

# Liver Cell Line Derived Conditioned Medium Enhances Myofibril Organization of Primary Rat Cardiomyocytes

Jinseok Kim<sup>1,2,3,7</sup>, Yu-Shik Hwang<sup>1,2,4,7</sup>, Alice Mira Chung<sup>1,2</sup>, Bong Geun Chung<sup>1,2,5</sup>, and Ali Khademhosseini<sup>1,2,4,6,\*</sup>

Cardiomyocytes are the fundamental cells of the heart and play an important role in engineering of tissue constructs for regenerative medicine and drug discovery. Therefore, the development of culture conditions that can be used to generate functional cardiomyocytes to form cardiac tissue may be of great interest. In this study, isolated neonatal rat cardiomyocytes were cultured with several culture conditions *in vitro* and characterized for cell proliferation, myofibril organization, and cardiac functionality by assessing cell morphology, immunocytochemical staining, and time-lapse confocal scanning microscopy. When cardiomyocytes were cultured in liver cell line derived conditioned medium without exogenous growth factors and cytokines, the cell proliferation increased, cell morphology was highly elongated, and subsequent myofibril organization was highly developed. These developed myofibril organization also showed high level of contractibility and synchronization, representing high functionality of cardiomyocytes. Interestingly, many of the known factors in hepatic conditioned medium, such as insulin-like growth factor II (IGFII), macrophage colony-stimulating factor (MCSF), leukemia inhibitory factor (LIF), did not show similar effects as the hepatic conditioned medium, suggesting the possibility of synergistic activity of the several soluble factors or the presence of unknown factors in hepatic conditioned medium. Finally, we demonstrated that our culture system could provide a potentially powerful tool for *in vitro* cardiac tissue organization and cardiac function study.

## INTRODUCTION

A major limitation in treating cardiac injury is the failure of current therapies to induce myocardium regeneration and the limited availability of donors. One possible avenue for remedying this situation is to optimize cardiomyocyte culture conditions to generate engineered cardiac tissues that can enhance cardiac function (Langer and Vacanti, 1993). The transplanted cardiomyocytes may be derived from various sources, such as adult stem cells as well as fetal or neonatal tissue. Although cardiomyocytes are pivotal elements of myocardial tissue structure and function, it has been considered that mammalian cardiomyocytes become terminally differentiated in the development of heart, after which the cells stop proliferating and only increase in cell size (Poss et al., 2002). However, it has been recently shown that neonatal cardiomyocytes still retain a certain amount of proliferation capability and a low level of proliferating populations of cardiomyocytes in post natal mammalian heart has been identified (Grounds et al., 2002; LaFramboise et al., 2007; Reinlib and Field, 2000; Soonpaa and Field, 1998). For cell-based therapy, primary cardiomyocytes that can proliferate may be required to repair damaged heart muscles (Grounds et al., 2002; LaFramboise et al., 2007). Thus, culture conditions that can enhance both proliferation and functionality of cardiomyocytes will be of great interest.

To date, many attempts have been made to develop culture conditions to expand and differentiate primary neonatal cardiomyocytes. Several factors, such as Insulin-like growth factor II (IGFII), macrophage colony-stimulating factor (MCSF), leukemia inhibitory factor (LIF), and cholesterol, have been identified to induce proliferation and myofibril organization of cardiomyocytes (Grounds et al., 2002; LaFramboise et al., 2007; Poss et al., 2002; Reinlib and Field, 2000; Soonpaa and Field, 1998; Vitello et al., 2004). In other approaches, conditioned medium

<sup>1</sup>Center for Biomedical Engineering, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA, 02139, USA, <sup>2</sup>Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA, 02139, USA, <sup>3</sup>Center for Bionics, Biomedical Research Institute, Korea Institute of Science and Technology, Seoul 136-791, Korea, <sup>4</sup>Department of Maxillofacial Biomedical Engineering and Institute of Oral Biology, School of Dentistry, Kyung Hee University, Seoul 130-701, Korea, <sup>5</sup>Department of Bionano Engineering, Hanyang University, Ansan 426-791, Korea, <sup>6</sup>Wyss Institute for Biologically Inspired Engineering at Harvard University, Boston, MA 02115, USA, <sup>7</sup>These authors contributed equally to this work.

\*Correspondence: [alik@rics.bwh.harvard.edu](mailto:alik@rics.bwh.harvard.edu)

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from different cell types has been applied to induce proliferation and myofibril organization. For instance, it has been reported that cardiac fibroblast or macrophage cell derived conditioned medium improved function of cardiomyocytes or myoblasts (LaFramboise et al., 2007; Vitello et al., 2004). Recently, human hepatocarcinoma cells (HepG2) have been used to induce cardiogenic differentiation of embryonic stem cells (ESCs), which reported that the number of beating colonies composed of mESC-derived cardiomyocyte distinctly increased after treatment with HepG2-conditioned medium. This may be due to a secretion pattern of soluble signaling factors that are partially similar to the signals from visceral endoderm, resulting in enhanced derivation of cardiomyocytes from embryonic stem cells (Mummery et al., 2002). However, to our knowledge, the effect of HepG2 cell line on primary isolated cardiomyocytes has not been studied.

In this paper, isolated neonatal rat cardiomyocytes were cultured with HepG2-conditioned medium (HepG2-CM) to evaluate the effect on primary isolated cardiomyocyte's proliferation and myofibril organization. The cell proliferation, myofibril organization, and cardiac functionality were also characterized by analyzing cell morphology obtained from immunocytochemical staining and time-lapse confocal scanning images.

## MATERIALS AND METHODS

### Isolation of neonatal rat cardiomyocytes and flow cytometric analysis

To isolate the cardiomyocytes, hearts were aseptically isolated from 1 day old neonatal Sprague-Dawley rats and washed with Hank's balanced salt solution (HBSS, Gibco, USA). After trimming the ventricles, the tissues were minced and incubated in a 0.3 mg/ml collagenase solution containing 0.6 mg/ml of pancreatin (Sigma, USA). The myocytes were dissociated in digestion buffer and collected in cold medium to inactivate digestion process. The isolated cells, which were a mixture of myocytes and non-myocytes, were suspended by plating in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) and plated onto culture dishes for 30 minutes to reduce contamination with cardiac fibroblasts. Isolated cells were separated on a discontinuous Percoll gradient (Top layer 1.070 g/ml and bottom layer 1.090 g/ml) by spinning at  $1,900 \times g$  for 30 min that banded the cardiomyocytes at the interface between the two layers and the fibroblast cells moved toward the top of the gradient. Cardiomyocytes were collected, washed, and plated at  $5.2 \times 10^4$  cells/cm<sup>2</sup> on 24-well tissue culture plates in normal cardiomyocyte medium consisting of high glucose-DMEM supplemented with 10% (v/v) fetal bovine serum (FBS: Gibco, USA) 100 units/ml penicillin and 100 µg/ml streptomycin, and 1 mM L-glutamine (Engelmann et al., 1990).

