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Markers are shared between adipogenic and osteogenic differentiated mesenchymal stem cells

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The stem cell differentiation paradigm is based on the progression of cells through generations of daughter cells that eventually become restricted and committed to one lineage resulting in fully differentiated cells. Herein, we report on the differentiation of adult human mesenchymal stem cells (hMSCs) towards adipogenic and osteogenic lineages using established protocols. Lineage specific genes were evaluated by quantitative real-time PCR relative to two reference genes. The expression of osteoblast-associated genes (alkaline phosphatase, osteopontin, and osteocalcin) was detected in hMSCs that underwent adipogenesis. The expression of adipocyte marker genes (adiponectin, fatty acid binding protein P4, and leptin) increased in a time-dependent manner during adipogenic induction. Adiponectin and leptin were also detected in osteoblast-induced cells. Lipid vacuoles that represent the adipocyte phenotype were only present in the adipogenic induction group. Conforming to the heterogeneous nature of hMSCs and the known plasticity between osteogenic and adipogenic lineages, these data indicate a marker overlap between MSC-derived adipocytes and osteoblasts. We propose a careful consideration of experimental conditions such as investigated time points, selected housekeeping genes and the evidence indicating lack of differentiation into other lineages when evaluating hMSC differentiation.

Key words: Mesenchymal stem cell, differentiation markers, cell plasticity, differentiation.

INTRODUCTION

The human bone marrow stroma contains multipotent mesenchymal cells that give rise to adipocytes and osteoblasts, as well as many other lineages (Caplan and Dennis, 2006). Cells isolated based on adherence to the tissue culture substrate do not represent a homogenous population of mesenchymal progenitors rather subpopulations of cells with variable differentiation potential (Muraglia et al., 2000; Pittenger et al., 1999). Most of the clones derived from bone marrow stromal cells possess osteogenic and adipogenic differentiation potential but some are only able to differentiate towards osteoblasts. The mechanisms of the differentiation process from precursor to fully differentiated mature cells are still not fully understood (Discher et al., 2009; Hwang et al., 2008; Scadden, 2006).

Methods have been developed for differentiating cells into specific differentiated cell types expressing the markers and phenotypes of the desired tissues (Pittenger et al., 1999). New materials-based and soluble factor-based differentiation protocols are constantly being developed to control the differentiation potential of all stem cell types (Fekete et al., 2012; Hoshiba et al., 2012; Keskar et al., 2009; Kollmer et al., 2012; Vater et al., 2011). Many of these protocols are validated to confirm the pre-

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sence of specific differentiation markers, but frequently alternative differentiation pathways are not excluded. In addition, many in the field of tissue engineering utilize a small subset of differen-tiation markers (Bakhshandeh et al., 2012; Choi et al., 2010; He et al., 2012; Henderson et al., 2008; Hess et al., 2012; Marion et al., 2006; Pountos et al., 2007; Wiren et al., 2011; Zhang et al., 2012) despite the potential for expression of these markers in other lineages.

There are several examples in the literature showing the expression of a differentiation marker by multiple cell types. Leptin, an adipokine produced by adipocytes was observed on the mRNA level in human osteoblasts during the mineralization period (Reseland et al., 2001) as well as in hMSCs that underwent osteogenesis (Noh, 2012). Leptin has pleiotropic effects on other bone marrow cells, including osteoblasts (Noh, 2012; Nuttall and Gimble, 2004) and was shown to promote osteogenesis and to inhibit adipogenesis in immortalized human marrow stromal cells (Thomas et al., 1999). This could be paracrine communication controlling the growth and differentiation of adipocytes and signaling osteogenesis when sufficient adipocytes are present.

