Effect of Electric Stimulation on Differentiation and Hypertrophy of Fat Precursor Cells

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ABSTRACT

An effect of electric stimulation with pulses on differentiation and hypertrophy of fat precursor cells has been investigated *in vitro*. 3T3-L1 (mouse fat precursor cells) was seeded at the concentration of 1000 cells per cm^2, and cultured in Dulbecco's Modified Eagle Medium with 10 percent of fetal bovine serum. Differentiation of cells was induced with insulin. An electric stimulator was used to generate electric pulses with period of 1 s, pulse width of 0.0025 s, and current amplitude of 0.03 A. The electric pulses were applied to the medium through the electrode for thirty minutes per day. The cells were observed with an inverted phase contrast microscope for 24 days. At the end of the culture, the tissue was observed with the microscope, after the tissue was stained with oil red O. The experiment shows that both differentiation and growth of 3T3-L1 are delayed with electric pulses.

Keywords: Biomedical Engineering, 3T3-L1, Electric Pulses, Cell Growth and Differentiation.

1. INTRODUCTION

Excess adipose tissue cause several disease in human body. Many methods have been investigated to control adipose tissue. Several methods include stimulation of electric pulses. Electric pulses are applied to electrodes, which are attached on the skin of human body. Electro-acupuncture might improve metabolic disturbances [1].

Cell culture technique has been developed and several methodologies might clinically be applied to regenerative medicine [2, 3].

3T3-L1 was used for cell culture in the experiment. 3T3-L1 is a cell line derived from cells of mouse 3T3. The cells have a fibroblast-like morphology. They fill the intercellular space in the tissue, when some parenchymal cells disappear. Under appropriate conditions, 3T3-L1 differentiates into an adipocyte-like phenotype. When cells enter a resting state, they accumulate triglyceride and convert to adipose cells [4, 5]. Insulin has been used to induce 3T3-L1 to an adipocyte-like phenotype [6].

In the present study, the effect of electric stimulation with pulses on differentiation and hypertrophy of fat precursor cells has been investigated *in vitro*.

2. METHODS

Electrode

A dish of 35 mm diameter was used for the cell culture. A cap with a couple of electrodes is made of Polydimethylsiloxane (PDMS) (Fig. 1). The electrodes are made of platinum wire of 0.2 mm diameter. The electrodes are mounted in the cap of PDMS (Fig. 2). The electrodes are dipped into the medium, when the cap is set on the dish.

A silicon wafer (Type N, Matsuzaki Seisakusyo, Co., Ltd., Tokyo, Japan) was used for a mold for the upper wall of the cap. The diameter of the wafer is 50 mm.

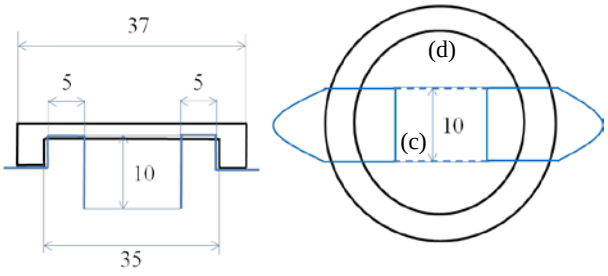

Fig. 1: Cap with a couple of electrodes (blue lines). Dimension in mm. (c) and (d) show the position for observation of cells (Figs. 11-15).

