Analyzing the behavior of normoxic and hypoxic cells through the use of microfluidic devices

Kellen Chen
Department of Bioengineering, University of California, Berkeley, California

Megan Rexius
Department of Bioengineering, University of Illinois at Chicago, Illinois

David T. Eddington
Department of Bioengineering, University of Illinois at Chicago, Illinois

Abstract

Current cellular exposure to atmospheric normoxic (21%) oxygen concentrations have been proven to be physiologically inaccurate since the human body only ranges between 1%-13% in the body. We wish to observe how human mesenchymal stem cells (hMSCs) and human lung micro vascular endothelial cells (HLMVECs) interact with one another and their behavior when exposed to either hypoxic (defined as being less than normoxic) or atmospheric normoxic concentrations. The cells were grown and cultured on microfluidic devices—a relatively cheap and easily fabricated method of experimental testing that can lend itself to mass production and cellular analysis techniques. The analyses mainly focus on quantifying the amount of hypoxia-inducible factor-1 (HIF-1) present in the cells—this factor is responsible for activating countless transcription factors within the cell. Overall, these methods and tests have provided evidence to the fact that hypoxic conditions increase cellular growth, migration, proliferation, and growth factor production by almost two times.

1. Introduction

The current standard for cell culture involves sterile hood techniques and incubation in order to promote proper cell growth *in vitro*, but ignores the fact that oxygen concentrations within the human body are much lower than that of the atmosphere—varying from 1% in the cartilage to 7% in the bone marrow to 10-13% in the arteries, lungs, and liver.[1] Thus, cells cultured under atmospheric conditions may not behave effectively during *in vitro* tests. Recent studies have begun to acknowledge this fact, and there has been testing done on tissue cultures of hMSCs under hypoxia with 1% oxygen.[2] Although these various studies have been unclear on whether or not this effect is beneficial or negative, they have shown that hypoxia indeed effects the differentiation and cellular growth of hMSCs.

While those studies have taken a step in the right direction, further studies must be done to address the amalgamation of cells and oxygen concentrations in the human body. In order for *in vitro* experiments to be as physiologically relevant as possible, they must also take into account the fact that cells in the body are neighbored by other types of cells which also may be exposed to different oxygen gradients. Specifically, the following studies aim to look at these effects on wound healing. When a surface wound occurs, some of the cells within the wound may be exposed to normoxic conditions which still neighbor hypoxic cells—the exact details are unclear.

In the following studies, hMSCs and HLMVECs were seeded (sometimes adjacently) and allowed to signal each other as they reached confluence. hMSCs were chosen due to previous studies involving hypoxia and also because MSCs can differentiate into a myriad of structural cells; HLMVECs were chosen due to their role in angiogenesis. Both cells are important during wound repair.[3] Using a novel microfluidic design, both cell types could be grown inside a microfluidic device while also being exposed to different oxygen concentrations. The devices can thusly mimic more natural conditions and also work easily with standard cell culture techniques—it is relatively inexpensive and also easy to collect cells for cellular analysis (western blotting, quantitative polymerase chain reaction (qPCR), immunofluorescence). The following experiments explore the process of microfabrication as well as the effects that different permutations of oxygen concentration and cell type can have on cell growth, proliferation, migration, and growth factor formation. Specifically, it will look at the presence of hypoxia-inducible factor-1 (HIF-1), which is the master regulator of hypoxic vascular responses and activates countless transcription factors that promote angiogenesis and cellular growth. For qPCR, quantities of glucose transporter 1 (GLUT1) were analyzed within the cells and normalized with glyceraldehydes 3-phosphate dehydrogenase (GADPH). GLUT1 aids in the transportation of glucose throughout the body. It is hypoxia responsive and affected by HIF-1 activity[4]. GADPH is a non-hypoxia affected transcription factor that needed to be quantified in order to compare with the amount of GLUT1 present.

