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CARACTERIZACIÓN DE CÉLULAS MESENQUIMALES DE OVINO INFECTADO CON SCRAPIE

Memoria presentada por

Diego Rubén Mediano Martín-Maestro

Para optar al grado de Doctor por la Universidad de Zaragoza

Zaragoza, Mayo de 2016

Departamento de Patología Animal
Universidad Zaragoza

Departamento de Anatomía, Embriología y Genética Animal
Universidad Zaragoza

Dña. **Inmaculada Martín Burriel**, Profesora Titular del Departamento de Anatomía, Embriología y Genética Animal, y Dña. **Rosa María Bolea Bailo**, Profesora Titular del Departamento de Patología Animal de la Facultad de Veterinaria de la Universidad de Zaragoza,

CERTIFICAN QUE:

D. Diego Rubén Mediano Martín-Maestro ha realizado, bajo nuestra dirección, los trabajos correspondientes a su Tesis Doctoral titulada «***Caracterización de células mesenquimales de ovino infectado por scrapie***» que corresponde con el proyecto de tesis aprobado por la Comisión de Doctorado en noviembre de 2013 y cumple los requisitos mínimos exigidos para optar al Grado de Doctor por la Universidad de Zaragoza, por lo que autorizan su presentación para que pueda ser juzgado por el Tribunal Correspondiente.

Lo que suscribimos como directoras del trabajo en Zaragoza, a 19 de mayo de 2016.

Fdo.: Inmaculada Martín Burriel

Fdo.: Rosa María Bolea Bailo

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ANTECEDENTES

En este primer apartado se describirán distintos aspectos relativos a las encefalopatías espongiformes transmisibles, principalmente del scrapie ovino, y a los modelos celulares desarrollados para el estudio de estas enfermedades. Dentro de estos modelos se profundizará en la posible aplicación de las células madre en el desarrollo de los mismos. Por último se detallarán los tipos y características de las células madre mesenquimales y la utilidad de los cultivos celulares para estudiar la toxicidad del prion patológico.

1 ENCEFALOPATÍAS ESPONGIFORMES TRANSMISIBLES

Las enfermedades priónicas son un conjunto de enfermedades caracterizadas por el plegamiento erróneo de una proteína que produce neurodegeneración (Prusiner, 1991; Aguzzi *et al.*, 2008). Las enfermedades priónicas pueden presentarse de forma hereditaria, debidas a la mutación del gen que codifica la proteína prión (***PRNP***); adquiridas, cuando son el producto de una infección con el agente causal; y esporádicas, sin una causa aparente conocida (Brown, 2008; Yokoyama & Mohri, 2008; Tranulis *et al.*, 2011).

Estas enfermedades comparten el mal plegamiento de la proteína prión celular **(PrP^{C}),** que resulta en la aparición de proteína prión patológica **(PrP^{Sc})**, cuyo superíndice procede de scrapie, así denominada al ser descrita por primera vez en las especies ovina y caprina (Prusiner *et al.*, 1998). Normalmente la proteína prión celular posee una forma predominantemente en forma de hélice alfa (Prusiner, 2001), mientras que la proteína patológica presenta grandes regiones en forma de lámina beta plegada (Pan *et al.*, 1993).

Estas formas aberrantes forman agregados y son resistentes a la digestión celular (Caughey *et al.*, 1990), acumulándose en tejido linfoide y nervioso, principalmente en el sistema nervioso central (**SNC**) (DeArmond *et al.*, 1985). Además de esta resistencia a la digestión celular, también son resistentes a la digestión *in-vitro* mediante proteinasas, como la proteinasa K (McKinley *et al.*, 1983; Oesch *et al.*, 1985).

Respecto a las enfermedades priónicas que afectan a los animales, se describen la enfermedad caquectizante crónica de los ciervos, la encefalopatía transmisible del visón, la encefalopatía espongiforme felina, el scrapie ovino y caprino y la encefalopatía espongiforme bovina (**EEB**) (Imran & Mahmood, 2011). El análisis histopatológico del SNC de los animales afectados por EEB reveló una gran similitud de las lesiones neuropatológicas con aquellas encontradas en el SNC de los animales infectados con scrapie. Por este motivo, existen teorías han considerado el scrapie como el origen de la EEB (Prusiner, 1998). A pesar de la asociación epidemiológica que corrobora este origen (Wilesmith *et al.*, 1988), hasta la actualidad no se ha conseguido desarrollar experimentalmente la EEB administrando el agente del scrapie ovino por vía oral a la especie bovina (Konold *et al.*, 2013) a pesar de que sí se ha conseguido la transmisión mediante inoculación intracraneal (Clark *et al.*, 1995).

La EEB, además de afectar al ganado bovino, ha dado lugar a una patología zoonótica humana, la nueva variante de la Enfermedad de Creutzfeldt-Jakob **(vCJD)** (Will *et al.*, 1996). El origen de la vCJD se ha atribuido a la exposición, en altas concentraciones, a productos contaminados con el agente de la EEB a finales de los años 80, que permitió al agente infeccioso atravesar la barrera de especie entre el ganado vacuno y la especie humana. Estudios epidemiológicos demostraron que el vehículo de infección de la EEB fueron las harinas de carne y hueso utilizadas en los piensos en granjas de rumiantes. La hipótesis más común es que estos suplementos estaban contaminados con el agente causante de la enfermedad en ovino. Es probable que el reciclaje del material infectado provocara una contaminación masiva de estas harinas, dando lugar a una epidemia de EEB con un máximo de casos a principios de los años 90 en el Reino Unido. Su extensión a otros países de Europa fue probablemente debida principalmente a la exportación de harinas contaminadas desde países con EEB, principalmente del Reino Unido.

Al parecer, la similitud estructural de los priones de ambas especies podría haber facilitado la transmisión de la enfermedad a los humanos (Ironside & Head, 2004; Smith *et al.*, 2004; Watts *et al.*, 2006; Eggenberger, 2007). Todas las investigaciones neuropatológicas realizadas sobre sistema nervioso de animales y humanos llevaron a concluir que los distintos procesos neurológicos poseían una base molecular muy similar. En todas las muestras analizadas se detectó una degeneración espongiforme

característica. Por esta capacidad de transmisión a este grupo de enfermedades se le denominó como encefalopatías espongiformes transmisibles (**EET**) (Riesner, 2002).

Las EET se caracterizan por presentar un curso progresivo y fatal, mostrando las siguientes características:

- Tienen períodos de incubación largos y variables (de meses a años), dependientes del huésped, de la cepa del agente causal y de la vía de transmisión.
- Están causadas por un agente no convencional altamente resistente a procedimientos físicos y químicos que inactivan bacterias, virus o viroides, y sensible a tratamientos que alteran las proteínas (DeArmond & Prusiner, 1995).
- Tienen carácter transmisible en forma experimental o natural (Ryder *et al.*, 2009; Miyazawa *et al.*, 2011).
- No se ha observado una respuesta inflamatoria clásica, mediada por antígeno, detectable en el huésped (Porter *et al.*, 1973), aunque sí se produce neuroinflamación (Carroll *et al.*, 2015).
- Producen un cuadro lesional caracterizado por una vacuolización espongiforme localizada principalmente en el SNC (Budka, 2003; Jeffrey & Gonzalez, 2004).

Entre las enfermedades priónicas que afectan a la especie humana se incluyen la enfermedad de Creutzfeldt-Jakob **(CJD)**, el kuru, el síndrome de Gerstmann-Sträussler-Scheinker y el insomnio familiar fatal (Imran & Mahmood, 2011). Las enfermedades priónicas están relacionadas con un grupo de enfermedades denominadas «conformacionales» en la especie humana como son la enfermedad de Alzheimer, Parkinson, o enfermedad de Huntington (Prusiner, 2012). Estas enfermedades surgen como consecuencia del plegamiento anómalo de una proteína específica. Este cambio de conformación causa la enfermedad, bien por la adquisición de una actividad tóxica, o por la pérdida de función de la proteína normal (Soto, 2003).

1.1 Agente causal: de virus a priones

Los veterinarios conocen la enfermedad desde que se detectaran los primeros casos de scrapie en Europa central e Inglaterra en 1730 (Araujo, 2013). Cuando se

comenzó a investigar el agente causal, se asumió que el agente responsable de las EET era un virus atípico perteneciente a la categoría de los lentivirus (Sigurdsson, 1954; Gajdusek & Gibbs, 1968; Thormar, 1971). Sin embargo, en 1967 el Dr. Pattison y colaboradores comprobaron que el agente era resistente al calor y formaldehido (Pattison & Jones, 1967), dos tratamientos que inactivan a la mayoría de los virus. A partir de los experimentos con radiación ionizante y ultravioleta, el Dr. Alper y colaboradores concluyeron que la replicación del agente causante de scrapie no debía depender de ácido nucleico (Alper *et al.*, 1967). Con estos resultados, el Dr. Griffith postuló la hipótesis de una proteína capaz de replicarse a sí misma (Griffith, 1967).

Posteriormente, el equipo de investigación del Dr. Prusiner, tras varios años de experimentos biofísicos y bioquímicos, consiguió aislar una glicoproteína resistente a proteasas a partir de encéfalos de hámster infectados por priones. Esta proteína era la predominante de la partícula infecciosa y se acumulaba en tejido nervioso de animales afectados, a veces en forma de placas amiloides. El Dr. Prusiner introdujo el término «prión» para distinguir al agente de otros patógenos como virus o viroides y lo definió como «pequeña partícula infecciosa proteica que es resistente a procesos de inactivación de ácidos nucleicos» (Prusiner, 1982). El Dr. Prusiner fue galardonado con el premio Nobel en 1997 por su trabajo en este campo.

El componente proteico aislado y purificado del tejido nervioso afectado tiene un peso molecular de 27-30 kDa y es totalmente resistente a la digestión con proteinasa K, por ello se le denominó $PrP^{27\text{-}30}$ o **PrP^{res}**. Las diferentes propiedades de la proteína normal y la patológica son debidas a las diferencias en la conformación tridimensional (Figura 1). La determinación de algunos aminoácidos del extremo N-terminal de esta proteína permitió la clonación del **cDNA** (Ácido desoxirribonucleico complementario) codificante. El gen de PrP^{res} fue localizado en el genoma del propio huésped y se descubrió que este péptido era en realidad un fragmento de otra molécula mayor (33-35 kDa) llamada PrP^{Sc} (Oesch *et al.*, 1985). Además, el gen *PRNP* se encontró en todas las especies susceptibles de contraer una EET y se comprobó que la proteína se expresaba de forma fisiológica en muchos tejidos de organismos sanos, con niveles más elevados en el SNC (Robakis *et al.*, 1986). La proteína presente en los animales sanos es sensible a proteasas y se la conoce como PrP^{sen} o PrP^{C}. Su secuencia primaria es idéntica a la de PrP^{Sc} y están codificadas por el mismo gen (Basler *et al.*, 1986).

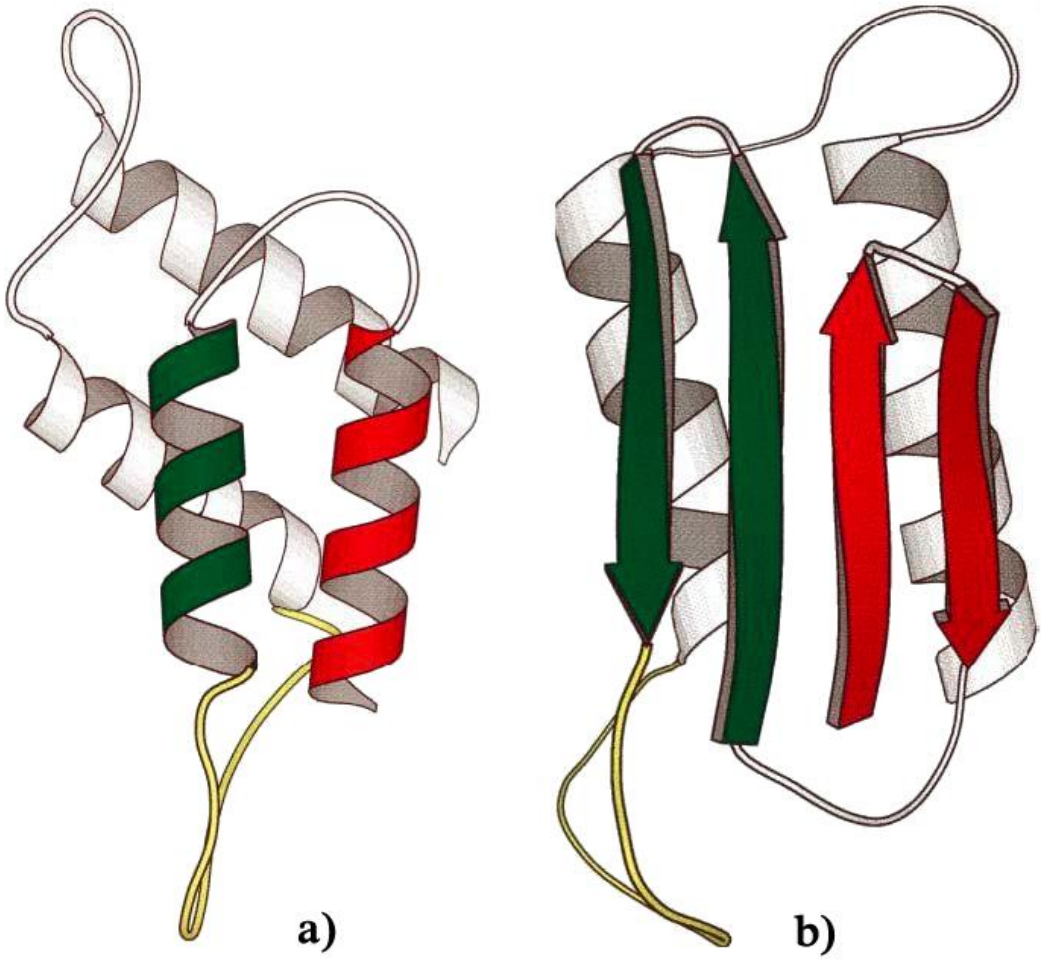

Figura 1. Modelos de las conformaciones terciarias de la proteína prión celular (PrP^{C}) (a) y de la proteína prión patológica (PrP^{Sc}) (b). (Cohen & Prusiner, 1998).

Investigaciones más recientes constituyen un nuevo sustento a la hipótesis priónica, ya que por fin se demuestra la generación *in vitro* de partículas infecciosas. Como ejemplo, una proteína PrP^{C} recombinante en ratón producida en *Escherichia coli* fue convertida en PrP infecciosa capaz de causar un fenotipo similar a la enfermedad priónica en ratones (Legname *et al.*, 2004). Además, el Dr. Castilla y colaboradores consiguieron generar partículas PrP^{res} *in vitro* mediante una técnica de amplificación cíclica (**PMCA**: *Protein Misfolding Cyclic Amplification*) y la inoculación de estas partículas en hámster provocaron una patología típica de scrapie e idéntica a la desencadenada por material infeccioso de tejido nervioso en los mismos animales (Castilla *et al.*, 2005).

1.2 Replicación y expansión de PrP^{Sc}

El mecanismo por el cual la proteína patológica induce la formación de nuevas partículas infecciosas a partir de la proteína normal PrP^{C} sigue siendo objeto de investigación. A partir de la teoría del origen priónico de las EET se han propuesto dos modelos principales (Figura 2) que se describen a continuación: el modelo de replegamiento o catalítico (Prusiner, 1991; Cohen, 1999) y el modelo de polimerización dependiente de nucleación (Lansbury & Caughey, 1995).

- **Modelo de replegamiento catalítico:** Sugiere que la PrP^{Sc} es capaz de emplear la PrP^{C} como sustrato y producir una cascada de mal plegamiento de PrP^{C}. La PrP^{Sc} sería responsable de la disminución de la energía requerida que evita la transformación espontánea de PrP^{C} en PrP^{Sc} (Aguzzi & Calella, 2009). Este proceso de conversión incluye varios fragmentos de PrP^{Sc} intermedios generados mediante un complejo mecanismo de oligomerización y reensamblaje en protofibrillas que crecerían en forma de fibras amiloides (Govaerts *et al.*, 2004; Silveira *et al.*, 2005). Las fibras más largas se romperían de forma natural produciendo fragmentos más pequeños, llamados semillas, que propagarían *de novo* el prión patológico (Baskakov *et al.*, 2001).

- **Modelo de nucleación:** Este modelo describe una polimerización no catalítica y termodinámicamente controlada, en la cual la conversión de PrP^{C} en PrP^{Sc} es un proceso reversible. El equilibrio favorece que existan formas PrP^{C} y únicamente ocurriría la transformación al existir contacto con agregados de PrP^{Sc}. La nueva PrP^{Sc} formada se añadiría a los agregados ya existentes de PrP^{Sc}. Una consecuencia de este modelo es que la infectividad depende de la presencia de oligómeros de PrP^{Sc}, ya que los monómeros no serían infecciosos (Aguzzi & Calella, 2009), siendo incluso sensibles a proteasas (Aguzzi & Lakkaraju, 2016).

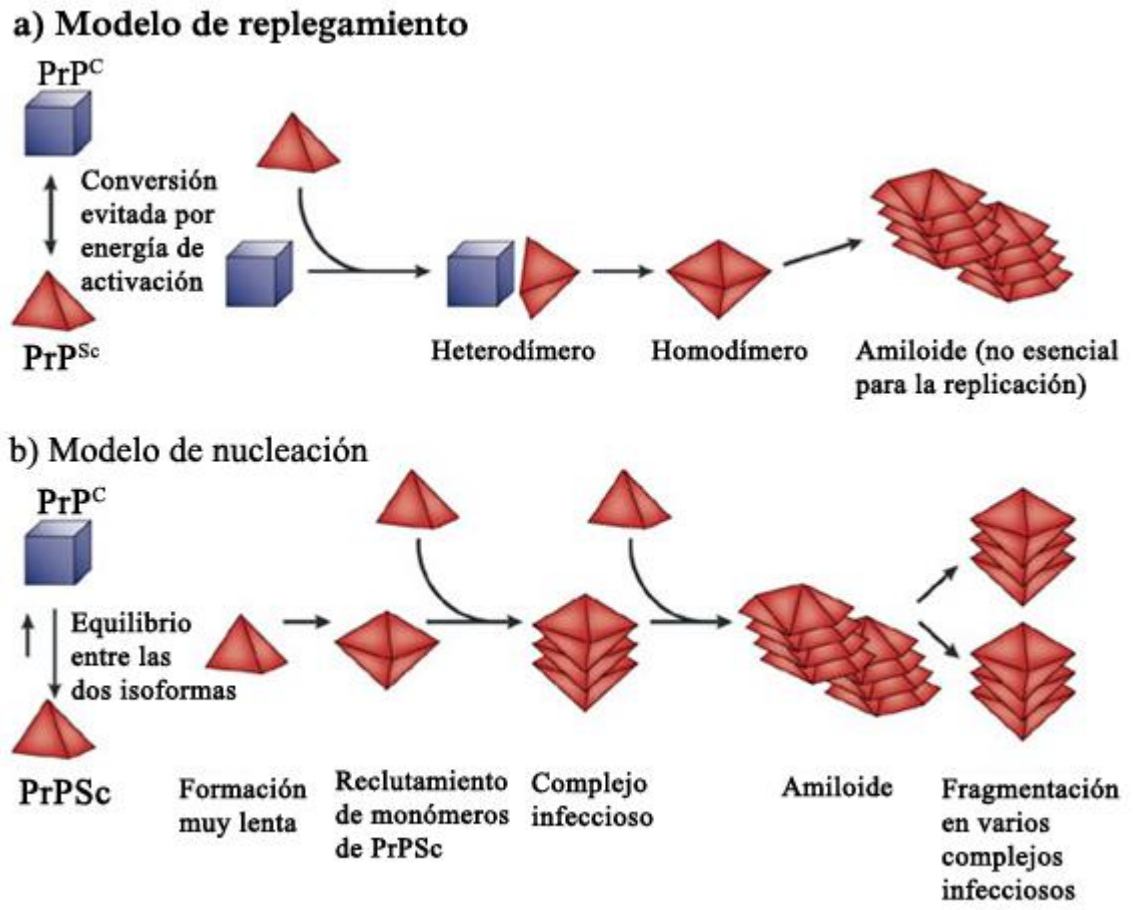

Figura 2. Modelos de replegamiento catalítico (a) y de nucleación (b) (basado en Aguzzi & Sigurdson, 2004).

Sin embargo, estos modelos deben ser investigados ya que no explican las variaciones que existen entre los componentes infecciosos. Por ejemplo, la existencia de múltiples isoformas de PrP^{Sc}, llamadas cepas, con propiedades únicas de infectividad, tropismo de especie, patología, neurotropismo y características biofísicas particulares (Morales *et al.*, 2007).

Independientemente del modo de replicación, la acumulación y expansión de PrP^{Sc} parece estar relacionada con los procesos de envejecimiento celular y pérdida de capacidad para eliminar estas formas proteicas anormales (Aguzzi & Lakkaraju, 2016). La expansión célula a célula puede tener lugar mediante varios mecanismos que se detallan a continuación:

- **Exosomas**: Los exosomas actuarían como «caballo de Troya» entre las células infectadas y las células aceptoras. Los exosomas son liberados de las células cuando los cuerpos multivesiculares se fusionan con la membrana plasmática de la célula y están formados por compuestos citosólicos e intracelulares. Tanto PrP^{C} como PrP^{Sc} están asociados con los cuerpos multivesiculares y exosomas (Yim *et al.*, 2015). Recientemente se ha podido detectar la salida de agregados de proteína Tau en ratones afectados experimentalmente por Alzheimer (Polanco *et al.*, 2016). A pesar de esta relación, no existe una evidencia clara hasta la fecha de que la transmisión de oligómeros o agregados patológicos sea a través de exosomas en la enfermedad de scrapie.
- **Mecanismos no convencionales**: Se trata de un término muy amplio que incluye mecanismos de secreción de proteínas de forma no convencional. La autofagia es un proceso fundamental en la defensa contra proteínas intracelulares mal plegadas, en las EET los procesos de autofagia se desregulan favoreciendo la acumulación de proteína patológica (Menzies *et al.*, 2011). La desregularización de la autofagia favorece la toxicidad celular y podría facilitar la salida de oligómeros unidos a membranas. Así se facilitaría la salida desde el interior celular al espacio extracelular de oligómeros y agregados mayores.
- **Intercambio mediante nanotúbulos**: Los nanotúbulos son conexiones frágiles y temporales que crean canales entre células, incluyendo neuronas; y que permiten el intercambio directo de proteínas, endosomas, mitocondrias, lisosomas o vesículas de Golgi (Marzo *et al.*, 2012). La PrP^{Sc} podría emplear estos nanotúbulos para propagarse entre células (Gousset *et al.*, 2009).

1.3 Funciones de la proteína prión

Los estudios de las funciones de la PrP^{C} se centraron en un principio en el estudio del SNC de individuos adultos, ya que la proteína prión celular se encuentra más abundantemente en neuronas (Linden *et al.*, 2008). Sin embargo, la PrP^{C} se expresa en grandes cantidades durante el desarrollo embrionario tanto en SNC como en sistema nervioso periférico (Manson *et al.*, 1992). Experimentos con ratones *knockout* para el gen *Prnp* demostraron la alteración en la expresión de 263 genes responsables de factores de crecimiento y receptores para factores de crecimiento, esto refuerza la idea

de que la PrP^C juega un papel importante en la regulación de las cascadas asociadas con el desarrollo embrionario (Khalife *et al.*, 2011). A continuación se describen algunas de las funciones que se han atribuido a esta proteína.

1.3.1 Regulación en la auto-renovación de células madre

La presencia de PrP^C se pudo confirmar en células progenitoras hematopoyéticas humanas hace más de una década (Liu *et al.*, 2001). Se comprobó experimentalmente que los ratones carentes del gen *Prnp* no poseían la capacidad de renovar la población celular a largo plazo, siendo la PrP^C fundamental para la auto-renovación de esta población celular (Zhang *et al.*, 2006).

Se ha visto que la expresión de PrP^C es necesaria para los procesos de división celular y expansión necesarios para el correcto desarrollo del SNC (Steele *et al.*, 2006). Estudios *in vitro* han demostrado que la formación de neuroesferas (sistema de cultivo consistente en agrupaciones redondeadas de células madre neuronales o **NSC**) es menos eficiente en ratones *knockout* durante la fase fetal (Santos *et al.*, 2011) y postnatal (Prodromidou *et al.*, 2014). El concepto de que la PrP^C contribuye a la proliferación de las células madre se extiende a otros tejidos como se ha podido comprobar recientemente en las células mesenquimales humanas procedentes de médula ósea (Mohanty *et al.*, 2012).

1.3.2 Diferenciación de células madre

La expresión de PrP^C está aumentada durante la diferenciación de células madre embrionarias humanas y murinas (Lee & Baskakov, 2010; Miranda *et al.*, 2011). De forma recíproca, la inducción mediante PrP^C de células madre embrionarias produce diferenciación (Lee & Baskakov, 2013). Parece que los niveles de expresión de PrP^C determina el destino de la diferenciación de las células progenitoras hematopoyéticas, ya que se encuentra PrP^C en la superficie de linfocitos T y B, monocitos, células dendríticas, es muy abundante en megacariocitos y plaquetas, pero no se encuentra en eritrocitos o granulocitos (Linden *et al.*, 2008). La PrP^C juega un papel fundamental en la diferenciación neuronal (Santos *et al.*, 2013) y la polarización y maduración sináptica (Kanaani *et al.*, 2005). Se ha encontrado un incremento de expresión de PrP^C durante la diferenciación y maduración neuronal, mientras que es la presencia de PrP^C en células

de la glía durante el desarrollo embrionario no es detectable (Steele *et al.*, 2006). Sin embargo, las cinéticas de desarrollo de astrocitos y oligodendrocitos se retrasan en ratones carentes del gen *Prnp* (Arantes *et al.*, 2009; Bribian *et al.*, 2012), reforzando la idea de que la PrP^C es esencial para el normal desarrollo del SNC.

1.3.3 Relación con procesos oncogénicos

Además de regular la proliferación y diferenciación, la PrP^C regula muchos tipos celulares, entre los que se incluyen las células con potencial oncogénico. La presencia de PrP^C en líneas celulares procedentes de tumor gástrico acelera la transición del ciclo celular G1 a S y promueve la proliferación (Liang *et al.*, 2007). Además, la PrP^C parece conferir cierta resistencia ante agentes citotóxicos (Mehrpour & Codogno, 2010), así como procurar propiedades invasivas (Pan *et al.*, 2006). La PrP^C interactúa con la proteína de superficie CD44 y parece promover la proliferación y migración en las células procedentes de cáncer de mama resistentes a quimioterapia (Cheng *et al.*, 2014). Además, la proteína CD44 se sobreexpresa en varios tipos celulares capaces de iniciar tumores, los cuales guardan ciertas similitudes con las células embrionarias y células madre adultas (Medema, 2013). Estas alteraciones en las funciones de la PrP^C se han visto también en procesos de neurodegeneración (Hirsch *et al.*, 2014) y es similar a los producidos durante el envejecimiento celular. Estos cambios pueden llevar a la desregularización en la interacción de las células madre con su nicho, hecho especialmente relevante en los procesos de envejecimiento celular (Martin-Lanneree *et al.*, 2014).

1.3.4 Regulación inmune

La PrP^C es una proteína ubiquitaria, aunque parece expresarse en mayor medida en lugares inmunológicamente quiescentes, como en SNC (Taraboulos *et al.*, 1992a), células de la glía en SNC (Moser *et al.*, 1995) y otros tejidos como testículos, ojo, placenta y útero (Tanji *et al.*, 1995; Johnson *et al.*, 2014). Como se ha visto anteriormente, la PrP^C se expresa también en células madre hematopoyéticas y su papel principal en estos órganos inmunoprivilegiados podría ser la protección frente al daño inflamatorio y el mantenimiento de la homeostasis inmunológica (Bakkebo *et al.*, 2015).

En experimentos llevados a cabo en ratones *knockout* para el gen *Prnp,* a los que se sometió a diversos procesos inflamatorios como colitis o encefalomielitis autoinmune, o se les provocó isquemia cerebral, se demostró que la ausencia de PrP^C exacerba el daño inflamatorio producido (Onodera *et al.*, 2014). La PrP^C parece tener un efecto supresor en la neuroinflamación mediada por células T, y su ausencia puede causar un mayor daño por inflamación y mayor susceptibilidad al estrés oxidativo (Tsutsui *et al.*, 2008).

1.3.5 Neuroprotección

La PrP^C parece tener un papel importante en la protección frente al estrés celular, así como en la supervivencia neuronal, diferenciación y proliferación. La sobreexpresión de PrP^C ha demostrado tener efectos protectores en líneas celulares primarias ante estímulos apoptóticos (Roucou *et al.*, 2003). Además, la PrP^C regula cascadas de señalización intracelular relacionadas con la supervivencia celular (Lo *et al.*, 2007). Cuando la co-chaperona citoplasmática llamada *stress-inducible-phosphoprotein 1* (STI1) es secretada al espacio extracelular se une a la región central de la PrP^C y promueve la supervivencia neuronal (Zanata *et al.*, 2002). Se ha descrito que la PrP^C puede ejercer un papel en el desarrollo del SNC y en la neurogénesis, posiblemente debido a su unión con la molécula de adhesión celular neural (N-CAM) (Chen *et al.*, 2003; Santuccione *et al.*, 2005). También promueve la auto-renovación de las células madre neuronales (Santos *et al.*, 2011), un proceso que se corresponde con el papel previamente descrito de regulación durante la neurogénesis.

1.3.6 Acción antiapoptótica

Uno de los ejemplos más claros del efecto citoprotector de la PrP^C es su habilidad para proteger las células neuronales frente a la apoptosis inducida por proteína X asociada a Bcl-2 (**BAX**) (Bounhar *et al.*, 2001; Roucou *et al.*, 2003). Este efecto parece ser específico contra BAX ya que ante la inducción de apoptosis por otros factores como del antagonista homólogo Bcl-2/*Killer*1 (**BAK**), de la estaurosporina o de la tapsigargina no se produce efecto citoprotector (Bounhar *et al.*, 2001; Roucou *et al.*, 2003; Roucou *et al.*, 2005). También actuaría atenuando los efectos apoptóticos de

la proteína llamada doppel (Anderson *et al.*, 2004). Del mismo modo, se ha visto que una sobreexpresión de PrP^C es capaz de reducir la respuesta de apoptosis inducida por factor de necrosis tumoral alfa (**TNF-α**) (Diarra-Mehrpour *et al.*, 2004).

Se han propuesto varios mecanismos mediante los cuales la PrP^C inhibiría la acción de BAX. Uno de ellos resultaría de la inhibición directa de la actividad de BAX impidiendo su translocación mitocondrial, cambio conformacional u oligomerización. Otra posible vía es la inhibición de activadores BH3 (dominio homólogo 3 de Bcl-2) de BAX, o promoviendo la asociación entre BAX y reguladores antiapoptóticos como **Bcl-2** (célula B CLL/Linfoma 2) o Bcl-XL (Regulador apoptótico Bcl-X) (Westergard *et al.*, 2007). Finalmente, la PrP^C podría tener efectos en la actividad de BAX relacionada con la liberación de calcio y respuesta a la desnaturalización de proteínas en el retículo endoplasmático a través de receptores transmembrana (Scorrano *et al.*, 2003; Hetz *et al.*, 2006). Se ha podido demostrar que la PrP^C inhibe el cambio conformacional de BAX cuando es activada fuera de la membrana mitocondrial (Roucou & LeBlanc, 2005). De forma contraria, otros estudios muestran que la PrP^C podría tener un efecto tóxico al unirse e interactuar secuestrando la Bcl-2 (Westergard *et al.*, 2007). El papel de la PrP^C en las vías de apoptosis y supervivencia celular requiere de más estudios.

1.3.7 Protección frente al estrés oxidativo

En cuanto a la protección frente al estrés oxidativo, el SNC es rico en iones metálicos y grasas insaturadas, lo cual lo hace especialmente sensible al estrés oxidativo (Halliwell, 1992). La PrP^C posee función antioxidante, esta función puede deberse a que las especies reactivas de oxígeno (**ROS**) promueven la apoptosis (Halliwell, 2006), y como se ha visto anteriormente, la PrP^C tiene efectos antiapoptóticos. La hipótesis de que la PrP^C actúe directamente sobre las ROS y tenga un efecto parecido al de la enzima superóxido dismutasa ha sido descartada (Jones *et al.*, 2005). La proteína prión sí que actuaría sobre las ROS, pero actuando como regulador del nivel de cobre intracelular (Brown *et al.*, 2001), de modo que actuaría aumentando la actividad citosólica de la superóxido dismutasa dependiente de cobre y zinc (Cu/ZnSOD) (Brown & Besinger, 1998). Se ha visto que la falta de expresión de PrP^C produce una disminución de esta enzima (Brown *et al.*, 1997).

