Lysosome Dispersion in Osteoblasts Accommodates Enhanced Collagen Production during Differentiation

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Lysosomes are essential organelles for intracellular degradation and are generally sequestered near the cell center to receive vesicles with contents targeted for destruction. During ascorbic acid (AA)-induced differentiation of osteogenic cells (Beck, G. R., Jr., Zerler, B., and Moran, E. (2001) Cell Growth Differ. 12, 61–83), we saw a marked increase in total lysosome organelles in osteoblastic cells, in addition to an enhanced endocytic rate. Interestingly, lysosomes were dispersed toward the cell periphery in differentiating osteoblasts. We determined that lysosome dispersion in differentiated osteoblasts required intact microtubules for long range transport and was dependent on kinesin motors but did not involve cytosolic acidification. Impairment of lysosome dispersion markedly reduced AA-induced osteoblast differentiation. Lysosomes were not secreted in differentiated osteoblasts, implicating them instead in intracellular degradation. We assayed the degradative capacity and saw a significant increase in DQ-ovalbumin fluorescence in differentiated osteogenic cells compared with undifferentiated control cells. Osteogenic cells are specialized for type I collagen production, and we noted enhanced secreted and intracellular collagen in AA-differentiated osteoblasts versus control cells. Importantly, osteoblasts displayed procollagen-containing vesicles that were distributed throughout the cytoplasm, a portion of which colocalized with lysosomes. Treatment of cells with 2,2'-dipyridyl to inhibit procollagen trimerization enhanced colocalization of lysosomes with procollagen-containing organelles, implicating dispersed lysosomes in collagen processing in osteogenic cells.

Proper organelle positioning is essential in all eukaryotic cells for inter-organelle trafficking, protein targeting, cellular homeostasis, and response to extracellular stimuli (2). The intracellular organelar architecture is dictated by the protein scaffold of the cell, namely the cytoskeleton. The major cytoskeletal component that dictates the final location of organelles is the microtubule (MT)5 cytoskeleton (3), with intermediate filaments also playing a role for certain organelles (4). MTs are long polarized polymers that interact indirectly with organelles through motor and adaptor proteins (5). The relative abundance and/or activity of motor proteins on the organelles is believed to determine whether the organelle will be positioned in the cell center (toward the MT minus ends) or toward the plasma membrane (at MT plus ends). Organelles, such as mitochondria and melanosomes, are held toward the cell periphery through the actions of the kinesin motor (6, 7), whereas the minus end-directed motor, dynein, is responsible for holding the nucleus, Golgi complex, lysosomes, and recycling endosomes within the cell interior (8).

Although most eukaryotic cells have a similar spatial organization of organelles, there are cell-specific differences in organelle quantities and positioning. The relative abundance of organelles can vary from cell to cell as required for cell specializations. For instance, changes in the amount and morphology of the endoplasmic reticulum and mitochondria occur during development, suggesting that the amount and structure of these organelles are both temporally and spatially regulated (9). In certain circumstances, organelle positioning and abundance can change within the same cell in response to receptor signaling events. Reorganization of the Golgi occurs during cell migration and phagocytosis as well as during the course of muscle cell differentiation in vitro (10–12).

In the majority of eukaryotic cells, lysosomes are situated near the cell center. The molecular mechanism that clusters lysosomes around the nucleus has been elegantly described. Specifically, the presence of the small GTPase Rab7 on late endosomes/lysosomes recruits an effector, Rab7-interacting lysosomal protein (RILP), which mobilizes dynein on lysosomal membranes to induce translocation of these organelles to a perinuclear location (13, 14). Lysosomes are the penultimate organelle in the endocytic pathway and the central site for intracellular degradation in eukaryotic cells (15). Lysosomes also participate in the heterophagy of phagosomal components and can degrade intracellular proteins and cyttoplasmic...
organelles through a process known as autophagy (16). Recently, attention has turned toward the role of lysosomes in mediating degradation of misfolded proteins and protein aggregates, notably within neurological disorders (17).

Osteoblast proliferation, differentiation, and mineralization stages are characterized by dramatic changes in both gene expression and cellular architecture (1, 18). Despite recent advances in the understanding of functional aspects of osteoblast differentiation, particularly how proteins are deposited and mineralized, there has been little reported on the intracellular changes that underlie osteoblast differentiation. In this study, we investigated organelle distribution in osteoblasts during differentiation. We observed a dramatic redistribution of lysosomes following 5 days of ascorbic acid (AA) treatment. We employed multiple fluorescent technologies, including confocal, epifluorescence, and total internal reflection fluorescent (TIRF) microscopy to delineate the mechanism of lysosome dispersion and its function during osteoblast differentiation. A clear understanding of intracellular vesicle trafficking in osteogenic cells is essential to resolve the cellular defects underlying bone-wasting disorders.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Fetal bovine serum and RPMI 1640 containing 25 mM HEPES (HPMI) were purchased from Wisent Inc. α-Minimal essential medium without ascorbic acid, 4',6-diamidino-2-phenylindole, Alexa Fluor 555 dye, Alexa Fluor 546 transferrin, DQ-ovalbumin, Alexa Fluor 488 dextran, tetramethylrhodamine-dextran, and 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluoresceintetrakis (acetoxymethyl) ester (BCECF-AM) were from Invitrogen. FuGENE-6 was purchased from Roche Diagnostics. Antibodies were obtained as follows. Rat polyclonal antibody (ID4B) against lysosome-associated membrane protein 1 (LAMP1) and anti-kinesin blocking antibody (SUK4) were from the Developmental Studies Hybridom Bank (Iowa City, IA). Anti-early endosomal antigen-1 (EEA1) (N-19) goat polyclonal IgG antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), cathepsin D mouse polyclonal antibody was from Abcam (Cambridge, UK), and mouse monoclonal anti-α-tubulin antibody was from Sigma. Type I collagen antibodies that recognized procollagen and secreted forms were obtained from Jaro Sodek (University of Toronto). All fluorescently labeled and horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The Pierce chemiluminescence kit was obtained from Thermo Scientific (Rockford, IL). All other reagents were purchased from Sigma.

