Enhanced Tissue Production through Redox Control in Stem Cell-Laden Hydrogels

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Cellular bioenergetics and redox (reduction–oxidation) play an important role in cell proliferation and differentiation, key aspects of building new tissues. In the present study, we examined the metabolic characteristics of human adipose-derived stem cells (hASCs) during proliferation and differentiation in both monolayer and three-dimensional biomaterial scaffolds. In monolayer, hASCs exhibited higher glycolysis and lower ox-phos as compared to both adipogenic and osteogenic differentiated cells, and hASCs demonstrated the Warburg effect (aerobic glycolysis). However, reactive oxygen species (ROS) levels increased during adipogenic differentiation, but decreased during osteogenic differentiation. Similarly, a decrease in ROS levels along with a higher mitochondrial membrane potential and viability was observed in hASCs encapsulated in poly(ethylene glycol) (PEG) hydrogels containing an adhesion peptide (RGD), compared to PEG hydrogels with a scrambled control peptide (GRD), demonstrating that adhesion-dependent signaling can also regulate ROS production and bioenergetics. As a result, we hypothesized that we could modulate osteogenesis in PEG hydrogels containing the adhesion peptide (RGD) by further reducing ROS levels using a small therapeutic molecule, L-carnitine, a metabolite with purported antioxidant effects. We observed reduced ROS levels, no effect on mitochondrial membrane potential, and increased osteogenic differentiation and tissue production in cells in the presence of L-carnitine. These results suggest the potential to manipulate tissue production by modulating cellular metabolism.

Introduction

THE FIELDS OF TISSUE ENGINEERING and regenerative medicine aim to restore tissue form and function lost to disease, trauma, or congenital abnormalities.¹ In general, tissue engineering employs biomaterial scaffolds that are combined with cells and biological signals designed to support cell proliferation, differentiation and, ultimately, new tissue growth. Researchers in tissue engineering focus heavily on new biomaterials development, stem cell research, and biological cues.² However, a critical area that rarely has been considered in tissue-engineering research is cell metabolism, including bioenergetics and cellular redox (reduction–oxidation) state³ (Fig. 1). In the early 1950s, Warburg proposed that cancer cells rely primarily on aerobic glycolysis instead of oxidative phosphorylation, a phenomenon now called “the Warburg effect.”^{3,4} This has been extensively studied in the context of metabolic aberrations in cancer cells, but its role in stem cells has recently surfaced.^{5–9} Understanding the role of metabolism in stem cell differen-

tiation, cell–biomaterial interactions and, ultimately, new tissue development will be key to designing strategies for improving tissue engineering and repair.

There are a number of modalities in which, metabolism may impact tissue engineering. Proliferating cells metabolize the majority of their glucose to lactate, favoring aerobic glycolysis over oxidative phosphorylation (oxphos), while differentiated cells use oxphos almost exclusively.⁴ Mitochondrial respiration has been associated with reactive oxygen species (ROS) production,¹⁰ which affects cell signaling and regulation of immune response, apoptosis, and a number of transduction cascades.¹¹ Recent studies indicate that ROS also influences stem cell differentiation. For example, in one study, ROS generation was found to be a causal factor in promoting human mesenchymal stem cell (hMSC) differentiation into adipocytes,¹² while others reported that ROS inhibited the osteogenic differentiation of hMSCs.¹³ In the present study, human adipose-derived stem cells (ASCs), whose metabolism has not been characterized, were evaluated for metabolic characteristics during

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FIG. 1. Basic components of cellular metabolism and methods used to investigate their changes during tissue development. Color images available online at www.liebertpub.com/tea

differentiation and tissue production in both monolayer and three-dimensional (3D) culture.^{12–14}

Hydrogels provide a useful platform for the 3D culture of cells to provide an environment more representative of that found *in vivo*, compared to a 2D culture system.¹⁵ Poly(ethylene glycol) (PEG) hydrogels have been used for cell culture and tissue engineering with a number of different cell types.^{16,17} These hydrogels are considered nonadhesive to cells and proteins, but have been modified with the tripeptide sequence, arginine–glycine–aspartic acid (RGD), to provide adhesion sites for cells.¹⁸ The adhesion peptide RGD has been shown to influence a number of cell processes, such as proliferation, migration, differentiation, and tissue production.¹⁹ In particular, incorporating RGD into PEG hydrogels significantly improved bone production by MSCs.²⁰ Although RGD peptide incorporation in PEG hydrogels is known to improve viability in adhesion-dependent stem cells,²¹ its role in stem cell metabolism with possible implication on differentiation has not been elucidated.

