

Nuclear Receptor Profile in Calvarial Bone Cells Undergoing Osteogenic Versus Adipogenic Differentiation

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ABSTRACT

Nuclear receptors (NRs) are key regulators of cell function and differentiation. We examined NR expression during osteogenic versus adipogenic differentiation of primary mouse calvarial osteoblasts (MOBs). MOBs were cultured for 21 days in osteogenic or adipogenic differentiation media. von Kossa and Oil Red O staining, and qRT-PCR of marker genes and 49 NRs were performed. PCR amplicons were subcloned to establish correct sequences and absolute standard curves. Forty-three NRs were detected at days 0–21. Uncentered average linkage hierarchical clustering identified four expression clusters: NRs (1) upregulated during osteogenic, but not adipogenic, differentiation, (2) upregulated in both conditions, with greater upregulation during adipogenic differentiation, (3) upregulated equally in both conditions, (4) downregulated during adipogenic, but not osteogenic, differentiation. One-way ANOVA with contrast revealed 20 NRs upregulated during osteogenic differentiation and 12 NRs upregulated during adipogenic differentiation. Two-way ANOVA demonstrated that 18 NRs were higher in osteogenic media, while 9 NRs were higher in adipogenic media. The time effect revealed 16 upregulated NRs. The interaction of condition with time revealed 6 NRs with higher expression rate during adipogenic differentiation and 3 NRs with higher expression rate during osteogenic differentiation. Relative NR abundance at days 0 and 21 were ranked. Basal ranking changed at least 5 positions for 13 NRs in osteogenic media and 9 NRs in adipogenic media. Osteogenic and adipogenic differentiation significantly altered NR expression in MOBs. These differences offer a fingerprint of cellular commitment and may provide clues to the underlying mechanisms of osteogenic versus adipogenic differentiation. *J. Cell. Biochem.* 105: 1316–1326, 2008. © 2008 Wiley-Liss, Inc.

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Reduced bone formation resulting from osteoporosis, ovariectomy, skeletal unloading, glucocorticoid therapy or normal aging is characterized by significantly decreased osteoblast number and increased bone marrow adipocyte number [Meunier et al., 1971; Wang et al., 1977; Burkhardt et al., 1987; Justesen et al., 2001; Marie and Kaabeche, 2006]. Because adipocytes and osteoblasts share a common progenitor [Bellows and Heersche, 2001], it is hypothesized that osteoporosis and osteopenia occur when progenitors preferentially differentiate toward adipocytes

rather than osteoblasts [Meunier et al., 1971; Beresford et al., 1992]. Drug discovery research aimed at shifting this differentiation balance towards osteoblasts is yielding promising results [see review by Nuttall and Gimble, 2000].

Calvaria-derived osteoblasts are one of the most widely used primary osteoblast models. The pool of cells collected from collagenase-digested 1-week-old mouse calvariae is enriched in osteoblasts that form mineralized nodules and express lineage-specific markers when cultured in osteogenic media [Garcia et al.,

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2002; Kalajzic et al., 2002]. The overwhelming majority of calvaria-derived cells (>80%) are committed osteoblasts with a small population of progenitors that differentiate toward osteoblasts, adipocytes and chondrocytes [Bellows and Heersche, 2001; Zhang et al., 2006].

Osteoblasts and adipocytes share a common progenitor whose fate correlates with the expression of *cbfa1/runx2* or *PPAR γ* , respectively [Tontonoz et al., 1995a; Ducy et al., 1997]. *Cbfa1/runx2* is a runt homology domain transcription factor that triggers expression of key osteoblastic genes [Ducy, 2000]. *PPAR γ* is a nuclear receptor (NR) that targets adipogenic genes and plays a major role in metabolic diseases [Lehrke and Lazar, 2005].

The NR superfamily has 49 members that are subdivided into seven families based on sequence homology and function [Robinson-Rechavi et al., 2003]. Among the functions performed by NRs is regulation of cellular differentiation both prenatally and postnatally. Indeed, NRs are critical for normal functioning in several organ systems, which makes them especially attractive drug targets when disease strikes these systems [see Anon., 2006].

NRs are also important osteoblast regulators, with several steroid hormone receptors affecting bone growth and bone mineral density [Gimble et al., 2006]. Due to their importance in cell differentiation and their role in fat and bone metabolism and the close relationship between osteoblasts and adipocytes [Gimble et al., 2006], we investigated the expression patterns of all the known NR family members in calvarial cell undergoing differentiation into osteoblastic or adipogenic phenotypes. NR profiling of adipocytic 3T3-L1 cells, macrophages and mouse tissues highlight the power of this technique to not only assay differences in NR expression among tissues but also to provide insights into the molecular regulators that may contribute to cellular differentiation [Barish et al., 2005; Fu et al., 2005; Bookout et al., 2006]. We cultured primary mouse calvarial cells in osteogenic and adipogenic media for 21 days and assayed mRNA levels for all 49 NRs by real-time PCR (qRT-PCR). Statistical analyses were then performed on the qRT-PCR data to identify patterns of NR gene expression as a function of condition and/or time.

MATERIALS AND METHODS

REAGENTS

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO).

CELL CULTURE

Primary mouse calvariae cells (MOBs) from 6- to 8-day-old CD-1 neonatal mice (Charles River laboratories, Inc., Boston, MA) [Tetradis et al., 2001a] were isolated and plated at a concentration of 100,000 cells/ml in DMEM with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 50 μ g/ml streptomycin (Mediatech, Inc Herndon, VA. Upon confluency (day 0), cells were treated with osteogenic [Kalajzic et al., 2002] or adipogenic [Cheng et al., 2003] differentiation media. Media was changed twice a week and at least 2 days prior to RNA extraction.

