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Differentiation of pluripotent stem cells into cardyomyocytes is influenced by size of embryoid bodies

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Aim. To find the relationship between the size of embryoid bodies and the efficiency of pluripotent stem cells differentiation into cardiomyocytes. **Methods.** Transgenic murine iPSC line AT25 and D3 ESC line αPIG (clone 44) were differentiated into cardiomyocytes in AggreWell plates containing microwells which cause the pluripotent stem cells to aggregate into EBs of an appropriate size. Both cell lines were genetically modified and expressed IRES-flanked enhanced green fluorescent protein (eGFP) under the control of cardiac alpha myosin heavy chain promoter. We applied flow cytometry and fluorescence microscopy to test the efficiency of the differentiation processes. **Results.** The efficiency of differentiation of embryoid bodies formed from iPSC line AT25 and containing 250 and 1000 cells was found to be lower as compared to embryoid bodies formed of 500 and 750 cells. The number of eGFP+ cells derived from embryoid bodies of 500 cells was 8.5 times higher compared to embryoid bodies of 250 cells (2.86 ± 0.30 % cardiomyocytes per embryoid bodies of 500 cells vs. only 0.34 % cardiomyocytes per embryoid bodies containing 250 cells); the difference was 4.7 times higher in comparison with embryoid bodies formed from 1000 cells. **Conclusions.** The size of embryoid bodies can affect differentiation of pluripotent stem cells into cardiomyocytes. Among the embryoid bodies formed from 250 to 2000 cells per embryoid body, the highest percentage of eGFP+ cells was obtained from 500-cell embryoid bodies.

Keywords: pluripotent stem cells, induced pluripotent stem cells, embryoid bodies, cardiomyocyte, differentiation.

Introduction

The ability of pluripotent stem cells (PSC) to proliferate *in vitro* and differentiate into any cell of three germ layers [1], such as hepatocytes [2], beta cells of the pancreas [3], cardiomyocytes [4], osteoblasts [5], endothelial cells [6], neurons [7] and other makes them a target of up-to-date research. Moreover, PSC can be used for disease modeling, drug toxicity screening or drug discovery, gene therapy and cell replacement therapy [8]. One of the main hurdles to overcome for achieving these goals is the determination of controlled conditions required for the process of differentiation.

It is known that the process of differentiation is affected by various environmental stimuli such as matrix components and cell-matrix interactions, growth factors, cytokines, signaling molecules providing intercellular contacts [9, 10]. During embryogenesis, the cell-cell interactions and cell position are the key factors which direct the differentiation. The same aspects influence the process *in vitro* [11].

The Embryoid body (EB) formation is often used as a method for initiating differentiation. EBs are three-dimensional aggregates, representing the early stages of embryo development. However, because the EB formation depends on various factors, it is

much more disorganized in comparison with real embryo. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) form the aggregates covered with a layer of visceral endoderm on the surface and an inner layer filled with primitive ectoderm cells, when the factors supporting PSC in undifferentiated state are removed from the media. This arrangement is similar to the early stages of embryonic development, where the endoderm tissue, which is in direct contact with the mesoderm, stimulates its development into cardiomyocytes [12]. It was proven that despite the differences in spatial organization most of the cell types are formed inside EBs, including nerve cells, cardiomyocytes, hematopoietic cells and others. Due to the similarities between embryogenesis and EB formation, many of the same growth factors are involved at the same stages [13, 14].

In order to improve the efficiency of differentiation of PSCs two main approaches are used. The first one is to supply cultivation medium with the factors involved in the process of formation, maturation and proliferation of the certain cell type. The second strategy is to adjust the physical parameters that influence the formation of cell aggregates, such as the size and shape of EBs [10, 8]. S. M. Dang *et al* [12] demonstrated that homogenization of EB population can significantly improve the efficiency of PSC differentiation into cardiomyocytes. Little is known about the basis of interconnection between EB size and shape and PSC direct differentiation. In order to reveal this mechanism, the profound molecular and cell research is required. Therefore it is important to find the relationship between the size of embryoid bodies and the efficiency of PSC differentiation into cardiomyocyte.

