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Enhancing the Cardiogenic Potential of Human Mesenchymal Stem Cells via Extracellular Matrix Proteins

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ABSTRACT

Current *in vitro* models of cardiogenic differentiation include a variety of manipulations and stimulating agents, which interfere with the application of such models for preclinical drug testing. So, the aim of this study was to develop an approach for cardiogenic differentiation *in vitro* with a minimum of manipulations and to assess the influence of the extracellular matrix protein collagen IV on the cardiogenic potential of human mesenchymal stem cells (MSCs). Cardiogenic markers were analyzed by immunofluorescence staining and Western blot analysis. The results showed that collagen IV increased the cardiac marker GATA4 and altered the level of muscle actin isoforms, α-smooth muscle actin and α-cardiac muscle actin, in two different lines of human MSCs. The results indicate that the use of matrices containing collagen IV may increase the cardiogenic potential of human MSCs and may be a promising approach to obtain an *in vitro* model for cardiogenic differentiation suitable for preclinical drug discovery.

Keywords: Human mesenchymal stem cell, cardiogenic potential, extracellular matrix, collagen IV, matrigel

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Introduction

Cardiomyocytes (CMs) are the heart muscle cells that are responsible for contractile function. During embryonic development of the mammalian heart, CMs proliferate extensively, but sometime after birth they undergo terminal differentiation, which is accompanied by exit from the cell cycle and loss of the ability to divide (1). This limits the availability of a stable cardiomyocyte line for testing various drugs targeting cardiac pathologies. In cardiac tissue, endogenous c-*kit*+cardiac stem cells are considered a potential source of new cardiomyocytes, although their role in cardiac regeneration is controversial (2). In this context, numerous research efforts are underway to differentiate various cells into CMs to develop *in vitro* models, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), as well as various non-muscle cells derived from heart tissue. However, all current methods of cardiogenic differentiation have certain limitations (3) and include a large number of manipulations, in particular, the introduction of various soluble stimulating factors, such as fibroblast growth factor (4,5,6), ascorbic acid (5,7), retinoic acid (8,9), 5-azacytidine (10,11), dimethyl sulfoxide (DMSO) (9,11) which is commonly used to induce cardiogenic differentiation of embryonic carcinoma stem cells through the formation of embryoid bodies (11), etc. Moreover, the most promising methods involve additional activation of various signaling pathways (Nodal/activin, BMP4 and Wnt/β-catenin) at different stages of cell differentiation, including the introduction of transcription factors into cells using viral delivery systems (12). All these manipulations result in models that are difficult to standardize and cannot be used for assessing the isolated effects of individual drugs. In this regard, there remains a need for a cardiogenic differentiation method that includes a minimum number of manipulations/stimulation factors and is easy to standardize.

According to the literature data, the differentiation of ESCs is less efficient compared to other stem cells (3,13). iPSCs are considered the most promising cells for cardiogenic differentiation, but the process to produce these cells itself includes complex manipulations with retroviral introduction of transcription factors (14). In turn, primary cultures of non-muscle cardiac cells, which may have an initial cardiogenic potential and respond well to cardiogenic differentiation stimuli, are difficult to obtain and even more difficult to standardize. Given these data, MSCs seem to be the optimal source for developing a simplified method for cardiogenic differentiation that requires minimum of manipulation and can be standardized.

In the past decade, a sufficient amount of data has accumulated to indicate that, in addition to the soluble factors, the extracellular matrix (ECM) is an important regulator of cardiogenic differentiation *in vivo* (15). Moreover, cardiac ECM components are essential for cardiac regeneration and repair after injuries, in particular, myocardial infarction (for a review see (16)). Such effects of the ECM may be attributed to the process of mechanotransduction mediated by integrins. Integrins are transmembrane receptors that are able to transfer the mechanical signals of the ECM into intracellular signaling cascades (17). The ECM of the cardiac tissue comprises a large number of different proteins, such as type I and III collagen, fibronectin, elastin, laminins, etc. (18). In addition to the common ECM, cardiomyocytes have their own basement membrane, a highly organized layer of extracellular matrix located on the outside of the sarcolemma and composed mainly of type I laminin and type IV collagen proteins (19, 20). Cardiac ECM has been shown to

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improve the effectiveness of cardiogenic differentiation methods in mouse embryonic stem cells (21) and adult bone marrow-derived stem cells (22).

Therefore, the aim of this study was to assess the influence of ECM components characteristic of cardiomyocytes in cardiac tissue on the cardiogenic potential of human MSCs in order to develop an approach for cardiogenic differentiation *in vitro* that involves a minimum of cell manipulations.

