posterior and distal positional identities by the cells and the molecules, governing this process is still a challenge for future experiments.

However, it is generally assumed that positional identity might be assigned to the cells by the same genes that control limb patterning.

1.4 Requirements for initiation and maintenance of regeneration

Apparently, after limb amputation regeneration occurs in response to a number of signals provided by the stump, since simple wounding does not lead to a formation of a new limb at the place of lateral injury. However, the first phase of regeneration, named preparation appears to be similar to some extent to the wound healing phenomena, as they both include trauma, formation of WE and the same molecular machinery involved.

1.4.1 Dermal factors

Formation of a wound epidermis (WE) is essential for both regeneration and wound healing to proceed to the next phase. Blastema formation is inhibited when formation of the wound epidermis is prevented by covering the amputation surface with a full-thickness skin flap or inserting the skinned amputated limb tip into the coelom [77], [78]. Removal of the wound epidermis at various stages of blastema growth and inserting the limb into the coelom or enclosing epidermis-free blastemas in fin tunnels leads to truncation of the regenerate skeletal pattern in the PD axis [77], [79]. This and other results suggest that wound epidermis is required to maintain cells in the cell cycle and prevent their differentiation. Evidence that the wound epidermis performs this function is that innervated blastemas in vitro undergo premature differentiation in the absence of epidermis [80].

1.4.2 Neural factors

The wound epidermis of regenerating urodele limbs is invaded by sprouting sensory axons within 2-3 days after amputation, while other sensory axons and motor axons make intimate contact with mesenchyme cells as the blastema forms [81], [82]. Total denervation of an amputated urodele limb does not prevent migration of wound epidermis, histolysis, or dedifferentiation, but mitosis of dedifferentiated cells does not take place and a blastema fails to form [83]. The nerve effect is quantitative; regeneration can be supported by either sensory or motor fibers, as long as the number of fibers is at or above a certain threshold [84], [85]. While the developing limb bud starts to grow in the absence of innervation, the initial growth of the blastema requires an adequate level of nervous supply [86], [87]. If the limb is denervated and amputated, blastemal cells can accumulate but do not proliferate. However, if the limb is denervated after a blastema has formed, regeneration will progress but the regenerated limb will be smaller in size. Therefore, regeneration depends on the presence of

the nerve only during the phase of rapid proliferation of blastemal cells. It has been suggested that the newt type III neuregulin, glial growth factor (GGF) and FGF2 might be among the factors secreted by the nerve, which either directly or indirectly control blastemal cell proliferation [88], [89], [90]. Another candidate is transferrin which assumed to be the only factor present in limb nerves that meets all the criteria for being a blastema cell survival/proliferation factor [91].

Prod1 is also implicated in the mechanism of nerve dependence. A protein called newt anterior gradient (nAG), a member of the AG family, was identified as a binding partner of Prod1, and its overexpression is able to rescue a denervated blastema and allow it to regenerate in the absence of the nerve [92]. Thus, more work will have to be carried out to fully define the molecular basis of nerve dependency.

1.4.3 AP/DV discontinuity. Meinchardt model

Satoh et al showed that skin wounding on the lateral side of the amphibian limb accompanied by a nerve deviation is essential for dedifferentiation, mobilization, and migration of dermal fibroblasts to form an ectopic blastema which is equivalent to the amputation blastema [93]. However, nerve-induced blastema didn't proceed to the next step and eventually regressed, suggesting that there must be something else necessary.

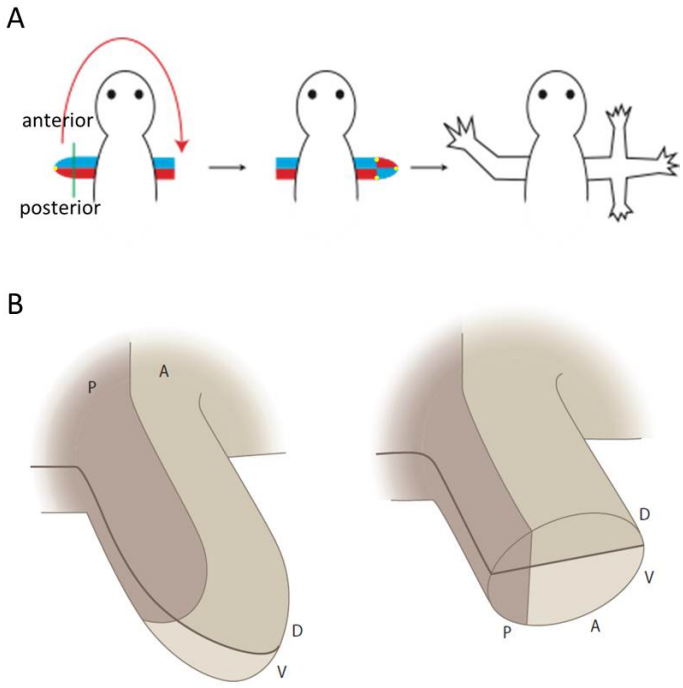


Figure 9. **A -** Iten & Bryant experiment. Blastema form one limb was amputated, rotated on 180° and transplanted to the contralateral stump, which resulted in outgrowth of supernumerary limbs. **B** - Meinchardt boundary model. Figure was adapted from Nacu&Tanaka review, 2011

In numerous blastema rotation experiments it was shown that juxtaposition of anterior and posterior limb cells is a crucial cue for growing out a properly patterned limb in the areas of anterior-posterior boundary (Figure 9, A) [95]. These results indicate that mature salamander limb tissue retains spatial coordinate information (positional information) that is activated and used during regeneration to define where a limb should grow out. Based on this and other results Meinhardt in 1983 assumed that production of distalizing morphogen caused by intersection of AP and DV boundaries that occurs at the stump surface as a consequence of cell migration [96]. After an amputation, the skin cells crawl over the wound and cells from the most anterior part of the limb meet cells from the posterior in what is termed *anterior-posterior discontinuity*. (Figure 9, B). In other words, interactions between cells derived from positions that are greater than half of the limb circumference apart are required to induce formation of a new limb. This hypothesis was termed "Boundary model". In agreement with this model supernumerary limbs do not outgrow in the place of lateral injury, where no intersection of boundaries occurs upon wound healing.

1.5 Requirements for ectopic limb formation. Accessory limb model.

The ability of urodeles to form accessory limbs from wounds on the sides of limbs, rather than the ends, provides the opportunity to break the regeneration process into simple components and study each of them separately. As an assay for the signals that control wound healing, dedifferentiation, growth, and pattern formation, this model system offers the important advantage of testing for a positive response in an organism in which all the necessary components for limb regeneration are known to be present. This model system is also important in that it allows for the experimental dissection of the multiple steps occurring during the early stages of regeneration.

Endo et al (2004) proposed Accessory Limb Model (ALM), which characterizes major signaling events and the early steps in limb regeneration controlled by them (Figure 10) [94]. This model suggests three steps required for *de novo* formation of accessory limb: skin injury, nerve damage and deviation of its end to the wound in conjunction with intersection of AP and DV boundaries, accomplished by grafting of the dermal cells to the lateral wound from the opposite side of the limb (in these experiments and henceforth it is assumed that dorsal and ventral segments intersect in the site of lateral injury and only anterior-posterior boundary has to be established).

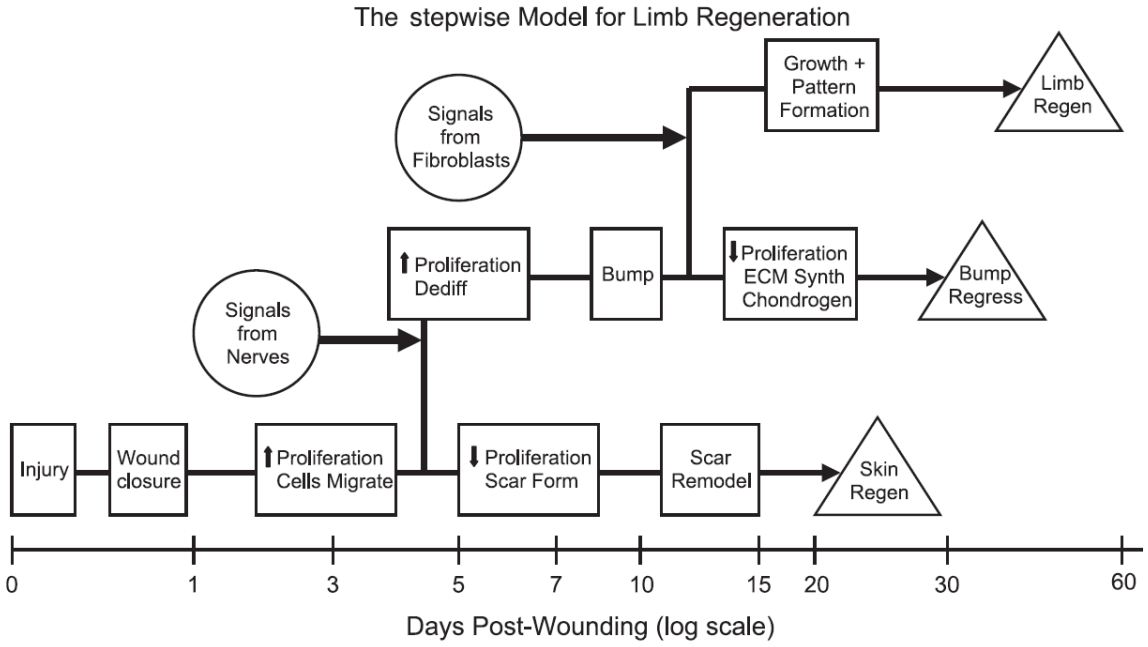


Figure 10. Accessory limb model by Endo et al. 2004

This model was confirmed by Satoh et al (2007) in experiments, where formation of ectopic blastema and distalization events, such as expression of hand identity factor *HoxA13* (Figure 11, D) and lower arm identity factor *HoxA11* in the injury on the upper arm, were detected in wounds with deviated nerve whether or not they had received a contralateral skin graft (Fig 11) [93]. Therefore, activation of Hox-dependent positional information occurs independently of APDV discontinuity.

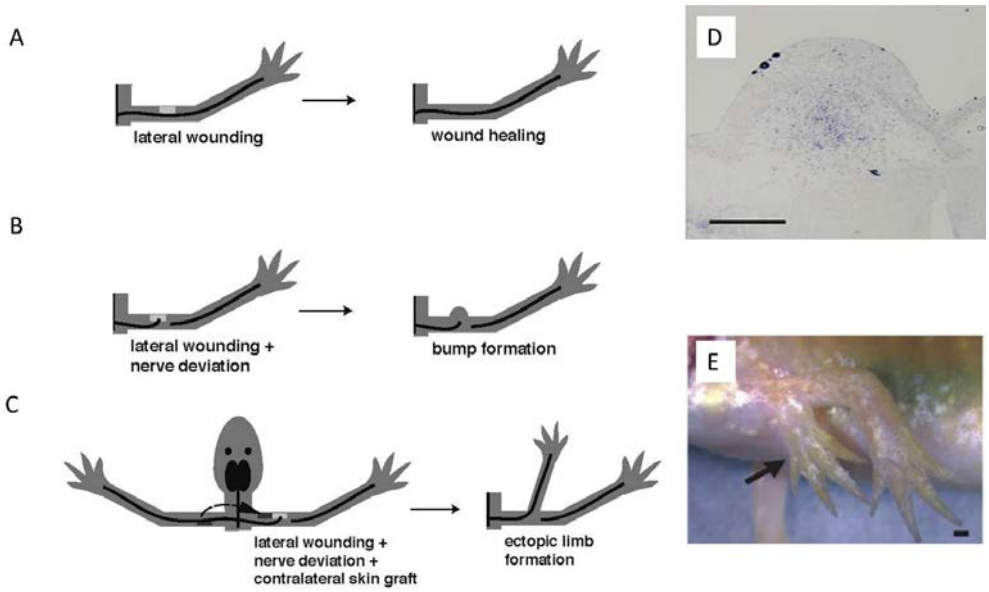


Figure 11. Satoh experiments. **A**-upon lateral wounding simple healing occurs**. B**- nerve deviation to the lateral wound results in bump formation. **C** –skin graft transplanted next to the lateral wound with deviated nerve results in Accessory Limb formation. **D –** *HoxA13* is expressed in the nerve induced ectopic blastema. **E** – Accessory limb. Figure was adapted from Kragl et al, 2009

Moreover, recently it was shown that expression factors of Hox family *HoxA9* and *HoxA11*, responsible for upper and lower arm identity respectively, are upregulated upon tissue injury and do not require axon damage, whereas the homeobox protein MEIS responsible for the upper arm identity is upregulated upon axon injury and does not require interaction with cells derived from the opposite side of the limb. [Eugeniu Nacu, unpublished results]. However, as Satoh experiments showed, the accessory limb in the **n**erve **i**nduced **u**pper **a**rm **l**ateral **w**ound on the **a**nterior side (NI-UALWA) forms only in the presence of posterior skin. These observations implies that regeneration is initiated by wounding and nerve signals, and requires activation of AP and DV positional information for maintenance of regeneration and further growth of the limb.

2. The aims of the thesis

It is crucial to understand which particular factors are able to create AP discontinuity and hence maintain regeneration. It is already known, that deflected nerve to the lateral wound results in formation of blastema, indicating that for induction of regeneration process a certain amount of nerve signals and signals from wound epidermis are required. For further maintenance of regeneration a graft of skin of posterior identity transplanted next to the anterior wound was shown to be effective. However, molecular factors, derived from this posterior skin graft and presumably involved in AP patterning and/or possessing posterior positional identity were not identified. The aim of this master thesis is to identify substances, that may induce AP discontinuity when delivered to the anterior side of the forelimb, thereby resulting in outgrowth of accessory limb from the **n**erve-**i**nduced **u**pper **a**rm **l**ateral **w**ound **a**nterior (NI-UALWA).

3. Material and methods

3.1 Animals procedures

Axolotls (Ambystoma mexicanum) used in all experiments were bred in captivity in our facility, were maintained at 17–20°C and fed with artemia. Larval of between 2 and 7cm from snout to tail were used in all experiments. To initiate regeneration, limbs were amputated at different levels along the PD axis. For all experimental procedures, axolotls were anesthetized in 0.03% benzocaine.

3.2 Cyclopamine and agonist treatment.

Cyclopamine was purchased from Toronto Research Chemicals. Hedgehog agonist was purchased from EMD Millipore. Larval axolotls were exposed to cyclopamine and the hedgehog agonist directly after limb amputation or wounding. Cyclopamine-treated axolotls were kept in 20 ml water plus 200, 300, 600 nmol/l cyclopamine. Control animals were kept in 20 ml water plus the cyclopamine-equivalent amount of DMSO. Agonist-treated axolotls were kept in 20-60 ml of water plus 4, 5, 10, 25, 40 nmol/l agonist. Control animals were kept in 20-60 ml of water. All animals were kept in the dark.

3.3 Surgical procedures

Lateral anterior and posterior wounds were created by cutting out square piece of skin from the mid-upper arm of anesthetized animals. Two ventral and two or one dorsal nerves were dissected free, severed at the elbow level and deflected to the wound. Two types of wounds were created: with and without muscle injury.

3.4 Electroporation

Upper arm and lower arm blastemas were electroporated 6-7 days post amputation. Ectopic blastemas were electroporated on 3rd day after wounding. Animals were anesthetized and immobilized on the silicon plate. Injection of plasmid mix was carried out under Olympus SZX10 optical stereomicroscope using glass microneedles for microinjection connected to the pneumatic Pico pump PV 830. Mixture of plasmids expressing CMV_dHAND VP16/CMV_Shh-N/CMV_Shh-full/CAGGS_C01-GFP and CAGGS_C02-Cherry plasmids were injected in UA and LA blastemas or UA mature limb or directly into UA ectopic

blastema. Right after injection the limbs/stumps were immersed in 1x PBS solution between tweezers "Tweezertrodes" (Harvard Apparatus) with stainless steel circular disk electrodes 1cm in diameter inserted at the tip. Tweezertrodes connected to the Electro Square Porator ECM 830 (BTX Harvard Apparatus). Five pulses of 25-75 V/cm with duration of 50ms were applied with the interval of 1s. Mouse dHAND -VP16 plasmid was kindly provided by Eric N Olson and mouse Shh-full and Shh-N plasmids were provided by Susanne Eaton.

3.5 Alcian blue/Alizarin red staining of limb regenerates

The regenerates were fixed overnight in 1x MemFa (1x MEM, 3.7 FA) and washed in PBS. The limbs were then dehydrated in 25% and 50% ETOH 10 min each. They were then placed in Alcian blue solution and kept at 37°C approximately 1 hour, until all cartilage elements were visibly stained. Then the limbs were washed in EtOH/acetic acid mix for 1 hour, 95% ETOH for 15 minutes and twice in 1%KOH solution, 10 minutes each time, until the soft tissue become clear. Then they were placed in Alizarin red solution, and stained at room temperature approximately 25 minutes, until ossified parts of the bones become visibly stained. Then they were washed in 1%KOH/glycerol mix. Finally, the limbs were placed in 20% glycerol/PBS solution and imaged under Olympus stereo microscope.

3.6 Cryosectioning

The limbs with AL were collected and fixed in 1x MemFa (1x MEM, 3.7 FA) over night at 4°C. Then the limbs were washed 2×5 min and 4×10 minutes in 1x PBS and placed in 20% Sucrose solution over night at 4°C. Afterwards they were embedded in O.C.T. and frozen in a plastic blocks. The cryosections of 10 μm depth were cut out and put on the Superfrost-plus microscopy slide (Menzel-Gläser). The sections were allowed to air dry for 1 hour and were then covered with glycerol and a coverslip placed on top.

3.7 Immunostaining

For Meis immunostaining we used Meis antibodies as a primary antibodies and goat anti-mouse Fc1 coupled with Alexa647 as a secondary antibodies. For visualization of nerves we used β-tubulin as a primary antibody and secondary goat anti-mouse coupled with Cy3 dye. For muscle staining we applied onto myosin heavy chain (MHC) antibody coupled with FITC.

3.8 Imaging

Fluorescent images of sections were made on the Zeiss Axioobserver using AxoVision software. Images were processed with Fiji software.

4. Results

4.1 Identification of candidates

What molecules can induce the outgrowth of an accessory limb from an anterior wound in the upper arm? We supposed that this ability might have the factors involved in AP patterning and control exactly posterior fate of the cells. As we discussed above, there are several morphogenes that are known to control formation of posterior structures. In chicken embryo it was demonstrated that Shh is expressed exclusively within the ZPA and is capable of recapitulating the polarizing potential of the ZPA [49], suggesting that Shh is indeed the posteriorizing factor. The limbs of urodele amphibians are homologous to those of other vertebrates, and it is to be expected that they will share conserved molecular mechanisms of development. Indeed, similar to the vertebrate development expression pattern of Shh was shown in axolotl developing and regenerating limb [97]. Application of beads soaked in recombinant SHH to the anterior margin of the limb results in mirror-image digit duplications [49].

Data from knockout, overexpression and chromatin immunoprecipitation (ChIP) experiments are consistent with the conclusion that dHAND acts as a key positive regulator of Shh expression in the limb bud [44], [41], [45]. Misexpression of dHAND in the anterior limb mesoderm of mouse or chick embryos also is sufficient to induce ectopic SHH and mirror image duplications of posterior skeletal elements [44], [41].

Based on these and other data on limb development and urodele limb regeneration we identified two major candidates for our study: dHAND and Shh.

