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Characterization of mesenchymal stem cells in sheep naturally infected with scrapie

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Mesenchymal stem cells (MSCs) can be infected with prions and have been proposed as *in vitro* cell-based models for prion replication. In addition, autologous MSCs are of interest for cell therapy in neurodegenerative diseases. To the best of our knowledge, the effect of prion diseases on the characteristics of these cells has never been investigated. Here, we analysed the properties of MSCs obtained from bone marrow (BM-MSCs) and peripheral blood (PB-MSCs) of sheep naturally infected with scrapie — a large mammal model for the study of prion diseases. After three passages of expansion, MSCs derived from scrapie animals displayed similar adipogenic, chondrogenic and osteogenic differentiation ability as cells from healthy controls, although a subtle decrease in the proliferation potential was observed. Exceptionally, mesenchymal markers such as *CD29* were significantly upregulated at the transcript level compared with controls. Scrapie MSCs were able to transdifferentiate into neuron-like cells, but displayed lower levels of neurogenic markers at basal conditions, which could limit this potential. The expression levels of cellular prion protein (PrP^C) were highly variable between cultures, and no significant differences were observed between control and scrapie-derived MSCs. However, during neurogenic differentiation the expression of PrP^C was upregulated in MSCs. This characteristic could be useful for developing *in vitro* models for prion replication. Despite the infectivity reported for MSCs obtained from scrapie-infected mice and Creutzfeldt–Jakob disease patients, protein misfolding cyclic amplification did not detect PrP^{Sc} in BM- or PB-MSCs from scrapie-infected sheep, which limits their use for *in vivo* diagnosis for scrapie.

INTRODUCTION

Prion diseases or transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative diseases characterized by the accumulation of pathological isoforms (PrP^{Sc}) of the cellular prion protein (PrP^C) in the brain, but also in lymphoid tissue and to a lesser extent in other tissues (Safar *et al.*, 1993).

Stem cell-based therapies have emerged as possible strategies to treat diseases of the central nervous system (Lindvall & Kokaia, 2010). Mesenchymal stem cells (MSCs) display certain characteristics that make them good candidates for the treatment of neurodegenerative diseases. For example, MSCs can transdifferentiate into neuron and glial cells (Chen *et al.*, 2001), although the functionality of differentiated cells is controversial (Przyborski *et al.*, 2008). In addition, these cells release angiogenic, neurogenic, neuroprotective, synaptogenic and scarring inhibition factors, which could exert a neuroprotective effect (Chen & Chopp, 2006). Transplantation of human MSCs in mice infected with prions does not arrest disease progression, but increases survival times (Song *et al.*, 2009), and brain extracts from prion-infected mice promote chemotaxis of MSCs *in vitro*. The chemokine receptors involved in the migration of human MSCs to brain lesions have also been identified (Song *et al.*, 2011).

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In addition to their use as therapy, stem cells are being used in prion research as possible *in vitro* models for prion propagation. A wide variety of stem cells express PrP^C, including MSCs (Mohanty *et al.*, 2012), and the function of this protein has been linked to stem cell biology (Lee & Baskakov, 2013; Zhang *et al.*, 2006). In recent years, three bioassays have used murine cells with mesenchymal characteristics to multiply different prion strains previously adapted in mice (Akimov *et al.*, 2008, 2009; Cervenakova *et al.*, 2011). The development of such *in vitro* models in species naturally susceptible to the disease would avoid the species barrier. Our group presented the first *in vitro* characterization of peripheral blood-derived ovine MSCs (PB-MSCs) and demonstrated that these cells express PrP^C, at least at the transcript level (Lyahyai *et al.*, 2012).

Finally, the determination of infectivity in MSCs obtained from individuals affected with prion diseases has been proposed for *in vivo* diagnosis for TSEs. Murine models experimentally infected with prions accumulate PrP^{Sc} in bone marrow-derived MSCs (BM-MSCs) and this infection could precede prion accumulation in brain (Takakura *et al.*, 2008). MSCs can also be found in peripheral blood, and the presence of PrP^{Sc} has been demonstrated in several fractions of human and ovine blood (Andréoletti *et al.*, 2012), in plasma and cells from different haematopoietic lineages. Whether circulating MSCs are infective or not is still unknown.

In addition to the therapeutic potential for their use in autologous transplants, the effect of prion disease in the characteristics of MSCs has never been investigated. Here, to the best of our knowledge, we present the first work focused on the characterization of BM- and PB-MSCs obtained from sheep naturally infected with scrapie – a prion disease that affects sheep and goats (Detwiler, 1992). Changes in PrP^C expression either related to the disease or induced by the neurogenic differentiation process have been evaluated in the present work, as well as the possible presence of PrP^{Sc} in both types of cells.

RESULTS

Mesenchymal characteristics of scrapie-derived MSCs

The mesenchymal characteristics concerning proliferation, differentiation potential and expression of mesenchymal cell surface markers were evaluated in BM- and PB-MSCs of healthy (H) and scrapie-infected (Sc) sheep in a clinical phase of the disease.

Isolation of MSCs and their proliferation potential

Plastic-adherent fibroblast-like cells were observed in all donor samples obtained from bone marrow aspirates and peripheral blood within the first days of culture. A great variability was observed in the number of adherent cells obtained at the end of passage 0, and no statistically significant differences were observed between cultures derived from healthy and scrapie-infected individuals (Table 1).

The proliferation ability of PB- and BM-MSCs obtained from healthy and scrapie-infected sheep was analysed during the first three passages. Mean culture time to complete a passage was 6.2 days for PB-MSCs and 7.8 days for BM-MSCs. Cell doubling (CD) and doubling time (DT) results are shown in Table 1. CD was significantly lower in Sc-PB-MSCs than in H-PB-MSCs at passages 1 and 3. In the same way, Sc-BM-MSCs displayed a decrease of CD, and this change was significant at passages 2 and 3 compared with H-BM-MSCs. In spite of these changes, DT results did not show statistically significant differences between healthy and scrapie MSCs.

Expression of cell surface markers

Quantitative real-time (qRT)-PCR was used to analyse seven mesenchymal and two haematopoietic surface markers; results are shown in Fig. 1. The amplification of the MSC-specific surface markers *CD29*, *CD36*, *CD73*, *CD90* and *CD166* was successful in both PB-MSCs and BM-MSCs obtained from healthy or scrapie-infected sheep. Differences

Table 1. Proliferation of MSCs derived from healthy and scrapie-infected sheep

Number of adherent cells at the end of passage 0 and CD/DT for the three first passages are shown (mean ± SD).

