Gene Expression in Thyroxin-Induced Metamorphosing Axolotl Hearts

Dipak K Dube1, 2, Aruna Narshi1, Matthew D. McLean2, Syamalima Dube3 and Bernard J. Poiesz1

1Department of Medicine, SUNY Upstate Medical University, USA
2Department of Developmental Biology, SUNY Upstate Medical University, USA

Introduction

The Mexican axolotl (Ambystoma mexicanum) is a unique model to study vertebrate heart development for several reasons. In addition to the wild-type animal, there is also an embryonic lethal condition caused by a homozygous recessive mutation in cardiac gene “c” [1,2]. These mutant embryonic hearts do not contract, are deficient in tropomyosin, and lack organized myofilbrils [3,4], making them an excellent model to study the process of cardiac myofilbrillogenesis [5,6]. In contrast to most other model organisms currently being utilized, axolotl embryos are comparatively large (2mm diameter), hence, they can be studied with relative ease. Being amphibians, axolotl embryos mature externally within jelly coats. Therefore, unlike using mammalian embryos, it is unnecessary to sacrifice the parent. Moreover, an average of a hundred axolotl embryos are produced from a single spawning and these embryos develop relatively slowly which is useful for studying vital developmental stages of interest [7]. In fact the timing of developmental stages of interest may be controlled to some extent, as the rate of development for these poikilothermic animal embryos is temperature dependent [8].

Moving from an aquatic environment to a terrestrial environment will expose an animal to different atmospheric pressure and respiration requirements. An advantage the axolotl has over other amphibians such as Xenopus for studying metamorphosis is its neoteny. In Xenopus, metamorphosis is the natural developmental pathway. The Mexican axolotl is an inducible obligate neotene, meaning it rarely metamorphoses in the wild and retains larval features, such as gills, into sexually mature adulthood. However, it may be induced to metamorphose by thyroid function manipulation [9]. It is thought that there is a deficiency in secretion of thyroid-stimulating hormone (TSH), possibly due to a poor response of the pituitary to thyrotrophic releasing hormone, which does not allow the axolotl to naturally metamorphose [9]. However, it can be experimentally induced to metamorphose using tri-iodo-L-thyronine (T3), thyroxin (T4) or TSH [10,11] to become a terrestrial amphibian. Once metamorphosis is induced, the axolotl goes through several morphological changes, culminating in complete metamorphosis in around 18 to 30 days, depending on the age and size of the animals [12-14].

Post metamorphosis, several anatomical alterations have been observed (Figure 1). To accommodate lung ventilation, Coleman & Hessler [15] found: (1) lung volume increased to more than three times that of neotenous form; (2) lung wall thickness reduced by approximately one-third (as the surface area of the lumen increased); (3) there was approximately 12% increase in the length of the lung; and (4) many new small blood vessels vascularised the metamorphosed lung. These changes increase the effectiveness of oxygen and carbon dioxide gas exchange concomitant with lung-dependent respiration. Associated with these morphological changes in the respiratory organs are marked changes in the associated cardiovascular system, where the branchial arches supplying the gills regress and are replaced by internal and external carotid arteries [16].

The heart muscle shows an increase in trabeculation [14,17] which seems appropriate for coping with the increase in metabolic demands of more musculature on terrestrially adapted limbs [18,19]. The neotenous heart has a “spongy” ventricle with less organized myofilbrils (Figure 2). Since the neotenous axolotls have gills and can respire cutaneously too, it is not so important that the heart be as functionally effective as that of a metamorphosed axolotl. The metamorphosed axolotl becomes a committed lung breather and so would need a more robust pump to circulate oxygenated blood. Consequently, the metamorphosed heart shows more organized myofilbrils and increased trabeculation in comparison with the neotenous salamander (Figure 2).

For these respiratory and cardiovascular changes to occur there must be some associated neurological changes too. It has been found that 18% of neurons from the dorsal vagal motonucleus (located in the medulla oblongata) migrate to a new ventrolateral position over the metamorphic course in axolotls [14]. Intriguingly, this migration of neurons was found to occur in human embryos and foetuses too [20,21]. It is hypothesised that this relocation could relate to the evolution of heart rate variability and that this region may be equivalent to a primitive form of the nucleus ambiguus found in higher organisms [22, 23]. The nucleus ambiguous appears to be involved in respiratory and cardiac rhythms, whereas neurons found in the dorsal vagal motonucleus are not involved in the respiratory cycle [24]. Therefore, this ventrolateral location may be involved in the ontogeny and evolution of lung breathing (and/or its control).

