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Identification of an aberrant cell line among human adipose tissue-derived stem cell isolates

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Abstract

Adipose tissue-derived stem cells (ADSC) are isolated from the stromal vascular fraction (SVF) of adipose tissue and considered an excellent cell source for regenerative medicine. During the isolation and propagation of several human ADSC cell lines, we observed the emergence of an unusual cell line designated HADSC-6. Although initially fibroblast-like as typical ADSC are, HADSC-6 cells became homogeneously cuboid in shape, had very little cytoplasm, and formed aggregates with capsule-like boundary. Proliferation assay showed that HADSC-6 grew much faster than typical ADSC cell lines, such as HADSC-20. Immunocytochemistry showed that HADSC-6 did not express endothelial markers CD31 and vWF, and matrigel tube formation assay showed that it was unable to form endothelial-like tube structures. However, LDL uptake, a reliable endothelial marker, was positively identified. Chromosomal analysis showed that HADSC-6 cells were hypertriploid, and soft agar colony formation assay showed that they were able to proliferate and form large colonies in an anchorage-independent manner. However, tumorigenicity test showed that HADSC-6 was unable to form tumors in athymic mice. RT-PCR analysis showed that both HADSC-6 and HADSC-20 expressed VEGF-A, VEGF-B, VEGF-D, and VEGFR1 but not VEGFR2 or VEGFR3. VEGF-C, however, was expressed at a high level in HADSC-20 but undetectable in HADSC-6. In the IGF system, IGF-1 was abundantly expressed in HADSC-20 but marginally detectable in HADSC-6, and IGF-1R was abundantly expressed in HADSC-6 but not detectable in HADSC-20. In the FGF system, bFGF was abundantly expressed in HADSC-20 but marginally detectable in HADSC-6, and FGFR1 was abundantly expressed in both. Taken together, these results suggested that HADSC-6 cells were spontaneously transformed from the endothelium; therefore, they were further compared to previously published data of four naturally occurring human angiosarcoma cell lines. The results showed that the established angiosarcoma cell lines exhibit considerable variations among themselves and HADSC-6 displayed most of these variable characteristics.

Keywords

Adipose tissue-derived stem cells; Endothelial; Tumor; Angiosarcoma

1. Introduction

Stem cells, being able to self-renew and differentiate into various cell types, hold great promise for regenerative medicine and tissue engineering. Adipose tissue-derived stem cells (ADSC) are an attractive stem cell source because of the ease of access to adipose tissue and the

uncomplicated enzyme-based isolation procedures. Several head-on comparison studies have shown that ADSC and bone marrow stem cells are similar in cell-surface expression profile, immunophenotype, differentiation potential, and therapeutic efficacy (Strem et al., 2005; Gimble et al., 2007; Valina et al., 2007).

ADSC are isolated from the stromal vascular fraction (SVF) of adipose tissue. Freshly isolated, they are a heterogeneous mixture of endothelial cells, smooth muscle cells, pericytes, fibroblasts, mast cells, and pre-adipocytes (Pettersson et al., 1984). Culturing of these cells under standard condition eventually (within the first few passages) results in the appearance of a relatively homogenous population of mesodermal or mesenchymal cells (Zuk et al., 2001). However, the exact cellular identity of this seemingly homogeneous population remains controversial. For example, Planat-Benard et al. (2004) showed that at passage 0 (cells cultured for 72 h) the great majority of the population (90–99%) of human or murine ADSC was composed of undifferentiated cells expressing CD34 but not CD31. On the other hand, Mitchell et al. (2006) showed that human ADSC at passage 0 are $59 \pm 25\%$ positive for CD34 and $24 \pm 17\%$ positive for CD31. Furthermore, the latter study showed that CD31 expression remained essentially unchanged at passages 3 and 4, and all other endothelial cell-associated markers (CD144, vascular endothelial growth factor receptor 2, and von Willebrand factor) were also stably maintained. However, despite these disagreements, three recent review articles all placed ADSC in the CD31-negative and thus nonendothelial category (Gimble et al., 2007; Helder et al., 2007; Schaffler and Buchler, 2007).

