Identification and Characterization of Adult Stem Cells From Human Orbital Adipose Tissue

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Purpose: To identify pluripotential stem cells from human orbital adipose depots.

Methods: Pluripotential adipose-derived stem cells were isolated from human orbital adipose during routine blepharoplasty surgery. Fresh adipose tissue was separated in nasal fat and central (preaponeurotic) fat. Individual adipose depots were minced, enzymatically digested, and plated on plastic culture dishes. Adherent populations of cells were expanded in culture, characterized by flow cytometry, and assayed for the potential to differentiate in different cell lineages.

Results: Orbital adipose-derived cells from the nasal and central adipose depots showed the potential to differentiate into the adipocyte, smooth muscle, and neuronal/glial lineages and expressed a CD marker protein profile consistent with that observed for adipose-derived stem cells from other adipose depots.

Conclusions: A population of adherent cells capable of pluripotential differentiation in vitro exists within adult human orbital adipose tissue. These cells are similar to those described in other adipose depots and will help facilitate understanding of orbital diseases and may provide a novel tissue source for the development of ocular regenerative medicine therapies.

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Stem cells are a unique population of cells that have garnered widespread lay and scientific attention. The common theme among all stem cells is the ability to self-replicate and the ability to differentiate in multiple cell fates.[1] Stem cells can be broadly classified in totipotential and pluripotential subtypes. Totipotent cells such as the controversial embryonic-derived stem cells have the ability to differentiate in all cell types and can give rise to a whole organism. In contrast, adult tissue-derived stem cells are pluripotential, that is, they have the ability to differentiate in many but not all cell types.

The reason for the existence of pluripotential stem cells in many adult tissues is unknown but is postulated to play a role in cellular renewal after trauma, disease, or aging. A prime example of this regenerative role is the presence of hematopoietic stem cells (HSCs) that are present in the bone marrow. HSCs play a regenerative role by continuously renewing circulating red and white blood cells. When bone marrow stem cells (MSCs) are transplanted in animals depleted of red and white cells, there is complete reconstitution of the hematopoietic and lymphocytic system.[2] Further study of bone marrow cells identified another population of stem cells known as mesenchymal or MSCs. MSCs have been shown in vitro to differentiate in mesenchymal tissues such as muscle, bone, cartilage, adipose, and stroma.[3–5] In vitro MSC isolation studies have shown that these cells are a rare population of cells in the bone marrow, accounting for about 0.001% to 0.01% of nucleated cells and ~10-fold less abundant than HSCs.[6]

Isolation of HSCs and MSCs requires bone marrow aspiration, which can be associated with low numbers of cells isolated, pain, and morbidity. Along this line, adipose tissue throughout the body is primarily derived from embryonic mesenchyme and contains easily harvested stroma. Surgeons routinely perform body adipose liposuction, and volumes of several hundred milliliters can be obtained during a single procedure. Using this rationale, Zuk et al.[7] analyzed adipocyte stromal cells obtained from human lipoaspirates and assayed for the presence of stem cells. They found a population of adherent cells similar to marrow-derived MSCs that showed similar cell surface marker expression. Furthermore, they showed that these cells were capable of differentiating toward muscle, bone, cartilage, and adipose cell fates.

We directed our attention to periocular adipose tissue as a potential source of adult regenerative cells. Adipose tissue throughout the body is generally derived from mesodermal tissues, and, hence, it is plausible that cells similar to MSCs constitute a significant fraction of the nonadipocytic cells obtained from these tissues. Cells of both ectodermal and mesodermal origin contribute to the development of the ocular system. Mesoderm contributes less to ocular development and is limited to the fibers of the extraocular muscle, endothelial lining of vessels, temporal sclera, and the vitreous. In contrast, most ocular tissues are derived from ectoderm and, in particular, neural crest-derived cells. Orbital adipocytes and the adipocytes surrounding the paratracheal region are unique in the body in that they are derived solely from neural crest cells and not the mesoderm.[8]

On the basis of the embryologic properties of orbital adipocytes, we postulated that orbital adipose might contain a similar but potentially unique population of regenerative cells. Using orbital fat obtained during routine blepharoplasty, we identified a population of orbital adipose-derived stem cells (ASCs) that have the ability to undergo differentiation toward multiple cell phenotypes. Furthermore, differences observed in ASCs obtained from adipose of different embryonic origin support the hypothesis that these cells may be a relevant source of regenerative cells for ocular-based cell therapy.

