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Adipogenic Human Adenovirus Ad-36 Induces Commitment, Differentiation, and Lipid Accumulation in Human Adipose-Derived Stem Cells

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Abstract

Human adenovirus Ad-36 is causatively and correlatively linked with animal and human obesity, respectively. Ad-36 enhances differentiation of rodent preadipocytes, but its effect on adipogenesis in humans is unknown. To indirectly assess the role of Ad-36-induced adipogenesis in human obesity, the effect of the virus on commitment, differentiation, and lipid accumulation was investigated in vitro in primary human adipose-derived stem/stromal cells (hASC). Ad-36 infected hASC in a time- and dose-dependent manner. Even in the presence of osteogenic media, Ad-36-infected hASC showed significantly greater lipid accumulation, suggestive of their commitment to the adipocyte lineage. Even in the absence of adipogenic inducers, Ad-36 significantly increased hASC differentiation, as indicated by a time-dependent expression of genes within the adipogenic cascade—CCAAT/Enhancer binding protein- β , peroxisome proliferator-activated receptor- γ , and fatty acid-binding protein—and consequentially increased lipid accumulation in a time- and viral dose-dependent manner. Induction of hASC to the adipocyte state by Ad-36 was further supported by increased expression of lipoprotein lipase and the accumulation of its extracellular fraction. hASC from subjects harboring Ad-36 DNA in their adipose tissue due to natural infection had significantly greater ability to differentiate compared with Ad-36 DNA-negative counterparts, which offers a proof of concept. Thus, Ad-36 has the potential to induce adipogenesis in hASC, which may contribute to adiposity induced by the virus.

Keywords

Obesity; Adiposity; Adipogenesis; Infectobesity; Lipogenesis; Adipocyte progenitors

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Introduction

Although obesity is recognized as a disease of multiple etiologies, microbial infection as an etiological factor has received consideration relatively recently [1–3]. Not all obesity can be explained by viral infections. However, if certain pathogens promote human obesity, recognizing their role is the first important step in addressing the infection and its pathogenesis. An accurate understanding of the varied etiological factors of obesity may lead to cause-specific treatments and, consequentially, its successful management. Seven viruses, a scrapie agent, a parasite, and gut microflora have been reported to cause obesity in animal models [4–16]. Whether these pathogens cause obesity in humans remains to be determined. Ethical reasons preclude experimental infection of humans. Determination of the causative role of a virus in humans will have to depend on indirect evidence, such as elucidating the mechanism of adipogenic action in tissue culture and animal models and applying it to observations in humans.

So far, adenoviruses are the only adipogenic viruses [9–11,13] linked with human obesity [12,17] and therefore form prime targets for determining the role of adipogenic viruses in human obesity. Although we have extensively documented a cause-and-effect relationship of Ad-36 with obesity in animal models [9,10,13,18] and demonstrated an association of Ad-36 with human obesity [17], the exact contribution of Ad-36 in human obesity has not yet been determined. Ad-36 enhances rodent preadipocyte differentiation and lipid accumulation [19]. Therefore, we hypothesized that in vivo, Ad-36 recruits adipocyte progenitors to undergo adipogenesis, which contributes to adiposity induced by the virus. We tested the adipogenic effect of Ad-36 on adipocyte progenitors—primary human adipose-derived stem/stromal cells (hASC), which retain multipotency [20]. The experiments described here show that Ad-36 increases commitment of hASC to the adipogenic lineage and induces differentiation and lipid accumulation. Moreover, as a proof of concept, we observed that hASC from humans naturally infected with Ad-36 show significantly greater lipid accumulation.

Materials and Methods

The following is an outline of the experiments conducted. hASC were isolated from lipoaspirates from subcutaneous adipose tissue sites of human volunteers and used as adipocyte progenitors for the experiments. Studies were reviewed and approved by the Institutional Review Board of the Pennington Biomedical Research Center. First, the potential of Ad-36 to infect hASC was ascertained. Next, the ability of Ad-36 to induce hASC to accumulate lipid and thus be committed to adipogenic lineage was determined in the presence of osteogenic media. Differentiation of hASC induced by Ad-36 was tested by determining the expression of several key genes of the adipogenic cascade. The virus dose-response and time course of lipid accumulation were determined. As a proof of concept, adipogenic potential was tested in hASC isolated from humans with or without natural Ad-36 infection. A detailed description of the assays and techniques is presented below.

hASC Extraction

Liposuction aspirates from subcutaneous adipose tissue sites were obtained from subjects (Table 1; six women, two men; age [mean $\pm$ SD], 37 $\pm$ 12 years; body mass index [BMI], 28 $\pm$ 3 kg/m²) who were free of metabolic diseases and undergoing elective plastic surgery. Lipid accumulation and gene expression time course and protein abundance experiments were repeated for at least three subjects. The samples were received without identifying markers. Tissue was washed three times with phosphate-buffered saline (PBS) and suspended in an equal volume of PBS supplemented with 0.1% collagenase type I (catalog no. LS004196; Worthington Biochemical, Lakewood, NJ, <http://www.worthington-biochem.com>) and 1% bovine serum albumin (BSA; catalog no. A6003; Sigma-Aldrich, St. Louis,

<http://www.sigmaaldrich.com>) for 1 hour at 37°C under mild controlled agitation at approximately 75 rpm. Tubes were centrifuged at 300g at room temperature for 5 minutes, followed by 10 seconds of vigorous shaking, and the centrifugation step was repeated. Supernatant was removed, and the cell pellet was resuspended in 10 ml of sterile PBS with 1% BSA, followed by centrifugation at 1,200 rpm for 5 minutes at room temperature. The cell pellet was resuspended in stromal media, and only 200 μ l of cell suspension was exposed to Red Blood Cell Lysing Buffer Hybri-Max (catalog no. R7757; Sigma-Aldrich) for 20 minutes, followed by a cell count using a hemocytometer and 0.4% trypan blue solution (catalog no. T8154; Sigma-Aldrich).

Inoculation of hASC Virus Dose-Response Experiment

hASC were cultured in stromal media until reaching 80% confluence. Cells were inoculated with either Ad-36 at a multiplicity of infection (MOI) of 1.9–243.2 or media Control [CON]. Stromal medium was replaced every 3 days for 9 days. For the following experiments, a dose of 2.7 MOI was selected for the infection of hASC (unless specified otherwise). Ad-2, a nonadipogenic virus [19,21], was used as a negative control.

Determination of Commitment of hASC to Adipogenic Lineage in the Presence of Osteogenic Media

Approximately 80%–90% confluent hASC were inoculated with media (CON) or Ad-36 as described above. Next, the medium was replaced with osteoblast induction medium (Dulbecco's modified Eagle's medium [DMEM] with 10% fetal bovine serum [FBS], 10 mM β -glycerophosphate, 0.15 mM ascorbate-2-phosphate, 10 nM dexamethazone, 100 U/ml penicillin, and 100 μ g/ml streptomycin), as previously described [22]. Cells were maintained in culture for 9 days post-osteogenic induction.

