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Characterization of free-floating spheres from human trabecular meshwork (HTM) cell culture in vitro

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Abstract

It has been observed in several tissues that direct isolation of cells in serum-free media and on nonadhesive substrates results in the formation of spherical clusters of cells known as free-floating spheres. Such free-floating spheres have been hypothesized to contain undifferentiated multipotent progenitor cells. Our goal was to isolate and characterize such free-floating spheres from HTM cell primary cultures.

For this purpose, HTM cells were incubated in serum-free media and on a nonadhesive substrate. Individual free-floating spheres generated in these conditions were isolated in 96-well plates, and their proliferative capacity was evaluated by monitoring their size increase over time. The expression of the TM markers, MGP and CHI3L1, was examined using recombinant adenoviruses containing the respective promoters. Morphology of the free-floating spheres was analysed in semithin sections, and the gene expression profile was obtained using Human Genome U133 Plus 2.0 Affymetrix microarrays.

HTM cells incubated in serum-free media and on nonadhesive substrate generated free-floating spheres that could be grown for more than 3 months. Addition of serum to the culture media promoted the attachment of the spheres to the substrate, migration of cells from the spheres, and differentiation into cells phenotypically similar to normal TM cells. Gene profiling analysis demonstrated strong similarities between the gene expression profiles of the spheres and HTM cell monolayers. Both infection with the recombinant adenoviruses and gene array analysis demonstrated the expression of CHI3L1 and MGP, indicating that free-floating spheres likely originate from HTM cells. Gene array analysis also showed expression of the marker for neural precursor cells nestin, as well as leukemia inhibitory factor, a gene involved in the maintenance of the undifferentiated state of progenitor cells. Analysis of semithin sections indicated that these TM free-floating spheres were highly dynamic structures demonstrating a distinct radial gradient of cell proliferation, survival, and apoptosis. Extensive up- and down-regulation of gene expression was associated with the processes of sphere attachment and cell migration after the addition of serum.

These results suggest that HTM primary cultures might contain relatively undifferentiated or progenitor cells. The availability of TM progenitor cell cultures could constitute a useful tool to investigate cell therapy approaches targeting the TM in glaucoma.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exer.2005.10.006

Keywords

trabecular meshwork; gene/expression; free-floating spheres

1. Introduction

Many specialized tissues in the adult organism have been shown to contain populations of either adult stem cells (ASCs) or progenitor cells that self-renew as well as differentiate to regenerate the tissues (LaBarge and Blau, 2002; Beltrami et al., 2003; Wulf et al., 2003). ASCs are multipotent and can differentiate into different mature cell types. In contrast, 'progenitor' or 'precursor' cells have a more restricted lineage commitment and lower self-renewal capacity than ASCs. Both ASCs and progenitor cells are believed to play an important role in the maintenance of adult tissues by generating new cells that replace those lost as a result of injury or normal wear and tear (Loeffler and Roeder, 2002). It has been hypothesized that a decline in the ability of these cells to keep up with the rate of cell death in tissues may be a major factor in the loss of tissue functionality in certain age-related disorders (Van Zant, 2003; Van Zant and Liang, 2003).

Currently, the idea that progenitor cells may be present in the trabecular meshwork (TM) is supported only by limited data. This evidence comes primarily from experiments in which a recovery in cell density is observed after cell death caused by either phagocytic stress in rats (Johnson et al., 1989) or laser trabeculoplasty (LTP) in perfused human eyes (Acott et al., 1989). Additionally, in vitro LTP experiments have shown that most of the cell division associated with regeneration of the TM takes place in the nonfiltering meshwork near Schwalbe's line (SL), suggesting that the SL region may harbour TM progenitor cells (Acott et al., 1989). Based on their distinct ultrastructural features, the cells in this portion of the TM have been hypothesized to be a different subtype from those in the filtering meshwork (Raviola, 1982). A better understanding of the presence of progenitor cells in the TM and the dynamics of cell turnover in the TM may be important to help clarify the mechanisms involved in the functional failure of this tissue in glaucoma. The TM is exposed to chronic insults, such as oxidative, mechanical, and phagocytic stress, that can result in the death of TM cells (Spector and Garner, 1981; Johnson et al., 1989; Green, 1995;). A decline in the ability of the TM tissue to replace lost cells with new cells could be a contributing factor to the malfunction of this tissue in glaucoma. This idea is supported by the observation of decreased cellularity in the TM in several late-onset glaucomas (Alvarado et al., 1984), and by alterations in SL cells in dogs with congenital glaucoma (Samuelson et al., 2001).

