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## Mesenchymal Stem Cells Respond to Hypoxia by Increasing Diacylglycerols

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### Abstract

Mesenchymal stem cells (MSC) are currently being tested clinically for a plethora of conditions, with most approaches relying on the secretion of paracrine signals by MSC to modulate the immune system, promote wound healing, and induce angiogenesis. Hypoxia has been shown to affect MSC proliferation, differentiation, survival and secretory profile. Here, we investigate changes in the lipid composition of human bone marrow-derived MSC after exposure to hypoxia. Using mass spectrometry, we compared the lipid profiles of MSC derived from five different donors, cultured for two days in either normoxia (control) or hypoxia (1% oxygen). Hypoxia induced a significant increase of total triglycerides, fatty acids and diacylglycerols (DG). Remarkably, reduction of DG levels using the phosphatidylcholine-specific phospholipase C inhibitor D609 inhibited the secretion of VEGF and Angiopoietin-2, but increased the secretion of interleukin-8, without affecting significantly their respective mRNA levels. Functionally, incubation of MSC in hypoxia with D609 inhibited the potential of the cells to promote migration of human endothelial cells in a wound/scratch assay. Hence, we show that hypoxia induces in MSC an increase of DG that may affect the angiogenic potential of these cells.

### Keywords

MESENCHYMAL STEM CELLS; HYPOXIA; DIACYLGLYCEROLS

Mesenchymal stem cells (MSC) were originally identified as a rare subset of non-hematopoietic stromal cells in the bone marrow, with multipotent differentiation and self-renewal potentials [Owen and Friedenstein, 1988]. Now, MSC are considered to reside surrounding blood vessels in various tissues and can be isolated from virtually all postnatal organs [da Silva Meirelles et al., 2006]. As numerous studies have reported their beneficial effect in tissue repair, MSC have become a popular candidate for cell therapy.

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### SUPPORTING INFORMATION

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MSC are currently being tested in hundreds of clinical trials [Mendicino et al., 2014] for a wide array of conditions, including chronic ischemic heart failure, type 1 diabetes, osteoarthritis, and acute respiratory distress syndrome just to mention a few. Of note, most of these trials do not rely on the differentiation potential of MSC, but rather focus on the paracrine signals that MSC secrete [Caplan and Correa, 2011], which promote survival of neighboring tissue [Gnecchi et al., 2006], modulate the immune system [Aggarwal and Pittenger, 2005], and promote angiogenesis [Kinnaird et al., 2004], among others. In many of the clinical or experimental settings MSC are used to promote revascularization at areas with poor circulation, and hence, are injected into an ischemic tissue environment, where they are exposed to a lower oxygen concentration than in their usual *ex vivo* culture conditions. As reviewed by Das et al. [2010], hypoxia exerts strong effects on MSC, affecting their proliferation, differentiation, and migration. We have recently shown that incubation of MSC for 48 h or more in 1% oxygen, induces metabolic changes that promote survival of MSC both in vitro and in vivo [Beegle et al., 2015]. In addition, it has also been shown that MSC respond to hypoxia altering their secretome [Potier et al., 2007]. Further understanding MSC biology and response to hypoxia, especially in terms of the secretion of angiogenic factors could be remarkably useful for enhancing the efficacy of MSC-based therapy.

It has been long established that lipids are not only essential building blocks of biological membranes and responsible for energy storage in the cell, but also have important regulatory and signaling functions in almost all cellular processes [Hannun and Obeid, 2008]. Changes in lipid levels that result in functional consequences eventually gave rise to the idea of “bioactive lipids” [Hannun and Obeid, 2008]. For example, inositol phospholipids have been shown to modulate acetyl-choline induced intracellular signaling [Hokin and Hokin, 1953], hydrolysis of phospholipids induces Protein Kinase C (PKC) activation [Nishizuka, 1992] and eicosanoids play a crucial role in signaling inflammation [Serhan and Savill, 2005]. Accordingly, it has become evident that lipids are crucial first and second messenger molecules [van Meer et al., 2008]. It has been also shown that lipids can be involved in hypoxic signaling. Ceramides, for example, are increased in renal tubular epithelial cells under hypoxia [Ueda et al., 1998] and fetal asphyxia induces changes in the ceramide metabolism of rat brains [Vlassaks et al., 2013]. It has also been shown that the cell lines Hela and 293T increase diacylglycerol (DG) levels when exposed to hypoxia, affecting the activity of the transcription factor hypoxia inducible factor 1 (HIF-1) [Temes et al., 2004]. Very little is known about the overall lipidomics [Dennis, 2009] of human MSC, or changes in lipid composition in response to stimuli such as hypoxia. In consequence, our aims were (1) to determine the lipid composition of human bone marrow derived MSC, (2) to identify changes induced by exposure to hypoxia, and (3) to evaluate whether these changes have a role in the angiogenic potential of MSC.