Alkaline phosphatase, widely used as a biochemical marker of bone turnover, also plays a role in adipogenesis. Inhibition of tissue-nonspecific alkaline phosphatase resulted in a decreased accumulation of lipid vacuoles during adipogenic differentiation of a murine preadipocyte cell line (Ali et al., 2005). Similarly, osteopontin (OPN) is not solely a key regulator of bone development, rather a multifunctional extracellular matrix (ECM) associated protein involved in inflammatory processes, tumorigenesis, cardiac fibrosis and obesity (Sodek et al., 2000). Upregulated OPN mRNA levels have been detected in adipose tissue of obese patients (Chapman et al., 2010). Osteocalcin, a non-collagenous protein found in mineralized adult bone, is another widely used bone marker. However, constitutive osteocalcin mRNA and protein expression by adipose stromal cells implicates that nonosteogenic cells of the marrow stroma also secrete osteocalcin (Benayahu et al., 1997). Exposure to the glucocorticoid dexamethasone which is a constituent of both, osteogenic and adipogenic differentiation media, has been shown to increase osteocalcin expression in cultured stromal cells (Leboy et al., 1991).

We can infer from these reports that those markers need further validation as tissue-specific differentiation markers. In the present study, we assessed the suitability of fatty acid binding protein 4, adiponectin and leptin as adipogenic differentiation markers and alkaline phosphatase, collagen type I, osteocalcin and osteopontin as osteogenic differentiation markers by evaluating the expression of these markers during adipogenic and osteogenic culture conditions. We show that these markers are not selectively expressed when cells are differentiated using common differentiation protocols.

MATERIALS AND METHODS

Human mesenchymal stem cell isolation and differentiation

Human bone marrow aspirates were obtained from AllCells, LLC (Emeryville, CA) and isolated by density gradient centrifugation utilizing Ficoll-Paque™ PLUS solution followed by cell-surface marker negative selection with RosetteSep® Human Mesenchymal Stem Cell Enrichment Cocktail (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's protocol. For each experiment, hMSCs isolated from one of three donors (non-smoker males ranging in age from 20 to 31 years old) were used with no cells used beyond passage four.

Cells were harvested using 0.25% trypsin with 1.0 M EDTA, centrifuged, and expanded in basal medium which consists of high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin and 100 unit/mL streptomycin (basal medium). Medium was changed every third day. Adipogenic differentiation was initiated by culturing 2×10^5 hMSCs in a well of a 6 well plate in MesenCult® adipogenic induction medium (Stem Cell Technologies, Vancouver, BC, Canada). The composition of the adipogenic medium is proprietary.

Adipogenic differentiation protocols routinely involve combinations of dexamethasone, 3-isobutyl-1-methyl-xanthine (IBMX), insulin, and indomethacin (Vater et al., 2011). Osteogenic differentiation was initiated by culturing 3×10^4 hMSCs in a well of a 6 well plate in Poietics™ osteogenic induction medium (Lonza, Walkersville, MD, USA) containing dexamethasone, ascorbic acid and β-glycerophosphate supplements (Pittenger et al., 1999; Vater et al., 2011). Controls were maintained in basal medium for the entire culture period of 4 weeks.

Quantitative real-time PCR

After 14 and 28 days, total RNA was extracted using the TRIzol® reagent (Life Technologies, Grand Island, NY, USA) in combination with the PureLink™ RNA Mini Kit (Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. To diminish genomic DNA contamination, RNA was treated with Turbo™ DNAse (Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. The purified RNA (10 ng/mL) was reverse transcribed with the High Capacity cDNA Reverse Transcription (RT) Kit (Life Technologies, Grand Island, NY, USA) under the following conditions: 25°C for 10 min, 37°C for 120 min followed by 85°C for 5 min.

To identify potential genomic DNA contamination, controls with no enzyme were evaluated. The PCR reactions were performed on an Applied Biosystems StepOnePlus™ PCR machine using 5 µL SYBR® Green PCR Master Mix (Life Technologies, Grand Island, NY, USA), 2 µL sequence specific primers (0.5 mM, *GAPDH* was used at 0.25 mM) (Table 1) and 3 µL cDNA (cDNA dilutions: *ADIPOQ, ALPL, FABP4, OPN*: 10 fold*, BGLAP*: five-fold*, COL1A1, LEP*: 31 fold) under the following conditions: 95°C for 10 min followed by 40 cycles of 15 s of denaturation at 95°C and 60 s of annealing and elongation at 60°C.

