Comparative Analysis of 3D-Culture System for Murine Neonatal Heart Regeneration: A Systematic Approach for Big Gene Expression Data

Julia Tzu-Ya Weng[1(✉)], Yi-Cheng Chen[2], Pei-Chann Chang[3], Shin-Ping Huang[1], and Yu-Wei Chiu[4]

[1] Department of Computer Science and Engineering, Yuan Ze University, Taoyuan, Taiwan
julweng@saturn.yzu.edu.tw, sl026015@mail.yzu.edu.tw
[2] Department of Computer Science and Information Engineering, Tamkang University, New Taipei City, Tamsui, Taiwan
ycchen@mail.tku.edu.tw
[3] Department of Information Management, Yuan Ze University, Taoyuan, Taiwan
iepchang@saturn.yzu.edu.tw
[4] Division of Cardiology Department of Internal Medicine, Far-Eastern Memorial Hospital, Taipei, Taiwan
dtmed005@yahoo.com.tw

Abstract. Cardiovascular diseases are the leading cause of death worldwide. Loss or dysfunction of cardiomyocytes is associated with many forms of heart disease. The adult mammalian heart has a limited regenerative ability after damage, leading to the formation of fibrotic scar tissues, hypertrophy, contractile dysfunction and ultimately, organ failure. In contrast, neonatal mammalian cardiomyocytes retain a significant replenishing potential briefly after birth. There is increasing enthusiasm to grow neonatal cardiomyocytes in 3D culture systems to artificially restore heart function. Various scaffolds and matrices are available, but the molecular and cellular mechanisms underlying proliferation and differentiation of neonatal mammalian cardiomyocytes are not very well understood. Here, we utilize a systematic strategy to analyze the extensive genome-scale gene expression profiles of two different 3D constructs. We present a comprehensive comparison that may help improve the protocols for growing cardiomyocytes in a 3D culture system.

Keywords: Cardiomyocytes · Regeneration · 3-D culture system · Gene expression · Bioinformatics

1 Introduction

Cardiovascular diseases (CVDs) are the number one cause of death throughout the world [1], with the loss or dysfunction of cardiomyocytes being the key event leading to the formation of fibrotic scar tissues, pathological hypertrophy, contractile

W.-C. Peng et al. (Eds.): PAKDD 2014 Workshops, LNAI 8643, pp. 754–764, 2014.
DOI: 10.1007/978-3-319-13186-3_67

dysfunction, and eventually resulting in heart failure [2]. In 2008, CVDs already account for 30 % of the total global deaths [1]. It is estimated that by 2030, more than 23 million people worldwide would die from CVDs [3].

Unfortunately, current pharmacological or surgical therapies for CVDs can slow the disease progression, but are unable to fully ameliorate cardiomyocyte loss or dysfunction [4]. Despite improvements in treatment, patients are still susceptible to an increased risk of chronic cardiac failure as scarring develops after injury [5]. The high disease burden, both from the perspectives of the healthcare system and the patients' quality of life, demands new therapeutic strategies to treat CVDs.

Advances in stem cell research and cell biology techniques have provided a wide variety of approaches to regenerate the heart from progenitor and cardiomyocytes derived from embryonic, neonatal, and adult cells. Though adult cardiomyocytes have been shown to have regenerative ability after injury, proliferation and differentiation occur at a much lower rate compared to embryonic and neonatal cells [6]. It is still unclear how and whether efficient cardiac regeneration can be stimulated and regulated in the adult mammalian heart.

In contrast, significant cardiac regeneration has been observed in the neonatal mammalian heart. In the mouse heart, surgical amputation of the ventricular apex has been shown to initiate cardiomyocyte proliferation, suggesting that the neonatal heart is capable of endogenous regenerative response [7]. However, such event only sustains for a brief period after birth. The cellular and molecular underpinnings of postnatal cell cycle arrest in cardiomyocytes and loss of cardiac regenerative capacity in the mammalian heart still remain elusive. Enhanced understanding of these key molecular events may offer novel insights for the development of ways to manipulate or extend the neonatal cardiac regenerative potential into adulthood.