The vascular endothelium distributes widely throughout the body, yet it rarely gives rise to malignant tumors in humans. Such tumors are called angiosarcoma or hemangiosarcoma and account for only 2% of soft-tissue sarcomas (Fata et al., 1999). Naturally occurring human angiosarcoma cell lines, that is, without cell fusion or gene transfection, are even rarer—only four have been reported so far. The first human angiosarcoma cell line, EVC304, is a spontaneously transformed cell line fortuitously identified among endothelial cell isolates obtained from 600 umbilical cords (Takahashi et al., 1990). The other three are isolated from angiosarcomas of the liver (Hoover et al., 1993) and scalp (Masuzawa et al., 1999; Krump-Konvalinkova et al., 2003).

During the past 3 years, we have isolated a total of 26 human ADSC cell lines. Routine inspections during their isolation and propagation led us to become suspicious of a cell line designated HADSC-6. Although initially similar to other ADSC cell lines in morphology and growth rate, HADSC-6 gradually transitioned from the typical fibroblast-like to cuboid shape and seemingly without cytoplasm. This endothelial-like morphology, its vascular origin, and its aggressive growth behavior led us to suspect the emergence of a naturally occurring human endothelial tumor cell line. Therefore, HADSC-6 was further compared to four established human angiosarcoma cell lines. Interestingly, these established angiosarcoma cell lines exhibit considerable variations among themselves and HADSC-6 displayed most of these variable characteristics.

2. Materials and methods

2.1. ADSC isolation

Adipose tissue samples were obtained from patients during routine abdominoplasty or penile prosthesis implantation following informed patient consent and according to the guidelines set by our institution's Committee on Human Research. The tissue samples were processed for ADSC isolation as described previously (Ning et al., 2006). Briefly, the tissue was rinsed with phosphate-buffered saline (PBS) containing 1% penicillin and streptomycin, minced into small pieces, and then incubated in a solution containing 0.075% collagenase type IA (Sigma-Aldrich, St. Louis, MO) for 1 h at 37 °C with vigorous shaking. The top lipid layer was removed

and the remaining liquid portion was centrifuged at 220g for 10 min at room temperature. The pellet was treated with 160 mM NH_4Cl for 10 min to lyse red blood cells. The remaining cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), filtered through a 40- μm cell strainer (BD Biosciences, Bedford, MA), and plated at a density of 1×10^6 cells in a 10-cm dish. After reaching 80% confluence, the cells were harvested and stored in liquid nitrogen at a density of 5×10^5 cells per ml of freezing media (DMEM, 20% FBS, and 10% DMSO). Cells were thawed and re-cultured as needed.

2.2. Cell culture

Human umbilical vein endothelial cell strain, HUVEC, was purchased from Lonza Biologics Inc. (Portsmouth, NH) and cultured in EGM2 medium (Lonza Biologics Inc.). Human prostate cancer cell line PC-3 was purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acid, 1% PSF (10,000 units/ml penicillin, 10,000 mcg/ml streptomycin SO_4 , and 0.025 $\mu\text{g}/\text{ml}$ fungizone), and 110 $\mu\text{g}/\text{ml}$ sodium pyruvate. All human ADSC cell lines, including HADSC-6 and HADSC-20, were cultured in the same medium as PC-3. Culture incubator was set at 37 °C with 5% CO_2 .

2.3. Immunocytochemistry and fluorescence microscopy

Cells were seeded onto a coverslip inside each well of a 6-well plate at 40–60% confluence in DMEM. The next day, the cells were rinsed with PBS and fixed with ice-cold methanol for 5 min. The cells were rinsed with PBS again and permeabilized with 0.05% triton X-100 for 8 min. After another PBS rinse, the cells were incubated with 5% horse serum for 1 h and then with anti-CD31 antibody CD31 (sc-1506, Santa Cruz Biotechnology, Santa Cruz, CA), anti-vWF antibody (ab6494-100, Abcam Inc., Cambridge, MA), or anti- β -actin antibody (A5441, Sigma-Aldrich, St. Louis, MO) for 1 h. After three rinses with PBS, the cells were incubated with FITC- or Texas red-conjugated goat anti-rabbit IgG or rabbit anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. After three rinses with PBS, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI, for nuclear staining, 1 $\mu\text{g}/\text{ml}$, Sigma-Aldrich, St. Louis, MO) for 5 min. The stained cells were examined with the Nikon Eclipse E600 fluorescence microscope and the images recorded with the Retiga 1300 Q-imaging camera.

