Research article

DIFFERENTIAL REDOX POTENTIAL PROFILES DURING ADIPOGENESIS AND OSTEOGENESIS

BARRY R. IMHOFF and JASON M. HANSEN*
Division of Pulmonary, Allergy/Immunology, Cystic Fibrosis and Sleep,
Department of Pediatrics, Emory School of Medicine, Emory University
Atlanta, 2015 Uppergate Drive Suite 350, Altanta, GA 30322, USA

Abstract: Development is an orderly process that requires the timely activation and/or deactivation of specific regulatory elements that control cellular proliferation, differentiation and apoptosis. While many studies have defined factors that control developmental signaling, the role of intracellular reduction/oxidation (redox) status as a means to control differentiation has not been fully studied. Redox states of intracellular couples may play a very important role in regulating redox-sensitive elements that are involved in differentiation signaling into specific phenotypes. In human mesenchymal stem cells (hMSCs), which are capable of differentiating into many different types of phenotypes, including osteoblasts and adipocytes, glutathione (GSH), cysteine (Cys) and thioredoxin-1 (Trx1) redox potentials were measured during adipogenesis and osteogenesis. GSH redox potentials (E_h) during both osteogenesis and adipogenesis became increasingly oxidized as differentiation ensued, but the rate at which this oxidation occurred was unique for each process. During adipogenesis, Cys E_h became oxidized as adipogenesis progressed but during osteogenesis, it became reduced. Interestingly,

* Author for correspondence. e-mail: jhansen@emory.edu, phone: 404-727-3145, fax: 404-712-0920

intracellular Trx1 concentrations appeared to increase in both adipogenesis and osteogenesis, but the E_h was unchanged when compared to undifferentiated hMSCs. These data show that hMSC differentiation into either adipocytes of osteoblasts corresponds to a unique redox state profile, suggesting that differentiation into specific phenotypes are likely regulated by redox states that are permissive to a specific developmental process.

Key words: Mesenchymal stem cells, Adipogenesis, Osteogenesis, Glutathione, Cysteine, Thioredoxin-1, Redox status

INTRODUCTION

Development is characterized by important events such as, cellular proliferation, differentiation and apoptosis. Timely and orderly activation/deactivation of elements involved in these processes is critical to ensure normal development. Moreover, untimely activation/deactivation of these signals can lead to abnormal differentiation, dysmorphogenesis or spontaneous abortion, suggesting that these signals are highly conserved and tightly controlled during development. Various signals that regulate cell specific-directed differentiation have been previously described, but a more ubiquitous regulatory factor that is not fully understood is that of intracellular redox states.

During mouse embryonic 3T3-L1 adipogenesis, glutathione (GSH)/glutathione disulfide (GSSG) redox potential (E_h) progressively becomes more oxidized. Using the Nernst equation to calculate E_h, GSH/GSSG concentrations demonstrate an approximate shift of + 25 mV [1, 2], suggesting that adipogenic progression favors a more oxidizing intracellular environment. Similarly, differentiation of colon adenocarcinoma HT-29 cells also demonstrated a shift to a more oxidizing intracellular GSH E_h, as indicated by a shift of nearly + 60 mV [3]. While the general oxidation of GSH is observed in many *in vitro* models of differentiation, there is evidence that the type of cellular differentiation may be promoted by specific redox environments. Using mouse C2C12 myoblasts as a model of myogenesis, GSH E_h progressively became more oxidized (approximately + 25 mV), but myogenic differentiation was actually enhanced under more reducing conditions, where the GSH E_h was only + 12 mV [4], suggesting that developmental signals involved in myogenesis may prefer more reducing environments.

While GSH E_h has been more extensively studied, there are some reports of other thiol-based redox couples states during cellular differentiation. During human adenocarcinoma HT-29 differentiation, glutathionylation of cellular proteins (formation of Pr-SSG) increased [5] and is likely the product of an increased intracellular GSH E_h [3]. However, while Cys E_h was not directly measured, cysteinylation of proteins (Pr-SCys) did not change, suggesting that the intracellular Cys E_h was unchanged [5]. Additionally, human intestinal adenocarcinoma Caco-2 differentiation coincides with an increase in GSH E_h values, but during these same periods of differentiation, E_h values of the

oxidoreductase Trx1 were unchanged throughout [6]. Because each redox couple is independently regulated it has been hypothesized that each couple is capable of controlling specific redox-sensitive elements and may contribute to the regulation of a more specified redox response [7].

