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3D Microfluidic Approach to Mechanical Stimulation of Osteocyte Processes

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Abstract—It has been well recognized that bone adapts its structure to best meet its mechanical environment. However, the cellular mechanism underlying bone adaptation is not well understood. Significant research efforts have been made towards understanding the cellular mechanotransduction mechanism in bone by studying cells in monolayer. These *in vitro* studies have indeed shown that bone cells are mechanosensitive and contributed critical insights into the mechanism of transduction. However, the monolayer approach may be limited in that it does not reflect the interaction of the osteocyte with its extracellular environment. It is in this context, we developed a microchamber system to mimic the *in vivo* extracellular environment in bone. Specifically, the osteocyte process appears to have unique ultrastructural characteristics which are potentially the molecular sites of cellular mechanosensitivity. In this study we describe a microfluidics approach aimed at replicating the bone canalicular system both in terms of geometry and fluid flow driven by exogenous loading. We demonstrate that individual osteocytes can be successfully cultured in this system for extended periods and extend processes similar to those observed *in vivo*. Our hope is that this approach will allow the role of flow over the osteocyte process to be better delineated.

Keywords—Osteocyte, Osteocyte process, Mechanotransduction, Microfluidics, Fluid flow.

INTRODUCTION

Mechanical loading is an important regulator of bone metabolism.[4,9] However, the cell-level mechanism that allows bone cells to respond to mechanical loading remains a mystery. Osteocytes, the cells entrapped in bone matrix (Fig. 1), have been suggested as the mechanical sensor cells.[1–3] Pericellular flow over the osteocyte caused by loading has been proposed to be one of the physical signals that osteocytes sense.[3,8] Many *in vitro* studies have shown that osteocytes respond to loading induced fluid flow.[5,6,11] However, the fluid flow exposure systems utilized in most *in vitro* studies lack the geometric and physical characteristics of the osteocyte's extracellular environment which may be important in the mechanotransduction mechanism. Such characteristics include the small cavities, lacunae, containing the osteocyte body, and very thin interconnecting canals, canaliculi, containing the osteocyte process, and the pericellular matrix connecting the osteocyte process membrane to the canalicular wall (Fig. 1). A more realistic *in vitro* loading system would allow investigators to determine the significance of these features in mechanotransduction. More specifically, as discussed by You *et al.*,[10] the mechanical signal that osteocytes detect might be the fluid flow experienced by the osteocytic cell process membrane rather than the cell body. Therefore, in order to further understand the mechanism of mechanotransduction on osteocytes, we have designed a microfluidics system which mimics the pericellular micro-architecture so that osteocyte processes can be exposed to fluid flow and the cellular response can be studied.

In this study we created several prototype microfluidics systems and demonstrated the validity of culturing osteocytes with MLO-Y4 cells. We present evidence showing that the cells remained viable in the artificial lacunae and, with time in culture, extended processes into the engineered canaliculus.

METHODS

Microfabrication of the 3D Microfluidics System

To create the microfluidics system we utilized the soft lithography approach in four steps: pattern design,

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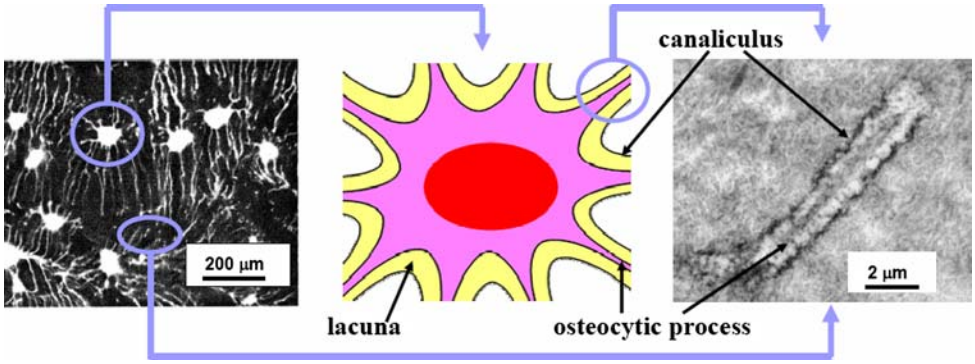

FIGURE 1. Left, photomicrograph of osteocytes encased in bone matrix; Middle, illustration of one osteocyte in a lacuna with its processes extended into the canaliculi; Right, electron photomicrograph showing a longitudinal section of an osteocyte process.

mask making, master making, and printing on polydimethyl siloxane (PDMS). The structural pattern was first created in L-edit design software (Fig. 2). This microchamber system was designed to contain multiple microchambers of 20 μm in diameter interconnected by canaliculus-like channels 1 μm wide and larger channels 20 and 40 μm wide. All microchambers and channels were designed to be 10 μm deep. This design allows the fluid to be delivered from the inlet/out regions and be driven through the different levels of channels and finally into the canaliculi like channels and lacunae like microchambers. Note that due to the change of dimension between the canaliculi like channels and the lacunae like microchambers, the fluid flow will be about 10 times slower in the lacunae than in the channels.