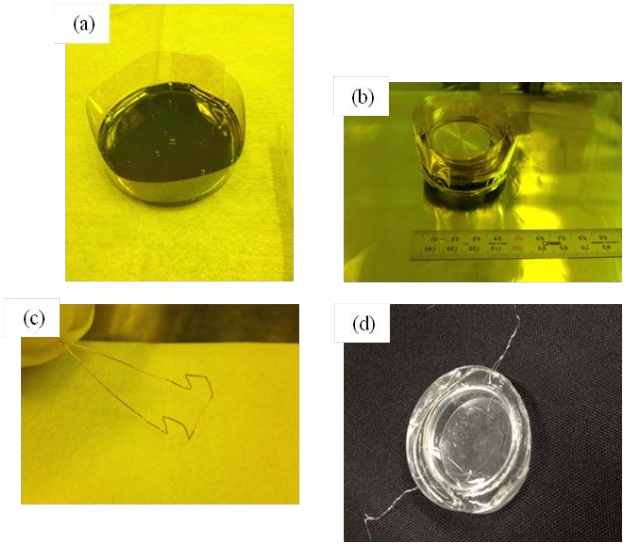

Fig. 2: Cap of PDMS with a couple of electrodes of Platinum wire. Top plate of cap (a), peripheral wall of cap (b), electrode of platinum wire (c), electrodes are fixed on cap (d).

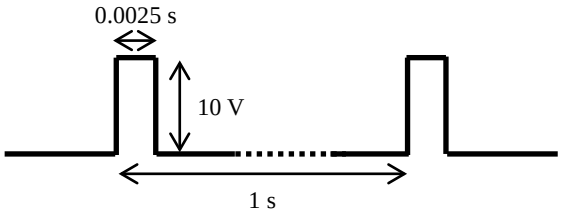

Fig. 3: Electric pulse.

After the wafer was enclosed with a peripheral wall of polyimide, 3 cm^3 of PDMS (Dow Corning Corporation, MI, USA) was poured with the curing agent on the wafer (Fig. 2(a)). PDMS was baked at 373 K for one hour in an oven. The base of dish (35 mm diameter) with the peripheral wall of polyimide was placed at the center of the disk of PDMS (Fig. 2(b)). PDMS of 7 cm^3 was poured into the donut canal between the walls of polyimide to make the peripheral wall of the cap, and baked at 333 K for 30 minutes in the oven. An electrode is formed with a platinum wire (Fig. 2(c)). A pair of electrodes was placed on the inside of the cap with the counter position. PDMS of 1 cm^3 was poured to cover the root of electrode wire for fixation, and baked at 393 K for one hour in an oven (Fig. 2(d)).

Electric stimulation

The electric pulses (period 1 s, amplitude 10 V, duration 0.0025 s) were generated with an electric stimulator (SEN5201, Nihon Kohden Corporation, Japan) (Fig. 3). The stimulator was connected to the electrodes, and the pulses (E) were applied to cells through the medium (Z) for thirty minutes per day. An electric resistance of 30 ohm (r) is serially inserted between the electrode and the stimulator (Fig. 4). The electric signals (V_1, V_2) were monitored by an oscilloscope during electric stimulation to cultured cells (Fig. 8).

The interval between electric stimulations is 23 hours and a half. One of the signals is the voltage between terminals of stimulator (V_1), and the other signal is the voltage (V_2) between terminals of the resistance (r). The electric current (I) is calculated by the following equation.

$$I = V_2 / r \quad (1)$$

Cell Culture

3T3-L1 (a cell line derived from cells of mouse 3T3) of the fifth passage was used for the cell culture. D-MEM (Dulbecco's Modified Eagle Medium) containing 10% decomplemented FBS (Fetal Bovine Serum) and 1% penicillin/ streptomycin (P/S) was used for the medium. The medium was refreshed every two days during cell culture. The cells were seeded on the dish with the density of 1000 cells/cm^3. A hemocytometer (Burker-Turk) was used to count number of cells. Cells were cultured in the incubator at 310 K with 5% CO_2 for 24 days.

The protocols of electric stimulation are classified into three groups: A, B, and C.

In group A, the electric stimulation was applied after 24 hours from cell seeding. In group B, the electric stimulation started on the second day after confluent state of the cells. Both in group A and in group B, the electric stimulation was subsequently applied every day (Figs. 5&6). In group C, cells are cultured without electric stimulation. In every group, insulin was applied to the medium for differentiation on the second day after confluent state of the cells. The insulin of 0.01 mg is dissolved in the medium (D-MEM+FBS+P/S) of 1 cm^3.

