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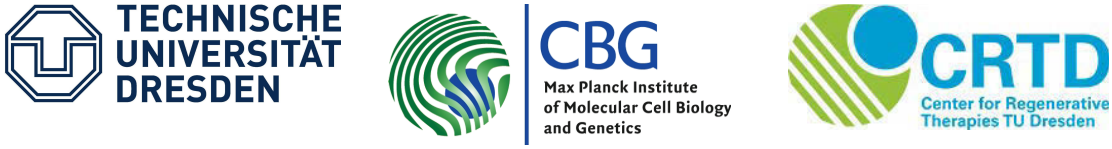
Patterning of stem cells during limb regeneration in *Ambystoma mexicanum*



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List of Abbreviations

Am	*Ambystoma mexicanum*
A/P	anterior/posterior
BrdU	Bromdesoxyuridin
cDNA	complementary Deoxyribonucleid Acid
DNA	Deoxyribonucleid Acid
D/V	dorsal/ventral
eGFP	endogenous Green Fluorescent Protein
EST	Expressed Sequence Tags
FGF	Fibroblast Growth Factor
GST	Glutathione-S-Transferase
GFP	Green fluorescent protein
Hox	*Homeobox genes*
LPM	Lateral plate mesoderm
MEF2C	Myocyte Enhancer Factor 2C
Meis	*Meis homebox gene*
MHC	Mysosin Heavy Chain
Myf5	*Myogenic factor 5*
Msx1	*msh homeobox-1*
p.a.	post-amputation
Pax7	*Paired box protein Pax-7*
PCM	Polar coordinate model
PCR	Polymerase Chain Reaction
P/D	proximo-distal
Prx1	*Paired related homeobox-1*
PSM	Presomitic mesoderm
RNA	Ribonucleic Acid
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
5'RACE	Rapid Amplification of cDNA ends
sc-PCR	single cell – Polymerase Chain Reaction
Tbx5	*T-box transcription factor TBX5*

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Summary

1 Motivation

Regenerative medicine is an emerging field of research with the promise of 'unlocking' the body's natural ability of self-repair. People suffering from organ failure, third-degree burns or limb amputation would greatly benefit from the ability to regenerate lost or damaged tissue structures. It would therefore be a significant 'breakthrough' to repair complex body structures using the molecules and concepts identified by studying regeneration in cellular reprogramming, stem cell biology and tissue engineering. Initially, it will be crucial to understand the fundamental mechanisms and signaling pathways that can stimulate our bodies to regenerate themselves. Mammals are generally accepted to be incapable of regenerating lost tissue structures and are unable to heal their wounds without scarring, which makes mammalian models unsuitable for studying regeneration. Currently, one of the main approaches to mammalian regenerative medicine is the isolation of stem cells, followed by their manipulation with the goal of directing their differentiation towards the morphogenesis of complex body structures (Bianco and Robey, 2001). In contrast to mammals, there is a group of vertebrates, called *urodeles,* that can faithfully restore various tissues following amputation. Many aspects of tissue repair in urodele amphibians, such as axolotls (*Ambystoma mexicanum*), resemble those occurring in mammals, but there is a characteristic distinction in the ability to restore the shape and pattern during tissue repair and regeneration between the two classes. During the process of mammalian tissue repair the shape is not restored, whereas during axolotl regeneration an excellent copy of the missing tissue is restored functionally and shapewise. Evolutionary considerations suggest that regeneration might be a biological principle that also underlies human wound healing. For decades, biologists have been extremely enthusiastic to understand the impressive regenerative ability of urodele amphibians to restore an entire organ because it is one of the most alluring examples of cellular plasticity. The axolotl represents a prime example: it provides an extraordinary system that can be used to elucidate cell and molecular mechanisms involved in the recruitment of progenitor/stem cells. Further, it is the only vertebrate that can regenerate multiple structures such as limbs, tail, spinal cord, jaws and skin at both larval and adult stages. Using the axolotl to study regeneration might give an insight into new promising strategies as well as clinically relevant therapies to induce human tissue repair.

During the process of regeneration, wound healing and the blastema cells are unique whereas dedifferentation and outgrowth are considered to be similar to the events that occur during vertebrate limb development. One fascinating aspect of the axolotl limb regeneration is that the progenitor/stem cells used for regeneration are of local origin; close to the amputation plane. Furthermore, as a characteristic regenerative response, they generate a population of undifferentiated, proliferative cells, the so called blastema cells, which gather at the distal part of the stump. The proximal limb tissue that remains after amputation is called the stump. The blastema contains cells of different tissue origins and with different differentiation potential. By self-renewal, establishment of pattern and differentiation, they form the replica of the limb including all different tissue types such as skin, muscles, peripheral nerves, blood vessels and connective tissue. It was shown by Kragl et al. in 2009 (Kragl et al., 2009) that the blastema is in fact a heterogenous pool of restricted progenitor cells. The lateral late mesoderm-derived cells (LPM) build the cartilage and connective tissue while a separate pool of presomitic mesoderm-derived cells (PSM) build the limb muscle. The finding that limb blastema cells have restrictions similar to those of developing limb bud cells suggests that the signaling events in a blastema probably resemble those of limb development. A key question is not only which cells give rise to the blastema but also how? Therefore, refined lineage tracing of cells is required to understand the molecular mechanism of blastema cell generation.

One of the questions that I have adressed is whether blastema cells are the progeny of reserve (stem) cells or of mature cells that undergo dedifferentiation? Initially, I focused on the question of whether proliferative cells that provide the raw material for regeneration arise from a population of resident stem cells or from dedifferentiation of mature cells. Muscle tissue is a complex structure composed of muscle cells, fibroblasts, blood cells, blood vessels and Schwann cells. Muscle cells were of particular interest because both muscle stem cells and mature muscle dedifferentiation have previously been implicated in salamander regeneration. Despite intensive studies (Slack, 2006; Hay, 1958; Calve and Simon, 2011), quantitative evidence of dedifferentiation was still missing due to a lack of techniques to perform long-term fate mapping of endogenous muscle fibers. Furthermore, the characteristic ability of salamander muscle fiber dedifferentiation has been accepted as a regeneration-specific ability. However, the experiments that supported muscle dedifferentiation were not shown in real-time *in vivo*. Therefore, it would be a major finding to confirm or even disprove muscle fiber dedifferentiation by studying this phenomenon under natural conditions *in vivo*. Previous *in vivo* sudies from my colleagues (Kragl et al., 2009) showed that blastema cells, derived from different limb tissues, remain restricted to their

lineages related to their embryonic origin by using an integrated GFP-transgene. It was also reported that during regeneration muscle makes muscle but not cartilage or epidermis. However, the experiments by Kragl et al., labeled both satellite cells (resident muscle stem cells) and mature muscle fibers; it was not resolved whether regenerated muscle tissue is derived only from stem cells, from dedifferentiated fibers or both. Our challenge was to investigate this long-outstanding question using genetic fate-mapping *Cre-loxP* (site-specific recombinase technology) to determine if muscle dedifferentiation occurs or if muscle tissue arises from a resident stem cell population ((Sandoval-Guzman et al., 2014) [KR1]).

Another important investigation for this field is to determine whether or not mature cells in the axolotl limb retain some stem cell-like properties or if blastema cells are genetically reactivated *de novo* during regeneration. A more precise understanding of limb regeneration will require the ability to molecularly identify different types of blastema cells. Another challenge is to identify molecular markers associated with progenitor cell identity are required to promote proliferation and patterning of the limb ((Kragl et al., 2013) [KR2]). So far, we know that the blastema is a pool of heterogenous progenitor cells (Kragl et al., 2009) and that they retain a memory of their lineage, but it is important to determine if any newly identified molecular markers can be used to distinguish LPM- (lateral plate mesoderm) or PSM- (pre-somitic mesoderm) derived cells or other lineages. It is also important to determine if molecular markers associated with limb bud development are also associated with limb regeneration. There are a number of blastema cell markers such as *Msx1* (Carlson et al., 1998; Koshiba et al., 1998; Simon et al., 1995), *Tbx5* (Khan et al., 2002) and *Prrx1* (Satoh et al., 2007; Suzuki et al., 2005) that have already been identified. At early stages, the blastema contains mainly dermis-derived cells and is enriched with cells expressing these markers, which are found in the limb bud as well. Connective tissue-derived cells most likely reactivate the expression of these marker genes (including Hox genes), but it is unknown if this represents differentiation and reactivation of embryonic mechanisms or if the resident stem cells already harbor this information.

We decided to include the putative homologue of Twist as a candidate gene, even though it is known that Twist, as an early marker of limb blastema, is expressed in mesodermal cells during development, as shown in other species (Tavares et al., 2001). Focusing on our newly isolated Twist sequences, we were encouraged to study the molecular profile of the blastema cells using several progenitor cell markers in order to observe the specific tissue types involved in blastema formation. Therefore, we molecularly profiled individual blastema cells using the newly employed single-cell PCR (sc-PCR) analysis (developed by Martin Kragl). By using sc-PCR, individual cells of the regenerating limb (GFP^+ labeled dermis,-muscle- and cartilage derived cells) are dissociated and analyzed for their expression combined with

several progenitor cell markers. We further linked their expression profile to their embryonic lineage by cell tracking experiments.

Investigations of how the resident stem cells in the adult urodele amputated limb become patterned are indeed crucial. In general, patterning is the process in which the positions and identities of cells with different fates are laid down. Remarkably, if a fully developed adult limb is amputated through the upper arm, only the distal part of the upper arm along with the complete lower arm and hand segments will be regenerated. The cells at the amputation plane will always regenerate elements more distal; a phenomenon that has been termed as 'the rule of distal transformation' (Butler, 1955; Rose, 1962). Additionally, transplanting a whole hand blastema onto an upper arm stump results in regeneration; the positional information between the stump and the hand blastema is intercalated. The intercalated tissue was shown to arise from the proximal stump (Maden, 1980). These results indicated that the cells at the amputation plane have an identity associated with their position along the proximo-distal (P/D) axis. But how do blastema cells form the exact missing portion of the limb independently from the level of amputation along the proximo-distal axis? The ability of mature cells to know their position within the proximo-distal axis of the limb and the ability of blastema cells to know what pattern they have to re-form is called positional information (Wolpert, 1969). The cells aquire the positional information along the proximo-distal axis of the limb from the shoulder (most proximal) to the tip of the fingers (most distal). Blastema cells (limb bud-like mesenchymal cells) as well as developing limb buds possess this information very early to form exactly the missing structure. The main focus of my project was to study how the mechanism of P/D patterning occurs during limb regeneration in comparison with development. Is regeneration of the appropriate limb segments (upper arm, lower arm and hand) occuring in a sequential order as it is proposed during development? Or is intercalation (insertion of the intermediate element-lower arm) at later stages the mechanism for segment formation during limb regeneration? It had been suggested for a long time that, independent of the level of amputation, the first cells that arise at the amputation plane are specific to hand cells creating a gap in positional identity, which triggers the progenitors of the intermediate element from the stump (Gardiner et al., 1995). Based on *whole-mount HoxA* gene expression data, Gardiner et al. (Gardiner et al., 1995) concluded that patterning during regeneration is fundamentally different from that during development. The authors (Gardiner et al., 1995) go on to propose the 'distal-first' hypothesis based on the finding that the early blastema is composed of cells with distal-most (hand identity) and proximal identities (upper arm identity) postulating the following intercalation of the intermediate element (lower arm identity). But clear evidence supporting or even disproving this model is lacking. In my study ((Roensch et al., 2013) [KR3]), we aimed to

achieve a qualitatively higher molecular and cellular resolution by section *in situ* hybridizations in comparison to *whole mount* hybridizations (Gardiner et al., 1995). Furthermore, the early limb blastema - 1 and 2 days post-amputation (p.a.) is a liquid infiltrate of inflammation and clotting associated material so the signal could have orginated from immune cells rather than prospective blastema cells (Lawrence et al., 1996), or the probe could have been trapped in the cavity. Due to the availability of the techniques, we focused on the critical early patterning events by using markers for different limb segments. This included our newly isolated specific posterior axolotl *HoxA9, HoxA11* and *HoxA13* sequences as markers of the future upper arm, lower arm and hand identity respectively. Our challenge was to study in which order the spatial domains are re-established during proximo-distal patterning in comparison to development.

2 State of the Art

2.1 The origin of progenitor cells in the limb blastema

Urodele limb regeneration occurs through different morphological changes and three characteristic steps, which are 1) wound healing and blastema formation, 2) proliferation and limb outgrowth, 3) differentiation and patterning of the missing structure (Figure 2.1-1 (Goss, 1969)) .