**Cell Culture and Osteoblast Treatments**—MC3T3-E1 subclone 4 was obtained from ATCC (American Type Culture Collection, Manassas, VA). The MC3T3-E1 preosteoblast cell line was routinely cultured in α-minimal essential medium supplemented with heat-inactivated 10% fetal bovine serum and incubated in a humidified atmosphere at 37 °C with 5% CO₂. Primary osteoblasts from mouse calvaria were isolated from 2–4-day-old CD1 mice pups based on described techniques (19, 20). Cells were grown for 5 days in α-minimal essential medium supplemented with 1 × 10⁻⁸ M dexamethasone, 10% fetal bovine serum, and antibiotics (100 µg/ml penicillin plus 50 µg/ml streptomycin sulfate). For differentiation, MC3T3-E1 and CD1 primary osteoblasts were grown with 50 µg/ml AA added to medium. Other substances used to differentiate MC3T3-E1 cells were 1 µM statin (21) and 20 µM myricetin (22). MC3T3-E1 cells were transfected using the FuGENE-6 protocol according to the manufacturer’s instructions. DNA constructs used were LAMP1-GFP, RILP-GFP, monomeric red tubulin, and KIF3A-DN-GFP. For cytoskeleton analysis, control and 5-day AA-differentiated osteoblasts were treated with 10 µM nocodazole, 20 µM taxol, and 20 µM cytochalasin D for 30 min. To inhibit procollagen peptide hydroxylation, control and AA-treated MC3T3-E1 cells were incubated with 0.3 mM 2,2′-dipyridyl for 1 h. For microinjection experiments, AA-treated MC3T3-E1 cells plated on 25-mm round coverslips were transferred to a stainless steel chamber, bathed in HPMI medium, and mounted on an inverted microscope stage. A 1:1 mixture of monoclonal anti-kinesin SUK4 antibody (4.3 mg/ml) and Alexa Fluor 555 dye in microinjection buffer was microinjected into MC3T3-E1 cells at room temperature using FemtoJet 5247 V1.06, InjectMan NI 2, and sterile Femtotips (Eppendorf, Hamburg, Germany). Cells were allowed to recover in a humidified 37 °C CO₂ incubator in media for 2 h prior to analysis.

**Immunostaining and Picrosirius Red Staining**—Control and AA-treated MC3T3-E1 and primary osteoblasts were fixed in 4% paraformaldehyde for 20 min and permeabilized in 0.1% Triton X-100 in PBS containing 100 mM glycine for another 20 min at room temperature. They were then blocked in 5% fetal bovine serum in PBS for 1 h at room temperature prior to staining with primary antibodies (anti-α-tubulin (1:10,000), anti-LAMP1 ID4B (1:4), anti-type I collagen (1:50), and EEA1 antibody (1:50)) for 1 h. Cells were subsequently washed and incubated with compatible fluorescence-conjugated secondary antibodies (1:1000) and/or phalloidin (1:500) for detection. The cells were then incubated in PBS with 4’,6-diamidino-2-phenylindole (1:10,000) for 10 min for nuclear staining. Images were taken using an inverted Axiovert 200M microscope equipped with differential interference contrast and epifluorescence optics along with Axiovision software (Carl Zeiss Microimaging Inc.) and postprocessed using Axiovision and Adobe Photoshop CS (Adobe Systems, Inc.). Whole neonatal mouse calvarial bones, partially cleaned by gently teasing away surrounding soft tissues, were also immunostained using a standard immunostaining protocol, except that the incubation times for permeabilization, blocking, and primary and secondary antibodies were increased to 1 h, 2 h, overnight, and 1 h, respectively. The calvariae were stained with anti-LAMP1 antibodies and phalloidin and mounted onto a coverslip, and confocal Z-stacks were acquired on a Zeiss LSM 510 confocal microscope. Picrosirius Red staining was performed as previously described (23). Collagen secretion in differentiated (50 µg/ml of AA) and control undifferentiated MC3T3-E1 cells was analyzed after 3, 5, 7, 14, and 21 days in culture. Cells were fixed with Bouin’s fluid for 1 h and stained with 100 mg/ml Sirius red dye in saturated aqueous picric acid for 1 h with mild shaking and washed thoroughly with 0.01 N hydrochloric acid to remove any nonspecific staining. The slides were then mounted on
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glass slides and viewed under a bright field Zeiss microscope with a color AxioCam HRm camera. Images were taken with a ×40 oil immersion objective.

Determination of Perinuclear Belt of Clustered Lysosomes for Quantification—For lysosome dispersion, we first established a “perinuclear belt” of fixed and defined dimensions in control (undifferentiated) osteoblasts and then counted the total number of lysosomes outside the belt. Initially, to establish the perinuclear belt, we examined TIF images of LAMP1 immunostaining from 50 randomly selected undifferentiated cells (with perinuclear clustered lysosomes) and measured the area around the nucleus that contained the majority of the lysosomes. This region was determined to be an almost circular area around the typically oval-shaped nucleus with a small radius equal to 0.5R (R being the largest radius of the oval cell nucleus) and a big radius equal to 1.3r (r being the small radius of the cell nucleus). Nuclei were identified by 4'6-diamidino-2-phenylindole staining. Once this perinuclear belt size was determined, it was used for all subsequent quantification of lysosomes in control and differentiated osteoblasts. The number of lysosomes residing outside the lysosome belt was counted using ImageJ, a Java-based image-processing package from the National Institutes of Health.

Experimental Manipulation of Cytosolic pH—Low confluence osteoblasts were grown on 25-mm coverslips in control or differentiation medium for 5 days. They were then washed in regular Ringer’s solution, pH 7.4 (155 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 mM NaH₂PO₄, 10 mM HEPES buffer, pH 7.2, 5 or 10 mM glucose, 0.5 mg/ml bovine serum albumin), and incubated in either prewarmed alkalinized Ringer’s solution, pH 7.9 (NH₄Cl (10–30 mM) in normal bicarbonate-free Ringer’s), or acetate Ringer’s solution, pH 6.7 (80 mM NaCl, 70 mM sodium acetate, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 mM NaH₂PO₄, 10 mM HEPES buffer, pH 6.9, 5 or 10 mM glucose), in a 37 °C incubator for 15 min (24). The cells were then fixed in 4% paraformaldehyde, permeabilized, blocked, and immunostained for LAMP1 as described above and imaged using epifluorescence microscopy.

