

## COMPARISON OF METHODS FOR CARDIOMYOCYTE DIFFERENTIATION OF MURINE EMBRYONIC STEM AND INDUCED PLURIPOTENT CELLS

*Hanging drop and mass culture methods for differentiation of genetically modified embryonic stem cells line aPIG44 and induced pluripotent cell line At25 towards cardiomyocytes were compared. Both cell lines express enhanced green fluorescent protein (GFP) under the control of  $\alpha$ -myosin heavy chain promoter. FACS analysis and fluorescent microscopy were used to investigate the effectiveness of both methods. As a result the differentiation curve for 2 types of cell lines was obtained. The maximum rate of GFP-positive beating cardiomyocytes was reached on day 11. Attachment of cells during differentiation by hanging drop method on day 2 was inferior if compared to day same parameters on day 5 and earlier attachment prolonged the overall process of differentiation. It was figured out that hanging drop method produced large beating areas in comparison with mass culture method, but could not be applied for large scale production. Mass culture method produced higher yield of embryoid bodies, but due to the heterogeneity of formed spheroids the effectiveness of differentiation of individual embryoid body was lower.*

**Keywords:** cardiomyocytes, induced pluripotent stem cells, hanging drop method, mass culture method, embryoid bodies.

### Introduction

Heart disease and failure are often characterized by a loss of functioning cardiomyocytes, which are terminally differentiated and show a very limited capacity for regeneration. Cardiac transplantation is currently the treatment of choice for end-stage heart failure; however, the number of available donors limits this treatment option for majority of patients. The development of new therapeutic paradigms for heart failure and alternative therapies, such as cardiac cell replacement, has therefore become imperative [1].

Cardiac replacement therapy can be done with the help of embryonic stem (ES) cell. ES cells can differentiate into derivatives of all three primary germ layers, mesoderm, ectoderm and ectoderm from which various cell types including cardiomyocytes can be differentiated depending on the on the cultivation conditions [2]. However the development of ES cells for regeneration therapy has two main disadvantages. First of all, ethical considerations make must be take in to account while new human ES cell lines are generated, as they are derived from preimplantation human embryos. Second, ES cells do not show the autologous genotype of patients [3].

Both this disadvantages can be solved with the methodology of induced pluripotent stem (iPS) cells. Recently, new insights in iPS cell technology showed that differentiation and lineage commitment are not irreversible processes and this has opened

new avenues in stem cell research. Hence, culture systems for expansion and differentiation of iPS cells can also apply methodologies developed with ES cells, although direct evidence of their use for iPS cells is still limited and needs further investigation [3].

The most popular method for cardiomyocyte differentiations in laboratories is the formation of embryoid bodies (EB), an aggregates of ES cells that mimic early stages of the embryonic development. Generally, there are two main ways to induce EB formation: in hanging drops and in liquid suspension culture in nonadherent dishes on the orbital rotary shaker called mass culture [4].

Usually the hanging drop method is composed of two steps; the aggregation of ES cells in drops and maturation of aggregates to EBs in suspension culture using low adherence bacterial Petri-dishes. Several elements of the method may be troublesome such as losses of EBs during picking up the formed EBs by pipette and attachment of premature EBs on Petri-dishes [5].

Orbital rotary shakers have been used to produce EBs as the constant circular motion provided by this simple system is good for improving the efficiency of EB formation. The advantages of this technique include accommodation of cell culture dishes on the rotary platform, easily allowing production of numerous parallel samples and allowing comparison of different experimental parameters [5].

The quality of formed EBs effects further differentiation occurring in EBs afterwards; this fea-

ture differs between EBs depending on the methods, because culture conditions such as cell density, culture period, and culture vessel are not the same. Heterogeneity in the quality of EBs may have detrimental effects on the synchronism of differentiation. To guarantee the homogeneity of EBs a reliable method is required for EB formation with reproducibility [4].

