Original Research

Metabolic utilization of human osteoblast cell line hFOB 1.19 under normoxic and hypoxic conditions: A phenotypic microarray analysis

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Impact statement

Currently, researchers understand that bone cells experience hypoxia during bone injury or fracture. Such stress condition exerts effect on bone regeneration and repair. However, there is limited knowledge on the metabolites and metabolism changes that occur in osteoblast cells when they undergo inherent regeneration and repair under hypoxia. This manuscript describes the use of Phenotype MicroArrays to observe the response of human osteoblast cells under normoxic and hypoxic conditions in terms of cell growth and utilization of metabolites. The human osteoblast cultured under these two different oxygen concentrations showed different growth curve and utilization of metabolites, suggesting oxygen levels play a role in bone repair and healing. We have deduced the main metabolites for osteoblast cells to produce energy under normoxic and hypoxic conditions. The new findings in this research help researchers to understand how hypoxia can influence utilization of metabolites in osteoblast cells, which serve as important knowledge to improve methods for bone regeneration.

Abstract

Osteoblasts play an important role in bone regeneration and repair. The hypoxia condition in bone occurs when bone undergoes fracture, and this will trigger a series of biochemical and mechanical changes to enable bone repair. Hence, it is interesting to observe the metabolites and metabolism changes when osteoblasts are exposed to hypoxic condition. This study has looked into the response of human osteoblast hFOB 1.19 under normoxic and hypoxic conditions by observing the cell growth and utilization of metabolites via Phenotype MicroArrays™ under these two different oxygen concentrations. The cell growth of hFOB 1.19 under hypoxic condition showed better growth compared to hFOB 1.19 under normal condition. In this study, osteoblast used glycolysis as the main pathway to produce energy as hFOB 1.19 in both hypoxic and normoxic conditions showed cell growth in well containing dextrin, glycogen, maltotriose, D-maltose, D-glucose-6-phospate, D-glucose, D-mannose, D-Turanose, D-fructose-6-phosphate, D-galactose, uridine, adenosine, inosine and α-keto-glutaric acid. In hypoxia, the cells have utilized additional metabolites such as α-D-glucose-1-phosphate and D-fructose, indicating possible activation of glycogen synthesis and glycogenolysis to metabolize α-D-glucose-1-phosphate. Meanwhile, during normoxia, D-L-α-glycerol phosphate was used, and this implies that the osteoblast may use glycerol-3-phosphate shuttle and oxidative phosphorylation to metabolize glycerol-3-phosphate.

Keywords: Osteoblast, hypoxic, glycolysis, glycerol-3-phosphate shuttle, phenotype microarray

***Experimental Biology and Medicine* 2021; 246: 1177–1183. DOI: 10.1177/1535370220985468**

Introduction

Bone is an organ that offers protection to the soft tissue in human, assists in locomotion, as well as to controlling calcium absorption.[1] Known as an organ that can regenerate itself by regulating bone regeneration and repair, one key element in this process is the presence of osteoblast. The formation of bone cells is facilitated via osteoblasts by producing extracellular organic matrix that undergo mineralization, trap within the tissue and change to become osteocytes.[2] Osteoblasts are not only active in bone regeneration and repair, but also participate in bone homeostasis and metabolism.

When bone fracture occurs, the blood supply is disrupted and causes hypoxia surrounding the fracture site.[3] The hypoxic condition around the fracture area triggers the production of inflammational signals and mechanical stimuli, and this will activate bone repair.[4] Hypoxia is usually associated with delayed bone healing.[5,6] However, a study by Wagegg *et al.* has instead shown that hypoxic condition encouraged osteogenic differentiation of human mesenchymal stem cells (MSCs) into osteoblasts.[7]

The osteoblasts participate in bone repair by formation of woven with or without lamellar matrix conformations and this is facilitated by the presence of MSCs and osteoblasts.[8] Since the osteoblasts have important role in assisting bone repair, it is interesting to see how the osteoblast utilizes the metabolites in order to grow in hypoxic condition. Therefore, the current study was carried out by using Phenotype Microarrays™ to assess the utilization of metabolites under normoxic and hypoxic conditions.