Bioenergetics and cellular redox state can affect tissue engineering through modulation of cell phenotypes and biological cues that regulate tissue development. Here we examined the metabolic characteristics of ASCs in an undifferentiated state and during osteogenic and adipogenic differentiation to identify a unique bioenergetics/redox signature. We then explored the metabolic effects of incorporating RGD in a 3D hydrogel environment with undifferentiated ASCs, and, finally, modulated osteogenic tissue production using an antioxidant, small metabolite, L-carnitine.

Materials and Methods

Cell culture

Expansion of ASCs. ASCs were isolated, as described by Mitchell *et al.*,²² and were received via a material transfer agreement. The cells were passaged by trypsin (0.025%; Gibco®, Life Technologies) digestion and plated at a density of 5000 cells/cm² in the ASC medium composed of the high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen™, Life Technologies), 10% Hyclone Defined fetal bovine serum (FBS; Thermo Fisher Scientific), 100 units/mL penicillin and 100 mg/mL streptomycin (Invitrogen™), and

1 ng/mL basic fibroblast growth factor (bFGF; PeproTech). The medium was changed every 2–3 days. Passage 4–6 cells were used for all experiments.

Adipogenesis. ASCs were plated at 5000 cells/cm²; after 24 h, the ASC medium was removed and replaced with the adipogenic medium, which was composed of the high-glucose DMEM (Invitrogen™), 10% Hyclone Defined FBS (Thermo Fisher), 100 units/mL penicillin and 100 mg/mL streptomycin (Invitrogen™), 1 μM dexamethasone (Sigma-Aldrich), 100 μM indomethacin, 500 μM isobutylmethylxanthine (IBMX), and 10 μg/mL bovine insulin (Sigma-Aldrich). The medium was changed every 2–3 days.

Osteogenesis. ASCs were plated at 5000 cells/cm²; after 24 h, the ASC medium was removed and replaced with the osteogenic medium, which was composed of the high-glucose DMEM (Invitrogen™), 10% Hyclone Defined FBS (Thermo Fisher), 100 units/mL penicillin and 100 mg/mL streptomycin (Invitrogen™), 100 nM dexamethasone (Sigma-Aldrich), 50 μM ascorbic acid-2-phosphate (Sigma-Aldrich), and 10 mM β-glycerophosphate (Sigma-Aldrich). The medium was changed every 2–3 days.

Carnitine. L-carnitine was provided by Sigma-Tau. Either 32 mM or no L-carnitine was added with media changes.

Measurements of bioenergetics (*O*₂ consumption and extracellular acidification rates)

A Seahorse Bioscience XF96 instrument was used to measure the rate of change of dissolved O₂ in each well (termed as the oxygen consumption rate [OCR]) and change in pH in the media, termed as the extracellular acidification rate (ECAR), which is also a determinant of glycolysis. The O₂ concentration and pH were measured three times for each condition, with the measurement time of 5 min followed by 2 min of mixing time. The respiratory rates were measured (*n* = 3) as basal rates (without any compound), after injection of port A (containing chemical compound), and after port B injection (containing another chemical compound). The respiratory rates were calculated as mean ± SD and measurements were repeated at least three times, with final representation of data as mean and standard error of the mean (SEM).

For bioenergetics measurements, 10,000 cells were plated in a 96-well XF cell culture plate (Seahorse Bioscience) in respective 100 μL cell culture media for 24 h. Cells were then washed twice with specialized media—DMEM (Seahorse Bioscience Company) and 150 μL of this specialized DMEM containing the same media constituents as respective cell culture media (except buffers) were added to each well for 30 min. The cell culture plate was kept at 37°C in a non-CO₂ incubator before commencing the bioenergetics assay. The compounds injected into each well were prepared in stock solutions and dissolved in the assay media just before the experiment. Twenty-five μL of the specialized media—DMEM (containing respective compounds)—were injected in each port of the sensor cartridge (Seahorse XF-96). Oligomycin (2 μM final well concentration; O4867 Sigma-Aldrich) was injected through Port A (to monitor oligomycin-sensitive respiration), followed by injection of iodoacetate

through Port B (600 μ M final well concentration; I2512 Sigma-Aldrich, which inhibits GPDH) to inhibit ECAR. Oxidative phosphorylation was calculated by measuring oligomycin-sensitive respiration, while ECAR inhibition with iodoacetate was used to calculate glycolysis.