Measurements of Intracellular pH in Osteoblasts—Low confluence MC3T3-E1 cells were grown on 25-mm glass coverslips for 5 days in control and differentiating media containing 50 µg/ml AA. Measurements of intracellular pH were done according to established methodology using a pH-sensitive fluorescent dye (25). A stock solution of the pH-sensitive fluorescent dye BCECF-AM at a final concentration of 1 mmol/liter was prepared in DMSO. The osteoblast cells were washed with PBS and incubated in prewarmed isotonic Na⁺ solution (130 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 20 mM HEPES, pH 7.4) containing 2 µM BCECF-AM for 20 min at 37 °C. After incubation, the cells were washed twice with isotonic Na⁺ solution and mounted on a prewarmed heating stage at 37 °C of a Nikon TMD-Diaphot microscope equipped with a Nikon Fluor oil immersion objective and Hoffman modulation optics with a thermostated Leiden holder. A chopping mirror was used to direct the excitation light alternately to two excitation filters (490BP10 and 440BP10) in front of a xenon lamp. For each coverslip, current measurements over an interval of 60 s were recorded. To minimize dye bleaching and photodynamic damage, neutral density filters were used to reduce the intensity of the excitation light reaching the cells. The excitation light was directed to the cells via a 510-nm dichroic mirror, and fluorescence emission was collected at 535BP25 filter. Photometric data were acquired at 10 Hz using Oscar software (Photon Technologies Inc.). To determine the cytosolic pH, cells were then incubated in isotonic K⁺ solutions (143 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES) with known pH gradients of 7.63, 7.35, 6.88, and 6.51 plus 10 µg/ml nigericin. To measure cell pH, a 490:425 ratio signified as y was plotted against the known pH gradient. The linear plot with an equation of y = mx + b was used to calculate the averaged cell intracellular pH signified as x.

Quantification of Endocytosis and Lysosomes in Osteoblasts—To label endosomes, control and AA-treated osteoblasts were serum-starved for 1 h. The cells were then incubated in serum-free medium supplemented with 50 µg/ml Alexa Fluor 546 transferrin for 5 min at 37 °C. Cells were then washed twice with cold 1× PBS, pH 4.5, to remove surface-associated Alexa Fluor 546 transferrin. The cells were then washed with regular cold 1× PBS and fixed for quantification. Total lysosome count was tabulated from control and AA-treated cells, which were immunostained with LAMP1. Lysosome number and transferrin uptake were quantified from TIF images of 20 cells for each experiment for three replicate trials. ImageJ was used to quantitate pixels corresponding to total EEA1/LAMP1 in control and AA-differentiated MC3T3-E1 and primary osteoblasts. The cell boundary was established from differential interference contrast images. To determine the levels of endocytosis, control and AA-treated osteoblasts were fixed and immunostained with EEA1 antibody. To analyze catalytic degradation in lysosomes in osteoblasts, glass coverslips or 96-well plates were coated with 0.01% poly-L-lysine for 1 h and 2 µl of 1 mg/ml DQ-ovalbumin and allowed to air dry. Primary and MC3T3-E1 osteoblasts (with and without 50 µg/ml AA for 5 days) were trypsinized, and equal cell numbers were seeded in the wells (70% confluence) and incubated overnight. The fluorescence emission in fluorescence intensity units was measured by means of ELISA using a FLUOstar OPTIMA luminescence microplate reader with an excitation filter of 485 nm and emission filter of 520 nm. Each value of fluorescence intensity represented a mean value obtained from three independent experiments. To examine the colocalization of degraded DQ-ovalbumin with lysosomes, cells were pulsed with 50 µg/ml tetramethylrhodamine-dextran for 2 h, prior to overnight plating on DQ-ovalbumin-coated coverslips. The following day, epifluorescent images of live cells were captured using epifluorescence microscopy. The weighted colocalization coefficient, the sum of intensities of colocalizing pixels relative to the overall sum of pixel intensities above the threshold (or background), was used in confocal slices of untreated and treated MC3T3-E1 cells to determine the relative levels of LAMP1-positive collagen vesicles. The advantage of a weighted colocalization coefficient is that differences in pixel intensity are taken into account (i.e. not all pixels contribute equally to the final colocalization coefficient value). In this regard, collagen vesicles showing a
weighted coefficient greater than or equal to 0.3 with LAMP1 were scored as positive within each cell/treatment.

**TIRF Imaging of Lysosomes**—For TIRF imaging of live cells, control and AA-differentiated osteoblasts were grown on 25-mm round glass coverslips for 5 days. On the 4th day, cells were pulsed with 50 μg/ml Alexa Fluor 488 dextran for 2 h. Subsequent washes with PBS were performed to remove extracellular residues of dextran. The dextran tracer was chased overnight to late endosomes or lysosomes (26). Cells were stimulated for secretion with 10 μM of ionomycin in a 2 mM calcium buffer for 5 min during imaging. MC3T3-E1 and primary osteoblast cells were imaged using TIRF with an α-Plan-Fluor ×100/1.45 objective, a 488-nm excitation filter, and a 100-milliwatt multiline argon-ion laser and TIRF slider (Zeiss). Images were acquired every second for up to 5 min.

**Western Blot Analysis**—To determine the levels of collagen type I and cathepsin D secreted from control and AA-treated MC3T3-E1 cells, Western immunoblotting was performed on the cell lysate and conditioned media from the cell cultures at day 5 of incubation. Protein was isolated by solubilizing the samples in radioimmune precipitation buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, pH 7.4) containing 2 μg/ml phosphatases and protease inhibitors. The protein concentration was determined by using a Bio-Rad protein assay kit. 70 μg of protein was boiled in sample buffer consisting of 2% SDS, 10% glycerol, 60 mM Tris, pH 8.8, and 0.001% bromphenol blue. The proteins were electrophoresed in 8% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Millipore, Bedford, MA) according to the manufacturer’s directions. The membranes were blocked in T-TBS (0.1% Tween 20 in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl) with 5% skim milk powder. After washing in T-TBS, the blots were incubated with anti-collagen antibody (1:1000) or anti-cathepsin D antibody (1:1000) overnight at 4 °C. Blots were washed and incubated with anti-sheep or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:1000), respectively, and positive bands were detected using the Pierce chemiluminescence kit.

