BIOSCAFFOLD FROM MOUSE EMBRYONIC FIBROBLAST MAINTAINS THE PLURIPOTENCY OF MOUSE EMBRYONIC STEM CELLS

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ABSTRACT

Cell culture using a 3D method provides a similar condition to the internal body environment. Various cell culture strategies have been developed using synthetic or biological materials; most existing publications use many reagents. Bioscaffold from mouse embryonic fibroblast (MEF) enhances cell attachment, interaction, and production of growth factors. Since bioscaffolds could maintain and stimulate pluripotency of stem cells, we conducted this study to prove bioscaffold function. Bioscaffold was prepared from MEF cultured in DMEM complete medium supplemented with dextran sulfate and L-ascorbic acid to increase extracellular matrix production. This medium acts as an embryo stem cell (ESC) culture medium. We used a Tali-cytometer to identify and quantify stem cells based on Sox2 and Oct4 proteins, markers of stemness. ESC culture using bioscaffold maintained the pluripotency of ESC as indicated by the presence of Oct 4 and Sox2 as ESC markers compared to MEF culture. From this research, the bioscaffold from MEF can be developed as media for ESC to improve propagation. Furthermore, it is a model for tissue engineering and *in vitro* organ development.

Keyword : Bioscaffold, Stem Cell, Mouse Embryonic Fibroblast, Decellularization, Oct4, Sox2

INTRODUCTION

Stem cells are unique cells in multicellular organisms, which can be developed into various types of cells and renew themselves. Stem cells are obtained from embryonic and adult cells. Embryonic stem cells (ESCs) are pluripotent cells that can multiply and produce all types of cells in the body. Stem

cells have three types: multipotent, pluripotent, and totipotent (Slack, 2021). Stem cells are important in medicine because they can repair themselves, showing their potential medical application in repairing tissues and organs (Chouw et al., 2018; Ulloa et al., 2005). A stem cell can be defined based on two properties, its possible limitless self-renewal and pluripotency and its ability to differentiate one or more specialized cell types. A protein marker important for pluripotent stem cells, i.e., "nuclear transcription factors," consisting of Oct3/4, Sox2, Klf, and Nanog (Zhao et al., 2018). Pluripotency of stem cells is formed from mouse embryonic fibroblasts (MEF) by combining the expression of four factors, namely Oct4, c-Myc, Sox2, and Klf4; this type of pluripotent cell is called induced pluripotent stem cell (Takahashi et al., 2006). As a supporting factor of stem cells, the biomaterial of the scaffold is physiologically safe.

So it is given as a drug to the patient that easily blends with the area to be repaired. It provides tissue integrity for cell attachment, interaction, and growth factors (Y & Ragunath, 2011). In addition, bioresorbable inorganic materials, polymers (natural and synthetic), and their hybrids, respectively, have also been developed as scaffolds. In this study, decellularized tissues serve as scaffold biomaterials that can boost structural, mechanical, and biological properties. This method shows excellent application potential in tissue engineering, which can be implanted directly in a patient with defective cells (Sandra et al., 2019).

Molecular crowding is a method used to accelerate the biochemical reaction by inserting inert macromolecules in a solution to occupy a significant volume of medium (Minton, 2019). Studies of crowding conditions using macromolecules might affect the structure of conformational stability, binding of small molecules, protein folding and shape, enzymatic activity, the interaction of the protein with protein, a protein with nucleic acid, and the pathological aggregation (Kuznetsova et al., 2014). The intracellular environment condition is crowded with volume occupancy of 5–40% molecules (Ellis & Allen, 2003), thereby creating a crowded (solid) medium with a minimal amount of free water (Zimmerman & S.O, 1991)**.** By contrast, the usual culture media used as cell culture media obtained different results.

In this research, bioscaffolds are expected to maintain and stimulate the pluripotency of stem cells. Therefore, this study is conducted to prove the function of the bioscaffold.

METHODS

This research started from January to May 2021 in the Laboratory of Virology and Cancer Pathobiology Research Center, Faculty of Medicine, Universitas Indonesia. MEF and ESC cells in liquid nitrogen stock were used for this research.

