UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE MEDICINA

BIOMEDICINA

EVALUACIÓN DE LA CAPACIDAD DE CÉLULAS TRONCALES MESENQUIMALES HUMANAS PROVENIENTES DE CULTIVOS A ESCALA CLÍNICA PARA MANTENER LA FORMACIÓN DE CÉLULAS PROGENITORAS HEMATOPOYÉTICAS

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

FAJARDO ORDUÑA GUADALUPE ROSARIO

TUTOR PRINCIPAL DE TESIS: DR. JUAN JOSÉ MONTESINOS MONTESINOS
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COORDINACIÓN

Lic. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que el Subcomité de Biología Experimental y Biomedicina del Posgrado en Ciencias Biológicas, en su sesión ordinaria del día 22 de agosto de 2016, aprobó el jurado para la presentación del examen para obtener el grado de **DOCTORA EN CIENCIAS** dela alumna **FAJARDO ORDUÑA GUADALUPE ROSARIO** con número de cuenta **300284847**, con la tesis titulada **"EVALUACIÓN DE LA CAPACIDAD DE CÉLULAS TRONCALES MESENQUIMALES HUMANAS PROVENIENTES DE CULTIVOS A ESCALA CLÍNICA PARA MANTENER LA FORMACIÓN DE CÉLULAS PROGENITORAS HEMATOPOYÉTICAS"**, realizada bajo la dirección del **DR. JUAN JOSÉ MONTESINOS MONTESINOS:**

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Suplente:	DR. EDELMIRO SANTIAGO OSORIO

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Cd. Universitaria, Cd, Mx., a 23 de septiembre de 2016

DRA. MARÍA DEL CORO ARIZMENDI ARRIAGA
COORDINADORA DEL PROGRAMA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

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ÍNDICE

LISTA DE ABREVIATURAS

CD:	grupo de diferenciación*
CFC:	célula formadora de colonias
CFC-E:	célula formadora de colonias eritroides
BFC-E:	célula formadora de explosión eritroide*
CFC-M:	célula formadora de colonias de monocitos
CFC-GM:	célula formadora de colonias de granulocitos y monocitos
CMNs:	células mononucleares
GVHD:	enfermedad de injerto contra hospedero*
SSC:	cultivo a pequeña escala*
CSC:	cultivo a escala clínica
HPC:	célula progenitora hematopoyética*
HSC:	célula troncal hematopoyética*
ISCT:	sociedad internacional de terapia celular*
MEC:	matriz extracelular
MO:	médula ósea
MSC:	célula troncal mesenquimal*
SP:	sangre periférica
SCU:	sangre de cordón umbilical
HSCT:	trasplante de células troncales hematopoyéticas*

*****Por sus siglas en inglés

RESUMEN

El potencial de diferenciación, capacidad de soporte hematopoyético y las propiedades inmunomoduladoras de las células troncales mesenquimales (MSCs, del inglés *Mesenchymal Stem Cells*) de médula ósea (MO), las convierte en agentes terapéuticos atractivos para un amplio rango de enfermedades. Actualmente, el principal uso terapéutico de las MSCs en la aplicación clínica ha sido para mejorar el trasplante de células troncales hematopoyéticas (HSCT, del inglés *Hematopoietic Stem Cells Transplantation*) y para tratar la enfermedad de injerto contra hospedero (GVHD, del inglés *Graft vs Host Desease*). Por otro lado, cultivos a escala clínica (CSCs, del inglés *Clinical Scale Cultures*) han sido usados para expandir MSCs de MO para su uso en protocolos de terapia celular, sin embargo, se conoce poco acerca de la funcionalidad de las células expandidas. El principal objetivo de este estudio fue evaluar las características funcionales de MSCs de MO expandidas a partir de CSCs para determinar la calidad de las células para protocolos de terapia celular, principalmente para aquellos de recuperación hematopoyética y tratamiento de la GVHD en el HSCT. Para realizar este proyecto, se analizó la morfología, inmunofonotipo, potencial de diferenciación (adipogénico, osteogénico y condrogénico), capacidad de soporte hematopoyético y capacidad de inmunosupresión de MSCs de MO provenientes de cultivos a pequeña escala (SSCs, del inglés *Short Scale Cultures*) y de CSCs en una manera comparativa. Después de 12 días de cultivo en CSCs, se obtuvieron 125.52 x 10^6 ± 25.6 x 10^6 MSCs de MO, número celular que corresponde al número de MSCs requeridas para HSCT (aproximadamente 1.7 x 10^6 MSCs/kg para un paciente de 70-kg de peso). Después de la expansión, las MSCs de MO mantuvieron un inmunofenotipo característicos de este tipo de células, sin embargo, el CSCs disminuyó su capacidad de diferenciación hacia los linajes adipogénico, osteogénico y condrogénico y su habilidad para inhibir la proliferación de linfocitos T comparado con MSCs de SSCs. Es importante notar que las MSCs de CSCs mantuvieron la habilidad para soportar la proliferación y expansión de células progenitoras hematopoyéticas y la capacidad para producir moléculas involucradas en la regulación de la hematopoyesis. Este estudio, resalta la importancia de evaluar las propiedades funcionales de MSCs de MO cultivadas a gran escala, para verificar su calidad con fines de terapia celular.

ABSTRACT

The differentiation capacity, hematopoietic support and immunomodulatory properties of human bone marrow mesenchymal stromal cells (BM-MSCs) make them attractive therapeutic agents for a wide range of diseases. The principal therapeutic use of MSCs in clinical application have been to improve the hematopoietic stem cells transplantation (HSCT) and to treat the graft versus host disease (GVHD). On the other hand, clinical scale cultures (CSCs) have been used to expand BM-MSCs for their use in cell therapy protocols, however, little is known about the functionality of the expanded cells. The main goal of the present study was to evaluate the functional characteristics of BM-MSCs expanded from CSCs to determine the quality of the cells for cellular therapy protocols, mainly for hematopoietic recovery and treatment for GVHD in the HSCT. To address this issue, we analyzed the morphology, immunophenotype, differentiation potential (adipogenic, osteogenic and chondrogenic), hematopoietic support and immunosuppressive capacity of BM-MSCs from short scale cultures (SSCs) and CSCs in a comparative manner. After 12 days of culture in CSCs, BM-MSCs reached cell numbers of 124.4 x 10^6 ± 27.2 x 10^6 MSCs, which corresponded to the number of cells required for transplantation (approximately 1.7 x 10^6 MSCs/kg for a 70-kg patient). After expansion, BM-MSCs expressed the characteristic inmunophenotype of MSCs, however, expansion decreased their differentiation capacity towards the adipogenic, osteogenic and chondrogenic lineages and their ability to inhibit T cell proliferation compared with SSCs-MSCs. Importantly, CSCs-MSCs maintained the ability to support the proliferation and expansion of hematopoietic progenitor cells and the capacity to express and produce molecules involved in the regulation of hematopoiesis. Our study highlights the need to evaluate the functional properties of the expanded BM-MSCs to verify their quality for cell therapy protocols.

CAPÍTULO I

INTRODUCCIÓN GENERAL

1. Células troncales mesenquimales, identificación y caracterización.

Las células troncales mesenquimales (MSCs del inglés *Mesenchymal Stem Cells*), también llamadas células estromales mesenquimales, son células troncales adultas, que fueron descubiertas en la médula ósea (MO) por Friedenstein y colaboradores en la década de los años sesenta (Friedenstein et al, 1966). En la actualidad, se conocen tres propiedades biológicas y funcionales principales de las MSCs, la cuales se han demostrado *in vitro* e *in vivo*. La primera propiedad es el potencial multilinaje, ya que las MSCs tienen la capacidad de diferenciarse hacia adipocitos, osteoblastos, condroblastos, cardiomiocitos, células epiteliales y células neurales (Kim and Cho, 2013). La segunda propiedad es la capacidad de soporte hematopoyético, ya que, las MSCs producen moléculas que favorecen la formación de células hematopoyéticas (Fajardo-Orduña et al, 2015). La tercera propiedad es la capacidad inmunosupresora mediada por su interacción con los linfocitos y otras células del sistema inmune (Castro-Manreza et al, 2014; Castro-Manrreza and Montesinos, 2015).

Debido a que no se ha identificado una molécula que caracterice de manera específica a las MSCs y que permita obtenerlas de manera selectiva, en el año 2006, la Sociedad Internacional de Terapia Celular (ISCT, del inglés *International Society for Cellular Therapy*), propuso a la comunidad científica adoptar medidas estándares para la identificación de MSCs. Así, la ISCT propone tres criterios: primero, las células deben ser adherentes al plástico cuando son mantenidas en cultivo; segundo, deben ser positivas para los antígenos CD105, CD73 y CD90, adicionalmente deben ser negativas para la expresión de antígenos hematopoyéticos tales como CD45, CD34, CD14, CD11b, CD79 y CD19 así como HLA-DR, y tercero, deben ser capaces de diferenciarse hacia adipocitos, osteoblastos y condroblastos (Dominici et al, 2006).

2. Capacidad de soporte hematopoyético de las células troncales mesenquimales

Las células de la sangre o células hematopoyéticas tienen un tiempo de vida finito, por lo que mantener sus números en condiciones estándar o de amplificación en respuesta a

estrés hematológico, requiere de una actividad hematopoyética constante, experimentando una serie de divisiones clonales a lo largo de la vida de un individuo (Szilvassy, 2003). Para mantener esta actividad constante, las células hematopoyéticas residen en un ambiente especializado llamado microambiente hematopoyético, que regula el proceso de la hematopoyesis (formación de células sanguíneas) a través de interacciones que ocurren entre las células hematopoyéticas y las células del sistema estromal (Adams and Scadden, 2006). El sistema estromal es un conjunto de células que producen moléculas de la matriz extracelular (MEC), moléculas de adhesión y citocinas que regulan la hematopoyesis. Entre los tipos celulares que constituyen el sistema estromal se encuentran los macrófagos (de origen hematopoyético), células endoteliales, adipocitos, osteoblastos y células reticulares, las cuales se originan a partir de las MSCs (Mayani et al, 2007; Fajardo-Orduña et al, 2015).

Dentro del microambiente hematopoyético se ha postulado la existencia de sitios específicos llamados "nichos" de células troncales hematopoyéticas (HSCs por su siglas en inglés), que regulan su autorenovación y diferenciación (Ehninger and Trumpp 2011). A partir de investigaciones realizadas principalmente en modelos murinos, se ha obtenido evidencia de que las MSCs son componentes importantes del microambiente hematopoyético dentro de la MO, y a que además de originar a células del sistema estromal, se ha observado que distintas sub-poblaciones de MSCs se localizan dentro de los diferentes nichos de las HSCs (Fajardo-Orduña et al, 2015), mostrando así un papel importante en la regulación de estas últimas. Las MSCs producen un rango de moléculas reguladoras de la hematopoyesis, dentro de las que se encuentran aquellas que regulan la actividad y favorecen el mantenimiento y autorenovación de las HSCs, así como citocinas involucradas en el proceso de compromiso y maduración de las células hematopoyéticas. Las MSCs también expresan moléculas de adhesión importantes en el contacto con células hematopoyéticas primitivas (Figura1, Fajardo-Orduña et al, 2015). Mientras que se demostró con estudios en modelos animales, la capacidad de las MSCs para mejorar el injerto y la producción de células hematopoyéticas después de la depleción de la MO (Delalat et al , 2009; Angelopoulou et al , 2003; Kim et al , 2006; Muguruma et al, 2006; Almeida-Porada et al, 2000; Liu et al, 2005).

Las MSCs también se han utilizado en sistemas de co-cultivo *in vitro* como capa alimentadora de células hematopoyéticas, con el objetivo de favorecer la expansión de HSCs y células progenitoras hematopoyéticas (HPCs), ello con la finalidad de tener el número suficiente de células para ser utilizadas en el trasplante de células troncales hematopoyéticas (HSCT, por sus siglas en inglés) en humanos adultos (Flores-Guzmán et al, 2009; Mehrasa et al, 2014). Estas células hematopoyéticas provenientes de sistemas de co-cultivos con MSCs ya han sido utilizadas en la clínica obteniendo resultados favorables en la recuperación hematopoyética. Así, las MSCs también pueden mejorar el HSCT de manera indirecta, es decir, incrementando el número de células hematopoyéticas y mejorando su capacidad de injerto previo al trasplante (De Lima et al, 2012).

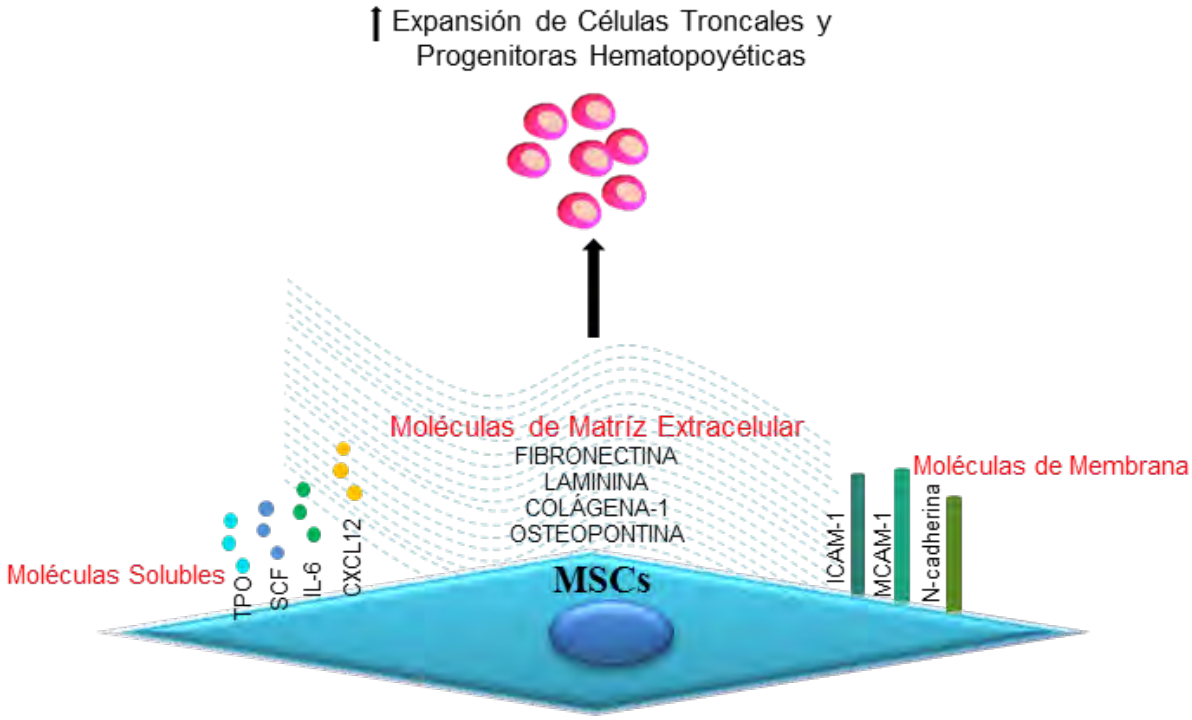

Figura 1. MSCs incrementan la expansión de HPCs y HSCs mediante la expresión de moléculas de membrana, producción de citocinas solubles y de moléculas de la matriz extracelular.

El HSCT es un tratamiento con fines curativos en pacientes con enfermedades hematológicas, el éxito del mismo depende en gran medida del número de HSCs / HPCs

trasplantadas y de la ausencia del rechazo del injerto (Ringdén, 2009). Debido a que las MSCs generan células estromales de la MO, forman parte del nicho de las HSCs, producen moléculas reguladoras de la hematopoyesis y se demostró su capacidad de soporte hematopoyético tanto in vitro como en modelos animales, se han utilizado MSCs en la clínica para mejorar el HSCT. Investigaciones recientes demuestran que las MSCs favorecen el injerto y una recuperación hematopoyética más rápida (número de neutrófilos y plaquetas) en pacientes sometidos a HSCT (Fajardo-Orduña et al, 2015), en donde los beneficios de las MSCs sobre el injerto se relaciona con su capacidad de soporte hematopoyético, especialmente cuando el número de HSCs / HPCs es limitado (Wu et al, 2013 a y b). Se sabe que el estroma de la MO en los pacientes con enfermedades hematológicas, sufre daño importante y prolongado por las altas dosis de quimioterapia y radioterapia antes del HSCT, lo que puede retrasar el injerto de las células hematopoyéticas (Wu et al, 2013 b). Con los datos previos se ha sugerido que las MSCs son capaces de reconstituir el sistema estromal de la MO de los pacientes después de un tratamiento ablativo (Hou et al, 2010; Wang et al, 2013), tal como se reportó en modelos murinos (Muguruma et al, 2006). A pesar de los reportes que indican que las MSCs favorecen el HSCT, también existen reportes en donde la recuperación hematopoyética en los pacientes sometidos a HSCT con MSCs no presentan mejora significativa en comparación con pacientes a los que no se les trasplantaron MSCs (MacMillan et al, 2009; Gonzalo-Daganzo et al, 2009; Bernardo et al, 2011; Fajardo-Orduña et al., 2015), debido a lo cual se requiere más información en este campo.

