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Identification and Cloning of a Human Urea Transporter HUT11, Which Is Downregulated During Adipogenesis of Explant Cultures of Human Bone

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Abstract Bipotential cells in human trabecular bone explant cultures that express osteoblast characteristics are able to undergo adipogenesis in the presence of 3-isobutyl-1-methylxanthine plus dexamethasone (Nuttall et al. [1998] J Bone Miner Res 13:371–382). The initial studies of these bipotential cells in explant cultures have been extended to examine differential gene expression during osteoblast/adipocyte *trans*differentiation. Using differential display, we have identified a gene expressed in trabecular bone explant cultures that is downregulated as these cells differentiate from an osteoblast to an adipocyte phenotype. Homology searching identified this gene as the human urea transporter HUT11. The expression and downregulation of HUT11 have been observed in multiple patient bone explant cultures. The size of the bone explant-derived HUT11 mRNA is ~4.4 kb, which is identical to the largest splice variant reported. In this article, we report the cloning and sequencing of this gene from primary human osteoblasts. In addition, we report tissue distribution for the bone explant-derived form of HUT11 mRNA and show a reciprocal relationship between the expression of HUT11 and the nuclear hormone receptor peroxisome proliferator-activated receptor gamma 2, which is a marker of adipocyte differentiation. Because the control of osteoblast/adipocyte *trans*differentiation is unknown, selective downregulation of HUT11 during adipogenesis suggests that HUT11 expression may be a marker of the switch from an osteoblast to an adipocyte phenotype. Understanding the role of HUT11 in osteoblasts may provide insights into the mechanism controlling osteoblast and adipocyte differentiation. J. Cell. Biochem. 76:639–650, 2000.

Key words: adipogenesis; urea transporter; HUT11; bone; osteoblast; marrow

It is well known that the decrease in bone volume associated with osteoporosis and age-related osteopenia is accompanied by increased marrow adipose tissue formation [Burkhardt et al., 1987; Meunier et al., 1971]. The reversal of this process may provide a novel therapeutic approach to either the prevention or treatment of these disorders [Burkhardt et al., 1987; Meunier et al., 1971; Student et al., 1980]. An increase in marrow adipocytes is observed in a variety of conditions that lead to bone loss, such as ovariectomy [Martin et al., 1990; Wronski et al., 1986], immobilization [Miniare et al., 1974], or treatment with glucocorticoids [Wang et al., 1977].

There is accumulating evidence for the existence of a multipotential mesenchymal stem cell as defined by lineage-specific gene expression and enzyme activities [Beresford et al., 1992; Dani et al., 1997; Friedenstein, 1990; Kelly et al., 1998; Nuttall et al., 1998; Park et al., 1999; Quarto et al., 1997] that gives rise to both the osteogenic and adipocytic lineage. Furthermore, evidence supporting the *trans*differentiation of these cells, suggests a large degree of plasticity between osteoblasts and adipocytes [Nuttall et al., 1998; Park et al., 1999;

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Pittenger et al., 1999]. In general, the relationship between adipogenesis and osteoblastogenesis appears to be reciprocal [Beresford et al., 1992; Dorheim et al., 1993].

In the mouse multipotential stromal cell line BMS-2, it has been shown that 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$] induces osteoblast differentiation with a corresponding inhibition of adipocyte differentiation [Kelly et al., 1998; Kelly and Gimble, 1998]. In co-culture with hematopoietic cells, BMS-2 cells can support the terminal differentiation of osteoclasts, which has been suggested as a role for bone marrow adipocytes [Kelly et al., 1998]. It has also been suggested that adipocytes play a purely passive role by filling marrow cavities and that they are not required for active hematopoiesis [Gimble, 1990; Tavassoli, 1984]. Other investigators suggest that adipocytes play a role in lymphohematopoiesis [Gimble et al., 1992; Pietrangeli et al., 1998] or serve an active role in the energy metabolism of the resorbing osteoclast. Adipocytes may participate in the animal's overall metabolism by clearing and storing circulating triglycerides [Hussain et al., 1989], as fatty acid oxidation appears to be the major source of acetyl-CoA to support a predominantly oxidative metabolism [Dodds et al., 1994]. These roles may not be mutually exclusive and it is possible that the role of marrow adipocytes changes in response to age, menopause, or emergency situations affecting either hematopoiesis or osteogenesis, or both.

It is well established that cultured explants of human trabecular bone (hOB) provide a means for obtaining large numbers of primary human cells that express the osteoblast phenotype [Beresford et al., 1984; Gundle et al., 1995; Gundle and Beresford, 1995; MacDonald et al., 1984; Mills et al., 1979]. These cells have been shown to produce type I collagen and express high levels of the osteoblast-specific marker, osteocalcin, and the bone/liver/kidney form of alkaline phosphatase. Both osteocalcin expression and alkaline phosphatase activity are further elevated in response to 1,25$(OH)_2D_3$ [Ashton et al., 1985; Beresford et al., 1986; Subramaniam et al., 1992; Weinreb et al., 1990]. In addition, these cells have the capacity to mineralize and produce all the major noncollagenous proteins of the extracellular bone matrix [Beresford et al., 1984; Gundle et al., 1994; Robey and Termine, 1985]. hOB preparations within diffusion chambers, which prevent mixing with host cells, form bone when implanted in vivo [Gundle et al., 1995]. By contrast, hOBs in the presence of dexamethasone (dex) plus 3-isobutyl-1-methylxanthine (IBMX) become rounded, small perinuclear granules appear and, over a 2- to 3-week period, fuse to form highly refractile vacuoles [Nuttall et al., 1998]. These vacuoles stain positive for neutral lipids by oil Red O and express adipocyte lineage-specific enzymes and genes, such as α-glycerophosphate 3-dehydrogenase activity, fatty acid binding protein aP2, and lipoprotein lipase. Adipogenesis can be inhibited in these cultures by either TNF-α or TGF-β. However, the mechanism by which adipogenesis is regulated in this model system remains unknown.

