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Murine bone marrow stromal cell culture with features of mesenchymal stem cells susceptible to mouse-adapted human TSE agent, Fukuoka-1

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Abstract

Transmission of transmissible spongiform encephalopathies (TSEs)/prion diseases through transplantation of bone marrow (BM) has never been reported in humans. However, the use of fetal bovine serum in current protocols for generating mesenchymal stem cells (MSCs) carries the risk of iatrogenic spread. We developed a cell model from murine BM-derived MSCs and tested its susceptibility to Fukuoka-1 (Fu) strain of TSEs. The adherent cells expressed significant levels of normal prion protein, PrP^c, at the time when they became immortalized. The cell culture underwent spontaneous transformation following inoculation with Fu-infected brain homogenate and became persistently infected after re-inoculation with Fu agent. Extensive analysis of the original and two Fu-exposed cell cultures revealed a phenotype characteristic of MSCs with a majority of cells being positive for stem cell antigen, Sca-1. Taken together, our results demonstrate that BM-derived MSCs can be infected with TSE agents under certain conditions ex vivo. Comprehensive studies should be undertaken to address the safety of cell-based therapies in regard to iatrogenic transmission of TSEs. BM-derived cell cultures can be used for studies of molecular mechanisms underlying the cells' susceptibility to various strains of TSEs, their propagation ex vivo, and for screening of potential anti-TSEs therapeutics.

Key words: *transmissible spongiform encephalopathies, prion diseases, cell culture, bone marrow, mesenchymal stem cells, PrP, Sca-1, VCAM-1, c-myc, nestin.*

Introduction

Cell cultures represent invaluable tools for the study of transmissible spongiform encephalopathies (TSEs), also known as prion diseases. TSEs constitute a group of fatal neurodegenerative disorders of humans and animals. The nature of infectious TSE agents is not fully understood. According to the "prion hypothesis", TSEs are caused by "prions", proteinaceous infectious particles resistant to procedures that modify nucleic acids [35]. Prions are hypothesized to consist of conformational variants of normal cellular prion protein PrP^c, which is expressed in various tissues of all mammalian species. Aberrant proteins bear designations that reflect the disease with which they are associated: PrP^{Sc} in the case of scrapie and PrP^{TSE} in general reference to the class of TSE diseases. Prions are apparently

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able to convert PrP^{C} into newly misfolded pathologic proteins [36]. However, earlier definition of the causative agents as a slow unconventional virus [15] is still not rejected by some researchers [25,26]. A "unified theory" postulating that both PrP^{TSE} and nucleic acids are involved in prion propagation was suggested to solve the dispute [48].

A number of cell cultures of neuronal and non-neuronal origin, mostly susceptible to mouse-adapted scrapie agent derived from sheep, have been developed to study TSE pathogenesis, including susceptibility to infection, molecular and biochemical mechanisms of PrP^{TSE} propagation, biological properties of prion strains, and mechanisms of neurodegeneration [2,44]. Persistently infected cell cultures have served as experimental models for *ex vivo* screening of various compounds as potential therapeutics [44]. More recently, genetically modified cell cultures were employed in developing susceptible bioassays for demonstrating infectivity [21,24]. We recently reported generation of murine spleen-derived stromal cell culture, tSP-SC, with features of mesenchymal stem cells (MSCs) [1], which was successfully infected with a mouse-adapted variant Creutzfeldt-Jakob disease (mo-vCJD) agent and a mouse-adapted isolate from the brain of a Gerstmann-Sträussler-Scheinker (GSS) disease patient, Fukuoka-1 (Fu) agent [42].

The research in development of clinical applications of MSCs for treatment of various diseases has increased dramatically in the past few years. MSCs represent multipotent stromal cells that adhere to plastic, express a specific profile of surface markers, and can differentiate *in vitro* under appropriate conditions at least along mesodermal lineages (osteoblastic, chondrocytic, adipocytic) [12]. Preparation of clinical grade MSCs from bone marrow (BM) requires *in vitro* propagation of derived cells in media containing fetal bovine serum (FBS) to achieve a high yield of cells in the final preparation [11,16]. In some instances, cells may be cryopreserved in medium containing FBS [11,23]. FBS represents a potential source of contamination with bovine spongiform encephalopathy (BSE) agent. The risk of iatrogenic transmission of TSEs associated with MSCs transplantation is unknown.

