Cardiosphere-derived cells demonstrate metabolic flexibility which is influenced by adhesion status

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- **SUPPLEMENTAL METHODS**
- **SUPPLEMTAL REFERENCES**

Supplemental Methods

Human MSCs and NRVM culture: Bone marrow derived human mesenchymal stem cells (MSCs) were cultured in STEMPRO® MSC SFM medium obtained from ThermoFischer. Cells were plated on gelatin coated 96 wells-Seahorse cell culture plates at a seeding density of 5000 cells/well. Cells were dissociated from ventricles of 2 day old neonatal rat pups (Sprague-Dawley rats obtained from Harlan, Indianapolis, Ind) using trypsin (US Biochemicals) and collagenase (Worthington), as previously described[1]. NRVMs were suspended in medium (M199 culture medium from ThermoFischer) with 10% fetal bovine serum and 40,000 cells were plated on fibronectin coated 96 wells-Seahorse cell culture plates.

Lactate Measurement: Since CO_2 generation by OxPhos can contribute to extracellular acidification (ECAR)[2], we also measured lactate levels directly, using the Lactate Assay Kit (Abcam-ab65331). Cells were plated as monolayers on TC treated plates, at a density of 1×10^6 cells/10cm^2, and cultured in DMEM containing 10% FBS, 2mM glutamine and glucose (25mM). Media was collected after 1 hour of oligomycin treatment. A standard curve was generated for lactate, and lactate levels (from medium) were quantified using the protocol recommended by the manufacturer.

Glucose (^{18}FDG) uptake: One day prior to the experiment, one set of CDCs was plated at a density of 1×10^5 cells/well as monolayers or 3×10^5 cells/20µL hydrogel for 24h samples[3]. Prior to labeling, cells were washed twice with PBS containing Ca^{2+} and Mg^{2+} and culture medium was changed to glucose free-DMEM containing 10% FBS for 1h. CDCs were cultured in T75 flasks for 1h and 3h experiments. In order to measure effects of PI3K-Akt inhibition on glucose uptake, 3h prior to the experiment, half the cells were treated with MK-2206 (10uM) or Ly294002 (10uM)

in IMDM medium without FBS for 1h. All cells were trypsinized and separated into three groups – suspension, adherent, and hydrogel in 500 µL of glucose free-DMEM.

For hydrogel experiments, control hydrogels were prepared without cells to quantify radionuclide trapping in hydrogels. For hydrogel synthesis, HA-NHS was dissolved in PBS containing Ca^{2+}/Mg^{2+} without addition of glucose prior to mixing with serum and lysed blood.

To measure glucose uptake, 500 µL glucose free-DMEM containing ^{18}FDG (final concentration of 74 kBq/ml) was added to each sample and control well for 1h to measure glucose uptake. Subsequently, cells/hydrogels were washed twice with cold PBS to remove any remaining free ^{18}FDG and lysed with proteinase K solution (10 mM Tris, 1 mM EDTA, 0.1% Trition X-100, and 0.1 mg/mL proteinase K). Counts were recorded in a gamma-counter (Perkin Elmer). After the gamma counting, radioactivity in the samples was allowed to decay by storing them in the -20°C freezer for 2 days. The hydrogels were manually crushed using a small piston (Sigma) and digested in proteinase K solution at 50°C for 24h. Double stranded DNA content was determined using the Quant-iT PicoGreen dsDNA Reagent and Kit (Invitrogen).

2-Photon Microscopy: Mitochondrial membrane potential and α_5 integrin-eGFP expression were examined using an Olympus FV1000 MP microscope and 20X water immersion lens. Mitochondrial membrane potential ($\Delta\Psi_m$) was monitored by labeling cells with 100nM of tetramethylrhodamine methyl ester (TMRM) for 30 minutes[4] and excitation wavelength of 800 nm; α_5 integrin-eGFP transduced cells were excited at 900 nm. Emitted light was collected by 3 photomultiplier tubes fitted with bandpass filters. 512x512 pixel images were collected at 37ºC and image analysis was performed using Image J (NIH, http://rsb.info.nih.gov/ij/) as described previously[4]. For hydrogels, a series of 512x512 pixel images of 200 µm (Z-stack) were taken at multiple regions in the hydrogels with at least 20 slices in each stack. Signal quantification was

performed by drawing a region of interest (ROI) and calculating mean fluorescence intensity which was normalized to fluorescent area.