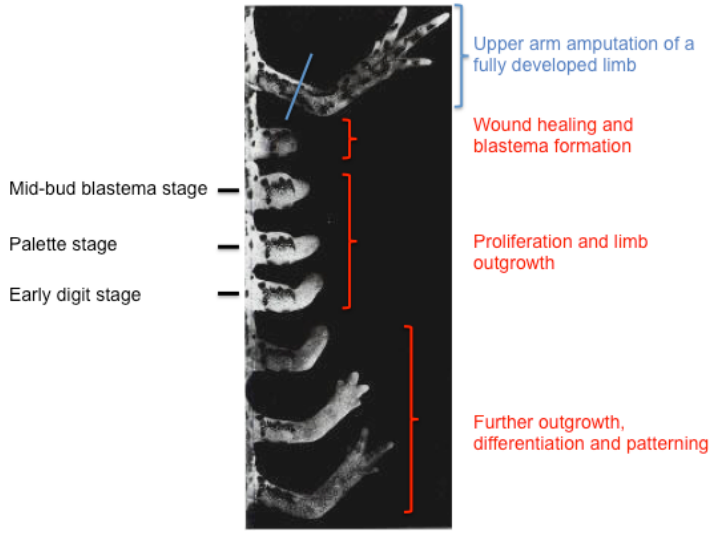


Figure 2.1-1 Urodele limb regenerates after upper arm amputation. Initially, the wound is covered by wound epidermis. A blastema forms adjacent to the wound epidermis and expands due to cell proliferation. After the early digit stage the differentiation becomes obvious, the regenerate further grows and a fully patterned limb is formed. Blue line marks the plane of amputation at the level of humerus. Adapted from (Goss, 1969).

After limb amputation, the exposed stump is covered by a wound epidermis and the blastema is formed underneath (Hay and Fischman, 1961). Within hours after amputation, a simplified epithelium crawls over the stump. Underneath the epidermis, cells derived from serveral mesodermal adult tissues undergo an extended period of proliferation to form a blastema. Depending on the size and age of the urodele amphibians, the blastema is visible between 3 and 7 days post-amputation (p.a.). The blastema, a zone of mesenchymal progenitor cells that accumulates underneath the wound epidermis, proliferates and will completely replace the missing structure – the original fully patterned limb. In general, the blastema cell is defined as a cell in the mesenchymal portion of the blastema. Blastema includes cells that have different tissue origins and differentiation potential. Until the late

midbud stage of regeneration, the blastema has a cone-like shape and resembles morphologically the developing limb bud.

The process of blastema formation has not been thoroughly elucidated, but it is known to arise from of various fully differentiated mesenchymal tissue types at the amputation plane, such as dermis, muscle, bone or Schwann cells. How blastema cells originate from the mature cells in the stump is still unknown. Possibly, blastema cells arise as a result of activation and proliferation of resident stem cells or as a result of dedifferentiation of local differentiated cells. Evidence of blastema cells arising as a result of dedifferentiation came from studying muscle fibers in salamander regeneration.

2.2 The origin of regenerated skeletal muscle tissue

It was shown, that muscle is an important contributor to blastema formation (Brockes, 1997). The skeletal muscle fiber in is a multinucleate cell type and its differentiation during embryonic development is characterized by the cellular fusion of somite-derived precursor cells (Buckingham, 2001; Tajbakhsh, 2005). One fascinating aspect is the reversal of differentiation during salamander appendange regeneration. The hypothesis that blastema cells arise as a result of dedifferentiation of resident stem cells was based on the morphological changes in muscle fibers during regeneration (Hay, 1958) observed by electron microscopy. In this study, they detected mononucleated cells, which seemed to break off from existing muscle fibers and enter the blastema. Another study used *in vitro* cultured *newt* myotubes, which were labeled with dye or transfected with virus, to show that myotubes are able to fragment and contribute to the regenerate after reimplanting them back into the regenerating limb blastema (Kumar et al., 2000; Lo et al., 1993).

In contrast to salamanders, adult mammalian skeletal muscle regenerates after injury without forming a blastema (Charge and Rudnicki, 2004). Also, the mammalian skeletal muscle regeneration does not include the cellularization of mononucleated cells. Instead, a stem cell population (satellite cells), which expresses Pax7 (generic marker of satellite cells), reenters the cell cycle, proliferates and incorporates into nascent or preexisting myofibers during mammalian muscle regeneration (Cornelison and Wold, 1997; Collins et al.,2005).
Evidence that resident stem cells most likely participitate in regeneration was demonstrated in *Xenopus* tail regeneration (Chen et al., 2006; Gargioli and Slack, 2004). When only muscle fibers of the *Xenopus* were genetically altered to constitutively express GFP, no blastema cells expressed GFP upon tail amputation. However, when the experiment was repeated in animals where both muscle fibers and satellite cells expressed GFP constitutively, cells

expressing GFP were observed in the blastema, indicating that satellite cells are the main soure of muscle progenitors. Other studies in salamander also demonstrated that $PAX7^+$ cells exist in mature muscle tissue and become proliferative after limb amputation (Morrison et al., 2006). They used BrdU to track cultured *newt* muscle satellite cells ($PAX7^+$) *in vivo* to show that BrdU positive cells labelled the cartilage, muscle and epidermis after complete regeneration. Most likely, BrdU could have been transfered from transplanted cells to the host cells (Burns et al., 2006). Interestingly, until that time it was widely accepted that dedifferentiation of muscle cells are the source of blastema cells. The distinctive characteristic of muscle fibers to dedifferentiate was thought to be a salamander-specific regenerative ability. Skeletal muscle has been intensively studied (Slack, 2006), but so far the quantitative evidence of dedifferentiation has been missing due to the inability to perform long-term fate mapping of endogenous muscle fibers.

Therefore, it is still debatable as to whether or not muscle tissue acquires a broad plasticity (Echeverri and Tanaka, 2002). Further studies based on short-term muscle labeling indicated that during the first weeks after amputation of a limb or tail, multinucleated myofibers and implanted myotubes dedifferentiate into mononuclear, proliferative cells (Calve and Simon, 2011; Echeverri et al., 2001; Lo et al., 1993). The *newt* myotube nuclei were stimulated *in vitro* to re-enter the cell cycle (Tanaka et al., 1997) and it was further demonstrated that several conditions could induce myofiber fragmentation into smaller myotubes or even mononuclear cells (Calve et al., 2010; Duckmanton et al., 2005; Kumar et al., 2004; Odelberg et al., 2000). Short-term experiments that traced dextran-injected myotubes implanted into a *newt* limb blastema showed that transplanted cells incorporated into cartilage, suggesting that muscle cells dedifferentiate into multipotent progenitor cells (Lo et al., 1993). However, the suggested muscle cell plasticity could have been a result of *in vitro* conditions and may not occur *in vivo*. At this time it was crucial to permanently label different cell types using an integrated GFP-transgene (Kragl et al., 2009). Using this transgenic tool of genetic fate mapping, my colleagues demonstrated that mature muscle cells form regenerated muscle tissue, but not cartilage or epidermis. However, it remained unclear whether regenerated muscle tissue is derived from satellite cells, from dedifferentiation of muscle cells or even both. In fact few studies have investigated the contributing tissues at a cellular level to this day (Iten and Bryant, 1973). A lack of molecular blastema markers has also impeded the perspective isolation of blastema progenitors. It is therefore still unclear if differentiated cells reverse their mature phenotypes or to what extent undifferentiated cells, such as stem cells, localized within differentiated tissue, become activated.

2.3 Molecular basis of blastema formation: Twist genes as lineage specific blastema cell marker

The developmental potential of the progenitor cells within the limb blastema are similar to the progenitor cells in the limb bud. It was shown that lateral plate mesoderm-derived cells form the cartilage and the dermis whereas a separate pool of presomitic mesoderm-derived cells build the muscle during limb regeneration (Kragl et al., 2009). It is known that Twist, as one early marker of limb blastema mesenchyme, is expressed in mesodermal cells during development, as shown in other species such as chicken (Tavares et al., 2001). To what extent are they molecularly recapitulated during blastema formation? Studying the expression pattern during the course of limb regeneration would provide insight into whether or not *Twist-1* and *Twist-3* are expressed in distinct spatial domains during development in comparison to regeneration. Furthermore, determining the pattern of Twist expression in different progenitor cell populations during limb development versus limb regeneration could reveal to what extent the regenerative process reflects or even recapitulates the process of limb development.

Twist-1 was shown to be expressed in the early limb bud. It plays a crucial functional role in growth and differentiation as well as in regulation of limb bud patterning (Krawchuk et al., 2010; Loebel et al., 2012; O'Rourke and Tam, 2002; Tavares et al., 2001). Loss of function leads to pleiotropic defects in limb growth and patterning (O'Rourke and Tam, 2002). Furthermore, *Twist-1* is an essential mesenchymal transcription factor to maintain epithelial-mesenchymal signalling during limb bud progression. The limb outgrowth of a Twist-1$^{-/-}$ knock-out demonstrated that the mouse forelimb bud was impaired and smaller compared with wild type (Chen and Behringer, 1995; Zuniga et al., 2002). Further studies of *Twist-1*$^{-/-}$ knock-out mice showed reduced FGF-10 expression and absence of FGF-4 expression (O'Rourke et al., 2002). During early chicken embryonic development, *Twist-1* was found in the somites, lateral plate mesoderm and limb mesenchyme. At later stages the expression was detected in the sclerotome, dermatome, limb bud mesenchyme and interdigital regions (Tavares et al., 2001), which are regions that give rise to the developing limb bud. In mice, *Twist-1* gene expression was detected in head neural crest, sclerotome dermatome of the somites, in the lateral plate mesoderm and in the developing limb bud (Li et al., 1995; Wolf et al., 1991). Therefore, *Twist-1* was identified as an important early molecular regulator during limb development that is expressed in undifferentiated mesenchymal tissue of the developing limb bud. It represents a useful candidate marker for a large population of limb blastema cells.

Expression analysis of *Twist-2* and *Twist-3* (also called *Dermo1*) in mice and avians showed that both are initially present in subectodermal mesenchyme from early dermal differentiation and later in the development of skeletal elements (Chen and Behringer, 1995; Li et al., 1995; Scaal et al., 2001). Furthermore, it is important to consider the phylogenetic analysis of the Twist genes. Nine representative species were used to study the vertebrate Twist family and three family members were found: *Twist-1*, *Twist-2* and *Twist-3*. Twist genes are highly conserved, but the copy number and function have diverged. During vertebrate evolution, multiple deletions and duplications of Twist genes occurred in various evolutionary lineages (Gitelman, 2007). For example, chicken and fish have retained all Twist genes whereas mammals have lost *Twist-3* and preserved *Twist-2*. In *Xenopus*, a member of the amphibians, *Twist-2* was lost but the *Twist-3* paralog aquired *Twist-2* functions. The two paralogs (*Twist-2* or *Twist-3*) appear to play similar roles and show similar expression patterns.

So far, in axolotl, only one partial Twist-like sequence has been studied (Satoh et al., 2008). Satoh et. al. isolated a partial Twist sequence from axolotl, containing the highly conserved bHLH region. The first axolotl orthologue of Twist was sequenced by that time, but the exact orthology remained unclear. Satoh and colleagues described its expression pattern during limb regeneration and Twist was shown to be a marker for blastema cell dedifferentiation of the connective-tissue derived cells during limb regeneration. Further investigations of the connective tissue cells during P/D patterning of the limb are indeed crucial.

2.4 Patterning events during regeneration

Structures more distal to the amputation plane are restored during regeneration. Historically, scientists performed rudimentary experiments to describe the fundamental characteristics of a regenerated limb. For example, if a fully developed adult limb is amputated through the upper arm, the distal part of the upper arm, elbow, lower arm, wrist and hand will be regenerated. When the amputation happens at the level of the hand- only the hand element will regenerate. The cells at the amputation plane will always regenerate limb elements more distal to that location, a phenomenon termed ‘the rule of distal transformation’ (Figure 2.4-1). The phenomenon was demonstrated by creating a limb that was inverted along the P/D axis (Butler, 1955; Rose, 1962). A hand stump was sutured to the body trunk of the same animal and allowed to heal. The distal element (hand stump) ended up being proximal to the elbow. Amputation of the limb through the upper arm in both cases lead to regeneration of structures distal to the amputation plane. The results demonstrated that cells at the level of amputation posses an identity associated with their position along the P/D axis. Therefore,

the resident cells of a fully developed limb need to know their position within the limb and cells which enter the blastema need to know which element of the P/D axis they have to rebuild (Wolpert, 1969).

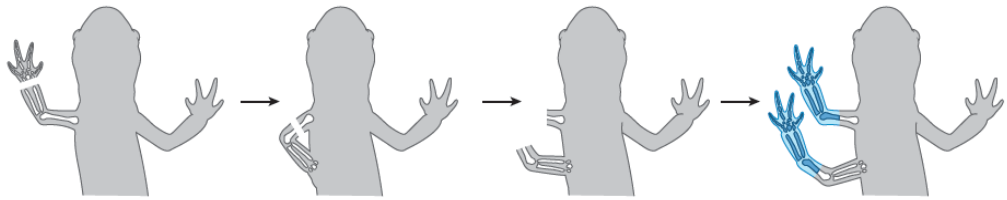


Figure 2.4-1 A regenerated limb harbours more distal structures. The hand stump was sutured to the body trunk. After healing, the limb was amputated through the upper arm. The elements distal of their origin regenerated in both cases-from the normal upper arm stump and from the inverted limb. Original experiment described in (Butler, 1955), Adapted from (Nacu and Tanaka, 2011).