1.4 Toxicidad de PrP^{Sc}

El acúmulo de PrP^{Sc} es necesario para que se manifieste la patología. Las alteraciones producidas por la enfermedad pueden ser a consecuencia de la falta de función protectora de PrP^{C}, o de los efectos tóxicos producidos por PrP^{Sc} (Rambold *et al.*, 2008; Bolea *et al.*, 2010). Existen casos en los cuales no se detecta un gran acúmulo de proteína patológica al presentarse la enfermedad (Lasmezas *et al.*, 1997). Por el contrario, en otros casos se ha encontrado un gran acúmulo de PrP^{Sc} sin que se observen signos clínicos (Piccardo *et al.*, 2007). Esto parece indicar que la capacidad infectiva y patogénica de la proteína prión son características independientes entre sí.

Estudios realizados en cultivos celulares han demostrado que tanto los oligómeros de PrP^{Sc} (Kazlauskaite *et al.*, 2005) como los agregados amiloides (Novitskaya *et al.*, 2006) producen toxicidad. La presencia de PrP^{C} intracelular es necesaria para que se produzca esta toxicidad, y si se reduce la cantidad de PrP^{C} disponible se reduce también la sensibilidad neuronal ante la toxicidad de PrP^{Sc} (Bueler *et al.*, 1994). A pesar de que existen muchas evidencias experimentales que sugieren que la neurotoxicidad de las enfermedades priónicas esta mediada por oligómeros de PrP^{Sc} (Hu & Huang, 2013), el mecanismo mediante el cual se produce la muerte neuronal dista de estar resuelto.

Uno de los posibles mecanismos de toxicidad se ha asociado con la presencia de PrP^{Sc} y la generación de poros en la membrana celular (Solomon *et al.*, 2010). Esto resulta en un desequilibrio eléctrico entre el medio intracelular y extracelular provocando una disfunción celular y la activación de señales proapoptóticas (Lashuel *et al.*, 2002; Quist *et al.*, 2005; Solomon *et al.*, 2012). En ovino infectado con scrapie natural, se ha demostrado la correlación entre los depósitos de PrP^{Sc} y la inducción de la proteína proapoptótica BAX (Lyahyai *et al.*, 2006; Lyahyai *et al.*, 2007), a pesar de que esta inducción no se traducía en evidencias claras de apoptosis, posiblemente debido a la activación de mecanismos neuroprotectores (Serrano *et al.*, 2009; Serrano *et al.*, 2011).

Otra hipótesis propuesta sugiere que la presencia de PrP^{Sc} provocaría una actividad excesiva en el receptor de N-metil-D-aspartato produciendo citotoxicidad y daño neuronal (Khosravani *et al.*, 2008). Por último, la presencia de PrP^{Sc} se ha asociado con la activación de respuesta neuroinflamatoria (Soto & Satani, 2011).

Además de su efecto directo en las neuronas, la acumulación de PrP^{Sc} puede participar indirectamente en el proceso de degeneración mediante la inducción de gliosis reactiva. La presencia de PrP^{Sc} induce la secreción por parte de la glía de algunas citoquinas y mediadores inflamatorios que acaban participando en la patogénesis de estas enfermedades (Heppner *et al.*, 2001). Estudios realizados por nuestro grupo en ovino infectado con scrapie demostraron la relación entre la expresión de factores involucrados en apoptosis e inflamación y la presencia de gliosis reactiva (Serrano *et al.*, 2009).

2 SCRAPIE

El scrapie es la primera EET reconocida en los mamíferos y constituye el prototipo de estas patologías. A pesar de haber sido una enfermedad endémica en muchos países desde hace más de 300 años, nunca ha supuesto un riesgo para la salud, e incluso los animales afectados por la enfermedad eran sacrificados y destinados al consumo humano sin que exista una asociación entre el aumento de enfermedades neurodegenerativas y el consumo de productos procedentes de la ganadería ovina (Schneider *et al.*, 2008). Fue a partir de la epidemia de la EEB en la década de los años 80, y su posible origen en piensos contaminados con restos de animales infectados con scrapie, cuando se renovó el interés por la enfermedad en su forma natural, ya que es considerada como modelo para el estudio de las EET. En la actualidad el scrapie ha sido descrito en casi todos los países del mundo, únicamente Australia y Nueva Zelanda están actualmente declarados libres de la enfermedad.

El período de incubación de scrapie varía entre 14 y 22 meses, y la mayoría de los casos clínicos se presentan en animales adultos entre 2 y 5 años de edad. Las ovejas afectadas pueden vivir entre 1 y 6 meses tras la aparición de los síntomas clínicos, y el desenlace siempre es la muerte del animal. La sintomatología puede variar ampliamente entre los animales enfermos, posiblemente debido a que se vean afectadas diferentes regiones cerebrales, y a la diversidad de cepas priónicas causantes de la enfermedad (Collinge & Clarke, 2007).

2.1 Patogenia

De forma natural la transmisión en el rebaño ocurre de forma vertical entre animales susceptibles. La oveja puede transmitir la enfermedad a su descendencia a través de la placenta y fluidos placentarios durante el parto. Puede producirse transmisión horizontal durante el periodo de nacimiento de corderos debido a la ingesta de los restos del parto o la contaminación de las camas por los mismos (Touzeau *et al.*, 2006). La transmisión a través de la leche y el calostro representa un riesgo adicional de transmisión para los corderos de rebaños afectados (Konold *et al.*, 2008).

La entrada al organismo se produce vía oral (Gough & Maddison, 2010). Tras la ingestión la PrP^{Sc} es capaz de atravesar la barrera intestinal a través de los enterocitos y pasar rápidamente a sangre y linfa. La PrP^{Sc} se acumula en un primer momento en las células dendríticas foliculares de los animales susceptibles y la primera replicación del prión ocurre en el sistema linforreticular, principalmente en las placas de Peyer a nivel del íleon (Jeffrey *et al.*, 2006). A través de los órganos linfoides el prión es capaz de replicarse y distribuirse ampliamente sin provocar respuesta humoral ni síntomas. El prión se puede detectar en sangre asociado a linfocitos T, monocitos y células mononucleares, con lo que no puede descartarse como medio de invasión la vía hematógena (Dassanayake *et al.*, 2016). Finalmente, el prión es capaz de alcanzar el SNC avanzando de forma ascendente desde el sistema nervioso entérico.

2.2. Neuropatología

La alteración más típica en el cerebro de las enfermedades priónicas es la vacuolización, dándole al encéfalo la apariencia de una esponja, y por ello el nombre de encefalopatías espongiformes. La degeneración espongiforme consiste en la aparición de pequeñas vacuolas redondas u ovaladas, difusas o focalmente localizadas en el neuropilo de las capas profundas del córtex, córtex cerebeloso o materia gris subcortical (Jeffrey *et al.*, 1992). Las zonas afectadas por la vacuolización y su distribución suelen emplearse para determinar las cepas del agente infeccioso (Morales *et al.*, 2007).

Los mecanismos moleculares responsables de la vacuolización pueden corresponderse con una permeabilidad anormal de la membrana y aumento del contenido de agua en las neuronas (Kovacs & Budka, 2008) o con procesos de autofagia

(Liberski *et al.*, 2004). La vacuolización podría ser un cambio no específico, ocurriendo de forma secundaria a la disrupción del transporte axonal (Williams *et al.*, 1994). La inflamación neuronal ocurrida en el transcurso de las EET ocurre mediante la activación de astrocitos y microglía, con poca o nula infiltración linfocitaria (Perry *et al.*, 2002). Las células T activadas pueden atravesar la barrera hematoencefálica producir citoquinas proinflamatorias y activar la microglía (Aloisi *et al.*, 2000). La activación de la microglía puede ejercer efectos neuroprotectores al promover la fagocitosis y presentación de antígeno, sin embargo, una activación crónica y excesiva puede favorecer el daño por estrés oxidativo y estrés celular, produciendo neurotoxicidad y neurodegeneración (Vidal *et al.*, 2009; Soto & Satani, 2011).

2.2.1. Muerte neuronal en scrapie

Las enfermedades priónicas cursan con fenómenos apoptóticos y autofágicos en el SNC, y una elevada producción de ROS, la cual no puede ser compensada por las células neuronales (Liberski *et al.*, 2008). El estrés oxidativo parece ejercer un papel en la patogenia y se ha visto que es un proceso común en las EET, sin embargo todavía no hay pruebas evidentes de si se trata de un suceso inicial o primario, o es un colaborador tardío en el desarrollo de la disfunción neuronal. Algunos estudios ligan el aumento de ROS como parte en sí misma de conversión de PrP^{C} a PrP^{res} en las EET (Wolschner *et al.*, 2009). Este proceso podría verse incrementado debido a la pérdida de las funciones antioxidantes de la PrP^{C} y sus efectos neuroprotectores (Véase apartado **1.3**).

Se ha visto que en el transcurso de estas enfermedades se produce un aumento de factores proapoptóticos como *BAX* (Lyahyai *et al.*, 2007). Se activan también rutas dependientes de BAK, que no pueden ser bloqueadas por inhibidores de caspasas (McCarthy *et al.*, 1997). En la especie ovina se ha descrito la sobreexpresión del factor inductor de apoptosis (**AIF**), factor que regula este tipo de apoptosis, indicando que podrían estar sucediendo procesos de apoptosis independiente de caspasas (Hedman *et al.*, 2012). Por otra parte, también se han detectado cambios en la expresión de genes involucrados en la apoptosis que podrían tener efecto neuroprotector. Por ejemplo, se ha observado un incremento de ***MCL1*** (Proteína marcadora de leucemia celular mieloide inducida 1) en encéfalos de ovino infectado con scrapie natural (Serrano *et al.*, 2009),

MCL1 es un gen de respuesta ante BAX y BAK que se ha visto tiene una función antiapoptótica (Cuconati *et al.*, 2003).

La apoptosis en las EET parece ser un proceso constante pero lento y que a veces cursa sin que se dé un aumento de marcadores específicos de apoptosis (Woodhouse *et al.*, 2006). Esto podría deberse a que se dan procesos de apoptosis y de compensación y protección neuronal al mismo tiempo. Estudios realizados por nuestro grupo en ovino afectado por scrapie natural se describió la ausencia de neuronas en apoptosis a pesar de la clara inducción de factores proapoptóticos (Lyahyai *et al.*, 2006; Lyahyai *et al.*, 2007; Hedman *et al.*, 2012), una regulación de factores pro- y anti-apoptóticos (Serrano *et al.*, 2009) y la posible protección de grupos neuronales específicos por la inducción de chaperonas (Serrano *et al.*, 2011).

2.3 Diagnóstico

El diagnóstico clínico puede resultar difícil ya que se trata de una enfermedad que no puede ser distinguida de otras patologías o encefalopatías mediante la observación de los signos clínicos. Los primeros síntomas en aparecer incluyen cambios en el comportamiento y en el temperamento, aparecen alteraciones locomotoras (ataxia cerebelosa), presentándose sobre todo en las extremidades posteriores. Las alteraciones sensoriales provocan rechinar de dientes, nerviosismo y prurito, dando lugar a la pérdida de lana y a la aparición de lesiones en la piel, sobre todo en la zona lumbar. Otros signos descritos son posturas anormales, hiperestesia, temblores musculares, nistagmo y depresión. En estados más avanzados se observa pérdida de peso, ataxia severa, parálisis con incapacidad para incorporarse y, finalmente, la muerte del animal (Pattison & Millson, 1960).

Cuando se sospecha que los signos clínicos son compatibles con scrapie existe un reducido número de técnicas que pueden confirmar la presencia de PrP^{Sc} en el animal vivo. La biopsia de tejido de membrana nictitante (Vargas *et al.*, 2006), tonsilas (van Keulen *et al.*, 2002), o mucosa rectal (Monleon *et al.*, 2005), pueden confirmar el diagnóstico. Sin embargo, estas pruebas presentan una baja sensibilidad, dando lugar a falsos negativos. Esto se debe a que la distribución del prión a través del sistema linfoide, nervioso o sangre depende de la resistencia genética del animal, de la exposición y de la cepa priónica. Se ha utilizado la técnica de la PMCA para amplificar

con más éxito la PrP^{Sc} en fluidos como la orina, la sangre o líquido cefalorraquídeo, donde el nivel estimado de la proteína es del orden de picogramos por mililitro (Properzi & Pocchiari, 2013).

Existen técnicas que surgieron como respuesta a la necesidad de técnicas rápidas y fiables para detectar a los animales enfermos de EEB a causa de la epidemia de esta enfermedad acontecida en Reino Unido y posteriormente en diferentes países europeos. Las técnicas rápidas autorizadas por la Unión Europea son distintos formatos de ELISA (Ensayo por inmunoabsorción ligado a enzimas), que consiste en la desnaturalización y concentración de la muestra previa incubación con anticuerpos anti-proteína prión, y la técnica de *Western Blotting* tradicional modificada, permitiendo acortar tiempos y aumentar el número de muestras utilizando anticuerpos monoclonales (Bolea *et al.*, 2005; Filali *et al.*, 2013).

Independientemente del diagnóstico *in vivo* o las técnicas rápidas, siempre es necesaria la confirmación. Tradicionalmente el diagnóstico de referencia ha sido el diagnóstico por las lesiones histopatológicas que se pueden apreciar en el SNC (Miller L.D., 1985; Bolea *et al.*, 2005). Pero debido a la imposibilidad de diagnóstico en casos en los que los animales presentan lesiones mínimas o insuficientes (Begara-McGorum *et al.*, 2000) o en los que el SNC ha sido congelado, la Organización Mundial de Sanidad Animal (OIE) amplió a otras técnicas rápidas los métodos de diagnóstico de referencia.

El diagnóstico inmunohistoquímico es un procedimiento que permite identificar la presencia y distribución de acúmulos de PrP^{Sc} de forma muy eficaz y sensible, incluso en encéfalos autolíticos (Debeer *et al.*, 2001; Monleon *et al.*, 2003). El diagnóstico mediante *Western Blotting* permite detectar la proteína patológica en tejido fresco o congelado en función de su resistencia a proteasas, su peso molecular y su reacción frente a anticuerpos específicos. Se realiza mediante la aplicación de una electroforesis en gel de poliacrilamida (Riek *et al.*, 1996) seguidas de un *Western Blotting* (Pan *et al.*, 1993). Esta técnica permite separar los constituyentes de la PrP^{Sc} en función del peso molecular de las diferentes formas (no glicosiladas, mono-glicosiladas y di-glicosiladas), y posteriormente ser incubados con un anticuerpo anti-PrP. Esta técnica presenta una elevada sensibilidad y una incidencia muy baja de falsos negativos.

Además, como se ha descrito anteriormente, la PMCA se ha empleado en investigación para la detección *in vivo* de la PrP^{Sc} ya que permite amplificar una cantidad inicial indetectable de proteína patológica haciéndola detectable. Se trata de una técnica altamente sensible que permite la detección incluso en animales preclínicos (Soto *et al.*, 2005). Se basa en ciclos de replicación de la PrP^{Sc} sobre un sustrato de PrP^{C} y una posterior sonicación para disgregar los polímeros formados y que actúen como «semillas» para continuar la replicación (Castilla *et al.*, 2006). La PMCA permite la cuantificación de la PrP^{Sc} amplificada (Chen *et al.*, 2010) al existir una relación directa entre la cantidad de PrP^{Sc} inicial en una muestra y el número de ciclos de PMCA necesarios para detectar la proteína patológica.

2.4 Cepas

El Dr. Pattinson y colaboradores fueron los primeros en observar que existían diferentes cepas productoras de la misma enfermedad, pero que presentaban sintomatología diferente. Concretamente en la especie caprina observaron dos síndromes clínicos, uno que denominaron *drowsy* (somnoliento) y otro *scratching* (rascado vigoroso) (Pattison & Millson, 1961). Estas cepas eran capaces de mantener sus características en infecciones consecutivas. Se ha postulado que las variaciones conformacionales en la PrP^{Sc} serían las responsables de las distintas variaciones observadas en las cepas (Tanaka *et al.*, 2004).

Se han aislado más de 20 cepas productoras de scrapie clásico en la especie ovina y caprina (Dickinson & Fraser, 1972; Dickinson, 1976; Carp *et al.*, 1987). Las cepas clasificadas como clásicas poseen características moleculares, de transmisión y fenotípicas que se corresponden con la enfermedad endémica de una población (Biacabe *et al.*, 2004), al contrario que las cepas consideradas como atípicas, las cuales poseen características moleculares y epidemiológicas tales que les permite ser diferenciadas de las cepas clásicas (Benestad *et al.*, 2003).

Durante la década de los noventa, los estudios de tipificación estuvieron basados en las características de incubación, síntomas clínicos y perfiles lesionales que las cepas producían en ratones inoculados experimentalmente, ya que las cepas mantienen sus características al dar el salto a otra especie (Bruce, 2003). El problema asociado a la denominada «barrera de especie» son los largos periodos de incubación y baja susceptibilidad a la enfermedad, para ello se han desarrollado modelos murinos

transgénicos capaces de expresar proteína prión celular del huésped original, reduciendo los tiempos de incubación (Thackray *et al.*, 2007). Sin embargo a veces no es suficiente para evitar la barrera de especie ya que el gen *PRNP* es un factor determinante, pero no el único, que parece establecer la susceptibilidad a distintas cepas priónicas (Telling, 2000).

La descripción de patrones lesionales y periodos de incubación tanto en especies naturalmente afectadas por las EET como en modelos murinos para la identificación de cepas han dado paso a las técnicas actuales de identificación. Las cepas priónicas se clasifican según los patrones de depósito de proteína prión revelados mediante inmunohistoquímica (Jeffrey *et al.*, 2001a; Jeffrey *et al.*, 2001b), llegándose a describir más de doce patrones lesionales según el acúmulo y localización de proteína prión (Gonzalez *et al.*, 2003). Se ha tratado de diferenciar las cepas mediante sus diferencias en los patrones de glicosilación, ya que parecen ser los responsables de la variación entre cepas (Kascsak *et al.*, 1986). Sin embargo, no parece ser el único factor, ya que se pueden producir cambios port-translacionales que alteren las características de las cepas (Cancellotti *et al.*, 2013), o producirse interacciones con otras moléculas que actúen como cofactores (Poggiolini *et al.*, 2013). Por ello, las características de las distintas cepas no corresponderían únicamente a la conformación terciaria de la PrP^{Sc}.

Una de las cepas más estudiadas es el «scrapie atípico». Se describió por primera vez en Noruega, en el año 1998 (Benestad *et al.*, 2003). Esta forma de scrapie producida por la cepa **Nor98** difiere del scrapie clásico tanto en el cuadro patológico como en el perfil genético. El scrapie atípico es capaz de afectar a animales genéticamente resistentes al scrapie clásico (Saunders *et al.*, 2006; De Bosschere *et al.*, 2007). En muchas ocasiones se detecta mediante vigilancia activa, ya que a diferencia de la forma clásica, apenas hay vacuolización en el óbex o médula oblonga, ni se encuentran depósitos de PrP^{Sc} en esta zona. No se ha encontrado acúmulo de PrP^{Sc} fuera del SNC (Andreoletti *et al.*, 2011) y la mayoría de los animales afectados exhiben vacuolización y acúmulo de PrP^{Sc} en el cerebelo y córtex cerebral (Greenlee & Greenlee, 2015). Además, el patrón obtenido por *Western Blotting* también es diferente, ya que se observa un perfil de 5 bandas, con la banda predominante de 12 kDa y la PrP^{Sc} en scrapie atípico es relativamente sensible a proteinasas (Klingeborn *et al.*, 2006). Se suele encontrar en individuos aisladamente, con lo que parece tener origen esporádico y no ser contagioso o ser su transmisión muy ineficiente en condiciones naturales

(Fediaevsky *et al.*, 2010). Sin embargo se ha conseguido la transmisión experimental de scrapie atípico mediante inoculación intracraneal (Simmons *et al.*, 2007) o vía oral (Simmons *et al.*, 2011), con lo cual no puede descartarse totalmente que se pueda producir una transmisión natural o a través de alimentos contaminados.

2.5 Genética de scrapie

El gen que codifica para la PrP^C, llamado *PRNP*, se localiza en el cromosoma 13 de los rumiantes y posee una longitud de 31.412 pares de bases en la especie ovina. Se han observado distintas mutaciones en este gen, algunas de ellas son las responsables de algunas diferencias observadas en el periodo de incubación y susceptibilidad de la enfermedad.

Las secuencias del gen *PRNP* ovino y caprino presentan un alto grado de conservación genética. La mayoría de polimorfismos descritos en ovino y caprino se localizan en la parte C-terminal de la proteína PrP, entre los codones 98 y 234 (revisado por Baylis & Goldmann, 2004). A pesar de la gran similitud, las alteraciones de aminoácidos asociadas con cambios en la susceptibilidad a scrapie parecen ser distintas en las dos especies. Actualmente se acepta la idea de que ambos factores, un genotipo susceptible y la exposición a un agente infeccioso, son requeridos para el desarrollo de la enfermedad en estas especies (revisado por Hunter, 2007).

2.5.1 Susceptibilidad genética a scrapie ovino

El gen *PRNP* ovino presenta un alto grado de polimorfismo, con 37 mutaciones descritas y cambio aminoacídico en 27 codones distintos (Goldmann, 2008). La mayoría son mutaciones puntuales y no se relacionan con la susceptibilidad a la enfermedad. En cambio, diversos ensayos han revelado una compleja relación entre los genotipos de PrP en los codones 136, 154 y 171, la cepa del agente infeccioso y la ruta de exposición (Goldmann *et al.*, 1994).

Las variantes V_{136}, R_{154}, Q_{171} y H_{171} (Valina$_{136}$, Arginina$_{154}$, Glutamina$_{171}$, Histidina$_{171}$) se asociaron a la susceptibilidad a scrapie, mientras que A_{136}, H_{154} y R_{171} (Siendo A alanina) se relacionaron con resistencia al padecimiento de la enfermedad.

De las doce posibles combinaciones de estas variantes alélicas, sólo cinco se detectan frecuentemente en la especie ovina: ARR, ARQ, AHQ, ARH y VRQ. Considerando estos cinco haplotipos, puede encontrarse un total de 15 genotipos del gen *PRNP* ovino con clara asociación con la susceptibilidad a padecer la enfermedad (Belt *et al.*, 1995; Ikeda *et al.*, 1995). El resto de haplotipos presentan una frecuencia muy baja y su asociación con la enfermedad es desconocida (Acin *et al.*, 2004a; Goldmann *et al.*, 2005; Alvarez *et al.*, 2006).La forma ARR corresponde al haplotipo más resistente y la VRQ se relaciona con el más sensible frente al scrapie. El haplotipo más frecuente es el ARQ en razas mediterráneas (Acutis *et al.*, 2004; Serrano *et al.*, 2007). También es el más frecuente en ovino español (Acin *et al.*, 2004a), siendo este haplotipo también el mayoritario en los animales diagnosticados con scrapie clásico (Acin *et al.*, 2004b).

Se ha observado que animales del mismo genotipo y raza, en el mismo rebaño, son susceptibles a algunas cepas de scrapie pero resistentes a otras (Goldmann *et al.*, 1994). Se desconoce si las diferencias observadas en la susceptibilidad a la infección son una consecuencia de las diferencias genéticas entre razas y poblaciones, o son debidas a la tendencia de las distintas cepas del agente por infectar determinadas poblaciones. Por tanto, a pesar de que el gen *PRNP* se puede considerar como un gen mayor que afecta a la susceptibilidad a scrapie, esta base genética por sí sola no explica la gran variabilidad observada en los distintos individuos.

En cuanto a las características genéticas que distinguen al scrapie atípico, se destaca la frecuencia elevada de genotipos con cierta resistencia a scrapie clásico, particularmente el haplotipo AHQ (Benestad *et al.*, 2003). Además suele aparecer asociado al alelo fenilalanina (F) en el codón 141, que normalmente codifica el aminoácido leucina (L) (Moum *et al.*, 2005). También es muy frecuente la asociación de las formas atípicas con genotipos resistentes ARR/ARR (Saunders *et al.*, 2006) o de cierta resistencia como ARR/ARQ, además de los genotipos con el haplotipo AHQ (ARR/AHQ, AHQ/AHQ o AHQ/ARQ) (Buschmann *et al.*, 2004; Moum *et al.*, 2005).

En cuanto a la EEB, se ha visto que el genotipo ARQ/ARQ, con cierta resistencia a la infección natural por scrapie clásico comparado con las variantes más susceptibles VRQ/-, es el más sensible a la exposición experimental ante EEB (Goldmann *et al.*, 1994). Asimismo, se ha demostrado la sensibilidad de animales ARR/ARR a la exposición oral e intracerebral del agente de EEB (Houston *et al.*, 2003; Andreoletti *et al.*, 2006). Más recientemente han aparecido algunos casos de scrapie

clásico portando este genotipo resistente ARR/ARR (Groschup *et al.*, 2007). Estos casos, que no se corresponden con la clasificación de riesgo a padecer scrapie, junto con la posibilidad de ser infectados por EEB, pueden poner en duda la relevancia de los programas europeos de selección genética frente a scrapie, ya que todos ellos seleccionan genotipos que son susceptibles a la cepa de scrapie atípico Nor98, y pueden serlo también a scrapie clásico o EEB (revisado por Hunter, 2007). Sin embargo, como se ha descrito anteriormente, el scrapie atípico afecta de forma esporádica y al parecer no contagiosa al ganado ovino, y la infección por EEB se ha conseguido únicamente de forma experimental. El scrapie clásico sigue siendo la forma de la encefalopatía que afecta a mayor número de animales y por ello se sigue actuando para su control y erradicación.

3 MODELOS CELULARES

Tal y como se ha ido describiendo en los apartados anteriores, aunque en los últimos años el avance en las investigaciones en enfermedades priónicas ha sido muy importante, todavía existen muchas incógnitas con respecto a los mecanismos de replicación del prión, de toxicidad celular, y de la susceptibilidad genética de los individuos en función de su genotipo *PRNP* a padecer la enfermedad dependiendo de las cepas de priones o la naturaleza misma de estas cepas.

El estudio de estos aspectos conlleva la utilización de modelos animales, generalmente murinos, que requieren una inversión importante en tiempo y dinero, con el agravante de utilizar especies que no son las susceptibles de forma natural a estas enfermedades. Cuando se utilizan modelos animales de especies susceptibles el tiempo requerido y la inversión es todavía mayor, al ser animales de mayor tamaño. Por ello se hace necesario el desarrollo de modelos celulares que nos permitan estudiar estos parámetros *in vitro*. Además, estos modelos se pueden utilizar tanto para el estudio de aspectos básicos de la enfermedad (Beranger *et al.*, 2001) como para testar posibles tratamientos (Ghaemmaghami *et al.*, 2007) o diagnosticar las distintas cepas de scrapie (Nishida *et al.*, 2000).

A pesar de que recientemente se han generado distintos métodos que permiten amplificar o multiplicar el prión *in vitro* sin necesidad de utilizar cultivos celulares, como la PMCA (Saborio *et al.*, 2001; Castilla *et al.*, 2005), estos métodos libres de células no permiten analizar el efecto de la infección del prión en la célula, ni son muchas veces viables en laboratorios de diagnóstico.

Los modelos celulares han permitido aumentar nuestro conocimiento sobre aspectos clave como la replicación del prión o sus efectos tóxicos a nivel celular (Hu & Huang, 2013; Krauss & Vorberg, 2013). En 1970 se consiguió la primera infección de cultivos celulares por el agente de scrapie (Clarke & Haig, 1970), incluso antes de que la PrP^{Sc} fuera identificada como un marcador de las EET y el posible agente causal. Sin embargo, la mayoría de líneas celulares que expresan PrP^{C} no pueden ser infectadas por el prión por razones desconocidas (Beranger *et al.*, 2001; Piccardo *et al.*, 2011). Sólo unas pocas líneas celulares han podido ser infectadas por priones y mostrar acumulación de PrP^{res} y/o infectividad. La mayoría de las líneas celulares utilizadas son de origen murino y su infección requiere la adaptación del agente infeccioso a esta especie. A continuación se describen los distintos modelos celulares empleados.

3.1. Líneas celulares neuronales murinas

Los primeros estudios se centraron en líneas de origen neuronal como son las líneas celulares de neuroblastoma (Race *et al.*, 1987) o células de Schwann (Follet *et al.*, 2002) o en líneas derivada de encéfalo de ratón (Birkett *et al.*, 2001). Gracias a las células de neuroblastoma se ha descubierto que el prión puede ser propagado *ex vivo* de forma indefinida. La propagación sucede entre la célula madre a las células hijas de forma que la división celular diluye la carga de partículas infecciosas de cada célula hija a la mitad (Ghaemmaghami *et al.*, 2007). El prión no se propaga únicamente a partir de células progenitoras, también existe transmisión horizontal que puede ocurrir por la secreción de priones al sobrenadante, mediante la participación de exosomas o nanotúbulos (Véase apartado **1.2**). Además, se han utilizado para estudiar los mecanismos de generación y tráfico de la PrP^{Sc} (Taraboulos *et al.*, 1992b), y para evaluar el potencial terapéutico de diferentes agentes (Caughey *et al.*, 1994; Caughey *et al.*, 1998).

Los modelos celulares murinos presentan varios problemas que el Dr. Solassol y colaboradores resumieron en:

- Limitada susceptibilidad a ciertas cepas de prión debido a la barrera de especie.
- Solo algunas células del cultivo se infectan.
- La propagación de priones induce cambios muy sutiles en el fenotipo de los cultivos infectados.

Existen modelos en los que se ha conseguido propagar el prión a pesar de la barrera de especie existente entre el cultivo y el inóculo. Estas líneas celulares generalmente son también de origen murino y propagan únicamente las cepas priónicas que han sido adaptadas experimentalmente a la infección en roedores (Solassol *et al.*, 2003).

Las dificultades encontradas en los modelos murinos se han intentado subsanar creando líneas celulares que expresen una PrP homóloga a la del inóculo (Vilette *et al.*, 2001). La creación de ratones transgénicos que expresan la PrP ovina, bovina o humana ha facilitado la creación de líneas celulares portadoras de PrP homólogas al inóculo (Laude *et al.*, 2002; Archer *et al.*, 2004). La creación de animales transgénicos es un método caro, laborioso y de larga duración. Además, en especies como la ovina las variantes genéticas tienen mucha influencia en la susceptibilidad de los individuos a las distintas cepas de scrapie clásico o atípico (Hunter, 1997; Benestad *et al.*, 2003). Esta metodología supondría la creación de tantos ratones como haplotipos descritos para el gen *PRNP* ovino.

3.2. Cultivos neuronales primarios

La obtención de células para este tipo de cultivos se realiza a partir de muestras de SNC de animales y requiere el sacrificio de los mismos. La infección de estos cultivos obtenidos a partir de neuronas de embriones o ratones y ratas neonatos ha demostrado ser un modelo de gran valor para el estudio de la patogénesis de virus neurotrópicos. En particular, para distinguir las lesiones causadas por el agente infeccioso o por la respuesta del huésped (Kaul *et al.*, 2001).

A diferencia de las líneas celulares neuronales murinas, donde no se detecta un efecto citopatológico ligado a la propagación del agente infeccioso de las EET a pesar de la infección persistente (Follet *et al.*, 2002), en células neuronales primarias infectadas se ha podido observar como la infección tanto de neuronas como de astrocitos condujo a una pérdida neuronal progresiva tras la propagación activa del prion (Cronier *et al.*, 2004). También ha permitido demostrar que los cultivos neuronales infectados son más sensibles al estrés oxidativo (Milhavet *et al.*, 2000), posiblemente ligado a la modificación de las funciones fisiológicas de la PrP^C (Véase apartado **1.3**).

Sin embargo, estos modelos están desarrollados en la especie murina y requieren de una adaptación previa del prión por ratón o de la creación de animales transgénicos que expresen la proteína prion celular de la especie origen del prion que sea el objeto de estudio (Cronier *et al.*, 2004), con las posibles variaciones sobre el agente original que ello conlleva. En especies como la humana, este tipo de cultivos son de difícil aplicación ya que requieren acceso a muestras de SNC del individuo y la infección empleando líneas celulares humanas con el agente de la CJD sólo se ha conseguido una vez (Ladogana *et al.*, 1995).

