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RNA-sequencing transcriptomic analysis of scrapie-exposed ovine mesenchymal stem cells

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ABSTRACT

In neurodegenerative diseases, including prion diseases, cellular models arise as useful tools to study the pathogenic mechanisms occurring in these diseases and to assess the efficacy of potential therapeutic compounds. In the present study, a RNA-sequencing analysis of bone marrow-derived ovine mesenchymal stem cells (oBM-MSCs) exposed to scrapie brain homogenate was performed to try to unravel genes and pathways potentially involved in prion diseases and MSC response mechanisms to prions. The oBM-MSCs were cultured in three different conditions (inoculated with brain homogenate of scrapie-infected sheep, with brain homogenate of healthy sheep and in standard growth conditions without inoculum) that were analysed at two exposure times: 2 and 4 days post-inoculation (dpi). Differentially expressed genes (DEGs) in scrapie-treated oBM-MSCs were found in the two exposure times finding the higher number at 2 dpi, which coincided with the inoculum removal time. Pathways enriched in DEGs were related to biological functions involved in prion toxicity and MSC response to the inflammatory environment of scrapie brain homogenate. Moreover, RNA-sequencing analysis was validated amplifying by RT-qPCR a set of 11 DEGs with functions related with prion propagation and its associated toxicity. Seven of these genes displayed significant expression changes in scrapie-treated cells. These results contribute to the knowledge of the molecular mechanisms behind the early toxicity observed in these cells after prion exposure and to elucidate the response of MSCs to neuroinflammation.

1. Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are a group of neurodegenerative diseases that affect both humans and animals. Animal TSEs include, among others, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE), and chronic wasting disease in cervids (CWD). They are caused by the accumulation in the central nervous system (CNS) of the misfolded form (PrP^{Sc}) of the cellular prion protein (PrP^{C}) (Soto and Satani, 2011). Sheep naturally infected with scrapie has been considered an excellent natural model for the study of the neuropathology associated with these diseases (Pattison and Jones, 1967). The main horizontal transmission route of the classical form of scrapie is the ingestion of straw bedding contaminated by remains of placenta expelled by infected animals during delivery, although other infection routes exist, like vertical transmission through the ingestion of milk and colostrum (Acín et al., 2021). Despite scrapie being an acquired TSE, the genetic susceptibility of individuals to infection and disease development is strongly linked to polymorphisms in the *PRNP* gene, which encodes PrP^{C} (Acín et al., 2021).

In addition to prion diseases, other human neurodegenerative diseases, including Alzheimer's, Parkinson's and Huntington's diseases, and Amyotrophic lateral sclerosis, are characterized by a chronic progressive degeneration of the structure and function of the CNS. This degeneration is due to the aggregation and accumulation of misfolded proteins in the CNS, leading to cellular dysfunction, loss of synaptic connections, and brain damage, which is a hallmark event in these

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pathologies that are also known as prion-like diseases (Soto and Pritzkow, 2018). Unfortunately, the exact pathogenic mechanisms occurring in these diseases are not fully understood, and there are no current effective treatments to stop the disease progression (Schlachetzki et al., 2013). In this regard, cellular in vitro models appear as useful tools to investigate underlying molecular mechanisms and the specific roles of certain molecules and compounds in these diseases, and to screen potential pharmacological targets (Schlachetzki et al., 2013).

In prion diseases, there are various murine cell lines that can replicate mouse-adapted prion strains (Butler et al., 1988; Klöhn et al., 2003; Mahal et al., 2007; Nishida et al., 2000). These strains are not naturally occurring, and they may not accurately express the characteristics of the original disease-causing strains (Krance et al., 2020). Therefore, it is critical to use cell models from natural susceptible species that replicate natural strains to reliably study disease characteristics and develop potential therapies (Krance et al., 2020).

Several cellular models from naturally infected animals and humans have been developed. MDBK bovine-derived kidney cells (Tark et al., 2015) and MDB cells derived from mule deer brain (Raymond et al., 2006) are able to replicate BSE and CWD prions, respectively. These cell culture models (neuronal and non-neuronal) are useful for prion propagation, but they do not exhibit a prion-specific neurodegenerative phenotype, with the exception of primary neurons or brain slice cultures (Hannaoui et al., 2013; Le et al., 2019). The latter require the sacrifice of animals for their obtainment, which is a limitation for their use in naturally susceptible species like domestic and wild ruminants or humans.

Mesenchymal stem cells (MSCs) are adult stem cells that can be easily isolated from accessible tissues like adipose tissue or bone marrow and can differentiate into other cell types, including neuronal-like cells (Pittenger et al., 1999; Zhao et al., 2002). These cells express the cellular prion protein (PrP^{C}) (Lyahyai et al., 2012; Zhang et al., 2006), and murine MSCs can be infected with prions and propagate PrP^{Sc} (Akimov et al., 2008, 2009; Cervenakova et al., 2011; Takakura et al., 2008).

In addition to their use in vitro for prion replication, these cells have also been proposed as candidates for developing new treatments for prion diseases. Although the effect of treatment in murine models has been modest, the transplantation of human MSCs prolonged survival (Song et al., 2009) and autologous murine MSCs enhanced microglial activation (Shan et al., 2017). MSCs also appear to migrate to the CNS of infected animals in response to chemoattractive factors (Song et al., 2011). The therapeutic properties of MSCs are mainly based on their immunomodulatory abilities. MSCs derived from murine adipose tissue have been shown to be effective in decreasing inflammation in an in vitro model of prion-induced gliosis (Hay et al., 2022).

We have previously evaluated the potential of ovine mesenchymal stem cells (oMSCs) as a cellular model for the study of prion diseases in a naturally susceptible species. The infectivity reported for MSCs obtained from scrapie-infected mice and Creutzfeldt–Jakob disease patients (Takakura et al., 2008) was not observed in oMSCs obtained from the bone marrow and peripheral blood of scrapie-infected sheep (Mediano et al., 2015). These cells are unable to propagate PrP^{Sc} when cultured with scrapie brain extracts under growth conditions (García-Mendívil et al., 2021). However, both oMSCs isolated from scrapie-infected animals (Mediano et al., 2015) and cells exposed to scrapie extracts (García-Mendívil et al., 2021) display a slight decrease in proliferation. This temporary loss of proliferation potential could be a consequence of scrapie toxicity in these cells.

