Terapia celular para lesiones que afectan a la médula espinal

Trasplante de células mesenquimales estromales o células de la glia envolvente del bulbo olfativo como tratamiento para lesiones de médula espinal

Abel Torres Espín

Tesis Doctoral

Dirigida por:

Dr. Xavier Navarro Acebes

Director de Tesis: Doctorante:

Dr. Xavier Navarro Acebes **Abel Torres Espín**

Departament de Biologia Cel·lular, de Fisiologia i d'Immunologia
Facultat de Medicina, Institut de Neurociències,
Universitat Autònoma de Barcelona

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INc Institut de Neurociències

UAB Universitat Autònoma de Barcelona

"Todos somos muy ignorantes. Lo que ocurre es que no todos ignoramos las mismas cosas."

Albert Einstein

Agradecimientos

En el diccionario de la RAE se define agradecer como "mostrar gratitud o dar gracias". Gracias proviene del latín *gratia*, la cual deriva de gratus y significa agradable, agraciado. Ambas formas surgen de la raíz indoeuropea *gwere* que resultará en varias palabras en castellano (grato, gracia, gracias, agradar, gratitud, ingrato, congratular y gratis) o en céltico como *bard* que a su vez dará lugar a la palabra bardo[1]...¿Pero esto no es una tesis sobre terapia celular? Pues sí. Durante la realización de este manuscrito he descubierto que lo más divertido de escribir una tesis es... la bibliografía e introducir las referencias (aunque lo hagas mediante software específico), así que no he podido evitar poner una más y sí, en los agradecimientos.

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Tanto en el teatro, como en el cine o la música de orquestra el director es fundamental y engrandece el resultado de la obra representada. La ciencia no puede ser menos y detrás de los grandes trabajos suele haber un gran director. No se si esta tesis será grande o no, pero puedo decir que ha sido dirigida por un Grande. Sin duda alguna, GRACIAS por ser mi maestro. Espero impaciente el día en que llegue a una centésima parte de tus conocimientos. Además, los grandes directores (ya se, hay sinónimos para grande pero me gusta más así...) gobiernan con sabiduría tribus enteras de becarios y doctores jovenzuelos. Y lo reconozco, podemos ser la causa del mayor dolor de cabeza, pero ¿que harías sin nosotros? Mejor no contestes... Por supuesto, el reparto de esta obra es amplio y todos los que formáis esa tribu sois los actores principales. GRACIAS a todos por el apoyo, las risas, los consejos e ideas, sin vosotros no habría yo, y sin yo esta tesis seria de otro.

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[1]Corominas, J., *Diccionario crítico etimológico castellano e hispánico,* Editorial Gredos, Madrid, 1980.

Índice de contenido

Resumen

La médula espinal puede verse dañada por patologías no traumáticas como tumores, infecciones, enfermedades autoinmunes y enfermedades degenerativas, y por lesiones traumáticas, tanto por acción directa como indirecta. Las lesiones traumáticas que dañan la medula espinal constituyen unas de las mayores causa de discapacidad física persistente al largo de toda la vida del paciente. A día de hoy, no hay tratamiento eficaz para este tipo de dolencias y las aplicaciones terapéuticas se basan en sacar el máximo potencial a las funciones conservadas. En los esfuerzos por encontrar tratamientos para éstas lesiones, el trasplante de diferentes tipos celulares constituye uno de los pilares de la investigación experimental. Dentro de esta estrategia dos de las poblaciones de células más usadas son las células de la glia envolvente que se encuentran en el bulbo olfatorio (OEC) y las células estromales mesenquimales (MSC). Ambos tipos celulares han demostrado mejorar la recuperación funcional y proteger parte del tejido dañado después de ser trasplantadas en modelos animales de lesiones de médula espinal. A pesar de ello, los mecanismos que subyacen a su acción han sido poco estudiados. Por este motivo, los trabajos incluidos en la presente tesis buscan la respuesta a un aspecto desconocido de estas terapias, como es la comparación del potencial terapéutico de las OEC y las MSC para las lesiones de médula espinal por contusión, tanto en su efecto como en su mecanismo. Los datos mostraron como, a pesar de que ambas células promovían una protección de tejido, las mejoras funcionales encontradas fueron limitadas y tan solo el trasplante de MSC en tiempos inmediatos a la lesión resultó en una mejora significativa. La poca supervivencia de las células dentro de la médula espinal lesionada podría ser uno de los motivos que explicaría la falta de un efecto beneficioso a nivel funcional. Un análisis de los cambios génicos inducidos por el trasplante reveló que la muerte de las células trasplantadas podría ser consecuencia de un rechazo inmunológico. La administración de un fármaco inmunosupresor a lo largo del seguimiento de los animales confirmó esta hipótesis, extendiendo la supervivencia de las células, pero con mejoras funcionales aun escasas, sugiriendo que la poca supervivencia de las células no es la única causa de la limitada mejora funcional encontrada. Por otro lado, el estudio génico también mostró como ambas células inducen la protección del tejido potenciando vías de reparación tisular. Mientras que las MSC modulaban estos procesos de reparación, aumentándolos en los tiempos tempranos después de la lesión y normalizándolos más tarde, las OEC actuaban tan solo en los tiempos tempranos, con reducidos efectos en los tiempos tardíos después de la lesión. Además, se ha indagado en la utilidad de las MSC como coadyuvante para el tratamiento de las lesiones de médula espinal por avulsión de raíz ventral. Este trabajo demostró como la presencia de las MSC en la médula espinal después de una avulsión mejoraba la supervivencia de motoneuronas y, en combinación con la reparación quirúrgica, aumentaba la velocidad de regeneración axonal y de reinervación muscular. En conclusión, la terapia celular con OEC o con MSC para lesiones de médula espinal puede ayudar al tratamiento de estas patologías, aunque por si solas las mejoras obtenidas son insatisfactorias. Una mejor comprensión de los mecanismos por los cuales las células trasplantadas ejercen su acción protectora permitiría optimizar su uso y establecer tratamientos combinados que respondan a varios objetivos terapéuticos.

INTRODUCCIÓN

1. La médula espinal

La médula espinal es la estructura del sistema nervioso central (SNC) situada en el interior de la columna vertebral. Su función principal es la de transmitir los impulsos nerviosos que comunican el cerebro con el resto del organismo, además de poder generar respuestas propias como es el caso de los reflejos espinales. La información sensorial que se recoge del organismo mediante los nervios periféricos viaja a través de las vías ascendentes de la médula espinal hasta el cerebro. Por otro lado, la información para el control motor y autonómico generada en el cerebro es conducida hasta los órganos efectores por las vías espinales descendentes. Tanto la información sensorial como la motora y autonómica es transmitida hacia o desde la médula espinal mediante los nervios espinales. Cada par de nervios espinales inerva una porción concreta del organismo obedeciendo a una estructura segmentaria de la médula espinal, tanto en arquitectura como en función, que divide el control nervioso en regiones diferenciadas del cuerpo. Los nervios espinales conectan con el segmento correspondiente de la médula espinal mediante la raíz dorsal o posterior, que contiene fibras sensoriales, y la raíz anterior o ventral, formadas por fibras motoras. Los cuerpos de las neuronas sensitivas se encuentran en los ganglios de las raíces dorsales, mientras que los cuerpos de las neuronas motoras se sitúan dentro de la médula espinal. La estructura interna de la médula espinal la podemos dividir entre la sustancia blanca, más periférica, y la sustancia gris (Fig I.1).

1.1. Sustancia blanca

La sustancia blanca está organizada en regiones o columnas: anterior o ventral, posterior o dorsal y lateral. Cada subdivisión de la sustancia blanca contiene haces de axones que se extienden a lo largo de la médula espinal, formando las vías ascendentes (fibras sensitivas) y descendentes (fibras motoras) (Dafny, 2008; Felten y Józefowicz, 2003; Tracey, 1985) (Fig I.2).

Vías ascendentes. A través de la columna dorsal se extienden los fascículos ascendentes grácil y cuneado, que transmiten la información relacionada con tacto, discriminación entre dos puntos, vibración, posición y sentido del movimiento consciente. En la columna lateral se localizan el tracto espinotalámico lateral, que lleva la información de dolor, temperatura y tacto grosero, y el tracto espinocerebeloso dorsal y ventral, que conduce la información de propiocepción inconsciente procedente de los músculos y las articulaciones. En la columna ventral hay cuatro tractos prominentes: el tracto espinotalámico anterior, que lleva información de dolor, temperatura y relacionada con tacto a núcleos del tronco del encéfalo y tálamo, el tracto espinoolivar, que lleva información de los organos de Golgi al cerebelo, el tracto espinoreticular y el espinotectal.

Vías descendentes. Éstas incluyen los tractos corticoespinal y rubroespinal, localizados en la columna lateral, en el humano. El tracto corticoespinal en ratas se encuentra localizado en la columna dorsal, siendo ésta la única diferencia existente entre la distribución de los tractos espinales entre hombres y ratas (Schwartz et al., 2005). Estos tractos llevan información asociada al movimiento voluntario. Otros tractos como el reticuloespinal, vestibuloespinal y el tracto corticoespinal anterior median balance y movimientos posturales. El tracto de Lissauer, situado entre el asta dorsal y la superficie de la médula espinal, lleva fibras descendentes del funículo dorsolateral, el cual regula las sensaciones entrantes de dolor a nivel espinal, y las fibras intersegmentales.

Figura I.1. Arquitectura de la sustancia blanca y sustancia gris de la médula espinal

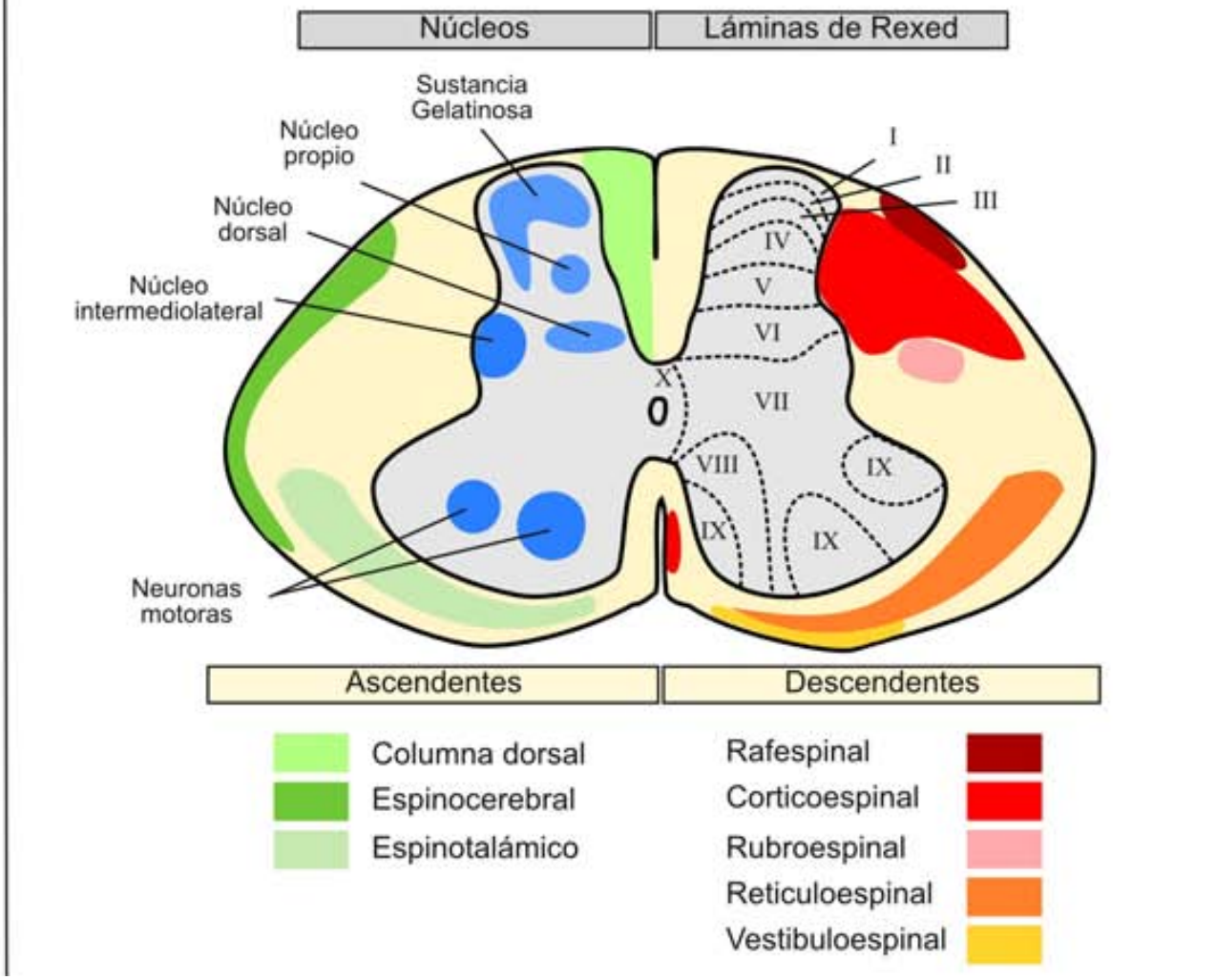

Figura I.1. Representación de una sección transversal de médula espinal humana. A la izquierda se muestran los núcleos de neuronas (azul) situados en el interior de la sustancia gris y la distribución de los tractos espinales ascendentes dentro de la sustancia blanca (tonos verdes). En la parte derecha de la sección se delimitan las zonas correspondientes a las láminas de Rexed (líneas punteadas) dentro de la sustancia gris y la organización de los tractos espinales descendentes (tonos rojos).

1.2. Sustancia gris

En la sustancia gris se sitúan los cuerpos neuronales, divididos en 10 agrupaciones somatotópicas denominadas láminas de Rexed. Dentro de la sustancia gris podemos distinguir dos zonas, asta dorsal o posterior, que engloba las 6 primeras láminas de Rexed y asta ventral o anterior, donde se encuentran las láminas de la VII a la IX. La lámina X se ubica en el centro de la médula espinal, rodeando el canal central (Brichta y Grant, 1985; Felten y Józefowicz, 2003; Dafny, 2008)(Fig I.1).

Lámina I: da origen a la vía que lleva información sobre dolor y estímulos termales al tálamo.

Lámina II: entrada aferente a la médula espinal. También reciben información del fascículo

descendente dorsolateral y envía axones a las láminas III y IV.

Lámina III: entrada aferente a la médula espinal. Está compuesta de células de tamaño variable y reciben sinapsis axodendríticas de las fibras Aß procedentes de la raíz dorsal. Además, contiene dendritas de las láminas IV, V y VI. Las neuronas de esta lámina funcionan como células propioespinales/interneuronas.

Lámina IV: recibe axones Aß, proyecta a la lámina II y responde a estímulos de tacto. Algunas células proyectan al tálamo vía tracto espinotalámico contralateral e ipsilateral.

Lámina V: está compuesta por neuronas cuyas dendritas se encuentran en la lámina II y que reciben información monosináptica nociceptiva de axones Aß y C procedente de órganos viscerales. Además, las células de esta lámina se proyectan al tronco del encéfalo y al tálamo vía tracto espinotalámico contralateral e ipsilateral.

Lámina VI: con mayor desarrollo en los engrosamientos cervical y lumbar, es amplia y se encuentra dividida en la region medial y en la lateral. Los axones aferentes la provenientes de los husos musculares terminan en la parte medial de la lámina, entre los niveles segmentales C8 a L3, y son el origen de las vías espinocerebrales ipsilaterales. En la zona lateral de la lámina proyectan las vías descendentes del tronco del encéfalo. Muchas de las neuronas pequeñas que se observan en la lámina, son interneuronas que participan en los reflejos espinales.

Lámina VII: ocupa una región amplia y heterogénea y es conocida como el núcleo intermedio lateral. Su forma y límites varían a lo largo de la médula espinal. Las neuronas de esta lámina reciben información de las láminas II a VI al igual que de las fibras viscerales aferentes. Estas neuronas sirven como relevo intermedio en la transmisión de impulsos de motoneuronas viscerales. El núcleo dorsal de Clarke forma una prominente columna oval de células desde C8 hasta L3. Sus grandes células dan origen a fibras nerviosas no cruzadas del tracto espinocerebeloso y células en las láminas V a VII, las cuales no forman un núcleo discreto, dan origen al tracto espinocerebeloso ventral. Las células en el asta lateral de la médula en los segmentos T1 a L3 dan origen a fibras preganglionares simpáticas al igual que las de los segmentos S2 a S4.

Lámina VIII: incluye un área en la base del asta ventral que varía de forma a lo largo de la médula espinal. En los engrosamientos de la médula, la lámina ocupa sólo la parte medial del asta ventral y es ahí donde terminan las fibras vestibuloespinales y reticuloespinales descendentes. Las neuronas de esta lámina modulan la actividad motora vía motoneuronas gamma, las cuales inervan las fibras intrafusales musculares.

Lámina IX: contiene varios grupos de grandes motoneuronas alfa y pequeñas motoneuronas gamma y beta, las cuales se encuentran organizadas somatotópicamente. El tamaño y la forma de esta lámina difiere a varios niveles espinales. En los engrosamientos de la médula, el número de motoneuronas alfa aumenta. Éstas motoneuronas alfa son celulas grandes multipolares que dan origen a fibras de las raíces ventrales que inervan las fibras extrafusales del músculo esquelético, mientras que las pequeñas motoneuronas gamma inervan las fibras intrafusales.

Lámina X: se encuentra alrededor del canal central y contiene neuronas que proyectan al lado opuesto de la médula.

2. Lesión medular

La médula espinal puede verse dañada por patologías no traumáticas como tumores; infecciones como la meningitis y la poliomielitis; enfermedades autoinmunes y enfermedades degenerativas como la esclerosis lateral amiotrófica y la atrofia muscular espinal. Las lesiones traumáticas que afectan a la médula espinal pueden derivar de un daño directo como la compresión o la laceración de la médula tras la rotura de las vértebras, así como por consecuencias indirectas tras una lesión de raíces espinales o nervios periféricos.

2.1. Lesión medular directa

La arquitectura de la médula espinal se puede ver seriamente comprometida después de una lesión directa, teniendo como consecuencia la pérdida completa o parcial de sus funciones nerviosas. Inmediatamente después de la lesión medular, los pacientes sufren el denominado shock medular (Bach-y-Rita y Illes, 1993), un proceso que suprime los reflejos espinales y bloquea la conducción ascendente y descendente a lo largo de la médula espinal, causando una pérdida en las funciones sensoriales, motoras y de control visceral por debajo del nivel segmentario de la lesión. Sin embargo, dependiendo del sitio, la naturaleza y la gravedad de la lesión, en una fase

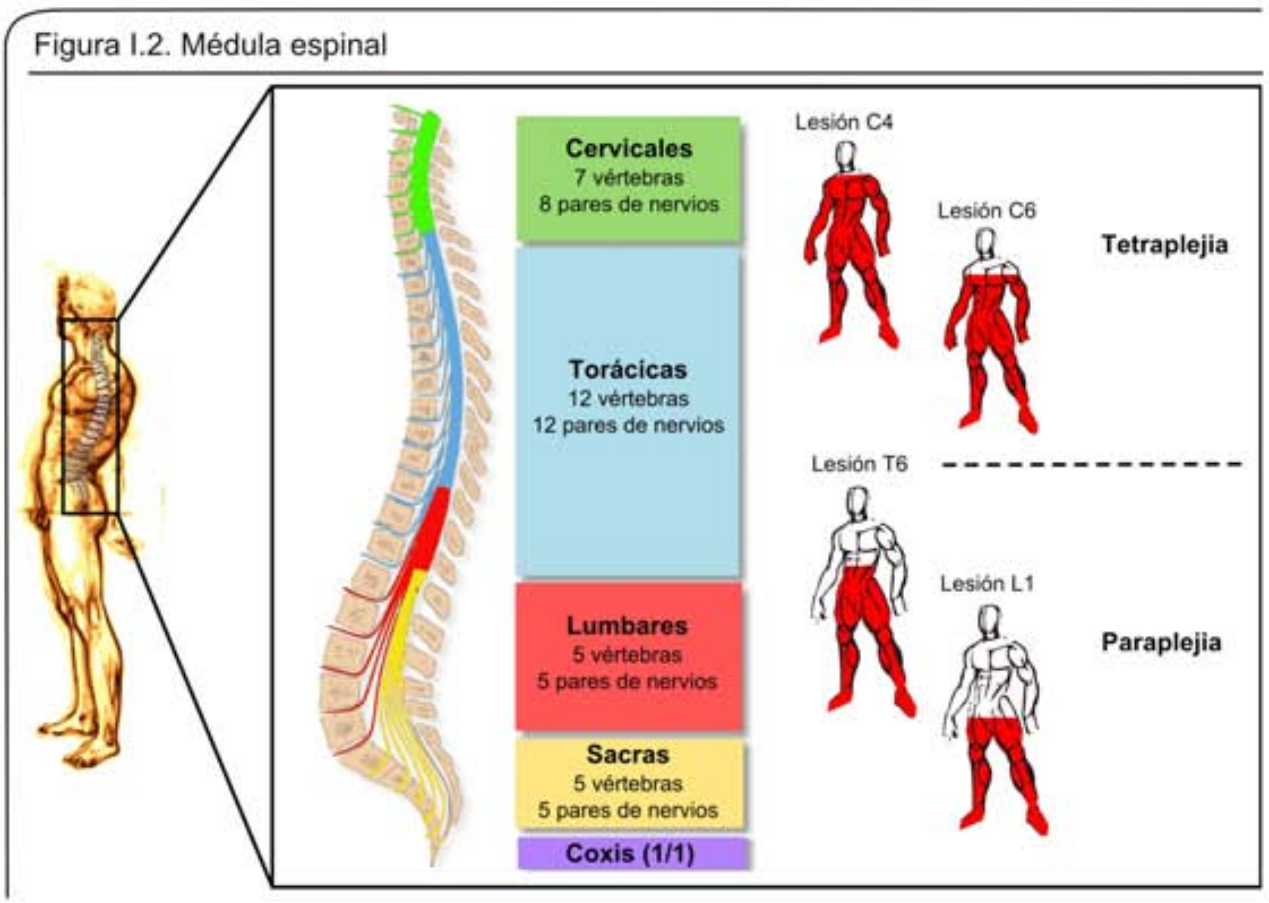

Figura I.2. Columna vertebral y médula espinal. Las lesiones que afectan los segmento medulares superiores como C4 o C6 producen parálisis en las cuatro extremidades (tetraplejia), mientras que las lesiones en los segmentos torácicos o lumbares conducen a una parálisis de las extremidades inferiores (paraplejia).

ulterior los pacientes pueden recuperar parcialmente las funciones perdidas después de un trauma. Cuanto mayor sea la severidad de la lesión menor será la recuperación espontánea, padeciendo de forma crónica parálisis o paresia, anestesia o hipoestesia y disfunciones autonómicas en las regiones corporales cuya inervación corresponde a segmentos medulares caudales al sitio de la lesión (Fawcett et al., 2007) (Fig. I.2).

La etiología de las lesiones de médula espinal es variada aunque mayoritariamente son causadas por accidentes de tráfico y en menor medida por lesiones deportivas, accidentes industriales y violencia. Se estima una incidencia anual de entre 15 y 30 casos de lesiones traumáticas de médula espinal por millón de habitantes, con una prevalencia de 2,5 millones de personas afectadas (Thuret et al., 2006; Wyndaele y Wyndaele, 2006). Se ha calculado que más de 300.000 personas viven con lesiones medulares en los estados miembros de la Unión Europea. En España la incidencia de estas dolencias es de aproximadamente 20 casos por cada millón de habitantes y año. Debido a que las lesiones de la médula espinal generalmente afectan a personas jóvenes (media entre los 28 y los 38 años) y a la falta de tratamientos eficientes, los déficits funcionales causados por la lesión medular, persistentes a lo largo de la vida del paciente (con una esperanza de vida media estimada aproximadamente de 30 años), constituyen una importante causa de discapacidad física, con un elevado coste social y económico (Thuret et al., 2006; Wyndaele y Wyndaele, 2006).

2.1.1 Fisiopatología de las lesiones traumáticas de médula espinal

La respuesta endógena después de una lesión de médula espinal se puede dividir en dos fases consecutivas. El impacto mecánico directo produce un daño físico primario causando necrosis de los segmentos afectados, disparando toda una secuencia de procesos secundarios que agravan los efectos del daño directo. Durante la primera fase, el impacto directo provoca muerte celular, de neuronas y glía en el lugar de la lesión, junto con daño axonal que interrumpe las vías espinales ascendentes y descendentes. El bloqueo de la conducción nerviosa genera parálisis y una pérdida temporal de todas las funciones de control neural por debajo de la zona de lesión, en la fase de shock espinal (Bach-y-Rita y Illes, 1993). Además, el daño vascular directo provoca disrupción de la barrera hematoespinal. Todo ello da lugar a la activación de procesos fisiopatológicos como son la hemorragia, la inflamación, la isquemia, la excitotoxicidad y el estrés oxidativo induciendo una muerte celular secundaria y expandiendo la zona de lesión (Fig. I.3). La extensión del daño tisular a través de los procesos secundarios está directamente relacionada con la magnitud del impacto mecánico inicial (Blight y Decrescito, 1986).

Daño vascular e isquemia. Inmediatamente después del impacto, la disrupción vascular causa hemorragia, edema y trombosis (Tator y Fehlings, 1991). Para contrarrestar el edema se liberan varios factores vasoactivos incluyendo tromboxano, leucotrienos, factores de agregación plaquetaria, serotonina y opioides endógenos. Esta situación conduce a una isquemia por hipoperfusión, hipoxia e hipoglucemia (Tator y Fehlings, 1991; Mautes et al., 2000; Oudega, 2012). La isquemia causa muerte celular por necrosis en el epicentro de la lesión, siendo un mecanismo importante para la expansión del daño tisular por inducción de cascadas de señalización destructivas (Profyris et al., 2004). Después de la isquemia, puede producirse un periodo de

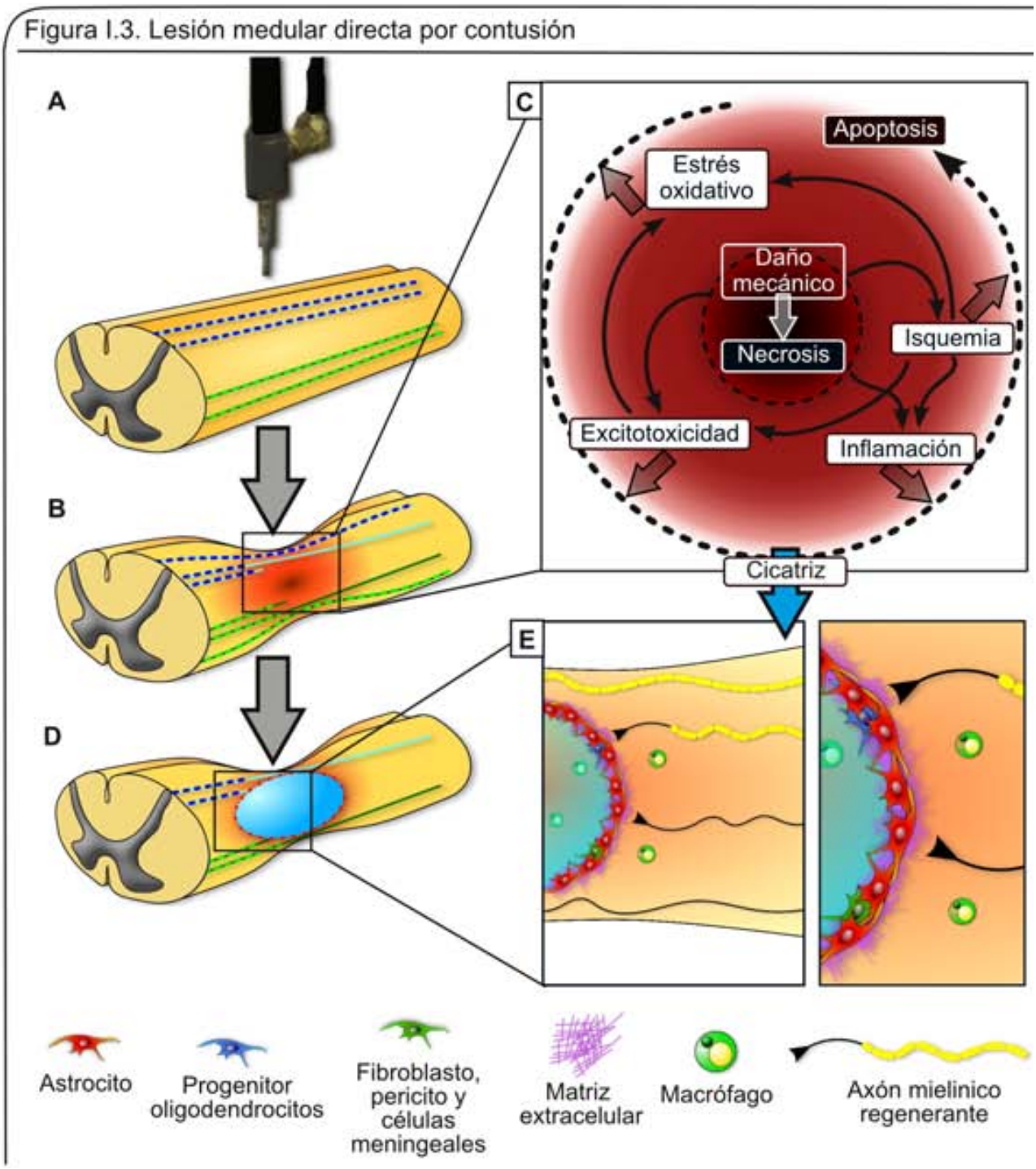

Figura I.3. Lesión de médula espinal directa por contusión. Los modelos de lesión medular por contusión consisten en percutir la superficie medular mediante la caída de peso o por la acción de un pistón (A). El impacto directo produce una zona de muerte neurótica inicial como consecuencia del daño mecánico (B). Este daño primario da paso a procesos secundarios incluyendo estrés oxidativo, isquemia, excitotoxicidad e inflamación que expanden la zona de lesión y produciendo una oleada de muerte secundaria mayoritariamente por apoptosis (C). La cronificación de la lesión da paso a la formación de una cavidad cística (E) delimitada por una cicatriz glial. La cicatriz constituirá una de las barreras más importantes para el crecimiento axonal.

reperfusión que causa un incremento de especies reactivas de oxigeno (ROS por sus siglas en inglés), contribuyendo también a la lesión secundaria (Basu et al., 2001). Por otro lado, la hipoxia e hipoglucemia local pueden disparar un incremento en la liberación de glutamato y otros aminoácidos excitadores conduciendo a una muerte celular por excitotoxicidad.

Estrés oxidativo. Las ROS como los iones de oxigeno, los radicales libres y los peróxidos son moléculas que tienen un alto poder oxidante debido a su orbital de electrones impares. El incremento de estas moléculas causa oxidación de lípidos, proteínas y ADN, resultando en una disfunción molecular, denominada estrés oxidativo (Lewen et al., 2000). Estos elementos oxidantes impiden la función de proteínas claves para la homeostasis celular como son las bombas iónicas y los transportadores de glucosa (Mattson, 2003). Además, el estrés oxidativo puede disminuir el transporte de glutamato en los astrocitos y las neuronas (Kwon et al., 2004; Fleming et al., 2006), promoviendo excitotoxicidad como resultado del incremento del glutamato extracelular. De la misma manera, la peroxidación lipídica mediada por estas moléculas resulta en la pérdida de la integridad de membrana en las células adyacentes a la lesión primaria (Lewen et al., 2000; Adibhatla y Hatcher, 2010). Todo ello comporta más disfunción celular dando lugar a una nueva situación de excitotoxicidad, muerte celular y estrés oxidativo que dispara una reacción en cadena, expandiendo el daño varios segmentos desde el epicentro de la lesión (Lewen et al., 2000).

Excitotoxicidad. La disrupción de la membrana celular, la muerte celular primaria y la isquemia dan lugar a la liberación de glutamato al espacio extracelular y alteraciones en la homeostasis iónica. Los elevados niveles de glutamato extracelular causan una despolarización persistente disparando una cascada de eventos que conduce a la muerte celular (Park et al., 2004). La despolarización se inicia debido a la activación de receptores AMPA, seguida por la activación de los canales de NA^{+} dependientes de voltaje, resultando en una masiva entrada de iones Na^{+} a la célula (Doble, 1999). El incremento intracelular de Na^{+} induce una despolarización persistente, alterando el balance iónico, seguido de una entrada pasiva de iones Cl^{-} (Rothman, 1985) y a su vez entrada de agua. El resultado final es una lisis celular osmótica y una expulsión del contenido celular al medio extracelular. Además, la prolongada despolarización y la activación de los receptores de NMDA por glutamato provocan una entrada de Ca^{++} (Doble, 1999). En situaciones normales, los niveles intracelulares de Ca^{++} se mantienen muy bajos, pero cuando la célula se despolariza en exceso, su concentración aumenta rápidamente. Los iones Ca^{++} estimulan la activación de múltiples enzimas y proteínas que pueden ser detrimentales para la homeostasis celular, como la activación de nucleasas que rompen la organización de la cromatina en el núcleo y fragmentan el ADN (Farber, 1990; Doble, 1999), la activación de proteasas citosólicas y mitocondriales, que inducen una disfunción del citoesqueleto y de los orgánulos (Duchen, 2012), las quinasas dependientes de Ca^{++}, como la PKC (proteína quinasa C) que altera los estados de fosforilación de proteínas citoplasmáticas (Favaron et al., 1990), y la fosfolipasa A2, un enzima que media la producción de moléculas perjudiciales (López-Vales et al., 2011).

Inflamación. Las lesiones en el SNC causan rápidas respuestas inflamatorias. Estas respuestas involucran la participación de componentes celulares como son neutrófilos, macrófagos, microglia y linfocitos, así como componentes humorales como interleuquinas, interferones y

prostaglandinas. La necrosis inicial y los restos celulares inducen a la microglia a secretar diferentes citoquinas pro-inflamatorias, siendo de las más importantes TNFα, IL-1β y IL-6 (Bartholdi y Schwab, 1997). Estas citoquinas promueven el reclutamiento de neutrófilos y macrófagos a las pocas horas después de la lesión, contribuyendo al proceso inflamatorio (Bartholdi y Schwab, 1997). La presencia de macrófagos en el tejido dañado se detecta a los 2 días, siendo el pico máximo entre los 5 y 7 días después de la lesión, y persiste durante meses en el área lesionada (Popovich et al., 1997; Carlson et al., 1998). La naturaleza de estos macrófagos puede ser o bien por infiltración desde el sistema inmunitario o por transformación de la microglia residente en el área dañada (Popovich et al., 1999, 2002). Independientemente de su origen, los macrófagos son los mayores sintetizadores de mediadores neurotóxicos que causan muerte celular, desmielinización y la formación de ROS durante la lesión secundaria (Carlson et al., 1998). No obstante, la presencia de macrófagos tiene también un papel beneficioso eliminando los restos de tejido dañado y secretando citoquinas que promueven la supervivencia celular (Schwartz, 2003). Se ha demostrado experimentalmente que la eliminación de monocitos y/o macrófagos activados por la lesión reduce la desmielinización secundaria y la pérdida axonal, incrementando la recuperación de las funciones neurológicas (Popovich et al., 1999; Mabon et al., 2000). Se ha barajado la hipótesis que el tiempo y la duración de la activación de macrófagos puede ser critica para su función beneficiosa o detrimental, desempeñando un papel esencial en la progresión dinámica de las afectaciones del SNC (Dougherty et al., 2000; Yin et al., 2003; Frank-Cannon et al., 2009). Por otro lado, la presencia de linfocitos T, detectada al rededor de una semana después de la lesión (Fleming et al., 2006), sugiere una respuesta autoinmunitaria debido a la rotura de la barrera hematoespinal. Aunque el papel de los linfocitos en la progresión de la lesión está aun en discusión, podrían estar contribuyendo a la expansión del daño.

Cicatriz glial e inhibición de la regeneración axonal. La eliminación del tejido necrótico y dañado por la acción fagocítica permite la formación de una cavidad cística que se extiende desde el epicentro de la lesión. Esta cavidad está encerrada por una formación fibrótica o cicatriz glial, separando el tejido lesionado del intacto y funcional. La formación y elongación de este quiste puede provocar siringomielia, un trastorno que conduce a déficits funcionales adicionales por incremento de la presión interna de la médula espinal (Brodbelt y Stoodley, 2003). Para la formación de esta cicatriz se requieren varias semanas, contribuyendo a ésta astrocitos activados por la lesión, fibroblastos y pericitos infiltrados, oligodendrocitos, microglia y la construcción de una densa matriz extracelular (Silver y Miller, 2004). La invasión de moléculas exógenas en el SNC después de la rotura de la barrera hematoespinal, así como el aumento de TGF-β (Moon y Fawcett, 2001), IL-1 (Giulian et al., 1988) e IFN-γ (Yong et al., 1991), inducen la activación de los astrocitos y la formación de la cicatriz. Estos astrocitos activados presentan hipertrofia como resultado del incremento de la expresión de proteínas asociadas al citoesqueleto, como GFAP y vimentina (Fawcett y Asher, 1999). Aunque la cicatriz glial formada presenta una acción beneficiosa, limitando el tejido lesionado e impidiendo en cierto modo la expansión del daño (Renault-Mihara et al., 2008), también ejerce una barrera fisicoquímica para el crecimiento axonal. Los astrocitos, conjuntamente con las células meningiales, la microglia y algunos precursores

neurales en la médula espinal, tienen una importante relevancia en la inhibición del crecimiento axonal ejercida por la cicatriz glial (Rolls et al., 2009). Además, el incremento de la expresión de moléculas relacionadas con el crecimiento axonal durante la embriogénesis, como efrinas y semaforinas, contribuye a esta inhibición. Las efrinas ejercen señales quimiorepelentes para el crecimiento del axón, siendo además un regulador de la adhesion y migración celular (Bolsover et al., 2008; Klein, 2012). El incremento de los receptores de efrinas después de la lesión puede adicionalmente favorecer la implicación de estas moléculas en el fallo de la regeneración axonal. Durante el desarrollo, las semaforinas están involucradas en la guía axonal, la ramificación y la formación de sinapsis (Roth et al., 2009). Después de la lesión hay también una regulación al alza de ambas formas de semaforinas, las secretadas y las ancladas a membrana, como la semaforina 3A, en fibroblastos y astrocitos. Las zonas de mayor impedimento del crecimiento axonal son altamente ricas en semaforinas indicando la importancia de éstas en la naturaleza inhibitoria de la cicatriz glial para la regeneración axonal (De Winter et al., 2002). Por otro lado, los proteoglicanos, moléculas estructurales de la matriz extracelular, también exhiben un papel detrimental para la regeneración axonal, siendo los proteoglicanos ricos en condrotin-sulfato (CSPG) unos de los más inhibitorios (Silver y Miller, 2004; Schwab et al., 2006). Además del papel limitante de la cicatriz glial, algunas moléculas asociadas con la mielina y oligodendrocitos se han descrito como agentes inhibidores para el crecimiento axonal. Por lo menos tres familias de moléculas son los inhibidores más potentes asociados con la mielina: Nogo (A, B y C), glicoproteinas asociadas a la mielina (MAG) y glicoproteinas de los oligodendrocitos (OMGp) (Zörner y Schwab, 2010).

2.1.2. Terapias para las lesiones medulares directas

A diferencia del sistema nervioso periférico (SNP), el SNC de mamíferos adultos ha limitado la capacidad de auto-reparación espontánea. Esto se debe a la escasa capacidad intrínseca de los axones centrales para regenerar (Goldberg et al., 2002) y la neurogénesis insuficiente para reemplazar las neuronas muertas (Yang et al., 2006; Vessal et al., 2007). Además, el ambiente negativo inducido por la expresión reducida de factores de crecimiento, la liberación de moléculas inhibidoras y la formación de una cicatriz glial en el sitio de la lesión también son importantes contribuyentes a la falta de regeneración (Fawcett, 1997). Todo ello conduce a una reparación incompleta de las lesiones de médula espinal, resultando en la cronificación del daño y la pérdida funcional permanente. Actualmente no existen tratamientos clínicos efectivos para retrasar, reducir o prevenir esta pérdida funcional. La comprensión de los mecanismos celulares y moleculares implicados en los procesos de degeneración que siguen al daño primario ha permitido el desarrollo conceptual de tres tipos de estrategias terapéuticas: 1) la neuroprotección del tejido preservado, reduciendo los procesos fisiopatológicos secundarios; 2) facilitar, potenciar y generar un ambiente propicio para el crecimiento y la regeneración axonal; 3) reemplazar las neuronas muertas, ya sea por potenciación de la neurogénesis endógena como por el trasplante celular. Estos objetivos se pueden abordar mediante terapias farmacológicas, celulares, rehabilitadoras o diferentes combinaciones de ellas.

Terapias neuroprotectoras farmacológicas. El daño causado por la lesión primaria es inevitable debido a que es el resultado directo del trauma inicial sobre la médula espinal. Sin embargo, los mecanismos dañinos producidos durante la lesión secundaria pueden ser prevenidos o reducidos. Así, en las dos últimas décadas la investigación se ha focalizado en el desarrollo de fármacos que, administrados tempranamente después de la lesión, sean capaces de interferir o reducir algunos de los mecanismos perjudiciales de la lesión secundaria. La administración aguda de metilprednisolona y naloxona, con acción inmunomoduladora y anti-inflamatoria, o la administración de MK801 y gaciclidina, bloqueantes de los receptores de NMDA, mejora la preservación de tejido espinal y reduce la pérdida secundaria de neuronas y glia en modelos experimentales de lesión de médula espinal (Baptiste y Fehlings, 2006). No obstante, el uso clínico de estos fármacos no ha demostrado efectos significativos, con la excepción de la metilprednisolona, hasta ahora el único fármaco recomendado para lesiones de médula espinal. A pesar de ello, la eficacia de la metilprednisolona ha sido extensamente cuestionada (Coleman et al., 2000; Hurlbert, 2000), lo que ha llevado a varios países a eliminar su uso para este tipo de lesiones. Recientemente, diferentes estudios han descrito una serie de nuevos fármacos con propiedades neuroprotectoras en modelos experimentales, aunque ninguno ha llegado, de momento, al uso en la práctica clínica. Algunos de estos fármacos son los inhibidores de la cicloxigenasa 2 (COX-2), como el NS398 o la indometacina (Schwab et al., 2004; López-Vales et al., 2006), inhibidores de la óxido nítrico sintasa como la aminoguanidina (López-Vales et al., 2006), y agentes anti-inflamatorios, como la IL-10 (Zhou et al., 2009) y la minociclina (Lee et al., 2003; Stirling et al., 2004).

Terapias regenerativas farmacológicas. Se ha postulado que el fallo en la regeneración de los axones del SNC es debido, en parte, a la insuficiente expresión de factores neurotróficos después de la lesión. Estos factores son moléculas que promueven la supervivencia, el crecimiento y la diferenciación de las neuronas, además de regular procesos como la plasticidad sináptica en el sistema nervioso. Existen una gran variedad de estudios en la literatura que demuestran como la aplicación de uno o varios de estos factores, como el FGF, NGF, EGF, BDNF, GDNF, VEGF, PDGF, NT3 y NT4/5, incrementa la supervivencia de neuronas y estimula el crecimiento axonal, tanto de las vías motoras como las sensitivas (Lu y Tuszynski, 2008; Gordon, 2009). Sin embargo, la aplicación local de factores de crecimiento dificulta el crecimiento de los axones de manera direccionada, limitando su extensión. Es por ello que las terapias enfocada a reducir el ambiente inhibidor de la regeneración han tomado gran relevancia en las investigación preclínica. De este modo, la administración local de condroitinasa ABC, que hidroliza los CSPG inhibidores presentes en la cicatriz glial, promueve la regeneración axonal y permite una mejora funcional después de su aplicación en lesiones de médula espinal (Bradbury et al., 2002; García-Alías et al., 2009; Bradbury y Carter, 2010). Por otro lado, respecto a los factores inhibidores asociados a la mielina, varios estudios han mostrado que la aplicación de IN-1, un anticuerpo que bloquea la actividad de Nogo, permite la regeneración de axones motores mejorando la función motora en modelos de lesión medular (Brosamle et al., 2000; Fouad et al., 2001). Algunos descubrimientos recientes indican que los diferentes inhibidores asociados a la mielina actúan activando el mismo receptor (NGR) y por la misma vía de señalización (RhoA) (David y Lacroix, 2003; Koprivica et al., 2004). Esto sugiere

que el bloqueo de RhoA o NGR, ambos bloqueando la acción inhibitoria de Nogo, MAG y OMGp, puede producir mayores efectos que la administración de IN-1 o NOGO-66. Algunos estudios han mostrado que la inhibición de RhoA, induce regeneración axonal y neurprotección después de una lesión de médula espinal (Dergham et al., 2002; Fournier et al., 2003).

Terapia rehabilitadora. La regeneración de las vías ascendentes y de las descendentes puede no ser suficiente para devolver las funciones sensoriomotoras, debido a los cambios plásticos inducidos en los segmentos medulares caudales a la lesión. Tras la fase de shock medular inicial, los segmentos conservados de la médula espinal también muestran cambios importantes en su actividad fisiológica. La interrupción de las conexiones sinápticas descendente causa la desinhibición de las neuronas motoras y la reorganización de los circuitos propioespinales. Estos cambios subyacen y están implicados en la fisiopatología de la hiperreflexia, la espasticidad y el dolor neuropático. Los circuitos de control reflejo pueden ser modulados y dirigidos hacia la mejora de la función mediante estrategias de rehabilitación de la marcha, de estimulación eléctrica funcional y una modulación dependiente de actividad. Además, las terapias rehabilitadoras destinadas a prevenir la atrofia muscular, la disreflexia autonómica y los daños en la piel, así como para mejorar las funciones que se han mantenido o recuperado parcialmente después de la lesión, se deben considerar de manera adicional a cualquier otro tratamiento de recuperación (Thuret et al., 2006).

2.2. Lesión medular indirecta: avulsión de raíces espinales

Entendemos por avulsión de las raíces espinales la desconexión, por estiramiento, de dichas raíces de la superficie de la médula espinal. Las causas más frecuentes son accidentes de tráfico en adultos jóvenes y complicaciones durante el parto en bebés, que conllevan un traumatismo generalmente en los hombros, provocando la separación de las raíces de su inserción en la médula espinal. Esto da lugar a una pérdida de la función en los órganos originalmente inervados por las raíces avulsionadas. La pérdida crónica de la inervación en estos órganos implica su atrofia, agravando los efectos directos de la lesión. Además, como consecuencia de la axotomía y el daño en la superficie medular, se suceden una serie de procesos patológicos que afectan directamente a la citoarquitectura de la médula espinal en los segmentos lesionados. En el caso de las lesiones por avulsión que implican a las raíces ventrales, el daño cursa con la muerte progresiva de las motoneuronas afectadas, perdiéndose entre un 50 y un 80% de éstas a las pocas semanas después de la avulsión (Koliatsos et al., 1994; Martin et al., 1999; Natsume et al., 2002; Hoang et al., 2003; Penas et al., 2009).