We therefore extended our initial studies [Nuttall et al., 1998] to investigate regulated gene expression in hOBs undergoing adipogenesis using differential display [Liang and Pardee, 1992], which permits identification of both upregulated and downregulated genes. We report the identification of a gene expressed in explant cultures from multiple patient samples that is downregulated as the cells undergo adipogenesis. After identification of full-length clones and homology searching, we identified this gene as the human urea transporter HUT11 [Lucien et al., 1998; Olives et al., 1994, 1996; Xu et al., 1997]. Urea is formed as a product, along with ornithine, after the catalysis of arginine via arginase within the urea cycle. Our data suggest that the downregulation of HUT11 gene expression may be a marker of the switch from the osteoblast to the adipocyte phenotype.

MATERIALS AND METHODS

Cell Culture and hOB Differentiation

Specimens of human trabecular bone were obtained with informed consent immediately after knee joint replacement surgery. Explants were isolated and cultured as described previously [Beresford et al., 1984; Nuttall et al., 1998]. hOBs, MG-63 [Billau et al., 1977], and ST2 [Whitlock et al., 1987] cultures were grown in Eagle's modified minimal essential medium (EMEM) (Gibco-BRL, Grand Island, NY). Saos2 [Fogh et al., 1977], HeLa [Gey et al., 1952], and TF274 [Prabhakar et al., 1998] cultures were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL). Jurkat [Weiss et al., 1984] and THP-1 [Tsuchiya et al., 1980] cultures were grown in RPMI medium 1640 (Gibco-BRL). Primary human chondrocyte cultures

were grown in 50% DMEM and 50% F12 Nutrient Mixture (Ham) (Gibco-BRL). All media were supplemented with 10% heat-inactivated charcoal-stripped fetal calf serum (FCS) (Hyclone, Logan, UT), penicillin (5 U)-streptomycin (5 µg) (Gibco-BRL), and L-glutamine (2 mM) (Gibco-BRL). Adipocyte differentiation of hOB cultures was induced by IBMX (100 µg/ml) (Sigma Chemical Co., St. Louis, MO) plus dex (1 µM) (Sigma). The cytokines TGF-β (100 ng/ml) (Genzyme, Boston, MA) and tumor necrosis factor-α (TNF-α) (100 ng/ml) (Genzyme, Boston, MA) were added to parallel cultures containing IBMX and dex. Fresh media and compounds were added every 3 days.

Differential Display

Total RNA was isolated from experimental cultures using Tri Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol [Chomczynski, 1993]. Messenger RNA (mRNA) was isolated using the PolyA Tract Isolation System (Promega, Madison, WI). Differential display (DD) was performed on mRNA isolated from hOBs cultured for 3 days both in the presence and in the absence of IBMX plus dex treatment, using "RNAmap" [Liang and Pardee, 1992] (Genhunter, Brookline, MA) as described by the manufacturer. A total of 80 different primer combinations were examined. The original DD band was identified with primers T12MA and AP-16. This DD band was initially sequenced with primer AP-16, followed by subcloning into the vector pCR 2.1 (Invitrogen, San Diego, CA). After cloning, the original DD band was resequenced using M13 universal forward and reverse sequencing primers.

Northern Blot Analysis

Total RNA samples were denatured in 7.7% formaldehyde and 50% formamide, followed by agarose gel electrophoresis [Kroczek and Siebert, 1989]. The RNA was transferred to Zeta Probe membranes (Bio-Rad, Hercules, CA) by vacuum blotting [Kroczek and Siebert, 1989] and cross-linked to the membrane in a Stratalinker (Stratagene, La Jolla, CA). Multiple tissue Northern blots were purchased from Clontech Laboratories (Palo Alto, CA). Hybridization with labeled probes was performed in Express Hyb (Clontech) and washed according to the manufacturer's protocol. Hybridized membranes were exposed to storage phosphor screens for an appropriate length of time (2–24 h) and then analyzed on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

cDNA Probes

cDNA probes were labeled with α-^{32}P-dCTP (New England Nuclear, Boston, MA) using a random primer labeling kit (Pharmacia, Piscataway, NJ) according to the manufacturer's protocol. In addition to the cloned DD band, a plasmid containing the HUT11 3′ UTR was obtained from the Image consortium (Image Clone 1010178) (NCBI:868756, accession no. AA228899) (Research Genetics, Huntsville, AL). A probe to β-actin (Clontech) was used to confirm equal loading of RNA on Northern blots.

Reverse Transcription-Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction (RT-PCR) amplification was performed using GeneAmp reagents (Perkin-Elmer, Foster City, CA), Taq DNA polymerase (United States Biochemical, Cleveland, OH), and dNTPs (Promega). $MgCl_2$ concentrations were optimized for each primer set. The program was as follows, 95°C, 30 s; 52°C, 1 min; 72°C, 1 min. The primers used: forward-AAGCCTTTGGAACTATGA, reverse-GTTCCATTTACAGATTTG yielded a 285-bp product when using the cloned cDNA as the template. Parallel RT-PCR reactions were performed using primers to β-actin (Clontech) to confirm equal loading of template into the RT-PCR reactions. PCR amplifications were performed in 1.5 mM $MgCl_2$ for 25, 30, 35, and 40 cycles to ensure that amplification was within the linear range. The data shown are amplicons from 25 cycles of RT-PCR.

