Characterization of mesenchymal stem cells in sheep naturally infected with scrapie

Diego R. Mediano,1† David Sanz-Rubio,1† Rosa Bolea,2 Belén Marín,2 Francisco J. Vázquez,1 Ana R. Remacha,1 Óscar López-Pérez,1,2 Natalia Fernández-Borges,3 Joaquín Castilla,3,4 Pilar Zaragoza,1 Juan J. Badiola,2 Clementina Rodellar1 and Inmaculada Martín-Burriel1,2

Correspondence
Inmaculada Martín-Burriel
minma@unizar.es

1Laboratorio de Genética Bioquímica, Instituto de Investigación Agroalimentaria (IA2), IIS Aragón, Universidad de Zaragoza, Zaragoza, Spain
2Centro de Investigación en Encefalopatías y Enfermedades Transmisibles Emergentes, Instituto de Investigación Agroalimentaria (IA2), IIS Aragón, Universidad de Zaragoza, Zaragoza, Spain
3CIC bioGUNE, Parque Tecnológico de Bizkaia, Derio, Spain
4IKERBasque, Basque Foundation for Science, Bilbao, Spain

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, synapticogenic 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).

†These authors contributed equally to this paper.
In addition to their use as therapy, stem cells are being used in prion research as possible \textit{in vitro} models for prion propagation. A wide variety of stem cells express PrP\textsuperscript{C}, including MSCs (Mohanty \textit{et al.}, 2012), and the function of this protein has been linked to stem cell biology (Lee \\& Baskakov, 2013; Zhang \textit{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 \textit{et al.}, 2008, 2009; Cervenakova \textit{et al.}, 2011). The development of such \textit{in vitro} models in species naturally susceptible to the disease would avoid the species barrier. Our group presented the first \textit{in vitro} characterization of peripheral blood-derived ovine MSCs (PB-MSCs) and demonstrated that these cells express PrP\textsuperscript{C}, at least at the transcript level (Lyahyai \textit{et al.}, 2012).

Finally, the determination of infectivity in MSCs obtained from individuals affected with prion diseases has been proposed for \textit{in vivo} diagnosis for TSEs. Murine models experimentally infected with prions accumulate PrP\textsuperscript{Sc} in bone marrow-derived MSCs (BM-MSCs) and this infection could precede prion accumulation in brain (Takakura \textit{et al.}, 2008). MSCs can also be found in peripheral blood, and the presence of PrP\textsuperscript{Sc} has been demonstrated in several fractions of human and ovine blood (Andréololetti \textit{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\textsuperscript{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\textsuperscript{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).

<table>
<thead>
<tr>
<th>Passage</th>
<th>Parameter</th>
<th>BM-MSCs</th>
<th>PB-MSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Healthy</td>
<td>Scapie</td>
</tr>
<tr>
<td>0</td>
<td>Adherent cells (× 10(^3))</td>
<td>379 ± 231</td>
<td>385 ± 363</td>
</tr>
<tr>
<td>1</td>
<td>CD</td>
<td>3.06 ± 0.57</td>
<td>2.98 ± 0.75</td>
</tr>
<tr>
<td></td>
<td>DT (days)</td>
<td>2.18 ± 0.60</td>
<td>2.42 ± 1.45</td>
</tr>
<tr>
<td>2</td>
<td>CD</td>
<td>2.9 ± 0.35</td>
<td>1.9 ± 0.84*</td>
</tr>
<tr>
<td></td>
<td>DT (days)</td>
<td>2.42 ± 0.35</td>
<td>3.57 ± 1.71</td>
</tr>
<tr>
<td>3</td>
<td>CD</td>
<td>2.61 ± 0.18</td>
<td>1.97 ± 0.50*</td>
</tr>
<tr>
<td></td>
<td>DT (days)</td>
<td>3.32 ± 0.68</td>
<td>2.55 ± 1.14</td>
</tr>
</tbody>
</table>

\( ^* \text{P}<0.05 \) (Student’s \( t \)-test).
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 maker \(CD29\), 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 \(CD14\), \(CD34\) and \(CD73\) (data not shown). Immunoreactivity against the haematopoietic marker \(CD45\) was not detectable (Fig. 2).