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METHODS

Source of Adipose-derived Stem Cells (ASCs). All human adipose tissue used in this study was obtained from the central (preaponeurotic) and nasal fat depots that are routinely discarded after removal during upper eyelid blepharoplasty. Nasal fat was distinguished from central fat by its location and relative pale color. If there was any cross-contamination or mixing of samples in the surgical field, the tissue was not processed further. All patients provided full informed consent, and this study was approved by the University of California, San Diego Institutional Human Research Protections Program and conforms to the principles outlined in the Declaration of Helsinki.

Isolation of Adipose-derived Regenerative Cells and ASCs. Freshly obtained adipose tissue was homogenized with scissors and scalpel blade and washed with sodium chloride 0.9%. The cumulative nucleated nonadipocyte cell population, or adipose-derived regenerative cells (ADRCs), were proteolytically released from adipose matrix by collagenase digestion. The buoyant layer of mature adipocytes was removed by aspiration, and the remaining cells were then filtered first through a 100-μm pore size filter and then through a 40-μm pore size filter. The remaining cells were centrifuged for 5 minutes at 400 *g*. Resuspended cell number and viability were determined using a fluorescent live/dead cell assay. Cells were plated at between 50,000 and 100,000 cells/cm^2 initially in 6-well culture plates in Dulbecco's modified eagle medium: F12 media plus 10% fetal bovine serum with antibiotic/antimycotic (growth media) and incubated at 37°C, 5% CO_2. Cells were fed every 3 to 4 days by replacing 80% of the media. The resultant adherent culture expanded cells are referred to as ASCs and 4 cell lines derived from these primary cultures were characterized further.

Colony Forming Cell Assay. Isolated ADRCs from processed ocular lipectomies were plated in 6-well plates at 1000, 5000, or 10,000 cells per well and cultured in Dulbecco's modified eagle medium: F12 media with 10% fetal bovine serum, 1% antibiotic, antimycotic for ~2 weeks in a humidified incubator at 37°C. After cell culture, colonies were fixed with 10% neutral-buffered formalin for an hour, washed with 1× phosphate-buffered saline and then stained with Mayer's hematoxylin for 2 minutes. Colonies were observed and counted on an inverted microscope using a 4× objective. Groups of adherent cells that were obviously separate from (i.e., greater than 200 μm from the nearest adjacent cell group) and that contained more than 20 cells were counted as a colony.

CD Marker Characterization of Ocular ASCs. ASCs at either passage 2 or 3 were stained for expression of CD11b, CD14, CD31, CD34, CD45, CD90, CD105, and CD106 cell surface marker proteins and analyzed using a Becton Dickinson (Franklin Lakes, NJ, U.S.A.) Aria fluorescence-activated cell sorting instrument. Cells were stained with relevant immunoglobulin G isotope control antibodies.

Differentiation of ASCs to Adipocytes. ASCs were grown to 80% to 90% confluence before each passage. ASCs at either passage 2 or 3 were plated at 300,000 cells per well in 6-well plates in new growth media. After 24 to 48 hours, cells either received new growth media or adipogenic differentiation media (Zen Bio, Durham, NC, U.S.A.) for 4 days. After day 4, the differentiation media were removed, and cells were incubated with adipocyte maintenance media (Zen Bio, Durham, NC, U.S.A.) for 1 day. Cells were then reincubated with adipogenic differentiation media for 4 days. Differentiation media were removed and cells in both control wells and differentiated wells were further incubated for 6 days in adipocyte maintenance media. Cells were then assayed for adipogenic differentiation with oil red O staining as previously described.[7]

Differentiation of ASCs to Smooth Muscle Cells. ASCs at passage 2 or 3 were plated at a density of 3,000 cells/well in 12-well tissue culture plates. Cells were incubated for 3 weeks in control growth media or smooth muscle growth media (SmGM, Clonetics Corp, San Diego, CA, U.S.A.). Cells were then fixed and immunofluorescently stained with antibodies against human smooth muscle proteins.

