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ORIGINAL

Comparison of characteristics of mouse immortalized normal endothelial cells, MS1 and primary cultured endothelial cells.

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ABSTRACT : Tumor blood vessels support the progression of tumors by providing nutrition and oxygen required for growth. By acting as gatekeepers, they allow the metastasis of tumors to secondary locations. An important strategy in cancer therapy has been to target tumor blood vessels consequently inhibiting tumor angiogenesis. To date, antiangiogenic therapy being employed for cancer treatment have yielded profoundly good results. However, it has been shown that current antiangiogenic drugs have several problems, such as adverse side effects and drug resistance. Tumor endothelial cells (TEC), which line the inner layer of blood vessels of the tumor stromal tissue, are the main targets of the antiangiogenic therapies. TEC have been reported to differ significantly from endothelial cells resident in normal blood vessels. These differences provide a window through which TEC can be targeted solely with little or no impact on normal endothelial cells (NEC). Currently, as part of new antiangiogenic drug discovery processes, cell-based screening is being performed using thousands of small chemical compounds. For the success of such screening purposes, there is a need to obtain the right kind of cells and in adequate quantities. Primary-cultured endothelial cells isolated from murine / human blood vessels are the preferred choice. However, maintenance of the primary-cultured endothelial cells is costly and overtime these cells become senescent and perish. As a result, MS1, SV40 immortalized islet-derived endothelial cells, have been used in place of the primary-cultured cells. MS1 is commercially available with comparatively cheaper cell culture requirements.

In this study, we compared the characteristics of MS1 and primary-cultured endothelial cells; NEC and TEC to investigate the possibility of using MS1 cells for chemical screening in search of a new antiangiogenic drug. MS1 cells proliferate faster compared to TEC and upregulated the mRNA expressions of CD133 and Sca-1 genes. However, mRNA expression of most of the other genes, which were upregulated in TEC compared to NEC, were also expressed at lower levels in the MS1 cells. Furthermore, MS1 migrated at a slower rate and did not form tubes on matrigel, as opposed to the function of TEC. In conclusion, MS1 did not completely resemble NEC, nor TEC in function and gene expression. It is suggested that for chemical screening, primary-cultured TEC and the corresponding NEC would be a more ideal choice of cells.

Key Words : Tumor endothelial cell, MS1, stem cell, antiangiogenic therapy, chemical screening

INTRODUCTION

endothelial growth factor (VEGF) inhibitor has improved the prognosis of various cancer patients¹⁾. However, these anti-VEGF drugs sometimes cause adverse effects such

Recently, antiangiogenic therapy such as anti-vascular

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as hypertention or delayed wound healing since VEGF is also important for physiological angigogenesis in normal tissue². It was initially presumed that tumor blood vessels may be the same as normal ones. However, currently it has been elucidated that tumor blood vessels differ morphologically and phenotypically from their normal counterpart. Furthermore, endothelial cells which line inner blood vessels also differ between in tumor blood vessels (Tumor endothelial cells : TEC) and in normal ones (Normal endothelial cells : NEC)³⁾. We have isolated and cultured TEC and reported that TEC are different from NEC in many aspects. Most strikingly, we have found that there is chromosomal abnormality even in TEC which is in tumor stroma^{4, 5)}. In addition, we have reported that TEC showed anti-cancer drug resistance, and they expressed some of stem phenotype, including expression of ABC transporter, *P-glycoprotein* (p-gp)^{6, 7)}, Aldehyde dehydrogenase (ALDH)⁸⁾ and osteoblast differentiation and sphere formation⁹⁾. Development of novel and safer tumor antiangiogenic agents is necessary to target tumor blood vessels more specifically. This has become an important objective in cancer therapy but its success will depend on an understanding of the biology of TECs.

We have reported several TEC markers by DNA microarray analysis comparing the gene expressions between TEC and $NEC^{10.13}$.

Recently, intensive high-throughput compound screenings have been common strategy for anti-cancer drug discovery. Accordingly, by intensive high-throughput compound screenings that target key molecules involved in those signaling pathways, a number of chemical compounds have been identified that specifically target tumor cells. Indeed, by this strategy, several drugs have been successfully invented, and some of them are now clinically used. To test the inhibitory effect of thousands of chemicals, it is necessary to recruit proper cells and prepare enough number of these cells. Mostly, for anticancer drug discovery, the effect of chemicals on signaling pathways and cell function have been clarified the differences between normal and tumor cells.