2.4. Proliferation assay

Cell proliferation assays were performed with the CellTiter-96 kit of Promega Inc. (Madison, WI). Briefly, HADSC-6 and HADSC-20 cells were seeded into 96-well plates at 2000 cells per well and incubated at 37 °C. At 0, 24, 48, 72, 96, and 120 h, an aliquot of 20 μl of CellTiter-96 reagent was added to each well. After 2 h of further incubation at 37 °C, the plate was scanned in a plate reader (Molecular Devices Corp., Sunnyvale, CA) at 490-nm absorbance. All assays were performed in triplicate in each experiment and all data presented in Section 3 are the average of three independent experiments.

2.5. Soft agar colony formation assay

Soft agar colony formation assay was used to assess the ability of cells to grow anchorage independently. We conducted this assay according to previous angiosarcoma studies (LaMontagne et al., 2000; Thamm et al., 2006). Briefly, a bottom layer of agar was formed inside a 60-mm culture dish by pouring 4 ml of 0.6% low-melting agarose (Sigma-Aldrich, St. Louis, MO) diluted in DMEM with 10% FBS. A top layer of agar was then formed by pouring 2 ml of 0.3% low-melting agarose containing 5×10^4 cells of HADSC-6 or HADSC-20 in DMEM with 10% FBS. The dishes were incubated at 37 °C in a humidified incubator with 5%

CO₂ and inspected daily. At day 14, the cells were stained with 0.5 ml of 0.005% crystal violet for 1 h, examined under a microscope, and photographed.

2.6. Low-density lipoprotein (LDL) uptake

Cells were seeded into 6-well plates at of 5×10^4 cells per well in DMEM or EGM2 medium and incubated at 37 °C. The next day, 10 µg/ml of acetylated low-density lipoprotein DiI complex (DiI AcLDL, Invitrogen Corporation, Carlsbad, CA) was added to the culture medium. The next day, after the removal of the medium, the cells were washed three times with PBS, examined by phase-contrast and fluorescence microscopy, and photographed.

2.7. Matrigel-based capillary-like tube formation assay

Cells (HADSC-6 and HADSC-20) were cultured in EGM2 for 6 days prior to the assay. HUVEC was always maintained in EGM2. The assay was initiated by coating a 4-well CultureSlide (BD Biosciences, San Jose, CA) with 150 µl of growth factor-reduced matrigel (BD Biosciences) per well. Approximately 5×10^4 cells of HADSC-6, HADSC-20, or HUVEC in 500 µl of EGM2 were then seeded into each well and incubated at 37 °C. Sixteen hours later, development of capillary-like networks was examined by phase-contrast microscopy and photographed.

2.8. Tumorigenicity test

The experimental protocol and animal care were approved by our institutional committee on animal research. Male homozygous (nu/nu) athymic nude mice, 4 weeks of age, were purchased from Simonsen Laboratories (Gilroy, CA). Each mouse received subcutaneous inoculation on the right flank of approximately 1×10^6 cells of PC-3 (two mice), HADSC-6 (three mice), or HADSC-20 (three mice) in 300 µl of PBS. The mice were observed for tumor formation weekly and photographed at the end of week 4.

2.9. Chromosomal analysis

Cells were seeded at 2×10^5 per 60-mm dish overnight. After treatment with 0.1 µg/ml of demecolcine (Sigma-Aldrich, St. Louis, MO) for 20 min, the cells were harvested, centrifuged, and resuspended in 200 µl of DMEM. The resuspended cells were then reconstituted in 10 ml of 75 mM KCl and incubated for 15 min in a 37 °C water bath. After the addition of three drops of freshly prepared fixative (3:1 methanol:acetic acid, glacial), the cells were centrifuged at 250g for 10 min and resuspended in 10 ml of fixative for 5 min. After repeating this fixation process two more times, the cells were centrifuged at 250g for 10 min and resuspended in 1 ml of fixative. The fixed cells were stored at 4 °C for later use or immediately used for chromosome spread, which was done by applying two drops of cell suspension to a pre-chilled (~4 °C) glass slide. Afterwards, the slides were kept at room temperature for 2 days, baked at 60 °C for 18 h, and stained with Giemsa (Invitrogen, Carlsbad, CA) for 10 min. After rinsing and drying, the slides were examined under a microscope in bright field; chromosome spreads with no or minimal overlaps were then photographed.