MEF culture under Dulbecco's modified

Eagle medium (DMEM) and crowding medium

MEF was seeded on a 12-well plate (Corning, USA) at 50,000 cells per well suspended in DMEM (SAFC, Switzerland), with 10% FBS, 2.5% HEPES, 2.5% sodium bicarbonate, and 1% penicillin-streptomycin solution (~Gibco, USA). After 76 h, cells were cultured at 37 °C under 5% CO_2 and then added with a crowding medium (500 kDa of dextran sulfate (Himedia, India), 100 µm of L-ascorbic acid 2-phosphate (Sigma Aldrich, USA)). Afterward, the cells are incubated for 24 h.

Decellularisation of cells to obtain bioscaffold

A 0.5% sodium deoxycholate (Himedia, India) suspended in phosphate-buffered saline (Gibco, USA) was added to the culture for 45 min incubation, followed by one-hour incubation in DNAse I (6 U/µL solutions). After this process, a bioscaffold was formed and ready to be used for culture.

Tali cytometer for fluorescent cell count

ESC in the extracellular matrix scaffold was cultured for 5–7 days. Afterward, cells are harvested using Trypsin EDTA (Gibco, USA), collected into a centrifuge tube (Corning, USA), and washed with flow cytometry buffer. Oct4 or Sox2 antibodies are added, followed by incubation for one hour. After washing, anti-mouse or rabbit anti-goat FITC antibodies were added as secondary antibodies, followed by one-hour incubation, and finally stored in 1% formaldehyde solution until reading using a Tali cytometer (Invitrogen, USA).

RESULTS

Cells isolated from 10-day-old mouse embryos were cultured in DMEM after being removed from liquid nitrogen storage. After a few days, The semi-floating spheroid or grape-like shape ECSs are observed in the culture. The ECS formed a spheroid or grape-like shape, semi-floating in the culture (Fig. 1a), whereas the MEF adhered to the bottom of the plate (Fig. 1b). In addition, MEF is indicated by the structure (morphology) having a flat elongated cytoplasm surrounding the nucleus.

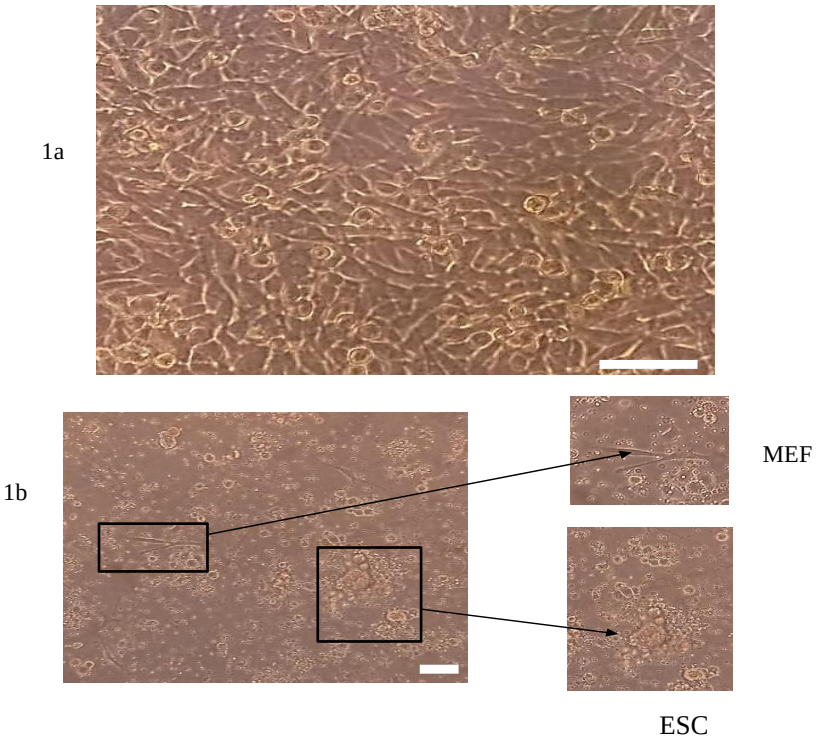


Figure 1. Mouse Embryonic Fibroblast (MEF, 1a) for preparing bioscaffold andMouse Embryonic Stem Cell (ESC, 1b) in DMEM culture media. Cells were documented under an inverted microscope. White bar: 50 µm.

