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Growth of primary embryo cells in a microculture system

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Abstract

We present optimal perfusion conditions for the growth of primary mouse embryonic fibroblasts (mEFs) and mouse embryonic stem cells (mESCs) using a microfluidic perfusion culture system. In an effort to balance nutrient renewal while ensuring the presence of cell secreted factors, we found that the optimal perfusion rate for culturing primary embryonic fibroblasts (mEFs) in our experimental setting is 10 nL/min with an average flow velocity 0.55 $\mu\text{m}/\text{sec}$ in the microchannel. Primary mEFs may have a greater dependence on cell secreted factors when compared to their immortalized counterpart 3T3 fibroblasts cultured under similar conditions. Both the seeding density and the perfusion rate are critical for the proliferation of primary cells. A week long cultivation of mEFs and mESCs using the microculture system exhibited similar morphology and viability to those grown in a petri dish. Both mEFs and mESCs were analyzed using fluorescence immunoassays to determine their proliferative status and protein expression. Our results demonstrate that a perfusion-based microculture environment is capable of supporting the highly proliferative status of pluripotent embryonic stem cells.

Keywords

Embryonic Stem Cells; Embryonic Fibroblasts; Perfusion Microculture System

1. Introduction

Embryonic stem cells are a promising source for tissue to use in transplantation therapy.¹ The study of stem cells has greatly contributed to the understanding and treatment of diseases such as Leukemia,² Parkinsons,³ and Diabetes.⁴ In addition, embryonic stem cells and recently described induced patient-specific pluripotent stem (iPS)^{5,6,7} cells provide the starting material for assessment of disease development using *in vitro* differentiation paradigms.^{8,9,10,11} The use of pluripotent cells as a therapeutic agent depends greatly on the ability to control self-renewal capability and direct lineage specific differentiation.

Stem cells can be directed to either self-renew or differentiate through environmental cues,¹² but the highly transient and localized cellular events involved in stem cell proliferation and directed differentiation are hardly predictive in nature or in culture dishes. The complexity of the microenvironment found in stem cell niches^{13,14} is difficult to reproduce *in vitro*; however, recent advances in microfluidic systems have demonstrated the potential to partially mimic the chemical conditions found in these niches. Such systems offer accurate control of the cell microenvironment under consideration.^{15,16} For example, microsystems have been used to generate chemical gradients for the directed differentiation of adult stem cells,^{17,18,19,20} to control cell-cell contact of mouse embryonic stem cells in a culture dish,²¹

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guide the axonal growth of neurons,²² and show gene regulation in response to a transient chemical stimulus.²³ The greatest advantage of a microculture system is the ability to manipulate and assess local culture conditions and configure the microenvironment. Culture conditions can be adjusted through the control of medium flow, temperature and pH, concentration gradients of exogenous growth factors, and possible electrical and optical stimulations. Importantly, a microculture system consumes very little biochemical reagents and allows the study of both isolated and combinatorial cell culture paradigms.

Although many studies have demonstrated the ability of microsystems to support immortal cell types, investigations of primary microcultures, including embryonic stem cells are relatively few.²⁴ Primary cells are derived directly from dissociated tissue. Immortal cells were primary cells that have been cultured through many passages to induce irreversible genetic changes,^{25,26} and thereby exhibit rapid growth and decreased reliance on cell secreted factors when compared with their source cells. In general, primary cells are more sensitive to the variation of culture conditions,^{27,28} and can be more representative of *in vivo* response. In this paper, we present the culture conditions and growth characteristics of primary mouse embryonic fibroblasts (mEFs) and primary mouse embryonic stem cells (mESCs) in a poly-dimethylsiloxane (PDMS) microculture system. We found that the optimal perfusion rate in our microculture system is 10 nL/min with an average flow velocity 0.55 $\mu\text{m}/\text{sec}$. From basic mass transfer theory, we speculate on how the perfusion flow balances the level of nutrient and cell secreted factors. The effects of seeding density and perfusion rate on cell growth and viability were investigated. Both mEFs and mESCs were analyzed using fluorescence immunoassays to determine their proliferative status and protein expression levels. The system we developed has a very simple channel design and is capable of maintaining a relatively uniform culture environment to facilitate the self-renewal of undifferentiated mESCs. We cultured mEFs alone and studied their growth characteristics, and cocultured mEFs and mESCs, using mEFs as a feeder layer to keep the embryonic stem cells in a primordial state. Primary mEFs and mESCs cultured in the microsystem exhibited similar morphology and viability to their growth in a conventional culture dish. Our study supports further investigation into the controlled differentiation of both embryonic stem cells or alternatively iPS cells in a microculture system.

