Universidad Zaragoza
1542

Tesis Doctoral

CÉLULAS MADRE MESENQUIMALES EQUINAS: OBTENCIÓN Y ANÁLISIS DE SUS PROPIEDADES IN VITRO

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2012

MEMORIA PARA OPTAR AL GRADO DE DOCTORA POR LA UNIVERSIDAD DE ZARAGOZA:

CÉLULAS MADRE MESENQUIMALES EQUINAS: OBTENCIÓN Y ANÁLISIS DE SUS PROPIEDADES *IN VITRO*.

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1. RESUMEN

Las células madre mesenquimales (MSCs) presentan una capacidad de autorrenovación y diferenciación a linajes derivados del mesodermo que las hacen idóneas para su aplicación en el tratamiento de lesiones del aparato locomotor en la especie equina. Las lesiones más comunes que sufren los caballos de carreras afectan a tejidos como el tendón o cartílago, que presentan una capacidad muy limitada de reparación. Las MSCs pueden contribuir a la formación de un tejido con propiedades similares a las del tejido sano y evitar así el riesgo de recaídas.

Para poder aplicar con garantías las MSCs a la clínica equina es fundamental el conocimiento de sus características. Por ello, en el presente trabajo de Tesis Doctoral se han estudiado las características de las MSCs equinas derivadas de médula ósea (BM-MSCs) y tejido adiposo (AT-MSCs) tanto en condiciones estándar de cultivo (20% O_2) como en condiciones denominadas de hipoxia (5% O_2), más similares a la concentración de oxígeno fisiológica que rodea a las células *in vivo*.

Antes de caracterizar las MSCs se realizó un ensayo para valorar la influencia del tiempo que transcurre entre la toma de muestras y el aislamiento de las células sobre su capacidad de autorrenovación. El retraso sufrido en el aislamiento de las células supuso una pérdida de MSCs, ya que el número de colonias obtenidas fue significativamente mayor cuando el aislamiento se llevó a cabo inmediatamente después a la obtención del tejido que 24 horas después.

A continuación se estudió la capacidad de proliferación de las MSCs y su potencial de diferenciación hacia linajes osteogénico y adipogénico. Los resultados mostraron que las AT-MSCs dieron lugar a mayor número de células en cultivo que las BM-MSCs. Ambos tipos celulares se diferenciaron hacia osteoblasto, presentando un limitado potencial adipogénico. Posteriormente se determinó el fenotipo de las BM-MSCs y AT-MSCs respecto a la expresión de ciertos marcadores de superficie por citometría de flujo y PCR cuantitativa en tiempo real. Las MSCs equinas mostraron un

perfil de expresión similar al de la especie humana, observándose diferencias en la expresión de ciertos marcadores entre BM-MSCs y AT-MSCs.

Por otra parte, el cultivo en condiciones de hipoxia no modificó la capacidad de expansión, fenotipo o potenciales osteogénico y adipogénico de las BM-MSCs. Sin embargo, la condrogénesis se vio potenciada por esta condición. El estudio de la evolución del ciclo celular y la viabilidad de las MSCs de ambos orígenes en las dos condiciones de oxígeno mostró que los cultivos en normoxia presentaron mayor número de células, debido a una mayor actividad en la división celular en las BM-MSCs y a una mayor viabilidad de los cultivos de las AT-MSCs. Además, las MSCs mostraron una tendencia a una mayor expresión de marcadores de pluripotencia en hipoxia, lo que indicaría que estas células se encontrarían en un estado menos diferenciado.

Las diferencias en las características de las MSCs *in vitro* podrían estar relacionadas con variaciones en su capacidad terapéutica *in vivo*. Por ello y como parte de un estudio pre-clínico de tratamiento de lesiones tendinosas en caballos, se analizó el fenotipo y potencial de diferenciación osteogénico, adipogénico y condrogénico de las BM-MSCs y AT-MSCs utilizadas en dicho estudio. Los resultados confirmaron los obtenidos anteriormente para el fenotipo y potencial osteogénico. Además, ambos tipos celulares mostraron similar capacidad de diferenciación adipogénica. Sin embargo, en nuestras condiciones experimentales, aunque las BM-MSCs se diferenciaron claramente hacia condrocito, las AT-MSCs mostraron un limitado potencial de diferenciación hacia ese linaje.

SUMMARY

Mesenchymal stem cells (MSCs) display abilities of self-renewing and differentiation towards mesoderm derived lineages which make them suitable for the application on the treatment of locomotor system injuries in the equine species. Most common types of injuries that racehorses suffer affect tissues such as tendon or cartilage, which have limited capacity for tissue repair. MSCs might contribute to the restoration of a tissue with similar properties to the healthy tissue and to reduce the risk of re-injury.

In order to apply the MSC-based treatments safely to the equine clinical field it is essential to understand their basic characteristics. Therefore, in the present Ph.D. project the characteristics of equine MSCs derived from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) have been studied, in standard culture conditions (20% O2) and in conditions designated as hypoxia (5% O2), which more resemble the physiological oxygen tension that surrounds the cells *in vivo*.

Before characterisation of the MSCs, an assay was performed to evaluate the influence of the time between the sample harvesting and the cell isolation on the self-renewing ability. It was shown that if a delay occurred before the cell isolation this lead to a loss of MSCs.The number of colonies obtained was significantly higher when the isolation was carried out straight after the harvesting than if it was done 24 hours later.

Subsequently, MSC proliferation as well as osteogenic and adipogenic differentiation potentials were studied. The results indicated that AT-MSCs yield higher number of cells in culture than BM-MSCs. Both cell types differentiated efficiently into osteoblast but displayed a limited adipogenic potential. Subsequently, the phenotype of BM-MSCs and AT-MSCs with respect to expression of certain cell surface markers was determined by flow cytometry and quantitative real time PCR. Equine MSCs showed an expression profile comparable to those of humans. However, differences in the expression of some markers between BM-MSCs and AT-MSCs were observed.

Hypoxic culture conditions did not modify the expansion ability, phenotype, or osteogenic and adipogenic potentials of BM-MSCs. However, chondrogenesis was enhanced by low oxygen levels. The study of cell cycle progression and viability of MSCs in the two oxygen conditions showed that normoxic cultures displayed higher number of cells. This was because of higher cell division activity in the BM-MSCs and higher viability in AT-MSC cultures in normoxia. Moreover, a tendency towards a higher expression of pluripotency markers was observed in hypoxic MSCs, which may suggest that these cells would remain in a more undifferentiated state.

The *in vitro* differences observed in the characteristics of the MSCs might bear a relevance to their *in vivo* therapeutic capacity. Consequently, the phenotype, as well as osteogenic, adipogenic and chondrogenic differentiation potentials of BM-MSCs and AT-MSCs were analysed as a part of a pre-clinical trial to treat tendon injuries in horses. The results confirmed the previous ones obtained for the phenotype and osteogenic potential. In addition, both cell types showed a similar adipogenic differentiation ability. However, in our experimental conditions, even though the BM-MSCs were obviously able to differentiate into chondrocytes, the AT-MSCs showed a limited potential of differentiation towards this lineage.

2. INTRODUCCIÓN Y OBJETIVOS

2.1. INTRODUCCIÓN

El impacto económico que las actividades relacionadas con el caballo tiene en los países desarrollados no se puede comparar con el de ninguna otra especie. La industria relacionada con el caballo mueve aproximadamente 100 billones de dólares en Estados Unidos, 5.000 millones de euros en Reino Unido y 900 millones en España [1, 2].

El potente aparato locomotor de estos animales es fundamental para el desarrollo de competiciones deportivas. La aparición de lesiones que afecten a tejidos de este sistema va a tener como resultado que el caballo no pueda llevar a cabo de forma normal su actividad o que incluso esté comprometida su permanencia en ella obligando a su prematura retirada [3].

El principal problema que presentan los tejidos como el tendón es su limitada capacidad de regeneración. La cicatriz que se forma tras una lesión presenta una arquitectura de fibras que no se corresponde con la del tendón nativo, consecuencia del proceso de fibrosis que tiene lugar [4]. Las características del tendón cicatrizado, tanto biomecánicas (de elasticidad y fuerza) como moleculares (proteínas que conforman la matriz extracelular de los tendones), son inferiores a las del tendón normal, provocando que el caballo no vuelva a alcanzar su nivel de actividad anterior a la lesión y aumentando también el riesgo de recaída [5].

Finalmente, el caballo además de ser paciente es un animal modelo para el estudio de lesiones en tendones y ligamentos en la especie humana debido a que la naturaleza de las lesiones en los tendones flexores en caballo es similar a las del tendón de Aquiles en atletas humanos [6]. Además también es considerado como animal modelo para el estudio de problemas en cartílago articular debido a que la composición molecular y el grosor del tejido es similar al de la especie humana [6]. Finalmente, el caballo es el modelo animal más próximo al humano ya que el caballo puede ser

sometido a un proceso de rehabilitación tras la aplicación de la terapia al igual que en humanos, seguimiento que es imposible de aplicar en animales modelo de otras especies.

Por estas razones es necesaria la búsqueda de un tratamiento que regenere los tejidos afectados por lesiones para devolverles las propiedades y la funcionalidad normales. El tratamiento mediante terapia celular es una alternativa a los tratamientos convencionales de estos defectos. La terapia celular basada en la utilización de células madre tiene como objetivo restaurar las propiedades de los tejidos lesionados. Aunque existen diferentes tipos de células madre, las células madre adultas no plantean problemas éticos y además pueden obtenerse de los mismos individuos que requieren el tratamiento, eliminando así el problema del rechazo en los trasplantes [7]. Las células madre mesenquimales (MSCs) son un tipo de células madre adultas que presentan la característica de diferenciarse a todos los tejidos que derivan de la capa embrionaria del mesodermo, la misma capa de las que proceden los tejidos que conforman el sistema músculo esquelético, por lo tanto estas células se perfilan como las mejores candidatas a utilizarse en la terapia para el tratamiento de lesiones de este sistema [8].

Aunque en la actualidad existen tratamientos basados en la utilización de MSCs en la especie equina [9], los mecanismos por los que las células llevan a cabo la regeneración no están del todo claros. El estudio de las características moleculares de las MSCs es fundamental para conocer la forma de aplicación más eficiente en la clínica. El estudio *in vitro* de las MSCs engloba la búsqueda de las fuentes de tejidos que proporcionen MSCs y la caracterización a nivel molecular de las mismas.

Dentro del análisis de sus características, es esencial conocer su capacidad de proliferación con el objetivo de poder obtener un número elevado de células para su uso en terapia. También es necesario establecer el fenotipo que presentan estas células para los diferentes marcadores de superficie para poder seleccionarlas de entre una población heterogénea de células. Finalmente se requiere verificar si poseen capacidad de diferenciación hacia los tipos celulares de tejidos derivados del mesodermo porque esta característica podría ser fundamental para su función en la regeneración de tejidos dañados.

Así mismo, es necesario establecer cuales son las condiciones de cultivo más adecuadas durante la expansión para potenciar las propiedades beneficiosas de estas células antes de su uso en terapia celular. El oxígeno es un parámetro determinante para procesos biológicos y moleculares [10]. Las MSCs cuando se encuentran en sus nichos en el interior de los organismos están rodeadas por un ambiente de oxígeno considerablemente más bajo que el porcentaje de oxígeno atmosférico que se utiliza en los incubadores habitualmente [11]. Al someter a las células a un entorno diferente al fisiológico sus características podrían resultar alteradas.

En el trabajo presentado para optar al grado de Doctora hemos analizado las propiedades de las MSCs equinas derivadas de médula ósea y tejido adiposo en cultivos *in vitro*. Inicialmente, se determinó el momento óptimo de aislamiento de las MSCs de los tejidos de origen. A continuación, estas células se caracterizaron, analizando su capacidad de proliferación, el fenotipo que presentan con respecto a la expresión de marcadores de superficie celular, su potencial de diferenciación hacia los linajes osteogénico, adipogénico y condrogénico y la expresión de marcadores de pluripotencialidad. Además, también se determinó la variación de estas características al utilizar una condición de oxígeno más parecida a la fisiológica durante la expansión de las mismas (5% O_2) comparándolas con MSCs expuestas a 20% de O_2. Finalmente, esta investigación se aplicó a un estudio clínico con el objetivo de determinar si las variaciones observadas *in vitro* se ven reflejadas en la capacidad terapéutica de estas células *in vivo*. Para ello se caracterizaron *in vitro* las BM-MSCs y AT-MSCs utilizadas en un estudio pre-clínico de tratamiento de lesiones tendinosas inducidas artificialmente.

2.2. OBJETIVOS

El objetivo general planteado en la presente memoria de Tesis Doctoral es conocer las características *in vitro* de las células madre mesenquimales equinas utilizadas habitualmente en terapia celular de lesiones del aparato locomotor. Para la consecución de este objetivo general se han propuesto los siguientes objetivos específicos:

1. Determinar el momento óptimo de aislamiento de MSCs a partir de los tejidos de origen.
2. Estudiar los potenciales de proliferación y diferenciación hacia linajes ostegénico, adipogénico y condrogénico de las MSC derivadas de médula ósea y tejido adiposo.
3. Analizar el fenotipo de estas células respecto a la expresión de marcadores de superficie y marcadores de pluripotencialidad.
4. Evaluar el efecto de la expansión en distintas atmósferas de oxígeno sobre las características *in vitro* de las MSCs equinas.

3. REVISIÓN BIBLIOGRÁFICA

3.1. CÉLULAS MADRE

Se define como célula madre a aquella célula no diferenciada que es capaz de autorrenovarse y de diferenciarse en otros tipos celulares.

Las células madre presentan dos tipos de divisiones: la simétrica y la asimétrica. La característica de autorrenovación viene dada a través de la división simétrica, las células madre se dividen para dar dos células hijas que conservan las mismas propiedades de célula madre. Mientras que la división asimétrica da lugar a células progenitoras en un estado más diferenciado que la célula madre inicial, proporcionando la capacidad de diferenciación de las células madre.

Las células madre se pueden clasificar en función de su potencial de diferenciación en:

- Totipotenciales: muestran la capacidad de dar lugar a tejidos embrionarios (como placenta o cordón umbilical) y de cualquiera de las tres capas embrionarias.
- Pluripotenciales: tienen la habilidad de diferenciarse en cualquier célula de las tres capas embrionarias.
- Multipotenciales: su capacidad de diferenciación se ve restringida a sólo uno de los linajes.
- Somáticas: sólo serán capaces de diferenciarse al tipo celular del tejido donde residen para mantener la homeostasis del mismo.

Otra forma de clasificar a las células madre es teniendo en cuenta el origen del que proceden:

- Células madre embrionarias: están presentes en las primeras etapas de desarrollo del embrión, en el estado de blastocisto. Son células pluripotenciales.
- Células madre germinales: se aíslan de los esbozos gonadales de los embriones y son células pluripotenciales.

- Células madre fetales: están en los tejidos y órganos fetales y tienen características similares a sus homólogas en tejidos adultos. Su capacidad de diferenciación no está del todo clara.
- Células madre adultas: están en los tejidos y órganos de los individuos adultos. Son células multipotenciales.
-

3.1.1. CÉLULAS MADRE EMBRIONARIAS.

Las células madre embrionarias son consideradas pluripotentes ya que presentan la capacidad de diferenciarse a tejidos de las tres capas embrionarias [12]. Tienen también la capacidad de proliferar continuamente gracias a un perfil transcripcional único que las mantiene en un estado indiferenciado [13]. Los marcadores genéticos específicos de las células madre embrionarias son *SSEA-3*, *SSEA-4*, *OCT-4*, *SOX-2* y *NANOG*, a los que se les denomina marcadores de pluripotencia, estos factores de transcripción van a regular la supresión de los genes que promueven la diferenciación [14]. Las células madre embrionarias además presentan la característica de pasar desapercibidas por el sistema inmune debido la etapa tan temprana del desarrollo del desarrollo en la que se encuentran [15].

3.1.2. CÉLULAS MADRE GERMINALES

Las células madre germinales se localizan en la cresta gonadal de los fetos. Este lugar es un esbozo de las gónadas y es donde tiene lugar la diferenciación de la línea germinal hacia óvulos y espermatozoides. Estas células son pluripotenciales y tienen una capacidad de diferenciación similar a las embrionarias ya que son capaces de diferenciarse a células de las tres capas embrionarias, pero su aislamiento resulta más difícil [16].

3.1.3. CÉLULAS MADRE FETALES

Las células madre fetales se encuentran formando parte de los tejidos que forman los órganos fetales. Su potencial de diferenciación es similar al de las células

madre adultas, aunque parecen mostrar mayor capacidad de expansión y diferenciación que éstas al encontrarse en un estado más primitivo [17].

3.1.4. CÉLULAS MADRE ADULTAS

Las células madre adultas van a ser capaces de diferenciarse *in vivo* en células de los tejidos en los que residen. Estas células se puede encontrar en la mayoría de los órganos [18-20], pero sin duda, el tejido considerado como la mayor fuente de células madre adultas es la médula ósea [21].

Las células madre adultas se encuentran dentro de tejidos en espacios denominados nichos. Son capaces de autorrenovarse y mantener la homeostasis celular en el tejido a través de procesos de diferenciación. Por medio del reemplazamiento de las células muertas por células recién diferenciadas los tejidos se encuentran siempre en constante renovación.

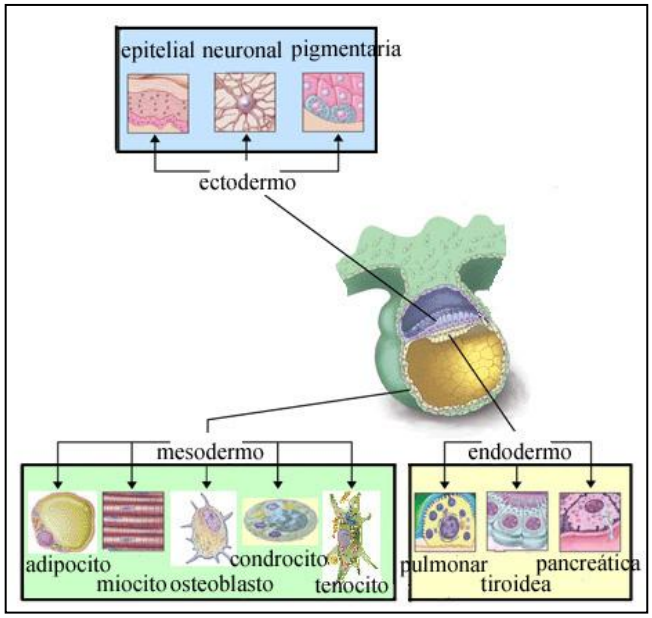


Figura 1: Diagrama de la distribución de las tres capas germinales y los tipos celulares que derivan de cada una de ellas. (© 2001 Terese Winslow, Lydia Kibiuk)

Algunas células madre adultas no sólo se especializan a células del mismo tejido en el que residen, sino también a otras células que derivan de la misma capa embrionaria de la que proceden [22]. De modo que, por ejemplo, células madre

procedentes de la capa ectodérmica van a ser capaces de especializarse a células epiteliales, o células procedentes del endodermo podrán dar lugar a células pancreáticas (Figura 1).

En algunos casos, parece que la plasticidad que presentan las células madre adultas no se detiene en la diferenciación a células de su misma capa embrionaria, ya que, por ejemplo, células derivadas de la capa mesodérmica han demostrado ser capaces de diferenciarse a células del endodermo, como los hepatocitos [23].

En mamíferos, la principal fuente de células madre adultas es la médula ósea. Este tejido está compuesto por una red tridimensional de estroma y muchos tipos celulares distintos (Figura 2). En la médula ósea se encuentran las células madre hematopoyéticas (Hematopoietic Stem Cells, HSCs), que son las responsables de la generación de todas las células sanguíneas y del sistema inmunitario. Desde el descubrimiento de las propiedades regenerativas de las HSCs hace 50 años [24], se han caracterizado y utilizado en trasplantes para el tratamiento de diversas patologías como leucemias, linfomas, anemias aplásicas, inmunodeficiencias primarias y enfermedades hereditarias que afectan a células hematopoyéticas.

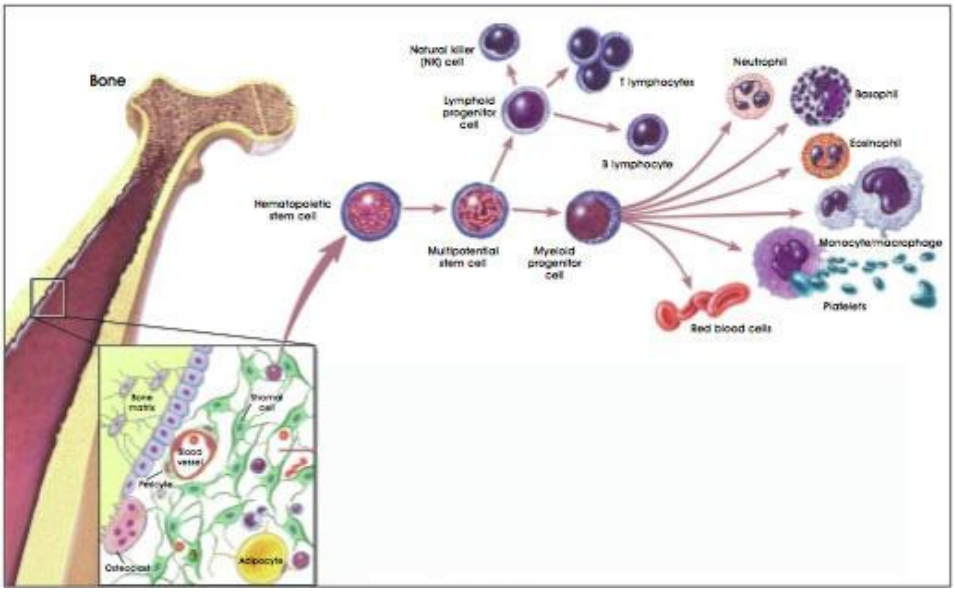


Figura 2. Estructura de la médula ósea y de la diferenciación celular de las HSCs. (© 2001 Terese Winslow, Lydia Kibiuk)

3.2. CÉLULAS MADRE MESENQUIMALES

Las células madre mesenquimales son células madre adultas que derivan de la capa embrionaria del mesodermo (Figura 1), por lo que van a ser capaces de diferenciarse a células que conforman los tejidos derivados de esa misma capa, como son hueso, cartílago, músculo, estroma medular, tendones, ligamentos, grasa y otros tejidos conectivos [8]. La capacidad de diferenciación de estas células no parece limitarse a estos tejidos, ya que también se ha demostrado capacidad de diferenciación a células de otras capas, como por ejemplo tejido neurogénico [25], células pancreáticas [26] o células hepáticas [27].

Además, estas células presentan también la propiedad característica de las células madre de autorrenovación, aunque a diferencia de las embrionarias, esta habilidad es finita y parece que las células acaban por detener su crecimiento *in vitro* tras largos periodos en cultivo [28].

3.2.1. CÉLULAS MADRE MESENQUIMALES DERIVADAS DE MÉDULA ÓSEA

Las HSCs no son las únicas células madre que se encuentran en la médula ósea. En 1970 Friedenstein y colaboradores descubrieron una población de células con morfología de fibroblasto y capacidad de formar colonias sobre la superficie de las placas de cultivo [29]. Por este motivo, inicialmente fueron denominadas como CFU-F (unidades formadoras de colonias de morfología de fibroblasto), aunque han sido varios los nombres que este tipo de células han recibido a lo largo del tiempo: células estromales de la médula, células precursoras estromales o células madre mesenquimales. Para clarificar la nomenclatura de estas células, la Sociedad Internacional de Terapia Celular (ISCT) propuso aplicar el acrónimo MSC (Mesenchymal stem/stromal cell) para referirse a ellas [30].

Sin embargo, las MSCs no están formadas solamente por una población de células, sino que son una mezcla heterogénea de poblaciones. Este hecho sumado a que no existe sólo un marcador de superficie para su identificación positiva entre los otros

tipos celulares de la médula ósea lleva a que sea difícil definir las MSCs. Por ello, la ISCT definió en 2006 [31] una serie de estándares que las células aisladas de médula ósea humanas deben cumplir para puedan ser denominadas como MSCs:

1. Capacidad de adherencia al plástico del material de cultivo
2. Expresión de ciertos marcadores de superficie: más de un 95% de las células en cultivo deben mostrar en su superficie las moléculas CD105 (endoglina), CD73 (SH2 y SH3) y CD90 (Thy-1); y menos de un 2% de las mismas deben ser positivas en la detección de CD45 (antígeno común de leucocitos), CD34 (marcador de células precursoras de células hematopoyéticas), CD14 ó CD11b y CD79α ó CD19 ó HLA-DR.
3. Multipotencialidad de diferenciación a osteoblasto, adipocito y condrocito.

Los estudios realizados sobre el análisis de estas características y otras más se desarrollan más ampliamente en el Apartado 5.

La proporción de MSCs en la médula ósea es baja respecto del total de células nucleadas [32], y disminuye a medida de que avanza la edad del individuo. En el caso de humanos la proporción de células varía desde 1/10.000 (MSCs/células de médula ósea) en los recién nacidos hasta 1/2.000.000 en personas de la tercera edad [8]. Esta disminución en la proporción de MSCs sería una de las causas de la lentitud a la hora de reparar tejidos dañados en las personas de avanzada edad, como por ejemplo roturas de huesos. El origen del control de la proporción de MSCs y su disminución con el tiempo en la médula ósea se desconoce.

3.2.2. CÉLULAS MADRE MESENQUIMALES DERIVADAS DE TEJIDO ADIPOSO.

Durante mucho tiempo, la médula ósea fue la principal fuente de obtención de células madre mesenquimales (BM-MSC). Sin embargo, estudios realizados durante la pasada década revelaron el tejido adiposo como fuente alternativa de células madre mesenquimales (AT-MSC) [33].

El tejido adiposo está constituido por adipocitos, preadipocitos, fibroblastos, células endoteliales e inmunes [34]. Además, la cantidad de células obtenidas puede ser

mayor debido a la posibilidad de trabajar con mayor cantidad de tejido de partida [35] y a que parece que las AT-MSCs muestran mayor tasa de proliferación que las MSCs que residen en la médula ósea [14]. Además, al igual que las BM-MSCs, la proporción de estas células en el tejido adiposo también parece disminuir con la edad del individuo [36].

Las AT-MSCs, de la misma manera que sus equivalentes de médula ósea, se pueden establecer en cultivos *in vitro* y también son capaces de diferenciarse hacia los linajes osteogénico, adipogénico y condrogénico [37], así como a tejidos procedentes de otras capas germinales como tejido neurogénico [38].

3.2.3. CÉLULAS MADRE MESENQUIMALES DERIVADAS DE OTROS TEJIDOS

La médula ósea y el tejido adiposo son los principales tejidos de donde se obtienen MSCs, pero no son los únicos, durante los últimos años han sido descritos el aislamiento y caracterización de células mesenquimales de otros tejidos adultos como sangre periférica [39], pulmón [40], músculo [41], líquido sinovial [42] y pulpa dental [43]. Además de en estos tejidos, también se han aislado MSCs de tejidos no adultos como sangre del cordón umbilical [44], tejido del cordón umbilical [45], líquido amniótico y placenta [46].

3.3. TERAPIA CELULAR

La terapia celular se define como el trasplante de células vivas a un organismo con el propósito de reparar un tejido o funciones perdidas. Las células que son utilizadas en este tipo de terapia son empleadas como agentes terapéuticos para la reparación de grupos de células o de tejidos que han sufrido algún daño y se plantean como alternativa de terapia para aquellos procesos que no poseen tratamientos en la actualidad o los que existen no son efectivos.

En los últimos años las células madre están siendo objeto de estudio para su utilización en terapia celular. Las células madre embrionarias se han considerado como

las más interesantes para su utilización por su capacidad de diferenciarse a todos los tejidos adultos. Sin embargo su utilización se encuentra limitada por los problemas de carácter ético y moral que conlleva el trabajar con ellas, además de por el riesgo de formación de teratomas al inyectar estas células como terapia [47]. Por ello, las células madre adultas suponen una alternativa al uso de las embrionarias. Además presentan la ventaja de que se pueden obtener del mismo individuo al que se va a tratar. Alternativamente a las células madre adultas, las células madre derivadas de tejidos no adultos suponen también una fuente de células importante para su utilización de forma alogénica, debido a que se encuentran en un estado más primitivo que las adultas y no plantean serios problemas éticos.

Las MSCs han demostrado ser una gran herramienta en la terapia celular debido a su potencial de diferenciación y a su capacidad de autorrenovación. La plasticidad que presentan estas células a la hora de diferenciarse las hace idóneas para reparar y regenerar tejidos dañados, siendo sobretodo la habilidad de diferenciación a tipos celulares de tejidos conectivos la que hace que las MSCs sean las candidatas ideales como fuente de células para la terapia celular de enfermedades relacionadas con el sistema musculoesquelético. La autorrenovación permite la obtención de las grandes cantidades de células que son necesarias para la aplicación en terapia. Además las MSCs también presentan la propiedad de pasar desapercibidas para el sistema inmunológico, ya que el huésped no genera ninguna respuesta frente a ellas, lo cual permite el trasplante de estas células de forma tanto autóloga y como alogénica, es decir, entre pacientes o incluso entre especies diferentes de los que proceden las células (xenotrasplante) [48]. Además debido a propiedades inmunológicas de las MSCs presentan, éstas están siendo utilizadas en ensayos clínicos para el tratamiento de enfermedades autoinmunes como la enfermedad de injerto contra huésped (GVHD) [49] o la enfermedad de Crohn [8].

Desde que se aislaron por primera vez las MSCs, se ha estudiado cómo estas células son capaces de reparar los tejidos de forma natural. A pesar del éxito de la utilización de las MSCs como agente terapéutico en animales modelos para el tratamiento de lesiones de tejidos, el nivel de inserción de las MSCs y la capacidad de diferenciarse *in vivo* en el tejido dañado es reducido [50]. Parece que la habilidad de regeneración de las MSCs a través de la inserción-diferenciación en el tejido es

secundaria, por lo que el principal sistema por el cual las células llevarían a cabo el proceso de regeneración sería alterando el microambiente del tejido través de la secreción de factores solubles. El mecanismo que gobernaría en la regeneración no está del todo claro, pero parece ser que las MSCs trasplantadas secretarían factores bioactivos que inhibirían la cicatrización y la apoptosis del tejido y estimularían la angiogénesis y la mitosis de las células madre o progenitoras intrínsecas del tejido dañado para regenerarlo. Esta actividad de secreción de factores por parte de las MSCs se denominaría actividad trófica [8].

3.3.1. TERAPIA CELULAR EN ANIMALES MODELO Y ENSAYOS CLÍNICOS.

Hasta el momento y debido a que la capa de origen embrionario de la que proceden las MSCs es el mesodermo, las mayores aplicaciones para tratamientos clínicos de estas células radican en su utilización para la regeneración de tejidos que derivan también de esa misma capa, como hueso, cartílago, músculo o tendón. Sin embargo, las MSCs han demostrado también su eficacia en numerosas estratégicas terapeúticas en el tratamiento de diversos desórdenes, no solamente relacionados con tejidos del sistema musculoesquelético.

A continuación se detallan algunos ejemplos de patologías en los que se ha aplicado terapia celular utilizando diversas fuentes de células, tratando en mayor profundidad aquellos realizados con MSCs.

3.3.1.1. TRATAMIENTO EN LESIONES ÓSEAS

Uno de las terapias con MSCs más importantes realizados hasta hoy es el uso de este tipo de células como terapia en la osteogénesis imperfecta. Ésta es una enfermedad congénita caracterizada por la malformación de los huesos y otros tejidos causada por la mutación del gen del colágeno tipo I, como consecuencia de ello los enfermos que padecen esta enfermedad sufren frecuentes fracturas de huesos. Los primeros estudios de tratamiento con MSCs realizados en un modelo murino mostraron tras de la infusión de las células se observó una recuperación funcional del hueso y el cartílago [51]. Este tipo de terapia se ha llevado acabó con éxito en la especie humana. La aplicación de

MSCs alogénicas sobre niños con esta enfermedad, produjo nueva formación de hueso, un aumento de la mineralización y una disminución en la frecuencia de las fracturas solamente tres meses después del tratamiento [52].

3.3.1.2. TERAPIA EN LESIONES CARTILAGINOSAS

El cartílago tiene una capacidad limitada de reparación intrínseca, pequeños daños se pueden reparar espontáneamente a través de la producción de cartílago hialino, pero los defectos grandes se reparan por medio de la formación de tejido fibroso o fibrocartílago cuyas propiedades bioquímicas y biomecánicas son diferentes al hialino. Como consecuencia de esta sustitución de tejido, el cartílago presenta una degeneración que puede derivar en osteoartritis [53].

Los tratamientos habituales para lesiones en el cartílago de las articulaciones están basados en: métodos quirúrgicos para promover la estimulación de células de la médula ósea y potenciar la secreción de citoquinas en el tejido [54], autotrasplantes de injertos de cartílago sano del propio paciente (mosaicoplastia) [55] o la implantación de condrocitos autólogos (ACI) [56]. Sin embargo estas técnicas o son invasivas como en la estimulación y en el ACI o requieren cantidades de cartílago sano escaso en el individuo como en la mosaicoplastia. Además el cartílago resultante de estos tratamientos tampoco llega a alcanzar las características del cartílago nativo [57].

La terapia celular basada en la utilización de las MSCs es menos invasiva, ya se realiza mediante inyección intraarticular [58]. Los resultados en animales modelos como la cabra son positivos, en ellos se observa una regeneración del menisco y una disminución de la degeneración del cartílago [59]. Sin embargo, en los ensayos clínicos realizados en pacientes de osteoartritis los resultados obtenidos no muestran mejora clínica significativa, aunque los síntomas de los pacientes parecen disminuir [60].

3.3.1.3. TRATAMIENTO DE LESIONES TENDINOSAS

Las tendinopatías son habituales en el campo ortopédico, más de 30 millones de lesiones de este tipo se registran cada año en la especie humana [61] como consecuencia de traumatismos, procesos inflamatorios, lesiones crónicas provocadas por el estrés de la repetición mecánica o de la excesiva tensión. Los tendones están formados

fundamentalmente por fibras de colágeno alineadas (95% colágeno tipo I, 5% colágeno tipo III y IV), y a pesar de encontrarse irrigados por vasos sanguíneos, esta vascularización puede ser insuficiente en algunas zonas de los mismos [62]. Como consecuencia de esto los tendones presentan también una limitada capacidad de regeneración propia [63]. La cicatriz que se genera en la reparación tiene propiedades biomecánicas inferiores a las del tendón nativo, debido a una incorrecta alineación de las fibras de colágeno cuando se reestructura la matriz extracelular que conforma el tendón [64].

Los tratamientos que se han estado llevando a cabo para curar este tipo de lesiones se han basado en autotrasplantes, alotrasplantes, xenotrasplantes de partes de tendón sano y prótesis, pero todos estos métodos han fallado a la hora de devolver la funcionalidad y las propiedades mecánicas adecuada al tendón a largo plazo [65].

Las terapias actuales que se está llevando a cabo están obteniendo resultados positivos en modelos animales. El objetivo de estos estudios es que el tendón recobre la fuerza y elasticidad que poseía antes de que apareciera la lesión. La utilización de factores de diferenciación y factores de crecimiento tales como IGF, con actividad mitogénica para reparar el tejido, han mejorado la capacidad de curación de tendones en conejos por medio de un aumento en la proliferación de tenocitos en el tendón, y de la síntesis de COL1A1 y proteoglicanos [66]. En tendones de Aquiles de rata lesionados de forma artificial han incrementado la fuerza de tracción biomecánica del tendón semanas después de la administración [67].

Otro de los tratamientos más extendidos es el uso de plasma rico en plaquetas (PRP) porque posee una concentración elevada de factores de crecimiento, es fácil y es barato de obtener. Además de los factores de crecimiento, también contiene fibrina, fibronectina y vitronectina, importantes para la adhesión celular y la migración epitelial [68]. Tras de la administración de PRP se observa un incremento de células infiltrándose en el sitio del tendón a reparar [69]. El PRP también proporciona un aumento en la resistencia y la elasticidad del tendón [70]. Sin embargo en la especie humana, la administración de PRP en el tendón de Aquiles ha dado lugar a resultados dispares. En algunos casos no se observó cambio alguno ni en la actividad ni en el dolor

ni en la curación final del tendón [71], y en otros la recuperación de la movilidad fue rápida y no hubo complicaciones de ningún tipo tras la lesión [72].

El tratamiento de tendinopatías con MSCs ha dado resultados prometedores en el conejo, ya que se ha observado aumento de la calidad histológica y de la fuerza biomecánica del tendón lesionado y tratado [73]. Las MSCs autólogas son capaces de recuperar un 20% de las funciones nativas del tendón aunque de forma dependiente de la dosis [74]. Además, también se ha observado una mejora del 37% en la biomecánica y la arquitectura del tejido en tendones de Aquiles [75]. Si las MSCs se cultivan *in vitro* con factores como el BMP-12, implicado en la tenogénesis, la capacidad de regenerar el tendón se ve incrementada por medio del aumento del número de células con fenotipo de tenocito, la elongación y el alineamiento de las fibras, y una mayor deposición de componentes de la matriz extracelular [76].

3.3.1.4. TRATAMIENTO DE LESIONES CARDÍACAS

El músculo cardíaco es un tejido con una pobre capacidad de regeneración tras haber sufrido isquemia. Este fenómeno produce la muerte de cardiomiocitos tras sufrir un infarto lo que puede terminar derivando en un fallo cardíaco. Debido a esto existe un elevado interés en encontrar células capaces de restaurar el tejido sano del miocardio y con ello mejorar la función del corazón. Se ha estudiado la posibilidad de utilizar varios tipos celulares para el tratamiento de defectos en el corazón, como el trasplante autólogo de células musculares satélite [77] o el de mioblastos [78]. Sin embargo las células ideales para aplicar al tratamiento serían aquellas en un estadío menor de diferenciación para que pudieran dar lugar a una diferenciación cardiomiogénica completa. Las MSCs derivadas de médula ósea podrían ser estas candidatas ya que han demostrado la habilidad de diferenciarse hacia linaje miogénico *in vivo* [79]. Estudios realizados en ratón revelan la capacidad de las MSCs para introducirse en el tejido dañado y adquirir el fenotipo de cardiomiocito [80]. En ensayos realizados en humano la infusión de MSCs autólogas ha mejorado la función contráctil del corazón 3 meses después del tratamiento [81], aunque parece que estas mejoras se deben más a la actividad trófica de las células que a su diferenciación [82], ya que muy pocas células parecen capaces de integrarse en el tejido [80].

3.3.1.5. TERAPIA INMUNOMODULADORA

La enfermedad de injerto contra huésped (GVHD) es una de las complicaciones más frecuentes asociadas al trasplante alogénico de médula ósea. En esta patología las células del injerto atacan a las células del huésped como resultado de la activación de las células T del injerto. Con los tratamientos inmunosupresores convencionales no siempre se puede controlar esta respuesta, por lo que hay que encontrar medios adicionales para disminuir la reactividad de las células T, como la eliminación de la población de células T del injerto a trasplantar [83] o únicamente de las alorreactivas [84]. Rrecientemente, el descubrimiento de las propiedades inmunosupresoras de las MSCs *in vivo* e *in vitro* [85], acompañadas de la falta de inmunogeneicidad que presentan estas células [86], ha hecho que se empiecen a utilizar en ensayos clínicos para el tratamiento de esta enfermedad. Cuando se realiza el trasplante de médula ósea en los pacientes se co-trasplantan también MSCs procedentes del mismo donante para que controlen la actividad de las células T y así evitar la aparición GVHD [87].

Existen más ejemplos de enfermedades relacionadas con el sistema inmune en el que se están aplicando terapias basadas en MSCs. Uno de ellos es el lupus eritematoso sistémico, en que se han llevado a cabo trasplantes de MSCs alogénicas en ratones modelos, obteniendo una inhibición *in vivo* de la proliferación y función de linfocitos B y T [88]. Otro patología sería la enfermedad de Crohn, en la que la piel dañada por la aparición de fístulas se podría regnerar por medio de terapia celular [89].

3.3.2. TERAPIA CELULAR EN CABALLOS

El caballo es un animal que se utiliza como modelo para la investigación en terapia celular, especialmente para aquellos tratamientos orientados hacia la regeneración de lesiones de los tejidos musculoesqueléticos. Este animal es considerado como modelo para el estudio de defectos en las articulaciones debido a que el grosor del cartílago articular y la composición molecular es similar al del cartílago humano [90]. También es considerado como animal modelo para el estudio de lesiones que se producen en tendones y ligamentos [91], la naturaleza de estas lesiones son similares a las que se producen en atletas humanos, siendo los problemas del tendón de Aquiles los

de mayor similitud con los de los tendones que comúnmente se lesionan en los caballos [92]. Otra de las razones para considerar el caballo como modelo animal es que el caballo puede ser sometido a periodos de rehabilitación posteriores a la terapia, procedimiento realizado en humanos después de que se les sea aplicada la terapia.

Sin embargo, el caballo no sólo sirve como animal modelo para el tratamiento de enfermedades relacionadas con humanos, sino que debido a su importancia económica constituye también un paciente para la terapia celular. En Estados Unidos la industria relacionada con el caballo mueve 102 billones de dólares (80.397 millones de €) al año y en Reino Unido 5.030 millones de € [2]. En España, en el año 2001, el mundo del caballo movió 900 millones de € a través de carreras, competiciones deportivas como doma clásica, exhibiciones o actividades de ocio y sector ganadero [1]. Por todo ello, las lesiones musculoesqueléticas en estos animales pueden provocar una grave pérdida económica.

Las lesiones articulares son la causa de un 60% del total de las cojeras en caballo [93], mientras que las lesiones que afectan al tendón son el mayor riesgo de baja de los caballos de competición [5]. La terapia celular aplicada al campo de las lesiones del aparato locomotor de caballos es esperanzadora. Durante la pasada década, varios ensayos con células madre han dado resultados positivos en el tratamiento de este tipo de lesiones en caballos. Como consecuencia de ello han surgido compañías comerciales con origen en grupos de investigación universitarios dedicadas al tratamiento de estas lesiones en caballos atletas [94] por medio de la utilización de MSCs expandidas *in vitro*.

La Figura 3 muestra un esquema del proceso completo del tratamiento que recibe un caballo cuando es tratado con terapia celular de MSCs. En primer lugar se toma una muestra de los tejidos que contienen MSCs del caballo lesionado, generalmente de médula ósea o tejido adiposo. Después, las MSCs se aíslan de los tejidos, se expanden durante varios pases en un medio de cultivo adecuado, se reimplantan en la lesión del animal por ecografía y finalmente, el animal sigue un programa de ejercicios de recuperación en el que la intensidad se incrementa gradualmente con los meses.

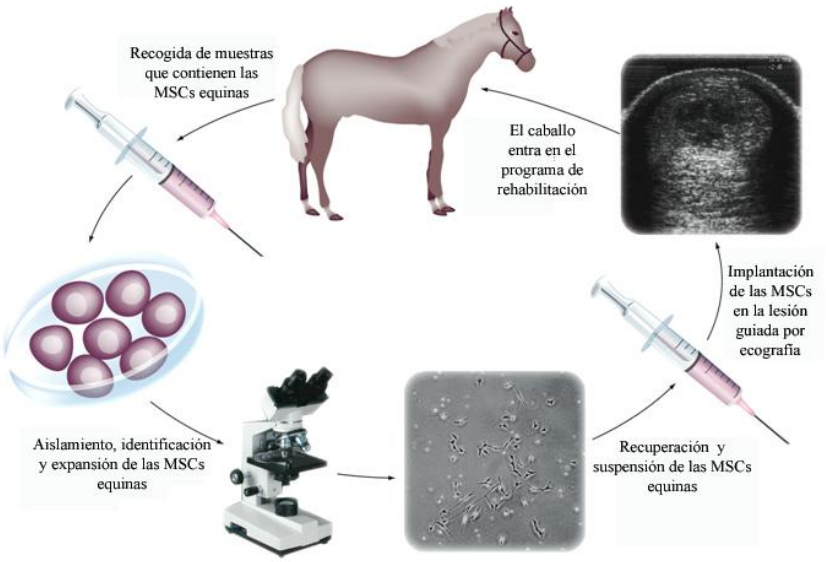


Figura 3. Esquema del tratamiento con MSCs a caballos con lesiones relacionadas con el sistema locomotor. Adaptado de VetCell MS-EQ™.

3.3.2.1. TRATAMIENTO DE LESIONES DE CARTÍLAGO

La etiología de las lesiones en el cartílago articular en caballo incluye el padecimiento de enfermedades (como la osteoartritis) y los traumatismos. La consecuencia en ambos casos es la aparición de cojera en el animal y por tanto un pobre desarrollo de su actividad, que puede terminar obligando al animal a retirarse de la competición [95]. Los tratamientos agresivos como la microfractura, consistente en la perforación del hueso subcondral para atraer a células y factores de crecimiento hasta la lesión, no han dado buenos resultados [96]. Por el contrario, la utilización de la terapia celular para el tratamiento de articulaciones con condrocitos sanos [97] o condrocitos acompañados del IGF-I [98] estimula la producción de matriz extracelular y preserva el fenotipo de los condrocitos en el tejido.

Sin embargo, la terapia celular basada en el implante de condrocitos autólogos presenta limitaciones, como la obtención de las células y el número limitado de pases que pueden estar estas células en cultivo antes de desdiferenciarse. La utilización de MSCs se plantea como la terapia celular con grandes posibilidad de éxito en caballos. Hasta el momento se han utilizado MSCs procedentes de distintas fuentes. Wilke y colaboradores, realizaron inyecciones intraarticulares de BM-MSCs junto con fibrina en lesiones inducidas obteniendo una mejora de las características de la articulación a corto

plazo con la inyección con células respecto a los controles de fibrina sola. Sin embargo, esta diferencia desparecía a largo plazo, posiblemente debido a la muerte de las MSCs inyectadas [99]. Frisbie y colaboradores también indujeron de forma artificial lesiones en las articulaciones de caballos e inyectaron BM-MSCs y fracción del estroma vascular del tejido adiposo (SVF), observando una mayor magnitud en la restauración de las propiedades del tejido por parte de las BM-MSCs [94]. La seguridad de la terapia con MSCs en el tratamiento de estas patologías también ha sido objeto de estudio en la especie equina. Carrade y colaboradores para comprobar la respuesta inmune de articulaciones sanas tras el tratamiento con MSCs autólogas y alogénicas derivadas de placenta. Aunque detectaron inflamación tras la inyección de ambos tipos celulares, el estudio citológico y clínico no detectó ningún tipo de infección ni rechazo [100].

3.3.2.2. TRATAMIENTO DE LESIONES TENDINOSAS

Los tendones son los tejidos que unen los músculos y los huesos y que estabilizan las articulaciones. En el caballo, cuyas extremidades delanteras son más alargadas, los tendones adquieren grandes dimensiones, como ocurre en el caso del tendón flexor digital superficial (SDFT) y del tendón flexor digital profundo (DDFT) (Figura 4). Estos tendones están localizados en los extremos de las patas y están expuestos a un peso muy elevado. Cuando el caballo galopa absorben y liberan gran cantidad de energía elástica para llevar a cabo el movimiento, llevando al límite el esfuerzo que realiza el tendón [101]. Como consecuencia de la tensión a la que se ve sometido el tendón cuando el animal está realizando esta actividad, pueden aparecer sobrecargas en el tejido o tendinitis, alterando la función de elasticidad que es crítica para la resistencia y velocidad del caballo [5]. El tendón más afectado por este fenómeno en caballos es el SDFT.

A nivel molecular, antes de que se produzca la lesión existe una fase previa de degeneración del tendón durante la cual se debilita, de forma que la práctica habitual del ejercicio empuja al tendón a trabajar más allá de su límite provocando el daño [102].

La curación espontánea es lenta y se lleva a cabo por medio de un proceso de fibrosis [103], el tejido lesionando se va rellenando de fibras de colágeno tipo III en lugar de colágeno tipo I dando lugar a un tejido menos entrecruzado y por lo tanto

menos fuerte [4]. Como consecuencia de esto el tejido nuevo se genera en forma de cicatriz, con una arquitectura de fibras diferente a la normal del tendón, haciendo que, aunque el tendón esté ya curado, sea funcionalmente deficiente. Esto se traduce en que el caballo no pueda realizar el nivel de actividad previo a la lesión, aumentando el riesgo de recaída [104].

