Charles University
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Hierarchical levels of organization and pattern formation during amphibian limb regeneration

Hierarchické úrovně patrností a organizace během regenerace končetiny obojživelníka

Bachelor´s thesis

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Poděkování:

Ráda bych poděkovala svému školiteli za rady při vypracovávání práce. Také děkuji své rodině za neutuchající podporu, především své sestře, která mě podporovala z domova, i ze zahraničí. Dále nemohu ani zapomenout na mé přátele, hlavně na důvěru v mou práci od kamarádky Laury.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně za použití literatury a informačních zdrojů, na základě konzultací se svým školitelem. V práci jsou uvedeny všechny zdroje a neobsahuje text vygenerovaný umělou inteligencí. Tato práce nebyla zpracována k získání jiného či stejného akademického titulu.

V Praze, 7. 8. 2024 Gabriela Klézlová

Abstract

Nucleic acids are considered to be the principal information polymers, yet from genetic information alone, it is impossible to predict the outcome of morphogenesis. Even as our knowledge of biochemical signaling expands, the regenerative mechanisms reconstructing the amphibian limb demand, in addition to the established morphogen model, a more complex framework. In contrast to limb development in regeneration cells' lineage and in the case of connective tissue-derived cells, even positional memory is maintained. The intention of my thesis it to explain the instructive cues provided by the properties of limb tissue at a steady state, such as epigenetic modifications, membrane-bound proteins, composition of extracellular matrix, and mechanical signaling. Cell plasticity is required for limb regeneration, but the precise reproduction of the structure depends on the conservation of pattern. Connective tissue-derived cells retain and relay the positional information to their progeny, and through the expression of surface molecules, the cells sort within the tissue. These superior pattern-forming cells communicate with the pattern-following cells and thus constitute a structured system.

Keywords:

positional information, pattern formation, positional memory, limb regeneration, epigenetic profile, connective tissue cells

Abstrakt

Ačkoliv je přisuzována nukleovým kyselinám role hlavních informačních polymerů, je nemožné na základě jejich sekvence samotné předvídat konečný výsledek morfogeneze. Proces regenerace končetin obojživelníků čerpá z rozšiřujícího se množství vědomostí o signalizačních molekul, jeho objasnění však vyžaduje komplexnější pojetí, které se nezakládá pouze na učení o morfogenech. Od vývoje končetiny se její regenerace liší tím, že buňky si zachovávají paměť o linii, ze které pochází, a buňky původem z pojivové tkáně dokonce uchovávají poziční informaci. Ačkoliv schopnost dediferenciace je nezbytná, nepodává instrukce k rekonstrukci vzoru končetiny. Má práce podává přehled o postoji, který vysvětluje vznik struktury při regeneraci končetiny na základě vlastností neporušené tkáně. Mezi tyto vlastnosti patří epigenetické modifikace, povrchové proteiny, složení mezibuněčné hmoty a a signalizaci využívající mechanických stimulů. Konečná morfologie regenerace končetiny u obojživelníků je zajištěna výsadně postavenými buňkami, které disponují poziční informací a předávají ji dceřinným buňkám. Na základě exprese povrchovým molekul se tyto buňky třídí. Buňky původem z jiných tkání, než pojivové zaujímají místo buněk následujících pokynů výše postavených buněk, jejichž vzájemná komunikace tvoří víceúrovňový systém.

Klíčová slova:

poziční informace, patrnost, poziční paměť, regenerace končetiny, epigenetický profil, buňky pojivové tkáně

Table of Contents

1 Introduction

Regeneration is a morphogenetic process that has been a subject of great fascination for scientists and common people alike. The captivation by the ability to restore lost body parts dates back to ancient civilizations, with myths of Prometheus and the Aztec god Xolotl, and continues to manifest in modern culture through the popularity of characters such as Wolverine or BBC's Doctor Who. While the latter fictional specimen undergoes a drastic change in appearance and morphology, the factual process involves creating a precise replica of missing structures.

The term regeneration traditionally refers to the replacement of lost or damaged tissues and organs and is a widespread phenomenon across the phylogenetic tree of life. However, tetrapods possess only very limited regenerative capacity, with amphibians, especially urodeles, being a striking exception as they are able to regenerate complex three-dimensional structures such as limbs.

Amphibians undergo a type of regeneration called epimorphosis; in other words, epimorphic regeneration differs from other types by reconstruction of a lost body part by the proliferation of dedifferentiated cells, which form a tissue known as blastema[1]. After dedifferentiation and cell division, this seemingly uniform pool of progenitor cells diversifies to reconstitute a replica of missing structures. To form an adequate proportion and pattern, limbs must be patterned along three axes – anterior/posterior (A/P), dorsal/ventral (D/V) axis, and proximal/distal (P/D) axis. Cells' position along each axis designates their function within the limb for which the term positional identity has been adopted. Two principles have been deemed significant for restoring the limb's constitution defined by positional identities - the rule of distal transformation and intercalation[2–5].

The rule of distal transformation describes a constant phenomenon that takes place after limb amputation that shows that only structures whose position is more distal relative to the amputation plane are regenerated[2]. The second one, intercalation, states that when tissues and their cells possess conflicting positional identities, such as when they are not found close to one another in an undamaged limb, their contact induces a morphogenetic conflict, which is a situation during which the form is restored by generating intermediate positional identities until there is all disparity is resolved[2,6,7].

To intercalate a positional identity has been thought to depend on a quality linked to cells[5,6]. Limb development, however, is in contrast to limb regeneration and has primarily been interpreted morphogenesis as a result of interaction among diffusible molecules such as retinoic

acid, sonic hedgehog, and fibroblast growth factors, and these two processes are often compared and likened to one another[8]. This close comparison omits the fact that regeneration is extremely context-dependent, unlike limb development, which operates with a hard-wired body plan and patterning field. Limb regeneration differs from development in the aspect of extensive remodeling of the disrupted tissue, which shows more resemblance to maintenance of tissue homeostasis as both homeostasis and regeneration compensate for the need to accommodate for the influence of an organism's environment[9].

Lastly, regeneration is commonly compared with cancer, which is marked by a lack of functional tissue organization and ceaseless uncooperative growth of tumor cells, which serves their but not the organism's, needs. Tumor cells do not acquire a stable cell fate and designation; figuratively, they have 'no memory of their purpose' for the organism[9–11].

While the feature of blastema cells towards reverting to greater stemness remains the most extensively studied topic, stemness doesn't account for the organization of cells with respect to one another. A significant discovery was achieved by the revelation that blastema cells are not as uniform as it had been thought and that one cell type is capable of retaining developmental history. This trait is ascribed to connective tissue (CT) cells and is integrated into a concept of positional memory, which is upheld by researchers' studies based on CT cells' position-specific epigenetic profile and also cell surface molecules[12–15].

A substantial amount of evidence also points out that CT cells work together with developmental signaling to coordinate pattern formation[15,16]. In my thesis I intend to illustrate that positional information enabling organization during limb regeneration is communicated through a system in which exists a hierarchical structure. The existence of this structure, where cells occupy different levels of control over the course of regenerative pattern formation, highlights CT cells' role and their spatial interaction with molecules, cells, and tissue-specific parameters of the regenerating limb.

2 Positional information in regeneration

Positional information is considered to be the central determinant of cellular behavior in living systems including systems undergoing regeneration. Following aquisition of positional information cells figuratively recognize their place within the organism [4].

2.1 Historical conception of positional information

Although Lewis Wolpert was the first to establish the concept of positional information, he was not the only scientist striving to capture the logic underlying the question of how and what concrete information gives rise to pattern. "The Chemical Basis of Morphogenesis" published in 1952 by Alan Turing is considered a pivotal work illustrating mechanisms of pattern formation in nature. Both Wolpert's and Turing's models assert that pattern, in Wolpert's work positional information, emerges via spatial interaction which is specific as interacting molecules differ in their concentration and diffusion rates over time and are localized within an established threedimensional system; in mathematical models, referred to as reaction-diffusion models [4,17,18].

To address how Wolpert linked his understanding of positional information to cells of the developing organisms can be explained by his 1971 work where he suggested a view that cells "are assigned positional information which effectively gives them their position in a coordinate system,"(Wolpert 1971) that essentially determines their following cell fate, cell type to differentiate into and relation to other cells in the developing organism. It is important to note that Wolpert's notion of signaling molecules termed morphogens is not identifiable with Turing's patterns as morphogens are not uniformly dispersed, but are defined as diffusing from nonrandom signaling centers of the organism in which cells register concentration of a specific morphogen and act on the positional information it provides [3,4,17]. (Alan Turing 1952; Wolpert 1969, 1971).

However, epimorphic regeneration of urodele organism's limbs is a phenomenon Wolpert regarded as exceptional in its nature and presumed that the influence of soluable morphogens alone could not be sufficient in precise construction of such complex structure [3].

