Endogenous Estrogen-Mimetic Compounds in Cell Culture Media Influence Human Mesenchymal Stromal Cell (hMSC) Processes and Differentiation in a Sex-Biased Manner

John C Bradford[1], Jennifer L Robinson[2,3]
[1]Bioegnineering Department, University of Washington, [2]Mechanical Engineering Department, University of Washington, [3]Orthopaedic Surgery and Sports Medicine Department, University of Washington

Abstract

Estrogens are global steroids that control cellular signaling pathways and impact cellular processes, phenotype, and homeostasis. Traditional cell culture media contains multiple sources of exogenous estrogens with known estrogenic effects yet how these exogenous estrogenic mimics are biasing *in vitro* studies, particularly when considering sex as a biological variable, is unknown. This is particularly of note in human mesenchymal stromal cells (hMSCs) where perturbations to their culture conditions are known to impact differentiation which is critical for cell therapy and tissue engineering considerations. Currently, the effect of exogenous estrogens from cell culture media on male and female hMSCs isn't considered, with most clinical trials including some form of exogenous estrogens in during culture and processing. Further, a baseline understanding of estrogen mimetic media influences on hMSCs is critical for adequate study design to specifically determine the impact of sex hormones on cell behavior. To this end, we investigated the effects of phenol red and unfiltered fetal bovine serum on the proliferation, metabolism, senescence, and differentiation capacity of male and female hMSCs. Phenol red significantly impacted proliferation, differentiation, and gene expression. Unfiltered FBS significantly impacted metabolism, senescence-associated staining, differentiation, and gene expression. Interestingly, treating with estrogen at the levels found in FBS did not recover most of the hMSC metrics tested, suggesting that estrogen signaling is not the only process impacted. Donor sex influenced significant differences in the response to proliferation, senescence associated staining, differentiation, and gene expression. Our work highlights sex-biased effects of estrogen-mimetic compounds in traditional culture media, highlighting a current gap in considering sex as a biological variable in cell therapy and tissue engineering products. The impact of exogenous estrogens must be considered in study design due to significant impacts on key indicators for hMSC cell processes, stemness and differentiation capacity.

Introduction

Men and women have varying exposure levels to estrogens across the lifespan (Yu et al., 2022). Beyond the exposure to estrogens, male and female tissues have differing responses to similar concentrations of estrogens (Vrtačnik et al., 2014). Estradiol is one of the main mechanisms through which male/female categorization is made, however, in reality estradiol exposure (current and previous) is just one variable that distinguish males and females, and not always a reliable one (Greenblatt et al., 1976). There are numerous

effects that estradiol has on the human body beyond what is well known, estradiol is known to impact almost every tissue including reproductive, nervous, musculoskeletal, and immune systems. Estradiol is produced in and affects both male and female cells, with the majority of estradiol signaling in men and post-menopausal women coming from local tissue production (Chakraborty et al., 2023; Knewtson et al., 2022; Vrtačnik et al., 2014). In these tissues estradiol has multiple mechanisms of action, including canonical genomic action, DNA methylation and epigenetic regulation, and non-genomic membrane-initiated steroid signaling (MISS). These actions are both nascent, direct genomic/MISS, and long-term, epigenetic regulation. Because of this, care must be taken when analyzing human data to consider both the recent levels of estradiol exposure, and the historical levels of estradiol exposure to fully account for differences.

Due to women's historical exclusion from research, the NIH implemented the sex as a biological variable (SABV) policy to increase the inclusion of female samples in research. This has been a strong start, with increasingly more papers citing sex as a biological variable as having an impact in their research, however the methodology is often flawed (Knewtson et al., 2022; Pape et al., 2024; Rich-Edwards & Maney, 2023). Despite this increase, the actual number of studies utilizing sufficient numbers of each sex, or not taking actions that obfuscate sex such as pooling cells is rather low (Knewtson et al., 2022). It is critical to understand the impact of exogenous hormones on both male and female cells and their presence may mask or exacerbate sex based differences and unintentionally bias studies. Male and female baseline hormonal exposure is different, and exposure to the same levels of estradiol may have varying impacts (Knewtson et al., 2022). Exposure of male and female cells to different levels of estradiol has distinct and decided impacts (Knewtson et al., 2022). In order to truly understand and study differences in the human condition and indeed work more closely towards personalized medicine, we must respect sex as the bimodal distribution (not binary) that it is. To this end, the ability to isolate these variables to the extent that is possible is critical, especially with many of the differences in biological sex being difficult to control such as the extent of X chromosome inactivation, or epigenetic impacts. E2 concentrations and their effects on cells need to be carefully considered when designing a study as the same E2 concentration may have different effects on male and female cells and controlling for that difference will induce different off target effects, limiting the efficacy of studies. This study represents a small portion of the work that needs to be done to be able to begin to understand the effects of exogenous estrogens on different cell types *in vitro* so that we may begin to de-couple many of the sex-based variables that cause difference that are

often seen utilizing SABV. Beyond the sufficient utilization of SABV as a research tool, care is not currently taken to consider the cells current and historical exposure to estradiol and estradiol mimetics.

Traditional cell culture media contains multiple forms of exogenous estrogens in the form of direct estrogens from fetal bovine serum, and an estrogen mimetic in the case of phenol red (**Figure 1A**), despite this and increasing acknowledgment of sex as a biological variable they remain a ubiquitous component of cell culture media (Berthois et al., 1986; Morgan et al., 2019; Welshons et al., 1988, Lennon et al., 1996). Exposure to these components of cell culture media may have both nascent and long-term epigenetic impacts. Human mesenchymal stromal cells (hMSCs) have the potential to promote repair and regeneration for degenerative conditions and are a viable target for cell therapy for a variety of tissues, including musculoskeletal, neural, and immune tissues/systems (Han et al., 2022; Wilson et al., 2015; Zheng et al., 2014). Male and female cells have varying response and exposure to estrogens transiently across the lifespan (Frederiksen et al., 2020). Thus, when studying sex specific repair strategies there is a need to examine cellular response to estrogens that they are regularly exposed to. While some attention has been given to this issue, previous results investigating phenol red come either from only male cells (Lysdahl et al., 2013) or from non-human sources (Hong et al., 2009). Additionally, these previous studies were limited by the inclusion of a single donor preventing the de-coupling of data from individual donor differences to demonstrate sex related differences. While FBS donor variability is known to effect hMSCs, no studies have investigated directly the effects of hormone stripping FBS for hMSC culture in male and female cells (Guiotto et al., 2020; Sikora et al., 2016). Frustratingly, other research has gone into intentionally utilizing unfiltered FBS for adipogenic and osteogenic differentiation (Tekkatte et al., 2012). To this end we present data from male and female hMSCs (N=4) on their proliferation, metabolism, senescence, differentiation, and gene expression in response to phenol red, unfiltered fetal bovine serum (FBS), and/or estradiol supplementation. Phenol red significantly increased proliferation in both male and female donors without altering senescence or metabolic profiles significantly. Filtered FBS is associated with more ‘stem like outcomes’ including lower senescence associated staining and a more glycolytic metabolic profile, particularly in female cells. When it comes to differentiation, filtered appears to inhibit female cells adipogenesis, while promoting it in male cells. According to our results, the optimal media formulation for attenuating senescence while maximizing proliferation is phenol red containing media with charcoal/dextran filtered FBS. Care must be

taken when deriving data from male and female cells differentiation as their baseline may be biased by the composition that is chosen.

The impact that these estrogens and estrogen mimetics have on various cell types is not thoroughly understood. Human mesenchymal stem cells (hMSCs) are of particular interest in the field of tissue engineering for their multi-lineage differentiation capability and their anti-inflammatory and pro-regenerative secretory factors that drive advantageous paracrine effects. Male and female cells have varying response and exposure to estrogens transiently across the lifespan. During pre-pubescence, women have higher levels of E2 than men due to higher aromatase activity in the tissues with this being exacerbated during pubescence with increased production of circulating E2 in the ovaries(Igarashi et al., 2021; Vrtačnik et al., 2014). During perimenopause and menopause E2 levels become highly variable, and while they tend to decrease, they can spike to 2.5-3 times higher than their peak levels seen in traditional ovulatory cycles (Gordon & Sander, 2021; Joffe et al., 2020). Following menopause women revert to their pre-pubescent methods of estradiol production, with 100% of it being due to tissue specific estradiol production (Vrtačnik et al., 2014). These varying levels affect both the nascent phenotype of hMSCs and the long term epigenetic programming of them, which needs to be considered both for the application of autogenic cell therapy, and for the influence it has on primary cells utilized for research. Indeed, 71% of clinical trials or pre-clinical results used to support clinical trials utilizing hMSCs that have posted results utilized some form of exogenous estrogens including phenol red and unfiltered FBS, most commonly both (**Supplementary Table 1**).

