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by

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Abstract

Lymph hearts are pulsatile organs present in lower vertebrates that propel lymph throughout the body and into the venous system, assisting in the maintenance of fluid homeostasis. In organisms such as frogs, several pairs of lymph hearts develop amidst the somites during the early tadpole stages. Due to the unique structure and function of lymph heart musculature—exhibiting characteristics of both skeletal and cardiac muscle—the origin of these cells remains highly controversial. Studies have found that in *Xenopus*, the *engrailed* gene is expressed explicitly in lymph heart muscle cells throughout development. Through designing a transgenic construct containing the *engrailed* promoter and a fluorescent reporter gene, this study seeks to monitor the growth and development of the lymphatic heart in vivo by incorporating the transgene into the genome of *Xenopus* embryos via transgenesis procedures.

Introduction

Lymphatic system in vertebrates

Among vertebrates, the lymphatic system—composed of lymph nodes, lymph vessels, and organs for circulating lymph fluid—serves a crucial role in the maintenance of fluid homeostasis (Kampmeier 1969). Within the vertebrate body, as blood flows through the capillaries, plasma filters into interstitial spaces, where it is subsequently absorbed by tissue cells or reabsorbed by the blood before it flows out of the tissue (Solomon et al. 2005). However, a small amount of this interstitial fluid is left behind (Solomon et al. 2005). If left uncollected, the pooling of this fluid may result in massive edema, tissue destruction, or even death (Peyrot et al. 2010). Such a problem is avoided in vertebrates with the presence of lymph vessels, which act as a drainage mechanism to collect the excess fluid and return it to the venous blood before it reaches the heart (Sabin 1913).

Within mammals, skeletal muscle contractions circulate lymph throughout the body (Kampmeier 1969). However, in lower vertebrates, such as fish, reptiles, birds, and amphibians, lymphatic hearts are also present to facilitate lymph circulation (Rumyantsev & Shmantzar 1967; Kampmeier 1969). Lymphatic hearts, which are pulsatile organs located at the junctions of the lymphatic and venous systems, serve as a mechanism to propel lymph throughout the lymphatic system and into the veins (Kampmeier 1969; Peyrot et al. 2010).

Lymph hearts in frogs

In frogs, several pairs of lymphatic hearts develop during embryonic development (Sabin 1913). An anterior pair, located dorso-caudal to the pronephroi, arises between the myotomes and skin during the tailbud stage (Rumyantsev & Shmantzar 1967; Kampmeier 1969; Peyrot et al. 2010). During the later tadpole stages, four more pairs of posterior lymph hearts develop and connect to the dorsal subcutaneous lymph sacks during metamorphosis. In the adult frog, the anterior pair of lymph hearts regresses, leaving the four pairs of posterior lymph hearts remaining (Kampmeier 1969).

Lymph heart structure

Structurally, the lymph heart is comprised of three tissue layers: the inner tunica intima, the middle tunica media, and the outer tunica externa (Satoh & Nitatori 1980). The inner tissue layer consists of an endothelial cell lining with connective tissue, the middle layer contains the lymph heart musculature, and the outermost layer is comprised of fibroelastic tissue (Peyrot et al. 2010). Within both mammals and frogs (specifically, *Xenopus*), the endothelium of the lymphatic vessels has been shown to be derived from the blood vasculature (Sabin 1913). However, the origin of lymph heart musculature, although widely studied, remains controversial.

Controversy of lymph heart musculature origin

The debate over lymph heart origin focuses upon which cell types during embryonic development are the progenitor cells for lymph heart musculature. In the developing vertebrate embryo, somites are masses of mesoderm which are distributed along the two sides of the neural tube (Satoh & Nitatori 1980). In *Xenopus*, the pair of anterior bilaterally symmetrical lymph hearts, which develop during the tailbud stage, are found adjacent to the trunk somites 3 and 4 (Sabin 1913; Kampmeier 1969). During development, the somites will differentiate into three tissue layers: the dermatome, myotome, and sclerotome (Satoh & Nitatori 1980). Since the anterior lymph hearts arise in a space nestled between two separate regions (one that forms the dermatome, and one that forms skeletal muscle cells—specifically, the ventral body wall limb muscle), it is unknown which mechanisms underlie the specification of certain cell types to form the lymph heart musculature.