Cloning

In order to obtain additional sequence information, "RACE Marathon PCR" (Clontech) was used to extend the complementary DNA (cDNA) sequence. PCR primers were designed from the sequence of the DD-RT-PCR product. PCR was performed using the Clontech Marathon-Ready cDNA from prostate and skeletal muscle and Advantage KlenTaq polymerase (Clontech). The PCR products were cloned directly into the pCR2.1 "TA" vector (Invitrogen, Carlsbad, CA). Colonies were screened using an oligonucleotide probe matching the extreme 5′ end of the known DD-RT-PCR product sequence. Several

positively hybridizing clones were isolated. The insert DNA was sequenced by dye terminator cycle sequencing (Perkin-Elmer) using an Applied Biosystems model 373 automated DNA sequencer. A second method was used to isolate additional upstream sequence of the cDNA. Pooled arrays of clones from a fetal liver cDNA plasmid library were screened using a PCR-based method (Mooney et al., manuscript in preparation). The PCR primers were designed from the known DD-RT-PCR product sequence. Three rounds of PCR resulted in the isolation of a clone which had a sequence overlap of 330 base pairs (bp) with the clone isolated by Marathon PCR.

TaqMan RT-PCR Quantitation

TaqMan real-time quantitative RT-PCR was performed according to the manufacturer's protocol (Perkin-Elmer Applied Biosystems) with the following modifications [Heid et al., 1996]. For each sample, 0.5 μg of total RNA was reverse transcribed according to the manufacturer's protocol (Promega) in a final volume of 50 μl. The RT reactions were then diluted to 500 μl in water. 5 μl of stock cDNA template was used per well for quantitation on an ABI PRISM sequence detection system. Amplification of the mRNA for the ribosomal protein RL-19 (accession no. S566985) was used to normalize HUT11 and nuclear hormone receptor peroxisome proliferator activated receptor gamma 2 (PPARγ2) expression. Human male genomic DNA (cat. number G1471; Promega) was used as a positive control and copy number standard. The absolute concentrations used were in 10-fold dilutions from 10^6 to 10^0 copies per reaction (using the ratio of 1 copy/3.5 pg of genomic DNA for a single copy gene) [Sambrook et al., 1989]. The sequences for the primers and probe used for each of the genes analyzed were as follows. HUT11: Forward Primer GAGGTGGAAAGATCGCTTGTG, Reverse Primer TTGAGATAGGGTCTTGCTCGG, Probe 6FAM-ATGATCATGTCACTGCACTCCAGCCTGTTAMRA.

PPARγ: Forward Primer CACAGGCCGAGAAGGAGAA, Reverse Primer TCAGCGGACTCTGGATTCAG, Probe 6FAM-TCGATATCACTGGAGATCTCCGCCAAC-TAMRA. Although this primer probe set does not distinguish between PPARγ1 and PPARγ2, it was previously shown in hOB cultures that PPARγ2 is upregulated during adipogenesis [Nuttall et al., 1998; Tontonoz et al., 1995a,b]. RL-19: Forward Primer CAAGCGGATTCTCATGGAACA, Reverse Primer TGGTCAGCCAGGAGCTTCTT, Probe 6FAM-TCCACAAGCTGAAGGCAGACAAGGC-TAMRA; Cycle conditions were as follows: 50°C, 2 min; 95°C,10 min; followed by 95°C,15 s; and 58°C (HUT11) or 60°C (PPARγ and RL-19) –1 min at 40 cycles.

RESULTS

Identification of a Gene Downregulated in Human Trabecular Bone Explant Cultures Undergoing Adipogenesis

Previously our laboratory described the differentiation of bipotential precursor cells present in hOB cultures to either the osteoblastic or adipocytic phenotype [Nuttall et al., 1998]. We used this differentiation system to search for genes involved in the process of adipocyte differentiation. Using differential display (DD), we compared the pattern of gene expression in human trabecular bone cell cultures (hOBs) with parallel cultures induced to undergo adipogenesis after treatment with IBMX plus dex for 72 h. This treatment period was selected in an effort to identify genes that were differentially expressed early in the commitment to differentiation, or as a marker of the switch from osteoblastogenesis to adipogenesis.

A total of six transcripts were found to be differentially expressed, one upregulated and five downregulated. One of the downregulated transcripts was chosen for in-depth analysis based on its strong expression in osteoblasts. Northern blot analysis demonstrated a single mRNA species of approximately 4.4 kb in control cultures (Fig. 1), which was downregulated after treatment with IBMX plus dex for 28 days. The hOB cultures had undergone adipogenesis as determined by the accumulation of refractile vacuoles of neutral lipid (Fig. 2). Sequencing and initial database analysis of the cloned cDNA revealed no homology with any known protein (Fig. 3). Further screening of the National Center for Biotechnical Information (NCBI) expressed sequence tag (EST) database identified homology with seven ESTs. Six of these ESTs were from NCBI prostate tissue libraries. A clone for one of these ESTs, NCBI: 868756, was obtained from the Image Consortium and used to determine the expression and tissue distribution pattern using a series of human multiple tissue Northern blots (Fig. 4). A 4.4-kb transcript was observed in prostate

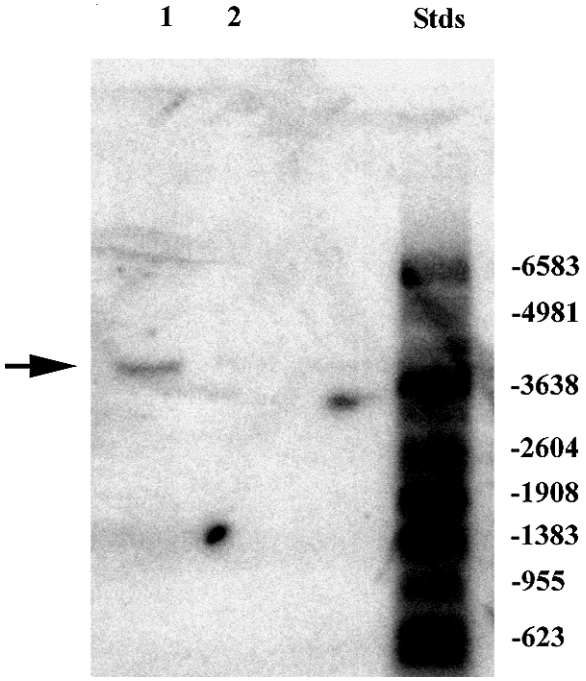

Fig. 1. Northern blot probed with cloned differential display (DD) band. hOB cultures were differentiated and Northern blots performed as described under Materials and Methods. **Lane 1**, untreated cultures, 4.4-kb form of HUT11 (arrow); **lane 2**, 3-isobutyl-1-methylxanthine (IBMX) plus dex-treated cultures. The probe used was the cloned cDNA from the original DD band, the sequence of which can be seen in Fig. 2. The lanes were evenly loaded as judged by ribosomal RNA staining and β-actin probing (not shown).

