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Culture Surfaces Induce Hypoxia-Regulated Genes in Human Mesenchymal Stromal Cells

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Abstract

Culturing human Mesenchymal stromal cells (hMSCs) *in vitro* in hypoxic conditions resulted in reduced senescence, enhanced pluripotency and altered proliferation rate. It has been known that *in vitro* hypoxia affects expression of cell surface proteins. However, the impact of culture surfaces on the hypoxia-regulated genes (HRG) have not yet been reported. This study utilized Next-Generation sequencing to analyse the changes in the gene expression levels of HRG for hMSCs cultured on different culture surfaces. The samples, which were cultured on four different synthesized surfaces (treatments) and tissue culture plate (control), resulted in a difference in growth rate. The sequencing results revealed that the transcription of a number of key genes involved in regulating hypoxic functions were significantly altered, including *HIF2A*, a marker for potency, differentiation, and various cellular functions. Significant alternations in the expression levels of previously reported oxygen-sensitive surface proteins were detected in this study, some of which closely correlate with the expression levels of *HIF2A*. Our analysis of the hMSCs transcriptome and HRG mapped out a list of genes encoding surface proteins which may directly regulate or be regulated by *HIF2A*. The findings from this study showed that culture surfaces have an impact on regulating the expression profile of HRG. Therefore, novel culture surfaces may be designed to selectively activate *HIF2A* and other HRG and pathways under *in vitro* normoxia. The understanding of the crosstalk between the regulating genes of hypoxia and culture surfaces may be utilized to strengthen desired hypoxic functions.

1 Introduction

The culturing of human Mesenchymal stromal cells (hMSCs) in hypoxia has shown benefits in reducing senescence, preserving pluripotency, and improving anti-oxidative defence [1-5]. However, contradictory evidence has been reported on the rate of proliferation [1, 3, 6-10] and the differentiation potential [1, 11-16] of hMSCs cultured in hypoxia. Similarly, the production of reactive oxygen species (ROS) has been the subject of some debate in hypoxic conditions [17-19]. These contrasting results may well have arisen from a wide range of factors, including for example, different levels of hypoxia, cell source, cell pool heterogeneity, donor-to-donor variation, culture conditions and extraction techniques [20-23].

Recent developments in the field of materials science and engineering have facilitated the use and study of different biomaterials to regulate stem cells proliferation and differentiation [24-26]. For instance, Lee *et al.* demonstrated that bone-marrow-derived hMSCs cultured on biomimetic polyacrylamide based hydrogel substrates were able to differentiate without the use of expensive and unreliable media supplements [27]. Furthermore, a significant number of reports have demonstrated that by fine tuning the biomaterial substrate properties, such as topography, chemistry, stiffness, etc., it is possible to modulate MSCs responses [28-33]. Cukierman *et al.* reported enhanced cell biological activities and narrowed integrin usage of cells merely by switching to a three-dimensional substrate topography [34]. Furthermore, Guvendiren and Burdick revealed that the spatial and temporal alignment and organization of hMSCs can be reversibly controlled by the topography and substrates of the culture surfaces [35]. Similarly, Phillips *et al.* demonstrated the induction of osteogenic and adipogenic differentiation in hMSCs by functionalizing the culture surface with amine groups [36]. The modification of surface properties may be inter-dependent in modulating the fate of hMSCs by unconventional mechanisms. Zhang *et al.* reported a synergistic effect of substrate topography and chemistry on the adhesion and differentiation of canine bone-marrow-derived MSCs [37]. Several other notable investigations have also described the effects of other materials properties on stem cells [28, 32, 38, 39].

To date, only a few culture parameters have been studied to regulate hMSCs behaviour, i.e. oxygen tension, metabolic feed, and cell-surface interaction. hMSCs are plastic adherent by nature, and the cell-surface interaction is primarily dictated by the characteristics of the applied surface in a static culture environment. The cellular effects induced by these three parameters are expected to be interlinked, as shown in Figure 1. Hypoxia has been shown to alter the rate of metabolic uptake and favour the glycolytic pathway for energy production; in addition, survival under hypoxia is strictly