2. Materials and Methods

Microsystem Fabrication

The microculture system was made by the soft lithography technology.^{29,30,31} Briefly, a printed mask was used to pattern a 150 μm film of photoresist (SU-8 2075, Microchem), which was deposited on a 10 cm silicon wafer using a spin coater (Laurell). After UV exposure and feature development, the master was used to make a PDMS (Sylgard 184, Dow Corning) mold, which was cured at 150 $^{\circ}\text{C}$ for 15 minutes. The inlet and outlet ports were punched with a tubing corer (Technical Innovations). Along with the PDMS mold, a #1.5 coverslip was simultaneously exposed to air plasma (Harrick Plasma) for 5 minutes. The two plasma-treated surfaces were quickly brought into contact to form a covalent bond. Poly-etheretherketone (PEEK) tubing (Upchurch) was used to connect the channel inlet to a 250 μL syringe (Hamilton). The syringe was filled with culture medium and loaded on a programmable syringe pump (Picoplus, Harvard Apparatus). A three way valve was fitted on the syringe tip to facilitate debubbling of the medium. Silicon tubing was found to have a detrimental effect on primary cell viability. This may be due to leaching of a toxic solvent or nonspecific absorption of nutrient to the tubing.¹⁷ The use of PEEK tubing significantly improved cell viability in our microsystem.

Cell Culture

All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Connecticut and conform to National Institute of Health guidelines. For the study of proliferation of mEFs, primary mEFs were harvested from a CD1 female at stage E13.5. Aliquots of 1 mL of $\sim 10^6$ cells/mL were prepared in cryopreservation medium and subsequently frozen at -128°C for later use. In each experiment, cells were thawed in a 37°C water bath and plated in a 100 mm dish for 1 day. Following a wash with PBS, cells were incubated with trypsin (Invitrogen) for 3 minutes and spun down. The pellet was resuspended in warm culture media and cells were seeded at the desired density in the microsystem and dish. Primary mEFs were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 0.1 mM non-essential amino acids (Invitrogen), 2 mM Glutamax (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.55 μM 2-mercaptoethanol (Sigma), and penicillin/streptomycin/-neomycin (Invitrogen). All cultures were maintained in a humidified incubator at 37°C and 5% CO_2 . The experimental setup is shown in Fig. 1. The microchamber was sterilized by flushing 2 mL of 70% ethanol followed by 10 mL of PBS and repeated three times. To facilitate cell adhesion to the substrate, both the microsystem and dish controls were coated with 0.01% poly-L-lysine (Sigma) for 4 hours and 0.1 mg/mL fibronectin (Invitrogen) overnight. For static experiments in the microchambers, the ports were underfilled with culture medium and covered with small PDMS blocks to form a reversible bond. This arrangement prevented the formation of gas bubbles during the static experiment and allow access for daily medium change and later immunostaining.

For culturing mESCs with mEFs as the feeder layer, JAX mESCs³² were maintained on a layer of mitomycin-C treated mEFs. The mESCs were cultured in mEF medium described above supplemented with 15% fetal bovine serum (Hyclone) and Leukemia inhibitory factor (1000 U/mL, Chemicon). The medium inside the syringe of the perfusion system was changed every other day to prevent possible degradation of the culture medium.

Immunocytochemistry

Following culture, cells were washed with PBS and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 30 minutes at room temperature. Careful introduction of reagents to the microchamber is necessary to ensure that cells are not removed in the process. Cells were permeabilized and blocked with a solution of 10% goat serum in 0.1% Triton X-100 (Sigma, T-8787)/PBS solution for 1 hour.

