REVERSIN INCREASE THE PLASTICITY OF BONE MARROW-DERIVED MESENCHYMAL STEM CELL FOR GENERATION OF CARDIOMYOCYTE IN VITRO

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ABSTRAK

Efisiensi rendah pengobatan sel punca adalah masalah besar dalam pengobatan sel punca dalam Myocardial Infarction akut. Transdifferentiation dari sumsum tulang yang diturunkan sel batang mesenchymal menjadi kardiomiosit mengambil waktu lama dalam kultur jaringan eksperimental. Tujuan dari penelitian ini adalah untuk mempercepat transdifferentiation sumsum tulang yang berasal sel punca menjadi kardiomiosit in vitro dengan mengpuncasi dedifferentiation sumsum tulang yang berasal sel batang mesenchymal, sebelum puncasi dengan 9 μM5-aza-2'-deoxycytidine ke kardiomiosit. Dalam studi ini dua-tiga bulan 2,5 kg berat badan pria dewasa Selandia Baru Kelinci yang anethezied dengan eter, tulang paha yang dipotong, dan tulang sel-sel sumsum diperoleh dengan aspirasi. Dalam percobaan kami setelah 1 minggu kultur sel batang mesenchymal, 20 nM reversin diberikan untuk mengpuncasi dediferentiation dan setelah paparan 24 jam dengan 9 μM5-aza-2-deoxycytidine, fase awal diferensiasi kardiomiosit itu muncul sebagai kultur sel yang sangat positif untuk gata-4 dan lemah yang positif untuk MLC-2α, meskipun kardiomiosit pemukulan belum muncul pada akhir percobaan. Dalam percobaan ini juga menunjukkan CD34 + dan c ditandai-kit + ekspresi gen pada pemeriksaan RT-PCR. Sebagai kesimpulan, plastisitas meningkatkan reversine sumsum tulang yang berasal sel batang mesenchymal untuk menghasilkan kardiomiosit setelah 9 μM5-aza-2'-deoxycytidine puncasi. CD34 + dan c-kit + dapat menjadi penanda untuk sel-sel progenitor kardiomiosit

ABSTRACT

Low efficiency of stem cell treatment is a big problem in the treatment of stem cell in Acute Myocardial Infarction. Transdifferentiation of Bone marrow-derived mesenchymal stem cells into cardiomyocyte took long time in experimental tissue culture. The purpose of the study is to speed up transdifferentiation of bone marrow-derived stem cells into cardiomyocyte in vitro by inducing dedifferentiation of bone marrow-derived mesenchymal stem cell, before induction by 5-aza-2'-deoxycytidine into cardiomyocyte. In this study two-three months old 2.5 kg weight adult male New Zealand Rabbits were anethezied with ether, thigh bones were excised, and bone marrow cells were obtained by aspiration. In our experiments after 1 week of mesenchymal stem cell cultures, 20 nM reversin was given to induce dediferentiation and after 24 hours exposure with 9 μM 5-aza-2-deoxycytidine, early phase of cardiomyocyte differentiation was appeared as cultured cell were strongly positive for GATA-4 and weakly positive for MLC-2α, although beating cardiomyocyte has not yet been appeared at the end of experiment. In these experiment also showed a marked CD34+ and c-kit+ gene expression on RT-PCR examination. In conclusion, reversine increase plasticity of bone marrow-derived mesenchymal stem cell to generate cardiomyocyte after 5-aza-2'-deoxycytidine induction. CD34+ and c-kit+ may be a marker for cardiomyocyte progenitor cells

***Keywords**: bone marrow-derived mesenchymal stem cells, cardiomyocyte, reversine, plasticity, 5-aza-2'-deoxycytidine*

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INTRODUCTION

Cardiovascular diseases like myocardial infarction, and subsequent heart failure are a leading cause of morbidity and mortality. Despite the enormous advances in the understanding and treatment of heart failure that have taken place during the past years, this condition remains a serious, and in fact, a growing problem worldwide (Braunwald & Bristow 2000). The human heart has a limited capacity for self-repair or regeneration after myocardial infarction, the irreversible loss of muscle, and accompanying contraction and fibrosis of myocardial scar can lead to progressive ventricular remodeling of nonischaemic myocardium, and the ventricular remodelling can result in progressive ventricular dilatation and heart failure. Heart transplantation has been a therapy for these severe cases for several decades, however, with an ever increasing shortage of donor organs for heart transplantation, its usage is much limited. There is a significant need for

