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Analyzing Collagen Alpha 1 (XI) Using a Zebrafish Model System

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ABSTRACT

Zebrafish (*Danio rerio*) are progressively becoming more popular as a model organism for research. The use of zebrafish is advantageous for several reasons. Zebrafish are small in size, exhibit high fecundity, rapidly generate, are transparent during development and are easily maintained in large numbers.
Using zebrafish as a model organism, the aim of this project is to determine the function of Collagen Type XI alpha I (COL11A1) during early development. To begin with, it is imperative that the zebrafish are kept healthy. Hence, we control the water quality and nutrition. The healthy zebrafish are then able to breed. The embryos are collected and their RNA is extracted for RT-PCR in order to determine when COL11A1 is being expressed (temporal expression pattern). In addition, anti-sense RNA ribo-probes are used to determine where the COL11A1 gene is being expressed (spatial expression pattern). To study the function of COL11A1, we microinject anti-sense morpholinos into 1-2 cell stage embryos. This diminishes the expression of *col11a1* and then the resulting morphants are compared to control zebrafish at the time points of interest. The ultimate goal of this research is to understand the function of COLL11A1 during early development. In doing so, we can gain insight into the Type II Stickler syndrome and Marshall syndrome.

BACKGROUND

Collagen type XI is a heterotrimeric molecule composed of three α chains, α1(XI), α2(XI), α3(XI). Collagen XI belongs to the fibrillar collagen family and is organized into cartilage fibrils with collagens type II and IX (Fig. 1). In some other cases, it also forms heterotrimer with collagen type V. Collagen XI is also involved in the normal differentiation and spatial organization of growth plate chondrocytes.

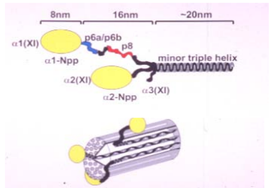

Figure 1: Schematic representation of the α1 chain of Collagen type XI containing the amino propeptide (NPP) and the variable region (VR) . This region will be released from the fibril by enzymes, forming a matured collagen fibril.

In humans, mutations in COL11A1 are associated with the birth defects of Type II Stickler syndrome (Fig. 2) and Marshall's syndrome (Fig. 3). The manifestations of Stickler syndrome include flattened facial appearance caused by underdeveloped bones in the middle of the face, as well as both impaired vision and hearing. Individuals with Marshall's syndrome phenotypically display by a flat or retracted midface, large eyeballs, a thick calvaria, abnormal frontal sinuses, and intercranial calcifications.

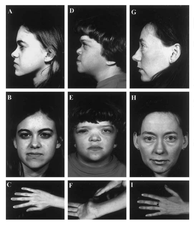

Figure 2: One characteristic feature of Stickler's syndrome is a flattened facial appearance.

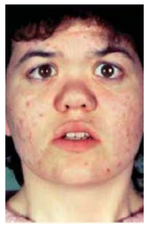

Figure 3: Characteristic facial structure associated with Marshall's syndrome.

To better understand the collagenopathy of COL11A1, zebrafish (*Danio rerio*) were used as a model organism for our research. The embryos produced are transparent, and have fewer cells than many other vertebrates. Also, the existence of the embryo outside of the mother allows for direct access. The embryos develop from a single cell to a small fish within 72 hours (Fig. 4), which allows the research process to advance quickly. Adult zebrafish are tropical freshwater fish that are 1-2 inches long. The average lifespan of zebrafish is 42 months. While zebrafish reach sexual maturity at 10-12 weeks, breeding is most successful at the ages of 7-18 months. Female zebrafish are more plump and have more of a silver tone than male zebrafish, which are slender and have a yellow hue on their ventral surface (Fig. 5).

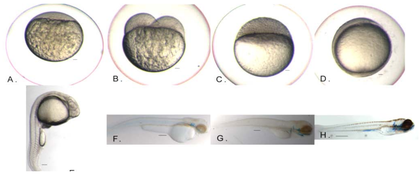

Figure 4: Zebrafish development. A) One-cell embryo, B) Two-cell embryo, C) Four hours post fertilization (hpf), D) 10.3 hpf, E) 24 hpf, F) 48 hpf, G) 72 hpf.

Figure 5: Adult zebrafish, male (left) and female (right).

MATERIALS AND METHODS

Zebrafish Husbandry

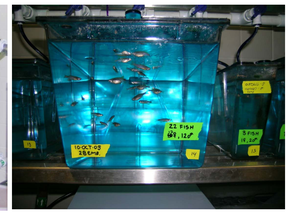

Figure 6: Aquatic Habitat™ system used to house zebrafish.

The male and female zebrafish are housed separately in 10L, 3L, and 1.5L tanks (Fig. 6). Ten liter tanks house 12 to 14 fish, 3L tanks house 6 to 8 fish, and 1.5L tanks house 4 to 5 fish. The number of fish per tank also depends on the sex of the zebrafish. Male zebrafish are smaller than female zebrafish, and therefore do not require as much space.

The zebrafish system constantly circulates artificial pond water (64ml Solution A, 64ml Solution B, 16L d-H_2O. Solution A= 175g NaCl, 35g $CaCl_2$, 2L d-H_2O. Solution B= 5g $NaHCO_3$, 2L d-H_2O) through three different filters: a prefilter, a cartridge filter, and a carbon filter. The prefilter is rotated in the morning and in the evening, and replaced every other day. The cartridge filter is replaced once a week. The carbon filter is replaced once a month.