Materials and methods

Cell cultures

All cell lines were provided by the shared research facility "Vertebrate cell culture collection (VCCC)" supported by the Ministry of Science and Higher Education of the Russian Federation (agreement NO. 075-15-2021-683), Institute of Cytology, Russian Academy of Sciences (https://incras-ckp.ru/upload/iblock/catalog/CCCV-2022-eng.pdf). Three cell lines of human MSCs characterized in VCCC and tested for bacterial, fungal and mycoplasmal contamination were used in this study: MSCWJ-1 (origin: human MSCs from the Wharton's jelly of the umbilical cord (23)); FetMSC (origin: human MSCs from the bone marrow of a 5-6 week old embryo (24)); M-FetMSC (origin: human MSCs from the limb muscle of a 5-6 week old embryo (25)).

Cells were cultured in a growth medium containing 90% DMEM/F12 with L-glutamine (1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 Nutrient Mixture, Biolot, Russia) and 10% sterile-filtered fetal bovine serum (FBS) (Hyclone, USA). All lines were maintained at 5% CO2, 37 °C and 90% humidity. Microbiological analysis confirmed the absence of bacterial, fungal and mycoplasmal contamination in each cell line at the beginning and end of each experiment. Cells were subcultured at 80-90% confluence (every 2-3 days) using a trypsin-EDTA solution. Cells at 3rd passage were used for the experiments. Morphological changes were observed with an inverted microscope (NICON, Japan).

Culturing of MSCs on ECM-coated surfaces or in the presence of soluble FGF

Two commercial preparations of Matrigel, manufactured by Corning Life Sciences, USA, with different characteristics, namely Standard Matrigel Formulation and Matrigel optimized for embryonic stem cells (hESC-qualified Matrigel), were used in this study for the preliminary assessment of the effect of basement membrane proteins on human MSCs. To obtain Matrigel-coated surfaces, Matrigel Standard or hESC-Matrigel was applied to coverslips and left overnight at 4 ℃, then washed several times with a sterile PBS solution. Cells were seeded in a complete growth medium on coverslips pretreated with one of the Matrigel types, as well as control (untreated) coverslips.

To obtain collagen-coated Petri dishes (for further analysis using Western blotting) or coverslips (for further immunofluorescent analysis), a solution of a type IV collagen (0.3 mg/mL, Sigma-Aldrich, USA) was applied to Petri dishes and coverslips and left overnight at 4 ℃, then washed several times with sterile PBS. Cells were seeded in a complete growth medium on coverslips or dishes pretreated with collagen IV, as well as control (untreated) coverslips or dishes.

For a general assessment of the effect of soluble inducers on the cardiogenic potential of MSCWJs, FetMSCs and M-FetMSCs, cells cultured on non-covered coverslips were treated with fibroblast growth factor (FGFb, 5 ng/mL, Sigma-Aldrich, USA).

Indirect immunofluorescence method and confocal microscopy

[DOI: 10.22088/IJMCM.BUMS.13.4.337]

Cells on coverslips were fixed with 4% paraformaldehyde solution at room temperature (RT) for 10 min, then washed 3 times with PBS and treated with 0.1% solution of Triton X-100 in PBS for 3 min at RT. After multiple washes, the cells were incubated with a 1% solution of bovine serum albumin (BSA, Biolot, Russia) at 37 °C for 30 min, followed by washing with PBS.

To detect early markers of cardiogenic differentiation, cells were stained with rabbit polyclonal antibodies against GATA4 (1:500, Sigma-Aldrich, USA) or with monoclonal mouse antibodies against αSMA (1:200, Sigma-Aldrich, USA) for 1h at RT. Goat anti-rabbit or anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibodies (1:200, Sigma-Aldrich, USA), respectively, were applied for 1h as secondary antibodies.

To reveal contractile structures, cells were treated with rhodamine-phalloidin (Sigma, USA) (26) at a dilution of 1:30 (in PBS) for 15 min (in the dark, RT), and then washed 3 times with PBS. To stabilize the fluorescent label, cells were placed in a special Mounting medium (Pharmacia Biotech, Sweden) or Fluoroshield containing 4′,6-diamidino-2-phenylindole (DAPI) dye for staining cell nuclei (Fluoroshield™ with DAPI, Sigma-Aldrich, USA). The preparations were stored at 4 ℃ and protected from light. Preparations were analyzed using a LEICA TCS SL laser scanning confocal microscope (Leica, Germany) with a 40x objective magnification. Images at each wavelength (488 nm for FITC-conjugated secondary antibodies, 543 nm for Rhodamine phalloidin, and 405 nm for DAPI) were acquired sequentially. Optical sections were processed using Leica LAS AF Software for confocal systems (Leica, Germany).