To further complicate matters, lymph heart muscle cells have features characteristic of both cardiac and skeletal muscle cells. Like cardiac muscle cells, lymph heart cells beat at the same rate as the circulatory heart, have a thin branched structure, and contract rhythmically without nerve cell innervation (Satoh & Nitatori, 1980). However, besides developing in a region that largely forms skeletal muscle, studies have shown that lymph heart muscle cells express markers of skeletal muscle fate (such as *myoD* and 12/101), but not those of cardiac fate or differentiation (such as *cardiac troponin* and GATA genes) (Peyrot et al. 2010). Thus, lymph heart muscle cells have been described as cardiac-like skeletal muscle cells, although this claim has not been met without controversy (Peyrot et al. 2010).

Various researchers have proposed different hypotheses regarding the origin of lymph heart musculature. Knower (1908) described lymph heart muscle cells as arising from adjacent

mytomes. Kampmeier (1969), however, contested this hypothesis, instead describing them as mesenchymal cells that lie lateral to the myotomes (Peyrot et al. 2010). Other studies on lymph heart development in chicks suggest that the endothelium and musculature of the lymph heart arise from the somites (Wilting et al. 2006; Valesek et al. 2007). A more recent study conducted by Peyrot et al. (2010) proposes that lymph heart muscle cells are under developmental control from the lymphatic endothelium. Thus, the origin of lymph heart muscle cells remains widely controversial as an array of hypotheses is further investigated. Oftentimes, the model organism used to test these hypotheses is the South-African clawed frog, *Xenopus laevis*.

Xenopus laevis as a model organism

There are various advantages to using the organism *Xenopus laevis* for the transgenesis procedure in this study. Largely used as a model system for vertebrate development, *Xenopus* are easily housed in the lab, have a short generation time (ranging from 3-24 months depending on the species), and exhibit a rapid rate of development, thus enabling experimental results to be obtained relatively quickly (Kroll & Amaya 1996; Hirsch et al. 2002; Ishibashi et al. 2009). In addition, adult females may be induced to ovulate large quantities of eggs year-round (Pan et al. 2006). The embryos develop outside of the mother, and the eggs are relatively large (approximately 1 mm in diameter in *Xenopus laevis*) and easy to manipulate, making them well suited for manipulations of gene activity via microinjection (Sparrow et al. 2000; Ogino et al. 2006; Sinzelle et al. 2006). Microinjected eggs have been demonstrated to heal extremely well after microsurgery (Ogino 2007). Furthermore, *Xenopus* tadpoles have transparent skin, allowing internal structures to be easily visualized in live embryos (Offield et at. 2000). This particular feature of *Xenopus* is especially useful in this study, as it will allow fluorescently-

labeled lymph heart muscle cells to be visualized within live embryos throughout the developmental stages.

Engrailed promoter

In order to visualize the developing lymph heart in live tadpoles, it was critical to find a gene—expressed explicitly within only lymph heart muscle cells—that could be labeled with a fluorescent probe for the transgenesis procedure. Studies have suggested that the *engrailed* gene found in *Xenopus* is required for the development of lymph heart musculature, but not lymph heart endothelium (McGrew et al. 1999; Peyrot et al. 2010). In an *in situ* hybridization study conducted by Peyrot and colleagues (2010), weak expression of the *engrailed* gene was first visualized in the clefts of the anterior trunk somites 3 and 4 at stage 28 in cross sections of fixed tapdoles (Figure 1). At later developmental stages, *engrailed* expression intensified and then condensed in a small region ventral to the notochord, where the lymph heart would later develop (Figure 1) (Peyrot et al. 2010). Researchers concluded that *engrailed* marks lymph heart myoblasts (Peyrot et al. 2010). Due to the selective nature of *engrailed* expression within lymph heart cells, the promoter region for the *engrailed* gene was selected for this transgenesis procedure. In addition, the oligonucleotide sequences flanking the region of the *engrailed-2* promoter have been identified (McGrew et al. 1999). Thus, the transgenic construct used in this study consists of the 2.7 kb *engrailed-2* promoter region inserted upstream of the fluorescent reporter gene for Green Fluorescent Protein (GFP), which will enable the real-time monitoring of transgene expression within live transgenic embryos.