Materials and methods

Materials

Phenotype microarray PM-M panels PM-M1, PM-M2, PM-M3, PM-M4, Biolog Redox dye Mix MB (6×) (BIOLOG) and Biolog IF-M1 were purchased from BIOLOG. DMEM/F12 was obtained from Gibco. G-418 sulfate was purchased from Promega. Fetal bovine serum (FBS) was obtained from TICO EUROPE. Meanwhile, the trypsin-EDTA (0.25%) and trypan blue solution were purchased from Naccalai (Japan).

Cell culture

Human osteoblast hFOB 1.19 was obtained from Centre for Stem Cell Research, University Tunku Abdul Rahman (UTAR), Malaysia. The cell was cultured in 25 cm^2 flask at 37°C in humidified atmosphere with 5% CO_2. The hFOB 1.19 was grown in DMEM/F12 (Gibco) supplemented with 10% FBS and 0.3 mg/mL antibiotic G-418 sulfate (Promega).

Cell growth curve

The cell growth curve was studied to observe the effect of different incubation conditions of normoxia and hypoxia on hFOB 1.19 cell proliferation. The setting for normoxic condition was programmed at 37°C with 5% CO_2 and 21% O_2, while the setting for hypoxic condition was adjusted to incubation at 37°C with 5% CO_2 and 1% O_2. Osteoblast cells were seeded in 24-well plates at a density of 0.8×10^4 cells per well and incubated at the respective settings of normoxic and hypoxic conditions.

The cell growth curve was evaluated by counting the cells every two days for a consecutive of 10-day culture. The cells were harvested by trypsinization using 200 μL of 0.25% trypsin-EDTA and incubated at 37°C for 5 min. Then, 500 μL of complete media was added and the cell suspension was transferred into a 1.5 mL centrifuge tube. The cells were centrifuged at 8000 r/min for 5 min and the supernatant was discarded. The cell pellet was suspended in new complete media and the cell count was performed using trypan blue exclusion method. The cell counts were recorded for every two days, and cell growth curve was plotted.

Phenotype microarray

The PM-M plates used were PM-M1, PM-M2, PM-M3, and PM-M4. PM-M1 contains carbohydrate and carboxylate substrates, while PM-M2, PM-M3, and PM-M4 contain single L-amino acids and most dipeptide combinations.[9] The procedure for Biolog Phenotype microarray was carried out according to the manufacturer's instruction for "Phenotype MicroArrays™ panel PM-M1 to PM-M14" provided by BIOLOG. MC-0 assay medium used for the phenotype microarray was prepared by the addition of antibiotic G-418 sulfate, 200 nM glutamine, and 5% FBS in BIOLG IF-M1.

The cultured hFOB 1.19 were harvested by removing the media into 15 mL centrifuge tubes before washing with PBS twice and trypsinized with trypsin-EDTA for 2 min. The trypsinization process was stopped by adding the saved media from 15 mL centrifuge tubes before centrifuged for 10 min at 350×*g*. After centrifugation, the cell supernatants were discarded and 10 mL of PBS were added into cell pellets and resuspended before centrifuged for 10 min at 350×*g*. Then, the supernatants were removed and MC-0 assay medium (BIOLOG) was added to the cell pellets. The cell pellets were suspended in MC-0 assay media by the pipetting method.

Then, 50 μL of cell suspension in MC-0 assay media were added to each well with seeding number for each plate as follows; PM-M1 and PM-M2 were seeded with 12,000 cells per well, while 10,000 cells per well were seeded in each of PM-M3 and PM-M4. The normoxic PM-M1 to PM-M4 plates were incubated at 37°C with 5% CO_2 and 21% O_2, while the hypoxic PM-M1 to PM-M4 plates were incubated at 37°C with 5% CO_2 and 1% O_2. All the PM-M plates were incubated for 48 h to allow the cells utilize all the metabolites available in MC-0 assay media Bochner, Siri.[9]

After 48 h incubation, 10 μL Biolog Redox dye Mix MB was added to each well of all PM-M plates. Then, the plates were sealed with sterile sealing tape for 96-well plates to prevent CO_2 loss. Then, the PM-M plates were incubated for 48 h inside Omnilog Incubator/Reader to monitor the reduction of dye. The PM-M plates were photographed and kinetic plots were generated at the end of incubation period.