ASCs and progenitor cells from several tissues form floating colonies known as spheres when cultured in serum-free media and on nonadhesive substrates. These sphere-forming culture conditions have been used to isolate ASCs and precursor cells from several murine organs including skin (Fernandes et al., 2004), bone marrow (Krause et al., 2001), central nervous system (Chiasson et al., 1999; Lobo et al., 2003), pancreas (Seaberg et al., 2004), inner ear (Li et al., 2003), skeletal muscle (Vourc'h et al., 2004), and cornea (Yoshida et al., 2005). Isolation of free-floating spheres from human tissues has been more limited, probably because of restricted tissue availability (Svendsen et al., 1998; Roisen et al., 2001; Bez et al., 2003; Lobo et al., 2003; Uchida et al., 2005; Yokoo et al., 2005). Therefore, we tested the ability of primary HTM cell cultures to generate free-floating spheres in serum-free media and nonadhesive cell culture conditions, and analyse the morphological features and gene expression profile of the TM cell spheres.

2. Materials and methods

2.1. Cell culture

Primary cultures of HTM cells were prepared from cadaver eyes as previously described (Stamer et al., 1995) and maintained at 37 °C in a humidified atmosphere of 5% CO₂ in low glucose Dulbecco's modified eagle medium (DMEM) with L-glutamine and 110 mg/l sodium pyruvate, supplemented with 10% fetal bovine serum (FBS), 100 µM nonessential amino acids, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate and 0.25 µg/ml amphotericin B. Reagents were purchased from Invitrogen Corporation (Carlsbad, CA). For the generation of free-floating spheres, cells were maintained in StemSpan™ Serum-Free Expansion Medium (SFEM) (StemCell Technologies, Seattle, WA). Attachment of the spheres was induced by replacing the serum-free media with DMEM containing 10% FBS. The protocols involving the use of human tissue were consistent with the tenets of the declaration of Helsinki.

2.2. Semithin sections

Cells were washed in PBS and fixed in cold 1% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 for 2 hr at 4 °C. After rinsing two times for 15 min in 0.1 M cacodylate buffer, cells were post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr and a half, dehydrated through graded ethanol series and propylene oxide, and embedded in Spurr's low-viscosity resin. Semithin sections (1 µm thick) were stained with 1% toluidine blue and examined under a light microscope.

2.3. Analysis of Ch3L1 and MGP promoter expression

Cells were infected with 10⁷ pfu of either the AdCh3L1-*LacZ* or the AdMGP-*LacZ* recombinant adenovirus (Gonzalez et al., 2004; Liton et al., 2005). An empty recombinant adenovirus was used as a negative control. Two days after infection, cells were fixed in 1% paraformaldehyde, 0.2% glutaraldehyde, 0.02% NP40, and 0.01% sodium deoxycholate in PBS. After two washes with PBS, β-galactosidase activity was detected by overnight incubation at 37 °C in 1 mg/ml 5-bromo-4-chloro-3 β-D-galactoside, 5 mM K₃Fe(CN), 5 mM K₄Fe(CN)6-3H₂O, and 2 mM Mg₂Cl in PBS.

2.4. RNA extraction and quality analysis

Total RNA was isolated using RNeasy kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol. After DNase treatment, RNA yields were determined using the RiboGreen® fluorescent dye (Molecular Probes, Inc, Eugene, OR). RNA quality was confirmed using the Agilent 2100 Bioanalyzer.

