TISSUE ENGINEERING: Part C
Volume 20, Number 12, 2014

DOI: 10.1089/ten.tec.2013.0683

Age-Related Effects on the Potency of Human Adipose-Derived Stem Cells: Creation and Evaluation of Superlots and Implications for Musculoskeletal Tissue Engineering Applications

Josephine C. Bodle, MEng,[1] Stephanie D. Teeter, BS,[1] Brandon H. Hluck, BS,[1] Joseph W. Hardin, BS,[1] Susan H. Bernacki, PhD,[1] and Elizabeth G. Loboa, PhD[1,2]

Human adipose-derived stem cells (hASC) are now a prevalent source of adult stem cells for studies in tissue engineering and regenerative medicine. However, researchers utilizing hASC in their investigations often encounter high levels of donor-to-donor variability in hASC differentiation potential. Because of this, conducting studies with this primary cell type can require extensive resources to generate statistically significant data. We present a method to generate pooled donor cell populations, termed ''superlots,'' containing cell populations derived from four to five age-clustered donors. The goal of generating these superlots was to 1) increase experimental throughput, 2) to utilize assay resources more efficiently, and 3) to begin to establish global hASC differentiation behaviors that may be associated with donor age. With our superlot approach, we have validated that pooled donor cell populations exhibit proliferative activity representing the combined behavior of each individual donor cell line. Further, the superlots also exhibit differentiation levels roughly approximating the average combined differentiation levels of each individual donor cell line. We established that high donor-to-donor variability exists between the pre-, peri-, and postmenopausal age groupings and that proliferation and differentiation characteristics can vary widely, independent of age. Interestingly, we did observe that cell lines derived from postmenopausal donors demonstrated a relatively high proclivity for osteogenic differentiation and a relatively lowered proclivity for adipogenic differentiation as compared with cells derived from pre- and perimenopausal donors. In general, superlots effectively represented the average differentiation behavior of each of their contributing cell populations and could provide a powerful tool for increasing experimental throughput to more efficiently utilize resources when studying hASC differentiation.

Introduction

The study of human adipose-derived stem cells (hASC) has been a rapidly expanding area of research due to the potential implementation of hASC in a wide range of clinical applications. Both *in vitro* and *in vivo* studies have demonstrated the ability of these cells to differentiate into a variety of mesodermal lineages such as adipogenic, osteogenic, chondrogenic, myogenic, and vasculogenic cell types.[1–5] Despite their multilineage differentiation potential, hASC exhibit a high level of donor-to-donor variability.[6–9] This particular characteristic presents a challenge for studying and manipulating hASC on the bench top, as well as employing their widespread use in regenerative medicine applications in the clinic.

With this high level of donor variability, it is necessary to consider the possible sources of donor-specific hASC differentiation capacity when selecting appropriate experimental donor cell lines. To obtain data representing the consensus behavior of differentiating hASC, it is critical to incorporate both experimental technical replicates within each donor cell line and use the statistically appropriate number of donors. Generating this data can become quite experimentally expensive and may not yield reports of consensus data, as accessible patient history is often limited to gender and age. Furthermore, this experimental challenge translates into a

[1]Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill, North Carolina State University, Raleigh, North Carolina.
[2]Department of Materials Science and Engineering, North Carolina State University, Raleigh, North Carolina.

larger clinical barrier to understanding how to appropriately utilize hASC in a regenerative medicine capacity.

To circumvent the need to run multiple replicates for various cell lines derived from individual donors, some laboratories and companies now generate pooled cell lines derived from a number of donors, termed ''superlots'', in their studies.[10,11] The rationale for superlots, or pooled donor cell lines, is that the number of samples necessary to generate consensus data on hASC differentiation behavior can be minimized. However, this method of pooling cell lines has not been thoroughly investigated. It is well established that age affects the stem cell activity *in vivo* and *in vitro*; however, characterizing potency changes in aging stem cells is a complex process.[9,12–15] A number of investigators have reported conflicting data on whether adipose and/or mesenchymal stem cell potency varies with age.[9,12,16,17] This may be due to variations in donor health, isolation, and culture procedures, but may also depend on the assays used for characterization. Further complicating the consensus data in the application of hASC for musculoskeletal tissue engineering, investigators have reported that mesenchymal and adipose-derived stem cells isolated from patients or animal models of osteoarthritis have full capacity to differentiate into osteogenic, chrondrogenic, and adipogenic tissues.[18,19] Additionally, there is evidence that rabbit ASC derived from osteoporotic rabbits have a comparable capacity for *in vitro* osteogenic differentiation as compared with healthy rabbits, suggesting that a disease such as osteoporosis does not preclude the use of ASC in autologous therapies.[20] However, what a majority of investigators do agree upon is that high donor-to-donor variability is a barrier to understanding the consensus behaviors of hASC differentiation and, moreover, a barrier to their widespread clinical use.

In this study, we present a method by which we generate hASC superlots derived from four to five individual donors per superlot group, with each group delineated by age, with the goal of further understanding and potentially streamlining research approaches in studying hASC for tissue engineering applications. The donor lines were clustered based on the estimated average age of menopause in Western societies being 51 years.[21] As all the donors were female, they were generally categorized into three age groups based on the availability of cells in a specific donor range in our cell bank: premenopausal (24–36 years), perimenopausal (48–55 years), and postmenopausal (60–81 years). It is important to note that the ages and number of donor cell lines incorporated in this study were based on the availability of donor cell lines within our laboratory bank of cells. Due to isolating and maintaining our own cell bank, the number of contributing donor cell lines and the range of ages incorporated are limitations of this study. Our overall objective was to present a method for generating hASC superlots, and to validate the approach of generating age-matched pooled samples to obtain consensus data on age-matched hASC.

Materials and Methods

hASC isolation and propagation

hASC were isolated from excess adipose tissue derived from anonymous patients undergoing elective surgery (Office of Human Research at UNC, IRB exemption protocol #10-0201), at the University of North Carolina hospitals (Chapel Hill, NC). Anonymous patient data obtained included age, gender, ethnicity, and the procedure from which the tissue was collected. Isolation of hASC was performed according to previously established protocols from our laboratory,[22] adapted from methods initially reported by Zuk *et al.*[2] Once hASC were extracted from $\sim$50 g of tissue, they were allowed to propagate in culture in a complete growth medium (CGM) until $\sim$80% confluency (or up to 2 weeks). Cells were expanded in the CGM containing Minimum Essential Medium, alpha modified supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The hASC were then trypsinized and frozen down at passage 0 (p0). All hASC used in these experiments were derived from female donors (ages 24–81 years).

Potency characterization: osteogenic, adipogenic, and chondrogenic differentiation

At the time of each individual donor hASC isolation, the cell isolates were characterized based on their growth and differentiation potential. Each donor cell line was characterized as p0 cells and cultured in the CGM, adipogenic differentiation medium (ADM), and osteogenic differentiation medium (ODM). Differentiation media contained inductive supplements to the aforementioned CGM. The ADM was composed of the CGM combined with 1 μM dexamethasone, 5 μg/mL h-insulin, 100 μM indomethacin, and 500 μM isobutylmethylxanthine. The ODM was composed of the CGM with the addition of 50 μM ascorbic acid, 0.1 μM dexamethasone, and 10 mM β-glycerolphosphate.