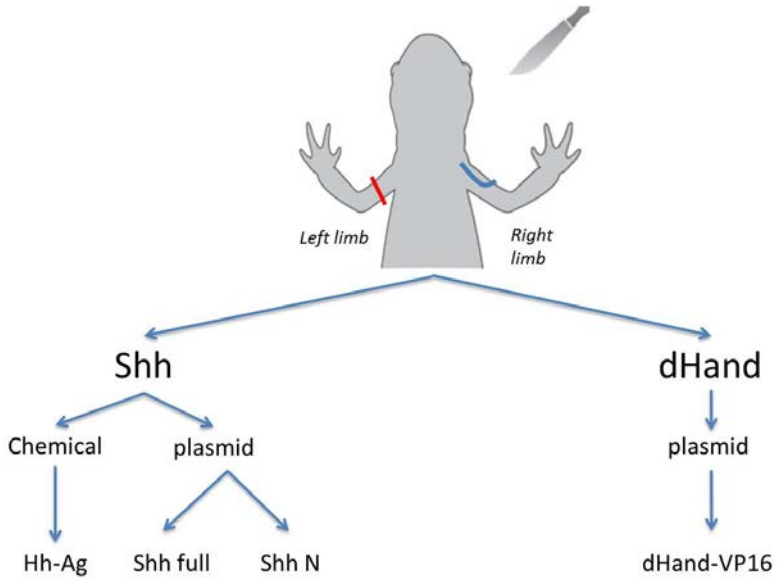


Figure 12. Plan of the experiments

For experiments we used mouse dHAND and mouse Shh plasmids since they are more than 90% conserved with newt or axolotl molecules. We also used chemical drug, which acts as Hedgehog-pathway agonist (Hh-Ag) (Figure 12).

4.2 Electroporation experiments

4.2.1 Introduction

To answer the questions "can Shh reproduce the results we got with Hh-Ag and induce limb outgrowth from an anterior lateral wound in the upper arm?" and "What effect will cause dHAND in the anterior LW?" we had to make the cells around the LW express these genes.

To induce expression of Shh and dHAND genes in the anterior cells we had to deliver them inside the cells. Several methods are used to transfer polar molecules like DNA into host cells microprecipitates, microinjection, liposomes, biological vectors and in vivo electroporation. The last one is well established in the model organisms.

Electroporation is a method to introduce foreign gene or protein material into a host cell. The concept of electroporation capitalizes on the relatively weak nature of the phospholipid bilayer's hydrophobic/hydrophilic interactions and its ability to spontaneously reassemble after disturbance [98]. By applying an external electric field, which just surpasses the capacitance of the cell membrane, transient and reversible breakdown of the membrane can be induced. This transient, permeabilized state can be used to load cells with a variety of different molecules, either through simple diffusion in the case of small uncharged molecules [99], or through electrophoretically driven processes allowing passage through the

destabilized membrane – as is the case for DNA transfer [99], [99]. Electroporation has both advantages and disadvantages compared to other methods. One of the advantages is its versatility: Electroporation is effective with nearly all cell and species types [101]. Besides, the amount of DNA required is smaller than for other methods [102]. As for the disadvantages, the most important is undoubtedly cell damage**:** If the pulses are of the wrong length or intensity, some pores may become too large or fail to close after membrane discharge causing cell damage or rupture [103].

In a number of works it was shown that electroporation works well in axolotl limbs and good conditions were developed [104], [76].

4.2.2 Electroporation set up

Tweezers with disk electrodes were wrapped in the insulating material to prevent direct contact of metal parts with the animal body thus avoiding skin burns. Electrodes were fixed at 0.5 cm distance between each other and clamped in the micromanipulator. The limb/stump was installed vertically with help of paper role and pushed through a little whole between electrodes (Figure 13). The space between electrodes was field with 1x PBS and then pulses were applied.

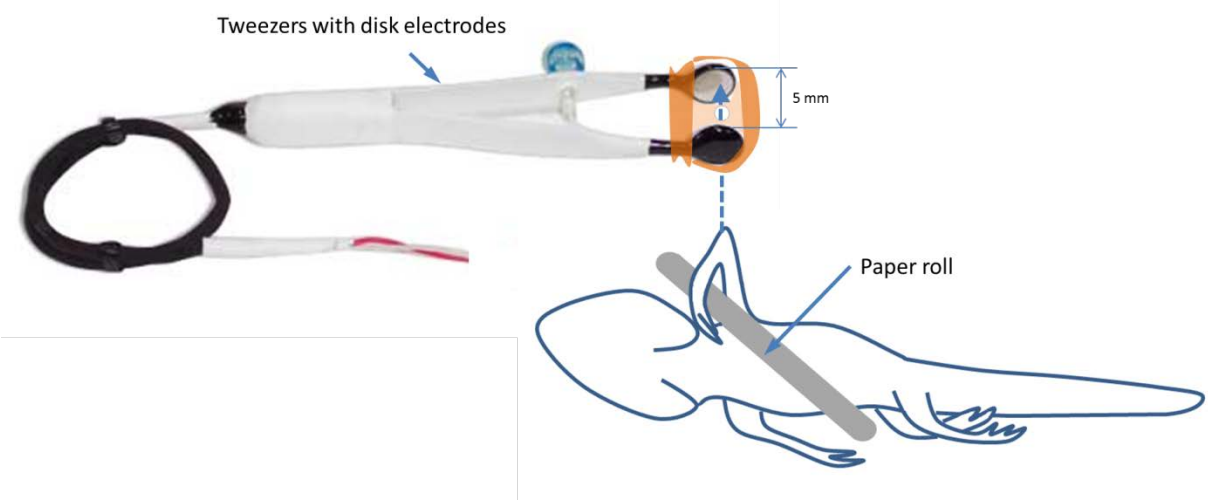


Figure 13. Electroporation set up.

4.2.3 Electroporation efficiency

First of all we tested the efficiency of electroporation with parameters used by our colleagues to electroporate axolotl limbs: 5 pulses of 25 V/cm and duration of 50ms with 1s pause between each pulse. However, the outcome of electroporation into forelimb blastema using these conditions wasn't satisfactory. We carried out a series of experiments sequentially

increasing the voltage and came to the conclusion that the most effective voltage value for blastema electroporation was 75 V/cm or 150 V with distance between electrodes 0.5 cm. (Figure 14).

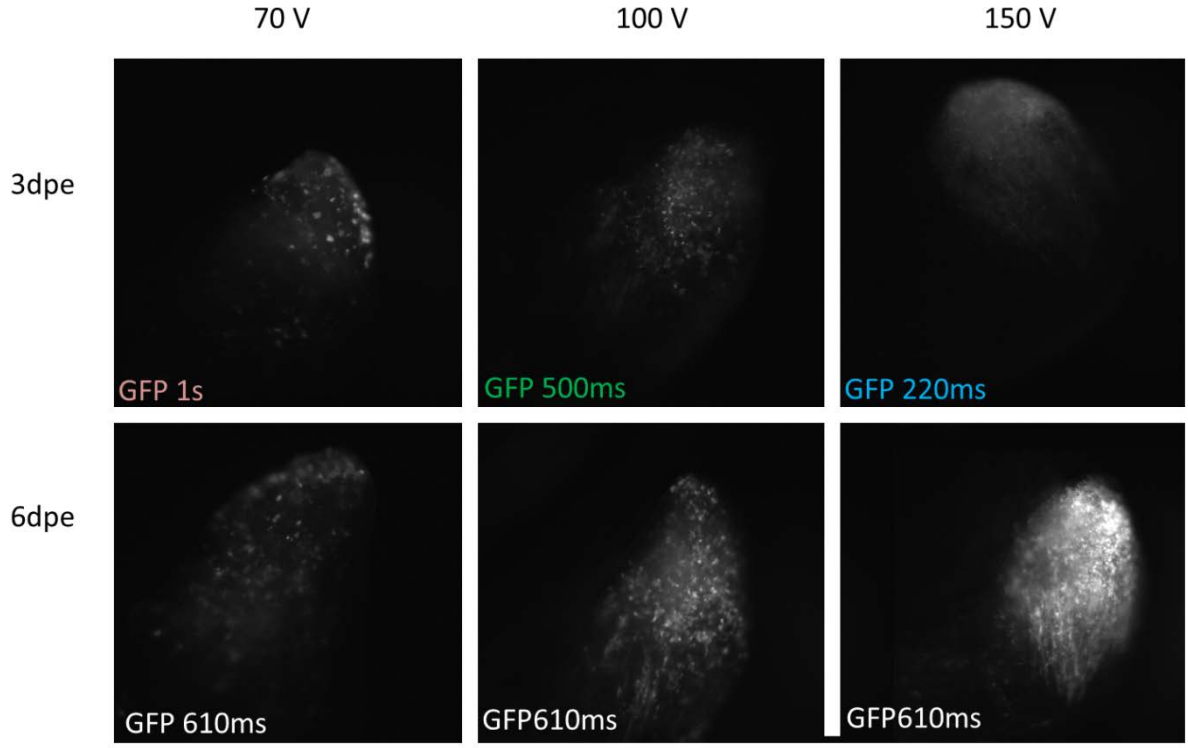


Figure 14. Comparison of electroporation effectiveness. Forelimb lower arm blastemas were electroporated with C02 cherry plasmid using distance between electrodes 0.5 cm and three different voltages: 70V, 100V and 150V. The pictures were taken on the 3rd and 6th day post electroporation (dpe). In the left bottom corner exposure time is shown.

All limbs looked normally after electroporation and regeneration progressed at a usual rate, except few limbs in the 150V group that showed signs of blastema degradation and low number of labeled cells, obviously because of extensive cell death. We proposed that these limbs were placed too close to the electrodes or even accidentally touched them during pulsing which resulted in burned tissue.

To have an idea of what percentage of the cells in blastema was electroporated we collected the most successful limbs, sectioned them and analyzed under fluorescent microscope (Figure 15). According to our estimate roughly10-20% of cells became under e best conditions (150V).

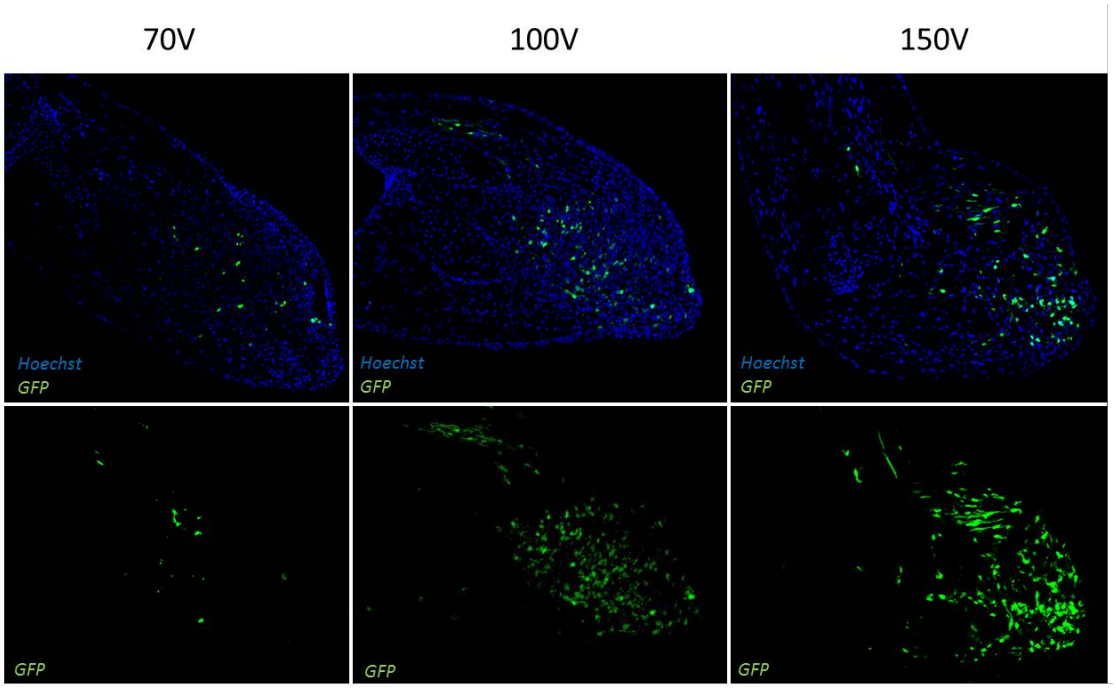


Figure 15. Fluorescent images of blastemas, electroporated with 70V, 100V and 150V.

For the mature limb electroporation we tried 70V, 90V and 150V with distance between electrodes 0.5cm. Animals in all groups look good, the limbs after electroporation did not show any evidences of necrosis and tissue destruction. The results are present in Figure 16. As it is noticeable from the images – under 70 and 90V the majority of cells expressing fluorescent marker seem to be muscle cells and only a few spindle-like spots resemble the shape of fibroblast cells. In contrast, at 150 V the amount of labeled cells that has spindle shape is comparable or maybe even more than amount of cells representing muscle fibers.

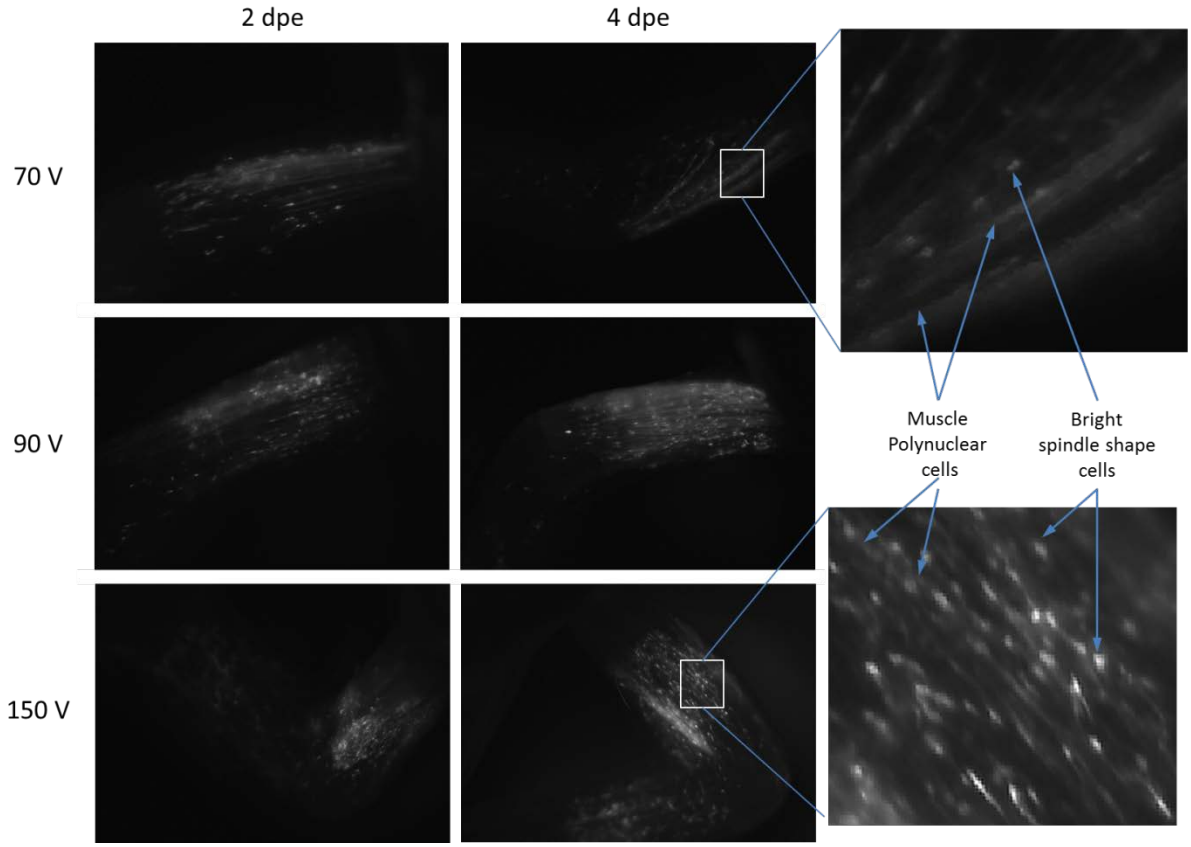


Figure 16. Comparison of mature UA electroporation using 70, 90 and 150 V with the distance between electrodes 0.5cm. The images were taken on the 2nd and 4th day post electroporation (dpe). The magnified views of the 150V and 70V samples are depicted in the right most columns, where the arrows point different types of labeled cells.

For our purposes we aimed to target fibroblast cells, since the axolotl limb connective tissues, particularly the dermal fibroblast, are a major source of blastema cells and overcontribute to the blastema cell population in comparison with the cells from other tissues [3]. In addition, it is believed that fibroblasts are responsible for the patterning of the limb. [Eugen Nacu results], [105]. Therefore we concluded from these experiments that the best voltage value for mature limb electroporation is 150V since more fibroblasts were affected using this voltage.

4.2.4 Effects of dHAND electroporation

To make sure that efficiency of dHAND electroporation is enough and gives visible effect on the limb patterning, it was reasonable to check first its ability to alter normal regeneration of the limb. For that purpose dHAND had to be electroporated into UA and LA blastemas or mature limb with subsequent amputation and observation of the outcome of regeneration. But due to the lack of time we performed these experiments in parallel with electroporation into NI-UALWA, hoping that it will induce formation of AL or at least some outgrowth.

In the first experiment we electroporated dHAND twice in mature UA with 4 days break between electroporation using 150 V. However, due to unknown reason why this time the voltage turned out to be too high and extensive damage of tissue was observed on the second day after second electroporation and even some cases of loose limbs. Due to tissue necrosis the majority of the electroporated cells died.

Next time we repeated all the same procedures, except we electroporated the limbs only once and reduced voltage to 120 V (Figure 17). In total we electroporated 18 experimental limbs and 16 control limbs. On the fourth day after electroporation 7 experimental limbs and 6 control limbs again showed evident tissue damage and were amputated at mid upper arm level, where the number of electroporated cells seemed to be high. The other limbs looked well and were used for upper arm lateral wound with nerve deviation. The results are present in the Figure 18.

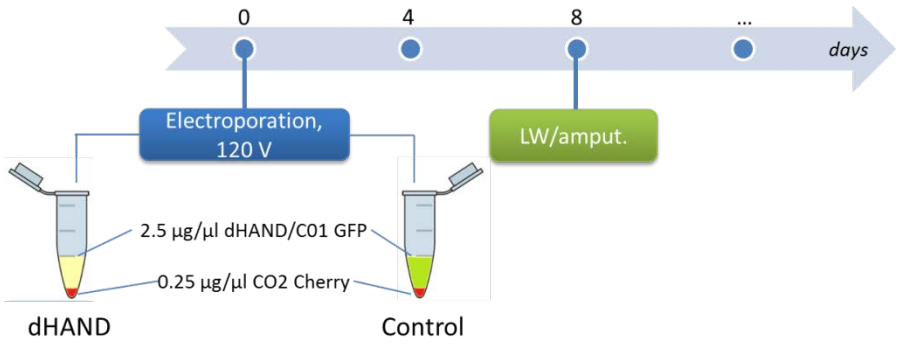


Figure 17. The experimental set up. LW – lateral wound.

On the third day after wounding nine experimental limbs out of 11 formed well defined bump in the place of injury. At the 8th day after wounding most of bumps reduced in size, however several bumps were still growing and evidently regressed only on 23rd day (Figure 18).

Among control animals 10 out of 11 limbs formed bump on the 3rd day, which progressed up to the 8th day and then started reducing and vanished completely on the 23rd day. As it is seen from the Figure 18 the fluorescent label was expressed in the regions far from the bump, what might become a reason why bump did not develop further.