**Real Time Reverse Transcription-PCR**—Total RNA was extracted with Trizol reagent from MC3T3-E1 osteoblast cells. Samples of total RNA (1.5–3 μg) were reverse transcribed, and semiquantitative real time PCR was performed on control, AA-treated, and two RILP-GFP-stable cell populations (27). The primers used were as follows: L32-F, 5′-CACAATGTC-AAGGAGCTGGAAGT-3′; L32-R, 5′-TCTACAAATGGGCT-TTTGGTCTT-3′; OCN-F, 5′-CGACCTACAGATCC-CAAGC-3′; OCN-R, 5′-GATGTTGAGATAGCTGCTAC-AAG-3′; BSP-F, 5′-CAGGAGGAGGTGAGTCT-3′; BSP-R, 5′-GTGTTGAGAAGTGGCGTT-3′; ALP-F, 5′-CACAATTTGTGGCCAGAGA-3′; ALP-R, 5′-TGCAT-ATTGTTGAGCTTTT-3′; OPN-F, 5′-AGCAGAGAAA-CTTTCAAGCA-3′; OPN-R, 5′-GATGATTGGCAACCTGCGTT-3′; LAMP1-F, 5′-CAGGGAGGCAGTGACTCTTC-3′; LAMP1-R, 5′-CAGGGAGGCAGTGACTCTTC-3′. Amplimers were quantified in triplicate samples in each of three independent experiments for each gene and normalized to corresponding L32 ribosomal protein values, and these data were then normalized to control untreated cells.

**Statistical Analysis**—Data are presented as the mean ± S.E. All experimental data were repeated in at least triplicates, taking into account 20 cells in each treatment (control and AA-treated and drug treatments). To avoid bias in our quantifications, cells from each treatment were randomly picked for imaging, and the resulting images were processed following a “blinded” approach in which the operator was unaware of the sample identity. Statistical comparisons were made by a two-tailed Student’s t test. Differences with p values of <0.05 were considered statistically significant. Results for PCR analysis were expressed as the mean ± S.E. and analyzed statistically by one-way analysis of variance and a post hoc Bonferroni test.

**RESULTS**

**Enhanced Endocytosis and Lysosomes in AA-treated Osteoblasts**—AA (reduced vitamin C) has been shown to be essential for the in vitro differentiation of many mesenchyme-derived cell types, including osteoblasts (28). Undifferentiated osteoblast precursors, following exposure to AA for several days, up-regulate expression of numerous genes that are key for collagen synthesis, the primary role of mature osteogenic cells (29). Since endocytic organelles had not previously been studied in this cell type, we began our study examining the levels of endocytosis in undifferentiated (control) osteoblast precursors versus osteoblasts that had been differentiated with AA for 5 days. Early endosomes were quantified in fixed cells via immunostaining with an antibody to EEA1. Cells were scored for total EEA1 fluorescence/cell, and AA-treated osteoblasts showed enhanced levels of early endosomes, compared with control cells (Fig. 1A). Uptake of fluorescently labeled transferrin was also increased in AA-stimulated cells (not shown), which may reflect AA-mediated up-regulation of transferrin receptors (30).

EEA1 immunostaining revealed enhanced early endosomes in AA-treated osteoblasts. We next investigated the relative abundance of later endocytic compartments, namely lysosomes, during osteoblast differentiation. We quantified the late endocytic compartments by immunostaining control and AA-treated MC3T3-E1 and primary calvarial osteoblast cells with LAMP1, a marker for lysosomes and late endosomes (31). Fig. 1C shows an epifluorescent image of the LAMP1-positive organelles in control and AA-treated primary osteoblastic cells. Since the osteoblastic cells were extremely flat at this stage, epifluorescence microscopy ably captured the majority of stained organelles in these cells. Quantification of the total fluorescent pixels in TIF images of these cells showed a significant increase in the total number of lysosomes and late endosomes in AA-treated cells compared with control cells (Fig. 1D). Interestingly, we also noted a striking change in the distribution of the lysosomes in these cells, with differentiated osteoblasts containing lysosomes that were spread throughout the cytoplasm.
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FIGURE 1. Effect of AA differentiation on endocytosis and total lysosome production in osteoblasts. A, representative fluorescent images of control and AA-treated MC3T3-E1 osteoblast cells fixed and immunostained with EEA1 to label early endosomes. B, quantification of total EEA1-positive vesicles in control and AA-treated MC3T3-E1 osteoblast cells from three independent experiments (n = 20); *, p < 0.05. C, immunofluorescent images of control and AA-treated primary osteoblast cells showing LAMP1-positive lysosomes/late endosomes. D, quantification of total LAMP1-positive organelles in control and AA-treated MC3T3-E1 and primary osteoblast cells from three independent experiments (n = 20 in each); *, p < 0.05. E, control and 5-day AA-treated MC3T3-E1 osteoblastic cells showing lysosomes labeled with tetramethylrhodamine (TR)–dextran (in red) and cleaved fluorescent DQ-ovalbumin (in green). The arrows point to the almost complete colocalization of lysosomes with fluorescent DQ-ovalbumin. F, quantitative analyses of DQ-ovalbumin fluorescence in control and AA-treated MC3T3-E1 osteoblast cells using ELISA. The y axis is the average fluorescent intensity produced by cleaved DQ-ovalbumin in arbitrary fluorescence intensity units (FIU). Data shown represent mean ± S.E. from three independent experiments (n = 20 in each). *, p < 0.05. Scale bars, 15 μm.