CELL STAINING

Von Kossa stain: MOBs were fixed with 1% formaldehyde, washed in cacodylate buffer pH 7.4, incubated in saturated lithium carbonate and subsequently incubated in 5% silver nitrate for 1 h under ultraviolet light. Cells were rinsed with water, incubated with 5% sodium thiosulfate, rinsed again with water and air-dried.

Oil Red O stain: MOBs were fixed in 60% isopropanol for 10 min and then stained with 0.3% Oil Red O in 60% isopropanol for 15 min. Subsequently, cells were rinsed with 60% isopropanol and air-dried.

REAL-TIME PCR (qRT-PCR)

Total RNA was collected at days 0, 3, 5, 7, 14, and 21 using Trizol reagent according to the manufacturer's protocol (Life Technologies, Inc., Grand Island, NY). To insure removal of genomic DNA, samples were then DNase-treated using the DNA-Free kit (Ambion Inc., Austin, TX). Three μ g total RNA were reverse-transcribed with RNA-dependent polymerase Moloney murine leukemia virus (Promega) according to manufacturer's protocol.

qRT-PCR of each gene was performed in triplicate for at least four independent experiments. One microliter (1 ng) of reverse-transcribed product was amplified with iQ SYBR Green master mix (BioRad, Hercules, CA) and gene-specific primers (Supplemental Table I) using the iCycler System and iCycler iQ Optical System software (BioRad). The amplification program was set for 1 cycle of 95°C for 3 min followed by 37 cycles of 95°C, 30 sec; 58–67°C, 20 sec (see Supplemental Table I for annealing temperature for specific genes); 72°C, 30 sec. Relative gene induction was determined by the $2^{-\Delta\Delta C_t}$ method using β -actin as control and was expressed as \log_2 fold induction [Livak and Schmittgen, 2001]. Genes that did not reach threshold by cycle 35 were considered to be not amplified.

To generate real-time PCR standard curves for each NR, adipogenic markers (lipoprotein lipase (LPL) and CD36 [Gregoire et al., 1995; Ibrahimi et al., 1996]) and osteogenic markers (alkaline phosphatase (ALP) and osteocalcin (OCN); [Aubin et al., 1995]) from bone, brain, liver and kidney was amplified using gene-specific primers (Supplemental Table I). PCR products were subcloned into a TA vector (Invitrogen Inc., Carlsbad, CA) and sequenced (Davis Sequencing, Davis, CA). Plasmid concentrations were determined and diluted to generate standard curves.

STATISTICAL ANALYSES

Hierarchical clustering was performed using CLUSTER and TREEVIEW software [Eisen et al., 1998]. Genes were clustered using uncentered Average Linkage Clustering.

Two-way ANOVA with interaction model utilized JMP6 software (SAS Institute Inc., Cary, NC). This model estimates the independent effects of culture media and time and the interaction effect of culture media and time on NR gene mRNA levels. More specifically, this analysis assesses differences in the mean NR expression for osteogenic versus adipogenic culture media over all time points (media effect), differences in the mean NR expression for each time point averaged over both culture media (time effect), and whether the time effect varies by media (media \times time effect). Fold induction was expressed as \log_2 fold induction. The significance threshold was set at 0.05.

To determine gene expression for each NR in osteogenic and adipogenic media, data for osteogenic and adipogenic induction were analyzed using one-way ANOVA with contrast. day 0 was compared to days 7, 14, and 21 using JMP6 software (SAS Institute).

RANKING

NR gene expression level was calculated based on gene-specific standard curves, normalized to the length of each amplicon and expressed relative to β -actin. The NRs were then ranked within each experiment according to their expression abundance by multiplying their original rank by 100 and dividing by 43 (the number of NRs that were regulated in the study). A minimum of three experiments containing at least 40 genes was utilized to generate the average gene ranking.

RESULTS AND DISCUSSION

VERIFICATION OF MOB OSTEOGENIC AND ADIPOGENIC POTENTIAL

MOBs are osteoblastic cells with adipocytic potential [Zhang et al., 2006]. To confirm this, MOBs cultured in osteogenic or adipogenic media were assayed for mineralization or adipogenesis at 0, 7, 14, and 21 days. MOBs cultured in osteogenic, but not adipogenic, media demonstrated mineralization by Von Kossa staining at 14 and 21 days (Fig. 1A). On the other hand, MOBs cultured in adipogenic, but not osteogenic, media exhibited positive Oil Red O staining beginning at day 7 (Fig. 1B), thus confirming the dual phenotypic differentiation capacity of primary MOBs.

We next determined if the osteogenic and adipogenic phenotypes correlate to specific osteoblast and adipocyte molecular markers. mRNA levels for osteoblast (ALP and OCN) and adipocyte (CD36 and LPL) markers were measured by qRT-PCR following osteogenic and adipogenic differentiation for 0–21 days. These data were expressed as \log_2 -fold induction and were analyzed by two-way ANOVA to detect differences due to culture media, time or culture media \times time. ALP mRNA levels were not affected by culture media (Fig. 1C,G), while OCN mRNA levels increased in osteogenic media and decreased in adipogenic media (Fig. 1D,G). CD36 and LPL were upregulated in both culture conditions; however, the level of induction for each gene was significantly higher in adipogenic media compared to osteogenic media (Fig. 1E–G).

Like culture media, time also differentially affected mRNA levels for OCN, CD36 and LPL, but not ALP (Fig. 1). While culture media and time independently affected only OCN, CD36 and LPL mRNA levels, the interaction of culture media \times time showed that all the markers were significantly regulated. mRNA levels for ALP and OCN were higher in osteogenic media over the time period studied, while LPL and CD36 mRNA levels were higher in adipogenic media during the same time period (Fig. 1).