Statistical analysis

The statistical analysis for the cell proliferation of hFOB 1.19 was carried out by F-test to determine the variance between the two sets of data. Since the variance between cell proliferation of normoxic and hypoxic groups is equal, *t*-test was then carried out. Statistical significance (*) is achieved if *P*-value is less than 0.05.

Results and discussion

Cell viability of hFOB 1.19 under normoxic and hypoxic conditions

The cell viability tests of hFOB 1.19 were carried out under two different conditions, i.e. hypoxic and normoxic conditions. The incubator for hypoxic condition was set by adjusting the level of oxygen to 1%, while the setting for normoxic condition followed the default setting for normal proliferation for cell culture, i.e. 21% O_2. The cell growth curve was plotted as time against cell count in cell per mL (Figure 1). Under hypoxic condition, hFOB 1.19 showed increase in cell growth in which the cell number increased from 5.0×10^3 cells/mL on day 2 to 10,000 cells/mL on day 4 and subsequently 1.5×10^4 cells/mL, 1.75×10^4 cells/mL and 4.25×10^4 cells/mL on day 6, day 8, and day 10, respectively. As for normoxic condition, the cell growth for hFOB 1.19 was increased from day 2 at 2.5×10^3 cells/mL, 1.25×10^4 cells/mL at day 4, and 1.5×10^4 cells/mL at day 6. At day 8, hFOB 1.19 showed a decrease in its growth to 1.0×10^4 cells/mL; however, there was an increase of cell growth at day 10 with a total of 3.75×10^4 cells/mL. Statistical tests showed *P*-value of more than 0.05, indicating that although cell proliferation of hFOB 1.19 under normoxic condition is better compared to hypoxic condition, the difference is not statistically significant.

In this study, the cell proliferation of hFOB 1.19 was also observed under microscope. The microscopic examination showed that hFOB 1.19 under hypoxic condition demonstrated better growth compared to hFOB 1.19 under normoxic condition (Figure 2). There are studies that showed the hypoxic condition promotes cell proliferation of certain cells. For example, study by Tuncay *et al.*[10] showed hypoxic condition enhanced proliferation of osteoblast-enriched culture isolated from fetal rat calvariae. Hypoxic condition was also reported to enhance proliferation of mesenchymal stem cells and osteogenic differentiation.[11] In addition, Hirao *et al.*[12] have also shown that low oxygen tension promotes differentiation of osteoblast to osteocytes.

There are few methods to measure cell proliferation and viability including trypan blue exclusion and colorimetric assays. Both of these methods have different approaches in measuring cell viability and proliferation. Trypan blue exclusion method utilizes trypan blue solution which is a dye that penetrates into the non-viable cells, staining the cells blue, while viable cells will not be stained. The colorimetric assays such as MTT assay (3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) use the reduction of MTT to formazan and measure the formation of formazan.[13] The formazan formation reflects the enzyme activity of the viable cells.

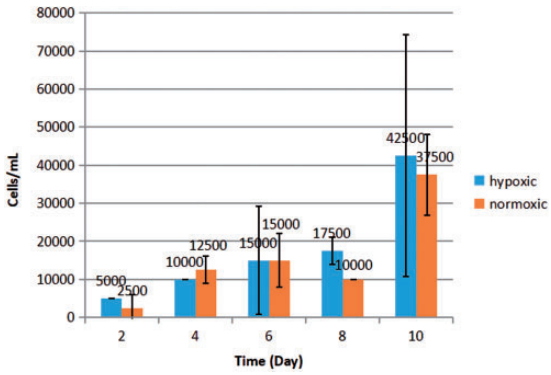

Figure 1. Cell viability of hFOB 1.19 under normoxic and hypoxic conditions. In general, hFOB 1.19 under hypoxic condition showed an increase growth compared to hFOB 1.19 under normal condition. On day 6, the cell growth for both conditions was similar. On day 8, the cell growth under normoxic condition showed a decrease (about 10,000 cells/mL) before it increased to 37,500 cells/mL. (A color version of this figure is available in the online journal.)