To investigate whether enhanced lysosome numbers in osteogenic cells correlated with their degradative abilities, we examined the efficiency of lysosome-mediated degradation of endocytosed materials. To this end, we utilized a fluorescent ovalbumin conjugate (DQ-ovalbumin) to quantify lysosomal degradation in AA-treated osteoblasts, versus control cells. DQ-ovalbumin is a self-quenched ovalbumin substrate for proteases, which, when cleaved, becomes fluorescent with excitation and emission maxima of ~505 and 515 nm, respectively, that can be detected with specific ELISA plate reader filters (32). Equal numbers of control and AA-treated MC3T3-E1 cells were seeded onto coverslips in 96-well plates coated with poly-l-lysine and DQ-ovalbumin and incubated overnight. Lysosomes were labeled in some cells by an overnight chase with fluorescent dextran (26). The next day, cells were imaged live using epifluorescence microscopy. Fluorescent vesicles indicative of the degradation of DQ-ovalbumin were primarily clustered around the nucleus in control cells but displayed a more scattered distribution in AA-treated MC3T3-E1 osteoblasts (Fig. 1E). A strong overlap between lysosomes and DQ-ovalbumin fluorescence was observed in control and differentiated osteoblasts (Fig. 1E). We quantified the total amount of LAMP1-positive organelles that were dispersed throughout the cytoplasm (Fig. 2, A and B). Quantification of lysosomes located outside the defined perinuclear belt showed a significant increase in dispersed lysosomes in AA-treated osteoblasts compared with untreated control cells for both MC3T3-E1 and primary osteoblasts (Fig. 2D), without a significant change in cell size/shape during the 5-day period of differentiation. Osteoblast differentiation induced by statin and myricetin also showed increased lysosome dispersion (Fig. 2D), indicating that lysosome redistribution during differentiation is specific but not limited to ascorbic acid. Removal of ascorbate at day 4 did not revert the dispersed lysosome phenotype, compared with untreated control osteoblasts (Fig. 2 D, WO). Lysosome dispersion was also analyzed and observed in osteoblasts treated with AA for up to 21 days (not shown); however, 5 days was the minimum time of differentiation that resulted in this phenotype, and consequently we used this time point for the rest of the analyses.

To determine whether the observations in vitro correlate with those in osteoblasts in vivo, we determined the localization of lysosomes/late endosomes in calvarial osteoblasts by doing whole mount immunostaining of mouse calvaria. Calvariae were double-labeled with LAMP1 for lysosomes/late endosomes and phalloidin to detect F-actin to demarcate the cell

Dextran/DQ-ovalbumin

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versus a perinuclear clustered pattern in undifferentiated cells (Fig. 1C).

Lysosome Distribution in Control and AA-differentiated Osteoblasts—To more fully understand spatial changes in lysosome patterning during osteoblast differentiation, lysosomes and late endosomes were detected using immunostaining with a LAMP1 antibody and epifluorescent microscopy, followed by inversion of captured images to accentuate organelle positioning (Fig. 2, A and B). In both MC3T3-E1 and primary osteoblastic cells from mouse calvaria, untreated cells showed LAMP1-positive organelles that largely clustered around the nucleus (Fig. 2, A and B). A perinuclear belt was determined based on the region around the nucleus that contained the bulk of lysosomes from 50 randomly chosen control osteoblasts (Fig. 2C; see "Experimental Procedures"). In contrast, AA-treated osteoblasts showed
peripheries and then analyzed in Z-sections using confocal microscopy. As expected, as we progressed through the Z-stacks, we observed by their morphological characteristics and tissue distribution a variety of cell types, including osteoblasts (cuboidal cells), osteocytes (stellate cells), and osteoclasts (large multinucleated cells) in the calvaria (Fig. 2E; the entire Z-stack is included as supplementalMovie 2E_mov). The lysosome distribution in osteoblasts was dispersed, with lysosomes observed throughout the cell cytoplasm toward the actin-rich cell cortex. These data strongly suggest that lysosome dispersion also occurs \textit{in vivo} in osteoblasts as it does \textit{in vitro}.

**Role of Cytosolic pH in Lysosome Distribution in Osteoblasts**—To begin deciphering the mechanism of lysosome dispersion during AA-induced osteoblast differentiation, we asked whether altering the cytosolic pH affects lysosome distribution, which has previously been reported in fibroblasts and macrophages (24). Control MC3T3-E1 cells and cells after 5-day incubation with AA were placed in Ringer’s solution of varying pH for 15 min, after which they were fixed and immunostained for LAMP1 (Fig. 3A). Incubation of cells in Ringer’s solution (pH 7.4) did not substantially affect lysosome distribution in either control or AA-treated MC3T3-E1 osteoblasts (Fig. 3A). Incubation of cells in Ringer’s solution containing NH₄Cl (pH 7.9) and 5 mM of glucose did not significantly affect lysosome distribution in control or AA-treated osteoblasts (Fig. 3B). However, experimentally decreasing the cytosolic pH by incubating cells in medium containing acetate (pH 6.7), significantly increased the amount of LAMP1 organelles outside the lysosome belt in control AA-treated osteoblasts, causing dispersion of lysosomes to the periphery of the cells (Fig. 3B). When osteoblasts were incubated in Ringer’s solution containing 10 mM glucose, there was a basal reduction in dispersed lysosomes under all conditions (Fig. 3C). As well, AA-treated osteoblasts were more sensitive to NH₄Cl treatment, showing significantly reduced dispersed lysosomes, compared with AA-treated cells in Ringer’s solution alone (Fig. 3C). Both control and AA-treated osteoblasts showed enhanced lysosome dispersion following acetate treatment (Fig. 3C).
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FIGURE 3. Effects of cytosolic pH changes on lysosome distribution in AA-treated MC3T3-E1 osteoblastic cells. A, inverted fluorescent image of AA-differentiated osteoblasts incubated in regular medium (pH 7.4) or after a 15-min incubation in Ringer’s solution (pH 7.4), 15-min incubation in acetate solution (pH 6.7), or 15-min incubation in alkalinized solution (pH 7.9). Examples of perinuclear belts used for quantification purposes are illustrated (dashed circles). Scale bars, 15 μm. B, quantification of LAMP1-positive organelles outside of the perinuclear belt in Ringer’s solution containing 5 mM glucose. C, representative quantification of the number of LAMP1-positive organelles outside the perinuclear belt in Ringer’s solution containing 10 mM glucose, following experimental pH changes in control and AA-treated MC3T3-E1 osteoblasts. All data are reported as mean ± S.E. from three independent experiments (n = 20). *, p < 0.05. D, representative average cytosolic pH of control and AA-treated MC3T3-E1 cells from BCECF experiments. The data are reported as mean ± S.E. from three replicate trials.

