Mesothelial cells: A cellular surrogate for tissue engineering of corneal endothelium

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Abstract:

Purpose: To evaluate whether mouse adipose tissue mesothelial cells (ATMCs) share morphological and biochemical characteristics with mouse corneal endothelial cells (MCECs) and to evaluate their capacity to adhere onto the decellularized basal membrane of human anterior lens capsules (HALCs) as a potential tissue engineered surrogate for corneal endothelium replacement. **Methods:** ATMCs were isolated from the visceral adipose tissue of adult mice and their expression of several corneal endothelium markers was determined with qRT-PCR and immunofluorescence. ATMCs were shortly cultured in a Mesothelial Retaining Phenotype Media (MRPM) and further seeded and cultured on top of the decellularized basal membrane of HALCs. ATMCs-HALCs composites were evaluated by optical microscopy, immunofluorescence and transmission electron microscopy. **Results:** Freshly isolated and MRPM cultured ATMCs expressed the corneal endothelium markers COL4A2, COL8A2, SLC4A4, CAR2, Na⁺/K⁺-ATPase, β -catenin, Zona Occludens-1 and N-cadherin at both the mRNA and protein levels. Furthermore, ATMCs displayed strong adhesion capacity onto the basal membrane of HALCs and formed a confluent monolayer within 72 hours of culture in MRPM. Ultrastructural morphological and marker characteristics displayed by ATMCs monolayer onto HALCs clearly indicate that ATMCs retained their original phenotype of squamous epithelial-like cells. **Conclusions:** MCECs and ATMCs share morphological and markers similarities. ATMCs adhered and formed structures mimicking focal adhesion complexes with the HALCs basal membrane. Monolayer structure and achieved density of ATMCs support the proposal of using adult human MCs as morphological and functional surrogate of damaged corneal endothelium.

Introduction

Tissue engineering is a complex interdisciplinary field gathering principles and methods of bioengineering, material science, and life sciences aimed to build biological surrogates which may be able to substitute for the native tissue functions lost after disease or traumatic processes¹⁻³. The election of a suitable cellular phenotype displaying the functions of the damaged cells in combination with the use of an adequate biological support or scaffold mimicking finely the biophysical properties of the tissue (i.e matrix protein composition and stiffness) are two major parameters that need to be carefully determined to engineer successful tissues biomimetics and ensure their long-term patency after transplantation.

The cornea is an extraordinary example of fine natural engineering, with its sophisticated disposition of collagen lamellae and cells with the lack of blood vessels in order to shape a total clear lens⁴. In this disposition, the corneal endothelium with its single layer of flat hexagonal cells attached firmly to the Descemet's membrane play an important role in regulating the state of corneal stromal hydration by a Na⁺/K⁺-ATPase endothelial pump and focal tight junctions that allows permeability to nutrients and other molecules from the aqueous humor. Furthermore, corneal endothelial cells (CECs) are metabolically very active, with large numbers of mitochondria to provide the high amount of energy required to pump water efficiently⁴.

Cumulative evidence suggests that the postnatal corneal endothelium lacks regenerative capacities and compensates its gradual loss of cellularity along life through hypertrophy of preexisting cells⁴⁻⁶. A critical loss of corneal endothelium cellularity caused either by accidents, surgical trauma or diseases may no longer ensure a proper regulation of stromal hydration, leading to severe corneal swelling, loss of stromal transparency and severe visual impairment. The only effective treatment so far is corneal transplantation to restore normal vision. Descemet stripping automated endothelial keratoplasty (DSAEK)

allows a selective substitution of the damaged corneal endothelium achieving very good results^{7, 8}. Unfortunately, the development of DSAEK is largely constrained by the limited availability of donor corneas.

