Human adipose-derived adult stem cells upregulate palladin during osteogenesis and in response to cyclic tensile strain

Michelle E. Wall,1* Andrew Rachlin,2* Carol A. Otey,2 and Elizabeth G. Loboa1

1Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Raleigh; and 2Department of Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

Submitted 16 February 2007; accepted in final form 6 August 2007

Wall ME, Rachlin A, Otey CA, Loboa EG. Human adipose-derived adult stem cells upregulate palladin during osteogenesis and in response to cyclic tensile strain. Am J Physiol Cell Physiol 293: C1532–C1538, 2007. First published August 8, 2007; doi:10.1152/ajpcell.00065.2007.—Cell morphology may be an important stimulus during differentiation of human adipose-derived adult stem (hADAS) cells, but there are limited studies that have investigated the role of the cytoskeleton or associated proteins in hADAS cells undergoing differentiation. Palladin is an actin-associated protein that plays an integral role in focal adhesion and cytoskeleton organization. In this study we show that palladin was expressed by hADAS cells and was modulated during osteogenic differentiation and in response to cyclic tensile strain. Human ADAS cells expressed the 90- and 140-kDa palladin isoforms and upregulated expression of both isoforms after culture in conditions that promoted osteogenesis. Palladin mRNA expression levels were also increased in hADAS cells subjected to cyclic tensile strain. Knockdown of the palladin gene during osteogenesis resulted in decreased actin stress fibers and decreased protein levels of Eps8, an epidermal growth factor receptor tyrosine kinase that colocalizes with actin. Silencing the palladin gene, however, did not affect hADAS cells’ commitment down the osteogenic lineage.

adipose-derived adult stem cells; mechanobiology; Eps8; actin cytoskeleton; mesenchymal stem cells

WHILE MESENCHYMAL STEM CELLS are undergoing osteogenic differentiation, the actin cytoskeleton reorients itself (23), and the cells change from a fibroblast-like to a more cuboidal morphology. It has been proposed that stem cell morphology and cytoskeletal tension play a role in stimulating stem cells to differentiate (15, 23). RhoA, a Ras-like GTPase involved in regulation of the actin cytoskeleton (12), has been linked to stem cell commitment (15). Similarly, palladin, which also regulates normal actin cytoskeleton formation, has been found to play a role in the differentiation of Rcho-1 stem cells into tropheoblast giant cells (20). Palladin is also upregulated during differentiation of monocytes into dendritic cells (17) and during neurite outgrowth of cultured cortical neurons (4). Palladin, like RhoA, could affect stem cell lineage commitment because of its role in actin cytoskeleton formation and thus cell morphology, but the presence and the function of palladin within adipose-derived adult stem (ADAS) cells has yet to be determined.

Palladin is a member of the palladin-myotilin-myopalladin gene family (19). There are three palladin isoforms that are most commonly found in both mouse and human tissues. The 90- to 92-kDa isoform is the most abundant isoform in cells and is ubiquitously expressed in most embryonic tissues. The 140-kDa isoform is also expressed in most embryonic tissues and in adult smooth muscle tissues. The 200-kDa isoform expression is limited primarily to the heart and bone (17, 20, 22). Palladin binds to multiple actin-associated proteins, including vasodilator-stimulated phosphoprotein (3), α-actinin (20, 25), ezrin (17), Lasp-1 (22), profilin (2), ArgBP2 (24), and Eps8 (9). In addition to regulating normal actin cytoskeleton formation, palladin also regulates focal adhesion formation, and thus modulates cell morphology and migration (20).

The purpose of this study was to investigate the presence of palladin in hADAS cells and the effects of osteogenic differentiation and mechanical load on palladin expression levels. It was hypothesized that hADAS cells would express palladin and that osteogenic differentiation and cyclic tensile strain would upregulate palladin expression levels. It was also hypothesized that knockdown of palladin would alter the actin cytoskeleton organization and thus alter osteogenic differentiation.