## Materials and methods

### Cell culture

Transgenic murine embryonic stem (ES) and induced pluripotent stem (iPS) cell lines expressing enhanced green fluorescent protein (EGFP) under the control of  $\alpha$ -myosine heavy chain ( $\alpha$ -MHC) promoter ( $\alpha$ -MHC-EGFP) were used in our experiments. iPS cell lines AT25 was derived by Azra Fatima and coworkers in the laboratory of Tomo Saric. The murine ESC line D3- $\alpha$ PIG44 [6] was used as a control was modified by Kolossov. iPS and ES cells were cultivated on murine embryonic fibroblasts (MEFs) to maintain the undifferentiated state in Dulbecco modified Eagle medium with 15 % fetal calf serum, 1 % nonessential amino acids, 0.1 mM  $\beta$ -mercaptoethanol (all purchased from Invitrogen, Karlsruhe, Germany), and 1000 U/ml LIF (Esgro<sup>®</sup>; Millipore, Billerica, MA, USA). MEFs were prepared from Him:OF1 outbred mice at embryonic day (E)14.5 and inactivated by mitomycin C treatment (10  $\mu$ g/ml; SERVA Electrophoresis, Heidelberg, Germany). Next, iPS and ES cells were passaged every 2 d; cells were trypsinized (0.05 %) and counted, and then  $5 \times 10^5$  cells were added to a 6-cm dish with preplated growth arrested MEFs ( $0.8 \times 10^5$  feeder cells/6-cm dish).

### Differentiation toward cardiomyocytes Hanging drop method

ES and iPS cells were differentiated as three-dimensional multicellular EBs using the hanging drop method. Briefly, upon reaching ~ 70 % confluence, ES cells were trypsinized and a single cell suspension of  $2.5 \times 10^4$  cells/ml was prepared in EB medium consisting of Iscove's Modified Dulbecco's Medium supplemented with 20% fetal calf serum, 1 % nonessential amino acids, 0.1 mM  $\beta$ -mercaptoethanol (all purchased from Invitrogen, Karlsruhe, Germany). ES or iPS cells were aggregated in hanging drops containing 500 cells/20  $\mu$ l suspension on the inner surface of the lid of a 10 cm bacteriological non-adherent Petri dish filled with 5 ml of phosphate buffered saline to prevent the evaporation of the drops and placed in the humidified incubator with 5 % CO<sub>2</sub> in the atmosphere, at the temperature of 37°. After two days the multicellular aggregates were transferred into a new bacteriological dish using EB medium and cultured

in suspension until day 5. On day 5 they were transferred to dishes coated with 0,1 % gelatin and cultured for a further 8 days as adherent EBs. Otherwise multicellular aggregates were attached on 0,1 % gelatin covered Petri dishes on day 2. Monitoring of the beating of EBs was performed from day 6 until day 15. On day 9 medium change was performed.

Appearance of one beating colony in 1 EB was counted as one beating area despite of the location and the size of the beating area within the EB.

### Mass culture method

Cardiac differentiation of the iPS and ES cells was performed in mass culture. Confluent undifferentiated ES or iPS cells were trypsinized into a single cell suspension. Suspension with  $1 \times 10^6$  cells per 13 ml of EB medium composed of Iscove's modified Dulbecco's medium supplemented with 20 % fetal calf serum, 1 % nonessential amino acids, 0.1 mM  $\beta$ -mercaptoethanol (all purchased from Invitrogen, Karlsruhe, Germany) were placed per 1 10 cm<sup>2</sup> bacteriological nonadherent Petri dish and maintained on a horizontal shaker to allow EB formation. After 2 days they were diluted to ~2000 embryonic bodies/10cm<sup>2</sup> dish, and maintained on the shaker until day 15.

### Microscopy and fluorescent microscopy

Apotome Axiovert Zeiss microscope with IMC optics, FITC filter set (AF Analysentechnik, Stuttgart, Germany) and x4 or x10 objectives was implemented for monitoring of differentiation process. Canon EOS 300D was used as capture device.