To identify chemicals which has antiangiogenic effects, endothelial cells should be tested in the screening. However, there are bottlenecks in maintaining enough number of primary cultured endothelial cells. For example, isolation from endothelial cells from tissue is technically complicating, and endothelial cell specific culture medium is costly. In addition, a shortage of cell numbers caused by cellular senescence in endothelial cells may be another issue. On the other hand, there are immortalized endothelial cells which are commercially available. MS1 is one of those option and they are mouse islet-delived normal endothelial cells which are immortalized by SV40 large T antigen¹⁴⁾. MS1 cells are well growing and the cost of culture them is much cheaper than those of primary cultured cells. Thus, it was assumed that it may be possible to apply MS1 cells for chemical screening assay. However, there has been no study to address whether MS1 resembles to TEC or to NEC.

In this study, to address the possibility of using MS1 cells for chemical screening, we compared the characteristics of MS1 and primary-cultured endothelial cells; TEC and NEC which we have isolated.

Materials and Method

Cell lines

The super-metastatic human malignant melanoma cell line A375SM was a kind gift from Dr. Isaiah J. Fidler (MD Anderson Cancer Center, Houston, TX, USA). The cells were cultured in minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% heatinactivated fetal bovine serum (FBS ; PAA Laboratories, Pasching, Austria). MS1 (mouse immortalized islet-derived normal endothelial cells) was obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Antibodies and reagents

The following antibodies were used : Purified Rat Anti-Mouse CD31 antibody (BD Pharmingen, San Diego, CA, USA), Purified Rat Anti-Mouse CD144 antibody (BD Pharmingen), Goat Anti-Rat Alexa Fluor 647 Antibody (BioLegend, San Diego, CA), Goat Anti-Rat Alexa Fluor 488 Antibody (Invitrogen, Tokyo, Japan), Alexa Fluor 647 *Griffonia simplicifolia* 1 isolectin B4 (GS1B4) (Life Technologies), fluorescein isothiocyanate (FITC) *-Bandeiraea simplicifolia* lectin isolectin B4 (BS1-B4 ; Vector Laboratories, Burlingame, CA, USA).

Animals

6-week-old female nude mice (BALB/c AJcl-nu/nu) were purchased from Clea Japan. A week later, we injected A375SM (1×10^6 cells) subcutaneously into the

right flanks of nude mice. All procedures for animal experiments were approved by the local animal research authorities, and animal care was in accordance with the institutional guidelines of Hokkaido University.

Isolation of mouse TECs and NECs

As described previously^{4, 9}, TECs were isolated from human tumor xenografts (A375SM) in nude mice. NECs were isolated from their dermis as controls. Briefly, xenografted A375SM tumors in 10 nude mice and the dermis of normal nude mice were minced. ECs were sorted as CD31+ cells using MACS magnetic separation system according to the manufacturer's instructions (Milltenyi Biotec, Bergisch Gladbach, Germany). Isolated ECs were plated onto fibronectin ($10\mu g/mL$)-coated plates and cultured in EGM-2 MV (Lonza, Basel, Switzerland) and 15% FBS. Diphtheria toxin (Calbiochem, San Diego, CA) (500 ng/mL) was added to TEC subcultures to kill any remaining human tumor cells. The isolated ECs were purified by a second round of purification using FITC-BS1-B4.

All purified ECs were cultured in EGM-2 MV at 37 $^{\circ}$ C in a humidified atmosphere of 5% CO₂ and 95% air. After TECs and NECs were characterized that they are positive for EC markers (CD31, CD105, CD144, VEGFR1, VEGFR2) and negative for hematopoietic markers (CD11b, CD45) and human cells gene (human HB-EGF) by real-time PCR, the cells were used in experiments.

