

SHORT REPORT

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# Increased oxygen consumption and OXPHOS potential in superhealer mesenchymal stem cells

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## Abstract

**Background:** Cell-based therapies show promise in repairing cardiac tissue and improving contractile performance following a myocardial infarction. Despite this, ischemia-induced death of transplanted cells remains a major hurdle to the efficacy of treatment. 'Superhealer' MRL/MpJ mesenchymal stem cells (MRL-MSCs) have been reported to exhibit increased engraftment resulting in reduced infarct size and enhanced contractile function. This study determines whether intrinsic differences in mitochondrial oxidative phosphorylation (OXPHOS) assist in explaining the enhanced cellular survival and engraftment of MRL-MSCs.

**Findings:** Compared to wild type MSCs (WT-MSCs), mitochondria from intact MRL-MSCs exhibited an increase in routine respiration and maximal electron transport capacity by 2.0- and 3.5-fold, respectively. When routine oxygen utilization is expressed as a portion of maximal cellular oxygen flux, the MRL-MSCs have a greater spare respiratory capacity. Additionally, glutamate/malate- and succinate-supported oxygen consumption in permeabilized cells was elevated approximately 1.25- and 1.4-fold in the MRL-MSCs, respectively.

**Conclusion:** The results from intact and permeabilized MSCs indicate MRL-MSCs exhibit a greater reliance on and capacity for aerobic metabolism. The greater capacity for oxidative metabolism may provide a protective effect by increasing ATP synthesis per unit substrate and prevent glycolysis-mediated acidosis and subsequent cell death upon transplantation into the glucose- and oxygen-deprived environment of the infarcted heart.

**Keywords:** Energetics, Mitochondria, Oxidative phosphorylation, Stem cells

## Findings

### Background

Advances in stem cell therapy for treating a myocardial infarction (MI) are impeded by inadequate survival and engraftment of implanted cells in the host tissue. Up to 99% of mesenchymal stem cells (MSCs) experience cell death following administration into the infarcted heart [1,2]. A predominant factor for the poor survival is ischemia [3,4]. Ischemia results in ATP depletion, a reduction in mitochondrial oxidative phosphorylation (OXPHOS) and increased reliance on glycolysis leading to cellular acidosis and cell death [4].

The bone marrow-derived 'Superhealer' MRL/MpJ MSC (MRL-MSC) has been reported to exhibit greater engraftment in the infarcted heart [5]. This improved retention promotes superior cardio-protection as indicated by increased angiogenesis, reduced infarct size and improved contractility in a murine MI model [5]. The primary contributor of MRL-MSC-mediated wound repair and cardiac contractile improvements was identified as the paracrine/autocrine factor, secreted frizzled related receptor protein 2 (sFRP2) [5,6]. sFRP2 is a member of the sFRP family that exhibits a cysteine-rich domain that binds Wnt glycoproteins [7]. sFRP binding diminishes Wnt interactions with frizzled receptors, which propagate apoptosis through the canonical  $\beta$ -catenin pathway [8]. In addition to its involvement in apoptosis, Wnt/ $\beta$ -catenin signaling promotes a switch in glucose metabolism from OXPHOS to glycolysis [9].

Given the infarcted region of the heart receives impaired oxygen and nutrient supply, the current study

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evaluates MSC OXPHOS to determine whether innate differences in the MRL-MSC oxygen utilization and mitochondrial energetics could explain their enhanced viability.

## Methods

### MSCs and cardiac myocytes

Procedures were approved by the University of Calgary and Vanderbilt University Animal Care and Use Committees. Murine WT- and MRL-MSCs were generated and expanded from C57BL6 and MRL/MpJ strains, respectively, as described [5,10]. Briefly, WT- and MRL-MSCs were cultured in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C in low glucose Dulbecco's Modified Eagle Medium (DMEM; GIBCO) containing 1000 mg/L glucose, 110 mg/L sodium pyruvate, 10% defined fetal bovine serum (FBS; HyClone), 10% penicillin-streptomycin, 10% fungizone and 10 µg/L platelet-derived growth factor-ββ (PDGF-ββ; R&D Systems). The immunophenotype of MSCs were CD44<sup>+</sup>, LY6A/E/Sca1<sup>+</sup>, CD45<sup>-</sup>, CD14<sup>-</sup>, CD11b<sup>-</sup>, CD16/32<sup>-</sup>, CD144<sup>-</sup> and CD146<sup>-</sup> as previously reported [5]. MSC capacity for trilineage differentiation was confirmed as previously described [5]. Passage 4 through 14 were used for all measurements. Primary cardiac myocytes from 10wk old, male, CD-1 mice were isolated using a collagenase digestion method as previously described [11].