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1 dependent on glucose availability [40-42]. Glucose has also been found to induce the production of
2 ROS and to regulate the expression of *HIF1A*, a pivotal transcription factor in the biological response
3 to hypoxia [43, 44]. Similarly, the impact of culture surfaces on glucose uptake, and *vice versa*, have
4 been reported [45, 46]. Hypoxic conditions and the presence of elevated ROS were found to alter
5 surface proteins and the arrangement of the extra-cellular matrix [47-50]. However, the impact of
6 culture surface on regulating hypoxic pathways has not been investigated. Azevedo *et al.* prepared a
7 type of “bioactive glass” that can induce hypoxic responses based on the dissolution of Cobalt ions
8 [51]. The stabilization effect observed in their study was chemically induced, by a hypoxia mimicking
9 agent, not caused by the alteration of surface interactions. The possibility of culture surfaces
10 regulating the expression of hypoxia-regulated genes (HRG) remains to be elucidated. This may be due
11 to the challenges in capturing a global representation of the induced changes by culture surfaces from
12 traditional cell culture surfaces. With the development of Next-Generation sequencing (NGS) the
13 expression levels of the entire transcriptome can be easily measured, effectively capturing genome-
14 wide changes in gene expression [52, 53]. A number of studies and reviews have addressed the relative
15 advantages of this technique [54-56].

Figure 1: Current knowledge on the interactions between the three key culture parameters [40-50].

This study aims to address the missing knowledge gap on whether culture surfaces can induce HRG, which in turn can be used to emphasize specific hypoxic functions in the future. The commercial hMSCs were cultured on four surfaces which were prepared in-house. The NGS technique was applied to obtain a global gene expression of each sample [32], from which the HRG were analysed. This study provides novel insights into the novel application of designing culture surfaces to selectively activate hypoxia-regulated functions.

Methods

Sample preparations

Details of cell culture, surface characterization and RNA extraction have been reported previously [32]. Four surfaces (S1-S4) were synthesized and the tissue culture plate (Control) was prepared to culture hMSCs [32]. Briefly, the synthesized surfaces were prepared from poly(L) Lactic acid (PLA) material. Two flat surfaces (S1 and S2) were made by pressing PLA granules at 210 °C for 15 min using a portable hot press. Nonwoven fibrous matrices (S3-S4) were made by electrospinning (an in-house setup) a 7.5% PLA solution. Subsequently, PLA substrates, both flat and fibrous (S2 and S4), were subjected to aminolysis reaction in 5% hexamethylenediamine solution (w/v, in isopropanol) for 30 min at 30 °C under mild agitation, in order to introduce amine groups that alters the surface from hydrophobic to hydrophilic [57]. *The flat PLA surface mimics a conventional two-dimensional cell culture plate surface topography, while, the fibrous PLA topography imitates the latest three-dimensional cell culture matrix and/or native extracellular matrix architecture. The pristine PLA surface denotes a hydrophobic surface - typical of synthetic polymers, while, the aminated PLA surface signifies a hydrophilic surface - typical of natural polymers.*

Human bone marrow-derived mesenchymal stem cells (hMSCs) (Lonza, UK) were incubated under 37 °C, 20% oxygen, 5% CO₂, with saturated humidity. The hMSCs were seeded onto the four different synthesized substrates (i.e. flat, pristine (S1); flat, aminated (S2); fibrous, pristine (S3); fibrous, aminated (S4)) at 50,000 cells per sample across 2 cm discs and cultured for 7 days with media changed at day 1 and then every other day. Standard tissue culture polystyrene substrate was used as control. Cell viability was measured at day 3, 5 and 7 using the Alamar blue based non-destructive assay (Thermo Scientific, UK). *A standard curve was prepared by taking known concentration of cells in serial dilutions. The absorbance of the test samples was compared with the standard curve in order to convert absorbance values into cell number.* Total RNA from each sample was extracted with the RNeasy Mini Kit (Qiagen, UK). NanoDrop UV/VIS spectrophotometer (1000, Thermo Scientific) and RNA agarose gel electrophoresis methods were used to assess the quality and quantity of the total extracted RNA [32].

Sequencing data analysis

RNA sequencing was completed on the Day 3 samples. The sequencing experiment and the upstream bioinformatics pipeline has been reported previously [32], further analysis and additional tests were done in this study. Genes with over 100% change in their expression levels against control,

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1 and a false discovery rate less than 0.001 were considered to be differentially expressed (DEG). A
2 threshold of expression level (FPKM >1) was set to filter out genes with low abundance.

3 **Analysis of hypoxia-regulated genes**

4 The gene set describing the HRG was obtained from an online database (HypoxiaDB,
5 www.hypoxiadb.com) [58]. The transcriptome datasets of the five samples (NCBI sample number:
6 SRS2493619; SRS2493628; SRS2493629; SRS2493630; SRS2493885) were compared and analysed for
7 the HRG. Hierarchical cluster analysis compared the gene expression profile for the ensemble HRG
8 between samples.