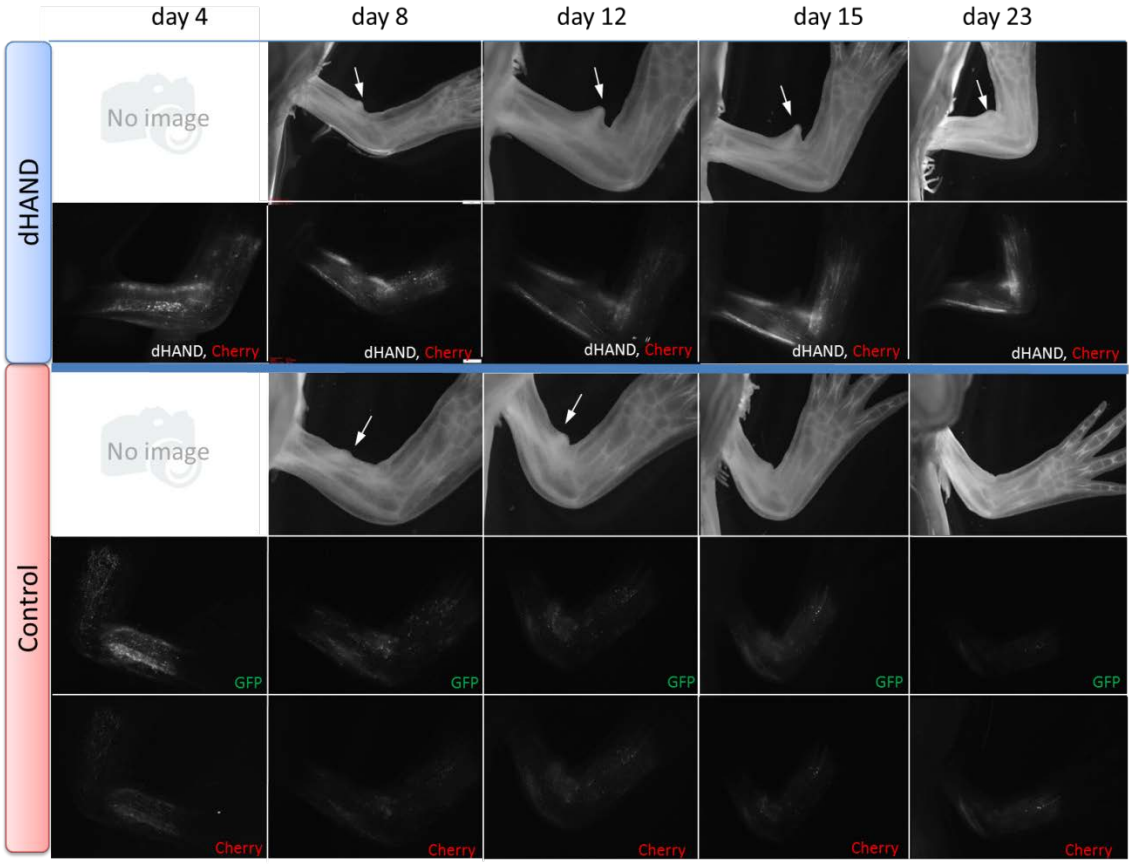


Figure 18 Representative limbs from the experiment with dHAND mature electroporation followed by creating on the upper arm nerve supplied lateral wound. Experimental animals (upper most panel) were electroporated with 2.5 µg/µL dHAND VP 16 plasmid + 0.25 µg/µL C02 cherry plasmid. Control animals (lower panel) were electroporated with 2.5 µg/µL C01 GFP plasmid + 0.25 µg/µL C02 cherry plasmid. The bumps are highlighted with white arrows.

Those limbs that were damaged by electroporation and subsequently amputated have regenerated normally and almost no fluorescently labeled cells were detected in regenerates.

In the next experiment NI-UALWA was conducted on 20 limbs. On the third day after wounding they formed small bumps in the place of injury (Figure 20, 3d column). On the same day the limbs were electroporated right into bumps. Ten limbs were electroporated with plasmid mix, containing 1.5 µg/µl dHAND VP 16+1.5 µg/µl of CO1 GFP plasmid. The rest ten limbs were used for control and their bumps were electroporated with A74/C01 plasmid mix in the same proportion like dHAND/C01 mix, where A74 is a vector for dHAND gene (Figure 19).

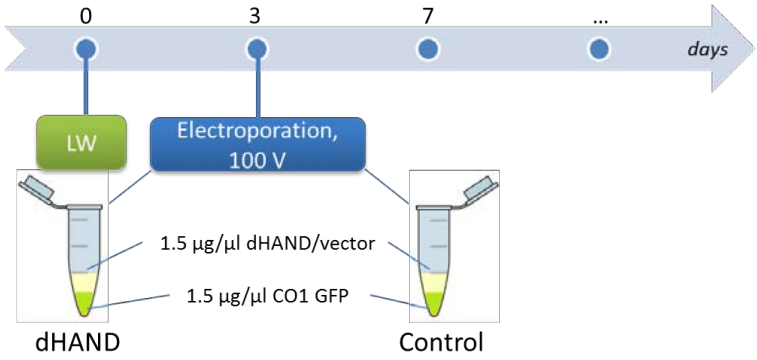


Figure 19. The experimental set up. LW – lateral wound.

During the next days bump continued to grow in both control and dHAND animals. However in the dHAND group electroporation looked less efficient and only a few cells in the bump region were expressing fluorescent marker (Figure 20, upper panel). On 14th day after wounding (10th day after electroporation) bump begun to reduce.

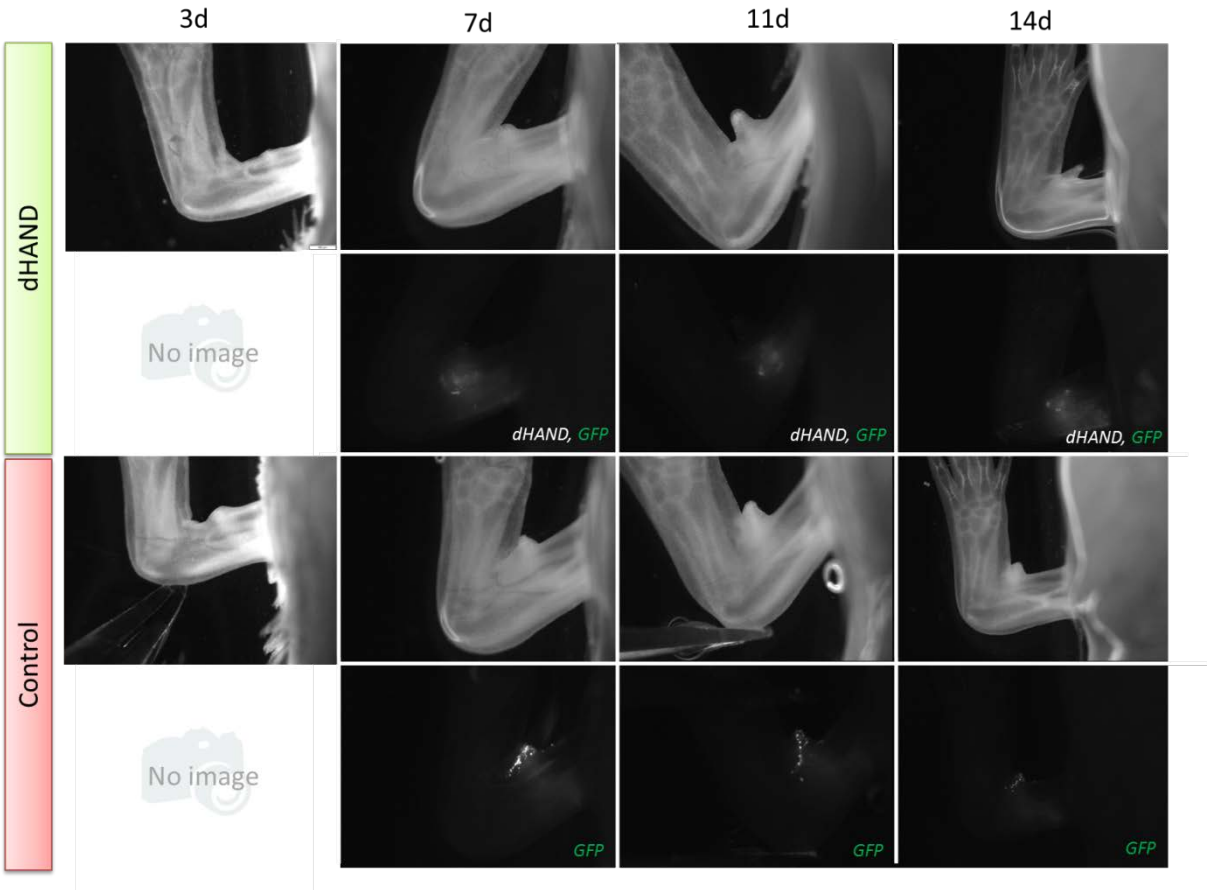


Figure 20. Results of dHAND electroporation into bump. On the upper arm of axolotl forelimb nerve-induced anterior wound was created. On third day after wounding (3d) bump formed at the place of injury. Plasmid mix of dHAND VP 16 and C01 GFP in concentrations 1.5 µg/µl was electroporated into bumps of experimental animals (group dHAND). Equivalent to dHAND VP 16 plasmid, lacking dHAND gene – A74 together with C01 in concentrations 1.5 µg/µl was electroporated into the bumps of control animals. Dark field and GFP images were taken on 7,11 and 14 days after wounding .

In parallel we performed another experiment where dHAND plasmid was electroporated into UA blastema using 150V and all the other parameters were the same as in previous experiments (5 pulses with duration 50 ms and 1s pause between each pulse). After electroporation tissue degraded, unbarring the humerus bone. The sticking out of the stump part of humerus was trimmed to ease and accelerate formation of WE. Therefore in remaining stump we detected only a scarce amount of cells, expressing the fluorescent label. However the animals were let recover for 3 weeks. The phenotype of regenerated limbs was normal; the majority of cells expressing fluorescent dye were located in the proximal part of the upper arm (Figure 21). From this experiment we could not conclude anything since cells expressing dHAND apparently did not contribute to the regeneration blastema. Obviously this happened because of tissue degradation, which destroyed the majority of cells, expressing dHAND. The reason why electroporation at 150V did not cause tissue destruction earlier was not found. We proposed that this could be because of some defect in the electroporation equipment, settings failure or electrodes degradation since they were used very often during that time.

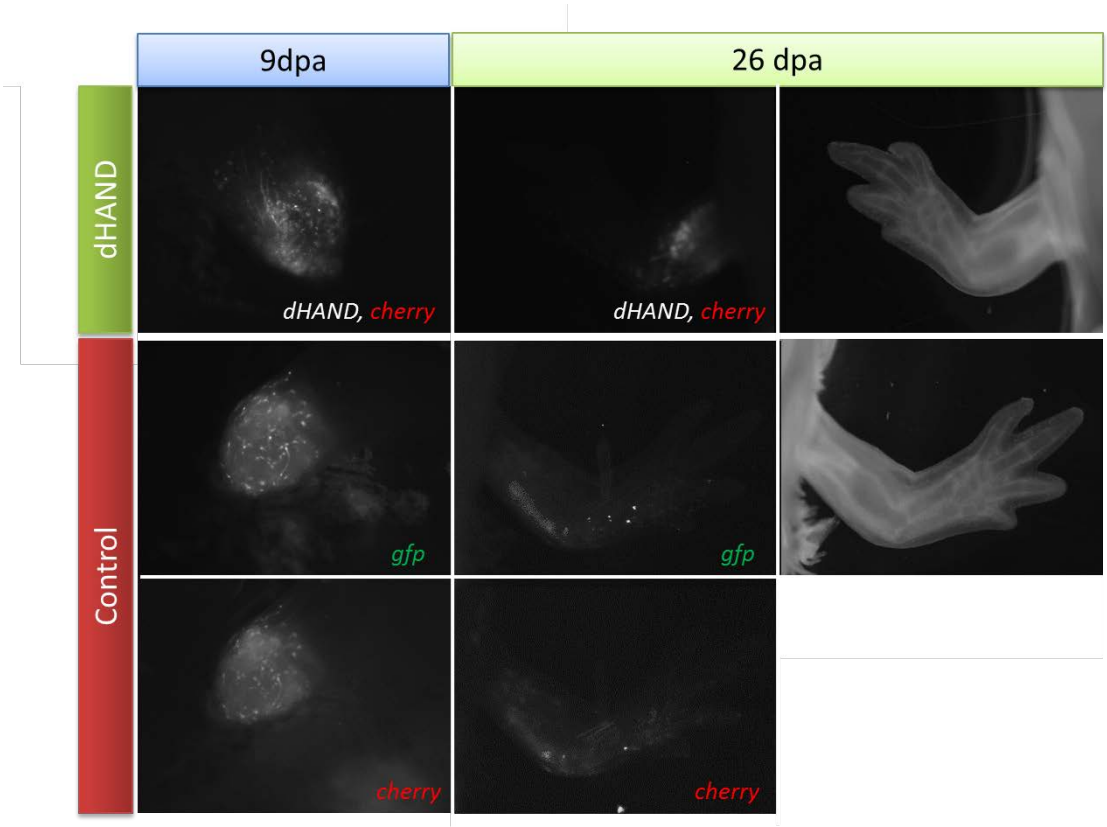


Figure 21. dHAND electroporation into UA blastema. Limbs were electroporated on 6th day after amputation at the UA level. dHAND group was electroporated with 2.5 µg/µl of dHAND VP 16 plasmid in mixture with 0.25 µg/µl C02 cherry plasmid. Control group was electroporated with 2.5 µg/µl of C01 GFP plasmid in mixture with 0.25 µg/µl C02 cherry plasmid. Fluorescent and dark field images were made on 26 day post amputation.

When we repeated this experiment using lower voltage of 100V for the upper arm (UA) and lower arm (LA) blastemas abundant fluorescent signal was observed in blastemas and no evident tissue damage. Although these limbs didn't finish regeneration yet, it is likely that they will pattern normally. That implies that dHAND is not expressed in the cells that were electroporated, although the fluorescent protein is expressed. As the next step it is reasonable to look at dHAND downstream markers to find out if plasmid is working, or try another plasmid.

4.2.5 Effects of Shh plasmids electroporation

To check the efficiency of Shh plasmid electroporation it was first electroporated into LA blastema. For that experiment 12 animals were used. All 24 limbs were amputated and formed blastemas. On the 6th day after amputation 8 limbs were electroporated with the mixture of Shh N plasmid in concentration 2.5 µg/µl and C02 cherry plasmid in concentration 0.25 µg/µl. Another 8 limbs were electroporated with the same mixture, except that Shh full plasmid was used instead of Shh N (Figure 22). Control 8 limbs were electroporated with mixture of C01 GFP plasmid in concentration 2.5 µg/µl and C02 cherry plasmid in concentration 0.25 µg/µl. On 27th day regeneration was complete and fluorescent and dark field images of the regenerates were taken (Figure 23).

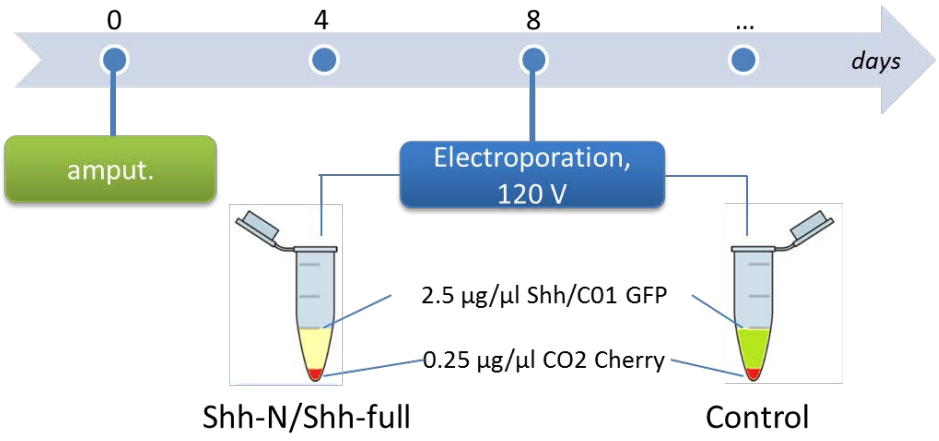


Figure 22. Diagram of experimental set up.

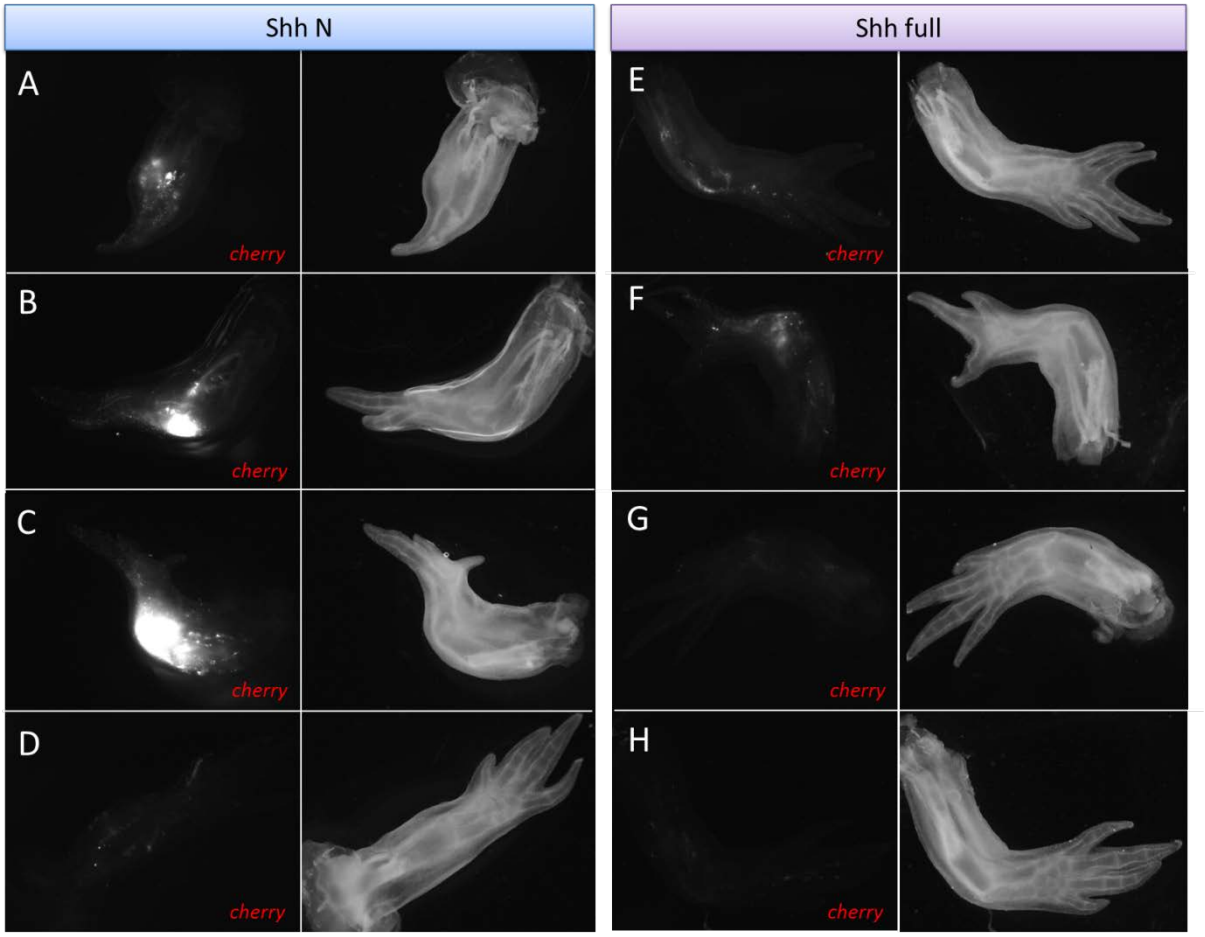


Figure 23 Results of LA blastema electroporation with Shh-N and Shh-full plasmids. On the panels **A-C** and **E-F** shown dark field images of malformed limbs (right image) and cherry expression pattern of the same limbs (left image). Panels **D,G** and **H** show normally patterned limbs with very low amount of cells, expressing Shh/cherry.

As it is seen from the Figure, the regenerates that expressed high amount of fluorescent label are mispatterned: lack digits, have digit bifurcations or even have a shape of spike (Figure 23, Panels A-C, E; F). The limbs that were electroporated less efficiently regenerated normally (Figure 23, Panels D, G; F).

In another experiment, where mature limbs were electroporated with Shh-full plasmid and later amputated at the LA level similar results were observed (Figure 24). As it is well seen from the Figure 25 electroporation of Shh full resulted in regeneration of mispatterned limbs. The regenerates acquired additional digits or developed malformed digits.