Phenol red is known to act in a similar manner to estradiol, however the full effects of it are not well understood. It has long been known that phenol red enhances the proliferative capacity of cells, particularly those that express estrogen receptors such as MCF-7 (breast cancer) cells (Berthois et al., 1986). Phenol red is also known to increase the expression of the progesterone receptor, indicating that it is likely acting through the estrogen receptor alpha (ER-α) (Berthois et al., 1986; Welshons et al., 1988). Phenol red is present in cell culture media at levels much higher than traditional serum levels of estradiol, 15-45 micromolar depending on which basal media formulation you are using, compared to the pico-nanomolar levels that are seen in human serum (Berthois et al., 1986). However, the action of phenol red is likely not 1:1 with that of estradiol, with Welshons et al. determining that the relative binding affinity of phenol red with the ER-α receptor is around three orders of magnitude lower than that of estradiol (Welshons et al., 1988). There is evidence that phenol red may contribute

to differentiation of MSCs, with a specific focus on the adipogenic and osteogenic fates, making it less ideal for chondrogenic studies (Lysdahl et al., 2013a). Phenol red has also been shown in enhance osteoblast proliferation *in vivo*, mimicking the actions of estradiol (Ernst et al., 1989). Charcoal dextran filtered FBS (CD-FBS) has not received as much attention, only one paper was found in the literature dealing with differentiation and CD-FBS concluding that it is a major contributor to metabolic profiles, proliferation, and differentiation in osteoblasts, however this work was not done in hMSCs. Despite this previous research, no work has been done on human hMSCs to understand the impacts of these estrogens and estrogen analogs. Understanding the effects of these estrogens on both male and female hMSCs is critical to understanding sex specific research in tissue engineering.

Herein we investigate the effect of phenol red, and Normal FBS on male and female hMSCs proliferation, metabolism, senescence, and adipogenic/osteogenic differentiation potential. We utilized 8 donors, 4 male 4 female, aged 19-23 derived primarily from bone marrow (with one female donor being derived from adipose due to age limitations). PicoGreen was utilized to assay proliferation, PrestoBlue (Resazurin/Resorufin) was utilized to assay metabolic capacity (**Figure 1B**). Basal metabolic rate was measured utilizing a seahorse XF analyzer. Senescence was determined using senescence associated staining of β-galactosidase. Differentiation was assayed utilizing quantitative polymerase chain reaction (qPCR), oil red o staining, and alizarin red staining. This work provides a baseline for the effects of common exogenous estrogens in cell culture media and can be utilized by other researchers as a baseline for determining which sources of exogenous estrogens are permissible for their given application. It also serves as a template for other cell types including iPSCs, ESCs, and primary cells for some of the critical outcomes researchers should check before designing studies with SABV in mind.

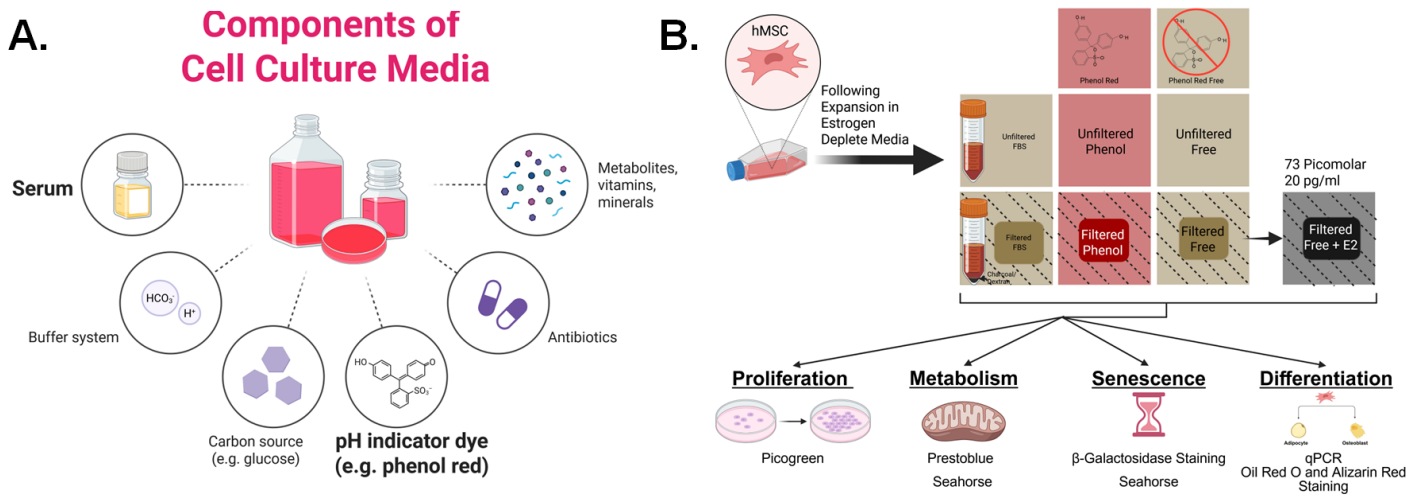

Figure 1. The common components of standard cell culture media, with components that contribute estrogens or estrogen mimetics highlighted (A) Study design with color and shading scheme for media compositions (B). Schematics made in Biorender.

Methods

Cell Culture

Cells were acquired from RoosterBio, ATCC, and StemBioSys with 4 male donors ranging from 19-22 and 4 female donors ranging from 20-23. All but one donor was bone marrow derived and the remaining was from adipose tissue. Cells were cultured in phenol red free, low glucose DMEM (Thermo Fisher #11054020) supplemented with 10% FBS (Atlanta Biologics) and L-glutamine (Thermo Fisher #21051024). Cells were expanded for 1 passage before being plated at 9,000 cells/cm^2. Cells were allowed to adhere for 24 hours before being changed into their respective media conditions.

Table 1: hMSC Donor Details

Donor: Age (years),Sex	Source
19, Male	RoosterBio, Bone Marrow
21, Male	RoosterBio, Bone Marrow
21, Male	StemBioSys, Bone Marrow
22, Male	RoosterBio, Bone Marrow
20, Female	RoosterBio, Bone Marrow
21, Female	StemBioSys, Bone Marrow
21, Female	Stemcell Tech, Adipose
23, Female	ATCC, Bone Marrow

Picogreen

After five days in culture cells were lysed with 1% Triton X-100 and manually agitated to ensure complete lysis of the cells. Invitrogen™ Quant-iT™ PicoGreen™ dsDNA Assay Kits were utilized to determine cell number.

PrestoBlue

Each 24 hours before the media change 10% HS PrestoBlue reagent (Thermofisher PN# P50201) was added to phenol red free charcoal dextran filtered FBS media and allowed to incubate for 1 hour. Following the incubation cells fluorescence was read at 560/590 in a Tecan plate reader.

qPCR

Cells were grown for 5 days in their respective media conditions then lysed according to manufacturer's protocol (Thermofisher, PureLink™ RNA Mini Kit). Samples were eluted into 50 µL ultrapure water and reverse transcribed to cDNA using the Applied Biosystems high-capacity cDNA reverse transcription kit. Samples were analyzed utilizing tapestation for representative RIN values and Qubit was run for RNA quantification. cDNA libraries were diluted to utilize 10 ng cDNA per reaction and assayed for GAPDH (Hs02786624_g1), Col10a1 (Hs00166657_m1), SOX9 (Hs00165814_m1), RUNX2 (Hs01047973_m1), ACAN (Hs00153936_m1), PPARG (Hs01115513_m1), and FABP4 (Hs01086177_m1). Samples normalized to pooled cDNA from phenol red unfiltered FBS hMSCs across all donors.

β-galactosidase staining

Cells were fixed on day 5 and stained with Cell Signaling Technology Senescence β-Galactosidase Staining Kit (Cat#9860) for senescence associated signaling in a 48 well plate. Images were taken utilizing a Revolve Revolution microscope of the entire well and exported to ImageJ utilizing a custom macro. Cells were outlined and then assayed for blue content, output is #cells with blue staining/total number of cells for percent senescence associated signaling.

Lineage Staining

Oil Red O

Cells were differentiated for 2 weeks in adipogenic media containing 1 uM Dexamethasone (PN# A17590.03), 500 uM Isobutylmethylxanthine (PN# J64598.MC), 5 ug/ml Insulin (PN# 12585014), and 200 ug/ml of Ascorbic Acid (PN# 49752). Image analysis was performed utilizing an image J macro. Briefly, the images are lightly blurred to help abstract their staining and remove extremely small particles interference before being run through a custom color deconvolution to pull out the specific color of positively stained lipid droplets. This image is then run through auto-local threshold before being watershed to isolate individual droplets and counted using analyze particles with a circularity gate to prevent off target staining from being counted.

Alizarin Red

Cells were differentiated for 2 weeks in osteogenic media containing 100 nM Dexamethasone (PN# A17590.03), 10 mM Beta-Glycerophosphate (PN# L03425.36), and 50 ug/ml Ascorbic Acid (PN# 49752). Image analysis was performed utilizing an image J macro. Briefly, the images are lightly blurred to help abstract their staining and remove extremely small particles interference before being run through a custom color deconvolution to pull out the specific color of positively stained calcium deposits. Analyze particles was then used to determine the number of positively stained calcium deposits with a size gate on analyze particles to prevent anything too small from being counted.