2.2.1. Fisiopatología de las lesiones por avulsión de raíces espinales

La lesión por avulsión de raíz causa una afectación de las diferentes partes del sistema nervioso periférico y central, como son la propia raíz y los segmentos medulares pertinentes. Después de la avulsión se da una degeneración de axones sensitivos y motores dependiendo de las raíces afectadas, así como una pérdida de sinapsis, una muerte neuronal (Koliatsos et al., 1994; Martin et al., 1999; Penas et al., 2009), una reacción glial (Reier et al., 1983; Oorschot & Jones, 1990; Carlstedt, 1997) que forma una cicatriz, y una respuesta inflamatoria que exacerba los efectos primarios de la lesión (Olsson et al., 2000). En el caso de la avulsión de raíces

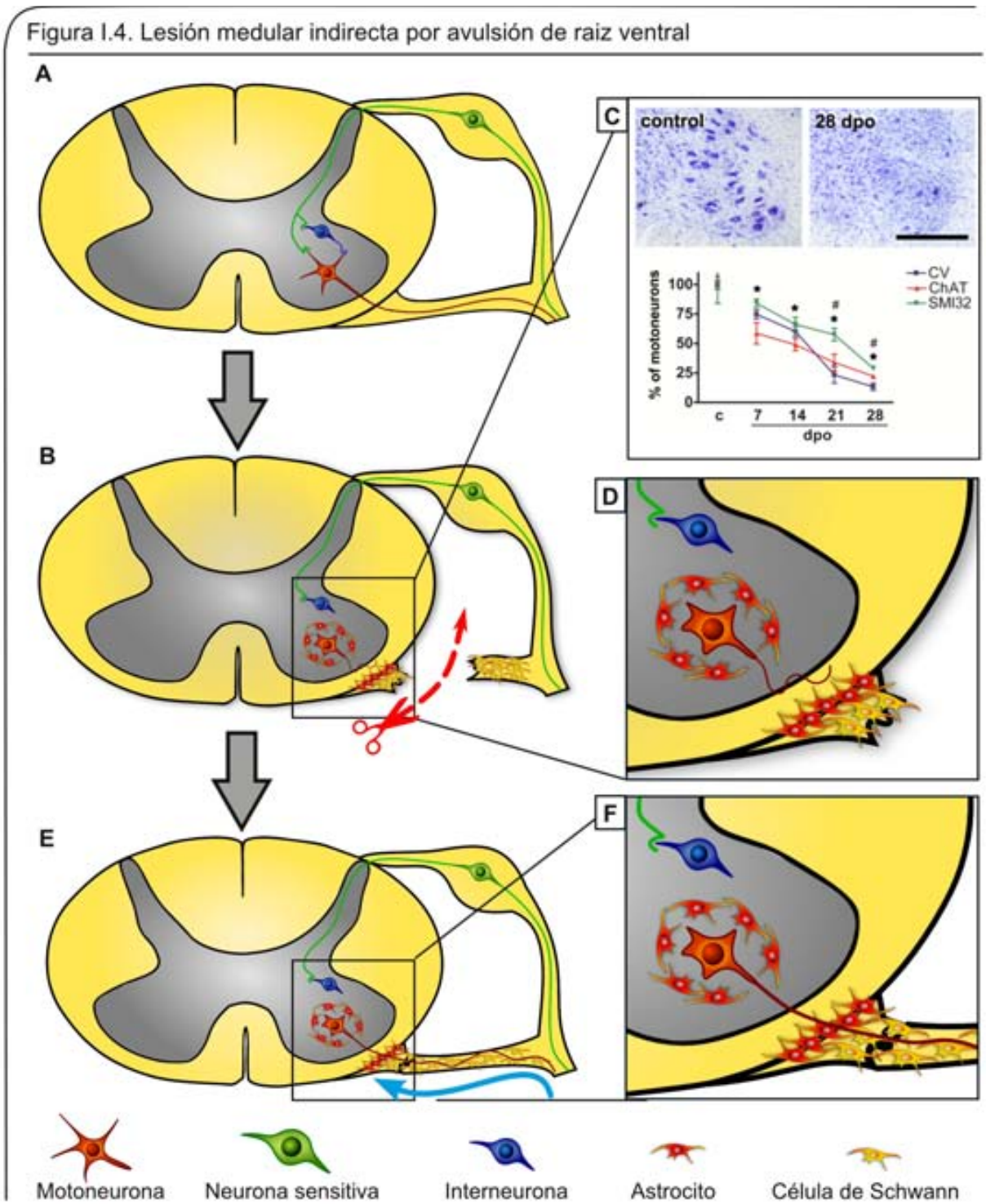

Figura I.4. Lesión medular causada por avulsión radicular ventral. En condiciones normales las neuronas sensitivas proyectan sus axones a través de las raíces dorsales y las neuronas motoras a través de las raíces ventrales (A). Después de una desconexión de la raíz ventral se suceden una serie de procesos que afectan tanto a las neuronas desconectadas como a los extremos proximales y distales de la raíz lesionada (B). Dos de los procesos más característicos son una muerte progresiva de las motoneuronas afectadas (C) y la formación de una barrera celular que impide una correcta regeneración axonal (D). La reimplantación de la raíz avulsionada permite un direccionamiento de los axones en regeneración hacia los órganos diana (E y F).

ventrales, una serie de cambios intrínsecos en las motoneuronas (MN) axotomizadas dan lugar a la atrofia y muerte de las mismas (Koliatsos et al., 1994; Martin et al., 1999; Penas et al., 2009). Ello conduce a la denervación muscular y consiguiente parálisis y atrofia de los músculos afectados (Fig. I.4).

Cambios en las MN axotomizadas. El cambio más notorio después de una avulsión de raíz ventral es una rápida muerte de las MN afectadas (Koliatsos et al., 1994; Martin et al., 1999; Natsume et al., 2002; Hoang et al., 2003; Penas et al., 2009). La desconexión de los órganos inervados, que interrumpe el aporte de factores tróficos, el trauma vascular, que induce excitotoxicidad, y la respuesta inflamatoria (Olsson et al., 2000), contribuyen a esta muerte. La muerte de las MN depende de varios factores como son la edad, la especie y el tiempo después de la lesión, siendo consecuencia tanto de procesos necróticos como apoptóticos (Li et al., 1998; Martin et al., 1999; Park et al., 2007; Penas et al., 2011). En tiempos tempranos después de lesión, la desintegración de la membrana celular de las MN, así como la presencia de macrófagos y la activación del sistema de complemento, sugieren una muerte necrótica (Ohlsson et al., 2006). Por otro lado, algunas MN lesionadas muestran signos de una muerte programada, como la fragmentación del ADN, cromatolisis y acumulación de neurofilamento fosforilado (Martin et al., 1999). Paralelamente, la respuesta temprana de las MN supervivientes a la avulsión engloba una retracción del soma y de las dendritas, reduciendo el número de conexiones sinápticas, mayormente las excitadoras (Lindå et al., 1985). Además de estos cambios morfológicos, las MN avulsionadas incrementan la expresión de genes apoptóticos como caspasas, Bax, ligandos de Fas y la óxido nítrico sintasa (He et al., 2003; Martin et al., 2005), acompañado de una reducción de la expresión de genes implicados en el metabolismo energético y la exocitosis (Hu et al., 2002). Estos eventos causan un cambio en las MN pasando de un estado comunicativo y de transmisión nerviosa a un fenotipo de supervivencia y regeneración. Esta transformación va asociada a un incremento de la expresión de factores de crecimiento, como el BDNF (Hammarberg et al., 2000) y sus receptores, una reducción de los receptores de NMDA, protegiendo a las MN de la excitoxicidad (Piehl et al., 1995), la expresión de chaperonas, como la Hsp27 en respuesta al estrés producido por la lesión (He et al., 2003), el aumento de metaloproteinasas, que podrían ejercer un papel en la remodelización de la matriz extracelular necesaria para la regeneración y una activación enzimática de proneurotrofinas con efectos beneficiosos para las MN (Hu et al., 2002), así como proteínas asociadas el crecimiento axonal como la GAP-43 (Olsson et al., 2000).

Cambios en la región de transición. El estrés mecánico producido por la tracción de la raíz conduce a una serie de cambios en la superficie medular y especialmente en la región intermedia entre el SNC y el SNP o región de transición. La avulsión induce la formación de una cicatriz constituida mayoritariamente por astrocitos reactivos (Nomura et al., 2002), aunque también por células meningeales y células fagocíticas e inflamatorias, rodeadas de una matriz extracelular rica en colágeno (Risling et al., 1993). La cicatriz formada en la región de transición es un obstáculo para el crecimiento de fibra nerviosas. Así, en el caso de lesiones que afectan a las raíces dorsales, los axones centrales de las neuronas sensoriales pueden crecer a lo largo del segmento de la raíz, pero no consiguen atravesar la zona de entrada de las raíces dorsales en el parénquima medular (Carlstedt, 1997; Navarro et al., 1999). En esta

cicatriz, a pesar de la ausencia de células de Schwann (SC), se han encontrado moléculas con acción regenerativa, como laminina, tenascina y colágeno, en una distribución tubular y rodeando axones en crecimiento (Risling et al., 1993; Frisen et al., 1995). Por otro lado, los astrocitos reactivos expresan receptores para neurotrofinas (Frisen et al., 1992; Frisen et al., 1993), sugiriendo la posibilidad de su recaptación y entrega a los axones en crecimiento, importante durante los procesos de regeneración. Además, en la cicatriz se sobreexpresa IGF-1 (Hammarberg et al., 1998), un factor de crecimiento con un potente efecto trófico en neuronas y promotor de la regeneración axonal (Allodi et al., 2012). A pesar de ello, también se han encontrado en la cicatriz algunas proteínas con carácter repulsivo como las semaforinas. No obstante, se cree que la liberación de VEGF en la matriz (Lindholm et al., 2004), un factor trófico reconocido por el mismo receptor que las semaforinas (Soker et al., 1998), podría reducir la acción repulsiva de éstas. Adicionalmente, el VEGF es un potente inductor de la angiogénesis e impulsor de la regeneración axonal, particularmente en MN (Oosthuyse et al., 2001). Pese a esta permisividad, la muerte de oligodendrocitos puede provocar la liberación de moléculas inhibidoras del crecimiento axonal asociadas a la mielina (Zörner y Schwab, 2010), reduciendo la capacidad de los axones para regenerar. Todo ello determina una interacción compleja entre las fibras nerviosas en crecimiento y el entorno generado en la matriz.

Cambios en las raíces avulsionadas. La pérdida de la conexión entre el axón y el soma neuronal inicia la denominada degeneración walleriana (Waller, 1850), una respuesta secuencial que conduce a la desaparición de los axones dañados en el segmento distal y a crear un ambiente favorable para la regeneración. La degeneración se inicia probablemente por un impedimento del transporte axonal (Mack et al., 2001), seguido por un incremento del calcio intracelular libre y la activación de calpaínas, todo ello causando la degradación de los microtúbulos y neurofilamentos, y con el tiempo, la fragmentación de los axones afectados (Beirowski, 2005; Coleman, 2005). Al mismo tiempo, la pérdida del contacto con el axón conlleva a una activación de las SC, iniciando la fagocitosis de sus propias vainas de mielina y la liberación de citoquinas atrayentes para neutrófilos y macrófagos. Así, las SC contribuirían a la respuesta inflamatoria mediada principalmente por macrófagos infiltrados desde el torrente sanguíneo (Stoll y Muller, 1999). La función principal de estos macrófagos es eliminar los restos de axones y mielina por fagocitosis. Además, se ha demostrado que la expresión de factores tróficos por parte de los macrófagos induce la proliferación de SC y fibroblastos, necesaria para los procesos de regeneración (Perry y Brown, 1992). En tiempos más tardíos, después de la recuperación de la barrera hemato-nerviosa, la mayoría de macrófagos mueren, mientras que unos pocos persistentes en el nervio, donde podrían ejercer una función moduladora de los axones regenerados (Leskovar et al., 2000). Además, la pérdida de contactos en las SC induce su proliferación y un cambio en su fenotipo. Esta transformación se caracteriza por una regulación a la baja de genes asociados a la mielina y un aumento de la expresión de genes relacionados con la regeneración, como NGF, BDNF, GDNF, NT-3 y moléculas de adhesión (Gillen et al., 1997; Gordon et al., 2003). Durante su proliferación, las SC se alinean dentro de los túbulos endoneurales en columnas denominadas bandas de Büngner, estimulando y guiando el crecimiento de los axones en regeneración. Por lo tanto, la presencia

de SC es esencial para la regeneración axonal mediante una acción tanto trófica como trópica. De hecho, una denervación crónica conduce, con el tiempo, a una reducción de la capacidad del nervio para sustentar la regeneración axonal, probablemente asociado a la reducción de la expresión de neurotrofinas por parte de las SC (Fu y Gordon, 1995; Sulaiman y Gordon, 2000; Gordon et al., 2003).

2.2.2. Tratamiento de la avulsión de raíces ventrales

La reconexión de los nervios o raíces espinales avulsionadas con los los segmentos medulares correspondientes, bien por reparación directa de las raíces arrancadas o bien mediante un injerto de nervio periférico, previene parcialmente de la muerte de MN y es indispensable para posibilitar la regeneración de los axones lesionados y la reinervación de los órganos diana. Sin embargo, a pesar de los recientes avances en la reparación quirúrgica, la recuperación funcional sigue siendo escasa. Es por ello que la investigación destinada a mejorar la supervivencia de las MN, potenciar el crecimiento axonal y que permita una correcta reinervación de los órganos correspondientes, es necesaria con el fin de proporcionar un mejor pronóstico a estas afectaciones.

Reparación quirúrgica. Las diferentes respuestas de las MN a la axotomía proximal por avulsión de raíz y a la axotomía distal por lesión de nervio sugirieren que los componentes periféricos son cruciales para la supervivencia de las MN. La reparación quirúrgica permite abordar dos objetivos, la supervivencia de las MN y la regeneración de axones hacia el nervio diana. Se ha demostrado que las MN poseen una capacidad intrínseca de regenerar axones, aun después de largos periodos de denervación (Gordon et al., 2003) o en ausencia de nervio periférico, prolongándolos dentro de la médula espinal (Havton y Kellerth, 1987). Por otro lado, las MN pueden formar dendraxones, axones extendidos desde las dendritas (Linda, 1985; Fenrich et al., 2007). Consecuentemente, cuando se facilita un substrato por donde regenerar, como los nervios transplantados y las raíces reimplantadas, las MN pueden prolongar sus axones dentro de él. Después de una denervación por lesión de nervio las SC proliferan y pasan a un fenotipo proregenerativo. Los factores liberados por las SC desde el nervio o la raíz reparada ejercen un papel tanto neurotrófico, esenciales para la supervivencia de MN y la regeneración y mielinización de axones (Li y Raisman, 1994; Frostick et al., 1998), como neurotrópico, favoreciendo no solo el crecimiento axonal, sino que además ejerciendo una fuerza de atracción para un crecimiento direccionado (Brushart, 1987), evitando así la formación de neuromas. Por lo tanto, la reparación quirúrgica permite a las MN regenerar y reinervar los músculos diana, favoreciendo la recuperación funcional (Cullheim et al., 1989; Carlstedt et al., 1993, Carlstedt et al., 1995). Así, el reimplante de raíces ventrales después de una avulsión cervical resulta en una recuperación de la función motora, acompañado de respuestas electromiográficas 20 semanas después de la cirugía (Gu et al., 2004). Del mismo modo, la reparación quirúrgica en avulsiones que afectan al cono medular o la cola de caballo permite la reinervación funcional del tracto urinario inferior (Hoang et al., 2006). En el caso de las lesiones que implican al plexo lumbar, aunque el reimplante permite el crecimiento axonal y la reinervación muscular, la recuperación funcional hasta ahora alcanzada es insignificante (Blits et al., 2004; Eggers et al., 2010). Las diferencias en el pronóstico entre las avulsiones del plexo braquial y del plexo lumbar después de la reparación se deben, básicamente, a la distancia

que los axones tienen que regenerar para alcanzar su diana (Gordon et al., 2003; Eggers et al., 2010). En el caso de las avulsiones de raíces lumbares el tiempo necesario para que las MN reinerven el músculo es elevado. Cuanto más tarde el músculo en ser reinervado, más grave será la atrofia que éste sufra, dificultando la recuperación funcional del mismo. Además, se ha descrito que la denervación crónica reduce la capacidad del nervio para mantener la regeneración axonal, debido mayormente a la reducción de la expresión de factores tróficos por parte de las SC (Fu y Gordon, 1995; Sulaiman y Gordon, 2000; Gordon et al., 2003). Por otro lado, a pesar de la mayor supervivencia inicial de MN promovida por el reimplante, esta supervivencia no se mantiene y con el tiempo la mayoría de las MN desconectadas mueren (Egger et al., 2010). Aunque la reparación quirúrgica es imprescindible, en algunos casos la retracción del muñón distal de las raíces avulsionadas hace imposible su reimplante. En estos casos el injerto autólogo de un segmento de nervio periférico sensitivo puede ser una solución. No obstante, se ha demostrado que la eficiencia de la regeneración a través del reimplante directo de la raíz avulsionada es mayor que en el caso del transplante de nervio periférico sensitivo (Chu et al., 2008). Esto podría deberse a una capacidad mayor de promover el crecimiento de axones motores por parte de los nervios motores que los sensitivos (Nichols et al., 2004; Hoke et al., 2006). Estas diferencias se podrían explicar por la existencia de dos fenotipos de SC, uno motor y otro sensitivo (Allodi et al., 2012), sugiriendo un trofismo y tropismo mayor a favor de las fibras nerviosas correspondientes.

Factores tróficos. La administración de factores de crecimiento como BDNF y GDNF en modelos experimentales de avulsión permite una mayor supervivencia de las MN (Li et al., 1995; Novikov et al.,1997). La combinación de GDNF con riluzol, un fármaco con acción neuroprotectora, demostró ser efectiva en cuanto a la protección de las MN frente a la muerte (Bergerot et al., 2004). A si mismo, en ese trabajo se describió un efecto sinérgico en la supervivencia de MN con la combinación del reimplante quirúrgico inmediato y la administración de GDNF o de riluzol, preservando el 80% de MN a las dos semanas después de la lesión. No obstante, a los tres meses el efecto protector tan solo se mantuvo en los animales que habían recibido el reimplante y la combinación de GDNF y riluzol. Los autores demostraron que estos efectos eran acompañados de una mejora funcional, favorecida por la supervivencia de MN, el incremento de la regeneración axonal promovido por el GDNF, y por un incremento del número de dendritas en las MN inducido por el riluzol. En otros experimentos, el reimplante se completó con la administración de neurotrofinas mediante vectores virales adenoasociados codificantes para GDNF o BDNF. Estos trabajos demostraron una mayor expresión de estos factores en aquellas MN que habían sobrevivido 16 semanas después del tratamiento. Debido a la potente acción proregenerativa de estos factores, su liberación prolongada y en elevadas concentraciones limitó el crecimiento de los axones a cortas distancias, favoreciendo la formación de neuromas. Se ha demostrado que altas concentraciones de neurotrofinas en el asta ventral promueve la formación de brotes colaterales, pero impide la extensión de los axones a largas distancias por la falta de una dirección concreta para el crecimiento (Blits et al., 2004).

3. Terapia celular en lesiones que afectan a la médula espinal

La terapia celular es una estrategia emergente con amplias aplicaciones médicas incluyendo la reparación de las lesiones del SNC. Las lesiones de médula espinal constituyen un reto para las estrategias de reparación celular que tratan de recuperar una tejido neural funcional. El interés de injertar células se basa en la posibilidad de abarcar varios propósitos con la misma estrategia terapéutica. Así, los objetivos principales para el trasplante celular son: modificar el ambiente de la lesión, modulando los procesos del daño secundario, rellenar una cavidad y crear un puente entre el tejido preservado, reemplazar las células muertas, ya sean neuronas u oligodendrocitos, y crear un ambiente favorable para la regeneración axonal. Otro punto de interés es la posibilidad de utilizar diferentes tipos de células como fuente de factores tróficos, ya sea por su producción intrínseca o por modificación genética de las células trasplantadas. De esta manera, el uso de células como vehículo de agentes farmacológicos evitaría algunas limitaciones como la administración repetida de estos factores. En las siguientes secciones se resumen los trabajos realizados en terapia celular para lesiones de médula espinal, a excepción de los que atañen a las células envolventes del bulbo olfatorio (OEC, capítulo 4) y células mesenquimales estromales (MSC, capítulo 5) que se detallan en los capítulos correspondientes.

3.1. Terapias celulares preclínicas para lesiones directas de médula espinal

El trasplante de células para el tratamiento de lesiones de médula espinal ha sido el objeto de numerosos estudios en las últimas dos décadas. Una amplia variedad de células se ha utilizado en base a su potencial para formar mielina, promover y dirigir el crecimiento axonal, crear un puente pro-regenerativo en el sitio de lesión o diferenciarse en células neuronales o gliales. Adicionalmente, muchos de estos tipos celulares tienen una acción paracrina, secretando factores tróficos y otras sustancias, que puede modificar el ambiento de la lesión y ejercer un efecto neuroprotector del tejido medular afectado. Seguidamente haremos un repaso a las estrategias más estudiadas en función del tipo celular o injerto (Fig. I.5).

Trasplante de nervio periférico. Aunque el trasplante de un segmento de nervio no puede considerarse propiamente como terapia celular, los primeros trabajos realizados durante los años 1980, demostraron la capacidad de los axones del SNC para crecer dentro del nervio injertado (Richardson et al., 1980). Posteriormente, la combinación de esta estrategia con distintas sustancias consiguió producir una mejora funcional en animales de experimentación (Cheng et al., 1996; Cheng, 1997; Lee et al, 2002; Lee et al., 2004). No obstante, el trasplante de nervio en pacientes con lesiones incompletas ha demostrado ser poco satisfactorio (Levi et al., 2002). A pesar de los resultados preclínicos obtenidos, la investigación clínica de este tipo de trasplantes es escasa. Por ello es necesario una mayor cantidad de estudios experimentales y analizar el posible beneficio de esta estrategia en lesiones completas, así como la combinación con otras terapias.

Trasplante de tejido nervioso fetal. El trasplante de médula espinal fetal después de lesiones completas en ratas y gatos ha resultado ser prometedor (Kunkel-Bagden y Bregman, 1990; Reier et al., 1992; Bregman et al., 1993), permitiendo la regeneración de un pequeño numero de axones de la médula huésped, conectando ambos extremos de la lesión y permitiendo mejoras funcionales significativas.

Figura I.5. Células más usadas en terapia celular para lesiones de médula espinal

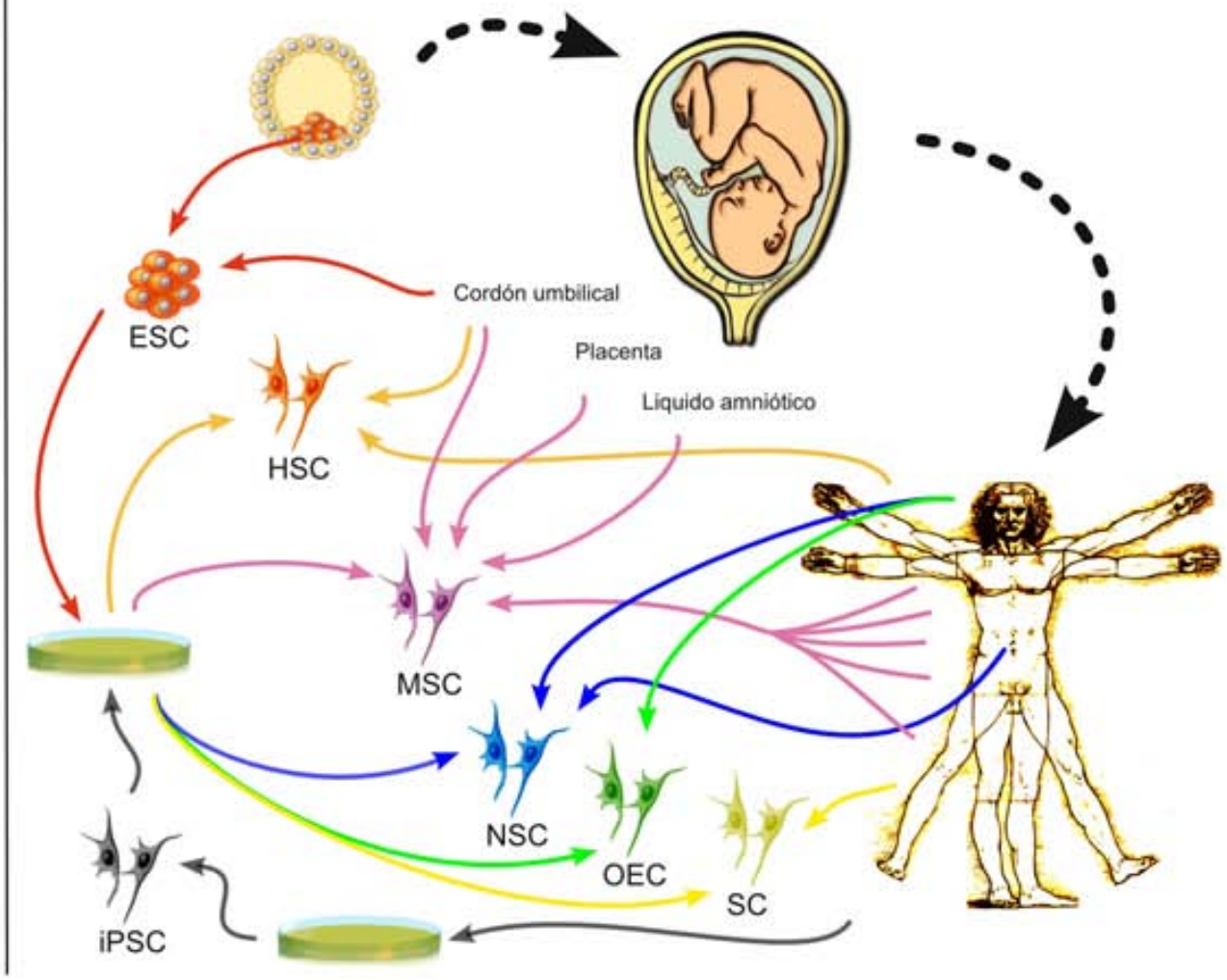

Figura I.5. Fuente de obtención de células para terapia celular en lesiones de médula espinal. En la investigación preclínica destinada a estudiar la terapia celular como tratamiento para lesiones de médula espinal se han usado diferentes tipos de células. Estos tipos los podemos dividir en dos grandes grupos, células obtenidas de estadios embrionarios y fetales o células obtenidas de individuos adultos. Algunos de estos tipos celulares como las HSC y las MSC se pueden derivar de un gran número de tejidos. Con la tecnología creciente en cultivos celular y control de los procesos de determinación fenotípica en la actualidad todos estos tipos celulares pueden obtenerse *in vitro* derivadas de ESC o de iPSC. ESC (embrionic stem cells), HSC (hematopoietic stem cells), MSC (mesenchymal stromal/stem cells), NSC (neural stem cells), OEC (olfactory ensheathing cells), SC (Schwann cells), iPSC (induced pluripotent stem cells).

Además, el tejido trasplantado puede estar secretando factores tróficos o incrementar la conducción de axones preservados en el caso de lesiones incompletas (Bergman et al., 2002). El trasplante intraespinal en pacientes con siringomelia ha demostrado ser satisfactorio, reduciendo el tamaño del quiste. Sin embargo, la dificultad para obtener tejido fetal no ha permitido la implantación de esta estrategia en la práctica clínica para este tipo de dolencias.

Trasplante de células gliales. Los dos tipos de células gliales más usados como terapia celular

en lesiones de médula espinal son las SC y las OEC. Las SC son células gliales responsables de la formación de las vainas de mielina alrededor de los axones periféricos, y establecen una íntima interconexión con los axones que permite el mantenimiento de éstos y la conducción de los impulsos. Los trabajos publicados sobre el trasplante de las SC para reparar las lesiones de médula espinal son extensos. La mayoría de estos trabajos muestran que las SC trasplantadas son capaces de secretar numerosos factores tróficos, incluyendo NGF, BDNF, GDNF y CNTF (Pellitteri et al., 2006), remielinizar axones dañados (Kohama et al., 2001), guiar los axones en regeneración (Zheng et al., 2000) o facilitar la invasión de las SC endógenas en los segmentos de la médula espinal lesionada (Hill et al., 2006). A pesar de estos cambios, el trasplante de SC parece promover el crecimiento axonal limitado a la zona del injerto, ya que los axones regenerados dejan de crecer cuando tienen que volver a entrar en el tejido huésped, más allá del trasplante, debido a la naturaleza inhibidora de la cicatriz glial circundante (Lakatos et al., 2000; García-Alías et al., 2004). Estos efectos pueden conducir a una cierta recuperación funcional (Takami et al., 2002), aunque no todos los estudios han demostrado una mejora (Pearse et al., 2007). Siendo así, se han intentado combinaciones terapéuticas para hacer el microentorno local más receptivo a la regeneración. De este modo, se han descrito beneficios, en cuanto a la capacidad locomotora, con la combinación de las SC embebidas en matrigel, una matriz polimérica, más condroitinasa (Fouad et al., 2005). Además, la combinación con neurotrofinas (Xu et al., 1995), metilprednisolona e IL-10 (Pearse et al., 2010), o un co-trasplante con OEC (Pearse et al., 2007) ha resultado también en diferentes grados de mejoría.

Trasplante de células madre. El término "célula madre" engloba a todo tipo celular con dos capacidades básicas: autorenovarse de manera indefinida y diferenciarse a otro tipo celular. Las células madre las podemos clasificar en función de si son embrionarias (ESC) o procedentes de individuos adultos. Las ESC son células pluripotentes presentes en los estadios embrionarios con capacidad de diferenciación a una gran parte de las células que componen el organismo adulto. Las células madre adultas son aquellas procedentes de los tejidos del individuo adulto. Su papel fisiológico es el de reemplazar las células que se van perdiendo en el órgano en que se encuentran situadas y repararlo en caso de daño. El uso de estás células supone una ventaja en cuanto a su aprobación ética, además de permitir un trasplante autólogo, reduciendo las posibilidades de rechazo. Las células madre adultas más usadas como terapia en lesiones de médula espinal la podemos distinguir en función de su procedencia: células madre neurales (NSC), células madre hematopoyéticas (HSC), células estromales/madre mesenquimales (MSC) y células madre de la sangre del cordón umbilical (UCB).

ESC: estas células pueden promover una recuperación funcional mediante la reconstrucción de circuitos dañados, por remielinización de axones y potenciando la regeneración axonal (McDonald et al., 1999). Además, las ESC procedentes de tejido fetal humano son capaces de sobrevivir en el tejido huésped y diferenciarse a células con características de oligodendrocitos y neuronas (Cummings et al., 2005; Iwanami et al., 2005), substituyendo así el tejido nervioso perdido. El conocimiento adquirido en los últimos años sobre la biología de estas células ha permitido hacer un uso más específico de ellas. Así, la pre-diferenciación de las ESC en el laboratorio permite el trasplante de ESC destinadas a un linaje celular

concreto. El trasplante de precursores neurales o gliales procedentes de ESC permite un cierto grado de recuperación funcional mediante su integración en el tejido medular, además de favorecer la preservación y la regeneración axonal (Herrera et al., 2001; Han et al., 2004; Hill et al., 2004). A pesar de las potenciales aplicaciones de estas células, el problema ético que suscita su obtención ha limitado el desarrollo de esta estrategia terapéutica (Thomson et al., 1998; Rossant, 2008).

NSC: las NSC son progenitores neurales situados en la capa subgranular de la circunvolución dentada del hipocampo, en la zona subventricular de los ventrículos laterales (Gage, 2000), en el epitelio olfativo y en las cercanías del conducto central de la médula espinal (Weiss et al., 1996; Johansson et al., 1999). Aunque las NSC son capaces de diferenciarse tanto a neuronas como a células gliales, los procesos de cultivo in vitro inducen su derivación principalmente a células con características gliales. Del mismo modo, después de ser trasplantadas, las NSC se diferencian principalmente en células astrogliales y en menor medida a oligodendrocitos (Cao et al., 2001; Karimi-Abdolrezaee et al., 2006; Vroemen et al., 2007). A pesar de ello, el injerto de las NSC se ha asociado con una mejora significativa de la recuperación locomotora (Cummings et al., 2005; Hofstetter et al., 2005; Karimi-Abdolrezaee et al., 2006; Ziv et al., 2006; Parr et al., 2008), aunque acompañada de un incremento de dolor neuropático en algunos casos (Hofstetter et al., 2005). A pesar de los importantes avances en la terapia con NSC, una mayor comprensión de su biología y la optimización de los procesos de aislamiento y cultivo son necesarias para el desarrollo de una aplicación terapéutica. No obstante, las NSC adultas representan una segura, no tumorigénica, fuente de soporte trófico que puede ser beneficioso.

HSC: las células madre hematopoyéticas residen principalmente en la médula ósea y se encargan de generar toda la línea celular sanguínea como son eritrocitos, leucocitos y megacariocitos. El trasplante de estas células en modelos animales ha demostrado un efecto beneficioso en cuanto a la protección de tejido y a la recuperación funcional (Koshizuka et al., 2004; Koda et al., 2005; Sigurjonsson et al., 2005; Wright et al., 2011). Aunque la acción de estas células dentro de la médula espinal lesionada está poco estudiada, la producción de factores como la angiopoyetina-1, con efectos neurotróficos, contribuiría a la preservación del tejido dañado (Takakura et al., 2000; Valable et al., 2003). Por otro lado, se ha sugerido la posibilidad de que las HSC se integren en el tejido neural. La expresión de marcadores gliales y neurales por parte de las HSC después del trasplante apoyarían esta hipótesis (Koshizuka et al., 2004). No obstante, la fusión de las HSC con células del tejido huésped ha sido demostrada (Terada et al., 2002; Alvarez-Dolado et al., 2004), dando una explicación a la presencia de estos marcadores en las células trasplantadas. Estás células se pueden obtener fácilmente mediante aspirados de médula ósea y sin la necesidad de cultivarlas previamente antes del trasplante, lo que supondría una ventaja para los tratamientos autólogos en los tiempos agudos después de la lesión. Sin embargo, su presencia en la médula ósea es de tan solo un 0,1%, dificultando la obtención de una cantidad terapéutica suficiente y limitando así su uso. El descubrimiento de HSC en la sangre del cordón umbilical, así como la estandarización de su obtención, la creación de bancos celulares y su uso habitual en la clínica, ha permitido en las últimas décadas usar el cordón umbilical como una nueva fuente de HSC para aplicaciones de terapia

celular.

UCB: la sangre del cordón umbilical se ha convertido en una importante fuente para el trasplante de células madre. La sangre restante del cordón después del nacimiento puede ser fácilmente recogida y almacenada, lo que abre la posibilidad de trasplantes autólogos durante la vida del donante. Las UCB no son una línea celular propiamente dicha, sino un conjunto de células en el que podemos encontrar una mezcla de HSC, células madre endoteliales, y en menor medida ESC, MSC y células somáticas. Debido a su heterogeneidad, las UCB son capaces de dar lugar a linajes hematopoyético, epitelial, endotelial y neuronal, tanto *in vitro* como *in vivo* (Harris, 2009). No es de extrañar pues, que más de 70 enfermedades diferentes hayan sido tratadas con trasplantes de células procedentes de la sangre del cordón umbilical (Paspala et al., 2009). En cuanto al uso de esta fuente de células para las lesiones de médula espinal, uno de los primeros trabajos demostró que leucocitos derivados de sangre de cordón umbilical humana conseguían reducir los efectos adversos de la lesión (Saporta et al., 2003). En los últimos años se ha demostrado la versatilidad de algunas células madre procedente de las UCB, comparándolas con células procedentes de otras fuentes. Así, animales trasplantados con UCB CD34+ humanas presentaron una mejora funcional mayor que los animales tratados con MSC procedentes de médula ósea humana (Zhao et al., 2004), demostrando que el trasplante intramedular de estas células podría ser una buena opción como tratamiento para las lesiones de médula espinal. Por otro lado, algunos estudios señalan la capacidad de las UCB humanas para diferenciarse en diversas células neuronales después del trasplante, promoviendo la restauración parcial del tejido medular y contribuyendo a la mejora de las funciones motoras (Kuh et al., 2005; Nishio et al., 2006; Dasari et al., 2008). Con esta idea, ratas trasplantadas con NSC derivados de las UCB humanas mostraron una recuperación de los potenciales evocados somatosensoriales, y la diferenciación fenotípica de éstas a oligodendrocitos (Dasari et al., 2008). Por último, el trasplante intraespinal de HSC procedentes del cordón umbilical también ha demostrado promover mejoras funcionales después de lesiones parciales de médula espinal (Zhao et al., 2004).

Células madre pluripotentes inducidas (iPSC). En 1962 John B. Gurdon demostró que cualquier célula adulta contenía la información necesaria para generar otro tipo celular, rompiendo con la teoría de que las células especializadas eran irreversibles. Casi 40 años después, el descubrimiento de como generar iPSC ha revitalizado el campo de la medicina regenerativa. Una tecnología descrita inicialmente por Takahashi y Yamanaka (Takahashi y Yamanaka, 2006), se basa en la reprogramación génica *in vitro* de células somáticas a células pluripotenciales con las mismas características que las ESC. De este modo, las iPSC presentan patrones idénticos en expresión génica, en metilación de la cromatina, y en formación de quimeras viables que las ESC (Amabile y Meissner, 2009), y son capaces de diferenciarse hacia todos los tipos celulares, incluyendo el linaje neural (Dimos et al., 2008; Wernig et al., 2008). Estas células han sido obtenidas de varios tejidos, tanto fetales como adultos, y de diferentes especies como ratones (Takahashi y Yamanaka, 2006), ratas (Liao et al., 2009), monos (Liu et al., 2008) y humanos (Takahashi et al., 2007; Yu et al., 2007; Lowry et al., 2008; Park et al., 2008). Uno de los primeros estudios en lesiones de médula espinal evaluó el trasplante en ratones de NSC derivadas de iPSC (Tsuji et al., 2010). Aunque los resultados fueron

satisfactorios en cuanto a la mejora funcional, los autores insistieron en la necesidad de un análisis cuidadoso de las células obtenidas antes de su aplicación clínica. Respecto a la metodología, aunque inicialmente estas células se obtenían mediante infección vírica o por medio de transgenes que limitaba su uso clínico, los últimos métodos de derivación usando técnicas no virales o proteínas recombinantes han conseguido solventar este problema (Kaji et al., 2009; Woltjen et al., 2009; Zhou et al., 2009). No obstante, la posibilidad de formar teratomas y la reprogramación incompleta o aberrante son obstáculos que aun deben superarse antes de trasladar las iPSC a la clínica. A pesar de estas limitaciones, la comunidad científica ha puesto grandes expectativas en esta emergente tecnología, como muestran alrededor de los 1000 artículos publicados tan solo en 2012 en relación a las iPSC.

3.2. Terapias celulares preclínicas para lesiones medulares por avulsión

A diferencia de las lesiones de médula espinal por trauma directo, la terapia celular es aun una estrategia poco estudiada en la búsqueda de tratamientos efectivos para las lesiones de avulsión radicular. No obstante, existen un conjunto de trabajos en los que el trasplante de algún tipo celular ha resultado beneficioso, tanto en lesiones que implican las raíces dorsales como las ventrales. Como ya hemos visto, el reimplante de la raíz avulsionada o la transferencia de nervio periférico son esenciales para la recuperación funcional. Aunque no consideraremos la reparación quirúrgica como terapia celular, los efectos beneficiosos de esta estrategia son mediados por las células del sistema nervioso periférico, principalmente las SC. Dado que en muchos casos el trasplante celular se ha acompañado de la reparación directa de la raíz dañada, debemos considerar por un lado la acción propia del trasplante y la mediada indirectamente por el nervio reimplantado y los elementos que lo componen.

Células embrionarias. El primer trabajo que se realizó con el trasplante de células en lesiones de raíz espinal fue en 1990, a manos de Kliot y colaboradores (Kliot et al., 1990). En dicho trabajo la administración de astrocitos embrionarios junto a una matriz polimérica permitió la regeneración de una mayor cantidad de axones en la médula espinal después de una lesión de la raíz dorsal. Los autores sugirieron que un trasplante embrionario podía modificar el ambiente detrimental de la lesión, permitiendo mayor crecimiento axonal. Aunque el trasplante de astrocitos no ha sido la estrategia seguida, diversos trabajos han optado por el uso de células embrionarias para este tipo de dolencias. Algunos de estos estudios se han centrado en el trasplante de motoneuronas procedentes de tejido embrionario con la finalidad de analizar la capacidad de éstas para sobrevivir en la médula lesionada, para reemplazar las MN huésped desaparecidas, para integrarse en el tejido huésped y para reinervar los músculos denervados (Nógrádi et al., 2011). La supervivencia del trasplante puede estar influenciada por la edad del tejido, siendo entre los días E11 a E13 los más óptimos en la rata, por el ambiente desfavorable de la lesión y un rechazo inmunitario (Theele et al., 1996), y por la posibilidad de alcanzar una diana (Nógrádi et al., 2011). En cuanto a la capacidad de reinervación, en trabajos previos donde el trasplante de MN embrionarias en la médula espinal eran acompañado de la transferencia de nervios motores, estas células conseguían extender sus axones hasta los músculos inervados por los nervios transferidos (Sieradzan y Vrbová,

1989; Clowry y Vrbová, 1992; Nógrádi y Vrbová, 1994). No obstante, no se observó en ninguno de los casos crecimiento axonal a través de la salida natural de axones motores en el hasta ventral. Posteriormente, la combinación del injerto de MN embrionarias con el reimplante de raíces avulsionadas resultó más eficiente en cuanto a la regeneración (Nógrádi y Vrbová, 1996; Nógrádi et al., 2011). El 20% de los axones que llegaban a reinervar el nervio reimplantado eran procedentes de las células injertadas y el 75% de los axones regenerantes lograban alcanzar el músculo diana, induciendo una mejora en la potencia de contracción muscular. Además, se demostró que estas MN injertadas eran capaces de establecer conexiones con el tejido neural preservado. Por otro lado, el trasplante de tejido sensorial embrionario también ha sido estudiado. Aunque en este caso no se utilizó un modelo de avulsión, tras eliminar los ganglios dorsales de L4 y L5 en ratas, estos fueron substituidos por ganglios de la raíz dorsal procedentes de embriones humanos (Levinsson et al., 2000). En estos estudios se pudo observar axones procedentes del injerto, tanto en la médula como en el nervio ciático, acompañado de la recuperación de la respuesta electrofisiológica, demostrando la capacidad de las neuronas sensitivas para regenerar y reestablecer conexiones con el tejido huésped.

Células madre neurales. El uso de estas células en lesiones de avulsión ha sido poco estudiado. Gao y colaboradores (Gao et al., 2005) trasplantaron NSC humanas previamente derivadas a neuronas colinérgicas en un modelo de deficiencia crónica de motoneuronas por axotomia de ciático en animales recién nacidos. Una vez trasplantadas en el animal adulto, las células se aposentaban en el tejido medular, proyectando axones a través del ciático, alcanzando los músculos diana y promoviendo cierta recuperación de la función motora. Más recientemente se ha demostrado que el tiempo óptimo para el trasplante de NSC en una lesión de avulsión de raíz ventral cervical es a las dos semanas después de la lesión, encontrando mayor diferenciación a neuronas que con el trasplante inmediato o a las 6 semanas (Su et al., 2011). Además, los autores observaron la aparición de neuronas $ChAT^{+}$, marcador característico de MN, asociado a un incremento de BDNF y GDNF tan solo con el trasplante a las dos semanas. En cuanto a las lesiones de raíz dorsal, a pesar de los escasos estudios, los resultados son controvertidos. Las NSC humanas trasplantadas después de una lesión de la raíz dorsal se integran en el tejido huésped, sobreviviendo, diferenciándose a neuronas y rodeándose de astrocitos, sugiriendo la interacción con éstos (Akesson et al., 2008). Estos resultados fueron similares con la administración de las NSC en el líquido cefaloraquídeo a través del 4º ventrículo (Ohta et al., 2004). En este caso, las NSC migraron a la zona de lesión, se integraron con el tejido y además consiguieron potenciar el crecimiento axonal. No obstante, un estudio más reciente, donde las NSC fueron inyectadas en la zona de salida de la raíz dorsal después de una axotomía y reimplante, demostró que estas células se diferenciaban mayormente a células gliales sin observarse regeneración axonal ni mejora funcional. A pesar de los escasos trabajos, los progenitores neurales pueden ser prometedores para la substitución de neuronas perdidas en estos tipos de lesiones.

4. Glia envolvente del bulbo olfatorio

Las neuronas olfativas primarias están localizadas en la mucosa nasal y proyectan sus axones hacia el bulbo olfativo situado en parte anterior del cerebro. La mucosa esta constituida por un neuroepitelio y por la lámina propia, un tejido conectivo separado del epitelio por la lámina basal. El epitelio contiene un conjunto de células incluyendo las neuronas primarias, células basales, células de sostén y las células que forman los conductos de las glándulas de Bowman. Las neuronas olfativas primarias son células bipolares que proyectan sus dendritas ciliadas hacia la mucosa y sus axones hacia el bulbo olfatorio atravesando la lámina propia. Cuando los axones entran en la lámina propia estos son agrupados por las OEC, formando largos fascículos nerviosos y guiando los axones hasta el bulbo. Estos nervios están constituidos por paquetes de axones encerrados por un tubo continuo de OEC interconectadas (Fig. I.6). Estas células, además, proyectan procesos citoplasmáticos dentro de cada fascículo, separándolos en pequeñas agrupaciones de axones amielinicos. La superficie externa del tubo formado por las OEC está cubierta por una lámina basal constituida por fibroblastos, aislando las células gliales del colágeno presente en el espacio extracelular. Una vez que los axones llegan al bulbo olfatorio atravesando el hueso etmoides entran por la capa nerviosa olfatoria. En este punto los fibroblastos presentes en el nervio interaccionan con las células meningeales y las OEC establecen contactos con los astrocitos en las partes más profunda de la capa nerviosa. Los axones de las neuronas primarias se proyectan hacia capas más profundas del bulbo para establecer sinapsis con otras neuronas. La vida media de estas neuronas primarias es aproximadamente de un mes, siendo substituidas por nuevas neuronas que derivan de las células madre situadas en la parte basal del epitelio olfativo. Durante los procesos de neurogénesis las neuronas inmaduras proyectan sus axones a través de la lámina propia, finalizando la maduración cuando estos axones establecen contactos en el bulbo. El crecimiento, la guía y la elongación de estos axones es asistida por las OEC, interaccionando con los astrocitos al llegar al bulbo y permitiendo su paso hasta las capas más profundas (Raisman G, 1985; Doucette, 1995). Esta característica y única habilidad para guiar el crecimiento de axones periféricos dentro del SNC ha constituido la base de la terapia celular con OEC para afectaciones del SNC. Las OEC, al igual que las SC, son capaces de secretar factores neurotróficos necesarios para el desarrollo neuronal y la regeneración axonal (Wewetzer et al., 2002). Tanto las OEC como las SC comparten una serie de marcadores morfológicos y moleculares, pero tienen diferentes orígenes embrionarios: OEC derivan de la placoda olfatoria y las SC de la cresta neural. Ambos tipos de células expresan p75, GFAP y S100, moléculas de adhesión celular tales como L1 y N-CAM, y componentes de matriz extracelular como la fibronectina y la laminina (Wewetzer et al., 2002). Pero como ya se ha comentado, a diferencia de las SC, las OEC son capaces de migrar dentro del SNC y convivir en un ambiente rico en astrocitos (Franssen et al., 2007). Las OEC de varias especies como la rata (Ramon-Cueto y Nieto-Sampedro, 1992), el ratón (Richter et al., 2008), el cerdo (Imaizumi et al., 2000), algunos primates (Rubio et al., 2008) y los humanos (Barnett et al., 2000), han sido aisladas y los protocolos para su cultivo han sido estandarizados. Hasta la fecha, las OEC han sido uno de los tipos celulares más utilizados para el trasplante experimental en lesiones medulares.

Figura I.6. Sistema nervioso olfativo

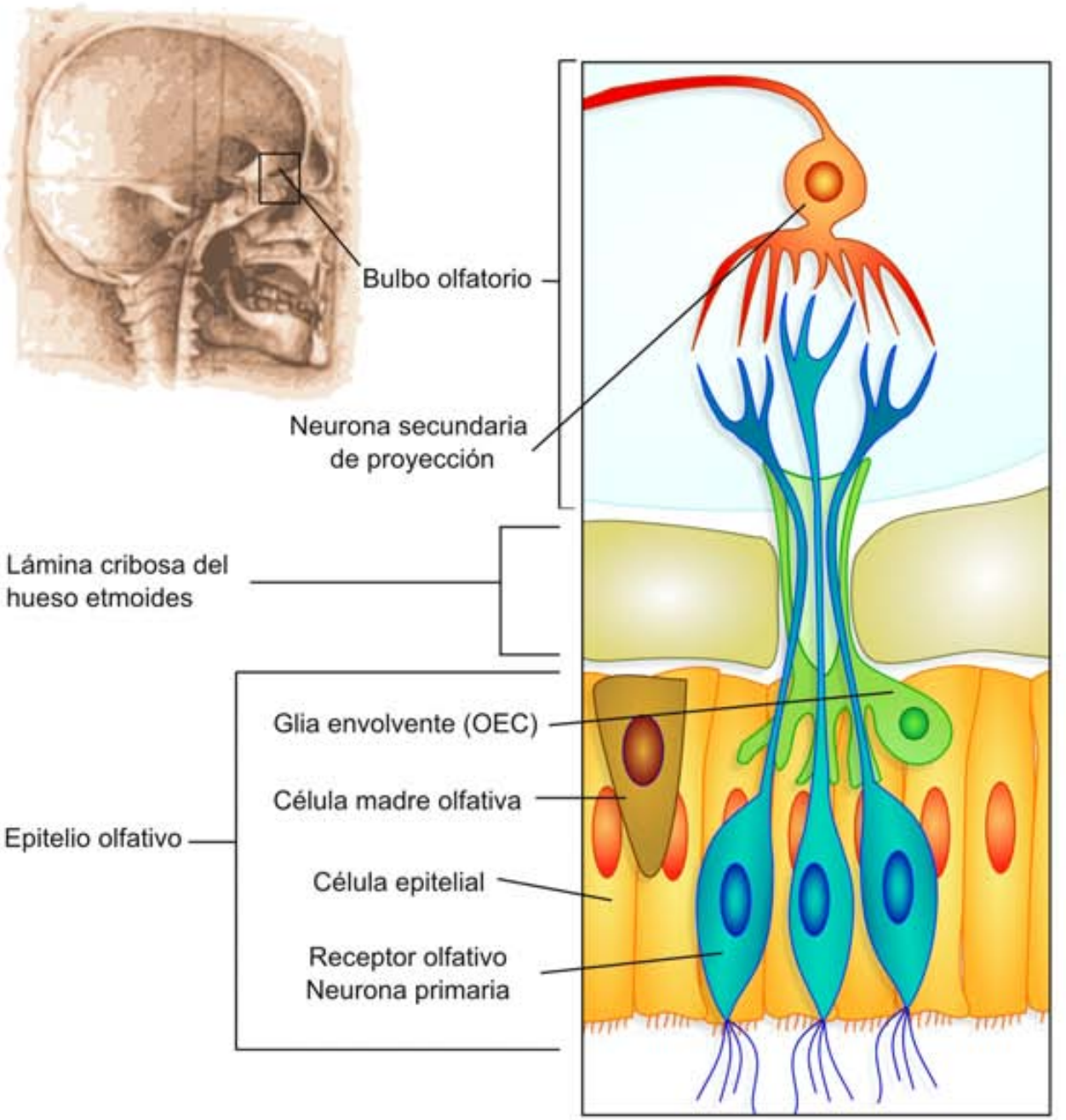

Figura I.6. Situación de las OEC en el sistema nervioso olfativo. Las células de la glia envolvente se encargan de empaquetar los axones sensitivos de las neuronas olfativas primarias y guiarlos desde el epitelio olfativo hasta entrar en el bulbo olfativo.

