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Cardiomyogenic differentiation potential of human lipoaspirate-derived stem cells on hyaluronic acid/gelatin plasma gels

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Abstract: Cardiomyogenic differentiation from mesenchymal stem cells has emerged as a novel approach for repair of damaged myocardium. Cell transplantation through direct cell injection is not an optimal method due to the lack of cell–extracellular matrix interactions. In the present study, differentiation potential of human adipose-derived stem cells (ASCs) to cardiomyocytes has been investigated by growing them on hyaluronic acid/gelatin (HA/G) plasma gels and coverslips and supplementing the growth medium with chemical modifiers (activin-a, BMP-4, insulin, valproic acid, and 5-azacytidine) in various combinations. The HA/G plasma gels were produced from human blood plasma-derived fibrinogen, gelatin, and human umbilical cord-derived hyaluronic acid. A network-based approach was employed to select marker genes for cardiomyogenic differentiation, and the expression levels of three markers (*GATA4*, *TBX5*, and *cTnI*) were followed by RT-qPCR to investigate the cardiomyogenic differentiation potential of ASCs. Results indicated that each combination of chemical modifiers led to different expression levels in the aforementioned cardiac markers, and this was material-dependent, too. The cardiac gene expression on HA/G plasma gels in the presence of activin-a + BMP-4 or insulin + valproic acid was more pronounced than in the presence of 5-azacytidine only, and scaffold and chemical modifier combinations were crucial for cardiomyogenic differentiation.

Key words: Cardiomyogenic differentiation, stem cells, human adipose-derived stem cells, HA/G plasma gel, biomaterials, tissue engineering

1. Introduction

Heart failure, which is characterized by a gradual loss of cardiomyocytes, and ischemia, which is characterized by reduced blood supply to the heart, are major cardiovascular health problems worldwide (Segers and Lee, 2008). The increase in morbidity and mortality rates related to cardiomyocyte loss leads to enhancement of cellular therapies since donor hearts for transplantation are in shortage, too. Previous studies on cell replacement therapies have achieved muscle regeneration and increased cardiomyocyte numbers in parallel with increased functionality by preventing hypertrophy at deficient tissues (Mummery et al., 2010).

Diverse types of stem and progenitor cells have been shown to improve cardiac function through various mechanisms, including formation of new endothelial cells, vascular smooth muscle cells, and myocytes, as well as through paracrine effects (Vunjak-Novakovic et al., 2009). In addition, different types of cells such as skeletal myoblasts, bone marrow-derived progenitor cells, embryonic or neonatal cardiomyocytes, adult cardiac progenitor cell populations, induced pluripotent stem cells, reprogrammed somatic cells, and embryonic stem cells (ESCs) have been investigated as candidates or models for regenerating damaged cardiomyocytes (Vunjak-Novakovic et al., 2009).

Adipose is an abundant tissue with a high proportion of adult stem cells, and these cells can be easily obtained from the lipoaspirate material. It has been reported that adipose-derived stem cells (ASCs) have the ability to differentiate along various mesenchymal lineages in the presence of induction factors (Zuk et al., 2002). ASCs have also been shown to be capable of in vitro differentiation into cardiomyocytes (Rangappa et al., 2003; Planat-Benard et al., 2004; Palpant and Metzger, 2010). Transplantation of adipose-derived cardiomyocytes onto an infarcted mouse heart contributed to cardiac repair (Bai et al., 2010). In cell transplantation, the major advantage of employing adipose-

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derived cardiomyocytes is the accessibility of the patients' own adipose by liposuction for autologous transplantation (Palpant and Metzger, 2010). Treatment with temporal activin-a and BMP-4 has led to an efficient cardiomyogenic differentiation from human ESCs (Kattman et al., 2006; Laflamme et al., 2007). In addition, in vitro treatment of adult bone marrow cells with 5-azacytidine led to the expression of cardiomyogenic genes and protein (Makino et al., 1999). Several studies have reported successful differentiation of rabbit or mouse ASCs, but not human ASCs, into contractile cardiomyocyte-like cells (Rangappa et al., 2003; Strem et al., 2005; Lee et al., 2009). In cardiac tissue, cardiomyocytes are surrounded by a basement membrane containing collagen type IV, fibronectin, laminin, and proteoglycans, and the extracellular space consists mainly of interstitial collagen types I and III, with many other macromolecules (Vanwinkle et al., 1996). In terms of niche, these extracellular matrix (ECM) components are essential in heart development and in mechanoelectrical function (Parker and Ingber, 2007). The high water content of hydrogels creates a tissue-like three-dimensional environment (Tibbitt and Anseth, 2009) and therefore hydrogels are used extensively to mimic ECM.

Hyaluronic acid (HA) is a nonsulfated glycosaminoglycan and is distributed throughout the ECM of all connective tissues in humans and other mammals (Yoo et al., 2005). Due to its high biocompatibility and low immunogenicity, HA is gaining popularity as a biomaterial for tissue engineering and tissue regeneration (Zheng et al., 2004; Leach and Schmidt, 2005). Furthermore, HA is of particular interest due to its ability to promote cell migration (Yoo et al., 2005).

For the purpose of cell delivery, fibrin glue is an extensively studied hydrogel system that is formed by mixing fibrinogen and thrombin via similar mechanisms involved in normal blood clotting (Christman et al., 2004; Martens et al., 2009). The major advantage of fibrin gels over the other gels is that it can be prepared in an autologous manner. Adipose-derived mesenchymal stem cells (MSCs) delivered in fibrin gel to treat myocardial infarction led to increase in arteriole densities and thus to improved heart function (Zhang et al., 2010).

In the present study, differentiation potential of human ASCs to cardiomyocytes has been investigated by growing them on HA/gelatin (HA/G) plasma gels and coverslips. The HA/G plasma gels were produced from fibrinogen precipitated from human blood plasma, gelatin, and human umbilical cord-derived HA and were crosslinked with glutaraldehyde. ASCs were isolated from human lipoaspirates and characterized with flow cytometry at their third passage, and also by differentiation into osteogenic and adipogenic lineages. The effect of chemical modifiers, including activin-a, BMP-4, insulin, valproic acid, and 5-azacytidine, were also studied by addition into to the growth medium in various combinations. A network-based approach was employed to select marker genes for cardiomyogenic differentiation. To investigate the cardiomyogenic differentiation potential of ASCs, the gene expression levels of three marker genes were examined after 2 and 3 weeks of incubation.