Results

Proliferation

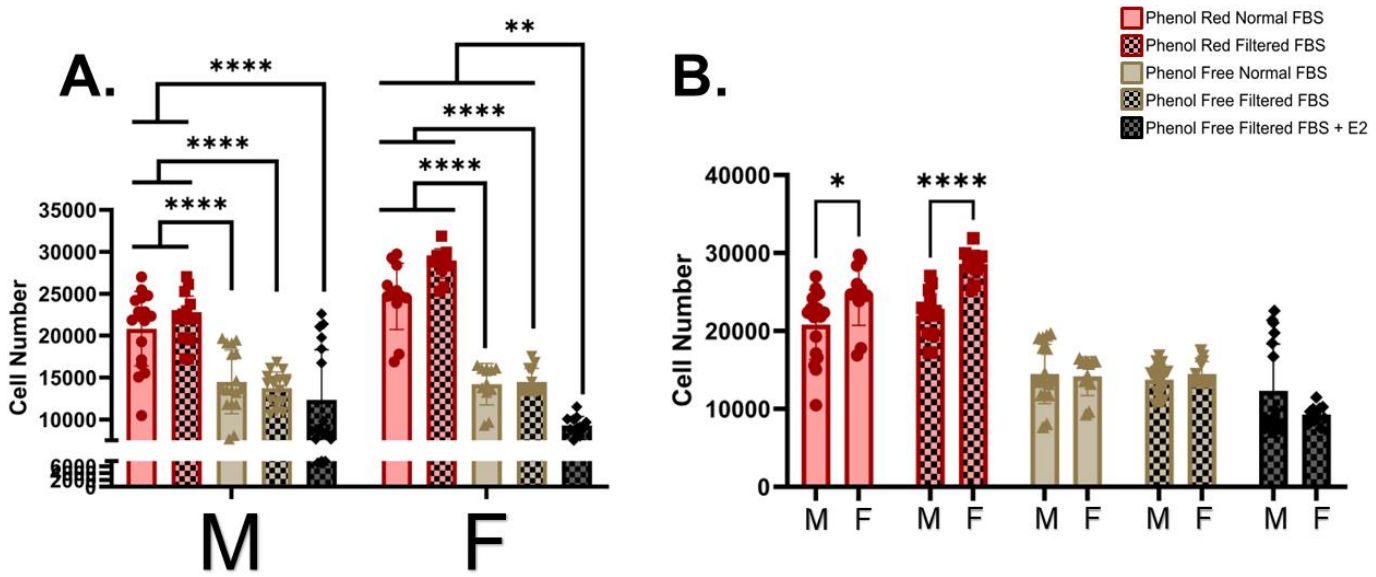

Figure 2. hMSC cell numbers at Day 5 from PicoGreen dsDNA assessment to determine media impacts on proliferation. Statistical comparisons done within donor sex (A) Statistical comparisons done between donor sexes (B). All data tested for normality utilizing the Shapiro-Wilk test (α = .05) and analyzed utilizing 2-way ANOVA and Bonferroni multiple comparison testing. ANOVA Statistics Interaction: P<0.0001, Row Factor (Sex): P<0.0079, Column Factor (Treatment): P<0.0001. * P =< .05, ** P =< .01, *** P =< .001, **** P =< .0001. Each dot represents a different well in a tissue culture plate, 3 wells per donor, 4 donors, for a total of 12 points per condition.

Phenol red containing media was the primary driver of cell proliferation across all donors **(Figure 2A**). Overall, female cells proliferated significantly more in the presence of phenol red compared to male cells (**Figure 2B**), an effect not observed in phenol free conditions (**Figure 2A&B**). Estradiol supplementation suppressed growth in female cells, but not in male cells when compared to the matched phenol free filtered FBS group. However, this effect was not significantly different when comparing the two populations directly (**Figure 2A**). Filtered vs normal FBS did not significantly impact proliferation. The interaction between sex and treatment was significant, indicating that sex significantly impacts the response of these cells to phenol red in a manner beyond which is explained simply by the sex differences alone without treatment.

Metabolism

The PrestoBlue assay was used to measure the hMSC populations' capacity to convert resazurin to resorufin, which is an indicator of cells' oxidative capacity to covert NADPH to NADP (Xu et al., 2015). This assay was used to assess the reductive capacity of hMSCs across 5 days of culture. FBS filtration was the major driver in metabolic activity (**Figure 3A**). Normal FBS significantly increased the metabolic activity of hMSCs in both male and female cells with the presence of phenol red (**Figure 3A**). In female cells, the metabolic differences were non-significant in phenol red free media; however, this could be driven by an individual female donor that was particularly metabolically active (**Figure 3A and Supplemental Figures**). Low level estradiol supplementation did not recover metabolic activity in hMSCs (**Figure 3A**). Female hMSCs had significantly higher metabolic activity in the phenol free filtered FBS media with minimal exogenous estrogens (**Figure 3B**). PrestoBlue time courses (**Figure 3C and 3D**) demonstrate that female cells overall had a much higher capacity for metabolic activity. ANOVA indicated significant effects from sex and treatment on the reductive capacity of male and female cells. However, the interaction parameter was non-significant indicating that the effect of exogenous estrogens on the reductive capacity of hMSCs is mostly explained by baseline sex differences and the levels of exogenous estrogens but the response to these exogenous estrogens is similar in male and female cells.

Because of this surprisingly stark shift in metabolic profile, a deeper metabolic profile utilizing the glycolysis stress test kit of the Seahorse XF Analyzer was done. Interestingly, the basal metabolic profile, namely the ratio of the oxygen consumption rate (OCR, indicative of oxidative phosphorylation) to extracellular acidification rate (ECAR, indicative of glycolysis), was significantly different only in female cells **(Figure 4A**).

Specifically, normal FBS caused an increase in the prevalence of oxidative phosphorylation in female hMSCs (**Figure 4A**). Female donors had a significantly lower OCR/ECAR in phenol red filtered FBS when compared to matched male donors and significantly higher OCR/ECAR in phenol free normal FBS indicating their sensitivity to these exogenous estrogens (**Figure 4B**). Low-level estradiol supplementation did not promote a significantly higher OCR/ECAR metabolic balance indicating this mechanism may not be solely mediated by estrogen. Female cells had significantly lower OCR/ECAR ratio in phenol red filtered conditions, but significantly higher OCR/ECAR in phenol free normal FBS conditions, indicating the impact of these exogenous estrogens may be more intense for the metabolic profile of female cells (**Figure 4B**). Based on comparisons between donor sex,