Electrophoresis and Western Blotting

Cells at the selected time points were lysed on ice in 100 µL of lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 1% protease inhibitor mixture (Sigma-Aldrich, USA) and centrifuged at 500 g. 2x Laemmli sample buffer (90 °C, 5 min) (27) was added to supernatants. The protein concentration in the lysates was equalized using a dot-blot method on an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich, USA) using Coomassie R-250 dye (Sigma-Aldrich, USA). The comparison and adjustment of protein concentration was carried out using the Quantity One 4.6.6 software.

Proteins in lysate samples were separated by electrophoresis in 12% polyacrylamide gel under denaturing conditions in the presence of SDS (27) in a Bio-Rad system, USA, at 40 mA for 1.5h, and transferred to Immobilon-P PVDF membranes (Sigma-Aldrich, USA) with a Bio-Rad wet transfer system, USA, at 20mA overnight in Tris-glycine buffer (pH 8.3) containing 10% ethanol and 0.1% SDS. After transfer, the membranes were blocked for 1 h at RT with a 5% solution of bovine serum albumin in a PBS containing 0.05% Tween 20 and then incubated overnight at +4℃ in the presence of one of the following primary antibodies, specific for a particular actin isoform: monoclonal mouse antibodies against alpha-smooth muscle actin (1:600, Sigma-Aldrich, USA), monoclonal mouse antibodies against alpha-cardiac actin (1:400, Sigma-Aldrich, USA), or monoclonal mouse antibodies against beta-cytoplasmic actin (1:800, Sigma-Aldrich, USA) used as a control of sample loading. Goat anti-mouse IgG conjugated with horseradish peroxidase (1:10000, Pierce, USA) was added for 40 minutes as a secondary antibody. SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific, USA) was used to detect peroxidase activity. Chemiluminescence was recorded using the ChemiDoc system (Bio-Rad, USA). Western blot results were

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processed and quantified relative to loading control (beta-actin) using Quantity One 4.6.6 software (Bio-Rad Laboratories, USA). The obtained data were analyzed and plotted as mean ± SD of three experiments with Microsoft Excel (Microsoft, USA). The level of statistical significance was expressed as a p-value ($p \leq 0.05$).

Results

Assessment of morphological changes in MSCs cultured on Matrigel or in the presence of FGF

Three MSC lines of different origin (see Methods section) used in this work are shown in Figure 1. All MSCs were monolayer cells characterized by a fibroblast-like morphology, according to the specifications of cell lines: MSCWJ-1 (Figure 1a), FetMSC (Figure 1b), M-FetMSC (Figure 1c).