## **MATERIALS AND METHODS**

### **ISOLATION AND CULTURE OF MESENCHYMAL STROMAL CELLS**

MSC were isolated as previously described [Beegle et al., 2015]. Briefly, bone marrow aspirates from healthy human donors (Stem Express, Placerville, CA) were passed through

90 µm pore strainers for isolation of bone spicules. Then, strained bone marrow aspirates were diluted with equal volume of phosphate-buffered saline (PBS) and centrifuged over Ficoll (GE Healthcare, Waukesha, WI) for 30 min at 700 g. Next, mononuclear cells and bone spicules were plated in plastic culture flasks using standard culture medium (Minimum Essential Medium  $\alpha$  (MEM  $\alpha$ , Thermo Scientific, South Logan, UT), supplemented with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 1% Penicillin-Streptomycin and 1% L-glutamine (Thermo Scientific). Culture medium was changed three times a week. MSCs were passaged after every 4–6 days in culture. MSCs from passage 4–7 were used for all experiments. For studies with hypoxia, cell cultures were placed in incubators (MCO-18M Sanyo) at 37°C with 5% CO<sub>2</sub>, humidified atmosphere and dedicated oxygen level, as established by replacement with nitrogen injections.

### QUADRUPOLE TIME-OF-FLIGHT TANDEM MASS SPECTROMETRY (QTOF-MS/MS)

After culture in either 20% or 1% O<sub>2</sub> for 48 h, MSC were trypsinized (Thermo Scientific) and stored at –80°C. Then, samples were processed for total lipid extraction and mass spectrometry analysis as previously described [Fiehn and Kind, 2007]. After quenching the cells, 1 × 10<sup>6</sup> dried cells were added to a 1.5 ml Eppendorf tube, placed on dry ice for 20 min to completely freeze and then thawed on ice. This freeze-thaw step was repeated twice. Then, 1 ml of pre-chilled (–20°C) extraction solvent was added to the cells (acetonitrile: isopropanol: water 3:3:2 v/v/v) and the freeze-thaw step repeated two more times. Samples were then vortexed for 10 s, shaken for 5 min (4°C), and centrifuged for 2 min at 14,000g. A total of 500 µl of the supernatant was evaporated in a cold trap concentrator (Lapconco Centrivap, Kansas City, MO) to complete dryness.

Lipidomics data were acquired using ultrahigh pressure liquid chromatography (UHPLC; Waters Acquity UPLC CSH C18 column). Chromatographic separation was followed by electrospray ionization (ESI) in both positive (Agilent 6530 QTOF MS, Agilent Technologies, Santa Clara, CA) and negative mode (Agilent 6550 QTOF MS) and QTOF-MS/MS.

Data were analyzed in a four-stage process. First, raw data were processed in an untargeted (qualitative) manner by MassHunter Qual (v. B.05.00, Agilent, Santa Clara, CA) to find peaks in up to 300 chromatograms. Peak features were then imported into Mass Profiler Professional (Agilent) for peak alignments to seek which peaks are present in multiple chromatograms, using exclusion criteria by the minimum percentage of chromatograms (30%) in which these peaks are positively detected. Peaks were then manually collated and constrained within the MassHunter quantification software (v. B.05.01) on the accurate mass precursor ion level, using the MS/MS information and the LipidBlast library to identify lipids with manual confirmation of adduct ions and spectral scoring accuracy. The following normalization steps were performed: “vector normalization” in which the sum of all peak heights was calculated for all identified metabolites (but not the unknowns) for each sample. Such peak-sums were called “mTIC” (“total ion chromatogram/current”). Subsequently it was determined, whether mTIC averages were significantly different between treatment groups. Because the average mTIC will be different between series of analyses (due to differences in machine sensitivity, tuning, maintenance status and other

parameters), additional normalizations were performed. For this purpose, identical samples (“QC samples”) were analyzed multiple times in all series of data acquisitions (a suitable QC sample was used for every 11th injection). Internal standards were used for absolute quantifications, with a standardized equation for peak height normalizations. Presented data are “absolute quantifications,” meaning they are normalized to the best suited internal standard for which the absolute concentration which was used in the spiking process is known. The best suited internal standard is defined as the internal standard that belongs to the same lipid class as the metabolite that needs to be normalized. Annotated lipids are abbreviated as: [Lipid class] [total carbon number in the chains]/[total number of double bonds in the chains].