alternative therapies for cardiovascular diseases. Transplantation of stem cells to the injured heart can have a favourable impact on tissue regeneration and contractile performance of the infracted heart (Wu et al. 2006). Cell-based therapy is a promising option for treatment of ischemic diseases. Several cell types have experimentally been shown to increase the functional recovery of the heart after ischemia by physically forming new blood vessels, differentiating to cardiac myocytes and—additionally or alternatively—by providing proangiogenic and antiapoptotic factors promoting tissue repair in a paracrine manner (Dimmler et al. 2008).

In recent years, there has been a tremendous increase in the understanding of stem cell biology. There are two characteristics that distinguish stem cells from other types of cells. These are (1) stem cells can self-renew; and (2) they can differentiate into other types of cells. Stem cells are unspecialized cells that renew themselves for long periods through cell division, and given certain conditions, can be induced to become cells with special and unique functions (Rehman et al. 2003).

Success of stem cell treatment depend on several factors for examples the correct stem cell source, optimal stage of stem cell development (progenitor cell). In in acute myocardial infarction timing of injection and homing factor may be important factors. Usually very difficult to develop cardiomyocyte in vitro from bone marrow-derived mesenchymal stem cell.. First step was to induce dedifferentiation (imm,otalization) by several passaging for 4 months followed by inducing differentiation into cardiomyocyte with 24 hours exposute to 5-azacytidine.

Table 1. Primers used for cardiomyocyte marker and genetic expression.

	Sequence	Size in bp. of amplified cDNA product
C-KIT F	5'-GGCTCATAAATGGCATGCTC -3'	219 bp
C-KIT R	5'-CTTCCATTGTACTTCATACATG -3'	
CD34 F	5'-GACTATGGTCAACTTTACAGTA -3'	235 bp
CD34 R	5'-AGATGATGTGTAAGCATATGGC -3'	
$GATA_4$ F	5'-AAGACGCCAGCAGGTCCTGCTGG -3'	275 bp
$GATA_4$ R	5'-CGCGGTGATTATGTCCCCATGACT -3'	
MLC_{2a} F	5'-ATCTGCAAGGCAGACCTGA-3'	286 bp
MLC_{2a} R	5'-CAGGAGAAGCTGCTTGAAC-3'	

Beating cardiomyocyte was appeared after ythe next 3 weeks (Makino et al. 1999). Recently dedifferentiation could be employed by 48 hours exposure to a small molecule reversine that reverse lineage-committed murine myoblast to a more primitive multipotent state. When cultured under osteoblast-inducing condition or adipocye-inducing condition, reversin-treated cell increase differentiation of osteoblast compared to control (DMSO only) (20-50 % vs 0 %) and adipocyte (15-42 % vs 0 %) (Chen et al. 2007).

The purpose of this experiment we to induce dedifferentiation by 48 hours exposure to reversin before emplying differentiation with 5-deoxy-2'-azacytidine to generate cardiomyocyte in vitro. We hope generation of cardiomyocyte take shorter time and more efficiently compare to control (without reversine).

MATERIALS AND METHODS

Two-three months old 2.5 kg weight adult male New Zealanad Rabbits were anethezied with ether, thigh bones were excised, and bone marrow cells were obtained. The procedure were performed by in accordance with the guidence for animal experiment of Faculty of Veterinary Medicine Airlangga University. Isolation of mononuclear stem cells was performed on 3 ml bone marrow solution with density-gradient ultracentrifugation (1.077 ficoll histopaque at 15o C at 1600 rpm for 10 minutes). Buffy coat was isolated and wash in α medium MEM contain 20% FBS. Pellet resuspended and plated at 10(5) of 10 ml disposable petri disk containing complete growth medium, incubated at 37o C with 5 % CO2. After 90 % confluenced, floating cells were discarded and attached cells were tripsinized with 1 % EDTA and splitted into 4 petridisc 2 x 10(5) cell with complete growth medium containing 20 % FBS, penstrep and fungizone.