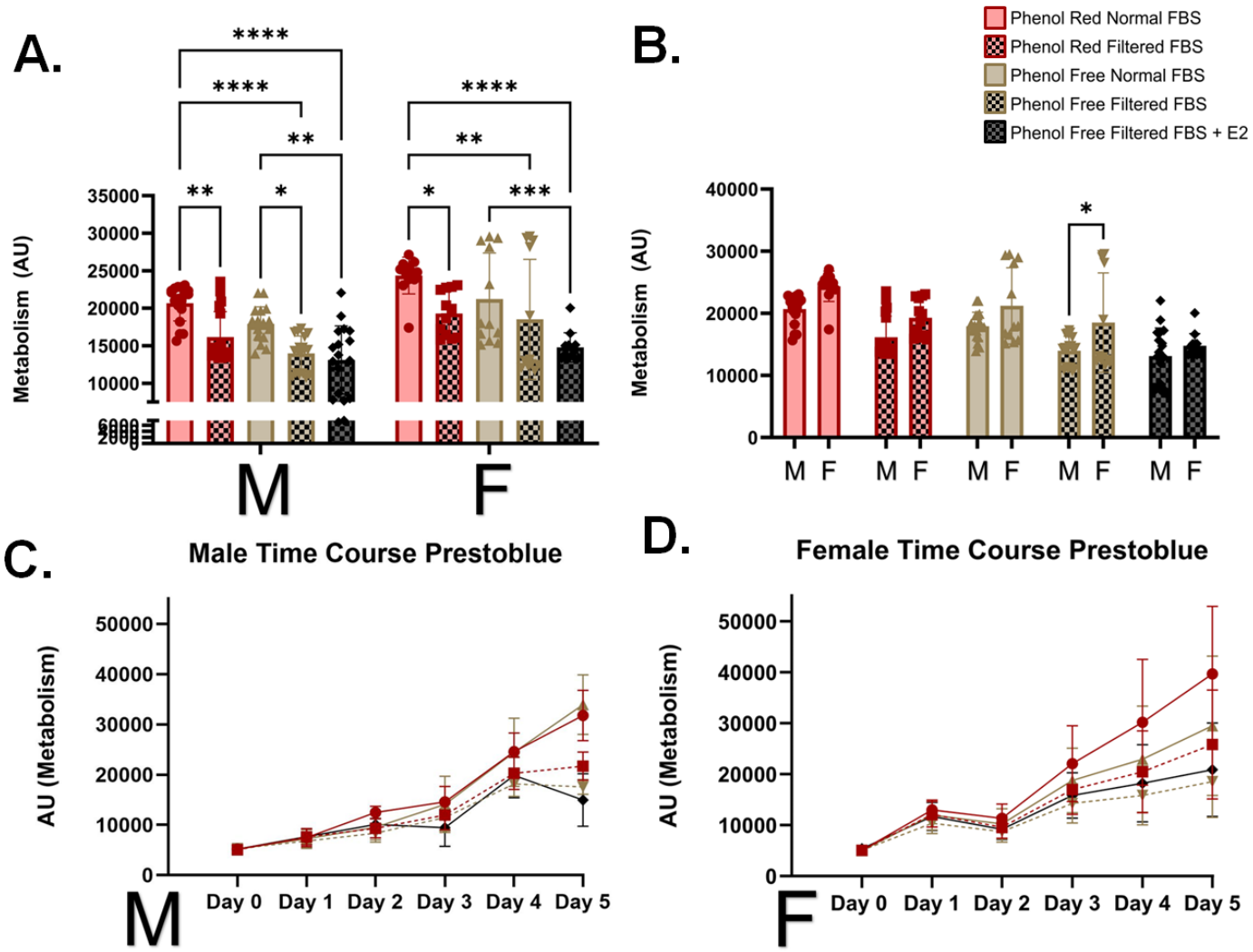

Figure 3. hMSC PrestoBlue metabolism on Day 5. Statistical comparisons done within donor sex (A) Statistical comparisons done between donor sexes (B) Male time course metabolism data through Day 5 (C) Female time course metabolism data through Day 5 (D). All data tested for normality utilizing the Shapiro-Wilk test (α = .05) and analyzed utilizing 2-way ANOVA and Bonferroni multiple comparison testing. ANOVA Statistics Interaction: P=0.7292, Row Factor (Sex): P<0.0001, Column Factor (Treatment): P<0.0001. * P =< .05, ** P =< .01, *** P =< .001, **** P =< .0001. Each dot represents a different well in a tissue culture plate, 3 wells per donor, 4 donors, for a total of 12 points per condition.

sex does not synergistically affect metabolism, rather there are baseline differences in metabolism based on sex.

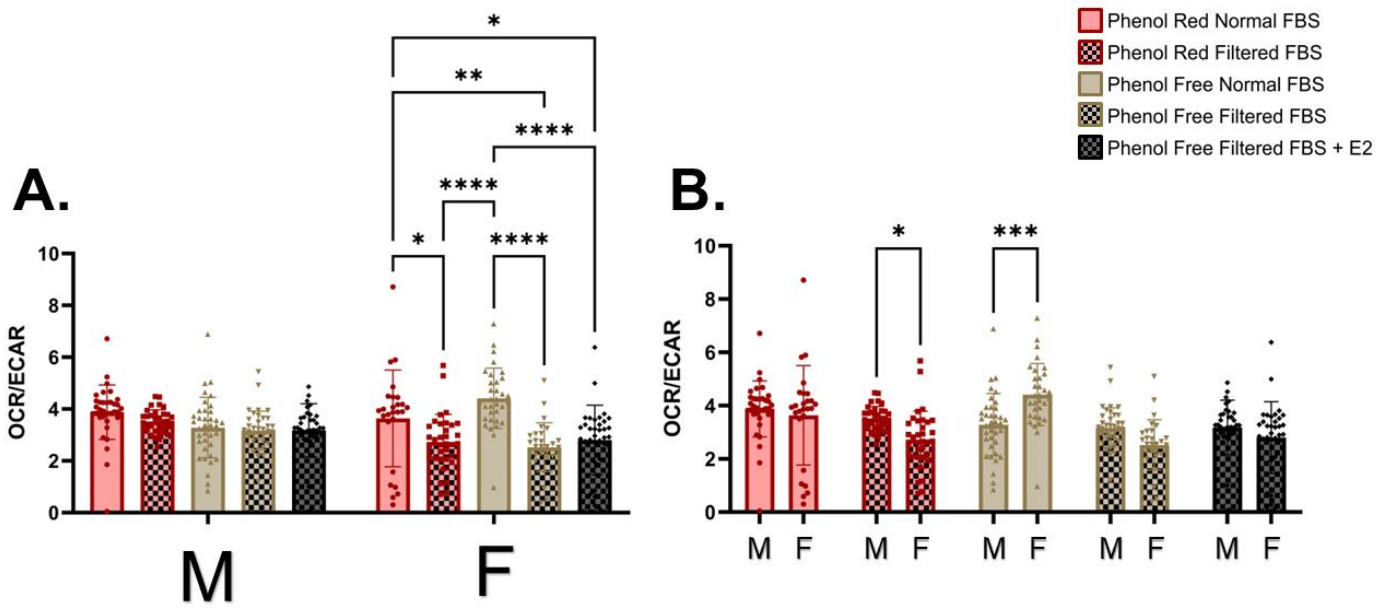

Figure 4. Seahorse Basal Metabolic Rate Data on Day 5 Statistical comparisons done within donor sex (A) Statistical comparisons done between donor sexes.(B) All data tested for normality utilizing the Shapiro-Wilk test (α = .05) and analyzed utilizing 2-way ANOVA and Bonferroni multiple comparison testing. ANOVA Statistics Interaction: P=0.4159, Row Factor (Sex): P<0.0001, Column Factor (Treatment): P<0.0001. * P =< .05, ** P =< .01, *** P =< .001, **** P =< .0001. Each dot represents a different well in a tissue culture plate, 3 wells per donor, 4 donors, for a total of 12 points per condition.

Senescence

Senescence based on β-galactosidase staining was conducted to determine whether the media impact on proliferation was due to an increase in senescence. Overall, senescence associated β-galactosidase staining levels were low, with the most activated group approaching 1% of cells associated with significant levels of staining (**Figure 5**). Senescence associated beta-galactosidase staining indicated a similar pattern to that of the basal metabolic rate. Normal FBS significantly increased senescence associated β-galactosidase staining in female donors. In contrast to the basal metabolic rate data, Normal FBS also increased the senescence associated β-galactosidase staining in the phenol red, but not the phenol free groups in the male cells. Low level estradiol supplementation significantly increased senescence associated β-galactosidase. Male cells exhibit significantly higher senescence associated staining in estradiol deplete conditions, whereas female cells exhibit

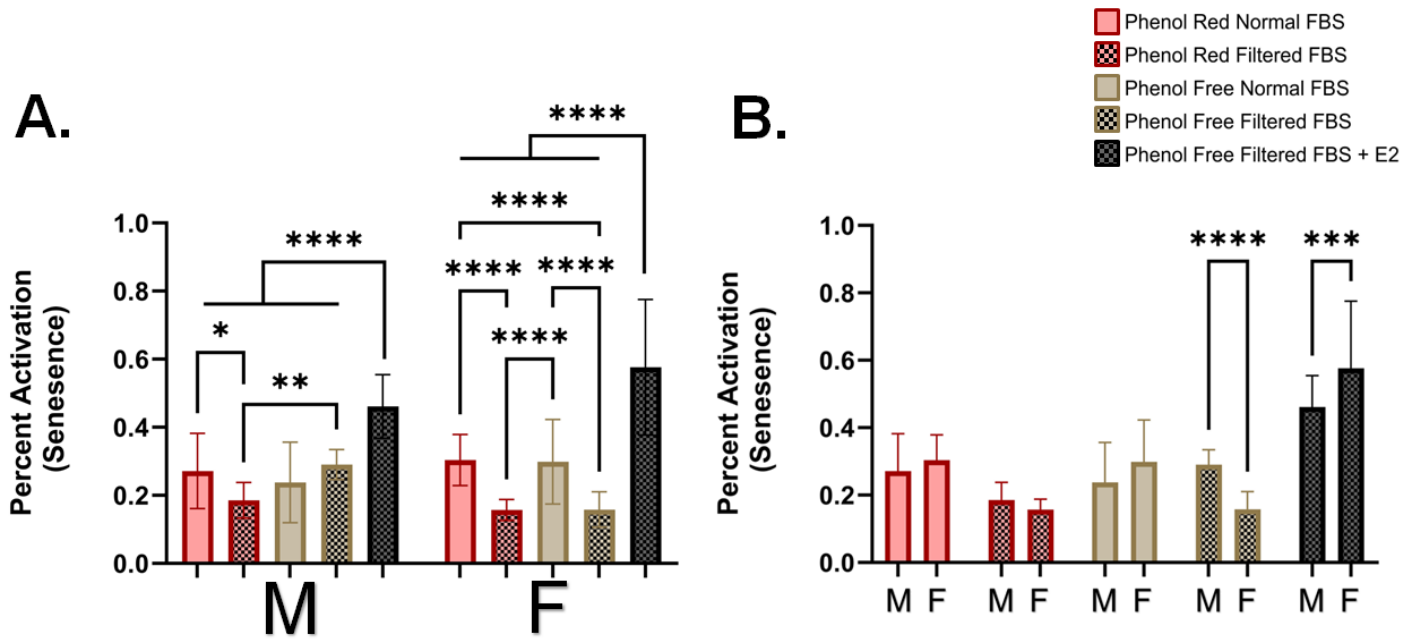

Figure 5. β-galactosidase-associated senescence staining on Day 5. Statistical comparisons done within donor sex (A) Statistical comparisons done between donor sexes. (B) Analyzed utilizing 2-way ANOVA and Bonferroni multiple comparison testing. ANOVA Statistics Interaction: P=0.0001, Row Factor (Sex): P<0.4408, Column Factor (Treatment): P<0.0001. * P =< .05, ** P =< .01, *** P =< .001, **** P =< .0001. Each dot represents a different well in a tissue culture plate, 3 wells per donor, 4 donors, for a total of 12 points per condition.

higher staining in low level estradiol supplementation groups. Female cells appear to be more vulnerable to senescence activation based on exogenous estradiol content. Sex alone did not have a significant effect on senescence associated staining. However, sex does contribute to significant differences in senescence staining profile in the presence of exogenous estrogens.