In regenerative cell biology research, 3D cultures are gaining attention since they allow for the generation of 3D architecture that resembles more closely to the native environment of the tissue. Indeed, cells cultured in 3D and 2D conditions have been shown to differ in gene expression profile and phenotype [8]. Important markers of development and response to hormonal stimulation are more readily observed in 3D systems. 3D cultures have also been found to activate cell proliferation more efficiently compared to 2D designs [9]. It appears that 2D cultures may not be as flexible for manipulations or modulations, and thus, lack translational potential.

Owing to its increasing popularity in regenerative medical research, 3D constructs are now available with variable physical, chemical, and biological properties that can be manipulated to suit different purposes. For example, extracellular matrix prepared from decellularized cardiac tissue could be fabricated into 3D scaffolds and matrices, and injected to treat postmyocardial infarction injury [10–12]. Hydrogels and synthetic biodegradable materials can also be fabricated to create customized patterns and constructs [13, 14]. In fact, hydrogels have been combined with cell agents to successfully improve repair of cardiac function post injury [15–18].

Similar to the difference between 3D and 2D culture systems, different properties of 3D constructs may result in varying levels of success in cardiac tissue regeneration. We have compared among two 3D systems, PuraMatrix™ (BD Bioscience, U.S.A.) and Go-Matrix (Bio-Byblos Biomedical Co., Ltd., Taiwan), and the traditional 2D-cell culture system. The PuraMatrix™ construct is composed of peptide hydrogel with

irregular pore arrangements, whereas Go-Matrix is made of gelatin from porcine skin with regular pore patterns. Compared to the more normal looking 2D-culture system and Go-Matrix, we observe irregular formation of muscle fibers that resembles myocardial fibrosis, as well as abnormal beating of the cardiac tissue manufactured from PuraMatrix™ (unpublished results).

In the present study, we set to compare the gene expression differences between PuraMatrix™ and Go-Matrix with microarray technology, using the 2D architecture as a baseline reference. The reason for including the 2D culture is that we have repeatedly and successfully generated cardiac tissues from this system. The next step in our research is to construct a 3D model that holds much more promises for clinical applications. Therefore, the 2D construct serves as a good normalization control for our analysis.

Microarray gene expression profiles comparing three different cell culture models, with each probe on the bio-chip repeatedly targeting (at least 10 times) each of the approximately 25,000 transcripts in the mouse genome. Such profiles represent a big dataset with great complexity and require a systematic pipeline combining the available bioinformatics tools to extract important biological meanings.

Here, we present an integrative system flow involving various tools for the analysis of gene expression differences between PuraMatrix™ and Go-Matrix. These resources are integrated on the WEB-based Gene SeT AnaLysis Toolkit (WebGestalt) [19] and mirWalk [20], and include curated information from Gene Ontology (GO) [21], Kyoto Encyclopedia of Genes and Genomes (KEGG) [22], Pathway Commons [23], WikiPathway [24], miRBase [25], Diana-microT [26], miRanda [27], miRDB [28], PicTar [29], PITA [30], RNA22 [31], and TargetScan [32]. We believe a combinatorial analysis utilizing publicly available bioinformatics tools should provide a better overview of the significance in the experiment design. Identifying the biological significance underlying the molecular differences between the two different 3D cell culture systems may help facilitate improvements in protocols for mending the “broken” heart.

2 Methods and Materials

2.1 System Flow

The system flow of our study is depicted in Fig. 1. Gene expression microarray experiment is performed to identify differentially expressed transcripts among two 3D cell culture systems, PuraMatrix™ and Go-Matrix, with the traditional 2D culture as a normalization control. Differentially expressed were categorized into up- and down-regulated genes, and divided into PuraMatrix- and Go-Matrix-specific groups. The differentially expressed genes are subsequently subjected to bioinformatics analysis WebGestalt [19] and mirWalk [20].

2.2 Cardiomyocyte Isolation

All procedures were approved by the Institutional Animal Care and Use Committee of the Far-Eastern Memorial Hospital. Neonatal B6 mice were sacrificed by de-capitation