and spleen. In addition, this 4.4-kb transcript was also expressed in spinal cord, and bone marrow (data not shown). A less abundant, approximately 3.0-kb, transcript was observed in thymus. No hybridization was detected to mRNA from the a variety of normal tissues including, testis, ovary, small intestine, colon mucosal lining, peripheral blood leukocytes, stomach, lymph node, trachea, adrenal gland, or uterus.

Identification of the cDNA as HUT11

A longer cDNA clone was identified in a fetal liver library that contained an identical sequence overlap with the human gene RACH1 (accession no. HSU35735), a gene capable of rescuing a check point deletion mutant in *Schizosaccharomyces pombe* in the presence of urea [Davey and Beach, 1995] (Fig. 3). The sequence to RACH1 was then used to search the databases and was found to be identical to the gene HUT11 (accession no. L36121), except that its sequence contained an extension of the 3′ UTR to that of the available HUT11 sequence. HUT11 is one of two known urea transporters initially identified as being expressed in erythrocytes and the kidney. The present HUT11 transcript size is 4.4 kb, with three splice variants reported in the literature of <3, 3.0–3.9, and 4–4.5 kb [Lucien et al., 1998; Olives et al., 1994, 1996]. The published sequence of HUT11 is only 1.6 kb in length [Olives et al., 1994; accession no. L36121). Thus, with a coding sequence of 1.2 kb, almost the entire 3′ UTR for the 4.4-kb transcript remained unknown in the Genbank sequence database for HUT11. Since the initial clone corresponds to the unpublished 3′UTR of HUT11 we therefore did not initially identify this cDNA as HUT11.

Expression of HUT11 During Osteoblast/Adipocyte *Trans*differentiation From Multiple Patient Samples

The supply of RNA obtained from individual patient bone explants was limited. Therefore, RT-PCR and TaqMan RT-PCR were performed to screen multiple patient hOB culture preparations. Specific oligonucleotide primers were designed based on the DD sequence (Fig. 3). Using these primers, the expected size of the amplification product is 285 bp. The RT-PCR reactions were controlled by the parallel amplification of β-actin, using specific oligonucleotide primers. HUT11 expression was detected in the RNA of explant cultures from two different patient samples (Fig. 5). By contrast, it was downregulated in RNA from parallel cultures treated with IBMX plus dex for 28 days. RNA from explant cultures of four additional patient samples was quantitated for the expression of HUT11 and PPARγ2, a gene shown to be upregulated as hOBs undergo adipogenesis [Nuttall et al., 1998; Tontonoz et al., 1995a,b]. HUT11 was detected and quantitated at similar levels in all four patient samples, while HUT11 was downregulated in each parallel culture after treatment with IBMX plus dex for 28 days (Fig. 6). In seven of seven individual patient samples, the HUT11 gene was downregulated in cultures that had been induced to undergo adipogenesis (Figs. 1, 5, 6). In addition, a reciprocal relationship was observed between downregulation of HUT11 and upregulation (three of four patient samples) of PPARγ2 mRNA expression (Fig. 6).

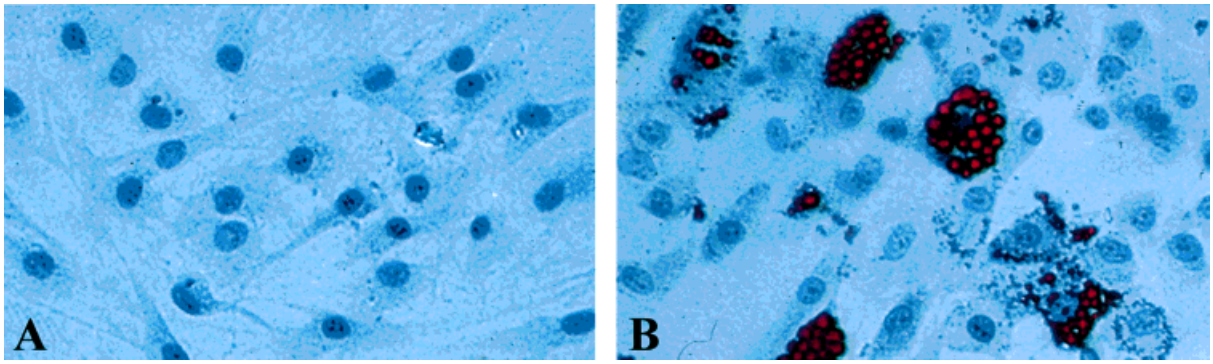

Fig. 2. Control and 3-isobutyl-1-methylxanthine (IBMX) plus dex-treated hOBs cultures. **A:** Control hOBS after 4 weeks of culture. **B:** IBMX plus dex-treated hOBs cultures, demonstrating the accumulation of refractile vacuoles stained positive for triglycerides by oil red O staining [Nuttall et al., 1998].