Cells were observed with an inverted phase-contrast microscope (IX71, Olympus Co., Ltd., Tokyo) every day. For comparison, typical figures not only of the center part (Fig. 1(c)) but also of the peripheral part (Fig. 1(d)) on the bottom of the dish are exemplified in Figs. 11-15. For quantitative evaluation of differentiation, the summation area of the rounded cell is calculated by number of pixels in the contrasted microscopic image (Fig. 7).

For chemical confirmation of differentiation, the cells on the bottom of the dish were stained with Oil Red O ($C_{26}H_{24}N_4O$, 1-(2,5-dimethyl-4-(2-5-dimethylphenyl) phenyldiazenyl) azonapthalen-2-ol) at the end of the experiment.

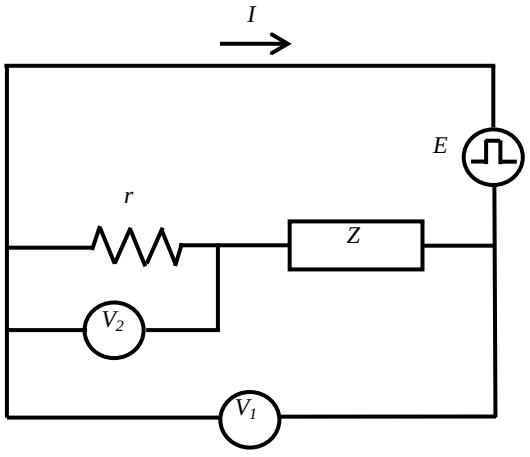

Fig. 4: Electric circuit: E, pulse generator; Z, medium; r, resistance; I, current; V, voltage.

Fig. 5: Electric stimulation for thirty minutes per day.

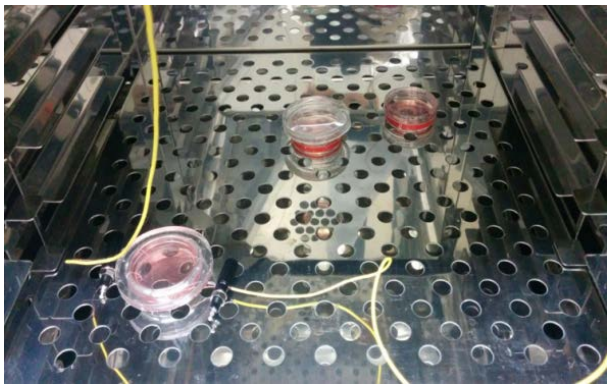

Fig. 6: Cell culture dish with electrodes in incubator.

3. RESULTS

Fig. 8 shows the electric signals monitored by an oscilloscope during electric stimulation to cultured cells. At the display of oscilloscope (Fig. 8), the voltage tracings show that the voltage (V_2 in Fig. 3) between terminals of resistance (r in Fig. 4) of 30 ohm is 0.8 V. These values make the electric current of 0.03 A calculated by Eq. 1.

Fig. 9 shows cells on the second day of culture. The right figure (Fig 9(b)) shows cells exposed to electric pulses for thirty minutes. Cells adhere to the bottom of the dish, and extend to show the linear shape. Some floating cells show the spherical shapes.

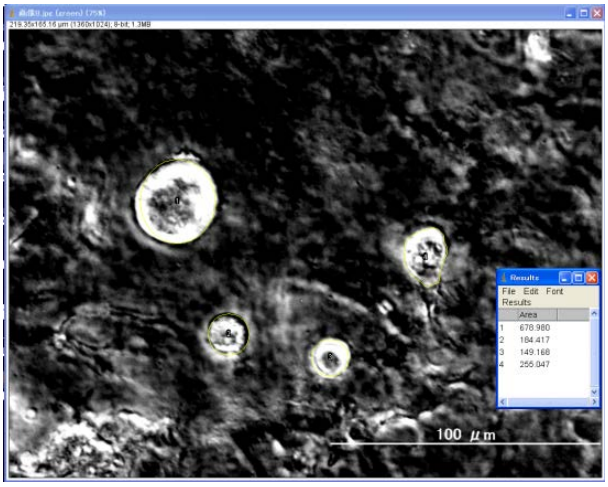

Fig. 7: Measurement of area of lipocyte.

