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PRELIMINARY INVESTIGATIONS ON HEART REGENERATION IN OBESE ZEBRAFISH, *DANIO RERIO*

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ABSTRACT
Human cardiomyocytes undergo cardiac hypertrophy after ischemic injury while zebrafish, *Danio rerio*, successfully regenerates ventricular cardiomyocytes. Understanding zebrafish cardiomyocyte proliferation, de-differentiation and regulation by MicroRNAs (miRNAs) may reveal novel therapeutic targets for treating myocardial infarction and other cardiac ailments in humans. **Objectives:** We utilized diet-induced obese zebrafish to investigate the molecular mechanisms underlying cardiac regeneration ϵ after cardiac injury in obese patients for regenerative medicine. Myocardial injury was inflicted on control and obese zebrafish heart by ventricular resection and expression of molecular factors driving cardiomyocyte regeneration were elucidated using semi-qRT-PCR. **Results:** Complete recovery from ischemic injury occurred after 57 ± 6 days post injury (dpi) in controls but was significantly ($P<0.05$) prolonged (79 ± 7 dpi) in obese zebrafish heart. Ventricular cardiomyocytes initiated proliferation after 7 ± 2 and 15 ± 4 dpi in control and obese zebrafish, respectively. Alpha 2 macroglobulin (*α2M*), *Gata4*, miR-133a, and *IGF2* were significantly ($P<0.05$) upregulated after 15 dpi whereas miRNA-99 and miR100 levels were completely down-regulated following injury. Interestingly, expression of *Gata4* was significantly delayed during initial stages of differentiation in obese zebrafish heart compared to controls. Only ~42% of lost tissues were replaced after 38 ± 4 dpi in obese heart which may be cited as possible reasons for delayed cardiomyocyte dedifferentiation than the control. **Conclusion:** Obese zebrafish may serve as an excellent vertebrate model for understanding expression of genes and molecular factors involved in cardiac regeneration. Reasons for delayed proliferation, differentiation and role of transcription factors and miRNAs in zebrafish cardiac regeneration are discussed.

KEY WORDS: Cardiac regeneration, obesity, myocardial injury, *IGF2*, miR133a, miR99, miR100.

INTRODUCTION
Obesity is a multifactorial, master metabolic disorder manifesting in clinical complications like Type II Diabetes Mellitus (T2DM), hypertension and several cardiovascular diseases.[1, 2] Acute myocardial infarction is the leading cause of death in humans worldwide that requires immediate intervention. Among developing nations, India has become the leading capital of obesity and cardiovascular diseases including myocardial infarction affecting even young adolescents.[3, 4] Mammalian cardiomyocytes regenerative ability to repair damaged heart is limited hence undergoes hypertrophy, a drastic increase in cell size rather than cell division as they are terminally differentiated.[5] Recently, Nam et al. (2013)[6] showed that cardiomyocytes could be directly programmed to regenerate from existing cardiac fibroblasts both *in vitro* and *in vivo* by overexpressing specific transcription factors. Cardiac regenerative capability of vertebrates were restricted to differentiated cardiomyocytes and not pericardial cells as evidenced by fate mapping studies by labelling cardiac myosin light chain binding protein (*cmlc2*) in mouse and zebrafish.[7-9] However, onset of cardiomyocyte proliferation and cell signalling during de-differentiation in cardiac hypertrophy and ventricular regeneration are less understood.[10] Our understanding of the molecular mechanism of cardiomyocyte regeneration in obese patients remain obscure, dissecting the molecular pathways with inexpensive genetic models may help develop therapies for acute myocardial infarction and cardiac hypertrophy in humans.[11,12]