Time Course of HUT11 Downregulation During Adipogenesis

To examine time-dependent regulation of HUT11 mRNA expression in differentiating cultures, hOBs were treated with IBMX plus dex to induce adipogenesis in the presence or absence of either TGF-β or TNF-α for 7, 14, or 28 days (Fig. 7). TaqMan quantitative RT-PCR analysis demonstrated that, in differentiating cultures, HUT11 mRNA was markedly downregulated after 7 days and was undetectable over the remaining time course (Fig. 7A). No change in HUT11 expression levels was observed in the absence of IBMX plus dex. TGF-β and TNF-α have both been shown to inhibit the adipogenic differentiation of hOB cultures [Nuttall et al., 1998]. The addition of TGF-β or TNF-α failed to prevent the downregulation of HUT11 mRNA expression (Fig. 7A). To confirm that the hOBs have undergone adipogenesis, expression of PPARγ2 a critical gene in the differentiation and maintenance of the adipocyte phenotype, was examined [Kliewer et al., 1999]. In contrast with HUT11, and consistent with the differentiation of the hOB cultures to adipocytes, the adipocyte marker gene PPARγ2 was upregulated after IBMX plus dex treatment. After 28 days, the PPARγ2 mRNA levels were induced nearly eightfold (Fig. 7B). The induction of PPARγ2 in response to IBMX plus dex treatment is totally inhibited by TGF-β (100 ng/ml) treatment at all time points (Fig. 7B), whereas TNF-α (100 ng/ml) prevented the induction of PPARγ2 after 28 days of treatment to less than 50% of that seen with IBMX plus dex treatment alone.

In hOB cultures, it is known that both IBMX and dex are required for the induction of the adipocyte genes lipoprotein lipase (LPL) and fatty acid binding protein (aP2) [Nuttall et al., 1998]. Furthermore, treatment with dex alone failed to induce either of these late adipocyte differentiation markers [Nuttall et al., 1998]. Therefore, the effect of dex treatment on HUT11 mRNA expression was examined (Fig. 8A). After 24-h treatment, HUT11 mRNA expression was decreased by 75% (Fig. 8A). However, dex did not completely downregulate HUT11, as was observed with IBMX plus dex treatment (Fig. 7A). Interestingly, dex treatment alone induced PPARγ2 gene expression by 7 days (Fig. 8B), suggesting that the downregulation of HUT11 occurs early in the differentiation cascade, before PPARγ2 mRNA induction.

Osteoblast Expression of HUT11

Since the hOB preparations are a mixed population of cells, we investigated whether osteoblastic cell lines express HUT11 mRNA by TaqMan analysis. The expression of HUT11 in osteoblast-like cells was observed in the human cell line MG-63 and murine cell line ST2 (Fig. 9). Non-osteoblastic human cell lines, HeLa, Jurkat, THP-1, and primary human chondrocytes do not express HUT11 (Fig. 9). In addition, the human osteoblast cell lines Saos2 and TF274 do express HUT11 mRNA (data not shown). Thus, based on the results from the multiple tissue Northern blots, database searching, and RT-PCR, the expression of this splice variant of HUT11 would appear to be restricted to only a few tissues and cell types. These data suggest that osteoblasts and their progenitors within the human explant cultures express HUT11 mRNA and that differentiated adipocytes do not express detectable HUT11 mRNA.

