INTRODUCTION

A naturally-occurring homozygous recessive lethal heart mutation was discovered in axolotl, *Ambystoma mexicanum*, by Humphrey (1) in which the heart forms in developing embryos, but fails to contract. The mutant hearts show a lack of myofibrils and drastic reductions of sarcomeric tropomyosin as well as cardiac troponin T (2). Lemanski (3) showed through electron microscopic studies that failure of the heart to beat was due to a lack of myofibril formation in the developing mutant hearts. A series of heart organ culture experiments were set up to evaluate whether the mutant hearts could be rescued by culturing with conditioned medium from normal embryonic anterior endoderm (4). These experiments showed that when mutant hearts were cultured with total RNA isolated from human fetal or human adult heart but not with skeletal muscle RNA. It is concluded that the human hearts, both fetal and adult, may contain an RNA that mimics the MIR from normal axolotl embryonic anterior endoderm, suggesting the possible presence of an MIR functional homologue in humans.

Key words
Myofibrillogenesis; Tropomyosin; Cardiac mutant axolotl; Organ culture, Human adult heart RNA; Human fetal RNA; Human skeletal muscle RNA

Abstract
The Mexican axolotl (*Ambystoma mexicanum*) carries a lethal cardiac mutation resulting in mutant embryos with no heartbeat. This homozygous recessive genotype results in a tropomyosin deficiency and the absence of organized myofibrils in the mutant hearts. Co-culturing mutant hearts with bioactive RNA, termed myofibril-inducing RNA (MIR), from normal axolotl embryonic anterior endoderm causes the mutant hearts to synthesize tropomyosin, form well-organized myofibrils, and develop a heartbeat. Present studies demonstrate that mutant hearts can be rescued by culturing with total RNA isolated from human fetal or human adult heart but not with skeletal muscle RNA. It is concluded that the human hearts, both fetal and adult, may contain an RNA that mimics the MIR from normal axolotl embryonic anterior endoderm, suggesting the possible presence of an MIR functional homologue in humans.
The ‘active RNA’ causing the rescue (termed, MIR for myofibril-inducing RNA) was sequenced and found to be 166 nucleotides in length (5-7). The RNA is unique in that it does not show significant homology to any known sequences in the databases. In comparing the MIR sequences obtained from normal and mutant embryos, a single point mutation was found at base 93 of the mutant MIR nucleotide sequence (5, 6). An RNA secondary structure prediction program, Genebee (Moscow State University, Russia) showed a conformational difference between the bioactive MIR and the mutant MIR, suggesting that the secondary MIR structure might be important in the mutant rescue process (6).

In the present study, we have found that human heart RNA also has the ability to promote normal myofibril formation and restores function to the mutant axolotl hearts suggesting that a functional homologue of the axolotl MIR could be present in human fetal and adult heart tissue. Identifying a human version of the MIR that can promote myofibrillogenesis and essentially turn non-muscle cells into cardiac muscle has potentially important implications for future treatment of myocardial infarcts, cardiomyopathies and other congenital or acquired myocardial diseases in humans.

**MATERIALS AND METHODS**

**Human fetal and adult heart total RNA**

Human fetal heart total RNA (BioChain Institute, Inc., Hayward, CA) and human adult heart total RNA (Applied Biosystems/Ambion, Austin, TX) were used to treat the embryonic axolotl mutant hearts in organ culture. Ethanol precipitations were performed to further purify commercially available RNA samples.

**RNase treatment of human heart total RNA**

As negative controls for the fetal and adult human heart RNA rescue of mutant hearts, the total RNA samples were treated with RNase using standard methods. Briefly, 40 µg of RNA samples from both fetal and adult human hearts were subjected to RNase treatment following the manufacturer’s protocol (Promega, Madison, WI). After ethanol precipitation, the digested RNA was resuspended in 20 µl of DEPC water and stored at -80°C until used. RNA samples were run on 8% polyacrylamide gels to verify the purity of RNA samples from both fetal and adult human hearts were subjected to RNase treatment following the manufacturer’s protocol (Promega, Madison, WI). After ethanol precipitation, the digested RNA was resuspended in 20 µl of DEPC water and stored at -80°C until used. RNA samples were run on 8% polyacrylamide gels to verify the purity of RNA samples.