Several studies have reported the tissue engineering of corneal endothelium biosubstitutes as an alternative issue to a corneal transplantation: Cultured HCECs transplanted onto chitosan-based membranes^{9, 10}, Descemet's membrane¹¹⁻²¹, collagen matrix²², human corneal stromal discs^{23, 24}, gelatin hydrogel discs^{25, 26}, acellular porcine corneal matrix²⁷, pericellular matrix prepared from human decidua-derived mesenchymal cells²⁸ and plastic compressed collagen²⁹. Alternatively, the lack of source of HCECs and their limited proliferation capacity in vitro also led to several researchers to evaluate the generation in vitro of CEC-like cells from distinct cell types such as embryonic neural crest cells¹⁰, corneal stroma stem cells³⁰, umbilical cord blood mesenchymal stem cells³¹ or human ESCs³². Despite these interesting results highlighting the different potential of these stem cells in regeneration of the corneal endothelium, their clinical application is however facing different problems related to immune rejection, ethic or limited tissue accessibility. The need to overcome these limitations will imply further efforts to identify novel sources of autologous cells that are phenotypically and functionally as close as possible to native CECs and that should be in addition, preferentially isolated from a source that is extraocular, abundant and clinically accessible.

The mesothelium is the outermost tissue layer lining the parietal surface of coelomic cavities (pleural, pericardial and peritoneal) and the visceral organs where they are housed³³. It was first described by Bichat in 1827 as a tissue displaying features of simple squamous epithelium³⁴. Although mesothelial cells (MCs) originate from the embryonic mesoderm^{33, 35}, they rather display morphological and biochemical characteristics consistent with simple squamous epithelial cells^{33, 36}. Among their main biological functions

described so far is to secrete glycosaminoglycans and lubricants to provide a protective and slippery surface for the optimal sliding of visceral organs inside coelomic cavities, such as the beating heart or the expanding lungs^{31, 36-38}. In addition, MCs also play a central role in a variety of intraserosal and submesothelial processes, including the transport of water and solutes, inflammation, host response, angiogenesis, tissue repair, and extracellular matrix remodeling^{31, 36-40}.

A careful examination and comparison of the morphological and biochemical hallmarks specific to the mesothelium and corneal endothelium leads to the conclusion that both tissues share many similarities. Hence, they are both composed of a single monolayer of flattened cells tightly compacted together and that is anchored onto a basement membrane that functions as a scaffold to keep orderly tissue structures^{4, 37, 41}. Developmentally, the corneal endothelium is derived from the cranial neural crest^{42, 43}. In contrast to vascular endothelial cells, HCECs lack significant expression of the vascular ECs markers von Willebrand Factor (Factor VIII) and CD31^{44, 45}. Phenotypically, HCECs rather resemble to human MCs, and as such, both cell types constitutively express cytokeratin 18⁴⁶, an intermediate filament that is however absent in vascular endothelial cells⁴⁷. Furthermore, HCECs also express significant levels of the mesothelial proteins HBME1, mesothelin and calbindin 2⁴⁸. Functionally, the corneal endothelium and mesothelium are both semi-permeable membranes involved in electrolytes and water transport, mechanisms which are mainly mediated through their significant Na⁺/K⁺-ATPase pump activity^{4, 49, 50}. Taken in account these findings, we hypothesized that MCs may represent a valuable cellular surrogate of CECs and should serve to build corneal endothelium biomimetics.

Unpublished results from CABIMER and NewBiotechnic researchers indicate that mouse adipose tissue MCs (ATMCs) can attach to different biomaterials, retaining both the

capability to proliferate in a monolayer until they fit the whole area. Once attained a full coverage, ATMCs display contact inhibition of proliferation. Working on this basis, we hypothesized that the adult mesothelium should represent a valuable source with capacity to substitute structurally and biochemically the damaged corneal endothelium. In this study, we devised a methodology to achieve a full mesothelialization of the decellularized basal membrane of human anterior lens capsules using mouse ATMCs.

Material and Methods

Isolation of mouse ATMCs

ATMCs were isolated from CD1 adult female mice. Guidelines for the animal research protocols were established and approved by the Animal Experimentation and Ethics Committee of CABIMER. Briefly, uterine cords and adipose tissue were surgically separated. Enzymatic detachment of ATMCs from fat pads was performed with minor modifications as previously reported for the isolation of mouse uterine MCs⁵¹.