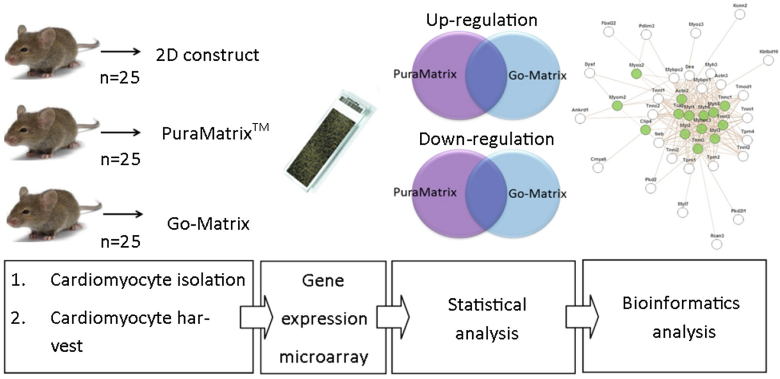

Fig. 1. System flow of our wet bench and bioinformatics analyses.

on postnatal day 3. 25 neonatal mice underwent the surgical procedures for cardiomyocyte isolation. The hearts were quickly excised and transferred into ice-cold phosphate buffer saline (PBS, Sigma), and quickly minced in a solution of colla-genase A + B solution (10 mg/ml in PBS). Cells in the supernatants were resuspended in claycomb medium with 10 % fetal bovine serum (FBS, Gibco) and plated for 1.5 h at 37 °C. The attached fibroblasts and endothelial cells were removed and the cardiomyocytes were isolated in the suspension solution.

2.3 Cell Seeding of Constructs

Cardiomyocytes were cultured on gelatin/fibronectin-coated tissue-culture flasks in claycomb medium at 37 °C in a CO_2 incubator. Cells harvested and cultured in this method served as the 2D control group. To prepare PuraMatrix™, 1 % stock solution was mixed with 20 % sucrose (Sigma–Aldrich, Sweden AB) and DMEM containing Ca^{2+} and Mg^{2+} in a 2:1:1 ratio, giving 0.5 % hydrogel. Cardiomyocytes were detached from the culture flasks with trypsin/EDTA, concentrated in claycomb medium and immersed with PuraMatrix™ peptide hydrogel. Go-Matrix is a gelatin scaffold fixed on tissue-culture flasks. The claycomb medium containing cardiomyocytes was dropped onto the dry Go Matrix. In total, 106 cells were cultured in each group.

2.4 RNA Isolation

RNA was isolated from harvested cardiomyocyte cells. RNA quality was determined by an OD 260/280 ratio ≥ 1.8, and OD 260/230 ratio ≥ 1.5 on a spectrophotometer and by the intensity of the 18S and 28S rRNA bands on a 1 % formaldehydeagarose gel. RNA quantity was detected by a spectrophotometer. RNA integrity was examined on an Agilent Bioanalyzer. RNA with a 2100 RIN (RNA integrity number) ≥ 6.0 and 28S/18S > 0.7 was subjected to microarray analysis.

2.5 Gene Expression Analysis

Total RNA samples from 25 mice in each construct are pooled and subsequently subjected to SurePrint G3 Mouse GE 8x60 K Microarray (Agilent Technologies, U.S.A.). Data are analyzed using R/Bioconductor. PuraMatrix™ and Go-Matrix expression profiles were normalized against that of the cardiomyocytes prepared from the traditional 2D cell culture system. Genes showing significant differential expression between normalized PuraMatrixTM and Go-Matrix (absolute value of $\log_2$ ratio > 1.0, FDR < 0.05) were categorized into PuraMatrix™ -specific down- and up-regulated genes, and Go-Matrix-specific down- and up-regulated genes.

2.6 Bioinformatics Analysis

A flow chart describing our bioinformatics analysis is given in Fig. 2. Differentially expressed genes were used as input for the bioinformatics analysis of gene ontology (GO) enrichment analysis and pathway enrichment analysis. Multiple testing bias is adjusted by a Bonferroni threshold of $p < 0.05$. These analyses are performed on the open analytical platform in WebGestalt [19], which integrates GO [21], KEGG [22], Pathway Commons [23], and WikiPathways [24]. microRNA target prediction analysis is conducted in mirWalk [20], which, in addition to the developers' own analytical algorithms, also makes comparisons with other microRNA databases, including miRBase [25], Diana-microT [26], miRanda [27], miRDB [28], PicTar [29], PITA [30], RNA22 [31], and TargetScan [32].