We demonstrate here a remarkable plasticity of MOBs cultured in either osteogenic or adipogenic media. Importantly, striking osteogenic and adipogenic phenotypes paralleled this genotype plasticity. These results support the conclusion that MOBs can acquire an adipocyte-like genotype and phenotype. Osteoblast acquisition of an adipocytic phenotype [Nuttall et al., 1998; Ahdjoudj et al., 2004], and vice versa [Justesen et al., 2004; Li et al.,

2005], has been reported. This plasticity is the basis for drug development designed to shift bone marrow cellularity away from adipocytes toward osteoblasts in osteoporosis patients [Nuttall and Gimble, 2004; Rosen and Bouxsein, 2006].

NR PROFILING IN MOBs

Having verified the differentiation fate of MOBs cultured in osteogenic and adipogenic media, we next measured NR gene expression in MOBs cultured under osteogenic and adipogenic conditions. We elected to measure NR gene expression using qRT-PCR rather than microarray analysis [Beck et al., 2001; Balint et al., 2003; Billiard et al., 2003; de Jong et al., 2004; Schroeder et al., 2007]. Our interest was in changes in the NR family of transcription factors, rather than the entire genome. While microarray chips contain NR family members, NR expression levels or changes in expression could be below microarray threshold. In fact, this is the case, as several reports on osteoblast differentiation using microarray failed to identify all but one NRs undergoing significant changes in expression [Balint et al., 2003; Billiard et al., 2003; de Jong et al., 2004; Schroeder et al., 2007]. Also, we were interested in quantitating expression changes between the NR family members during osteogenic and adipogenic differentiation. While more labor intensive, the profiling approach offers a clearer, albeit less global, picture of gene expression patterns during MOB differentiation. Since NRs are critical for differentiation in a number of cell types, including osteoblasts [Gimble et al., 2006], we elected to use the profiling approach to study the entire NR family in MOBs.

To perform the NR profiling experiment, we used qRT-PCR to measure NR gene expression at days 0, 3, 5, 7, 14, and 21 in MOBs cultured under osteogenic and adipogenic conditions (Fig. 2, Supplemental Figs. 1–5). PXR, CAR1, TLL, FTZ-F1, DAX-1 and PNX were not detected at any time point in either osteogenic or adipogenic culture media; thus, they were not included in subsequent analyses.

FOUR PATTERNS OF NR GENE EXPRESSION WERE DETECTED IN MOBs CULTURED IN OSTEOGENIC AND ADIPOGENIC MEDIA

Fold induction of the remaining 43 NRs were calculated and expressed as \log_2 . These data were then subjected to hierarchical clustering analysis (Fig. 2A). Four main patterns of NR gene expression were detected. Pattern 1: NRs upregulated during osteogenic, but not adipogenic, differentiation (Fig. 2B, Supplemental Fig. 1). Pattern 2: NRs upregulated in both culture media but with a higher upregulation during adipogenic versus osteogenic differentiation (Fig. 2C, Supplemental Fig. 2). Pattern 3: NRs upregulated during both adipogenic and osteogenic differentiation (Fig. 2D, Supplemental Fig. 3). Pattern 4: NRs downregulated during adipogenic differentiation, but not during osteogenic, differentiation (Fig. 2E, Supplemental Fig. 4). Five NRs (VDR, HNF4 α , FXR β , COUP-TFI and ERR β) did not fall into any of the four patterns (Fig. 2A, Supplemental Fig. 5).

PATTERN 1: NRs UPREGULATED DURING OSTEOGENIC, BUT NOT ADIPOGENIC, DIFFERENTIATION

Twenty NRs were significantly upregulated throughout osteogenic differentiation (Table I). Noteworthy is the increased expression of