Previous experiments have shown that the essential patterning information is already established very early in the blastema at the stage when the cells are still undifferentiated (Dearlove and Stocum, 1974; Michael and Faber, 1961; Stocum, 1968). This was demonstrated by transplantation of the midbud blastema stage to a neutral environment, such as the dorsal fin, where it resulted in the formation of all limb elements that would have formed *in situ* (Stocum, 1968). Another study (Echeverri and Tanaka, 2005) from our lab found that patterning information is required very early during the course of regeneration by performing cell labeling and inter-blastema transplantations experiments. The cells in the proximal and distal region were labeled by electroporation a plasmid that expresses constitutively GFP. Depending on where the cells were labeled (proximal vs. distal) they ended up in their region of origin-the labeled cells were found in the regenerated upper arm and hand. The authors concluded from the results that the blastema is probably a mixed pool of cells with different positional identities that sort themselves out at later stages during regeneration. When fluorescent cells from the distal tip of a 4 day hand blastema stage were transplanted to the proximal region of an 8 day blastema, the labeled cells contributed to the regenerated hand, indicating that at that time the cells were already specified to their distal cell fate. Another sudy demonstrated by transplanting a hand blastema onto an upper arm stump results in intercalation of the intermediate (lower arm) missing structures from the stump cells (Maden, 1980). Intercalation has been defined on a tissue level phenomenon. On a cellular level, intercalation would imply that progenitor cells at the amputation plane have a graded property manifested on their surface. When most proximal and distal cells ‘meet’ each other- the cells respond by proliferation and producing cells that ‘fill in’ the missing part. Intercalation does not occur when a hand blastema was grafted onto the lower arm blastema- cells will migrate to the wrist of the regenerate and will form an ectopic hand, indicating the critical role of cell surface interactions in pattern formation. The experiments

from Echeverri et al. (Echeverri and Tanaka, 2005) and Maden (Maden, 1980) supported the model where the most distal structures are specified first during regeneration and the values between the stump (proximal) and the distal cells are intercalated.

From these studies, a new question arose: Is positional identity tissue specific? In 2009, Kragl et al. (Kragl et al., 2009) showed that fingertip GFP^+ labeled cartilage cells were transplanted into the upper arm of a white host. After healing and folllowing amputation, GFP^+ cells were not found in the upper arm nor in the lower arm, but only in the hand region. Cartilage cells cannot produce more proximal cell types then their position of origin and, hence, they obey the rule of distal transformation. In contrast, upon similar transplantations, Schwann cells were found along the entire P/D axis of the limb and therefore do not obey that rule. Furthermore, cartilage-derived cells express molecular markers of P/D positional identity such as *HoxA13* whereas Schwann cell-derived cells do not. Nacu et al. (Nacu et al., 2013) demonstrated that connective tissue cells, and not muscle or Schwann cells, are involved in the establishment of the P/D patterning. Taken together these results indicate that connective tissue cells are the main cellular determinants of limb P/D patterning during regeneration.

2.5 Hox genes as marker of proximo-distal limb segments

How is positional identity established in order to give rise to more distal parts of the limb? And how are limb segments re-specified after amputation? A likely contributor to these processes is the HOX family of homeodomain transcription factors, which play an important role in pattern formation of the primary and secondary body axis during development. Mammals have four HOX clusters- HoxA, HoxB, HoxC and HoxD. The expression of genes from the HoxA locus is generally collinear, which means that during limb development they are expressed in order of their location on the chromosome. The 3' Hox genes are expressed first whereas the 5' located Hox genes are expressed later and more distally of the limb bud. HoxA and HoxD genes (paralog genes) are useful markers to study P/D positional identity, showing similar but not identical expression domains. During early limb bud development, the Hox9 paralogs are detected throughout the entire limb bud, Hox11 paralogs are expressed in future lower limbs and hands and Hox13 paralogs are expressed in the future carpus and digits exclusively (Zakany and Duboule, 2007). In later stages of development the Hox genes have distinct expression domains (Nelson et al., 1996; Tabin and Wolpert, 2007). A functional double knock-out of *hoxa11* and *hoxd11* in mice resulted in an absence of the radius and ulna (Davis et al., 1995) (Figure 2.5-1). A combined knock-out of *hoxa13* and *hoxd13* showed drastic malformations of the hand and digits (Fromental-Ramain et al.,

1996b), demonstrating the important role of Hox elements during limb development. Since it is well established that the HoxA family can function as a marker of proximo-distal positional identities, we are able to investigate the progression of limb segment identities during development as well as during regeneration.

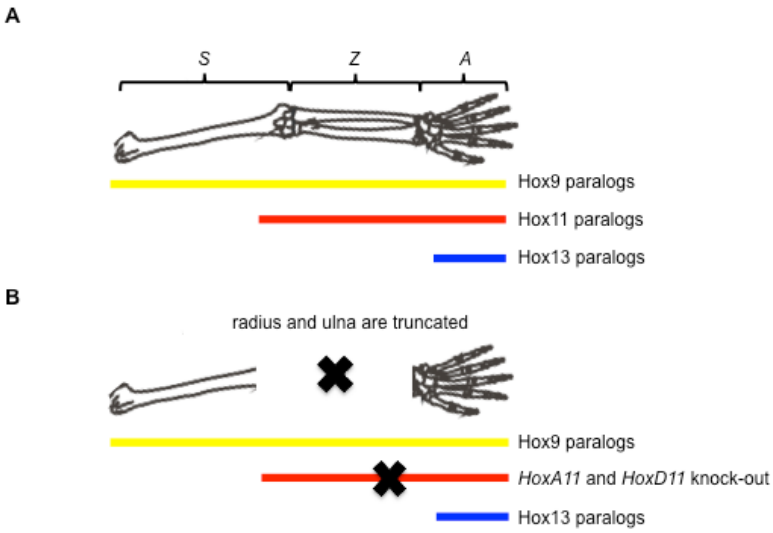


Figure 2.5-1: A functional double knock-out of hoxa11 and hoxd11 resulted in the absence of the radius and ulna. (A) S- stylopod comprising humerus, defining upper arm; Z- zeugopod comprising radius and ulna, defining lower arm; A – autopod comprising carpus and digits. Hox9 paralogs during early limb development will give rise to the cells of the whole limb (stylopod, zeugopod and autopod), Hox11 paralogs will mark the cells of the future zeugopod and autopod and Hox13 paralogs will give rise to the cells of the future digits of the autopod. (B) In the absence of both HoxA11 and HoxD11 the zeugopod is truncated. Stylopod is normal and autopods detected with minor defects.

2.6 Models of P/D patterning during development and regeneration

Based on the studies of markers for P/D positional identities, several models have been proposed to explain in which order the proximo-distal structures are specified during development and regeneration.

2.6.1 Models of limb development

Morphologically, vertebrate limbs consist of three major segments: upper arm, lower arm and hand. Already before these structures become recognizable, developing vertebrate limb buds can be subdivided into three distinct regions based on the expression of the limb segment markers (posterior HoxA family). The zone expression *HoxA9*$^{+}$*/HoxA11*$^{-}$*/HoxA13*$^{-}$ will give rise to the progenitor cells of the upper arm, *HoxA9*$^{+}$*/HoxA11*$^{+}$*/HoxA13*$^{-}$ of the lower arm, and *HoxA9*$^{+}$*/HoxA11*$^{+}$*/HoxA13*$^{+}$ will form the hand element (Fromental-Ramain et al., 1996a; Fromental-Ramain et al., 1996b; Zakany and Duboule, 2007).

Two main classes of models have been proposed in the past to explain how the proximo-distal structures are specified during development. The first class of models propose that the progenitor cells are specified in a proximal to distal order during limb development. This is supported by the 'progress zone model' (progressive specification) (Summerbell et al., 1973). According to the model, signals from the overlaying AER (Apical Ectodermal Ridge), a thickened epithelium, keep the cells in an undifferentiated state. The proximo-distal values are specified by the period of time cells spend in the undifferentiated zone (the progress zone). The distal cells in the progress zone autonomously undergo a progressive change of proximo-distal positional specification from more proximal to distal cell fates, for as long as they are within the progress zone. The 'progress zone model' is consistent with the molecular expression data that indicates that progenitor cells express *HoxA9*, followed by the sequential expression *of HoxA11* more distally and the nested expression *of HoxA13* in the distalmost tip, as the developing limb bud emerges shown in other vertebrates such as mice and chicken (Haack and Gruss, 1993; Nelson et al., 1996; Yokouchi et al., 1991). This expression data combined with functional studies suggested that the progenitor cells are specified in a proximal to distal order during limb development.

The second class of models, such as the 'early specification model', suggests that all proximo-distal limb segments are specified early during development (Dudley et al., 2002). The segment-specific progenitor pools expand during limb bud expansion and the distal limb mesenchyme becomes progressively determined. The model was developed to explain the distal truncations observed by AER removal. As the limb bud emerges, a 200-µm domain of

cell death becomes a proportionally smaller portion of the total limb bud, resulting in progressively less distal truncations in skeletal pattern. By fate mapping experiments, it was demonstrated that marked distal cells are not incorporated into skeletal structures after AER removal (Dudley et al., 2002). Therefore, the skeletal trunctions follwed by AER removal would reflect the apopotic loss of already specified elements.

Further 'opposing gradient models' take into account other observations. For example, FGFs have been shown to restrict the RA effects in the distal region of the developing limb bud (Mariani et al., 2008; Mercader et al., 2000). An endogenous morphogen, such as retinoic acid, produced proximal to the limb bud, is also known to be involved in limb patterning (Mandal et al., 2013; Rosello-Diez et al., 2011) by acting as an inducer of proximal characteristics (Mercader et al., 2000; Yashiro et al., 2004).

Both the 'early specification model' and the 'progress zone model (described above) are based on historical data. However, when considering more recent molecular data, neither of these models can explain current observations. Lewandoski et al. in 2000 (Lewandoski et al., 2000) demonstrated that FGF-8 signaling from the AER is indeed necessary for the limb development. These days, a new way of conceptualizing proximo-distal specification (Tabin and Wolpert, 2007) was proposed in 2007 by the 'differentiation-front model' (Tabin and Wolpert, 2007). It postulates that P/D identities are determined as the proliferating mesenchyme leaves the undifferentiated zone- the timepoint when mesenchyme is no longer under the influence of the proximalizing action of retinoic acid (RA) and the proliferative and distalizing influence of the AER-FGF signaling (Mercader et al., 2000). As the limb bud emerges and cells leave the range of RA-signaling, the cells distalize, whereas the proximal cells are progressively determined as indicated by limb segment-specific marker genes. The cells distal of the Meis (marker of the future stylopod) (Mercader et al., 2005) zone express *HoxA11* whereas the cells at the tip of the limb bud have the most distal identity and express *HoxA11* and *HoxA13*. During later stages of limb development, the distal cells only express *HoxA13*.

In principle, regeneration does not necessarily create a whole limb; it just regenerates the missing structures distal of the amputation plane. Therefore, regenerative proximo-distal identity specification is possibly different from development. We need to consider several models of forming proximo-distal identities.

2.6.2 Models of forming proximo-distal identities during regeneration

Several models that describe the regeneration of the different limb identities need to be considered in the case of how early patterning events might occur (Figure 2.6-1). This is

because there are some alternative models postulated of how the blastema is formed at early stages and expands during limb outgrowth.

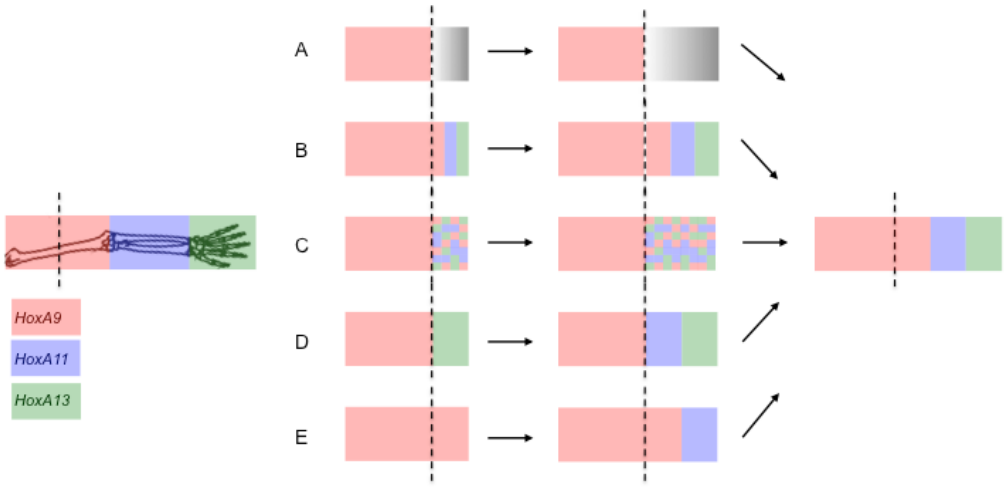


Figure 2.6-1 Theoretical models of how P/D identities could be re-established during regeneration by HoxA gene expressing populations. (A) A morphogen gradient in the blastema determines the positional identity of the cells. (B) All positional identities are specified at the same time and they are organized in their domains and expand during regeneration. (C) The blastema is a mixed pool of different positional identities (randomly distributed) that are sorted into their specific domains at later stages. (D) Distal identities are specified initially after amputation and the intermediate elements are intercalated at later stages through cell surface interactions. (E) The proximo-distal positional identities are specified sequentially similar to development. Red marks the stylopod and future stylopod cells, Blue - the zeugopod and future zeugopod cells, Green- the autopod and future autopod cells. Dashed perpendicular line marks the plane of amputation at the level of humerus.

(A) Initially, we have to consider the possibility that there is a signal produced, most likely from the AER, which forms a concentration gradient as it spreads out into the surrounding blastema (Figure 2.6-1 (A)). The graded signalling molecule acts directly on cells in a concentration dependent manner to specify gene expression changes and cell fate selection (Tabata and Takei, 2004).