Cellular models have provided very useful information into redox potential changes during differentiation, but limitations of these cellular models, where they are usually capable of differentiating into only one terminal phenotype, has made it difficult to determine how redox states of different couples are regulated during differentiation that may be more relevant to development *in vivo*. In this report, we use multipotent human mesenchymal stem cells (hMSCs) that are capable of differentiating into many different phenotypes *in vitro*, including osteoblasts and adipocytes [8], as a more suitable cellular model of differentiation. Using hMSCs allows for the common, basal starting redox potential in undifferentiated cells and provides a means by which redox profiles related to a specific stem cell fate can be determined. Here, we show that the combined redox profiles of GSH, Cys and Trx1 are unique to each osteogenic and adipogenic processes, suggesting that modification of a single redox couple may alter terminal differentiation.

MATERIALS AND METHODS

Cells and cell culture
Human mesenchymal stem cells (hMSCs) were purchased from the mesenchymal stem cell repository at the Texas A&M University Institute for Regenerative Medicine and were cultured according to instructions from the provider. In brief, cells were grown in alpha minimum essential medium (αMEM) supplemented with 16.5% fetal bovine serum, 2-4 mM glutamine and antibiotics. On day one post-confluence, initiation of differentiation was commenced by replacing growth media with differentiation media. For adipogenesis, the growth medium was supplemented with 0.5 μM dexamethasone, 0.5 μM isobutylmethylxanthine and 50 μM indomethacin. For osteogenesis, growth medium was supplemented with 10 nM dexamethasone, 20 mM β-glycerolphosphate and 50 μM ascorbic acid 2-phosphate. Media was changed every 2-3 days for up to 21 days of differentiation.

Assessment of differentiation
Following 21 days in differentiation media, cultures were washed and then fixed with 10% buffered formalin for 1 h. For osteogenic cultures, mineral deposits were visualized by staining with Alizarin Red S (1% Alizarin Red S in deionized water, pH 4.2) for 20 min. hMSCs undergoing adipogenesis were stained with freshly prepared Oil Red O stain (0.3% Oil Red O in 60% isopropanol). Both cultures were washed to remove excess stain and then viewed and photographed via light microscopy.

Differentiation was also assessed via the expression of markers of terminal differentiation for adipocytes and osteoblasts, namely fatty acid synthase (FAS) and osteopontin, respectively. RNA was collected through the differentiation period with the RNEasy RNA collection kit (Qiagen). cDNA was generated with the QuantiTect Reverse Transcriptase cDNA synthesis kit (Qiagen) per the manufacturer's instructions and then used in quantitative real-time fluorescence PCR analysis using SYBR Green detection (SABiosciences) with an Opticon 2 Real Time PCR cycler (Bio-Rad). Primers for fatty acid synthase, osteopontin and β-actin (for standardization) were purchased from SABiosciences. Changes in expression were quantified via the $\Delta\Delta Ct$ method and are expressed as fold changes in expression from day 0 of differentiation (undifferentiated).

GSH and Cys analysis

Cytosolic GSH, GSSG, Cys and cystine (CySS) were assayed by HPLC as S-carboxymethyl, N-dansyl derivatives using γ-glutamylglutamate as an internal standard as described by Jones [9] and Jiang [10]. In brief, hMSCs were washed in ice cold PBS and the collected in 5% perchloric acid containing the internal standard and proteins were precipitated. Samples were centrifuged and the soluble fraction containing free GSH, GSSG, Cys and CySS was derivatized. Cellular volumes were based on ratios of cytosol to protein concentrations as determined elsewhere [3]. To calculate the GSH E_h, GSH and GSSG concentrations were used in the Nernst equation, where $E_h = E_o - 30$ x $(\ln ([GSH]^2/[GSSG]))$ and $E_o = -264$ at a pH of 7.4. To calculate the Cys E_h, Cys and CySS concentrations were used in the Nernst equation, where $E_h = E_o - 30$ x $(\ln ([Cys]^2/[CySS]))$ and $E_o = -250$ at a pH of 7.4.