2.2 Models of pattern formation in regeneration

Thomas Hunt Morgan, Wolpert's contemporary, known primarily as a Nobel-prize laureate and ‚Father of experimental genetics', was likewise quite interested in studying regeneration. He was also the one to coin the term epimorphosis [1]and cause subsequent usage of the term and its derived forms. Bryant and French used this definition, but more importantly were heavily inspired by Wolpert's understanding of abstract coordinates demarcating cells in organisms in construction of the polar coordinate model (PCM) [5,6].

PCM attributes values to cells along the proximodistal (P/D) axis as well as to cells in remaining two body axes. At the amputation plane the latter two axes assign every cell a value that is to be the closest, but not identical to the values of neighbouring cells. After amputation cells are capable of perceiving discrepancy between their value and their neighbours' and incite intercalary growth to generate cells with missing intermediate values. The intercalation takes place first within the circular plane and once intermediate values are replenished cells apply the same procedure to missing values of the P/D axis and generate cells with the closest distal values. Cells located in the new section again preferentially intercalate appropriate values within the plane and this cycle continues till the completion of the structure, such as a limb of axolotl (Figure 1) [5,6] .

An important premise of PMC is that cells possess intrinsic positional information – pre-existing positional identity, perceive positional information values of in their vicinity and actively engage in resolution of positional disparities [5,6].

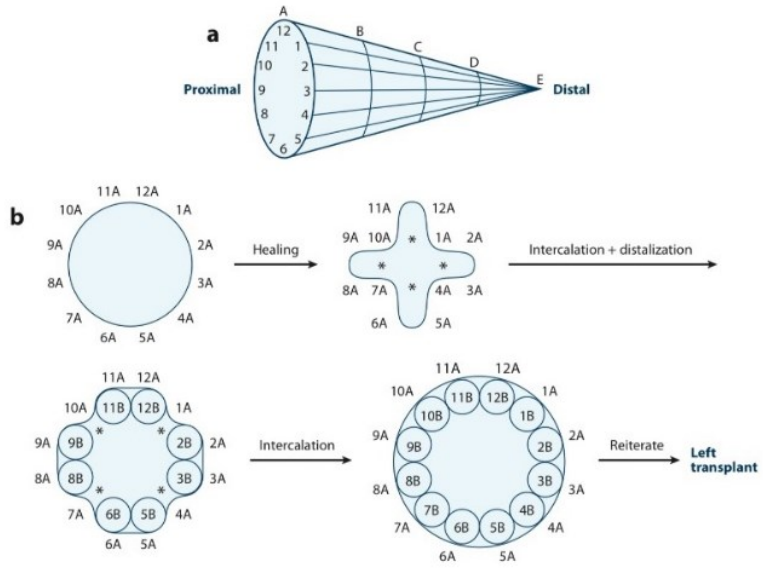


Figure 1- The polar coordinate model (PCM). *(a) Polar coordinates of positional information in the limb. Proximodistal positional information is represented by concentric circles (A–E). Circumferential positional information is represented by numbers on the circumference (1–12). (b) Distalization after amputation of normal limbs. Taken from* [8]

PCM as a model of regenerative pattern formation has been shown as quite useful [19–21], but not entirely reliable in its predictions [22–24]. In addition, an aspect in which the model falls short is a lack of concrete detectable material evidence of their theory [6], and as a response, another patterning model emerged – the boundary model [25,26]. The boundary model embraced the knowledge obtained from experiments on insect imaginal discs [27–29] to form an analogy for vertebrate limb, promoting the role of morphogen gradient in the process of distal outgrowth and specification of proximodistal cellular identities [25,26]. To compensate for cells which possess missing positional values at the circumferential plane, the model supports intercalation as described by the previous model but proposes that the execution of intercalation is limited to singular planes and does not interfere with proximodistal identity [6,25,26,30].

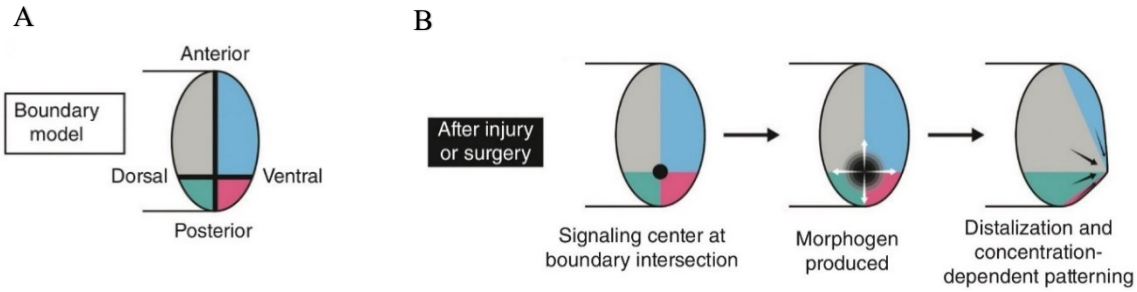


Figure 2 - Features of Meinhardt's boundary model (1983). *(a) Positional values are distributed in quadrants separating the anteroposterior and dorsoventral axes of the limb. Larger anterodorsal and anteroventral domains abut smaller posterodorsal and posteroventral domains at boundaries (thick black lines). (b) After injury or surgery, a distalizing morphogen is secreted by wound epidermis cells (also referred to as apical ectodermal ridge or cap) and induces proliferation and patterning of underlying blastema cells. Taken from*[15]

While the model did provide a more mechanistic explanation, there exist data refuting its claims. However, the key assumption the model shares with PCM which is the necessity of preexisting positional identity was not invalidated [6,26].

PCM and the boundary model are not the only attempts at elucidation of regenerative appendages pattern formation of appendages [16,31] – yet in spite of all their endeavor and progress in the field the framework which would account for shortcomings of presently known model has not been found [32].

2.3 The concept of positional memory

Despite the current lack of a satisfactory model, the emergence of the concept of positional memory represents a solid construct that appears promising for future efforts to formulate a comprehensive theory [15]. It is estimated that the term was introduced to the regeneration research by Bruce Carlson during the late 1970 [33]. The characteristic of blastema to always regenerate more distal structures relative to the amputation plane even when the piece

of blastema is transplanted to another location in the body, which has supported the suggestion that the cells in blastema demonstrate autonomy [34,35] and that there must be cells which possess positional memory [14,15,33,36,37].

L. Otsuki and E. M. Tanaka propose that the term positional memory is, in its essence, a trait of steady-state cells of a specific cell type, but it does not merely encompass possessing spatially diverse cell-intrinsic information. Cells with positional memory must be capable of generating pattern by providing cells with instructive signals. And lastly, the nature of the signal must allow for its reception and interpretation. Although this set of assumptions and the publication is quite recent, the authors supplement the claims with empirical evidence gathered from early to present studies and methods [15].

3 Surgical induction of pattern

Early regeneration research relied on tissue amputation and displacement assays, but also techniques such as irradiation to investigate formation of pattern and despite the progress in biotechnology surgical disruption has always been a cornerstone technique in the vertebrate regeneration biology field [15,32].

3.1 Graft and amputation assays

Experimental data of Carlson himself hinted at the possibility of specific cell types generating pattern - in his assay, he tested displacement of tissues on axolotls and found that neither epidermis nor bone could induce morphogenetic conflict. On the other hand, dermis and muscle were capable of supporting pattern formation [38].

Additionally, outcomes of grafting experiment conducted by Susan and Peter Bryant together with Vernon French of demonstrated that posterior limb tissue possesses more positional information as amputated limbs of blastemas composed of either only double posterior, or double anterior tissue displayed a great difference in complexity of produced pattern [6].

Carlson's results concur with the requirement for pattern-provoking capacity and restricted it to cells in muscle and dermis, meanwhile the data of the aforementioned trio of researchers highlight that cells at steady state harvested from different sites may contain diverse positional information without focusing on the cell type [6,38].

3.2 The accessory limb model

Although the regeneration research did not stay behind the methods of the era of molecular biology, The accessory limb model (ALM) presents a milestone procedure that has persisted since its conception and publication in 2004 [20]. The cornerstone of ALM is the deviation of the brachial nerve to a lateral wound, followed by implantation of a skin graft composed of epidermis and dermis layer from the opposite position with respect to the wound site, the graft's final implantation site. Without innervation, a bump can be observed at the implantation site, which grows for 19 days and then recedes, but by obeying all the requirements, an ectopic limb equivalent to the naturally regenerated limb is formed [20,39].