Induction of Adipocyte Differentiation

The adipocyte differentiation medium (DMEM-Ham's F-12 medium + 3% FBS + 1 $\times$ antibiotic solution + 33 μ M biotin + 17 μ M pantothenate + 100 nM human insulin + 1 μ M dexamethasone + 250 μ M 3-isobutyl-1-methylxanthine [IBMX] + 5 μ M rosiglitazone) was added to almost confluent hASC. Three days later, the medium was changed to adipocyte medium (DMEM-Ham's F-12 medium + 3% FBS + 1 $\times$ antibiotic solution + 33 μ M biotin + 17 μ M pantothenate + 100 nM insulin + 1 μ M dexamethasone) and replaced every 3 days thereafter. Reagents were as follows: DMEM/Ham's F-12 medium nutrient mixture (1:1) and FBS (catalog no. SH30070.03; HyClone, Logan, UT, <http://www.hyclone.com>); 1 $\times$ antibiotic-antimycotic (catalog no. A-5955; Sigma-Aldrich) containing 10,000 U of penicillin, 10 mg of streptomycin, and 25 μ g of amphotericin B per ml; D-biotin (catalog no. 47,868; Sigma-Aldrich); D-pantothenic acid (catalog no. P3161; Sigma-Aldrich); insulin (catalog no. I5500; Sigma-Aldrich); dexamethasone (catalog no. D1756; Sigma-Aldrich); IBMX (catalog no. I5879-1G; Sigma-Aldrich); and rosiglitazone (Avandia, Alaska Scientific, Inc., Anchorage, AK, <http://www.alaskascientific.net>).

Determination of Lipid Accumulation

Oil red O, a lipid-specific dye, was used to determine lipid accumulation in hASC as described [23]. Cells were fixed for 1 hour with 10% formalin solution (catalog no. HT551128; Sigma-Aldrich), washed with water, and stained for 2 hours with oil red O (catalog no. BP 112-10; Fisher Scientific, Pittsburgh, <http://www.fishersci.com>), followed by exhaustive rinsing with water. The dye was extracted with isopropyl alcohol (catalog no. 190764; Sigma-Aldrich), and its absorbance was read at 510 nm.

DNA and RNA Extraction and cDNA Synthesis

Total DNA was extracted using the DNeasy Tissue Mini Kit (catalog no. 69504; Qiagen, Hilden, Germany, <http://www1.qiagen.com>) and stored at -80°C until it was used for amplification. RNA was extracted using the RNeasy Mini Kit (catalog no. 74104; Qiagen). Residual DNA was eliminated by amplification-grade deoxyribonuclease I (catalog no. 18,068-015; Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>). One microgram of total RNA was reverse-transcribed to cDNA using the iScript cDNA Synthesis Kit (catalog no. 170-8890; Bio-Rad, Hercules, CA, <http://www.bio-rad.com>). Samples were stored at -80°C until they were used for amplification.

Quantitative Real-Time Polymerase Chain Reaction

A standard was generated using cDNA or DNA (depending on the experimental design) pooled from the experimental samples. An ABI Prism 7700 sequence detector (Applied Biosystems, Branchburg, NJ, <http://www.appliedbiosystems.com>) and a SYBR Green detection system (catalog no. 170-8880; Bio-Rad) were used for quantitative real-time (qRT) polymerase chain reaction (PCR). Both samples and standards were run in duplicate, and each transcript level was adjusted to a housekeeping gene (Cyclophilin B). Primer sequences used for the qRT-PCR are shown in supplemental online Table 2. In addition, the following premade primers were used: human RunX2 (catalog no. Hs00231692_m1; Applied Biosystems), human CCAAT/Enhancer binding protein (C/EBP)- β (catalog no. Hs00270923_s1; Applied Biosystems), and human peroxisome proliferator-activated receptor- γ (PPAR- γ ; catalog no. Hs00234592_m1; Applied Biosystems).

Viral Infection by Immunofluorescence

Subconfluent hASC in 96-well plates were infected at various MOIs (0, 1.9, 3.8, 7.6, 15.2, 30.4, 60.8, 121.6, and 243.2) and fixed 3 days later. Another 96-well plate was infected with Ad-36 at 3.8 MOI. The cells were fixed with 4% paraformaldehyde for 20 minutes 3, 5, 7, and 9 days postinfection. Directly after fixing, all cells were processed for immunohistochemistry. They were permeabilized in 0.1% Triton PBS, blocked in 10% goat serum in 1% BSA, and incubated overnight with a rabbit antibody for Ad-36 hexon protein, followed by incubation with a secondary Alexa Fluor goat anti-rabbit IgG (red). 4,6-Diamidino-2-phenylindole (DAPI) staining of the same cells was used to calculate the percentage of infected cells as described in the Immunofluorescence section, below.

Immunofluorescence

hASC were grown on chamber slides (Lab-Tek, Nunc, Rochester, NY, <http://www.nuncbrand.com>) or 96-well plates (Corning Costar, Acton, MA, <http://www.corning.com/lifesciences>) to 70%–80% confluence, serum-starved overnight, infected with Ad-36 at various MOIs (as required for the experiment), fixed in 4% paraformaldehyde, and processed for immunofluorescence. Lipid droplets were identified using the fluorescent neutral lipid dye BODIPY 493/503 (Invitrogen). Ad-36 viral particles were identified using anti-Ad-36 rabbit polyclonal antibodies that we had generated. Lipoprotein lipase (LPL) was detected using anti-LPL mouse monoclonal antibodies (Abcam, Cambridge, MA, <http://www.abcam.com>). Secondary antibodies were goat anti-rabbit Alexa Fluor 594 (Invitrogen) and goat anti-mouse Alexa Fluor 488 (Invitrogen), respectively. Slides were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen). Images were acquired on a Zeiss Axioplan 2 (Carl Zeiss, Jena, Germany, <http://www.zeiss.com>; Everest) and Zeiss Axiovert 40 CFL using a $\times 63$ Zeiss Plan-Apochromat, $\times 20$ LD Plan Neofluar with $\times 20$ and $\times 2$ objectives, and a Photometrics Cool Snap HQ camera. All images were processed using WICF ImageJ software (NIH) with the Nuclear Counter plug-in used for semiquantitative (visually corrected) analysis of images. One image of the entire well ($\times 2$ objective) was taken

per well in triplicate. For LPL study, images were deconvoluted using Autodeblur software (Media Cybernetics, Crofton, MD, <http://www.mediacy.com>).

Association of Natural Ad-36 Infection with Greater Lipid Accumulation in hASC

Given that experimental infection of animals with Ad-36 increases adiposity, we hypothesized that natural Ad-36 infection in humans increases the adipogenic potential of preadipocytes in vitro. Natural Ad-36 infection was determined by screening subcutaneous abdominal adipose tissue of 34 obese, normoglycemic Pima Indians (18 males, 16 females; age [mean + SD], 27 ± 7 years; BMI, 36 ± 5 kg/m²; body fat, 36% ± 5% as determined by DXA) for Ad-36 DNA, as described below. Lipid accumulation due to differentiation of hASC in these subjects was determined (described below) in a blinded manner and compared between the Ad-36 DNA-positive and -negative groups.