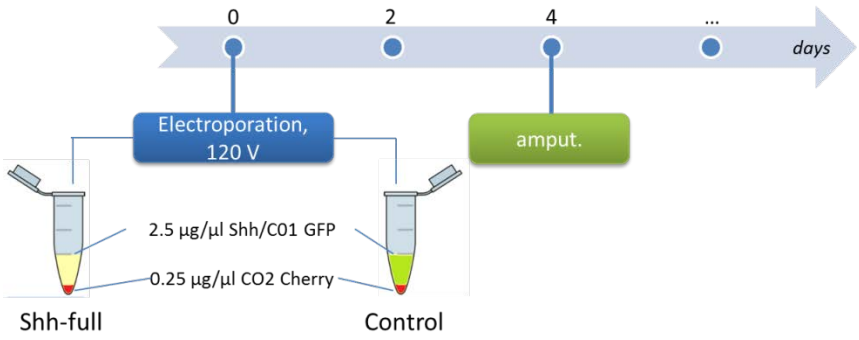


Figure 24 Experimental set up

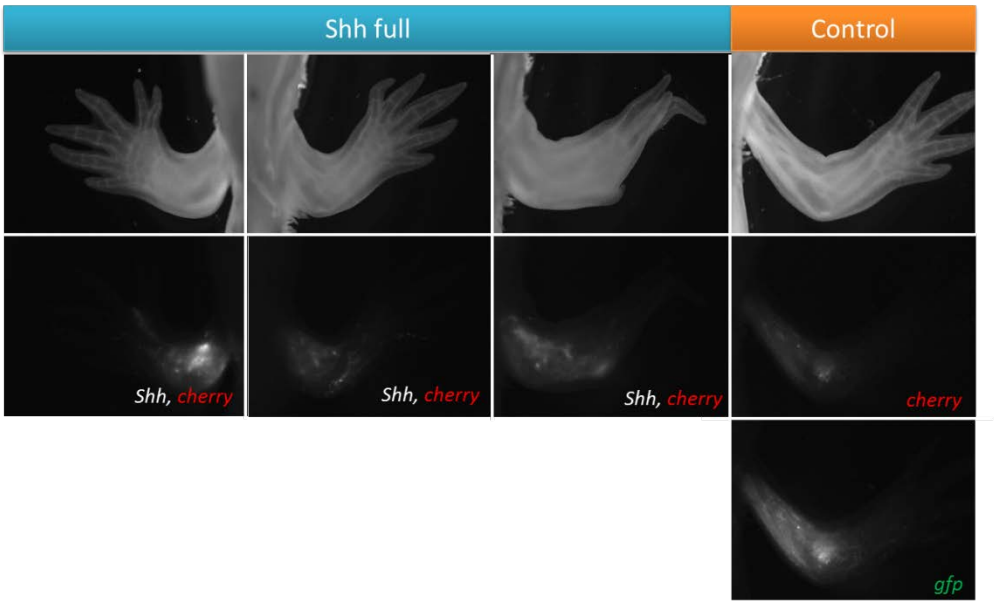


Figure 25 Shh full electroporation results. Mix, containing Shh full plasmid in concentration 2.5 µg/µl and C02 cherry plasmid in concentration 0.25 µg/µl was electroporated in mature UA of forelimbs. In control limbs mix containing C01 GFP and C02 cherry plasmids in same proportions was electroporated. 4 days after electroporation limbs were amputated at the LA level. Fluorescent and dark field images of regenerates were taken on 30 day after electroporation.

Altogether these data indicate that electroporation of Shh with used concentration of plasmids and electroporation parameters works efficiently and results in mispatterned regenerates of the limbs.

As the next step Shh full plasmid was electroporated first in mature limb and after 6 days post electroporation NI-UALWA was created. In 3 days when well defined bump formed in the place of injury electroporation was repeated, but this time injection was made directly into bump. The same plasmids in the same concentrations and electroporation parameters were used as in the previous experiment. The results are present in the Figure 26. Already in a couple of days after second electroporation the limbs looked weird, they were very rigid, fixed in one position and immobile, and it was hard to straighten them. Apparently, the amount of Shh expressed by the cells turned out to be toxic and caused cell death. The

control limbs looked well and flexible, thus we concluded it was not the effect of double electroporation.

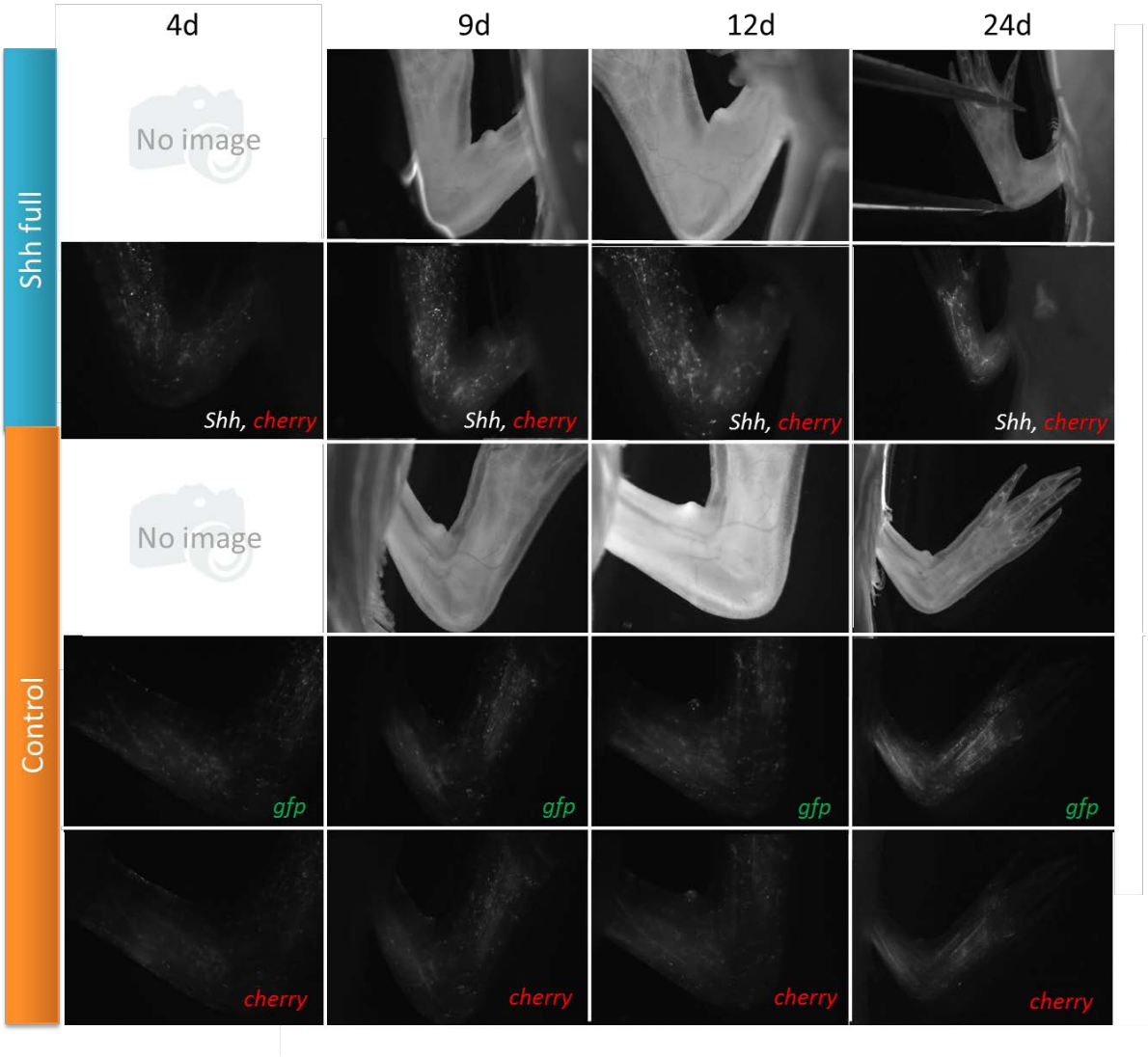


Figure 26. Shh full electroporation with subsequent creation of NI-UALWA and second electroporation into bump. Fluorescent images were take on the 4th day after first electroporation (4d). NI-UALWA were created on the 6th day after first electroporation. Three days later, when bump at the place of wounding was formed 2nd electroporation was performed directly into bump. Dark field and fluorescent images were taken on 9, 12 and 24 days after 1st electroporation.

From this experiment we concluded, that double electroporation of Shh full plasmid in concentration 2.5 µg/µl creates too high level of expression, which is obviously causes cell death and thus has to be reduced.

4.3 Soluble Hh-Ag is able to produce accessory limb from the anterior nerve-induced lateral wound

4.3.1 Introduction

Hedgehog (Hh) proteins, including Sonic Hedgehog (Shh), communicate to cells through receptor proteins on the cell surface that activate an intracellular signal-transduction pathway culminating in the regulation of gene expression (Figure 27) [106]. Key players are two transmembrane proteins. The first, Patched (Ptc), is the receptor to which Hh binds, and the second, Smoothened (Smo), which is structurally similar to seven-transmembrane proteins, initiates intracellular signaling in response to the Hh signal. In the absence of Hh ligand, Ptc restrains Smo activity; binding of Hh to Ptc releases Smo, which then transmits the intracellular signal. The regulation of Smo activity therefore occupies a pivotal position in the cellular response to Hh. Previous work had identified small molecules that inhibit or activate Hh signaling by interacting with Smo [107], [108], [109]. These include the antagonist cyclopamine as well as Smoothened agonist (SAG) (also called Hedgehog-pathway agonist – Hh-Ag).

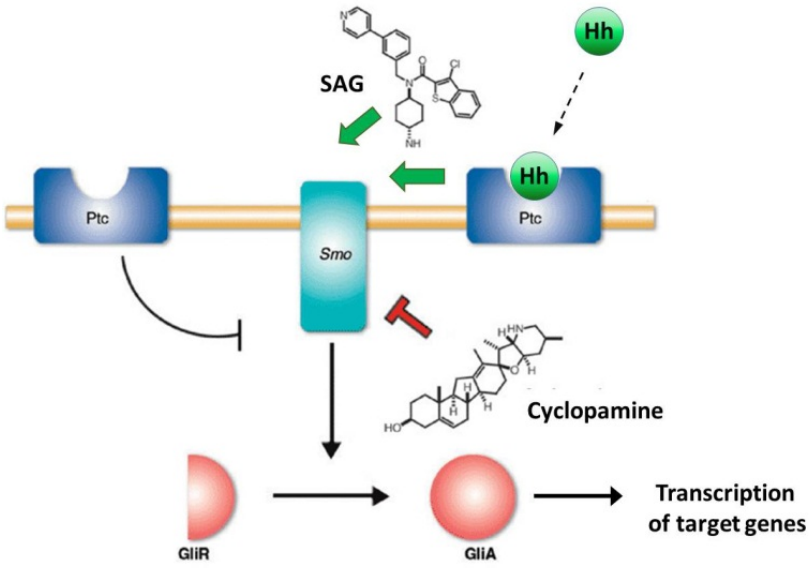


Figure 27. In the absence of Hedgehog (Hh) ligand, the transmembrane protein Patched (Ptc) inhibits Smoothened(Smo). Hh binds to Ptc, and this relieves repression on Smo, which signals intracellularly to promote the generation of Gli transcription activators (GliA) instead of transcriptional repressors (GliR). The structurally distinct molecules Smoothened agonist (SAG) can activate Hh signaling by targeting Smo, whereas the antagonist Cyclopamine inhibits Hh signaling (Image adapted from J. Briscoe,2006).

Previously it was shown that treatment with cyclopamine induces loss of posterior structures during limb regeneration [110], [111], [112]. (Figure 28)

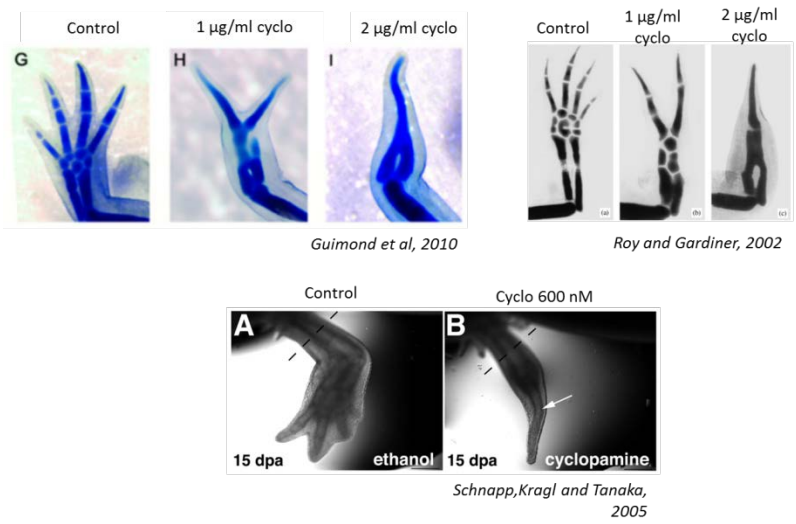


Figure 28 Cyclopamine effect on limb regeneration

As it was mentioned above, Shh participates in AP patterning. It is secreted by the posteriorly located ZPA and diffuses anteriorly with quenching concentration. In numerous experiments with ectopic application of Shh and ZPA transplantation the mirror image duplication of posterior structures was observed [59], [113]. Thus, Shh was considered as a candidate for our experiments. Since Hh-Ag is also able to induce Hh-pathway and is available in soluble form – we conducted several experiments using Hh-Ag.

4.3.2 Effectiveness of Hh-Ag drug

To test effectiveness of Hh-Ag it was first applied to the amputated limbs simultaneously with cyclopamine treatment. Since cyclopamine inhibits endogenous Shh (Figure 27), it is expected that AP patterning will be impaired upon cyclopamine treatment and restored, at least partially, upon exogenously applied Hh-Ag. For positive control of cyclopamine effectiveness we also amputated tails of the animals, since it was reported that cyclopamine specifically inhibits hedgehog signaling pathway during the tail regeneration [111].

For this experiment we took 27 axolotl larvae of approximate length 4cm from snout to tail tip. All left limbs were amputated at the level of mid-lower arm, whereas all right limbs were amputated at the level of upper arm, on ¾ parts proximally from the elbow. The tails were amputated on 1cm distance from the tail tip (Figure 29, C). All animals were randomly sorted into 4 groups (Figure 29, A).

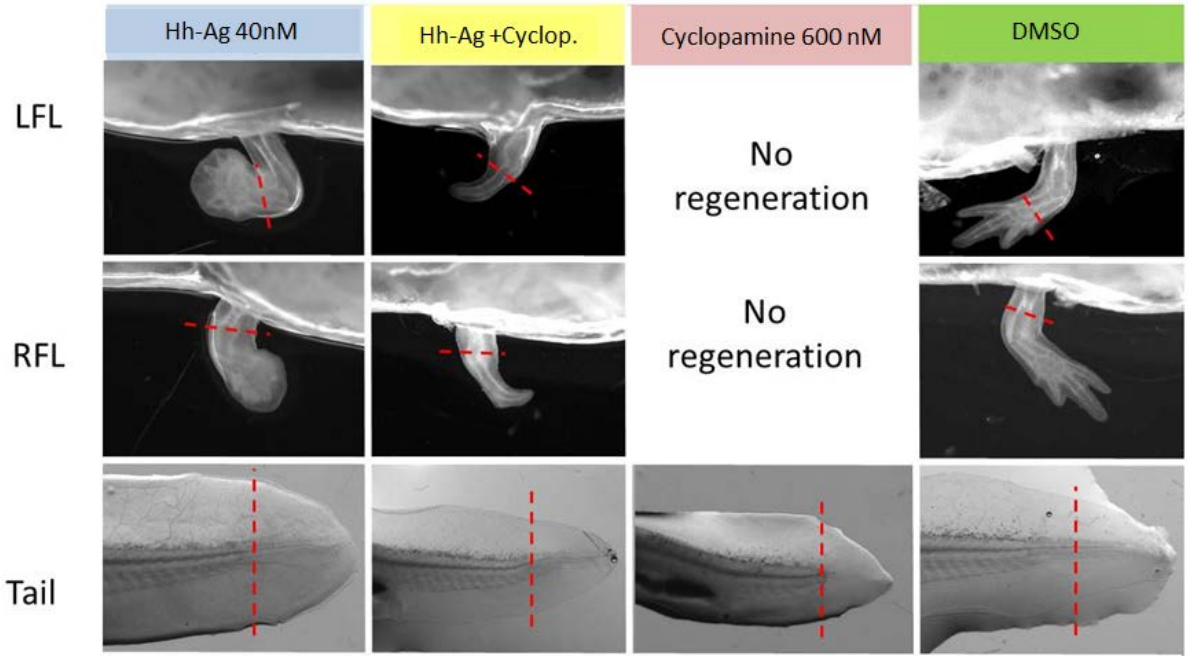


Figure 29. Effectiveness of Hh-Ag. Examples of limb and tail regenerates from different groups. Amputation plane is indicated with red dashed line. LFL – left forelimb, RFL –right forelimb.

All animals of control group showed normal regeneration, proper digit patterning and formation of bones in the limbs and normal regeneration of all structures in tail (spinal cord, chord, nerves, vessels and fin). All animals treated with 600 nM of cyclopamine did not regenerate their limbs. Only formation of wound epithelium was observed on the stumps of these animals and in tail regeneration of all structures except fin also failed. This is consistent with the loss of Shh signaling. Cyclopamine acts as inhibitor of Hh pathway. Loss of Hh signaling inhibits limb and tail regeneration.

In the group treated with both Hh-Ag and cyclopamine we observed for all animals loss of wrist elements, ulna and radius are hard to distinguish or absent, no digit patterning, formation of cartilaginous single or split asymmetric spikes (not segmented along the proximo-distal and anterior-posterior axes), whereas in tail regeneration of spinal cord and chord structure. This implies that Hh-Ag can partially rescue cyclopamine and therefore it works.

In the group treated with Hh-Ag only we detected clearly seen malformation of the regenerated limbs. Abnormal regeneration included deformation of digits, loss of digit segmentation, fusion of two fingers on their tips, distortion of AP patterning, deformation of wrist bones. However, in case of amputation through upper arm we observed normal

regeneration of radius and ulna, or slight shortening of these bones. This allows concluding that Hh-Ag induces extra digits as expected of activation of Hh-pathway, hence it is working.

4.3.3 Effect of different concentrations of Hh-Ag on regeneration

As the next step we tested the influence of Hh-Ag of different concentrations on normal regeneration of the axolotl forelimb, when added to the water where animals were kept. For that we took animals of 5.0-5.5 cm from snout to tail tip. Left arms of all animals were amputated through the upper arm level on ¾ parts distally from the shoulder, whereas right arms of all animals were amputated through the lower arm, just proximal of wrist. Then all animals were randomly sorted into3 groups. First group was treated with 4nM Hh-Ag, second group was treated with 25nM Hh-Ag and third group was kept in normal tap water. After 21 day of Hh-Ag treatment the pictures of regenerates were taken with stereo microscope (Figure 30)

We detected slight malformation of digits in case of 4 nM of Hh-Ag and strong deformations in case of 25 nM Hh-Ag in comparison to normal regeneration (control group). We concluded that even low concentration of Hh-Ag is able to affect normal regeneration of the limbs.

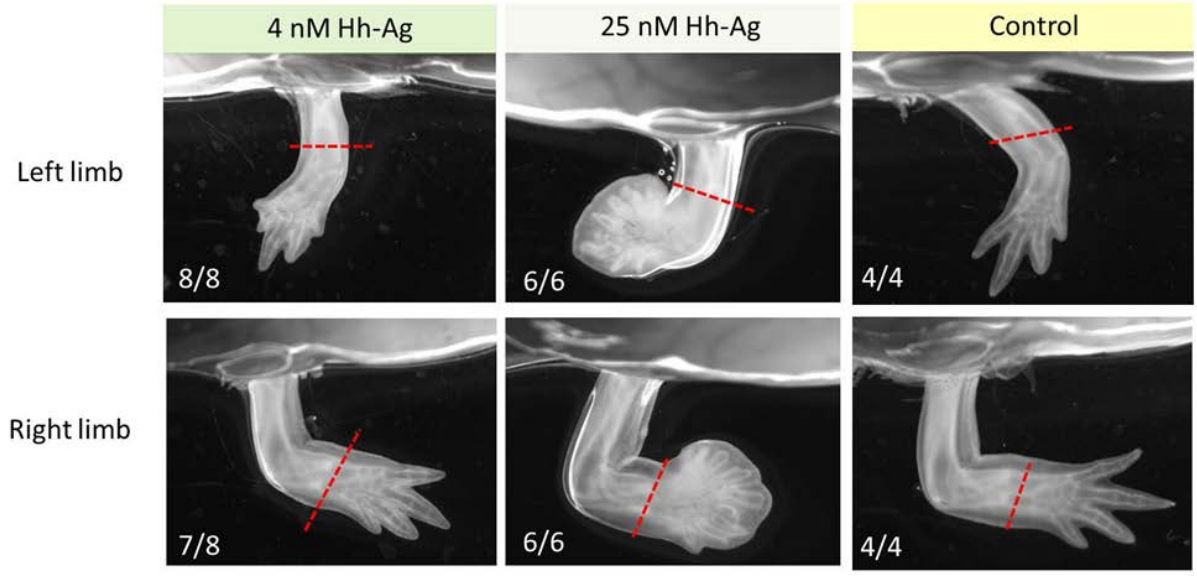


Figure 30. Effect of Hh-Ag of different concentrations on normal limb regeneration. Example limbs, treated with 4 nM, 25 nM Hh-Ag after amputation and untreated normally regenerated limbs(control column). Amputation plane is indicated with red dashed line.