3.3. Cultivos de células troncales neuronales

Más recientemente, el Dr. Milhavet y colaboradores propusieron el uso de células troncales neuronales (**NSC**) para la propagación *in vitro* del prión (Milhavet *et al.*, 2006). Estas células pueden diferenciarse a distintos tipos celulares del SNC (Reynolds *et al.*, 1992) y pueden multiplicar la proteína prión a partir de muy poca dosis de homogeneizado de encéfalo infectado. Las NSC en cultivo forman neuroesferas que han podido ser infectadas con priones (Giri *et al.*, 2006; Herva *et al.*, 2010). Presentan una serie de ventajas como modelos celulares para la propagación del prión: constituyen un cultivo que se auto-renueva, se puede obtener de cerebro fetal de ratones normales o transgénicos con secuencias de PrP^C de distintos genotipos o especies en el caso de animales transgénicos; y las neuroesferas diferenciadas son más similares a los tipos celulares presentes en el encéfalo que las líneas tumorales. Las neuroesferas se diferencian a neuronas y a astrocitos, los cuales representan el principal tipo celular infectado por el prión *in vivo* (Herva *et al.*, 2010).

A pesar de lo prometedor de estas líneas, existen muy pocos trabajos que muestren el aislamiento de progenitores neuronales de especies de interés en los estudios sobre priones como son los rumiantes (Duittoz & Hevor, 2001) o la especie humana. Estas células se obtienen a partir de embriones, con los problemas éticos que ello supone en la especie humana (Schwartz *et al.*, 2003). Las células troncales también están presentes en el SNC adulto (Reynolds & Weiss, 1992), principalmente en el hipocampo y la zona ventricular (Lie *et al.*, 2004) pero también se ha descrito la presencia de estas células en la médula espinal (Danilov *et al.*, 2006). La inaccesibilidad de las fuentes de NSC en el encéfalo limita severamente la utilidad de las mismas. Más recientemente se han podido diferenciar a neuroesferas, mediante transdiferenciación, células madre mesenquimales (**MSC**) obtenidas a partir de tejido adiposo en humanos (Yang *et al.*, 2015), lo que puede facilitar su acceso para su uso en investigación.

3.4. Cultivos de células troncales mesenquimales

En los últimos años se han descrito dos bioensayos destinados a la multiplicación de distintas cepas priónicas utilizando células con características mesenquimales obtenidas a partir del estroma de bazo (Akimov *et al.*, 2008) y médula ósea de ratón (Akimov *et al.*, 2009). En ellos se han conseguido multiplicar con éxito una cepa de la vCJD y otra de Gerstmann-Sträussler-Scheinker adaptadas a ratón.

Se ha demostrado que se puede detectar la presencia de PrP^{res} en modelos murinos infectados experimentalmente y que la infección de las MSC *in vivo* podría preceder a la acumulación de PrP^{Sc} en el SNC (Takakura *et al.*, 2008). Para multiplicar el prión no parece necesaria la diferenciación de las MSC a células neurales, siendo posible la infección de MSC en líneas murinas (Cervenakova *et al.*, 2011).

La presencia de PrP patológica en las MSC obtenidas a partir de médula ósea tomadas en autopsias de pacientes fallecidos por CJD se ha propuesto como un método alternativo de diagnóstico precoz de la enfermedad (Takakura *et al.*, 2008). Estos autores proponen que las MSC de médula ósea expresarían PrP^{Sc} a largo plazo, con lo cual podrían ser un reservorio para los agentes de las EET. En ese caso, las células sanguíneas podrían infectarse al entrar en contacto con las MSC. Sin embargo, en la especie humana la presencia de infectividad en la médula ósea de pacientes con EET ha sido negativa en algunos estudios (Brown *et al.*, 1994), por lo tanto, la presencia de

prión en las MSC podría depender del tiempo en el que se tome la muestra durante el transcurso de la enfermedad. En la especie ovina se ha conseguido la detección de agregados de PrP en sangre circulante de animales afectados por scrapie (Bannach *et al.*, 2012) y se ha investigado la capacidad infectiva de las transfusiones de diferentes fracciones sanguíneas en ovino (Andreoletti *et al.*, 2012). Sin embargo, no se ha investigado hasta la fecha la presencia de prión en MSC de animales afectados por scrapie.

3.5 Cultivos celulares para el estudio de la toxicidad del prión

Tal y como se ha señalado en apartados anteriores, sólo unos pocos modelos celulares son capaces de replicar y propagar la proteína prión patológica. La mayoría de estos modelos no muestran signos de toxicidad una vez infectados con el prión. Por ejemplo, el empleo de cultivos celulares de neuronas inmortalizadas o de líneas de la neuroglia no parece ser efectivo en el estudio de los efectos de apoptosis de las EET *in vitro,* ya que no parecen mostrar signos de citotoxicidad (Solassol *et al.*, 2003). Sin embargo, las células de cultivos neuronales primarios y astrocitos sí serían apropiadas para el estudio de apoptosis neuronal (Cronier *et al.*, 2004). Estos cultivos muestran infectividad al ser expuestos al agente causante de scrapie, tanto ovino, como de cepas adaptadas a ratón, y mostrarían signos de degeneración neuronal.

Algunos de los cultivos que muestran signos de citotoxicidad han servido para comprender distintos procesos que dan lugar a la muerte neuronal en las enfermedades priónicas. Por ejemplo, el péptido PrP106-126 induce apoptosis neuronal directa en cultivos primarios neuronales de córtex de ratón mediante el aumento de la producción de ROS (Carimalo *et al.*, 2005). Se había comprobado el aumento en la producción de radicales de oxígeno como respuesta al péptido, pero requiriendo siempre la presencia de microglía (Brown *et al.*, 1996; Brown, 2001).

Gracias a cultivos de líneas neuronales diferenciadas a partir de neuroesferas se ha estudiado que la acumulación de $PrPr^{es}$ induce una temprana respuesta lisosomal de ROS que ocurre a la vez que el inóculo infeccioso se interna en la célula y se almacena en vesículas ácidas. Se trata de una respuesta adaptativa y rápidamente se reestablece el equilibrio reducción-oxidación. A pesar de esta aparente normalidad, la propagación de PrP^{Sc} continúa. Con el paso del tiempo esta respuesta de adaptación falla y se produce

daño perioxidativo en los componentes lipídicos que conducen a la activación de la muerte celular (Haigh *et al.*, 2011).

Los cultivos neuronales primarios también se han utilizado para comprobar que el efecto citotóxico depende tanto de los tipos celulares, como de la cepa priónica empleada. La propagación del prión cursa con una primera fase de replicación, seguida por una fase citotóxica. De hecho, la mayor neurotoxicidad parece ocurrir cuando la acumulación de PrP anormal alcanza una meseta (Hannaoui *et al.*, 2013).

Finalmente, los estudios *in vitro* que utilizan células infectadas han mostrado ser útiles a la hora de estudiar moléculas antipriónicas y antiapoptóticas (Carimalo *et al.*, 2005; Trevitt & Collinge, 2006). Así, conocer los procesos tempranos de apoptosis o citotoxicidad ocurridos durante el transcurso de las EET, cuando la apoptosis aun es reversible, podría dar lugar a nuevas dianas terapéuticas en la lucha contra la pérdida neuronal.

4 CÉLULAS MESENQUIMALES

Se define como célula madre a aquella célula no diferenciada procedente del embrión, feto o individuo adulto, que es capaz de auto-renovarse indefinidamente, o en el caso de las MSC, durante la vida del organismo y es capaz de diferenciarse en otros tipos celulares especializados de diferentes tejidos y órganos. Las células de origen embrionario son totipotenciales, pudiendo diferenciarse a cualquier tipo celular del organismo, y pueden auto-renovarse en cultivo por periodos indefinidos de tiempo, al contrario que las MSC adultas, que presentan una capacidad finita de proliferación *in vitro* y son multipotenciales, su capacidad de diferenciación se ve restringida en principio a sólo uno de los linajes celulares.

El Dr. Friedenstein y colaboradores describieron por primera vez en 1970 este tipo de células aisladas a partir de médula ósea de ratones y cobayas. Estas células que se adherían al plástico y eran capaces de formar colonias con una morfología de fibroblasto, por ello se las denominó **CFU-F** (*Colony-Forming Unit like Fibroblast*) (Friedenstein *et al.*, 1970). Posteriormente, se estudiaron las capacidades de las CFU-F

aisladas de la médula ósea respecto a su capacidad de proliferación, auto-renovación y diferenciación a los distintos linajes posibles (Castro-Malaspina *et al.*, 1980; Kaneko *et al.*, 1982). Las denominadas MSC son un tipo de células madre adultas indiferenciadas que se encuentran en los tejidos y órganos de individuos adultos. Poseen las capacidades de auto-renovación *in vitro* (Ksiazek, 2009) y de dar lugar a células diferenciadas del tejido en el que residen, además de a un número limitado de tipos celulares de otras capas embrionarias (Almalki & Agrawal, 2016).

La capacidad de diferenciación de las células madre adultas depende del origen embrionario de las mismas (Figura 3). Las MSC son consideradas células troncales multipotenciales, ya que son capaces de generar células maduras de su misma capa embrionaria. Al proceder de la capa embrionaria mesodermal, las MSC pueden diferenciarse en los tejidos que derivan de ella: adipocitos, mioblastos, osteoblastos, condrocitos y tenocitos.

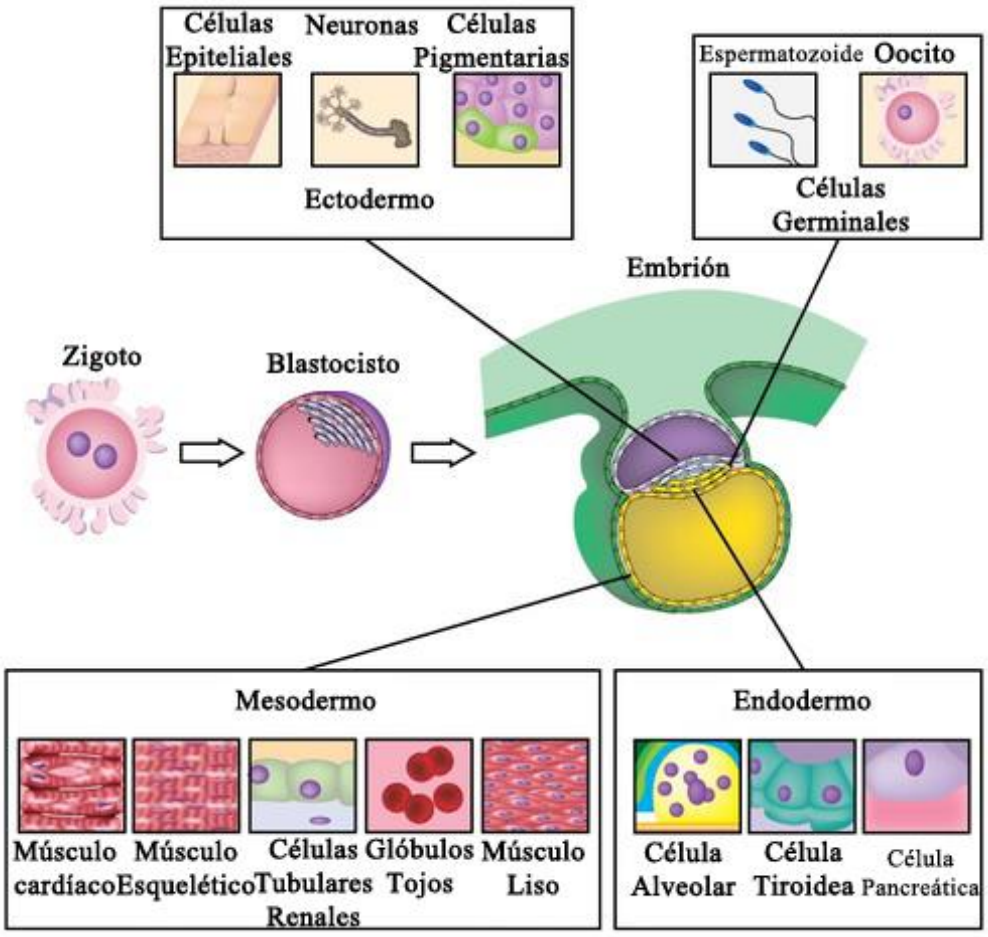

Figura 3. Diagrama de las capas embrionarias y células que derivan de cada una de ellas (Basado en Terese Winslow y Caitlin Duckwall, 2001).

4.1 Fuentes de obtención de MSC

Se han aislado células madre adultas de la mayoría de los tejidos vasculares (da Silva Meirelles *et al.*, 2006), aunque el tejido considerado como la mayor fuente de MSC es la médula ósea (Friedenstein *et al.*, 1970). Hasta la actualidad se han conseguido aislar MSC de numerosos tejidos adultos como son la sangre periférica (Bunnell *et al.*, 2008), pulmón (Zvaifler *et al.*, 2000), músculo (Lama *et al.*, 2007), líquido sinovial (Adachi *et al.*, 2002), hueso, cartílago, tendón (Bernard-Beaubois *et al.*, 1997), el tejido vascular (Galmiche *et al.*, 1993) y la pulpa dental (Sekiya *et al.*, 2011). En el caso de las MSC ovinas, existen trabajos previos que describen el aislamiento de MSC a partir de médula ósea (McCarty *et al.*, 2009; Rentsch *et al.*, 2010) o tejido adiposo (Niemeyer *et al.*, 2010).

4.2 Caracterización de las MSC

Existe disconformidad en cuanto a los marcadores de superficie que definen a las MSC frente a otros tipos celulares que son capaces de adherirse al plástico y que pueden encontrarse en algunos tejidos de los que se extraen las MSC. Así, la Sociedad Internacional de Terapia Celular determinó en 2006 unos criterios mínimos que debían cumplir las células aisladas a partir de médula ósea humana para poder ser consideradas MSC (Dominici *et al.*, 2006):

- Capacidad de adherencia al plástico en condiciones estándares de cultivo.
- Expresión de ciertos marcadores de superficie analizados mediante citometría de flujo: más de un 95% de **CD105** (endoglina), **CD73** (nucleotidasa 5' terminal) y **CD90** (Thy-1); y menos de un 2% de **CD45** (proteína tirosina fosfatasa), **CD34** (marcador de células precursoras hematopoyéticas), CD14 o CD11b, CD79α o CD19 y HLA-DR.
- Ser capaces de diferenciarse a células de linaje mesodérmico *in vitro*: osteoblasto, adipocito y condrocito.

Estos criterios son generalmente válidos para definir las MSC de otras especies distintas a la humana, aunque el criterio de los antígenos de superficie es, en ocasiones, difícil de determinar en alguna de ellas debido a la falta de especificidad de los

anticuerpos comerciales (Ranera *et al.*, 2011a). Para caracterizar las MSC de especies como el ovino se estudian una serie de parámetros relacionados con sus diferentes capacidades en cuanto a proliferación, expresión de marcadores de superficie y diferenciación a los linajes mesenquimales.

4.2.1 Capacidad de proliferación

Las MSC son un interesante modelo de estudio debido a la facilidad para establecer cultivos *in vitro* y realizar ensayos de proliferación con ellas (Bianco *et al.*, 2001). Estas células son capaces de expandirse en cultivo a un ritmo entre 20 y 50 doblajes en 10 semanas (Lee *et al.*, 2003). En el caso de la especie humana, el tiempo de doblaje normal para las MSC es de unos 4 días en los primeros pases, que aumenta a medida que van avanzando dichos pases en cultivo (Piccinato *et al.*, 2015). Cada especie tiene una tasa de crecimiento propia (Martinez-Lorenzo *et al.*, 2009), en la especie ovina se ha descrito una gran variabilidad en la proliferación de las MSC obtenidas de médula ósea entre individuos, independiente de su edad y concentración inicial de células mononucleadas obtenidas (Rhodes *et al.*, 2004). Esto indicaría que no se puede establecer una estimación predictiva del valor de un determinado individuo como donante a la hora de MSC para el desarrollo de un modelo de estudio o terapia.

4.2.2 Fenotipo

El reconocimiento e identificación de las MSC por su fenotipo específico en cuanto a la expresión de marcadores de superficie celular es importante para poder distinguirlas de otros tipos celulares que las acompañan y puedan contaminar el cultivo. No existe un marcador inequívoco para las MSC aisladas de diferentes tejidos de origen (Baksh *et al.*, 2004). Por ello, la forma de caracterizarlas es mediante la búsqueda combinada de la presencia de ciertos antígenos de superficie y la ausencia de otros característicos de las células hematopoyéticas o endoteliales (Tabla 1). La expresión de estos marcadores puede variar a lo largo del cultivo *in vitro* debido al enriquecimiento y homogeneización del mismo (Mitchell *et al.*, 2006). También puede variar según el tejido de origen de las MSC (Noel *et al.*, 2008).

Tabla 1. Principales marcadores de superficie analizados en MSC humanas de médula ósea. (Modificado de Deans & Moseley, 2000; Dominici *et al.*, 2006).

Tipo de marcador	Antígeno	Detección
Endoglina	CD105	Positiva
Nucleotidasa 5' terminal	CD73	Positiva
Thy-1	CD90	Positiva
Proteína tirosina fosfatasa	CD45	Negativa
Antígeno células precursoras hematopoyéticas	CD34	Negativa
Receptor LPS/ Mac 1	CD14/CD11b	Negativa
IGα asociada/ CVID3	CD79α/CD19	Negativa
Complejo mayor de histocompatibilidad	HLA-DR	Negativa
OTRAS MOLÉCULAS DE ADHESIÓN		
HCAM	CD44	Positiva
NCAM	CD56	Positiva
VCAM	CD106	Positiva
ALCAM	CD166	Positiva
PECAM-1	CD 31	Negativa
OTRAS INTEGRINAS		
VLA-α1	CD49a	Positiva
VLA-α2	CD49b	Positiva
VLA-α3	CD49c	Positiva
VLA-α4	CD49d	Negativa
VLA Subunidad β	CD29	Positiva

En el caso de *CD34* se ha visto que no presenta expresión en las células procedentes de la médula ósea humana, pero sí de forma leve en las de tejido adiposo (Noel *et al.*, 2008). Al contrario, con *CD106* se detecta expresión en células procedentes de médula ósea humana, pero no en las obtenidas de grasa (Kern *et al.*, 2006). En la especie ovina existen pocos trabajos que analicen el fenotipo para las MSC, sin embargo, en los últimos años se ha descrito la correspondencia entre los marcadores

empleados en la especie humana y su empleo para caracterizar las MSC ovinas, siendo positivas para CD44, CD105, CD29 y CD166, y negativas para CD34, CD45, CD14, CD106, CD31 y STRO1 (Mrugala *et al.*, 2008; McCarty *et al.*, 2009). En el caso de CD 106 y Stro-1 vemos que se dan resultados opuestos entre los criterios para la caracterización de MSC humanas y los resultados en la caracterización de MSC ovinas.

4.2.3 Potencial de diferenciación

Las MSC derivan de la capa mesodermal del embrión, por lo que poseen el potencial de diferenciación a osteocito, condrocito, adipocito, tenocito, células del estroma y mioblasto (Figura 4). En sus nichos, las MSC permanecen quiescentes en un estado G0/G1 del ciclo celular a la espera de un estímulo externo que les indique que deben comenzar a proliferar y diferenciarse posteriormente (Baksh *et al.*, 2004). *In vitro* es posible conseguir la diferenciación de las MSC a los tipos celulares derivados del mesodermo mediante la adición de medios de cultivo específicos con los inductores adecuados.

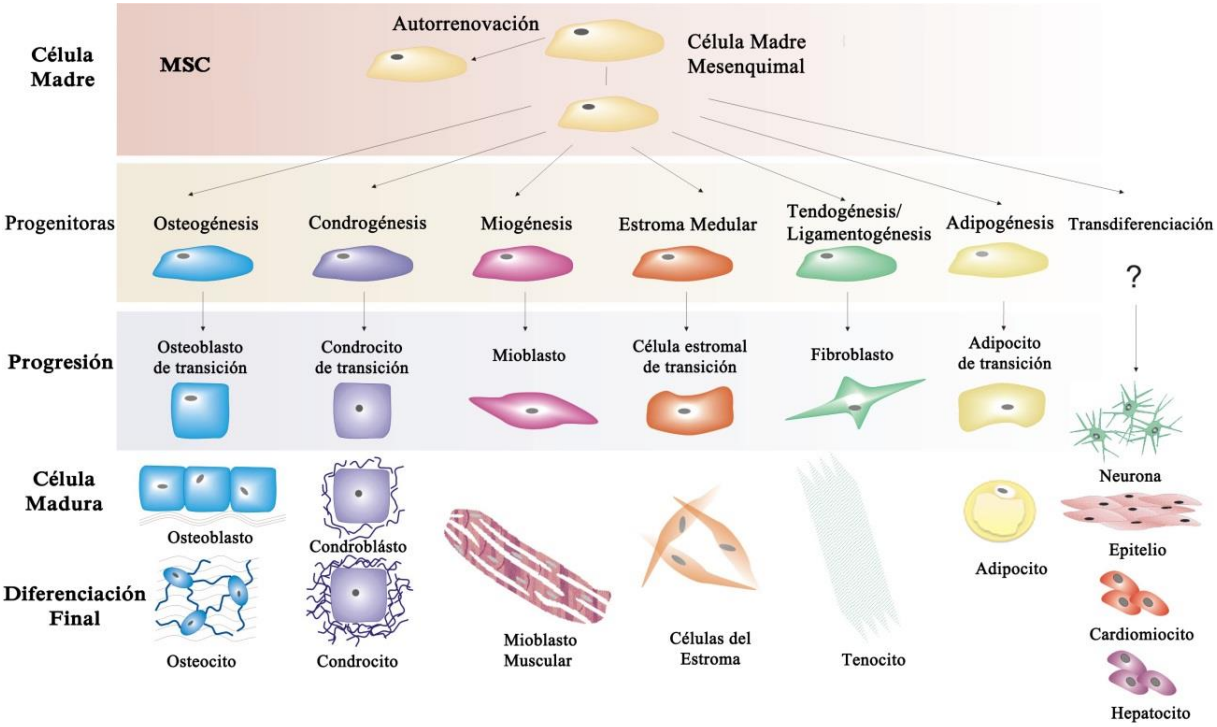

Figura 4. Potencial de diferenciación en diferentes linajes de las MSC (Basado en Caplan y Bruder, 2001).

4.2.3.1 Diferenciación condrogénica

La diferenciación condrogénica de las MSC se ha comprobado repetidas veces empleando moléculas como el factor de crecimiento transformante beta 3 (**TGF-β3**) (Barry, 2003). El TGF-β3 junto a la proteína morfogenética del hueso 6 (**BMP6**) son capaces de incrementar el depósito de matriz cartilaginosa (Sekiya *et al.*, 2001). La diferenciación a condrocitos se ha descrito en cultivos en monocapa (Jager *et al.*, 2006), aunque se han observado procesos de desdiferenciación o transdiferenciación a células con morfología de fibroblasto una vez que estas células en monocapa son puestas en suspensión (Haudenschild *et al.*, 2001). Generalmente la diferenciación condrogénica se realiza en cultivo en 3D, en pellet, ya que un entorno tridimensional junto con una situación de hipoxia favorecen esta diferenciación (Xu *et al.*, 2007).

La diferenciación condrogénica está regulada por varios factores de transcripción como la familia Sox (Ylostalo *et al.*, 2006). La *Sox5* y *Sox6* se expresan durante la diferenciación condrogénica, siendo *Sox9* el factor expresado en condrocitos diferenciados (Lefebvre *et al.*, 2001). La presencia de proteína morfogenética del hueso 2 (**BMP2**) produce la sobreexpresión de estos factores (Uusitalo *et al.*, 2001). La *Sox9* se considera la principal proteína reguladora de la diferenciación ya que regula la activación de otros genes como el agrecano, el colágeno tipo II (***COL2A1***) y la ***COMP*** (proteína oligomérica de la matriz del cartílago) (Ylostalo *et al.*, 2006; Augello & De Bari, 2010). Estos genes constituirán las principales proteínas de la matriz extracelular del cartílago (Hennig *et al.*, 2007). Para comprobar la diferenciación, además de la detección de la expresión de estos genes mediante reacción en cadena de la polimerasa (**PCR**) en tiempo real (**RT-PCR**) o *Western Blotting* (Marion & Mao, 2006), para observar la diferenciación a nivel microscópico es necesario realizar la tinción de los glucosaminoglicanos presentes en él mediante la tinción con *Alcian Blue* (Ranera *et al.*, 2012).

En el caso de la especie ovina se ha descrito que TGF-β3 induce la diferenciación condrogénica, además de poseer gran variabilidad individual en su potencial de diferenciación condrogénica (Mrugala *et al.*, 2008). Se ha utilizado la inoculación de MSC prediferenciadas a condrocito como tratamiento en modelos ovinos de lesiones articulares (Marquass *et al.*, 2011). Debido al tamaño y peso de la oveja adulta, así como las similitudes estructurales, bioquímicas, fisiológicas e inmunológicas,

hacen que sea un animal modelo para lesiones articulares y en pruebas de futuras aplicaciones clínicas (Pilichi *et al.*, 2014)

4.2.3.2 Diferenciación adipogénica

Para que las MSC entren en diferenciación adipogénica, se emplean normalmente cultivos en monocapa con medios complementados con inductores relacionados con ligandos de receptores de hormonas asociados con la adipogénesis, como son la dexametasona (ligando del receptor de glucocorticoides), la insulina, o el factor de transcripción del receptor gamma activado por el factor proliferador de peroxisomas (***PPARγ***) (Suva *et al.*, 2004; Zhang *et al.*, 2009). Estos inductores producen en las células alteraciones en la transcripción génica que conllevan un aumento de la expresión de factores de transcripción como *PPARγ* o el ***C/EBPα*** (proteína estimuladora ligante a CCAAT). PPARγ se expresa durante la diferenciación adipogénica y la supresión de este factor de transcripción inhibe la adipogénesis (Zhuang *et al.*, 2016).

Estos factores de transcripción desencadenan a su vez la activación de genes relacionados con la adipogénesis como ***aP2*** (proteína de unión específica a ácidos grasos), ***LPL*** (lipoproteina lipasa), ***GLUT4*** (transportador de glucosa tipo 4), perilipina (***PLIN1***), adiponectina (***ADIPOQ***) y adipsina (***CFD***) entre otros. Los factores de transcripción *PPARγ* y *GULT4*, junto con *LPL*, se consideran marcadores de diferenciación temprana y se expresan durante las primeras fases de diferenciación adipogénica (Sekiya *et al.*, 2004; Fernyhough *et al.*, 2007). El resto de marcadores se expresan en fases intermedias o tardías de la adipogénesis (Katz *et al.*, 1999; Kang *et al.*, 2005). La expresión de estos genes puede comprobarse mediante RT-PCR (Pittenger *et al.*, 1999)

La activación y expresión de estos genes provoca un cambio morfológico: las MSC cambian su forma típica de fibroblasto por una más redondeada, que acumula paulatinamente gotas lipídicas. Estas gotas lipídicas aumentan su tamaño a medida que acumulan más grasa y pueden ser observadas mediante la tinción con *Oil Red-O* (Marion & Mao, 2006).

Por último, la diferenciación de MSC en adipocitos y osteoblastos está regulada y balanceada (Gregory *et al.*, 2005). La osteogénesis y la adipogénesis son procesos íntimamente relacionados ya que si se produce uno de ellos, se mantiene inhibido el otro debido a la implicación de PPARγ (Akune *et al.*, 2004) La dexametasona puede inhibir o promover la diferenciación adipogénica dependiendo de la dosis: promueve la diferenciación osteogénica en dosis bajas, y sin embargo estimula la adipogénesis a dosis altas (Bruder *et al.*, 1997). En ovino se ha descrito la diferenciación a osteoblastos a partir de MSC obtenidas de médula ósea (Mrugala *et al.*, 2008) y sangre de cordón umbilical (Jager *et al.*, 2006).

4.2.3.3 Diferenciación osteogénica

La diferenciación osteogénica fue la primera identificada (Friedenstein, 1995) posiblemente debido a que las MSC procedentes de médula ósea se encuentren listas para diferenciarse a osteoblasto (Marion & Mao, 2006). El cultivo se realiza en monocapa y los reactivos añadidos a los medios son el ascorbato, dexametasona y β-glicerofosfato. Son sustancias que promueven el depósito de matriz ósea y la expresión de marcadores osteogénicos (Chung *et al.*, 1992; Jaiswal *et al.*, 1997; Alhadlaq & Mao, 2003). El ascorbato, por ejemplo, aumenta la expresión de la fosfatasa alcalina (marcador intermedio de diferenciación), de osteocalcina (***BGLAP***) y osteopontina (***SPP1***), marcadores tardíos de diferenciación (Beck *et al.*, 2000). La dexametasona puede inhibir o promover la diferenciación osteogénica, a dosis bajas promueve la diferenciación osteogénica (Bruder *et al.*, 1997), como se ha visto en el apartado anterior.

La diferenciación osteogénica de las MSC se comprueba gracias a una serie de cambios genéticos y una remodelación de la matriz extracelular con la aparición de nódulos y cristales minerales. El marcador de osteogénesis temprana es el factor de transcripción Runx2 que promueve la osteogénesis e inhibe la condrogénesis o adipogénesis (Komori, 2006). Runx2 provoca la expresión de marcadores tardíos de diferenciación como la *BGLAP*, *SPP1,* y la síntesis de colágeno tipo I (***COL1A1***) (Augello & De Bari, 2010). El cambio en la matriz extracelular es apreciable mediante microscopio óptico gracias a la presencia de componentes de calcio y fosfato mediante tinción de *Alizarin Red S*. Esta observación es útil para la caracterización, pero no

suficiente por sí sola, se necesita la confirmación mediante RT-PCR de la expresión del perfil de expresión de los genes de diferenciación (Frank *et al.*, 2002).

Como se ha definido con anterioridad, el ovino es un animal modelo ampliamente utilizado para el estudio de la aplicación de terapia celular en el tratamiento de lesiones del aparato locomotor (Feitosa *et al.*, 2010; Zannettino *et al.*, 2010; Marquass *et al.*, 2011). Las MSC ovinas obtenidas de tejido adiposo y médula ósea presentan esta capacidad de diferenciación a tejido óseo (Niemeyer *et al.*, 2010).

4.3 Plasticidad de las MSC: diferenciación neuronal

Las MSC son capaces, en ciertas condiciones, de diferenciarse en otros tipos celulares además de adipocitos, condrocitos y osteoblastos, como son miocitos (Rowlands *et al.*, 2008) o cardiomiocitos en co-cultivo con células neonatales (Ramkisoensing *et al.*, 2011). Además, las MSC son capaces de diferenciarse en células que no proceden del mesodermo, como son las células neurales y del endotelio (ectodermo) (Deng *et al.*, 2006; Ramkisoensing *et al.*, 2011) y las células hepáticas (endodermo) (Karnieli *et al.*, 2007), capacidad que se conoce como plasticidad. Asimismo, parece que la plasticidad de las células troncales es más extensa de lo que se creía en un principio, la diferenciación hacia osteocito, condrocito u adipocito podría no ser definitiva, dándose procesos de transdiferenciación de un tipo celular a otro entre células totalmente diferenciadas (Song & Tuan, 2004).

El scrapie ovino ejerce sus efectos patológicos en el SNC, por ello nos centramos en el potencial de las MSC de transdiferenciación a neurona. Para la diferenciación a neuronas *in vitro* se han empleado medios enriquecidos con mercaptoetanol, alfa-MEM o ácido retinoico y medios con dimetilsulfóxido (**DMSO**) junto a beta-hidroxianisol (**BHA**). Estos factores inducen cambios en las MSC y se vuelven inmunorreactivas al antígeno nuclear neuronal (***NEUN***), la enolasa específica de neurona (***NSE***), nestina ***(NES)*** y la proteína ácida fibrilar glial (***GFAP***). Sin embargo, se ha observado una gran variabilidad en la expresión de marcadores neuronales en las MSC transdiferenciadas a neurona (Montzka *et al.*, 2009).

También se observan cambios morfológicos en los cultivos diferenciados como son la retracción del citoplasma, cuerpos celulares esféricos, protrusiones celulares y

una detención de la proliferación. Las células se muestran al microscopio con elongaciones ordenadas en dos o tres polos y con formaciones ramificadas en sus extremos (Ryu *et al.*, 2012).