Primary antibodies were made in 10% goat serum to prevent nonspecific cross reactivity and incubated at 4°C overnight before rinsing with PBS: (a) rabbit anti-Ki67 IgG (Novocastra, 1:1000, NCL-Ki67p) to label proliferative mEFS, (b) mouse anti-Caspase 3 IgG (Millipore, MAB4703, 0.3 $\mu\text{g/mL}$) to label mEFs undergoing apoptosis, (c) mouse anti-SSEA-1 IgM (Millipore, MAB4301, 1:100) to label undifferentiated mESCs, and (d) rabbit anti-Oct4 IgG (Millipore, AB3209, 1:200) to label undifferentiated mESCs. Cells were then rinsed three times in PBS for five minutes each to remove primary antibodies. Secondary antibodies were made in a 10% goat serum/PBS solution and incubated for 4 hours on cell culture microchambers followed by three more five-minute washes with PBS: (a) goat anti-rabbit IgG Alexafluor 546 (Invitrogen, A-11010), (b) goat anti-mouse IgG Alexafluor 488 (Invitrogen, A-11001), (c) goat anti-mouse IgM Alexafluor 546 (Invitrogen, A-21045), and (d) goat anti-rabbit IgG Alexafluor 488 (Invitrogen, A-11008). All secondary antibodies were used at a dilution of 1:500. DAPI was added to visualize nuclei for cell counting. Mountant was not used in the microchambers as it removed cells due to its high viscosity. Processed microchannels were filled with PBS and the ports were covered with small PDMS slabs for later observation.

Microscopy

Immunostained cells were visualized on a fluorescence microscope (Zeiss Axoskop) equipped with a CCD camera (Retiga EX, QImaging). Images were captured with OpenLab (Improvision) on a desktop computer. Phase contrast microscopy was performed on an inverted microscope (Unico) equipped with a CCD camera (Micropublisher RTV 3.3, QImaging) using Q-Capture (QImaging) on a desktop computer. Image analysis was done with ImageJ (NIH) for cell counting. Electron micrographs were obtained on a field emission scanning electron microscope (Zeiss 982 Gemini).

3. Results & Discussion

Seeding Density and Gas Transport

It has been shown that primary mEFs in a culture dish grew slower, or not at all, at low seeding densities (0–5,000 cells/cm²), and grew faster at higher densities (>20,000 cells/cm²) until confluency.²⁵ Seeding density should also affect the response of primary cells in a microculture system. We varied the seeding density within the microchamber and observed the fold increase in cell number after 3 days without perfusion. We found a similar response at low densities (0–5,000 cells/cm²) in the microsystem, slow growth or none at all (Fig. 2). At the lower limit, cells are most likely constrained by the low concentration of cell secreted factors present in the culture medium. On the other hand, at high seeding densities (>30,000 cells/cm²) cells also exhibited slow growth or none at all. This response differs from what was observed in a culture dish.²⁵ It has been suggested that a buildup of growth inhibiting factors will constrain the growth of cells in a microsystem.³³ Alternatively, it is likely that high cell densities in the microsystem are constrained by nutrient depletion due to the large cell culture area to volume ratio. For our system this ratio is at least an order of magnitude greater than a 35 mm culture dish. Although the medium was replaced each day, nutrients can be consumed quickly. Continuous perfusion provides constant replenishment and should support a broader range of cell densities.