4.3.4 Effect of Hh-Ag on the maintenance of anterior ectopic blastema

This experiment aimed to test if Hh-Ag is able to establish AP discontinuity and thus maintain blastema growth and limb patterning from the ectopic blastema, induced by

wounding and deviation of nerve tracts to the wound. We created wounds on the first batch of animals, and deviated one sciatic nerve tract to the wound surface, but none of them eventually formed bumps in the place of injury. We proposed that neural signaling from a single nerve filament didn't achieve a threshold, necessary for inducing blastema formation. Taking this into account all following lateral wounds were created ventrally with injury of two ventral nerves and one or two dorsal nerves (Figure 31).

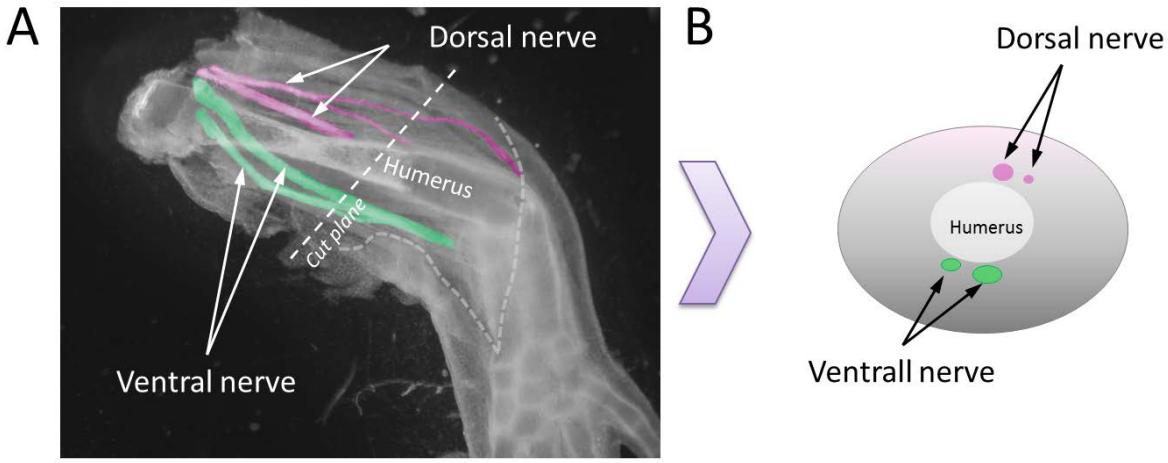


Figure 31 Arrangement of the nerves in the upper arm in the axolotl forelimb. **A**-skin cut on the UA, with located beneath it ventral and dorsal nerves. **B** – Schema of the limb cross section at the level of UA

My supervisor, Dr. Eugen Nacu, created ventral anterior lateral wounds on the right upper arms (UALW-A) of 22 animals and amputated all left limbs at the level of upper arm on ¾ parts proximally from the elbow. The left limbs served as the controls that Hh-Ag was working on the animal. Ten of them we treated with 10 nM Hh-Ag and kept in dark, since the drug is light sensitive. Twelve randomly selected animals served as controls and were kept in normal tap water. Images were taken every week using the stereomicroscope (Figure 32).

On 9 day after wounding/amputation in control group, kept in tap water we detected 8-10 nerve induced bumps (Figure 32, B). Most of these bumps regressed completely two weeks later, and only some of them were still recognizable, but none of them showed further growth during the next weeks. Among Hh-Ag treated animals we detected 7 bumps out of 10 created wounds and 6 of them continued growing and eventually formed accessory limbs. After 3 weeks of Hh-Ag treatment it was terminated and AL were left to finish their patterning under normal conditions (Figure 32, A).

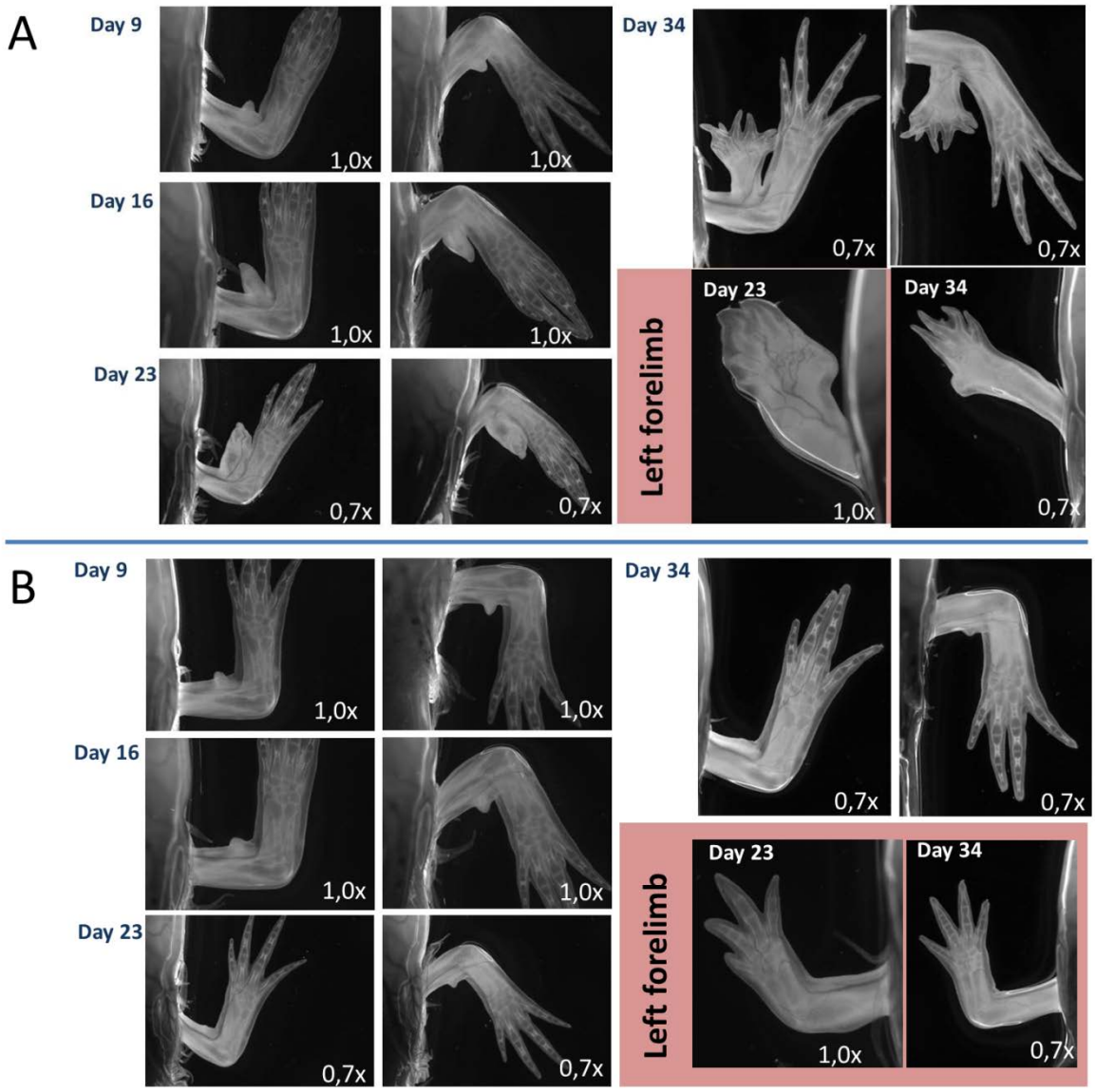


Figure 32 Panel A – limbs treated with Hh-Ag in concentration 10 nM for three weeks formed accessory limb from the NI-UALWA. For positive control left limb was amputated. Panel B – control animals with NI-UALWA kept in tap water formed small bump, which eventually regressed.

These results suggest that activation of hedgehog signaling by Hh-Ag induces limb outgrowth from an anterior lateral wound in the upper arm.

To find out the limb segments that have regenerated we amputated AL and stained their skeletal elements with Alcian blue and Alizarin red stain (Figure 33).

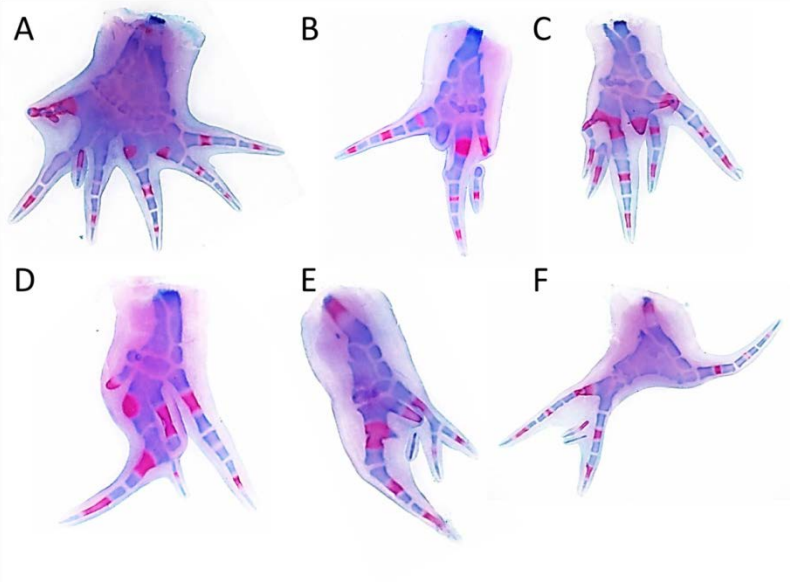


Figure 33 Accessory limbs. Alcian blue/Alizarin red skeletal staining

In the Figure 33 blue color depicts cartilage elements, whereas red marks already ossificated fragments of skeleton. In all accessory limbs we can clearly define patterned digits and several skeletal structures of hand. Limbs B, E and F as it is seen from the Figure have single bone in the basis, which resembles humerus, whereas limbs A,C and D have several bones. However it is not really clear whether the skeleton of lower arm and upper arm is present.

For further analysis of the structures, that constitute AL we sectioned limbs them and stained sections for the marker of upper arm cell identity - Meis, β-tubulin for visualization of nerves and myosin heavy chain to ascertain presence of muscle tissue in AL (Figure 34).

We analyzed in total 5 accessory limbs. In all ALs we observed Meis in dermis, however it is a migrating tissue and presence of Meis in it does not indicate proximal identity. When we looked at skeletal elements we did not observe any Meis expression in 3 out of 5 limbs. In rest two ALs we detected Meis-positive cartilage, which resembles more an nodule rather than an elongated upper arm bone. This indicates that UA structure was not formed in the ALs and only a few limbs have cartilaginous nodule of proximal identity isolated from the other AL skeleton. Perhaps no proximal structures were formed because the wound was created at the distal part of the upper arm. Another explanation could be that Shh promotes the outgrowth of only distal structures [42]. In rest the ALs seem to be very close to the normal forelimb structure. They formed hand skeleton and digits, muscles and nerves, as it is seen from the Figure 34 (D and C).

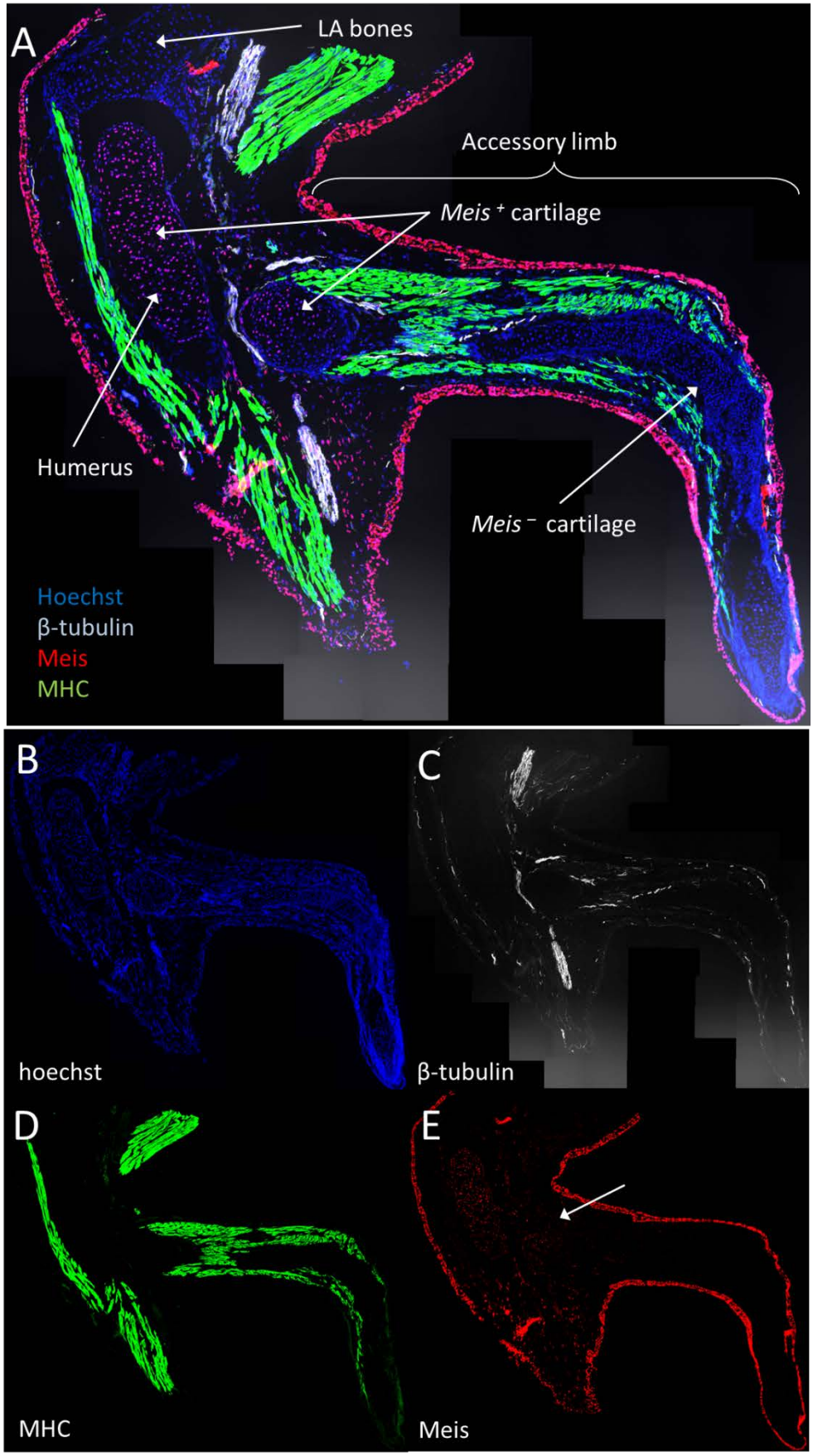


Figure 34 Fluorescent image of AL section staining. **A** – composite, **B** – nucleus stained with hoechst, **C** –nerve tissue, visualized by β-tubulin staining, **D** –muscle tissue, visualized by myosin heavy chain (MHC) staining, **E** – Meis staining

4.4 Possible mechanism by which Hh-Ag induces Al outgrowth

When we got the outgrowth of the accessory limb upon treatment of NI-UALWA with Hh-Ag the question arose: what is the mechanism of Hh induced formation of AL? We proposed three possible ways (Figure 35):

1. Shh plays role in determining posterior positional identity in regeneration, thus its presence in the wound causes a portion of anterior cells to convert to the cells with posterior identity therefore creating AP discontinuity.
2. Shh acts through maintaining FGF expression in the wound epidermis, as in development Shh was shown to promote FGF expression [55].
3. Combination of 1 and 2.

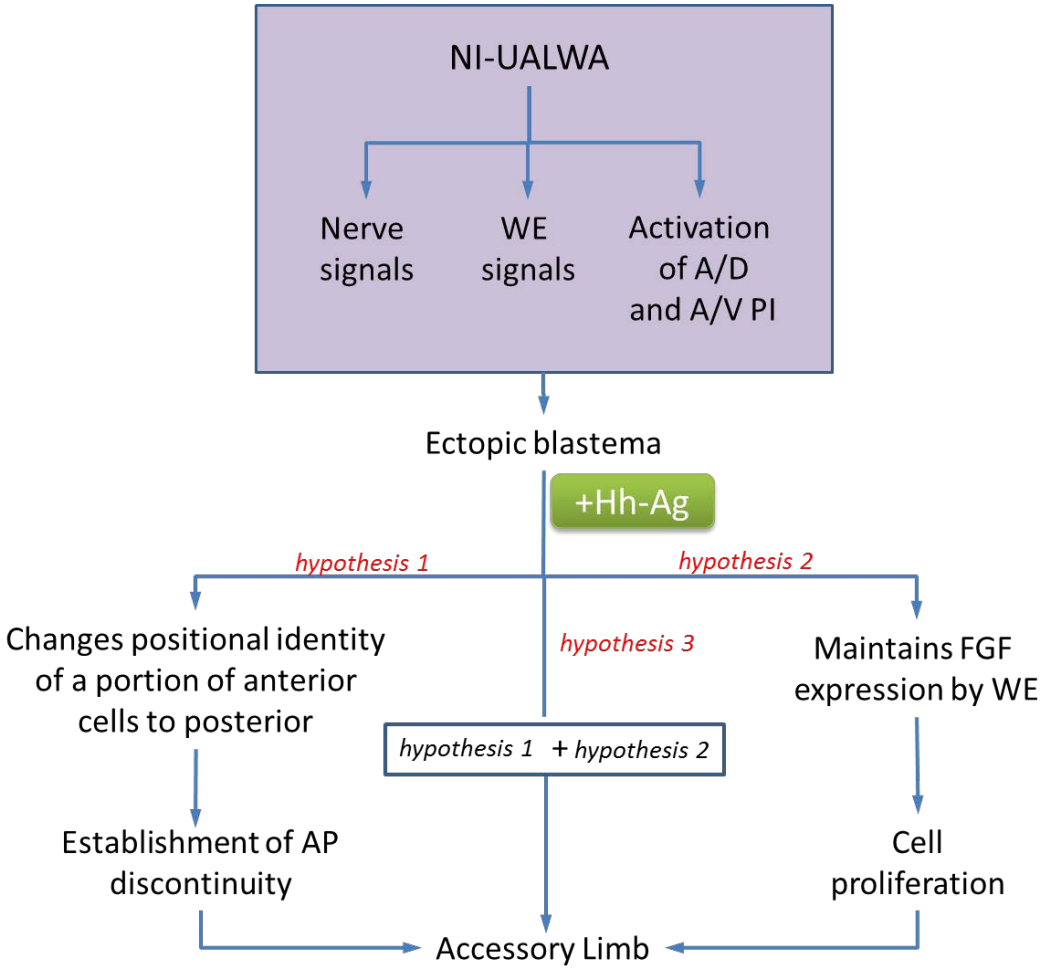


Figure 35 Proposed hypothesis of mechanism of Hh-Ag induction of AL from NI-UALWA

The first hypothesis can be tested in several ways: by performing cellular and molecular assay. As for the molecular assay it is reasonable to check, which positional identity have acquired the cells in the AL, in particular, do they express molecules of anterior positional identity, like Gli3, molecules of posterior positional identity, like dHAND and Shh,

and what is the pattern of FGF expression. This could be done by *in situ* hybridization method. As for the cellular assay it is interesting to test behavior of the cells treated with Hh-Ag. We accomplished this in two ways: by transplantation of AL skin cells and by series of repeated amputation-regeneration of the limb formed in the presence of Hh-Ag and in the inhibition of endogenous Shh.