Cell proliferation: Cell proliferation was monitored using the WST8 assay (Dojindo Laboratories). Cells were plated in 96 well plates (1000cells/well) and medium was changed every other day. Each condition had 6 wells per day, and one row was used to obtain a reading on a daily basis. Medium was changed after 24 hours of plating to DMEM (Catalog# 17-207-CV, Corning) containing glutamine (2mM) and FBS (10%); glucose (25mM), pyruvate (25mM) or (25mM) glucose+(1mM) DMOG (Dimethyloxallyl Glycine) with and without oligomycin (4uM) for 07 days. Medium was changed every other day. WST 8 reagent (10uL) was added 2h prior to reading the plate using SpectroMax2 and 450 absorbance. Subsequent rows were scanned on a daily basis to plot a proliferation curve.

Western blot: Western blots for AMPK, Akt, HIF-1α, FLAG and Cleaved Caspase Substrate Motif were performed as follows. Cells were washed twice using ice-cold PBS and lysed using RIPA buffer containing proteinase and phosphatase inhibitors. Cell lysates were clarified by centrifugation at $>10^4$ g for 10 min at 4°C. Protein estimation of cell lysate was done using the BCA assay (Pierce-Thermoscientific). Equal amounts of proteins were electrophoresed on precast Nupage 4-12% Bris-Tris gels (Invitrogen-Life technologies) and transferred to nitrocellulose membranes. The membranes were first incubated with the following primary antibodies: Akt (Cell signaling Technologies, Catalog#9272), p-Akt (Cell signaling Technologies, Catalog#9276), HIF1α (Sigma, Catalog # SAB1403916), AMPK (Cell signaling Technologies, Catalog# 2532) , p-AMPK (Cell signaling Technologies, Catalog#2535), Cleaved Caspase Substrate Motif [DE(T/S/A)D] MultiMab™ (Cell signaling Technologies, Catalog#8698) or FLAG (Sigma, #F7425). GAPDH (Cell signaling Technologies, Catalog#2118) from Cell Signaling

Technologies, was used as a loading control. Subsequently, horseradish peroxidase-conjugated secondary antibody was used to conjugate the primary antibodies and chemiluminescence solution (SuperSignal West Femto Chemiluminescent Substrate- Thermoscientific) was used to visualize target proteins. Image J (NIH) was used for semi-quantitative analysis. Phosphorylation of protein was calculated against total amount of respective protein. Results are presented as densitometry levels with respect to their relevant control. Minimum of three samples for each condition were run to permit statistical analysis; values were calculated as mean $\pm$ SD.

Cell surface GLUT1 expression: HEK293 cells were used for transient transfection of FLAG-GLUT1[5] constructs using Lipofectamine 2000 (Invitrogen). FLAG-GLUT1 is an exofacial construct which was used to detect surface levels of GLUT1. Cells were analyzed using FACscan (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Inc., Ashland, OR). Cells were kept in suspension for 1h, 3h and 6h followed by washing with Ca^{++} and Mg^{++}-free phosphate-buffered saline (PBS). Anti-Fc (BD Biosciences PharMingen, San Diego, CA) was used for blocking. Subsequently, rabbit anti-FLAG (Sigma-Aldrich) was added followed by Alexafluor-588 donkey anti-rabbit (Molecular probes-Invitrogen) to determine FLAG surface expression. The relative intensity calculated reflected mean cell fluorescence of cell surface GLUT1 levels. To normalize for variable FLAG levels, the surface expression was normalized to the ratio of FLAG/Actin as described previously[5].

Flow Cytometry for quantification of mitochondrial number: We used MitoTracker Green FM (Cat#M7514-ThermoFischer) which labels mitochondria regardless of mitochondrial membrane potential[6]. Cells were washed with PBS, harvested and incubated with 75nM MitoTracker Green at 37°C for 30 min before washing them twice with PBS and analyzing the

mean fluorescence intensity of the dye in cells, using FACscan (BD Biosciences, San Jose, CA), as previously described[6].