Culture of ATMCs

ATMCs were seeded (35000 cells/cm²) into T-25 flask (136196, Nunc) into 5 ml of a Mesothelial Retaining Phenotype Media (MRPM) consisting of a DMEM low glucose GlutaMax media (21885-05, Gibco) supplemented with 2% FBS (Lonza), 1% B27 supplements (17504, Gibco), 1% penicillin-streptomycin (Gibco), 100 µM β-mercaptoethanol (31350-010, Gibco) and 1 µg/ml of hydrocortisone (Sigma Aldrich). ATMCs were cultured for 2 days in MRPM (5% CO₂, 21% O₂ and 37°C) and harvested with trypsin (15400-054, Gibco).

Murine corneal endothelial cells culture

Primary murine corneal endothelial cells (MCECs) were isolated by collecting corneas from two to three adult CD1 mice (2-6 months old). Corneal endothelium layer was gently stripped off with the help of a scalpel. MCECs clusters released through stripping were then subjected to primary explant culture during 7 days in an incubator (5%

CO₂, 21% O₂, 37°C) in a medium consisting of a DMEM low glucose GlutaMax media (21885-05, Gibco) containing 5% FBS (Lonza), 1% penicillin-streptomycin (Gibco), 1% non-essential amino acids (Gibco), 100 µM β-mercaptoethanol (31350-010, Gibco), 1X ITS (41400045, Gibco), 10 ng/ml recombinant murine bFGF (450-33, PeproTech), 10 ng/ml recombinant murine EGF (315-09, PeproTech) and 1 µg/ml hydrocortisone (Sigma Aldrich). Primary expanded MCECs were further subcultured three times at 5000 cells/cm² during 4-5 days into T-75 flasks (156499, Nunc). MCECs in subculture passage 3 used in the study were termed P3 MCECs.

Immunofluorescence

For immunofluorescence, cells were cultured into µ-Dish (45079, Ibidi GmbH, Germany). For cell-surface antigens detection, cells were fixed with 4% PFA (P6148, Sigma Aldrich) and blocked in PBS containing 3% BSA (PBS-BSA). For intracellular antigens detection, cells were permeabilized with 0.5 % Triton X-100 (Sigma, T8787) or cold methanol (-20°C) and then blocked into PBS-BSA. Antibodies used in this study are described in supporting information Table S1. Nuclei were counterstained with 1 µg/ml Hoechst 33342 (Sigma, 14533). Fluorescence images were captured with an inverted fluorescence microscope Olympus IX71 (Olympus. www.olympus.co.uk).

Quantitative reverse Transcription-Polymerase Chain Reaction

Total RNA content was extracted with RNeasy Mini Kit (74104, QIAGEN), and reverse-transcribed into cDNA by using MMLV reverse transcriptase (Promega, Madison, WI, USA)). Quantitative real-time PCR was performed using SYBR-Green and detected using an ABI Prism 7500 system (Applied Biosystems, Foster City, CA, USA). Gene expression was normalized to HYWAZ mRNA (TATAA Reference Gene Panel, ref. D101-D136, TATAA Biocenter AB, Göteborg, Sweden). Stripped corneal endothelium served as the calibrator sample. Primers sequences are in listed in supporting information Table S2.

Human anterior lens capsules

Adjustment of HALCs

A total of 34 human anterior lens capsules (HALCs) of 5 mm diameter were obtained after patient informed consent during normal cataract surgery procedures. HALCs were directly stored in distilled water to accomplish a total decellularized basal membrane. To note, HALCs were invariantly rolled or folded due to their natural convex structure, being their inner side corresponding to the decellularized epithelial side. HALCs were further distributed into 35 mm cell culture dishes vented with 4 inner rings (627170, Greiner CELLSTAR®) into 100 µl of distilled water. HALCs were correctly oriented with their outer side facing plastic surface. Their complete flattening was achieved by eliminating gradually water with the use of a pipette and needles.

Seeding of HALCs with ATMCs

An initial volume of 60µl MRPM containing 10^5 ATMCs was dropped onto HALCs. Culture dishes containing the seeded HALCs were then placed in a 140 mm Petri dish containing pieces of tissue paper soaked in water to maintain optimal humidification and were finally transferred in an incubator (5% CO₂, 21% O₂, 37°C). Around 80-90% of ATMCs were already firmly adhered after 2 hours of culture. An additional volume of 120 µl of MRPM was then carefully added to the rings. After 6 hours, a final volume of 1.2 ml of MRPM was carefully added out of the rings. After 24 and 48 hours, MRPM (1.5 ml) was exchanged. After 72 hours, HALCs typically displayed complete cellular coverage and termed ATMCs-HALCs composites.

