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ATF5, a Possible Regulator of Osteogenic Differentiation in Human Adipose-Derived Stem Cells

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ABSTRACT

The regulatory pathways involved in maintaining the pluripotency of embryonic stem cells are partially known, whereas the regulatory pathways governing adult stem cells and their ''stem-ness'' are characterized to an even lesser extent. We, therefore, screened the transcriptome profiles of 20 osteogenically induced adult human adipose-derived stem cell (ADSC) populations and investigated for putative transcription factors that could regulate the osteogenic differentiation of these ADSC. We studied a subgroup of donors' samples that had a disparate osteogenic response transcriptome from that of induced human fetal osteoblasts and the rest of the induced human ADSC samples. From our statistical analysis, we found activating transcription factor 5 (ATF5) to be significantly and consistently down-regulated in a randomized time-course study of osteogenically differentiated adipose-derived stem cells from human donor samples. Knockdown of ATF5 with siRNA showed an increased sensitivity to osteogenic induction. This evidence suggests a role for ATF5 in the regulation of osteogenic differentiation in adipose-derived stem cells. To our knowledge, this is the first report that indicates a novel role of transcription factors in regulating osteogenic differentiation in adult or tissue specific stem cells. J. Cell. Biochem. 113: 2744–2753, 2012.

KEY WORDS: ADIPOSE DERIVED STEM CELLS; ATF5; OSTEOGENIC DIFFERENTIATION

Recent studies on cell populations obtained from adipose tissue collectively confirm the hypothesis that there are resident adult stem cells in adipose tissue. These adipose-derived stem cells (ADSCs) are able to differentiate to cells that expressed osteogenic [Leong et al., 2006a], chondrogenic [Liu et al., 2007], neurogenic [Zuk et al., 2002] and cardiomyocytic [Yamada et al., 2007] characteristics. Late passage ADSC did not elicit a proliferative response from allogeneic T cells in culture studies which hinted

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towards the ability of ADSC to cross traditional histocompatibility barriers, suggesting that allogeneic transplant of human ADSC might be possible [McIntosh et al., 2006]. At the potentially therapeutic front, Cowan et al. [2004] showed the potential of using these cells to repair critical-sized calvarial defects. Besides non-weight bearing bone sites, ADSCs shows good promise as tissue engineered grafts implants for cartilage defects [Li et al., 2012]. Despite the wealth of data on the expanding array of differentiation capabilities of ADSCs, there is little existing knowledge pertaining to the mechanistic regulation of ADSC differentiation or "stem-ness."

Oct4 has been established as the master transcription factor in regulating pluripotency in embryonic stem cells. Oct4-deficient embryos were able to develop to the blastocyst stage. However, the inner cell mass could not differentiate into more mature but still pluripotent inner cell mass cells, demonstrating that Oct4 was essential in the establishment of pluripotency in embryonic stem cells [Nichols et al., 1998].

The importance of Oct4 in regulating pluripotency of adult stem cells like bone marrow-derived mesenchymal stem cells (MSCs) is however not as conclusive. There are conflicting reports of whether bone marrow mesenchymal stem cells expressed Oct4 [Lamoury et al., 2006; Roche et al., 2007]. Interestingly, Oct4 is consistently expressed in adipose-derived stem cells for at least up to 30 passages [Izadpanah et al., 2006], yet recent experiments show low or no expression of Oct4 and Sox2 in a longer list of mesenchymal derived stem cells [Jaramillo-Ferrada et al., 2011]. Therefore, the effect of this interplay of transcription factors in the classical tug-of-war between proliferation and differentiation process remains a thought-provoking and expanding research topic.

To gain an insight into this mechanistic basis of regulation of differentiation in ADSCs, we compared transcriptome profiles of differentiated and undifferentiated ADSCs of 20 randomly selected human donors who underwent cosmetic abdominal liposuction. The expression level of the putative transcription factor(s) would be expected to decreasing through the course of differentiation. Therefore, we hypothesized that as ADSCs differentiates, the putative adult "stem-ness" transcription factor would be down-regulated.

Following these specific criteria, we uncovered a transcription factor, ATF5 that might be involved in maintaining proliferative potential of ADSCs. ATF5 was found to be consistently down-regulated after osteogenic differentiation stimuli. Knockdown of ATF5 with siRNA sensitized ADSCs towards osteogenic stimulation. This data suggests an important role of ATF5 in how ADSCs switch between proliferation and differentiation commitment according to the chemical stimuli.

MATERIALS AND METHODS

CELL ISOLATION AND CULTURE PROCEDURES

Lipoaspirates were obtained from abdominal regions of healthy adult patients after informed consent and approval by the Institutional Review Board, National University Hospital. The samples were then processed, as previously described [Leong et al., 2005]. The adipose tissue obtained was first washed with phosphate buffered saline solution (PBS) then digested with 0.075% Collagenase Type I (Gibco, Invitrogen) for 2 h at 37°C with gentle continuous shaking. The adipose-derived stem cells (ADSC) were pelleted at 500*g* and then plated on culture flasks (TPP, Trasadingen, Switzerland). Cells were grown in culture media (Dulbeco's modified Eagle's media supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 U/ml streptomycin). Media change was performed every 4 days.