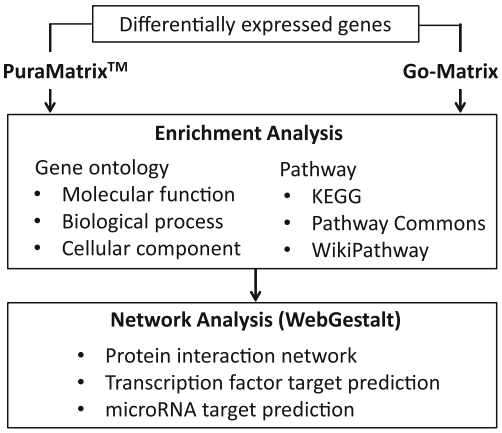

Fig. 2. Bioinformatics analysis workflow

3 Results and Discussions

In total, there are 516 up-regulated and 338 down-regulated genes, and 401 up-regulated and 292 down-regulated genes specific to the PuraMatrix™ and Go-Matrix expression profiles, respectively (Fig. 3). It appears that the Go-Matrix expression profile may be

more similar to the traditional 2D culture system. For both 3D constructs, the top three most enriched biological processes are: biological regulation, metabolic process, and response to stimulus. The top three most important cellular components include the membrane, nucleus, and macromolecular complex. For molecular functions, both expression profiles are enriched in protein binding, ion binding, and metabolite binding.

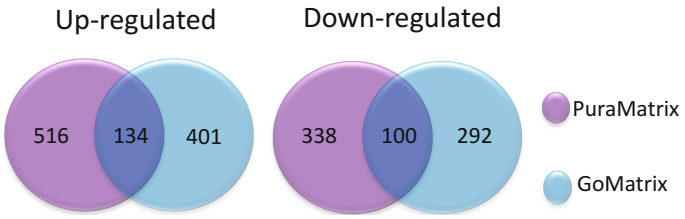

Fig. 3. Number of Differentially expressed genes from the PuraMatrix™ and Go-Matrix expression profiles

PuraMatrix™ and Go-Matrix expression profiles appear to be similar in muscle contraction pathways, though the gene expression patterns are different. In particular, the striated muscle contraction pathway that is enriched in both PuraMatrix™ and Go-Matrix gene list. However, the two 3D cell culture systems differ in the genes that are differentially expressed in this pathway.

Though similar in some pathways, PuraMatrix™ and Go-Matrix expression profiles appear to be enriched in different types of cellular pathways. For instance, most of the differentially expressed genes in the PuraMatrix™ construct seem to belong to muscle contraction and cell adhesion related pathways (Table 1). In contrast, Go-Matrix-specific differentially expressed genes are enriched in pathways involving membrane receptor interactions.

Cell culture observations indicate that the PuraMatrix™ cell culture system tends to generate tissue with abnormal muscle fiber arrangements and contractions (unpublished results). This is consistent with our gene expression profile analysis in that most of the differentially expressed genes in the PuraMatrix™ design belong to cardiomyopathy pathways. For example, among the list of PuraMatrix™-specific up-regulated genes involved in dilated cardiomyopathy, the ryanodine receptor 2 (*Ryr2*) gene, when silenced by RNAi, protects rat cardiomyocytes from simulated ischemia-induced injury [33]. The *Pln* (phospholabam) gene, when deleted or suppressed, is known to restore normal cardiomyocyte contraction and slow the progression towards cardiomyopathy [34]. This suggests that the observed phenotype and gene expression changes associated with the PuraMatrix™ construct of cardiomyoctes may serve as a potential basis for the future development of a cardiomyopathy related disease model.

In contrast, the differentially expressed genes in the Go-matrix seem to be related to the development of cardiac cells and the establishment of cell-cell contact (Table 1). This corresponds to the ordered arrangements of muscle fibers and regular muscle contractions of the Go-Matrix cardiomyocyte culture (unpublished results). The enrichment analysis result is also consistent with similar findings in fetal sheep, in which neuroactive-ligand-receptor interaction, cytokine-cytokine receptor interaction,

Table 1. List of top 3 most enriched pathways in PuraMatrix™ and Go-Matrix expression profiles as identified by KEGG and WikiPathway.