The bioenergetics data were normalized to the cell number by first fixing the cells with 4% paraformaldehyde immediately after the end of the Seahorse XF Analyzer run, and then labeling the cells in the XF 96-well plate with Hoechst (33258-Molecular Probes, Invitrogen™) for 10 min. Each well was then washed with phosphate-buffered saline (PBS) twice before imaging with a Zeiss Axio Observer fluorescent microscope (Carl Zeiss Microscopy) with a 4 $\times$ lens. The cell number in each well was quantified using the particle counter in ImageJ (U.S. National Institutes of Health, Bethesda, MD) to count each cell nucleus from the captured images.

Peptide conjugation to PEG

The cell-binding peptide YRGDS (Biomatik) or YGRDS (scrambled peptide control for no cell adhesion; Biomatik) was reacted with acryloyl-PEG-N-hydroxysuccinimide (acryloyl-PEG-NHS, 3500 Da; [JenKem Technology]) in a 1:2.5 molar ratio in 50 mM TRIS buffer (pH 8.2) in the dark for 2 h at room temperature. Excessive acryloyl-PEG-NHS was used to assure full reaction of the peptide. The product was dialyzed with a 1000-Da molecular weight cutoff membrane (Spectrum Laboratories), lyophilized, and stored at -20°C .

Hydrogel encapsulation and cultivation

Polymer was prepared by mixing 3.4 kDa poly(ethylene glycol)-diacrylate (PEGDA; Sunbio) and 2.5 mM YRGDS-PEG-acrylate or 2.5 mM YGRDS-PEG-acrylate (PEG control for no cell adhesion) in sterile PBS (Invitrogen™), along with 0.05% (w/v) photoinitiator, Irgacure D2959 (Ciba Specialty Chemicals), all thoroughly mixed to make a 15% (w/v) hydrogel. ASCs were homogeneously suspended in the hydrogel solution to make a concentration of 20 million cells/mL. Hydrogels for two-photon microscopy were made with 25 μ L of the cell-polymer mixture loaded into cylindrical molds (6 mm in diameter) and exposed to UV light (365 nm, 3 mW/cm²) for 5 min to achieve gelation. Hydrogels for biochemical assays were made with 100 μ L of the cell-polymer mixture and polymerized, as described above. The hydrogels were cultured in respective media at 37°C in 5% CO₂.

Microscopy

Two-photon microscopy was used to monitor the cellular redox state and ROS using an Olympus FV1000 MP with a 20 $\times$ water immersion lens (Olympus America). Each image was collected at a 10 μ m/s scan speed and a 512 $\times$ 512 pixels optical section was used for analysis by ImageJ. Z-stacks were taken for cell-laden hydrogels with a slice size of 2 μ m, and a 3D reconstruction of the image was done with ImageJ. For signal quantification, a Z projection of each image was produced with the region of interest drawn around each cell, and mean fluorescence intensity was calculated (in single-cell analysis). Mean and standard deviation were calculated from each image and the final data are represented as mean $\pm$ SEM, as each study was repeated at

least twice and three samples ($n=3$) were taken for each sample in each study.

Cells were labeled with dyes in cell culture media at 37°C for 30 min and washed with PBS (with Ca and Mg; Invitrogen™) before imaging. A special chamber/plastic mold was created for cell-laden constructs and the top of the chamber/plastic mold was covered with a glass cover slip before using the water immersion lens. ROS were measured after labeling cells with 5 μ M DCF (chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate-CM-H₂DCFDA-C6827; Molecular Probes, Invitrogen™) and 5 μ M of MitoSOX Red (M36008-Molecular Probes, Invitrogen™). MitoSOX Red allows detection of mitochondrial superoxide, while DCF permits detection of intracellular hydrogen peroxide. $\Delta\psi_m$ (mitochondrial membrane potential) was monitored with 100 nM of monovalent cationic fluorescent dye tetramethylrhodamine, methyl ester (TMRM-T-668; Molecular Probes, Invitrogen™).