Here we report the first successful generation of a murine BM-derived spontaneously immortalized stromal cell culture with features of MSCs, which was spontaneously transformed following inoculation with Fu agent and became persistently infected with Fu upon re-inoculation. We provide extensive phenotypic characterization of the original immortalized cell culture, BMSC/336, transformed Fu agent-exposed uninfected cell culture, BMSC/336-Fu, and persistently infected with Fu agent cell culture, BMSC/336-Fu2, of BM origin. Our data reveal some differences between those cell cultures in morphology, in representation of several subpopulations of cells, and in expression of certain proteins. However, the observed phenotypic changes can be attributed rather to the change from untransformed to transformed status of the cell cultures than to their susceptibility to infection or their infectious status.

Materials and Methods

Experimental animals and sources of inoculum

SJL/OlaHsd (Ola) female mice used for cell isolation were from Harlan Laboratories (Bicester, UK).

Fu strain propagated through multiple passages in Swiss mice at NIH was a gift of Dr. P. Brown. It was further propagated in Swiss mice in our laboratory.

Experimental studies in mice were approved by the Institutional Animal Care and Use Committee of the American Red Cross Holland Laboratory.

Cell cultures

NIH 3T3 cells (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated FBS (Lonza, Walkersville, MD), and 1% penicillin-streptomycin (Invitrogen).

Generation of bone marrow-derived stromal cell cultures

BM cells were isolated from an Ola female mouse intracerebrally inoculated under isoflurane anesthesia with 0.1% normal bovine brain homogenate diluted into physiological saline. At the time of euthanasia, the mouse had multiple spontaneous tumors involving lymph nodes, Peyer's patches, and spleen. The BM tissue was flushed out of femurs and tibiae and vigorously pipetted to achieve a homogeneous cell suspension. Cells were harvested by centrifugation and further maintained in complete growth medium (CGM) containing Iscove's Modified Dulbecco's Medium (IMDM) supplemented with HEPES and L-glutamine (Lonza), 10% heat-inactivated fetal bovine serum (FBS, Lonza), 5% medium conditioned by X63

Ag8-653 myeloma cells transfected with a vector expressing mouse interleukin 3 [20], 10% BIT 9500 (StemCell Technologies, Vancouver, Canada), 5 ng/ml sodium selenite (Sigma-Aldrich, St. Louis, MO), 50 µM 2-mercaptoethanol (Invitrogen, Carlsbad, CA), and 1% penicillin-streptomycin (Invitrogen). Cells were split 1 : 3 approximately twice per week and became immortalized within 8 weeks.

Immortalized cells were frozen in growth medium supplemented with 20% FBS and 10% DMSO (Sigma-Aldrich, St. Louis, MO). After recovery from frozen stocks, cells were maintained in the CGM.

Infection of cell cultures with Fu agent and following propagation

A 10% brain tissue homogenate was prepared from the pooled brains of terminally sick Swiss mice intracerebrally inoculated with Fu agent, in sterile 0.9% NaCl solution by successive passages through needles decreasing in gauge from 18 to 28. BM-derived adherent cells (2×10^5) were plated into 25 cm^2 culture flasks one day before infection. Inoculation was performed according to Vorberg & Priola [46]. Cells were harvested and tested for the presence of PrP^{TSE} at 96 hours and every 5 passages after inoculation.

Fluorescence-activated cell sorting (FACS) and analysis

FACS analysis and corresponding antibodies listed in Table II have been previously described [1] except a fluorescein isothiocyanate (FITC)-conjugated CD45 (clone 30-F11) antibody purchased from BD Pharmingen.

Immunocytochemistry

Immunocytochemical detection of nestin was performed using 10 µg/ml mouse anti-nestin antibodies (Neuromics, Edina, MN) as described elsewhere [49].