Flow Cytometry for viability was performed using a FACscan instrument (LSRII-Becton Dickinson, San Jose, CA, U.S.A.) and FlowJo software (Tree Star, Ashland, OR, U.S.A.) for analysis. Cell viability was assessed following cell labeling with annexin V and propidium iodide using BD Bioscience flow cytometry equipment. Annexin V was diluted at a concentration of 1 mg/ml in binding buffer and cells ($1x10^6$ cells) were suspended in 1 ml of freshly prepared solution. Cells were incubated for 10 min in the dark at room temperature in solution containing Annexin V; PI solution (0.1ml to give final conc. of 1mg/ml) was added 5 min prior to flow cytometry analysis where 10,000 events were collected per sample. Cell debris was excluded by scatter gating.

Alpha$_5$ integrin localization: CHO-K1 cells were transfected with plasmids expressing α_5 integrin-eGFP using Lipofectamine 2000. The plasmid was purchased from Addgene (Plasmid 15238) and has a Neomycin-resistance gene. Cells were selected for 5 days using 500µg/ml of G418 Neomycin to obtain >95% GFP positive cells prior to experiments. Imaging was performed using a 2-photon laser scanning fluorescence microscope.

PI3K-Akt Inhibition: In order to evaluate the effect of Akt inhibition on cell metabolism and ^{99m}Tc-pertechnectate uptake, 10µM-LY294002 (Sigma-L9908) a reversible PI3K inhibitor, 1µM-Wortmannin (Sigma-W1628) an irreversible PI3K inhibitor and 10µM-MK-2206 (Selleck Chemical) a reversible Akt inhibitor were used. Akt inhibition was confirmed by western blot for p-Akt. Prior to performing experiments using MK2206, effect of the drug on cell viability was

assessed using 2 photon microscopy. CDCs were treated for 1 hr with MK-2206 (10μM); subsequently, MK-2206 was washed by rinsing cells in PBS containing Mg^{+2}/Ca^{+2}. Cells were then labeled with calcein and propidium iodide and imaged by 2-photon microscopy. Cell viability in hydrogels after MK-2206 pre-treatment was 93±4.29% at 24h, which was similar ($p>0.45$) to controls (~95%).

RNA Isolation and RT-PCR: CDC were lysed under regular culture condition of 10% FBS in IMDM medium to obtain mRNA using AllPrep DNA/RNA minikit (Qaigen) following instructions from the kit provider. RT-PCR was done by Qaigen one-step PCR kit in Biorad iCycler thermo cycler. Primers were designed by Primer 3 and following primers were used; Glut 1 forward (ATAAAAAGGCAGCTCCGCGCG) reverse (TGGAGTTCCGCCTGCCAAAGC), Glut 4 forward (ATAAAAAGGCAGCTCCGCGCG) reverse (TGGAGTTCCGCCTGCCAAAGC)

Lentivirus synthesis: As described previously[7, 8], the cDNA encoding the hNIS gene was sub-cloned in place of eGFP into the vector RRLsin18.cPPT.CMV.eGFP.Wpre, resulting in plasmids designated cpPPT.CMV.hNIS Viral vectors were produced by Lipofectamine 2000 (Invitrogen) transfection of 4 lentiviral vector plasmids into HEK293T cells (ATCC, Manassas, VA, USA). Vector-containing supernatant was collected at 24 hours and 48 hours after transfection, filtered, and concentrated (Centricon Plus-70, Millipore, Billerica, MA, USA). Viral titer was assigned on concentrated supernatant by HIV-1 p24 ELISA (Dupont, Wilmington, DE, USA). For genetic labeling, rCDCs were transduced at a multiplicity of infection of 20 yielding transduction efficiencies of >95% for hNIS expression. NIS expression was confirmed by flow cytometry using a monoclonal mouse anti-hNIS antibody (Abcam, Cambridge, MA, USA) and by

in-vitro ^{99m}Tc-pertechnetate uptake. We have previously demonstrated that transduction of CDCs with hNIS at an MOI (multiplicity of infection) of 20 does not affect cell proliferation, using in vitro studies.[7, 9] Percentage of transduced cells was calculated by flow cytometry prior to cell transplantation.