In this study, hFOB 1.19 cultured under hypoxic condition showed higher cell growth than hFOB 1.19 under normoxic condition by comparing the cell growth curve and microscopic examination of cells under both conditions. However, in order to study the utilization of carbon and nitrogen sources that might be influenced by the level of oxygen availability, the HFOB 1.19 were examined via phenotype microarray (BIOLOG) under two conditions (21% O_2 and 1% O_2).

Phenotype microarray assessment of metabolites utilization by hFOB 1.19 under normoxic and hypoxic conditions

To investigate the utilization of metabolites by hFOB 1.19 under hypoxic and normoxic conditions, the metabolism of hFOB 1.19 was identified using phenotype microarray PM-M plates (BIOLOG).

Each of the PM-M plates has three wells as negative control (without any metabolites) and positive control (coated with D-glucose). In order to observe the utilization of metabolites by hFOB 1.19, the cells were incubated under normoxic and hypoxic conditions for two days. After incubation, the BIOLOG dye was added to observe the reduction activity of dye caused by the energy produced through cell catabolism. The BIOLOG dye was added after the incubation to ensure the cell utilized all the fetal bovine serum provided by the media. When cells utilized the metabolites, Biolog Redox dye Mix MB would be reduced and developed into a purple solution in the PM-M which reflects the energy release due to catabolic pathways.[9] These changes can be measured using Biolog's OmniLog PM Instrument. The depletion of the serum was observed in the negative control wells in which the colorless wells indicate no reduction of the BIOLOG dye.

In this study, the cell growth was detected in the PM-M1, whereas the PM-2, PM-M3, and PM-M 4 showed low to no cell growth compared to the control wells (Figure 3). There are 14 wells containing carbon sources that showed cell growth in both hypoxic and normoxic condition, which includes dextrin (well A5), glycogen (well A6), maltotriose