2.5. Oligonucleotide microarray analysis

Total RNA (10 µg) from free-floating spheres, attached colonies, and migrating cells was hybridised to Affymetrix Human Genome U133 Plus 2.0 microarrays following the manufacturer's instructions. Data analysis was performed using the GeneSpring Software 7.0 (Silicon Genetics, Redwood City, CA). Raw data from the three hybridisations were normalized to 50th percentile per chip and to median per gene. Genes with a differential gene expression of two-fold were selected and then filtered on flags to retain the genes that were presented in at least one of the conditions. Since, some genes were represented in the arrays in more than one spot, we verified a consistent differential expression in all the spots to eliminate false positives.

2.6. Quantitative real-time PCR

First strand cDNA was synthesized from total RNA 0.5 µg by reverse transcription using oligodT primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time PCR reactions were performed in a 20 µl mixture containing 1 µl of the cDNA preparation, 1× iQ SYBR Green Supermix (Biorad, Hercules, CA), and 500 nm of each primer, in the BIO-RAD iCycler iQ system (BioRad, Hercules, CA) using the following PCR parameters: 95 °C for 5 min followed by 50 cycle of 95 °C for 15 sec, 60 °C for 15 sec, and 72 °C for 15 sec. The fluorescence threshold value (Ct) was determined using the iCycle iQ system software. The absence of nonspecific products was confirmed by both analysis of the melt curves and electrophoresis in 3% Super AcrylAgarose gels. β-Actin was used as an internal standard of mRNA expression. Normalized gene expression is represented as $\text{Inv}(\Delta\text{Ct})$, where $\Delta\text{Ct} = \text{Ct}_{\text{gene}} - \text{Ct}_{\text{actin}}$. The sequences of the primers used for the amplifications are shown in Table 1.

3. Results

3.1. Isolation and expansion of HTM free-floating spheres

A small fraction (approximately 10%) of primary human TM (HTM) cultures generated in our laboratory showed the presence of dense three-dimensional clusters of cells. The formation of such clusters was particularly abundant in HTM cell lines from three donors ages 17, 25 and 58 years old at passages 3, 4 and 5. These clusters of cells were also observed in late passage cultures after most cells had died or reached terminal replicative senescence. Isolated clusters from these senescent cultures showed the ability to grow in a pattern characteristic of cells lacking contact inhibition and typical of progenitor cells (Fig. 1(A)–(C)). Apart from this pattern of growth, these cells contained abundant refringent bodies and were more resistant to trypsin digestion than normal HTM cells. Analysis at higher magnification demonstrated the presence of cells migrating from the clusters and acquiring a morphology indistinguishable from that of cultured HTM cells (Fig. 1(C)).

To investigate whether these clusters of cells could generate free-floating spheres in the absence of serum, HTM primary cultures from one of the cell lines containing three-dimensional colonies (donor age 25) were trypsinized normally at passage three to remove the HTM cell monolayer; the remaining attached colonies were then cultured in serum-free media. Two days later, we observed in the culture the occurrence of both free-floating spheres (Fig. 1(D) and (E)) and spheres partially adhered to the plate (Fig. 1(F)). Free-floating spheres presented different sizes: larger spheres had generally a dark core and a well-defined spherical shape (Fig. 1(D)), while smaller ones, some of them consisting in just a few cells, appeared more translucent and irregular (Fig. 1(E)). Sphere cells were passaged by diluting ten times the cell cultures once a week with fresh culture media; spheres partially attached became floating generating more free-floating spheres. We also evaluated the proliferative capacity of the cells inside the sphere by monitoring over time the size increase of individual spheres cloned by limited dilution in 96-well plate (Fig. 2).

Free-floating spheres could be expanded in vitro for 3 months and could be cryopreserved in serum-free media supplemented with 10% DMSO. Culturing the cells for longer periods of time and cryopreservation diminishes the proliferative potential.