PDMS is known to be highly permeable to gases such as CO₂ and O₂.³⁴ To be certain that the PDMS layer does not limit gas transport during cell culture, we fabricated circular membranes with diameter 3 cm and thickness from 1 to 15 mm, supported by several 150 µm tall columns to permit a confined medium space when the membrane was placed in a culture dish. Cells were seeded throughout the confined space between the PDMS layer and the substrate of the culture dish and were cultured as in a regular culture dish. No difference in cell viability was observed between cultures either without the PDMS membrane or with membranes of varying thickness from 1 to 15 mm. The similar results suggest that PDMS in this range of thicknesses does not restrict gas transport in our system. The oxygen diffusivity in PDMS is about 3.4×10^{-9} m²/s,³⁴ the same order of magnitude as the diffusivity in a culture media. Assuming the saturated O₂ concentration in PDMS is also similar to the culture media, around 170 nmol/mL, the thickness of PDMS membrane or the diffusion length is less than 15 mm, and oxygen is completely depleted at the substrate, the corresponding oxygen flux across the diffusion length would be higher than 4×10^{-8} mol/s/m². For an observed high cell density of approximately 4×10^4 cells/cm² in our microculture system, this diffusion flux is sufficient to feed primary mEFs with an oxygen consumption rate about 0.1 nmol/s/10⁶ cells.³⁵

Proliferation of Primary mEFs

MEFs have a flat fusiform morphology *in vitro* and are a highly mobile cell type. To confirm that cell growth in the perfused microculture system is similar to that expected in a culture dish, eight microsystems and eight dishes were cultured for comparison. Each day, cells in a microsystem and a dish were stained to determine the cell density, death and

proliferation over a period of 8 days. Cells in the microchamber were seeded at a density around 11,000 cells/cm² and perfused with medium at a flow rate of 10 nL/min. This flow rate corresponds to a replacement time of 500 minutes (~3 replacements per day) and was found to provide adequate replenishment of nutrient and retention of cell secreted factors. Secretion dependent growth and inhibition are important for self organization of tissue in a developing embryo. When perfused at a higher flow rate (>10 nL/min) cell death occurred over the course of a day, likely due to the removal of cell secreted factors. The flow rate of 10 nL/min is an order-of-magnitude lower than the maximal flow rate found for the immortal line 3T3 fibroblasts cultured in a similar channel configuration.³⁶ This is perhaps because 3T3 cells, which are derived from mEFs, have a lower dependence on cell secreted molecules.²⁵ At a very low flow rate (1 nL/min) cell death increased over the course of a few days. A tunable flow rate corresponding to cell density is an alternative approach. However, the constant flow rate of 10 nL/min worked well for primary mEFs at density range around 10,000 to 30,000 cells/cm. We observed fewer dead cells during the perfusion experiment than in the static microchannel experiment, indicating that the faster replacement time is sufficient for cells to uptake nutrients.

Primary mEFs exhibited a healthy morphology throughout the 8 days of culture. Cells formed a confluent monolayer on day 3. As cell number increased, the monolayer was more densely packed, without showing alignment associated with the flow direction (Fig. 3b). Studies have found fibroblasts to collectively align with the flow direction in response to shear stress.^{37,38} In our study, the hydrodynamic shear stress is too low to direct cell alignment. Primary mEFs are uniformly distributed throughout the chamber even if the seeding is initially nonuniform.

To assess cell proliferation, cells in the microchamber were fixed and stained, shown in Fig. 4. Ki-67 expression, a marker for cell undergoing mitosis, was observed mainly on the first two days in the microsystem and conventional culture dish. This suggests contact inhibition or a buildup of secreted molecules occurred after two days as the cells reached confluency. Importantly, apoptosis was largely absent in both the microchannel and dish, as indicated by active Caspase-3 (Cas-3) staining. Morphological examination also showed that cell cultures in both the microchannel and dish appeared viable with a low number of detached dying cells.

The growth of mEFs in the microchamber can be modeled by the logistic equation:³⁹

$$\frac{dN}{dt} = kN \left(1 - \frac{N}{N_{\infty}} \right), \quad (1)$$

where N is cell density [cells/cm²], k is the growth rate [1/hr], and N_{∞} is known as the carrying capacity or the saturated cell density. The exact solution of the logistic equation is given by

$$N(t) = \frac{N_0 N_{\infty}}{N_0 + (N_{\infty} - N_0)e^{-kt}} \quad (2)$$

where t is time and N_0 is the initial cell density. The solution describes an exponential growth phase, followed by a linear phase until saturation of the cell population. This result is used to determine the cell growth rate from experimental data. The growth rate k was obtained by counting DAPI stained nuclei using ImageJ (Fig. 4). Cell growth exhibited