Hydrogel preparation: Carboxyl groups in hyaluronic acid (HA, MW 16 kDa; LifeCore Biomedical) were modified to amine-reactive N-hydroxysuccinimide esters as previously described[3, 10]. Peripheral blood from Wistar Kyoto rats (Charles Rivers) was collected using heparinized and serum tubes. For serum isolation, blood was centrifuged at 1500 rpm for 10min. In order to generate lysed blood, whole blood was frozen at -20 and subsequently thawed. All blood products were stored at -20°C until further use. Animals were euthanized according to the Johns Hopkins Animal Care and Use Guidelines.

HA:Bl:Ser hydrogels were prepared by mixing 1:1 of 10 w/v% HA-NHS dissolved in PBS containing 25mM glucose, with equal volume of lysed blood:serum (1:1) derived from rats. All polymerization was carried out at pH 7-7.4 and at room temperature for 3min. We chose a 1:1 (v/v) ratio for lysed blood and serum and HA in order to maximize high adhesivity of HA-NHS, growth factor concentration and adhesion motifs from blood products. In order to ensure functionality of the NHS groups, hydrogels were synthesized within 15-20 min of dissolving HA in glucose-containing PBS.

In vitro ^{99m}Tc-pertechnectate uptake[7, 8]**:** CDCs were transduced with a 3rd generation lentivirus expressing the human sodium-iodide symporter (Lv-CMV-hNIS) at an MOI of 20. ^{99m}Tc-pertechnetate (11.1 kBq/mL) was added for 1h in cell/hydrogel culture media. Subsequently,

cells/hydrogels were washed twice with cold PBS to remove any remaining free ^{99m}Tc-pertechnetate. Cells/hydrogels were lysed with proteinase K solution (10 mM Tris, 1 mM EDTA, 0.1% Trition X-100, and 0.1 mg/mL proteinase K). Counts were recorded using a gamma-counter (Perkin Elmer). After the gamma counting, radioactivity in the samples was allowed to decay by storing them in the -20°C freezer for 2 days. The effect of perchlorate (100μM), a specific NIS blocker on ^{99m}Tc-pertechnetate uptake was measured by adding perchlorate to some wells prior to the addition of ^{99m}Tc-pertechnetate. Total dsDNA was determined using Picogreen assay as described[3].

In vivo studies:

Animal surgery

Anesthesia was induced in male Wistar Kyoto rats (200-250 g, Charles River) with 5% isoflurane flowed in with 2 L/min oxygen, and maintained with 2% isoflurane. The heart was exposed through a left lateral thoracotomy in the 4th and 5th intercostal space. One million NIS^{+} CDCs were encapsulated in hydrogels and applied epicardially. Subsequently, the chest was closed and the animals were allowed to recover prior to SPECT/CT imaging at 1 hour and 24 hours post-transplantation.

In vivo SPECT/CT imaging[3]

In order to determine the effect of cellular metabolism and Akt inhibition, 1x10^{6} NIS^{+}-CDCs encapsulated in HA:Bl:Ser hydrogels were applied epicardially to the anterior wall of the left ventricle in non-infarcted WK rats following a left sided thoracotomy. Two groups of animals were studied: Group 1 consisted of NIS^{+}-CDCs encapsulated in HA:Bl:Ser hydrogels and Group 2 consisted of NIS^{+}-CDCs pre-treated with reversible Akt inhibitor for 1h followed by washout prior to encapsulation in HA:Bl:Ser hydrogels. $^{99m}TcO_4^-$ (^{99m}Tc labeled technetium-pertechnetate;

630±75 MBq) and ^{201}TlCl (^{201}Tl labeled thallous chloride; 70±4 MBq) were injected intravenously via the tail vein immediately after cell transplantation; isoflurane was turned off and the animals were monitored for spontaneous breathing and allowed to fully recover prior to imaging. *In vivo* dual isotope SPECT imaging was performed 1h after injection of ^{99m}Tc-pertechnetate (to visualize transplanted NIS^{+}-CDCs) and ^{201}TlCl (to visualize myocardium). CT imaging was performed prior to SPECT imaging. Both scans were performed on a small animal SPECT/CT system (X-SPECT-CT from Gamma Medica Inc.) using inhalational isoflurane as the anesthetic agent, administered via a nose cone. Animals were allowed to recover in their cages after completion of imaging on d0. The same rats were re-injected with ^{99m}Tc-pertechnetate (630±75 MBq) and ^{201}TlCl (70±4 MBq) via the tail vein and *in vivo* dual isotope SPECT-CT imaging was performed on d1 post-transplantation.