9 A list of the hypoxia-altered surface proteins in hMSCs were compiled from the literature
10 database and checked against the detected expressions in this study. Gene expression correlation and
11 cluster analysis were both conducted with the criteria of Pearson coefficient (R) > 0.9 or < -0.9 and p
12 < 0.01.

13 The genes encoding hMSCs surface proteins were obtained from the compiled literature
14 surface proteome [59-61]. Pathway enrichment analysis and the identification of functional gene sets
15 (e.g., genes involved in each cell cycle stages) were conducted utilizing the KEGG database [62]. Gene
16 ontology was done using the DAVID database [63].

18 **Quantitative real-time PCR**

19 RNA extracted from the Day-5 samples were used for qRT-PCR testing of expression levels of
20 *HIF2A* (also known as *EPAS*). The RNA samples were reversely transcribed, with a genomic DNA
21 wipeout step, using the QuantiTect Reverse Transcription Kit (Qiagen, Stanford, USA) according to the
22 manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using Rotor-Gene
23 6000 (Qiagen, Stanford, USA), with the following cycle parameters: enzyme activation at 95°C for 2
24 min, 40 cycles of denaturation for 5 seconds of 95°C followed by 60°C for 25 seconds. The fluorescence
25 acquisition was performed with the SyGreen Blue Mix Lo-ROX (Pcrbiosystems, UK) following the
26 manufacturer's instructions. 3 repeats were run for each sample-gene pair. The fold change was
27 calculated using the delta-delta-Ct method with *GAPDH* as the reference gene. The primers were
28 designed using Primer-BLAST (ncbi.nlm.nih.gov/tools/primer-blast/) and ordered from Sigma, UK. The
29 tested primer sequences are listed below as: *HIF2A*-f: GCGCTAGACTCCGAGAACAT; *HIF2A*-r:
30 TGGCCACTTACTACCTGACCCTT; *GAPDH*-f: GGATTTGGTCGTATTGGG; *GAPDH*-r:
31 GGAAGATGGTGATGGGATT.

Results

hMSCs were cultured on 5 different surfaces for 3 days before total RNA was extracted and processed for NGS. The overall sequencing quality was confirmed by mapping the saturation and distribution of the reads [32]. A total of 2,147 HRG were detected, with 1,873 genes exceeding a threshold expression level (Supplementary Information table S1). The HRG were analysed, to examine their dependency on culture surface conditions, unless otherwise specified.

Cell culture data

The samples seeded on different culture surfaces resulted in significant differences in growth rate over the culture period, as shown in Figure 2. The control surface supported the fastest proliferation rates and had a much shorter lag phase compared to the cells cultured on the synthesized surfaces (until day 3). Compared to the samples cultured on flat surfaces (S1 and S2), fibrous surfaces (S3 and S4) resulted in higher levels of growth suppression and resulted in the slowest growth rate. The modification of surface chemistry induced negligible changes on cell growth. Readers interested in cell culture and material characterization data can refer to the Supplementary Information or our earlier work [32].

Key cell cycle regulators and hallmark genes involved in different cell cycle stages were analysed (Supplementary Information table S2). Despite the treated samples experienced slower growth rate than the Control, several positive cell-cycle regulating genes were up-regulated, including *CCND1*, *CCNE2*, *CCNB2*, *E2F1*. Meanwhile, some other genes involved in cell cycle progression were found to be down-regulated, e.g. *TGFB2*, *INHBA*, *THBS1*, etc. The genes involved in the G1 phase were relatively stable with only 2 genes (*CDC6* and *TBRG4*) changing by more than 50%; none of the G1 phase genes were highly expressed. No genes involved in the S phase transition was differentially expressed. Contrastingly, the expression levels of 7 genes involved in regulating the M phase (*AURKA*, *MAD2L1*, *KPNA2*, *TGFB1*, *PPP5C*, *NOLC1*, *PAM*) were significantly altered, suggesting a greater variation during phase progression between samples which may have contributed to the observed differences in cell growth rate, as shown in Figure 2.

Figure 2: Cell growth data for samples cultured on the five different surfaces. The cell number (mean) on each day is represented with standard error. The difference between the Control and the treated samples are statistically different ($p < 0.001$). The difference between S1 and S3, and S2 and S4 are also statistically different (marked with asterisk) ($p < 0.001$). Figure adapted from data presented in [32].

