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Genomics and epigenomics of axolotl regeneration

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ABSTRACT The axolotl (*Ambystoma mexicanum)* has been a widely studied organism due to its capacity to regenerate most of its cells, tissues and whole-body parts. Since its genome was sequenced, several molecular tools have been developed to study the mechanisms behind this outstanding and extraordinary ability. The complexity of its genome due to its sheer size and the disproportionate expansion of a large number of repetitive elements, may be a key factor at play during tissue remodeling and regeneration mechanisms. Transcriptomic analysis has provided information to identify candidate genes networks and pathways that might define successful or failed tissue regeneration. Nevertheless, the epigenetic machinery that may participate in this phenomenon has largely not been studied. In this review, we outline a broad overview of both genetic and epigenetic molecular processes related to regeneration in axolotl, from the macroscopic to the molecular level. We also explore the epigenetic mechanisms behind regenerative pathways, and its potential importance in future regeneration research. Altogether, understanding the genomics and global regulation in axolotl will be key for elucidating the special biology of this organism and the fantastic phenomenon that is regeneration.

KEY WORDS: *Ambystoma mexicanum, regeneration, molecular and epigenetic processes*

General characteristics of *Ambystoma mexicanum* (axolotl)

The genus *Ambystoma* comprises 33 species of salamanders found from southern Alaska to southern Mexico. Out of these, 17 species inhabit the mountains of Central Mexico. They are commonly known as "axolotls" (from Nahuatl language: atl "water" and xolotl "monster", also known as smooth water-skinned animal). These animals are native to canals of Mexico City (Xochimilco and Chalco lakes). Interestingly, no much data exists pertaining to the mortality rate of axolotls, some scientific reports have been published that in the wild their lifespan ranges from 5 to 7 years; while in captivity it ranges from around 10 to 15 years (Farkas and Monaghan, 2015), being the maximum life expectancy up to 25 years (Vieira *et al.,* 2020). Particularly, the Centro de Investigaciones Biológicas y Acuícolas de Cuemanco in Mexico City (CIBAC; http://www2.xoc.uam.mx/investigacion/cibac/) has in captivity several living specimens of native axolotls (*Ambystoma mexicanum*) from Xochimilco, whose age ranges between 15 and 16 years. Those axolotls are currently registered according to the Mexican program of the Ministry of the Environment and Natural resources (SEMARNAT; Registry number: SGPA/DGVS 06264).

Unfortunately to axolotls, due to human needs, their habitat has been manipulated and modified. Although few individuals remain in their natural habitats, axolotls are widely distributed in aquariums, pet shops, and recently, they have been used as an animal model that is gaining momentum with new genomic and experimental tools around the world. The axolotl was introduced to the scientific community by the French herpetologist August Duméril, who classified 7 specimens of *Ambystoma mexicanum* (six wild-type and one white mutant). These had been captured by French explorers in Lake Xochimilco and relocated to Paris, in the middle of the 19th century. In the 1960s, an albino tiger salamander (*Ambystoma tigrinum*), among a few more specimens, were added

Abbreviations used in this paper: AGSC, Ambystoma Genetic Stock Center; DRG, dorsal root ganglion; HDAC, histone deacetylase; LINE, long interspersed nuclear element; LTR, long terminal repeat; WE, wound epithelium.

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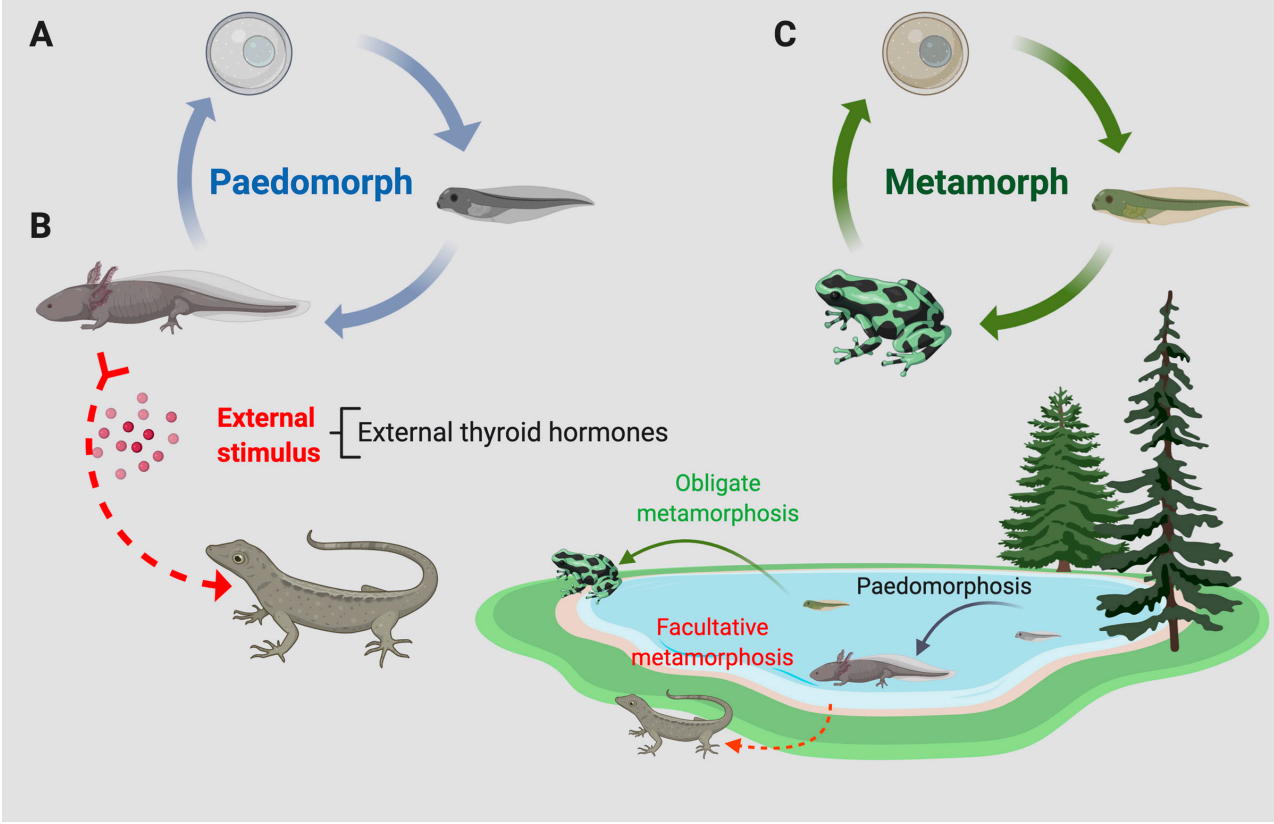