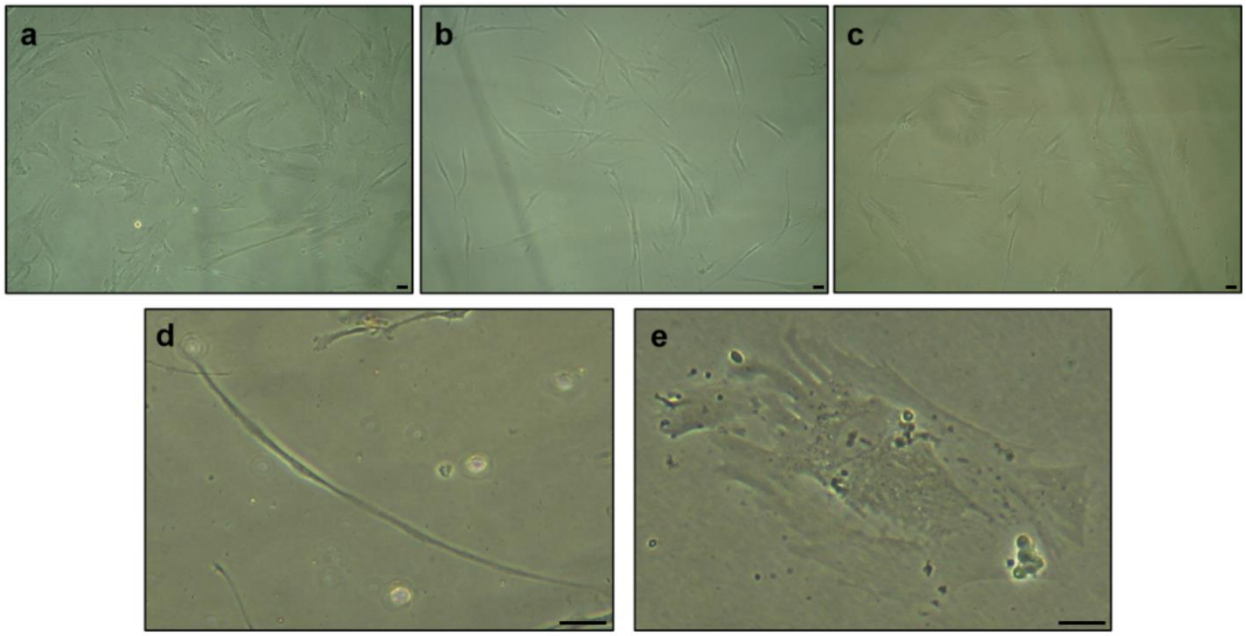

Fig. 1. Morphological changes in MSCs cultured under different conditions; inverted microscope; a) control MSCWJs-1, b) control FetMSCs, c) control M-FetMSCs, d) FGF-stimulated FetMSCs, e) M-FetMSCs on Matrigel Standard. The scale bar is 20 µm.

To assess the effect of ECM, which is rich in basement membrane proteins, on the cardiogenic potential of MSCs, 2 types of commercial Matrigel, a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, were used in the current study. For a general comparison of the effect of ECM with the isolated action of soluble inducers, cells were treated with a basic fibroblast growth factor (FGFb), which is often used as a key component of the induction media for cardiogenic cell differentiation (4,6).

Visible morphological changes were observed only in FetMSCs in the presence of FGF and M-FetMSCs on the Matrigel Standard preparation. FGF-stimulated FetMSCs were strongly elongated as compared to control cells and formed thin strands (Figure 1d), while M-FetMSCs on Matrigel Standard spread more intensively and acquired a polygonal shape (Figure 1e), which is typical, in particular, for cardiogenic differentiation of various cells *in vitro*. In MSCWJ-1 cells, cultured on both Matrigel preparations or in the

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presence of FGF no visual differences were observed as compared to corresponding control cells (data not shown).

Results of detection of the early marker of cardiogenic differentiation GATA4 and the actin cytoskeleton in M-FetMSCs cultured on Matrigel

Cardiogenic differentiation of non-muscle cells *in vitro* is accompanied by significant changes in their morphology, including increasing cell size and acquiring a polygonal shape. Since the morphological changes observed in FetMSC cells in the presence of FGF are not characteristic of cardiogenic cell differentiation, only M-FetMSC cells cultured on Matrigel Standard were selected for further analysis in this preliminary experiment.

Many well-known markers of CMs are usually used to assess the cardiogenic potential of cells. One of the key early markers of cardiogenic differentiation is the GATA4 transcription factor. Alongside the expression of GATA4, cardiogenic differentiation in heart tissue is accompanied by changes in the organization of actin contractile structures with the gradual formation of a striated myofibrillar apparatus in cells. In this regard, to assess the cardiogenic potential, the cells were stained with antibodies against GATA4, as well as the rhodamine phalloidin dye to reveal the organization of actin contractile structures.