## MEASUREMENT OF DG LEVELS BY ELISA

To measure DG levels in MSC, we used the Human Diacylglycerol ELISA Kit (Biotrend Chemicals LLC, Destin, FL), following manufacturer’s instructions. MSC (10,000cells/cm<sup>2</sup>) were cultured in 6-well plates in normoxia (21% Oxygen), hypoxia (1% Oxygen), and with or without D609 (50 µM). In addition, MSC in normoxia were also tested in presence of Cobalt Chloride (100 µM). After 48h, cells were lifted by trypsinization and stored at –80°C as dry cell pellets. Total lipids were extracted as previously described [Bligh and Dyer, 1959; Petkovic et al., 2005]. Briefly, cell pellets were re-suspended in PBS (100,000 cells/100 µl). Lipids were extracted from cell suspension by addition of 375 µl chloroform: methanol mixture (1:2 v/v), and vortex for 2 min. Then, 125 µl chloroform were added, and extracts were vortexed for 30 s. Finally, samples were washed with 125 µl of 1.5 M NaCl, vortexed for 30 s, and centrifuged at 500g for 10 min. Organic phase (lower phase) was used for analysis and measured in triplicate.

## DETECTION OF ANGIOGENIC FACTORS

In order to detect secretion levels of vascular endothelial growth factor (VEGF), interleukin-8 (IL-8/CXCL-8), and angiopoietin-2 (Ang-2), MSC (13,000cells/cm<sup>2</sup>) were cultured for 48 h in 12-well plates in either normoxia or hypoxia and in either presence or absence of 50 µM D609 (O-(octahydro-4,7-methano-1H-inden-5-yl) carbon potassium dithioate). Then, supernatants (i.e., conditioned media) were collected and stored at –80°C. Quantification of angiogenic factors was performed by enzyme-linked immunosorbent assay (ELISA) using the respective DuoSet kits (R&D Systems, Minneapolis, MN), following manufacturer’s instructions.

To measure mRNA levels of VEGF, IL-8, and Ang-2, cells were cultured as above, but for 12 h. Total RNA was extracted using Quick-RNA MicroPrep kit (Zymo Research, Irvine, CA). Reverse transcription was performed with 1 µg RNA using TaqMan Reverse Transcription Reagents kit (Life Technologies, Grand Island, NY). For semi-quantitative detection of mRNA levels of VEGF, IL-8, Ang-2, and GAPDH (internal control) real time PCR (RT-PCR) was done using TaqMan primers/probes (Life Technologies): Hs00900055\_m1, Hs00174103\_m1, Hs01048042\_m1, and Hs99999905\_m1, respectively. Special attention was placed on having only minimal variation on GAPDH mRNA levels, in between samples.

## WOUND/SCRATCH ASSAY

Conditioned media of MSC was prepared and stored as described above. Human umbilical vein endothelial cells-derived VeraVecs [Seandel et al., 2008] (Angiocrine Bioscience, New York, NY) were cultured in EndoGro medium (Millipore, Billerica, MA). For wound/scratch assays, VeraVecs were plated at 79,000 cells/cm<sup>2</sup> in 24-well plates containing plastic inserts that leave a homogeneous 500 μm gap in between confluent monolayers of cells (Cytoselect 24-well wound healing assay [Cell Biolabs, San Diego, CA]). The next day, inserts were removed and media was changed to the conditioned media from MSC. Regular MSC culture medium was used as negative control. Pictures were taken immediately and 10 h after adding conditioned media. Pictures were then analyzed using TScratch software [Geback et al., 2009] (ETH Zürich). Wound closure was determined as [open image area after 10h/initial open image area]\* 100. Values represent the average of three independent experiments.