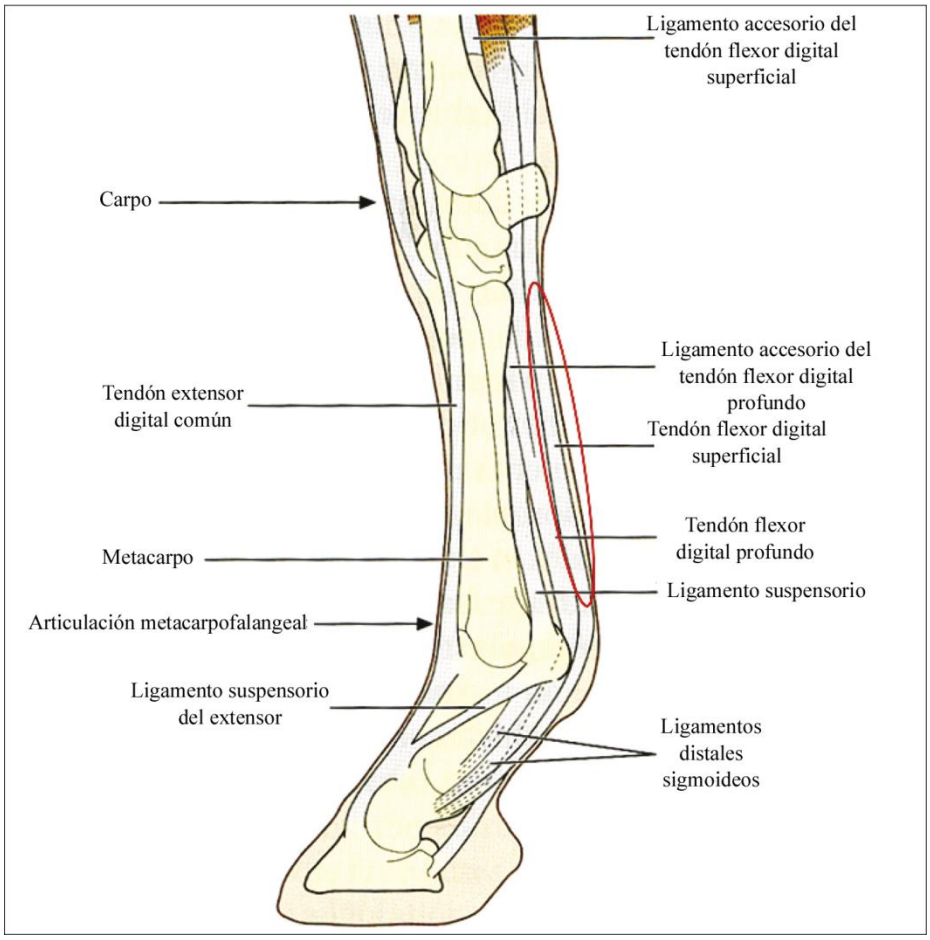


Figura 4. Anatomía de los ligamentos y tendones de la extremidad distal del caballo.

El objetivo de la regeneración *in vivo* de los tendones en équidos es el de recuperar la funcionalidad del tejido a través de la restauración de las propiedades mecánicas y biológicas que tenía previamente a la lesión. Por tanto el objetivo que se plantean las estrategias regenerativas para el tratamiento de estas lesiones es el de utilizar células y factores que ayuden a la regeneración de la matriz extracelular que conformará el tejido para que lo hagan de la forma más aproximada posible a la matriz que poseía inicialmente el tendón. Smith y colaboradores, en 2003, fueron los primeros en utilizar BM-MSCs resuspendidas en sobrenadante de médula ósea autólogo en el

tratamiento de lesiones tendinosas centrales espontáneas en el SDFT, tras el tratamiento no se observaron reacciones adversas ni formación de tumores [105]. La eficacia de este tratamiento quedó demostrada en un el seguimiento realizado tras la administración de las células, que mostró que el 98,2% de caballos de carreras con lesiones en el SDFT volvieron a competir con un bajo índice de recaída en la lesión (27,4%) [9]. En ensayos clínicos controlados, las BM-MSCs se han convertido en las células más utilizadas para investigar el uso de la terapia celular en lesiones en el SDFT. Estas células han sido administradas en suspensión con distintos factores como fibrinógeno [106], suero autólogo [107], BM-MSCs modificadas genéticamente que sobreexpresan la IGF-I [108], factor de crecimiento capaz de estimular la curación del tendón por sí solo [66]. Los resultados obtenidos en estos ensayos muestran una mejora ecográfica en la alineación de las fibras en el tendón respecto a los controles y cambios significativos en la histología de los tejidos tratados, sin embargo no se observan diferencias significativas en la expresión de genes relacionados con el metabolismo del tendón [108].

También se ha aplicado terapia celular con células procedentes de otros orígenes, Nixon y colaboradores administraron SVF derivadas de tejido adiposo en un modelo de lesión inducida con colagenasa. Aunque observaron pocos cambios ecográficos y de expresión de marcadores genéticos entre tendones control y tratados, la histología reveló diferencias significativas [109]. Watts y colaboradores en cambio optaron por la administración de células fetales embrionarias equinas en el mismo modelo de lesión, obteniendo también una mejora en la alineación de las fibras en los tendones de los animales tratados pero, al igual que en casos anteriores, sin correspondencia en la expresión de genes marcadores [110].

Por tanto, aunque los resultados que se van obteniendo presentan ventajas respecto a los tratamientos tradicionales para las lesiones del aparato locomotor, todavía quedan incógnitas sin resolver, entre otras la dosis óptima de MSCs a administrar, el número de administraciones, el medio de suspensión para hacerlo y el momento preciso para llevarlo a cabo. La mayoría de los trabajos se centran en el efecto de las BM-MSCs, y no exploran otras fuentes alternativas de células para investigar su potencial regenerativo.

3.4. CARACTERIZACIÓN DE LAS CÉLULAS MADRE MESENQUIMALES

A pesar de que el tratamiento con MSCs ya es una realidad, todavía no se conocen en profundidad las propiedades que presentan estas células y las diferencias que presentan cuando son obtenidas de diferentes fuentes. Por ello, antes de ser utilizadas de forma masiva en el tratamiento de pacientes es necesario el desarrollo de estudios básicos para su caracterización y el desarrollo de metodologías adecuadas para su expansión y cultivo.

3.4.1. POTENCIAL DE AUTORRENOVACIÓN Y PROLIFERACIÓN

La autorrenovación es la habilidad de las células para generar idénticas copias de ellas mismas durante mucho tiempo y sin perder su estado no diferenciado. Debido a la heterogeneicidad de poblaciones que presentan las MSCs, los resultados de los estudios sobre la capacidad de proliferación han sido muy variables, aunque todos ellos demuestran que las MSCs son capaces de mantenerse largos periodos de tiempo en cultivo *in vitro* sin diferenciarse. En la especie humana se ha descrito que las BM-MSC pueden doblarse aproximadamente 38 veces en cultivo, lo que correspondería con unos 15 pases [111]. Aunque la tasa de proliferación de las AT-MSCs es mayor en la misma especie [112], el tiempo que llegan a permanecer en cultivo es parecido, ya que se han conseguido expandir también hasta el pase 15 [113].

De forma habitual las MSCs se cultivan *in vitro* utilizando medios de cultivo, como por ejemplo *Dulbecos's modified Eagle's médium* (DMEM) suplemententados con suero fetal bovino como sustrato para el crecimiento. Pero si a los cultivos de MSCs, derivadas tanto de médula ósea como de tejido adiposo, se les añaden factores de crecimiento que potencien la división mitótica, como el factor de crecimiento de fibroblasto 2 (FGF-2), el potencial de crecimiento de las MSCs aumenta y las células puede llegar a superar los 70 doblajes en las derivadas de médula [114] y hasta 150 en las derivadas de tejido adiposo [115].

3.4.2. FENOTIPO DE MARCADORES DE SUPERFICIE

Las HSCs pueden ser seleccionadas positivamente dentro de la médula ósea por la presencia en su superficie del marcador CD34 [116]. Sin embargo, no existe un marcador único en la superficie para selección de las MSCs [117], por lo que se recurre a la confección de un perfil de expresión mediante una batería de marcadores completándose además por la ausencia de antígenos relacionados con las células hematopoyéticas o endoteliales.

Los antígenos mostrados en la Tabla 1 muestran los principales marcadores que se examinan por citometría de flujo para la determinación del inmunofenotipo de las MSCs humanas. Entre las moléculas habituales que se examinan se encuentran integrinas (CD49a-f, STRO-1, CD29), cadherinas (CD144), enzimas (CD73, CD13), receptores de superficie (CD44), glicoproteínas (CD105), moléculas de adhesión (CD54, CD106, CD166, CD146), y los complejos mayores de histocompatibilidad MHC-I y MHC-II. Aunque la batería de marcadores positivos que se analizan varía entre distintos trabajos, la mayoría de ellos coinciden en la elección de los marcadores de HSCs CD34 y CD45 como negativos.

El patrón de expresión de los marcadores de superficie es bastante similar entre las BM-MSCs y las AT-MSCs. Los antígenos CD73, CD90 y CD105 señalados como marcadores positivos para la definición de las BM-MSCs en humano [31], se encuentran también presentes en AT-MSCs [118], y en MSCs derivadas de placenta [119], sangre [39] e incluso endometrio [120]. Pero existen diferencias en la presencia de algunos de ellos [121]. La molécula CD34 descrita como marcador de selección positiva para las HSCs en la médula ósea, se encuentra ausente en las BM-MSCs, pero sin embargo se ha detectado en AT-MSCs [122] y MSCs derivadas de sangre periférica [39]. La expresión de este marcador tiende a disminuir a medida que avanzan los pases en el cultivo, indicando que la expansión conduciría a un cultivo de MSCs más homogéneo. De igual forma, los antígenos CD49d y CD106 que se consideran marcadores diferenciadores en MSCs humanas, están expresados en las BM-MSCs pero no en las AT-MSCs [123].

Tabla 1. Marcadores de superficie analizados en MSCs humanas

Tipo de marcador	Antígeno	Detección
SH2 y SH3	CD73	Positiva
Endoglina	CD105	Positiva
STRO-1		Positiva
ALCAM	CD166	Positiva
VLA-α1	CD49a	Positiva
VLA-α2	CD49b	Positiva
VLA-α3	CD49c	Positiva
VLA-α4	CD49d	Negativa
VLA-α5	CD49e	Positiva
VLA-α6	CD49f	Positiva
Cadherina 5	CD144	Negativa
PECAM-1	CD31	Negativa
ICAM-1	CD54	Positiva
ICAM-2	CD102	Positiva
ICAM-3	CD50	Positiva
HCAM	CD44	Positiva
VCAM	CD106	Positiva
NCAM	CD56	Negativa
Cadena LFA-α1	CD11a	Negativa
Cadena LFA-β1	CD18	Negativa
Cadena CR4 α	CD11c	Negativa
Mac 1	CD11b	Negativa
	CD34	Negativa
Antígeno común leucocitario	CD45	Negativa
Thy-1	CD90	Positiva
MUC18	CD146	Positiva
ITGB1	CD29	Positiva
ANPEP	CD13	Positiva
MHC-I		Positiva
MHC-II		Negativa

Adaptado de [124]

Existen diversos factores que parecen influenciar la presencia de estos antígenos en la superficie celular de las MSCs. Entre ellos se encuentra el suero utilizado para suplementar el medio de cultivo o la variabilidad entre individuos [125].

La caracterización de los marcadores de superficie a través de citometría de flujo supone un reto para las MSCs derivadas de animales, ya que los anticuerpos comerciales están diseñados para reaccionar contra antígenos humanos y murinos, mostrando, en general, una baja reactividad frente a las moléculas de otras especies animales [126].

3.4.3. POTENCIALES DE DIFERENCIACIÓN

Las MSCs al derivar de la capa embrionaria del mesodermo presentan la capacidad de diferenciarse a células especializadas de tejidos que también derivan de ella. Tanto *in vivo* como *in vitro* se ha demostrado la capacidad de diferenciación de las MSCs a hueso [111], cartílago [127], tendón [128], músculo [129], tejido adiposo [130] y estroma de la médula ósea [21] (Figura 5).

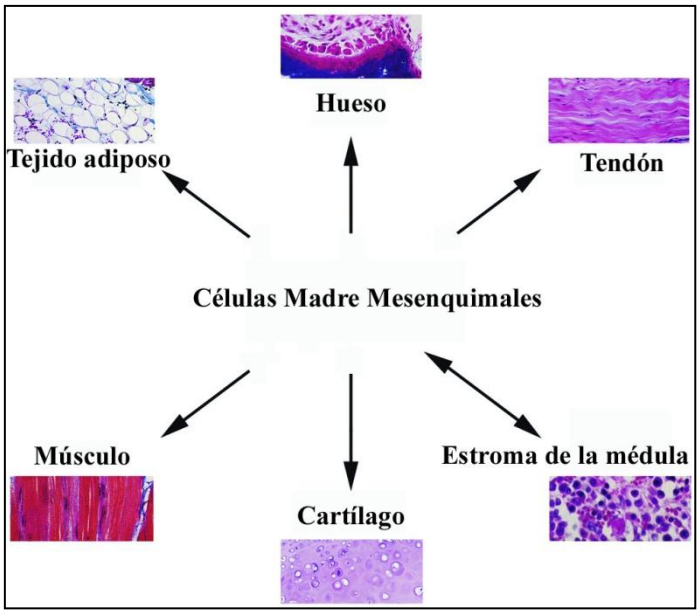


Figura 5: Esquema de los diferentes linajes que resultan de la diferenciación de las BM-MSCs.

La heterogenicidad de poblaciones de las MSCs también se ve reflejada en la capacidad de diferenciación a los diferentes linajes. Estudios realizados en la especie humana han demostrado que un tercio de los clones iniciales que se adhieren a la placa de cultivo después del aislamiento de las células muestran capacidad de diferenciar hacia los tres linajes (osteogénico, condrogénico y adipogénico) [131]. Sin embargo,

alrededor de aproximadamente un 80% de los clones presentan la bipotencialidad de diferenciarse hacia condrocito y osteoblasto [132].

A medida que el cultivo de MSCs va avanzando en el número de pases, los potenciales de multidiferenciación *in vitro* se pierden, siendo comúnmente las capacidades de diferenciación hacia linajes adipogénicos y condrogénicos las que desaparecen, quedando la osteogénesis como único linaje al que las MSCs son capaces de diferenciarse [132].

3.4.3.1. DIFERENCIACIÓN ADIPOGÉNICA

El compromiso y diferenciación de las MSCs hacia adipocito requiere la expresión y acción secuencial en la célula de varios factores reguladores. La activación conjunta de los factores de transcripción PPARγ (receptor gamma activado por proliferador de peroxisoma) y C/EBPα (proteína ligante a CCAAT) es la desencadenante del inicio de la diferenciación. Estas dos proteínas se unen a secuencias específicas de DNA para activar la expresión de genes diana, que van a crear y mantener el fenotipo de adipocito y van a inducir el arresto del ciclo celular. Los genes que codifican para estas moléculas se regulan positivamente durante las primeras etapas de la adipogénesis y por ello se consideran de expresión temprana [133].

PPARγ y C/EBPα también regulan la expresión de genes relacionados con el metabolismo de lípidos. Estos marcadores adipogénicos van a ayudar en el seguimiento de las diferentes etapas de la diferenciación adipogénica y entre ellos vamos a encontrar marcadores de expresión temprana, intermedia y tardía. Por ejemplo, la LPL (lipoprotein lipasa), la enzima que cataliza la hidrólisis de los triglicéridos para controlar la acumulación de lípidos, se considera marcador temprano [134]. También se activa la expresión de genes que codifican para proteínas que no están relacionadas con el metabolismo de lípidos, como es el caso de la proteína aP2 (proteína de unión específica a ácidos grasos) que está involucrada en el transporte intracelular de ácidos grasos, y va a ser considerado un marcador intermedio de adipogénesis [135]. Los genes que codifican para la los factores de secreción adipsina y la leptina por parte de los adipocitos maduros, y la ACBP (proteína de unión acil-coenzima A) relacionada con el metabolismo de la acil-CoA, se consideran marcadores tardíos de adipogénesis [136].

La inducción de las MSCs en cultivo hacia el linaje adipogénico se consigue a través de la utilización de sustancias agonistas como suplemento en el medio de cultivo. Estas moléculas van a activar distintas vías metabólicas. Por ejemplo, la dexametasona estimula la adipogénesis vía receptor de glucocorticoides, el 3-isobutyl-1-methylxantine (IBMX) promueve la diferenciación vía de la proteín-quinasa dependiente de cAMP y la potencia la adipogénesis a través de la unión al receptor IGF-1 que activa diferentes señales de transducción para la formación de grasa [137].

El seguimiento de la diferenciación adipogénica de las MSCs se puede realizar a través del análisis de la presencia de los diferentes marcadores comentados anteriormente a nivel proteico o a nivel de tránscritos. Además, existen tintes con afinidad específica hacia los ácidos grasos (como rojo oleoso O ó rojo nilo) que proporcionan tinciones específicas que permiten visualizar la formación de gotas lipídicas en el interior de las células diferenciadas.

3.4.3.2. DIFERENCIACIÓN OSTEOGÉNICA

En el caso de la diferenciación osteogénica los factores que se van a expresar para que las MSCs se especialicen hacia osteoblastos (las células funcionales que conforman el hueso) son los reguladores transcripcionales RUNX2 (factor de transcripción 2 relativo a runt) y Osterix [138]. Estos marcadores tempranos de la diferenciación osteogénica van a activar la expresión de genes relacionados con la formación de la matriz extracelular que es el principal proceso que se produce durante la osteogénesis.

Aunque no está del todo claro el proceso por el cual las MSCs se diferencian hacia osteoblasto, se conocen los genes que se regulan positivamente durante el proceso de mineralización de la matriz extracelular. La proteína estructural de la matriz extracelular es el COL1A1 (colágeno tipo I) y su presencia en la matriz extracelular aumenta a medida que la diferenciación progresa [139]. La ALP (fosfatasa alcalina) se considera un marcador intermedio y está relacionada con la hidrólisis de la glucosa-6-fosfato para liberar el fosfato. El calcio presente en el medio se une al fosfato precipitando y dando lugar nódulos de hidroxiapatita. Otras moléculas involucradas en el proceso de mineralización de la matriz extracelular son la OC o BGLAP

(osteocalcina) y la BMP-2 (proteína morfogénica ósea 2) [140]. Además, hay otras proteínas que tienen función de adhesión como la SPARC (osteonectina), considerada como marcador temprano, que está involucrada en la unión de los cristales de mineral formados con el colágeno. Por su parte la BSP (proteína siálica del hueso) y la OP o SPP1 (osteopontina) tiene la función de unir las células diferenciadas con la matriz extracelular a través de las integrinas [141].

La inducción de la diferenciación hacia linaje osteogénico de MSCs en cultivo se consigue a través de la exposición de las células a la dexametasona, que se une a los factores de transcripción relacionados con la ostegénesis, al ácido ascórbico que desempeña su función en el metabolismo del colágeno [142] y al β-glicerofosfato, que promueve la mineralización de la matriz extracelular aportando fosfato modulando la actividad metabólica [143].

Para analizar la diferenciación *in vitro* hacia osteoblasto comúnmente se llevan a cabo tinciones específicas (Von Kossa, rojo de alizarina) que muestran afinidad por el calcio depositado en la matriz extracelular por las células diferenciadas. Pero este método resulta insuficiente a la hora de cuantificar la diferenciación [144], por ello se analiza también la expresión de los genes sobreexpresados durante la osteogénesis descritos anteriormente o se determina la actividad de la fosfatasa alcalina.

3.4.3.3. DIFERENCIACIÓN CONDROGÉNICA

La diferenciación condrogénica de las MSCs conlleva la rápida biosíntesis de glicosaminoglicanos y la generación de una matriz extracelular, todo ello acompañado con una alteración de la morfología celular. El factor de transcripción desencadenante de estos procesos es SOX9, que va a regular la expresión del resto de genes relacionados con la condrogénesis.

Tras la inducción condrogénica, la expresión del marcador temprano SOX9 [145] va acompañada de una condensación de las células y un aumento en la expresión y secreción de componentes cartilaginosos a la matriz extracelular similares a los componentes que conforman el cartílago hialino *in vivo*. La proteína de mayor presencia en este tipo de tejido es el COL2A1 [146] (colágeno tipo II), sin embargo se considera

como marcador tardío de diferenciación ya que se expresa más tarde que otros componentes de la matriz. El COL2A1 aumenta progresivamente junto con la proteína de adhesión condroadherina [147]. En la matriz extracelular van a estar presentes diversos proteoglicanos como el agrecano, con función estructural para resistir la compresión, y cuya expresión aparece desde los primeros días de la diferenciación y también va en aumento paulatinamente [147]. Acompañando al agrecano, se encuentran otros proteoglicanos como es el caso de la fibromodulina, la decorina o el biglicano, que se encargan de unir las fibras de colágeno de la matriz. Estas glicoproteínas se expresan de una manera más rápida y en fases del cultivo más avanzadas. Otra proteína que se encuentra asociando las fibras de colágeno y los agrecanos es la COMP (proteína oligomérica de la matriz del cartílago), cuya expresión se detecta desde los primeros días de la diferenciación [148]. El colágeno tipo X comienza a expresarse más tarde que el resto de colágenos y está asociadoa a condrocitos hipertróficos [149], ya que suele aparecer durante las últimas etapas de formación de hueso *in vivo* a partir del cartílago *in vivo*.

A diferencia de las inducciones adipogénica y osteogénica que se llevan a cabo en células cultivadas en monocapa, la diferenciación condrogénica, habitualmente, se desarrolla en tres dimensiones. El objetivo de realizar la diferenciación en este sistema de micromasas, es el de generar un ambiente de hipoxia, ya que el cartílago está pobremente vascularizado y por lo tanto contiene niveles muy bajos de oxígeno [150]. La presencia de miembros de la familia TGF-β (factor de crecimiento transformante) es determinante para promover la condrogénesis en las MSCs [147], siendo las isoformas 2 y 3 las que causan mayor efecto, induciendo de una forma más rápida la acumulación de proteoglicanos y COL2A1. La familia de las BMP (proteínas morfogénicas de hueso) son capaces de potenciar el efecto que generan los TGF- β, siendo más efectivo el efecto de la isoforma 2 que de la 6 [151].

Al igual que en la diferenciación a los otros linajes, existen distintas formas de analizar y cuantificar la diferenciación condrogénica. Mientras que tinciones como azul alcian, azul de tolouidina y safranina O tiñen los glicosaminoglicanos (GAG) de la matriz extracelular, que se pueden cuantificar también a través de la reacción con DMMB (azul de dimetilmetileno) [152]. Se pueden determinar por inmunohistoquímica las moléculas COL2A1 y otras glicoproteínas de la matriz extracelular. También el

análisis del aumento de expresión de proteínas durante la condrogénesis descritas anteriormente, indica el grado de diferenciación que las MSCs han alcanzado.

3.4.3.4. DIFERENCIACIÓN A LINAJES DE OTRAS CAPAS EMBRIONARIAS

En los últimos años se están explorando cada vez más la posibilidad de que las MSCs no sólo se diferencien a los linajes que proceden de la misma capa embrionaria de las que ellas derivan, sino que presentan el potencial de diferenciación para dar lugar a células presentes en tejidos que derivan de las capas embrionarias endodérmica y ectodérmica, en un proceso denominado transdiferenciación [153].

Una de las diferenciaciones de las MSCs sobre la que más se investiga es la diferenciación hacia tipos celulares neurales (derivados del ectodermo) debido a la aplicación que podrían tener para el tratamiento de enfermedades neurodegenerativas. Los protocolos de diferenciación que se aplican para conseguir este objetivo varían desde la inducción química utilizando suplementos adecuados [154], transfección génica de moléculas participes en la neurogénesis [155] o directamente el cultivo de MSCs con células neurales [156]. El perfil de expresión génica de las MSCs en respuesta a la inducción hacia célula neural muestra un aumento de genes neurogénicos relativos a la transmisión sináptica como la calcio/calmodulina dependiente de serin-protein quinasa (CaMKII) y la sintaxina 1 (STX1). Además se observa también aumento de expresión de genes asociados a precursores neuronales como el supresor de señales de citoquina 2 (SOCS2) y el factor de crecimiento neuronal 1 (NGF) [157].

También ha sido descrita la capacidad de las MSCs de diferenciarse hacia linajes celulares derivados del endodermo, como es el caso de diferenciaciones hacia células pancreáticas [26] o hepatocitos [27] . A través de manipulación genética de MSCs se ha conseguido crear células productoras de insulina lo que puede suponer un gran avance para la terapia de reemplazamiento en el tratamiento de la diabetes [158]. La transdiferenciación de las MSCs hacia células similares a hepatocitos genera una amplia expectativa en el campo de los trasplantes debido a la escasez de órganos disponibles [159].

3.4.4. PROPIEDADES INMUNOLÓGICAS

Otra de las características de las MSCs que se están estudiando en la actualidad es la relación de estas células con el sistema inmune. Las MSCs muestran un efecto inmunomodulatorio sobre células del sistema inmunitario a través de la interacción con linfocitos B y T, células NK, células dendríticas, macrófagos y neutrófilos [160]. La Figura 6 muestra un esquema de como las MSCs realizan estos efectos inmunosupresores a través de la inhibición de la proliferación de las células T, y de la producción de TNF-α y INF-γ y aumentando los niveles de IL-10, ya sea por medio de la secreción de moléculas o por medio del contacto célula-célula [161].

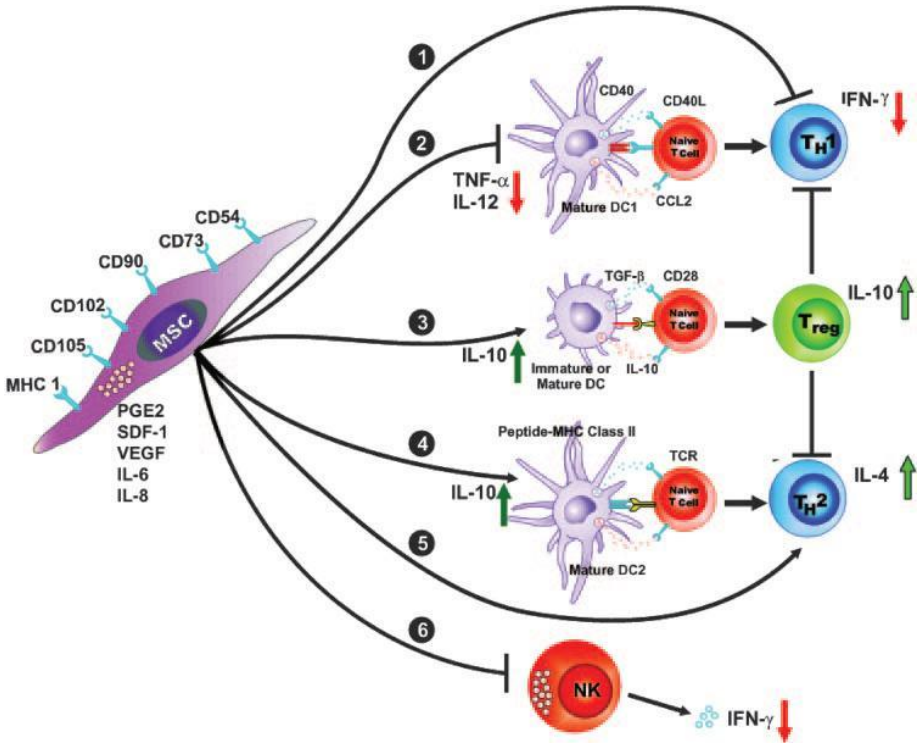


Figura 6. Esquema de los mecanismos de inmunoregulación de las MSCs sobre diferentes células del sistema inmunológico. Figura original de [161].

Además las MSCs se considera que son “inmunoprivilegiadas”, al no expresar en su superficie el complejo mayor de histocompatibilidad de clase II (MHC-II) [100], molécula implicada en el rechazo de trasplantes. Como resultado de la ausencia del MHC-II el tratamiento *in vivo* con MSCs no parece provocar respuestas inflamatorias ni rechazos, aunque aún se están llevando a cabo muchas investigaciones al respecto.

3.5. CÉLULAS MADRE MESENQUIMALES EQUINAS

La terapia celular, como se ha comentado en el Apartado 3.2., es de suma importancia para el tratamiento de lesiones en caballos que de otra manera tienen un mal pronóstico de recuperación. Pero de igual importancia es determinar la naturaleza y el fenotipo de las células que se están utilizando en la terapia, es fundamental conocer sus características moleculares para comprender cómo actúan durante los procesos de reparación y de esa forma potenciarlas para conseguir resultados clínicos favorables.

Al inicio de este trabajo de Tesis Doctoral los estudios desarrollados sobre la caracterización de las células madre mesenquimales equinas eran escasos. Pero en los últimos años debido al creciente interés por su utilización en terapia celular para el tratamiento de lesiones, el número de publicaciones orientadas hacia el análisis de las propiedades *in vitro* de las MSCs equinas ha aumentando aportando información sobre sus características.

Las MSCs equinas, de igual forma que las humanas, pueden obtenerse de diferentes tejidos, siendo los principales la médula ósea [162] y el tejido adiposo [163]. Pero también han despertado interés las MSCs procedentes de sangre periférica [164], cordón umbilical [165], sangre del cordón umbilical [166], de encías y ligamento periodontal [167]. En todos estos casos la selección de las células se ha realizado por medio de la capacidad de adherencia al plástico que poseen estas células. Una vez en cultivo la morfología que adquieren las MSCs equinas es la típica de forma de huso extendiendo procesos y mostrando un crecimiento en monocapa [168].

3.5.1. POTENCIAL DE AUTORRENOVACIÓN Y PROLIFERACIÓN

Las MSCs equinas son capaces de formar colonias cuando se siembran a densidades bajas confirmando así su capacidad de autorrenovación [169]. Tienen una capacidad de expansión similar a las MSCs humanas, pudiéndose doblar más de 30 veces, incrementando este número si el cultivo se suplementa con FGF-2 [170]. Entre las MSCs obtenidas de diferentes orígenes en el caballo, las derivadas de tejido adiposo son las que presentan mayor potencial de proliferación [170], mientras que las derivadas

de sangre periférica son las de menor, ya que su capacidad de expansión se limita a 5 ó 6 pases, y además mostrando una elevada sensibilidad a la tripsinización [164].

3.5.2. FENOTIPO DE MARCADORES DE SUPERFICIE

Los criterios de mínimos propuestos por la ISCT son válidos para la definición de las MSCs humanas, sin embargo, hasta la fecha no existen criterios universales para la caracterización de las MSCs de todas las especies. El principal problema que presenta la caracterización del fenotipo de las MSCs equinas es el de la falta de anticuerpos comerciales que muestren reactividad con los epítopos de las moléculas de superficie de la especie equina [171]. Durante el desarrollo de este trabajo de Tesis Doctoral, se han descrito anticuerpos contra antígenos que la ISCT propone como marcadores de MSCs en la especie humana que presentaban reactividad con las moléculas equinas, como por ejemplo CD105 [172] y CD90 [173, 174]. Otras moléculas que también se analizan como antígenos presentes en las MSCs humanas de forma habitual en la determinación del inmunofenotipo, también muestran reactividad con las equinas, como es el caso de CD44 [173] y CD29 [175]. La Tabla 2 muestra los marcadores de superficie para los cuales se ha estudiado reactividad con anticuerpos comerciales.

Cómo técnica alternativa a la citometría de flujo, la PCR cuantitativa permite determinar la expresión de los genes correspondientes a los marcadores de superficie cuando no se encuentran anticuerpos comerciales disponibles [126]. Por medio de esta técnica se han confirmado resultados obtenidos por la citometría de flujo para las moléculas CD90, CD105 y CD45 [172], y hallado la expresión de otras como CD73 para la que la citometría daba un resultado negativo [172, 174]. Por este motivo hay que tomar con precaución los resultados negativos que se puedan obtener por medio de citometría de flujo, ya que falta de especificidad de los anticuerpos comerciales por los epítopos equinos puede conducir a falsos negativos.

Tabla 2. Marcadores de superficie analizados por citometría de flujo en MSCs equinas.

Tipo de marcador	Antígeno	Detección	Referencia
SH2 y SH3	CD73	Negativa	[175, 176]

Endoglina	CD105	Positiva	[165, 167, 172]
STRO-1		Positiva	[174]
HCAM	CD44	Positiva	[173-177]
Cadena LFA-α1	CD11a	Negativa	[172]
Antígeno común leucocitario	CD45	Negativa	[172, 177]
Thy-1	CD90	Positiva	[167, 172-178]
ITGB1	CD29	Positiva	[175, 176]
ANPEP	CD13	Positiva	[173, 177]
MHC-I		Positiva	[176]
MHC-II		Negativa	[176]
	CD14	Negativa	[176]
	CD164	Negativa	[177]

3.5.3. POTENCIAL DE DIFERENCIACIÓN

Las MSCs equinas pueden ser inducidas hacia linajes osteogénico, adipogénico y condrogénico, requisito de la ISCT para definir las MSCs humanas, aunque el potencial de diferenciación de las MSCs equinas varía en función del origen del cual proceden las células.

3.5.3.1. DIFERENCIACIÓN ADIPOGÉNICA

La inducción *in vitro* hacia este linaje es la más difícil de conseguir en el caso de las MSCs equinas, los protocolos estandarizados para la diferenciación de MSCs humanas (con IBMX, indometacina, insulina y dexametasona) no son eficaces para generar la diferenciación a adipocito [126]. La utilización de suero de conejo como potenciador de la adipogénesis parece ser clave para alcanzar la diferenciación debido a su alto contenido en ácidos grasos [179]. Aunque la inducción se ha conseguido en las MSCs procedentes de todos los tejidos, para algunos orígenes como la sangre periférica o las encías, y en algunos de los trabajos publicados [169, 174] sólo se ha logrado la diferenciación en una etapa temprana del proceso, en la cual se puede apreciar un cambio morfológico acompañado por la formación de pequeñas gotas lipídicas en el interior de las células pero sin llegar a fusionarse entre ellas para generar otras más grandes [164, 167].

La valoración del potencial de diferenciación se lleva a cabo de igual forma que lo señalado anteriormente para las MSCs humanas, por medio de la tinción de los ácidos grasos almacenados en el interior de las células con rojo oleoso O. Además también se observa el aumento en la expresión de los genes relacionados con la adipogénesis *PPARγ* y *LPL* [172].

3.5.3.2. DIFERENCIACIÓN OSTEOGÉNICA

La diferenciación hacia osteoblasto ocurre de forma más rápida a la descrita en otras especies [162]. Las células se empaquetan en múltiples capas dando lugar a nódulos, lo cual está asociado a una mayor formación de matriz extracelular. Los factores que se utilizan para conseguir la inducción son los mismos que los utilizados en la especie humana: dexametasona, β-glicerofosfato y ácido ascórbico. Las células procedentes de médula ósea parecen ser las que poseen mayor capacidad osteogénica [180, 181], aunque las derivadas de tejido adiposo también muestran una habilidad de diferenciación notable [181, 182] si bien algo más lenta [178]. Las MSCs procedentes de sangre periférica, cordón umbilical y sangre de cordón umbilical también son capaces de diferenciarse hacia linaje osteogénico aunque su potencial es menor que el de los dos casos anteriores [180, 181].

La monitorización de la diferenciación se realiza por las tinciones habituales de la matriz extracelular con rojo de alizarina o von Kossa, y para la cuantificación se han estudiado varios genes relacionados con la osteogénesis, como la osteopontina, el colágeno tipo I [172] o la osteonectina [170].

3.5.3.3. DIFERENCIACIÓN CONDROGÉNICA

La diferenciación condrogénica requiere unos tiempos similares a los descubiertos para la especie humana y se realiza con el mismo sistema de pellets. El inductor principal de la condrogénesis es también el mismo, el TGF-β. Tanto la isoforma TGF-β1 como la TGF-β3 son capaces de inducir la condrogénesis en MSCs procedentes de médula ósea y tejido adiposo [175, 178]. El potencial condrogénico parece ser mayor en las BM-MSCs que en las AT-MSCs [183], aunque las MSCs derivadas de cordón umbilical muestran en otro estudio mayor potencial que las

derivadas de médula [184]. Las MSCs procedentes de sangre periférica presentan una capacidad limitada a la hora de llevar a cabo esta diferenciación [164, 180].

Las tinciones que se utilizan son Safranina O, azul de alcian y azul de tolouidina también son las habituales para determinar la diferenciación condrogénica de los pellets de las MSCs equinas. La cuantificación del potencial se puede determinar a través de inmunohistoquímica de las moléculas nombradas con anterioridad del colágeno tipo II y otras glicoproteínas de la matriz extracelular [185], la cuantificación de los GAG generados [183], o por medio del análisis de la expresión de genes relativos a la condrogénesis [172].

3.6. CULTIVO DE LAS MSCs.

Para que las MSCs puedan ser utilizadas en terapia celular generalmente se tienen que obtener un número elevado de estas células. Sin embargo el número de MSCs que se aíslan de los tejidos de origen es bajo, por lo que es necesaria la expansión de estas células en cultivo *in vitro* de las MSCs. Como se menciona el apartado 4.1. en el cultivo de las MSCs se utiliza habitualmente medios de cultivos, estos medios están formados de un medio comercial base que aporta el pH y los aminoácidos necesarios para el crecimiento, una mezcla de antibióticos que suelen ser penicilina/estreptomicina y suero fetal bovino.

3.6.1 FACTORES QUE AFECTAN AL CULTIVO

El crecimiento de las MSCs en cultivo *in vitro* es altamente artificial, el entorno nuevo que rodea a las células es muy distinto al que están acostumbradas *in vivo*. El crecimiento en una superficie bidimensional conduce a las células a adoptar morfologías distintas a las que normalmente se encuentran [153]. Por ello, el mayor reto en el cultivo de las MSCs es el desarrollo de sistemas de cultivo que sean óptimos para la expansión efectiva y menos altere las propiedades de estas células.

En los siguientes apartados se detallan la influencia que pueden diversos factores sobre el cultivo de las MSCs.

3.6.1.1. SUERO FETAL BOVINO

. Las MSCs *in vitro* se ven expuestas a medios de cultivo que intentan aportar lo necesario para su crecimiento, pero que distan de la composición adecuada y aporte continuo de nutrientes y factores que poseen cuando están en sus nichos. Para intentar paliar este hecho, los medios de crecimiento están suplementados, habitualmente, con suero fetal bovino (FBS), rico en factores de crecimiento, nutrientes, hormonas y vitaminas. De forma habitual los medios de crecimiento de las MSCs se suplementan añadiendo un 10% de FBS [186], aunque mayores concentraciones de FBS pueden potenciar un aumento en el crecimiento de las MSCs [187]. Sin embargo, existe una elevada variabilidad entre los diferentes lotes comerciales. Estas diferencias pueden alterar el patrón de crecimiento de las células y los perfiles de expresión de moléculas de adhesión [188] entre lote y lote, y con respecto a las células en su estado nativo. Además, existe el riesgo añadido de transmisión de enfermedades infecciosas a través del FBS [189].

3.6.1.2. OXÍGENO

Otro factor que suele ser diferente en los cultivos de MSCs respecto al que se encuentra en los organismos es el oxígeno. Esta molécula está implicada en importantes fenómenos fisiológicos y patológicos como el desarrollo embrionario [190], la formación de tumores [191] o la reparación de tejidos [192]. El oxígeno también forma parte de procesos celulares como los de apoptosis, metabolismo, diferenciación y migración [11], todos ellos coordinados a través de la estabilización del factor de hipoxia inducible 1 α (HIF1α) [193]. El oxígeno también es capaz de inducir cambios en respuestas transcripcionales, modificaciones post-traduccionales y en el tráfico intracelular de proteínas [11].

Habitualmente, los incubadores que se emplean para el crecimiento de las células utilizan el aire atmosférico como fuente de oxígeno, por lo que las células se encuentran creciendo en su interior a un porcentaje de oxígeno de entorno al 20%. Sin embargo, el ambiente que rodea a las MSCs cuando están en sus nichos es bastante más bajo, encontrándose entre 1 y 7% en la médula ósea [194] y entre 2 y 8% en el tejido adiposo [195] (Figura 7).

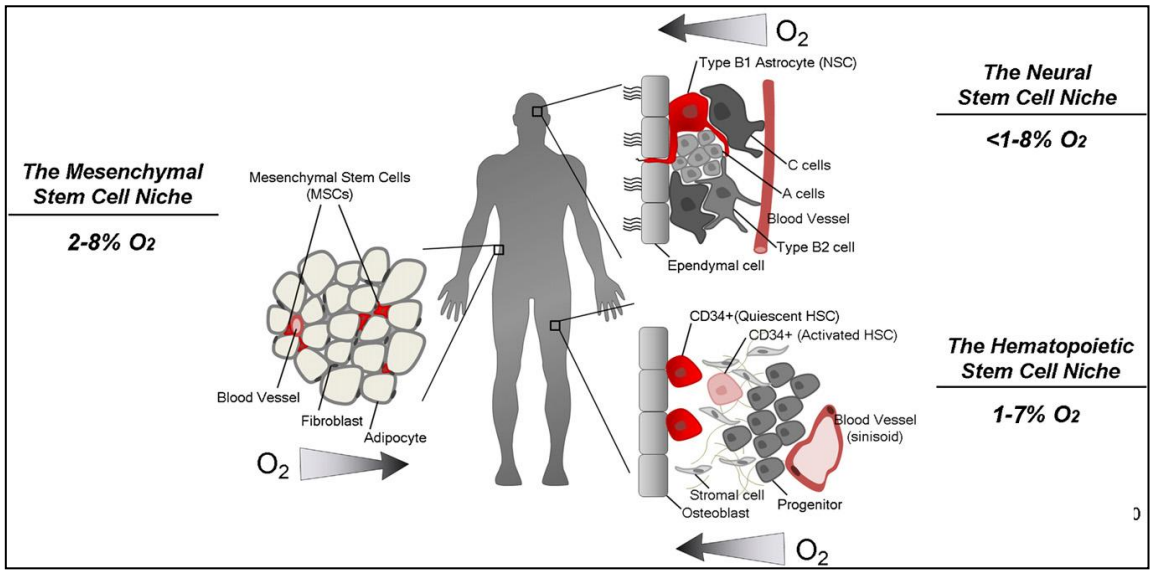


Figura 7. Esquema del porcentaje de oxígeno en diferentes nichos de células madre en la especie humana. Adaptado de [10]

Los trabajos realizados sobre cómo afecta la hipoxia (nivel bajo de oxígeno en cultivo *in vitro*) a las diferentes características de las MSCs están llenos de controversia, ya que existe una elevada heterogeneidad a la hora de llevarlos a cabo. Dentro de los parámetros que varían entre experimentos se encuentra el porcentaje de oxígeno a los que las células son expuestas que se mueve en el rango entre 1% y el 8% [196, 197] o el pase en el que se encuentran las MSCs, que varía entre los primeros pases [198] y el pase 8 [199]. Además, los trabajos se han desarrollado en diferentes especies como la murina [197], la humana [200] o la canina [201] y, por lo tanto, las MSCs presentar diferente sensibilidad hacia el oxígeno. Todo ello hace que los resultados que se han obtenido hasta el momento en el estudio del efecto de la hipoxia sobre las MSCs no sean homogéneos y en algunos casos contradictorios.

La mayoría de los estudios sobre la caracterización de las MSCs que se detallan a continuación han sido llevados en las especies humana y murina. Sin embargo, hasta ahora, en todos los trabajos desarrollados para la caracterización de las MSCs equinas ninguno de ellos se ha orientado hacia la determinación del efecto de la hipoxia sobre estas características.

3.6.1.2.1. Efecto del oxígeno en la proliferación celular

Los mecanismos moleculares por los cuales el porcentaje de oxígeno puede regular la proliferación celular no se conocen con exactitud. Sin embargo, HIF 1α parece estar relacionado con la expresión de los inhibidores de quinasas ciclina-dependientes p21 y p27 envueltas en la regulación del ciclo celular [202, 203] y también con la de expresión de factores anti-apoptóticos tales como Bcl-2 y survivina [204]. La proliferación se ve afectada por cambios en la presión de oxígeno, aunque no hay un acuerdo general sobre si está potenciada o inhibida. Es lógico pensar que las MSCs cultivadas en hipoxia al encontrarse en un entorno más parecido al fisiológico presenten mayor capacidad de proliferación, y así es como se demuestra en estudios realizados con MSCs humanas en los que se observan: un aumento en el potencial de proliferación de las MSCs en un pase determinado [196], un aumento en el tamaño y número de colonias que se forman (CFU-F) [205] y en la aparición más tardía de la senescencia [206] en comparación con MSCs expuestas a 20% O_2. Sin embargo, existen estudios que contradicen los resultados anteriores tanto en la especie humana [200] como para otras especies domésticas como el perro [201].

3.6.1.2.2. El oxígeno no modifica el fenotipo de marcadores de superficie

El fenotipo determinado que muestran las MSCs cultivadas con un 20% O_2 se mantiene independientemente de que las células estén expuestas a bajas presiones de oxígeno. Las MSCs cultivadas en condiciones de hipoxia siguen cumpliendo los criterios de expresión de marcadores propuestas por la ISCT [31], y también mantienen la expresión de los demás marcadores que habitualmente se estudian para establecer el fenotipo [207].

3.6.1.2.3. Influencia del oxígeno en el potencial de diferenciación

Al igual que en el caso de la proliferación, no existe un acuerdo general sobre el efecto de la hipoxia sobre los potenciales de diferenciación de las MSCs.

Las MSCs parecen conservar su potencial de diferenciación hacia osteoblasto en ambientes bajos en oxígeno [198], aunque en condiciones de oxígeno demasiado bajas (en torno al 1% de O_2) esta capacidad tiende a inhibirse. La capacidad osteogénica puede ser nuevamente restaurada a concentraciones más elevadas de oxígeno (3%) [200]. Este resultado estaría respaldado por el hecho de que para la diferenciación hacia osteoblasto *in vivo* en el hueso vascular se requiere presiones de oxígeno elevadas [208].

La diferenciación hacia linaje adipogénico muestra también resultados dispares cuando las MSCs se cultivan a concentraciones bajas de oxígeno. Mientras que algunos autores remarcan el entorno favorable que una tensión baja de oxígeno proporciona para potenciar la adipogénesis [209, 210], otros no detectan ningún cambio remarcable en la capacidad de diferenciación de las MSCs [196, 211].

En cambio, el oxígeno parece desempeñar un papel más importante en la condrogénesis que en las dos anteriores diferenciaciones. La formación del cartílago durante el desarrollo embrionario tiene lugar en condiciones de hipoxia [212] y el cartílago adulto es un tejido no vascularizado que presenta una presión de oxígeno cercana al 1% de O_2 [213], por lo que un ambiente de hipoxia favorecería la diferenciación de las MSCs hacia un fenotipo condrogénico. Así lo demuestran los estudios que describen una potenciación de la condrogénesis cuando las MSCs se cultivan en condiciones de hipoxia [214, 215].

4. MATERIAL Y MÉTODOS

A continuación se procederá a enumerar las técnicas y materiales utilizados durante el desarrollo del trabajo de Tesis Doctoral. Se hará una pequeña descripción de cada uno de ellos a la vez que se indicará con qué objetivo concreto y en qué manuscritos han sido utilizados.

4.1 ANIMALES

Los animales utilizados para la obtención de las MSCs que se utilizaron para la realización de la presente memoria fueron distintos según los trabajos.

En los manuscritos I, II y III los caballos eran pacientes del Hospital Clínico Veterinario de la Universidad de Zaragoza con diferentes tipos de lesiones de tendón y ligamento. Las edades de los caballos variaron entre los 4 y los 17 años, y había variedad tanto en el sexo como en la raza. Las muestras de médula ósea y tejido adiposo fueron obtenidas bajo consentimiento del dueño y de acuerdo a la normativa sobre el bienestar animal.

En los manuscritos IV, V y VI los caballos de los que se obtuvieron las muestras biológicas pertenecían a un proyecto del Ministerio de Educación y Ciencia concedido a nuestro grupo de investigación. Se trataban de machos castrados y con un rango de edad entre 4 y 7 años.

Todos los estudios fueron aprobados por el Comisión Ética Asesora para la Experimentación Animal de la Universidad de Zaragoza PI36/07

4.2. CULTIVO DE MSCs

Para el desarrollo de este trabajo de Tesis Doctoral se llevaron a cabo técnicas de cultivo de MSCs. El manejo de las células se realizó en condiciones de esterilidad

haciendo uso de una cabina de flujo laminar. A continuación se explican brevemente las etapas que fueron necesarias para el cultivo celular.