Thioredoxin-1 analysis

Trx1 E_h was determined via redox western methodology as previously described [11]. In brief, detection of oxidized and reduced Trx1 was achieved by iodoacetic acid labeling followed by separation by a non-reducing, non-denaturing PAGE. Separation is based on charge, where the reduced (labelled) form migrates more rapidly through the gel. After electrophoresis, samples are immunoblotted to nitrocellulose, blocked in Li-Cor blocking buffer for 1 h, and probed with a goat anti-human Trx1 polyclonal antibody for 1 h. An AlexaFluor680 donkey anti-goat antibody was used for secondary detection. Visualization was performed with the Li-Cor Odyssey Infrared Detection System (v1.2). Band densitometries were measured with the Odyssey detection software and were used in the Nernst equation, where $E_h = E_o - 30$ x $(\ln ([Trx1_{red}]/[Trx1_{ox}]))$ and $E_o = -256$ at a pH of 7.4.

RESULTS

Differentiation

Fatty acid synthase expression significantly increased on day 10 of differentiation compared to expression levels on day 0. Osteopontin expression levels significantly increased earlier on day 7. On day 21, staining showed evidence of

both osteogenesis and adipogenesis (Fig. 1A). Differentiation was assessed through the expression of fatty acid synthase or osteopontin over a 21 d period (Fig. 1B). These results demonstrate the differentiation potential of hMSCs and correlate well with other differentiation studies using these cells [8].
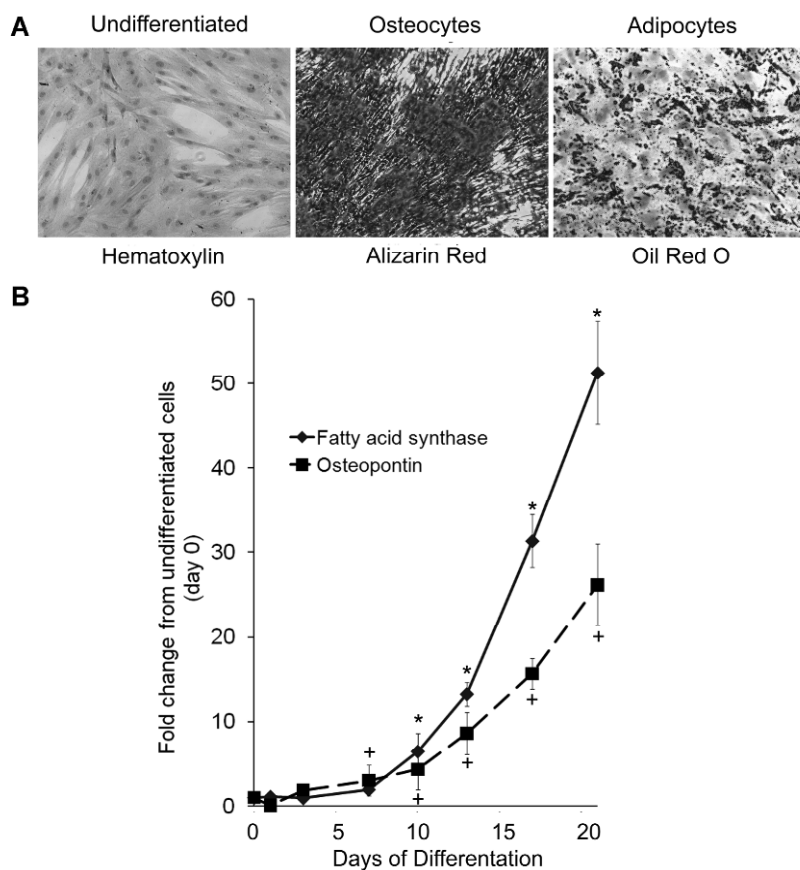

Fig. 1. Differentiation of hMSCs. A – hMSCs were differentiated over a 21 d period and then stained with either Alizarin Red (osteocytes) or Oil Red O (adipocytes) and then counterstained with hematoxylin. B – RNA was collected and analyzed for markers of differentiation (Fatty acid synthase [FAS] and osteopontin) throughout the 21 d period of differentiation. Asterisks denote a significant statistical change ($p < 0.05$) in FAS expression from day 0 and crosses denote a significant statistical change ($p < 0.05$) in osteopontin expression from day 0. Data is the result of at least three independently performed experiments.