4.1. Tratamiento de la lesión medular directa con OEC

Una variedad de modelos de lesión medular han sido usados para estudiar los efectos del trasplante de OEC en la regeneración de los tractos espinales como estrategia terapéutica. Muchos de estos estudios muestran efectos positivos de las OEC en cuanto al crecimiento axonal y la recuperación funcional (Li et al., 1997, Ramón-Cueto et al., 2000, Lu et al., 2002; Verdú et

al., 2003; García-Alías et al., 2004; López-Vales et al., 2006). Sin embargo, hay un buen número de trabajos recientes que contradicen estos resultados, mostrando un beneficio escaso o inexistente con el trasplante de estas células, y cuestionando la capacidad de las OEC para promover la regeneración axonal en lesiones medulares (Takami et al., 2002; Boyd et al., 2004; Lee et al., 2004; Lu et al., 2006; Ramer et al., 2004b; Riddell et al., 2004; Collazos-Castro et al., 2005; Steward et al., 2006). Aunque las diferentes estrategias experimentales pueden explicar la disparidad en los resultados, experimentos similares también han mostrado efectos diferentes (Takami et al., 2002; Plant et al., 2003; Steward et al., 2006; Barnett y Riddell, 2007). Cinco son los mecanismos propuestos que contribuyen al efecto beneficioso del trasplante de OEC incluyendo la estimulación del crecimiento axonal, una interacción con la cicatriz glial, una neuroprotección, la inducción de la angiogénesis y la remielinización de los axones espinales.

Crecimiento axonal. En los primeros estudios sobre trasplante de OEC se mostraron resultados sobre los efectos de tales células promoviendo el crecimiento axonal y una mejoría en la recuperación funcional (Ramon-Cueto y Nieto-Sampedro, 1994; Li et al., 1997, 1998; Ramon-Cueto et al., 1998, 2000). Después de una sección completa de médula espinal y el trasplante de OEC, los axones descendentes y los ascendentes cruzaron la zona de lesión alcanzando distancias de entre 1,5 y 2,5 cm. Estas son las mayores distancias descritas de axones centrales regenerantes después del trasplante de OEC. En estos estudios, el nivel de recuperación de la función locomotora y de los reflejos sensorimotores parecen estar correlacionados con la distancia de regeneración (Ramon-Cueto et al., 1998, 2000). Sin embargo, un buen número de estudios posteriores han cuestionado que el trasplante de OEC promueve la regeneración axonal (Takami et al., 2002; Resnick et al., 2003). Las diferencias de resultados pueden deberse a varios factores como el tiempo de trasplante y el modelo de lesión. El tiempo después de la lesión en que se realiza el trasplante puede ser esencial para el éxito de la terapia. En un lesión de los tractos corticoespinales por calor, el trasplante de OEC 8 semanas después del daño produjo las mismas mejoras que el trasplante agudo (Li et al., 1997; Keyvan-Fouladi et al., 2003). En el caso de lesiones de sección completa de la médula espinal, el trasplante de OEC 6 semanas después de la lesión también promovió la regeneración axonal, acompañada de una ligera recuperación de actividad motora, no obstante, los resultados fueron más satisfactorios con un trasplante inmediato o a la semana de la lesión (López-Vales et al., 2006, 2007). En contra, otro estudio mostró mejores resultados con el trasplante crónico que el agudo (Plant et al., 2003). A pesar de estas diferencias, parece que el trasplante de OEC puede inducir el crecimiento axonal en modelos de sección, por lo que, a pesar de que el efecto tiende a reducirse con el intervalo de tiempos entre la lesión y el trasplante, el tiempo de trasplante no puede explicar por completo la marcada disparidad de resultados entre estudios señalada anteriormente. En cuanto al modelo de lesión, en contraste con los estudios que han empleado modelos de secciones netas, una buena cantidad de estudios no han conseguido demostrar ningun efecto sobre el crecimiento axonal ni sobre la recuperación funcional después de una lesión por contusión (Takami et al., 2002; Resnick et al., 2003; Barakat et al., 2005; Collazos-Castro et al., 2005). La contusión es, probablemente, el paradigma de lesión más complejo y de consecuencias más graves. Sin embargo, es el modelo de lesión que más se asemeja a la

mayoría de lesiones sufridas en humanos, con procesos patológicos similares (Bunge et al., 1997). No obstante, también se han publicado mejoras en la regeneración de axones supraspinales y recuperación funcional después del trasplante de OEC en un modelo de contusión (Plant et al., 2003). La limitada supervivencia de las células trasplantadas en lesiones inducidas por contusión podría explicar la falta de efectos positivos en cuanto a la regeneración axonal (Hill et al., 2006; Pearse et al., 2007).

Interacción con la cicatriz glial. Un componente esencial del mecanismo de las OEC para promover crecimiento axonal es su potencial para migrar a través de la cicatriz glial, De forma que, desde el lugar de inyección, las OEC pueden formar un puente a través de la lesión, ejerciendo la función de andamio para la regeneración axonal. Esta hipótesis implica que las OEC implantadas adquieren propiedades que no tienen en su nicho natural. Sin embargo, en los trabajos donde se hizo un seguimiento creíble de las OEC implantadas, mediante marcaje con vectores virales (Ruitenberg et al., 2003; Lakatos et al., 2003a;) o usando partículas magnéticas (Lee et al., 2004; Ramer et al., 2004a), no se observó migración de las OEC o ésta era escasa. En tiempos tardíos después del trasplante las OEC implantadas parecen rellenar el lugar de lesión, aunque se ha postulado que esto puede ser debido más a fenómenos de difusión y no de migración (Lu et al., 2006). Recientemente se ha relacionado la limitada migración de las OEC dentro de la médula espinal a la liberación de Nogo, un inhibidor del crecimiento axonal asociado a mielina, que promueve la adherencia de las OEC a la matriz. Las OEC *in vitro* expresan el receptor de Nogo y el bloqueo de este receptor mejora la migración de las OEC en ambientes ricos en mielina (Su et al., 2007). A pesar de su limitada migración en el ambiente de lesión, las OEC parecen interaccionar con los astrocitos de manera distinta a las SC. Mientras que las SC no son capaces de mezclarse con los astrocitos, formando acúmulos aislados, las OEC establecen estrechas interconexiones con ellos (Lakatos et al., 2000). Además, la interfase entre los astrocitos y las SC induce expresión de CSPG (Lakatos et al., 2003a; Verdú et al., 2003; García-Alías et al., 2004; Ramer et al., 2004a), un proteoglicano inhibidor del crecimiento axonal, pero no las interacciones con las OEC. La degradación de CSPG incrementa la interacción entre astrocitos y SC, indicando el papel de estos proteoglicanos en la relación de los astrocitos con otras células. Así, la interacción de las OEC con los astrocitos parece ser uno de los mecanismos por el cual estas células crean un ambiento permisivo para la regeneración, formando canales permisivos a la regeneración axonal (Li et al., 2005b; Raisman y Li, 2007). Además, se ha sugerido que la interacción entre astrocitos y OEC permitiría la migración de SC (Cao et al., 2007), favoreciendo su entrada desde las raíces espinales en el tejido lesionado.

Neuroproteción y angiogénesis. Se ha demostrado que la presencia de las OEC en el ambiente de la lesión puede promover la preservación de tejido, incluyendo la supervivencia de neuronas espinales, contribuyendo así a una mejoría funcional (Plant et al., 2003; Ruitenberg et al., 2003, 2005; Verdú et al., 2003). Esta acción puede ser mediada por el conjunto de factores tróficos que las OEC liberarían después del trasplante. Por otro lado, algunos estudios han demostrado un incremento de nuevos vasos sanguíneos después del trasplante de OEC en lesiones de médula espinal (Li et al., 1998; López-Vales et al., 2004; Ramer et al., 2004a,b; Richter et al., 2005). La angiogénesis es imprescindible durante los procesos generales de reparación

tisular. Además del beneficio obvio de una mayor cantidad de vasos sanguíneos en el ambiente isquémico de la lesión, se ha propuesto que estos vasos podrían formar una guía para el crecimiento de nuevos axones (Ramer et al., 2004a; Richter et al., 2005). El mecanismo por el cual las OEC promueven angiogénesis podría ser mediante la expresión de VEGF después del trasplante (Au y Roskams, 2003; López-Vales et al., 2004).

Remielinización. En su ambiente natural, las OEC no mielinizan los axones de las neuronas olfatorias primarias. No obstante, las OEC son capaces de mielinizar neuritas en cultivos de ganglios de la raíz dorsal (Devon y Doucette, 1992), aunque en un trabajo similar posterior se describió que las OEC no mielinizaban las neuritas, sino las SC contaminantes procedentes del cultivo de ganglio (Plant et al., 2002). *In vivo*, en estudios de trasplante de OEC en lesiones desmielinizantes, la presencia de estas células permite una remielinización de las zonas dañadas, con un consiguiente incremento de la velocidad de conducción (Franklin et al., 1996; Imaizumi et al., 1998; Kato et al., 2000; Lakatos et al., 2003b; Akiyama et al., 2004; Radtke et al., 2004; Sasaki et al., 2006a). A pesar de estas evidencias, no está muy clara la implicación directa o indirecta de las OEC en este proceso. Se ha sugerido que son las SC endógenas que invaden la zona lesionada, y no las OEC, las que mielinizan los axones. La expresión de factores tróficos por parte de las OEC implantadas, así como su interacción con la cicatriz glial, permitiría una mayor invasión de SC y la remielinización de los axones (Ramer et al., 2004a; Boyd et al., 2005). Además, se ha propuesto que las OEC estarían englobando paquetes de axones mielinizados por las SC, similar a los fascículos que forman en el sistema olfatorio (Boyd et al., 2005). Por otro lado, la remielinización después de una sección de nervio óptico ha sido observada con el trasplante de SC pero no de OEC (Li et al., 2003, 2007a). Sin embargo, algunos estudios han demostrado una implicación directa de las OEC en la remielinización, tanto después de una lesión de nervio ciático (Dombrowski et al., 2006) como de médula espinal (Sasaki et al., 2006b).

4.2. Tratamiento de lesión medular por avulsión con OEC

Diversos trabajos han implantado OEC en lesiones por avulsión o sección de la raíz espinal dorsal con resultados contradictorios. En 1994 Ramón-Cueto y colaboradores (Ramón-cueto et al., 1994) demostraron que la inyección en el asta dorsal de OEC después de la rizotomía y reparación quirúrgica de la raíz dorsal en rata promovía el crecimiento de axones desde la raíz implantada hasta la médula espinal. Posteriormente, en un estudio similar de rizotomía en niveles lumbares, nuestro laboratorio relacionó la regeneración axonal inducida por las OEC con la recuperación de los reflejos espinales, indicando la reconexión de estos axones con la circuitería espinal (Navarro et al., 1999). Un trabajo posterior observó resultados similares en rizotomía lumbo-sacra (Taylor et al., 2001). Más recientemente se demostró la recuperación de la función de pinza de las manos de ratas después de una avulsión dorsal a nivel de plexo braquial. El reimplante de las raíces avulsionadas acompañado del trasplante de OEC humanas en rata consiguió reestablecer la sinapsis de las aferencias sensitivas, necesarias para la función compleja de las patas anteriores (Ibrahim et al., 2009). En lesiones que afectan segmentos inferiores de la médula espinal el trasplante de OEC también ha resultado efectivo. Después de una lesión de las raíces dorsales de los segmentos L6, S1 y S2, el reimplante de las raíces dañadas acompañado de la inyección de OEC alrededor del núcleo parasimpático sacro

resultó en una regeneración de las aferencias primarias procedentes de la vejiga, acompañado de una recuperación de la función de micción perdida por la lesión (Pascual et al., 2002). La facilidad para promover el crecimiento de los axones sensitivos de las raíces implantadas puede deberse a la capacidad de las OEC para interaccionar con la cicatriz glial. Así, el trasplante de las OEC en la zona de entrada de los axones sensitivos en el asta dorsal formaría una interfase entre los astrocitos de la médula y las SC de la raíz, abriendo "canales" de paso para los axones regenerantes (Li et al., 2004). A pesar de estos resultados, otros autores no han conseguido replicar los beneficios del trasplante, llegando a la conclusión de que el trasplante de OEC después de rizotomías de raíz dorsal y reparación no impulsaba un mayor crecimiento axonal ni una mejora funcional (Riddell et al., 2004; Ramer et al., 2004). No obstante, recientemente se ha publicado que el trasplante OEC en la médula espinal, una semana después de una lesión por compresión de las raíces dorsales C7 y C8, redujo la producción de dolor neuropático y mejoró la función motora de las extremidades anteriores (Wu et al., 2011).

En el caso de las lesiones que afectan a las raíces ventrales, tan solo un estudio ha sido publicado con el trasplante de OEC como terapia. En este trabajo se administró OEC después de una avulsión de raíz ventral y reimplante a nivel de S1, mejorando la reinervación de la raíz lesionada (Li Y et al., 2007).

5. Células estromales/madre mesenquimales

Durante el desarrollo embrionario, en la capa mesodérmica residen progenitores multipotentes que generarán los tejidos mesenquimales tales como el tejido conjuntivo, óseo, cartilaginoso, muscular, adiposo y hematopoyético. En el organismo adulto una pequeña porción de células madre multipotentes procedentes del mesodermo, o células madre mesenquimales, se localizan en algunos de los órganos que forman estos tejidos. Inicialmente estas células fueron identificadas como una subpoblación de células de la médula ósea con capacidad de diferenciarse a osteoblastos. Esta potencialidad fue demostrada mediante trasplantes heterotópicos y por la presencia de células no hematopoyéticas en cultivos de extractos de médula ósea capaces de generar colonias (Friedenstein et al., 1968). Años más tarde, Caplan y colaboradores (Caplan, 1991) propusieron que estas células eran en realidad células madre capaces de diferenciarse a todas las células del linaje mesenquimal, como se demostró posteriormente (Pittenger, 1999). A pesar de sus características, las divergentes definiciones de estas células durante las décadas de 1990 y 2000 han dificultado la comparación de los trabajos publicados. Por este motivo la International Society for Cellular Therapy (ISCT) propuso considerarlas como células estromales/madres mesenquimales (MSC), dado que no todas ellas son células madre, y estableció un estándar para su definición (Horwitz et al., 2005; Dominici et al., 2006). Los criterios mínimos para considerar MSC incluían la adhesión al plástico y la diferenciación in vitro a los linajes adipogénico, condrogénico y osteogénico. Además, se incluyó una lista de marcadores de superficie, no específicos de las MSC, en que la presencia de unos y la ausencia de otros permiten caracterizar el fenotipo de estas células. En los últimos años se han identificado células con características similares en otros tejidos como el adiposo (Zuk et al., 2001), la placenta (In't Anker et al., 2004), la sangre del cordón umbilical (Erices et al., 2000), las células perivasculares del cordón umbilical (Sarugaser et al., 2005), la pulpa dental

(Gronthos et al., 2000), el líquido amniótico (Nadri y Soleimani, 2007), la membrana sinovial (De Bari et al., 2001), y el tejido mamario durante la lactancia (Patki et al., 2010). Respecto a otros tipos celulares, las MSC tienen ventajas significativas en terapia celular, tales como la falta de controversia ética respecto a su fuente y la posibilidad de trasplantes autólogos, evitando así riesgos de rechazo o de efectos secundarios asociados a la inmunosupresión. Por otra parte, las células derivadas de la médula ósea se han utilizado durante años en el tratamiento de enfermedades hematopoyéticas, por lo que los protocolos para el aislamiento, aplicación y seguridad están bien establecidos en el uso clínico (Battiwalla et al., 2009; Wright et al., 2011).

5.1. Tratamiento de lesión medular directa con MSC

Las MSC son probablemente las células más ampliamente usadas como tratamiento para las lesiones directas de médula espinal (Tetzlaff et al., 2010). A nivel experimental, el trasplante intraparénquima de MSC ha demostrado proporcionar una mejoría funcional y una mayor preservación de tejido después de lesiones de médula espinal (Chopp et al., 2000; Hofstetter et al., 2002; Neuhuber et al., 2005; Himes et al., 2006; Deng et al., 2008). El trasplante de MSC realizado a través de otras vías diferentes como son la inyección intravenosa, por punción lumbar o en el cuarto ventrículo, también ha resultado ser efectivo en la mejora de las funciones locomotoras y en la preservación de tejido, concluyendo que las células son capaces de viajar a la zona de lesión y establecerse para ejercer su función protectora (Ohta et al., 2004; Bakshi et al., 2006; Paul et al., 2009). Incluso se han realizado estudios en animales de mayor tamaño como el cerdo (Zurita et al., 2008) o el mono rhesus (Deng et al., 2006). A pesar de estos resultados y de la cantidad de trabajos publicados, los mecanismos por el cual el trasplante de MSC reduce los efectos detrimentales de la lesión medular han sido poco estudiados. Las hipótesis que se discuten abarcan desde la modulación del ambiente de la lesión mediante una acción paracrina, el potenciar el crecimiento axonal ya sea de manera directa o creando un ambiente más permisivo para la regeneración, o la transdiferenciación e integración de las MSC trasplantadas en el tejido huésped.

Neuroprotección. En la mayoría de los trabajos previos el trasplante se ha realizado en un tiempo agudo o subagudo después de la lesión, encontrando una disminución en la cantidad de tejido lesionado (Chopp et al., 2000; Hofstetter et al., 2002; Neuhuber et al., 2005; Himes et al., 2006; Amemori et al., 2010; Quertainmont et al., 2012). Esto ha sugerido que las MSC podrían ejercer un papel neuroprotector, reduciendo o inhibiendo las procesos secundarios a la lesión mediante una acción paracrina. Las propiedades inmunomoduladoras (Nauta y Fibbe, 2007) y neurotróficas (Caplan y Correa, 2011) de estas células apoyarían esta hipótesis. Las capacidades inmunomoduladora e inmunosupresora de las MSC fueron demostradas *in vitro* (Nauta and Fibbe, 2009), con la inhibición de la proliferación de linfocitos T, e *in vivo*, regulando el rechazo injerto contra huésped de los trasplantes de médula ósea en pacientes de leucemia (Le Blanc et al., 2004). En otros modelos experimentales como la isquemia cerebral, el trasplante de MSC produce una mejora funcional y una reducción del tejido lesionado como resultado de un cambio en la activación de macrófagos y microglia, mediado por la secreción de factores anti-inflamatorios y anti-inmunitarios, regulando la magnitud y el tiempo de los procesos inflamatorios (Ohtaki et al., 2008). Estas acción inmunomoduladora contribuiría a

disminuir la respuesta inflamatoria aguda después de la lesión de médula espinal, reduciendo la activación de los astrocitos y de la microglia/macrófagos (Abrams et al., 2009). No obstante, a pesar de la cantidad de trabajos que apuntan a está hipótesis, la respuesta inflamatoria aguda inducida por el trauma no solo contribuye a los procesos patológicos secundarios, sino que interviene además en los procesos de reparación tisular. Recientemente se ha publicado que la presencia de las MSC después de la lesión promueve no tanto la reducción de la activación de macrófagos, como un cambio en su fenotipo en favor de los macrófagos m2 necesarios durante los procesos de reparación tisular (Nakajima et al., 2012). Por lo tanto, es necesario un conocimiento más amplio sobre el efecto de las MSC en la respuesta inflamatoria, teniendo en cuenta tanto la supresión de algunos procesos como el incremento de otros. En cuanto a su acción trófica, las MSC secretan un conjunto de factores de crecimiento como son el BDNF, el NGF y el VEGF, entre otros (Crigler et al., 2006). Estos factores podrían ayudar a proteger el tejido neural, promoviendo la supervivencia de células afectadas por la lesión secundaria y contribuyendo a la angiogénesis (Himes et al., 2006; Quertainmont et al., 2012).

Regeneración axonal. Algunos de los factores con propiedades neurotróficas secretados por las MSC pueden potenciar la regeneración axonal y la plasticidad neuronal. El co-cultivo de neuronas con MSC o con un medio condicional procedente de éstas promueve el crecimiento de neuritas *in vitro* (Wright et al., 2007). *In vivo*, las MSC injertadas en lesiones de nervio periférico también han demostrado ser capaces de incrementar el crecimiento axonal (Marconi et al., 2012). En lesiones de médula espinal, la recuperación funcional después de un trasplante durante la fase crónica del trauma suscita un incremento del número de fibras nerviosas que cruzan la lesión (Zurita y Vaquero, 2004; Vaquero et al., 2006). Pese a estos resultados, el estímulo ejercido por las MSC para el crecimiento de axones parece ser insuficiente para sobreponerse al ambiento limitante de la cicatriz glial y otros factores inhibidores. Otra explicación que se ha propuesto es que las MSC ejercen de puente para los axones regenerantes permitiendo que crucen la zona de lesión (Hofstetter et al., 2002). En co-cultivos se ha demostrado que las MSC confieren puentes celulares permitiendo el crecimiento de neuritas en matrices inhibitorias (Wright et al., 2007). Esta acción puede ser mediada por la expresión de moléculas de adhesión y receptores de membrana incluyendo Ninjurin1 y 2, Netrina 4 (Crigler et al., 2006), y factores de guía axonal como robo1 y 4 (Phinney et al., 2006). Paralelamente, las MSC pueden modificar la matriz extracelular inhibitoria formada después de la lesión, expresando metaloproteinasas como la MMP1 y la MMP2, que la degradan (d'Ortho et al., 1997, Son et al., 2006) o proteínas de matriz como laminina, fibronectina y colágeno, más permisivas para la regeneración axonal.

Transdiferenciación. Una de las hipótesis que más controversia ha levantado es la transdiferenciación e integración de las MSC en el tejido nervioso. Algunos autores han publicado la presencia de marcadores neurales en las MSC trasplantadas, sugiriendo la diferenciación de éstas a células neurales (Mezey et al., 2000; Kadoya et al., 2009). No obstante, diversos estudios demuestran que la expresión de estos marcadores puede deberse bien a procesos de fusión entre las MSC y las células del tejido huésped, o bien a una expresión intrínseca de estos marcadores por parte de las MSC sin que ello indique un fenotipo nervioso. Pese a ello, recientemente se han

publicado algunos estudios en los que se demuestra que las MSC se diferenciaron en cultivo a células con una respuesta electrofisiológica típica neuronal (Wislet-Gendebien et al., 2005) o características de células gliales (Deng et al., 2005; Kamada et al., 2005). La prediferenciación de las MSC a células neurales abre nuevas fuentes para obtener neuronas y glia. El trasplante de SC (Kamada et al., 2005) o neuronas (Deng et al., 2005) derivadas de MSC también promueven la regeneración axonal y al recuperación funcional.

Todas estas posibilidades han inspirado una creciente (y apasionante) discusión sobre la respuesta dinámica de las MSC frente a los ambientes a los que han sido sometidas tras el trasplante. El cultivo de MSC con extractos de médulas lesionadas induce la síntesis de varias citoquinas como la IL-6, IL-7 y VEGF (Zhukareva et al., 2010), indicando que las MSC son capaces de responder al ambiente de la lesión. Así, el estudio ecológico de estas células, analizando su comportamiento interrelacionado con el ambiente que les rodea es imprescindible para conocer el significado biológico de todos los cambios complejos observados después de su trasplante.

5.2. Tratamiento de lesión medular por avulsión con MSC

A pesar del creciente interés de las células mesenquimales en todos los campos de la medicina, tan solo un trabajo previo ha sido publicado con el trasplante de MSC en una lesión por avulsión de raíz ventral (Chitarra et al., 2009). En este trabajo ratas adultas a las que se les había practicado una avulsión unilateral de las raíces ventrales L3, L4 y L5, el trasplante de MSC consiguió rescatar entre el 60 y el 70% de las MN, al menos durante las dos primeras semanas después de la lesión. Además, los autores observaron una reducción de la reactividad astrocitaria y una recuperación de la expresión de sinaptofisina, proteína presente en la vesículas sinápticas, sugiriendo una menor pérdida de sinapsis en el asta ventral. Aunque estos resultados son prometedores, en el trabajo se limitaron al trasplante sin reparación por el reimplante quirúrgico, lo que no permite saber si la mayor supervivencia de MN implica también un aumento en la regeneración axonal, en la reinervación y en la función motora. Dada la capacidad de las MSC para secretar factores tróficos como BDNF y GDNF, la supervivencia de MN podría deberse a esta acción paracrina.

6. Ensayos clínicos de terapia celular

En la actualidad exiten 1209 estudios clínicos en patologías de médula registrados en ClinicalTrials.gov, un registro y una base de datos de los resultados de estudios clínicos, tanto privados como públicos, realizados en todo el mundo. De estos estudios 465 están enfocados en lesiones traumáticas de la médula espinal, de los cuales en 22 se ensaya o se ha ensayado algún tipo de terapia celular. En 15 de estos estudios el tratamiento son MSC expandidas en cultivos, procedentes del cordón umbilical o extractos directos de la médula ósea o de la grasa y tan solo en 1 se han utilizado las OEC. Hay que tener en cuenta que no todos los estudios clínicos están registrados en esta base de datos y otros trabajos no indexados en ClinicalTrials.gov han sido publicados. En cuanto a la avulsión, no hay registro alguno de ensayos clínicos relacionados con el trasplante celular. A continuación se presenta un resumen de los ensayos que han terminado y cuyos resultados se han publicado.

6.1. Ensayos clínicos con OEC

Uno de los primeros aspectos a considerar cuando discutimos el potencial clínico de las OEC es la procedencia de estas células en humanos. Algunas vías han sido estudiadas y se han considerado relativamente accesibles para la obtención de OEC viables, incluyendo fetos abortados, biopsias de mucosa o donantes fallecidos (Huang et al., 2003; Bianco et al., 2004; Miedzybrodzki et al., 2006). Se ha considerado el trasplante autólogo como la mejor opción para reducir el posible rechazo del injerto. A pesar de los resultados contradictorios observados en los estudios preclínicos, el trasplante de OEC para lesiones de médula espinal ha sido llevado a la clínica (Huang et al., 2003; Feron et al., 2005; 2006a,b; Lima et al., 2006). Un ensayo clínico de fase I fue realizado para analizar la seguridad y facilidad del trasplante autólogo de OEC en pacientes de lesión medular (Feron et al., 2005). En otro estudio con trasplante autólogo de OEC, se publicó mejoras funcionales en la escala de valoración motora ASIA, sin embargo el estudio fue diseñado sin controles (Lima et al., 2006). Por otro lado, un estudio de observación independiente publicó procedimientos inadecuados por parte de Huang y colaboradores, concluyendo que los procesos de implante no seguían el estándar internacional para ensayos de eficacia y seguridad (Dobkin et al., 2006). En este estudio, pocos días después de la intervención se encontraron mejoras funcionales, aunque estas fueron relacionadas más con la propia intervención quirúrgica que con el efecto las OEC (Dobkin et al., 2006; Huang et al., 2006a). Además, las OEC fueron obtenidas de tejido fetal, donde la identificación de las mismas es algo dificultoso y las células trasplantadas podrían haber sido astrocitos o células inmaduras (Dobkin et al., 2006). Un prerequisito antes de empezar los ensayos clínicos debería ser que existieran resultados consistentes y convincentes en estudios con animales. Ello implica la necesidad de rigurosos ensayos preclínicos y trabajos enfocados en estudiar los mecanismos de acción y resolver la falta de consistencia en los experimentos de trasplante de OEC (Fawcett et al., 2007; Lammertseet al., 2007; Steevesetal., 2007; Tuszynski et al., 2007). Paralelamente, el efecto beneficioso del trasplante de OEC debe ser comparado con otras células con la intención de distinguir sus propiedades únicas y poder establecer las mejores condiciones para el trasplante (Lu et al., 2006).

6.2. Ensayos clínicos con MSC y derivados

La aplicación clínica más estudiada para lesiones de médula espinal implica el uso de toda la fracción mononuclear (MCP) de células procedentes de la médula ósea (Park et al., 2005; Callera, 2006; Syková et al., 2006; Chernykh et al., 2007; Yoon et al., 2007; Deda et al., 2008; Geffner et al., 2008; Kumar et al., 2009; Pal et al., 2009; Kishk et al., 2010). La MCP engloba células hematopoyéticas en diferentes estados de diferenciación, células endoteliales, HSC y MSC. Aunque en la clínica no se han realizado comparaciones entre las preparaciones de MCP y de MSC purificadas, estudios preclínicos demostraron que no había diferencia en cuanto eficacia, preservación del tejido medular y reducción de la cicatriz glial (Samdani et al., 2009). La razón de mayor peso para el uso de MCP es la facilidad de obtención mediante aspirados de médula ósea y centrifugación, en contra de la necesaria expansión de varios días de las MSC. Esto proporciona dos ventajas, la preparación de una gran cantidad de células, necesaria en el caso de los pacientes, y la posibilidad de realizar un trasplante agudo sin

esperar a la expansión *in vitro* de las células. En dos de los estudios donde usaron las MCP, el trasplante se combinó con la administración del factor de estimulación de granulocitos (GM-CSF). Se había descrito previamente que este factor, administrado después de una lesión de médula espinal en ratones, movilizaba las MCP endógenas hasta la zona de lesión y promovía una mejora funcional (Koda et al., 2007). Para la combinación en los ensayos clínicos se propuso que el GM-CSF podía, a parte de promover la movilización endógena, aumentar la supervivencia de las MCP trasplantadas (Park et al., 2005; Callera, 2006; Syková et al., 2006; Yoon et al., 2007). En el primer ensayo en que se usó esta combinación, la administración se realizó dentro de los primeros siete días después de la lesión directamente en la zona dañada (Park et al., 2005). Cinco de los seis pacientes tratados presentaron una leve mejora en las funciones neurológicas. En la actualidad el estudio se ha ampliado con más pacientes tratados en fase aguda y se han incluido nuevos grupos de tratamiento subagudo (de 14 días a 8 semanas después de la lesión) y crónico (más de 8 semanas de lesión) (Yoon et al., 2007). Además, se incluyó un grupo control en el que los pacientes eran tratados de manera convencional con descompresión y cirugía para la inmovilización de la columna en la zona afectada. En este estudio, 10 meses después de la administración aproximadamente el 30% de los pacientes con tratamiento agudo y subagudo presentaron una mejora neurológica frente al 0% con el trasplante crónico y al 8% en el grupo control. No obstante, en algunos pacientes no quedó claro si la mejora fue inducida por el trasplante de las MCP o por la evolución propia de la lesión. En otro estudio se usó la misma combinación pero administrando las MCP mediante punción lumbar (Callera, 2006). Diez pacientes fueron tratados 4 horas después del aspirado de médula ósea mediante inyección en el líquido cefalorraquídeo de 100 millones de MCP. Después de 12 semanas de seguimiento no se observaron efectos adversos (Callera, 2006). En otro estudio, 20 pacientes entre 10 y 467 días de la lesión medular fueron trasplantados mediante administración intravenosa o intraarterial 5 horas después del aspirado, demostrando mejoras tan solo en un paciente (Syková et al., 2006). Mejoría funcional y de la calidad de vida han sido también reseñadas en otro estudio, donde 4 pacientes en fase aguda y 4 en fase crónica de la lesión fueron tratados con una administración múltiple de 800 millones de MCP, 200 millones inyectados directamente en la zona de lesión, 300 millones administrados por punción lumbar y 300 millones más vía intravenosa (Geffner et al., 2008). Por otro lado, 9 pacientes crónicos mostraron mejoras funcionales después de la administración intramedular de MCP previamente congeladas y descongeladas, sugiriendo que este proceso no afecta al efecto beneficioso de estas células (Dada et al., 2008). No obstante, este estudio se realizó, como muchos otros, sin la inclusión de un grupo control, dificultando la validación de los resultados. En el caso del trasplante de MSC, se publico un caso de trasplante mediante punción lumbar a un paciente 13 días después de la lesión de médula espinal donde 6 meses después de la intervención no se observaron efectos adversos y las funciones neurológicas mejoraron paulatinamente (Saito et al., 2008). Sin embargo, en pacientes agudos no hay que descartar las mejoras producidas por los procesos intrínsecos de reparación. En un estudio reciente de administración de MSC por punción lumbar, solo los paciente de trasplante agudo mostraron una mejora en su calidad de vida, mientras que los pacientes crónicos no mostraron cambios aparentes (Pal et al., 2009).

7. Conclusión

Las lesiones de médula espinal implican un proceso biológico complejo y multifactorial, por lo que seguramente no existirá un tratamiento único y eficaz para la restauración completa de las funciones perdidas. La terapia basada en el trasplante celular puede proporcionar beneficios al actuar sobre varios objetivos, como son la modulación de la reacción inflamatoria, la neuroprotección durante la fase aguda, la remielinización de los tractos nerviosos preservados, la creación de un ambiente favorable para la regeneración axonal y la substitución del tejido neural destruido. Sin embargo, las mejoras limitadas en los resultados funcionales obtenidos apuntan a la necesidad de la combinación con otros tratamientos, como farmacológicos y de rehabilitación, con un diseño cuidadoso de la aplicación de cada uno de ellos (Fawcett, 2006; Schwab et al., 2006; Bunge, 2008; Kubasak et al., 2008; Rowland et al., 2008; Karimi-Abdolrezaee et al., 2010; Tetzlaff et al., 2010). Algunos nuevos enfoques están incluyendo terapias basadas en trasplante de células madre complementada con moléculas que promueven la plasticidad y la regeneración axonal (Kadoya et al., 2009; Karimi-Abdolrezaee et al., 2010). Teniendo en cuenta la evolución dinámica de los procesos patológicos intrínsecos a un daño de médula espinal, no es difícil pensar en la co-aplicación de diferentes estrategias, tanto celulares como farmacológicas y rehabilitadoras, aplicadas de manera complementaria al mismo tiempo o en fases distintas de la lesión. Así, la investigación preclínica intenta desentrañar nuevas estrategias terapéuticas combinadas que pueden facilitar la neuroprotección, la regeneración y la recuperación funcional sin inducir efectos secundarios adversos. A pesar de las esperanzas puestas en estas estrategias, la diversidad de trabajos y resultados obtenidos hacen difícil focalizar el estudio, la optimización y la traslación de los resultados a su aplicación en pacientes. Es por ello que se han propuesto algunas directrices necesarias para agrupar el desarrollo de las terapias preclínicas y maximizar así las posibilidades de éxito antes de embarcarse en ensayos clínicos. En los últimos años algunos de las terapias más prometedores han sido llevadas a pruebas clínicas prospectivas, informando de resultados prometedores. Pese a ello, hay que destacar la falta de grupos controles, importante para determinar las mejorías intrínsecas a la intervención, como podrían ser las causadas por la descompresión asociada a la inyección intramedular de suspensiones celulares. Además, debido a la dificultad de valorar las mejoras en la función neurológica, se requerirá una mayor cantidad de pacientes de los incluidos hasta ahora en la mayor parte de estudios. La falta de información en la mayoría de estudios sobre los tratamientos de rehabilitación que siguen los pacientes en paralelo, que podría ser determinante en las mejoras observadas, también dificulta la interpretación de los resultados. Con la intención de solventar estos problemas, la International Campaign for Cures of Spinal Cord Injury Paralysis (ICCP) ha publicado una guía para el diseño de futuros ensayos clínicos (Fawcett et al., 2007) (www.campaignforcure.org).

OBJETIVOS

Objetivo general:

Estudiar el efecto del trasplante celular como tratamiento para lesiones que afectan a la médula espinal, ya sean por contusión traumática directa como por mecanismos indirectos como la avulsión radicular.

Objetivos específicos:

- Obtener y optimizar cultivos de células mesenquimales estromales (MSC) procedentes de la médula ósea y de células gliales envolventes procedentes del bulbo olfatorio (OEC) de rata.
- Optimizar el trasplante celular dentro del parénquima medular, con particular interés en el marcaje de las células a trasplantar, la localización de las células trasplantadas y los efectos nocivos del proceso de trasplante.
- Comparar los beneficios del trasplante celular para lesiones de médula espinal en función de diferentes parámetros como la edad del donante o la criopreservación de las células cultivadas.
- Comparar el trasplante de MSC y el de OEC como tratamiento agudo para lesiones de médula espinal por contusión.
- Estudiar los mecanismos que subyacen a los posibles efectos del trasplante de MSC o OEC mediante el análisis comparativo del transcriptoma.
- Optimizar el trasplante celular para lesiones medulares por contusión en función de los datos resultantes de los objetivos anteriores.
- Estudiar el trasplante celular en un modelo de lesión medular indirecta por avulsión de raíz y su combinación con la reparación quirúrgica.

DESARROLLO EXPERIMENTAL

En este apartado se describe de manera resumida el desarrollo experimental de las distintas fases de esta tesis. Se ha de notar que el material y los métodos se detallan en cada capítulo de resultados y que esta sección es tan solo una guía que engloba el seguimiento de los experimentos que atañen a los objetivos planteados.

Objetivo 1: Obtener y optimizar cultivos de células mesenquimales estromales (MSC) procedentes de la médula ósea y células gliales envolventes procedentes del bulbo olfatorio (OEC) de rata.

Dado que los resultados de un trasplante celular dependen en gran parte de las características de las células usadas y éstas a su vez de los procesos de cultivo, consideramos importante inicialmente estandarizar estos cultivos. Con la intención de comparar el trasplante de MSC con el de OEC, ambos cultivos fueron puestos a punto procedentes de los mismos animales, a la edad post-natal p22. Todos los estudios de trasplante de MSC para lesiones de médula espinal han sido, hasta la fecha, realizados con MSC procedentes de individuos adultos, por lo que parecía pertinente una caracterización de los cultivos de MSC procedentes de nuestros animales jóvenes. Para ello se analizó la curva de crecimiento de estos cultivos, así como su inmunofenotipo y su capacidad de diferenciación, de igual manera que se ha descrito para las MSC adultas. En el caso de los cultivos de OEC, uno de los puntos claves es la purificación de estas células. Aunque nuestro laboratorio ya había usado extensamente las OEC y el proceso de cultivo estaba estandarizado, se planteó optimizar la purificación de estos cultivos mediante el uso del anticuerpo anti-p75 unido a partículas metálicas microscópicas. Los resultados se detallan en el capítulo 1.

Objetivo 2: Optimizar el trasplante celular dentro del parénquima medular: marcaje de las células a trasplantar, localización de las células trasplantadas y efectos nocivos del proceso de trasplante.

Otros parámetros que pueden influir en el resultado final de las terapias celulares son los que conciernen a la preparación de las células para el trasplante y el propio proceso de trasplante. Con la intención de observar las células trasplantadas, localizarlas y poder realizar su seguimiento, tanto las MSC como las OEC cultivadas fueron marcadas con la proteína verde fluorescente (GFP), evaluando su expresión. Además, las células fueron marcadas con PKH26, una tinción fluorescente, previamente a su inyección en la médula espinal. Las células se trasplantaron en animales con una lesión medular por contusión y se estudió su presencia en la médula espinal. Por otro lado, con la intención de valorar el efecto del proceso de trasplante en las células, los preparados celulares para el trasplante se sometieron al método de inyección y se cuantificó su supervivencia. Por último, animales no lesionados fueron inyectados con el vehículo de los preparados celulares para comprobar los posibles daños intrínsecos al trasplante intramedular. Los resultados obtenidos se describen en el capítulo 1.

Objetivo 3: Comparar los beneficios del trasplante celular para lesiones de médula espinal en función de diferentes parámetros como la edad del donante o la criopreservación del cultivo.

Antes de entrar a analizar de manera extensiva los trasplantes de MSC y OEC en lesiones de médula espinal, varios parámetros correspondientes a la preparación de las células requerían ser evaluados. Por un lado, se compararon trasplantes de MSC procedentes de animales jóvenes y de animales adultos con el

objetivo de averiguar si la edad del donante ejercía influencia en los efectos del trasplante. Por otra lado, dado que se baraja la posibilidad de la creación de bancos celulares de estas células para su disponibilidad en la práctica clínica, nos interesamos por la posibles diferencias entre trasplantar células procedentes de un cultivo fresco y células que habían sido previamente criopreservadas, simulando el mantenimiento en los bancos celulares. De este modo, los diferentes preparados celulares (MSC frescas o descongeladas y OEC frescas o descongeladas) fueron trasplantados en animales con una lesión medular por contusión. En todos los casos, a los animales se les sometió a un seguimiento de las capacidades locomotoras y se analizó la cantidad de daño tisular en la médula espinal. Los resultados obtenidos de estos experimentos se detallan en el capítulo 1.

Objetivo 4: Comparar el trasplante de MSC y el de OEC como tratamiento agudo para lesiones de médula espinal por contusión.

Existe un extenso número de trabajos en los que el trasplante de MSC o OEC ha resultado beneficioso para el tratamiento de lesiones que afectan a la medula espinal. Sin embargo, la discusión de cuál de los tipos celulares es el más adecuado para este tipo de lesiones no está exento de controversia. Dada la falta de trabajos dedicados a comparar estos dos tipos celulares, nos planteamos estudiar el efecto del trasplante de MSC y OEC obtenidas en las mismas condiciones y analizadas en paralelo. Para ello se eligieron la mejores condiciones resultantes de los tres primeros objetivos. Se realizaron lesiones de la médula espinal por contusión en ratas adultas y se aplicaron tres inyecciones intramedulares de uno de los siguientes tratamientos:

•Inyección aguda, inmediatamente después de la lesión, del vehículo en el cual se prepararon las células para el trasplante.

•Trasplante agudo de MSC procedentes de animales p22 y de un cultivo fresco.

•Trasplante agudo de OEC procedentes de animales p22 y de un cultivo fresco.

•Inyección retardada, 7 días después de la lesión, del vehículo.

•Trasplante retardado de MSC procedentes de animales p22 y de un cultivo fresco.

•Trasplante retardado de OEC procedentes de animales p22 y de un cultivo fresco.

Los animales fueron evaluados durante 6 semanas después de la lesión, analizando la recuperación de las habilidades locomotoras, la sensibilidad frente a estímulos mecánicos y térmicos, las respuestas electrofisiológicas y la extensión del daño tisular de la médula espinal. Adicionalmente se realizaron los mismos grupos de trasplante pero con la inyección de células previamente marcadas con GFP. Estos animales se sacrificaron a diferentes tiempos después de la lesión para el análisis de la localización y la supervivencia de las células trasplantadas. Los resultados obtenidos se desarrollan extensamente en el capítulo 2.

Objetivo 5: Estudiar los mecanismos que subyacen a los posibles efectos del trasplante de MSC o OEC mediante el análisis comparativo del transcriptoma.

Siguiendo con el estudio comparativo del trasplante de MSC y OEC para el tratamiento de lesiones de médula espinal por contusión, quisimos averiguar los efectos tempranos del trasplante en cuanto a la expresión génica. Para ello se realizaron los mismos grupos que en el diseño anterior, es decir, el trasplante de MSC o OEC o la inyección de vehículo en un tiempo agudo o bien retardado 7 días después de una lesión medular por contusión. Cada grupo experimental se dividió en dos subgrupos, uno donde los animales se sacrificaron a los 2 días

después del trasplante y otro a los 7 días. Además, se añadió un grupo de animales intactos que establecimos como condición basal. De cada uno de los animales se extrajo el segmento medular correspondiente a la lesión y se procesaron las muestras para la obtención del RNA. Una vez acumuladas todas las muestras, se realizó un estudio de microarray y un extenso análisis de los cambios de expresión génica encontrados. Los resultados de dicho análisis se encuentran resumidos en el capítulo 3.

Objetivo 6: Optimizar el trasplante celular para lesiones medulares por contusión en función de los datos resultantes de los objetivos anteriores.

De los resultados de la comparación celular pudimos concluir que el trasplante de MSC en agudo y bajo nuestras condiciones parecía ser el que mayor beneficio otorgaba como tratamiento (ver capítulo 2). Además, uno de los resultados más destacables del análisis génico fue una temprana activación de vías metabólicas relacionadas con un rechazo inmunitario hacia las células trasplantadas (ver capítulo 3). Por ello el siguiente experimento consistió en combinar el trasplante agudo de MSC con un tratamiento inmunosupresor, con la intención de averiguar si dicha inmunosupresión permitía una mayor supervivencia del injerto y una mejora de los resultados obtenidos con el trasplante solo. Para ello se realizaron 4 grupos experimentales de ratas adultas con lesión medular por contusión y se les administró uno de los siguientes tratamientos inyectados en tres puntos intramedulares:

- Inyección aguda de vehículo.
- Trasplante agudo de MSC procedentes de animales jóvenes y de un cultivo fresco.
- Inyección aguda de vehículo más la administración diaria de FK506 como agente inmunosupresor.
- Trasplante agudo de MSC procedentes de animales jóvenes y de un cultivo fresco más la administración de FK506 como agente inmunosupresor.

De igual manera que en los experimentos anteriores, a todos los animales se les realizó un seguimiento de 6 semanas evaluando la recuperación de la función locomotora, la respuesta frente a estímulos mecánicos y térmicos, las respuestas electrofisiológicas y se analizó el grado de daño tisular de la médula espinal. Además, se realizaron dos grupos de animales con lesión medular, con trasplante de MSC marcadas con GFP con o sin el tratamiento inmunosupresor. Estos animales fueron sacrificados a varios tiempos post lesión para analizar la localización y supervivencia del injerto. Los resultados derivados de estos experimentos se exponen en el capítulo 4.

Objetivo 7: Estudiar el trasplante celular en un modelo de lesión medular indirecta por avulsión de raíz y su combinación con la reparación quirúrgica.

En este objetivo empleamos un modelo de lesión que afecta a la medula espinal de manera indirecta, como es la avulsión de raíces espinales, para comprobar los posibles efectos directos de la terapia celular sobre la neuroprotección y la promoción de la regeneración axonal. El tratamiento de estas lesiones mediante terapia celular ha sido poco estudiado y tan solo un trabajo previo utilizó trasplante de MSC en un modelo experimental de avulsión radicular. En los experimentos iniciales los animales que habían recibido una avulsión unilateral de las raíces de segmentos medulares L4, L5 y L6 fueron trasplantados con MSC o bien inyectados con vehículo en los segmentos medulares afectados. Se realizó una cuantificación del número de motoneuronas que sobrevivían a la lesión con o sin tratamiento, así como un análisis de la reactividad glial en las zonas medulares dañadas. Una vez demostrado el efecto positivo de las MSC, combinamos el tratamiento celular con el

reimplante quirúrgico de las raíces ventrales avulsionadas. Así, tras la avulsión de raíces L4-L6, se procedió a la reinserción de las raíces en su segmento de salida en la superficie de la médula espinal, combinándolo con un trasplante de MSC o con la inyección de vehículo intramedular. Cada grupo se dividió en dos, sacrificando un subgrupo al mes y el otro subgrupo a los dos meses después de la lesión. Durante el seguimiento se evaluó la función motriz de la extremidad afectada y las respuestas electrofisiológicas, y se realizó un análisis espacio-temporal de la regeneración axonal a través de las raíces implantadas. Los resultados se muestran en el capítulo 5.

RESULTS: CHAPTER 1

Optimization of cell-based therapy for spinal cord injury

Introduction

Cell therapy is one of the most promising strategies in regenerative medicine, becoming a relevant focus in the research for numerous diseases, including spinal cord injury (SCI). Several cell types have been evaluated for SCI treatment; among them adult Mesenchymal Stromal Cells (MSC) derived from the bone marrow and Olfactory Ensheathing Cells (OEC) from the olfactory bulb have received considerable attention, considering the positive results of several experimental studies and the feasibility of their use for autologous trasplantation (Syková et al., 2006; Sahni and Kessler, 2010; Hernández et al., 2011; Mothe and Tator, 2012). Although numerous studies have reported beneficial effects of MSC (Chopp et al., 2000; Hofstetter et al., 2002; Neuhuber et al., 2005; Himes et al., 2006; Amemori et al., 2010; Quertainmont et al., 2012; Ohta et al., 2004; Paul et al., 2009) or OEC (Li et al., 1997, Ramón-Cueto et al., 2000, Lu et al., 2002; Verdú et al., 2003; García-Alías et al., 2004; López-Vales et al., 2006) transplanted in spinal cord injured animals, there are other contradictory and divergent results about the repair potential of these engrafted cells (Takami et al., 2002; Resnick et al., 2003; Barakat et al., 2005; Collazos-Castro et al., 2005; Pearse et al., 2007; Tewarie et al., 2009). The results and the success of the cell therapy may be dependent on several factors, including time of transplantation (Hofstetter et al., 2002; López-Vales et al., 2006), delivery methods (Paul et al., 2009), type and severity of the injury (Himes et al., 2006), donor variation (Phinney et al., 1999; Neuhuber et al., 2005), culture condition of the cells (Kawaja, 2009; Novikova et al., 2011), methodology of assessment, and the laboratory that performs the experiments (Steward et al., 2006; Franssen et al., 2007).