Differentiation

To assess the impacts of these metabolic and proliferative shifts on the differentiation potential of male and female hMSCs, cells were differentiated osteogenic and adipogenic media for 2 weeks and stained with oil red o and alizarin red to assess lipid droplets and calcium deposits, respectively. Staining for alizarin red (**Figure 6)** revealed an increased in osteogenic differentiation in phenol red media with unfiltered FBS when compared to filtered FBS in both male and female hMSCs. In male hMSCs, this was the only condition that was significantly different from others whereas in female donors the phenol free conditions were both higher than the phenol red filtered FBS conditions. Additionally, in female donors the estradiol supplemented condition was significantly

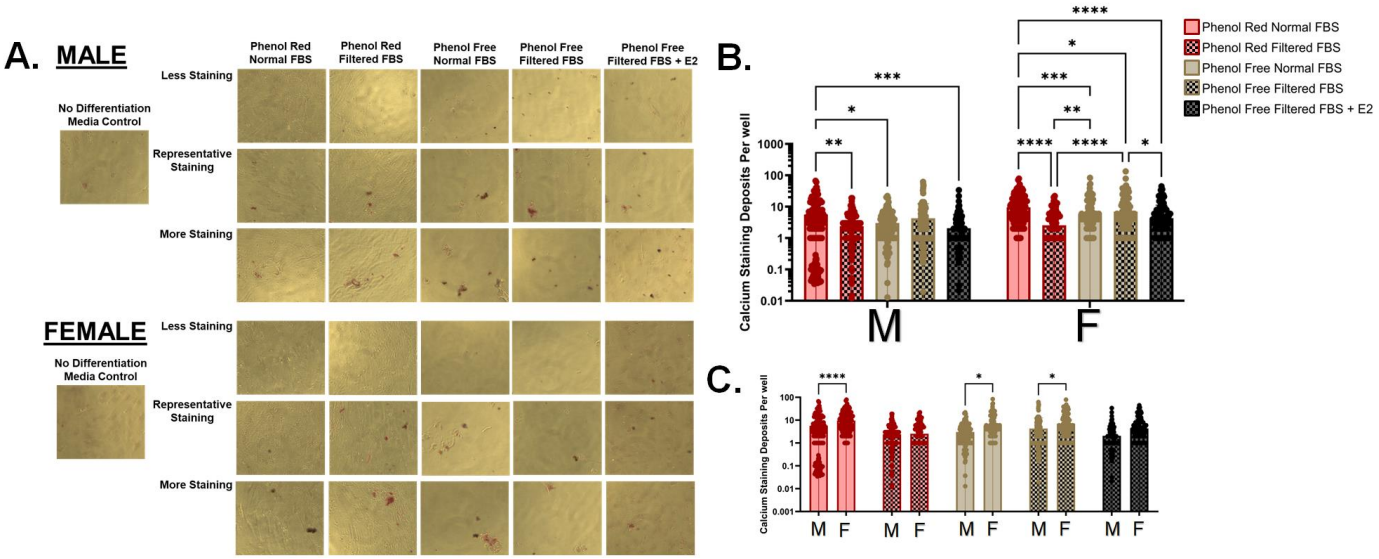

Figure 6. Alizarin Red staining to assess media impacts on osteogenic differentiation. Representative images ranging from less to more staining for each condition in male and female hMSCs. (A) Statistical comparisons done within donor sex. (B) Statistical comparisons done between donor sexes. (C) Analyzed utilizing 2-way ANOVA and Bonferroni multiple comparison testing. ANOVA Statistics Interaction: P=0.0565, Row Factor (Sex): P<0.0001, Column Factor (Treatment): P<0.0001. * P =< .05, ** P =< .01, *** P =< .001, **** P =< .0001.

lower in calcium deposition staining than the phenol free filtered FBS condition, indicating that in female cells picomolar estradiol supplementation inhibits osteogenic differentiation. Female donors had significantly higher expression of calcium staining in phenol red unfiltered media as well as phenol free filtered/unfiltered FBS. Overall, osteogenic differentiation was increased by normal FBS in phenol red containing media in male and female donors indicating higher levels of exogenous estrogens may accelerate osteogenic differentiation at day 14.

Adipogenic differentiation revealed that phenol red conditions, particularly filtered FBS were significantly higher than phenol free conditions, in male cells (**Figure 7A and 7B**). In female cells, this difference was reduced to phenol free unfiltered FBS and the E2 supplementation. This indicates that, in female cells, picomolar concentrations of estradiol present in FBS may work to inhibit adipogenic differentiation. Female cells had higher amounts of adipogenic staining in phenol red conditions as well as the phenol free unfiltered conditions. The fact that the phenol free unfiltered FBS condition and E2 supplementation condition trended similarly indicates that this effect may be largely due to the picomolar presence of estradiol, and the higher amounts in phenol red containing media indicates that phenol red may be rescuing this pattern of behavior. ANOVA parameters indicate that treatment, baseline sex difference, and the contribution of sex to the response of cells to treatment were all significant.

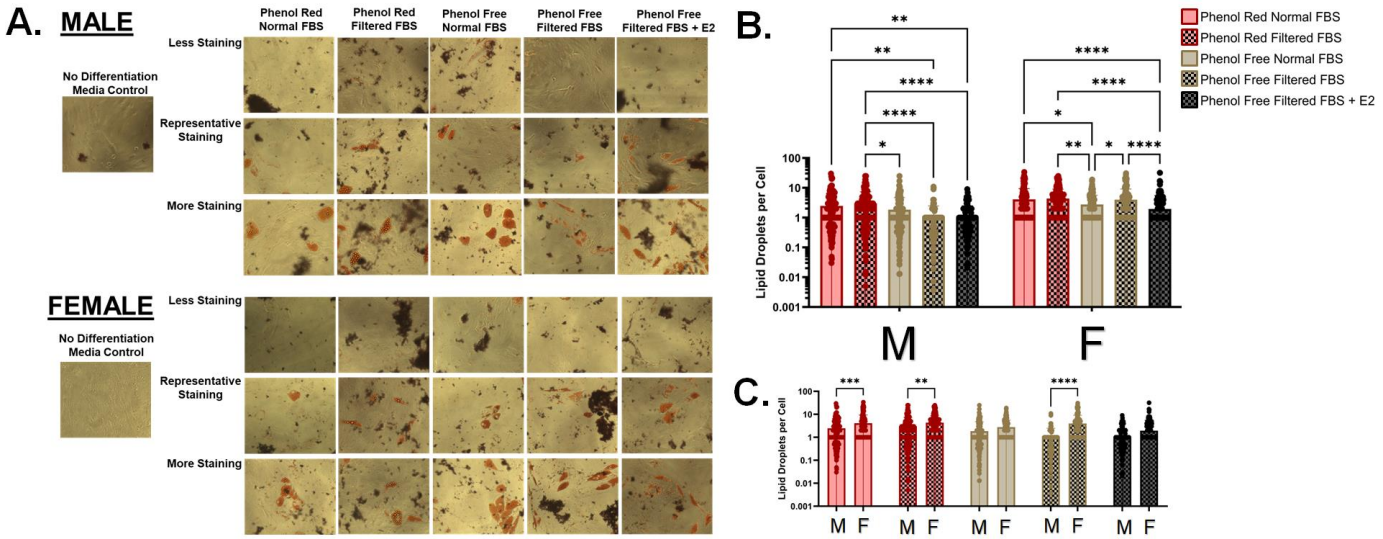

Figure 7. Oil Red O staining to assess media impacts on adipogenic differentiation. Representative images ranging from less to more staining for each condition in male and female hMSCs. (A) Statistical comparisons done within donor sex. (B) Statistical comparisons done between donor sexes. (C) Analyzed utilizing 2-way ANOVA and Bonferroni multiple comparison testing. ANOVA Statistics Interaction: P=0.0029, Row Factor (Sex): P<0.0001, Column Factor (Treatment): P<0.0001. * P =< .05, ** P =< .01, *** P =< .001, **** P =< .0001.

qPCR analysis was performed for 6 key genes, 2 genes per traditional tri-lineage path incorporating 1 transcription factor and 1 key expressed protein expressed by each lineage based on prior literature recommendations (Daprà et al., 2022). Individual donor graphs are in Supplementary (**Figures 1-8**). Samples for qPCR were taken from non-differentiation media, meaning that increased in mRNA expression of these target genes is solely due to the differences in the media conditions and how they are priming hMSCs for future differentiation rather than compounds in the differentiation media. Osteogenic differentiation was assessed by RUNX2 and Col10a1 expression, adipogenic differentiation was assessed by FABP4 and PPARG, and chondrogenic differentiation was assessed by SOX9 and Col2a1. Col2a1 failed to amplify in any of the tested conditions and was therefore excluded from the final data.