Glutathione redox states
Glutathione redox potential in undifferentiated hMSCs was measured to be - 259 mV ([GSH] = 3.22 mM (± 0.14); [GSSG] = 15.8 μM (± 0.92); total GSH [GSH] + ([GSSG] x 2) = 3.24 mM (± 0.24)). In cultures stimulated to undergo adipogenesis, the GSH E_h became significantly oxidized by day 3, shifting

nearly + 15 mV ([GSH] = 3.13 mM (± 0.14); [GSSG] = 44 μM (± 0.92); total GSH [GSH] + ([GSSG] x 2) = 3.25 mM (± 0.24)) (Fig. 2). On days 5 to 13, a steady increase in GSH E_h was observed, changing by up to + 40 mV ([GSH] = 2.72 mM (± 0.20); [GSSG] = 234 μM (± 10.5); total GSH [GSH] + ([GSSG] x 2) = 3.19 mM (± 0.35)) by day 13. These changes highlight changes to the GSH redox potential but not to total GSH concentrations, thus, these findings constitute a general oxidative shift of GSH that is independent of concentrations. On days 17 and 21 of adipogenic differentiation, the GSH E_h leveled and did not significantly change from day 13. In hMSCs stimulated to undergo osteogenesis, GSH E_h also increased, becoming more oxidizing over time (Fig. 2). Unlike adipogenesis, the GSH E_h in osteogenic hMSCs remained relatively unchanged during the earlier, initial periods of differentiation (days 0 to 7). Significant changes were not observed until day 10, when the GSH E_h increased + 15 mV ([GSH] = 3.12 mM (± 0.18); [GSSG] = 44 μM (± 12.2); total GSH [GSH] + ([GSSG] x 2) = 3.20 mM (± 0.30)). On subsequent days of differentiation (days 13-21), there was a steady increase in GSH redox potential, and by day 21, the GSH E_h was nearly identical to that in adipogenic cultures, measured at - 226 mV ([GSH] = 2.87 mM (± 0.1); [GSSG] = 161 μM (±51); total GSH [GSH] + ([GSSG] x 2) = 3.21 mM (± 0.25)).
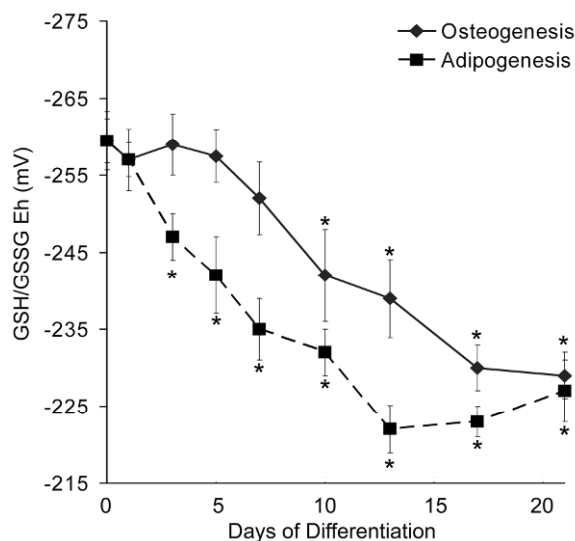

Fig. 2. GSH/GSSG E_h during adipogenesis and osteogenesis of hMSCs. Redox potentials shift in both adipogenesis and osteogenesis. Asterisks denote a statistically significant change ($p < 0.05$) from day 0. Cells undergoing adipogenesis show a more rapid oxidation of the GSH/GSSG E_h, beginning at day 3. Interestingly, significant changes to the GSH/GSSG E_h during osteogenesis did not occur until day 10. Data is the result of at least three independently performed experiments.