The pattern was then transferred to a chrome mask with a direct laser writing system. To transfer the pattern from the mask to a master, we used SPR220-7, a positive photoresist. It was spin-coated onto a silicon wafer at 1700 rpm to yield a 10 μm thick film, which was then exposed to ultraviolet light through the mask. The wafer was then treated with developer such that the uncrosslinked SPR220-7 not exposed to UV was dissolved and washed away from the wafer. To facilitate the separation of the PDMS from the master, a thin layer of Cr–Au was sputter-coated onto the entire master after developing. PDMS prepolymer and curing agent was mixed and degassed under vacuum. The inlet and outlet channels were then aligned vertically over the wafer where the inlet/outlet of the microfluidics system was patterned. The PDMS mixture was then poured onto the master, cured at 55 °C overnight and carefully removed. The result of this process was a microfabricated microfluidics system with inlet and outlet channels and multiple microchambers 10 μm deep and 20 μm in diameter, replicating the lacunae, each connected to two 10 μm deep and 1 μm wide channels, representing the canaliculi.[12]

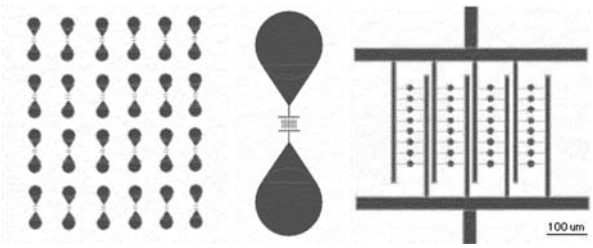

FIGURE 2. The L-edit design of the microchambers. Left, an array of microchambers on a single silicon wafer. Note the large lobes are the inlet/outlet regions for fluid delivery; Middle, a single microchamber system; Right, higher magnification depicting an array of lacunae.

Fluid Flow Delivery in the Microfluidics System

The completed microfluidics system was obtained by applying the PDMS piece to a glass slide (75 × 38 mm) and connecting the outlet of the microfluidics system to a vacuum pump. We utilize vacuum both help seal the PDMS to the glass slide as well as pull fluid from the inlet across through the chamber. Fluid can be loaded by adding the desired solution to the system inlet with flow driven by vacuum. Fluorescent beads were used to monitor the fluid flow in the microchannels. No leakage was observed during the fluid flow delivery.

Bone Cell Culture in the Microfluidics System

We coat the microfluidics system with fibronectin by vacuum driving a fibronectin solution (100 μg/mL) into the system and allowing it remain in the microfluidics system at 37 °C for 2 h. Thus, only the microchannels and microchambers are coated. The glass slide was then removed and the PDMS piece was washed with phosphate buffer solution (PBS). To ensure that cells grow in the microchambers only,

we employed two different approaches. (1) Bulk cell transferring approach. We seeded MLO-Y4 (Gift of Dr. Bonewald, University of Missouri-Kansas City) cells on the PDMS substrate at 300,000 cells/mL and cultured them at 37 °C for 30 min to allow them to attach to the fibronectin coated area (i.e., microchannels and microchambers). We then rinsed the PDMS with PBS to remove the nonadherent cells that had been deposited on non-fibronectin-coated parts of the PDMS piece. Such an approach allowed us to seed cells in microchamber and microchannels only by exploiting selective adhesion to the fibronectin coating. (2) Individual cell transferring approach. Under the light microscope, MLO-Y4 cells were aspirated into a micropipette and transferred individually into microchambers, with one cell in each chamber, and the chamber system was sealed with a fibronectin coated glass slide. For both approaches, the cells were stained with CellTrackerTM green CMFDA at 1 μM and observed with a fluorescent microscope at 24 h post seeding.