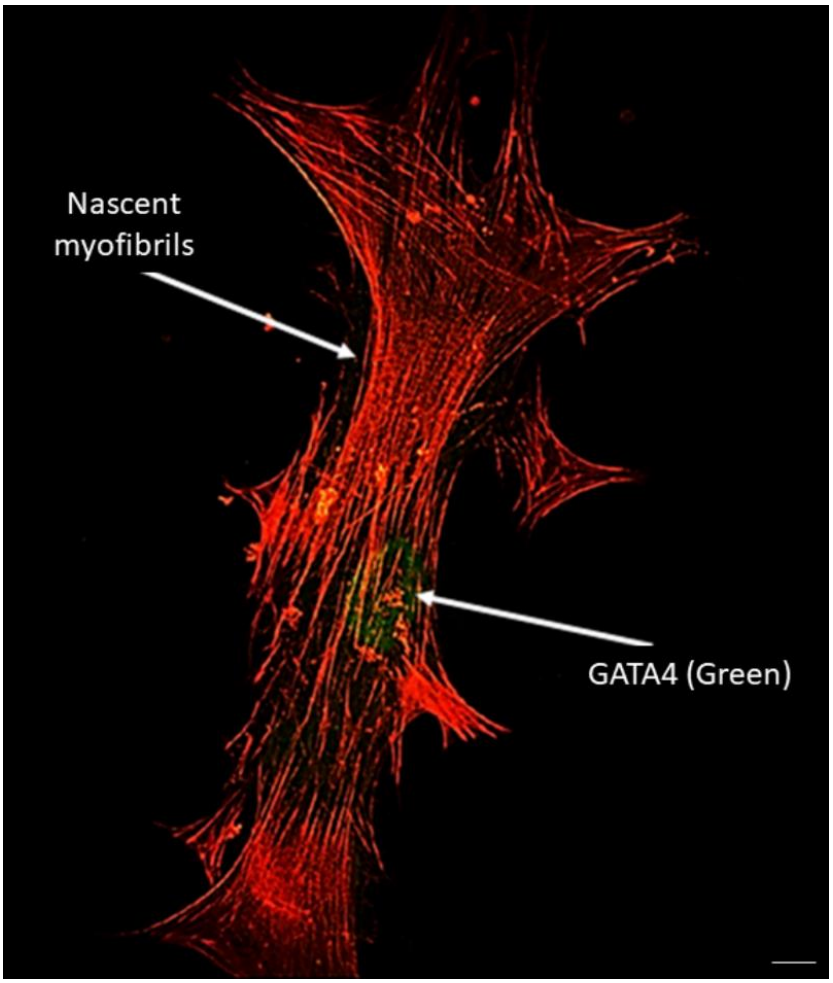

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Fig. 2. Detection of actin structures and GATA4 in M-FetMSCs cultured on Matrigel Standard. Cells were stained with anti-GATA4 antibodies (green) and rhodamine-phalloidin (red). The scale bar is 10 µm.

According to the results of immunofluorescent staining, in the vast majority of cells the pronounced reorganization of their actin cytoskeleton was observed along with the staining for GATA4, which correlated with morphological changes described above (Figure 1e). In single cells, the formation of striated structures, which are characteristic of the myofibrillar apparatus of muscle cells, was also observed (Figure 2).

Results of detection of cardiogenic markers in FetMSCs and M-FetMSCs cultured on collagen IV

Since Matrigel is a complex preparation consisting of many components, it was necessary to study the effect of individual extracellular matrix proteins characteristic of the basement membrane of cardiomyocytes *in vivo*. In this regard, a commercial preparation of purified collagen IV (Sigma-Aldrich, USA) was used. Two lines of MSCs, FetMSCs and M-FetMSCs, were selected for comparison in this experiment.

For both cell types cultured on collagen IV, there were no obvious morphological differences from the control cells, except for an increase in cell density in the presence of collagen IV. According to the results of immunofluorescent analysis, GATA4 was essentially not detected in control FetMSCs (Figure 3a), whereas in FetMSCs cultured on collagen IV, weak staining for GATA4 was observed in some nuclei, as can be seen in Figure 3b.

In control M-FetMSCs, some nuclei showed weak staining for GATA4 (Figure 3c). In cells cultured on collagen IV, more pronounced staining of the nuclei with anti-GATA4 was observed (Figure 3d).

In addition to the appearance of GATA4, the differentiation of CMs in heart tissue is accompanied by a coordinated switching of actin isoforms (28), which is the key protein of cell contractile apparatus. At the early stages of cardiogenesis, transient expression of alpha-smooth muscle actin (αSMA) is observed, which is subsequently replaced by mature isoform, alpha-cardiac actin (αCAA) (28, 29, 30). Since the appearance of αSMA is a prerequisite for further cardiogenic differentiation (31), we additionally stained cells with anti-αSMA antibodies to assess their cardiogenic potential.

In control FetMSCs, αSMA was essentially not detected (Figure 3e). However, when FetMSCs were cultured on collagen IV, slight staining for αSMA was observed in some cells (Figure 3f).

Interestingly, in the control M-FetMSC culture, a significant percentage of cells was stained with antibodies against αSMA, where diffuse staining of the cytoplasm was observed (Figure 3g). When cells were cultured on collagen IV, no visible increase in color intensity was observed, however, in addition to the diffuse staining of the cytoplasm, staining of the cell contractile structures was detected (Figure 3h).