2. Materials and methods

2.1. ASC isolation, expansion, and characterization

ASC isolation from subcutaneous fat of the abdominal region was performed according to our previous study (Korurer et al., 2014). The procedures were performed in accordance with the ethical guidelines of the Kocaeli University Medical Ethics Committee. Briefly, adipose tissue was obtained by lipoaspiration material from female donors; fatty portions were collected with pipette and digested with 0.1% (w/v) collagenase (GIBCO). Cell suspension was centrifuged and washed with phosphate-buffered saline (GIBCO). The pellet was resuspended in basal medium (DMEM/F12; GIBCO) containing 10% fetal bovine serum (FBS; Invitrogen/GIBCO), 1% L-glutamine, 1 ng/mL bFGF (GIBCO), and 0.1% primocin (InvivoGen) and then filtered through a strainer (BD Biosciences). The isolated ASCs were plated in a 25-cm^2 tissue culture flask (BD Biosciences) and were cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

The ASCs were characterized with flow cytometry at their third passage, and also were differentiated into osteogenic and adipogenic lineages. Flow cytometry was performed using FACS Calibur (BD Biosciences). The data were analyzed with Cell Quest software (BD Biosciences), and the forward and side scatter profiles were gated out of debris and dead cells. The surface antigens (CD13, CD29, CD44, CD90, CD146, CD166, HLA ABC, CD3, CD8, CD11b, CD14, CD15, CD19, CD33, CD34, CD45, CD117, and HLA-DR) of the cells were characterized. The osteogenic and adipogenic differentiation was performed as described in our previous studies (Karaoz et al., 2010; Korurer et al., 2014). For in vitro osteogenic differentiation of ASCs, the osteogenic medium composed of minimum essential media (MEM; Invitrogen/GIBCO) supplemented with 10 nM dexamethasone (Sigma-Aldrich), 50 μg/mL ascorbate-2-phosphate (Wako Chemicals), 10 mM β-glycerophosphate (Sigma-Aldrich), 0.1% primocin (InvivoGen), and 10% FBS (Invitrogen/GIBCO) was used. At the end of the second week, osteogenic differentiation was assessed by staining with alizarin red (Sigma-Aldrich). To induce adipogenic differentiation of ASCs, MEM supplemented with 10% FBS (Invitrogen/GIBCO), 0.5 mM isobutyl-methyl xanthine (IBMX, Sigma-Aldrich), 10^{-6} M dexamethasone (Sigma-Aldrich), 10 μg/mL insulin (Invitrogen/GIBCO), 200 μM indomethacin (Sigma-

Aldrich), and 0.1% primocin (InvivoGen) was added to the cells during 2 weeks. Intracellular lipid vesicles indicating adipogenic differentiation were confirmed by Oil Red O (Sigma-Aldrich) staining.

2.2. Preparation and characterization of HA/G plasma gels

HA/G plasma gels were prepared and characterized using the previously described protocol (Korurer et al., 2014). Briefly, umbilical cord-derived HA and G were crosslinked using ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride/N-hydroxyl succinimide (EDC/NHS). The final HA:G ratio in the hydrogel was set to 2:98 (w/w). HA/G plasma gel was formed by mixing 120 µL of concentrated human fibrinogen (480 mg/mL) precipitated from human blood plasma, 30 µL of crosslinked HA/G solution, and 30 µL of 40 mM $CaCl_2$ solution in each well of a 48-well plate. Later, 50 µL of thrombin (40 U/mL) was added to each well and mixed quickly. The mixture was stored at 37 °C for 2 h for polymerization and was crosslinked with glutaraldehyde.

The characterization studies consisted of scanning electron microscopy (SEM) imaging, compression tests, porosity and pore size determination of gels, and cell viability assay on the gels (Korurer et al., 2014). The morphologies of HA/G plasma gels were observed with SEM (QUANTA 400F Field Emission). Compression test of the gels (9.2 mm in diameter) was conducted with an LR 30 K mechanical tester (Lloyd Instruments). The gels were stained with Coomassie brilliant blue (Merck) to determine their porosity and pore size through microscopy. Gel porosity and pore sizes were determined from these images taken under a light microscope by using Scion Image software. The viability, adhesion, and proliferation rate of ASCs on HA/G plasma gels were determined by tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, and monosodium salt test (WST-1; Roche) for 1, 5, 7, 14, and 21 days (Korurer et al., 2014).

2.3. Cardiomyogenic differentiation of ASCs on the gels

The cardiomyogenic differentiation potential of ASCs on HA/G plasma gels and coverslips was investigated by using three different differentiation media (InsuM, Bmp4M, and 5AzaM) and a control medium (ContM) (Table 1). ASCs were seeded at a density of 7.5×10^3 cells/gel on the HA/G plasma gels and 7.5×10^3 cell/cm^2 on the coverslips in 48-well plates. When the cells on the coverslips reached 70% confluence, chemical modifiers were added to the ASCs on the gels and coverslips. The media were replaced twice a week. At the end of the 2nd and 3rd weeks, cell lysis was performed by applying cell lysis buffer (Roche RNA Isolation Kit) to the cells in each group. Lysed cells were stored at −80 °C.

2.4. Selection of marker genes for cardiomyogenic differentiation

A network-based approach was developed to select appropriate marker genes associated with cardiomyogenic differentiation. Candidate genes were obtained from a comprehensive literature survey. Protein–protein interaction subnetworks around proteins encoded by candidate genes were reconstructed using data from a previous study (Karagoz et al., 2015) and visualized via Cytoscape (Smoot et al., 2011). The DAVID Functional Annotation Bioinformatics Microarray Analysis tool (Alvord et al., 2007) was employed to map the granular annotations of genes and corresponding proteins represented in the subnetworks. The significantly enriched Gene Ontology (GO) terms and KEGG pathways were determined by hypergeometric test, and 0.05 was selected as the P-value threshold. Genes with significantly enriched GO terms associated with cardiomyogenic differentiation were selected as marker genes and their expression profiles were followed.

2.5. Gene expression analysis via RT-qPCR

Total RNA was extracted from lysed cells using the High Pure RNA Isolation Kit (Roche). cDNA was synthesized using a cDNA synthesis kit (Fermentas). Real-time PCR was performed under a 2 × 4 × 2 experimental design with a total of 16 different samples (Table 2) in order to comparatively analyze the effects of support material (i.e. HA/G plasma gel or coverslip), the media containing chemical modifiers (InsuM, Bmp4M, 5AzaM, ContM) and culture time (14 or 21 days). Cardiac gene expression was examined through mRNA expression profiles of selected markers genes, i.e. *GATA4*, *T-box5* (*TBX5*), and cardiac troponin I type 3 (*cTnI*). Primers of these genes

Table 1. Compositions of differentiation and control media employed in the present study.