## STATISTICAL ANALYSIS

Values are given as mean ± SEM. A paired Student's *t*-test was used to determine statistically significant differences established as *P* < 0.05.

## RESULTS

### LIPID COMPOSITION OF MSC

To our knowledge, neither an analysis of total lipid composition nor changes in lipid composition induced by hypoxia in human MSC have been previously reported. In order to address this, we isolated MSC from five different donors and cultured them for 48 h in either normoxia (140 mmHg in the culture media of cells [Beegle et al., 2015]) or hypoxia (1% O<sub>2</sub>, equivalent to 10 mmHg) and then processed cells for total lipid analysis by mass spectrometry. Using a MALDI-TOF lipidome analysis, a total of 1965 different molecular ions were detected; 1,444 by positive ion mode and 521 by negative ion mode. Out of these, 1841 molecular ions (93.7%) were detected in all five donors of MSC. Most of these molecular ions are not annotated and are only referred to by an identification number. However, 390 ions (21.2%) could be identified as putative lipid species using LipidBLAST [Kind et al., 2013] (Fig. 1A and Supplementary Table S1). From these, phosphatidylcholine (PC) species accounted for 76.5% of all identified lipids, followed by sphingomyelin (SM; 11%), phosphatidylethanolamine (PE; 2%), and triglyceride (TG; 2%) species. Altogether, PC, SM, PE, and TG accounted for over 90% of all annotated lipids detected in MSC (Fig. 1B). The lipid detected most abundantly was PC (18:1/16:0) accounting for 13.1% of all annotated lipids (not shown) measured.

### HYPOXIA INDUCES CHANGES IN THE LIPID COMPOSITION OF MSC AND SIGNIFICANTLY INCREASES DIACYLGLYCEROLS LEVELS

In order to identify changes in MSC in lipid composition induced by hypoxia, we performed two types of analysis. First, we grouped the detected species by lipid class and performed a Student's *t*-test to identify significant differences by class. We found that TGs, DGs, and fatty acids (FA) were significantly increased in MSC exposed to hypoxia by 4.8, 1.8, and

1.3-fold, respectively, as compared to MSC in normoxia (Fig. 1C and D). We then compared differences in the individual lipid species. From the 390 annotated lipids, only two (0.5%) were found to be significantly lower in hypoxia, which may fall in the category of false positive results. In contrast, 41 lipid species (10.5%) were significantly increased in hypoxic MSC as compared to controls. Remarkably, we found that each of the seven detected DG species were significantly increased in MSC under hypoxia (Fig. 2A). Altogether, our lipidomic analysis revealed a strong up-regulation of many lipids in MSC exposed for 48 h to hypoxia and especially noteworthy was the increase of all detected DG. This increase of DG levels by hypoxia was further confirmed using ELISA (Fig. 2B). In addition, we found that exposure for 48 h to the hypoxia-mimicking agent Cobalt Chloride ( $\text{CoCl}_2$ ) [Goldberg et al., 1987; Schuster et al., 1989] exerted a similar effect, inducing an increase of DG levels in MSC. To evaluate the possible effects of increased DG levels in MSC in hypoxia, we used D609, a well-known inhibitor of PC-PLC and SMS, hence reducing overall DG levels [Adibhatla et al., 2012]. Interestingly, we found that addition of 50  $\mu\text{M}$  D609 reduced DG levels in MSC cultured under hypoxia but not in MSC under normoxia, although these differences were not statistically significant.