2. Methods

2.1 Microfabrication

The microfluidic devices were designed by combining four differing layers of polydimethylsiloxane (PDMS)—which is a silicone elastomer used in soft lithography[5]—the bottom foundation, the microfluidic network, the gas permeable membrane, and the well to contain the cells (Figure 1). An important detail to note was that the bottom foundation will either be composed of a glass slide or PDMS strips. The glass slides were necessary to perform immunofluorescence tests while the PDMS foundations were needed for PCR and Western Blotting.

The well and network parts first needed to be designed through photo lithography. SU-8 was spun onto a silicon wafer and placed on a hotplate at 65 ^{o}C for 5 minutes and then 95 ^{o}C for 60 minutes. The wafer was placed under an ultraviolet (UV) light with a UV resistant mask over it. The mask had open areas designating the design of the respective layer for the device, and these open areas were consequently cross-linked once exposed to the UV light for a set amount of time. These wafers would then be put on a hotplate again at 65 ^{o}C for 5 minutes and then 95 ^{o}C for 25 minutes, and then placed on a shaker in a solution of SU-8 developer to wash off all non cross-linked SU-8. The resultant mold would be placed in a petri dish and covered with PDMS (soft lithography) and cured at 85 ^{o}C for 60 minutes. The PDMS was created at a 10:1 ratio of pre-polymer and curing agent (Sylgard 184 kit, Dow Corning). The cured pieces were cut out and bonded through oxygen plasma treatment. More in depth details on the fabrication process can be found in a previously published paper by S. C. Oppegard and D.T. Eddington[6].

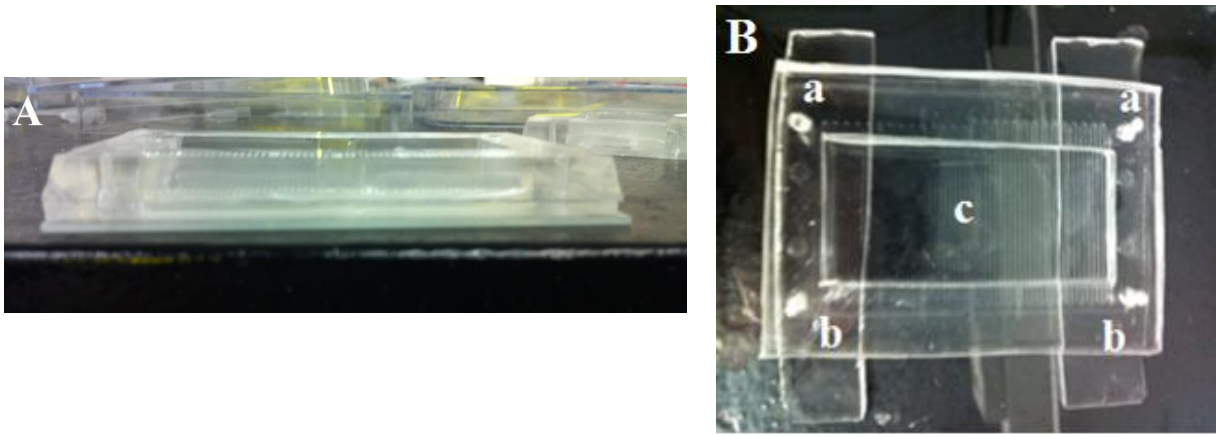

Figure 1: (A) The microfluidic device was composed of four layers: (from the bottom going up) a foundation layer, the microfluidic network so that oxygen could flow across the surface of the devices, the gas permeable membrane so that the oxygen could diffuse to the cells on top, and the well to keep the cells and media within a confined area. (B) Oxygen from tanks would be inserted at the (a) inlets and travel across half the devices before exiting the (b) outlets. (c) The cells would reside within the well.