By comparing differences in gene expression patterns, transcriptomic analyses are helpful tools for studying cell biology and for understanding the physiological and pathological processes involved within a cell (Chen et al., 2016). RNA-sequencing (RNA-seq) is a transcriptome profiling technology that uses next-generation sequencing platforms, allowing the investigation of differential expression patterns between various conditions such as disease and healthy status (Chen et al., 2016; Courtney et al., 2010; Marguerat et al., 2008). This technology has been used to explore the transcriptome of several neurodegenerative diseases, including Alzheimer's and Parkinson's diseases, at a tissue and cellular level, in order to find genes and pathways altered or involved in these pathologies (Guennewig et al., 2021; Olah et al., 2020; Smajic et al., 2022; Soreq et al., 2014). In prion diseases, such as CJD and scrapie, RNA-seq transcriptomic approaches have also been employed to uncover the underlying molecular mechanisms associated with TSEs (Bartoletti-Stella et al., 2019; Carroll et al., 2020).

In the present study, RNA-seq of ovine bone marrow-derived mesenchymal stem cells (oBM-MSC) cultured in contact with scrapie brain extracts was performed to identify differentially expressed genes and molecular pathways potentially involved in the response of ovine MSCs to scrapie prions. After the transcriptomic analysis, a set of differentially expressed genes was selected for a validation study using quantitative real-time PCR to verify the results obtained in the RNA-seq analysis. The aim of this study is to investigate the early toxicity of scrapie in MSCs and the molecular response of these cells to scrapie-infected brain extracts. This will help assess their potential use as inflammatory modulating therapy in prion diseases.

2. Materials and methods

2.1. Ovine mesenchymal stem cell culture

A bone marrow sample was obtained from a one-year-old female Rasa Aragonesa sheep carrying the ARQ/ARQ genotype for the *PRNP* gene. This genotype is homozygous for Alanine (A), Arginine (R), and Glutamine (Q) at codons 136, 154 and 171, respectively, and is the most susceptible genotype to scrapie in this breed (Acín et al., 2004). After sedation with xylazine and local anaesthesia with lidocaine, a bone marrow aspirate was harvested as previously described (García-Mendívil et al., 2021). All procedures were carried out under project licence PI44/18, approved by the Ethical Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed in accordance with the Spanish Policy for Animal Protection, RD53/2013, which meets European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

MSC isolation from the bone marrow aspirate (2–3 mL) was performed using the previously described protocol (Ranera et al., 2012), which is based on the separation of the mononuclear fraction after density gradient centrifugation in Lymphoprep (STEMCELL Technologies) and further isolation due to the ability of MSCs to adhere to plastic. After isolation, cells were expanded up to passage 2 in basal medium, consisting of low glucose Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS), 1 % L-glutamine (Sigma-Aldrich) and 1 % streptomycin/penicillin (Sigma-Aldrich).

2.2. Exposure of ovine mesenchymal stem cells to scrapie

To analyse the response of oBM-MSCs to scrapie exposure, cells at passage 3 were seeded at 9000 cells/cm^2 in T25 flasks and cultured with brain inocula obtained from a healthy sheep (normal brain homogenate, NBH) and from a sheep naturally infected with scrapie (scrapie-treated, SCR) for 2 days (2 days post inoculation, 2dpi). After that time the inocula were removed and the cells were maintained in growth conditions for two more days (4dpi). These cells were compared to oBM-MSCs cultured in growth medium only (medium only, MO).

The inocula were prepared using CNS samples of a healthy sheep and a sheep infected with classical scrapie, both carrying the ARQ/ARQ genotype for the *PRNP* gene. The inocula were preserved at the tissue bank of the Centre of Encephalopathies and Emerging Transmissible Diseases (CEETE; University of Zaragoza). The presence/absence of PrP^{Sc} in the CNS tissues was confirmed following the protocols used in previous works (Bolea et al., 2005), which consisted of two rapid

diagnostic tests (Prionics-CheckWestern blotting; ThermoFisher Scientific and Idexx HerdChek; IDEXX, Westbrook, ME, USA) and confirmation by immunohistochemical examination of the CNS tissue. CNS samples were homogenized and diluted 1:10 (g/mL) in physiological saline solution (Braun). Afterwards, samples were incubated at 70 °C for 10 min before adding streptomycin sulphate (100 μg/mL) and benzylpenicillin (100 μg/mL). The safety of the inocula was evaluated as previously described (García-Mendívil et al., 2021).

The inocula were diluted 1:10 in DMEM medium (10 % FBS, 1 % L-glutamine and 1 % streptomycin/penicillin) and added to the cells, which were maintained in this medium for 48 h. Subsequently, the cells were washed twice with PBS, the medium was replaced with growth medium only, and the cells were left for another 2 days in culture.

2.3. RNA extraction of ovine mesenchymal stem cells

For RNA extraction, cells at different conditions (SCR, NBH and MO) and times (2 dpi and 4 dpi) were retrieved by trypsinization and subsequent centrifugation. Afterwards, RNA was extracted using the Direct-zol RNA MiniPrep kit (Zymo Research) following the manufacturer's instructions.

Before library preparation, the integrity of the RNA samples was checked with Agilent 2100 Bioanalyzer, RNA purity was evaluated with agarose gel electrophoresis and NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the quantity of RNA was determined using NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer. Approximately 2 μg of total RNA per sample were used for RNA-sequencing.

2.4. RNA-sequencing and bioinformatic analysis

2.4.1. Library preparation, quality control, mapping, and quantification

The cDNA library construction was performed at Novogene in Cambridge (UK). Quantified libraries were pooled and sequenced on an Illumina platform, according to effective library concentration and data amount, and paired-end reads were generated. The quality control of raw reads was made using in-house perl scripts, and clean reads were obtained by removing reads containing adapters, reads containing poly-N and low quality reads. Q20, Q30 and GC content of the clean data were then calculated. After the quality control, the paired-end clean reads were aligned to the ovine reference genome oar_rambouillet-v1.0 (Ensembl) using the software Hisat2 v2.0.5. The mapped reads of each sample were assembled by StringTie v1.3.3b (Pertea et al., 2015) in a reference-based approach. For transcript quantification, the software FeatureCounts v1.5.0-p3 was used and FPKM (Fragments Per Kilobase of transcript per Million mapped reads) of each gene was then calculated based on the length of the gene and read count mapped to this gene.

2.4.2. Differential expression analysis and GO and KEGG enrichment analyses

Differential expression analysis was performed using the DESeq2 R package v1.20.0. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (FDR). Genes with an adjusted P-value <0.05 and a log2(fold change) (log2FC) > 0.58 (upregulated) or < −0.58 (downregulated) found by DESeq2 were assigned as differentially expressed (Backer et al., 2023; Chernov and Shubayev, 2021; Love et al., 2014; Perez-Roman et al., 2022; Wang et al., 2022; Yan et al., 2023). Volcano plots of the differentially expressed genes (DEGs) were drawn using SRplot online platform (Tang et al., 2023).