The study protocol was approved by the Institutional Review Board of the National Institute of Diabetes and Digestive and Kidney Diseases and by the Tribal Council of the Gila River Indian Community. All subjects gave written informed consent before participation. Samples of subcutaneous abdominal adipose tissue were obtained from obese Pima Indians with normal glucose tolerance by aspiration biopsy, and hASC were isolated as described [24]. The aspirates were screened for Ad-36 DNA by nested PCR as described [25]. The hASC were typically cultured for 14 days before induction of differentiation. At confluence, hASC were induced to differentiate by the addition of Medium 199 supplemented with 10⁻⁸ M insulin, 10⁻⁶ M hydrocortisone, and 0.5 mM 3-isobutyl-1-methylxanthine for 5 days. Subsequently, the culture was maintained in medium containing 3% FBS, 10⁻⁸ M insulin, and 10⁻⁶ M hydrocortisone. Primary human skin fibroblasts that were cultured in a similar manner served as a negative control for this differentiation procedure.

The differentiation of preadipocytes into lipid-filled cells with adipocyte morphology was monitored morphologically until 18 days after induction of differentiation. At this time, the cell monolayer was fixed and stained with a lipid-specific dye, oil red O. Representative color pictures representing 4.5-mm² areas were taken at a magnification of ×40 using a Polaroid Microcam (Polaroid, Cambridge, MA, <http://www.polaroid.com>) on an inverted microscope (Eclipse TE200; Nikon, Tokyo, <http://www.nikon.com>). The photomicrographs served as templates for determining the percentage of preadipocyte differentiation, which was calculated as the ratio of the number of lipid-filled cells to the total cell number multiplied by 100%.

Statistical Analysis

Statistical differences in group means were determined by Student's *t* test, and the differences were considered significant at *p* < .05. Overall, because of the novel nature of the study, several rigorous measures were used to ascertain and confirm the findings. To ensure a relatively generalizable response, the experiments were conducted with hASC obtained from different volunteers. Commitment of hASC to adipogenic lineage due to Ad-36 infection was determined in the presence of osteogenic media. mRNA expression and protein levels of multiple key genes in the adipogenic cascade were determined to ensure differentiation. Ability of the Ad-36-differentiated cells to function as adipocytes was determined by detailed lipoprotein lipase studies and by documenting intracellular lipid accumulation by using two different assays.

Results

Infection of hASC

Documenting successful cellular entry by a virus is important in investigating its effects on cells. The increase in Ad-36 and Ad-2 gene expression and viral DNA content over time in infected hASC demonstrated successful viral entry and replication in the cells (Fig. 1A, 1B).

The percentage of hASC getting infected increased in proportion to the viral dose (MOI) used to inoculate the cells (Fig. 1C, 1D). The total number of cells per plate decreased at extremely high MOI (Fig. 1D). At a lower MOI (3.8), the cell population was stable throughout the experiment, and the percentage of Ad-36-infected cells increased from 0.5% on day 3 postinoculation to 20% on day 7 (Fig. 1E, 1F), indicating the spread of infection with time.

Ad-36 Induces Commitment of hASC to Adipocyte Lineage

As expected from osteogenic ability of multipotent hASC [26,27], the osteogenic media increased RunX2 expression, an indicator of induction of osteogenic commitment (Fig. 2A). Despite the presence of osteogenic media, Ad-36 attenuated RunX2 expression (Fig. 2A) and instead diverted the commitment to the adipogenic lineage, as suggested by greater expression of PPAR- γ , a key regulator of adipogenic pathway (Fig. 2B), and consequentially greater lipid accumulation 9 days postinoculation (Fig. 2C). These data suggest that the adipogenic effect of Ad-36 is robust enough to induce adipogenic program in hASC, despite the presence of the osteogenic media. On the other hand, in the absence of any inducers, Ad-2 failed to increase lipid in hASC (Fig. 2D), suggesting that the adipogenic effect of Ad-36 is not common to all adenoviruses. Because of the lack of adipogenic effect of Ad-2 on hASC, additional experiments focused only on the effects of Ad-36.

Ad-36 Induces Spontaneous Differentiation of hASC to Adipocytes

C/EBP- β , PPAR- γ , and key genes involved in differentiation of preadipocyte are possible targets for Ad-36-induced lipid accumulation. Fatty acid-binding protein (aP2) is extensively used as a marker of differentiation of uncommitted cells to mature adipocytes. A time course showed that Ad-36 significantly increased C/EBP- β (Fig. 3A) and PPAR- γ (Fig. 3B) gene expressions 9 days postinfection. Moreover, Ad-36 progressively increased the expression of aP2 5 ($p < .01$), 9 ($p < .05$), and 12 ($p < .01$) days postinoculation (Fig. 3C). As a representative key regulator of adipogenesis, protein abundance of PPAR- γ was determined; it was significantly increased by Ad-36 (Fig. 3D). It is noteworthy that the effect of Ad-36 was observed in the absence of adipogenic inducers, which shows that the virus induces spontaneous differentiation of hASC.

LPL contributes to lipid uptake in adipocytes. Inactive LPL is stored in the perinuclear region, which is a heparin-unreleasable (HUR) fraction. For lipid uptake, activated LPL translocates extracellularly and can be eluted by heparin treatment (heparin releasable [HR] fraction). Ad-36 increased LPL expression in a time-dependent manner (Fig. 4A). Approximately 80% of LPL in mock-infected hASC remained as HUR fraction, whereas 97% of the enzyme in the Ad-36-infected group was extracellular, HR fraction (Fig. 4B–4D).

Within a group of Ad-36-infected hASC, not all cells support viral entry or replication, which provides an excellent opportunity to observe differences in the response of cells with and without Ad-36 infection in the same group. Figure 4E shows extensive translocation of LPL away from the perinuclear region to the periphery only in the cells infected with Ad-36. The change of subcellular localization of LPL was accompanied by a change in the electrophoretic mobility of the protein (Fig. 4B–4D), suggesting post-translational modification and activation of LPL by Ad-36, indicative of a functioning adipocyte.

Ad-36 Induces Lipid Accumulation in hASC

Differentiated adipocytes accumulate lipid, which was determined in hASC in response to an increasing viral dose. Ad-36 increased lipid accumulation in a dose-dependent manner (Fig. 5A). At 2.7 MOI of Ad-36, hASC accumulated threefold more lipid compared with control. The slight decrease in lipid accumulation at higher MOI is perhaps due to lifting of lipid-laden cells during medium changes and handling. Despite the loss of cells, the total amount of lipid per well was significantly greater in Ad-36-infected groups at all MOIs tested.

Next, a time course experiment showed that Ad-36 significantly increased lipid accumulation in hASC 9 and 12 days postinfection, as determined by oil red O assay (Fig. 5B) in the absence of differentiation inducers. hASC obtained from donors belonging to a wide range of ages and BMIs (age range, 22–57 years; BMI range, 26–32 kg/m²) showed similar results, suggesting the effect to be BMI- and age-independent (data not shown).

The effect of Ad-36 on lipid accumulation by hASC was also determined when adipogenesis was induced by differentiation media. Compared with the uninfected control, Ad-36 significantly increased lipid accumulation on day 5 postinoculation (Fig. 5C). As expected, treatment with differentiation media eventually induced adipogenesis in the uninfected hASC, and the lipid content per cell was similar for the two groups 9 days postinoculation. A time course of lipid accumulation by Ad-36 in the presence or absence of adipogenic induction is shown pictorially in Figure 5D. These results show that even in the presence of the differentiation media, the onset of lipid accumulation was earlier in the Ad-36-induced hASC.