Materials and methods

Transgenic murine iPSC line AT25 was engineered from murine iPSC line TiB7.4 in Center for Physiology and Pathophysiology, Institute for Neurophysiology, Medical Faculty, University of Cologne, Cologne, Germany (Fatima *et al,* manuscript in preparation). iPSC line TiB7.4 was kindly provided by Rudolf Jaenisch and Alexander Meissner [15]. Transgenic murine D3 ESC line αPIG (clone 44) was described earlier [16]. Both cell lines were genetically modified and express the puromycin resistance gene N-acetyl-aminotransferase and the IRES-flanked enhanced green fluorescent protein (eGFP) under the control of cardiac alpha myosin heavy chain promoter (αMHC). The ability of cardiomyocytes to express eGFP under the control of cardiac-α-MHC promoter gave us the opportunity to apply flow cytometry and fluorescence microscopy to test the efficiency of the differentiation processes [16]. These cells were maintained on irradiated mouse embryonic fibroblasts (MEF) in Dulbecco's minimal essential medium containing 15 % fetal bovine serum (FBS), 1x non-essential amino acids (NEAA), 2 mM L-glutamine, 50 μM β-mercaptoethanol (β-ME) and 1000 U/ml leukemia inhibitory factor (LIF, ESGRO, Chemicon/Merck Millipore, Billerica, MA, USA). Unless otherwise specified, all cell culture reagents were obtained from Life Technologies (Carlsbad, CA, USA). Both stem cell lines iPSC and ESC were used in the experiments starting from the passage 5.

AggreWell™400 (Stem Cell Technologies) plates were used to start cardiomyocyte differentiation in differentiation medium. It consists of Iscove's modified Dulbecco's medium containing 20 % FBS, 1x NEAA, 50 μM β-ME. These plates contain microwells (400 μm in size) which cause the pluripotent stem cells (iPSC and ESC) to aggregate into EBs. The procedure was done accordantly to the manufacturer's instructions from the AggreWell manual. Briefly 0.5 ml of differentiation medium was placed into each well of an AggreWell™400 plate, centrifuged at 2000g for 10 min in a swinging bucket rotor that was fitted with a plate holder to remove any small bubbles from the AggreWell plates. PSC were added to each well at concentrations of 3×10^5 cells/ml, 6×10^5 cells/ml, 9×10^5 cells/ml, 1.2×10^6 cells/ml and 2.4×10^6 cells/ml to make EBs with 250, 500, 750, 1000, and 2000 cells respectively. From each well of the AggreWell™400 plate we obtained 1200 EBs. The AggreWell™400 plate was centri-

fuged at 200 g for 3 min to capture the cells in the wells. EBs were maintained under standard CO_2 incubator conditions (37 °C, 5 % CO_2) for 48 h.

EBs were counted under a light microscope Axiovert 10 (ZEISS, Germany) after 2 days of cultivation. Then they were transferred into Petry dishes with fresh differentiation medium and maintained on a shaker GFL 3006 (GFL, Braunschweig, Germany) under continuous horizontal agitation. Differentiation was continued without medium change until the 9th day and afterwards the medium was changed every 2–3 days.

Efficiency of cardiac differentiation was analyzed by determining beating EBs and the fraction of eGFP-positive cardiomyocytes on days 6, 9, 11, 13 and 15 of differentiation by flow cytometry and fluorescence microscopy. EBs were examined using Zeiss Axiovert 200M fluorescence microscope and analyzed with Zeiss Axiovision 4.5 software (Carl Zeiss, Jena, Germany). Single cell suspension was prepared for flow cytometry. Cells were analyzed by FACScan (BD Pharmingen). Cell debris was gated out and 10000 events were acquired for analysis. The presence of dead cells was determined by propidium iodide staining (Sigma, Germany). Data analysis was performed using FSC Express 4 Flow Research Edition (De Novo Software, USA) software.