4.2.1. OBTENCIÓN DE MUESTRAS Y AISLAMIENTO DE MSCs

Las muestras biológicas a partir de las que se han aislado las MSCs y la metodología utilizada para el procesado de las mismas fueron:

- Aspirados de médula ósea del esternón: se obtuvieron con una aguja 4 11G Jameshi con heparina. Las células mononucleares de los aspirados se aislaron por gradiente de centrifugación con Lymphoprep (Atom). Las células fueron lavadas con PBS y sembradas a una densidad de 2.000.000 de células mononucleadas/cm^2.
- Tejido adiposo subcutáneo del área supragluteal cercana a la cola del caballo: la SVF se aisló por digestión con colagenasa tipo I (Sigma-Aldrich), las células se lavaron con PBS y se sembraron a una densidad de 100.000 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 10% de FBS (Gibco). 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 condiciones en las que se mantuvieron las MSCs fueron a 37ºC, 5% de CO_2 y 20% de O_2 para todos los trabajos, y además a 37ºC, 5% de CO_2 y 5% de O_2 para los manuscritos IV y V.

4.2.2. EXPANSIÓN Y CRIOPRESERVACIÓN

Para la realización de experimentos que componen esta memoria fue necesario trabajar con un número elevado de células, objetivo que se consiguió a través de la expansión de MSCs a lo largo de varios pases. A continuación se resume la metodología utilizada para ello.

Las MSCs de ambos orígenes se cultivaron realizando cambios de medio cada 3 días hasta alcanzar aproximadamente el 80% de confluencia. Posteriormente, las células se despegaron de la superficie de la placa de cultivo por tratamiento con un 0,25% de

tripsina/EDTA (Sigma-Aldrich) y se contaron con un hemocitómetro o un contador de partículas Z2 Coulter. En este punto, una fracción de células fue re-sembrada a una densidad de 5.000 MSC/cm^2, que constituyó un nuevo pase, y el resto de las células fueron criopreservadas para la realización de la caracterización que aparece en los manuscritos II, III y VI. El medio de congelación en el que las células fueron resuspendidas estaba formado por un 90% de FBS y 10% de DMSO. Esta metodología se repitió hasta el pase 3 para cada uno de los animales.

4.2.3. ENSAYOS DE PROLIFERACIÓN

La proliferación celular se determinó de dos formas distintas a lo largo de la presente memoria.

En el manuscrito II las células se sembraron en placas de 96 pocillos y durante un pase entero se determinó el número de células viables para construir una curva de proliferación mediante el ensayo MTT (Sigma-Aldrich) siguiendo las especificaciones del fabricante.

En el manuscrito V las células fueron sembradas en placas de 6 pocillos por triplicado y durante un pase entero fueron contadas en un contador de partículas Z2 Coulter para elaborar una curva de proliferación.

4.2.4. ENSAYOS DE DIFERENCIACIÓN

La inducción de las MSCs hacia linajes mesodérmicos es fundamental para su caracterización, a continuación se indican las condiciones, tinciones espécificas y cuantificaciones que se llevaron a cabo para valorar las diferenciaciones durante la realización de esta memoria.

4.2.4.1. CONDICIONES DE DIFERENCIACIÓN

Las condiciones de cultivo para los ensayos de diferenciación llevados a cabo en los manuscritos II, III, IV y VI fueron las siguientes:

- Diferenciación osteogénica: las células se sembraron a una densidad de 20.000 células/cm^2 durante 7-9 días. El medio de inducción estaba formado por medio de crecimiento suplementado con 10 nM de dexametasona, 10 mM de β-glicerofosfato y 100 μM de ascorbato-2-fosfato.
- Diferenciación adipogénica: las células se sembraron a una densidad de 5.000 células/cm^2 durante 14 días. 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.
- Diferenciación condrogénica: aproximadamente 500.000 MSCs se dispusieron en forma de pellet mediante centrifugación en tubos cónicos de polipropileno durante 21 días. El medio condrogénico estaba compuesto por DMEM rico en glucosa (Gibco) suplementado con 10% de FBS, 10 ng TGF-β3 (R&D Systems), ITS+ premix (BD), 40 μg/mL prolina (Sigma-Aldrich), 50 μg/mL de ascorbato-2-fosfato y 0.1 μM dexametasona.

4.2.4.2. TINCIONES ESPECÍFICAS

Para valorar la diferenciación de las MSCs se llevaron a cabo las siguientes tinciones específicas para cada una de las diferenciaciones:

- Tinción osteogénica: las células se fijaron con etanol frío durante 1 hora, se lavaron con agua destilada y se incubaron durante 10 minutos con rojo de alizarina al 2% y pH 4,6. Posteriormente el exceso de tinción se eliminó con lavados de agua destilada.
- Tinción adipogénica: las células se fijaron con formalina al 10% (Sigma-Aldrich) durante 15 minutos, después se lavaron con PBS y se tiñeron durante 30 minutos a 37 ºC con rojo oleoso al 0,3% en isopropanol. Finalmente, el exceso de tinción se eliminó lavando con agua destilada.
- Tinción condrogénica: los pellets diferenciados se fijaron con formalina al 10%, se embebieron en bloques de parafina y fueron cortados en secciones de 5 μm de grosor. A lo largo del estudio se fueron poniendo a punto diversas tinciones para valorar la diferenciación condrogénica. Los cortes se tiñeron con azul alcián y safranina O y también se realizó inmunohistoquímica de la proteína S-100 con el anticuerpo

policlonal de conejo IR504 (DAKO). Además se valoró la histología de los pellets con la tinción clásica hematoxilina y eosina.

4.2.4.3. CUANTIFICACIÓN

Las diferenciaciones hacia los distintos linajes fueron cuantificadas por diversas técnicas:

- Diferenciación osteogénica: se cuantificó la actividad de la ALP. Después de lisar las células con tampón RIPA, el lisado celular fue incubado con el sustrato p-NPP. La aparición del producto amarillo p-NP como consecuencia de la transformación de p-NPP por la enzima, fue medida en un espectofotómetro a 405 nm. Los resultados se normalizaron con la cantidad total de proteína del lisado calculado por BCA. Este ensayo se llevó a cabo para cuantificar la osteogénesis en los manuscritos II, IV y VI.
- Diferenciación adipogénica: el rojo oleoso que tiñe los lípidos formados en las células en diferenciación fue extraído con isopropanol y medido a 510 nm. Los resultados fueron normalizados con la cantidad total de proteína obtenida mediante tinción con Ponceau.
- Diferenciación condrogénica: se cuantificaron los sGAG generados en la matriz extracelular. La cantidad de sGAG se determinó por reacción con la solución DMMB y midiendo la absorbancia a 530 nm en un espectofotómetro Biotek Synergy HT. Las cantidades de sGAG se normalizaron con el contenido en DNA de las muestras obtenido mediante el ensayo del Hoechst 33258.

4.3. CITOMETRÍA DE FLUJO

La citometría de flujo se basa en la detección por medio de fluorescencia de células marcadas, bien con anticuerpos conjugados con fluoróforos o bien con fluoróforos unidos a partes de la célula como el núcleo o la membrana celular. La señal emitida por las células excitadas a una determinada longitud de onda es recogida por un detector que genera un histograma con los datos. Durante el desarrollo de esta memoria el citómetro de flujo empleado fue el FACSARIA (BD Biosciences) perteneciente al

Servicio de Separación del Instituto Aragonés de Ciencias de la Salud se y utilizaron diversos fluoróforos con diferentes objetivos que pasamos a detallar a continuación.

4.3.1 VIABILIDAD CELULAR

La viabilidad de los cultivos celulares se determinó identificando la proporción de células, viables, apoptóticas y necróticas en los cultivos. La incubación de las células con Annexina V (AnV) permitió identificar las células apoptóticas ya que la molécula tiene afinidad por la fosfatidilserina translocada a la superficie de la membrana celular en células apoptóticas. Estas células se diferenciaron de las necróticas mediante tinción con ioduro de propidio (PI), esta molécula es capaz de unirse al DNA cuando las membranas de las células están alteradas como consecuencia de la necrosis. De esta forma, las células en un estado de apoptosis temprana eran positivas para AnV y negativas para PI, las células no viables eran positivas para PI y AnV, mientras que las viables eran negativas para las dos moléculas. La metodología utilizada se llevó a cabo siguiendo las instrucciones del fabricante. Esta técnica fue utilizada en los manuscritos II y V.

4.3.2. INMUNOFENOTIPO

La presencia de los marcadores de superficie de MSCs se determinó incubando las células con anticuerpos monoclonales de ratón anti-humano de diversas casas comerciales siguiendo las instrucciones especificadas para cada uno de los casos. Se utilizaron controles de isotipo como negativos y tinción de células muertas con SYTOX™ (Molecular Probes) antes de analizar los marcadores en el citómetro de flujo. Esta técnica fue utilizada para establecer el inmunofenotipo en los manuscritos III y V.

4.3.3. CICLO CELULAR

La progresión del ciclo celular de las MSCs en cultivo fue determinada mediante tinción del DNA utilizando PI de las células fijadas previamente con etanol frío al 70%. Los datos recogidos por el citómetro mostraron la proporción de células en las diversas

fases del ciclo celular (G_0/G_1, S ó G_2/M) siendo la cantidad de fluorescencia recogida proporcional a la cantidad de DNA en cada una de ellas.

4.4. EXTRACCIÓN DE RNA Y SÍNTESIS DE DNA COMPLEMENTARIO (cDNA)

La extracción de RNA y la síntesis de DNA complementario se realizaron en todos los trabajos como paso previo al análisis de expresión de los distintos marcadores. La metodología para la extracción y síntesis cDNA varió según la cantidad de células de partida.

- Para cultivos con más de 500.000 células, las células fueron tratadas con tripsina, lavadas con PBS, transferidas a tubos eppendorf, centrifugadas y congeladas a -80 ºC. 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 el tratamiento con DNAse turbo (Ambion) de acuerdo con las instrucciones de los fabricantes. La síntesis de cDNA se llevó a cabo con hexámeros aleatorios utilizando el kit SuperScript First-Strand Sintesis System (Invitrogen) siguiendo las recomendaciones del fabricante.
- Para cultivos con menos de 500.000 células, las células fueron lavadas con PBS y congeladas directamente en las placas de cultivo a -80 ºC. 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.

4.5. REACCIÓN EN CADENA DE LA POLIMERASA (PCR)

La PCR es una técnica utilizada habitualmente a lo largo de todo el desarrollo de la memoria que se presenta. Además de la técnica convencional, también se utilizó la PCR cuantitativa.

4.5.1 PCR CONVENCIONAL

La técnica consiste en la amplificación específica de un fragmento de DNA o cDNA mediante una serie de repeticiones de ciclos de tiempo y temperatura.

Está técnica se empleó para la validación de los cebadores diseñados durante del desarrollo de todos los trabajos expuestos en esta tesis.

4.5.2. PCR CUANTITATIVA

Esta metodología se utilizó en la práctica totalidad de los manuscritos y, permitió estudiar y comparar la expresión de marcadores de diferenciación entre células diferenciadas y no diferenciadas, así como determinar y comparar la expresión de los marcadores de superficie celular y pluripotencialidad entre células procedentes de distintos orígenes y cultivadas en distintas condiciones de oxígeno.

La técnica se fundamenta en el uso de fluoróforos que emiten distinta cantidad de fluorescencia en función del número de copias del fragmento amplificado. El número de copias del fragmento se corresponde en un determinado ciclo, ciclo umbral (threshold cycle, Ct), con la cantidad de molde en la muestra original. Cuando se cuantifica la expresión de un gen se utilizan genes normalizadores (housekeeping genes) para disminuir el error entre muestras generado por pasos previos a la cuantificación, como diferencias en la síntesis de cDNA o errores de pipeteo. Además, si el número de muestras a analizar no puede ser cuantificado en una sola reacción, las muestras pueden ser amplificadas en reacciones distintas y normalizar los resultados utilizando muestras intercalibradoras en cada reacción (internal run calibrators) que eliminarían las variaciones aparecidas entre las distintas reacciones.

Para el desarrollo de los manuscritos que se exponen en esta memoria el fluoróforo utilizado fuela molécula intercalante de DNA SYBRGreen, cuya detección es directamente proporcional a la cantidad de producto de PCR en la reacción.

4.6. SECUENCIACIÓN AUTOMÁTICA

La secuenciación de fragmentos se ha utilizado en el desarrollo del trabajo de Tesis Doctoral para la comprobación de los productos de PCR de los genes del estudio para los que se diseñaron los cebadores. A continuación se describe brevemente los pasos que se siguieron para las secuenciaciones de las muestras.

Los productos de PCR fueron purificados con ExoSAP-IT (USB) para eliminar nucleótidos, cebadores y otros componentes residuales. Posteriormente los productos purificados fueron secuenciados en reacciones separadas para cada uno de los cebadores utilizando el Big Dye Terminador Cycle Sequencing Kit v.3.1. Finalmente, las secuencias fueron purificadas con el kit X-Terminator y sometidas a electroforesis capilar en el sistema de secuenciación automática ABI PRISM 310 (Applied Biosystems). Todos los kits se utilizaron siguiendo las recomendaciones del proveedor.

4.7. 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 caballo, diseño de cebadores para su uso en PCR, análisis de las amplificaciones y, en general, para realizar el tratamiento estadístico comparativo entre distintos tipos celulares o distintas condiciones.

A continuación aparecen los distintos programas utilizados en la elaboración de esta tesis.

- Búsqueda y recuperación de datos en sistemas integrados de búsqueda y bases de datos: ENTREZ (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 (Applied Biosystems).
- Visualización de secuencias y alineamiento: BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).
- Análisis de secuencias y productos amplificados de RT-qPCR: StepOne™ software (Applied Biosystems).

- Análisis estadístico: Paquete estadístico SPSS 15.0, 18.0 y 19.0 para Windows.

5. RESULTADOS Y DISCUSIÓN

Como consecuencia del trabajo desarrollado en la realización de la Tesis Doctoral que se presenta se han generado varios manuscritos que están publicados, en fase de revisión o a la espera de ser valorados. Además de estos manuscritos se ha incluido un trabajo que forma parte de un estudio pre-clínico.

En primer lugar, buscamos el momento óptimo de aislamiento de las BM-MSCs de la médula ósea para obtener el mayor número de colonias posibles al comienzo de la expansión de las células **(Manuscrito I)**. Para ello analizamos el efecto que ejerce el retraso de 24 horas entre toma de muestras y procesado de las mismas en la obtención de colonias.

Una vez que determinamos que el aislamiento de células inmediatamente tras la extracción proporcionaba más colonias de partida, procedimos a la caracterización de las MSCs derivadas de médula ósea y tejido adiposo. Estos tejidos fueron elegidos por ser los más habituales para la obtención de MSCs en individuos adultos de la especie humana. Las propiedades de las MSCs que analizamos inicialmente fueron la capacidad de proliferación y la viabilidad de las células de ambos orígenes *in vitro*, además de los potenciales de diferenciación que mostraban hacia los distintos linajes mesodérmicos **(Manuscrito II)**. Las células derivadas de ambos tejidos se establecieron y proliferaron en los cultivos con una similar viabilidad. Las MSC equinas mostraron un buen potencial de diferenciación a osteoblasto y condrocito utilizando condiciones descritas en la bibliografía para otras especies. Sin embargo, fue necesario testar hasta 16 medios de diferenciación distintos para alcanzar la diferenciación adipogénica.

Establecidas las condiciones óptimas de cultivo y diferenciación pasamos a analizar el fenotipo para antígenos de superficie celular de las BM-MSCs y AT-MSCs **(Manuscrito III)**. Inicialmente intentamos determinar el inmunofenotipo por medio de citometría de flujo, pero tras la evaluación de 5 anticuerpos comerciales frente a los marcadores de MSCs más utilizados en la especie humana encontramos que sólo 2 de ellos mostraron reactividad contra los epítopos de las MSCs equinas. Por tanto, para completar el análisis del fenotipo de estas células utilizamos la RT-qPCR, y de esta

manera obtuvimos el perfil de expresión génico de los marcadores de MSCs en las células equinas y establecimos diferencias en la expresión de ciertos marcadores.

Estos estudios de caracterización los llevamos a cabo mediante el cultivo de células en condiciones estándar, es decir, a 37 ºC, 5% CO_2 y 20% O_2. Sin embargo, nos interesamos por conocer el efecto del oxígeno sobre las características analizadas utilizando unas condiciones de cultivo más similares a las fisiológicas, es decir, en condiciones de hipoxia (5% O_2). Analizamos la capacidad de expansión de las BM-MSCs bajo las dos atmósferas de oxígeno y los potenciales de diferenciación hacia osteoblasto, adipocito y condrocito con las condiciones establecidas en el manuscrito II **(Manuscrito IV)**. Además, determinamos de qué modo el oxígeno afectaba al cultivo de las AT-MSCs y BM-MSCs analizando la viabilidad y la progresión del ciclo celular y estudiamos si el fenotipo y la expresión de marcadores de pluripotencia de las MSCs equinas resultaban alterados por la distinta condición de oxígeno **(Manuscrito V)**.

Por último, aplicando las técnicas de cultivo y moleculares puestas a punto a lo largo de los anteriores manuscritos, llevamos a cabo la caracterización de BM-MSCs y AT-MSCs utilizadas en un estudio pre-clínico para el tratamiento de lesiones tendinosas inducidas **(Manuscrito VI)**. Analizamos y comparamos entre los dos tipos celulares el fenotipo de los marcadores de superficie y la habilidad para diferenciarse hacia linajes osteogénico, adipogénico y condrogénico. Los resultados obtenidos de la caracterización de estas MSCs se engloban dentro del estudio pre-clínico que está siendo realizado en colaboración con el Hospital Clínico Veterinario de la Universidad de Zaragoza y que tiene como objetivo establecer una correlación entre ellos y otros datos funcionales, ecográficos, moleculares, histológicos y ultraestructurales que se están analizando.

A continuación se enumeran los manuscritos a los que se ha hecho mención:

I. Ranera B, Remacha AR, Álvarez-Arguedas S, Romero A, Zaragoza P, Martín-Burriel I, Vázquez FJ and Rodellar C. **Effect of time on equine bone marrow derived mesenchymal stem cells isolation.** *Veterinary Record* (Enviado)

II. Ranera B, Ordovás L, Lyahyai J, Bernal ML, Fernandes F, Remacha AR, Romero A, Vázquez FJ, Osta R, Cons C, Varona L, Zaragoza P, Martín-Burriel I and Rodellar C. **Comparative study of equine bone marrow and adipose tissue-derived mesenchymal stromal cells.** *Equine Veterinary Journal 2012 Jan;44(1):33-42.*

III. Ranera B, Lyahyai J, Romero A, Vázquez FJ, Remacha AR, Bernal ML, Zaragoza P, Rodellar and Martín-Burriel I. **Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue.** *Veterinary Immunology and Immunopathology 2011 Nov 15;144(1-2):147-54.*

IV. Ranera B, Remacha AR, Álvarez-Arguedas S, Castiella T, Vázquez FJ, Romero A, Zaragoza P, Martín-Burriel I, Rodellar C. **Expansion under hypoxic conditions enhances the chondrogenic potential of equine bone marrow-derived mesenchymal stem cells.** *The Veterinary Journal* (En revisión).

V. Ranera B, Remacha AR, Álvarez-Arguedas S, Romero A, Vázquez FJ, Zaragoza P, Martín-Burriel I, Rodellar C. **Effect of hipoxia on equine mesenchymal stem cells derived from bone marrow and adipose tissue.** *BMC Veterinary Research* (Enviado).

VI. **Characterization of the equine MSCs used in a preclinical trial to evaluate the capacity of MSCs for healing induced tendón injuries.**

5.1. MANUSCRITO I

Effect of time on equine bone marrow derived mesenchymal stem cells isolation.

Beatriz Ranera, Ana Rosa Remacha, Samuel Álvarez-Arguedas, Antonio Romero, Pilar Zaragoza, Inmaculada Martín-Burriel, Francisco José Vázquez, Clementina Rodellar.

Superficial digital-flexor tendon (SDFT) from racehorses usually suffers over-strain injuries consequence of overuse during the performance and the effect of aging (Williams and others 2001). Tendon healing takes place through a process of fibrosis (Diegelmann and Evans 2004) that results in a tendon scar with inferior biochemical and mechanical properties (Leadbetter 1992). Consequently, returning to the same performance level previous to the injury is committed, the risk of the re-injury increases (Crevier-Denoix and others 1997) and leads in some occasions to the premature retirement of racehorses.

Conventional treatments for this kind of injury have failed in restoring the SDFT functionality (Dyson 2004). However cell therapy based on the application of BM-MSCs has shown efficacy in the treatment of spontaneous tendinitis in racehorses (Godwin and others 2011). These promising results have encouraged the setting up of commercial companies which are based on the use of expanded BM-MSC to deal with equine locomotor injuries.

Bone marrow from the injured horse is collected by the veterinarian and sent in containers at 4 ºC to the laboratory. Within 48 hours of aspiration, samples are usually received in the laboratories where BM-MSCs will be isolated and expanded. Once there are enough BM-MSCs, they are recovered and transported back to the veterinarian for implantation in the horse injury (Smith 2008).

To our knowledge there are not previous reports about how the delay in BM-MSCs isolation due to the shipping of bone marrow can affect the samples. The aim of the present work was to determinate the effect of the time on the ability to form BM-MSC CFUs (colony forming units) when the isolation is performed straight from the harvesting and 24 hours later.

Bone marrow was obtained from the sternebrae of 5 castrated male horses aged 4 to 7 years. All procedures were carried out under Project Licence PI36/07 approved by the Ethic Committee for Animal Experiments from the University of Zaragoza. The care and use of animals was performed in accordance with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

A total volume of 20 mL of bone marrow was collected from the horses and subsequently split in two fractions of 10 mL. BM-MSCs were straight isolated from one of the fractions, which were denominated as “0 hours” (0h) cultures, and the remaining fraction was placed in a polystyrene box with two ice block mimicking the manner of delivery, and were denominated as “24 hours” (24h) cultures. BM-MSCs from both fractions were isolated as previously described (Ranera and others 2011a; Ranera and others 2011b) and set in T75 flask with growth media, consisting of low glucose DMEM supplemented with 10% FBS, 1% Glutamine and 1% Streptomycin/Penicillin. Before setting in culture, a 50µL aliquot of the mononuclear cells was counted in a haemocytometer. Mononuclear cells were washed twice with PBS after 24, 48 and 72 h of incubation at 37 ºC at 5% CO_2. Afterwards, the media was changed twice a week.

After 10 days of culture, a CFU assay was performed. The cells were fixed with 10% paraformaldehyde and stained with 10% Giemsa in phosphate buffer. Pictures from the flasks were taken using a Canon G11 camera and the colonies were counted using cell counter tool from Image J software. Differences in mononuclear cells and number of colonies between 0h and 24h cultures were evaluated with the Student’s t test using the SPSS Statistic 19 software.

The average of mononuclear cells obtained after the straight isolations was $12.5 \cdot 10^6 \pm 7.02 \cdot 10^6$ (mean ± standard deviation), while the count of the mononuclear cells obtained 24 hours after the aspiration was $5.81 \cdot 10^6 \pm 3.75 \cdot 10^6$. Therefore, the viability of the mononuclear cells dropped a 46.48% in the samples treated 24 hours after collection from the animal and maintained in shipping conditions. The percentage of loss is greater than the 9% decrease reported for expanded BM-MSCs during a period of 72h when they are sent back to the veterinarian for re-implantation in the injury (Godwin and others 2011).

In both cultures first colonies appeared approximately 4 days after isolation and the cells displayed the typical spindle shaped morphology (Vidal and others 2006). After 10 days, the cultures were stopped and the CFU assay was performed. A significantly higher number of colonies was detected in 0h (383.60 ± 149.32) than in 24h (156.2 ± 81.32) cultures (FIG. 1), which means a 59.28% less CFU. The differences in the formation of CFU between the two cultures were evident macroscopically (FIG.

2). Therefore, the lapse of time between the collection of bone marrow and the isolation of BM-MSCs might be crucial in the quantity of colonies formed, and consequently in the final number of BM-MSCs at the end of the time of culture.

As the purpose of the application of BM-MSCs in cell therapy is treating spontaneous injuries in horses; obtaining the greater amount of cells as soon as possible is imperious. The presence of the animal away from the laboratory where the BM-MSC isolation takes place makes necessary the transport of the bone marrow by delivery system. As a result, a lapse of time appears between these two stages, affecting the viability of the mononuclear cells and the ability to form colonies as we show in this report. Our data suggest that sample transport at 4 ºC might not be the most accurate, although low temperatures slows down the metabolism as it is proved in the shipping of other cell types (Parks 1997). To obtain a higher number of cells and compensate the loss of viability during the transport, the isolation of BM-MSCs might be made from a larger volume of bone marrow. For instance, 60 mL seem to be sufficient to yield a considerable number of cells (Toupadakis and others 2011). Further studies are necessaries to establish which conditions would be the best to increase the viability of mononuclear cells and obtain higher number of CFU. While these conditions are achieved, the study of the MSC characteristics, as differentiation potentials, of MSCs isolated at different times would be interesting.

Acknowledgements

This work was performed as part of the AGL2008-02428/GAN (MICINN/FEDER) project and was partially financed by the Gobierno de Aragón (Grupo de Excelencia LAGENBIO) and InstitutoAragonés de Ciencias de la Salud (I+CS). B. Ranera was supported by a doctoral grant from DGA.

FIGURE LEGENDS

FIG. 1. Mean ± standard deviation of the number of colony forming units (CFU) of BM-MSCs after 10 days of culture. The bar on the left represents the colonies counted of BM-MSCs from straight isolated cultures and the bar on the right from 24 hours delayed cultures.

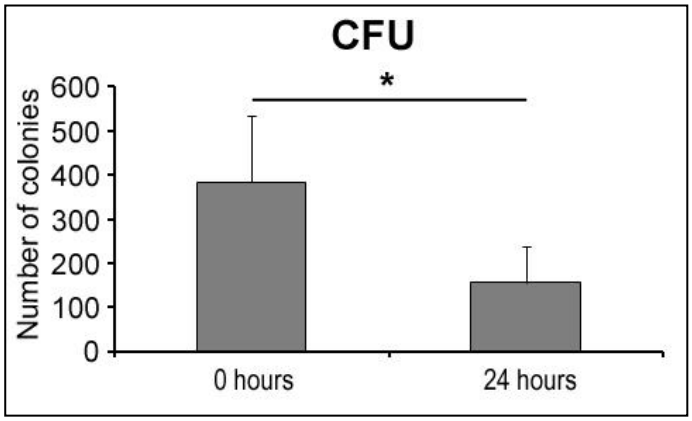
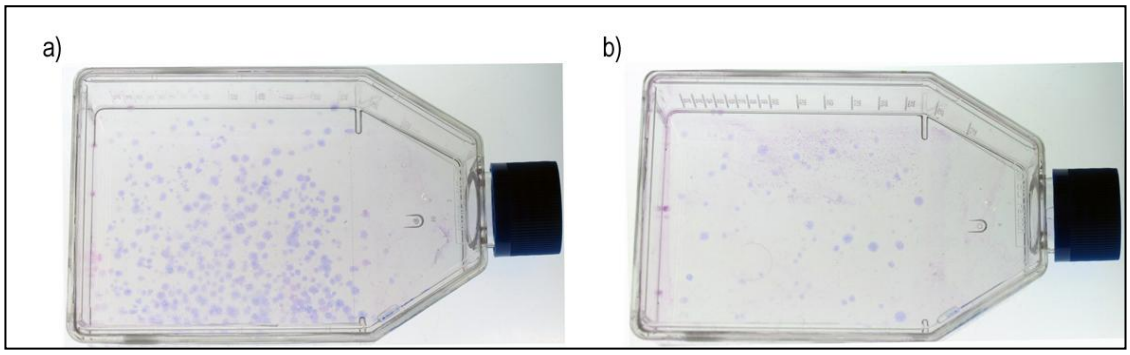


FIG. 2. Representative CFU assays after 10 days. a) Stained colonies of straight isolated cultures in a T75 flask. b) Stained colonies of 24 hours delayed cultures in a T75 flask.



REFERENCES

CREVIER-DENOIX, N., COLLOBERT, C., POURCELOT, P., DENOIX, J. M., SANAA, M., GEIGER, D., BERNARD, N., RIBOT, X., BORTOLUSSI, C. & BOUSSEAU, B. (1997) Mechanical properties of pathological equine superficial digital flexor tendons. Equine Vet J Suppl, 23-26

DIEGELMANN, R. F. & EVANS, M. C. (2004) Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci 9, 283-289

DYSON, S. J. (2004) Medical management of superficial digital flexor tendonitis: a comparative study in 219 horses (1992-2000). Equine Vet J 36, 415-419

GODWIN, E. E., YOUNG, N. J., DUDHIA, J., BEAMISH, I. C. & SMITH, R. K. (2011) Implantation of bone marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon. Equine Vet J 44, 25-32

LEADBETTER, W. B. (1992) Cell-matrix response in tendon injury. Clin Sports Med 11, 533-578

PARKS, J. E. (1997) Hypothermia and mammalian gametes. San Diego, Accademic Press

RANERA, B., LYAHYAI, J., ROMERO, A., VAZQUEZ, F. J., REMACHA, A. R., BERNAL, M. L., ZARAGOZA, P., RODELLAR, C. & MARTIN-BURRIEL, I. (2011a) Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue. Vet Immunol Immunopathol 144, 147-154

RANERA, B., ORDOVAS, L., LYAHYAI, J., BERNAL, M. L., FERNANDES, F., REMACHA, A. R., ROMERO, A., VAZQUEZ, F. J., OSTA, R., CONS, C., VARONA, L., ZARAGOZA, P., MARTIN-BURRIEL, I. & RODELLAR, C. (2011b) Comparative study of equine bone marrow and adipose tissue-derived mesenchymal stromal cells. Equine Vet J 44, 33-42

SMITH, R. K. (2008) Mesenchymal stem cell therapy for equine tendinopathy. Disabil Rehabil 30, 1752-1758

TOUPADAKIS, C. A., WONG, A., GENETOS, D. C., CHEUNG, W. K., BORJESSON, D. L., FERRARO, G. L., GALUPPO, L. D., LEACH, J. K., OWENS, S. D. & YELLOWLEY, C. E. (2011) Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue. Am J Vet Res 71, 1237-1245

VIDAL, M. A., KILROY, G. E., JOHNSON, J. R., LOPEZ, M. J., MOORE, R. M. & GIMBLE, J. M. (2006) Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. Vet Surg 35, 601-610

WILLIAMS, R. B., HARKINS, L. S., HAMMOND, C. J. & WOOD, J. L. (2001) Racehorse injuries, clinical problems and fatalities recorded on British racecourses from flat racing and National Hunt racing during 1996, 1997 and 1998. Equine Vet J 33, 478-486

5.2. MANUSCRITO II

Comparative study of equine bone marrow and adipose tissue-derived mesenchymal stromal cells.

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Equine Veterinary Journal

Equine Veterinary Journal ISSN 0425-1644
DOI: 10.1111/j.2042-3306.2010.00353.x

Comparative study of equine bone marrow and adipose tissue-derived mesenchymal stromal cells

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Summary

Reasons for performing study: Mesenchymal stromal cells (MSCs) represent an attractive source for regenerative medicine. However, prior to their application, fundamental questions regarding molecular characterisation, growth and differentiation of MSCs must be resolved.
Objectives: To compare and better understand the behaviour of equine MSCs obtained from bone marrow (BM) and adipose tissue (AT) in culture.
Methods: Five horses were included in this study. Proliferation rate was measured using MTT assay and cell viability; apoptosis, necrosis and late apoptosis and necrosis were evaluated by flow cytometry. The mRNA expression levels of 7 surface marker genes were quantified using RT-qPCR and CD90 was also analysed by flow cytometry. Differentiation was evaluated using specific staining, measurement of alkaline phosphatase activity and analysis of the mRNA expression.
Results: High interindividual differences were observed in proliferation in both cell types, particularly during the final days. Statistically significant differences in viability and early apoptosis of cultured AT- and BM-MSCs were found. The highest values of early apoptosis were observed during the first days of culture, while the highest percentage of necrosis and late apoptosis and lowest viability was observed in the last days. Surface marker expression pattern observed is in accordance to other studies in horse and other species. Osteogenic differentiation was evident after 7 days, with an increasing of ALP activity and mRNA expression of osteogenic markers. Adipogenic differentiation was achieved in BM-MSCs from 2 donors with one of the 16 media tested. Chondrogenic differentiation was also observed.
Conclusions: Proliferation ability is different in AT-MSCs and BM-MSCs. Differences in viability and early apoptosis were observed between both sources and CD34 was only found in AT-MSCs. Differences in their osteogenic and adipogenic potential were detected by staining and quantification of specific tissue markers.
Potential relevance: To provide data to better understand AT-MSCs and BM-MSCs behaviour *in vitro*.

Keywords: horse; mesenchymal stromal cells; regenerative medicine; AT-MSCs; BM-MSCs

Introduction

The use of mesenchymal stem cells (MSCs) has been recently shown as a therapeutic alternative that could help in the treatment of tendon and ligament lesions (Murphy *et al*. 2003; Smith *et al*. 2003; Angele *et al*. 2008).

Cell-based therapeutic options favour the use of autologous cells such as a population of bone marrow cells or adipose tissue cells that have been characterised as mesenchymal stromal cells (MSCs) (Herzog *et al*. 2003). Cultured MSCs exhibit good multipotency and they can differentiate both *in vitro* and *in vivo* into several tissues, including bone (Holtorf *et al*. 2005), cartilage (Bernardo *et al*. 2007), tendon, skeletal muscle (Dezawa *et al*. 2005), adipose tissue and cardiac muscle (Orlic *et al*. 2001). This multi-differentiation potential of MSCs provoked clinical interest in whether these cells could be used for regenerative medicine (Horwitz *et al*. 1999; Bobis *et al*. 2006). In horses, to date, several reports have analysed both bone marrow (BM) and adipose tissue (AT) derived MSCs as potential therapeutic agents for horse musculoskeletal diseases (Smith *et al*. 2003; Richardson *et al*. 2007) such as tendon lesions (Violini *et al*. 2009), suspensory ligament rupture (Smith and Webbon 2005) and bone and joint related conditions in the equine athlete (Wilke *et al*. 2007). These lesions result in high morbidity and often compromise full recovery (Smith *et al*. 2003).

However, the efficacy of MSC therapy in both animals and human patients is sometimes extremely low (Brooke *et al*. 2007; Granero-Molto *et al*. 2008). Indeed, in bone repair, utilising MSC subpopulations with poor osteogenic potential and a short lifespan decreases the therapeutic efficacy (Liu *et al*. 2009).

MSC specific markers have been characterised in man and some laboratory animals using techniques such as the phenotypic analysis of cell surface markers by flow cytometry and indirect immunofluorescence (Koch *et al*. 2009). Limited work about equine MSC membrane surface markers has been done. A recent work describes the immunophenotype of equine AT-derived MSCs for CD13, CD44 and CD90 (de Mattos Carvalho *et al*. 2009).

Recently, several reports have focused on characterising equine MSCs obtained from different sources (Vidal *et al*. 2006, 2007; Colleoni *et al*. 2009; Mambelli *et al*. 2009). Further characterisation is necessary to predict the potential and suitability of a specific cell population for application in equine cellular therapy. The aim of our work was to analyse the proliferation capacity and cell viability of equine BM- and AT-MSCs. We examined the expression of specific cell surface markers and the tri-lineage differentiation ability of the MSCs into osteoblasts, adipocytes and chondrocytes by staining procedures and evaluated the utility of several molecular markers for monitoring MSC differentiation.

Materials and methods

Animals

Five horses (400–450 kg bwt, age: 8–12 years) were used to characterise equine MSCs. The horses were clinical patients suffering from varying types of tendon and ligament injuries and biological samples (bone marrow and adipose tissue) were obtained with owner consent and according to local animal welfare regulations. The study was approved by the ethics committee of the University of Zaragoza.

MSC isolation, culture and expansion

Bone marrow aspiration and isolation: Each BM aspirate, collected as previously described by Orsini and Divers (1998), was diluted 1:3 with PBS[1]

and then layered over Lymphoprep[2] and centrifuged for 20 min at 1700 ***g***. The MSC-enriched cell population above the Lymphoprep layer was aspirated and washed twice with PBS. The pellet was resuspended in 10 ml basal medium, consisting of low glucose Dulbecco Modified Eagle's Medium (DMEM)[3] and supplemented with 10% fetal bovine serum, 1% glutamine[3] and 1% streptomycin/penicillin[3]. Cells were counted, plated at a density of 2×10^6 nucleated cells/cm^2 in 6-well plates and incubated at 37°C, 5% CO_2.

Adipose tissue harvest and isolation: Adipose tissue samples were collected, as previously described by Vidal *et al.* (2007), from the supragluteal subcutaneous area of the same 5 animals. The tissue was digested with 0.01% of activated collagenase (*type I*)[3] for 30 min at 37°C with continuous shaking. Subsequently, samples were centrifuged, the supernatant removed and the cells washed with PBS by further centrifugation. Finally, the pellet was resuspended in 10 ml basal medium (DMEM), counted and plated at a density of 10^5 nucleated cells/cm^2 in 6 well plates and incubated at 37°C, 5% CO_2.

Cell culture, cryopreservation and thawing: For culture expansion, cells were maintained in basal medium at a density 5000 cells/cm^2 in T75 or T175 culture flask[4]. The medium was changed every 3 days until cells reached approximately 80% confluence. Subsequently, cells were detached by treating with 0.25% tryspin-EDTA[3] and counted with a haemocytometer Z2 Coulter particle count and size analyser[5]. Cells were passaged until passage 3. Then, they were frozen in 10^6 all quots in crystals[6] with freezing medium, consisting of 90% FBS and 10% DMSO. MSC characterisation assays were performed with cryopreserved cells at passage 3. Cells were thawed at 37°C and set in culture for 3 days to readjust the culture conditions prior to be used on the different experiments

MSC proliferation, viability, apoptosis and necrosis

MTT assay: A MTT assay[3] was applied to analyse the proliferation characteristics of cultured BM-MSCs (n = 5) and AT-MSCs (n = 4). Eight 96 well plates were seeded on Day 0, and 8 replicas of each horse and source at a density of 5×10^3 cells/cm^2 in basal medium and allowed to proliferate over the course of 8 days. On each of these 8 days, one plate was used to measure proliferation using the MTT assay (Mosmann 1983). Optical density (570 nm) for each well was determined using a Biotek Synergy HT spectrophotometer. Linearity of the assay was tested for the range of cell densities expected for the experiment for both BM- and AT-MSCs. Briefly, 0–15,000 cells/cm^2 (8 replications of each density) from each source were seeded in a 96 well plate and incubated for 24 h in basal medium. The MTT assay was performed and average absorbance for each density used to construct a standard curve. Viable cell numbers for the different samples and days were determined using the calibration curve.

Cell doubling time at this passage was calculated according to the formulae:

$$CD = ln\frac{\frac{Nf}{Ni}}{ln2} DT = \frac{CT}{CD}$$

DT = cell doubling time; N_f = final number of cells; N_i = initial number of cells; CD = cell doubling number.

Flow cytometry: Flow cytometry was used to evaluate cell viability, apoptosis and necrosis of cultured MSCs. AT-MSCs and BM-MSCs from 2 animals were seeded at a density of 1500 cells/cm^2 and cultured in 6 well plates for 8 days. Cells were trypsinised daily for flow cytometry analysis. Apoptosis was measured using the FITC-conjugated Annexin V kit (Immunostep) according to the manufacturer's protocol. Briefly, Annexin V-FITC (AnV) bounded to cells which showed phosphatidylcholine and sphingomyeline in their membranes, which is associated with early apoptosis. Propidium iodide (PI) bounded to necrotic cells because of their membranes have lost the integrity. Fluorescence was read by flow cytometry using FAC Saria[4] in accordance with the manufacturer's protocol.

Expression of cell surface marker genes

The expression levels of 7 genes coding for 4 MSC surface markers (*CD73*, *CD90*, *CD105* and *CD166*), 2 haematopoietic markers (*CD34* and *CD45*) and an endothelial marker (CD31) were evaluated in cultured AT-MSCs and BM-MSCs from passage 3, using RT-qPCR. Total RNA was isolated from approximately 40,000 BM and AT-MSCs using Cell-to-cDNA II[7] according to the manufacturer's instructions. RNA obtained from equine peripheral blood and whole bone marrow was used as a positive control to validate the primers for the study of haematopoietic markers.

Primers were designed using Primer Express 2.0 software[8] based on known equine sequences. Primer details, accession numbers for equine mRNA sequences and amplicon sizes are shown in Table 1.

Real-Time RT-PCR (RT-qPCR) was performed and monitored using the StepOne Real Time PCR System[8]. All reactions were carried out in a total volume of 10 µl with 2 µl of cDNA as a template and Fast SYBR Green Master Mix[8]. cDNA was amplified following the manufacturer's protocol: 20 s at 95°C of an initial activation and denaturation, 45 cycles consisting of 3 s at 95°C and 30 s at 60°C. Each cDNA sequence was tested in triplicate and a dissociation curve protocol run after every PCR reaction. The levels of gene expression were determined by the comparative Ct method. A normalisation factor (NF) was used to determine the expression level of each gene in each sample. The NF was calculated as the geometric mean of the quantity of 2 housekeeping genes (*GAPDH* and *B2M*). Primers used for housekeeping gene amplification were previously described (Kolm *et al.* 2006).

CD90 (Thy-1) was also analysed by flow cytometry. Briefly, 50,000 BM-MSCs and AT-MSCs in passage 3 were incubated at 4°C for 15 min with 5 µl of mouse anti-human CD90-PE monoclonal antibody[10] (BD Pharmingen). Cells were analysed by a fluorescence activated cell sorter (Facsaria)[4].

Osteogenic differentiation

Osteogenic differentiation was induced by culturing thawed AT- and BM-MSC from passage 3. Cells were plated at a density of 20,000 cells/cm^2 in triplicate (in 24 well plates) in differentiation medium containing basal medium supplemented with 10 nmol/l dexamethasone, 10 mmol/l β-glycerophosphate and 100 µmol/l ascorbate-2-phosphate (all from Sigma-Aldrich)[3] (Koerner *et al.* 2006). Differentiation culture was maintained for 7 days.

Alizarin red staining: To evaluate calcium deposition, cells were fixed on the last day of culture with 70% ethanol and stained with 2% Alizarin Red dye[3] pH 4.6 for 10 min and washed with PBS for 15 min.

Alkaline phosphatase assay: The activity of alkaline phophatase (ALP) was evaluated for every 2 days in culture. Cells were lysed with RIPA buffer (NaCl 150 mmol/l, TRIS-HCl 50 mmol/l, NP-40 1%, Sodium deoxicolate 0.25%, EDTA 1 mmol/l, PMSF 100 mmol/l and Sodium ortovanadate 100 mmol/l) for 45 min at 4°C. A fraction of the lysate was used for the ALP assay using p-nitrophenyl phosphate (pNPP)[3] as a substrate. Absorbance of p-nitrophenol (pNP) was measured at 405 nm in a Biotek Synergy HT spectrophotometer.

The ALP activity value was quantified by comparison with a standard curve of commercial pNP[3]. ALP activity, expressed as mmol/l pNP/µg total protein, was normalised using the remaining lysis fraction to calculate the total protein content by the BCA method[5].

Osteogenic marker gene expression: Osteogenic differentiation was monitored every 2 days by analysing the mRNA expression of osteogenic markers, alkaline phosphatase (*ALP*), osteocalcin (*BGLAP*), collagen *type I* (*COL1A1*), runt-related transcription factor 2 (*RUNX2*) and osteopontin (*SPP*). Briefly, mRNA was extracted from differentiated and control AT- and BM-MSC samples using the same protocol as described for the analysis of surface membrane markers (see above). Specific primers for the genes are shown in Table 1.

TABLE 1: Cell surface, osteogenic and adipogenic markers analysed by RT-qPCR. GenBank accession numbers of the sequences used for primers design. Primers (F: Forward and R: Reverse) and length of the amplicon in base pair (bp)

Genes	Accesion number	Primer sequence	Amplicon size
CD31	NM_001101655	F:TCTAGAACGGAAGGCTCCCT R:TGGGAGCAGGGCAGGTTCA	145
CD34	XM_001491596	F:CACTAAACCCTCTACATCATTTTCTCCTA R:GGCAGATACCTTGAGTCAATTTCA	150
CD45	AY_114350	F:TGATTCCCAGAAATGACCATGTA R:ACATTTTGGGCTTGTCCTGTAAC	100
CD73	XM_001500115	F:GGGATTGTTGGATACACTTCAAAAG R:GCTGCAACGCAGTGATTTCA	90
CD90	EU881920	F:TGCGAACTCCGCCTCTCT R:GCTTATGCCCTCGCACTTG	93
CD105	XM_001500078	F:GACGGAAAATGTGGTCAGTAATGA R:GCGAGAGGCTCTCCGTGTT	100
CD166	XM_001503380	F:GTCTGGTCTTCTGCCTCTTGATC R:TCGGCAAGGCATGATAATAGTG	103
ALP	XM_001504312	F:GATGGCCTGAACCTCATCGA R:AGTTCGGTCCGGTTCCAGAT	92
BGLAP	DQ_007079	F:GGCAGAGGTGCAGCCTTC R:CTCCAGAGGGTCCGGGTAG	114
COL1A1	AF_034691	F:ACACAGAGGTTTCAGTGGTTTGG R:CACCATGGCTACCAGGTTCAC	89
RUNX2	XM_001502519	F:CTCCAACCCACGAATGCACTA R:CGGACATACCGAGGGACATG	80
SPP1	XM_001496152	F:CTCACATCACCTGTGGAAAGCA R:CACGGCTGTCCCAATCAGA	104
LPL	XM_001489577	F:TGTATGAGAGTTGGGTGCCAAA R:GCCAGTCCACCACAATGACAT	70
PPARγ	XM_001492411	F:TGCAAGGGTTTCTTCCGGA R:GCAAGGCATTTCTGAAACCG	104
GAPDH	NM_001163856	F:GGCAAGTTCCATGGCACAGT R:CACAACATATTCAGCACCAGCAT	128
B-2M	NM_001082502	F:TCGTCCTGCTCGGGCTACT R:ATTCTCTGCTGGGTGACGTGA	102

Adipogenic differentiation

Adipogenesis was induced by culturing thawed, AT- and BM-MSC from passage 3. Cells were plated in triplicate at a density of 20,000 cells/cm^2 in 24 well plates. Sixteen adipocyte differentiation media were tested for differentiation of BM- and AT-MSCs. Five of these media were previously described for the differentiation of equine MSCs Table 2 the remainder were modifications based on them. Only basal medium supplemented with 1 μmol/l dexamethasone[3], 500 μmol/l 3-isobutyl-1-methylxanthine[3], 200 μmol/l indomethacin[3] and 15% rabbit serum[3] (highlighted medium) was able to induce adipogenic differentiation. Cultures were maintained for 15 days.

Oil red O staining: Adipogenic differentiation was examined by performing Oil Red O staining. On Day 15, cells were fixed with 10% formalin[3] for 15 min at room temperature, stained with 0.3% Oil Red O dye (dissolved in 60:40; isopropanol : distilled water) and washed with distilled water.

Adipogenic marker expression: Adipogenic differentiation was monitored by analysing the expression of lipoprotein lipase (*LPL*) and peroxisome proliferator-activated receptor gamma (*PPARγ*) markers every other day throughout the differentiation process. Briefly, mRNA extraction, RT-qPCR and evaluation of results were conducted as described above. Specific primers for the adipogenic markers are shown in Table 1.

Chondrogenic differentiation

Chondrogenic differentiation was performed in pellet culture. Approximately 3 × 10^5 cells were placed in a 15 ml conical polypropylene tube, centrifuged at 1700 ***g*** for 5 min and resuspended in chondrogenic medium, consisting of DMEM high glucose supplemented with 10% FBS, 10 ng/ml TGFβ-3[9], ITS+ premix[4], 40 μg/ml proline[3], 50 μg/ml ascorbate-2-phosphate[3], 0.1 μmol/l dexamethasone[3]. Differentiation culture was maintained for 21 days

Histology

Pellets were fixed in 10% formalin[3], embedded in paraffin and sectioned into 5 μm sections. The sections were hydrated with increasing amounts of alcohols, stained with Mayer's haematoxylin and 3% Alcian Blue dyes, rinsed with distilled water, dehydrated with decreasing amounts of alcohols and mounted.