Fig. 1. Illustration depicting the differences between paedomophosis and metamorphosis. (A) *Paedomorphosis is a phenomenon observed in axolotls, where they retain their aquatic juvenile stage throughout their life cycle.* **(B)** *The transition from larva to adult form can be induced in response to exogenous thyroid hormones (red spheres) named as facultative metamorphosis.* **(C)** *Metamorphosis involves the initiation in larvae of behavioral, morphological, physiological, biochemical and genetic programs leading to color change, and the loss and gain of traits necessary for adaptation to the terrestrial habitat; also known as "obligate metamorphosis". Created with BioRender.com.*

to the colony. In fact, most modern-day laboratory axolotls descend directly from the same founders (Farkas and Monaghan, 2015). Thus, several conservation actions have focused on stopping the black market of axolotls, inbreeding of the species and harboring valuable information about wild versions to labs worldwide. In spite of this, the Indiana University started in 1960 the axolotl colony, which was extended years later by the University of Kentucky to create the Ambystoma Genetic Stock Center (www.ambystoma.org; https://ambystoma.uky.edu/genetic-stock-center/). To date, AGSC is considered as the most extensive colony of laboratory axolotls. However, a review of the AGSC pedigree analysis made by Woodcock and colleagues, showed that all individuals of the axolotl population have approximately 5.8 (+/-1) % of the tiger salamander DNA (Woodcock *et al.,* 2017). It is not clear why the historical axolotl lines were lost, but the authors speculate that because the native Xochimilcan axolotls have declined precipitously, this made it difficult to introduce new biological material into the collection. In addition, A. *Tigrinum* hybrids have proven to be more viable and fertile for stock production (Woodcock *et al.,* 2017).

Unlike many amphibians, axolotls do not undergo metamorphosis under physiological conditions, they are neotene, completing their full life cycle in the aquatic juvenile stage, a phenomenon known as paedomorphosis (Fig. 1A). However, a transition from larval to adult form may be induced in response to exogenous thyroid hormones (THs), since the axolotl have insufficient concentration of thyroxine hormone (T4), which determines a low rate of secretion of thyroid stimulating hormone (TSH) (Fig. 1B). Metamorphosis induces changes that larvae initiate behavioral, morphological, physiological, biochemical and genetic programs which will later manifest color change, loss and gain of traits necessary to transition from the aquatic habitat to a terrestrial habitat (Fig. 1C) (Coots and Seifert, 2015). Experimentally induced metamorphosis is also characterized by a reduction in the regeneration process leading to higher rate of minor morphological errors, while the facultative paedomorphic axolotls show spectacular capacities to restore missing parts of the body throughout the life-long lasting neoteny (Monaghan *et al.,* 2014).

The sexual dimorphism can be observed in the slimmer and longer bodies of adult males, compared to females. In the adult stage, males weigh average is 125-130 g, and females are typically about 170-180 g. Anatomically, it is a primitive tetrapod which possesses a calcified skeleton with cartilaginous joints, true teeth, and a complex olfactory system (Farkas and Monaghan, 2015). Axolotls have extraordinary mechanisms for gas exchange: cutaneous and pulmonary respiration, and gills and their skin are highly vascularized for this purpose, they present dermal and epidermal layers formed by loose connective tissue, collagen fibers, and fibroblasts (Seifert *et al.*, 2012). Their lungs are elongated, run parallel to the spinal cord, and can provide 40-60% of the axolotl's oxygen via surface breathing. Three external gills, which extend as branches,

protrude from the neck region on each side of their heads (Fig. 2A). They are covered by filaments used for respiration (Farkas and Monaghan, 2015). *A. mexicanum* has a global popularity not only for its biological and physiological characteristics, but also because of its remarkable ability to fully regenerate many body parts, perhaps the main reason why it has been so widely studied.

Regeneration is a common feature of many multicellular organisms. In 1768, Lazzaro Spallanzani showed in a brief publication entitled *Prodromo,* the regenerative abilities of worms, snails, tadpole and salamander tails and limbs and salamander jaw regeneration. In fact, appendage regeneration were the first discoveries to show that a vertebrate could regenerate and restore complex form. Notwithstanding, during the 18th century the mechanism of regeneration vertebrate appendix was under debate because it was not supposed to be possible. To date, it is well known that salamanders have the potential to regenerate complex structures (e.g. entire limbs, tail, substantial parts of the central nervous system, etc.) (Endo *et al.,* 2004). Particularly, *Ambystoma mexicanum* has emerged as the prime salamander model used in regeneration and repairing research due to its remarkable and highly successful ability to regenerate many of their body parts. Although axolotls retain the capability of regeneration throughout adulthood the rate of the phenomenon diminishes with age, from weeks in larval to months in sexually mature adults (Vieira *et al.,* 2020). Regeneration of the limb has been the structure most extensively studied, providing the basis and the cellular and molecular mechanisms that regulate and remodel tissues and organs (Endo *et al.,* 2004). Therefore, analyzing the axolotl genome and understanding the molecular and epigenetic mechanisms of regenerative potential, could also provide clues about the genes and new molecular perspectives related to function during successful, failed or aberrant tissue regeneration. This review focuses on molecular mechanisms that enable axolotl regeneration and highlights areas where further research is needed.

The mechanism of tissue regeneration in axolotls from evolutionary and molecular perspectives

Regeneration is a physiological mechanism which implies the renewal of tissues and functional restoration process of organ or appendage after injury. While, the vertebrates urodeles (newts and salamanders) there is remarkable ability to regrow tissues, organs and multiple body parts throughout their lives. Compared to other species, the process of regeneration in *Ambystoma mexicanum* is characterized by the capability to form a specialized structure called blastema, considered a crucial step during regeneration followed by injury (Endo *et al*., 2004). Throughout the course of evolution, the regeneration of appendages has been present in tetrapods, however in some of them the genetic programs may be quite different, probably because they have evolved independently.