Además de estos marcadores, y cambios morfológicos, estudiando sus capacidades electrofísicas se ha visto que expresan dos rectificadores tardíos de canales de K^+ (eag1 y eag2), los cuales son fundamentales para restaurar el potencial negativo requerido por la neurona para ejercer su actividad (Mareschi *et al.*, 2006). A pesar de que las MSC se han podido diferenciar a elementos neuronales *in vitro* (Sanchez-Ramos *et al.*, 2000; Deng *et al.*, 2001; Woodbury *et al.*, 2002), su capacidad para diferenciarse en neuronas funcionales y células de la glía sigue siendo fuente de controversia (Hardy *et al.*, 2008).

4.4 Potencial terapéutico en enfermedades del SNC

Las MSC se han propuesto como terapia potencial frente a lesiones del SNC ya que colaboran en procesos de regeneración axonal (Mantovani *et al.*, 2012), expresan marcadores neuronales y presentan un efecto antiinflamatorio (Ryu *et al.*, 2012). Además, las MSC secretan factores neurotróficos que podrían prevenir la degradación neuronal, además de promover la neurogénesis de las MSC del SNC (Munoz *et al.*, 2005). Las MSC parecen migrar a zonas dañadas del SNC y mejoran la recuperación tras un proceso isquémico (Savitz *et al.*, 2003; Wan *et al.*, 2014) y se han propuesto como terapia en lesiones medulares y de nervios periféricos(Naghdi *et al.*, 2009; Casanas *et al.*, 2014).

En el caso de enfermedades neurodegenerativas, se ha experimentado con el trasplante de MSC por vía intracraneal, intravenosa o intraperitoneal (Colpo *et al.*, 2015). El uso de MSC parece mejorar la función de las motoneuronas en la enfermedad de Parkinson estudiada en modelos animales (Paul & Anisimov, 2013). Del mismo modo, las MSC mejoran la función motora, supervivencia neuronal y reduce la neuroinflamación en modelos murinos de esclerosis lateral amiotrófica (Vercelli *et al.*, 2008).

Hasta el momento son limitados los estudios que han analizado el potencial terapéutico de estas células en enfermedades priónicas. Sin embargo, se ha descrito el

efecto beneficioso del trasplante vía intracraneal e intravenosa de MSC humanas en ratones infectados por el scrapie. Los animales tratados con MSC aumentaron su periodo de supervivencia, a pesar de que no se detuvo el progreso de la enfermedad (Song *et al.*, 2009). Las quimiocinas responsables de la migración de las MSC hacia áreas lesionadas del SNC tras la infección con priones han sido también descritas por el mismo grupo (Song *et al.*, 2011).

La capacidad de diferenciación de las MSC adultas a células del SNC y su potencial terapéutico frente a lesiones y procesos de degeneración del SNC las convierte en modelos celulares candidatos al estudio de las enfermedades priónicas. Asimismo, presentan la ventaja añadida de la facilidad de obtención de las MSC a partir de médula ósea u otros tejidos de fácil acceso en individuos adultos, evitando la necesidad de tener que recurrir al asilamiento de células troncales neuronales a partir de tejidos embrionarios.

OBJETIVOS

A pesar de la capacidad de las MSC murinas para multiplicar la infección por priones *in vitro* y la propuesta como terapia celular en las patologías humanas, las MSC no se han estudiado en profundidad en las especies que son naturalmente susceptibles a las enfermedades priónicas. El objetivo general de este trabajo de tesis doctoral es el estudio y caracterización de MSC en ovino control y afectado con scrapie y el estudio de su uso potencial como modelo *in vitro* para la multiplicación del prión y como método alternativo de diagnóstico de scrapie *in vivo*. El desarrollo de este estudio en ovino, además de su interés en el ámbito de la Medicina Veterinaria, supone un punto de partida para el desarrollo de modelos celulares humanos, ya que estas células se pueden obtener fácilmente de individuos sanos y/o enfermos. Para la consecución de este objetivo general se han planteado los siguientes objetivos específicos:

- Aislar y caracterizar las células mesenquimales ovinas procedentes de sangre periférica de ovino sano, incluido su potencial neurogénico.

- Determinar el posible efecto de la enfermedad en las características de las células mesenquimales procedentes de médula ósea y sangre periférica de ovino con scrapie.

- Analizar la presencia de prión en las MSC obtenidas de ovino con scrapie para determinar su potencial diagnóstico *in vivo*.

- Estudiar la capacidad de las MSC ovinas para multiplicar la infectividad del prion *in vitro*.

- Analizar los efectos de la infección con priones en la viabilidad de las MSC *in vitro* y estudiar el posible efecto tóxico de la infección.

MATERIAL Y MÉTODOS

En este apartado se detallan los materiales y métodos utilizados en este trabajo. Se hará una breve descripción de la metodología seguida en el trabajo de la presente memoria y se indicará, ya que los resultados son presentados en forma de manuscritos, el objetivo de cada apartado y el artículo en que aparecen.

1 CULTIVO DE MSC

El manejo de las células se realizó en condiciones de esterilidad haciendo uso de una cabina de flujo laminar y en laboratorio de bioseguridad P3 del Centro de Investigación en Encefalopatías y Enfermedades Transmisibles Emergentes (**CIEETE**) debido a las características de las células o a los inóculos utilizados. A continuación se explican brevemente los diferentes pasos requeridos para el cultivo celular.

1.1 Obtención de muestras y aislamiento de MSC ovinas

Para llevar a cabo este trabajo se obtuvieron muestras a partir de ovinos procedentes del rebaño experimental del CIEETE, cuyos animales proceden de rebaños regionales afectados por la enfermedad de scrapie.

Declaración Ética

El cuidado y uso de los animales se realizó de acuerdo con la Política Española de Protección Animal RD1201/05, que cumple con la directiva de la Unión Europea 86/609 relativa a la protección de los animales utilizados para experimentación y otros fines científicos.

Todos procedimientos de uso y toma de muestras de los animales empleados durante los experimentos están aprobados por el Comité de Ético de Experimentación Animal de la Universidad de Zaragoza. (PI06/12).

- **Animales:** Durante la realización de este trabajo se han obtenido muestras biológicas de un total de 30 ovejas, con edades comprendidas entre 1 y 6 años,

de raza Rasa Aragonesa y distintos genotipos para el gen *PRNP*. El uso y toma de muestras de estos animales fue aprobado por la comisión ética de la Universidad de Zaragoza (PI38/10). Se obtuvo médula ósea y la sangre periférica de 10 animales sanos y 20 animales afectados por scrapie.

- **Aspirados de médula ósea de cabeza humeral:** Las intervenciones se realizaron bajo neuroleptoanalgesia (sedación profunda con xilacina intravenosa y anestesia local en la zona de punción). Para minimizar el sufrimiento y estrés innecesario de los animales, los procedimientos se realizaron en las instalaciones donde los animales se encuentran estabulados habitualmente. Cuando la neuroleptoanalgesia surtió efecto, el animal se colocó sobre una mesa móvil en decúbito lateral, en la que se preparó asépticamente la zona de la punción.

  El lugar escogido para la extracción de médula ósea fue la cabeza humeral, siendo el punto de entrada el lado cráneo-lateral del húmero proximal, distal al tubérculo mayor. Se utilizó una aguja de 13G Jamshidi (Care Fusion), por lo que no fue necesaria incisión previa ni sutura posterior. En ningún caso fue necesario revertir la sedación con atipamezol intravenoso. El volumen aspirado de médula ósea se recolectó en una jeringuilla precargada con heparina y varió entre 3 y 15 ml. Éste se homogeneizó y las células mononucleares se aislaron por centrifugación en gradiente, utilizando Lymphoprep (Atom), a 400 g sin aceleración ni deceleración. Las células fueron lavadas dos veces con tampón fosfato salino **(PBS)** (Gibco). Para el contaje de células mononucleadas se eliminaron los glóbulos rojos utilizando Red Blood Cell Lysis Buffer (SIGMA) y fueron sembradas a una densidad de 2.000.000 de células mononucleadas/cm^2.

- **Obtención de sangre procedente de vena yugular:** Durante la extracción de sangre no fue necesaria la anestesia ni sedación, ya que la extracción de sangre es un procedimiento rutinario y no doloroso. El lugar escogido fue la vena yugular, y se obtuvieron unos 25 ml de sangre periférica por animal, recolectando la muestra en tubos heparinizados (Becton Dickinson). La sangre fue diluida en un volumen 1:1 de PBS y posteriormente las células mononucleares se aislaron por centrifugación en gradiente de Lymphoprep (Atom) a 400 g sin aceleración ni deceleración. Las células fueron lavadas con

PBS dos veces y sembradas a una densidad de 2.000.000 de células mononucleadas/cm^2.

Las células de ambos orígenes se mantuvieron en medio de crecimiento, compuesto por DMEM bajo en glucosa (Sigma-Aldrich), suplementado con 1% de L-glutamina (Sigma-Aldrich), 1% de penicilina/estreptomicina (Sigma-Aldrich) y según su origen, se suplementó con un 10% de suero fetal bovino (**FBS**) (Gibco) (células procedentes de médula ósea), y con un 20% de FBS (células obtenidas a partir de sangre periférica). Después de la extracción, a las 24, 48 y 72 horas, las células se lavaron con PBS y el medio se cambió para eliminar las células no adherentes. Las MSC se mantuvieron en condiciones de 37ºC y 5% de CO_2 para todos los procesos realizados.

1.2 Expansión y criopreservación de MSC

Las MSC de ambos orígenes se cultivaron realizando cambios de medio dos veces por semana, hasta alcanzar aproximadamente un 80% de confluencia. Posteriormente, las células se separaron de la superficie de la placa de cultivo por tratamiento con 0,25% tripsina/EDTA (Sigma-Aldrich) y se contaron empleando la cámara de Neubauer. Una fracción de células fue resembrada a una densidad de 5.000 MSC/cm^2, constituyendo un nuevo pase. Las células se expandieron hasta pase 3, y posteriormente todas las células fueron criopreservadas y mantenidas en nitrógeno líquido para la realización de experimentos posteriores. El medio de congelación en el que las células fueron resuspendidas estaba compuesto por un 90% de FBS y 10% de DMSO.

1.3 Caracterización de las MSC

Antes de estudiar su posible aplicación como modelos celulares para el estudio de scrapie, fue necesario comprobar que las células aisladas eran MSC. (Manuscritos II y III) Para ello se caracterizaron mediante el análisis de su capacidad de proliferación y expresión de los marcadores de superficie e inducción de las MSC hacia los linajes mesodérmicos utilizando diferentes condiciones de cultivo en función de los

requerimientos específicos de cada una de ellas. La caracterización en cuanto a potencial de diferenciación ha sido más exhaustiva para las MSC derivadas de sangre periférica, ya que este trabajo es el primero que describe el aislamiento de este tipo de células. Así, en las **PB-MSC** (Células mesenquimales de sangre periférica) se ha analizado la cinética de diferenciaciones mediante marcadores moleculares, mientras que en **BM-MSC** (Células mesenquimales de médula ósea) sólo se han estudiado en el punto final.

1.3.1 Capacidad de proliferación

La capacidad de proliferación se evaluó estimando los parámetros del doblaje celular (**CD**) y del tiempo de doblaje (**DT**). Para ello se contaron las células tras el aislamiento (pase 0) hasta el pase en que se congelaron todas. El contaje se realizó en cámara de Neubauer. Los valores de CD y DT fueron calculados empleando las fórmulas:

$$CD = ln\frac{\frac{Ni}{Nf}}{ln\,(2)} \qquad\qquad DT = \frac{CT}{CD}$$

Siendo *CD* el doblaje celular, *Ni* el número inicial de células en un determinado pase, *Nf* el número de células al final del pase, *DT* el tiempo de doblaje y *CT* el tiempo de cultivo en días.

1.3.2 Expresión de marcadores de superficie

El estudio basado en la reacción en cadena de la polimerasa cuantitativa o en tiempo real (**RT-qPCR**) se ha usado para cuantificar los niveles de expresión del ácido ribonucleico mensajero (**mRNA**) para seis genes de antígenos de superficie. Se han analizado los marcadores mesenquimales *CD29*, *CD73*, *CD90 y CD105* y los marcadores hematopoyéticos *CD34* y *CD45*. La metodología más detallada para la realización de este análisis se describe en el **apartado 2**.

Además, se comprobó la expresión de estos marcadores a nivel de proteínas mediante citometría de flujo. Debido a que no se disponía de anticuerpos diseñados específicamente para ovino se probó la reactividad de un panel de anticuerpos diseñados para reconocer epítopos de marcadores de MSC humanas frente a BM-MSC y PB-MSC ovinas. Se analizaron los marcadores mesenquimales CD19, CD36, CD49d, CD73, CD90, CD105 y CD166 y los marcadores hematopoyéticos CD34 y CD45. La metodología y anticuerpos han sido descritos previamente por nuestro grupo de trabajo (Ranera *et al.*, 2011a). La inmunoreactividad se determinó mediante citometría de flujo en el servicio de Citómica de los Servicios de Apoyo a la Investigación de la Universidad de Zaragoza/CIBA (citómetro FACSARIA, BD Biosciences). Previamente se comprobó la viabilidad celular mediante SYTOX® *Blue Dead Cell Stain* (Molecular ProbesTM). Esta metodología se llevó a cabo en el trabajo descrito en el Manuscrito III.

1.3.3 Capacidad de diferenciación a linajes mesodérmicos

Las diferenciaciones de PB-MSC y BM-MSC a linajes mesodérmicos se realizaron por duplicado empleando medios específicos que se describen a continuación. Para todas las diferenciaciones se realizaron controles de MSC de ambos orígenes en las mismas condiciones de siembra y mantenidas en el medio de crecimiento de expansión descrito en **apartado 1.1**.

Diferenciación adipogénica

Las PB-MSC y las BM-MSC se sembraron a una densidad de 5.000 MSC/cm^2 en placas de 24 pocillos y se mantuvieron durante 14 días en medio adipogénico. El medio de diferenciación estaba compuesto por medio de crecimiento suplementado con 1 µM de dexametasona, 500 µM de IBMX, 200 µM de indometacina y 15% de suero de conejo (Ranera *et al.*, 2012) y fue renovado dos veces por semana. Los lípidos intracelulares formados en el interior de las MSC diferenciadas fueron visualizados a los días 7 y 14 de cultivo. Para ello los cultivos se fijaron con formalina al 10% (Sigma-Aldrich) durante 15 minutos y se realizó una tinción con *Oil Red-O* 0,3% (Sigma-Aldrich), que tiñe de rojo los ácidos grasos. Se estudió la expresión mediante RT-qPCR de los marcadores adipogénicos: receptor gamma activado por el factor proliferador de

peroxisomas (*PPARγ*), esteaoril-CoenzimaA desaturasa (***SCD)***, e interleuquina 6 ***(IL-6)***.

Diferenciación osteogénica

Las células se sembraron a una densidad de 20.000 MSC/cm^2 en placas de 24 pocillos y se cultivaron durante 28 días, según protocolos empleados para otras especies (Ranera *et al.*, 2011b). El medio de inducción osteogénica estaba formado por medio de crecimiento suplementado con 10 nM de dexametasona, 10 mM de β-glicerofosfato y 100 μM de ascorbato-2-fosfato y fue renovado dos veces por semana. Para la valoración de la osteogénesis, las células se fijaron con etanol frío al 70% los días 7, 14 y 21 de la diferenciación y se incubaron durante 10 minutos con *Alizarin Red S* 2% (Sigma–Aldrich) y pH 4,6. Esta molécula presenta afinidad por el calcio depositado en la matriz extracelular generada por las células diferenciadas. Además se estudió la expresión de los marcadores osteogénicos *COL1A1* (colágeno tipo 1) y *BGLAP* (osteocalcina) mediante RT-qPCR.

Diferenciación condrogénica

La diferenciación condrogénica se realizó en cultivos en monocapa sembrados en una placa de 24 pocillos. Las células se sembraron a una densidad de 100,000 MSC/cm^2 y se mantuvieron durante 21 a 28 días en cultivo con medio condrogénico. Éste estaba compuesto por DMEM bajo en glucosa (Gibco) suplementado con 10% de FBS, 1,8 ng/ml TGF-β3 (R&D Systems), un 10% de ITS + Universal Culture Suplement Premix (BD), 56 μg/ml de ascorbato-2-fosfato y 0,1 μM de dexametasona (Jager *et al.*, 2006). El medio se renovó dos veces por semana. Las células se fijaron los días 14 y 21 con un 10% formalina (Sigma-Aldrich) durante 10 minutos y los cultivos se tiñeron con *Alcian Blue* 1% (Sigma-Aldrich) diluido en ácido clorhídrico 0,1 M, a pH final de 1, para revelar los glucosaminoglicanos depositados en la matriz extracelular. A los 14 y 21 días de cultivo se midieron los marcadores colágeno tipo 2 (*COL2A1*), biglicano ***(BGN)*** y lumicano (***LUM)*** mediante RT-qPCR en las PB-MSC y el día 21 en las BM-MSC.

1.4 Capacidad de transdiferenciación neurogénica

Se probó la plasticidad de las MSC induciendo su diferenciación neurogénica. Esta prueba se llevó a cabo durante la realización de los Manuscritos II y III. Las células se sembraron a una densidad de 5.000 MSC/ cm^2. El medio de diferenciación empleado fue el Medio Neurogénico HyClone (Thermo Scientific) que se renovó dos veces por semana. La diferenciación se comprobó mediante la observación directa al microscopio los días 3 y 6, y mediante el análisis por RT-qPCR en ambos tipos celulares de los marcadores neurogénicos: proteína 2 asociada a microtúbulos (***MAP2)***, factor hormonal liberador de hormonas leutinizantes embriónico nasal ***(NELF),*** nestina (*NES*), polipéptido medio de neurofilamento (***NEFM)*** y tubulina beta clase 3 (***TUBB3)***. También se analizó la expresión de *PRNP*.

2 ANÁLISIS DE LA EXPRESIÓN GÉNICA

Para realizar el análisis de la expresión génica de los distintos marcadores en todos los manuscritos fueron necesarios los procedimientos que a continuación se describen.

2.1 Extracción de RNA y síntesis de DNA complementario

La extracción de RNA y la síntesis de ácido desoxirribonucleico complementario (cDNA) se realizaron como paso previo al análisis de expresión de los distintos marcadores. La metodología para la extracción y síntesis de cDNA fue la siguiente:

- Para cultivos con más de 500.000 células, la extracción de RNA se llevó a cabo con el *kit* comercial RNA spin mini (GE Healthcare) y la posible contaminación con DNA genómico se eliminó mediante tratamiento con DNAse Turbo (Ambion) de acuerdo con las instrucciones de los fabricantes. La síntesis de cDNA se llevó a cabo utilizando el *kit* SuperScript First-Strand Sintesis System

(Invitrogen), siguiendo las recomendaciones de uso. Los extractos de cDNA se diluyeron 1:5 en agua ultrapura para su posterior análisis mediante RT-qPCR.

- Para cultivos sobre placas de 24 pocillos, la extracción de RNA y síntesis de cDNA se realizaron utilizando el *kit* comercial Cell to cDNA II (Ambion) de acuerdo con las instrucciones del fabricante. Los extractos de cDNA se diluyeron 1:5 en agua ultrapura para su posterior análisis mediante RT-qPCR.

2.2 Reacción en cadena de la polimerasa

La PCR cuantitativa o en tiempo real (RT-qPCR) permitió estudiar y comparar la expresión de marcadores de superficie celular en los cultivos para comprobar su naturaleza, así como comparar la expresión de marcadores después de las diferenciaciones con los observados en los cultivos control.

2.2.1 Diseño y optimización de cebadores

Para la realización de este trabajo se han utilizado algunos cebadores diseñados previamente por nuestro grupo de trabajo (Lyahyai *et al.*, 2010) y nuevos cebadores diseñados específicamente para este estudio empleando bases de datos específicas como *GeneBank* y software para la creación de cebadores como BLAST® (*Basic Local Alignment Search Tool*) y Primer Express 2.0 (Applied Biosystems). Las secuencias de los cebadores utilizados se describen en los correspondientes manuscritos.

2.2.2 PCR convencional

Una vez diseñados los cebadores su especificidad tuvo que ser validada mediante PCR convencional y posterior electroforesis en gel de agarosa al 2% para comprobar que se producía la amplificación de un único fragmento.

2.2.3 RT-qPCR

Todas las reacciones se realizaron por triplicado empleando 2 µl de cDNA diluido 1:5 en agua ultrapura y 0,15 µl de cada cebador (20 pM), junto a 2,5 µl de agua purificada y 5 µl de Fast SYBR® Green Master Mix (Life Technologies) en un volumen total de 10 µl.

El método de cuantificación utilizado ha sido el comparativo o ΔΔCt (Pfaffl, 2004). Los valores obtenidos de la amplificación del cDNA se normalizan empleando genes normalizadores. Los genes normalizadores (*housekeeping genes*) empleados en el estudio fueron Glucosa-6-Fosfato Deshidrogenasa (***G6PDH***) e Hipoxantina-Guanina Fosforribosiltransferasa (***HPRT***). El factor de normalización al que referir la expresión de los genes de interés en cada muestra se calculó como la media geométrica de las dos medidas de expresión de *G6PDH* y *HPRT*. Una vez normalizados los valores se calculan las diferencias entre los Ct de las muestras y los controles o **ΔCt** (*Cycle threshold*, ciclo a partir del cual la fluorescencia supera el valor de fondo), y el resultado es el llamado ΔΔCt. El cálculo de *fold-change* en la expresión de un gen entre dos muestras equivale a 2^(-ΔΔCt).

Finalmente, se utilizó la prueba estadística *t* de Student o la prueba U de Mann-Whitney, en función de la normalidad de los datos, considerándose estadísticamente significativos los resultados cuya $p < 0.05$.

2.3 Análisis de expresión de PrP^C mediante Dot Blotting

La técnica de *Dot Blotting* se utilizó para comprobar la expresión de PrP^C en los cultivos celulares de MSC previamente a la infección. (Manuscrito III). Se utilizaron los reactivos del *kit* Prionics® -Check Western (Prionics). Se homogeneizaron alícuotas de 10^6 células, consevadas a -80ºC, en 500 µl de tampón de homogenización Prionics® -Check Western (Prionics). La concentración de proteína total fue cuantificada por el método del ácido Bicinconínico (BCA (Sigma Aldrich)). Posteriormente, el extracto celular se centrifugó a 10.000 g durante 60 minutos a 4ºC para precipitar las proteínas, se eliminó el sobrenadante y el precipitado se diluyó en agua ultrapura para obtener una concentración final de 25 µg/µl. Se depositó un total de 50 µg de proteína sobre una membrana de 0,2 µm Immun-Blot® PVDF (Bio-Rad), y, una vez adsorbida la muestra,

la membrana se bloqueó durante 1 hora a temperatura ambiente (**RT**) empleando leche desnatada en polvo resuspendida al 5% en TBST (Tampón Salino Tris con Tween 20 al 0.05 %). Las membranas se incubaron durante 1 hora a RT con el anticuerpo monoclonal de ratón anti-PrP IgG_1 6H4 (Prionics) diluido 1:5000 en tampón de bloqueo previamente descrito. Tras tres lavados con TBST, la membrana se incubó 1 hora a RT con el anticuerpo secundario de cabra anti-IgG de ratón conjugado con fosfatasa alcalina (Prionics), diluido 1:5000 en tampón de bloqueo. El revelado se realizó mediante el revelador CDP-Star® (Tropix), y las imágenes de quimioluminiscencia se obtuvieron mediante el revelador digital cuantificador Versa-Doc™ Imaging System Modelo 4000 (Bio-Rad).

3 INFECCIÓN DE LAS MSC

En este apartado se describe la metodología utilizada para la infección de las BM-MSC y las PB-MSC con extractos de encéfalos de ovino afectados por scrapie y la detección de la PrP^{Sc} en cultivos celulares (Manuscrito IV).

3.1 Inoculación con scrapie

Para probar la susceptibilidad a la infección de las MSC por aislados priónicos se utilizaron inóculos del encéfalo de animales sanos, como control negativo, y de animales enfermos. Las muestras obtenidas post-mortem se homogenizaron y diluyeron en una solución de suero salino fisiológico (Braun) en una proporción 1:10 (g/ml). Para evitar posibles contaminaciones en los cultivos, las muestras se sometieron a un tratamiento antibiótico con sulfato de estreptomicina (100 μg/ml) junto a bencil-penicilina (100 μg/ml). Además se sometió a la muestra a un tratamiento térmico a 70ºC durante 10 minutos. Antes de proceder a la inoculación se testaron las muestras mediante cultivo en agar sangre (5% sangre ovina), durante 5-7 días a 37ºC, y se confirmó la falta de crecimiento.

Las células fueron sembradas a una densidad de 5.000 MSC/cm^2, a continuación se mantuvieron 24 h en cultivo para permitir su adhesión a la placa, y se añadió el

inóculo al medio de cultivo al 1%. Se dejó el inóculo en contacto con las MSC durante 48 horas antes de renovar el medio de cultivo. Posteriormente el medio se renovó dos veces por semana. En cada pase las células se mantuvieron en cultivo hasta alcanzar aproximadamente el 80% de confluencia.

3.2 Determinación de PrP^{Sc} mediante *Western Blotting*

La técnica de *Western Blotting* se utilizó para confirmar la presencia de PrP^{Sc} en los cultivos de MSC. Las muestras se procesaron utilizando el *kit* de reactivos para la confirmación *in vitro* de muestras sospechosas positivas a EET, TeSeE™ Western Blot (Bio-Rad) siguiendo las instrucciones del fabricante. Brevemente, aproximadamente 800-1.000 µg de proteína total se sometieron al proceso de identificación de la proteína prión tras su digestión con proteinasa K. El marcador de talla empleado fue el MagicMark™ XP Western Standard (Invitrogen™) y el control de transferencia utilizado fue el Kaleidoscope™ Prestained Standards (Bio-Rad), siguiendo las instrucciones del fabricante. La electroforesis se realizó en gel al 12% de Bis-Tris poliacrilamida. Tras la migración, las proteínas se transfirieron a una membrana de nitrocelulosa de 0,20 µm (Bio-Rad). La presencia de PrP^{Sc} se determinó mediante el anticuerpo primario monoclonal del *kit* TeSeE™ Western Blot (Bio-Rad). Por último se utilizó el revelador Luminata Crescendo Western HRP (Merk Millipore). Las imágenes de quimioluminiscencia se obtuvieron con el lector Versa-Doc™ Imaging System Modelo 4000 (Bio-Rad).

3.3 PMCA

La PMCA es una replicación *in-vitro* que permite detectar la presencia de PrP^{Sc} y cuantificarla tras la realización de un *Western Blotting*. Los ensayos por PMCA se llevaron a cabo en los laboratorios del Dr. Castilla en el bioGUNE. Brevemente, 10^6 MSC de ambos orígenes fueron centrifugadas y resuspendidas en 120 mL de homogeneizado de cerebro de ratón transgénico Tg338 VRQ ovino. Se realizan cinco ciclos de 24 h de sonicación (S-700MPX; QSonica) e incubación a 37-38ºC, las muestras incluyen controles negativos sin MSC. Tras una digestión con proteinasa K las

muestras fueron analizadas mediante *Western Blotting* para comprobar la presencia o ausencia de PrP^{Sc}.

3.4 Estudio de marcadores involucrados en la regulación de apoptosis

Con el fin de determinar un posible efecto tóxico de la inoculación con extractos de encéfalos de ovino afectados por scrapie en los cultivos, se analizó la expresión de genes involucrados en la regulación de la apoptosis mediante RT-PCR (Manuscrito IV). La infección con inóculo de animales enfermos y sanos como control se realizó de la forma descrita anteriormente. Posteriormente los cultivos de BM-MSC obtenidos de ovejas sanas y afectadas por scrapie se mantuvieron durante una semana en cultivo. Los marcadores proapoptóticos seleccionados fueron *AIF* (factor inductor de apoptosis), *BAK* (antagonista homólogo Bcl-2/*Killer*1), *BAX* (proteína X asociada a Bcl-2), *BCL-2* (célula B CLL/Linfoma 2) y *FAS* (Receptor de muerte celular de superficie Fas). Los marcadores anitapoptóticos seleccionados fueron ***BCL2L1*** (Proteína 1 Reguladora de Apoptosis *Bcl-2-Like*) y *MCL1* (Proteína marcadora de leucemia celular mieloide inducida 1). Estos marcadores han sido descritos anteriormente por nuestro grupo de investigación (Lyahyai *et al.*, 2006; Lyahyai *et al.*, 2007; Serrano *et al.*, 2009). La metodología utilizada para la realización de la RT-qPCR es la descrita en el apartado **2.2.4**.

4 HERRAMIENTAS BIOINFORMÁTICAS

En el desarrollo de este trabajo se han utilizado diversos programas y bases de datos para la búsqueda de secuencias génicas específicas de oveja, diseño de cebadores para su uso en PCR, análisis de las amplificaciones y resultados. A continuación se detallan los programas y bases de datos utilizados en la presente memoria.

- Búsqueda y recuperación de datos en sistemas integrados de búsqueda y bases de datos: (http://www.ncbi.nlm.nih.gov/).

- Alineamiento y búsqueda de secuencias: BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).
- Diseño de cebadores: Primer Express Software 2.0 (Applied Biosystems).
- Análisis de productos amplificados de RT-qPCR: StepOne™ software (Applied Biosystems).
- Análisis estadísticos: SPSS statistics ver.15.0 (IBM)

RESULTADOS Y DISCUSIÓN

Como resultado del trabajo realizado durante la presente memoria de Tesis Doctoral se han generado varios manuscritos que están publicados o van a ser enviados para su publicación. Antes de presentar los artículos se hará una breve descripción de ellos.

En primer lugar se realizó una revisión bibliográfica del estado actual de los estudios *in vitro* para el estudio de scrapie y otras enfermedades priónicas. Se hizo especial hincapié en los estudios que utilizan células troncales mesenquimales para la multiplicación del prión o para la terapia en este tipo de enfermedades y otras enfermedades neurodegenerativas (**Manuscrito I)**.

Previamente a nuestro trabajo, distintos estudios habían caracterizado las MSC de médula ósea de ovino. Sin embargo, aunque la presencia de MSC en sangre periférica de humanos y distintas especies animales se había descrito con anterioridad, no existía ningún estudio de este tipo en ovino, especie objeto de nuestro trabajo. Al ser la sangre periférica un tejido fácilmente accesible, su uso como fuente potencial de MSC sería de gran utilidad tanto para nuestro estudio como para su posterior aplicación en la especie humana. Por tanto, nuestro siguiente paso fue establecer las condiciones de aislamiento, cultivo y caracterización de MSC de sangre periférica de ovino sano. Para ello se analizó su potencial de diferenciación a linajes mesodérmicos, la presencia de marcadores mesenquimales, su potencial neurogénico y la expresión del gen *PRNP*, características importantes para valorar su potencial como método *in vitro* de estudio de las EET (**Manuscrito II**).

Tal y como se ha comentado en apartados anteriores, las MSC se han propuesto como potencial tratamiento para las enfermedades priónicas. Antes de su uso en terapia, nos planteamos analizar el posible efecto de la enfermedad en las características mesenquimales de las MSC obtenidas de médula ósea y sangre periférica (**Manuscrito III**). Para ello analizamos la expresión de marcadores de superficie, la capacidad de proliferación y diferenciación a linajes mesodérmicos y el potencial neurogénico de las células procedentes de ovino con scrapie en fase clínica y las comparamos con las características de las MSC procedentes de ovino control. En este mismo trabajo, se confirmó la expresión a nivel proteico de PrP^C en las MSC obtenidas de animales sanos y con scrapie y se valoró si la transdiferenciación hacia linajes neuronales aumentaba

los niveles de expresión de la misma Finalmente, se analizó la presencia de proteína prión patológica en células mesenquimales procedentes de médula ósea y sangre circulante para valorar su potencial como método de diagnóstico.

Por último se llevó a cabo la infección de BM-MSC procedentes de ovino sano con extractos de encéfalo de ovinos afectados por scrapie clásico para comprobar si dichas células eran capaces de infectarse y así propagar la infección *in vitro* a lo largo de los pases en cultivo celular (**Manuscrito IV**). Asimismo, se analizó el potencial efecto tóxico de la infección por proteína priónica en estos cultivos celulares, analizando su capacidad de proliferación y la expresión de genes involucrados en la regulación del proceso apoptótico.