Statistical analysis

Differences in viability, apoptosis and necrosis over time were evaluated by a multiple linear regression, which considers a specific slope for each combination of tissue (AT and BM) and individual:

$$y_{ijk} = \mu + b_i x_{ij} + e_{ijk}$$

where y_{ijk} is the percentage of necrotic, late apoptotic, viable or early apoptotic cells for the ith combination of tissue and individual (AT-1, AT-2, BM-1, BM-2), the jth recording date (Days 1–10) and the kth replicate (13), b_i is the specific slope for each tissue and individual combination, x_{ij} is the day after seeding and e_{ijk} is the residual. Thus, a general mean and 4 different regression

TABLE 2: Media used for adipogenic differentiation including basal media, supplements and sera

	DMEM	Dexamethasone	IBMX	Indomethacin	Insuline	Pantothenate	Biotin	Rosiglitazone	Sera	Human
1	LG	1 µmol/l	500 µmol/l	200 µmol/l					10% FBS	+
2	LG	1 µmol/l	500 µmol/l	200 µmol/l					5% RS 10% FBS	
3	LG	1 µmol/l	500 µmol/l	200 µmol/l					15% RS 10% FBS	
4	LG	1 µmol/l	500 µmol/l	200 µmol/l					10% HS 10% FBS	
5[1]	LG	1 µmol/l	500 µmol/l	200 µmol/l	10 µg/µl				15% RS 10% FBS	
6	LG	1 µmol/l	500 µmol/l	200 µmol/l	10 µg/µl				10% FBS	+
7[2]	DMEM/F12	1 µmol/l	500 µmol/l	200 µmol/l	10 µg/µl				10% FBS	
8[3]	DMEM/F12	1 µmol/l	500 µmol/l		100 nmol/l	17 µmol/l	33 µmol/l	5 µmol/l	5% RS 3% FBS	+
9	LG	1 µmol/l	500 µmol/l		100 nmol/l	17 µmol/l	33 µmol/l	5 µmol/l	5% RS 3% FBS	+
10	LG	1 µmol/l	500 µmol/l		100 nmol/l	17 µmol/l	33 µmol/l	5 µmol/l	5% RS 10% FBS	
11	LG	1 µmol/l	500 µmol/l		100 nmol/l	17 µmol/l	33 µmol/l	5 µmol/l	15% RS 10% FBS	
12	LG	1 µmol/l	500 µmol/l		100 nmol/l	17 µmol/l	33 µmol/l	5 µmol/l	10% HS 10% FBS	
13	HG	1 µmol/l	500 µmol/l	100 µmol/l	10 µmol/l				10% FBS	+
14[4]	DMEM/F12	1 µmol/l	500 µmol/l	100 µmol/l	10 µg/µl				10% FBS	+
15[5]	HG	1 µmol/l	500 µmol/l	100 µmol/l	10 µg/µl				2% KSR	
16	HG	1 µmol/l			5 µg/µl				20% FBS	

(+) Indicates media tested in human MSCs. Highlighted medium is the final medium used in the present work; [1]Koch *et al.* 2007; [2]Koerner *et al.* 2006; [3]Vidal *et al.* 2006; [4]Arnhold *et al.* 2007; [5]Mambelli *et al.* 2009; FBS, Fetal calf serum; HG = high glucose DMEM; HS = horse serum; KSR = knockout serum replacement; LG = low glucose DMEM; RS, Rabbit serum.

slopes were estimated for each process (necrosis, late apoptosis, viability and early apoptosis).

Differences in osteogenic and adipogenic marker gene expression and ALP activity between control and treated cultures were evaluated with the Student's *t* test. In addition, a 2-way ANOVA test was performed to identify the effect of time and animal in the expression or activity of these markers during the differentiation period. When significant main effects or an interaction between the main effects was found, specific comparisons were made with paired Student's *t* tests. P values <0.05 were considered significant. Finally, Pearson's correlation was used to analyse the association between the expression of every pair of osteogenesis differentiation markers and ALP activity. Statistics were assessed using SPSS 14.0 software.

Results

Isolation and proliferation of MSCs

Plastic-adherent fibroblast-like cells were observed in all donor samples obtained from both bone marrow aspirates and adipose tissue within the first days of culture. Cells were expanded until passage 3 and frozen. After thawing, cells maintained their morphology and proliferation capacity.

The MTT assay was applied to analyse the proliferation characteristics of cultured BM-MSCs and AT-MSCs. The total number of viable cells for the different samples and days were determined by extrapolation from the calibration curves: $y = 1.91 \cdot 10^{6}\, x + 0.0384$, $r^2 = 0.978$ for BM-MSCs and $y = 1.77 \cdot 10^{6}\, x + 0.0272$, $r^2 = 0.991$ for AT-MSCs, where y is the quantity of cells and x the optical density of the well. Mean values of the number of cells per well from BM- and AT-MSCs are presented in Figure 1. The lag phase extended until the second day post seeding. Afterwards, AT-MSCs started growing at a consistent rate until Day 8. The BM-MSC growing slowed down on Days 4 and 5, showing differences within both types with a residual significance (Student's *t*, P = 0055 and P = 0.063, respectively). Since that moment, on average, AT-MSC culture had a higher cell number than BM-MSC culture, although great interindividual differences were observed in both cell types, particularly during the final days. The higher differences of the cell number of AT-MSCs vs. BM-MSCs were more evident when comparing cells of each origin from the same animal (data not shown).

Cell doubling time were also calculated being similar in both cell type, 2.47 ± 0.4 days for BM-MSC and 2.2 ± 0.2 days for AT-MSC.

Viability, apoptosis and necrosis of MSCs

The Annexin V assay was used to determine the percentage of viable, early apoptotic, necrotic + late apoptotic and necrotic cells during 8 days of culture. The highest percentage of viable cells, propidium iodide negative and annexin V negative (PI-, AnV-), was observed at Day 1 for both MSC types (61.23% in BM and 62.75% in AT) and the lowest percentages were found at Day 5 in BM-MSC (38.2%) and at Day 8 in AT-MSC (18.72%). The percentage of necrotic cells (PI+ AnV- cells) was very low, ranging from 0.48–2.37% in BM-MSC and from 0.75–2.12% in AT-MSC at Days 0 and 1, respectively. The highest percentage of early apoptotic cells (PI-, AnV+) was observed the second day of culture (22.75% in BM- and 21.73% in AT-MSC) and the lowest at Day 7 (9.62% for BM and 3.02% for AT). Finally, necrotic + late apoptotic cells (PI+, AnV+) displayed the lowest percentage the first day of culture (21.87% in BM and 21.4% in AT) and the highest value was observed at Day 5 in BM (48.03%) and Day 8 in AT (76.18%).

Differences in the kinetic of the viability changes between MSC sources and animals were assessed by multiple linear regressions. Slope values show the decrease or increase of each parameter as time goes by (Table 3). Whereas necrosis + late apoptosis was increasing during the culture period (positive slope), the other parameters were decreasing (negative slope). For necrosis + late apoptosis, we did not find any significant differences between regression slopes. Neither significant differences were found in necrosis between individuals or MSC source. Nevertheless, the percentage of early apoptosis along the course of the experiment decreased

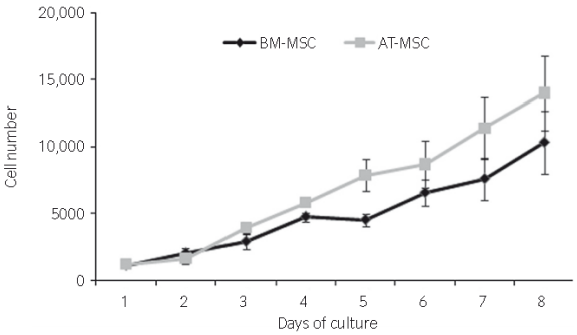

Fig 1: BM and AT-MSC proliferation along time evaluated with the MTT assay. Mean ± s.e. of cell number are shown.

TABLE 3: Behaviour of viability, necrosis + late apoptosis, early apoptosis and necrosis along time for each sample origin (BM or AT) and individual (1 or 2)

Traits	Slope	Estimate	s.e.	Conf. interval
Necrosis	b_{BM1}	-0.031	0.027	[-0.084, 0.022][a]
	b_{AT1}	0.012	0.027	[-0.041, 0.065][a]
	b_{BM2}	-0.071	0.027	[-0.124, -0.018][a]
	b_{AT2}	-0.038	0.027	[-0.091, 0.015][a]
Necrosis + late apoptosis	b_{BM1}	3.801	1.294	[1.265, 6.337][a]
	b_{AT1}	3.869	1.259	[1.401, 6.337][a]
	b_{BM2}	2.804	1.259	[0.336, 5.272][a]
	b_{AT2}	5.123	1.259	[2.655, 7.591][a]
Viable cells	b_{BM1}	-2.989	0.385	[-3.744, -2.234][a]
	b_{AT1}	-2.382	0.385	[-3.137, -1.627][a]
	b_{BM2}	-0.637	0.385	[-1.392, 0.118][b]
	b_{AT2}	-3.626	0.385	[-2.871, -4.381][a]
Early apoptosis	b_{BM1}	-0.677	0.194	[-1.057, -0.297][a]
	b_{AT1}	-1.488	0.194	[-1.868, -1.108][b]
	b_{BM2}	-0.149	0.194	[-0.529, 0.231][a]
	b_{AT2}	-1.445	0.194	[-1.825, -1.065][b]

Slope estimates, standard error (s.e.) and confidential interval of the slopes. Same letter ([a or b]) indicates overlapping intervals (no significant differences).

significantly faster in AT than in BM MSC (P<0.05). However, this result did not correspond with an increase of viability in AT-MSCs. As shown in Table 3, only BM-MSC from the second individual had a less marked slope in the decrease of viability along the course of culture.

Cell surface antigen gene expression

BM-MSCs and AT-MSCs expressed transcripts for *CD73*, *CD90*, *CD105* and *CD166* (Fig 2). The levels for these MSCs marker expressions were similar for both sources of MSCs. *CD34* was significantly enriched in AT-MSCs compared to BM-MSCs (P<0.01), which did not express this cell marker. Equine MSCs did not express haematopoietic (CD45) and endothelial (CD31). In the same RT-qPCR assays, *CD34* in bone marrow and *CD31* and *CD45* in peripheral blood displayed a positive amplification with a relative expression value of 1.

CD90 flow cytometry analysis showed the presence of this molecule in a high percentage in BM-MSCs (93.05 ± 2.30) and AT-MSCs (96.07 ± 0.76) (data not shown).

Osteogenic differentiation

Osteogenic differentiation was achieved for both sources of MSCs, which showed multiple Alizarin Red-stained calcium deposits (Fig 3). During osteogenic induction, ALP activity was measured on Days 0, 3, 5 and 7 of

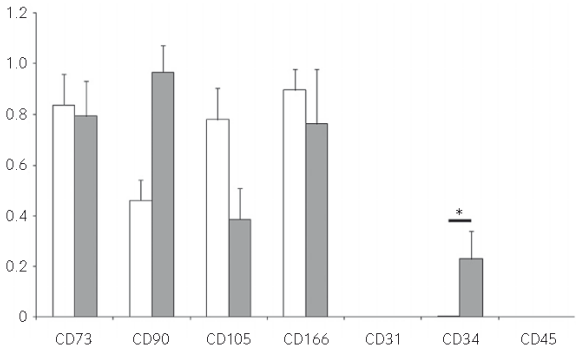

Fig 2: Mean ± s.e. of relative mRNA expression (y axis) of 6 antigens surface markers (x axis). White bars represent BM-MSC and grey bars are AT-MSC. *P<0.05, n = 5.

culture. ALP activity increased at Day 5 for both BM-MSCs and AT-MSCs and increased further still by Day 7 (Fig 4). Differences in ALP activity between Days 0 and 7 were significant for BM-MSCs (P<0.001) and AT-MSCs (P<0.05).

Transcript levels of 4 osteogenic markers were analysed on Days 0, 3, 5 and 7 of culture (Fig 4). *RUNX2* expression levels started to increase at Day 3 in osteogenic cell cultures, and were significantly compared to controls at Day 7 in both cell types. *SPP1* mRNA expression reached its maximum at Day 5 for BM-MSCs (146-fold higher than in control cultures) and decreased afterwards, although at Day 7 its expression was still significantly higher in differentiated cells than in control cultures. The highest level of *SPP1* expression in treated AT-MSCs was found on Day 7 (19.4-fold higher than in controls), although not significant difference was observed.

BGLAP mRNA expression increased slightly in treated AT-MSCs. At Day 5 in differentiated BM-MSCs, significant differences were observed with respect to control cells and the highest expression (13.4-fold higher) was on Day 7. *ALP* mRNA increased during the early stage of osteogenesis for both types of cells. In addition, a correlation between *ALP* gene expression level and activity was observed for BM-MSCs ($r = 0.429$, P<0.01). Finally, *COL1A1* expression levels in osteogenic cultures were similar or lower than controls for both cell types. The expression of *RUNX2* correlated with the expression of *ALP* and *SSP1* in both MSC sources. Table 4 shows these and other significant Pearson correlation coefficients that were obtained. *COL1A1* expression did not positively correlate with any of the other osteogenic markers.

Adipogenic differentiation

Sixteen different media and several seeding conditions were tested for their ability to induce the adipogenic differentiation of equine MSCs (Table 2). Under the conditions tested, only one medium could induce differentiation in BM-MSCs from 2 donors. No differentiation was observed in any of AT-MSCs. Lipid droplets appeared at Day 4 and increased in size over time. On Day 15 of culture, the oil red O staining showed intracellular accumulation of red-stained lipid droplets (Fig 3). The remaining samples were subjected to the same culture conditions, but their morphology did not change during the differentiation process.

Expression of adipogenic markers was analysed every other day for 15 days. *PPARγ* and *LPL* expression profiles in BM-MSCs are shown in Figure 5. *PPARγ* and *LPL* mRNA expression levels increased 64- and 354-fold, respectively, on the last day of culture with respect to control cells. These markers were also analysed for cells that did not exhibit morphological signs of differentiation. RT-qPCR results confirmed the lack of differentiation because no significant changes were observed in their expression (data not shown).

Chondrogenic differentiation

Equine MSCs were able to differentiate into chondrocytes (Fig 3). Haematoxylin and Alcian blue stainings were performed on Day 21 of culture showed the characteristic chondrogenic phenotype with lacunae formation throughout the pellet and the glycosaminoglycans in the extracelullar matrix appeared stained in blue.

Discussion

Characteristics of marker expression, proliferation and differentiation show significant variations between analogous cell populations (Conget and Minguell 1999; Larson *et al.* 2008). Prior to the application of stromal cell populations in regenerative medicine, fundamental questions about *in vitro* growth and differentiation of mesenchymal stromal cell populations must be resolved (Ciba *et al.* 2009). In this work, we extensively characterise 2 types of MSCs (AT-derived MSCs and BM-derived MSCs), evaluating their proliferation potential, viability, gene expression of membrane surface markers and ability to differentiate into osteogenic, adipogenic and chondrogenic cells.

Different attempts to monitor the growth of equine BM- and AT-MSCs have been reported (Vidal *et al.* 2006, 2007; Mambelli *et al.* 2009). Our work compares the proliferation capacity and viability of both sources of MSCs using the MTT approach at passage 3. Vidal *et al.* (2006, 2007)

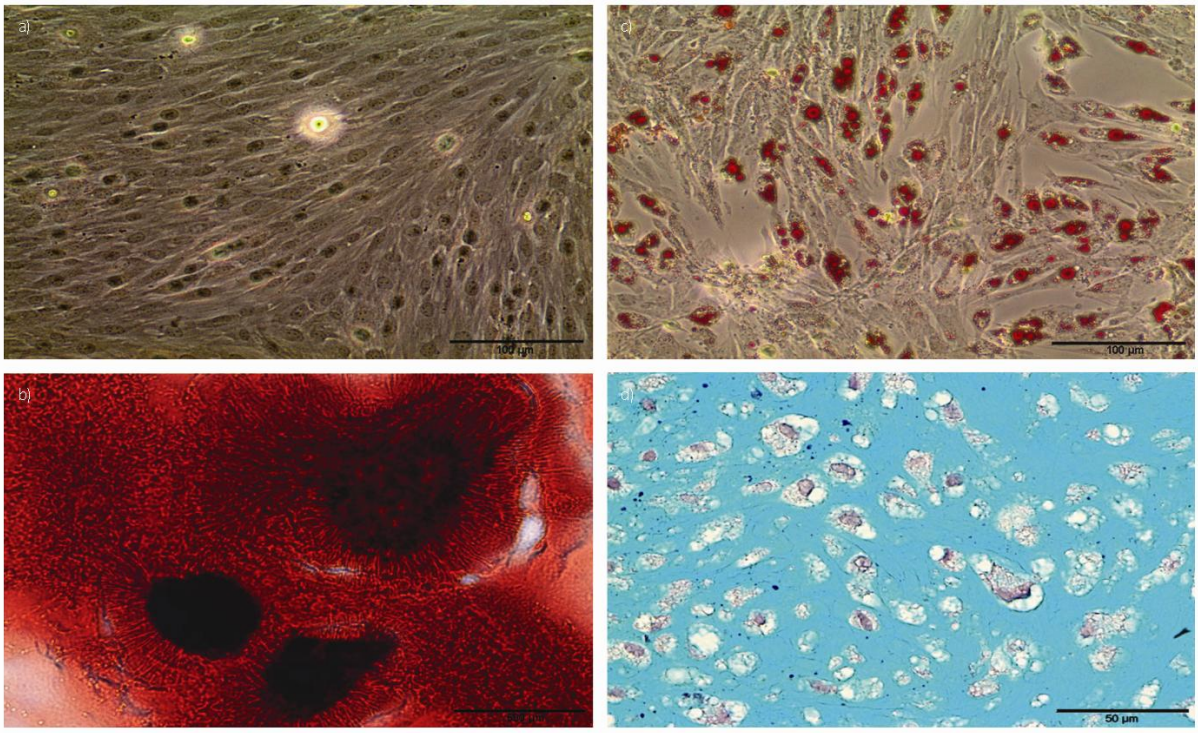

Fig 3: Staining for osteogenic, adipogenic and chondrogenic differentiation in BM-MSC. a) Control MSCs maintained in basal medium (magnification 10×). b) Alizarin red staining of cells cultured for 7 days in osteogenic differentation medium (magnification 4×). c) Oil red O staining of cells cultured for 15 days in adipogenic differentiation medium (magnification 10×). d) Haematoxylin Mayer's and Alcian Blue stainings of pellets cultured for 21 days in chondrogenic medium (magnification 40×).

reported a cell doubling time for AT-MSCs that was higher than BM-MSCs for at passage 3. Under our conditions, AT-MSCs had a slightly lower cell doubling time than BM-MSCs, but the differences were not significant. A slowing down in the cell proliferation of BM-MSC was observed at Days 4 and 5. For this reason the number of cells in the AT-MSC was higher than BM-MSC culture since these days. This result was more evident for cells from the same animal, indicates the existence of interindividual differences and confirms the results of other studies that demonstrate a large variation between MSC populations with regard to growth rate (Sen *et al.* 2001; Izadpanah *et al.* 2006).

In agreement with MTT results, viability and necrosis + late apoptosis percentage displayed the lowest and highest values, respectively on Day 5. To our knowledge, this is the first study that analyses the viability, apoptosis and necrosis of equine MSCs in culture. Given the limited size of the experiment, we cannot infer a general pattern of evolution over time, but we detected evidence of variability in viability and early apoptosis between cells from different sources. The highest values of early apoptosis were observed during the first days of culture that could correspond with the lag phase, when cells are readjusting to the culture. In AT-MSCs, early apoptosis decreased faster than in BM-MSCs while necrosis + late apoptosis increased following the same tendency (faster in AT-MSC than in BM-MSC); these 2 findings could explain that not significant differences in viability were found between cells from the 2 sources. Future research must be done to clarify the sources of this variability and improve the ability to predict the specific pattern of evolution of viability, apoptosis and necrosis over time. Nevertheless, for their use in cell therapy, the culture of AT-MSC should be stopped before the culture is close to confluence, as the highest percentage of necrotic + late apoptotic cells and lowest viability was observed on the last day of culture.

There have been recent studies on the immunophenotype of equine AT-derived MSCs for CD13, CD44 and CD90 (de Mattos Carvalho *et al.* 2009) and equine BM-derived MSCs for CD29, CD44, CD90 and CD45 (Radcliffe *et al.* 2010). The determination of equine CD markers using anti-human antibodies is difficult because of the small reactivity of available antibodies with equine epitopes (Ibrahim and Steinbach 2007). In an attempt to determine the expression of well known MSC markers in man (CD73, CD105, CD90 and CD166) and haematopoietic and endothelial markers (CD34, CD35 and CD431), we used RT-qPCR. Although this technique shows the mRNA expression of different markers, this expression is not always correlated with the presence of protein. Protein detection by other methods may possibly complement the results we have obtained. Data obtained by flow cytometry for the CD90 antigen supported data from RT-qPCR. In agreement with previous reports in horses (Arnhold *et al.* 2007; de Mattos Carvalho *et al.* 2009; Radcliffe *et al.* 2010) CD90 is an antigen present in established culture of equine MSC. The expression profile of these markers in equine BM- and AT-MSCs in passage 3, was in accordance with the immunophenotype reported for human MSCs (Dominici *et al.* 2006; Kern *et al.* 2006; Jarocha *et al.* 2008; Liu *et al.* 2008), including the differences observed for the expression of CD34 between BM- and AT-MSCs. Because the immunoreactivity for CD34 in human AT-MSCs declines with passages (Mitchell *et al.* 2006; Noel *et al.* 2008), further analysis is necessary to confirm that the loss of CD34 in equine AT-MSCs is similar to that in human cells.

One of the main characteristics of MSCs is their capacity to differentiate into multiple lineages. We have analysed the osteogenic, adipogenic and chondrogenic potential of equine BM- and AT-MSCs. MSC differentiation into osteoblasts was confirmed by staining calcium deposits with Alizarin red and measuring the increase of ALP activity and the expression of osteogenic markers over time. The osteogenic differentiation in equine MSCs was faster than in other species, including human (Liu *et al.* 2009), porcine (Zou *et al.* 2008) and bovine (Bosnakovski *et al.* 2005) and similar to previous reports in horses (Vidal *et al.* 2006).

The use of molecular markers allows osteogenic differentiation to be monitored and indicates which genes are involved in the process. Here, we present for the first time the expression profiles of a battery of osteogenic markers in both types of equine MSCs. In agreement with data found in

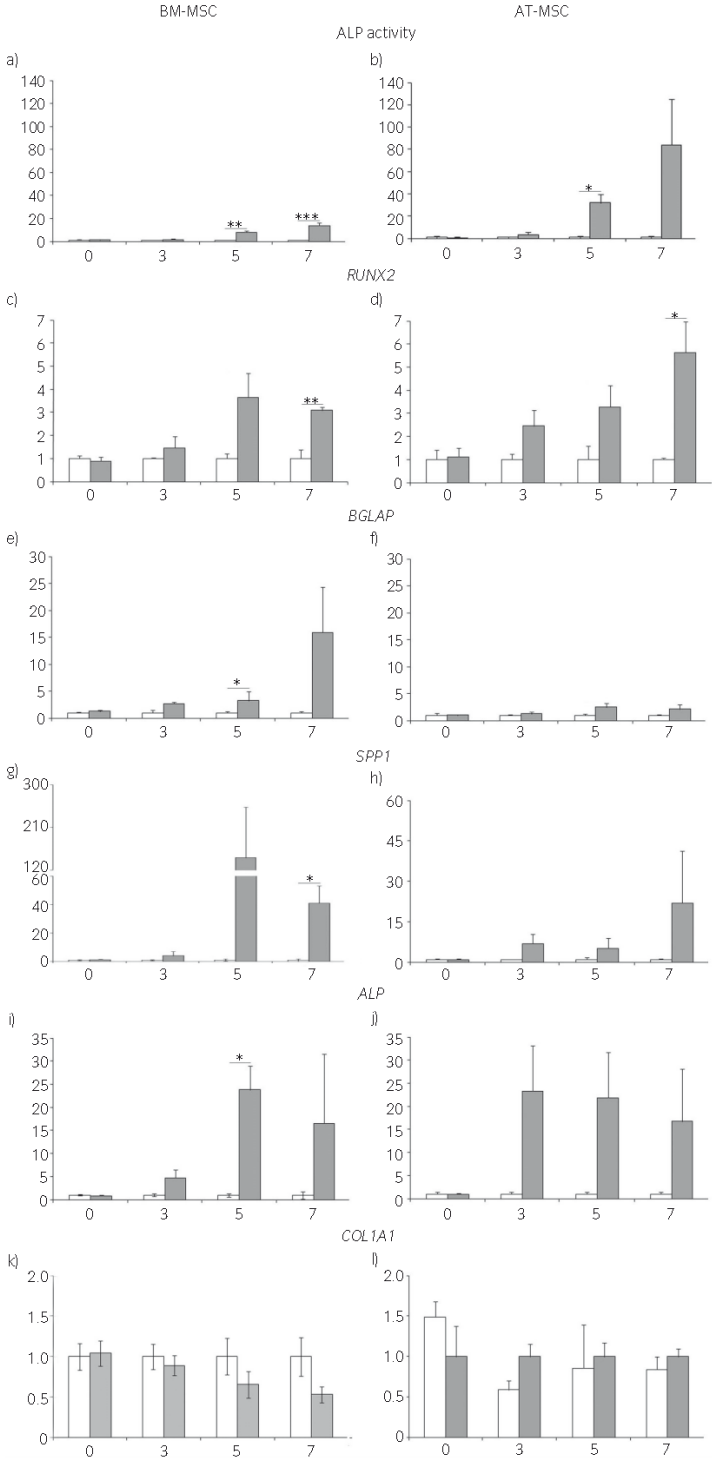

Fig 4: Alkaline phosphatase activity (a,b) and osteogenic marker expression levels (c–l) in controls (white bars) and differentiated (grey bars). BM-MSC (left) and AT-MSC (right). X axis represents culture days. Y axis represents the mean ± s.e. of relative mRNA expression data for RUNX2 (c,d), BGLAP (e,f) SPP1 (g,h) ALP (i,j) and COL1A1 (k,l) genes. *P<0.05 **P<0.01 ***P<0.001, n = 5.

TABLE 4: Correlation between osteogenic marker expression and ALP activity. No significant correlations are not shown

		ALP	BGLAP	COL1A1	RUNX2	SPP1	ALP activity
BM-MSC	ALP				0.527**	0.448**	0.429**
	BGLAP						0.559**
	COL1A1						-0.320*
	RUNX2	0.527**				0.584**	0.702**
	SPP1	0.448**			0.584**		
	ALP Activity	0.429**	0.559**	-0.320*	0.702**		
AT-MSC	ALP		0.472**		0.332*		
	BGLAP	0.472**					
	COL1A1						
	RUNX2	0.332*				0.609**	0.727**
	SPP1				0.609**		0.861**
	ALP Activity				0.727**	0.861**	

*P<0.05; **P<0.01.

other species (Bosnakovski *et al.* 2005; Zou *et al.* 2008), 4 genes (*BGLAP*, *SPP1*, *ALP* and *RUNX2*) were upregulated during osteogenesis. Moreover, the expression of some these genes was correlated with ALP activity, indicating that they are involved in the differentiation process. Our findings suggest that osteogenic differentiation of equine BM- and AT-MSCs occurs by upregulating a cascade of specific genes, including *RUNX2*, *BGLAP*, *SSP1* and *ALP* but not *COL1A1*. While this osteogenic marker seems to be upregulated in other species (Bosnakovski *et al.* 2005) and is commonly used in human MSC characterisation (De Bari *et al.* 2008), other authors have reported no remarkable increase of *COL1A1* mRNA after osteogenic differentiation in human (Liu *et al.* 2008) and porcine (Zou *et al.* 2008) MSCs.

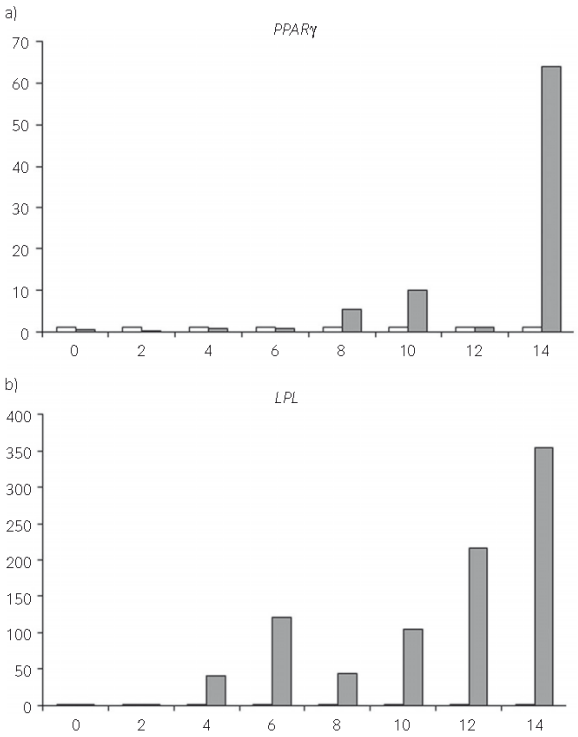

Fig 5: mRNA expression levels of adipogenic marker in controls (white bars) and differentiated (grey bars) BM-MSC evaluated by RT-qPCR. X axis represents culture days and Y axis represents realtive mRNA expression of the PPARγ(a) LPL (b) genes.

We cultured BM- and AT-MSCs in different adipogenic media. Previous reports found that rabbit serum enhanced adipogenesis *in vitro* for human (Janderova *et al.* 2003), rat and mouse (Diascro *et al.* 1998) MSCs. Rabbit serum has a high content of free fatty acids, which are putative ligands of PPARγ and may thus enhance adipogenesis. When we compared 16 different kinds of adipogenic induction media, only the medium with a similar composition to the medium used for human BM- and AT-derived MSCs (Pittenger *et al.* 1999) and supplemented with 15% rabbit serum was able to induce adipogenic differentiation. Other authors did not find it necessary to add rabbit serum to achieve any reliable adipogenesis (Mambelli *et al.* 2009). The intracellular accumulation of red-stained lipid droplets on Day 15 of culture was indicative of adipogenic differentiation. However, cytoplasmic droplets were already visible within the first few days of culture. This characteristic was in agreement with findings observed by other authors (Vidal *et al.* 2007; Mambelli *et al.* 2009). Our results indicate that equine MSCs display an adipogenic potential lower than other species (Bosnakovski *et al.* 2005; Zou *et al.* 2008). Cells from one of the animals whose adipogenic differentiation was possible were used to monitor the process with molecular markers. Both *PPARγ* and *LPL* genes were upregulated under adipogenic conditions compared to controls, indicating that these markers are involved in the differentiation process. The increase was more evident on the last day of culture.

As a final step in the characterisation process, we demonstrated equine MSCs had tri-lineage potential since cells were able to differentiate into chondrocytes.

In summary, here we present the characterisation of equine BM- and AT-MSCs, comparing their proliferation activity, viability, apoptosis, necrosis and differentiation. We also describe a battery of molecular markers for phenotype characterisation and monitoring osteogenic and adipogenic differentiation, which will contribute to a more extensive characterisation of equine MSCs prior to their use in cell therapy.

Acknowledgements

This work was performed as part of the AGL2008-02428/GAN (MICINN) and PAMER Pipamer 09/019 projects and was partially financed by the Gobierno de Aragón (Grupo de Excelencia LAGENBIO) and Instituto Aragonés de Ciencias de la Salud (ICS). B. Ranera was supported by a doctoral grant from the DGA.

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References

Adachi, N., Ochi, M., Deie, M. and Ito, Y. (2005) Transplant of mesenchymal stem cells and hydroxyapatite ceramics to treat severe osteochondral damage after septic arthritis of the knee. *J. Rheumatol.* **32**, 1615-1618.

Angele, P., Johnstone, B., Kujat, R., Zellner, J., Nerlich, M., Goldberg, V. and Yoo, J. (2008) Stem cell based tissue engineering for meniscus repair. *J. Biomed. Mater. Res. A.* **85**, 445-455.

Arnhold, S.J., Goletz, I., Klein, H., Stumpf, G., Beluche, L.A., Rohde, C., Addicks, K. and Litzke, L.F. (2007) Isolation and characterization of bone marrow-derived equine mesenchymal stem cells. *Am. J. vet. Res.* **68**, 1095-1105.

Bernardo, M.E., Emons, J.A., Karperien, M., Nauta, A.J., Willemze, R., Roelofs, H., Romeo, S., Marchini, A., Rappold, G.A., Vukicevic, S., Locatelli, F. and Fibbe, W. (2007) Human mesenchymal stem cells derived from bone marrow display a better chondrogenic differentiation compared with other sources. *Connect. Tissue Res.* **48**, 132-140.

Bobis, S., Jarocha, D. and Majka, M. (2006) Mesenchymal stem cells: characteristics and clinical applications. *Folia Histochem. Cytobiol.* **44**, 215-230.

Bosnakovski, D., Mizuno, M., Kim, G., Takagi, S., Okumura, M. and Fujinaga, T. (2005) Isolation and multilineage differentiation of bovine bone marrow mesenchymal stem cells. *Cell Tissue Res.* **319**, 243-253.

Brooke, G., Cook, M., Blair, C., Han, R., Heazlewood, C., Jones, B., Kambouris, M., Kollar, K., McTaggart, S., Pelekanos, R., Rice, A., Rossetti, T. and Atkinson, K. (2007) Therapeutic applications of mesenchymal stromal cells. *Semin. Cell. Dev. Biol.* **18**, 846-858.

Ciba, P., Sturmheit, T.M., Petschnik, A.E., Kruse, C. and Danner, S. (2009) In vitro cultures of human pancreatic stem cells: gene and protein expression of designated markers varies with passage. *Ann. Anat.* **191**, 94-103.

Colleoni, S., Bottani, E., Tessaro, I., Mari, G., Merlo, B., Romagnoli, N., Spadari, A., Galli, C. and Lazzari, G. (2009) Isolation, growth and differentiation of equine mesenchymal stem cells: effect of donor, source, amount of tissue and supplementation with basic fibroblast growth factor. *Vet. Res. Commun.* **33**, 811-821.

Conget, P.A. and Minguell, J.J. (1999) Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J. Cell. Physiol.* **181**, 67-73.

De Bari, C., Dell'Accio, F., Karystinou, A., Guillot, P.V., Fisk, N.M., Jones, E.A., McGonagle, D., Khan, I.M., Archer, C.W., Mitsiadis, T.A., Donaldson, A.N., Luyten, F.P. and Pitzalis, C. (2008) A biomarker-based mathematical model to predict bone-forming potency of human synovial and periosteal mesenchymal stem cells. *Arthr. Rheum.* **58**, 240-250.

Dezawa, M., Ishikawa, H., Itokazu, Y., Yoshihara, T., Hoshino, M., Takeda, S., Ide, C. and Nabeshima, Y. (2005) Bone marrow stromal cells generate muscle cells and repair muscle degeneration. *Science* **309**, 314-317.

Diascro, D.D., Jr, Vogel, R.L., Johnson, T.E., Witherup, K.M., Pitzenberger, S.M., Rutledge, S.J., Prescott, D.J., Rodan, G.A. and Schmidt, A. (1998) High fatty acid content in rabbit serum is responsible for the differentiation of osteoblasts into adipocyte-like cells. *J. Bone Miner. Res.* **13**, 96-106.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D. and Horwitz, E. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**, 315-317.

Granero-Molto, F., Weis, J.A., Longobardi, L. and Spagnoli, A. (2008) Role of mesenchymal stem cells in regenerative medicine: application to bone and cartilage repair. *Expert Opin. Biol. Ther.* **8**, 255-268.

Herzog, E.L., Chai, L. and Krause, D.S. (2003) Plasticity of marrow-derived stem cells. *Blood* **102**, 3483-3493.

Holtorf, H.L., Jansen, J.A. and Mikos, A.G. (2005) Flow perfusion culture induces the osteoblastic differentiation of marrow stroma cell-scaffold constructs in the absence of dexamethasone. *J. Biomed. Mater. Res. A.* **72**, 326-334.

Horwitz, E.M., Prockop, D.J., Fitzpatrick, L.A., Koo, W.W., Gordon, P.L., Neel, M., Sussman, M., Orchard, P., Marx, J.C., Pyeritz, R.E. and Brenner, M.K. (1999) Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat. Med.* **5**, 309-313.

Ibrahim, S. and Steinbach, F. (2007) Non-HLDA8 animal homologue section anti-leukocyte mAbs tested for reactivity with equine leukocytes. *Vet. Immunol. Immunopathol.* **119**, 81-91.

Izadpanah, R., Trygg, C., Patel, B., Kriedt, C., Dufour, J., Gimble, J.M. and Bunnell, B.A. (2006) Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. *J. Cell. Biochem.* **99**, 1285-1297.

Janderova, L., McNeil, M., Murrell, A.N., Mynatt, R.L. and Smith, S.R. (2003) Human mesenchymal stem cells as an in vitro model for human adipogenesis. *Obes. Res.* **11**, 65-74.

Jarocha, D., Lukasiewicz, E. and Majka, M. (2008) Adventage of mesenchymal stem cells (MSC) expansion directly from purified bone marrow CD105+ and CD271+ cells. *Folia Histochem. Cytobiol.* **46**, 307-314.

Kern, S., Eichler, H., Stoeve, J., Kluter, H. and Bieback, K. (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* **24**, 1294-1301.

Koch, T.G., Heerkens, T., Thomsen, P.D. and Betts, D.H. (2007) Isolation of mesonchymal stem cells from equine umbilical cord blood. *BMC Biotechnol.* **7**, 26.

Koch, T.G., Berg, L.C. and Betts, D.H. (2009) Current and future regenerative medicine - principles, concepts, and therapeutic use of stem cell therapy and tissue engineering in equine medicine. *Can. vet. J.* **50**, 155-165.

Koerner, J., Nesic, D., Romero, J.D., Brehm, W., Mainil-Varlet, P. and Grogan, S.P. (2006) Equine peripheral blood-derived progenitors in comparison to bone marrow-derived mesenchymal stem cells. *Stem Cells* **24**, 1613-1619.

Kolm, G., Knapp, E., Wagner, R. and Klein, D. (2006) Increased interleukin-1beta mRNA expression in skin biopsies of horses with Culicoides hypersensitivity following challenge with Culicoides nubeculosus extract. *Vet. Immunol. Immunopathol.* **113**, 90-98.

Larson, B.L., Yiostalo, J. and Prockop, D.J. (2008) Human multipotent stromal cells undergo sharp transition from division to development in culture. *Stem Cells* **26**, 193-201.

Liu, F., Akiyama, Y., Tai, S., Maruyama, K., Kawaguchi, Y., Muramatsu, K. and Yamaguchi, K. (2008) Changes in the expression of CD106, osteogenic genes, and transcription factors involved in the osteogenic differentiation of human bone marrow mesenchymal stem cells. *J. Bone Miner. Metab.* **26**, 312-320.

Liu, H., Toh, W.S., Lu, K., Macary, P.A., Kemeny, D.M. and Cao, T. (2009) A subpopulation of mesenchymal stromal cells with high osteogenic potential. *J. Cell. Mol. Med.* **13**, 2436-2447.

Mambelli, L.I., Santos, E.J., Frazao, P.J., Chaparro, M.B., Kerkis, A., Zoppa, A.L. and Kerkis, I. (2009) Characterization of equine adipose tissue-derived progenitor cells before and after cryopreservation. *Tissue Eng. Part C Methods* **15**, 87-94.

de Mattos Carvalho, A., Alves, A.L., Golim, M.A., Moroz, A., Hussni, C.A., de Oliveira, P.G. and Deffune, E. (2009) Isolation and immunophenotypic characterization of mesenchymal stem cells derived from equine species adipose tissue. *Vet. Immunol. Immunopathol.* **132**, 303-306.

Mitchell, J.B., McIntosh, K., Zvonic, S., Garrett, S., Floyd, Z.E., Kloster, A., Di Halvorsen, Y., Storms, R.W., Goh, B., Kilroy, G., Wu, X. and Gimble, J. (2006) Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* **24**, 376-385.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63.

Murphy, J.M., Fink, D.J., Hunziker, E.B. and Barry, F.P. (2003) Stem cell therapy in a caprine model of osteoarthritis. *Arthr. Rheum.* **48**, 3464-3474.

Noel, D., Caton, D., Roche, S., Bony, C., Lehmann, S., Casteilla, L., Jorgensen, C. and Cousin, B. (2008) Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials. *Expt. Cell Res.* **314**, 1575-1584.

Orlic, D., Kajstura, J., Chimenti, S., Limana, F., Jakoniuk, I., Quaini, F., Nadal-Ginard, B., Bodine, D.M., Leri, A. and Anversa, P. (2001) Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc. Natl. Acad. Sci. USA* **98**, 10344-10349.

Orsini, J.A. and Divers, T.J. (1998) Bone marrow biopsy. In: *Manuel of Equine Emergencies: Treatment and Procedures*. 1st edn., Eds: J.A. Orsini and T.J. Divers and W.B. Saunders, Philadelphia, USA. pp 27-28.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S. and Marshak, D. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147.

Radcliffe, C.H., Flaminio, M.J. and Fortier, L.A. (2010) Temporal analysis of equine bone marrow aspirate during establishment of putative mesenchymal progenitor cell populations. *Stem. Cells Dev.* **19**, 269-282.

Richardson, L.E., Dudhia, J., Clegg, P.D. and Smith, R. (2007) Stem cells in veterinary medicine - attempts at regenerating equine tendon after injury. *Trends Biotechnol.* **25**, 409-416.

Sen, A., Lea-Currie, Y.R., Sujkowska, D., Franklin, D.M., Wilkison, W.O., Halvorsen, Y.D. and Gimble, J.M. (2001) Adipogenic potential of human adipose derived stromal cells from multiple donors is heterogeneous. *J. Cell. Biochem.* **81**, 312-319.

Smith, R.K. and Webbon, P.M. (2005) Harnessing the stem cell for the treatment of tendon injuries: heralding a new dawn? *Br. J. Sports Med.* **39**, 582-584.

Smith, R.K., Korda, M., Blunn, G.W. and Goodship, A.E. (2003) Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment. *Equine vet. J.* **35**, 99-102.

Vidal, M.A., Kilroy, G.E., Johnson, J.R., Lopez, M.J., Moore, R.M. and Gimble, J.M. (2006) Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. *Vet. Surg.* **35**, 601-610.

Vidal, M.A., Kilroy, G.E., Lopez, M.J., Johnson, J.R., Moore, R.M. and Gimble, J.M. 2007) Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells. *Vet. Surg.* **36**, 613-622.

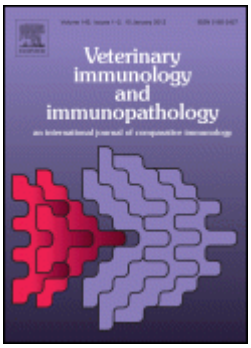
Violini, S., Ramelli, P., Pisani, L.F., Gorni, C. and Mariani, P. (2009) Horse bone marrow mesenchymal stem cells express embryo stem cell markers and show the ability for tenogenic differentiation by in vitro exposure to BMP-12. *BMC Cell Biol.* **10**, 29.

Wilke, M.M., Nydam, D.V. and Nixon, A.J. (2007) Enhanced early chondrogenesis in articular defects following arthroscopic mesenchymal stem cell implantation in an equine model. *J. Orthop. Res.* **25**, 913-925.

Zou, L., Zou, X., Chen, L., Li, H., Mygind, T., Kassem, M. and Bunger, C. (2008) Multilineage differentiation of porcine bone marrow stromal cells associated with specific gene expression pattern. *J. Orthop. Res.* **26**, 56-64.

Author contributions B. Ranera carried out the isolation and expansion of the MSCs, the differentiation assays and histology and the writing of the manuscript. L. Ordovas participated in the expansion of the MSCs, and carried out the proliferation and visibility, apoptosis and necrosis assays. J. Lyahyai performed the RT-qPCRs. M.L. Bernal participated in the writing of the manuscript. F. Fernandes participated in the expansion of MSCs. A.R. Remacha participated in the differentiation assays and histology. A. Romero carried out the aspiration of the bone marrow and the harvesting of the adipose tissue. F.J. Vásquez participated in the aspiration of the bone marrow and the harvesting of the adipose tissue. R. Osta participated in the design of the study. C. Cong participated in the isolation and expansion of MSCs. L. Varona performed the statistical analysis. P. Zaragoza participated in the design of the study and the writing of the manuscript. I. Martin-Burriel participated in the design of the study and in the writing of the manuscript. C. Rodellar coordinated the design of the study, supervised the work and participated in the writing of the manuscript.

5.3. MANUSCRITO III



Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue.

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Veterinary Immunology and Immunopathology 144 (2011) 147–154

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Short communication

Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue

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ARTICLE INFO

Article history:
Received 22 November 2010
Received in revised form 17 June 2011
Accepted 27 June 2011

Keywords:
Horse
MSC
Cell surface markers
Immunophenotype
Gene expression

ABSTRACT

Bone marrow and adipose tissue are the two main sources of mesenchymal stem cell (MSC). The aim of this work was to analyse the immunophenotype of 7 surface markers and the expression of a panel of 13 genes coding for cell surface markers in equine bone marrow and adipose tissue-derived MSCs obtained from 9 horses at third passage. The tri-lineage differentiation was confirmed by specific staining. Equine MSCs from both sources were positive for the MSC markers CD29 and CD90, while were negative for CD44, CD73, CD105, CD45 and CD34. The gene expression of these molecules was also evaluated by reverse transcriptase real-time quantitative PCR along with the expression of 5 other MSC markers. Both populations of cells expressed *CD13*, *CD29*, *CD44*, *CD49d*, *CD73*, *CD90*, *CD105*, *CD106*, *CD146* and *CD166* transcripts. Significant differences in gene expression levels between BM- and AT-MSCs were observed for *CD44*, *CD90*, *CD29* and *CD34*. Both cell types were negative for *CD45* and *CD31*. The surface antigens tested revealed a similar phenotypic profile between horse and human MSCs, although specific differences in some surface antigens were noticed.

1. Introduction

The use of stem cell in therapy and tissue engineering in equine medicine is relatively new, but it is an exciting research field that is beginning to rapidly expand (Smith et al., 2003; Crovace et al., 2007; Richardson et al., 2007). Due to similarities in size, load and types of joint injuries suffered by horses and humans, a U.S. Food and Drug Administration (FDA) report concluded that the horse was the most appropriate model animal for testing the clinical effects of mesenchymal stem cell (MSC)-based therapies for certain types of injuries in humans, especially joint injuries (Cellular, Tissue and Gene Therapies Advisory Committee, 2005). In addition, the economic and welfare costs of performance-related injuries in horses have stimulated interest in stem cell-based regenerative medical techniques to accelerate and improve healing (Paris and Stout, 2010). Therefore, the horse can be considered not only as an animal model for human injuries and osteoarthritis (Goodrich et al., 2007) but also as a patient itself.

Bone marrow and adipose tissue are the main sources of MSCs for the treatment of equine orthopaedics (Smith et al., 2003; Koch et al., 2008), although alternative sources for MSC isolation, such as umbilical cord or peripheral blood,

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doi:10.1016/j.vetimm.2011.06.033

have been described (Koerner et al., 2006; Hoynowski et al., 2007). Recent studies have demonstrated that MSCs are very heterogeneous; there are subpopulations of cells that have different shapes and varying proliferation and differentiation abilities (Zhang and Chan, 2010). Clear characterisation of MSCs is extremely relevant for their identification before use in therapy (Tarnok et al., 2010).

Because MSCs are becoming tools utilised in equine regenerative medicine, it is important to define equine-specific markers to precisely characterise this cell population (Koch et al., 2009). Mesenchymal stem cells and other stem cell lineages can be identified by the expression of specific "stemness" marker proteins and other stem cell epitopes that are not expressed by somatic cells. A unique MSC marker has not yet been identified, in contrast to the antigen CD34 that is used for positive immunoselection of haematopoietic stem cells (Tuan et al., 2003). The different subpopulations of adipose tissue are difficult to characterise due to the rapid nature of adipose stromal vascular cells to adopt a mesenchymal phenotype *in vitro* and the complex organisation of stromal cells surrounding the small vessels (Zimmerlin et al., 2010). Moreover, it has been proposed that the expression level of stem cell markers is also related to the method of cell isolation (Deans and Moseley, 2000; Panchision et al., 2007; Martinez-Lorenzo et al., 2009). Human MSCs are the best characterised, and the International Society for Cellular Therapy has established a minimal criteria for defining this type of cells (Dominici et al., 2006). First, MSCs should adhere to plastic; second, MSCs should express CD105, CD73 and CD90 and should not express CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR; and finally, MSCs should be able to differentiate into osteoblasts, adipocytes and chrondoblasts *in vitro*. There is also general agreement that MSCs lack CD31 and CD14 expression (Kern et al., 2006) and express the antigens CD44 (Tarnok et al., 2010), CD106 (Pittenger et al., 1999) and CD166 (Mitchell et al., 2006) and other cell adhesion molecules, such as CD13, CD29, CD49f, CD54, CD59, CD63 and CD146 (Deans and Moseley, 2000; Kern et al., 2006). These antigens can be detected by flow cytometry, although this method has not been used to validate most of these molecules in equine MSCs. In addition to flow cytometry, the gene expression of these antigens can be detected quantitatively using reverse transcriptase real-time quantitative PCR (RT-qPCR) (Radcliffe et al., 2010).