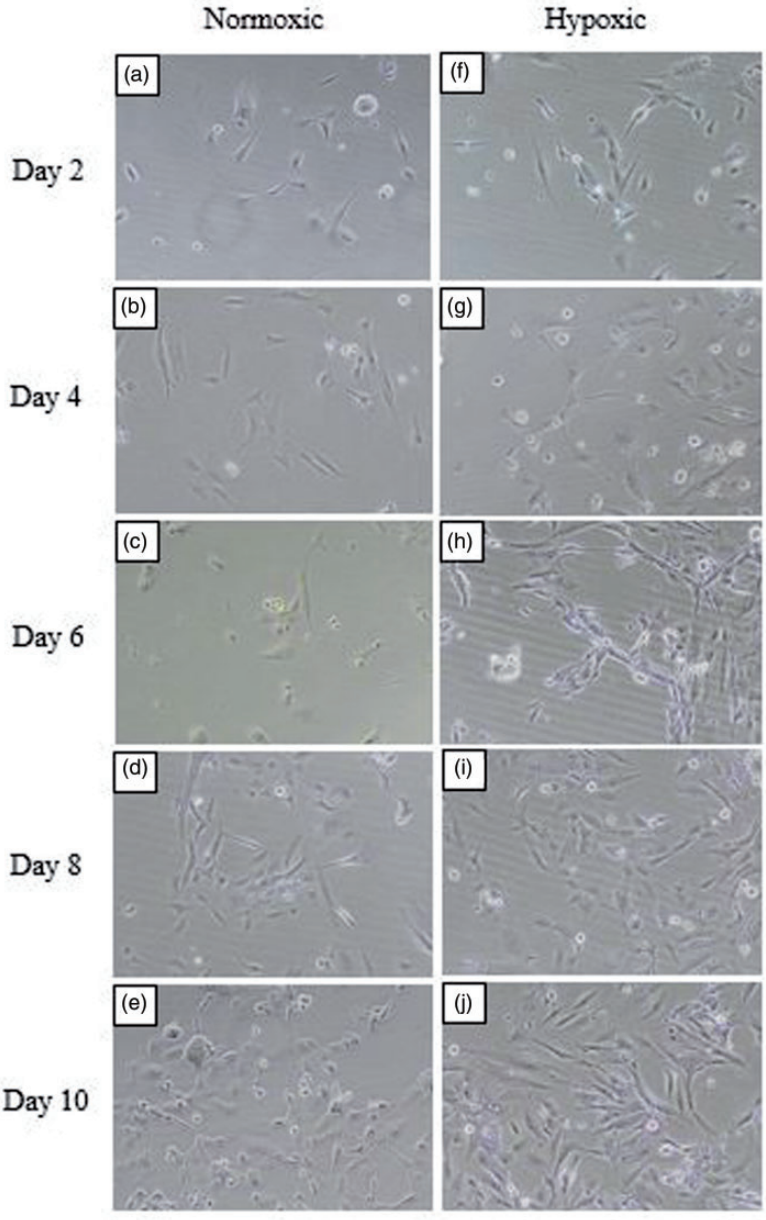

Figure 2. Observation of hFOB 1.19 for 10 days under a microscope (mag. 100X). (a–e) hFOB 1.19 under normoxic condition from Day 2 to Day 10. (f–j): HFOB 1.19 under hypoxic condition from Day 2 to Day 10. In this study, the cell growth of hFOB 1.19 under hypoxic condition showed greater proliferation compared to cell growth of hFOB 1.19 under normoxic condition.

(well A8), D-maltose (well A9), D-glucose-6-phospate (well B1), D-glucose (well B4,B5 and B6), D-mannose (well C5), D-Turanose (well C12), D-fructose-6-phosphate (well D6), D-galactose (well E3), Uridine (well E10), adenosine (well E11), inosine (well E12), and α-keto-glutaric acid (well G6). In addition, hFOB 1.1.9 under hypoxic condition showed two wells containing α-D-glucose-1-phosphate (well B2) and D-fructose (well D7) were metabolized under low oxygen availability compared to normoxic condition. As for hFOB 1.19 under normoxic condition, only one well coated with D-L-α-glycerol phosphate (well F10) was shown to be utilized by hFOB 1.19.

Osteoblasts use glycolysis as the main pathway to produce energy.[14] Previous studies showed that glucose plays a role in osteoblast differentiation[15] and metabolism of epiphyseal-metaphyseal bone slices *in vitro*.[16] However, it is interesting to observe which metabolites will be utilized by osteoblasts if the osteoblasts are grown under hypoxic condition compared to normoxic condition. In this study, osteoblast in both conditions showed strong utilizing of metabolites associated with glycolysis such as glucose, D-glucose-6-phosphate, fructose and mannose; disaccharides, i.e. maltose, turanose, tagatose, and galactose with other polysaccharides such as glycogen and dextrin. This study shows concurrence with a study reported by Flanagan and Nichols,[17] which described an *in vitro* experiment on human bone metabolism, and showed that the human bone has affinity to using glucose as a source of energy.