Cysteine redox states

Cysteine redox potentials were also measured throughout differentiation. For both cells undergoing adipogenesis or osteogenesis, Cys E_h was measured. Cys redox potentials in undifferentiated cells (day 0) were measured between - 155 and - 160 mV ([Cys] = 137 μM (± 5.9); [CySS] = 77 μM (± 29.7); total Cys ([Cys] + ([CySS] x 2) = 323 μM (± 100)) (Fig. 3). In hMSCs stimulated to undergo adipogenesis, cells became significantly more reducing on day 1 and decreased by -8 mV ([Cys] = 186 μM (± 9.8); [CySS] = 65 μM (± 27.3); total Cys ([Cys] + ([CySS] x 2) = 316 μM (± 57.1)). However, the Cys E_h returned to undifferentiated values until day 13. On day 13, became significantly more oxidizing as compared to day 0 values, increasing by approximately + 15 mV ([Cys] = 97 μM (± 6.24); [CySS] = 95 μM (± 9.5); total Cys ([Cys] + ([CySS] x 2) = 286 μM (± 22.1)). This same redox potential was maintained throughout the remainder of the culture period. Interestingly, during osteogenesis, hMSCs showed a very distinct change in redox potential as compared to adipogenic redox profiles. From the initial commencement of osteogenesis, intracellular Cys E_h decreased and continued to decrease to day 17. Day 17 showed the greatest change in Cys E_h, which had decreased by - 35 mV from day 0 ([Cys] = 260 μM (± 15.6); [CySS] = 21 μM (± 7.0); total Cys ([Cys] + ([CySS] x 2) = 302 μM (± 13.4)). On day 21, the Cys E_h increased slightly but was still significantly reduced compared to day 0.
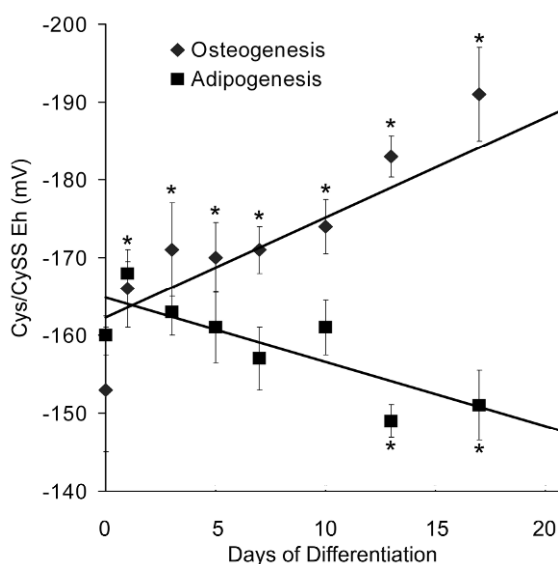

Fig. 3. Cys/CySS E_h during adipogenesis and osteogenesis. Asterisks denote a statistically significant change ($p < 0.05$) in Cys/CySS E_h from day 0. During adipogenesis, intracellular Cys/CySS E_h increases, becoming significantly more oxidizing by day 13. Conversely, during osteogenesis, redox potentials decrease, demarcating a significant shift to a more reducing state on day 1 and continues to become increasingly more reduced as osteogenesis ensues. Data is the result of at least three independently performed experiments.