The data on cell numbers are represented as mean ± standard deviation for 3 samples. Statistical significance was determined using the Student t-test at $P < 0.05$.

Results and Discussion

Pluripotent stem cells, which include ESCs and iPSCs, have the ability to differentiate into the cells of all three germ lineages including ectoderm, mesoderm and endoderm. A lot of differentiation protocols start with the formation of 3-dimensional aggregates of cells called embryoid bodies. The formation of EBs heterogeneous in size and shape is a cause of inefficient and uncontrolled differentiation. In order to get homogeneous population of EBs, the AggreWell plates were used, each well of which

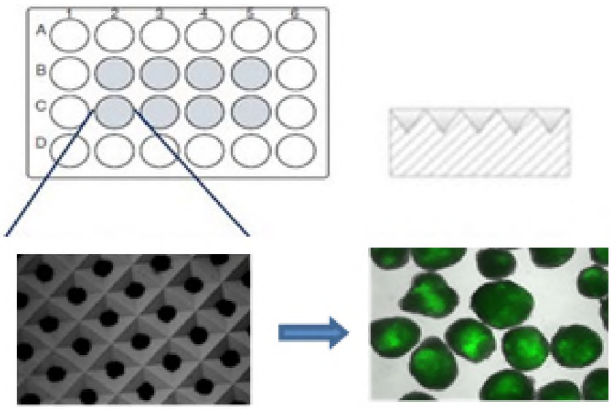

Fig. 1. AggreWell™ contains microwells to make uniform cell aggregates

contains a certain number of microwells (Fig. 1). After transferring the single cell suspension, EBs of a defined size are formed.

The plates mentioned above provide the opportunity to get EBs of different sizes - from 250 to 2000 cells per EB. At the first stage the efficiency of differentiation into cardiomyocytes was tested with EBs of 250, 100 and 2000 cells/EB. The iPSC cell line AT25 was used. Homogeneous EBs of identical size were formed in each well of AggreWell plates on the second day of differentiation (Fig. 2).

However, EBs formed from 2000 cells had irregular form, without clearly defined smooth edges, with dark opaque color. On the 3d day of differentiation it was clear that most of EBs formed from 2000 cells were destroyed. The amount of nonviable cells was more than 95 %.

EBs formed with 250 and 1000 cells had typical round shape. They had the surface layer formed with ectodermal cells and the inner layer with endodermal cells. The amount of differentiated cells was higher in EBs formed with 1000 cells. The first GFP+ cells were observed on the 8-th day of differentiation. The flow cytometry analysis showed that on the 9-th day of differentiation the amount of cardiomyocytes was 0.19 ± 0.02 % of cell population, on the 11-th day the amount was 0.58 ± 0.01 % of GFP+ cells. The highest number of differentiated cells was observed on the 13-th day of differentiation. It was 0.60 ± 0.03 % of eGFP+ cells. The