(B) Another possibility could be that during early patterning, the upper arm, lower arm and hand progenitor cells at the amputation plane are organized initially in their appropriate domains (Figure 2.6-1 (B)) accoring to the ‘early specification model’.

(C) Or they are randomly distributed and subsequently sorted into their appropriate domains (Figure 2.6-1 (C)).

(D) Alternatively, the first blastema cells formed after amputation may have the most distal identity and this is followed by the subsequent intercalation of the intermediate P/D identities

occuring within the blastema during the process of regeneration (Figure 2.6-1 (D)) proposed by Gardiner et al in 1995 (Gardiner et al., 1995) by the 'distal-first' hypothesis. On the cellular level, intercalation would imply that the resident progenitor cells can form an express gradient of molecules on their cell surface.

(E) Lastly, the process of blastema patterning might be similar to the mechanism of patterning of the limb bud in development. Cells first retrieve the identity appropriate to the amputation plane (upper arm) followed by the sequential progressive specification of the more distal identities (Figure 2.6-1 (E)). Echeverri et al. (Echeverri and Tanaka, 2005) performed cell labelling via electroporation of a constitutively expressing GFP plasmid at different zones of the blastema. Proximal labeled cells in a blastema form proximal structures whereas distal parts of the blastema generate distal structures. They excluded from their studies that the blastema is a mixed pool of progenitor cells with different positional identities that reorganise themselves at later stages of development (Figure 2.6-1 (C)).

The most plausible model that has been accepted for a long time was the model of intercalary regeneration (Figure 2.6-1 (D)). The understanding of how patterning occurs during limb regeneneration was drastically influenced by tissue grafting experiments in insect and salamander legs, which exhibit disparate responses of limb cells when their neighbors are changed. For example, in cockroach they showed (Bohn, 1976) that grafting a distal piece of a foreleg onto a proximal hind leg stump resulted in restoration of the missing intermediate segments. They referred to this as 'intercalation model' based on the morhology of the outgrowth, not on processes at the cellular level. Tissue grafting experiments in salamander limbs indicated that such patterning events may be more conserved. This is because when a hand blastema (distal blastema) was grafted onto an upper stump, it resulted in intercalation of the intermediate (lower arm) missing element. The intermediate element ensued from the stump tissue (Maden, 1980; Pesciteli and Stocum, 1980). These experiments support the idea that the distal structures are specified first during regeneration and that the values between the cells of the stump and distal cells are intercalated via cell surface interactions (Figure 2.6-1 (D)). When an upper arm blastema was transplanted onto a wrist stump, an upper arm regenerated from the wrist autonomously without tissue intercalation between the wrist and the upper arm – demonstrating a converse result to the described insect limb grafting experiments.

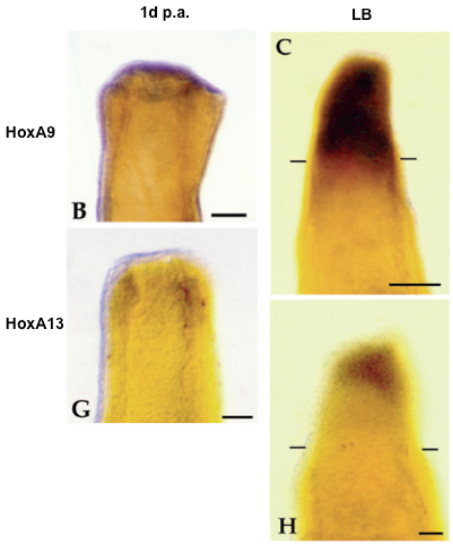


Figure 2.6-2 Re-expression of HoxA9 and HoxA13 during regeneration. Synchronous HoxA9 and HoxA13 re-expression at early stages during regeneration starting at 1 day p.a. at the level of the humerus. The expression domains of HoxA9 and HoxA13 become spatially distinct at the late limb bud stage (LB). Adapted from (Gardiner et al., 1995).

Based on *whole-mount* experiments, Gardiner et al. (Gardiner et al., 1995) concluded (Figure 2.6-2) that the order of HoxA gene expression during regeneration is converse from that during development. This led to Gardiner et al.'s 'distal-first' hypothesis (*hoxa13* upregulation at 1 day p.a.), supporting the models (Figure 2.6-1 (A-D)) where the distal blastema cells are specified early during regeneration. The results, however, did not show convincing cellular resolutions by whole mount *in situ* hybridizations, so the precise location of the signal and whether it is truly in blastema cells remains unclear. Due to technical limitations, it has remained unresolved whether salamander limb blastema cells become specified in a progressive, proximal-to-distal order or distal first, followed by intercalation.

3 Objectives

One fascinating aspect of using the limb to study the phenomenon of regeneration is how appendage amputation stimulates recruitment of cells next to the amputation plane to generate a population of undifferentiated, proliferative cells called the blastema. The blastema has all the information required to generate the correct differentiated cell types and correct pattern to regenerate only the missing portion of the limb.

Specific aims

1. One aim of my work was to understand how cells at the amputation plane assemble the blastema. Does the blastema arise from resident stem cells or by cellular dedifferentiation?

We labeled and traced the endogenous muscle fibers during axolotl limb regeneration to determine if muscle dedifferentiation occurs or if muscle tissue arises from a resident stem cell population.

(1) We addressed the question by using the *Cre-loxP* system to long-term fate map endogenous muscle fibers and follow them during the process of regeneration.

(2) The specificity of the *Cre-loxP* system was confirmed using well-characterized germline transgenic animals to perform cell-fusion-mediated muscle-specific labeling of blastema grafting experiments.

(3) The specificity of the muscle labeling was confirmed by analyzing the expression of muscle-specific markers such as MHC, MEF2C and PAX7.

Specifically, I contributed to the studies in which we determined whether axolotl $PAX7^{+}$ satellite cells contribute to the regenerated muscle and muscle stem cells.

2. Another goal of my project was to identify molecular markers that provide insight into the relationship of cell differentiation and cell specification in the regenerate. In order to achieve this aim we investigated connective tissue-derived blastema cells, since this is the main cell population that plays a role in patterning of the regenerated limb.

(1) I used the newly isolated Twist genes as molecular markers of the connective tissue-derived blastema cells and investigated their expression in embryonic development and in limb regeneration.

(2) To determine the spatial localization of their mRNAs I performed section *in situ* hybridizations on embryos of different stages.

(3) I examined the expression pattern during different stages of limb bud development in comparison to limb regeneration by *in situ* hybridizations.
(4) We analyzed *Twist-1* and *Twist-3* expression status and compared the expression level with lineage-specific genes like *Myf5* and *Sox9* in LPM- versus PSM-derived cells using single-cell PCR analysis.
(5) To distinguish from which lineage the regenerating cells are derived, I performed *in situ* hybridizations on LPM- versus PSM specific labeled tissue. We achieved the specific labelling of the major limb tissue by grafting the embryonic GFP-lateral plate mesoderm (LPM) or GFP^-PSM presomitic mesoderm from GFP^+ transgenic donors into white (GFP^-) host embryos.

3. The main aim of my project was to understand the mechanism of proximo-distal patterning underlying normal limb regeneration. Gardiner et al. (Gardiner et al., 1995) have previously proposed the 'distal-first' hypothesis based on *whole mount in situ* hybridizations. The hypothesis states that after amputation through the upper arm the early blastema is only composed of cells with 'distal-most' (hand) identity and proximal identities (upper arm), while the intermediate element (lower arm) arises later from an interaction between the distal cells and proximal identities (intercalation). To further explore this hypothesis we investigated the critical early patterning events using higher qualitative molecular and cellular resolution to analyze the positional identity markers *HoxA9*, *HoxA11* and *HoxA13*. The expression pattern of these genes mark the future upper arm, lower arm and hand identity, which allowed us to examine the progression of the limb identities during limb development and regeneration.
(1) We isolated full-length cDNA clones of axolotl *HoxA9*, *HoxA11* and *HoxA13*. I investigated their expression pattern by *in situ* hyridization. I generated polyclonal antibodies against the proteins and used additionally immunohistochemical analysis at different stages of limb development to characterize the antibodies.
(2) I studied the progression of HOXA protein expression in the regenerating limb at early time points. Due to the absence of a morphologically defined blastema at 1 day p.a., we alternatively used limb samples in which connective tissue derived blastema precursors had been labeled with GFP-expression.
(3) I developed a transplantation assay to functionally assess the order of limb blastema cell specification. More specifically, I used this assay to determine if early upper arm blastema cells already possess hand identity as predicted by the intercalation model.

4 Own research results

4.1 Myofibers do not contribute to limb regeneration

The goal of this work ((Sandoval-Guzman et al., 2014) [KR1]) was to permanently label and trace muscle fibers during regeneration in axolotl, in order to address the long debated question of whether proliferative cells arise from a population of resident stem cells or from dedifferentiation of mature cells.

To assess whether axolotl myofibers contribute to limb regeneration, we performed fate mapping experiments using the *Cre/loxp* system. The labeled YFP expression associated with myofibers in the axolotl where exclusively restricted to the upper arm region (Fig.3 [KR1]). Conclusively, myofibers do not contribute to the limb regeneration. We used the powerful system of germline transmission transgenesis (Sobkow et al., 2006) to confirm the observation that myofibers do not contribute to muscle regeneration. The transgenic *loxP* reporter animals (*CAGGS:loxp-GFP-STOP-loxp-Cherry*) and tamoxifen-inducible *Cre* line (*CAGGS:ert2-cre-ert-T2A-nucGFP)* were used (Khattak et al., 2013). Our team performed cell-fusion-mediated muscle-specific labeling of the genotype *CAGGS:loxP Cherry* with the genotype yielding *CAGGS:ert nucGFP* myoblasts-the nuclei of these two genotypes would share one cytoplasm (Fig. S4 [KR1]). The shared cytoplasm which allows the inducible CRE protein from one nucleus to enter the *CAGGS:loxP Cherry* transgenic nucleus. By tamoxifen induction, the recombination would occur showing Cherry expression throughout the myofiber cytoplasm. We gained cell-fusion-mediated muscle-specific labeling of *CAGGS:loxP Cherry* and *CAGGS:ert nucGFP* myoblasts by grafting an upper arm blastema from one genotype to the upper arm of the host animal of the other genotype. After complete limb regeneration, no $Cherry^{+}$ myofibers were found in the mononucleated cells, whereas after tamoxifen injection the myofibers exhibited strong Cherry expression (Fig. 4 [KR1]). The muscle marker, MHC, was used to determine the specificity of Cherry expression (Fig. S5 [KR1]).

To further investigate if myofibers contribute to the limb regenerate, we amputated the limb through labeled fibers at the level of humerus and followed the fate of $Cherry^{+}$ myofibers. They were restricted to the upper arm stump, no $MHC^{+}/Cherry^{+}$ myofibers were found in the lower arm or in the hand suggesting that myofibers do not contribute to the regenerated limb (Fig 4 [KR1]) visualized by whole-mount microscopy.

However, a Cherry$^+$ signal that could possibly be generated by mononucleate cells was exclusively present in close proximity to the amputation plane suggesting a possible contribution of muscle-derived mononucleated, proliferative cells. Additionally by immunohistochemistry performed on sections of a 10 day limb blastema, the Cherry$^+$ signal did not colocalize with the proliferation marker PCNA and no Cherry$^+$ cells colocalized with PAX7$^+$ blastema cells (Fig. S7 [KR1]). It has been shown that myofibers at the amputation plane undergo morphological changes, but there was no evidence of myofiber contribution to proliferative cells.

To investigate the source of cells for muscle regeneration, we used GFP-labeled PSM embryonic transplants to follow the contribution of these cells to the regenerate. As demonstrated by Kragl et al. 2009 (Kragl et al., 2009), we showed that the GFP$^+$ signal was colocalized with MEF2C$^+$ and PAX7$^+$ signal (Fig. 5 [KR1]), indicating that PAX7$^+$ satellite cells are the main source of regenerated muscle. To confirm that satellite cells participate to muscle regeneration, I quantitatively traced the PAX7$^+$ cells in the blastema, where almost all GFP$^+$ cells of the GFP-PSM transplanted animal expressed the protein PAX7 (Fig. 5 [KR1]). By analysing which GFP$^+$ cells colocalized with the EdU nucleotide (EdU pulse injection before blastema was collected) and PCNA, we found that the majority of GPF$^+$ cells also expressed the proliferation marker PCNA (Fig. 5 [KR1]). The results demonstrated that PAX7$^+$ satellite cells from the mature tissue give rise to proliferative muscle progenitor cells of the limb blastema. Lastly, molecular profiling of the GFP-labeled cells that were isolated from the satellite cell-derived limb blastema revealed expression of *Pax7* and *Myf5 as* detected by RT-PCR.

Thus, by using *Cre/loxP*-based genetic fate mapping in transgenic axolotl, it was indirectly assumed that PAX7$^+$ satellite stem cells could be the major source of regenerated myogenic tissue and no muscle dedifferentiation occurs.

4.2 *Twist-1* and *Twist-3* show lineage specific expression pattern

It is known that Twist, as an early marker of limb blastema, is expressed in mesodermal cells during development, as shown in other species (Tavares et al., 2001). We aimed to molecularly study individual blastema cells with several progenitor cell markers by using the tools of section- and whole-mount *in situ* hybridizations as
well as single-cell PCR (sc-PCR) ((Kragl et al., 2013) [KR2]). In general, the low number of cells presents at this early stage, which makes surgical manipulations impossible, limits the analysis of early events and cell fate decisions during blastema formation. We determined

the expression pattern of *Twist-1* and *Twist-3* during development and regeneration. Further, we characterized their embryonic origin in order to compare their expression pattern with lineage specific genes using LPM- and PSM- derived limb blastema cells.