2.2 Device Functionality

After a sufficient amount of devices were created, one of them needed to be tested to ensure proper functionality. The device was attached to two gas tanks, one for each inlet. The three combinations of gas tested were 1) both inlets at atmospheric 21% oxygen, 2) both inlets at hypoxic 0% oxygen gas, and 3) one inlet at 0% and one at 21% (dual conditions). Using the program Metamorph, the device was viewed under a microscope with a moving platform. The device was calibrated and multiple pictures were taken across the device while the gas was flowing through them. The camera took pictures of fluorescence given off by platinum(II) octaethylporphine ketone (PtOEPK), which naturally fluoresces but is quenched in the presence of oxygen. Thusly, the side exposed to atmospheric concentrations should fluoresce at a lower intensity than that of the hypoxic condition.[7] The PtOEPK was spun on a silicon wafer with a PDMS layer and then cured at 85 ^{o}C. Strips of it were cut off and place on the device (Figure 2).

2.3 Cell Culture

hMSCs and HLMVECs were cultured and passaged (P9-12) in Endothelial Cell Based Medium-2 (EBM-2) and MEM Alpha Medium (MEM-α), respectively. When enough devices were completed, the devices were autoclaved for 30 minutes at 121 ^{o}C and then seeded. The seeding procedure first involved removing the media from the cultures and exposing them to a two-time 5 mL wash of Dulbecco's Phosphate Based Saline (DPBS) and then 4 mL of trypsin-CDTA. The cells were momentarily incubated to allow the enzyme to detach them and then 6 mL of the respective media was added to neutralize the trypsin. The solution was centrifuged to pellet the cells and the supernatant aspirated. The cells were resuspended in 1-4 mL of their respective media and counted. For the devices, a total of approximately one million cells per device were seeded and then allowed to grow.

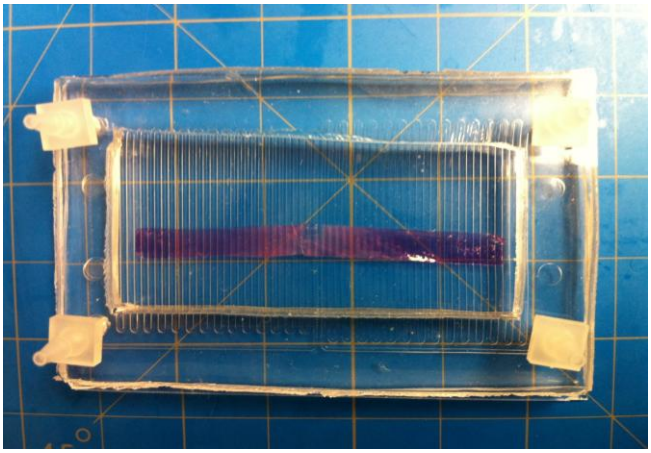

Figure 2: One of the microfluidic devices with a pink strip of cured PtOEPK lying on the center to detect the presence of oxygen (inverse relationship between fluorescence intensity and amount of oxygen).

2.4 Experimental Setup

After devices became fully confluent with cells, plastic tubing was attached to the inlets and led out of the incubator. The tubing was connected to a rotometer—responsible for managing air flow—which subsequently was attached to a gas tank (either 0% or 21%). With multiple devices, we were able to test a variety of conditions. The HLMVECs were run for 24 hours, and the MSCs were run for 20 hours based off previous research.

2.5 Immunofluorescence Tests

Immunofluorescence tests can only be performed on devices with a glass foundation, due to the application of 4% paraformaldehyde to "fix" the cells to the device. The device was first exposed to a 3x PBS wash before the 4% paraformaldehyde. They were then washed with 0.2% Tx-100 (triton) in PBS for 10 minutes and another PBS wash was applied before incubating the device with anti-HIF-1 α antibody overnight. A 3x Tris-Buffered Saline & Tween 20 (TBST) wash was performed and then the device was incubated with the secondary antibody for two hours at room temperature. Finally, after a 3x TBST wash, the device was stained with 4',6-diamidino-2-phenylindole (DAPI ; a fluorescent stain with a 0.4 μL:2 mL ratio of Hoechst 33342 trihydrochloride trihydrate:blocking buffer). The cells could then be imaged with a microscope to detect fluorescence.