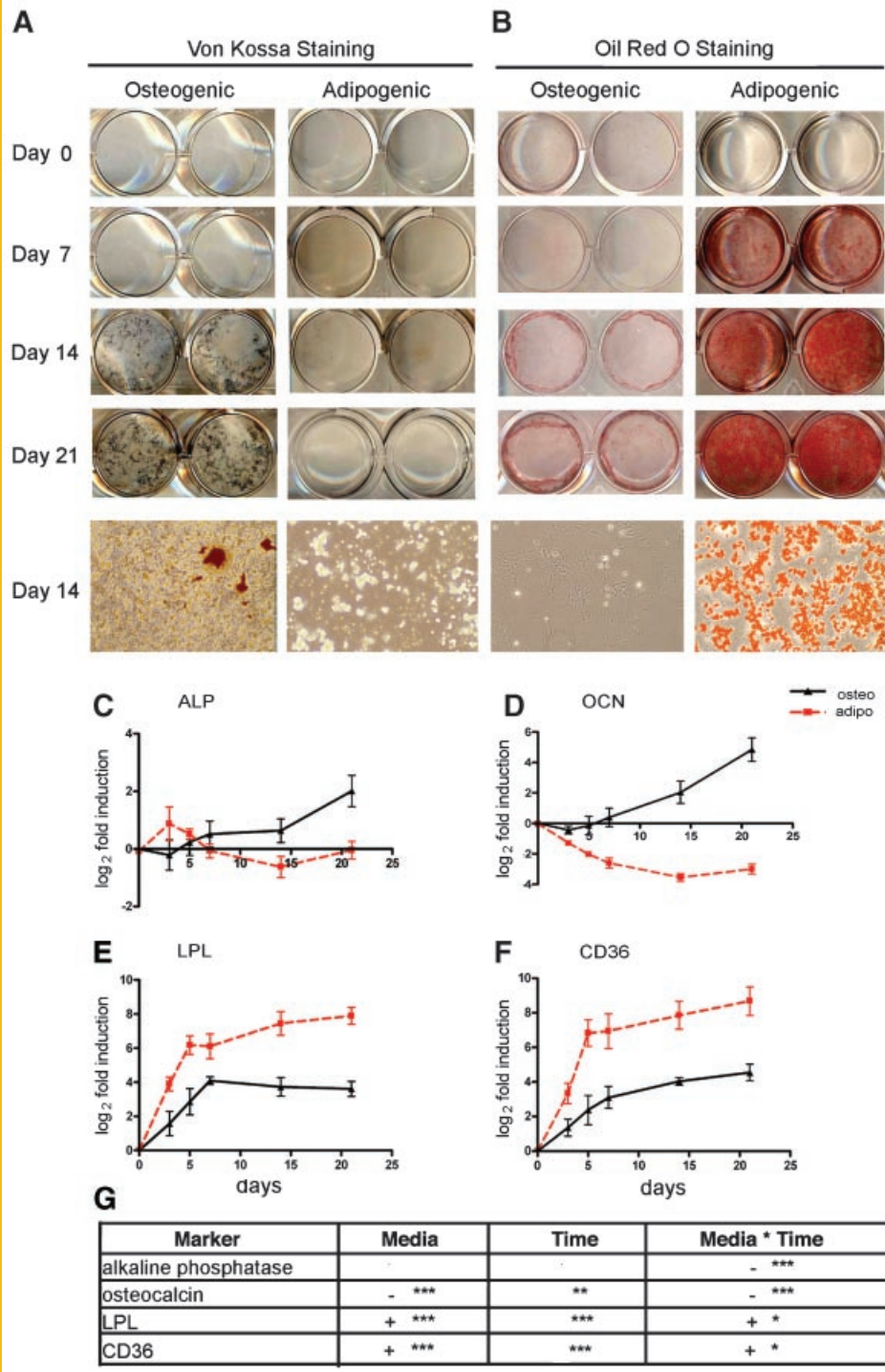


Fig. 1. Osteogenic and adipogenic differentiation. A: von Kossa stain of MOBs maintained under osteogenic or adipogenic conditions for 0, 7, 14, and 21 days. B: Oil Red O stain of MOBs maintained under osteogenic or adipogenic conditions for 0, 7, 14, and 21 days. mRNA levels for: (C) ALP, (D) OCN, (E) LPL, (F) CD36 as determined by qRT-PCR of MOBs maintained under osteogenic or adipogenic conditions for days 0, 3, 5, 14, and 21 days. Graphs of mean \pm SEM normalized to β -actin expressed as \log_2 fold induction. G: Effects of culture media, time and media \times time in the two-way ANOVA with interaction. (+) Significantly higher in adipogenic culture media. (-) Significantly higher in osteogenic culture media. All significant time effects showed increased gene expression over time. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