MATERIALS AND METHODS

Cell isolation, culture, and characterization. Excess human adipose tissue from abdominoplasty procedures was obtained from three female donors (45-year-old African American, 31-year-old Cauca- sian, and 35-year-old Caucasian) in accordance with an approved IRB protocol. Human ADAS cells were isolated from the tissue using a method based on density and differential adhesion, as described by Zuk et al. (30). Briefly, adipose tissue was digested with 0.075% collagenase type I (Worthington Biochemical, Lakewood, NJ) for 30 min, and then the ADAS cell-rich dense cell fraction was pelleted by centrifugation at 12,000 g. Blood cells were lysed by incubation and resuspension of the pelleted cells in 160 mM NH4Cl. Stromal cells were then pelleted by centrifugation at 12,000 g for 10 min and then resuspended in α-MEM supplemented with 10% fetal bovine serum (FBS) (lot selected; Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (growth medium). After 24 h, the cell monolayer was washed twice with phosphate-buffered saline to remove nonadherent cells, and fresh growth medium was added. Human ADAS cells were then characterized via immunohistochemical analysis of surface markers that have been found to be present (CD73, CD105, and CD166) and absent (CD34 and CD45) in hADAS cells and by their ability to differentiate down osteogenic and adipogenic pathways. All cell culture chemicals

* M. E. Wall and A. Rachlin contributed equally to this study.

Address for reprint requests and other correspondence: E. G. Loboa, Joint Dept. of Biomedical Engineering at UNC-Chapel Hill and NC State Univ., 2142 Burlington Laboratories, Campus Box 7115, NC State Univ., Raleigh, NC 27695-7115 (e-mail: egloboa@ncsu.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
and supplies were purchased from Mediatech (Herndon, VA) and GIBCO-BRL (Grand Island, NY) unless otherwise noted.

Osteogenic differentiation. Cells were plated at 50,000–100,000 cells/10 cm² and grown until they reached 100% confluency. Cells were then cultured for 2 wk in growth or osteogenic medium. Osteogenic medium consisted of growth medium supplemented with 50 μM ascorbic acid, 0.1 μM dexamethasone, and 10 mM β-glycerophosphate (5, 10–11, 18, 27). Extent of osteogenic differentiation was determined by deposition of calcium. Calcium deposits were visualized by staining with Alizarin Red S.

Fabrication of collagen gels. Human ADAS cells were seeded into collagen gels consisting of 70% type I collagen (BD Biosciences, San Jose, CA) (pH adjusted to 7.0), 20% 5× MEM, and 10% FBS at 60,000 cells/200 μL gel solution. The cell-seeded gel solutions were loaded into TissueTrain collagen I-coated six-well culture plates (Flexcell International, Hillsborough, NC) to create linear three-dimensional collagen constructs.

Application of tensile strain. Cell-seeded constructs were subjected to 14 days of 10% cyclic uniaxial tensile strain at 1 Hz for 4 h/day using a Tissue Train Flexcell Strain Unit (FX-4000, Flexcell International).

RNA isolation and real-time RT-PCR analysis. Total RNA was purified using Eppendorf Perfect RNA mini-columns (Hamburg, Germany) according to the manufacturer’s recommended protocol for
used for gene expression analysis of palladin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the endogenous control. Expression levels were determined with the \( \Delta\Delta C_t \) method (14).

**Immunohistochemical analysis.** Cells were fixed with 10% formalin. Cell membranes were permeabilized using 0.2% Triton X-100 and 0.5% BSA. Three primary antibodies were utilized with specificity to different palladin isoforms. These antibodies included a mouse monoclonal anti-palladin antibody against all isoforms (IE6) (20), a rabbit polyclonal anti-palladin antibody against all isoforms, and a rabbit polyclonal anti-palladin antibody against the 140- and 200-kDa isoforms (4IgNT) (22). Cells were counterlabeled with appropriate secondary polyclonal antibodies conjugated with either Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes) to visualize palladin. Cells were also stained with Alexa Fluor 594 phalloidin (Molecular Probes) and DAPI to label f-actin and nuclei, respectively. Cells were viewed at room temperature with a Leica DM LFSA epifluorescent microscope (Wetzlar, Germany) equipped with a ×40 water immersion, UV objective lens; a Hamamatsu ORCA ERG deep-cooled, high-resolution camera (Hamamatsu City, Japan); and SimplePCI image capture and analysis software (Compix, Sewickley, PA).