Thioredoxin-1 redox analysis

Trx1 redox status was determined via redox Western methodologies (see materials and methods section), allowing for the separation and quantification of oxidized and reduced Trx1. Relative amounts of reduced and oxidized are then used to determine the E_h. While the total amount of Trx1 appeared to increase (Fig. 4A), the separation of oxidized and reduced Trx1 throughout both adipogenesis and osteogenesis revealed no significant changes in the Trx1 redox status as a consequence of differentiation (Fig. 4B). On day 0, undifferentiated cells showed a Trx1 E_h of - 278 mV and while subsequent measures fluctuated slightly, these changes were not significantly different from the initial Trx1 E_h measurement.
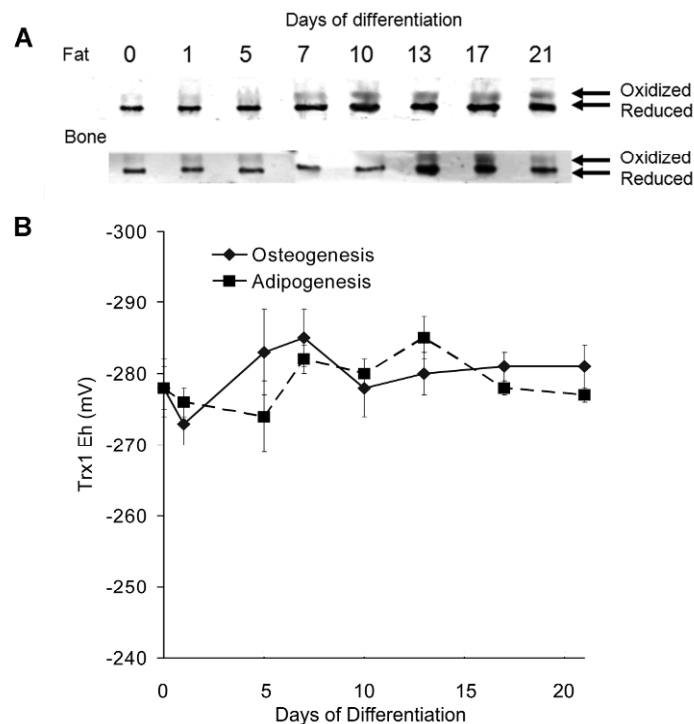

Fig. 4. Trx1 E_h during hMSC differentiation. Trx1 redox potentials were determined via redox Western blotting as described in the methods section. A – Separation of the reduced and oxidized forms of Trx1 is shown. The upper band represents oxidized Trx1, and the lower band depicts the reduced. B – Densitometric band measurements are then put into the Nernst equation and redox potentials are obtained. During both adipogenesis and osteogenesis, Trx1 redox state is unchanged and remains constant throughout. Data is the result of at least three independently performed experiments.

DISCUSSION

Development and cellular differentiation is a highly conserved process. Events occur in a timely and orderly fashion to promote normal organogenesis. While many molecular events are involved in the specification of certain tissues, very little is known about how more global regulators of signaling pathways, like redox states, are regulatory. Here, we present the novel findings, demonstrating unique patterns between various redox couples and various terminal differentiation states, suggesting that explicit redox states regulate differentiation. The use of human mesenchymal stem cells allows for the proper evaluation of redox states during differentiation. Due to hMSCs pluripotent nature, multiple cell types can be evaluated originating from a common undifferentiated cell [8]. Characterization of hMSCs has been widely performed, of which adipogenesis and osteogenesis are included [12-14].

There are many redox couples that contribute to the overall intracellular redox environment. Of particular interest are those that contain thiols, such as cysteine, glutathione and thioredoxin-1. Under more reducing states, these couples promote the reduction of redox-sensitive elements (i.e. the reduction of regulatory cysteine residues in proteins [Pr-SH]). Conversely, while under more oxidizing states, these couples promote protein oxidation (Pr-SSR or Pr-SOH). It is through these modifications, that the activity of redox-sensitive proteins is regulated.

These thiol-containing couples were initially believed to be in equilibrium but recently, data has shown that these couples are in fact independently regulated. For example, in heavy metal toxicity, certain metals (cadmium, mercury and arsenic) cause preferential oxidation of Trx1 but GSH is relatively unaffected [15]. Conversely, iron and nickel alter GSH redox states but not Trx1. In a more physiologic example, epidermal growth factor (EGF)-stimulated growth in keratinocytes requires intracellular ROS generation, but GSH analysis shows no oxidation [16, 17]. Interestingly, only cytosolic Trx1 becomes oxidized with EGF treatment [16]. The independence of thiol containing redox couples suggest that redox-sensitive elements are under the individual control of specific redox couples, not under global redox status [7]. Thus, in development, developmental signaling may be regulated by the status of a specific redox couple and promote a specific cell type.