Immunocytochemistry

MS1 was cultured on cover glass and fixed in 100% methanol for CD31 staining, isolectin GS-IB4 staining, and in 4% parafolmaldehyde (PFA) for CD144. After cells were blocked with 5% goat serum in PBS for 1h at room temperature, cells were incubated with primary antibodies or lectin for 16h at 4°C. Then cells were immunostained with the secondary antibody for 2h at room temperature. All samples were counterstained with 4, 6-diamidino-2-phenylindole (DAPI; Roche, Indianapolis, IN) and visualized using an FV1000 confocal microscope (Olympus, Tokyo, Japan). Images were analyzed with Fluoview FV10-ASM Viewer software (Olympus).

Real-time RT-PCR

Total RNA was extracted and first-strand complementary DNA (cDNA) was synthesized using ReliaPrep RNA Cell Miniprep System (Promega) and first-strand complementary DNA (cDNA) was synthesized from 1 μ g

of RNA using ReverTra Ace, RT Buffer (ToYoBo, Osaka, Japan). Oligo dT primer, Random primer, dNTP mixture (Takara Bio, Shiga, Japan). The primers used for RT-PCR are indicated below.

mouse GAPDH :

Fw: 5'- GGGTGGTGGACCTCATGGCCTACAT -3',

Rv: 5'- CGAGTTGGGATAGGCCTCTCTTGC -3',

mouse vascular endothelial growth factor \boldsymbol{A} (VEGFA) :

Fw: 5'- CCTGCCGAAGCTCTCCACGATTTG -3',

Rv: 5'- AGAACACTTGTTGCAGGCAGCGG -3',

mouse VEGF receptor 1 (VEGFR1) :

Fw: 5'- GAGGTAGTGCTAGTGGTGGTGG -3',

Rv : 5'- TCCCCTCCTGCTTCTGCTTG -3', mouse VEGFR2 :

Fw: 5'- GCCCTGCTGTGGTCTCACTAC -3',

Rv: 5'- CAAGCATTGCCCATTCGAT -3',

mouse Aldehyde dehydrogenase (ALDH) :

Fw : 5'- TCCGTCATGACCAGGTGCTTTCCA -3', Rv : 5'- ACAACACCTGGGGAACAGAGCAGC -3', mouse CD90 :

Fw: 5'- TGTAGTGAGGGTGGCAGAAGAA -3',

Rv : 5'- AGGGGCAAGGGAAAGAAGAATA -3', mouse stem cell antigen-1 (Sca-1) :

Fw: 5'- GAAGAGGCAGAATTCCAAGG -3',

Rv : 5'- ATGTGGGAACATTGCAGGAC -3', mouse CD133:

Fw: 5'- GCAAAAGCAAACCAGTTGCCTG -3',

Rv : 5'- TCAGTGATTGCTGCACAGGAGG -3', mouse interleukin-6 (IL-6) :

Fw: 5'- CTGATGCTGGTGACAACCAC -3',

Rv: 5'- TCCACGATTTCCCAGAGAAC -3',

mouse differentially expressed in FDCp 6 homolog (Def6) :

Fw: 5'- CACCAACGTGAAACACTGGAATG -3',

Rv: 5'- CGGGTCAGGCGCTTTAGAGA -3',

mouse transmembrane protein 176b (Tmem176b) :

Fw: 5'- CCCTTGCAATCAAGCATGGAC -3',

Rv : 5'- GCATGATGCAGAAGGCTAGGAA -3', mouse Biglycan :

Fw : 5'- AACTCACTGCCCCACACAGCTTC -3', Rv : 5'- GCGGTGGCAGTGTGCTCTATCCATC -3', mouse lysyl oxidase (LOX) :

Fw: 5'- GCACACACACAGGGATTGAG -3',

Rv : 5'- CCAGGTAGCTGGGGTTTACA -3', mouse prostacyclin receptor (PTGIR) :

Fw : 5'- AGGCAGAGGTGCTGGAGGGTCTAGA -3',

Rv : 5'- TGCCAGTCAATTCCCAGTTGCCCG -3', mouse cyclooxygenase-2 (COX-2) :

Fw: 5'- AGGAACTCAGCACTGCATCCTGCC -3',

Rv : 5'- GAGCAGCACAGCTCGGAAGAGCATC -3', mouse suprabasin (SBSN) :

Fw: 5'- CCCATGAGGATCCCGTTGA -3',

Rv: 5'- CCAGCTTGAGTGATTCCGTTATTG -3',

mouse C-X-C chemokine receptor type 7 (CXCR7) :

Fw: 5'- GTTGTCCTCACCATCCCAGT -3',

Rv: 5'- CTCATGCACGTGAGGAAGAA -3'

Real-time PCR was conducted using KAPA SYBR Fast qPCR kit (NIPPON Genetics Co, Ltd, Japan). Cycling conditions were set according to the manufacturer' s instructions based on the use of CFX Manager (Bio-Rad, Hercules, CA). mRNA expression levels were normalized to GAPDH levels and analyzed using delta-delta-Ct method.

