Responses of Cells to Flow in Vitro

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ABSTRACT

The response of cells to the flow has been studied *in vitro*. The response of cells was examined in two types of flow channels: in a donut-shaped open channel, and in a parallelepiped flow channel. Variation was made on the material of the parallelepiped channel to study on adhesion of cells to the plates: glass and polydimethylsiloxane. Behavior of cells on the plate was observed under a flow of a medium with an inverted phase-contrast-microscope. The shear stress on the wall is calculated with an estimated parabolic distribution of the velocity between the parallel plates. The adhesion of cells was evaluated with the cumulated shear, which is a product of the shear stress and the exposure time. The experimental results show that cells are responsive to the flow, which governs orientation, exfoliation, and differentiation. The response depends on the kinds of cells: endothelial cells orient along the stream line, although myocytes orient perpendicular to the stream line. The adhesion depends on the combination between scaffold and cell: myocytes are more adhesive to glass than cartilage cells, and fibroblasts are more adhesive to polydimethylsiloxane than glass.

Keywords: Biomedical Engineering, Cell Culture, Flow, Orientation, Adhesion and Shear Stress.

1. INTRODUCTION

Cells are responsive to various environmental factors, such as electric [1, 2], magnetic [3, 4] and mechanical [5-18] fields.

The cell culture technique has been developed and applied to many fields: regenerative medicine, diagnostics, etc. The acceleration technique for orientation and differentiation of cells has been studied to make biological tissue *in vivo* and *in vitro* [1, 5-8]. Control methodology for orientation and differentiation of cells would be applied to regenerative tissue technology.

The mechanical stress is one of the interested points in the environment of cells, because they receive mechanical forces *in vivo*. Several methods have been designed to apply mechanical stress to cells [6-18]. A transmission point of stress to a specimen is important. In the most of studies, the stress is applied to a scaffold. When fixation between the cell and the scaffold is not enough, the stress is not transmitted to the cell. A flow can be used, on the other hand, to apply a stress field to a specimen [1, 7-13]. The whole specimen directly receives the shear stress in the shear flow. In the present study, the response of cells to the flow has been studied *in vitro*.

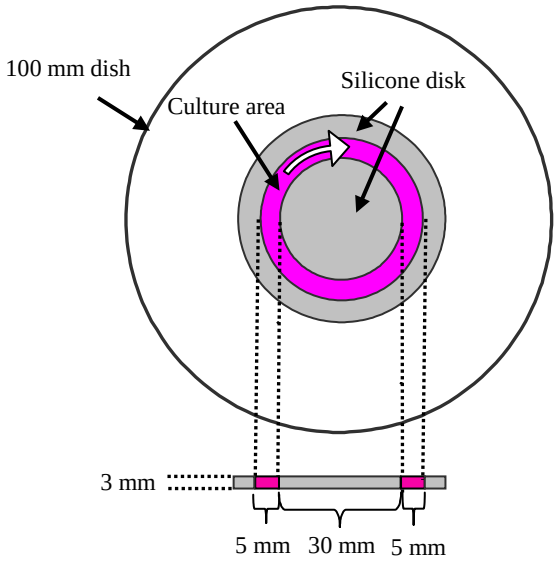

Fig. 1: Donut-shaped open channel system.

Fig. 2: Culture dish on swinging plate in incubator.

2. METHODS

Donut-Shaped Open Channel

A donut-shaped open channel system for the cell culture has been designed to apply a vortex flow on cells *in vitro*. A polystyrene culture dish was used. A silicone rubber disk of 3 mm thick (K-125, Togawa Rubber Co., Ltd., Osaka) is attached on the inner bottom of the culture dish to restrict the space for the flow of the medium (Fig. 1). The silicone rubber disk is stuck on the bottom of the dish with affinity between their surfaces without adhesive.

Two types of silicone disk were prepared. The first one is as follows. The silicone disk of 30 mm diameter is attached at the center of the culture dish of 52 mm internal diameter without collagen coating.