A melting curve analysis was performed after each run to confirm product specificity. The delta-delta-Ct method (Livak and Schmittgen, 2001) was employed to determine the relative gene expression level of the gene of interest normalized to the endogenous controls glyceraldehyde-3-phosphate (*GAPDH*) and ribosomal protein L13A (*RPL13A*). Statistical significance was determined using first a two-way ANOVA comparing the treatments and time followed by Newman-Keuls' post-hoc comparison of groups.

Table 1. Genes and primers used for qPCR.

Gene	Full Name	Sequences 5´-> 3´	Accession number/ Reference
ADIPOQ	Adiponectin	For: AGG GTG AGA AAG GAG ATC C Rev: GGC ATG TTG GGG ATA GTA A	NM_004797
ALPL	Alkaline Phosphatase	For: ATT TCT CTT GGG CAG GCA GAG AGT Rev: ATC CAG AAT GTT CCA CGG AGG CTT	NM_000478.4
BGLAP	Bone gamma-carboxyglutamate (Osteocalcin)	For: CAG CGA GGT AGT GAA GAG AC Rev: TGA AAG CCG ATG TGG TCA G	NM_199173
COL1A1	Collagen type I	For: TGT GGC CCA GAA GAA CTG GTA CAT Rev: ACT GGA ATC CAT CGG TCA TGC TCT	NM_000088
FABP4	Fatty acid binding protein 4	For: TGG TTG ATT TTC CAT CCC AT Rev: TAC TGG GCC AGG AAT TTG AC	NM_001442
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	For: TTC GAC AGT CAG CCG CAT CTT CTT Rev: GCC CAA TAC GAC CAA ATC CGT TGA	NM_002046.4
LEP	Leptin	For: CTG ATG CTT TGC TTC AAA TCC A Rev: GCT TTC AGC CCT TTG CGT T	NM_000230
OPN	Osteopontin	For: AGA ATG CTG TGT CCT CTG AAG Rev: GTT CGA GTC AAT GGA GTC CTG	NM_001251830
RPL13α	Ribosomal protein L13 α	For: CAT AGG AAG CTG GGA GCA AG Rev: GCC CTC CAA TCA GTC TTC TG	NM_012423

Immunofluorescence

Briefly, the samples were fixed in 4% paraformaldehyde, rinsed with 1X PBS and incubated with blocking solution (1% BSA in 1X PBS) for 30 min on a shaker. Osteocalcin mouse monoclonal IgG antibody (25µL; 100 µg/mL; Santa Cruz Biotech, Santa Cruz, CA, USA) was added directly to 500 µL blocking solution and samples were further incubated for 3 h at room temperature (RT). Samples were washed with 1X PBS and incubated with 5 µL AlexaFluor® 488-labeled goat anti-mouse secondary antibody (2 mg/mL; Molecular Probes, Carlsbad, CA) in 500 µL 1X PBS at RT and protected from light. After 25 min, 0.5 µL H33258 (1 mg/mL; Life Technologies, Grand Island, NY, USA) was added to the solution and incubated for an additional 5 min at RT. The secondary antibody solution was discarded after 30 min and the samples were washed with 1X PBS. Images were taken with an Olympus IX70 inverted microscope and processed using QCapture Pro software.

Sudan III staining

To stain lipid vacuoles, the samples were rinsed with 1X PBS (pH 7.4) and incubated with Sudan III solution (0.3% w/v of Sudan III in 70% ethanol) for 3 min. After several washes with double deionized water (DDIW), Harris hematoxylin solution was added and incubated for 1 min. Samples were destained in fresh acid ethanol (0.5% 1 N HCL in 70% EtOH) for 1 min. Afterwards, the wells were rinsed with DDIW until the water ran clear. Images were taken under bright field with an Olympus IX70 inverted microscope.

RESULTS

Depending on the reference genes, sometimes referred to as housekeeping genes, used in the qPCR analysis, different gene expression patterns have been observed during osteogenic differentiation (Quiroz et al., 2010). Instabilities in gene expression levels during cell differentiation must be taken into account. The genes of interest were normalized to the most commonly used reference genes, *GAPD*H and *RPL13A*. These reference genes were also chosen based upon their recent assessment as reference genes in hMSCs (Curtis et al., 2010).