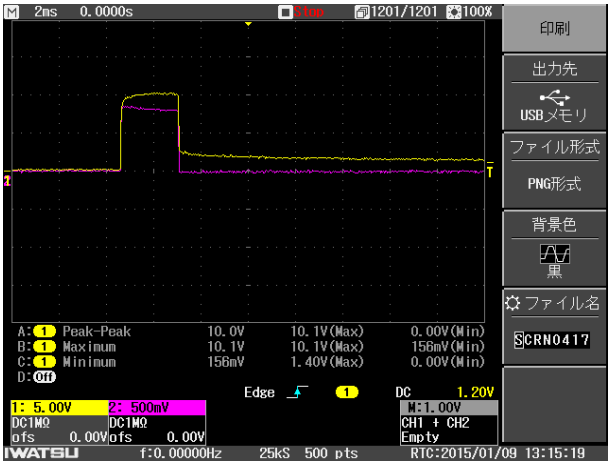

Fig. 8: Voltage tracings of electric pulse.

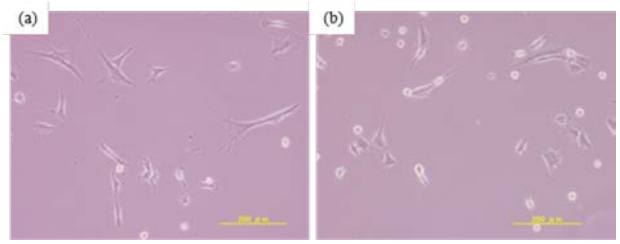

Fig. 9: Cells on day 2. Group C (left), and group A (right). Dimension from left to right is 1 mm in each section.

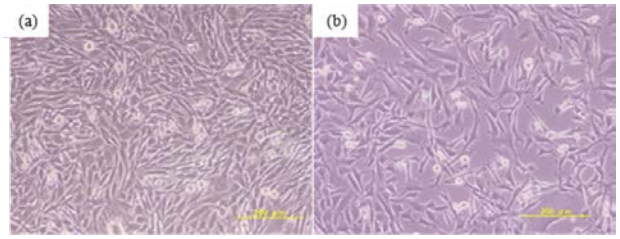

Fig. 10: Cells on day 6. Group C (left), and group A (right). Dimension from left to right is 1 mm in each section.

Fig. 10 shows cells on the sixth day of culture. Although several vacancies are left among cells on the bottom of the dish in the group A with electric stimulation (Fig. 10(b), the cells proliferate to confluent state without electric stimulation (Fig. 10(a)).

The cells proliferated to confluent state on the sixth day of culture in the group C without electric stimulation so that the medium was changed to that with insulin on the eighth day of culture in every group. The protocol of group B also started on the eighth day of culture.

Fig. 11 shows cells on the eighth day of culture. The figure shows several flat cells, which are exemplified with the circles in Fig. 11.

Fig. 12 shows cells on the twelfth day of culture. The figure shows several spherical cells, which include lipid droplets, which are exemplified with the circles.

Fig. 13 shows cells on the 22nd day of culture. The figure also shows several spherical cells, which include lipid droplets, which are exemplified with the circles.

On the 24th day, cell culture was stopped and the cells on the bottom of the dish were stained (Fig. 14).

The number of lipid droplet is maximum in the group C without electric stimulation. The data of area of the rounded cell are distinguished by the contrasted microscopic image, and distributed between 0.00012 mm^2 and 0.0057 mm^2.