After three passaging and 90 % confluence, attached cells were tripsinized, suspended in 3 ml medium. After washing, 10(3) cells were plated at 24 wells microplate and culture with complete medium, incubated at 37o C and 5 % CO2 for 3 days. 20 nM reversin was added for each well to induce dedifferentiation for 24 hours. After washing, replace with new complete growth medium containing 9 μM 5-aza-2-deoxycytidine to induce difffrentiation toward cardiomyocytes. Cells incubated again for 24 hors and growth medium replace every 2 days until 21 days. Mesenchymal stem cells were observed by phase-contarst microscope before reversine, after reversine and serially after exposure to 5-aza-2-deoxycytidine serially until 21 days. Characterization of cardiomyocyte progenitor cells by RT-PCR/ Total RNA was extracted from cultured cells by Trizol Reagents (Invitrogen). Reverse transcriptase (RT)-PCR of stem cell marker for cardiomyocyte (CD34+ and c-kit+) and cardiomyocyte expresing gene (GATA-4 and MLC-2a) were performed using 1 μg of total RNA. Dnase I was applied.

RESULTS

Figure 1. Isolation of bone marrow-derived stem cells by density-gradient centrifugation with ficol histopaque at 1.077 density (left) and mixed stem cells (hemopoietic and mesenchymal stem cells) under phase-contrast microscope.

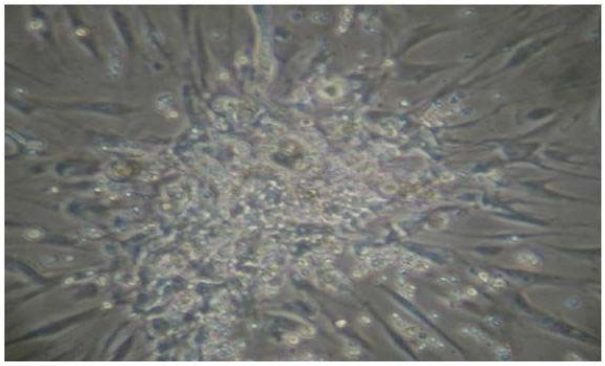

Figure 2. Culture of mesenchymal stem cell, 3 days after separation with hemopoietic stem cells.

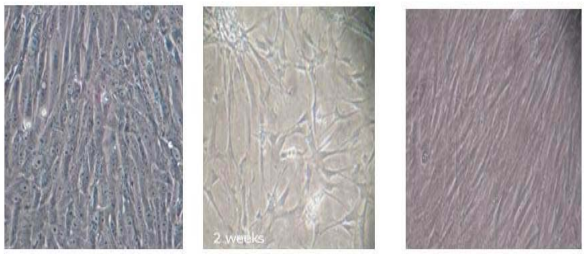

Figure 3. Mesenchymal stem cells after 3 passaging on complete grwth medium before induction of dedifferentiation with reversin. A. Rabbit bone marrow-derived mesenchymal cell. B. Human adipose tissue- derived mesenchymal stem cell. C. Human bone marrow-derived mesenchymal stem cell.

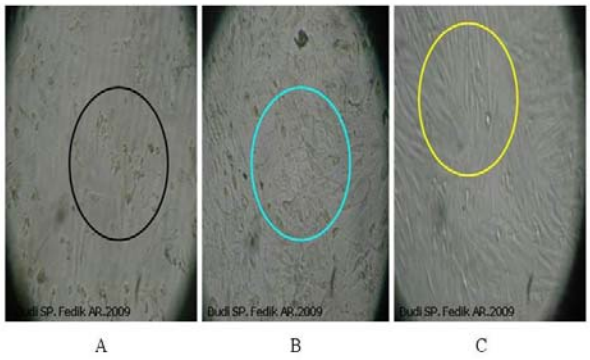

Figure 4. Rabbit bone marrow-derived Human bone marrow-derived Human Adipose tissue mesenchymal stem cell mesenchymal stem cell -derived mesenchymal stem cells

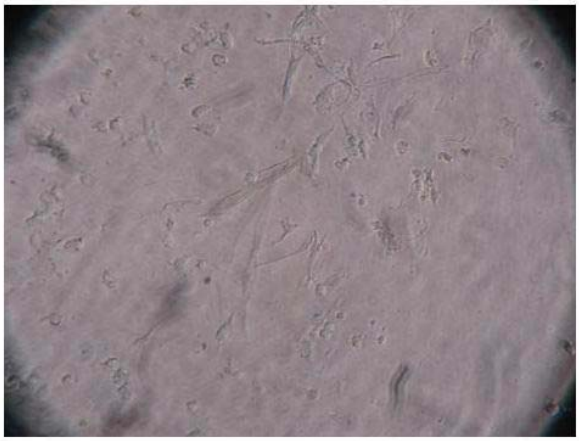

Figure 5A. Rabbit bone-marrow-derived mesenchymal stem cells.