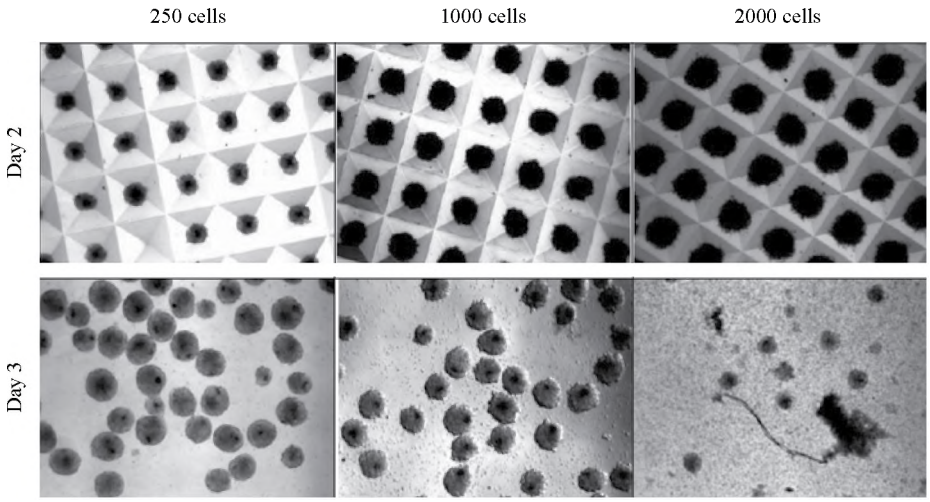

Fig. 2. Embryoid bodies of different size in AggreWell plates on the 2[d] and 3[d] days of differentiation, x4.

amount of cardiomyocytes identified in EBs formed with 250 cells was growing from the 9-th until the 13-th day of differentiation: 0.04 ± 0.01 % of eGFP+ cells were identified on the 9-th day, 0.19 ± 0.01 % – on the 11-th day and 0.34 ± 0.02 % – on the 13-th day. There were no eGFP+ cells in EBs formed with 2000 cells.

Differentiation of PSC into cardiomyocytes obtained from EBs in the range from 500 cells to 750 cells per EB was more efficient (Fig.3, 4).

It was found that the efficiency of differentiation of EBs with 250 and 1000 cells is lower compared with EBs of 500 and 750 cells. So, on the 13-th day of differentiation when the highest amount of cardiomyocytes was obtained, the number of eGFP+ cells derived from EBs of 500 cells was 8.5 times higher compared to EBs of 250 cells (2.86 ± 0.30 % of the cardiomyocytes per 500 cells EBs versus 0.34 % of eGFP+ cells per EBs containing 250 cells); when compared with 1000 cells EBs the difference was 4.7 times higher (the amount was 0.60 ± 0.03 % of differentiated cells). The difference between EBs of 750 cells and EBs of 250 and 1000 cells was higher by 6.4 and 3.6 times, respectively (the number of eGFP+ cells of the first EB size was 2.16 ± 0.02 %, for 250 cells EBs - 0.34 ± 0.02 %, and for 1000 cells EBs – 0.60 ± 0.03 %).

Comparison of the efficiency of differentiation of EBs formed with 500 and 750 cells per EB revealed that the difference between them varied from 1.3 times

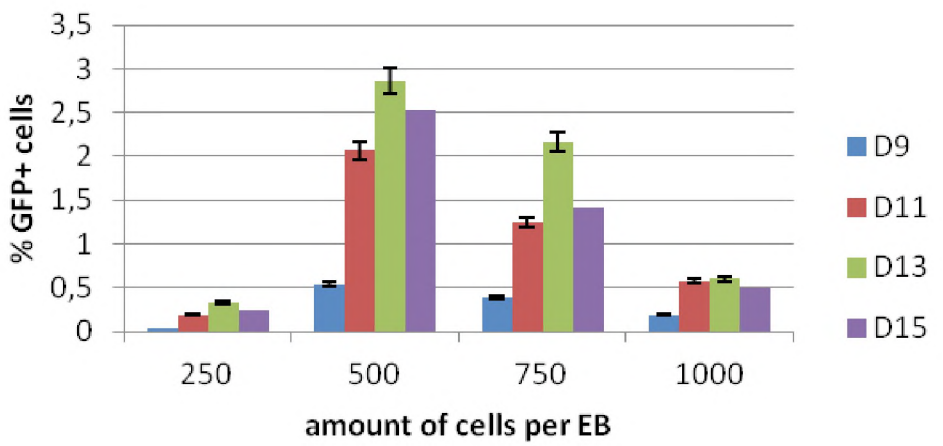

Fig. 3. The efficiency of cardiomyocyte differentiation depending on the size of EBs.