**Axolotl embryonic heart dissection**

The embryos at stages 35-38 were anesthetized with Finquel (MS-222; Argent Chemical Laboratories,) in Steinberg’s solution (1:5000) and carefully staged to ensure uniformity. The embryos were then dissected in Steinberg’s solution in a 100mm sterile Petri dish with four layers of Parafilm previously sterilized 20 minutes by ultraviolet light. The inner chest cavities were exposed with micro-dissecting forceps and, the hearts were removed and placed temporarily in a separate Petri dish until used in the bioassay.

**Axolotl embryonic heart bioassay**

The dissected hearts were individually transferred into 96-well plates (Falcon) containing a 20 µl droplet of Steinberg’s solution containing 2 µg of RNA or in a 20 µl droplet of Steinberg’s solution only or Steinberg’s solution containing Lipofectin. The 96-well plates were then covered and placed in a glass bowl containing a layer of paper towels soaked with Steinberg’s solution supplemented with antibiotic/antimycotic solution (Invitrogen, Chicago, IL) to provide a sterile and moist environment. The bioassays were placed in a sealed container and incubated at 15°C for six days.

**Preparation of embryonic hearts for electron microscopic analysis**

Embryonic hearts were extirpated from embryos after anesthetizing in MS-222 and were processed for electron microscopy by methods we have used routinely and published (4-8). Briefly, the hearts were placed in a phosphate buffered paraformaldehyde-glutaraldehyde solution and fixed at room temperature for 12 hours. The tissues were rinsed briefly in phosphate buffer then postfixed in a phosphate buffered 1% osmium tetroxide solution for one hour at 0°C, rinsed briefly in a buffer and dehydrated through a graded series of ethanol solutions to 100%. After clearing the tissues in propylene oxide for several hours the samples were embedded in plastic resin (EMS), thin-sectioned (700Å) with a diamond knife, stained in lead citrate and uranyl acetate and viewed at 60 or 80kV in a JEOL electron microscope.

**Immunostaining of hearts for confocal microscopic analysis**

Immunostaining of hearts was carried out with CH1 monoclonal anti-tropomyosin antibodies (CH1 antibodies were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa) followed by secondary antimouse antibodies conjugated with Oregon green (Invitrogen, Chicago, IL) following our published protocols (5, 6).
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contain organized myofibrils complete with Z lines (Figure 3A). Mutant sibling heart cells, on the other hand, fail to form sarcomeric myofibrils and instead show amorphous materials collected at the peripheries of cells where myofibrils would normally form (Figure 3B). Electron microscopy further reveals that mutant hearts treated with MIR (myofibril-inducing RNA) are “rescued” and begin to form myofibrils complete with Z lines (Figure 3C).

Confocal microscopic analysis was used to examine the presence of tropomyosin in cultured axolotl hearts in bioassays. Figure 4 shows clear tropomyosin staining in axolotl embryonic normal heart cultured in Steinberg’s solution (Figure 4A). The normal hearts demonstrated the existence of many organized sarcomeric myofibrils at the peripheries of the cardiac cells. The untreated mutant hearts showed only trace amounts of tropomyosin staining but no evidence of organized sarcomeric myofibrils (Figure 4B). However, a significant presence of tropomyosin along with organized sarcomeric myofibrils was observed in mutant hearts cultured in Steinberg’s solution containing MIR (Figure 4C). MIR was used as a positive control since MIR was known to rescue mutant hearts in our previous studies (5, 6). Human fetal heart RNA also rescued the mutant hearts. The mutant hearts rescued by human fetal heart total RNA showed clear sarcomeric myofibril formation and the immunostaining of tropomyosin (Figure 4D) which were very similar to the positive control mutant hearts rescued by MIR. Digestion of the human fetal heart total RNA by RNase appears to destroy its rescuing ability of the mutant heart; tropomyosin did not appear and organized sarcomeric myofibrils failed to form in mutant hearts incubated with RNase-treated human fetal heart total RNA (Figure 4F). Addition of RNase-treated RNA neither increased nor inhibited contractility function in the normal hearts and well-organized myofibrils remained similar to the normal hearts in organ cultures receiving no treatment. Human adult heart total RNA rescued the mutant hearts as well (Figure 4E). Tropomyosin was synthesized and organized sarcomeric myofibrils were formed in the mutant heart cells treated with human adult heart total RNA (Figure 4E). Mutant hearts cultured with RNase-treated human adult heart total RNA showed no evidence of increased tropomyosin staining or the presence of organized sarcomeric myofibrils; these controls mimic the appearance of mutant hearts cultured in Steinberg’s solution with no treatment. Furthermore, no tropomyosin staining was observed in mutant hearts after treatment with human skeletal muscle RNA (data not shown).