MANUSCRITO I

REVIEW

The Potential of Mesenchymal Stem Cell in Prion Research

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Impacts

- Research in transmissible spongiform encephalopathies is mainly developed in murine models, either *in vivo* or *in vitro*. It requires time to adapt natural prion strains to mouse. Development of models using cells from natural hosts could facilitate *in vitro* infection, prion strain typing and toxicity studies.
- Mesenchymal stem cells (MSCs) express PrP^C, can be infected by prions and can differentiate into neuronal-like cells. These characteristics can be

Keywords:
Mesenchymal stem cell; prion; *in vitro*; transmissible spongiform encephalopathies; scrapie

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I. Summary

Scrapie and bovine spongiform encephalopathy are fatal neurodegenerative diseases caused by the accumulation of a misfolded protein (PrP^{res}), the pathological form of the cellular prion protein (PrP^C). For the last decades, prion research has greatly progressed, but many questions need to be solved about prion replication mechanisms, cell toxicity, differences in genetic susceptibility, species barrier or the nature of prion strains. These studies can be developed in murine models of transmissible spongiform encephalopathies, although development of cell models for prion replication and sample titration could reduce economic and timing costs and also serve for basic research and treatment testing. Some murine cell lines can replicate scrapie strains previously adapted in mice and very few show the toxic effects of prion accumulation. Brain cell primary cultures can be more accurate models but are difficult to develop in naturally susceptible species like humans or domestic ruminants. Stem cells can be differentiated into neuron-like cells and be infected by prions. However, the use of embryo stem cells causes ethical problems in humans. Mesenchymal stem cells (MSCs) can be isolated from many adult tissues, including bone marrow, adipose tissue or even peripheral blood. These cells differentiate into neuronal cells, express PrP^C and can be infected by prions *in vitro*. In addition, in the last years, these cells are being used to develop therapies for many diseases, including neurodegenerative diseases. We review here the use of cell models in prion research with a special interest in the potential use of MSCs.

Introduction

Transmissible spongiform encephalopathies (TSE) or prion diseases are fatal neurodegenerative diseases that include Creutzfeldt-Jakob disease (CJD) in humans, the bovine spongiform encephalopathy (BSE) in cattle and scrapie (Prusiner, 1998) in sheep and goats. The animal TSE with the highest economic and health interest was the BSE. Described for the first time in United Kingdom in 1987 (Wells et al., 1987), there is not experimental evidence to show that variant CJD (vCJD) is caused by BSE prions; however, epidemiological, biochemical and neuropathological data strongly suggest the zoonotic nature of this disease (Will et al., 1996; Aguzzi and Calella, 2009). Conversely,

ovine scrapie was the first-known TSE; it was first described in 1732 in the UK and Germany and has spread worldwide. Nowadays, only two countries seem to be scrapie free, Australia and New Zealand (MacDiarmid, 1996). Scrapie is not considered as zoonosis, but it is one of the best-known TSE and might be used for basic research as a natural model of these diseases (Lyahyai et al., 2007; Serrano et al., 2009; Filali et al., 2011).

Prion diseases are related to other conformational disorders such as Alzheimer (AD), Parkinson's or Huntington's diseases, all of them consequence of specific protein misfolding (Aguzzi and Haass, 2003). Protein structural changes lead to the development of these diseases due to the toxic activity of the new isoform or to the loss of function of the normal protein (Soto, 2003). Abnormal proteins often accumulate in brain tissues as amyloid fibrils (Carrell and Lomas, 1997; Dobson, 1999; Soto, 2001). Prion diseases are characterized by the aggregation of PrP^{res}, the pathological form of the cellular prion protein PrP^{C} (Prusiner and DeArmond, 1994), mainly in the central nervous system (CNS) but also in the lymphoreticular system in scrapie and Creutzfeldt-Jakob disease variant (DeArmond and Prusiner, 1995; Prusiner, 1998).

PrP^{C} is a glycoprotein anchored to the cell membrane and is expressed in a large class of cells although its physiological function remains unknown in detail (Aguzzi et al., 2008). However, the central role of PrP^{C} and PrP^{res} in the TSEs has been demonstrated. Transgenic mice with the gene encoding a disrupted prion protein (*PRNP*) are resistant to prion infection, preventing the formation of PrP^{res} (Bueler et al., 1993). The transformation of PrP^{C} into PrP^{res} entails the conversion of alpha-helices into beta-sheet features (Pan et al., 1993). This structural change confers PrP^{res} resistance to physical and chemical agents. *In vivo* diagnosis of prion diseases is based on clinical aspects; however, the final confirmation must be done *post-mortem* with the detection of PrP^{res} in the CNS.

The *PRNP* gene shows a high degree of polymorphism in many species. In human and sheep, some *PRNP* variants have been associated with the outcome, susceptibility and lesion degree of TSEs (Tranulis, 2002). Those polymorphisms can affect the conversion of PrP^{C} into PrP^{res} (Bossers et al., 1997). The exact mechanisms involved in the genetic differences of susceptibility, incubation time or neuropathy remain elusive, but some mutations in specific regions may play a key role in the early stages of pathogenic conversion (Legname, 2012). In the last decade, breeding programs based on the selection against susceptible genotypes have been established in sheep (EU, 2003; Melchior et al., 2010) and, in a lesser degree, in goats (Goldmann et al., 2011). However, ovine genotypes resistant to classical scrapie have proved to be sensitive to the infection of atypical/nor98 scrapie strain (Saunders et al., 2006; Fediaevsky et al., 2010).

Individuals naturally affected by TSEs display different disease phenotypes, which are attributed to different prion strains (Jeffrey and Gonzalez, 2007; Benestad et al., 2008). In the absence of reliable technology to fully characterize the agent, classification of disease phenotype has been used as a strain-typing tool that can be applied to any host (Corda et al., 2012).

Prion strains were first defined by their incubation times in $Sinc^{s7}$ and $Sinc^{p7}$ (acronym for scrapie incubation) inbred mice, and the types and patterns of brain lesions induced at the terminal stage of disease in these mice (Dickinson and Meikle, 1971). These murine alleles were later associated with two variants (a and b) of the *Prnp* gene (Westaway et al., 1987).

Studies in mice have revealed considerable strain variation in the agents causing TSEs. TSE strains can keep their identity after propagation in different host species or *Prnp* genotypes, showing that these agents carry their own strain-specific information (Carp et al., 1989; Kimberlin et al., 1989; Bruce, 1993). In contrast, there are TSE isolates that change their properties when propagated in different hosts (Kimberlin et al., 1987). It is not known whether this information resides in specific self-perpetuating modifications of PrP, or whether a separate informational molecule is required (Bruce, 2003). The use of murine models has shown that the strain causing BSE in cattle has also infected domestic cats, exotic ungulates and vCJD human patients (Aldhous, 1990; Barria et al., 2014). In contrast, different TSE strains are associated with sporadic CJD and sheep scrapie (Bruce et al., 1994).

Biochemical analysis of the resultant PrP^{res} after mice infection complements the murine assays providing a correlation between prion strain phenotype and the molecular nature of different PrP conformers (Thackray et al., 2008). Murine infection for prion strain characterization requires the stabilization of the agent into the new host by means of serial passages in mice (Bruce, 2003). Such analysis can last many years due to prolonged incubation periods. However, prion strain characterization in mice could be shortened; a recent work demonstrates that the PrP^{res} patterns produced by one serial passage in wild type mice of bovine or ovine BSE were consistent, stable and showed minimal and predictable differences from mouse-stabilized reference strains. This biological property makes PrP^{res} deposition pattern mapping a powerful tool in the identification and definition of TSE strains on primary isolation (Corda et al., 2012).

Distinct prion strains may bear highly divergent risks of transmission to humans: Sheep scrapie-derived strains may be innocuous (Schneider et al., 2008), whereas BSE-derived strains appear to induce vCJD in humans (Aguzzi, 2008; Chen et al., 2013a). Also, two subtypes of sporadic CJD have been recently demonstrated to coexist in humans

II. (Polymenidou et al., 2005). Therefore, strain discrimina- tion is not only a curious academic enigma but is also cru- cial for prion diagnostics and public health (Aguzzi, 2008). Prion strain typing using *in vitro* cultured cells might be a tool that would make the process of characterization faster and cheaper than murine infections.

Although during the last decades prion research has greatly progressed, there are still many unknown questions about prion replication mechanisms, cell toxicity, differ- ences in genetic susceptibility related to prion strains or the nature of these prion strains. These studies are usually developed in murine models that require a great economic and time-consuming investment. In addition, these animals are not naturally susceptible to TSEs. When using suscepti- ble species, the time required is even greater (Kimberlin, 1990; Foster et al., 1993; Jeffrey et al., 2006). Consequently, it is necessary to develop *in vitro* models that would allow the study of these parameters. Although techniques like protein misfolding cyclic amplification (PMCA) allow the *in vitro* amplification and multiplication of the prion protein (Saborio et al., 2001; Castilla et al., 2005), these cell-free methods do not permit to study the effect of prion infection and are not often available in diagnostic laboratories. The combination of *in vitro* infectivity titration cell assays and PMCA for cell-free biochemical measurement of prion associated seeding activity could replace prion bioassays in rodents (Boerner et al., 2013). Moreover, development of *in vitro* models based on cell culture can be useful for basic research (Beranger et al., 2001), but also for the creation of possible therapies or prion strain diagnosis (Nishida et al., 2000).

Cell Models in Prion Diseases

Since the 1970s, *ex vivo* cell culture models for the detection and multiplication of prion strains have been developed, representing valuable tools for the study of TSEs. Strain-specific biological characteristics, including clinical manifestation, incubation period and pathological profiles remain unchanged after passages in cell cultures (Arima et al., 2005). Neuronal cultures were the first to be infected by the scrapie agent (Clarke and Haig, 1970). However, so far, only a few cell lines can be infected by prions and show accumulation of PrP^{res} and/or infectivity.

The first studies were focused on neuronal cell lines derived from neuroblastoma (Race et al., 1987; Butler et al., 1988), Shwann cells (Follet et al., 2002), the murine brain-derived line SMB (Birkett et al., 2001), or later from glioblastoma (Kikuchi et al., 2004). These cell lines showed persistent infection (Race et al., 1987; Schatzl et al., 1997) and have been useful to evaluate the therapeutic potential of antibodies against the prion protein (Enari et al., 2001; Kim et al., 2004; Perrier et al., 2004; Oboznaya et al., 2007; Wei et al., 2012) and drugs used for other diseases (Marella et al., 2002; Turnbull et al., 2003; Sandberg et al., 2004; Yung et al., 2004; Gayrard et al., 2005; Nunziante et al., 2005). *In vitro* studies have revealed that effective compounds against PrP^{res} from one species or strain cannot be assumed to be active against others (Kocisko et al., 2005).

Cell lines have also permitted to study the mechanisms of prion conversion, trafficking and cell-to-cell spread of prion infection (Taraboulos et al., 1992; Nishida et al., 2000; Atarashi et al., 2006; Paquet et al., 2007). A scrapie cell assay based on the infection of N2A sublines was described as sensitive as the mouse bioassay, 10 times faster, more than two orders less expensive and suitable for robotization (Klohn et al., 2003). Subcloning has been one of the strategies used to enhance the susceptibility of murine cell lines to prion infection because there is great variability within cell lines, where only a few cells get infected (Elleman, 1984; Solassol et al., 2003), which leads to a great heterogeneity with regard to prion susceptibility in tumour cell sublines (Bosque and Prusiner, 2000; Mahal et al., 2007). A study carried out in scrapie-infected neuroblastoma cells (ScN2a) evidenced that the highest levels of PrP^{res} were found in slow-growing cells and in retinoic acid treated cells, a compound associated with neuronal differentiation that increases the amount of PrP^{C} on non-infected cells (Bate et al., 2004). However, other studies have demonstrated that there is little correlation between total PrP^{C} expression and cell responsiveness to prion infection, suggesting that the differential expression of other auxiliary factors might play a role in rendering a cell susceptible to prion (Prusiner, 1991). Whether this variability is due to epigenetic differences or chromosomal alterations remains unknown (Mahal et al., 2007). These differences in susceptibility between and within lines have been used to determine prion strains (Ghaemmaghami et al., 2007; Mahal et al., 2007; Aguzzi, 2008; Oelschlegel et al., 2012).

Although most of the studies have been developed in cell lines of neural origin, susceptibility of a cell line to TSE infection cannot be predicted on the basis of its tissue origin or its level of PrP^{C} expression. A common fibroblast cell line (Vorberg et al., 2004), rabbit epithelial cells RK13 (Courageot et al., 2008) or muscle cells (Dlakic et al., 2007) have been used to prion propagation.

Due to the species barrier, murine cell models display limited susceptibility to certain prion strains; consequently, a previous adaptation of the prion strain in mouse (2003) is necessary. The use of cell lines expressing a PrP homologous to that of the inoculum species has tried to mitigate the difficulties encountered in murine models. The expression of a heterologous PrP in a refractory system can be enough to cross the species barrier *ex vivo* (Vilette et al., 2001). The creation of transgenic mice expressing ovine,

bovine or human PrP has facilitated the production of cell lines carrying PrP homologous to the inoculums (Laude et al., 2002; Archer et al., 2004; Lawson et al., 2008). However, the creation of transgenic mice is an expensive, laborious and long process, especially in species such as sheep, whose genetic variants have great influence on the susceptibility of individuals to different strains of scrapie (Hunter, 1997; Benestad et al., 2003); this methodology would mean the preparation of as many mice as sheep *PRNP* gene haplotypes.

Other limitation of murine cell lines is that prion infection induces subtle changes in the phenotype of infected cultures (Nishida et al., 2000; Solassol et al., 2003). Very few cell lines have shown the cytotoxic effects associated with prion propagation, and primary neuronal cultures have also been used to assess *in vitro* neural tropism and toxicity of prion strains (Hannaoui et al., 2013). Infection of primary neuron and astrocyte culture leads to cell death after the active propagation of the prion (Cronier et al., 2004) and increases its sensitivity to oxidative stress (Milhavet et al., 2000). Prion strains also display different tropism *in vitro*, the affinity and cytotoxic effect vary among strains and neuronal types (Hannaoui et al., 2013). Like cell lines, these *in vitro* models are developed in the murine species and require previous adaptation of the prion strain or the construction of transgenic mice that bear the protein of primary prion species (Cronier et al., 2004). This type of *in vitro* models has little application in naturally susceptible species like human or sheep.

Milhavet et al. (2006) proposed the use of neural stem cells (NSCs) for prion propagation *in vitro*. These cells can differentiate into different CNS cell types (Reynolds et al., 1992; Uchida et al., 2000) and can multiply the prion protein from a little dose of infected brain homogenates. The use of stem cells from the affected species could eliminate specie barrier problem and open new lines of research for diagnosis and therapy development. Neural stem cells form neurospheres in culture and, once differentiated, are more similar to the cell types present in brain than tumour cell lines (Herva et al., 2010). The undifferentiated neurospheres have also been infected with prions (Giri et al., 2006), although neurogenic differentiation may shorten the time of getting infected (Herva et al., 2010).

Apart from these promising lines, there are very few studies showing the isolation of neuronal progenitors of prion natural host species such as sheep (Duittoz and Hevor, 2001) or humans. These cells are derived from embryos, with the ethical issues involved, or from adult CNS tissues (Reynolds and Weiss, 1992), mainly in the hippocampus and ventricular zone (Lie et al., 2004), but also in the spinal cord (Danilov et al., 2006). The inaccessibility of the NSCs niches in the brain severely limits their clinical application. The use of adult stem cells might solve these problems; we will review the potential of mesenchymal stem cells (MSCs) to the development of *in vitro* assays for prion replication and therapy below.

Mesenchymal Stem Cells

Mesenchymal stem cells are a type of adult stem cells with self-renewal and differentiation capacities. Conversely to embryo stem cells, MSCs present *in vitro* finite proliferation ability after long-term cultures (Ksiazek, 2009). Although MSCs have been isolated from vascular tissues of almost all organs (da Silva Meirelles et al., 2006), they were first identified in bone marrow (Friedenstein et al., 1970), which is considered the major MSC source. Bone marrow contains two populations of stem cells, those of the hematopoietic lineage (HSC) that continually repopulates the blood cells and stromal cells or MSCs (Friedenstein et al., 1966).

Most protocols to isolate MSCs are based on their ability to plastic adherence; however, other cell types present in some MSC niches also display this ability. Because of the lack of a unique marker to define and select the MSCs from other plastic adherent cells, the International Society of Cell Therapy (ISCT) determined in 2006 (Dominici et al., 2006) some minimal criteria for human bone marrow-derived cells to be considered as MSCs. These progenitor cells must show plastic adherence under standard culture conditions; express certain surface markers such as CD105 (endoglin), CD73 (5^0 nucleotidase) and CD90 (Thy-1) but not CD45 (Protein tyrosine phosphatase), CD34 (Hematopoietic Progenitor Cell Antigen), CD14 (Myeloid Cell-Specific Leucine-Rich Glycoprotein) or CD11b (Neutrophil Adherence Receptor), CD79a (Immunoglobulin-Associated Alpha) or CD19 (B-Lymphocyte Surface Antigen B4) and HLA-DR (Major histocompatibility complex, class II); and differentiate *in vitro* into osteoblast, adipocyte and chondrocyte lineages. These critera have been extended to define more species apart from human (De Schauwer et al., 2011). However, the lack of specific commercial antibodies for the different species complicates their detection and subsequent study (Ranera et al., 2011).

As mesoderm-derived cells, they can differentiate *in vitro* into osteogenic, chondrogenic, adipogenic and other mesenchymal lineages (Friedenstein, 1976; Caplan, 1991; Kuznetsov et al., 1997). But exposing the cells to specific inductive conditions, they can also differentiate into cells belonging to different embryo layers such as neural cells (ectoderm; Sanchez-Ramos et al., 2000; Deng et al., 2001; Woodbury et al., 2002; Zhao et al., 2002) and hepatic cells (endoderm; Karnieli et al., 2007). This ability is known as plasticity, which is actually more extensive than it was initially thought. Differentiated MSCs into neural cells expresses neuronal cell surface markers such as the

hexaribonucleotide binding protein (NEUN), neuron-specific enolase (NSE), nestin (NES) or glial fibrillary acidic protein (GFAP). Additionally, they present K^+ transport, essential to restore the negative potential in the neuron (Mareschi et al., 2006). The differentiation ability of MSCs to adult CNS cells makes them cellular model candidates for the study and treatment of neurodegenerative diseases, with the added advantage of the easiness to obtain adult MSCs from bone marrow or even peripheral blood. However, further investigations will be necessary to elucidate if MSC-derived neuron-like cells exhibit the adequate neuronal functions to become clinically relevant for use in neural repairs (Liu et al., 2012). Compared with foetal NSCs, adult MSCs show a limited neurogenic potential using the current differentiation protocols (Lepski et al., 2010).

In addition to differentiation capacities, MSCs display a particular relationship with immune system that makes them a suitable therapeutic option (Al Jumah and Abumaree, 2012). Mesenchymal stem cells are considered immunoprivileged, because they express the major histocompatibility complex class I (MHC-I) but not the molecules expressed by the antigen-presenting cells: MHC-II (Bartholomew et al., 2002; Jones et al., 2007; Li et al., 2007; Deuse et al., 2011), and the co-stimulatory antigens CD89m CD86 and CD40, that without them the immune response cannot be triggered (Mok et al., 2013).

As well as the lack of immunogenicity, MSCs have a potent capacity to inhibit the proliferation of stimulated immune cells (Crop et al., 2009). Bone marrow-derived MSCs suppress the immune responses of allogeneic lymphocytes (Bartholomew et al., 2002; Tse et al., 2003), and recently, it has been demonstrated that dental pulp MSCs inhibit the proliferation of peripheral blood mononuclear cells (Tomic et al., 2011). Therefore, MSCs derived from different sources have an immunomodulatory role.

On the other hand, other studies report MSCs as immunostimulatory cells that may play a role of antigen-presenting cells and induce the immune response (Le Blanc et al., 2003; Potian et al., 2003; Meirelles Lda et al., 2009). The underlying mechanisms are not completely understood yet; several studies have shown that the immunosuppressive effect of MSCs may be exerted by cell-to-cell interactions (Augello et al., 2005; Nasef et al., 2007) either by soluble factors secreted by MSCs or lymphocyte-affected MSCs (Cai and Rao, 2002; Yang, 2007; Tasso and Pennesi, 2009). Further studies will be necessary to elucidate the whole picture of the immunoregulatory properties of the MSCs.

Taking all the properties described above together with the ability to migrate to sites of tissue injured like bone fractures, cerebral ischaemia or infarcted heart (Caplan and Bruder, 2001; Tomita et al., 2002), MSCs are a strong candidate for cellular therapy (Doering, 2008). In humans, clinical trials using MSCs have been targeted towards a broad spectrum of diseases with promising and discouraging results because the culture environment which MSCs are subjected to, the timing and tissue site specificity of MSCs delivery will affect treatment efficacy (Mok et al., 2013).

For the last years, veterinary medicine has been important for the development of stem cell therapies. In addition to the use of large animals as experimental models for human diseases, a relatively liberal legal and ethical regulation has facilitated the clinical translation of these therapies (Volk and Theoret, 2013). However, drug agencies could soon control the veterinary uses of stem cells, including MSCs (Cyranoski, 2013). A great part of the MSC-based therapies in Veterinary Medicine is addressed to the treatment of locomotor lesions in equine (Carvalho et al., in press; Kang et al., 2013; Pigott et al., 2013; Ricco et al., 2013) species where MSCs are relatively well characterized *in vitro* (Arnhold et al., 2007; Vidal et al., 2007; de Mattos Carvalho et al., 2009; Coli et al., 2011; Ranera et al., 2011, 2012, 2013; Seo et al., in press). Stem cell therapies are also being developed for the treatment of osteoarthritis, bound healing and spinal cord injuries in dogs (Ryu et al., 2012; Chung et al., 2013; Vilar et al., 2013) and chronic kidney disease in cats (Quimby et al., 2013). We will briefly review the use of MSCs in the treatment of neurodegenerative diseases and their potential use in the study of prion diseases and the development of *in vitro* models for the use of these diseases affecting both, humans and animals.

MSCs as Therapy for Prion and Other Neurodegenerative Diseases

Stem cell-based therapies have emerged as possible strategies to treat diseases of the CNS (Lindvall and Kokaia, 2010). Paul and Anisimov (Paul and Anisimov, 2013) have reviewed the properties of MSCs which could explain CNS repair. Whereas the plasticity of MSCs to differentiate into functional neurons and glial cells is controversial (Hardy et al., 2008), these cells could exert their neuroprotective effect by releasing angiogenic, neurogenic, neuroprotective, synaptogenic and scarring inhibition factors (Chen and Chopp, 2006). This paracrine action suggests that MSCs could act without cell-to-cell contact, in which case the engraftment of MSCs would not been necessary (Bai et al., 2012); therefore, an intravenous injection of MSCs could result in neuroprotection and repair (Uccelli and Prockop, 2010). Finally, the immunomodulatory role of these cells could also be critical for the neuroprotective effect of these treatments (Kassis et al., 2008).

Mesenchymal stem cells from different sources (bone marrow, adipose tissue, umbilical cord, etc.) and several delivery methods (intravenous, intracarotid, intracerebroventricular, etc.) have been used for cell therapy in animal

models of CNS disorders and neurodegenerative diseases (Paul and Anisimov, 2013). Many studies have been focused on the treatment of the damage produced by ischaemic stroke (for review Wan et al., 2014). Mesenchymal stem cells grafted into the brain of ischaemic models would abrogate neurological deficits (van Velthoven et al., 2013), reduce the infarct size by modulating the apoptosis in the infarct and peri-infarct area (Leu et al., 2010; Chen et al., 2013b) and modulate the activation of glial cells towards their immunomodulatory activity (McGuckin et al., 2013). Other mechanisms have been also proposed to explain the therapeutic benefits of MSCs in the stroke treatment (Paul and Anisimov, 2013). In addition, before engraftment or delivery, MSCs can be genetically modified to overexpress neuroprotective factors and provide sustained and long-term delivery of these molecules at supraphysiological levels (Kocsis and Honmou, 2012).

Parkinson's disease (PD) has been also a target for many MSC-therapy-based treatments. Transplant of placental-derived MSCs into rat models of PD provided experimental evidence of dopaminergic differentiation of the transplanted progenitors and treated animals displayed a minor, but significant, amelioration (Park et al., 2012). Other works have used naïve MSCs or epigenetically modified MSCs displaying in most cases motor improvement of the animals treated (Paul and Anisimov, 2013).

Mesenchymal stem cell-based therapies for Huntington's and Alzheimer diseases, which course with accumulation in the CNS of a polyglutamine expanded form of the huntingtin protein or amyloid-beta deposition, respectively, are being also investigated (Ma et al., 2013; Rossignol et al., 2014). The development of this type of therapies constitutes a promising option for these types of diseases in which effective treatments do not exist.

Besides the great number of works focused on the use of MSCs in the treatment of other neurodegenerative diseases, only one group has investigated the effect of human MSC transplantation in mice infected with prions (Song et al., 2009). In that work, the authors demonstrated that brain extracts from prion-infected mice promote chemotaxis of human MSCs *in vitro* and identified the transplanted MSCs *in vivo* in the areas of brain lesions in infected mice. Small populations of these MSCs differentiate into cells expressing neuronal, astrocyte or oligodendrocite markers. Finally, microenvironments in the brain lesions stimulate MSCs to produce trophic factors. The sum of this mechanisms prolonged survival of prion-infected mice although MSCs could not arrest the disease progression. In a later study, the same authors identified the chemokine receptors involved in the migration of human MSCs to brain lesions (Song et al., 2011). These results are promising and, like their use in other neurodegenerative diseases, suggest that therapies based on MSCs may provide an effective treatment for prion diseases. Scrapie-infected sheep may be a good animal model for the evaluation of these treatments prior to their use in humans.

MSC as Cell Model for Prion Studies

Cellular prion protein is expressed in a wide variety of stem cells, including embryonic and hematopoietic stem cells and its function has been linked to stem cell biology (Zhang et al., 2006; Lee and Baskakov, 2013), modulating the proliferation and self-renewal of these cells (Steele et al., 2006; Zhang et al., 2006). Bone marrow-derived MSCs also express PrP^{C} and its expression decreases with passages (Mohanty et al., 2012), as well as the capacity to proliferate (Wagner et al., 2009).

In recent years, three bioassays have described the proliferation of different prion strains in cells with mesenchymal characteristics derived from mouse mesenchymal stromal spleen (Akimov et al., 2008) and bone marrow (Akimov et al., 2009; Cervenakova et al., 2011). These cells successfully multiplied a variant strain of Creutzfeldt-Jakob disease and another of Gerstmann-Sträussler-Schneiker previously adapted in mouse.

Bone marrow stromal cells do not need to differentiate into neural cells to multiply prions. Murine models experimentally infected with prions accumulate PrP^{res} in MSCs and this infection could precede prion accumulation in brains (Takakura et al., 2008). The same study demonstrated the presence of PrP^{res} in bone marrow-derived MSC obtained from CJD patients, and it was proposed as an alternative method for diagnosis (Takakura et al., 2008). These authors hypothesized that bone marrow MSCs would long-term express PrP^{res} and could be a reservoir for prion agents, and blood cells could get infected after contact with MSCs. However, there are contradictory results on that issue; a different research group analysed the infectivity of bone marrow in TSE patients obtaining negative results (Brown et al., 1994), suggesting that the infectivity of this tissue would depend on the moment of sampling during the course of disease. In large animal models for TSE, the infectivity of bone marrow does not seem to be a general feature in prion diseases. Whereas in experimentally infected cattle, BSE prions have been found in bone marrow (Wells et al., 1999); PrP^{Sc} deposits were not observed in this tissue in natural scrapie-infected sheep (Caplazi et al., 2004), although infectivity of bone marrow was previously reported in this natural model (Hadlow et al., 1982).

To our knowledge, the presence of PrP^{res} in MSCs in naturally infected hosts apart from humans has never been investigated. Sheep infected with classical scrapie are good models for the study of the pathogenesis of vCJD in humans and to assess risk through blood transfusion route

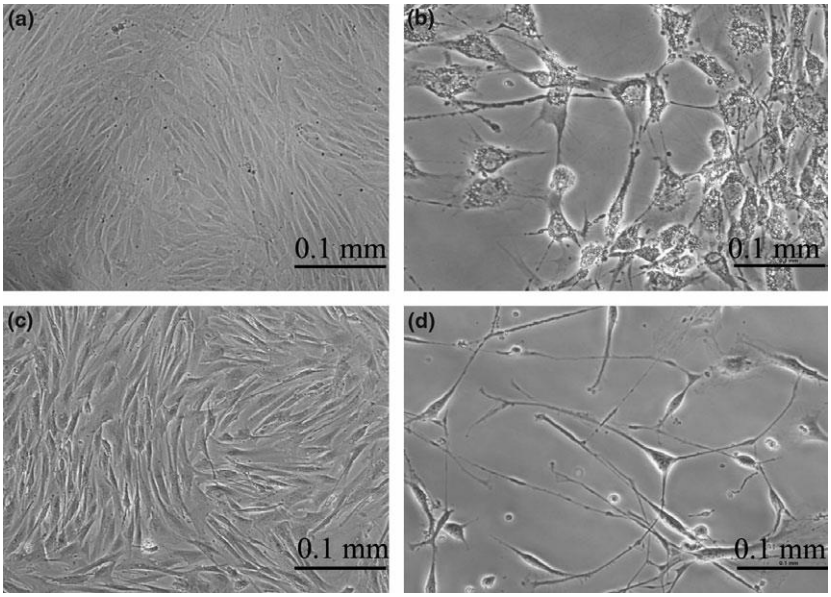

Fig. 1. Neurogenic differentiation of ovine mesenchymal stem cell (MSC) derived from bone marrow (a, b) and peripheral blood (c, d). Image shows ovine MSC cultured 3 days under standard conditions (a, c) and under neurogenic conditions (b, d). Control cells show a fibroblastic morphology while differentiated cells show neuron compatible characteristics such as shiny bodies and branched elongations.

III. (Ironside et al., 2000, 2006; Andreoletti et al., 2012). These studies would contribute not only to the establishment of new diagnosis test, especially if the infectivity was detected in peripheral blood MSCs, but also to assess TSE transmis- sion risk by the use of allogeneic MSC in cell therapy.

The potential of ovine MSCs (oMSCs) to multiply prions has neither been investigated. Murine MSC infection requires the use of prion strains adapted to mouse. Cell models based on natural host-derived MSCs would allow the use of inocula without previous passages in mice, facili- tating transmission studies. Ovine MSCs have been used for research in tissue engineering (Caplan, 1991) or cardio-myopathies (Psaltis et al., 2010) and bone marrow and adipose tissue-derived oMSCs are relatively well character-ized regarding their *in vitro* (Rentsch et al., 2010) and *in vivo* (Niemeyer et al., 2010) differentiation potential and their phenotype for mesenchymal surface cell markers (Martinez-Lorenzo et al., 2009). We have recently described the isolation and characterization of MSCs from ovine peripheral blood (Lyahyai et al., 2012). In addition to the other mesenchymal characteristics, both bone marrow- and peripheral blood-derived MSCs are able to trans-differenti-ate into neuronal-like cells (Fig. 1) and express PrP^C at least at the transcript level (Lyahyai et al., 2012). The expression of PrP^C makes these cells good candidates to develop *in vitro* systems for the study of prion infectivity and multiplication. In addition, during the neurogenic differentiation process, the expression of PrP^C increases (Lyahyai et al., 2012) as PrP^C participates in neuritogeneis (Loubet et al., 2011), this increase could facilitate the infection of these cells to develop *in vitro* models for prion replication.

Besides these problems that need to be solved, the use of MSCs from natural hosts could facilitate *in vitro* studies of transmissibility, species barrier or genetic resistance to TSEs.

The experiments developed in murine models and the characteristics described for MSC-derived from animal species naturally susceptible to prion infection, that is, the expression of PrP^C and their neurogenic differentiation potential, strongly suggest that these cells can be good candidates for developing cell culture-based models. These models would not need a prior adaptation of the prion isolate and would facilitate the studies related to species barrier, genetic resistance or toxicity.

Conclusions

Mesenchymal stem cells express prion protein and studies developed in mice have shown that these cells can multiply the pathological form of the prion protein. In addition, ovine MSCs can also been infected with scrapie agent although further studies are necessary to prolong infectivity along passages. Finally, MSCs are being used for the devel-opment of cell therapies in many neurodegenerative dis-eases. These characteristics make MSCs good candidates to develop *in vitro* models for prion research and cell-based therapies.