Transmission electronic microscope

ATMCs-HALCs composites (n=8) were fixed with 1.6% glutaraldehyde in 0.15M sodium cacodylated buffer (pH 7.3) for 1 hour at 4°C. HALCs were then post fixed in 1% OsO₄ in the same buffer, dehydrated in ethanol and embedded in epoxy resin. Thin sections were cut and stained with uranyl acetate and lead citrate to be examined with a PHILIPS CM-10 transmission electron microscope operating at 80 kV. The electron

microscopy data were collected after identification of transversal cuts of ATMCs-HALCs composites at primary magnification 5,000x and pictures were taken at enlargements of 20,000x.

Immunohistochemistry

Mouse corneas from CD1 adult mice were fixed with 4% PFA, permeabilized and blocked into PBS-Tx-BSA and finally embedded in OCT cryostat sectioning medium. Alternatively, corneas were fixed and permeabilized in cold methanol for Na⁺/K⁺-ATPase and β -catenin detection. Thin sections (15 μ m) were mounted onto poly-L-lysine coated glass slides and incubated with primary and secondary antibodies (supporting information Table S1).

MetaMorph-based quantification of cellularity

The cellularity of ATMCs-HALCs composites was quantified with Meta Imaging Software MetaMorph Offline version 7.5.1.0 (MDS Analytical technologies, Sunnyvale, CA, USA). Squares areas of 40 000 μ m² (n= 12) were extracted from phase contrast pictures of ATMCs lining HALCs and numbers of cells in each square were calculated with “manually count objects” function. For cell density quantification of the adult mouse corneal endothelium, intact corneas were fixed with PFA and stained with Hoechst 33342 before being mounted in sandwich between thin glass coverslips. Fluorescence pictures of the corneal endothelium layer were taken. Numbers of cells in squares areas of 40 000 μ m² were determined by calculating numbers of nuclei. Mean numbers of cells in squares were used to deduce the mean $\pm$ SD number of cells/mm².

Statistical analysis

Values are presented as mean $\pm$ SD. Statistical significance was calculated by using an unpaired Student's t-test, p <0.05 was considered significantly different.

Results

Characterization of ATMCs cultured in MRPM

A gentle trypsinization of the adipose tissue could efficiently and selectively release its outermost MCs layer (Fig. 1A). Adipose tissue MCs (ATMCs) cultured for 48 hours in MRPM typically generated a cobblestone-like monolayer of flattened polygonal epitheloid cells (Fig. 1B). Consistent with their mesothelial phenotype, the MRPM cultured ATMCs displayed typical intercellular expression of β -catenin and zona occludens-1 (ZO-1). Furthermore, they also displayed wide nuclear expression of Wilm's Tumor Protein (WT1) and membrane expression of mesothelin (data not shown). The MRPM cultured ATMCs lacked detectable expression of the pan-endothelial cells marker CD31. Furthermore, and consistent with their mesothelial nature, the MRPM cultured ATMCs did not exhibit stress fibers positive for F-actin and α -SMA. Finally, ATMCs culture displayed some nuclei Ki-67 positive, indicating that a pool of ATMCs was proliferating.

Isolation and characterization of mouse CECs

Our stripping procedure typically resulted in the detachment of large clusters of the corneal endothelium layer from the Descemet's membrane (Fig. 2A and 2B). Immunofluorescence analysis of fragments of stripped Descemet's membrane could confirm that they were mainly devoid of their corneal endothelium layer (Supplementary Fig. S1). Clusters of MCECs subjected to explant culture could successfully adhere, spread and proliferate to form a compact monolayer with cobblestone morphology within 5-7 days of culture (Fig. 2C). The establishment of highly homogeneous cultures of polygonal MCECs could be achieved through sequential subculture of MCECs until passage 3 (P3 MCECs) (Fig. 2D-F).