**Figure 1.** Gross morphology of normal (+/+) and cardiac lethal mutant (c/c) siblings at Harrison’s stage 40. Mutant embryos are shorter in length than normal and display ascites and microcephaly. (This figure was reprinted from: Lemanski, LF: Morphology of developing heart in cardiac lethal mutant Mexican axolotls, Ambystoma mexicanum. Develop. Biol. 1973; 33:312-333) x 7.

**Figure 2.** Gross morphology of the hearts of the embryos in Fig. 1 after removal of the overlying epidermis. The normal heart (2A) shows distinct chambers: bulbus arteriosus (b), conus arteriosus (c), ventricle (v), atrium (a) and sinus venosus (s). The mutant heart (2B) is billowy in appearance (arrows) and the chambers cannot be distinguished with assurance. (This figure was reprinted from: Lemanski, LF: Morphology of developing heart in cardiac lethal mutant Mexican axolotls, Ambystoma mexicanum. Develop. Biol. 1973; 33:312-333) x 60.

**Figure 3.** Electron micrographs of axolotl embryonic hearts. A. Portions of normal (+/+) heart cells containing well organized sarcomeric myofibrils (MF) and Z bands (Z). B. Mutant heart cells (c/c) without any treatment showing no obvious myofibrils, only amorphous material (AM). C. Mutant heart cells treated with axolotl Myofibril-Inducing RNA (MIR) show organized sarcomeric myofibrils complete with Z bands.
DISCUSSION

Our results show that total RNA from both human fetal and adult hearts, but not from skeletal muscle, have the capability of rescuing the mutant axolotl hearts in organ culture, suggesting that these RNAs contain or act as functional homologues of the MIR (myofibril-inducing RNA) derived from normal embryonic axolotl anterior endoderm (4-6). The mutant heart rescue was demonstrated by immunostaining techniques. These studies showed accumulation of tropomyosin and formation of sarcomeric myofibrils in the mutant hearts as a result of the treatment with RNA from human fetal or adult hearts. The levels of tropomyosin expression and myofibril formation are similar to what was observed in MIR rescued mutant hearts (5,6).

The mutant embryonic axolotl hearts showed very little tropomyosin staining in this study, consistent with our previous reports that only trace amounts of tropomyosin were synthesized by the mutant heart (5-7, 9). However, the mutant hearts treated with MIR developed organized sarcomeric myofibrils and were able to beat. These results corroborate our earlier studies (4-6). RNase digestion of the RNAs eliminated the rescuing ability of mutant hearts by human heart RNA. The RNase-treated RNA had no adverse effect on normal hearts in culture since these hearts could beat and develop sarcomeric myofibrils in the same way as untreated normal hearts in organ culture.

Visual analysis of the sarcomeric tropomyosin staining showed that rescue of mutant hearts by fetal or adult human heart RNA treatment is similar to the rescue by axolotl MIR. This study suggests that the mechanism for heart differentiation in axolotls might have been preserved during evolution (7). However, the possible mechanisms by which MIR (6), sheep heart RNA (7), or human heart RNA rescue the mutant heart condition are still unknown and require further study.

Thus, both fetal and adult human heart RNAs possess the capability of rescuing the naturally-occurring recessive cardiac lethal heart mutation in axolotls in a manner similar to the rescue observed with MIR obtained from normal axolotl embryos. This would seem to predict the existence of a functional human homologue to the axolotl MIR.