SPECT/CT image acquisition and processing[3]*:* The SPECT module X-SPECT-CT system is composed of two gamma camera heads each consisting of a pixelated NaI(Tl) with a total area of 125 mm × 125 mm, divided into 80 × 80 number of pixels with 1.56 mm pitch. Low-energy knife-edge pinhole collimators were used with a pinhole aperture of 1 mm diameter and a focal length of 9 cm; a radius-of-rotation of 5.42 cm was used in the SPECT data acquisition. Each camera head acquired 128 projections over a 180-degree range, with an acquisition time of 30s for each projection for all scans. Data was acquired in listmode and subsequently rebinned into two energy windows ("75 keV +10%/−10%" and "140 keV +10%/−10%") to obtain separate sets of ^{201}Tl and ^{99m}Tc projections. The ^{99m}Tc and ^{201}Tl projection datasets were reconstructed using a 3D pinhole ordered-subset expectation-maximization (OS-EM) imaging reconstruction algorithm with 8 and 4 updates, respectively with an isotropic reconstructed image voxel size of 0.7 mm.

X-ray computed tomography (CT) was performed on the microCT module with an X-ray tube voltage of 75 kVp. A total of 512 projections were acquired over a 360-degree range. The projections with 1,184 × 1,120 isotropic pixels (100 μm) were reconstructed into a CT volume of 512^3 isotropic voxels with 170 μm pixel size. The SPECT and CT were then registered using rigid body transform, with preset parameters specific to the system[3].

SPECT image quantification[3]*:* For absolute quantification, a calibration factor (CF) was calculated from an experimental study by inserting a small hollow sphere filled with a known amount of radioactivity of ^{99m}Tc or ^{201}Tl in water in an averaged rat-size water-filled cylindrical phantom to simulate a rat scan. Dose-response plot for ^{99m}Tc-pertechnetate was obtained by dual isotope SPECT imaging of varying doses of ^{99m}Tc-pertechnetate and ^{201}Tl and then used to calculate the calibration factor. SPECT data for phantom were acquired using exactly the same acquisition settings as those used in the animal experiments. In this case, CF (MBq/i.i.) was defined as the quotient of the known activity concentration (MBq/ml) within the radioactive sphere in the phantom divided by the measured mean image intensity (i.i/cm^3) within a ROI drawn over the small sphere in the SPECT image of the phantom.

To quantify tracer uptake *in vivo*, regions-of-interest (ROI) were manually defined in a region of increased focal tracer uptake and in a contralateral normal region of a mid-myocardial section. In the case of no observable increased focal myocardial tracer accumulation, an ROI was placed on the distal anterior wall. The total radioactivity in the region of interest was calculated by the image intensity within the ROI multiplied by the CF. The radioactivity concentration (MBq/mL) within the ROI was calculated by the total activity divided by the volume of the ROI. The background activity was calculated by placing an ROI in the basolateral wall of the heart.

Supplemental References

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Online Appendix

Supplemental Figure Legends:

Figure S1: OxPhos inhibition does not induce HIF-1α stabilization: Western blot reveals absence of HIF-1α activation during aerobic glycolysis and following oligo treatment for 12h during aerobic glycolysis. Hypoxia (1% O_2, 5% CO_2, 94% N_2) and DMOG lead to HIF-1α activation /stabilization.

Figure S1

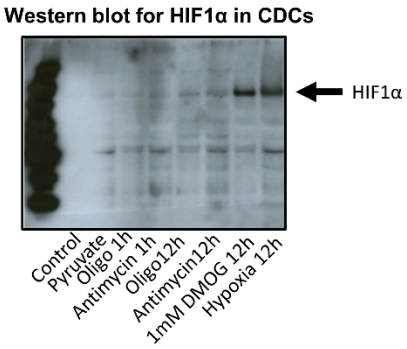

Figure S2A: Western blot to assess Akt phosphorylation following treatment with Ly294002 (10µM) and MK2206 (10µM): PI3K/Akt inhibition leads to progressive Akt de-phosphorylation over 6h.