3.2. Generation of cell monolayers from free-floating spheres

Incubation of free-floating spheres in the presence of serum (10%) and onto cell-treated plastic quickly promoted the attachment of the spheres to the plate (Fig. 3). After 24 hr, the presence of cells migrating out of the attached spheres was already evident (Fig. 3(A) and (B)). By 72 hr, all the spheres had practically disappeared, and cells had evolved towards a

true monolayer with morphology indistinguishable from that of typical TM cells (Fig. 3(C) and (D)). Incubation of TM free-floating spheres in the presence of serum on a nonadherent substrate also resulted in adhesion of the spheres and the formation of a cell monolayer (data not shown).

3.3. Semithin sections of free-floating spheres and attached spheres

Toluidine blue-stained semithin sections of free-floating spheres demonstrated a noncompact multicellular structure of viable cells and an abundant extracellular matrix (ECM) (Fig. 4(A)–(D)). Apoptotic phenomena were identified in the inner layers and rarely seen at the periphery of the sphere (Fig. 4(B)). Mitotic figures in all phases of the cell cycle were found peripherally and never noticed in the inner part of the spheres (Fig. 4(C)). The outer layers were also characterized by the presence of bilobal nuclei, giant multinuclear cells, and extensive vacuolisation (Fig. 4(D)).

Semithin sections of spheres attached to the substrate after the addition of serum to the culture revealed a looser structure with less ECM as cells migrated out of the sphere (Fig. 4(E)–(H)). Mitotic events and vacuolisation were much less frequent than in the free-floating spheres. In addition, attached spheres showed extracellular granules intensively stained with toluidine blue (Fig. 4(H)).

3.4. Expression of HTM markers: matrix gla protein (MGP) and chitinase 3-like 1 (CH3L1)

To investigate the expression of the markers for HTM cells, MGP and CH3L1, free-floating spheres were transduced with the previously reported recombinant adenoviruses, AdMGP-*LacZ* or AdCH3L1-*LacZ*, containing the *LacZ* gene under the control of either MGP promoter or CH3L1 promoter, respectively (Gonzalez et al., 2004; Liton et al., 2005). As shown in Fig. 5, cells on the surface of the spheres demonstrated positive β -galactosidase activity after infection with either the AdMGP-*LacZ* or the AdCH3L1-*LacZ* adenovirus.

3.5. Gene expression profile analysis

To gain insight into the potential origin of free-floating spheres, we compared the gene expression profile of the spheres with that previously reported for cultured HTM cells (Liton et al., 2005). As shown in Table 2, most of the genes highly expressed in HTM cells also showed high levels of expression in free-floating spheres, including tissue inhibitor of metalloproteinases 1 (TIMP1), chitinase 3-like 1 (CH3L1), vimentin, and thymosin β 4. Only a few genes, such as prostaglandin D2 synthase, transgelin, and biglycan, demonstrated down-regulation in the free-floating spheres compared to HTM cells.

In addition, we analysed the gene expression profile of spheres attached to the substrate after the addition of serum, as well as that from the cells migrating out of the spheres. Attachment of the spheres after the addition of 10% serum was associated with the up-regulation of 716 genes and the down-regulation of 1467 genes. Similarly, 2498 genes and 762 genes were up-regulated and down-regulated, respectively, in the cells migrating from the attached spheres compared to the cells contained in the attached spheres.

To facilitate the interpretation of the data, genes showing differential gene expression in any of the stages were clustered into four different patterns of expression (Fig. 6). The first cluster included the genes (57 genes) that were consistently down-regulated during the process of attachment and migration. With the exception of vimentin, none of the genes reported to be highly expressed in cultured TM cells were present in this group. The second cluster included genes (33 genes) that showed a consistent up-regulation during the attachment and migration process. This group included transgelin, brain-derived neurotrophic factor (BDNF), and several genes involved in the TGF- β signalling, such as