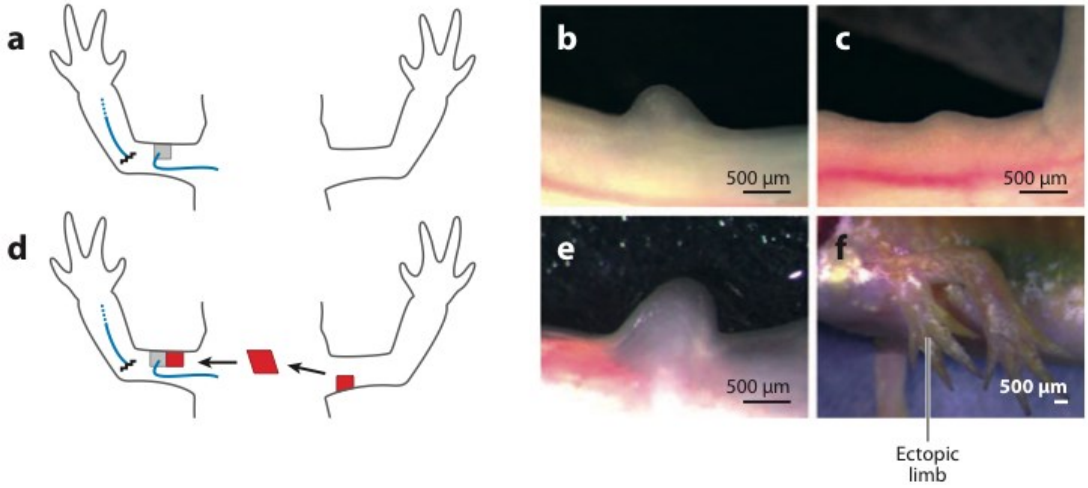


Figure 3 - The accessory limb model. *(a–c) Deviation of a nerve to a lateral wound (a) results in formation of a bump (b), which eventually regresses (c). (d–f) Deviation of a nerve to a lateral wound coupled with transplantation of a piece of skin from the opposite side of the contralateral limb (d) results in bump formation (e), which eventually forms an ectopic limb (f). Taken from* [8]

Several crucial implications arose from this gain-of-function assay. Firstly, the pattern can be formed at an apriori existing limb field. And that regeneration permissive environment provided by nerve supply was a sufficient background for the positional confrontation of pattern-provoking tissues of the graft and host site. The positional confrontation at last was shown to require only two conflicting boundaries which conflicted the condition of three boundaries set by the boundary model [20,25,39].

4 The potential of recurrent limb regeneration

Carlson's proposed trigger for the generation of ectopic pattern - displacement of positional identities was conceived with the contemporary regard of tissue manipulation

experiments, but since then novel technologies have been deployed, and the general usage of ALM revealed that exogenous application of fibroblast growth factor 8 (FGF8) and sonic hedgehog protein (SHH) could drive ectopic limb formation in the absence of anteroposterior (A/P) tissue confrontation needed in the original assay. Furthermore, the engineered limbs were subsequently subjected to amputation to elucidate the mechanistic basis of positional identity [15,20,38,40].

Albeit the aforementioned artificial limb exhibited a great degree of morphological complexity, their composition was lacking in structures along the P/D axis and natural asymmetry of the limb's A/P pattern. And strikingly, their amputation demonstrated that they were missing conflicting pattern-provoking cues as the fact the amputation did not result in a successful regeneration was considered an indication that the regenerates were lacking in the inheritable disparity of positional information, which would sustain blastema's patterning capability [40].

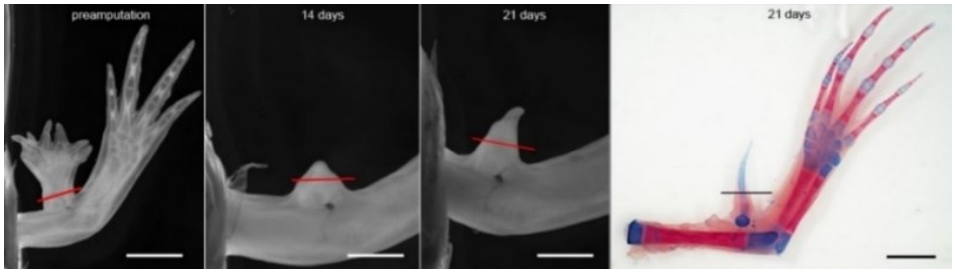


Figure 4 – Lacking patterning capability of ectopic limbs. *Shh-induced ectopic limbs fail to regenerate in response to amputation, indicating a lack of positional diversity in these structures. No further growth observed 21 days post-amputation. Alcian blue/anizarin red staining of cartillage and bone, respectively. Scale bar, 2 mm.* [40]

4.1 The positional identity of cells in engineered limbs

To discern what tissue could provide the necessary regenerative and patterning context, full-thickness skin grafts, in other words grafts containing epidermal, but also dermal layer of tissue from the artificial limbs were used as donor implants for ALM. The host site on either the anterior or posterior side of a limb was both supplied with the skin transplant and then monitored to decide which donor tissue promoted limb outgrowth and patterning. Indeed, only one of the host limbs produced viable blastema and a patterned limb outgrowth. The sustained limb formation at the posterior side was, therefore, an indicator that the skin graft contained

anterior positional identity and unavailability of the opposite identity prohibited natural regeneration (Figure 5) [40].

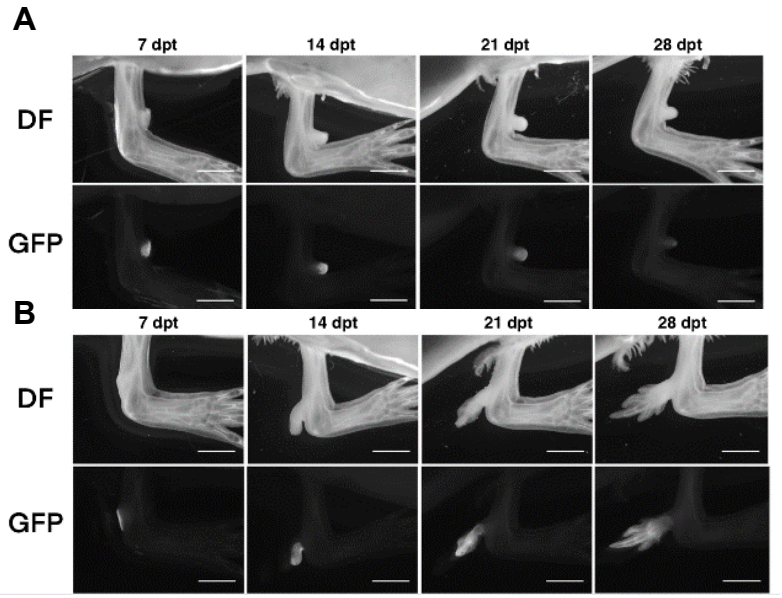


Figure 5 *– Positional identity of artificially engineered limbs. (a) Anterior host site with a skin transplant from an engineered limb shows no accessory limb outgrowth, indicating no determination of posterior identity during ectopic induction of outgrowth (n = 0 of 22). (b) Posterior host site with a skin transplant from the same limb as in c results in new accessory limb outgrowth, revealing the anterior identity of the transplanted skin (n = 15 of 21). DF, dark field images. GFP panels show the transplanted skin. dpt, days post transplantation of skin. Scale bars, 2 mm. Taken from* [40]

4.2 Reprogramming of positional information

Generating ectopic limbs using aforementioned signaling molecules failed to establish the posterior positional identity of the blastema cells, indicating the absence of a factor that could achieve reprogramming cells' positional information [40].

A great amount of evidence has supported the view that retinoic acid (RA) and its derivates can modify positional information in amphibians [41]. Early examples of retinoids' specific effect on blastema implied that they were capable of proximalizing positional information in urodeles, invoking duplication of proximodistal structures in limbs [42–44]. However, this potency of retinoids was proven to be limited to a time frame within which the cells were sensitive to its influence [43,44], which contributed to a hypothesis that cells are not receptive to respecification unless they are in a dedifferentiated state when positional information is not yet stabilized [45].

In addition to proximalization, it is asserted that RA alters pattern in the limb regenerates by ventralizing and posteriorizing the positional identity of blastema cells[46].

Experiments featuring RA application were conducted in order to elucidate rules governing complete limb patterning, relying on a search for the requirements for engineering completely patterned limbs with equal ability to regenerate pattern as natural urodele limbs. One of them administered RA to innervated wounds which yielded limbs fulfilling the listed requirements, but only if administered to anterior and dorsal wound sites [47]. Another even accomplished these requirements in the absence of a nerve from a naturally non-regenerating anterior wound site, substituting the function of nerves with fibroblast growth factor 2 (FGF2), FGF8, and bone morphogenetic protein 2 (BMP2) (Figure 6) [48].

Figure 6 – Retention of pattern-forming capacity in RA induced limbs*. RA induced structures demonstrate regenerative capacity, indicating the establishment and stabilization of new positional identities within the RA-induced outgrowths. Taken from Vieira et al., 2019*

The important insight from artificially induced limbs is that after their amputation, RA does not simply grant cells positional information but operates with preexisting positional information, which must be spatially diverse. The limb's tissue, whose positional identity was anterior, was unable to induce and sustain morphogenetic conflict, nor act on positional cues such as RA and interestingly similar reaction, respectively a supposed lack of reprogramming, was presumed by Vieira et al. [48] after treating mature limb tissue with RA and observing no expression of A/P markers. The ability to induce morphogenetic conflict repeatedly is a key trait of cells which are assumed to possess positional memory and results of researchers such as Carlson indicated that only certain cell types display these capabilities. However, the matter of identifying these cell types appears to have been resolved [14,49,50].

