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A histological atlas of the tissues and organs of neotenic and metamorphosed axolotl

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ABSTRACT

Axolotl (*Ambystoma Mexicanum*) has been emerging as a promising model in stem cell and regeneration researches due to its exceptional regenerative capacity. Although it represents lifelong lasting neoteny, induction to metamorphosis with thyroid hormones (THs) treatment advances the utilization of Axolotl in various studies. It has been reported that amphibians undergo anatomical and histological remodeling during metamorphosis and this transformation is crucial for adaptation to terrestrial conditions. However, there is no comprehensive histological investigation regarding the morphological alterations of Axolotl organs and tissues throughout the metamorphosis. Here, we reveal the histological differences or resemblances between the neotenic and metamorphic axolotl tissues. In order to examine structural features and cellular organization of Axolotl organs, we performed *Hematoxylin & Eosin*, *Luxol-Fast blue*, *Masson's trichrome*, *Alcian blue*, *Orcein* and *Weigart's* staining. Stained samples from brain, gallbladder, heart, intestine, liver, lung, muscle, skin, spleen, stomach, tail, tongue and vessel were analyzed under the light microscope. Our findings contribute to the validation of the link between newly acquired functions and structural changes of tissues and organs as observed in tail, skin, gallbladder and spleen. We believe that this descriptive work provides new insights for a better histological understanding of both neotenic and metamorphic Axolotl tissues.

1. Introduction

Metamorphosis term is used to define the innate process of amphibian transition from larval stage to adult form (Shi, 2000). This transformation provides an excellent model system to understand vertebrate organogenesis and remodeling of the organs. During and following this transformation, commonly observed phenotypical changes are anatomical and histological reconstitution of the organs as well as appendages to function properly in terrestrial life conditions. Regression, disappearing and/or remodeling of the existing organs as well as formation of new organs are the observed adjustments of metamorphosis (reviewed in (Brown and Cai, 2007)). For the description of changes at organ and system level, *Xenopus leavis* is the widely used organism among the amphibians (Burggren and Warburton, 2007; Colombo et al., 2015). Previous studies have demonstrated that from tadpole to adult frog transformation, most of the organs undergo remodeling such as skin (Yoshizato, 1996), lung (Dodd and Dodd, 1976) and liver (Atkinson et al., 1998). The external gills of the tadpoles, which are the primary site for respiration in aquatic environment, disappear at the end of the metamorphosis (Ishizuya-Oka et al., 2010). Bone marrow, functional limbs and glands in skin and stomach are the examples of newly formed cells, tissues and structures with metamorphosis. Timing and rate of this complex process is regulated by hormonal activity and several external factors such as temperature (Hayes et al., 1993), density of population (Semlitsch and Caldwell, 1982), threat of predator presence and food levels (Kupferberg et al., 1994). In terms of hormonal regulation, this remodeling cascade starts with production and secretion of thyroid hormones (THs). It has been found that, THs levels in amphibians are low at early larval stage and peak at metamorphic climax (Mondou and Kaltenbach, 1979).

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THs are formed by coupling of iodine with tyrosine residues and consequently condensation of two aromatic rings of the tyrosine (Hulbert, 2000; Nussey and Whitehead, 2013)). There are two active forms of THs; the thyroxine or T4 which has four iodine at aromatic rings (3, 5, 3′ and 5′ respectively) and T3, which has three iodine at aromatic rings (3, 5 and 3′). T4 hormone is the main hormone produced by thyroid gland in most of the species, and it is converted to T3 in peripheral organs (Nussey and Whitehead, 2013). Since T3 has almost 10 times higher affinity for its receptors than T4, it is accepted as biologically active TH (Hulbert, 2000). Secretion of THs to blood is pursued by uptaking into the cells. Once TH locates within a cell, it binds to its receptors called as thyroid hormone receptors (TRs) which are a sub class of nuclear receptor family proteins (Huang et al., 2010). In most of the vertebrates there are two paralogous of this gene; TRa and TRb (Escriva et al., 2002; Paris and Laudet, 2008). In the absence of TH, these receptors are suppressed by corepressor proteins and therefore target genes can not be transcribed. Whereas binding of TH to TRs brings about the conformational change of the TRs, and releasing of corepressor enhances binding of TRs to hormone response elements (HREs) on DNA by interacting with retinoid X receptor (RXR) (Kliewer et al., 1992). Binding to DNA triggers the recruitment of coactivator proteins, and hence, expression of the target genes in the presence of TH is achieved (Buchholz et al., 2006). Expression of the genes with TH is essential for remodeling of the organs during metamorphosis of amphibians, and according to microarray studies a large number of genes are differentially expressed with the increased TH activity (Das et al., 2006; Yen et al., 2003). Although TRs and RXR proteins are highly conserved among the vertebrates, TH induced gene expression profile remarkably differs between the animals (Bertrand et al., 2004). In spite of presence of conserved coactivator, corepressor and nuclear receptors between the amphibians and mammals (Furlow and Neff, 2006) limited overlap in physiological response to TH between these animals indicates specialized function of TH in amphibians.