Results of the detection of actin isoforms in FetMSCs and M-FetMSCs cultured on collagen IV

In control FetMSCs, both alpha-actin isoforms, αSMA and αCAA, were essentially absent according to the Western blot results, while in FetMSCs cultured on collagen IV, the appearance of the early marker of cardiogenic differentiation αSMA (Figure 4a-b) was shown, which is consistent with the results of immunofluorescent staining (Figure 3f). Moreover, Western blot revealed the appearance of the late cardiomyocyte marker αCAA in these cells (Figure 4a-c).

On the contrary, in control M-FetMSCs, the initial high level of αSMA was observed (Figure 4a-b), which confirms the results of immunofluorescent staining. At the same time, the low level of α-cardiac

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isoform was also detected in control cells (Figure 4a-c), which significantly increased in M-FetMSCs cultured on collagen IV (Figure 4a-c). Interestingly, in these cells, a decrease in the amount of αSMA was

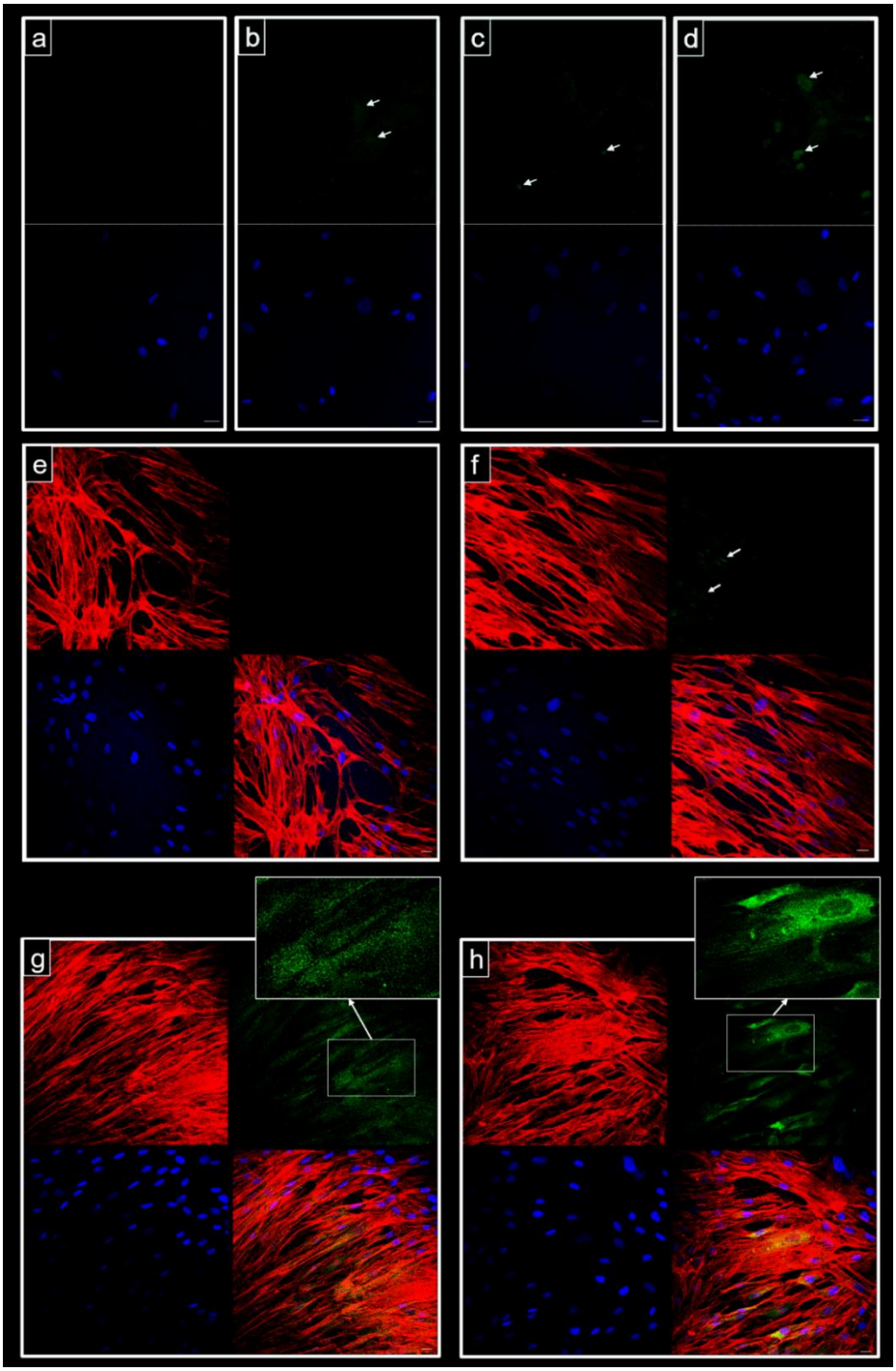

Fig. 3. Cardiogenic markers in MSCs, immunofluorescent staining. a, b, c, d) GATA4 – green, nuclei (DAPI dye) – blue; a) Control FetMSCs; b) FetMSCs on collagen IV; c) Control M-FetMSCs; d) M-FetMSCs on collagen IV. Arrows show GATA4-positive cells. The scale bar is 20 µm. e, f, g, h) Actin structures (rhodamine-phalloidin) – red, αSMA (anti-αSMA antibodies) – green, nuclei (DAPI