Biochemical assays

Hydrogel samples were harvested for calcium and DNA assays. The DNA assay was performed as previously described.²³ For quantitative calcium measurement, lyophilized hydrogel samples were homogenized in 0.5 M HCl and vigorously vortexed for 16 h at 4°C. The supernatant was collected for the calcium assay following the manufacturer's protocol (QuantiChrom™). Wet weights and dry weights were obtained from all constructs for normalization of extracellular matrix contents after 48 h of lyophilization.

Histology

At the end of 3 weeks, hydrogels were fixed overnight in 4% paraformaldehyde at 4°C and transferred to 70% ethanol until being embedded in paraffin, according to standard histological techniques. Sections were stained with Alizarin Red for calcium deposition and mineralization.

Statistical analysis

Data were analyzed with either a one-way ANOVA followed by a Tukey *post hoc* test (for three groups) or an unpaired, two-tailed Student's *t*-test (for two groups). Statistical significance was determined and represented with asterisks: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Results

Bioenergetic characteristics of ASC differentiation in monolayer

The bioenergetic state of stem cells is correlated with differentiation.^{5,13} For instance, hMSCs shift from glycolytic metabolism to mitochondrial oxphos with differentiation.¹³ Here, we investigated the changes in the bioenergetics that occur with differentiation of human ASCs into osteogenic and adipogenic lineages (Fig. 2A–C). Osteogenesis and adipogenesis were confirmed with alkaline phosphatase staining and Oil Red O staining, respectively. The OCR and ECAR, determined using a Seahorse XF-96 analyzer, changed as cells transitioned from a growth to a differentiation phenotype. We observed a threefold increase in oxphos with osteogenesis and approximately a twofold increase during

FIG. 2. Change in bioenergetics with stem cell differentiation. Cell morphology after 1 week in culture conditions for (A) growth (undifferentiated ASCs), (B) osteogenesis (alkaline phosphatase staining), and (C) adipogenesis (Oil Red O staining). (D) Oxidative phosphorylation increased with stem cell differentiation, (E) while glycolysis decreased. Results are mean $\pm$ SEM ($n=3$). Data were analyzed with a one-way ANOVA followed by the Tukey *post hoc* comparison test. Statistical significance was determined and represented with asterisks: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Scale bars: 100 μ M. ASCs, adipose-derived stem cells; GM, growth media; ECAR, extracellular acidification rate; OCR, oxygen consumption rate; SEM, standard error of the mean. Color images available online at www.liebertpub.com/tea

adipogenesis, as compared to growth conditions (Fig. 2D). Glycolysis rates, determined by inhibiting ECAR with iodoacetate (inhibits GPDH), also changed with the ASC differentiation state. Glycolytic metabolism decreased after osteogenic and adipogenic differentiation compared to ASCs in growth conditions before differentiation (Fig. 2E).

Change in cellular redox state during adipogenesis and osteogenesis in monolayer

The metabolic states of the ASCs in growth and differentiation conditions were further characterized by evaluating the $\Delta\Psi_m$ (mitochondrial membrane potential) and ROS using two-photon microscopy. Mitochondrial membrane potential ($\Delta\Psi_m$) forms a component of proton motive force (along with a pH gradient), which is required for ATP synthesis and is also indicative of a mitochondrial role in bioenergetics.²⁴ Mitochondrial membrane potential, assessed using the cationic dye TMRM, revealed a higher fluorescence intensity in osteogenic and adipogenic cells, as compared to undifferentiated ASCs (Fig. 3A). The significant increase in $\Delta\Psi_m$ with stem cell differentiation correlates with the increased oxidative phosphorylation previously shown in Figure 2A.

ROS and intracellular hydrogen peroxide levels, measured after labeling cells with MitoSox and DCF, respectively, differed depending on differentiation toward an osteogenic or adipogenic lineage. Mitochondrial superoxide levels decreased $\sim 50\%$ with osteogenesis, while they significantly increased twofold with adipogenesis, when compared to undifferentiated ASCs (Fig. 3B). Intracellular hydrogen per-

oxide followed a similar trend, with decreased levels found with osteogenesis and significant increases observed with adipogenesis (Fig. 3C). These results show that although the bioenergetics of osteogenesis and adipogenesis are similar, the redox states of the cells in these two lineages is very different (Fig. 3D).