Dissociated cells were washed with phosphate-buffered saline (PBS: Gibco, USA) and fixed with 4% paraformaldehyde. The cells were permeabilized by using 0.4% Triton X-100 in PBS containing 4% bovine serum albumin (BSA: Sigma, USA) and incubated with the primary mouse monoclonal anti-β myosin heavy chain (β-MHC) antibody (Abcam, JPN) for overnight at 4°C. After washing with PBS, the cells were incubated with secondary FITC-conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology, USA) for 1 h at 37°C. Finally, the cells were washed in PBS and analyzed. Flow cytometric analysis was performed with Beckman Dickinson FACscan (Beckman Dickinson, USA). Negative control was prepared with normal mouse IgG<sub>1</sub> (Santa Cruz Biotechnology, USA) isotype controls and same FITC-conjugated secondary antibody.

**Table 1.** The list of medium used in this study

Medium	Component
G1. Cardiac fibroblast conditioned medium	Conditioned medium from Cardiac fibroblast culture for 4 days in Cardiac fibroblast medium
G2. HepG2 conditioned medium	Conditioned medium from HepG2 culture for 4 days in HepG2 medium
G3. 50% HepG2 conditioned medium	1:1 Mixture of cardiomyocyte medium and HepG2 conditioned medium

### Preparation of cardiac fibroblast and HepG2-conditioned medium

The conditioned media from HepG2 in this study were prepared from each separate culture prior to experiment. Although the biological mechanism (i.e. how hepatic cell line can influence cardiac mesoderm formation or cardiogenic differentiation) has not been well elucidated, several studies have used the conditioned medium from HepG2 cell line culture to enhance cardiogenic differentiation from stem cells (Hwang et al., 2006; Lake et al., 2000). In this study, to evaluate the effect of HepG2-CM on cardiomyocyte behavior, the HepG2 conditioned medium was used according to previous research protocol (Hwang et al., 2006; Lake et al., 2000). Briefly,  $5 \times 10^4$  cells/cm<sup>2</sup> of HepG2 cells (ATCC HB-8605, USA) were seeded and cultured in T75 tissue-culture flasks in a 5% (v/v) CO<sub>2</sub> humidified incubator at 37°C by using high glucose-DMEM supplemented with 10% (v/v) FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1 mM L-glutamine. The conditioned medium was collected after 4 days of culture, sterilized with a 0.22 µm filter, and stored at 4°C prior to use.

### Experimental design

In this experiment, the effect of HepG2-CM on proliferation and myofibril organization of isolated rat cardiomyocyte was evaluated. The culture conditions are as follows: (G1) cardiomyocytes were cultured on tissue culture plates in 50/50 HepG2-CM and normal cardiomyocyte culture medium, (G2) cardiomyocytes were cultured on tissue culture plates in 100% HepG2-CM, (G3) cardiomyocytes were culture on tissue culture plates in normal cardiomyocyte medium. All media used in this study are listed in Table 1. The experiment was repeated three times independently. The other test groups were summarized in the section of supplemental materials and methods.

### Immunocytochemical characterization

Cells were fixed with 4% paraformaldehyde (Sigma, USA) for 10 min, washed three times with PBS, followed by permeabilization with 0.1% triton X-100 (Sigma, USA), and blocking of non-specific binding by incubation with 10% (w/v) normal goat serum (Invitrogen, USA) in PBS. The primary antibodies, anti-sarcomeric β-actinin (Sigma, USA), anti tropomyosin (Sigma, USA), anti-connexin43 (Ab cam, JPN), and anti prolyl 4-hydroxylase (Ab cam, JPN) were diluted as 1:80 with 4% BSA solution, and were incubated at 4°C overnight. Following a wash with PBS, the secondary Alexa Fluor 546 conjugated antibody (Invitrogen, USA) and the secondary FITC-conjugated antibodies (Santa Cruz Biotechnology, USA) were incubated for 1 h at room temperature. The cells were counterstained with DAPI (Vecta Shield: Fisher Scientific, USA) to visualize the cell nucleus and immuno-stained cells were observed by using Nikon Eclipse TE2000-U epifluorescence microscope (MVi, USA).

### Time-lapse confocal laser scanning microscopic observation of calcium ion fluctuation

[Ca<sup>2+</sup>] was monitored in single cardiac cells by using the fluorescent dye fluo-4 AM (Molecular Probes, USA). The 10 μM fluo-4 AM was loaded into the cells for 15 min with DMEM containing dimethyl sulfoxide (0.1%) and 0.025% pluronic F-127 (Molecular Probes, USA). The coverslip was rinsed with the standard extracellular solution and transferred to the well. Fluo-4 fluorescence was monitored by an inverted confocal laser scanning microscope (CLSM 510, Zeiss, Germany) with a 100× objective numerical aperture (0.80, Neofluar, Zeiss, Germany). For fluorescence excitation, the 488-nm band of an Argon laser was used and emission was recorded by using a long-pass LP 515 filter set. Contractions of cardiomyocytes were monitored by changing light diffraction in the transmission image.

### Quantitative analysis to compare morphology of cell culture

Computer-assisted image analysis was used to compare the morphology of cardiomyocytes cultured in normal cardiomyocyte culture medium or HepG2 conditioned medium for 5 days. A mixture of cardiac fibroblasts and cardiomyocytes were plated at  $5.2 \times 10^4$  cells/cm<sup>2</sup> on 24-well tissue culture plates. Immunocytochemical staining of cardiomyocytes was conducted as described in section 2.4. Red channel was split from the original fluorescent images to separate the fluorescence images of the cellular sarcomeres. Using conventional image processing program (Corel Photo-Paint, Version 13.0.0.667, Corel Corporation, USA), edge detect filter was applied to the images. Aligned cardiomyocyte cluster was identified by the sarcomere alignment from filtered images. A group of cells that did not show significant alignment was identified as one cluster. Morphological characteristic and the degree of elongation of the identified clusters were quantified by fitting ellipse to the clusters using an Image J program (Abramoff et al., 2004). The cluster degree of elongation was indicated by the aspect ratio of the major and the minor axes of their fitted ellipses. Statistical significance was evaluated by performing a Student's paired *t*-test with two-tailed distribution.