3. Capacidad de inmunosupresión de las células troncales mesenquimales

Las MSCs poseen propiedades inmunoreguladoras demostradas tanto *in vitro* como *in vivo* en modelos murinos, mismas que actúan sobre células del sistema inmune innato (células asesinas naturales, NK por sus siglas en inglés) y adaptativo (células dendríticas, linfocitos B y linfocitos T; Bernardo et al, 2015). En la respuesta inmune, las MSCs se comunican con el microambiente inflamatorio e interactúan activamente con las células del sistema inmune, expresando moléculas de superficie que incluyen miembros de la familia de integrinas y moléculas de adhesión que promueven interacciones celulares vía unión con su receptor sobre células inmunes (Najar et al, 2016). La capacidad de inmunoregulación de las MSCs se presenta de manera inducida por la presencia de

citocinas inflamatorias secretadas principalmente por células activadas del sistema inmune. Las citocinas influyen en los efectos inmunoreguladores de las MSCs dependiendo del tipo de citocinas y su concentración, indicando que la función inmunoreguladora de las MSCs es plástica (Najar et al, 2016). La presencia de IFN-γ y/o TNF-α pueden influir en las propiedades inmunosupresoras de las MSCs y producir diferentes efectos sobre su función. Mientras que en la presencia de un ambiente inflamatorio las MSCs se activan y adoptan un fenotipo inmunosupresor (MSCs tipo 2), caracterizado por la secreción de concentraciones altas de factores solubles como indolamina 2,3-dioxigenasa (IDO), prostaglandina E2 (PGE2), óxido nítrico (ON), factor de crecimiento transformante β (TGF-β) y el factor de crecimiento de hepatocitos (HGF), en ausencia de un ambiente inflamatorio, las MSCs pueden adoptar un fenotipo pro-inflamatorio (MSCs tipo 1) e incrementar la respuesta de linfocitos T mediante la secreción de citocinas que reclutan linfocitos en sitios de inflamación. El balance entre estas dos rutas ayudan al control de la homeostasis en los tejidos por la promoción de la defensa del huésped o la reparación del tejido (Bernardo et al, 2015).

Por medio de estudios *in vitro* se sabe que tanto MSCs autólogas como alogénicas son capaces de suprimir la proliferación de linfocitos T inducida por aloantígenos, mitógenos y anticuerpos agonistas de CD3 y CD28 (Le Blanc et al, 2003; Di Nicola et al, 2002; Tse et al, 2003; Krampera et al, 2003). Los efectos inmunosupresores de las MSCs se han observado utilizando poblaciones totales de células mononucleadas (CMNs) de sangre periférica (SP) y poblaciones enriquecidas en linfocitos T $CD3^+$, $CD4^+$ o $CD8^+$ y en cada caso se ha demostrado la capacidad de las MSCs para disminuir su proliferación (Castro-Manreza and Montesinos 2015). Los mecanismos a través de los cuales las MSCs llevan a cabo la supresión de la activación y proliferación de linfocitos T involucran la secreción de factores solubles como TGFB1, HGF, IDO, ON, PGE2, interleucina -10 (IL-10) y HLA-G5, además de mecanismos dependientes del contacto MSCs-linfocito T mediante la expresión del ligando de muerte programada-1 (PD-L1) y HLA-G1 (Figura 2. Castro-Manreza and Montesinos 2015). Estudios previos demostraron que las MSCs inhiben la expresión de moléculas de activación tempranas como CD25 y CD69 en células T $CD4^+$ y $CD8^+$ activadas con fitohematoglutinina (PHA; LeBlanc et al, 2004; Groh et al, 2005).
Se sabe que la inhibición de la proliferación de los linfocitos T por parte de las MSCs no se ha asociado con la inducción de apoptosis, sino porque las mantiene en fase G0/G1

del ciclo celular (Le Blanc et al, 2003; Tse et al, 2003). Por otro lado, se reportó que las MSCs son capaces de inhibir la diferenciación de monocitos y células progenitoras hematopoyéticas $CD34^+$ hacia células dendríticas $CD1a^+$, además de disminuir la capacidad de estas células para inducir la activación de linfocitos T. De la misma manera, las MSCs cultivadas con células dendríticas maduras favorece la inducción de células reguladoras presentadoras de antígeno, a través de las cuales pueden suprimir indirectamente la proliferación de linfocitos T (Nauta et al, 2006; Jiang et al, 2005).

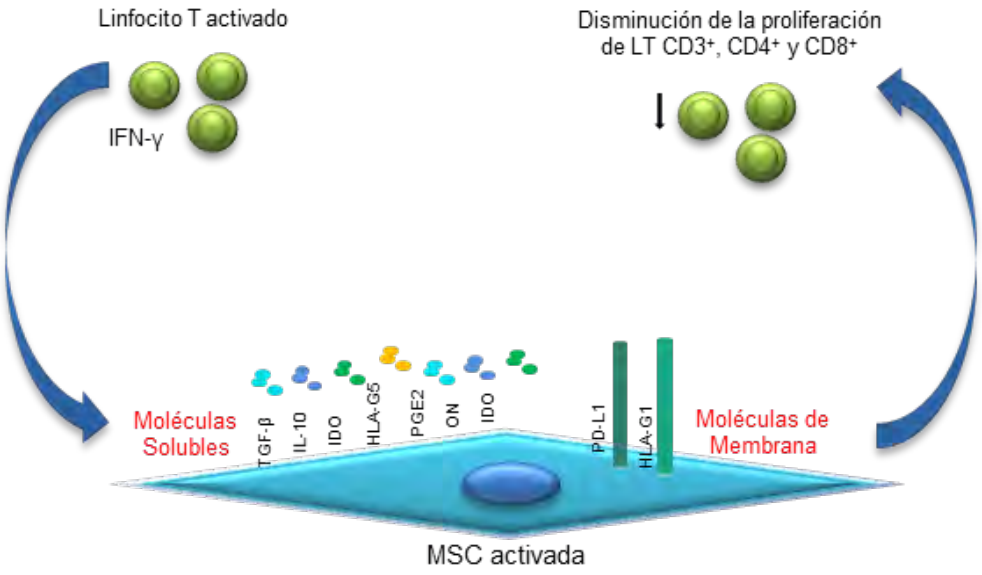

Figura 2. MSCs activadas por un microambiente inflamatorio, inhiben la proliferación de linfocitos T CD3, CD4 y CD8 mediante la producción de citocinas solubles y la expresión de moléculas de membrana.

Mediante estudios *in vitro* también se demostró que las MSCs son capaces de suprimir la proliferación, diferenciación y producción de citocinas de linfocitos B (Corcione et al, 2006; Krampera et al, 2006) y suprimen la citotoxicidad y proliferación de células NK (Sotiropoulou et al, 2006). Además, las MSCs tienen la capacidad de polarizar linfocitos T hacia un fenotipo regulador, efecto dependiente de la presencia de monocitos, que representa un mecanismo importante por el cual las MSCs inhiben la inflamación (Burr et al, 2013).

Por otro lado, aunado a la capacidad de soporte hematopoyético, las MSCs también favorecen el HSCT mediante la producción de un microambiente anti-inflamatorio que reduce la posibilidad de rechazo del injerto (Ball et al, 2007). Cabe destacar que las MSCs no expresan moléculas co-estimuladoras (CD40, CD80 y CD86) necesarias para la activación de linfocitos T (Majumdar et al, 2003; LeBlanc et al, 2003; Chen et al, 2015).

Así, debido a la capacidad de inmunosupresión de las MSCs, principalmente a la supresión de la proliferación y activación de los linfocitos T, se han utilizado durante o después del HSCT con el fin de prevenir o tratar la enfermedad de injerto contra hospedero (GVHD, del inglés Graft versus Host Disease) que se desarrolla como efecto asociado al trasplante de células hematopoyéticas y que ocurre entre el 30% y el 80% de los receptores, dependiendo del grado de histocompatibilidad con el donador (Castro-Manreza and Montesinos 2015; Zhao et al, 2015). La GVHD se caracteriza por la activación de los linfocitos T provenientes del donador, los cuales desarrollan una reacción en contra del organismo del receptor provocándole un daño severo principalmente a la piel, tracto gastrointestinal, hígado y pulmón (Goker et al, 2001; Aggarwald and Pittenger Blood 2005; Chen et al, 2015). La mayoría de los estudios en donde se han utilizado MSCs para tratar la GVHD, muestran que estas son efectivas entre el 45% al 70% de los casos. Sin embargo, también se ha reportado a pacientes que no presentan mejoría después de que se les ha trasplantado MSCs como tratamiento para la GVHD, por lo que aún existe controversia al respecto (Zhao et al, 2015).

Dentro de las causas que pudieran determinar el efecto de las MSCs en el tratamiento de la GVHD, así como en la recuperación hematopoyética, puede ser la edad y el estado crítico del paciente, el número de MSCs, el número de dosis de MSCs, el grado de GVHD desarrollado en el paciente y la manipulación *in vitro* de las mismas previo a su trasplante, (Zhao et al, 2015; Chen et al, 2015).

4. Cultivo a escala clínica de las células troncales mesenquimales

El número de MSCs presentes en la MO (principal fuente de éstas células para su uso en trasplantes) o en otros tejidos adultos o neonatales es muy bajo, por lo que son insuficientes para su aplicación clínica sin previa expansión *ex vivo*. Las MSCs se

encuentran en una frecuencia de 1 MSC por $0.031x10^6$ CMNs de MO (Montesinos et al, 2009), mientras que para su aplicación en el HSCT se utilizan de $1x10^6$ hasta $5x10^6$ MSCs por kg de peso del paciente (Fajardo-Orduña et al, 2015). Debido a lo anterior, se necesita la expansión *in vitro* de MSCs para obtener el número de células suficientes para su aplicación clínica. Algunos estudios han tratado de optimizar las condiciones de expansión *in vitro* de las MSCs, incluyendo el uso de sustitutos de productos de origen animal como el suero fetal bovino (SFB) mediante el uso de lisado de plaquetas o suero sangre de cordón umbilical o de sangre periférica de humano, aunque las MSCs que se han aplicado en HSCT a la fecha, han sido expandidas con SFB (Azouna et al, 2012; Chase et al, 2012; Cooper et al, 2010). También se han utilizado distintos tratamientos de superficie en las placas de cultivo (Dolley-Sonneville et al, 2013) y sistemas de expansión automatizados como bioreactores (Dos Santos et al, 2011; Martin-Manso y Hanley, 2015; Carmelo et al, 2015). Con lo anterior, se ha logrado incrementar la producción de MSCs (Fekete et al, 2012, Bara et al, 2014), sin embargo, algunos grupos reportan como consecuencia la pérdida de su multipotencialidad y capacidad de proliferación, por lo que las condiciones de cultivo (medio de cultivo, suplementos, superficie de crecimiento, etc.) pueden afectar las características biológicas y funcionales de las MSCs que deberían ser consideradas al expandir a las células en una escala clínica (Bara et al, 2014).

PLANTEAMIENTO DEL PROBLEMA

Actualmente, el HSCT es uno de los principales tratamientos con fines curativos en pacientes con enfermedades hematológicas, sin embargo, su éxito depende del número de células hematopoyéticas trasplantadas, de su capacidad para injertar en la MO y de la ausencia del rechazo del injerto. Además de ello, en los mismos pacientes con enfermedades hematológicas, el estroma de la MO también sufre un daño de gran importancia después de ser sometidos a altas dosis de quimioterapia y radioterapia, por lo que se hace necesario reconstituir el sistema estromal de la MO después de un tratamiento ablativo. Aunado a esto, después del HSCT, el paciente puede desarrollar GVHD dependiendo del grado de incompatibilidad con las células provenientes del donador. Por todo lo anterior, son varios los retos para que el HSCT sea un tratamiento exitoso. A este respecto, se ha reportado que las MSCs de MO tienen la capacidad de incrementar la expansión de HSCs y HPCs, así como de inhibir la proliferación de linfocitos T *in vitro*. Asimismo, se ha reportado que MSCs trasplantadas con células hematopoyéticas pueden mejorar el injerto mediante una recuperación hematopoyética más rápida (recuperación del número de neutrófilos y plaquetas) debido a su capacidad de soporte hematopoyético, así como inhibir el rechazo del injerto y prevenir o tratar la GVHD debido a su capacidad para suprimir a las células del sistema inmune, tanto en modelos murinos como en pacientes trasplantados con células hematopoyéticas. Por todo lo anterior, las MSCs de MO han resultado una herramienta prometedora para mejorar el HSCT, sin embargo, también existen reportes que indican que las MSCs no favorecen la recuperación hematopoyética o no eliminan la GVHD hasta en un 50% de los pacientes con HSCT. Por otro lado, debido a que la presencia de MSCs en la MO es escasa (1 MSC / 30,000 CMNs), se tiene la necesidad de expandirlas en cultivos a escala clínica para obtener un número suficiente de ellas para su uso en el HSCT (1-5X10^6 MSCs / kg peso del paciente). Sin embargo, no hay estudios en donde se comparen las características bilógicas, la capacidad de soporte hematopoyético y la capacidad para inhibir la proliferación de linfocitos T de MSCs de MO antes y después de someterlas a un proceso de expansión a escala clínica, por lo que no se sabe si este proceso modifica de alguna manera su biología y funcionalidad, y por lo tanto las MSCs pierdan su capacidad para favorecer el HSCT. Debido a lo anterior, en este trabajo pretendemos evaluar y comparar características biológicas (inmunofenotipo, morfología y capacidad de diferenciación) y

funcionales (capacidad par a s oportar l a he matopoyesis y capacidad de s uprimir l a proliferación de linfocitos T) de MSCs provenientes de cultivo a pequeña escala y cultivo a escala clínica.

HIPÓTESIS

El c ultivo a es cala c línica no m odificará la c aracterísticas bi ológicas ni l a c apacidad de soporte hematopoyético y de inmunosupresión de las MSCs de médula ósea.

OBJETIVOS

Objetivo general

Determinar y comparar la capacidad de MSCs de médula ósea provenientes de cultivos a pequeña escala (SSC) y a escala clínica (CSC) para soportar la formación de HPCs de sangre de cordón umbilical y disminuir la proliferación de linfocitos T de sangre periférica.

Objetivos particulares

1. Determinar y comparar el i nmunofenotipo, la m orfología y l a c apacidad d e diferenciación adipogénica, osteogénica y condrogénica de MSCs provenientes de SSC y CSC.

2. Determinar y comparar la capacidad de MSCs provenientes de SSC y CSC para favorecer l a p roliferación y ex pansión de H PCs (CFC, c élulas C D34^{+}CD38^{-}Lin^{-} y LTC-IC) a partir de la población enriquecida en células CD34^{+}CD38^{-}Lin^{-} de SCU, así c omo s u c apacidad para pr oducir citocinas (SCF, T PO, IL -6 y G M-CSF), expresar m oléculas de m embrana (Jagged-1, N -cadherina, M CAM e I CAM-1), SDF-1 intracelular y t ranscritos de moléculas de M EC (FN, LA MA y C ol-I) importantes en la regulación de la hematopoyesis.

3. Determinar y comparar la capacidad de MSCs provenientes de SSC y CSC para disminuir la proliferación de linfocitos T CD3^{+}, CD4^{+} y CD8^{+} de sangre periférica.

CAPÍTULO II

Stem Cells and Development
ORIGINAL ARTICLE

Bone Marrow Mesenchymal Stromal Cells from Clinical Scale Culture: In Vitro Evaluation of their Differentiation, Hematopoietic Support and Immunosuppressive Capacities.

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Bone Marrow Mesenchymal Stromal Cells from Clinical Scale Culture: In Vitro Evaluation of Their Differentiation, Hematopoietic Support, and Immunosuppressive Capacities

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The differentiation capacity, hematopoietic support, and immunomodulatory properties of human bone marrow mesenchymal stromal cells (BM-MSCs) make them attractive therapeutic agents for a wide range of diseases. Clinical scale cultures (CSCs) have been used to expand BM-MSCs for their use in cell therapy protocols; however, little is known about the functionality of the expanded cells. The main goal of the present study was to evaluate the functional characteristics of BM-MSCs expanded from CSCs to determine the quality of the cells for cellular therapy protocols. To address this issue, we analyzed the morphology, immunophenotype, differentiation potential (adipogenic, osteogenic and chondrogenic), hematopoietic support, and immunosuppressive capacity of BM-MSCs from short scale cultures (SSCs) and CSCs in a comparative manner. After 12 days of culture in CSCs (HYPERFlask System), BM-MSCs reached cell numbers of $125.52\times10^6\pm25.6\times10^6$ MSCs, which corresponded to the number of cells required for transplantation ($\sim1.7\times10^6$ MSCs/kg for a 70-kg patient). After expansion, BM-MSCs expressed the characteristic markers CD73, CD90, and CD105; however, expansion decreased their differentiation capacity toward the adipogenic, osteogenic, and chondrogenic lineages and their ability to inhibit T-cell proliferation compared with SSCs-MSCs. Importantly, CSCs-MSCs maintained the ability to support the proliferation and expansion of hematopoietic progenitor cells and the capacity to express the molecules, cytokines, and extracellular matrix proteins involved in the regulation of hematopoiesis. Our study highlights the need to evaluate the functional properties of the expanded BM-MSCs for verification of their quality for cell therapy protocols.