Medium	Content
InsuM	100 µg/mL insulin (Sigma), 2 mM valproic acid, 10% FBS, 0.1% primocin, L-DMEM
Bmp4M	Activin-a for 24 h and then 10 ng/mL BMP-4, 2 mM valproic acid, 10% FBS, 0.1% primocin, L-DMEM
5AzaM	10 µg/mL 5-azacytidine (Sigma), 10% FBS, 0.1% primocin, L-DMEM for 24 h
ContM	10% FBS, 0.1% primocin, L-DMEM

Table 2. Experimental design for real-time PCR studies.

Sample ID	Support material	Medium	Culture time (days)
1	HA/G plasma gel	InsuM	14
2	Coverslip	InsuM	14
3	HA/G plasma gel	Bmp4M	14
4	Coverslip	Bmp4M	14
5	HA/G plasma gel	5AzaM	14
6	Coverslip	5AzaM	14
7	HA/G plasma gel	ContM	14
8	Coverslip	ContM	14
9	HA/G plasma gel	InsuM	21
10	Coverslip	InsuM	21
11	HA/G plasma gel	Bmp4M	21
12	Coverslip	Bmp4M	21
13	HA/G plasma gel	5AzaM	21
14	Coverslip	5AzaM	21
15	HA/G plasma gel	ContM	21
16	Coverslip	ContM	21

were designed via Roche Probe Finder software (Table 3). Primers were diluted with pure water to obtain 20 mM primer concentration. PCR was performed using optimized amplification conditions, namely 0.4 µL of reference and target primers, 0.4 µL of reference and target probe, 3 µL of pure water, 10 µL of master mix, and 5 µL of cDNA per single well in a 96-well plate. As housekeeping genes, *10-GUSB*, *17-GUSB*, and *81-GUSB* were used for *cTnI*, *GATA4*, and *TBX5*, respectively. The real-time PCR assay was performed using the Dual Color Hydrolysis Probe with a Roche Light Cycler 450II device. Quantification of the results was done using the $2^{-\Delta\Delta CT}$ technique (Livak and Schmittgen, 2001), which compares the CT values of the reference and target genes. All real-time PCR experiments were performed in triplicate (3 independent experiments) and three trials for each sample were performed (a total of 48 well plates for each experiment).

2.6. Statistical analysis

All experiments were performed in triplicate. Data were reported as 95% confidence intervals for sample means (i.e. µ ± 95% CI). In addition, the statistical significance was analyzed using one-way analysis of variance (ANOVA)

Table 3. Primers for real-time PCR.

Primer		Length (bp)	Position	Tm (°C)	GC (%)	Sequence
cTnI (NM_000363.4)	Left	21	672–692	59	52	aagaaggaggacaccgagaag
	Right	20	718–737	60	50	cagtgcatcgatgttcttgc
GATA4 (NM_002052.3)	Left	20	1513–1532	60	50	ggaagcccaagaacctgaat
	Right	19	1592–1610	59	53	gttgctggagttgctggaa
TBX5 (NM_000192.3)	Left	22	609–630	59	50	gaagaggtgggatagttggaga
	Right	18	718–735	60	56	ggcaggtcttttgcgtca

test followed by post-Tukey test. The statistical significance of same experimental groups depending on the time using paired sample t-test was applied. Differences between the experimental and control groups were regarded as statistically significant when $P < 0.05$. All statistical analyses were performed using SPSS 14.0. Statistical analyses are presented in Supplementary Tables 1 and 2 (on the journal's website).

3. Results

3.1. Isolation and characterization of human ASCs

The ASCs, which were isolated from human lipoaspirate material, were attached to the flask surface and displayed a fibroblast-like spindle-shaped morphology during their early days of incubation (Figures 1A and 1B). For further characterization, these primary cells were cultured until 70% confluence, subcultured, and cryopreserved. Differentiation of ASCs into osteogenic (Figures 1C and 1D) and adipogenic lineages (Figures 1E and 1F) were confirmed by immunohistochemical and immunofluorescence staining. The flow cytometry results indicated undifferentiated MSC surface antigen profile (Figure 2).

3.2. Characterization of HA/G plasma gels

Cardiomyogenic differentiation potential of stem cells may depend on cell niche and the components of the ECM play a pivotal role in the differentiation process. The HA/G plasma gels produced in the present study were 9.2 mm in diameter, compact, and easy to handle (Figure 3A). The gels were stained with Coomassie brilliant blue to determine their porosity and pore sizes, and the transverse lengths of the pores were measured to get an idea about the pore sizes of the scaffolds (Figure 3B). The porosity and average pore diameter of the HA/G plasma gels were calculated as 71.05 ± 5.39% and 132 ± 19.07 μm, respectively. Elastic modules of HA/G plasma gels were calculated as 3.53 ± 0.22 kPa. SEM was used to observe the porous and fibrous microstructure, surface topography, and pore size distribution of the scaffolds (Figure 3C) and the morphology of unseeded (Figure 3D) and ASC-seeded HA/G plasma gels after 21 days (Figure 3E). ASCs metabolized the gel as there was a substantial increase in pore size on the top portion of the cell seeded gels. ASC proliferation was determined as a function of time on coverslips (as a control) and HA/G plasma gels (Figure 3F). Viability, adhesion, and proliferation of cells on these gels were investigated with respect to time; the amount of cells on coverslips was much more than that on the gels on the first day. However, cell numbers on the gels increased later in culture ($P < 0.05$).