When normalized against *GAPDH*, there is significant variation in the results and two of the three genes examined lacked statistical significant (Figure 1A, C and E). When normalized against *RPL13A*, adipocyte differentiation markers (*ADIPOQ*, *FABP4*; *LEP*) were significantly upregulated ($P < 0.5$) under adipogenic conditions at day 28 (Figure 1B, D and F). Consistent with the postulate of an inverse relationship between adipo- and osteogenesis (Nuttall and Gimble, 2004), adipocyte-associated genes were not significantly upregulated in osteogenic differentiation groups. *ADIPOQ* and *LEP* were still detectable in cells that underwent osteogenic differentiation (Figure 1B, C, D and F), but at a much lower level. Lipid vacuoles that represent the adipogenic phenotype were only present in the adipogenic induction group (Figure 2). No lipid vacuoles could be detected in the osteogenic induction groups at any time point.

In contrast, osteogenic genes were upregulated during adipogenesis. With both reference genes, alkaline phosphatase (*ALPL*) mRNA levels were upregulated under adipogenic and osteogenic differentiation conditions

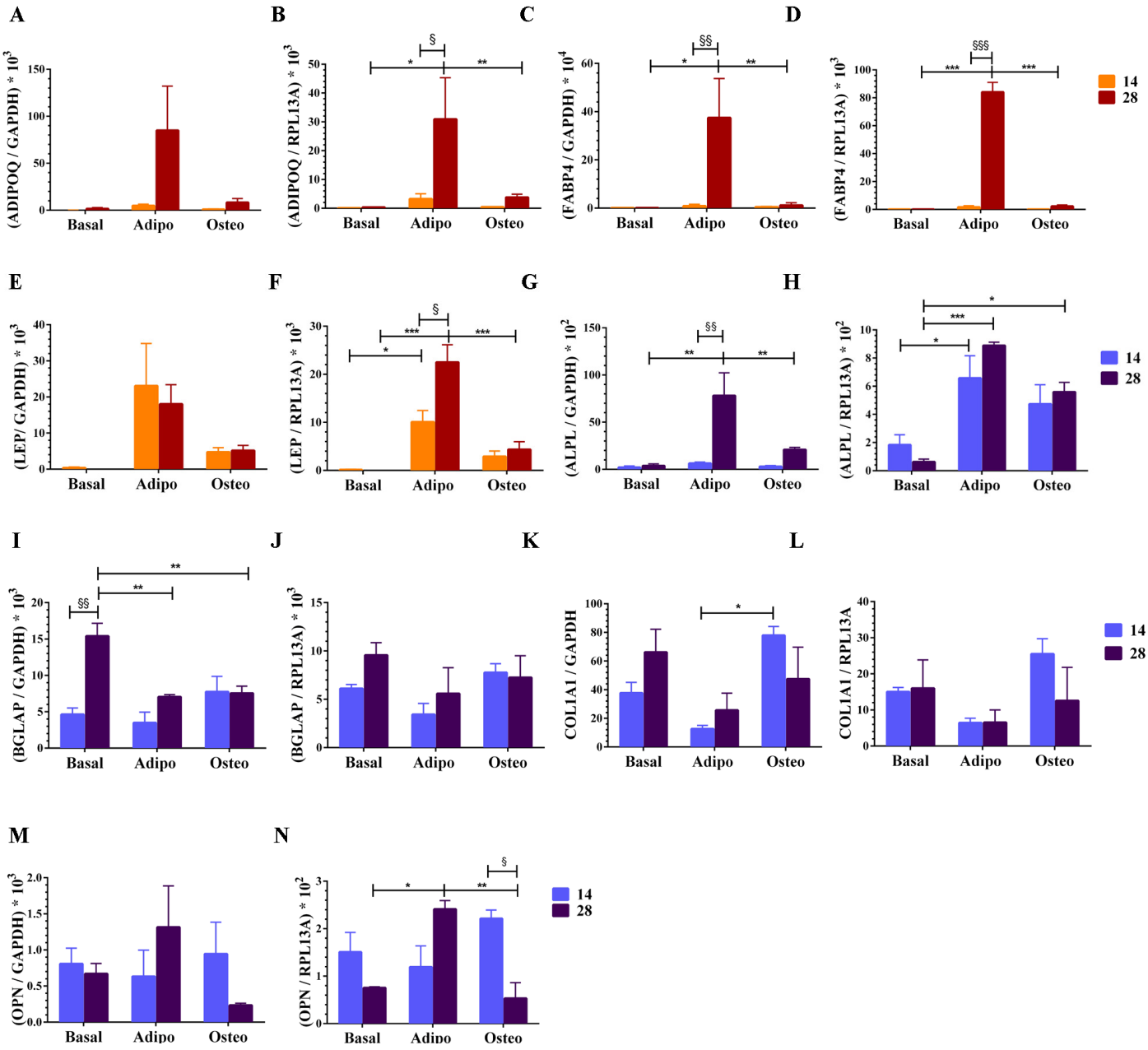

Figure 1. Expression profiles of genes encoding for adipocyte- and osteoblast-related genes. Expression of adipocyte marker genes (A-B) adiponectin (*ADIPOQ*), (B-C) fatty acid binding protein 4 (*FABP4*), (D-E) leptin (*LEP*), and osteoblast marker genes (G–H) alkaline