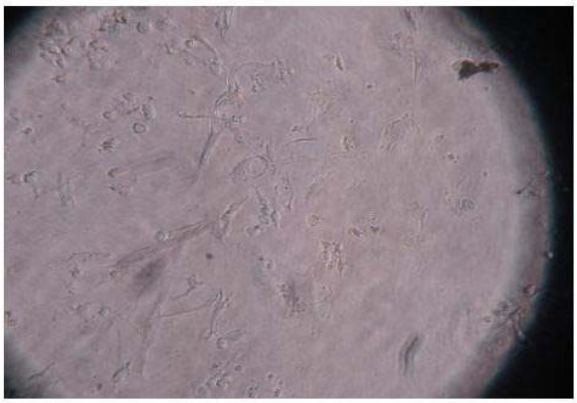

Figure 5B. Human bone marrow-derived mesenchymal stem cells.

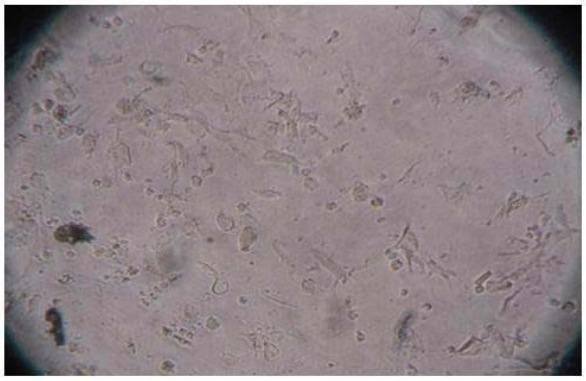

Figure 5C. Adipose tissue-derived mesenchymal stem cells.

Results of RT-PCR :

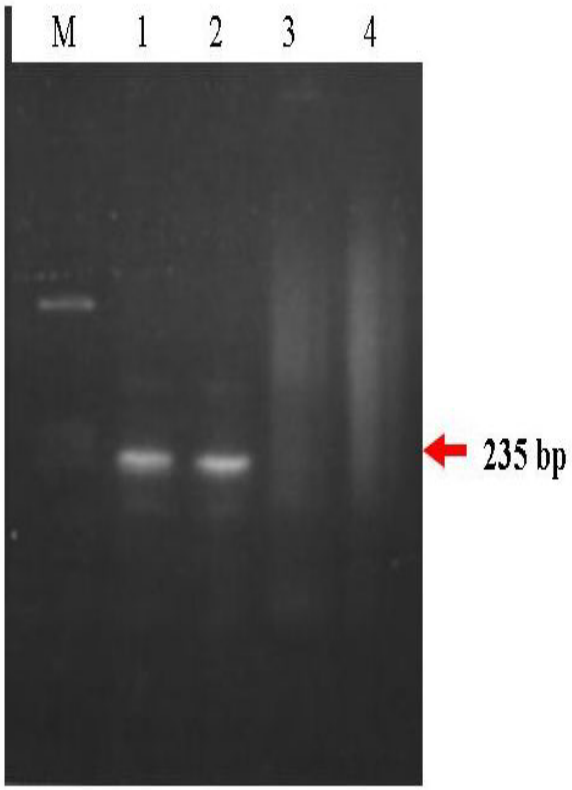

Figure 6. Analysis of RT-PCR product of specific receptor CD34+ by gel electrophoresis stained with ethium bromide.. M: Marker. Lane 1 and 2 : after reversine & 5-aza-2-deoxycytidine. Lane 3: after reversin only. Lane 4 : after 5-aza-2-deoxycytidine only.