2.6 Quantitative Polymerase Chain Reaction (qPCR)

To prepare the cells for PCR, the media was aspirated off and the device was given a 2x PBS wash. We then cut the device into 3 equal pieces and added 300 μL of a 50 μL:5 mL ratio of mercapto-ethanol to lysis buffer to each piece. The cells were scraped off and transferred to DNA/RNA free tubes. Then, the DNA was converted to cDNA using a high-capacity cDNA reverse transcription kit and then left in a warm bath. 2.5 μL of each set of cDNA was then added to a SYBR Green template, which consists of 12.5 μL master mixture (AB4309155), 1.25 μL of forward primer, 1.25 μL of reverse primer, and 7.5 μL dH_2O. The DNA was then placed in a PCR machine for actual analysis.

3. Results

3.1 Device Functionality

Proper functionality was observed throughout the devices as shown in Figure 3. The results were calibrated to display the percentage of oxygen concentration detected by the PtOEPK. They show that when both inlets are inserted with the same gas (either 0% or 21% oxygen), the gas will be properly delivered almost uniformly throughout the device. Also, when one inlet is hypoxic (the left in this case) and one atmospheric normoxic, the device will properly receive the different gasses on each respective half.

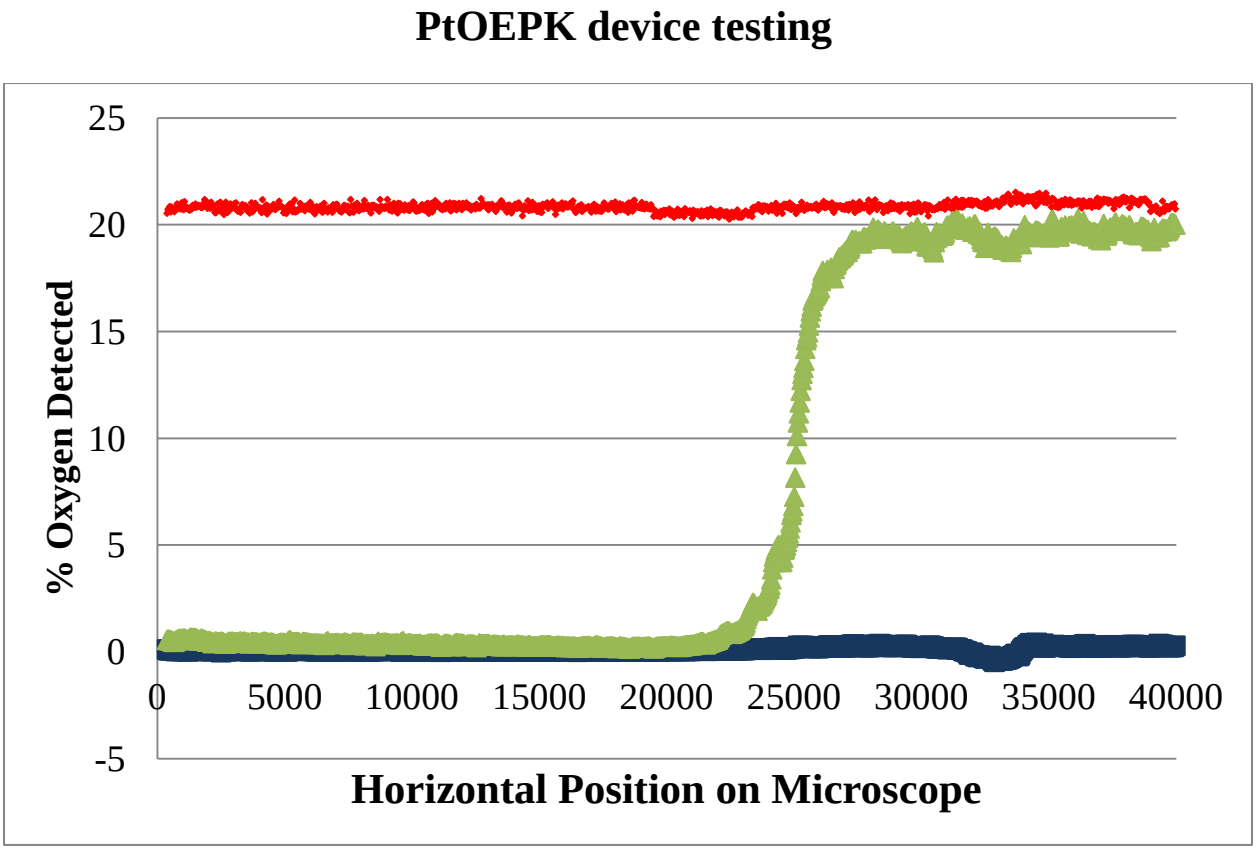