The sarcopterygians (lobe finned vertebrates) that grouped the salamanders and lungfishes are the only animals with the capacity of paired appendage regeneration, after endoskeleton amputation and regardless the level of damage. While, among the actinopterygian (ray finned fishes), the species that regenerate paired fins, including endoskeleton after amputation are the polypterid fishes (Clastridia). Currently, attempts have been made to clarify whether paired fins and limb regeneration processes share a common origin. In this context, Darnet and colleagues provided evidence of a wide phylogenetic distribution of paired-fin regeneration after endoskeleton amputation across fish lineages. Thus, by regeneration assays and RNA-sequencing (RNA-seq) analysis, they demonstrated that common genetic pathways and expression profiles are deployed during regeneration axolotls and *Polypterus* blastemas. Furthermore, transcriptome comparisons in early-stages blastemas revealed significant similarities in gene-expression profiles between axolotls and *Polypterus,* which means that both species *s*hare a regeneration-specific genetic program. The gene analysis of differential gene expression profiles in blastema in *axolotls* and *Polypterus,* revealed that 35.31% of the up-regulated genes in these species possess homologs in the axolotl. While, 67.54% are enriched in gene ontology categories such as morphogenesis, extracellular matrix organization, and chromatin remodeling in both blastema transcriptomes. Several genes have been reported as possible candidates or to be critical for proper and successful regeneration during axolotl regeneration, for more information see the references (Hass and Whited, 2017; Sanor *et al*., 2020), however information about the function of many genes has not been fully experimentally demonstrated until now. Darnet and colleagues hypothesized that the regeneration genetic program in vertebrates has a deep evolutionary origin, which evolved from the paired fin regeneration of the fish ancestors, while there are lineages with a regeneration-incompetent program, such as the amniota (Darnet *et al*., 2019). This proposal has become a guide to identify the genetic signature of vertebrate appendage regeneration, and which is based on the comparison to the functions of homolog genes in other organisms. The regeneration process implies mechanisms such as morphogenesis, cell proliferation and differentiation, which are present in the molecular programs of embryos of other species, or wound healing and inflammatory response, which can also be found in adults. Thus, identifying genes implied in such processes in well-studied organisms and elucidate how they are regulated, may provide some insights into candidate regeneration genes in the axolotl.

Ambystoma mexicanum has the capacity of regenerating tissues of a wide range of body structures, which makes it a wonderful and key model to study the regenerative processes. Nevertheless, some tissues are limited to some stages of development, such as the case of the regeneration of eye lens which occurs during the first two to three weeks after hatching, but this ability is lost thereafter (Suetsugu *et al.,* 2012). Additionally, the axolotl adults regenerate lost cells in neural tissues, including brain regions and spinal cord, both in terms of structure and function. In this regard, an important challenge has been to define whether the reappearance of a perfect neuronal units are also functional copies of the original structure. Amamoto *et al.,* addressed this problem by removing a large portion of the telencephalon, showing that the original neuronal diversity even at the level of subpopulations was regenerated, and suggesting that the brain axolotls can sense which types of neurons are damaged. Even though the neuronal diversity was restored, projections were not faithful replication of the original (Amamoto *et al.,* 2016). The reason for this uncoupling neuronal diversity and physiological circuits is not yet known. Other types of injury models have also addressed how neuronal cells regenerate followed by chemical ablation of dopaminergic (DA) neurons; showing that neurons are able to regenerate in several brain regions together with remarkable recovery of the locomotor activity, after DA ablation in individual neuronal subtypes. This occurs

presumably by activation and proliferation of the ependymoglial cells, proposed as the main source of new neurons and which are the equivalent of glial cells in mammals (Parish *et al.,* 2007). Another amazing aspect in *Ambystoma mexicanum* is that after tail amputation the spinal cord is fully repaired. Both rostral and caudal sides are reconnected, together with the correct number of segmented vertebrae, myotomes and dorsal root ganglia (DRG). In 2012, Tanaka´s group demonstrated that regeneration of the central and peripheral nervous system (CNS, PNS) occurred during axolotl tail regeneration, together with a substantial portion of DRG and Schwann cells, which arise from cell pools associated to the central regenerating spinal cord; suggesting the existence of cells with spinal cord neural stem cell properties (McHedlishvili *et al.*, 2012).

Successful limb regeneration requires the formation of an undifferentiated cellular structure named blastema, which contains a collection of progenitor cells highly proliferative (Kragl *et al.*, 2009). A difference from any other undifferentiated mass, the blastema is characterized by its essential functional attributes for establishing its developmental potential, its lineage-restricted and positional cellular properties. In urodeles, the cells that form the blastema are derived by natural reprogramming of skeletal, muscle cells, Schwann cells and fibroblasts to a less differentiated state. Thus, after amputation shortly the blastema is formed by a layer of epithelial tissue that covers all tissues of mesodermal origin, over the wound of a severed limb, with the subsequent progenitor cells activation by differentiation process. Once it reaches a critical size, the bulb flattens to a "palette" stage, and cells meant to become cartilage coalesce and condense, and the various tissue types appear, including dedifferentiated cells which form a fully functional limb with the size and age of the axolotl (Fig. 2B) (Endo *et al.,* 2004; Kragl *et al.*, 2009; Haas and Whited, 2017).

Morphologically, axolotl limbs contain different tissue types: neural, myogenic, epidermal, and connective tissue. Together, these comprise three main segments: upper and lower arms, and hand, so when an *Ambystoma* suffers tissue damage, a thin layer of cells migrates from the stump epidermis to cover the site of amputation. Within the next few days after the injury, progenitor cells in surrounding tissue are activated and re-enter the cell cycle. This mechanism induces cellular proliferation and migration of epidermal cells to form the specialized epithelium named wound epithelium (WE), which is structurally and molecularly distinct new tissue. The early WE develops into the new thickened epidermis called the apical epidermal cap (AEC), the WE/AEC consists only of an epithelial layer and lacks of basal lamina. The absence of basal lamina and dermis allows direct contact between WE cells and the underlying tissues, proposed to facilitate bidirectional critical signaling for blastema formation and maintenance (Tas-