It appears that there has been a conservation of mechanism from amphibians to mammals, including humans, that permits the rescue of mutant hearts by promoting the formation of sarcomeric myofibrils. Previous studies in our laboratory (6) suggest that the secondary structure of the MIR is important for binding to essential proteins that may lead to normal myofibrillogenesis.

The discovery of a single nucleotide point mutation in the mutant axolotl MIR sequence and possible differences in RNA secondary structure between normal and mutant MIR have led us to believe that the potential of the normal RNA to rescue and the mutant RNA not to rescue is based on their inherently different secondary structures. Furthermore, it is also clear that the mutant MIR does not bind to specific protein(s) in the axolotl hearts as the normal MIR does (6). Additional studies will be required to determine if the rescuing human heart RNAs bind to these specific proteins.

Since axolotl MIR, sheep heart total RNA, as well as fetal and adult human heart total RNA rescue the axolotl embryonic mutant hearts, it is possible that all of these RNAs possess the necessary secondary structures needed to bind to the protein(s) which may be essential for rescue activities. The RNAs may have their distinct characteristics and sequences but may also share a region that has the appropriate stoichiometry to bind to an essential protein(s). This is one possible mechanism whereby sheep heart RNA (7) as well as the human heart RNA may serve as functional homologues to the axolotl MIR.

Another possible mechanism for the rescue of mutant hearts by axolotl MIR, sheep RNA or human RNA may be related to its gene regulation capabilities. Zhang et al. (7) have reported the possible existence of a TATAbox and a polyadenylation site in the MIR sequence. However, the MIR is a non-coding RNA. These findings could suggest that the MIR could be a microRNA and this may regulate the mRNAs and the proteins for which they originally code. In modifying the mRNA, the proteins needed for myofibrillogenesis and synthesis of tropomyosin may be modified or
regulated due to the point mutation found in the mutant MIR sequence. Based on this hypothesis, the normal MIR should possess a functional microRNA sequence that allows transcriptional regulation leading to the promotion of myofibrillogenesis.

Sempere et al. (10) suggest that some microRNAs are conserved across species. The microRNA rescue model is consistent with the fact that the sheep and human heart RNAs are functional homologues to axolotl MIR. Although Zhang et al. (6) could not find homologies to MIR in the databases, the fact that axolotl MIR (5, 6), sheep heart RNA (7) and now human heart RNA all can rescue this genetically defective axolotl heart by promoting myofibrillogenesis strongly suggests an evolutionary conservation of some common mechanisms involving a small RNA. It is possible that these RNAs possess the proper microRNA sequence that allows the onset of myofibrillogenesis and heart function. If the specific RNAs causing the rescue can be isolated and characterized from sheep heart and human fetal and adult hearts, it may turn out that the same RNA characteristics possessed by the axolotl MIR are present as well in these RNAs. The basic studies to determine the mechanism(s) by which axolotl MIR, sheep heart RNA, and human fetal and adult heart RNA rescue the axolotl embryonic mutant heart condition may serve as a foundation for future studies leading to clinical trials using gene therapy for patients suffering from cardiac muscle damage due to myocardial infarction, congenital defects or various cardiomyopathies. Adult stem cells might also, in future studies, be induced by these active RNA components to differentiate into functional muscle cells at the sites of heart muscle damage and thus, serve as a basis to develop clinical treatments for cardiac diseases.

CONCLUSION
Human adult heart RNA and human fetal heart RNA restore function in the cardiac mutant axolotl hearts. Previous studies showed that bioactive axolotl myofibril-inducing RNA (MIR) and sheep heart RNA rescued hearts in mutant axolotl. Treatment with human heart RNA restores tropomyosin expression and myofibril formation in the hearts of mutant axolotl embryos. Database searches of the human genome does not reveal significant homology sequences with the axolotl MIR. It is hypothesized that human RNA is a “functional homologue” of the MIR and perhaps possesses the necessary secondary structure needed to bind to the protein(s) which may be essential for rescue activities. This finding may lead to regeneration of damaged or diseased human cardiac muscle and restoration of normal heart function.

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REFERENCES