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Does Non-Coding RNA Play a Critical Role in Sarcomeric Tropomyosin Expression and Subsequent Myofibrillogenesis in Axolotl Heart?

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The Mexican axolotl, *Ambystoma mexicanum*, provides a useful model to study heart development because some of the animals carry a cardiac lethal mutation in gene "c", [1,2]. Electron microscopic analyses of homozygous (c/c) mutant embryos have shown mutant embryos do not form organized myofibrils and, as a result, the hearts fail to beat. They die soon after hatching from a lack of circulation. Although most sarcomeric proteins are present at near normal levels, tropomyosin, a component of the thin filament, is drastically reduced in mutant cardiac myocytes [3]. The defect can be corrected in mutant hearts grown in whole organ culture in the presence of total RNA from axolotl anterior endoderm or endoderm mesoderm-conditioned medium [4].

Long before the discovery that non-coding RNAs play critical roles in fine-tuning gene expression patterns during heart development and cardiac lineage expression [5-8], Lemanski et al. reported that a small non-coding RNA (166 nt long) cloned from axolotl endoderm mesoderm induced sarcomeric tropomyosin protein in mutant axolotl hearts *in situ*. This same RNA promoted myofibril formation and rescued mutant hearts [9]. Zhang et al. subsequently named this non-coding bioactive RNA as Myofibril Inducing RNA (MIR) and evaluated MIR gene expression in mutant (c/c) and normal (+/+) axolotls at different developmental stages [10,11]. As determined by RT-PCR, MIR was found to be expressed only in the heart and expression levels were not different in mutant and normal hearts [10]. However, a point mutation in MIR RNA (^{93}G–>^{93}U) from mutant embryos was detected. Computational analyses of both normal and mutant MIR RNAs showed a significant alteration in the secondary structure in mutant MIR RNA (^{93}G–>^{93}U). As a result, binding pattern of mutant MIR RNA to its putative binding proteins was altered. Most importantly, the mutant MIR failed to rescue mutant hearts *in situ*, did not induce sarcomeric TM, and did not promote fibril formation in mutant hearts [10]. Zhang et al. [11] isolated and sequenced the MIR gene and performed a comprehensive study on the effect of MIR on sarcomeric TM expression, contractility and myofibrillogenesis in axolotl hearts. The series of experiments performed can be summarized as follows [11]:

1. Transfection of double stranded MIR (ds MIR) into normal axolotl hearts *in situ* resulted in normal hearts mimicking the mutant phenotype. In other words, the knockdown of MIR expression caused the disarray of the organized myofibrils in normal hearts and significantly decreased their contractility.

2. In axolotl hearts, three sarcomeric TM isoforms are expressed. Of the three, TPM1α and TPM1α are the alternatively spliced products of the *TPM1* gene [12,13] and TPM4α is the sarcomeric isoform of the *TPM4* gene [14]. Transfection of dsMIR caused a considerable decrease in the expression of all sarcomeric TM isoforms in normal axolotl hearts. However, qRT-PCR analyses of various sarcomeric TM transcripts using isoform-specific primer-pairs with RNA from axolotl hearts treated and untreated with dsMIR showed that transcript levels of TPM1α, TPM1α, and TPM4α did not change considerably Zhang et al. concluded fairly that the inhibition of sarcomeric TM expression in normal axolotl hearts after knocking down MIR expression by ds MIR, was not due the down regulation of transcription of *TPM1* and *TPM4* genes.

3. Transfection of sense- and antisense MIR in normal hearts did not cause any significant decrease at the transcript level of TPM1α, TPM1α, and TPM4α. Most importantly, qRT-PCR results showed that the level of TPM1α, TPM1α, and TPM4α in normal and mutant hearts were comparable although total sarcomeric TM protein was decreased in mutant axolotl hearts. Hence, the decrease in sarcomeric TM expression in mutant axolotl hearts is not due to a deficiency at the transcription level.

4. Zhang et al. performed an impressive 2D western blot analysis with protein extracts from normal and mutant axolotl hearts with and without treatment with sense or anti-sense MIR. Western blot analysis was carried out with CH1 monoclonal antibody that recognizes all sarcomeric TM. The 2D western results convincingly showed 5-different spots of sarcomeric TM (or TM isoforms) in normal and mutant axolotl hearts. However, the expression level of all TM isoforms was significantly lower in mutant hearts. The authors concluded that MIR promotes myofibrillogenesis in mutant axolotl hearts by augmenting sarcomeric TM protein production.