Western blotting

For detection of PrP, cells were grown in 25 cm^2 flasks to confluent density and then lysed with 1 ml lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate, 10 mM Tris, pH 7.5) for 1 hr with continuous shaking on wet ice. Cells were harvested with a cell scraper and insoluble material was removed by centrifugation at 10,000 g for 5 minutes. Two 400-µl aliquots of supernatant were treated with 5 µg/ml proteinase K (PK) (Invitrogen) followed by incubation at 37°C for 30 minutes while one 150-µl aliquot was left PK-untreated. Next, proteins were precipitated with methanol at –80°C. Precipitates were centrifuged at 10,000 g for 30 minutes. Pellets were dissolved in Laemmli Sample Buffer (Bio-Rad, Hercules, CA) with 5% 2-mercaptoethanol (Sigma-Aldrich) and heated at 95°C for 10 minutes before loading into the gel.

For detection of other proteins, including nestin, SDF-1, c-myc, and α-tubulin, cell lysates were prepared using ~10^6 cells as described in [33]. Nestin was also tested in the intermediate filament fraction [33] to verify results obtained from total cell lysates.

Protein detection by western blotting was performed as described earlier [5]. Detection of PrP was performed using monoclonal 6D11 antibody (SIGNET, Dedham, MA) and corresponding antibodies were used for detection of nestin (Rat-401), SDF-1 (FL-93), c-myc (N-262) (all from Santa Cruz Biotechnology,

Table I. List of cell cultures

Cell culture	Description
BMSC/336 (non-transformed, not exposed to Fu)	BM Stromal Cells derived from Ola mouse inoculated with 0.1% normal bovine brain homogenate and maintained in CGM. No PrP^C was detected in cells at the time of isolation or 3 days later. High levels of PrP^C were demonstrated 2 months after isolation at the time of immortalization and through following passages.
BMSC/336-Fu (transformed, not propagating Fu)	BMSC/336 cell culture inoculated on passage 6 with 0.1% Fu/Swiss brain homogenate and became spontaneously transformed on passage 7. No PrPres was detected by western blotting starting at passage 5 after the inoculation and through at least 55 passages.
BMSC/336-Fu2 (transformed, propagating Fu)	BMSC/336-Fu cell culture re-inoculated with 0.1% Fu/Swiss brain homogenate on passage 20 after first inoculation. PrPres was demonstrated through at least 85 passages following re-inoculation.

Table II. Immunophenotyping by FACS analysis of spontaneously immortalized cell cultures derived from bone marrow of Ola mouse

Marker	Analyzed cell culture, % of positive cells		
	BMSC/336	BMSC/336-Fu	BMSC/336-Fu2
Sca-1	92.1 ± 1.1	65.4 ± 2.5	74.4 ± 3.4
CD90 (Thy1.1)	4.6 ± 1.6	5.1 ± 0.4	4.1 ± 2.1
CD34	7.2 ± 0.8	3.7 ± 1.2	5.7 ± 1.4
CD106 (VCAM-1)	98.3 ± 0.9	4.9 ± 1.3	6.3 ± 2.2
CD44 (H-CAM)	100	100	100
CD45R/B220	8.3 ± 3.1	9.9 ± 2.7	7.7 ± 2.5
6D11 (PrP)	80.9 ± 2.3	98.0 ± 1.2	93.3 ± 1.7

The Sca-1, CD34, CD106 (vascular cell adhesion molecule-1 [VCAM-1]), CD45R/B220, and 6D11 antibodies used were FITC-conjugated; the CD90 (Thy1.1) and CD44 (homing-associated cell adhesion molecule [H-CAM]) antibodies used were phycoerythrin-conjugated. All antibodies were from BD Pharmingen, except for CD34 (AbD Serotec), and anti-PrP specific monoclonal antibody 6D11 (SIGNET). Analysis was performed by FACSCanto™ flow cytometer using FACSDiva software (BD Biosciences).

Santa Cruz, CA), and α-tubulin (Cell Signaling Technology, Danvers, MA).

Results

Development of an immortalized BM-derived cell culture

BM cells isolated from an Ola mouse (a strain closely related to the SJL/J strain, that is known to develop spontaneous B cell lymphomas at 8 months of age and older [32,41]) were maintained as a stromal adherent culture. After approximately eight weeks, cells escaped from proliferative crisis and showed stable continued growth indicating spontaneous immortalization. The cells displayed fibroblast-like polygonal shape (Fig. 1). At the confluent stage, lipid vacuoles were frequently observed in the cytoplasm, indicating spontaneous adipocyte differentiation (data not shown). The immortalized cell culture was termed BMSC/336 (Table I). No PrP^{c} was detected in cells at the time of isolation or 3 days later (data not shown). High levels of PrP^{c} were demonstrated at the time when cells became immortalized and through the subsequent propagation (Fig. 2).