### Cardiac fibroblast and cardiomyocyte proliferation assay

For cell proliferation studies of cardiac fibroblast, cardiac fibroblasts were seeded onto the plates at the concentration of  $1 \times 10^4$  cells/cm<sup>2</sup> and were cultured for 1, 4, and 7 days in normal cardiomyocyte culture medium or 50% HepG2 conditioned medium at 37°C in a 5% CO<sub>2</sub> humidified incubator. Unattached cells were removed by washing with PBS. Cell proliferation was evaluated by quantifying the metabolically active proliferating cells by measuring the level of the endogenous mitochondrial dehydrogenase by addition of 200 μl/well of DMEM without phenol red (Gibco) and 40 μl/well of mitochondrial dehydrogenase substrate (Promega, USA). After incubation for 2 hours, mitochondrial dehydrogenase activity was stopped by addition of 50 μl/well 10% (w/v) Sodium Dodecyl Sulfate (SDS; Sigma, USA) in PBS. The plates were read at a 490 nm wavelength using a microplate reader (MRX II, Dynex Technology, USA). Statistical significance was evaluated by performing a Student's paired *t*-test with two-tailed distribution (*n* = 6).

For cell proliferation studies of cardiomyocyte, cardiomyocytes were seeded onto the plates at the concentration of  $5 \times 10^5$  cells/cm<sup>2</sup> and were cultured for 1, 3, 5 and 7 days in normal cardiomyocyte culture medium or 50% HepG2 conditioned medium at 37°C in a 5% CO<sub>2</sub> humidified incubator. Unattached cells were removed by washing with PBS. Cell proliferation was

evaluated by BrdU incorporation assay (Merck, USA) according to manufacturer's instruction. Briefly, the cells were incubated in BrdU containing medium for 24 h, were fixed with the Fixative/Denaturing solution for 30 mins, and reacted with 1:100-diluted mouse anti-BrdU antibody (Calbiochem, USA) for 1 h at room temperature. After washing with wash buffer, the cells were reacted with Peroxidase Goat Anti-Mouse IgG for 30 min at room temperature. After washing with wash buffer, the cells were incubated in substrate solution in the dark at room temperature for 15 min, and then the plates were read at dual wavelengths of 450-540 nm using a microplate reader (MRX II, Dynex Technology, USA). Statistical significance was evaluated by performing a Student's paired *t*-test with two-tailed distribution (*n* = 6).

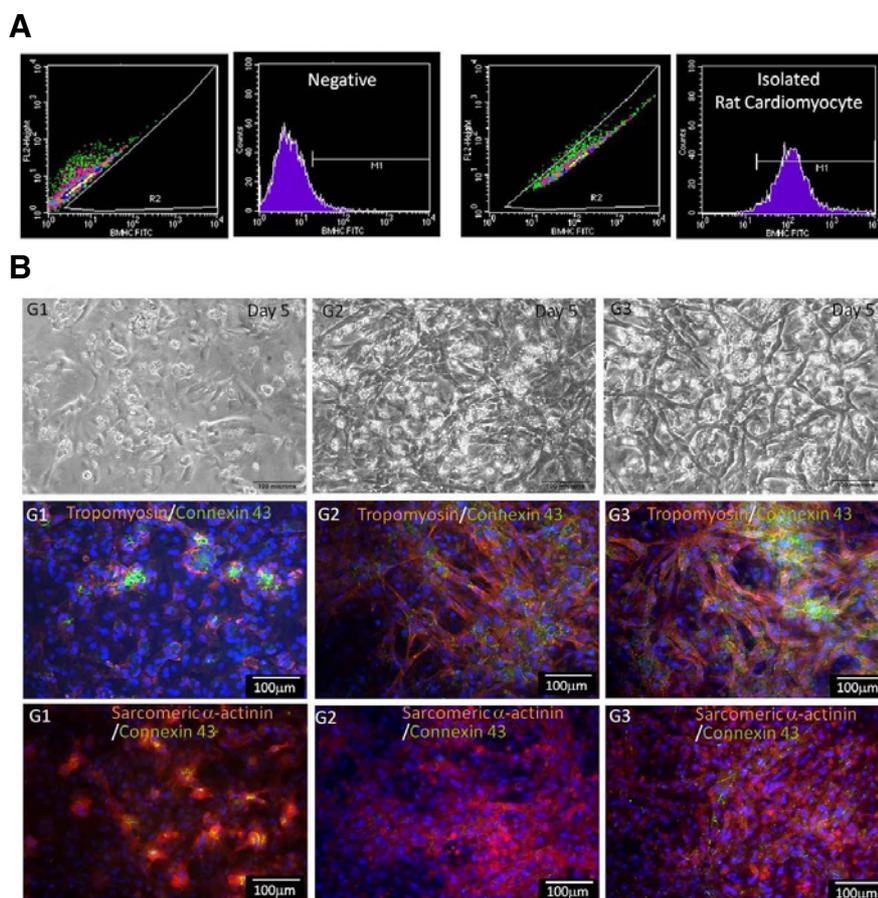
### The effect of individual soluble factors on myofibril organization

LIF (Chemicon, USA), MCSF (Sigma, USA), IGF II (Peprotech, USA), Follistatin (Peprotech, USA) and Oncostatin M (Peprotech, USA) were selected as potent candidates to enhance proliferation and myofibril organization. Several concentrations of each factor (1, 10, and 100 ng/ml) were tested to evaluate the effect of individual growth factors on cardiomyocyte morphology and myofibril organization as compared to cardiomyocyte culture in normal cardiomyocyte medium and HepG2-CM.

## RESULTS

### The conditioned medium from HepG2 cell line induced myofibril organization of primary isolated neonatal rat cardiomyocytes

We isolated neonatal rat cardiomyocytes purified from rat hearts after collagenase treatment and Percoll gradient centrifugation. After isolation, the purity of isolated rat cardiomyocyte was characterized by flow cytometric analysis. The β-MHC positive population showed 95% positive cell population (Fig. 1A), and left histogram in Fig. 1A represented the isotype control, normal mouse IgG<sub>1</sub>. The isolated cardiomyocytes in various culture conditions and analyzed the resulting cell morphology by immunocytochemical staining. When cardiomyocytes cultured on tissue culture plates in normal cardiomyocyte medium (DMEM containing 10% FBS and L-Glutamine), we found irregular shaped cell colonies composed of cardiomyocytes (Fig. 1B, G1). In contrast, when cardiomyocytes were cultured in 50% and 100% HepG2-CM without any exogenous growth factors and cytokines, highly elongated cell morphology was visible in the entire culture and subsequently organized myofibrils were detected (Fig. 1B, G2 and G3). Such morphological change of cardiomyocyte in HepG2-CM and cell to cell interactions were also characterized by immunocytochemical staining of cardiac actin, myosin, and connexin 43. We observed that tropomyosin and sarcomeric β-actinin was regionally expressed around cell colonies in normal cardiomyocyte medium (Fig. 1B, G1). In comparison, cardiomyocytes expressed tropomyosin and sarcomeric β-actinin along the organized myofibril in both 50% and 100% HepG2 conditioned medium (Fig. 1B, G2 and G3). We also found that connexin 43 expression was restricted around cardiomyocyte colonies in normal cardiomyocyte medium (Fig. 1B, G1), however, in cultures containing the HepG2 conditioned medium it was expressed along the organized myofibrils (Fig. 1B, G2 and G3).