For optimal zebrafish health, the pH of the water must be approximately 7.4. The alkalinity is measured approximately every other day. When the pH is below 7.4, alkalinity is adjusted by adding ±2.0 g $NaHCO_3$. The temperature of the water is maintained at approximately 28ºC by a water heater. The water system also has a UV light in order to grow nitrogen-fixing bacteria.

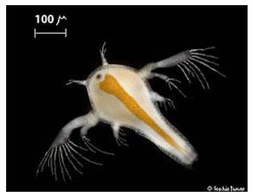

Figure 7: Adult brine shrimp (*Artemia salina*)

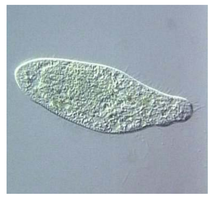

Figure 8: *Paramecium multimicronucleatum*

The adult zebrafish are fed live adult brine shrimp (*Artemia salina*) (Fig. 7) in the morning and dry fish food (Adult Zebrafish Diet by Zeigler®) in the evening. Zebrafish embryos are nourished by their yolk sac for three days post fertilization. Zebrafish that are 4 to 9 days old are fed only paramecia (*Paramecium multimicronucleatum*) (Fig. 8), and then are gradually introduced to the adult zebrafish diet.

Zebrafish breed at "sunrise" after ten hours of darkness. Randomly selected fish are placed in a breeding chamber at a ratio of two males to one female. The males chase after the females, and in response, the females release eggs and the males release sperm. Then the fertilized embryos are collected.

Molecular Techniques

RNA Extraction

RNA is extracted from embryos at various hours post-fertilization (hpf) times. Embryo cells are immersed in an RNA stabilization reagent, and lysed with a buffer. The resulting solution is homogenized and the viscosity of the lysate is reduced. Ethanol is added in order to adjust the binding conditions. The solution is then spun, causing the RNA to absorb into the membrane. Contaminants are removed by wash spins. RNA elutes to water and can be removed (Fig. 9).

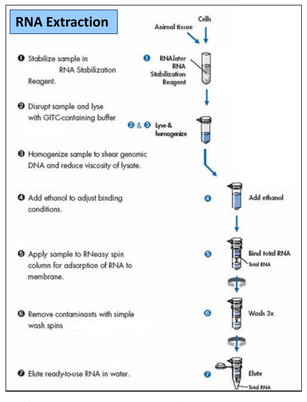

Figure 9: RNA extraction process

Reverse Transcriptase/Polymerase Chain Reaction

The extracted RNA is then converted to cDNA (complementary DNA) via reverse transcription. In reverse transcription, a primer is annealed to mRNA. Reverse transcriptase converts mRNA into cDNA, starting at the 3' end of the annealed primer (Fig. 10).
RT-PCR (reverse transcription-poly-chain reaction) amplifies the cDNA. RT-PCR starts by denaturing the sample with heat. Then the sample is cooled, and the added primers anneal to the denatured strands. At 72ºC the strand extends by the DNA polymerase, making a new copy. RT-PCR is cycled by up to thirty times (Fig. 11).
Gel electrophesis is performed on the cDNA samples, and the presence of expected band (targeted DNA fragment) will indicate when COL11A1 is transcribed in the embryo.

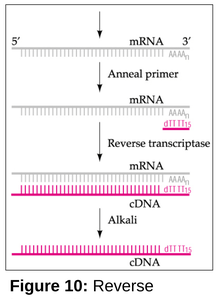

Figure 10: Reverse transcription process

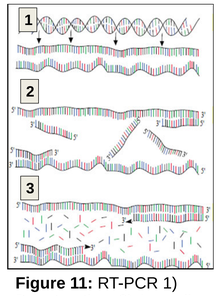

Figure 11: RT-PCR 1) Denaturation, 2) Annealing, 3) Extension

In situ Hybridization

In situ hybridization is also performed. Embryos with various hpf (hours post fertilazation) times are emerged into a solution of anti-sense RNA riboprobes. The presence of the target mRNAs will be visualized by color development by targeting the riboprobes, which are specially labeled. Alternatively, a direct fluorescent labeled riboprobe could be used, and signal can be detected by fluorescent microscope (Fig. 12).

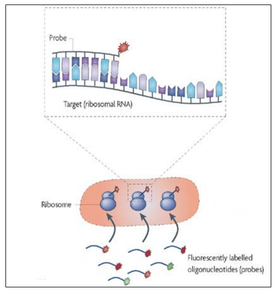

Figure 12: *In situ* hybridization process.

Microinjection

Anti-sense morpholinos, slightly modified oligonucleotides, are microinjected into 1-2 cell stage embryos. The anti sense morpholinos bind to RNA, and hence knock down the production of COLL11A1 protein or its spliced forms. The resulting phenotypes of the morphants are compared to the phenotypes of control embryos at 72 hpf. This process determines the function of COL11A1 in early development.

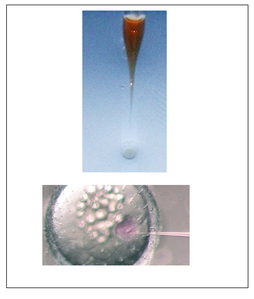

Figure 13: Microinjection into early zebrafish embryo

APPLICATIONS

By determining when and where Collagen Type XI alpha I is expressed during early development, and the purpose that it serves, we are gaining insight into the birth defects of Type II Stickler Syndrome and Marshall syndrome.

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