linear behavior for a period of two days and then the growth rate reduced until cell density reached saturation at day 4 (Fig. 5). We did not observe the exponential growth phase suggested by the logistic law perhaps due to insufficient cell secreted molecules at low cell density. Using an initial seeding density of 11,300 cells/cm² and the observed saturation density of 34,600 cells/cm², the growth rate for primary mEFs cultured in the microchannel is 0.051 hr⁻¹. This result is similar to the growth rate found for primary adult human foreskin fibroblasts, 0.040 hr⁻¹, cultured in a microchannel at a flow rate of 166 nL/min.³⁸ The nonlinear fit in Fig. 5 has a correlation coefficient 0.98.

Primary mEFs are important as a feeder cell for cultured embryonic stem cells, and fibroblasts are the main cell type used for generating iPS cells, implicating their use as a cell type of choice in microculture systems. The culture of mouse embryonic stem cells (mESCs) within a microsystem is an essential initial goal for the study of self-renewal and differentiation of embryonic stem cells. Next, we describe the growth characteristics of mESCs in a microculture system.

Self-Renewal of mESCs

We cocultured mEFs with mESCs at a flow rate of 10 nL/min. Feeder layer mito-C-treated, nondividing mEFs were seeded in the microchannel at a density ~20,000 cells/cm² and cultured overnight. The following day, mESCs were seeded on top of the mEFs at a density of ~8,000 cells/cm² (the dark spots in Fig. 6a). At day 2, mESC colonies were visible and by day 3 they formed larger aggregates (Fig. 6b). The colonies were well organized and appeared spheroid, though they did not reach the top wall of the channel. Fibroblasts were dispersed throughout the channel and some formed a bird's nest pattern surrounding the stem cell colonies (Fig. 6c & 6d). It is likely that fibroblasts are attracted to cytokines secreted by stem cells. The surrounding mEFs secrete molecules such as FGF-2 that are important for stem cell self-renewal.⁴⁰ It is interesting to note that human ES cells cultured in the absence of a feeder layer will partially differentiate into fibroblast-like cells, producing their own feeder cells.⁴¹ mESCs in the perfusion microsystem appeared to grow at a similar rate as those kept in a conventional culture dish. This is likely because mESCs have a faster metabolism compared to mEFs, indicated by a faster pH change of the culture medium. The flow rate of 10 nL/min, while adequate for the growth of mEFs may not support mESCs at a similar cell density. As demonstrated previously, mESC colony size increases with increasing flow rate when cultured in a microchannel,³⁶ indicating a tunable flow rate can support a broader range of stem cell densities.

On day 5, mESCs were fixed and stained to determine whether cells had remained pluripotent or had differentiated. There was little morphological indication of differentiation under phase contrast microscopy, which would be noticeable by a departure from the characteristic spheroidal colony geometry. Figure 7 shows mESC microcultures stained positive for the pluripotent markers Oct-4 and SSEA-1, indicating undifferentiated mESCs. Nearly all mESC colonies stained positive for both markers, indicating a high degree of self-renewal capacity in the microchannel.

Shear Stress and Mass Transfer Characteristics

The culture chamber has dimensions 17 mm in length, 2 mm in width, and 150 μm in height. For a volume flow rate of 10 nL/min, the average flow velocity is 0.55 μm/sec. The corresponding Reynolds number using the channel height and the dynamic viscosity ~10⁻³ kg/m/s is 8.3×10⁻⁵. Due to the small Reynolds number the entrance length of the microchannel is negligible compared to the total channel length. There exists no momentum boundary layer in the microchannel. The velocity field of a steady, fully developed, pressure driven flow in a three-dimensional rectangular channel can be found in standard text.⁴² The

corresponding shear stress applied to the substrate or adherent cells is $\sim 2.3 \times 10^{-4}$ dyn/cm², corresponding to the low end of values reported for the culture conditions of several cell types.²⁴ It is expected that the shear stress is fairly uniform for almost 80% of the substrate area. The nonuniform stress near the side walls vanishes at the corners and the influence to cells should be negligible. An important aspect of the flow stress calculation is that with the addition of a cell monolayer of 15 μ m thickness, the increase of shear stress is only about 0.5%. Therefore the presence of the mEF monolayer should hardly influence the flow stress on cells. For large stem cell colonies that may occlude the microchannel the shear stress distribution is more complex.