Sample variations of HRG expression levels

The expression variations of HRG across the different samples are shown in Figure 3. Samples with similar expression patterns and small Euclidean distances were paired. The HRG expression profile of the cells cultured on the synthesized surfaces exhibited a significant difference compared to the control sample. Small expression variations were observed between S1 and S2, and between S3 and S4, the two pairs were cultured on flat and fibrous surfaces, respectively. As described previously for all genes [32], the alteration of the surface topography resulted in a greater impact on cell HRG expression profile than the changes made on surface chemistry. Similar markers (e.g., frequency of gene expression levels, the distribution of coefficient of variation, etc.) were found to be comparable between the HRG and the complete transcriptome profile.

Figure 3: Dendrogram representing hierarchical cluster distributions of the samples cultured on the four synthesized surfaces and Control for the 1,873-detected hypoxia-regulated genes. The Euclidean distance is shown on the vertical axis. Samples with smaller pairwise difference is grouped together.

Compiled expression of oxygen-sensitive surface proteins

In the literature, various surface proteins were affected by *in vitro* hypoxic cell culture. It is important to determine whether the expression of these proteins were altered in this study. A list of 15 reported hypoxia-altered surface proteins in hMSCs is shown in Table 1, their respective expression levels from samples in this study are shown. The expression levels for six of these genes, i.e., *CD93*, *PLCAP*, *ESAM*, *SMOC2*, *CDH* and *PLEKHA6*, were below the threshold for further analysis in all samples. The measured level of *ITGB1* and *ITGA5* were consistently detected.

Table 1: The compiled expression of surface proteins which were altered under *in vitro* hypoxia. The expression of the two hypoxic hallmark genes, *HIF1A* and *HIF2A* (EPAS) are also shown. The measured gene expression levels from the control (C) and the four synthesized surfaces are reported. Up- and down-regulated genes are highlighted in green and red, respectively. The Pearson coefficient (*R*) and *p* values for the expressions of each gene are shown.

Entrez ID	Gene	<i>R</i>	<i>p</i>	S1	S2	S3	S4	C	Ref.
83483	PLVAP	-	-	-	-	-	-	-	[16]
90952	ESAM	-	-	0	0.1	0	0.1	0.1	[16]
22918	CD93	-	-	0	0	0	0	0	[16]
22874	PLEKHA6	-	-	0.1	0.1	0	0.1	0.3	[16]
64094	SMOC2	-	-	0	0.2	0	0	0	[16]
999	CDH	-	-	0.2	0	0	0	0.1	[64]
3688	ITGB1	-0.62	0.26	788	802	943	834	820	[64]
7412	CD106	0.89	0.04	10.1	13.2	7	10	24.2	[16]
1000	N-CAD	0.97	0.01	69.6	75.1	44.9	52.5	83.7	[64]
3693	ITGB5	-0.62	0.26	144	126.3	127	128	88	[64]
3678	ITGA5	-0.83	0.08	165	135.9	192	159	121	[64]

3673	ITGA2	-0.86	0.06	2.9	3.3	6.9	4	1.7	
2335	FN1	-0.83	0.08	7149	7037	15,579	14,297	8124	[64, 65]
3690	ITGB3	-0.93	0.02	5.6	5.4	17.8	13.9	3.3	[64]
6387	CXCL12	0.42	0.48	82.4	78.9	45.4	44.2	53.4	[65]
3091	HIF1A	-0.83	0.08	140	169	186	184	148	-
2034	HIF2A (EPAS)	1	0	137	135	48.3	56.8	214	-

The two hypoxic hallmark genes, *HIF1A* and *HIF2A (EPAS)* were detected in all samples in this study, with *HIF1A* being consistently expressed. *HIF2A* was down-regulated in cells cultured on the synthesized surfaces, including a 4-fold decrease in samples cultured on fibrous surfaces, similar to the trend for *N-CAD*. Interestingly, the remaining eight genes exhibited large expression variations across samples. The genes with up- or down-regulation of greater than 1.5-fold change are highlighted in green and red in Table 1, respectively. The samples cultured on fibrous surfaces, were found with significantly higher expressions of *ITGA2*, *ITGB3* and *FN1*, than the control. Conversely, *N-CAD*, a differentiation marker [66-68] was found to be reduced by 50%. *CXCL12*, a chemokine to increase hMSCs homing and the interaction with hematopoietic stem cells [69, 70], was detected with a 2-fold increase in samples cultured on flat surfaces over those on fibrous surfaces. The expression level of a typical MSCs marker, *CD106*, was found to be suppressed in all samples cultured on the synthesized surfaces, whereas, *ITGB5* was found to be consistently upregulated.