Gene Expression and Apoptosis in Metamorphic Axolotl Hearts

The animal body undergoes rapid and dramatic changes during amphibian metamorphosis. Although a large body of data exists on changes induced in Xenopus during metamorphosis both at the physiological and molecular levels, little is known about urodeles with the exception of a few [25,26], even though they offer the unique situation of controlled inducible metamorphosis. Changes occurring in Xenopus gut, liver, and skin are well characterized, but there are no reports of any changes occurring in the hearts of metamorphosing animals, including the axolotl.

We first reported a dramatic increase in the expression of HoxA5 gene in axolotl hearts as determined by RT-PCR and in situ hybridization during spontaneous metamorphosis [27]. Subsequently,

*Corresponding author: Dipak K Dube, Department of Medicine, SUNY Upstate Medical University, USA, Tel: 315 464-5440; E-mail: dubed@upstate.edu

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we studied the temporal and spatial expression pattern of *HoxA5* in cardiac tissues during thyroxin-induced metamorphosis of the Mexican axolotl, and a similar increase in *HoxA5* expression was found. The complete process of metamorphosis in the axolotl took 24-25 days. The transcript level of *HoxA5* started to increase, compared to the control, at 4 h after thyroxin injection and plateaued by 48 h. It returned to the normal/control level at day 28 after thyroxin injection. We performed in situ hybridization analyses for expression analyses of *HoxA5* transcripts on sections from different regions of hearts from neotenous and thyroxin-induced metamorphosing axolotls. Staining for *HoxA5* was seen in the cells of the epicardium but no staining was detected in the myocardium or endocardium. In contrast, the ventricle from a metamorphic juvenile axolotl was performed after staining with *HoxA5* antibody. It is to be noted that juvenile axolotls (2-3 inches long) undergo complete metamorphic changes at a much faster rate compared to adult animals. After thyroxin injection, it takes about 15-18 days for the completion of the entire process of metamorphosis in juveniles. Immunohistochemical results showed no positive signal for *HoxA5* in control hearts whereas there was a very weak signal of *HoxA5* in day 3 post-injection hearts. At day 10 and day 14 (around halfway to three-quarters into the metamorphosis), there was a significant increase in *HoxA5* protein expression in hearts of thyroxin injected juveniles. *HoxA5* protein was localized to all sections of the z-series in a diffuse pattern. Therefore, it appears that the process of metamorphosis induces the expression of *HoxA5* in cardiac tissues, which is not seen in the neotenous hearts.

*HoxA5* is a member of the Hox group of the homeobox genes. The Hox genes encode transcription factors that are involved in regulating body formation during development. In vertebrates there are four Hox gene clusters known as HoxA, HoxB, HoxC, and HoxD. *HoxA5* is the member of HoxA cluster. Aubin et al. [28] created *HoxA5* homozygous mutant mice. Analysis of the new born homozygous mutants found improper trachea and lung morphogenesis, which strongly suggests that *HoxA5* is involved in appropriate lung and trachea development [29, 30].

Hall et al. [29] identified common genes and signalling pathways whose expression was associated with reversal of heart failure and restoration of ventricular function in human patients with non-ischaemic refractory heart failure after treating with a novel combination therapy. The combination therapy consisted of a left ventricular assist device (LVAD) combined with pharmacologic therapy including a selective 2-agonist. In the study, microarray analysis is using RNA samples taken at the time of the LVAD implant, and then at LVAD removal following recovery of ventricular function, was performed. They identified a total of 263 genes, which were significantly up- or down-regulated in the recovered hearts (paired T-test, P < 0.01). Amongst these genes, *HoxA5* was found to be down-regulated 2.99 fold in recovery patients following combination therapy. Although the researchers noted the significant down regulation of *HoxA5* expression during recovery, they did not discuss its significance. So the question still remains as to whether *HoxA5* plays any significant role in heart failure.

In another study, Dewy et al. [30] using gene co-expression network analysis, defined the gene expression network topology of cardiac hypertrophy and failure, and the extent of recapitulation of fetal gene expression programs in failing and hypertrophied adult myocardium. They used unbiased marginal module analysis of gene co-expression networks from all murine myocardial transcript abundance data available in the published literature to formally evaluate the hypotheses that there are gene programs unique to fetal myocardium and these gene programs are re-capitulated in myocardial adaptation. The authors concluded that modules common to developing and failing myocardium were enriched in targets of *HoxA5*, a transcription factor.
with known roles in lung development in mammals and apoptotic heart morphogenesis in amphibians.