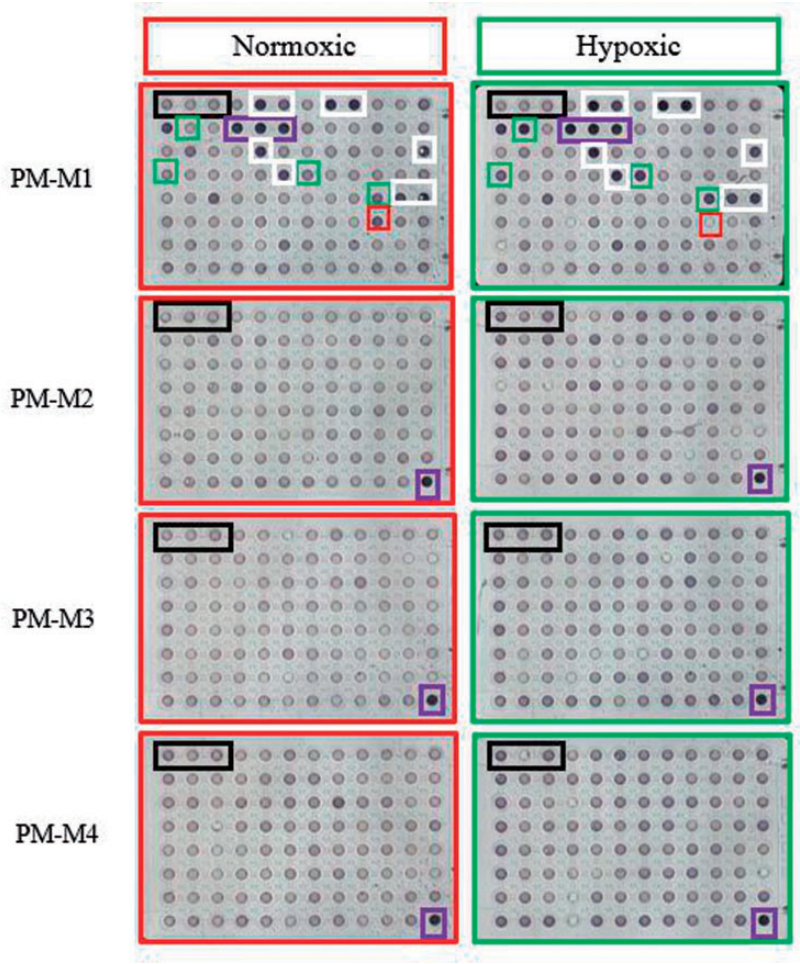

Figure 3. Photographs of hFOB 1.19 cells after 48 h of incubation under normoxic condition and hypoxic conditions in the Biolog's Omnilog PM Instrument. The plates were arranged from top, PM-M1 to bottom plates labeled as PM-M4. Negative control wells (black boxes) were wells without biochemical substrate, while positive control wells (purple boxes) were the wells supplemented with D-glucose. The utilization of metabolites was highlighted by green boxes (represent high utilization of metabolites used by hFOB 1.19 in hypoxic condition compared to normoxic condition) and red boxes (represent high utilization of metabolites used by hFOB 1.19 in normoxic condition compared to hypoxic condition). The white boxes represent metabolites utilized by hFOB 1.19 in both hypoxic as well as normoxic condition. (A color version of this figure is available in the online journal.)

In bone fracture, when the blood supply is disrupted, the depletion of oxygen in fracture area occurred and this caused hypoxic condition to the bone tissue.[18] When the hypoxic condition in fracture site occurred, this will hinder the healing of bone fracture.[19] As the hypoxic condition is present in the fracture site, it also triggers the osteoclast differentiation and bone resorption activity by activation of hypoxia-inducible factor (HIF) in mature osteoblasts.[20] In this study, osteoblasts in hypoxic condition showed greater growth compared to those in normoxic condition except for well containing D-L-α-glycerol phosphate (Figure 4). A study conducted by Hung *et al*.[11] showed that the proliferation of human mesenchymal stem cell (MSCs) was increased in hypoxic condition and also promoted osteogenic differentiation of MSCs. Hypoxic condition can trigger osteoblast cells to facilitate bone remodeling by producing pro-osteoclastogenic cytokines such as RANKL, IL-6, IL-11, etc.[21]

Our study showed that oxygen availability could change metabolite utilization. The results indicated that α-D-glucose-1-phosphate and D-fructose were metabolized by osteoblast in hypoxic condition, while D-L-α-glycerol phosphate was utilized by osteoblast in normoxic condition. Glucose-1-phosphate is the intermediate substrate used for the glycogen synthesis[22] and glycogenolysis.[23] There was an interesting observation in the utilization of glycogen and glucose-1-phosphate by osteoblasts. The osteoblasts in both hypoxic and normoxic condition showed positive result in utilization of glycogen; however, only osteoblast in hypoxic condition showed the utilization of glucose-1-phosphate. The lack of growth supplement from FBS to the osteoblast may induce the osteoblast to utilize glycogen to proliferate. On the other hand, the oxygen deprivation influenced the osteoblasts to utilize glucose-1-phosphate and may induce the glycogen synthesis. Pescador *et al*.[24] study on muscle under hypoxic condition showed the C2C12 myotubes accumulation of glycogen in the cells, inducing the muscle glycogen synthase and proved that HIF have a role in accumulation of glycogen in muscle cell.

On the other hand, the osteoblasts in hypoxic condition showed more reduction activity in well containing fructose compared to osteoblasts in normoxic condition.