connective tissue growth factor (CTGF), retinoic acid induced 3, retinoic acid receptor responder 1, and transforming growth factor beta-induced (68 kDa). The third cluster included genes (183 genes) that showed increased expression subsequent to the addition of serum, followed by a decreased expression in the migrating cells. This group was characterized by the presence of several genes involved in the proinflammatory response, such as IL-6, IL-1 β , IL-7, colony-stimulating factor 3, chemokine (C-X-C motif) ligand 2, chemokine (C-X-C motif) ligand 3, chemokine (C-X-C motif) ligand 5, and chemokine (C-X-C motif) ligand 6, as well as genes implicated in ECM remodelling, like matrix metalloproteinase 1 (MMP1), matrix metalloproteinase 3 (MMP3), a disintegrin-like and metalloprotease with thrombospondin type 1 motif 17 (ADAMTS17), and a disintegrin-like and metalloprotease with thrombospondin type 1 motif 20 (ADAMTS20). Finally, the fourth cluster grouped genes (685 genes) down-regulated in the attached spheres whose expression increased during the migration. This group included a large number of genes involved in regulation and signal transduction, including members of the Rho family, protein kinases, and protein phosphatases. The full lists of genes present in each cluster are included as Supplemental material.

To validate the data from the gene array analysis, the expression changes of several genes included in the different clusters presented in Fig. 6 were confirmed by RT-PCR (Fig. 7). The selected genes included: Protein kinase (cAMP dependent) inhibitor beta (cluster 1), a gene involved in the maintenance of barrier function in vascular cells, (Lum et al., 2002); transgelin (cluster 2), a marker for smooth muscle cell differentiation (Owens et al., 2004); BDNF (cluster 2), believed to modulate neural progenitor cell proliferation and to enhance the maturation of neurons derived from EGF-generated neural stem cells (Schinstine and Iacovitti, 1997); IL-6 (cluster 3), known to act synergistically with stem cell factors and required in vivo for the regulation of stem cells and committed progenitors of the hematopoietic system (Bernad et al., 1994); colony-stimulating factor 3 (cluster 3), involved in the mobilization of peripheral blood stem cells with long-term repopulating potential (Molineux et al., 1997); and leukemia inhibitory factor (LIF, cluster 3), which has been associated with the maintenance of the undifferentiated state of progenitor cells (Pitman et al., 2004; Tarasenko et al., 2004). We also confirmed in both floating spheres and normal HTM cells the expression of nestin considered a neural cell progenitor marker (Lendahl et al., 1990).

4. Discussion

Our results showed that primary HTM cell cultures contain cells with a distinct pattern of growth typical of cells without contact inhibition. The spontaneous presence of clusters of cells with this apparent loss of contact inhibition may initially result from either growth of cell colonies or cell aggregation. However, the observation that isolated clusters of cells have the ability to generate extensive growth after most cells in the culture have reached replicative senescence indicates that, regardless of their initial origin, the clusters contain cells with high replicative potential. Furthermore, these cells have the ability to grow in the form of free-floating spheres when cultured in serum-free media. In addition, when the TM free-floating spheres were incubated with serum, they evolved into monolayers of cells morphologically indistinguishable from typically cultured TM cells, indicating the possibility that they might differentiate spontaneously into TM cells. This characteristic pattern of growth has been associated with the presence of ASCs or precursor cells in several tissues (Krause et al., 2001; Roisen et al., 2001; Li et al., 2003; Lobo et al., 2003; Fernandes et al., 2004; Seaberg et al., 2004; Vourc'h et al., 2004) and suggests that such ASCs or precursor cells may be present in primary HTM cell cultures.

To ensure that the sphere-forming cells were generated from cells present in the TM, dissections of the TM tissue used to generate primary cell cultures were conducted under a stereomicroscope taking special care to exclude any non-TM material.