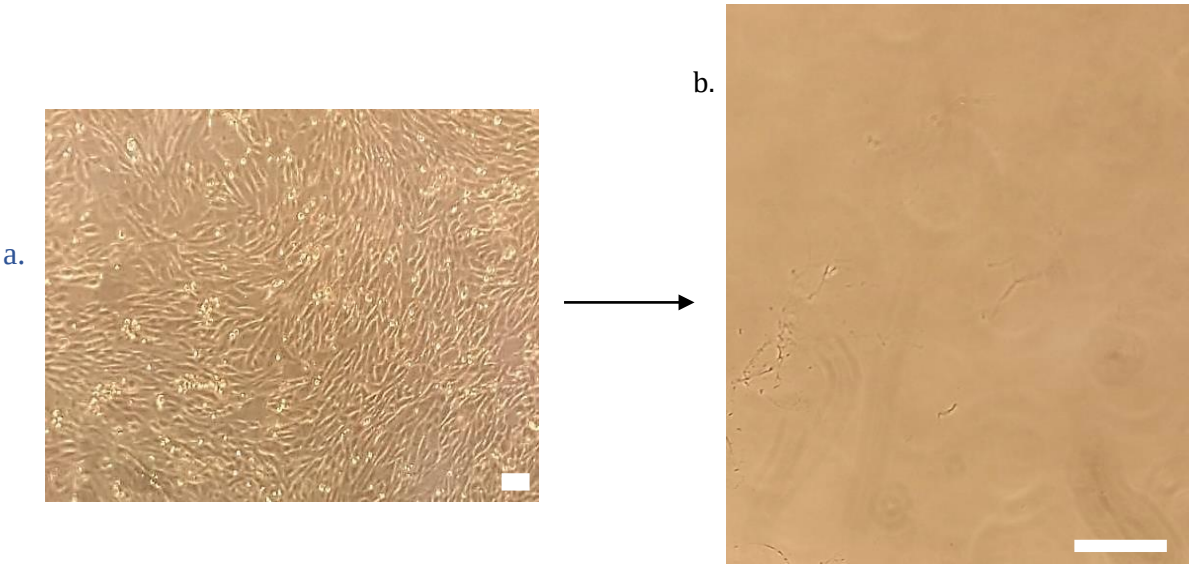
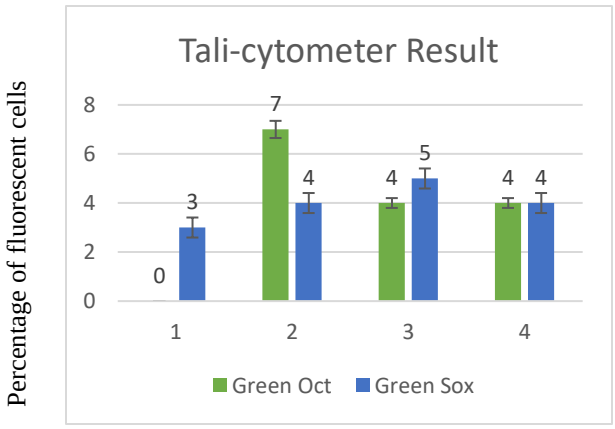


Figure 2. Bioscaffold formed by the extracellular matrix after decellularization of confluent Mouse Embryonic Fibroblast (MEF, a). MEF culture was crowded with dextran sulfate and supplementedwith ascorbic acid to enhance the production of the extracellular matrix. Then, cells are decellularized using sodium deoxycholate, resulting in a bioscaffold for ESC culture (b). Cells are documented under an inverted microscope. White bar: 50 µm.