3.3. Selection of marker genes for cardiomyogenic differentiation

A network-based approach was employed to select appropriate marker genes to follow cardiomyogenic differentiation. Candidate genes, which were commonly employed in gene expression analysis to follow

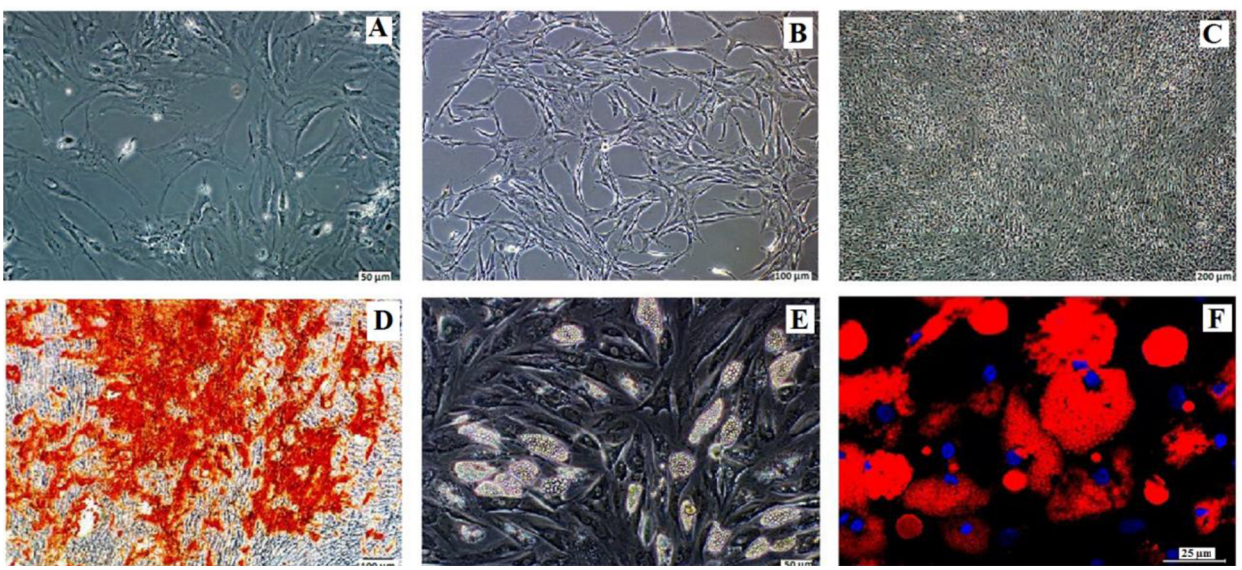

Figure 1. Characterization data of ASCs. Morphology of ASCs phase-contrast microscopy, representative flow cytometry analysis of cell-surface markers on ASCs and staining for differentiations. **(A)** Passage (P) 0 at day 6. **(B)** ASCs exhibited large flattened or fibroblast-like morphology during P2 at day 2. **(C, D)** Phase-contrast microscopy alizarin red staining control, after osteogenic differentiation. **(E)** Phase-contrast microscopy image after 21 days of adipogenic differentiation control. **(F)** Oil Red O staining immunofluorescence image after 21 days of adipogenic differentiation. Original magnifications: A: 20×, B: 10×, C: 4×, D: 10×, E: 20×, F: 40×.

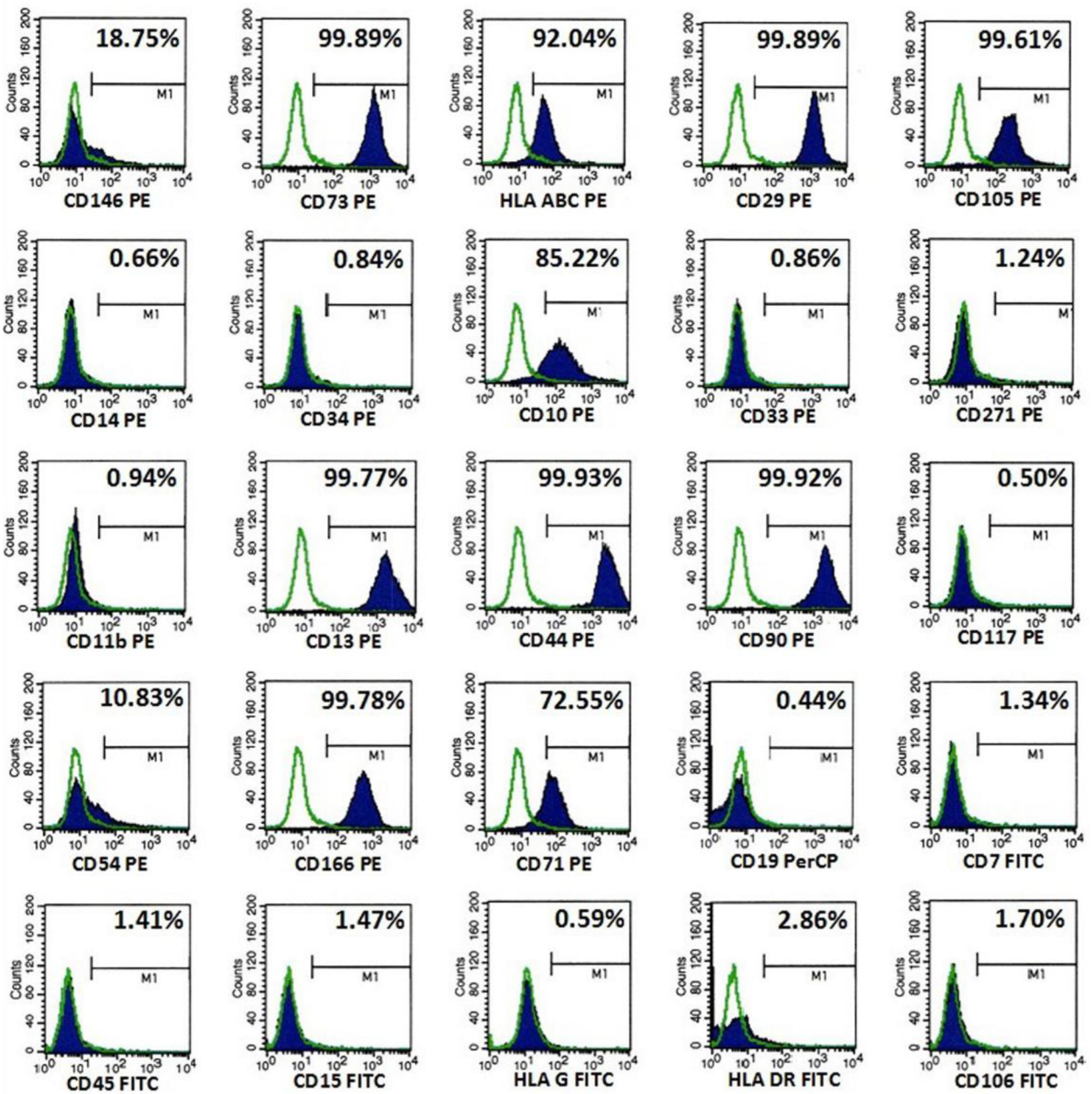

Figure 2. Representative flow cytometry analysis of cell-surface markers on ASCs.