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of DEGs were implemented by the enrichment tool ShinyGO v0.77 (Ge et al., 2020). GO terms and KEGG pathways with FDR < 0.05 were considered significantly enriched.

2.5. Quantitative real-time PCR

To validate the results observed in the RNA-sequencing analysis, a set of 11 genes was selected among the significant DEGs that showed higher changes in expression (fold change) between MSCs exposed to scrapie (SCR-MSCs) and to normal brain homogenate (NBH-MSCs) at 2 dpi. This validation step aims to confirm the reliability and reproducibility of the findings obtained from the RNA-sequencing assay.

Retrotranscription was performed from 200 ng of total RNA using qScript™ cDNA Supermix (Quanta Biosciences™), according to the manufacturer's instructions. Resulting cDNA was diluted 1:5 in water and gene expression was quantified by quantitative real-time PCR (RT-qPCR) using the Fast SYBR™ Green Master Mix (Applied Biosystems, Thermo Fisher Scientific) in a StepOne Plus Real-Time PCR instrument (Applied Biosystems). Each sample was amplified by triplicate. The comparative quantification of the results was standardized by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), using the geometric mean of *SDHA*, *G6PDH* and *GAPDH* housekeeping genes (Lyahyai et al., 2010) as a normalizer. Student's *t*-test was applied to identify differences between groups, which were considered significant at $P < 0.05$. Table 1 lists the primers used for the RT-qPCR assay.

3. Results

3.1. Clean data quality and mapping results

After filtering the raw reads, clean reads had an error rate lower than 1 % and GC content distribution was equal and stable in all sequenced samples. Around 98 % of the bases had a quality score greater than 20 and around 94 % greater than 30 (Supplementary Table S1).

Approximately 97 % of the clean reads were aligned to the reference sheep genome, being around the 87–89 % aligned to unique positions and 8–9 % to multiple locations in the genome (Supplementary Table S2). The majority of the reads were mapped in exon regions (67.04 % ± 0.68).

3.2. Distribution of gene expression levels

The distribution of gene expression levels among the different samples was similar (Supplementary Fig. S1). The correlation coefficient of samples within groups was greater than 0.92 with a $R^2 > 0.8$, which

Table 1
Sequences of primers used in the RT-qPCR assay. Fw = Forward primer and Rv = Reverse primer.

Gene	Primer sequences
RPS15A	Fw: TGCTCCAAAGTCATCGTCAG
	Rv: CAAATCTGGGGCTGATCACT
MMP1	Fw: TGCTCATGCTTTTCAACCAG
	Rv: GAGCAAGCTGAACATCACCA
PGAP4	Fw: TGTAAAATGGGGACCGTGAT
	Rv: CAGCTGCTCCATAGGTGTCA
SERPINB2	Fw: GTTTGTGGCAGACCATCCTT
	Rv: GCCACACCATGTCTGTGTTC
PDK4	Fw: AGCTGACCCAGTCACCAATC
	Rv: ACTGCCACCACATCACAGTT
MMP12	Fw: TATGGCCTCGAGATGGAAAC
	Rv: ATTTTCAACATCCGGCACTC
PRR11	Fw: GAAAGCACTTCAGGCTGGAC
	Rv: CTTTGGACTTCGGTTTCAGG
SAA3	Fw: AGCCCTGTCATTCTTGGTTG
	Rv: TCCCTGGTCATACCCTTGAG
PTGS1	Fw: AAAGAAGCTGGTTTGCCTCA
	Rv: TCCACACTGATGCTCTCGAC
CYP1A1	Fw: GGGCACGTGCTGACCTT
	Rv: GTGAAGCTGTAGAGGTCGGG
F13A1	Fw: GACTGCATTGTGGGGAAGTT
	Rv: TGACCATAGCTCCAGCTCCT

showed close expression patterns (Supplementary Fig. S2). Intergroup differences were evaluated by principal component analysis (PCA), revealing that samples between groups where dispersed while the samples within groups gathered together (Fig. 1). Moreover, a total of 11,926 genes were co-expressed in the three groups of samples (SCR, NBH, MO) taking together the two study times (Fig. 2). While the two experimental times in the MO samples hardly differentiated the culture groups, time was the main separating factor in the inoculated samples. Although SCR-MSCs and NHB-MSC were differentiated, both groups were grouped by time.

3.3. Differential gene expression analysis

Differentially expressed genes were found between the different groups at the two study times T1 (2 dpi) and T2 (4 dpi). The higher number of DEGs was observed at the time of removing brain inocula (2 dpi). Between SCR and NBH-exposed cells, a total of 185 genes were differentially expressed (110 up-regulated and 75 down-regulated) at 2 dpi (Table 2, Fig. 3a, Supplementary Table S3), whereas only 70 DEGs (13 up-regulated and 57 down-regulated) were found at 4 dpi (Table 2, Fig. 3b, Supplementary Table S4). Out of the 13 up-regulated genes in SCR-MSCs, one gene (*ELN*) also showed upregulation at 2 dpi. The remaining 12 genes were newly regulated at 4 dpi. Among the 57 down-regulated genes, 50 were newly regulated at 4 dpi, while 7 genes (*MMP12*, *MMP1*, *PLTP*, *ESM1*, *ETV4*, *SAA3* and *CPXM1*) were also downregulated at 2 dpi.

Regarding the DEGs observed in SCR-MSCs when compared with MO-MSCs, 1616 and 378 genes were differentially expressed at 2dpi

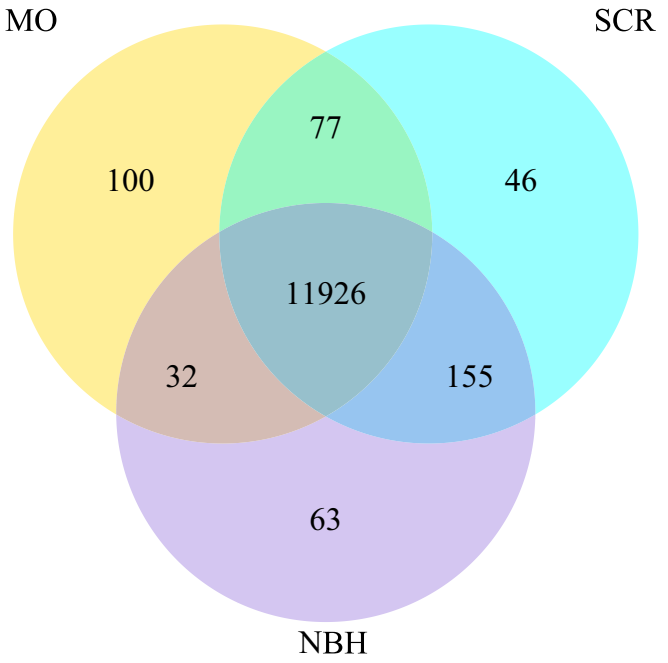

Fig. 2. Venn diagram representing the number of co-expressed genes between SCR (scrapie treated), NBH (exposed to Normal Brain Homogenate) and MO (growth in Medium Only) groups.