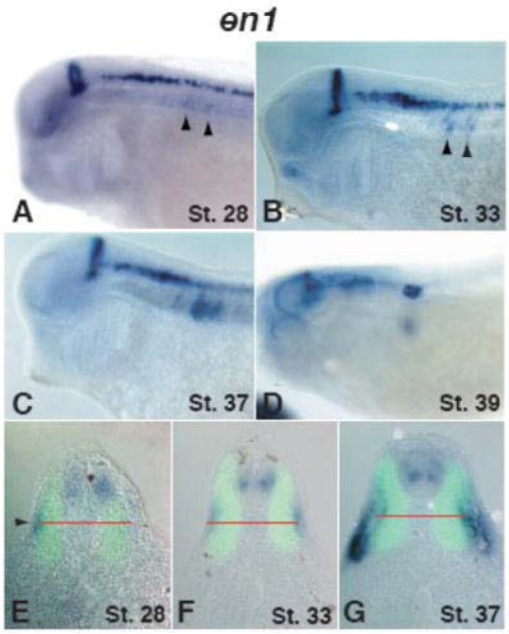

Figure 1. Image reprinted from Peyrot et al. (2010) of in situ hybdridization with the *engrailed* gene in *Xenopus laevis* tadpoles throughout various stages of development. The *engrailed* gene labels the developing lymph heart musculature, and expression is localized to the mid-hindbrain boundary, spinal interneurons, and anterior somites. Somatic expression is initiated at stage 28 (A) in a superficial region on a horizontal plane with the notochord (E, arrowhead, middle of notochord is indicated by red line). Lateral views indicate early expression in anterior somites (A-B, arrowheads), which intensifies and then moves ventrally to occupy the final position of the lymph heart (C-D, G). The transverse cross section of a *Xenopus* tadpole at stage 33 (F) reveals that the intensity of *engrailed* expression is increasing (Peyrot et al. 2010).

Transgenesis procedure

A critical advancement for *Xenopus* studies has been the development of a simple and efficient transgenesis procedure (Warkman & Krieg 2006). The original procedure, designed by Kroll and Amaya (1996), involves the transplantation of sperm nuclei (containing the transgenic construct) within an unfertilized egg (Kroll & Amaya 1996; Warkman & Krieg 2006). Various modifications to this transgenic procedure have since been developed, including those employing the use of ΦC31 integrase, the *I-SceI* meganuclease, the Sleeping Beauty transposon system, and restriction enzyme mediated insertion (REMI) (Sparrow et al. 2000; Ivics et al. 2004; Allen &

Weeks 2005; Ogino et al. 2006; Pan et al. 2006; Sinzelle et al. 2006). While the changes to the original transgenesis were designed to optimize the procedure in general, they also served to enhance the long-term viability of the transgenic embryos, resulting in a higher rate of survival to reproductive adulthood rather than the production of the maximum numbers of transgenic embryos (Hirsch et al. 2002). Due to the size of the transgenic construct utilized in this study, as well as the relative ease and efficiency of the procedure, the specific transgenesis procedure utilized in this study is a modified version of REMI (Ogino 2007). Although the procedure has an overall efficiency of only 10%, the relative ease of experimental duplication after the initial sperm and ooctye extract preparations will enable large quantities of transgenic embryos to be produced relatively quickly in future studies.

Objectives

The main objectives of this research focus upon investigating the controversial nature of the lymph heart musculature development. Which genes are specific to lymph heart development, as well as which cells form the precursors to lymph heart musculature, are pivotal questions this study seeks to address. The main approach utilized is a modified restriction enzyme mediated insertion transgenesis procedure in *Xenopus laevis* tadpoles. Specifically, through fluorescently labeling the promoter of a gene found only in lymph heart muscle cells (*engrailed*), the development of the lymph heart musculature may be visualized in live embryos throughout tadpole maturation.