phosphatase (*ALPL*), (I–J) osteocalcin (*BGLAP*), (K-L) collagen type I (*COL1A1*), (M-N) osteopontin (*OPN*) in hMSCs cultured in basal, adipogenic (adipo) and osteogenic (osteo) induction medium for 14 and 28 days; mRNA levels were normalized to the expression of endogenous control genes glyceraldehyde-3-phosphate (*GAPDH*) and ribosomal protein L13A (*RPL13A*). Values are presented as mean plus or minus (±) standard error of the mean (n=3 donors). Statistical significance is indicated as (*) for differences between treatment groups at day 14 and 28, respectively and (§) for the difference between day 14 and 28 for a given treatment and the number of symbols indicating level of significance with one, two, and three symbols indicating $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

(Figure 1G, H). Significantly elevated osteopontin (*OPN*) mRNA levels ($P < 0.5$) were observed in cells that were cultured in adipogenic medium for 28 days compared to the non-induced control and the osteogenic induction group when *OPN* was normalized against *RPL13A* (Figure 1N). At day 14, *OPN* levels were higher in the osteogenic induction group. Early bone marker, collagen type I (*COL1A1*) and mRNA levels were significantly elevated ($P < 0.5$) in the osteogenic induction group compared to the adipogenic group at day 14 when normalized to *GAPDH* (Figure 1K). Due to variations between donors, no statistically significant differences in the expres-