Figure 3: The red line represents the normoxic conditions, the green line the dual conditions, and the blue line the hypoxic conditions. This shows that when the same gas was inserted into both inlets (either 0% or 21%), the device received an almost uniform distribution of that respective concentration across the network. Also, when the left inlet was inserted with 0% and the right inlet with 21% (dual conditions), the PtOEPK successfully detected the proper concentrations on their respective halves.

3.2 Immunofluorescence

The cells were viewed under a microscope under the proper fluorescence wavelengths to view cells (Figure 4). Pictures were taken of the cells on both the hypoxic and normoxic sides of the devices through HIF-1 staining and show an increase in HIF-1 production in hypoxic conditions. Further studies must be done to quantify these values and compare them to other results. GLUT1 staining was also performed (Figure 5) on MSCs, showing a larger amount of GLUT1 production in the hypoxic conditions as compared to those in normoxic conditions.

3.3 qPCR

The cells were amplified through PCR until the amount of DNA reached a certain threshold (C_t). The numbers were normalized by calculating $2^{\Delta Ct}$, in which ΔC_t = (C_t of GADPH)

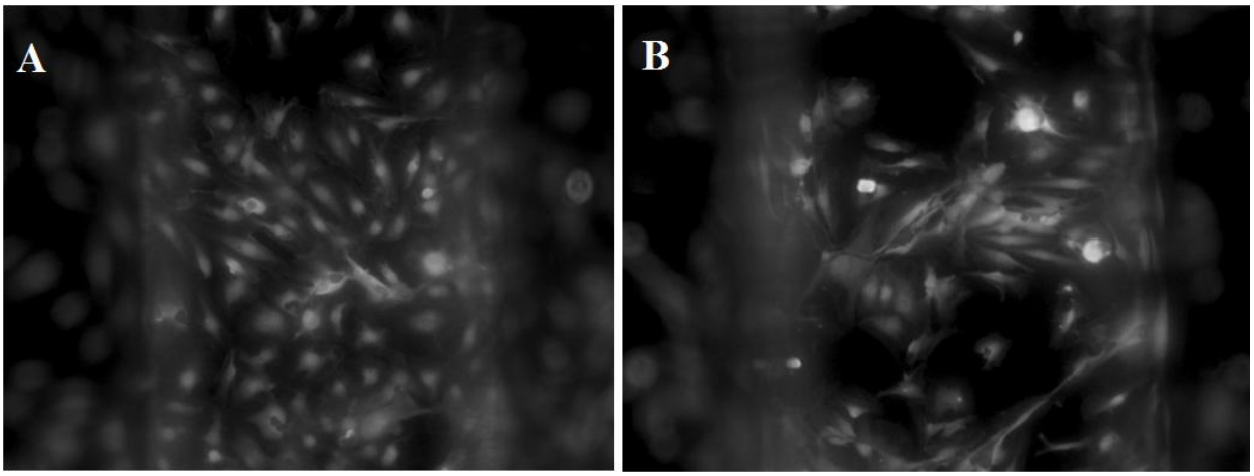

Figure 4: Images of a device showing the HIF-1 staining of (A) hypoxic conditions and (B) normoxic conditions. The two show that there is a higher amount of HIF-1 production in hypoxic conditions. Further analysis must be done to quantify this amount.