Passage	Parameter	BM-MSCs		PB-MSCs	
		Healthy	Scrapie	Healthy	Scrapie
0	Adherent cells ($\times 10^3$)	379 ± 231	385 ± 363	596 ± 427	630 ± 369
1	CD	3.06 ± 0.57	2.98 ± 0.75	2.92 ± 0.31	1.70 ± 1.12*
	DT (days)	2.18 ± 0.60	2.42 ± 1.45	1.88 ± 0.56	10.15 ± 14.64
2	CD	2.9 ± 0.35	1.9 ± 0.84*	2.14 ± 0.79	2.06 ± 0.63
	DT (days)	2.42 ± 0.35	3.57 ± 1.71	4.17 ± 3.56	2.79 ± 0.59
3	CD	2.61 ± 0.18	1.97 ± 0.50*	2.51 ± 0.35	1.84 ± 0.38*
	DT (days)	3.32 ± 0.68	2.55 ± 1.14	2.63 ± 0.87	2.99 ± 0.76

*$P<0.05$ (Student's *t*-test).

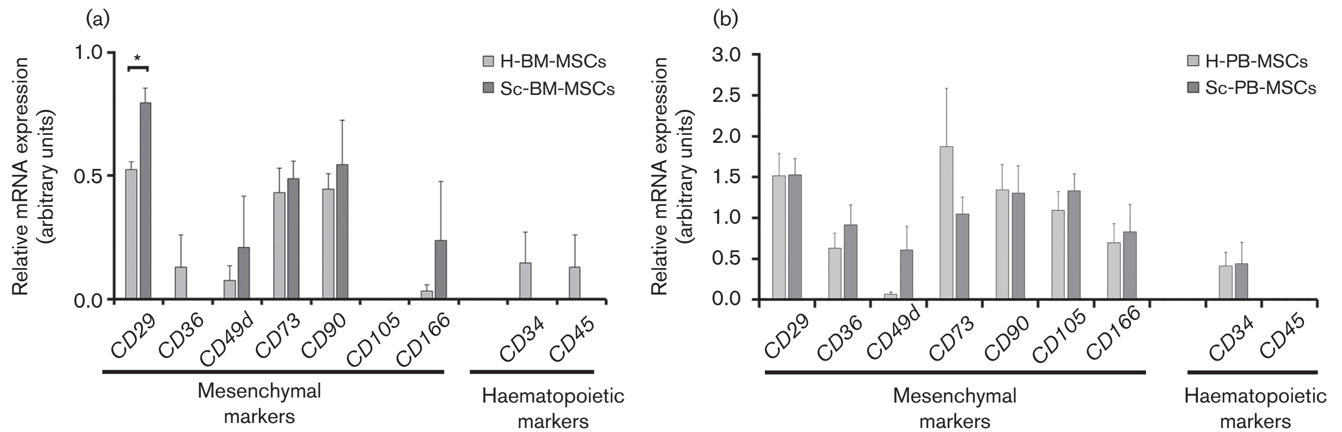


Fig. 1. Expression of cell surface markers. Gene expression of mesenchymal and haematopoietic cell surface markers quantified by qRT-PCR in (a) BM-MSCs and (b) PB-MSCs. Relative mRNA expression levels are expressed as mean±SEM. Significant differences between healthy and scrapie-derived cultures were calculated with Student's *t*-test (*$P<0.05$).

between tissue sources were observed for the expression of the *CD105* MSC marker. This marker was not detectable in BM-MSC cultures, but it was properly amplified in PB-MSCs (Fig. 1). The expression of haematopoietic markers was also analysed; whereas *CD45* was absent in all PB-MSCs and only one H-BM-MSC culture showed mRNA expression for this marker, the expression of *CD34* was variable for the different cultures (four of five H-BM-MSCs, one of four Sc-BM-MSCs, two of five H-PB-MSCs and two of five Sc-BM-MSCs; data not shown). When the expression of these markers was compared between healthy and scrapie-infected MSCs, only *CD29* was significantly upregulated by Sc-BM-MSCs and the remaining markers showed no differences related to the disease condition.

Cross-reactivity of seven anti-human MSC marker antibodies was tested in two H-BM-MSC, two Sc-BM-MSC and one Sc-PB-MSC cultures. Ovine MSCs displayed large size and complexity, and a lack of immunoreactivity was observed for the isotype controls for each mouse mAb (Fig. 2). Ovine MSCs derived from the two sources were robustly positive for the typical MSC marker CD29, showing >97 % of positive cells. On the contrary, these cultures displayed low and variable percentages of CD90 and CD105 immunoreactive cells (Fig. 2), which may have resulted from unspecific antibody reactions. We could not expand the putative markers set with other antibodies as the cultures were negative for CD14, CD19 and CD73 (data not shown). Immunoreactivity against the haematopoietic marker CD45 was not detected in any case.

The immunophenotype for CD29 was evaluated in nine PB-MSC (four scrapie-infected and four healthy) and in seven BM-MSC cultures (four scrapie-infected and three healthy). In accordance with gene expression, the percentage of $CD29^{+}$ cells and the fluorescence intensity were higher in PB-MSCs than in BM-MSCs ($P<0.05$), but significant differences were not observed between cultures derived from scrapie-infected and healthy sheep (Fig. 2).

Adipogenic, osteogenic and chondrogenic differentiation

The adipogenic differentiation potential was analysed by specific Oil Red O dying and microscopic observation. Translucent intracellular droplets of variable size and number were observed across their differentiation process; Oil Red O staining confirmed their lipid nature (Fig. 3). In addition, the expression of *GPAM* (mitochondrial glycerol-3-phosphate acyltransferase), *PPARG* (peroxisome proliferator-activated receptor γ) and *SCD* (stearoyl-CoA desaturase) was analysed by qRT-PCR at the end of the differentiation process (day 14 of culture for BM-MSCs and day 21 of culture for PB-MSCs). Although high variability was observed within groups of cultures, a statistically significant difference between differentiated and undifferentiated cells was observed for the upregulation of the adipogenic marker *PPARG* in Sc-BM-MSCs ($P<0.05$), and *GPAM* in differentiated H-PB-MSCs ($P<0.01$) and Sc-PB-MSCs ($P<0.05$). PB-MSCs took longer to differentiate into adipocytes (21 versus 14 days for BM-MSCs).