cardiomyogenic differentiation, were obtained from a comprehensive literature survey (Asumda et al., 2012; Zhou et al., 2012; Hartung et al., 2013; Pisano et al., 2013). These included *GATA4*, *Nkx 2.5*, Troponin T (*cTnT*), a-myosin heavy chain (*aMHC*), cardiac troponin I (*cTnI*), and *TBX5*. Protein–protein interaction subnetworks around proteins encoded by candidate genes were reconstructed and enrichment analysis was employed to map the granular annotations of genes and corresponding proteins in the subnetworks. Subnetworks that were significantly enriched with GO terms directly associated with early-phase cardiomyogenic differentiation were then identified and candidate genes, around which these subnetworks were reconstructed, were selected as markers to be employed in RT-qPCR analysis.

When the protein–protein interaction subnetworks were analyzed, three genes, i.e. *GATA4*, *TBX5*, and *cTnI*, came into prominence.

Since proteins encoded by *GATA4* (Entrez id: 2626) and *TBX5* (Entrez id: 6910) are physically interacting, their subnetworks were interconnected. According to enrichment analysis of the integrated subnetwork around

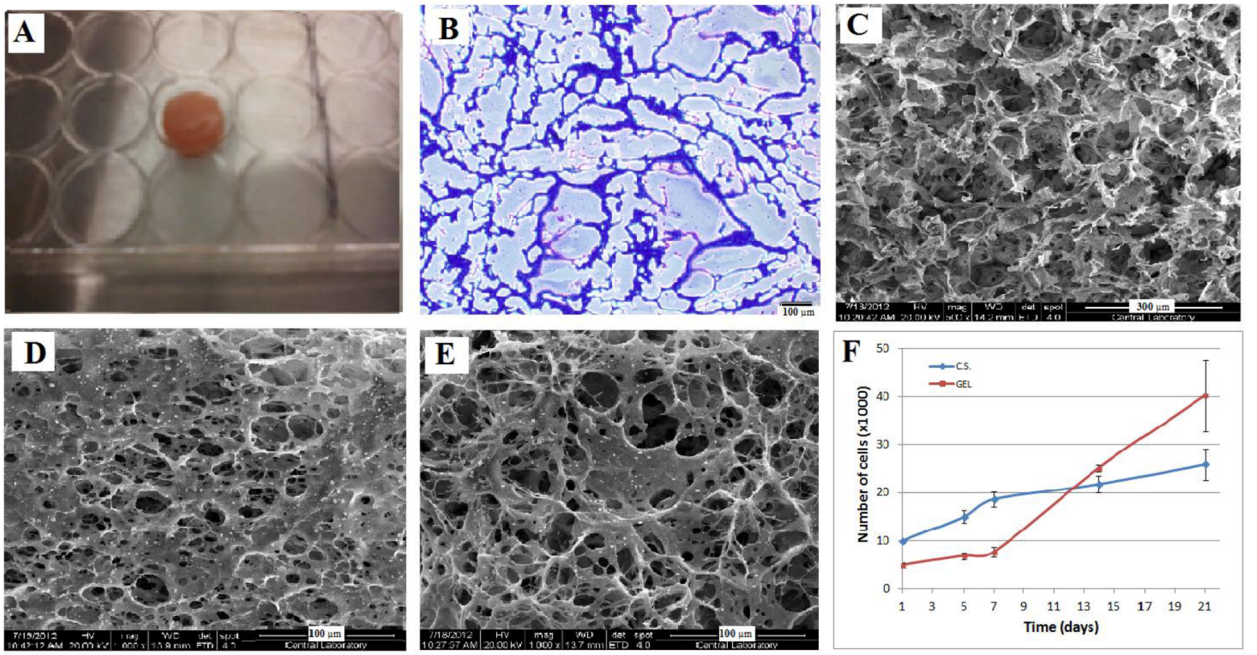

Figure 3. Characterization studies of HA/G plasma gel. **(A)** Macrograph of the gel, **(B)** pore distribution of the gel stained via Coomassie brilliant blue, **(C)** SEM micrograph of the HA/G plasma gel on day 1, **(D)** SEM micrograph of the unseeded HA/G plasma gel on day 21, **(E)** SEM micrograph of the cell-seeded HA/G plasma gel on day 21, **(F)** WST-1 proliferation assay results of ASCs on HA/G plasma and coverslip.

GATA4 and *TBX5*, the statistically enriched biological processes, cellular components, molecular function terms, and KEGG pathways were all related to embryonic development of heart. The statistical enrichment of molecular function terms related to transcription activities and chromatin binding supported the idea that these genes are associated with transcription level of cell regulation. As a result of KEGG pathway enrichment analysis, cancer-related pathways were also emphasized as significant pathways related to these genes. At a subnetwork of genes associated with embryonic development, the enrichment of cancer pathways is expected since embryonic processes may cause occurrence of cancer, because processes occurring during tumorigenesis may be similar to processes occurring in early development (Monk and Holding, 2001). According to enrichment analysis of the subnetwork around *cTnI* (Entrez id: 7137), biological processes, cellular components, molecular function terms, and KEGG pathways were related to the muscle contraction system process. The enrichment of structural and functional terms associated with the muscle contraction process indicated a tendency towards more specific cardiomyogenic differentiation process.

The statistically significant increase in gene expression levels of these genes, which are associated with biological terms related to the embryonic development of heart and muscle contraction process, could be interpreted as an indicator for progress towards cardiomyogenic differentiation of ASCs. Therefore, these three genes were employed in RT-qPCR analysis as cardiac makers.

3.4. Cardiomyogenic differentiation of ASCs on the gels

After RNA isolation, the amount of RNA of each experimental group was determined as 10–100 ng/µL. cDNA was synthesized from RNA and RT-qPCR analyses were conducted for 16 experimental groups (Table 2). The comparative expressions of selected cardiac marker genes were investigated to follow related cardiomyocyte development. ASCs on coverslips grown in control medium for 14 and 21 days were used as controls (sample id: 8 and 16, respectively), and the average CT value of these controls was used as the reference value. Comparative analysis using $2^{-\Delta\Delta CT}$ technique indicated that differential expression levels were observed for the investigated genes after 14 and 21 days of culture (Figure 4). Please see Supplementary Tables 1 and 2 (on the journal's website) for further information about the P-values of the experimental groups.