HoxA5 is known to act as a positive regulator for the p53 gene that protects cells against malignant transformation through apoptosis [31]. Apoptosis or programmed cell death is a genetically controlled response for cells to commit suicide. Disruption of apoptosis has been described as a fundamental pathogenic mechanism in a variety of human diseases including various cardiovascular diseases like myocardial infarction, heart failure, and atherosclerosis [32]. During metamorphosis, amphibians undergo remodelling of various organs [33]. Previously, we reported an increase of p53 expression in cardiac tissue in axolotls during metamorphosis after injecting thyroxin [13]. A significant up-regulation of HoxA5 [12] and p53 expression [13] suggest an increase in apoptosis via p53 in thyroid-induced metamorphic axolotl hearts. Recently, we have evaluated the apoptosis in heart sections from control and thyroxin-induced metamorphosed axolotls by using CardioTACS™ In Situ Apoptosis Detection Kit ( Trevigen). We found an approximate 3-fold increase in apoptosis in sections from metamorphic animals compared to the control (unpublished Narshi and Dube). We hypothesize that thyroxin induces HoxA5, which in turn augments the expression of p53 gene and subsequently brings about higher levels of apoptosis in metamorphosing axolotl hearts.

Our gene expression studies over the years show that the expression of various homeobox genes like HoxA5 [12] and Nkx2.5 [13], -MyHC [34], and p53, undergo significant changes in T3-induced metamorphic axolotl hearts. Several sarcomeric protein genes like tropomodulin and myosin binding protein C do not undergo significant modulation in metamorphic axolotls. T3, the active form of the thyroid hormone, serves as a ligand for its receptors, which are known as thyroid hormone receptors (TRs). There are two such receptors designated as TRα and TR. These receptors are nuclear transcription factors responsible for activating or repressing transcription of the genes containing thyroid responsive elements (TRE) in a thyroid-dependent manner [35]. Myosin heavy chain (MyHC) genes have TREs at their upstream regulatory regions [36]. Thyroid hormones upregulate α-MyHC, whereas -MyHC is down regulated. On the contrary, the mechanism by which HoxA5 without TRE at the upstream promoter region is upregulated in axolotl hearts during metamorphosis is not clear.

Wang and Shi [37] reported upregulation of thyroid receptor-α (TRα) and retinoic acid receptor (RXRα) in the tail during metamorphosis in frogs. RXRα alone does not activate the gene(s) containing retinoic acid responsive element (RARE) [38]. For its effective binding with DNA and subsequent activation of transcription, it requires the formation of a heterodimer with another closely related protein RXR. The heterodimer RXRα/RXR is actually the effective inducer of the gene(s) with RARE [38]. It is unknown whether RXR is also induced along with RXRα in thyroid hormone induced metamorphosed frog tail [37]. If both RXRα and RXR are induced in metamorphic axolotl hearts, these receptors can form a heterodimer that subsequently activates the HoxA5 gene, which has RARE, but not TRE, at the upstream promoter region. The elevated level of HoxA5 protein may in turn induce p53-independent apoptosis in metamorphic axolotl hearts. In fact, Chen et al [39] reported that HoxA5 can act directly downstream of RAR and may contribute to retinoid-induced antancer and chemo-preventive effects by inducing apoptosis in MCF10A breast cancer cells. As p53 expression is up regulated in metamorphosing axolotl hearts, HoxA5 may induce apoptosis by both p53 independent and dependent pathways.

The other possibility is that thyroxin may induce another protein, with or without homeobox, which in turn activates the HoxA5 gene in axolotl heart during metamorphosis. In fact, the relatively slow rate of induction of the HoxA5 transcripts and its translational product, which takes several days after thyroxin treatment, strongly argues in favor of the involvement of some other intermediary regulatory protein(s) in the subsequent augmentation of HoxA5 in metamorphosing hearts [12].

Concluding Remarks

The axolotl is an apt animal model to study the ontogeny of heart rate variability and related neural changes, as it predominantly uses its gills when in the larval form, and upon metamorphosis, becomes a committed lung breather. The central nervous system is likely to undergo essential modifications in association with lung, and possibly heart, innervation and gill degeneration.

Many genes that are associated with molecular and morphological events during metamorphosis have been identified from studies of anurans, and in particular Xenopus laevis. In contrast, little is known about patterns of gene expression during salamander metamorphosis. Page et al [40] reported a detailed molecular analysis on the effect of thyroid hormone on gene expression in metamorphic axolotl using microarray analyses with RNA from control and metamorphic skins. However, to the best of our knowledge, no such analyses has been reported on metamorphosing and metamorphic axolotl hearts that may explain better the morphological as well molecular changes that have been observed. Such future studies might explain the higher level of apoptosis that we have observed in metamorphic axolotl hearts.

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