Ex-vivo inoculation of BM-derived cells with Fu and subsequent propagation

Previously we reported that stromal tSP-SC cell culture originated from the spleen of an Ola mouse

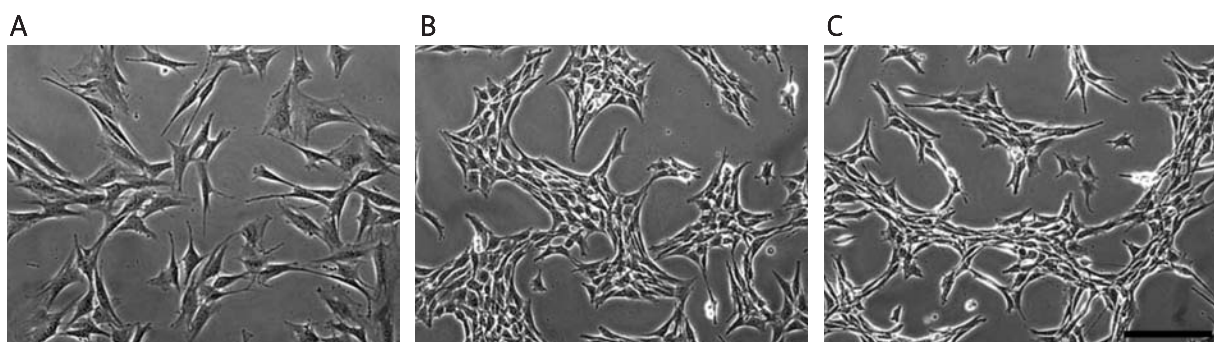

Fig. 1. Morphology of spontaneously immortalized BM stromal cell cultures derived from SJL/Ola mice. Untransformed BMSC/336 cells (A) have a fibroblast-like polygonal shape. Transformed BMSC/336-Fu cells (B) display significant morphological changes with about 5-fold decrease in cell spreading, and Fu infected BMSC/336-Fu2 cells (C) maintain the same morphology as parental BMSC/336-Fu cell culture. Scale bar, 200 µm.

persistently propagates mo-vCJD or Fu agents after inoculation *ex vivo* [1]. To test whether a cell culture derived from BM of an Ola mouse is susceptible to Fu infection, we inoculated BMSC/336 cell culture with 0.1% Fu/Swiss brain homogenate. The inoculated cells, termed BMSC/336-Fu (Table I), produced no detectable PrP^{res} throughout at least 57 passages as demonstrated by western blotting (Fig. 2) even though the PrP^{res} was demonstrated in the cell culture at 96 hours following the inoculation (data not shown). We observed spontaneous transformation of the cells by passage 7 following inoculation, characterized by a significant (approximately 7-fold) increase in cell proliferation rate, loss of contact inhibition of cell proliferation, and considerable changes in cell morphology (Fig. 1). The extent of cell spreading decreased about 5-fold compared to non-transformed parental BMSC/336 cells. Transformed status of these cells was confirmed by soft agar assay (data not shown). The transformed cell culture was re-inoculated with 0.1% Fu/Swiss brain homogenate at passage 20 after transformation and was designated as BMSC/336-Fu2 (Table I). No change in the cell morphology was observed (Fig. 1). The re-inoculation of cells led to stable infection and propagation of PrPres through at least 85 passages (Fig. 2).

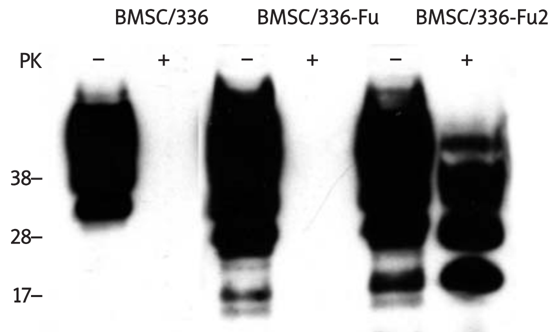

Fig. 2. Immunoblot for PrP from spontaneously immortalized BM-derived stromal cell cultures (Table I). Presence of total PrP and PrPres is evidenced in PK-untreated samples (–) and samples treated with PK (+), respectively. Note that the PK-treated samples (+) are five-fold more concentrated than samples not treated with PK (–). The membrane was developed using 6D11 antibody. The molecular mass standard in kilodaltons is shown on the left.