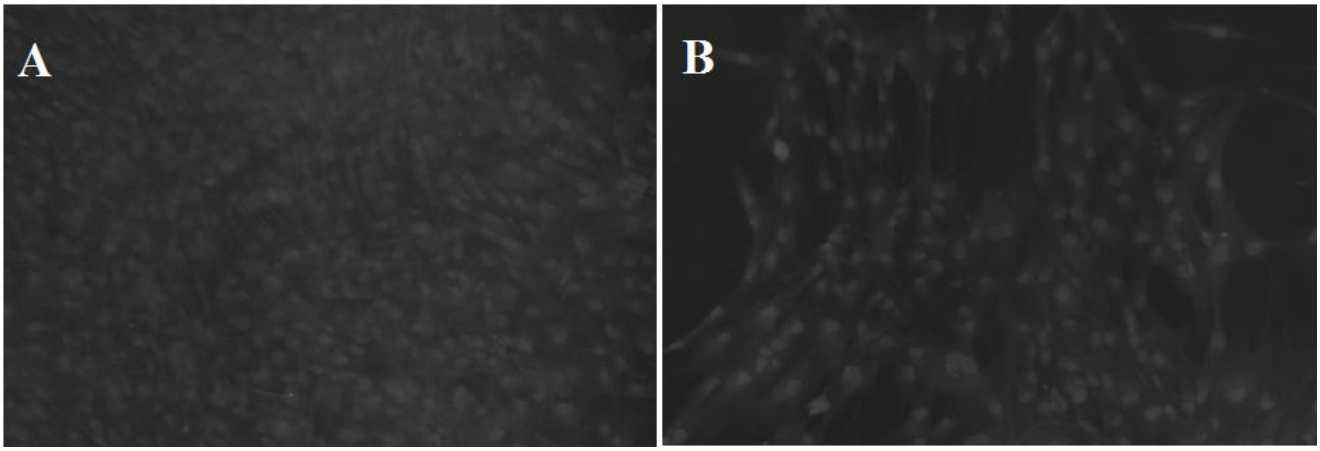

Figure 5: GLUT1 staining images in (A) hypoxia and (B) normoxia. The hypoxic hMSCs show an increase in GLUT1 production, which correlates to an increase in HIF-1 production as well as cellular proliferation, migration, and growth.

– (C_t of GLUT1). These newly calculated numbers were then divided by the average of the $2^{\Delta Ct}$ values for the normoxic devices in order to normalize them. The subsequent graph outputs the amount of GLUT1 as a ratio to the other devices, with the normoxic devices set to 1 (Figure 6).

4. Discussion and Future Work

Through the various tests and continual assembly line production of microfluidic devices, we can safely conclude that our devices perform at their intended functionality and can be fully utilized for their intended usage. Any gas that is inputted at the inlets will properly flow through its respective half of the device, exposing that half of the cells to a specific oxygen concentration. An important issue to note with the devices is that roughly 10-20% of completed devices are