### High-resolution respirometry

Cellular respiration was measured using the Oxygraph-2k (Oroboros Instruments) at 37°C. Experiments evaluating intact cell respiration were performed with MSCs suspended in culture medium consisting of low glucose Dulbecco's Modified Eagle Medium (DMEM; GIBCO) containing 1000 mg/L glucose and 110 mg/L sodium pyruvate. Experiments evaluating permeabilized cell oxygen consumption were performed with MSCs suspended in a mitochondrial respiration medium (MiR05) containing 0.5 mM EGTA, 3 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 20 mM taurine, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM HEPES, 1 g/L BSA, 60 mM potassium-lactobionate, 110 mM sucrose, pH 7.1 [12].

A phosphorylation control protocol was performed to assess intact cell respiration [13]. Without substrate addition, routine respiration ( $R_{DMEM}$ ) was determined. Oligomycin (2 µg/ml) evaluated futile oxygen flux (LEAK) [14]. Maximal capacity of the electron transfer system (ETS) was evaluated via the step-wise (0.5 µM steps) titration of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). All respiratory states were corrected for residual/non-mitochondrial oxygen consumption (0.5 µM rotenone and 2.5 µM antimycin A).

Digitonin-permeabilized cell analysis was employed to assess complexes I and II. Optimal digitonin concentrations required to permeabilize MSCs were determined

as previously outlined [15]. Cardiac myocytes were permeabilized with digitonin as indicated by Liu et al. (1991) [16]. Routine respiration ( $R_{MiR05}$ ) was identified in MiR05. State 2 respiration ( $V_{CI}$ ) was assessed by the addition of complex I substrates glutamate (10 mM) and malate (2 mM). ADP (5 mM) induced state 3 respiration/maximal oxygen consumption ( $V_{MAX-CI}$ ). A subsequent titration of succinate (10 mM) allowed for evaluation of state 3 respiration by convergent electron input. Convergent electron input is defined as electrons being passed through a series of oxido-reduction reactions from both complex I and II to the Q-junction which is followed by the flow of electrons through complex III, cytochrome *c* and complex IV [17]. Inhibition of complex I with 0.5 µM rotenone revealed succinate-supported respiration ( $V_{MAX-CII}$ ). The acceptor control ratio (ACR) was defined as  $V_{MAX-CI}$  relative to  $V_{CI}$ . A titration of 10 µM cytochrome *c* evaluated intactness of the outer mitochondrial membrane and provided quality control for the digitonin-permeabilized cell preparations.

### Nutrient deprivation and ischemic culture conditions

WT-and MRL-MSCs were plated at a density of 3000 cells/cm<sup>2</sup> on 58 cm<sup>2</sup> dishes (Nunc) for two days under the previously described conditions. The cells were washed with Dulbecco's phosphate buffered saline (GIBCO) and randomly segregated into three groups: control, glucose-deprived and ischemic. Control cells were cultured in a humidified atmosphere of 5% CO<sub>2</sub> and 37°C in low glucose DMEM (GIBCO) containing 1000 mg/L glucose, 110 mg/L sodium pyruvate, 10% defined fetal bovine serum (FBS; HyClone), 10% penicillin-streptomycin, 10% fungizone and 10 µg/ml platelet-derived growth factor-ββ (PDGF-ββ; R&D Systems). Glucose-deprived cells were cultured at 5% CO<sub>2</sub> and 37°C in no glucose DMEM (GIBCO) supplemented with 10 mg/L glucose, 1.1 mg/L sodium pyruvate, 10% penicillin-streptomycin, 10% fungizone creating conditions of 1% glucose and pyruvate compared to the control group. Ischemic conditions were identical to the glucose-deprived MSCs with the addition of 12 ml of sterile mineral oil (Sigma) creating an oil layer of approximately 5 mm over the cells to induce an oxygen-deprived environment. The WT-and MRL-MSCs remained under these conditions for 48 hr prior to further analysis. The length of culture conditions was chosen to provide the most relevance to cell-based therapy for the infarcted heart. Intramyocardial injection of stem cells into the acutely infarcted heart has been reported to result in approximately 40-50% retention (of total transplanted cells) 1-2 hours following transplantation [4,18,19]. The initial attrition of cells within two hours of delivery via direct intramyocardial injection is largely the consequence of cell leak from the injection site, being "washed away" by local bleeding resulting from the injection and/or being

expelled by myocardium contraction [19]. Of the cells remaining in the cardiac tissue, further cell death/loss occurs in the first 48 hr (1-25% of total transplanted cells are retained) with cell death stabilizing by seven days following delivery [4,18,19].

