ABSTRACT
A new model is proposed which describes the spontaneous detachment of vascular smooth muscle cells induced by surface curvature. Growing tubular structures from smooth muscle cells (SMCs) in vitro is a key challenge in microvascular tissue engineering. SMC growth is however significantly suppressed on curved substrates. We show that this is caused by mechanical interaction between adhering cells and the surrounding geometry, which compromises the adhesion of growing tissue. Our model opens up new strategies for engineering luminal vasculature in microdevices, and gives new insights for controlling tissue formation in micro environments.

KEYWORDS: Vascular smooth muscle cell, Curvature, Detachment

INTRODUCTION
Our research is motivated by the recent progress in tissue engineering which has led to clinical successes for several functional tissues [1]. However, the restrictions of passive diffusion of biomolecules pose a major challenge against producing thick tissues. Thus, there is a need for micro vasculature to construct large organs in vitro. Though we have reported a basic coculture system of vascular smooth muscle cells (SMCs) and endothelial cells (ECs) utilizing a separable microchip [2], the curious phenomenon that growth of SMCs is significantly suppressed by curvature remains elusive [3]. To construct multilayered luminal vasculature inside a microchip for practical use in tissue engineering (Fig. 1), the mechanism how SMCs interact with the surrounding structure must be clarified. In this report, we investigated how SMCs act on curvature, which finally determines tissue morphology.

THEORY
SMCs are spindle-shaped muscle cells that provide mechanical stability to the vascular wall. Although they form luminal vasculature with ECs in the living body, their growth is significantly suppressed on curved substrates whose diameter is several 100 µm (Fig. 2). Assuming that their strong contractility detaches themselves and finally terminates their growth, we propose a simplified detaching model (Fig. 3a). In this model, cells or tissue on curvature are described by one-dimensional contractile elements between adhesion points that can detach from the substrate due to their contractility. First, the probability of detachment $P_D$ is determined by the force balance in the following equation,
where $F_D$ and $F_A$ are detaching force and adhering force, respectively. Next, $F_D$ is determined by the contraction force $\gamma$ of the linear element and the angle $\alpha$ between the surface and direction of force (Fig. 3b). It can be finally expressed using curvature of surface $\kappa$ and element length $L$ as:

$$F_D = 2\gamma\sin\alpha = \gamma L$$  \hspace{1cm} (2)

In addition, $\gamma$ can be expressed using a contraction constant $c$ in following equation, assuming that the contraction force generated by cells or tissues is proportional to their length:

$$\gamma = cL$$  \hspace{1cm} (3)

Combining Eq. 2 and Eq. 3 with Eq. 1, $P_D$ can be finally expressed as:

$$P_D = \frac{cL^2}{F_A}$$  \hspace{1cm} (4)

This equation predicts that $P_D$ is enhanced by larger $c$ and $\kappa$. In order to verify this model, the effects of these parameters on cellular detachment were investigated throughout the following experiment.

**EXPERIMENTAL**

Primary human arterial SMCs (HASMCs) (Kurabo, Japan) between passages 6 - 10 and smooth muscle cell growth medium (Lonza, Switzerland) were used for the experiment. First, a glass substrate with microchannels of different curvature (Fig. 4a) was sequentially coated by 0.1 % Poly-L-lysine and 0.3 mg/mL type I collagen solution for 2 hours and overnight, respectively. Next, HASMCs were seeded onto the substrate at the density of $5 \times 10^4$ cells/cm$^2$. After 2 hours of incubation, the substrate was placed in the medium to which contraction inhibitor or enhancer was added. In order to observe $P_D$ under different cellular contractility, 5 conditions were prepared. 2 and 20 $\mu$M of Blebbistatin were added to reduce $c$, and 5 and 25 nM of TGF-b were added to increase $c$. In the remaining condition, none of them was added for control. After 48 hours of cell culture, the actin cytoskeleton and focal adhesions were stained by immunofluorescence to visualize tissue morphology following immobilization. Finally, the substrates were inversely placed onto glass coverslips and their structure was observed by confocal laser scanning microscopy.