In the reported literature data, a few of these genes were found to be altered by hypoxia. In Mathew *et al.*, expression levels of *FN1*, *N-CAD*, *ITGB1*, *ITGB3*, *ITGB5*, *CDH1*, and *ITGA2* were reported with 1.5- to 2.5-fold increase in hypoxic culture; the increase with *CDH1* and *ITGA2* were not statistically significant [64]. The increase in the hypoxic expression of *FN1* and *CXCL12* was also reported [65]. The signal measured via flow cytometry for *CD106* was greater in 1.5% oxygen culture condition. The induced fold change, because of *in vitro* hypoxia, is in a similar magnitude as induced by culture surfaces in this study.

HIF2A is regulated by surface proteins

Table 1 compiled the reported hMSCs genes which encode surface protein and are sensitive to culture oxygen tension. This study utilized the NGS to show a change in culture surfaces could also alter the expression levels of genes encoding these oxygen-sensitive surface proteins. The expression level of *HIF2A*, a hallmark hypoxic gene, was measured to be significantly down-regulated. From the

1 results reported in the literature [71, 72] and from this study, HIF2 α is hypothesized to be a key
2 mediator in both regulating and being regulated by different surface proteins.

3 The Pearson coefficient (*R*) and the *p*-value across the samples are also presented in Table 1.
4 Expression levels of *CD106* and *N-CAD* were found to be statistically significant and positively
5 correlated with the expression profile of *HIF2A* across the tested samples, which may suggest a direct
6 up- or downstream regulation between the two and *HIF2A*. The positive correlation between *CD106*
7 and *N-CAD* expression was reported by Aomatsu *et al.* [73]. In their study, the overexpression of *N-*
8 *CAD* was able to rescue the suppressed expression of *CD106*, and the overexpression of a dominant
9 negative *N-CAD* reduced the level of *CD106*. Yamashita *et al.* discovered the direct regulation of *HIF2A*
10 on *CD106* via *HIF2A* knockdown and chromatin immunoprecipitation assays [71]. The downregulation
11 of *CD106* expression is therefore hypothesized to occur downstream to that of *HIF2A* and *N-CAD*.
12 Leonel showed that the secretion of VEGF, a downstream HIF2 α target, significantly increased after
13 the incubation of bone-marrow derived MSCs with additional N-cadherin in the solution [74]. Despite
14 the results demonstrated by Leonel, no direct gene expression level correlation between *N-CAD* and
15 *HIF2A* has been reported, it remains to be elucidated that whether *N-CAD* is altered upstream to *HIF2A*.

16 Additionally, *N-CAD* has been reported to regulate the differentiation potential of MSCs [75-
17 78]. Liu *et al.* found that the expression level of *CD106* was greatly reduced after differentiation while
18 the expression levels of other MSCs markers remained stable [79]. *HIF2A* has been reported to
19 contribute to the maintenance of cell pool stemness [80, 81]. *CD106* and *N-CAD* may contribute to the
20 regulation of cell pool potency via pathways involving *EPAS* (*HIF2A*). Additionally, the high *CD106*
21 expressing population of MSCs have demonstrated strong elevated angiogenic capacity with
22 numerous marker genes and produced cytokines being significantly upregulated [82].

23 On the other hand, *ITGB3* (*CD61*) negatively correlated with the expression of *HIF2A* (Table 1).
24 Various studies have found that *ITGB3* has a regulatory potential in controlling tumour growth and
25 angiogenesis, whereas the inhibition of *ITGB3* has reduced cellular angiogenic potential [83-87].
26 Furthermore, we found that the expression of *VEGFA* was found to be positively correlated with *ITGB3*,
27 showing a negative correlation with that of *HIF2A*. In the first 3 days of culture, other pathways may
28 be the more dominant factors in affecting the expression of *VEGF* other than *HIF2A*. Kracun *et al.*
29 reported the overexpression of the *ITGB3* protein receptor, β 3-endonexin, was found to negatively
30 regulate the expression of HIF1 α mRNA under hypoxia; HIF2 α protein, however was not affected [88].
31 Therefore, incorporated with the findings from this study, the surface-induced negatively correlated

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ITGB3 expression, may be a result of the downstream *HIF2A* regulation. The impact of *ITGB3* on MSCs have not been reported; its reported functions in regulating growth and angiogenesis in tumour cells [89, 90] are expected to be analogous to those in MSCs.