**Fig. 1.** Morphological and immunocytochemical characterization of primary neonatal rat cardiomyocytes in various culture conditions: (A) FACS analysis for  $\beta$ -MHC positive cardiomyocyte population after purification by Percoll gradient centrifuge. (B) Morphology of cardiomyocytes cultured in various culture conditions after 5 days of culture and immunocytochemical characterization of cardiomyocyte cultures with cardiomyocyte specific antibodies against tropomyosin and sarcomeric  $\alpha$ -actinin and gap junction antibody against connexin 43. G1: Cardiomyocyte cultured in normal cardiomyocyte medium. G2: Cardiomyocyte cultured in 50% conditioned medium collected from HepG2 cell line culture. G3: Cardiomyocyte cultured in 100% conditioned medium collected from HepG2 cell line culture. Scale bars are 100  $\mu$ m.

### The conditioned medium from HepG2 cell line did not influence the interaction between cardiac fibroblasts and cardiomyocytes through gap junctions and cardiac fibroblast proliferation

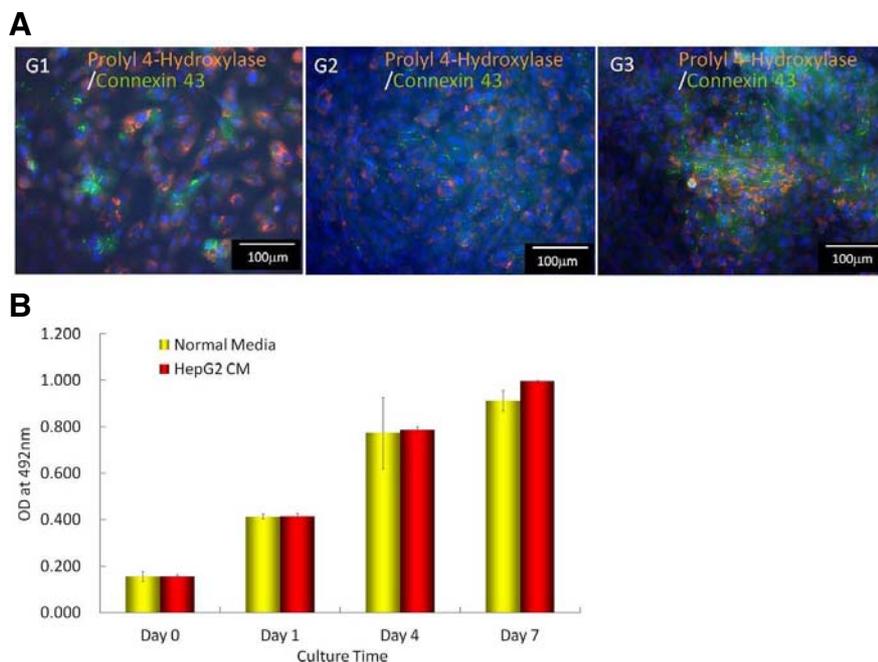
Despite the purification processes in our cardiac isolation protocols, fibroblasts still remained in the enriched cultures. Since it is known that cardiac fibroblasts interact with cardiomyocytes to synchronize contraction through gap junction molecules (Oyamada et al., 1994), the mode of contact between cardiac fibroblasts and cardiomyocytes was characterized by immunocytochemical staining with prolyl 4-hydroxylase (Bai et al., 1986), a marker of fibroblast, and connexin 43, a marker of gap junction between cardiomyocytes, antibodies. As shown in Fig. 2A, the positive reaction with prolyl 4-hydroxylase antibody was detected in areas around cardiomyocytes. The gap junction proteins (connexin 43) were also expressed in cardiomyocytes cultured in normal cardiomyocyte medium and HepG2-CM. The connexin 43 expression was developed along with the elongated cardiomyocytes cultured in HepG2-CM. In a parallel study, the proliferation of cardiac fibroblasts was evaluated in normal cardiomyocyte medium and HepG2-CM. As shown in Fig. 2B, we did not observe significant differences in the proliferation of cardiac fibroblasts between the cultures in normal medium and in HepG2-CM, which indicated that HepG2-CM did not influence the proliferation of cardiac fibroblast.

It has been well known that cardiac fibroblast influences cardiomyocyte morphology and cell physiology *via* direct contact interaction (Oyamada et al., 1994) and the release of paracrine

factors (LaFramboise et al., 2007). In order to compare the effect of HepG2-CM with cardiac fibroblast-based culture conditions, other culture conditions were evaluated. When cardiomyocytes were cultured on a monolayer of pre-plated live or fixed cardiac fibroblasts in normal cardiomyocyte culture medium and also on tissue culture plate in cardiac fibroblast-conditioned medium, cardiomyocytes showed irregular shaped cell colonies (Supplementary Data 1). In addition, we observed that tropomyosin and sarcomeric  $\beta$ -actinin was regionally expressed around cell colonies in culture containing monolayers of pre-plated cardiac fibroblasts (G4), fixed cardiac fibroblasts (G5) or cardiac fibroblast-conditioned medium (G6). Interestingly, larger cardiomyocyte colonies were found in cultures containing pre-plated cardiac fibroblasts, while the colonies in cardiac fibroblast conditioned medium showed relatively round morphology. In addition, connexin 43 were also restructly expressed around cardiomyocyte colonies in other cardiomyocyte culture conditions; culture on monolayers of pre-plated cardiac fibroblasts (G4) and culture in cardiac fibroblast-conditioned medium (G6). However, only a relatively few connexin 43 expression was detected in cultures on a monolayer of fixed fibroblasts (G5) in comparison with other groups (Supplementary Data 1C).