The expression level of the *GATA4* gene was elevated in all experimental groups at day 21 when compared to day 14 (Figure 4). The *GATA4* gene expression level of ASCs on the gels was also higher than those cultured on coverslips for all experimental groups. The maximum *GATA4* gene expression level of ASCs was observed at day 21 on the gels induced with BMP-4 (sample id: 11). The

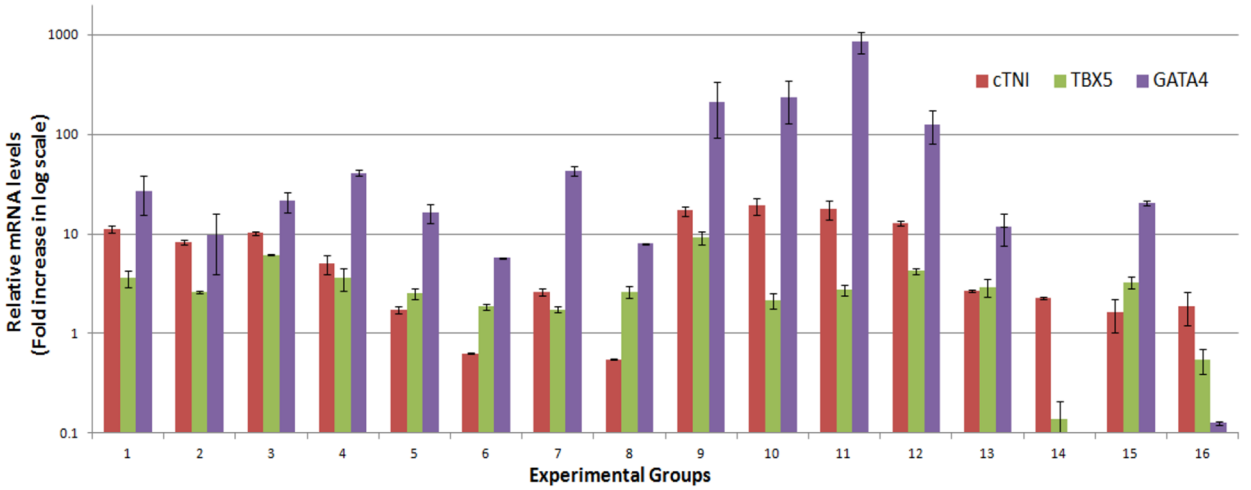

Figure 4. Comparative analysis of differential expression levels for *GATA4*, *TBX5*, and cTnI genes. All real-time PCR experiments were performed in triplicate (3 independent experiments). **1**: HA/G plasma gel, InsuM, 14 days; **2**: coverslip, InsuM, 14 days; **3**: HA/G plasma gel, Bmp4M, 14 days; **4**: coverslip, Bmp4M, 14 days; **5**: HA/G plasma gel, 5AzaM, 14 days; **6**: coverslip, 5AzaM, 14 days; **7**: HA/G plasma gel, ContM, 14 days; **8**: coverslip, ContM, 14 days; **9**: HA/G plasma gel, InsuM, 21 days; **10**: coverslip, InsuM, 21 days; **11**: HA/G plasma gel, Bmp4M, 21 days; **12**: coverslip, Bmp4M, 21 days; **13**: HA/G plasma gel, 5AzaM, 21 days; **14**: coverslip, 5AzaM, 21 days; **15**: HA/G plasma gel, ContM, 21 days; **16**: coverslip, ContM, 21 days.

GATA4 expression level of ASCs on the HA/G plasma gels induced with BMP-4 was 21 times higher when compared to expression of this gene by the cells on coverslips at day 14 (sample id: 3). However, this ratio increased to 863 at the end of 21 days (sample id: 11). *GATA4* expression level of ASCs on the gels induced with insulin (sample id: 2) was 3 times higher than that on the coverslips (sample id: 1) at day 14.

When experimental groups are analyzed in terms of *TBX5* gene expression, the gene expression ratios of ASCs on the gels were higher than those on the coverslips (Figure 4). *TBX5* expression level of ASCs induced with BMP-4 medium on the gel (sample id: 11) was less than that on the coverslip (sample id: 12) at day 21, but this difference was not statistically significant ($P > 0.05$). *TBX5* expression level of ASCs induced with insulin medium on the gel (sample id: 9) was highest when compared to the other experimental groups at day 21.

cTnI expression levels of ASCs induced with BMP-4 and insulin on the gels and coverslips were higher than the expression levels of other experimental groups and this expression ratio was significantly increased at day 21 (Figure 4). While *cTnI* expression level of ASCs on the gels was higher than that of ASCs on coverslips, this difference was not statistically significant under insulin induction (sample id: 9 and 10, respectively) ($P > 0.05$). On the other hand, expression level of the *cTnI* gene was induced significantly with insulin.

4. Discussion

Myocardial infarct or myocardial ischemia-like diseases are associated largely with loss of cells and cardiac tissue engineering is considered as a feasible approach to provide a tissue replacement in such cases to ensure the integrity and regeneration of the damaged tissue. Cardiac tissue patch is obtained in the laboratory by combining cardiomyocyte-like cells with a 3D scaffold with an ultimate aim of providing a functional replacement for the tissue loss. In cardiac tissue engineering and cardiac regenerative medicine studies, scaffolds have been produced with different methods and various different cell types have been used (Leor et al., 2005; Sterodimas et al., 2010). The chemical modifiers, mimics of ECM, physical inducers, and coculture affect the cardiomyogenic differentiation process of MSCs (Dimarakis et al., 2006). The niche has a crucial effect on stem cell differentiation and commitment to self-renewal pathways. Fundamentally, the niche is the microenvironment of cells and it shapes the behavior of cells with its structural, biological, and mechanical features. The target cell differentiation is more efficiently achieved by provision of both growth factors and various microenvironmental features to the stem cells in the laboratory.