Figure S2B: Oligo treatment after Akt inhibition leads to depolarization of $\Delta\Psi_m$ and reduction in CDC viability. Calibration bar represents 50µm

Figure S2

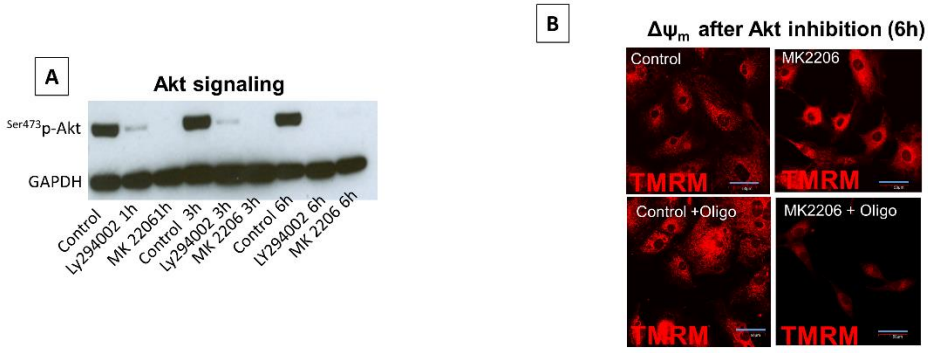

Figure S3: **GLUT1 expression and translocation**

A: **PCR**: GLUT1 is the main glucose transporter in CDCs.

B: **Normalization of total cellular FLAG-GLUT1 levels by western blot for FLAG, using beta-actin as the loading control**. PI3K-Akt inhibition +/- oligo for 6h does not affect total cellular FLAG-GLUT1 protein expression indicating that reduced cell surface expression of FLAG-GLUT1 (Fig2E) was not due to reduction of total cellular protein levels.

Figure S3

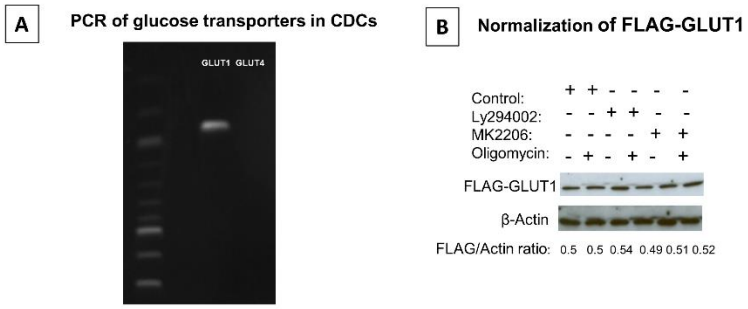

Fig S4A-B. Respirometry plots following treatment with metabolic inhibitors in MSCs demonstrate coupled respiration of <60% and high ECAR which is similar to results obtained in

ASCs and CDCs. NRVMs demonstrate >85% coupled respiration and low glycolysis (ECAR). One-way ANOVA was used to determine statistical significance. Data is presented as mean ± SEM; n=6, each experiment was repeated twice.

Fig S4C: Mitochondrial quantification using MitoTracker Green revealed that NRVMs have the highest numbers of mitochondria per cell, followed by CDCs, ASCs and MSCs. One-way ANOVA was used to determine statistical significance. Data is presented as mean ± SD; n=6.