Martin *et al.* reported that HIF2 α binds to the promoter of *CXCL12* under hypoxia and may be used to regulate angiogenesis [91]. HIF2 α has been observed to induce FN1, which also regulates cytoskeleton arrangement, under hypoxia in lung cancer endothelial cells [92], and melanoma cells [93]. A partial impact of *HIF2A* on *ITGB1* and *ITGA5* was reported by Hughes *et al.* in cancer cell lines [94]. The four types of synthesized surfaces selectively altered the expression of *HIF2A* and the gene expression profile, which may in turn, differentially regulate the respective downstream hypoxic signalling pathways and functions. From the experimental data reported in the literature and this study, the change in HIF2A expressions is hypothesized to be the result of different activated surface proteins.

Mapping *HIF2A* correlated genes and functions

In order to infer how the synthesized surfaces altered the HRG, four gene sets were studied, including the genes encoding hypoxia-sensitive surface proteins (Table 1), all detected HRG, hMSCs cell surface proteome markers [59-61] and the detected genes from this study that are correlated to the expression of *HIF2A*. 573 genes were found to be correlated with the expression of HIF2A (full details shown in Supplementary table S3). Figure 4 shows the number of unique and shared genes from the four gene sets. The genes that are shared by three sets are listed in the diagram, e.g. hypoxia-regulated and *HIF2A*-correlated surface proteins: *CD47*, *HM13*, *COL6A1*, *TGFBR1*, *CD44*, and *PVR*. This finding provides insights for future studies to validate specific gene and pathway interactions between the interaction of surface and hypoxia signalling.

Figure 4: Venn diagram indicating the number of shared genes between the four gene sets. The genes with the expression levels correlating with that of HIF2A in samples cultured on the five surfaces, the detected hypoxia-regulated genes, genes encoding hMSCs surface proteome and the literature reported oxygen-sensitive surface proteins.

Across the samples cultured on the five types of surfaces, 1,873 of the 2,289 HRG were detected. These HRG significantly enriched key signalling, adhesion, and cancer related pathways, e.g., FoxO, PI3K-Akt, MAPK and HIF1A signalling, focal adhesion, cell cycle, colorectal, pancreatic cancer and leukaemia pathways (Table 2). 102 HRG were found to be differentially expressed in more than two sets of surfaces, demonstrating the effective alteration in the expression of HRG (Supplementary Information table S4).

Among the genes encoding the 15 oxygen-sensitive surface proteins in Table 1, only *VCAM1*, *ITGB1*, *CXCL12* and *FN1* were both detected in our samples and reported in the hypoxia database. The other four detected genes encoding integrin subunits were not found in the database; among the six undetected genes, only *CD93* was referenced to be regulated by hypoxia.

Comparing with the expression levels of *HIF2A* across samples, 321 positively and 252 negatively correlated genes were found. *N-CAD*, a hypoxia-sensitive differentiation marker, positively correlated with the expression of *HIF2A* with a 1.5-fold expression reduction in the samples cultured on fibrous surfaces. 147 of the correlated genes were found to be also regulated by hypoxia (Supplementary Information table S5), and 24 genes were detected in the hMSCs surface transcriptome (Supplementary Information table S6); *CD44*, *CD47*, *HM3*, *COL6A1*, *TGFBR1* and *PVR* were found in both lists. Figure 5A shows the gene ontology results for the 37 correlated HRG (with the legend shown in Figure 5B) contributing to the functions of extracellular matrix organization, cell

Certain correlated genes were differentially expressed across the samples cultured on different surfaces. 33 and 17 DEGs were found in the positively and negatively correlated genes, respectively. The induced signalling responses from the culture surfaces are more pronounced on the expression of certain genes, significantly up- or down-regulate their expression. Two pathways were found to be significantly enriched by these DEGs, i.e. Hippo signalling pathway, and the pathways in cancer. The genes encoding two surface proteins, *LGALS3* and *TFGBR1*, were differentially expressed.

