Diversity of respiratory parameters and metabolic adaptation to low oxygen tension in mesenchymal stromal cells

Kim Olesen [a,b,1], Noah Moruzzi [c,1], Ivana Bulatovic [a], Clifford Folmes [d], Ryounghoon Jeon [d], Ulrika Felldin [e], Andre Terzic [d], Oscar E. Simonson [e,f], Katarina Le Blanc [g], Cecilia Österholm [a], Per-Olof Berggren [c], Tomas Schiffer [h], Sergey Rodin [e,f,i], Andreas Tilevik [b], Karl-Henrik Grinnemo [e,f,*]

[a] *Department of Molecular Medicine and Surgery, Karolinska Institutet, 171 76, Stockholm, Sweden*
[b] *School of Bioscience, University of Skövde, 541 28, Skövde, Sweden*
[c] *The Rolf Luft Research Center for Diabetes and Endocrinology, Department of Molecular Medicine and Surgery, Karolinska Institutet, 171 76, Stockholm, Sweden*
[d] *Departments of Cardiovascular Medicine, And Biochemistry and Molecular Biology, Mayo Clinic, Scottsdale, AZ, 85259, USA*
[e] *Department of Surgical Sciences, Uppsala University, 751 85, Uppsala, Sweden*
[f] *Department of Cardiothoracic Surgery and Anesthesiology, Uppsala University Hospital, 751 85, Uppsala, Sweden*
[g] *Department of Laboratory Medicine, Karolinska Institutet, Karolinska University Hospital, 141 86, Stockholm, Sweden*
[h] *Department of Physiology and Pharmacology, Karolinska Institutet, 171 77, Stockholm, Sweden*
[i] *Endocrinology Research Centre, Moscow, 115478, Russia*

A R T I C L E   I N F O

A B S T R A C T

Objective: Cell metabolism has been shown to play an active role in regulation of stemness and fate decision. In order to identify favorable culture conditions for mesenchymal stromal cells (MSCs) prior to transplantation, this study aimed to characterize the metabolic function of MSCs from different developmental stages in response to different oxygen tension during expansion.

Materials and methods: We cultured human fetal cardiac MSCs and human adult bone-marrow MSCs for a week under hypoxia (3% O_2) and normoxia (20% O_2). We performed mitochondrial characterization and assessed oxygen consumption- and extracellular acidification-rates (OCR and ECAR) in addition to oxygen-sensitive respiration and mitochondrial complex activities, using both the Seahorse and Oroboros systems.

Results: Adult and fetal MSCs displayed similar basal respiration and mitochondrial amount, however fetal MSCs had lower spare respiratory capacity and apparent coupling efficiency. Fetal MSCs expanded in either hypoxia or normoxia demonstrated similar acidification rates, while adult MSCs downregulated their aerobic glycolysis in normoxia. Acute decrease in oxygen tension caused a higher respiratory inhibition in adult compared to fetal MSCs. In both sources of MSCs, minor changes in complex activities in normoxic and hypoxic cultures were found.

Conclusions: In contrast to adult MSCs, fetal MSCs displayed similar respiration and aerobic glycolysis at different O_2 culture concentrations during expansion. Adult MSCs adjusted their respiration to glycolytic activities, depending on the culture conditions thus displaying a more mature metabolic function. These findings are relevant for establishing optimal *in vitro* culturing conditions, with the aim to maximize engraftment and therapeutic outcome.

1. Introduction

Rather than being a mere consequence of the cell energy requirements, metabolism is now believed to play an active role in determining cell fate and in the regulation of stemness, including cell renewal and differentiation [1]. As an example, a shift in balance from oxidative phosphorylation to glycolysis occurs during the reprogramming of somatic cells into pluripotent stem cells (PSCs) [2,3]. The

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metabolism of stem cells and adult resident progenitors have therefore gained attention in order to understand the *in vivo* homeostasis and the impact of *in vitro* culturing along with engraftment outcomes.