The authors justifiably concluded that MIR, the non-coding RNA from axolotl, boosted sarcomeric TM expression in mutant axolotl hearts *in situ* by facilitating translation of various TM transcripts already present. MIR did not act at the transcription level.

Recently, Kochegarov et al. [15] reported a unique form of microRNA, MicroRNA-499c, from human fetal heart, which matched, in partial sequence alignment, to human microRNA-499a and b, and promoted the formation of myofibrils in cardiac mutant axolotl hearts in organ culture. Confocal microscopy confirmed the augmentation of sarcomeric TM expression in mutant hearts. Analyses for transcripts of various cardiac specific genes including TPM4α using qRT-PCR with gene/isoform-specific primers showed a substantial increase in TPM4α

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transcripts in mutant hearts lipofected with microRNA-499c. However, there are some significant differences between the findings of this study and the study by Zhang et al. [11].

1. The qRT-PCR data, presented by Zhang et al. [11], showed little difference between the transcript levels of various sarcomeric TM isoforms (TPM1α, TPM1α, and TPM4α) in normal and mutant axolotl hearts. In comparison, qRT-PCR data of Kochegarov et al. (2013) showed that mutant hearts contained 10-20% lower levels of TPM4α transcripts compared to normal axolotl hearts. In this context, it is worth mentioning that similar to Zhang et al. [10], conventional RT-PCR analyses by Spinner et al. [14] also did not find significant differences in transcript levels of TPM4α between normal and mutant axolotl hearts. Additionally, *in situ* hybridization of normal and mutant hearts with antisense and sense TPM4α probes suggested the expression level of TPM4α transcripts were comparable between normal and mutant hearts. These findings were in line with those of Zhang et al. [10,11]. In addition, Denz et al. [16] cloned and sequenced the upstream regulatory region of the *TPM4* gene from normal and mutant axolotls and did not find any differences in the nucleotide sequences. The results suggest there is no mutation in the upstream regulatory region of the *TPM4* gene in mutant animals that may contribute to decreased transcript levels in mutant hearts. However, other differences in the transcription machinery are not ruled out that may decrease transcript levels of TPM4α in mutant axolotl heats.

2. Zhang et al. [11] performed qRT-PCR with RNA from untreated and MIR-treated mutant hearts. Although lipofection of MIR sense strand of MIR augmented sarcomeric TM production significantly in mutant hearts, there was no obvious increase in the transcript levels for any one of the three known sarcomeric TM isoforms. In contrast, the data presented by Kochegarov et al. (2013), showed about an 80% increase in TPM4α transcripts in mutant hearts when lipofected with human microRNA-499c. They, however, did not quantify transcripts of other sarcomeric TM including TPM1α and TPM1α. The qRT-PCR and confocal microscopic analyses imply that microRNA-499c promoted myofibril formation in mutant axolotl hearts *in situ* by boosting the expression of TPM4α transcripts and ultimately increased the production of TPM4α protein. One may agree with the statement made by Kochegarov et al. [15] that axolotl MIR and human microRNA-499c are functional homologs because both non-coding RNAs promote myofibril formation in mutant axolotl hearts *in situ*. However, altogether the published data do not support that these two non-coding RNAs operate on the same immediate target gene.

It is well established that various non-coding RNAs play pivotal role(s) in cardiogenesis, cardiac regeneration, cardiac homeostasis, gene regulatory networks and gene silencing, [16-19]. A number of recent studies have convincingly demonstrated that non-coding RNA networks participate to control gene expression. Non-coding RNAs (ncRNAs) including microRNA appear to control every aspect of gene regulatory network activity, including transcriptional control, post-transcriptional gene regulation and epigenetic targeting. Non-coding regulatory RNA can broadly be divided into two groups: 1) small non-coding RNA and 2) long non-coding RNA. MicroRNA (miRNA) and anti-sense RNA are part of the small non-coding RNA group [20]. In addition to the small ncRNAs, several studies utilizing high-throughput genomic screens have demonstrated that mammalian genomes produce thousands of long transcripts that have no significant protein coding potential [21,22]. These transcripts are collectively known as long (or large) non-coding RNAs (lncRNAs) because these RNA molecules are more than 200 nucleotides long. They are typically polymerase II transcribed, 5'-capped, alternatively spliced and polyadenylated [23].