To assess multipotency, p0 hASC were grown in each medium condition for 14 days. Staining and monitoring of morphological changes through microscopy were used to evaluate the adipogenic and osteogenic differentiation potential. Cells were seeded in six-well plates (5×10^4 cells/well) and were grown to 90–100% confluency in the CGM (1–3 days). Upon confluency, hASC were cultured in their treatment media, ODM or ADM (14 days), for osteogenic or adipogenic differentiation induction, respectively, as well as the CGM for a baseline comparison. Evidence of differentiation was visualized with phase-contrast microscopy and using Alizarin Red S for calcium accumulation (osteogenesis) and Oil Red O for lipid droplet accumulation (adipogenesis) as visualized on a dissecting microscope (Leica Microsystems, Inc.). Cross staining of Alizarin Red S in ADM- and CGM-treated wells and Oil Red O staining in ODM- and CGM-treated wells was performed to ensure that hASC yield an appropriate differentiation response (Fig. 1). Any cell lines that exhibited aberrant staining such as lipid accumulation under culture with the osteogenic induction medium or calcium accretion under culture with the adipogenic induction medium were excluded.

For an expanded study of multipotency, we also characterized our hASC lines for their chondrogenic differentiation potential. To induce chondrogenesis, hASC were cultured in chondrogenic induction media (Dulbecco's Modified Eagle's media, high glucose without glutamine, supplemented with 1% FBS, 100 U/mL penicillin, 100 μg/mL

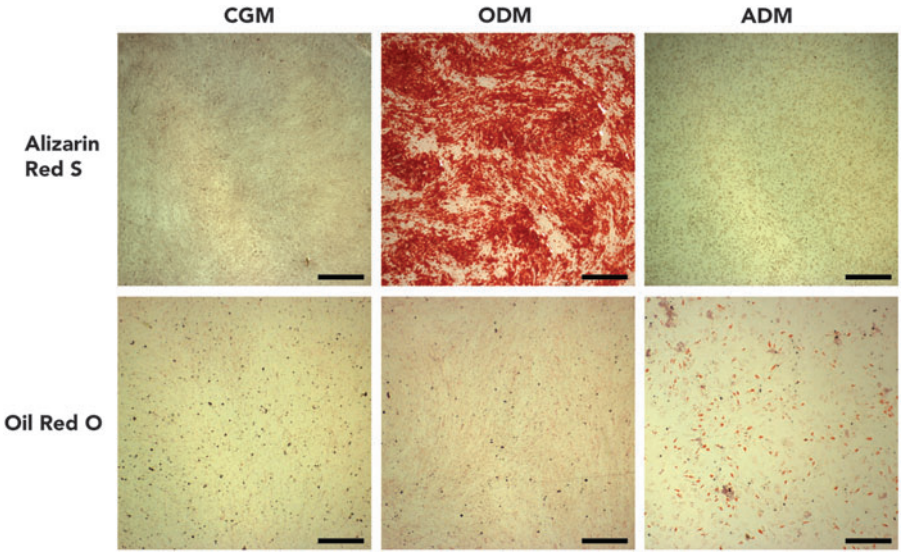

FIG. 1. Characterization and validation procedure used to evaluate freshly isolated human adipose-derived stem cells (hASC) lines. hASC seeded in a six-well plate are grown in the complete growth medium (CGM), osteogenic differentiation medium (ODM), and adipogenic differentiation medium (ADM), two wells per medium treatment, in columns. Following 14 days of culture, the hASC are then fixed and stained with Alizarin Red S indicating cell-mediated calcium accretion or Oil Red O indicating formation of lipid droplets, hallmarks of osteogenesis and adipogenesis, respectively. CGM wells provide a benchmark control to evaluate hASC responses to differentiation media and to ensure hASC are not undergoing random differentiation in the absence of induction factors. To further confirm the hASC are undergoing appropriate differentiation, cross staining of Alizarin Red S to ADM-cultured hASC and Oil Red O to ODM-cultured hASC was used to check for aberrant staining. Scale bar = 500 μm. hASC, human adipose-derived stem cells. Color images available online at www.liebertpub.com/tec

streptomycin, 4 mM L-glutamine, 6.25 μg/mL h-Insulin, 10 ng/mL transforming growth factor-β3, and 50 nM ascorbate-2-phosphate) for 3–4 weeks and were fixed, embedded in paraffin, and sectioned for analysis. Sections of the pellets were stained with Safranin O or Alcian Blue to characterize levels of hASC chondrogenesis (data not shown).

Age-matched approach to hASC superlots

It is well known that bone density changes with age. Furthermore, during and after menopause, a decrease in bone density is frequently observed. Additionally, the development of osteoporosis is often associated with the occurrence of menopause.[23] Therefore, in an attempt to take this information into consideration, we generated pooled donor hASC superlots. We categorized donor groups by the average reported ages of pre-, peri-, and postmenopausal women.[21] The premenopausal superlot was composed of cells derived from donors of age 24–36 years, the perimenopausal superlot was composed of cells derived from donors of age 48–55 years, and the postmenopausal superlot was composed of cells derived from donors of age 60–81 years. It should, however, be noted that these groupings were based on average reported data and the availability of donor cells within each age group, and were not selected based on the reported menopausal phase of the specific donor as that information was not, and is not typically, reported with the anonymous patient data.

Generating hASC superlots

The goal of creating a hASC superlot is to increase the throughput of experimental data, to acquire true consensus hASC growth and differentiation characteristics to best harness their potential for clinical applications, and to streamline the bench-to-bedside process to produce hASC-derived therapeutics that behave in a predictable regenerative capacity *in vivo.* In general, high donor-to-donor variability exists among hASC donor cell lines. In a laboratory setting, it is simple enough to select lines that generally express a high level of potency toward osteo- and/or adipogenic lineages. In contrast, when considering autologous replacement therapies in a clinical setting, one does not have the luxury of selecting for potency. It is therefore important to evaluate hASC with both high and low differentiation potential for osteogenic, adipogenic, and/or other lineages of interest.

Four to five donor cell lines were chosen as contributing cell populations to each superlot. Before selecting the specific donor hASC lines, candidate cells were qualitatively rated as strong, moderate, or poor differentiators as designated by their characteristic staining for osteogenic differentiation or adipogenic differentiation with Alizarin Red S or Oil Red O, respectively (Fig. 2). Following this qualitative ranking, a combination of strong, moderate, and poor differentiator hASC lines was selected to compose each age-grouped superlot with the goal of generating a cell population that would represent the average hASC differentiation potential of a particular age group, that is, pre-, peri-, and postmenopausal (Table 1 and Figure 3).