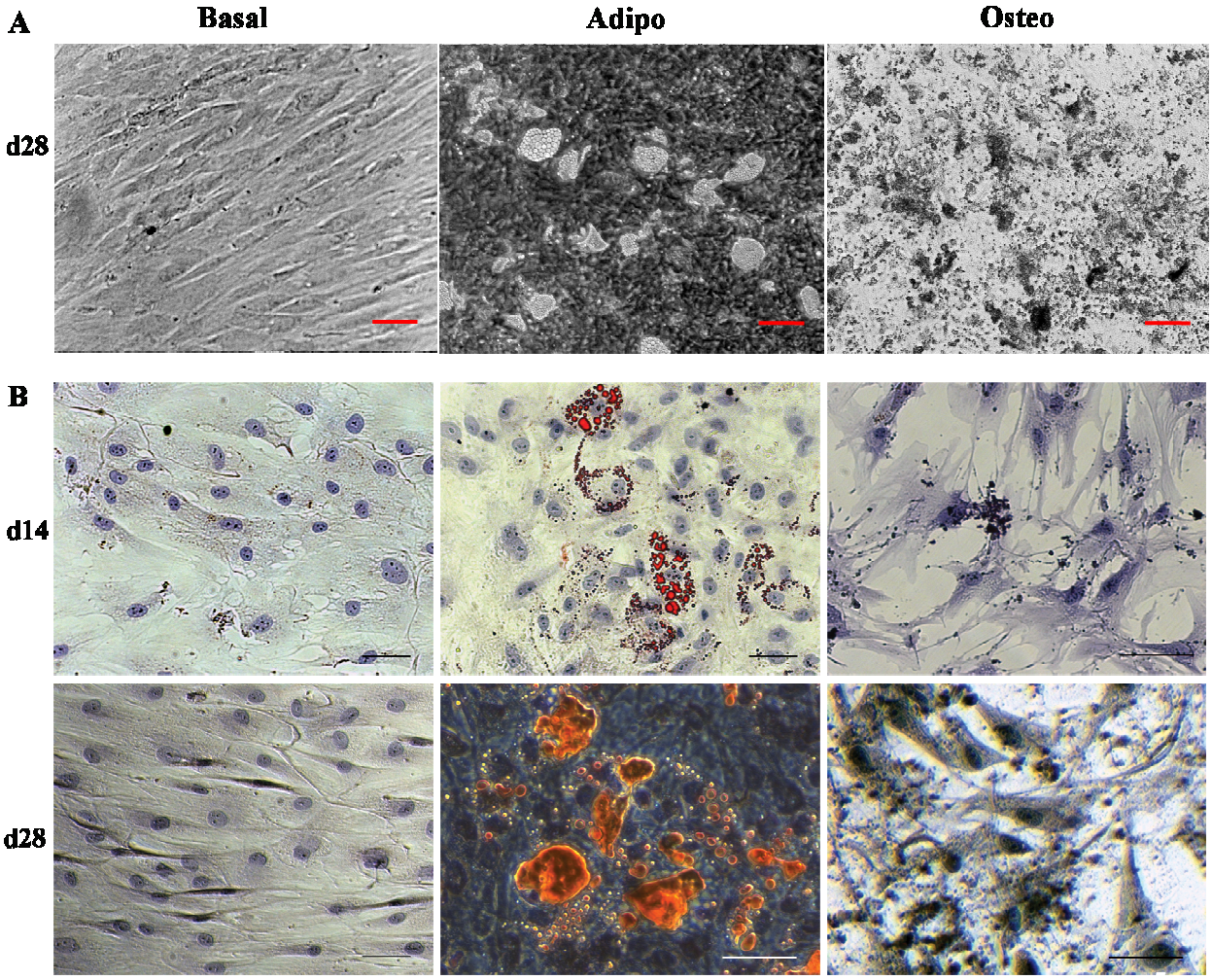

Figure 2. Morphology of differentiated cells. **(A)** Cells in the untreated control group (basal) maintained an undifferentiated phenotype with fibroblast-like cells, black regions within the cell monolayer indicate calcification in the osteogenic induction group (osteo) and lipid vacuoles were visible in the adipogenic induction group (adipo) at day 28 of differentiation. **(B)** To evaluate adipogenic differentiation, Sudan III staining was conducted. The presence of lipid vacuoles was observed in the adipo group at day 14 and 28. No lipid vacuoles could be seen in the basal and in the osteo group. The scale bar in each image is 50 µm.

sion of the late osteoblast marker osteocalcin (*BGLAP*) were observed between adipogenic and osteogenic treatment groups (Figure 1I, J). Similar to the *COL1A1* expression pattern, *BGLAP* appears to be lower in adipocyte cultures at day 14. Interestingly, *BGLAP* was significantly upregulated under basal conditions at day 28 when normalized to *GAPDH* (Figure 1I). Quiroz et al. (2010) obtained similar results due to the high variability of *GAPDH* under basal culture. The relative quantification with *RPL13A* seemed to produce lower standard deviations in the expression levels of adipogenic genes and thus gave more significance to the results. The overall expression patterns of adipogenic and osteogenic genes were similar with both housekeeping genes.