### Flow cytometry

Flow cytometry (Guava easyCyte 6HT 2 L; Millipore) was employed to measure the level of apoptosis induced by simulated glucose deprivation and ischemia. FlowCelect MitoLive Kit (Millipore) was performed according to the manufacturer's instructions to detect three cellular states: Healthy, viable cells (intact mitochondrial membrane potential and intracellular esterase activity), early apoptosis (impaired mitochondrial membrane potential) and late apoptotic/cell death events (impaired mitochondrial membrane potential and intracellular esterase activity). Mitosense red with an excitation/emission wavelength of 640/650 nm is employed by the FlowCelect MitoLive Kit (Millipore) to evaluate mitochondrial membrane potential. Calcein, acetoxymethylester (AM) is cell permeant non-and non-fluorescent. This compound is hydrolyzed by intracellular esterases to a fluorescent anion calcein with an

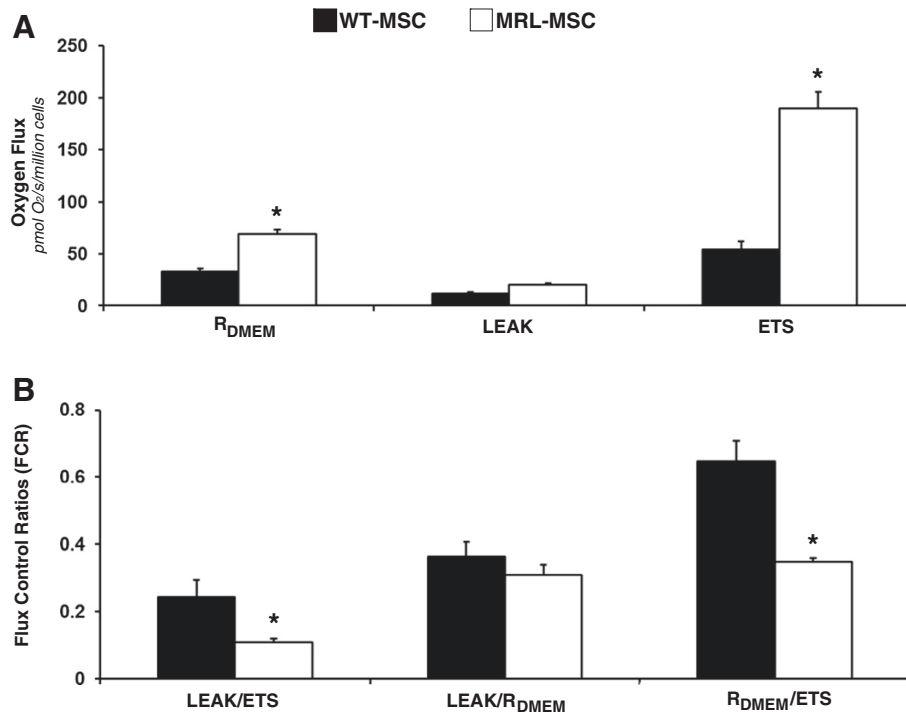
excitation/emission wavelength of 488/525 nm which is utilized by the FlowCelect MitoLive Kit (Millipore) to assess cellular vitality.

### Statistical analyses

Two-way repeated measures ANOVAs were utilized to assess statistical differences in high-resolution respirometry experiments ( $p < 0.05$ ). One-way ANOVAs were performed for all other experimental measures. Differences within the ANOVAs were determined using Tukey's post hoc. Data are reported as means  $\pm$  SEM.

### Results

The initial incubation in culture medium allowed for determination of  $R_{\text{DMEM}}$ .  $R_{\text{DMEM}}$  in WT-MSCs was  $33.1 \pm 2.5$  pmol  $\text{O}_2/\text{s}/10^6$  cells (Figure 1A). MRL-MSC  $R_{\text{DMEM}}$  was elevated at  $69.4 \pm 3.9$  pmol  $\text{O}_2/\text{s}/10^6$  cells (Figure 1A). Oligomycin-induced LEAK respiration allowed for determination of non-ADP phosphorylating oxygen utilization. LEAK respiration was similar between WT-MSCs compared to the MRL-MSCs,  $11.7 \pm 1.2$  vs.  $20.0 \pm 1.6$  pmol  $\text{O}_2/\text{s}/10^6$  cells (Figure 1A). Maximal capacity of the electron transport system (ETS)