### Cardiac cells in HepG2-conditioned medium were more elongated and spontaneously contracted

During the culture, contracting (beating) cardiomyocytes were easily found in all culture groups. These spontaneous contrac-



**Fig. 2.** The characterization of the distribution of cardiac fibroblasts and cardiomyocytes, and the proliferation of cardiac fibroblasts in different culture conditions (A) Immunocytochemical staining of cardiomyocytes and cardiac fibroblasts in various culture conditions after 5 days of culture with fibroblast specific antibody against prolyl 4-hydroxylase and gap junction antibody against connexin 43. Scale bars are 100  $\mu$ m. (B) Proliferation of cardiac fibroblasts in normal cardiomyocyte medium and 100% HepG2 conditioned medium. Error bars are standard deviation (Student's paired *t*-test with two-tailed distribution, *n* = 6).

tions became increasingly synchronized between each cardiomyocytes within 5 days of culture in HepG2-CM. As shown in Figs. 3A and 3B, cardiomyocyte morphology was found to be highly elongated in HepG2-CM and rounded in normal cardiomyocyte medium. In addition, gap junction protein (connexin 43), which is known to play a role of synchronizing contractions between cardiomyocytes was found to be expressed between elongated, cardiomyocytes expressing highly developed sarcomeric  $\alpha\beta$ -actinin, which was not found in cardiomyocytes cultured in normal cardiomyocyte medium. To further evaluate the contraction activity of cardiomyocytes in HepG2-CM, the contraction activity was characterized by observing the cell contraction-mediated calcium ion flux under time-lapse confocal scanning microscope. Calcium ion flux was detected by using a fluorescent calcium indicator, Flu-4 AM. The bright red intensity indicates the distinct increase of calcium concentration within cardiomyocytes during contraction. Time-lapse confocal images (Figs. 3C and 3D) showed time dependent increase of calcium concentration within each cardiomyocyte in both normal cardiomyocyte medium and HepG2-CM, but the pattern in synchronization of contractions was found to be different between cardiomyocytes in HepG2-CM and cardiomyocytes in normal cardiomyocyte medium. In normal cardiomyocyte medium, contraction-mediated calcium fluctuation, signal change from green to red, was only observed within a cardiomyocyte, and synchronization of beating was hardly found in neighbouring cardiomyocytes (Fig. 3C). In contrast, such contraction-mediated calcium fluctuation was detectable within all cardiomyocytes with well developed myofibrils in HepG2-CM, and showed synchronization between neighbouring cardiomyocytes, which also correlates to the expression of connexin 43 by elongated cardiomyocytes (Fig. 3B).

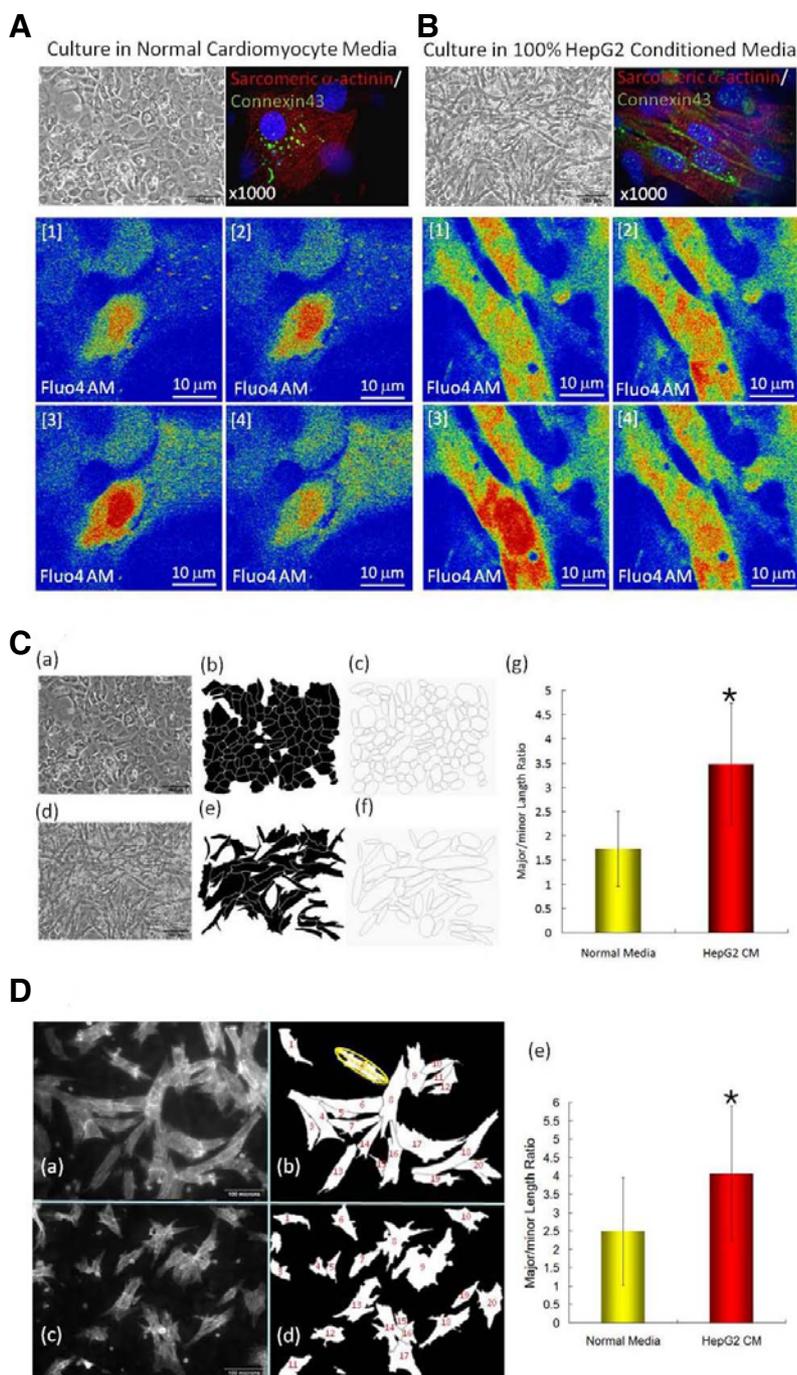
To quantitatively analyze the morphology of the cardiomyocytes cultured in HepG2-CM and normal cardiomyocyte medium, we measured the ratio of longitudinal and latitudinal axial lengths of each cluster using images under light and fluorescence microscopy after immunocytochemical staining. When

cardiomyocytes form myofibrils, they show a more elongated shape and a more striated morphology. Ellipse fitting method enables the analysis of the morphological difference in a quantitative manner. As shown in Figs. 3C(a)-3C(f) and Figs. 3D(a)-3D(d), outline trace and ellipse fitting were performed to analyze individual aggregated cardiomyocyte clusters. The longitudinal direction of a fitted ellipse was defined as a major axis and the latitudinal direction as a minor axis. The ratio of major axis to minor axis was used as a quantitative standard of morphological information of the aggregated cardiomyocyte cluster (Bashur et al., 2006). A larger aspect ratio indicates a more elongated shape of the cells. As shown in Fig. 3C(g), the aspect ratio of the cardiomyocytes had the average values of 1.73 and 3.47 when they were cultured in normal medium and HepG2-CM ( $p < 0.01$ , *n* = 113 and 45 respectively). In the quantitative analysis of cell morphology after immunocytochemical staining, the aspect ratio was 2.49 and 4.06, when they were cultured in normal medium and HepG2-CM ( $p < 0.01$ , *n* = 68 and 168 respectively) [Fig. 3D(e)]. This quantitative analysis indicates that cardiomyocytes in the HepG2-CM were more stretched and rod-like in their morphology.