CELL DIFFERENTIATION

At 80–90% confluence, cells were trypsinized and replated at 5,000 cells/cm^2. Cells in the uninduced group were fed with maintenance culture media (DMEM supplemented with 10% FBS, 50 U/ml penicillin and 50 U/ml streptomycin). Cells in the induced groups were fed with osteogenic media (maintenance culture media + osteogenic cocktail consisting of 50-μM L-ascorbic acid–2-phosphate (Sigma–Aldrich, A8960), 10 mM β-glycerophosphate (Sigma–Aldrich, 6376), 0.01 μM 1α, 25-dihydroxycholecalciferol (Sigma–Aldrich, D1530) as previously described [Leong et al., 2006b]. For the experiments, Passage 3 ADSCs were used. Human fetal osteoblasts (hFOBs-CRL-11372 from ATCC), were also maintained and induced with the same induction cocktails used for the ADSCs. Human embryonic kidney (HEK) 293 cells and human skin fibroblasts cells were also included in the study.

ALIZARIN RED S STAINING FOR CALCIFIED MATRIX

After washing in PBS, cells were fixed in 10% formalin solution for 10 min and stained with 2% Alizarin red S (Sigma–Aldrich, A5533) for 5 min. The cells were washed thoroughly with distilled water. Samples were evaluated under a bright-field light microscope for identification of reddish-stained calcified nodules.

IMMUNOSTAINING FOR BONE MATRIX-RELATED PROTEINS

Culture media was removed completely and the samples washed twice with PBS. Fixation was achieved with −20°C methanol for 10 min and blocking was done with 10% goat serum (Dako, X0907) for 1 h. Incubation with primary antibodies against osteopontin (1:500, Chemicon AB1870), osteonectin (1:1,000, Chemicon AB1858), and osteocalcin (1:500, Chemicon AB1857) was carried out for 90 min at room temperature. Subsequently, the samples were washed thrice with PBS and incubated with the appropriate secondary antibodies (Dako EnVision kit, K4011) for 30 min at room temperature. All samples were counterstained with hematoxylin, mounted and viewed under a bright-field light microscope.

WESTERN BLOTTING

Cells were lysed in ice-cold lysis buffer (50 mM Tris–HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail). Lysates were sonicated and the protein concentrations determined using the Bradford assay. Fifty microgram of protein from each group was separated by SDS–PAGE and transferred onto nitrocellulose membranes. After blocking with 5% non-fat dry milk/TBST, individual membranes were probed with antibodies specific to osteopontin (Chemicon AB1870) or β-actin (Bethyl labs; A300-491A) for 2 h at room temperature with shaking.

After primary antibodies incubation and washing with TBST, horseradish peroxidase-conjugated secondary antibodies were added. Immunoreactive bands on the membrane were detected by chemiluminescence using an enhanced ECL detection kit (Pierce #34077).

TOTAL RNA EXTRACTION

After 2 and 28 days of induction, cells were lysed and total RNA was extracted with the Trizol and chloroform method. Total RNA was precipitated and cleaned up with RNeasy columns (Qiagen, 74106).

TOTAL RNA PREPARATORY PROCEDURES FOR AFFYMETRIX GENECHIP

First strand cDNA was synthesized from 5 μg of total RNA using a $T7(dT)_{24}$ primer and SuperScript II reverse transcriptase at 42°C for 1 h. The use of the $T7(dT)_{24}$ primer in the first strand cDNA synthesis incorporated a T7 RNA polymerase promoter into the cDNA for the future initiation site for T7-mediated in vitro transcription. The second strand cDNA was then synthesized using *E. coli* DNA ligase, DNA polymerase I and RNase H (Affymetrix GeneChip® One-Cycle cDNA Synthesis Kit). Double-stranded cDNA was cleaned up with the GeneChip cDNA cleanup module (Affymetrix). In vitro transcription was carried out for 16 h using the Affymetrix GeneChip® IVT Labeling Kit and cleaned up. Subsequently, 20 μg of biotinylated-cRNA was fragmented at 94°C for 35 min and then kept at −80°C. Prior to hybridization, fragmented biotinylated-cRNA were mixed with control oligonucleotide B2, eukaryotic hybridization controls (both from GeneChip® Hybridization Control Kit), herring sperm DNA and bovine serum albumin in a 2-(*N*-Morpholino)ethanesulfonic acid buffer, heated to 99°C for 5 min and equilibrated to 45°C before hybridizing to the Affymetrix GeneChip® Plus 2.0 array for 16 h at 45°C. Arrays were then washed and stained using the Affymetrix® Fluidics Station 450. The stained arrays were scanned using the Affymetrix® GeneChip® scanner 3000.

MICROARRAY DATA ANALYSIS

A total of 54,675 probe sets on the Affymetrix Plus 2.0 were analysed performed using the Agilent Technologies Genespring GX 7.3 software. Cell file data was preprocessed with robust multi-array analysis (RMA) algorithm [Irizarry et al., 2003]. Global normalization was performed by first setting all values below 0.01 to 0.01 and then dividing each gene of all samples by its median. Genes with median <10 had their median adjusted to 10. Present, marginal and absent flags identified by Affymetrix MicroArray Suite v5 were not included in further analysis. Sample data set were group into Day-0, Day-2, or Day-28 samples. Probe sets significantly perturbed over background were filtered using Bonferroni correction on *t*-test *P*-values $<5 \times 10^{-6}$ in any of the three time points. The 11,148 genes that were significantly perturbed above background were filtered further by looking for genes with at least 1.7-fold change and a *P*-value <0.05. A one-way ANOVA at $P < 0.05$ was used further reduced the list. With a focus on only the down-regulated genes [Induced (I^+)/Uninduced $(I^-) < 1$] the list was narrowed down further. Raw and normalized data files were curated at EMBL-EBI MIAMExpress (accession number: E-MEXP-1216).