The chondrogenic differentiation potential of MSCs was evaluated by Alcian Blue G dying, which stains glycosaminoglycans generated during chondrogenesis. The differentiation process was arrested when solid formations were observed on the plate (28 days for BM-MSCs and 21 days for PB-MSCs). These formations were stained in blue, indicating a correct differentiation process, although the staining was more intense in BM-MSCs than in PB-MSCs (Fig. 3). In addition, chondrogenic markers *BGN* (biglycan), *COL2A1* (collagen, type II, α1) and *LUM* (lumican) were analysed by qRT-PCR assay. In cultures obtained from healthy sheep, *BGN* was significantly downregulated in differentiated BM-MSCs ($P<0.05$) and upregulated in differentiated PB-MSCs ($P<0.05$) (Fig. 3). These differences could reflect two different moments in the kinetics of the chondrogenic process. The comparison between scrapie-infected and healthy

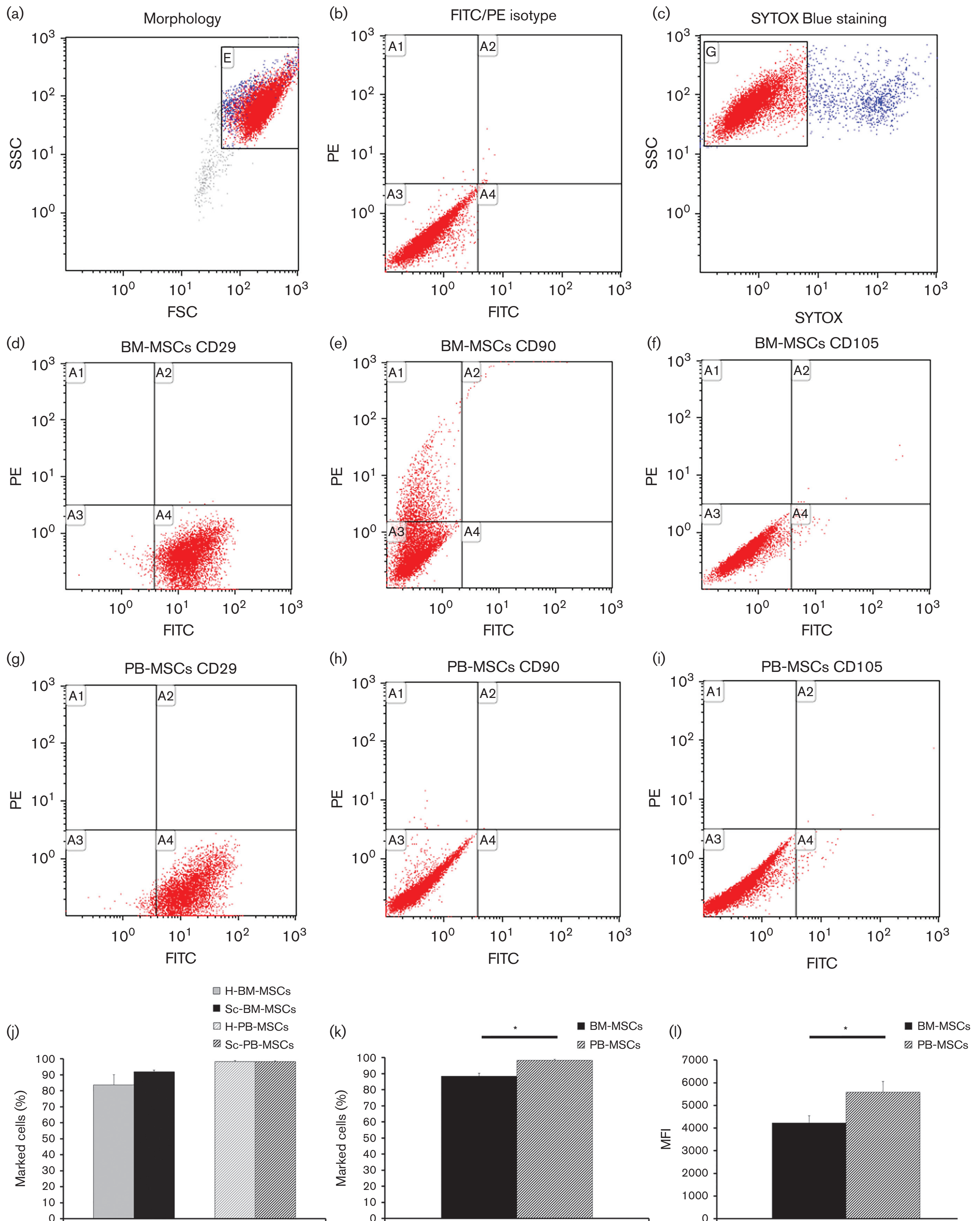

(a)
Morphology
SSC
FSC
E
(b)
FITC/PE isotype
PE
FITC
A1
A2
A3
A4
(c)
SYTOX Blue staining
SSC
SYTOX
G
(d)
BM-MSCs CD29
(e)
BM-MSCs CD90
(f)
BM-MSCs CD105
(g)
PB-MSCs CD29
(h)
PB-MSCs CD90
(i)
PB-MSCs CD105
(j)
H-BM-MSCs
Sc-BM-MSCs
H-PB-MSCs
Sc-PB-MSCs
Marked cells (%)
(k)
BM-MSCs
PB-MSCs
Marked cells (%)
(l)
BM-MSCs
PB-MSCs
MFI

Fig. 2. Representative flow cytometry plots showing (a) ovine MSC morphology, (b) isotype controls of mouse mAbs for FITC and phycoerythrin (PE), (c) SYTOX Blue staining for dead cells, and results for the markers CD29, CD90 and CD105 in (d–f) BM-MSCs and (g–i) PB-MSCs. The bar charts below the plots show (j) percentages of CD29 immunoreactive cells in scrapie-infected (Sc) and healthy (H) BM- and PB-MSCs, and (k) percentages of CD29 immunoreactive cells and (l) mean fluorescence intensity (MFI) in BM- and PB-MSCs. Data are presented as mean ± SEM. Significant differences between bone marrow and peripheral blood were calculated with Student's *t*-test (*$P<0.05$).

cultures only revealed a significant downregulation of *BGN* in Sc-BM-MSCs at basal conditions.

The osteogenic differentiation ability of MSCs was confirmed using Alizarin Red S staining to detect calcium deposits. Red-dyed nodular aggregations were observed, demonstrating the mineralization process (Fig. 3). Quantification of osteogenic marker expression by qRT-PCR revealed a significant downregulation of *COL1A1* (collagen type I, α1) in differentiated Sc-BM-MSCs ($P<0.05$). The variability observed between groups did not allow the detection of significant differences between scrapie-infected and control cultures.

Transdifferentiation into neuronal-like cells

The potential of MSCs to transdifferentiate into neuronal-like cells was evaluated *in vitro* in cells obtained from bone marrow and peripheral blood from healthy and scrapie-infected sheep. Direct observation by microscopy showed morphological changes after 3 days of culture under neurogenic conditions. MSCs changed their fibroblast-like appearance into sharply defined shapes and retracted toward the nucleus, with some neurite-like processes (Fig. 4). Neurogenic differentiation was also evaluated using qRT-PCR analysis of the neurogenic markers *NEFM* (neurofilament, medium polypeptide), *NES* (nestin) and *TUBB3* (tubulin, β3 class III). Although morphology was clearly altered in the differentiated cultures, the expression of neurogenic markers in differentiated cells did not display statistically significant changes at day 3; only *NES* and *TUBB3* were significantly downregulated in differentiated Sc-PB-BMCs ($P<0.05$) and H-BM-MSCs ($P<0.01$), respectively (Fig. 4). Downregulation of these neurogenic markers was also observed in MSC cultures derived from scrapie-infected sheep at basal levels: *NES* in Sc-BM-MSCs ($P<0.05$) and *TUBB3* in Sc-PB-MSCs ($P<0.05$) (Fig. 4).