RUNX2 expression was significantly higher in the phenol free unfiltered FBS condition when compared to E2 supplementation in both male and female cells (**Figure 8A and 8B**). In female cells, this effect was expanded with the estrogen deplete condition being significantly higher than both other filtered FBS conditions. Thus, in female cells phenol red may be acting in a similar manner to estradiol supplementation, while unfiltered FBS counteracts this effect. Female cells had a significantly lower expression of RUNX2 in phenol red filtered FBS conditions. The phenol free filtered FBS condition, particularly in females, was significantly upregulated in both when compared to other filtered FBS conditions. ANOVA parameters for RUNX2 expression revealed that

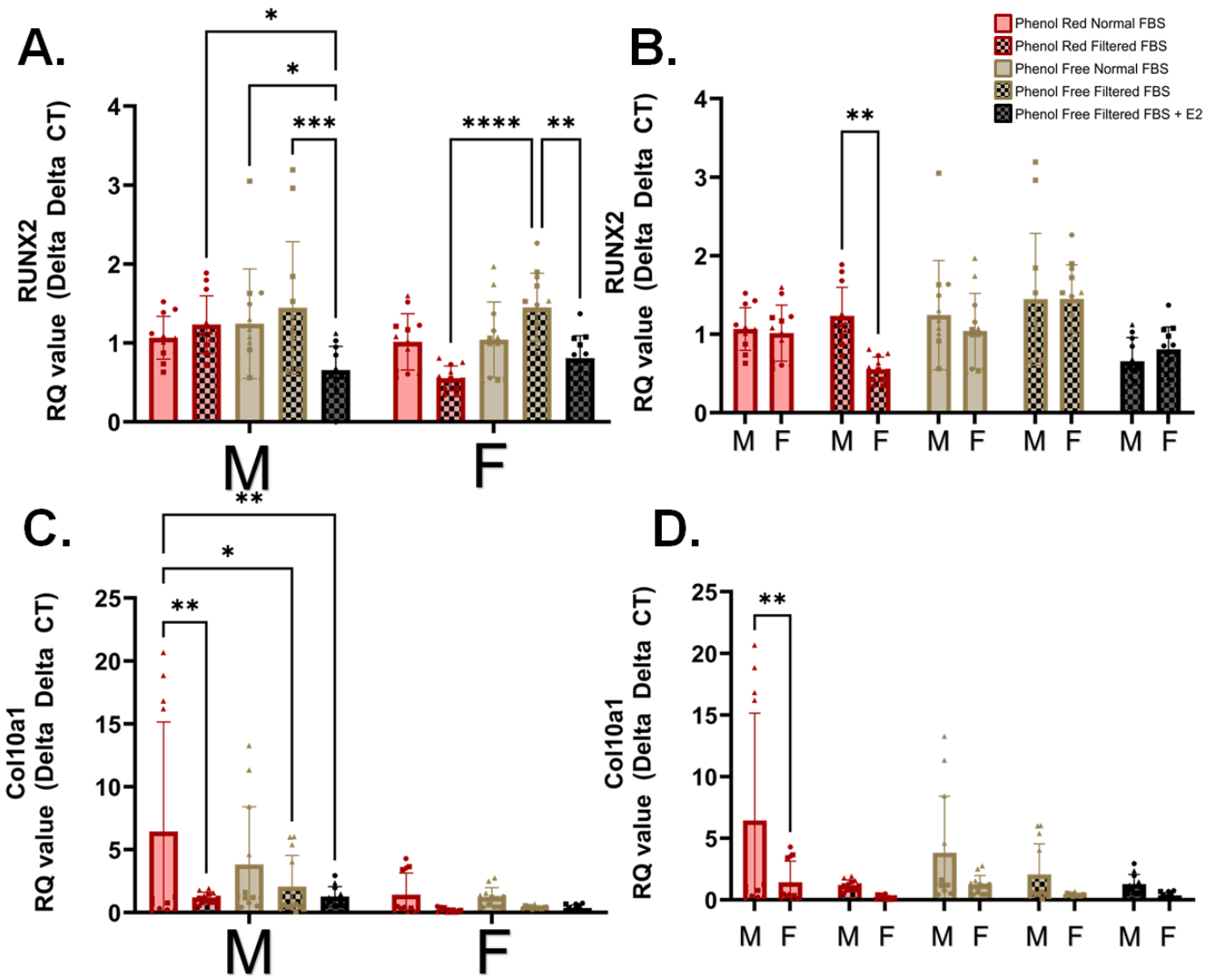

Figure 8. Osteogenic markers Runx2 and Col10a1. A. Statistical comparisons done within donor sex for Runx2 (A) and Col10a1 (C). Statistical comparisons done between donor sexes for Runx2 (B) and Col10a1 (D). Analyzed utilizing 2-way ANOVA and Bonferroni multiple comparison testing. RUNX2 ANOVA Statistics Interaction: P=0.0262, Row Factor (Sex): P=0.0665, Column Factor (Treatment): P<0.0001. Col10a1 ANOVA Statistics Interaction: P=0.1793, Row Factor (Sex): P=0.0003, Column Factor (Treatment): P=0.0032. * P =< .05, ** P =< .01, *** P =< .001, **** P =< .0001. Each dot represents a different well in a tissue culture plate, 4 wells per donor, 3 donors, for a total of 12 points per condition.

the amount of exogenous estrogens (treatment) and the interaction of donor sex with that treatment was significant, but the baseline differences between male and female donors was not. This indicates that all sex differences in RUNX2 expression are due to the differential response of male and female cells to either phenol red or unfiltered FBS.

Interestingly, the Col10a1 expression pattern did not match with the expression pattern of its matched transcription factor RUNX2 (**Figure 8C and 8D**). Col10a1 expression was significantly spiked in the male cells in the phenol red unfiltered FBS condition, with this being significantly higher than all the filtered FBS conditions. Expression in female donors was low and non-significant between groups. No direct sex differences were observed within groups. Sex did significantly contribute to the variance via ANOVA statistics, and treatment with

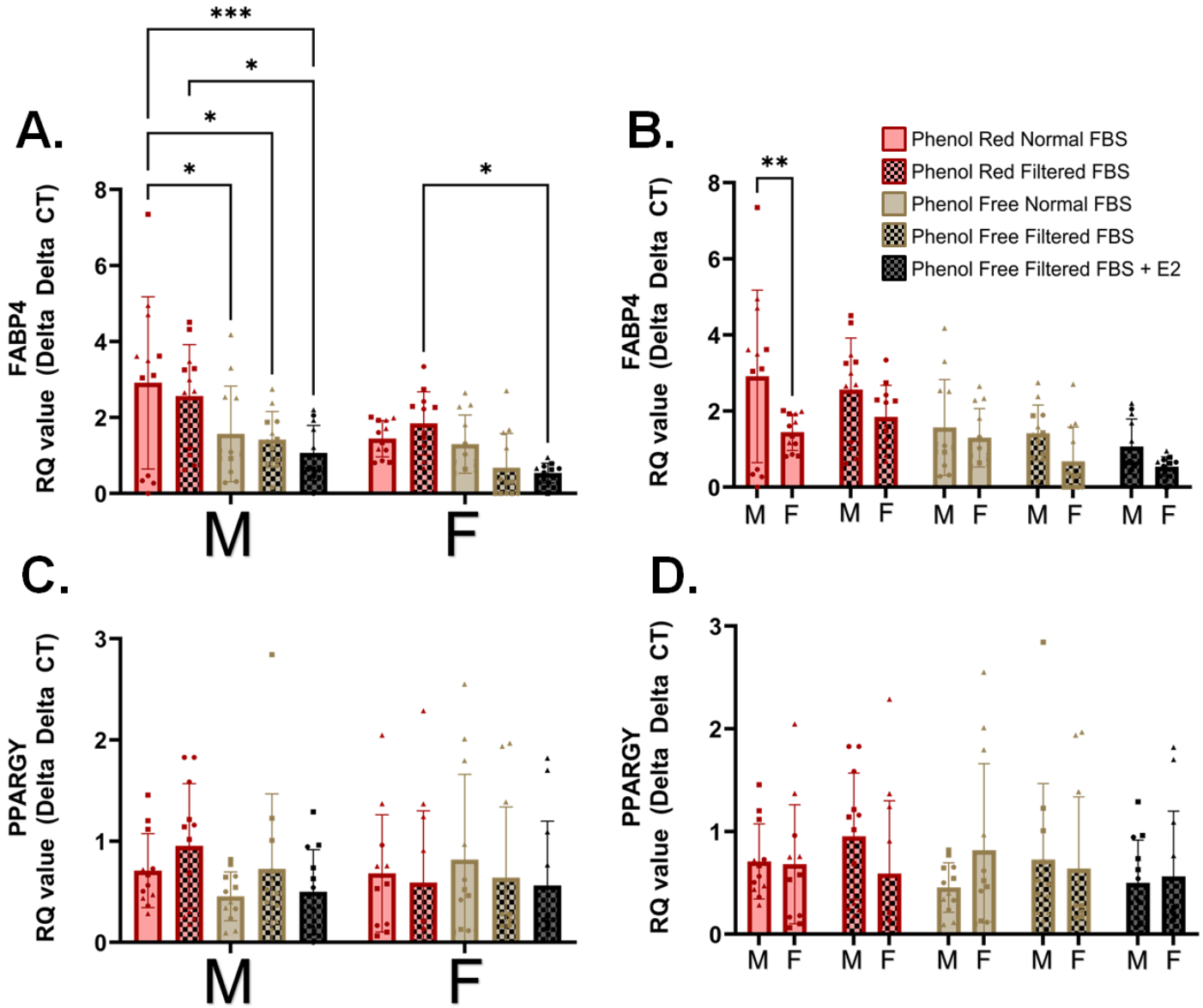

Figure 9. Adipogenic markers FABP4 and PPARG. Statistical comparisons done within donor sex for FABP4 (A) and PPARGY (C). Statistical comparisons done between donor sexes for FABP4 (B) and PPARGY (D). Analyzed utilizing 2-way ANOVA and Bonferroni multiple comparison testing. FABP4 ANOVA Statistics Interaction: P=0.4108, Row Factor (Sex): P=0.0003, Column Factor (Treatment): P=0.0032. PPARG ANOVA Statistics Interaction: P=0.3563, Row Factor (Sex): P=0.9237, Column Factor (Treatment): P=0.7332. * P =< .05, ** P =< .01, *** P =< .001, **** P =< .0001. Each dot represents a different well in a tissue culture plate, 4 wells per donor, 3 donors, for a total of 12 points per condition.

exogenous estrogens was significant as well however, the interaction between sex and treatment was non-significant.