#### Myofibril organization was dependent on cardiomyocyte density in HepG2-conditioned medium

After one day of seeding, a higher number of cardiomyocytes with elongated morphology was observed in HepG2-CM. Hence, to evaluate the effect of HepG2-CM on cardiomyocyte proliferation capacity with morphological change, the proliferation of cardiomyocytes that were cultured in normal cardiomyocyte medium and HepG2-CM was evaluated. As shown in Figs. 4A and 4B, higher proliferation activity of cardiomyocytes was observed in cells that were cultured in HepG2 conditioned medium throughout whole culture period for 7 days ( $p < 0.05$ , *n* = 6).

To assess the effect of cell density on myofibril organization, morphological and immunocytochemical evaluation of the myofibril organization in different cardiomyocyte densities in normal cardiomyocyte medium and 100% HepG2-CM were performed.



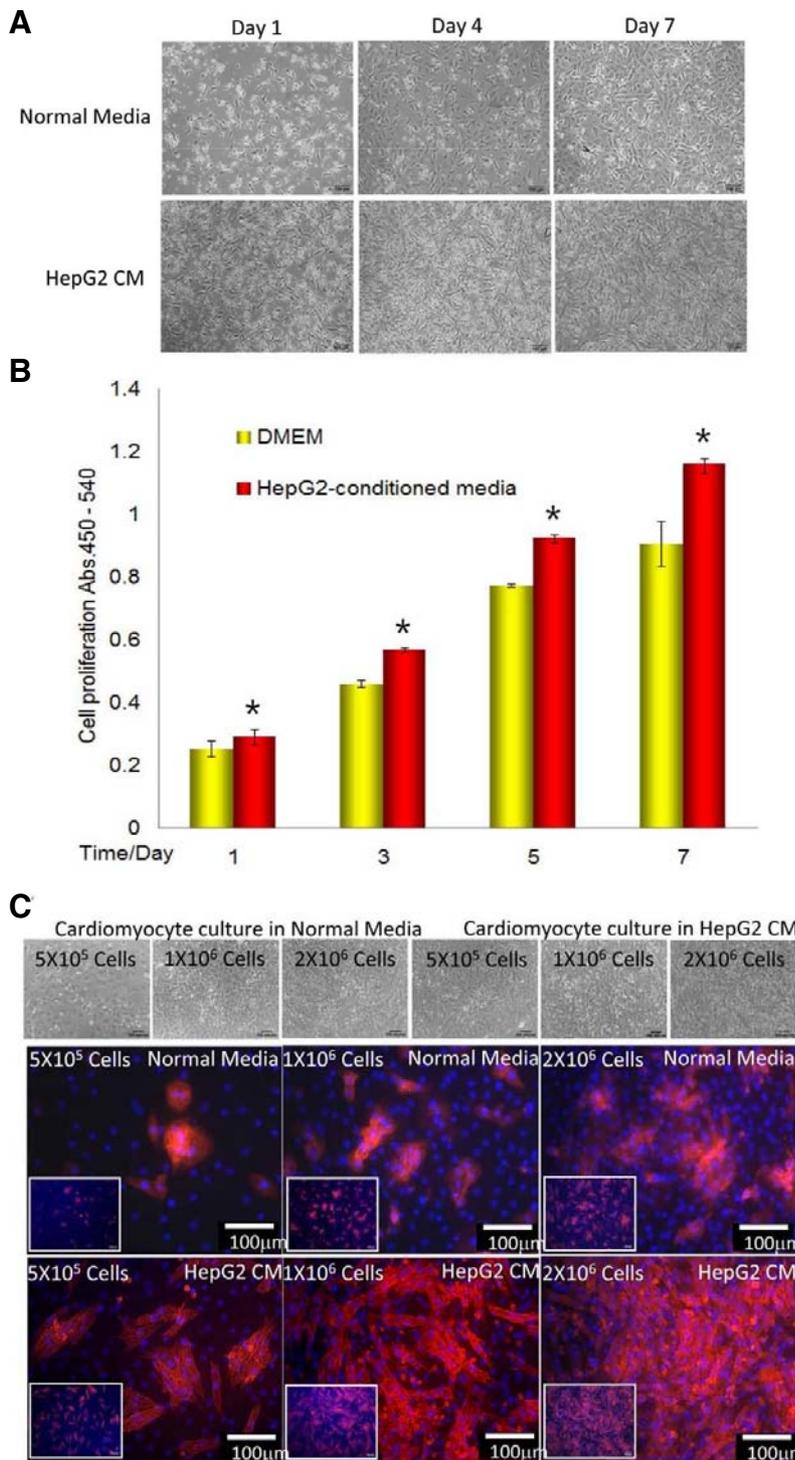
**Fig. 3.** The confocal scanning microscope images of cardiomyocyte beating mediated by calcium fluctuation using calcium indicator (Fluo-4 AM): (A) Time-lapsed images of calcium fluctuation in cardiomyocyte culture with normal cardiomyocyte medium. (B) Time-lapsed images of calcium fluctuation in cardiomyocyte culture with 100% HepG2 conditioned medium. Each confocal image ([1-4]) was captured at interval of 400 ms. (C) Light microscope images and quantitative analysis to compare morphology of cardiomyocytes in different culture conditions: (a) and (d) are light microscope images of cardiomyocytes cultured with normal cardiomyocyte medium and 100% HepG2 conditioned medium, respectively. (b), (c), (e) and (f) are images after the outline trace. Each cluster was identified and processed through ellipse fitting. (g) Aspect ratio of cardiomyocyte aggregate clusters cultured with normal cardiomyocyte medium and HepG2 conditioned medium (\* represents  $p < 0.01$ , Student's paired  $t$ -test with two-tailed distribution). (D) Quantitative analysis to compare immunostained cell morphology after immunocytochemical characterization: (a) and (c) are the microscopic images of cardiomyocytes cultured with 100% HepG2 conditioned medium and normal cardiomyocyte medium, respectively. (b) and (d) are the images after the outline trace. Each cluster was identified and processed through ellipse fitting. (e) Aspect ratio of cardiomyocyte aggregate clusters cultured with normal cardiomyocyte medium and HepG2 conditioned medium (\* represents  $p < 0.01$ , Student's paired  $t$ -test with two-tailed distribution).