ATMCs share phenotypic marker similarities with MCECs

qPCR analysis

ATMCs cultured for 2 days in MRPM and P3 MCECs were analyzed by qPCR for

their expression of several corneal endothelium markers (Fig. 3). Collagen alpha-2 IV and VII chains (COL4A2 and COL8A2, respectively), solute carrier family 4 (anion exchanger), member 4 (SLC4A4), carbonic anhydrase II (CAR2), Na⁺/K⁺-ATPase subunit alpha-1 (ATP1A1) and N-cadherin (CDH2) were expressed in significantly higher extent in the stripped corneal endothelium than in whole corneas.

Interestingly, COL4A2 and N-cadherin genes were found to be expressed many times higher in P3 MCECs than in the stripped corneal endothelium (freshly isolated MCECs), evidencing thus phenotypic changes undertaken by MCECs upon culture. In this way, COL8A2 and CAR2 genes were in contrast strongly down-regulated in P3 MCECs as compared with freshly isolated MCECs, confirming hence a partial loss of the CECs phenotype upon subculture.

Relevantly, we found that COL4A2, SLC4A4, CAR2 and Na⁺/K⁺-ATPase genes were expressed at higher levels in freshly isolated ATMCs than in the stripped corneal endothelium, suggesting hence that the mesothelium tissue share phenotypic and functional similarities with the corneal endothelium. COL8A2, a highly specific corneal endothelium marker⁵² was in contrast expressed to a lower extent in freshly isolated ATMCs ($P<0.03$). As previously found for cultured MCECs, COL4A2 and N-cadherin genes were also strongly up-expressed in the MRPM cultured ATMCs ($P<0.03$ for both genes), suggesting hence phenotypic changes suffered by ATMCs in response to culture conditions. Despite this, the expression of the corneal endothelium genes COL8A2, SLC4A4, CAR2 and Na⁺/K⁺-ATPase was found to be almost similar in the freshly isolated and MRPM cultured ATMCs.

Comparison of the MRPM cultured ATMCs and P3 MCECs indicated that they displayed similar expression pattern for COL4A2, COL8A2, SLC4A4 and N-cadherin genes. By contrast, Na⁺/K⁺-ATPase was however expressed in significantly higher extent in the MRPM cultured ATMCs than in the P3 MCECs ($P<0.03$).

Immunofluorescence analysis

We next used immunofluorescence procedures to determine the expression pattern of corneal endothelium markers in the MRPM cultured ATMCs and P3 MCECs (Fig. 4 and Table 1). Positive immunoexpression of COL8A2, N-cadherin, ZO-1, β -catenin, Na^+/K^+ -ATPase and SLC4A4 could be accordingly detected in the endothelial layer of mouse corneas sections and were widely expressed in P3 MCECs (Fig. 4). Of major relevance, ATMCs cultured in MRPM were also found to immunoexpress these corneal endothelium markers in an extent almost similar to P3 MCECs, indicating hence that ATMCs share many phenotypic similarities with MCECs. Furthermore, ATMCs and MCECs display very similar surface marker patterns as they displayed no expression for CD31 and CD45, low expression for CD44 and high expression for CD29 and CD54 (Table 1).

Characterization of decellularized HALCs

HALCs obtained by capsulorhexis were transparent sheets of around 5 millimeters diameter and 20 μ m thickness. Transmission electronic microscope analysis of decellularized HALCs could confirm that their decellularization was fully accomplished within sterile water (Supplementary Fig. S2).

Characterization of ATMCs-HALCs composites.

Microscopic analysis

Adhesion of ATMCs on top of HALCs was mostly achieved within only 2 hours of culture (data not shown). After 24 hours of culture, ATMCs were firmly adhered on top of HALCs and a subset of them were actively proliferating as indicated by the presence of small round and refringent cells (Fig. 5A). ATMCs gradually reached confluence on top of HALCs between 24 and 72 hours of culture in MRPM (Fig. 5B). Noticeably, confluent ATMCs lacked α -SMA and F-actin positive stress fibers, indicating hence they did not undergo significant EMT (Fig. 5C). Cell density quantification of the 72 hours cultured ATMCs-HALCs composites indicated that the monolayer established by ATMCs reached a

mean cell density of 1378 ± 187 cells/mm² (Fig. 5D). By comparison, the native adult mouse corneal endothelium displayed a mean cell density of 3188 ± 277 cells/mm² (Fig. 5D and Supplementary Fig. S3).