REAL-TIME PCR: DEOXYRIBONUCLEASE I (DNASE I) TREATMENT AND REVERSE TRANSCRIPTION

Before reverse transcription, total RNA samples were subjected to DNase I (Fermentas, #EN0521) treatment to remove genomic DNA carryover to the real-time PCR. DNA digestion was carried out at 37°C for 30 min. The samples were then mixed with 70% ethanol and repurified with RNeasy columns and quantified. Purified RNA was reverse transcribed to cDNA using MuLV reverse transcriptase RNase H^- (Fermentas, #EP0451).

PRIMER SEQUENCES

The following primer sequences were used; Osteocalcin (5′ gcagagtccagcaaaggt 3′; 5′ cagccattgatacaggtagc 3′); ATF5 (5′ tggcttctctgactggatga 3′; 5′ tccatctgttccagctcctt 3′).

PCR STANDARDS PREPARATION

PCR was done to amplify the cDNA product. The single amplified PCR product was verified based on size in a 3% agarose gel under UV illumination. The standards were prepared as previously described [Leong et al., 2007].

QUANTITATIVE REAL-TIME PCR

After assuring the suitability of the primers for their uniqueness to amplify a single PCR product, quantitative real-time PCR was done using the Quantitect SYBR Green PCR kit (Qiagen, 204143) on a thermocycler (Stratagene Mx 3000 P). The PCR + dissociation routine used were as follows: 95°C for 10 min, 45 cycles of 94°C 30 s, 60°C 45 s, 72°C 30 s, *95°C 1 s, 60°C 30 s, slow ramp up to 95°C at 0.5°C per second with continuous measurement, 95°C 10 s, 25°C 30 s*, end (dissociation phase in italics).

KNOCKDOWN OF ATF5

ADSCs were plated at 8,000 cells/cm^2 into T-75 culture flasks. A day later, ATF5 was knocked down with ATF5 Stealth™ Select RNAi collection (Invitrogen; 1299001). Negative RNAi control used was Stealth™ RNAi Negative Control Med GC (12935300). RNAi oligos were transfected into the various ADSC samples using Lipofectamine RNAiMax (Invitrogen 13778) according to the manufacturer's recommendations. After 8 h of incubation with the transfection mixture, the cells were washed with sterile PBS and then induced with various concentrations of induction cocktails (100% and 10% concentrations). At 48 h post-induction, total RNA was extracted as described earlier.

RESULTS

DONORS' CELLS RESPOND DIFFERENTLY TO IDENTICAL INDUCTION STIMULUS

Mineralization capacity was different for different donors' cells (Fig. 1A). After 28 days of induction, more mineralization in the cells of donor IDs DON2 and DON16 were evident compared to that of DON3 and DON13 (Fig. 1A). After an induction regime of 28 days, ADSCs were able to express key osteo-proteins including osteocalcin, osteopontin and osteonectin (Fig. 1B) with increased extracellular matrix formation. However, different donors' cells responded different even though they were subjected to the same