FABP4 expression (**Figure 9A and 9B**) showed a significant increase in the phenol red filtered FBS conditions, in agreement with the semi-quantification of Oil Red O staining when compared to the phenol free unfiltered + E2 condition. Male cells additionally had significantly higher expression of FABP4 in both phenol red conditions, indicating phenol red may pre-prime hMSCs for adipogenic differentiation. Female donors in the phenol red unfiltered FBS conditions had significantly lower expression of FABP4 compared to male cells. ANOVA indicated significant differences based on sex and treatment, but not their interaction, indicating that male cells may simply have a higher baseline for FAPB4 expression *in vitro*.

PPARG showed no significant differences in treatment or sex (**Figure 9C and 9D**). Individual donors do show significant changes between the treatment conditions, however these effects are not seen when data is aggregated by sex, indicating that PPARG response to exogenous estrogens may be donor dependent, and not sex dependent.

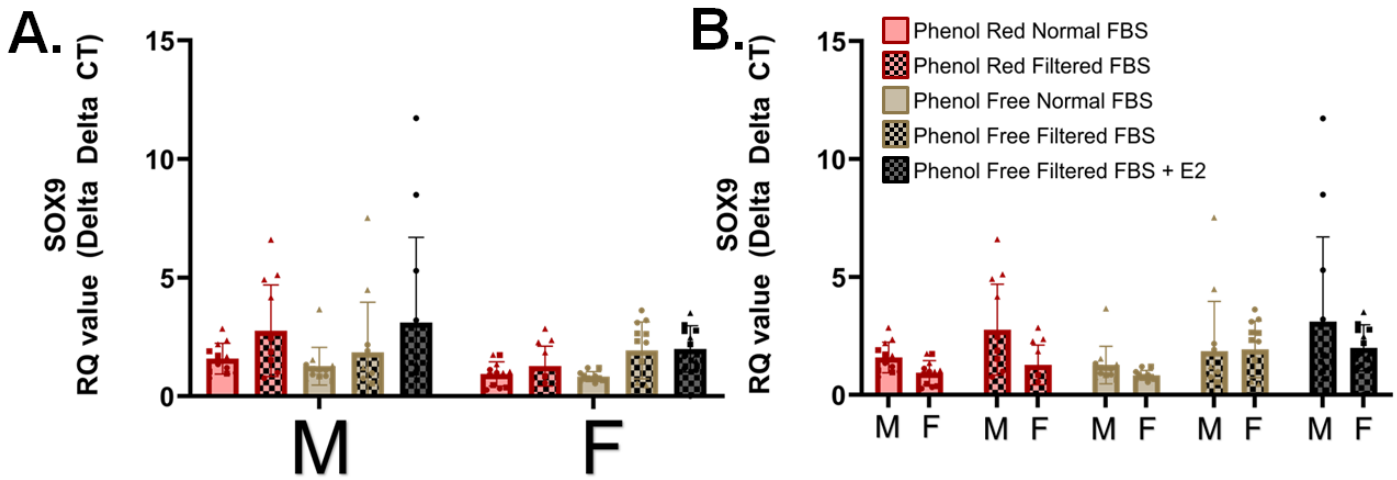

Figure 10. Chondrogenic marker Sox9. Statistical comparisons done within donor sex (A) Statistical comparisons done between donor sexes (B). Analyzed utilizing 2-way ANOVA and Bonferroni multiple comparison testing. SOX9 ANOVA Statistics Interaction: P=0.4873, Row Factor (Sex): P=0.0155, Column Factor (Treatment): P=0.0111. * P =< .05, ** P =< .01, *** P =< .001, **** P =< .0001. Each dot represents a different well in a tissue culture plate, 4 wells per donor, 3 donors, for a total of 12 points per condition.

qPCR for SOX9 did not reveal any statistically significant direct comparisons **(Figure 10)**. However, ANOVA indicated that both sex, and the treatment did significantly impact the variance. The interaction parameter was non-significant indicating sex differences would be largely due to baseline expression differences.

Discussion

Phenol Red

Phenol red and normal FBS have differing impacts on male and female cells *in vitro.* The PrestoBlue assay is often utilized for an overall approximation of cell number, however utilizing this method as a cell number proxy can be inaccurate (Vieira-da-Silva & Castanho, 2023). Herein, we utilize PrestoBlue to understand the reductive capacity of the cells. Phenol red is a driving influence of proliferation but has little effect on metabolism or senescence. Additionally, phenol red does cause female cells to proliferate significantly more than male cells in a manner that is not explained solely by baseline sex differences. Given the results of Berthois et al, that demonstrated an increase in proliferation due to phenol red only in cells that had widespread expression of the ER-α receptor, indicates that phenol red is increasing proliferation through ER-α in both male and female cells and that it is mimicking the action of estradiol at concentrations much higher than the supplemented value of 20 pg/mL (Berthois et al., 1986). While phenol red can act as a non-steroidal estrogen, it appears that even with the increased concentration present in cell culture media, for routine growth the inclusion of phenol red in standard cell culture media is warranted for its increase in cellular proliferation without any deleterious effects on metabolism, senescence, or stemness.

Osteogenic differentiation at 14 days showed phenol red can increase calcium deposition in the presence of unfiltered FBS, which is similar to the results achieved by (Lysdahl et al., 2013b) with the alizarin red staining at 14 days being non-significantly different and their alkaline phosphatase being increased by phenol red significantly at day 14. Lysdahl *et al.* expanded timepoints go on to show decreases in the phenol red conditions at further timepoints, warranting future studies as this study only included one male donor. Phenol red alone did not have a significant impact on Col10a1 or RUNX2 expression, similar to the day 14 results from Lysdahl *et al*., However, in female cells with filtered FBS, phenol red did show a significant decrease in RUNX2 expression compared to the phenol free condition which was also true of its associated osteogenic staining. The pattern of RUNX2 expression follows a similar one to that of the alizarin red staining, particularly in regards to the female donors phenol red filtered FBS condition being low expressing and the phenol free filtered FBS condition being high expressing, indicating that estrogen deplete media may enhance osteogenesis which is supported by the literature (Schiavi et al., 2021). The direct sex differences observed in the phenol red filtered FBS conditions did not also appear in the alizarin red staining analysis and vice versa.

Phenol red also significantly increased the adipogenic differentiation however in contrast to osteogenic differentiation this effect was significantly higher in filtered FBS conditions in female donors. This increase was also removed with picomolar estradiol supplementation indicating the estradiol component of FBS may be the cause of this difference in female donors. Female donors had significantly higher amounts of adipogenic differentiation compared to male donors in phenol red conditions. The increased adipogenic staining was accompanied by an increase in FABP4 expression in filtered FBS conditions.

Unfiltered FBS

Unfiltered and filtered FBS had the greatest impacts on hMSC metabolism, senescence, and differentiation. Normal FBS increased the reductive capacity of hMSCs in male and female cells in the presence of phenol red. In phenol red free media, it significantly increased the reductive capacity of male, but not female, hMSCs. This is possibly because female cells had higher variance in their metabolic profiles. When analyzed via Seahorse XF analyzer, the basal metabolic rate of hMSCs varied drastically in female, but not male cells. The metabolic profiles of hMSCs can be indicative of their stem-like potential (Sánchez-Aragó et al., 2013) hMSCs with a higher reliance on glycolytic metabolism have been shown to exhibit enhanced stem-like properties (Estrada et al., 2012). On the other hand, hMSCs with a higher reliance on oxidative phosphorylation indicating that un-filtered FBS maybe be causing differentiation in hMSCs prior to the intentional induction of differentiation cues, particularly in female cells. Filtered FBS returned female hMSCs to a more stem-like metabolic profile. Male cells did not see significant metabolic effects in their basal metabolic rates. Individual female donors were again highly variable in this data, with some donors having extremely low OCR/ECAR even in the phenol red normal FBS condition. Filtered FBS not only rescued the female donors to a more stem like metabolic profile, it also significantly reduced the expression of senescence associated β-galactosidase staining in a similar manner. Male cells also showed a reduction in senescence associated β-galactosidase staining in the phenol red, but not the phenol free condition.