Mesenchymal stromal/stem cells (MSC) can be derived from various tissues, exhibiting slightly different properties. MSC are most commonly derived from the bone marrow (BMSC) (Friedenstein et al., 1968), but they can be obtained also from other sources, including adipose tissue (Zuk et al., 2001; Boquest et al., 2006), skin, placenta (In't Anker et al., 2004), amniotic fluid (Nadri and Soleimani, 2007) and umbilical cord (Erices et al., 2000). The established criteria to define stromal cells refer to their adherent ability to uncoated plastic during tissue culture, capability of differentiating into multiple mesodermal tissues, and characteristic phenotype like CD34+/CD90+ or CD45- (Dominici et al., 2006). BMSC are a mixture of stromal and hematopoetic cells from the bone marrow, including mononuclear cells such as macrophages, and non-hematopoetic mesenchymal cells which maintain and regulate the growth of hematopoetic stem cells. Regarding to the contradictory results found using BMSC as a treatment for SCI, the intrinsic characteristics of donors can contribute to the inconsistencies between studies (Phinney et al., 1999; Neuhuber et al., 2005). The capacity of hMSC to enhance neurite outgrowth in DRG cultures and to improve functional recovery after their transplantation in a spinal cord hemisection injury are subjected to significant donor variation (Neuhuber et al., 2005). Moreover, the donor source also influences the in vitro growth properties of MSC, the osteogenic differentiation and the *in vivo* bone formation by hMSCs (Phinney et al., 1999). These differences may be derived from donor-related variations in cytokines and growth factors expression (Neuhuber et al., 2005). Interestingly, it was reported an age-dependent variation in the amount of MSC in the bones and their proliferation capacity (Baxter et al., 2004; Mareschi et al., 2006), as well as changes in some markers expression (Scharstuhl et al., 2007) with an increase of donor age. Also, the senescence of MSC cultures increases with the age of the donor, that is directly related with more apoptosis and less number of cell forming colonies (Stolzing and Scutt, 2006; Tokalov et al., 2007). Moreover, MSC show

an age-related decrease in capacity to protect in front of oxidative stress, probably by reduction in their proteosomical activity (Sotlzing and Scutt, 2006). In contrast, some studies have shown that the differentiation potential of MSC in culture is not dependent on donor age (Soltzing and Scutt, 2006; Tokalov et al., 2007).

Olfactory ensheathing cells (OEC) are a special type of glia located in the olfactory system. The axons of the new olfactory sensory neurons are enveloped by OEC that guide and support their elongation, crossing from the PNS of the olfactory mucosa to the CNS of the olfactory bulb, where axons make new synaptic connections with other neurons (Raisman, 1985; Doucette, 1995). There are some differences between OEC subtypes depending on the origin, lamina propia of the olfactory mucosa or olfactory bulb (Richter et al., 2005), and the cell properties can be altered depending on the culture conditions (Radtke et al., 2010). These differences can contribute to the divergent results reported regarding OEC transplantation for SCI.

Cell transplantation requires a large number of cells. While MSC can be easily expanded *in vitro*, OEC cultures have a limited capability to proliferate and therefore require growth factors and longer time cultures. The age of the cells in culture and the purification method can influence the neuroprotective effect exhibited by OEC (Novikova et al., 2011). In fact, long culture periods reduced the remyelinating potential of OEC transplanted in injured spinal cord (Radtke et al., 2010). In contrast to rodents, OECs obtained from dogs (Techangamsuwan et al., 2008) and primates (Rubio et al., 2008) can stay up to three months *in vitro* without entering in senescence. It has been proposed the creation of OEC cell banks to maintain the cells cryopreserved until their potential use, solving the difficulty to obtain a sufficient number of cells for transplantation (Ramón-Cueto and Muñoz-Quiles, 2011). Although many of the OEC transplantation studies used cryopreserved and thawed cells, there are no studies on the comparative effect of fresh or thawed OEC for the repair of SCI induced by contusion.

The application of cell therapy requires the optimal characterization and management of the cells, as well as the control of several factors that may have an influence on the outcome of the transplant, such as the nature of the cells, donor variations, cell culture methods and cell delivery methods. Thus, the aims of this first chapter include:

1. Set up and characterization of the bone marrow derived MSC cultures from young donors.
2. Set up and characterization of the olfactory bulb derived OEC cultures.
3. Optimization of the cell transplantation into the spinal cord after contusion injury.
4. Comparison of the effects of MSC from young or adults donors transplanted after spinal cord injury.
5. Comparison of the effects of fresh or thawed cells for spinal cord injury treatment.

Material and Methods

MSC cultures

Primary MSC cultures were obtained from femurs and tibias of P22 male Sprague-Dawley rats. Rats were euthanized with CO2. Tibias and femurs were placed on cool phosphate buffered saline (PBS) and epiphyses were removed. The diaphyses of bones were flushed using a syringe with a 21G needle with PBS and the marrow was mechanically homogenised. The cell mixture was filtered through a 70µm nylon mesh and recovered by centrifugation for 10min at 1500rpm. The pellet was resuspended in growth medium: α-MEM with L-glutamine (Life Technologies, Grand Island, NY, USA) supplemented with 20% heat-inactivated pre-testate fetal bovine serum (FBS) (Lonza, Verviers, Belgium), 2mM L-glutamine (Life Technologies) and 100 units/ml penicillin-streptomycin (Life

Technologies, 100x); and plated in 100mm culture dishes (Iwaki, Asahi Technoglass, Chiba, Japan) at a density of 5•106 cells¬/cm2. After 24h, the supernatant containing non-adherent cells was removed and fresh medium was added. When the culture was near confluence, every 4-5 days, the cells were detached using PBS with 0.05% trypsin (Life Technologies) and 0.04% EDTA (Sigma, St. Louis, MO) and re-plated at 5,000 cells/cm2. Cells were passaged 3-4 times, expanded to 80-90% of confluence. The cells were frozen in medium made of 80% normal growth medium plus 20% DMSO. The cells were kept frozen in liquid nitrogen.

Growth of MSC cultures. The BMSC at passage 4 were plated at 200 cells/cm2 in 30mm culture dishes (Iwaki). At days of culture 1, 3, 5, 7, 10 and 12 the cells were detached using PBS with 0.05% trypsin and 0.04% EDTA, the non-viable cells were labeled with trypan-blue and the viable cells were counted under microscope using a Neubauer chamber. Three dishes were detached at every time point and the number of cells counted. The double leveled population (PDL) was calculated using the formula: PDL= (Log Nt/Log N0)*3.33 where Nt is the final number of cells in at time t and N0 the number of cells at the initial time.

MSC immunophenotype characterization. For phenotype characterization a cell suspension of bone marrow extract and MSC after 4 passages in culture were labeled for 30 min using antibodies against CD90, CD29, CD11b and CD45, conjugated with FITC (BD Bioscience, Erembodegem, Belgium; 1:200 v/v). After incubation, the cells were centrifuged for 5 min at 1300rpm and resupended in PBS. The analysis of surface markers was performed using flow citometry (FACSCalibur, Becton Dickinson) and FACSDiva software (BD Bioscience).

MSC differentiation assays. The capacity of the BMSC for differentiation to adipocytes and osteocytes was assessed. MSC at passage 4 were plated in growth medium at 5000 cells/cm2 in 6-well plates. After 24-48h, when the culture was near confluence, the medium was replaced by a differentiation induction medium, which was replaced every 72h until 21 days. The adipogenic medium consisted in DMEM (Life Technologies) supplemented with 10% FBS (Lonza), 1µM dexametasone (Sigma) and 10µg/ml insulin (Sigma). For osteogenic differentiation, the cells were cultivated with DMEM (Life Technologies) containing 10% FBS (Lonza), 0.1µM dexametasone (Sigma), 0.02mM bisphosphate ascorbic acid (Sigma) and 0.01M bisphosphate glicerol (Sigma). As a negative control the cells were maintained with normal growth medium. For analysis of adipogenesis, the cells were fixed for 20 min with 4% paraformaldehyde in phosphate buffer (PB) and labeled using 60% Oil red O stock solution (0.5% Oil red O in isopropanol, Sigma) for 15 min and washed with distilled water. For osteogenic labeling, cells were fixed using 70% ethanol pre-cooled for 1h at 4ºC, washed and incubated during 30 min with 0.1mg/ml Alizarin red solution (Sigma) in distilled water.

OEC culture

Olfactory bulbs were aseptically removed and stored in cold Hank's balanced salt solution (HBBS) with calcium and magnesium. The meningeal layer was stripped off with a fine forceps, and tissue was enzymatic (tripsin 0.25%, collagenase A 1mg/ml, and DNAsa I 1mg/ml) and mechanically dissociated. The cells were recovered by centrifugation in Dulbecco's minimum essential medium nutrient mixture F-12 Ham (DMEM) and seeded onto 25 cm2 flasks coated with poly-L-lisine and incubated in 5% CO2 at 37ºC. Culture medium was DMEM supplemented with 10% FBS (Life Technologies). Cells were kept in culture at least for 7 days. For purification, the cells were incubated with mouse anti-p75NGFR antibody (1:100, Chemicon, ref. MAB365), then immunopurified attaching them to goat anti-mouse IgG microbeads (Miltenyi Biotec) using the MACS separation (Miltenyi Biotec) according to

manufacturer protocols. The cells were frozen in medium made of 80% of normal growth medium plus 20% of DMSO, and kept frozen in a liquid nitrogen container.

OEC immunophenotype characterization. For immunocitochemistry, the OEC cultures were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 minutes, then the samples were incubated for 24h with primary antibodies mouse anti-p75NGFR and anti-S100 (1:200, DiaSorin, ref. 22520). After washing, the samples were incubated 1h with secondary antibodies Cy3 conjugated donkey anti-mouse (1:200, Jackson IR) or Alexa 488 donkey anti-mouse (1:200, Invitrogen). DAPI staining was used to stain nuclei of all the cells present in the culture (1:1000, Sigma). Slides were dehydrated and mounted with Citoseal 60 (Thermo Fisher Scientific, Madrid, Spain).

Cell labeling for transplantation

For identification of the cells after grafting we used two different approaches, green fluorescence protein (GFP) expression and labeling with PKH26 (Sigma). MSC were transfected with a lentiviral vector encoding for GFP under EF1α promoter. Cells in passage 2 were plated at 2000 cells/cm2 and incubated with lentiviruses at MOI of 10 during 48h. Then, the medium was changed and the cells cultured as described above. OEC transfection was done at 2-3 days of selection at MOI of 50 for 24 hours. The infected cells were checked using an antibody against GFP (1:200, Abcam). The efficacy of infection was measured by flow cytometry (FACSCalibur, Becton Dickinson) and FACSDiva software (BD Bioscience). The medium was replaced by complete culture medium and the cells were cultured for other 5 days. For the PKH26 marker, both MSC-GFP and OEC-GFP were labeled before transplantation according to manufacturer indications.

Spinal cord injury and transplantation

Adult Sprague-Dawley female rats (9 weeks old; 250-300g) were used in the SCI experiments. The animals were housed with free access to food and water at a room temperature of 22 ± 2ºC under a 12:12 light-dark cycle. The experimental procedures were approved by the ethical committee of the Universitat Autònoma de Barcelona in accordance with the European Directive 86/609/EEC. Under anesthesia with ketamine (90mg/kg) and xylacine (10mg/kg) and aseptic conditions, a longitudinal dorsal incision was made to expose T6-T10 spinous processes. A laminectomy was performed in T8-T9 vertebra and a cord contusion was induced by either weight drop of a 10g impounder from 20 mm above the spinal cord surface or contusion of 200Kdyns using the Infinite Horizon Impactor device (Precision System and Instrumentation, Kentucky, USA). The cells for transplantation were suspended in L15 medium (Life Technologies) at 50,000 cells/µl and maintained in ice during the time of surgery. Using a glass needle (100µm internal diameter, Eppendorf, Hamburg, Germany) coupled to a 10µl Hamilton syringe (Hamilton #701, Hamilton Co, Reno, NV, USA), 3µl of the corresponding cell suspension or vehicle (L15) were intraspinally injected in 2 or 3 points, with a total of 150,000 cells per injection. A perfusion speed of 2µl/min was controlled by an automatic injector (KDS 310 Plus, Kd Scientific, Holliston, MA, USA), and the tip was maintained inside the tissue 3min after each injection to avoid liquid reflux. The wound was sutured by planes and the animals allowed to recover in a warm environment. Bladders were expressed twice a day until reflex voiding of the bladder was re-established. To prevent infection, amoxicilin (500 mg/l) was given in the drinking water for one week.

Cell survival during transplantation

In order to quantify the possible effect of the transplantation process on cell survival, fresh or

thawed MSC and OEC were resuspended in L15 at the same concentration used for transplantation. The cells were submitted to injection as described above and recollected in a tube after passage through the glass needle. After labeling with trypan-blue the viable cells were counted using a Neubauer chamber. The results were expressed as a percentage of viable cells compared to the amount of alive cells before the procedure. The cells were placed in ice and the same analysis was performed at 30 min and 8 hours after the cell preparation to simulate the time between the first and last rats transplanted in a surgery day.

Functional assessments

Open-field locomotion assessment. Motor behavior was tested before surgery and at 3, 7, 14, 21, 28, 35 and 42 days postoperation (dpo). Animals were placed individually in a circular enclosure and allowed to move freely for 5 minutes. Two observers evaluated locomotion during open-field walking and scored the hindlimb performance, according to the BBB-scale, ranging from 0 (no movement of the hindlimbs) to 21 (normal movement) (Basso et al., 1995).

Treadmill locomotion assessment. The maximal walking speed that the rats were able to sustain on a treadmill was assessed at end time point, 42 days after surgery, using a Digigait Imaging system (Mouse Specifics Inc., Boston, MA) as previously described (Redondo-Castro et al., 2013). Briefly, a high-speed video camera mounted below a transparent treadmill belt captured ventral images of the animal at 140 frames per second, and a minimum of 8 sequential step cycles were recorded. For the maximal speed analysis the treadmill speed was progressively increased from 0 cm/s until the animal was not able to run at the selected speed.

Histology

Tissue processing. The rats were deeply anesthetized (pentobarbital 60 mg/kg i.p.) and intracardially perfused with 4% paraformaldehyde in PBS. The spinal cord segment from 1 cm rostral to 1 cm caudal to the injury epicenter (2 cm total length) was harvested and post-fixed in the same fixative solution for 24h and cryopreserved in 30% sucrose. For evaluation of the injury transversal spinal cord sections 30μm thick were cut with a cryotome (Leica CM190, Leica Microsystems, Wetzlar, Germany) and distributed in 15 series of 24 sections (separated by 450μm) each. For GFP+ cell localization 30μm thick longitudinal sections of the spinal cord segment were cut and distributed in 12 series of 8 sections (separated by 360μm) each.

Hematoxylin-eosin staining. One series of 24 transverse sections was re-hydrated 5 min in water, then submerged in hematoxylin Harris solution (Fluka, Sigma) according to the manufacturer instructions for 5 min. The tissue sections were washed in water followed by 1% HCL in ethanol solution during 30s. The sections were washed with water again and stained with Eosin Y (Merck Millipore, Dramstadt, Germany) during 6 min. The sections were dehydrated and mounted with DPX (Sigma).

Immunohistochemestry. Spinal cord sections of GFP+ cell transplanted rats were processed for immunohistochemestry against GFP. Tissue sections were blocked with TBS-0.3% Triton-5% fetal bovine serum and incubated for 24h at 4ºC with the antibody rabbit anti-GFP (1/200 Life Technologies). After washes, sections were incubated for 2h at room temperature with secondary antibody donkey anti-rabbit AlexaFluor 488 (1/200, Jackson Immunoresearch, West Grove, PA, USA). Slides were dehydrated and mounted with Citoseal 60 (Thermo Fisher Scientific, Madrid, Spain).

Histological analysis. Images were obtained with a digital camera (Olympus DP50) attached to the microscope (Olympus BX51). Analysis of the GFP+ area was performed using 8 spinal cord sections (separated by 360μm between pairs) of each animal. Consecutive images of the spinal

cord injured segment were taken at 40x with the same setting, and the total section was mounted using Photoshop software (Adobe Systems Inc.). Transversal section images were taken at 40x with the same setting. The area of spared tissue was delineated and measured using ImageJ software for each section. The volume of the spared tissue was calculated using the Cavalieri's estimator of morphometric volume (Rosen and Harry, 1990).

Statistical and data analysis

Quantitative data of spared tissue and BBB score were analyzed by two way ANOVA for repeated measures. Data of maximal treadmill speed was evaluated as survival curves by log-rank test. Tissue volume quantification results were statistically assessed using one way ANOVA. In all the comparisons, α of 5% was considered as significant. Bonferroni's post hoc tests for comparative pairs of groups were used.

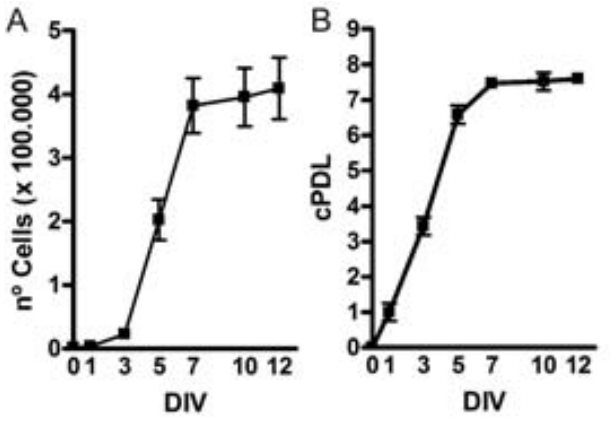

Figure 2. Kinetics proliferation of young MSC in vitro. (A) Growth curve of MSC for 12 DIV and an initial density of 200 cells/cm2. With X formula, we can calculate the growing rate (μ) and the time of division (t). (B) Population doubling level (PDL) accumulated during the 12 days of culture. In the PDL formula N0 represents the initial number of cells and N the number of cells at each time point. Data shown as mean ± SEM.

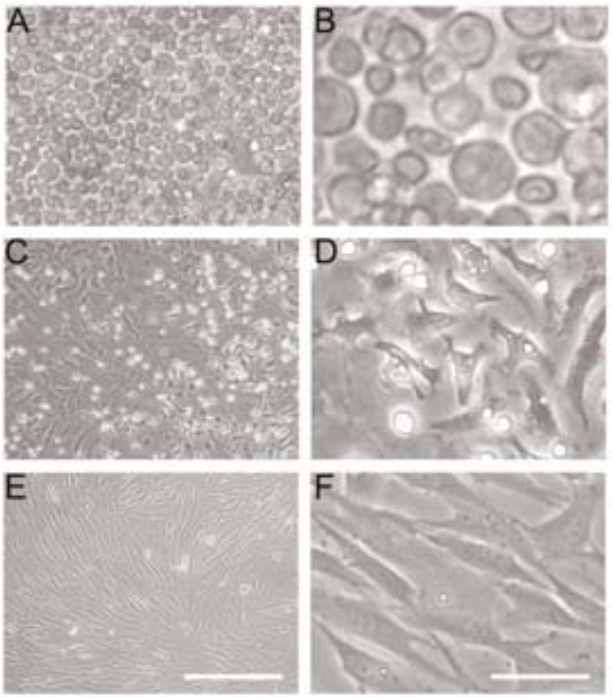

Figure 1. Evolution of MSC culture. Phase-contrast micrography of the direct bone marrow extract (A and B). After 3DIV (C and D) adherent cells acquired a spindle-like shape while others remained in suspension. At the third passage, 21 days after extraction, most cells present in the culture were fusiform (E and F). Scale bar= 200μm in A, C and E, 50 μm in B, D and F.

Results

Young Bone Marrow derived Mesenchymal Stromal Cells

Primary culture of young MSC. The purification of MSC from the bone marrow extract is possible by the adherence ability of these cells to the culture plate surface. With successive passages fibroblast-like fusiform cells appeared and the number of round cells in suspension decreased (Fig. 1A-D). The homogeneity of the culture was reached progressively, to a maximum after three passages (21 DIV) (Fig. 1C-D), when we found a more homogeneous cell population with an elongated spindle morphology.

Proliferation of MSC cultures. The growth of the culture at the same passage exhibit three differentiated stages. Initially, the cells showed a

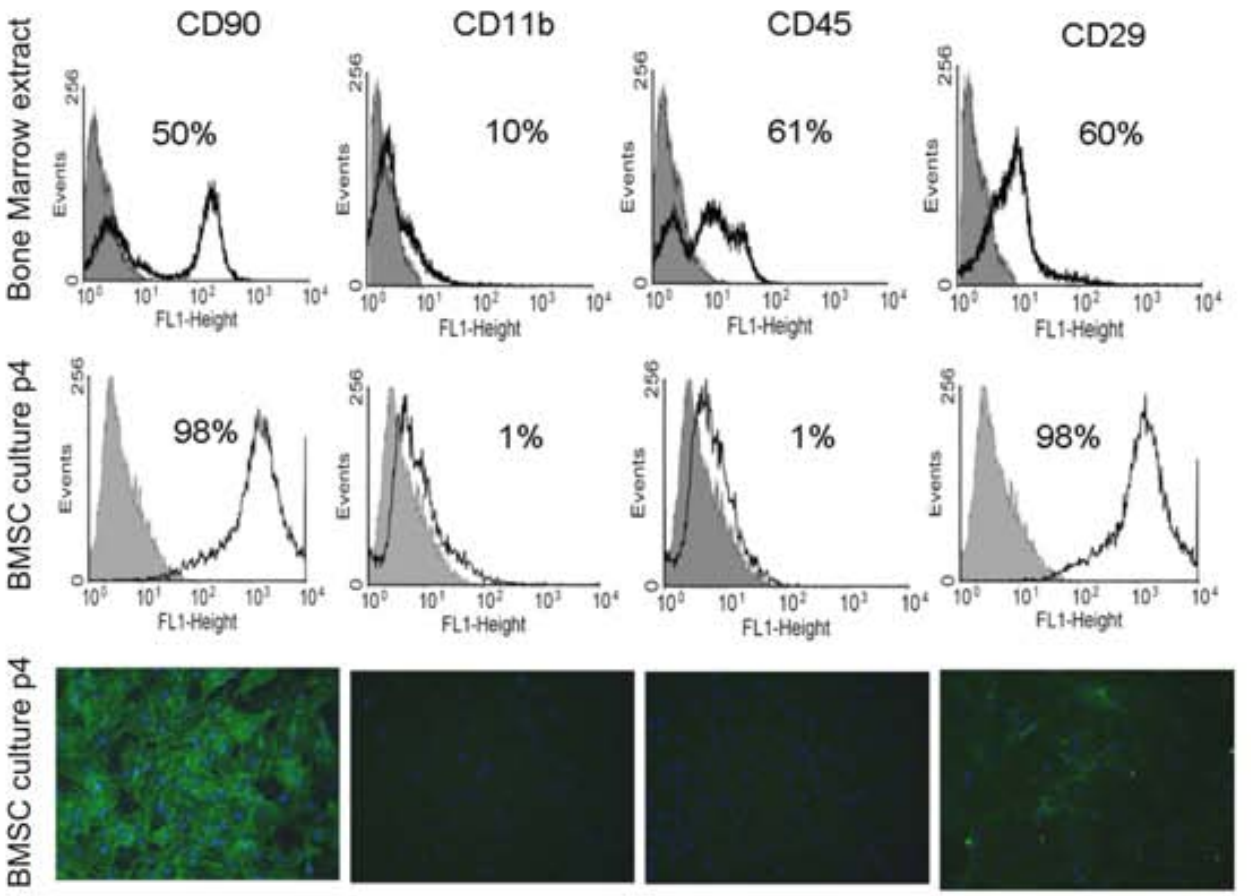

Figure 3. Immunophenotypic profile of MSC analyzed by flow cytometry. The graphics of fluorescence levels for each marker (CD90, CD11b, CD45 and CD29) show the proportion of cells positive for these proteins in the direct extract from bone marrow and after 4 passages (p4) in vitro. In grey the level of fluorescence corresponding to the control antibody and in white the fluorescence for each marker. Immunocytochemistry against these proteins show the expression of CD90 and CD29, but not CD11b nor CD45 by MSC after 4 passages in culture. Green: FITC conjugated antibody against each marker indicated in the top of each column; Blue: DAPI for labeling the nuclei.

slow proliferation rate until 3 days in culture. Then, an exponential growth phase was observed reaching a maximum rate of division (slope of the exponential phase) between 3 and 5 days with a cell density of 20,000 cells/cm2 at day 4, corresponding to 50% confluence. This exponential growth phase ended at 6-7 days of culture achieving an stationary phase characterized by a low proliferation rate (Fig. 2A). The population doubling level (PDL) represents the number of times that cells doubled their number as a function of time in culture. This parameter depends basically on the mean division time of the cells and the number of dead cells at each time. The MSC reached the maximum overlap between 3 and 5 days in culture with a minimum of 18.3 h. From day 7 to the end of follow-up the cell population duplicated between 7.3 and 7.9 times with respect to the initial plated density (Fig 2B).

Immunophenotypic characterization of the MSC. The expression of different surface markers and the non-expression of others are typically used for characterizing the phenotype of the MSC. Flow cytometry analysis (Fig. 3) showed that the

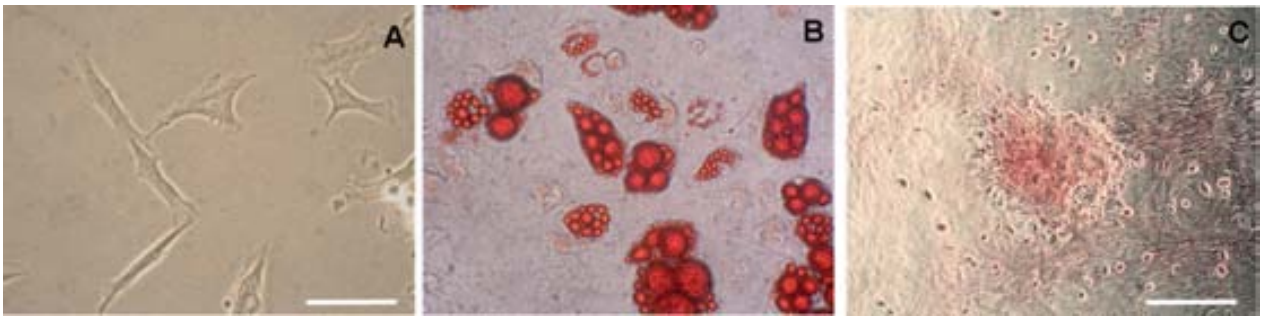

Figure 4. Phase-contrast micrography of MSC differentiated in vitro at passage 4. (A) MSC grown with standard growth medium. (B) MSC cultured with adipogenic medium and stained with Oil Red (red: lipid vesicles in adipocytes). (C) MSC cultured with osteogenic medium and stained with Alizarin Red (red: clusters of calcium). Scale bar = 100µm in A-B and in 200µm C.

population obtained from direct extraction of bone marrow expressed CD90, CD11b, CD45 and CD29 in a proportion of 50%, 10%, 61% and 50% of cells respectively (Fig. 3A). After four passages in vitro 98% of the cells expressed CD90 and CD29, whereas only 1% showed expression of CD11b and CD45 (Fig. 3B). These observation were corroborated by immunocytochemestry of the cells at passage 4 against the same markers (Fig. 3C).

In the differentiation assays we observed adipogenic (Fig. 4B) and osteogenic cells (Fig. 4C) at passage 4, but also cells without morphological changes after mitogenic treatment, indicating that a subpopulation of MSC remained as stem cells in culture.

Olfactory Ensheathing Cells from olfactory bulb

OEC obtained from the olfactory bulb grew in an elongated shape and forming colonies with some cells extending from between colonies (Fig. 5A). Nine days after extraction, p75+ cells were approximately 45% of the total cells in the cultures. After purification of the culture by positive selection against p75+, the proportion of cells that were expressing this marker were higher than 75% (Fig. 5C). The purified cultures were also labeled by immunocytochemistry against S100, another characteristic marker of OEC, showing positive labeling more than 75% of the cells(Fig. 5E and H).

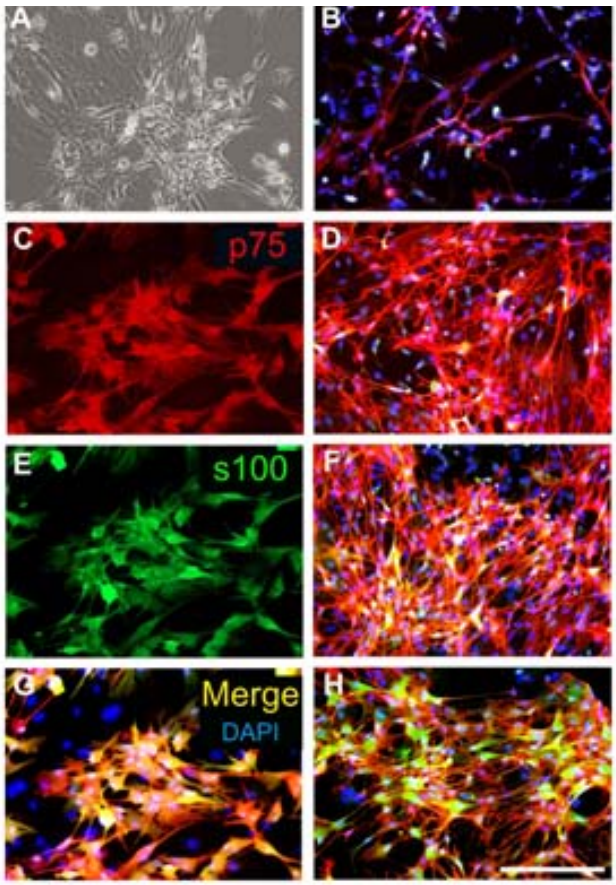

Figure 5. Photomicrographies of OEC cultures. (A) Phase-contrast micrography of OEC growing in clusters. A large proportion of cells in the culture were positive for p75(C) and S100 (E). Both markers co-localized in the same cells (G). During culture time, a progressive increase of p75+ cells was observed (B: 3DIV, D: 5DIV, F: 7DIV). After immunopurification, the percentage of p75+ and S100+ cells was higher (H) than before purification (F). Scale bar=200µm.

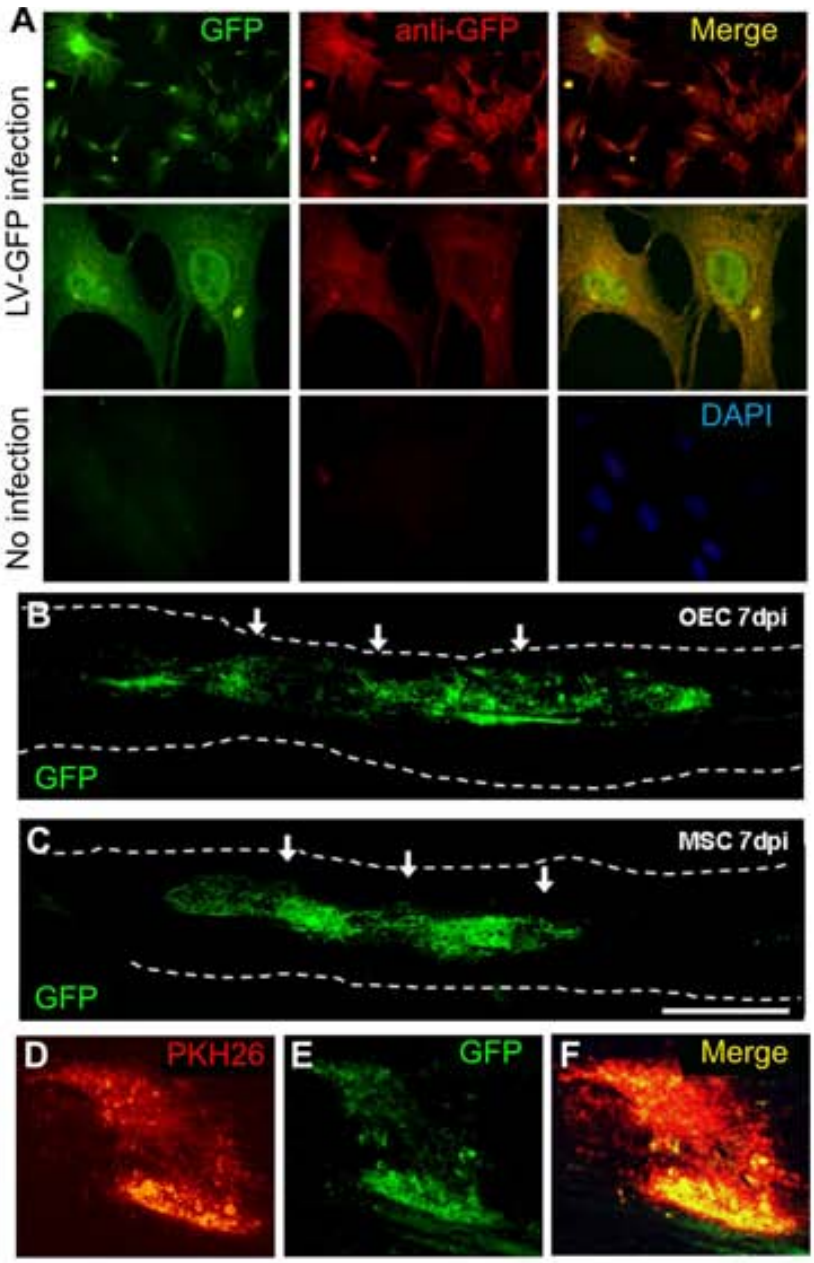

Figure 6. GFP labeling for engrafted cell following after injection into the injured spinal cord. Before transplantation, the cells were infected with LV-GFP and the GFP expression evaluated by GFP autofluorescence and by immunofluerescence using an anti-GFP antibody (A). One week after transplantation, both GFP+ BMSC and OEC were located in the spinal cord injury area and around the injection sites (B). For ensuring the localization of the engrafted cells, GFP+ cells were also labeled with PKH26 (D). Both markers, PKH26 and GFP (E), co-localized (F), in the transplanted cells. Scale bar = 1000μm in B, C. Dotted line in B-C delimit the spinal cord longitudinal section.

Cell preparation for transplantation

Cell labeling and graft localization. The cells for transplantation were labeled with LV-GFP. Moreover, before transplantation the cells were also labeled with PKH26. GFP fluorescence co-localized with labeling by anti-GFP antibody, indicating that the green fluorescence is due to the GFP (Fig. 6A). Using flow cytometry we found that around 90% of the cells in the infected culture were positive for GFP.

To test the engraftment, 4 rats were subjected to SCI and the cells were transplanted in the spinal cord parenchyma (2 rats received MSC-GFP and another 2 rats OEC-GFP). After transplantation GFP+ cells were located at the injury zone and at the injection sites (Fig. 6 B-C). We observed that both GFP (Fig. 6D) and PKH26 (Fig. 6E) markers co-localized (Fig. 6F), confirming that the fluorescent cells were the engrafted ones.

Effect of injection in the spinal cord. In order to check the harmfulness of the injection into the spinal cord, two groups of intact rats were submitted to surgery, exposing the spinal cord, but without contusion; in one an intraparenchyma injection of vehicle was made whereas the other was not injected. One week after the procedure, animals showed no significant changes in locomotion (BBB values of 17.4 ± 1.96 in animals injected and 18.25 ± 1.23 in animals without injection), indicating that the injection performed did not produce significant damage.

Cell survival during the transplant method. Table 1 summarizes the cell viability after the injection procedure. Just after cell preparation for transplantation, there were around 97% alive cells in both MSC and OEC fresh cultures. In the case of cells that were previously frozen and thawed, the survival was significantly reduced with respect to fresh cultures (for both MSC and OEC viability was approximately 85%). After submitting the cells to the injection process there was a decrease in survival to 95% in both fresh cultures and to 80% in both thawed cultures. Eight hours after cell preparation and injection, the viability of both fresh and thawed cultures was reduced, to 83% and 66% respectively. These results indicate that transplantation process reduces the number of alive engrafted cells in a similar magnitude in MSC and OEC. Cell death was more important in the cultures that were frozen and thawed, indicating a negative effect of cryopreservation.

Young vs. adult source of MSC for SCI transplantation

To compare the effect of the MSC donor age, MSC cultures were obtained from young rats (22 days of age) and adult rats (10 weeks of age) and the cells were transplanted after SCI. A total of 20 rats were injured using the impactor device and injected in 3 points, at the epicenter, one rostres and another caudal, both at 2mm from de

Table 1. Percentage of surviving cells before and after submitting them to the injection method.

	MSC		OEC	
	Fresh	*Thawed*	*Fresh*	*Thawed*
Before	97.5% ± 2.1	86.2% ± 2.2	97.3% ± 1.8	85.4% ± 1.7
After	95.3% ± 0.7	82.1% ± 3.1	94.7% ± 2	80% ± 2.1
After 8h	84.4%± 1.3	66.6% ± 1.5	82.5% ± 2.1	65.5% ± 0.8

epicenter. The rats were divided in three groups of treatment: one group was injected with vehicle (L15 medium) (VE group, n=8), a second group received MSC from young donors (yMSC group, n=8), and the third group of rats were transplanted with MSC from adult donors (aMSC group, n=4). The animals were followed during 42 days after injury and transplantation by locomotion assessment. At the end of the follow up the animals were sacrificed and histological analyses were performed to quantify the amount of tissue damage.

Locomotion. Immediately after injury all rats were completely paraplegic. Between 3-7 days after surgery, the injured animals showed slight or extensive movements of the three hindlimb joints, achieving BBB scores of 4-7 points. After the first week, the locomotor performance improved progressively until plantar placement of the paw with weight support in stance only (BBB score 9) or weight support with occasional/frequent plantar stepping without fore-hind limb coordination (BBB score 10-11) (Fig. 7). There were no significant differences between groups, although the aMSC grafted animals showed lower scores than the other rats during the recovery phase. Remarkably, aMSC rats were not able to support their weight until 21 days after injury, whereas the rats of vehicle and yMSC groups supported their weight from 14 days after injury (Fig. 7A). At the end of the follow-up, the maximal speed that animals were able to run was measured under treadmill condition (Fig. 7B). The proportion of animals that were able to run decreased as treadmill speed increased. Animals injected with vehicle or transplanted with aMSC achieved a median speed of 20 cm/s. In

yMSC treated rats, the proportion of animals that run at faster speeds was higher, reaching a median of 37.5 cm/s.

Histological analysis. There were no significant differences in the area of tissue sparing at any distance from the epicenter between aMSC and vehicle groups. In contrast, the animals treated with yMSC showed larger spared tissue with respect to the vehicle group both rostrally and caudally to the epicenter (Fig. 8). Although these differences were also observed between yMSC and aMSC, the means were only statistically different at the caudal segment. These results suggest that MSC from young donors exhibit greater tissue protection than MSC obtained from adult donors.

Fresh vs. thawed cultures for SCI transplantation

Both MSC and OEC cultures were prepared for transplantation from fresh cultures or from cultures that were previously frozen and then thawed before transplantation. The study was divided in two parts, one part for fresh and thawed MSC comparison and another part for fresh and thawed OEC comparison.

In the case of MSC transplantation, 15 rats were injured in the spinal cord using the weight drop method and injected in 2 points, one rostral and another caudal, both at 2mm from the epicenter. Then the rats were divided in three groups: 2 injections of vehicle (VE group, n=5), 2 injections of fresh MSC (f-MSC group, n=5) or 2 injections of thawed MSC (t-MSC group, n=5). The animals were followed during 35 days.

In the case of OEC transplantation, 20 rats were injured using the impactor device and transplanted in 3 points of the injured spinal cord (at the epicenter, one point rostral and another point caudal, both at 2mm from the epicenter). The rats were divided in three groups: 3 injections of vehicle (VE group, n=8), 3 injections of fresh OEC (f-OEC, n=8) or 3 injections of thawed OEC (t-OEC). The animals were followed during 42 days.

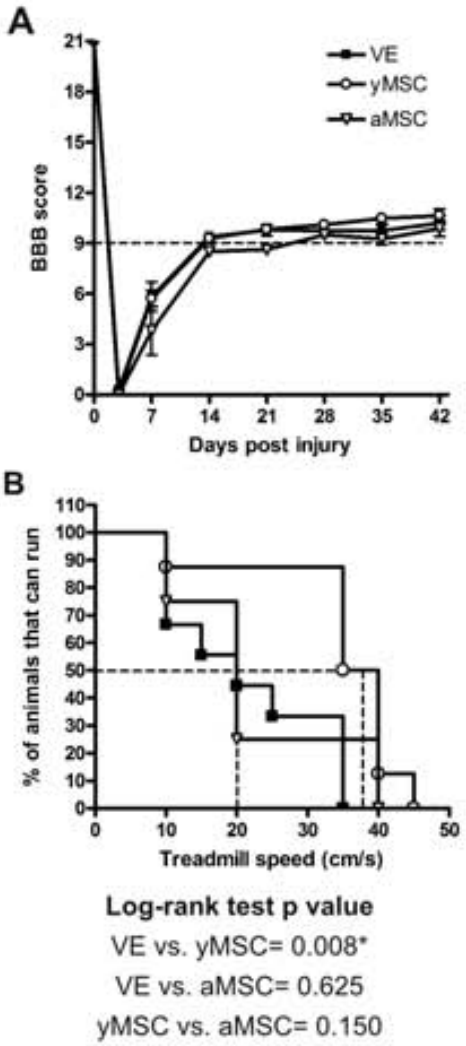

Figure 7. Locomotor performance of spinal cord injured rats treated with MSC. All the animals showed complete paralysis of the hindlimbs at 3 days after injury, followed by a recovery phase reaching a BBB score between 10 and 11 points (A). At the end time point, the maximal speed that animals were able to run was evaluated under treadmill condition (B). Log-rank tests, used to analyse the treadmill speed plots, indicated better performance of the yMSC group compared with VE and aMSC groups (p=0.008).

All the animals were assessed by BBB score and histological analysis of the spared tissue was performed at the end of the follow up.

Locomotion. Deficits and recovery of locomotion were similar to that described above. Briefly, after both types of SCI, induced by weight drop or by impactor, the animals showed early

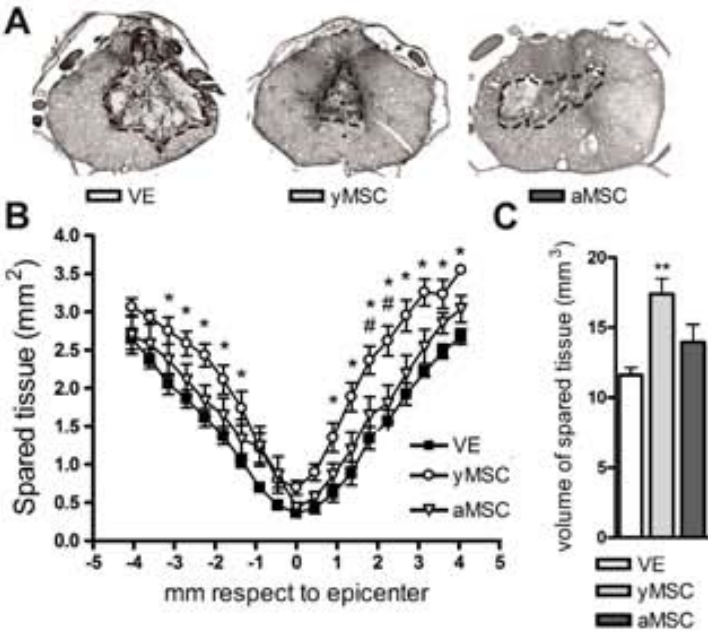

Figure 8. Histological analysis of the injured spinal cord after transplantation. Using transversal section series of the spinal cord injured segment (A), the area of spared tissue from 4 mm rostral to 4 mm caudal to the lesion epicenter (B), and the accumulative volume were calculated (C). Group yMSC showed higher amount of spared tissue than group VE.

complete paraplegia followed by recovery achieving a plateau of 10-11 points in the BBB scale. In MSC transplantation, no differences were observed between groups in the outcome (Fig. 9A). Regarding the OEC transplantation, we found slight better locomotion score in the vehicle treated animals than in both OEC groups at one week after surgery. Although the differences were not significant, it was observed that from 14 days until the end of follow-up the t-OEC engrafted animals were not able to support their weight during walking, or they performed locomotion with dorsal stepping (9 points in BBB score), whereas f-OEC rats could support weight during voluntary locomotion and made plantar stepping occasionally (BBB 10 points) (Fig. 9B).

Histological analysis. Quantification of the spared tissue was performed using transversal sections of the spinal cord segment that included the injury. In the case of MSC treatment, no differences were observed between animals injected with vehicle and animals with f-MSC transplant. In contrast, the transplantation of t-MSC induced less tissue preservation, specially in the caudal segments from the epicenter. The volume of spared tissue showed a reduction of the preserved tissue in the animals treated with t-MSC compared to the other two groups. Similarly, for the OEC transplantation t-OEC produced less amount of preserved tissue with respect to the correspondent vehicle group and f-OEC treated animals. In addition, the spared tissue was larger in the animals injected with f-OEC that in those administered with vehicle. These results suggest that both, MSC or OEC, injected from thawed cultures provide a negative effect regarding tissue protection.

Conclusions

•MSC obtained from young rats exhibit similar characteristics in culture than those established for MSC from adults rats (Neuhuber et al., 2008; Dominici et al., 2006) regarding plastic adhesion capability, fast proliferation with a maximum population doubling time less than 20h, expression of CD90 and CD29 but not expression of CD11b and CD45, and differentiation to mesodermal tissue as adipocytes and osteoblasts.

•The results of MSC cultures allow the estimation of the cell number that can be obtained at certain time and seeding density. Thus, we can

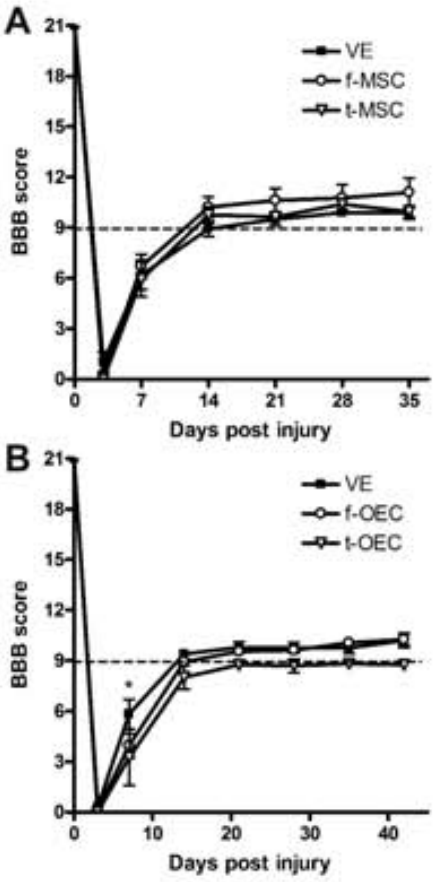

Figure 9. Locomotor assessment after SCI and cell trasplantation. 3 days after injury, all the animals were paraplegic. During the following weeks, the injured rats showed progressive recovery of locomotor skills. In the study of the MSC transplantation, no differences in the recovery were found (A). In the case of OEC only at 7 days the differences were significant (B). *p<0.05 VE group vs f-OEC and t-OEC group.