2.10. RT-PCR analysis

Cells were homogenized in Tri-Reagent RNA extraction solution (Molecular Research Center, Cincinnati, OH). The extracted RNAs were further treated with DNase I to remove traces of contaminating DNA. Quantity and integrity of RNAs were examined by a spectrophotometer and agarose gel electrophoresis, respectively. The RNAs were reverse transcribed into a “library” of complementary DNAs (cDNAs) using SuperScript reverse transcriptase and its accompanying reagents (Invitrogen, Carlsbad, CA). Briefly, 2.5 µg of RNA was annealed to 0.4 µg of oligo-dT primer in a 12-µl volume. A volume of 4 µl of 5 × buffer, 2 µl of 0.1 M DTT, 1 µl of 10 mM dNTP, and 1 µl of reverse transcriptase were then added to bring the final

reaction volume to 20 μ l. After 1 h of incubation at 42 °C, the mixture was incubated at 70 °C for 10 min to inactivate the reverse transcriptase. A volume of 80 μ l of TE buffer was then added to make a 5 $\times$ diluted library. A portion of this library was further diluted to various concentrations (up to 100 $\times$ dilution). One microliter of each dilution was then used in a 10- μ l polymerase chain reaction (PCR) to identify the optimal input within the linear amplification range. In addition to the 1- μ l diluted library, the PCR mixture consisted of 10 ng of each of a primer pair (Table 1) and reagents supplied with the Taq polymerase (Invitrogen). PCR was performed in DNA Engine (MJ Research, Inc., Watertown, MA) under calculated temperature control. The cycling program was set for 35 cycles of 94 °C, 5 s; 55 °C, 5 s; 72 °C, 10 s, followed by one cycle of 72 °C, 5 min. The PCR products were electrophoresed in 1.5% agarose gels in the presence of ethidium bromide, visualized by UV fluorescence, and recorded by a digital camera connected to a computer.

3. Results

3.1. Cell morphology and growth characteristics

Consistent with previous reports, our human ADSC cell lines were mostly fibroblast like at passage 0 (initial adherence to culture dishes). However, during subsequent cell passages, it became increasingly apparent that a distinctive cell population was gradually dominating over the fibroblast-like cell population within the HADSC-6 cell line (Fig. 1A). This unusual cell population was cuboid in shape, had very little cytoplasm, and formed aggregates with a capsule-like boundary (Fig. 1B). At passage 6, this cell population appeared to have completely overtaken the fibroblast-like cells and thus become homogeneous (Fig. 1A). Proliferation assay showed that HADSC-6 grew much more rapidly than typical HADSC cell lines, such as HADSC-20 (Fig. 2).

3.2. Endothelial characteristics

Because HADSC-6 was originated from the stromal vascular structure of adipose tissue and its morphology was suggestive of an endothelial origin, we tested whether it possessed endothelial characteristics. Immunocytochemistry showed that it did not express endothelial markers CD31 and vWF (Fig. 3). Matrigel tube formation assay showed that it was unable to form endothelial-like tube structures (Fig. 4). However, LDL uptake, which has been shown to be the most reliable in vitro endothelial characteristic (Craig et al., 1998), was positively identified (Fig. 5). The specificity of the LDL-uptake test was supported by the positive results with HUVEC endothelial cells and the negative results with HADSC-20 and PC-3 prostate cancer cells.

3.3. Tumorigenic characteristics

HADSC-6 cells' rapid growth rate (Fig. 2) and apparent lack of contact inhibition (cells pile up in extended culture, data not shown) raised the possibility that they were tumorigenically transformed, and this was tested by three standard methods. Firstly, chromosomal analysis showed that HADSC-6 cells were hypertriploid, having chromosomal numbers ranging from 75 to 79 (Fig. 6). Secondly, soft agar colony formation assay showed that HADSC-6 cells were able to proliferate and form large colonies in an anchorage-independent manner, as in contrast to HADSC-20 cells, which did not form colonies (Fig. 7). Thirdly, tumorigenicity test showed that HADSC-6 was unable to form tumors in athymic mice, as in contrast to PC-3 cancer cells, which did form tumors (Fig. 8).