Fig. 15 shows relation between the areas (S [mm^2]) of rounded cell and the culture days. Each figure shows maximum (A), mean (B) and minimum (C) area, respectively. In each figure, data of circle belongs to the group C, data of square belongs to the group A, and data of triangle and cross belong to the group B. Data of triangle belongs to the position of c in Fig.1 and data of cross belongs to the position of d in Fig 1. The area extends earlier without electric stimulation than with electric stimulation (day 11 in Fig. 15). The growth of rounded cell tends to decrease under electric stimulation.

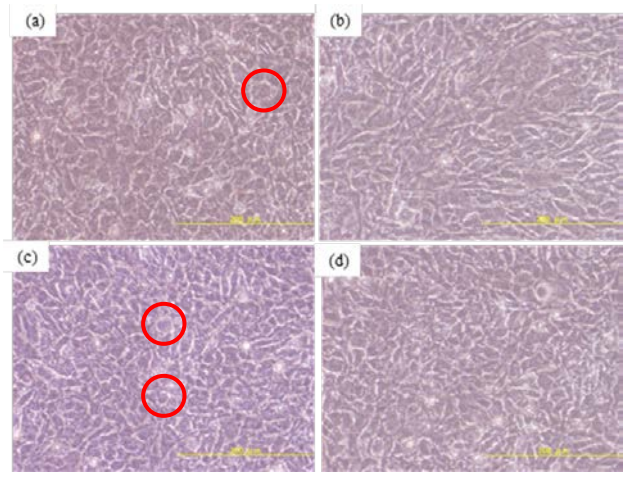

Fig. 11: Cells on day 8. Group C (a), group A (b) and group B (c: center, d: peripheral (Fig. 1)). Dimension from left to right is 0.5 mm in each section.

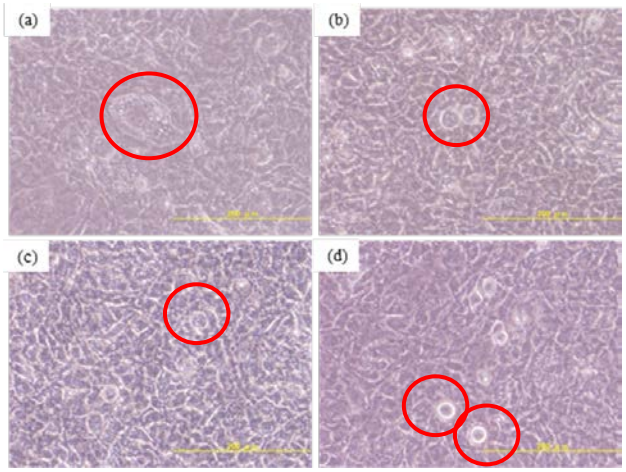

Fig. 12: Cells on day 12. Group C (a), group A (b) and group B (c: center, d: peripheral (Fig. 1)). Dimension from left to right is 0.5 mm in each section.

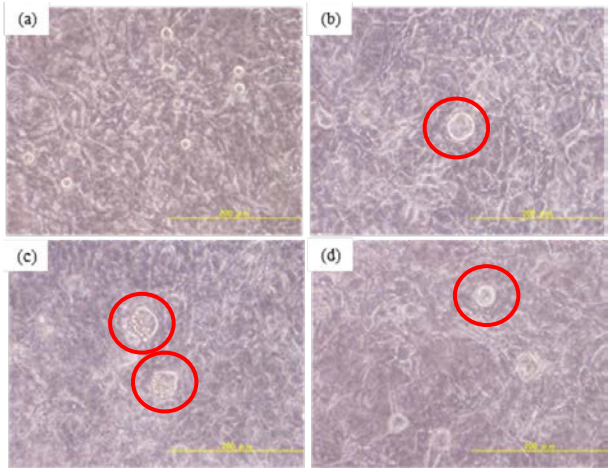

Fig. 13: Cells on day 22. Group C (a), group A (b) and group B (c: center, d: peripheral (Fig. 1)). Dimension from left to right is 0.5 mm in each section.