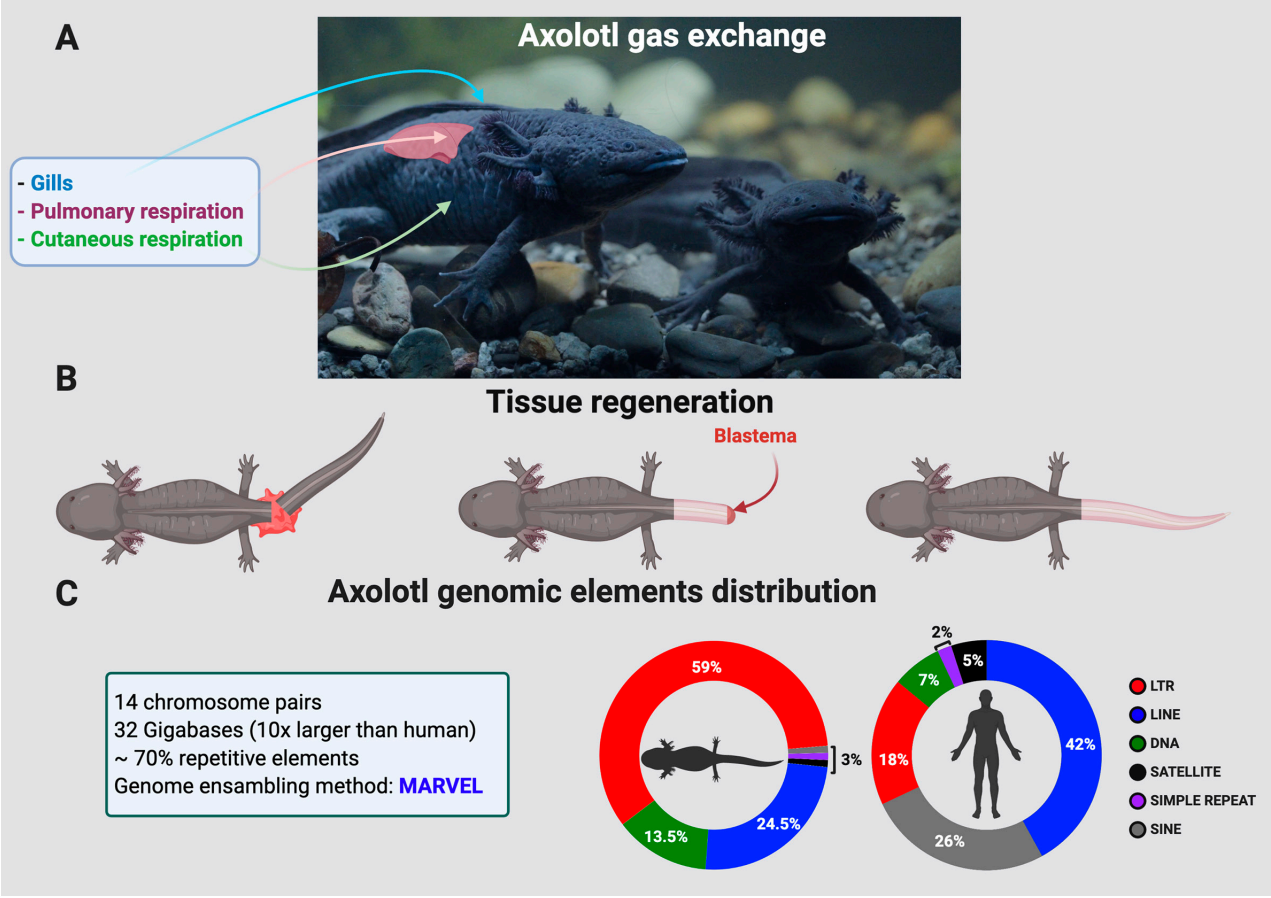

Fig. 2. Key features in *Ambystoma mexicanum*. (A) *The axolotl has three mechanisms of gas exchange: gills, pulmonary, and cutaneous respiration.* **(B)** *Process of tissue regeneration following limb injury, through the formation of a blastema and consequent tissue differentiation.* **(C)** *Comparison of axolotl and human genomic element distribution. Axolotl genomes contain a large proportion of repetitive elements, namely LTRs, SINEs, LINEs, satellites, and simple repeats; adapted from Nowoshilow* et al., *2015. Created with BioRender.com.*

sava and Garling, 1979; Campbell and Crews, 2008). Until now, it has been widely accepted that this thickened WE is required for the regenerative process, as well the interaction with regrowing nerves and to recruit regeneration-competent cells for successful blastema formation (Endo *et al.,* 2004; Campbell and Crews, 2008; Haas and Whited, 2017). Revisiting the classical experiments by immediate insertion of amputated limb into body cavity in newts, the internal tissue proliferation has been shown to initially occur independently of WE formation, however blastema was not formed and regeneration did not occur. Highlighting the importance of injury, nerves and WE in controlling the successful regeneration (Tassava and Loyd, 1977; Haas and Whited, 2017). It is important to mention, that significant progress has been made in the elucidation of blastema formation, however the origin of blastemal cells and the synchronized signaling mechanisms between different tissues and cell types to initiate the blastema remain to be unclear (Kragl *et al*., 2009; Sandoval-Guzmán *et al*., 2014; Leigh *et al.,* 2018; Gerber *et al.,* 2018). During appendage regeneration, remains debatable whether blastema cells arise by a type of stem cells (muscle satellite cell) or by cellular dedifferentiation. Particularly, the skeletal muscle cells have been the focus of these studies due to the lack of quantitative evidence for muscle dedifferentiation, especially to follow for a long-term the fate of endogenous muscle fibers. Studies conducted in two salamanders by a Cre-loxP reporter-based fate mapping experiments of muscle during limb regeneration demonstrated that in the newt, the muscle differentiation makes significant contribution to muscle regeneration. While in the axolotl myofibers made no contribution to limb regeneration, and the recruitment of abundant $PAX7^+$ satellite cells were the main contributor to mature tissue into blastema during limb muscle regeneration. This unexpected difference in the occurrence of myofiber dedifferentiation showed that even in two species of salamanders closely related there is a flexibility in the cellular and regenerative mechanisms (Sandoval-Guzmán *et al*., 2014).