Second hypothesis can be tested by treating with Hh-Ag **posterior** nerve-induced upper arm lateral wound (NI-UALW**P**). If the second hypothesis is true, then Hh-Ag will maintain FGF expression by WE and AL will outgrow also at posterior side of the limb.

4.5 Testing 1st hypothesis.

4.5.1 AL tissue assay

Our first hypothesis proposes that under Hh-Ag treatment at least a certain amount of the anterior cells acquires a posterior positional identity. If this is indeed so, we assumed, that these cells due to having posterior identity can induce formation of AL if transplanted next to the NI-UALWA, analogically to the Satoh experiment, where posterior skin transplanted to the anterior wound can induce formation of an AL [94].

For this assay skin pieces from circumference of the AL were transplanted by my supervisor to the NI-UALWA of the host animals (Figure 36). This way we were aiming to answer two questions : “does the AL able to regenerate after amputation?” and “does the AL skin piece that originates from anterior limb field but formed under influence of Hh-Ag able to create AP discontinuity and hence induce outgrowth of AL when transplanted onto the host animals next to the NI-UALWA?”

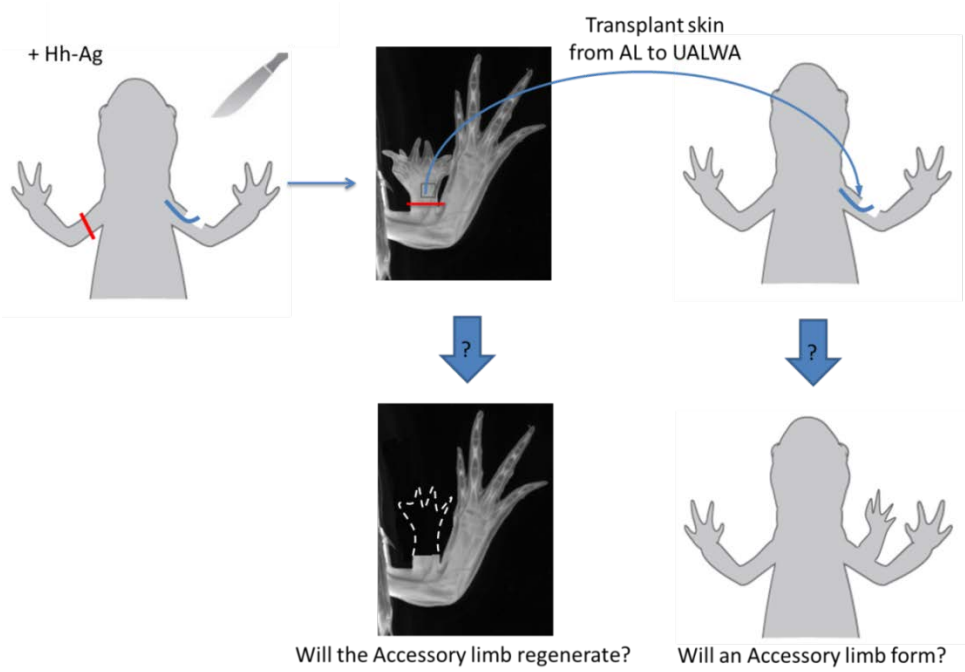


Figure 36 Schematic representation of arising issues and proposed solutions for them. The ALs amputation checks their ability to regenerate. The skin from the amputated ALs transplanted next to the NI-UALWA of the host animals tests its ability to create AP discontinuity and therefore induce formation of AL.

The design of the experiment is present in the Figure 37. For skin transplantation we used 5 AL. Skin from each AL was transplanted to one of the limbs of 2 host animals next to the NI-UALWA. In total we had 12 limbs with NI-UALWA and transplanted skin. The results are present in the Figure 38 Panels A and B.

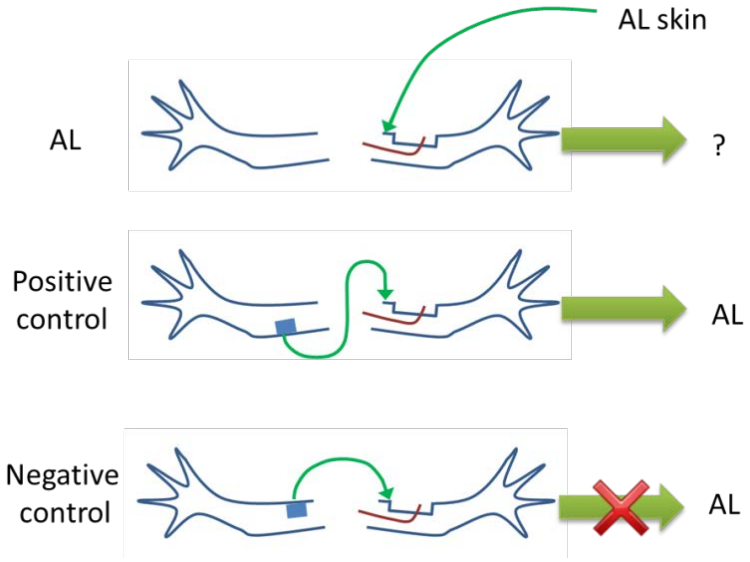


Figure 37 AL skin transplantation experiment design

On all limbs with UALWA and transplanted skin the bump formed on the 7th day after wounding and skin transplantation. 6 bumps disappeared on the 21st day, 4 bumps

reduced in size and 2 were still expanding in width, however none of them eventually resulted in AL outgrowth.

For the negative control we transplanted anterior skin of 4 white animals next to the nerve-induced UALWA on the right limb of 8 host animals. All limbs out of 8 formed bumps on 7th day after injury. In the next two weeks no further growth or reduction of bump has been noticed and none of them resulted in AL (Figure 38, Panel C).

For positive control normal posterior skin was transplanted next to the NI-UALWA on 4 host limbs in total. All of them formed bumps on the 7th day. On the 21st day 3 bumps were still present, and one reduced and disappeared. No AL outgrowth was observed (Figure 38, Panel D).

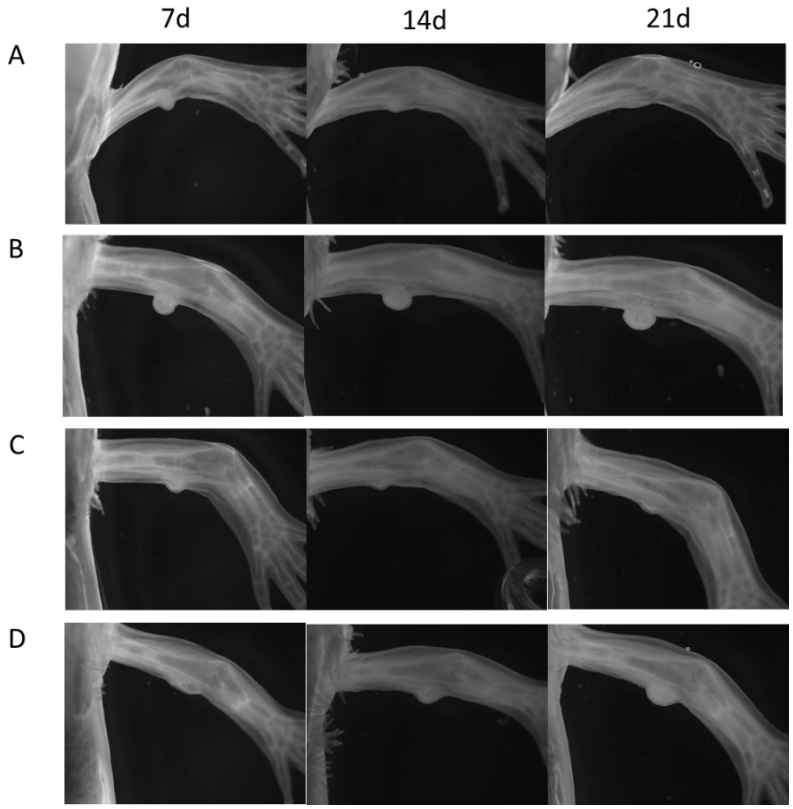


Figure 38. Results of AL skin transplantation. Dark field images of the limbs were taken using stereomicroscope on 7th, 14th and 21st days (7d, 14d, 21d) after wounding and skin transplantation. Panels **A** and **B** represent host animals, on the UA of which a piece of skin from AL was transplanted and NI-UALWA introduced. Panel **C** shows the example limb from negative control group. On the UA of these animals anterior lateral wounds, accomplished with nerve deviation were created and skin from anterior side of donor limb was transplanted next to the wound. Panel **D** represents positive control. 4 limbs were used to create NI-UALWA and posterior skin from donor animal was transplanted to the host limbs with LW.

Since Al formation failed in the positive control, we can't rely on the results of this experiment and draw any conclusions. Possible reason of experiment failure is probably in the complicacy of transplantation technique and weak adhesion of skin graft. Therefore we need to optimize this procedure.

After amputation of AL we let the remaining stump to regenerate for 4 weeks and then collected the whole limbs with AL stumps and stained for skeletal elements (Figure 39).

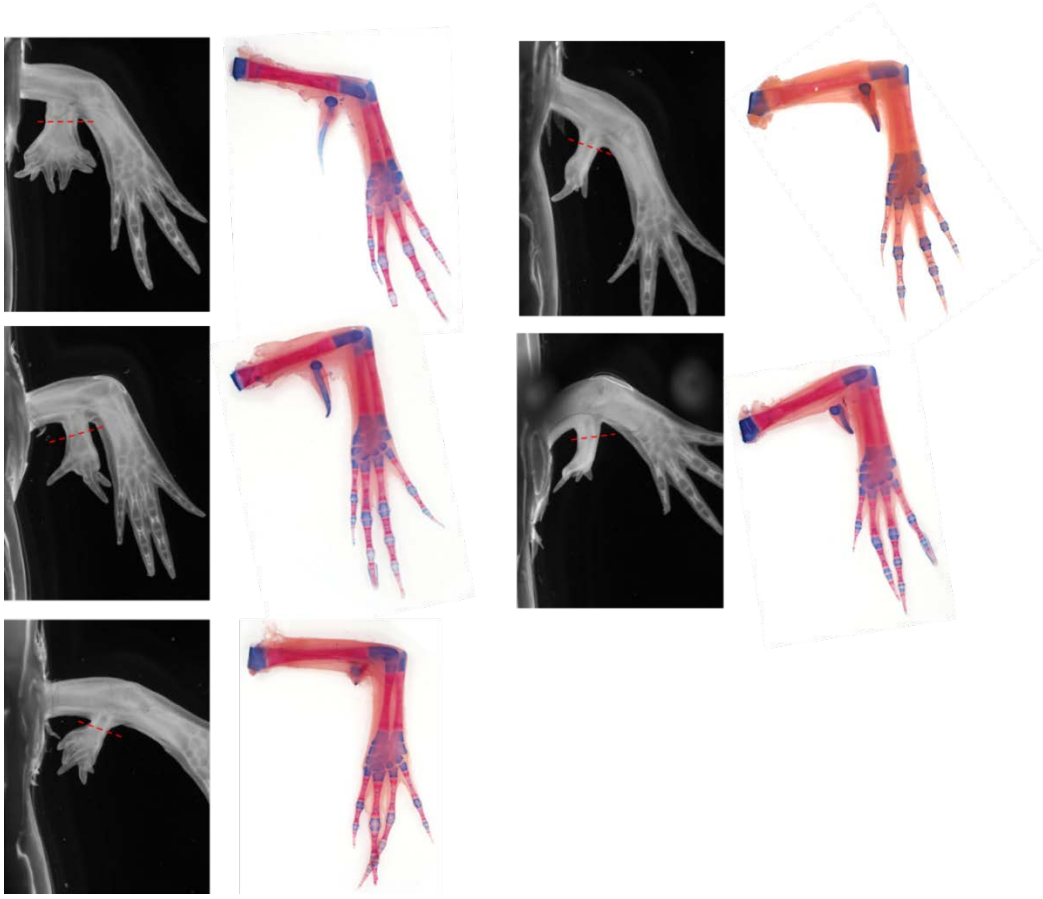


Figure 39 Alcian blue/Alizarin red staining of the limbs 4 weeks later after amputation of AL. Red dashed line depicts the plane of amputation.

At the place of AL we observed formation of spikes instead of regeneration of limb. This could have two explanations. First assumption is consistent with our 1st hypothesis. It implies that all cells, that constitute the AL were posteriorized by the Hh-Ag, thus the AP discontinuity required for maintenance of regeneration was lacking in the stump tissue. Second assumption is consistent with our 2nd hypothesis and implies that AP discontinuity wasn't established by Hh-Ag, instead of that AL outgrowth was induced by maintaining of FGF secretion by WE. In this case after amputation of AL no regeneration could take place, since only anterior cells are present in the stump.

4.5.2 Amputation- regeneration assay

After we have tested Hh-Ag efficiency on the amputated limbs and have inhibited endogenous Shh by cyclopamine the mispatterned malformed forelimbs were observed as a result. It was interesting to see if the cells that undergone a course of Hh-Ag/cyclopamine

treatment can restore normal limb patterning after second amputation or their properties and hence behavior were stably changed by the drug. To explore the nature of Shh influence on the cells we designed two series of experiments named “Branch Hh-Ag” and “Branch cyclopamine”, where we tested if the cell behavior was altered by blocking or activating Shh pathway in the cells.

Branch Hh-Ag

In series of experiment called “Branch Hh-Ag” we amputated axolotl limbs at the shoulder level and treated them with 25 nM Hh-Ag during regeneration period. Depending on the duration of Hh-Ag treatment we divided all animals in two groups: “3-weeks group” and “4-weeks group”. 3-weeks group consisted of 18 animals which were treated with 25nM Hh-Ag for 3 weeks after amputation and 19 control animals which were let to regenerate at normal conditions. After first round of regeneration all these animals were moved to new clean boxes and kept in tap water, which was systematically replaced with fresh to rinse the traces of Hh-Ag. On day 35 mispatterned regenerates were amputated at a lower level then first amputation: left limb was amputated a bit distal of the shoulder, right limb at the wrist level (Figure 40). Then all limbs were let to regenerate for 3 weeks.

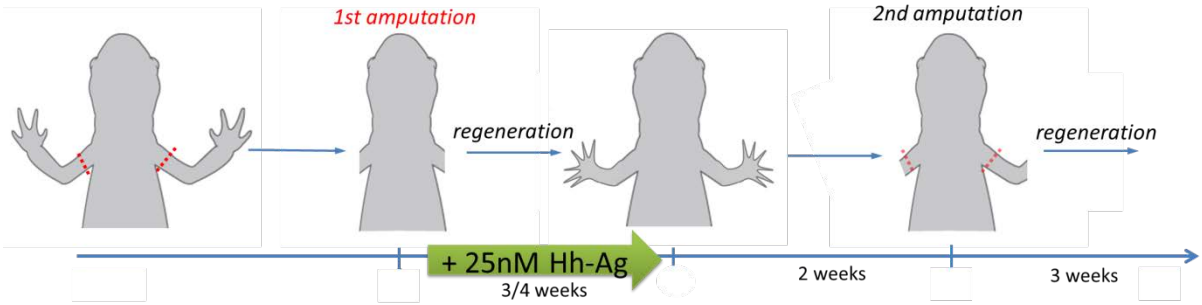


Figure 40. Design of the “Hh-Ag branch” experiment. Amputated at the shoulder limbs were treated with 25 nM of Hh-Ag for 3 /4 weeks. 2 weeks later after termination of drug treatment the regenerates were amputated second time. Left limb was amputated at the UA a bit lower than before. Right limb was amputated at the wrist level.

Second group named “4-weeks” consisted of ten control animals and ten animals which have undergone all the same manipulations as the animals of the group “3-weeks”, except that they were treated with Hh-Ag one week longer.

Amputated regenerates were collected and stained for cartilage elements with Alcian blue stain. After second round of regeneration limbs were amputated at the shoulder level and stained for cartilage and bones with Alcian blue and Alizarin red stains. Results are present in the Figure 41 and Figure 42 for group “3-weeks” and Figure 43 for group “4-weeks”.

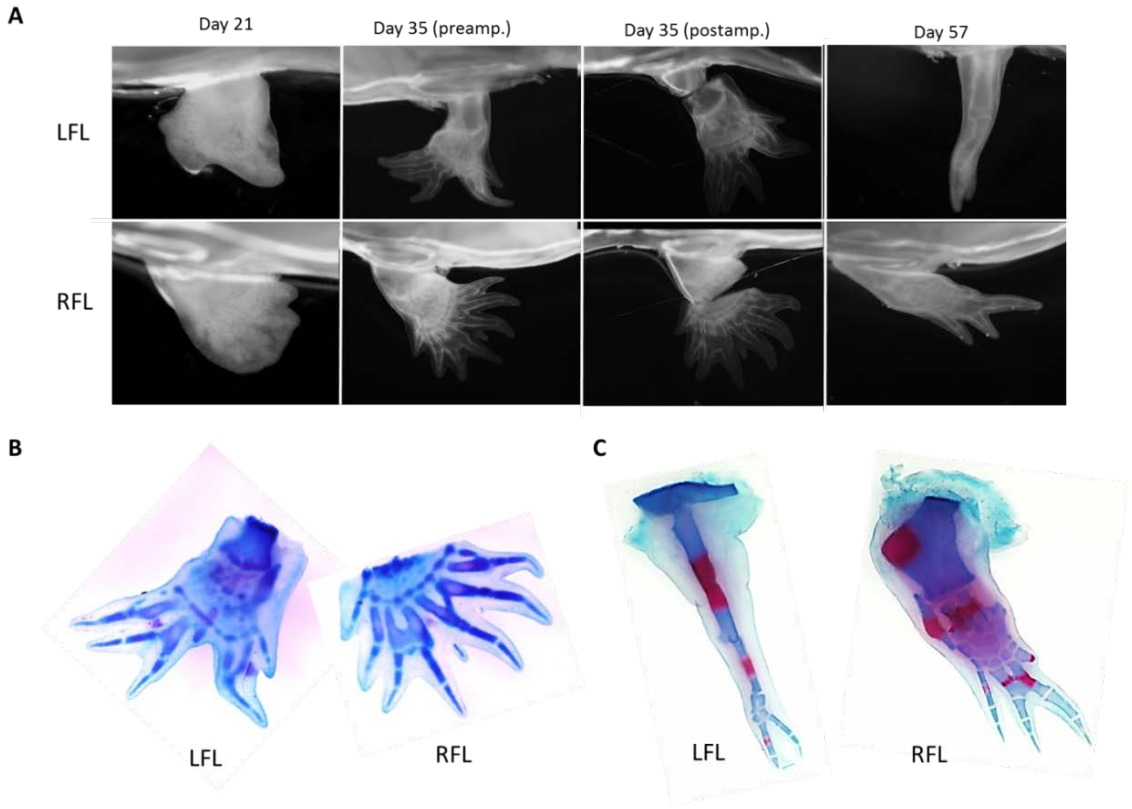


Figure 41 Results "3-weeks group" of Branch Hh-Ag experiment. Panel **A** represents the limbs, which formed after three weeks of Hh-Ag treatment (Day 21). The Hh-Ag treatment was stopped on 21st day. In two weeks (day 35) the limbs were amputated 2nd time. Left forelimb (LFL) was amputated at the UA level, right forelimb (RFL)- at the wrist level. The pictures of 2nd round of regeneration were taken on day 57. Panel **B** represents Alcian blue staining of the limbs, amputated on day 35. Panel **C** represents Alcian blue/Alizarin red skeletal staining of the final regenerates (day 57), where blue color marks cartilage, red color - bone.

As it is clear from the Figure 41 and Figure 42 (Panels A, day 35; Panels B), with the use of Hh-Ag formed abnormal mispatterned and malformed limbs. Panels B represent skeletal staining of these limbs. Both left and right limbs have grown additional digits, the upper arm segment was shortened as well as the lower arm bones, which were difficult to distinguish but for sure exceed normal amount of two bones (one radius and one ulna). Some digits fused with each other or bifurcate on the tip. In the control group (Figure 42, Panels D and E) all limbs had normal patterning. Thus, Hh-Ag affects normal lower arm and hand regeneration and impairs AP patterning.

After flushing of the system from Hh-Ag by keeping animals in tap water for 2 weeks (Figure 40) and amputation of malformed regenerates at a lower level the remaining in the stump cells, portion of which was influenced by Hh-Ag couldn't restore normal patterning of the forelimbs. The example limbs resulted from the second round of regeneration are present in the Panels A (day 57) and Panels C.