**Western blot analysis.** Cells were collected by scraping into a lysis buffer (0.5% DOC, 0.1% SDS, 0.1% Nonidet-p-40, 10 mM EDTA, 150 mM KCl, 20 mM HEPES, pH 7.4, with the Sigma protease inhibitor cocktail for mammalian cells). Lysates were gently rotated at 4°C and then centrifuged for 15 min at 15,000 g. Supernatants were collected and protein concentrations calculated by BCA assay (Pierce, Rockford, IL). Supernatants were then boiled for 5 min in Laemmli sample buffer, resolved on 4–12% 1.0 mm Tris-Bis acrylamide gels (Invitrogen), and electroblotted onto nitrocellulose. The membrane was stained with Ponceau S to check for correct protein loading; blocked overnight in 2% gelatin in Tris-buffered saline (TBS); and stained with monoclonal anti-actin (1:10,000; Sigma, St. Louis, MO), and rabbit anti-tubulin (1:10,000; Chemicon, Temecula, CA), monoclonal anti-actin (1:10,000; Sigma, St. Louis, MO), and rabbit anti-palladin serum (1:5,000) in TBS with 0.05% Tween. After 1 h of incubation, the membrane was washed three times with TBS-Tween and then incubated for an hour with IRdye700 and IRdye800 infrared-tagged anti-mouse (1:20,000) and anti-rabbit (1:15,000) secondary antibodies. The membrane was then washed three times in TBS-Tween and then briefly washed in TBS before being imaged with the infrared imaging Odyssey scanner (LI-COR Biosciences; Lincoln, NE). Protein levels were determined using LI-COR’s Odyssey Application software version 1.2, after background levels were corrected for using the “median; right and left” setting. This analysis provided arbitrary intensity units for each band (tubulin, actin, and palladin).

**siRNA knockdown of palladin.** Small interfering RNA (siRNA) duplex 19-base oligonucleotides were purchased from Dharmacon Research (Lafayette, CO) (target sequence: UCACUACACAUCAAAAGA). The control was siCONTROL Non-Targeting siRNA #1 (Dharmacon). On day 0, cells were transfected using the transfection reagent TransIT siQuest (Mirus Bio, Madison, WI). Two wells from each donor received 50 nM of either the palladin or control siRNA. On day 2, six wells from each treatment group were switched to osteogenic medium. On day 9, the siRNA transfection was repeated by using the same conditions as on day 0. On day 16, the cells were harvested, and protein was collected for Western blot analysis or RNA was harvested for RT-PCR analysis.

**Statistical analysis.** For mRNA and protein expression levels, data were subjected to a two-tailed Student’s \( t \)-test or rank sum \( t \)-test to determine significant differences between control and osteogenic conditions on appropriate days (\( P < 0.05 \)). To determine significant differences among hADAS cells subjected to both mechanical and chemical stimuli, data were subjected to a one-way ANOVA followed by a two-tailed Student’s \( t \)-test (\( P < 0.05 \)). Data are presented as means ± SD.

**RESULTS**

Western blot results indicated that hADAS cells in monolayer culture, which were positive for the stem cell markers...
CD105 and CD166, constitutively expressed the 90- and 140-kDa palladin isoforms but not the 200-kDa isoform (Fig. 1, A and B). Immunohistochemical staining with phalloidin and either an antibody against all three palladin isoforms or an antibody against the two larger isoforms showed that palladin was primarily present in the cells in linear bundles (Fig. 1, C and D) and occasionally as punctate localizations within the cytoplasm (Fig. 2B). Palladin bundles were located throughout the cytoplasm and along actin-rich stress fibers (Fig. 1, C and D). To determine whether the 90- and 140-kDa isoforms were located within similar regions of the cells, hADAS cells were double labeled with an antibody against both palladin isoforms or an antibody against the 140-kDa isoform. Human ADAS cells had colocalized expression of both the 90- and 140-kDa palladin isoforms (Fig. 2A). However, the 90-kDa palladin isoform was not always colocalized with the 140-kDa isoform (Fig. 2B), and some cells expressed primarily the 90-kDa but not the 140-kDa isoform (Fig. 2C).

Nondifferentiated hADAS cells did not express the 200-kDa isoform, which has been found in the neonatal bone (22).