In osteogenic differentiation of male and female hMSCs unfiltered FBS cause an increase in alizarin red staining compared to filtered FBS in phenol red conditions. Additionally, only in unfiltered FBS conditions did male cells see a significant increase in Col10a1 expression, matching the significant differences seen in

osteogenic staining however this affect was largely driven by a single donor. RUNX2 expression was not significantly different due to FBS conditions.

Adipogenic differentiation showed only an increase in lipid droplet accumulation in phenol free conditions with female donors. All other conditions showed no significant differences between filtered and unfiltered FBS in the same phenol conditions. Filtered FBS particularly in phenol red conditions shows higher adipogenic staining and expression of FABP4 for female cells compared to the phenol free normal FBS and estradiol control conditions. Additionally female donors with filtered FBS show significantly higher baseline staining in phenol red and phenol red free conditions.

Sex Differences

Sex differences observed by direct comparison and previously noted in either the phenol red or unfiltered FBS sections above are summarize in **Figure 11** the discussion in this section is largely dedicated to the interpretation of ANOVA parameters and what they tell us about how sex is altering hMSC cellular response. To properly

Table 2: ANOVA Interaction Parameters Informing Sex Differences

Test	Interaction	Sex Differences	Treatment
Proliferation	P<0.0001	P=0.0079	P<0.0001
Redox Activity	P=0.7292	P<0.0001	P<0.0001
Metabolism	P=0.4159	P<0.0001	P<0.0001
Senescence	P<0.0001	P=0.4408	P<0.0001
Osteogenic Staining	P=0.0565	P<0.0001	P<0.0001
Adipogenic Staining	P=0.0029	P<0.0001	P<0.0001
Sox9 Expression	P=0.4873	P=0.0155	P=0.0111
RUNX2 Expression	P=0.0262	P=0.0665	P<0.0001
Col10a1 Expression	P=0.1793	P=0.0003	P=0.0032
FABP4 Expression	P=0.4108	P=0.0003	P=0.0032
PPARG Expression	P=0.3563	P=0.9237	P=0.7332

assess differences due to sex, we must consider both the inherent differences due to sex (row factor in our analysis) and the contribution of sex to the differential response of cells (interaction parameter). ANOVA parameters (**Table 2**) for proliferation indicate that there are both inherent differences in proliferation within the

same treatment groups, and that the treatment combined with sex create a significantly different effect than what the sex differences or treatment alone would cause. For redox activity assayed by the PrestoBlue assay, both

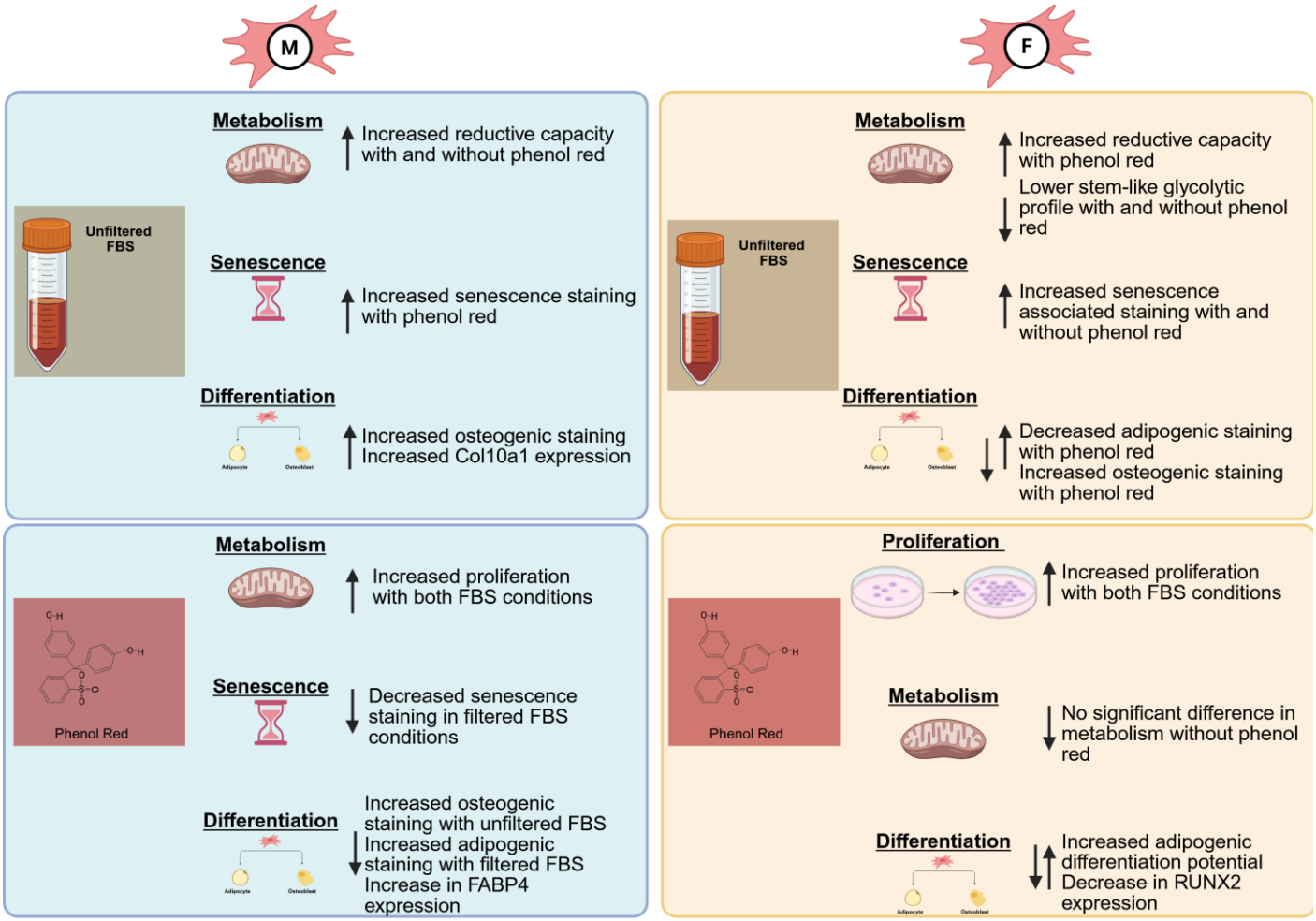

Figure 11. Visual summary of observed differences based on sex, phenol red, and unfiltered FBS

sex and treatment caused significant effects, however they did not interact indicating that basal sex differences explain largely the difference between the sexes rather than their differential response to the treatment. Metabolism assayed by seahorse had a similar response, sex was inherently different, and treatment cause

significant differences but differences between the samples was explained solely by the effect of treatment or sex. Senescence however was not significantly different based on sex, but sex did cause significantly different impacts of the treatments on senescence associated staining. Direct differentiation of donors showed that adipogenic differentiation has both baseline sex differences, and a significant contribution from sex to the reaction to exogenous estrogens. Osteogenic differentiation showed a significant baseline difference in sex but was slightly over the traditional threshold for significance for the interaction between sex and treatment. SOX9, Col10a1, and FABP4 expression was all significantly different based on donor sex for baseline expression, but

not significantly different in response to treatment. RUNX2 expression which mapped well onto the osteogenic staining results indicated a significant difference in both baseline sex differences and response to exogenous estrogens based on donor sex. Given the RUNX2 and alizarin red data support each other well, particularly for female cells, it is fair to conclude that osteogenic differentiation is significantly affected by the donor response to exogenous estrogens.

Conclusion

Much work needs to be done to understand how sex differences impact regeneration and repair for the field of tissue engineering. One of the variables that needs to be well characterized is the differential response of cells to hormones, specifically estrogens. To understand the differential response of cells to estradiol, the exogenous estradiol compounds commonly present in cell culture and the response of cells to them must be understood. This work presents a brief survey of the effects of exogenous estrogens commonly present in cell culture media on hMSCs, a common cell type investigated for tissue engineering applications. We have demonstrated that phenol red significantly increases proliferation without any clear deleterious effects on cells, however caution should still be given to its inclusion as the long-term epigenetic effects warrant future study. Unfiltered fetal bovine serum caused cells to lose their stem-like metabolic profile and increase senescence-associated staining. Baseline sex differences in redox activity and metabolism were observed, while sex was found to significantly alter cell response to phenol red induced proliferation as well as senescence associated staining due to unfiltered fetal bovine serum. Donor sex additionally significantly impacted adipogenic differentiation, both baseline and in response to exogenous estrogens, and impacted osteogenic differentiation baseline. Gene expression analysis revealed differential response to osteogenic and adipogenic markers. Chondrogenic marker SOX9 was also significantly impacted by the amount of exogenous estrogens. Moving forward, these results should be considered whenever designing a study investigating sex-biased effects. Future work should be done to investigate the long-term epigenetic effects of exogenous estrogens as estradiol is known to affect DNA methylation and other epigenetic regulatory mechanisms that may be altered long term in culture based on limited exposure.

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