PrP^C gene and protein expression

The expression of the prion protein was confirmed at the transcript level by qRT-PCR in the different cell types (Fig. 5). The expression was quantified at basal conditions and after 3 days of culture under neurogenic conditions. Changes in *PRNP* expression related to the disease were not observed. A dot-blotting assay confirmed the expression of PrP^C by ovine MSCs, displaying high variability between cultures. This assay validated the lack of differences in PrP^C expression between healthy and scrapie-infected cultures at basal conditions (Fig. 5).

The expression of the *PRNP* gene was not significantly modified after neurogenic differentiation either in BM- or PB-MSCs, although a trend to increase its expression was observed mainly in differentiated BM-MSCs from both control and scrapie-infected sheep (Fig. 5). Dot-blotting was performed at day 9 of neurogenic differentiation in a reduced number of cultures. The expression of PrP^C was analysed in four BM-MSC cultures (two from healthy sheep and two from scrapie-infected sheep). Three of these cultures appeared to increase the expression of PrP^C under neurogenic conditions; however, the PrP^C signal decreased in one of the scrapie-infected cultures under neurogenic differentiation (Fig. 5). A similar assay was performed in six PB-MSC cultures (three from healthy sheep and three from scrapie-infected sheep). In this case, differentiated cultures obtained from scrapie-infected sheep displayed a significant PrP^C upregulation ($P<0.05$) compared with their controls under growth conditions (Fig. 5).

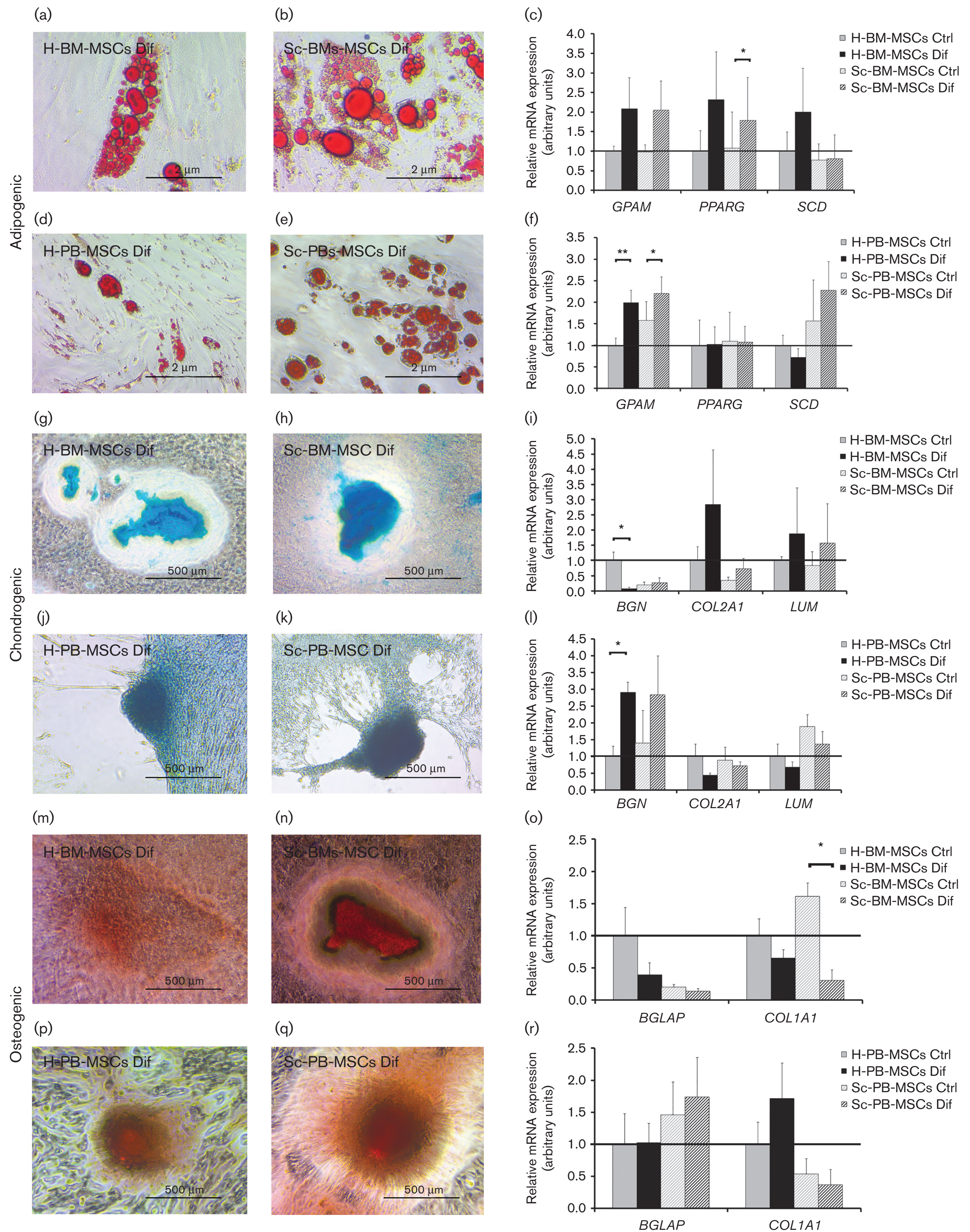
Determination of PrP^{Sc} in MSCs

A blind protein misfolding cyclic amplification (PMCA) analysis was developed to detect PrP^{Sc} in BM- and PB-MSC cultures obtained from healthy and scrapie-infected sheep at passage 3. None of the cultures displayed a seed able to amplify PrP^{Sc} after five rounds of PMCA. Under the same conditions, this technique amplified a 10^{-12} dilution of a scrapie ARQ 10 % brain homogenate. To test if PrP^{Sc} was lost during MSC expansion, bone marrow mononuclear cells and passage 1 BM-MSCs derived from two scrapie-infected sheep were also analysed by PMCA. None of them displayed detectable replication of PrP^{Sc}.

DISCUSSION

MSCs have been proposed as good candidates for cell therapy in neurodegenerative diseases. MSCs can be isolated from different tissues, including the accessible bone marrow or even peripheral blood. The key benefit of adult stem cells such as MSCs is their potential use in autologous therapies, avoiding the ethical concerns and risks of embryonic stem cells (Dantuma *et al.*, 2010). During recent years, the potential of MSCs for the treatment of neurodegenerative pathologies such as Alzheimer's disease, Parkinson's disease or amyotrophic lateral sclerosis (ALS) has been investigated (for review, see Tanna & Sachan, 2014).