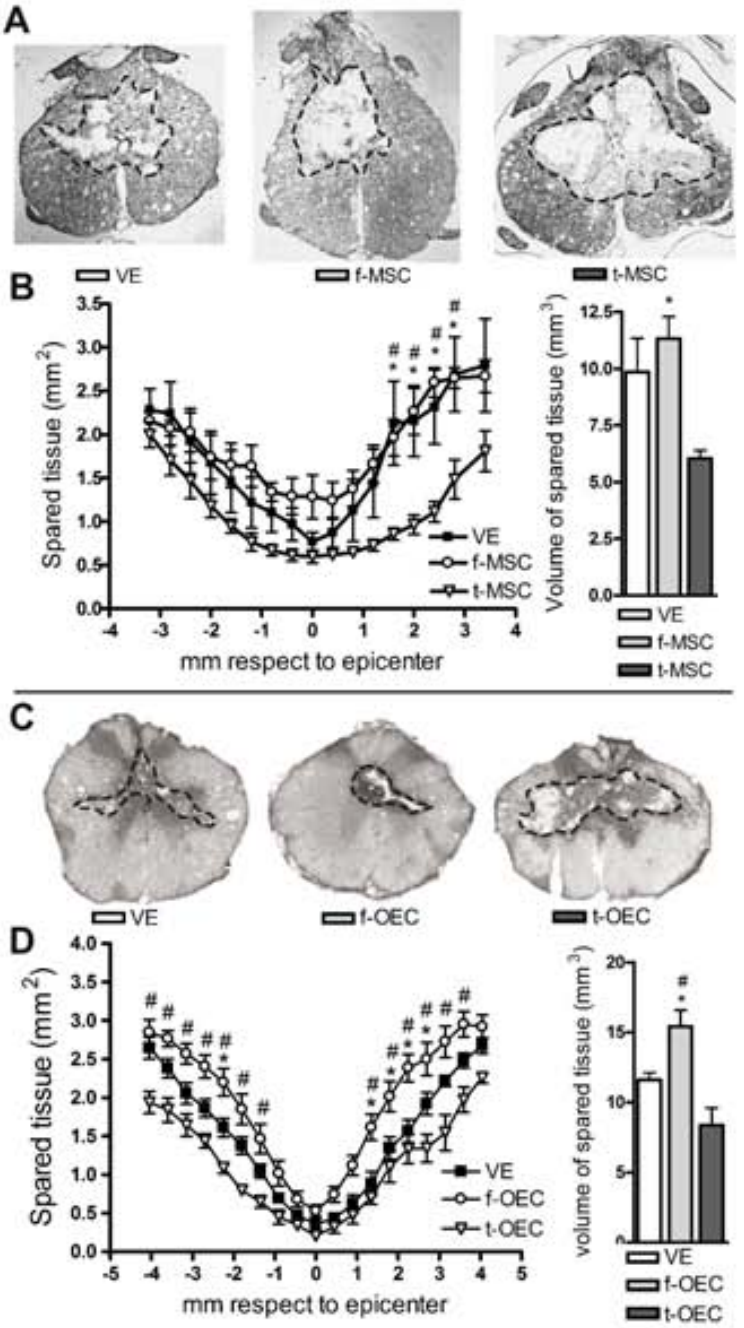

Figure 10. Histological analysis of injured spinal cord. The amount of preserved tissue after MSC treatment (A and B) and OEC treatment (C and D) was quantified on transversal sections of the spinal cord (A and C, respectively). The area of spared tissue and the total preserved volume of the studied spinal cord segment (B and D) were calculated. In B: #p<0.05 VE group vs. t-MSC group, *p<0.05 f-MSC group vs. t-MSC group. In D: #p<0.05 f-OEC group vs. t-OEC group, *p<0.05 f-OEC group vs. VE group.

standardize and coordinate the culture growth with the transplantation day.

•OEC cultures from olfactory bulb could be purified by anti-p75 attached microbeads to a final proportion of S100+/p75+ cells higher than 75%.

•Both MSC and OEC cultures could be infected with a letiviral vector encoding for GFP with an efficiency around 90%. At least seven days after transplantation the engrafted cells maintained the

GFP expression and were localized inside the injured area and surrounding the injection points.

•The injection procedure in the spinal cord does not affect the neurological functions of intact rats.

•At least 83% of both MSC and OEC in fresh cultures survive to injection procedure during the surgery time. In the case of the frozen and thawed cells only 66% of the cells survived.

•The transplantation of MSC from young donors after injury provides greater locomotion recovery and tissue protection than MSC transplants from adult donors.

•The transplantation of both, MSC and OEC, seems to be more beneficial, or at least no harmful, if the cells were transplanted from fresh cultures than from cultures that were frozen and thawed.

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RESULTS: CHAPTER 2

Bone marrow Mesenchymal Stromal Cells and Olfactory Ensheathing Cells transplantation after spinal cord injury: a morphological and functional comparison in rats

Bone marrow Mesenchymal Stromal Cells and Olfactory Ensheathing Cells transplantation after spinal cord injury: a morphological and functional comparison in rats

Abel Torres-Espín, Elena Redondo-Castro, Joaquim Hernandez, Xavier Navarro

Group of Neuroplasticity and Regeneration, Institute of Neurosciences, Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Bellaterra, Spain.

Corresponding author: Xavier Navarro, Unitat de Fisiologia Mèdica, Edif. M, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain. Tel: +34-935811966, Fax: +34-935812986, E-mail: xavier.navarro@uab.cat

Abstract

Background: Cell therapy for spinal cord injury is a promising strategy for clinical application. Both Bone marrow Mesenchymal Stromal Cells (MSC) (also known as bone marrow-derived "mesenchymal stem cells") and Olfactory Ensheathing Cells (OEC) have demonstrated beneficial effects following transplantation in animal models of spinal cord injury. However, due to the large number of affecting parameters that determine the therapy success and the lack of methodological consensus, the comparison of different works is difficult.

Methods: We compared the effects of MSC and OEC transplants at early or delayed time after a spinal cord contusion injury in the rat. Functional outcomes for locomotion, sensory perception and electrophysiological responses were assessed. Moreover, the grafted cells survival and the amount of cavity and spared tissue were studied.

Results: The findings indicate that grafted cells survived until 7 days post-injection but markedly disappeared in the following two weeks. Despite the low survival of the cells, MSC and OEC grafts provided tissue protection after early and delayed transplantation. Nevertheless, only acute MSC grafts improved locomotion recovery in treadmill condition and electrophysiological outcomes with respect to the other injured groups.

Discussion: These results, together with previous works, indicate that the MSC seem a better option than OEC for treatment of contusion injuries.

Key words: cell therapy, mesenchymal stromal cells, olfactory ensheathing cells, spinal cord injury, transplantation.

Introduction

Spinal cord injury (SCI) leads to partial or complete loss of motor, sensory and autonomic impairments and dysfunctions below the injury level, due to the damage to the local circuitry of the spinal cord and the interruption of ascending and descending neural pathways. In the search for potential treatments that may promote repair and recovery from the injury, cell-based therapies have demonstrated beneficial effects using different type of cells in pre-clinical models (1-4). Two of the most extensively studied cells used for SCI repair are bone marrow mesenchymal stromal cells (MSC) and olfactory ensheathing cells (OEC).

MSC have been shown to provide some functional recovery and tissue protection after SCI

by acute or delayed administration directly into the spinal cord (5-10), by intrathecal injection or intravenous perfusion (11,12). The mechanisms underlying the beneficial effects of MSC transplants may include neuroprotection by secreting or inducing the expression of neuroprotective molecules into the injured tissue (8,10), modulation of the neuroinflammatory process (13,14), and their contribution to a more permissive environment for axonal regeneration and neural tissue reconstruction (6,15,16).

OEC are glial cells present in the olfactory system. Their function is to guide and support the elongation of newly growing sensory axons from the olfactory epithelium to the central olfactory bulb, where axons make new synaptic connections (17,18). Acute transplantation of these cells into partial or complete spinal cord lesions reduced the volume of injured tissue, induced improvement in functional outcome, and promoted axonal regeneration (19-24). The proposed mechanisms of action of grafted OEC include the interaction with astrocytes (25,26), promotion of angiogenesis and modulation of the early neuroinflammatory response (27,28), remyelination (29) and enhancing axonal regeneration (19,24,30,31).

Despite the positive outcomes found after treatment with both type of cells, some results are controversial and different findings have been reported depending on the time of transplantation (6,27), delivery methods (12), type and severity of the injury (8), donor variation (7,32), culture condition of the cells (33) and the laboratory that performed the experiments (25,34). Therefore, the objective of this work was to compare the effects on tissue protection and functional recovery of MSC or OEC transplantation after a contusive SCI, using the same animal model, assessment methodologies and culture conditions, by the same experimenters. The results may likely contribute to the knowledge for choosing the best option for cell therapy in SCI.

Material and Methods

Primary cell cultures

Primary cultures of MSC and OEC were set up from P22 male Sprague-Dawley rats. The animals were euthanized with CO2. From each animal tibias and femurs were removed for MSC culture and the olfactory bulbs for OEC culture.

MSC culture and characterization

Tibias and femurs were placed on cool phosphate buffered saline (PBS) and epiphyses were removed. The diaphyses of bones were flushed with PBS using a syringe and the marrow was homogenized. The extract was filtered through a 70µm nylon mesh and recovered by centrifugation for 10min at 1500rpm. The pellet was resuspended in growth medium: α-MEM with L-glutamine (Life Technologies, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Lonza, Verviers, Belgium), 2mM L-glutamine (Life Technologies) and 100 units/ml penicillin-streptomycin (Life Technologies, 100x); and plated in 100mm culture dishes (Iwaki, Asahi Technoglass, Chiba, Japan) at a density of 5•106 cells¬/cm2. After 24h, the supernatant containing non-adherent cells was removed and fresh medium was added. When the culture was near confluence, every 4-5 days, the cells were detached using PBS with 0.05% trypsin (Life Technologies) and 0.04% EDTA (Sigma, St. Louis, MO) and re-plated at 5,000 cells/cm2. Cells were passaged 3-4 times, expanded to 80-90% of confluence.

For phenotype characterization a cell suspension of bone marrow extract and MSC after 4 passages in culture were labeled for 30 min using antibodies against CD90, CD29, CD11b and CD45, conjugated with FITC (BD Bioscience, Erembodegem, Belgium; 1:200 v/v). After incubation, the cells were centrifuged for 5 min at 1300rpm and resupended in PBS. Analysis of surface markers was performed using flow citometry (FACSCalibur, Becton Dickinson) and

FACSDiva software (BD Bioscience). The capacity of the MSC for differentiation to adipogenic and osteogenic cells was assessed as described before (35). For analysis of adipogenesis, cells were fixed for 20 min with 4% paraformaldehyde in phosphate buffer (PB); the adipogenic cells were labeled using 60% Oil red O stock solution (0.5% Oil red O in isopropanol, Sigma) for 15 min and washed with distilled water. For osteogenic cells labeling, cells were fixed using 70% ethanol pre-cooled for 1h at 4ºC, washed and incubated during 30 min with 0.1mg/ml Alizarin red solution (Sigma) in distilled water.

OEC culture and characterization

Olfactory bulbs were aseptically removed and stored in cold Hank's balanced salt solution (HBBS) with calcium and magnesium. The meningeal layer was stripped off with a fine forceps, and tissue was enzymatically (trypsin 0.25%, collagenase A 1mg/ml, and DNAsa I 1mg/ml) and mechanically dissociated. The cells were recovered by centrifugation in Dulbecco's minimum essential medium nutrient mixture F-12 Ham (DMEM) and seeded onto 25 cm2 flasks coated with poly-L-lisine and incubated in 5% CO2 at 37ºC. Culture medium was DMEM supplemented with 10% FBS (Life Technologies). Cells were kept in culture at least for 7 days. For purification, the cells were incubated with mouse anti-p75NGFR antibody (1:100, Chemicon, ref. MAB365), then immunopurified with goat anti-mouse IgG microbeads (Miltenyi Biotec) using the MACS separation (Miltenyi Biotec). OEC purity was at least 75%. For immunocytochemistry OEC cultures were fixed with 4% paraformaldehyde in PBS for 30 minutes, then incubated for 24h with primary antibodies mouse anti-p75NGFR and anti-S100 (1:200, DiaSorin), and after washing incubated 1h with secondary antibodies Cy3-conjugated donkey anti-mouse (1:200, Jackson IR) or Alexa 488 donkey anti-mouse (1:200, Invitrogen).

Cell labeling

For identification of the cells after grafting we used a green fluorescence protein (GFP) lentiviral vector. MSC were transfected with a lentiviral vector encoding for GFP under EF1α promoter. Cells in passage 2 were plated at 2000 cells/cm2 and incubated with lentiviruses at MOI of 10 during 48h. Then, the medium was changed and the cells cultured as described above. OEC transduction was done at 2-3 days of selection at MOI of 50 for 24 hours. Then the medium was replaced by complete culture medium and the cells cultured for 5 more days.

Spinal cord injury and cells transplantation

Adult female rats (9 weeks old; 250-300g) were used. The animals were housed with free access to food and water at room temperature of 22 ± 2ºC. The experimental procedures were approved by the ethical committee of the Universitat Autònoma de Barcelona in accordance with the European Directive 86/609/EEC.

Under anesthesia with ketamine (90mg/kg) and xylacine (10mg/kg) and aseptic conditions, a longitudinal dorsal incision was made to expose T6-T10 spinous processes. A laminectomy was performed in T8-T9 vertebra and a cord contusion of 200Kdyns was induced using an Infinite Horizon Impactor (Precision System and Instrumentation, Kentucky, USA). The animals were divided in 6 groups. Three groups of rats were transplanted acutely, 30 min after operation (0dpo), with vehicle (VE-0dpo, n=8), with MSC (MSC-0dpo, n=8) or with OEC (OEC-0dpo, n=8). Other three groups of rats were transplanted at 7 days postoperation with vehicle (VE-7dpo, n=7), MSC (MSC-7dpo, n=7) or OEC (OEC-7dpo, n=7). The cells for transplantation were suspended in L15 medium (Life Technologies) at 50,000 cells/µl and maintained in ice during the time of surgery. Using a glass needle (100µm internal diameter, Eppendorf, Hamburg, Germany) coupled to a 10µl Hamilton syringe (Hamilton #701, Hamilton Co,

Reno, NV, USA), 3µl of the corresponding cell suspension or vehicle (L15) were intraspinally injected at the epicenter and at 2mm rostrally and caudally, for a total of 450,000 cells per rat. A perfusion speed of 2µl/min was controlled by an automatic injector (KDS 310 Plus, Kd Scientific, Holliston, MA, USA), and the tip was maintained inside the tissue 3min after each injection to avoid liquid reflux. The wound was sutured and the animals allowed recovering in a warm environment. Bladders were expressed twice a day until reflex voiding of the bladder was re-established. To prevent infection, amoxicillin (500 mg/l) was given in the drinking water for one week.

Locomotor and sensory function assessment

Open-field locomotion. Motor behavior was tested before surgery and at 3, 7, 14, 21, 28, 35 and 42 days postoperation (dpo). Animals were placed individually in a circular enclosure and allowed to move freely for 5 minutes. Two observers evaluated locomotion during open-field walking and scored the hindlimb performance, according to the BBB-scale, ranging from 0 (no movement) to 21 (normal movement) (36).

Treadmill locomotion. The maximal walking speed, gait motion and interlimb coordination at end time point were assessed under treadmill condition using a Digigait Imaging system (Mouse Specifics Inc., Boston, MA) as previously described (37). Briefly, a high-speed video camera mounted below a transparent treadmill belt captured ventral images of the animal at 140 frames per second, and a minimum of 8 sequential step cycles were recorded. For the maximal speed analysis the treadmill speed was progressively increased from 0 cm/s until the animal was not able to run at the selected speed. For gait and interlimb coordination the animals were recorded at 20 cm/s, only if the animals were able to run at this speed. The videos were analyzed as described elsewhere (37). The start and ending times of the stance phase of each step were extracted by analyzing the videos using ImageJ software (NIH, Bethesda MA, USA), and foot gait diagram was depicted (38). Quantitative values for the gait parameters (stride duration, stance duration and swing duration) and the stance/swing ratio were calculated using a Visual Basic macro for Excel® software (Microsoft Corporation) with the values previously extracted. To assess the coordination of locomotion the regularity index (RI) was calculated. A regular step pattern implies fully coordinated locomotion, in which each paw is exactly placed one time every four footprints, and is placed or raised at a given time. The RI is calculated as the percentage of correct step sequences with respect to the total number of step cycles. Thus, the lower RI, the larger the number of missteps not following the correct pattern (39,40).

Sensory tests were performed before surgery and at 42 dpo in both hindpaws. The rats were habituated in the testing chamber for 10 minutes. The response to mechanical stimuli was evaluated by an electronic Von Frey algesimeter (Bioseb, Chaville, France). The spring metallic filament connected to a force sensor was applied in the medial part of the hindpaw, the pressure gradually increased and the threshold was determined as the force at which the animal withdrew the paw. The response to heat stimuli was determined by using a Plantar algesimeter (Ugo Basile, Comerio, Italy). The rats received a radiant hot stimulus in the hindpaw and the latency time until the animal withdrew the limb was used to determine the hot pain threshold. A cut-off time was set at 20s to prevent tissue damage. Five trials separated by 10 min resting periods for each hindpaw were performed in both sensory tests. The value for each test was the mean of both hindpaws.

Electrophysiological studies

For the electrophysiological tests the animals were anesthetized with pentobarbital (30 mg/kg i.p.), placed prone onto a metal plate and skin temperature maintained above 32°C. An

electromyograph (Sapphire 4ME, Vickers) was used.

Central conduction tests consisted in the evaluation of motor evoked potentials (MEPs). MEPs were elicited by transcranial electrical stimulation of the brain. Two needle electrodes were placed subcutaneously over the skull, the anode over the sensorimotor cortex and the cathode on the nose. Single electrical pulses of supramaximal intensity (25 mA, 100 μs) were applied, and the MEPs were recorded with monopolar needle electrodes from the tibialis anterior (TA), gastrocnemius medialis (GM) and plantar (PL) muscles (23,41).

Peripheral motor nerve conduction tests were performed by stimulating the sciatic nerve with single electrical pulses (100 μs at supramaximal intensity) delivered by needles inserted percutaneously at the sciatic notch, and recording the compound muscle action potentials (CMAP) of TA, GM and PL muscles by means of needle electrodes. The active electrode was inserted on the belly of the muscle and the reference at the fourth toe. The direct M wave and the reflex H wave (electrophysiological analogue of the stretch reflex) were recorded in the same sweep. The peak latency and the onset-to-peak amplitude of the maximal M and H waves were measured. The H/M amplitude ratio was then calculated, as it provides an index of the proportion of motor units activated by the monosynaptic reflex relative to the total pool of spinal motoneurons (41,42).

Histology

Tissue processing. The end time point of the animals for treatment study was 42 days after injury. For cell tracking, subgroups of animals injected with GFP-MSC or GFP-OEC were sacrificed at 7, 14 and 21 days after injection (n=2 for each time point). The rats were deeply anesthetized (pentobarbital 60 mg/kg i.p.) and intracardially perfused with 4% paraformaldehyde in PBS. The spinal cord segment from 1cm rostral to 1cm caudal of the injury epicenter (2cm total length) was harvested and post-fixed in the same fixative solution for 24h and cryopreserved in 30% sucrose. For evaluation of the injury transversal spinal cord sections 30μm thick were cut with a cryotome (Leica CM190, Leica Microsystems, Wetzlar, Germany) and distributed in 15 series of 24 sections (separated by 450μm) each. For cell survival study 30μm thick longitudinal sections of the spinal cord segment were cut and distributed in 12 series of 8 sections (separated by 360μm) each.

Hematoxylin-Eosin staining. One series of 24 transverse sections was re-hydrated 5 min in water, then submerged in hematoxylin Harris solution according to the manufacturer instructions (Fluka, Sigma) for 5 min. The tissue sections were washed in water followed by 1% HCL in ethanol solution during 30s. The sections were washed with water again and stained with Eosin Y (Merck Millipore, Dramstadt, Germany) during 6 min. The sections were dehydrated and mounted with DPX (Sigma).

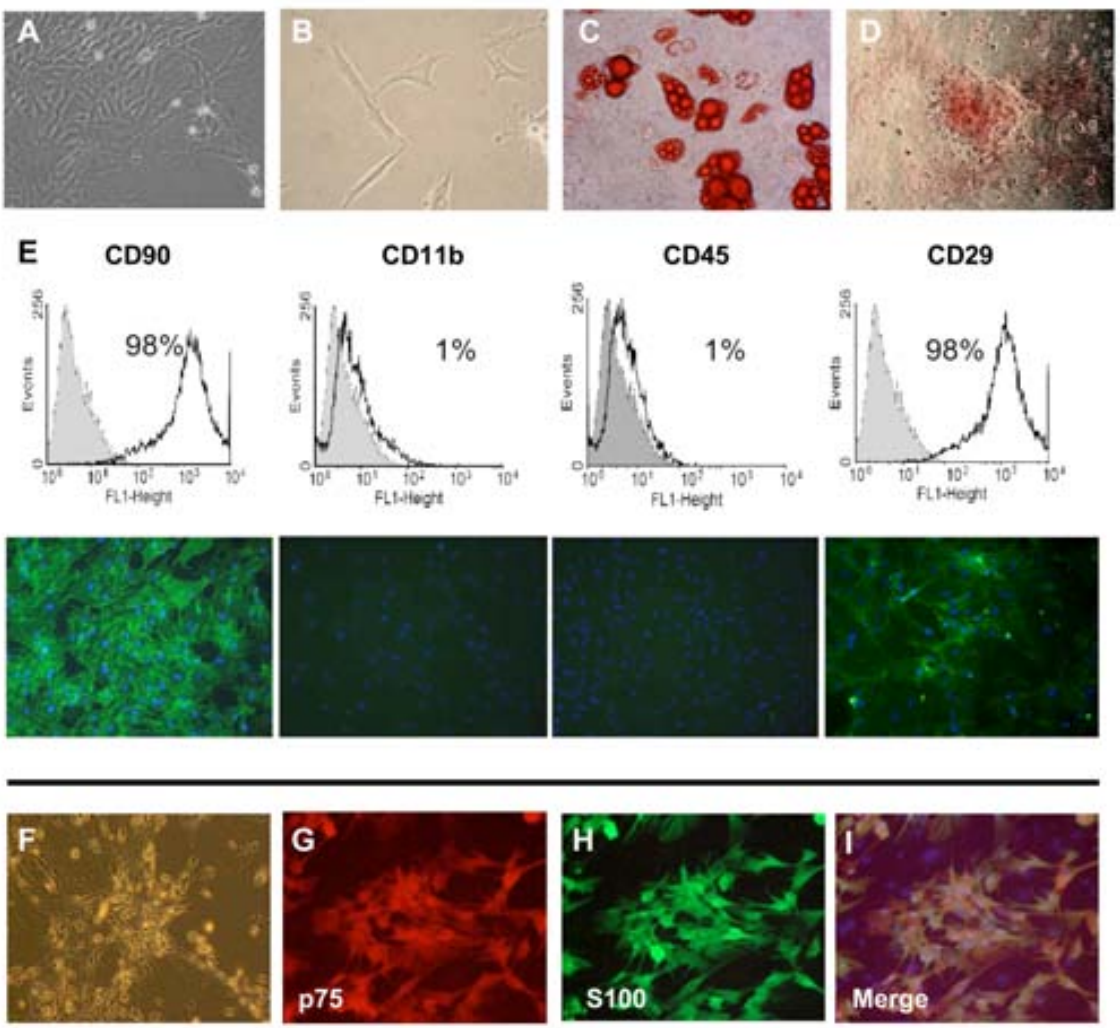
Immunohistochemestry. Spinal cord sections of GFP+ cell transplanted rats were processed for immunohistochemestry against GFP. Tissue sections were blocked with TBS-0.3% Triton-5% fetal bovine serum and incubated for 24h at 4ºC with the antibody rabbit anti-GFP (1/200 Life Technologies). After washes, sections were incubated for 2h at room temperature with secondary antibody donkey anti-rabbit AlexaFluor 488 (1/200, Jackson Immunoresearch, West Grove, PA, USA). Slides were dehydrated and mounted with Citoseal 60 (Thermo Fisher Scientific, Madrid, Spain).

Histological analysis. Images were obtained with a digital camera (Olympus DP50) attached to the microscope (Olympus BX51). Analysis of the GFP+ area was performed using 8 spinal cord sections (separated by 360μm between pairs) of each animal. Consecutive images of the spinal cord injured segment were taken at 40x with the same setting and the total section was mounted using Photoshop software (Adobe Systems Inc.).

The microphotographs were analyzed using ImageJ software. The GFP labeled area in each section was measured after defining a threshold for background correction. Analysis of spared tissue and injury size was made using 19 transversal cord sections (separated by 450µm between pairs) of each animal. The transversal section images were taken at 40x with the same setting. The area of spared tissue, cavity and total spinal cord section were delineated and measured using ImageJ software for each section. The volume of the graft, spared tissue, cavity and total spinal cord injured segment were calculated using the Cavalieri's estimator of morphometric volume (43).

Statistical and data analysis

Quantitative data of gait analysis, sensory tests and electrophysiology tests were analyzed by one way ANOVA. Data of graft volume were analyzed using two way ANOVA. Statistical analyses of spared tissue, cavity and total section area and BBB score were performed by two way ANOVA of repeated measures. Bonferroni's post hoc test for comparative pairs of groups was used. In all the comparisons, α of 5% was considered as significant and mean ± SEM were represented. Data from maximal treadmill speed was evaluated as survival curves by log-rank test.



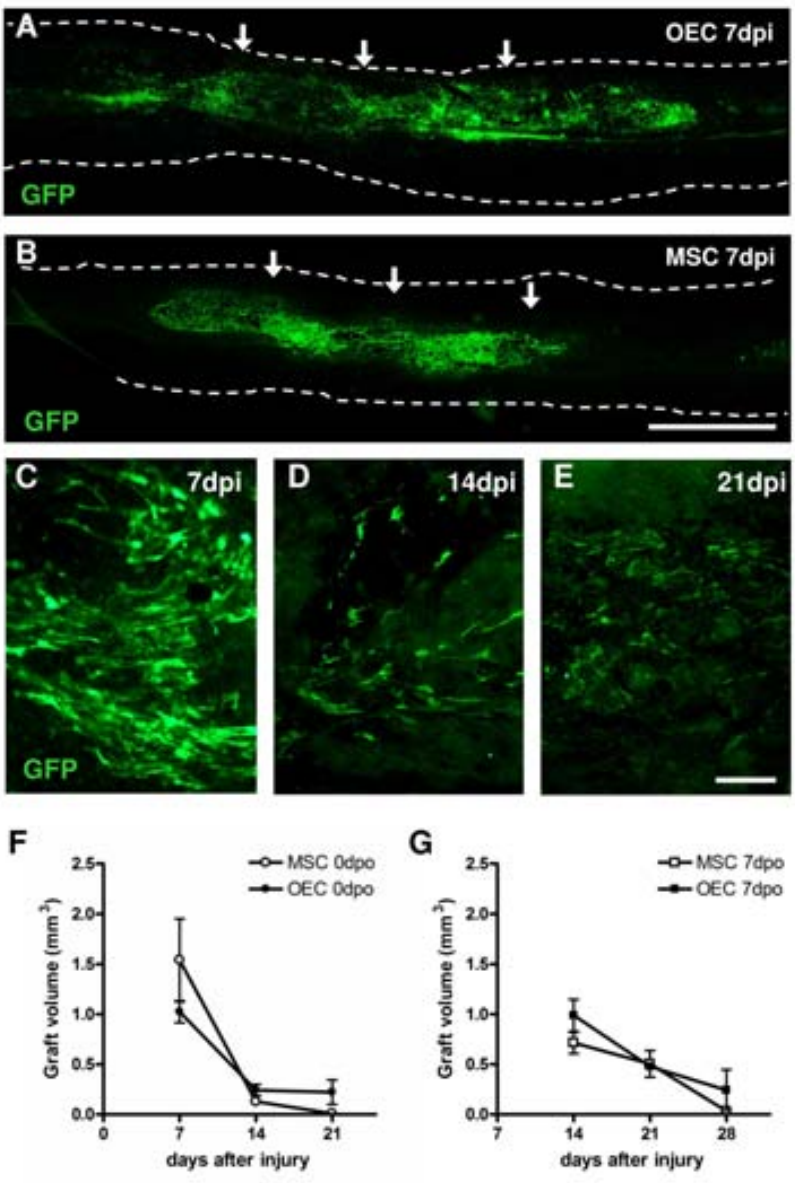


Fig. 1. Primary cell culture characterization. Micrographs showing MSC primary cultures (A, B). The pluripotency of the MSC was assessed with specific medium that induced their differentiation into adipocytes (labeled with Oil red staining) (C) or osteoblasts (labeled with Alizarin Red) (D). Phenotypic characterization (E) was performed against CD90, CD11b, CD45 and CD29 by FACS (E, top panels) and immunocytochemistry (E, bottom panels). Micrographs showing OEC primary cultures (F-I). Immunolabeling against p75 (G) and S100 (H) demonstrated that most cells co-express (I) these two typical OEC markers.

Fig. 2. Grafted cell survival in the injured spinal cord using GFP labeling. OEC (A) and MSC (B) GFP+ grafted cells (green) were localized 7 days after transplantation into the spinal cord surrounding the injection sites (white arrows). Independently of the type of cells and time of injection, the density of GFP+ cells decreased from 7 days (C) to 14 days (D) and 21 days after injection (E), as reflected by the volume occupied by grafted cells in the spinal cord in both acute (F) and delayed (G) transplants. Scale bar = 1000µm in A, B; 100µm in C-E. Dotted line in A-B delimit the spinal cord longitudinal section.

Results

Cell culture characterization

The MSC were checked by their capability for differentiation to other cell types, such as adipocytes and osteoblasts. Figure 1 shows MSC in primary culture under normal growth condition (Fig. 1A,B). The addition of morphogens in the medium induced the transformation of MSC to adipocytes, with lipid accumulation inside the liposomes (Fig. 1C), and osteoblasts, forming cell clusters rich in calcium deposits (Fig. 1D). Moreover, MSC cultures were characterized by the positive expression of CD90 and CD29, in 98% of the cells, and the negative expression of CD11b and CD45, in only 1% of the cells, confirmed using FACS and immunocytochemestry (Fig. 1E). OEC cultures were labeled by immunocytochemistry for p75 and S100, two typical markers for these cells (Fig. 1F-G). Most OEC (95%) expressed both antigens and only a low number presented immunolabeling for one but not the other marker (Fig. 1I).

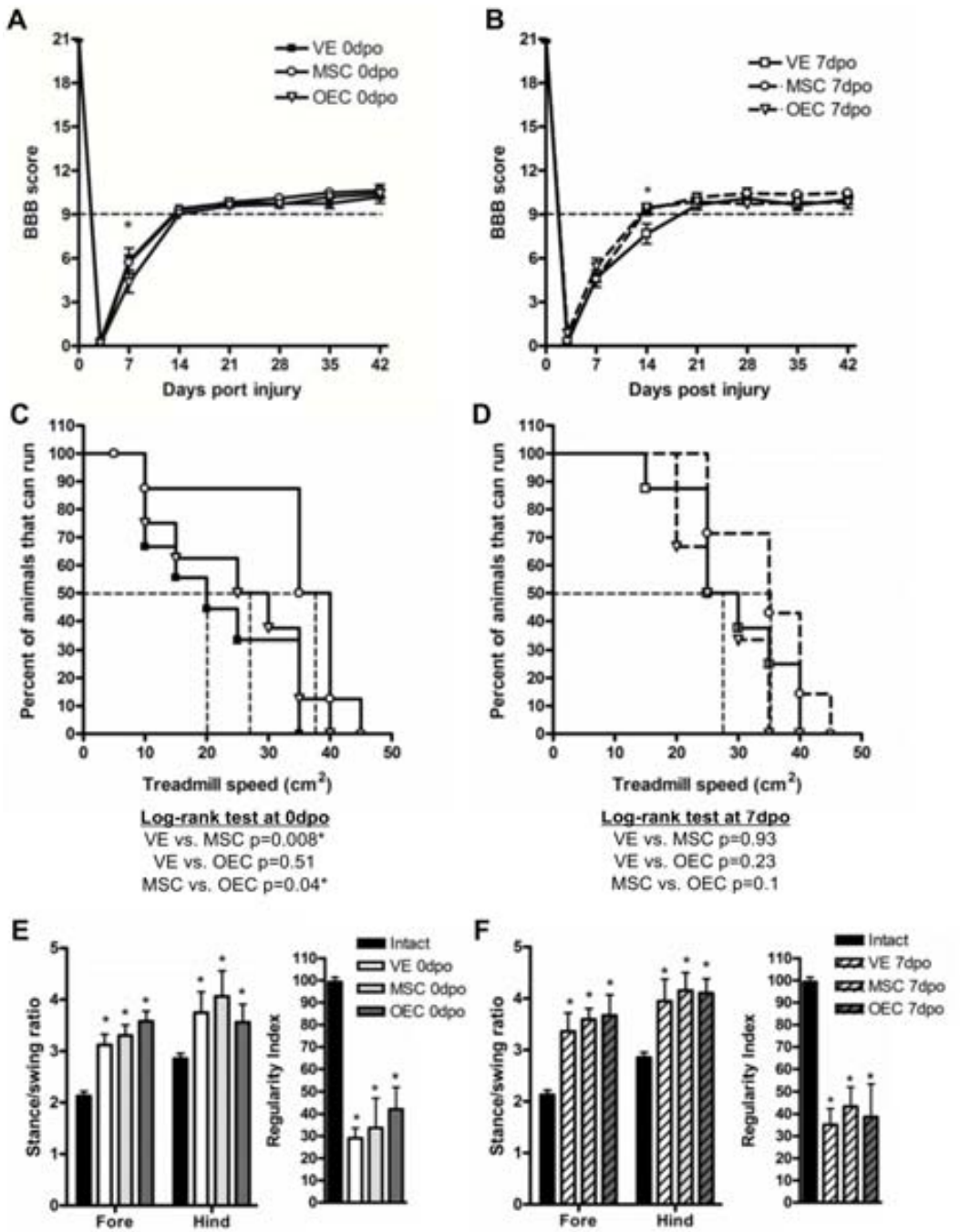


Fig. 3. Locomotion test results. The open field locomotion was evaluated weekly after the injury using the BBB score in acute (A) and delayed treatment (B). After injury all the rats showed paralysis of the hindlimbs, with partial recovery durig the next 2-3 weeks. The dotted line indicates a relevant point in the BBB scale (score of 9 points) regarding the capacity of the rats to support body weight. There were slight improvements with the delayed MSC and OEC transplants at 14 days compared to VE group. In A, * $p<0.05$ VE-0dpo and MSC-0dpo groups vs. OEC-0dpo group. In B, * $p<0.05$ OEC-7dpo and MSC-7dpo groups vs. VE-7dpo group. The percentage of animals that were able to run at increasing treadmill speeds was analyzed as survival curves (C and D). The p value for each comparison represent the statistical significance of the log-rank test. The gait performance during treadmill walking was represented by the stance/swift ratio for which all the SCI animals showed similar disturbances (E and F, left panels). The interlimb coordination, assessed using the regularity index, showed marked reduction in all the injured rats (E and F, right panel). In E and F, * $p< 0.05$ SCI groups vs. intact control rats.

Graft survival into the spinal cord

Independently of the time of injection or the cell type, at 7 days after transplantation a high accumulation of GFP labeled cells was observed, with a similar graft volume in all the groups (Fig. 2A,B). The cells were found surrounding the epicenter of the injury and clustering near the rostral and caudal injection sites. A strong reduction of the amount of GFP was observed at 2 weeks after grafting in all groups, that continued at 3 weeks after transplantation (Fig. 2C-G). These observations indicate that the survival of grafted cells was relatively short in time and decreased markedly two weeks after the transplant.

Locomotor function

The BBB open field locomotion score was used to test the gross voluntary locomotion activity after SCI. In all the injured groups there was paralysis of the hindlimbs (0 score) at 3 days post-injury, followed by recovery during the next two weeks (Fig. 3A,B). The animals achieved a plateau without significant recovery during the last 3 weeks of follow-up with a BBB score about 10-11 points. During the early phase, there were only a few differences between groups; the acute MSC transplant and the vehicle groups showed higher BBB score than the OEC transplant group at 7 days, whereas both subacute cell transplants had higher scores than vehicle at 14 days. No significant differences were found between the acute and the sub-acute injected groups during the plateau phase.

Digigait. Non-injured animals were able to run at maximal treadmill speed about 80 cm/s. In all injured groups, the rats supported their weight in stance and all were able to run at 5 cm/s treadmill speed. In acute treatment significantly more rats of the MSC group were able to sustain walking at higher speeds than in the groups OEC and VE (log-rank p value 0.008 and 0.04, respectively), with a higher median speed (MSC-0dpo: 37.5 cm/s; OEC-0dpo: 30 cm/s; VE-0dpo: 20 cm/s) (Fig. 3C). No statistical differences were detected between OEC and VE acute groups. With subacute treatment, no significant differences were found between the three groups, although the MSC transplant preformed slightly better than the other two groups.

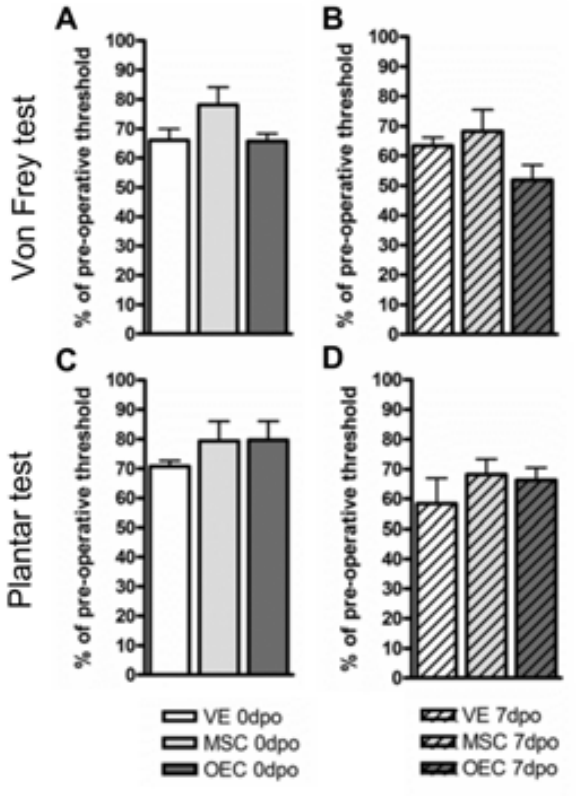


Fig. 4. Sensory test results. The responses to noxious mechanical stimulus assessed using an electronic Von Frey device (A and B) and to heat stimulus using the plantar test (C and D). The threshold values are represented in percentage with respect to pre-operative values for each treatment group. After the injury, the thresholds of both sensory tests were reduced in all treatment conditions, indicating hypersensitivity in front to noxious mechanical and thermal stimuli. No differences were observed between acute or delayed transplant groups.

The analysis of the gait parameters and interlimb coordination were performed at 20 cm/s treadmill speed (37). After SCI, the animals increased the forelimb step and reduced the hindlimb step number. As a consequence, the

stance and swing time for each step decreased in forelimbs, while the stance time increased in hindlimbs. These changes were reflected in the stance/swing ratio that increased for both fore and hindlimbs after the injury in comparison with intact rats. There were no significant differences between treatment groups in the gait parameters (Fig. 3E,F). On the other hand, the regularity index for alternating step pattern, used as a measure of global coordination, was markedly reduced in all the groups after injury.

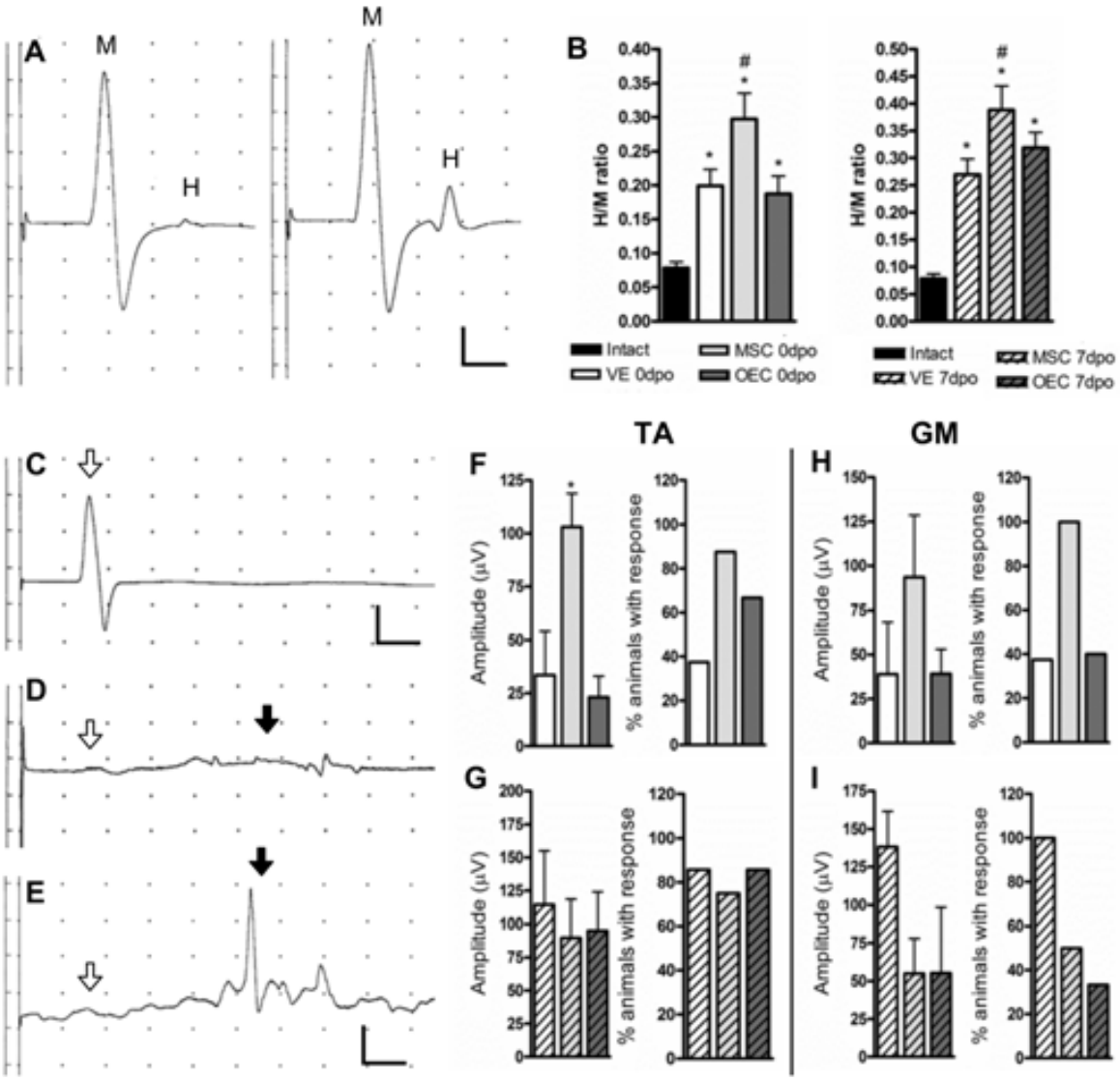


Fig. 5. Electrophysiological results. CMAP recorded in plantar muscle before (A, left panel) and after (A, right panel) SCI. After SCI no changes were observed in the amplitude of the M wave, but the H wave increased. The increased H/M ratio after injury indicates hyperreflexia in the spinal segments below the damage. The H/M ratio was higher after both acute and delayed MSC groups compared with OEC and VE groups (B). Representative recordings of MEPs of the tibialis anterior (TA) muscle before injury (C), after injury and VE injection (D), and after injury and MSC injection (E). In intact rats MEPs had a latency about 6ms (C, with arrow). After injury this early response disappeared, and a second slower response was recorded with a latency about 20ms (D and E, black arrow). The amplitude of the late MEP component and the percentage of animals that presented this response from TA and GM muscles are represented for the acute (F and H, respectively) and delayed (G and I, respectively) treatments. In B and C, * $p<0.05$ injured groups vs. intact rats; # $p<0.05$ MSC group vs. OEC and VE groups. In A, vertical bar = 2mV, horizontal bar = 2ms. In C, vertical bar = 10mV, horizontal bar = 5ms. In D and E, vertical bar = 50μV, horizontal bar = 5ms.

Sensory function

The sensory responses to mechanical and heat noxious stimuli were evaluated at the end of the follow-up with Von Frey and Planter algesimetry tests respectively. The withdrawal thresholds, normalized to the pre-operative values for each animal, showed a decrease in both tests after the spinal cord contusion, indicative of hyperalgesia (Fig. 4). There were no significant changes between any of the acute or sub-acute treatment groups.

Electrophysiology results

Spinal reflexes. Electrical stimulation of the sciatic nerve evoked in all the animals two consecutive muscle responses, the M and the H waves. At the end of the follow-up, 6 weeks after injury, neither the amplitude nor the latency of the M waves were significantly changed in the SCI rats. In contrast, the H wave amplitude increased after injury (Fig. 5A). Thus, the H/M amplitude ratio dramatically increased after injury in all animals with respect to preoperative values, indicating hyperreflexia. This increase was significantly higher in acute MSC than in OEC and VE treated groups, and in subacute MSC than in OEC and VE groups (Fig. 5B).

MEP. Prior to the injury, MEPs were recorded as a single wave, with a mean latency of ~8, ~6 and ~6 ms, and mean amplitude of ~0.22, ~14 and, ~8.1 mV, in PL, TA and GM muscles respectively (Fig. 5C). In all the injured rats the normal MEP response was abolished during the follow-up, and only a few animals presented a very small response (less than 50μV) at the normal latency at 42 days post-injury (Fig. 5D). A late MEP response (latency ~20ms) and polyphasic appeared after injury in TA and GM muscles (Fig. 5D,E). In the acute treatment groups, the mean amplitude of this second component was higher in MSC than OEC and VE groups (Fig. 5F,H). On the other hand, although the mean amplitude was higher in the delayed VE group compared to both MSC-7dpo and OEC-7dpo groups in the GM muscle, no statistical differences were found between groups.

Histological results

We evaluated the amount of injured and spared tissue at 42 days after surgery. In this analysis we considered separately the acute and sub-acute treated groups. In the acute transplants, the amount of spinal cord spared tissue was higher in the two cell treated groups than in the vehicle injected group. However, while MSC injection significantly preserved more tissue in both rostral and caudal areas to the epicenter, in the OEC grafted group tissue preservation was only significant in the caudal region (Fig. 6A). At the center of the injury, around the epicenter, there were no differences between groups in the amount of spared tissue. We found a higher total volume of spared tissue with both MSC (17.4±3.1 mm3) and OEC (15.4±3.3 mm3) transplants in comparison to vehicle injection (11.6±1.5 mm3). Regarding the total area in cross-section of the spinal cord, after injury we observed a progressive reduction from both rostral and caudal segments to the epicenter. With transplantation of both MSC and OEC, the cord transverse area was increased with respect to the control group, with a significantly increased volume in the total spinal cord injury segment (MSC-0dpo: 20.6±2.6 mm3; OEC-0dpo: 19.0±3.1 mm3; VE-0dpo: 15.9±0.9 mm3) (Fig. 6D).

On the other hand, after sub-acute transplantation of the cells no differences for spared tissue measures were found between the three injured groups. However, in contrast with acute transplants, a strong reduction in the amount of the cavity was observed after sub-acute MSC and OEC injection (Fig. 6G). In consequence, the volume of injured tissue was reduced in both MSC and OEC treated animals (MSC-7dpo: 3.9±0.8 mm3; OEC-7dpo: 3.2±0.6 mm3; VE-7dpo: 7.2±1.5 mm3). Thus, the total section area and the total volume of the cord segment were higher in vehicle injected rats than in MSC and OEC treated rats

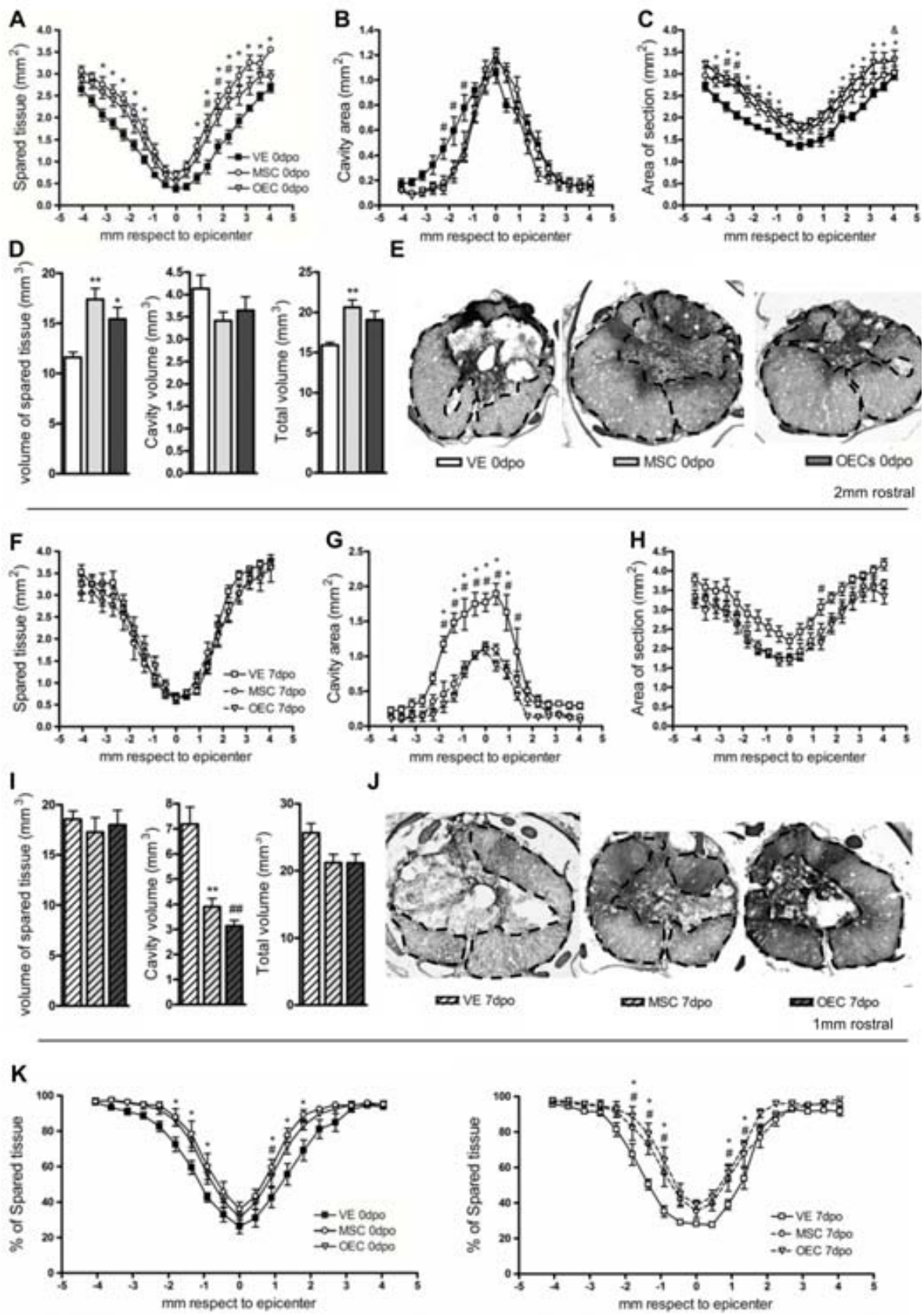

A
Spared tissue (mm²)
mm respect to epicenter
VE 0dpo
MSC 0dpo
OEC 0dpo
B
Cavity area (mm²)
mm respect to epicenter
C
Area of section (mm²)
mm respect to epicenter
D
volume of spared tissue (mm³)
Cavity volume (mm³)
Total volume (mm³)
E
VE 0dpo
MSC 0dpo
OECs 0dpo
2mm rostral
F
Spared tissue (mm²)
mm respect to epicenter
VE 7dpo
MSC 7dpo
OEC 7dpo
G
Cavity area (mm²)
mm respect to epicenter
H
Area of section (mm²)
mm respect to epicenter
I
volume of spared tissue (mm³)
Cavity volume (mm³)
Total volume (mm³)
J
VE 7dpo
MSC 7dpo
OEC 7dpo
1mm rostral
K
% of Spared tissue
mm respect to epicenter
VE 0dpo
MSC 0dpo
OEC 0dpo
% of Spared tissue
mm respect to epicenter
VE 7dpo
MSC 7dpo
OEC 7dpo

Fig. 6. Histological analysis. The analysis was divided according to acute (A-E) and delayed (F-J) transplant after SCI. The measures of spared tissue (A and F), cavity (B and G) and total section (C and H) areas were made on hematoxylin-eosin stained transversal sections (E and J) of the injured spinal cord segment. The volumes of the three measures were also calculated (D and I). The spared tissue area was also represented as the percentage with respect to the total section area for both acute (K, left panel) and delayed (K, right panel) transplants (K). * $p<0.05$ group MSC vs. group VE; # $p< 0.05$ group OEC vs. group VE.

due to the increased size of the central cavity (Fig. 6H-J).