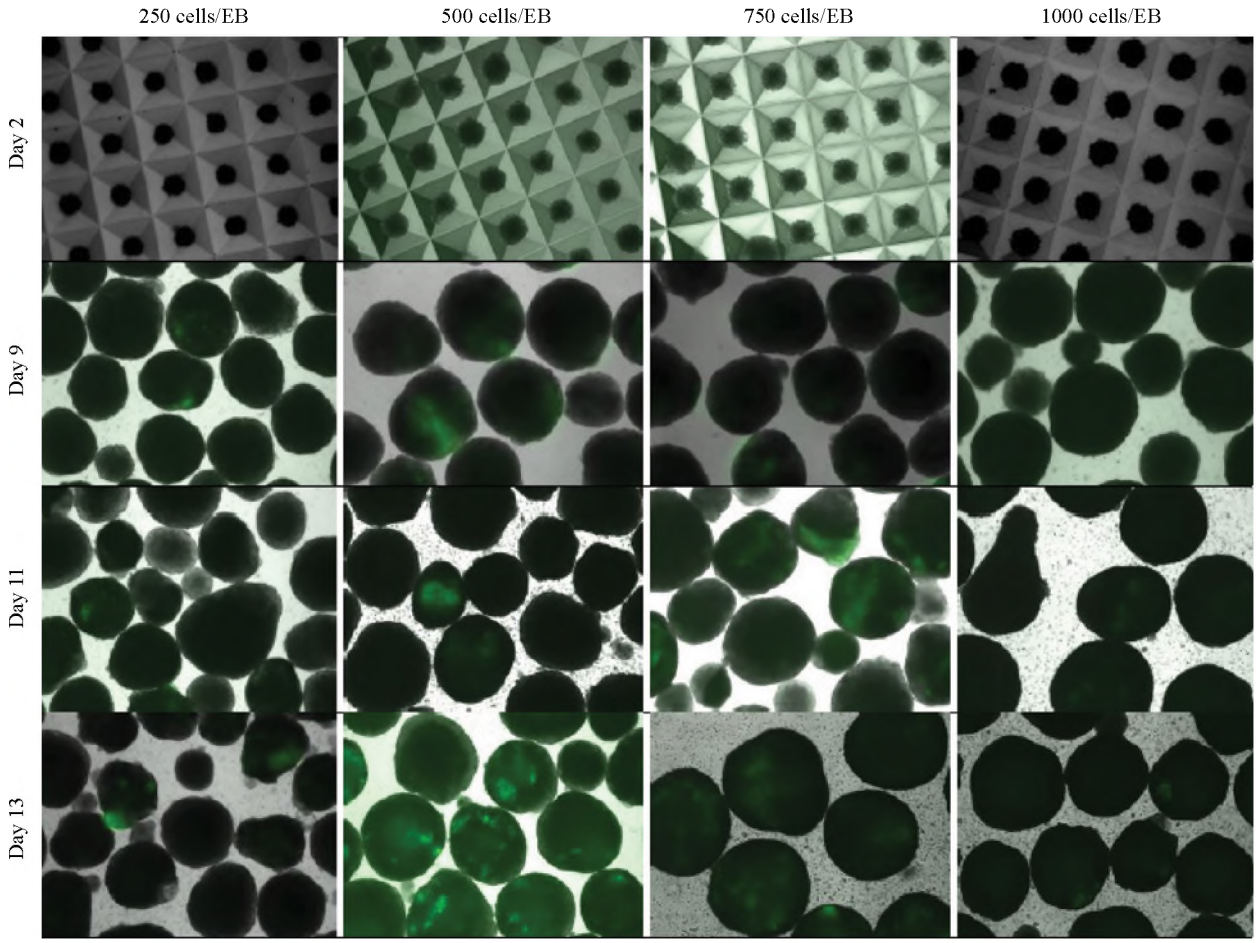

Fig. 4. iPSCs differentiation into cardiomyocytes with AggreWell plates (fluorescence microscopy, x4)