We isolated for the first time, the axolotl full-length *Twist-1* (*AmTwist-1*) and *Twist-3* (*AmTwist-3*) cDNA sequences by screening our long insert cDNA library. Based on our cDNA sequence of *Twist-1* and *Twist-3*, we performed cell-tracing experiments using single cell PCR combined with *in situ* hybridization. Initially, we investigated the *Am (Ambystoma mexicanum) Twist-1* and *AmTwist-3* expression pattern during development and regeneration. To determine the individual expression pattern during embryonic development, we showed by whole-mount *in situ* hybridization using embryos at different stages (Fig.1 [KR2]) that in the early neurula stage, *AmTwist-1 is* expressed in the axial, paraxial and lateral plate mesoderm but no expression was found in the ectodermal layer. Furthermore, at later stages the transcript was detected in head neural crest, notochord, somitic mesoderm and sclerotome whereas no transcript was detected in the myogenic region of the somitic mesoderm as confirmed by *Myf5* expression. *AmTwist-3* transcript was localized at later stages in development in a ventromedial population of somites, supposable as the axolotl sclerotome (Fig.1 [KR2]). We showed that the expression of *AmTwist-1* is analogous to the expression pattern of *Twist-1* orthologs in other vertebrates.

During embryonic limb development, *AmTwist-1* was detected in the mesenchym of the early and medium limb bud stage and in the layer underneath the epidermis in the distal portion of the emerging limb bud by section *in situ* hybridization (Fig. 2 [KR2]). The same expression pattern was found for the *Twist-3* transcript, except that it was more restricted to a thin sub-epidermal layer. By analyzing both expression patterns during limb regeneration (amputation at the level of humerus) [Fig. 3 (KR2)], we found *Twist-1* expression throughout the early limb blastema (7 day p.a.) while *Twist-3* was not detected. By 8 days p.a., *Twist-1* was highly expressed in the tip of the blastema and further proximally in a domain underneath the epidermis. In contrast, *Twist-3* was found only in a thin layer underneath the proximally epidermis but not in the tip of the blastema. By a later blastema stage (12 days p.a.), the *Twist-1* transcript was detected in the region of cartilage formation whereas *Twist-3* was expressed in the thin layer underneath the epidermis in a more proximal region. Conclusively, the expression pattern of *Twist-1* and *Twist-3* during limb development is similar to that during limb regeneration suggesting that *Twist-1* marks undifferentiated progenitors whereas *Twist-3* is a marker for dermal precursor cells in agreement with published Twist studies (Scaal et al., 2001).

Next we compared the expression of Twist with lineage specific genes like *Sox9* (chondrogenic marker) and *Myf5* (myogenic marker) in the blastema cells, derived from different origins such as dermis, cartilage and muscle, which were generated using embryonic transplantation methods of GFP^+ tissue (Sobkow et al., 2006). This was achieved using sc-PCR to analyse cells, which were dissociated and FACS sorted prior to PCR. Analysis of GFP^+ labeled dermis-derived blastema revealed that all cells express *Twist-1,* whereas *Twist-3* was only express in a subset of *Twist-1*$^+$ cells (Fig.4 [KR2]). For cartilage-derived, GFP^+ blastema cells, the majority of cells co-express *Sox9* and *Twist-1*, whereas no *Twist-3*$^+$ cells express *Sox9*. Lastly, a small number of *Myf5*$^+$ cells that coexpress *Twist-1* but not *Twist-3* were detected in the muscle-derived blastema cell population. We also investigated whether the cells derived from GFP-LPM or GFP-PSM coexpress *Twist-1* and *Twist-3* by *in situ* hybridization. We found *Twist-1*$^+$ and *Twist3*$^+$ cells in LPM-derived GFP^+ blastema cells but no coexpression with the PAX7 myogenic marker (Fig.5 [KR2]). Further, we observed only a small number of *Twist-1*$^+$ cells colocalized with PSM-derived GFP^+ blastema cells (Fig.6 [KR2]), which confirmed the results of our sc-PCR. No coexpression of *Twist-3* and PSM-derived GFP+ cells was detected.

Lastly, using sc-PCR method on dissociated mature limb cells, we investigated if there is a population of *Twist-1*$^+$ cells, present in the mature limb, which could be a precursor for limb blastema cells. By analyzing mature limbs, we found that a small number of *Twist-1*$^+$ population decreases from larval to adult stages (with age). Contrary, a large percentage of *Twist-1*+ cells was found in the regenerating limb blastema (12 day p.a.) of LPM-and PSM-derived cells (Fig. 7 [KR2]).

Upon limb amputation, *Twist-1* and *Twist-3* mRNA expression reflects cell-type specific and spatial expression patterns found during limb development. This observation indicates that blastema cells re-use molecular machinery similar to that used during development. The molecular studies we have performed using Twist as a marker supported our recent knowledge that the connective tissue-derived blastema cells are critical for the study of limb patterning. Furthermore, cartilage-derived cells express molecular markers of P/D positional identity like *HoxA13* (Nacu et al., 2013).

4.3 *HoxA* gene reinduction found by molecular assays

To gain insight into how P/D patterning of the appropriate limb segments (upper arm, lower arm and hand) occurs, we analyzed the progression of segment identities during development in comparison to regeneration. Gardiner et al. (Gardiner et al., 1995) concluded

based on *whole-mount HoxA* gene expression data, that patterning during regeneration is fundamentally different from that during development. The authors (Gardiner et al., 1995) proposed the 'distal-first' hypothesis based on the finding that the early blastema is composed of cells with distal-most (hand identity) and proximal identities (upper arm identity) postulating the following intercalation of the intermediate element (lower arm identity). But clear evidence supporting or even disproving this model was lacking. In my third publication ((Roensch et al., 2013) [KR3]), we present qualitatively higher molecular and cellular resolution of the critical early patterning events by focusing on newly isolated specific posterior axolotl *HoxA9, HoxA11* and *HoxA13* sequences as marker of the future upper arm, lower arm and hand identity respectively.

Due to the availability of a cDNA library (Habermann et al., 2004) in our lab, full-length axolotl *HoxA13* was initially cloned. Furthermore, we obtained the full-length sequence of *HoxA9* and *HoxA11* by 5' RACE using limb bud total RNA. The complete *HoxA9* coding region was PCR amplified from limb bud cDNA. For cloning axolotl full-length *HoxA11*, a long insert library was screened. The antibodies against HOXA9 and HOXA13 were prepared as described in previously published work from our lab (McHedlishvili et al., 2012). For HOXA11 antibody production, we created a GST-HOXA11 fusion protein descriped in Supplementary Material [KR3].

We demonstrated for the first time that during development the HoxA genes in axolotl are expressed in a spatial and temporal manner termed colinearity shown by expression of HOXA9, HOXA11 and HOXA13 protein during development (Fig. 1 [KR3]). It was further demonstrated that *HoxA9* transcripts were expressed throughout the limb bud first, followed by more distal expression of *HoxA11* and finally *HoxA13* is expressed in the distal-most tip of the developing limb bud (Fig. S2 [KR3]). The expression pattern of the HoxA genes is similar to the expression pattern found in other vertebrates (Haack and Gruss, 1993; Nelson et al., 1996; Yokouchi et al., 1991). These findings demonstrated that limb development occurs in a progressive specification in a proximal to distal order in axolotl.

We investigated in which order the sequence of HOXA protein expression occurs in the regenerating upper arm blastema, including the cricical early blastema stages by using tissue sections to gain a high cellular resolution in comparison to whole-mount. We generated animals with a GFP-labelled dermis by transplanting GFP^+ embryonic lateral plate mesoderm (LPM) that forms the connective tissue in the limb (Sobkow et al., 2006). Connective tissue cells, which are derived from LPM, obey the rule of distal transformation. They mark the relevant blastema precursors and can only form limb segments distally to their original

identity (Nacu et al., 2013). These previous results from my colleagues indicate that connective tissue-derived blastema cells are the critical cells to focus on when studying patterning (Kragl et al., 2009; Nacu et al., 2013).

Our amputations for the molecular assays were performed uniformly at the level of the humerus (upper arm). By using connective tissue labeled limbs, we did not observe a HOXA9, HOXA11 or HOXA13 signal at 1 day p.a. (Fig. 2 and Fig. S3 [KR3]). Furthermore, there was no HOXA13 colocalization with GFP^+ cells. Even at 4 days p.a. we did not observe a HOXA13 immunofluorescence signal at a high cellular level. By investigating an early blastema stage (6 days p.a.) we found that HOXA9 and HOXA11 expression was present throughout the entire blastema, whereby HOXA13 expression is absent (Fig.2 [KR3]). In addition, we studied medium-bud (8 days p.a.) and late-bud (12 days p.a.) blastema stages where we observed a clear nested expression pattern of HOXA9, HOXA11 and HOXA13. A HOXA9 signal was observed in the entire blastema, HOXA11 was expressed in a distinct domain and the first timepoint where we detected a robust signal of HOXA13 expression was at the distal tip of a blastema 8 days p.a. (Fig. S4 [KR3]). The nested expression pattern of *HoxA9*, *HoxA11* and *HoxA13* in mesenchymal cells as well as the late onset of *HoxA13* mRNA expression was confirmed by *in-situ* hybridization using longitudinal sections (Fig. S5 [KR3]). Addressing the long debated question of which order the limb specification occurs during limb regeneration, we demonstrated that the process occurs in a proximal to distal sequence just like the colinear expression pattern observed during limb development.

4.4 The sequential order of limb segment specification confirmed by functional transplantation assay

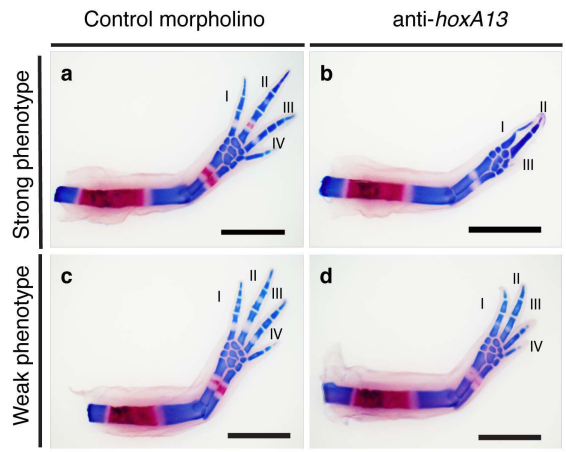
To functionally assess the order of blastema cell specification, we developed a transplantation assay to investigate whether upper arm blastema cells already harbour hand identity, as it would be assumed by the intercalary regeneration model (Haack and Gruss, 1993). Initially, we examined the specific expression of HOXA13 in the hand identity using immunostaining on sections of hand blastema. The onset of HOXA13 expression was initially detected at 4 days p.a. and was increased at 6 days p.a. (Fig. S6 [KR3]). Further, to confirm the sensitivity and the specificity of HOXA13 detection method, we treated our limbs with retinoic acid (RA). It is known that RA converts a hand blastema into an upper arm blastema (Crawford and Stocum, 1988; Maden, 1982; Mercader et al., 2005; Saxena and Niazi, 1977) - it breaks the ‘rule of distal transformation’. By increasing the concentration of retinol palmitate treatment and amputation at the wrist level, the structures more proximal than the level of amputation will regenerate (Maden, 1983). Due to the proximalizing role of RA,

HOXA13 expression could not be detected after treating the hand blastemas with RA, confirming the sensitivity of the HOXA13 antibody.

To confirm the order of blastema cell specification and the fact that early upper-arm blastema cells already possess hand identity we performed a functional transplantation assay. We used eGFP expressing connective tissue derived cells, which were labelled by embryonic grafting of GFP tissue, as donor tissue. As a control we used an 8 days p.a. hand blastema. Transplanting 8 day HOXA13-expressing blastema cells (Fig. S6 [KR3]) into a 6 day upper arm blastema of a white host contributed only to the regenerated hand fate. Transplantation of distal cells of 8 day upper arm blastema resulted in contributions similar to that of hand blastema transplantations, indicating that distal cells (HOXA13 positive) at this stage form the hand identity. By taking the proximal cells from an 8 day upper arm blastema, the GFP signal was found in the upper arm, lower arm and hand of the recipient, indicating that HOXA9 positive, HOXA13 negative cells localize in that region and therfore the HOXA9 expression domain is located throught the entire P/D axis of the limb. Transplantation of the distal cells of a HOXA13 negative blastema stage (4 day upper arm blastema) led to GFP^+ cells in the upper arm and hand identities rather than in the regenerated hand only. The transplantation assay indicates that blastema cells show a hierarchy of positional values. Amputation at the upper arm level results in a blastema harboring cells that convert to form a lower arm and hand, whereas amputations at the hand level generates hand blastema cells that are restricted and normally do not become upper arm cell fates.