Prion diseases are fatal neurodegenerative pathologies that affect humans and animals. As there is no effective treatment for this group of diseases, they are candidates for stem cell therapy. Human MSCs inoculated in mice infected with scrapie migrate to prion lesions in the brain, differentiate


(a)
H-BM-MSCs Dif
2 μm
(b)
Sc-BMs-MSCs Dif
2 μm
(c)
Relative mRNA expression (arbitrary units)
H-BM-MSCs Ctrl
H-BM-MSCs Dif
Sc-BM-MSCs Ctrl
Sc-BM-MSCs Dif
GPAM
PPARG
SCD
(d)
H-PB-MSCs Dif
2 μm
(e)
Sc-PBs-MSCs Dif
2 μm
(f)
H-PB-MSCs Ctrl
H-PB-MSCs Dif
Sc-PB-MSCs Ctrl
Sc-PB-MSCs Dif
GPAM
PPARG
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Adipogenic
(g)
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500 μm
(h)
Sc-BM-MSC Dif
500 μm
(i)
BGN
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(j)
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Sc-BMs-MSC Dif
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BGLAP
COL1A1
(p)
H-PB-MSCs Dif
500 μm
(q)
Sc-PB-MSCs Dif
500 μm
(r)
BGLAP
COL1A1
Osteogenic

Fig. 3. Adipogenic, chondrogenic and osteogenic differentiation of BM- and PB-MSCs of healthy and scrapie-infected sheep. Oil Red O staining of (a) H-BM-MSCs, (b) Sc-BM-MSCs, (d) H-PB-MSCs and (e) Sc-PB-MSCs cultured under adipogenic differentiation conditions. Alcian Blue staining of (g) H-BM-MSCs, (h) Sc-BM-MSCs, (j) H-PB-MSCs and (k) Sc-PB-MSCs cultured in chondrogenic medium. Alizarin Red staining of (m) H-BM-MSCs, (n) Sc-BM-MSCs, (p) H-PB-MSCs and (q) Sc-PB-MSCs cultured in osteogenic differentiation medium. Quantification by qRT-PCR of (c, f) adipogenic, (i, l) chondrogenic and (o, r) osteogenic markers. Significant differences between expression levels were calculated with Student's *t*-test (*$P<0.05$; **$P<0.01$). Dif, Differentiation; Ctrl, control.

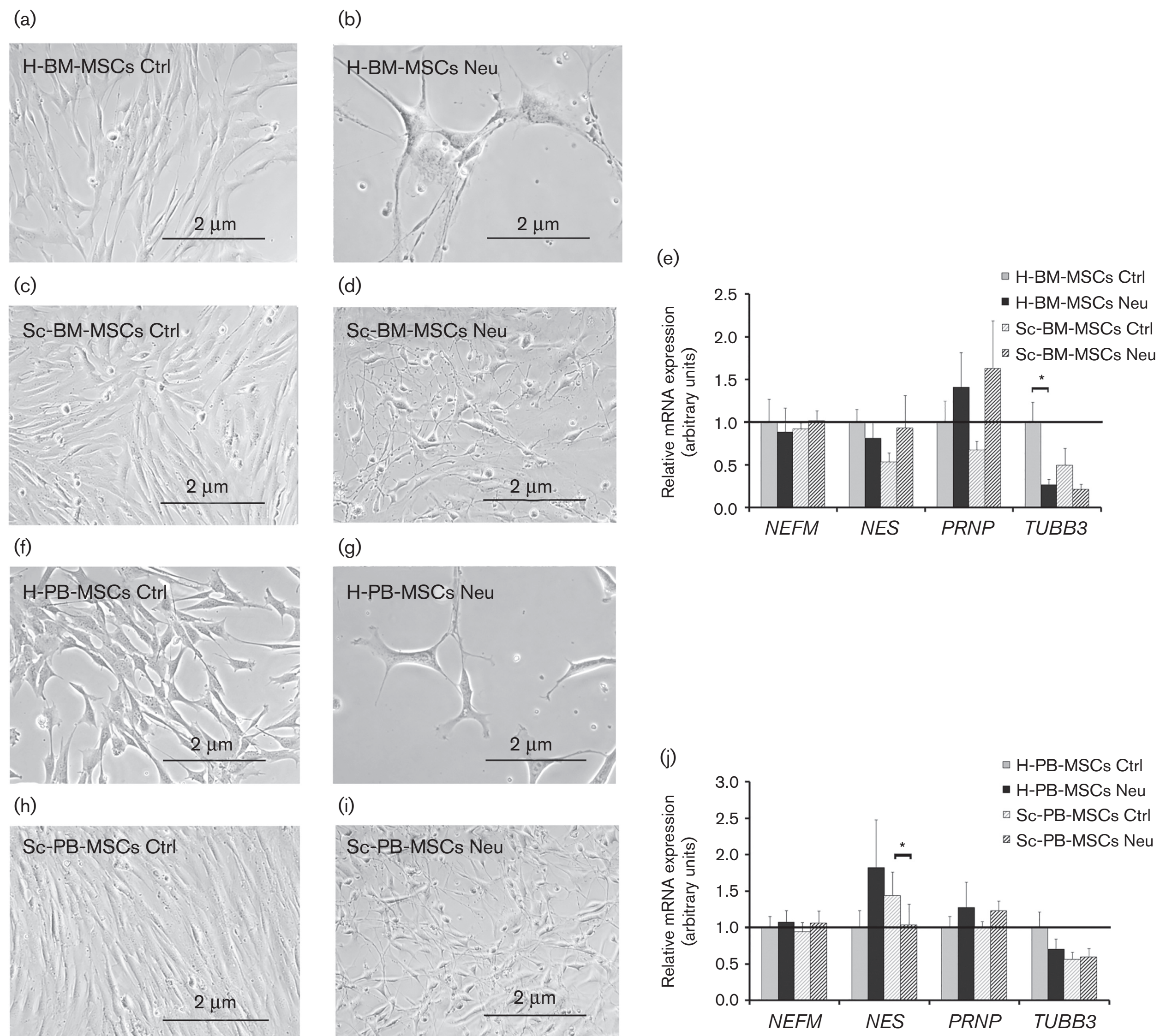

Fig. 4. Neurogenic differentiation of BM- and PB-MSCs of healthy and scrapie-infected sheep. Phase-contrast micrographs of H-BM-MSCs under (a) basal and (b) neurogenic conditions, (c) basal Sc-BM-MSCs, (d) differentiated Sc-BM-MSCs, (f) basal H-PB-MSCs, (g) differentiated H-PB-MSCs, (h) basal Sc-PB-MSCs, and (i) differentiated Sc-PB-MSCs. Bar charts show the relative expression levels of neurogenic markers for (e) BM-MSCs and (j) PB-MSCs quantified by qRT-PCR. Significant differences were calculated with Student's *t*-test (*$P<0.05$). Ctrl, Control (basal); Neu, neurogenic.