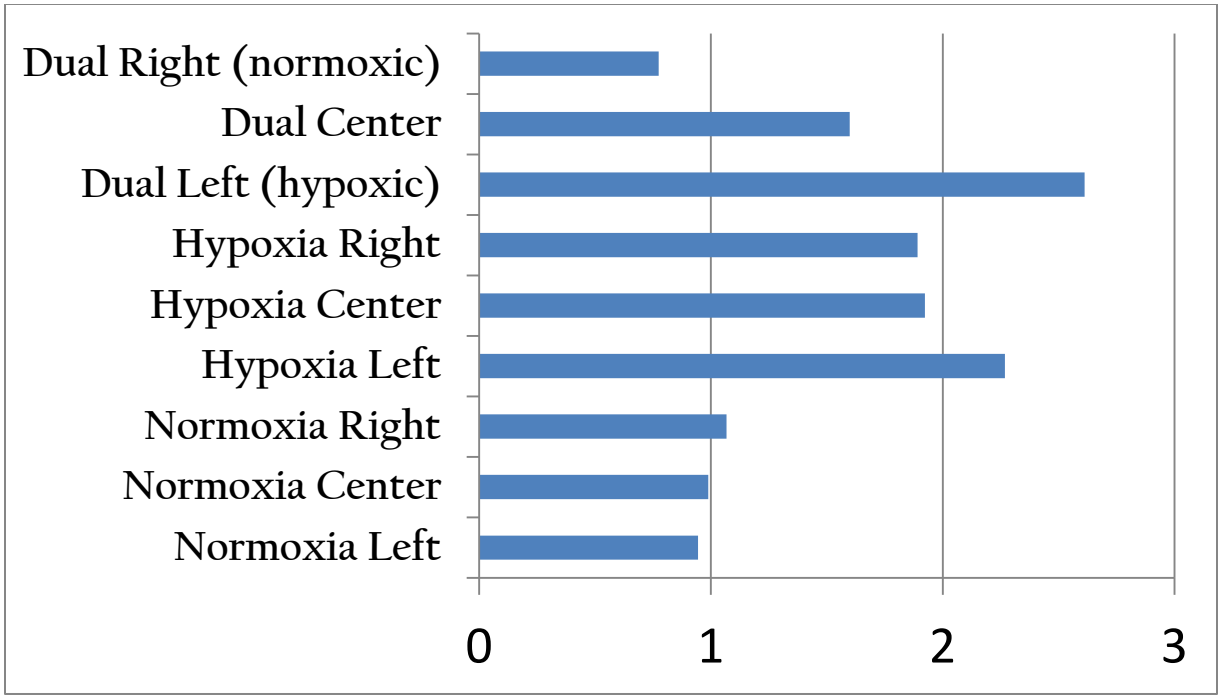

Figure 6: The devices exposed to atmospheric normoxic conditions (21% oxygen) were normalized to 1, and it can be observed that the hypoxic devices had almost twice as much GLUT1 as the normoxic devices (x axis describes a ratio). In the dual condition device, it is apparent that the right atmospheric normoxic side expressed much less GLUT1 than the left hypoxic side, giving further data supporting the benefits of hypoxic conditions.

dysfunctional due to faults in the PDMS curing or bonding issues. Further testing must be done to solve this problem. The images that have been collected pertaining to the amount of HIF-1 in those cells show that there is a difference in the quantity of HIF-1 and GLUT1 between the hypoxic and normoxic sides of the devices. Further analysis must still be done on the HIF-1 and GLUT1 images to fully quantify the amount of transcription factor that is present. The qPCR results show that a fully hypoxic device outputs about twice as much GLUT1 (which correlates to more cellular activity) as a fully normoxic device, and also gives us evidence that the cells will grow faster and proliferate more on the hypoxic side of a dual condition device.

5. Conclusion

Overall—although further tests must still be performed—we can safely conclude that culturing cells under hypoxic conditions definitely increases transcription factor production, which consequently should lead to an increase in cellular growth, proliferation, and migration. These results show that our novel microfluidic design can properly deliver differing gas concentrations the cells on each half of the devices. Both cell lines respond by up-regulating HIF-1 in hypoxic conditions. These findings will greatly affect future cell culture procedures

worldwide due to the need to accurately mimic human physiology. The results show that cells respond positively when exposed to 0% hypoxic conditions, and we should make use of this in the future.

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