Mammalian cardiomyocytes retain their inherent ability to re-divide with limitations but, zebrafish responds to ischemic injury by regenerating ~20% of the lost ventricular cardiomyocytes which makes them an excellent vertebrate model for cardiac regeneration research.[13] Temporal expression pattern of specific

transcription (*Gata4*) and regulatory factors, cell signalling (*Wnt/β*-catenin/*IGF*) and miRNAs involved in cardiac regeneration in zebrafish are still under investigation. Alpha2 Macroglobulin (*α2M*) belongs to Thio-ester Protein (TEP) superfamily of protease inhibitors with potential role in liver, gut development and immunity.[14,15] Increasing levels of *α2M* during cardiac hypertrophy in AIDS patients confirmed its reliability as a biomarker for myocardial insults in humans.[16] Recently, role of novel Small non-coding RNAs (sncRNAs) like miRNAs, known to play potential role in post-transcriptional gene silencing and regulation of gene expression from nematodes to humans were described.[17, 18] Several miRNAs such as miR-33, miR-99 and miR100 regulates expression of specific transcription factors essential for cardiac repair and regeneration.[12] Silencing of miR99/100 expression in cardiomyocytes indirectly up-regulated expression of *Gata4*, a cell proliferation marker and *Fntβ* and *Smarca5* required for cardiomyocyte proliferation.[9, 11]

Zebrafish, *Danio rerio* (Hamilton, 1822)[19] exhibits ~74% genetic homology to humans and availability of complete genome sequence makes them extremely useful in genetic studies for elucidating pathophysiological pathways in cardiovascular and metabolic disorders.[20,21] External fertilization, transparent embryos and larvae, fully functional organs with structural and functional similarity to humans and their amenability to genetic manipulation techniques, makes them an ideal vertebrate for investigating molecular mechanisms of cardiac proliferation, differentiation and regeneration.[10, 11, 21, 22] Very few authors have confirmed the putative role of miRNAs in zebrafish cardiac regeneration. Zebrafish mutant's *ncp* and *nbl* which were unable to regenerate their fins also failed to regenerate their heart, clearly indicating the involvement of similar set of genes in fin and cardiac regeneration.[22,13] Hence, studies on effects of ischemic injury upon cardiomyocyte proliferation and differentiation associated gene expression in zebrafish during wound repair and regeneration were required. In the present study, our objectives were to (i) inflict myocardial injury by ventricular resection in zebrafish (ii) identify molecular factors critical for cardiac regeneration in obese zebrafish and (iii) characterize novel miRNAs in proliferating cardiomyocytes after injury by semi-qRT-PCR analyses.

MATERIAL AND METHODS

Zebrafish husbandry

Wild type zebrafish isogenic lines were raised following optimized protocols.[20, 23] Control zebrafish were fed pelleted diet (Aqua feed, Madurai) and maintained at 12:12 light:dark (LD) conditions while, experimental fish were fed 6% cholesterol (Sigma, USA) filled pellets for >6 weeks to induce obesity, as described.[20] For regeneration experiments, obese and control wild type zebrafish (n=8 each) were selected from the young age groups (12-18 months) and used for the present study. Fishes were raised in sterile rearing medium after dissection to prevent infection and sacrificed after the experiments to harvest visceral organs for RNA isolation and histopathology.

Myocardial injury-ventricular resection

Fishes were anesthetized in 0.1% Tricaine Methane Sulfonate (MS222, Sigma Aldrich, USA) and held dorsal side up with cotton. Small portion (~2mm) near the chest cavity was cut open with a sterile surgical blade to expose the heart. The ventricular portion of the heart was sliced (~10-20%) with a fine cut using sterile DEPC treated surgical steel blade following established protocols.[24] To prevent bleeding, injured heart was pressed briefly with damp cotton soaked in sterile water containing antibiotic. The dissected animals were allowed to recuperate in sterile water bath for 2 -3 days, with feeding once a day, until further experiments. All fishes were sacrificed to harvest heart and visceral organs after 14, 28, 56 and 86 days post injury (dpi) for total RNA and miRNA isolation for semi-qRT-PCR analyses.