3.4. Expression of vascular-related genes

Despite lacking tumorigenicity, HADSC-6 cells' aneuploidy, ability of LDL uptake, and ability of anchorage-independent growth were strong indications for them being angiosarcoma-

like cells. To gain further support, their expression of vascular-related genes was analyzed by RT-PCR, in which HADSC-20 was used as non-angiosarcoma control. The results show that (1) both cell lines expressed VEGF-A, VEGF-B, VEGF-D, and VEGFR1 but not VEGFR2 or VEGFR3, (2) VEGF-C was expressed at a high level in HADSC-20 but undetectable in HADSC-6, (3) IGF-1 was abundantly expressed in HADSC-20 but marginally detectable in HADSC-6, (4) IGF-1R was abundantly expressed in HADSC-6 but not detectable in HADSC-20, (5) bFGF was abundantly expressed in HADSC-20 but marginally detectable in HADSC-6, and (6) FGFR1 was abundantly expressed in both.

To ascertain that the expression profile of HADSC-20 was representative of HADSC cell lines in general, we tested three additional HADSC cell lines (HADSC-1, HADSC-18, and HADSC-21) and obtained identical results (data not shown). Therefore, it can be concluded that (1) HADSC cells isolated from different individuals exhibited identical vascular-related gene expression profile and (2) HADSC-6 differed from normal HADSC cell lines in this regard.

3.5. Comparison to established human angiosarcoma cell lines

The above experimental results suggested that HADSC-6 cells were spontaneously transformed from the endothelium; therefore, they were further compared to previously published data of four naturally occurring human angiosarcoma cell lines. The results (Table 2) showed that the established angiosarcoma cell lines exhibit considerable variations among themselves and HADSC-6 displayed most of these variable characteristics (see Discussion for details).

4. Discussion

HADSC-6 cell line was established fortuitously from the adipose tissue of a patient who showed no sign of harboring tumors. Morphologically the original HADSC-6 cell isolate was similar to our other human ADSC isolates. However, as cell passages were carried on, it became increasingly clear that an unusual cell line was emerging. As shown in Fig. 1, at passage 3, a cell population whose morphology was drastically different from typical ADSC was becoming predominant, and at passage 6, it has become homogeneous. Because HADSC-6 was derived from the vascular fraction of adipose tissue and had an endothelial appearance, it was presumed to originate from one or a few tumor cells of the vascular endothelium in the patient's adipose tissue sample. Alternatively, it could have arisen through spontaneous transformation in culture from normal endothelial cells present in the SVF. Also possible is that it might have resulted from the fusion of normal endothelial cell and a normal ADSC. Whether it possesses multipotent differentiation ability has not been tested, as we focused on the characterization of its angiosarcoma potential.

To verify the endothelial tumor identity of HADSC-6, we conducted a series of experiments aiming at obtaining evidence that these cells possess both endothelial and tumor properties. The results were then compared to published data of four naturally occurring angiosarcoma cell lines (Table 2). With regard to endothelial properties, HADSC-6 cells did not express endothelial markers CD31, CD34, and vWF; this is also found in the majority (three out of four) of the established angiosarcoma cell lines. HADSC-6 and all four established angiosarcoma cell lines do express two important vascular markers, VEGF-A and VEGFR-1, but expression of these two genes was also observed in HADSC-20. The strongest evidence for HADSC-6's endothelial identity was perhaps the ability of DiI-Ac-LDL uptake, which has been shown to be the most reliable endothelial characteristic (Craig et al., 1998) and which was positively identified in three of the four established angiosarcoma cell lines but not in HADSC-20.

With regard to tumor properties, HADSC-6 and two of the established angiosarcoma cell lines had abnormal chromosomal make-up. On the other hand, HADSC-6 is the only cell line that displayed anchorage-independent growth (Table 2). More precisely, HADSC-6 not only grew in soft agar but also proliferated. This proliferative ability differentiated HADSC-6 from HADSC-20, which also grew (survived) in soft agar but did not proliferate—a property of ADSC, as has been reported previously (Lin et al., 2005). Previous studies (Baserga, 1997) have also shown a strong correlation between anchorage independence and expression of IGF1R, a property of HADSC-6 but not HADSC-20 (Fig. 9). Finally, while two of the established angiosarcoma cell lines are tumorigenic, HADSC-6 and the other two established angiosarcoma cell lines were not (Table 2). This lack of tumorigenicity in spite of having abnormal chromosomal make-up has been demonstrated previously (Boukamp et al., 1997). Thus, HADSC-6 is similar to the established angiosarcoma cell lines in both endothelial and tumor properties. However, while three of the four established angiosarcoma cell lines were isolated from angiosarcoma tumors, HADSC-6 and one of the established angiosarcoma cell lines (ECV304) were isolated from tissue samples with no known angiosarcoma history. Therefore, whether HADSC-6 is an angiosarcoma cell line cannot be definitively proven.