Cell proliferation assay

 1×10^3 cells per well were seeded into 96-well dishes in EGM-2 MV (TEC and NEC) and DMEM supplemented with 10% FBS (MS1). Cell proliferation was measured every day for 3 d by MTS assay (Promega, Tokyo, Japan).

Wound scratch assay

 4×10^5 cells per well were seeded into 6 well dishes. MS1 was cultured in DMEM with 10% FBS or EGM-2 MV. TEC were cultured in EGM-2 MV. When cells reached confluence, ECs were wounded by scraping using p200 tip. Cell movements were observed every 4h for 24h.

Tube formation assay

Growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) was placed in each well of a 96-well dish and incubated at 37° C for 30 min. Each cells were resuspended in EBM-2 without FBS and seeded at 1.6×10^4 cells per well, and incubated at 37° C for 12h.

Statistical analysis

Differences between groups were evaluated by oneway ANOVA, and with Tukey-Kramer to test multiple comparisons. Differences were considered significant if p<0.01.

RESULTS

Endothelial cell markers expression in MS1 cells

Expression of endothelial cell markers in MS1 cells was analyzed by immunofluorescent staining. Cell adhesion molecule, CD31 and CD144 expression were confirmed in MS1 and these molecules were localized in cell membrane when the cells were contacted to each other. Furthermore, GS1B4 lectin binding, which is one of endothelial specific phenotype, was observed (Fig. 1).

CD31 GS1B4 CD144

Fig. 1 Endothelial cell marker expressions in MS1 cells. Immunocytochemisty of CD31 (red) and CD144 (green) in MS1. Endothelial cell specific lectin, GS1 B4 binding was shown (red). Scale bar, 50μm.

MS1 grows faster compared to primary-cultured endothelial cells.

TEC proliferated faster than NEC in consistent with our previous reports. MS1 showed even higher proliferation rate compared to TEC. Compared to the number of cells at the beginning, 72h after cell seeding, the cell number was 6 times and 3 times more in MS1 and TEC, respectively. These results suggested that MS1 proliferate faster compared to TEC (Fig. 2).





mRNA Expressions of stem markers.

Since it is reported that MS1 cells form benign tumor (hemangioma) when they are inoculated subcutaneously in mouse and they have tumorigenesis¹⁴, expression of stem-related marker expression was analyzed in MS1, TEC and NEC. mRNA expression levels of Sca-1, CD90, CD133, IL-6 and ALDH were compared among these endothelial cells. These genes were significantly upregulated in TEC compared to NEC in consistent with our previous reports^{8, 15)}. MS1 showed significantly lower expression level of CD90, IL-6 and ALDH mRNAs, compared to TEC, as NEC did. On the other hand, Sca-1 and CD133 were significantly upregulated in MS1 even compared to NEC (Fig. 3).



Fig. 3 mRNA expressions of stem markers. mRNA expression levels of stem markers ; Sca-1, CD90, CD133, IL-6 and ALDH were analyzed by Real-time PCR. n=3. Error bar shows standard deviation. (*P<0.01)</p>

mRNA expression of angiogenesis related genes.

We have reported that TEC showed proangiogenic phenotype via autocrine loop of upregulated angiogenesis related-genes, such as VEGFA^{12, 15, 20)}. mRNA levels of VEGFA and its receptors, VEGFR1 and VEGFR2 were significantly higher in TEC compared to NEC, consistentently with our previous reports¹⁵⁾. mRNA levels of VEGFA and VEGFR1 were lower in MS1 than in NEC. On the other hand, VEGFR2 expression levels were significantly higher in MS1 compared to TEC and NEC (Fig. 4).