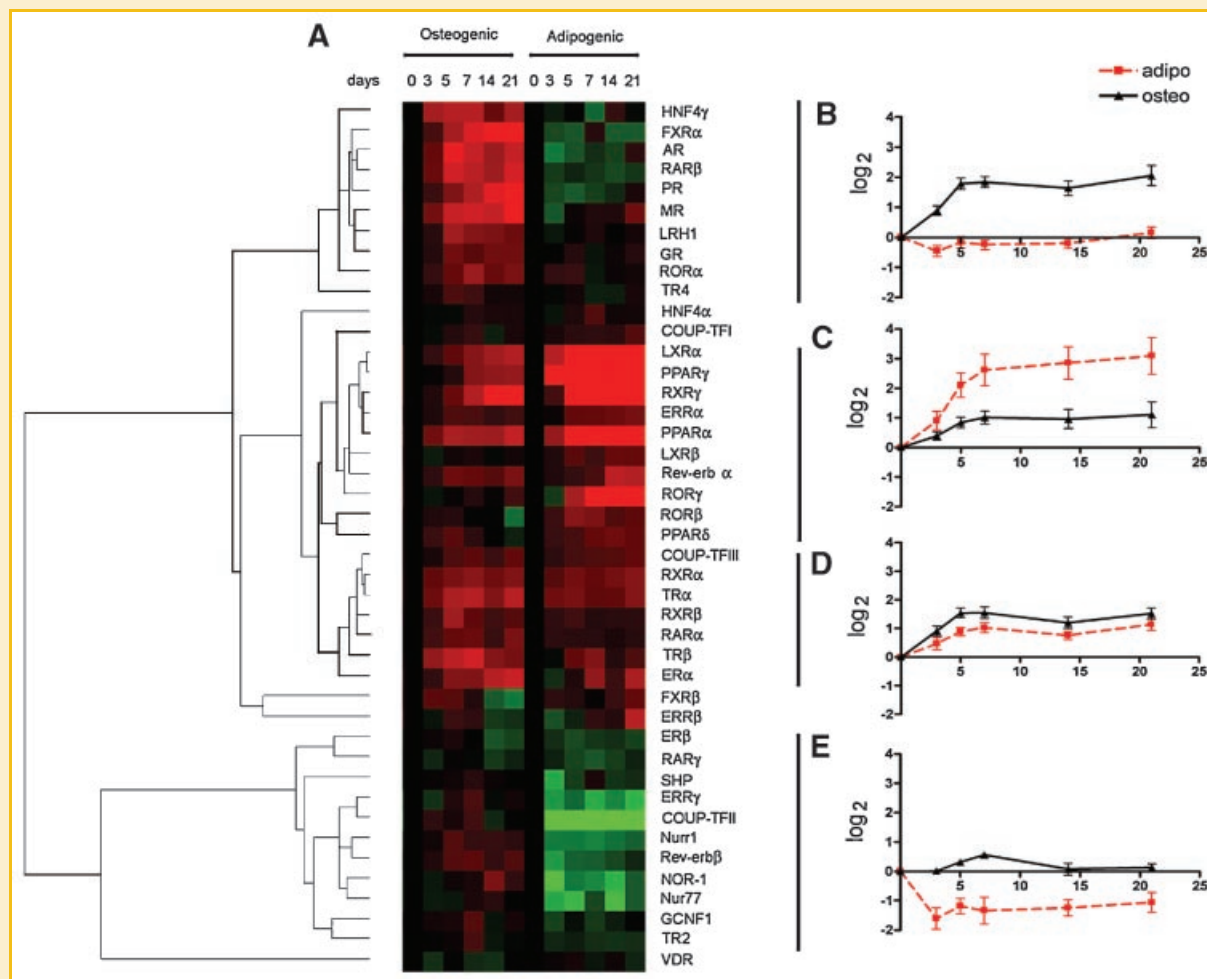


Fig. 2. Cluster analysis of NR expression during osteogenic or adipogenic differentiation. A: Log₂ fold induction ratios of gene expression relative to day 0 were used for hierarchical cluster analysis. Red represents expression levels greater than those on day 0; green represents expression levels less than those on day 0; black represents expression levels equal to those on day 0. Intensity reflects magnitude of expression. Each column is the average of at least four individual experiments. Clustering identified four main expression patterns across time and condition. B–E: Plot of mean \pm SEM of NRs in the group expressed as log₂ fold induction for days, 0, 3, 5, 7, 14, and 21. B: NRs upregulated during osteogenic, but not during adipogenic, differentiation. C: NRs upregulated in both conditions but with a higher induction in adipogenic differentiation. D: NRs upregulated in both conditions. E: NRs downregulated during adipogenic differentiation, but not during osteogenic, differentiation. HNF4 α , COUP-TF-I, FXR β , ERR β and VDR were excluded from the graphs as they did not fall into one of the four primary clusters.

all four members of the NR3C family (GR, MR, PR, AR) and the modest, but significant, increased expression of PPAR γ , PPAR α and LXR α . No NRs were downregulated during osteogenic differentiation.

Testosterone activates AR to produce significant anabolic effects in bone at the expense of adipose tissue [Yeh et al., 2002; Singh et al., 2003; Notini et al., 2007]. Thus, our data are consistent with published findings for AR's anabolicity (Fig. 2; Tables I and II). However, our data contradict data showing that AR expression increases during adipogenic differentiation of 3T3-L1 cells [Fu et al., 2005]. This may reflect a difference in lineage commitment between calvarial bone cells and preadipocytic 3T3-L1 cells.

Although PR was significantly induced in osteogenic media, it was among the least abundant NRs basally, and culturing for 21 days in osteogenic media did little to change its relative

abundance (Fig. 2; Tables I–III). Thus, it should not be surprising that PR null mice do not exhibit a skeletal phenotype [Ishida et al., 1996; Pei et al., 2003; Pei et al., 2006]. FXR α is best known for its role in controlling serum bile acid, cholesterol and triglyceride levels, but it is also implicated in adipocyte differentiation and intracellular lipid utilization to counterbalance LXR α activity [Sinal et al., 2000; Cariou et al., 2006; Kalaany and Mangelsdorf, 2006; Rizzo et al., 2006]. This is consistent with our finding that FXR α expression was induced in osteogenic media while LXR α was most strongly induced by adipogenic media (Fig. 2, Table I).

MR is expressed in osteoblasts and adipocytes [Beavan et al., 2001; Caprio et al., 2007]. Interestingly, MR levels increase biphasically in 3T3-L1 cells during adipogenic differentiation, with peak expression within 1 h of differentiation and a second peak 9–10 days later [Fu et al., 2005]. Thus, our finding that MR was the

TABLE I. One-Way ANOVA With Contrast of NR Gene Expression Under Osteogenic or Adipogenic Conditions

Formal name	Nuclear receptor	Osteogenic Media	Adipogenic Media
NR1A1	TR α	4.3***	2.9*
NR1A2	TR β	4.4***	
NR1B1	RAR α		
NR1B2	RAR β	5.9**	
NR1B3	RAR γ		
NR1C1	PPAR α	4.2***	14.0***
NR1C2	PPAR δ		2.3***
NR1C3	PPAR γ	4.6*	37.0***
NR1D1	Rev-erb α	2.4**	4.5*
NR1D2	Rev-erb β		
NR1F1	ROR α	3.1**	
NR1F2	ROR β		
NR1F3	ROR γ		
NR1H2	LXR β		
NR1H3	LXR α	4.9**	45.4***
NR1H4	FXR α	8.2***	
NR1H5	FXR β		
NR1I1	VDR		
NR2A1	HNF4 α		
NR2A2	HNF4 γ	4.1***	
NR2B1	RXR α	3.2**	2.8***
NR2B2	RXR β		
NR2B3	RXR γ	8.5***	43.0***
NR2C1	TR2		
NR2C2	TR4		
NR2F1	COUP-TFI		1.7**
NR2F2	COUP-TFII		
NR2F6	COUP-TFIII	2.2*	2.1***
NR3A1	ER α	4.4***	
NR3A2	ER β		
NR3B1	ERR α	1.7**	2.5***
NR3B2	ERR β		2.9*
NR3B3	ERR γ		0.7*
NR3C1	GR	2.3***	
NR3C2	MR	8.7***	
NR3C3	PR	7.8***	
NR3C4	AR	5.7***	
NR4A1	Nurr77		
NR4A2	Nurr1		0.5**
NR4A3	NOR-1		0.4**
NR5A2	LRH1	3.1*	
NR6A1	GCNF1		
NR0B2	SHP		

NR gene expression during days 7, 14, and 21 was compared to day 0 utilizing one-way ANOVA with contrast. Values are fold change values comparing days 7–21 versus day 0.