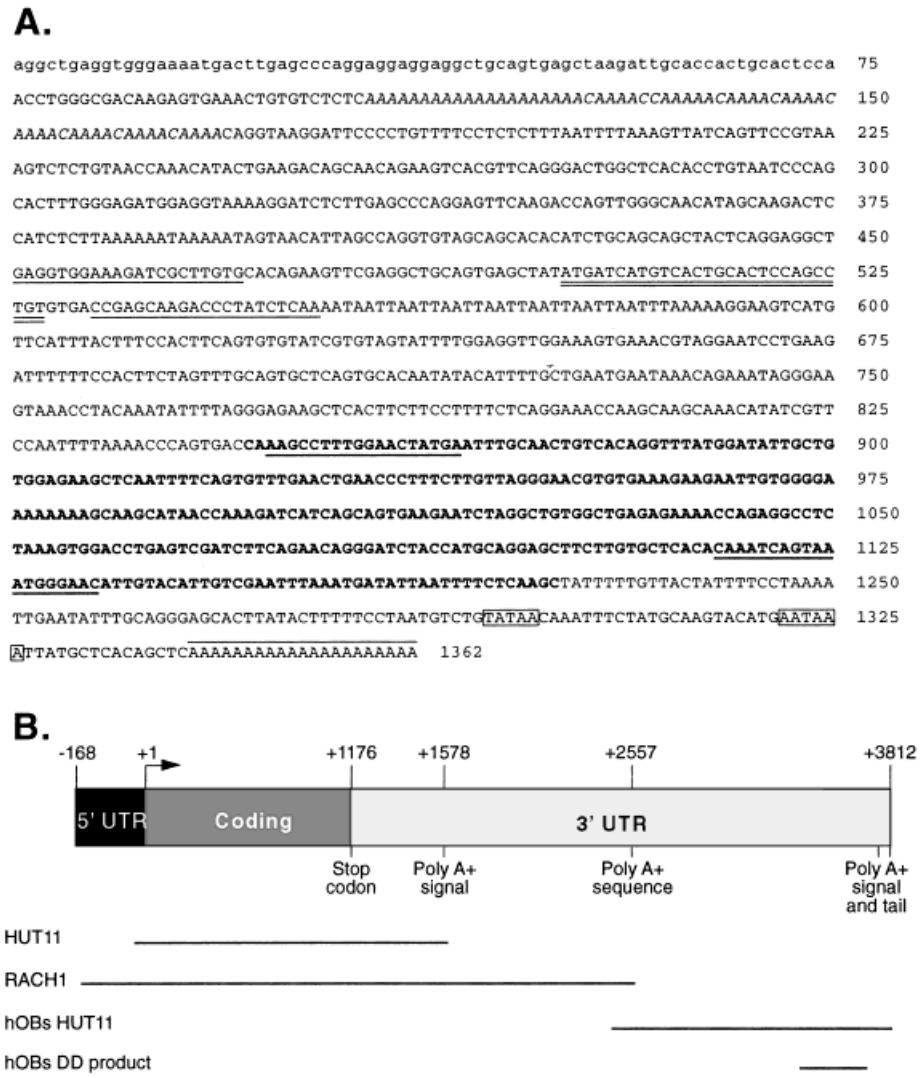

Fig. 3. Sequence extension and composite diagram of full-length HUT11 mRNA. **A:** Sequence extension for the 4.4-kb form of HUT11 from the point at which it overlaps with RACH1 (lowercase) to the poly A^+ tail (overlined). The highlighted sequences correspond to the cloned differential display (DD) band (bold), PCR primers within DD band used for reverse transcription-polymerase chain reaction (RT-PCR) (thick underline), primers (thin underline), and probe (double underline) used for TaqMan real-time quantitative RT-PCR analysis, Poly A sequence 3′ of RACH1 (italics), and polyadenylation signals (boxed). **B:** Composite diagram to 4.4 kb HUT11 mRNA. The composite is a product of the sequences from HUT11 (accession no. L36121), RACH1 (accession no. U35735), hOBs HUT11 3′ UTR (Fig. 3A), and the hOBs DD product.

DISCUSSION

These results demonstrate that the urea transporter, HUT11, expressed in human trabecular bone explant cultures, is downregulated as these cells undergo adipogenesis. Downregulation of HUT11 appears to occur early in the differentiation process, before the increase in expression of PPARγ2 mRNA. In the differentiating mouse preadipocyte cell line 3T3-F442A, FCS and insulin stimulate PPARγ2 mRNA expression after 1 day, well before the appearance of lipid vesicles [Tontonoz et al., 1994]. The adipogenic process of hOBs takes longer (17–21 days) than in the 3T3-F442A cell system, presumably because hOBs are primary human cultures. Using hOBs, we previously showed that the upregulation of PPARγ2 mRNA occurs within 3 days after IBMX plus dex treatment [Nuttall et al., 1998]. We now present data indicating that HUT11 is downregulated 1 day after dex treatment. Although we demonstrate a reciprocal relationship between the downregulation of HUT11 and upregulation of PPARγ2, downregulation of HUT11 is not a result of the

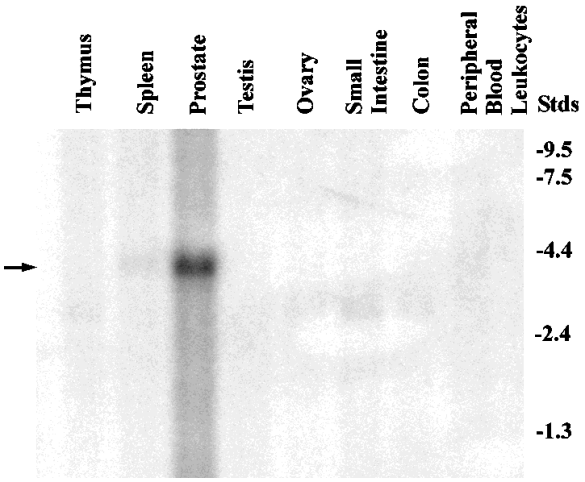

Fig. 4. Multiple tissue Northern blot probed for HUT11 with Image Clone 1010178. The 4.4-kb form of HUT11 is indicated by an arrow. The multiple tissue Northern blot was purchased from Clontech Laboratories and probed as described under Materials and Methods. The blot was judged to be evenly loaded after probing for β-actin (data not shown).

induction of PPARγ2. This is because the downregulation of HUT11 occurs before the induction of PPARγ2. Further support is generated from the fact that PPARγ2 induction is inhibited by TGF-β and TNF, whereas neither cytokine was capable of preventing the downregulation of HUT11. These data suggest that HUT11 may be a very early marker of adipogenic differentiation and perhaps the earliest indicator of the switch from osteoblastogenesis to adipogenesis. Inhibition studies will determine whether HUT11 downregulation is central for adipogenesis.

The expression and downregulation of mRNA coding for the urea transporter HUT11, in human bone explants stimulated to undergo adipogenesis, suggest that urea may play a role in the biology of mesenchymal stem cells. Urea is a product, along with ornithine, from a pathway that competes for arginine as a substrate. It is well established that the cycling of arginine (and citrulline) is critical in maintaining substrate for the generation of nitric oxide (NO). NO is an important mediator of osteoblast activity and a stimulator of bone formation [Macpherson et al., 1999]. NO is also known to play distinct roles in the biochemistry of chondrocytes, osteoblasts, and adipocytes [Cipolletta et al., 1998; Gaudiot et al., 1998; Macpherson et al., 1999]. The formation of arginine in the urea cycle leads metabolically to the synthesis of