### REDUCTION OF DG LEVELS WITH D609 AFFECTS SECRETION OF ANGIOGENIC PROTEINS IN MSC

MSC promote angiogenesis *in vivo* through secretion of angiogenic signals such as VEGF [Kinnaird et al., 2004; Williams and Hare, 2011]. Indeed, MSC usually reside around blood vessels as pericytes [Crisan et al., 2008] promoting blood vessel maturation [Pedersen et al., 2014]. To evaluate the possible effects of increased DG levels in MSC in hypoxia, we used D609, a well-known inhibitor of PC-PLC, hence reducing overall DG levels [Adibhatla et al., 2012]. After incubation of MSC for 48 h in normoxia or hypoxia, with or without D609 (50  $\mu\text{M}$ ), supernatant conditioned media were collected and amount of secreted VEGF, CXCL8/IL-8, and Angiopoietin-2 (Ang-2) were measured by ELISA. In line with previous publications [Dai et al., 2007; Martin-Rendon et al., 2007; Burlacu et al., 2013], we observed a significant increase in VEGF secretion in MSC exposed to hypoxia, whereas addition of D609 (i.e., reduction of DG) caused a reduction of VEGF levels in both MSC in normoxia and hypoxia (Fig. 3A). In contrast, secreted IL-8 levels were reduced by hypoxia, whereas addition of the inhibitor D609 increased IL-8 secretion in both MSC in normoxia and hypoxia (Fig. 3B). Secretion levels of Ang-2 by MSC were overall very low. However, we detected a slight decrease in Ang-2 secretion in MSC exposed to hypoxia, as compared to controls, whereas addition of D609 reduced Ang-2 secretion even further in both MSC in normoxia and hypoxia (Fig. 3C). Overall, these trends are very similar at mRNA levels, measured after incubation for 12 h in hypoxia (Supplementary Fig. S1). However, although mRNA levels of VEGF, IL-8 and Ang-2 showed a similar trend to the respective secreted protein levels (Fig. 3). Our results show that VEGF and Ang-2 are both regulated transcriptionally by hypoxia, while all other differences in between conditions were not significant. These results suggest that some of the changes on VEGF, IL-8 and Ang-2 might be attributed to action on a transcriptional level, but most probably post-transcriptional mechanisms (of either protein synthesis or protein secretion) are also involved. Altogether, our results suggest that DG are involved in the secretion of VEGF, IL-8, and Ang-2 in MSC in both normoxic and hypoxic conditions.



## REDUCTION OF DG IN MSC INHIBITS THEIR POTENTIAL TO INDUCE MIGRATION OF ENDOTHELIAL CELLS

To further investigate whether the increase of DG in MSC under hypoxia could exert a functional effect, we prepared conditioned media of MSC cultured under either normoxia or hypoxia and in the presence or absence of D609. We then tested whether these conditioned media affected the migration of human endothelial cells in vitro. As shown in Figure 4, conditioned media from MSC cultured under hypoxia promotes endothelial cell migration to a similar extent than MSC under normoxia. However, under hypoxia, addition of D609 reduced the migration of endothelial cells, suggesting that the increase of DG in MSC under hypoxia is an important mechanism to alter the angiogenic secretome of MSC.

## DISCUSSION

Using a MS lipid analysis, nearly two thousand molecular ions were detected in MSC isolated from five different human donors. From these, 390 molecular ions could be identified as annotated lipid species using LipidBLAST [Kind et al., 2013]. A previous study used MALDI-TOF to identify the lipid composition of ovine MSC [Fuchs et al., 2008], whereas Kilpinen et al. compared phospholipids by MS/MS mass spectrometry and fatty acids by gas chromatography of MSC derived from five young and five old donors [Kilpinen et al., 2013]. Recently, Lee et al. measured metabolic changes (including several lipids) in clonally-isolated human MSC during cellular senescence using ultraperformance liquid chromatography/quadrupole time-of-flight mass spectrometry [Lee et al., 2014] and Bergante et al. studied changes in glycosphingolipid in human MSC undergoing osteogenesis [Bergante et al., 2014]. To our knowledge, our report is the most comprehensive analysis of lipid composition of human MSC to date.

Our lipidomic analysis revealed a strong up-regulation of many lipids in MSC exposed for 48 h to hypoxia, similarly to other cell types [Briggs and Glenn, 1976; Gordon et al., 1977]. As previously described in fibroblasts [Gordon et al., 1977], TGs and FAs were significantly increased under hypoxia, a phenomenon that could be explained by the inability of hypoxic cells to support the oxygen-dependent fatty acid oxidation [Bhatnagar, 2003].

In addition, here we show that hypoxia consistently induced an increase of all DG detected. DG production in the cell can occur through several different mechanisms: during both the biosynthesis and catabolism of TG, or through phosphatide phosphatase enzyme activity, which uses phosphatidic acid to form DG, as a key step in the formation of PC, PE and Phosphatidylserines (PS). DG is also produced by the enzymatic cleavage of PI or PC in the cell membrane, by either PI- or PC-specific phospholipase C. The enzyme sphingomyelin synthase (SMS) uses ceramide and the phosphocoline head group of PC to produce DG and SM.