Functions	G.O. Number	Gene Count	%	p	q
Extracellular matrix organization	0030198	14	9.5	1.40E-08	1.80E-05
Cell adhesion	0007155	15	10.2	4.10E-05	2.70E-02
Regulation of cell growth	0001558	7	4.8	6.40E-05	2.80E-02
Response to hypoxia	0001666	9	6.1	1.20E-04	3.80E-02
Angiogenesis	0001525	10	6.8	1.30E-04	3.40E-02
Negative regulation of angiogenesis	0016525	6	4.1	1.90E-04	4.10E-02

Figure 5: *HIF2A*-correlated and hypoxia-regulated genes. A) The 37 positive and negative correlations are shown in green and red, respectively. Different functions from gene ontology analysis are labelled, and each unique gene is numbered with the gene names shown in B). The genes reported for the positive and negative regulation of angiogenesis are shown in part C).

Quantitative PCR validation of *HIF2A* expression

Quantitative PCR was performed to confirm the trend observed in the sequencing results using Day-5 samples. The Ct values and the fold change for *EPAS* (*HIF2A*) against Control are shown in Figure 6. The reduced expression of *HIF2A* on the synthesized surfaces was confirmed with qRT-PCR. A modest down-regulation in S1 and S2, and a significant down-regulation in S3 and S4 samples were confirmed for, as previously shown in Table 1. The comparable results between NGS and qPCR using Day-3 and Day-5 samples, respectively, demonstrated stable down-regulation of *EPAS* (*HIF2A*) expression on fibrous surface.

Figure 6: Quantitative real-time PCR results showing the Ct values (A) and the relative fold change of the samples compared to the Control (B), $n=3$. The results from RNA sequencing is shown for each sample-gene pair.

Discussion

Hypoxic cell culture has exhibited outstanding impacts on the rate of proliferation, stemness, differentiation and other capacities of hMSCs. However, by far, the deterministic impact of hypoxia is difficult to be drawn from experimental results, due to the different culture parameters used [2, 95]. Although various studies have reported the alterations in expression levels of surface proteins under *in vitro* hypoxia [16, 48, 50, 96], the understanding of whether different culture surfaces affect the expression of HRG remains to be elucidated (Figure 1). With the available knowledge in the field, it is not feasible to design a controlled experiment to test the induction of surface proteins on hypoxic responses. This study utilized the advantage of RNA sequencing in capturing the entire transcriptomic expressions to address this challenge. The change in the HRG expression in the hMSCs cultured on the four different synthesized surfaces were examined. By addressing this missing link, it may shed insights into addressing some controversies on the effects of hypoxia on MSCs. In addition, certain HRG and

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hypoxia-regulated pathways may be selectively enhanced or suppressed by different culture surfaces. Certain benefits from *in vitro* cell culture may be specifically induced without subjecting cells to a hypoxic environment.

In this study, the hMSCs cultured on four different surfaces were analysed for the induced HRG and enriched pathways. The expression levels of several key hypoxia functional genes were significantly altered, e.g. *HIF2A*, *VEGFB*, *THBS1*, etc. Various surface proteins and their encoded genes may have enacted upstream of *HIF2A*, which led to the regulation of *HIF2A*, as shown in Figure 7; *CD106* is hypothesized to be downstream of *HIF2A* and *NCAD*. This finding suggests the effect of surface protein activations on oxygen-sensing/hypoxia-regulating pathways, providing supporting evidence and encouraging future studies to fill the missing knowledge gap in Figure 1. *HIF2A* is hypothesized to play a key intermediate role in linking the surface sensing and hypoxic response pathways.

The expression level of *HIF1A* was comparable across different samples. The capacity of HIF1 α in responding to different oxygen levels and regulating hypoxic functions are carried out at the protein level, its gene expression is expected to be relatively stable [97-99]. Different experiments in the literature have reported that HIF2 α protein is less sensitive to the protein-level degradation as HIF1 α [80, 100, 101], therefore, a change in its gene expression is expected to be reflected at the protein level. Different stem cell markers and the rate of proliferation have been traced back to *HIF2A* activity [102-106]. This study observed the reduction in *HIF2A* gene expression in hMSCs cultured on all the synthesized surfaces. Alterations in pluripotency and differentiation markers were also observed in a surface-dependent fashion. The variation in these markers was selective to specific surfaces, which may have contributed to some controversial observations on their differentiation capacities [3, 13-16, 107].

Figure 7: A hypothesis of HIF2A-regulated functions in the hMSCs cultured on different culture surfaces.