To create each superlot, each individual donor cell line was expanded to 80% confluency in the CGM with medium changes every 3–4 days. Once each cell line reached 80% confluency, cells were trypsinized, counted, and combined in equal proportions into a mixed superlot cell population. For one single 75 cm^2 flask, 1×10^5 total hASC were seeded

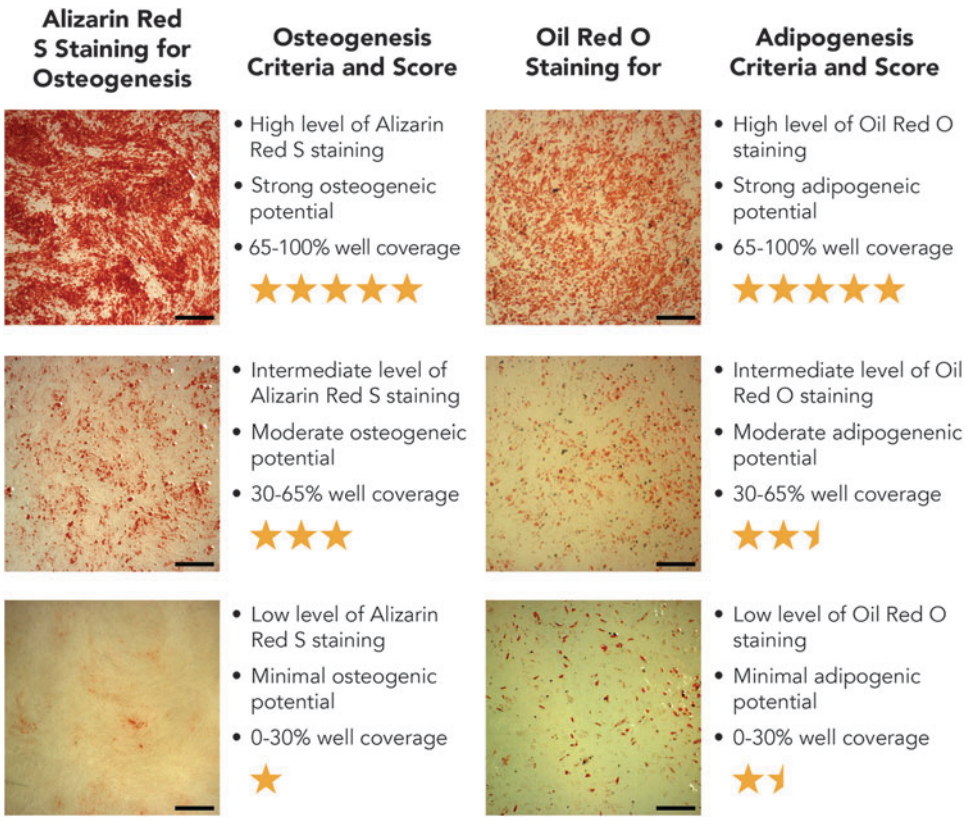

FIG. 2. Qualitative potency characterization system. Once a freshly isolated donor hASC line has been validated, its differentiation potential is qualitatively ranked on a scale from one to five stars. Chart shows examples of high-, intermediate-, and low-level differentiators, from top to bottom, and their relative osteogenic (left) and adipogenic (right) potency ranking. Scale bar = 500 μm. Color images available online at www.liebertpub.com/tec

per flask; therefore, 2×10^4 cells from each hASC donor cell line were added to create a five-donor cell line superlot for each of the pre- and postmenopausal superlots (Fig. 3). For the perimenopausal four-donor superlots, the proportions were adjusted accordingly with 2.5×10^4 cells per line to a total of 1×10^5 cells in one 75 cm^2 flask.

Validating superlots

The differentiation potential and proliferative activity of each individual donor and superlot cell line were then evaluated to validate the representative behavior of the pooled donor superlots. The proliferative activity was

Table 1. Donor Patient Profile and hASC Characterization Following Isolation

Donor ID	*Age (years)*	*Gender*	*Ethnicity*	*Tissue origin and procedure—if reported*	*Initial osteogenic differentiation (scale 1–5)*	*Initial adipogenic differentiation (scale 1–5)*
Premenopausal						
1	36	F	Other	Adipose	1.5	3.5
2	36	F	Caucasian	Adipose	3.5	3
3	24	F	Caucasian	Adipose (liposuction)	1.5	4
4	29	F	Native American	Adipose	2	2.5
5	34	F	Caucasian	Adipose	1.5	3
Perimenopausal						
6	48	F	Caucasian	Adipose (liposuction)	1	1.5
7	50	F	Caucasian	Adipose	2	2
8	55	F	Unreported	Adipose	2	1
9	49	F	Caucasian	Adipose	3	2
Postmenopausal						
10	60	F	Caucasian	Adipose (surgical removal)	3	3
11	60	F	African American	Adipose (panniculectomy)	1	2
12	70	F	Unreported	Adipose (abdominoplasty)	1.5	3.5
13	81	F	Caucasian	Adipose	3.5	2
14	61	F	Caucasian	Adipose (abdominoplasty)	3	1

Age and gender information was supplied for all hASC lines used in this study. Characterization of all 14 donor lines, as described in Figure 1, was used to validate hASC lines. Donor hASC differentiation potential was ranked on a scale from 1 to 5 as represented in Figure 2. Information on patient ethnicity and the surgical procedure from which the hASC were derived from were included in the chart, where available.

hASC, human adipose-derived stem cells.

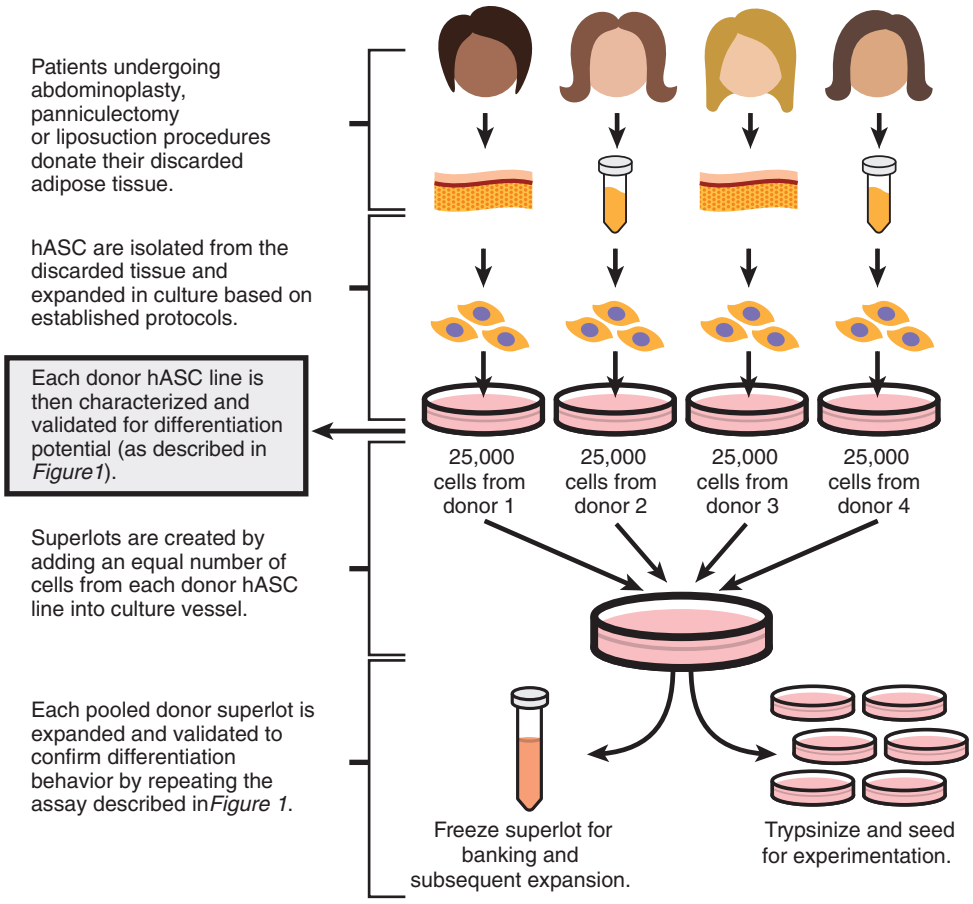

FIG. 3. Schematic describing isolation procedure of individual hASC donor cell lines, their characterization, and subsequent generation of age-matched superlots. Color images available online at www.liebertpub.com/tec