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 staining 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 glycero-3-phosphate acyltransferase), \(PPARG\) (peroxisome proliferator-activated receptor \(\gamma\)) 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 dyeing, 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, \(\alpha1\)) 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

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**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 (*\(P<0.05\)).
Morphology

BM-MSCs CD29

PB-MSCs CD29

BM-MSCs CD90

PB-MSCs CD90

BM-MSCs CD105

PB-MSCs CD105

SYTOX Blue staining

FITC/PE isotype

FITC

Sc-BM-MSCs

H-BM-MSCs

Sc-PB-MSCs

H-PB-MSCs

Marked cells (%)

MFI

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PrP 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 PrPC by ovine MSCs, displaying high variability between cultures. This assay validated the lack of differences in PrPC 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 PrPC was not significantly downregulated 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).

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...
Relative mRNA expression (arbitrary units)

- GPAM
- PPARG
- SCD
- BGN
- COL2A1
- LUM
- BGLAP
- COL1A1

H-BM-MSCs Ctrl
Sc-BM-MSCs Ctrl
Sc-PB-MSCs Ctrl
H-BM-MSCs Dif
Sc-BM-MSCs Dif
Sc-PB-MSCs Dif
**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.
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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 dis-
ease 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 regen-
erative medicine.

Brains from prion-infected mice segregate chemoattractive
factors that attract MSCs to brain lesions associated with
prion replication (Song 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 dis-
eases 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 signifi-
cantly 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 contami-
nation 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 a 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 com-
patible 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 anti-
odies did not display cross-reactivity against ovine cell sur-
face 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

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**Fig. 5.** Expression of PrP<sub>C</sub> by MSCs isolated from healthy and scrapie-infected sheep under basal and neurogenic conditions. (a) Dot-blot showing PrP<sub>C</sub> expression in H-BM-MSCs and Sc-BM-MSCs under basal conditions, and bar chart showing the PrP<sub>C</sub> quantification in healthy and scrapie-infected groups of samples after normalization. (b) PrP<sub>C</sub> dot-blots of BM-MSC cultures under growth and neurogenic conditions, and their individual quantification. (c) PrP<sub>C</sub> 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.
study, PrP<sup>C</sup> contributes to neuronal polarization through spatially organizing β<sub>1</sub>-integrins (CD29) at the plasma membrane but does not modify total β<sub>1</sub>-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 down-regulation 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<sup>C</sup> 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<sup>C</sup> 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<sup>C</sup> protein in both, BM- and PB-MSCs. The disease appeared not to modify the expression of PrP<sup>C</sup> either at the transcript or at the protein level. Both qRT-PCR and dot-blotting assays revealed a high variability in PrP<sup>C</sup> expression between cultures. Heterogeneity in PrP<sup>Sc</sup> 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<sup>C</sup> levels (Prusiner, 1991).

Neuro2A cells treated with retinoic acid, a compound used for neuronal differentiation, overexpressed PrP<sup>C</sup> 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<sup>C</sup> 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<sup>Sc</sup> 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<sup>Sc</sup>. 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 prionemia 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<sup>Sc</sup> infectivity, and they cannot be used for in vivo diagnosis of ovine scrapie. However, the increase of PrP<sup>C</sup> 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.

**METHODOLOGY**

**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 symptoms 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

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confirmed by determination of PrPSc 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 Jameshidi needle and 10 ml syringes previously loaded with 5000 IU sodium heparin. All procedures were carried out under Project Licence P906/12 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 x g. Mononuclear cells were plated at 106 cells cm2 in six-well plates with a low-glycine modified Eagle’s medium (DMEM; Sigma-Aldrich), Viable and non-viable cells were discriminated using the anti-human haematopoietic markers CD34 and CD45 (LCA). The anti-human integrin-1, H-CAM, and endoglin (CD90) and endoglin (CD105) antibodies (Lytahyai et al., 2012). Both CD markers was defined as the emission of a fluorescence signal previously (Ranera et al., 2011). Before the FACS analysis (FACSAria; BD Biosciences), viable and non-viable cells were discriminated using the biotinylated 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).

**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.

**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 8 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) 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) 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) 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) after 28 days of chondrogenic culture for BM-MSCs and 21 days for PB-MSCs.

**PrPSc gene and protein expression.** The expression of PrPSc 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 PrPSc protein determination, 106 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 PrPSc 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, 106 mesenchymal cells from scrapie-infected (Sc-BM-MSCs n=4, Sc-PB-MSCs n=6) or healthy (BM-MSCs n=5, H-PB-MSCs n=5) sheep were centrifuged and pellets were resuspended in 120 µl Tg338 VRQ ovine transgenic micro 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 PrPSc 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 PrPSc 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–Wilks 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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