Introduction

Mesenchymal stromal cells (MSCs) are a population of multipotent cells within the bone marrow (BM) that possess the following three main biological properties: a differentiation capacity toward the adipogenic, osteogenic, and chondrogenic lineages [1]; a hematopoietic support capacity because they produce molecules that favor the self-renewal and maintenance of hematopoietic stem cells (HSCs) and the proliferation and expansion of hematopoietic progenitor cells (HPCs) [2]; and immunosuppressive properties through which they exert immunoregulatory effects on different immune

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system cells, including T cells [3]. Based on these properties, MSCs have been recognized as promising candidates for clinical use in cell therapy, particularly in hematopoietic stem cell transplantation (HSCT) [1,4]. In HSCT, graft improvement and faster hematopoietic recovery have been observed in patients transplanted with MSCs [1], including the prevention and treatment of graft-versus-host disease (GVHD) [3].

The number of MSCs obtained from BM is limited. Therefore, the cells must be expanded in clinical scale cultures (CSCs) to obtain a sufficient number for use in HSCT (cell doses up to 5 million MSC/kg body weight) [2]. Although encouraging results have been obtained using MSCs in HSCT, their application is not effective in GVHD in all cases [1,5]. The differences in these results that related to the clinical application of MSCs reported by several groups may be due to the applied MSC expansion conditions in the CSCs and even the number of passages and the utilized doses.

Currently, several groups are attempting to optimize CSC conditions to improve MSC expansion and quality, and good results have been reported [6–8]. Other studies have provided evidence demonstrating changes in the immunophenotype of expanded MSCs, as well as the loss of their multipotency and proliferation capacity [9–10]. Therefore, culture conditions (eg, culture media, supplements, or growth surface used) and clinical expansion itself can affect the functional properties of MSCs and should be considered to determine the most suitable in vitro MSC expansion system that does not change the properties required for cell therapy protocols.

In general, most experiments have only studied the maintenance of the stem cell phenotype and the differentiation potential of MSCs following CSCs and have not investigated the functional properties of MSCs before [short scale cultures (SSCs)] and after CSCs. Therefore, examining the differentiation, hematopoietic support, and immunosuppressive abilities of MSCs before and after subjecting them to CSCs is essential. To date, little is known regarding this aspect; thus, it is important to establish whether these characteristics are altered in CSCs-MSCs before they can be applied clinically.

Materials and Methods

Isolation of BM-MSCs and SSCs

BM samples were obtained from hematologically healthy donors according to the Declaration of Helsinki and the Local Ethics Committee of Villacoapa Hospital, Mexican Institute for Social Security (IMSS). After written informed consent, human BM aspirates were obtained from five donors as we previously reported [11]. Briefly, mononuclear cells (MNCs) were obtained from BM samples by density gradient centrifugation (Ficoll-Paque PLUS; Sigma-Aldrich, St. Louis, MO) and were seeded at a density of 0.2×10^6 MNCs/cm^2. The cells were resuspended in low-glucose Dulbecco's modified Eagle's medium (Lg-DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Gibco), 10 µL /mL of L-glutamine (200 mM; Gibco), 10 µL/mL of penicillin-streptomycin (10,000 U/mL; Gibco), and 2 µL/mL of gentamicin (50 mg/mL, Gibco). Four days later, nonadherent cells were removed, and fresh medium was added. Upon reaching 80% confluence, adherent cells were detached with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; Gibco) and were reseeded at a density of 2×10^3 cells/cm^2 into 55 cm^2 Petri cell culture dishes (Corning; Corning, Inc., New York, NY). These MSCs corresponded to SSCs.

Clinical scale cultures of MSCs

CSCs of MSCs were performed as follows. MSCs from SSCs were seeded at a density of 2×10^3 cells/cm^2 into 1,720 cm^2 HYPERFlask cell culture vessels (Corning, Inc.) in Lg-DMEM (Gibco) supplemented with 10% FBS (Gibco), 10 µL/mL of L-glutamine (200 mM; Gibco), 10 µL/mL of penicillin-streptomycin (10,000 U/mL; Gibco), and 2 µL/mL of gentamicin (50 mg/mL; Gibco). Upon reaching 80% confluence, the adherent cells were detached with trypsin-EDTA (Gibco) and reseeded at a density of 2×10^3 cells/cm^2 into eight new HYPERFlasks (Corning, Inc.; a total of 13,760 cm^2) with the aim of obtaining $\sim70\times10^6$ to 140×10^6 MSCs ($1–2\times10^6$ MSC/kg for a 70 kg patient). The medium change was performed on the sixth day of culture and cells were harvested on day 12. These MSCs corresponded to CSCs.

Morphological characterization of MSCs

To observe structural differences between MSCs obtained from SSCs and CSCs, the cells were grown in a Petri dish (Corning, Inc.) at a density of 4×10^3 cells/cm^2. After 6 days of culture, the cells were stained with toluidine blue (Sigma-Aldrich) and examined under a phase contrast microscope.

Immunophenotyping of MSCs

Immunophenotypic analysis of MSCs was performed by flow cytometry as we previously reported [11]. Monoclonal antibodies against CD14, CD31, CD34, CD45, CD105, HLA-DR (Caltag Laboratories, Thermo Fisher Scientific), CD13, CD73, CD90, and HLA-ABC (Becton Dickinson, Franklin Lakes, NJ) conjugated with FITC (fluorescein isothiocyanate), PE (phycoerythrin), or APC (allophycocyanin) were used. Previously blocked with Fc receptor blocker (Human Blocking Reagent; Miltenyi Biotec, Bergisch Gladbach, Germany), $1–2\times10^5$ cells were resuspended in 100 µL of phosphate-buffered saline (PBS; Gibco) supplemented with 3% FBS and 1 mM EDTA and incubated with the appropriate antibodies for 20 min. The cells were subsequently washed with 1 mL of PBS (supplemented with 3% FBS and 1 mM EDTA) and fixed with FACS Lysing Solution (Becton Dickinson). The cells were acquired using a FACSCanto II (Becton Dickinson). At least 10,000 events were collected per sample, and the data were analyzed with the FlowJo 7.6.1 software (FlowJo LLC, Ashland, OR).

Adipogenic, osteogenic, and chondrogenic differentiation of MSCs

The osteogenic and adipogenic differentiation capacities were induced with Stem Cell Kits™ (STEMCELL Technologies, Inc., Vancouver, Canada) on days 21 and 14, respectively. The chondrogenic capacity was induced using Chondrogenic Differentiation Medium (Cambrex; Cambrex Bio Science Walkersville, Inc., Walkersville, MD) supplemented with 10 ng/mL transforming growth factor beta (TGF-β; Cambrex) for 28 days as previously reported [12]. The differentiation capacity was determined by cytochemistry, immunostaining, and the detection of transcripts associated with each lineage by real-time polymerase chain reaction (PCR).

Adipogenic differentiation was determined by cytochemistry and observing lipid vacuoles stained with Oil Red O (Sigma-Aldrich) and detecting the peroxisome proliferator-activated receptor (PPAR)-γ and lipoprotein lipase (LPL) transcripts. Osteogenic differentiation was determined by detecting alkaline phosphatase (ALP) activity (SIGMAFAST BCIP/NBT; Sigma-Aldrich), performing immunocytochemistry for osteopontin (OPN; Abcam, Inc., Cambridge, MA) and pro-collagen I (pro-Col-I; Santa Cruz Biotechnology, Inc., Dallas, TX) and detecting the ALP, OPN, and collagen I (Col-I) transcripts. Chondrogenic differentiation was evaluated by the formation of micromasses. Histological sections were prepared for immunohistochemistry detection of collagen II (Col-II; Abcam, Inc.). The Aggrecan (ACAN) and SRY-box 9 (SOX9) transcripts were also detected. All transcripts were evaluated by real-time PCR (see the Real-Time PCR section for details).

Coculture of MSCs/umbilical cord blood-hematopoietic progenitor cells

Umbilical cord blood (UCB) samples were collected according to the Institutional Declaration of Helsinki and the Local Ethics Committee of Troncoso Hospital, IMSS. After written informed consent, UCB samples were obtained from five normal full-term deliveries. $CD34^+CD38^-Lin^-$ HPCs were enriched from MNCs by negative selection using StemSep™ kit (STEMCELL Technologies) according to the kit's instructions as we previously reported [13,14]. MSC layers at 80% confluence were incubated with 0.3 μg/mL of mitomycin C (Mitolem, Lemery, DF., Mexico) to inhibit cell growth. Ten thousand enriched $CD34^+CD38^-Lin^-$ cells were seeded onto MSC layers in six-well plates (Corning, Inc.) in Stemline medium (Sigma-Aldrich) with or without the early-acting cytokines thrombopoietin (TPO), Flt-3 ligand (FL), stem cell factor (SCF), and interleukin-6 (IL-6) all at a concentration of 10 ng/mL (all from PeproTech, Rocky Hill, NJ). Cultures were collected on day 14, and the medium was changed on day 7.

Proliferation of hematopoietic cells

The total numbers of nucleated and viable cells from the cultures were determined with a hemocytometer using Turk's solution and trypan blue stain (Gibco), respectively [13,14].

Colony-forming cell assays

To determine HPC expansion, the presence of colony-forming cells (CFCs) was analyzed using the methylcellulose assay (MethoCult™; STEMCELL Technologies) as we previously reported [13,15]. After 14 days of culture, CFCs were counted with the aid of an inverted microscope. CFCs were classified as follows: erythroid colonies, including colony-forming cell erythroids (CFC-Es) and burst-forming cell erythroids (BFC-Es), and myeloid colonies, including CFC-Gs, colony-forming cell myeloids (CFC-Ms), and CFC-GMs.

Long-term culture-initiating cell assays

Primitive HPCs were detected using the long-term culture-initiating cell (LTC-IC) assay [pre-colony-forming cells (pre-CFCs)] based on the method described by Eaves and colleagues [16,17] as we previously reported [14]. Briefly, after coculture with MSCs for 14 days, hematopoietic cells were cultured with the M210B4 stromal line as a feeder layer for 35 days. Subsequently, MNCs were harvested and seeded in cultures with methylcellulose for CFC quantification. The eight CFC/1 LTC-IC [14,16,17] proportion was used.

Quantification of $CD34^+CD38^-Lin^-$ cells

To determine primitive HPC expansion, the frequency and fold increase of $CD34^+CD38^-Lin^-$ cells were analyzed by flow cytometry. $2–3\times10^5$ MNCs were incubated with antibodies against CD34, CD38, CD14, CD16, CD19, CD41a, and CD71 conjugated with FITC, PE, or APC (Becton Dickinson). The cells were acquired on a FACSCanto II (Becton Dickinson). At least 10,000 events were collected per sample, and the data were analyzed with the FlowJo 7.6.1 software (FlowJo LLC).

Assessment of molecules involved in MSC-hematopoietic cell interaction

To detect the expression of molecules involved in MSC-hematopoietic cell interaction, MSCs were analyzed by flow cytometry. Monoclonal antibodies against CD146, CD54 (Becton Dickinson), Jagged-1, N-cadherin, and intracellular stromal cell-derived factor 1 (SDF-1; R&D Systems, Inc., Minneapolis, MN) conjugated with carboxyfluorescein (CFS), FITC, PE, or APC were used. Cytofix/Cytoperm buffer (Becton Dickinson Pharmingen) was used according to the supplier's instructions to permit the detection of intracellular SDF-1. Cells were acquired in a FACSCanto II (Becton Dickinson). At least 10,000 events were collected per sample, and the data were analyzed with the FlowJo 7.6.1 software (FlowJo LLC).

Assessment of cytokines in MSC conditioned culture media

To quantify the secreted cytokine concentrations, supernatants were obtained from MSC cultures. MSCs were seeded in a six-well plate (Corning, Inc.) and grown to 80% confluency; thereafter, the culture media were exchanged for Stemline medium (Sigma-Aldrich). After 4 days, the conditioned medium was carefully harvested and centrifuged for 10 min at 2,500 rpm to remove debris. Cytokine analyses were performed with a cytometric flex bead array (CBA; Becton Dickinson Biosciences) for IL-6 and granulocyte macrophage colony-stimulating factor (GM-CSF) and enzyme-linked immunosorbent assay (ELISA Kit; R&D Systems, Inc.) for TPO and SCF according to the suppliers' instructions.

Assessment of extracellular matrix molecules expressed by MSCs

To quantify the expression of extracellular matrix molecules in MSCs, we detected fibronectin (FN), laminin (LAMA), and Col-I transcripts by real-time PCR (see Real-Time PCR section for details).

Real-time PCR

Total RNA was extracted from MSCs with the TRIzol reagent (Invitrogen, Thermo Fisher Scientific). Complementary

DNA (cDNA) was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Abingdon, United Kingdom). The cDNA samples were analyzed by relative quantification using the LightCycler® 480 Probes Master Kit (Roche Applied Science, Indianapolis, IN) and universal probes (Roche Applied Science) following the manufacturer's protocol in a LightCycler 480 System (Roche Applied Science). The primers obtained from IDT are listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd). All expression levels were normalized to the expression of the endogenous control [hypoxanthine phosphoribosyltransferase (HPRT) or GAPDH]. The units used to describe the expression were CP (crossing points; it is defined as point at which the fluorescence rises appreciably above background fluorescence, CP is obtained using second Derivative Maximum method). The calculations of expression were performed by the ddCt; Cp was calculated using Fit Point method in the LightCycler 480 SW 1.5 (Roche Applied Science) [18]. In adipogenic, osteogenic, and chondrogenic differentiation assays, the relative expression corresponded to the ratio to housekeeping genes in differentiated cells. In quantification of the expression of transcripts for extracellular matrix (ECM) proteins, the relative expression corresponded to the ratio to housekeeping genes in undifferentiated cells.

Coculture MSCs/peripheral blood MNCs

MNCs were obtained from peripheral blood (PB) samples by density gradient centrifugation (Ficoll-Paque PLUS; Sigma-Aldrich). Cocultures of MSCs/MNCs were performed in a 1:2 ratio (1×10^5 MSCs:2×10^5 MNCs) in 24-well plates (Corning, Inc.). MNCs were activated with 5 μg/mL of phytohemagglutinin (PHA) (Sigma-Aldrich) in 50% RPMI (Gibco) and 50% Lg-DMEM (Gibco) media supplemented with 10% FBS (Gibco), 10 μL/mL of L-glutamine (200 mM; Gibco), 10 μL/mL of penicillin-streptomycin (10,000 U/mL; Gibco), and 2 μL/mL of gentamicin (50 mg/mL, Gibco). MNCs activated in the absence of MSCs were used as positive controls for proliferation. The cultures were maintained for 7 days to permit concurrent evaluation of $CD3^+$, $CD4^+$, and $CD8^+$ cell proliferation.

Proliferation of $CD3^+$, $CD4^+$, and $CD8^+$ cells

MNCs were stained with 5 μM carboxyfluorescein succinimidyl ester (CFSE) before activation and coculture with MSCs. The proliferation of $CD3^+$, $CD4^+$, and $CD8^+$ cells was analyzed by flow cytometry. PE-conjugated monoclonal antibodies against CD3, CD4, and CD8 (Becton Dickinson) were used. The cells were acquired using a FACSCanto II (Becton Dickinson). At least 10,000 events were collected per sample, and the data were analyzed with the FlowJo 7.6.1 software. MNCs activated in the absence of MSCs were used as positive controls and set at 100% proliferation. The levels of proliferation observed in the cocultures were normalized to this control [11].

Statistical analysis

The mean ± standard deviation or standard errors of the mean of the experiments conducted are reported. The Student's *t*-test or one-way analysis of variance and Kruskal–Wallis test were employed using the IBM SPSS Statistics 22 software. Statistical significance was considered when the *P* value was <0.05.

Results

SSC-MSC and CSC-MSC characteristics

To obtain MSCs from CSCs, BM-MSCs were seeded at a density of 2×10^3 cells/cm^2 in eight HYPERFlask cell culture vessels with Lg-DMEM supplemented with 10% FBS. After 12 days, we obtained an average of $15.69\times10^6\pm3.2\times10^6$ MSCs from each HYPERFlask. Thus, we obtained a total of $125.52\times10^6\pm25.6\times10^6$ CSC-MSCs, which corresponded to the number of cells required for the transplantation of $\sim1.7\times10^6$ MSCs/kg for a 70 kg patient. The MSC yield in HYPERFlask was $9{,}990\pm900$ cells/cm^2 after 12 days in culture, which was lower than that obtained from normal cell culture flasks, because in a shorter time (6 days in culture) it was $9{,}122\pm1{,}860$ cells/cm^2. To determine structural differences between MSCs obtained from the SSCs and CSCs, the cells were grown in a Petri dish, stained with toluidine blue and examined under a phase contrast microscope. Figure 1A, a shows the typical fibroblastoid morphology of SSC-MSCs [12], whereas in the CSC-MSCs (Fig. 1A, b) we observed not only fibroblast cells but also a higher content of "large cells" with abundant cytoplasm. Similar to the MSCs from SSCs as previously reported [12], MSCs from CSCs expressed markers characteristic of MSCs, such as CD73, CD90, CD105, CD13, and HLA-ABC. The expression of hematopoietic markers (CD14, CD34, and CD45) was not observed; CD31 and HLA-DR were also not present (Supplementary Table S2).