Expression analysis of the MGP and CH3L1 promoters is consistent with the concept that the free-floating spheres are indeed of TM origin. These two promoters have been previously shown to be preferentially expressed in outflow pathway cells in perfused anterior segments of human eyes (Gonzalez et al., 2004; Liton et al., 2005). Although not all cells showed expression of both promoters, it has to be taken into account that adenoviral vectors may not be able to penetrate easily inside the spheres, and that given the relatively fast rate of replication in the spheres, many cells may have lost the adenoviral transgene between the time of infection and that of the analysis as a result of cell replication.

Additional support for the TM origin of the free-floating spheres was also provided by the overall similarity found between the gene expression profile of the free-floating spheres and those previously reported for TM cell monolayers (Liton et al., 2005). The differences in gene expression observed in these analyses do not appear strong enough to indicate that the spheres were generated as a result of contamination of cells from non-TM tissue, and could therefore reflect different levels of cell differentiation. The expression in HTM spheres of LIF, a gene involved in the maintenance of the undifferentiated state of embryonic stem cells (Pitman et al., 2004; Tarasenko et al., 2004), is consistent with this concept. HTM spheres also expressed nestin, which is considered a marker for neural precursor cells (Lendahl et al., 1990). Interestingly, the expression of nestin was detected in HTM cell monolayers but not in gene arrays of HTM tissue from postmortem eyes (Gonzalez et al., 2005), suggesting that HTM cells grown in standard cell culture conditions may remain in a relatively undifferentiated state compared to those in the TM tissue.

The ability of TM cells to form multicellular spheroids in cell culture using nonadherent substrates was reported by Matsuo and Matsuo (1996, 1997). However, in these studies, TM cells were incubated in 15% serum. Under these conditions, no actual growth of the cell aggregates was observed, and no mitotic figures were present in the cell aggregates. Serum deprivation appears to be an important factor to prevent cell differentiation and maintain growth of undifferentiated ASC and precursor cells (Svendsen et al., 1998; Krause et al., 2001; Toma et al., 2001; Suslov et al., 2002; Bez et al., 2003; Li et al., 2003; Lobo et al., 2003; Fernandes et al., 2004; Seaberg et al., 2004; Vourc'h et al., 2004; Zhang et al., 2004; Uchida et al., 2005; Yokoo et al., 2005; Yoshida et al., 2005). Our own results showed that the absence of serum played a more critical role than cell attachment withdrawal in the maintenance of the free-floating sphere growth. Unlike the aggregates reported by Matsuo and Matsuo (1996, 1997), spheres grown in the absence of serum were capable of detectable growth for at least 3 months and showed frequent mitotic figures in the semithin sections.

Although the proliferative capacity of the HTM sphere cells appears to be lower than that observed in similar spheres from murine tissues, it has to be taken into account that sphere colonies isolated from several human tissues have consistently shown a much lower self-renewal capacity than those derived from rodent tissues (Svendsen et al., 1998; Toma et al., 2001; Uchida et al., 2005). Sphere colonies are not thought to contain exclusively undifferentiated cells, but to be composed by heterogeneous cell populations including immature cells, lineage-restricted progenitors, and postmitotic cells (Suslov et al., 2002). While most studies performed on rodents indicate that sphere colonies obtained from various tissues contain immature cells, results from similar experiments conducted with human tissues suggest that it is difficult to maintain undifferentiated human cells in culture (Svendsen et al., 1998).

Currently, our knowledge about the specific signalling mechanisms governing the maintenance, migration, and differentiation of progenitor cells is very limited. The extent of the changes in gene expression associated with attachment, migration, and, potentially, differentiation observed by gene array analysis, suggests that regulation of these processes may involve multiple signalling pathways. The observation that the absence of serum rather than cell attachment withdrawal is critical in preventing the cells from the spheres to migrate and, potentially, differentiate indicates that these signalling pathways may be regulated by yet-to-be identified factors present in the serum. TM floating spheres may provide a practical experimental model for the study of such regulatory factors.