Differentiation of ASCs to Neuronal Cells. ASCs were plated in 4-well chamber slides in Dulbecco's modified eagle medium: F12 plus 10% fetal bovine serum at a concentration of 12,000 cells per slide in 2.5-ml media/slide. Cells were allowed to adhere for 48 hours in culture. Media were then replaced with either fresh growth media or neuro/glial differentiation media (neurobasal media supplemented with B27 [Invitrogen, Carlsbad, CA, U.S.A.]), plus basic fibroblast growth factor (20 ng/ml), brain-derived neurotrophic factor (10 ng/ml), and epidermal growth factor (20 ng/ml) (all from R and D Systems, Minneapolis, MN, U.S.A.). Cells were incubated for 5 days in neuro/glial differentiation media and changed to neurobasal media supplemented with B27 only for an additional 10 days. Cells were then fixed and immunostained with antibodies against neuronal and glial marker proteins.

RESULTS

Yield of ADRCs From Orbital Adipose Tissue. The average mass of tissue processed for ADRCs was 0.329 and 0.486 g for the nasal and central adipose depots, respectively. The mean number of total ADRCs was 7.1×10^5 and 15.6×10^5 cells/g of processed tissue for the nasal and central adipose depots, respectively. The mean viability of the cells was greater than 94% for both populations. The number of colony forming adherent cells from ocular adipose was in the range of 0.01% to 0.1% of total plated cells, and this is ~1 to 2 logarithms less adherent cells than reported for adipose tissue obtained from other depots.[9] There was no statistically significant difference between colony forming cell values for the nasal and central ocular depots.

Morphology of Orbital Adipose and ASCs. During routine upper eyelid blepharoplasty, the nasal adipose depot has a more pale appearance compared with the yellowish central adipose depot. After blepharoplasty, central and nasal adipose tissues were separately isolated and subjected to orbital ASC isolation as described in the Methods section. Figure 1 shows the morphology of orbital ASCs isolated from the central (A) and nasal adipose depots (B). Cells from both populations, particularly the nasal-derived cells, exhibit long cellular processes reminiscent of cultured oligodendrocytes.

Phenotypic Characterization of Orbital ASCs. To characterize the orbital ASCs, we performed flow cytometry using commercially available markers to cell surface proteins. Immunostaining with antibodies for CD11b, CD14, CD45, and CD106 (markers of lymphoid and myeloid cells) was negative on orbital ASCs isolated from central and nasal adipose depots. In addition, staining for CD31 expression (a marker for endothelial cells) was negative. Both CD90 and CD105, typical markers for adult mesenchymal stem cells, stained positively on nasal and central ASCs. Flow cytometry with CD34, a marker of HSCs, endothelial cells, and some mesenchymal stem cells, was expressed on 2-fold more cells in the nasal ASC lines compared with the central ASC lines.

Adipogenic Potential of Orbital ASCs. We tested the ability of orbitally derived ASCs to differentiate in adipocytes using previously described methods.[10] Nasal and central ASCs were incubated in either control media or adipogenic differentiation media for 2 weeks and stained with oil red O to determine lipid accumulation. Figure 2 shows accumulation of lipid as evidenced by oil red O staining when ASCs are incubated with adipogenic differentiation media (right panel). There was no oil red O staining when ASCs were cultured in control media (left panel). Quantitatively, the percent positive oil red O staining cells ranged from 11% to