In cellular models of osteogenesis, MC3T3-E1 differentiation was significantly decreased with prior subtoxic treatment of hydrogen peroxide, and mineralization was decreased by nearly half [18]. Markers of osteogenic differentiation, bone sialoprotein (BSP), osteocalcin (OS) and Runx2, were decreased as well, indicating a failure of cells to differentiate normally. Similarly, in other studies using both MC3T3-E1 and M2-10B4 cells, direct addition of hydrogen peroxide or generation of peroxide through a xanthine/xanthine oxidase system caused a significant, dose-dependent decrease in osteogenic differentiation [19].

In cellular models of adipogenesis, many papers have shown the inhibitory effects of reactive oxygen species on differentiation [20]. However, these approaches use high levels of peroxide or extracellular peroxide-inducing systems (i.e. glucose oxidase) to illustrate the sensitivity of adipogenic processes to oxidative insult. While these systems clearly increase ROS availability, it is unknown how they affect intracellular redox states of the various redox couples studied here. Our laboratory has found that extracellular redox states alters 3T3-L1 adipogenesis, where more reducing extracellular redox states inhibited adipogenesis, while more oxidizing conditions promoted it [1]. While the (de)regulatory effects of ROS and/or redox status during cellular differentiation are not fully understood, it is clear that the molecular mechanisms that govern redox regulation of differentiation require further study.

The primary purpose of this study is to evaluate the redox states during adipogenesis and osteogenesis of hMSCs. A general oxidation of GSH was observed in cells undergoing adipogenesis and osteogenesis. The concomitant shift to a more oxidizing intracellular environment with differentiation has been described previously with other cell types, however, of interest here, is the unique profile of cells undergoing adipogenesis compared to those undergoing osteogenesis. Adipogenic cells become oxidized very quickly and then are maintained in a more oxidized GSH state, while osteogenic cells remain reduced during the initial periods of differentiation and then rapidly oxidized during terminal differentiation. These data suggest that redox-sensitive elements that are regulated by GSH redox state are more highly controlled early in adipogenesis and late in osteogenesis.

While GSH comprises the greater portion of non-protein thiol in most cells, another, less understood pool is the cysteine pool. Very little is known about Cys during the differentiation of any cell type, but it has been suggested to be a very important node of redox control in cells (Jones). Due to the relatively common natures of GSH and Cys, the results here were somewhat surprising, where adipogenic cells became more oxidizing but osteogenic cells became more reducing. The polarity of these findings suggests a very important role of the Cys redox couple in differentiation, at least when osteogenesis is compared to adipogenesis. In previous work in cellular differentiation models, cytosolic HT-29 glutathionylated protein levels increased, but cytosolic HT-29 cysteinylated protein concentrations were unchanged [5], demonstrating the independent mechanism of protein modification by GSH and Cys.

Thioredoxin-1 is unique compared to GSH and Cys. Oxidation of Trx1 forms an intramolecular disulfide, while GSH and Cys form intermolecular disulfides. Trx1 has been shown to act on specific proteins, most notably NF-κB. The p50 subunit of NF-κB contains a redox-sensitive cysteine ($Cys^{61/62}$) residue that regulates its DNA binding [21]. Trx1 has been shown to participate in the reduction of $Cys^{61/62}$ during severe oxidative stress, preserving NF-κB signaling [22]. Preservation of Trx1 redox status may be critical in the progression of differentiation. Previous studies show that overexpression of thioredoxin binding

protein-2 (TBP2) decreased expression and activity of Trx1 [23]. Moreover, through TBP2 overexpression, altered Trx1 function and expression were shown to negatively regulate osteoclast differentiation [24]. Here, we show that regardless of the terminal cell phenotype, Trx1 redox status was unaltered. However, while GSH and Cys are dynamically controlled during differentiation, the Trx redox couple may ultimately prove equally as critical in the regulation of developmental events.

These data support the idea of the redox regulation of differentiation in a specific, not generalized, manner. Understanding what types of redox changes occur during the differentiation of various cellular phenotypes may provide a better understanding of directed differentiation of stem cells and how these elements may be susceptible to environmental insults and dysregulation. Unique redox profiles suggest specificity toward certain cell phenotypes and processes; however, the elements that are regulated by these changing redox states are currently unknown but are clearly an open area of future study.

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