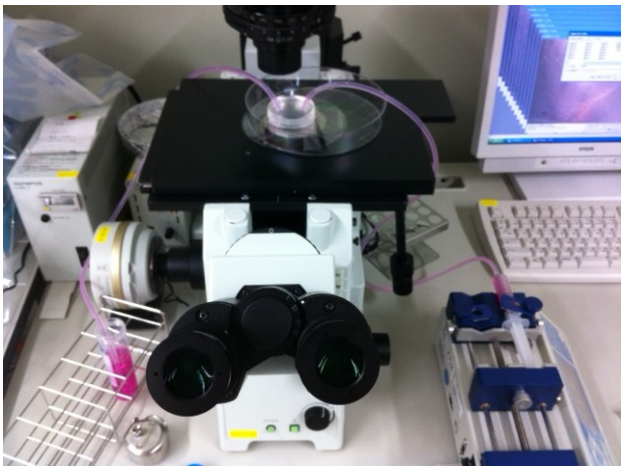

Fig. 3: Parallelepiped flow channel system: flow chamber and microscope (middle), syringe pump (right).

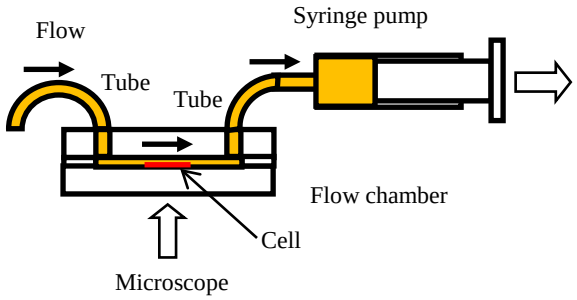

Fig. 4: One-way flow system.

The second one is as follows. The silicone disk of 30 mm diameter and the silicone ring are attached at the center of the culture dish of 100 mm internal diameter with collagen coating. A silicone ring has the inner hole of 40 mm diameter. The centers of the disks are adjusted to the center of the dish. Cells are cultured in the donut shape interspace between silicone disks.

The culture dish is placed on a plate, which inclines at 0.1 rad of the horizontal plane (Fig. 2). The plate rotates to generate a swing motion (WAVE-SI, Taitec, Co., Ltd., Koshigaya). The rotating speed of the plate is 20 revolutions per minute (rpm) (2.1 rad/sec). The motion produces a one-way clockwise vortex flow in the medium in the donut-shaped open channel.

The continuously swinging plate is placed in an incubator, where both temperature of 37 degrees Celsius and carbon dioxide partial pressure of 5 percent are maintained.

Parallelepiped Channel

A one-way flow system was designed to observe responses of cells to a fluid shear stress *in vitro*. The system consists of a flow chamber, a syringe pump, tubes and a microscope (Figs. 3&4). TE-331S (Terumo Co., Ltd. Tokyo) or Micro-syringe-pump (ISIS Co., Ltd.) was used for the syringe pump. A plastic tube of 2 mm internal diameter and of 3 mm external diameter was used for the connector to a flow chamber.

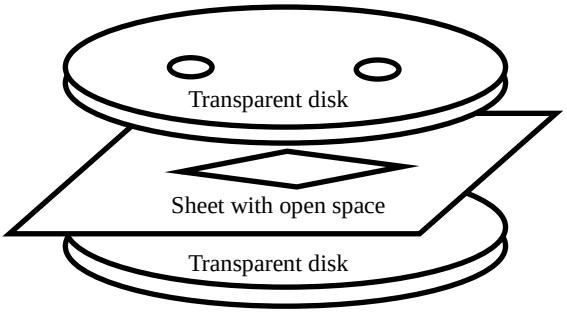

Fig. 5: Flow chamber consists of two transparent plates and a thin silicone rubber sheet.

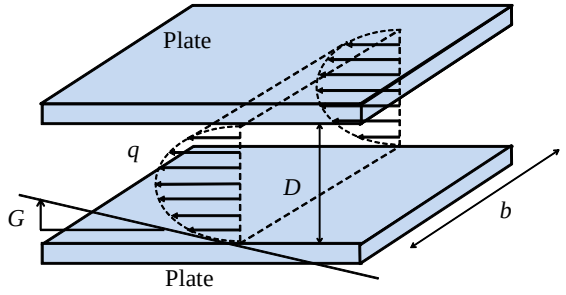

Fig. 6: Parabolic distribution of the velocity between parallel plates.

The flow chamber consists of two transparent plates and a thin silicone rubber sheet (Fig. 5). Two kinds of material are alternatively used for the transplant plates: glass or polydimethylsiloxane (PDMS).