Immunophenotyping of BM-derived immortalized and transformed cell cultures

With the aim of establishing lineages of the BM-derived stromal cell cultures listed in Table I, we performed immunophenotyping of cells by FACS analysis using a variety of antibodies. Complete analysis of the data is summarized in Table II. In all the tested cell cultures, the majority of cells were positive for stem cell antigen Sca-1 with a higher population (above 90%) in untransformed BMSC/336 cell culture. In the transformed persistently infected BMSC/336-Fu2 and in transformed but not infected BMSC/336-Fu cell cultures, less than 75% of cells were positive for stem cell antigen 1 (Sca-1), a marker of multipotent hematopoietic stem cells (HSC). Each cell culture had a very small subpopulation (less than 8%) of cells positive for HSC/mesenchymal stem cell marker CD90.1 and the HSC/progenitor cell marker CD34.

All the BM-derived cultures were 100% positive for homing-associated cell adhesion molecule (H-CAM), which is expressed on mesenchymal stem cells. Another cell surface receptor typical for BM stromal cells, vascular cell adhesion molecule-1 (VCAM-1), was expressed on 98% of BMSC/336 cells, but only small subpopulations of VCAM-1-positive cells were present in transformed BMSC/336-Fu and BMSC/336-Fu2 cell cultures. Minor populations of the analyzed cells were positive for pro-B cell marker CD45R/B220 (Table II); however, no correlation with the transformed status of the cell cultures or their susceptibility to Fu infection was observed. The cell cultures were negative

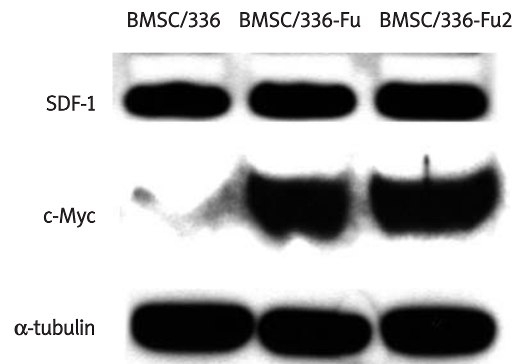

Fig. 3. Immunoblots for stromal derived factor-1 (SDF-1) and c-myc from spontaneously immortalized BM stromal cell cultures (Table I). Immunoblot for α-tubulin was used as a protein load control. The membranes were developed using corresponding rabbit polyclonal antibodies.