Total RNA and miRNA isolation and cDNA library construction

For isolating total RNA from the heart, TRiZol method was followed. Briefly, selected fish were sacrificed followed by decapitation to remove heart and visceral organs with DEPC treated sterile surgical blades. Total RNA was isolated from zebrafish hearts following standard procedures[25] and RNAs smaller than 200 bases were isolated using miRNEasy kit (Qiagen, USA) and subsequently purified in 12.5% PAGE for isolating smaller miRNA population by homo-polymer (PolyA) tailing and used for semi-qRT-PCR analyses. First strand cDNA was synthesized from the isolated total RNA pool with Revert Aid First Strand cDNA synthesis kit (Qiagen, USA), PCR amplified (25 cycles) and stored in freezer (-80C).

Semi qRT –PCR

Primers were designed for miRNA-99, miR-100, miR-133a, Alpha 2 Macroglobulin (*α2M*), *Gata4*, *IGF2* and housekeeping gene β-actin based on previously reported sequences in PUBMED. Semi-qRT PCR was carried out in a thermal cycler (Applied Bio-Systems, ABI, USA). PCR amplified products were separated in 2.5% Agarose gel and imaged under a Gel documentation unit (BioRad, USA). Using Image J software the level of expression was measured by comparing light intensity of each amplicons against the house keeping gene β-actin as described.[25]

RESULTS

Diet-induced Obese Zebrafish

Diet-induced obese (DIO) zebrafish generated by feeding high-cholesterol diet (HCD) for > 8 weeks exhibited increased adiposity in abdominal regions.[2] Our earlier studies indicated that gut microbes from Phylum *Firmicutes spp.* increased in number and accelerated lipid metabolism, thereby inducing obesity. Gnotobiotic

zebrafish strains succumbed due to lack of immunity and dysregulated metabolism.[20] Body Mass Index (BMI) values were significantly higher for the DIO zebrafish than the controls and females outweighed males. Obese zebrafish also had delayed motor response and altered behaviour pattern compared to controls.[26]

Heart Regeneration

Myocardial injury by ventricular resection was successful in removing ~15-20% ventricular tissues from the zebrafish heart, as confirmed by histological analysis. Initiation of cardiomyocyte proliferation in obese zebrafish heart was significantly (P<0.05) delayed and failed to complete cardiac repair and development within the expected time frame (~60 dpi), compared to the control (Fig. 1). Cardiomyocyte proliferation and dedifferentiation began at 7 dpi in controls which took ~57 ± 6 dpi to completely regenerate but obese heart took 79 ± 7 dpi to complete cardiac repair.[27] Initiation of cardiomyocyte proliferation in obese zebrafish began >14 dpi and took ~86 dpi to complete regeneration and become fully functional.

Semi qRT-PCR analyses

Expression analyses of transcription factor *Gata4*, a reliable marker for confirming cardiomyocyte proliferation and *IGFI* for cell signalling to determine cardiomyocytes de-differentiation during cardiac regeneration revealed upregulation of *IGFI* after injury in both control and obese hearts (Fig. 1). While *Gata4* expression was upregulated immediately after injury in the control but was significantly (P< 0.05) delayed (~14 days) in obese zebrafish heart.

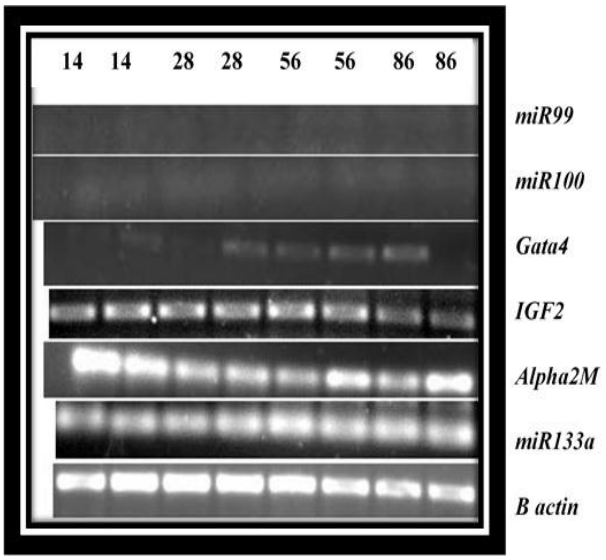

Figure 1. Semi-qRT-PCR analyses of regenerating zebrafish heart at different time intervals after myocardial injury. Expression level of genes were calculated using Image J software against the housekeeping gene, β-actin.