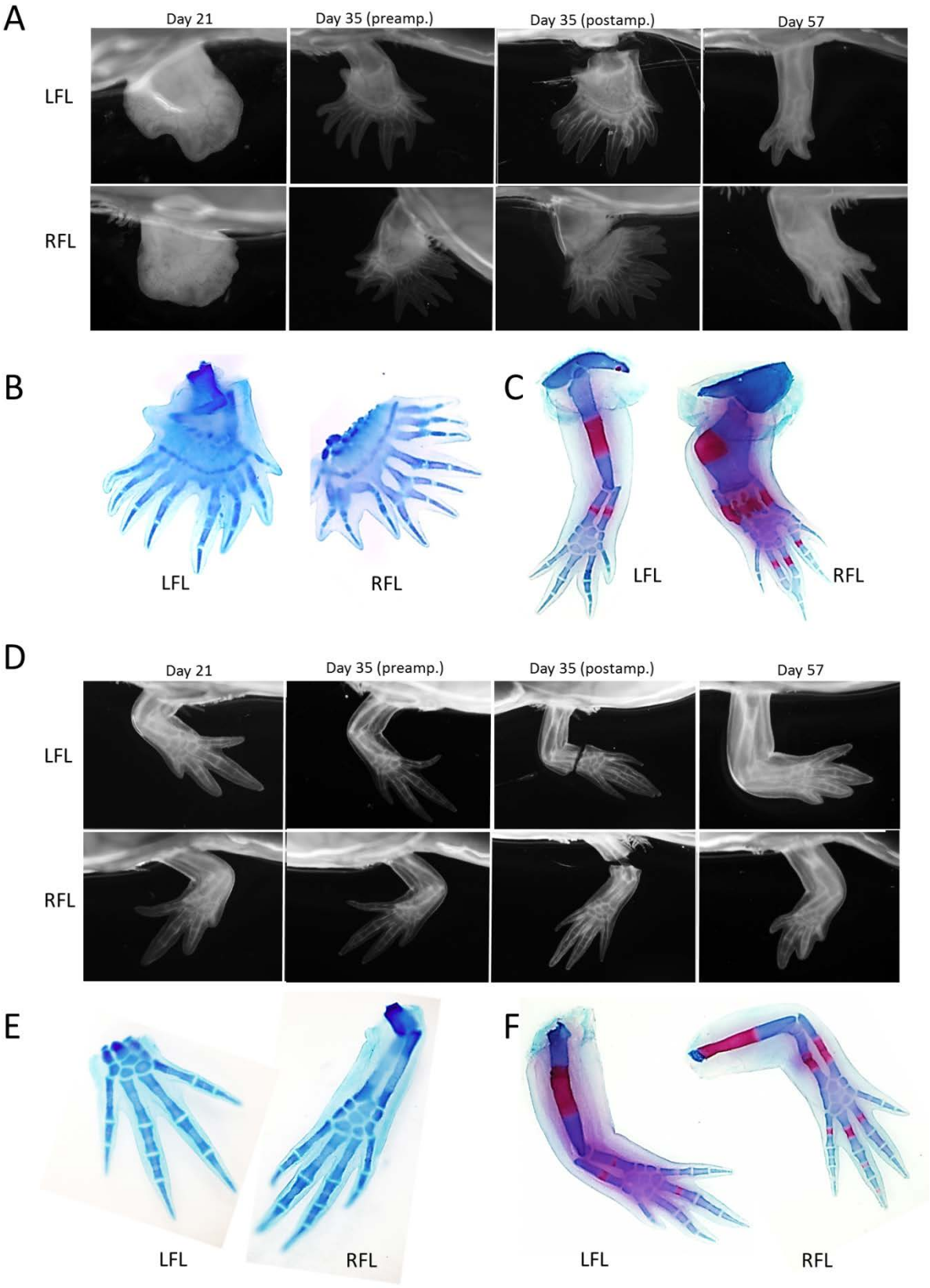


Figure 42 Results„3-weeks group". Panels **A**, **B** and **C** represent another example of limbs, treated with Hh-Ag. Panels **D, E** and **F** represent control limbs. LFL-left forelimb, RFL-right forelimb. Panels **B** and **E** represent Alcian blue staining of the limbs, amputated on day 35. Panels **C** and **F** represent Alcian blue/Alizarin red skeletal staining of the final regenerates (day 57), where blue color is the cartilage, red color - bone.

All right forelimbs (RFL), that regenerated after amputation at the wrist level showed similar structure. In the lower arm of these limbs up to five bones were formed (in presence of Hh-Ag), based on morphology they are supposedly one radius and several ulnas. After second amputation through numerous carpal bones the hand regenerated again abnormally. Digit number was ranging from 2 to 5. Some digits fused with each other, some lacked phalanges. The limbs that were amputated at the UA level (left forelimbs, LFL) formed one to two short digits, reduced number of carpal bones and one LA bone, based on morphology we suspect it is ulna,. This indicates that they lack anterior structures. Three LFLs made and exception by restoring absolutely normal skeleton after second amputation (Figure 42, C) comparable with control limb skeleton (Figure 42 F). This exception probably occurred because of shortening of humerus during Hh-Ag affected regeneration, what made it difficult to determine the level which is distal than the first amputation level. In this case regeneration proceeded from cells that were mature during Hh-Ag treatment period and it is possible that Hh-Ag can posteriorize only blastema cells.

In the 4-weeks group we got similar results (Figure 43).

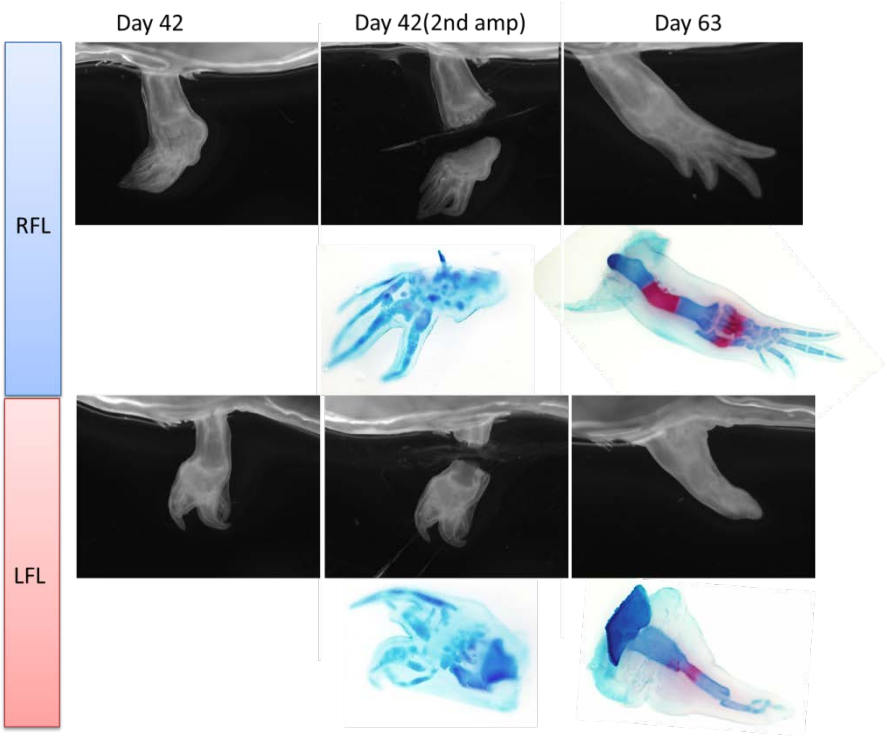


Figure 43 „4-weeks group“. Amputated at the shoulder level limbs were treated with Hh-Ag 25 nM for 4 weeks. 2 weeks after termination of Hh-Ag treatment dark field images were taken and limbs were amputated 2nd time. LFL was amputated at UA level a bit distal then before. RFL was amputated at the wrist level. Amputated limbs were stained for skeletal elements.

In the right forelimb skeleton we observed up to 4 lower arm bones, formed in the presence of Hh-Ag. After second amputation at the wrist level 2 to 4 digits were formed, obviously due to already existing morphology (numerous lower arm bones) (Figure 43, RFL). Left limb, that was amputated at the upper arm level second time regenerated only a few structures, more likely of posterior character (Figure 43, LFL), or didn't regenerate at all (not shown).

Concluding from all these results, we assume that Hh-Ag have changed the identity of the cells, by influencing some cell property. This resulted in altered behavior of affected cells even after termination of Hh-Ag treatment. Perhaps the ratio of anterior and posterior cells in the regenerate was shifted towards predominance of posterior cells upon Hh-Ag treatment and caused formation of the limbs consisting of mostly posterior bones, as in the left limb case. Upon long term Hh-Ag treatment (4-weeks group) all cells of the limb could theoretically be posteriorized, thus causing the arrest of regeneration, since no AP discontinuity will exist after amputation of such limb. That possibility explains why several limbs in the 4-weeks group didn't regenerate after 2nd amputation.

Branch Cyclopamine

Exploring the role of Shh in defining posterior identity we also performed a series of experiments where we inhibited Hh-pathway by cyclopamine drug and observed how the cell behavior and regeneration have changed. In previous studies [110] it was demonstrated that the inhibition or loss of Shh signaling induces defects in the anterior-posterior pattern of axolotl regenerating limbs. Earlier we used cyclopamine in concentration 600 nM and observed complete block of regeneration. We decided to reduce concentration to get any outgrowth and used 300 and 200 nM cyclopamine.

As a first step LFLs and RFLs of 29 animals were amputated at the shoulder level. Ten animals were treated with 300nM cyclopamine for three weeks, nine animals with 200nM cyclopamine for the same period of time and the rest ten animals were used as a control and got DMSO (solvent for cyclopamine) into water during three weeks. After that we left all animals in the new boxes in normal tap water, which was changed approximately twice a week to flush the system and wash out all traces of drug (Figure 44).

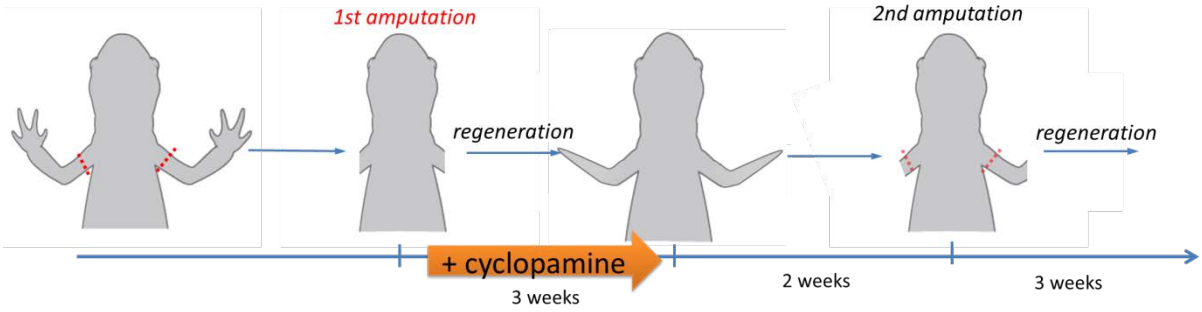


Figure 44 Design of experiment „Branch cyclopamine". Amputated at the shoulder limbs were treated with cyclopamine (200 and 300 nM) for 3 weeks. 2 weeks later after termination of drug treatment the regenerates were amputated second time. Left limb was amputated at the UA a bit lower than before. Right limb was amputated at the LA/wrist level.

Five weeks after 1st amputation on day 39 we observed formation of spikes in the group, treated with 300 nM cyclopamine and split spikes in the 200 nM group (Figure 45, A).

The spikes were amputated second time at different levels: LFL at the UA level, a bit distal then the level of first amputation; RFL at the lower arm level or wrist (due to modified by cyclopamine limb anatomy it is hard to define this structures in regenerate). Then the limbs were left to recover in normal conditions (i.e. tap water).

Three weeks later we observed completely different patterns of left and right limb regeneration. Left limb which were amputate through the UA in both 300nM and 200nM groups formed absolutely normal limbs, compared to the control limb (Figure 45, Panel A, 60dpa column).

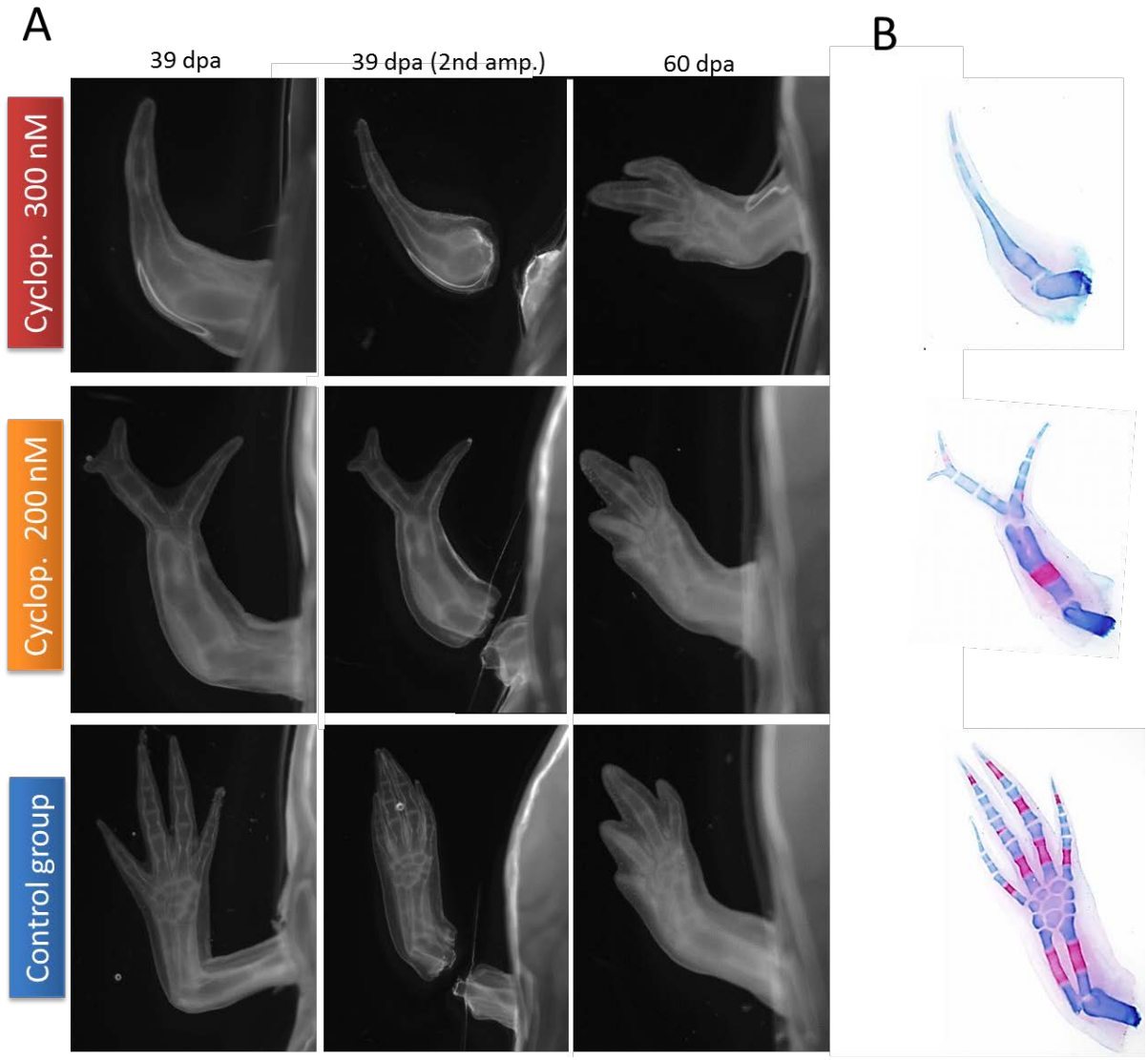


Figure 45 Results of "Branch cyclopamine" experiment. Left limb regeneration. Panel **A** represents left limb from the groups treated with 300 nM cyclopamine (upper row) and 200 nM cyclopamine (middle row) for three weeks and left limb from control group (lower raw). The pictures of regenerates were taken on 39 day post amputation (39 dpa) and amputated second time at the UA level. Panel **B** represents skeletal elements of amputated on day 39 regenerates (upper and middle images correspond to 300 nM and 200 nM group respectively, lower image represents control limb)

Our assumption is that Hh-pathway does not play role in the determination of upper arm structures, thus no cells in the UA were affected by cyclopamine treatment and hence normal regeneration followed after amputation at the UA level. However another explanation is possible. The regeneration from the UA level takes longer, thus even if a few cells still expressing Shh were present in the UA, they could be able to compensate AP patterning, whereas the regeneration from the wrist or LA level takes much less time which might be not enough for compensation of posterior structures.

As for the left limb, the whole picture looks a bit more complicated. In the presence of cyclopamine just like the left forelimb right limbs formed spikes in group treated with 300nM of drug and split spikes in the 200 nM group (Figure 46, 39dpa).

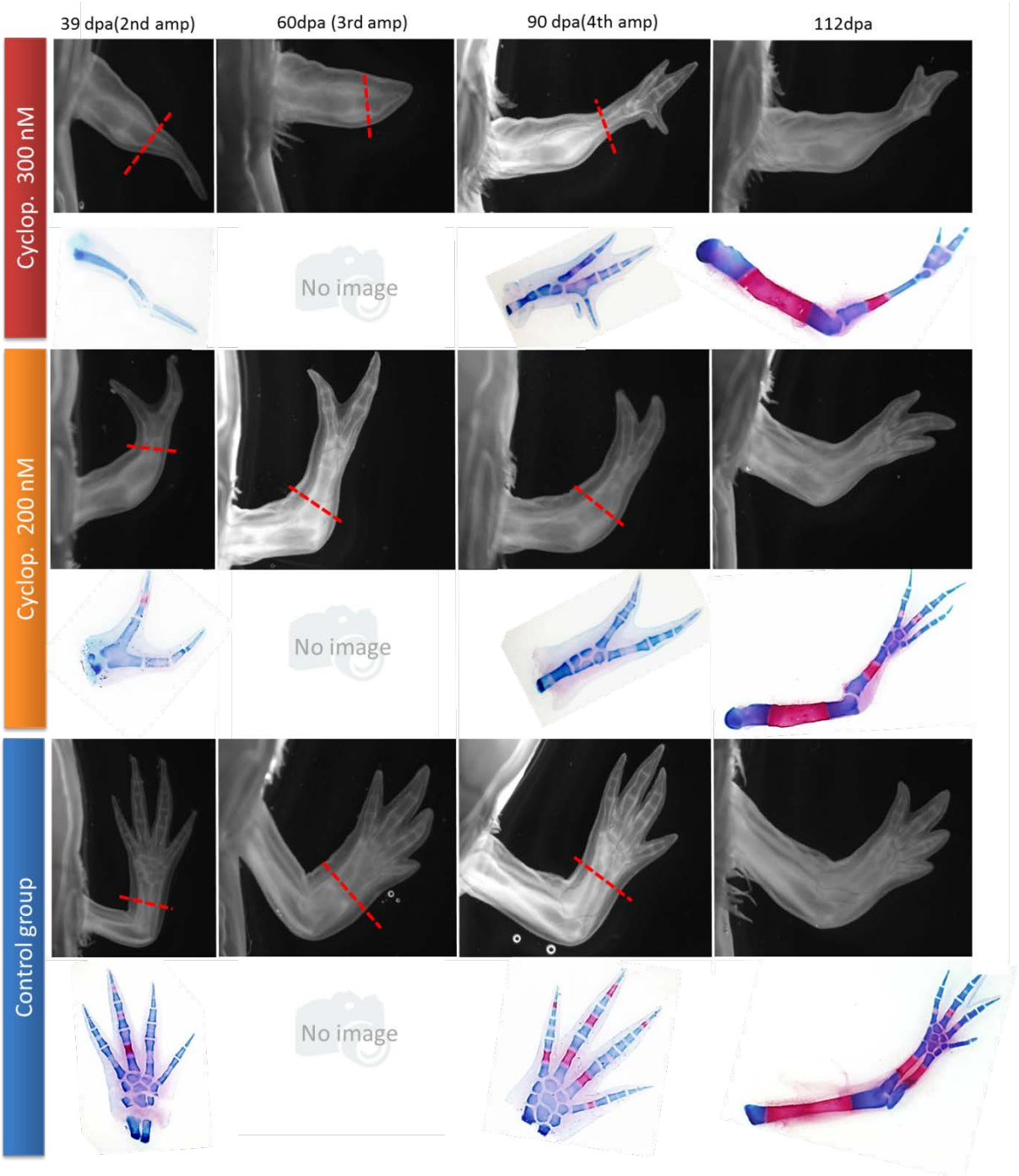
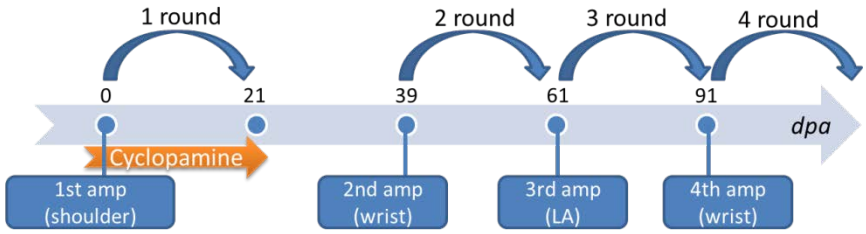


Figure 46 Results of "Branch cyclopamine" experiment. Right limb regeneration. Red dashed line shows the level of amputation. " 2nd amputation was performed a bit proximally from the wrist. 3rd - at the mid upper arm level. 4th – at the wrist level. Amputated limbs stained with Alcian blue/Alizarin red located beneath the dark field image of the same limb.