Amongst stem cells, mesenchymal stromal cells (MSCs) constitute an attractive clinical tool due to their immunomodulatory and regenerative properties, and are relatively easy to harvest and expand [4,5]. However, MSCs represent a heterogeneous population of cells that can display different phenotypic and functional characteristics. Depending on the developmental stage and expansion conditions, the cells might exhibit very different degree of survival after transplantation into living hosts. In our study, we have taken advantage of metabolic analyses to characterize human MSCs from different developmental stages, exemplified by first trimester fetal hearts and adult bone marrow. We analyzed their metabolic parameters and adaptability to oxygen tension of the environment, being either normoxia (20% O_2) or physoxia (3% O_2) [6,7], which we refer to *in vitro* as hypoxia. Our findings add relevant information for adjusting *in vitro* expansion protocols for MSCs in regards to their source.

2. Research design and methods

2.1. Generation and characterization of fetal and adult human mesenchymal stromal cells

Human fetal cardiac MSCs were derived and expanded as previously described [8] from abortion material, gestational week 4,5–6 (ethical permit Dnr: 2015/1369-31/2). Human bone-marrow MSCs were isolated from iliac crest bone marrow aspirates from healthy donors after informed consent and approval from the Regional Ethics Board in Stockholm (ethical permit Dnr: 2016/1582-31) using a similar protocol as previously described [9]. The expanded MSCs fulfill the International Society for Cellular Therapy (ISCT) guidelines [10] of being mesenchymal stromal cells by expressing mesenchymal surface markers, in combination with their clonogenic expansion potential and multi-lineage differentiation capacity [8,10].

2.1.1. Mitochondrial characterization

Mitochondria were stained for 1h with 500 nM MitoTracker Red (TermoFisher, Waltham, MA USA) and visualized with LSM 780 confocal microscope, objective alpha Plan-Apochromat $100\times/1.46$ Oil DIC M27, Ex 561/488/405, Em 671/534/448. Electron microscopy (EM) was performed as previously described [11]. Mitochondrial quantification was performed by calculating the ratio of nuclear and mitochondrial DNA by qPCR QuantStudio5 thermocycler (Thermo-Fisher) after DNA isolation using DNeasy Kit (Qiagen). Primer pairs: mitochondrial ND1: F_TTCTAATCGCAATGGCATTCCT, R_AAGGGTTG-TAGTAGCCCGTAG; ND5 F_TTCATCCCTGTAGCATTGTTCG, R_GTTGGAATAGGTTGTTAGCGGTA; nuclear TYR3M F_AGGG-TATCTGGGCTCTGG, R_GGCTGAAAAGCTCCCGATTAT; B2M F_TGCTGTCTCCATGTTTGATGTATCT, R_TCTCTGCTCCCCACCTC-TAAGT. 1 µl of DNA were loaded into a 96-well plate together with 10 µM primer pairs and SYBR™ Green PCR Master Mix (ThermoFisher).

2.2. Extracellular flux analysis (Seahorse)

In order to adapt the MSCs to hypoxic or normoxic conditions, the fetal and adult MSCs were cultured in either normoxia (20% O_2) or hypoxia (3% O_2) before Seahorse measurements. Oxygen consumption rates (OCR) and extracellular acidifications rates (ECAR) were measured using a XF24 Extracellular Flux Analyzer (Agilent, Santa Clara, CA, USA). 30 000–50 000 cells/well coated with Geltrex™ (ThermoFisher) were seeded the day before analysis, which was carried out in XF assay medium (25 mM glucose, 2 mM glutamax, 1 mM pyruvate, 1x non-essential amino acids, 1% FBS). Serial injections of oligomycin (final concentration 0.5 mg/ml), Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone FCCP (1 µM) and rotenone (0.5 µM) plus antimycin

(1 µM) were carried out. The following indexes were calculated: Coupling efficiency ((1-(oligomycin sensitive/basal))*100), Spare respiratory capacity (FCCP respiration/Basal) and OCR/ECAR (basal OCR/ basal ECAR). Each value was normalized to total protein quantified using a Bradford protein assay (Bio-rad, Hercules, CA, USA).