Materials and Methods

Plasmid construct

The plasmid construct used in this study was M23 pEnP1 (7.5 kb, obtained from Addgene, Inc.), containing the *engrailed-2* promoter (2.7 kb) from *X. laevis*, flanked by 5' *XhoI*

and 3' *HindIII* cloning sites, with a luciferase tag adjacent to the 3' *HindIII* site (Figure 2) (McGrew et al. 1999). Due to the lower levels of fluorescence typically associated with the luciferase fluorescent probe compared to the GFP reporter gene, it was decided that the *engrailed-2* promoter should be cloned into a vector containing GFP to enable better visualization of the fluorescent transgene in live embryos (McGrew et al. 1999; Hirsch et al. 2002; Sobkow et al. 2005).

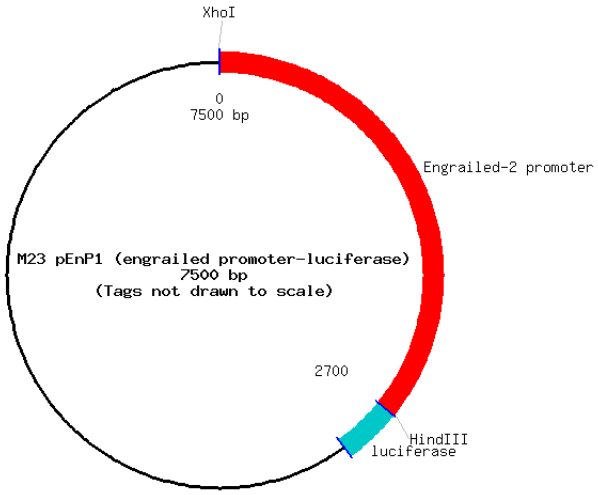

Figure 2. Schematic of the M23 pEnP1 plasmid (7.5 kb) containing the *engrailed-2* promoter region (2.7 kb) and luciferase reporter gene (Addgene). Image reprinted from McGrew et al. (1999).

Using the oligonucleotide primers 5'-ULX, 5'-GGCTCGAGAGATCTCTGGAAGTCTCCATA-3' and S/B-3', 5'-CACACACACACTCTCTCCAAGCTTGGG-3', the *engrailed-2* (2.7 kb) promoter region of pEnP1 was PCR amplified using the Advantage 2 10X DNA Polymerase mix (Clontech), in a PTC200 gradient cycler with the conditions listed below (Table 1) (McGrew et al. 1999).

Process	Temperature (°C)	Time	Cycles
Initial Denaturization	95	2 min	1
Denaturization	95	30 sec	30
Annealing	55	30 sec	30
Extension	72	2 min	30
Final Extension	72	10 min	1
	4	--	1

Table 1. PTC200 gradient cycler conditions used in the PCR amplification of the *engrailed-2* promoter region (2.7 kb) of pENP1, using Advantage 2 10X DNA Polymerase mix (Clontech) and primers 5'-ULX and S/B-3'.

PCR products were verified on a 0.8% agarose gel (Figure 3), followed by gel purification of the 2.7 kb bands using the Roche Applied Science Agarose DNA Gel Extraction Kit.