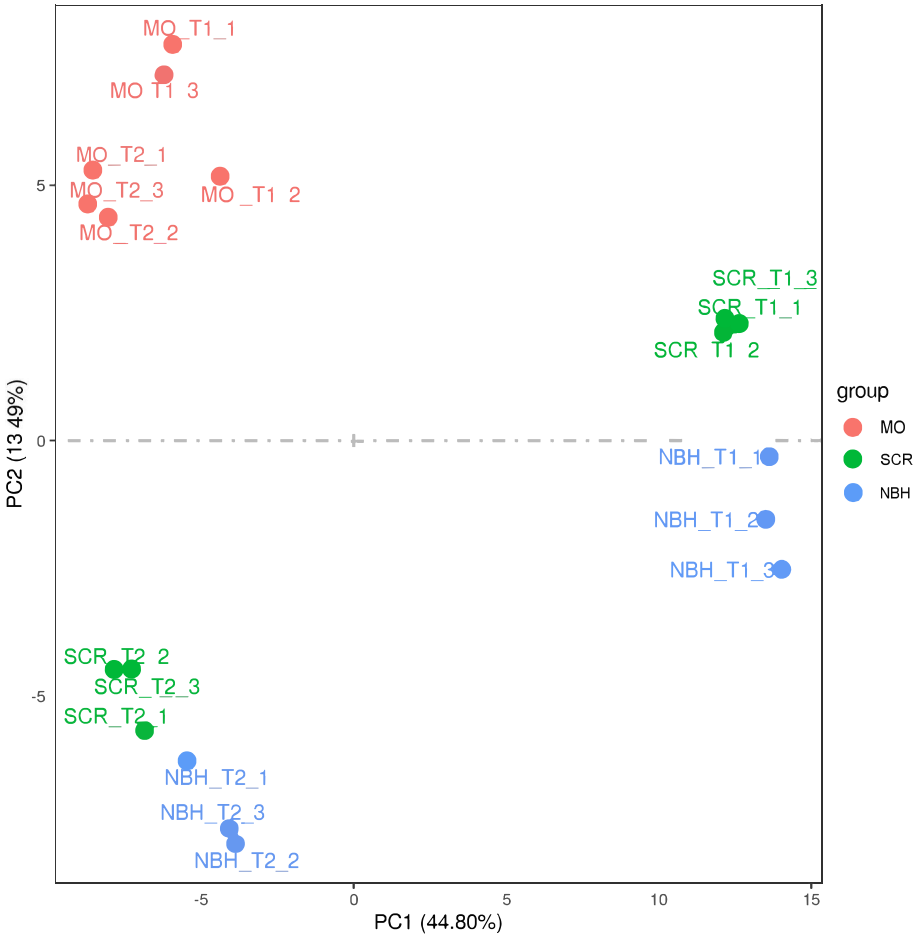

Fig. 1. Principal component analysis showing the different sample groups clustered separately. Cell conditions: SCR (scrapie treated), NBH (exposed to Normal Brain Homogenate), MO (growth in Medium Only). Time point: T1 (2 dpi) and T2 (4 dpi).

Table 2
Number of differentially expressed genes found in all compared groups.

Compared groups[1]	All DEGs[2]	Up DEGs[3]	Down DEGs[4]
SCR T1 vs NBH T1	185	110	75
SCR T1 vs MO T1	1616	834	782
NBH T1 vs MO T1	2105	901	1204
SCR T2 vs NBH T2	70	13	57
SCR T2 vs MO T2	378	250	128
NBH T2 vs MO T2	674	407	267

[1] Compared groups: Compared group names.
[2] All DEGs: The total number of differentially expressed genes in the compared groups.
[3] Up DEGs: The up-regulated number of differentially expressed genes in the compared groups.
[4] Down DEGs: The down-regulated number of differentially expressed genes in the compared groups.

(Table 2, Supplementary Table S5) and 4 dpi (Table 2, Supplementary Table S6) respectively. In addition, 2105 DEGs were found at 2 dpi (Table 2, Supplementary Table S7) and 674 at 4 dpi (Table 2, Supplementary Table S8) between NBH-MSCs and MO-MSCs. Hierarchical clustering analysis of DEGs was also carried out, showing genes and samples with similar expression patterns clustering together (Fig. 4). Similar to the PCA study, all MO cells clustered together whereas exposed cells were grouped according to the analysis time.

3.4. Enrichment analysis of differentially expressed genes

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses were performed in order to find biological functions and pathways significantly associated with the identified DEGs. The DEGs observed between SCR-MSCs and NBH-MSCs, and between exposed (SCR and NBH) and non-exposed cells (MO-MSCs), at both times, were enriched in several significant biological pathways and functions.

3.4.1. KEGG enrichment

KEGG is a collection of databases that provides resources on genomic, biological pathways and disease information (Kanehisa and Goto, 2000). Pathway enrichment analysis identifies significantly enriched metabolic or signal transduction pathways associated with differentially expressed genes, comparing them to the whole genome background. KEGG pathways with an FDR < 0.05 are considered significantly enriched.

Comparing inoculated cells with those that were not, in terms of KEGG pathways, we found that DEGs in both SCR and NBH cells exhibited both shared and unique pathways. At 2 dpi, up-regulated DEGs in SCR-MSCs were enriched in pathways related to cell cycle regulation, cell adhesion, and proinflammatory mechanisms. Up-regulated DEGs in NBH-MSCs also showed enrichment in the cell cycle pathway, along with associations with proinflammatory pathways. Similarly, down-regulated DEGs in both SCR and NBH cells were associated with pathways related to cell-matrix interaction, protein absorption, and stem cell pluripotency regulation (Supplementary Tables S9 and S10).

At 4 dpi, no significant KEGG enrichment was found in up-regulated DEGs of SCR-MSCs compared to MO-MSCs. However, up-regulated DEGs in NBH-MSCs were associated with glycolysis/gluconeogenesis and HIF-1 pathways. Down-regulated DEGs in both groups were associated with cell-matrix interaction and focal adhesion pathways (Supplementary Tables S11 and S12).

On the other hand, up-regulated DEGs identified in SCR-MSCs compared to NBH-MSCs (Supplementary Table S13) showed no significant KEGG enrichment at 2 dpi. However, down-regulated DEGs were associated with ferroptosis and several proinflammatory pathways, including the IL-17 signalling pathway, the Toll-like receptor signalling pathway and the TNF signalling pathway. Among the down-regulated genes involved in these pathways, we found *IL6* and *MMP1*.