2.3. Oroboros respirometry

Before Oroboros measurements, the fetal and adult MSCs were adapted to hypoxic (3% O_2) or normoxic (20% O_2) cultures for one week. The cell respiration was measured with high resolution respirometry (O2–K, Oroboros, Innsbruck, Austria) at 37 °C, at 20% and 3% O_2 tension. Complex activities were measured after cell permeabilization with digitonin at 20% O_2 in EGTA 0.5 mM, MgCl$_2$ 3 mM, K-lactobionate 60 mM, Taurine 20 mM, KH$_2$PO$_4$ 10 mM, HEPES 20 mM, Sucrose 110 mM and BSA fatty acid free 1 g/L. Complex I (CI) state 2 was measured after addition of Pyruvate (5 mM) and Malate (2 mM), then state 3 was induced by the addition of ADP (2.5 mM). Maximal respiration (state 3, CI + Complex II (CII)) was measured by the addition of succinate (10 mM), and CII state 3 after adding CI inhibitor rotenone (0.5 µM). Antimycin (2.5 µM) was added to control for non-mitochondrial respiration. Ratios of Complex-activities were calculated by dividing CI-linked OCR with CI + CII-linked OCR (CI) and CII-linked OCR divided by CI + CII-linked OCR (CII).

2.4. Statistics

Statistical analysis was carried out on Seahorse data, after outliers removal, using GraphPad prism 8 (San Diego, CA, USA), using paired or unpaired T-Test depending on the data distribution on the technical replicates to avoid loss of information between wells. Three biological replicates (donors) were used for the experiments. Statistical analyses on Oroboros and gene quantity data were performed using R V.3.6.3 (R Foundation for Statistical Computing, Austria). The data were analyzed with Welch's t-tests or paired t-tests when appropriate. All experiments were performed with three biological replicates. All tests were two-sided, and a p-value < 0.05 was considered to be statistically significant.

3. Results and discussion

In this study, we used human MSCs from two different developmental stages that fulfilled the ISCT MSC-criteria [8,10]: first trimester fetal heart- and adult bone marrow- derived MSCs, respectively. The heart is the first organ to develop from the splanchnic mesoderm during the first trimester of human embryogenesis [12,13]. Thus, MSCs isolated from first trimester hearts constitute the earliest developmental source of MSCs, thereby representing the most naïve state. In order to understand differences and similarities in metabolic adaptation of adult and fetal MSCs in relation to oxygen tension, both sources were adapted for one week to either normoxia (20% O_2) or hypoxia (3% O_2), which more closely resemble physiological conditions [6,7] (Fig. 1A). We performed metabolic functional measurements using Seahorse XF24, which enables the simultaneous assessment of two major cellular energy-producing pathways, namely mitochondrial respiration and aerobic glycolysis.