Although MSCs derived from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) seem to be closely related, differences in their biological characteristics have been reported (Im et al., 2005; Puissant et al., 2005). Both AT-MSCs and BM-MSCs share the expression of most surface antigens, but some markers are expressed differentially. For example, in humans, AT-MSCs express CD34, whereas no expression is detected in BM-MSCs; conversely, the expression of the CD106 marker has been found in BM-MSCs but not in AT-MSCs (De Ugarte et al., 2003; Gangenahalli et al., 2006).

Although there are several groups working on the characterisation of adult equine MSCs (Koerner et al., 2006; Vidal et al., 2006, 2007, 2008; Arnhold et al., 2007; Colleoni et al., 2009; Violini et al., 2009), only three recent studies (de Mattos Carvalho et al., 2009; Mambelli et al., 2009; Radcliffe et al., 2010) have reported the analysis of surface antigens by flow cytometry.

The lack of reactivity between commercial monoclonal antibodies and epitopes on equine cells makes it difficult to establish the phenotype of equine MSCs (Taylor et al., 2007) using flow cytometry as the sole technique. However, RT-qPCR allows monitoring of the expression levels of these markers in a limited number of cells, so the combined use of both techniques could facilitate the study of the phenotype of horse MSCs. The purposes of this work are to analyse the phenotype of both BM-MSCs and AT-MSCs with regard to 7 membrane cell surface markers using flow cytometry and also to extend the analysis to 6 more markers by RT-qPCR.

2. Materials and methods

2.1. Animals and cell isolation and expansion

Aspirates from bone marrow of nine horses were harvested using a 4″ 11G Jameshdi needle with 2500 UI of sodium heparin. Mononuclear cells were isolated by gradient centrifugation on Lymphoprep (Atom) for 20 min at 1700 rpm. The cells were rinsed twice with PBS (Gibco), counted, and plated at 2×10^6 nucleated cells/cm^2 in 6-well plates (Becton Dickinson) in growth medium consisting of low glucose Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% Foetal Bovine Serum, 1% Glutamine (Sigma) and 1% Streptomycin/Penicillin.

Samples of subcutaneous adipose tissue from near to the dorsal gluteal muscle below the tail were collected from eight horses. The stromal vascular fraction (SVF) was isolated by digestion with 0.01% collagenase (Type I, Sigma–Aldrich) for 30 min at 37 °C with continuous shaking, followed by centrifugation at 1700 rpm for 5 min. The cells were washed twice with PBS, counted, and seeded in growth medium at 10^5 nucleated cells/cm^2 in 6-well plates.

Both mononuclear and SVF cells were washed twice with PBS after 24, 48 and 72 h of incubation at 37 °C at 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 in T75 or T175 flasks (Becton Dickinson) at 5000 cells/cm^2. The cells were trypsinised repeatedly until the third passage and then were cryopreserved in FBS with 10% DMSO. Approximately 10^6 cells from passage three were thawed at 37 °C and plated in a T75 flask for three days to re-adjust prior to being used for the different analyses.

2.2. Differentiation assays

2.2.1. Osteogenic differentiation

Cells were plated at 20,000 cells/cm^2 in 12-well plates and cultured under osteogenic conditions for 9 days. Differentiation medium consisted of growth medium supplemented with 10 nM dexamethasone, 10 mM β-glycerophosphate and 100 μM ascorbate-2-phosphate. To assess their osteogenic differentiation, cells were fixed in

70% ethanol for 1 h and stained with 2% alizarin red S (Sigma–Aldrich) for 10 min.

2.2.2. Adipogenic differentiation

Cells were plated at 2500 cells/cm^2 in 12-well plates and cultured for 14 days with adipogenic medium, consisting of growth medium supplemented with 1 μM dexamethasone, 500 μM IBMX, 200 μM indomethacin and 15% rabbit serum. To examine their adipogenic differentiation, the cells were fixed with 10% formalin (Sigma) for 15 min and stained with 0.3% oil red O for 30 min at 37 °C.

2.2.3. Chondrogenic differentiation

Approximately 500,000 cells were pelleted and placed into 15 mL conical polypropylene tubes with chondrogenic medium, consisting of high glucose DMEM supplemented with 10% FBS, 10 ng/mL TGFβ-3 (R&D Systems), ITS + premix (BD), 40 μg/mL proline (Sigma), 50 μg/mL ascorbate-2-phosphate and 0.1 μM dexamethasone. The culture was maintained for 21 days. To assess the chondrogenic differentiation of these cells, pellets were fixed with 10% formalin, embedded in paraffin and cut into 5 μm sections. Finally, sections were stained with haematoxylin and alcian blue dyes.

2.3. Immunophenotyping

A total of 7 surface markers were analysed by flow cytometry, the mesenchymal cell markers CD29 (Integrin β-1), CD44 (H-CAM), CD73 (ecto-5′-nucleotidase), CD90 (Thy-1) and CD105 (Endoglin), and the haematopoietic markers CD34 and CD45 (LCA).

Cells were suspended in PBS/2 mM EDTA at 10^6 cells/mL. Fifty microliter aliquots of cells were transferred to flow cytometry tubes and incubated for 15 min at 4 °C with mouse anti-human CD29-FITC (Caltag Laboratories), CD34-PE (Becton Dickinson), CD44-FITC (Immunostep Research), CD45-APC (Becton Dickinson), CD73-PE (BD Pharmingen), CD90-PE (BD Pharmingen) or CD105-FITC (R&D Systems) monoclonal antibodies. Negative control staining was performed using a FITC-conjugated mouse IgG1 κ isotype, a PE-conjugated mouse IgG1 κ isotype, a PERCP-Cy 5.5-conjugated mouse IgG1 κ isotype and an APC-conjugated mouse IgG1 isotype antibody (all from BD Biosciences). Subsequently, cells were washed with PBS and diluted in 500 μL of PBS/2 mM EDTA.

Before the analysis with the fluorescence-activated cell sorter (FACSARIA, BD Biosciences), 0.5 μL of SYTOX® Blue dead cell stain (Molecular Probes™) was added to the cell dilution to get a 1 μM final concentration of dye. Samples were analysed after 5 min of incubation at room temperature. This staining discriminates between viable and non-viable cells. Living cells were gated in a dot-plot of side scatter signals versus SYTOX staining. At least, 3000 gated events were acquired on a biexponential fluorescence scale. 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 the software FACSDIVA 5.0.1 (BD Biosciences).

2.4. Gene expression analysis of cell surface markers

The expression of 13 genes coding for cell surface antigens was analysed by RT-qPCR on both types of equine MSCs. These markers included the 7 molecules analysed by flow cytometry (see above) as well as *CD13* (aminopeptidase), *CD31* (PECAM), *CD49d* (α-4 integrin), *CD106* (VCAM-1), *CD146* (MCAM) and *CD166* (ALCAM).

2.5. RNA extraction and reverse transcription

Cells were seeded on 24-well plates at 76,000 cells/well for 24 h in triplicate. Afterwards, the cells were washed with PBS and stored at −80 °C. Cell to cDNA II kit (Ambion) was used for total RNA isolation according to the manufacturer's instructions. Briefly, the cells were thawed at 0 °C, rinsed with ice-cold PBS, and 100 μL of ice-cold Cell Lysis II Buffer was added to each well. Samples were transferred to a 96-well plate and incubated at 75 °C for 15 min. Then 2 μL of DNAse I was added, and the reaction was incubated at 37 °C for 15 min and 75 °C for 5 min. Afterwards, the reverse transcriptase master mix reaction, consisting of buffer 10×, dNTPs, random decamers, RNAse inhibitor and M-MLV retrotranscriptase, was added to 10 μL of the samples. Finally, the samples were maintained for 60 min at 42 °C for cDNA synthesis and heated for 10 min at 95 °C.

2.6. Real-time quantitative PCR analysis

The cDNA generated was analysed by real-time PCR. The primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). Primer information, accession numbers for equine mRNA sequences and amplicon sizes are shown in Table 1. The amplification reaction was performed in triplicate using the Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and the StepOne™ Real Time PCR System device (Applied Biosystems, Foster City, CA, USA). All of the reactions were performed in a total volume of 10 μL with 2 μL of cDNA as template and 300 nM of forward and reverse primers. Amplification of the cDNA was achieved following the manufacturer's conditions: an initial activation and denaturation step of 20 s at 95 °C followed by 45 cycles consisting of 3 s at 95 °C and 30 s at 60 °C. A dissociation curve protocol was run after every reaction to identify the presence of spurious PCR bands or high levels of primer dimers. The levels of gene expression were determined by the comparative Ct method. A normalisation factor (NF) calculated as the geometric mean of the quantity of two housekeeping genes (*GAPDH* and *B2M*) was used to normalise the expression of each gene. The primers, probes and PCR conditions for the amplification of housekeeping genes was described previously (Kolm et al., 2006).

2.7. Statistic analysis

The software SPSS 15.0 was used for the statistical analysis. Data obtained from flow cytometry and RT-qPCR were

Table 1
Genes analysed, GenBank accession numbers, primer sequences for reverse transcriptase RT-PCR (forward and reverse) and amplicon sizes in base pairs (bp).

Gene	Accession number	Primer sequence (5′–3′)	Amplicon size
CD13	XM_001487923	F: TGACCCTGACCCCAACGT R: CGCCCTGCAGCCAGTATT	110
CD29	XM_001492665	F: GTAAAAAGTCTTGGAACCGATCTGAT R: CACAAATGAGCCAAACCCAATT	81
CD31	NM_001101655	F: TCTAGAACGGAAGGCTCCCT R: TGGGAGCAGGGCAGGTTCA	145
CD34	XM_001491596	F: CACTAAACCCTCTACATCATTTTCTCCTA R: GGCAGATACCTTGAGTCAATTTCA	150
CD44	NM_001085435	F: CCCACGGATCTGAAACAAGTG R: TTCTGGAATTTGAGGTCTCCGTAT	95
CD45	AY114350	F: TGATTCCCAGAAATGACCATGTA R: ACATTTTGGGCTTGTCCTGTAAC	100
CD49d	XM_001917601	F: CATCGGCCTTCTCACAGAGAA R: GCCATTATTGTCTGCATCAATTTG	101
CD73	XM_001500115	F: GGGATTGTTGGATACACTTCAAAAG R: GCTGCAACGCAGTGATTTCA	90
CD90	EU881920	F: TGCGAACTCCGCCTCTCT R: GCTTATGCCCTCGCACTTG	93
CD105	XM_001500078	F: GACGGAAAATGTGGTCAGTAATGA R: GCGAGAGGCTCTCCGTGTT	100
CD106	NM_001101650	F: CATCGTGACCTGTGGGCATA R: TGGGTTTCCCTCCACTAGCA	111
CD146	XM_001917594	F: CTGGACTTGGAAACCACAACATC R: CAGGTCTCACTCGGACATCAGA	85
CD166	XM_001503380	F: GTCTGGTCTTCTGCCTCTTGATC R: TCGGCAAGGCATGATAATAGTG	103

analysed for normality with the Shapiro-Wilk test Differences in gene expression and reactivity levels between BM- and AT-MSCs were determined using the unpaired non-parametric Mann–Whitney test. For both test, $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. MSC isolation and differentiation

The minimal criteria established to define the human MSCs (Dominici et al., 2006) are: the capacity for attachment to plastic, the expression of certain markers in their cell surface and the ability of differentiation into osteoblast, adipocyte and chondrocyte.

In this study, colonies of fibroblast-like cells were observed in all of the cultures the day following isolation and plating (Fig. 1a). These cells were expanded until reaching passage three, and then they were frozen. The tri-lineage differentiation ability was confirmed in equine MSCs (Fig. 1). Specific Haematoxylin and alcian blue staining of sections obtained from pellets of cells undergoing chondrogenic differentiation showed lacunae formation, which is a typical characteristic of the chondrogenic phenotype; moreover the proteoglycans produced in the extracellular matrix during cartilage differentiation were stained in blue. Calcium deposits formed during osteogenic differentiation were stained in red by alizarin red, whereas control cells did not display any deposit. Finally, oil red O-stained lipid droplets appeared inside of the cells under adipogenic induction while the control cultures did not show any change. Therefore, the equine cells used for further analysis met the minimal criteria concerning the plastic attachment and pluripotency.

3.2. Immunophenotype

Most of the cell surface markers used to sort subpopulations of human mesenchymal stem cells using flow cytometry have not been validated in horses, and there is evidence that some of these markers do not cross-react with horse antigens. In this work, we have analysed 7 surface markers by flow cytometry.

Equine MSCs displayed large size and complexity (Fig. 2a) and a lack of immunoreactivity was observed for the isotype controls for each mouse monoclonal antibody (Fig. 2b–e). Cells negative for SYTOX® staining were then included in the cytometry analysis as viable cells (Fig. 2f). In accordance with the immunophenotype described for human MSCs (De Ugarte et al., 2003; Kern et al., 2006; Liu et al., 2008), our flow cytometry results revealed that horse MSCs derived from the two sources were robustly positive for the typical MSC markers CD29 and CD90 (Fig. 2g and h), with more than 90% of positive cells and no statistically significant differences between cell sources (Fig. 2). Despite using a different antibody, our results confirmed the expression of CD90 by equine AT- and BM-MSCs as reported recently (de Mattos Carvalho et al., 2009; Radcliffe et al., 2010). There are contradictory findings with respect to the CD29 immunophenotype of equine MSCs, the immunoreactivity observed in our work is in agreement with the results obtained for equine BM-MSCs (Radcliffe et al., 2010), but not with those reported previously for equine AT-MSCs (Mambelli et al., 2009). Although we must

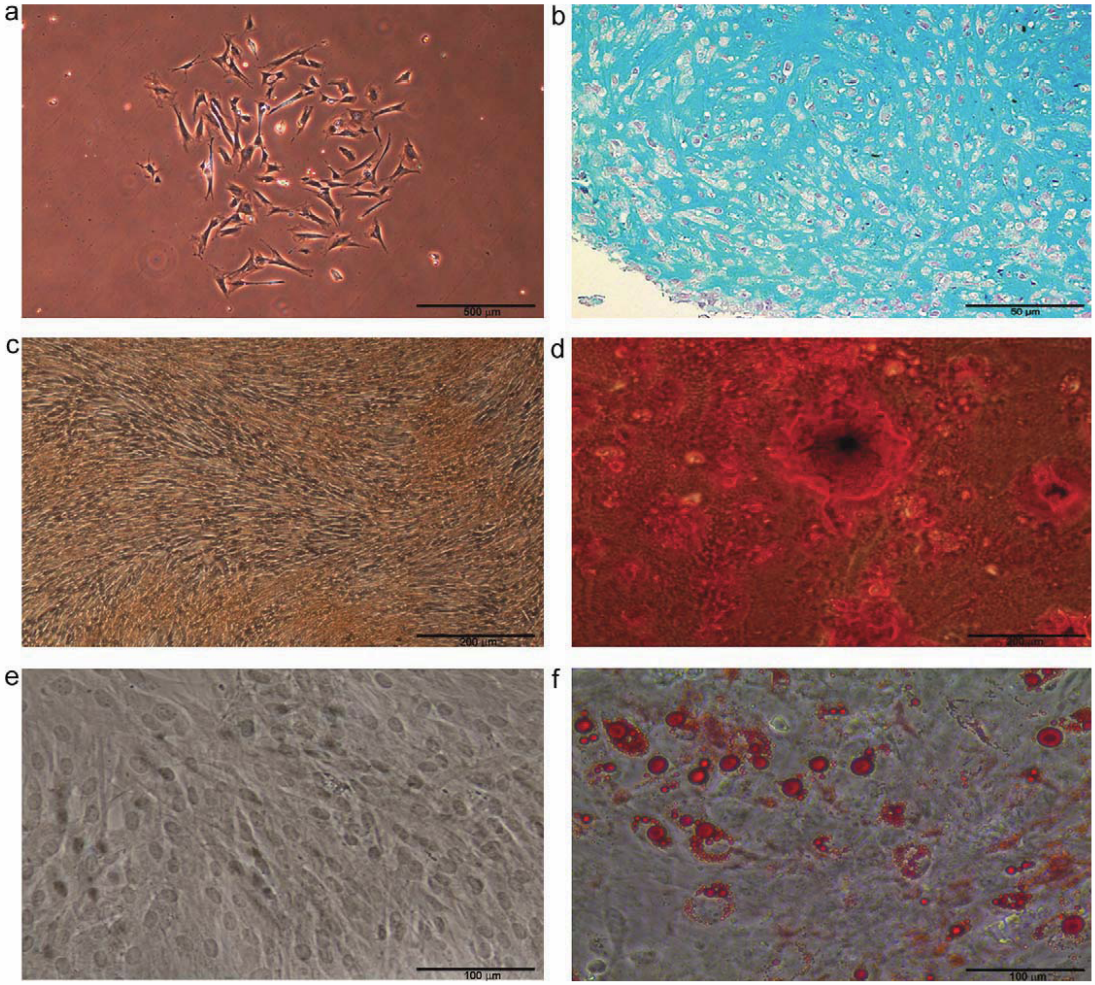


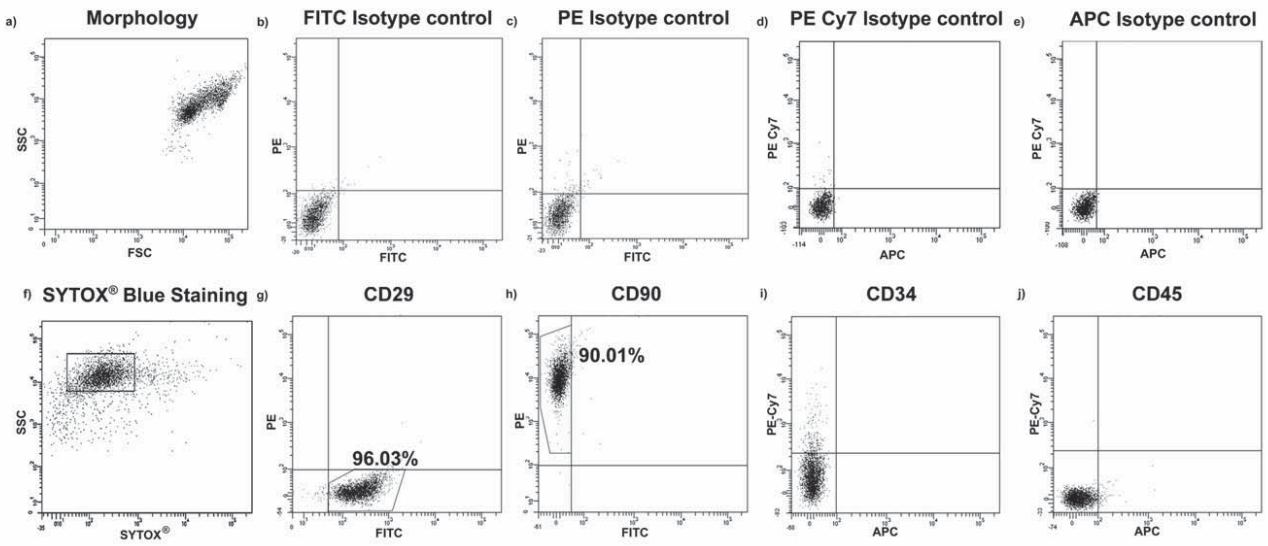
Fig. 1. Isolation and differentiation of equine MSCs. (a) A colony of MSCs after eight days of culture post-isolation (4× magnification). (b) Chondrogenic differentiation. A pellet section stained with haematoxylin and alcian blue (20× magnification). (c) Osteogenic differentiation. Lack of alizarin red staining in control cultures (4× magnification) and (d) calcium deposits stained in red in differentiated cells (4× magnification). (e) Adipogenic differentiation. Control cultures showing the absence of staining (20× magnification) and (f) oil red O dye-stained lipid droplets produced inside differentiated cells (20× magnification).

bear in mind that the antibodies used in this and other studies are not specific to the equine species, confirming the immunoreactivity for CD29 and CD90 in different works using different antibodies gives more reliability to these results.

Unfortunately, we could not expand the putative markers set with other antibodies as both BM- and AT-MSCs were negative for CD44, CD73 and CD105 (data not shown). Negative results for CD73 have also been reported in rabbit and sheep, although these species displayed cross-reactivity with anti-human CD44 and CD105 antibodies (Martinez-Lorenzo et al., 2009).

Reactivity against the haematopoietic antigens CD45 and CD34 (Fig. 2h and i), considered negative markers for human MSC (De Ugarte et al., 2003), was not detected for either of the two cell types, which is consistent with the results obtained in other species such as sheep or rabbit (Martinez-Lorenzo et al., 2009). Nevertheless, this result should be interpreted with caution, as the negative reactions for these markers are not truly negative because cross-reactivity between human and horse marker antibodies has not been confirmed. The expression of these antigens could be different between species and between cells with different origins. For example, haematopoietic stem cells (HSCs) from murine bone marrow and from human liver express CD45 but differ in the expression of CD34 and CD133 (Tarnok et al., 2010).

In order to clarify if the absence of immunostaining for the markers analysed was due to dissimilarities in the epitope sites between horse and human, we compared the public protein sequences of these two species using the BLAST tool. Percentages of identities ranged between 69% with a 100% query coverage (QC) for CD34 and 95% with a 97% QC for CD29. The remaining markers displayed the following identity percentages: 72% with 100% QC for CD105, 84% with 77% QC for CD90, 85% with 55% QC for CD44, and 89% with 90% QC for CD73. It was not possible to complete this analysis for CD45 as only a small sequence of the equine marker was found. These BLAST comparisons did not revealed a clear relationship between



BM-MSC (*n*=9)	96.03 ± 0.40	90.01 ± 3.65	0.30 ± 0.02	1.50 ± 0.09
AT-MSC (*n*=8)	97.22 ± 1.09	95.12 ± 1.13	3.07 ± 0.12	0.07 ± 0.03

Fig. 2. Flow cytometry representative plots *n* = *1*. (a) Plot showing equine MSCs morphology. (b–e) Isotype controls of mouse monoclonal antibodies for FITC, PE, PE-Cy7 and APC, respectively. (f) Plot showing SYTOX® Blue staining for dead cells. (g–j) Plots showing flow cytometry results for the markers CD29, CD90, CD34 and CD45, respectively. Below the plots are shown the means ± s.e. of percentage of positive cells for these markers in BM-MSC (*n* = *9*) and AT-MSC (*n* = *8*).

identity and immunoreactivy, although we might expect that if homology between human and horse molecules is 100% it is very likely that the anti-human antibody detects the corresponding horse protein, and that the staining pattern of the other antibodies remain questionable as long as no proof of specificity for the horse molecules exists.

3.3. Gene expression of cell surface markers

Because there are few specific markers for equine MSCs and many of the positive stem cell markers described for other species show little or no cross-reactivity with horses (Ibrahim and Steinbach, 2007; Smith, 2008), gene expression-based technologies can help in the identification of other possible molecules described as MSC markers (Rallapalli et al., 2009; Radcliffe et al., 2010). In our study, reverse transcriptase RT-qPCR was performed to quantify the mRNA expression of the cell surface antigen genes analysed by flow cytometry and other 6 molecules considered as positive or negative MSC markers in human.

These markers analysed by flow cytometry in our study are commonly used as markers to define human MSCs (Dominici et al., 2006; Mitchell et al., 2006). In accordance with our cytometry results, equine cells expressed *CD29* and *CD90* (Fig. 3). Although both AT and BM-MSCs expressed these markers, their mRNA expression was significantly higher in AT-MSCs than in BM-MSCs ($p < 0.05$ and $p < 0.01$, respectively). However, in contrast to the negative results obtained by flow cytometry, the real time PCR study revealed the amplification of *CD73*, *CD105* and *CD44* transcripts in both types of cells. Whereas AT and BM-MSCs displayed similar expression levels for *CD73* and *CD105*, *CD44* expression was significantly higher in AT-MSC than in BM-MSCs ($p < 0.001$). Therefore, the negative immunophenotyping observed in this work might be due to a lack of cross-reactivity of the antibodies used to the equine antigens rather than to the absence of these antigens in the cell surface. As in human MSCs, CD73, CD105 and CD44 molecules might also be considered as markers to identify equine MSCs.

The expression of the haematopoietic *CD34* marker was only observed in AT-MSCs, in contrast with the lack of immunoreactivity detected by flow cytometry but in agreement with human AT-MSC phenotyping (Noel et al., 2008). As the expression of this marker tends to decrease with the number of passages (Mitchell et al., 2006), our expression results indicate that equine AT-MSCs at passage three still express *CD34*.

Both cell types were negative for the haematopoietic marker *CD45*, which confirmed the negative result observed in flow cytometry. Therefore, as in humans (De Ugarte et al., 2003; Kern et al., 2006), equine MSCs do not expresses CD45 in established MSC cultures. Our results confirmed those previously reported (Radcliffe et al., 2010). Similarly, the endothelial marker *CD31* was not expressed by equine MSCs in agreement with the findings reported for human MSCs (Noel et al., 2008).

The antigens *CD166*, *CD13*, and *CD146* are considered positive markers for human MSCs from both origins (De Ugarte et al., 2003), in agreement with this both equine MSC types expressed these genes without significant differences between both cell sources.

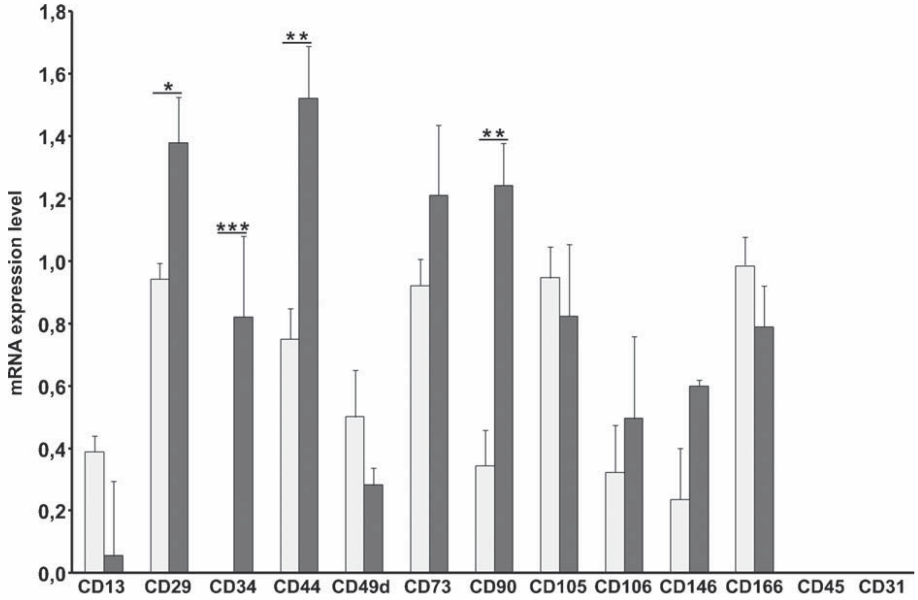


Fig. 3. mRNA expression of the *CD13, CD29, CD34, CD44, CD49d, CD73, CD90, CD105, CD106, CD146, CD166, CD45* and *CD31* cell surface markers. Relative mRNA expression levels are expressed as mean ± s.e. White bars correspond with BM-MSCs ($n = 9$) and grey bars with AT-MSC ($n = 8$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

CD49d and *CD106* have been reported as differential markers for human BM- and AT-MSCs (De Ugarte et al., 2003; Kern et al., 2006; Noel et al., 2008). In our real time PCR analysis both cell sources displayed similar transcript levels for these markers. Consequently, neither *CD49d* nor *CD106* would be considered key markers to differentiate MSCs from different sources in the horse, suggesting that the pattern of marker expression established for human MSC may not always be followed by MSCs of other species.

In summary, despite the lack of antibodies that cross-react with horse epitopes to identify markers that allow one to define MSCs by flow cytometry, it is possible to establish a profile of markers through gene expression analysis using RT-qPCR. This technique detects the expression of markers in the cells without the problem of specificity. And it also makes possible to quantify the expression levels between the different types of cells, which cannot be assessed by flow cytometry because this methodology only reflects the percentage of cells expressing antigens without quantifying the intensity of signal. The panel of surface antigens tested revealed a similar phenotypic profile between horse and human MSCs, although specific differences in some surface antigens were noticed. A similar cell surface profile was also observed between BM-MSCs and AT-MSCs, with CD34 emerging as a key molecule to differentiate cells derived from bone marrow and adipose tissue. The present study could help researchers identify these cells more quickly before using them for cellular-based therapies in equine medicine. However, many questions still remain, and further investigation will be necessary to clarify the mechanisms and functions of stem cell epitopes, such as the effect of marker expression variation on the pluripotency of MSCs or the study of their expression by cells from different passages.

Acknowledgements

This work was performed as part of the AGL2008-02428/GAN (MICINN/FEDER) and PAMER Pipamer 09/019 projects and was partially financed by the Gobierno de Aragón (Grupo de Excelencia LAGENBIO) and Instituto Aragonés de Ciencias de la Salud (I+CS). B. Ranera was supported by a doctoral grant from the DGA.

References

Arnhold, S.J., Goletz, I., Klein, H., Stumpf, G., Beluche, L.A., Rohde, C., Addicks, K., Litzke, L.F., 2007. Isolation and characterization of bone marrow-derived equine mesenchymal stem cells. Am. J. Vet. Res. 68, 1095–1105.

Cellular, Tissue and Gene Therapies Advisory Committee, 2005. Cellular products for joint surface repair In FDA Center for Biologics Evaluation and Research. March 3–4.

Colleoni, S., Bottani, E., Tessaro, I., Mari, G., Merlo, B., Romagnoli, N., Spadari, A., Galli, C., Lazzari, G., 2009. Isolation, growth and differentiation of equine mesenchymal stem cells: effect of donor, source, amount of tissue and supplementation with basic fibroblast growth factor. Vet. Res. Commun. 33, 811–821.

Crovace, A., Lacitignola, L., De Siena, R., Rossi, G., Francioso, E., 2007. Cell therapy for tendon repair in horses: an experimental study. Vet. Res. Commun. 31 (Suppl 1), 281–283.

de Mattos Carvalho, A., Alves, A.L., Golim, M.A., Moroz, A., Hussni, C.A., de Oliveira, P.G., Deffune, E., 2009. Isolation and immunophenotypic characterization of mesenchymal stem cells derived from equine species adipose tissue. Vet. Immunol. Immunopathol. 132, 303–306.

De Ugarte, D.A., Alfonso, Z., Zuk, P.A., Elbarbary, A., Zhu, M., Ashjian, P., Benhaim, P., Hedrick, M.H., Fraser, J.K., 2003. Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. Immunol. Lett. 89, 267–270.

Deans, R.J., Moseley, A.B., 2000. Mesenchymal stem cells: biology and potential clinical uses. Exp. Hematol. 28, 875–884.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., Horwitz, E., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. Cytotherapy 8, 315–317.

Gangenahalli, G.U., Singh, V.K., Verma, Y.K., Gupta, P., Sharma, R.K., Chandra, R., Luthra, P.M., 2006. Hematopoietic stem cell antigen CD34: role in adhesion or homing. Stem Cells. Dev. 15, 305–313.
Goodrich, L.R., Hidaka, C., Robbins, P.D., Evans, C.H., Nixon, A.J., 2007. Genetic modification of chondrocytes with insulin-like growth factor-1 enhances cartilage healing in an equine model. J. Bone Joint Surg. Br. 89, 672–685.
Hoynowski, S.M., Fry, M.M., Gardner, B.M., Leming, M.T., Tucker, J.R., Black, L., Sand, T., Mitchell, K.E., 2007. Characterization and differentiation of equine umbilical cord-derived matrix cells. Biochem. Biophys. Res. Commun. 362, 347–353.
Ibrahim, S., Steinbach, F., 2007. Non-HLDA8 animal homologue section anti-leukocyte mAbs tested for reactivity with equine leukocytes. Vet. Immunol. Immunopathol. 119, 81–91.
Im, G.I., Shin, Y.W., Lee, K.B., 2005. Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? Osteoarthritis Cartilage 13, 845–853.
Kern, S., Eichler, H., Stoeve, J., Kluter, H., Bieback, K., 2006. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 24, 1294–1301.
Koch, T.G., Berg, L.C., Betts, D.H., 2008. Concepts for the clinical use of stem cells in equine medicine. Can. Vet. J. 49, 1009–1017.
Koch, T.G., Berg, L.C., Betts, D.H., 2009. Current and future regenerative medicine – principles, concepts, and therapeutic use of stem cell therapy and tissue engineering in equine medicine. Can. Vet. J. 50, 155–165.
Koerner, J., Nesic, D., Romero, J.D., Brehm, W., Mainil-Varlet, P., Grogan, S.P., 2006. Equine peripheral blood-derived progenitors in comparison to bone marrow-derived mesenchymal stem cells. Stem Cells 24, 1613–1619.
Kolm, G., Klein, D., Knapp, E., Watanabe, K., Walter, I., 2006. Lactoferrin expression in the horse endometrium: relevance in persisting mating-induced endometritis. Vet. Immunol. Immunopathol. 114, 159–167.
Liu, F., Akiyama, Y., Tai, S., Maruyama, K., Kawaguchi, Y., Muramatsu, K., Yamaguchi, K., 2008. Changes in the expression of CD106, osteogenic genes, and transcription factors involved in the osteogenic differentiation of human bone marrow mesenchymal stem cells. J. Bone Miner. Metab. 26, 312–320.
Mambelli, L.I., Santos, E.J., Frazao, P.J., Chaparro, M.B., Kerkis, A., Zoppa, A.L., Kerkis, I., 2009. Characterization of equine adipose tissue-derived progenitor cells before and after cryopreservation. Tissue Eng. Part C Methods 15, 87–94.
Martinez-Lorenzo, M.J., Royo-Canas, M., Alegre-Aguaron, E., Desportes, P., Castiella, T., Garcia-Alvarez, F., Larrad, L., 2009. Phenotype and chondrogenic differentiation of mesenchymal cells from adipose tissue of different species. J. Orthop. Res. 27, 1499–1507.
Mitchell, J.B., McIntosh, K., Zvonic, S., Garrett, S., Floyd, Z.E., Kloster, A., Di Halvorsen, Y., Storms, R.W., Goh, B., Kilroy, G., Wu, X., Gimble, J.M., 2006. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells 24, 376–385.
Noel, D., Caton, D., Roche, S., Bony, C., Lehmann, S., Casteilla, L., Jorgensen, C., Cousin, B., 2008. Cell specific differences between human adipose-derived and mesenchymal–stromal cells despite similar differentiation potentials. Exp. Cell Res. 314, 1575–1584.
Panchision, D.M., Chen, H.L., Pistollato, F., Papini, D., Ni, H.T., Hawley, T.S., 2007. Optimized flow cytometric analysis of central nervous system tissue reveals novel functional relationships among cells expressing CD133, CD15, and CD24. Stem Cells 25, 1560–1570.
Paris, D.B., Stout, T.A., 2010. Equine embryos and embryonic stem cells: defining reliable markers of pluripotency. Theriogenology 74, 516–524.
Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., Marshak, D.R., 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284, 143–147.
Puissant, B., Barreau, C., Bourin, P., Clavel, C., Corre, J., Bousquet, C., Taureau, C., Cousin, B., Abbal, M., Laharrague, P., Penicaud, L., Casteilla, L., Blancher, A., 2005. Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. Br. J. Haematol. 129, 118–129.
Radcliffe, C.H., Flaminio, M.J., Fortier, L.A., 2010. Temporal analysis of equine bone marrow aspirate during establishment of putative mesenchymal progenitor cell populations. Stem Cells Dev. 19, 269–282.
Rallapalli, S., Bishi, D.K., Verma, R.S., Cherian, K.M., Guhathakurta, S., 2009. A multiplex PCR technique to characterize human bone marrow derived mesenchymal stem cells. Biotechnol. Lett. 31, 1843–1850.
Richardson, L.E., Dudhia, J., Clegg, P.D., Smith, R., 2007. Stem cells in veterinary medicine – attempts at regenerating equine tendon after injury. Trends Biotechnol. 25, 409–416.
Smith, R.K., Korda, M., Blunn, G.W., Goodship, A.E., 2003. Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment. Equine Vet. J. 35, 99–102.
Smith, R.K., 2008. Mesenchymal stem cell therapy for equine tendinopathy. Disabil. Rehabil. 30, 1752–1758.
Tarnok, A., Ulrich, H., Bocsi, J., 2010. Phenotypes of stem cells from diverse origin. Cytometry A 77, 6–10.
Taylor, S.E., Smith, R.K., Clegg, P.D., 2007. Mesenchymal stem cell therapy in equine musculoskeletal disease: scientific fact or clinical fiction? Equine Vet. J. 39, 172–180.
Tuan, R.S., Boland, G., Tuli, R., 2003. Adult mesenchymal stem cells and cell-based tissue engineering. Arthritis Res. Ther. 5, 32–45.
Vidal, M.A., Kilroy, G.E., Johnson, J.R., Lopez, M.J., Moore, R.M., Gimble, J.M., 2006. Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. Vet. Surg. 35, 601–610.
Vidal, M.A., Kilroy, G.E., Lopez, M.J., Johnson, J.R., Moore, R.M., Gimble, J.M., 2007. Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells. Vet. Surg. 36, 613–622.
Vidal, M.A., Robinson, S.O., Lopez, M.J., Paulsen, D.B., Borkhsenious, O., Johnson, J.R., Moore, R.M., Gimble, J.M., 2008. Comparison of chondrogenic potential in equine mesenchymal stromal cells derived from adipose tissue and bone marrow. Vet. Surg. 37, 713–724.
Violini, S., Ramelli, P., Pisani, L.F., Gorni, C., Mariani, P., 2009. Horse bone marrow mesenchymal stem cells express embryo stem cell markers and show the ability for tenogenic differentiation by in vitro exposure to BMP-12. BMC Cell Biol. 10, 29.
Zhang, L., Chan, C., 2010. Isolation and enrichment of rat mesenchymal stem cells (MSCs) and separation of single-colony derived MSCs. J. Vis. Exp. 22, 1852.
Zimmerlin, L., Donnenberg, V.S., Pfeifer, M.E., Meyer, E.M., Peault, B., Rubin, J.P., Donnenberg, A.D., 2010. Stromal vascular progenitors in adult human adipose tissue. Cytometry A 77, 22–30.

5.4. MANUSCRITO IV

Expansion under hypoxic conditions enhances the chondrogenic potential of equine bone marrow-derived mesenchymal stem cells.

Beatriz Ranera, Ana Rosa Remacha, Samuel Álvarez-Arguedas, Tomás Castiella, Francisco José Vázquez, Antonio Romero, Pilar Zaragoza, Inmaculada Martín-Burriel, Clementina Rodellar.

Abstract

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are widely used in regenerative medicine in horses. Most of the molecular characterisations of BM-MSCs have been made at 20% O_2, higher oxygen level than surrounds the cells inside the bone marrow. The present work compares the lifespan and the tri-lineage potential of equine BM-MSCs expanded in normoxia (20% O_2) and hypoxia (5% O_2). No significant differences were found in long-term cultures, in osteogenesis and in adipogenesis between normoxic and hypoxic expanded BM-MSCs. An up-regulation of the chondrogenesis-related genes (*COL2A1*, *AGN*, *LUM*, *BGL* and *COMP*) and an increase of the extracellular sGAG content (sulphated glycosaminoglycans) were found in cells that were expanded under hypoxia. These results suggest that the expansion of BM-MSCs in hypoxic conditions enhances chondrogenesis in equine BM-MSCs.

Keywords: Equine; MSCs; Bone marrow; Hypoxia; Chondrogenesis.

Physiological oxygen concentrations surrounding cells are much lower than the oxygen concentrations used in culture. Traditional incubators are supplied by room air that has an oxygen content of approximately 20% (referred to as “normoxia” according to the conventional terminology). This is in contrast with the oxygen tension inside the MSC niches bone marrow (BM) which ranges between 1% and 7%, (Mohyeldin et al., 2010).

Hypoxia is important during the formation of cartilage during foetal development. In vitro MSC chondrogenesis is controversial; in some studies it is enhanced (Zscharnack et al., 2009) and in other studies the opposite effect is described (D'Ippolito et al., 2006).

The use of MSCs as a therapeutic tool in the treatment of musculoskeletal defects in horses has increased during the last few years (Frisbie and Smith, 2010). **Most in vitro studies** of equine MSCs conducted recently (Ranera et al., 2011b) have been carried out in traditional incubators in an attempt to know as much as possible of MSC characteristics for use them in cell therapy. However, how low oxygen tension affects the expansion of equine MSCs is still unknown.

The aim of this work was to analyse the effect of oxygen tension on the proliferation and differentiation potentials of equine MSCs derived from bone marrow (BM-MSCs), comparing cells expanded under hypoxic and normoxic conditions.

Bone marrow aspirates were obtained from a total of 5 castrated male horses. All procedures were carried out under Project Licence PI36/07 approved by the Ethic Committee for Animal Experiments from the University of Zaragoza. The care and use of animals was performed in accordance with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

Samples were collected and MSC were isolated as previous described (Ranera et al., 2011a; Ranera et al., 2011b). Cultures were maintained at 37ºC in either normoxic (20% O_2) or hypoxic conditions (5% O_2) in growth media. Growth potential of equine BM-MSCs was determined for ten passages (Supplementary material). Equine BM-

MSCs were also expanded until passage two for both oxygen conditions. Then, the cells were cryopreserved for further analysis.

Osteogenic and adipogenic differentiation analyses were performed (Supplementary material). For chondrogenic differentiation, BM-MSCs were thawed and 500,000 BM-MSCs were pelleted and exposed to chondrogenic induction media (Ranera et al., 2011a; Ranera et al., 2011b) for 21 days at 20% O_2. After the induction period, the micromasses were sectioned and examined by alcian blue and safranin O staining. Immunohistochemistry for S-100 protein was performed using the polyclonal rabbit antibody IR504 (DAKO). On days 0, 7, 14 and 21 sulphated glycosaminoglycans (sGAG) and gene expression of chondrogenesis-related and HIF-1α genes quantifications were assessed. The RNA spin mini (GE Healthcare) and the Superscript kit (Invitrogen) were used for total RNA isolation and reverse transcription, following manufacturer's instructions. Table 1 shows the names of the genes analysed by real-time quantitative PCR (RT-qPCR). The levels of gene expression were determined as previously described (Ranera et al., 2011a; Ranera et al., 2012). Statistics were assessed using PSAW Statistics 18 software. Differences in quantitative analyses between BM-MSCs expanded in normoxic and hypoxic conditions were evaluated with the Student's *t* test, ANOVA test was carried out to analyse the effect of time along the course of differentiations. Statistical significance was defined as $P < 0.05$.

Lifespan, osteogenic and adipogenic potentials of BM-MSCs were not affected by hypoxia. No significant differences were detected between normoxic and hypoxia cells in these assays (Supplementary material).

In contrast, chondrogenesis seemed to be enhanced by hypoxia. Fig. 1 shows the fold change of the chondrogenic quantifications with respect to the undifferentiated cells from day 0. The production of sGAG by hypoxic BM-MSCs during differentiation was greater than that of normoxic BM-MSCs (Fig. 1a). This increase was significant on the last day of culture with respect to day 0. The fold change of expression of *COL2A1* and *ACAN* on day 21 with respect to day 0 of differentiation was higher in BM-MSCs expanded under normoxic condition (Fig. 1b-c), although significant differences between the expression levels on these days were only detected in hypoxic BM-MSCs. This contradiction was due to the expression of these genes was three times more up-

regulated on day 0 in BM-MSCs expanded under low oxygen condition (Supplementary Table 1). This fact might be the result of an enhancement of *SOX9* in hypoxic cultures (Robins et al., 2005) which is a trigger of the chondrogenic genes *COL2A1* and *AGN* (Barry et al., 2001).

The mRNA expression levels of the *LUM*, *BGN* and *COMP* genes were higher in MSCs from cultures expanded at 5% O_2 than those that were expanded at 20% O_2 over the course of differentiation. But only significant differences were detected between pellets from hypoxic and normoxic BM-MSCs on day 14 for *LUM* and on day 21 for *COMP*. Significant expression differences of these markers were observed for hypoxic expanded BM-MSCs on day 21 relative to days 0 and 7 for *LUM* and relative to days 0, 7 and 14 for *COMP*, suggesting that the differentiation of equine BM-MSCs into chondrocytes is favoured in a low-oxygen environment. These results agree with other reports in bovine, human and murine species (Markway et al., 2010; Robins et al., 2005; Zscharnack et al., 2009) which describe an enhancement of chondrogenesis in MSCs exposed to hypoxia.

The gene expression of *HIF-1α* increased gradually during chondrogenesis in BM-MSCs expanded in hypoxia, significant expression differences were observed on day 21 relative to days 0, 7 and 21 (Fig. 1g). The up-regulation of *HIF-1α* in normoxic BM-MSCs was not as marked as in hypoxic BM-MSCs over the chondrogenic induction. In addition, the difference on the *HIF-1α* expression was significantly higher in hypoxic cells than in normoxic cells on day 21 (Supplementary Table 1). The up-regulation of chondrogenic-related genes and *HIF-1α* during chondrogenic induction in hypoxic BM-MSCs might indicate *HIF-1α* is an essential factor to achieve chondrogenesis in MSCs. Taken together these results, *HIF-1α* would play a role in extracellular matrix formation regulating up-stream the expression of the chondrogenic-related genes. This result agrees with cartilage formation models that point out *HIF-1α* as a key factor in cartilage development and differentiation (Schipani, 2005).

Chondrogenesis was confirmed by the glucosaminoglycan-specific stainings with alcian blue and safranin O (Figure 2a-b and 2c-d) that showed a greater accumulation of these molecules in the extracellular matrix in BM-MSCs expanded

under hypoxic conditions. Additionally, more chondrocyte differentiated cells were detected in hypoxic pellets by S-100 protein immunostaining (Figure 2e-f).

Microenvironment has been also proved as decisive in the chondrogenic differentiation of equine MSCs (Lettry et al., 2010). The co-culture of equine MSCs and mature articular chondrocytes led to the up-regulation of the chondrogenesis-related genes, showing an enhancement of chondrogenesis.

In horses, MSCs have been used to treat musculoskeletal injuries (Frisbie and Smith, 2010); however, these cells had been exposed to 20% O_2 during the expansion, which is not their physiological oxygen condition. Pre-culturing cells in an environment similar to the stem cell niche prior to transplant enhances the therapeutic potential of the cells due to low oxygen tension, which preserves the stemness of the cells and increases cell engraftment and motility (Rosova et al., 2008).

Our results indicate that hypoxic culture conditions enhance equine BM-MSC chondrogenesis, while osteogenesis, adipogenesis and proliferation are not affected. Although further studies are necessary to determine the effect of hypoxia in MSC cultures in depth and how it might affect in an in vivo treatment, the expansion of BM-MSCs under hypoxia seems to be more accurate culture condition than the common normoxic expansion prior to clinical applications.

Conflict of interest statement

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

Acknowledgements

This work was performed as part of the AGL2008-02428/GAN (MICINN/FEDER) project and was partially financed by the Gobierno de Aragón (Grupo de Excelencia LAGENBIO) and InstitutoAragonés de Ciencias de la Salud (I+CS). B. Ranera was supported by a doctoral grant from DGA. We also want to apologize for those authors whose relevant work was not cited due to size restrictions

References

Barry, F., Boynton, R.E., Liu, B., Murphy, J.M., 2001. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res 268, 189-200.

D'Ippolito, G., Diabira, S., Howard, G.A., Roos, B.A., Schiller, P.C., 2006. Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells. Bone 39, 513-522.

Frisbie, D.D., Smith, R.K., 2010. Clinical update on the use of mesenchymal stem cells in equine orthopaedics. Equine Vet J 42, 86-89.

Lettry, V., Hosoya, K., Takagi, S., Okumura, M., 2010. Coculture of equine mesenchymal stem cells and mature equine articular chondrocytes results in improved chondrogenic differentiation of the stem cells. Jpn J Vet Res 58, 5-15.

Markway, B.D., Tan, G.K., Brooke, G., Hudson, J.E., Cooper-White, J.J., Doran, M.R., 2010. Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures. Cell Transplant 19, 29-42.

Mohyeldin, A., Garzon-Muvdi, T., Quinones-Hinojosa, A., 2010. Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell 7, 150-161.

Ranera, B., Lyahyai, J., Romero, A., Vazquez, F.J., Remacha, A.R., Bernal, M.L., Zaragoza, P., Rodellar, C., Martin-Burriel, I., 2011a. Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue. Vet Immunol Immunopathol.