From the perfusion rate we also speculate the mass transfer characteristics about the delivery of nutrients and the retention of cell secreted factors. The length of the concentration boundary layer can be estimated by equating the convective time scale in the streamwise direction and the diffusion time scale across the channel height, i.e.,

$$\frac{L}{U} \sim \frac{h^2}{D}, \quad (3)$$

where L is the developing length of the concentration boundary layer, U is the average or characteristic velocity, h is the channel height, and D is the molecular diffusivity. Assuming $D \sim 10^{-11}$ m²/s for small macromolecules such as FGF, for a flow rate of 10 nL/min the concentration profile generated by either cell uptake or secretion at the substrate will have a developing length ~ 1.2 mm. As the developing length is much less than the length of the culture chamber (17 mm), the concentration boundary layer along the substrate is negligible. However if the flow rate increases 10-fold, the developing length becomes 12 mm, which is closer to the chamber length. The presence of a concentration boundary layer will compress the species concentration gradient and increase mass flux to and from the cells. Although the viscous boundary layer vanishes, a diffusion boundary layer at high flow rate is expected to play an essential role in nutrient delivery and the removal of secreted proteins in the microculture chamber.

It is expected that the primary mEFs will exhibit a strong dependence on secreted molecules for growth, thus ideally there is an optimal balance between nutrient delivery and the removal of secreted molecules in order to maintain the solution conditions required for cell growth. Assisted by the scaling analysis, next we consider 8 limiting cases for the possible concentration fields of nutrient and secreted molecules within the microchannel (Fig. 8). The limiting cases outline desirable and undesirable conditions for cell growth, helping to explain experimental results in terms of the flow rate and channel configuration.

If the mass transfer of nutrients and secreted molecules are reaction limited (slower cell uptake/secretion kinetics), depicted in Fig. 8a to 8d, there will be excess nutrient and a limited amount of secreted molecules available to cells. Cases *a* and *b* have a gradient of nutrient and secreted molecules across the channel height without a concentration boundary layer due to the low flow rate. At a high flow rate, cases *c* and *d*, the concentration gradient is confined to the boundary layer. In cases *a* through *d*, cell uptake and secretion are assumed constant and the mass flux at the substrate is fixed. On the other hand, if the mass transfer is transport limited (faster cell uptake/secretion kinetics), depicted in Fig. 8e to 8h, there will be limited nutrient and an excess amount of secreted molecules available to cells. For cases *e* and *g*, nutrient is depleted at the surface, while cases *f* and *h* the concentration of the secreted molecules are assumed to be saturated at the surface. Under transport limited conditions, both nutrient and secretion flux significantly decreases downstream for all cases

e, *f*, *g*, and *h*. At high flow rate, the boundary layer enhances the mass flux to and from cells at the substrate. An ideal perfusion rate for primary mEF and mESC culture should be a compromise between the limiting cases *c* and *f*. Case *c* maximizes the transport of nutrients to cells, while case *f* maintains a high level of secreted molecules at the surface due to a minimal out flux. The optimal flow rate 10 nL/min is experimentally derived, and it implies that case *f*, the retention of secreted molecules, is a primary concern in this study. The optimal perfusion rate gives an appropriate balance of cell uptake/secretion kinetics and mass transport.