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dye) – blue; e) Control FetMSCs; f) FetMSCs on collagen IV, arrows show αSMA-positive cells; g) Control M-FetMSCs; h) M-FetMSCs on collagen IV. The scale bar is 20 μm.

shown (Figure 4a-b), which corresponded to the intracellular redistribution of αSMA according to the immunofluorescent data (Figure 3g-h).

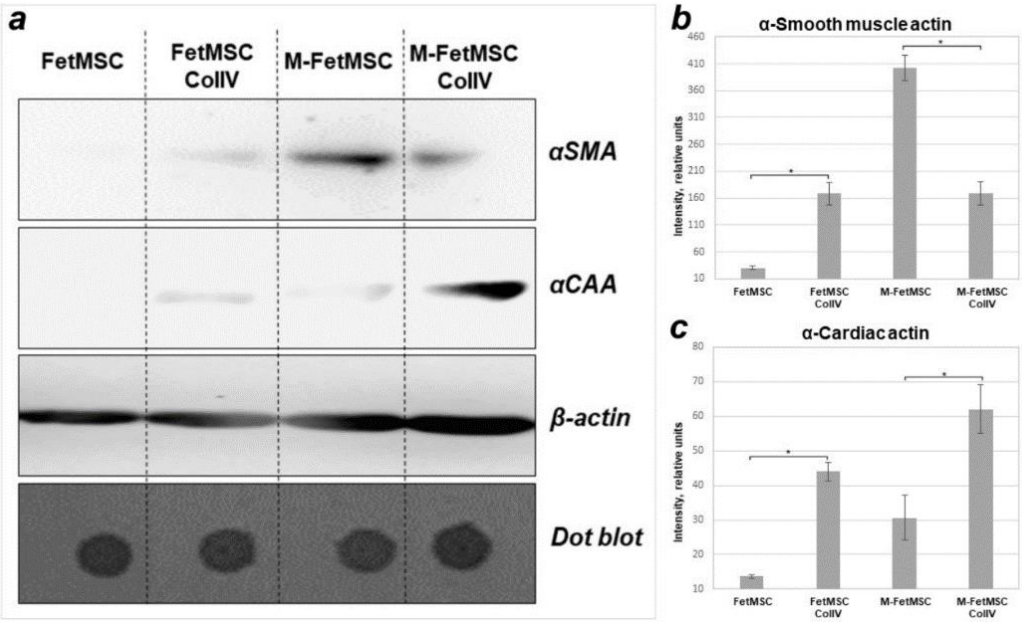

Fig. 4. Change in the level of α-smooth muscle actin (αSMA) and α-cardiac actin (αCAA) in FetMSCs and M-FetMSCs cultured on collagen IV (ColIV). a) Representative image of Western blot results, β-actin was used as an internal loading control along with the Dot-blot results. b) The level of α-smooth muscle actin in control FetMSCs (FetMSC), control M-FetMSCs (M-FetMSC), FetMSCs cultured on collagen IV (FetMSC ColIV), and M-FetMSCs cultures on collagen IV (M-FetMSC ColIV). c) The level of α-cardiac actin in control FetMSCs (FetMSC), control M-FetMSCs (M-FetMSC), FetMSCs cultured on collagen IV (FetMSC ColIV), and M-FetMSCs cultured on collagen IV (M-FetMSC ColIV). Data are shown as mean ± SD (*$p \leq 0.05$).