on the 13-th day of differentiation to 1.8 times on the 15-th day. For EBs formed with 500 cells the amount of cardiomyocytes was 2.07 ± 0.01 % on the 11-th day, 2.86 ± 0.30 % on the 13th day and 2.53 ± 0.04 % on the 15th day of differentiation. The number of cardiomyocytes derived from EBs formed with 750 cells was significantly lower ($P < 0.05$). It was 1.25 ± 0.01 % on 11-th day, 2.16 ± 0.02 % on the 13th day and 1.41 ± 0.03 % on the 15th day of differentiation.

Thus, the experiments show that the size of EBs significantly affects the efficiency of cell differentiation. The highest percentage of eGFP+ cells was obtained from EBs formed with 500 cells.

No eGFP+ cells or beating areas were observed when AggreWell plates were used for the ESCs line differentiation. EBs of different size and shape were identified on the 2-d day of differentiation (Fig. 5). According to our previous results [17] the murine ESC line had high ability to differentiate into cardiomyocyte by a "hanging drop" method (EBs are attached to the gelatin-covered Petry dishes) and by a mass culture method (EBs aggregate spontaneously by cultivation in nonadherent Petry dishes). During the first 2 days EBs in AgreWell plates form regular EBs similar to those obtained by the "hanging drop" method; starting from the third day they are cultivated in suspension likely to the mass culture method. Therefore, we assume that the AgreWell method is linespecific and is insufficient to produce cardiomyocytes from the applied ESC cell line.