Because differences in the spinal cord segment size may induce wrong interpretation of the measurements, the amount of spared tissue was normalized with respect to the total section area and represented in percentage (Fig. 6K). In acute transplantations, MSC preserved more percentage of tissue than the control group in both rostral and caudal regions between 1 and 2 mm from the epicenter. The OEC treated rats showed more percentage of spared tissue but significantly only around 1mm caudal to the epicenter. The same analysis for sub-acute transplants resulted in higher percentage of spared tissue in both cell treated groups than in the control, with significant differences in rostral and caudal segments between 1 and 2 mm from the epicenter.

Discussion

Cell therapy for SCI is a widely debatable issue. Whereas some experimental studies have shown therapeutic effects, other reported no significant improvement and even the appearance of undesirable secondary effects (1-4). Several factors can influence the potential therapeutic benefit of cell grafts following a SCI, such as the nature of the cells, age and donor variation, injury type and severity, cell culture methods, cell delivery methods, and the specific techniques used to evaluate the outcomes, thus making it complicated the comparison of published works from different laboratories. In this context, we aimed to compare the effects of MSC and OEC transplantation, two of the most promising cell types for SCI repair, in order to determine the best option in moderate spinal cord contusion injuries. The results of this study show a similar amount of tissue protection provided by grafted MSC or OEC, both in acute and subacute treatments. Unfortunately, the increased tissue preservation was not accompanied by significant improvement in functional outcomes in the animals treated with OEC. The engraftment with MSC induced higher hyperreflexia, but only in the acute transplant the animals achieved higher speed treadmill running and recovery in MEPs. Furthermore, the grafted cell survival study revealed a fast reduction of both MSC and OEC within the injured spinal cord.

Thoracic spinal cord injury by a moderate contusion leads to a drastic destruction of the spinal cord that compromises practically all the parenchyma at the epicenter and extends tissue damage along several spinal cord segments. This affectation prevents the transmission of the motor descending information, as shown by the abolition of MEPs, resulting in marked locomotor deficits, characterized by reduced score in the BBB scale that measures the overground walking skills of the rats (36), decrease in the speed running on a treadmill and uncoordinated locomotor pattern. The partial protection of spinal cord tissue by MSC or OEC transplantation after the injury was not reflected in improved recovery of both overground locomotion nor gait parameters and coordination. Only an improvement in the running speed was observed in the acute MSC transplanted rats. The slight enhancement of motor performance provided by the acute MSC graft may be related to the recovery of responses in MEPs observed in these animals. Nevertheless, this recovery of MEPs was low in comparison to intact animals, and the BBB score achieved by the acute MSC treated group was similar to the other groups. A 200kdyn contusion injury destroys the ventral and lateral funiculi of the white matter (44). The ventrolateral

white matter contains descending pathways important for the control of hindlimb motor function, as the reticulospinal tract (45) whose preservation is critical for locomotion and MEP responses (44,46,47). In our study, MSC or OEC transplantation was not successful regarding cord tissue preservation at the center of the injury. Consequently, the ventrolateral white matter remained severely affected compromising the gross locomotion performance. On the other hand, SCI induce an amplification of the spinal reflex activity in segments caudal to the lesion. This hyperreflexia is consequence basically of the loss of inhibitory descending pathways (48) and changes in the spinal neurons excitability (49). The transplantation of MSC, independently of the time of injection, induced higher hyperreflexia than in OEC transplanted and in control injured rats. The increase in spinal reflexes may contribute to the recovery of locomotion after SCI (50). In fact, functional recovery after incomplete SCI has been related with the increase of spinal H reflex responses (51). Under treadmill condition, the activation of afferent inputs enhances the locomotion movements in spinalized animals (52). Thus, the exacerbated hyperreflexia may increase the sensitivity of spinal circuitries to afferent inputs leading to better locomotion recovery (51,53). The increased hyperreflexia in the acute MSC grafted rats together with the partial recovery of MEPs may explain the improvement of these animals on the treadmill testing. Regarding the sensory perception, no differences between treatments were found in the algesimetry tests. Although the cell grafts did not revert the increased hyperalgesia after injury, they neither increased hypersensitivity to mechanical and thermal stimuli with respect to control injured rats, a detrimental effect observed with other cell types such as neural stem cells (54).

The histological results show that the transplantation of MSC or OEC after SCI slightly protected against tissue damage. There were no significant differences between acute and subacute cell transplants. Tissue sparing induced by the cell transplant was in both distal and rostral areas from the epicenter. Nevertheless, at the lesion epicenter the cord tissue was similarly disrupted in all the SCI groups. Thus, the number of engrafted cells into the epicenter seemed insufficient to counteract the damage events. Some studies indicated that MSC (10,13,15,55) and OEC (22,23) transplants exert tissue protection after SCI, but others did not find this effect (56-58). The different types of lesion induced, as well as methodological variations in measuring the amount of spared tissue may contribute to these discrepancies. In our case, for example, if we focused only in the absolute amount of spared tissue there were no differences between the subacute treatments, despite the higher proportion of preserved tissue with respect to the total area of the spinal cord with cell transplants.

Localization of the transplanted cells indicated similar amounts of MSC and OEC during the first week after injection, but a marked decrease in the GFP signal measured the two following weeks. As previously described, the survival of transplanted cells within the spinal cord is limited independently of cell type, which indicates that the spinal cord environment after contusion is hostile to the grafted cells (13,14,59,60). Although immunoprivileged properties of MSC were described (61), reduced survival of engrafted MSC from rats (13) and human donors (8) has been previously reported. Similarly, the limited survival of the OEC transplant in contusion injury was also described (59,60). Furthermore, our data show no differences in time-survival between MSC and OEC, neither in acute nor in subacute engraftment. Therefore, although the survival of the grafted cells could be important for the treatment success, this factor does not explain the differences found between MSC and OEC transplants.

MSC vs. OEC for spinal cord contusion injuries

Our findings demonstrate the capacity of both MSC and OEC graft to provide beneficial effects

after spinal cord contusion. While a number of works previously described tissue sparing and functional recovery with MSC transplantation after contusion injuries (5-8,10), the effects are not robust after OEC treatment in the contusion model. Several studies of acute and delayed OEC transplantation after contusion injury showed no effects on locomotion recovery (50,56,57,59,62). In contrast, after complete transection injuries the effects of OEC on axonal growth and functional improvement have been more consistent (19,20,24,25,30). Contusion is the most complex and severe type of lesion to the spinal cord, and generally results in the formation of large cystic cavities, extensive hemorrhage and secondary loss of tissue. Furthermore, the hostile environment after contusion seems detrimental for the graft survival. However, spinal cord contusion is clinically the most relevant injury model, since it mimics the neuropathology of most human SCI (63). The easier procurement and expansion of the MSC, in addition to the better functional effects found in the MSC grafted groups, make them more feasible for cell therapy in spinal cord contusion than OEC.

On the other hand, the post-injury time of intervention is an important point to consider for cell therapy. The earliest times seem to be better to prevent damage and protect the tissue (24,30). However, the detrimental ambience during the first days of injury could reduce the efficacy of the transplant. Moreover, the clinical application of acute cell transplants may be complicated. Some works have reported better results with delayed than with early cell transplantation (6,24), but another described more positive effects in acute than in delayed treatment (58) in accordance to our present study for the MSC grafts. Again, the controversial results may depend on the different experimental conditions. Further investigations to compare the type of cells regarding mechanisms of action, the time treatment window, the number of cells injected and the possible combined treatments are still necessary for determining the best cell therapy strategy for SCI.

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RESULTS: CHAPTER 3

Mesenchymal stem cells or olfactory ensheathing cells transplantation after spinal cord injury: gene expression changes

Gene expression changes in the injured spinal cord following transplantation of mesenchymal stem cells or olfactory ensheathing cells

Abel Torres-Espín, Joaquim Hernandez, Xavier Navarro

Group of Neuroplasticity and Regeneration, Institute of Neurosciences, Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Bellaterra, Spain.

Corresponding author: Xavier Navarro, Unitat de Fisiologia Mèdica, Edif. M, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain. Tel: +34-935811966, Fax: +34-935812986, E-mail: xavier.navarro@uab.cat

Abstract

Transplantation of bone marrow derived mesenchymal stromal cells (MSC) or olfactory ensheathing cells (OEC) have demonstrated beneficial effects after spinal cord injury (SCI), providing tissue protection and improving the functional recovery. However, the changes induced by these cells after their transplantation into the injured spinal cord remain largely unknown. We analyzed the changes in the spinal cord transcriptome after a contusion injury and MSC or OEC transplantation. The cells were injected immediately or 7 days after the injury. The mRNA of the spinal cord injured segment was extracted and analyzed by microarray at 2 and 7 days after cell grafting. The gene profiles were analyzed by clustering and functional enrichment analysis based on the Gene Ontology database. We found that both MSC and OEC transplanted acutely after injury induce an early up-regulation of genes related with tissue protection and regeneration. In contrast, cells transplanted at 7 days after injury down-regulate genes related with tissue regeneration. The most important changes after MSC or OEC transplant were a marked increase in expression of genes related with foreign body response and adaptive immune response. These data suggest a regulatory effect of MSC or OEC transplantation after SCI regarding tissue repair processes, but a fast rejection response to the graft cells. Our results provide an initial step to determine the mechanisms of action and to optimize cell therapy for SCI.

Key words: spinal cord injury, cell therapy, mesenchymal stem cells, olfactory ensheacing cells, microarray

Introduction

Spinal cord injury (SCI) leads to partial or complete loss of motor, sensory and autonomic functions and secondary impairments below the injury level, due to damage on the local circuitry of the spinal cord and the interruption of ascending and descending neural pathways. SCI results in a sequence of coordinated changes in gene and protein expression profile associated with physiopathological events including hemorrhage, inflammatory and immune activation, excitotoxicity, oxidative stress, and neuronal activity imbalances (Aimone et al., 2004; Afjehi-Sadat et al., 2010; Carmel et al., 2001; De Biase et al., 2005; Di Giovanni at al., 2003).

Cell therapy has become a promising approach for repairing the injured spinal cord (Coutts and Keirstead, 2008; Hernández et al., 2011; Mothe

and Tator, 2012; Sahni and Kessler, 2010; Tetzlaff et al., 2011). Pre-clinical studies have demonstrated that transplantation of mesenchymal stromal cells (MSC) (Chopp et al., 2000; Himes et al., 2006; Hofstetter et al., 2002; Neuhuber et al., 2005; Quertainmont et al., 2012) or olfactory ensheathing cells (OEC) (García-Alías et al., 2004; Li et al., 1997; López-Vales et al., 2006; Lu et al., 2002; Ramón-Cueto et al., 2000; Verdú et al., 2003) reduces tissue damage and improves functional performance in different paradigms of SCI, although other studies failed to replicate such beneficial results (Barakat et al., 2005; Collazos-Castro et al., 2005; Pearse et al., 2007; Steward et al., 2006; Takami et al., 2002). Little is known about the mechanisms underlying the benefits after cells grafting into the injured spinal cord. Regarding the MSC it has been suggested that their effects are due to their capability to secrete and/or induce the expression of protective molecules such as BDNF and GDNF (Himes et al., 2006; Quertainmont et al., 2012); to modulate inflammation (Abrams et al., 2009; Nakajima et al., 2012) and to generate a more permissive environment for axonal regeneration and neural tissue reconstruction (Ankeny et al., 2004; Himes et al., 2006; Hofstetter et al., 2002; Koda et al., 2007). The beneficial actions of OECs include the ability of these cells to modulate and interact with reactive astrocytes (Chuah et al., 2011; Franssen et al., 2007), to induce neoangiogenesis (López-Vales et al., 2004; Richter et al., 2005), to remyelinate naked axons (Imaizumi et al., 1998), to modulate the immune response (Chuah et al., 2011; López-Vales et al., 2004; Richter et al., 2005) and to promote axonal regeneration (Li et al., 1998; López-Vales et al., 2006, 2007; Stamegna et al., 2011).

Despite a number of studies have investigated the changes in gene expression profile after different types of SCI in laboratory animals, no studies have focused on the analysis of gene expression changes triggered by transplanted cells in the lesioned spinal cord. Such information may be of importance to better understand the cellular and molecular mechanisms modulated by the transplanted cells. In the present work, we analysed for the first time the gene expression profiles of the spinal cord that received an acute or 7 days delayed graft of MSC or OEC following a contusion injury. Our results confirm that SCI causes several changes in gene transcription, and the injection of cells significantly modified some pathways affected after injury. Transplantation of both MSC and OEC leads to over expression of genes involved in tissue repair during the acute phase of injury, and the decline during subacute time. Our results further indicate how these cells contribute to regulate the wound repair response after SCI, and could explain the beneficial effects provided by the transplantation. On the other hand, a large number of genes associated to the immune response were also found up-regulated, indicative of cell rejection.

Material and methods

Primary cell cultures

Primary cultures of MSC and OEC were set up from P22 male Sprague-Dawley rats. The animals were euthanized with CO2.

MSC culture and characterization. Tibias and femurs were placed on cool phosphate buffered saline (PBS) and epiphyses were removed. The diaphyses of bones were flushed with PBS using a syringe and the marrow was homogenized. The extract was filtered through a 70μm nylon mesh and recovered by centrifugation for 10min at 1500rpm. The pellet was resuspended in growth medium: α-MEM with L-glutamine (Life Technologies, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Lonza, Verviers, Belgium), 2mM L-glutamine (Life Technologies) and 100 units/ml penicillin-streptomycin (Life Technologies, 100x); and plated in 100mm culture dishes (Iwaki, Asahi Technoglass, Chiba, Japan) at a density of

5•106 cells¬/cm2. After 24h, the supernatant containing non-adherent cells was removed and fresh medium was added. When the culture was near confluence, every 4-5 days, the cells were detached using PBS with 0.05% trypsin (Life Technologies) and 0.04% EDTA (Sigma, St. Louis, MO) and re-plated at 5,000 cells/cm2. Cells were passaged 3-4 times and expanded to 80-90% of confluence. The cultured MSC were characterized by their expression of CD90 and CD29 but not of CD11b and CD45 surface markers, and their differentiation capability to adipocytes and osteoblasts using methods previously described (Harting et al., 2008). For analysis of adipogenesis, cells were fixed for 20 min with 4% paraformaldehyde in phosphate buffer (PB); the adipocytes were labeled using 60% Oil red O stock solution (0.5% Oil red O in isopropanol, Sigma) for 15 min and washed with distilled water. For osteocytes labeling, cells were fixed using 70% ethanol pre-cooled for 1h at 4ºC, washed and incubated during 30 min with 0.1mg/ml Alizarin red solution (Sigma) in distilled water.

OEC culture and characterization. Olfactory bulbs were aseptically removed and stored in cold Hank's balanced salt solution (HBBS) with calcium and magnesium. The meningeal layer was stripped off with a fine forceps, and tissue was enzymatically (trypsin 0.25%, collagenase A 1mg/ml, and DNAsa I 1mg/ml) and mechanically dissociated. The cells were recovered by centrifugation in Dulbecco's minimum essential medium nutrient mixture F-12 Ham (DMEM) and seeded onto 25 cm2 flasks coated with poly-L-lisine and incubated in 5% CO2 at 37ºC. Culture medium was DMEM supplemented with 10% FBS (Life Technologies). Cells were kept in culture at least for 7 days. For purification, the cells were incubated with mouse anti-p75NGFR antibody (1:100, Chemicon, MAB365), then immunopurified with goat anti-mouse IgG microbeads (Miltenyi Biotec) using the MACS separation (Miltenyi Biotec). OEC purity was at least 75%. For immunocytochemistry OEC cultures were fixed with 4% paraformaldehyde in PBS for 30 minutes, then incubated for 24h with primary antibodies mouse anti-p75NGFR and anti-S100 (1:200, DiaSorin), and after washing incubated 1h with secondary antibodies Cy3-conjugated donkey anti-mouse (1:200, Jackson IR) or Alexa 488 donkey anti-mouse (1:200, Invitrogen).

Spinal cord injury and cell transplantation

Adult Sprague-Dawley female rats (9 weeks old; 250-300g) were used. The animals were housed with free access to food and water at room temperature of 22 ± 2ºC. The experimental procedures were approved by the ethical committee of the Universitat Autònoma de Barcelona in accordance with the European Directive 86/609/EEC.

Under anesthesia with ketamine (90mg/kg) and xylacine (10mg/kg) and aseptic conditions, a longitudinal dorsal incision was made to expose T6-T10 spinous processes. After laminectomy of T8-T9 vertebra, the spinal cord was subjected to a contusion of 200Kdyns using the Infinite Horizon Impactor (Precision System and Instrumentation, Kentucky, USA). The animals were divided in 6 groups. Three groups of rats were transplanted acutely, 30 min after operation (0dpo), with vehicle (VE-0dpo, n=8), with MSC (MSC-0dpo, n=8) or with OEC (OEC-0dpo, n=8). Other three groups of rats were transplanted at 7 days postoperation with vehicle (VE-7dpo, n=8), MSC (MSC-7dpo, n=8) or OEC (OEC-7dpo, n=8). The cells for transplantation were suspended in L15 medium (Life Technologies) at 50,000 cells/µl and maintained in ice during the time of surgery. Using a glass needle (100µm internal diameter, Eppendorf, Hamburg, Germany) coupled to a 10µl Hamilton syringe (Hamilton #701, Hamilton Co, Reno, NV, USA), 3µl of the corresponding cell suspension or vehicle (L15) were intraspinally injected at the epicenter and at 2mm rostrally and caudally, for a total of 450,000 cells per rat. A

perfusion speed of 2µl/min was controlled by an automatic injector (KDS 310 Plus, Kd Scientific, Holliston, MA, USA), and the tip was maintained inside the tissue 3min after each injection to avoid liquid reflux. The wound was sutured and the animals allowed recovering in a warm environment. Bladders were expressed twice a day until reflex voiding of the bladder was re-established. To prevent infection, amoxicillin (500 mg/l) was given in the drinking water for one week.

Sample preparation and mRNA extraction

The rats of each experimental group were sacrificed randomly at 2 (n=4) or 7 (n=4) days post cell injection (dpi) by decapitation after deep anesthesia, and a spinal cord segment (5mm long) centered in the contusion epicenter was harvested and maintained in RNA-latter solution (Qiagen, Barcelona, Spain). A spinal cord segment of intact animals (n=4) was also obtained. The samples were processed for mRNA analysis following the manufacturer instructions. The total RNA of each sample was extracted with RNeasy mini kit (Qiagen), including a DNase step (RNase free DNase set, Qiagen, Barcelona, Spain).

Microarray

The microarray hybridization and the statistical processing of raw data were performed by specialized service (Scientific and Technical Support Unit and Statistics and Bioinformatics Unit, Vall d'Hebron Research Institute). For the gene expression an Affymetrix RAT Exon/Gene 1.1 ST chip array was used according to the manufacturer protocol.

Data analysis. The images of hybridized microarrays were processed with the Expression Console software (Affymetrix). Raw expression values obtained directly from .CEL files were pre-processed using the RMA method (Irizarry et al., 2003), a three-step process that integrates background correction, normalization and filtering of probes values. Data were first submitted to non-specific filtering to remove low signal genes (those genes whose mean signal in each group did not exceed a minimum threshold) and low variability genes (those genes whose standard deviation between all samples did not exceed a minimum threshold). The selection of differentially expressed genes between conditions was based on a linear model analysis with empirical Bayes moderation of the variance estimated following the methodology developed by Smyth (Smyth, 2004) and implemented in the limma Bioconductor package. To determine the main effects of the injury, each gene expression profile of injured groups was compared to non injured animals and a cut-off P value <0.05 and fold change (FC) >1.5 were applied to select the differentially expressed genes. To determine the gene expression changes after cell transplantation, each gene expression profile of MSC or OEC groups was compared to the correspondent vehicle group. In this case, the cut-off for differentially expressed genes was a P value <0.05 and a FC >1.3.

All the statistical analysis were done using the free statistical language R and the libraries developed for microarray data analysis by the Bioconductor Project (www.bioconductor.org).

Microarray validation

To validate the results obtained by microarrays analysis, in silico comparison of the SCI differential expressed genes lists and RT-PCR of target genes were performed.

In silico validation. Three different data profiles were selected in Gene Expression omnibus database (GEO, National Centre of Biotechnology Information): GSE464 (spinal cord injury contusion and regeneration time course in rats: above T9 (RG-U34A)), GSE5296 (spinal cord injury contusion model in mice: time course), and GSE22161 (comparative gene expression analysis of thoracic spinal cord from G93A SOD1 mutant rats and from wild type littermates following mild compression injury). For the comparison of the

GEO profile with our results, each up-regulated and down-regulated list of genes were obtained from the GEO data analysis tools, filtered with our microarray profile to eliminate the probes not assessed in our chip, and compared with correspondent up-regulated and down-regulated lists that we obtained from our samples. The percentages of concordant and discordant genes with respect to the total changed genes in the GEO data up-regulated and down-regulated profile were calculated. Thus, if one gene appeared up-regulated at the same time in the GSE464 and in our results this gene contributed to the percentage of concordance. Genes that were not coincident in the compared list contributed to the percentage of discordance, in which we distinguished the genes that changed in the GEO profile but not in our data and the genes that changed in opposite direction than our results.

RT-PCR. 1 µg RNA of each sample was reverse-transcribed using 10 µmol/L DTT, 200 U M-MuLV reverse transcriptase (New England BioLabs, Barcelona, Spain), 10 U RNase Out Ribonuclease Inhibitor (Invitrogen), 1µmol/L oligo(dT) and 1 µmol/L of random hexamers (BioLabs, Beverly, MA, USA). The reverse transcription cycle conditions were 25°C for 10 min, 42°C for 1 h and 72°C for 10 min. We analyzed the mRNA expression by means of specific primer sets (supplementary data, Table S1). Glyceraldehyde 3-phosphate dehydrogenase (GADPH) expression was used to normalize the expression levels of the different genes of interest. Gene-specific mRNA analysis was performed by SYBR-green PCR using the MyiQ5 PCR detection system (Bio-Rad Laboratories, Barcelona, Spain). We previously fixed the optimal concentration of the cDNA to be used as template for each gene analysis to obtain reliable CT (threshold cycle) values for quantification. Four samples were used per condition and each one was run in duplicate. The thermal cycling conditions comprised 3 min polymerase activation at 95°C, 40 cycles of 10 s at 95°C for denaturation and 30 s at 62°C for annealing and extension, followed by a DNA melting curve for determination of amplification specificity. CT values were obtained and analyzed using BioRad Software. Fold change in gene expression was estimated using the CT comparative method normalizing to GADPH CT values and relative between pairs of samples.

Analysis of biological meaning

To investigate the biological meaning, term enrichment analysis in the Gene Ontology (GO, http://www.geneontology.org/) and functional annotation GO term clustering analysis were performed (Huang et al., 2009). The Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.7) (National Institute of Allergy and Infectious Diseases (NIAID); http://david.abcc.ncifcrf.gov/home.jsp) was used for DAVID's GO biological process FAT (GOTERM_BP_FAT). The GO terms were classified by functional annotation clustering analysis, where the list of selected genes (the sample) were compared to a reference set (the whole probes in the Affymetrix chip used) with the following options: similarity term overlap=3; similarity threshold=0.5; initial group membership=2; final group membership=2; multiple linkage threshold=0.05; EASE=0.05. The functional annotation clusters were ranked from largest to smallest enrichment score (ES) and the GO terms associated to every cluster were ranked from smallest to largest P value. Moreover, every cluster was labeled with a representative name of the GO terms included in the cluster. Besides the functional classification of the GO terms, to determine which of these annotation clusters were the most important regarding the amount of changes, two parameters were defined: the number of GO terms included in every group, and the G>30, defined as the number of the up and down-top 30 differential expressed genes present in every cluster. The G>30 allows to know which of the most changed genes are associated with others, therefore giving

an overview of the most changed genes associated with the altered process by the treatments. A summery of the amount of up and down regulated genes, the number of over represented GO terms associated with these genes and the quantity of GO functional clusters are included in the supplementary data. The whole information of the functional annotation cluster classification, with the GO terms included in every cluster (and no clustered GO terms, NC), the number of genes in every enriched GO term (G) and the p value of the clustering, is also presented in the supplementary data (Tables S3-S17), whereas Tables 1, 2, 3 and 5 show a summary of these clusters. Moreover, the symbol of the G>30 clustered genes, their logFC and the correspondent clusters are included in the Figures 2-5.

Results

Transcriptional profile after spinal cord injury and microarry validation

About 5000 genes significantly changed their expression after spinal cord contusion compared to non-injured cord. Between 2000 to 2500 genes were up-regulated and down-regulated for each time point after injury. These results confirm that SCI induces considerable changes in the gene transcription profile (Fig. 1A-D).

Microarray data was validated by both in silico and RT-PCR quantitative analyses. For the in silico comparison, the transcriptional profile of the SCI samples were contrasted with three microarrays data published in the GEO database: GSE464, GSE5296 and GSE22161 (Fig. 1E). After filtering genes that were differentially expressed, we found that the genes that were up-regulated and down-regulated in our experiments were very similar to those found in a dataset obtained from rat spinal cord following a mild contusion injury (GSE464 profile) (Fig. 1E). Indeed, the matching of genes up-regulated and down-regulated was 88.5% and 79.1%, respectively at day 2, and 90.8% and 75.3%, respectively, at day 7 post-injury. Our results were also analogous to those obtained after mild spinal cord contusion in mice (GSE5296 profile) since the matching of genes that were up-regulated and down-regulated at day 7 post-injury was 79.4% and 84.1%, respectively. Interestingly, we observed that less than 50% of the genes (37.6% and 45% for up- and down-regulated genes, respectively) coincided with the gene expression profile obtained 7 days after spinal cord compression injury (GSE22161 profile). Therefore, these results reveal that the transcriptome changes that occur in the spinal cord largely depend on the type of the primary lesion.

To validate the gene expression changes observed in the rat spinal cord after contusion and cell treatment, we analyzed by RT-PCR 9 targeted genes identified in the microarray. The expression changes of these 9 genes were compared to their expression changes in the microarray for different comparison and time in a total of 102 probes (Fig. 1F). The results revealed a high correlation in the expression changes of these 9 genes comparing PCR and microarray analysis results (Pearson's $r = 0.87$, $p<0.0001$; supplementary data), and thus, evidenced the reliability of the our microarray data.

Biological changes after MSC cell transplantation

Acute transplant of MSC. Functional annotation clustering analysis indicated that acute transplantation of MSC induced changes in several physiopathological responses of the spinal cord (Table 1, Fig. 2; see also Tables S3 and S4). At day 2 post-grafting, the acute MSC graft induced the expression of genes related to tissue morphogenesis and development, extracellular matrix (ECM) reorganization and response to nutrient levels, and to a lesser degree the up-regulation of genes related to cell proliferation and adhesion, angiogenesis, wound healing and inflammation, vasoconstriction, and response to hypoxia (Table 1 and Table S3). Genes present in the up-top30 list (Fig. 2A) include the enhanced

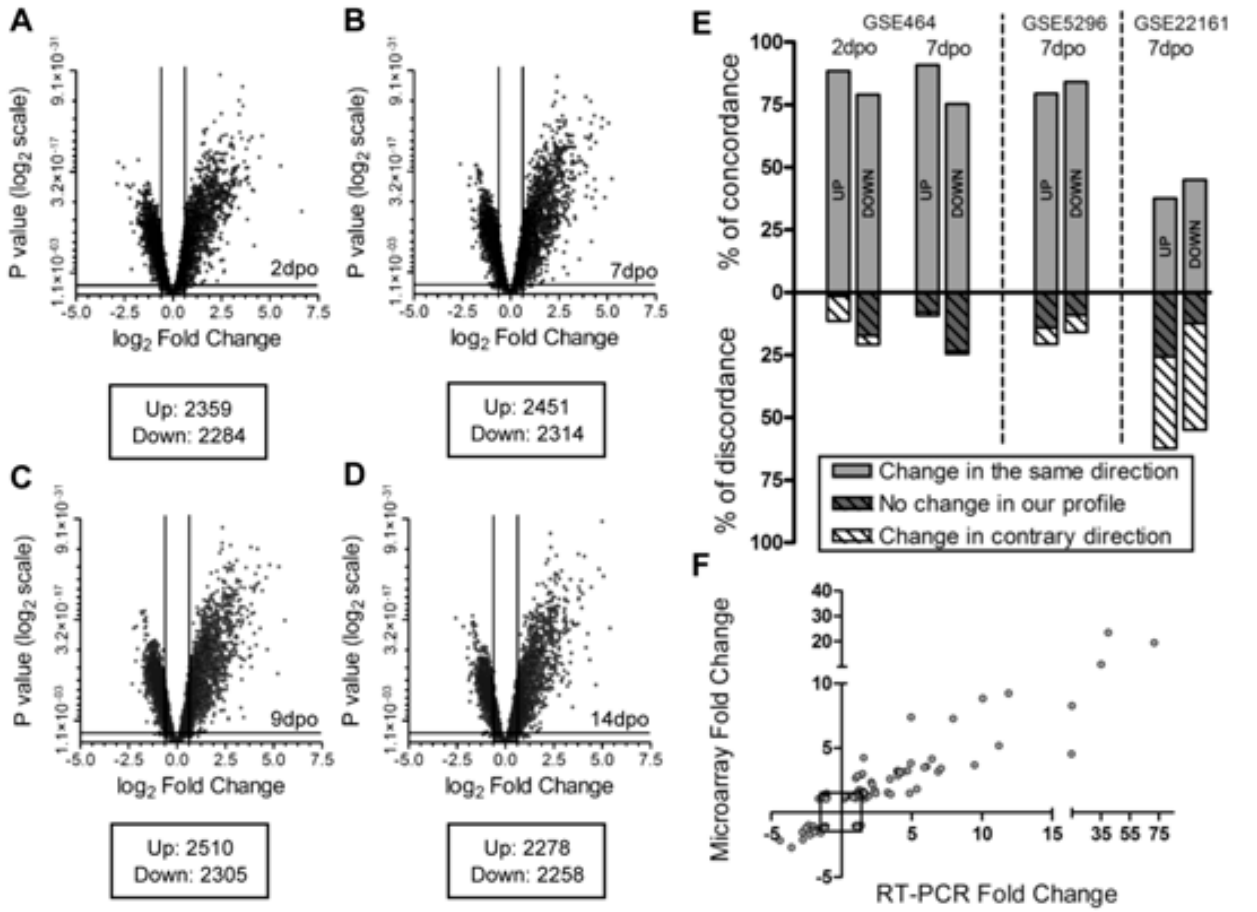


Fig 1. Microarray data validation. Spinal cord injury by contusion induced several gene changes with respect to the non injured spinal cord. More than 2000 probes were found up-regulated and similar number down-regulated at 2 (A), 7 (B), 9 (C) and 14 (D) days after injury. The *in silico* comparison of our gene array results showed a high concordance of up-regulated genes and down-regulated genes with other previously published (E) after the same type of injury in rats (GSE464) and in mice (GSE5296), but not with other type of SCI in rats (GSE22161). The validation of some target genes by RT-PCR indicated a good concordance between expression changes obtained by microarray and by RT-PCR (F). In this comparison a few discrepancies were observed but only in target genes that have not achieved the cut-off of significant changes (square in F).

transcription of Col1a1 and Col1a2, that codify for pro-collagen type I, Col3a1 (pro-collagen type III) and the Mmp13 (collagenase III). Other genes associated to the ECM, such as Postn (periostation) and Fgf7 (fibroblast growth factor 7 or keratinocyte growth factor) were found strongly up-regulated. Moreover, some genes related to inflammation such as Mmp12 (macrophage elastasa), Ccr2 (C-C chemokine receptor type 2), a receptor of for chemokine Ccl2 (chemokine ligand 2) involved in monocyte chemotaxis, the chemokine Ccl7, and Ptgs2 (prostaglandin-endoperoxide synthase 2 or Cox-2), involved in the prostaglandin synthesis were also found in the up-top 30 list (Fig. 2A), suggesting that acute MSC transplant was triggering greater wound and inflammatory response (Table 1 and Table S3, cluster 7). In addition, another interesting gene that was found up-regulated was Wnt5a (wingless-type MMTV integration site family, member 5A), which has a role in cell proliferation; and Enpp1 (ectonucleotide pyrophosphatase/phosphodiesterase 1), which interacts with insulin receptor.

On the other hand, the acute MSC transplant reduced gene expression at day 2 following grafting, especially of genes involved in

catecholamine secretion (Table 1 and S4). However, we observed that the only clustered gene of the down top 30 list (Fig. 2A) was Cidea (cell death-inducing DFFA-like effector a), an activator of apoptosis, that together with some cell death non-clustered associated genes found on the top down 30 list (data not shown), suggest that MSC may protect against cell death.

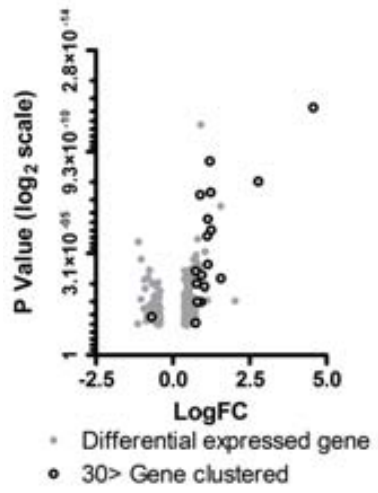
One week after acute treatment with MSC, the largest class of genes induced by the cell grafting was related to adaptive immune response, antigen processing and leukocyte migration (Table 1 and S5, cluster 2 and 3). Those genes include the chemokines CCL9, CXCL13 (chemokine, C-X-C motif, ligand 13), the chemokine receptors CCR2, the protein CD4 (Cluster of differentiation 4) present in the surface of lymphocytes T helper, monocytes and dendritic cells, and the genes RT1 class Ib and RT1 class II, two epitopes of the major histocompatibility complex (MHC) I and II, respectively (Fig. 2B). On the other hand, the acute MSC graft reduced the biological processes related to ion and cell homeostasis, and regulation of neurotransmission at this time point (Table 1 and S6, cluster 1, 3 and 5). Among them the genes Agtr1a (angiotensin II receptor, type 1a), which has a role in blood pressure and sodium homeostasis regulation; Hspa1l (heat shock 70 kDa protein 1L), a regulator of cell stress, and Cgrp (Calcitonin-related polypeptide α), which participates in regulating neurological vascular activities. Genes related to neurotransmission that are down-regulated by the acute MSC graft are Gabra5 (GABA A receptor, α 5), Scn2b (sodium channel subuni β-2) and Slc6a11 (solute carrier family 6, member 11) (Fig. 2B).

Delayed transplant of MSC. In contrast to acute MSC graft, the main changes observed at day 2 following a delayed MSC graft in the contused spinal cord were found in overrepresented GO terms of genes down-regulated. The biological processes for genes down-regulated were associated to tissue morphogenesis and organ development, response to nutrient levels, neuron development, ECM organization, response to hypoxia, cell proliferation, migration and adhesion, response to wound and inflammatory response and lipid biosynthesis (Table 2 and S7). The most important down-

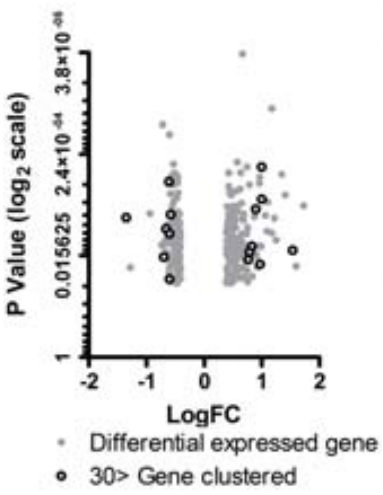
Table 1. Fucntional annotation clusters summary: Acute MSC vs VHC

2dpo			
UP genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Cell adhesion	4,13	4	2
2. ECM and collagen organization	3,47	6	15
3. Face and head development	3,43	2	2
4. Blood vessel development	2,57	5	5
5. Response to vitamin and nutrient	2,52	7	14
6. Cell proliferation and regeneration	2,3	4	5
7. Response to wounding and inflammatory process	2,06	5	4
8. Response to hypoxia	1,89	3	2
9. Prostaglandin biosynthesis	1,87	1	6
10. Tissue morphogenesis and organ development	1,8	9	23
11. Vasoconstriction	1,52	1	2
NC	-	-	7
		Total	**87**
DOWN genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Regulation of catecholamine secretion	2,04	1	9
NC	-	-	2
		Total	**11**

7dpo			
UP genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Wound healing and coagulation	3,39	1	5
2. Adaptive immune respone and antigen processing	3,19	7	36
3. Leukocyte migration	1,73	2	7
4. Response to LPS	1,63	0	3
NC	-	-	6
		Total	**57**
DOWN genes Functional clustering	**ES**	**30> G**	**nº GO**
1. Regulation of neurotransmission	3,67	2	6
2. GABA signaling and anion transport	3,61	1	4
3. Sodium and potassium ion transport	3,47	2	6
4. Locomotor behavior	3,04	3	5
5. Ion homeostasis	2,07	4	14
6. Secretion	1,51	1	2
NC	-	-	3
		Total	**40**



	Gene	logFC	P.Value	Cluster n°
UP	Mmp13	4,59	1,1E-11	2-5-8-10
	Postn	2,80	2,1E-08	1-2
	Mmp12	1,59	4,2E-04	6-10
	Col1a1	1,27	3,0E-06	2-3-4-5-10
	Enpp1	1,25	6,4E-08	4-5
	Wnt5a	1,22	2,6E-09	5-10
	Ptgs2	1,16	1,0E-04	6-7-9-11
	Col1a2	1,16	9,7E-07	2-4-6-10
	Plod2	1,14	5,5E-06	8
	Col6a3	1,05	9,3E-04	5
	Aldh1a3	0,96	3,0E-04	3-10
	Ccr2	0,94	4,6E-03	4-5-7-8-10
	Wisp1	0,91	8,2E-08	1
	Msln	0,82	4,6E-03	1
	Col3a1	0,80	7,2E-04	1-2-4-5-6-7-10
	Fgf7	0,77	2,0E-04	2-6-7-10
	Ccl7	0,76	4,0E-02	7
DOWN	Cidea	-0,67	2,1E-02	1



	Gene	logFC	P.Value	Cluster n°
UP	Cxcl13	1,55	1,3E-02	2
	Cd4	1,01	4,3E-04	2
	Il8ra	1,01	1,6E-03	3
	RT1-Da	0,98	2,3E-02	2
	Ccl9	0,91	2,4E-03	2
	Pf4	0,84	1,1E-02	1-2-3
	RT1-EC2	0,80	1,4E-02	2
	Ccr2	0,78	1,9E-02	2
DOWN	S100a9	-1,34	3,4E-03	4
	Agtr1a	-0,69	1,7E-02	4-5-6
	Gabra5	-0,65	5,4E-03	1-2-3
	Hspa1l	-0,60	7,9E-04	5
	Scn2b	-0,60	7,8E-04	3
	Calca	-0,59	6,6E-03	4-5
	Cd55	-0,59	4,2E-02	5
	Slc6a11	-0,57	3,0E-03	1

Fig 2. G>30 clustered genes after acute MSC transplantation. The volcano plots represent the graphical distribution of the G>30 genes that were present in one or more functional clusters of the biological mining analysis over the total of differential expressed genes. The G>30 clustered genes, their fold change, the p value and the cluster number (see table 1) are shown for samples obtained 2 days (A) and 7 days (B) after SCI and acute MSC transplantation.

regulated gene was Pla2g2a (secreted Phospholipase A2 group IIA) involved in phospholipid degradation. Other 2 genes related to lipid homeostasis, Lpl (lipoprotein lipase), involved in lipoproteins uptake and Ptgis (Prostaglandin-I synthase), involved in the synthesis of the cholesterol and other lipids, were found within the top 30 down-regulated genes (Fig. 3A). Moreover, Cdh1 (Cadherin-1), which encode for a calcium-dependent cell-cell adhesion glycoprotein involved in cell adhesion and ECM related process; Wisp2 (WNT1-inducible-signaling pathway protein 2) and Upk1b (Uroplakin 1B), which are involved in cell development, proliferation and activation; and Agtr1a which plays a role in the regulation of blood pressure and sodium homeostasis, were also down-regulated (Fig. 3A). Interestingly, some genes involved in the inflammatory response that were up-regulated at day 2 following acute MSC transplant, such as Cxcl10, Lbp (Lipopoly-saccharide-binding protein), and Itgax (CD11c), were down-regulated in the contused spinal cords following delayed MSC graft (Fig. 3A).

The genes up-regulated at day 2 after delayed MSC transplant were largely related to the immune response (the most important event in terms of G>30), but also to the response to hypoxia, mechanical and abiotic stimulus, as well as to

locomotor behavior (Table 2 and S6). The most over-expressed gene was Lcn2 (lipocalin-2), an iron chelattor with activity during inflammation and a mediator of the innate immune response (Trude et al., 2004). Other genes related to the immune response in the top up-regulated list are Ccl2, Ccl6, expressed in neutrophils and macrophages, Nos2 (nitric oxide synthase 2 inducible), a marker for m1 macrophages phenotype, Gal (galanin), a neuropeptide with a function during inflammation and granulation of the tissue after injuries (Yamamoto et al., 2011), Lig-4 (ligase IV) and Il1rpal1 (interleukin 1 receptor accessory protein-like 1), that encode a protein member of the inteleukin 1 receptor family. In addition, the Mmp13, one of the top 30 up-regulated genes after acute transplantation of MSC, was increased after the delayed grafting (Fig. 3A).

Seven days after delayed transplantation of MSC, virtually all the increased biological processes were associated to the immune response (Table 2 and S8). Among the most up-expressed genes (Fig. 3B) we found chemokines such as Cxcl9, Cxcl3, Cxcl11, Cxcl10, Ccl2 and Ccl5, genes associated with MHC such as RT1-Da, RT1-Ba, RT1-N1, RT1-EC2, RT1-Bb, RT1-N3 and CD74, C3(Complement component 3), which play key role in the complement system, and Fcnb (Ficolin b), an activator of the complement via lectin pathways (Endo et al., 2012). Similarly to acute MSC treatment, the activation of these genes at day 7 following the delay MSC transplantation suggests that immune rejection of the grafted cells occurred.

In the down-regulated genes, most of the represented events were related to regulation of foamy cell activation, blood coagulation, inflammation and lipid related process (Table 2 and S9). Thus, the under-expression of genes (Fig. 3B) such as fabp4 (fatty acid binding protein 4) involves in the binding and transport of fatty acids, Lpl, which was also reduced at early time following delayed MSC transplant, Clec5a (C-type lectin domain family 5, member A), which has a role in immune response, and CD163, a surface protein of the M2 macrophages phenotype, might indicate that the delayed MSC transplant reduced the formation of foaming macrophages at day 7 post-grafting.

Biological changes after OEC cell transplantation

Acute OEC transplantation. The acute injection of OEC induced marked changes in the gene expression profile at day 2 postlesion. The enriched GO terms for up-regulated genes were included in 27 functional clusters. Most of the enriched cluster were related to immune response and leukocyte mediated immunity, tissue morphogenesis and organ development, cell growth and proliferation, response to organic substances and nutrient levels, angiogenesis, response to hypoxia and ECM re-modeling (Table 3 and S10). Among the genes related to immune response and leukocyte activity we observed the induction of cytokines such as Cxcl2, Cxcl9, Cxcl13, Cxcl12, and the cytokine receptor Ccr2 and Clec4d (C-type lectin domain family 4, member D), which are all associated with myeloid cells (Fig. 4A). These genes, together with others related to the adaptive immunity, such as CD8a found on cytotoxic lymphocytes and Sh2dlb (SH2 domain-containing protein 1B) (Fig. 4A), which plays a role in the transduction of antigen presenting cells, suggest that grafted OEC may burst the recruitment of immune cells within the contused spinal cord at early time points following transplantation. Moreover, the presence of other macrophage and neutrophil markers, such as Nos2 and Itgax, and ECM organization associated genes, such as Mmp13, Mmp9 and Postn (Fig. 4A), might also indicate the activation of tissue repair mechanisms.

On the other hand, the gene list over-represented in DOWN GO terms was classified in 4 functional clusters involved in endocytosis, phosphorylation, regulation of phosphorylation and

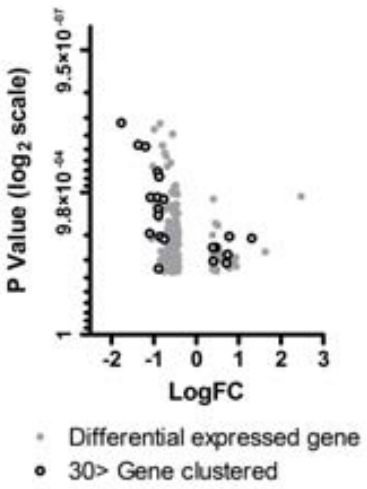
Table 2. Fucntional annotation clusters summary: Delayed MSC vs VHC

2dpi			
UP genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Defense and immune response	2,85	6	2
2. Response to mechanical and abiotic stimulus	2,12	4	2
3. Locomotory behavior	1,67	4	2
4. Response to hypoxia	1,58	3	2
NC	-	-	3
		Total	**11**
DOWN genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Negative regulation of cell communication	4,44	0	2
2. Cell proliferation	3,67	3	2
3. Response to nutrient levels	2,71	6	7
4. Cell adhesion	2,65	4	2
5. Blood vessel development	2,52	1	2
6. ECM organization	2,07	2	2
7. Tissue and organ development	2,06	6	20
8. Response to hypoxia	1,99	1	2
9. Mesenchyme development	1,76	0	5
10. Retinoic acid and vitamin A process	1,73	0	10
11. Cell migration	1,57	1	3
12. Response to wounding and inflammatory response	1,53	2	4
13. Neuron development	1,52	3	10
14. Regulation of Wnt receptor pathway	1,47	0	2
15. Lipid biosynthesis	1,39	2	2
NC			8
		Total	**83**

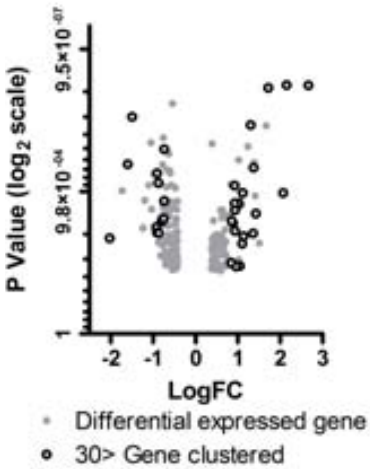
7dpi			
UP genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Defense and immune response	7,55	18	12
2. Leukocyte mediated immunity and adaptive immune response	4,14	10	16
3. Cell migration, chemotaxis and response to cytokine	3,21	6	16
4. Regulation of leukocyte activation	3,05	10	46
5. Regulation of cell migration	2,17	3	6
NC			5
		Total	**101**
DOWN genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Regulation of hormone levels	2,94	0	3
2. Lipid storage	2,88	1	4
3. Regulation of foam cell differentiation	2,67	4	4
4. Lipid catabolic process	2,58	2	2
5. Blood coagulation and inflammatory response	2,47	4	7
6. Response to retinoic acid and vitamin A	2,21	0	6
7. Response to hormone	2,19	4	3
8. Iron ion transport	1,87	1	3
9. Cell adhesion	1,73	2	2
10. Immune response and leukocyte chemotaxis	1,64	2	5
NC			3
		Total	**42**

synaptic transmission (Table 3 and S11). The only clustered gene in the down-top30 list was Nrbp2 (nuclear receptor binding protein 2), recently described as a mediator in neural progenitor cells survival (Fig. 4A).