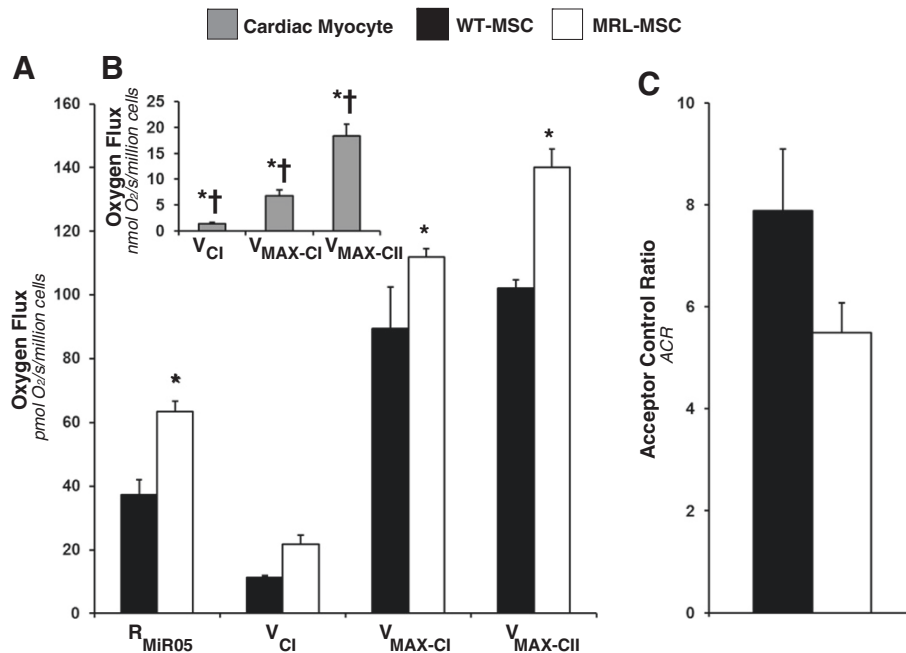


**Figure 1 Oxygen flux in intact wild-type mesenchymal stem cells (WT-MSC) and MRL/MpJ mesenchymal stem cells (MRL-MSC). (A)**  $R_{\text{DMEM}}$ , LEAK and ETS oxygen consumption in intact WT-MSC and MRL-MSC.  $R_{\text{DMEM}}$  represents routine respiration, LEAK represents oxygen consumption independent of ADP phosphorylation and ETS indicates maximal electron transport capacity. **(B)** Flux control ratios (FCR). Ratios of oxygen flux in differing respiratory control states, normalized to a defined reference respiratory state.  $n=6$ , data are mean  $\pm$  S.E.M. \* $p < 0.05$  vs. WT-MSC.

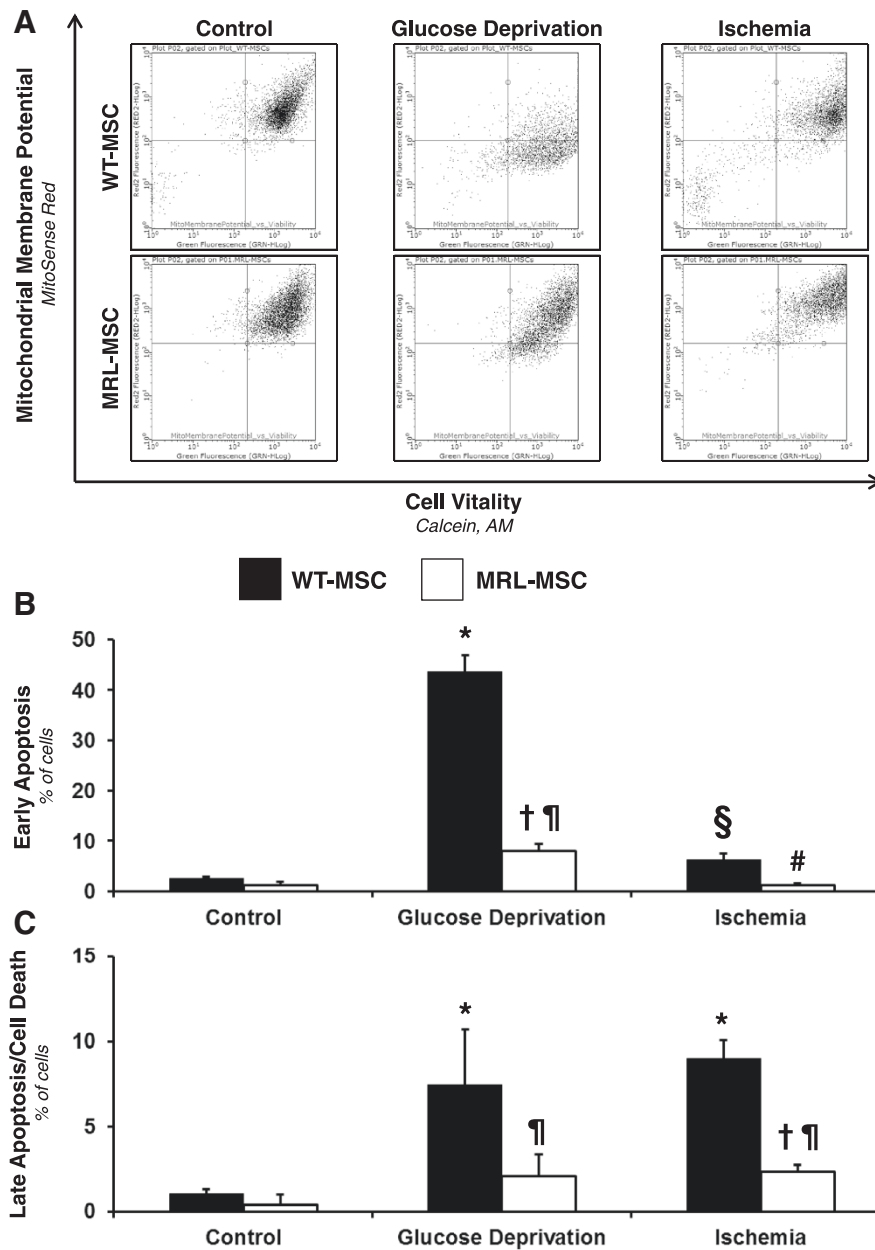
differed between cell types. In intact WT-MSCs, the ETS of  $54.4 \pm 7.4$  pmol  $O_2/s/10^6$  cells was lower than the MRL-MSCs ETS of  $190.1 \pm 15.3$  pmol  $O_2/s/10^6$  cells (Figure 1A). These results indicate that absolute oxygen flux in the routine respiratory state and maximal respiratory rate are elevated in the MRL-MSCs.