The increase in DG content during hypoxia had been previously reported in HeLa and 293-T cells, and this increase was mainly dependent on PC-PLC/SMS activity [Temes et al., 2004]. These authors also proposed a role of DG in the regulation of HIF-1 activity, as the inhibition of PC- PLC/SMS enzymes abrogated both DG increase and HIF-1 activation in hypoxia in both cell types, with the effect being independent of PKC activity. DG



accumulation in hypoxia was also observed in rat smooth muscle cells from precapillary pulmonary arteries, with DG likely activating TRPC6 channels, thereby mediating acute hypoxic pulmonary vasoconstriction [Weissmann et al., 2006]. It also has been shown that myocardial ischemia caused a rapid increase in DG content in rabbit hearts [Gysembergh et al., 2000]. Altogether, our findings and these previous publications, suggest that in addition to the accumulation of TG and FA, also DG are commonly increased in cells or tissues exposed to hypoxia.

In this study, we did not address the specific mechanism(s) responsible for the accumulation of DG under hypoxia in MSC. In order to determine possible consequences of having increased DG levels in MSC under hypoxia, we used the PC-PLC/SMS inhibitor D609, which has been shown to decrease DG levels [Hillemeier et al., 1996; Walter et al., 1996; Bettaieb et al., 1999; Antony et al., 2001; Zhang et al., 2001]. We found that after exposure for 48 h to D609, only MSC under hypoxia showed reduced DG levels, suggesting that the increase of DG in hypoxia may relate to the activity of enzymes PC-PLC or SMS. Although overall VEGF, IL-8, and Ang-2 levels strongly varied among the individual donors, our results were consistent among all 6–8 individual experiments. For both VEGF and IL-8, D609 induced the opposite effect than hypoxia, whereas secretion of Ang-2 was decreased by both hypoxia and D609. Of note, D609 affected VEGF, IL-8, and Ang-2 secretion in both normoxia and hypoxia, suggesting that DG play a general role in the secretion of these proteins. In addition, the effect of D609 was often milder in hypoxia, possibly due to enhanced production of DG. Alternatively, compensatory mechanisms might be in place since, for example, DG promote HIF-1 activity [Temes et al., 2004] and VEGF is a downstream target gene of HIF-1.

Endothelial cell migration is a critical step during the formation of new blood vessels [Ridley et al., 2003]. Multiple factors secreted by MSC are possibly involved in this process, where remarkably D609 only affected the pro-angiogenic effect of MSC cultured in hypoxia. This suggests that the specific signals of MSC promoting migration of endothelial cells are distinct under normoxia and hypoxia, where under hypoxia signals are more dependent on DG levels. Future experiments need to further identify DG-mediated angiogenic factors secreted by MSCs under hypoxia.

Altogether, our results show that MSC respond to hypoxia by increasing the expression of several lipids. In particular, a consistent increase in DG was found, which we showed, affected the angiogenic potential of the cells. The changes in lipid composition presented here offer new insight into the changes that MSC undergo under hypoxia, which can impact the therapeutic use of these cells in patients.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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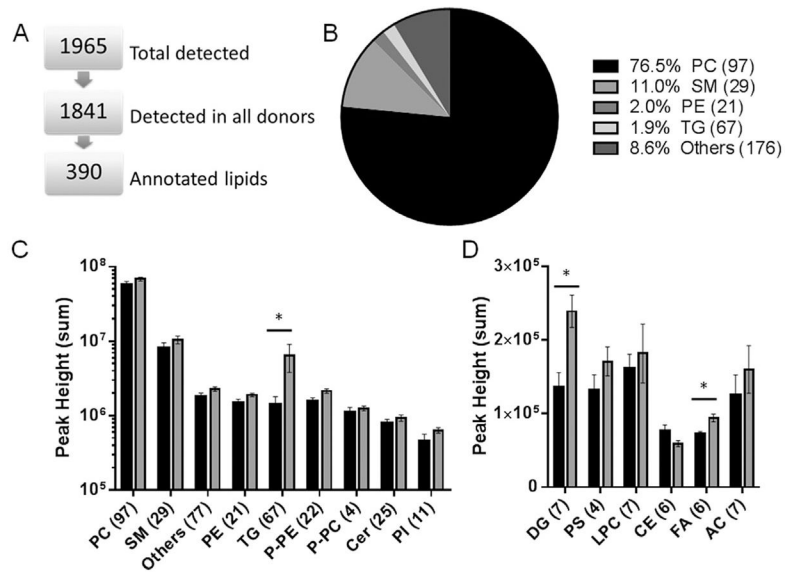
This work was performed at the Institute for Regenerative Cures, University of California Davis, Sacramento, CA.