In this study, ECM structure was imitated with HA/G plasma gels and cardiomyogenic differentiation was investigated under different media supplemented with several chemical modifiers in different proportions. The

plasma gels were produced from fibrinogen precipitated from human blood plasma, gelatin, and human umbilical cord-derived HA and were crosslinked with glutaraldehyde. Glutaraldehyde was used to increase the stability of hydrogels. Considering its induction potential in in vivo vascularization and repair of damaged heart tissue (Li and Guan, 2011) as well as human myofibroblast proliferation and tissue regeneration and/or development (Ye et al., 2011), fibrinogen was preferred. HA is gaining popularity as a biomaterial for tissue engineering and tissue regeneration due to its high biocompatibility and low immunogenicity (Zheng et al., 2004; Leach et al., 2015) as well as its ability to promote cell migration (Yoo et al., 2005). Previously, it has been shown that rate of cell proliferation and cardiomyogenic differentiation of C2C12 myoblasts was significantly increased with a film like scaffold, which was produced by mixing alginate and gelatin (Rosellini et al., 2009), and films including gelatin in high proportions were proposed as ideal scaffolds. Considering the previous findings and gelatin's effect in induction of cardiomyogenic differentiation, as well as its positive effects on cell attachment and expansion, gelatin concentration in the HA/G plasma gels was kept higher than the HA concentration in the present study.

The stem cells are considered invaluable as a cell source, since they are expanded easily in the laboratory and can be differentiated into many cell types. Nowadays, MSCs are considered as the most safe stem cells to be used in medicine. Some MSC types, such as bone marrow-derived, umbilical cord blood-derived, etc., have been investigated in preclinical and clinical applications. Through their secreted growth factors and cytokines, MSCs can decrease cell death in damaged tissue, accelerate vascularization, and differentiate into target cells in vivo, and, when they are used as allogenic cells, even immune rejection can be suppressed. It has been reported that ASCs have the ability to differentiate along various mesenchymal lineages in the presence of induction factors (Zuk et al., 2002). ASCs have also been shown to be capable of in vitro differentiation into cardiomyocytes (Rangappa et al., 2003; Planat et al., 2004; Palpant et al., 2010) and transplantation of adipose-derived cardiomyocytes onto an infarcted mouse heart contributed to cardiac repair (Bai et al., 2010). When ASCs are transplanted, these cells differentiated into vascular cells and angiogenesis was improved in damaged tissue with cardiac factors (Bai et al., 2010). By producing HA/G plasma gel via the precipitation of fibrinogen from autologous human plasma, immune reaction probability may be decreased.

The effects of five chemical modifiers (i.e. activin-a, BMP-4, insulin, valproic acid, and 5-azacytidine) were also investigated in this study. Treatment with temporal activin-a and BMP-4 has led to efficient cardiomyogenic differentiation from human ESCs with a significant increase in the percentage of human ESC-derived cardiomyocytes in the culture from 1% to 30% (Kattman et al., 2006; Laflamme et al., 2007). In addition, in vitro treatment of adult bone marrow cells with 5-azacytidine led to expression of cardiomyogenic genes and proteins (Wakitani et al., 1995; Makino et al., 1999). Moreover, the early development of avian pericardial mesoderm has been found to be regulated by insulin and insulin-like growth factors (Heng et al., 2004). The cardiac differentiation studies related to use of insulin and valproic acid were not reported. Since MSCs express genes related to pluripotency, the protocol (Steven et al., 2008) leading to efficient cardiomyogenic differentiation of ESCs with BMP-4 and activin-a was used in this study. 5-Azacytidine, which leads to cardiomyocyte-like cell production from MSCs in vitro (Rosca and Burlacu, 2010; Martinez et al., 2011), was also included in the study.

Several genes have been employed in the determination of cardiomyogenic differentiation in the literature. Similar to other biological processes cardiomyogenic differentiation should also be governed by an organization of proteins mediated through protein–protein interactions (Sevimoglu and Arga, 2014). Therefore, a network-based approach, which considers the interacting partners of a protein encoded by a candidate gene and their molecular functions as well as roles in biological processes, was employed to select appropriate marker genes to follow cardiomyogenic differentiation in the present study. Significant enrichment results with biological terms related to embryonic development of heart and muscle contraction process were searched. Among the commonly used genes, three genes, i.e. *GATA4*, *TBX5* and *cTnI*, came into prominence as cardiac markers. *GATA4* encodes a member of the GATA family of zinc finger transcription factors, which regulates genes involved in embryogenesis and in myocardial differentiation and function (Perrino et al., 2006). *GATA4* is a critical transcription factor for proper mammalian cardiac development and essential for survival of the embryo, and it promotes cardiac morphogenesis and cardiomyocyte survival and maintains cardiac function in the adult heart (Perrino et al., 2006). T-Box transcription factor *TBX5* is involved in the regulation of developmental processes. The encoded protein may play a role in heart development and specification of limb identity (Takeuchi et al., 2003). Troponin I (*TnI*), which is a troponin complex, is a heteromeric protein playing an important role in the regulation of skeletal and cardiac muscle contraction (Gomes et al., 2002). The statistically significant increase in gene expression levels of these genes could be interpreted as an indicator for progress towards cardiomyogenic differentiation of ASCs. The *GATA4*, *TBX5*, and *cTnI* gene expression levels of ASCs on the gels was also higher than

those cultured on coverslips for all experimental groups. The cardiomyogenic differentiation level increased with time. However, *GATA4* expression level of ASCs on the gels induced with insulin (sample id: 2) was 3 times higher than that on the coverslips (sample id: 1) at day 14. *TBX5* expression level of ASCs induced with insulin medium on the gel (sample id: 9) was the highest when compared to the other experimental groups at day 21. *cTnI* expression levels of ASCs induced with BMP-4 and insulin on the gels and coverslips were higher than the expression levels of other experimental groups and this expression ratio was significantly increased at day 21. Generally, cardiomyogenic differentiation marker expressions of ASC were increased in the presence of activin-a and BMP-4 or insulin- and valproic acid-including medium. As a result the cardiac gene expression on HA/G plasma gels in the presence of activin-a and BMP-4 or insulin and valproic acid was more potent than in the presence of 5-azacytidine alone.

In conclusion, differentiation potential of human mesenchymal stem cells to cardiomyocytes was investigated by growing them simultaneously on gels with inductions by various chemical modifiers in the present study. Combination of HA/G plasma gel and growth factors such as insulin and valproic acid or activin-a and BMP-4 was assigned for investigations related to cardiomyogenic differentiation of MSCs. Various chemical modifiers show different effects on the cardiac marker gene expressions of ASCs on gels and coverslips, so gene expression profiles at a broader range must be analyzed using high-throughput techniques (such as microarrays or next-generation sequencing) in order to understand and optimize the differentiation process at the molecular level. In further studies, it is aimed to increase the percentage of differentiated cardiomyocytes through scaffold-based mechanical effects and chemical modifiers. The data obtained from the present study will be an enlightening source for in vitro functional 3D heart muscle production for tissue engineering applications.