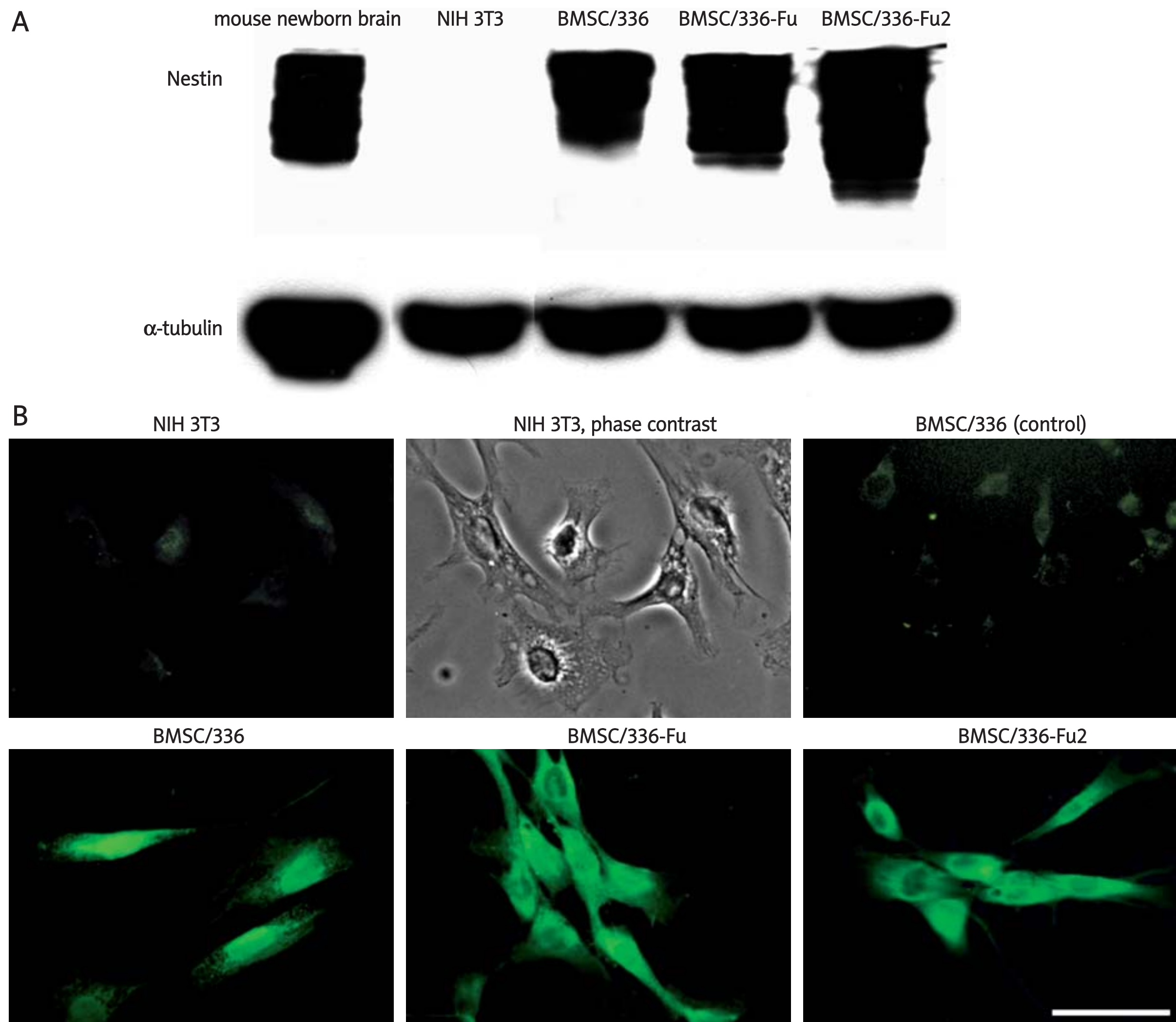

Fig. 4. Detection of nestin in immortalized BM stromal cell cultures (Table I). A. Immunoblot for nestin in total cell lysates performed with anti-nestin mouse monoclonal antibody. NIH 3T3 cells were used as a negative control. Lysate of mouse newborn brain was used as a nestin positive control. Immunoblot for α-tubulin with corresponding rabbit polyclonal antibody was used as a protein load control. B. Immunostaining for nestin from permeabilized NIH 3T3 cells and BM stromal cell cultures using corresponding mouse monoclonal antibody. BMSC/336 cells incubated with secondary FITC-conjugated goat-anti-rabbit antibodies were used as a negative control to confirm specificity of the staining in BMSC cell cultures. Scale bar, 50 µm.

for leukocyte common antigen CD45, for lineage markers of myeloid and monocytic cells CD11b and Gr-1, and for one of the endothelial cell markers, platelet endothelial adhesion molecule-1 (PECAM-1).

Analysis also revealed that transformed BMSC/336-Fu and Fu-infected BMSC/336-Fu2 cell cultures had greater than 93% PrP-positive cells. The untransformed and uninfected BMSC/336 cell culture displayed slightly lower percentage of PrP-positive cells (Table II).

Characterization of the BM-derived cell cultures for selected markers

Expression of selected stromal/stem cell markers in the developed cell cultures was analyzed by western blotting. The cultures were found to express stromal cell derived factor-1 (SDF-1) (Fig. 3), confirming the stromal phenotype of the cells. They were also positive for the intermediate filament protein nestin, a marker

of neuroepithelial stem cells (Fig. 4A). The results demonstrating nestin expression obtained using total cell lysates were further confirmed by western blotting of the intermediate filament protein fractions (data not shown), and also by immunocytochemical staining (Fig. 4B). Control NIH 3T3 mouse fibroblasts were negative for nestin as shown by both methods (Fig. 4A-B).