Differentiation capacity of SSC-MSCs and CSC-MSCs

After 14 days of induction, adipogenic differentiation was detected based on the presence of positive lipid vacuoles after Oil Red O staining and PPAR-γ and LPL expression by real-time PCR. CSC-MSCs presented the formation of lipid vacuoles positive for Oil Red O staining; however, the formation of adipocytes was lower compared with SSC-MSCs (Fig. 1B, a, b). No PPAR-γ messenger RNA (mRNA) expression was detected in the CSC-MSCs, whereas an expression level of 0.22 ± 0.26 was observed in the SSC-MSCs (Fig. 1C). In addition, the LPL mRNA expression was significantly lower ($P<0.05$) in the CSC-MSCs compared with the SSC-MSCs (25.9 ± 12.8 and 224.1 ± 97.6, respectively; Fig. 1C).

After 21 days of induction, the osteogenic differentiation capacity was determined by detecting ALP activity and performing immunocytochemistry to assess the presence of OPN and pro-Col-I; in addition, the ALP, OPN, and Col-I expression levels were measured by real-time PCR. Both SSC-MSCs and CSC-MSCs presented ALP activity and OPN and pro-Col-I positivity by immunocytochemistry (Fig. 2A, a–f). ALP and OPN mRNA expression showed a tendency to decrease in the CSC-MSCs compared to the SSC-MSCs (0.53 ± 0.15 and 1.0 ± 0.49 for ALP and 0.32 ± 0.23 and 0.85 ± 0.59 for OPN, respectively, Fig. 2B). Conversely, the Col-I mRNA expression showed a tendency to increase in the CSC-MSCs compared with the SSC-MSCs (0.86 ± 0.36 and 0.36 ± 0.10, respectively; Fig. 2B).

After 28 days of induction, histological sections were prepared to analyze the chondrogenic differentiation capacity.

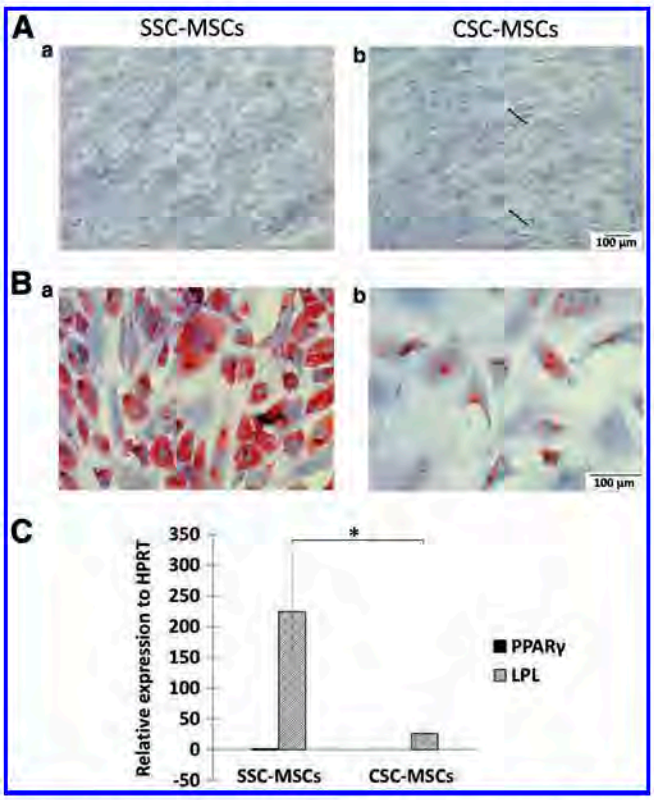

FIG. 1. Morphology and adipogenic differentiation capacity are modified in MSCs from CSCs. **(A)** Morphological appearance under the microscope of MSCs from **(a)** SSCs and **(b)** CSCs stained with toluidine blue (magnification 10×). Large cells with abundant cytoplasm were predominantly observed in CSC-MSCs (*arrows*). **(B)** Adipogenic differentiation was indicated by the accumulation of neutral lipid vacuoles stained with Oil Red O: **(a)** SSC-MSCs and **(b)** CSC-MSCs (magnification 20×); one representative experiment is shown. **(C)** Real-time PCR to analyze adipogenic differentiation-associated gene expression. The values shown are relative to HPRT expression. The data are shown as the mean ± SEM. $n = 5$ (*$P < 0.05$). CSC, clinical scale culture; HPRT, hypoxanthine phosphoribosyltransferase; LPL, lipoprotein lipase; MSCs, mesenchymal stromal cells; PCR, polymerase chain reaction; PPAR-γ, peroxisome proliferator-activated receptor gamma; SEM, standard error of the mean; SSC, short scale culture. Color images available online at www.liebertpub.com/scd

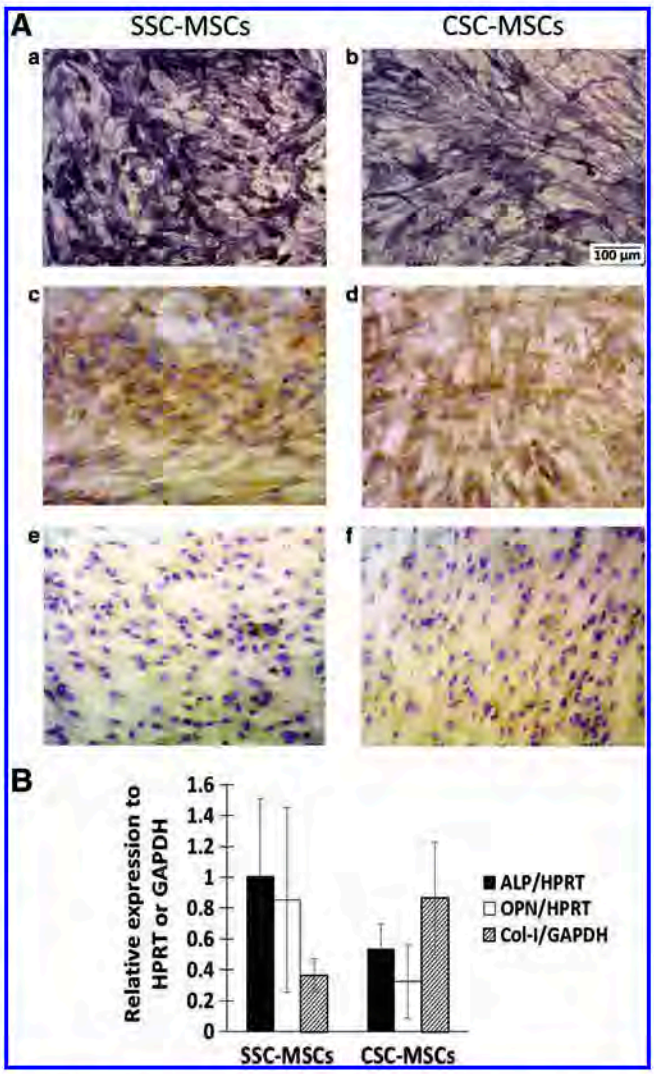

FIG. 2. Osteogenic differentiation capacity in MSCs from CSCs. **(A)** Osteogenic differentiation was indicated by ALP staining **(a, b)** and immunocytochemistry for OPN **(c, d)** and pro-Col-I **(e, f)** detection. One representative experiment is shown (magnification 20×). **(B)** Osteogenic differentiation-associated gene expression analysis by real-time PCR. The values shown for ALP and OPN are relative to HPRT expression, whereas the Col-I value is relative to GAPDH expression. The data are shown as the mean ± SEM. $n = 5$. ALP, alkaline phosphatase; Col-I, collagen I; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OPN, osteopontin. Color images available online at www.liebertpub.com/scd

Col-II positivity by immunohistochemistry was determined in the micromasses, and SOX9 and ACAN expression was measured by real-time PCR. Both SSC-MSCs and CSC-MSCs were able to form micromasses, but Col-II positivity by immunohistochemistry was decreased in the micromasses from CSC-MSCs in contrast to SSC-MSCs (Fig. 3A, a, b). SOX9 and ACAN mRNA expression was decreased in the CSC-MSCs compared with the SSC-MSCs (1.94 ± 0.69 and 4.66 ± 1.88, respectively, for SOX9 and 0.12 ± 0.11 and 0.92 ± 0.19, respectively, for ACAN, $P < 0.05$) (Fig. 3B).

Capacity of SSC-MSCs and CSC-MSCs for hematopoietic proliferation

We previously defined proliferation as the production of new cells from a cell population regardless of the type of cells produced [15]. Thus, the proliferation of the hematopoietic progenitor-enriched cell population was determined based on the total cell number generated in the culture with a hemocytometer, Turk's solution, and trypan blue staining. Figure 4A shows hematopoietic cell cultures in the absence of MSCs or with MSCs from SSCs or CSCs (Fig. 4A, a–c, respectively).

As shown in Fig. 4B, the hematopoietic cells were unable to proliferate in the absence of both MSCs and human recombinant cytokines (control); in this culture, the total number of nucleated cells decreased gradually and disappeared by day 14. On day 14, a fold increase in the total number of hematopoietic cells was observed in the cocultures in the absence of

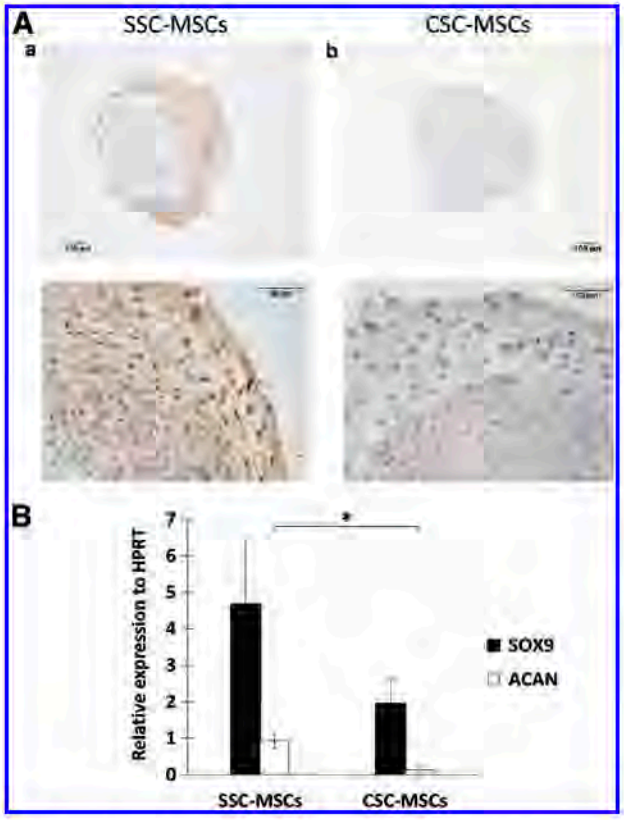

FIG. 3. Chondrogenic differentiation capacity is decreased in MSCs from CSCs. **(A)** Chondrogenic differentiation was indicated by immunohistochemistry for Col-II detection (**a, b**); one representative experiment is shown (magnification 10× and 40×). **(B)** Chondrogenic differentiation-associated gene expression analysis by real-time PCR. The values shown are relative to HPRT expression. The data are shown as the mean ± SEM, $n = 5$ ($^*P < 0.05$). ACAN, aggrecan; Col-II, collagen II; SOX9, SRY-box 9. Color images available online at www.liebertpub.com/scd

cytokines in the presence of MSCs from CSCs and SSCs (6.19 ± 2.6 and 5.07 ± 1.88, respectively; Fig. 4B). Interestingly, when cytokines (FL, SCF, TPO, and IL-6) were added to the cocultures, significantly greater fold increases ($P < 0.05$) were observed with MSCs from CSCs and SSCs (74.5 ± 26.4 and 110 ± 52.7, respectively) compared with the cultures with only cytokines (25 ± 10.7) or MSCs (Fig. 4B). No significant differences were detected in the number of hematopoietic cells obtained in cocultures from CSC-MSCs and SSC-MSCs.

Hematopoietic progenitor cell expansion capacity of SSC-MSCs and CSC-MSCs

We previously defined cellular expansion as the production of cells that maintained specific characteristics of the population of cells from which they originated [15]. In this study, we assessed the expansion of progenitors capable of forming both myeloid and erythroid colonies in semisolid cultures. Myeloid colonies included CFC-G, CFC-M, and CFC-GM and erythroid colonies included CFC-E and BFC-E (Fig. 5A). On day 14, slight fold increases in CFC-Ms were observed in cultures in the absence of cytokines and in the presence of SSC-MSCs or CSC-MSCs (1.26 ± 1.04 and 1.42 ± 1.41, respectively; Fig. 5B, a). Interestingly, when cytokines were added to the cocultures, the fold increases of CFC-Ms were 150.2 ± 85.62 and 217.6 ± 109.1 with MSCs from CSCs and SSCs, respectively; these increases were significantly greater ($P < 0.05$) than the increases in cultures with cytokines alone (24.4 ± 8.4; Fig. 5B, a). Similarly, slight fold increases in CFC-Es were observed in the presence of CSC-MSCs and SSC-MSCs (0.89 ± 0.94 and 0.83 ± 0.65, respectively; Fig. 5B, b). When cytokines were added to the cocultures, the fold increases of CFC-Es with CSC-MSCs and SSC-MSCs (77.32 ± 43.45 and 109.1 ± 65.1, respectively) were significantly greater ($P < 0.05$) compared with cultures with cytokines alone (15.3 ± 10.6; Fig. 5B, b). No significant differences were detected in the number of myeloid and erythroid progenitors obtained in cocultures from CSC-MSCs and SSC-MSCs.

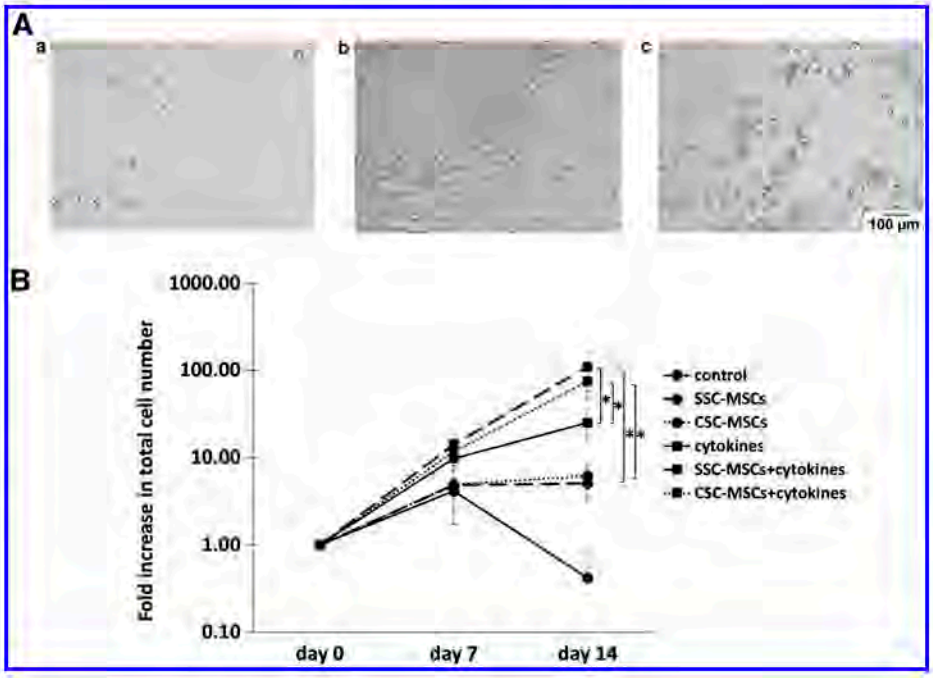

FIG. 4. CSCs do not modify the capacity of MSCs to increase the proliferation of hematopoietic cells. **(A)** Culture of the population enriched in $CD34^+CD38^-Lin^-$ cells in the presence of cytokines: **(a)** without MSCs; **(b)** with SSC-MSCs; and **(c)** with CSC-MSCs. One representative experiment is shown (magnification 20×). **(B)** Kinetics of the proliferation of the population enriched in $CD34^+CD38^-Lin^-$ cells in the absence or presence of SSC-MSCs or CSC-MSCs and in the absence or presence of cytokines. The data are shown as the mean ± SEM. $n = 5$ ($^*P < 0.05$).