evaluated using the alamarBlue® assay (AbD Serotech). The differentiation potential was measured qualitatively using Alizarin Red S to stain calcium deposits indicating osteogenesis, and Oil Red O to stain lipid droplets, indicating adipogenesis, following 14 days of differentiation and fixation in a 10% buffered formalin solution. Differentiation images were captured using a Leica EZ4 D Digital Stereomicroscope (Leica Microsystems, Inc.). Additionally, colorimetric absorbance assays were used to quantitatively measure osteogenesis and adipogenesis on a Tecan Microplate Reader (Tecan Group Ltd.) using the Magellan™ Data Analysis Software (Tecan Group Ltd.). To measure osteogenesis, cell lysates were collected in 0.5 N HCl, and calcium was extracted through mild agitation overnight at 4°C. To measure adipogenesis, a lipid extraction buffer was used to extract lipids from accumulated lipid vacuoles in adipogenically differentiated hASC. A Calcium LiquiColor® Assay (StanBio) was used to analyze osteogenesis through calcium accretion, and a colorimetric adipogenesis assay (BioVision) was used to measure lipid accumulation. The levels of calcium accretion and lipid accumulation of the individual cell lines were compared with those of the superlots to investigate the behavior of the pooled samples relative to hASC from each individual donor.

Measuring DNA and protein content

The total DNA and protein content were measured to analyze the total cell number and physiological changes in osteogenically differentiated hASC in relation to both age and culture within a superlot. To measure the DNA content, osteogenically differentiated hASC were lysed in a papain digest DNA lysis buffer (5 mM EDTA, 5 mM cysteine HCl, and 2.5 U papain/mL in phosphate-buffered saline). The DNA content was measured under fluorescence on a Tecan Microplate Reader (Tecan Group Ltd.) using 0.2 µg/mL Hoechst 33258 dye (Molecular Probes®). To measure the protein content, cells were collected in 0.5 N HCl and the protein content of hASC lysates was measured using a colorimetric BCA absorbance assay (Thermo Scientific Pierce).

Statistical analysis

Statistical analysis was performed using Prism 5 for Mac OS X, Version 5.0a (GraphPad Software, Inc.). A one-way analysis of variance with a Newman–Keuls multiple comparison *post hoc* test was used to analyze statistical difference and to identify statistical significance among sample conditions. For each individual donor condition, $n=3$ replicates. Pre- and postmenopausal groups had $n=5$ individual donors and perimenopausal had $n=4$ individual donors. Statistical significance is indicated as follows: $*p<0.05$, $**p<0.01$, and $***p<0.001$.

Results

To ensure that all cell lines were propagating in the pooled superlot cultures, we analyzed the proliferation profiles of individual cell lines in addition to the profiles of

the superlots. We analyzed the pre-, peri-, and postmenopausal age groups to see if there were any notable differences in the proliferation rates as measured by alamarBlue, indicative of metabolic activity (Fig. 4). In general, the proliferation profile of the superlots fell roughly within the range of all the individual cells lines for the pre-, peri-, and postmenopausal clusters (Fig. 4). There was no noticeable difference in the proliferation rate between the two age groups; however, there were differences among specific donor cell lines in pre-, peri-, and postmenopausal groups, suggesting that hASC proliferation is donor specific as opposed to age related.

High donor-to-donor variability generally existed in both osteogenic and adipogenic differentiation levels among all the three age groupings (Figs. 5 and 6). Osteogenic levels for individual donors ranged from 0.0179 to 11.63 calcium/DNA for premenopausal cell lines, from 0.548 to 2.489 calcium/DNA for perimenopausal cell lines, and from 0.548 to 16.030 calcium/DNA for postmenopausal cell lines (Fig. 5). Adipogenic values for individual donor cell lines ranged from 0.8 to 5.0 pmol lipids/μg protein for premenopausal cell lines, from 0.7 to 2.9 pmol lipids/μg protein for perimenopausal cell lines, and from 0.0 to 0.6 pmol lipids/μg protein for postmenopausal cell lines (Fig. 6). Individual cell lines did not consistently demonstrate multipotency, but rather, particular lines exhibited a proclivity for differentiating toward a particular lineage.

Calcium accretion (Fig. 5) and lipid accumulation levels (Fig. 6) of each of the age-grouped superlots fell within the range of differentiation levels of each individual donor cell line contributing to the superlot. This result was generally observed for all age groups in osteogenesis and adipogenesis, roughly validating the principle of pooled donor cell lines exhibiting the average differentiation behavior of the combined donor cells. In general, the superlots roughly approximated the combined average adipogenesis levels of the individual donor lines. This also followed for the osteogenesis of the perimenopausal superlot, however, the pre- and postmenopausal superlot osteogenic levels were the exception to this trend with the combined donor averages (4.035 calcium/DNA and 6.11 calcium/DNA, respectively) predicting a value twice the superlot calcium accretion levels of the premenopausal superlot and two-thirds that of the postmenopusal superlot (1.7822 calcium/DNA and 9.605 calcium/DNA, respectively) (Fig. 5a, c).

Although the high level of donor variability was observed across all age groups, some general differentiation trends were observed among age groups. Surprisingly, the postmenopausal group exhibited higher osteogenic calcium accretion levels as compared with the pre- ($p<0.001$) and perimenopausal superlots ($p<0.001$) (Fig. 7a, c–e). Conversely, the perimenopausal group had approximately half the adipogenic lipid accumulation levels of the premenopausal superlot ($p<0.01$), and the postmenopausal superlot had nearly half of the perimenopausal lipid accumulation levels (Fig. 7b, f–h).

As hASC undergo osteogenic differentiation, continued proliferation persists as part of the osteogenic phenotype. Furthermore, as osteogenesis proceeds, hASC begin to deposit extracellular matrix proteins. To evaluate this, we measured the total DNA and protein content following 14 days of osteogenic differentiation. We found that the total DNA yield decreased with age (Fig. 8a, c), however, the total protein yield following osteogenic differentiation increased with age (Fig. 8b, d). This trend was observed both in the combined donor averages and superlots (Fig. 8). The consistent trends between superlots and average donor data further validate the use of superlots to represent the average