Since we observed a dependence between lysosomal dispersion and cytosolic pH, we theorized that differentiation of osteoblasts may include a progressive change in cytosolic pH. We assessed the cytosolic pH directly in control and AA-treated MC3T3-E1 osteoblasts using the fluorescent pH sensor, BCECF (33). Surprisingly, we determined the cytosolic pH in both control and AA-treated osteoblasts to be very similar and within the range of pH 7.6–7.8 (Fig. 3D). Consequently, changes in cytosolic pH do not account for the lysosome redistribution in AA-differentiated osteoblasts.

MTs, but Not Actin, Are Important for Lysosome Dispersion in AA-treated MC3T3-E1 Osteoblasts—In order to further understand the individual components responsible for the dramatic dispersion of lysosomes in differentiated osteoblasts, we investigated the role of the cytoskeleton in the translocation and positioning of lysosomes in osteoblasts. The actin and MT cytoskeleton and associated motor proteins were our initial targets. Epifluorescent images of both AA-differentiated and control MC3T3-E1 cells immunostained with LAMP1 and α-tubulin revealed a colocalization of lysosomes and late endosomes with MTs (Fig. 4A). Live imaging of transfected cells with monomeric red tubulin and LAMP1-GFP constructs also confirmed that lysosomes were situated on MTs in osteoblasts (not shown). To test the MT dependence of the lysosomal distribution in AA-differentiated osteoblasts, cells were treated with 10 μM nocodazole for 30 min. Depolymerization of MTs did not markedly alter the cell shape of the osteoblasts; however, it did cause a collapse of lysosomes toward the cell center and increased lysosomal clustering (Fig. 4B). There was a significant reduction in lysosomes outside the perinuclear belt following quantification of LAMP1-positive organelles in AA-treated MC3T3-E1 cells (Fig. 4E). To assess whether lysosome positioning relies on MT dynamics, we stabilized MTs in AA-stimulated MC3T3-E1 osteoblasts using a 30-min treatment with 20 μM taxol. Cells were fixed and immunostained for LAMP1 and α-tubulin, and epifluorescent images were captured (Fig. 4C). Quantification of LAMP1-positive organelles outside the perinuclear belt in taxol-treated cells did not reveal a statistically significant change in lysosome distribution in either control or AA-treated MC3T3-E1 cells (Fig. 4E), indicating that MT turnover is not essential for lysosome positioning in osteoblasts. Collectively, these findings indicate that intact MTs are required for the directed translocation and positioning of late endosomes and lysosomes in AA-differentiated osteoblasts. Organelle tethering at the cell periphery has been shown to be mediated by cortical F-actin (34). We examined the role of actin cytoskeleton in lysosome positioning in osteoblasts by incubating cells in 20 μM of cytochalasin D for 30 min to disrupt F-actin. Treated control and AA-differentiated MC3T3-E1 cells were fixed and immunostained for LAMP1 and α-tubulin and examined with epifluorescent microscopy (Fig. 4D). Cytochalasin D treatment resulted in a slight retraction in the cytoplasm.
lysosomes within the perinuclear belt, compared with untreated cells (Fig. 5D). In conclusion, the dispersed lysosome phenotype observed in AA-differentiated MC3T3-E1 cells is abrogated by kinesin inhibition, implicating this motor in lysosome positioning in osteoblasts.

Lysosomes and Lysosome Contents Are Not Secreted in AA-differentiated Osteoblasts—Our immunofluorescence analysis of AA-differentiated osteoblasts revealed lysosomes that frequently localized at the cell periphery. Since fusion of the lysosomes with the plasma membrane has been observed in some cell types (36), we utilized TIRF microscopy in an attempt to visualize lysosome secretion in osteoblasts. TIRF microscopy restricts the excitation plane such that fluorophores are excited within a thin region of the cell opposed to the coverslip and thus is ideal for imaging secretory events at the plasma membrane. We pulse-chased Alexa Fluor 488 dextran into AA-treated MC3T3-E1 cells (Fig. 6A and supplemental Movie 6A_mov). TIRF imaging revealed many dextran-positive vesicles within the evanescent wave in AA-treated osteoblasts (Fig. 6A). Cells were imaged every second for a period up to 10 min, and while lysosomes approached the plasma membrane, secretion of dextran was never observed during these time frames (Fig. 6A and supplemental Movie 6A_mov). We further stimulated the cells with 10 μM ionomycin in a 2 mM calcium buffer to increase intracellular Ca2+, but lysosome secretion was still not observed by TIRF microscopy (Fig. 6B and supplemental Movie 6B_mov). We also immunostained unpermeabilized osteoblasts for external LAMP1 on the plasma membrane; however, no detectable fluorescence of LAMP1 was observed in AA-treated osteoblasts (not shown). Moreover, immunoblotting for cathepsin D in conditioned media of control and AA-treated osteoblasts did not reveal secretion of lysosomal enzymes in osteoblasts (not shown). Collectively, these findings indicate that peripheral lysosomes in differentiated osteoblasts are not competent for secretion.

Lysosome Dispersion Is Involved in Differentiation of Osteoblasts—To investigate whether lysosome redistribution is required for AA-mediated osteoblast differentiation, we engineered MC3T3-E1 cells stably expressing RILP-GFP to cluster lysosomes (Fig. 7A). RILP-GFP cells were exposed to AA for 5 days, and gene expression was compared with untransfected control and AA-stimulated MC3T3-E1 osteoblasts. Total mRNA levels of specific bone cell markers, including Col1a1 (collagen type 1 a1), Alp (alkaline phosphatase), Bsp (bone sia-
enhance collagen translation, processing of procollagen chains, and fibril assembly (37, 38). Consistent with this, we observed a slight, but not significant increase in Col1a1 mRNA by AA exposure, which was not significantly reduced by RILP-GFP expression (Fig. 7C). In contrast, the up-regulation of Bsp, Ocn, and Alp observed in untransfected AA-treated cells was significantly reduced following stable transfection of RILP-GFP into osteoblastic cells (Fig. 7, D–F). There was no significant difference between RILP clones in any of the genes tested (not shown).