Expectedly, levels of *α2M,* a stress biomarker of cardiac hypertrophy was significantly upregulated after 4 dpi in both control and obese (remained elevated until 56 dpi in obese heart). Expression of miR-133a increased immediately after injury while, miR-99 and 100 were significantly (P<0.05) down regulated and were rarely detectable compared to housekeeping gene β-actin in both control and obese hearts (Fig.1).

DISCUSSION

Obesity a non-communicable metabolic disorder culminating in chronic conditions like non-alcoholic fatty liver disease (NAFLD), Non-alcoholic Steato-Hepatosis (NASH), Liver cirrhosis and Hepato-Cellular Carcinoma (HCC), if left unattended.[2] Obesity is a major risk factor for several cardiovascular disorders (CVDs) including myocardial infarction and cardiac hypertrophy hence, increases the morbidity and mortality in young adolescents.[28] Mammalian cardiac regeneration is limited and human heart lacks the capability to dedifferentiate and regenerate. Though, mammalian cardiomyocytes have the ability to regenerate, zebrafish successfully regenerated (~20%) their lost ventricular tissues.[9, 10, 11, 29] Very few authors have successfully inflicted myocardial injury in zebrafish to gain insight into regenerative capability and the molecular pathways of cardiac regeneration.[9, 11, 30] Zebrafish, *D. rerio* exhibits ~74% genomic and organ similarity to humans and availability of complete genome sequence makes them extremely useful in genetic studies for dissecting cardiovascular and metabolic disorders.[21] Wild type zebrafish regenerated lost ventricular cardiomyocytes after ischemic injury[13] while, in diet induced obese zebrafish regeneration was delayed and failed to complete cardiac repair within the time period, compared to the controls (Fig. 1). Reasons for delayed regeneration may be attributed to delay and significantly down regulated expression of transcription factors and genes involved in DNA synthesis, cardiomyocyte proliferation and differentiation.[11, 31]

Histological analyses of regenerating zebrafish heart showed that cardiomyocytes from primordial layer alone underwent hypertrophy after injury while, cortical layer divided contributing to nascent cardiomyocytes and epicardial layer to vascularization of newly formed cardiomyocytes, similar to humans.[7, 8, 10] For instance, adaptive and physiological or pathological hypertrophy is induced by strenuous exercise or cardiac insults, respectively.[16] Although, in both cases, heart undergoes enlargement, former is a healthy adaptation and later is detrimental. Since, cardiomyocytes are terminally differentiated cells that cannot undergo division hence, can only enlarge; ischemic injury in humans always results in irreversible loss of cardiomyocytes and scaring.[5,13] Several studies indicated that cardiomyocytes at wound site responds to *IGF1/2* signalling leading to up/down regulation of major transcriptional regulators i.e. *Gata4, Hand3, Mef2, Nkx2.5, Tbx5 and Tbx20,* preceding DNA synthesis.[6,7, 10, 11] Similarly, in the present study, expression of developmental markers associated with cell proliferation

like *Gata4* was significantly delayed and down regulated (>14dpi) in obese heart. Also, by histological analyses, we found that newly formed sarcomeres underwent considerable remodelling suggesting involvement of identical canonical pathways in heart regeneration, in consistent with previous reports from mouse and humans.[32,33] While lower order vertebrates could rapidly regenerate lost organs including heart, inability of humans to regenerate despite conserved extrinsic and intrinsic pathways, remains unknown.[9, 11, 13] Increased expression of miR99 and miR100 inhibited expression of *Fntβ* and *Smarca5* preventing cardiomyocyte proliferation and regeneration in obese zebrafish heart, confirming that *Fntβ* and *Smarca5* are direct targets of miR99 and miR100 (Fig. 1).