* $P < 0.05$.
 ** $P < 0.01$.
 *** $P < 0.001$

most strongly induced NR in osteogenic media, but was not significantly induced in adipogenic media (Table I), was unexpected.

PATTERN 2: NRs UPREGULATED IN BOTH CULTURE MEDIA BUT WITH A HIGHER UPREGULATION DURING ADIPOGENIC VERSUS OSTEOGENIC DIFFERENTIATION

The NRs that showed the highest fold-induction were among the group expressed more in adipogenic media than osteogenic media. LXR α , PPAR γ and RXR γ were induced over 37-fold, while PPAR α and Rev-erb α were induced 14- and 4.5-fold, respectively, in adipogenic media (Table I). With the exception of PPAR α , these genes are markedly regulated during 3T3-L1 adipogenic differentiation, with LXR α , RXR γ and PPAR γ expression rising late in differentiation and Rev-erb α having both early and late expression

TABLE II. Two-Way ANOVA of NR Expression During Osteogenic and Adipogenic Differentiation

Formal name	Nuclear receptor	Media	Time	Media \times time
NR1A1	TR α		**	
NR1A2	TR β	–*		
NR1B1	RAR α			
NR1B2	RAR β	–***		–*
NR1B3	RAR γ			
NR1C1	PPAR α	+**	***	
NR1C2	PPAR δ	+**		+**
NR1C3	PPAR γ	+***	***	+*
NR1D1	Rev-erb α		***	
NR1D2	Rev-erb β	–***		
NR1F1	ROR α	–**		
NR1F2	ROR β	+*		
NR1F3	ROR γ	+**	**	+*
NR1H2	LXR β	+*		
NR1H3	LXR α	+***	***	+**
NR1H4	FXR α	–***		–**
NR1H5	FXR β			+***
NR1I1	VDR			
NR2A1	HNF4 α			
NR2A2	HNF4 γ	–*		
NR2B1	RXR α		**	
NR2B2	RXR β			
NR2B3	RXR γ	+**	***	
NR2C1	TR2			
NR2C2	TR4	–*		
NR2F1	COUP-TFI	+*		
NR2F2	COUP-TFII	–**		
NR2F6	COUP-TFIII		**	
NR3A1	Er α		***	
NR3A2	Er β	–*	***	
NR3B1	ERR α		***	
NR3B2	ERR β			+***
NR3B3	ERR γ	–***		
NR3C1	GR	–***	*	
NR3C2	MR	–***	***	
NR3C3	PR	–***	***	–***
NR3C4	AR	–***	**	
NR4A1	Nurr77	–***		
NR4A2	Nurr1	–***		
NR4A3	NOR-1	–***		
NR5A2	LRH1	–***		
NR6A1	GCNF1			
NR0B2	SHP			

Culture media, time and media \times time effects in two-way ANOVA models. Significantly higher in adipogenic media (+) or osteogenic media (–). All significant time effects were increasing (overall level for condition effects, rate of increase or interaction effects).

* $P < 0.05$.
 ** $P < 0.01$.
 *** $P < 0.001$.

peaks [Fu et al., 2005]. The strong induction of these classic adipogenic genes in MOBs cultured in adipogenic media supports the genotypic plasticity of osteoblasts.

LXR α regulates cholesterol homeostasis, bile acid metabolism, lipid metabolism and adipocytic differentiation [Tontonoz et al., 1995b; Juvet et al., 2003; Ulven et al., 2005; Cummins and Mangelsdorf, 2006]. LXR α and its isoform LXR β are both expressed in bone, although LXR β is the more strongly expressed of the two, which we confirm here [Robertson et al., 2006] (Table III). Surprisingly, given their relative expression levels in bone, it is LXR α $-/-$, rather than LXR β $-/-$, mice that have a bone phenotype [Robertson et al., 2006]. LXR α null females have increased cortical bone suggesting that LXR α may inhibit bone formation. These data may also suggest that LXR α preferentially promotes adipocytogenesis over osteoblastogenesis. Indeed, our data that LXR α is

TABLE III. NR Ranking by Expression Abundance

Formal name	Nuclear receptor	Day 0	Adipogenic (Day 21)	Osteogenic (Day 21)
NR2C1	TR2	1	5	1
NR2F6	COUP-TFIII	2	4	4
NR2F2	COUP-TFII	3	6	6
NR2B1	RXR α	4	3	7
NR2F1	COUP-TFI	5	7	8
NR1B3	RAR γ	6	12	2
NR1I1	VDR	7	8	18
NR4A1	Nur77	8	19	14
NR1A1	TR α	9	11	3
NR1H2	LXR β	10	10	9
NR1C3	PPAR γ	11	1	5
NR1D1	Rev-erb α	12	9	13
NR2B2	RXR β	13	16	10
NR1C2	PPAR δ	14	15	12
NR3B1	ERR α	15	13	19
NR2C2	TR4	16	17	21
NR1D2	Rev-erb β	17	20	11
NR3C1	GR	18	14	17
NR1H3	LXR α	19	2	16
NR1B1	RAR α	20	22	15
NR4A2	Nurr1	21	26	23
NR0B2	SHP	22	24	30
NR1F1	ROR α	23	21	22
NR1C1	PPAR α	24	18	20
NR3C4	AR	25	23	25
NR5A2	LRH1	26	27	24
NR6A1	GCNF1	27	28	26
NR3A2	ER β	28	31	27
NR2A1	HNF4 α	29	32	36
NR1A2	TR β	30	29	29
NR4A3	NOR-1	31	36	31
NR1F2	ROR β	32	33	34
NR1F3	ROR γ	33	25	40
NR3A1	ER α	34	37	32
NR3B2	ERR β	35	30	42
NR3C2	MR	36	34	35
NR1B2	RAR β	37	38	37
NR1H4	FXR α	38	39	33
NR2B3	RXR γ	39	35	28
NR3C3	PR	40	40	38
NR3B3	ERR γ	41	41	39
NR1H5	FXR β	42	42	43
NR2A2	HNF4 γ	43	43	41