At 4 dpi, KEGG enrichment revealed that in scrapie-exposed cells (SCR-MSCs) up-regulated DEGs were associated with the term "phagosome", while down-regulated DEGs were associated with the p53 signalling pathway and cellular senescence.

3.4.2. GO enrichment

GO is a classification system used to unify the presentation of gene

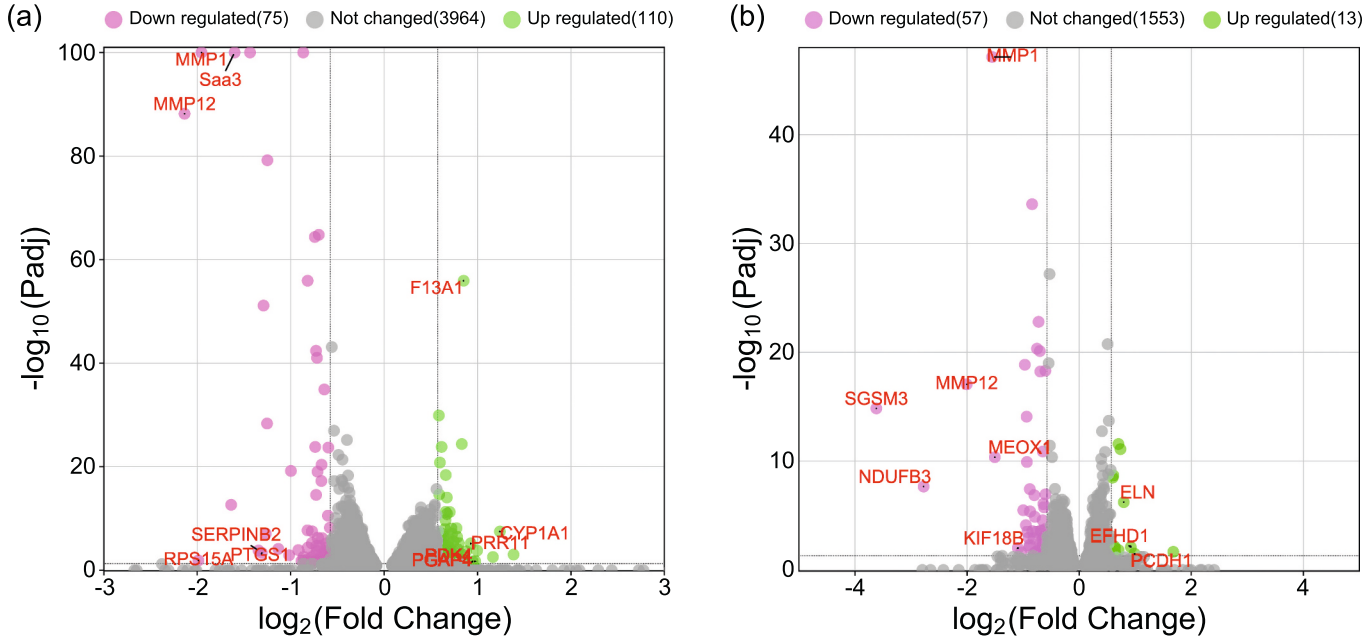

Fig. 3. Volcano plots showing the overall distribution of differentially expressed genes between scrapie-exposed cells (SCR) and cells treated with negative-inoculum (NBH) at (a) 2 dpi (T1) and (b) 4 dpi (T2). The x-axis shows the fold change in gene expression (log2FoldChange), and the y-axis shows the statistical significance (-log10Padj). Green dots represent up-regulated genes and pink dots represent down-regulated genes. The grey dashed line indicates the threshold line for differential gene screening criteria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