After one week of normoxic culture, we found a similar basal respiration between fetal and adult cells (Fig. 1B). However, fetal cells displayed higher oligomycin sensitive respiration ($p<0.01$) (Fig. 1B) and a lower coupling efficiency compared to adult MSCs ($p<0.01$) (Fig. 1C). Moreover, fetal cells displayed lower maximal respiration ($p<0.01$) (Fig. 1B) and spare respiratory capacity ($p<0.001$) (Fig. 1D). After normoxic culture, there was no difference between cells in the ECAR and in the ratio between respiration and anaerobic glycolysis (Fig. 1E and F). Taken together, after normoxic culture the basal metabolic parameters of adult and fetal MSCs are similar. However, the respiration coupled to ATP production is significantly lower in the fetal cells, which also displayed a lower maximal respiratory capacity than the adult cells
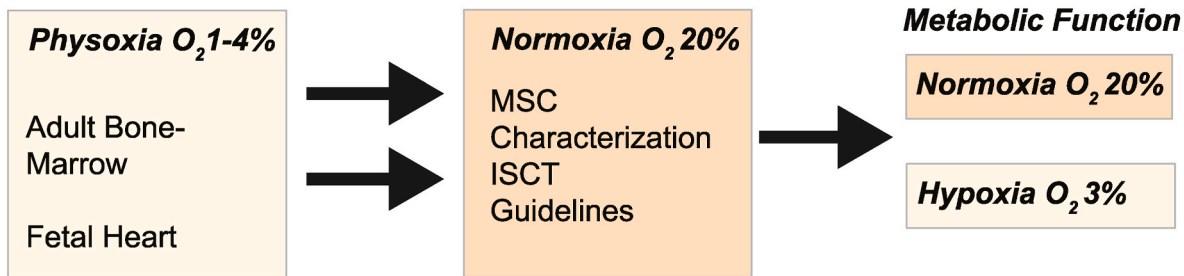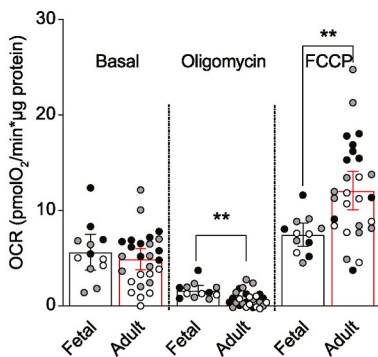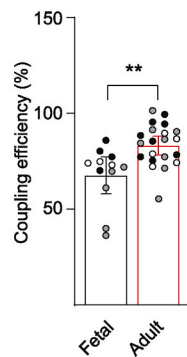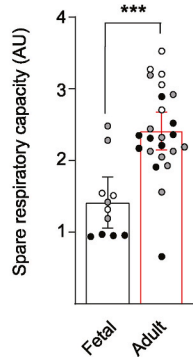
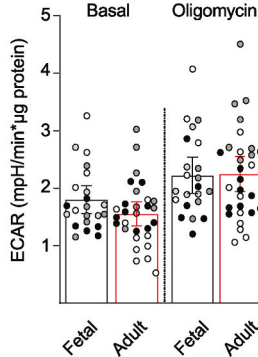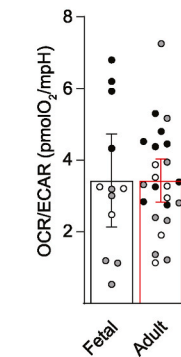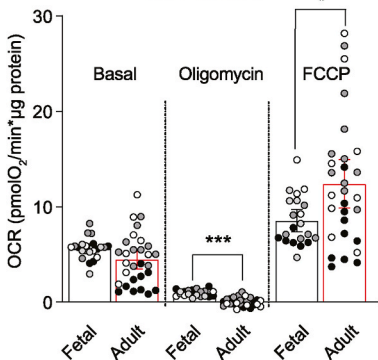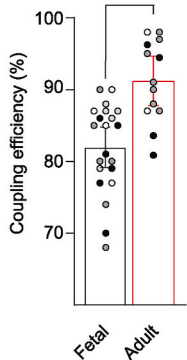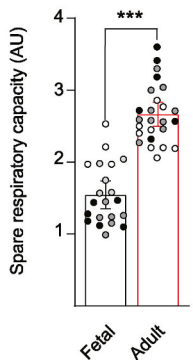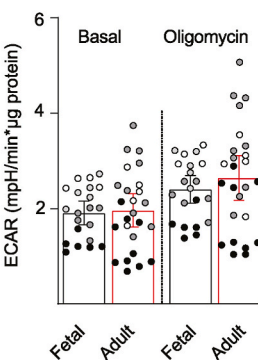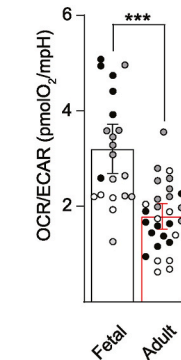*(caption on next page)*