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Supplementary Table 1. The statistical significance of experimental groups using one-way analysis of variance (ANOVA) (gel: HA/G plasma gel, cs: coverslip).

		14 days			21 days		
		GATA4 P-values	*TBX5* P-values	*cTNI* P-values	*GATA4* P-values	*TBX5* P-values	*cTNI* P-values
ContM-cs	ContM-gel	0.000	0.332	0.007	0.000	0.001	1.000
	InsuM-cs	1.000	1.000	0.000	1.000	0.083	0.000
	InsuM-gel	0.008	0.212	0.000	0.008	0.000	0.000
	Bmp4M-cs	0.000	0.201	0.000	0.000	0.000	0.000
	Bmp4M-gel	0.103	0.000	0.000	0.103	0.009	0.000
	5AzaM-cs	0.999	0.521	1.000	0.540	0.990	1.000
	5AzaM-gel	0.540	1.000	0.233	0.999	0.004	1.000
ContM-gel	ContM-cs	0.000	0.332	0.007	0.000	0.001	1.000
	InsuM-cs	0.000	0.330	0.000	0.000	0.383	0.000
	InsuM-gel	0.033	0.003	0.000	0.033	0.000	0.000
	Bmp4M-cs	1.000	0.003	0.001	1.000	0.540	0.000
	Bmp4M-gel	0.003	0.000	0.000	0.003	0.953	0.000
	5AzaM-cs	0.000	1.000	0.010	0.000	0.000	1.000
	5AzaM-gel	0.000	0.445	0.561	0.000	0.996	0.997
InsuM-cs	ContM-cs	1.000	1.000	0.000	1.000	0.083	0.000
	ContM-gel	0.000	0.330	0.000	0.000	0.383	0.000
	InsuM-gel	0.020	0.214	0.000	0.020	0.000	0.887
	Bmp4M-cs	0.000	0.203	0.000	0.000	0.013	0.023
	Bmp4M-gel	0.214	0.000	0.007	0.214	0.934	0.987
	5AzaM-cs	0.977	0.518	0.000	0.789	0.018	0.000
	5AzaM-gel	0.789	1.000	0.000	0.977	0.771	0.000
InsuM-gel	ContM-cs	0.008	0.212	0.000	0.008	0.000	0.000
	ContM-gel	0.033	0.003	0.000	0.033	0.000	0.000
	InsuM-cs	0.020	0.214	0.000	0.020	0.000	0.887
	Bmp4M-cs	0.082	1.000	0.000	0.082	0.000	0.234
	Bmp4M-gel	0.879	0.000	0.515	0.879	0.000	1.000
	5AzaM-cs	0.003	0.005	0.000	0.289	0.000	0.000
	5AzaM-gel	0.289	0.147	0.000	0.003	0.000	0.000
Bmp4M-cs	ContM-cs	0.000	0.201	0.000	0.000	0.000	0.000
	ContM-gel	1.000	0.003	0.001	1.000	0.540	0.000
	InsuM-cs	0.000	0.203	0.000	0.000	0.013	0.023
	InsuM-gel	0.082	1.000	0.000	0.082	0.000	0.234
	Bmp4M-gel	0.007	0.000	0.000	0.007	0.110	0.110
	5AzaM-cs	0.000	0.005	0.000	0.001	0.000	0.000
	5AzaM-gel	0.001	0.139	0.000	0.000	0.214	0.000
Bmp-gel	ContM-cs	0.103	0.000	0.000	0.103	0.009	0.000
	ContM-gel	0.003	0.000	0.000	0.003	0.953	0.000
	InsuM-cs	0.214	0.000	0.007	0.214	0.934	0.987
	InsuM-gel	0.879	0.000	0.515	0.879	0.000	1.000
	Bmp4M-gel	0.007	0.000	0.000	0.007	0.110	0.110
	5AzaM-cs	0.041	0.000	0.000	0.944	0.002	0.000
	5AzaM-gel	0.944	0.000	0.000	0.041	1.000	0.000
5AzaM-cs	ContM-cs	0.999	0.521	0.233	0.999	0.990	1.000
	ContM-gel	0.000	1.000	0.561	0.000	0.000	1.000
	InsuM-cs	0.977	0.518	0.000	0.977	0.018	0.000
	InsuM-gel	0.003	0.005	0.000	0.003	0.000	0.000
	Bmp4M-cs	0.000	0.005	0.000	0.000	0.000	0.000
	Bmp4M-gel	0.041	0.000	0.000	0.041	0.002	0.000
	5AzaM-gel	0.282	0.653	0.307	0.282	0.001	1.000
5AzaM-gel	ContM-cs	0.540	1.000	0.999	0.540	0.004	1.000
	ContM-gel	0.000	0.445	0.000	0.000	0.996	0.997
	InsuM-cs	0.789	1.000	0.977	0.789	0.771	0.000
	InsuM-gel	0.289	0.147	0.003	0.289	0.000	0.000
	Bmp4M-cs	0.001	0.139	0.000	0.001	0.214	0.000
	Bmp4M-gel	0.944	0.000	0.041	0.944	1.000	0.000
	5AzaM-cs	0.282	0.653	0.282	0.282	0.001	1.000

Supplementary Table 2. The statistical significance of same experimental groups depending on time using paired sample t-test (gel: HA/G plasma gel, cs: coverslip, 14: 14 days, 21: 21 days).

Compared groups	*GATA4* P-values	*TBX5* P-values	*cTNI* P-values
ContM-cs-14 – ContM-cs-21	0.000	0.009	0.075
ContM-gel-14 – ContM-gel-21	0.019	0.034	0.170
InsuM-cs-14 – InsuM-cs-21	0.060	0.211	0.025
InsuM-gel-14 – InsuM-gel-21	0.098	0.015	0.009
Bmp4M-cs-14 – Bmp4M-cs-21	0.074	0.305	0.000
Bmp4M-gel-14 – Bmp4M-gel-21	0.021	0.002	0.063
5AzaM-cs-14 – 5AzaM-cs-21	0.000	0.004	0.001
5AzaM-gel-14 – 5AzaM-gel-21	0.009	0.307	0.018