TEC marker expressions in MS1 cells.

We have reported that several molecules which are highly upregulated in TEC compared to NEC, as TEC markers^{10, 13, 16, 17)}. TEC marker expression levels were compared among endothelial cells. MS1 expressed significantly lower level of LOX, Biglycan, Def6, Tmem176b and SBSN compared to TEC as well as NEC (Fig. 5).



Fig. 4 mRNA expressions of angiogenesis -related genes. mRNA expression levels of VEGFA,VEGFR1 and VEGFR2 were analyzed by Real-time PCR. n=3. Error bar shows standard deviation. (*P<0.01)</p>



Fig. 5 mRNA expressions of TEC markers.

mRNA expression levels of LOX, Biglycan, Def6, Tmem176b and SBSN were analyzed by Real-time PCR. n=3. Error bar shows standard deviation. (*P<0.01)

Expression of inflammatory related TEC marker in MS1 cells

We have reported that TEC upregulated inflammatory related genes; COX-2¹⁸⁾, CXCR7^{19, 20)}, and PTGIR¹¹⁾ compared to NEC. IL-6 is also inflammatory marker as well as stem marker. IL-6 expression level was lower in MS1 as described before. COX-2 and PTGIR mRNA levels were lower but CXCR7 mRNA level was





Fig. 6 mRNA expressions of inflammatory-related TEC markers. mRNA expression levels of COX-2, CXCR7 and PTGIR were analyzed by Real-time PCR. n=3. Error bar shows standard deviation. (*P<0.01)</p>



Fig. 7 Cell migration and tube formation

- Cell migration was analyzed by wound scratch assay in MS1 and TEC.
- b. Tube formation on matrigel by MS1 and TEC. Representative figures are shown.

Cell migration and tube formation in MS1 cells.

The results aforementioned above suggested that MS1 cells maintain NEC phenotype, although they are immortalized and have tumorigenesis. To analyze function of endothelial cells, cell migration was compared between MS1 and TEC by wound scratch assay. TEC migrated faster and the space made by scratch was almost completely closed 24h after. In contrast, MS1 did not migrate neither in DMEM with 10% FBS nor in EGM-2MV and the space were remained (Fig. 7a). Next, tube formation on matrigel was analyzed in these cells. It has been reported that TEC and NEC form tubes during culture, suggesing that they maintain endothelial cell function¹⁵⁾. MS1 did not make tubes in DMEM with 10% FBS which is regular culture medium for them. Furthermore, MS1 did not show tube formation even in EGM-2MV which is culture medium containing several angiogenic growth factors and is medium for TEC, neither (Fig. 7b).

DISCUSSION

Angiogenesis is the process of formation of new blood vessels and is required for tumor growth as tumor blood vessels provide nutrition and oxygen to the tumor. They act as gatekeepers of tumor metastasis. In normal adult tissue, blood endothelial cells usually divide only once in more than 100 days and most of them are quiescent. In adult tissue, angiogenesis is induced by angiogenic factors (e.g. VEGF) secreted from ischemic tissue, such as wound healing. Tumor angiogenesis is the most important as pathological angiogenesis²¹⁾. Currently, most of antiangiogenic drugs target VEGF or its signaling pathway. The therapeutic effects of anti-VEGF therapy was obtained when they are applied with chemotherapy. VEGF inhibiton causes not only inhibiting tumor growth by starving them but also converting immature and leaky tumor blood vessels to mature vessels with pericyte coverage. When tumor blood vessels become normalized, blood supply and drug delivery are regained. This is one of mechanism for antiangiogenic therapy's additional therapeautic effect when combined with anticancer drugs²²⁾. Humanized anti-VEGF antibody, bevacizumab has been widely used for cancer therapy, such as colorectal cancer, non-small cell lung cancer, breast cancer, malignant glioblastoma and ovarian cancer. However, the therapeautic efficacy is not enough and limited, despite of expectation based on the results of pre-clinical studies using animals. In addition, it is now observed that anti-VEGF drugs have severe side effects, including lethal hemoptysis and intestinal perforation²³. This is attributed to the fact that most current antiangiogenic drugs are inhibitors of VEGF/VGEFR pathway, which is important for even NEC survival^{24, 25)}.