The obtained data were processed to calculate the volume ratio of detaching parts (Fig. 4b). In brief, the surface shape of substrate was extracted from the stack of fluorescent images. Then, the number of fluorescent voxels close to the surface of substrate $N_A$ was counted as the volume of adhering cells. On the other hand, the number of fluorescent voxels far from the surface of substrate $N_D$ were counted as the volume of detaching cells. The criterial length to judge close/far from surface was determined by the thickness of the monolayer of SMCs on the reference flat part. Finally, the volume ratio of the detaching part $R_D$ was calculated from $N_A$ and $N_D$ as:

$$R_D = \frac{N_D}{N_A + N_D}$$  \hspace{1cm} (5)

In this calculation, $R_D$ corresponds to averaged $P_D$ of intended area which is described in simplified detaching model.

**Figure 3:** Schematics of simplified detaching model. (a) Basic idea of this model. Probability of detachment is determined by the balance of detaching and adhering forces acting on cell or tissue. (b) Description of detaching force generated by contraction of theoretical chords. (c) Description of contraction force.

**Figure 4:** Schematics of experiments. (a) Glass substrate with different curvature. (b) Numerical calculation. The number of fluorescent voxels near/far from the surface were distinguished and compared. Thickness of adhering part was determined from the reference part in flat area.
RESULTS AND DISCUSSION

The summarized result is shown in Fig. 5. There is a general tendency that larger $\kappa$, which means smaller radius of microchannel, induces detachment of SMCs from curvature. On the other hand, when focusing on cellular tension, the presence of blebbistatin clearly suppresses the detachment of SMCs. Especially in the case of 20 $\mu$M, no SMCs detached from the surface even on the largest $\kappa$. In contrast, the detachment of SMCs was dramatically promoted by TGF-β. Under the presence of 25 nM of TGF-β, detachment was observed even in the smallest $\kappa$: almost all SMCs detached and finally formed a bridging structure across the microchannel. Though the control of $c$ was not quantitative enough to validate a linear relationship to $P_D$, this result clearly shows that stronger contractility $c$ and surrounding curvature $\kappa$ cooperatively induce tissue detachment, which accords to the proposed detaching model. It also implies that the force-geometry balance would be a more severe problem when using smaller environments or more contractile cells. In the case of constructing luminal microvascular tissue from HASMCs in microchip, reducing $c$ is necessary. Adding contraction inhibitors or reducing the rigidity of the substrate [4] would be effective to suppress cellular contractility, however this might potentially affect other cellular processes.

In this preliminary model, each parameter described above was assumed to be independent of $\kappa$. However, recent progress in molecular biology and biophysics have revealed that cellular functions actively respond to mechanical force [5]. Though detachment behavior of SMCs could be basically explained by the model, there is still a possibility that curvature potentially affects other parameters and functions of SMCs.

CONCLUSION

Previous researchers have optimized various physical and chemical substrate properties for micro tissue modeling. Curvature on a scale far larger than a single cell was found to promote the growth of bone tissue [6]. We firstly report that in the case of smooth muscle cells, curvature can also inhibit tissue formation by contractility-induced detachment. We conclude that the balance of force and geometry is an important factor for micro tissue engineering. This knowledge opens up new strategies for engineering luminal vasculature in microdevices, and gives new insights for designing complex tissues utilizing intrinsic cellular contractility.

ACKNOWLEDGEMENTS

This work was supported by Grant-in-Aid for JSPS Fellows (22・9034), JSPS Grants-in-Aid for Young Scientist (A) (21681019), Grant-in-Aid for Challenging Exploratory Research (21651049), the JSPS Core-to-Core program (20002) and the EU Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 327065.

REFERENCES


CONTACT

T. Yamashita, tel: +81-3-5841-7233; yamashita@icl.t.u-tokyo.ac.jp