Figure S4

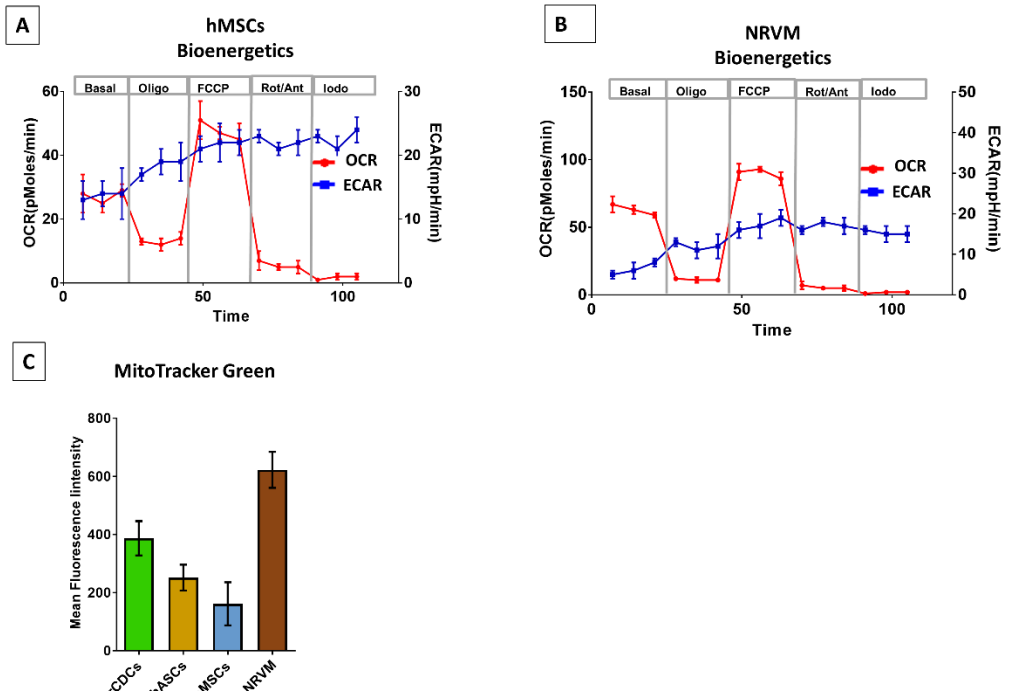

Figure S5: Cell adhesion status influences mitochondrial membrane potential. Suspension led to $\Delta\Psi_m$ depolarization and re-plating restored $\Delta\Psi_m$. Calibration bar represents 50µm.

Figure S5

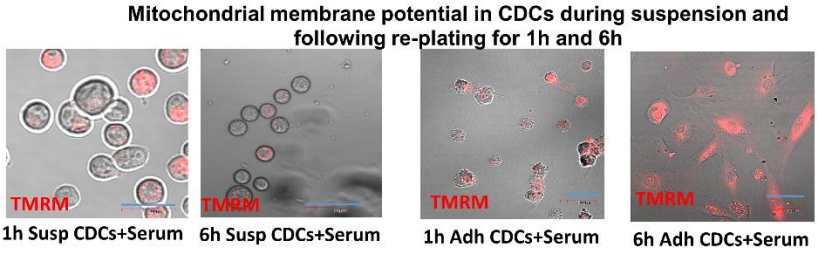

Figure S6: Akt phosphorylation in cells cultured as adherent monolayers for 24h, encapsulated in hydrogels for 1, 3, 6, 24h in the presence/absence of MK2206 treatment/washout, and suspended for 1, 2 and 3h. Encapsulation in hydrogels led to rapid increase in Akt phosphorylation. Reversible Akt inhibition by MK2206 (treatment/washout) delayed Akt activation following encapsulation. (Pretx- pretreatment).

Figure S6

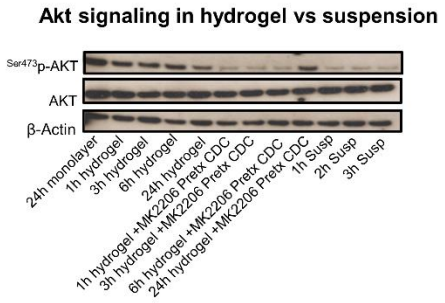

Figure S7: In vitro hydrogel results

A: CDC pretreatment with MK2206 for 1h prior to washout and encapsulation in HA:Bl:Ser hydrogels led to lower OCR and oligo-sensitive OCR when compared to control (vehicle-treated group). Metabolism was restored in MK2206-treated cells at 24h following encapsulation, resulting in similar levels of OCR in MK2206 treated/un-treated cells. One-way ANOVA was used

to determine statistical significance. Data is presented as mean ± SEM; n=6, each experiment was repeated three times.

B: Pretreatment of CDCs with MK2206 led to reversible reduction in total cellular ATP levels. Comparison was made with 1h sample using student's t-test. Data is presented as mean ± SEM; n=6, each experiment was repeated twice.

C: Acute inhibition PI3K (Ly294002, Wortmannin) or Akt (MK2206) reduced in vitro ^{99m}Tc-pertechnetate uptake in adherent NIS^{+}CDCs. Statistical comparison was performed using student's t-test. Data is presented as mean ± SD; n=6.

*p<0.05.

Figure S7