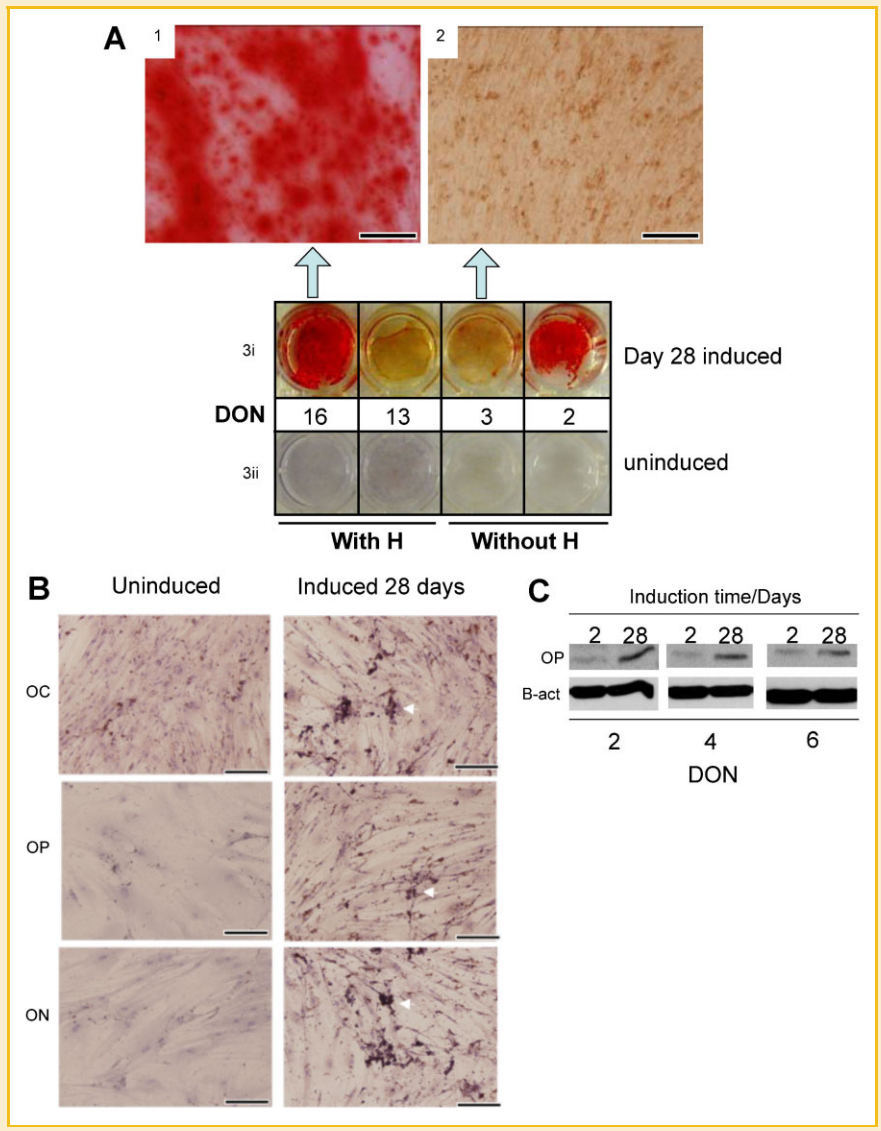

Fig. 1. Donor dependent response to the same osteogenic stimulus. A: Differential Alizarin Red S staining for mineralization seen in a range of samples. Panels 1 and 2—scale bar = 200 μm. Panel 3i and 3ii panel—macroscopic view of the wells containing cells from different Donor samples (DON 2,3,13,16). Cells were first stained with Alizarin Red S and thereafter counterstained with and without hematoxylin (H) staining. B: Immunohistochemistry of ADSC at 28 days after an osteogenic induction, were able to express osteocalcin, osteopontin and osteonectin proteins (white arrows). Scale bar represents 200 μm. C: Immunoblots of ADSC from various donors (DON2, DON4, and DON6) subjected to osteogenic induction showing a increased expression of osteopontin over the time course of 2 and 28 days. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

induction stimulus (Fig. 1C). However, immunoblotting against osteopontin after a time course induction regime on three respresentative donor cell populations (DON2, DON4, and DON6) showed an increased expression of osteopontin from day 2 to day 28 of induction (Fig. 1C). The data supports a perturbation of the transcriptional landscape as the stem cells were driven towards the osteogenic lineage, with osteogenic induction up to 28 days.

DIFFERENTIAL GLOBAL GENE EXPRESSION RESPONSES TO INDUCTION AMONGST THE DONOR POPULATION CELLS BASED ON MICROARRAY ANALYSIS

The transcriptome of these cells subjected to the various time course of differentiation using genome-wide microarrays were analysed to probe for perturbations of the transcriptional landscape. The entire normalized data set (65 samples and their corresponding 54,675 genes) demonstrated the median for each sample was centered around ~1 (Fig. S1). This is indicative that the bulk of the transcriptome data points were statistically normalized.

Filtering out background expressions by accepting genes with *t*-test *P*-value $<5 \times 10^{-6}$, the number of genes reduced from 54,675 to 11,148. Further analyses were carried with these 11,148 genes. Preliminary filtering of genes with a minimum of 1.7-fold-differences between induced (I^+) and non-induced (I^-) groups narrowed down the list to 73 genes. Testing only these 73 genes for all the samples within the Induced (I^+) and uninduced (I^-) groups for statistical significance (one-way ANOVA test with variances not

assumed to be equal, *P*-value cutoff at 0.05 and Bonferroni correction) further reduced the list to 46 genes (Table I). qPCR validation of a portion of the genes in Table I was summarized in Figure S2.

Hierarchical clustering of the 11,148 genes indicated good segregation between the induced (Day 28) and uninduced (Day 0) groups (Fig. 2). Interestingly, there was a subgroup of induced D28 samples that clustered closer to the uninduced group (D0) and two major clusters of induced samples (Fig. 2). As expected, the uninduced (D0) control samples human fetal osteoblasts (hFOBs), human embryonic kidney cells (HEK 293), and human dermal fibroblasts (HDF) were clustered distinctly differently from the main two uninduced and induced ADSCs samples (Fig. 2).

DETERMINATION OF ATF5 AS A POTENTIAL GENE OF INTEREST

Focusing only on genes related to transcription and differentiation, we note ATF5 to be significantly downregulated from the uninduced to induced state (Table I). Independent real-time PCR validation of ATF5 and other significantly changed genes from this list of 46 genes could be found in Figure S2 respectively.

ATF5 SIGNIFICANTLY DOWNREGULATED WHEN ADSC DIFFERENTIATED DOWN THE OSTEOGENIC LINEAGE

From the microarray data, activating transcription factor 5 (ATF5) was found to be downregulated in 19 of the 20 donor sample groups throughout osteogenic differentiation (Fig. 3A). This was confirmed with real-time PCR assay on the same donor samples (Fig. 3B). Interestingly, a human pre-osteoblastic cell line (hFOB) did not show a similar trend of decrease when subjected to the same osteogenic differentiation regime. This suggested that ATF5 was important in regulating the osteogenic induction of ADSCs or even non-osteoblastic cell types.