The full length MIR RNA is more than two hundred nucleotides long with a poly A tail, poly adenylation signal, and a open-reading frame with maximum 35 amino acid residues. The anatomy of the full length MIR fits well with the current definition of long non-coding RNA (lncRNA). The transfection of full length MIR [11] or a smaller fragment of MIR [9,10] does have the ability to promote myofibrillogenesis in mutant axolotl hearts. The smaller fragment of the active MIR does not have any open reading frame. Hence, the myofibril promotional activity of MIR does not require coding of any protein by itself. It is not known whether the full length MIR upon transfection into axolotl hearts undergoes any cleavage by an unknown cellular process(es) yielding a 166 nt long active core. MIR was shown to bind with more than one cellular protein. It is not clear whether MIR alone or in combination with its putative binding protein(s) promotes sarcomeric TM expression in axolotl heart. Interestingly, Zhang et al. [10] discovered that (^{79}G–>^{79}U) MIR from mutant hearts failed to perform the rescue activity and also its binding pattern with cellular proteins were altered. The ^{79}G–>^{79}U mutation caused a drastic change in the secondary structure of MIR. The authors rightfully speculated that this secondary structure of MIR is essential for its myofibril promotional activity.

Using RT-PCR with RNA from normal and cardiac mutant hearts (from several spawnings) Zhang et al. [10] demonstrated that mutant MIR is expressed only in hearts of cardiac mutant axolotl. However, experiments were not conducted to demonstrate the presence of this mutation in the mutant axolotl genome. This is an essential prerequisite if one claims that this G–>U mutation is responsible for cardiac mutant phenotype.

Experimental evidence demonstrates that both noncoding RNAs, MIR and microRNA-499c, fix the mutant phenotype of axolotl hearts *in situ*. The primary question remaining is the mechanism(s) by which these two noncoding RNAs perform the rescuing activity. Zhang et al. [11] claimed that MIR is essential for the expression of sarcomericTM in axolotl hearts. The treatment of mutant hearts with sense MIR augments the sarcomeric TM expression directly or indirectly by an influence at the translational level. On the other hand, the data presented by Kochegarov et al. [15] suggests microRNA499-c induced the transcription of the TPM4 gene thereby producing more TPM4α transcripts in mutant axolotl hearts

The next question to ask is, what are the immediate target genes of these two non-coding RNA? MicroRNA-499c is the most recent member of the MicroRNA 499 family of core 22 bp nucleotide sequence are conserved in all family members. Kochegarov et al. [15] have presented a detailed literature survey on the important roles microRNA-499 may play in cardiac differentiation and cardiogenesis during development. Also, they have cited the works of Wilson et al. [24] where the authors claimed over-expression of microRNA-499 enhanced expression of myocyte-specific enhancer factor 2C, the transcription factor which is involved in cardiac morphogenesis and myogenesis and vascular development. Its now well established that MicroRNAs are noncoding RNAs, which regulate the translation of genes by binding to untranslated sites (UTRs) in their targets. Several important issues need to be addressed – (1) whether or not microRNA.499-c exists in axolotl. Probably it does because microRNA-499 sequence is conserved in the genome of a variety of vertebrate species for example, human, mouse,

rat, bovine, xenopus and zebrafish [24]. If microRNA-499c is present in axolotl, one should find out whether there is any alteration/mutation in microRNA-499c sequence in cardiac mutant axolotl; (2) whether transfection of human or axololt microRNA-499a or microRNA-499b can rescue mutant axolotl hearts *in situ*; (3) what is the target gene in axololt heart whose expression is repressed by microRNA-499c resulting in an augmented expression of TPM4α. (4) whether knocking down of microRNA-499c in normal/wild type axolotl mimics the cardiac phenotype.

Although the effect of MIR on rescuing the cardiac phenotype is well characterized, final proof of the involvement of MIR in sarcomeric TM expression and subsequent myofibril formation in axolotl heart will come from some in vivo experimental results (for example, creating transgenic mutant axolotl expressing MIR in a cardiac specific manner or transgenic wild type axolotl knocking down MIR). Of course, identification of the immediate target gene(s) whose expression is controlled by MIR, warrant priority as well.

In summary, two non-coding RNAs without significant homology between themselves have been shown to rescue the cardiac mutant axolotl phenotype *in situ*. One, MIR, was originally found in the axolotl itself, but to date has not been reported in any other species. The other, microRNA-499c was cloned from human cardiac muscle tissue and likewise its presence in other vertebrates is unknown. Whether either or both together explain the cardiac mutant axolotl genetic disease is currently unclear. It is possible that they represent laboratory phenomena rather than physiologically relevant molecules. However, further investigation is certainly warranted to explore their potential role in vertebrate heart development and disease.

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