Because HADSC-6 was originally isolated to be a stem, not tumor, cell line, its subsequent characterization as a tumor cell line was carried out with HADSC-20 that served as a non-tumor cell control. The results showed that HADSC-6 had higher proliferation rate, was able to proliferate in soft agar, and lacked the ability to form endothelial-like structures. At the gene expression level, the two cell lines were similar but differed significantly in the expression of VEGF-C, IGF-1, IGF-1R, and bFGF. To ascertain that these differences were consistent, we tested three additional HADSC cell lines (HADSC-1, HADSC-18, and HADSC-21) and obtained identical results (data not shown). Therefore, it can be concluded that (1) HADSC cells isolated from different individuals exhibited identical vascular-related gene expression profile and (2) HADSC-6 differed from normal HADSC cell lines in this regard.

The clinical applicability of ADSC has been tested in several trials with favorable outcomes (Lin et al., 2008). However, the identification of HADSC-6 as a possible angiosarcoma cell line introduces a negative factor into the planning for future therapies with ADSC. This problem is especially acute if SVF cells are to be used because our data show that cells with tumorigenic potential became evident only after a few passages. Our data also show that homogeneously grown HADSC-6 and HADSC-20 (as well as other normal HADSC lines) had different vascular-related gene expression profiles. Thus, it may be possible to screen SVF cell isolates for “tumorigenic markers” such as negative (or low-level) expression of VEGF-C, IGF-I, and bFGF and positive expression of IGF-1R (Fig. 9). However, because we did not have a chance to test the SVF cell isolates, we are not sure whether such screening would be sensitive enough to identify the existence of a small number of tumorigenically transformed cells. Thus, it may be necessary to assemble a human SVF cell bank and screen its deposited cell samples for the aforementioned tumorigenic markers. The screening results would provide a basis for the risk-verses-benefit assessment of the clinical applicability of ADSC.

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Fig. 1. Cell morphology. (A) HADSC-6 and HADSC-20 cells at passage 3 (P3) and passage 6 (P6) are shown. Note the presence of both fibroblast-like and cuboid-shaped cells in HADSC-6/P3, while only the cuboid-shaped cells were present in HADSC6/P6. Original magnification was 40 $\times$. (B) HADSC-6 and HADSC-20 cells at passage 6 were stained for β -actin (red) and the nucleus (blue). Original magnification was 200 $\times$.

Fig. 2.

Cell growth rate. HADSC-6 and HADSC-20 cells at passage 6 were assayed for growth rate with the CellTiter-96 kit (Promega Inc., Madison, WI). The assays were performed in triplicate in each experiment and the data presented are the average with standard deviation of three independent experiments.

Fig. 3. Expression of endothelial markers. HADSC-6 and HADSC-20 cells at passage 6 were examined for expression of endothelial markers CD31 and vWF. HUVEC and PC-3 cells served as positive and negative controls, respectively. Green color indicates expression of CD31 or vWF; blue color indicates cell nuclei. Original magnification was 200 $\times$.

Fig. 4. Ability to form capillary-like tubes. HADSC-6 and HADSC-20 cells at passage 6 were examined for ability to form capillary-like tubes on matrigel-coated surface. HUVEC cells served as positive control.

Fig. 5. Ability of LDL uptake. HADSC-6 and HADSC-20 cells at passage 6 were examined for ability of LDL uptake. HUVEC and PC-3 cells served as positive and negative controls, respectively. Red color indicates the presence of LDL, which was in a conjugated form with the red fluorescence dye DiI. Original magnification was 40 $\times$.

Fig. 6. Chromosomal analysis. HADSC-6 and HADSC-20 cells at passage 6 were examined for chromosomal make-up. Ten metaphases of each cell line were examined. The average chromosomal count was 77 (75–79) and 46 (45–47) for HADSC-6 and HADSC-20, respectively. A representative chromosomal spread of each cell line is shown.

Fig. 7. Ability to grow anchorage independently. HADSC-6 and HADSC-20 cells at passage 6 were examined for ability to grow anchorage independently. Cells were suspended in soft agar, incubated for 2 weeks, and stained with crystal violet.