### Flow cytometry

Single cell suspension was prepared by trypsinization. Cell clumps were removed by passing through cell filter strainer cap of a round bottom tube from Falcon<sup>®</sup> (BD, Heidelberg, Germany). Propidium iodide (PI) staining (Sigma) was included to exclude dead cells. Acquisition of 10 000 live (PI negative) cells was made with FACScan (BD Biosciences), and the data analysis was carried out with CellQuest software (BD).

### Results

First the formation of EBs from iPS and ES cell line by mass culture method was compared. Both cell lines express enhanced green fluorescent protein (GFP) under the control of  $\alpha$ -myosine heavy chain promoter. The analysis of cardiomyocyte differentiation was made by monitoring of spontaneous contraction using bright field and fluorescent microscopy, and FACS analysis of GFP<sup>+</sup> cells. Both cell lines started to show spontaneous contraction

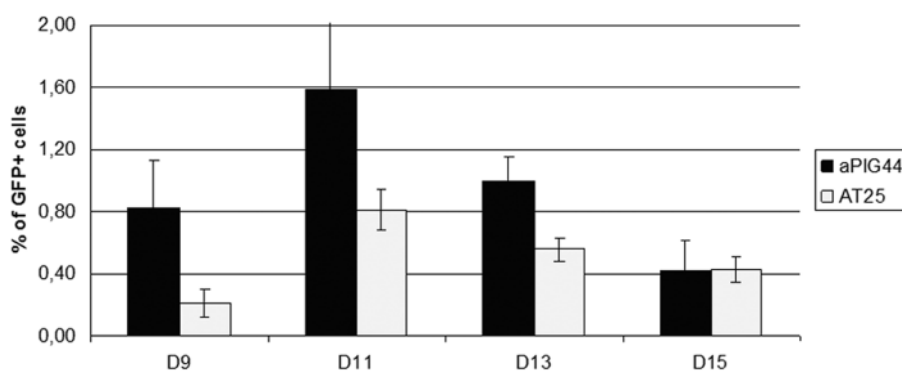


Fig. 1. Percentage of GFP<sup>+</sup> cells obtained by FACS analysis

from day 8. Amount of beating EBs with GFP<sup>+</sup> cells increased and reached its maximum until day 11 (see fig. 1). The percentage of GFP<sup>+</sup> cells measured by FACS on day 11 was  $1,6 \pm 0,6$  for aPIG44 cell line. Maximum numbers of beating cells for iPS cell line was also measured on day 11, but was lower in comparison with ES cell line. It's rate was  $0,8 \pm 0,13$  % of GFP<sup>+</sup> cells. After day 11 the number of beating EBs slowly decreased. But some GFP<sup>+</sup> cells could still be detected even after a month of cultivation in EB medium.

On day 3 both cell lines differentiated by mass culture method formed heterogeneous population of EBs with different size and form of EBs (see fig. 2). But formation of EBs by iPS cell line was more homogeneous in comparison to ES cell line. On latter stages of differentiation towards cardiomyocytes, clearer differences in EB heterogeneity could be observed, especially in aPIG44 cell line. Heterogeneity of the size and form of EBs can be the reason for big variation of differentiation rate of aPIG44 cell line.

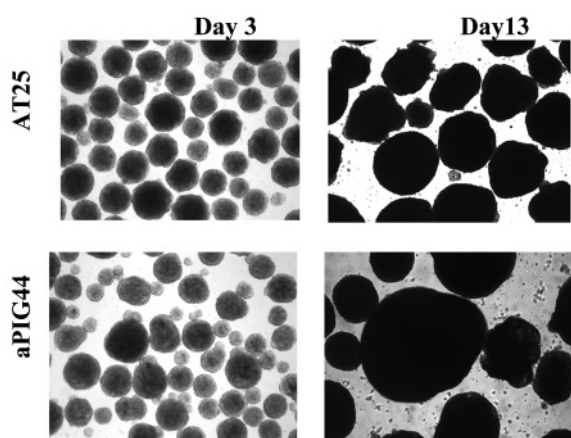


Fig. 2. Comparison of the morphological appearance of EBs formed by ES cell line aPIG44 and iPS cell line AT25 on day 3 and day 13 of differentiation by mass culture method, magnification x4