Axolotl limb regeneration has been proposed to follow the rule of distal transformation referring to the regeneration of distal structures in the amputation plane (Mercader *et al.,* 2005). Mercader and coworkers found that the axolotls Meis homeobox family overexpression play an important role in the proximodistal patterning during limb regeneration, due to induces relocation of distal blastema cells to more proximal regions locations in the regenerated limb, and which is prompted by retinoic acid (RA) pathway activation. RA-Meis pathway is not only crucial during limb development, but also is essential to specify proximal fates during limb regeneration (Mercader *et al.,* 2005). Thus, connective cells have been proposed to form the patterned limb skeleton that serves as a guideline for the rest of the tissues (Gerber *et al*., 2018; Leigh *et al.,* 2018). Obeying this rule, the display of nuclear Meis homeobox genes has been identified as an upper arm regulator (Mercader *et al.,* 2005), while myogenic cells do not obey the rule and their nuclear Meis homeobox genes appear at any proximodistal level. The cells descending from lateral plate mesoderm together with blastema contribute to detect the site of injury, and induce regeneration of bone, cartilage, tendons, periskeleton, and dermal and interstitial fibroblasts. This makes connective tissue cells an important target for molecular regeneration programs (Kragl *et al*., 2009; *G*erber *et al*., 2018; Leigh *et al.,* 2018). Additionally, cartilage-derived blastema cells present positional identity expressing Meis, HoxA9, and HoxA13 genes and following the rule of distal transformation; whereas Schwann-derived cells do not present any of the above (Mercader *et al.,* 2005; Gerber *et al*., 2018; Leigh *et al.,* 2018). Muscle, cartilaginous tissue, and Schwann cells give rise mainly to the same cell lineage but there is no evidence if muscle cells may dedifferentiate into stem cells (Kragl *et al*., 2009). Interestingly, the chondrocytes have been suggested to be susceptible to proliferative signals from the amputation site, however they retain differentiated morphology and position, and no migration into the regenerated site has been observed. In contrast, the pericytes proliferate and migrate into blastema, giving rise solely to pericytes. But they are not the main contributors to skeleton and skeleton digit regeneration. While, cell recruitment to form the blastema has spatio-temporal features requires that periskeletal and dermal fibroblasts set the guide by building the skeleton, followed by migration of dermal fibroblasts found within 500 μm of the site of amputation, to form the soft connective tissue. Regarding the muscle cells mechanisms, contradictory results have shown in salamanders (newt and axolotl). An important contribution of myofibers $PAX7^-$dedifferentiate into proliferative blastema during regenerated newt limb was observed, while in axolotl myofibers neither generate proliferating cells nor contribute to limb muscle regeneration. However, multiple $PAX7^+$ satellite cells were found to regenerate muscle in axolotl (Sandoval-Guzmán *et al*., 2014). Suggesting that clear differences in the cellular processes for blastema formation and muscle differentiation have seemed between these two species that share the ability of adult limb regeneration. This could bear significance for the loss of regenerative ability in some species of urodele, or simply new strategies have been developed or modified among salamanders to suit their regenerative needs after any type of injury.

To study the amazing regenerative mechanisms, several tools have now been developed, such as localized genome editing through retroviral infection or CRISPR/Cas, with successful genomic integration in embryos and larval and adult limbs (Fei *et al*., 2018). Albeit reverse genetic engineering seems a promising strategy (Sanor *et al*., 2020), the transcriptomic analysis has provided sequence data and molecular information to identify the genes networks involved in axolotl regeneration. Which we will address in the next section.

The transcriptomics of the axolotl

Despite progress in axolotl sequencing, several problems remain to be solved before it is likely to create a complete assembly of the axolotl genome. Therefore, at the moment and historically, transcriptomics remains to be the tool that will continue to provide the majority of significant advances in the genetics of limb regeneration (Nowoshilow *et al.,* 2018; Smith *et al.,* 2019). Due to this, the tools that have been used to understand the networks of genes and the pathways of the regenerative mechanisms of the axolotl have focused on studies of proteomics, transcriptomics using RNA-Seq and microarrays techniques (Bryant *et al.,* 2017; Brown and Peirson, 2018). To date there are some methodologies or different analysis tools that have been tested and accepted for the study of limb regeneration in the axolotl genome. Within advances in omics studies, a deep RNA sequencing analyzes of blastema in a time course experiment allowed the discovery of genes candidates involved in the regenerative process (Stewart *et al*., 2013). In this regard, recently a study was carried out that collectively collects all the transcriptome data that come from expression microarrays

and RNA seq in axolotl, in order to obtain a top 100 of genes involved, filtering the data despite the methodological discrepancies. To answer this kind of question, one of the key methodologies that has been employed is integrative data analysis (IDA) (Sibai *et al.,* 2019). This methodology is applied in many scientific disciplines and focuses on deriving a scientific consensus on a particular research question (Brown and Peirson, 2018; Walsh *et al.,* 2015).

A typical problem that arises in this type of case is the cost of using new "omic" technologies, often focused on collecting a limited number of biological replicas. Due to the complexity, researchers face challenging statistical problems that arise from having such limited replications, which usually generate problems that are manifested in high false positive and false negative observations (Benedetti *et al.,* 2014; Brown and Peirson, 2018). However, the RNA expression studies from Expression Microarrays and the subsequent RNA-seq studies served to assemble the transcriptome *de novo* (Bryant *et al.*, 2017). Which was a great step to boost the study of the axolotl and its regeneration, and finally to generate the first draft of its genome.

The genome of *Ambystoma mexicanum*

In 2018, the *Ambystoma mexicanum* genome was sequenced and assembled, comprising 32 gigabases (Gb) distributed in 14 chromosome pairs (Nowoshilow *et al.*, 2018). Recent efforts have yielded genome assemblies consisting of thousands of unordered scaffolds that resolve gene structures, but do not yet permit large-scale analyses of genome structure and function (Smith *et al.*, 2019). As an experimental approach they combined long-read sequencing, optical mapping, and developed a new genome assembler known as MARVEL (Fig. 2C). The 32 Gb of axolotl genome is 10 times larger than the human genome and is currently the largest genome ever sequenced (Nowoshilow *et al.*, 2018). Nevertheless, the complexity of the sequencing process was not only due to its large size, but also because around 70% of the genome is composed of repetitive elements such as long interspersed nuclear elements (LINEs) and mainly the LTR retrotransposons, compared to *Homo sapiens* who presents a different distribution of these repetitive elements (Fig. 2C). This presumably reflects the evolutionary diversification of mobile elements that accumulated during an ancient episode of genome expansion. These genomic characteristics have been poorly described in animals, which has complicated its study at the genomic level, but could probably be the key to one of the most important miracles of this organism: tissue regeneration (Keinath *et al*., 2015).