ALTERING ATF5 LEVELS CHANGES DIFFERENTIATION COMMITMENT OF STEM CELLS AND MATURE OSTEOBLASTS

After effectively knocking down ATF5 levels (Fig. 4A) with siRNA, ADSCs were sensitized towards osteogenic induction, shown by the

TABLE I. List of Genes That Were Significantly Changed Over the Course of differentiation. Activating Transcription Factor 5 Is Highlighted (Induced = I^+; Uninduced = I^-)

Fold change	*P*-value	Description
8.225	1.85E−07	Olfactomedin-like 2A
7.363	7.78E−05	Similar to KIAA0386
4.953	2.34E−05	CDNA FLJ46440 fis, clone THYMU3016022
4.252	2.44 E−05	Laminin, alpha 1
3.538	1.86E−05	CDNA FLJ26188 fis, clone ADG04821
3.183	1.35E−05	Zinc finger, CCHC domain containing 14
2.953	1.19E−05	CDNA FLJ43311 fis, clone NT2RI2009855
2.778	0.00216	Synaptotagmin-like 4 (granuphilin-a)
2.286	2.81E−08	Transient receptor potential cation channel, subfamily M, member 7
2.221	2.221	PCTAIRE protein kinase 2
2.196	2.32E−06	Hypothetical protein FLJ10211
2.17	0.0444	Polypyrimidine tract binding protein 2
2.063	3.17E−05	MSTP150
2.039	0.0117	Muscleblind-like (Drosophila)
2.038	1.27E−05	Transcribed locus, moderately similar to XP_517655.1
1.984	0.0363	CDNA FLJ31010 fis, clone HLUNG2000174
1.944	0.0185	Baculoviral IAP repeat-containing 6 (apollon)
1.911	0.0352	IQ motif and WD repeats 1
1.887	0.032	AT rich interactive domain 2 (ARID, RFX-like)
1.869	0.00729	CDNA FLJ13202 fis, clone NT2RP3004503
1.784	0.0498	Hypothetical gene CG012
1.76	5.18E−06	600944774T1 NIH_MGC_17 Homo sapiens cDNA clone IMAGE:2960610 3′
1.753	0.00285	Ataxin 1
0.554	0.00178	LOC284561
0.543	0.00059	EH-domain containing 1
0.536	1.75E−05	Human DNA sequence from clone RP1-287G14 on
0.512	6.92E−06	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)
0.466	0.0118	Seryl-tRNA synthetase
0.448	3.32 E−05	Phosphodiesterase 1C, calmodulin-dependent 70 kDa
0.438	0.000127	SH2 domain containing 4A
0.415	1.50E−05	yz36g02.s1 Morton Fetal Cochlea Homo sapiens cDNA clone IMAGE:2851703′
0.406	1.71 E−05	Chromosome 13 open reading frame 12
0.402	0.0135	Eukaryotic translation initiation factor 4E binding protein 1
0.363	0.0404	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)
0.361	0.0255	Tribbles homolog 3 (Drosophila)
0.335	0.0147	Activating transcription factor 5
0.324	0.000384	Chromosome 20 open reading frame 50
0.29	0.000113	Nipsnap homolog 3A (C. elegans)
0.238	1.28 E−06	Down Syndrome critical region gene 1
0.268	0.0169	Solute carrier family 7, (cationic amino acid transporter, y+system) member 11
0.257	0.00872	ab38f03.s1 Stratagene HeLa cell s3 937216 Homo sapiens cDNA clone
0.24	0.000172	Kelch domain containing 7B
0.23	3.14E−05	Human DNA sequence from clone RP4-774124 on chromosome 1q24.1-24.3
0.196	0.000217	CDNA FLJ43039 fis, clone BRTHA3003023
0.18	0.000451	Heat shock 27 kDa protein family, member 7 (cardiovascular)
0.0831	0.000109	Inhibitor of DNA binding 1, dominant negative helix–loop–helix protein