LEAK respiration operates at  $0.25 \pm 0.05$  of ETS in the WT-MSCs compared to  $0.11 \pm 0.01$  of ETS in MRL-MSCs (Figure 1B). This indicates a higher portion of maximal electron transport system capacity is used for futile reactions in WT-MSCs. The LEAK/ $R_{DMEM}$  ratio was not altered between cell types. LEAK oxygen flux was  $0.36 \pm 0.04$  of WT-MSC  $R_{DMEM}$  compared to  $0.31 \pm 0.02$  of MRL-MSC  $R_{DMEM}$  (Figure 1B).  $R_{DMEM}$  functions at  $0.65 \pm 0.06$  of ETS in WT-MSCs which was significantly higher than the  $R_{DMEM}/ETS$  ratio of  $0.35 \pm 0.01$  exhibited by MRL-MSCs (Figure 1B). The lower  $R_{DMEM}/ETS$  ratio in the MRL-MSCs indicates that the 'Superhealer' cell type display a greater potential electron transport capacity in excess of routine requirements.

Oxygen consumption in permeabilized cells was evaluated to obtain a greater understanding of the OXPHOS function. Suspension of intact MSCs in MiR05 did not change routine respiration.  $R_{MiR05}$  remained significantly lower in the WT-MSCs,  $37.3 \pm 4.7$  vs.  $63.5 \pm 3.3$  pmol  $O_2/s/10^6$  cells (Figure 2A). State 2 respiration ( $V_{CI}$ ), was similar between WT- and MRL-MSCs. WT-MSC state 2 respiration was  $11.4 \pm 0.6$  pmol  $O_2/s/10^6$  cells compared to the MRL-MSC respiration of  $21.8 \pm 2.7$  pmol  $O_2/s/10^6$  cells (Figure 2A). Following the addition of ADP, state 3 ( $V_{MAX-CI}$ ) was lower in the WT-MSCs compared to the MRL-MSCs,  $90.0 \pm 13.0$  vs.  $112.0 \pm 2.7$  pmol  $O_2/s/10^6$  cells (Figure 2A). In addition, state 3 in WT-MSCs supported by succinate alone ( $V_{MAX-CII}$ ) was  $102.0 \pm 2.8$  pmol  $O_2/s/10^6$  cells. This was significantly lower than the  $V_{MAX-CII}$  exhibited by MRL-MSCs,  $140.1 \pm 5.7$  pmol  $O_2/s/10^6$  cells (Figure 2A). As a comparison, isolated cardiac myocyte oxygen flux was higher than that of both WT- and MRL-MSCs. Isolated, cardiac myocyte  $V_{CI}$ ,  $V_{MAX-CI}$  and  $V_{MAX-CII}$  were  $1.44 \pm 0.24$ ,  $6.84 \pm 1.08$  and  $18.33 \pm 2.18$  nmol  $O_2/s/10^6$  cells, respectively (Inset, Figure 2B). Additionally,