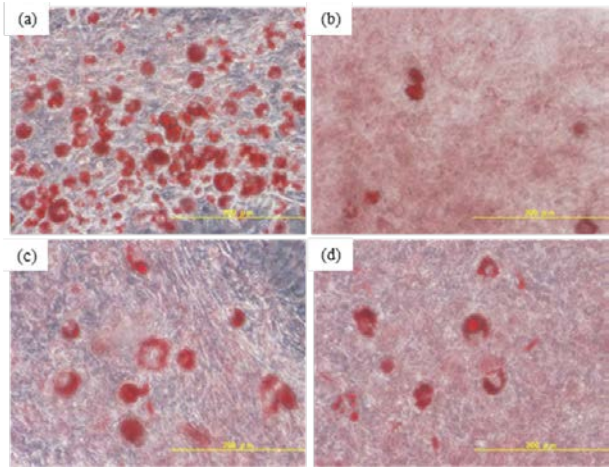

Fig. 14: Cells stained with Oil Red O on 24th day. Group C (a), group A (b) and group B (c: center, d: peripheral (Fig. 1)). Dimension from left to right is 0.5 mm in each section.

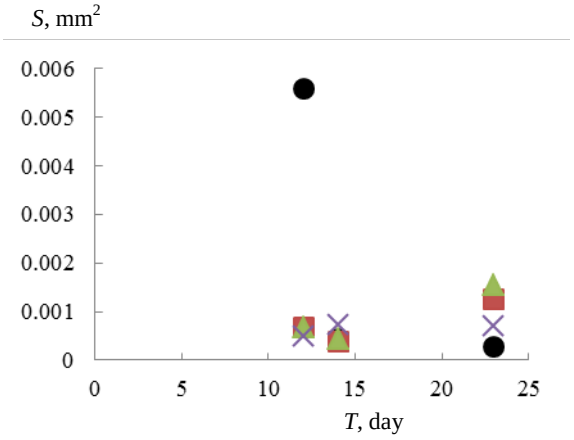

Fig. 15A: Maximum area (S [mm^2]) of rounded cell vs. culture days. Group C (circle), group A (square) and group B (c: triangle, d: cross (Fig. 1)).

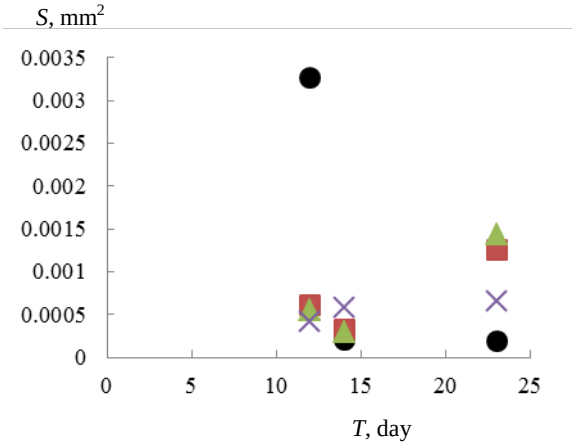

Fig. 15B: Mean area (S [mm^2]) of rounded cell vs. culture days. Group C (circle), group A (square) and group B (c: triangle, d: cross (Fig. 1)).

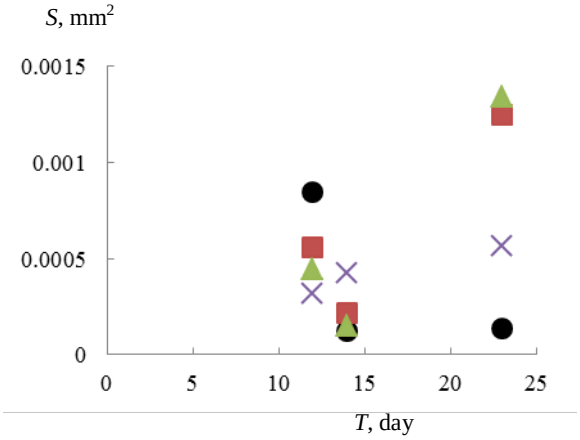

Fig. 15C: Minimum area (S [mm^2]) of rounded cell vs. culture days. Group C (circle), group A (square) and group B (c: triangle, d: cross (Fig. 1)).