Cell-biomaterial interactions influence cell metabolic state

Moving to a 3D environment better mimics the native tissue environment and is required for engineering bulk tissues. Previous reports have demonstrated that incorporating an adhesive peptide sequence, such as RGD into a PEG scaffold, enhances hMSC viability.²¹ Studies suggest that RGD provides cues and interactions that regulate proliferation and differentiation; however, the mechanism is not clearly understood.¹⁹ We evaluated the influence of the adhesion peptide and increased cell-material interactions on cellular metabolism. Using two-photon microscopy, we examined the metabolic characteristics of ASCs encapsulated in a hydrogel with and without RGD (Fig. 4A). After 5 days of culture in PEG-RGD scaffolds, DNA (cell number) was significantly greater when compared to PEG scaffolds without the adhesion peptide (Fig. 4B). Furthermore, the mitochondrial membrane potential ($\Delta\Psi_m$) was significantly higher in RGD-modified gels (Fig. 4C). To further characterize how RGD influences cell-biomaterial interactions and cell signaling, the level of ROS was evaluated.¹¹ The presence of RGD adhesion peptides in the hydrogel significantly

FIG. 3. Differential cellular redox state during osteogenesis and adipogenesis. Mean fluorescence intensity per cell from two-photon microscopy of live cells in monolayer to assess: **(A)** $\Delta\psi_m$ (TMRM), **(B)** superoxide produced by mitochondrial (MitoSox Red), and **(C)** intracellular hydrogen peroxide (DCF) at 1 week. **(D)** Schematic showing differential redox profile of osteogenesis and adipogenesis. Results are mean $\pm$ SEM ($n=3$). Data were analyzed with a one-way ANOVA followed by the Tukey post-comparison test. Statistical significance was determined and represented with asterisks: *** $p < 0.001$. Scale bars: 20 μ M. ROS, reactive oxygen species; TMRM, tetramethylrhodamine, methyl ester. Color images available online at www.liebertpub.com/tea

decreased both mitochondrial superoxide and intracellular hydrogen peroxide levels, particularly during the times immediately after cell encapsulation (Fig. 4D, E). Incorporating RGD into the scaffold not only influenced cell viability (DNA content), but also multiple metabolic parameters that play a role in cell signaling. Interestingly, undifferentiated ASCs in RGD-modified gels do not proliferate after 5 days of culture, as the percent of total DNA to initial DNA is still 100% (± 5). However, cells in the scrambled (GRD) control gels undergo apoptosis, as DNA goes down to 82% (± 2), suggesting that the presence of the adhesion motif RGD acts as a prosurvival signal (Fig. 4B).

Small metabolite (L-carnitine) modulates redox state and tissue production

ROS levels decreased with osteogenic differentiation, as well as when ASCs were encapsulated in RGD-modified PEG hydrogels. To further decrease ROS levels and potentially increase osteogenesis, ASCs were cultured with a small metabolite, L-carnitine, which exhibits antioxidant properties.²⁵

After 3 weeks of culture, the L-carnitine-treated hydrogel constructs increased bone tissue production, as characterized by Alizarin staining, DNA content, and calcium deposition (Fig. 5A, B). While L-carnitine did not affect $\Delta\psi_m$ (Fig. 5C), it did reduce ROS levels significantly. Mitochondrial superoxide levels decreased 31% with L-carnitine treatment, accompanied by an approximately twofold decrease in intracellular hydrogen peroxide (Fig. 5D, E). These findings suggest that L-carnitine and PEG-RGD hydrogels can modulate redox status and increase tissue production.

Discussion

Tissue engineers are continually searching for strategies to improve tissue growth and repair. Here our goal was to identify a unique metabolic/redox signature in differentiating ASCs that can be employed for enhanced tissue production. Although biomaterials, cell type, and biological signals (including growth and differentiation factors) have been shown to determine progenitor cell survival, differentiation, and tissue production along multiple lineages,^{26,27}