4.5 HOXA13 is functionally involved in the formation of future autopod cells during regeneration

Our molecular data as well as the cell tracing results suggested that *HoxA13* is functionally involved in the formation of hand cells during limb regeneration. To confirm the function of *HoxA13*, we knocked-down its gene expression by electroporation of anti-*hoxA13* morpholino (5' AGGAGCACTGAAGCTGTCATAGCCC 3') into a 6 day blastema and allowed regeneration to occur for 30 days (*Data not published).* 45% of the regenerates that had been electroporated with anti-*hoxA13* morpholinos resulted in malformations of hand bone elements (N=53), while the upper arm and lower arm showed no obvious phenotype. The defects ranged from hands missing at least one digit, forming fewer metacarpals (17%) up to mild defects such as shorter digit length (Figure 4.5-1 (b),(d)).



Experiment	Total Number of limbs	Strong phenotype No. (%)	Weak phenotype No. (%)	No phenotype No. (%)
1	10	3 (30)	4 (40)	3 (30)
2	5	2 (40)	2 (40)	1 (20)
3	5	0 (0)	2 (40)	3 (60)
4	33	4 (12)	7 (21)	22 (67)
Total	**53**	**9 (17)**	**15 (28)**	**29 (55)**

Figure 4.5-1 HOXA13 knock-down resulted in hand defects. Regenerated limbs after morpholino electroporation of anti-hoxA13 and control morpholinos into 6 day upper arm blastemas. Alcian Blue: Cartilage, Alcian Red: Ossified bone. (a),(c) Limbs transfected with control morpholino. (b), (d) Limbs transfected with anti-hoxA13 morpholino. Strong phenotype seen in (b) with missing hand skeletal elements. Weak phenotype seen in (d) with shorter digits compared to contralateral control. Abbreviations: I-IV, digit numbers from anterior to posterior. Scale bar, 1mm.

Some of the variability in phenotype may arise due to the number of cells successfully electroporated with the morpholino. The control, contralateral limbs that had been electroporated with a non-hybridizing morpholino did not exhibit malformations in the hand identity (Figure 4.5-1 (a),(c), N=55). These observations are consistent with mouse knock-out experiments, where homozygous deletion of *HoxA13* in mice results in the absence of anterior digits and fused carpal elements (Fromental-Ramain et al., 1996b). Our knock-down experiment indicates that during axolotl limb regeneration, *HoxA13* is functionally involved in the formation of future autopod cells during regeneration, which is similar to development.

5 Discussion

5.1 Myofiber contribution to regenerated muscle is divergent in two different species

The goal of the work [KR1] was to permanently label and trace muscle fibers during regeneration in axolotl. By using *Cre/loxP*-based genetic fate mapping in transgenic axolotl, it was indirectly assumed that PAX7^{+} satellite stem cells are the major source of regenerated myogenic tissue and no muscle dedifferentiation occurs. We were able to compare the results in collaboration with the group of András Simon (Karlinska Institute, Sweden) to another salamander species- the *newt* (*Notophthalmus viridescens*) (Figure 5.1-1) using Cre/loxP genetic fate mapping as well. Surprisingly, in the *newt,* muscle dedifferentiation contributes to regenerated muscle, whereas in the axolotl, myofibers do not contribute to limb regeneration. Instead, PAX7^{+} cells are most likely to be the main contributor to muscle regeneration. In *newt*, myofiber fragmentation results in *Myf5*$^{+}$, PAX7^{-} proliferating cells in the blastema, which give rise to future skeletal elements.

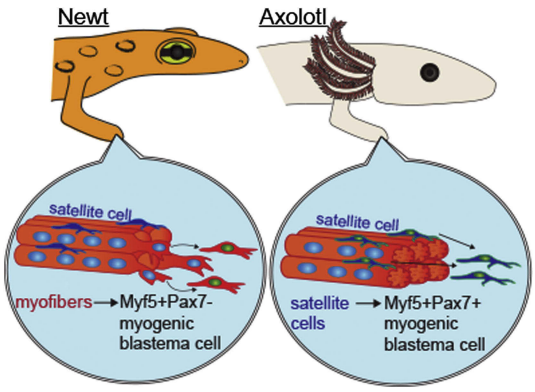


Figure 5.1-1: Myofiber contribution to regenerated muscle is divergent in two different species. In newt, myofiber fragmentation results in Myf5+, PAX7- proliferating cells, which give rise to the muscle in the regenerated limb. In axolotl, PAX7+ satellite stem cells are suggested to be the main source for myogenic tissue (Myf5+) in the regenerated limb. (Adapted from: http://dx.doi.org/10.1016/j.stem.2013.11.007)

For the first time, it was shown that there is an unexpected diversity concerning the mechanism of muscle regeneration among two salamander species. The lack of myofiber contribution in axolotl was proven by comparing the efficiency of muscle labeling with that of the newt. By using germline transgenically integrated cassettes (loxP expression cassette) the labeling efficiency in axolotl and *newt* was similar. Considering that the axolot is neotenic (i.e. it retains larval feature throughout its entire life) the same experiments were carried out

using postmetamorphic axolotls. A contribution to labeled myofibers was not observed. Under these conditions, no contribution of $PAX7^+$ cells was found when investigating the larval *newt*. These findings indicate that there is a significant difference at the molecular level for blastema build-up between these two salamander species. It further demonstrates that there is a flexibility of cellular mechanims used during limb regeneration, even among two closely related species. Finally, if one salamander species uses a mammalian regenerative strategy (Cornelison and Wold, 1997; Collins et al.,2005) involving stem cells and another uses a dedifferentiative strategy, this raises the question of whether there are other fundamental aspects of regeneration that could be further anomalous. This hypothesis is promising since there could be more than one possible mechanism to induce mammalian regeneration.

5.2 Developmental program is recapitulated during regeneration shown by *Twist-1* and *Twist-3* expression pattern

The developmental potential of the progenitor cells within the limb blastema are similar to the progenitor cells in the limb bud. It has been shown previously that lateral plate mesoderm-derived cells form the cartilage and the dermis whereas a separate pool of presomitic mesoderm-derived cells builds the muscle during limb regeneration (Kragl et al., 2009). It is known that Twist, as an early marker of limb blastema mesenchyme, is expressed in mesodermal cells during development, as shown in other species (Tavares et al., 2001). We isolated for the first time, the axolotl full-length *Twist-1* (*AmTwist-1*) and *Twist-3* (*AmTwist-3*) cDNA sequences. The primers for *Twist-1* were designed based on a partial EST sequence (Habermann et al., 2004), whereas the primers for *Twist-3* were designed based on the partial sequence published by Satoh et al. (Satoh et al., 2008). The sequence from this publication contained only the bHLH domain, so it was not clear if this sequence belonged to the ortholog of *Twist-1* or *Twist-2/3*. However, from the sequence we identified in our screen, we conclude that the isolated clone from this publication is the *Twist-3* ortholog (Satoh et al., 2008). We did not attempt to identify a *Twist-2* ortholog since it has not been found in *Xenopus laevis* and therefore it is likely that it was lost during evolution.

Using the newly isolated sequences of *Twist-1* and *Twist-3*, we performed *in situ* hybridizations that demonstrated that embryonic players are reused during regeneration by reactivating the distinct position- and tissue-specific developmental gene programs. We showed that *Twist-1* and *Twist-3* expression patterns during development are recapitulated during blastema formation. We showed that *Twist-1* and *Twist-3* are expressed in LPM-derived GFP^+ and $PAX7^-$ cells of the limb blastema visualized by *in situ* hybrizations

combined with immunohistochemistry. *Twist-1* is not expressed in most of the GFP$^+$, PAX7$^+$ cells whereas *Twist-3* does not correlate with these two markers at all. Remarkably, the PSM-GFP$^+$ cells did not reach the *Twist-1* expression domain in the distal tip of the limb blastema. Instead, they were more proximally and medially localized in comparison to the GFP$^+$ cells of the LPM-derived blastema cells. Nevertheless, their expression status correlates with that of other vertebrates and it supports our sequence analysis that *Twist-1* and *Twist-3* are axolotl specific orthologs.

Lastly, we used sc-PCR method on dissociated mature limb cells, to investigate if there is a population of *Twist-1*$^+$ cells present in the mature limb that could act as a precursor for limb blastema cells. By analyzing mature limbs, we found that a small *Twist-1*$^+$ population decreases from larval to adult stages (with age). Indeed, further studies are neccessary to identitfy other processes that trigger the regenerative capacity at the larval stage, such as changes in oxygen consumption, enhanced differentiation potential or a less innervated state.

Our results demonstrated that connective tissue is a major contributor to the limb blastema cells. Other studies performed in my group investigated connective tissue subtypes using single cell transcriptomics and identified *Twist-3* as a specific marker for dermal fibroblasts (Prayag Murawala, *unpublished data*). Our *in situ* hybridization experiments of *Twist-3* in the blastema cells also showed specific expression of *Twist-3* in dermal fibroblasts. Based on these studies, GFP knockin of the *Twist-3* locus was generated and confirmed that *Twist-3* specifically labels connective tissue. This suggests that *Twist-3* is an important marker for connective tissue-derived blastema cells.

5.3 P/D patterning occurs in a progressive specification

In the past, it was proposed that P/D patterning in general is different from that during regeneration (Gardiner et al., 1995). The 'distal-first' hypothesis based on the finding that the early blastema is composed of cells with distal-most (hand identity) and proximal identities (upper arm identity) by whole-mount *in situ* hybridizations, postulating the following intercalation of the intermediate element (lower arm identity). The hypothesis therefore postulates that intercalation of the intermediate element (lower arm identity) occurs later. But sufficient evidence either confirming or disproving this model was lacking due to technical limitations Thus, it has remained unresolved whether salamander limb blastema cells become specified in a progressive, proximal-to-distal order or distal first, followed by intercalation. Our results generated from HoxA gene analysis indicated that P/D patterning of the appropriate limb segments (upper arm, lower arm and hand) occurs in a progressive specification (in a proximal to distal order) rather than by intercalation [KR3]. It further demonstrates that the patterning events used during regeneration are similar to that during limb development (Figure 5.3-1).

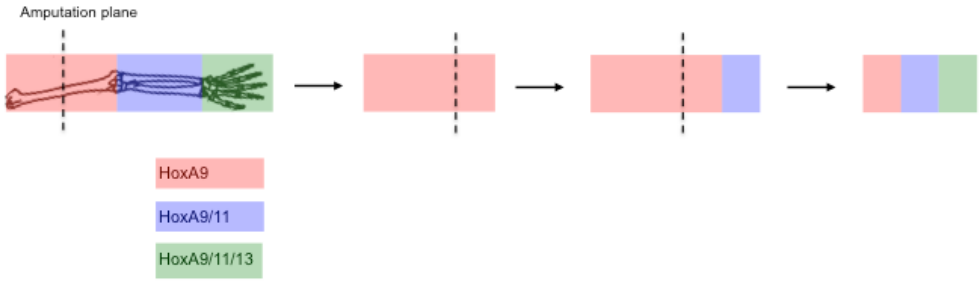


Figure 5.3-1: Axolotl limb blastemas acquire their positional identity in a proximal to distal sequence. The proximo-distal positional identities are specified sequentially similar to development shown by in situ hybridizations and immunohistochemistry on sections as well as functional transplantation assays. Red marks the stylopod and future stylopod cells, blue marks the zeugopod and future zeugopod cells andgreen marks the autopod and future autopod cells. Dashed perpendicular line marks the plane of amputation.

Using high molecular and cellular resolution analysis of HOXA protein and mRNA expression, we showed for the first time that axolotl limb blastema cells acquire their positional identity in a proximal to distal sequence. We found a hierarchy of cellular restrictions in positional identities. Amputation at the level of the upper arm showed that the blastema harbours cells, which convert to lower arm and hand. The nested expression pattern of *HoxA9*, *HoxA11* and *HoxA13* in mesenchymal cells as well as the late onset of *HoxA13* mRNA expression was confirmed by *in-situ* hybridization using longitudinal sections. We showed that *HoxA11* mRNA, but not protein, is observed in the *HoxA13*-expressing domain (Fig 1 and Fig. S2 [KR3]). It indicates that *Hox* genes might be activated post-

transcriptionally. Possibly, more distally localized HOX proteins act as negative regulators of the more proximally localized HOX protein translation. In the past, it was shown that *HoxA13* causes homeotic transformation of lower arm bones into carpals (Yokouchi et al., 1995). It is possible that *HoxA13*, alone or in combination with *HoxD13,* inhibits *HoxA11* expression in the cells present at the tip of the blastema, possibly explaining why HOX protein expression does not overlap.

We developed a transplantation assay to functionally assess if the early upper arm blastema cells already harbour hand identity. We used eGFP expressing connective tissue-derived blastema cells as donor cells because it is known that they are the critical cells when studying P/D patterning (Kragl et al., 2009; Nacu et al., 2013). As a control we used an 8 days p.a. hand blastema (HoxA13 expressing cells present). Transplanting 8 day HOXA13-expressing blastema cells into a 6 day upper arm blastema of a white host contributed only to the regenerated hand fate. Transplantation of distal cells of 8 day upper arm blastema resulted in contributions similar to that of hand blastema transplantations, indicating that distal cells (HOXA13 positive) at this stage form the hand identity. By taking the proximal cells from an 8 day upper arm blastema, the GFP signal was found in the upper arm, lower arm and hand of the recipient, indicating that HOXA9 positive, HOXA13 negative cells localize in that region and therfore the HOXA9 expression domain is located throught the entire P/D axis of the limb. Transplantation of the distal cells of a HOXA13 negative blastema stage led to GFP^+ cells in the upper arm and hand identities rather than in the regenerated hand only. The transplantation assay indicates that blastema cells show a hierarchy of positional values. Amputation at the upper arm level results in a blastema harboring cells that convert to form a lower arm and hand, whereas amputations at the hand level generates hand blastema cells that are restricted and normally do not become upper arm cell fates. It is known that myogenic cells break the rule of distal transformation (Nacu et al., 2013). Based the findings that muscle cells can get proximalized and break ‘the rule of distal transformations’, the same setup of transplantation experiments is possible by using myogenic-derived blastema cells (label muscle tissue by transplanting GFP^+ embryonic presomitic mesoderm PSM) as donor tissue. As it would be expected, by transplanting the GFP^+ tissue of a hand blastema into upper arm blastema, the cells would be present in the upper arm, lower arm and hand indicating that myogenic blastema cells break the rule of distal transformation- contrary to the cells from connective tissue.