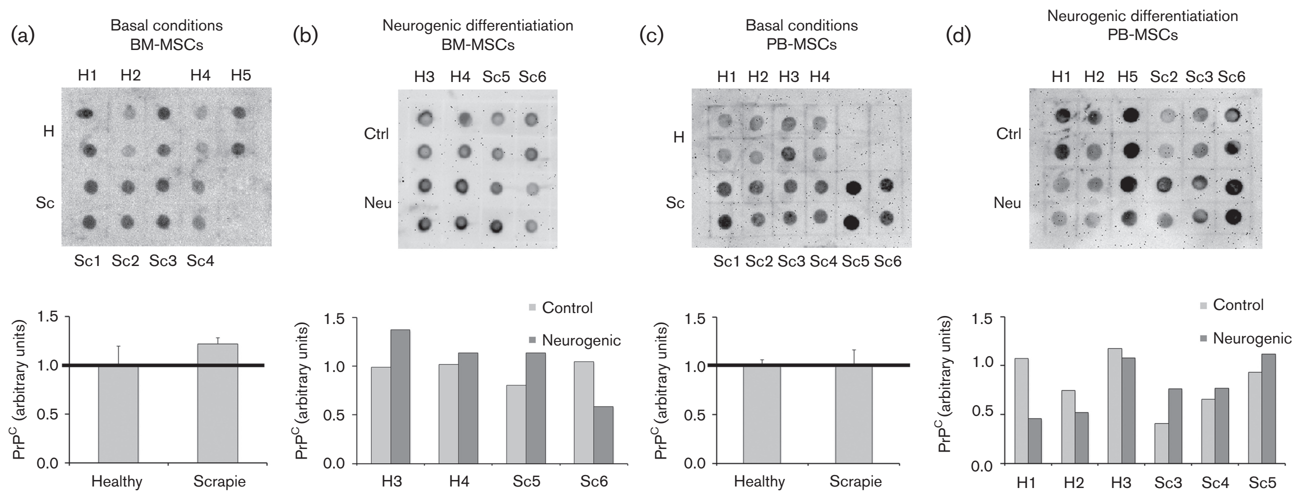

Fig. 5. Expression of PrP^C by MSCs isolated from healthy and scrapie-infected sheep under basal and neurogenic conditions. (a) Dot-blots showing PrP^C expression in H-BM-MSCs and Sc-BM-MSCs under basal conditions, and bar chart showing the PrP^C quantification in healthy and scrapie-infected groups of samples after normalization. (b) PrP^C dot-blots of BM-MSC cultures under growth and neurogenic conditions, and their individual quantification. (c) PrP^C dot-blots of H-PB-MSCs and Sc-PC-MSCs under basal conditions, and quantification by groups after normalization. (d) Dot-blots of PB-MSC cultures under growth and neurogenic conditions, and their individual quantification. Each sample was tested in duplicate. Ctrl, Control (growth); Neu, neurogenic.

into cells with neuronal and glial characteristics, and increase the lifespan of scrapie-infected mice (Song *et al.*, 2009). Before their use in autologous therapy, the effect of the disease on the characteristics of MSCs should be investigated. To the best of our knowledge, this is the first study analysing the mesenchymal characteristics of BM- and PB-MSCs in a prion disease model. Scrapie in sheep can be regarded as a good model for the human prion diseases and could be used for testing new therapies, such as those based on regenerative medicine.

Brains from prion-infected mice segregate chemoattractive factors that attract MSCs to brain lesions associated with prion replication (Song *et al.*, 2009, 2011). Our results suggest that MSCs are not mobilized in the natural disease because we obtained adherent fibroblast-like cells from peripheral blood and bone marrow of all the animals analysed, and no differences related to the disease.

A lower growth potential has been suggested in MSCs obtained from patients with other neurodegenerative diseases, such as ALS (Bossolasco *et al.*, 2010; Ferrero *et al.*, 2008) or multiple sclerosis (Mallam *et al.*, 2010); however, this decrease has always been not significant and in other diseases like Parkinson's, MSCs from patients did not differ from control cultures (Zhang *et al.*, 2008). In our study, PB- and BM-MSCs from scrapie-infected sheep displayed a significantly lower CD parameter during the three first passages, which could indicate a lower proliferation potential, although this reduction was not confirmed with the DT parameter.

All cells from both origins and disease status expressed the MSC surface markers *CD29*, *CD73* and *CD90* at the transcript level. The expression of haematopoietic marker *CD45* mRNAs in one of the H-BM-MSC cultures could be due to contamination with haematopoietic cells, although its expression has been described in MSCs from haematologic disease patients and, under certain culture conditions, this marker can be expressed by MSCs (Yeh *et al.*, 2006). Similarly, although *CD34* is considered an haematopoietic progenitor marker, there is evidence of *CD34* expression by MSCs derived from different tissues, including bone marrow or peripheral blood (Lin *et al.*, 2012; Lyahyai *et al.*, 2012; Ranera *et al.*, 2011). As a consequence, BM- and PB-MSCs from healthy and scrapie-infected sheep displayed a gene expression profile compatible with the mesenchymal origin of these cells.

The expression of these markers was evaluated at the protein level by flow cytometry. Due to the lack of specific antibodies for ovine epitopes, several studies have tested the cross-reactivity between anti-human antibodies and ovine cell surface proteins (Boxall & Jones, 2012; McCarty *et al.*, 2009; Mrugala *et al.*, 2008). We used a panel of seven anti-human antibodies, some of which have shown immunoreactivity against epitopes from other mammalian species, such as horses (Ranera *et al.*, 2011). This analysis confirmed the high expression of CD29 by ovine MSCs; however, as in other works, most of the anti-human antibodies did not display cross-reactivity against ovine cell surface markers (Boxall & Jones, 2012).

The only difference observed between healthy and scrapie-infected cultures was a slight increase of *CD29* transcripts in Sc-BM-MSCs. This difference was not confirmed at the protein level due to the high variability observed along the

study. PrP^C contributes to neuronal polarization through spatially organizing β_1-integrins (CD29) at the plasma membrane but does not modify total β_1-integrin expression levels (Loubet *et al.*, 2012). Further investigations are necessary to confirm if the variability observed in CD29 protein levels can affect the ability of these cells to differentiate into neuronal-like cells.

MSCs from ALS and Parkinson's disease patients display abilities to differentiate into the three mesodermal lineages (Bossolasco *et al.*, 2010; Zhang *et al.*, 2008). In our study, cells were maintained under differentiation conditions until they showed morphology and staining compatible with adipogenic, chondrogenic or osteogenic differentiation. In general, high variability was observed within groups of donors. The heterogeneity in differentiation potential between MSC donors has been described in many species (Lei *et al.*, 2013; Lyahyai *et al.*, 2012; Ranera *et al.*, 2012; Siegel *et al.*, 2013). This variability makes the comparison of MSCs from scrapie and healthy animals difficult. Nevertheless, the few significant changes related to the disease represented a downregulation of differentiation markers (*BGN* and *COL1A1*) in cells obtained from affected individuals; this fact could reflect a slight loss of differentiation potential.