5 Positional memory-encoding cell types

The breakthrough in the elucidation of the behavior of individual cell types during regeneration was achieved by Elly M. Tanaka's research team [14]. Together with demonstration that dedifferentiated blastema cells revert to their original lineage the researchers found that dermis and tendon-derived cells translocate to their original region along the P/D - the donor

grafts labeled with Green Fluorescent Protein (GFP) [51] were implanted into the host limb in a manner that grafts' position in the host limb matched their original site in the donor limb (Figure 7) [14].

The cells derived from connective tissue make up to 78% of the sum of cells in blastema [52], but CT-derived cells were not the only candidate for cell type displaying positional preference [14]. It was hypothesized that since denervation leads to regeneration failure [53,54], Schwann cells could exhibit preference for a position in the regenerate based on their previous location in tissue. However, implanted GFP-labeled Schwann cells were dispersed throughout the host limb independently of their previous position along the P/D axis (Figure 7)[14].

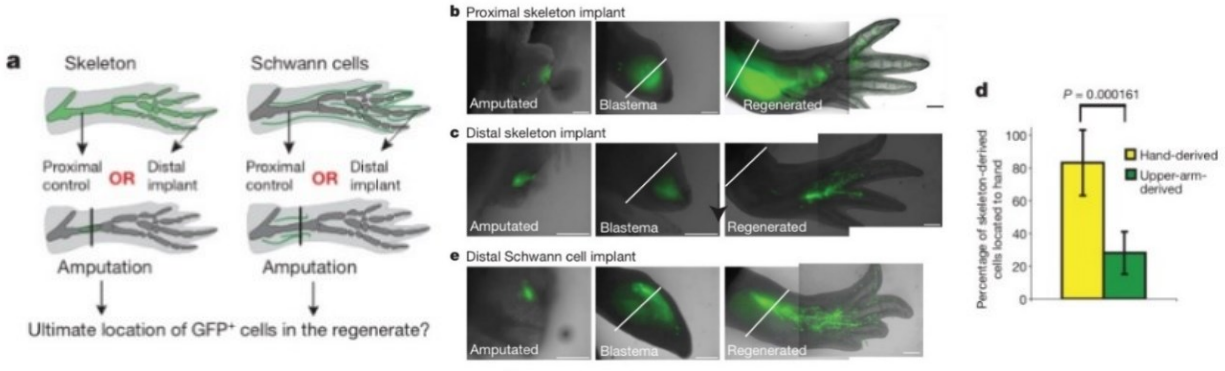


Figure 7 - Schwann cell-derived blastema cells do not possess proximo- distal positional identity but cartilage-derived cells do. *a, Schematic to show the experimental design. b, c, e, Time course through regeneration of the indicated graft type. Progeny of distal skeletal cells localize distally while Schwann-cell-derived cells show no positional preference. n 5 14 (b), 20 (c) and 15 (e) limbs. Scale bars: 0.5 mm. d, Percentage of cartilage-derived progeny originating from upper arm (six limbs) or hand transplants (seven limbs) that contribute to hand skeleton after regeneration..Taken from* [14]

The sense of position, observed in CT-derived cells migrating to the limb segment of their origin [14], was also noted by Rinn et al. in human dermal fibroblasts as their extraction site determined the possibility of palmoplanar differentiation of epidermal cells the mature fibroblasts were later co-cultured with. The results were claimed to be connected to cells' epigenetic profiles acquired during limb development, asserted to reflect differential spatiotemporal gene expression, and to correspond to mature fibroblasts' position in the body [55].

5.1 Epigenetic basis of positional memory

Epigenetic regulation presents a distinct category of gene expression regulatory mechanisms whose nature lies in the alteration of chromatin accessibility, excluding changes to the DNA sequence itself. This modulation of gene activity includes histone modifications, variety in histone proteins, and DNA methylation [11,56,57], which ensure the generation of tissue-

specific cell identities and were shown to operate at the Hox D cluster of limb bud cells, but also at morphogenetic gene loci of mature and blastema cells [58,59].

5.1.1 Chromatin states underlying positional identities in limb cells

During limb development, transcription factors of homeobox genes, notably gene products of HoxD and HoxA cluster, specify the cellular identity of limb bud cells [24,59,60]. Homeobox genes, including Hox genes, encode homeoproteins whose name is derived from a DNA-binding domain known as the homeodomain, which is crucial for TFs to govern gene expression during the morphogenesis of individual anatomical segments and structures [60,61]. In developing limbs gene expression along the P/D axis demarcates three specific regions - future stylopod, zeugopod, and autopod which are designated by homeoproteins Meis1 and 2, Hoxa11 and Hoxa13, respectively [62–65].

The loss of regenerative capacity upon metamorphosis in amphibians is closely connected to failure in limb segmentation and has been long documented in observation of limb amputation in Xenopus froglets as this act causes them to replace the appendage with a meager linear spike structure with cartilaginous "backbone" [66–68]. Yakushiji et al. [69] detected an important difference between Xenopus tadpoles and regeneration incompetent froglets, though - the methylation status of the Shh enhancer as is its hypomethylated state that allows posterior limb bud cell-specific Shh expression [70], the lack of methylation at the enhancer was observed in axolotls as well and linked to Shh expression [69].

A significant study concerning the impact of epigenome on the recapitulation of developmental patterning processes was undertaken, which investigated the role of histone modifications in regulation of positional information [71]. Distribution of two types of modification in the sequences of morphogenetic genes was examined – in relation to chromatin accessibility, permissive trimethylation at histone 3 at lysine 4, and repressive trimethylation at the same histone, but attached to lysine 27 (H3K27me3) [72,73].

Sequences of interest included HoxA11 and HoxA13 gene loci, but also T-box genes 5 (tbx5) and 4 (tbx4), whose expression is characteristic for cells in forelimb and hindlimb, respectively, and is activated by Hox homeoproteins [74]. For chromatin immunoprecipitation sequencing (ChIP-seq), samples from fore and hindlimb blastema and limb bud cells were collected [71].

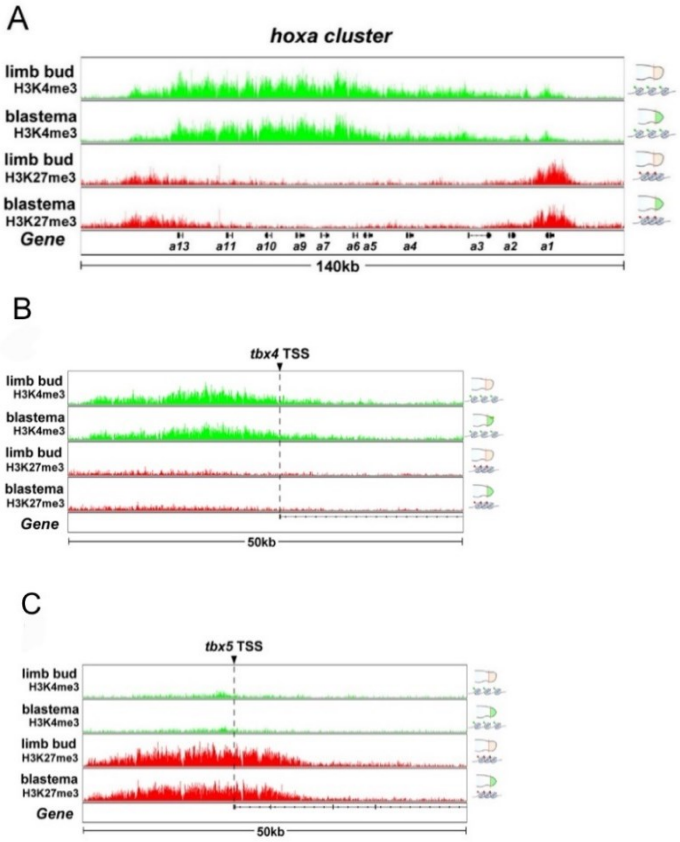


Figure 8 - Histone modi cation of morphogenetic genes in X. tropicalis hindlimb bud regeneration. *(A) Read mapping shows epigenetic regulation of the hoxa cluster. K4 trimethylation peaked from hoxa5 to hoxa13, while K27 trimethylation peaked at hoxa1, indicating strong expression of 5′ hox genes. Modi cation pro les were similar in the stage 53 limb bud (0 dpa) and blastema amputated at the ankle level (7 dpa). The genome sequence is indicated in 140 kb. Read mapping range: 0 60. (B) and (C) For tbx5, which is speci cally expressed in the forelimb, a high peak of H3K27me3 but not H3K4me3 indicated epigenetic repression of tbx5 in the hindlimb bud. In (C), a high peak of H3K4me3 but not H3K27me3 at tbx4, which is specifically expressed in the hindlimb, indicates chromatin regulation to an active state. Thus, ChIP-seq analysis re ected intrinsic hindlimb features. Limb bud (0 dpa) and blastema (7 dpa) histone modification profiles are similar in each panel. Transcription start sites are indicated by a vertical dotted line. Taken from* [71]

From ChIP-seq data, it was ascertained that hindlimb bud and blastema cells' epigenetic profiles bore a strong resemblance to one another as HoxA11 and HoxA13 gene loci were enriched in H3K4me3 marking chromatin open for transcription, which was also the case with tbx4. The opposite chromatin state was noted in tbx5 gene loci where hindlimb bud and blastema cells amassed H3K27me3, restricting forelimb formation (Figure 8) [71]. The implied recollection of former position by hindlimb blastema cells was regarded as an argument in favor of intrinsic positional memory corresponding to chromatin states of the developing limb bud [33,71].