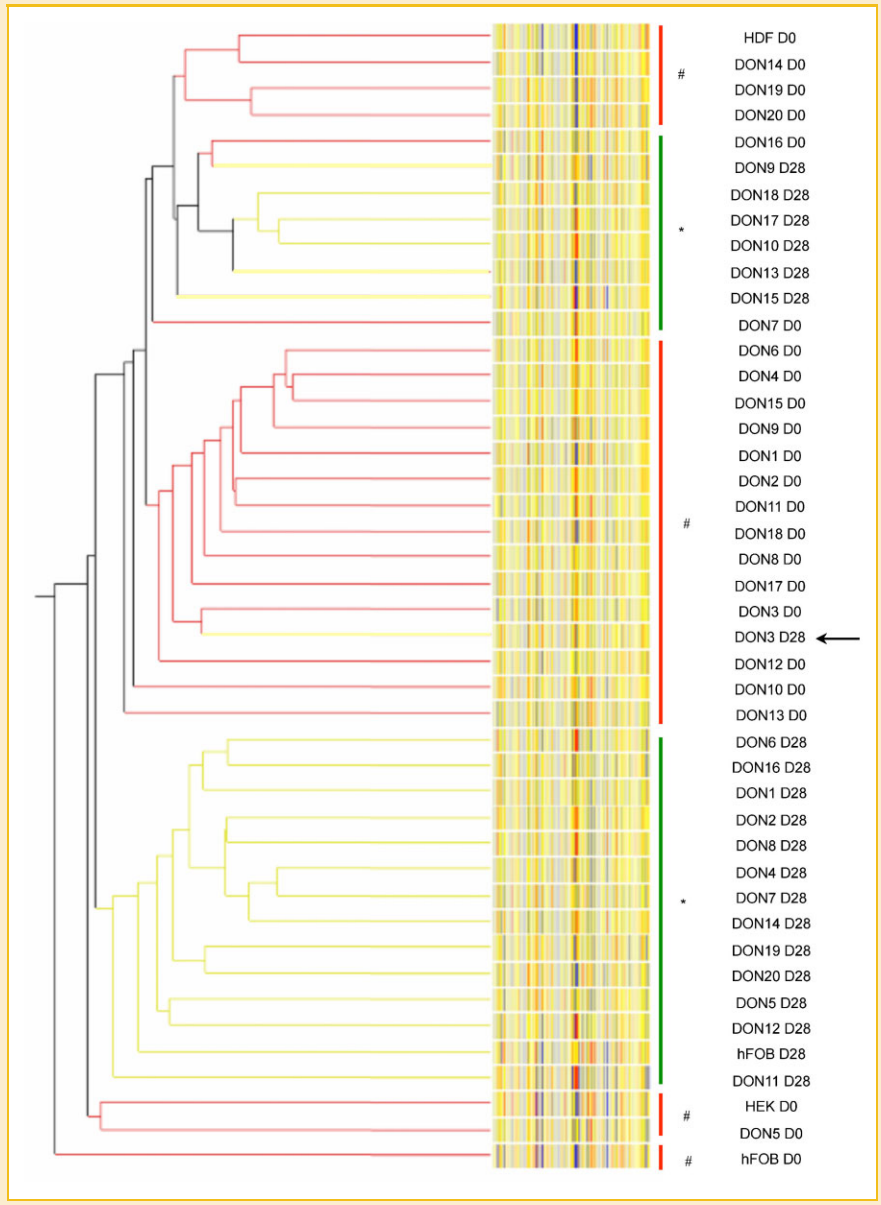

Fig. 2. Condition tree of groups based on significantly expressed genes. Clear clustering between induced (D28) and uninduced (D0) samples were observed with one exceptions (black arrow). There were however distinctly two groups of osteogenic induced ADSC samples (D28-*). Likewise a smaller subcluster of D0 samples were distinct from the larger subcluster of uninduced samples (D0-#). The non-osteogenic control samples (HEK D0, HDF D0) and osteogenic controls (hFOB D28) were clustered separately as expected. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

positive response to a tenth of the concentration of induction media, which would otherwise have no observable effect on osteocalcin (OC) expression (Fig. 4B). We repeated the knockdown experiments on three other donors' ADSCs (not entered into the initial microarray screen) and observed a similar phenomenon (Fig. 4C,D).

DISCUSSION

Pochampally et al. [2004] described an increase in ATF5 gene expression, as assayed with Affymetrix arrays, when surviving MSCs were subjected to a serum deprivation culture. The surviving MSCs also expressed Oct4 and hTERT and were described to be indicative of early progenitors. Their report reinforced our current observations that there is higher ATF5 expression in the undifferentiated state of MSCs.

In our study, adiposed-derived stems cells from a range of human samples, were found to have differentially gene expression when all were similarly induced osteogenicially over 28 days. This finding was supported by immunohistology and immunoblotting (Fig. 1A,B), mineralization assay (Fig. 1A) and microarray data (Fig. 2). Quantitatively, a significant percentage (8 out of 20, or 40%) of the donors' ADSC samples had a disparate osteogenic response after 28 days of induction using the same osteogenic induction. The

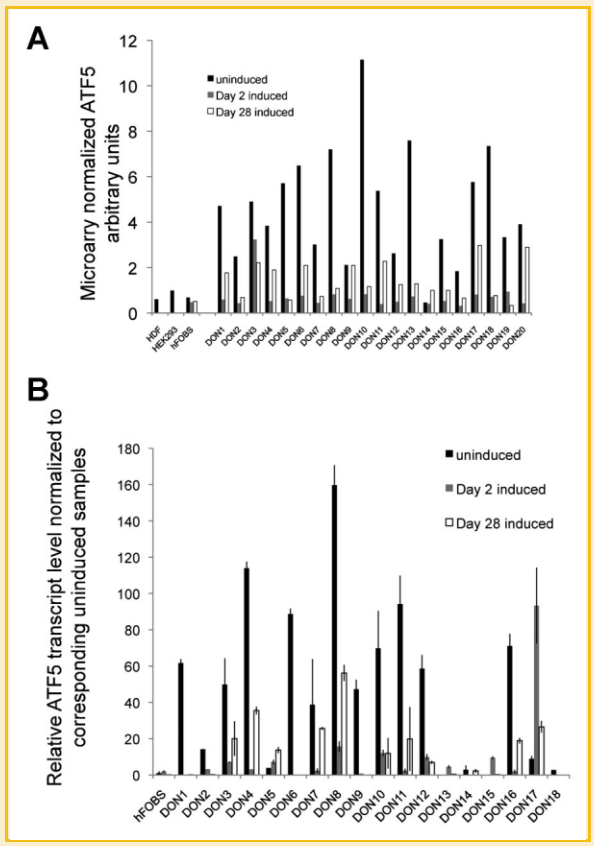

Fig. 3. ATF5 is consistent downregulated amongst ADSC samples subjected to osteogenic induction. Graphs summarizing (A) normalized ATF5 expression data from microarray analysis for each donor sample at uninduced, day 2 induced and day 28 induced state. B: The same sampling of the same samples as (A) but using real-time reverse-transcriptase PCR assay and then normalized to hFOBs levels. The uninduced sample for most donors had higher expression of ATF5 than the corresponding days 2 and 28 induced samples.