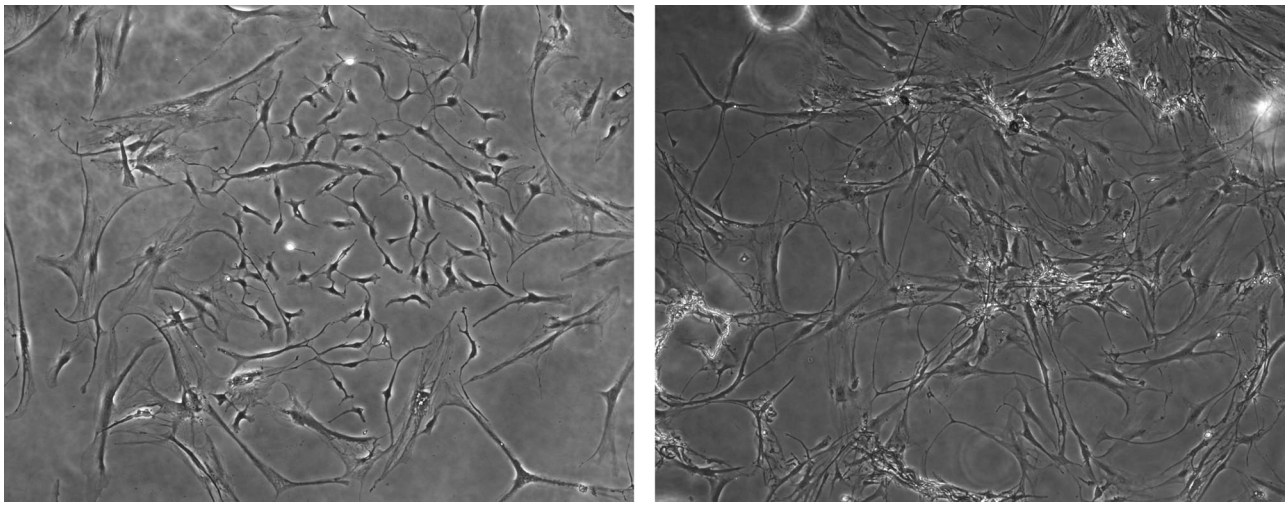

FIG. 1. Phase contrast microscopy of orbital adipose-derived stem cells after cellular processing. Orbital adipose-derived stem cells derived from central adipocyte depots are shown on the left; nasal adipocyte depots are shown on the right. Nasal orbital adipose-derived stem cells seem to have longer and finer cellular processes resembling neuronal cells compared with central depot-derived adipose-derived stem cells.

22%, and there was no statistically significant difference between the adipogenic potential of nasal versus central-derived ASCs. As a positive control, ASCs derived from abdominal adipose tissue showed a 70% to 80% adipogenic potential (data not shown).

Smooth Muscle Potential of Orbital ASCs. Next, we assayed the ability of orbital-derived ASCs to express proteins associated with the smooth muscle lineage. Central and nasal ASCs were incubated in a smooth muscle differentiation assay for 21 days as described in the Methods section. ASCs were immunostained with antibodies directed against smooth muscle cell markers. Figure 3 shows staining for smooth muscle actin, an early maker of smooth muscle differentiation in ASCs, incubated under stimulated conditions compared with control media. However, staining with mature smooth muscle cell markers such as actinin, vimentin, and smooth muscle myosin were seen in less than 1% of orbital ASCs under differentiation conditions.

Neuronal and Glial Potential of Orbital ASCs. Orbital ASCs from both central and nasal depots were examined for neurogenic and glial lineage differentiation potential by culturing the cells in the presence of neurotrophic factors.[10] Centrally derived ASCs stained positive for mature neuronal makers NeuN, TH, and β-tubulin III, astrocytic marker GFAP, and oligodendrocyte markers O4 and CNPase. Nasal ASCs on the other hand, stained positively with β-tubulin III and O4. Nestin, a neural stem cell marker stained positive only with central ASCs. Figure 4 shows representative immunofluorescent staining of neuronal and glial marker protein expression on central ASCs.

DISCUSSION

In this study, we developed a method to isolate and identify a subpopulation of cells in orbital adipose tissue that have the capacity to develop in different cell types in vitro. To