Immunofluorescent analysis showed that in hASC inoculated with Ad-36, lipid accumulation was induced only in the cells supporting viral replication (Fig. 5E). As expected, the infected cell developed hypertrophy of the nucleus upon viral particle and lipid accumulation. Since no adipogenic inducers were used, lipid accumulation was not expected in other cells in the field that remained uninfected (Fig. 5E). These data show that within the infected group of cells, only those cells expressing viral proteins (Ad-36-infected) showed lipid accumulation. This evidence supports a direct effect of Ad-36 on lipid accumulation in individual hASC.

Natural Ad-36 Infection Is Associated with Greater Lipid Accumulation in hASC

hASC cultures isolated from the adipose depot of Ad-36 DNA + subjects ($n = 9$ [27%]; mean age, 28; mean BMI, 36 kg/m²) showed a significant increase in adipogenic potential compared with those from Ad-36 DNA– subjects ($n = 24$ [73%]; mean age, 29; mean BMI, 36 kg/m²) (Fig. 6). These results suggest that natural infection with Ad-36 enhances the adipogenic response of preadipocytes to adipogenic stimuli in humans.

Discussion

The exact mechanism of Ad-36-induced adiposity is unknown. The amount of viral DNA in the adipose tissue of the experimentally infected animal correlates with the mass of the adipose tissue [10] and in vitro infection of rodent preadipocytes by Ad-36 enhanced lipid accumulation [19]. These findings suggested that adiposity induced by Ad-36 in animal models involves a direct interaction of the virus with adipose tissue. Considering the strong potential of adipose tissue-resident stem cells to replicate and differentiate into adipocytes, we hypothesized that the observed in vivo adipogenic effect of the virus may involve recruitment of these cells. We tested the hypothesis in hASC, the human adipose tissue-resident multipotent stem cells [28–31].

Adipose-derived stem/stromal cells (ASC) are derived from the stromal vascular fraction cell population; however, as we previously described [32,33], they are the adherent population that has been expanded in culture and, based on their surface immunophenotype, represent <5% of

the original stromal vascular fraction cells. Clonal analysis of the culture-expanded hASC shows that up to 52% of clonal cells were capable of differentiation into two or more lineages [34,33]. Under appropriate stimuli, these cells commit to the adipocyte lineage and accumulate lipid [35]. To establish human relevance, the cells were obtained from human subjects. Admittedly, intersubject variability is a limitation for human primary cells extracted from lipoaspirate [36–39]. However, the protein expression of hASC is highly homogeneous in different donors, and expression of cell surface proteins is similar [20]. More importantly, we focused on the adipogenic response with or without infection in the same individual instead of interindividual comparison. Samples from different age and BMI groups (Table 1) exhibited similar behavior when exposed to Ad-36, minimizing the concerns about intersubject variability. Multiple subjects were used to ensure that the cellular response to Ad-36 was not an individual aberration. Also, the cells isolated from an individual are not adequate to conduct all experiments reported here without expanding by passaging several times. To avoid multiple passaging, cells from an individual were used for a limited number of experiments. Critical experiments were repeated in cells obtained from multiple individuals. It is worth noting that cells from all individuals studied showed a consistent response to Ad-36.

Demonstration of permissiveness of hASC to Ad-36 or Ad-2 infection is important to investigate the effect of these viruses on the cells. For instance, Ad-36 enhances differentiation of 3T3-L1 cells (a rodent preadipocyte cell line), but the infection is abortive [19], whereas Ad-2 requires Coxsackie virus adenovirus receptor for cell entry, and unless overexpressed in 3T3-L1 cells [40], its infectivity of the cell line is poor. A time-dependent increase in viral gene expression and DNA amount indicated successful entry and replication of Ad-36 and Ad-2 in hASC. It is noteworthy that despite a successful entry and replication in hASC, Ad-2 did not induce adipogenesis, which is consistent with earlier observations of the lack of its adipogenic ability in 3T3-L1 [19] and in animals [21]. This indicated that the adipogenic effect of an adenovirus is not due merely to the cell entry but depends on its subsequent specific interaction with host cell machinery. A complete lack of adipogenic effect of Ad-2 on hASC allowed us to focus only on the adipogenic effects of Ad-36 on commitment, differentiation, and lipid accumulation. Unlike A549 cells (human lung cancer cell line), which show characteristic cytopathic effect (CPE) and cell lysis in response to adenovirus infection, no CPE was observed in Ad-36-infected hASC even up to 2 weeks postinfection. Considering the affinity of adenoviruses for the respiratory tract, viral replication and lysis in lung cells are expected. Since increasing amounts of viral mRNA and DNA indicated successful viral replication in hASC, we believe the response of hASC to adenovirus infection may be peculiar to the cell type.

Similar to the approach of McBeath et al. [41], we used osteogenic media to determine the potency of stimulus by Ad-36 to drive commitment of hASC to adipogenic lineage. Despite the presence of osteogenic media, which induced RunX2, a marker of osteogenic induction in uninfected control cells, Ad-36 induced commitment of hASC to adipocyte lineage. It should be noted that the Ad-36-induced adipogenesis may occur through the process of initiating commitment to the adipocyte lineage by previously undifferentiated ASC and/or by accelerating differentiation of cells that are already committed to the adipocyte lineage.

During the process of preadipocyte differentiation, expressions of C/EBP- β and C/EBP- δ are followed by those of C/EBP- α and PPAR- γ and culminate in lipid accumulation [42]. C/EBP- β expression is critical for activation of PPAR- γ and other downstream proadipogenic genes [43]. PPAR- γ expression, together with that of C/EBP- α , leads to activation of several downstream genes, including glycerol 3-phosphate dehydrogenase and aP2 [42,44,45], and consequent completion of the differentiation process. Robust upregulation of expression of these key indicators of adipogenic pathway convincingly demonstrated the induction of adipogenesis by Ad-36 in hASC. In addition to a direct effect on the cellular adipogenic

program, Ad-36 may contribute to adipogenesis by modulating inflammatory cytokine response, which was not tested in this study.

LPL is a key enzyme for uptake of triglyceride-delivered fatty acids to adipose or muscular tissue [46,47]. Increased adipocyte LPL activity increases lipid storage in adipocytes and clears free fatty acid from circulation [48,49]. Activation of LPL, suggested by its extracellular translocation, shows transformation of Ad-36-infected hASC to cells with potentially functional capabilities as adipocytes.

Because of the potential significance of the findings, Ad-36-induced lipid accumulation was determined in multiple experiments and by using oil red O and BODIPY assays. The oil red O assay allowed quantification of lipid after extraction from cells, whereas BODIPY helped visualize and quantitate lipid in intact cells. Viral dose determined the amount of lipid accumulated and the number of infected cells. At a given viral dose, infection spread with time. Lipid accumulation per cell was also increased in a time-dependent manner for a given MOI. This increase may be due to accumulation of more lipid in an infected cells and/or recruitment of more cells due to the spreading infection. Collectively, this suggests a time- and virus dose-dependent increase in adiposity in vivo.