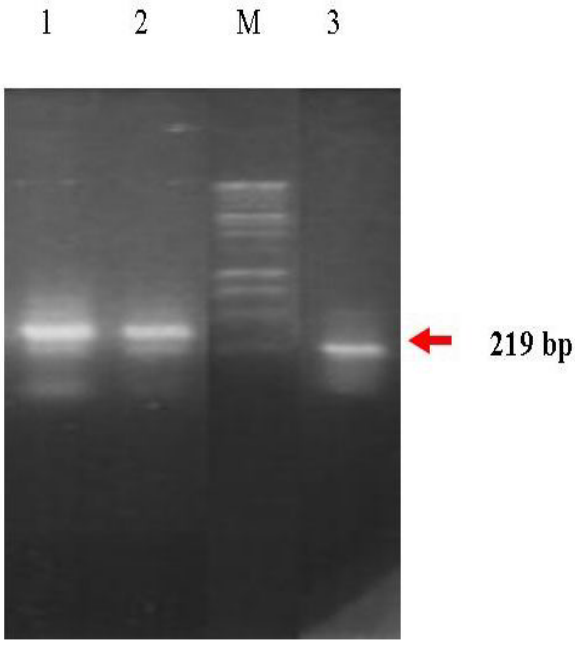

Figure 7. Analysis of RT-PCR products of specific receptor c-kit+ by gel elctrophoresis stained with ethium bromide.:Lane 1 and 2 : after reversine & 5-aza-2-deoxycytidine. M Marker. 3 : after 5-aza-2-deoxycytidine only.

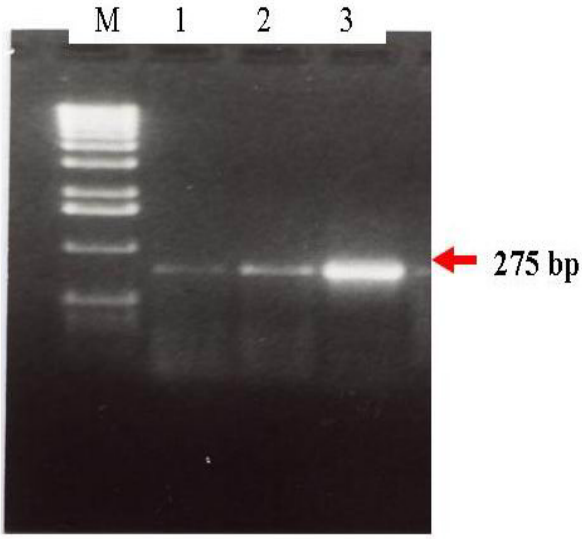

Figure 8. Analysis of RT-PCR products of cardiomyocyte genetic expression of GATA4 after 28 days of cultures by gel elctrophoresis stained with ethium bromide. Lane 1-3: cDNA spesifik GATA4, M: Marker.

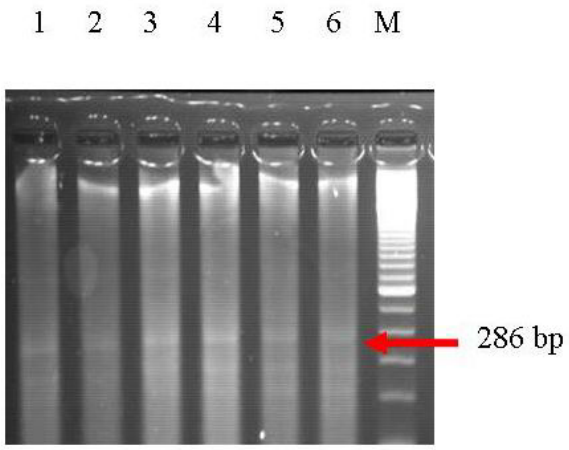

Figure 9. Analysis of RT-PCR product of cardiomyocyte genetic expression MLC-2a after 28 daysa of cultures by gel electrophoresis stained with ethium bromide.. Lane 1-6: cDNA spesifik MLC-2a, M: Marker.