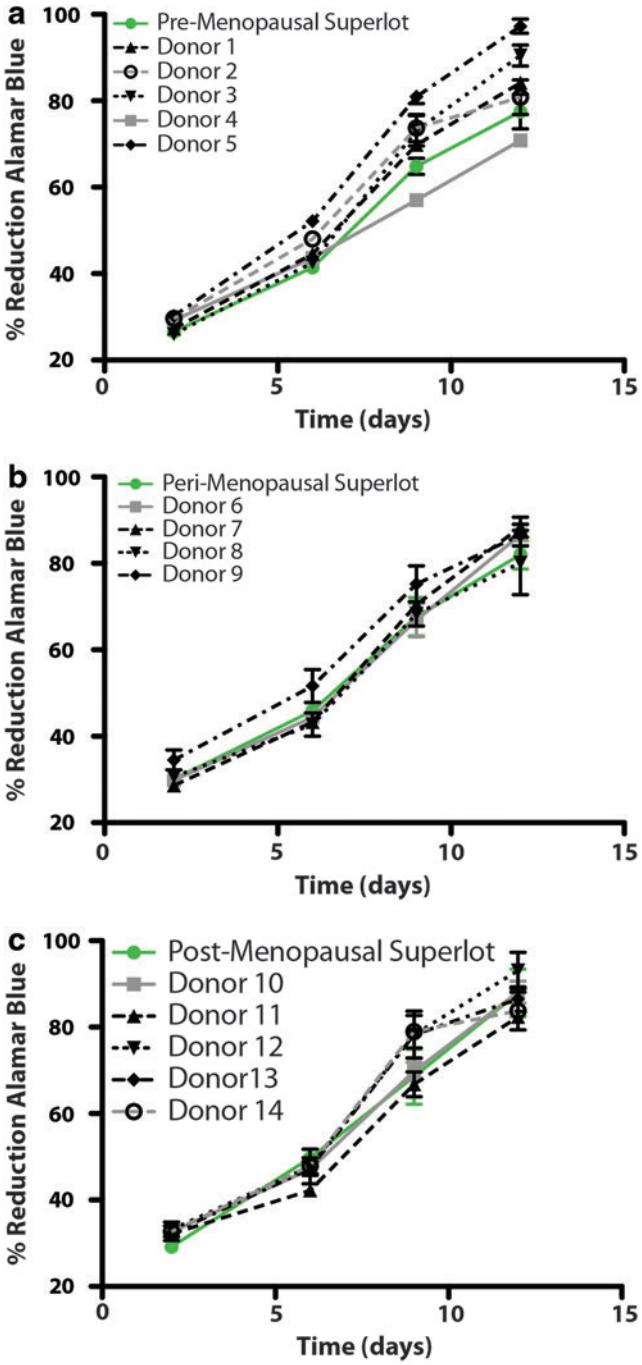

FIG. 4. Proliferation profiles of pre- **(a)**, peri- **(b)**, and postmenopausal **(c)** donor and superlot cell lines for up to 12 days in an expansion medium (CGM). The proliferation activity is measured by percent reduction of alamarBlue, indicative of metabolic activity and increased proliferation. $n=3$ replicates, error bars represent SEM. SEM, standard error of the mean. Color images available online at www.liebertpub.com/tec

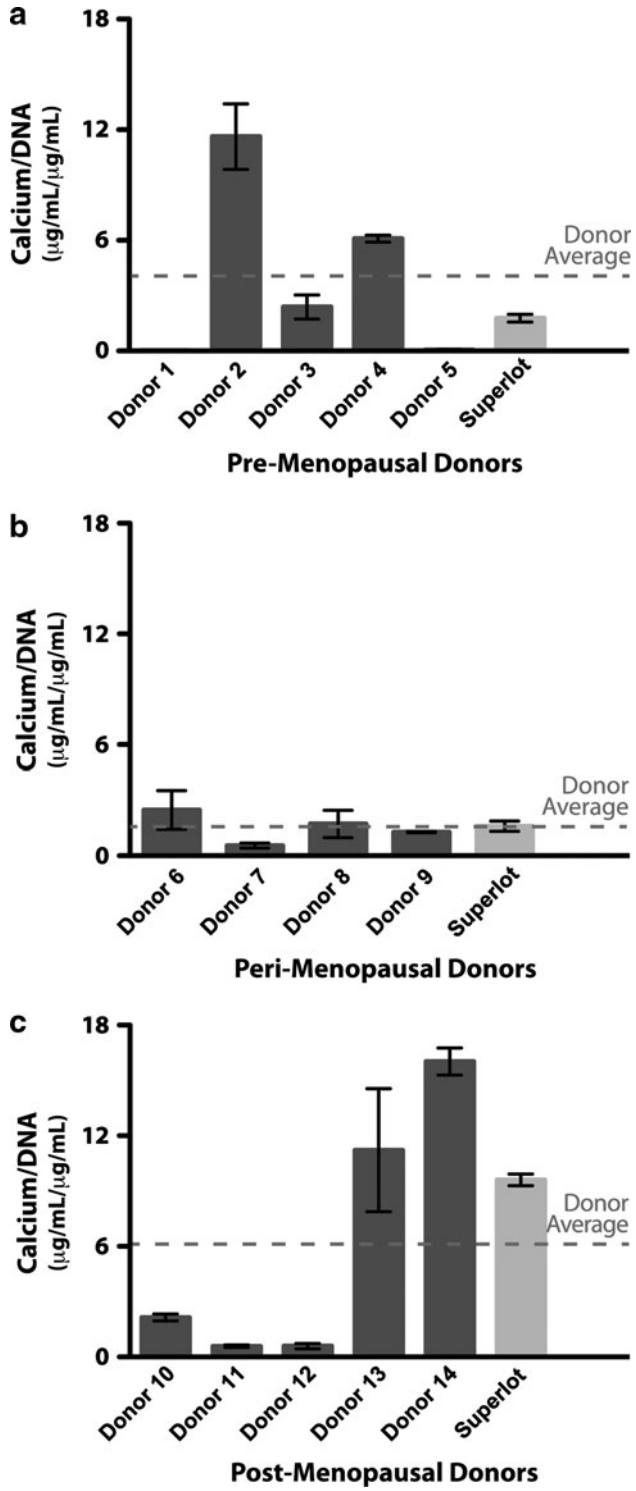

FIG. 5. Osteogenic differentiation of pre- **(a)**, peri- **(b)**, and postmenopausal **(c)** donor lines and superlots following 14 days of culture in ODM. Calcium accretion is normalized to DNA content for individual donor lines contributing to each age-matched superlot. Red line indicates the combined average calcium/DNA value of the contributing donor lines, which represents the theoretical reference value for the calcium/DNA superlot value. High donor-to-donor variation is observed across age groups. $n = 3$ replicates per donor or superlot, error bars represent SEM.