Next step was to compare differentiation by hanging drop method. The appearance of beating areas started on day 7 for both cell lines in the experiments where EBs were transferred on gelatin-covered plates on day 5. Until day 11 most of EBs started contract spontaneously and had at least one beating area per EB (see fig. 3). Beating areas of PIG44 cell line covered the whole EB and synchronous contraction of hole EB could be easily seen under light microscopy. For AT25 cell line most common was presence of several small beating areas with different frequency of contraction within the same EB. The amount of beating areas had a tendency to decrease slightly after day 11, but the difference was not significant. Such tendency was admitted not only for iPS cell line, but for ES cells also.

The rate of differentiation was high in aPIG44 cells in comparison with AT25 cell line. Beating areas could be observed in  $68 \pm 4,7$  % of EBs formed from the aPIG cell line. Amount of produced cells with spontaneous contraction by AT25 cell line was at  $45 \pm 3,7$  %. Such fact means that AT25 cell line has lower cardiomyocyte differentiation potential then established ES cell line, and requires modification of differentiation method in order to produce a large numbers of active cardiomyocytes.

Time when EBs attached to the surface dramatically influenced the amount of differentiated cardiomyocytes. In both cell lines there was a tendency that EBs attached on earlier stages (on day 2) produced less cardiomyocytes. EBs formed by aPIG44 cell line started to form first beating areas only on day 8, and the peak of EBs with beating areas was reached only on D13, that means that attachment of not completely formed EBs on earlier stages prolonged the differentiation process and makes the protocol more time-consuming. For iPS cell line AT25 the tendency for prolonged differentiation after earlier attachment was also observed, but the difference between EBs with spontaneous contractility on day 11, 13 and 15 was not significant.

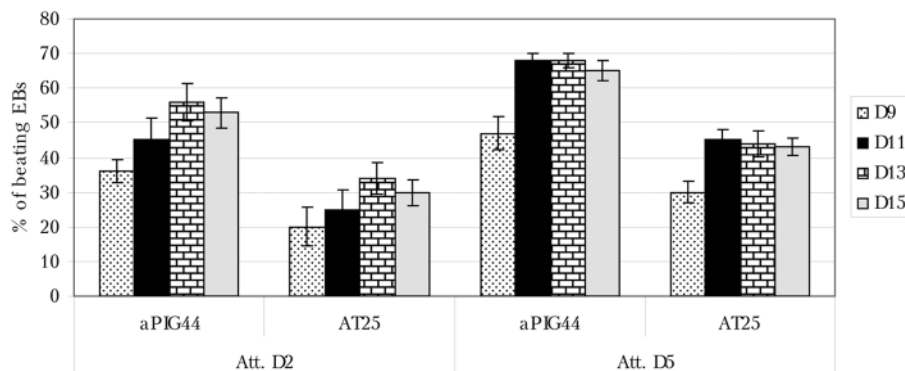


Fig. 3. Percentages of beating areas in EB formed by hanging drop method

### Discussion

Hanging drop and mass culture methods introduced for cell differentiation into cardiomyocytes were developed for embryonic stem cell lines. Application of both of these methods for differentiation of murine iPS cells lead to formation of EBs. GFP<sup>+</sup> cells with spontaneous contractility under appropriate conditions were present in obtained EBs. Such result means that both methods used in experimental work can be applied for iPS cell differentiation into cardiomyocytes. But obtained results show that AT25 cell line produce less cardiomyocytes under standard differentiation conditions. In order to get more cells for heart recovery therapy new modification of protocol should be applied.