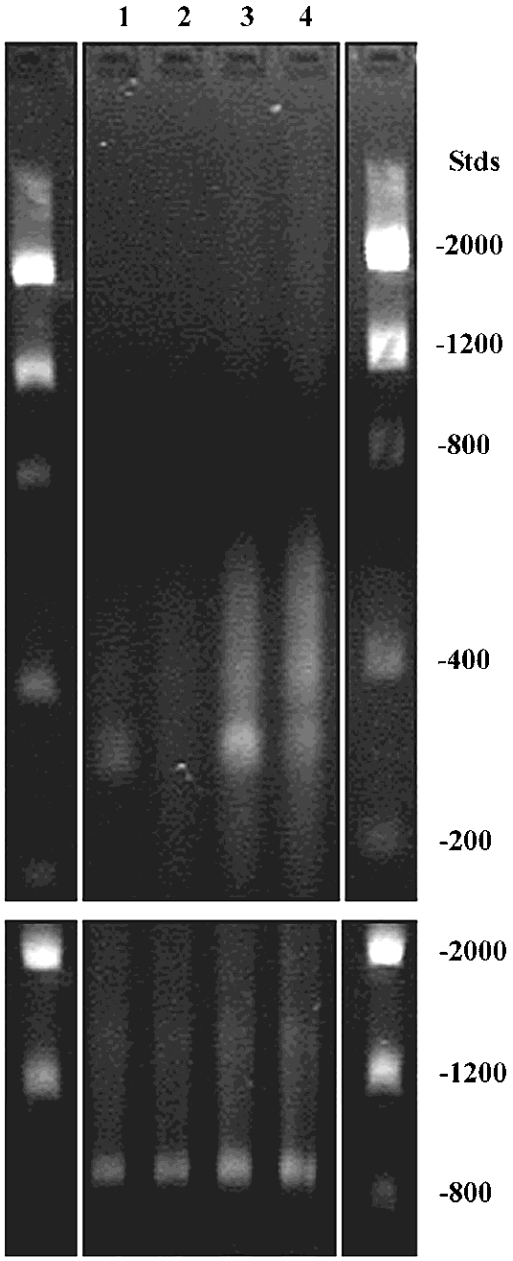

Fig. 5. RT-PCR amplification of HUT11 from total RNA of control and 3-isobutyl-1-methylxanthine (IBMX) plus dex-treated hOB cultures. **Lanes 1, 2**, control and IBMX plus dex-treated cultures, respectively, from same patient. Likewise, **lanes 3, 4**, control and IBMX plus dex-treated cultures, respectively, from a second patient. Primers and conditions were as described under Materials and Methods and in Fig. 2.

polyamines via the rapidly inducible ornithine decarboxylase [Miyanka et al., 1998; Promeneur et al., 1996; Xu et al., 1997], which, interestingly, is co-localized with HUT11 in the renal outer medulla. This enzyme converts ornithine to putrescine, which, in turn, is converted to spermidine and spermine. Polyamines, which

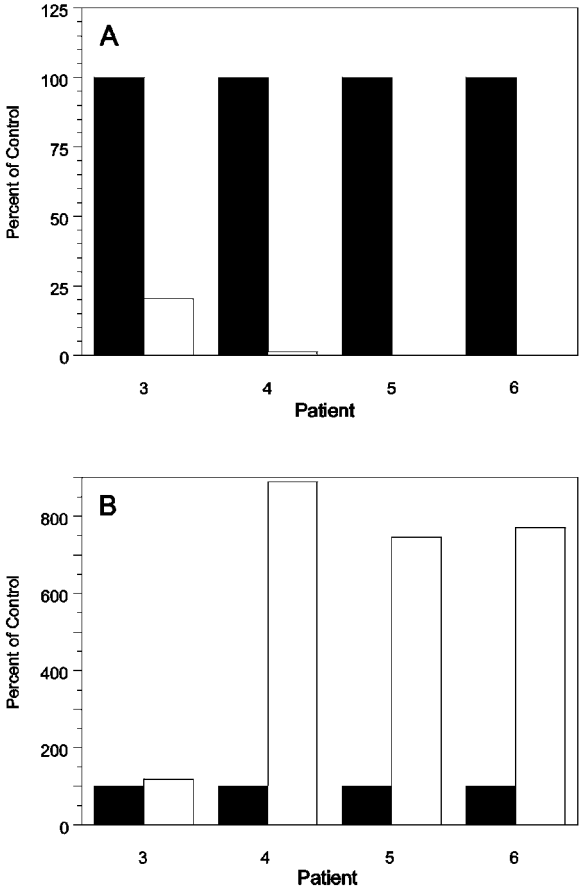

Fig. 6. Additional patient samples of hOBs were examined for mRNA expression of HUT11 and PPARγ2 by TaqMan real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). The conditions used were as described under Materials and Methods. **A:** HUT11 primers and probe were used to detect the cDNA of HUT11. **B:** PPARγ primers and probes were used to detect cDNA of PPARγ2. Bars correspond to cultures treated as follows: ■, control; □, IBMX plus dex-treated.

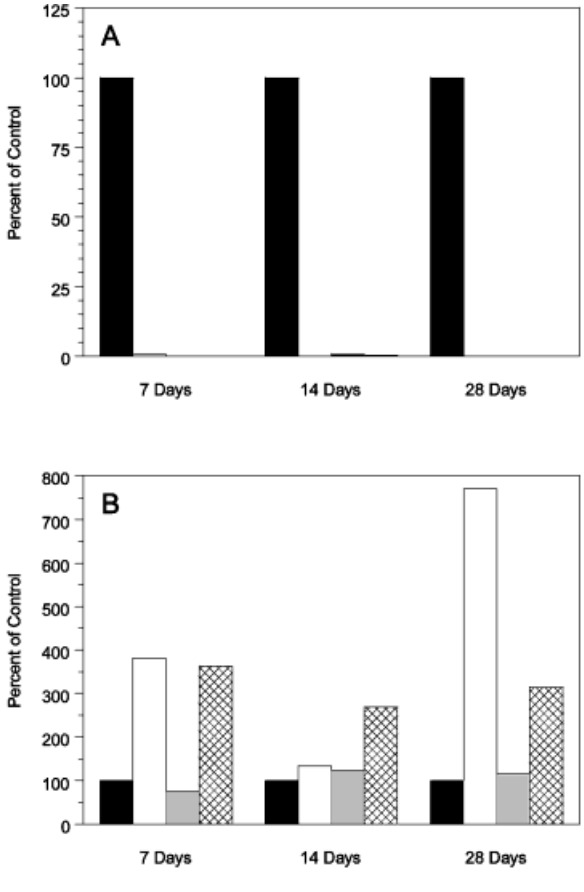

Fig. 7. Treated hOBs cultures were examined for mRNA expression of HUT11 and PPARγ2 by TaqMan real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). The conditions used were as described under Materials and Methods. **A:** HUT11 primers and probe were used to detect the cDNA of HUT11. **B:** PPARγ primers and probe were used to detect cDNA of PPARγ2. Bars correspond to cultures treated as follows: ■, control; □, 3-isobutyl-1-methylxanthine (IBMX) plus dex-treated; ▨, IBMX plus dex and TGFβ, (▩), IBMX plus dex and TNFα.

are small aliphatic polyionic cations, are known to be involved in proliferation and differentiation of a number of cell types [Heby, 1981] and could therefore play an early role in this differentiation switch.