Fig. 1. Schematic workflow, metabolic flux analysis and mitochondrial morphology. A) Schematic overview of the workflow from isolation of cells and characterization according to ISCT guidelines, in addition to bioenergetics analysis using the Seahorse and Orobo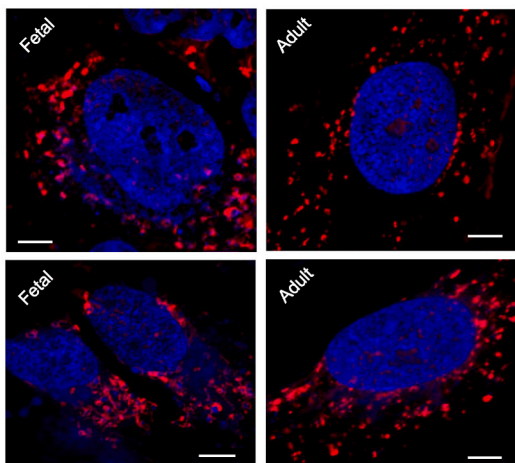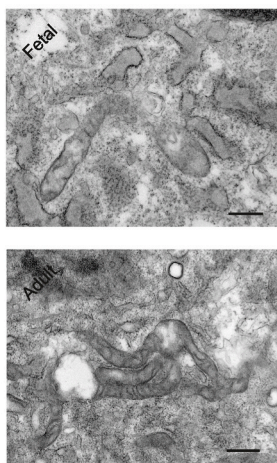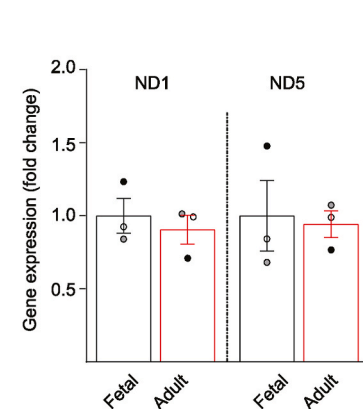ros system. Analysis was performed 1 week after either normoxia or hypoxia culture, representing oxygen tension close to physoxia, versus the standard culture conditions of MSCs *in vitro*. **B)** Protein normalized OCR recording of fetal and adult MSCs after 1 week of culture in normoxia using XF24 Flux Analyzer at baseline and after injections of oligomycin, FCCP and rotenone-antimycin. **C)** Apparent coupling efficiency calculated using the data in Fig1B. **D)** Spare respiratory capacity calculated using the data in Fig.1B. **E)** Protein normalized ECAR recording of fetal and adult MSCs after 1 week of culture in normoxia using XF24 Flux Analyzer at baseline and after injections of oligomycin, **F)** Basal OCR/ECAR ratio calculated using the data in Fig.1B,E. **G)** Protein normalized OCR recording of fetal and adult MSCs after 1 week of culture in hypoxia using XF24 Flux Analyzer at baseline and after injections of oligomycin, FCCP and rotenone-antimycin. **H)** Apparent coupling efficiency calculated using the data in Fig.1G. **I)** Spare respiratory capacity calculated using the data in Fig.1G. **J)** Protein normalized ECAR recording of fetal and adult MSCs after 1 week of culture in hypoxia using XF24 Flux Analyzer at baseline and after injections of oligomycin, **K)** Basal OCR/ECAR ratio calculated using the data in Fig.1G,J. *Data are expressed as mean ± CI (n≥11).* **L)** Representative images of cells stained with mitotracker, visualized by confocal microscopy, in fetal and adult MSCs. *Scale bar = 5 μm.* **M)** Representative images of transmission electron microscopy of mitochondria in fetal and adult MSCs. *Scale bar = 0.5 μm.* **N)** Mitochondrial DNA versus nuclear DNA normalized on fetal MSCs measured by qPCR. *ND1* and *ND5* represent two mitochondrial genes. *Data are expressed as mean ± SD (n=3).*

* = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001. **B–N** Empty white bar = fetal MSCs; empty red bar = adult MSCs. Bar plots have where each donor is represented by different color-filled circles. Abbreviations: ECAR = extracellular acidification rate; FCCP = Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; ISCT = international society of cell therapy; MSC = mesenchymal stromal cell; OCR = oxygen consumption rate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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indicating less mature mitochondria.