A recent study, "A chromatin code for limb segment identity in axolotl limb formation" [13] corroborates earlier conjectures [11,33,37,71] by providing data arguing in favor of intrinsic instructive information in limb segments in regenerative pattern formation which the study ascribes to CT cells directly for their previously documented position specificity [14,49,55].

Harvested CT cells of juvenile axolotls were found to be " mature" based on the pattern of limbs and divided into cells from the upper (UA) and lower limb (LA) and hand, also referred to as autopod. For their isolation from other cell types fluorescence-activated cell sorting was used. Chromatin profiling demonstrated that H3K27me3 is the fundamental mark preserving developmental history in mature CT cells by localizing H3K27me3 at HoxD13 (Figure 10) and HoxA13 (Figure 9, 10) in UA cells' epigenetic signature while autopod CT cells were enriched in H3K27me3 at HoxD9 gene loci (Figure 9, 10) [13].

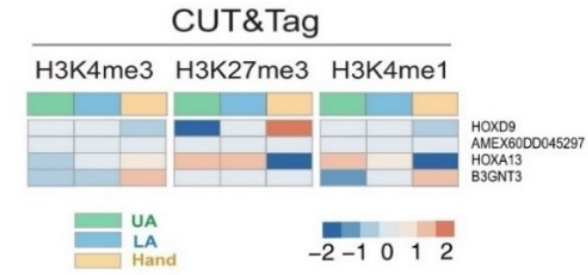


Figure 9 - Heatmap of promoter peaks (within ±2 kb from TSSs) showing differential accessibility and their associated modified histone levels. *Values (dark blue to red) calculated based on log2 fold-change Hand/UA of genomic coverage for genes showing a significant chromatin-state switch. HoxD9 and HoxA13 are among those showing strongest differential accessibility and differential histone H3K27me3. Taken from Kawaguchi et al., 2024*

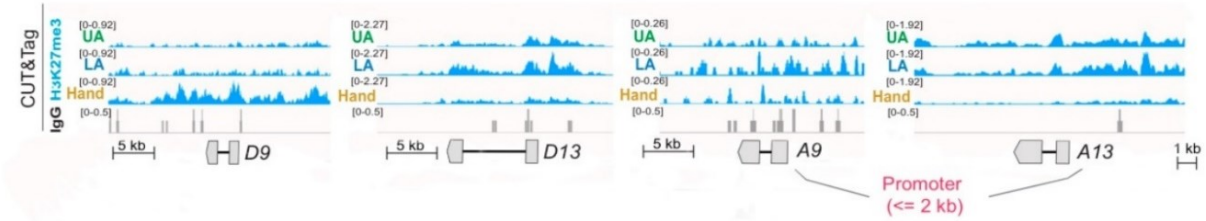


Figure 10 - Profiles for accessible chromatin and histone modifications at the HoxD9 and HoxD13 , and at the HoxA9 and HoxA13 loci show segment-specific differences. *Chromatin accessibility was marked by distribution of H3K27me3 with blue, and IgG control marked with light gray. Upper arm (UA). Lower arm (LA). Taken from* [13]

The H3K27me3 mode of function, in theory, is explained on processes after amputation of the entire arm and on amputation from wrist down. Following surgical removal of all three limb segments, early blastema consists of cells restricted from expressing autopod-associated homeoproteins by the accumulation of H3K27me3, which leads to selective expression of Meis as its gene is accessible for transcription. Only during the transition to the successive blastema stage can HoxA13 expression emerge at the distal tip of blastema as its characteristics become

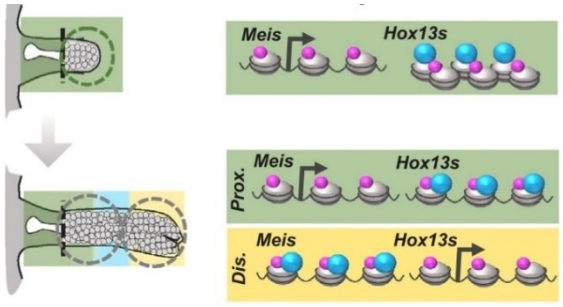


Figure 11 – Model of selective upper arm program initiation based on limb segment-specific chromatin states of patterning genes by differential occupation of chromatin by H3K27me3 marks *(depicted as blue spheres). Early UA blastema expression histories are maintained by epigenetic history and later occuring late blastema reactivation of segment specific epigenetic modifications along P/D axis at homeoproteins. Upper arm (UA), HoxA13 and HoxD13 (Hox13s). Trimethylation at histone 3 at lysine 27 (H3K27me3), Taken from* [13]

more analogous to developing limb buds. However, the study includes research into regeneration-specific elements, which are indispensable for the process (Figure 11) [13].

Amputation of axolotl autopod, on the other hand, selectively triggers HoxA 13 expression while Meis genes retain remembrance of their position, marked by H3K27me3-caused chromatin condensation. The repression of the upper limb program is, in addition, executed by HOXA13, for it does not act purely as a TF in autopod transcriptional program but also directly antagonizes expression of upper-limb specific genes. Noticeably, the proposed mechanism of restriction is in accordance with the rule of distal transformation [2,13,35].

5.1.2 Maintenance of chromatin states

To secure replication of the parental state of chromatin structure at cell division, chromatin-modifying enzymes are recruited, and their activity was shown to target histone marks and DNA in the case of DNA methyltransferases. The fidelity and changes in chromatin landscape are relevant to regeneration as both limb and lens regeneration were found to be accompanied by upregulation of genes associated with changes in chromatin accessibility (Day and Beck 2011; Pearl et al. 2008).

The activity of histone deacetylases (HDACs), a class of enzymes that remove acetyl marks and cause compaction of chromatin associated with gene repression [77], was registered in Xenopus tail regeneration, respectively upregulation of HDAC1 expression [78.] Subsequently, Taylor and Beck conducted a study using HDAC inhibitors throughout the course of both regeneration and development of limbs and tails [79]. The most important chemical – valproic acid (VPA), was tested and proven to specifically inhibit HDAC activity [80,81].

To observe the impact of disruption of HDAC activity hindlimbs of stage 52 Xenopus tadpoles, respectively, on limb buds, were divided into three groups. Two groups of tadpoles underwent amputation, which marked a level of the future knee. One of them served as a control group and was not exposed to the VPA solution. The third group consisted of tadpoles with uncut developing hindlimbs and, together with the second group, was treated with VPA solution at a concentration of 0,2 mg/ml for 48 hours, in case of the regenerating tadpoles starting from the time of surgery to 48 hours post-amputation[79].

Uncut tadpoles' hindlimbs' pattern demonstrated only negligible deviation from species' limb morphology as the amputated control hindlimbs' regeneration exhibited naturally occurring defects. The amputated hindlimbs kept in the VPA solution on the other hand were severely deformed, (Figure 13) featuring only one digit and malformed tarsus. These results suggested that developmental patterning is not influenced by impairment of HDAC activity which was also supported by an unperturbed development of tail buds removed at stage 32 embryo in a culture with VPA [79,82].

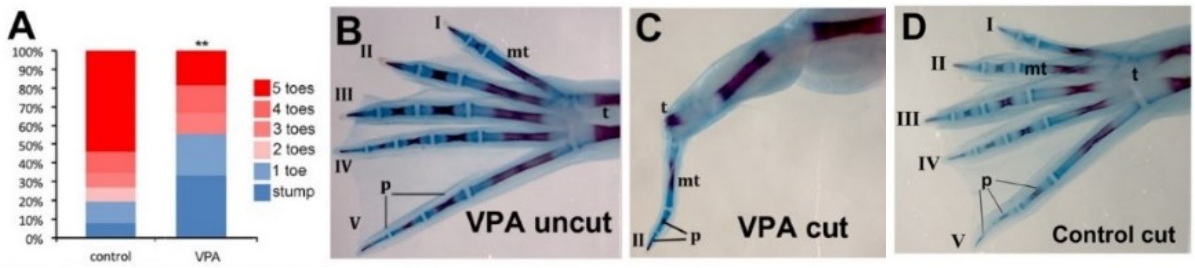


Figure 12 - VPA reduces hind limb regeneration in stage 52 Xenopus tadpoles. *In (B–D) digit numbers are indicated (I–V), p: phalanges, mt: metatarsal, t: tarsus. (A) Stacked column graph showing the percentage of left hind limbs that had failed to regenerate (stump), or that had regenerated 1–5 toes by stage 58. The left hind limbs of stage 52 tadpoles were amputated at the approximate level of the future knee. The group treated with 0.2 mg/ml VPA regenerated significantly fewer toes than the control, untreated group (B) VPA treated limb that was not amputated showing perfect development. (C) VPA treated limb after regeneration, in this example only digit II is present and the single tarsus is deformed. (D) Control limb after regeneration, in this example one phalange is missing from digit IV showing that control regeneration is often imperfect. Taken from* [79]

Taylor and Beck interpreted their research as indicating that HDACs are required for regeneration but not for processes of standard limb and tail development and patterning. The end of their study insinuates that the activity of histone deacetylases may be responsible for re-activating and stabilizing the expression of genes associated with developmental patterning [79].