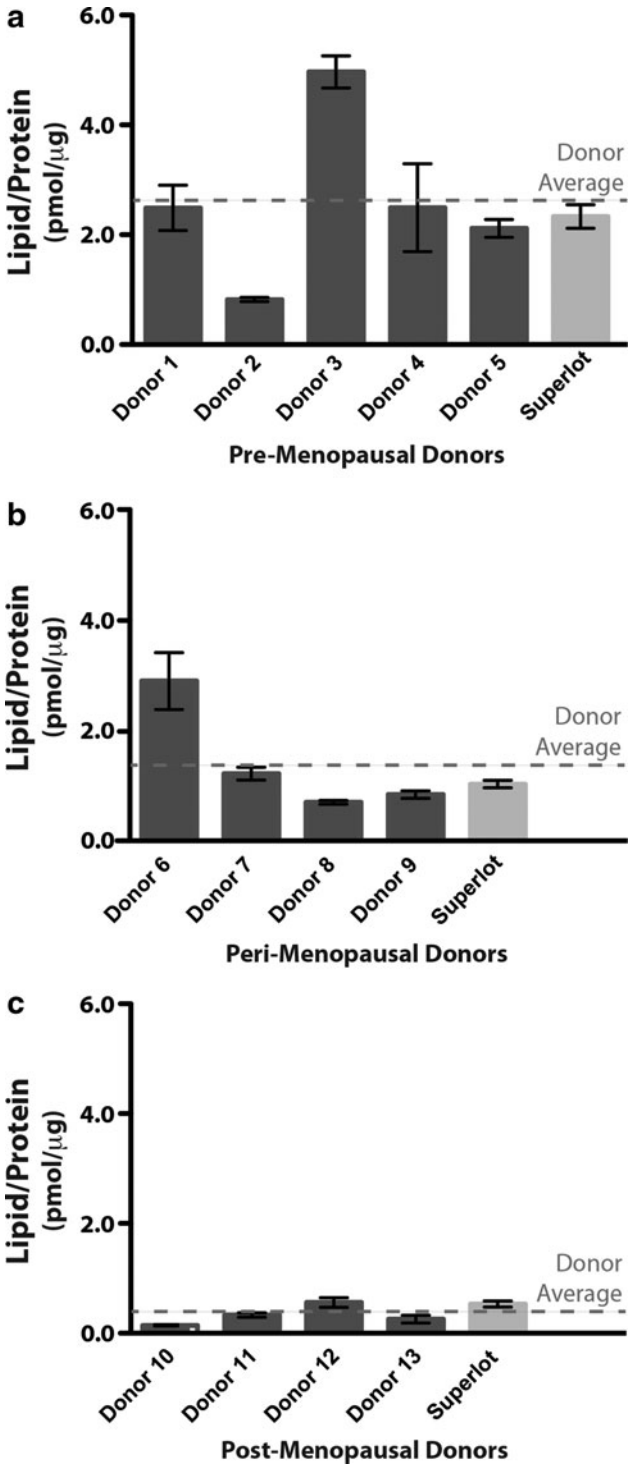

FIG. 6. Adipogenic differentiation of pre- **(a)**, peri- (**b**), and postmenopausal **(c)** donor lines and superlots following 14 days of culture in ADM. Lipid accumulation levels normalized to protein for individual donor lines contributing to each age-matched superlot. Red line indicates the combined average lipid/protein value of the contributing donor lines, representing the theoretical value for the lipid/protein superlot value. High donor-to-donor variation is observed within age groups. Generally, donor hASC derived from older patients expressed reduced potential for adipogenic differentiation, particularly, the postmenopausal group. $n = 3$ replicates per donor or superlot, error bars represent SEM.

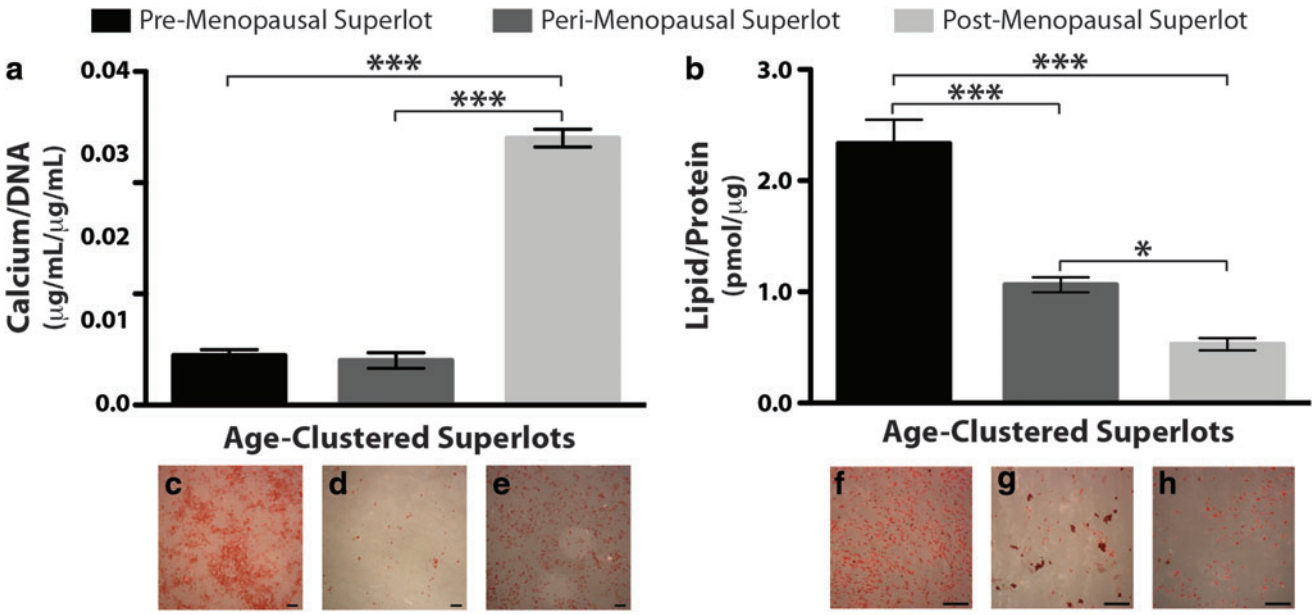

FIG. 7. Summary of pre-, peri-, and postmenopausal superlot potency. Osteogenic differentiation as measured quantitatively by Calcium Liquicolor Assay® **(a)** and adipogenic differentiation as measured by lipid accumulation assay **(b)**, following 14 days of culture in ODM and ADM, respectively. Alizarin Red S staining to visualize superlot osteogenesis through calcium accretion of pre- **(c)**, peri- **(d)**, and postmenopausal **(e)** superlots. Oil Red O staining to visualize superlot adipogenesis through lipid droplet staining of pre- **(f)**, peri- **(g)**, and postmenopausal **(h)** superlots. $n = 3$ replicates per superlot, error bars represent SEM. Scale bar = 500 μm. $^{*}p < 0.05$; $^{***}p < 0.001$. Color images available online at www.liebertpub.com/tec

behavior of individual donors. They also provide a useful tool in highlighting age-related differences in the physiology of hASC undergoing osteogenic differentiation (Fig. 8).

Discussion

The goal of this article was to evaluate and present a method to more efficiently execute studies using hASC to facilitate their eventual use in clinical applications. More specifically, we presented an approach to globally understand hASC as a cell type and to more effectively evaluate their potential as an autologous cell source in tissue replacement therapies. A majority of studies reporting data on hASC use preselected donor cell lines known to be strong differentiators and often hASC characterized as poor differentiators are excluded from experimental cell sources.[24,25] More recently, some investigators have been utilizing commercially available hASC superlots or have been preparing them in-house; however, there is a dearth of data characterizing the effects of propagating these mixed donor cell populations. Clearly, studying pooled cell populations will significantly increase throughput, but validating their differentiation potential in a mixed donor population is critical to drawing results from more sophisticated hASC superlot studies.

To effectively understand the physiological behavior of hASC for clinical applications, it is necessary to study the range of their differentiation capacities. To address this issue, we have used the approach of generating superlots, containing four to five age-clustered pooled cell populations. In our superlots, we preselected cell lines that were characterized to include low-, medium-, and high-level differentiators (Fig. 2 and Table 1), to determine whether they would accurately exhibit a differentiation pattern characteristic of the combined average of the individual cell lines. When compared with the osteogenic and adipogenic differentiation levels of each individual donor, the superlot differentiation levels generally approximated the average value of all the individual donors, with the exception of calcium accretion of the pre- and perimenopausal superlots (Figs. 5 and 6). For most part, we confirmed our expected result, suggesting that there is likely a minimal reactivity between cells from different donors. Moreover, this also suggests that the proportion of each donor cell population remains roughly equal following propagation and differentiation. This is further supported by only nominal differences in the proliferation profiles among hASC donors across age groups and the fact that the superlot proliferation profiles fit within the range of donor profiles (Fig. 4).