Comparing the same cells after one week of culture in hypoxia, we found a similar phenotype as after normoxic culture. In fact, we found a decrease in oligomycin sensitive respiration, coupling efficiency as well as maximal respiration and spare respiratory capacity in the fetal cells (Fig. 1G–J). Interestingly, after hypoxic culture we found a significant difference in the ratio between OCR and ECAR between the two cell sources (Fig. 1K), suggesting a higher glycolytic flux as energy source in the adult cells when grown in hypoxia compared to the fetal cells. The lower maximal respiratory capacity and coupling efficiency of the fetal cells grown in either normoxia or hypoxia could indicate a difference in mitochondrial number, morphology or network compared to adult cells. However, in line with the similarities of the fetal and the adult MSCs in basal respiration, we did not find any significant difference in mitochondrial morphology (Fig. 1L-M) and numbers (Fig. 1N). Although, we see clustering of data within the respiratory experiments that indicates a donor-variability, which is commonly seen in MSC-cultures, the differences in metabolic function seem to be more related to the developmental stage of the MSCs and the oxygen tension in the environment. Thus, the culturing conditions of MSCs might have more impact on the therapeutic outcome than the donor-variability.

To better understand the metabolic adaptation to culture conditions in each cell type, we compared the metabolic changes between the adult and the fetal cells when cultured in either normoxia or hypoxia and we found an increase in coupling efficiency in both cell types after culture in hypoxia ($p<0.05$) (Fig. 2A). This was probably due to the necessity to enhance the ATP production with lower oxygen levels or due to the scarcity of cellular ATP, which might increase proton flux and decrease membrane potential. Moreover, we found an unexpected increase in the spare respiratory capacity in the fetal cells when grown in hypoxia ($p<0.01$) (Fig. 2B). A more efficient ETC or substrate delivery might partly explain the increased spare respiratory capacity in the fetal cells. Interestingly, we found an increased basal ($p<0.01$) and maximal ($p<0.05$) ECAR in the adult cells only (Fig. 2C), as well as a lower OCR/ECAR ratio ($p<0.001$) (Fig. 2D). This indicates a significant increase in the basal glycolytic activity after hypoxic growth, reflecting an adaptation to the environment and a change in pyruvate handling. The fetal cells on other hand, did not significantly adjust the relationship between OCR and ECAR in response to oxygen tension.

To complement the data, we cultured both types of cells under normoxic and hypoxic conditions for a week and placed them into the Oroboros system measurement chamber that allows decrease in oxygen concentration from 20% to 3% while measuring respiration. By calculating the relative drop of respiration between 20% and 3%, we found no significant differences depending on culture conditions prior the assay between adult and fetal MSCs (Fig. 2E). However, after preculture in normoxia, fetal MSCs preserved a significant higher basal OCR when oxygen was lowered to 3% than adult MSCs, with a similar trend after

preculture in hypoxia (Fig. 2E). This suggests a different oxygen-dependent metabolic function between the two cell types, which is consistent with previous results acquired using the Seahorse system. Accordingly, adult MSCs increased their glycolytic flux, and consequently decreased the ratio between respiration and glycolysis after hypoxic culture.