Current knowledge on the conservation of chromatin landscapes, especially in the research of regenerative morphogenesis, is lacking, but from inquiries into the maintenance of H3K27me3 marks at Hox clusters, the involvement of polycomb repressive complexes 1 and 2 is assumed to play a role in regeneration because of their presumed activity during the course of embryonic development [13,16,33].

6 Instructive signals guiding regenerative pattern formation

The last chapter is dedicated to CT-derived cells' relationship to one another, but not in the context of blastema as it did not account for the extracellular environment of the tissue with the entirety of the cells in blastema. The complexity of the background in which patterning and morphogenetic processes take place extends beyond biochemical signaling, and this fact is well

highlighted in the meticulous review "Programmed and self-organized flow of information during morphogenesis": *"In all cases, cells change shape, divide and move with respect to one another. All these processes require mechanical forces, such as tension through contraction of actomyosin networks, cell–cell and cell–substrate adhesion, cell protrusive forces or cell growth. These active forces must be organized in space and time to correctly orient and execute these different morphogenetic processes"*[83]. Accordingly, I aim to point out instructive forces which are considered to play an important role in organization of limb regeneration.

6.1 Proximal identity-conferring molecules

The gene expressing the first known protein associated with position specificity was discovered in the red-spotted newt (*Notophthalmus viridescens*), named Prod1, and is found exclusively in urodeles [84].

The protein is transcribed by Schwann cells, which express different levels of Prod1 as the level expressed by cells in the upper arm is 1.7 times greater than that of cells in the autopod area. Experiments following its discovery have noted that *Notophthalmus* PROD1 is membrane-bound – by a glycosylphosphatidylinositol anchor to the cell surface. Similarly, as in an intact limb, proximal blastema cells transcribe 1,7x higher level of Prod1 than their distal counterparts, which is a signature corresponding to the steady decline of RA concentration towards the distal blastema. The assumption that Prod1 bestows proximal cellular identity upon cells was tested on axolotl blastema cells by exogenous retinoid treatment aimed at distal blastema cells. The increase in expression of Prod1 caused by retinoids in distal blastema cells and their subsequent transplantation corresponded with the assumption that after transplantation of the treated cells, it was observed that the previously distal blastema cells translocated to proximal blastema segment and did not contribute to the distal structures of axolotl regenerates (Figure 13) [85].

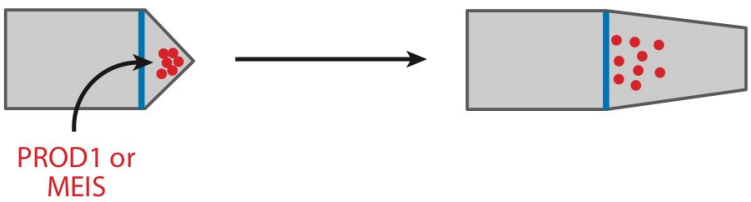


Figure 13 - Distal blastema cells electroporated with plasmids expressing MEIS or PROD1 are found in proximal regions of the regenerate. *Taken from* [8]

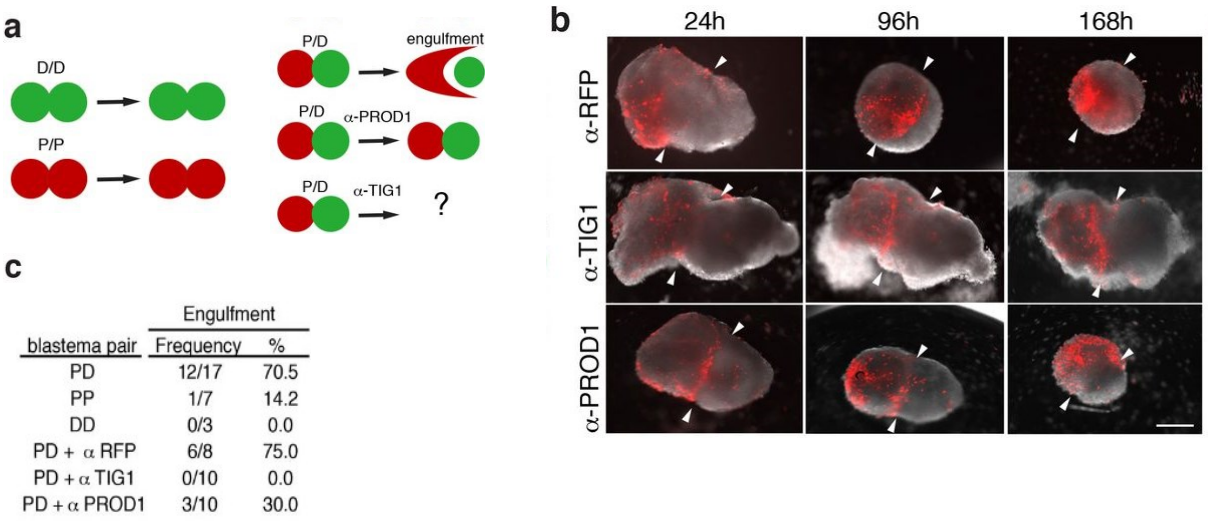
As for the protein's properties, da Silva et al. suggested that Prod1 could be responsible for intercalation but were unable to show clear evidence. However, they have assessed that there's clear evidence pointing at selective adhesive properties of the molecule as an antibody

against Prod1 inhibited the natural engulfment of distal blastema cells by proximal in hanging drop culture (Figure 14) [84].

Only after almost 20 years another gene conferring proximal identity was detected in axolotls – Tarzarotene-induced gene 1 (Tig1) [12]. It is implied that properties of the protein expressed by Tig1 are analogous to those of Prod1 encoded protein in newts – since both proteins are expressed at the cell surface and TIG1 content is likewise slopping from its peak concentration in the limb tissue connected to the trunk to minimal presence in the most distal limb segments. Also, the expression of Tig1 by cells facilitates tissue sorting processes and inhibition of engulfment via anti-TIG1 antibodies as in accordance with the assay design of da Silva et al. [84]demonstrated an identical phenomenon – disruption of natural engulfment of distal blastema by proximal blastema cells which was ascribed to a change in surface tension preventing natural engulfment (Figure 14). After amputation, RA acts as an up-regulator of Tig1, causing the amount of expressed protein to be the greatest in proximal blastema cells and regress distally, which corresponds to the spatial pattern of Prod1 expression [12].

Tig1's similarities to newt Prod1 are not coincidental as overall findings about Prod1 in urodeles were not absolute and nonconflicting. For instance, axolotl Prod1 does not exhibit all properties observed in *Notophthalmus viridescens*. However, the encoded protein was not found to be anchored to the axolotl cell surface, and yet selective adhesion was still reported in axolotl blastema[86]. The mode of action of Prod1 is also regarded as elusive since Kumar et al. reported that only after amputation does its expression relocate and is present in alleged blastema cells of CT origin [87]. Furthermore, results of a later experiment disclosed that the outcome of the conducted Prod1 loss-of-function assay was incongruent with PROD1's proximalizing effect because the outcome noted an absence of lower arm as well as loss of phalange patterning [88].

In contrast to Prod1, Tig1 gene is evolutionarily conserved, does not conflict the possibility of differential tension blastema organization by membrane-bound molecules[86], and allegedly unlike Prod1 is expressed in CT cells at steady state, which concurs with the notion of intrinsic positional memory of CT-derived cells [15,89]. It is thus assumed that TIG1 is a conserved part of a proximalizing network that includes RA and its effector TF MEIS that could have been modified by additional integration of Prod1 in urodeles [15].



c

	Engulfment	
blastema pair	Frequency	%
PD	12/17	70.5
PP	1/7	14.2
DD	0/3	0.0
PD + α RFP	6/8	75.0
PD + α TIG1	0/10	0.0
PD + α PROD1	3/10	30.0

Figure 14 - Tig1 mediates proximo-distal cell-surface interactions. *a Schematic of the engulfment assay. Juxtaposition of proximal (P) and distal (D) blastemas results in the engulfment of the distal one by its proximal counterpart. b Representative images of juxtaposed proximal (red) and distal (white) blastemas at the indicated times post juxtaposition, and after the indicated treatments. Note lack of engulfment following treatment with anti-TIG1 (α-TIG1) antibodies raised against two extracellular epitopes. Scale bar: 500 μm. c Quantification of engulfment for the indicated pairs (pooled data from three independent experiments). For α-Prod1, α-Tig1 and α-TMEFF1, [antibody] = 20 μg/ml. Taken from* [112]

6.2 Tissue microenvironment

Previously mentioned impact of physical interactions was ascribed to cell surface tension, but the composition of the tissue microenvironment itself has been shown to be crucial for the control and determination of cell behavior and cell fate [10,90,91]. Its influence in amphibian regeneration can be well demonstrated in relation to cancerous morphogenesis, which produces a disorganized mass whose unsuppressed growth and plasticity inherent to the process do not permit proportionate pattern formation and termination of cell division [10].