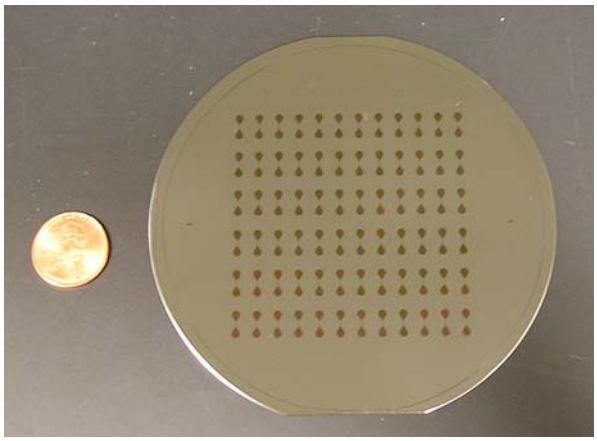

FIGURE 3. The silicon wafer with the designed flow system pattern transferred.

RESULTS

The result of the photolithography technique is illustrated in Fig. 3 on a 4″ silicon wafer. This wafer then serves as a negative master so that multiple copies of the PDMS channel systems can be fabricated. Electron microscopy of the PDMS castings demonstrated excellent preservation of the channel systems (Fig. 4) and we did not observe significant deformation of the PDMS features or collapse of the channels. Figure 5 demonstrates several prototype microfluidics systems on one PDMS substrate. One of the systems has its inlet and outlet connected to fluid delivery and vacuum tubes. Figure 6 demonstrates MLO-Y4 cells seeded and grown in the microchambers using the bulk seeding and differential adhesion approach described above. Photomicrographs obtained with epifluorescence illumination one day after seeding demonstrate that the cells remain viable while in the microchamber system. Figure 6 also shows that while many cells were located in the artificial "lacunae," cells were also found outside of the individual microchambers. The micropipette approach for seeding cells was developed to address this problem. Figure 7 shows the result of cell seeding in microchambers using the micropipette approach immediately after

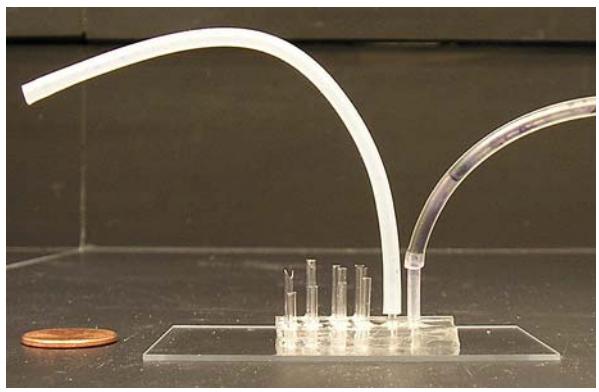

FIGURE 5. The closed microchamber system.

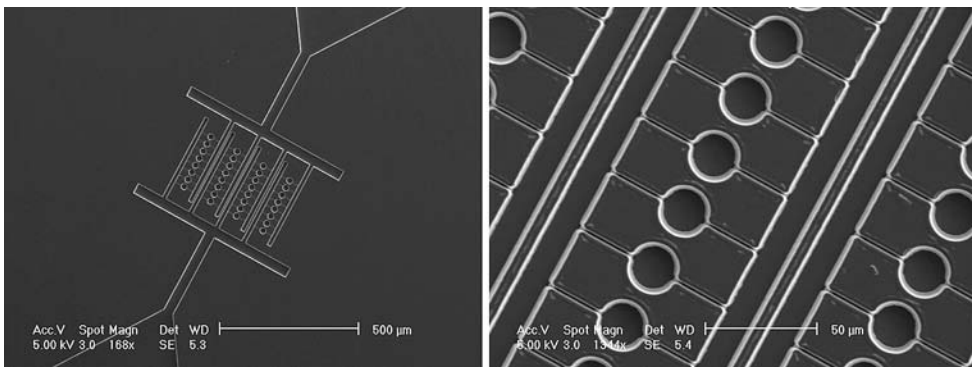

FIGURE 4. Scanning electron microscope image of the microchambers. Left, a single microchamber system; Right, higher magnification showing the geometry of the lacunae and canaliculi each intended to accommodate a single cell.

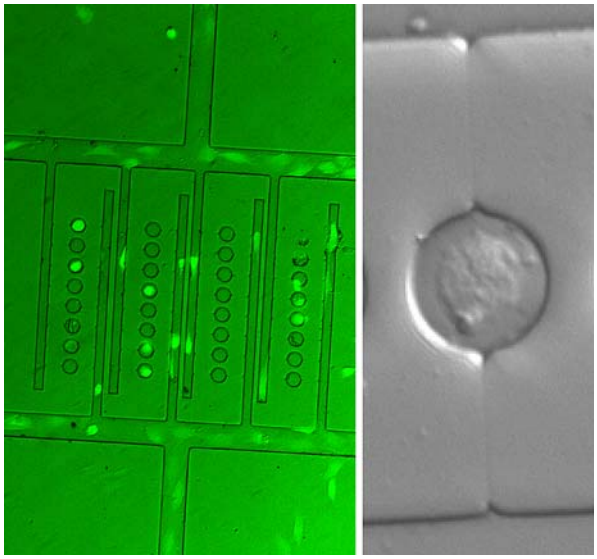

FIGURE 6. MLO-Y4 cells seeded into the microchambers using differential adhesion of the cells to fibronectin. Left, fluorescent image of cells stained with cytoplasmic Fluorescein-AM (CMFDA). Right, light microscope image of one cell grown in a microchamber.