---

Fig. 4. Knockdown of palladin alters f-actin expression. A and B: images of untreated hADAS cells and hADAS cells transfected with a control small interfering RNA (siRNA) or palladin siRNA and cultured in growth (A) or osteogenic media (B) for 3 days. hADAS cells transfected with palladin siRNA had shorter actin bundles near the cell periphery (arrows) and less definable stress fibers throughout the cell body (arrowheads). Red is phalloidin-stained actin filaments, and blue is DAPI-stained nuclei. Scalebar is 100 μm.

Fig. 5. Knockdown of palladin does not affect osteogenesis. A: Alizarin red S-stained calcium deposits in cultures of hADAS cells transfected with a control siRNA or palladin siRNA and cultured in osteogenic media for 14 days. Cells counterstained with hematoxylin. Scale bar is 100 μm. B: relative change in osteopontin protein expression in hADAS cells transfected with a control siRNA or palladin siRNA and cultured in osteogenic media for 14 days. Values were normalized to tubulin.
Therefore, hADAS cells in monolayer culture were chemically promoted down an osteogenic pathway and were analyzed for changes in palladin mRNA and protein expression levels to determine whether this larger isoform could be expressed and to determine whether the expression of the other palladin isoforms were altered during differentiation. Analysis of palladin mRNA expression levels indicated that palladin was significantly upregulated by twofold at 24 h of differentiation and was continuously upregulated throughout 2 wk of differentiation in hADAS cells in both monolayer (Fig. 3A) and three-dimensional culture (Fig. 3B). Western blots confirmed that hADAS cells in monolayer culture significantly upregulated the 90-kDa palladin isoform by twofold after 1 and 2 wk of culture in osteogenic media (Fig. 3, C and D). Human ADAS cells had similar increases in protein levels of the 140-kDa isoform but to a lesser extent (data not shown). The 200-kDa isoform of palladin was not expressed during these initial 2 wk of differentiation. Staining of cells with Alizarin Red S indicated that cells in osteogenic media, but not complete growth media, deposited calcium within 2 wk, indicating that the cells were undergoing osteogenesis (data not shown).

Alterations in palladin expression in response to mechanical load were also analyzed since uniaxial cyclic tensile strain has been found to promote bone marrow-derived mesenchymal stem cells down the osteogenic lineage (28). Human ADAS cells seeded in three-dimensional collagen gel constructs and stem cells down the osteogenic lineage (28). Human ADAS cells had similar increases in protein levels of the 140-kDa isoform but to a lesser extent (data not shown). The 200-kDa isoform of palladin was not expressed during these initial 2 wk of differentiation. Staining of cells with Alizarin Red S indicated that cells in osteogenic media, but not complete growth media, deposited calcium within 2 wk, indicating that the cells were undergoing osteogenesis (data not shown).

To determine whether palladin could affect actin organization in hADAS cells, the palladin gene was silenced by siRNA treatment. Cells were then cultured for 3 days in conditions that promoted osteogenesis, and the cytoskeleton was labeled with phallolidin and viewed by indirect immunofluorescence. There was no observable difference seen in the actin cytoskeleton organization between hADAS cells in growth media and those cells in osteogenic media (Fig. 4). Cells appeared to have long actin stress fibers that extended the length of the cell body. Knockdown of palladin appeared to decrease the number of long stress fibers within hADAS cells in both growth and osteogenic media as determined by visual assessment (Fig. 4). These cells had shorter actin bundles near the cell periphery (Fig. 4, arrow) and less definable stress fibers throughout the cell body (Fig. 4, arrowhead). However, these differences in cytoskeletal organization did not alter the ability of the cells to undergo osteogenesis since silencing the palladin gene did not alter calcium deposition or protein levels of osteopontin after 2 wk of culture in osteogenic media (Fig. 5).

In addition to altering actin organization, palladin could also affect other actin-associated proteins. Protein levels of Eps8 were analyzed in hADAS cells promoted down an osteogenic pathway for 2 wk to determine whether other actin-associated proteins were also modulated during osteogenesis. Like palladin, Eps8 expression was upregulated during osteogenesis (Fig. 6, lane 2). Knockdown of palladin during osteogenesis dramatically decreased Eps8 expression (Fig. 6, lane 4).