**Figure 2 Oxygen flux in permeabilized wild-type mesenchymal stem cells (WT-MSC) and MRL/MpJ mesenchymal stem cells (MRL-MSC).** (A) Routine respiration in MiR05 ( $R_{MiR05}$ ) in intact MSCs. State 2 oxygen consumption through Complex I supported by glutamate and malate ( $V_{CI}$ ) in digitoin-permeabilized MSCs. State 3 or maximal oxygen consumption through Complex I supported by glutamate and malate ( $V_{MAX-CI}$ ) in digitoin-permeabilized MSCs. State 3 or maximal oxygen consumption through Complex II supported by succinate ( $V_{MAX-CII}$ ) in digitoin-permeabilized MSCs. (B) Given MSCs are transplanted into the infarcted heart, isolated, primary cardiac myocyte oxygen utilization is provided as a metabolic reference state. Cardiac myocyte state 2 oxygen consumption through Complex I supported by glutamate and malate ( $V_{CI}$ ) in digitoin-permeabilized cardiac myocytes. State 3 or maximal oxygen consumption through Complex I supported by glutamate and malate ( $V_{MAX-CI}$ ) in digitoin-permeabilized cardiac myocytes. State 3 or maximal oxygen consumption through Complex II supported by succinate ( $V_{MAX-CII}$ ) in digitoin-permeabilized cardiac myocytes. (C) Acceptor control ratio (ACR; defined as  $V_{MAX-CI}/V_{CI}$ ). n = 6-9, data are mean  $\pm$  S.E.M. \*p < 0.05 vs. WT-MSC. †p < 0.05 vs. MRL-MSC.



**Figure 3** Effects of 48-hour glucose deprivation and ischemia on wild-type mesenchymal stem cell (WT-MSC) and MRL/MpJ mesenchymal stem cell (MRL-MSC) apoptosis. **(A)** Representative fluorescence-activated cell sorting analysis of apoptotic cells after mitosense red and calcein, AM staining. Healthy, viable MSCs are mitosense red<sup>+</sup>/calcein, AM<sup>+</sup>. The mitosense red<sup>+</sup>/calcein, AM<sup>+</sup> MSCs are in the early apoptotic phase, whereas the mitosense red<sup>+</sup>/calcein, AM<sup>+</sup> MSCs are in the late apoptotic/cell death phase. **(B)** Quantitation of MSC early apoptosis induced by 48 hr glucose deprivation and ischemia with mitosense red and calcein, AM staining. The graph shows the percentage of all MSCs that are mitosense red<sup>+</sup>/calcein, AM<sup>+</sup> MSCs after glucose deprivation and ischemia. **(C)** Quantitation of MSC late apoptosis/cell death induced by 48 hr glucose deprivation and ischemia with mitosense red and calcein, AM staining. The graph shows the percentage of all MSCs that are mitosense red<sup>+</sup>/calcein, AM<sup>+</sup> MSCs after glucose deprivation and ischemia. n = 6-7 independent experiments, data are mean ± S.E.M. \*p < 0.05 vs. WT-MSC Control. †p < 0.05 vs. MRL-MSC Control. ¶p < 0.05 vs. WT-MSC of identical experimental conditions. §p < 0.05 vs. WT-MSC Glucose Deprivation. #p < 0.05 vs. MRL-MSC Glucose Deprivation.

the ACR did not differ between WT- and MRL-MSCs (Figure 2C).

Manipulation of culture conditions to simulate 48 hr glucose deprivation exhibit a clear induction of early apoptosis defined by mitochondrial membrane potential impairment (Figure 3A & B). WT-MSCs under glucose deprivation-only conditions for 48 hr resulted in a drastic increase in early apoptosis ( $43.61 \pm 7.59\%$  vs.  $2.57 \pm 0.69\%$ ; Figure 3B). The effect of glucose deprivation on MRL-MSCs also resulted in an increased percentage of cells undergoing early apoptosis ( $8.02 \pm 1.37\%$  vs.  $1.16 \pm 0.69\%$ ; Figure 3B). However, the proportion of mitochondrial membrane impaired MRL-MSCs was significantly lower than that of WT-MSCs following glucose deprivation ( $8.02 \pm 1.37\%$  vs.  $43.61 \pm 7.59\%$ ; Figure 3B). Interestingly, ischemic culture conditions did not promote significant increases in early apoptotic WT-MSCs or MRL-MSCs compared to control MSCs (Figure 3B). Furthermore, the reduced oxygen availability may have provided a mitochondrial protective effect in the presence of glucose deprivation as the WT-MSCs ( $43.61 \pm 7.59\%$  vs.  $6.29 \pm 2.55\%$ ; Figure 3B) and MRL-MSCs ( $8.02 \pm 1.37\%$  vs.  $1.11 \pm 0.47\%$ ; Figure 3B) exhibited elevated early apoptosis in the glucose-deprived condition compared to the ischemic condition.