In light of the prevailing view that specific adipogenic inducers are required to commit hASC to the adipocyte lineage [20,31,36,38,50], the ability of Ad-36 to induce spontaneous lipid accumulation merits attention. In the presence of adipogenic inducers, Ad-36 accelerated lipid accumulation, which eventually occurred in the uninfected hASC. Overall, Ad-36 appears to be a robust inducer of commitment, differentiation, and lipid accumulation in hASC.

Adipose tissue-resident stem cells are an important source for recruitment to adipogenic lineage and conversion to adipocytes. Excessive or impaired adipogenesis leading to obesity or lipodystrophy, respectively, can play a major role in destabilizing insulin secretion and action, glucose and lipid metabolism, energy balance, immune functions, and reproduction [51]. This underscores the need to determine various intra- and extracellular regulators of the adipogenic process.

The experiment that screened human adipose tissue samples for natural Ad-36 infection was a blinded study conducted by coauthors in two locations. The presence of Ad-36 DNA in human adipose tissue was determined at one location, whereas the adipogenic potential of hASC obtained from the same adipose tissue samples was determined at the other location. Post hoc comparisons showed significantly greater differentiation potential of ASC from Ad-36 DNA + subjects. This is the first report of a functional difference associated with natural Ad-36 infection in humans, consistent with our similar observations in hASC experimentally infected with Ad-36.

In summary, these findings support our hypothesis that natural infection with Ad-36 commits ASC to the adipocyte lineage and increases adiposity. The findings facilitate further research to screen other human adenoviruses for their potential adipogenic effects and to screen and identify subgroups of humans with adiposity of adenoviral origin. We believe that recognizing novel etiological factors of human obesity will eventually lead to specific and more effective treatment and/or prevention strategies. In addition, these findings may have implications in other areas of stem cell metabolism, particularly in identifying regulatory controls of adult stem cell commitment. The ability of Ad-36 to induce adipogenic programming in uncommitted cells may provide additional tools to elucidate some of the as yet unknown pathways and the basis to more effectively manipulate adult stem cell commitment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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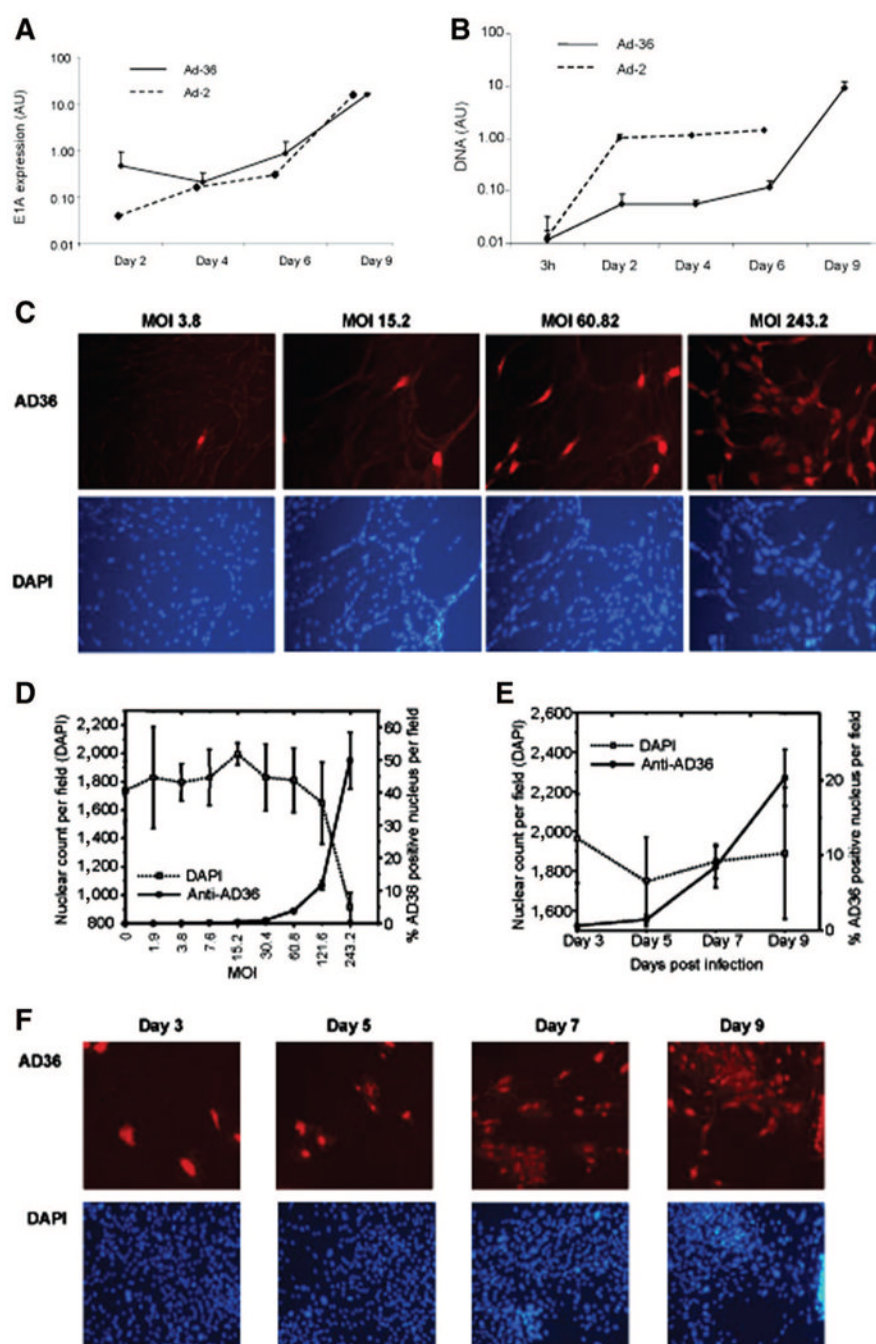


Figure 1.

Infectivity of Ad-36 and Ad-2. Human adipose-derived stem/stromal cells (hASC) were infected with Ad-36 or Ad-2, and mRNA and DNA were determined by quantitative real-time-polymerase chain reaction. Values are represented in logarithmic scale ($n = 3$; mean $\pm$ SD).

(A): Ad-36 E1A and Ad-2 E1A mRNA were expressed and increased over time. **(B):** Ad-36 and Ad-2 genomic DNA increased over time. **(C):** hASC were inoculated with increasing MOI of Ad-36. Three days postinoculation, cell nuclei were stained with DAPI stain, and the infected cells were identified by staining with Ad-36 hexon protein antibodies (magnification, $\times 10$).

(D): Change in percentage of infected cells and cell count 3 days postinoculation. **(E):** Progressive change in percentage of infected hASC (Ad-36/DAPI) and cell count

postinoculation with 3.8 MOI of Ad-36. **(F)**: Representative images of increasing number of Ad-36-infected cells on days 3, 5, 7, and 9 post-viral inoculation (3.8 MOI) (magnification, $\times 10$). Three experimental replicates were used for cells from one subject **(A–F)**. Abbreviations: AU, arbitrary units; DAPI, 4,6-diamidino-2-phenylindole; h, hours; MOI, multiplicity of infection.