Recently, there have been several trials to isolate TEC which is small population in tumor tissue (2% of tumor single cells). These studies have revealed that TEC differs from NEC in many aspects^{3, 26)}. Currently, as part of new drug discovery processes, cell-based screening

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is being performed using thousands of small chemical compounds. A number of chemical compounds have been identified that specifically target tumor cells or diseasespecific induced pluripotent stem (iPS) cells. Indeed, by this strategy, several drugs have been successfully invented, and some of them are now used in clinic. For the success of such screening purposes there is a need to obtain the right kind of cells and in adequate quantities. For development of novel antiangiogenic drugs which target TEC more specifically, cell-based assay screening system is one of strategy. Primary-cultured endothelial cells isolated from tumor blood vessels are the preferred choice. However, to obtain TEC for screening has several issues to be overcome. TEC is very few population in tumor tissue. In addition, to maintain TEC in culture required expensive endothelial cell specific culture medium. MS1, SV40 immortalized mouse isletderived endothelial cells are commercially available with comparatively cheaper cell culture cost¹⁴⁾. It is known that MS1 maintain common endothelial marker expressions such as CD31. Thus, MS1 cells have been often used in vascular research. We also have used them as positive control for analysis of endothelial cell marker expression.

In this study, to address the possibility of using MS1 cells for chemical screening, we compared the characteristics of MS1 and primary-cultured endothelial cells ; TEC and NEC which we have isolated. We found that MS1 proliferate fast and they express stem markers, Sca-land CD133, which suggests that MS1 cells are similar to TEC, not to NEC. However, angiogenesis-related gene expression levels were lower in MS1 than those in NEC (except VEGFR2). In addition, stem cell markers ; ALDH, CD90, IL-6 were not upregulated in MS1 unlike TEC. IL-6 is not only stem marker also inflammatory marker. Inflammation related genes, including IL-6, which have been reported to be upregulated in TEC, were expressed in MS1 at significantly lower level compared to TEC, although only CXCR7 was upregulated like TEC. Furthermore, LOX, Biglycan, Def6, Tmem176b and SBSN, which have been reported as TEC markers, were not expressed in MS1. These results suggested that MS1 resemble to NEC more, rather than TEC. Since functional validation is important for cell-based screening, cell migration and tube formation, which are key functions for endothelial cells, were analyzed in MS1 and TEC. TEC migrates fast in consistent with our previous observation, but MS1 did not migrate in wound scratch assay. It is already reported that TEC and NEC can make tubes on matrigel during in vitro culture, however, MS1 did not show tube formation neither in DMEM with 10% FBS nor in EGM-2 MV. These results suggest that MS1 does not completely resemble primary cultured NEC nor TEC in function and gene expression. Since it was assumed that these phenotypical differences between MS1 and primary-cultured endothelial cells may accounts for different culture condition (culture medium for MS1 does not contain endothelial cell growth factors), we tried to maintain MS1 in EGM-2 MV which is rich for angiogenic factors for analysis. However, growth speed of MS1 became slower under EGM-2 MV, and MS1 showed senescence as shown β -gal positive staining (data not shown). The difference in original organs which endothelial cells were isolated, might be another reason ; MS1 was isolated from pancreatic islet and primary cultured endothelial cells were from dermis or subcutaneous tumors. Further research will be required to elucidate the mechanism for different phenotype between MS1 and NEC/TEC.

MS1 has been often used in functional analysis in angiogenesis research. To evaluate function of target molecule in endothelial cells, gain of function, or loss of function experiments might be possible in MS1. However, for cell-based screening of chemicals, cells which represent in vivo tumor angiogenesis are required. Based on our results aforementioned, primary cultured TEC and NEC may be more suitable for these screening. To establish the method of maintaining enough number of TEC/NEC after isolation from tissues, further optimization will be needed.

CONCLUSION

Mouse immortalized normal endothelial cell, MS1 showed upregulation of some of stem markers and fast cell proliferation like TEC, however, most of TEC marker expressions were lower compared to TEC, showing NEC phenotype. Strictly speaking, MS1 did not completely resemble NEC nor TEC in function and gene expression. It is suggested that for chemical screening, primarycultured TEC and the corresponding NEC would be a more ideal choice of cells.

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