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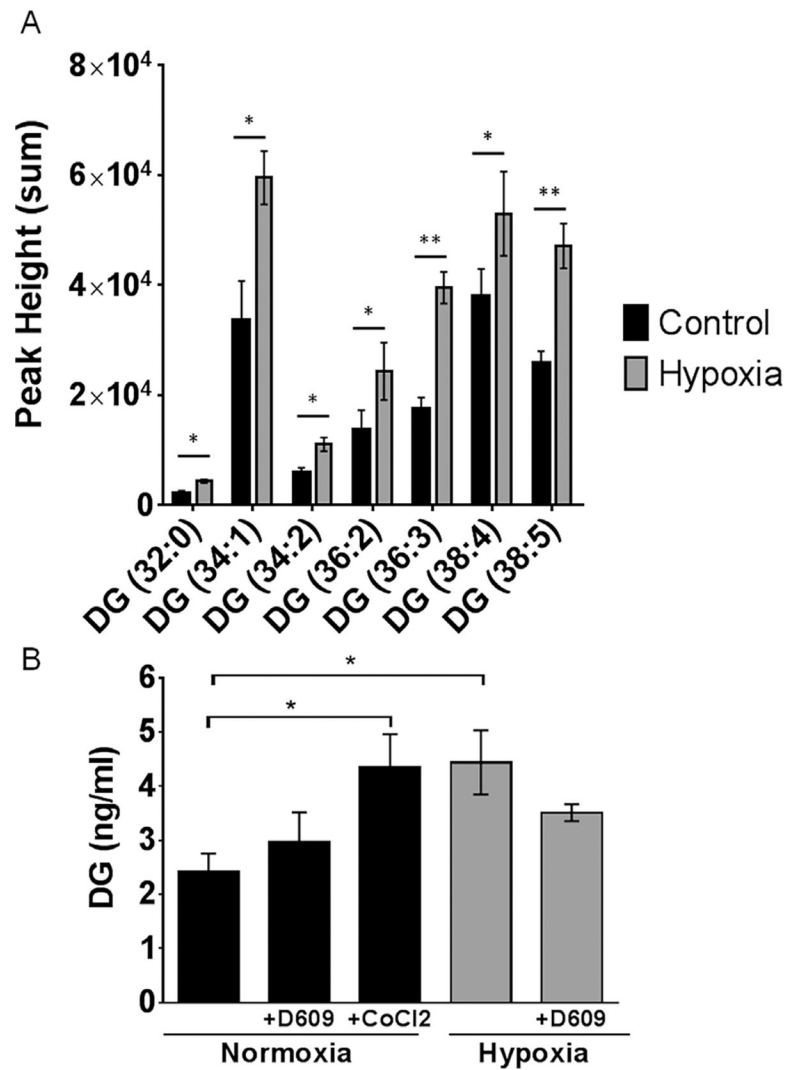
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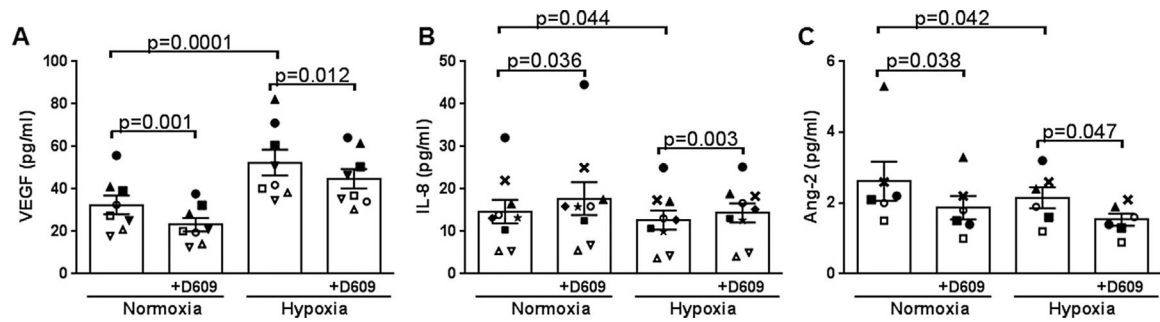
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**Fig. 1.**