Seven days after acute OEC transplantation, the changes in gene expression profile were modest. Nevertheless, we found 29 over-represented UP GO terms that were classified in 3 functional clusters related to response to organic substance and estrogens, response to LPS and regulation of blood vessels (Table 3 and S12). The most over-expressed transcript was Inmt (indolethylamine N-methyltransferase), which codifies for the protein that synthesizes dimethyltryptamine (DMT), an endogen ligand of Sigma-1 receptor (Mavlyutov et al., 2012) that confers protection against oxidative stress (Pal et al., 2012; Penas et al., 2011). Within the up-top30 gene list the 9 clustered genes were related to estrogen response and sexual tissue development (Fig. 4B), but some of the genes, such as Mmp13, Mmp8, Ccl5 and Bmp4 are also implicated in aspects of the tissue repair response, like tissue remodelling (GO: 0048771) and ECM organization (GO:0030198), enriched GOs but not clustered in the functional annotation analysis (Table S12). Interestingly, most of the genes related to the immune response, and that were up-regulated at day 2 following acute OEC transplant, were normalized at day 7. Only some leukocyte markers for T helper cells, such as CD4, were still up-regulated. Fewer changes were found in down-regulated list of genes and only 3 DOWN GO terms were enriched without any functional clustering (Table 3 and S13). Indeed, none of the down-top



	Gene	logFC	P.Value	Cluster n°
UP	Mmp13	1,33	9,5E-03	2-4
	Ccl2	0,80	8,7E-03	1-2-3-4
	Ccl6	0,76	2,1E-02	1-3
	Nos2	0,73	3,2E-02	1-4
	Gal	0,48	1,5E-02	1-3
	Pcdh15	0,42	2,9E-02	2-3
	Lig4	0,42	1,5E-02	1-2
	Il1rapl1	0,41	1,5E-02	1
DOWN	Pla2g2a	-1,75	3,4E-05	2-3-7
	Itgax	-1,35	1,0E-04	4
	Msln	-1,18	1,1E-04	4
	Rsad2	-1,08	7,5E-03	7
	Lpl	-1,06	1,3E-03	13-15
	Smoc2	-0,90	1,3E-03	6
	Lbp	-0,89	3,7E-04	3-12-13
	Cdh1	-0,88	2,3E-03	3-4-6-7-13
	Wisp2	-0,88	3,1E-03	2-4-7
	Cxcl10	-0,87	4,2E-02	2-3-11-12
	Ptgis	-0,86	4,7E-04	8-15
	Alpl	-0,83	8,8E-03	3
	Upk1b	-0,75	1,4E-03	7
	Agtr1a	-0,74	9,9E-03	3-5-7



	Gene	logFC	P.Value	Cluster n°
UP	Cxcl9	2,68	5,8E-06	1-5
	RT1-Da	2,17	5,7E-06	1-2-4
	Cxcl13	2,09	1,1E-03	1-4
	RT1-Ba	1,74	6,5E-06	1-2-4
	Fcnb	1,44	3,0E-03	1-2-4
	RT1-N1	1,39	3,2E-04	1-2
	Cxcl11	1,38	7,8E-03	1
	Cd74	1,32	4,0E-05	1-2-4
	Cxcl10	1,15	9,0E-03	1-3-4-5
	Ccr2	1,13	1,1E-03	1-3-4-5
	S100a9	1,12	1,3E-02	3
	Mmp13	1,06	3,8E-02	3
	RT1-EC2	1,04	1,8E-03	1-2
	RT1-Bb	0,97	3,9E-02	1-2
	Ccl2	0,95	2,5E-03	1-3-4
	Irf1	0,95	6,9E-03	4
	C3	0,94	7,6E-04	1-2-4
	RT1-N3	0,94	1,8E-03	1-2
	Ccl5	0,90	4,7E-03	1-3
	Gbp5	0,87	4,3E-03	1
	Rsad2	0,86	3,3E-02	1-2
DOWN	Serpina3n	-2,01	9,9E-03	7-5
	Fabp4	-1,58	2,7E-04	7
	Lpl	-1,48	2,7E-05	2-3-4
	Pf4	-0,91	5,8E-03	3-5-10
	Cd163	-0,89	4,2E-04	3
	Clec5a	-0,89	7,6E-03	3
	Lbp	-0,86	6,7E-04	5-7-10
	Gcgr	-0,85	7,7E-03	7
	Adam8	-0,78	4,3E-03	9
	F5	-0,73	3,8E-03	9-5
	Dgat2	-0,72	1,3E-04	4
	Heph	-0,72	1,6E-03	8

Fig 3. G>30 clustered genes after delayed MSC transplantation. The volcano plots represent the graphical distribution of the G>30 genes that were present in one or more functional clusters of the biological mining analysis over the total of differential expressed genes. The G>30 clustered genes, their fold change, the p value and the cluster number (see table 2) are shown for samples obtained 2 days (A) and 7 days (B) after SCI and delayed MSC transplantation.

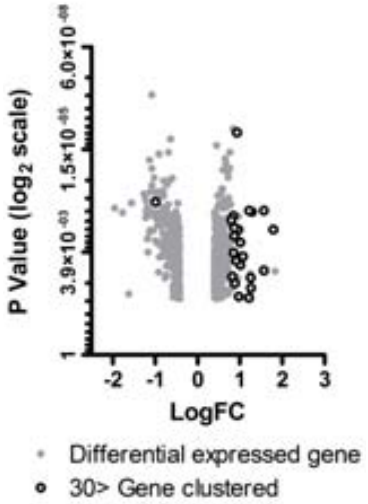
Table 3. Fucntional annotation clusters summary: **Acute OEC vs VHC**

2dpi			
UP genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Response to wounding ans inflammatory response	4,14	4	4
2. Bone remodeling	3,03	3	2
3. Immune response and Leukocyte activation	2,99	7	8
4. Response to hypoxia	2,91	6	4
5. Face and head development	2,74	1	2
6. Blood vessel development	2,64	2	3
7. ECM and collagen organization	2,56	3	11
8. Regulation of cell proliferation	2,38	4	2
9. Cell adhesion	2,21	2	2
10. Hemopoiesis	2,20	5	4
11. Skeletal and cartilage development	2,17	2	2
12. Leukocyte mediated immunity and phagocytosis	2,15	6	29
13. Response to estrogen and nutrient	2,09	6	18
14. Regulation of metabolic process	2,03	1	20
15. Embryonic development	2,02	1	4
16. Protein kinase pathway	2	0	2
17. Immune response-regulating cell surface receptor	1,91	1	2
18. Regulation of leukocyte activation	1,89	3	15
19. Retinoic acid metabolic process	1,88	1	3
20. leukocyte mediated cytotoxicity	1,83	0	2
21. Tissue morphogenesis and organ development	1,83	3	24
22. Antigen processing and presentation	1,83	0	3
23. Cell growth and proliferation	1,71	5	17
24. Negative regulation of cell communication	1,70	0	2
25. Leukocyte chemotaxis	1,56	1	2
26. Ribonucleoprotein complex	1,52	1	2
27. Circulation system process	1,49	1	3
28. Leukocyte activation during immune response	1,49	0	2
29. Mononuclear cell proliferation	1,46	3	3
NC			11
		Total	**208**
DOWN genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Endocytosis	1,79	0	5
2. Phosphorylation	1,72	1	4
3. Regulation of phosphorylation	1,53	0	3
4. Positive regulation of synaptic transmission	1,38	0	2
NC	-	-	1
		Total	**15**

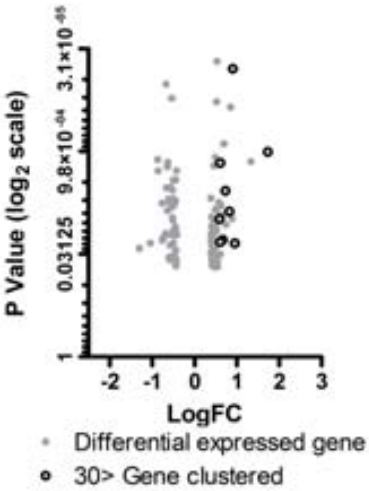
7dpi			
UP genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Response to organic substance and estrogen	2,07	9	19
2. Resposne to LPS	1,83	1	2
3. Regulation of blood vessel size	1,75	0	3
NC	-	-	5
		Total	**29**
DOWN genes Functional clustering	**ES**	**G>30**	**nº GO**
NC	-	-	3
		Total	**3**

30 genes was clustered in any GO terms (Fig. 4B).

Delayed OEC transplantation. At 2 days following delayed grafting of OEC into the spinal cord there were small changes in the gene expression profile. Only the neuron differentiation GO term (GO:0030182) was found to be enriched in the list of up-regulated genes (Table 4 and S14). Indeed 4 genes from the up-top30 list: slitrk6 (SLIT and NTRK-like protein 6), related to neural and neuritogenic activity (Aruga and Mikoshiba, 2003), Pcdh15 (protocadherin-15), Klhl1 (Kelch-like protein 1) and Chrdl1 (chordin-like 1 or neuralin 1), which inhibit glial differentiation but promote neuronal differentiation were associated with this GO term.



	Gene	logFC	P.Value	Cluster n°
UP	Cxcl9	1,81	1,2E-03	3-4-12
	Postn	1,59	4,3E-04	7-9
	Nnat	1,59	1,1E-02	12-13
	Nos2	1,30	4,6E-04	1-2-4-6-8-26-29
	Sfrp2	1,28	2,8E-02	13-15
	Mmp13	1,27	1,6E-02	4-7-11-12-13-23
	Ccr2	1,24	4,3E-04	1-2-3-4-6-10-13-21-27
	Cxcl13	1,23	4,8E-02	3-10
	Cxcl2	1,09	5,1E-03	1-3-25
	Cyp1b1	1,03	2,4E-03	13-19
	Sh2d1b	1,03	8,2E-03	12-14-17-18
	Cd8a	0,99	4,5E-02	1-3-10-12-18
	Terc	0,98	1,2E-03	4-8-10-12-29
	Il11	0,95	6,4E-06	8-14
	Itgax	0,93	6,5E-03	9
	Mmp9	0,90	2,2E-02	2-4-7-11-13-18-23
	Aldh1a3	0,89	1,1E-03	5-19-21-23
	Col8a1	0,89	1,7E-03	3-29
	Wisp2	0,87	4,2E-03	8-9-23
	Calcb	0,86	5,7E-04	27
	Clec4d	0,84	1,5E-02	3
	Ifi204	0,82	7,3E-04	10-21-23
DOWN	Nrbp2	-0,97	2,7E-04	2



	Gene	logFC	P.Value	Cluster n°
UP	Mmp13	1,75	1,0E-03	1
	Mmp8	0,97	2,2E-02	1
	H19	0,92	6,2E-05	1
	Ccl5	0,84	7,6E-03	1-2
	Bmp4	0,75	3,8E-03	1
	Cdc25b	0,69	2,0E-02	1
	Cd4	0,63	2,1E-02	1
	Thra	0,62	1,5E-03	1
	Rbm3	0,61	9,8E-03	1

Fig 4. G>30 clustered genes after acute OEC transplantation. The volcano plots represent the graphical distribution of the G>30 genes that were present in one or more functional clusters of the biological mining analysis over the total of differential expressed genes. The G>30 clustered genes, their fold change, the p value and the cluster number (see table 3) are shown for samples obtained 2 days (A) and 7 days (B) after SCI and acute OEC transplantation.

The main changes observed in the contused spinal cord two days after delayed OEC graft were associated to gene transcription suppression, which led to 70 enriched GO terms and 4 functional annotation clusters. These clusters were related to cell migration and proliferation, wound healing and immune response, ion homeostasis and lipid metabolism, and glycerolipid metabolic process (Tables 4 and S15). Indeed, in the down-top30 list we found a large number of genes related to wound and immune response such as Sept5 (septin-5) that encodes the septin family protein with a role in membrane compartmentalization, Prg4 (proteoglycan 4), Pf4 (Platelet factor 4 or Cxcl4), a proteoglycan associated factor that attracts neutrophils, macrophages and fibroblasts, Plau (urokinase-type plasminogen activator), Lbp, Vnn1(pantetheinase) that plays a role in hematopoietic cell trafficking and triggering inflammatory reaction, Itgal (Integrin, α L or CD11a), part of the lymphocyte function-associated antigen 1 involved in recruitment of immune cells and binding T helper cells to antigen-presentation cells, and RT1-T24-1 (RT1 class I, locus T24, gene 1), a component of the MCH I (Fig. 5A). Other genes in the down-top30 list are associated to ion

and cell homeostasis such as Agtr1a and Casr (calcium-sensing receptor), activator of the phospholipase C pathway in presence of extracellular calcium, and another associated to lipid metabolism such as Apoc1 (apolipoprotein C-I), Lbp and Pnlip (pancreatic lipase) (Fig. 5A). Thus, our data suggest that one week delayed transplantation of the OEC in SCI suppresses some biological processes involved in tissue repair at early time points following grafting.

At 7 days after delayed OEC transplant, 14 Go terms clustered in 4 functional annotation categories were associated to up-regulated genes (Tables 4 and S16). Similar to delayed MSC transplant at day 7 post-grafting, most of the genes induced in the spinal cord after delayed OEC graft were related to antigenic immune response, such as the chemokines Cxcl9, Cxcl10, Cxcl11 and the MCH components RT1-Bb, RT1-Da, RT1-Ba and RT1-N1 (Fig. 5B). Interestingly, the expression of Inmt, one of the most up-regulated genes observed at day seven following acute OEC transplant, was also found to be over-expressed after delayed OEC transplant.

We also found that the delayed OEC graft induced suppression of several genes classified in 14 GO terms and clustered in 5 functional packages. Similarly to results at day 2 following delayed OEC graft, most of the genes down-regulated in the spinal cord at day 7 after delayed OEC transplantation were related to repair processes (Tables 4 and S17). In the down-top30 list we observed some genes involved in regulation of cell growth, such as Htra3 (high-temperature requirement factor A3), a serine protease that cleaves beta-casein/CSN2, as well as several extracellular matrix (ECM) proteoglycans, and inhibitors of TGF-β signalling, such as Igfbp5 and Igfbp6 (insulin-like growth factor-binding protein 5 and 6). Moreover, genes associated to endogenous stimulus response, Serpina3n (serine protease inhibitor A3N), Lox (lysyl oxidase) involved in collagen and elastin stabilization, Hmgcs2 (3-hydroxy-3-methylglutaryl-CoA synthase 2) that catalyzes the first reaction of ketogenesis, and Lbp, were also found in the down-top30 gene list (Fig. 5B).

Discussion

Several studies have shown that transplantation of MSC and of OEC after SCI exerts beneficial effects on functional recovery, tissue protection, axonal regeneration and remyelination. However, the mechanisms and changes triggered by the grafted cells on the spinal cord are still poorly understood. The results of the present study contribute to the knowledge of the role played by MSC and OEC transplants in the injured spinal cord, and provide important information regarding the genes and pathways modified by these cells when grafted into the injured spinal cord.

Cell therapy and tissue healing

Endogenous repair processes or wound healing are activated quickly, synchronically and sequentially after tissue damage in order to avoid the expansion of the injury and to provide a suitable environment for tissue regeneration (Teller and White, 2009; Velnar et al., 2009). The repair dynamic events include: blood cloating and fibrin formation to reduce oedema, recruitment of inflammatory cells to phagocyte the cell and myelin debris and start the inflammatory reaction, angiogenesis to restore the damaged blood vessels and to increase oxygen and nutrient supply to hypoxic areas, ECM synthesis and collagen deposition to allow tissue remodeling, and cell proliferation to replace the lost cells (Teller and White, 2009; Velnar et al., 2009). All these events have to be precisely regulated, otherwise they can induce undesirable effects in the damaged tissue, such as cyst formation, tissue fibrosis and prevention of regenerative processes (Velnar et al., 2009). Chronification of the wound healing process occurs after injuries to the CNS, and thus,

Table 4. Fucntional annotation clusters summary: **Delayed OEC vs VHC**

2dpi			
UP genes Functional clustering	**ES**	**G>30**	**nº GO**
NC	-	-	1
		Total	**1**
DOWN genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Regulation of cell migration and proliferation	2,21	7	13
2. Response to wounding and immune response	2,01	8	24
3. Response to endogenous stimulus: ion homeostasis and lipid metabolism	1,95	8	26
4. glycerolipid metabolic process	1,78	2	6
NC	-	-	1
		Total	**70**

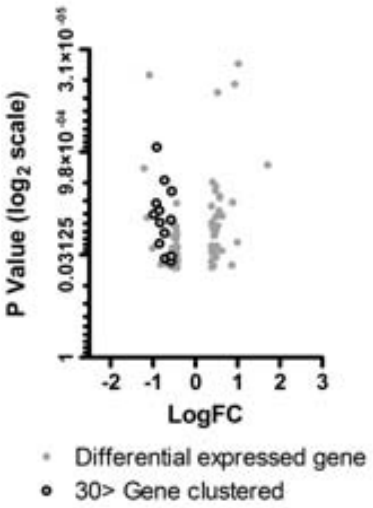
7dpi			
UP genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Immune response	2,83	8	2
2. Antigen processing and presentation	2,71	5	7
3. Circadian rhythm	1,76	2	2
4. Chemotaxis	1,48	3	2
NC	-	-	1
		Total	**14**
DOWN genes Functional clustering	**ES**	**G>30**	**nº GO**
1. Regulation of cell growth	3,61	3	2
2. Response to endogenous stimulus	2,01	4	4
3. Negative regulation of cell proliferation	1,99	2	2
4. Regulation of cell adhesion	1,89	2	2
5. ECM organization	1,53	2	2
NC	-	-	2
		Total	**14**

therapies aimed at inducing the remission of these events is expected to induce neuroprotection and a better milieu for axonal regeneration. The results showed herein suggest that both MSC and OEC can promote different aspects of tissue repair mechanisms, which may explain, in part, their beneficial effects of their grafting.

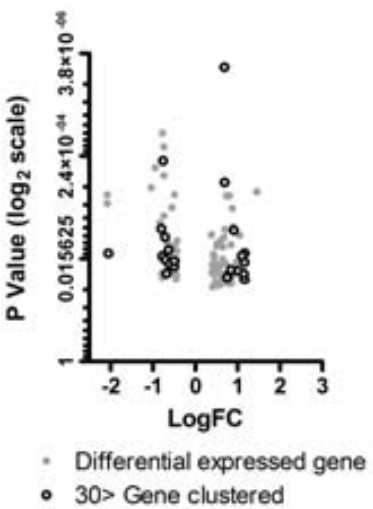
Our microarray data showed that acute transplantation of OEC induced larger changes in gene expression profile in SCI than acute transplantation of MSC. Both cell treatments induced early gene expression related to tissue morphogenesis, ECM organization, angiogenesis, response to hypoxia and low nutrient levels, cell proliferation, and recruitment of inflammatory cells, whereas they suppress gene expression associated to neurotransmission. Acute MSC treatment, however, reduced some genetic pathways related to cell death, phospholipase activity and lipid storage whereas most of the events selectively suppressed by acute OEC were related to phosphorylation and endocytosis. On the other hand, when MSC and OEC are transplanted in subacute SCI, most of the effects induced by both cell grafts are related to the suppression of biological events, especially, those mechanisms related to tissue repair.

MSC and tissue repair. As previously mentioned, the main early changes induced by acute transplantation of MSC were associated to wound healing processes, which are necessary for effective tissue repair (Teller and White, 2009; Velnar et al., 2009). Acute MSC grafts enhance the expression of genes related to ECM organization and collagen synthesis, in particular related with collagen I deposition such as the two genes coding for collagen type I (col1a1 and col1a2), Plod2, Mmp13 and Mmp12, indicating a possible effects on matrix re-modeling. Moreover, the acute MSC graft induced up-regulation of genes involved in the recruitment of inflammatory cells, angiogenesis, vasoconstriction and response to hypoxia as shown by the enrichment GO terms analysis. All those process are necessary during tissue repair (Teller and White, 2009; Velnar et al., 2009), suggesting that the presence of MSC increased wound healing pathways.

On the other hand, the gene expression profile observed after delayed MSC transplant indicated an opposite dynamic compared to the acute graft, since we found reduction in tissue repair mechanisms, such as tissue morphogenesis, ECM remodeling, angiogenesis and response to hypoxia. Interestingly, the inflammatory process



	Gene	logFC	P.Value	Cluster nº
DOWN	Lpl	-0,97	8,2E-03	1-4
	Agtr1a	-0,90	5,7E-03	3
	Sept5	-0,89	8,5E-04	2-3
	Prg4	-0,83	7,2E-03	2
	Pf4	-0,83	2,2E-02	1-2-3
	Igfbp3	-0,82	1,1E-02	1
	Plau	-0,71	1,5E-02	1-2
	Casr	-0,71	2,6E-03	1-3
	Apoc1	-0,70	3,6E-02	3-4
	Lbp	-0,57	3,4E-02	2-3
	Vnn1	-0,56	9,9E-03	1-2
	Pnlip	-0,55	4,0E-02	3
	Itgal	-0,55	3,4E-02	1-2-3
	RT1-T24-1	-0,54	3,8E-03	2



	Gene	logFC	P.Value	Cluster nº
UP	S100a9	1,19	1,3E-02	2-4
	RT1-Bb	1,18	1,9E-02	1-2
	Cxcl9	1,17	3,7E-02	1
	Cxcl11	1,17	3,0E-02	1
	RT1-Da	1,12	1,4E-02	1-2
	Cxcl10	1,02	2,6E-02	1-4
	Il8ra	0,93	5,1E-03	4
	RT1-N1	0,86	2,5E-02	1-2
	Oasl	0,78	3,4E-02	1
	RT1-Ba	0,77	3,4E-02	1-2
	Thra	0,71	7,3E-04	3
	Dbp	0,71	6,9E-06	3
DOWN	Serpina3n	-2,04	1,3E-02	2
	Lox	-0,79	4,8E-03	2-5
	Hmgcs2	-0,76	1,4E-02	2
	Tnfrsf11b	-0,74	3,1E-04	5
	Fbln2	-0,70	6,8E-03	4
	Col8a1	-0,68	1,7E-02	4
	Dpt	-0,66	3,0E-02	3-5
	Htra3	-0,62	1,1E-02	1
	Lbp	-0,59	2,0E-02	2
	Igfbp6	-0,48	2,2E-02	1
	Igfbp5	-0,48	1,8E-02	1-3

Fig 5. G>30 clustered genes after delayed OEC transplantation. The volcano plots represent the graphical distribution of the G>30 genes that were present in one or more functional clusters of the biological mining analysis over the total of differential expressed genes. The G>30 clustered genes, their fold change, the p value and the cluster number (see table 4) are shown for samples obtained 2 days (A) and 7 days (B) after SCI and delayed OEC transplantation.

related to wound healing appeared to be enriched in both up and down list of genes, suggesting that this process was modulated (Abrams et al., 2009; Dayan et al., 2011; Nakajima et al., 2012). Collectively, our data suggest a regulatory role for MSC when transplanted into the injured spinal cord, boosting the restorative mechanisms at early phases following SCI but inducing resolution at later time points and accelerating the homeostasis of the injured tissue. The potentiation of repair processes could be the most probable mechanisms underlying the beneficial effects of the MSC transplantation after SCI regarding tissue sparing (Ankeny et al., 2004; Himes et al., 2006; Nandoe Tewarie et al., 2009; Quertainmont et al., 2012), neoangiogenesis (Quertainmont et al., 2012) and inflammatory modulation (Abrams et al., 2009; Nakajima et al., 2012). Some authors have suggested that MSC are one of the main players during the physiological repair of any injured

tissues (Caplan, 2008; Caplan and Correa, 2011; Valtieri and Sorrentino, 2008). In vivo, MSC appear to enhance the regenerative potential of multiple tissues as a result of paracrin mechanisms that become activated when exposed to an injury environment (Bieback et al., 2012; Caplan and Correa, 2011; Jackson et al., 2012). During the inflammation phase of wound healing, proinflammatory mediators can activate regulatory functions in MSCs leading to secretion some inlfammatory mediators (Németh et al., 2009) and inducing a shift in favor of anti-inflammatory cytokines (Ren et al., 2012). As a result, the activity of MSC in the injured tissues potentiates wound healing and promotes tissue regeneration (Jeon et al., 2010; Peranteau et al., 2008). During the proliferation phase, paracrine factors secreted by the MSC, including bFGF, VEGF, HGF, IL10 and MMP-9 (Chen et al., 2008; Kim et al., 2011), stimulate survival and proliferation of resident cells (Kinnaird et al., 2004; Park et al., 2010), angiogenesis (Gruber et al., 2005; Kaigler et al., 2003) and vascular stability (Lozito et al., 2009). Therefore, MSC can also contribute to generation of a revascularized tissue, remodeling the ECM, re-epithelization and attenuate of fibrotic scar (Bieback et al., 2012; Jackson et al., 2012). Moreover, it has been proposed that perivascular cells named pericytes are indeed MSC that readily infiltrate tissues after blood vessel breakdown, acting as a cellular sensor of damage and secreting mediators for tissue repair (Caplan, 2008). Pericytes have been found to play a crucial role in repair processes after SCI (Göritz et al., 2011). This can explain, in part, the potential benefits of the therapeutic transplantation of MSC (Bieback et al., 2012; Keating, 2012; Wang et al., 2012).

OEC and tissue repair. Similarly to the MSC transplant, the acute presence of the OEC in the injured spinal cord induced the expression of genes related to tissue repair associated process including tissue morphogenesis and organ development, cell growth and proliferation, response to organic substances and nutrient levels, angiogenesis, response to hypoxia and ECM remodelling, whereas a reduction of some of these pathways was observed after delayed transplantation. Of interest, most of the early changes observed after acute OEC injection were related to gene associeted with inflammatory and immune response. These results are in agreement with previous data from our laboratory describing an earlier and higher recruitment of microglia/macrophages induced by acute OEC graft in the injured spinal cord of rats (López-Vales et al., 2004). In addition, we also observed increased angiogenic activity in acute OEC transplanted cords as previously described (Lépez-Vales et al., 2004). The neoangiogenic process triggered by OECs is likely dependent on VEGF induced by COX-2 and iNOS activity, since administration of COX-2 and iNOS inhibitors reduced angiogenic activity of OECs (López-Vales et al., 2004). Although this three genes did not appear within the top30 gene list, both, COX-2 and VEGF genes were found up-regulated in our microarray. In agreement with our results, gene expression analyses have shown that OEC express a large range of genes involved in wound healing, ECM remodelation, cell adhesion and angiogenesis (Franssen et al., 2008). Despite the marked early changes in expression profile induced by acute OEC transplant following SCI, very few changes were observed at day 7 following the acute transplantation. This could be due to rejection of the trasplanted cells in the spinal cord since we also found an early activation of adaptive immune response in the gene array, as well as to the poor integration and migration but strong boundering (Lu et al 2006; Ruitenberg et al., 2002) of the OEC in the injured spinal cord.

Delayed transplantation of OEC in SCI induced few changes in the gene expression profile. Among the modulated mechanisms, we found that delayed OEC grafts reduced inflammatory wounding

associated genes, suggesting an immuno-modulatory capability of OECs when transplanted in subacute SCI. Interestingly, the few genes early up-regulated by delayed OEC injection were mostly related to neuron differentiation. A recent study demonstrated that OEC conditioned medium induced cultured neural stem cells towards neuronal formation (Duan et al., 2011). However, the molecules released by OEC involved in this action are largely unknown.

The results showed herein suggest that the early presence of MSC or OEC in the injured spinal cord may potentiate or accelerate some of the tissue repair mechanisms. This could enable a faster homeostasis recovery, enhancing ECM remodeling, limiting the lesion expansion and creating a favorable environment for tissue regeneration, while in the delayed times the transplanted cells seem to reduce the tissue repair response to inhibit chronification of the wound healing process.

Grafted cell rejection

One of the most remarkable results observed at seven days after acute or delayed cell transplantation was the predominant up-regulation of genes involved in the response against foreign organisms. Indeed, gene expression profile was enriched in cluster of processes such as immune and defense response, leukocyte activation, chemotaxis and migration, leukocyte mediated immunity, antigen processing and presentation, and adaptive immune response. In the list of genes associated with these processes we found an over expression of rat major histocompatibilty complex (MHC) class I such as RT1-N1, RT1-N3, and RT1-EC2, and class II such as RT1-Da, RT1-Ba and RT1-Bb (Dressel et al., 2001; Günther and Walter, 2001). The expression of these genes may indicate the presence of antigen presenting cells (APC) and that these cells were presenting MHC class I and class II antigens. The presence of MHC class I antigen stimulate the immature CD8+ lymphocytes to became mature cytotoxic T lymphocytes (Cole et al., 2012; Heather and Carbone, 2001; Holling et al., 2004) that induce apoptosis in the grafts. In our gene array we found an over expression of CD8 and CTLa2a (cytotoxic T lymphocyte-associated protein 2 alpha) (not presented in top 30 genes list, data not shown), indicating the presence of cytotoxic T lymphocytes in the injured spinal cord tissue. In addition, the MHC class II antigen presented by APC interact with CD4+ immature lymphocytes inducing their maturation to mature T-helper CD4+ lymphocytes (Holling et al., 2004). T-helper CD4+ lymphocytes are the mediators cells that regulate these immune response by cytokines expression. Besides MHC class II genes, we also found an up-regulation of CD74, involved in the formation and transport to membrane of the MHC class II, indicative of the presence of MCH class II, CD4 and some chemokines that act as chemoattractant to T-cells, such as Cxcl9, Cxcl10 and Cxcl11 (Müller et al., 2010; Oo et al., 2010; Rot and Von Adrian, 2004), and B-cells, such as Cxcl13 (Oo et al., 2010; Rot and Von Adrian, 2004), suggesting recruitment of CD4+ T-cells and B-cells. Collectively, these results may indicate a host rejection response against the grafted cells initiated at least at day 7 post-transplantation of MSC and OEC. Rejection of the grafted cells may explain, in part, the limited survival of OEC and MSC when transplanted into injured spinal cords (Abrams et al., 2009; Barakat et al., 2005; Nakajima et al., 2012; Pearse et al., 2007). Survival of the grafted cells largely influences the treatment success, and thus, the combination of cell grafts with immunesupressant agents will be probably needed to optimize the efficacy of cell therapies.

Conclusion

Our data provide an overview of the mechanisms modulated by MSC and OEC when transplanted after an SCI. The changes observed in gene expression profile suggest that transplantation of both, MSC and OEC,

accelerates and/or enhances tissue repair events during the early stages of the injury, while it tends to resolve these processes at later time points. However, the greater activation of the adaptive immune response in the grafted spinal cords at one week after transplantation may be indicative that rapid cell rejection occurs in the injured spinal cord. Further investigation is needed to better understand the neuroprotective actions of MSC and OEC grafts, as well as to avoid cell rejection to optimize cell therapies for clinical application in SCI.

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RESULTS: CHAPTER 3

Supplementary data

Table S1. Primers used for RT-PCR and microarray validation

Mmp13	Forward: ...GAAGATGTCAGGCATAAAGG... (21bp) Reverse: ...TTCTCCATCTCTGTGTCCTC... (20bp)
Hgf	Forward: ...GGCATTCCAACACAAACAAC... (20bp) Reverse: ...ATCTGTTTGCGTTTCTCCTC... (20bp)
Lcn2	Forward: ...GCGAATGCGGTCCAGAAAG... (19bp) Reverse: ...CCTGACGAGGATGGAAGTG... (19bp)
Plod2	Forward: ...TGAGTGGCTCTTTGAGATGG... (20bp) Reverse: ...TCCTCTCTCTTCTTCTTCAACC... (22bp)
Bcl2	Forward: ...GCCTTCTTTGAGTTCGGTG... (19bp) Reverse: ...GCCAGGAGAAATCAAACAGAG... (21bp)
Itgax	Forward: ...GACGGAAGATACCCAGCAG... (19bp) Reverse: ...AGTCATCTGTGAGCCTCC... (18bp)
Scarb1	Forward: ...CCCAGATGTCACACTGTCC... (19bp) Reverse: ...GACGGAGAAAGTCAGGAGC... (19bp)
Hif1a	Forward: ...AGTGAACAGGATGGAATGGAG... (21bp) Reverse: ...GGTTTCTGCTGCCTTGTATG... (20bp)
Lpl	Forward: ...GCTGGTGGGAAATGATGTG... (19bp) Reverse: ...TCATCAGGAGAAAGGCGAC... (19bp)

Table S2. Number of genes changed, associeted GO terms and clusters for every comparison

vs VHC		UP genes	DOWN genes	UP GO terms	DOWN GO terms	UP clusters	DOWN clusters
MSC	Acute 2dpi	173	99	87	11	11	1
	Acute 7dpi	163	157	57	40	4	6
	Delayed 2dpi	29	164	11	83	4	15
	Delayed 7dpi	91	123	101	42	5	10
OEC	Acute 2dpi	315	284	208	15	29	4
	Acute 7dpi	68	51	29	3	3	0
	Delayed 2dpi	37	55	1	70	0	4
	Delayed 7dpi	59	42	14	14	4	5

Table S3. Functional annotation cluster: Acute MSC vs VHC 2dpi UP

Functional annotation cluster (enriched score)	G	P Value	Functional annotation cluster (enriched score)	G	P Value
1. Cell adhesion (4,13)			**7. Response to wounding and inflammatory process (2,06)**		
GO:0007155~cell adhesion	15	7,3E-05	GO:0009611~response to wounding	14	2,6E-04
GO:0022610~biological adhesion	15	7,3E-05	GO:0006954~inflammatory response	7	1,9E-02
2. ECM and collagen organization (3,47)			GO:0032496~response to lipopolysaccharide	5	2,9E-02
GO:0030198~extracellular matrix organization	10	1,2E-07	GO:0002237~response to molecule of bacterial origin	5	3,8E-02
GO:0043062~extracellular structure organization	10	6,8E-06	**8. Response to hypoxia (1,89)**		
GO:0030199~collagen fibril organization	5	4,2E-05	GO:0001666~response to hypoxia	7	1,0E-02
GO:0010810~regulation of cell-substrate adhesion	6	4,8E-05	GO:0070482~response to oxygen levels	7	1,4E-02
GO:0030155~regulation of cell adhesion	8	1,4E-04	**9. Prostaglandin biosynthesis (1,87)**		
GO:0010811~positive regulation of cell-substrate adhesion	5	1,4E-04	GO:0001516~prostaglandin biosynthetic process	3	6,2E-03
GO:0032964~collagen biosynthetic process	3	6,0E-04	GO:0046457~prostanoid biosynthetic process	3	6,2E-03
GO:0032963~collagen metabolic process	4	8,7E-04	GO:0006692~prostanoid metabolic process	3	1,1E-02
GO:0044259~multicellular organismal macromolecule metabolic process	4	1,0E-03	GO:0006693~prostaglandin metabolic process	3	1,1E-02
GO:0044236~multicellular organismal metabolic process	4	1,9E-03	GO:0046456~icosanoid biosynthetic process	3	3,2E-02
GO:0043588~skin development	4	2,7E-03	GO:0006636~unsaturated fatty acid biosynthetic process	3	3,4E-02
GO:0045785~positive regulation of cell adhesion	5	2,9E-03	**10. Tissue morphogenesis and organ development (1,80)**		
GO:0008544~epidermis development	6	3,2E-03	GO:0001655~urogenital system development	9	1,5E-04
GO:0007398~ectoderm development	6	4,7E-03	GO:0051216~cartilage development	6	5,3E-04
GO:0048730~epidermis morphogenesis	3	1,7E-02	GO:0060541~respiratory system development	6	5,3E-03
3. Face and head development (3,43)			GO:0048729~tissue morphogenesis	8	6,7E-03
GO:0060324~face development	4	2,6E-04	GO:0050678~regulation of epithelial cell proliferation	5	9,9E-03
GO:0060322~head development	4	5,1E-04	GO:0048565~gut development	4	1,0E-02
4. Blood vessel development (2,57)			GO:0019748~secondary metabolic process	5	1,1E-02
GO:0001568~blood vessel development	11	7,9E-05	GO:0048732~gland development	7	1,5E-02
GO:0001944~vasculature development	11	1,0E-04	GO:0048608~reproductive structure development	6	1,9E-02
GO:0001974~blood vessel remodeling	3	1,8E-02	GO:0030324~lung development	5	2,1E-02
GO:0048771~tissue remodeling	4	2,4E-02	GO:0030323~respiratory tube development	5	2,2E-02
GO:0048514~blood vessel morphogenesis	6	3,5E-02	GO:0034754~cellular hormone metabolic process	4	2,4E-02
5. Response to vitamin and nutrient (2,52)			GO:0001822~kidney development	5	3,0E-02
GO:0033273~response to vitamin	8	1,0E-04	GO:0006776~vitamin A metabolic process	3	3,0E-02
GO:0007584~response to nutrient	10	1,9E-04	GO:0035295~tube development	7	3,4E-02
GO:0010033~response to organic substance	22	2,5E-04	GO:0016101~diterpenoid metabolic process	3	3,4E-02
GO:0031667~response to nutrient levels	11	4,4E-04	GO:0001523~retinoid metabolic process	3	3,4E-02
GO:0009991~response to extracellular stimulus	11	7,6E-04	GO:0048598~embryonic morphogenesis	8	3,6E-02
GO:0031214~biomineral formation	4	3,0E-03	GO:0006721~terpenoid metabolic process	3	4,1E-02
GO:0033189~response to vitamin A	5	3,9E-03	GO:0021983~pituitary gland development	3	4,3E-02
GO:0001503~ossification	6	4,5E-03	GO:0030326~embryonic limb morphogenesis	4	4,7E-02
GO:0060348~bone development	6	7,1E-03	GO:0035113~embryonic appendage morphogenesis	4	4,7E-02
GO:0048545~response to steroid hormone stimulus	9	7,6E-03	GO:0046660~female sex differentiation	4	4,8E-02
GO:0032526~response to retinoic acid	4	1,6E-02	**11. Vasoconstriction (1,51)**		
GO:0009725~response to hormone stimulus	11	2,8E-02	GO:0045907~positive regulation of vasoconstriction	3	1,8E-02
GO:0031960~response to corticosteroid stimulus	5	3,8E-02	GO:0019229~regulation of vasoconstriction	3	4,8E-02
GO:0008285~negative regulation of cell proliferation	7	4,4E-02	**NC**		
6. Cell proliferation and regeneration (2,30)			GO:0001501~skeletal system development	12	4,7E-05
GO:0007167~enzyme linked receptor protein signaling pathway	10	1,6E-03	GO:0042127~regulation of cell proliferation	17	8,5E-04
GO:0008284~positive regulation of cell proliferation	12	1,7E-03	GO:0034097~response to cytokine stimulus	5	2,3E-02
GO:0031099~regeneration	6	5,5E-03	GO:0060688~regulation of morphogenesis of a branching structure	3	2,4E-02
GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway	7	1,1E-02	GO:0048592~eye morphogenesis	4	3,1E-02
GO:0031100~organ regeneration	4	1,8E-02	GO:0001889~liver development	4	3,1E-02
			GO:0010876~lipid localization	5	4,3E-02

Table S4. Functional annotation cluster: Acute MSC vs VHC 2dpi DOWN

Functional annotation cluster (enriched score)	G	P Value	Functional annotation cluster (enriched score)	G	P Value
1. Regulation of catecholamine secretion (2,04)			GO:0051899~membrane depolarization	3	1,3E-02
GO:0051046~regulation of secretion	6	2,8E-03	GO:0043279~response to alkaloid	3	3,4E-02
GO:0050433~regulation of catecholamine secretion	3	4,3E-03	GO:0007631~feeding behavior	3	3,8E-02
GO:0060341~regulation of cellular localization	6	4,9E-03	GO:0051046~regulation of secretion	6	2,8E-03
GO:0007610~behavior	7	6,2E-03	**NC**		
GO:0051952~regulation of amine transport	3	8,1E-03	GO:0044242~cellular lipid catabolic process	3	3,4E-02
GO:0014070~response to organic cyclic substance	5	9,4E-03	GO:0044057~regulation of system process	5	3,7E-02

Table S5. Functional annotation cluster: Acute MSC vs VHC 7dpi UP

Functional annotation cluster (enriched score)	G	P Value	Functional annotation cluster (enriched score)	G	P Value
1. Wound healing and coagulation (3,39)			GO:0002694~regulation of leukocyte activation	6	1,2E-02
GO:0050817~coagulation	6	2,3E-04	GO:0050865~regulation of cell activation	6	1,4E-02
GO:0007596~blood coagulation	6	2,3E-04	GO:0031349~positive regulation of defense response	4	1,7E-02
GO:0007599~hemostasis	6	2,7E-04	GO:0019884~antigen processing and presentation of exogenous antigen	3	1,8E-02
GO:0042060~wound healing	8	6,9E-04	GO:0032103~positive regulation of response to external stimulus	4	1,8E-02
GO:0050878~regulation of body fluid levels	6	1,6E-03	GO:0019882~antigen processing and presentation	4	2,3E-02
2. Adaptive immune respone and antigen processing (3,19)			GO:0050870~positive regulation of T cell activation	4	2,5E-02
GO:0006955~immune response	23	1,4E-12	GO:0002673~regulation of acute inflammatory response	3	2,6E-02
GO:0006952~defense response	17	1,2E-07	GO:0050727~regulation of inflammatory response	4	2,9E-02
GO:0009611~response to wounding	17	3,9E-07	GO:0002253~activation of immune response	4	3,5E-02
GO:0002684~positive regulation of immune system process	12	3,1E-06	GO:0051249~regulation of lymphocyte activation	5	3,5E-02
GO:0006954~inflammatory response	11	1,2E-05	GO:0002526~acute inflammatory response	4	3,6E-02
GO:0002250~adaptive immune response	7	1,9E-05	**3. Leukocyte migration (1,73)**		
GO:0002460~adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	7	1,9E-05	GO:0006935~chemotaxis	5	5,2E-03
GO:0048584~positive regulation of response to stimulus	11	1,9E-05	GO:0042330~taxis	5	5,2E-03
GO:0002443~leukocyte mediated immunity	7	4,0E-05	GO:0050900~leukocyte migration	4	1,2E-02
GO:0016064~immunoglobulin mediated immune response	6	4,9E-05	GO:0007626~locomotory behavior	6	2,9E-02
GO:0019724~B cell mediated immunity	6	6,0E-05	GO:0030595~leukocyte chemotaxis	3	3,8E-02
GO:0002449~lymphocyte mediated immunity	6	1,6E-04	GO:0060326~cell chemotaxis	3	4,4E-02
GO:0002252~immune effector process	7	4,8E-04	GO:0006928~cell motion	8	4,8E-02
GO:0002696~positive regulation of leukocyte activation	6	2,1E-03	GO:0006935~chemotaxis	5	5,2E-03
GO:0050867~positive regulation of cell activation	6	2,4E-03	**4. Response to LPS (1,63)**		
GO:0050729~positive regulation of inflammatory response	4	2,6E-03	GO:0032496~response to lipopolysaccharide	5	1,7E-02
GO:0048002~antigen processing and presentation of peptide antigen	4	3,3E-03	GO:0002237~response to molecule of bacterial origin	5	2,3E-02
GO:0050778~positive regulation of immune response	6	5,7E-03	GO:0009617~response to bacterium	6	3,3E-02
GO:0002495~antigen processing and presentation of peptide antigen via MHC class II	3	6,2E-03	**NC**		
GO:0019886~antigen processing and presentation of exogenous peptide antigen via MHC class II	3	6,2E-03	GO:0001775~cell activation	7	1,5E-02
GO:0002504~antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	3	7,1E-03	GO:0015837~amine transport	5	1,7E-02
GO:0002675~positive regulation of acute inflammatory response	3	7,1E-03	GO:0002520~immune system development	7	2,3E-02
GO:0051251~positive regulation of lymphocyte activation	5	9,7E-03	GO:0045087~innate immune response	4	3,2E-02
GO:0002478~antigen processing and presentation of exogenous peptide antigen	3	1,1E-02	GO:0043085~positive regulation of catalytic activity	9	3,5E-02
Continue in the other column			GO:0006959~humoral immune response	3	4,8E-02

Table S6. Functional annotation cluster: Delayed MSC vs VHC 2dpi UP

Functional annotation cluster (enriched score)	G	P Value	Functional annotation cluster (enriched score)	G	P Value
1. Defense and immune response (2,85)			**4. Response to hypoxia (1,58)**		
GO:0006952~defense response	5	1,3E-03	GO:0001666~response to hypoxia	3	2,5E-02
GO:0006955~immune response	5	1,5E-03	GO:0070482~response to oxygen levels	3	2,8E-02
2. Response to mechanical and abiotic stimulus (2,12)			**NC**		
GO:0009612~response to mechanical stimulus	3	4,2E-03	GO:0001775~cell activation	7	1,3E-02
GO:0009628~response to abiotic stimulus	4	1,4E-02	GO:0015837~amine transport	5	1,6E-02
3. Locomotory behavior (1,67)			GO:0045087~innate immune response	4	3,0E-02
GO:0007610~behavior	4	1,6E-02			
GO:0007626~locomotory behavior	3	2,9E-02			