An open space of rectangular cross section is cut off in a thin sheet of silicone rubber being 0.1 mm thick, and sandwiched between the plates. The open space forms a parallelepiped flow channel with the cross section of 1-3 mm width × 0.1 mm. The three plates stick together with their surface affinity without an adhesive. In the case of PDMS disk, the inner surface of PDMS of the chamber was exposed to the oxygen gas in a reactive ion etching system to be characterized as hydrophilic, before assembled.

At the upper plate, two holes were machined to be connected to the plastic tubes. One of the tubes is connected to the plastic syringe pump (Fig. 4). The room temperature was maintained at 25 degrees Celsius. The chamber is placed on the inverted phase-contrast microscope (IX71, Olympus Co., Ltd., Tokyo).

Cell

Six kinds of cells were used in the experiment: C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse), normal cartilage cell (collected from costal cartilage of Sprague Dawley rat, Takara-bio), L6 (rat skeletal muscle cell), A7r5 (rat aortic smooth muscle cell), CS-2P2-C75 (primary normal porcine aortic endothelial cell), and L929 (fibroblast-like, mouse connective tissue, RCB1422, Riken Bio Resource Center, Tsukuba).

Flow Test in Donut-Shaped Open Channel

In the flow test with donut-shaped open channel, each kind of cells was alternatively suspended in the Dulbecco's Modified Eagle's Medium (D-MEM) with density of 1.0×10^6 cells per cm^3. Fetal bovine serum (FBS) was added to the medium with the volume rate in 10 percent of FBS and in 90 percent of D-MEM. The suspension was poured into the dish and cultured in the incubator for three hours without flow stimulation. After the cultivation for three hours, the cells were cultured with flow stimulation for five days. The continuous rotation of the plate makes a steady flow in the medium through channel.

The volume of the medium is adjusted to cover whole surface of the bottom of the canal in the culture dish, and not to flow over the superior surface of the silicone disk during the swing motion of the plate. The cells were cultured in the vortex flow of the medium, while the plate was continuously rotating at 37 degrees Celsius in the incubator. The medium was refreshed every two days. The directions of cells were observed with an inverted phase-contrast microscope every 24 hours. The results were compared with the control test without flow.

Flow Test in Parallelepiped Channel

In the flow test with parallelepiped channel, the channel with the suspension was placed in the incubator for several hours (24 hours for the glass, 3 hours for PDMS) to make cells adhere to the plate of the chamber before the flow test.

After the chamber was set on the microscope out of the incubator, the constant flow of the medium was applied to adhered cells with the syringe pump (Fig. 3). The flow path was carefully examined to avoid mixing of air bubbles, which might stir the medium in the flow chamber and induce exfoliation of cells. The behavior of cells on the plate of the chamber was observed with the microscope. The photos of cells were taken during the flow test for one hour. Variation was made in flow rate between 1×10^{-9} m^3 s^{-1} and 8×10^{-9} m^3 s^{-1}.

Shear Stress on Wall of Parallelepiped Channel

The shear rate [G, s^{-1}] on the wall of the plate is calculated by Eq. 1, which is assuming a parabolic distribution of the velocity between parallel plates (Fig. 6).

$$G = 6\, q / (b\, D^2) \qquad (1)$$

In Eq. 1, q is the flow rate [m^3 s^{-1}], b is width of the canal [m] and D is distance [m] between two parallel walls. In the present study, D is 0.1 mm, and b ranges from 1 mm to 3 mm.

The shear stress T [Pa] is the product of viscosity N [Pa s] of the fluid and the shear rate G [s^{-1}] of the flow (Eq. 2).

$$T = N\, G \qquad (2)$$

The viscosity of the medium was measured with the cone and plate type of a viscometer (TVE-22L, Toki-Sangyo Co., Ltd. Tokyo).

3. RESULTS

The experimental results with C2C12 of donut shaped canal show that cells adhere adjacent to the inner circle in the donut shape of the canal between the silicone disks. The cells proliferate in the vortex flow of the medium. The array of myotubes grows around the silicone disk, and the alignment curves to the radial direction. The longitudinal axes of cells orient to the direction perpendicular to the flow, and the orientation develops from the inner circle to the outer circle (Fig. 7). The experimental results show that cells extend to the area, where cells have not adhered yet. The results with normal cartilage cell, with L6, and with L929 were similar to that with C2C12. They develop orientation perpendicular to the flow. Flow stimulates differentiation of C2C12 to myotubes.