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RESEARCH ARTICLE Open Access

Isolation and characterization of ovine mesenchymal stem cells derived from peripheral blood

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43. **Abstract**

Background: Mesenchymal stem cells (MSCs) are multipotent stem cells with capacity to differentiate into several mesenchymal lineages. This quality makes MSCs good candidates for use in cell therapy. MSCs can be isolated from a variety of tissues including bone marrow and adipose tissue, which are the most common sources of these cells. However, MSCs can also be isolated from peripheral blood. Sheep has been proposed as an ideal model for biomedical studies including those of orthopaedics and transmissible spongiform encephalopathies (TSEs). The aim of this work was to advance these studies by investigating the possibility of MSC isolation from ovine peripheral blood (oPB-MSCs) and by subsequently characterizing there *in vitro* properties.

Results: Plastic-adherent fibroblast-like cells were obtained from the mononuclear fraction of blood samples. These cells were analysed for their proliferative and differentiation potential into adipocytes, osteoblasts and chondrocytes, as well as for the gene expression of cell surface markers. The isolated cells expressed transcripts for markers *CD29*, *CD73* and *CD90*, but failed to express the haematopoietic marker *CD45* and expressed only low levels of *CD105*. The expression of *CD34* was variable. The differentiation potential of this cell population was evaluated using specific differentiation media. Although the ability of the cultures derived from different animals to differentiate into adipocytes, osteoblasts and chondrocytes was heterogeneous, we confirmed this feature using specific staining and analysing the gene expression of differentiation markers. Finally, we tested the ability of oPB-MSCs to transdifferentiate into neuronal-like cells. Morphological changes were observed after 24-hour culture in neurogenic media, and the transcript levels of the neurogenic markers increased during the prolonged induction period. Moreover, oPB-MSCs expressed the cellular prion protein gene (*PRNP*), which was up-regulated during neurogenesis.

Conclusions: This study describes for the first time the isolation and characterization of oPB-MSCs. Albeit some variability was observed between animals, these cells retained their capacity to differentiate into mesenchymal lineages and to transdifferentiate into neuron-like cells *in vitro*. Therefore, oPB-MSCs could serve as a valuable tool for biomedical research in fields including orthopaedics or prion diseases.

Keywords: Sheep, Mesenchymal stem cell, Peripheral blood, Neurogenesis

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44. Background

Mesenchymal stem cells (MSCs) are morphologically fibroblast-like cells that are characterized by their ability to both self-renew and differentiate into tissues of meso- dermal origin (osteoblasts, adipocytes, chondrocytes and myocytes) [1]. However, MSCs can also give rise to other cell types such as astrocytes and neurons [2,3]. This indicates cellular pluripotency and suggests that MSCs are responsible for the normal turnover and mainten- ance of adult mesenchymal tissues [4].

Sheep is an ideal model for bone tissue engineering [5] and has been proposed as an animal model for a wide range of applications in biomedical research, such as for the studies of respiratory diseases [6], cardiomyopathies [7,8], neurological disorders [9] and prion diseases [10,11]. Although MSCs are generally obtained from the bone marrow [12], they can also be isolated from other sources such as adipose tissue, umbilical cord blood and foetal tissues [13,14]. The isolation of MSCs from per- ipheral blood (PB-MSCs) has been reported for a variety of mammals including guinea pigs, rabbits, dogs, mice, rats, horses and humans [15-19]. Because blood harvest- ing is a less invasive procedure to obtain stem cells, this method would represent a significant advantage for patients and, therefore, would be an ideal candidate technique to obtain PB-MSCs for future clinical applica- tions. Moreover, monitoring the presence and the pro- portional quantity of MSCs in the peripheral blood could possibly help in the understanding of the patients'reaction to a disease.

The isolation procedure of ovine PB-MSCs (oPB- MSCs) would facilitate the sampling of these progenitor cells for use in a wide variety of applications, including fundamental and applied studies of orthopaedics or prion diseases. Here, we present the first study describ- ing the isolation and characterization of oPB-MSCs. The osteogenic, chondrogenic and adipogenic differentiation potential of oPB-MSCs was analysed *in vitro* and moni- tored by specific staining and molecular differentiation markers. We also demonstrate the capacity of these cells to differentiate into neuron-like cells and the expression of the gene coding for the prion protein (*PRNP*) in both regular and differentiated cells.

45. Results

Isolation and characterization of peripheral blood derived fibroblast-like cells

Isolation and expansion of peripheral blood derived fibroblast-like cells

Plastic-adherent fibroblast-like cells were observed within the first days of culture of the nucleated cell frac- tion of peripheral blood obtained from total six sheep. Although the volume of blood collected was similar for all animals (approximately 25 mL), the number of peripheral blood nucleated cells (PBNC) obtained was variable, ranging from $0.594\ 10^6$ to $1.9\ 10^6$ PBNC/mL, with mean $1.36\ 10^6 \pm 682646$. After the isolation process, a mean of 281400 ± 178051 adherent cells were obtained from each individual, varying between 2.7 and 9.3 adher- ent cells for every 1000 PBNC (mean: 5.85 ± 2.7).

Cells were expanded until the second passage and then frozen. The proliferation capacity of the adherent cells was measured during the first two passages. An average of 12.6 days was necessary to complete the first passage. Mean cell doubling during the first passage was 2.29 ± 0.887 and the doubling time was 5.99 ± 1.86 days. Time required to complete the second passage was shor- tened to 7.33 days, cell doubling decreased to 1.84 ± 0.975 and the doubling time was 4.88 ± 2.68 days.

After thawing, the cells from passage 2 were expanded for two more passages to obtain sufficient amount of cells for the differentiation assays. The cells were then characterized by analysing the expression of cell surface markers and the tri-lineage differentiation potential into adipocytes, osteoblasts and chondrocytes.

Expression of mesenchymal cell surface markers

To initiate the characterization of oPB-MSCs, the ex- pression of six cell surface markers specific for mesen- chymal and haematopoietic cells were first analysed at the transcript level by quantitative real time PCR (RT- qPCR). All analysed cultures expressed *CD29* (integrin β1), *CD73* (ecto-5'-nucleotidase) and *CD90* (Thy-1), whereas the expression of *CD34* (CD34 molecule) was detected in five out of six of these cultures. The amplifi- cation of the hematopoietic marker *CD45* (protein tyro- sine phosphatase, receptor type, C) was not detected and *CD105* (endoglin) was only weakly amplified at thresh- old cycles above 35.

Adipogenic potential

Cells cultured under adipogenic conditions presented cytoplasmic lipid droplets under light microscope, al- though the size of the droplets was variable depending on the donor animal. To confirm that the contents of the droplets were lipids, the cultures were stained with oil red O (Figure 1A and B). The expression of adipo- genic markers was analysed on days 7 and 14 of post-in- duction. The expression profiles of *PPARG* (peroxisome proliferator-activated receptor gamma), *SCD* (stearoyl-CoA desaturase) and *IL6* (interleukin 6) are shown in Figure 2. During the induction of differentiation, the *PPARG* and *SCD* mRNA expression levels increased to 7.3- and 20.8-fold, respectively. However, these changes were not statistically significant due to the high variabil- ity observed between animals. A significant downregula- tion of *IL6* (−31-fold, $P < 0.05$) was detected after two weeks of culture (Figure 2A).

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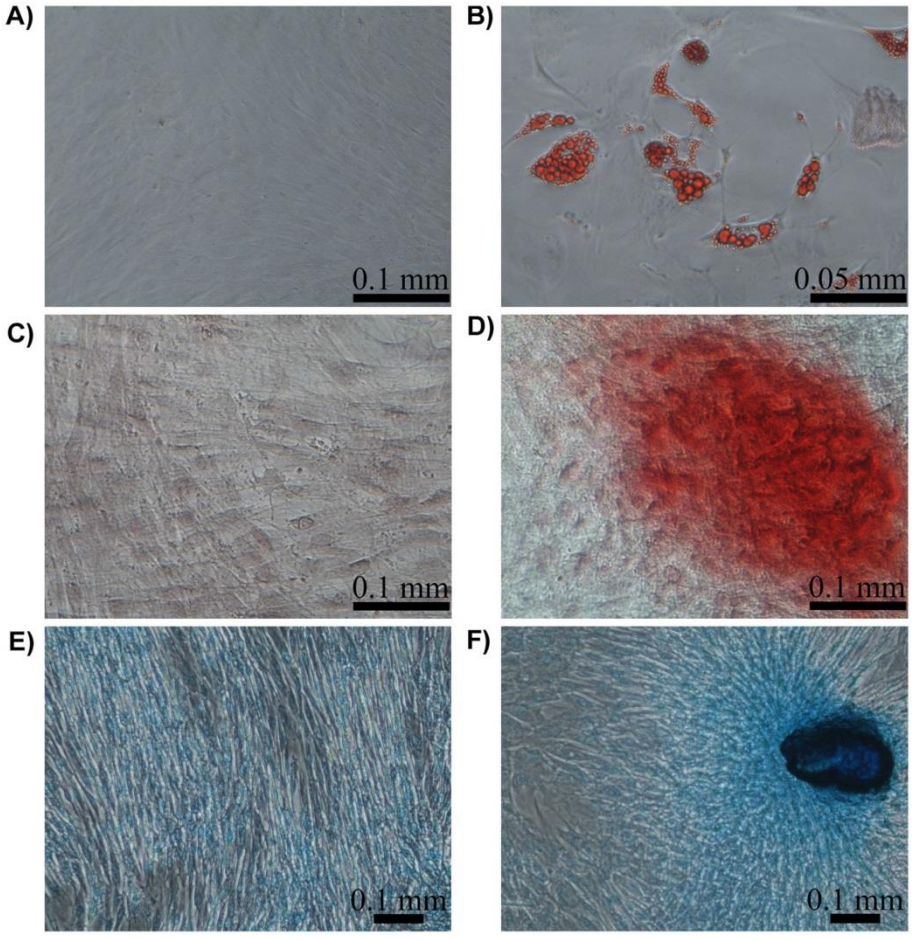

Figure 1 Staining for adipogenic, osteogenic and chondrogenic differentiation of PB-MSCs. Oil red O staining of cells cultured for 15 days in basal (A) and adipogenic differentiation medium (B). Alizarin red staining of cells cultured for 21 days in basal (C) and osteogenic differentiation medium (D). Alcian blue staining of cells cultured for 21 days in basal (E) and chondrogenic medium (F).

Osteogenic potential

The ability of oPB-MSCs to differentiate into osteoblasts was demonstrated using alizarin red staining (Figure 1C and D). Nodule-like aggregations stained in red appeared in the osteogenic media on the 21st day of culture exclusively, indicating that these cultures were mineralized at a relatively late stage. However, the cells from different animals displayed variable osteogenic potential. The expression of osteogenic markers was evaluated in the cultures that displayed positive staining (n = 2). The expression levels of *COL1A1* (collagen, type 1, α 1) were not altered during the first 2 weeks in osteogenic media. However, a strong downregulation of *COL1A1* was observed at 3 weeks of culture. In contrast, the expression

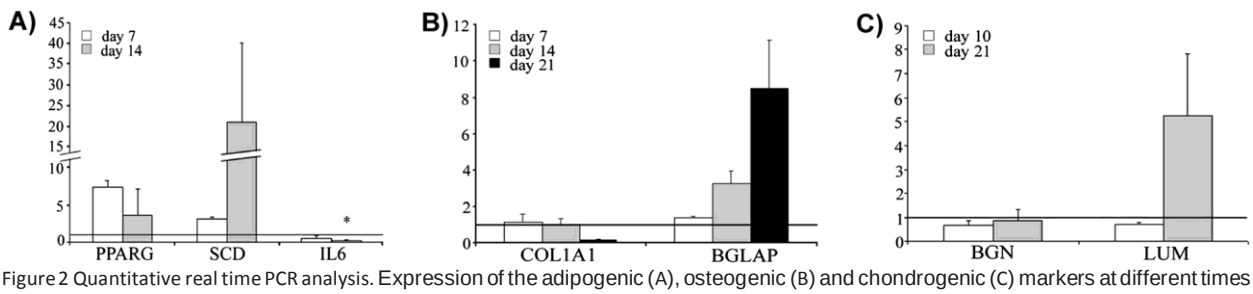

Figure 2 Quantitative real time PCR analysis. Expression of the adipogenic (A), osteogenic (B) and chondrogenic (C) markers at different times of the differentiation process relative to the levels observed in control cultures (values set to 1, horizontal line). Data are shown as mean ± standard errors. Statistically significant differences between differentiated and control cells were determined by Student t test (*P<0.05).

levels of *BGLAP* (bone gamma-carboxyglutamate (gla) protein, or osteocalcin) increased drastically throughout the culture period (Figure 2B).

Chondrogenic potential

The chondrogenic potential was evaluated in monolayer cultures. Ovine PB-MSCs formed nodule-like aggregations in both control and induced conditions. However, the oPB-MSCs in chondrogenic media displayed a stron- ger staining with alcian blue (Figure 1F). Although the chondrogenic marker expression analysis did not reveal variations in the gene expression levels of the *BGN* (biglycan), *LUM* (lumican) was found to be upregulated on the 21st day of culture (Figure 2C).

Neuronal differentiation of oPB-MSCs

The ability of the isolated cells to transdifferentiate into neuronal cells was evaluated *in vitro*. The cells cultured under neurogenic conditions displayed distinctly altered morphology after the first 24 hours of induction. Differentiated cells were sharply defined, retracted towards the nucleus displaying phase-bright bodies, and some neurite-like processes (thin, long, and often branched) became apparent (Figure 3B,C). Neuronal differentiation was also demonstrated using RT-qPCR analysis. Control cells displayed none or very low levels of *NELF* (nasal embryonic LHRH factor) expression on 3 and 6 days of culture, while low expression levels of the remaining markers (*MAP2* [microtubule-associated protein 2], *NES* [nestin], *NEFM* [neurofilament, medium polypeptide], *TUBB3* [tubulin, beta 3]) were observed. The expression of these markers increased in neurogenic conditions, with a peak of expression on day 6 post-induction. Sta- tistically significant changes were found for *NELF* on day 3 of culture (5.85 fold induction, $P < 0.001$) and an over-expression tendency was observed for *MAP2* on day 6 (2.4 fold induction, $P < 0.1$). Moreover, oPB-MSCs expressed transcripts of the prion protein (*PRNP*), which increased up to 5 times during the neurogenic period (Figure 3D).

46. Discussion

Despite the importance of ovine as a large animal model for many conditions (i.e., orthopaedic injuries or Transmissible Spongiform Encephalopathies) the characterisation of ovine MSCs (oMSCs) is still limited. During the last decade, there has been an important effort within the scientific community to focus on the characterisation of MSCs obtained from different species, including the sheep. However, most research on MSCs has been per- formed on cells derived from bone marrow and, to a lesser degree, adipose tissue. The osteogenic and chon- drogenic differentiation potential of MSCs *in vitro* [20-22] and *in vivo* [23,24] is currently relatively well

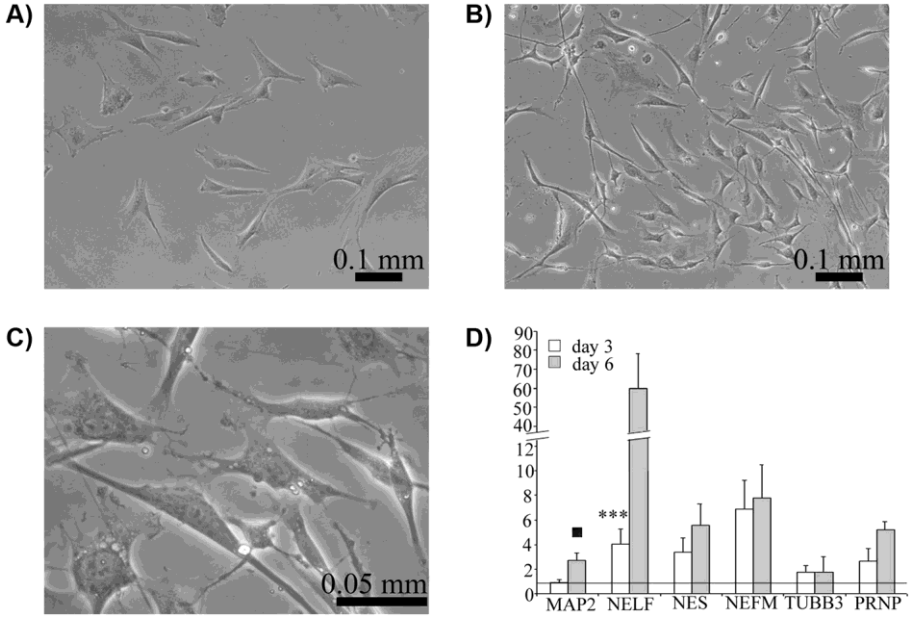

Figure 3 Neurogenic differentiation of PB-MSCs. Phase contrast micrograph of ovine PB-MSCs at passage 3 cultured on basal and neurogenic medium for 3 days. Control cells (A) showed a fibroblast-like shape whereas differentiated cells (B) displayed neuronal-like features such as phase-bright bodies, long multipolar extensions and branching ends (C: higher magnification). Increase of the expression levels of neurogenic markers after 3 (white bars) and 6 (grey bars) days of induction relative to the levels observed in cells cultured on basal medium (values set to 1, horizontal line) as assessed by real time PCR. Data are shown as mean ± standard errors. Statistically significant differences between differentiated and control cells were determined by Student t test (▪ $P<0.10$, *** $P<0.001$).

understood. Their phenotype for mesenchymal surface cell markers has also been analysed [25], and their proliferative potential has been shown to be heterogeneous [26]. Although the existence of MSCs in peripheral blood has been demonstrated in many species [17,18], this work represents the first report describing the isola- tion of these cells from sheep circulation.

The minimal criteria to define human MSCs proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy are: (1) plastic-adhesion when maintained in standard culture conditions; (2) expression of CD105, CD73 and CD90, and lack expression of the haematopoietic markers CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules and; (3) ability to differenti- ate to osteoblasts, adipocytes and chondroblasts *in vitro* [27]. In our study, plastic-adherent cells with a fibroblast-like morphology were obtained from all ex- perimental sheep and were further analysed to deter- mine the expression of mesenchymal markers and their ability to differentiate into adipocytes, osteoblasts and chondrocytes.

In other domestic species [28] the proportion of MSCs in the peripheral blood is low, which is in agreement with the few colonies of MSCs detected in our original oPB-MSCs cultures. Although the proliferation ability of oPB-MSCs was very different between individuals, the doubling time was generally longer than in other species, such as horse [29]. This difference may be due to the higher percentage of FBS used in the isolation of equine PB-MSCs (30%) and also due to the addition of dexa- methasone to the growth media, which has been demon- strated to favour the expansion of MSCs [30]. The variability observed in this work is in accordance with the high heterogeneity in the proliferative potential of oMSCs obtained from bone marrow (oBM-MSCs) [26].

The absence of a well-defined immunophenotype for PB-MSCs renders the comparison of studies difficult. Moreover, most of the cell surface markers utilized to sort subpopulations of human MSC by flow cytometry have not been validated in sheep [21]. Gene expression- based technologies may be useful for the identification of possible molecules described as MSC markers [31,32]. In our study, RT-qPCR was performed to quantify the mRNA expression levels of six cell surface antigens con- sidered as either positive (*CD29*, *CD73*, *CD90* and *CD105*) or negative (*CD34* and *CD45*) MSC markers in humans.

In accordance with the immunophenotype described for human PB-MSCs [33-35], our expression analysis revealed significant amplification of the typical MSC markers, *CD29*, *CD73* and *CD90*, and a weak signal for *CD105*. In contrast, the haematopoietic marker *CD45* was not expressed. To our knowledge, there are no published data concerning the gene expression of cell surface markers in oMSCs obtained from other tissues. However, we have observed amplification of *CD29*, *CD73* and *CD90* in oBM-MSCs, as well as the lack of *CD34* and *CD45* expression (unpublished work from our group). Using flow cytometry, the presence of CD29 and CD105 has also been detected in oBM-MSCs [21,36]. Additionally, oMSCs isolated from adipose tissue (oAT- MSCs) display high expression of CD90 and low immu- noreactivity for CD105 [25]. The immunophenotypes of oBM-MSCs [36] and oAT-MSCs [25] are negative for the haematopoietic CD34 marker. However, this marker is expressed at low levels in human PB-MSCs [37] and in equine MSCs derived from adipose tissue [38] as demonstrated by RT-qPCR. We detected *CD34* expres- sion in 5 out of 6 cultures, which may indicate individual variability. Finally, the cells analysed were negative for the haematopoietic marker *CD45*, as are human MSCs [16]. We have previously found a good correlation be- tween MSC marker gene expression and the immuno- phenotype detected by flow cytometry in equine MSCs [39]. Although flow cytometry analysis is necessary to validate the immunophenotype of the isolated cells, the gene expression profile observed in this work strongly suggests that the peripheral blood derived fibroblast-like cells obtained as described would fulfill the requirements to be considered as MSCs.

Ovine BM-MSCs can be differentiated into adipocytes, showing lipid droplets in their cytoplasm and the induc- tion of adipogenic markers [20,36]. Similarly, adipogenic differentiation has been achieved here in all peripheral blood derived cell cultures, although great variability in the size of lipid droplets was observed. The expression of two adipogenic markers was evaluated in the cultures using RT-qPCR. PPARG is considered the master regula- tor of adipogenesis [40,41] and is up-regulated in MSCs under adipogenic conditions [42]. SCD is expressed uniquely in adipocytes and catalyzes the rate-limiting step in the synthesis of poly-unsaturated fatty acids, thereby exhibiting a pivotal role in adipocyte metabolism [43]. Inter-individual variability was also noticeable in the expression of these adipogenic markers, which explains the lack of statistically significant differences in *PPARG* and *SCD* expression results despite the strong overexpression observed throughout the culture period. We also determined the expression of IL-6, which main- tains the proliferative and undifferentiated state of bone marrow-derived MSCs [44] and is down-regulated dur- ing lineage-specific differentiation [45]. In accordance with these reports, a significant decrease was detected in the expression of *IL6* in the differentiated cultures. Therefore, using specific staining and gene expression profiles of adipogenic markers, we have confirmed the adipogenic potential of oPB-MSCs.

Similar to the adipogenic analysis, a great individual variation was also observed in the osteogenic potential. Osteogenic mineralization was confirmed on the last day of culture in osteogenic conditions (21 days) by staining calcium deposits with alizarin red. The induction period necessary for visualization of matrix mineralisation in oMSCs varies among different studies. The period reported for oBM-MSC mineralisation ranges from 21 days [36] to 5 weeks [21], while 4 weeks are required to differentiate periodontal oMSCs [46]. The weak ali- zarin red staining observed in some of our experiments could be due to the relatively short period of induction.

Although COL1A1 is considered an early marker of osteoprogenitor cells [47], we observed either no changes or a strong down-regulation on the 21st day of culture. Besides displaying a rapid mineralisation, oBM- MSCs cultured under osteogenic conditions express increased or declined levels of COL1A1 depending on the differentiation moment [21]. Other authors have reported either no significant increase in *COL1A1* mRNA expression levels after osteogenic differentiation in human [48], porcine [49] and equine [39] MSCs, or a down-regulation of this marker in human PB-MSCs dur- ing osteogenesis [50]. Therefore, *COL1A1* may not be suitable for monitoring osteogenesis in oPB-MSCs. In contrast, *BGLAP* was upregulated during the differenti- ation process and was maximally expressed on the last day of culture (day 21), coinciding with the positive ali- zarin red staining. This is in accordance with the role of BGLAP as a late marker of developing osteoblasts [51].

The sheep has been used as a large animal model for the studies of chondrogenesis both *in vivo* [52] and *in vitro* [22]. The chondrogenic potential of oMSCs has been evaluated mainly in micromass cultures of cells derived from bone marrow [20,21,36]. Chondrogenesis was evaluated in our study using a bidimensional culture with a high cell concentration seeding, according to the protocol described by Jäger et al. [53] for chondrogenic differentiation of ovine umbilical cord blood-derived MSCs. Chondrogenic nodules were observed in both control and chondrogenic media, although the staining was stronger in the induced cultures. The confirmation with molecular markers was not straightforward as the expression of the two components of the extracellular matrix *BGN* and *LUM* changed in opposite directions during chondrogenic differentiation. In accordance to our results, the lack of strong *BGN* overexpression has been reported for chondrogenic induced micropellets of oBM-MSCs [36]. However, further analysis is necessary to fully confirm the ability of oPB-MSCs to differentiate into chondrocytes.

During the last decade, many reports have described the *in vitro* neural transdifferentiation of MSCs derived from a range of species [2,54,55] but, to our knowledge, this has never been investigated in oMSCs. Neurogenic capacity of PB-MSCs would offer exciting possibilities for autologous therapeutic treatments for a variety of neurological disorders. As ovine is a natural model for prion diseases, the transdifferentiation of MSCs into neural cells could provide an excellent *in vitro* model for the study of these pathologies. Here, we described altera- tions in the morphology and expression profiles of neurogenic markers (*MAP2*, *NEFM*, *NELF*, *NES* and *TUBB3*) that are consistent with neural differentiation. In addition, we detected up-regulation of *PRNP*, which could also be involved in the morphological changes as the cellular prion protein seems to be necessary for neuritogenesis [56]. The variable success in the ability to transdifferentiate MSCs to a neural phenotype could be influenced by the inter-donor variability of expression of neural-related markers in MSCs prior to differentiation [57]. Nevertheless, our study shows that oPB-MSCs re- tain the ability to transdifferentiate. Finally, although murine bone marrow stromal cells express the prion protein [58], this has not been previously shown in species susceptible to prion diseases. In the present work, we have demonstrated the expression of *PRNP* in oPB-MSCs and its overexpression during neuronal differentiation.

47. Conclusions

In this study we describe, for the first time, the isolation of mesenchymal stem cells from ovine peripheral blood. These cells express mesenchymal markers and retain the ability to differentiate into adipocytes and osteoblasts. Although oPB-MSCs seem to differentiate into chondro-cytes, further studies are necessary to confirm the suit-ability of these cells for chondrogenesis studies. Finally, these cells can transdifferentiate into neuron-like cells and express *PRNP*.

48. Methods

Animals and MSC isolation

Peripheral blood (25 mL) was obtained from a total of 6 sheep aged 1.5 to 6 years. The animals belonged to the Rasa Aragonesa breed and came from regional flocks. The procedure for blood collection from commercial farm animals was performed according to the recom- mendations of the Joint Working Group on Refinement [59]. The ethics committee of the University of Zaragoza approved the study (PI38/10). The blood was collected in 5 mL tubes with sodium heparin. Immediately after, blood was diluted in 1 volume of PBS and layered over Lymphoprep (Atom) in a 1:1 proportion. The mono- nuclear fraction was harvested after a density gradient centrifugation step of 20 min at 1600 g. Mononuclear cells were rinsed twice in the same volume of PBS by centrifugation for 5 min at 1600 g. The cells were

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resuspended, counted and plated at 10^6 cells/cm^2 in 6-well plates with basal medium consisting of low glucose Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 20% foetal bovine serum (FBS), 1% L-glutamine (Sigma-Aldrich) and 1% streptomycin/ penicillin (Sigma-Aldrich).

Non-adherent cells were removed washing the mononuclear cells twice with PBS after 24, 48 and 72 h of incubation at 37°C and 5% CO_2 and were maintained in growth medium until reaching approximately 80% confluence. The cells were then treated with trypsin (Sigma Aldrich) and plated either in T75 or T175 flasks (Becton Dickinson) at 5000 cells/cm^2 in basal medium with 10% FBS. The cells were trypsinised until the second passage (P2) and then cryopreserved in FBS with 10% DMSO.

The yield of adherent cells during these two passages was used to characterize the self-renewal capacity of the cells isolated towards the estimation of the cell doubling (CD) and the doubling time (DT) parameters. These values were calculated using the formula: CD = ln (Nf/ Ni)/ ln2; DT: time (days)/ CD, Nf being the final number of cells in the culture, and Ni the initial number.

Approximately 10^6 cells from passage two were thawed at 37°C and plated in a T75 flask. Cells were grown for two more passages prior to being used for the differentiation analyses.

Adipogenic differentiation

The cells obtained from the 6 sheep were seeded at 5000 cells/cm^2 in 24-well plates with a previously described adipogenic medium [39]. Four replicates were seeded for each sheep, two were cultured with growth (control) medium and the other two with the adipogenic medium. The medium was changed every 3 days, and the differ- entiation was maintained for 14 days. To analyse the adi- pogenic differentiation, cells were fixed in 10% formalin

Table 1 Cell surface, adipogenic, osteogenic, chondrogenic and neurogenic markers analysed by RT-qPCR

Genes	Accession number	Primer sequences		Amplicon size (bp)
		Forward (5' → 3')	Reverse (5' → 3')	
Cell Surface Markers				
CD29	AF349461	GTGCCCGAGCCTTCAATAAAG	CCCGATTTTCAACCTTGGTAATG	87
CD34	AB021662	TGGGCATCGAGGACATCTCT	GATCAAGATGGCCAGCAGGAT	107
CD45	NM_001206523	CCTGGACACCACCTCAAAGCT	TCCGTCCTGGGTTTTATCCTG	101
CD73	BC114093	TGGTCCAGGCCTATGCTTTTG	GGGATGCTGCTGTTGAGAAGAA	115
CD90	BC104530	CAGAATACAGCTCCCGAACCAA	CACGTGTAGATCCCCTCATCCTT	96
CD105	NM_001076397	CGGACAGTGACCGTGAAGTTG	TGTTGTGGTTGGCCTCGATTA	115
Differentiation Markers				
PPARG	NM_001100921	GCCCTGGCAAAGCATTTGTA	TGTCTGTCGTCTTTCCCGTCA	94
SCD[1]	AJ001048	CCCAGCTGTCAGAGAAAAGG	GATGAAGCACAACAGCAGGA	115
IL6	FJ409227.1	CAGCAAGGAGACACTGGCAG	TGATCAAGCAAATCGCCTGAT	101
COL1A1	AF129287	CCTGCGTACAGAACGGCCT	ACAGCACGTTGCCGTTGTC	93
BGLAP	DQ418490	CCCAGGAGGGAGGTGTGTG	CTAGACCGGGCCGTAGAAGC	99
BGN	NM_001009201.1	AACATGAACTGCATTGAGATGGG	GCGAAGGTAGTTGAGCTTCAGG	93
LUM	NM_173934.1	AAGCAATTGAAGAAGCTGCACA	TTAGTGAGCTGCAGGTCCACC	92
NES	194665083	CAAATCGCCCAGGTCCTG	GCCTCTAGGAGGGTCCTGTATGT	95
NEFM	194669578	GCTCGTCATCTGCGAGAATACC	CACCCTCCAGGAGTTTCCTGTA	91
NELF	27806522	CGCTATGCAGGACACAATCAAC	GGGTCTCCTCACCTTCCAAGA	161
TUBB3	116004470	GACCTCGAGCCTGGAACCAT	GCCCCACTCTGACCAAAGATG	92
MAP2	194664873	TGTCCCAGTGGAGGAAGGTTT	TCTTGTCTAGTGGCTCGGCTG	95
PRNP	BC119821	CGCAGAAGCAGGACTTCTGAA	TGGATTTGTGTCTCTGGGAAGA	86
Housekeeping genes				
G6PDH[2]	AJ507200	TGACCTATGGCAACCGATACAA	CCGCAAAAGACATCCAGGAT	76
HPRT[3]	EF078978	AGGTGTTTATTCCTCATGGAGTAATTATG	GGCCTCCCATCTCCTTCATC	79

GenBank accession numbers of the sequences used for primer design. Primer sequences (F: Forward and R: Reverse) and the length of the amplicon in base pairs (bp).
[1] Primers described in Dervishi et al. [60].
[2] Primers described in García-Crespo et al. 2005 [61].
[3] Primers described in Lyahyai et al. 2010 [62].

(Sigma-Aldrich) for 15 min, and lipid droplets formed inside the cells were stained with 0.3% oil red O (Sigma-Aldrich). The expression of the adipogenic markers *PPARG*, *SCD* and *IL6* was analysed at days 7 and 14 of culture using RT-qPCR.

Osteogenic differentiation

Cells from the 6 sheep were plated at 2 x 10^4 cells/cm^2 in 24-well plates and cultured under osteogenic condi- tions (two replicates) or with growth medium (two repli- cates) for 21 days as previously described [39]. To assess their osteogenic potential, cells at days 7, 14 and 21 were fixed in 70% ethanol for 1 h and stained with 2% Ali- zarin Red S (Sigma Aldrich) for 10 min. The transcript expression of the osteogenic markers *COL1A1* and *BGLAP* was evaluated by RT-qPCR at days 7, 14 and 21 of culture.