PuraMatrix™ enriched pathways	Go-Matrix enriched pathways
KEGG (up-regulation)	
Dilated cardiomyopathy	Neuroactive-ligand-receptor interaction
Hypertrophic cardiomyopathy	Cytokine-cytokine receptor interaction
Pathways in cancer	Complement and coagulation cascades
KEGG (down-regulation)	
Vascular smooth muscle contraction	Axon guidance
Neuroactive-ligand-receptor interaction	Notch signaling pathway
Protein digestion and absorption	Melanogenesis
WikiPathway (up-regulation)	
Striated muscle contraction	Complement and coagulation cascades
Focal adhesion	Non-odorant G-protein coupled receptors
PPAR signaling pathway	Striated muscle contraction
WikiPathway (down-regulation)	
Striated muscle contraction	Delta-Notch signaling pathway
Integrin-mediated cell adhesion	Cholesterol biosynthesis
Focal adhesion	Complement and coagulation cascades

complement and coagulation cascades, etc. were associated cardiogenesis [35]. In particular, the Notch signaling pathway has been implicated in development of cardiac neural crest cells and heart valves [36].

Interestingly, differentially expressed genes in both PuraMatrix™ and Go-matrix profiles are enriched in the striated muscle contraction pathway, though these genes play differing roles in this particular process (Fig. 4). This indicates that differences in the modulation of gene expression have specific effect on the muscle contraction phenotype. Such differences may contribute to the phenotypic variations between the cardiomyocytes grown in these two culture systems.

The interactions among the differentially expressed genes involved in the striated muscle contraction pathway may reveal the molecular underpinnings of the differences in muscle contraction between cardiomyoctyes constructed by PuraMatrixTM and Go-Matrix. Protein-interaction and microRNA target analysis suggest that three genes would be strong potential candidates for further functional study of cardiac development. These genes are *Myh6* (myosin, heavy polypeptide 6, cardiac muscle, alpha), which is expressed in the atria and associated with congenital heart defects [37]; *Tnnc1* (troponin C type 1), which plays a regulatory role in the beating of the early developing heart [38]; *Des* (desmin), which is an intermediate filament gene important for muscle architecture [39].

Our results suggest that that *Myh6*, *Tnnc1*, and *Des* are likely interacting with each other, as well as with other important molecular signatures of the cardiac contraction pathway (Fig. 5). Furthermore, their expression may be regulated by several microRNAs that have been implicated to play important roles in cardiac function. For instance, the mir-29 family is responsible for modulating the development of cardiac

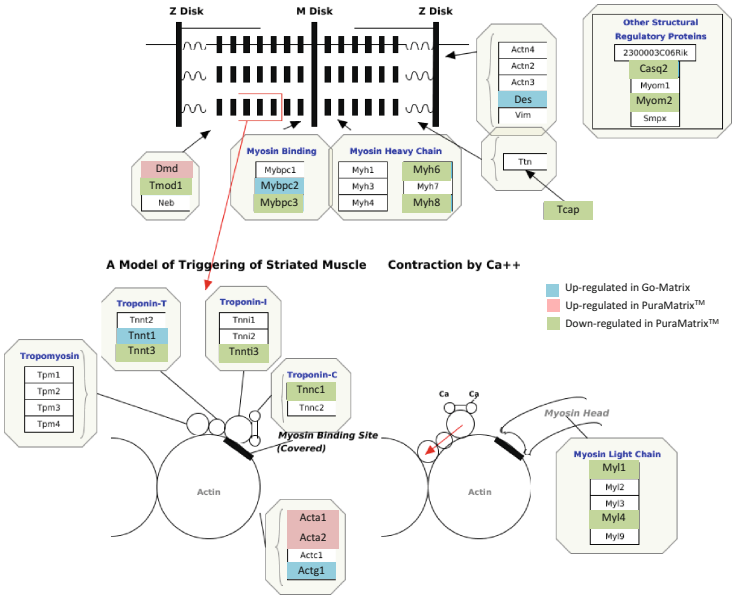

Fig. 4. PuraMatrix™-specific and Go-Matrix-specific differentially expressed genes involved in the striated muscle contraction pathway

fibrosis after injury [40]. In addition, mir-208a is known to be a regulator of cardiac stress response [41]. The interaction network presented in Fig. 5 may represent a potential modulatory mechanism underlying the differences the in muscle contraction phenotype between PuraMatrix™ and Go-Matrix.