Discussion

To make a preliminary assessment of whether basement membrane proteins influence cariogenic differentiation of MSCs, cells were cultured on two types of Matrigel preparations. The results of the morphological assessment, as well as immunofluorescence analysis, demonstrated a significant influence of the Matrigel Standard on the cardiogenic potential of M-FetMSCs, with pronounced changes in their morphology being observed in the vast majority of cells including the acquisition of a polygonal shape, which is particularly typical of cardiogenic cell differentiation *in vitro.* Moreover, in addition to GATA4 staining, rearrangement of the actin cytoskeleton and even the appearance of striated myofibrillar structures were observed in individual cells. GATA4 is an important early marker of cardiogenic differentiation, which plays a crucial role in the regulation of cardiac gene expression and is expressed in both embryonic and adult cardiomyocytes (32). It was shown that the overexpression of GATA4 in embryonic stem cells can accelerate cardiogenesis and increase the number of terminally differentiated cardiomyocytes (33). In this regard, despite the fact that myofibrillar structures may be observed not only in cardiac but also in skeletal muscles, the staining of the nuclei of M-FetMSCs cultured on collagen IV for GATA4 suggests their cardiogenic

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rather than skeletal differentiation. It is worth noting that our experiments were intended to assess only the short-term effect of ECM after 5 days of culturing. It is possible that long-term culturing of M-FetMSCs, as well as FetMSC, on Matrigel Standard, would result in a more pronounced effect, including the appearance of a large number of striated cells.

Matrigel is a complex preparation derived from the basement membrane of mouse Engelbreth-Holm-Swarm sarcoma (EHS) which includes, in addition to proteins characteristic of the CMs basement membrane, many other ECM components and growth factors anchored on it. In cell cardiogenic differentiation protocols, Matrigel has been mainly included in the methods for generating hiPSC-derived cardiomyocytes (34), however, there are controversial data on its efficiency and appropriateness in such protocols due to its tumor origin and undefined composition (35,36). Therefore, it was necessary to study the effect of preparations with a known composition and concentration based on individual matrix proteins, characteristic of the CMs basement membrane. Since it was shown that the incorporation of collagen IV (ColIV) into the three-dimensional substrates enhanced mouse ESCs differentiation into cardiovascular progenitor cells (37), and the exposure of iPSCs to ColIV induced the expression of cardiovascular and hematopoietic markers (38), a commercial preparation of collagen IV was chosen for further analysis.

In heart tissue, cardiogenesis is accompanied by a coordinated switching of actin isoforms. The actin family in vertebrates consists of six closely related isoforms that are encoded by separate genes and are highly conserved (39). α-Actins are tissue-specific isoforms of actin and are characteristic of muscle cells. Myofibrils of mature cardiac muscles contain predominantly α-cardiac actin (αCAA). However, in the early stages of differentiation of cardiac muscle cells, transient expression of alpha-smooth muscle actin (αSMA) is observed, which protein is characteristic of vascular smooth muscle and myoepithelial cells (28). At later stages of cardiogenesis, αSMA is replaced by a mature isoform, αCAA (28, 29, 30). During stem cell differentiation towards cardiomyocytes, the same pattern of actin isoform expression is observed (31, 40). It was shown that inhibition of αSMA expression leads to the impaired differentiation of mouse ESCs towards CMs, confirming that the appearance of αSMA is a prerequisite for further cardiogenic differentiation (31).

In our study, the results of αSMA analysis with Western blot were consistent with the immunofluorescence data and showed an increase in αSMA level in FetMSCs cultured on collagen IV, along with the appearance of a later cardiac actin isoform (αCAA). These data, together with increased staining for GATA4, may indicate the initiation of cardiogenic differentiation in these cells.

At the same time, in M-FetMSCs cultured on collagen IV, a decrease in the level of αSMA was observed with its intracellular redistribution as compared to control cells. Since the maturation of cardiac cells is normally preceded by the expression of αSMA which is subsequently replaced by αCAA isoform, a decrease in αSMA accompanied by a significant increase in the level of αCAA and the intensity of nuclear staining for GATA4 probably indicates a progressive cardiogenic differentiation of M-FetMSC cells on collagen IV.

In conclusion, the results of this study suggest that basement membrane proteins can increase the cardiogenic potential of MSCs, which is consistent with data from studies on other types of stem cells. Based on the data obtained, we believe that culturing M-FetMSCs on well-defined matrices including collagen IV

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can be used as an approach to develop an *in vitro* model of cardiogenic differentiation suitable for preclinical drug discovery.

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Credit authorship contribution statement

Galina Chizhikova: Investigation, Methodology, Formal analysis, Writing – original draft

Mikhail Khotin: Fundraising, Project management, Resources

Natalya Bildyug: Conceptualization, Supervision, Investigation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interests

The authors declare no competing interests.

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