FIG. 4. Scaffold influence on stem cell metabolism. **(A)** Schematic of cells encapsulated in the PEG-RGD hydrogel. The red pendant groups represent RGD motifs. **(B)** The DNA content was higher in PEG-RGD hydrogels ($N=3$, mean $\pm$ SD). Mean fluorescence intensity per cell from two-photon microscopy of live cells in PEG hydrogels with RGD (cell adhesion peptide) covalently incorporated versus PEG hydrogels with a scrambled peptide (GRD for no adhesion) covalently incorporated to assess **(C)** $\Delta\psi_m$ (TMRM), **(D)** superoxide produced by mitochondrial (MitoSox Red), and **(E)** intracellular hydrogen peroxide (DCF). Two-photon microscopy results are mean $\pm$ SEM ($n=3$). Data were analyzed with a one-way ANOVA followed by the Tukey *post hoc* comparison test. Statistical significance was determined and represented with asterisks: *** $p < 0.001$, * $p < 0.05$. Scale bars: 50 μ M. PEG, poly(ethylene glycol). Color images available online at www.liebertpub.com/tea

FIG. 5. Carnitine's effect on stem cell metabolism and tissue production. **(A)** Increased Alizarin Red staining of osteogenesis in PEG-RGD hydrogel corresponds to increased mineralization and calcium deposition at 3 weeks. **(B)** Applying carnitine to ASC osteogenic differentiation in a PEG-RGD scaffold increased viability and calcium/dry weight at 3 weeks ($N=3$, mean $\pm$ SD). Mean fluorescence intensity per cell from two-photon microscopy of carnitine and osteogenesis in PEG-RGD hydrogels to assess **(C)** $\Delta\psi_m$ (TMRM), **(D)** superoxide produced by mitochondrial (MitoSox Red), and **(E)** intracellular hydrogen peroxide (DCF) at 1 week. Two-photon microscopy results are mean $\pm$ SEM ($n=3$). Data were analyzed with an unpaired, two-tailed Student's *t*-test. Statistical significance was determined and represented with asterisks: *** $p < 0.001$, * $p < 0.05$. Scale bars: **(A)**, 100 μ M; **(C–E)**, 50 μ M. Color images available online at www.liebertpub.com/tea

cellular metabolism has never been investigated to manipulate tissue production. For our study, we used ASCs, which display similar differentiation potential as bone marrow-derived stromal/stem cells.^{28,29} However, ASCs have a wider applicability because of the ease of retrieval in large numbers,²⁹ suitability for long-term culture and genetic stability in culture,^{30,31} and reduction in graft-versus-host immune reaction via an immunosuppression role,^{32,33} making them ideal for cell transplantation studies. We showed that

osteogenesis has a similar bioenergetic profile to that of adipogenesis, as both lineages show higher oxidative phosphorylation as compared to undifferentiated ASCs; however, their redox states are different. ROS levels during osteogenesis are significantly lower as compared to undifferentiated ASCs or ASCs undergoing adipogenesis, suggesting the biological importance of lower ROS levels during osteogenesis. Using a metabolic substrate, L-carnitine, an antioxidant has been previously demonstrated to decrease ROS levels in

cells,^{25,34} we saw enhanced tissue production during osteogenesis in a 3D scaffold, possibly because ROS levels are kept low. Although addition of L-carnitine decreased the cellular ROS levels, it did not change the mitochondrial membrane potential ($\Delta\Psi_m$), suggesting that it has no effect on mitochondrial respiration and bioenergetics.²⁴ This suggests that modulating the cellular redox state using a metabolic substrate enhances tissue production and provides a new understanding through which, redox manipulation can be used to increase mineralization during osteogenesis. Cellular ROS levels have been measured in the context of oxidative damage in pathologies,³⁵ but they also mediate a range of biological processes and can have a direct influence on tissue production pathways, which should be explored further.