4. DISCUSSION

The behavior of a biological cell depends on several factors: electrical [7-10], magnetic [11], and mechanical factors [12-15]. The microfluidic system has been fabricated to study the behavior of cells *in vitro* [16-19]. The pattern of the surface has been applied to study the surface effect of adhesion of cells [20-21]. Electric stimulation has been applied to human body in many cases. Some of them are applied to clinical treatment [1].

In the present study, the voltage of the electric pulse is selected lower than the voltage, which generates electrolysis of the medium. The medium does not show any chemical changes like bubble generation. The color the medium is maintained, which indicates the constant value of pH.

3T3-L1 is a cell line derived from cells of 3T3 mouse [4-6]. 3T3-L1 cells have a fibroblast-like morphology. The cells differentiate into an adipocyte-like phenotype under appropriate conditions. After differentiation, the cell become sphere, which includes lipid droplets. The morphological change from linear to flat is used as prelude for differentiation.

Oil red O ($C_{26}H_{24}N_4O$) is fat-soluble diazo dye. It is a lysochrome used for staining of neutral triglycerides and lipids on frozen sections and some lipoproteins on paraffin sections.

To evaluate the effect of electric stimulation on differentiation of 3T3-L1, electric stimulation is applied to 3T3-L1 before cells proliferate to the confluent state in Group A in the present study. Both number and growth of adipocyte-like cell tend to decrease with exposure to electric pulses.

The tendency is almost the same regardless the position from the electrode on the culture plate, which shows the uniform electric field on the plate in the present study.

5. CONCLUSION

An effect of electric stimulation with pulses on differentiation and hypertrophy of fat precursor cells has been investigated *in vitro*. The experiment shows that both differentiation and growth of 3T3-L1 are delayed with electric pulses.

6. ACKNOWLEDGMENT

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REFERENCES

[1] L. Mannerås, I.H. Jonsdottir, A. Holmaeng, M. Loenn and E. Stener-Victorin, “Low-Frequency Electro-Acupuncture and Physical Exercise Improve Metabolic Disturbances and Modulate Gene Expression in Adipose Tissue in Rats with Dihydrotestosterone-Induced Polycystic Ovary Syndrome”, **Endocrinology**, Vol. 149, No. 7, 2008, pp. 3559-3568.

[2] T. Shimizu, M. Yamato, A. Kikuchi and T. Okano, “Cell Sheet Engineering for Myocardial Tissue Reconstruction”, **Biomaterials**, Vol. 24, No. 13, 2003, pp. 2309-2316.

[3] G.D. Smith, S. Takayama and J.E. Swain, “Rethinking In Vitro Embryo Culture: New Developments in Culture Platforms and Potential to Improve Assisted Reproductive Technologies”, **Biology of Reproduction**, Vol.86, No. 3: 62, 2012, pp. 1-10.

[4] H. Green and O. Kehinde, “An Established Preadipose Cell Line and Its Differentiation in Culture. II. Factors Affecting the Adipose Conversion”, **Cell**, Vol. 5, No. 1, 1975, pp. 19-27.

[5] K. Zebisch, V. Voigt, M. Wabitsch and M. Brandsch, “Protocol for Effective Differentiation of 3T3-L1 Cells to Adipocytes”, **Analytical Biochemistry**, Vol. 425, No. 1, 2012, pp. 88-90.

[6] A.W. Harmon and J.B. Harp, “Differential Effects of Flavonoids on 3T3-L1 Adipogenesis and Lipolysis”, **American Journal of Physiology - Cell Physiology**, Vol. 280, No. 4, 2001, pp. C80-C813.

[7] S. Hashimoto, F. Sato, R. Uemura and A. Nakajima, “Effect of Pulsatile Electric Field on Cultured Muscle Cells in Vitro”, **Journal of Systemics Cybernetics and Informatics**, Vol. 10, No. 1, 2012, pp. 1-6.