Historically, the genetic research efforts in axolotl were focused in two classical recessive traits (white (d/d) and albino (a/a)) that are maintained in the domestic axolotl population. These traits possess loss-of-function pigmentation mutants that have been recently mapped. The white (d/d) was collected in Xochimilco (Mexico) from the original 33 founder axolotls and then shipped to Europe. The single locus recessive allele "d", and white axolotl phenotypes have been extensively used to understand vertebrate pigmentation. Currently, genetic studies have shown that white mutants' phenotype is a consequence of transcriptional defect in endothelin 3 (*edn3*). This gene encodes for a peptide factor that promotes pigment cell migration and differentiation in other vertebrates. Surprisingly, the albino phenotype "a", did not arise within the axolotl lineage but was instead established by interspecific hybridization. Albino phenotype has the capacity to cross with other metamorphic salamanders by *in vitro* fertilization (Woodcock *et al*., 2017). Such hybrids were crossed into many modern axolotl strains that are maintained by Ambystoma Genetic Stock Center (AGSC). It is in these hybrids in which most of the genomic research has been conducted, and it is important to understand that our current knowledge in axolotl genome is not fully based in a native Xochimilcan axolotl, so differences might yet exist compared to the wild species (Nowoshilow *et al*., 2018; Keinath *et al*., 2015).

Due to these historical aspects as a model that has facilitated research in multiple areas such as evolution, development, and regeneration; the genomic study of the axolotl was a necessary step to acquire further knowledge, and was imperative to understand the biological mechanisms behind axolotl secrets, but its elucidation needed the advance in molecular biology and genomic sequencing tools. Therefore, the study of the axolotl genome has been very intense, and because of its nature, it has been full of difficulties in recent years. The reason behind this, and the challenges presented at the efforts towards the integral assembly of genomes such as that of salamanders, is their large genome sizes and the enormous number of repetitive regions that they contain. This is also true for the axolotl's genome, where the large size was originally estimated from *A. tigrinum* (Keinath *et al*., 2015).

To achieve a full understanding of the size and characteristics of the axolotl's genome and to be able to assemble it on a chromosomal scale, there have been major hurdles towards obtaining a full assembly. This is due to the inability of acquiring sufficient read-length (Henson *et al.,* 2012; Bryant *et al*., 2017). The initial approximations derived from proximity ligation (despite extensive analysis of the resulting datasets) showed limitations and few useful results. For this reason, meiotic mapping methods were used as a tool to generate dense genome-wide scaffolding information. This led to the employment of hybrid crosses between *A. mexicanum and A. tigrinum* that were used to develop meiotic maps for the species and infer the positions of quantitative trait loci (QTL), sex, and Mendelian pigment mutants (Woodcock *et al*., 2017; Bryant *et al*., 2017). This was done by obtaining a reference genome with the application of the most modern technologies and techniques in sequencing and assembly (Kuleshov *et al*., 2016; Phillippy, 2017; Zheng *et al*., 2016). Together with the use of long reads sequencing instruments, the assembly of the complicated axolotl genome became a reality. Nowadays, the latest version of the assembly harbors annotated models for a vast majority of axolotl genes on scaffolds that typically exceed a megabase in length (N50 ~3 Mb) (Nowoshilow *et al*., 2018; Gerber *et al*., 2018; Keinath *et al*., 2015; Zheng *et al*., 2016; Smith *et al*., 2009). Yet, much research needs to be done to elucidate now more specific features of the axolotl genome, such as the identification of regulatory elements, genetic association studies, among others. But thanks to such advances in sequencing, assembly, and genetic manipulation, the genomic composition was confirmed (Keinath *et al*., 2015; Nowoshilow *et al*., 2018). To put the axolotl genome scale in perspective, it is 1.57 times larger than the loblolly pine genome (20.6 Gb). The size of its chromosomes varies from 3.14 Gb on its largest chromosome, to 0.66 Gb on its smallest chromosome (Smith *et al*., 2019), while human and mouse chromosome length average 128 Mb and 124 Mb, respectively; hence, one large axolotl's chromosome is around the size of the entire human genome (Nowoshilow *et al*., 2018).

One of the characteristics found in the axolotl genome that explains its enormous length, is that it is generally highly repetitive

and contains unusually long introns (Bryant *et al*., 2017; Smith *et al*., 2009). When compared to other genomes, axolotls exhibit extensive preservation between humans and chickens, where, interestingly, the axolotl homologous genome segments are on average 14 and 51 times longer, respectively (Voss *et al*., 2011). A possible hypothesis that arises from genomic results is that the introns are unusually long. This suggests that, in general, gene regulatory elements may be more separated in salamanders than in other vertebrates. One explanation of how axolotl gene regulation might deal with this issue could be by long range enhancer-promoter contact and high order chromatin structures that facilitate gene regulation (Smith *et al.,* 2009*;* Benedetti *et al*., 2014). However, these regulatory mechanisms are mainly unknown. Also, the size of axolotl genome is thought to be caused from an ancient episode of genome expansion resulting from the activity of mobile elements, like LTR retroelements classes, LINE 1/2 elements, *gypsy* and other endogenous retroviruses (Keinath *et al*., 2015; Nowoshilow *et al*., 2018). The axolotl genome is constituted by 18.6 Gb (66% of the contig assembly) of repetitive sequences, as LTRs and other retroviruses are the most abundant. Due to the presence of many repeated sequences, the average size of an intron is 22.8 kb, which is 16 and 13 times larger than the observed in mice (1.47kb) and humans (1.75kb), respectively (Fig. 2C) (Nowoshilow *et al*., 2018). From genomic and together with mRNA analysis, it has been now established that, as observed in other organisms, and despite the large size of its genome, a total of 23,251 protein-coding genes have been annotated. This result shows a similar number of coding genes to those found in other vertebrate genomes (Bryant *et al*., 2017; Smith *et al*., 2019; Brown and Peirson, 2018). Therefore, the genetic machinery underlying the development and characteristics of this organism is governed, as in other species, by a similar set of conserved genes. In short, a tremendous research effort has been made to understand and assemble the axolotl genome. Although the recent findings and mappings are not yet fully elucidated, they are sufficient to make way for studies of regulatory elements and other genetic mechanisms that could be used to help solve and understand phenomena that have generated great interest in this species, such as regeneration. So now there is a reference to compare to, and the future of the study of this organism is about to open new chapters.