A list of the 2,289 oxygen-sensitive genes was analysed in this study. Their dependency on oxygen tension may originate from their roles in the various oxygen sensing pathways. This study detected 1,873 of these genes, some of which demonstrated strong dependency to the culture surfaces hMSCs adhered to. Outstanding differences were observed for *ITGA2*, *FN1*, *ITGB3*, and *CXCL12*, in samples cultured on both fibrous and flat surfaces. The expressions of the genes encoding various hypoxia-regulated surface proteins were found to be closely related to that of *HIF2A*. Considering of the reported oxygen-sensitive surface proteins, this study found that culture surfaces regulate the gene expression of various surface proteins, some of which subsequently alter the expression of *HIF2A* and its downstream functions. Culture surfaces triggered part of the oxygen sensing and its downstream signalling pathways.

This study found *HIF2A* to be regulated by a change in activated surface proteins. The analysis of *HIF2A* expression across the samples cultured on different surfaces yielded 573 genes with significant expression correlations (Supplementary table S3), 147 of which have been reported with regulation dependency to hypoxia (Supplementary table S5). Among the correlated genes, the DEGs showed outstanding differences between the compared samples, including genes encoding hMSCs surface proteins. The significant reduction in the *HIF2A* gene expression in the samples cultured on fibrous surfaces (Table 1 and Figure 6) was induced by their surface topography, which regulated the expression of certain genes encoding surface proteins and led to the downstream alteration on *HIF2A*. Subsequently, *HIF2A* was transcribed and translated to regulate various crucial functions in hMSCs, such as survival [7]. Gene ontology analysis revealed the functions associated with the correlated HRG,

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including regulation on cell growth, hypoxic functions, angiogenesis, and cell surface interactions. *HIF2A* activity has been reported to promote hMSCs growth [101, 108]; in this study, the change in the measured *HIF2A* expression levels (Table 1 and Figure 6) is in line with the observed trend of cellular growth rate (Figure 2).

The rate of degradation of PLA was not expected to be significant in this experiment. All material was handled under the suggested conditions [109]. Therefore, the pH in culture medium was not expected to be affected by PLA degradation and the subsequent reduction of cell proliferation. No change in media colour was observed. In addition, culture media was changed every alternative day to reduce the impact of material degradation and to ensure the availability of metabolites and growth factors. Furthermore, with the moderate cell density, oxygen tension was not expected to be significantly different for the hMSCs cultured on different surfaces. The change in the expressions of HRG was considered to be independent of oxygen tension.

In storage, transportation, and therapeutic applications, maintaining a consistent hypoxic environment may be challenging, research has shown short-term exposure to normoxia can result in lasting changes [65, 110]. The findings from this study showed the possibility of using specially designed culture surfaces in regulating HRG and pathways in hMSCs under *in vitro* normoxia. A few other studies have reported on the altered gene expressions induced by culture surfaces [37, 111-115]. Furthermore, *in vitro* hypoxia and culture surface may interactively affect the regulation of surface proteins and their downstream pathways. This effect may have contributed to some contrasting results when comparing hypoxic experimental data from different research groups. Lastly, fibrous surfaces induced a more complex array of pathways which could play a major role in further reducing cellular growth rate. *In vitro* rate of proliferation dictates the required time for a large-scaled hMSCs production, it is important to establish a set of culture conditions (hypoxia, culture surfaces, etc.) which can optimize the process. Novel surfaces can be designed to up- or downregulate *HIF2A* and its downstream functions such as angiogenesis, cell migration, growth, etc. The preservation of hMSCs also need to be monitored during culture, hypoxia has been reported to effectively manage the process; specialized surfaces can be designed to reverse the undesired tendency. Future research will investigate on the potential of utilizing hypoxia and culture surfaces to collectively regulate hMSCs status.

Conclusions

The results from RNA sequencing demonstrated that culture surfaces play a role in altering the expressions of HRG and hypoxia-regulated pathways. The sensitivity of the incurred effects was found to be pathway and surface specific. Expression levels of key hypoxic functional genes were altered because of a change in culture surface. Fibrous surfaces induced more hypoxia regulated DEGs which encoded a complex array of cellular functions and resulted in slower rates of proliferation than hMSCs cultured on flat surfaces. The expression levels of several oxygen-sensitive surface proteins were found to be affected by a change in culture surfaces, some of which are hypothesized to act upstream of *HIF2A* and subsequently modulated its expression. Culture surfaces with different substrates hold great potential towards designing surfaces to selectively activate hypoxia-induced pathways under *in vitro* normoxia or to enhance specific functions under hypoxia.

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Conflict of Interests

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, and in the decision to publish the results.

Availability of data

All the processed data are presented in the manuscript or in the supplementary information. Information on raw data and materials are available from the NCBI BioProject number PRJNA395968 and from the corresponding authors upon request.

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