Chondrogenic differentiation

For chondrogenic differentiation in monolayer cultures (n = 5), 10^5 cells/cm^2 were seeded in 24-well plates with the chondrogenic media described by Jäger et al. [53] (two replicates) or with growth medium (two replicates). The culture was maintained for 21 days with the media being changed twice per week. To determine chondrogenic differentiation, the cultures were stained with alcian blue dye (Sigma-Aldrich). Briefly, cells were washed with PBS, fixed with 70% ethanol for 1 h at room temperature, washed three times with distilled water, stained with alcian blue stain diluted in methanol at a 1:1 proportion and washed with water until the ex- cess staining was removed. *BGN* and *LUM* transcripts were quantified at days 10 and 21 of chondrogenic in- duction using RT-qPCR.

Neuronal differentiation

The neurogenic potential of the isolated cells was tested in two cell lines. The cells were seeded at 2500 cells/cm^2 in 24-well plates with the neurogenic medium (Thermo Scientific) (two replicates) or under growth medium (two replicates) and maintained for 6 days, changing the media every 3 days. Differentiation was monitored by both light microscope and analysis of the mRNA expres- sion levels of neurogenic markers (*MAP2*, *NELF*, *NES*, *NEFM* and *TUBB3*) and *PRNP* by RT-qPCR at days 3 and 6.

Real Time quantitative PCR

The potential of cultured cells to differentiate into adipocytes, osteoblasts, chondrocytes and nervous cells was monitored via analysis of the expression levels of differentiation markers (Table 1) using RT-qPCR. The same methodology was used to evaluate the expression levels of cell surface markers for mesenchymal (*CD29*, *CD73*, *CD90* and *CD105*) and haematopoietic (*CD34* and *CD45*) stem cells in undifferentiated cells. The primers for RT-qPCR were designed using Primer Express 2.0 software (Applied Biosystems).

RNA extraction and cDNA synthesis were performed on both differentiated and control oPB-MSC cultures using the cells-to-cDNA kit (Ambion). The isolated cDNA was diluted 1:5 in water for further analysis. Amplification experiments were performed in triplicate using Fast SYBR Green Master Mix reagent (Life Tech- nologies) and the StepOne™ Real Time System (Life Technologies). The levels of gene expression were deter- mined using the comparative Ct method. A normalization factor (NF) calculated as the geometric mean of the quantity of two housekeeping genes (*GAPDH* and *HPRT*) was used to normalize the expression levels for each gene. Variations in gene expression between dif- ferentiated and control oPB-MSCs were evaluated with the Student's *t* test. Statistical significance was defined as $P < 0.05$.

Abbreviations

BGLAP: Bone Gamma-Carboxyglutamate (Gla) Protein; *BGN*: Biglycan; *CD105*: Endoglin; *CD29*: Integrin beta 1; *CD34*: CD34 molecule; *CD45*: Protein Tyrosine Phosphatase Receptor Type C; *CD73*: Ecto-5'-nucleotidase; *CD90*: Thy-1 cell surface antigen; *COL1A1*: Collagen type I alpha 1; FBS: Foetal Bovine Serum *IL6,* interleukin 6 (interferon, beta 2); *LUM*: Lumican; *MAP2*: Microtubule-Associated Protein 2; MSCs: Mesenchymal Stem Cells; *NEFM*: Neurofilament, Medium Polypeptide; *NELF*: Nasal Embryonic LHRH Factor; *NES*: Nestin; oAT-MSCs: ovine Adipose Tissue-Derived Mesenchymal Stem Cells; oBM-MSCs: ovine Bone Marrow-Derived Mesenchymal Stem Cells; oPB-MSCs: ovine Peripheral Blood-derived Mesenchymal Stem Cells; oMSCs: ovine Mesenchymal Stem Cells; *PPARG*: Peroxisome Proliferator-Activated Receptor Gamma; *PRNP*: Prion Protein; RT-qPCR: quantitative Real Time PCR; *SCD*: Stearoyl-CoA Desaturase (delta-9-desaturase); *TUBB3*: Tubulin Beta 3.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JL and DRM carried out the isolation and expansion of the cells, differentiation assays, gene expression analyses, statistical analysis and participated in drafting the manuscript. BR, AS and ARR helped in the culture and differentiation assays and manuscript drafting. RB performed the sample collections from the animals and participated in cell isolation. PZ and CR helped to draft the manuscript. IMB conceived the study, participated in its design and draft the manuscript. All authors read and approved the final manuscript.

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MANUSCRITO III

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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).

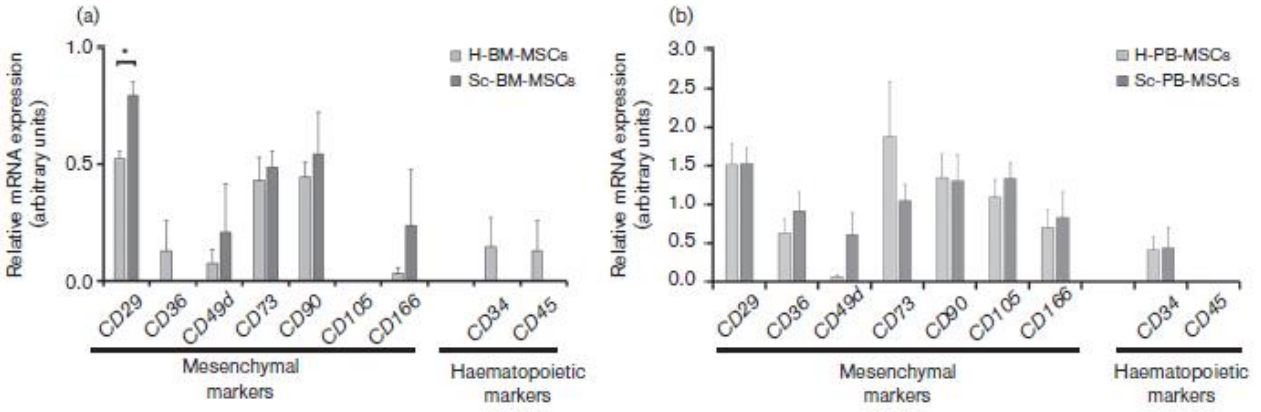


Fig. 1. Expression of cell surface markers. Gene expression of mesenchymal and haematopoietic cell surface markers quantified by qRT-PCR in (a) BM-MSCs and (b) PB-MSCs. Relative mRNA expression levels are expressed as mean ± SEM. Significant differences between healthy and scrapie-derived cultures were 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

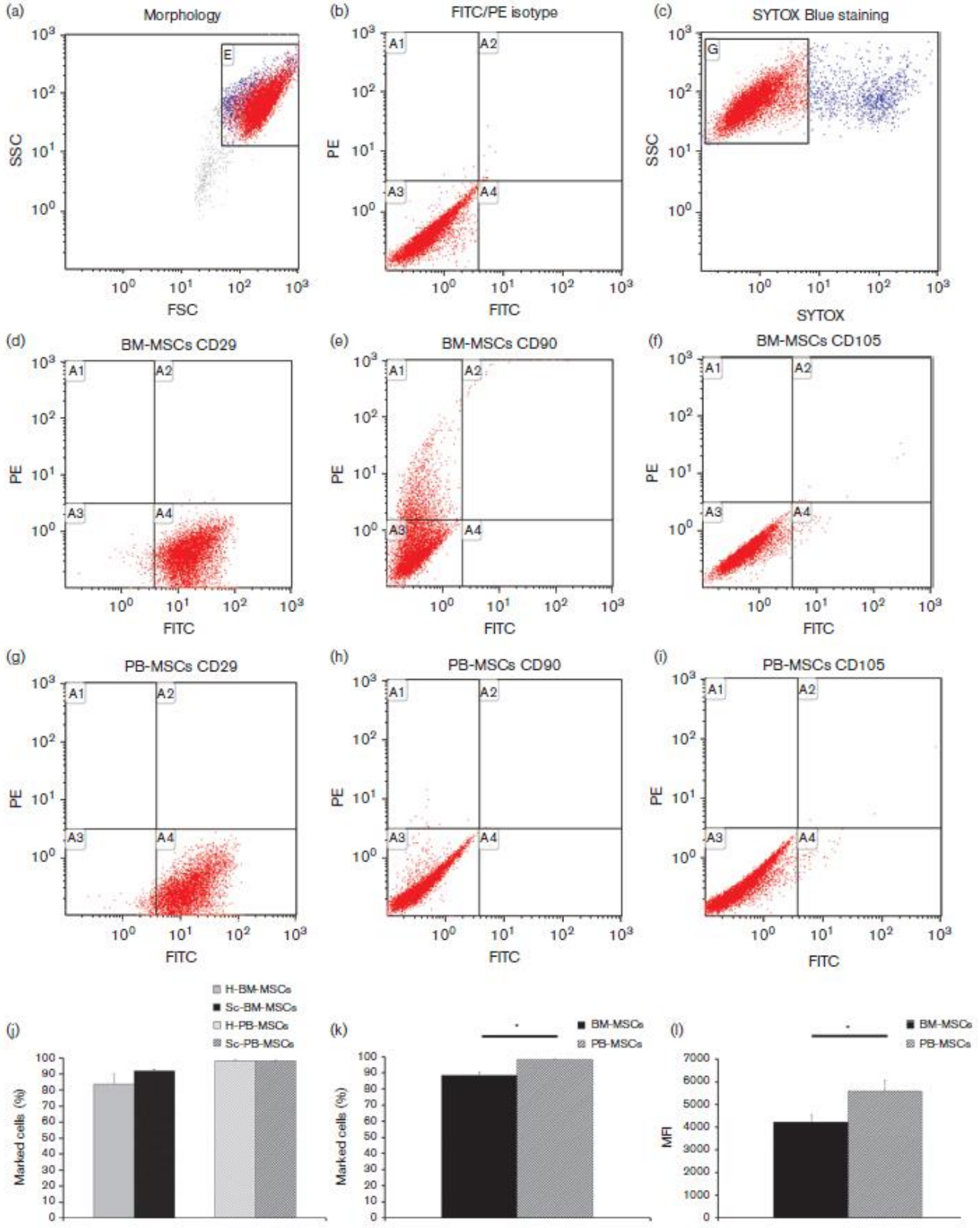

(a)
Morphology
SSC
FSC
R
(b)
FITC/PE isotype
PE
FITC
A1
A2
A3
A4
(c)
SYTOX Blue staining
SSC
SYTOX
G
(d)
BM-MSCs CD29
(e)
BM-MSCs CD90
(f)
BM-MSCs CD105
(g)
PB-MSCs CD29
(h)
PB-MSCs CD90
(i)
PB-MSCs CD105
(j)
H-BM-MSCs
Sc-BM-MSCs
H-PB-MSCs
Sc-PB-MSCs
Marked cells (%)
(k)
BM-MSCs
PB-MSCs
Marked cells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

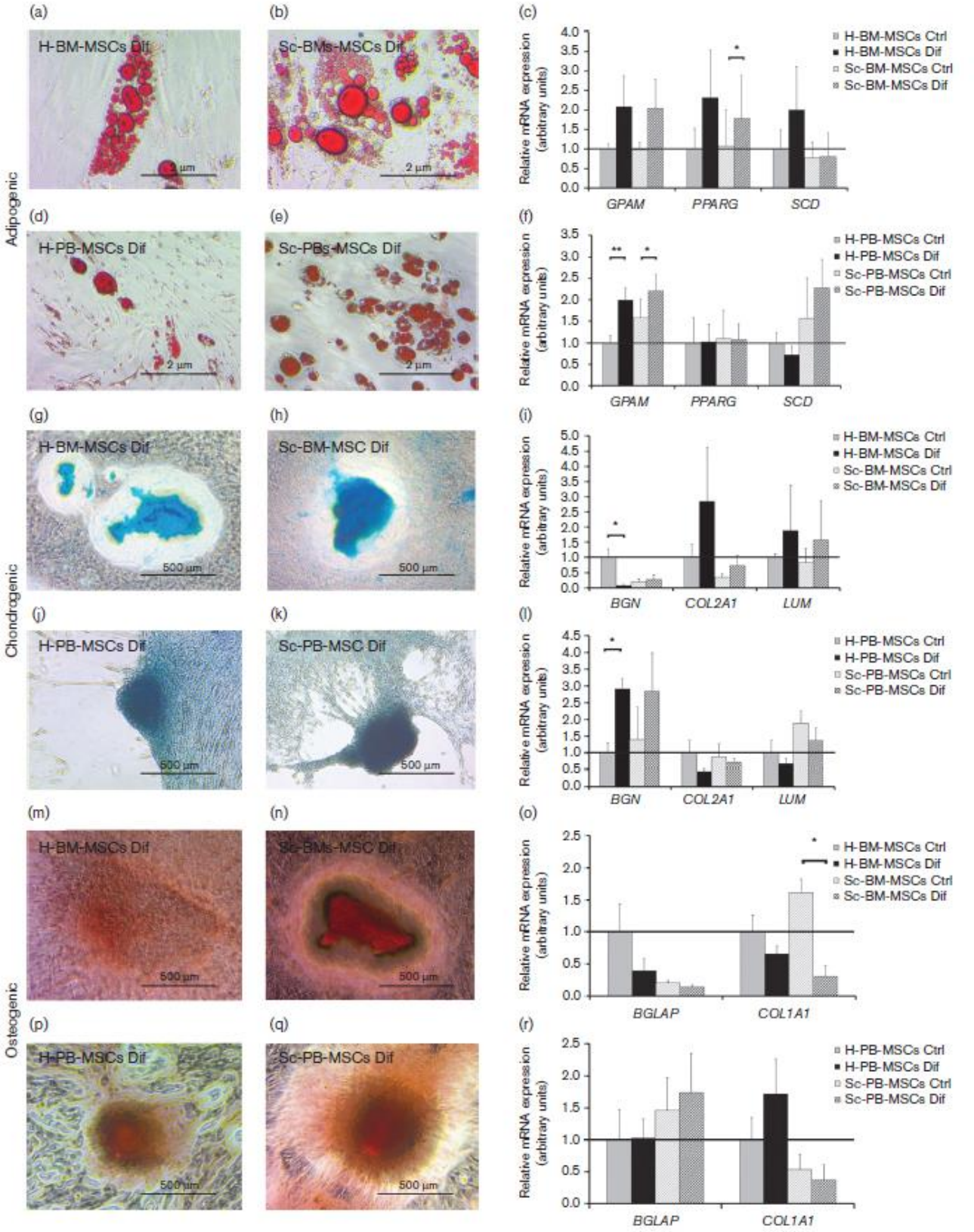
Determination of PrP^{Sc} in MSCs

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


Adipogenic
Chondrogenic
Osteogenic
(a)
H-BM-MSCs Dif
2 µm
(b)
Sc-BMs-MSCs Dif
2 µm
(c)
Relative mRNA expression (arbitrary units)
GPAM
PPARG
SCD
H-BM-MSCs Ctrl
H-BM-MSCs Dif
Sc-BM-MSCs Ctrl
Sc-BM-MSCs Dif
(d)
H-PB-MSCs Dif
2 µm
(e)
Sc-PBs-MSCs Dif
2 µm
(f)
GPAM
PPARG
SCD
H-PB-MSCs Ctrl
H-PB-MSCs Dif
Sc-PB-MSCs Ctrl
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(h)
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(i)
BGN
COL2A1
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H-BM-MSCs Dif
500 µm
(n)
Sc-BMs-MSC Dif
500 µm
(o)
BGLAP
COL1A1
(p)
H-PB-MSCs Dif
500 µm
(q)
Sc-PB-MSCs Dif
500 µm
(r)
BGLAP
COL1A1

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.

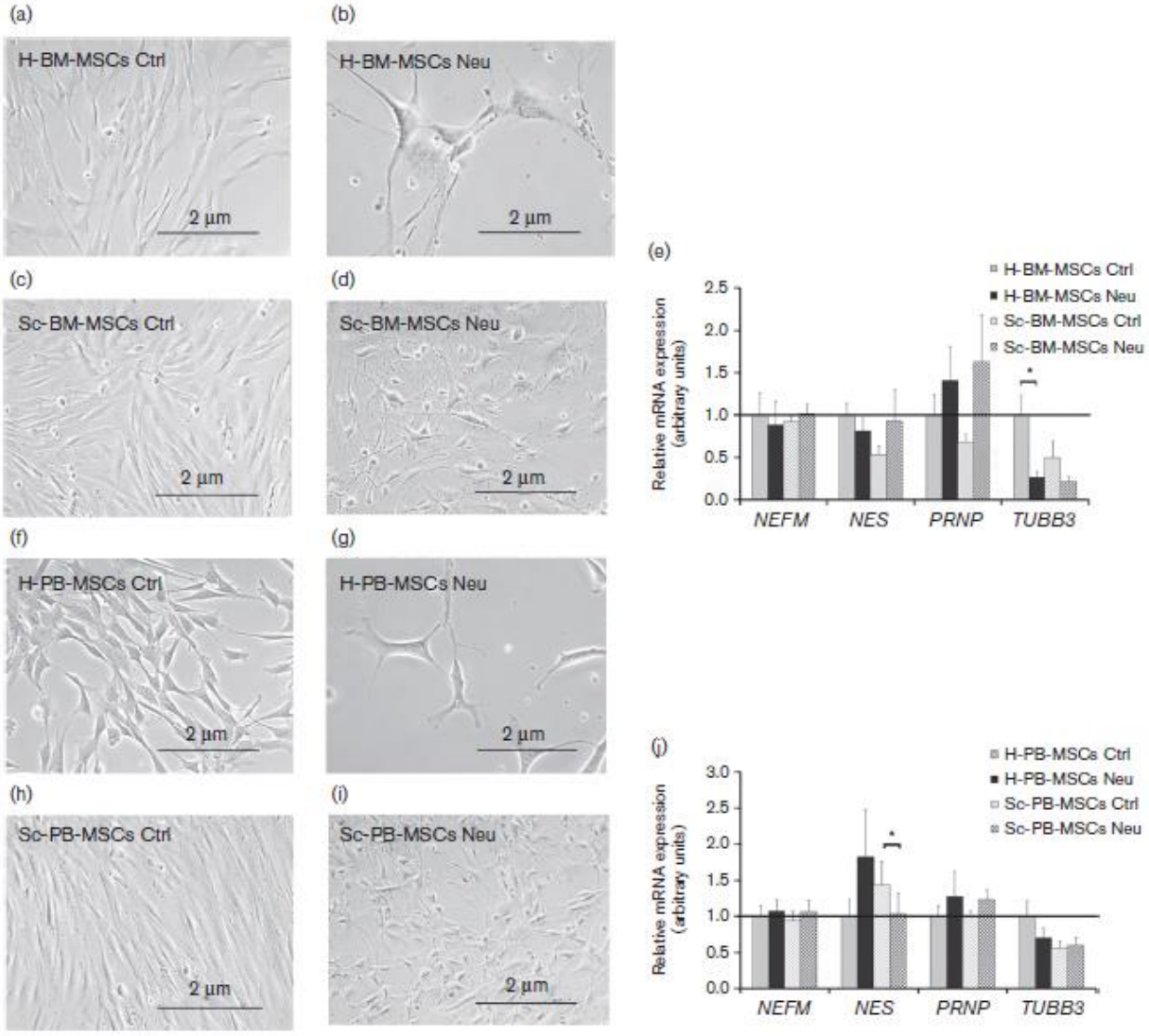

Fig. 4. Neurogenic differentiation of BM- and PB-MSCs of healthy and scrapie-infected sheep. Phase-contrast micrographs of H-BM-MSCs under (a) basal and (b) neurogenic conditions, (c) basal Sc-BM-MSCs, (d) differentiated Sc-BM-MSCs, (f) basal H-PB-MSCs, (g) differentiated H-PB-MSCs, (h) basal Sc-PB-MSCs, 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.

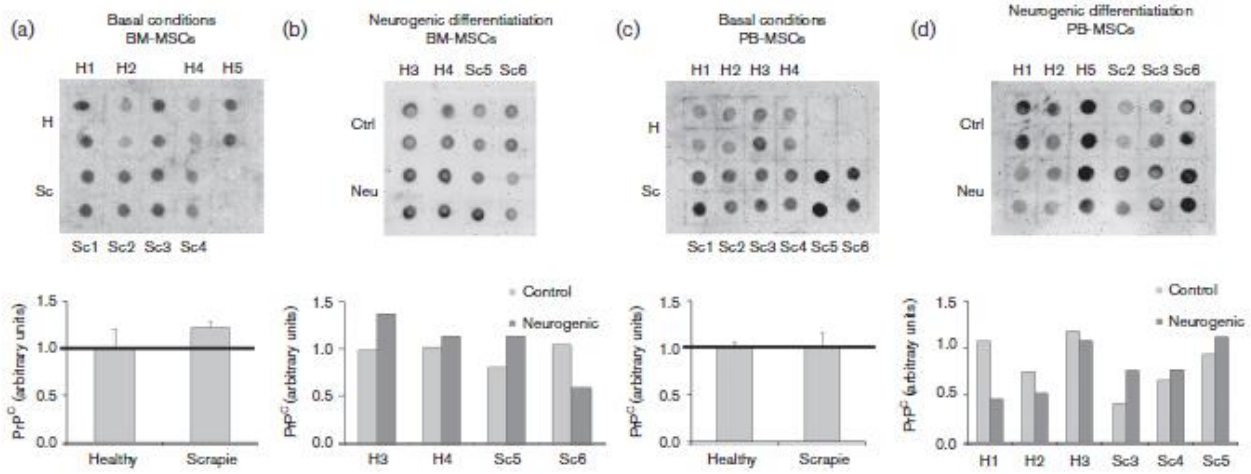

Fig. 5. Expression of PrP^C by MSCs isolated from healthy and scrapie-infected sheep under basal and neurogenic conditions. (a) Dot-blots showing PrP^C expression in H-BM-MSCs and Sc-BM-MSCs under basal conditions, and bar chart showing the PrP^C quantification in healthy and scrapie-infected groups 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^C 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′-CAAACGGTGGTTGCATTGACTT-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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MANUSCRITO IV

Bone marrow derived ovine mesenchymal stem cells do not propagate scrapie infection but show cytotoxicity

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Abstract

Scrapie is a prion disease that affects sheep and goats and it is considered the prototype of transmissible spongiform encephalopathies (TSEs). Mesenchymal stem cells (MSCs) have been described as candidates for developing *in vitro* models of prion diseases. Murine MSCs are able to propagate prions after adaptation to mouse. Although ovine MSCs (oMSCs) express the prion protein, their susceptibility to prion infection has never been investigated. We investigate here the potential of bone marrow derived oMSCs to be infected by natural scrapie and to propagate infectivity. In addition, the possible toxic effect of scrapie infection has been investigated analysing the proliferation potential of infected cells and the expression of genes involved in apoptosis regulation. Cultures were maintained for 48 hours in contact with ovine classical scrapie or control brain homogenates. Ovine MSCs do maintain detectable levels of PrP^{Sc} by Western Blotting on the three first passages, although the signal weakened and was not detectable afterwards. Ovine MSCs infected with scrapie displayed lower cell doubling and higher doubling time at the first passage after infection than those infected with control brain. Finally, the expression of apoptotic markers was analysed by real time quantitative PCR, significant upregulation of *AIFM1*, *BCL2L1*, *FAS* and *MCL1* was observed in scrapie infected oMSCs. Our results suggest that bone marrow derived oMSC do not replicate prions; on the contrary, the infection with scrapie seems to be toxic for these cells.

Keywords: Scrapie, prion, mesenchymal stem cell, sheep, infection**,** apoptosis

Introduction

Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases that affect both humans and animals. These diseases are characterized by the accumulation of PrP^{Sc}, an abnormal isoform of the cellular prion protein (PrP^{C}), mainly in the central nervous system (Prusiner, 1998). Scrapie is a TSE that affects sheep and goats and is considered the prototype of these diseases.

Cell cultures are valuable tools for the study of prion protein propagation in TSEs and the evaluation of the capacity of certain drugs to inhibit prion protein accumulation. However, few cell lines can be infected and show PrP^{Sc} accumulation and/or infectious capacity. Murine cell lines are used in most cases and previous mouse-adaptation of prion strain is required to eliminate the problem of species barrier (Solassol et al., 2003).

Mesenchymal stem cells (MSCs) are fibroblast-like cells characterized by their ability to both self-renewal and differentiation in mesodermal tissues (osteoblasts, adipocytes, chondrocytes and myocytes) (Pittenger et al., 1999). In addition, these cells can transdifferentiate *in vitro* into cells with neurogenic origin (Woodbury et al., 2002; Zhao et al., 2002) and undifferentiated cells express PrP^{C} (Takakura et al., 2008). Murine bone marrow derived mesenchymal stem cells (BM-MSCs) can be infected with a Gerstmann-Sträussler-Schneiker strain previously adapted in mouse *ex vivo* (Akimov et al., 2009) and maintain the infectivity along passages. The susceptibility of these cells to prion infection makes them good candidates to develop *in vitro* models for prion research (Mediano et al., 2015). The easy access to MSC niches would facilitate the development of *in vitro* models from naturally susceptible species like humans or ruminants, which would avoid the adaptation process.

We have recently described the isolation of ovine MSCs from peripheral blood (oPB-MSCs), these cells expressed PrP^C at the transcript level (Lyahyai et al., 2012). Our group also described the presence of PrP^C on bone marrow derived ovine MSCs (oBM-MSCs) at both transcript and protein levels (Mediano et al., 2015). In the same study, a diminished proliferation potential of scrapie sheep derived oBM-MSCs was observed when compared to healthy sheep derived oBM-MSC proliferation potential. The presence of PrP^{Sc} could not be confirmed after PMCA assay in scrapie sheep derived oBM-MSCs. However, their susceptibility to scrapie infection *in vitro* and their potential to replicate prions has never been investigated. The aim of the present study was to evaluate the potential of oBM-MSCs for prion infection and replication *in vitro* and the possible toxic and apoptotic effects of this infection.

Materials and methods

Scrapie inocula

Medulla oblongata belonging to a healthy (negative controls) and a classical scrapie infected sheep from the tissue bank of the Research Center on Encephalopathies and Transmissible Emerging Diseases (CIEETE; University of Zaragoza) were used to prepare inocula. The presence/absence of PrP^{Sc} in the medullas was studied following protocols reported in other works (Bolea et al., 2005), using immunohistochemical examination of brain tissue and two rapid diagnostic tests (Prionics-Check Western blot and Idexx Herd Chek®). Medulla oblongata samples were homogenized and diluted 1:10 (g/mL) in physiological saline solution (Braun). Afterwards, samples were treated at 70ºC for 10 minutes before adding Streptomycin sulphate (100 μg/mL) and Benzylpenicillin (100 μg/mL). In order to check the quality of the inocula once

generated, samples were incubated in blood agar plates, and the absence of any bacterial growing was confirmed.

Animals and sample collection

Bone marrow (n=9) samples were obtained from 9 adult *Rasa Aragonesa* female sheep with the ARQ/ARQ genotype for the *PRNP* gene, which is the most frequent genotype in the scrapie cases described in this breed (Acin et al., 2004). Three of the animals were scrapie free sheep and six were scrapie affected sheep.

After animal sedation (Xylazine IV) and local anaesthesia (Lidocaine) bone marrow aspirates were harvested from humeral head using a 13 G Jameshdi needle and 10 mL syringes previously loaded with 5000 UI of sodium heparin. All procedures were carried out under Project Licence PI06/12 approved by the in-house Ethic Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were 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.

Naturally scrapie affected sheep showed clinical signs of the disease and were sacrificed after sample collection. ELISA test was performed to confirm these sheep as scrapie affected animals. The three healthy animals did not display any clinical sign compatible with scrapie. Nevertheless, an *in vivo* test for PrP^{Sc} determination in third-eyelid biopsies was performed as previously described (Vargas et al., 2006) to avoid the use of scrapie infected preclinical sheep.

MSC culture

MSC cultures used in this work have been previously characterised (Mediano et al., 2015). Briefly, MSC isolation from bone marrow aspirates (3-5 mL) was performed as previously described (Lyahyai et al., 2012; Mediano et al., 2015; Ranera et al., 2012). This protocol is based on the separation of the mononuclear fraction after a density gradient centrifugation in Lymphoprep (Atom) and a further isolation thanks to the adherence of MSCs to plastic. Cells were expanded in basal medium, consisting of low glucose Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS), 1% L-Glutamine (Sigma-Aldrich) and 1% Streptomycin/Penicillin (Sigma-Aldrich), until passage 3 for further characterization and prion infection.

In addition to plastic-adherence in 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 ability to differentiate to mesodermal lineages and the expression of mesenchymal and haematopoietic markers was evaluated as previously described (Mediano et al., 2015).

The expression of PrP^C in oBM-MSCs was confirmed by RT-qPCR and Dot-Blotting following standard procedures as previously described (Mediano et al., 2015).

Scrapie infection of cell cultures and propagation

Three oBM-MSCs cultures at passage 3 were seeded at 5000 cels/cm^2 in P6 plates. After adhesion for 24h, basal media was substituted by inocula diluted in 1:10 in DMEM media (10% FBS, 1% L-Glutamine and 1% Streptomycin/Penicillin). Cells were maintained in this media for 48h. Afterwards, medium was changed twice a week.

Cultures were maintained until passage 3 post-infection, every passage was performed at around 80% confluence. Approximately 10^6 cells of passages 1, 2 and 3 post-infection were frozen at -80ºC for further PrP^{Sc} determination by Western-Blotting.

Western Blotting determination of PrP^{Sc}

Pellets of 10^6 cells were homogenized in 100 µL PBS. Afterwards, samples were analysed using the BSE Scrapie Discriminatory Kit (Bio-Rad) and treated following manufacturer recommendations. Electrophoresis was developed in 12% SDS-PAGE gels. Afterwards, protein was transferred to 0.20 µm nitrocellulose membrane (Bio-Rad).

Gene expression of apoptosis related genes

The possible cytotoxicity of prion infection was evaluated through the gene expression analysis of apoptotic markers in 3 oBM-MSCs cultures derived from healthy sheep and 6 cultures derived from scrapie affected sheep. Infection with control and scrapie sheep brain inocula was performed as described above and the cultures were maintained during a week after inoculation. Then 10^6 cells were frozen for further analysis. The apoptotic markers analysed were: Mitochondrion Associated Apoptosis-Inducing Factor 1 (*AIFM1*), Apoptosis Regulator Bcl-2-Like Protein 1 (*BCL2L1*), Fas Cell Surface Death Receptor (*FAS*), Myeloid Cell Leukemia 1 (*MCL1*) Apoptotic markers BCL2-Antagonist/Killer 1 (*BAK1*), BCL2-Associated X Protein (*BAX*) and B-Cell CLL/Lymphoma 2 marker (*BCL2*). The expression of apoptotic markers was evaluated at the transcript level by RT-qPCR using primers and conditions described previously (Lyahyai et al., 2006; Lyahyai et al., 2007; Lyahyai et al., 2012; Serrano et

al., 2009). Briefly, total RNA from approximately 200,000 cells was extracted and converted to cDNA using Cells-to-cDNA™ II Kit (Ambion). The synthesised cDNA was diluted 1:5 in water for further analysis. Amplifications were performed in triplicate using Fast SYBR Green Master Mix reagent (Life Technologies) and the StepOne™ Real Time System (Life Technologies). Gene expression was determined by comparative Ct method. A normalization factor calculated as the geometric mean of the quantity of two housekeeping genes (*G6PDH* and *HPRT*) was used to normalize the expression levels for each gene using primers and conditions described elsewhere (Lyahyai et al., 2012).

Differences in gene expression between groups were evaluated with the Student's t test and Mann-Witney U test depending on the parametric or non-parametric distribution of expression profiles. Statistical significance was defined as $P < 0.05$.

MSC proliferation potential

The effect of scrapie infection in the proliferation potential was evaluated in oBM-MSCs derived from control sheep. Adherent cells were counted through passages 1 to 3 after infection and Cell Doubling (CD) and Doubling Time (DT) features were calculated as previously described (Lyahyai et al., 2012). The results were evaluated with a paired Student's t test comparing the data from oBM-MSCs cultures after being exposed to healthy and scrapie affected sheep brain inocula.

Results

The mesenchymal characteristics of the cells used in this work were previously confirmed (Mediano et al., 2015). The tri-lineage differentiation ability was verified as well as the expression of mesenchymal markers *CD29*, *CD73*, *CD90* and *CD105* and the lack of expression of haematopoietic marker *CD45* and *CD34*. Therefore, the ovine cells used for further analyses met the minimal criteria of plastic attachment, pluripotency and expression of mesenchymal cell surface markers. In addition, the expression of the prion protein was confirmed by dot blotting showing high variability between cultures (Mediano et al., 2015).