Altogether, both genomic and transcriptomic data open a new window that will allow a better understanding of axolotl regeneration. This being the case, it leads to the question, how is a genome with such excessive dimensions regulated? the answer could be hidden in the epigenetic mechanisms and in the architecture of genomic contacts at a distance that must be generated at the nuclear three-dimensional level.

When the DNA sequence is not enough: the epigenetics of the axolotl

In 1942, Conrad Waddington coined the term “epigenetics” to explain the mechanism involved in the cell differentiation process. Waddington defined epigenetics as “the branch of biology that studies the causal interactions between genes and their products which bring the phenotype into being” (Rajagopal and Stanger, 2016). A substantial part of his work was based on the explanation of a model known as the “epigenetic landscape”, represented by a ball rolling down a hill with valleys and ridges that could affect its final ball position (Fig. 3A). This process results in the establishment of a cellular identity, where each “road” leads to a terminally differentiated cell. But it has been proposed that cellular identity can change by physiological mechanisms in an adult, as a response to injury. There are two main pathways for such cellular plasticity: firstly, it is related to cells that can adopt a progenitor-like phenotype, this mechanism is known as de-differentiation. The second process occurs when somatic cells transform directly into another mature cell by a trans-differentiation phenomenon (Fig. 3A). This cellular plasticity involves a series of events that alter both the transcriptome and the cellular proteome, which are mainly regulated by changes in their epigenome, as post-translational modifications of histones and epigenetic DNA modifications, that together generate changes in the epigenetic landscape (Fig. 3A and B). This model was used to explain the course of differentiation from a pluripotent to unipotent cell state and is currently being applied to explain different biological and physiological phenomena, such as tissue regeneration, as well as pathological ones, such as cancer (Baedke, 2013). Currently, one of the most accepted definitions of epigenetics is the inheritance of gene expression patterns without altering the underlying DNA sequence by adapting chromatin structure, which is the physiological form of our genetic information. The regulation of gene expression is intimately regulated through various epigenetic processes. Among them, the DNA methylation and the post-translational modifications of histones and non-coding RNA are the most conserved epigenetic mechanisms throughout species (Allis and Jenuwein, 2016).

DNA methylation and demethylation as a conserved epigenetic mechanism in most species

DNA methylation has been one of the most historically studied among the different epigenetic processes. Several reports have shown that this mechanism is conserved across species, even leading to very particular epigenetic systems for some of them (Zhong, 2016). The DNA methylation is a covalent modification. So far, three types have been described: N6-methyladenina (6mA), N4-methylcytosine (4mC) and 5-methylcytosine (5mC) (Greer *et al.,* 2015). While 6mA and 4mC are restricted to prokaryotes and some eukaryotes, 5mC is the most representative epigenetic mark in eukaryotes (Harris and Goldman, 2020). DNA methylation has been implicated in several biological processes including genome integrity, gene imprinting, tissue and organism development, gene silencing, X chromosome inactivation, suppression of retroviruses and transposons, and even in gene alternative splicing phenomena, among others (Nowoshilow *et al*., 2018; Allis and Jenuwein, 2016; Shayevitch *et al*., 2018). It was previously believed that DNA methylation as a covalent epigenetic mark was difficult to remove, until the enzymes responsible known as Ten-Eleven Translocation family proteins were discovered (Tahiliani, *et al*., 2009). These enzymes promote the oxidation of 5mC into 5-hydroxymethyl (5hmC), 5-formyl (5fC), and 5-carboxyl (5caC) (Wu and Zhang, 2017). This mechanism revolutionized the idea that was held about the establishment of epigenetic marks, thus giving them a biological dynamism. Particularly, the active demethylation of 5mC has been associated with different processes such as pluripotency of embryonic stem cells, neuron development, and tumorigenesis in mammals, among others (Shi *et al*., 2017). Mainly, the possibility of altering the methylation state of a cell can impact its genetic transcriptional regulation. The axolotl is no exception to this phenomenon. It has

been reported that 72 hours after limb injury there is a modulation in the expression in *de novo* DNA methyltransferase 3 (*DNMT3a*) (Fig. 3B). Axolotl wounds treated with a DNA methyltransferase inhibitor induce changes in gene expression and cellular response in regenerative processes, suggesting that DNA methylation and demethylation (or eraser) mechanisms could be influencing axolotl tissue regeneration (Fig. 3B) (Aguilar and Gardiner, 2015). It has also been reported that the axolotl can present a specific distribution of 5hmC and 5fC, which is conserved between amphibians and mammals (Fig. 3B). Neural cells, skin, and connective tissue have been reported to have a strong abundance of 5hmC compared to the rest of the tissues. These results suggest that many of the epigenetic marks remain conserved and could be the key to specific processes such as tissue regeneration (Almeida *et al.*, 2012; Alioui *et al.*, 2012). In the chromatin of axolotl oocytes, a reduction in the repressive histone marks such as H3K9me3 and in the protein of heterochromatin 1 alpha (HP1-α) has been reported, as well as an increase in the acetylation of H3 lysine 9 (H3K9ac) (Bian *et al.*, 2009). This suggests an epigenetic plasticity, which is not fully understood in axolotl somatic cells, which may be a crucial point in tissue regeneration (Fig. 3B). In this sense, a very elegant experimental approach showed that the disturbance of histone deacetylases (HDAC) by compounds inhibited tail regeneration in axolotl embryos treated for 7 days. These data strongly suggest that HDACs participate substantially in the regenerative process in response to injury (Voss *et al.*, 2019; Baddar *et al.*, 2021). These antecedents point that the axolotl is one of the few reported tetrapods which has the ability to regenerate structures such as limbs, tail, heart, eye lenses, brain, and spinal cord, where genetic and epigenetic molecular mechanisms could be involved in the tissue regeneration mechanism. Although regeneration of axolotl limbs is the most studied model, little has been addressed from an epigenetic perspective. There are several reasons that have led to this, particularly that its genome represents a significant genetic challenge. Finally, the currently poorly addressed epigenetic components of the axolotl may be responsible for transcriptional gene regulation.