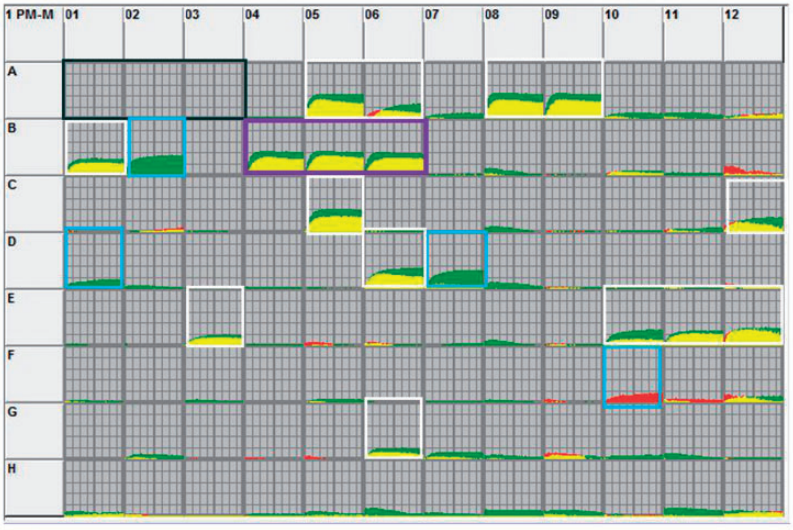

Figure 4. Metabolic changes of hFOB 1.19 incubated under normoxic (red) and hypoxic (green) conditions. The metabolic changes were monitored inside Omnilog, where signals from all the wells were subtracted by background signal in negative control wells (labeled with black box). Yellow represents the overlapping growth between hFOB 1.19 under normoxic and hypoxic conditions. Blue boxes represent high utilization of metabolites by hFOB 1.19 under either hypoxic condition or normoxic condition. White boxes represent the metabolites utilized by osteoblast cells in both conditions. Purple boxes are the wells containing D-glucose, which represent the positive controls. (A color version of this figure is available in the online journal.)

The metabolism of fructose usually takes place in the liver producing two intermediates for glycolysis, i.e. glyceraldehyde 3-phosphate and dihydroxyacetone-phosphate.[25] Fructose is metabolized by fructokinase to yield fructose 1-phosphate and these fructose 1-phosphate stimulate activity of alkaline phosphatase in osteoblast of neonatal rat calvaria.[26] In the case of fructose and hypoxic condition, Anundi *et al*.[27] showed the fructose protects liver cells from apoptosis due to the hypoxic condition.

D,L-α-glycerol phosphate, also known as glycerol 3-phosphate, has been shown to be participating in the several metabolic pathways, i.e. glycolysis, lipid metabolism, and glycerol-3-phostaphe shuttle.[28] In this study, the glycerol-3-phosphate was utilized by osteoblasts in normoxic condition only, and it is believed that glycerol-3-phosphate shuttle and oxidative phosphorylation may take part in the metabolism of glycerol-3-phosphate by osteoblasts in normoxic condition. Glycerol 3-phosphate shuttle utilized the glycerol 3-phosphate and converted it to dihydroxyacetone phosphate (DHAP) by mitochondrial glycerol-3-phosphate dehydrogenase 2 with simultaneous reduction of flavin adenine dinucleotide (FAD) to $FADH_2$ and it was used in mitochondrial oxidation phosphorylation to generate ATP.[29]

Conclusions

The current study showed that the osteoblasts can adapt in both normoxic and hypoxic conditions by slight modification in term of utilization of metabolites. This input of osteoblast metabolites utilization may be beneficial for future investigation to enhance the fracture healing by increasing osteoblast cell proliferation. However, since the healing of bone fracture is influenced by multiple aspects such as physiological, molecular, and cellular factors, cautions need to be addressed for the direct usage of these metabolites to enhance fracture healing. As this study only focused on the utilization of metabolites by osteoblast cells in hypoxic and normoxic conditions, future studies of the HIF and its connection with glycogen and glucose-1-phosphate utilization are suggested to assess their association at genetic level.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; YCC, YSQ, QW, and GB conducted the experiment, AP and SWT wrote the manuscript, KPA, NMJ, AKEH, AF, BA, MSAK, AVS, SKS, and PLM edited the manuscript. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank the Centre for Stem Cell Research, University Tunku Abdul Rahman (UTAR), Malaysia for providing human osteoblast hFOB 1.19.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

This project was supported by National Natural Science Foundation of China through Research Grant No. H81371943.

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(Received October 7, 2020, Accepted December 10, 2020)