In order to study the respiratory properties of the mitochondria isolated from the cellular metabolic regulations and if mitochondrial substrate oxidation could be affected by culture conditions, we measured complex I (CI) and II (CII) substrate-linked respiration. The cells were permeabilized during respirometry and the state 3 respiration of CI and CII was determined in relation to CI + CII respiration (Fig. 2F and G). Fetal MSCs showed slight but significantly higher CI-linked activity when cultured in normoxia in comparison to hypoxia ($p = 0.012$) while adult MSCs demonstrated an opposite trend, although with no statistical significance. The CII-linked activity was also not statistically different between the cell sources and oxygen tensions (Fig. 2F and G), even though adult MSCs in normoxia demonstrated a trend towards higher CII-linked activity. Since increased lipid oxidation is linked to CII activity [14,15], we speculate that, during normoxia, the adult MSCs downregulate glycolysis favoring fatty acid oxidation. The metabolic changes together with the absence of numeral and gross morphological changes of mitochondria, points toward a different cellular metabolic regulation during hypoxia in the fetal and adult cells rather than a change in mitochondrial content and their structural maturity.

In summary, we have shown that MSCs from two different developmental stages have different oxygen-dependent metabolism and differ in their ability to adapt their metabolisms to the environment. The fetal MSCs maintain similar respiration and aerobic glycolysis during both normoxic and hypoxic culture conditions, although with lower ATP-coupled respiration. In comparison, adult MSCs demonstrated metabolic features more similar to differentiated cells, such as high ATP-coupled respiration, higher spare respiratory capacity and capacity to modulate pyruvate conversion to lactate in determinate environmental condition compared to fetal cells. Accordingly, during normoxia adult MSCs down-regulate their glycolysis, which in turn might affect their capacity to preserve stem cell characteristics [16], while fetal MSCs instead retain aerobic glycolysis.
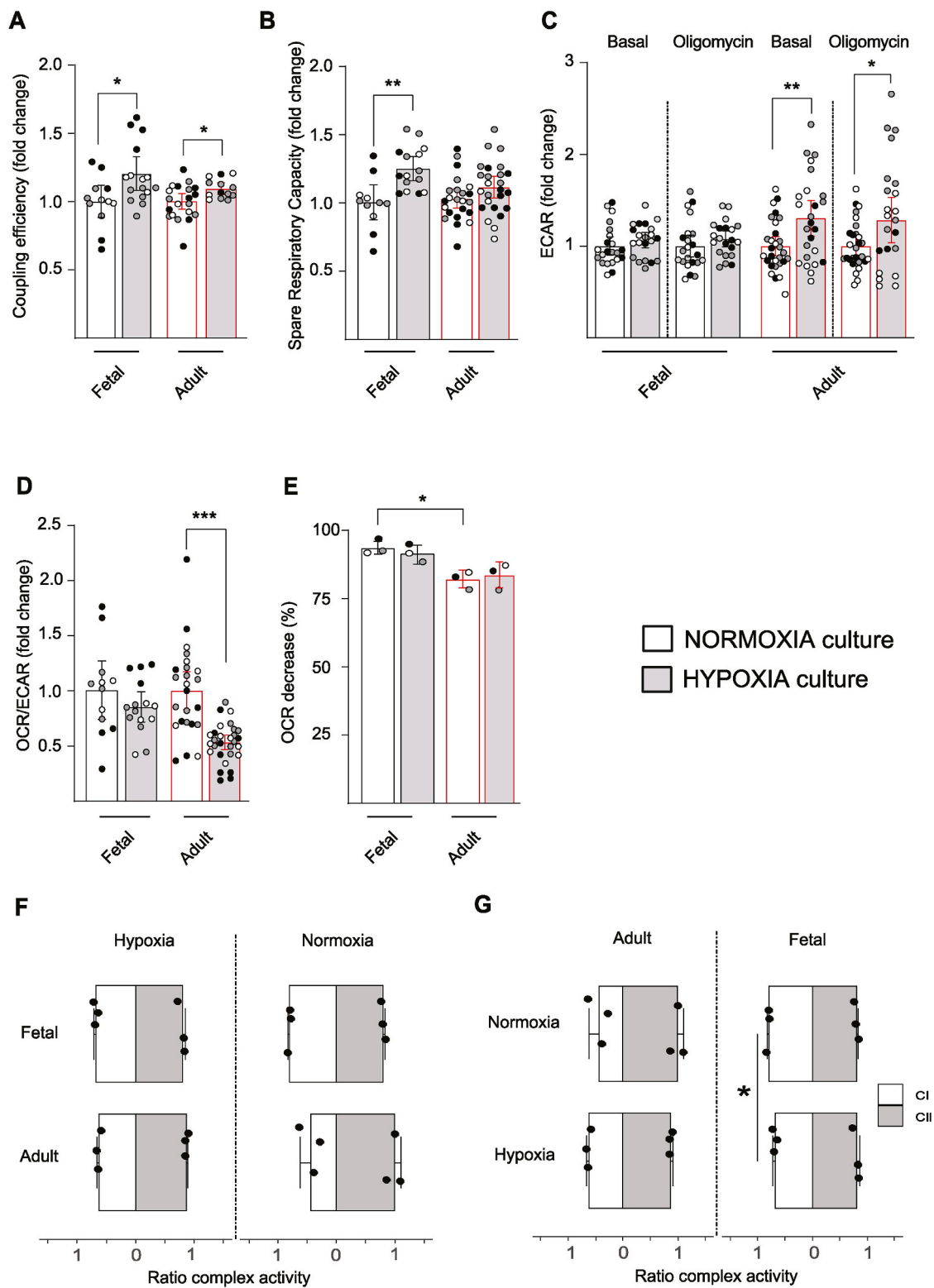