We tested BM-derived cell cultures for expression of transcription factor c-myc, which is frequently involved in cell transformation and hypothetically may contribute to susceptibility of the cells to prion infection. The BMSC/336-Fu and BMSC/336-Fu2 cell cultures displaying transformed phenotype showed similar levels of c-myc that were noticeably elevated compared to non-transformed BMSC/336 cells (Fig. 3).

Discussion

There are at least two potential routes of TSE transmission during MSCs transplantation: through cells from a donor carrying a subclinical disease and through contamination of MSCs during *ex vivo* propagation by animal derived products, for example, the BSE agent in FBS.

To our knowledge there has been only one study that tested the presence of infectivity in BM of patients afflicted with TSE, with a negative result [3], and only in one study has the presence of PrPres been demonstrated in MSCs isolated from BM of CJD patients at early stages of cell propagation [40].

To date, no transmission of any human TSE through BM transplantation has ever been reported. However, epidemiological studies in the UK have identified four vCJD-positive individuals (three of whom died as a consequence of the disease) who had been transfused with non-leukoreduced red blood cells from three donors who later died from vCJD [18]. This fact opened the possibility that BM of vCJD-infected individuals may contain low levels of infectivity due its immediate contact with blood.

To address the possibility of TSE-agent contamination of MSCs through *ex vivo* exposure to animal-derived products, we developed an adherent stromal cell culture from murine bone marrow, BMSC/336, with features of MSCs, and tested its susceptibility to Fu agent.

We did not detect PrP^{c} by western blotting of cell lysates prepared from BMSC/336 cell culture at early stages of propagation. However, significant expression of PrP^{c} was demonstrated at the time when the cells became immortalized. FACS analysis confirmed that the majority of the cells were positive for surface PrP^{c}. Further characterization of cells by FACS analysis revealed that their immunophenotype is consistent with mouse BM-derived stromal cells with features of MSCs [9,30]. Specifically, most of the analyzed cells expressed Sca-1, VCAM-1, and all cells were positive for H-CAM (Table II), while being negative for leukocyte common antigen CD45, myeloid and monocyte linage markers CD11b and Gr-1, and an endothelial marker, PECAM. In addition, we found that the generated cell cultures expressed significant levels of nestin, a marker of neuronal progenitors that was also found in immature MSCs [43,45]. This marker was slightly elevated in BMSC/336-Fu compared to BMSC/336 cells and was expressed at the highest level in BMSC/336-Fu2 cells. Elevated expression of nestin has been reported for neoplastic cells in high-grade astrocytomas [13]. PrP^{Sc} infection of neuronal cell lines is known to cause changes in the expression of multiple genes and as a result the alterations in the expression profiles vary between cell lines [17]. Persistent Fu infection could lead to up-regulation of nestin in BMSC/336-Fu2 cell culture compared to BMSC/336 and BMSC36-Fu cultures, either directly or through further dedifferentiation of the cells.

In further experiments, we investigated the susceptibility of the developed cell culture to TSE infection. On the first attempt we were not able to persistently infect spontaneously immortalized BMSC/336 cells with Fu agent even though the presence of PrP^{res} was demonstrated in the cell culture after 96 hours following inoculation. The inoculated but not infected cell culture, BMSC/336-Fu, subsequently underwent spontaneous transformation and became persistently infected with Fu agent, BMSC/336-Fu2, upon re-inoculation. Cell transformation following inoculation with mouse-adapted scrapie agent has been previously reported [27]. We have no evidence that the transformation event is related to TSE infection. Earlier, we reported the transformation of spleen-derived MSCs that originated from an Ola mouse, following *ex-vivo* inoculation with normal mouse brain homogenate [1]. Spontaneous transformation has been demonstrated after continued *in vitro* propagation of murine MSCs [39] and human adult MSCs [37].

The BMSC/336 cell culture has been derived from a mouse of the Ola strain, which is known to develop spontaneous B-cell lymphomas [41]. Therefore, the presence of a transforming virus in this cell culture cannot be excluded. This notion is supported by the

evidence that neoplasm formation in SJL/J mice, which are closely related to Ola mice, is associated with the presence of D1-murine leukemia virus [7,8]. It was recently reported that infection with Moloney murine leukemia virus strongly enhances the release of scrapie agent by co-infected cells [22]. In addition, we observed a robust accumulation of PrP^{res} in cancerous lymph nodes and spleens of Ola mice infected with either Fu or mo-vCJD agents [6].