rest of the samples had closer transcriptomes to our positive control sample of hFOBs (Fig. 2). If ATF5 is truly a key regulator of osteogenic differentiation in ADSCs, the divergence in differentiation response must therefore be the downstream of ATF5 signaling. This hypothesis is supported by the fact that 19 out of the 20 samples studied still showed a decreased expression of ATF5 through the course of differentiation.

ATF5 transcripts and protein are expressed in a wide variety of tissues during development and in the adult, with particularly high expression of transcripts in adult liver [Shimizu et al., 2009]. The ATF family members are transcription factors that recognize the consensus ATF/CRE site 5′-GTGACGT(A/G)(A/G)-3′ and have been implicated in stress responses [Hai et al., 1999], physiological processes like proliferation [Smith et al., 2003] and osteo-matrix formation [Takayanagi, 2007]. The sequence of the specific ATF5 DNA binding site was determined to be C(C/T)TCT(C/T)CCTTA [Li et al., 2009]. ATF5 was initially discovered in a yeast two-hybrid pull down system as an interacting partner of Cdc34 [Pati et al., 1999]. Interestingly, another ATF member, ATF4 is a substrate of ribosomal serine/threonine kinase 2 (RSK2). Collagen type I expression is regulated through the phosphorylation of ATF4 by RSK2. Without ATF4, the appearance of the bone trabeculae is delayed. This delay in osteogenesis suggested that ATF4 is required to mediate some aspects of RUNX2-initiated osteoblast differentiation. Osteocalcin, an important osteo-protein found in mature bone matrix, is also a target gene of ATF4 in vivo [Yang and Karsenty, 2004]. ATF4 transcript is ubiquitously expressed in all major tissues but its protein expression is limited to bone tissues [Yang et al., 2004]. The similarity between ATF4 and ATF5 reinforces the possible role of ATF5 in osteogenic differentiation of cells of non-osteoblastic origin. However, it is possible that ATF5's role is not only limited to osteogenesis but also neurogenesis as neural stem cells in the developing brain express high levels of ATF5.

Conversely, fully differentiated neurons, astrocytes and oligodendrocytes do not show any detectable expression of ATF5 in vivo [Angelastro et al., 2005]. Constitutive expression of ATF5 was able to maintain neural stem cells in their undifferentiated state, conversely knockdown or loss of function studies of ATF5 accelerated the differentiation of neural stem cells to form mature neurons and glial cells [Angelastro et al., 2005]. However, ATF5 is also highly expressed in a number of cancer types, including neural tumors such as neuroblastomas, medulloblastomas and glioblastomas [Greene et al., 2001], breast cancer [Li et al., 2009], liver cancer [Gho et al., 2008] and leukemia [Reddiconto et al., in press]. In addition, another closely related ATF family member, ATF7 was upregulated in Caco-2 cells, a human colonic adenocarcinoma cell line as they underwent spontaneous differentiation into intestinal epithelia [Peters et al., 2001].

Dexamethasone-induced osteogenic induction in pre-osteoblast cultures exerts an anti-proliferative effect; brought about by repressing ATF4-dependent cyclin A gene expression [Gabet et al., 2011]. Interestingly, cyclin D3, a regulator of the cell cycle, binds specifically to ATF5 and potentiates its transcriptional activity [Liu et al., 2003]. Extrapolating from the case of ATF4, ATF5 may be controlling proliferation of ADSC through cyclin D3. This pro-proliferative role of ATF5 is further observed in exclusive expression of ATF5 in cerebellar granule neuron progenitor cells which quickly become undetectable in post-mitiotic cerebellar granule neurons [Lee et al., 2011]. ATF5 is acetylated at its lysine-29 and this increases its interaction with p300 coactivator. This activated complex then binds to the Egr-1 promoter site and transcribes Egr-1 which in turns drives cyclin expression [Zhang et al., 2010; Liu et al., 2011] and results in increased proliferation.