Cultures derived from scrapie-infected sheep displayed a morphology compatible with neurogenic differentiation, and no clear differences were observed between scrapie-infected and healthy cultures during the differentiation process. Undifferentiated human MSCs express nestin mRNA (Montzka *et al.*, 2009) and the expression of the nestin gene increases progressively with the number of passages in rat MSCs (Wislet-Gendebien *et al.*, 2003), which has been suggested to be an important stage in the ability to differentiate into neuronal cells. Donor heterogeneity in the expression levels of neurogenic markers has been described in humans (Montzka *et al.*, 2009). Similarly, ovine MSC cultures analysed in our study displayed great variability in the basal levels of neurogenic markers. However, in addition to this variability, we found a statistically significant reduction of *NES* and *TUBB3* in scrapie-derived BM- and PB-MSCs, respectively, that could limit their therapeutic potential when used as autologous therapy.

BM-MSCs express PrP^C and its expression decreases with passage number (Mohanty *et al.*, 2012), as well as their capacity to proliferate (Wagner *et al.*, 2009). This characteristic has been used to develop *in vitro* models for prion multiplication based on the culture and infection of murine MSCs (Akimov *et al.*, 2008, 2009; Cervenakova *et al.*, 2011). We reported the expression of PrP^C in ovine PB-MSCs at the transcript level (Lyahyai *et al.*, 2012) and proposed ovine MSCs as good candidates to develop *in vitro* models for prion propagation in the natural host (Mediano *et al.*, 2015). In this work, we have confirmed the expression of the PrP^C protein in both, BM- and PB-MSCs. The disease appeared not to modify the expression of PrP^C either at the transcript or at the protein level. Both qRT-PCR and dot-blotting assays revealed a high variability in PrP^C expression between cultures. Heterogeneity in PrP^{Sc} susceptibility has been reported for subclones of tumour cell lines (Bosque & Prusiner, 2000; Mahal *et al.*, 2007); however, the susceptibility to prion infection was not correlated with PrP^C levels (Prusiner, 1991).

Neuro2A cells treated with retinoic acid, a compound used for neuronal differentiation, overexpressed PrP^C and were more susceptible to prion infection (Bate *et al.*, 2004). Previously (Lyahyai *et al.*, 2012), we observed an upregulation of *PRNP* transcripts during the neurogenic differentiation process of ovine PB-MSCs. Although showing high variation, most MSC cultures also increased the expression of PrP^C during neurogenic differentiation that could help in their further use for *in vitro* infection.

BM-MSCs from mice infected with prions and from Creutzfeldt–Jakob disease (CJD) patients show infectivity (Takakura *et al.*, 2008), although there is a certain controversy about the infectivity of bone marrow in human patients (Brown *et al.*, 1994). The infectivity of blood and blood cells has been reported in scrapie-infected sheep (Halliez *et al.*, 2014; Lacroux *et al.*, 2012) and CJD patients (Douet *et al.*, 2014). Our PMCA analysis did not detect the presence of PrP^{Sc} either in PB- or BM-MSCs derived from sick animals at passage 3. The infectivity does not seem to be lost during their proliferation in culture because bone marrow mononuclear cells and MSCs at passage 1 did not present PMCA-replicable PrP^{Sc}. Although the infectivity of bone marrow from scrapie-infected sheep has been reported (Hadlow *et al.*, 1982), our study does not corroborate the hypothesis of bone marrow being the source of the prionaemia described in sheep. Thus, infectivity of BM-MSCs cannot be considered as a general feature for prion diseases and their analysis cannot be used for *in vivo* diagnosis for scrapie.

To conclude, our study shows the characterization of MSCs obtained from individuals affected with prion diseases. BM- and PB-MSCs from scrapie-infected animals revealed subtle differences in proliferation, an increase in *CD29* expression at the transcript level, slight differences in the expression of osteogenic and chondrogenic markers, and downregulation of some neurogenic markers from basal levels. Whether these slight modifications could have an influence on their ability for autologous cell therapy needs further investigation. Finally, neither PB- nor BM-MSCs displayed PrP^{Sc} infectivity, and they cannot be used for *in vivo* diagnosis of ovine scrapie. However, the increase of PrP^C during the neurogenic differentiation process could help in developing new *in vitro* assays for the study of prion disease biology based on the culture and infection of MSCs.

METHODS

Animals and sample collection. A total of 20 adult Rasa Aragonesa female sheep were used in this study, 19 displayed the ARQ/ARQ genotype for the *PRNP* gene and one sheep showed the AHQ/AHQ genotype. Eleven of these sheep displayed clinical symptomatology of scrapie and nine were considered as healthy, without any clinical signs compatible with scrapie or with any other pathology. Animals that displayed neurological symptoms were sacrificed and diagnosis was

confirmed by determination of PrP^{Sc} in medulla oblongata samples as described previously (Bolea *et al.*, 2005).

Approximately 30 ml peripheral blood was collected from six scrapie-infected and five healthy sheep by jugular venepuncture in tubes with sodium heparin. After animal sedation (xylazine intravenously) and local anaesthesia (lidocaine), bone marrow aspirates were harvested from the humeral head of seven scrapie-infected and six healthy sheep using a 13G Jameshdi needle and 10 ml syringes previously loaded with 5000 IU sodium heparin. All procedures were carried out under Project Licence PI06/12 approved by the Ethic Committee for Animal Experiments from the University of Zaragoza. The care and use of animals was performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Isolation and expansion of MSCs. MSC isolation from peripheral blood (30 ml) and bone marrow aspirates (3–13 ml) was performed as described previously (Lyahyai *et al.*, 2012; Ranera *et al.*, 2012). Both protocols were based on the separation of the mononuclear fraction after a density gradient centrifugation in Lymphoprep (Atom) for 20 min at 400 ***g***. Mononuclear cells were plated at 10^6 cells cm^{-2} in six-well plates with basal medium, consisting of low-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 20 % FBS for PB-MSCs or 10 % FBS for BM-MSCs, and with 1 % L-glutamine (Sigma-Aldrich) and 1 % streptomycin/penicillin (Sigma-Aldrich). Cells were incubated at 37 °C and 5 % CO_2, expanded until passage 3, cryopreserved in FBS with 10 % DMSO and stored at −150 °C for further characterization.

MSC proliferation. Adherent cells were counted at the end of passage 0 using a Neubauer chamber. Afterwards, MSCs were counted through passages 0–3, and CD and DT parameters were calculated as described previously (Lyahyai *et al.*, 2012).