Ultrastructural analysis

Ultrastructural analysis of ATMCs-HALCs composites with transmission electron microscopy indicated the presence of numerous microvilli on the apical membrane of ATMCs (Fig. 6A), structures that are also particularly abundant in CECs²⁹. ATMCs also displayed abundant mitochondria (Fig. 6A) and rough endoplasmic reticulum (Fig. 6B), two organelles that are also particularly abundant in CECs⁴. ATMCs also displayed dense-electron junctional complexes at apicolateral cell-cell contact, a type of structure consistent with desmosomes (Fig. 6B). Interestingly, the basal membrane of ATMCs was found to be in close contact with the HALCs surface and displayed numerous invaginations (Fig. 6C). Other segments of the ATMCs basal membrane were in contrast rather exvaginated, thicker and more electron-dense, a type of structure that is consistent with the establishment of focal adhesion complexes with the HALCs surface.

Discussion

Stem cells–based tissue engineering of corneal endothelium equivalents holds the promise to provide definitive treatments for corneal endothelial disorders. In recent years, significant approaches have been carried out to identify suitable scaffolds and source of stem cells to bioengineer functional corneal endothelium equivalents. In most cases, the different scaffolds proposed were used in combination with primary HCECs^{14, 15, 17, 18, 20-27, 53}. Alternatively, different studies also reported the generation of CECs-like cells with distinct efficiency from corneal stromal cells³⁰ or extraocular stem cells such as neural crest cells¹⁰, umbilical cord blood stem cells³¹ and human ESCs³².

The use of heterologous HCECs to bioengineer functional corneal endothelium equivalents is undoubtedly promising⁷, but is however strongly limited by the important

shortage of suitable donor corneas and the difficulty to achieve their expansion in sufficient numbers without loss of original phenotypic properties due to their induction of EMT by serum components⁵⁴. Finally, and not less important, corneal endothelium equivalents build with heterologous cells are susceptible to immunological rejection, principally in patients with highly vascularized corneal beds⁵⁵.

In this study we show through marker analysis that ATMCs are phenotypically and functionally quite similar to MCECs and may therefore represent a useful cellular surrogate to bioengineer autologous non-immunogenic corneal endothelium equivalents. In additional support of their therapeutic use, previous studies already reported the successful cultivation of human ATMCs isolated from clinical samples of the greater omentum^{44, 45, 56, 57}, the largest visceral adipose tissue depot in humans. Of particular interest, it was indicated that each square centimeter of human omental tissue can provide around one million ATMCs⁵⁷. On this basis, and given that the adult human corneal endothelium is roughly composed by around 300.000 cells⁵⁸, the surgical isolation through laparoscopic surgery of reduced pieces of omental tissue should provide sufficient numbers of ATMCs to bioengineer several corneal endothelium equivalents. Alternatively, the reports that human peritoneal mesothelial cells (HPMCs) can be isolated from peritoneal fluids collected by needle aspiration^{42, 45} also suggest that this minimal invasive approach could also be a way to obtain MCs for tissue engineering of corneal endothelium equivalents.

Our results indicate that the adipose tissue mesothelium and corneal endothelium share morphologic and phenotypic similarities. Of particular relevance, the high expression of functional corneal endothelium markers such as SLC4A4, CAR2 and Na⁺/K⁺-ATPase in the freshly isolated ATMCs suggests that the mesothelium share functional similarities with the corneal endothelium. In support of this concept, two previous reports already indicated that HPMCs display significant Na⁺/K⁺-ATPase pump activity⁴⁹,

⁵⁰, a function that is critically required for the transport of fluids across the mesothelium membrane.