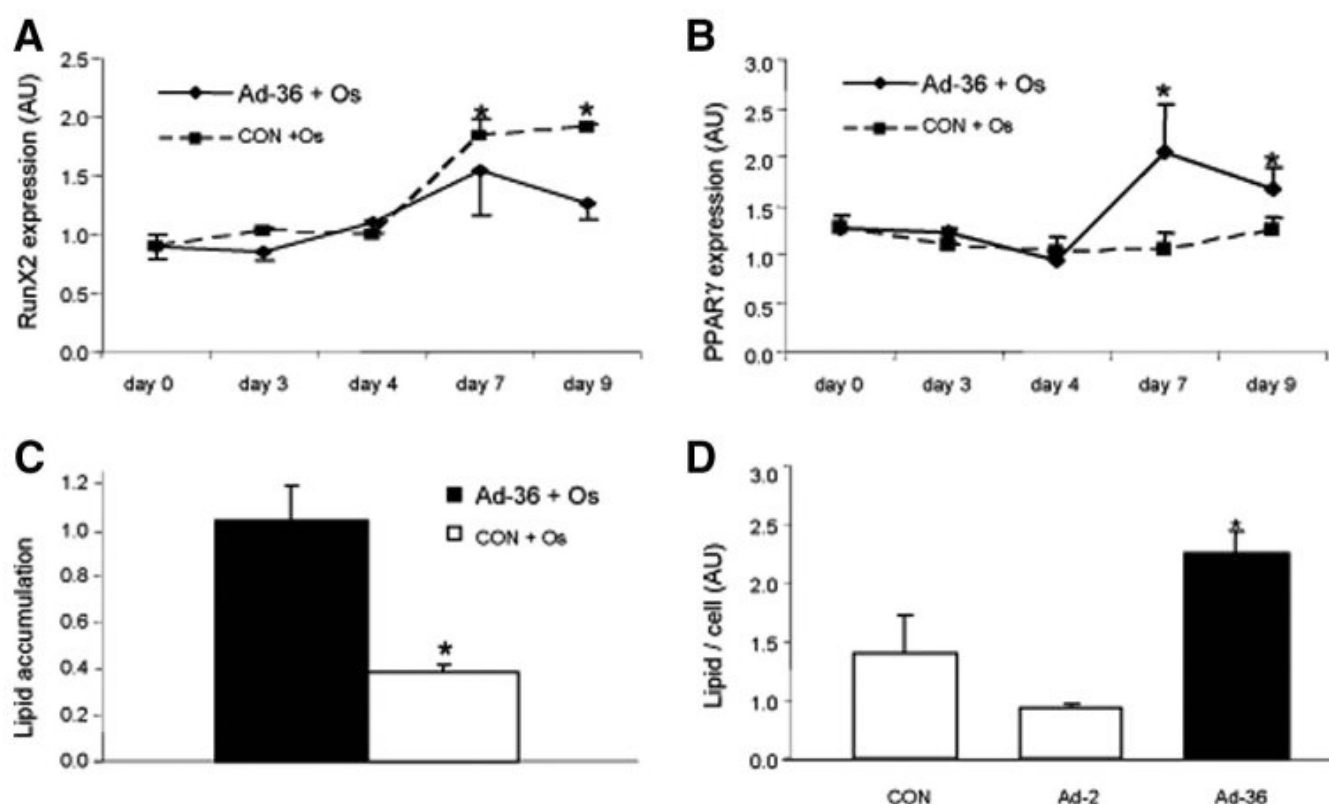


Figure 2.

Ad-36 commits human adipose-derived stem/stromal cells (hASC) to adipocyte lineage. hASC were inoculated with media (CON) or Ad-36 (Ad-36). Three days later, both groups were induced to Os for 6 days (CON + Os and Ad-36 + Os). Ad-36-inoculated group showed lower expression of RunX2 (*, $p < .05$ [day 7] and $p < .005$ [day 9]) (A) and greater peroxisome proliferator-activated receptor- γ expression (*, $p < .05$) (B), as determined by quantitative real-time polymerase chain reaction. Ad-36 inoculation, but not Ad-2 inoculation, increased lipid accumulation 9 days postinoculation, as determined by oil red O staining (C, D) (*, $p < .002$). Data are mean $\pm$ SD. Three experimental replicates were used for cells from one subject (A–C). The experiment shown in (D) was repeated in cells from two subjects. Representative data are presented. Abbreviations: AU, arbitrary units; CON, control; Os, osteogenic lineage; PPAR, peroxisome proliferator-activated receptor- γ .

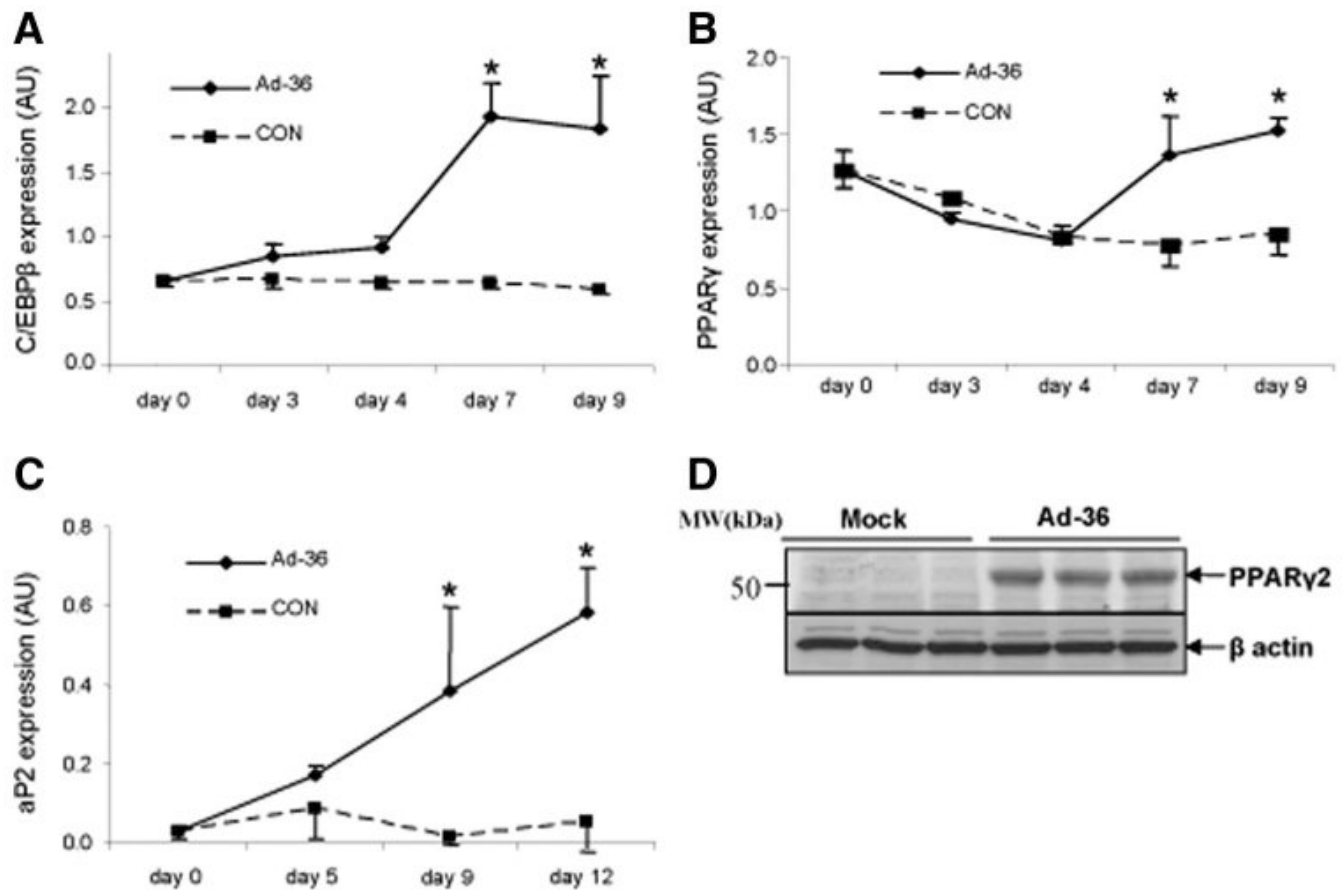


Figure 3.