Simulated 48 hr glucose deprivation and ischemia *in vitro* resulted in late apoptosis/cell death defined by impaired mitochondrial membrane potential and cellular activity (Figure 3A & C). WT-MSC exposure to reduced glucose for 48 hr showed an increase in late apoptosis ( $7.47 \pm 1.28\%$  vs.  $1.04 \pm 0.69\%$ ; Figure 3C). Glucose deprivation did not evoke alterations in the percentage of MRL-MSC undergoing late apoptosis ( $2.08 \pm 0.18\%$  vs.  $0.38 \pm 0.21\%$ ; Figure 3C). The combination of decreased oxygen and substrate availability showed an increase in WT-MSCs ( $9.01 \pm 2.19\%$  vs.  $1.04 \pm 0.69\%$ ; Figure 3C) and MRL-MSCs ( $2.33 \pm 0.87\%$  vs.  $0.38 \pm 0.21\%$ ; Figure 3C) late apoptosis/cell death compared to control conditions (Figure 3B). However, the proportion of non-viable MRL-MSCs was significantly lower than that of WT-MSCs following 48 hr ischemia ( $2.33 \pm 0.87\%$  vs.  $9.01 \pm 2.19\%$ ; Figure 3B). In summary, the aforementioned experiments evaluated WT-and MRL-MSCs early and late apoptosis following 48 hr of depressed substrate availability or ischemia *in vitro*. Our results suggest MRL-MSC exhibited a lower rate of apoptosis compared to WT-MSCs under nutrient deprivation. Such characteristics may be transferable to the *in vivo* conditions of the infarcted heart allowing for greater MRL-MSC survival and a subsequent increase in their therapeutic effects.

## Discussion

Upon transplantation, MRL-MSCs exhibit increased engraftment and survival in the infarcted heart compared

to WT-MSCs [5]. We aimed to evaluate innate differences in mitochondrial function between these cell types to explain differences in cell viability. Novel findings identify unique energetic characteristics of MRL-MSCs that would be protective in glucose-compromised and ischemic environments.

Assessment of oxygen utilization in the intact cell allowed for the determination of physiological respiratory states. MRL-MSCs displayed similar LEAK respiration, however, routine respiration and maximal electron transport capacity were greater. In order to eliminate the potential confounding influence of mitochondrial density, flux control ratios (FCR) were evaluated. Results revealed a lower LEAK and routine respiration as a proportion of maximal electron transport capacity in MRL-MSCs. This suggests the presence of higher electron transport capacity in MRL-MSCs. To identify regulatory factors responsible for the increased OXPHOS potential, two major sites of electron input into the electron transport system (complex I and complex II) were examined. In the presence of ADP, oxygen flux through complex I was higher in the MRL-MSCs. Because complex I is not the only site of electron input into the electron transport system, succinate-supported, maximal respiration was determined. This complex II-mediated oxygen flux was higher in the MRL-MSCs and indicates a greater aerobic energy production in the MRL-MSCs. Given the MSCs are transplanted into the heart, we also assessed the oxygen consumption of primary cardiac myocytes to provide a metabolic reference state to which MSC oxygen utilization could be compared. Relative to the WT-MSCs, cardiac myocyte  $V_{CI}$ ,  $V_{MAX-CI}$  and  $V_{MAX-CII}$  were 127-, 77- and 180-fold greater, respectively. This indicates the oxidative capacity of MSCs is relatively minor in comparison to that of cardiac myocytes and the MRL-MSC respiration is only modestly higher than WT-MSCs. The mechanism responsible for the increased intact cell oxidative function and permeabilized cell complex I and II activity in the MRL-MSCs is currently unknown. Given the role of sFRP2 in modulating apoptosis and glucose metabolism, future experiments evaluating the effect of sFRP2 on mitochondrial OXPHOS in the WT-and MRL-MSCs are notable future endeavours.

Several reports conclude WT-MSCs preferentially derive energy from glycolysis with little dependence on glucose oxidation [20-22]. We report MRL-MSCs exhibit elevated absolute OXPHOS function ( $R_{DMEM/MiR05}$ ,  $V_{MAX-CI}$ ,  $V_{MAX-CII}$  and ETS) and higher OXPHOS potential ( $R_{DMEM/ETS}$ ). Based on these observations we propose the slight elevation in OXPHOS displayed by the 'Superhealer' MRL-MSCs is advantageous. For instance, in MSCs, there is a shift towards OXPHOS dependence under glucose deprivation [23]. The increase in oxidative function is thought to be an adaptive