The experiment with CS-2P2-C75 shows that cells adhere to the bottom of the culture dish adjacent to the inner circle of silicone disk in 24 hours. The area of adherence extends to the radial direction. Cells elongate to the spindle shape, of which long axis tilts to the circumferential flow direction after the third day of culture (Fig. 8). The results with A7r5 were similar to that with CS-2P2-C75.

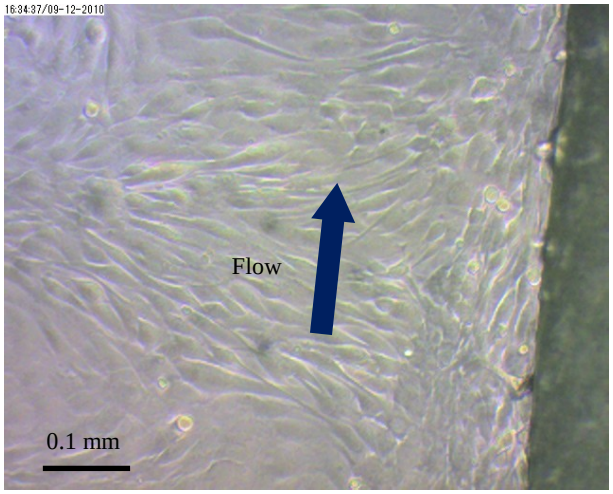

Fig. 7: C2C12 cultured for 3 days in flow near inner circle between silicone disks.

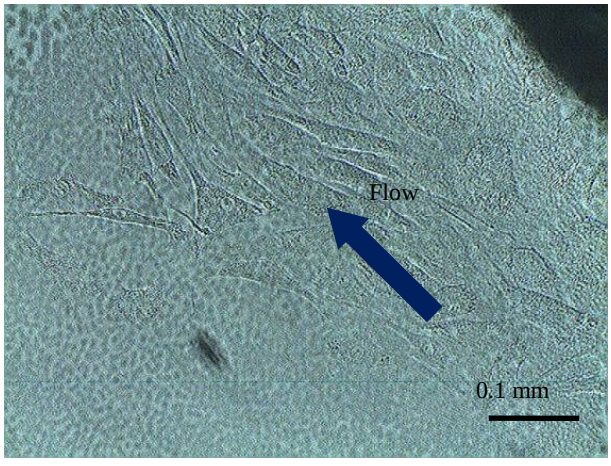

Fig. 8: CS-2P2-C75 cultured for 3 days in flow near inner circle of 30 mm silicone disk.

The result of measurement with the viscometer shows that the viscosity of the medium is 0.0010 Pa s at 25 degrees Celsius at the shear rate of 600 s^{-1}. The calculated shear rate on the glass wall of the flow chamber by Eq. 1 varies between 220 and 5000 s^{-1}, when the flow rate varies between 1×10^{-9} m^3 s^{-1} and 8×10^{-9} m^3 s^{-1}. The calculated shear stress, thus, varies between 0.2 and 5 Pa for viscosity of 0.001 Pa s, when the shear rate varies between 220 and 5000 s^{-1}.

Figs. 9-11 shows cartilage cells under the steady flow of 30 mL/hour, which generate a wall shear stress of 3 Pa estimated by Eqs. 1 & 2. The cell (A in Fig. 9) elongates to the downstream (Fig. 10) and exfoliates in three minutes (Fig. 11).

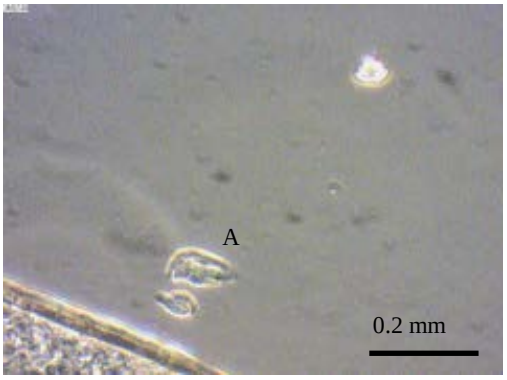

Fig. 9: Cartilage cells before flow stimulation.