A better understanding of the mechanisms involved in the regulation of the regenerative properties of the TM may provide important insight into the pathophysiological mechanisms involved in the failure of the TM to maintain normal levels of IOP in POAG. The reported decreased cellularity in the TM of POAG donors compared to normal age-matched controls (Alvarado et al., 1984) suggests that a decline in the regenerative potential of the TM may contribute to the progression of glaucoma. Such a regenerative loss may result from a combination of age-dependent quantitative and qualitative changes in tissue-specific precursor cells, as well as from alterations in the environmental cues that promote those cells to participate in tissue maintenance and repair. The observation that exposure of aged muscle progenitor cells (satellite cells) to serum from young mice, either in vivo by heterochronic parabiotic pairings or in vitro, rejuvenates their restorative potential (Conboy et al., 2005), suggests that, at least in some tissues, changes in the tissue microenvironment may indeed affect the functionality of progenitor cells. Therefore, it is possible that even old progenitor cells may be capable of maintaining and repairing aged tissues if provided with optimal environmental cues.

Additional studies will be necessary to determine whether free-floating spheres derived from HTM cell primary cultures contain ASC or progenitor cells. The availability of cell cultures containing such precursor cells might provide a particularly useful model for studying such environmental cues and signalling mechanisms involved in the dynamics of cell migration and differentiation involved in the maintenance of a normal cell population in the TM. Identifying and, ultimately, manipulating such mechanisms may have important clinical benefits in the treatment of age-related diseases like POAG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Light microscopy views of three-dimensional clusters of cells present in HTM cell primary cultures grown under normal conditions (A, B, C) and free-floating spheres generated in serum-free media (D, E, F). (A, B) Low magnification photographs demonstrating the change in size of three-dimensional cluster of cells. (C) High magnification photograph of a three-dimensional cluster of cells showing the migration of cells out of the cluster. (D) Large free-floating sphere with a well-defined shape and a dark core. (E) Small translucent free-floating spheres. (F) Partially attached spheres.

Fig. 2. Size increase over time of an isolated free-floating sphere cloned by limited dilution in 96-well plate (panels A–C). Average growth from measurements performed in six independent spheres of similar size is presented in panel D.

Fig. 3. Generation of cell monolayers from free-floating spheres. (A, B) Attachment of free-floating spheres and migration of cells 24 hr after addition of 10% serum. (C) Cell monolayer generated by 72 hr after incubation with 10% serum. (D) Confluent monolayer generated from a free-floating sphere stained for beta-galactosidase expression after infection with the AdCH3L1-*LacZ* virus to facilitate the visualization of the morphology of individual cells.

Fig. 4.

Toluidine blue-stained semithin sections of free-floating spheres (A–D) and attached spheres generated 24 hr after addition of 10% serum (E–F). (A) Low magnification of free-floating spheres. (B) Presence of apoptotic phenomena (a) in the inner layers. (C) Mitotic figures (m) and cells with large vacuolae (v) in the periphery of the sphere. (D) Presence of giant cells with either large irregular nuclei or multinucleated cells. (E) Low magnification of attached sphere 24 hr after addition of 10% serum. (F) Cells migrating out of the sphere. (G) Giant cells with bilobal nuclei. (H) Presence of abundant granules showing intense staining with toluidine blue.

Fig. 5.

Expression of MGP and CH3L1 promoters in HTM spheres. β -galactosidase staining 48 hr after transduction with the recombinant adenoviruses AdMGP-*LacZ* and AdCH3L1-*LacZ*.

Fig. 6. Cluster analysis of genes with similar expression changes associated with attachment and cell migration after addition of 10% serum. The *x*-axis depicts the three-time points (floating spheres, attached spheres, and migrating cells). The *y*-axis shows the normalized intensity values (log scale) for the genes included in each cluster, which provides a measurement of the expression levels for each specific gene. The full lists of genes present in each cluster are included in supplemental material.

Fig. 7.
Real-time PCR analysis of the expression of selected genes in floating spheres, attached spheres, migrating cells, and HTM cell monolayers.