[8] H. Kuriyama and T. Tomita, “The Responses of Single Smooth Muscle Cells of Guinea-pig Taenia Coli to Intracellularly Applied Currents, and Their Effect on the Spontaneous Electrical Activity”, **Journal of Physiology**, Vol. 178, No. 2, 1965, pp. 270–289.

[9] H. Park, R. Bhalla, R. Saigal, M. Radisic, N. Watson, R. Langer and G. Vunjak-Novakovic, “Effects of Electrical Stimulation in C2C12 Muscle Constructs”, **Journal of Tissue Engineering and Regenerative Medicine**, Vol. 2, No. 5, 2008, pp. 279-287.

[10] H. Kaji, T. Ishibashi, K. Nagamine, M. Kanzaki and M. Nishizawa, “Electrically Induced Contraction of C2C12 Myotubes Cultured on a Porous Membrane-Based Substrate with Muscle Tissue-Like Stiffness”, **Biomaterials**, Vol. 31, 2010, pp. 6981-6986.

[11] S. Hashimoto and K. Tachibana, “Effect of Magnetic Field on Adhesion of Muscle Cells to Culture Plate”, **Journal of Systemics Cybernetics and Informatics**, Vol. 11, No. 4, 2013, pp. 7-12.

[12] S. Hashimoto and M. Okada, “Orientation of Cells Cultured in Vortex Flow with Swinging Plate in Vitro”, **Journal of Systemics Cybernetics and Informatics**, Vol. 9, No. 3, 2011, pp. 1-7.

[13] S. Hashimoto, F. Sato, H. Hino, H. Fujie, H. Iwata and Y. Sakatani, “Responses of Cells to Flow in Vitro”, **Journal of Systemics Cybernetics and Informatics**, Vol. 11, No. 5, 2013, pp. 20-27.

[14] A. Kamiya, R. Bukhari and T. Togawa, “Adaptive Regulation of Wall Shear Stress Optimizing Vascular Tree Function”, **Bulletin of Mathematical Biology**, Vol. 46, No.1, 1984, pp. 127-137.

[15] F. Sato, S. Hashimoto, T. Yasuda and H. Fujie, “Observation of Biological Cells in Rhombus Parallelepiped Flow Channel”, **Proc. 17th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 1, 2013, pp. 25-30.

[16] D.C. Duffy, J.C. McDonald, O.J.A. Schueller and G.M. Whitesides, “Rapid Prototyping of Microfluidic Systems in Poly (dimethylsiloxane)”, **Analytical Chemistry**, Vol. 70, No. 23, 1998, pp. 4974-4984.

[17] S. Hashimoto, A. Mizoi, H. Hino, K. Noda, K. Kitagawa and T. Yasuda, “Behavior of Cell Passing through Micro Slit”, **Proc. 18th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2014, pp. 126-131.

[18] S. Hashimoto, Y. Takahashi, H. Hino, R. Nomoto and T. Yasuda, “Micro Hole for Trapping Flowing Cell”, **Proc. 18th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2014, pp. 114-119.

[19] A. Khademhosseini, J. Yeh, S. Jon, G. Eng, K.Y. Suh, J.A. Burdick and R. Langer, “Molded Polyethylene Glycol Microstructures for Capturing Cells within Microfluidic Channels”, **Lab on a Chip**, Vol. 4, No. 5, 2004, pp. 425-430.

[20] T. Okano, S. Nishiyama, I. Shinohara, T. Akaike, Y. Sakurai, K. Kataoka and T. Tsuruta, “Effect of Hydrophilic and Hydrophobic Microdomains on Mode of Interaction between Block Polymer and Blood Platelets”, **Journal of Biomedical Materials Research**, Vol. 15, No. 3, 1981, pp. 393-402.

[21] K. Noda, S. Hashimoto, T. Yasuda and H. Fujie, “Culture of Myoblast on Gold Film Sputtered on Polydimethylsiloxane Disk”, **Proc. 18th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2014, pp. 150-155.