The caveat to utilizing the superlots can be observed in the case of the pre- and postmenopausal superlots expressing calcium accretion levels notably below or above the predicted combined average, respectively. The premenopausal superlot underperformed, while the postmenopausal overperformed in representing the consensus osteogenic differentiation level of the group, with the premenopausal expressing approximately half the expected level of average calcium accretion (Fig. 5a) and the postmenopausal superlot expressing an $\sim 30\%$ increase compared with the predicted average (Fig. 5c). We speculate that the large difference between the expected average combined donor value and the superlot value of calcium may be skewed due to specific donor lines accreting little to no calcium over the course of a 14-day differentiation. This particularly comes into play when normalizing to the DNA content, which is

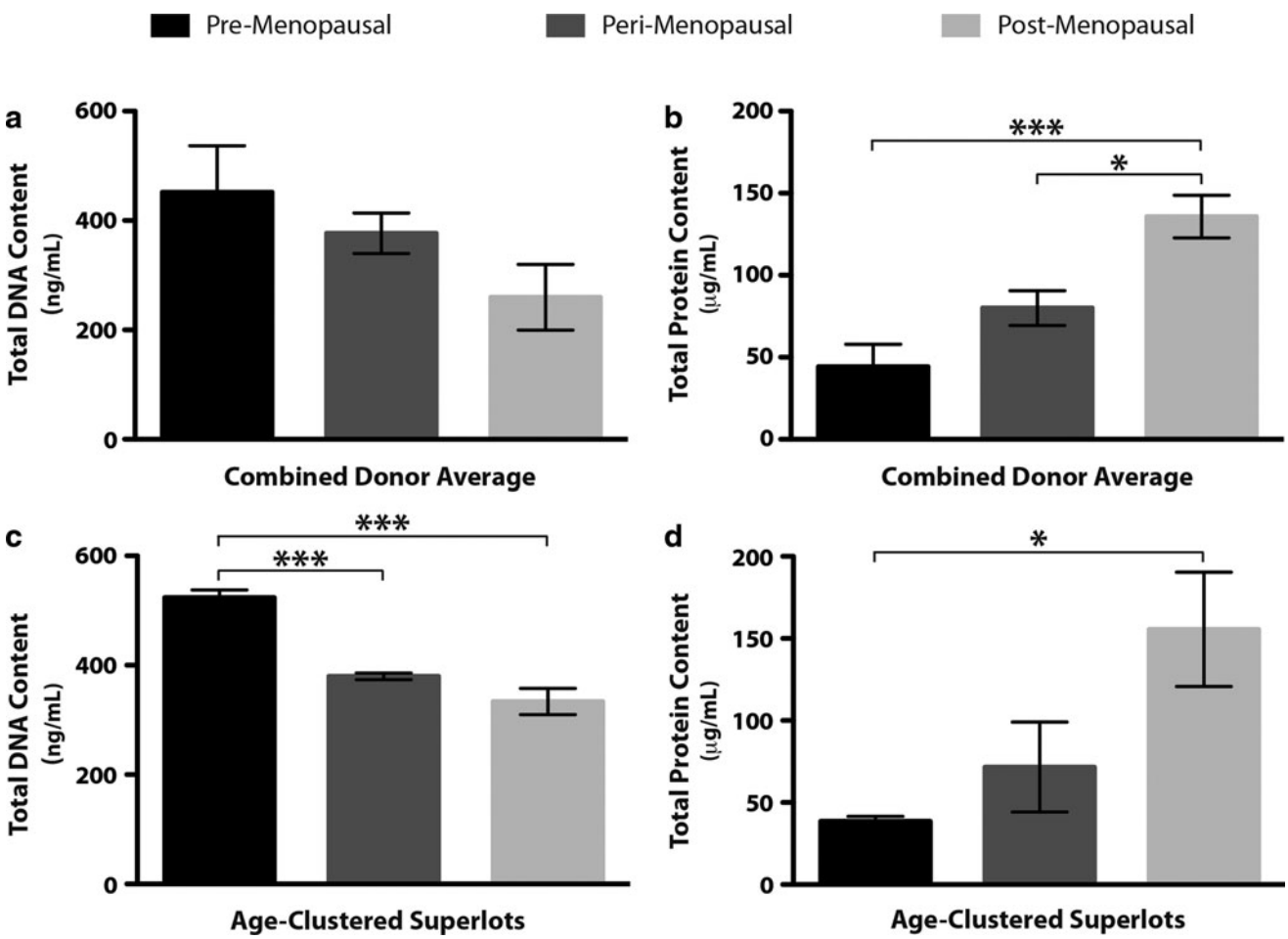

FIG. 8. Total DNA and protein content levels in age-clustered groups. Following 14 days of osteogenic differentiation, total DNA **(a, c)** and protein **(b, d)** content were measured for each individual donor cell line and superlots. Top row graphs represent the average DNA **(a)** and protein **(b)** content of individual cell lines ($n = 5$ donors for pre- and postmenopausal, $n = 4$ donors for perimenopausal). Bottom row graphs represent DNA **(c)** and protein **(d)** content of each each superlot ($n = 3$ replicates for each superlot). Error bars represent SEM.

proportional to the number of cells in culture. Human ASC growing in culture, expressing minimal calcium have the potential to significantly reduce the calcium/DNA ratio. Moreover, the general cell-lineage specification paradigm proposes that proliferation and differentiation are somewhat opposing processes.[26] Once a cell population begins to undergo differentiation, its proliferation activity is impeded. However, it may be possible that the proliferative activity remains higher through day 14 for donor hASC that show little to no evidence of osteogenic differentiation, skewing the calcium/DNA data. These results are corroborated by the high level of DNA content in premenopausal donors and the relatively low level of DNA in postmenopausal donors, following 14 days of differentiation (Fig. 8a, c). Interestingly, this issue did not affect donor lines undergoing adipogenic differentiation, but this may also be due to the lowered metabolic activity of the adipogenic phenotype and the media formulation. We have observed that hASC cultured under the ODM tend to persist in a proliferative capacity slightly longer than hASC cultured under the ADM. This could also be related to lineage-specific metabolic and physiologic processes resulting in hASC quiescence/senescence under induction of lineage commitment.[12,14] The skewing of the osteogenic superlot data may hint that hASC exhibiting minimal to no evidence of calcium accretion express cell physiologies preventing them from undergoing osteogenesis and/or accreting calcium. Therefore, it is critical that investigators utilizing pooled hASC superlots proceed with caution—acknowledging that the superlot cell populations have a higher level of heterogeneity compared with single donors, which can affect the outcome of studies.