Furthermore, the thickness of the concentration boundary layer can be scaled as

$$\frac{\delta_D}{z} \sim \left(\frac{h}{z}\right)^{2/3} \left(\frac{D}{u_{max}h}\right)^{1/3}, \quad (4)$$

where δ_D is the boundary layer thickness, h is the channel height, z is the streamwise distance to the entrance point, and u_{max} is the maximum velocity of the fully developed flow.⁴³ Because δ_D is proportional to $u_{max}^{-1/3}$, so if the flow rate increases 1000-fold, for instance from 10 nL/min to 10 μ L/min, the overall thickness of the diffusion layer will be compressed to only 10% of the original thickness, and the diffusion flux will increase by a factor of 10. The scaling law indicates that with the presence of a concentration boundary layer, the transport of both nutrients and secreted molecules increases slightly. However, the shear stress is proportional to the flow rate and will increase significantly. These are important concerns in designing new microculture system prototypes.

4. Conclusion

In summary, this study demonstrates that a perfusion microculture system is capable of growing primary cells (fibroblasts) and maintaining mESCs as pluripotent cells. We found that primary mEFs and mESCs grew in the perfusion microculture system at a rate similar to those in conventional cell culture dishes. Primary mEFs are likely to have a greater dependence on cell secreted factors when compared to their immortalized counterpart 3T3 fibroblasts cultured under similar conditions. This dependence affected both cell seeding density and the perfusion flow rate required for proliferating cultures. A flow rate of 10 nL/min was found to balance nutrient delivery and secretion removal to sustain mEF densities around $1\text{--}3 \times 10^4$ cells/cm² for at least 8 days. Under the same flow rate, mESCs were found to self-renew and remain viable for at least 5 days. The fibroblast cell type is an important model for future studies incorporating iPS cells in the microsystem, and the culture parameters developed here support this goal. In this study, the cell uptake rate of nutrient and secreted molecules are largely unknown, as these chemicals are difficult to measure *in situ*. Further work on proteomics and biochemical sensing is needed to validate the details of transport mechanisms for a better understanding of the stem cell microenvironment.

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Fig. 1.

(a) Schematic of the experimental setup. (b) The single channel design (2 mm in width, 17 mm in length, and 150 μm in height, representing a total volume of 5 μL) with seeding and perfusion inlets and an outlet connected to PEEK tubing. A total of 8 microculture systems via fluidic manifolds connected to four 250 μL syringes, which were loaded on two computerized precision syringe pumps and placed outside the incubator. Each channel was fed with continuous medium perfusion at a flow rate 10 nL/min.

Fig. 2.

Fold increase in cell number over 3 days of growth in a static microchamber versus the initial seeding density. Cell culture medium was replaced daily.

Fig. 3.
Optical micrographs of mEFs in the microchannel: (a) Day 1 (b) Day 2.

Fig. 4. Primary mMEFs in the perfusion microsystem at day 1 of culture. Ki-67 marks proliferative cells, DAPI stains nuclei, and Cas-3 stains apoptotic cells. The percentage of Ki-67 labeled cells shown is about 33%.

Fig. 5. Growth of mEFs in a microculture system over an 8 day period at a flow rate of 10 nL/min. The filled square at time=0 represents the initial seeding density, while the second square represents the density of adherent cells after 4 hrs of attachment. The following 8 data points represent results from cell staining of 8 parallel chambers sacrificed at daily intervals. The dash line illustrates the hypothetical exponential growth phase obtained from the logistic model.

Fig. 6. Single cell mESCs cocultured with mEFs in a microculture system at day 1 (a), by day 3 colonies of mESCs can be detected (b), by day 5 the colonies have increased in size (c). A higher magnification of mESC colonies at day 5 (d).

Fig. 7.
MESCs in a microculture system stained for undifferentiated stem cells at day 5.

Fig. 8.

Schematic of spatial variation of the concentration of nutrient (red, subscript n) and secreted molecules (blue, subscript s) within the microchannel. Total 8 hypothetical limiting cases are delineated by slow cell uptake/secretion (reaction limited) and fast cell uptake/secretion (transport limited) events. Further distinction is made by the presence of a concentration boundary layer (dash lines) at a relatively high flow rate. The boundary condition for cases a through d is a constant surface flux, and for cases e through h is a constant surface concentration.