Previously mentioned impact of physical interactions was ascribed to cell surface tension, but the composition of the tissue microenvironment itself has been shown to be crucial for the control and determination of cell behavior and cell fate [10,90–92]. Its influence in amphibian regeneration can be well demonstrated in relation to cancerous morphogenesis, which produces a disorganized mass whose unsuppressed growth and plasticity inherent to the process do not permit proportionate pattern formation and termination of cell division [10].

The role of bioelectricity in pattern formation has become increasingly more pronounced, and it was demonstrated that both blockage of ion flow and their increased permeability severely decreases morphological complexity and accuracy in affected regenerating tissue [93].

6.2.1 ECM composition

Backed by the premise that CT cells incorporate and mediate positional information, it was hypothesized that ECM, which these cells synthetise and break down, specifically heparan sulfate (HS), could pose as an instructive cue in pattern formation [94].

HS is found in heparan sulfate proteoglycans (HSPGs), which are deeply evolutionarily conserved biomolecules, one type among many proteoglycans where protein always acts as a scaffold to linear glycosaminoglycan (GAGs) polymers that can be bound to the cell surface, but also localized freely in ECM after cleavage of the protein scaffold. From a protein backbone via covalent bonds formed between serine hydroxyl groups and xylose units protrude linear heparan sulfate (HS) chains. Each HS chain initially consists of a 'linkage tetrasaccharide', which allows prolongation by alternating addition of N-acetylglucosamine and glucuronic acid units whose residues can undergo sulfation and other enzymatic reactions. The uneven distribution of charge, sulfation types, deacetylation and further possible modifications of HS chains [95]. create an immense variety in overall structure and biological activity which enables HS and HSPGs to interact with proteins and play a key part in signaling pathways out of which a significant number mediates regenerative and patterning processes [96–99]

Phan et al. performed a modified ALM assay which demonstrated that strictly decellularized natural and artificial ECM transplants used instead of regular tissue grafts have distinct effects on the probability of blastema and pattern formation. The outcomes of transplantations were, according to the authors, caused by a differential HS content in anterior and posterior limb tissue [94].

Every transplantation of an anterior ECM graft to anterior wounds failed to promote not only pattern induction but also prevented the development of blastema itself as opposed to innervated anterior wounds with no graft at all. Posterior ECM proved to be capable of inciting generation of pattern in almost a third of cases when blastema had formed [94], which is consistent with the findings implying posterior limb tissue preserves more positional information than anterior [23,52,100]. To confirm whether HS in ECM encodes unique positional information, differentiating anterior and posterior ECM, ECM grafts from both anterior and posterior sites underwent heparin lyase III treatment (HepIII) before grafting [20].

Anterior grafts's regenerative potential increased and the outcome of their transplantation was relatively comparable to absence of a graft. In contrast to anterior ECM, the implants of HepIII treated posterior ECM pointed at a twofold decrease in blastema formation and only one case displaying patterned outgrowth (Table 1)[94].

Graft type	Total	Blastema*	Pattern**
No ECM graft	23	21 (91%)	0
Anterior ECM	8	0	0
Anterior ECM grafted in posterior wound	5	5 (100%)	0
Posterior ECM	14	10 (71%)	3 (30%)
Heparin lyase III treated anterior ECM	15	14 (93%)	1 (7%)
Heparin lyase III treated posterior ECM	12	4 (33%)	1 (25%)

Table 1 – Position-specific effects of anterior/posterior ECM grafts on blastema formation are dependant on heparan sulphate

**percentage of total number of grafts that developed an ectopic blastema*

***percentage of ectopic blastemas that developed skeletal elements with pattern. Taken from Phan et al., 2015*

One more study examined the effect of HepIII on pattern formation by the same method (Endo et al. 2004), but in contrast to the previous study [94], the used grafts consisted of periosteal tissue of ulnar periosteum origin. The results of the transplantation assays marked a significant difference in pattern-forming capacity between treated and untreated tissue grafts (Figure 15). While the emergence of pattern was not completely inhibited by enzymatic activity of HepIII, HepIII caused a considerable decrease in complexity of induced pattern in ectopic limbs[101].

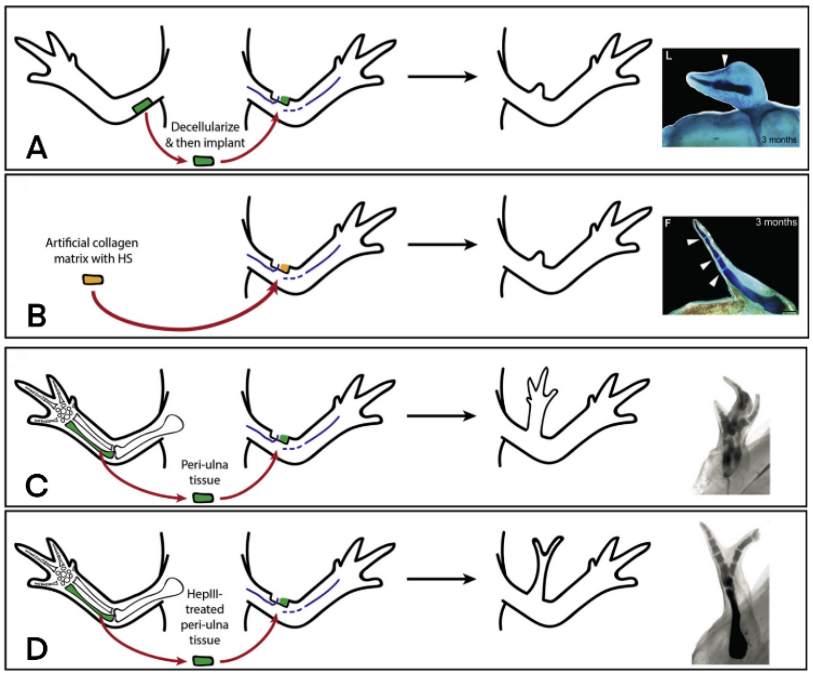
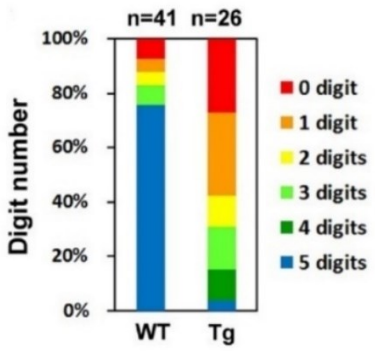


Figure 15 - HS mediated communication of positional information during regeneration. *A) Posterior de-cellularized ECM stimulates the formation of minimally patterned outgrowths, when grafted into an innervated, anterior wound sites. B) Artificial, HS-containing ECM elicits the formation of more complicated patterning relative to de-cellularized posterior ECM, when grafted into an anterior, innervated wound site. C) A complete limb can be patterned when ulna-derived periosteum tissue is grafted into an innervated wound site; however D) heparinase-III treatment of the periosteum reduces the complexity of the resultant pattern. Taken from Vieira et al., 2019. Figure modified from* [16]

6.2.2 Mechanotransduction

The changes in contact between cells themselves and the surrounding ECM are capable of altering cell behavior via recognition of mechanical and physical cues [102,103]. Cells' sense of external cues related to their microenvironment causes them to activate specific gene expression [104–106]. The nuclear factors that trigger corresponding gene expression remain to be properly characterized, however progress in research of regeneration in amphibians was achieved by specifying the Yes-associated protein 1 (Yap1), a transcription regulator of Hippo signaling, which is located in the cytoplasm in its inactivated form, but upon removal of a repressive phosphate Yap1 travels to the nucleus to enable target gene transcription [102,107].

In order to confirm whether the Hippo pathway contributes to regulation of regeneration in vertebrates, S. Hayashi et al. chose to examine conventionally studied Xenopus limb bud. Investigation of Yap1 function was carried out by inhibition of its endogenous expression in a transgenic Xenopus laevis strain, which expresses a dominant-negative form of Yap (dnYap). The mutant dnYap consists of the functional and repressor domain originated from mouse Yap1 and Drosophila engrailed, respectively. The introduced transgene [108]contained a promoter that allows heat-shock inducible overexpression of dnYap and a reporter gene for GFP to signify transgene's activation and therefore was regarded as suitable for temporary perturbation of Yap1 expression [109].