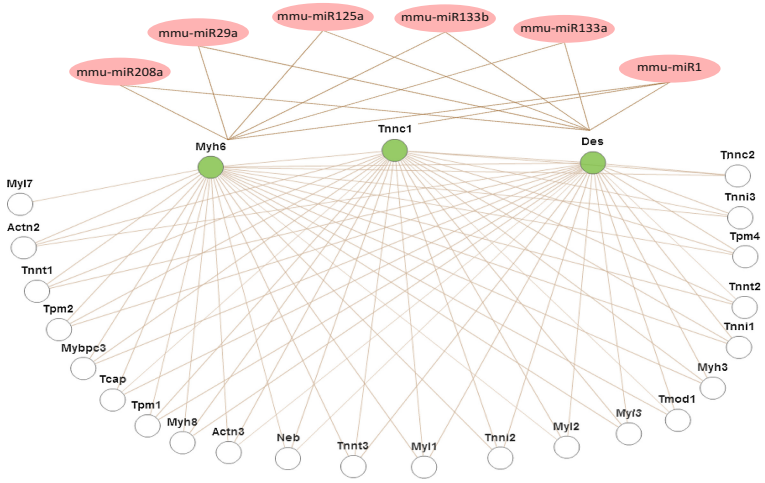

Fig. 5. Protein interaction network showing the interaction among Myh6, Tnnc1, Des with other genes and corresponding validated regulatory microRNAs from the striated muscle contraction pathway

4 Conclusion

3D cell culture systems are gaining attention in regenerative medical research. These constructs can be manipulated to suit different purposes, either for understanding development and disease mechanisms, or establishing a disease model. The ways in which these systems can be manipulated is of particular interest to regenerative medicine. Here, we present a systematic pipeline, integrating a wide variety of bioinformatics tools, to extract important biological meanings from the genome-scale differences between two 3D systems utilized for cardiomyocyte culturing. Our analysis presents a preliminary picture of the significant pathways and interactions associated with cardiomyocyte development in 3D. Our findings may have special implications for the future establishment of a better model for murine neonatal heart regeneration.

References

1. Organization, W.H.: Global status report on noncommunicable diseases 2010. World Health Organization (2011)
2. Kajstura, J., Urbanek, K., Perl, S., Hosoda, T., Zheng, H., Ogorek, B., Ferreira-Martins, J., Goichberg, P., Rondon-Clavo, C., Sanada, F., D'Amario, D., Rota, M., Del Monte, F., Orlic, D., Tisdale, J., Leri, A., Anversa, P.: Cardiomyogenesis in the adult human heart. Circ. Res. **107**, 305–315 (2010)
3. Mathers, C.D., Loncar, D.: Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med. **3**, e442 (2006)
4. Steinhauser, M.L., Lee, R.T.: Regeneration of the heart. EMBO Mol. Med. **3**, 701–712 (2011)
5. Bartunek, J., Behfar, A., Dolatabadi, D., Vanderheyden, M., Ostojic, M., Dens, J., El Nakadi, B., Banovic, M., Beleslin, B., Vrolix, M., Legrand, V., Vrints, C., Vanoverschelde, J.L., Crespo-Diaz, R., Homsy, C., Tendera, M., Waldman, S., Wijns, W., Terzic, A.: Cardiopoietic stem cell therapy in heart failure: the C-CURE (Cardiopoietic stem Cell therapy in heart failURE) multicenter randomized trial with lineage-specified biologics. J. Am. Coll. Cardiol. **61**, 2329–2338 (2013)
6. Hsieh, P.C.H., Segers, V.F.M., Davis, M.E., MacGillivray, C., Gannon, J., Molkentin, J.D., Robbins, J., Lee, R.T.: Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. Nat. Med. **13**, 970–974 (2007)
7. Porrello, E.R., Mahmoud, A.I., Simpson, E., Hill, J.A., Richardson, J.A., Olson, E.N., Sadek, H.A.: Transient regenerative potential of the neonatal mouse heart. Science **331**, 1078–1080 (2011)
8. Akins Jr., R.E., Rockwood, D., Robinson, K.G., Sandusky, D., Rabolt, J., Pizarro, C.: Three-dimensional culture alters primary cardiac cell phenotype. Tissue Eng. Part A **16**, 629–641 (2010)
9. Kellar, R.S., Landeen, L.K., Shepherd, B.R., Naughton, G.K., Ratcliffe, A., Williams, S.K.: Scaffold-based three-dimensional human fibroblast culture provides a structural matrix that supports angiogenesis in infarcted heart tissue. Circulation **104**, 2063–2068 (2001)
10. Singelyn, J.M., Christman, K.L.: Injectable Materials for the Treatment of Myocardial Infarction and Heart Failure: The Promise of Decellularized Matrices. J. Cardiovasc. Transl. **3**, 478–486 (2010)