As shown in Fig. 4B, cardiomyocytes formed subsequently organized myofibrils with relatively higher activity in accordance with higher cell densities when cultured in HepG2-CM. However, we did not observe significant difference in myofibril organization activity of cardiomyocytes between low and high cell density when cultured in normal cardiomyocyte medium.

**Myofibril organization was independent of FBS concentration and individual growth factors in HepG2-conditioned medium**

*In vitro* studies have shown various factors to enhance prolifera-

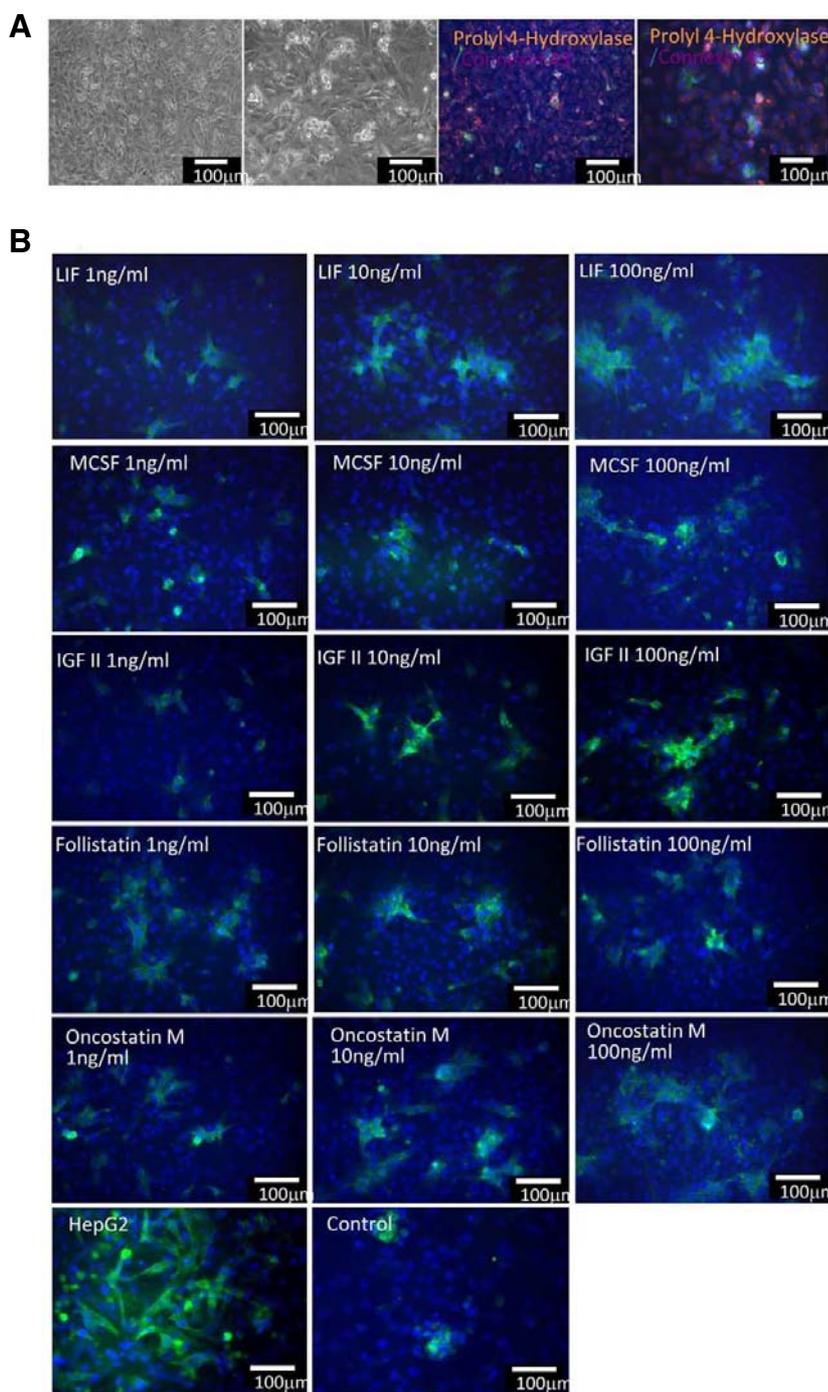
tion and myofibril organization from myoblasts and cardiac myocytes (Husmann et al., 1996; Okazaki et al., 2007; Pisconti et al., 2006; White et al., 2001). Cardiomyocytes showed highly developed myofibril organization as well as they enhanced proliferation. Given that the reduction of FBS concentration in HepG2-CM can influence myofibril organization of cardiomyocytes, cardiomyocytes were cultured in the culture condition of 50% reduced FBS. However, in these cultures we did not observe distinct subsequent myofibril organization in both normal culture medium (10% FBS) and culture medium with 5% FBS (Fig. 5A).



**Fig. 4.** Cardiomyocyte proliferation in different culture conditions and the morphological and immunocytochemical characterization of cardiomyocytes with different initial cell seeding densities: (A) Time course images of cardiomyocytes in normal cardiomyocyte medium and 100% HepG2 conditioned medium. Scale bars are 100  $\mu\text{m}$ . (B) Proliferation of cardiomyocyte in normal cardiomyocyte medium and 100% HepG2 conditioned medium. Error bars are standard deviation. (\*represents  $p < 0.05$ , Student's paired  $t$ -test with two-tailed distribution,  $n = 6$ ) (C) Morphological and immunocytochemical evaluation for myofibril organization with different cardiomyocyte densities in normal cardiomyocyte medium and 100% HepG2 conditioned medium. Cells were stained by sarcomeric  $\alpha$ -actinin. Each inset of fluorescent images indicates low magnification. Scale bars are 100  $\mu\text{m}$ .

From the information about soluble factors secreted from HepG2 cells, we selected several soluble factors as potent candidates to enhance proliferation and myofibril organization. We analyzed the effect of individual soluble factors with different concentrations (1, 10, and 100 ng/ml) on cardiomyocytes. As shown in Fig. 5B, cardiomyocytes showed distinct myofibril organization under different culture conditions supple-

mented with individual LIF, MCSF, IGF II, Follistatin, and Oncostatin M. The effect of each soluble factor on myofibril organization strongly appeared at the concentration of 100 ng/ml, especially in 100 ng/ml IGFII supplemented culture condition. However, in none of the conditions we observed well developed myofibril organization as compared to HepG2-CM.



**Fig. 5.** The effect of soluble factors on cardiomyocyte morphology and cardio-myofibril organization: (A) The effect of reduced FBS concentration on myofibril organization. Cells were cultured with 5% FBS. Scale bars are 100 μm. (B) The effect of LIF, IGF II, MCSF, follistatin, and oncostatin M on myofibril organization. Cells were stained by sarcomeric α-actinin. Scale bars are 100 μm.