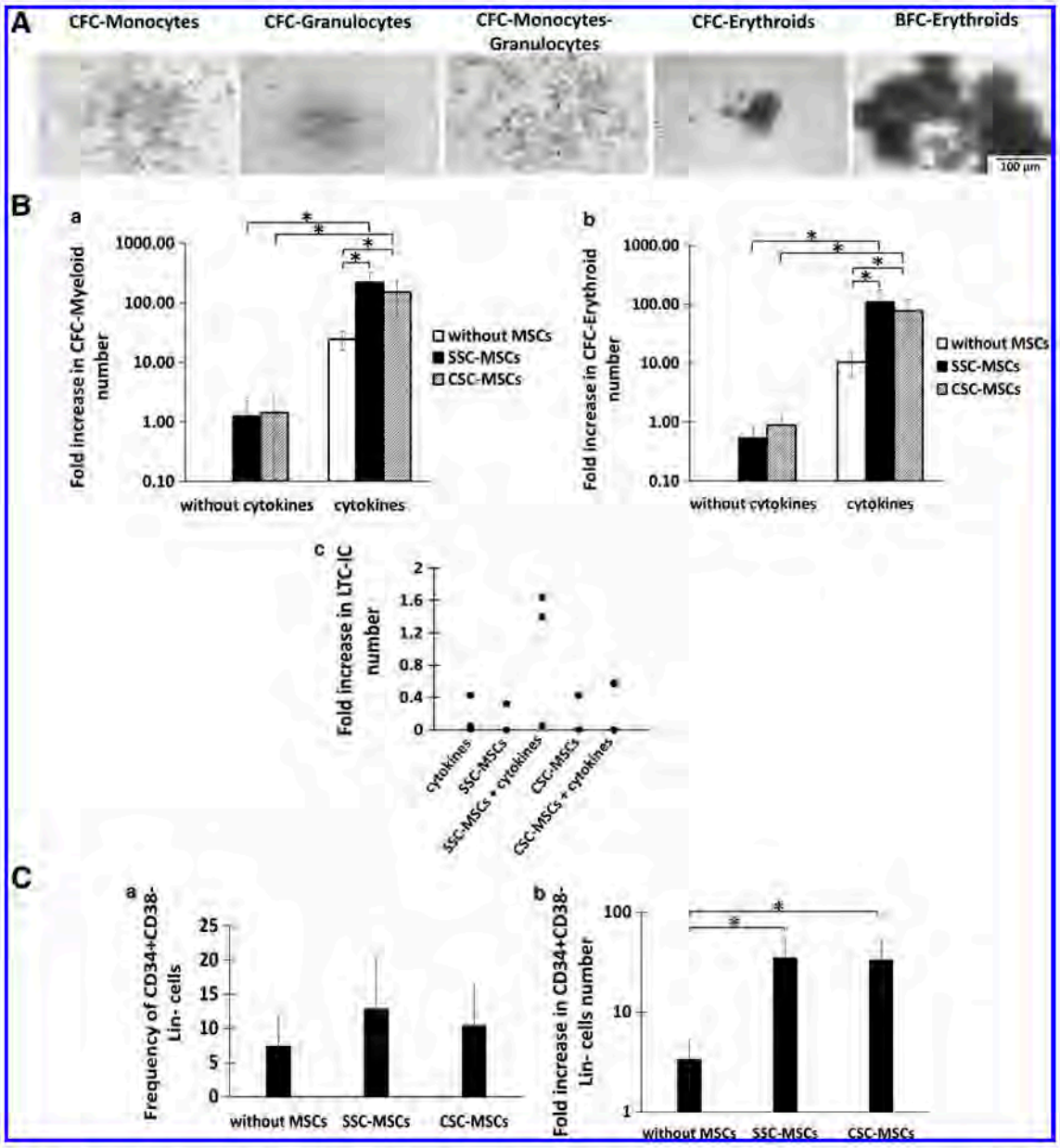

FIG. 5. CSC does not modify the capacity of MSCs to increase HPC expansion. **(A)** Colonies obtained on day 14 of culture: CFC-Monocytes, CFC-Granulocytes, CFC-Monocytes-Granulocytes, CFC-Es, and BFC-Es. One representative experiment is shown (magnification 20×). **(B)** Fold increases in the number of **(a)** CFC-M, **(b)** BFC- and CFC-Es; $n=5$, and **(c)** LTC-IC (pre-CFC); $n=3$, in cultures with or without MSCs from SSCs or CSCs in the absence and presence of cytokines. **(C)** Frequency per 2.5×10^5 hematopoietic cells **(a)** and fold increase **(b)** of $CD34^+CD38^-Lin^-$ cells in cocultures with cytokines with or without SSC-MSCs or CSC-MSCs; $n=5$. The data are shown as the mean ± SEM (*$P<0.05$). BFC-Es, burst- forming cell erythroids; CFC, colony-forming cell; CFC-M, colony-forming cell myeloid; CFC-Es, colony-forming cell erythroids; HPCs, hematopoietic progenitor cells; LTC-ICs, long-term culture initiating cells; pre-CFCs, pre-colony-forming cells.

Expansion of more primitive CFC (pre-CFC) and $CD34^+CD38^-Lin^-$ cells

We analyzed the effect of CSC-MSCs and SSC-MSCs on the formation of primitive HPCs with a LTC-IC capacity in the absence or presence of cytokines. Similar to the cultures with cytokines alone, the presence of LTC-ICs was observed on day 14 of some cultures with only CSC-MSCs and SSC-MSCs. When cytokines were added to the cocultures, slight fold increases of the LTC-IC numbers were observed with MSCs from SSCs compared with cultures with MSCs from CSCs (Fig. 5B, c). However, no significant differences were detected between the CSC-MSCs and SSC-MSCs.

Another parameter used to determine primitive HPC expansion is the increase in the number of cells with the $CD34^+CD38^-Lin^-$ immunophenotype, which we evaluated in this study. Cultures were generated with a population enriched in $CD34^+CD38^-Lin^-$ cells in the presence of cytokines and in the absence and presence of CSC-MSCs or SSC-MSCs. On day

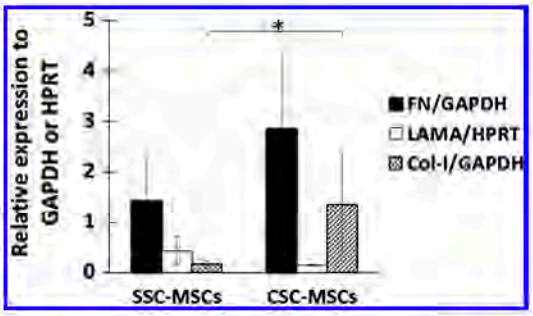

FIG. 6. Gene expression analysis of matrix extracellular proteins. Expression of FN, LAMA, and Col-I in SSC-MSCs and CSC-MSCs detected by real-time PCR. $n=5$ (*$P<0.05$). FN, fibronectin; LAMA, laminin.

14 of culture, increases were observed in the $CD34^+CD38^-Lin^-$ cell frequency in cultures with CSC-MSCs and SSC-MSCs (10.4 ± 6 and 12.9 ± 7.4, respectively), compared with cultures without MSCs (7.4 ± 4.2), although no significant differences were observed in the frequency values (Fig. 5C, a). However, the fold increases in the number of $CD34^+CD38^-Lin^-$ cells in cultures with CSC-MSCs or SSC-MSCs were significantly greater ($P<0.05$) (33.4 ± 19.3 and 35.3 ± 20.3, respectively), compared with cultures without MSCs (3.3 ± 1.9; Fig. 5C, b). No significant differences were detected in cell numbers between the cultures from CSC-MSCs and SSC-MSCs.

Expression of molecules, cytokines, and extracellular matrix that regulate hematopoiesis

As part of the analysis of the hematopoietic support capacity of the CSC-MSCs, we analyzed the expression and secretion of important molecules involved in the regulation of hematopoietic cell formation and compared them with the molecules expressed in SSC-MSCs. Among the molecules analyzed, we determined the expression of important molecules for cell–cell contact by flow cytometry. In SSC-MSCs and CSC-MSCs, we observed a half level in the expression of CD146 and N-cadherin (Supplementary Table S3). Jagged-1 expression was not detected in either type of MSCs. We also observed a tendency for an increase in CD54 in CSC-MSCs compared to SSC-MSCs. Furthermore, we observed high intracellular expression of SDF-1 in both SSC-MSCs and CSC-MSCs (Supplementary Table S3). There were no significant differences in the expression of these molecules between the two types of cultures.

Regarding cytokine secretion, we quantified TPO, SCF, IL-6, and GM-CSF in the conditioned medium by CBA or ELISA. TPO was produced in similar quantities in the SSC-MSCs and CSC-MSCs (Supplementary Table S4). We also observed a tendency for a decrease in the production of SCF and IL-6 in the CSC-MSCs compared to the SSC-MSCs; however, no significant differences were detected (Supplementary Table S4). Furthermore, GM-CSF was not detected in the MSC cultures.

We also quantified the expression of ECM transcripts by real-time PCR. We observed a tendency for an increase in the expression of FN in CSC-MSCs compared to SSC-MSCs (2.86 ± 1.5 and 1.42 ± 0.91, respectively; Fig. 6). LAMA expression was similar in the CSC-MSCs and SSC-MSCs (0.15 ± 0.01 and 0.44 ± 0.28, respectively), whereas Col-I was significantly higher in the CSC-MSCs compared to the SSC-MSCs (1.35 ± 1.04 and 0.16 ± 0.07, respectively; $P<0.05$; Fig. 6).

Immunosuppressive capacity of the SSC-MSCs and CSC-MSCs

The immunosuppressive capacity of the CSC-MSCs was evaluated in cocultures of MNCs from PB stimulated with

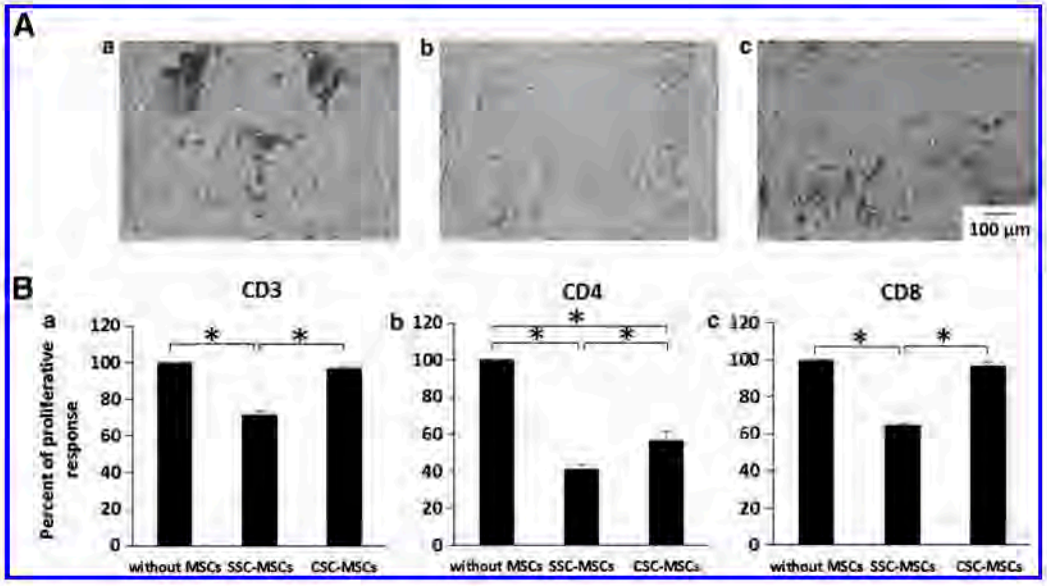

FIG. 7. CSCs decrease the capacity of MSCs to inhibit $CD3^+$, $CD4^+$, and $CD8^+$ T-cell proliferation. (**A**) Proliferation of PHA-activated MNCs in cultures: (**a**) without MSCs; (**b**) with SSC-MSCs; and (**c**) with CSC-MSCs. One representative experiment is shown (magnification 10×). (**B**) Proliferation of activated $CD3^+$ (**a**), $CD4^+$ (**b**), and $CD8^+$ T cells (**c**) in the presence of MSCs. Activated MNCs cultured in the absence of MSCs served as the positive control (100% proliferation). The data are shown as the mean ± SEM. $n=3$ (*$P<0.05$). MNCs, mononuclear cells; PHA, phytohemagglutinin.

PHA in the absence or presence of SSC-MSCs or CSC-MSCs (Fig. 7A, a–c). The proliferation of $CD3^+$, $CD4^+$, and $CD8^+$ T cells (stained with CFSE) was determined by flow cytometry. The proliferation of activated T cells cultured in the absence of MSCs was considered 100% of the proliferative response (positive control). The CSC-MSCs showed significantly less ability to inhibit $CD3^+$ T-cell proliferation compared to the SSC-MSCs (96.8%±1% and 71.7%±1.7%, respectively; $P<0.05$; Fig. 7B, a). Similar results were obtained for $CD8^+$ T-cell proliferation in the presence of CSC-MSCs (96.9%±1.6%) and SSC-MSCs (64.6%±1.25%; $P<0.05$; Fig. 7B, c). Interestingly, although $CD4^+$ T-cell proliferation was significantly reduced (56.27%±5.3%, $P<0.05$) in the presence of CSC-MSCs compared to the positive control, this inhibition was significantly less than that observed with SSC-MSCs (41.1%±2.3%; $P<0.05$; Fig. 7B, b).

Discussion

MSCs were first described by Friedenstein et al. almost four decades ago [19]. Since then, MSCs have become one of the most attractive cell types for cell therapy due to their capacity to differentiate toward different cell lineages (adipocytes, osteoblasts, chondrocytes, endothelial cells, and cardiomyocytes) [20], their immunosuppressive properties [3], and their hematopoietic cell support [2]. At present, several clinical trials involving the use of MSCs for hematopoietic cell transplant, GVHD, autoimmune diseases, and tissue regeneration are in progress [1,3,4]. However, the low frequency of MSCs in BM ($1/0.031\times10^6$ MNCs) [12] raises the need to expand the cells to reach the number needed for clinical applications ($1–5\times10^6$/kg) [2]. Different groups have been able to expand MSCs on a clinical scale and in sufficient numbers for their use in clinical trials [7,8,21–23]. However, the expanded cells may present differences in their functional characteristics due to the cell expansion procedure, and this issue has not been addressed by these studies. The aim of this study was to compare the morphology, immunophenotype, differentiation capabilities, hematopoietic support, and immunosuppression of BM-MSCs before (SSC-MSCs) and after (CSC-MSCs) their clinical scale expansion.

We found that the CSC-MSCs maintained a fibroblastic morphology and an immunophenotype similar to SSC-MSCs, which was consistent with previous reports [6,7,24–29]. However, we found a difference in the sizes of the cells. CSC-MSCs contained a higher proportion of cells with abundant cytoplasm, which we previously described as "large cells" [12]. The presence of "large cells" has been associated with decreased adipogenic and chondrogenic differentiation potential in MSC cultures [30,31], although further studies showed that such cells are associated with cellular senescence, which has been demonstrated by beta-galactosidase staining [32,33].

Regarding the differentiation potential of CSC-MSCs, several reports showed that they retained their adipogenic, osteogenic, and chondrogenic capabilities even at late passages [24–28], but there were also reports that the expansion of MSCs in CSCs diminished their ability to differentiate into these lineages [10,31,34]. In the present work, we evaluated the differentiation potential of CSC-MSCs using cytochemical, immunohistochemical, and gene expression analyses to help clarify this controversy. We found that the adipogenic potential of CSC-MSCs was drastically decreased when assessed by cytochemistry (Oil Red O staining) and real-time PCR (LPL and PPAR-γ). The osteogenic potential of CSC-MSCs was slightly reduced compared to SSC-MSCs, and we found decreased expression of ALP by cytochemistry and OPN by immunohistochemistry and real-time PCR. It is important to mention that high ALP activity has been observed at 14th day, but in our study, expression of ALP was determined at 14 and 21 days and there were no significant differences between both days of induction (data not shown). Similar ALP activity was observed at 14 and 21 days. Notably, the expression of pro-Col-I and Col-I was not reduced, but was higher than the expression in SSC-MSCs, although the difference was not significant. Regarding the chondrogenic differentiation, MSCs from CSCs had the ability to form micromasses; however, they exhibited reduced expression of Col-II and ACAN.

Decreased differentiation of CSC-MSCs toward adipocytes, osteoblasts, and chondrocytes was also observed in MSCs cultured under standard conditions after 8–10 passages [10]. This decrease has also been associated with the presence of "large cells" in the culture, which have a decreased differentiation capacity [30,31]. This finding may not impede the ability to use CSC-MSCs for cell therapy because the decreased differentiation potential has been shown to be reversible in the presence of morphogens and growth factors [34] and suggest that most clinical MSC applications do not rely on differentiating donor cells, but on the secretion of trophic factors that induce host tissue regeneration [35].

We also analyzed MSC hematopoietic support before and after their expansion in CSCs. We found that CSC-MSCs could support the proliferation and expansion of early HPCs (immunophenotype $CD34^+CD38^-Lin^-$), late HPCs (CFC-Ms and CFC-Es), and primitive HPCs (LTC-ICs). In agreement, we found that CSC-MSCs did not exhibit modified expression of the cell surface molecules (CD146, CD54, and N-cadherin), chemokines (SDF-1), cytokines (TPO, SCF, and IL-6), and extracellular matrix proteins (FN and LAMA) involved in the self-renewal, homing, proliferation, and differentiation of HSCs/HPCs [2]. Interestingly, we did not detect GM-CSF in the CSC-MSCs or SSC-MSCs supernatant in contrast to other reports [36,37]. However, the culture conditions were different from those evaluated in our study. The increased expression of Col-I transcripts in CSC-MSCs could be related to Col-I's ability to support hematopoiesis given its involvement in maintaining the functional characteristics of the HSCs in hematopoietic niches [2]. However, this result may also suggest the onset of a fibrotic reaction (related to senescence of MSCs) [38] and should be considered in future clinical applications, especially for treating diseases such as cardiac ventricular fibrosis after myocardial infarction and lung allografts [38,39]. To the best of our knowledge, this is the first study to determine the hematopoietic support of CSC-MSCs and establish that this capacity is not lost after their culture on a large scale. These results support the use of CSC-MSCs for hematopoietic recovery in patients undergoing HSCT, which to date have shown positive effects [2,40].