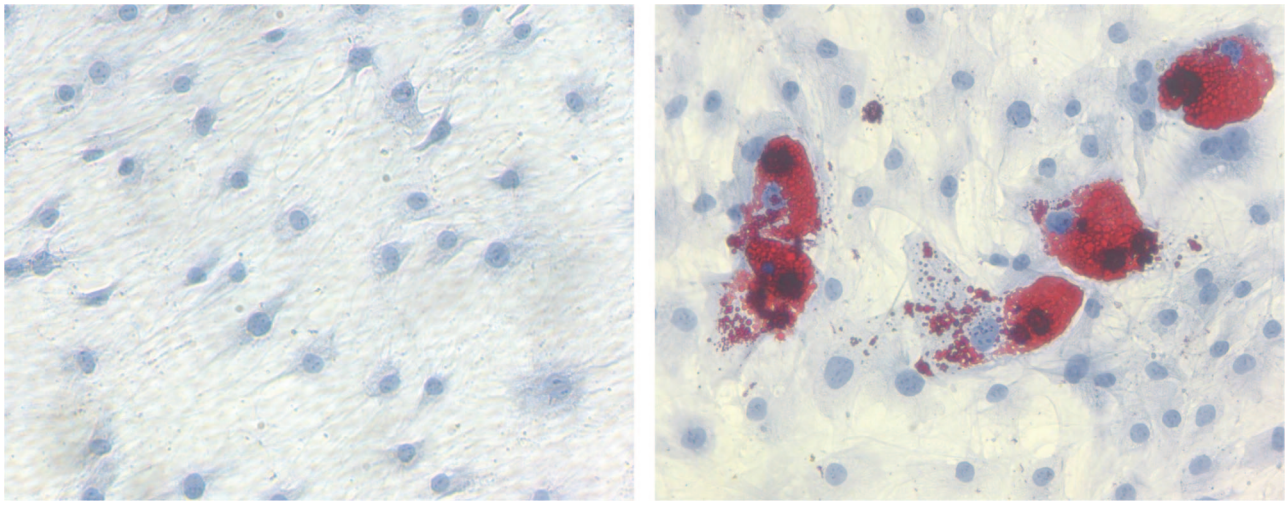

FIG. 2. Differentiation of orbital adipose-derived stem cells to adipocytes. Left panel shows differentiation with control media and right panel shows orbital adipose-derived stem cells stained with oil red O after incubation with adipogenic differentiation media for 14 days. No appreciable difference was seen in the adipogenic potential of nasal versus central orbital adipose-derived stem cells.

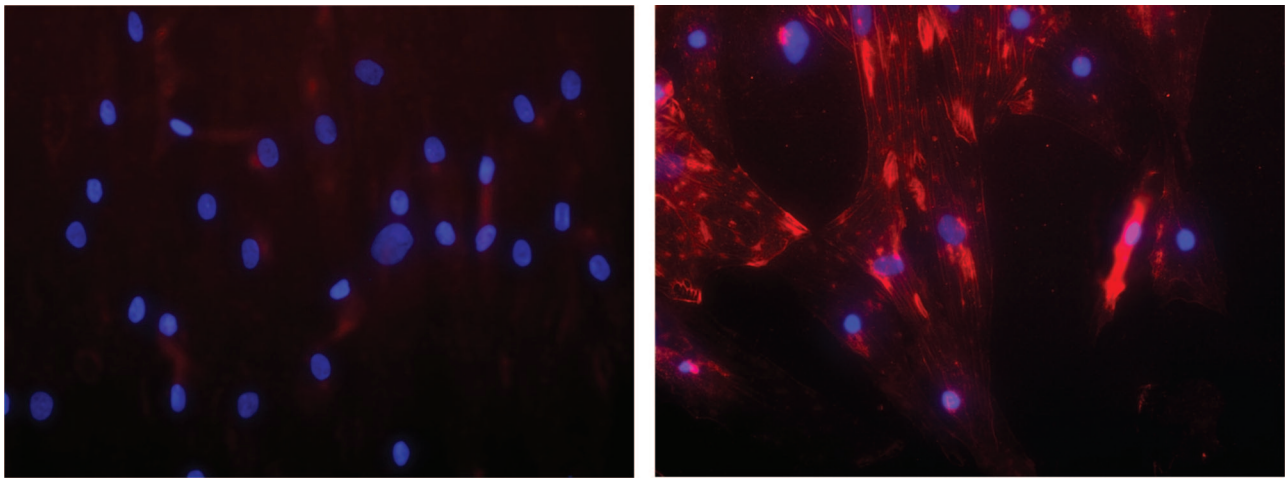

FIG. 3. Smooth muscle differentiation potential of orbital adipose-derived stem cells. Orbital adipose-derived stem cells were incubated in control media (left) and smooth muscle differentiation media (right) for 21 days and stained with monoclonal antibody to smooth muscle actin.

our knowledge, this is the first reported study to identify multipotent stem-like cells in human orbital adipose depots, although characterization of a preadipocyte fibroblast population has been previously performed.[11] These preliminary characterization studies using orbital fat obtained during blepharoplasty demonstrate that orbital ASCs express cell surface proteins and can express smooth muscle, neuronal and glial progenitor, and mature adipocyte phenotypes.