11. Godier-Furnemont, A.F.G., Martens, T.P., Koeckert, M.S., Wan, L., Parks, J., Arai, K., Zhang, G.P., Hudson, B., Homma, S., Vunjak-Novakovic, G.: Composite scaffold provides a cell delivery platform for cardiovascular repair. Proc. Natl. Acad. Sci. U.S.A. **108**, 7974–7979 (2011)
12. Singelyn, J.M., Sundaramurthy, P., Johnson, T.D., Schup-Magoffin, P.J., Hu, D.P., Faulk, D. M., Wang, J., Mayle, K.M., Bartels, K., Salvatore, M., Kinsey, A.M., Demaria, A.N., Dib, N., Christman, K.L.: Catheter-deliverable hydrogel derived from decellularized ventricular extracellular matrix increases endogenous cardiomyocytes and preserves cardiac function post-myocardial infarction. J. Am. Coll. Cardiol. **59**, 751–763 (2012)
13. Khademhosseini, A., Langer, R., Borenstein, J., Vacanti, J.P.: Microscale technologies for tissue engineering and biology. Proc. Natl. Acad. Sci. U.S.A **103**, 2480–2487 (2006)
14. Rane, A.A., Chuang, J.S., Shah, A., Hu, D.P., Dalton, N.D., Gu, Y., Peterson, K.L., Omens, J.H., Christman, K.L.: Increased infarct wall thickness by a bio-inert material is insufficient to prevent negative left ventricular remodeling after myocardial infarction. PLoS ONE **6**, e21571 (2011)
15. Garbern, J.C., Minami, E., Stayton, P.S., Murry, C.E.: Delivery of basic fibroblast growth factor with a pH-responsive, injectable hydrogel to improve angiogenesis in infarcted myocardium. Biomaterials **32**, 2407–2416 (2011)
16. Lu, W.N., Lu, S.H., Wang, H.B., Li, D.X., Duan, C.M., Liu, Z.Q., Hao, T., He, W.J., Xu, B., Fu, Q., Song, Y.C., Xie, X.H., Wang, C.Y.: Functional improvement of infarcted heart by co-injection of embryonic stem cells with temperature-responsive chitosan hydrogel. Tissue Eng. Part A **15**, 1437–1447 (2009)
17. Huang, N.F., Yu, J.S., Sievers, R., Li, S., Lee, R.J.: Injectable biopolymers enhance angiogenesis after myocardial infarction. Tissue Eng. **11**, 1860–1866 (2005)
18. Davis, M.E., Motion, J.P., Narmoneva, D.A., Takahashi, T., Hakuno, D., Kamm, R.D., Zhang, S., Lee, R.T.: Injectable self-assembling peptide nanofibers create intramyocardial microenvironments for endothelial cells. Circulation **111**, 442–450 (2005)
19. Wang, J., Duncan, D., Shi, Z., Zhang, B.: WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. Nucleic Acids Res. **41**, W77–W83 (2013)
20. Dweep, H., Sticht, C., Pandey, P., Gretz, N.: miRWalk–database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J. Biomed. Inform. **44**, 839–847 (2011)
21. Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M., Sherlock, G.: Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. **25**, 25–29 (2000)
22. Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., Katayama, T., Kawashima, S., Okuda, S., Tokimatsu, T., Yamanishi, Y.: KEGG for linking genomes to life and the environment. Nucleic Acids Res. **36**, D480–D484 (2008)
23. Cerami, E.G., Gross, B.E., Demir, E., Rodchenkov, I., Babur, O., Anwar, N., Schultz, N., Bader, G.D., Sander, C.: Pathway Commons, a web resource for biological pathway data. Nucleic Acids Res. **39**, D685–D690 (2011)
24. Pico, A.R., Kelder, T., van Iersel, M.P., Hanspers, K., Conklin, B.R., Evelo, C.: WikiPathways: Pathway editing for the people. PLoS Biol. **6**, 1403–1407 (2008)
25. Griffiths-Jones, S., Saini, H.K., van Dongen, S., Enright, A.J.: miRBase: tools for microRNA genomics. Nucleic Acids Res. **36**, D154–D158 (2008)