Table 1

Primer sequences used for the real-time PCR analysis

Gene	Primer sequences	
Protein kinase (cAMP dependent) inhibitor beta (PKIB)	Forward	5'-CGGCTGGAGTCATGCTATAC-3'
	Reverse	5'-TGAATCTGTCCTCATAGCAACA-3'
Transgelin (TAGLN)	Forward	5'-CAAGACTGACATGTTCCAGA-3'
	Reverse	5'-GCCGAGGTCGTCCGTAGCCT-3'
Brain-derived neutrophic factor (BDNF)	Forward	5'-ACCTCGTGCTTTAAGTGCCT-3'
	Reverse	5'-TTATCCAGAGGTGGGATGGT-3'
Interleukin 6 (IL6)	Forward	5'-CAAATTCGGTACATCCTCGACGGC-3'
	Reverse	5'-GGTTCAGGTGTTTTCTGCCAGTGC-3'
Colony-stimulating factor 3 (CSF3)	Forward	5'-CAGAGCTTCCTGCTCAAGTG-3'
	Reverse	5'-GCACACTCACTCACCAGCTT-3'
Nestin (NES)	Forward	5'-CAGAGGTGGGAAGATACGGT-3'
	Reverse	5'-AGCTCTGCCTCATCCTCATT-3'
Leukemia inhibitory factor (LIF)	Forward	5'-AACCAGATCAGGAGCCAACT-3'
	Reverse	5'-CCCTGGGCTGTGTAATAGAGA-3'
Actin, beta (ACTB)	Forward	5'-CCTCGCCTTGCCGATCCG-3'

Table 2

Expression levels in the free-floating spheres of the genes more highly expressed in HTM cells

Gene name ^a	Unigene	Signal ^b	Rank ^c
Ferritin, light polypeptide (FTL)	Hs.433670	27 202	2
Tissue inhibitor of metalloproteinases 1 (TIMP1)	Hs.446641	16 874	65
Glyceraldehyde-3-phosphate dehydrogenase (GAPD)	Hs.169476	23 110	19
Elongation factor EF-1-alpha (EEF1A1)	Hs.439552	24 938	12
Actin, beta (ACTB)	Hs.426930	29 090	3
Interferon induced transmembrane protein 1 (9-27) (IFITM1)	Hs.458414	18 872	49
Insulin-like growth factor binding protein 4	Hs. 1916	10 154	403
Clusterin	Hs. 436657	8791	568
Interferon induced transmembrane protein 3 (1-8U) (IFITM3)	Hs.374650	18 036	55
Vimentin (VIM)	Hs.435800	23 383	2
Ferritin, heavy polypeptide 1 (FTH1)	Hs.448738	29 152	17
Beta-2-microglobulin (B2M)	Hs.48516	24 975	10
Thymosin, beta 4 (TMSB4)	Hs.446574	32 542	1
Chitinase 3-like 1 (CH3L1)	Hs.382202	20 512	30
Guanine nucleotide binding protein, beta polypeptide 2-like 1 (GNB2L1)	Hs.5662	18 918	48
Annexin A2 (ANXA2)	Hs.462864	19 681	40
Lectin, galactoside-binding, soluble, 1 (LGALS1)	Hs.407909	24 556	13
Actin, gamma 1 (ACTG1)	Hs.14376	26 588	7
Laminin receptor 1 (LAMR1)	Hs.374553	17 160	61
Ubiquitin C (UBC)	Hs. 183704	19 628	41
Prostaglandin D2 synthase 21kDa	Hs. 446429	1157	7577
Transgelin	Hs. 433401	8701	8974
Ornithine decarboxylase antizyme 1	Hs. 446427	15 144	125
Nonmuscle/smooth muscle alkali myosin light chain (MYL6)	Hs.77385	16 810	68
Enolase 1 (ENO1)	Hs.433455	19 987	35

^a Genes more highly expressed in cultured HTM cells.^b Raw signal value in arrays hybridised with RNA from free-floating spheres.^c Expression rank in free-floating spheres.