attempt to maximize ATP synthesis in the nutrient shortage given OXPHOS produces approximately 32 moles of ATP per mole of glucose [24]. This is much greater than the 2 moles of ATP resulting from glycolysis [24]. To evaluate this hypothesis we cultured WT- and MRL-MSCs under glucose-deprived conditions for 48 hr where oxygen was not limiting. We found that WT- and MRL-MSCs displayed a reduction in mitochondrial membrane potential; an early indicator of apoptosis [25]. However, WT-MSCs exhibited a significantly higher proportion of cells with mitochondrial dysfunction compared to MRL-MSCs. Additionally, the percentage of late apoptotic cells was also higher in WT-MSCs compared to the MRL-MSCs following the reduced substrate conditions. The elevated routine oxygen flux and electron transport capacity in MRL-MSCs may provide benefits when faced with a lack of substrate by better accommodating the switch towards oxidative metabolism. This could assist in preservation of ATP levels and prevent energetic starvation. Furthermore, a potential for increased coupling of glycolysis to glucose oxidation in MRL-MSCs could minimize intracellular acid load and improve cell viability in the infarcted heart.

Recently, reports indicate that substrate deprivation is much more detrimental to MSC survival than hypoxia [20,22]. To expand on our glucose deprivation studies we assessed MSC apoptosis following 48 hr of ischemia (glucose and oxygen deprivation). Under the ischemia conditions WT- and MRL-MSCs showed an elevated proportion of late apoptotic/dead cells. However, there was no added effect of depressed oxygen availability on late apoptosis/cell death over reduced glucose availability. These findings are consistent with the reports that substrate availability and not hypoxia is more influential in MSC viability. Also, the percentage of late apoptotic cells was elevated in the WT-MSCs compared to the MRL-MSCs which are in agreement with studies indicating MRL-MSCs exhibit increased engraftment and survival in the infarcted heart [5].

Interestingly, ischemia had an unexpected influence on impaired mitochondrial membrane potential/early apoptosis. The number of early apoptotic WT- and MRL-MSCs was significantly lower following 48 hr of combined substrate and oxygen reduction compared to early apoptotic cells under decreased glucose conditions only. The mechanism for this finding is currently unknown. It is possible that the oxygen deprivation results in a compensatory reduction in MSC oxidative metabolism thereby exhibiting a protective effect on the MSCs by finding a unique compromise between OXPHOS and glycolysis. This speculative compromise may provide a more optimal balance between ATP synthesis, glycolysis-mediated acidosis from the lack of oxygen and reactive oxygen species-induced damage resulting from OXPHOS

under non-limiting oxygen concentrations. Alternatively, it cannot be ignored that ischemia may primarily induce late apoptotic events independent of early mitochondrial dysfunction. Of note, a limitation of this study is the use of mineral oil to induce hypoxia without measuring oxygen concentration in the culture medium. Future studies should accurately measure oxygen concentrations to identify the effect of various oxygen levels in combination with substrate deprivation on mitochondrial-induced apoptosis.

In conclusion, little attention has been given to the optimal metabolic characteristics that would allow transplanted cells to cope with oxygen and glucose deprivation. We found MRL-MSCs display elevated OXPHOS that may minimize metabolic stress and subsequently improve cell viability upon transplantation into the infarcted heart.

#### Abbreviations

ACR: Acceptor control ratio; DMEM: Dulbecco's modified eagle's medium; ETS: Electron transport system; FCCP: Cyanide-p-trifluoromethoxyphenylhydraz-one; 48 hr: Forty-eight hours; LEAK: Futile oxygen consumption; FCR: Flux control ratio; MI: Myocardial infarction; MiR05: Mitochondrial respiration medium; MSC: Mesenchymal stem cell; MRL-MS-C: MRL-MPJ mesenchymal stem cell; OXPHOS: Oxidative phosphorylation;  $R_{\text{DMEM}}$ : Routine respiration in dulbecco's modified eagle's medium;  $R_{\text{MiR05}}$ : Routine respiration in mitochondrial respiration medium; sFRP2: secreted frizzled related receptor protein 2;  $V_{\text{C1}}$ : State 2 respiration supported by complex I substrates;  $V_{\text{MAX-C1}}$ : State 3 respiration supported by complex I substrates;  $V_{\text{MAX-CII}}$ : State 3 respiration supported by complex II substrates; WT-MS-C: Wild type mesenchymal stem cell; 10 wk: Ten weeks.

#### Competing interests

The authors declare they have no competing interests.

#### Authors' contributions

All authors participated in experimental design. MA and PY isolated, cultured and differentiated the MSCs. DB isolated the murine cardiac myocytes. CH cultured the MSCs and performed the experimental assays. CH analyzed and interpreted the data and drafted the manuscript. All authors edited and approved the final manuscript.

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