Biomaterial scaffolds seeded with progenitor cells provide a strategy to repair and regenerate damaged tissue,^{36,37} but investigation of metabolic regulation of these cells in scaffolds is often ignored. Cells in 3D scaffolds behave differently than when in 2D and offer closer insight into the *in vivo* behavior of cells,¹⁵ as extracellular cues in 3D scaffolds can mimic the variable rigidity and adhesion sites for cells.³⁸ Synthetic biomaterials provide better alternatives to the natural biomaterials used to study biological processes, as the chemical composition, mechanical properties, and degradation rates of synthetic biomaterials can be defined in a reproducible manner.³⁹ Stem cells are adhesion dependent, and in the absence of cell-binding sites, they undergo apoptosis, a process known as anoikis,⁴⁰ which has also been described in PEG-based 3D scaffolds.^{41,42} We employed a PEG-based, defined synthetic hydrogel system with adhesion binding sites RGD, with a scrambled peptide (GRD) as a control, to evaluate changes in mitochondrial membrane potential (as an indicator of mitochondrial bioenergetics²⁴) and ROS with adhesion in undifferentiated ASCs. We have identified that with the nonadhesion scaffold (PEG-GRD), mitochondrial membrane potential is decreased, along with an eventual decrease in viability around day 5 (Fig. 4). A decrease in mitochondrial membrane potential is known to induce cell death with subsequent release of proapoptotic factors and activation of cell death pathways.^{43–45} Interestingly, viability was not reduced initially when cellular H_2O_2 levels were also high (in the PEG-GRD scaffold), suggesting a possible role of high H_2O_2 in ASC survival, as later on viability decreased along with H_2O_2 levels. The persistent increase in superoxide levels, but decreasing H_2O_2 levels [which is a biologically active and stable form⁴⁶ as compared to superoxide radicals (Fig. 4)] could explain higher detoxification of H_2O_2 from cells, but still increasing superoxide production. In cancer cells, higher H_2O_2 levels following cell detachment have also been linked to anoikis resistance, possibly through activation of prosurvival signaling cascades.^{47,48} It will be interesting to investigate whether the decrease in viability of undifferentiated ASCs is linked solely to declining mitochondrial membrane potential with subsequent bioenergetics stress and apoptosis, or to the loss of the H_2O_2 burst seen initially with ASC detachment.

The adhesion peptide sequence RGD has been used in hydrogel systems to promote cell adhesion and has been shown to influence other processes, such as proliferation, migration, and differentiation.⁴⁹ Kloxin *et al.*⁵⁰ observed a fourfold increase in glycosaminoglycan (GAG) production after removal of RGD from the encapsulated cell environment of hMSCs undergoing

chondrogenesis, while Hsiong *et al.*²⁰ found that the presentation of adhesion ligands regulates osteoprogenitor cell differentiation. As demonstrated in these examples, it appears that RGD has a differential effect on stem cell differentiation and tissue production, as the presence of RGD seems to enhance osteogenesis yet decrease chondrogenesis and GAG production. The mechanism explaining this differential effect of adhesion-mediated signaling on cell differentiation pathways is not completely understood. We have also previously seen higher osteogenesis with incorporation of the RGD-peptide into a PEG scaffold,⁵¹ and our current study suggests that PEG-RGD scaffolds keep lower redox levels, higher membrane potential, and higher viability in adhesion-dependent cell types (such as ASCs) during osteogenesis, while chondrogenesis, which requires an adhesion-independent scaffold⁵² depends on higher ROS levels.⁵³ These data suggest an influence of scaffold/biomaterial composition on ROS generation and, subsequently, on optimal differentiation of stem cells. Although matrix rigidity has been linked to stem cell survival and differentiation,⁵⁴ our study highlights that the biomaterial composition itself⁵¹ and ROS levels can influence progenitor cell differentiation and tissue production, which will be interesting to investigate further.

Although stem cells have been shown to demonstrate aerobic glycolysis (Warburg effect),^{6–9} we have shown that ASCs (Fig. 2), like other progenitor cells, show aerobic glycolysis, and with differentiation (Fig. 2), there is an increase in ox-phos and a decrease in glycolysis. Aerobic glycolysis is essential in proliferating cells as glycolysis not only provides ATP, but also macromolecules for cellular biosynthetic processes (pentose phosphate shunt).⁴ It has been suggested that aerobic glycolysis maintains lower ROS levels by decreasing mitochondrial respiration, as mitochondria are the biggest source of ROS generation in cells⁵⁵ and higher ROS levels during cell proliferation can lead to oxidative stress and DNA damage.^{56–58} However, our data suggest that although undifferentiated ASCs have higher glycolysis and lower ox-phos as compared to during osteogenesis and adipogenesis, cellular ROS levels are higher in adipogenesis and lower in osteogenesis (Fig. 3). This suggests a disconnect in mitochondrial respiration and ROS generation, highlighting the possible role of antioxidant enzymes during stem cell differentiation. This underscores the need that cellular bioenergetics and ROS levels are investigated separately to understand their implications in stem cell differentiation and tissue production, as adipogenesis requires higher ROS levels¹² and our data suggest that lowering the cellular ROS levels improves osteogenesis.

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Disclosure Statement

No competing financial interests exist.

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