Fig. 8.

Tumorigenicity test. HADSC-6 cells at passage 6 were examined for tumorigenicity. PC-3 cells served as positive controls. Each mouse received subcutaneous inoculation on the right flank of approximately 1×10^6 cells. The mice were observed for tumor formation weekly and photographed at the end of week 4.

Fig. 9.

Expression of vascular-related genes. HADSC-6 and HADSC-20 cells at passage 6 were examined for expression of vascular-related genes. Cellular RNAs were reverse transcribed into cDNAs, which were then run in PCR with gene-specific primers (Table 1). The PCR products were electrophoresed in 1.5% agarose gels in the presence of ethidium bromide, visualized by UV fluorescence, and photographed. Lane actin indicates expression levels of β -actin gene, which served as controls for equal RNA input. Lane M is 100-bp size marker, the 600-bp band of which is indicated. Lanes under VEGF and VEGFR are self-explanatory. IGF is IGF-1, IGFR is IGF-1R, FGF is bFGF, and FR is FGFR.

Table 1

Primers for RT-PCR analysis

Gene	Sequence	Product size, bp
VEGF-A	Forward: 5'-CTCCACCATGCCAAGTGGT	515
	Reverse: 5'-GGCTTGTCACATCTGCAAGT	
VEGF-B	Forward: 5'-GAGGAAAGTGGTGCATGGA	444
	Reverse: 5'-GTGTCTGGGTTGAGCTCTAA	
VEGF-C	Forward: 5'-TGAAATTACAGTGCCTCTCTC	534
	Reverse: 5'-TCTGTACATTACAGGCACAT	
VEGF-D	Forward: 5'-GCCAGTGAGCTGGGGAAGA	309
	Reverse: 5'-AGGACAGAGTTTCTTGAATG	
VEGFR1	Forward: 5'-ATGCTGGATTGCTGGCACA	323
	Reverse: 5'-TCAAACATGGAGGTGGCATT	
VEGFR2	Forward: 5'-GCCTTTGGCCAAGTGATTGA	479
	Reverse: 5'-TCCAAGGTCAGGAAGTCCTT	
VEGFR3	Forward: 5'-ATCACCGAGGCTGACGCTGA	261
	Reverse: 5'-GCCTTCTTGTCTATGCCTG	
IGF-1	Forward: 5'-TGCTTCCGGAGCTGTGATCT	528
	Reverse: 5'-CAGAGAGGAATTTAGTGCAAC	
IGF-1R	Forward: 5'-AAGTTCGTCCACAGAGACCT	471
	Reverse: 5'-CTTGTTCTCCTCGCTGTAGT	
bFGF	Forward: 5'-GCTTCTTCCTGCGCATCCA	355
	Reverse: 5'-CAGCTCTTAGCAGACATTGG	
FGFR1	Forward: 5'-GTGGCTGTGAAGATGTTGAA	344
	Reverse: 5'-GCCAGGTCTCGGTGTATGCA	
β -Actin	Forward: 5'-TCTACAATGAGCTGCGTGTG	368
	Reverse: 5'-AATGTCACGCACGATTTCCC	

Table 2
Comparison of HADSC-6 and HADSC-20 to established human angiosarcoma cell lines

Name of the cell line	ECV304	HAEND	ISO-HAS	AS-M	HADSC-6	HADSC-20
Reference	Takahashi et al. (1990)	Hoover et al. (1993)	Masuzawa et al. (1999)	Knump-Konvalinkova et al. (2003)	Present study	Present study
Tissue origin	Umbilical cord endothelium	Hepatic angiosarcoma	Cutaneous (scalp) angiosarcoma	Cutaneous (scalp) angiosarcoma	Subcutaneous fat	Subcutaneous fat
CD31 expression	-	-	+	+	-	-
CD34 expression	-	-	-	ND	-	-
vWF expression	-	-	+	+	-	-
VEGFR-1 expression	ND	+	+	ND	+	+
VEGFR-2 expression	ND	-	+	+	-	-
Dil-Ac-LDL uptake	-	-	+	+	+	-
Anchorage-independency	-	ND	ND	ND	+	Weakly +
Endothelial tube formation	ND	ND	+	ND	-	Weakly +
Karyotype	High trisomic	ND	ND	Hypodiploid	Hypertriploid	Diploid
Tumorigenicity	+	-	+	-	-	-