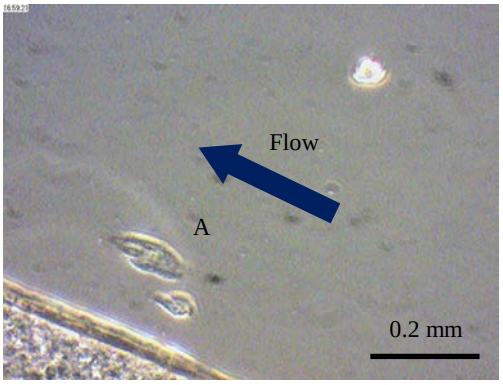

Fig. 10: Cartilage cells in the flow of 30 mL/hour for two minutes.

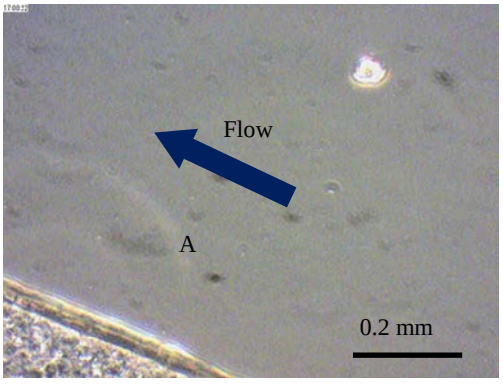

Fig. 11: Cartilage cells in the flow of 30 mL/hour for three minutes.

Figs. 12&13 show L929 under the flow of 18 mL/hour, which generate a wall shear stress between 2.9 Pa and 3 Pa estimated by Eqs. 1 & 2. The medium flows from left to right in Figs. 12&13. The distance from left to right is 0.5 mm in Figs. 12&13. The inlet of the chamber is located near left end of Figs. 12&13, and the width of the flow path linearly increases from 1.0 mm to 1.1 mm in the figures. As the increase of the width of the plow path, the wall shear stress decreases. The estimated shear stress varies from 3.0 Pa at left-end to 2.9 Pa at right-end. Some cells elongate to the downstream along the streamline of the flow in 40 min (left upper B in Fig. 12). Some cells (center C in Fig. 13) exfoliate, and flow to the downstream in 43 min.

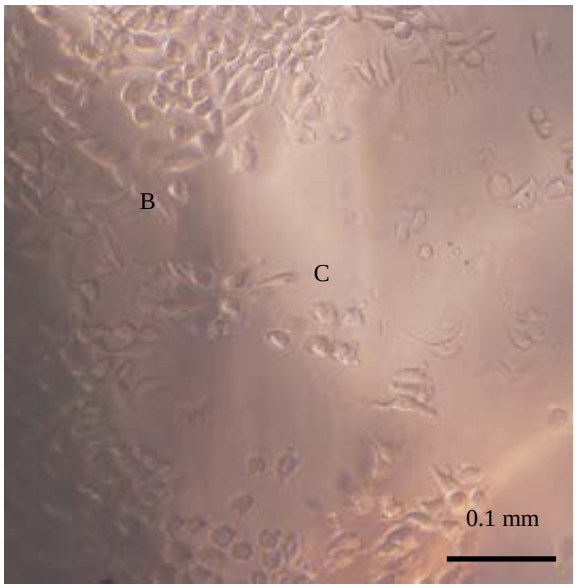

Fig. 12: L929 after flow stimulation of 18 mL/hour for 10 min.

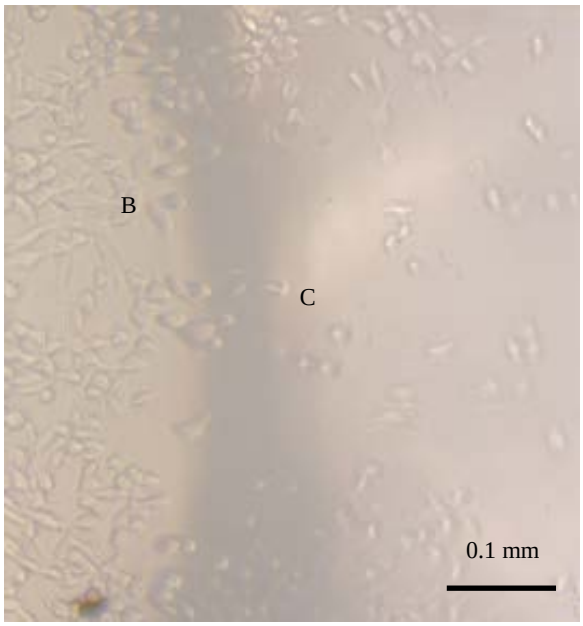

Fig. 13: L929 after flow stimulation of 18 mL/hour for 43 min.