Immunostaining was carried out to detect and visualize the expression of osteocalcin (Figure 3). Similar to the mRNA expression data, osteocalcin was detected in the adipogenic induction group as well as in the osteogenic induction group at both time points (Figure 3). Under both conditions, osteocalcin expression levels decreased from day 14 to day 28. In contrast to the mRNA data, no osteocalcin protein expression was detected in the basal control groups.

DISCUSSION

Dexamethasone is a mutual component of adipogenic and osteogenic induction medium and has been shown to increase osteocalcin and leptin production in hMSCs (Leboy et al., 1991; Noh, 2012). Although additional compounds are added to the differentiation cocktails, heterogeneity in the cultures still exists. (Pittenger et al., 1999; Vater et al., 2011). Whether this phenomenon

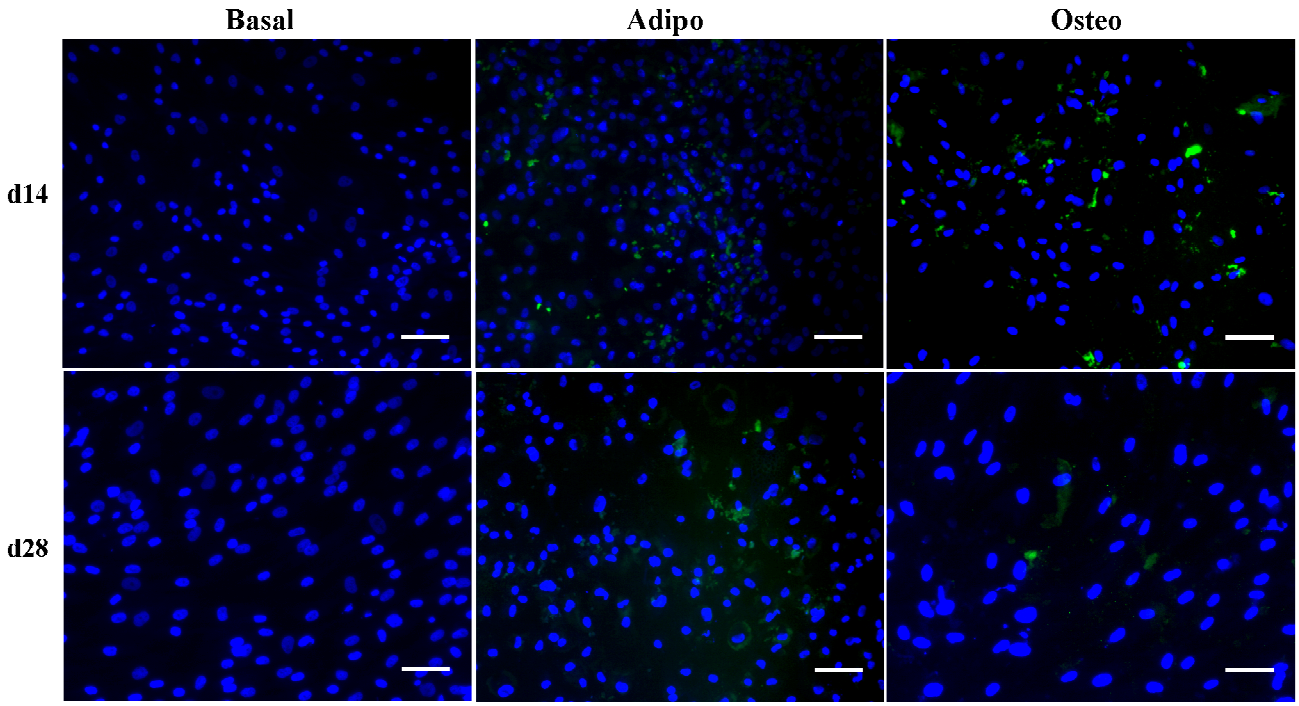

Figure 3. Representative immunofluorescence micrographs of osteocalcin expression in hMSCs, hMSC-derived adipocytes (adipo), and hMSC-derived osteoblasts (osteo). Osteocalcin expression (green) was detected to a comparable level in hMSC-derived adipocytes and osteoblasts. The expression in both groups decreased at day 28 compared to day 14. No osteocalcin was detected in the basal control. H33258 (blue) was used as a nuclear stain. Scale bar is 50 µm.

reprogress towards one lineage can transdifferentiate into another lineage, or whether the current set of markers is not definitive enough for one lineage, still needs further investigation. Similar to our observation, simultaneous expression of osteoblast markers (alkaline phosphatase and osteocalcin) and adipocyte markers (peroxisome proliferator-activated receptor γ 2 and lipoprotein lipase) was confirmed on a single cell level in hMSC-derived osteoblasts (Ponce et al., 2008).