**DISCUSSION**

This study investigated whether palladin was expressed by hADAS cells and altered during osteogenic differentiation and in response to mechanical load since palladin has been found to play a role in actin cytoskeleton reorganization. hADAS cells were found to express the two smaller palladin isoforms (90 and 140 kDa) but not the 200-kDa isoform. The expressed palladin isoforms were associated with actin stress fibers, as has been seen in other cell types (20), but these isoforms were not always colocalized within the cells. Taken together with a prior study (20) that shows differential expression of the palladin isoforms in various tissues, these results may indicate that the palladin isoforms have separate functions within cells as well as tissues. Alternatively, the variations in the localization of these two palladin isoforms among the hADAS cells may also result from heterogeneities in isolated hADAS cells.

Palladin was found to be upregulated in the hADAS cells during both osteogenesis and tensile strain. Furthermore, silencing the palladin gene decreased actin stress fibers, but it did...
not affect the ability of the cells to undergo osteogenesis. hADAS cells were still able to deposit calcium, and protein levels of osteopontin were not affected by palladin knockdown. Contrastingly, Rodriguez et al. (23) found that disruption of the actin cytoskeleton with cytochalasin D decreased calcium deposition and alkaline phosphatase activity in bone marrow-derived mesenchymal stem cells undergoing osteogenesis. Since the knockdown of palladin did not completely disrupt the cytoskeleton, and prior studies indicate that tension in the cytoskeleton affects stem cell lineage commitment (15), our data suggest that knockdown of palladin did not alter cytoskeletal tension enough to affect the ability of hADAS cells to undergo osteogenesis in response to chemical factors.

Knockdown of palladin did decrease Eps8 protein levels. Eps8 is an unusual protein that affects the actin cytoskeleton through two distinct pathways. First, Eps8 was shown to be part of a trimeric complex that activates the small GTPase Rac and thus stimulates the formation of actin-rich membrane ruffles (13, 26). In addition to this indirect pathway for influencing actin organization, Eps8 has also been shown to bind directly to actin filaments and cap their rapidly growing ends (6, 7). Thus Eps8 can play a direct role in organizing actin filament arrays in which the filaments are all the same length. The fact that palladin knockdown in stem cells resulted in decreased levels of Eps8 suggests that binding of palladin may increase the half-life of Eps8 in cells, a possibility that would have to be explored in future studies.

This study found that palladin was upregulated in hADAS cells subjected to cyclic tensile strain. Furthermore, there was an additive effect on palladin mRNA expression levels in hADAS cells cultured in osteogenic media while being subjected to tensile strain. Mechanical load can alter actin cytoskeleton organization (16). Additionally, cytoskeletal interactions can modulate the response of bone cells to mechanical signals (21, 29). To this end, cytoskeletal interactions could thus affect the response of hADAS cells to mechanical load. Recently, Engler et al. (8) demonstrated that naïve mesenchymal stem cells sense the mechanical properties of the environment, and that this is a major determinant of cell fate. The authors showed that the level of organization of the actin cytoskeleton and the degree of cell contractility (which is determined by the degree of stiffness of the extracellular matrix) drove stem cells to follow a neurogenic, myogenic, or osteogenic lineage. Mechanical signals can be detected by the cells through interactions among the matrix, integrins, and the cytoskeleton (1). If palladin affected these interactions, then palladin could modulate the response of the cell to mechanical load. However, this study only indicates that cyclic strain can upregulate palladin mRNA levels and does not indicate whether palladin can affect mechanotransduction in hADAS cells. Further studies would need to be conducted to determine this latter point.

This study is the first to show that palladin is present in hADAS cells and that palladin was upregulated in hADAS cells during osteogenic differentiation and during mechanical load. Although the function of palladin within hADAS cells is unknown, knockdown of palladin was found to decrease actin stress fibers and Eps8 expression without affecting the ability of hADAS cells to differentiate down the osteogenic pathway.

ACKNOWLEDGMENTS

The authors thank Drs. W. Losken and J. van Aalst for providing the adipose tissue samples and Dr. S. Bernacki, D. Aram, A. Finger, S. Goicoechea, A. Hanson, C. Haslauer, and P. Middlebrooks for technical assistance.

GRANTS

This study was supported by the Ralph E. Fowe Faculty Enhancement Award (to E. G. Loboa), National Institutes of Health Grants NS-43243 and GM-61743 (to C. A. Otey), and a North Carolina Biotechnology Center Multidisciplinary Research Grant (to E. G. Loboa).

REFERENCES