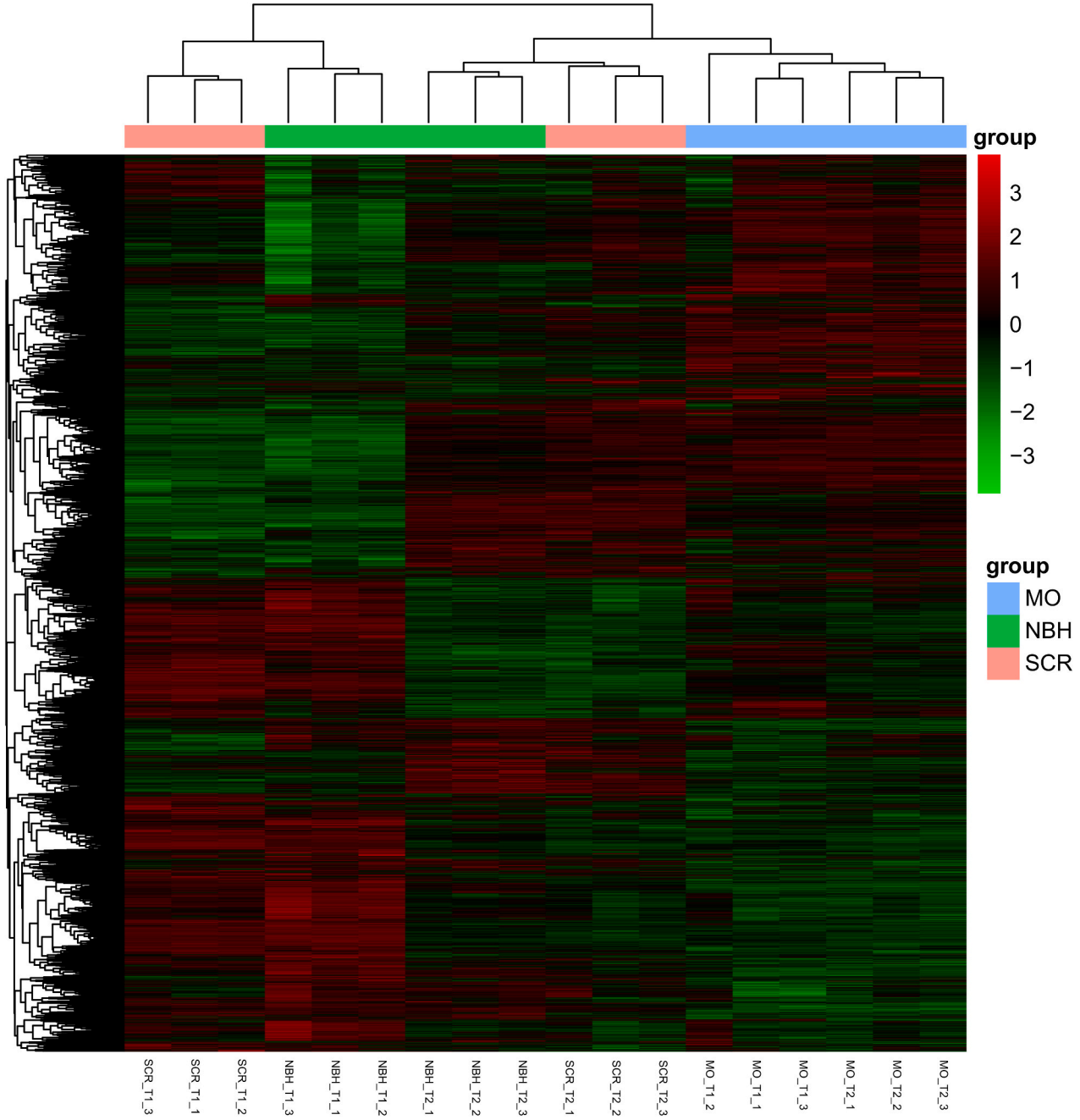

Fig. 4. Differential expression gene clustering heatmap clustered using the log2(FPKM+1) value. Red colour indicates genes with high expression levels, and green colour indicates genes with low expression levels. The colour ranging from red to green indicates that log2(FPKM+1) values where from large to small. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

properties across all species. It includes three main branches: cellular component, molecular function and biological process. GO terms with an FDR < 0.05 are considered significantly enriched.

In terms of GO processes, the comparison between exposed and non-exposed cell cultures at 2 dpi showed that up-regulated DEGs in SCR-MSCs were associated with cell division and cell cycle phase regulation. In NBH-MSCs, DEGs showed similar enrichment in these processes, along with an associations with inflammatory response pathways. Down-regulated DEGs in exposed cells were linked to cell population proliferation, cell projection and growth factor activities (Supplementary Tables S9 and S10).

At 4 dpi, DEGs in both SCR-MSCs and NBH-MSCs shared similar biological processes, such as cell population proliferation, signalling receptor activity and responses to growth factors and organic substances. Additionally, DEGs in SCR-MSCs were involved in the regulation of chemokine production, while in NBH-MSCs, DEGs were associated with cell migration and motility (Supplementary Tables S11 and S12).

Furthermore, up-regulated DEGs in SCR-MSCs compared to NBH-MSCs at 2dpi were only enriched in the cellular component “kinetochore microtubule”. In contrast, down-regulated DEGs were linked to the biological processes such as acute-phase response, regulation of acute inflammatory response, defence response, regulation of apoptotic process, regulation of programmed cell death and response to external stimulus. They were also associated with molecular functions including peroxidase and oxidoreductase activity, antioxidant activity and

metallocarboxypeptidase and metallopeptidase activities (Supplementary Table S13).

At 4 dpi, up-regulated DEGs were involved in processes related to the synthesis and modifications of macromolecules, such are hyaluronan synthase activity and coA carboxylase activity, as well as processes related to cell interaction and signalling, such as calcium and metal ion binding. They were also associated with enzymatic activities, including small molecule sensor activity, and energy metabolism, such as NAD+ kinase activity. Conversely, down-regulated DEGs were linked to processes involved in cell division and cytoskeleton organization, with enriched terms like microtubule binding, phosphatidylcholine and sphingolipid transporter activities and ATP-dependent activity (Supplementary Table S14).

3.5. RT-qPCR validation of RNA-sequencing analysis

The expression of 11 genes differentially expressed at 2 dpi between SCR- and NBH-MSC was quantified with RT-qPCR to assess the validity of the results of the RNA-sequencing transcriptomic analysis.

Most (9 out of 11) of the analysed genes showed expression trends concordant with those observed in the RNA-sequencing analysis (Table 3, Fig. 5). Three genes (*MMP1*, *SERPINB2* and *MMP12*) were significantly down-regulated in SCR-MSCs compared to the NBH-MSCs, whereas four genes (*PGAP4*, *PDK4*, *PRR11* and *F13A1*) were significantly up-regulated. The other four remaining genes (*RPS15A*, *SAA3*, *PTGS1* and *CYP1A1*) did not display significant expression changes, although *SAA3* and *PTGS1* displayed the same trend than the RNAseq analysis.

Overall, the RT-qPCR results were congruent with the RNA-sequencing profiling, supporting the validity of the transcriptomic analysis.

4. Discussion

In the current study, an RNA-seq transcriptomic analysis of ovine bone marrow-derived mesenchymal stem cells cultured during two days in contact with scrapie brain extracts was carried out to identify genes and biological pathways potentially involved in early prion toxicity and in the molecular response of these cells to priming with scrapie brain extracts.

A previous study by our group reported that oBM-MSCs exposed to ovine scrapie brain extracts were unable to maintain PrP^{Sc} over time, only those cultured under neurogenic conditions seemed to replicate scrapie prions (García-Mendívil et al., 2021). Interestingly, the viability or cell proliferation of oBM-MSCs exposed to scrapie brain extracts initially decreased during the period when the cells were in contact with the scrapie inoculum, suggesting a potential early toxic effect of prion exposure in these cells (García-Mendívil et al., 2021). Moreover, the study showed that the cell viability of oBM-MSCs exposed to either scrapie-infected or normal brain homogenates was higher compared to non-inoculated cultures, suggesting that brain inocula, regardless of being scrapie-infected or not, may contain factors that stimulate oBM-MSCs proliferation (García-Mendívil et al., 2021). In accordance with this, the DEGs identified in inoculated cells (SCR and NBH) showed enrichment in different pathways related to cell proliferation, such as cell cycle, cell division, cell population proliferation, cell projection, and signalling pathways regulating pluripotency of stem cells. Additionally, pathways related to growth factor activity were also enriched. Other studies have shown that growth and trophic factors can increase proliferation and survival of MSCs (Rodrigues et al., 2010). For instance, murine MSCs cultured under hypoxic conditions and supplemented with basic fibroblast growth factor show increased cell proliferation and maintain their multipotency through late passages (Caroti et al., 2017).

On the other hand, MSCs are capable of migrating to prion-caused brain lesions, being this migration mediated by different chemoattractive factors (Song et al., 2009, 2011). In our study, oBM-MSCs seem to respond to the inoculation with brain homogenates, as the DEGs of inoculated cells were associated with processes of cell migration and motility, regulation of chemokine production, cellular response to chemical stimulus and HIF-1 signalling pathway, which is a transcription factor that improves MSCs migration and proliferation (Ciria et al., 2017; Zhou et al., 2020).