However, the role of ATF5 may not be limited to proliferation but also differentiation since when ATF5 was knocked down with RNAi, we detected an increase in osteocalcin gene expression, the unique osteoblastic secreted protein, at a significantly lowered osteogenic induction concentration (Fig. 4B,D). In fetal mouse development, OC protein expression could only be detected 4–5 days after RUNX2 expression, the master transcription factor for osteoblast differentiation [Karsenty, 2008]. Without osteogenic induction, the silencing of ATF5 however, did result in upregulation of OC expression (Fig. 4B) but to a lesser extent compared to the osteoinduction group (Fig. 4B). This indirectly showed that ATF5 role may not only be linked to osteogenic differentiation or there are other factors that are required to lead to osteogenic differentiation. One such factor might be FoxO1 which interacts directly with ATF4 in osteoblasts and with

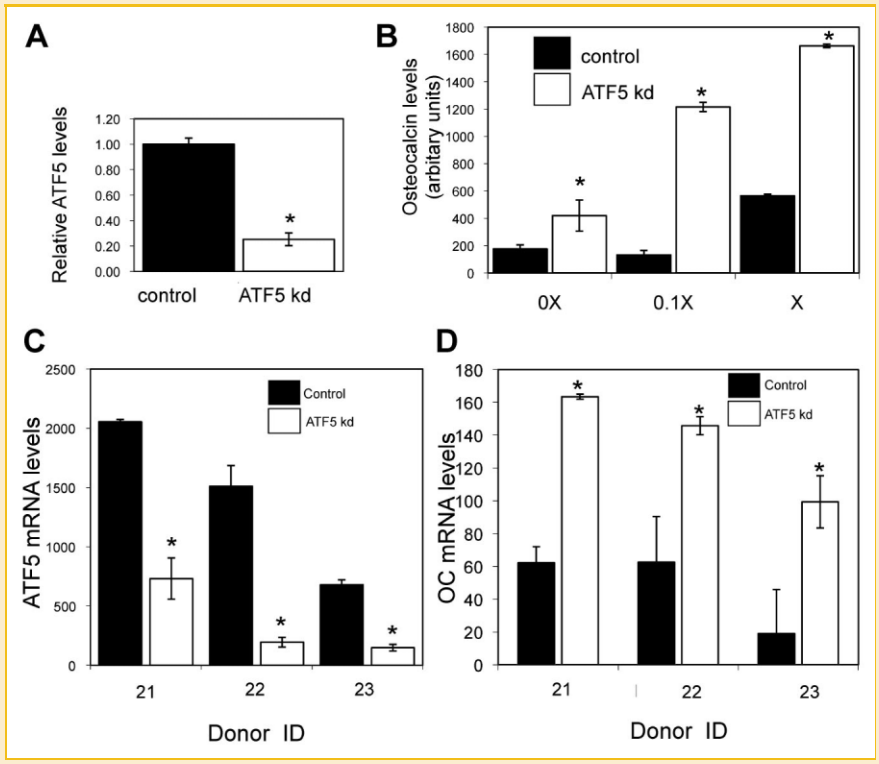

Fig. 4. ATF5 knockdown (kd) sensitizes ADSC to osteogenic induction (2 days of induction). A: Effective knockdown of ATF5 level by RNAi ($n = 3$). B: When endogeneous ATF5 was knocked down with RNAi (using DON6 cells), at the same concentrations of induction components, there were significantly increased levels of osteocalcin (OC) mRNA expression (*P-value <0.05 when compared with corresponding knockdown control). C: ATF5 normalized to β-actin copy numbers of samples induced with 10% of the standard induction dose (*P-value <0.05). D: Osteocalcin copy numbers normalized to β-actin at 10% of the standard the induction concentration. Other ATF5 knockdown cell populations from DON21, 22, and 23 consistently showed a significant increase in osteocalcin compared to the knockdown control (*P-value <0.05).

RUNX2 in mesenchymal stem cells and regulates their osteogenic differentiation process [Almeida, 2011]. This ties in with the discussion on ATF5 expression in neural stem cells. ATF5 may be a stem-ness gene; a "guardian" of adult stem cell stem-ness or it can be perceived that ATF5 is the "brake" in the differentiation machinery. When its expression drops, stem cells leave their undifferentiated stage and become responsive to signals and cutes and progresses into lineage differentiation. In our model, it is the induction media that causes ADSC's to differentiate down the osteogenic lineage and not ATF5.

Hong et al. [2005] described a 14-3-3 protein, TAZ as a transcriptional modulator of mesenchymal differentiation. The WW domain of TAZ binds strongly to the Pro-Pro-X-Tyr sequence of regulatory regions of Runx2 and PPARγ, as well as members of the Sox, SMAD, and Forkhead families [Hong and Yaffe, 2006]. There are however, no WW domain sequences on ATF5. Nonetheless, it is likely that ATF5 works in unison with other partners to exert its potential effect on regulating MSC differentiation.

Interestingly, recent data describing osteocalcin (OC) as a hormone secreted by osteoblasts, which could regulate adipocyte metabolic biology [Lee et al., 2007], might indicate a novel role of ATF5. Due to the heterogeneity of the cell population in our culture system, there would be osteoblastic cells and non-osteoblastic cells present at the same time after osteogenic induction. Therefore, it was also possible that ATF5 was inhibiting similar communication between these two cell types in culture. This hypothesis is somewhat supported by our data that the transcription of osteocalcin was higher after ATF5 knockdown compared to non-knockdown controls, even after lowering the osteogenic media supplement concentration by 90% (Fig. 4B).

In conclusion, ATF5 is a relatively unexplored transcription factor which has shown functionality in neural cancer stem cells, mesenchymal and ectodermal adult stem cells. Our results indicate that ATF5 is an interesting target for further studies elucidating its roles in osteogenic differentiation of MSCs. Future work which is focused on finding its binding partners and its exact DNA binding domain in the context of MSC differentiation will provide greater understanding of the complexities controlling the differentiation regulation of adult MSCs and hypothetically cancer stem cells.

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