26. Maragkakis, M., Reczko, M., Simossis, V.A., Alexiou, P., Papadopoulos, G.L., Dalamagas, T., Giannopoulos, G., Goumas, G., Koukis, E., Kourtis, K., Vergoulis, T., Koziris, N., Sellis, T., Tsanakas, P., Hatzigeorgiou, A.G.: DIANA-microT web server: elucidating microRNA functions through target prediction. Nucleic Acids Res. **37**, W273–W276 (2009)
27. Betel, D., Wilson, M., Gabow, A., Marks, D.S., Sander, C.: The microRNA.org resource: targets and expression. Nucleic Acids Res. **36**, D149–D153 (2008)
28. Wang, X.: miRDB: a microRNA target prediction and functional annotation database with a wiki interface. RNA **14**, 1012–1017 (2008)
29. Anders, G., Mackowiak, S.D., Jens, M., Maaskola, J., Kuntzagk, A., Rajewsky, N., Landthaler, M., Dieterich, C.: doRiNA: a database of RNA interactions in post-transcriptional regulation. Nucleic Acids Res. **40**, D180–D186 (2012)
30. Kertesz, M., Iovino, N., Unnerstall, U., Gaul, U., Segal, E.: The role of site accessibility in microRNA target recognition. Nat. Genet. **39**, 1278–1284 (2007)
31. Loher, P., Rigoutsos, I.: Interactive exploration of RNA22 microRNA target predictions. Bioinformatics **28**, 3322–3323 (2012)
32. Grimson, A., Farh, K.K.H., Johnston, W.K., Garrett-Engele, P., Lim, L.P., Bartel, D.P.: MicroRNA targeting specificity in mammals: Determinants beyond seed pairing. Mol. Cell **27**, 91–105 (2007)
33. Guo, Z., Wang, S., Jiao, Q., Xu, M., Gao, F.: RNAi targeting ryanodine receptor 2 protects rat cardiomyocytes from injury caused by simulated ischemia-reperfusion. Biomed. Pharmacotherapie **64**, 184–190 (2010)
34. Hoshijima, M., Ikeda, Y., Iwanaga, Y., Minamisawa, S., Date, M.O., Gu, Y., Iwatate, M., Li, M., Wang, L., Wilson, J.M., Wang, Y., Ross Jr., J., Chien, K.R.: Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via in vivo cardiac rAAV gene delivery. Nat. Med. **8**, 864–871 (2002)
35. Cox, L.A., Glenn, J.P., Spradling, K.D., Nijland, M.J., Garcia, R., Nathanielsz, P.W., Ford, S.P.: A genome resource to address mechanisms of developmental programming: determination of the fetal sheep heart transcriptome. J. Physiol-London **590**, 2873–2884 (2012)
36. Niessen, K., Karsan, A.: Notch signaling in cardiac development. Circ. Res. **102**, 1169–1181 (2008)
37. Granados-Riveron, J.T., Ghosh, T.K., Pope, M., Bu'Lock, F., Thornborough, C., Eason, J., Kirk, E.P., Fatkin, D., Feneley, M.P., Harvey, R.P., Armour, J.A., David Brook, J.: Alpha-cardiac myosin heavy chain (MYH6) mutations affecting myofibril formation are associated with congenital heart defects. Hum. Mol. Genet. **19**, 4007–4016 (2010)
38. Stoutamyer, A., Dhoot, G.K.: Transient expression of fast troponin C transcripts in embryonic quail heart. J. Muscle Res. Cell Motil. **26**, 237–245 (2005)
39. Sam, M., Shah, S., Friden, J., Milner, D.J., Capetanaki, Y., Lieber, R.L.: Desmin knockout muscles generate lower stress and are less vulnerable to injury compared with wild-type muscles. Am. J. Physiol-Cell Ph. **279**, C1116–C1122 (2000)
40. van Rooij, E., Sutherland, L.B., Thatcher, J.E., DiMaio, J.M., Naseem, R.H., Marshall, W.S., Hill, J.A., Olson, E.N.: Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc. Natl. Acad. Sci. U.S.A. **105**, 13027–13032 (2008)
41. Callis, T.E., Pandya, K., Seok, H.Y., Tang, R.H., Tatsuguchi, M., Huang, Z.P., Chen, J.F., Deng, Z.L., Gunn, B., Shumate, J., Willis, M.S., Selzman, C.H., Wang, D.Z.: MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. J. Clin. Invest. **119**, 2772–2786 (2009)