Knocking-down *HoxA13* expression by electroporation of anti-*hoxA13* morpholino indicated that this gene is functionally involve in the formation of the future autopod (hand) cells. These results were not published because it is difficult to reliably distinguish between specific and

non-specific effects using morpholino mediated knock-down models and a generally accepted technology to assess the side effects does not exist (Schulte-Merker et al., 2014). The recent development of reliable tissue-specific gene expression models in axolotl brings new possibilities to investigate regeneration process. For example specific CRISPR-mediated deletion of different positional identity markers like (Fei et al., 2014; Fei et al., 2016) *HoxA9*, *HoxA11* and *HoxA13* (single and combined knockouts with the genes of the HoxD locus) could help prove their functional role in the specific limb segment (upper arm, lower arm and hand) specification.

6 Resumé

Axolotl uniquely generates blastema cells as a pool of progenitor/stem cells to restore an entire limb, a particular property that other organisms, such as humans, do not have. What underlies these differences? Is the main difference that cells residing at the amputation plane (in the stump) undergo reprogramming processes to re-enter the embryonic program, which allows developmental patterning to start, or are there fundamental differences? There is also a significant debate about whether regeneration occurs via stem cell differentiation or by dedifferentiation of mature limb tissue. The aim of my thesis was to address following questions: Are the cells in the blastema reprogrammed or differentiated to regenerate? Are the blastema cells genetically reactivated *de novo* during regeneration? How does the amputated limb exactly know which part of the limb needs to be regenerate?

Using a novel technique of long-term genetic fate mapping, my team demonstrated that dedifferentiation in regenerated axolotl muscle tissue does not occur. Instead, $PAX7^{+}$ satellite cells indeed play an important role during muscle regeneration in the axolotl limb. Surprisingly, this is in contrast to the *newt*, which regenerates muscle cells through a dedifferentiation process. Therefore, there is a fundamental difference that underlies the regenerative mechanism ((Sandoval-Guzman et al., 2014) [KR1]). This demonstrates that there is an unexpected diversity and flexibility of cellular mechanims used during limb regeneration, even among two closely related species. Finally, if one salamander species uses a mammalian regenerative strategy (Cornelison and Wold, 1997; Collins et al., 2005) involving stem cells and another uses a dedifferentiative strategy, this raises the question of whether there are other fundamental aspects of regeneration that could also be anomalous. This hypothesis is promising since there could be more than one possible mechanism to induce mammalian regeneration.

The process of limb regeneration in principle seems to be more similar to those of limb development as historically assumed. We showed molecularly that embryonic players are reused during regeneration by reactivating the position- and tissue-specific developmental gene programs by using the newly isolated Twist sequences as early blastema cell markers ((Kragl et al., 2013) [KR2]). To gain insights into the molecular mechanisms of the P/D limb patterning in general, it was crucial to study the early patterning events of the resident progenitor/stem cells by using the specific blastema cell marker *HoxA* as a positional marker along the proximo-distal axis. Our HOXA protein analysis using high molecular and cellular resolution as well as transplantation assays demonstrated for the first time that axolotl limb blastema cells acquire their positional identity in a proximal to distal sequence. We found a

hierarchy of cellular restrictions in positional identities. Amputation at the level of the upper arm showed that the blastema harbors cells, which convert to lower arm and hand. We observed ((Roensch et al., 2013) [KR3]) for the first time that intercalation- the intermediate element (lower arm) arises later from an interaction between the proximal and distal cells identities- does not occur. Intercalation, which has been an accepted model for a long time, is not the patterning mechanism underlying normal (without any manipulation) limb regeneration that is unique to axolotl. We further demonstrated, using the Hox genes as markers that positional identity is cell-type specific since their effects were confirmed to be present in the lateral plate mesoderm- derived cells of the limb.

As our knowledge about limb blastemas expands concerning cell composition and molecular events controlling patterning, the similarity to development is becoming more and more clear. My work has resolved many ambiguities surrounding the molecularly identification of different types of blastema cells and how P/D limb patterning occurs during regeneration in comparison to development. It has highlighted the importance of combining high-resolution methods, such as *in situ* hybridizations, single-cell PCR (sc-PCR) of individual dissociated blastema cells and genetic labeling methods with grafting experiments to map cell fates *in vivo*.

In addition to understanding the processes of regeneration, another long-term goal in the regenerative medicine field is to identify key molecules that trigger the regeneration of tissues. Recently, my colleague Takuji Sugiura (Sugiura et al., 2016) observed that an early event of blastema formation is the secretion of molecules like MLP (MARCKS-like protein), which induces wound-associated cell cycle re-entry. Such findings further increase the enthusiasm of biologists to understand the underlying principles of regeneration. By building our knowledge of the molecules and pathways that are involved in tissue regeneration, we increase the possibility of identifying a way to ‘activate’ regenerative processes in humans and thus reach the final goal of regenerative medicine, which is to use the concepts of cellular reprogramming, stem cell biology and tissue engineering to repair complex body structures.

Summary [German]

Das multidisziplinäre Fachgebiet der regenerativen Medizin beschäftigt sich mit der Wiederherstellung von durch Krankheit oder Unfall geschädigten/funktionsgestörten Zellen, Geweben sowie kompletten Organen. Gegenwärtig suchen Forscher weltweit nach Möglichkeiten, die körpereigenen Regenerations- und Reparaturprozesse anzuregen. Mediziner und Forscher erhoffen sich, neue individuelle Therapieansätze zur Behandlung von schweren Verletzungen wie Querschnittslähmungen oder starken Verbrennungen sowie degenerativen Erkrankungen wie Alzheimer oder Multiple Sklerose zu finden, um den gesunden Originalzustand wiederherzustellen. Für die erfolgreiche 'Stimulation' des Regenerationsprozesses ist es entscheidend, die fundamentalen molekularen Mechanismen sowie deren komplexe Signalwege zu verstehen. Hoch entwickelte Organismen, wie der Mensch, haben ein beschränktes Potential, verloren gegangenes Gewebe (Gehirn, Herz oder Auge) wiederherzustellen. Des Weiteren kommt es bei größeren Wunden zur Narbenbildung. In diesem Zusammenhang gilt dem einzigartigen Modellorganismus der Regenerationsbiologie, dem Schwanzlurch Axolotl (*Ambystoma mexicanum*), ein besonderes Interesse, da aus dessen Regenerationsfähigkeit grundlegende Erkenntnisse gewonnen werden können. Der adulte Schwanzlurch ist in einzigartiger Weise in der Lage, verlorene Gliedmaßen und Rückenmark, perfekt (vollständig und funktional) und zeit seines Lebens, ohne Narbenbildung zu regenerieren.

Mit hohem Enthusiasmus sind Wissenschaftler derzeit weltweit bestrebt, die Regenerationsfähigkeit amputierter Gliedmaßen im Salamander zu 'entschlüsseln'. Das Ziel ist es, zu verstehen warum voll ausdifferenzierte adulte Zellen dazu fähig sind sich in Vorläuferzellen umzuwandeln und den fehlenden Abschnitt einer Gliedmaße exakt wiederherzustellen. Die residenten Stammzellen, die an der Amputationsstelle (distal) lokalisiert sind, erzeugen eine Zellpopulation bestehend aus undifferenzierten, proliferierenden Vorläuferzellen (s.g. Blastemzellen). Eine der gundlegenden Herausforderungen besteht darin, die zellulären und molekularen Mechanismen zu verstehen, die an der Rekrutierung der Vorläufer-/Stammzellen beteiligt sind. Durch deren Fähigkeit zur Selbsterneuerung ('self-renewal'), der räumlichen und zeitlichen Musterneubildung auf zellulärem Niveau sowie der gewebespezifischen Differenzierung, wird eine exakte Kopie des fehlenden Abschnittes einschließlich der verschiedenen spezifischen Gewebetypen (Nerven-, Skelett- und Muskelgewebe) erzeugt. Die Blastemzellen entwickeln

sich nicht, wie bisher angenommen in ein pluripotentes Stadium zurück, sondern behalten eine gewebespezifische Erinnerung an ihren vorherigen Differenzierungszustand (Kragl et al., 2009). Es wurde gezeigt, dass während der Regeneration verschiedener Gewebearten, beispielsweise aus ehemaligen Muskelzellen wiederum Muskelzellen aber keine Knorpel-, Knochen- oder Epidermiszellen entstehen. Bei dieser Studie wurden die Satellitenzellen (Muskelstammzellen) sowie auch die Myofibrillen mit GFP markiert, und somit blieb die Frage ungeklärt, ob regeneriertes Muskelgewebe von dessen Stammzellen (Satellitenzellen) abstammt oder durch Dedifferenzierung der Myofibrillen entsteht. Das Blastem ist somit eine heterogene Population von Vorläuferzellen mit limitiertem Entwicklungspotential (Kragl et al., 2009), d.h. die Blastemzellen behalten eine Erinnerung an ihren vorherigen Differenzierungszustand und besitzen somit ein molekulares Gedächtnis. Eine zentrale Fragestellung befasst sich nicht nur welche Zellen an der Blastementstehung beteiligt sind sondern vor allem wie.

Die entscheidende Fragestellung, ob neu gebildetes Muskelgewebe durch Dedifferenzierung vorhandener differenzierter Zellen und/oder durch Reaktivierung residenter Stammzellen gebildet wird, wurde erstmals im regenerierenden Muskelgewebe des Axolotls untersucht. Anhand eleganter und kürzlich integrierter Techniken im Axolotl (genetischen Kartierungssystems *Cre-loxP*) ist es erstmalig und weltweit möglich, Muskelgewebe sowie dessen Stammzellen farblich zu markieren. Parallel wurde diese Fragstellung von András Simon (Karlinska Institute, Schweden) am eng verwandten Lurch (*newt*) erforscht. Erstmalig konnten wir beim Axolotl zeigen, dass an der Amputationsstelle vorhandene spezifische $PAX7^{+}$ Satellitenzellen mutmaßlich für die Regenerationsaktivität verantwortlich sind ((Sandoval-Guzman et al., 2014) [KR1]). Beim *newt* hingegen wird die Neubildung der Muskelfasern durch Dedifferenzierung der Myofibrillen initiiert. Diese Ergebnisse zeigen eine unerwartete evolutionäre Diversität der zellulären Mechanismen der beiden eng verwandten Salamanderarten in Bezug auf die Regeneration von Muskelgewebe.

Eine weitere, zentrale Herausforderung der vorliegenden Arbeit bestand darin, molekulare Mechanismen eindeutig zu verstehen, die die ablaufende gewebespezifische Differenzierung der Blastemzellen reflektieren, sowie deren möglicher embryonaler Reaktivierung. Spiegelt die Regeneration möglicherweise den Prozess der Embryonalentwicklung der Gliedmaßen wieder? In diesem Zusammenhang wurden die Gene der Twist Familie von gewebespezifischen Vorläuferzellen verschiedener Blastemzellen profiliert. Erstmalig konnten wir die Genexpressionsmuster der homologen Gene *AmTwist1* und *AmTwist3* in der Embryonalentwicklung mit dem Genexpressionsmuster während der Regeneration einer Gliedmaße vergleichen ((Kragl et al., 2013) [KR2]). Die Genexpressionsmuster von

AmTwist1 sowie Am*Twist3* wurden räumlich (spatial) und zelltyp-spezifisch charakterisiert. Während des Regenerationsprozesses einer Gliedmaße kommt es zu wesentlichen Veränderungen der Genexpression, die den molekularen Mechanismen der embryonalen Entwicklung einer Gliedmaße sehr ähneln.