Remarkably, in the transcriptomic analysis, the highest number of differentially expressed genes between SCR- and NBH-MSCs was observed early after exposure to the prion-injured brain environment (2 dpi); coinciding with the time when oBM-MSCs had been in contact with the different inocula, which were removed afterwards. This suggests that oBM-MSCs react promptly to prion-infected brain tissues, triggering mechanisms that could be involved in prion toxicity or represent a differential response of MSCs to scrapie priming.

The down-regulated DEGs found in scrapie-exposed oBM-MSCs (SCR-MSCs) compared to NBH cells were enriched in multiple pathways associated with proinflammatory processes, including the IL-17, Toll-like receptor and the TNF signalling pathways, as well as cytokine-cytokine receptor interaction. These pathways indicate a response of MSCs to a neuroinflammatory environment, which is a known factor in the neuropathology of neurodegenerative diseases such as scrapie (Guijarro et al., 2021). In prion-infected brains, there is an increase in numerous proinflammatory cytokines and chemokines, including IL-1α and β and TNF (Carroll and Chesebro, 2019). Murine adipose-derived mesenchymal stromal cells have been shown to respond to these inflammatory cytokines and prion-infected brain homogenate by increasing production of anti-inflammatory molecules, thereby reducing inflammation in persistent-infected glial cells (Hay et al., 2022). Notably, IL-6, one of the cytokines downregulated in SCR-MSCs, has been identified as downregulated in activated microglia cultured with MSCs. Both the enhancement and suppression of IL-6 production by MSCs can be involved in immunomodulatory mechanisms. The reduction of IL-6 production creates a favourable environment for the secretion of anti-inflammatory cytokines (Kerkis et al., 2024) and could

Table 3
RT-qPCR validation results. Relative expression changes are expressed in terms of $2^{-\Delta\Delta Ct}$ in scrapie-treated cells (SCR) and normal brain homogenate-treated cells (NBH) at 2 dpi (T1). Significant changes are expressed as *P*-value. NS = non-significant.

Gene	$2^{-\Delta\Delta Ct}$ SCR_T1	$2^{-\Delta\Delta Ct}$ NBH_T1	RT-qPCR SCR_T1vsNBH_T1	RNAseq SCR_T1vsNBH_T1	Same expression trend	Validated gene (RT-qPCR P-value)
RPS15A	1.27	1	Up	Down	No	No (NS)
MMP1	0.27	1	Down	Down	Yes	Yes ($p = 0.001$)
PGAP4	1.37	1	Up	Up	Yes	Yes ($p = 0.1$)
SERPINB2	0.62	1	Down	Down	Yes	Yes ($p = 0.08$)
PDK4	1.45	1	Up	Up	Yes	Yes (p = 0.1)
MMP12	0.26	1	Down	Down	Yes	Yes (p = 0.001)
PRR11	1.63	1	Up	Up	Yes	Yes ($p = 0.01$)
SAA3	0.89	1	Down	Down	Yes	No (NS)
PTGS1	0.80	1	Down	Down	Yes	No (NS)
CYP1A1	0.85	1	Down	Up	No	No (NS)
F13A1	1.76	1	Up	Up	Yes	Yes ($p = 0.008$)

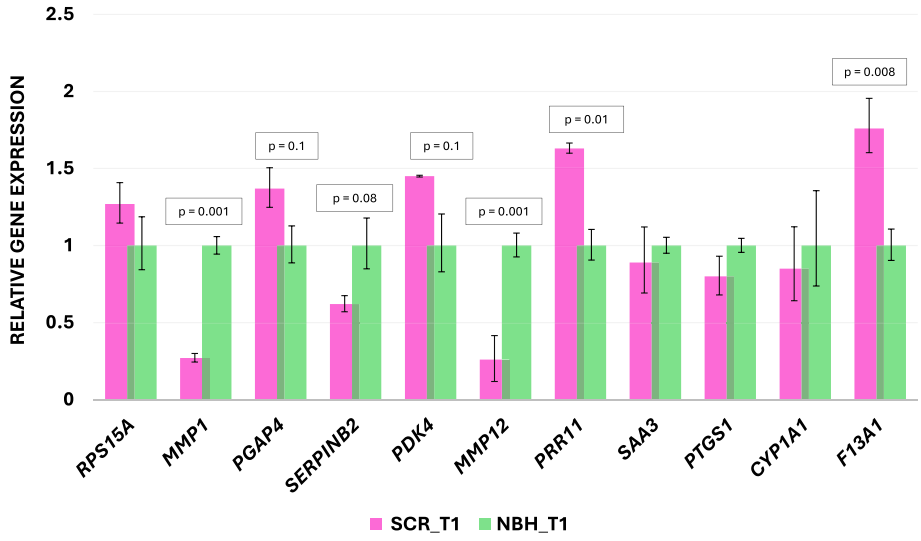

Fig. 5. Relative gene expression evaluated by real time PCR of the eleven differentially expressed genes selected for RNA-sequencing validation. Relative expression levels in scrapie-treated oBM-MSCs at 2 dpi (SCR_T1, pink bars) and oBM-MSCs exposed to normal brain (NBH_T1, green bars) are presented as $2^{-\Delta\Delta Ct}$ mean values ± standard error (SE). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

represent a positive response of oMSCs to modulate neuroinflammation in scrapie.

Programmed cell death processes, including apoptosis and ferroptosis, were terms also enriched in downregulated genes in SCR-MSCs. The apoptosis mechanism contributes to neurodegeneration in scrapie disease as a common form of neuronal cell death (Hedman et al., 2012; Lucassen et al., 1995; Serrano et al., 2009), while ferroptosis has been associated with the pathophysiology of different diseases, including prion-like neurodegenerative disorders (Reichert et al., 2020). These mechanisms observed in oBM-MSCs exposed to scrapie tissues could be related to the slight but significant decrease in viability of MSCs exposed to scrapie compared to the cells exposed to normal brain homogenate (García-Mendívil et al., 2021). However, these processes may also be involved in inflammatory responses (Deng et al., 2023), and proliferation can be affected by exposure to an inflammaroty environment (Li et al., 2023), which could also explain the reduction in viability or proliferation potential in scrapie-exposed cells.

After removing the scrapie inoculum, primed cells showed different transcriptome profiles compared to those cultured with control brains. Enriched GO terms in overexpressed genes suggest that these cells are involved in remodelling the extracellular matrix, cell adhesion and energetic metabolism, which could be linked to a response to maintain cellular homeostasis. Changes in the expression of extracellular matrix components have been described in other in vitro prion models (Marbiah et al., 2014). On the other hand, down-regulated genes at 4 dpi were related to the cell cycle, which could be associated with a decrease in cell proliferation, consistent with the decreased proliferation observed in our previous study (García-Mendívil et al., 2021).