MSC characterization. In addition to plastic adherence under standard culture conditions, the minimal criteria to define MSCs are the expression of certain cell surface markers, and the ability to differentiate into adipocytes, osteoblasts and chondroblasts *in vitro* (Dominici *et al.*, 2006).

The expression of mesenchymal (*CD29*, *CD36*, *CD49*, *CD73*, *CD90*, *CD166* and *CD105*) and haematopoietic (*CD34* and *CD45*) cell surface markers was evaluated at the transcript level by qRT-PCR. Total RNA was extracted and converted to cDNA from 200 000 previously frozen cells using a Cells-to-cDNA II kit (Ambion). Amplifications were performed using primers and conditions described previously (Lyahyai *et al.*, 2012).

The expression of the surface markers at the protein level was analysed by flow cytometry. First, a total of seven surface markers were examined in five cultures (four BM-MSC and one PB-MSC): the mesenchymal cell markers CD29 (integrin-1), CD44 (H-CAM), CD73 (ecto-5′-nucleotidase), CD90 (Thy-1) and CD105 (endoglin), and the haematopoietic markers CD34 and CD45 (LCA). The anti-human mouse antibodies and the methodology have been described previously (Ranera *et al.*, 2011). Before the FACS analysis (FACSAria; BD Biosciences), viable and non-viable cells were discriminated using the SYTOX Blue dead cell stain (Molecular Probes). Positive staining for the CD markers was defined as the emission of a fluorescence signal that exceeded levels obtained by >95 % of cells from the control population stained with matched isotype antibodies. Dot-plots were generated using FACSDiva 5.0.1 (BD Biosciences).

Afterwards, the expression of CD29 was evaluated by flow cytometry in three H-BM-MSC, four Sc-BM-MSC, four H-PB-MSC and five Sc-PB-MSC cultures by quantifying the fluorescence intensity and percentage of positive cells.

Adipogenic, osteogenic and chondrogenic differentiation was developed *in vitro* following the previously described methodology (Jäger *et al.*, 2006; Ranera *et al.*, 2012) for both PB- and BM-MSCs. Cells were maintained under differentiation conditions until most cultures from the same group (BM- or PB-MSCs) displayed a morphology compatible with differentiated cells. Adipogenic differentiation was confirmed using a 0.3 % Oil Red O (Sigma-Aldrich) specific stain at 14 (BM-MSCs) or 21 days (PB-MSCs) of culture. Chondrogenic differentiation was identified by Alcian Blue G dye (1 : 1 in methanol) (Sigma-Aldrich) after 28 days of chondrogenic culture for BM-MSCs and 21 days for PB-MSCs. Osteogenic differentiation was confirmed by 2 % Alizarin Red S staining (Sigma-Aldrich) at 28 days of culture in all cases. In addition, the expression of adipogenic (*GPAM*, *PPARG* and *SCD*), chondrogenic (*BGN*, *COL2A1* and *LUM*) and osteogenic markers (*BGLAP* and *COL1A1*) was analysed by qRT-PCR on the same day as staining. Primers used for *GPAM* qRT-PCR were (forward: 5′-CATACAAGCACCGAGGGT-3′; reverse: 5′-CAAACGGT-GGTTGCATTGACTT-3′), and primers and conditions for the amplification of remaining markers and housekeeping genes were those described previously (Lyahyai *et al.*, 2010, 2012).

Transdifferentiation into neuron-like cells. The capacity of MSCs to transdifferentiate into neuron-like cells was analysed by culturing the cells with HyClone Neural Differentiation kit medium (Thermo Scientific) as described previously (Lyahyai *et al.*, 2012). The cultures were studied by direct observation under a microscope at day 3 of neurogenic culture. Additionally, qRT-PCR was performed as described previously (Lyahyai *et al.*, 2012) to detect the expression of neurogenic markers *NEFM*, *NES* and *TUBB3* at day 3 of culture. Expression levels in the differentiated cultures were compared with cultures under basal conditions.

PrP^C gene and protein expression. The expression of PrP^C in PB- and BM-MSCs was evaluated under basal and neurogenic conditions by qRT-PCR and dot-blotting following standard procedures. Primers and conditions for the amplification of *PRNP* and the housekeeping genes were those described previously (Lyahyai *et al.*, 2010). The expression of this gene was evaluated under the same cell cultures as neurogenic markers (see above).

For PrP^C protein determination, 10^6 cells at basal conditions or after 9 days of neurogenic culture were homogenized in 500 µl PBS. Samples of 10 µg total protein were deposited by dropping 7 µl on a 0.2 µm Immun-Blot PCDF (Bio-Rad) membrane, and PrP^C was determined using the mouse mAb anti-PrP IgG1 6H4 (Prionics) and the alkaline phosphatase-conjugated goat anti-mouse IgG (Prionics) as secondary antibody. CDP-Star substrate (Tropix) was used to determine chemiluminescence in a Versa-Doc Imaging System (Bio-Rad). Chemiluminescence signals were evaluated using ImageJ 1.4.3.67 (Psion Image) as described previously (Filali *et al.*, 2013).

PMCA. *In vitro* prion replication experiments were performed as described previously (Castilla *et al.*, 2008). Briefly, 10^6 mesenchymal cells from scrapie-infected (Sc-BM-MSCs $n=4$, Sc-PB-MSCs $n=6$) or healthy (H-BM-MSCs $n=5$, H-PB-MSCs $n=5$) sheep were centrifuged and pellets were resuspended in 120 µl Tg338 VRQ ovine transgenic mice brain homogenate. The final volume was split in two 0.2 ml PCR tubes and samples were subjected to sonication (S-700MPX; QSonica). The sonicator settings were 20 s at a power setting of 70–80 % followed by 30 min of incubation for a total of 24 h for each round, performed at 37–38 °C. Up to five serial rounds of PMCA were performed and unseeded tubes were included as negative controls. To test for PrP^{Sc} presence, all sonicated samples were digested with 50–100 µg proteinase K ml^{-1} for 1 h at 42 °C and analysed by Western blotting. Blots were probed with anti-PrP mAb 9A2. The presence of PrP^{Sc} in bone marrow mononuclear cells

obtained from two scrapie-infected sheep and MSCs from these two sheep at passage 1 were also evaluated following the same procedure.

To ensure the proper sonicator operation and in order to discard potential cross-contaminations, standard PMCA of Tg338 brain homogenate seeded with ovine scrapie up to 10^{-12} dilution was performed at a later time.

Statistical methods. SPSS 15.0 was used for the statistical analysis. Data obtained from qRT-PCR, flow cytometry and Western blotting were analysed for normality with the Shapiro–Wilk test. Differences in gene expression, reactivity levels and dot intensity between scrapie-infected and healthy MSCs were determined using the unpaired non-parametric Mann–Whitney *U*-test or Student's *t*-test. For the different tests, $P<0.05$ was considered statistically significant.

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