The percentage of beating EBs formed by hanging drop method was higher than formed by mass culture method. Even though hanging drop method is commonly used to prepare uniform-sized EBs, this method has disadvantages in the mass preparation of EBs due to its labor-intensive procedure. Mass culture EB generation is easier for production of the large amounts of EBs. One drawback of this method, however, is that the EBs often fuse together to form large aggregates, which leads to formation of heterogeneous population of EBs. This has negative effects on cell proliferation and differentiation, as well as causing extensive cell death. But mass culture method can easily allow production of nu-

merous parallel samples what can be used for comparison of different experimental parameters.

### Conclusions

Differentiation of iPS cell line towards cardiomyocytes by hanging drop and mass culture methods is comparable with differentiation of established ES cell line.

Attachment of EBs on early stages of differentiation prolongs the whole process of differentiation towards cardiomyocytes and leads to less effective differentiation.

Amount of EBs with beating areas produced with hanging drop method is significantly higher when compared to the mass culture method, but this method is very time and labor consuming, and could not be applied for large scale production of cardiomyocytes.

Heterogeneity of produced population of EB by mass culture method results in decrease of its effectiveness.

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## ПОРІВНЯННЯ МЕТОДІВ ДИФЕРЕНЦІЮВАННЯ В КЛІТИНИ СЕРЦЯ ЕМБРІОНАЛЬНИХ СТОВБУРОВИХ ТА ІНДУКОВАНИХ ПЛЮРИПОТЕНТНИХ КЛІТИН МИШІ

Було порівняно метод висячої краплі та метод суспензійної культури для диференціації ембріональної стовбурової та індукованої плюрипотентної лінії в кардіоміоцити. Обидві лінії є генетично модифікованими та експресують зелений флуоресцентний протеїн (GFP) під контролем  $\alpha$ -MHC промотера. Для перевірки ефективності диференціації було застосовано методи проточної цитофлуориметрії та флуоресцентної мікроскопії. В результаті отримано криву диференціації двох типів клітин. Максимальний рівень GFP позитивних кардіоміоцитів, що скорочуються, зафіксовано на 11-й день диференціювання. При застосуванні методу висячої краплі прикріплення клітин під час диференціації на більш ранніх стадіях (на 2-й день на відміну від 5-ого дня диференціювання) пролонгує процес диференціації. Встановили, що метод висячої краплі продукує ділянки, що скорочуються, щодо великого розміру. Однак цей метод не може бути застосований для масового виробництва кардіоміоцитів. Методом культивування в суспензійній культурі можна створити більшу кількість ембріональних тіл, проте їх гетерогенність зменшує ефективність диференціації кожного окремого ембріонального тіла.

**Ключові слова:** кардіоміоцити, індуковані плюрипотентні клітини, метод висячої краплі, метод суспензійної культури, ембріональні тільца.

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## ПРОТИПУХЛИННА АКТИВНІСТЬ ЕМБРІОНАЛЬНИХ ПРОТЕЇНІВ КУРКИ У МИШЕЙ З КАРЦИНОМОЮ ЛЕГЕНІ ЛЬЮЇС

На моделі метастазуючої карциноми легені Льюїс показано, що найбільший протипухлинний та антиметастатичний ефект застосування ембріональних протеїнів курки спостерігається при їх введенні на 1-шу, 7-му та 14-ту доби після перещеплення або видалення пухлини.

**Ключові слова:** ембріональні протеїни курки, протипухлинна активність, антиметастатична активність.

Застосування протипухлинних вакцин (ПВ) є одним із потенційно потужних засобів біотерапії раку. Сьогодні за різними технологіями розроблена ціла низка ПВ [1, 2], значна кількість з яких проходить клінічні випробування, проте ефективність їх застосування залишається недостатньо високою. Однією з імовірних причин цього є толерантність імунної системи онкохворого до пухлинних антигенів, які є власними і в

переважній більшості немудованими білками організму [3]. Порівняно недавно в науковій літературі з'явилися повідомлення про те, що використання як ПВ ксеногенних гомологів пухлинних антигенів чи білків-учасників канцерогенезу здатне подолати імунологічну толерантність до відповідних білків організму хворого [3–5]. Насправді, за здатністю індукувати протипухлинну імунну відповідь ксеногенні гомологи