It has previously been shown that glutamate is a potential source of arginine via pyrroline-5-carboxylate synthase, ornithine aminotransferase, and the urea cycle [Murphy and Newsholme, 1998]. The glutamate transporter (GLAST), previously identified only in brain, has been cloned from bone [Mason et al., 1997]. This is a novel site for expression of GLAST. GLAST is expressed by osteocytes and is regulated in bone that is undergoing formation in response to mechanical loading [Patton et al., 1998]. It is interesting to speculate that a complete urea cycle in osteoblasts would permit the use of transported glutamate for the generation of arginine. The use of the entire urea cycle for the generation of arginine and ornithine, outside of the liver, is not without precedent. Sertoli cells use the urea cycle to generate arginine and ornithine [Tsukaguchi et al., 1997]. Therefore, the expression of HUT11 mRNA in osteoblasts suggests that osteoblasts may use the urea cycle for the generation of arginine as a substrate for both nitric oxide synthase (NOS) and polyamine synthesis.

We have shown that the human urea transporter, HUT11, is expressed by human osteoblasts in vitro. Osteoblast HUT11 mRNA expression is downregulated when explant cultures

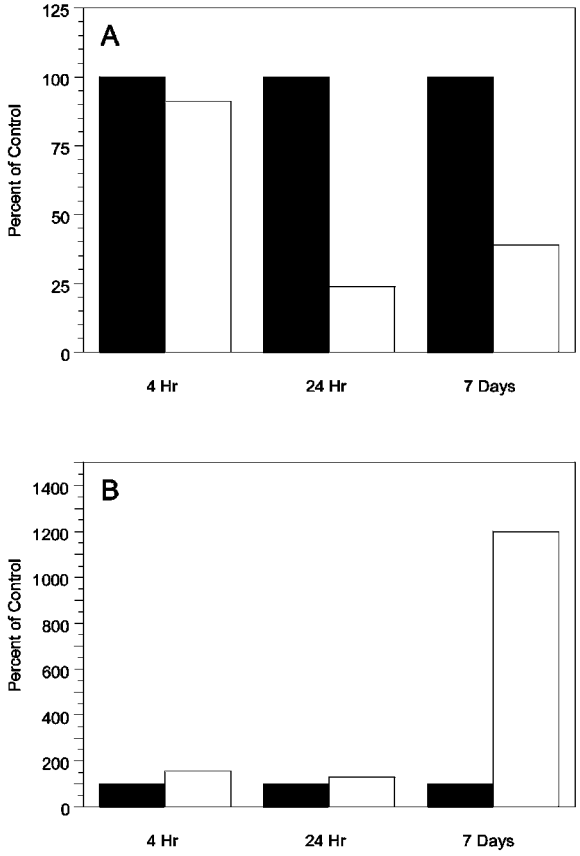

Fig. 8. Treated hOBs cultures were examined for mRNA expression of HUT11 **(A)** and PPARγ2 **(B)** by TaqMan real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). The conditions used were as described under Materials and Methods. Bars correspond to cultures treated as follows: ■, control; □, dex alone.

are driven toward adipogenesis with IBMX plus dex treatment. Downregulation of HUT11 occurs before the upregulation of PPARγ2 mRNA, suggesting that the loss of HUT11 expression may signal the switch from the osteoblast to the adipocyte phenotype. On the basis of Northern blot analysis and homology searching, the expression of this splice-variant of HUT11 appears to be restricted to only a few cell types. Although there is direct in vitro evidence for *trans*differentiation [Nuttall et al., 1998] it is also possible that these cultures are heterogeneous in nature and may contain stem-like cells which could be differentiated upon upregulation of PPARγ2. We believe that understanding the regulation of HUT11 expression and function may provide insights into the mechanisms responsible for the control of osteoblast/adipocyte differentiation.

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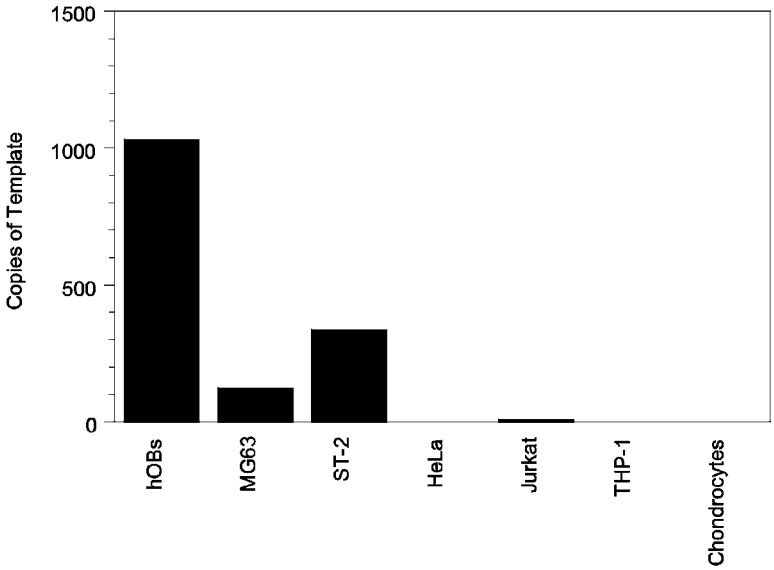

Fig. 9. Comparison of hOBs with osteoblast and non-osteoblast cell lines for expression of HUT11 mRNA by TaqMan real-time quantitative RT-PCR. The conditions used were as described under Materials and Methods.

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