Conclusions and Future Directions

Ambystoma mexicanum is considered an icon of Mexican culture, also known as the axolotl. Unfortunately, for different causes it is amongst the species that are racing towards extinction, as listed by the International Union for the Conservation of Nature and Natural

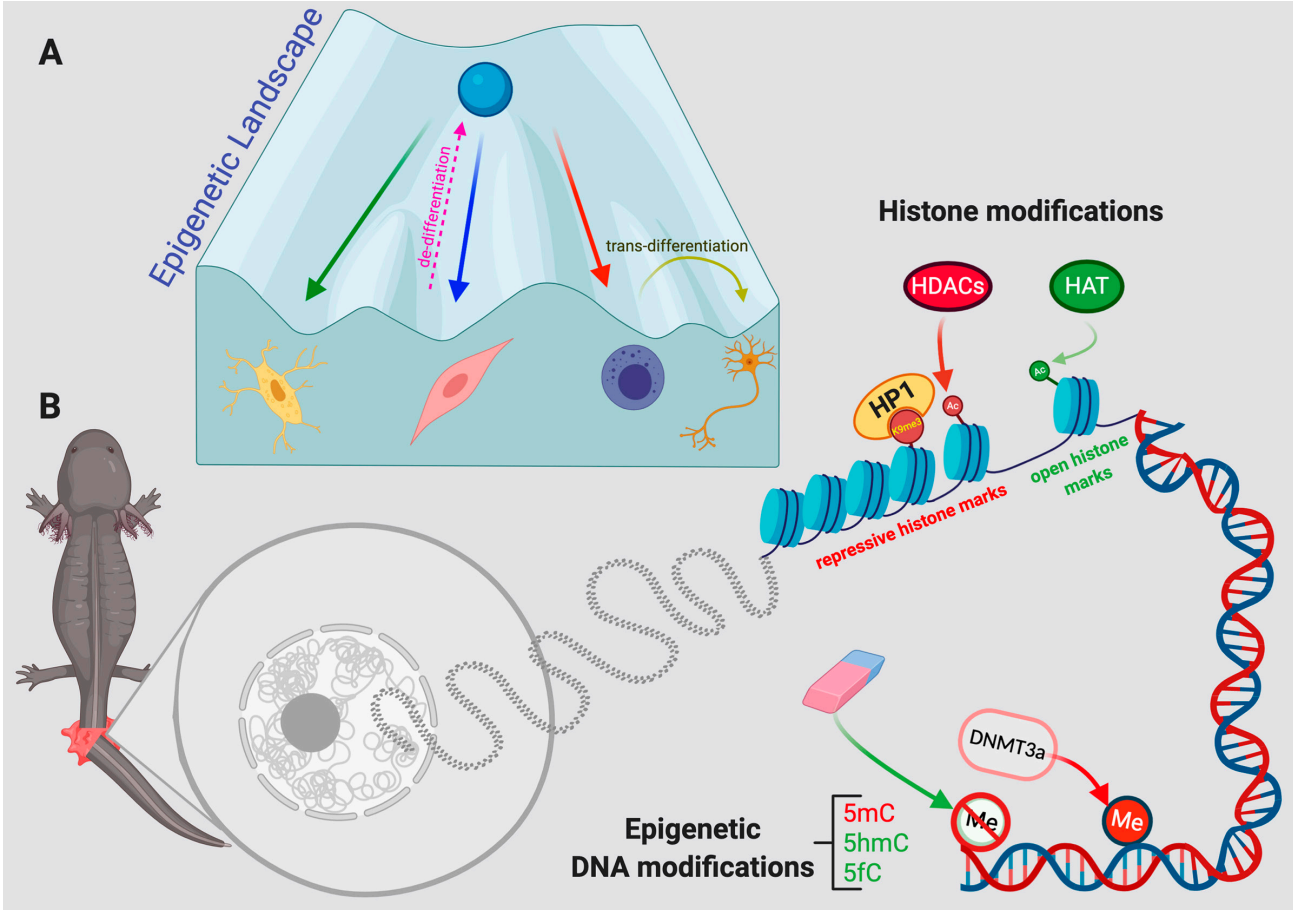

Fig. 3. Epigenetic processes reported in the axolotl. (A) *Waddington's epigenetic landscape, where the processes of cellular differentiation (green, blue and red lines), de-differentiation (dotted pink line) and trans-differentiation (yellow line) are shown.* **(B)** *An injury in axolotl may induce epigenetic changes related to the regeneration process, such as DNA methylation (5mC), DNA demethylation (5hmC and 5fC), repressive histone marks (H3K9me3 and HP1), and the acetylation (by histone acetyltransferase (HAT)) and deacetylation of histone (mediated by histone deacetylases (HDACs). Created with BioRender.com.*

Resources (IUCN) (https://www.iucn.org/). Curiously, *Ambystoma mexicanum* is one of the amphibians that can be found in research labs, aquariums and pet shops worldwide. However, the specimens found in these places possibly have a certain degree of inbreeding, since they come from the same population, which leads to vulnerability to certain diseases. In this sense, diverse institutions such as the University of Kentucky through the AGSC project maintains a breeding colony of axolotls and distributes them for research and teaching purposes. Despite the efforts of conservation programs, *Ambystoma mexicanum* continues to face problems to survive and breed in its natural environment.

Ambystoma mexicanum has become an ideal study model for research, given its unique physiological and morphological characteristics. Perhaps the most important contribution of the axolotl to medical research is related to its amazing capacity to regenerate cells and multiple tissues and organs. The axolotl has developed an extraordinary mechanism of regeneration, beginning with the blastema where newly de-differentiated cells congregate to later form a fully functional limb with perfect healing and no sign of previous damage. But, how does the regeneration pathway work? This is a question that is currently still unresolved. Regeneration research has been addressed toward the blastema formation, but most of the mechanisms behind this process are not fully understood. It has been observed that age is a determining factor in transcriptional gene regulation during tissue regeneration, including the transdifferentiation process, proposing that the environmental conditions, genetic and epigenetic components may be partly responsible for the tissue regeneration processes in axolotl. Therefore, the study of molecular biology, genomics, and epigenetic machinery underlying the regenerative mechanisms offers a novel approach to unravel this outstanding process.

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