## DISCUSSION

Deciphering the molecular cues that regulate the formation of the heart could be of benefit for repairing damaged heart muscles using various cell sources, such as primary cardiomyocytes, myoblasts, embryonic stem cells, and progenitor cells. For cardiac tissue engineering, the culture conditions that can enhance both proliferation and myofibril organization need to be optimized. Cardiomyocytes have become a powerful cell type

for investigating cardiac development and pathology, as well as facilitating screening of potential interventions (LaFramboise et al., 2007; Mummery et al., 2002; Reinlib and Field, 2000; Soonpaa and Field, 1998). To date, several approaches have been performed for studying the effects of various soluble factors and conditioned medium on cardiomyocyte proliferation and maturation. It has been well known that cardiac fibroblast influences cardiomyocyte morphology and cell physiology via direct contact interaction to synchronize contractions between

cardiomyocytes (Oyamada et al., 1994). In addition, LaFramboise et al. reported that various cytokines (vascular endothelial growth factor(VEGF), growth related oncogene (GRO/KC), monocyte chemoattractant protein-1, leptin, macrophage inflammatory protein-1 $\alpha$ , interleukin(IL)-6, IL-10, IL-12p70, IL-17, and tumor necrosis factor(TNF)- $\alpha$ , transforming growth factor(TGF)- $\beta$  and regulated upon activation, normal T-cell expressed and secreted (RANTES) were elevated in cardiac fibroblast-conditioned medium, and that only a factor, granulocyte macrophage colony-stimulating factor, decreased in comparison with fresh normal medium. Under the influence of fibroblast-conditioned medium, cardiomyocytes exhibited marked hypertrophy and diminished contractile capacity (LaFramboise et al., 2007). On the other hand, based on the biological role of endoderm on mesoderm formation and subsequent cardiogenesis during gastrulation process, endoderm like cell line (i.e. HepG2 cell line) has been used to induce cardiogenic differentiation *via* co-culture or the use of conditioned medium in the study of cardiogenic differentiation of embryonic stem cells (Mummery et al., 2002). However, the underlying mechanisms have not been elucidated. In this study, primary rat cardiomyocyte was cultured as a model for the study of the effect of HepG2-CM on the proliferation and myofibril organization.

In this study, the effect of HepG2-CM on primary isolated cardiomyocyte's proliferation and myofibril organization was evaluated majorly in comparison with normal cardiomyocyte culture medium, and also other culture conditions were evaluated in order to compare the effect of HepG2-CM with cardiac fibroblast-based culture conditions. From the results, we found that the HepG2-CM significantly enhanced the activity of subsequent myofibril organization and the cardiomyocyte proliferation as compared to other culture conditions. In addition, it was shown that cardiac fibroblasts did not influence the proliferation and myofibril organization, but the synchronization of contraction of neighboring cardiomyocytes was dependent on living cardiac fibroblasts, from the finding that there were many beating foci with different frequency in the culture of cardiomyocytes on the fixed cardiac fibroblast layer. Interestingly, the HepG2-CM selectively enhanced cardiomyocyte proliferation without significantly increasing cardiac fibroblast proliferation. Furthermore, the well organized myofibrils in HepG2-CM showed contracting activity, which was confirmed by time-lapse confocal scanning microscopic observation of calcium ion fluctuation. The myofibrils forming cardiomyocytes could interact with surrounding cardiac fibroblasts via gap junction, connexin 43.

Previous studies have shown the capacity of HepG2 cells to express multiple cytokines. It is known that HepG2 cells express mRNAs for interferon (IFN)-gamma, TNF-alpha, TGF-beta, macrophage colony-stimulating factor (M-CSF), oncostatin M (OSM), intercellular adhesion molecules (ICAM)-1 (CD54), IL4, IL5, IL7, IL10, IL11, IL12 and IL6 receptor (Mezzasoma et al., 1993; Nishimura and Naito, 2005; Oppmann et al., 1996; Se-menkova et al., 1997; Stonans et al., 1999). In addition, it has been reported that HepG2 cells are constitutive producers of LIF. In our study, from the finding of the enhanced proliferation and myofibril organization in HepG2-CM, several candidates (i.e. IGF II, M-CSF, leukemia inhibitory factor(LIF), oncostatin M and follistatin) were selected as based on literature review about soluble factors secreted from HepG2 cell line (Mezzasoma et al., 1993; Nishimura and Naito, 2005; Oppmann et al., 1996; Semenkova et al., 1997; Stonans et al., 1999). It has been reported that IGF II increased cell proliferation and induced the activation of myogenic transcription factors and myofibril organization (Husmann et al., 1996). It has also

been reported that LIF enhanced myoblast proliferation and increased the number and size of myofibrils (White et al., 2001). MCSF protected cardiomyocytes and myofibrils from cell death, and improved cardiac function by increasing VEGF production from cardiomyocytes (Okazaki et al., 2007). In addition, follistatin has been shown to regulate myofibril organization in response to deacetylase inhibitors (Pisconti et al., 2006). However, although each soluble factor showed concentration dependent induction of myofibril organization, this process was not as pronounced as compared to cardiomyocyte culture in HepG2-CM. These results suggest that there might be unknown factors or complex interactions between soluble factors in HepG2-CM. Although many biological studies have determined individual molecules that are important for cardiac cell function *in vitro*, it is becoming increasingly accepted that the wide array of signals in the cardiac microenvironment that can interact in a synergistic and antagonistic manner is strongly dependent on their temporal and spatial expression, dosage, and specific combinations. However, further studies about soluble factors secreted from HepG2 cells are required. Therefore, further study will be done to search for candidate molecules using cytokine array with hepatic conditioned medium and to identify the key factors *via* various approach such as immunodepletion or gene silencing study about each candidate molecule. Finally, our study suggest the use of high-throughput screening experiments for determining optimum mixture of soluble factors and for developing optimal culture medium containing recombinant growth factor reconstitution that can be used to enhance cardiomyocyte proliferation and myofibril organization.

## CONCLUSIONS

In this paper, we analyzed the response of primary rat cardiomyocytes in response to HepG2-CM as a function of and myofibril organization, proliferation, and cardiac function. We demonstrated the enhanced proliferation and and myofibril organization of cardiomyocytes cultured with HepG2-CM as compared to normal culture. We also found that cell morphology was highly elongated and subsequent myofibril organization was highly developed when cardiomyocytes were cultured with HepG2-CM. Furthermore, these subsequent organized myofibrils showed higher contractibility and functionality of cardiomyocytes. Our results suggest the possibility of synergistic activity of the several soluble factors or the presence of unknown factors in hepatic conditioned medium. Therefore, our culture system can provide a potentially powerful tool for *in vitro* cardiac tissue organization and cardiac function study.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

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