Relative expression levels for each NR were determined at days 0 and 21 of osteogenic or adipogenic culture.

upregulated in MOB s cultured in adipogenic, but not osteogenic, media supports a role for LXR α in directing the adipogenic phenotype.

Our finding that PPAR γ is upregulated in MOB s cultured in adipogenic media confirms previous findings [Diascro et al., 1998; Maurin et al., 2005]. There is evidence that the high PPAR γ levels that we detected are a major contributor to the adipocytic phenotype that MOB s acquire in adipogenic media [Kim et al., 2005]. In fact, PPAR γ 's potency as an adipogenic factor is so strong that an adipogenic phenotype results in several cell types in which PPAR γ is activated [for recent examples see Hazra et al., 2004; Semple et al., 2006; Singh et al., 2006; Yamanouchi et al., 2007].

Given the breadth of cell types responsive to PPAR γ activation, including osteoblasts [Li et al., 2006], it is likely that osteoporosis treatments designed to inactivate PPAR γ would lack specificity, resulting in potential unwanted side effects. Moreover, confirming previous observations [Diascro et al., 1998; Nuttall et al., 1998; Lecka-Czernik et al., 1999], the PPAR γ activators troglitazone and ciglitazone demonstrate dose-dependent effects in osteoblastic MC3T3-E1 cells [Jackson, 1991]. At high doses, both PPAR γ

activators inhibited mineralization and alkaline phosphatase activity. However, at low doses (<2.5 μ M), troglitazone and ciglitazone significantly increased mineralization and alkaline phosphatase activity. Taken together with our data that osteogenic media increased PPAR γ expression in osteoblasts, these data suggest that low-level PPAR γ activation may enhance osteoblastic activity.

RXR γ is highly expressed in muscle, anterior pituitary and brain, yet RXR γ deletion does not affect development, probably due to redundancy among the RXR α , β and γ isoforms [Krezel et al., 1996]. Supporting this is the finding that RXR γ mRNA levels increase in adipocytes in which RXR α is conditionally knocked-out [Metzger et al., 2005]. Importantly, RXR isoform redundancy appears to have functional specificity [Metzger et al., 2005]. RXR α deletion in mature adipocytes blocks excess lipid accumulation and RXR α deletion in RXR γ null adipocytes leads to cell death. Interestingly, PPAR γ deletion in mature adipocytes also leads to apoptosis, suggesting that PPAR γ -mediated adipocyte survival requires either RXR α or RXR γ and that PPAR γ -mediated lipid accumulation requires RXR α . If this were the case, then we would conclude that increased RXR γ in MOB s cultured in adipogenic media might not contribute to lipid accumulation (Fig. 1), but instead may contribute to cell survival.

PPAR α potentially regulates fatty acid oxidation (FAO) genes [Lee et al., 1995; Schoonjans et al., 1996]. Interestingly, adult female PPAR α null mice develop significant cortical bone porosity [Wu et al., 2000] and high-dose PPAR α activators increase *in vitro* osteogenesis [Jackson and Demer, 2000]. Taken together, these data suggest that PPAR α may regulate adipogenesis and osteogenesis by regulating cellular metabolism. Our finding that both osteogenic and adipogenic media induced PPAR α expression, albeit at different magnitudes, suggests that PPAR α induction in MOB s may allow these cells to adapt metabolically to varying signals.

Rev-erb α is an orphan nuclear receptor that is induced during adipocyte differentiation [Chawla and Lazar, 1993]. In MOB s , Rev-erb α was induced 4.5-fold in adipogenic media and showed a significant, but lower, induction in osteogenic media (Fig. 2; Table I). It is interesting to consider that Rev-erb α and Rev-erb β are transcribed from the antisense strand of the TR α gene and Rev-erb α expression inhibits TR α expression [Munroe and Lazar, 1991; Chomez et al., 2000]. Consistent with this, we found a reciprocal relationship between Rev-erb α and TR α expression in MOB s cultured in osteogenic and adipogenic media. While both genes have relatively high expression (TR α is ranked 9th and Rev-erb α is 12th; Table III), adipogenic media increased Rev-erb α 's ranking to 9th place, while TR α dropped to 11th. On the other hand, osteogenic media increased TR α 's position to 3rd and dropped Rev-erb α to 13th place. These data suggest that preferential transcription from this locus toward TR α will promote osteoblast differentiation, while Rev-erb α expression will promote adipocyte differentiation.

PATTERN 3: NRs UPREGULATED DURING BOTH ADIPOGENIC AND OSTEOGENIC DIFFERENTIATION

Among the genes equally upregulated in osteogenic and adipogenic media were COUP-TFIII, RXR α and TR α . Interestingly, RXR α and TR α are intermediate markers of adipogenic differentiation, having high basal expression that increases and plateaus 1–2 days after

differentiation is initiated [Fu et al., 2005]. We also found high basal expression for these genes, as well as for COUP-TFIII. In fact, COUP-TFIII, RXR α and TR α were, respectively, the 2nd, 4th, and 9th most highly expressed NRs at baseline (Table III).