Despite the skewed pre- and postmenopausal data, there is considerable value in employing superlots containing donor lines with differing potencies. The reasoning for selecting this range of potency levels within our cell lines was to begin to glean potency information of a typical hASC. Extending this thinking further, using superlots, may facilitate prediction of the differentiation potential for an average patient's hASC. Since hASC are so extensively studied for their promise as an autologous cell source for tissue replacement therapies, it is important to acquire practical data that can be more directly translated into the clinic. That is to say, every patient will not have hASC with a high level of multipotency or hASC that exhibit high proclivity toward

the desired cell lineage. Through studying the hASC superlot differentiation activity, investigators can begin to profile the average patient, and subsequently hone research approaches to address the limitations of the hASC source.

In addition to using a superlot approach to characterize the typical patient hASC population, we thought it necessary to highlight the extreme differences among hASC donor cell differentiation potential. This is not only clinically relevant information but suggests that as investigators we should evaluate reported data with a critical eye. In the future it may be necessary to develop a classification system to identify predictors of differentiation potential. Although some work has been done profiling hASC using cell surface markers,[6,27] microarray surveys of gene expression,[28,29] and microRNA profiles,[12] these data have not been extensively correlated with hASC potency. It is important to note that the ages and number of donor cell lines incorporated in this study were based on the availability of donor cell lines within our laboratory bank of cells. Due to isolating and maintaining our own cell bank, the number of contributing donor cell lines and the range of ages incorporated are limitations of this study. It is additionally important to consider the *in vivo* differentiation capacity of hASC. This study describes a method to consider donor-specific effects on hASC differentiation, toward the goal of clinically employing them in tissue engineering applications, however, we only consider their *in vitro* differentiation capacity. The differentiation behavior of hASC derived from donors of a specific age, and that of superlots, will need to be further investigated to elucidate the valid application of these data within *in vivo* model systems.

According to our data, which display a high degree of donor-to-donor variability, regardless of age, we suggest that a method for baseline characterization of hASC be reported along with donor patient data. Stark differences were observed among the relative differentiation capacities of pre-, peri-, and postmenopausal age groups and the trends were contrary to what was expected, showing the youngest group expressing the highest proclivity for adipogenic differentiation and the oldest group expressing the highest proclivity for osteogenesis (Fig. 7). These findings are consistent with other human and animal ASC studies exhibiting a decrease in adipogenesis with age.[16,17] Interestingly, our osteogenesis results showing increased calcium accretion in the postmenopausal age group are consistent with work in human mesenchymal stem cells derived from osteoporotic postmenopausal donors.[19] However, the effect of age on hASC differentiation, particularly osteogenesis, has been largely debated in the literature.[9,12,14,16]

In addition to variations in hASC potency with age, hASC also exhibit age-related changes in total DNA and protein content with age, following 14 days of osteogenic differentiation (Fig. 8). Generally, premenopausal hASC exhibited the highest DNA content following 14 days of osteogenesis, while postmenopausal cells had the highest level of protein. This may, in part, explain the lowered level of osteogenesis as this may indicate higher proliferation of hASC derived from younger patients, thus slowing the lineage commitment process, while hASC derived from postmenopausal donors slow their proliferation as evidenced by a relatively lowered DNA content (Fig. 8a, c). This is further corroborated by the fact that hASC derived from postmenopausal donors exhibited relatively higher total protein values than pre- and perimenopausal cell lines following 14 days of osteogenesis, which may be indicative of increased extracellular matrix production, a hallmark of osteogenesis (Fig. 8b, d). It is important to note that when hASC are cultured under expansion media for up to 12 days, the proliferation profile among age groups does not vary drastically (Fig. 4), suggesting that these changes are specifically related to hASC developing the osteogenic phenotype.

Our data suggest that there may be age-related changes in hASC potency, which are likely related to physiological changes associated with menopause. It should be noted that, with the limitations of our study and our currently catalogued cell bank, we only grouped our age-matched superlots based on the average purported age of menopausal phases, and supplied patient information did not include menopausal information. With that general information, we do hypothesize that these hASC potency changes may be related to the physiological changes in the body such as a lowering of bone density and osteoblast/osteoclast activity already known to affect women following menopause.[30] However, we propose that to generate more informative age-related studies in the future, it would be valuable to acquire female patient information on the menopausal phase. Having this information would allow hASC studies to report more definitive results on the differentiation potential of hASC and further the capacity and limitations of their therapeutic applications. Although there is a strong relationship between the menopausal phase and bone density and physiology, the age-matched hASC superlot approach proposes a streamlined tactic to predict the behavior of tissue-engineered constructs of other musculoskeletal tissues such as cartilage, tendon, and muscle. In the case of other tissues, it may be useful to cluster the ages of the donors around critical developmental phases for a given tissue and/or even to develop specific donor patient criteria to select for characteristics beneficial to a particular lineage. This study is the first step in building this approach.

It should be noted that this study focuses on *in vitro* behavior of superlots, however, it is unclear whether superlots can predict hASC differentiation when used in studies in which hASC are implanted *in vivo*. Exploring the use of superlots supports the goal of utilizing hASC for autologous tissue replacements although the cells are derived from many patients and there is potential for an immune response. Nonetheless, their potential to streamline hASC studies and increase throughput is clear, and thus their *in vivo* characterization may be a critical next step to validating their efficacy.

One final caveat to this study, and a majority of hASC studies in the literature that should be noted, is that hASC are largely sourced from overweight to severely obese patients. The hASC in our cell banks are obtained from discarded tissue derived from abdominoplasty, panniculectomy, or liposuction tissue. Although all three procedures are technically cosmetic surgery, we do not have patient body mass index (BMI) or weight data, and thus, little indication of the patient's health status. It is likely that hASC derived from healthy patients will exhibit a different differentiation profile from those derived from severely obese patients.[31,32] Other sources of variation may include the specific medical procedure with which a tissue was obtained, however, it is likely that patient physiology may have the greatest influence. This

may partially explain the high level of donor-to-donor variation across age groups. Understanding the source of the differential hASC potencies is essential to applying hASC in clinical procedures.

In conclusion, we have described a method for generating age-matched pooled donor cell lines to produce hASC superlots containing cells from four to five donors. We have demonstrated that the hASC can proliferate and differentiate in these pooled donor cell environments and that they exhibit the combined average osteogenic and adipogenic differentiation levels of each donor cell line. Furthermore, we highlight the high degree of donor-to-donor variation in differentiation capability that occurs among donor cell lines and suggest that superlots may be a way to circumvent this issue when generating experimental data. We do, however, suggest that researchers proceed with caution in determining whether their particular study is best suited for analyses using individual donor hASC lines or hASC superlots. Either approach will depend, of course, on the particular scientific question being asked. That being said, we propose that hASC superlots are a powerful tool to study hASC, and may provide an efficient way to increase data throughput to facilitate hASC use in clinical applications.

Acknowledgments

The authors would like to thank Dr. Liliana Mellor for her technical expertise. They would also like to acknowledge their funding sources: NIH/NIBIB grant R03EB008790 (E.G.L.), NIH/NCRR10KR51023 (E.G.L.), NC TraCS 10KR51023 (E.G.L.), NSF/CBET1133427 (E.G.L.), and the NSF Graduate Research Fellowship Program (J.C.B.).

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Elizabeth G. Loboa, PhD
Joint Department of Biomedical Engineering
University of North Carolina at Chapel Hill
North Carolina State University
911 Oval Drive
4208B EB3, Campus Box 7115
Raleigh, NC 27695-7115

E-mail: egloboa@ncsu.edu

Received: November 6, 2013
Accepted: March 12, 2014
Online Publication Date: April 28, 2014