DISCUSSION

Mesenchymal stem cell can be isolated from bone marrow, adipose tissue or other tissue (Wu et al. 2006, Dimmler et al. 2008). Progenitor stem cell can also isolated from peripheral blood after induction with G-CSF for 3 day by apheresis (Wu et al. 2006, Rehman et al. 2003). In this experiment we use rabbit bone marrow-derived mesenchymal stem cells. As a general rules of transdiferentiation, stem cell can be induced easily into several specific cell tissue by increasing potency of stem cell. Stem cells in early embryos give rise of all tissue of the adult body and are therefore termed pluripotent. Stem cells in some adult tissue, involved in tissue replacement and repair, usually give rise only to cell type already present in the surrounding tissue from which they are derived. Stem cells of the bone marrow, for example, give rise to hematopoietic cells. These adult stem cells are generally regarded as multipotent (Fukuda & Yuasa 2006). In some experiment these stem cells can also differentiate into other type cell as we called it transdifferentiation. Although all adults stem cell specifically mesenchymal stem cell or stromal cells are multipotent, in reality they have different multipotency. For example it is very difficult to induce transdifferentiation of bone marrow-derived mesenchymal stem cell into cardiomyoacyte in vitro. Makino et al (1999) showed that by inducing immortalization (dedifferentiation) of bone-marrow-derived mesenchymal stem cells by repeat passaging for 4 motnhs, stem cells be induced by 3 mol/L 5-azacytidine for 24 hours into beating cardiomyocyte within 2 weeks in vitro. In the contrary, fat tissue-derived mesenchymal stem cell can be easily differentiated into beating cardiomyocyte spontaneously within three week of culture although tne amount of cardiomyocyte was only 0.02-0.07 % of cultured cell culture (Planat-Benard et al. 2004).

Several methods have been employed to induce dedifferentiation, by the use of free cell extract embryo (Taranger et al. 2005, Rajasingh et al. 2008), genetic transduction (Takahashi et al. 2007, Zhang et al. 2009, Heng et al. 2004) and small molecule like reversin (Chen et al. 2007). A small molecule, reversine, was identified that reverses lineage-committed murine myoblasts to a more primitive multipotent state. Chen et al showed that reversine can increase the plasticity of C2C12 myoblasts at the single-cell level and that reversine treated cells gain the ability to differentiate into osteoblasts and adipocytes under lineage-specific inducing conditions. Moreover, reversine is active in multiple cell types, including 3T3E1 osteoblasts and human primary skeletal myoblasts (Chen et al. 2007).

In our trial after three times passaging, bone marrow-derived mesenchymal stem cells was treated with 20 nM reversine for 24 hours. to induced pluripotency. Cultured cell seem more homogen on phase contrast microscope. Induction of differentiation into cardiomyocyte was performed by exposaure to 9 μM 5-aza-2-deoxycytidine for 24 hours. After 21 days of induction, early phase of cardiomyocyte differentiation was appeared as cultured cell were positive for GATA-4 and weakly positive for MLC-2α, although beating cardiomyocyte has not yet been appeared. May be it need longer exposure to reversin for example 4 days or several induction with cardiomyocyte inducing agent (5-azacytidine or 5-aza-2-deoxycytidine to induce beating cardiomyocyte.

Administration of 20 nM reversin for 4 days before inducing osteogenesis and adipohgenesis, increase efficncy of differentiation from 3.4 % to 20-50 % and from 3.1 % to 15-42 % respectively (Chen et al. 2007). We did not perform quantitative measurement in our experiment. In previous experiment showed that the origin of cardiomyocyte were stem cell with CD34+ and c-kit+ (CD117+) markers (Limana et al. 2007, Ye et al. 2006, Fukuda & Yuasa 2006). Our experiment showed also positive for these markers.

CONCLUSION

Usually difficult to develop cardiomyocyte from bone marrow-derived mesenchymal stem cells in vitro. It need many passaging until 4 months to make bone marrow-derived mesenchymal stem cell become

immortal (dedifferentited). After inducing differentiation with 3 μM5-azacytidine for 24 hours, beating cardiomyocyte appeared after 3 weeks. In our experiments after 1 week of mesenchymal stem cell cultures, 20 nM reversin was given to induce dediferentiation and after 24 hours exposure with 9 μM 5-aza-2-deoxycytidine, early phase of cardiomyocyte differentiation was appeared as cultured cell were positive for GATA-4 and weakly positive for MLC-2α, although beating cardiomyocyte has not yet been appeared. In these experiment also showed that CD34+ and c-kit+ were marker for cardiomyocyte progenitor cells. May be it need longer exposure to reversin (for example 48 hours) or several time induction with 5-aza-2-deoxycytidine to develop beating cardiomyocyte.

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