Finally, we assessed whether the CSC modified the immunosuppressive capacity of MSCs. We analyzed the ability of CSC-MSCs to inhibit PHA-activated $CD3^+$, $CD4^+$, and $CD8^+$ T-cell proliferation. We observed that CSC-MSCs showed a reduced immunosuppressive capacity compared to SSC-MSCs because they were not able to inhibit the proliferation of $CD3^+$

and $CD8^+$ T cells. However, it is important to note that CSC-MSCs retained their ability to suppress the proliferation of $CD4^+$ T cells although to a minor extent compared to SSC-MSCs. The decreased ability of CSC-MSCs to inhibit T-cell proliferation may explain the cases where no favorable response is observed in the treatment of GVHD [1,5].

The presence of senescent cells (characterized by morphological alterations), the decrease in the differentiation potential and immunosuppressive capacity of MSCs from CSCs, suggests that MSCs expanded in CSCs reveal a gradual loss of functionality [10,32,33,41]. The mechanisms underlying MSCs replicative senescence may include telomere shortening, accumulation of DNA damage, and epigenetic changes [10]. Alves et al. demonstrated that MSCs were capable of accumulating DNA damage during in vitro culture; moreover, p16 and p21 expression was increased, resulting in the loss of their differentiation potential [42]. After large-scale in vitro expansion, MSCs acquired genetic alterations [43,44], but these alterations did not lead to malignant transformation [45,46]. It is important to mention that in our culture system, we have used xenogenic FBS as a supplement for the in vitro MSC expansion. However, it was demonstrated by several working groups that human serum and platelet lysate provide much better growth support (and less senescence) than FBS [26,47], although it has also been reported that the use of platelet lysate decreases the immunosuppressive capacity of MSCs [47]. We are currently doing research along this line.

In summary, our study shows that there is a differential regulation of CSC-MSC properties because the hematopoietic support capacity is not affected despite decreases in their ability to differentiate and cause immunosuppression. To our knowledge, this is the first study in which the hematopoietic support capacity and immunosuppressive properties of BM-MSCs from SSCs and CSCs are compared in a comparative manner. Our results suggest that large-scale MSC production for clinical use must include proper quality controls to ensure the efficacy of MSCs for use in patients.

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Author Disclosure Statement

The authors declare that they have no competing or financial interests.

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Supplementary Table S1. Polymerase Chain Reaction Primers

Gene symbol		Primers: Forward, reverse	Probe (UPL)
PPAR-γ	NM_015869.4	tccatgctgttatgggtgaa, tgtgtcaaccatggtcatttc	14
LPL	NM_000237.2	gtggccgagagtgagaaca, ggaaggagtaggtcttatttgtgg	13
ALP	NM_001127501.2	agaaccccaaaggcttcttc, cttggcttttccttcatggt	31
OPN	NM_001251830.1	gagggcttggttgtcagc, caattctcatggtagtgagttttcc	18
COL-I	NM_000089.3	ctggagaggctggtactgct, agcaccaagaagaccctgag	79
SOX9	NM_000346.3	gtacccgcacttgcacaac, tctcgctctcgttcagaagtc	61
ACAN	NM_001135.3	cctccccttcacgtgtaaaa, gctccgcttctgtagtctgc	76
FN	M10905.1	aagagcgagcccctgatt, atgaagattggggtgtggaa	60
LAMA5	NM_005560.3	cctcgtcctccaatgacac, gcgctgcagtcacaattc	32
HPRT	NM_000194.2	tgaccttgatttattttgcatacc, cgagcaagacgttcagtcct	73
GAPDH	NM_002046.3	agccacatcgctcagacac, gcccaatacgaccaaatcc	60

ACAN, aggrecan; ALP, alkaline phosphatase; COL-I, collagen I; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase; LAMA5, laminin 5; LPL, lipoprotein lipase; OPN, osteopontin; PPAR-γ, peroxisome proliferator-activated receptor gamma; SOX9, SRY-box 9; UPL, Universal Probe Library.

Supplementary Table S2. Ag Expression Profiles by Short Scale Culture Mesenchymal Stromal Cells and Clinical Scale Culture Mesenchymal Stromal Cells

Antigen	SSC-MSCs Positive (%)	CSC-MSCs Positive (%)
CD73	97.1±4.2	85.3±15.3
CD90	99.4±1.1	95.5±8.8
CD105	99.2±0.8	98.0±2.3
CD13	96.9±3.1	98.2±2.0
CD14	0.7±1	3.0±5.8
CD34	0.6±0.6	0.6±0.5
CD45	0.2±0.4	0.1±0.3
CD31	1.8±1.4	2.7±3.4
HLA-ABC	90.2±11.4	81.2±25.2
HLA-DR	1.6±1.4	8±11

Expression of cell markers was determined by flow cytometry. The results represent the mean±SD and correspond to the frequency (%) of cells positive for each particular Ag ($n=5$).

CSC-MSCs, clinical scale culture-mesenchymal stromal cells; SD, standard deviation; SSC-MSCs, short scale culture-mesenchymal stromal cells.

Supplementary Table S3. Molecules Expressed by Short Scale Culture Mesenchymal Stromal Cells and Clinical Scale Culture Mesenchymal Stromal Cells That Regulate Hematopoiesis

Ag	*SSC-MSCs (%)*	*CSC-MSCs (%)*
CD146 (MCAM)	35.4 ± 48.6	45.2 ± 29.3
CD54 (ICAM-1)	8.6 ± 3.3	35.4 ± 28.5
SDF-1 (CXCL-12; intracellular)	97.6 ± 1.9	82.1 ± 29.5
Jagged-1	0.3 ± 0.3	1.6 ± 2.8
N-cadherin	27.1 ± 9.5	29.7 ± 15.6

Expression of membrane molecules was determined by flow cytometry. The results represent the mean ± SD and correspond to the frequency (%) of cells positive for each particular Ag ($n = 3$).

SDF-1, stromal cell-derived factor 1.

Supplementary Table S4. Hematopoietic Cytokines Secreted by Short Scale Culture Mesenchymal Stromal Cells and Clinical Scale Culture Mesenchymal Stromal Cells

Cytokine	*SSC-MSCs (pg/mL)*	*CSC-MSCs (pg/mL)*
TPO	21.8 ± 7.0	23.1 ± 8.9
SCF	83.6 ± 31.9	53.4 ± 7.4
IL-6	3,088.1 ± 1,284.5	2,167.0 ± 1,521.7
GM-CSF	0	0

Secretion of molecules was determined by ELISA (TPO and SCF) and the flex bead system (IL-6 and GM-CSF). The results represent the mean ± SD and correspond to pg/mL ($n = 3$).

ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-6, interleukin-6; SCF, stem cell factor; TPO, thrombopoietin.

CAPÍTULO III

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REVIEW ARTICLE

Hematopoietic Support Capacity of Mesenchymal Stem Cells:
Biology and Clinical Potential

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Hematopoietic Support Capacity of Mesenchymal Stem Cells: Biology and Clinical Potential

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Mesenchymal stem cells (MSCs) play an important role in the physiology and homeostasis of the hematopoietic system. Because MSCs generate most of the stromal cells present in the bone marrow (BM), form part of the hematopoietic stem cell (HSC) niche, and produce various molecules regulating hematopoiesis, their hematopoiesis-supporting capacity has been demonstrated. In the last decade, BM-MSCs have been proposed to be useful in some *ex vivo* protocols for HSC expansion, with the aim of expanding their numbers for transplant purposes (HSC transplant, HSCT). Furthermore, application of MSCs has been proposed as an adjuvant cellular therapy for promoting rapid hematopoietic recovery in HSCT patients. Although the MSCs used in preliminary clinical trials have come from the BM, isolation of MSCs from far more accessible sources such as neonatal tissues has now been achieved, and these cells have been found to possess similar biological characteristics to those isolated from the BM. Therefore, such tissues are now considered as a potential alternative source of MSCs for clinical applications. In this review, we discuss current knowledge regarding the biological characteristics of MSCs as related to their capacity to support the formation of hematopoietic stem and progenitor cells. We also describe MSC manipulation for *ex vivo* HSC expansion protocols used for transplants and their clinical relevance for hematopoietic recovery in HSCT patients.

Introduction

Mesenchymal stem cells (MSCs) are adult stem cells that are initially isolated from bone marrow (BM) and must be positive for CD105, CD73, and CD90, express low levels of MHC-I, and be negative for MHC-II, CD11b, CD14, CD34, CD45, and CD31. Additionally, these cells must be capable of differentiation into osteoblasts, adipocytes and chondroblasts *in vitro* (1). Friedenstein's research group was the first to demonstrate the presence of MSCs in the BM capable of forming bone and cartilage and representing a minimum percentage of the total BM cell population (2). However, there was little interest in these BM cells for several years, and most research focused on understanding the mechanisms regulating blood cell production (hematopoiesis) through the interactions between hematopoietic and stromal cells, these latter cells with the capability of sustaining hematopoietic production. Greater importance is now placed on the participation of MSCs because it is now known that they are the origin of most stromal cells and furthermore by themselves constitute an essential Hematopoietic Stem Cell (HSC) niche component (3,4). MSCs are key in the study of hematopoiesis, and manipulation of these cells has been implicated in the development of HSC transplant (HSCT) strategies. In this context, MSCs have played a greater role, not only in relation to the biological mechanisms regulating hematopoiesis but also in their use as a tool in models of HSC expansion *ex vivo* and as cell therapy applied to aid in the recovery of HSCT patients (3). The purpose of this review is to describe and discuss the relevant aspects of the capability of MSCs to support hematopoiesis, in addition to their *ex vivo* manipulation and clinical importance.

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Role of MSCs in Hematopoiesis

Hematopoiesis is the process by which HSCs generate and replenish hematopoietic progenitors and all mature blood cells (4). Within the BM, hematopoiesis is the result of interaction between hematopoietic and stromal cells (5,6). In murine models, MSCs have been identified as important components of the BM hematopoietic microenvironment and as a source of cells of the stromal system (adipocytes, osteoblasts, and reticular cells). Specific sites within the hematopoietic microenvironment referred to as "niches" have been proposed to regulate quiescence, self-renewal, and differentiation of HSCs (4). Three kinds of hematopoietic niches have been proposed: a) osteoblastic or endosteal niche (on the trabecular endosteum surface) where quiescent HSCs are found; b) endothelial or vascular niche (within the perivascular sinusoids) where dividing HSCs are located (7,8) and c) reticular niche associated with HSC maintenance and viability via direct cell-to-cell contact with reticular cells (9,10). However, there is controversy regarding the true existence of different niches, mostly due to the close contact between the different cell types reported to make up each niche (4).

MSC Subpopulations

Different MSC subpopulations have been found within the various HSC niches (Figure 1), demonstrating their important role in regulating these hematopoietic cells (3,10). The vascular niche has been shown to produce factors

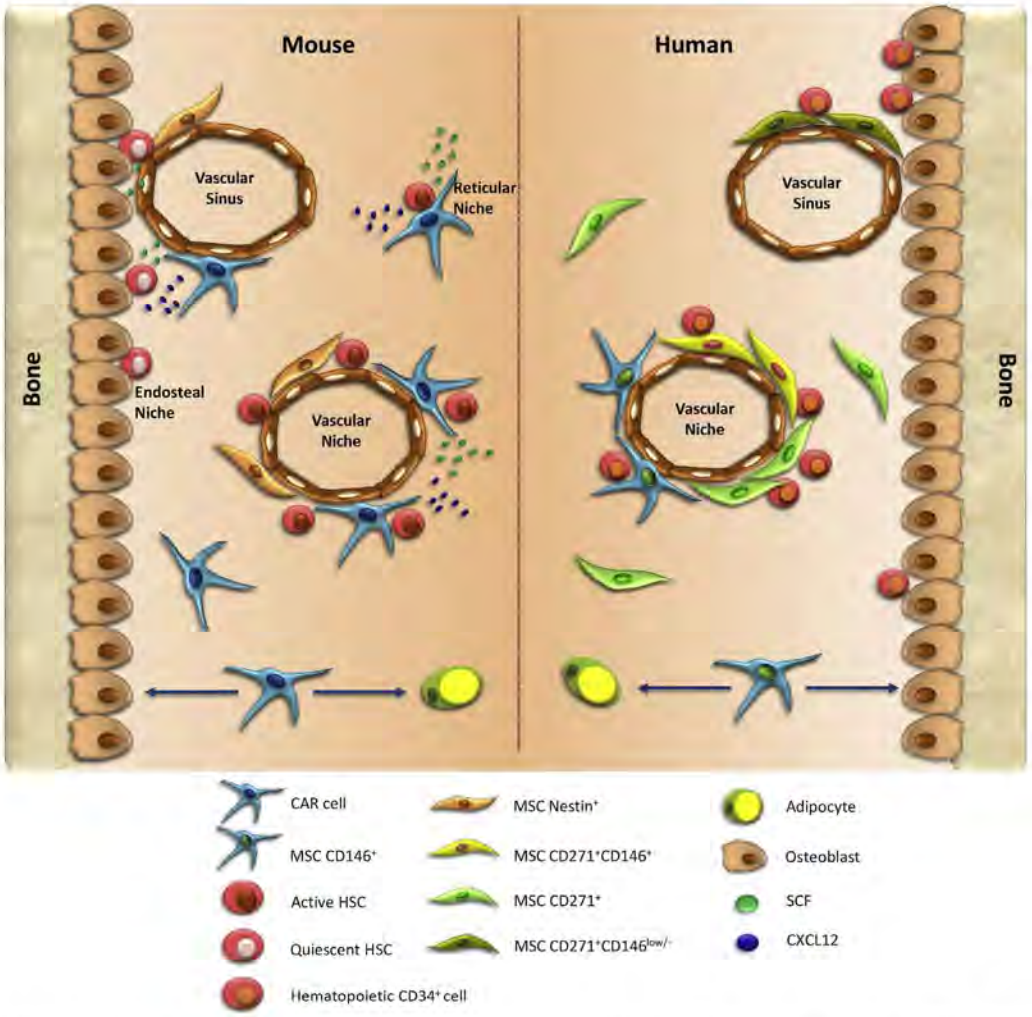

Figure 1. Model of the localization of mesenchymal stem cell (MSC) subpopulations within mouse and human bone marrow (BM). The endosteal, vascular, and reticular niches in mouse BM are shown. Two MSC subpopulations have been detected in the BM stroma: CAR cells, mainly located around the sinusoids and dispersed throughout the trabecular zone, and Nestin$^+$ MSCs, which are located mostly in the endosteal zone. In human BM, a CD146$^+$ MSC subpopulation has been detected, which has been suggested to be the human equivalent of murine CAR cells. CD271$^+$ and CD271$^+$CD146$^+$ MSCs have also been shown to be in a perivascular zone and CD271$^+$CD146$^{-/low}$ cells within the endosteal zone. (A color figure can be found in the online version of this article.)

important for mobilization, homing, and engraftment of HSC. Two perivascular cell groups that possess mesenchymal cell properties function as niche cells. One of these two cell groups has recently been shown to be high secretors of SDF-1 (CXCL12) and, as a result, have been named CXCL12 abundant reticular (CAR) cells. Phenotypically they express VCAM-1, CD44 and platelet-derived growth factor receptor (PDGFRα and PDGFRβ) (10). The other cell group found in mouse BM is a Nestin$^+$ subpopulation (Figure 1). In murine models, reticular cells are the major cellular component in the BM stroma and some of them as CAR cells, expressing CXCL12 at high levels, which is an essential chemokine in homing and HSCs maintenance (9,10). CAR cells are uniformly distributed throughout the BM around venous sinusoids and close to the endosteum. CAR cells constitute ~0.27% of the nucleated cells within the BM. There are an abundance of HSCs in close contact with them (9,10). CAR cells are also the main producer of stem cell factor (SCF) and are required for maintaining the number of erythroid progenitor cells and B cells and for keeping HSCs in an undifferentiated state (10). Most CAR cells possess the potential to differentiate into adipocytes and osteoblasts and are therefore considered to be a subpopulation of MSCs (10).

Using transgenic mice expressing GFP in Nestin$^+$ cells, Méndez-Ferrer et al. showed that the Nestin-GFP$^+$ cell subpopulation constitutes 0.04−0.08% of the nucleated cells in the BM. These Nestin-GFP$^+$ cells are considered to be a subpopulation of MSCs because they show an adipogenic and osteogenic potential (11). Nestin-GFP$^+$ cells exhibit a mostly perivascular distribution and near or adjacent to these cells there are a greater number of HSCs/HPCs (hematopoietic progenitor cells) compared with those found in the endosteal region. Nestin-GFP$^+$ cells exhibit high CXCL12 and SCF mRNA expression levels, similar to CAR cells, as well as Ang-1 (angiopoietin-1) and OPN (osteopontin). Within this Nestin-GFP$^+$ cell population, there are cells with adipogenic and osteogenic potential. Moreover, after Nestin-GFP$^+$ cell depletion, the number of HSCs/HPCs decreases by ~50% in the BM and increases in the spleen, whereas HSCs/HPCs homing to the BM is reduced by 90%. These findings suggest that Nestin-GFP$^+$ cells are involved in the homing and retention of HSCs in the BM (11).