Very recently, terminally differentiated somatic cells were reprogrammed into induced pluripotent stem cells (iPSCs) by over expressing 5 major transcription factors (*Gata4, Hand3, Mef2, Nkx2.5, Tbx5*) that differentiated into cardiomyocytes in humans and mechanism was similar in mice and zebrafish.[6,10,11] Interestingly, overexpression of miR-133 family significantly increased cardiac regenerative capacity, which may be attractive targets for regeneration therapy.[11,12,31] Treatment with cardiac stem cells, induced pluripotent stem cells or overexpressing critical miRNAs are promising avenues for developing personalized medicine for myocardial infarction and cardiac hypertrophy in obese humans.[10, 16 21] Therefore, a detailed study on *in vitro* and *in vivo* temporal expression pattern of *miRNAs* and regulation of transcription factors essential for cardiomyocyte dedifferentiation during cardiac regeneration in zebrafish using next generation sequencing (NGS) technologies may help unravel novel molecular biomarkers and therapeutic targets for therapy.[10, 11, 12, 32]

Embryonic mouse heart express low levels of miR-99/100 during development while, their expression was up-regulated in humans.[12] Silencing of such small non-coding RNAs with antagomirs have affirmed their role in regulation of cardiac regeneration in mouse heart; miR99/100 antagomirs significantly increased cardiomyocyte proliferation and ventricle size even in the absence of injury.[34] In the present study, expression of miR99/100 were rarely detectable compared to the controls indicating their direct role in cardiac regeneration (Fig. 1). However, silencing of miR99/100 in adult cardiomyocytes increased expression of *Gata4*, a biomarker for cardiomyocyte differentiation, which may be beneficial for targeted therapeutics. Our present study confirms previous reports by Zhang et al. (2013)[31] that down regulation of miR99/100 expression indirectly elevates the expression of *Gata4*, *Smarca5* and *Fntβ*.[34] Interestingly, expression of miR-133a were up regulated but miR-99/100 family members were down regulated during later stages of differentiation than during initiation, compared to controls, confirming their role in early cardiomyocyte differentiation, as in humans.[12, 34] Since, down regulation of miR99/100 correlated with increasing levels of Proliferating Cell Nuclear Antigen (PCNA), *Gata4* and Histone H3 phosphorylation in adult cardiomyocytes.[5, 11] Further, transcriptome profiling by RNA deep sequencing may uncover novel SncRNAs that may serve as potential targets for cardiac repair and regeneration in cardiovascular diseases.[5, 34]

Increased *IGF1/2* signalling indicates re-activation of zebrafish cardiomyocytes differentiation pathways to regenerate lost ventricular tissues, as in mouse models (Fig.1). Further, we also confirm earlier reports on altered expression of molecular markers such as *Gata4* and miR99/100 in obese zebrafish that were down regulated in cardiomyocytes, found adjacent to the wound sites, during initial stages of regeneration.[10, 13] Subbiah et al. (2010)[16] reported increased expression of *α2M* in cardiomyocytes of cardiac hypertrophy in AIDS patients. Novel isoforms of Alpha 2 macroglobulin (*α2M*) present in zebrafish[14,15], are stress marker proteins which were upregulated immediately after injury thus making them a reliable marker for myocardial insults. In the present study, both, ventricular injury and the stress to regenerate lost cardiomyocytes resulted in upregulation of *α2M* expression in zebrafish heart (Fig. 1). Elucidating temporal gene expression pattern of obese zebrafish heart during regeneration may shed light on wound response factors and intrinsic mechanisms during myocardial infarction in obese patients, beneficial for regenerative medicine therapy.

CONCLUSION

The present study illustrates the feasibility of using zebrafish as a genetic model for better understanding the molecular pathways involved in heart regeneration including specific miRNAs and stress proteins associated with dedifferentiation of adult cardiomyocytes after myocardial injury. Temporal expression of specific transcription factors under the regulation of miRNAs at the wound site determines the recovery period after ischemic injury in adult zebrafish and humans. We conclude that humanized zebrafish model may be useful for investigating the mechanism of cardiac regeneration to help develop targeted therapeutics and personalized medicine for several human cardiovascular disorders.

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