The relatively reduced percentage of positive differentiation events seen in ocular ASC cell lines may have a number of different causes. Three likely explanations may account for these differences. First, a coisolated, more rapidly proliferative, but nonstem cell population eventually overtakes the stem cell cultures by the time the differentiation experiments are performed. Second, the ASCs isolated from these fat depots are not as responsive to the differentiation media used for stem cells derived from other depots in the body. Third, the difference results from both coisolate contaminating cells and different responsiveness to differentiation cues. Future clonal analysis studies of primary ASC cultures may provide an answer.

Genotypic and Phenotypic Differences Between Nasal and Central Adipose Depots. During fetal development, the nasal and central orbital adipose depots likely develop from different tissue layers. The nasal fat pad is continuous with the intraconal fat and possesses similar gross morphologic appearance. Orbital connective tissue, as exquisitely studied by Johnston et al.,[8] was shown to originate from neural crest cells. Central orbital fat, by its more yellow appearance, however, grossly resembles adipose tissue from throughout the rest of the body and although not directly studied, is presumed to be derived from mesoderm, as are the rest of the body's yellow adipose tissues.

Despite the differences in color between the 2 depots, initial analysis of fatty acids and proteins showed no distinct differences.[12] More recently, Sires et al.[13] have found using high-performance liquid chromatography that central fat contains higher quantities of β-carotene and lutein than nasal fat. The physiologic significance of the differences in carotenoid levels between the 2 cell populations is as yet unknown.

In this study, ASC cell lines were established from both nasal and central adipose depots. The in vitro differentiation potential of the cell lines characterized here was similar; however, differential expression of cell surface proteins between ASCs of central and nasal depot origin was observed. Approximately twice the number of nasally derived ASCs expressed the progenitor marker protein CD34 on their surface compared with central depot-derived ASCs at passages 2 and 3. The lower level of expression in centrally derived ASCs is consistent with what is reported for early passage ASCs derived from other subcutaneous adipose depots. While classically a marker for HSCs, CD34 expression is also observed on vascular endothelial cells and has recently been correlated to HSC ability to differentiate in neuronal cells lineages as well.[14] Thus, CD34's sustained expression in these cultured may indicate the presence of a neuroprogenitor cell population.[15] Interestingly, the increased CD34 reactivity of the nasal depot ASCs is in accord with the neural crest derivation of their source tissue and could represent a more neuro-progenitor-like cell population.

Because these same cells were not found to express the CD31 antigen or the CD45 antigen, the increased level CD34 positive cells in the nasally derived cell lines was not likely due to an increased abundance of a contaminating vascular endothelial or hematopoietic cell populations. Furthermore, no gross differences in the vascularity of the different adipose depots were observed. A similar differential pattern of expression in the nasal and central depot-derived ASCs was observed for CD90 (Thy 1), a cell surface marker associated with both bone marrow-derived and ASCs, but which is also expressed on retinal ganglion cells and decreases in response to increased intraocular pressure.[16,17]

The dual expression of O4 epitope and neuronally associated β-tubulin III in response to in vitro neurogenic culture further supports the neurogenic potential of the nasally derived cells. Under neurogenic conditions used in this study, however, central depot cells seemed to express more neuronal and glial associated antigens than the nasal depot-derived cell lines. The reason for the disparity between the CD marker expression and neurodifferentiation data is not known. However, the results may simply reflect differences in differentiation signaling capacity inherent to the different developmental origins of the depots or may be due to differences in differentiation capacity acquired during in vitro cell culture.