**Lysosomes Colocalize with Procollagen-containing Vesicles in AA-treated Osteoblasts**—Enhanced collagen synthesis is a hallmark feature of differentiated osteoblasts (29, 39). We investigated extracellular and intracellular collagen production in control and AA-differentiated osteoblasts. To look at secreted collagen, we utilized Picosirius Red staining (Fig. 8A). Enhanced pink staining, indicative of secreted collagen, was observed in AA-treated osteoblasts versus undifferentiated control cells (Fig. 8A). Enhanced collagen secretion in AA-treated osteoblasts versus control cells was also confirmed by immunoblotting conditioned media with an anti-collagen antibody (Fig. 8B). Intracellular collagen was visualized using an anti-type I collagen antibody, which detects intracellular procollagen. We fixed and immunostained both control and AA-treated MC3T3-E1 osteoblast cells with LAMP1 and collagen type I antibodies (Fig. 8C). Prominent procollagen staining was detected in the perinuclear region of both control and AA-differentiated osteoblasts, which probably reflects procollagen within the Golgi apparatus. AA-differentiated osteoblasts had more prominent procollagen staining, some of which was dispersed throughout the cell (Fig. 8C). Lysosome dispersion in AA-treated cells was not due to space constraints in the perinuclear region caused by collagen synthesis, since blocking collagen production or Golgi trafficking still resulted in lysosome dispersion in AA-treated cells (supplemental Fig. 1). Closer examination of collagen vesicles and LAMP1 organelles in AA-treated osteoblasts revealed a partial overlap of these organelles (Fig. 8D). Interestingly, a significantly higher number of LAMP1-positive collagen vesicles was observed in differentiated osteoblasts versus control cells (Fig. 8F). To experimentally disrupt the helical structure of procollagen, control and AA-treated osteoblasts were treated with 0.3 mM 2,2′-dipyridyl for 1 h (40). Fig. 8E shows an AA-treated MC3T3-E1 cell treated with 2,2′-dipyridyl and immunostained for LAMP1 and procollagen. A region of the cell was magnified to demonstrate overlap of LAMP1-positive organelles and procollagen-containing vesicles. A significant increase in the number of LAMP1-positive procollagen vesicles was observed in cells treated with 2,2′-dipyridyl, compared with untreated cells (Fig. 8F). Collectively, these results demonstrate a spatial role for lysosomes in collagen processing during osteoblast differentiation.

**DISCUSSION**

Here we show that differentiation of osteoblasts results in the dispersion of lysosomes. This is the first description of lysosome redistribution in development. Removal of ascorbate during osteoblast differentiation did not restore lysosome perinuclear clustering, indicating that AA does not directly mobilize

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lroprotein), Ocn (osteocalcin), and Opn (osteopontin), were analyzed using reverse transcription-PCR (Fig. 7, B–F). Opn mRNA, which is enhanced after 20 days of AA stimulation (37, 38), was not induced in the 5-day AA period observed (Fig. 7B). Opn levels were variable but not significantly increased in two clones of RILP-GFP-expressing cells exposed to AA, compared with untransfected AA-treated cells (Fig. 7B). AA induces only a modest increase in Col1a1 mRNA and is instead thought to

**FIGURE 5. Effect of down-regulating kinesin activity on peripheral lysosome localization in AA-differentiated osteoblasts.** AA-treated MC3T3-E1 cells were microinjected with a kinesin-blocking antibody (SUk4, green) (A) or transfected with RILP-GFP (green) (B) or transfected with KIF3A-DN-GFP (green) (C). A–C, cells were then fixed and immunostained for α-tubulin (blue) and LAMP1 (red). Scale bars, 15 μm. D, quantitative analysis of the number of LAMP1-positive organelles outside the perinuclear belt (dashed circle) in untreated and kinesin-inhibited MC3T3-E1 osteogenic cells. Data are reported as mean ± S.E. from three independent experiments (*n* = 20 in each). *p < 0.05, mAb, monoclonal antibody.
lysosomes and that maintenance of lysosomes toward the cell periphery is a differentiation-induced phenotype. Moreover, we show that lysosome dispersion is required for production of osteogenic cells, providing the first direct link between spatial positioning of lysosome organelles with cell differentiation.

There has been extensive work characterizing the mechanism of inward lysosome migration and lysosome perinuclear clustering (13); however, much less is known about the mechanism and function of anterograde lysosome trafficking. We demonstrate that lysosome trafficking to the cell periphery involves MTs and kinesin motors (Fig. 9). Kinesin comprises a large family of ubiquitous molecular motors that transport intracellular cargo along MTs in an anterograde fashion using the energy derived from ATP hydrolysis (41). Various membranous organelles, such as precursors of plasma membrane, synaptic vesicles, and mitochondria, are conveyed in an anterograde manner at varying velocities through kinesin.

To confirm the role of kinesin motors in translocating lysosomes toward the cell periphery in AA-treated osteoblasts, we initially utilized kinesin-blocking antibodies and noted their disruptive effect on lysosome distribution. This observation was further buttressed by transfection of dominant negative KIF3A-GFP, since it has been previously reported that KIF3A/B is a heterodimeric kinesin superfamily protein that works as a microtubule plus end-directed motor for delivery of Golgi-derived organelles (42). Finally, RILP overexpression also blocked anterograde movement of lysosomes in osteoblasts, similar to its inhibition of kinesin-based Sif (Salmonella-induced filament) formation in epithelial cells (35).

Kinesin activity is known to be pH-sensitive (43), and lysosome distribution in cells can be altered by manipulating cytosolic pH (44). We did not see any changes of the cytosolic pH during AA-stimulated

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**FIGURE 6.** TIRF imaging of peripheral lysosomes in osteoblasts. A, still TIRF images of AA-treated MC3T3-E1 cells pulsed with 50 μg/ml Alexa Fluor 488 dextran for 2 h and chased into lysosomes overnight. B, TIRF images of dispersed dextran-containing lysosomes in AA-treated MC3T3-E1 cells, treated with ionomycin. The arrows point to lysosomes that were excited within the evanescent field but were not subsequently secreted during imaging. Scale bars, 15 μm.