A critical parameter in tissue engineering of corneal endothelium equivalents is the maintenance of the density of replacement cells. Herein, we demonstrate that murine ATMCs could adhere, proliferate and generate a confluent and compact monolayer of polygonal cells firmly attached on top of the HALC basal membrane. Interestingly, previous studies already indicated that HALCs represent an excellent substrate for the adhesion, growth and viability of primary HCECs^{59, 60}, limbal epithelial stem cells, human trabecular meshwork (HTM) cells^{61, 62}. The use of HALC as surrogate of the Descemet's membrane should be strongly supported by the evidence that both structures are smooth and transparent basal membranes permeable to water, electrolytes and nutrients from the aqueous humor. In addition, the HALC and Descemet's membrane display quite similar thickness and elasticity^{63, 64}. Furthermore, they are both principally composed by deposition of collagen fibers, being the lens capsule mainly composed by collagen type III and in less extent by collagen type I⁶⁵ whereas the Descemet's membrane is in contrast mainly composed by collagen type IV and VIII⁶⁶⁻⁶⁸. Comparative transplantations experiments of both HALC-based or Descemet's membrane-based corneal endothelium substitutes remain however to be performed to evaluate the performance of HALC as substrate surrogate of the Descemet's membrane.

Conclusion

Primary murine ATMCs share morphological and functional similarities with murine CECs, suggesting that ATMCs are useful cells to bioengineer non-immunogenic corneal endothelium equivalents. ATMCs could adhere and form adhesion structures with the HALCs basal membrane. Monolayer structure and density of the ATMCs-HALCs composites was better than expected. Further experiments to assess the functionality of human ATMCs as substitute of human CECs are under progress. In the future we propose

a similar technique described as Descemet Membrane Endothelial Keratoplasty technique that provides a fast and high visual rehabilitation⁶⁹.

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Figures legends:

Figure 1. Efficient recovery of adipose tissue mesothelial cells (ATMCs) by gentle trypsinization of the adult mouse visceral adipose tissue. (A) Left photograph shows representative aspect of uterine cords and laterally connected adipose fat pads (AT) after surgical separation. Middle photograph shows isolated visceral adipose fat pads. Right photograph shows the representative aspect of ATMCs released after trypsinization of adipose fat pads. ATMCs were mainly detached as small sheets that rapidly adopted a “grape-like” aspect after full cellular retraction. (B) Immunofluorescence characterization of ATMCs cultured for 48 hours in MRPM (Mesothelial Retaining Phenotype Media). Upper left image shows the typical mesothelial cobblestone-like morphology adopted by ATMCs cultures after 48 hours in MRPM. ATMCs displayed the typical epithelial intercellular expression (arrowhead) of the tight adhesion proteins β -catenin, ZO-1 (Zona Occludens-1). ATMCs displayed ring-like F-actin staining (arrowhead) and diffuse cytoplasmic expression of α -SMA (alpha-smooth muscle actin). ATMCs accordingly lacked immunoexpression of the pan-endothelial marker CD31. A significant number of ATMCs cultured in MRPM displayed nuclear expression of the proliferative marker Ki-67 (arrowheads). Nuclei are counterstained in blue with Hoechst 33342.

Figure 2. Isolation and establishment of MCECs cultures. Corneal endothelium stripping typically released large fragments of Descemet membrane (A) from which were detached large fragment of the corneal endothelium layer during the process of stripping. (B). (C) Adherent culture of MCECs clusters generated a compacted monolayer of MCECs exhibiting polygonal morphologies (see enlarged spot). (D) Morphology of MCECs at the end of their first subculture, with observable large clusters of tightly packaged MCECs. (E, F) Show representative aspect of MCECs morphologies at the end of their second and

third subculture steps, respectively. Note how MCECs retained original cobblestone morphologies through sequential subculture steps.

Figure 3. Quantitative RT-PCR analysis of corneal endothelium markers. Expression profile of COL4A2, COL8A2, SLC4A4, CAR2, Na⁺/K⁺-ATPase and N-cadherin genes was analyzed in whole corneas, stripped corneal endothelium, passage 3 subcultured MCECs (P3 MCECs), freshly isolated ATMCs (do ATMCs) and ATMCs cultured for 2 days into MRPM (d2 ATMCs). Stripped corneal endothelium isolated from six mice served as the calibrator sample (black bars). Gene expression was normalized against expression levels of YWHAZ (housekeeping gene). Results are mean fold change $\pm$ SD in mRNA expression relative to the calibrator sample (set as 1) calculated from three distinct isolation of corneas and three independent isolation and cultures of MCECs and ATMCs. Statistical significance (*P<0.05 and **P<0.03) was determined by using Student's t-test.