Lipid content detected in MSC cultured in either normoxia or hypoxia. (A) Schematic overview of total lipids detected, those detected consistently in MSC derived from all five donors and the fraction of those, with a common name (annotated). (B) Pie chart with major lipid groups found in MSC (cultured in either normoxia or hypoxia). See abbreviations in main text. (C) and (D) show average lipid amount detected in MSC under normoxia (black bars) or hypoxia (gray bars). Notice the logarithmic scale used for the most abundant lipids (C) and linear scale used for lipids in lower amounts (D). Statistical analysis was performed by paired Student's *t*-test to each individual lipid where  $*P < 0.05$ .



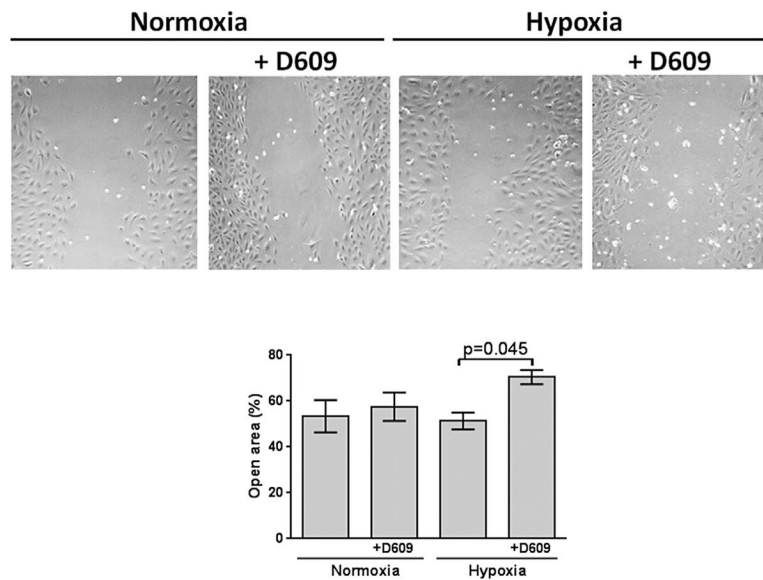
**Fig. 2.** Diacylglycerols (DG) are increased in MSC under hypoxia. (A) Individual DG species identified in MSC in normoxia (black bars) and hypoxia (gray bars). For complete list of individual lipids detected, please refer to Supplementary Table S1. (B) Increased DG levels by hypoxia were further confirmed using an ELISA-based method. Also, incubation for 48 h in presence of CoCl<sub>2</sub> (100  $\mu$ M), induces an increase in DG in MSC. In contrast, addition of D609 (50  $\mu$ M) to the culture media decreases DG levels in MSC under hypoxia, but not normoxia. In both experiments, statistical analysis was performed by paired Student's *t*-test, where \**P*<0.05 and \*\**P*<0.005.



**Fig. 3.**

Effect of hypoxia and D609 on secretion of angiogenic factors. After culture of MSC for 48 h in either normoxia or hypoxia and in the presence or absence of 50  $\mu\text{M}$  D609, cells were counted and supernatants were collected and levels of angiogenic factors determined by ELISA. Values represent the amount of angiogenic factor (VEGF, IL-8, or Ang-2) secreted by 1,000 cells over 48 h. Individual symbols represent single experiments performed with MSC derived from a different donor while bars represent the overall averages. Statistically differences were determined by paired Student's *t* tests comparing the indicated conditions, with *P* values indicated over respective brackets.





**Fig. 4.** Inhibition of DG synthesis by D609 reduces the angiogenic potential of MSC. Effect of supernatants of MSC cultured for 48 h in either normoxia or hypoxia and in the presence or absence of 50  $\mu$ M D609 on migration of endothelial cells. Upper pictures show representative images of the migration of endothelial cells in a wound/scratch assay, 10 h after exposure to supernatant of MSC. The histogram shows the averages of three independent experiments. Notice that a greater open area represents a lower level of migration of the endothelial cells. A statistically difference was determined by a paired Student's *t*-test.