One recently established microglial cell line, which has been infected with multiple scrapie strains and mouse-adapted BSE, was generated by transformation using c-myc oncogene [19]. In our study, the untransformed cell culture BMSC/336 had significantly lower levels of c-myc protein compared to the transformed cell cultures, BMSC/336-Fu and BMSC/336-Fu2. Recent evidence indicates that intracellular signaling pathways are involved in PrP^{Sc} formation in scrapie-infected GT1-1 cells (ScGT1-1) [29]. One of these, the extracellular-signal-regulated kinase (ERK) pathway operates through sequential activation of mitogen-activated protein kinases (MAPK), and regulates gene expression, including that of c-myc. Activation of the ERK pathway by brain-derived neurotrophic factor promotes formation of PrP^{Sc} while its inhibition by the mitogen-activated protein-kinase kinase (MEK1/2) inhibitors results in clearance of PrP^{Sc} from ScGT1-1 cells [28,29]. These data argue for a possible role of the ERK/MAPK pathway acting through regulation of transcription factors such as c-myc, in the propagation of PrP^{TSE}. The transformation event dramatically increases genomic instability and could cause activation of transcription factors through gene expression/repression or activation/inactivation of signal transduction pathways.

Extensive immunophenotyping by FACS of transformed, BMSC/336-Fu and BMSC/336-Fu2, and untransformed BMSC/336 cell cultures revealed few significant differences in representation of cell subpopulations. The expression of PrP^{C} is a determining factor for propagation of TSE agents *in vivo* and *ex-vivo* [4]. Somewhat increased representation of PrP^{C}-positive cells was demonstrated in transformed BMSC/336-Fu and BMSC/336-Fu2 (Table II) compared to untransformed BMSC/336 cell culture, but no significant difference was noticed between subpopulations in uninfected BMSC/336-Fu cell culture and infected BMSC/336-Fu2 cell culture (Table II).

The Sca-1- and VCAM-1-positive cells were highly represented in untransformed BMSC/336 culture (Table II). Transformed BMSC/336-Fu cell culture, which was exposed to Fu agent but did not become infected, had the lowest subpopulation of Sca-1-positive cells; however, a somewhat larger Sca-1-positive subpopulation was observed in Fu-infected BMSC/336-Fu2 cell culture. A dramatic decrease (more than 10-fold lower) of VCAM-1-positive cells was observed in both transformed cell cultures. The reason for the change in VCAM-1-positive cell subpopulation in transformed cell cultures is unknown but it can be related directly to the cell transformation event, as was reported for transformed murine myocardium-derived endothelial cell line [31].

In all tested cell cultures, fewer than 10% of cells were positive for CD45R/B220 and CD34 markers. It has been reported recently that bone marrow $CD34^{+}/B220^{+}$ progenitors have the potential to differentiate toward microglia [10]. Furthermore, the microglia displays some features of immature myeloid cells and BM progenitors that are considered to be the common precursors of these cells in the brain [38,34]. The microglial cell line MG20, developed from tga20 mice, which overexpress PrP^{C} [14], has been successfully infected with various mouse-adapted scrapie strains and mouse adapted BSE [19]. It is plausible that the $CD34^{+}/B220^{+}$ subpopulations are a primary target of TSE infection in BM-derived MSC culture, and that they sustain their division rate after being infected. Although the ability of a particular cell type to support TSE infection has already been suggested [47], additional studies are still required for addressing this possibility.

Lymphoreticular tissues play a crucial role in prion invasion and propagation; therefore, cell cultures developed from these tissues may represent the most adequate models for studying both cellular and molecular aspects of TSEs for which a parenteral route of exposure has been suggested. Therefore, we envision that the BMSC/336-Fu2 cell culture, which persistently propagates Fu agent, might serve as a useful tool for the *ex vivo* studies of TSEs and for screening of potential drugs.

In conclusion, we have shown that mouse-derived cell cultures of BM stromal origin with features of MSCs can be persistently infected with Fu agent under certain conditions *ex vivo*. Further studies are underway to explore whether such cell cultures and BM-MSCs of human origin could be infected with other TSE agents, including BSE and vCJD.

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