Concerning the MSC subpopulations found in human BM, a study performed by Saccheti et al. identified a CD146$^+$ (melanoma cell adhesion molecule, MCAM) MSCs subpopulation, which exhibits a subendothelial localization in the venous sinusoid walls and represents ~0.11% of nucleated BM cells. Some 99% of CD146$^+$ cells form CFU-Fs *in vitro* (12). These cells also express CD105, alkaline phosphatase and high levels of Jagged-1, N-cadherin, CXCL12, and SCF. Human CD146$^+$ MSCs subpopulations transplanted into immunodeficient mice form bone and adipocytes in addition to showing hematopoietic activity. Due to their multipotent nature, localization, and ability to induce hematopoietic activity, it has been suggested that these CD146$^+$ cells could be the human counterpart to mouse CAR cells (12).

A study performed by Tormin et al. identified CD271$^+$CD146$^+$ and CD271$^+$CD146$^-$ cell subpopulations with similar characteristics to the CD146$^+$ MSCs reported by Saccheti et al. However, unlike the latter study, Tormin et al. reported that the expression or lack of CD146 was related to the localization of MSCs in the BM. The CD271$^+$CD146$^+$ subpopulation was found in the perivascular region, whereas the CD271$^+$CD146$^{-/low}$ subpopulation was found in the endosteal region. Interestingly, the CD34$^+$ HSCs/HPCs are in close proximity to both MSC subpopulations (13). This finding agrees with another report stating that 86% of CD34$^+$ HSCs/HPCs are in direct contact with CD271$^+$ MSCs, although mainly showing a perivascular distribution (14). Several studies have described that MSCs express a wide range of hematopoiesis-regulating molecules. Such molecules are involved in distinct processes that regulate homing, adhesion, quiescence, maintenance, self-renewal and proliferation of HSCs/HPCs (Table 1).

MSCs Confer Hematopoietic Support and Improvement

Based on different studies in animal models (mainly murine models), the capacity of human MSCs to confer hematopoietic support and improvement has been demonstrated. Delalat et al. showed that transplanting CD34$^+$ cells from umbilical cord blood (UCB) and BM-MSCs into irradiated mice significantly increased the number of single lineage-committed hematopoietic progenitors called colony-forming cells (CFCs) in the host's spleen (39). Another study using human CD34$^+$ cells from peripheral blood (PB) and BM-MSCs transplanted into sublethally irradiated NOD/SCID mice revealed an increase in the number of grafted hematopoietic cells, characterized by a large number of myeloid cells and platelets in PB as well as myeloid and megakaryocyte progenitors in mouse BM (40). Using different proportions of the BM CD34$^+$ HSC:MSCs transplanted into sublethally irradiated NOD/SCID mice, Kim et al. determined that the grafting rate increased with the number of MSCs, showing an optimal ratio of 1:8, whereas a higher MSC dose reduced graft efficiency (41). Likewise, it has been found that MSC transplantation promotes the grafting of two UCB units in NOD/SCID mice, decreasing the predominance in the graft of only one of the two units and increasing the total grafting of hematopoietic cells compared with a transplant without MSCs (42). Another study in which BM-MSCs and UCB-CD34$^+$ cells were co-transplanted into NOD/SCID mice via intramedullary injection showed that from 4−10 weeks after transplantation the MSCs became grafted within the hematopoietic

Table 1. Hematopoiesis-regulating molecules secreted and/or membrane-expressed in MSCs

Molecules (receptor/ligand)	MSC source			Effect on hematopoietic cells	Reference
	BM	UCB	PL		
Soluble cytokines					
CXCL12 (SDF-1)/CXCR4	●	●	●	Maintenance, quiescence, survival, homing, and HSC and HPC expansion	(10,15–17)
TPO/MPL	●	●	ND	HSC quiescence, fate commitment and differentiation of thrombocytes and platelet release	(18–20)
SCF/c-kit (CD117)	●	●	●	HSC maintenance and expansion, HPC proliferation and differentiation toward myeloid and erythroid lineages, in combination with other factors.	(15,18,21–23)
FL/flt3	●	●	●	Survival, HSC maintenance and self-renewal, HPC proliferation and differentiation toward myeloid and erythroid lineages, in combination with other factors.	(18,21,23,24)
IL-6/IL-6R	●	●	●	Fate commitment, HPC proliferation and maturation toward myeloid and megakaryocytic lineages	(15,17,18,21)
GM-CSF/GM-CSFR	●	●	IL-1α, TNFα stimulated	Pleiotropic effects on hematopoietic cells, HPC fate commitment toward myeloid lineage	(17,18,21,25)
G-CSF/G-CSFR	IL-1α stimulated	ND	IL-1α, TNFα stimulated	HPC proliferation, differentiation and survival, terminal differentiation and maturation of granulocytes	(21,25,26)
M-CSF/M-CSFR	●	●	●	HPC proliferation, differentiation and survival, terminal differentiation and maturation of macrophages	(18,21,25)
Adhesion molecules					
N-cadherin/β-catenin	●	●	ND	HSC stemness and niche interaction	(12,17,27)
VCAM-1 (CD106)/integrins α4, β1, β2	●	●	ND	Stromal adhesion of hematopoietic cells	(17,28–30)
ICAM-1 (CD54)/integrins β1, β2	●	●	●	HSC homing	(29–31)
ALCAM (CD166)/ALCAM, CD6	●	●	●	HSC maintenance	(31,32)
LFA-3 (CD58)/integrins αLβ2	●	●	●	Stromal adhesion of hematopoietic cells	(29,31)
MCAM (CD146)/VEGF	●	ND	●	HSC and HPC maintenance	(12,33)
ECM molecules					
Collagen I/integrins β1, β2, CD44	●	ND	ND	HSC maintenance	(20,30,34)
Fibronectin/integrins β1, β2,CD44	●	ND	●	Reported to either promote or inhibit HSC proliferation, leading to controversy	(20,21,30,35)
Laminin/integrins α6, β1, β2	●	ND	●	Stromal adhesion of hematopoietic cells	(21,26,30)
Osteopontin/integrins β1, CD44	●	ND	ND	Maintenance regulation and HSC homing to the BM	(36,37)
Other molecules					
Jagged-1/Notch1,2	●	ND	ND	HSC self-renewal	(12,38)

BM, bone marrow; HSC, hematopoietic stem cells; HPC, hematopoietic progenitor cells; UCB, umbilical cord blood; PL, placenta; ND, not determined.
● Molecule expression in the indicated source.

microenvironment of the host mice and differentiated into pericytes, stromal cells, osteoblasts and endothelial cells, all of which are essential components of the hematopoietic microenvironment. Moreover, the presence of human MSCs in mouse BM increases the activity of human hematopoietic cells (43). There are also studies reporting the hematopoietic support capacity of MSCs in other animal models such as fetal sheep where the grafting of hematopoietic cells is promoted, and the presence of circulating donor cells is observed during gestation and after birth (44). Similarly, it has been shown that co-transplantation of MSCs and HSCs in Rhesus macaques improves the grafting of hematopoietic cells (45).

Ex vivo Manipulation of MSCs

MSCs have been used in co-culture systems as a feeder layer for hematopoietic cells with the purpose of promoting the expansion of HSCs/HPCs, particularly those from UCB, with the aim of obtaining a sufficient number of cells to be used for adult HSCT. Several groups have shown that the use of MSCs improves HSCs/HPCs expansion *ex vivo*, even

demonstrating that the expansion of hematopoietic cells is much greater in the presence of both MSCs and cytokines that are important in the maintenance of primitive cells compared with that obtained in the presence of either MSCs or cytokines alone. These results indicate a synergistic effect of these two components (MSCs and cytokines) in promoting hematopoiesis *ex vivo* as previously shown (46). Our group and others have expanded HSCs/HPCs from more homogenic and primitive populations such as $CD34^+$ and $CD34^+CD38^-Lin^-$cell populations enriched by positive and negative selection, respectively, obtaining a greater increase in the number of hematopoietic cells (Supplementary Table 1).

Interestingly, De Lima et al. reported transplantation of UCB hematopoietic cells previously cultured *ex vivo* with MSCs into patients with hematological disorders. The patients received two UCB units, one of which was previously expanded in culture with MSCs and another non-manipulated unit. The co-culture of UCB hematopoietic cells (total nucleated cells) with MSCs was performed for 14 days (before transplantation) in the presence of hematopoietic cytokines (SCF, FL, TPO, and G-CSF, 100 ng/mL each). Co-culture with MSCs increased the number of nucleated cells 12.2-fold, that of $CD34^+$ cells 30.1-fold, and the number of CFCs 17.5-fold. Patients received 8.34×10^7 nucleated cells/kg of weight and 1.81×10^6 $CD34^+$ cells/kg of weight on average, among which the minority came from the non-manipulated UCB unit and the majority came from the unit cultured with MSCs (2.28×10^6 and 0.38×10^6, respectively). In the patients transplanted with hematopoietic cells co-cultured with MSCs, the grafting of neutrophils and platelets was observed at an earlier time (15 and 42 days, respectively) compared with patients receiving non-manipulated cells (24 and 49 days, respectively) (47). This study provides evidence that MSCs can improve HSCT indirectly, i.e., increasing the number of hematopoietic cells and improving their grafting capacity prior to transplantation. Currently, application of hematopoietic cells previously cultured *ex vivo* with MSC is a little studied subject and only one study is registered in clinicaltrials.gov (48).

Clinical Application of MSCs in HSCT

Application of MSCs favors grafting and hematopoietic recovery in HSCT. HSCT is the transplantation of hematopoietic stem cells derived from the BM, PB or UCB. It may be autologous (the patient's own HSC are used) or allogeneic (the HSC come from a donor). It is most often performed for patients with multiple myeloma or leukemia. In these cases, BM cells are usually destroyed with radiation or chemotherapy before transplantation. Infection and graft vs. host disease (GVHD) are major complications of allogeneic transplantation. HSCT success depends on the number of transplanted HSCs/HPCs and graft resistance to host rejection to a large extent (49). In this regard, because of the ability of MSCs to generate BM stromal cells, form part of the hematopoietic niche and produce hematopoiesis-regulating molecules as well as their confirmed capacity to provide hematopoietic support both *ex vivo* and in animal models, they have been applied in clinical practice to improve HSCT (Supplementary Table 2).

Several studies have reported that MSCs favor grafting and rapid hematopoietic recovery (number of neutrophils and platelets) in HSCT patients (Supplementary Table 2). The benefit of their application in easing grafting may be related to their capacity for hematopoietic support, particularly when the number of HSCs/HPCs is low (50,51). MSCs can also improve HSCT by promoting the formation of an anti-inflammatory microenvironment, which reduces the probability of graft rejection (52). In some HSCT cases, mainly in breast cancer patients, autologous MSCs are used (53), unlike the allogeneic MSCs used in patients with hematological disorders (Supplementary Table 2). Interestingly, in a study that was performed in 14 pediatric patients with hematological disorders or immune deficiencies who were IV transplanted with allogeneic BM-MSCs compared with 47 patients subjected to the same transplant conditions but without MSCs, it was observed that neutrophil and platelet reconstitution was faster in patients treated with MSCs (52). Similar results have been reported by other groups (54,55). However, there are also reports in which the prompt hematopoietic reconstitution observed in MSC-HSCT patients is not much different from that of non-MSC-transplanted patients (56–58) (Supplementary Table 2).

It is likely that the differences in these results related to the clinical application of MSCs reported by several groups may be due to the applied MSC expansion conditions at the clinical scale and even to the number of passages and utilized doses. Moreover, no consensus has been reached regarding the possibility of applying successive doses to optimize the application of these cells. Currently, there are several groups attempting to optimize culture conditions to improve MSC expansion and quality, and good results have been reported using platelet lysates, autologous serum, and platelet-rich plasma in contrast to those obtained using animal serum (59,60). Other studies have provided evidence demonstrating the change in the immunophenotype of MSCs expanded *in vitro* as well as the loss of their multipotency and proliferation capacity. Therefore, culture conditions (e.g., culture media, supplements or growth surface employed) can affect the biological characteristics of MSCs and should be considered in the expansion of these cells at a clinical scale (60).

In patients with hematological disorders, the stroma of the BM also suffers significant and prolonged damage due to the high doses of chemotherapy and radiotherapy applied before HSCT, which can delay hematopoietic cell grafting (51). Galotto et al. in 1999 observed an up to

90% decrease in the frequency of CFU-Fs (MSCs) in HSCT patients, and no recovery of the frequency of these cells was detected, even 12 years post-transplantation. This reduction in the number of MSCs would affect the formation of stromal cells as well as the graft itself and the maintenance of HSCs/HPCs production, which could be related to the significant decrease in bone mineral density and LTC-IC levels (HSC/HPCs) found in these patients (61), resulting in deficient hematopoietic activity. In this regard, it is possible that transplanted MSCs may reconstitute the BM stromal system following an ablative treatment as reported in murine models because increases in the levels of SDF-1, TPO, and IL-11 have been detected in the serum of MSC-HSCT patients, which is important because these cytokines are essential for the maintenance of HSCs/HPCs and are produced mainly by MSCs and stromal cells (62). It is possible that MSCs are involved in stroma reconstitution in transplanted patients because donor MSCs have been detected in the patients' BM up to 6 months after HSCT with MSCs (63).

At present, 33 clinical studies from ~532 related to MSC application for several disorders have been registered about MSC-infusion to promote engraftment of HSCT and as treatment for GVHD (48).

MSCs sources other than BM and their clinical potential in HSCT. BM has been the main source of MSCs for application in HSCT. However, our group and others have shown that MSCs can be isolated from other tissues or organs in the adult stage, fetal sources (64) and from neonatal sources such as the umbilical cord (65), umbilical cord blood, and placenta (31).

We isolated MSCs from alternative sources to the BM, particularly from neonatal sources. It presents the advantage of being harmless to donors, exhibiting a lower risk of viral transmission and easy accessibility. MSCs obtained from such sources also meet the criteria defining MSCs, including showing a capacity for adhesion to the culture vessel, fibroblast-like morphology, expression of characteristic MSCs markers, and their differentiation potential (31). Similarly, it has been reported that MSCs from neonatal sources exhibit the capacity to produce molecules regulating hematopoiesis (Table 1), increasing HPCs expansion *ex vivo* (18,21) (Supplementary Table 1) and favoring hematopoietic grafting in murine models (66,67).

Application of MSCs from neonatal sources in HSCT such as those from the umbilical cord (UC) has recently been initiated. In this regard, three reports have been published with similar results (Supplementary Table 2) (50,65,68). Thus, UC-MSCs have been utilized in allogeneic HSCT of the BM, mobilized PB, and UCB, in dosages ranging from $0.8-10 \times 10^6$/kg of weight applied via the IV route hours before transplant. UC-MSCs ensured the grafting of hematopoietic cells and promoted a rapid recovery of neutrophils and platelets compared with HSCT patients without prior MSC treatment. These reports show more homogeneous results compared with those obtained from BM-MSC treatments because hematopoietic reconstitution is favorable in most treated patients. The last observation may suggest that MSCs obtained from neonatal sources are a viable option for the improvement of HSCT (50,65,68).

Conclusion

In addition to giving rise to stromal cells, MSCs themselves are essential components of the BM stroma and the HSC niche. MSC subpopulations are mainly found in the perivascular region and in close contact with BM-HSCs, which has been observed in both humans and mice. Due to their capacity to provide hematopoietic support, demonstrated both *ex vivo* and in animal models, MSCs have been clinically employed with the purpose of improving HSCT. However, some reports indicate that BM-MSCs do not significantly improve HSCT. Therefore, it is necessary to continue studying their biological and functional properties during expansion at a clinical scale and their manipulation *ex vivo* to determine whether these procedures have any influence on the functional characteristics of these cells. MSCs from neonatal sources have shown encouraging results in favoring HSCT. However, as in the case of BM-MSCs, it is necessary to further study their biological characteristics and find better conditions for their isolation and culture at a clinical scale *ex vivo* to obtain more favorable results for their application as a cellular therapy to improve HSCT.