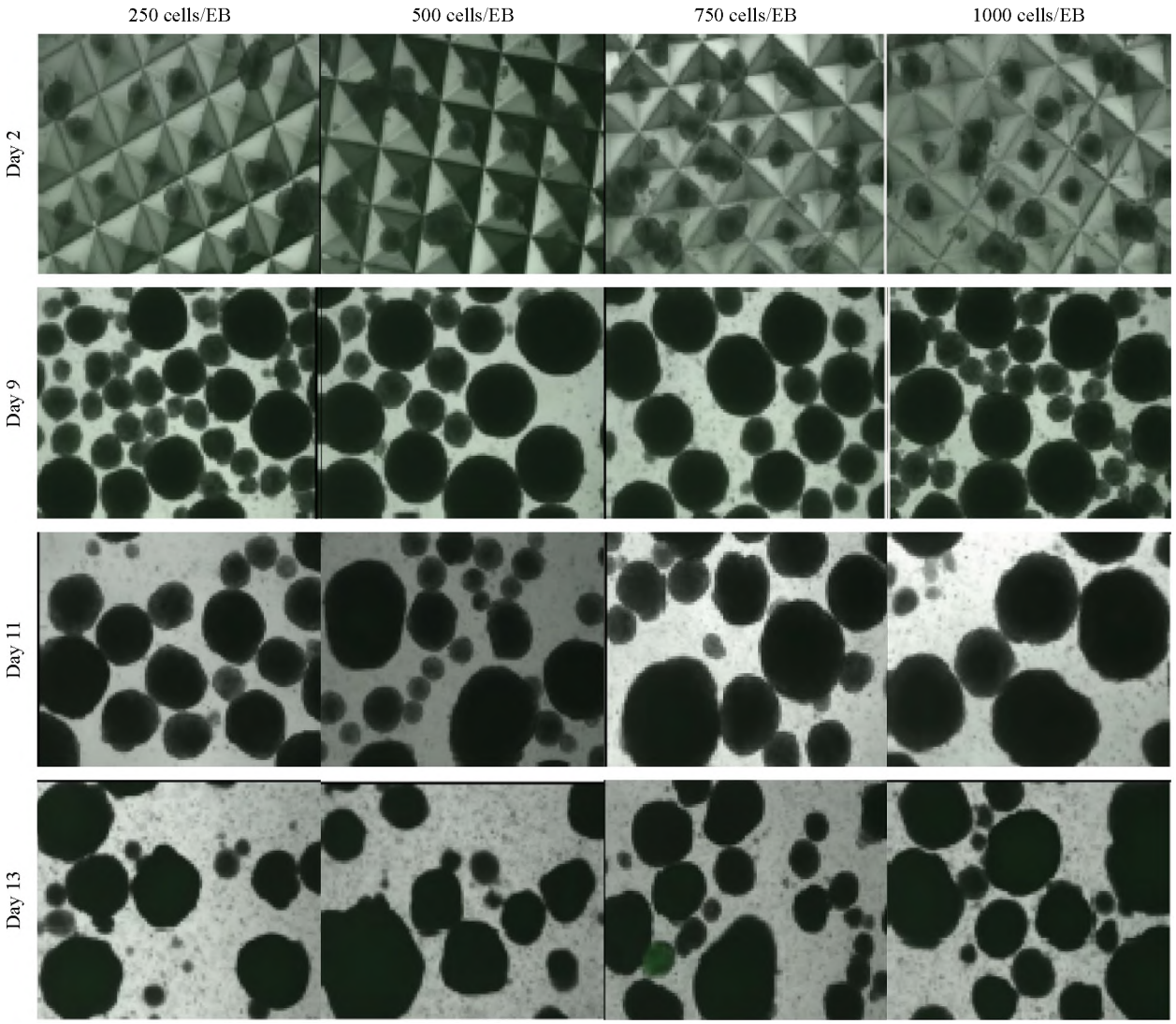

Fig. 5. ESC differentiation into cardiomyocytes with AggreWell plates

According to the outcome of our work it is possible to improve the efficiency of differentiation in cardio direction if the size of EB changes. It is known that for the normal embryonic development of the cardiovascular system different gradients of signaling molecules are required. For example, the factors secreted from neighboring lateral endoderm, such as BMP, stimulate cardiogenesis, whereas the canonical Wnt signals from neighboring neuroectoderm inhibit cardiogenesis of mesoderm [18]. Therefore, the ratio of the endoderm and neuroectoderm can affect the processes of differentiation. The size of EBs, in turn, can affect the value formed by different cell layers.

EBs are the aggregates formed from PSC that are covered with endodermal cells on the surface, containing nucleus formed from ectodermal cells in the middle of it, and a layer of mesodermal cells between two other layers. Modifying the EB size we change the amount of endoderm on its surface. So we can assume that increasing the number of endoderm cells we increase the number of mesoderm

cells. In turn mesoderm cells are transformed into cardiomyocytes by the factors secreted by endoderm cells. So we modulate cell position relatively to each other by changing the seeding density of cells. Moreover EB size, which depends mainly on the initial amount of PSC, forming EB, influences such parameters as diffusion of soluble adhesion molecules and intercellular interactions. Therefore, it is possible that EBs, containing 500 cells have the most appropriate ratio of the surface covered with endoderm cells to the surface of mesoderm cells with which they can interact. At the same time larger EBs become unable to support diffusion of cardiogenic factors, at the sufficient level for differentiation.

Conclusions

Modifying such parameter as EB size can beneficially affect the differentiation of PSC into cardiomyocytes. Among EBs formed within the range from 250 cells to 2000 cells per EB the highest percentage of eGFP + cells was obtained from EBs formed with 500 cells.