Es ist bereits bekannt, dass unabhängig von der Amputationsstelle einer Gliedmaße entlang der proximo-distalen Achse, ausschließlich die exakt fehlende Struktur ersetzt wird ('rule of distal transformation') (Butler, 1955; Rose 1962). Die zell-spezifische Eigenschaft (aus dem lateralen Mesoderm abstammendes Gewebe) konnte bereits in vorangegangen Publikationen gezeigt werden, wobei unter anderem die Reaktivierung der molekularen HoxA Maschinerie nachgewiesen wurde (Kragl et al., 2009; Nacu et al., 2013). In meiner Dissertation stand in diesem Zusammenhang eine weitere fundamentale Fragestellung im Vordergrund: Wie läuft der grundlegende Mechanismus der proximo-distalen Musterbildung ('patterning') der fehlenden Segmente einer Gliedmaße ab? Das zuvor weltweit akzeptierte Regenerationsmodell (Gardiner et al., 1995) der interkalaren Regeneration ('Einschub des fehlenden Segments') konnte mit dieser Studie erstmalig widerlegt werden. Gardiner et al. hatten die These aufgestellt, dass bei der Amputation eines Oberarms, die Vorläuferzellen an der Amputationsstelle die Identität der distalen Fingerspitzenidentität besitzen. Die Autoren spekulierten, dass die Wiederherstellung des proximo-distalen Musters, durch einen Einschub des fehlenden Abschnittes (Unterarm), zu einem späteren Zeitpunkt erfolgen muß. Diese These konnte jedoch nur anhand von whole-mount *in situ* Hybridisierung aufgestellt werden. Durch die von uns erzielte hohe molekulare und zelluläre Auflösung anhand von *in-situ* Hybridisierung dünner Gewebeschnitte im Vergleich zu whole-mount *in-situ* Hybridisierung (Gardiner et al., 1995) ist erstmalig eine detailierte HOXA Proteinanalyse der drei Segmente einer Gliedmaße (Oberarm, Unterarm, Hand) gelungen ((Roensch et al., 2013) [KR3]). Unsere Ergebnisse zeigten, dass die Musterbildung auf zellulärem Niveau nach Amputation einer Gliedmaße in spezifischer, sequentieller Reihenfolge von proximal nach distal (fortlaufende Spezifizierung) erfolgt, in Analogie zur Embryonalentwicklung. Dies impliziert einen fundamentalen Schritt in der regenerativen Medizin. Des Weiteren konnten wir anhand spezifischer Transplantationsexperimente mit dem Gewebe (abstammend aus dem lateralem Mesoderm) von transgenen GFP-Tieren die Ergebnisse der fortlaufenden Spezifizierung einer Gliedmaße während der Regeneration demonstrieren. Dabei konnte die zellspezifische Eigenschaft der Positionsinformation ('positional identity'), von den aus dem lateralen Mesoderm abstammenden Bindegewebszellen betätigt werden.

Dennoch bleibt bis heute ungeklärt, wie adulte Stammzellen entlang der proximo-distalen Achse einer Extremität die wichtigen Schlüsselfaktoren der Embryonalentwicklung

reaktivieren und sich in Blastemzellen umwandeln. Weiterhin ist es unumgänglich s.g. ‘Schlüsselmoleküle’ zu identifizieren, welche die Regeneration im Allgemeinen steuern. Meinem Kollegen, Takuji Sugiura, ist es kürzlich gelungen, eines dieser möglichen ‘Schlüsselmoleküle’ MLP (MARCKS-like protein) zu identifizieren. MLP ist maßgeblich an der Auslösung des initialen Wundverschlusses nach einer Amputation beteiligt (Sugiura et al., 2016). Bei der Blockierung von MLP konnte gezeigt werden, dass die Regeneration erheblich beeinträchtigt ist.

Durch fortführende Studien bezüglich der Eigenschaften von Stammzellen, die in der Lage sind, komplexe Körperteile zu regenerieren, versprechen sich Wissenschaftler weltweit zukünftig neue Erkenntnisse zu gewinnen. Des Weiteren wird mit hohem Enthusiasmus versucht zu ‘entschlüsseln’, wie eine Verletzung exakt die Stammzellen dazu veranlasst den exakt fehlenden Teil zu regenerieren, anstatt einfach nur Narbengewebe zu bilden. Dieses Wissen könnte möglicherweise weltweit in neuartige medizinische Therapiemöglichkeiten einfließen.

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List of Publications

All the publications presented in my thesis are here listed below.

[KR1] Fundamental differences in dedifferentiation and stem cell recruitment during skeletal muscle regeneration in two salamander species

***equal contribution**

DOI: http://dx.doi.org/10.1016/j.stem.2013.11.007

Tatiana Sandoval-Guzmán*	Experimental design Interpretation of the reported experiments and results Acquisition and analysis of data Drafting and revising the manuscript
Heng Wang*	Experimental design Interpretation of the reported experiments and results Acquisition and analysis of data Drafting and revising the manuscript
Shahryar Khattak	Experimental design Interpretation of the reported experiments and results Acquisition and analysis of data
Maritta Schuez	Performance of experiments
Kathleen Roensch	**Performance and quantification of experiments** **Experimental design**
Eugeniu Nacu	Performance of experiments
Akira Tazaki	Performance of experiments
Alberto Joven	Performance of experiments
Elly M. Tanaka	Experimental design Interpretation of the reported experiments and results Drafting and revising the manuscript Corresponding author

András Simon	Experimental design
	Interpretation of the reported experiments and results
	Drafting and revising the manuscript
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☐	6	CYTOTHERAPY	1465-3249	2906	3.055	3.275	0.659	123	4.9	0.00702	0.831
☐	7	EUR CELLS MATER	1473-2262	1620	4.558	5.702	0.565	46	4.9	0.00501	1.709
☐	8	J BIOMATER TISS ENG	2157-9083	12	0.476	0.476	0.051	39		0.00004	0.096
☐	9	J TISSUE ENG REGEN M	1932-6254	1470	2.826	3.699	0.902	102	3.5	0.00607	0.942
☐	10	REGEN MED	1746-0751	1169	3.873	3.784	0.613	62	3.5	0.00469	1.037
☐	11	STEM CELL RES	1873-5061	757	4.467	4.760	0.923	65	2.6	0.00388	1.522
☐	12	STEM CELL REV REP	1550-8943	956	4.523	4.453	0.474	116	2.1	0.00435	1.239
☐	13	STEM CELL TRANSL MED	2157-6564	33			0.340	94		0.00000	
☐	14	STEM CELLS	1066-5099	18253	7.701	8.368	1.297	269	5.0	0.06738	2.734
☐	15	STEM CELLS DEV	1547-3287	4295	4.670	4.700	0.849	304	3.2	0.01656	1.322
☐	16	TISSUE ENG	1076-3279	14767	4.065	4.623	0.727	374	4.6	0.04131	1.222
☐	17	TISSUE ENG REGEN MED	1738-2696	137	0.345	0.225	0.196	51	2.9	0.00044	0.047

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[KR2] Muscle and connective tissue progenitor populations show distinct Twist1 and Twist3 expression profiles during axolotl limb regeneration

***equal contribution**

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Martin Kragl*:	Experimental design Interpretation of the reported experiments and results Acquisition and analysis of data Drafting and revising the manuscript
Kathleen Roensch*:	Experimental design Interpretation of the reported experiments and results Acquisition and analysis of data Drafting and revising the manuscript
Ina Nüsslein:	Performance of Flow Cytometry
Akira Tazaki:	Interpretation of the reported experiments and results Acquisition and analysis of data Drafting and revising the manuscript
Yuka Taniguchi:	Performed and Analyzed Whole-mount *in situ* hybridization
Hiroshi Tarui:	Introduction of large scale single cell PCR to M.K.
Tetsutaro Hayashi:	Introduction of large scale single cell PCR to M.K.
Kiyokazu Agata:	Introduction of large scale single cell PCR to M.K.
Elly M. Tanaka:	Experimental design Interpretation of the reported experiments and results Drafting and revising the manuscript Corresponding author

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				Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	Articles	Cited Half-life	Eigenfactor® Score	Article Influence® Score
☐	1	ADV ANAT EMBRYOL CEL	0301-5556	352	3.200	3.208			>10.0	0.00049	1.180
☐	2	ANNU REV CELL DEV BI	1081-0706	8866	17.983	19.806	0.786	28	8.5	0.02601	11.113
☐	3	BIRTH DEFECTS RES A	1542-9768	2025	3.146	2.780	0.274	106	4.6	0.00699	0.888
☐	4	BIRTH DEFECTS RES C	1542-975X	994	4.442		0.346	26	5.2	0.00364	
☐	5	BMC DEV BIOL	1471-213X	2167	2.728	2.922	0.282	39	5.0	0.01101	1.194
☐	6	CELLS TISSUES ORGANS	1422-6405	1951	1.961	2.433	0.455	99	5.9	0.00485	0.703
☐	7	CURR TOP DEV BIOL	0070-2153	2246	6.912	6.467	0.762	42	4.9	0.01129	3.314
☑	8	DEV BIOL	0012-1606	31290	3.868	4.049	0.824	370	8.6	0.07480	1.737
☐	9	DEV CELL	1534-5807	20306	12.861	14.091	2.174	201	5.6	0.10605	8.205
☐	10	DEV DYNAM	1058-8388	10728	2.590	2.863	0.610	164	6.9	0.03284	1.233
☐	11	DEV GENES EVOL	0949-944X	1739	1.695	1.973	0.438	32	8.5	0.00377	0.822
☐	12	DEV GROWTH DIFFER	0012-1592	1974	2.397	2.341	0.586	70	6.2	0.00646	0.942
☐	13	DEV NEUROBIOL	1932-8451	1753	4.423	3.204	1.430	114	3.1	0.01147	1.351
☐	14	DEV NEUROSCI-BASEL	0378-5866	1859	3.413	3.250	0.050	40	8.2	0.00436	1.248
☐	15	DEV PSYCHOBIOL	0012-1630	2992	2.595	2.475	0.658	73	9.7	0.00528	0.844
☐	16	DEVELOPMENT	0950-1991	51191	6.208	6.888	1.258	434	9.2	0.12297	3.353
☐	17	DIFFERENTIATION	0301-4681	3042	2.855	3.011	0.861	79	7.9	0.00661	1.002

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☐	18	EVODEVO	2041-9139	149	3.914	3.943	0.308	26	1.8	0.00120	1.968
☐	19	EVOL DEV	1520-541X	1759	3.155	2.917	0.500	46	6.1	0.00603	1.195
☐	20	GENE DEV	0890-9369	57627	12.444	12.741	2.149	249	8.9	0.17180	7.123

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Journals 1 - 20 (of 41) [1 | 2 | 3] **Page 1 of 3**

[KR3] Progressive specification rather than intercalation of segments during limb regeneration

***equal contribution**

DOI: 10.1126/science.1241796

Kathleen Roensch*:	Experimental design Interpretation of the reported experiments and results Acquisition and analysis of data Drafting and revising the manuscript
Akira Tazaki*:	Experimental design Interpretation of the reported experiments and results Acquisition and analysis of data Drafting and revising the manuscript
Osvaldo Chara:	Performance and development of image analysis Statistical analysis
Elly M. Tanaka:	Experimental design Interpretation of the reported experiments and results Drafting and revising the manuscript Corresponding author

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				Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	Articles	Cited Half-life	*Eigenfactor*® Score	*Article Influence*® Score
☐	41	P ROY SOC A-MATH PHY	1364-5021	15105	2.378	2.244	0.664	214	>10.0	0.01997	1.218
☐	42	PHILOS T R SOC A	1364-503X	11509	2.891	3.108	1.576	295	8.2	0.03501	1.476
☐	43	PLOS ONE	1932-6203	133246	3.730	4.244	0.407	23406	2.4	0.77844	1.545
☐	44	R&D MAG	0746-9179	15	0.132	0.036	0.020	50		0.00004	0.012
☐	45	REND LINCEI-SCI FIS	2037-4631	131	0.915		0.056	36	3.0	0.00059	
☐	46	S AFR J SCI	0038-2353	1718	0.835	0.813	0.271	70	>10.0	0.00168	0.265
☐	47	SAINS MALAYS	0126-6039	270	0.408		0.071	211	2.7	0.00067	
☐	48	SCI AM	0036-8733	4991	1.478	1.875	0.379	103	>10.0	0.00602	0.798
☐	49	SCI ENG ETHICS	1353-3452	451	0.901	0.975	0.795	44	5.9	0.00130	0.369
☐	50	SCI REP-UK	2045-2322	1158	2.927	2.927	0.703	792	1.0	0.00564	1.576
☐	51	SCI WORLD J	1537-744X	2297	1.730	1.603	0.149	1149	4.1	0.00702	0.427
☑	52	SCIENCE	0036-8075	508489	31.027	33.587	6.691	832	9.7	1.35315	17.697
☐	53	SCIENCEASIA	1513-1874	208	0.398		0.017	60	4.6	0.00061	
☐	54	SCIENTIST	0890-3670	244	0.387	0.230	0.220	41	7.2	0.00065	0.107
☐	55	T ROY SOC SOUTH AUST	0372-1426	396	0.241	0.363	0.111	9	>10.0	0.00019	0.122
☐	56	TECHNOL REV	1099-274X	304	0.738	0.503	0.227	44	>10.0	0.00102	0.330

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Selbstständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbstständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

Dresden, 18. Januar 2017

Kathleen Rönsch

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Anlage 1

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2. Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts habe ich Unterstützungsleistungen von folgenden Personen erhalten:
Dunja Knapp, PhD
Osvaldo Chara, PhD
Prof. Elly Tanaka

3. Weitere Personen waren an der geistigen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die Hilfe eines kommerziellen Promotionsberaters in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

4. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

5. Die Inhalte dieser Dissertation wurden in folgender Form veröffentlicht:
Publikationen in folgender Fachliteratur:
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7. Ich bestätige, dass ich die Promotionsordnung der Medizinischen Fakultät der Technischen Universität Dresden anerkenne.

8. Ich habe die Zitierrichtlinien für Dissertationen an der Medizinischen Fakultät der Technischen Universität Dresden zur Kenntnis genommen und befolgt.

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Anlage 2

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Projektnummer 55-8811.71/103; 55-8811.71/96; 55-8811.71/210; 55-8811.71/205

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