Unlike the frogs, Axolotl represents larval characters beyond the larval stage, throughout its life. Inadequate conversion of T4 to T3, presence of inactive form of T3 (3,3′,5′- triiodothyronine) and expression of limited number of TRs contribute for lifelong lasting neoteny of Axolotl (Galton, 1992). Administration of T4 or T3 either by injection or immersion is sufficient to trigger the metamorphosis (Jacobs et al., 1988; Page and Voss, 2009). Weight loss, diminishment and disappearance of tail fin and gills and molting are the morphological signs for metamorphosis (Rosenkilde and Ussing, 1996). Availability of induction to metamorphosis offers the opportunity to utilize the metamorphosed Axolotl as a complementary system to Neotenic ones, since it is an accomplished model organism to study regeneration (Coots and Seifert, 2015; McCusker and Gardiner, 2011; Vincent et al., 2015), scarless wound healing (Denis et al., 2013; Seifert et al., 2012), cancer (Menger et al., 2010; Smith et al., 2000) and stem cells (Rodrigo Albors et al., 2015; Zielins et al., 2016). Particularly, remarkable regeneration capacity of this model holds a great promise to understand the molecular basis of regeneration and restoration considering its success in functional regeneration of the internal organs (Cosden-Decker et al., 2012), central nervous system (Amamoto et al., 2016; Maden et al., 2013; Zammit et al., 1993) and extremities (Kragl et al., 2009; Satoh et al., 2015) following the damage or amputation. Considering the evolutionary proximity between the amphibians and mammals, employment of Axolotl as a model system allows translation of acquired messages to Mammalians effectively. Although number of researches on Axolotl has been expanded, to our best knowledge, there is no extensive study to generate a histological map of its organs. Here, in this study we provide a histological atlas of Axolotl tissues and organs for both pre and post-metamorphic stages. All isolated tissues and organs were histologically analyzed for both neotenic and metamorphosed Axolotls. General and organ specific histological staining were performed to describe the similarities and variations between the pre and post-metamorphic animals. Our results demonstrate that remodeling of several organs is the primary source for adaptation to terrestrial life conditions. Disappearance of pre-existing and formation of new organs also maintains the sustainable survival in the new environmental conditions. In addition to revealing the histological differences and resemblances between pre and post-metamorphic Axolotls, this study serves as a general reference for histological information to use in further studies. It is well known that histological documentation of tissues and organs is tremendously useful to follow up the effects of any treatments at tissue and organ level. Therefore, we certainly believe that this reference map will be very beneficial and be widely used in Axolotl researches.

2. Materials methods

2.1. Ethical statement

Animal care and experimental procedures were approved by the Animal Research Ethics Committee of the Istanbul Medipol University (authorization number 38828770-E.2302) and the research was performed in accordance with the European Community guidelines for ethical animal care and use of laboratory animals.

2.2. Animal handling and induction of metamorphosis

Axolotls (*Ambystoma mexicanum*) were obtained from the Ambystoma Genetic Stock Center (AGSC) and bred in animal care facility of Istanbul Medipol University. Adult animals, 14–16 cm in length, were used in all experiments. Animals were maintained in individual aquarias at ~20 °C in Holtfreter's solution before sampling. Metamorphosis was induced by using L-thyroxine (Sigma-Aldrich, T2376) as described below: (Page and Voss, 2009). T4 solution with a final concentration of 50 nM was prepared by mixing L-thyroxine stock solution with modified Holtfreter's solution. Axolotls were transferred into containers (one Axolotl/container) having 50 nM T4 solution. T4 containing medium was changed every third day and animals were observed for morphological changes. After ~2–3 weeks of T4 administration, weight loss, disappearance of the fin and decrease in the gills size were apparent. Administration of the hormone was continued for another 3 weeks until fully metamorphosed Axolotls were obtained. Both neotenic and metamorphic Axolotls were sacrificed in 0.02% benzocaine (Sigma-Aldrich, E1501) and organs were isolated immediately after the sacrifice.

2.3. Histological analysis

Isolated organs (brain, gallbladder, heart, intestine, liver, lung, spleen, stomach and tongue), skin and tail were fixed in 10% neutral buffered formalin (NBF) for 48 h. Following the removal of fixative by washing the samples with tap water for 1 h, the organs were incubated in ascending alcohol series (70%, 90% and 100% ethanol) for 1 h at 60 °C. Incubation in 100% ethanol was repeated two more times. Then, samples were incubated in toluene for 30 min at room temperature twice. As a next step, samples were embedded to paraffin. Microtome was used to section the paraffin embedded organs in 4 μm thick tissue sections. Then, the sections were deparafinized by incubation in toluene (30 min at 60°), descending alcohol series (100%, 96% and 70%; 1 min at RT) and distilled water (1 min at RT). Paraffin sections of all organs were stained with Hematoxylin and Eosin (Bio-Optica Mayer's Hematoxylin and Eosin Y Plus) according to manufacturer's protocol to identify general