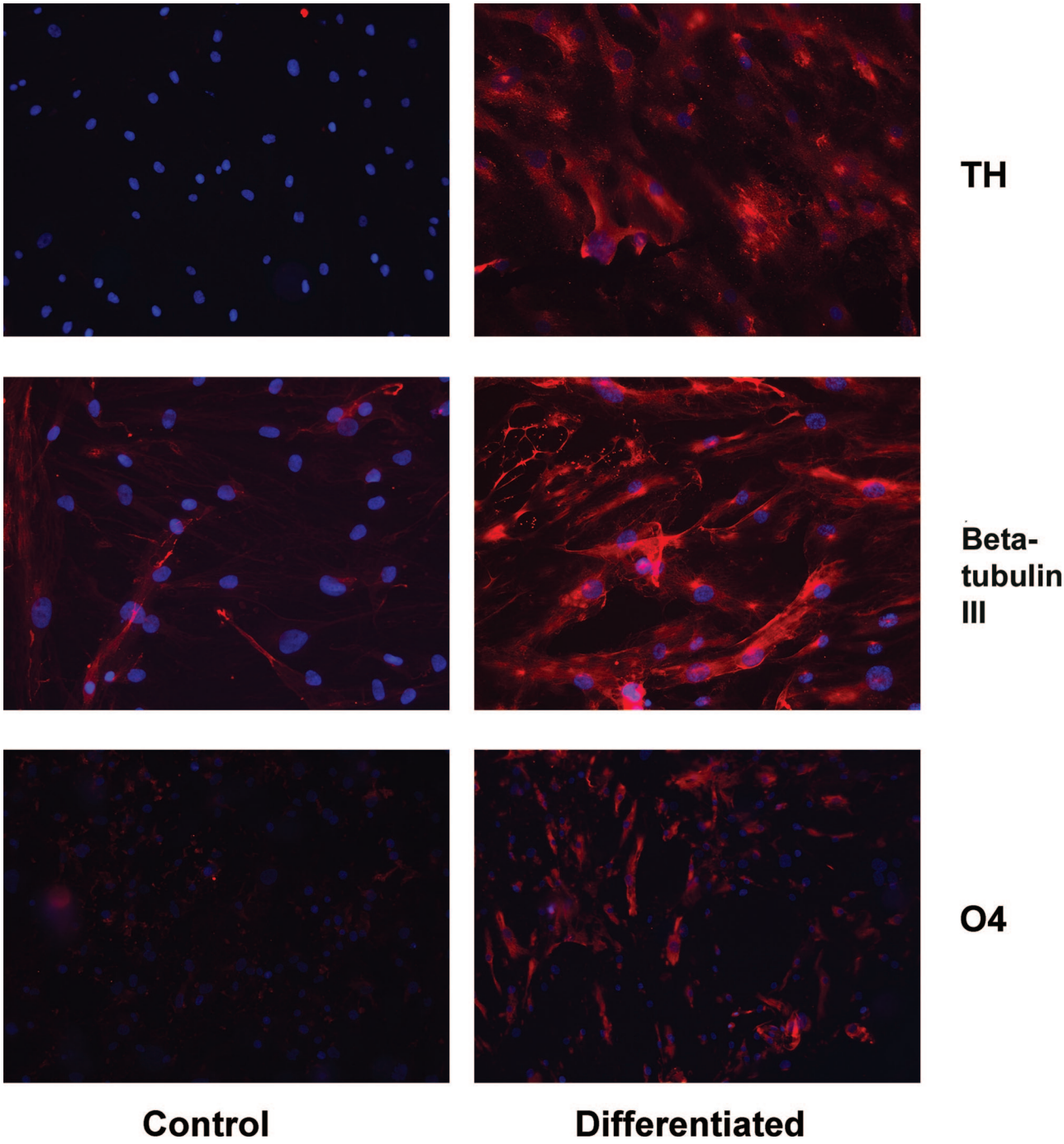

FIG. 4. Neuro/glial differentiation potential of orbital adipocytes-derived cells. Orbital adipocytes-derived cells were incubated in control media (left) and neurogenic differentiation media (right) and stained with monoclonal antibodies to neural/glial marker proteins. Top row: tyrosine hydroxylase; mid row: β-tubulin III; and bottom row: oligodendrocytic marker epitope, O4.

Ophthalmic Applications of ASCs. Ultimately, as ophthalmologists, we seek to improve the condition of ocular-related diseases. The therapeutic potential for ASC application is vast. Figure 5 shows some possible ASC-related clinical applications. ASCs isolated from patients with heritable diseases such as retinitis pigmentosa can be isolated, and in vitro studies can be performed to look for new drugs or therapeutics with no risk to patients. Cells derived from ASCs can be applied to a host of diseases ranging from retinal disease such as macular degeneration, heritable dystrophies, and retinal vascular diseases, glaucomatous conditions such as trabecular meshwork reconstruction and ganglion cell replacement, neuro-ophthalmic diseases such as traumatic and degenerative optic neuropathies, ocular surface conditions such as dry eye syndrome, keratoconjunctivitis sicca, and ocular surface burns.