The critical shear stress, at which the cells are exfoliated, is summarized in Table 1 with the cumulated shear. The cumulated shear is the integral of the shear stress during the exposure time. The results show that C2C12 is more adhesive than the other cells to the glass surface after the incubation for 24 hours.

The experimental results show that L929 is easily exfoliated from the glass plate. Adhesion between fibroblasts and PDMS (oxygenized), on the other hand, is much harder. Three hours is enough to make adhesion of L929 to PDMS, although 24 hours is not enough to make adhesion of L929 to glass.

Table 1. Shear stress of exfoliation (on glass)

Cell	Shear stress [Pa]	Cumulated shear [Pa min]
C2C12	6	18
Cartilage Cell	3	9
L6	0.4	8
A7r5	0.3	4.5
L929	*	*
L929**	3	129

* exfoliated immediately at the flow rate of 1×10^{-9} m^3 s^{-1}.
**on polydimethylsiloxane.

4. DISCUSSION

Several movements occur on adhered cells in the flow: deformation, tilting to downstream, elongation along the streamline, deformation to be rounded, exfoliation, rolling to downstream [10-12].

It is not easy to estimate the wall shear stress in the medium flow at the bottom of the donut-shaped open channel, which has free surface to the air. It is possible, on the other hand, to estimate the wall shear stress in the medium flow in the parallelepiped flow channel, where a parabolic distribution of the velocity can be assumed [7, 12]. The small Reynolds number of the present experiment supports the estimation. The similar response of the cell to the medium flow shows that the shear stress is in the same level between tests of two types of flow channel: tilting, orientation, and exfoliation.

The inner surface of the chamber was exposed to the oxygen gas in a reactive ion etching system (RIE-10NR, Samco Inc., Kyoto) to be characterized as hydrophilic (oxygen plasma ashing), before assembling. The oxygenized surface of PDMS might make strong affinity to L929. Micromachining technique might extend the fabrication of the flow channel for biological cells [14].

Both acceleration of proliferation and orientation of cells are important targets in the research field of regenerative medicine on cultured biological tissue. The previous study shows that electric stimulation enhances differentiation of muscle cells [1]. Another study shows mechanical stimulation improves a tissue-engineered human skeletal muscle [5]. Another previous study shows that muscle cells can adhere and proliferate under electric stimulation with periodical pulses, and that adhesion of muscle cells can be controlled with the amplitude of the pulse [1].

The previous studies show that a mechanical field, on the other hand, affects on cells' behavior. Erythrocytes are very flexible,

and are rolled and deformed in the shear flow [13]. The shear flow also affects on the orientation of endothelial cells [7, 9, 10, 15, 16]. The shear stress affects on the orientation of the smooth muscle cells in the biological tissue [17, 18]. The direction of the mechanical field affects orientation of fibroblasts [6].

Too strong mechanical stimulation damages cells. The moderate mechanical stimulation, on the other hand, might accelerate differentiation of cells [7]. The mechanical stimulation decreases proliferation of cells [8]. The mechanical stress also exfoliates several cells, which makes vacancy around the adhesive cell. The differentiation might be optimization of cells to changing environment. In the present study, the differentiation was confirmed that the developed myotubes show synchronous contraction with electric pulses, which applied to the culture medium.

The channels which have been used in the present study are useful to investigate the response of cells to the flow, because of the uniform direction of the stream line. The parallelepiped flow channel is available to quantify the effect of shear stress on the cell.

5. CONCLUSION

Response of cells to the flow has been studied *in vitro*. The experimental results show that cells are responsive to the flow, which governs orientation, exfoliation, and differentiation. The response depends on the kinds of cells: endothelial cells orient along the stream line, although myocytes orient perpendicular to the stream line. The adhesion depends on the combination between scaffold and cell: myocytes are more adhesive to glass than cartilage cells, and fibroblasts are more adhesive to polydimethylsiloxane than glass.

6. ACKNOWLEDGMENT

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