Prion infection

After incubation of oMSCs with the inocula for 48h., survival cells retained their ability to proliferate and were expanded until passage 3 post-infection. Western-Blotting analysis revealed the presence of PrP^{Sc} in the cultures during these three passages although the intensity of bands decreased with the number of passages (Fig1) and was lost in further subculture (data not shown).

Apoptosis markers

The expression of apoptotic markers was analysed in both healthy and scrapie affected sheep derived BM-MSCs after infection with scrapie and control inocula. A high variability was observed in the expression levels of these markers. Significant differences between control and scrapie infected cultures were only observed in oBM-MSCs derived from healthy sheep. In these cultures the pro-apoptotic markers *AIFM1* and *FAS* and the anti-apoptotic genes *BCL2L1* and *MCL1* were significantly up-regulated ($P<0.05$) in those infected with scrapie brain (Fig 2) and a trend to

upregulation ($P<0.1$) was observed for the pro-apoptotic gene *BAX* in the same cultures.

Proliferation of infected cells

As regulation of apoptosis genes was observed only in oBM-MSCs derived from healthy sheep, the effect of scrapie infection in the proliferation potential was analysed in this source of oBM-MSC. Through the analysis of CD and DT the proliferation potential was evaluated in cultures infected with brain homogenates obtained from healthy and scrapie infected sheep. Significant differences were found for both CD and DT at the first passage after infection. CD was higher and DT was lower in the cultures treated with the scrapie inoculum infected cells compared to those infected with healthy sheep brain inoculum (Table1).

Discussion

Prion diseases are fatal neurodegenerative processes that affect animals and humans. During the last decade a great effort has been made to obtain *in vitro* models for the study of these pathologies. Most of the cellular models are based on the culture of murine cell lines (Solassol et al., 2003) and require a previous adaptation of the strain to mouse, due to the well-known phenomenon of the species barrier. Then, *in vitro* models with natural host background would be very useful tools for research in many prion topics, e.g.: prion replication, toxicity, genetic susceptibility, differences in strain susceptibility and new treatment testing.

MSCs can be easily obtained from many accessible adult tissues like bone marrow or even peripheral blood (Kaneko et al., 1982; Zvaifler et al., 2000) and they show the ability to transdifferentiate into neuronal elements *in vitro* (Sanchez-Ramos et al., 2000; Woodbury et al., 2002). Several works have described the ability of stromal cells with a murine origin to propagate prion infectivity (Akimov et al., 2009; Akimov et al., 2008; Cervenakova et al., 2011; Takakura et al., 2008). As an example, murine bone marrow derived MSCs can be persistently infected with the Fu TSE strain (Cervenakova et al., 2011). Although MSCs derived from human, cattle and sheep express PrP^{C} (Mediano et al., 2015; Takakura et al., 2008), to the best of our knowledge, the potential of MSCs derived from naturally susceptible species to be infected and propagate prion infection has never been investigated.

In this work we infected ovine bone marrow derived MSCs whose mesenchymal properties were previously confirmed evaluating the tri-lineage differentiation ability and the expression of cell surface markers (Mediano et al., 2015). Once the expression of the prion protein was confirmed, cells were infected keeping in contact cultures with brain extracts. MSCs were maintained in culture and Western Blotting revealed the presence of PrP^{Sc} in scrapie infected cultures three passages after inoculation, which indicates that PrP^{Sc} is retained in the cell culture during these passages.

Stromal cells with a murine origin are able to propagate prions for many passages (Akimov et al., 2009; Akimov et al., 2008). On the contrary, oMSCs do not seem to be permissive to PrP^{Sc} infection as the presence of the pathologic protein seemed to weaken between passage 2 and 3 (Fig. 1). In some works, murine BM-MSCs infected with prions *in vitro* display very little or no PrP^{Sc} production during the first 10 or even 50 passages (Cervenakova et al., 2011; Takakura et al., 2008) and a stable and Western Blotting detectable production of PrP^{Sc} afterwards. Contrary to murine cells,

MSCs obtained from humans or unconventional model organisms can be maintained in culture for many fewer passages (Bonab et al., 2006; Calloni et al., 2014). In our conditions, at passage 10 oMSCs displayed the effects of aging (data not shown).

PrP^{Sc} seemed to be uptake by oMSC but it did not lead to successful prion infection. Ovine MSC cultures were maintained under expansion conditions and displayed a high proliferation rate, with an average doubling time for the culture maintained for prion propagation study of 2.7 ± 1.86 days. Cell division modulates prion accumulation in cultured cells (Ghaemmaghami et al., 2007) and direct proximity between donor and recipient cells increases the infection in other cell culture models (Kanu et al., 2002), the high proliferation rate observed in ovine MSCs could avoid transmission of PrP^{Sc} from infected cells to non-infected ones because they cannot be in contact enough time. Therefore, only the cells infected during the inoculation process and their daughters would show infection and it will get diluted in successive passages. Changes in culture conditions focused on slowing down proliferation rate could facilitate propagation.

On the other hand, we cannot discard that infection with scrapie inocula could result toxic for the culture. Although no toxicity was observed in murine MSCs infected with the CJD agent (Takakura et al., 2008), we have to bear in mind that our cells come from a naturally susceptible species.

Toxicity occurs in some primary neuronal cultures and neuronal cell lines after infection with different prion strains through the activation of apoptosis (Hannaoui et al., 2013). In our study we investigated a possible induction of apoptosis analysing the expression of genes involved in this programmed cell death. Significant overexpression of *AIFM1*, *BCL2L1*, *FAS* and *MCL1* was observed in healthy sheep derived oBM-

MSC infected with scrapie brain inoculum. Although both proapoptotic genes like *AIFM1* and *FAS* and antiapoptotic genes like *BCL2L1* and *MCL1* were modified, similar results have been observed in the central nervous system of sheep naturally infected with scrapie (Serrano et al., 2009), suggesting that a differential regulation of the apoptotic processes is occurring *in vitro* after inoculation with scrapie. A great number of studies performed both *in vivo* (Hedman et al., 2012; Lyahyai et al., 2006; Lyahyai et al., 2007; Shi et al., 2014) and *in vitro* (Wang et al., 2012) have demonstrated the induction of the mitochondrial pathway of apoptosis by prions through the induction of *BAX*. In our study, this pro-apoptotic gene displayed a trend to overexpression in cells infected with scrapie, which would be in accordance with a possible induction of apoptosis. On the other hand, it is known that overexpression of the proto-oncogene product Bcl-2 can protect neuronal cells against the apoptotic cell death induced by different agents (Adams and Cory, 2007; Cory and Adams, 2002; Lawrence et al., 1996; Michaelidis et al., 1996). Moreover, the level of Bcl-2 protein in neurons, infected with a prion fraction, drops to 82% after an incubation period of 9 days, after a transient increase at days 3 and 6 (Perovic et al., 1996; Perovic et al., 1997). The expression of this marker, similar to the findings observed in ovine scrapie (Lyahyai et al., 2006; Serrano et al., 2009) and human CJD (Siso et al., 2002), did not significantly modify its expression in infected cells.

Finally, we wanted to test if the differences observed in apoptosis marker expression could lead to a decrease in the number of cells analysing the proliferation potential of MSCs obtained from control sheep infected with scrapie. In our study CD was significantly higher in cells infected with healthy sheep brain inoculum and accordingly, DT was higher in cells infected with scrapie. Then, those cells exposed to the scrapie agent displayed lower proliferation potential similar to the findings observed

in MSCs obtained from scrapie sheep (Mediano et al., 2015), this could be a consequence of a lost of infected cells due to prion toxicity. Through passages, CD and DT differences were lower, together with the loss of PrP^{Sc} detection, it might indicate a restoration of the cell culture condition after elimination of PrP^{Sc} infected cells, increasing the proportion of non-infected cells with passages, which display higher proliferation potential. Further analyses are necessaries to confirm and identify apoptotic cells in ovine MSC cultures after scrapie infection.

Conclusions

This work describes for the first time the infection with scrapie of MSCs obtained from a natural host of the disease. In our conditions, ovine MSCs derived from bone marrow were not permissive to prion infection but seemed to be susceptible to prion toxicity. This ability could be useful for the study of differences in genetic susceptibility to different scrapie isolates or to analyse the molecular mechanisms of prion toxicity.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Figure legends

Fig1. Infection of ovine MSCs with scrapie. Determination of PrP^{Sc} by Western Blotting after proteinase K digestion of oBM-MSC from passage 1 to 3 infected with classical scrapie affected sheep brain inocula.

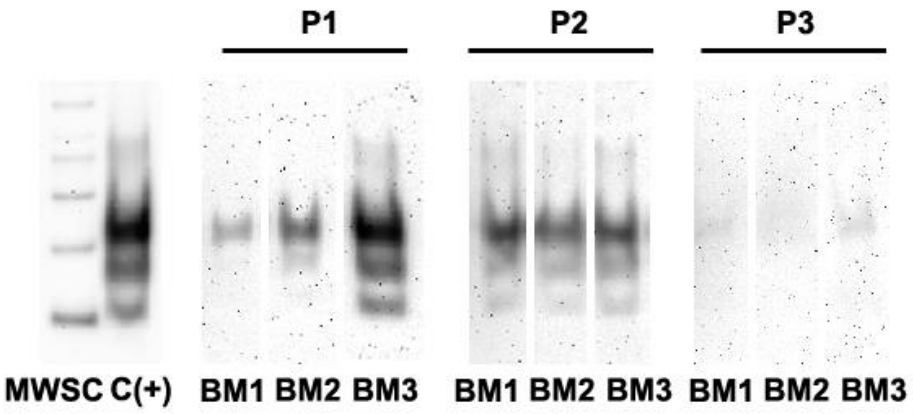

Fig2. Apoptotic markers quantified by Rt-qPCR in 3 healthy sheep derived oBM-MSC after being exposed to healthy sheep brain extract (H-oBM-I(-)) and scrapie affected sheep brain (H-oBM -I(+)). Relative mRNA expression levels are expressed as mean ± s.e. Significant differences were calculated with the Student t test (* P<0.05, ■ P<0.1) and Mann-Withney U test (* P<0.05).

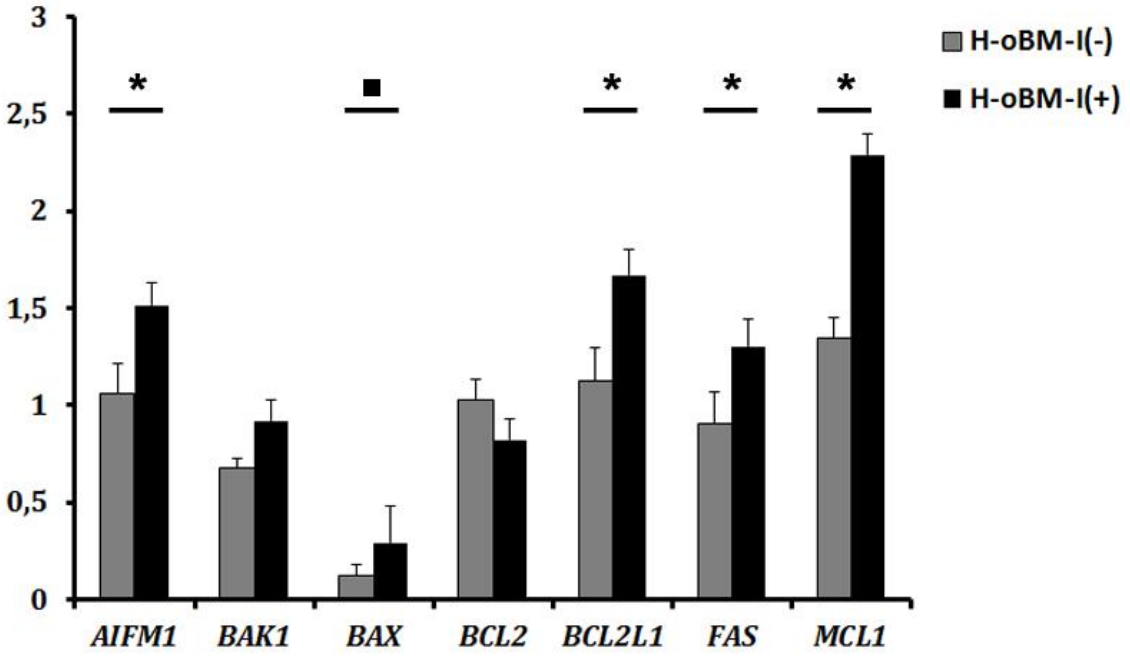

Table 1. CD and DT of o-BM-MSC through passages 1 to 3 post-infection. Significant differences were calculated with the Student t test (* P<0.05, **P<0.01)

Passage		Inoculum	
		Healthy	**Scrapie**
1	**CD**	3,150±0,286*	2,949±0,219*
	DT (days)	1,714±0,355**	1,825±0,343**
2	**CD**	3,22±0,651	2,870±0,531
	DT (days)	2,054±0,653	2,291±0,681
3	**CD**	1,93±0,390	1,807±0,027
	DT (days)	2,116±0,428	2,214±0,033

DISCUSIÓN GENERAL

El scrapie ovino es una enfermedad neurodegenerativa para la cual no existe tratamiento y que está causada por una acumulación de PrP^{Sc} en el SNC. A pesar de que en las últimas décadas se ha avanzado mucho en el conocimiento de esta enfermedad, varias incógnitas necesitan aún ser resueltas, como los mecanismos de replicación, toxicidad celular, las diferencias genéticas que causan diferentes grados de susceptibilidad, la barrera de especie o la naturaleza de las cepas priónicas. Los modelos celulares pueden facilitar las investigaciones necesarias para elucidar estas cuestiones. Hasta el momento, la mayoría de los cultivos celulares utilizados en el estudio de las enfermedades priónicas se han basado en líneas celulares de origen murino. Estos cultivos requieren una previa adaptación de los inóculos priónicos a esta especie. En este trabajo nos planteamos utilizar células mesenquimales ovinas para la posible replicación de scrapie, el estudio de la toxicidad de la infección o analizar sus características en individuos afectados por la enfermedad.

Antes de comenzar este trabajo de tesis doctoral, dos estudios habían demostrado que las MSC murinas expresaban PrP^{C} y podían ser infectadas por priones *in vitro* (Takakura *et al.*, 2008; Cervenakova *et al.*, 2011). En el **Manuscrito 1** de esta tesis doctoral se ha realizado un trabajo de revisión bibliográfica para analizar el potencial de las MSC como modelo de estudio para estas enfermedades neurodegenerativas. Las MSC procedentes de médula ósea son capaces de transdiferenciarse a neurona, característica importante para desarrollar modelos para el estudio de las EET. A pesar de que las MSC murinas son susceptibles a la infección por proteína priónica, y que se detectó la forma patológica del prión en MSC procedentes de dos pacientes afectados por CJD (Takakura *et al.*, 2008), la capacidad de las MSC ovinas para ser infectadas por proteína priónica o la presencia de la misma en células de animales naturalmente afectados por la enfermedad de scrapie no se había estudiado con anterioridad.

Asimismo, en el estudio presentado en el **Manuscrito 1** se revisó el potencial terapéutico de las MSC para el tratamiento de las enfermedades neurodegenerativas. Una de las ventajas de las MSC para ser utilizadas en este tipo de terapias es la posibilidad de su aislamiento a partir de numerosos tejidos de individuos adultos de fácil acceso. El grupo de investigación del Dr. Song describió el incremento en el tiempo de supervivencia de ratones infectados con scrapie tras el tratamiento con MSC

(Song *et al.*, 2009). Antes de la posible aplicación del tratamiento con MSC en la especie humana es necesario comprobar que la enfermedad no afecte a las características de estas células, lo cual complicaría el uso de trasplantes autólogos, y comprobar si las MSC pueden transportar proteína prión para poder ser administradas con seguridad en el caso de trasplantes alogénicos. En este trabajo hemos valorado su potencialidad tomando el ovino infectado con scrapie como modelo natural de EET.

Una fuente de fácil acceso para la obtención de MSC es la sangre periférica. Se ha documentado el aislamiento de MSC a partir de sangre periférica en varias especies (Zvaifler *et al.*, 2000; Koerner *et al.*, 2006). En el **Manuscrito 2** se describe por primera vez el aislamiento y caracterización de MSC ovinas a partir de este tejido. Las PB-MSC ovinas cumplen con los criterios mínimos que definen las MSC, como son la adherencia al plástico en condiciones estándar de cultivo, la expresión de marcadores como CD29, CD73, CD90 y CD105 a nivel de transcritos, la falta de expresión de marcadores hematopoyéticos como CD34 y CD45 y por último la capacidad para diferenciarse a células del linaje mesodérmico. La expresión de marcadores característicos de MSC a nivel proteico tanto de BM-MSC como de PB-MSC se confirmó durante la realización del **Manuscrito 3**, comprobándose la correlación entre los resultados obtenidos mediante RT-qPCR y citometría de flujo.

La proporción de MSC contenida en sangre fue muy variable entre individuos (**Manuscrito 2**), así como también la capacidad de proliferación de las PB-MSC en cultivo. Sin embargo, esta heterogeneidad se da también entre las BM-MSC ovinas (Rhodes *et al.*, 2004), lo cual parece indicar que esta heterogeneidad no depende del tejido, sino de que existe una gran variabilidad entre individuos de la misma especie. La capacidad de diferenciación de las PB-MSC a linajes mesodérmicos también mostró variabilidad individual. La diferenciación adipogénica, condrogénica y osteogénica de las PB-MSC se evaluó mediante la cuantificación de la expresión de marcadores específicos de diferenciación usando RT-qPCR, y mediante tinciones específicas.

La capacidad de las PB-MSC para transdiferenciarse a neurona se evaluó mediante la observación al microscopio de cambios morfológicos y la expresión de marcadores específicos mediante RT-qPCR (**Manuscrito 2**). Durante su estudio se ha comprobado que las PB-MSC diferenciadas a neurona aumentan la expresión de *PRNP*. Como consecuencia del desarrollo de este estudio podemos concluir que las MSC

pueden obtenerse a partir de sangre periférica también en ovino y podrían ser candidatas a la elaboración de modelos para el estudio de las EET. Es el primer trabajo de este tipo desarrollado en una de las especies susceptibles a las enfermedades priónicas, evitando así la necesidad de tener que adaptar el aislado priónico a la especie murina.

Las MSC han sido propuestas como un tratamiento potencial de trasplante autólogo para muchas enfermedades neurodegenerativas (Colpo *et al.*, 2015), incluidas las enfermedades priónicas (Song *et al.*, 2011). Para investigar la posible repercusión de las enfermedades priónicas en las características de estas células, se han caracterizado las MSC de ovinos afectados por la enfermedad de scrapie en fase clínica y se han comparado con las obtenidas de ovinos sanos (**Manuscrito 3**). Al comparar la cantidad de MSC obtenidas a partir de sangre periférica y de médula ósea no se observaron diferencias entre los animales sanos y los animales afectados por scrapie. Por tanto, las MSC no parecen movilizarse en la fase clínica de la enfermedad, a pesar de que se ha evidenciado que los encéfalos de ratón infectado por priones segregan factores de quimioatracción, que reclutan MSC en áreas del SNC lesionadas durante la replicación del prión (Song *et al.*, 2011). No obstante, para las BM-MSC y PB-MSC obtenidas a partir de animales afectados por scrapie se observaron diferencias significativas en la capacidad de proliferación celular, con un menor parámetro CD, durante los primeros tres pases de replicación. Esto indicaría una menor capacidad de proliferación de estos dos tipos de células.

Del mismo modo, no se observaron diferencias en los perfiles de expresión de la mayoría de los marcadores de superficie específicos para MSC a nivel de transcritos. Durante la realización del trabajo publicado en el M**anuscrito 3,** la única diferencia significativa en la expresión de marcadores de superficie para MSC fue una sobreexpresión de *CD29* en las BM-MSC obtenidas de animales afectados por scrapie. La PrP^C contribuye a la polarización neuronal a través de la organización espacial de las integrinas β1 (CD29) en la membrana plasmática, pero no modifica el nivel de expresión de este marcador (Loubet *et al.*, 2012). Las diferencias observadas a nivel de transcritos no pudieron ser confirmadas en los niveles de proteína mediante citometría de flujo.

Asimismo, se ha comprobado en el mismo trabajo (M**anuscrito 3**) que las BM-MSC y PB-MSC de ovinos sanos y afectados por scrapie eran capaces de diferenciarse

a linajes mesodérmicos. Las MSC de ambos orígenes presentaron una capacidad de diferenciación heterogénea (**Manuscritos 2 y 3**), lo cual hizo más difícil su comparación. Sin embargo, sí que se apreciaron algunos cambios significativos en los marcadores *BGN* y *COL1A1*. Estos marcadores aparecen significativamente más bajos durante la diferenciación osteogénica y condrogénica de las MSC obtenidas de animales afectados por scrapie, lo cual podría indicar una menor capacidad de diferenciación.

La capacidad de las MSC de transdiferenciarse a neurona, procedentes de ambos orígenes, ovinos sanos y ovinos afectados por scrapie, ha sido similar. Al igual que la diferenciación hacia linajes mesodérmicos, las MSC ovinas presentaron variabilidad en los niveles basales de marcadores neurogénicos. A pesar de ello, se observó una reducción significativa de *NES* y de *TUBB3* en los niveles basales de las MSC obtenidas de animales con scrapie, lo cual podría limitar su potencial de transdiferenciación y su uso como terapia autóloga.

En el M**anuscrito 2** describimos la expresión de *PRNP* a nivel de transcritos en las PB-MSC, y durante el trabajo de caracterización descrito en el **Manuscrito 3** se confirmó la expresión de PrP^{C} mediante *Dot Blotting* de las PB-MSC y BM-MSC sin que se haya visto diferencias en los niveles de expresión entre animales sanos y afectados por scrapie. A pesar de que existe una gran variabilidad en los resultados, se observó que la diferenciación neurogénica parece aumentar la cantidad de PrP^{C} expresada por las MSC. Esta característica podría ser de gran utilidad para la creación de modelos *in vitro* para el estudio de las enfermedades priónicas basados en el cultivo con MSC.

Las MSC ovinas parecen no poseer carga de proteína prión patológica en animales afectados por scrapie, ya que en ningún caso se pudo detectar proteína PrP^{Sc} mediante amplificación por PMCA en BM-MSC o PB-MSC. Este análisis se realizó en MSC después de su expansión *in vitro*. Con el fin de determinar si la infección se había perdido con los pases, se analizó la infectividad de la médula ósea de los individuos afectados. A pesar de que la infectividad de médula ósea de ovinos afectados por la enfermedad de scrapie se ha descrito con anterioridad (Hadlow *et al.*, 1982), no hemos podido corroborar la hipótesis de que la médula ósea es la fuente de la prionemia descrita en ovino. Por la tanto, la infección de BM-MSC no debe considerarse como una característica general de las enfermedades priónicas y su análisis no puede utilizarse como método diagnóstico *in vivo* de la enfermedad de scrapie.

Durante la realización del último estudio (**Manuscrito 4**) se comprobó la susceptibilidad de las MSC ovinas a ser infectadas por priones y su capacidad de replicar dicha infección. Mediante la técnica de *Western Blotting* determinamos que las BM-MSC retienen la infectividad durante los primeros pases tras la inoculación con extractos de encéfalo de ovino con scrapie. Sin embargo, la PrP^{Sc} parece desaparecer con cada pase celular. Esta pérdida de infectividad podría deberse a que las MSC en cultivo se dividen a un ritmo rápido, lo que no facilitaría el avance de la infección y transmisión horizontal entre células, produciéndose únicamente transmisión vertical de la célula madre a las hijas, diluyendo por lo tanto la carga priónica en los sucesivos pases.

Sin embargo, tampoco se debe descartar que el inóculo induzca efectos nocivos sobre las MSC, que produjeran la muerte de las células infectadas y, por tanto, impidieran la transmisión de infectividad en el cultivo. Para valorar esta hipótesis, se ha estudiado el posible efecto tóxico que la infección podría ejercer sobre las MSC (**Manuscrito 4)**. En nuestro estudio de caracterización de las MSC obtenidas de animales en fase clínica de scrapie (**Manuscrito 3**) se había observado una disminución en el potencial de proliferación de las MSC obtenidas de animales enfermos. En este último estudio se observó una disminución del potencial de proliferación de las MSC infectadas con extracto de scrapie. El análisis del CD en el pase 1 tras la infección mostró una reducción significativa y, consecuentemente, un aumento significativo en el DT en las células expuestas al inóculo de encéfalos de ovino afectado por scrapie. Durante los sucesivos pases las diferencias en el CD y DT fueron progresivamente menores. Estos resultados, junto con la pérdida de detección de PrP^{Sc}, parecen indicar que el cultivo de MSC se recupera, incrementando la proporción de células no infectadas por la proteína prión, que muestran un mayor potencial de proliferación.

La dilución de la infectividad y la menor proliferación celular sugieren que las células infectadas podrían estar sufriendo un proceso de muerte celular. Estudios anteriores de nuestro grupo han demostrado la regulación diferencial de la apoptosis en SNC de ovino infectado con scrapie (Lyahyai *et al.*, 2006; Lyahyai *et al.*, 2007; Serrano *et al.*, 2009; Hedman *et al.*, 2012). Con el fin de determinar si un proceso similar se estaba induciendo *in vitro*, en este trabajo analizamos los perfiles de expresión de una serie de genes involucrados en la regulación de la apoptosis en los cultivos de BM-MSC

infectadas con scrapie. Al igual que lo descrito *in vivo* (Lyahyai *et al.*, 2006; Lyahyai *et al.*, 2007), al comparar estos cultivos con cultivos infectados con extracto de encéfalos de ovino control, las MSC infectadas con scrapie mostraron una tendencia a la sobreexpresión del marcador proapoptóticos *BAX*. Asimismo, de forma similar a lo que ocurre en scrapie natural (Serrano *et al.*, 2009), se determinó una sobreexpresión significativa de los marcadores *AIF1*, *BCL2L1*, *FAS* y *MCL1*, lo que indica una regulación diferencial de la apoptosis producida por la presencia del prión.

En conclusión, las MSC procedentes de médula ósea no parecen propagar PrP^{Sc}, pero presentan susceptibilidad a la toxicidad ejercida por esta proteína. Esta capacidad puede ser utilizada para estudiar las diferencias en la susceptibilidad a diferentes cepas priónicas o para analizar los mecanismos moleculares de la toxicidad del prión.

CONCLUSIONES

1. Las células extraidas a partir de sangre periférica de ovino cumplen las características para ser consideradas MSC, expresan marcadores mesenquimales y, a pesar de la gran variabilidad observada en el potencial de diferenciación de estas células, mantienen su capacidad de diferenciarse a células de linaje mesodérmico.

2. Las células mesenquimales extraídas a partir de sangre periférica y médula ósea de ovino expresan la proteína prión celular y pueden transdiferenciarse a células neuronales convirtiéndolas en buenas candidatas para el desarrollo de modelos celulares de las EETs.

5. Las células mesenquimales de origen sanguíneo y medular procedentes de animales ovinos afectados por scrapie presentan un menor potencial de proliferación, una ligera sobreexpresión de transcritos de *CD29* y una disminución de la expresión de marcadores neurogénicos a nivel basal con respecto a las MSC de ovino control. Por tanto, la enfermedad altera las características de las MSC.

6. Las células mesenquimales de origen sanguíneo y medular procedentes de ovinos afectados por scrapie no pueden ser utilizadas como herramientas para el diagnóstico *in vivo* de la enfermedad de scrapie al no detectar la presencia de PrP^{Sc} mediante la técnica de amplificación PMCA.

7. Las células mesenquimales procedentes de médula ósea de ovino pueden ser infectadas con extractos de encéfalos de ovino afectados por scrapie, pero pierden la PrP^{Sc} a lo largo de los pases en cultivo celular indicando su falta de potencial para replicar la infectividad.

8. La infección con proteína priónica de células mesenquimales de médula ósea de ovino muestran signos de toxicidad, como la disminución de su potencial de proliferación y cambios en la expresión de genes involucrados en la regulación de la apoptosis.

RESUMEN

Las encefalopatías espongiformes transmisibles (EET) son enfermedades neurodegenerativas sin tratamiento posible. En las últimas décadas se han desarrollado modelos celulares tanto para el estudio de los mecanismos moleculares de la enfermedad como para su posible aplicación en el diagnóstico precoz y para la evaluación de tratamientos *in vitro*. Sin embargo, existen muy pocos modelos celulares capaces de multiplicar la proteína prión patológica, la mayoría son derivados murinos que requieren la adaptación previa del aislado priónico en ratón antes de su multiplicación.

Uno de los objetivos principales del presente estudio consiste en desarrollar un modelo celular que permita estudiar el scrapie en células procedentes del hospedador natural de la enfermedad, la especie ovina. Para ello se han estudiado y caracterizado las células mesenquimales (MSC) ovinas obtenidas a partir de sangre periférica y médula ósea. Se ha seleccionado la sangre periférica como tejido de fácil acceso, y la médula ósea por considerarse el lugar de mayor concentración de MSC en animales adultos. En las EET la proteína prión patológica se acumula principalmente en el Sistema Nervioso Central y es allí donde ejerce su acción patológica, por ello se ha analizado la plasticidad de las MSC ovinas para su diferenciación neurogénica. Por otra parte, se ha estudiado la susceptibilidad de estas células frente a la infección con el agente de la enfermedad de scrapie. Además, las MSC se han propuesto como posible terapia para las EET por lo que el segundo gran objetivo de esta tesis consistió en analizar los efectos que la enfermedad pueda desencadenar sobre las características de las MSC y su posible infectividad.

En este trabajo se describe por primera vez la posibilidad de aislar MSC a partir de sangre periférica de ovino. Tanto las MSC ovinas de este origen como las obtenidas a partir de médula ósea expresan proteínas y transcritos de marcadores de superficie característicos de células mesenquimales. Tal y como se ha descrito en otras especies, se ha observado gran variabilidad individual de las MSC ovinas, que parece afectar tanto a la capacidad de diferenciación como a la plasticidad de estas células. Ambos tipos de MSC expresan la proteína prión celular y son susceptibles a la infección con aislados priónicos procedentes de animales afectados por scrapie, si bien, a diferencia de lo que ocurre en MSC de ratón, no parecen propagar la proteína prión patológica. La infección con proteína priónica reduce la capacidad de proliferación de las MSC. Este hecho

podría deberse a un efecto tóxico del prión y a la inducción de procesos apoptóticos. Estas características indican el potencial de las MSC ovinas como modelo celular en el hospedador natural de la enfermedad de scrapie.

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ABREVIATURAS

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A

AIF	Factor inductor de apoptosis.

B

BAK	Antagonista homólogo Bcl-2/*Killer*1.
BAX	Proteína X asociada a BCL-2.
BCL-2	Célula B CLL/Linfoma 2.
BCL2L1	Proteína 1 Reguladora de Apoptosis Bcl-2-*Like*.
BGLAP	Osteocalcina.
BGN	Biglicano.

C

cDNA	Ácido desoxirribonucleico complementario.
CJD	Enfermedad de Creutzfeldt-Jakob.
COMP	Proteína oligomérica de la matriz del cartílago.
COL1A1	Colágeno tipo 1.
COL2A1	Colágeno tipo 2.

D

DMSO	Dimetilsulfóxido.

E

EEB	Encefalopatía espongiforme bovina.
EET	Encefalopatía espongiforme transmisible.

F

FAS	Receptor de muerte celular de superficie Fas

G

GFAP	Proteína ácida fibrilar glial.

L

LUM	Lumicano.

M

MAP2	Proteína 2 asociada a microtúbulos.
MCL1	Proteína marcadora de leucemia celular mieloide inducida 1.
MSC	Células madre mesenquimales.

N

NEFM	Polipéptido medio de neurofilamento.

NELF Factor leutinizante embriónico nasal.

NEUN Antígeno nuclear neuronal

NES Enolasa específica de neurona.

NES Nestina.

NCS Células troncales neuronales.

NSC Células madre neuronales.

P

PCR Reacción en cadena de la polimerasa.

PMCA *Protein Misfolding Cyclic Amplification.*

PPARγ Receptor gamma activado por el factor proliferador de peroxisomas

PRNP Proteína prión.

PRP^{C} Proteína prión celular.

PRP^{res} Proteína resistente a digestión con proteasas.

PRP^{Sc} Proteína prión patológica.

R

ROS Especies reactivas de oxígeno.

RT-qPCR PCR cuantitativa.

RT-PCR PCR en tiempo real.

S

SNC Sistema nervioso central.

T

TFG-β3 Factor de crecimiento transformante beta 3.

TNF-α Factor de necrosis tumoral alfa.

TUBB3 Tubulina beta clase 3.

V

vCJD Nueva variante de la Enfermedad de Creutzfeldt-Jakob