Ranera, B., Ordovas, L., Lyahyai, J., Bernal, M.L., Fernandes, F., Remacha, A.R., Romero, A., Vazquez, F.J., Osta, R., Cons, C., Varona, L., Zaragoza, P., Martin-Burriel, I., Rodellar, C., 2011b. Comparative study of equine bone marrow and adipose tissue-derived mesenchymal stromal cells. Equine Vet J.

Ranera, B., Ordovas, L., Lyahyai, J., Bernal, M.L., Fernandes, F., Remacha, A.R., Romero, A., Vazquez, F.J., Osta, R., Cons, C., Varona, L., Zaragoza, P., Martin-Burriel, I., Rodellar, C., 2012. Comparative study of equine bone marrow and adipose tissue-derived mesenchymal stromal cells. Equine Vet J.

Robins, J.C., Akeno, N., Mukherjee, A., Dalal, R.R., Aronow, B.J., Koopman, P., Clemens, T.L., 2005. Hypoxia induces chondrocyte-specific gene expression in mesenchymal cells in association with transcriptional activation of Sox9. Bone 37, 313-322.

Rosova, I., Dao, M., Capoccia, B., Link, D., Nolta, J.A., 2008. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem Cells 26, 2173-2182.

Schipani, E., 2005. Hypoxia and HIF-1 alpha in chondrogenesis. Semin Cell Dev Biol 16, 539-546.

Zscharnack, M., Poesel, C., Galle, J., Bader, A., 2009. Low oxygen expansion improves subsequent chondrogenesis of ovine bone-marrow-derived mesenchymal stem cells in collagen type I hydrogel. Cells Tissues Organs 190, 81-93.

SUPPLEMENTARY MATERIAL

Material and methods

Growth potential.

After the first treatment with trypsin, BM-MSCs cultured in each oxygen condition were seeded in triplicate in 6-well plates at a density of 5,000 cells/cm^2. This was considered passage one. The cells were maintained in culture for ten passages (approximately one hundred days) and were trypsinised at 90% confluence. At every passage, the BM-MSCs were counted in a haemocytometer Z2 Coulter particle count and size analyser, and reseeded at the initial density. The cells were fed every 3 days.

Population doubling was calculated every passage according to the formula:

$PD = \ln (N_f/N_i) / \ln 2$,

Where N_f is the final number of cells; N_i is the initial number of cells; and PD is the population doubling number.

Osteogenic differentiation.

Osteogenic differentiation was set as previous described. The differentiation induction was maintained for 9 days at 20% O_2 for cells expanded in both conditions. After that period, calcium deposits formed were stained by alizarin red 2%.

To quantify the osteogenic potential of BM-MSCs, the activity of alkaline phosphatase (ALP) was evaluated on days 0, 3, 6 and 9 as it was previous described .

Adipogenic differentiation.

Adipogenic differentiation was set as in previous works . Induction was maintained for 14 days at 20% O_2 for cells expanded in both conditions. To determine the adipogenic differentiation, lipid droplets formed inside the cells were stained using oil red O 0.3% staining.

To quantify the accumulation of lipids, the oil red O was extracted from differentiated BM-MSCs with isopropanol after every staining, and the absorbance was measured at 510 nm. Then, proteins from fixed cells were stained with Ponceau S dye

(Fluka) for 10 minutes to normalise oil red O measures. Ponceau was extracted from cells using PBS, and the absorbance was measured at 515 nm.

Results

Cell growth.

The cells were passaged ten times, which corresponds with nearly 100 days of culture. During that period, no significant difference was observed in the cell doubling between the BM-MSCs exposed to 5% or 20% O_2 (Supplementary Fig. 1). Both cell types showed a similar lifespan. At approximately day 80 of culture, the cells started to slow down the proliferation, reaching the plateau of the growth curve. The proliferative ability was similar at passage ten; normoxic and hypoxic cells displayed a cumulative population doubling of 31.28 ± 6.28 and 31.68 ± 5.10, respectively.

Osteogenic potential.

To examine the effect of low oxygen tension in osteogenic differentiation of MSCs, alkaline phosphatase activity (ALP Activity) and mRNA expression of runt-related transcription factor 2 (*RUNX2*) and alkaline phosphatase (*ALP*) were quantified on days 0, 3, 6 and 9 in the BM-MSCs cultured in induction and control media. Supplementary Fig. 2a-c shows the fold change of the osteogenic quantifications of both experimental groups relative to their respective controls.

The ALP activity was significantly higher in all time points relative to day 0 for both conditions ($P<0.05$). Significant differences ($P<0.05$) were found between normoxic BM-MSCs and their controls on days 3, 6, and 9 ($P<0.01$), whereas with hypoxic BM-MSCs, differences were only detected on day 9 ($P<0.05$).

The mRNA expression of *ALP* increased during the induction period for both conditions, being statistically significant ($P<0.05$) for cells expanded under normoxic conditions due to the differences found between the *ALP* expression on day 9 and the other three time points analysed. The expression of *RUNX2* in the cells expanded under both conditions was significantly different during the time of differentiation ($P<0.05$) because of the differences found between the last day of culture and day 0. The *ALP* expression levels in normoxic and hypoxic cells were significantly higher in induced cells on days 6 ($P<0.05$ and $P<0.01$, respectively) and 9 ($P<0.01$ and $P<0.05$,

respectively) than in the respective controls. Significant differences in the expression of *RUNX2* were also found in differentiated cells compared to non-induced cells on days 6 and 9 in normoxic BM-MSCs and on day 9 ($P<0.05$) in hypoxic BM-MSCs. The cells from the two experimental conditions did not display significant differences either in ALP activity or in the mRNA expression of the genes analysed.

In addition to the modifications of gene expression of osteogenesis-related genes, alizarin red staining confirmed the ability of the normoxic and hypoxic BM-MSCs to differentiate into osteogenic lineages (Supplementary Fig. 2d-e).

Adipogenic differentiation.

To assess adipogenic differentiation in the cells expanded in 20% O_2 and 5% O_2, oil red O staining and quantification of mRNA expression levels of lipoprotein lipase (*LPL*) and peroxisome proliferator-activated receptor γ (*PPARγ*) were carried out. (Supplementary Fig.3a-c) show the fold change of these quantifications of differentiated cells relative to control cells for both experimental groups. The amount of intracellular lipids increased during the induction period until day 9 in both cell types. Significant differences were found in oil red O quantification in hypoxic and normoxic MSCs in respect to their controls on days 5 and 9 ($P<0.05$) and day 9 ($P<0.01$), respectively. This increase was also significant at day 9 in hypoxic BM-MSCs with respect to day 0 ($P<0.05$). The foldchange of *LPL* and *PPARγ* mRNA expression in adipogenic cultures with respect to controls was statistically significant in normoxic expanded BM-MSCs on day 14 ($P<0.01$ and $P<0.001$, respectively). Hypoxic BM-MSCs did not display significant differences from controls, although the levels of expression of *LPL* and *PPARγ* were higher over the course of induction, except for the last day of culture. Significant differences were not found between cells expanded in the two oxygen conditions for any quantification.

Oil red O stained the lipid droplets formed during the induction, supporting the quantification data obtained (Supplementary Fig. 3d-e).

TABLES

Table 1

Chondrogenesis-related genes analysed by RT-qPCR. GenBank accession numbers of the sequences used for primers design. Primers (F: forward and R: reverse) and size of the amplicon (bp).

Genes	Accesion number	Primer sequence	Amplicon size
COL2A1	AF034691	F: CAGACGGGTGAACCTGGTAT R: TCTCCACGAGCACCTCTTTT	130
ACAN	AF019756	F: CTACGACGCCATCTGCTACA R: ACCGTCTGGATGGTGATGTC	96
BGN	AF035934	F: AAGGCCTCCAGCATCTCTATG R: GGAGATGTAGAGCTTCTGCAGC	107
LUM	AB292110	F: CTTGTCCATAGTGCATCTGCTTTAAG R: GAAAGTAAACGCACCTGGATTCA	100
COMP	AF325902	F: GGCGACGCGCAAATAGA R: GCCATTGAAGGCCGTGTAA	111
GAPDH [a]	NM_001163856	F:GGCAAGTTCCATGGCACAGT R:CACAACATATTCAGCACCAGCAT	128
B-2M [a]	NM_001082502	F:TCGTCCTGCTCGGGCTACT R:ATTCTCTGCTGGGTGACGTGA	102

[a] previously described in (Ranera et al. 2011a, Ranera et al. 2011b).

Supplementary Table 1

Fold change of sGAG content and mRNA expression of chondrogenic markers of BM-MSCs expanded under hypoxic condition with respect to BM-MSCs expanded under normoxic condition over the course of chondrogenic induction.

	Day 0	Day 7	Day 14	Day 21
sGAG	0.88697751	1.40130049	1.91805101	3.04567293
COL2A1	3.26061676	7.57473234	1.09065902	1.86996292
ACAN	3.45142561	2.16313025	19.6669121	2.41817827
LUM	0.97115285	0.78220806	5.52281672	2.33184727
BGN	0.41454571	1.09046634	4.19227171	1.20176531
COMP	0.96775935	2.16313025	19.6669121	2.41817827
HIF-1α	1.76238176	1.59523717	3.40116404	8.51620515

Figures legends

Fig. 1. Mean ± s.e of normalised data with undifferentiated cells (day 0) of BM-MSCs (n=4). White bars represent cells expanded at 20% O_2, and grey bars represent cells expanded at 5% O_2. a) sGAG formed over the course the induction in the extracellular matrix of the pellets. b-f) mRNA expression of chondrogenesis-related genes at days 0, 7, 14 and 21 of differentiation. g) mRNA expression of HIF-1α at days 0, 7, 14 and 21 of differentiation. * $P < 0.05$; # $P < 0.05$, ## $P < 0.01$ between normoxic and hypoxic BM-MSCs.

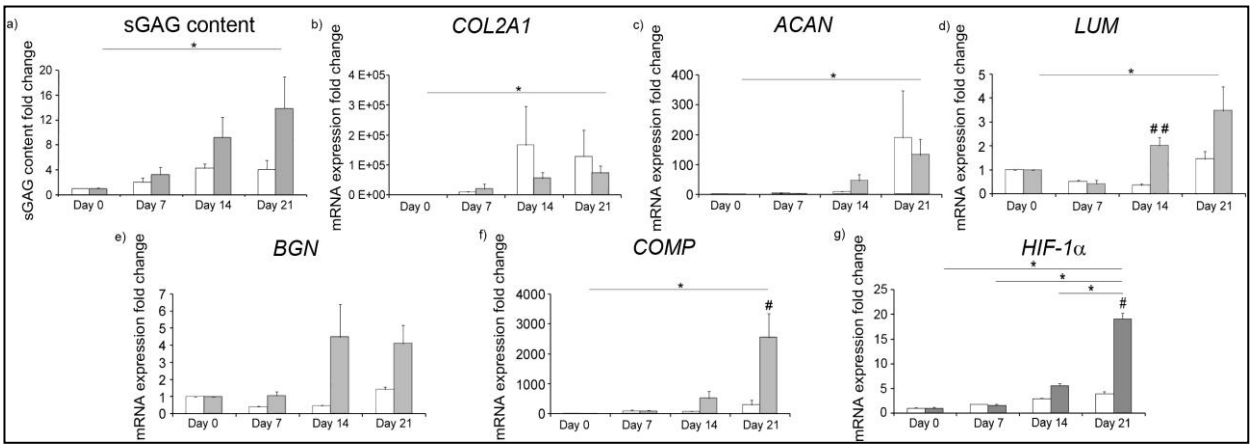
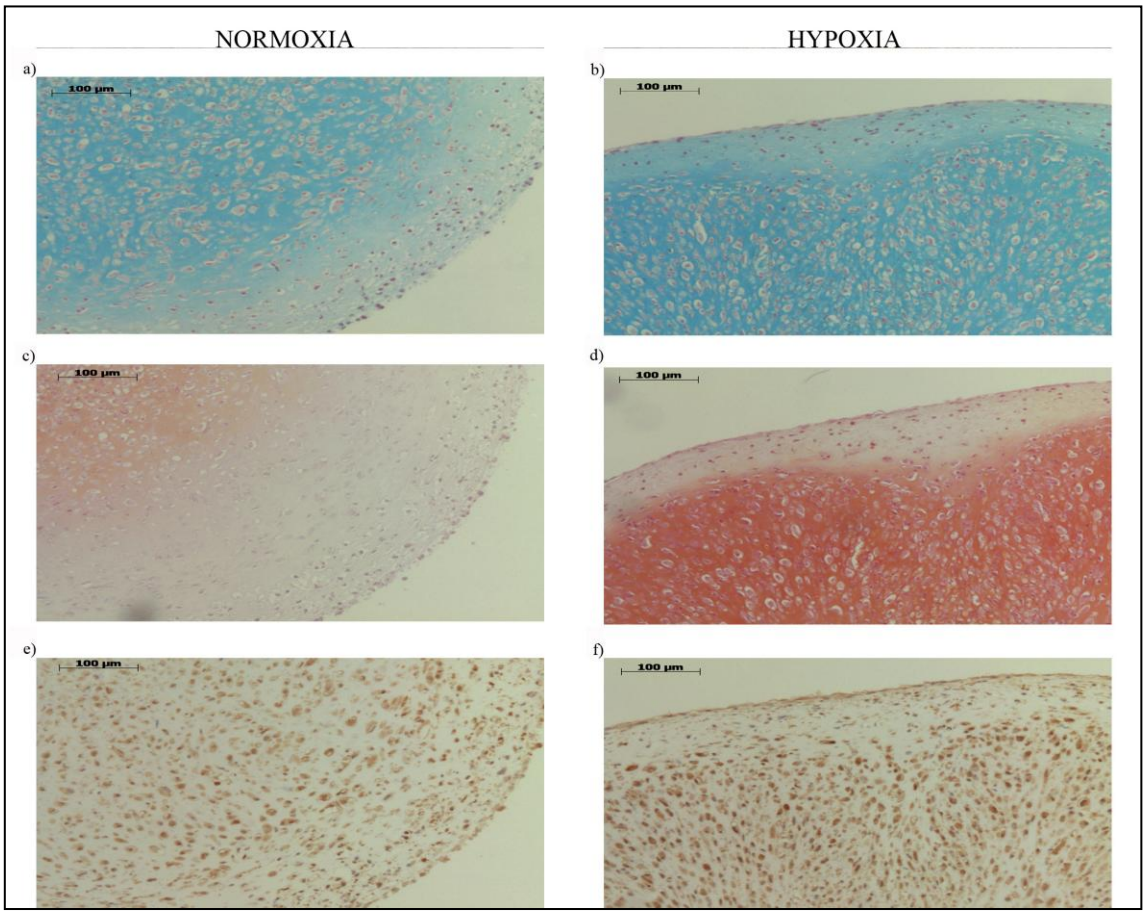
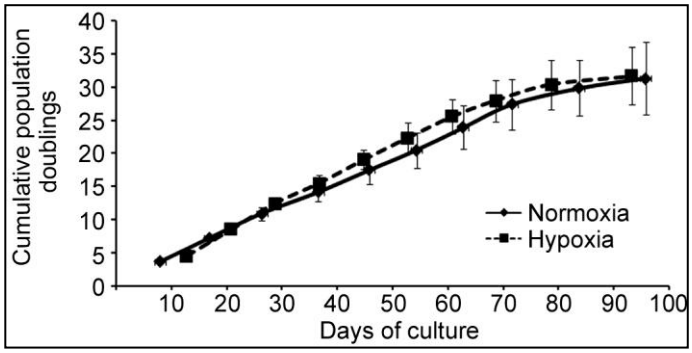


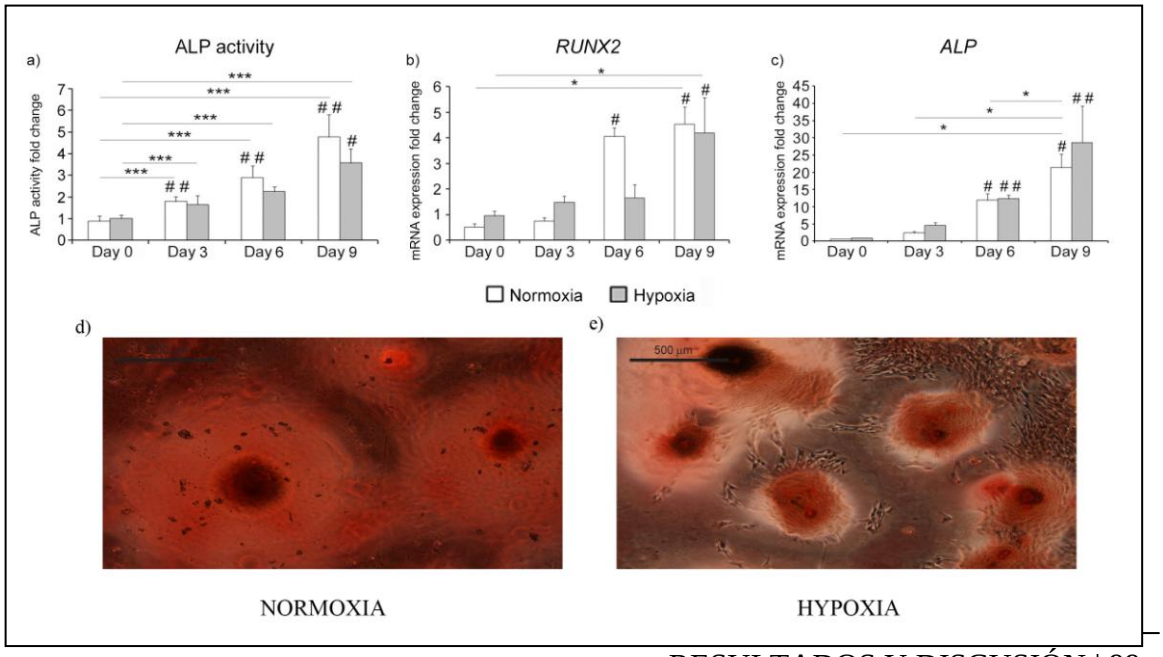
Fig. 2. Representative staining for chondrogenic differentiation in normoxic BM-MSCs (on the left) and hypoxic BM-MSCs (on the right). a) and b) extracellular glucosaminoglycans stained by alcian blue staining of sections on day 21 of chondrogenesis (magnification 5x). c) and d) extracellular glucosaminoglycans stained by safranin O staining of chondrogenic sections on day 21 (magnification 5x). e) and f) Immunohistochemistry of S-100 protein on differentiated chondrocytes on day 21.



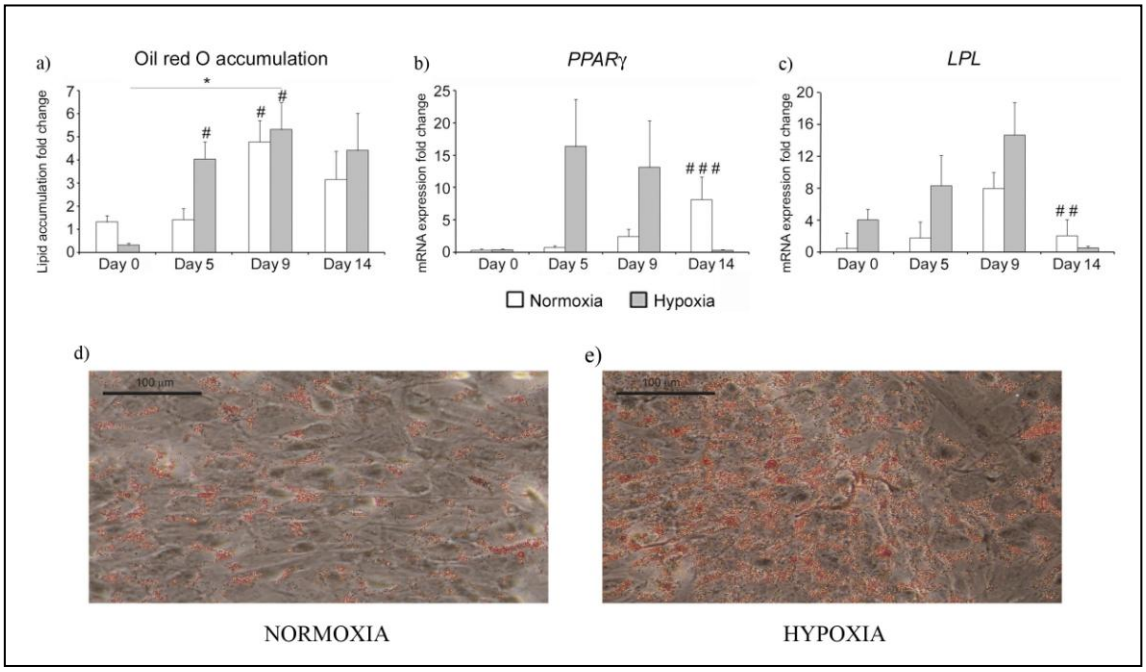
Supplementary Fig. 1. Mean ± s.e. of cumulative population doublings (CPD) of BM-MSCs (n=5) expanded for nearly 100 days under 20% O_2 (solid line) and 5% O_2 (striped line). From day 80 of culture, the growth of both cell types slowed down. The CPDs (cumulative population doubling) at the end of the culture were 31.28 ± 6.28 and 31.68 ± 5.10 for normoxic and hypoxic cells, respectively.



Supplementary Fig. 2. Mean ± s.e. fold change in mRNA expression of osteogenic differentiated BM-MSCs (n= 4) with respect to their controls for specific differentiation markers and representative alizarin red staining. White bars represent cells expanded at 20% O_2, and grey bars represent cells expanded at 5% O_2. Time course variations of alkaline phosphatase activity (a), *RUNX2* (b) and *ALP* (c) gene expression. d) and e) Calcium deposits formed at day 9 of osteogenic induction (magnification 4x) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ differentiated cells relative to the controls cells.



Supplementary Fig. 3. Mean ± s.e. fold change in mRNA expression of adipogenic differentiated BM-MSCs (n= 4) with respect to their controls for specific differentiation markers and representative oil red O staining. White bars represent cells expanded at 20% O_2, and grey bars represent cells expanded at 5% O_2. Time course variations of oil red O accumulation (a), *PPARγ* (b) and *LPL* (c) gene expression. d) and e) Lipid droplet accumulation inside the cells at day 9 of adipogenic differentiation (magnification 20x). * $P < 0.05$; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ differentiated cells relative to the controls cells.



5.5. MANUSCRITO V

BMC Veterinary Research

Effect of hypoxia on equine mesenchymal stem cells derived from bone marrow and adipose tissue.

Beatriz Ranera, Ana Rosa Remacha, Samuel Álvarez-Arguedas, Antonio Romero, Francisco José Vázquez, Pilar Zaragoza, Inmaculada Martín-Burriel, Clementina Rodellar.

Abstract

Background. Mesenchymal stem cells (MSCs) derived from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) are being applied to equine cell therapy. The physiological environment in which MSCs reside is hypoxic and does not resemble the oxygen level typically used in *in vitro* culture (20% O_2). This work compares the growth kinetics, viability, cell cycle, phenotype and expression of pluripotency markers in both equine BM-MSCs and AT-MSCs at 5% and 20% O_2.

Results. At the conclusion of culture, fewer BM-MSCs were obtained in hypoxia than in normoxiaas a result of significantly reduced cell division. Hypoxic AT-MSCs proliferated less than normoxic AT-MSCs because ofa significantly higher presence of non-viable cells during culture. Flow cytometry analysis revealed that the immunophenotypeof both MSCs was maintained in both oxygen conditions. Gene expression analysis using RT-qPCRshowed that statistically significant differences were only found for *CD49d* in BM-MSCs and *CD44* in AT-MSCs. Similar gene expression patterns were observed at both 5% and 20% O_2 for the remaining surface markers. Equine MSCs expressed the embryonic markers *NANOG*, *OCT4* and *SOX2* in both oxygen conditions. Additionally, hypoxic cells tended to display higher expression, which might indicate that hypoxia retains equine MSCs in an undifferentiated state.

Conclusions.Hypoxia attenuates the proliferative capacity of equine MSCs, but does not affect the phenotype and seems to keep them more undifferentiated than normoxic MSCs.

Keywords: hypoxia, horse, AT-MSC, BM-MSC, characterisation.

Background

In recent years, mesenchymal stem cells (MSCs) have become increasingly utilised in regenerative medicine and tissue engineering applications because of their properties for self-renewal, differentiation and immunoregulation [1].

To study these properties, MSCs must be isolated from their physiological niches and cultured *ex vivo*. The micro-environment that cells experience in laboratory culture is very different from their native settings; therefore, it is possible that the true *in vivo* properties of these cells might be modified by artificial culture. One environmental property that is commonly altered by the change of environment is the percentage of oxygen. Traditional incubators are supplied with atmospheric air that contains 20% oxygen (defined as "normoxia"), which is a not physiologically accurate for any kind of cell. Two common MSC sources are bone marrow and adipose tissue, in which the oxygen tension ranges from 1%-7% [2] and 2%-8% [3], respectively.

All nucleated cells are able to sense and respond to the availability of O_2 [4]. Rat MSCs modify the expression of molecules involved in cell proliferation and survival when they are exposed to low oxygen tensions that approximate physiological conditions [5]. Hypoxia inducible factor 1α (HIF-1α) regulates the expression of many cell cycle molecules, including p21, anti-apoptotic factors, such as Bcl-2 [6], and pro-apoptotic proteins, such as p53 [7]. Consequently, rat MSCs exhibit different proliferation rates when cell expansion under hypoxia and normoxia are compared; however, some controversy exists regarding whether low oxygen tension enhances [8] or suppresses proliferation [9]. Finally, oxygen plays an important role in the differentiation [10] and maintenance of stemness in MSCs [11].

Due to the inability of tendons and articulations to heal properly, MSC-based therapies have been utilised in horses to treat orthopaedic disorders resulting from sporting endeavours [12, 13]. Oxygen levels in cartilage are among the lowest throughout the body [14], and hypoxia appears to be essential for tendon repair [15]. In addition, hypoxic preconditioning improves the therapeutic potential of human MSCs [16]. Taken together, these facts suggest that horse MSCs cultured in hypoxia might

constitute a more relevant model for the treatment of injuries in low-oxygen tissues than those currently utilised, which are usually cultured in 20% O_2.

To improve the methodology for equine stem cell therapy, it is necessary to examine the characteristics and to compare the behaviour of MSCs in normoxic and hypoxic conditions. Specifically, this study contrasts the proliferation kinetics, viability, cell cycle progression, phenotype and stemness of MSCs derived from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) cultured in 5% and 20% O_2.

Results

Proliferation kinetics

The growth kinetics of BM- and AT-MSCs expanded in normoxia and hypoxia were monitored for 7 days.

Cells derived from bone marrow showed a similar lag phase (Figure 1a) from day 1 to 3 in both hypoxic and normoxic conditions. The logarithmic phase began on day 3 for BM-MSCs under both oxygen conditions, but lasted until day 5 in hypoxia and day 6 in normoxia. On day 5, hypoxic BM-MSCs reached a plateau in their growth curve, while normoxic BM-MSCs continued in the log phase for one additional day. On day 6, the proliferation of normoxic BM-MSCs slowed. Consequently, a higher final number of BM-MSCs were present in 20% O_2 culture.

Similar lag phase behaviour was observed in AT-MSCs exposed to both oxygen atmospheres (Figure 1b); however, unlike BM-MSCs, this growth continued until day 4. After day 4, normoxic and hypoxic AT-MSCs began the logarithmic phase; nevertheless, normoxic AT-MSCs grew faster than hypoxic AT-MSCs, yielding significant differences in cell number on day 5. AT-MSCs exposed to 5% O_2 reached a plateau on day 6, while AT-MSCs in 20% O_2 culture continued in the log phase until the conclusion of the culture period, resulting in a significantly higher number of normoxic AT-MSCs on day 7.

Cell cycle

To examine potential cell cycle differences resulting from differing oxygen conditions, cellular DNA content was evaluated in both oxygen cultures for the same time periods used in the proliferation study.

Cell cycle data obtained for BM-MSC cultures showed that normoxic cells were more active than hypoxic cells between day 2 and the conclusion of the assay. Cultures that were exposed to 5% O_2 had higher proportions of cells in G_0/G_1 phase; these differences were statistically significant on days 2, 3 and 4 (Table 1a). In support of this finding, a significantly lower percentage of hypoxic BM-MSCs were observed in S phase on days 2 and 4 and in G_2/M phases on day 2. However, when hypoxic and normoxic AT-MSCs were compared, any significant differences were observed over the course of the culture period (Table 1b).

In comparing normoxic cultures of MSCs from both bone marrow and adipose tissue on days 2 and 3, BM-MSCs displayed a significantly higher percentage of cells in G_0/G_1 and reduced frequency of cells in S phase compared with AT-MSCs. The proportions of cells in each phase of the cell cycle were comparable throughout the remaining time course (Table 1c).

In contrast to normoxic MSCs, hypoxic cells displayed greater differences in cell cycle progression, as shown in Table 1d. On the first day of culture, there were significantly higher percentages of AT-MSCs in G_0/G_1 phase and of BM-MSCs in S phase. However, in the days following, up until day 5, this behaviour was inverted: significantly more BM-MSC were in G_0/G_1, whereas AT-MSCs were more active in cell division and more abundant in S phase on days 2, 4 and 5 and in G_2/M phase on days 2 and 3.

Cell viability

Oxygen tension can influence the viability of cell cultures. Possible changes in apoptosis and viability were monitored during the 7-day culture using Annexin V

(AnV) to detect the levels of phosphatidylserine in the outer membrane and propidium iodide (PI) to evaluate the integrity of cellular membranes.

With regard to viability, BM-MSCs showed similar proportions of apoptotic (AnV^+ PI^-) and non-viable cells (PI^+) during throughout the full 7 days of culture for cells expanded in either oxygen tension (Table 2a). Conversely, AT-MSCs exposed to both oxygen conditions contained the lowest percentage of viable cells on the first day of culture.

Viability increased thereafter and eventually reached the levels observed in BM-MSCs at day 5. Statistically significant differences in viability between hypoxic and normoxic AT-MSCs were detected on days 1, 2, 3 and 5 (Table 2b). These differences were due to a high frequency of PI^+ cells in hypoxic conditions, as the proportion of AnV^+ PI^- cells was similar for both conditions and never higher than 10% of the total population. Hypoxic AT-MSCs displayed a significantly higher abundance of PI^+ cells than normoxic AT-MSCs on days 3, 4, 5 and 7.

When normoxic MSCs cultures from bone marrow and adipose tissue were compared (Table 2c), the proportions of viable BM-MSCs on days 1, 2 and 3 were significantly higher than those of AT-MSCs. However, this trend reversed on day 4, and on day 5, the proportion of viable cells was significantly higher in AT-MSCs than in BM-MSCs. The differences in viability between both cell types observed at early stages of culture resulted from a significantly higher proportion of PI^+ AT-MSCs on days 1 and 2; in contrast, a significantly higher percentages of apoptotic cells were detected in BM-MSCs relative to AT-MSC cultures on days 4, 5 and 6.

In comparing expanded BM-MSCs and AT-MSCs in 5% O_2 culture, the behaviour displayed by normoxic equivalents was maintained for the first few days of culture (i.e., significantly higher percentages of PI^+ and lower percentages of AnV^-PI^- cells were detected in AT-MSC cultures). However, unlike normoxic cells, these results were consistent throughout the entire culture period and were significantly different between days 1 and 5. In addition, the proportion of AnV^+PI^- was similar for both cell types, which contrasts with the results of comparisons between normoxic MSCs (Table 2d).

Immunophenotype and gene expression patterns of surface markers

The immunophenotype for the surface markers CD29 and CD90 was analysed using flow cytometry, which revealed that the cells displayed the same expression patterns independently of the MSC source or oxygen content (Table 3). In all cases, the percentage of positive cells was greater than 93%.

In addition to flow cytometry, real time quantitative PCR (RT-qPCR) was performed to assess the expression of *CD29* and *CD90,* as well as 8 additional surface antigens (Figure 2). Few significant differences in gene expression were found between cells from the same origin that were expanded in different oxygen conditions. The expression of the *CD49d* gene was significantly higher in normoxic BM-MSCs than in their hypoxic counterparts. In AT-MSCs, *CD44* expression was significantly higher in normoxia.

Further differences in gene expression were observed when cultures from different sources exposed to the same oxygen tension were compared. In general, there was a trend of higher gene expression for all surface markers analysed in AT-MSCs, with the exception of *CD49d* in normoxic conditions and *CD106* in both hypoxia and normoxia. Normoxic AT-MSCs cells displayed significantly higher expression of *CD44*, *CD90* and *CD105* than normoxic BM-MSCs. Hypoxic AT-MSCs also showed significantly higher expression of *CD44*, *CD29*, *CD34*, *CD90* and *CD146* than hypoxic BM-MSCs. Similar gene expression patterns of the surface antigens *CD73* and *CD166* were detected in the four conditions (two types of cells grown under two oxygen treatments). Although the level of *CD106* mRNA was very low in hypoxic AT-MSCs, any significant differences existed between the two tissue sources and oxygen conditions.

Pluripotency markers

The expression of pluripotency markers was measured in both normoxic and hypoxic cells using RT-qPCR.

Transcripts of the embryonic stem cell makers *OCT4*, *NANOG* and *SOX2* were detected in BM-MSCs and AT-MSCs expanded in both oxygen conditions (Figure 3a,

3b and 3c). The mRNA levels were consistently higher in MSCs derived from adipose tissue, with statistically significant differences for the *OCT4* gene in hypoxic BM-MSCs and AT-MSCs, and for the *NANOG* gene in normoxic BM-MSCs and AT-MSCs.

MSCs exposed to 5% O_2 expressed higher levels of the three genes than MSCs exposed to 20% O_2.

Discussion

In the equine veterinary field, orthopaedic injuries are a major cause of retirement of athletic horses [17]. As a result, it is not surprising that equine regenerative medicine is primarily focused on the treatment of musculoskeletal defects. The present cell therapy studies were carried out with MSCs [12, 13, 18] and non-adult stem cells [19-21]. To better understand the mechanisms of action of MSCs *in vivo*, a large number of studies to characterise equine MSCs have been reported over the last five years [22-26]. However, because the overall objective of regenerative treatments is the use of MSCs in live horses, it is important to determine all of the properties of MSCs in an oxygen environment that closely emulates the original physiological niche from which the cells derive. To our knowledge, this constitutes the first study to perform an analysis of the influence of oxygen tension on proliferation, viability, stemness and marker expression in equine MSCs.

The effects of hypoxia on MSC proliferation have been studied specifically in humans and mice. Enhancements in cell growth following exposure to hypoxia have been described [10, 11, 27]. However, there is no unanimous consent that this is the case, as Feher et al. (2010) reported no difference in the growth of normoxic and hypoxic cells, and Volker et al. (2010) reported similar numbers of cells for both oxygen conditions at the conclusion of the culture period. In addition, Holzwarth et al. (2010), Zeng et al. (2011) and Wang et al. (2005) reported that low oxygen tension inhibited the proliferation of MSCs. Similar results have been reported to occur in dogs, in which MSCs derived from bone marrow and adipose tissue exposed to atmospheric O_2 showed more proliferative capacity than those expanded from passage 1 to passage 3 under hypoxic conditions (1% or 5% O_2) [28]. In agreement with these findings, our results describing the proliferation of equine cells as a function of oxygen tension

showed that the growth of AT-MSCs was significantly higher at atmospheric oxygen tension, while BM-MSCs underwent also more proliferation in 20% O_2.

Differences in cell growth between cultures expanded under different oxygen conditions could result from cell cycle changes or alterations of cell viability. Human MSC populations derived from umbilical cord and bone marrow accumulate cells in G_0/G_1 phase under low oxygen tension [9, 29]. Similar to these experiments, we found that hypoxic BM-MSCs displayed a higher percentage of cells in G_0/G_1 phases than normoxic BM-MSCs throughout the entire culture period. Moreover, the significantly higher proportion of normoxic BM-MSCs involved in the active stages of cell division (S or G_2/M) during the median days of culture led to a higher number of cells at the conclusion of the culture period. Cellular arrest in G_0/G_1 phase in hypoxic BM-MSCs might be caused by up-regulation of cyclin-dependent kinase inhibitors that control the cell cycle checkpoint [30-32]

In contrast to BM-MSCs, differences observed in the proliferation of different AT-MSC cultures were not due to cell cycle variations, but to variations in cell viability. Rat MSCs undergo a reduction in cell viability when permanently exposed to hypoxia [33]. Similar results were obtained in our work in that the proportions of viable AT-MSCs in hypoxic cultures were always lower than those in normoxic cultures. Reduced viability in hypoxic conditions reflects insufficient adaptation of AT-MSCs at 5% O_2, as higher percentages of non-viable cells were found in hypoxic conditions relative to populations at 20% O_2. No detectable changes in apoptosis have been previously described for hypoxic MSCs [34, 35]; our data describing the proportion of AnV^+PI^- cells corroborates these reports because statistically insignificant differences existed between both MSC types at different oxygen tensions.

In our experiments, equine BM-MSCs and AT-MSCs exhibited different proliferative capacities under each oxygen condition; these differences resulted from changes in the cell cycle profiles for BM-MSCs (Table 1a) and in the viability of AT-MSCs (Table 2b).

Moreover, AT-MSCs under either oxygen tension adapted more poorly to the culture environment following trypsinisation than cells derived from bone marrow, as

shown by a significantly higher proportion of PI^+ AT-MSCs at days 1 and 2 for both 5% and 20% O_2. This is reflected in the increased lag phase displayed by AT-MSCs. However, AT-MSCs also showed a significantly increased proportion of cells undergoing cell division during the first days of culture, which indicates that viable cells in culture have higher proliferative capacities than BM-MSCs. The increase in cell division of viable AT-MSCs might compensate for cell death in the population because the final number of cells obtained at the end of the experiment was higher in AT-MSC cultures than in BM-MSC cultures. This result indicates a higher proliferative ability for AT-MSCs than BM-MSCs, which is in agreement with previous reports using horses [26] and other species [28]. In a previous study, we described the more rapid decrease of apoptosis in AT-MSCs compared with BM-MSCs in cultures at 20% O_2 using a limited number of animals (n=2) [26]. The current study confirms this finding because the only detectable significant differences in apoptotic cell death existed between normoxic BM-MSCs and AT-MSCs.

Flow cytometric immunophenotype analysis of horse MSCs revealed that the surface antigen CD90 was detectable in all MSC types [36-39]. In addition, cross-reactivity with human antibodies has been demonstrated for the CD29 antigen in a previous report from our group and also in other studies [40, 41]. Because in other species hypoxia does not alter the immunophenotype of MSCs with regard to CD29 [29] and CD90 [9, 27, 42], we attempted to characterise this phenotype in equine MSCs and to analyse the presence of these molecules in both BM-MSCs and AT-MSCs in hypoxia and normoxia. According to the literature, MSCs displayed the same immunophenotype for CD29 and CD90 independently of the cell source and oxygen tension.

The lack of immunoreactivity of commercial antibodies with equine MSC antigens remains a challenge in determining the immunophenotype of these cells by flow cytometry. As a supplement to this technique, RT-qPCR has been used to establish the expression profiles of various markers in equine MSCs [37, 40]. Similar gene expression patterns were demonstrated in AT-MSCs when they were compared to BM-MSCs in their respective oxygen conditions. AT-MSCs at both oxygen tensions expressed higher levels of *CD29*, *CD44*, *CD90*, *CD146* and *CD34* transcripts respect to BM-MSCs; in contrast, only normoxic AT-MSCs expressed less *CD49d* compared to

normoxic BM-MSCs. These results are in agreement with our previous report [41]. The differences in *CD105* expression, with respect to our previous work, might be due to individual differences because different animals were used in the present study. Hypoxia seemed to significantly modify mRNA levels of *CD49d* in BM-MSCs and *CD44* in AT-MSCs, which is in agreement with other studies that have described different expression profiles for *CD49d* [18] and *CD44* [43] in hypoxia. The remaining surface markers analysed in this study showed no difference in gene expression as a function of oxygen tension.

The expression of specific markers characteristic of embryonic stem cells have been described before for equine BM-MSCs [44] and AT-MSCs [38]. However, to our knowledge, this is the first work that compares the gene expression of the pluripotency markers *OCT4*, *NANOG* and *SOX2* in equine AT-MSCs and BM-MSCs that were exposed to different oxygen concentrations. In our experimental conditions, equine MSCs expressed all three pluripotency markers. In general, higher expression of each marker was detected in AT-MSCs and was statistically significant for *OCT4* in hypoxia and for *NANOG* in normoxia. The consistently higher expression of all genes in hypoxia might reflect the enhanced stemness of hypoxic equine MSCs [45]. These results corroborate other studies that have described up-regulation of pluripotency-associated markers of hypoxic MSCs [11, 46, 47], which might suggest that low oxygen tension helps maintain the undifferentiated stem cell phenotype.

Conclusions

Oxygen plays a deterministic role in equine MSC cultures. It is able to modify their proliferative capacity via cell cycle modification in BM-MSCs and alterations in cell viability in AT-MSCs. Moreover, the immunophenotype of both MSC types is not altered by hypoxia. However, hypoxia appears to be an important factor in the maintenance or acquisition of stemness in equine MSCs.

Material and methods

Animals

Biological samples were obtained from a total of 12 castrated male horses aged 4 to 7 years. All procedures were carried out under Project Licence PI36/07, which was approved by the Ethic Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed in accordance with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals for experimental and other scientific purposes.

MSC isolation, culture and expansion

Samples were collected as previously described [26, 41]. Briefly, bone marrow aspirates were harvested from the sternum of six horses. The mononuclear fractions were enriched with MSCs, which were isolated in a centrifugation gradient using Lymphoprep (Atom, Barcelona, Spain). Isolated MSCs were rinsed twice with PBS and plated at a concentration of 10^6 cells/cm^2 in growth medium, which consisted of DMEM Low Glucose (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with foetal bovine serum, L-glutamine (Sigma-Aldrich, St. Louis, Missouri, USA) and penicillin/streptomycin (Sigma-Aldrich, St. Louis, Missouri, USA).

Subcutaneous adipose tissues were collected from the dorsal gluteal muscle below the tail from six horses. The stromal vascular fractions (SVFs) were isolated by digestion with 0.01% collagenase (Type I, Sigma-Aldrich, St. Louis, Missouri, USA) for 30 min at 37°C with continuous shaking. The cells were rinsed twice with PBS and plated in growth medium at a concentration of 10^5 cells/cm^2.

Both MSC types were expanded for 4 weeks at 37ºC in either normoxic (5% CO_2 and 20% O_2) or hypoxic (5% CO_2 and 5% O_2) conditions. Cell growth kinetics, immunophenotype, viability and cell cycle analyses were carried out using newly passaged cells fresh cells. Aliquots of 10^6 cells were preserved at -150ºC at the final passage, as described above, for further gene expression studies.

Cell growth kinetics

Cells from bone marrow and adipose tissue were seeded in 6-well plates in triplicate at a density of 5,000 cells/cm^2. BM-MSCs (n=6) and AT-MSCs (n=6) were exposed to 20% O_2 or 5% O_2 atmospheres for 7 days. Every day, the cells were collected using 0.25% trypsin/EDTA, and an aliquot of 50 µL of each culture was counted in a haemocytometer Z2 Coulter particle count and size analyser to obtain growth curves.

Analysis of cellular DNA content

Half of the MSCs harvested from the proliferation assay were fixed in 70% ice-cold ethanol and treated with 0.02 mg/mL RNAse and EDTA. DNA was stained with 0.1 mg/mL propidium iodide (Sigma-Aldrich, St. Louis, Missouri, USA). Cells were incubated in the dark for 30 min, and samples were analysed on a FACSARRAY (BD Biosciences, East Rutherford, New Jersey, USA) cytometer using the MODIFIT 3.0 software.

Viability assay

The remaining fraction of MSCs harvested in the proliferation assay was used to determine MSC viability. Apoptosis was measured by the detection of phosphatidylserine on the outer leaflet of the plasma membrane with the fluorescent dye Annexin V-FITC (Immunostep, Salamanca, Spain) in accordance with the manufacturer's instructions. Briefly, cells were rinsed with ice-cold PBS and then resuspended in 200 µL of binding buffer. Subsequently, 10 µL of Annexin V stock solution was added to cells and incubated for 30 min at 4ºC. Non-viable cells were identified by incubation with 5 µL of propidium iodide, a dye that penetrates into the cell nucleus when the plasma and nuclear cell membranes are damaged. PI-stained cells were immediately analysed in a FACSARIA cytometer (BD Biosciences, East Rutherford, New Jersey, USA) using FACSDIVA 5.0.1 software.

Immunophenotyping

To determine the immunophenotype of BM-MSCs and AT-MSCs after hypoxic and normoxic culture, the expression of the MSC surface markers CD29 (Integrin β1) and CD90 (Thy-1) was assessed by flow cytometry as previously described [41] using mouse anti-human monoclonal antibodies CD29-FITC (Caltag Laboratories, Little Balmer, Buckingham, UK) and CD90-PE (BD Pharmingen, San Diego, California, USA.). Negative control staining was performed using a FITC-conjugated mouse IgG1 isotype and a PE-conjugated mouse isotype. The immunophenotype was determined with the cytometer and software described in section 5.5.

Gene expression analysis

The expression of 10 genes encoding cell surface molecules, including *CD29* and *CD90*, was determined by real-time quantitative PCR. Additional antigens examined were *CD34*, *CD44* (H-CAM), *CD49d* (α4 integrin), *CD73* (ecto-5'-nuclease), *CD105* (endoglin), *CD106* (VCAM 1), *CD146* (MCAM) and *CD166* (ALCAM). The gene expression levels of the pluripotency markers *OCT4*, *SOX2* and *NANOG* were also analysed using the same technique.

Total RNA was extracted using the RNA spin mini (GE Healthcare Lifesciences, Little Chalfont, UK) and DNAse turbo (Ambion, Foster City, California, USA.) kits; subsequently, the Superscript kit (Invitrogen, Carlsbad, CA, USA) was used for reverse transcription of 1.5 µg of total RNA into complementary DNA. All kits were used in accordance with the manufacturer's instructions.

Table 3 shows the names of the analysed genes, GenBank accession numbers for equine mRNA sequences, forward and reverse primer sequences and amplicon sizes. Amplifications were performed in triplicate using the Fast SYBR Green Master Mix reagent (Applied Biosystems, Foster City, California, USA) and the StepOne™ Real Time System (Applied Biosystems, Foster City, California, USA). The levels of gene expression were determined using the comparative Ct method. A normalisation factor was calculated as the geometric mean of the quantity of two housekeeping genes (*GAPDH* and *B2M*) and used to normalise the expression of each gene.

Statistical analyses

The software SPSS 19.0 (Armonk, Nueva York, USA) was used to perform statistical analyses. Data obtained from flow cytometry and RT-qPCR were analysed for normality with the Shapiro-Wilk test. Differences in gene expression and reactivity levels in BM- and AT-MSCs expanded under hypoxia and normoxia conditions were determined using unpaired non-parametric Mann–Whitney tests. Differences in proliferation, viability and cell cycle were evaluated with Student's *t*-test. For both tests, $P < 0.05$ was considered statistically significant.

Abbreviations

Mesenchymal stem cell: MSC
Bone marrow-derived mesenchymal stem cell: BM-MSC
Adipose tissue-derived mesenchymal stem cell: AT-MSC
Annexin V: AnV
Propidium iodide: PI
Apoptotic cells: $AnV^{+}PI^{-}$
Non-viable cells: PI^{+}
Viable cells: $AnV^{-}PI^{-}$
Real time quantitative PCR: RT-qPCR

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BR carried out the expansion of the cells, proliferation assays, gene expression analyses, statistical analysis and drafted the manuscript. ARR participated in the expansion of the cells and proliferation assays. SAA participated in the gene expression analyses. AR performed the sample collections from the horses. FJV participated in the sample collections from the horses. PZ helped to draft the manuscript. IMB conceived

the study, participated in its design and helped to draft the manuscript. CR conceived the study, participated in its design and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Javier Godino from the Cell Separation Service of the Instituto Aragonés de Ciencias de la Salud (I+CS) for his technical assistance with the immunophenotyping experiments, cell cycle determination and viability assays. This work was performed as part of the AGL2008- 02428/GAN (MICINN/FEDER) and PAMER Pipamer 09/019 projects and was partially financed by the Gobierno de Aragón (Grupo de Excelencia LAGENBIO) and Instituto Aragonés de Ciencias de la Salud (I+CS). B. Ranera was supported by a doctoral grant from the DGA.