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Supplementary Data

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Supplementary Table 1. *Ex vivo* studies on the effect of MSCs on the proliferation and expansion of hematopoietic cells

Source of hematopoietic cells	Source of MSCs	Culture conditions	Results (fold-increase)	Reference
UCB-MNC	BM	IL-3 (20 ng/mL), IL-6 (20 ng/mL), FL (100 ng/mL), SFC (100 ng/mL), G-CSF (20 ng/mL), EPO (0.1U/ml). For 12 days	Increased expansion of $CD34^+CD38^-HLADR^-$ cells (2.84 ± 0.29) and LTC-IC (7.7 ± 2.1) with MSCs plus cytokines vs. cytokines alone (2.09 ± 0.26 and 5.7 ± 1.4, respectively)	(69)
UCB-MNC	BM	SCF (100 ng/mL), G-CSF (100 ng/mL), MGDF (100 ng/mL). For 13 days	Increases in MNCs (10–20), myeloid-CFCs (7–18), HPP-CFCs (2–5) and $CD34^+$ (16–37) with MSCs plus cytokines	(70)
UCB-CD34+ cells	BM	SCF (50 ng/mL), FL (50 ng/mL), TPO (20 ng/mL). For 12 days	Greater increases in MNCs (65.25 ± 9.77), CFCs (22.27 ± 3.32), $CD34^+$ cells (7.46 ± 1.11) and LTC-IC (4.63 ± 1.6) with MSCs plus cytokines vs. cytokines alone (55.92 ± 9.54, 17.63 ± 3.05, 5.34 ± 0.91 and 3.54 ± 1.5, respectively)	(21)
UCB-CD34+ cells	PL	SCF (50 ng/mL), FL (50 ng/mL), TPO (20 ng/mL). For 12 days	Greater increases in MNCs (105.92 ± 16.55), CFCs (36.73 ± 5.79), cells $CD34^+$ (14.89 ± 2.32) and LTC-IC (7.43 ± 2.66) with MSCs plus cytokines vs. cytokines alone (55.92 ± 9.54, 17.63 ± 3.05, 5.34 ± 0.91 and 3.54 ± 1.5, respectively)	(21)
UCB-CD34+ cells	UCB	SCF (100 ng/mL), TPO (10 ng/mL), FL (50 ng/mL), IL-6	Greater increase in the proliferation of $CD34^+$ cells with	(18)

		(100 ng/mL). For 14 days	MSCs plus cytokines vs. cytokines alone. Greater CFC expansion with MSCs without cytokines.	
MPB-CD34+ cells	BM	TPO (10 ng/mL), FL (50 ng/mL), SCF (50 ng/mL), IL-6 (10 ng/mL). For 14 days	Higher frequency of $CD34^+$ cells with MSCs plus cytokines vs. cytokines alone at day 7, 85 ± 11% vs. 54 ± 8.9% and at day 14 of culture 40 ± 9% vs 3.4 ± 1.2%.	(71)
UCB-CD34+ cells	UCB	TPO (80 ng/mL), SCF (50 ng/mL), and FL (50 ng/mL). For 14 days.	Adhesion of $CD34^+$ cells, maintenance of CD34+CD38- cells and LTC-IC, showing the same result as in the BM-MSCs	(17)
UCB-CD34+CD38-Lin- cells	BM	SCF (10 ng/mL), TPO (10 ng/mL), FL (10 ng/mL), IL-6 (10 ng/mL). For 28 days	Greater increase in MNCs (2,500, day 28), myeloid-CFCs (360, day 21) and $CD34^+$ cells (40, day 14) with MSCs plus cytokines vs. cytokines alone (440 for MNCs, 16 for myeloid-CFCs and no increase in $CD34^+$ cells)	(46)
UCB-CD34+ cells	PL/UCB	IL-3 (100 ng/mL), SFC (100 ng/mL), TPO (50 ng/mL). For 14 days	Greater increases in proliferation of CD34+ cells and expansion of CFCs, $CD34^+$ cells, and CD34+CD38- cells with MSCs plus cytokines vs. cytokines alone	(72)
UCB-CD34+ cells	UC	TPO (25 ng/mL). For 7 days	Higher frequency of CD34+ and CD34+$CD41a^+$ cells with MSCs plus cytokines 32.8 ± 13.2% and 9.0 ± 5.1%, respectively vs. cytokines alone 19.8 ± 2.5% and 6.2 ± 0.5%, respectively.	(73)
UCB-CD34+ cells	BM	SCF (10 ng/mL), TPO (20 ng/mL), FGF-1 (10 ng/mL). For 7 days	Greater increase in the proliferation of CD34+ cells and the expansion of CFCs with MSCs plus cytokines vs. cytokines alone	(74)

UCB-CD34+ cells	BM	SCF (50 ng/mL), TPO (50 ng/mL), FL (40 ng/mL). For 14 days	Greater increases in MNCs (46.6 ± 5), CFCs (90 ± 24) and $CD34^+$ cells (43.25 ± 8) with MSCs plus cytokines vs. cytokines alone (32 ± 2, 87 ± 13, 20.5 ± 7.8, respectively). Also a higher percentage of apoptotic cells in culture with cytokines alone in comparison with those in presence of MSCs.	(75)

BM, bone marrow; UCB, umbilical cord blood (UCB); PL, placenta; UC, umbilical cord; ND, not determined.

Suplementary Table 2. Clinical studies on the effect of MSCs on the HSC grafting index and time of hematopoietic recovery in HSCT patients

Subjetcs	Number of patients	Source of HSCs/HPCs ($CD34^+$, $x10^6$/kg)	Source of MSCs (MSCs, $x10^6$/kg)	MSC timing	Time to recovery ANC $0.5x10^9$/L. Average (range days)	Time to recovery platelets $20x10^9$/L. Average (range days)	Effect	Reference
Breast cancer	28	Autologous PB	Autologous BM (1–2.2)	At the time of PB	8 (6–11)	8.5 (4–19)	Safe treatment and fast hematopoietic recovery	(53)
Hematological diseases	46	Allogeneic BM or PB	Allogeneic BM (1–5)	4 h pre-HCT	14 (11–26)	20 (15–36)	Fast hematopoietic recovery, but without a difference in the grafting rate compared with the controls	(76)
Hematological deseases	7	Allogeneic BM (2.65–3.39), MPB (7.2–68.9) or UCB (0.21)	Allogeneic BM (1)	0–4 h post HCT	12 (10–28)	12 (8–36)	Fast engraftment and 100% of donor chimerism, including in patients with first transplant failure	(54)
Hematological diseases and nonmalignant disorders (immune	14	Allogeneic MPB 21.5 (11.6–38.6)	Allogeneic BM 1.6 (1–3.3)	4 h pre-HCT	12 (10–17) vs. 13[a] (9–28)	10 (9–18) vs. 13[a] (9–100)	Transplantation successful in 100% of patients, with fast hematopoietic reconstitution compared	(52)

deficiency) in pediatric patients							with historical controls (15% transplant failure)	
Hematological diseases	10	Allogeneic BM 4.27 (0.27–10.11)	Allogeneic BM 0.34 (0.03–1.5)	4 h pre-HCT	16 vs. 15[a]	30 vs. 27[a]	Higher relapse than the control group	(77)
Pediatric acute leukemia	8	UCB	Allogeneic BM (0.9–5)	day 0	19	53	No difference in engraftment rate compared with historical controls	(56)
Hematological diseases, deficiency on hematopoietic recovery	6	Allogeneic MPB (2.1–5)	Allogeneic BM (1)	Post-HCT	5–15	12–21	2/6 patients showed rapid hematopoietic recovery	(55)
Hematological diseases	9	Allogeneic MPB 2.61 (2.4–3.3), UCB 0.12 (0.037–0.28)	Allogeneic BM, 1.2 (1.04–2.15)	Immediately after HSCT	12 (1–31) vs. 10[a] (9–36)	44 (27–98) vs. 32[a] (13–97)	No difference in grafting compared with the controls	(57)
Pediatric aplastic anemia	6	Allogeneic BM (7.98) and PB (7.25)	Allogeneic BM or UC, 1.4 (0.8–2.5)	Pre-HCT	12.3 (11–18)	13.8 (11–22)	Fast hematopoietic recovery, with all of the patients surviving follow-up a range of 6–29 months	(65)
Hematological diseases with primary or secondary	20	Allogeneic BM/PB, 5.51 (3.07–8.45)	Allogeneic BM 1 or 3 doses, one dose every 4	Post-HCT	14	14	17/20 patients showed reaction to MSC transplantation. Most	(78)

graft failure			weeks (1)				patients showed development of infection episodes (CMV or EBV). After 166–904 days, only 9 patients survived. MSCs are beneficial in the treatment of primary and secondary graft failure	
High-risk acute lymphoblastic leukemia and acute myeloid leukemia	8	UCB, 0.3 (0.25–0.61)	UC 7.1 (2.4–10.1)	4 h pre-HCT	12 (8–16) vs. 21[a] (17–43)	30 (20–45) vs. 73[a] (42–135)	Fast hematopoietic recovery, improved grafting	(50)
Severe aplastic anemia	17	Allogeneic BM 9.3 (6.3–13.2) and MPB 4.5 (2.8–8)	UC (2.8–10)	6 h pre-HCT	12 (11–21)	14 (11–75)	Improved patient survival	(68)

BM, bone marrow; UCB, umbilical cord blood; UC, umbilical cord; PB, peripheral blood; MPB, mobilized peripheral blood; HCT, hematopoietic cell transplantation; ANC, absolute neutrophil count.

[a]Control group.

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Discusión y conclusiones generales

En este trabajo se describen las propiedades de soporte hematopoyético y capacidad de inmunosupresión de las MSCs descritas *in vitro* e *in vivo* y por las cuales han representado una importante opción de tratamiento para mejorar la recuperación hematopoyética así como para prevenir o tratar la GVDH durante o después del HSCT. Las MSCs producen células que forman el estroma de las HSCs dentro de la MO, así como moléculas reguladoras de la hematopoyesis las cuales involucran citocinas solubles, moléculas de MEC y moléculas expresadas a nivel de membrana que permiten su contacto directo con células hematopoyéticas primitivas, y se encuentran presentes en los nichos de las HSCs favoreciendo el mantenimiento y autorenovación de estas últimas. Las MSCs también producen moléculas solubles que se expresan a nivel de membrana que permiten su contacto con células del sistema inmunológico y que inhiben su proliferación y activación, por lo que suprimen alguna reacción inmunológica presente. Debido a estas propiedades que presentan las MSCs, recientemente han iniciado a nivel mundial un importante número de ensayos clínicos que involucran su uso en el HSCT, mostrando a la fecha resultados alentadores para mejorar y promover una recuperación hematopoyéticas más rápida y para tratar la GVHD entre 30% y 60% de los pacientes, lo cual evita que un porcentaje importante de pacientes muera por fallo del injerto o por el desarrollo de la GVHD (Fajardo-Orduña et al, 2015; Castro-Manreza and Montesinos, 2015; Kuzmina et al, 2012; Kim and Cho, 2013). A pesar de las evidencias en reportes clínicos que muestran que las MSCs favorecen el HSCT, también existen reportes que muestran un importante porcentaje de los casos en los que estas células no mejoran la recuperación del paciente, por lo que es de gran importancia investigar y determinar las posibles causas que inducen el fallo de las MSCs en un número importante de pacientes.

Por otro lado, el número de MSCs que se obtiene a partir de la MO (principal fuente de estas células para su uso en terapia celular) u otros tejidos, es insuficiente para su aplicación clínica (Montesinos et al., 2009). Por lo que ha sido necesario realizar la expansión *in vitro* de MSCs para obtener el número de células suficientes para su aplicación en el HSCT.

Actualmente, se han reportado estudios que evalúan el inmunofenotipo y la capacidad de diferenciación de las MSCs después de someterlas a una expansión a escala clínica, sin embargo, existe controversia sobre si este tipo de cultivo *in vitro* modifica o no estas características biológicas (Azouna et al, 2012; Chase et al, 2012; Cooper et al, 2010; Dolley-Sonneville et al, 2013; Dos Santos et al, 2011; 2014; Martin-Manso and Hanley, 2015; Carmelo et al, 2015), aunado a esto, no existen reportes sobre si el cultivo a escala clínica de las MSCs modifica sus propiedades funcionales, principalmente capacidad de soporte hematopoyético y capacidad de inmunosupresión, ambos de gran importancia para su uso en el HSCT y el tratamiento contra la GVHD, por lo que es necesario analizar con el objetivo de conocer la calidad de las MSCs para trasplantar y asegurar que se lleve a cabo la recuperación esperada en los pacientes.

Por todo lo anterior, en este trabajo se determinó y comparó la capacidad de diferenciación, soporte hematopoyético e inmunosupresión de MSCs de MO antes y después de haberlas expandido a escala clínica dentro de cajas de cultivo HyperFlask. En nuestra investigación logramos expandir y obtener hasta 125.52 x 10^6 ± 25.6 x 10^6 MSCs a partir del cultivo a escala clínica, lo que correspondería al número de células requeridas para su uso en el HSCT de aproximadamente 1.7 x 10^6 MSCs por kg de un paciente de 70 kg de peso. Encontramos que el CSC mantiene el inmunofenotipo y en gran medida la morfología fibroblastoide de las MSCs, sin embargo, favorece el desarrollo de células grandes y anchas en comparación con MSCs de SSC. La capacidad de diferenciación adipogénica osteogénica y condrogénica se redujo de manera importante en las MSCs provenientes de CSC, lo que confirma datos reportados por otros grupos (Gharibi and Hughes, 2012; Narcisi et al, 2015; Neuhuber et al, 2008). Respecto a esto, se ha visto que MSCs grandes y anchas están relacionadas con una disminución de la capacidad de diferenciación adipogénica y condrogénica (Narcisi et al, 2015; Neuhuber et al, 2008), por otro lado, también se ha reportado que MSCs con estas características morfológicas (grandes y anchas) son células senescentes (Majore et al., 2009; Chang et al., 2015). Esto podría sugerir además, que el efecto de regeneración de tejidos que llevan a cabo las MSCs trasplantadas, se pudiera realizar a través de la secreción de factores que favorezcan la recuperación del tejido dañado y no por la diferenciación de las MSCs hacia células del linaje para lo que se requiere la regeneración, tal como ha sido propuesto por otros autores (Horwitz and Prather 2009).

Respecto a la capacidad de soporte hematopoyético, encontramos que las MSCs provenientes de CSC mantienen la capacidad para incrementar la proliferación de células hematopoyéticas totales (CMNs), la expansión de HPCs (CFC-Mieolides y CFC-Eritroides) y HPCs primitivas (CD34+CD38-Lin- y LT-CIC) a partir de la población enriquecida en células $CD34^{+}CD38^{-}Lin^{-}$ de SCU. También, encontramos que el CSC no modifica la capacidad de las MSCs para expresar moléculas de MEC, moléculas expresadas a nivel de membrana y producir citocinas importantes en la formación de células hematopoyéticas. Estos resultados podrían sugerir que de manera independiente a la expansión a escala clínica de las MSCs, pueden estar involucrados otros factores que interfieran en la recuperación hematopoyética de los pacientes con HSCT (Zhao et al, 2015; Chen et al, 2015). Otro aspecto que analizamos en las MSCs provenientes de CSC fue su capacidad de inmunosupresión, respecto a lo cual, encontramos que este afecta de manera negativa a las MSCs ya que pierden la capacidad para disminuir la proliferación de linfocitos T $CD3^{+}$ y $CD8^{+}$, y disminuyen su capacidad para suprimir la proliferación de linfocitos T $CD4^{+}$. Tal disminución en la capacidad de supresión de la proliferación de linfocitos en las MSCs después de someterlas a un CSC, podría afectar su potencial de inmunosupresión en los tratamientos clínicos, no sólo en el tratamiento para GVHD si no también en su uso como tratamiento de enfermedades autoinmunes (Castro-Manrreza y Montesinos, 2015), por lo que es necesario realizar más estudios al respecto.

La presencia de MSCs con morfología ancha, la disminución en las capacidades funcionales de las MSCs tales como capacidad de diferenciación adipogénica, osteogénica y condrogénica y capacidad de inmunosupresión, podría sugerir que las MSCs expandidas en CSC llegan a una senescencia replicativa, la cual se caracteriza por la reducción gradual de la proliferación, alteraciones morfológicas y finalmente la pérdida de sus funciones o senescencia funcional (Bonab et al, 2006; Gharibi and Hughes, 2012). El mecanismo detrás de la senescencia replicativa de las MSCs puede ser multifactorial y asociado con el acortamiento de los telomeros y la acumulación del daño al DNA y/o cambios epigenéticos, sin embargo, los mecanismos detrás de la senescencia funcional de las MSCs son menos conocidos (Gharibi and Hughes, 2012).

Por todo lo anterior, con este trabajo podemos concluir que el CSC modifica parte de la capacidad funcional de las MSCs de MO, disminuyendo su capacidad de diferenciación

adipogénica, osteogénica y condrogénica así como su capacidad de inmunosupresión. Por lo que es necesario estudiar más a fondo el efecto del CSC en la capacidad funcional de las MSCs, tal como en ensayos *in vivo*, y tratar de mejorar las condiciones y sistemas de cultivo de expansión a escala clínica para llevar a cabo una adecuada producción de MSCs con la finalidad de asegurar la obtención de células con buena calidad para aplicaciones de regeneración de tejidos como hueso y cartílago, así como, para su uso en donde se requiera su capacidad de inmunosupresión tal como el caso del tratamiento de la GVHD o de enfermedades autoinmunes, esto para asegurar su eficacia en los pacientes. No obstante debido a que el CSC no afecta la capacidad de soporte hematopoyético de las MSCs, éstas se pueden utilizar en pacientes con HSCT en donde el objetivo principal sea la recuperación hematopoyética rápida.

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