Changes in the expression of seven genes were validated by RT-qPCR. Two genes encoding two different matrix metalloproteinases were downregulated in scrapie-exposed oBM-MSCs: *MMP1* (Matrix metalloproteinase 1) and *MMP12* (Matrix metalloproteinase 12). Matrix metalloproteinases (MMPs) are extracellular endopeptidases involved in extracellular matrix (ECM) turnover and degradation of its constituents (Khokha et al., 2013; Minta et al., 2020). The ECM is a dynamic structure undergoing controlled remodelling, and the balance between the synthesis, development and degradation of its components is essential to ensure a normal physiology (Cox and Erler, 2011). Altered expression of matrix proteases can dysregulate this homeostatic balance leading to various pathological conditions (Cox and Erler, 2011). Dysregulation of genes encoding proteins involved in ECM remodelling have also been described in other in vitro models of prion diseases where inhibition of metalloproteinases 2–9 was related with an increase of prion propagation (Marbiah et al., 2014). Moreover, an excessive MMP proteolytic activity has been associated with the pathogenesis of several CNS diseases, such as cerebral ischemia, Parkinson's and Alzheimer's diseases, and traumatic injury (Crocker et al., 2004). The MMP1 and MMP12 enzymes are also implicated in the CNS inflammation process. *MMP12* is upregulated in aged brains of C57BL/6 J mice enhancing neuroinflammation (Liu et al., 2013) and increased *MMP1* expression in rat microglia treated with synthetic prion peptide 106–126 is accompanied by an increment of several cytokines and inflammatory mediators (Song et al., 2012). Downregulation of these genes in MSCs exposed to scrapie can be part of the response of these cells to neuroinflammation.

SERPINB2 (Serpin peptidase inhibitor clade B member 2), which was also downregulated in oBM-MSCs, may function as a stress response protein with cytoprotective activity. In mouse embryonic fibroblasts transfected with mutant huntingtin, *SERPINB2* modulates protein degradation capacity and protein aggregation, and protects cells from the proteotoxicity associated with protein misfolding and proteostasis dysfunction (Lee et al., 2015). In addition, this protein is a regulator of inflammatory processes and has been associated with a number of extrinsic inflammatory and autoimmune conditions (Schroder et al., 2010). Therefore, a downregulation of this gene after exposure to scrapie could be an indicator of cytotoxicity and a response to inflammatory conditions in oBM-MSC cultures.

PGAP4 is a gene encoding for post-GPI attachment to proteins GalNAc transferase 4, which is a GPI-specific GalNAc transferase that catalyses the first reaction for generating the GalNAc chain, a posttranslational modification found in different proteins including PrP^{C} (Hirata et al., 2018). This GPI-GalNAc side chain seems to be implicated in the conversion of PrP^{C} to PrP^{Sc} (Bate et al., 2016). A recent study reported that prion-infected *PGAP4* knock-out mice displayed shorter disease incubation periods than the wild type ones, indicating a possible protective role of GPI-GalNAc side chain and *PGAP4* against prion pathology (Hirata et al., 2022). The upregulation of *PGAP4* in oBM-MSCs could be a protective mechanism activated in response to scrapie exposure.

Another upregulated gene in SCR-MSCs was *PDK4*. In the hippocampus of prion-infected mice, an overexpression of *PDK4* has been described along with a decrease of glucose oxidative degradation and an increase in fatty acid-associated oxidative stress. Moreover, inhibition of *PDK4* extended the survival time of these mice (Arnould et al., 2021). The upregulation of *PDK4* could be related to oxidative stress conditions

in SCR cells but also to a response to the inflammatory environment created by the scrapie brain homogenate because PDK4 overexpression has also been linked to inflammation (Park and Jeoung, 2016).

The upregulation of two more genes was also validated in SCR-MSCs: *PRR11* (Proline-rich protein 11) and *F13A1* (Coagulation factor XIII A chain). *PRR11* is a tumor-related gene involved in cell cycle, tumorigenesis and metastasis that is upregulated in different types of cancer including gastric and lung cancers (Hu et al., 2018; Zhang et al., 2017; Zhou et al., 2019). In non-small-cell lung cancer cells, the silencing of *PRR11* induces autophagy and inhibits cell proliferation (Zhang et al., 2017). Interestingly, an impairment of the autophagy process has been described in sheep naturally infected with scrapie (López-Pérez et al., 2019) and in scrapie-infected transgenic mice (López-Pérez et al., 2020). The upregulation of *PRR11* observed in our study could affect the autophagic activity of the cells primed with scrapie or their cell proliferation potential. *F13A1* expression, otherwise, has been associated with pro-inflammatory and cell stress pathways in adipose tissue (Kaartinen et al., 2020, 2021). As an upregulation of this gene was found in oBM-MSCs, it can be thought that the pathways found in adipose tissue could also be triggered in these cells as a consequence of the prion brain exposure. These two genes have never been related to prion or other neurodegenerative diseases before. Further studies are necessary to elucidate their potential role in these diseases and in the response of oBM-MSCs to scrapie brain tissues.

Altogether, our findings demonstrate that ovine mesenchymal stem cells exposed to scrapie differentially express genes involved in inflammatory response pathways. Most of these differentially expressed genes are associated with proinflammatory pathways and are downregulated in scrapie-exposed cells. This suggests that the mechanisms observed in this transcriptomic study reflect the response of oBM-MSCs to inflammatory molecules that can be present in scrapie brain homogenate. Following this initial response, transcriptomic changes in MSCs exposed to scrapie appear to be related to extracellular matrix remodelling and decreased proliferation. Using ovine scrapie as a natural animal model of prion diseases, our study helps to elucidate the mechanisms behind the therapeutic potential of MSCs to regulate neuroinflammation during prion infection, including the downregulation of genes involved in proinflammatory pathways like *IL6* and *MMP1*. Further transcriptomic studies in oBM-MSCs differentiated into neuron-like cells and exposed to scrapie prions could be useful to identify mechanisms underlying neurotoxicity.

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CRediT authorship contribution statement

Adelaida Hernaiz: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Belén Marín:** Writing – review & editing, Investigation. **Francisco J. Vázquez:** Writing – review & editing, Investigation. **Juan J. Badiola:** Writing – review & editing, Resources, Funding acquisition. **Pilar Zaragoza:** Writing – review & editing, Resources, Funding acquisition. **Rosa Bolea:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Inmaculada Martín-Burriel:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT, an AI language model developed by OpenAI, to assist with the correction of English grammar and to enhance the clarity of the language. After using this tool/service, the authors reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of competing interest

The authors report that there are no competing interests to declare.

Data availability

The raw and processed transcriptomic data generated in this study have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) (GEO) and are accessible through GEO Series accession number GSE245610 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc = GSE245610).

Appendix A. Supplementary data

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