From a disease standpoint, we may find that certain diseases may involve ASCs directly. One such example is thyroid-related orbitopathy. In a subset of patients with thyroid-

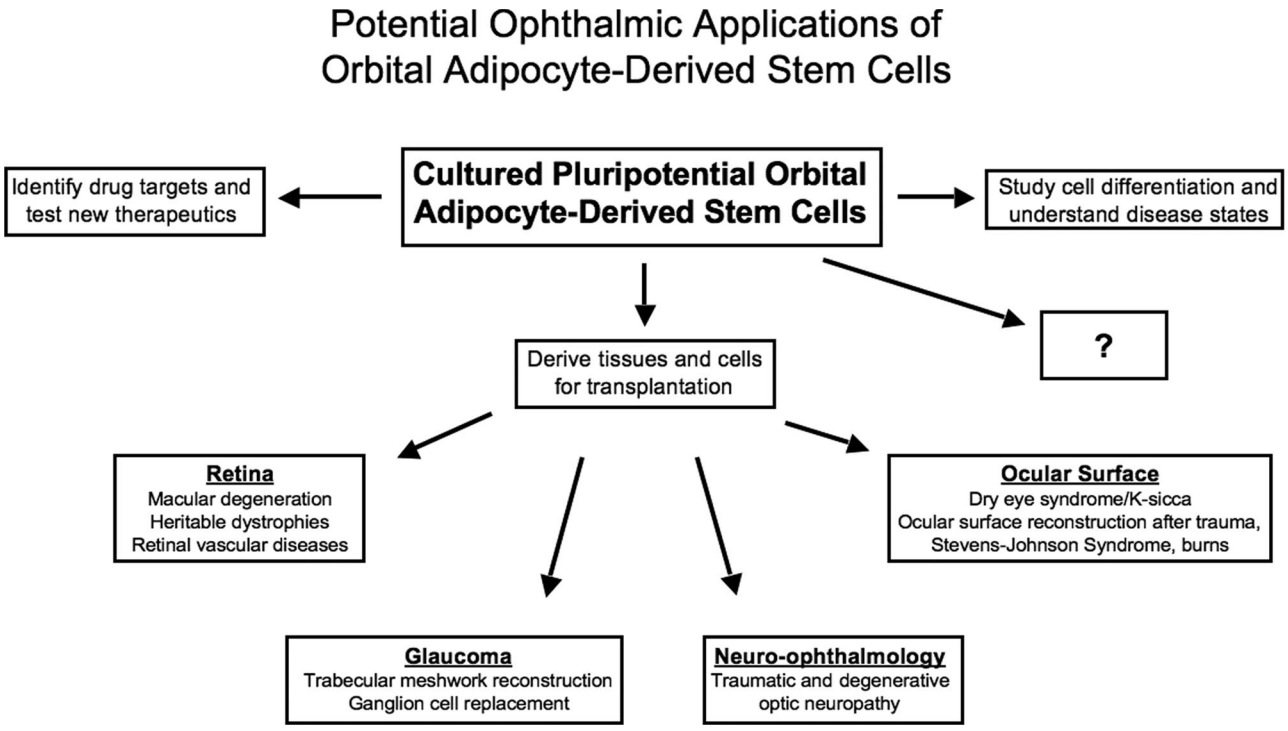

FIG. 5. Potential ocular applications of orbital adipocyte-derived stem cells.

related orbitopathy, there is selective expansion of the orbital fat compartment, the so-called type I patients.[18] We have already shown that in vitro, under conditions of adipogenic stimulation, ASCs can develop in oil red O staining adipocytes. One can postulate that the immune system is selectively targeting the adipogenic differentiation pathway in orbital ASCs. In vitro studies of ASCs from patients with thyroid-related orbitopathy may provide valuable insights into this disease.

In summary, we have identified a novel population of adult stem-like cells from human orbital fat that possess pluripotential capabilities in vitro. This finding opens the door to study of orbital diseases and the development of new therapeutic modalities. The next challenge will be to learn how to direct these ASCs to develop in specific cell types both in vitro and in vivo and apply them to ophthalmic diseases.

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