Graph 1 – Percentage of varying degrees of digit number regeneration in WT and dnYap tadpoles. *Taken from*[109]

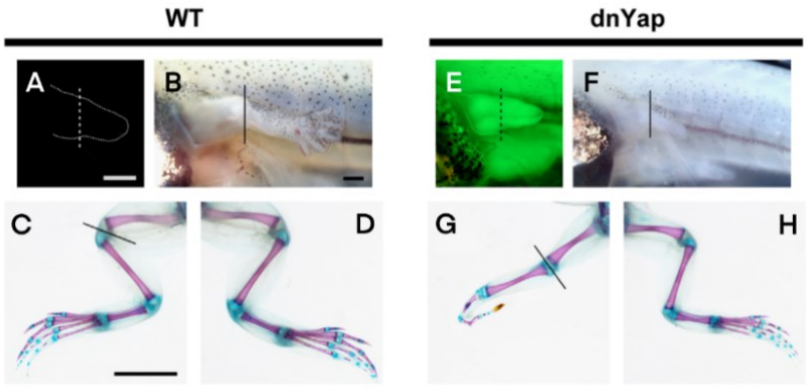


Figure 16 – Loss of Yap1 function caused limb bud regeneration defects. *a-d Examples of limb bud regeneration in WT. (a) GFP reporter expression was not observed after heat-shock in WT. (b) WT tadpole with 5 digits had regenerated at 14 dpa. c,d .. The WT amputated left hindlimb bud had completely regenerated and was equivalent to the intact right hindlimb. (e-f) Examples of limb bud regeneration in dnYap Tg. (e) The GFP reporter was strongly expressed after heat-shock in Tg tadpoles. (f) The regenerating limb was regressed, and no obvious primordia of multiple digits were observed at 14 dpa. (g,h) dnYap Tg animals showed regeneration defects: the number of digits was reduced in the regenerated limb,the intact right hindlimb did not show any obvious developmental defects-. Scale bar¼ 500 mm in (a, b, e, f) and 5 mm in (c, d, g, h). Taken and modified from* [109]

In contrast to WT froglets, dnYap froglets at 14 dpa exhibited drastic anomalies from the WT phenotype, such as missing skeletal structures and a decrease in their size – both reduction and absence of the zeugopod (Figure 16). The effects of Yap1 inhibition were well illustrated by a quantifiable trait - the number of digits. The WT froglets failed to regenerate all digits only in a quarter of cases. However, in the dnYap froglets, the percentage of individuals with less than five digits rose to 96,2 % (Graph 1).

Additionally, it is intriguing that while severe defects were documented in amputated limb buds of dnYap froglets, their contralateral limb buds were unaffected by Yap1 inhibition and developed fully functional limbs, indicating that the Hippo pathway is only necessitous for regenerative pattern formation (Figure 16) [109].

7 Hierarchy behind coordination of limb regeneration

The distinction between cells with intrinsic positional information and those without was confirmed by a number of studies, inferring that CT cells' positionally naïve counterparts are subordinate to them. In comparison to them, CT cells' duty is to retain, recognize, and relay signals to produce an adequate regenerate.

7.1 Functioning positional memory

Positional memory in mature CT cells is the prerequisite for constant ability to form a missing segment pertinent to the scale of injury, and while its retention is presumed to be based on the state of chromatin, only spatially diverse positional identities are capable of inciting blastema and pattern formation. Interestingly, the spatial diversity required for accurate outgrowth patterning appears to be constrained as amputation through a site where limb dermis and epidermis had been exchanged with a full-thickness skin graft excised from axolotl head and flank differ in the probability of successful regeneration [16,110].

The anatomical proximity of head and flank CT cells in relation to limb tissue appears to indicate that the disparity of positional identities itself does not drive regeneration and pattern formation unless the CT cells recognize and interpret the discrepancy of positional identities as relevant. CT cells contributing to axolotls' heads are regeneration competent as the jaw regeneration was documented in axolotls, but it is hypothesized that based on mature CT cells' positional identity specified at development [13,55] cells are differentially capable of interpretation and communication of the instructions needed to reconstruct the exact missing part. Resolution

of a discrepancy between head and limb CT cells provides limb CT cells with context they would never naturally come across and thus cannot comprehend [16].

7.2 Retinioc acid and other pattern forming molecules

RA is a key input necessary for reprogramming of positional identity and impacts chromatin dynamics, tissue-wide biochemical signaling, transcriptional control, and blastema's biomechanical properties. However, RA's reprogramming is limited to times when cells are responsive to it and do not possess stabilized positional information which is dictated by intrinsic developmental history retained by the cells of the limb segment. RA's interactions with chromatin landscape are not fully understood, but it has been proposed that RA's capability to alter positional identity infers it may be placed to the second level of hierarchy of limb pattern formation [13,15,16].

7.2.1 Nonheritable positional cues

In contrast to RA, both FGF8 and SHH do not alter intrinsic cellular positional identity and are assumed to generate pattern only momentarily. The control of Shh signaling is exerted by RA as it is upstream of Shh expression and the methylation state of its enhancer. Induction of an SHH signaling center was observed in posterior blastema upon confrontation of posterior with anterior cells, and later on, it appeared to be involved in a positive feedback loop with anterior expression of FGF8. As regeneration progresses, maintenance of this loop drives distal outgrowth mediated by FGF factors [16,40,62].

Anteriorly restricted FGF8 antagonizes RA's influence and its targets, but from Nacu et al., 2016 study it was asserted that even though RA interacts with FGF8 and SHH at transcriptional level to establish pattern, the molecules on their own influence the process of limb formation only temporarily. This implies that in the hierarchy of pattern formation, FGF8-SHH signaling is at the level below intrinsic cellular memory and RA signaling [16,40].

7.3 Interactions throughout blastema

Local and long-distance relay of positional information generated within the blastema presents the last level at which pattern is communicated. Intercellular bioelectricity and mechanotransduction are transmitted to all blastema cells, and their behavior is subsequently modified. The properties of ECM were proven to affect many morphogenetic processes, such as dedifferentiation and cell fate specification. HS chains in HSPGs are produced by CT cells,

deposited into ECM and their properties are associated with modulation of FGF signaling – therefore, the positional cues they provide cannot be considered as the most basal instructive influence and may take place in-between the last and the upper level of pattern formation control [33,93,94,111].

8 Conclusion

In conclusion, my thesis presents current findings to illustrate the view of a hierarchical status of cells within limb blastema, where CT-derived cells occupy a special position[15,16]. The characteristic feature of these cells is positional memory, which is reminiscent of spatiotemporal gene expression during limb development. Positional memory refers to mature CT cells' chromatin structure, which is spatially diverse and corresponds to cells' position in the limb and, at the same time, enables adequate regeneration[15]. The epigenetic profile of the CT limb cells appears to be primarily determined by repressive histone methylation, selectively driving appropriate transcriptional program of limb segment which had been lost[13]. Two molecules, Prod1 and Tig1, were found to encode proximal position along the proximodistal axis[12,84]. During regeneration, it is indicated that their expression in CT cells declines towards distal limb segments, and in the case of Tig1, this gradient can be observed in CT cells at the steady state. Furthermore, Prod1 and Tig1 are implied in the retinoic acid-mediated signaling network, which includes Meis transcription factors whose expression is impacted by the accessibility of chromatin[15]. Lastly, the proteins display the property of selective adhesion, which allows cell sorting of proximal and distal cells based on differential surface tension[12]. Tissue dynamics has been shown to significantly influence pattern formation in all blastema cells for anterior and posterior limb tissue differ in ECM composition, and decellularized ECM grafts demonstrate that ECM on its own, presumably mainly heparan sulfate, is involved in instructing the formation of pattern in the regenerate[94]. How cells perceive and act on mechanical cues was investigated and documented in a conserved mechanotransduction pathway – the Hippo pathway[111].

Although the exact nature of positional information appears elusive, the study of limb regeneration has demonstrated that control over pattern formation operates at several scales as mature CT cells represent a position and cell-type specific level of control independent of the injury. The imprint of their developmental history can be hereditarily modified to supply missing positional identities, and lastly, the correct limb morphology relies on signaling, which does not provide cells with diverse and hereditary positional identities[15,16].

9 Abbreviations

ALM – accessory limb model
A/P – anteroposterior
BMP-2 – bone morphogenic protein
CT – connective tissue
dnYap – dominant negative Yap mutant
D/V – dorsoventral
ECM – extracellar matrix
FGF – fibroblast growth factor
FGF2 - fibroblast growth factor 2
FGF8 - fibroblast growth factor 8
GAG - glycosaminoglycan
GFP – green fluorescent protein
H3K4me3 – trimethylation at histone 3 lysine 4
H3K27me3 – trimethylation at histone 3 lysine 27
HDAC – histone deacetylase
HepIII – heparan lyase III
HS – heparan sulfate
HSPG – heparan sulfate proteoglycans
LA – lower arm
P/D – proximodistal
PCM – polar coordinate model
RA – retinoic acid
SHH – sonic hedgehog
tbx4, tbx5 – T-box gene 4, T-box gene 5
TF – transcription factor
Tg - transgenic
Tig1 – Tarozene-induced gene 1
TSS - transcription start site
UA – upper arm
VPA – valproic acid
WT – wild type
Yap1 – Yes-associated protein 1

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