The spikes were amputated 2 weeks after termination of cyclopamine treatment a bit proximally form the wrist (Figure 46, 39 dpa, red dashed line). In contrast to left limbs, right

limbs again formed spikes after second round of regeneration (Figure 46, 60 dpa). We proposed that in the inhibition of endogenous Shh, caused by cyclopamine cells had lost posterior identity signaling. In order to find out, whether the compensation of AP patterning can occur, we repeated amputation-regeneration rounds two times, which makes in total 4 amputations of the right limbs (Figure 47, A). Figure 46 demonstrates examples of two last regeneration rounds in the columns 90dpa and 112dpa.

A



B

	animal	regeneration round			
	N	1	2	3	4
Cyclop. 300 nM	1	spike	normal	-	-
	2	spike	spike	4digits	4digits
	3	spike	spike	split spike	3 digits
	4	spike	spike	split/3 digits	3 digits
	5	spike	spike	4digits	4digits
	6	spike	normal	-	-
	7	spike	spike	split spike	split spike
	8	spike	spike	split spike	split spike
	9	spike	spike	split spike	spike
	10	spike	spike	3 digits	3 digits
Cyclop. 200 nM	1	split spike	split spike	4digits	4digits
	2	split spike	split spike	4digits	4digits
	3	split spike	split spike	4digits	4digits
	4	split spike	spike	3 digits	3 digits
	5	split spike	split spike	3 digits	3 digits
	6	split spike	spike	3 digits	3 digits
	7	split spike	split spike	split spike	3 digits
	8	split spike	split spike	4digits	4digits
	9	split spike	spike	split spike	3 digits

Figure 47 **A**. Timeline of cyclopamine treatment and right limb amputation events. **B**. Table of results from each round of regeneration of right limbs.

From the table in Figure 47, B it is noticeable that pattern of regeneration is inconsistent in every round after termination of cyclopamine treatment and perhaps also depends on drug concentration. Due to diversity of regeneration patterns it is hard to draw any conclusions from this experiment. However, we formulated three assumptions.

1. It is possible that Shh does not play role in the specification of cells in the UA (as it was proposed in development), thus all limbs amputated second time at the UA level regenerated normally. Moreover, in case of LA amputation performed at the relatively proximal level – a certain number of UA cells could contribute to the blastema and

result in partial compensation of posterior structures. This explains why in the second round of regeneration, where limbs were amputated far from the UA, majority of them formed spikes or split spikes (Figure 47, B). When those spikes were amputated more proximally (third round of regeneration) much more posterior structures were formed from the remaining stumps, possibly because of the influence of the UA cells.

2. Our second assumption implies that the main factor, which determines the behavior of cells, is the time during which a certain cell experienced cyclopamine effect. Taking in account, that proximal blastema cells differentiate earlier, the cells that have eventually composed distal structures could be exposed to cyclopamine for a much longer time, than proximal cells. Thus, the more proximally the cell is located, the more it can compensate the posterior structures.
3. Our third proposal considers the time necessary for compensation to happen. Possibly all cells along the PD axis are equally capable to compensate posterior structures, but since regeneration from different amputation levels takes different time and the rate varies a bit for different animals we observe different extent of compensation events. This explains such a big diversity of regeneration patterns.

All in all, we think that cyclopamine indeed changed cells' behavior, what supports the hypothesis that Shh-mediated cell specification occurs in sense of stable cell property, however, our results are not conclusive, since they could be interpreted ambiguously.

4.6 Testing 2nd hypothesis. Hh-Ag in the NI-UALWP.

According to our 2nd hypothesis Shh acts through maintaining FGF expression in the wound epidermis, as in development Shh was shown to promote FGF expression [55]. If this is true, than Hh-Ag should maintain regeneration on any side of the limb. To test this assumption my supervisor made NI-UALW on the posterior side of the limb. These animals were treated with Hh-Ag in concentration 10 nM for three weeks. However, only 6 limbs out of 18 formed little bump, which eventually regressed and disappeared (Figure 48).

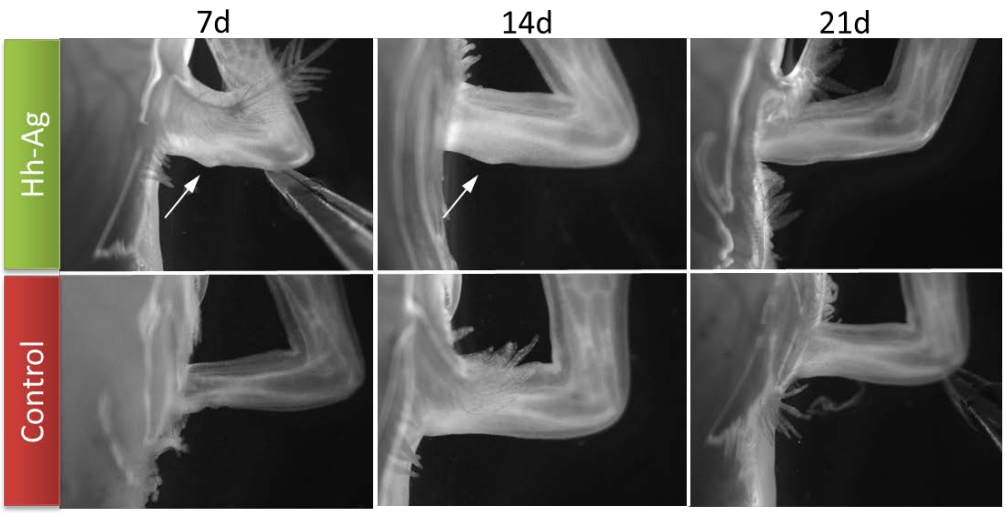


Figure 48 Hh-Ag effect on the posterior nerve-induced lateral wound. Posterior wound was created on the upper arm of the axolotl forelimb. Right after wounding the animals were treated with Hh-Ag in concentration 10nM for three weeks. Only some animals formed small bump, which eventually regressed.

The most possible reason is that signals from nerves didn't achieve necessary threshold, and hence ectopic blastema was not formed properly. This experiment have to be repeated with increased amount of nerves deviated to the wound to allow us drawing any conclusion.

4.7 Problems with reproducibility of AL results

To get more ALs for further structural and molecular analysis we repeated experiment where we treated NI-UALWA with 10 nM Hh-Ag for three weeks and got 6 ALs in the end.

I and my supervisor created UALW-A on 45 animals. Ten of them we treated for 3 weeks with 25 nM Hh-Ag and 27 with 10 nM of Hh-Ag also during 3 weeks after injury. Unfortunately we observed very low percentage of AL formation among all these animals. 17 animals treated with 10nM formed bumps. Three of them have grown into AL and one into spike. In the group of animals treated with 25 nM Hh-Ag 6 formed bump and one of them grown into AL. (Figure 49)

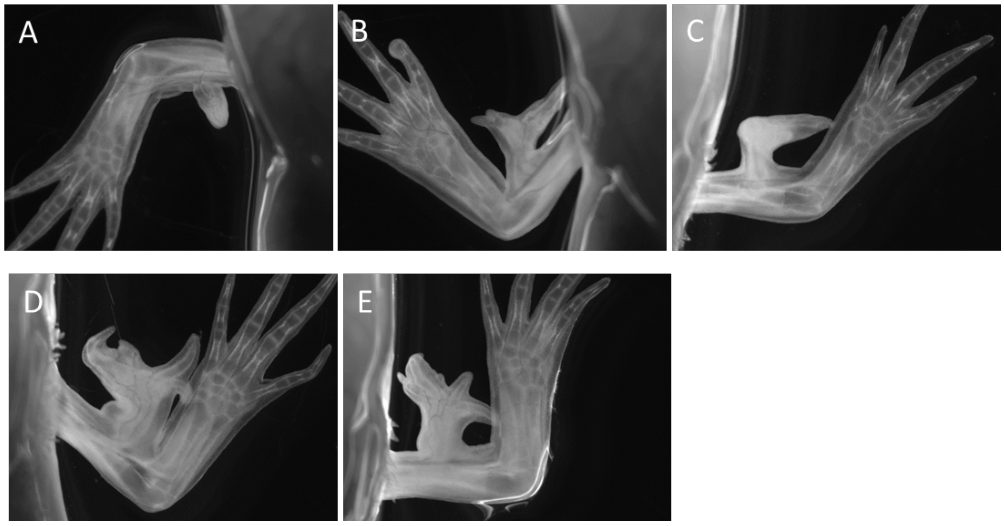


Figure 49 Accessory limbs. A-D formed upon 10 nM Hh-Ag treatment. E formed upon 25 nM Hh-Ag treatment.

Our assumption was that the exact place of lateral wound was slightly different in the last experiment. When first ALs were obtained the wound located a bit ventrally and distally, closer to the elbow, while on second batch of animals the wound was shifted proximally and dorsally. It is known that Shh comes up late in regeneration it is thought to be involved in LA and hand patterning, but not UA: Thus, close proximity to the LW could play crucial role for AL growth. Another assumption was that the size of wound plays role in AL formation.

In attempts to find out what was the exact reason of low frequency of AL outgrowth together with my supervisor we created UALW-A on the next batch of animals. Ten animals were used to create relatively small shifted a bit ventrally wound closer to the elbow. These animals were kept for 3 weeks in Hh-Ag 10 nM. Twelve animals were used to produce relatively big wound also in close proximity to the elbow and were kept for 3 weeks in Hh-Ag 10 nM. For each type of wound we also created some extra animals for control and kept them in normal tap water.

Surprisingly we again observed numerous bumps but only a few outgrowths (Figure 50).

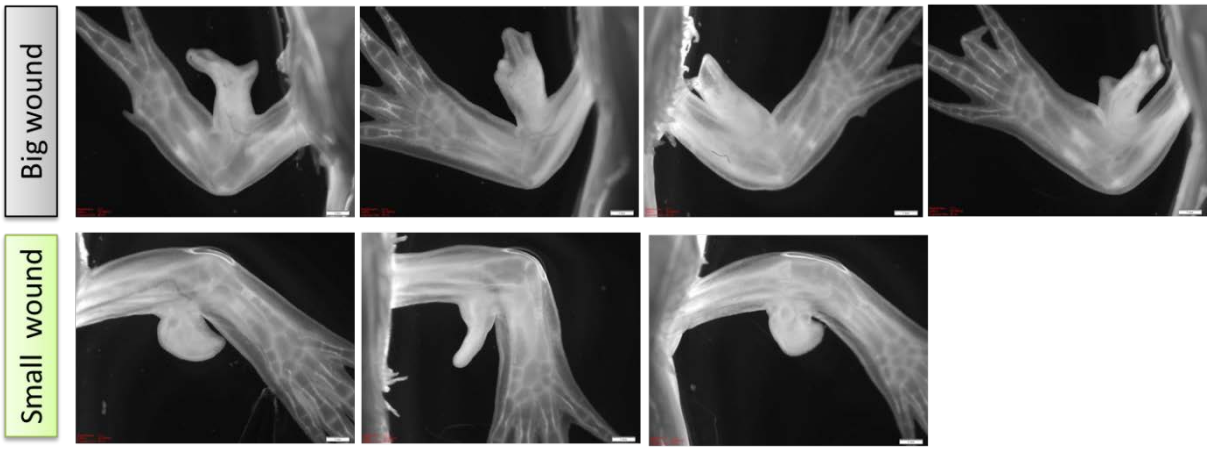


Figure 50. AL formed in the groups with big and small UALW-A

Taking into account that during the more or less successful experiment with AL outgrowth animals were fed on the light for 1-3 hours a day before putting into fresh solution and during later experiments they were always kept in dark– the next assumption was that the light could decrease the concentration of Hh-Ag in solution. As the next step we reduced concentration of Hh-Ag to 5 nM and kept animals in absolutely dark place in all the same other conditions. Unfortunately it didn't work, and instead of AL formation we observed only 5 thickened stable bumps and one spike out of 14 limbs with UALW-A of middle size, located distally (Figure 51). The other limb of every animal that was amputated at the UA level and used as a positive control showed evident mispatterning, indicating that Hh-Ag is working.

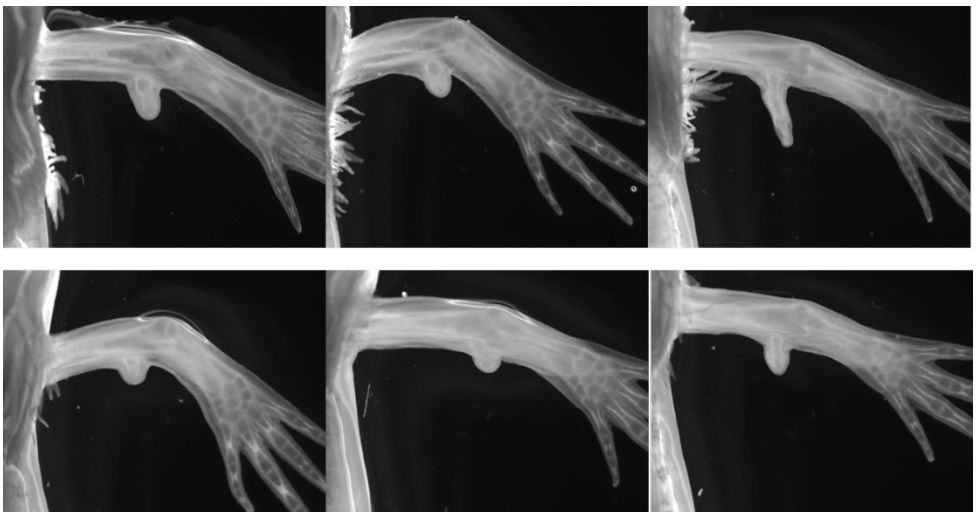


Figure 51 Bumps, grown upon 5 nM Hh-Ag treatment.

Our feeling in concern to this experiment, that Hh-Ag in concentration 5 nM was able to induce more bumps, which as we think could eventually result in AL, than in concentration 10 nM. However, due to unknown reason at a certain time point the efficiency of Hh-Ag

dramatically diminished or it was completely deactivated. This led to the arrest of bump progression, differentiation of blastema cells and thickening of the formed protuberance. However the influence of Hh-Ag on the amputated opposite limb was already enough to induce strong mispatterning. For more reliable data this experiment has to be repeated more accurately.

5. Discussion

In this study we aimed to detect the substances, able to induce outgrowth of ectopic accessory limb from the anterior nerve-induced lateral wound. Based on the AL model proposed by Endo and Satoh experiments, where they transplanted posterior skin to the NI-UALWA and got AL formation, we assumed that morphogenes, involved in AP patterning could be considered as the potential candidates. From the study of developmental limb patterning it was already known that Sonic Hedgehog is involved in specification of posterior structures of lower arm and formation of digits [48], [49], [50], [51], [52]. Its upstream regulator dHAND was shown to be the earliest player in the establishment of antero- posterior asymmetry [41]. We proposed that AP discontinuity at the injury site could be established under influence of these two molecules (Shh or dHAND) and result in AL outgrowth.

Since the dHAND gene is more than 90% conserved among vertebrate species, its function was proved in chicken and mouse, and its presence was shown in Xenopus [114], we postulated that dHAND might have the same function in regeneration as in development. We used in vivo electroporation method to deliver plasmid containing dHand into amputation blastemas and nerve-induced ectopic anterior bumps. From our results we can not conclude whether dHAND is able to establish AP discontinuity or not. Apparently the plasmid we used for electroporation is not working properly or even is not expressed in the cells. In support to this the early indications of LA and UA regeneration blastemas electroporated with dHAND do not show any signs of AP mispatterning and look normally. To test if dHAND is expressed in electroporated cells it is reasonable to look at downstream markers of dHAND. The *dHand* could be cloned into another vector and experiments could be repeated with new plasmid.

In our study we showed that activation of Shh-pathway is capable to induce an ectopic limb. Shh plasmid electroporation results look promising; however by this technique we didn't get an AL formation. The electroporation technique has to be further improved to achieve more precise and effective targeting of the cells in the ectopic bump, and perhaps different concentrations of Shh plasmid have to be tested. Hh-Ag treatment resulted in outgrowth of the limb consisting of the hand, digits and the structure, that seems to be the lower arm, but lacks upper arm components. This indicates that Shh is involved in patterning of only distal structures as it was earlier hypothesized.

We proposed that Shh could induce accessory limb formation by two mechanisms or their combination: switching positional identity of the cells from anterior to posterior by affecting cell surface properties and maintenance of FGF expression by WE.

To test first hypothesis we observed behavior of regenerates, treated with Hh-agonist and antagonist. In support of the first hypothesis we observed that regenerates, formed in presence of Hh-Ag or Hh-pathway inhibitor have retained some altered features even when drug treatment was terminated. Evident limb malformation was a result of regeneration originating from drug treated cells.

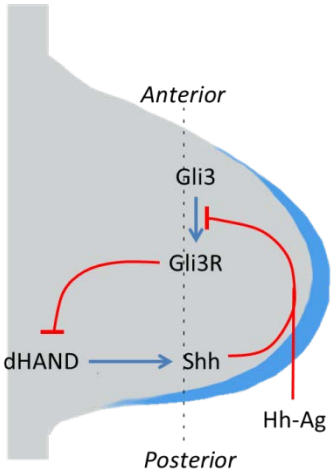


Figure 52 Proposed mechanism of molecular interactions in the regeneration blastema.

It was already shown that Sonic hedgehog (Shh), produced posteriorly, diffuses across the bud and prevents processing of full-length Gli3 protein to a short repressor form (Gli3R) [115]. Recently in developmental studies it was establish that GLI3-mediated transcriptional repression is crucial for restricting dHAND expression to the posterior mesenchyme concurrent with restriction of the competence to activate SHH signaling. Experimental evidence suggests that GLI3 might directly repress Hand2 transcription in early limb development [116]. Here we propose that in regeneration similar interaction could occur (Figure 52). Shh signaling attenuates Gli3 repressor production enabling the maintenance of *Hand2* expression, which in turn could be responsible for acquiring by the cells of posterior identity.

However, our results are not conclusive and could be interpreted ambiguously, thus further experiments have to be performed.

To test second hypothesis we tested if Hh-Ag is able to induce AL from the posterior nerve-induced lateral wound. For that we performed only one experiment, where we treated with Hh-Ag animals with NI-UALWP for three weeks. Unfortunately this experiment failed due to wounding technique. Apparently nerve signals didn't achieve threshold necessary for ectopic blastema formation. This experiment has to be repeated with deviation of additional nerves to the posterior wound.

As a future perspective in exploring the mechanism of AL formation it would be interesting to see what influence will make the FGF when added to the NI-UALWA: Also the dHAND ability to induce ectopic limb has to be tested. It will be interesting to add these molecules and Shh in different combinations to the NI-UALWA and observe their summarized effect.

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