PATTERN 4: NRs DOWNREGULATED DURING ADIPOGENIC DIFFERENTIATION, BUT NOT DURING OSTEOGENIC DIFFERENTIATION

Nurr1, NOR-1 and ERR γ were significantly downregulated in adipogenic media (Tables I and II). The appearance of Nurr1 and NOR-1 in this cluster was of particular interest to us, as we showed that parathyroid hormone (PTH) rapidly and transiently induces these genes in osteoblasts, thereby classifying them as primary response genes [Tetradis et al., 2001a,b; Pirih et al., 2003; Pirih et al., 2005]. Moreover, Nurr1 induces both osteopontin and osteocalcin, which are important osteoblast differentiation markers [Lammi et al., 2004; Pirih et al., 2004]. Consistent with their classification as primary response genes, Nur77, Nurr1 and NOR-1 are also rapidly and transiently induced following addition of adipogenic differentiation agents in 3T3-L1 cells [Fu et al., 2005]. ERR γ expression in osteoblasts has not been shown previously and its role in adipocyte function is primarily as an ERR α inducer [Liu et al., 2005].

NR EXPRESSION DURING OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION

NR expression levels plateaued on days 7–21. To avoid spurious results based on statistical fluctuations and to increase the power of our statistical methods, we grouped gene expression levels from days 7, 14, and 21 and performed one-way ANOVA with contrast between day 0 and the grouped data from days 7 to 21 for osteogenic and adipogenic culture conditions (Table I).

Twenty NRs were significantly upregulated throughout osteogenic differentiation. Noteworthy is the increased expression of all four members of the NR3C family (GR, MR, PR, AR) and the modest, but significant, increased expression of PPAR γ , PPAR α and LXR α . No NRs were downregulated during osteogenic differentiation.

On the other hand, 12 NRs were upregulated during adipogenic differentiation. These included PPAR γ , LXR α and RXR γ . Moreover, three NRs (ERR γ , Nurr1, NOR-1) were downregulated during adipogenic differentiation.

NR EXPRESSION AS A FUNCTION OF OSTEOGENIC AND ADIPOGENIC CULTURE MEDIA AND TIME

Having demonstrated that NR gene expression varies in MOB cultured in either osteogenic or adipogenic culture media, we next investigated in greater detail the effect of culture media, time and the interaction of culture media with time (media \times time) on NR expression in MOB by performing a two-way ANOVA with interaction analysis on our qRT-PCR data. Twenty-seven NRs were differentially expressed in osteogenic versus adipogenic culture media (Table II). Eighteen of these 27 NRs were significantly higher in osteogenic media, including, the NR3C and NR4A (Nur77, Nurr1, NOR-1) families and LRH1. The remaining nine NRs were higher in adipogenic media, including the NR1C (PPAR α , β , γ) and NR1H

(LXR α , LXR β , FXR α , FXR β) families. Interestingly two members of the NR1F family (ROR β , ROR γ) were higher in adipogenic media while one (ROR α) was higher in osteogenic media.

When the time effect was analyzed, 16 NRs were upregulated, while none were downregulated. Among these genes were all four members of the NR3C family and ER α , ER β , PPAR γ . The interaction of culture media with time (media \times time) revealed that nine NRs were significantly different. Six had higher expression in adipogenic media and three NRs had higher expression in osteogenic media over the time course (Table II).

RANKING OF NR GENE EXPRESSION

Fig. 2 and Tables I and II demonstrated changes in individual NR levels in MOB undergoing osteogenic versus adipogenic differentiation. To compare gene expression among NRs, we established the relative abundance of all NRs at culture day 0 and day 21 (Table III).

At day 21 of osteogenic differentiation, RXR γ , TR α , PPAR γ , RAR α and FXR ranked at least five positions higher, while VDR, SHP, ERR β , ROR γ , HNF4 α , Nur77 and TR4 ranked at least five positions lower, relative to their rankings on day 0. TR2 and RAR γ were the most highly expressed NRs in MOB following 21 days in osteogenic culture media.

LXR α , PPAR γ , ROR γ , PPAR α , and ERR β ranked at least five positions higher following adipogenic differentiation. Nur77, RAR γ , NOR-1, and Nurr1 ranked at least five positions lower relative to the ranking of day 0. PPAR γ and LXR α were the most highly expressed NRs in MOB after 21 days of culture in adipogenic media.

CONCLUSIONS

We have demonstrated that MOB cultured in osteogenic and adipogenic media acquired distinct phenotypes that correlated with distinct NR genotypes. Our data provide an initial fingerprint of NR gene expression in relation to osteoblastic differentiation. Additional investigation on NR protein expression, specific isoform regulation and functional analyses, should further provide evidence of NR involvement in cellular commitment and may offer clues to the underlying mechanisms of osteoblast differentiation and function. The remarkable plasticity of the osteoblast genome may also contribute to the consistent finding that marrow adiposity increases in patients with bone metabolic diseases such as osteoporosis. The possibility that selected NRs may be therapeutic targets to prevent and reverse the debilitating effects of bone loss warrants consideration. Indeed, NR-targeted therapies are being designed to treat osteosarcomas [Haydon et al., 2002; Lucarelli et al., 2002], highlighting the importance of this transcription factor superfamily in maintaining normal osteoblast function and the feasibility of designing efficacious drugs to alter NR function.

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