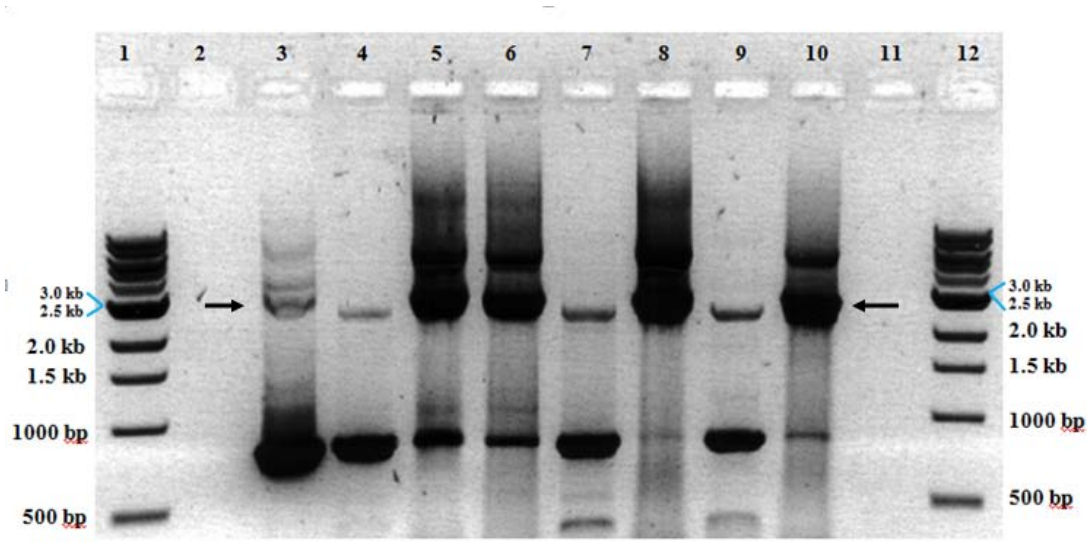

Figure 3. DNA gel electrophoresis image (0.8% agarose gel) of products obtained from the PCR amplification of the *engrailed-2* promoter region in pEnP1 using Advantage 2 10X DNA Polymerase mix and primers 5'-ULX and S/B-3'. Lanes 1 and 12 contain New England BioLab's 1 kb DNA ladder; Lanes 2 and 11 contain dH_2O; and Lanes 3-10 contain the products of the PCR amplification. As noted by the row marked with the two black arrows, a 2.7 kb fragment was successfully amplified in each pEnP1 sample. Light blue bars adjacent to the 3.0 kb and 2.5 kb labels on the DNA ladders indicate that complete separation of all the bands within the ladder was not visualized. Other bands of varying lengths which were amplified in the pEnP1 samples were not identified, and assumed to be due to binding of the primers to unforeseen regions of the plasmid. Image processing involved inversion of the look-up table.

DNA concentration of the products was measured using the NanoDrop 1000 Spectrophotometer. The DNA products were stored at -20°C until further use in the design of the transgene construct.

Transgenesis

All *Xenopus laevis* frogs were bred and raised in the laboratory. Oocyte extract was prepared by the method of Ogino (2007). The remainder of the transgenesis procedure will be completed according to the method of Ogino (2007) at a later date.

Discussion and Future Work

The utilization of transgenesis procedures within the research lab can provide powerful insight into the regulation of both when and where a transgene is expressed in a developing embryo (Warkman & Krieg 2006). Transgenic studies within *Xenopus laevis*, a model organism for vertebrate embryological development, can provide a wealth of insight into the genetic mechanisms underlying the partitioning of certain cell types throughout the stages of development.

Currently in this study, there are various steps which must be completed before the powerful tool of transgenesis may be utilized to monitor the development of the lymph heart musculature in live embryos via the visualization of localized fluorescence within the lymph heart region. First, designing the transgenic construct must be completed. Since the *engrailed-2* promoter region has been successfully amplified and isolated via PCR and Agarose DNA Gel Extraction, it will next need to be cloned into a vector containing the GFP reporter gene. Afterwards, for use in the transgenesis procedure, the *engrailed-2* promoter/GFP transgene construct will be linearized via restriction enzyme digestion (from the vector containing the transgene construct). This linearized DNA fragment, along with the prepared sperm nuclei and

oocyte extracts, will be microinjected into single-cell *X. laevis* embryos via the modified REMI methods of Ogino (2007). Through this procedure, it is expected that the transgene construct will be incorporated into the genomes of some of the *X. laevis* embryos, which will hopefully survive to reproductive adulthood.

As the transgenic tadpoles develop, it is expected that the cells which will form the lymph heart musculature will be visible *in vivo* via fluorescence. The expression of this transgene, and the migration of progenitor cells to the lymph heart region, may therefore be monitored throughout the developmental stages. Ideally, experimental data suggesting which specific areas (of the undifferentiated somites, connective tissue cells, etc.) are associated with the emergence and development of lymph heart muscle cells would provide valuable insight into the origin of lymph heart musculature. Since the most recent studies suggest the lymph heart muscle cells are under developmental control from the lymphatic endothelium, any experimental data obtained from this transgenic study would likely be valuable in either supporting or refuting this hypothesis (Peyrot et al. 2010). Other future work will focus upon establishing a homozygous line of transgenic *X. laevis* for in-depth analysis and investigation into the controversial origin of the lymph heart musculature cells.

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