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Диференціація плюрипотентних стовбурових клітин в кардіоміоцити залежить від розміру ембріоїдних тілець

Г. В. Будаш, Д. І. Білько, Н. М. Білько

Мета. Знайти зв'язок між розміром ембріоїдних тілець та ефективністю диференціювання плюрипотентних стовбурових клітин в кардіоміоцити. **Методи.** Трансгенні клітинні лінії індукованих плюрипотентних клітин AT25 та ембріональних стовбурових клітин D3 αPIG44 диференціювали в кардіоміоцити в AggreWell планшетах. Вище згадані планшети містять мікролунки, які дають можливість сформувати ембріоїдні тільця з плюрипотентних стовбурових клітини певного заданого розміру. Обидві клітинні лінії були генетично модифіковані і експресували IRES-фланкированый зелений флуоресцентний білок (eGFP), під контролем кардіоспецифічного α-MHC промотера. Для перевірки ефективність процесів диференціювання були застосовані методи проточної цитометрії та флуоресцентної мікроскопії. **Результати.** Було встановлено, що ефективність диференціювання ембріоїдних тілець отриманих з лінії індукованих плюрипотентних клітин лінії AT25 розміром 250 і 1000 клітин менша в порівнянні з ембріональними тільцями сформованими з 500 і 750 клітин. Кількість eGFP+ клітин, отриманих з ембріоїдних тілець розміром 500 клітин була 8,5 разів більшою ніж в порівнянні з ембріональними тільцями розміром 250 клітин (що становило 2,86 ± 0,30 % кардіоміоцитів для ембріоїдних тілець розміром 500 клітин, і лише 0,34 % eGFP+ клітин для ембріоїдних тілець розміром 250 клітин). **Висновки.** Впливати на ефективність диференціювання плюрипотентних стовбурових клітин в кардіоміоцити можна змінюючи початковий розмір ембріоїдних тілець. Серед ембріоїдних тілець, утворених в діапазоні від 250 до 2000 клітин, найвищий відсоток eGFP + клітин отримували з ембріоїдних тілець, утворених 500 клітинами.

Ключові слова: плюрипотентні стовбурові клітини, індуковані плюрипотентні стовбурові клітини, ембріоїдні тільця, кардіоміоцити, диференціювання.

Дифференцировка плюрипотентных стволовых клеток в кардиомиоциты зависит от размера эмбриоидных телец

Г. В. Будаш, Д. И. Билько, Н. М. Билько

Цель. Найти связь между размером эмбриоидных телец и эффективностью дифференцировки плюрипотентных стволовых клеток в кардиомиоциты. **Методы.** Трансгенные клеточные линии индуцированных плюрипотентных клеток AT25 и эмбриональных стволовых клеток D3 αPIG44 дифференцировали в кардиомиоциты в AggreWell планшетах. Упомянутые планшеты содержат микролунки, которые дают возможность сформировать эмбриоидные тельца из плюрипотентных стволовых клеток определенного заданного размера. Обе клеточные линии были генетически модифицированы и экспрессировали IRES-фланкированый зеленый флуоресцентный белок (eGFP), под контролем кардиоспецифического α-MHC промотера. Для проверки эффективности процессов дифференцировки были применены методы проточной цитометрии и флуоресцентной микроскопии. **Результаты.** Было установлено, что эффективность дифференцировки эмбриоидных телец полученных из линии индуцированных плюрипотентных клеток AT25 размером 250 и 1000 клеток меньше по сравнению с эмбриональными тельцами сформированными из 500 и 750 клеток. Количество eGFP + клеток, полученных из эмбриоидных телец размером 500 клеток была 8,5 раз больше чем по сравнению с эмбриональными тельцами размером 250 клеток (что составляло 2,86 ± 0,30 % кардиомиоцитов для эмбриоидных телец размером 500 клеток, и только 0,34 % eGFP+ клеток для эмбриоидных телец размером 250 клеток). **Выводы.** Изменение первоначального размера эмбриоидных телец влияет на эффективность дифференцировки плюрипотентных стволовых клеток в кардиомиоциты. Среди эмбриоидных телец, образованных в диапазоне от 250 до 2000 клеток, высокий процент eGFP + клеток получали из эмбриоидных телец, образованных 500 клетками.

Ключевые слова: плюрипотентные стволовые клетки, индуцированные плюрипотентные стволовые клетки, эмбриоидные тельца, кардиомиоциты, дифференцирование.

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