Media
1 : Bioscaffold from MEF supplemented with ascorbic acid
2 : Bioscaffold from MEF crowdedwith dextran and supplemented with ascorbi acid
3: Bioscaffold from MEF withoutcrowding
4. MEF feeder

alue					*p-Value*				
Oct4	1	2	3	4	Sox2	1	2	3	4
1		0.395	0.6	0.439	1		0.6	0.439	N/A
2	0.395		0.426	0.641	2	0.6		0.293	N/A
3	0.6	0.426		0.641	3	0.439	0.293		N/A
4	0.439	0.641	0.641		4	N/A	N/A	N/A	

Figure 3. Tali cytometer results for counting the number of fluorescent cells corresponding to the presence of Oct4 and Sox2. Cells are analyzed after staining with Oct4 or Sox2 primary antibodies, followed by the addition of a secondary antibody conjugated with a green fluorescent fluorophore (FITC).

After being cultured in the natural scaffold, ESC is observed using a Tali cytometer to analyze the presence or absence of stem cell protein markers, namely, Oct4 and Sox2. Even though it was not significantly different for pluripotent cells, using natural scaffold and crowding tended to increase the stem cell markers, Sox2 dan Oct4 (Fig. 3).

DISCUSSION

Stem cells show great application potential in cell therapy, tissue engineering, regenerative medicine, and biotechnology. ESC is usually cultured in two-dimensional culture plates as a monolayer, often added with xenogenic materials to attach substrates, cytokines, growth factors, and serum. The additional animal xenogenic materials potentially transmit pathogens, and it is also challenging to reach reproducibility between cultures caused by lot-to-lot fluctuations in the material used (Jason A & Vunjak-Novakovic, 2009). Natural 3D bioscaffold allows complex spatial interactions between cells, a component of ECM, andgradients of nutrients, oxygen, and waste (Lee et al., 2008). Bioscaffolds prepared from crowded MEF cells provide good 3D niches for ESC, thereby propagating and maintaining their pluripotency. The addition of nutrients, growth factors, and animal materials must be replaced for future applications. However, the difference among media 1, 2, 3, and 4 is still not statistically significant. Although ESC cultured in bioscaffold from MEF crowded with dextran supplemented with ascorbic acid and ESC cultured in MEF feeder

are not statistically different, they tend to increase pluripotency, as shown by the increase in the number of Sox 2 and Oct 4 protein. Based on previous reports, MEFs exhibited by structure (morphology) have a flat elongated cytoplasm surrounding the nucleus with a conditioned medium. They are commonly used as media for ESC and induced pluripotent stem cell culture (Jozefczuk et al., 2012; Seo et al., 2020). In addition, scaffold incorporates soluble or growth factors for proliferation and differentiation (Kraehenbuehl et al., 2011). However, adding growth factors is necessary to achieve a good environment. Some potential herbal extracts are investigated to induce proliferation and differentiation of ESC. Naringin to citrus fruit extract has increased human bone marrow mesenchymal cell proliferation and differentiation (Peng-Zhang & Yan, 2009).

In contrast, the additional leaf extract of *Olea europaea* can affect cell differentiation, shown by higher gene expression for vascular endothelial growth factor, platelet-derived growth factor receptor, and vascular endothelial factor receptor (Gomez et al., 2012). ESC are differentiated into specialized endothelial cells, one of the findings of neovascularisation repair, which can regenerate damaged tissue (Chouw et al., 2018; Gomez et al., 2012; Slack, 2021). In addition, the benefits of stem cells are that they can be reprogrammed, with specific genetic settings, to suit the patient's needs (Meiliana & Wijaya, 2011).

Bioscaffold is suggested to be applied in the following experiment for propagating, maintaining, and differentiating ESC with the development of media and growth factors. As the characteristic of ESC is pluripotent, these stem cells are developed into various types of cells by adding growth factors and modifying media. Thus, natural products such as herbal, active compounds, and plant extract are being explored for their potential application as growth factors (Lin et al., 2011; Saud et al., 2019).

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