When hMSCs differentiate into mature osteoblasts, they have to pass through several maturation stages that are characterized by a time-dependent expression of gene markers. Alkaline phosphatase and collagen type I mRNA levels are upregulated during early stages of bone formation (Jaiswal et al., 1997; Jikko et al., 1999). Osteopontin expression peaks twice during bone development, in the proliferation phase (~ day 4) and in the mineralization phase (~ day 14-21) (Aubin, 2001). Osteocalcin and osteopontin are highly expressed during the last stage of bone formation, the mineralization period. Although commonly used as an indicator for osteogenic differentiation, almost all of these markers are not bone specific. Alkaline phosphatase, an ubiquitous cellular protein, was upregulated during adipogenic and osteogenic differentiation (Figure 1B, H). Alkaline phosphatase does confirm initiation of differentiation but cannot be considered as lineage-specific. Collagen type I is the main component of bone extracellular matrix (ECM) but has been identified in a number of unrelated cell types (Hing, 2004). In our study, *COL1A1* expression was upregulated under osteogenic and downregulated under adipogenic conditions at day 14 (Figure 1K). At later time points, we observed no differences in the *COL1A1* expression levels between adipocyte and osteocyte cultures. Osteopontin cannot be considered as a bone-specific marker either since it regulates cell adhesion, migration and survival in other tissues as well (Sodek et al., 2000). *OPN* was detected in adipogenic and osteogenic lineages but gene expression patterns were distinct from each other with *OPN* being upregulated at earlier time points in osteoblast cultures and at later stages in adipocyte cultures (Figure 1N). In contrast to the protein levels (Figure 3), osteocalcin mRNA levels were not significantly upregulated during osteogenic induction (Figure 1I, J). We obtained similar results when hMSCs underwent osteogenic differentiation within a 3D hydrogel scaffold (Kollmer et al., 2012). Although Ca^{2+}-levels were significantly upregulated, no increase in osteogenic gene expression was observed. A discrepancy between mineralization and gene expression data indicated that an up regulation in osteogenic genes in hMSCs does not correlate with their ability to differentiate towards the osteogenic lineage (Shafiee et al., 2011). Glucocorticoid-mediated down regulation of osteocalcin mRNA levels has also been reported (Viereck et al., 2002).

The presence of osteocalcin in hMSC-derived adipocytes is in concert with a recently published study where steocalcin was detected in human preadipocytes and to a lesser extent in fully differentiated adipocytes (Foresta et al., 2010). Osteocalcin's role in human physiology has been further expanded as a circulating hormone influencing beta-cell proliferation, glucose intolerance, and insulin resistance has recently arisen (Lee et al., 2007).

Conclusion

The current work and previous reports clearly indicate that many of the markers used for determining the end fate of adipocytic and osteoblastic differentiation are shared between adipogenic and osteogenic differentiated hMSCs. Adipocytes and osteoblasts share a common pool of precursor cells and their plasticity is regulated by activation or silencing of genes, signaling molecules and transcription factors (Garces et al., 1997; Gimble et al., 1996). Our data indicate the need for a better understanding of the conditions and molecular regulators involved in controlling the plasticity of hMSCs. This knowledge is a prerequisite to manipulate adult stem cells for engineering functional tissues in regenerative medicine and to shed light into the pathogenesis of metabolic and skeletal disorders, like atherogenesis, diabetes and osteoporosis. Furthermore, the present study suggests that differentiation towards one lineage should be accompanied by evidence indicating lack of differentiation into other lineages.

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