**FIGURE 7.** Effect of lysosome clustering on osteoblast differentiation. A, epifluorescence image of one MC3T3-E1 clonal cell line stably expressing RILP-GFP. Scale bar, 15 μm. B–F, semiquantitative PCR analysis of osteoblast marker proteins, Opn, Col1a1, Alp, Bsp, and Ocn, with their signal intensity normalized against that of the ribosomal protein gene L32. Each sample was done in triplicate, and data are plotted as the mean ± S.E. of results from three independent experiments of control, AA-treated, and two RILP-GFP-stable clones. *, p < 0.005; **, p < 0.06 AA + RILP versus AA.
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Apart from tubular lysosome formation in macrophages, there are other instances where lysosomes have been observed to migrate toward the cell periphery. During experimental wounding of cells and during *Trypanosome* infection, lysosomes are recruited to the wound edge/site of pathogen adherence (36, 49). Recently, morphological and biochemical studies revealed that several cell types use lysosomes as regulatory secretory organelles. These cells include osteoclasts, melanocytes, and cells of the immune system (50). Our first indication that differentiated osteoblasts do not secrete lysosomes was the absence of an actin requirement for peripheral lysosome sorting. Secretory organelles are typically tethered at the cortex via cortical actin interactions and move to the plasma membrane by unconventional myosin-based transport (51). More convincing was the lack of lysosome secretion we observed during TIRF imaging. Therefore, instead of driving lysosomes completely to the plasma membrane, differentiation of osteoblasts appears to involve bringing lysosomes close to the cell edge without fusion.

What is the role of lysosome dispersion during osteoblast differentiation? It is possible that lysosomes move to the cell periphery to assist in endosomal maturation and eventual intracellular degradation of internalized material. We see enhanced endocytosis and pronounced degradative abilities in lysosomes in AA-stimulated osteogenic cells versus undifferentiated precursors. More compelling is the potential role of lysosomes in managing the enormous collagen production within osteogenic cells. We observed a colocalization of lysosomes with collagen in osteoblasts. Although this collagen is probably newly synthesized collagen *en route* for secretion, we cannot rule out the possibility that the collagen vesicles we detected were first secreted and then taken up by the cells. Collagen is known to be internalized into cells by a process known as “collagen phagocytosis” (52). However, we noticed enhanced lysosome overlap with collagen-containing vesicles when cells were treated with 2,2′-dipyridyl to impair collagen trimerization (53, 54). This suggests that procollagen-containing vesicles are targeted directly to lysosomes, which may have evolved to remove aberrantly assembled proteins during the mass production of collagen in osteogenic cells (Fig. 9). It is also possible that intracellular degradation of procollagen by lysosomes is a means to

osteoblast differentiation, in agreement with previous studies (45), although the lysosomes in these cells could be mobilized when acidification of the cytosol was induced experimentally. Therefore, similar to previous studies of tubular lysosomes in macrophages (46, 47), our results in osteoblasts suggest that under normal conditions, kinesin activity promotes anterograde movement in differentiated cells irrespective of cytosolic pH.

We also observed that increasing extracellular glucose levels modulates lysosome dispersion in osteoblasts. Prolonged treatment of osteoblasts with high levels (22 mM) of glucose blunts both osteoblast differentiation and mineralization (48). Within 24 h of elevated glucose exposure, both collagen I and osteocalcin levels are significantly reduced compared with osteoblasts exposed to physiological levels of glucose (5 mM) (48). The effects of hyperglycemia on lysosome redistribution in osteoblasts may represent a mechanism for quality control during metabolic stress.

**FIGURE 8.** Lysosomes co-localize with procollagen vesicles in AA-induced osteogenic cells. A, picrosirius red staining of collagen in control MC3T3-E1 cells and cells treated with AA for 5 days. B, collagen immunoblot analysis of cell lysates and conditioned media of untreated and AA-treated MC3T3-E1 cells. C, control and 5-day AA-treated MC3T3-E1 osteoblasts showing overlap of lysosomes in green with procollagen in red. Cell boundaries are shown by white dashed lines (D). C and D, confocal image of a control cell (C) and an AA-treated MC3T3-E1 osteoblast (D), with the boxed region magnified, showing lysosomes in green and procollagen in red, with arrows pointing to the colocalization of the two. E, fluorescent image, with magnified subset (box) of an AA-treated MC3T3-E1 osteoblast treated with 0.3 mM 2,2′-dipyridyl for 1 h and immunostained for lysosomes (green) and procollagen (red). The arrows indicate overlap of procollagen and LAMP1-positive organelles. F, quantification of the number of LAMP1-positive procollagen-containing vesicles in control and AA-treated MC3T3-E1 cells, without and with 2,2′-dipyridyl treatment for 1 h. Data are reported as mean ± S.E. from three independent experiments (n = 20); *p < 0.05. Scale bars, 15 μm.
regulate the net protein production during osteoblast differentiation. In fibroblasts, upward of 15% of newly synthesized collagen is degraded intracellularly rather than secreted (55). Studies of procollagen in fibroblasts showed a partial co-distribution or procollagen with lysosome markers (56), suggesting that lysosomes play an active role in the basal degradation of collagen.

Collagen secretion requires MTs (57), and dispersed lysosomes in osteogenic cells may situate the lysosomes along the same highway for rapid interactions with vesicles containing structurally abnormal procollagen. Beyond processing-defective collagen, lysosomes may also participate in the in vivo conversion of procollagen to collagen. The lysosomal enzyme cathepsin D has been shown to be involved in the removal of COOH-terminal propeptide from procollagen, thus mediating mature collagen production (58). In the absence of lysosome dispersion, collagen processing is not complete, and immature or dysfunctional collagen is secreted, which will limit collagen outside-in signals required for osteoblast differentiation (37). The prominent role for lysosomes in osteoblast function and differentiation may explain the redistribution of this organelle from a more confined perinuclear location to more accessible dispersed sites throughout the cell. This is the first description of MT-mediated lysosome movement to the cell periphery, not for secretion but instead to potentially accommodate the marked increase in collagen production during osteoblast differentiation.

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