References

1. Koch TG, Berg LC, Betts DH: **Current and future regenerative medicine - principles, concepts, and therapeutic use of stem cell therapy and tissue engineering in equine medicine**. *Can Vet J* 2009, **50**(2):155-165.
2. Harrison JS, Rameshwar P, Chang V, Bandari P: **Oxygen saturation in the bone marrow of healthy volunteers**. *Blood* 2002, **99**(1):394.
3. Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE, Rood JC, Burk DH, Smith SR: **Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response**. *Diabetes* 2009, **58**(3):718-725.
4. Semenza GL: **HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus**. *Cell* 2001, **107**(1):1-3.
5. Ohnishi S, Yasuda T, Kitamura S, Nagaya N: **Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells**. *Stem Cells* 2007, **25**(5):1166-1177.
6. Chacko SM, Ahmed S, Selvendiran K, Kuppusamy ML, Khan M, Kuppusamy P: **Hypoxic preconditioning induces the expression of prosurvival and proangiogenic markers in mesenchymal stem cells**. *Am J Physiol Cell Physiol* 2010, **299**(6):C1562-1570.
7. Zhu W, Chen J, Cong X, Hu S, Chen X: **Hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells**. *Stem Cells* 2006, **24**(2):416-425.
8. Ren H, Cao Y, Zhao Q, Li J, Zhou C, Liao L, Jia M, Cai H, Han ZC, Yang R *et al*: **Proliferation and differentiation of bone marrow stromal cells under hypoxic conditions**. *Biochem Biophys Res Commun* 2006, **347**(1):12-21.
9. Holzwarth C, Vaegler M, Gieseke F, Pfister SM, Handgretinger R, Kerst G, Muller I: **Low physiologic oxygen tensions reduce proliferation and**

differentiation of human multipotent mesenchymal stromal cells. *BMC Cell Biol* 2010, **11**:11.

10. Lennon DP, Edmison JM, Caplan AI: **Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis**. *J Cell Physiol* 2001, **187**(3):345-355.
11. D'Ippolito G, Diabira S, Howard GA, Roos BA, Schiller PC: **Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells**. *Bone* 2006, **39**(3):513-522.
12. Godwin EE, Young NJ, Dudhia J, Beamish IC, Smith RK: **Implantation of bone marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon**. *Equine Vet J* 2011.
13. Frisbie DD, Kisiday JD, Kawcak CE, Werpy NM, McIlwraith CW: **Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis**. *J Orthop Res* 2009, **27**(12):1675-1680.
14. Grimshaw MJ, Mason RM: **Bovine articular chondrocyte function in vitro depends upon oxygen tension**. *Osteoarthritis Cartilage* 2000, **8**(5):386-392.
15. Zhao J, Zhang P, Qin L, Pan XH: **Hypoxia is essential for bone-tendon junction healing: the molecular biological evidence**. *Int Orthop* 2010, **35**(6):925-928.
16. Rosova I, Dao M, Capoccia B, Link D, Nolta JA: **Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells**. *Stem Cells* 2008, **26**(8):2173-2182.
17. Berg L, Koch T, Heerkens T, Bessonov K, Thomsen P, Betts D: **Chondrogenic potential of mesenchymal stromal cells derived from equine bone marrow and umbilical cord blood**. *Vet Comp Orthop Traumatol* 2009, **22**(5):363-370.
18. Schnabel LV, Lynch ME, van der Meulen MC, Yeager AE, Kornatowski MA, Nixon AJ: **Mesenchymal stem cells and insulin-like growth factor-I gene-enhanced mesenchymal stem cells improve structural aspects of healing in equine flexor digitorum superficialis tendons**. *J Orthop Res* 2009, **27**(10):1392-1398.
19. Carrade DD, Owens SD, Galuppo LD, Vidal MA, Ferraro GL, Librach F, Buerchler S, Friedman MS, Walker NJ, Borjesson DL: **Clinicopathologic findings following intra-articular injection of autologous and allogeneic placentally derived equine mesenchymal stem cells in horses**. *Cytotherapy* 2010, **13**(4):419-430.
20. Guest DJ, Smith MR, Allen WR: **Equine embryonic stem-like cells and mesenchymal stromal cells have different survival rates and migration patterns following their injection into damaged superficial digital flexor tendon**. *Equine Vet J* 2010, **42**(7):636-642.
21. Watts AE, Yeager AE, Kopyov OV, Nixon AJ: **Fetal derived embryonic-like stem cells improve healing in a large animal flexor tendonitis model**. *Stem Cell Res Ther* 2011, **2**(1):4.
22. Vidal MA, Kilroy GE, Johnson JR, Lopez MJ, Moore RM, Gimble JM: **Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity**. *Vet Surg* 2006, **35**(7):601-610.

23. Arnhold SJ, Goletz I, Klein H, Stumpf G, Beluche LA, Rohde C, Addicks K, Litzke LF: **Isolation and characterization of bone marrow-derived equine mesenchymal stem cells**. *Am J Vet Res* 2007, **68**(10):1095-1105.
24. Mambelli LI, Santos EJ, Frazao PJ, Chaparro MB, Kerkis A, Zoppa AL, Kerkis I: **Characterization of equine adipose tissue-derived progenitor cells before and after cryopreservation**. *Tissue Eng Part C Methods* 2009, **15**(1):87-94.
25. Toupadakis CA, Wong A, Genetos DC, Cheung WK, Borjesson DL, Ferraro GL, Galuppo LD, Leach JK, Owens SD, Yellowley CE: **Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue**. *Am J Vet Res* 2010, **71**(10):1237-1245.
26. Ranera B, Ordovas L, Lyahyai J, Bernal ML, Fernandes F, Remacha AR, Romero A, Vazquez FJ, Osta R, Cons C *et al*: **Comparative study of equine bone marrow and adipose tissue-derived mesenchymal stromal cells**. *Equine Vet J* 2012.
27. Dos Santos F, Andrade PZ, Boura JS, Abecasis MM, da Silva CL, Cabral JM: **Ex vivo expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia**. *J Cell Physiol* 2010, **223**(1):27-35.
28. Chung DJ, Hayashi K, Toupadakis CA, Wong A, Yellowley CE: **Osteogenic proliferation and differentiation of canine bone marrow and adipose tissue derived mesenchymal stromal cells and the influence of hypoxia**. *Res Vet Sci* 2010.
29. Zeng HL, Zhong Q, Qin YL, Bu QQ, Han XA, Jia HT, Liu HW: **Hypoxia-mimetic agents inhibit proliferation and alter the morphology of human umbilical cord-derived mesenchymal stem cells**. *BMC Cell Biol* 2011, **12**:32.
30. Gardner LB, Li Q, Park MS, Flanagan WM, Semenza GL, Dang CV: **Hypoxia inhibits G1/S transition through regulation of p27 expression**. *J Biol Chem* 2001, **276**(11):7919-7926.
31. Iida T, Mine S, Fujimoto H, Suzuki K, Minami Y, Tanaka Y: **Hypoxia-inducible factor-1alpha induces cell cycle arrest of endothelial cells**. *Genes Cells* 2002, **7**(2):143-149.
32. Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC, Huang LE: **HIF-1alpha induces cell cycle arrest by functionally counteracting Myc**. *EMBO J* 2004, **23**(9):1949-1956.
33. Peterson KM, Aly A, Lerman A, Lerman LO, Rodriguez-Porcel M: **Improved survival of mesenchymal stromal cell after hypoxia preconditioning: role of oxidative stress**. *Life Sci* 2010, **88**(1-2):65-73.
34. Volkmer E, Kallukalam BC, Maertz J, Otto S, Drosse I, Polzer H, Bocker W, Stengele M, Docheva D, Mutschler W *et al*: **Hypoxic preconditioning of human mesenchymal stem cells overcomes hypoxia-induced inhibition of osteogenic differentiation**. *Tissue Eng Part A* 2010, **16**(1):153-164.
35. Lavrentieva A, Majore I, Kasper C, Hass R: **Effects of hypoxic culture conditions on umbilical cord-derived human mesenchymal stem cells**. *Cell Commun Signal* 2010, **8**:18.
36. Pascucci L, Curina G, Mercati F, Marini C, Dall'aglio C, Paternesi B, Ceccarelli P: **Flow cytometric characterization of culture expanded multipotent mesenchymal stromal cells (MSCs) from horse adipose tissue: Towards the definition of minimal stemness criteria**. *Vet Immunol Immunopathol* 2011.

37. Braun J, Hack A, Weis-Klemm M, Conrad S, Treml S, Kohler K, Walliser U, Skutella T, Aicher WK: **Evaluation of the osteogenic and chondrogenic differentiation capacities of equine adipose tissue-derived mesenchymal stem cells**. *Am J Vet Res* 2010, **71**(10):1228-1236.
38. Raabe O, Shell K, Wurtz A, Reich CM, Wenisch S, Arnhold S: **Further insights into the characterization of equine adipose tissue-derived mesenchymal stem cells**. *Vet Res Commun* 2011, **35**(6):355-365.
39. de Mattos Carvalho A, Alves AL, Golim MA, Moroz A, Hussni CA, de Oliveira PG, Deffune E: **Isolation and immunophenotypic characterization of mesenchymal stem cells derived from equine species adipose tissue**. *Vet Immunol Immunopathol* 2009, **132**(2-4):303-306.
40. Radcliffe CH, Flaminio MJ, Fortier LA: **Temporal analysis of equine bone marrow aspirate during establishment of putative mesenchymal progenitor cell populations**. *Stem Cells Dev* 2010, **19**(2):269-282.
41. Ranera B, Lyahyai J, Romero A, Vazquez FJ, Remacha AR, Bernal ML, Zaragoza P, Rodellar C, Martin-Burriel I: **Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue**. *Vet Immunol Immunopathol* 2011.
42. Nekanti U, Dastidar S, Venugopal P, Totey S, Ta M: **Increased proliferation and analysis of differential gene expression in human Wharton's jelly-derived mesenchymal stromal cells under hypoxia**. *Int J Biol Sci* 2010, **6**(5):499-512.
43. Deguchi JO, Yamazaki H, Aikawa E, Aikawa M: **Chronic hypoxia activates the Akt and beta-catenin pathways in human macrophages**. *Arterioscler Thromb Vasc Biol* 2009, **29**(10):1664-1670.
44. Violini S, Ramelli P, Pisani LF, Gorni C, Mariani P: **Horse bone marrow mesenchymal stem cells express embryo stem cell markers and show the ability for tenogenic differentiation by in vitro exposure to BMP-12**. *BMC Cell Biol* 2009, **10**:29.
45. Ezashi T, Das P, Roberts RM: **Low O2 tensions and the prevention of differentiation of hES cells**. *Proc Natl Acad Sci U S A* 2005, **102**(13):4783-4788.
46. Fehrer C, Brunauer R, Laschober G, Unterluggauer H, Reitinger S, Kloss F, Gully C, Gassner R, Lepperdinger G: **Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan**. *Aging Cell* 2007, **6**(6):745-757.
47. Grayson WL, Zhao F, Izadpanah R, Bunnell B, Ma T: **Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs**. *J Cell Physiol* 2006, **207**(2):331-339.

Figure legends

Figure 1.Growth kinetic curves of equine MSCs at different oxygen concentrations.

Growth kinetics of BM-MSCs (n = 6) (A) and AT-MSCs (n = 6) (B). The Y axis represents the number of cells, and the X axis represents the number of days in culture. Data are represented as the means ± standard deviation. Black lines correspond to MSCs exposed to 20% O_2, and grey lines to MSCs exposed to 5% O_2. (*P < 0.05)

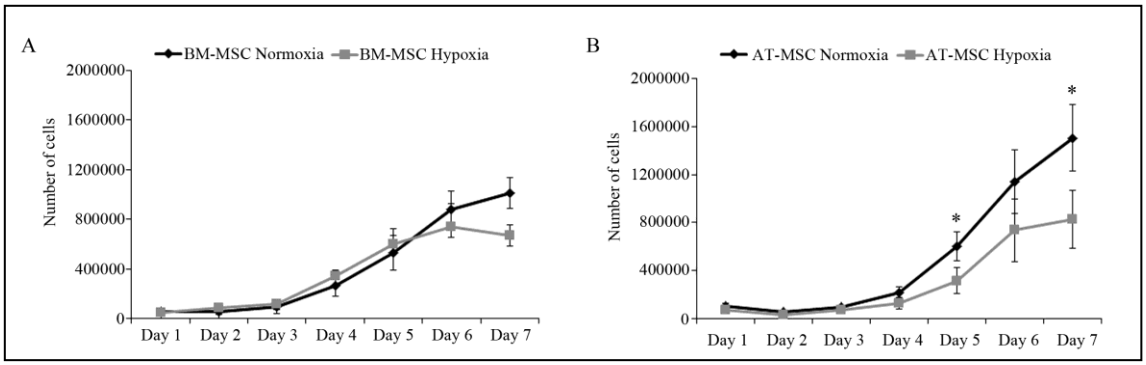


Figure 2.Gene expression of *CD29, CD34, CD44, CD49d, CD73, CD90, CD105, CD106, CD146* and *CD166* cell surface markers of equine MSCs.

Relative mRNA expression levels are expressed as the mean ± standard error. White bars correspond to normoxic BM-MSCs (n = 6), light grey bars with hypoxic BM-MSCs (n=6), dark grey bars with normoxic AT-MSCs (n = 6) and black bars with hypoxic AT-MSCs (n = 6). (*P < 0.05)

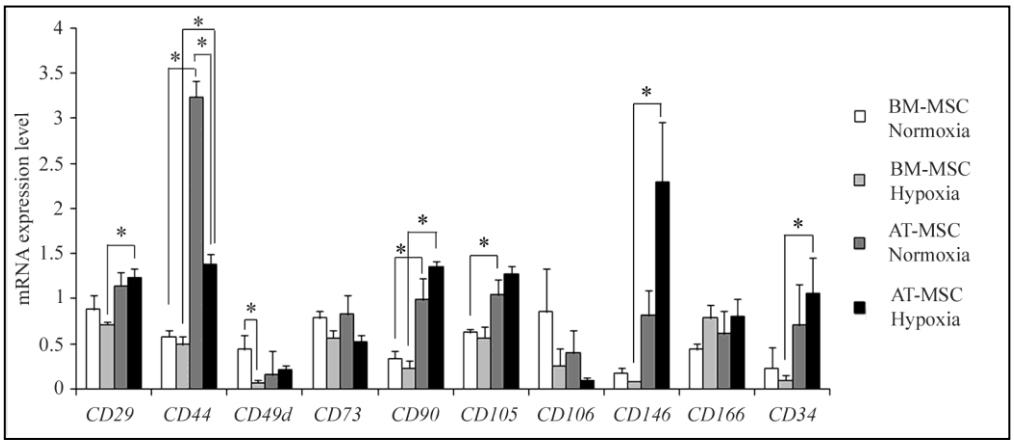


Figure 3.Gene expression of the pluripotency markers in equine MSCs.

Relative mRNA expression levels are expressed as the mean ± standard error for *OCT4* (A), *NANOG* (B) and *SOX2* (C) . White bars correspond to MSCs cultured under normoxia (n = 6), and grey bars correspond to MSCs cultured under hypoxia (n = 6). *$P < 0.05$

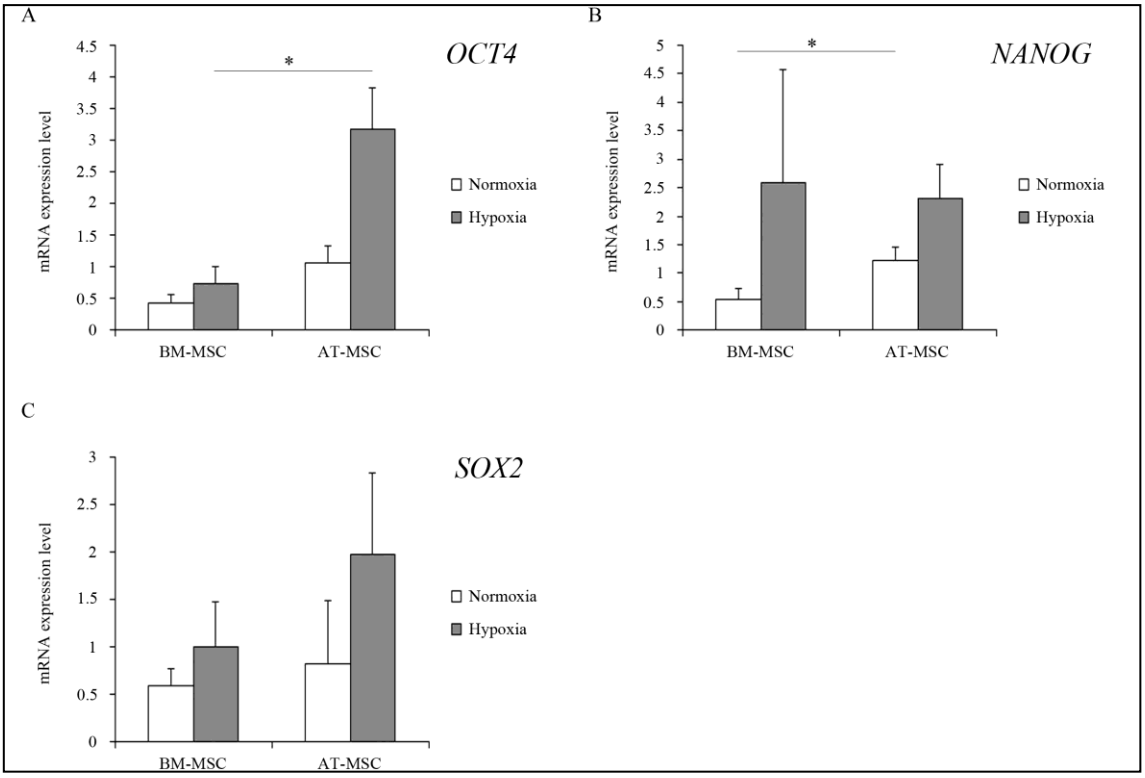


Table legends

Table 1.**Analysis of cell cycle in BM-MSCs (n = 6) and AT-MSCs (n = 6) cultured under hypoxic or normoxic conditions.**

The G_0/G_1,S and G_2/M cell cycle phases were analysed for 7 days. Cell cycle data are represented as the mean ± standard deviation. The “a” and “b” superscripted bold data are significantly different. (A) Compares normoxic and hypoxic conditions of BM-MSCs; (B) compares normoxic and hypoxic conditions of AT-MSCs; (C) compares BM-MSCs and AT-MSCs in normoxic conditions; and (D) compares BM-MSCs and AT-MSCs in hypoxic conditions.

a) b) c) d)

		BM-MSC		AT-MSC		Normoxia		Hypoxia	
		Normoxia	Hypoxia	Normoxia	Hypoxia	BM-MSC	AT-MSC	BM-MSC	AT-MSC
Day 1	G_0/G_1	81.88 ± 15.37	78.64 ± 10.64	84.33 ± 9.48	89.04 ± 5.98	81.88 ± 15.37	84.33 ± 9.48	**78.64 ± 10.64[a]**	**89.04 ± 5.98[b]**
	S	12.51 ± 10.53	14.51 ± 7.39	9.86 ± 7.17	4.99 ± 3.12	12.51 ± 10.53	9.86 ± 7.17	**14.51 ± 7.39[a]**	**4.99 ± 3.12[b]**
	G_2/M	5.61 ± 5.30	6.85 ± 4.70	5.80 ± 2.42	5.74 ± 3.10	5.61 ± 5.30	5.80 ± 2.42	6.85 ± 4.70	5.74 ± 3.10
Day 2	G_0/G_1	**49.32 ± 4.46[a]**	**62.64 ± 5.52[b]**	39.86 ± 3.26	41.33 ± 6.23	**49.32 ± 4.46[a]**	**39.86 ± 3.26[b]**	**62.64 ± 5.5[a]**	**41.33 ± 6.23[b]**
	S	**33.93 ± 6.05[a]**	**24.51 ± 5.29[b]**	39.07 ± 1.50	35.54 ± 7.66	**33.93 ± 6.05[a]**	**39.07 ± 1.50[b]**	**24.51 ± 5.29[a]**	**35.54 ± 7.66[b]**
	G_2/M	**17.89 ± 6.99[a]**	**12.85 ± 3.67[b]**	21.07 ± 3.52	23.13 ± 2,20	17.89 ± 6.99	21.07 ± 3.52	**12.85 ± 3.67[a]**	**23.13 ± 2,20[b]**
Day 3	G_0/G_1	**56.00 ± 5.85[a]**	**65.96 ± 5.83[b]**	45.37 ± 3.85	51.57 ± 5.86	**56.00 ± 5.85[a]**	**45.37 ± 3.85[b]**	**65.96 ± 5.83[a]**	**51.57 ± 5.86[b]**
	S	31.36 ± 8.24	22.53 ± 5.18	38.36 ± 4.49	31.42 ± 9.07	**31.36 ± 8.24[a]**	**38.36 ± 4.49[b]**	22.53 ± 5.18	31.42 ± 9.07
	G_2/M	12.94 ± 4.46	11.50 ± 3.31	14.60 ± 1.04	17.01 ± 6.58	12.94 ± 4.46	14.60 ± 1.04	**11.50 ± 3.31[a]**	**17.01 ± 6.58[b]**
Day 4	G_0/G_1	**56.30 ± 5.65[a]**	**74.13 ± 7.11[b]**	54.67 ± 2.64	52.74 ± 6.51	56.30 ± 5.65	54.67 ± 2.64	**74.13 ± 7.11[a]**	**52.74 ± 6.51[b]**
	S	**30.59 ± 10.05[a]**	**15.90 ± 6.12[b]**	30.24 ± 1.30	26.40 ± 3.42	30.59 ± 10.05	30.24 ± 1.30	**15.90 ± 6.12[a]**	**26.40 ± 3.42[b]**
	G_2/M	13.00 ± 5.00	9.97 ± 4.26	14.91 ± 2.34	20.19 ± 8.15	13.00 ± 5.00	14.91 ± 2.34	9.97 ± 4.26	20.19 ± 8.15
Day 5	G_0/G_1	69.77 ± 11.77	79.77 ± 7.22	65.83 ± 5.76	62.63 ± 9.25	69.77 ± 11.77	65.83 ± 5.76	**79.77 ± 7.22[a]**	**62.63 ± 9.25[b]**
	S	20.16 ± 13.11	11.18 ± 4.44	22.18 ± 3.15	23.85 ± 5.48	20.16 ± 13.11	22.18 ± 3.15	**11.18 ± 4.44[a]**	**23.85 ± 5.48[b]**
	G_2/M	10.07 ± 2.33	9.06 ± 5.04	11.99 ± 2.75	13.55 ± 4.23	10.07 ± 2.33	11.99 ± 2.75	9.06 ± 5.04	13.55 ± 4.23
Day 6	G_0/G_1	75.51 ± 15.79	83.07 ± 6.31	78.36 ± 1.89	71.49 ± 11.70	75.51 ± 15.79	78.36 ± 1.89	83.07 ± 6.31	71.49 ± 11.70
	S	16.08 ± 15.59	8.42 ± 3.02	13.16 ± 1.80	18.43 ± 9.18	16.08 ± 15.59	13.16 ± 1.80	8.42 ± 3.02	18.43 ± 9.18
	G_2/M	8.36 ± 1.67	8.35 ± 5.52	8.49 ± 1.01	10.35 ± 2.53	8.36 ± 1.67	8.49 ± 1.01	8.35 ± 5.52	10.35 ± 2.53
Day 7	G_0/G_1	81.71 ± 6.83	84.50 ± 8.33	85.78 ± 1.56	77.16 ± 12.67	81.71 ± 6.83	85.78 ± 1.56	84.50 ± 8.33	77.16 ± 12.67
	S	15.65 ± 16.21	7.78 ± 4.03	6.73 ± 1.32	14.06 ± 9.66	15.65 ± 16.21	6.73 ± 1.32	7.78 ± 4.03	14.06 ± 9.66
	G_2/M	2.64 ± 2.01	7.77 ± 4.92	7.50 ± 1.26	8.78 ± 3.20	2.64 ± 2.01	7.50 ± 1.26	7.77 ± 4.92	8.78 ± 3.20

Table 2.**Viability of BM-MSCs (n = 6) and AT-MSCs (n = 6) cultured under hypoxic or normoxic conditions.**

Viability data are represented as the mean ± standard deviation. Non-viable cells (PI^+), viable cells (AnV^-PI^-) and apoptotic cells (AnV^+PI^-) were analysed for 7 days. Superscripted bold data ("a" and "b") are significantly different. (A) Compares normoxic and hypoxic conditions of BM-MSCs; (B) compares normoxic and hypoxic conditions of AT-MSCs; (C) compares BM-MSCs and AT-MSCs in normoxic conditions; and (D) compares BM-MSCs and AT-MSCs in hypoxic conditions.

		a)		b)		c)		d)	
		BM-MSC		AT-MSC		Normoxia		Hypoxia	
		Normoxia	Hypoxia	Normoxia	Hypoxia	BM-MSC	AT-MSC	BM-MSC	AT-MSC
Day 1	PI^+	13.64 ± 2,03	12.06 ± 3.89	46.64 ± 26.38	72.24 ± 17.20	**13.64 ± 2,03**[a]	**46.64 ± 26.38**[b]	**12.06 ± 3.89**[a]	**72.24 ± 17.20**[b]
	AnV^-PI^-	81.92 ± 2.74	81.39 ± 8.51	**47.86 ± 23.61**[a]	**21.79 ± 12.35**[b]	**81.92 ± 2.74**[a]	**47.86 ± 23.61**[b]	**81.39 ± 8.51**[a]	**21.79 ± 12.35**[b]
	AnV^+PI^-	4.44 ± 1.27	6.52 ± 4.94	5.60 ± 2.69	6.05 ± 6.04	4.44 ± 1.27	5.60 ± 2.69	6.52 ± 4.94	6.05 ± 6.04
Day 2	PI^+	15,02 ± 3.05	13,97 ± 6.37	28.01 ± 11.11	52.85 ± 25.16	**15,02 ± 3.05**[a]	**28.01 ± 11.11**[b]	**13,97 ± 6.37**[a]	**52.85 ± 25.16**[b]
	AnV^-PI^-	81.91 ± 3.67	81.72 ± 7.00	**65.45 ± 12.05**[a]	**39.31 ± 22.23**[b]	**81.91 ± 3.67**[a]	**65.45 ± 12.05**[b]	**81.72 ± 7.00**[a]	**39.31 ± 22.23**[b]
	AnV^+PI^-	3.07 ± 0.94	4.53 ± 1.52	6.54 ± 2.53	9.31 ± 5.23	**3.07 ± 0.94**[a]	**6.54 ± 2.53**[b]	4.53 ± 1.52	9.31 ± 5.23
Day 3	PI^+	15.44 ± 7.98	14.07 ± 5.08	**21,29 ± 3.94**[a]	**45.97 ± 19.78**[b]	15.44 ± 7.98	21,29 ± 3.94	**14.07 ± 5.08**[a]	**45.97 ± 19.78**[b]
	AnV^-PI^-	77.84 ± 3.96	75.04 ± 11.65	**74.28 ± 3.13**[a]	**46.24 ± 18.6**[b]	**77.84 ± 3.96**[a]	**74.28 ± 3.13**[b]	**75.04 ± 11.65**[a]	**46.24 ± 18.61**[b]
	AnV^+PI^-	6.71 ± 2.16	10.86 ± 7.19	4.43 ± 1.62	7.83 ± 5.23	6.71 ± 2.16	4.43 ± 1.62	10.86 ± 7.19	7.83 ± 5.23
Day 4	PI^+	14.43 ± 6.25	14.60 ± 7.41	**14.64 ± 3.60**[a]	**25.39 ± 11.67**[b]	14.43 ± 6.25	14.64 ± 3.60	**14.60 ± 7.41**[a]	**25.39 ± 11.67**[b]
	AnV^-PI^-	76.82 ± 5.89	77.11 ± 7.49	80.98 ± 2.88	68.31 ± 11.32	76.82 ± 5.89	80.98 ± 2.88	77.11 ± 7.49	68.31 ± 11.32
	AnV^+PI^-	8.74 ± 4.06	8.31 ± 3.15	4.41 ± 2.40	6.30 ± 5.49	**8.74 ± 4.06**[a]	**4.41 ± 2.40**[b]	8.31 ± 3.15	6.30 ± 5.49
Day 5	PI^+	8.7 ±3.27	7.34 ± 2.61	**8.29 ± 1.80**[a]	**17.82 ± 7.72**[b]	8.7 ±3.27	8.29 ± 1.80	**7.34 ± 2.61**[a]	**17.82 ± 7.72**[b]
	AnV^-PI^-	83.03 ± 4.91	82.28 ± 8.29	**89.64 ± 1.54**[a]	**77.37 ± 6.67**[b]	**83.03 ± 4.91**[a]	**89.64 ± 1.54**[b]	**82.28 ± 8.29**[a]	**77.37 ± 6.67**[b]
	AnV^+PI^-	8.28 ± 3.49	10.37 ± 7.60	2.06 ± 0.73	4.81 ± 4.22	**8.28 ± 3.49**[a]	**2.06 ± 0.73**[b]	10.37 ± 7.60	4.81 ± 4.22
Day 6	PI^+	8.04 ± 4.37	10.27 ± 1.59	8.64 ± 1.51	14.14 ± 8,83	8.04 ± 4.37	8.64 ± 1.51	10.27 ± 1.59	14.14 ± 8,83
	AnV^-PI^-	84.79 ± 7.37	77.76 ± 9.44	89.18 ± 1.32	81.43 ± 10.06	84.79 ± 7.37	89.18 ± 1.32	77.76 ± 9.44	81.43 ± 10.06
	AnV^+PI^-	7.16 ± 3.46	11.98 ± 7.96	2.14 ± 0.66	4.42 ± 2.71	**7.16 ± 3.46**[a]	**2.14 ± 0.66**[b]	11.98 ± 7.96	4.42 ± 2.71
Day 7	PI^+	9.77 ± 5,27	10.95 ± 4.29	**8.05 ± 4.28**[a]	**15.69 ± 8.96**[b]	9.77 ± 5,27	8.05 ± 4.28	10.95 ± 4.29	15.69 ± 8.96
	AnV^-PI^-	83.29 ± 6.91	80.45 ± 5.98	85.67 ± 7.91	78.05 ± 7.77	83.29 ± 6.91	85.67 ± 7.91	80.45 ± 5.98	78.05 ± 7.77
	AnV^+PI^-	6.92 ± 3.40	8.65 ± 7.26	6.14 ± 4.32	6.96 ± 1.16	6.92 ± 3.40	6.14 ± 4.32	8.65 ± 7.26	6.96 ± 1.16

Table 3. Immunophenotype of BM-MSCs and AT-MSCs cultured under normoxic and hypoxic conditions.

	CD29	CD90
BM-MSC Normoxia	99.73 ± 0.03	97.40 ± 0.66
BM-MSC Hypoxia	99.41 ± 0.26	98.06 ± 0.60
AT-MSC Normoxia	97.93 ± 0.83	96.52 ± 1.01
AT-MSC Hypoxia	98.75 ± 1.33	93.65 ± 3.92

Data are expressed as the mean ± standard deviation of percentages of MSCs positive for CD29 and CD90 expression. The expression data were obtained using flow cytometry.

Table 4. Summary of gene information

Gene	Accession number	Primer sequence (5'-3')	Amplicon size (bp)
CD29 [a]	XM_001492665	F: GTAAAAAGTCTTGGAACCGATCTGAT R: CACAAATGAGCCAAACCCAATT	81
CD34 [a]	XM_001491596	F:CACTAAACCCTCTACATCATTTTCTCCTA R: GGCAGATACCTTGAGTCAATTTCA	150
CD44 [a]	NM_001085435	F: CCCACGGATCTGAAACAAGTG R: TTCTGGAATTTGAGGTCTCCGTAT	95
CD49d [a]	XM_001917601	F: CATCGGCCTTCTCACAGAGAA R: GCCATTATTGTCTGCATCAATTTG	101
CD73 [a]	XM_001500115	F: GGGATTGTTGGATACACTTCAAAAG R: GCTGCAACGCAGTGATTTCA	90
CD90 [a]	EU881920	F: TGCGAACTCCGCCTCTCT R: GCTTATGCCCTCGCACTTG	93
CD105 [a]	XM_001500078	F: GACGGAAAATGTGGTCAGTAATGA R: GCGAGAGGCTCTCCGTGTT	100
CD106 [a]	NM_001101650	F: CATCGTGACCTGTGGGCATA R: TGGGTTTCCCTCCACTAGCA	111
CD146 [a]	XM_001917594	F: CTGGACTTGGAAACCACAACATC R: CAGGTCTCACTCGGACATCAGA	85
CD166 [a]	XM_001503380	F: GTCTGGTCTTCTGCCTCTTGATC R: TCGGCAAGGCATGATAATAGTG	103
OCT4 [b]	XM_001490108	F: AGAGGCAACCTGGAGAACATG R: GGGCAATGTGGCTGATCTG	70
NANOG [b]	XM_001498808	F: TACCTCAGCCTCCAGCAGAT R: CAGTTGTTTTTCTGCCACCT	119
SOX2 [c]	FJ356148	F: TGGTTACCTCTTCCTCCCACT R: GGGCAGTGTGCCGTTAAT	178
GAPDH [a]	NM_001163856	F: GGCAAGTTCCATGGCACAGT R: CACAACATATTCAGCACCAGCAT	128
B2M [a]	NM_001082502	F: TCGTCCTGCTCGGGCTACT R: ATTCTCTGCTGGGTGACGTGA	102

[a] previously described in[225]

[b] previously described in[178]

[c] previously described in [249]

Genes analysed, GenBank accession numbers, primer sequences for reverse transcriptase RT-PCR (F: forward and R: reverse) and amplicon sizes in base pairs (bp).

5.6. MANUSCRITO VI

Characterisation of the equine MSCs used in a preclinical trial to evaluate the capacity of MSCs for healing induced tendón injuries.

INTRODUCTION

Cell therapy in the orthopaedic field in horses is becoming an important treatment of injuries related to the musculoskeletal system. During the last decade several authors have reported the use of mesenchymal stem cells (MSCs) to treat defects in tendons and joints[94, 105]. Unlike traditional treatments, the results obtained from cell therapy studies have shown a favourable evolution of the healing of injuries after the treatment and a reduction of re-injury risk[9].

MSCs are an interesting tool for cell therapy due to their self-renewing ability and their capacity to differentiate into cellular lineages from connective tissues. Therefore,it is essential to know the molecular characteristics of the MSCs to understand how the cells are able to repair the tissues, and then, try to enhance their properties to achieve better clinical results.

Equine MSCs have been isolated from peripheral blood [164], umbilical cord blood [166], gingiva and periodontal ligament [167]. However, bone marrow and adipose tissue derived MSC are the two main cell types use for cell therapy in horses [108, 109], and also the best characterized *in vitro* [169, 172]. Equine bone marrow MSCs (BM-MSCs) and adipose tissue MSCs (AT-MSCs) satisfy the minimal criteria of definition suggested by the ISTC [31].Both cell types express on their surface the antigens CD29, CD90, CD44 and CD105 [165, 167, 172, 175, 176] and, despite the lack of specific antibodies against equine epitopes, the gene expression of CD13, CD49d, CD73, CD106, CD146 and CD166 has been confirmed [220]. Tri-lineage differentiation towards osteoblast, adipocyte and chondrocyte has been also demonstrated in equine BM-MSCs and AT-MSCs [162, 172].

The aim of this work was to characterize and compare the differentiation potentials of equine BM-MSCs and AT-MSCs used in an experimental design of a pre-clinical study to treat induced injuries in the superficial digital flexor tendon.

MATERIAL AND METHODS

Animals

Biological samples were obtained from a total of 12 castrated male horses aged 4 to 7 years. All procedures were carried out under Project Licence PI36/07, which was approved by the Ethic Committee for Animal Experimentation from the University of Zaragoza. The care and use of animals were performed in accordance with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals for experimental and other scientific purposes.

MSC isolation, culture and expansion

Samples were collected as previously described [225]. Briefly, bone marrow aspirates were harvested from the sternum of six horses. The mononuclear fractions were enriched with MSCs, which were isolated in a centrifugation gradient using Lymphoprep (Atom, Barcelona, Spain). Isolated MSCs were rinsed twice with PBS and plated at a density of 10^6 cells/cm^2 in growth medium, which consisted of DMEM Low Glucose (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10% foetal bovine serum, 1% L-glutamine (Sigma-Aldrich, St. Louis, Missouri, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, Missouri, USA).

Subcutaneous adipose tissues were collected from the dorsal gluteal muscle below the tail from six horses. The stromal vascular fractions (SVFs) were isolated by digestion with 0.01% collagenase (Type I, Sigma-Aldrich, St. Louis, Missouri, USA) for 30 min at 37°C with continuous shaking. The cells were rinsed twice with PBS and plated in growth medium at a density of 10^5cells/cm^2.

Both BM-MSCs and AT-MSCs were expanded at 5.000 cells/cm^{2}in growth medium at 20% $O_2$and 5% CO_2.The medium was changed every 3 days until cells reached approximately 80% confluence. Subsequently, cells were treated with 0.25% trypsin-EDTA and counted. At passage three the cells were detached, one part of the cells were injected into the SDFT injury on the horse and the other part were used on the cell surface phenotyping and differentiation assays.

Cell surface markers

The mesenchymal stem cells markers CD29 (Integrin β1) and CD90 (Thy-1) were analysed in BM-MSCs and AT-MSCs by flow cytometry as we previously described [220]. The gene expression of *CD29*, *CD90* and eight more cell surface antigens was studied by real-time quantitative PCR (RT-qPCR). Additional molecules examined were *CD34*, *CD44* (H-CAM), *CD49d* (α4 integrin), *CD73* (ecto-5'-nuclease), *CD105* (endoglin), *CD106* (VCAM 1), *CD146* (MCAM) and *CD166* (ALCAM) [220].

Osteogenic differentiation

BM-MSCs and AT-MSCs were plated at 20.000 cells/cm^{2}and cultured during 9 days in differentiation medium containing growth medium supplemented with 10nM dexamethasone, 10 mM β-glycerophosphate and 100 mMascorbate-2-phosphate (all from Sigma-Aldrich). On days 0, 3, 6 and 9 the activity of alkaline was measured as previously described[251],and also the gene expression of the osteogenic markers runt-related transcription factor 2 (*RUNX2*), alkaline phosphatase (*ALP*) and osteopontin (*SPP1*)was analyzedby RT-qPCR following our previously described protocols [251]. Osteogenic differentiation was also monitored by alizarin red staining of the calcium deposits on the same days.

Adipogenic differentiation

Both cell types were seeded at 5,000 cells/cm^{2}and cultured for 14 days in adipogenic inductive media consisted of growth medium supplemented with1μM dexamethasone, 500 μM IBMX, 200 μM indomethacin and 15% rabbit serum (all from Sigma-Aldrich). The adipogenesis was studied on days 0, 5, 9 and 14 analysing the gene expression of the adipogenic markers lipoprotein lipase (*LPL*) and peroxisome proliferator-activated receptor gamma (*PPARγ*) by RT-qPCR and staining the lipid droplets with oil red O as previously reported [251].

Chondrogenic differentiation

Chondrogenesis was induced in 250,000 pelleted BM-MSCs and AT-MSCs for 21 days. Differentiation medium consisted of DMEM high glucose, 10% FBS, 10 ng/ml TGFβ-3, 1% ITS+ premix, 40 mg/ml proline, 50 mg/ml ascorbate-2-phosphate and 0.1 mM dexamethasone.Haematoxylin-eosin staining was performed and the gene expression of collagen type II (*COL2A1*), aggrecan (*ACAN*) and cartilage oligomeric protein (*COMP*) was analyzed by RT-qPCRon days 0, 7, 14 and 21.

RESULTS

Cell surface markers

The immunophenotype obtained for CD29 and CD90 by flow cytometry showed that more than 93% of BM-MSCs and AT-MSCs were positive for these markers. These data agree with our previous findings [220].

Due to the lack of immunoreactivity of commercial antibodies with equine epitopes to determine the phenotype of equine MSCs, RT-qPCR was used to complete the analysis of mesenchymal markers. AT-MSCs expressed higher levels of *CD29*, *CD44*, *CD73*, *CD90*, *CD146* and *CD34* transcripts respect to BM-MSCs, and *CD49d* was more expressed in BM-MSCs than in AT-MSCs. Both cell types did not express the haematopoietic marker *CD45*. These results confirm our previous work [220]. (y el submitted).

Osteogenic potential of MSCs

Both BM-MSCs and AT-MSCs displayed osteogenic potential (Fig. 1). The gene expression of *RUNX-2* increased until day 6for both cell types (Fig. 1a). In differentiated AT-MSCs the level of mRNA was significantly higher than control AT-MSCs on days 3 and 6while in differentiated BM-MSCs the gene expression was only significant on day 6. On day 9 the *RUNX-2* mRNA levels decreased for both cell types, although they were still significantly higher than controls in the case of induced AT-MSCs. Gene expression of *RUNX-2* in AT-MSCs was higher than in BM-MSCs during all the induction period, significant differences were found between both MSC types on

day 3.Although the mRNA levels of *RUNX-2* were similar in both cell types at day 6 and a marked down-regulation of this gene was observed in BM-MSCs on day 9. The kinetic of *RUNX-2* gene expression confirms the results obtained in previous work of our group for both cell types [251].

The gene expression of *SPP1* increased during the induction period for both cell types (Fig. 1b). However, the mRNA levels in BM-MSCs were significantly higher than in AT-MSCs from day 3. The *SPP1* up-regulation in BM-MSCs and AT-MSCs in this study agree with the findings made before [251].

The *ALP* gene expression and activity were higher in AT-MSCs than in BM-MSCs (Fig. 1c-d). The transcript level increased significantly on days 3 and 6 in BM-MSCs, and on days 6 and 9 in AT-MSCs. The up-regulation of *ALP* in BM-MSCs was accompanied with a gradual increase of the ALP activity. The statistically significant differences detected in *ALP* gene expression between BM-MSCs and AT-MSCs on day 3 would be reflected on the significantly higher ALP activity in AT-MSCs than in BM-MSCs at the same day. All these results confirm our previous findings [251].

Theo osteogenic differentiation showed by the quantification analyses of the gene expression of osteogenic markers was corroborated by the alizarin red staining performed. Osteogenic nodule formation was evident from day 3 in some BM-MSCs, and it was found in all the cases from day 6. At day 9, the BM-MSC induced cultures were completely covered by nodules and calcium deposits indicating that the differentiation was fully accomplished (Fig 1e-h). In AT-MSC induced cultures the appearance of nodules occurred later than in BM-MSCs, and although the osteogenic differentiation was evident at day 9, the calcium deposits appeared in a lesser extent (Fig. 1i-l).

Taken together these results, we have demonstrated that the equine BM-MSCs and AT-MSCs used for the treatment of tendon lesions display osteogenic potential. However, the monitoring of osteogenic marker expression showed that BM-MSCs are able to differentiate faster than AT-MSCs. The early marker *RUNX2* was down-regulated before in BM-MSCs than in AT-MSCs, while the gene expression of the late marker *SPP1* was always higher in BM-MSCs. The staining results and the *SPP1* gene

expressionsuggest that BM-MSCs exhibit higher osteogenic potential, which would be in agreement with other authors [180, 181].

Adipogenic potential

Adipogenic induction *in vitro* is difficult to achieve in equine MSCs [126].In our previous report we tested sixteen different adipogenic inductive media to differentiate BM-MSCs into adipocytes [251] and, finally, the addition of rabbit serum seemed to favour the adipogenesis in equine MSCs. In the present work, adipogenic differentiation was achieved for both BM-MSCs and AT-MSCs (Fig. 2). Gene expression of *PPARγ*increased progressively until day 9 in BM-MSCs, but the gene expression was only significantly higher than control cells on day 14 (Fig. 2a). On the contrary, induced AT-MSC cultures showed a significant up-regulation of *PPARγ*on days 5 and 9, although its expression was decreasing during the inductive culture period. On days 5 and 9 the *PPARγ*gene expression was significantly higher in AT-MSCs than in BM-MSCs. *LPL* gene expression (Fig.2b) showed a similar kinetic to *PPARγ*, an up-regulation was detected on days 5 and 9 in BM-MSCs, and then the gene expression diminished. In AT-MSCs cultures, the highest mRNA level of *LPL* was found on day 5, and it was significantly higher in comparison to the mRNA level in BM-MSCs, afterwards the gene expression decreased until the end of the time of culture.

The adipogenic compatible changes revealed by RT-qPCR were confirmed by staining. Oil red O staining (Fig. 2c-j) showed that the most MSCs in culture underwent morphological changes and abundant formation of small lipid droplets inside of the cells on day 5. However, during the time course of the adipogenic induction the cells with lipid droplets inside gradually decreased and more death cells were detected in culture.

These results agree with other authors who demonstrated the use of rabbit serum as culture supplement is able to induce adipogenesis in BM-MSCs [162] and in AT-MSC [172]. In equine MSCs, terminal adipogenesis is difficult to achieve, because after an initial period of induction the cells detach without completing the differentiation[172]. The molecular analysis by RT-qPCR of the adipogenic markers

might indicate AT-MSCs display more adipogenic potential than BM-MSCs. The current adipogenic marker gene expression profiles differs with our previous work [251]. The differences might be owing to the fact that in the present work we have analysed a more representative number of samples (n=6), whereas in our previous report the gene expression kinetics was determined only with one sample.

Chondrogenic potential

Adequate chondrogenic differentiation was only detected in BM-MSCs in our experimental conditions (Fig. 3). The gene expression of *COL2A1*, *ACAN* and *COMP* was gradually up-regulated during the induction period in BM-MSCs (Fig 3a-c). In contrast, weak amplifications of these markers were detected in AT-MSCs, finding significant differences with respect to BM-MSCs for the gene expression of *COL2A1* on days 7, 14 and 21, and for *ACAN* and *COMP* on days 14 and 21.

The differences in the chondrogenic potential of both cell types observed with the gene expression analysis were confirmed by the histological analysis. Haematoxylin-eosin staining showed the formation of hyaline extracellular matrix in BM-MSCs, the cells underwent morphological changes with the appearance of lacunae formation throughout the pellet sections (Fig. 3d-g). In contrast, AT-MSCs did not seem to change their morphology, it was not either observed extracellular matrix formation and picnotic nuclei were detected, which would indicate cellular necrosis (Fig. 3h-k).

Although the chondrogenic differentiation in equine AT-MSCs have been already demonstrated [172], other works have found higher chondrogenic differentiation potential in equine BM-MSCs than in AT-MSCs [183]. Our results are in agreement with these reports, since AT-MSCs were not able to differentiate into chondrocytes and generate a typical chondrogenic extracellular matrix, showing that AT-MSCs have limited chondrogenic potential in our experimental conditions.

In summary, equine MSCs used for the treatment of induced tendon lesions meet the minimum criteria to be considered mesenchymal stem cells. The cells displayed the mesenchymal stem cell surface markers and did not express the haematopoieticmarker *CD45*. However, equine MSCs showed different differentiation potential depending on

the source of isolation. Both cell types were able to differentiate into osteoblast, although BM-MSCs achieve it faster than AT-MSCs. Adipogenic differentiation ability was more evident during the first days of induction for both cell types, but AT-MSCs seemed to display greater adipogenic potential than BM-MSCs at molecular level. In contrast, chondrogenic differentiation was only accomplished in BM-MSCs, showing poor chondrogenic potential of AT-MSCs. Further analyses are necessaries to determine if the differences found in the MSC characteristics between these cell types *in vitro* might be reflected in their therapeutic effects *in vivo*.

FIGURES

Figure 1.Time course ofosteogenic differentiation of equine BM-MSCs and AT-MSCs. Mean ± standard error (s.e.) inmRNA expression levels of differentiated cells respect to control cultures is shown for *RUNX2* (a), *SPP1* (b) and *ALP* (c). Mean activity fold change of ALP ± s.e.(d). Black horizontal lines represent gene expression or ALP activity in control cultures (values set to 1). White bars represent BM-MSCs and grey bars AT-MSCs. Representative alizarin red staining of BM-MSCs during the induction period (e-h). Representative alizarin red staining of AT-MSCs during the induction period(i-l).Statistically significant differences between differentiated cells relative to the controls cells (* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$) and between BM-MSCs and AT-MSCs(# $p< 0.05$, ## $p< 0.01$).