Fig. 2. Metabolic flux analysis, oxygen-sensitive respiration and complex activity. Protein normalized values in fold change differences between normoxia and hypoxia cultures of adult and fetal MSCs, using XF24 Flux Analyzer in **A)** coupling efficiency, **B)** spare respiratory capacity, **C)** ECAR at baseline and after injection of oligomycin, **D)** OCR/ECAR ratio. **E)** Cellular respiration at 3% oxygen tension expressed in percentage compared to 20% oxygen tension using the Oroboros system. **F)** Complex I and II activities in relation to their combined state (CI & CII-linked respiration). * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001. **A-D** Empty white bar = fetal MSCs; empty red bar = adult MSCs. **A-E** Bar plots where each donor represented by different color-filled circles. **F-G** Bi-directional plots where black dots are representing individual donors. **E-G** was analyzed with Welch's *t*-test or paired *t*-test when appropriate, biological repeats indicated by number of dots in bar charts; n = 3. All bar charts are expressed as mean ± SD. Abbreviations: ECAR = extracellular acidification rate; MSC = mesenchymal stromal cell; OCR = oxygen consumption rate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

CRediT authorship contribution statement

Kim Olesen: Conceptualization, Investigation, Methodology, Formal analysis, Visualization, Data curation, Writing – original draft, Writing – review & editing. **Noah Moruzzi:** Visualization, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Ivana Bulatovic:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Clifford Folmes:** Data curation, Writing – review & editing. **Ryounghoon Jeon:** Investigation. **Ulrika Felldin:** Investigation. **Andre Terzic:** Supervision. **Oscar E. Simonson:** Writing – review & editing. **Katarina Le Blanc:** Resources. **Cecilia Österholm:** Supervision, Writing – review & editing. **Per-Olof Berggren:** Funding acquisition, Writing – review & editing. **Tomas Schiffer:** Investigation, Methodology, Data curation. **Sergey Rodin:** Methodology, Writing – review & editing. **Andreas Tilevik:** Supervision, Formal analysis, Writing – review & editing. **Karl-Henrik Grinnemo:** Conceptualization, Funding acquisition, Project administration, Resources, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

Karl-Henrik Grinnemo and Sergey Rodin are co-owners of the company AVulotion AB. Per-Olof Berggren is cofounder and CEO of Biocrine AB. The other authors declare no conflict of interest.

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