Figure 4. Comparative immunofluorescence analysis of corneal endothelium markers expression patterns into cultured MCECs and ATMCs. Left panel shows representative immunoexpression levels of F-actin and of the corneal endothelium markers COL8A2, N-cadherin, ZO-1, β -catenin, Na⁺/K⁺-ATPase and SLC4A4 in mouse cornea for reference. Middle and right panel shows respective immunoexpression patterns obtained in subcultured MCECs (passage 3) and MRPM cultured ATMCs. Nuclei are counterstained in blue with Hoechst 33342.

Figure 5. Morphological analysis of ATMCs seeded Human Anterior Lens Capsules. (A) Left image shows a 10X phase contrast picture of one ATMCs-seeded-HALC after 24 hours of culture in MRPM. White arrows point to the border of the HALC. Plastic surface with adhered ATMCs (left upper area) is observable as out of focus. Right upper images

show enlargement of blue and red spots drawn in left image. Proliferation at this step was evidenced by the presence of many duplets of small round and refringent proliferating cells (arrowhead). Other cells were in turn more flattened (arrow) and represent mature ATMCs. **(B)** Representative aspect of ATMCs-seeded-HALCs surface after 72 hours of culture. At this step HALCs surface was fully recovered by ATMCs that established tight contact between together (see arrow in enlarged black square). **(C)** Double immunofluorescence labelling of F-actin and α -SMA in ATMCs-HALCs composites showing ring like staining pattern of F-actin and absence of α -SMA positive stress fibers in ATMCs. **(D)** MetaMorph-based quantification of cellular density in the adult mouse corneal endothelium and ATMCs-HALCs composites. Data are expressed as the mean $\pm$ standard deviation of cell counts performed from distinct areas (n=12). Statistical significance was determined by using Student's t-test.

Figure 6. Transmission Electron Microscope analysis of ATMCs-HALCs composites.

(A) Thin transverse section through a ATMCs-HALCs complex. A total number of four ATMCs (black arrowheads) can be observed adhered on top of the HALC basal membrane side. Right image shows magnification of black spot. Note how ATMCs, like do MCECs, display typical apical membrane protrusions or microvilli on their apical surface (black arrows) and numerous mitochondria (red stars). **(B)** Higher magnification allowing the visualization of tight contact established between ATMCs. ATMCs typically displayed a large rough endoplasmic reticulum (yellow spot). Right image shows an enlargement of black spot drawn in left image and allows the visualization of electron-dense tight junction complexes at apicolateral intercellular contact between ATMCs (black arrowheads). **(C)** Left image shows representative aspect of ATMCs basal membrane in tight contact with HALCs surface. Right image shows an enlargement of black spot drawn in left image and allows the visualization of invaginated segments of ATMCs basal membrane (red arrows)

and of exvaginated membrane segments that are strongly electron-dense (black arrowheads) and featuring hemidesmosomes.

A

B

Stripped Descemet membrane

Corneal endothelium layer

Cornea

MCECs

ATMCs

F-actin

COL8A2

N-cadherin

ZO-1

β -catenin Na⁺/K⁺ATPase

SLC4A4

A

24 hours

B

72 hours

C

F-actin- α -SMA-nuclei

D

A

B

C

Table 1. Marker expression profile of mouse ATMCs and CECs

Antigen	Adipose tissue mesothelial cells	Corneal endothelial cells
β-catenin	++	++
ZO-1	++	++
COL8A2	+ / ++	++
SLC4A4	+ / ++	+
Na⁺/K⁺-ATPase	++	++
N-cadherin	+ / ++	++
CD54 (ICAM-1)	++	++
CD29 (Integrin β1)	++	++
CD31 (PECAM-1)	-	-
CD45 (LCA)	-	-
CD44 (HCAM)	+	+

(-): no expression; (+): low expression; (+ / ++): intermediate expression; (++) : high expression.

Abbreviations: COL8A2, Collagen alpha-2(8) chain; ICAM-1, intercellular cell adhesion molecule 1; LCA, leukocyte common antigen; PECAM-1, platelet endothelial cell adhesion molecule 1; SLC4A4, solute carrier family 4 (anion exchanger), member 4; ZO-1, zona occludens 1.