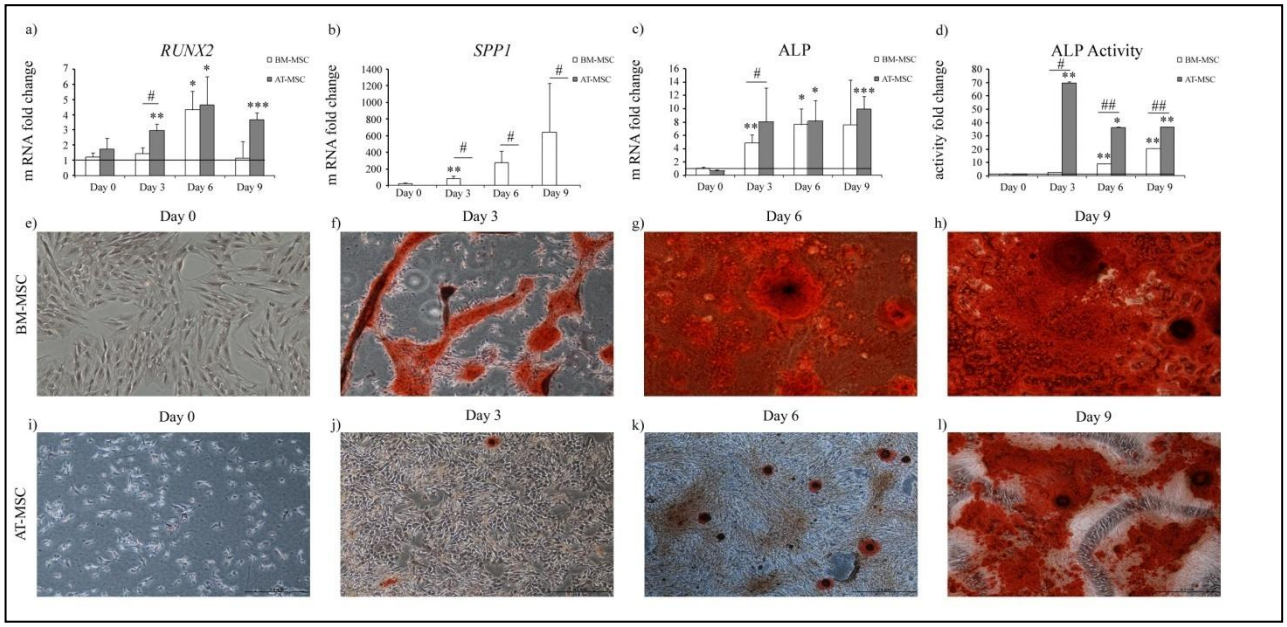


Figure 2.Time course of adipogenic differentiation of equine BM-MSCs and AT-MSCs. The mean ± s.e.of the mRNA expression levels of differentiated cells with respect to controls is shown for *PPARγ* (a) and *LPL* (b). Black horizontal line represents expression levels for control cultures (values set to 1). White bars represent BM-MSCs and grey bars AT-MSCs. Representative oil red O staining of BM-MSCs (c-f) and AT-MSCs (g-j) during the induction period. Statistically significant differences betweendifferentiated and controls cells (* $p < 0.05$) and between BM-MSCs and AT-MSCs (# $p < 0.05$).

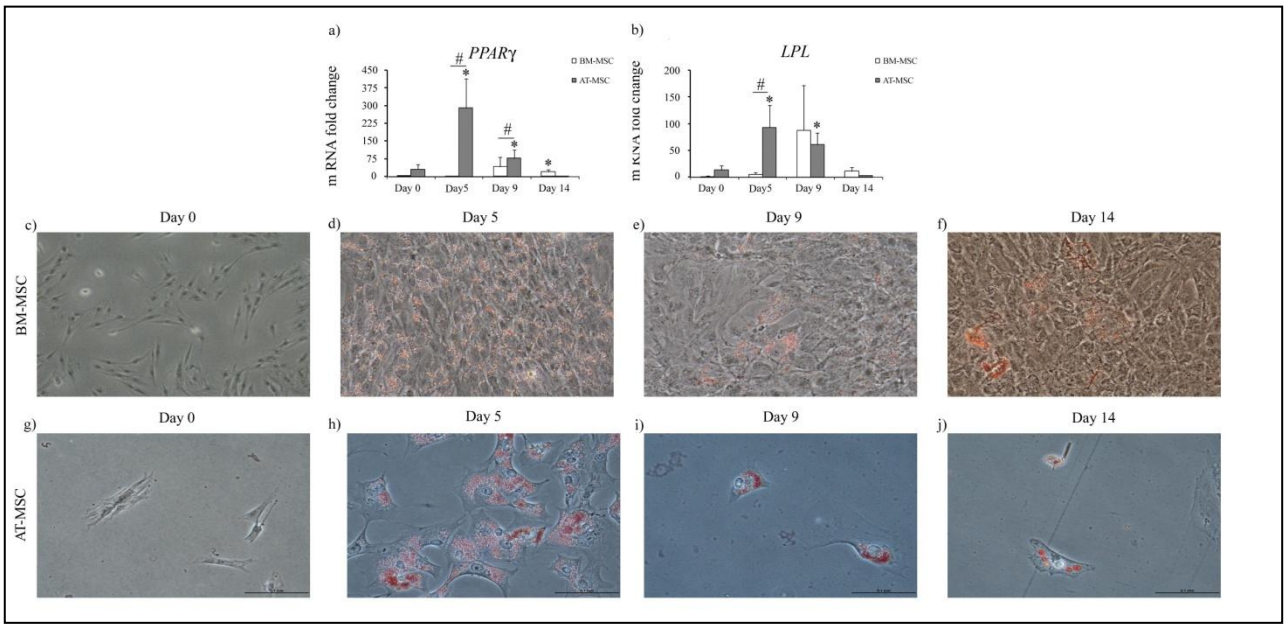
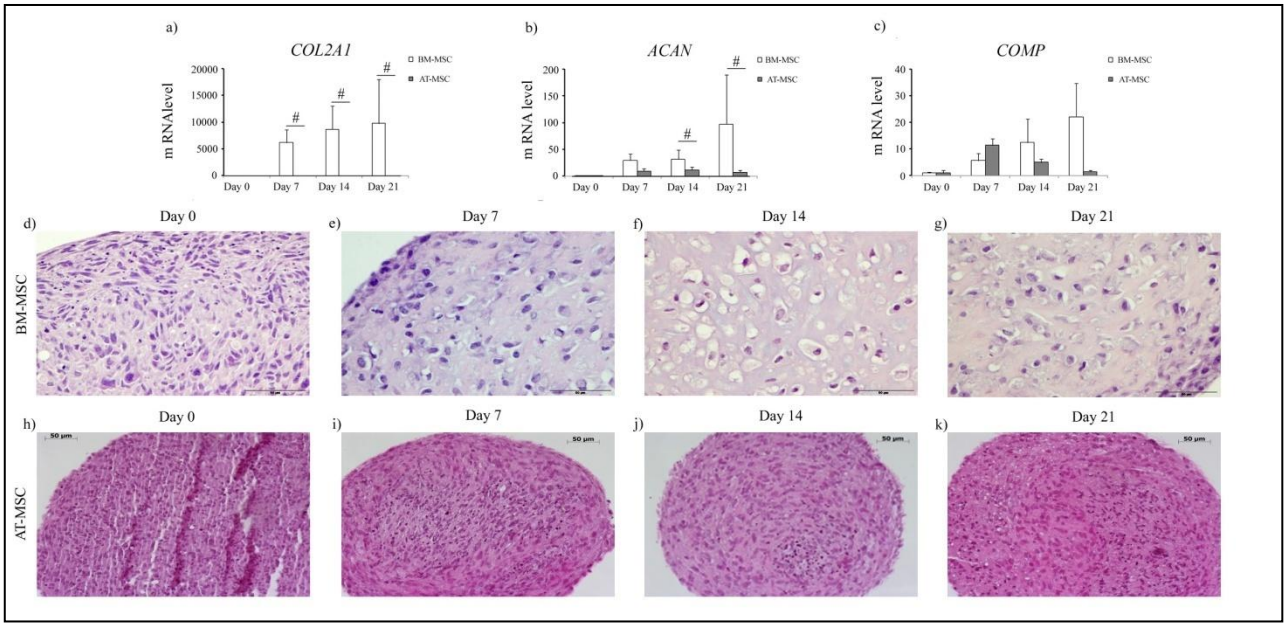


Figure 3. Time course of chondrogenic differentiation of equine BM-MSCs and AT-MSCs.

Mean ± standard error (s.e.) of the mRNA expression levels of differentiated cells with respect to controls is shown for *COL2A1* (a), *ACAN* (b) and*COMP* (c). White bars represent BM-MSCs and grey bars AT-MSCs. Representative Haematoxylin and eosin staining of BM-MSCs (d-g) and AT-MSCs (h-k) during the induction period. Statistically significant differences between BM-MSCs and AT-MSCs (# $p < 0.05$).



6. CONCLUSIONES

1. El incremento del tiempo que transcurre entre la extracción de la médula ósea y el aislamiento de BM-MSCs disminuye la formación de CFU en cultivo.
2. Las AT-MSCs muestran mayor capacidad de proliferación en cultivo que las BM-MSCs, si bien la viabilidad de ambos tipos celulares en cultivo es similar.
3. Aunque se observan grandes diferencias individuales, las MSCs equinas presentan elevado potencial osteogénico. La diferenciación hacia este linaje se produce de forma más rápida que en las BM-MSCs que en las AT-MSCs. De forma similar, el potencial de diferenciación condrogénico es mayor en las BM-MSCs que en las AT-MSCs. Por el contrario, el potencial adipogénico de las MSCs equinas es más limitado, pero similar para los dos tipos celulares.
4. Las BM-MSCs y AT-MSCs muestran un inmunofenotipo similar respecto a la expresión de antígenos de superficie. El análisis de la expresión génica confirmó la presencia de los marcadores mesenquimales y la ausencia de los marcadores hematopoyéticos y endoteliales en los dos tipos celulares, aunque se encontraron diferencias en la expresión entre MSCs de diferentes orígenes.
5. La expansión en hipoxia influye en la división celular de las BM-MSCs y en la viabilidad de las AT-MSCs, dando lugar a un número menor de células que en los cultivos en normoxia. Por otra parte, el nivel bajo de oxígeno en los cultivos de BM-MSCs no altera la capacidad de expansión, inmunofenotipo o potencial de diferenciación osteogénico y adipogénico; sin embargo, potencia su capacidad de diferenciación hacia condrocito.
6. Las MSCs equinas expresan a nivel de transcritos los marcadores de pluripotencialidad *SOX2*, *OCT4* y *NANOG*, siendo sus niveles generalmente mayores en las AT-MSCs que en las BM-MSCs. La expansión en hipoxia tiende a incrementar la expresión de estos marcadores lo que indicaría que las MSCs equinas cultivadas en estas condiciones se mantienen en un estado más indiferenciado.

CONCLUSIONS

1. The increased time between the bone marrow harvesting and the BM-MSC isolation decreases the number of CFU in the culture.
2. The AT-MSCs display higher proliferative ability than BM-MSCs in culture, although the viability of both cell types similar.
3. Even though large individual differences may be observed, the equine MSCs in general have high osteogenic potential. The differentiation towards this lineage is faster for BM-MSCs than for AT-MSCs. Similarly, chondrogenic potential is more pronounced in BM-MSCs than in AT-MSCs. In contrast, adipogenic potential of equine MSCs is more limited but similar for both cell types studied.
4. BM-MSCs and AT-MSCs display similar immunophenotype with respect to the expression of cell surface antigens. The gene expression analysis confirmed the presence of the mesenchymal markers and the absence of the hematopoietic and the endothelial markers in both cell types. However, relative differences in gene expression were found between the MSCs derived from different sources.
5. The cell expansion under hypoxic conditions influences on cell division in BM-MSCs and in the viability of AT-MSCs, yielding to a lower number of cells in hypoxic cultures. On the other hand, the low oxygen level in BM-MSCs cultures does not alter the expansion ability, immunophenotype or osteogenic and adipogenic potentials; however, hypoxia enhances the ability to differentiate into chondrocyte.
6. Equine MSCs express the pluripotency marker transcripts of SOX2, OCT4 and NANOG, their levels being generally higher in AT-MSCs than in BM-MSCs. The expansion in hypoxia tends to increase the expression of these markers, which may indicate that equine MSCs cultured in these conditions are maintained in a more undifferentiated state.

7. BIBLIOGRAFÍA

1. TRAGSEGA, S.A.y.S.G., *Estudio y caracterización del sector equino en España*. 2003.
2. www.horsecouncil.org. *National Economic Impact of the U.S. Horse Industry*. 2005.
3. Frisbie, D.D. and R.K. Smith, *Clinical update on the use of mesenchymal stem cells in equine orthopaedics.* Equine Vet J, 2010. **42**(1): p. 86-9.
4. Smith, R.K., *Mesenchymal stem cell therapy for equine tendinopathy.* Disabil Rehabil, 2008. **30**(20-22): p. 1752-8.
5. Richardson, L.E., et al., *Stem cells in veterinary medicine--attempts at regenerating equine tendon after injury.* Trends Biotechnol, 2007. **25**(9): p. 409-16.
6. Williams, I.F., A. Heaton, and K.G. McCullagh, *Cell morphology and collagen types in equine tendon scar.* Res Vet Sci, 1980. **28**(3): p. 302-10.
7. Koch, T.G., L.C. Berg, and D.H. Betts, *Concepts for the clinical use of stem cells in equine medicine.* Can Vet J, 2008. **49**(10): p. 1009-17.
8. Lo, B. and L. Parham, *Ethical issues in stem cell research.* Endocr Rev, 2009. **30**(3): p. 204-13.
9. Caplan, A.I., *Adult mesenchymal stem cells for tissue engineering versus regenerative medicine.* J Cell Physiol, 2007. **213**(2): p. 341-7.
10. Godwin, E.E., et al., *Implantation of bone marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon.* Equine Vet J, 2011. **44**(1): p. 25-32.
11. Mohyeldin, A., T. Garzon-Muvdi, and A. Quinones-Hinojosa, *Oxygen in stem cell biology: a critical component of the stem cell niche.* Cell Stem Cell, 2010. **7**(2): p. 150-61.
12. Csete, M., *Oxygen in the cultivation of stem cells.* Ann N Y Acad Sci, 2005. **1049**: p. 1-8.
13. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-6.
14. Chen, L. and G.Q. Daley, *Molecular basis of pluripotency.* Hum Mol Genet, 2008. **17**(R1): p. R23-7.
15. Verfaillie, C.M., *Adult stem cells: assessing the case for pluripotency.* Trends Cell Biol, 2002. **12**(11): p. 502-8.
16. Dattena, M., et al., *Sheep embryonic stem-like cells transplanted in full-thickness cartilage defects.* J Tissue Eng Regen Med, 2009. **3**(3): p. 175-87.
17. Shamblott, M.J., et al., *Derivation of pluripotent stem cells from cultured human primordial germ cells.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13726-31.
18. Bongso, A.L., E.H., ed. *Stem cells: their definition, classification and sources*. Stem Cells: From Benchtop to Bedside. Vol. 5. 2005, World Scientific.
19. Cattaneo, E. and R. McKay, *Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor.* Nature, 1990. **347**(6295): p. 762-5.
20. Bergmann, O., et al., *Evidence for cardiomyocyte renewal in humans.* Science, 2009. **324**(5923): p. 98-102.

21. Toma, J.G., et al., *Isolation of multipotent adult stem cells from the dermis of mammalian skin.* Nat Cell Biol, 2001. **3**(9): p. 778-84.
22. Prockop, D.J., *Marrow stromal cells as stem cells for nonhematopoietic tissues.* Science, 1997. **276**(5309): p. 71-4.
23. Weissman, I.L., D.J. Anderson, and F. Gage, *Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations.* Annu Rev Cell Dev Biol, 2001. **17**: p. 387-403.
24. Lagasse, E., et al., *Purified hematopoietic stem cells can differentiate into hepatocytes in vivo.* Nat Med, 2000. **6**(11): p. 1229-34.
25. Till, J.E. and C.E. Mc, *A direct measurement of the radiation sensitivity of normal mouse bone marrow cells.* Radiat Res, 1961. **14**: p. 213-22.
26. Bronzi, D., et al., *Neural markers expression in rat bone marrow mesenchymal stem cell cultures treated with neurosteroids.* Neurochem Res. **35**(12): p. 2154-60.
27. Karnieli, O., et al., *Generation of insulin-producing cells from human bone marrow mesenchymal stem cells by genetic manipulation.* Stem Cells, 2007. **25**(11): p. 2837-44.
28. Ishikawa, T., et al., *Stem cells for hepatic regeneration: the role of adipose tissue derived mesenchymal stem cells.* Curr Stem Cell Res Ther, 2011. **5**(2): p. 182-9.
29. Ksiazek, K., *A comprehensive review on mesenchymal stem cell growth and senescence.* Rejuvenation Res, 2009. **12**(2): p. 105-16.
30. Friedenstein, A.J., R.K. Chailakhjan, and K.S. Lalykina, *The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells.* Cell Tissue Kinet, 1970. **3**(4): p. 393-403.
31. Horwitz, E.M., et al., *Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement.* Cytotherapy, 2005. **7**(5): p. 393-5.
32. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.* Cytotherapy, 2006. **8**(4): p. 315-7.
33. Mafi, R., et al., *Sources of adult mesenchymal stem cells applicable for musculoskeletal applications - a systematic review of the literature.* Open Orthop J, 2011. **5 Suppl 2**: p. 242-8.
34. Zuk, P.A., et al., *Multilineage cells from human adipose tissue: implications for cell-based therapies.* Tissue Eng, 2001. **7**(2): p. 211-28.
35. Caspar-Bauguil, S., et al., *Adipose tissues as an ancestral immune organ: site-specific change in obesity.* FEBS Lett, 2005. **579**(17): p. 3487-92.
36. Schaffler, A. and C. Buchler, *Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies.* Stem Cells, 2007. **25**(4): p. 818-27.
37. Sharpless, N.E. and R.A. DePinho, *How stem cells age and why this makes us grow old.* Nat Rev Mol Cell Biol, 2007. **8**(9): p. 703-13.
38. Liu, T.M., et al., *Identification of common pathways mediating differentiation of bone marrow- and adipose tissue-derived human mesenchymal stem cells into three mesenchymal lineages.* Stem Cells, 2007. **25**(3): p. 750-60.
39. Bunnell, B.A., et al., *Differentiation of adipose stem cells.* Methods Mol Biol, 2008. **456**: p. 155-71.
40. Zvaifler, N.J., et al., *Mesenchymal precursor cells in the blood of normal individuals.* Arthritis Res, 2000. **2**(6): p. 477-88.

41. Lama, V.N., et al., *Evidence for tissue-resident mesenchymal stem cells in human adult lung from studies of transplanted allografts.* J Clin Invest, 2007. **117**(4): p. 989-96.
42. Adachi, N., et al., *Muscle derived, cell based ex vivo gene therapy for treatment of full thickness articular cartilage defects.* J Rheumatol, 2002. **29**(9): p. 1920-30.
43. Sekiya, I., et al., *[Articular cartilage regeneration with synovial mesenchymal stem cells].* Clin Calcium, 2011. **21**(6): p. 879-89.
44. Estrela, C., et al., *Mesenchymal stem cells in the dental tissues: perspectives for tissue regeneration.* Braz Dent J. **22**(2): p. 91-8.
45. Robinson, S.N., et al., *Mesenchymal stem cells in ex vivo cord blood expansion.* Best Pract Res Clin Haematol. **24**(1): p. 83-92.
46. Mitchell, K.E., et al., *Matrix cells from Wharton's jelly form neurons and glia.* Stem Cells, 2003. **21**(1): p. 50-60.
47. Klein, J.D. and D.O. Fauza, *Amniotic and placental mesenchymal stem cell isolation and culture.* Methods Mol Biol. **698**: p. 75-88.
48. Knoepfler, P.S., *Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine.* Stem Cells, 2009. **27**(5): p. 1050-6.
49. Grinnemo, K.H., et al., *Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium.* J Thorac Cardiovasc Surg, 2004. **127**(5): p. 1293-300.
50. Krampera, M., et al., *Mesenchymal stem cells: from biology to clinical use.* Blood Transfus, 2007. **5**(3): p. 120-9.
51. Phinney, D.G. and D.J. Prockop, *Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views.* Stem Cells, 2007. **25**(11): p. 2896-902.
52. Pereira, R.F., et al., *Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice.* Proc Natl Acad Sci U S A, 1995. **92**(11): p. 4857-61.
53. Horwitz, E.M., et al., *Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta.* Nat Med, 1999. **5**(3): p. 309-13.
54. Shelbourne, K.D., S. Jari, and T. Gray, *Outcome of untreated traumatic articular cartilage defects of the knee: a natural history study.* J Bone Joint Surg Am, 2003. **85-A Suppl 2**: p. 8-16.
55. Lotz, M., *Cytokines in cartilage injury and repair.* Clin Orthop Relat Res, 2001(391 Suppl): p. S108-15.
56. Hangody, L., et al., *Mosaicplasty for the treatment of articular defects of the knee and ankle.* Clin Orthop Relat Res, 2001(391 Suppl): p. S328-36.
57. Brittberg, M., et al., *Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation.* N Engl J Med, 1994. **331**(14): p. 889-95.
58. Tins, B.J., et al., *Autologous chondrocyte implantation in knee joint: MR imaging and histologic features at 1-year follow-up.* Radiology, 2005. **234**(2): p. 501-8.
59. Mizuno, K., et al., *Exogenous synovial stem cells adhere to defect of meniscus and differentiate into cartilage cells.* J Med Dent Sci, 2008. **55**(1): p. 101-11.
60. Murphy, J.M., K.W. Kavalkovitch, and D. Fink, *Regeneration of meniscal tissue and protection of articulat cartilage by injection of mesenchymal stem cells.* Osteoarthritis Cartilage, 2000. **8**(Supp. B S25).

61. Koga, H., et al., *Mesenchymal stem cell-based therapy for cartilage repair: a review.* Knee Surg Sports Traumatol Arthrosc, 2009. **17**(11): p. 1289-97.
62. Maffulli, N., J. Wong, and L.C. Almekinders, *Types and epidemiology of tendinopathy.* Clin Sports Med, 2003. **22**(4): p. 675-92.
63. Brockis, J.G., *The blood supply of the flexor and extensor tendons of the fingers in man.* J Bone Joint Surg Br, 1953. **35-B**(1): p. 131-8.
64. Kannus, P., *Structure of the tendon connective tissue.* Scand J Med Sci Sports, 2000. **10**(6): p. 312-20.
65. Favata, M., et al., *Regenerative properties of fetal sheep tendon are not adversely affected by transplantation into an adult environment.* J Orthop Res, 2006. **24**(11): p. 2124-32.
66. Goh, J.C., et al., *Tissue-engineering approach to the repair and regeneration of tendons and ligaments.* Tissue Eng, 2003. **9 Suppl 1**: p. S31-44.
67. Dahlgren, L.A., et al., *Insulin-like growth factor-I improves cellular and molecular aspects of healing in a collagenase-induced model of flexor tendinitis.* J Orthop Res, 2002. **20**(5): p. 910-9.
68. Dines, J.S., et al., *The effect of growth differentiation factor-5-coated sutures on tendon repair in a rat model.* J Shoulder Elbow Surg, 2007. **16**(5 Suppl): p. S215-21.
69. Eppley, B.L., J.E. Woodell, and J. Higgins, *Platelet quantification and growth factor analysis from platelet-rich plasma: implications for wound healing.* Plast Reconstr Surg, 2004. **114**(6): p. 1502-8.
70. Kajikawa, Y., et al., *Platelet-rich plasma enhances the initial mobilization of circulation-derived cells for tendon healing.* J Cell Physiol, 2008. **215**(3): p. 837-45.
71. Bosch, G., et al., *The effect of platelet-rich plasma on the neovascularization of surgically created equine superficial digital flexor tendon lesions.* Scand J Med Sci Sports, 2010. **21**(4): p. 554-61.
72. de Vos, R.J., et al., *Platelet-rich plasma injection for chronic Achilles tendinopathy: a randomized controlled trial.* JAMA. **303**(2): p. 144-9.
73. Sanchez, M., et al., *Comparison of surgically repaired Achilles tendon tears using platelet-rich fibrin matrices.* Am J Sports Med, 2007. **35**(2): p. 245-51.
74. Chong, A.K., et al., *Bone marrow-derived mesenchymal stem cells influence early tendon-healing in a rabbit achilles tendon model.* J Bone Joint Surg Am, 2007. **89**(1): p. 74-81.
75. Awad, H.A., et al., *Repair of patellar tendon injuries using a cell-collagen composite.* J Orthop Res, 2003. **21**(3): p. 420-31.
76. Young, R.G., et al., *Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair.* J Orthop Res, 1998. **16**(4): p. 406-13.
77. Lee, J.Y., et al., *BMP-12 treatment of adult mesenchymal stem cells in vitro augments tendon-like tissue formation and defect repair in vivo.* PLoS One, 2011. **6**(3): p. e17531.
78. Kessler, P.D. and B.J. Byrne, *Myoblast cell grafting into heart muscle: cellular biology and potential applications.* Annu Rev Physiol, 1999. **61**: p. 219-42.
79. Menasche, P., et al., *Myoblast transplantation for heart failure.* Lancet, 2001. **357**(9252): p. 279-80.
80. Saito, T., et al., *Myogenic Expression of Mesenchymal Stem Cells within Myotubes of mdx Mice in Vitro and in Vivo.* Tissue Eng, 1995. **1**(4): p. 327-43.

81. Toma, C., et al., *Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart.* Circulation, 2002. **105**(1): p. 93-8.
82. Chen, S.L., et al., *Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction.* Am J Cardiol, 2004. **94**(1): p. 92-5.
83. Miyahara, Y., et al., *Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction.* Nat Med, 2006. **12**(4): p. 459-65.
84. Horowitz, M.M., et al., *Graft-versus-leukemia reactions after bone marrow transplantation.* Blood, 1990. **75**(3): p. 555-62.
85. Dickinson, A. and A. Cant, *Haemopoietic stem-cell transplantation: improving immune reconstitution, avoiding graft-versus-host disease.* Lancet, 2002. **360**(9327): p. 98-9.
86. Maitra, B., et al., *Human mesenchymal stem cells support unrelated donor hematopoietic stem cells and suppress T-cell activation.* Bone Marrow Transplant, 2004. **33**(6): p. 597-604.
87. Le Blanc, K., *Immunomodulatory effects of fetal and adult mesenchymal stem cells.* Cytotherapy, 2003. **5**(6): p. 485-9.
88. Le Blanc, K., et al., *Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells.* Lancet, 2004. **363**(9419): p. 1439-41.
89. Deng, W., et al., *Effects of allogeneic bone marrow-derived mesenchymal stem cells on T and B lymphocytes from BXSB mice.* DNA Cell Biol, 2005. **24**(7): p. 458-63.
90. Weil, B.R., et al., *The immunomodulatory properties of mesenchymal stem cells: implications for surgical disease.* J Surg Res, 2011. **167**(1): p. 78-86.
91. Frisbie, D.D., M.W. Cross, and C.W. McIlwraith, *A comparative study of articular cartilage thickness in the stifle of animal species used in human pre-clinical studies compared to articular cartilage thickness in the human knee.* Vet Comp Orthop Traumatol, 2006. **19**(3): p. 142-6.
92. Fiester, A., H. Scholer, and A. Caplan, *Stem cell therapies: time to talk to the animals.* Cloning Stem Cells, 2004. **6**(1): p. 3-4.
93. Frisbie, D.D., *Future directions in treatment of joint disease in horses.* Vet Clin North Am Equine Pract, 2005. **21**(3): p. 713-24, viii.
94. Frisbie, D.D., et al., *Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis.* J Orthop Res, 2009. **27**(12): p. 1675-80.
95. Pool, R.R. and D.M. Meagher, *Pathologic findings and pathogenesis of racetrack injuries.* Vet Clin North Am Equine Pract, 1990. **6**(1): p. 1-30.
96. Frisbie, D.D., et al., *Arthroscopic subchondral bone plate microfracture technique augments healing of large chondral defects in the radial carpal bone and medial femoral condyle of horses.* Vet Surg, 1999. **28**(4): p. 242-55.
97. Barnewitz, D., et al., *Treatment of articular cartilage defects in horses with polymer-based cartilage tissue engineering grafts.* Biomaterials, 2006. **27**(14): p. 2882-9.
98. Nixon, A.J., et al., *Enhanced repair of extensive articular defects by insulin-like growth factor-I-laden fibrin composites.* J Orthop Res, 1999. **17**(4): p. 475-87.
99. Wilke, M.M., D.V. Nydam, and A.J. Nixon, *Enhanced early chondrogenesis in articular defects following arthroscopic mesenchymal stem cell implantation in an equine model.* J Orthop Res, 2007. **25**(7): p. 913-25.

100. Carrade, D.D., et al., *Clinicopathologic findings following intra-articular injection of autologous and allogeneic placentally derived equine mesenchymal stem cells in horses.* Cytotherapy, 2010. **13**(4): p. 419-30.
101. Wilson, A.M., et al., *Horses damp the spring in their step.* Nature, 2001. **414**(6866): p. 895-9.
102. Smith, R.K., et al., *The influence of ageing and exercise on tendon growth and degeneration--hypotheses for the initiation and prevention of strain-induced tendinopathies.* Comp Biochem Physiol A Mol Integr Physiol, 2002. **133**(4): p. 1039-50.
103. Dahlgren, L.A., H.O. Mohammed, and A.J. Nixon, *Temporal expression of growth factors and matrix molecules in healing tendon lesions.* J Orthop Res, 2005. **23**(1): p. 84-92.
104. Crevier-Denoix, N., et al., *Mechanical properties of pathological equine superficial digital flexor tendons.* Equine Vet J Suppl, 1997(23): p. 23-6.
105. Smith, R.K., et al., *Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment.* Equine Vet J, 2003. **35**(1): p. 99-102.
106. Crovace, A., et al., *Cell therapy for tendon repair in horses: an experimental study.* Vet Res Commun, 2007. **31 Suppl 1**: p. 281-3.
107. Pacini, S., et al., *Suspension of bone marrow-derived undifferentiated mesenchymal stromal cells for repair of superficial digital flexor tendon in race horses.* Tissue Eng, 2007. **13**(12): p. 2949-55.
108. Schnabel, L.V., et al., *Mesenchymal stem cells and insulin-like growth factor-I gene-enhanced mesenchymal stem cells improve structural aspects of healing in equine flexor digitorum superficialis tendons.* J Orthop Res, 2009. **27**(10): p. 1392-8.
109. Nixon, A.J., et al., *Effect of adipose-derived nucleated cell fractions on tendon repair in horses with collagenase-induced tendinitis.* Am J Vet Res, 2008. **69**(7): p. 928-37.
110. Watts, A.E., et al., *Fetal derived embryonic-like stem cells improve healing in a large animal flexor tendonitis model.* Stem Cell Res Ther. **2**(1): p. 4.
111. Bruder, S.P., N. Jaiswal, and S.E. Haynesworth, *Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation.* J Cell Biochem, 1997. **64**(2): p. 278-94.
112. Schaffler, A., et al., *Potential of adipocytokines in predicting peripancreatic necrosis and severity in acute pancreatitis: pilot study.* J Gastroenterol Hepatol, 2007. **22**(3): p. 326-34.
113. Oedayrajsingh-Varma, M.J., et al., *Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure.* Cytotherapy, 2006. **8**(2): p. 166-77.
114. Bianchi, G., et al., *Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2.* Exp Cell Res, 2003. **287**(1): p. 98-105.
115. Zaragosi, L.E., G. Ailhaud, and C. Dani, *Autocrine fibroblast growth factor 2 signaling is critical for self-renewal of human multipotent adipose-derived stem cells.* Stem Cells, 2006. **24**(11): p. 2412-9.
116. Tuan, R.S., G. Boland, and R. Tuli, *Adult mesenchymal stem cells and cell-based tissue engineering.* Arthritis Res Ther, 2003. **5**(1): p. 32-45.

117. Baksh, D., L. Song, and R.S. Tuan, *Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy.* J Cell Mol Med, 2004. **8**(3): p. 301-16.
118. Gronthos, S., et al., *Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow.* J Cell Sci, 2003. **116**(Pt 9): p. 1827-35.
119. Igura, K., et al., *Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta.* Cytotherapy, 2004. **6**(6): p. 543-53.
120. Kyurkchiev, S., A. Shterev, and R. Dimitrov, *Assessment of presence and characteristics of multipotent stromal cells in human endometrium and decidua.* Reprod Biomed Online. **20**(3): p. 305-13.
121. Puissant, B., et al., *Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells.* Br J Haematol, 2005. **129**(1): p. 118-29.
122. Noel, D., et al., *Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials.* Exp Cell Res, 2008. **314**(7): p. 1575-84.
123. Kern, S., et al., *Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue.* Stem Cells, 2006. **24**(5): p. 1294-301.
124. Deans, R.J. and A.B. Moseley, *Mesenchymal stem cells: biology and potential clinical uses.* Exp Hematol, 2000. **28**(8): p. 875-84.
125. Torensma, R., et al., *Ceramic hydroxyapatite coating on titanium implants drives selective bone marrow stromal cell adhesion.* Clin Oral Implants Res, 2003. **14**(5): p. 569-77.
126. De Schauwer, C., et al., *Markers of stemness in equine mesenchymal stem cells: a plea for uniformity.* Theriogenology, 2010. **75**(8): p. 1431-43.
127. Kadiyala, S., et al., *Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro.* Cell Transplant, 1997. **6**(2): p. 125-34.
128. Awad, H.A., et al., *Autologous mesenchymal stem cell-mediated repair of tendon.* Tissue Eng, 1999. **5**(3): p. 267-77.
129. Galmiche, M.C., et al., *Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway.* Blood, 1993. **82**(1): p. 66-76.
130. Dennis, J.E., et al., *A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse.* J Bone Miner Res, 1999. **14**(5): p. 700-9.
131. Pittenger, M.F., et al., *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. **284**(5411): p. 143-7.
132. Muraglia, A., R. Cancedda, and R. Quarto, *Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model.* J Cell Sci, 2000. **113 (Pt 7)**: p. 1161-6.
133. Sekiya, I., et al., *Adipogenic differentiation of human adult stem cells from bone marrow stroma (MSCs).* J Bone Miner Res, 2004. **19**(2): p. 256-64.
134. MacDougald, O.A. and M.D. Lane, *Transcriptional regulation of gene expression during adipocyte differentiation.* Annu Rev Biochem, 1995. **64**: p. 345-73.
135. Katz, A.J., et al., *Emerging approaches to the tissue engineering of fat.* Clin Plast Surg, 1999. **26**(4): p. 587-603, viii.

136. Kang, X., Y. Xie, and D.A. Kniss, *Adipose tissue model using three-dimensional cultivation of preadipocytes seeded onto fibrous polymer scaffolds.* Tissue Eng, 2005. **11**(3-4): p. 458-68.
137. Rosen, E.D. and B.M. Spiegelman, *Peroxisome proliferator-activated receptor gamma ligands and atherosclerosis: ending the heartache.* J Clin Invest, 2000. **106**(5): p. 629-31.
138. Romero-Prado, M., et al., *Functional characterization of human mesenchymal stem cells that maintain osteochondral fates.* J Cell Biochem, 2006. **98**(6): p. 1457-70.
139. Guillot, P.V., et al., *Comparative osteogenic transcription profiling of various fetal and adult mesenchymal stem cell sources.* Differentiation, 2008. **76**(9): p. 946-57.
140. Frank, O., et al., *Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro.* J Cell Biochem, 2002. **85**(4): p. 737-46.
141. Kassem, M., B.M. Abdallah, and H. Saeed, *Osteoblastic cells: differentiation and trans-differentiation.* Arch Biochem Biophys, 2008. **473**(2): p. 183-7.
142. Franceschi, R.T., *The role of ascorbic acid in mesenchymal differentiation.* Nutr Rev, 1992. **50**(3): p. 65-70.
143. Chung, C.H., et al., *Mechanism of action of beta-glycerophosphate on bone cell mineralization.* Calcif Tissue Int, 1992. **51**(4): p. 305-11.
144. Bonewald, L.F., et al., *von Kossa staining alone is not sufficient to confirm that mineralization in vitro represents bone formation.* Calcif Tissue Int, 2003. **72**(5): p. 537-47.
145. Bi, W., et al., *Sox9 is required for cartilage formation.* Nat Genet, 1999. **22**(1): p. 85-9.
146. Grigolo, B., et al., *Molecular and immunohistological characterization of human cartilage two years following autologous cell transplantation.* J Bone Joint Surg Am, 2005. **87**(1): p. 46-57.
147. Barry, F., et al., *Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components.* Exp Cell Res, 2001. **268**(2): p. 189-200.
148. Haleem-Smith, H., et al., *Cartilage oligomeric matrix protein enhances matrix assembly during chondrogenesis of human mesenchymal stem cells.* J Cell Biochem.
149. Rucklidge, G.J., G. Milne, and S.P. Robins, *Collagen type X: a component of the surface of normal human, pig, and rat articular cartilage.* Biochem Biophys Res Commun, 1996. **224**(2): p. 297-302.
150. Solursh, M., *Formation of cartilage tissue in vitro.* J Cell Biochem, 1991. **45**(3): p. 258-60.
151. Sekiya, I., et al., *Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma.* Cell Tissue Res, 2005. **320**(2): p. 269-76.
152. Neame, P.J. and F.P. Barry, *The link proteins.* Experientia, 1993. **49**(5): p. 393-402.
153. Maltman, D.J., S.A. Hardy, and S.A. Przyborski, *Role of mesenchymal stem cells in neurogenesis and nervous system repair.* Neurochem Int. **59**(3): p. 347-56.

154. Deng, Y.B., et al., *Implantation of BM mesenchymal stem cells into injured spinal cord elicits de novo neurogenesis and functional recovery: evidence from a study in rhesus monkeys.* Cytotherapy, 2006. **8**(3): p. 210-4.
155. Trzaska, K.A., et al., *Brain-derived neurotrophic factor facilitates maturation of mesenchymal stem cell-derived dopamine progenitors to functional neurons.* J Neurochem, 2009. **110**(3): p. 1058-69.
156. Sanchez-Ramos, J., et al., *Adult bone marrow stromal cells differentiate into neural cells in vitro.* Exp Neurol, 2000. **164**(2): p. 247-56.
157. Tondreau, T., et al., *Gene expression pattern of functional neuronal cells derived from human bone marrow mesenchymal stromal cells.* BMC Genomics, 2008. **9**: p. 166.
158. Li, Y., et al., *Generation of insulin-producing cells from PDX-1 gene-modified human mesenchymal stem cells.* J Cell Physiol, 2007. **211**(1): p. 36-44.
159. Puglisi, M.A., et al., *Adipose tissue-derived mesenchymal stem cells and hepatic differentiation: old concepts and future perspectives.* Eur Rev Med Pharmacol Sci. **15**(4): p. 355-64.
160. Brandau, S., et al., *Tissue-resident mesenchymal stem cells attract peripheral blood neutrophils and enhance their inflammatory activity in response to microbial challenge.* J Leukoc Biol. **88**(5): p. 1005-15.
161. Aggarwal, S. and M.F. Pittenger, *Human mesenchymal stem cells modulate allogeneic immune cell responses.* Blood, 2005. **105**(4): p. 1815-22.
162. Vidal, M.A., et al., *Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity.* Vet Surg, 2006. **35**(7): p. 601-10.
163. Vidal, M.A., et al., *Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells.* Vet Surg, 2007. **36**(7): p. 613-22.
164. Koerner, J., et al., *Equine peripheral blood-derived progenitors in comparison to bone marrow-derived mesenchymal stem cells.* Stem Cells, 2006. **24**(6): p. 1613-9.
165. Hoynowski, S.M., et al., *Characterization and differentiation of equine umbilical cord-derived matrix cells.* Biochem Biophys Res Commun, 2007. **362**(2): p. 347-53.
166. Koch, T.G., et al., *Isolation of mesenchymal stem cells from equine umbilical cord blood.* BMC Biotechnol, 2007. **7**: p. 26.
167. Mensing, N., et al., *Isolation and characterization of multipotent mesenchymal stromal cells from the gingiva and the periodontal ligament of the horse.* BMC Vet Res, 2011. **7**: p. 42.
168. Taylor, S.E., R.K. Smith, and P.D. Clegg, *Mesenchymal stem cell therapy in equine musculoskeletal disease: scientific fact or clinical fiction?* Equine Vet J, 2007. **39**(2): p. 172-80.
169. Arnhold, S.J., et al., *Isolation and characterization of bone marrow-derived equine mesenchymal stem cells.* Am J Vet Res, 2007. **68**(10): p. 1095-105.
170. Colleoni, S., et al., *Isolation, growth and differentiation of equine mesenchymal stem cells: effect of donor, source, amount of tissue and supplementation with basic fibroblast growth factor.* Vet Res Commun, 2009.
171. Ibrahim, S., et al., *Screening of anti-human leukocyte monoclonal antibodies for reactivity with equine leukocytes.* Vet Immunol Immunopathol, 2007. **119**(1-2): p. 63-80.

172. Braun, J., et al., *Evaluation of the osteogenic and chondrogenic differentiation capacities of equine adipose tissue-derived mesenchymal stem cells.* Am J Vet Res, 2010. **71**(10): p. 1228-36.
173. de Mattos Carvalho, A., et al., *Isolation and immunophenotypic characterization of mesenchymal stem cells derived from equine species adipose tissue.* Vet Immunol Immunopathol, 2009.
174. Pascucci, L., et al., *Flow cytometric characterization of culture expanded multipotent mesenchymal stromal cells (MSCs) from horse adipose tissue: towards the definition of minimal stemness criteria.* Vet Immunol Immunopathol, 2011. **144**(3-4): p. 499-506.
175. Radcliffe, C.H., M.J. Flaminio, and L.A. Fortier, *Temporal analysis of equine bone marrow aspirate during establishment of putative mesenchymal progenitor cell populations.* Stem Cells Dev, 2010. **19**(2): p. 269-82.
176. Guest, D.J., J.C. Ousey, and R.W. Smith, *Defining the expression of marker genes*
in equine mesenchymal stromal cells. Stem Cells and Cloning: Advances and Applications, 2008. **1**(1): p. 1-9.
177. Martinello, T., et al., *Cryopreservation does not affect the stem characteristics of multipotent cells isolated from equine peripheral blood.* Tissue Eng Part C Methods. **16**(4): p. 771-81.
178. Raabe, O., et al., *Further insights into the characterization of equine adipose tissue-derived mesenchymal stem cells.* Vet Res Commun, 2011. **35**(6): p. 355-65.
179. Janderova, L., et al., *Human mesenchymal stem cells as an in vitro model for human adipogenesis.* Obes Res, 2003. **11**(1): p. 65-74.
180. Ahern, B.J., et al., *Evaluation of equine peripheral blood apheresis product, bone marrow, and adipose tissue as sources of mesenchymal stem cells and their differentation potential.* Am J Vet Res. **72**(1): p. 127-33.
181. Toupadakis, C.A., et al., *Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue.* Am J Vet Res, 2011. **71**(10): p. 1237-45.
182. Mambelli, L.I., et al., *Characterization of equine adipose tissue-derived progenitor cells before and after cryopreservation.* Tissue Eng Part C Methods, 2009. **15**(1): p. 87-94.
183. Vidal, M.A., et al., *Comparison of chondrogenic potential in equine mesenchymal stromal cells derived from adipose tissue and bone marrow.* Vet Surg, 2008. **37**(8): p. 713-24.
184. Berg, L., et al., *Chondrogenic potential of mesenchymal stromal cells derived from equine bone marrow and umbilical cord blood.* Vet Comp Orthop Traumatol, 2009. **22**(5): p. 363-70.
185. Stewart, A.A., et al., *Effect of dexamethasone supplementation on chondrogenesis of equine mesenchymal stem cells.* Am J Vet Res, 2008. **69**(8): p. 1013-21.
186. Romanov, Y.A., et al., *Optimum conditions for culturing of human bone marrow and adipose tissue mesenchymal precursor cells.* Bull Exp Biol Med, 2006. **142**(4): p. 515-20.
187. Kosmacheva, S.M., et al., *In vitro growth of human umbilical blood mesenchymal stem cells and their differentiation into chondrocytes and osteoblasts.* Bull Exp Biol Med, 2008. **145**(1): p. 141-5.

188. Bieback, K., et al., *Altered gene expression in human adipose stem cells cultured with fetal bovine serum compared to human supplements.* Tissue Eng Part A. **16**(11): p. 3467-84.
189. Heiskanen, A., et al., *N-glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible.* Stem Cells, 2007. **25**(1): p. 197-202.
190. Ezashi, T., P. Das, and R.M. Roberts, *Low O2 tensions and the prevention of differentiation of hES cells.* Proc Natl Acad Sci U S A, 2005. **102**(13): p. 4783-8.
191. Harris, A.L., *Hypoxia--a key regulatory factor in tumour growth.* Nat Rev Cancer, 2002. **2**(1): p. 38-47.
192. Jauniaux, E., et al., *In-vivo measurement of intrauterine gases and acid-base values early in human pregnancy.* Hum Reprod, 1999. **14**(11): p. 2901-4.
193. Iyer, N.V., et al., *Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha.* Genes Dev, 1998. **12**(2): p. 149-62.
194. Harrison, J.S., et al., *Oxygen saturation in the bone marrow of healthy volunteers.* Blood, 2002. **99**(1): p. 394.
195. Pasarica, M., et al., *Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response.* Diabetes, 2009. **58**(3): p. 718-25.
196. Dos Santos, F., et al., *Ex vivo expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia.* J Cell Physiol, 2010. **223**(1): p. 27-35.
197. Ren, H., et al., *Proliferation and differentiation of bone marrow stromal cells under hypoxic conditions.* Biochem Biophys Res Commun, 2006. **347**(1): p. 12-21.
198. Grayson, W.L., et al., *Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells.* Biochem Biophys Res Commun, 2007. **358**(3): p. 948-53.
199. Volkmer, E., et al., *Hypoxic preconditioning of human mesenchymal stem cells overcomes hypoxia-induced inhibition of osteogenic differentiation.* Tissue Eng Part A, 2010. **16**(1): p. 153-64.
200. Holzwarth, C., et al., *Low physiologic oxygen tensions reduce proliferation and differentiation of human multipotent mesenchymal stromal cells.* BMC Cell Biol, 2010. **11**: p. 11.
201. Chung, D.J., et al., *Osteogenic proliferation and differentiation of canine bone marrow and adipose tissue derived mesenchymal stromal cells and the influence of hypoxia.* Res Vet Sci, 2010.
202. Gardner, L.B., et al., *Hypoxia inhibits G1/S transition through regulation of p27 expression.* J Biol Chem, 2001. **276**(11): p. 7919-26.
203. Koshiji, M., et al., *HIF-1alpha induces cell cycle arrest by functionally counteracting Myc.* EMBO J, 2004. **23**(9): p. 1949-56.
204. Chacko, S.M., et al., *Hypoxic preconditioning induces the expression of prosurvival and proangiogenic markers in mesenchymal stem cells.* Am J Physiol Cell Physiol, 2010. **299**(6): p. C1562-70.
205. Iida, K., et al., *Hypoxia enhances colony formation and proliferation but inhibits differentiation of human dental pulp cells.* Arch Oral Biol, 2010. **55**(9): p. 648-54.
206. Fehrer, C., et al., *Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan.* Aging Cell, 2007. **6**(6): p. 745-57.

207. Nekanti, U., et al., *Increased proliferation and analysis of differential gene expression in human Wharton's jelly-derived mesenchymal stromal cells under hypoxia.* Int J Biol Sci, 2010. **6**(5): p. 499-512.
208. Ma, T., et al., *Hypoxia and stem cell-based engineering of mesenchymal tissues.* Biotechnol Prog, 2009. **25**(1): p. 32-42.
209. Fink, T., et al., *Induction of adipocyte-like phenotype in human mesenchymal stem cells by hypoxia.* Stem Cells, 2004. **22**(7): p. 1346-55.
210. Valorani, M.G., et al., *Hypoxia increases Sca-1/CD44 co-expression in murine mesenchymal stem cells and enhances their adipogenic differentiation potential.* Cell Tissue Res, 2010. **341**(1): p. 111-20.
211. Martin-Rendon, E., et al., *Transcriptional profiling of human cord blood CD133+ and cultured bone marrow mesenchymal stem cells in response to hypoxia.* Stem Cells, 2007. **25**(4): p. 1003-12.
212. Haselgrove, J.C., I.M. Shapiro, and S.F. Silverton, *Computer modeling of the oxygen supply and demand of cells of the avian growth cartilage.* Am J Physiol, 1993. **265**(2 Pt 1): p. C497-506.
213. Grimshaw, M.J. and R.M. Mason, *Bovine articular chondrocyte function in vitro depends upon oxygen tension.* Osteoarthritis Cartilage, 2000. **8**(5): p. 386-92.
214. Markway, B.D., et al., *Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures.* Cell Transplant, 2010. **19**(1): p. 29-42.
215. Zscharnack, M., et al., *Low oxygen expansion improves subsequent chondrogenesis of ovine bone-marrow-derived mesenchymal stem cells in collagen type I hydrogel.* Cells Tissues Organs, 2009. **190**(2): p. 81-93.