Ad-36 induces adipogenic program in human adipose-derived stem/stromal cells (hASC). Cells were grown without adipogenic induction media. hASC inoculated with Ad-36 showed greater expression of C/EBP- β (**A**), PPAR- γ (**B**), and aP2 (**C**), as determined by quantitative real-time-polymerase chain reaction (*, $p < .01$). Data are mean $\pm$ SD. Western blot analysis showed greater PPAR- γ 2 protein abundance in hASC 3 days postinoculation with Ad-36. Three experimental replicates were used for cells from each subject (**A–C**). The experiments were repeated with cells from three separate subjects. Representative data are presented. (**D**): hASC were infected with Ad-36 at 3–8 MOI. Six days later, cells were harvested and Western blotted with anti-PPAR- γ 2 antibodies. Abbreviations: aP2, fatty acid-binding protein; AU, arbitrary units; CON, control; MW, molecular weight; PPAR- γ , peroxisome proliferator-activated receptor- γ .

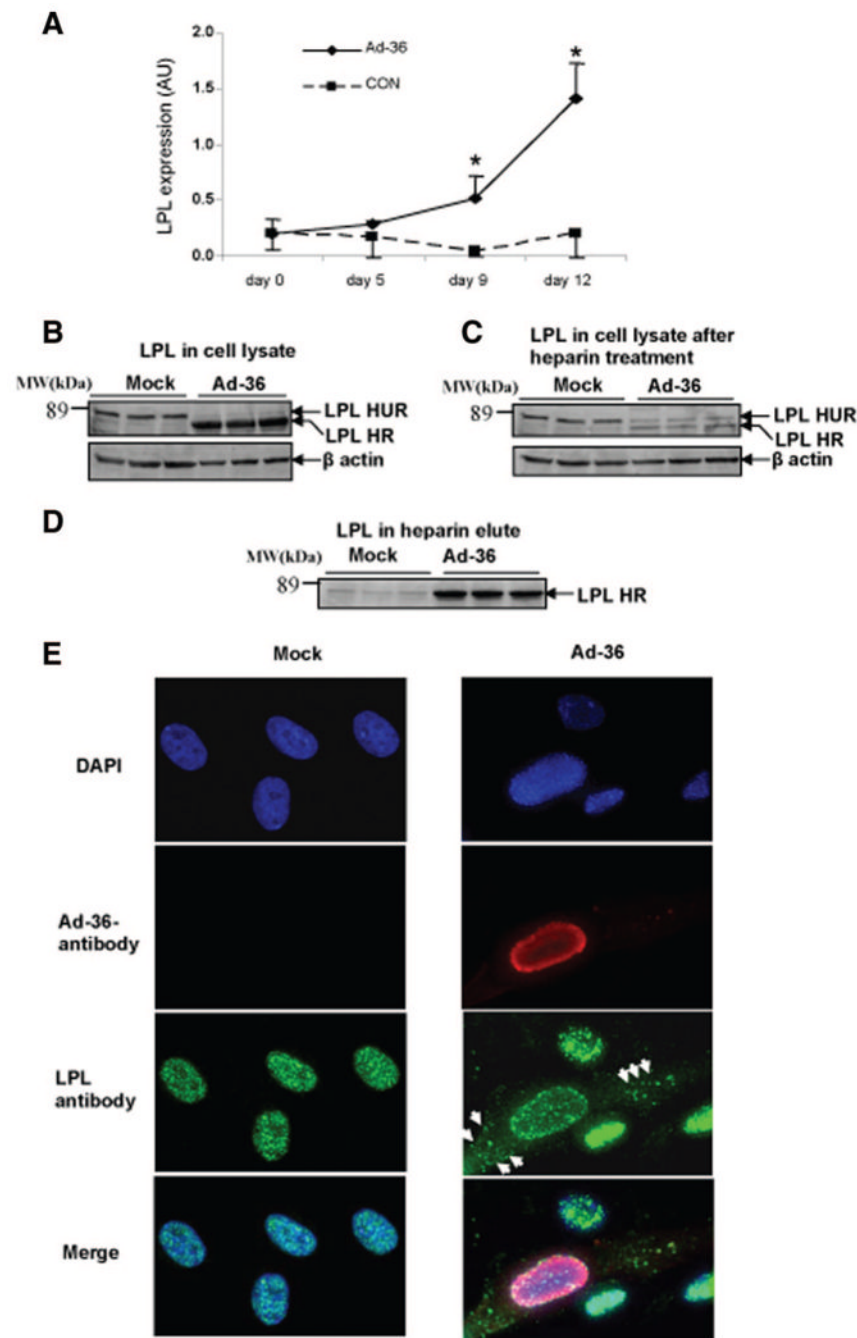


Figure 4.

Effect of Ad-36 on LPL. **(A):** Human adipose-derived stem/stromal cells (hASC) inoculated with Ad-36 showed greater expression of LPL, as determined by quantitative real-time-polymerase chain reaction (*, $p < .05$). Data are mean $\pm$ SD. Shown is a representative Western blot analysis of LPL fractions 6 days post-Ad-36 infection of hASC. **(B):** Total cell lysates were blotted with antibodies to LPL. Note that there were two distinct fractions, labeled HR and HUR. **(C):** Cells were pretreated with 200 U/ml heparin prior to Western blotting. Note the decrease of LPL HR fraction after treatment, particularly in the Ad-36 group. **(D):** Western blot of 200 U/ml heparin elute (post-heparin fraction); no HUR LPL was observed in the elute. Three experimental replicates were used for cells from each subject. The experiments were

repeated with cells from three separate subjects **(A-D)**. Representative data are presented. **(E)** Immunofluorescent images representing changes in LPL distribution during Ad-36 infection. hASC were infected with Ad-36 and 6 days later stained with antibodies to Ad-36, LPL, and DAPI. In the Ad-36 group, among the DAPI-stained nuclei, one nucleus showed the presence of Ad-36 infection and translocation of LPL away from the nucleus. Arrows indicate LPL-containing secretion granules in cytoplasm and premembrane space, whereas other nuclei in the field, as well as those in the mock-infected group, showed perinuclear localization of LPL (magnification, $\times 63$). Three experimental replicates were used for cells from one subject. Abbreviations: AU, arbitrary units; CON, control; DAPI, 4,6-diamidino-2-phenylindole; HR, heparin releasable; HUR, heparin unreleasable; LPL, lipoprotein lipase; MW, molecular weight.