FIGURE 7. MLO-Y4 cells seeded into the microchambers using the micropipette transfer seeding technique.

cell seeding. Here, one column of microchambers was seeded with MLO-Y4 cells with a 100% success rate. Figure 8 shows the SEM image of a cell in the microchamber 24 h after seeding. As one can see, the cell has extended multiple cell processes, one of which has extended some distance into the microchannel.

DISCUSSION

In the current study, we have built a 3D microfabricated microchamber system in which bone cells can be grown in an environment that simulates the geometry of the lacunae and canaliculi. To our knowledge, this is the first attempt to study bone cell mechanotransduction in a system designed to model the canalicular flow system. Recently Leclerc *et al.*[7] developed a micro device with MC3T3-E1 cells grown in microchannels. However, the dimensions of this microchannel system were 150–300 μm in width. These channels are several orders of magnitude larger than the canaliculus and do not allow for the specialized cell-matrix interactions seen to occur between the osteocyte process and the surrounding channel wall that may be the site of cellular mechanotransduction.[10,12] In the work presented here we were able to achieve a much smaller channel width. However, these channels are roughly one order of magnitude larger than the canalicular width. Using PDMS technology, it is not possible to further reduce the feature size without collapse. However it may be possible to utilize other polymer microfabrication techniques or the silicon structures themselves to overcome this limitation. Nonetheless, as Fig. 8 demonstrates, it does appear that although the channel width of our microchambers is too large, the cell process does form focal interactions with the channel wall that are reminiscent of those observed *in vivo*.

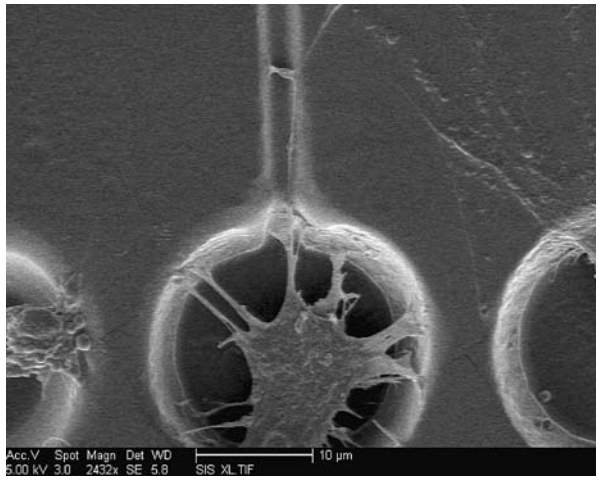

FIGURE 8. SEM of an MLO-Y4 cell in the microchamber illustrating cell process growth into the microchannel.

Two different approaches were used to seed the cells in the microchambers. For the first approach, as was shown in Fig. 6, not all of microchambers were occupied by cells and many cells were found outside of the artificial 'lacunae'. However, it may be possible to use this approach with single-cell readouts such as fluorescent intracellular calcium measurements where poorly placed cells or occluded channels can be ignored. The second approach utilized micropipette transferring of the cells to the microchambers. As can be seen in Fig. 7, this approach guarantees a high rate of success in placing cells in microchambers. However, this approach is limited in that it is labor intensive and time consuming. This may also make it impractical for cell-ensemble assays such as mRNA or protein based

measurements. Nevertheless, this approach should not be problematic for single-cell based investigations of mechanotransduction (e.g., single-cell intracellular calcium measurements).

In summary, this innovative approach allows us to culture osteocytes in a 3D environment that mimics the *in vivo* morphology of bone. Furthermore it allows us to localize osteocyte mechanotransduction to a potentially specialized cellular structure (the osteocyte process). We will next apply fluid flow to cells cultured in this system and analyzed the cellular response using single-cell based biochemical analysis technique. In the long term, this microfabricated device might be developed into an advanced artificial bone matrix with the bone cells seeded in biodegradable microfabricated microchambers interconnected through microchannels in a tissue engineering application.

ACKNOWLEDGMENT

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