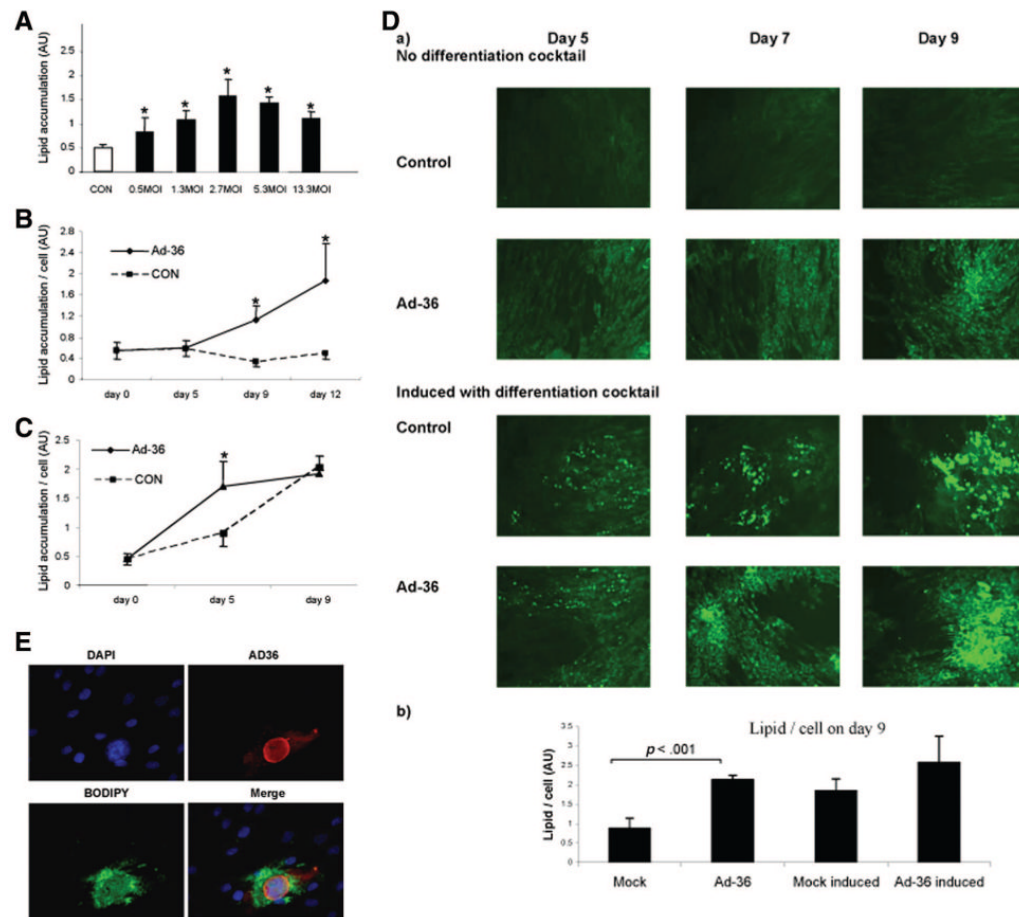


Figure 5.

Ad-36 induces as well as enhances lipid accumulation in human adipose-derived stem/stromal cells (hASC). Lipid accumulation (determined by oil red O staining) in hASC, 9 days postinoculation with Ad-36. Data are mean $\pm$ SD. **(A)**: Response to increasing MOI of Ad-36 (*, $p < .05$). **(B)**: Time course of lipid accumulation in Ad-36-infected cells (*, $p < .01$). **(C)**: In the presence of differentiation inducers, Ad-36-infected hASC showed enhanced lipid accumulation starting earlier in the time course. As expected, due to differentiation inducers, the uninfected control group eventually increased lipid accumulation (*, $p < .005$). **(Da)**: hASC were infected with Ad-36 at 3.8 MOI and fixed on days 5, 7, and 9 postinfection. Plates were stained with BODIPY FL 493/503 and DAPI (not shown). **(Db)**: Comparison of fluorescent signal of BODIPY FL between groups (data normalized to the nuclear count). **(E)**: An example of lipid accumulation specifically in Ad-36-infected hASC. A representative field shows DAPI stain for nuclei of multiple cells (blue), and Ad-36 hexon protein antibodies are seen in one infected cell (red). Lipid-specific BODIPY stain showed cellular lipid accumulation (green) only in the Ad-36-infected cell (merge) 6 days postinoculation. Three experimental replicates were used for cells from each subject. The experiments were repeated with cells from three **(B)** or two **(C)** separate subjects. Representative data are presented. Abbreviations: AU, arbitrary units; CON, control; DAPI, 4,6-diamidino-2-phenylindole; h, hours; MOI, multiplicity of infection.

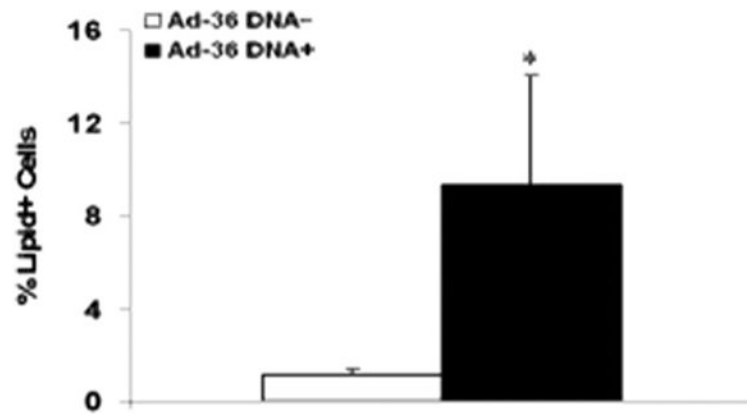


Figure 6.

Differentiation of human adipose-derived stem/stromal cells (hASC) from adipose tissue of naturally infected subjects. Differentiation of hASC cultures isolated from the adipose depot of Ad-36 DNA+ subjects ($n = 9$ [27%]) and Ad-36 DNA- subjects ($n = 24$) was monitored for 18 days, and cultures were stained with oil red O. Representative color pictures representing were taken at a magnification of $\times 40$ using a Polaroid Microcam on an inverted microscope (Eclipse TE200; Nikon). The photomicrographs served as templates for determining the percentage of preadipocyte differentiation, which was calculated as the ratio of the number of lipid-filled cells to the total cell number, multiplied by 100%. *, $p < .003$.

Table 1

Characteristics of the donors of human adipose-derived stem/stromal cells

Donor no.	Sex	Age (years)	BMI (kg/m ²)	Source of lipoaspirate
1	F	22	25.8	Abdomen, thigh
2	F	36	26.4	Abdomen, thigh
3	F	28	31.6	Thigh, axilla
4	F	51	29.4	Knees, elbows, upper arms
5	F	29	25.1	Abdomen, thigh
6	F	57	31.8	Thigh
7	M	37	32.8	Chest,
8	M	36	26.6	Abdomen, chest

Abbreviations: BMI, body mass index; F, female; M, male.