Table S7. Functional annotation cluster: Delayed MSC vs VHC 2dpi DOWN

Functional annotation cluster (enriched score)	G	P Value	Functional annotation cluster (enriched score)	G	P Value
1. Negative regulation of cell communication (4,44)			GO:0060485~mesenchyme development	4	1,1E-02
GO:0009968~negative regulation of signal transduction	11	2,1E-05	GO:0014033~neural crest cell differentiation	3	3,6E-02
GO:0010648~negative regulation of cell communication	11	6,3E-05	GO:0014032~neural crest cell development	3	3,6E-02
2. Cell proliferation (3,67)			**10. Retinoic acid and vitamin A process (1,73)**		
GO:0042127~regulation of cell proliferation	21	3,7E-06	GO:0034754~cellular hormone metabolic process	5	3,0E-03
GO:0008284~positive regulation of cell proliferation	10	1,2E-02	GO:0019748~secondary metabolic process	5	9,6E-03
3. Response to nutrient levels (2,71)			GO:0042573~retinoic acid metabolic process	3	1,0E-02
GO:0033273~response to vitamin	9	9,3E-06	GO:0010817~regulation of hormone levels	6	1,7E-02
GO:0007584~response to nutrient	9	7,0E-04	GO:0042445~hormone metabolic process	5	2,0E-02
GO:0010033~response to organic substance	20	9,9E-04	GO:0006776~vitamin A metabolic process	3	2,8E-02
GO:0031667~response to nutrient levels	9	4,9E-03	GO:0016101~diterpenoid metabolic process	3	3,2E-02
GO:0009991~response to extracellular stimulus	9	7,5E-03	GO:0001523~retinoid metabolic process	3	3,2E-02
GO:0048545~response to steroid hormone stimulus	8	1,9E-02	GO:0006721~terpenoid metabolic process	3	3,8E-02
GO:0033189~response to vitamin A	4	2,4E-02	GO:0006775~fat-soluble vitamin metabolic process	3	4,7E-02
4. Cell adhesion (2,65)			**11. Cell migration (1,57)**		
GO:0007155~cell adhesion	12	2,2E-03	GO:0030334~regulation of cell migration	6	1,9E-02
GO:0022610~biological adhesion	12	2,2E-03	GO:0051270~regulation of cell motion	6	3,1E-02
5. Blood vessel development (2,52)			GO:0040012~regulation of locomotion	6	3,2E-02
GO:0001944~vasculature development	9	1,6E-03	**12. Response to wounding and inflammatory response (1,53)**		
GO:0001568~blood vessel development	8	5,6E-03	GO:0009611~response to wounding	10	1,8E-02
6. ECM organization (2,07)			GO:0032496~response to lipopolysaccharide	5	2,5E-02
GO:0030198~extracellular matrix organization	5	8,1E-03	GO:0002237~response to molecule of bacterial origin	5	3,3E-02
GO:0043062~extracellular structure organization	6	8,8E-03	GO:0002526~acute inflammatory response	4	4,8E-02
7. Tissue and organ development (2,06)			**13. Neuron development (1,52)**		
GO:0035295~tube development	12	1,9E-05	GO:0030030~cell projection organization	10	6,4E-03
GO:0008285~negative regulation of cell proliferation	11	1,8E-04	GO:0032989~cellular component morphogenesis	9	2,5E-02
GO:0060429~epithelium development	9	2,1E-03	GO:0000904~cell morphogenesis involved in differentiation	7	2,5E-02
GO:0001655~urogenital system development	7	3,7E-03	GO:0051240~positive regulation of multicellular organismal process	7	3,1E-02
GO:0001822~kidney development	6	5,3E-03	GO:0045597~positive regulation of cell differentiation	7	3,3E-02
GO:0048729~tissue morphogenesis	8	5,3E-03	GO:0031175~neuron projection development	7	4,0E-02
GO:0030278~regulation of ossification	5	7,1E-03	GO:0000902~cell morphogenesis	8	4,1E-02
GO:0007423~sensory organ development	8	8,1E-03	GO:0048667~cell morphogenesis involved in neuron differentiation	6	4,3E-02
GO:0035239~tube morphogenesis	6	1,6E-02	GO:0048666~neuron development	8	4,3E-02
GO:0030324~lung development	5	1,8E-02	GO:0048812~neuron projection morphogenesis	6	5,0E-02
GO:0030323~respiratory tube development	5	1,9E-02	**14. Regulation of Wnt receptor pathway (1,47)**		
GO:0001503~ossification	5	2,0E-02	GO:0030178~negative regulation of Wnt receptor signaling pathway	3	2,2E-02
GO:0060688~regulation of morphogenesis of a branching structure	3	2,2E-02	GO:0030111~regulation of Wnt receptor signaling pathway	3	5,0E-02
GO:0060541~respiratory system development	5	2,3E-02	**15. Lipid biosynthesis (1,39)**		
GO:0001763~morphogenesis of a branching structure	5	2,4E-02	GO:0006633~fatty acid biosynthetic process	4	3,4E-02
GO:0060348~bone development	5	2,8E-02	GO:0008610~lipid biosynthetic process	7	4,8E-02
GO:0001654~eye development	5	4,4E-02	**NC**		
GO:0048732~gland development	6	4,4E-02	GO:0030155~regulation of cell adhesion	7	7,8E-04
GO:0048754~branching morphogenesis of a tube	4	4,5E-02	GO:0006955~immune response	12	1,4E-03
GO:0022612~gland morphogenesis	4	4,6E-02	GO:0009636~response to toxin	5	3,0E-03
8. Response to hypoxia (1,99)			GO:0033280~response to vitamin D	3	1,4E-02
GO:0001666~response to hypoxia	7	8,8E-03	GO:0051147~regulation of muscle cell differentiation	3	3,8E-02
GO:0070482~response to oxygen levels	7	1,2E-02	GO:0046677~response to antibiotic	3	3,8E-02
9. Mesenchyme development (1,76)			GO:0030509~BMP signaling pathway	3	3,8E-02
GO:0014031~mesenchymal cell development	4	9,9E-03	GO:0007507~heart development	6	5,0E-02
GO:0048762~mesenchymal cell differentiation	4	1,0E-02			
Continue in the other column					

Table S8. Functional annotation cluster: Delayed MSC vs VHC 7dpi UP

Functional annotation cluster (enriched score)	G	P Value	Functional annotation cluster (enriched score)	G	P Value
1. Defense and immune response (7,55)			GO:0051249~regulation of lymphocyte activation	8	8,5E-06
GO:0006955~immune response	31	9,6E-29	GO:0051251~positive regulation of lymphocyte activation	7	1,0E-05
GO:0006952~defense response	23	4,3E-18	GO:0050863~regulation of T cell activation	7	2,2E-05
GO:0006954~inflammatory response	14	3,6E-11	GO:0042110~T cell activation	6	2,4E-04
GO:0009611~response to wounding	14	1,4E-07	GO:0030217~T cell differentiation	5	2,9E-04
GO:0048584~positive regulation of response to stimulus	9	1,7E-05	GO:0002703~regulation of leukocyte mediated immunity	5	4,9E-04
GO:0032103~positive regulation of response to external stimulus	6	2,1E-05	GO:0045321~leukocyte activation	7	6,4E-04
GO:0032101~regulation of response to external stimulus	7	1,5E-04	GO:0002683~negative regulation of immune system process	5	8,5E-04
GO:0002673~regulation of acute inflammatory response	4	4,4E-04	GO:0050670~regulation of lymphocyte proliferation	5	1,1E-03
GO:0050729~positive regulation of inflammatory response	4	5,9E-04	GO:0032944~regulation of mononuclear cell proliferation	5	1,1E-03
GO:0050727~regulation of inflammatory response	5	6,7E-04	GO:0070663~regulation of leukocyte proliferation	5	1,2E-03
GO:0002675~positive regulation of acute inflammatory response	3	2,7E-03	GO:0030098~lymphocyte differentiation	5	1,2E-03
GO:0031349~positive regulation of defense response	4	4,2E-03	GO:0001775~cell activation	7	1,3E-03
2. Leukocyte mediated immunity and adaptive immune response (4,14)			GO:0042102~positive regulation of T cell proliferation	4	1,3E-03
GO:0019882~antigen processing and presentation	10	8,4E-11	GO:0046649~lymphocyte activation	6	1,5E-03
GO:0048002~antigen processing and presentation of peptide antigen	8	4,2E-10	GO:0048534~hemopoietic or lymphoid organ development	7	1,6E-03
GO:0002252~immune effector process	9	1,3E-07	GO:0002520~immune system development	7	2,0E-03
GO:0002495~antigen processing and presentation of peptide antigen via MHC class II	4	4,7E-05	GO:0002697~regulation of immune effector process	5	2,1E-03
GO:0019886~antigen processing and presentation of exogenous peptide antigen via MHC class II	4	4,7E-05	GO:0002704~negative regulation of leukocyte mediated immunity	3	2,3E-03
GO:0002504~antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	4	5,8E-05	GO:0002707~negative regulation of lymphocyte mediated immunity	3	2,3E-03
GO:0002478~antigen processing and presentation of exogenous peptide antigen	4	1,2E-04	GO:0002521~leukocyte differentiation	5	3,3E-03
GO:0019884~antigen processing and presentation of exogenous antigen	4	2,5E-04	GO:0050671~positive regulation of lymphocyte proliferation	4	3,5E-03
GO:0002443~leukocyte mediated immunity	5	5,8E-04	GO:0032946~positive regulation of mononuclear cell proliferation	4	3,7E-03
GO:0016064~immunoglobulin mediated immune response	4	1,8E-03	GO:0070665~positive regulation of leukocyte proliferation	4	4,1E-03
GO:0019724~B cell mediated immunity	4	2,1E-03	GO:0042129~regulation of T cell proliferation	4	4,2E-03
GO:0002449~lymphocyte mediated immunity	4	3,7E-03	GO:0002706~regulation of lymphocyte mediated immunity	4	4,2E-03
GO:0002474~antigen processing and presentation of peptide antigen via MHC class I	3	4,3E-03	GO:0030097~hemopoiesis	6	5,6E-03
GO:0002250~adaptive immune response	4	4,6E-03	GO:0002698~negative regulation of immune effector process	3	6,3E-03
GO:0002460~adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	4	4,6E-03	GO:0050777~negative regulation of immune response	3	1,2E-02
GO:0050778~positive regulation of immune response	4	3,2E-02	GO:0045582~positive regulation of T cell differentiation	3	1,3E-02
3. Cell migration, chemotaxis and response to cytokine (3,21)			GO:0008284~positive regulation of cell proliferation	7	1,4E-02
GO:0042330~taxis	7	3,6E-06	GO:0045621~positive regulation of lymphocyte differentiation	3	1,5E-02
GO:0006935~chemotaxis	7	3,6E-06	GO:0042127~regulation of cell proliferation	9	1,9E-02
GO:0007626~locomotory behavior	9	8,2E-06	GO:0045580~regulation of T cell differentiation	3	2,6E-02
GO:0007610~behavior	11	4,1E-05	GO:0051250~negative regulation of lymphocyte activation	3	2,8E-02
GO:0050900~leukocyte migration	5	2,0E-04	GO:0002695~negative regulation of leukocyte activation	3	3,0E-02
GO:0032496~response to lipopolysaccharide	6	3,2E-04	GO:0050866~negative regulation of cell activation	3	3,3E-02
GO:0002237~response to molecule of bacterial origin	6	4,6E-04	GO:0045619~regulation of lymphocyte differentiation	3	3,5E-02
GO:0009617~response to bacterium	7	6,9E-04	GO:0002822~regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	3	4,0E-02
GO:0030595~leukocyte chemotaxis	4	8,4E-04	GO:0002819~regulation of adaptive immune response	3	4,0E-02
GO:0060326~cell chemotaxis	4	1,1E-03	**5. Regulation of cell migration (2,17)**		
GO:0019221~cytokine-mediated signaling pathway	4	4,1E-03	GO:0040017~positive regulation of locomotion	5	1,7E-03
GO:0010033~response to organic substance	12	6,2E-03	GO:0040012~regulation of locomotion	6	2,5E-03
Continue in the nest page			GO:0030334~regulation of cell migration	5	9,5E-03

Table S8. Continue			GO:0030335~positive regulation of cell migration	4	1,1E-02
GO:0009612~response to mechanical stimulus	4	6,4E-03	GO:0051272~positive regulation of cell motion	4	1,4E-02
GO:0034097~response to cytokine stimulus	4	1,8E-02	GO:0051270~regulation of cell motion	5	1,4E-02
GO:0016477~cell migration	5	4,4E-02	**NC**		
GO:0006928~cell motion	6	4,9E-02	GO:0007159~leukocyte adhesion	4	2,2E-04
4. Regulation of leukocyte activation (3,05)			GO:0045087~innate immune response	4	8,5E-03
GO:0002684~positive regulation of immune system process	12	1,6E-08	GO:0006959~humoral immune response	3	1,9E-02
GO:0002696~positive regulation of leukocyte activation	8	1,1E-06	GO:0009615~response to virus	3	2,4E-02
GO:0050867~positive regulation of cell activation	8	1,4E-06	GO:0009725~response to hormone stimulus	7	4,3E-02
GO:0002694~regulation of leukocyte activation	9	1,5E-06			
GO:0050870~positive regulation of T cell activation	7	2,0E-06			
GO:0050865~regulation of cell activation	9	2,1E-06			
Continue in the other column					

Table S9. Functional annotation cluster: Delayed MSC vs VHC 7dpi DOWN

Functional annotation cluster (enriched score)	G	P Value	Functional annotation cluster (enriched score)	G	P Value
1. Regulation of hormone levels (2,94)			**6. Response to retinoic acid and vitamin A (2,21)**		
GO:0042445~hormone metabolic process	6	6,6E-04	GO:0033273~response to vitamin	6	6,9E-04
GO:0034754~cellular hormone metabolic process	5	7,1E-04	GO:0007584~response to nutrient	7	1,9E-03
GO:0010817~regulation of hormone levels	6	3,3E-03	GO:0033189~response to vitamin A	4	8,4E-03
2. Lipid storage (2,88)			GO:0031667~response to nutrient levels	7	8,8E-03
GO:0010886~positive regulation of cholesterol storage	3	4,2E-04	GO:0009991~response to extracellular stimulus	7	1,2E-02
GO:0010884~positive regulation of lipid storage	3	8,7E-04	GO:0032526~response to retinoic acid	3	4,7E-02
GO:0010885~regulation of cholesterol storage	3	1,5E-03	**7. Response to hormone (2,19)**		
GO:0010883~regulation of lipid storage	3	5,4E-03	GO:0009725~response to hormone stimulus	10	4,8E-03
3. Regulation of foam cell differentiation (2,67)			GO:0010033~response to organic substance	14	5,3E-03
GO:0045597~positive regulation of cell differentiation	8	1,1E-03	GO:0009719~response to endogenous stimulus	10	1,0E-02
GO:0010744~positive regulation of foam cell differentiation	3	1,2E-03	**8. Iron ion transport (1,87)**		
GO:0051094~positive regulation of developmental process	8	3,7E-03	GO:0000041~transition metal ion transport	4	5,5E-03
GO:0010743~regulation of foam cell differentiation	3	4,2E-03	GO:0006826~iron ion transport	3	1,1E-02
4. Lipid catabolic process (2,58)			GO:0030001~metal ion transport	7	3,7E-02
GO:0016042~lipid catabolic process	7	2,1E-04	**9. Cell adhesion (1,73)**		
GO:0008610~lipid biosynthetic process	6	3,2E-02	GO:0007155~cell adhesion	8	1,8E-02
5. Blood coagulation and inflammatory response (2,47)			GO:0022610~biological adhesion	8	1,8E-02
GO:0007596~blood coagulation	5	8,4E-04	**10. Immune response and leukocyte chemotaxis (1,64)**		
GO:0050817~coagulation	5	8,4E-04	GO:0006955~immune response	8	1,4E-02
GO:0007599~hemostasis	5	9,5E-04	GO:0007610~behavior	8	1,7E-02
GO:0009611~response to wounding	10	1,4E-03	GO:0030595~leukocyte chemotaxis	3	2,3E-02
GO:0050878~regulation of body fluid levels	5	4,0E-03	GO:0060326~cell chemotaxis	3	2,7E-02
GO:0042060~wound healing	5	2,6E-02	GO:0007626~locomotory behavior	5	4,2E-02
GO:0006954~inflammatory response	5	4,8E-02	**NC**		
			GO:0055114~oxidation reduction	12	7,9E-04
			GO:0042493~response to drug	8	4,6E-03
			GO:0006869~lipid transport	4	4,3E-02

Table S10. Functional annotation cluster: Acute OEC vs VHC 2dpi UP

Functional annotation cluster (enriched score)	G	P Value
1. Response to wounding ans inflammatory response (4,14)		
GO:0009611~response to wounding	23	3,0E-06
GO:0006952~defense response	20	3,4E-05
GO:0006954~inflammatory response	14	7,6E-05
GO:0002526~acute inflammatory response	7	3,5E-03
2. Bone remodeling (3,03)		
GO:0048771~tissue remodeling	8	9,7E-05
GO:0046849~bone remodeling	4	9,1E-03
3. Immune response and Leukocyte activation (2,99)		
GO:0006955~immune response	22	5,0E-06
GO:0030155~regulation of cell adhesion	10	1,6E-04
GO:0045321~leukocyte activation	12	9,0E-04
GO:0046649~lymphocyte activation	10	2,1E-03
GO:0001775~cell activation	12	2,6E-03
GO:0042110~T cell activation	8	2,8E-03
GO:0010810~regulation of cell-substrate adhesion	5	5,0E-03
GO:0045785~positive regulation of cell adhesion	5	1,9E-02
4. Response to hypoxia (2,91)		
GO:0001666~response to hypoxia	13	9,1E-05
GO:0070482~response to oxygen levels	13	1,7E-04
GO:0007568~aging	9	3,6E-03
GO:0040012~regulation of locomotion	8	4,1E-02
5. Face and head development (2,74)		
GO:0060324~face development	4	1,3E-03
GO:0060322~head development	4	2,5E-03
6. Blood vessel development (2,64)		
GO:0001568~blood vessel development	13	5,0E-04
GO:0001944~vasculature development	13	6,6E-04
GO:0048514~blood vessel morphogenesis	8	3,8E-02
7. ECM and collagen organization (2,56)		
GO:0032963~collagen metabolic process	5	2,8E-04
GO:0044259~multicellular organismal macromolecule metabolic process	5	3,5E-04
GO:0030198~extracellular matrix organization	8	5,6E-04
GO:0044236~multicellular organismal metabolic process	5	8,6E-04
GO:0032964~collagen biosynthetic process	3	1,8E-03
GO:0043062~extracellular structure organization	9	2,1E-03
GO:0048730~epidermis morphogenesis	4	4,8E-03
GO:0030199~collagen fibril organization	4	4,8E-03
GO:0043588~skin development	4	1,2E-02
GO:0008544~epidermis development	6	3,0E-02
GO:0007398~ectoderm development	6	4,1E-02
8. Regulation of cell proliferation (2,38)		
GO:0042127~regulation of cell proliferation	23	2,3E-03
GO:0008284~positive regulation of cell proliferation	15	7,3E-03
9. Cell adhesion (2,21)		
GO:0022610~biological adhesion	16	6,2E-03
GO:0007155~cell adhesion	16	6,2E-03
Continue in the next page		

Functional annotation cluster (enriched score)	G	P Value
GO:0032268~regulation of cellular protein metabolic process	14	2,7E-02
GO:0051248~negative regulation of protein metabolic process	8	2,9E-02
GO:0001932~regulation of protein amino acid phosphorylation	8	2,9E-02
GO:0048585~negative regulation of response to stimulus	6	3,1E-02
GO:0033135~regulation of peptidyl-serine phosphorylation	3	3,1E-02
GO:0042326~negative regulation of phosphorylation	4	3,2E-02
15. Embryonic development (2,02)		
GO:0043009~chordate embryonic development	16	1,9E-03
GO:0009792~embryonic development ending in birth or egg hatching	16	2,1E-03
GO:0001701~in utero embryonic development	9	4,5E-02
GO:0001824~blastocyst development	4	4,6E-02
16. Protein kinase pathway (2)		
GO:0007167~enzyme linked receptor protein signaling pathway	12	7,6E-03
GO:0007178~transmembrane receptor protein serine/threonine kinase signaling pathway	6	1,3E-02
17. Immune response-regulating cell surface receptor (1,91)		
GO:0002768~immune response-regulating cell surface receptor signaling pathway	5	8,3E-03
GO:0002764~immune response-regulating signal transduction	5	1,8E-02
18. Regulation of leukocyte activation (1,89)		
GO:0002684~positive regulation of immune system process	13	5,4E-04
GO:0050850~positive regulation of calcium-mediated signaling	4	3,0E-03
GO:0022407~regulation of cell-cell adhesion	4	4,1E-03
GO:0050848~regulation of calcium-mediated signaling	4	4,1E-03
GO:0051249~regulation of lymphocyte activation	8	1,3E-02
GO:0002683~negative regulation of immune system process	6	1,4E-02
GO:0050670~regulation of lymphocyte proliferation	6	1,8E-02
GO:0032944~regulation of mononuclear cell proliferation	6	1,9E-02
GO:0070663~regulation of leukocyte proliferation	6	2,1E-02
GO:0002694~regulation of leukocyte activation	8	2,2E-02
GO:0050865~regulation of cell activation	8	2,6E-02
GO:0051251~positive regulation of lymphocyte activation	6	2,7E-02
GO:0002696~positive regulation of leukocyte activation	6	3,7E-02
GO:0050867~positive regulation of cell activation	6	4,2E-02
GO:0050863~regulation of T cell activation	6	4,5E-02
19. Retinoic acid metabolic process (1,88)		
GO:0019748~secondary metabolic process	7	3,9E-03
GO:0006721~terpenoid metabolic process	4	1,8E-02
GO:0042573~retinoic acid metabolic process	3	3,1E-02
20. leukocyte mediated cytotoxicity (1,83)		
GO:0001909~leukocyte mediated cytotoxicity	3	1,0E-02
GO:0001906~cell killing	3	2,1E-02
21. Tissue morphogenesis and organ development (1,83)		
GO:0030278~regulation of ossification	8	4,5E-04
GO:0048565~gut development	6	1,1E-03
GO:0048732~gland development	11	2,6E-03
GO:0022612~gland morphogenesis	7	3,3E-03
GO:0048729~tissue morphogenesis	11	5,1E-03
GO:0060688~regulation of morphogenesis of a branching structure	4	8,1E-03
GO:0035295~tube development	11	9,2E-03
GO:0060541~respiratory system development	7	1,3E-02

Table S10. Continue		
10. Hemopoiesis (2,2)		
GO:0032844~regulation of homeostatic process	9	7,1E-04
GO:0048534~hemopoietic or lymphoid organ development	11	1,0E-02
GO:0002520~immune system development	11	1,4E-02
GO:0030097~hemopoiesis	10	1,5E-02
11. Skeletal and cartilage development (2,17)		
GO:0001501~skeletal system development	13	1,6E-03
GO:0051216~cartilage development	5	2,8E-02
12. Leukocyte mediated immunity and phagocytosis (2,15)		
GO:0006909~phagocytosis	7	3,3E-05
GO:0006910~phagocytosis, recognition	4	4,1E-04
GO:0006911~phagocytosis, engulfment	4	5,8E-04
GO:0051130~positive regulation of cellular component organization	11	1,5E-03
GO:0002444~myeloid leukocyte mediated immunity	4	2,5E-03
GO:0002819~regulation of adaptive immune response	6	4,2E-03
GO:0002822~regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	6	4,2E-03
GO:0010324~membrane invagination	9	4,6E-03
GO:0006897~endocytosis	9	4,6E-03
GO:0002706~regulation of lymphocyte mediated immunity	6	4,8E-03
GO:0050764~regulation of phagocytosis	4	6,3E-03
GO:0051050~positive regulation of transport	11	6,5E-03
GO:0002703~regulation of leukocyte mediated immunity	6	7,9E-03
GO:0008037~cell recognition	5	8,3E-03
GO:0050778~positive regulation of immune response	8	8,7E-03
GO:0002443~leukocyte mediated immunity	6	9,4E-03
GO:0002712~regulation of B cell mediated immunity	4	1,0E-02
GO:0002889~regulation of immunoglobulin mediated immune response	4	1,0E-02
GO:0048584~positive regulation of response to stimulus	10	1,6E-02
GO:0050777~negative regulation of immune response	4	1,8E-02
GO:0032101~regulation of response to external stimulus	8	2,0E-02
GO:0030100~regulation of endocytosis	5	2,1E-02
GO:0045576~mast cell activation	3	2,4E-02
GO:0016044~membrane organization	11	2,7E-02
GO:0002824~positive regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	4	2,8E-02
GO:0002821~positive regulation of adaptive immune response	4	2,8E-02
GO:0002697~regulation of immune effector process	6	3,7E-02
GO:0050766~positive regulation of phagocytosis	3	3,9E-02
GO:0050727~regulation of inflammatory response	5	4,6E-02
13. Response to estrogen and nutrient (2,09)		
GO:0043627~response to estrogen stimulus	11	1,8E-04
GO:0010033~response to organic substance	31	4,6E-04
GO:0033273~response to vitamin	9	6,0E-04
GO:0007584~response to nutrient	11	2,7E-03
GO:0048545~response to steroid hormone stimulus	13	3,8E-03
GO:0009991~response to extracellular stimulus	13	5,3E-03
GO:0001541~ovarian follicle development	5	9,0E-03
GO:0022602~ovulation cycle process	6	1,0E-02
GO:0009725~response to hormone stimulus	17	1,3E-02
GO:0007435~salivary gland morphogenesis	4	1,4E-02
GO:0002009~morphogenesis of an epithelium	8	1,7E-02
GO:0001655~urogenital system development	8	1,8E-02
GO:0021983~pituitary gland development	4	1,9E-02
GO:0007431~salivary gland development	4	2,1E-02
GO:0060429~epithelium development	10	2,4E-02
GO:0035272~exocrine system development	4	3,4E-02
GO:0070169~positive regulation of biomineral formation	3	3,5E-02
GO:0030501~positive regulation of bone mineralization	3	3,5E-02
GO:0030324~lung development	6	3,5E-02
GO:0030323~respiratory tube development	6	3,8E-02
GO:0021536~diencephalon development	4	3,9E-02
GO:0014031~mesenchymal cell development	4	4,6E-02
GO:0001763~morphogenesis of a branching structure	6	4,8E-02
GO:0048762~mesenchymal cell differentiation	4	4,9E-02
GO:0035239~tube morphogenesis	7	4,9E-02
22. Antigen processing and presentation (1,83)		
GO:0042590~antigen processing and presentation of exogenous peptide antigen via MHC class I	3	1,8E-03
GO:0002474~antigen processing and presentation of peptide antigen via MHC class I	3	4,3E-02
GO:0002478~antigen processing and presentation of exogenous peptide antigen	3	4,3E-02
23. Cell growth and proliferation (1,71)		
GO:0060348~bone development	9	1,1E-03
GO:0001503~ossification	8	2,7E-03
GO:0045786~negative regulation of cell cycle	6	4,2E-03
GO:0051726~regulation of cell cycle	11	1,1E-02
GO:0045597~positive regulation of cell differentiation	11	1,1E-02
GO:0030279~negative regulation of ossification	3	1,8E-02
GO:0001558~regulation of cell growth	8	2,8E-02
GO:0010942~positive regulation of cell death	12	2,8E-02
GO:0040007~growth	9	3,2E-02
GO:0007346~regulation of mitotic cell cycle	6	3,4E-02
GO:0048634~regulation of muscle development	4	4,1E-02
GO:0008361~regulation of cell size	8	4,2E-02
GO:0051094~positive regulation of developmental process	11	4,2E-02
GO:0016049~cell growth	4	4,4E-02
GO:0006917~induction of apoptosis	8	4,6E-02
GO:0012502~induction of programmed cell death	8	4,6E-02
GO:0032535~regulation of cellular component size	9	4,7E-02
24. Negative regulation of cell communication (1,7)		
GO:0010648~negative regulation of cell communication	10	1,7E-02
GO:0009968~negative regulation of signal transduction	9	2,3E-02
25. Leukocyte chemotaxis (1,56)		
GO:0030595~leukocyte chemotaxis	4	2,5E-02
GO:0060326~cell chemotaxis	4	3,0E-02
26. Ribonucleoprotein complex (1,52)		
GO:0022613~ribonucleoprotein complex biogenesis	7	2,2E-02
GO:0007435~salivary gland morphogenesis	4	1,4E-02
GO:0002009~morphogenesis of an epithelium	8	1,7E-02
GO:0001655~urogenital system development	8	1,8E-02

Table S10. Continue			GO:0000387~spliceosomal snRNP biogenesis	3	4,3E-02
GO:0046660~female sex differentiation	6	1,4E-02	**27. Circulation system process (1,49)**		
GO:0042698~ovulation cycle	6	1,5E-02	GO:0003014~renal system process	4	1,5E-02
GO:0048511~rhythmic process	8	1,7E-02	GO:0008015~blood circulation	7	4,7E-02
GO:0031667~response to nutrient levels	11	2,2E-02	GO:0003013~circulatory system process	7	4,7E-02
GO:0008585~female gonad development	5	3,4E-02	**28. Leukocyte activation during immune response (1,49)**		
GO:0009719~response to endogenous stimulus	17	3,5E-02	GO:0002366~leukocyte activation during immune response	4	3,2E-02
GO:0034097~response to cytokine stimulus	6	3,9E-02	GO:0002263~cell activation during immune response	4	3,2E-02
GO:0046545~development of primary female sexual characteristics	5	4,2E-02	**29. Mononuclear cell proliferation (1,49)**		
GO:0031099~regeneration	6	4,6E-02	GO:0008283~cell proliferation	10	2,9E-02
14. Regulation of metabolic process (2,03)			GO:0070661~leukocyte proliferation	4	3,7E-02
GO:0032270~positive regulation of cellular protein metabolic process	12	1,7E-03	GO:0032943~mononuclear cell proliferation	4	3,7E-02
GO:0031400~negative regulation of protein modification process	8	2,4E-03	**NC**		
GO:0051247~positive regulation of protein metabolic process	12	2,4E-03	GO:0002252~immune effector process	9	9,5E-04
GO:0010562~positive regulation of phosphorus metabolic process	8	2,7E-03	GO:0050795~regulation of behavior	5	1,2E-02
GO:0045937~positive regulation of phosphate metabolic process	8	2,7E-03	GO:0002437~inflammatory response to antigenic stimulus	3	1,2E-02
GO:0031401~positive regulation of protein modification process	10	3,9E-03	GO:0006260~DNA replication	7	2,1E-02
GO:0001934~positive regulation of protein amino acid phosphorylation	7	6,0E-03	GO:0009617~response to bacterium	9	2,9E-02
GO:0010563~negative regulation of phosphorus metabolic process	5	7,1E-03	GO:0007229~integrin-mediated signaling pathway	4	3,4E-02
GO:0045936~negative regulation of phosphate metabolic process	5	7,1E-03	GO:0008285~negative regulation of cell proliferation	10	3,5E-02
GO:0031399~regulation of protein modification process	12	9,5E-03	GO:0007264~small GTPase mediated signal transduction	9	3,8E-02
GO:0042327~positive regulation of phosphorylation	7	9,7E-03	GO:0051412~response to corticosterone stimulus	3	4,3E-02
GO:0033138~positive regulation of peptidyl-serine phosphorylation	3	1,2E-02	GO:0009620~response to fungus	3	4,3E-02
GO:0001933~negative regulation of protein amino acid phosphorylation	4	1,6E-02	GO:0045834~positive regulation of lipid metabolic process	4	4,6E-02
GO:0032269~negative regulation of cellular protein metabolic process	8	2,5E-02			
Continue in the other column					

Table S11. Functional annotation cluster: Acute OEC vs VHC 2dpi DOWN

Functional annotation cluster (enriched score)	G	P Value	Functional annotation cluster (enriched score)	G	P Value
1. Endocytosis (1,79)			**3. Regulation of phosphorylation (1,53)**		
GO:0043112~receptor metabolic process	4	4,1E-03	GO:0042325~regulation of phosphorylation	9	2,6E-02
GO:0031623~receptor internalization	3	4,2E-03	GO:0019220~regulation of phosphate metabolic process	9	3,2E-02
GO:0006898~receptor-mediated endocytosis	3	2,8E-02	GO:0051174~regulation of phosphorus metabolic process	9	3,2E-02
GO:0006897~endocytosis	5	4,6E-02	**4. Positive regulation of synaptic transmission (1,38)**		
GO:0010324~membrane invagination	5	4,6E-02	GO:0050806~positive regulation of synaptic transmission	3	3,8E-02
2. Phosphorylation (1,72)			GO:0051971~positive regulation of transmission of nerve impulse	3	4,6E-02
GO:0006796~phosphate metabolic process	15	1,1E-02	**NC**		
GO:0006793~phosphorus metabolic process	15	1,1E-02	GO:0010608~posttranscriptional regulation of gene expression	5	4,1E-02
GO:0006468~protein amino acid phosphorylation	11	3,2E-02			
GO:0016310~phosphorylation	12	3,5E-02			

Table S12. Functional annotation cluster: Acute OEC vs VHC 7dpi UP

Functional annotation cluster (enriched score)	G	P Value	Functional annotation cluster (enriched score)	G	P Value
1. Response to organic substance and estrogen (2,07)			GO:0051216~cartilage development	3	3,3E-02
GO:0001503~ossification	5	1,1E-03	GO:0009612~response to mechanical stimulus	3	3,9E-02
GO:0060348~bone development	5	1,7E-03	GO:0022602~ovulation cycle process	3	4,2E-02
GO:0045137~development of primary sexual characteristics	5	2,2E-03	GO:0042698~ovulation cycle	3	4,9E-02
GO:0008585~female gonad development	4	3,3E-03	**2. Responsne to LPS (1,83)**		
GO:0007548~sex differentiation	5	3,8E-03	GO:0032496~response to lipopolysaccharide	4	1,3E-02
GO:0046545~development of primary female sexual characteristics	4	4,1E-03	GO:0002237~response to molecule of bacterial origin	4	1,6E-02
GO:0001501~skeletal system development	6	4,4E-03	**3. Regulation of blood vessel size (1,75)**		
GO:0010033~response to organic substance	11	4,4E-03	GO:0050880~regulation of blood vessel size	3	1,7E-02
GO:0046660~female sex differentiation	4	5,0E-03	GO:0035150~regulation of tube size	3	1,7E-02
GO:0003006~reproductive developmental process	6	6,7E-03	GO:0003018~vascular process in circulatory system	3	1,9E-02
GO:0048545~response to steroid hormone stimulus	6	6,9E-03	**NC**		
GO:0008406~gonad development	4	1,3E-02	GO:0010810~regulation of cell-substrate adhesion	3	1,3E-02
GO:0043627~response to estrogen stimulus	4	2,3E-02	GO:0006955~immune response	6	2,6E-02
GO:0048511~rhythmic process	4	2,7E-02	GO:0048771~tissue remodeling	3	2,9E-02
GO:0048608~reproductive structure development	4	2,7E-02	GO:0043085~positive regulation of catalytic activity	6	4,0E-02
Continue in the other column			GO:0030198~extracellular matrix organization	3	4,8E-02

Table S13. Functional annotation cluster: Acute OEC vs VHC 7dpi DOWN

Functional annotation cluster (enriched score)	G	P Value
NC		
GO:0006800~oxygen and reactive oxygen species metabolic process	3	6,7E-03
GO:0051260~protein homooligomerization	3	2,5E-02
GO:0006873~cellular ion homeostasis	4	4,9E-02

Table S14. Functional annotation cluster: Delayed OEC vs VHC 2dpi UP

Functional annotation cluster (enriched score)	G	P Value
NC		
GO:0030182~neuron differentiation	4	3,2E-02

Table S15. Functional annotation cluster: Delayed OEC vs VHC 2dpi DOWN

Functional annotation cluster (enriched score)	G	P Value	Functional annotation cluster (enriched score)	G	P Value
1. Regulation of cell migration and proliferation (2,21)			**3. Response to endogenous stimulus: ion homeostasis and lipid metabolism (1,95)**		
GO:0045597~positive regulation of cell differentiation	7	2,0E-04	GO:0045834~positive regulation of lipid metabolic process	4	5,4E-04
GO:0051094~positive regulation of developmental process	7	6,0E-04	GO:0019216~regulation of lipid metabolic process	5	7,1E-04
GO:0042127~regulation of cell proliferation	9	1,6E-03	GO:0045806~negative regulation of endocytosis	3	9,1E-04
GO:0001558~regulation of cell growth	5	2,7E-03	GO:0042304~regulation of fatty acid biosynthetic process	3	2,0E-03
GO:0001503~ossification	4	6,1E-03	GO:0019722~calcium-mediated signaling	3	4,9E-03
GO:0060348~bone development	4	8,2E-03	GO:0060627~regulation of vesicle-mediated transport	4	6,3E-03
GO:0030336~negative regulation of cell migration	3	1,3E-02	GO:0009725~response to hormone stimulus	7	6,9E-03
GO:0040013~negative regulation of locomotion	3	1,5E-02	GO:0051129~negative regulation of cellular component organization	4	9,1E-03
GO:0051271~negative regulation of cell motion	3	1,6E-02	GO:0010033~response to organic substance	9	1,2E-02
GO:0030334~regulation of cell migration	4	1,8E-02	GO:0046890~regulation of lipid biosynthetic process	3	1,2E-02
GO:0040008~regulation of growth	5	1,9E-02	GO:0009719~response to endogenous stimulus	7	1,2E-02
GO:0051270~regulation of cell motion	4	2,5E-02	GO:0048545~response to steroid hormone stimulus	5	1,6E-02
GO:0040012~regulation of locomotion	4	2,5E-02	GO:0019217~regulation of fatty acid metabolic process	3	1,6E-02
2. Response to wounding and immune response (2,01)			GO:0006874~cellular calcium ion homeostasis	4	1,7E-02
GO:0009611~response to wounding	9	7,2E-05	GO:0055074~calcium ion homeostasis	4	1,8E-02
GO:0006955~immune response	8	3,7E-04	GO:0030100~regulation of endocytosis	3	1,9E-02
GO:0045087~innate immune response	4	2,8E-03	GO:0010565~regulation of cellular ketone metabolic process	3	2,0E-02
GO:0042060~wound healing	5	2,9E-03	GO:0006875~cellular metal ion homeostasis	4	2,1E-02
GO:0002526~acute inflammatory response	4	3,1E-03	GO:0055065~metal ion homeostasis	4	2,3E-02
GO:0002696~positive regulation of leukocyte activation	4	5,8E-03	GO:0060191~regulation of lipase activity	3	2,5E-02
GO:0006954~inflammatory response	5	5,9E-03	GO:0009890~negative regulation of biosynthetic process	6	2,7E-02
GO:0050867~positive regulation of cell activation	4	6,4E-03	GO:0030005~cellular di-, tri-valent inorganic cation homeostasis	4	3,1E-02
GO:0031099~regeneration	4	6,9E-03	GO:0055066~di-, tri-valent inorganic cation homeostasis	4	3,6E-02
GO:0002684~positive regulation of immune system process	5	7,4E-03	GO:0030003~cellular cation homeostasis	4	4,2E-02
GO:0048589~developmental growth	4	9,8E-03	GO:0008284~positive regulation of cell proliferation	5	4,4E-02
GO:0016477~cell migration	5	1,2E-02	GO:0051050~positive regulation of transport	4	4,4E-02
GO:0002694~regulation of leukocyte activation	4	1,8E-02	**4. glycerolipid metabolic process (1,78)**		
GO:0048870~cell motility	5	2,0E-02	GO:0006641~triglyceride metabolic process	3	1,3E-02
GO:0051674~localization of cell	5	2,0E-02	GO:0006639~acylglycerol metabolic process	3	1,6E-02
GO:0050865~regulation of cell activation	4	2,0E-02	GO:0046486~glycerolipid metabolic process	4	1,6E-02
GO:0001819~positive regulation of cytokine production	3	2,8E-02	GO:0006638~neutral lipid metabolic process	3	1,7E-02
GO:0042330~taxis	3	3,2E-02	GO:0006662~glycerol ether metabolic process	3	1,8E-02
GO:0006935~chemotaxis	3	3,2E-02	GO:0018904~organic ether metabolic process	3	1,9E-02
GO:0040007~growth	4	3,7E-02	**NC**		
GO:0006952~defense response	5	4,2E-02	GO:0050878~regulation of body fluid levels	3	4,7E-02
GO:0006928~cell motion	5	4,4E-02			
GO:0051251~positive regulation of lymphocyte activation	3	4,5E-02			
GO:0001775~cell activation	4	4,7E-02			

Table S16. Functional annotation cluster: Acute OEC vs VHC 7dpi UP

Functional annotation cluster (enriched score)	G	P Value	Functional annotation cluster (enriched score)	G	P Value
1. Immune response (2,83)			**3. Circadian rhythm (1,76)**		
GO:0006955~immune response	9	5,2E-05	GO:0048511~rhythmic process	4	1,6E-02
GO:0048584~positive regulation of response to stimulus	4	4,2E-02	GO:0007623~circadian rhythm	3	1,9E-02
2. Antigen processing and presentation (2,71)			**4. Chemotaxis (1,48)**		
GO:0002495~antigen processing and presentation of peptide antigen via MHC class II	3	1,1E-03	GO:0006935~chemotaxis	3	3,2E-02
GO:0019886~antigen processing and presentation of exogenous peptide antigen via MHC class II	3	1,1E-03	GO:0042330~taxis	3	3,2E-02
GO:0002504~antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	3	1,2E-03	**NC**		
GO:0019882~antigen processing and presentation	4	1,9E-03	GO:0005976~polysaccharide metabolic process	3	4,0E-02
GO:0002478~antigen processing and presentation of exogenous peptide antigen	3	2,0E-03			
GO:0019884~antigen processing and presentation of exogenous antigen	3	3,1E-03			
GO:0048002~antigen processing and presentation of peptide antigen	3	6,6E-03			

Table S17. Functional annotation cluster: Acute OEC vs VHC 7dpi DOWN

Functional annotation cluster (enriched score)	G	P Value	Functional annotation cluster (enriched score)	G	P Value
1. Regulation of cell growth (3,61)			**4. Regulation of cell adhesion (1,89)**		
GO:0001558~regulation of cell growth	6	6,4E-05	GO:0010810~regulation of cell-substrate adhesion	3	4,8E-03
GO:0040008~regulation of growth	6	9,1E-04	GO:0030155~regulation of cell adhesion	3	3,5E-02
2. Response to endogenous stimulus (2,01)			**5. ECM organization (1,53)**		
GO:0010033~response to organic substance	9	1,7E-03	GO:0030198~extracellular matrix organization	3	1,9E-02
GO:0009725~response to hormone stimulus	6	8,3E-03	GO:0043062~extracellular structure organization	3	4,5E-02
GO:0009719~response to endogenous stimulus	6	1,3E-02	**NC**		
GO:0042493~response to drug	4	4,8E-02	GO:0009611~response to wounding	5	2,1E-02
3. Negative regulation of cell proliferation (1,99)			GO:0032844~regulation of homeostatic process	3	3,3E-02
GO:0008285~negative regulation of cell proliferation	5	4,0E-03			
GO:0042127~regulation of cell proliferation	6	2,5E-02			

RESULTS: CHAPTER 4

Immunosuppression of allogenic mesenchymal stromal cells transplantation after spinal cord injury improves graft survival and functional outcomes

Immunosuppression of allogenic mesenchymal stem cells transplantation after spinal cord injury improves graft survival and functional outcomes

Abel Torres-Espín, Elena Redondo-Castro, Joaquim Hernandez, Xavier Navarro

Group of Neuroplasticity and Regeneration, Institute of Neurosciences, Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Bellaterra, Spain.

Corresponding author: Xavier Navarro, Unitat de Fisiologia Mèdica, Edif. M, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain. Tel: +34-935811966, Fax: +34-935812986, E-mail: xavier.navarro@uab.cat

Abstract

Cell therapy for spinal cord injury is a promising strategy for clinical application. Mesenchymal stem cells (MSC) have demonstrated beneficial effects following transplantation in animal models of spinal cord injury. However, although immunoprivileged properties of the MSC were described, their survival into injured spinal cord is reduced due to the detrimental milieu in the damaged tissue and immune rejection of the cells. The poor survival of the engrafted cells could be an affecting parameters that determine the therapy success. Therefore, we compared the effects of immunosuppressant treatment after early MSC transplants after a spinal cord contusion injury in the rat. Functional outcomes for locomotion, sensory perception and electrophysiological responses were assessed. Moreover, the grafted cells survival and the amount of cavity and spared tissue were studied. The findings indicate that grafted cells survived better with immunosuppressant treatment. Despite the improved survival of the cells, MSC grafts provided similar tissue protection after transplantation. Nevertheless, the immunosuppression enhanced the MSC grafts improved locomotion recovery in treadmill condition, but not in electrophysiological outcomes. These results, indicate that the MSC transplantation combined with immunosuppresion prolongs the survival of the engrafted cells but the achieved outcomes were limited.

Key words: spinal cord injury, cell therapy, transplantation, mesenchymal stem cells, olfactory ensheathing cells.

Introduction

Traumatic spinal cord injuries (SCI) cause direct tissue damage that initiates a cascade of secondary events resulting in expanded cord damage. Transplantation of mesenchymal stem cells (MSC) after SCI has been demonstrated to provide some beneficial effects (Sahni and Kessler, 2010; Hernández et al., 2011; Tetzlaff et al., 2011; Mothe and Tator, 2012) by promoting tissue sparing (Ankeny et al., 2004; Nandoe Tewarie et al., 2009; Quertainmont et al., 2012), modulating the inflammatory response (Abrams et al., 2009; Nakajima et al., 2012) and supporting axonal regeneration (Hofstetter et al., 2002). Nevertheless, MSC transplants only allow for limited recovery of the lost functions after SCI (Chopp et al., 2000;

Hofstetter et al., 2002; Himes et al., 2006; Quertainmont et al., 2012).

Regarding the feasibility of MSC transplants, the possibility of autologous transplantation has been proposed as an important consideration for the translation into the clinical setting. However, some aspects may pose difficulties for the autologous application of these cells after SCI. The characteristics of the MSC, such as expansion growth rate (Phinney et al., 1999), cytokine expression profile and response to inflammatory stimulus (Zhukareva et al., 2010), as well as the capability to stimulate axonal growth and produce beneficial effects after SCI (Neuhuber et al., 2005), are subjected to donor variation. On the other hand, the time needed for the in vitro expansion of the cells make impossible the autologous transplant during the acute face of the injury. Allogenic cell transplantation would solve this issues, provided that host rejection is avoided by an accurate selection of the donor and immunosuppression for ensuring the success of the treatment.

It has been reported that MSC are able to modulate the immunological activation of immune cells in vitro, including the suppression of T-cell proliferation and the inhibition of dendritic cells differentiation (Bartholomew et al., 2002; Tse et al., 2003; Nauta and Fibbe, 2009; Samuelsson et al., 2009). In addition, MSC are hypoimmunogenic due to their low expression of the major histocompatibility complex (MHC) class I and no expression of costimulatory molecules (Tse et al., 2003), thus not inducing the proliferative response of lymphocytes (Bartholomew et al., 2002; Tse et al., 2003). Indeed, the possibility of universal donor MSC for therapeutic applications was suggested (Nauta and Fibbe, 2009). Nevertheless, the immunomodulatory properties of MSC in vivo are under investigation and the mechanisms have not been well addressed. Transplantation of a MSC allograft into the intact spinal cord of rats leads to short-term survival of the graft that was prolonged by immunosuppressive treatment (Swanger et al., 2005), indicating an immunogenic response against MSC. In addition, we have recently found an increased expression of immunogenic reaction associated genes a few days after allogeneic MSC transplantation in the injured spinal cord. Thus, despite the relative immunoprivileged properties of MSC, the injured spinal cord of the host appears to produce an immunogenic reaction against the transplanted cells. This exacerbated immune response may explain the fast disappearance of the grafted allogenic MSC reported after SCI in rats (Abrams et al., 2009; Nandoe Tewarie et al., 2009) and dogs (Jung et al., 2009), as well as that of xenogenic human MSC in injured rats (Nakajima et al., 2012).

Therefore, the reduced survival of the grafted MSC after SCI seems a main factor limiting the success of the transplant to produce reparative effects. The aim of this work was to investigate if an immunosupressive treatment after allogenic MSC transplantation prolongs the survival of the cells after SCI, improves functional recovery and helps to reduce tissue secondary damage. For this aim, we selected to administer tacrolimus (FK506), a FDA-approved immunosuppressant drug, that in addition to efficiently prevent allograft rejection exerts neuroprotective and pro-regenerative actions (see reviews by Gold et al., 2004; Sosa et al., 2005; Glaus et al., 2011). In experimental animals, FK506 treatment has been consistently found to increase axonal regeneration after peripheral nerve injury (Gold et al., 1994; Udina et al., 2002, 2003a), allowing the use of allogenic cells for repairing long nerve defects (Udina et al., 2004). After SCI in rats FK506 exerts some protective effects from secondary injury and enhances axonal sprouting and regeneration (Madsen et al., 1998; Wang and Gold, 1999; Voda et al., 2005; López-Vales et al., 2005).

Material and Methods

MSC cultures and characterization

Primary cultures of MSC were set up from P22 Sprague-Dawley rats. The animals were euthanized with CO2. From each animal tibias and femurs were removed, placed on cool phosphate buffered saline (PBS) and epiphyses were cut. The diaphyses of bones were flushed with PBS and the marrow was mechanically homogenized. The cell mixture was filtered through a 70μm nylon mesh and recovered by centrifugation for 10min at 1500rpm. The pellet was resuspended in growth medium: α-MEM with L-glutamine (Life Technologies, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Lonza, Verviers, Belgium), 2mM L-glutamine (Life Technologies) and 100 units/ml penicillin-streptomycin (Life Technologies 100x); and plated in 100mm culture dishes (Iwaki, Asahi Technoglass, Chiba, Japan) at a density of 5•106 cells¬/cm2. After 24h, the supernatant containing non-adherent cells was removed and fresh medium was added. When the culture was near confluence, every 4-5 days, the cells were detached using PBS with 0.05% trypsin (Life Technologies) and 0.04% EDTA (Sigma, St. Louis, MO) and re-plated at 5000 cells/cm2. Cells were passaged 3-4 times, expanded to 80-90% confluence. The cultured MSC were characterized by the expression of CD90 and CD29 surface markers and not of CD11b and CD45, and their differentiation capability to adipocytes and osteoblasts using the method previously described (Harting et al., 2008). For analysis of adipogenesis, cells were fixed for 20 min with 4% paraformaldehyde in phosphate buffer (PB); the adipocytes were labeled using 60% Oil red O stock solution (0.5% Oil red O in isopropanol, Sigma) for 15 min and washed with distilled water. For osteocytes labeling, cells were fixed using 70% ethanol pre-cooled for 1h at 4ºC, washed and incubated during 30 min with 0.1mg/ml Alizarin red solution (Sigma) in distilled water.

Cell labeling

For identification of the cells after grafting we used two different approaches, green fluorescence protein (GFP) expression by the cells and labeling with PKH26 (Sigma). MSC were transfected with a lentiviral vector encoding for GFP under EF1α promoter. Cells in passage 2 were plated at 2000 cells/cm2 and incubated with lentiviruses during 48h. Then, the medium was changed and the cells cultured as described above. The cells for transplantation were detached and resuspended in L15 medium (Life Technologies) at 50,000 cells/μl and maintained in ice during the surgery.

Spinal cord injury and cells transplantation

Adult female Sprague-Dawley rats (9 weeks old; 250-300g) were used. The animals were housed with free access to food and water at a room temperature of 22 ± 2ºC under a 12:12 light-dark cycle. The experimental procedures were approved by the ethical committee of the Universitat Autònoma de Barcelona in accordance with the European Directive 86/609/EEC.

Under anesthesia with ketamine (90mg/kg) and xylacine (10mg/kg) and aseptic condition, a longitudinal dorsal incision was made to expose T6-T10 spinous processes. A laminectomy of T8-T9 vertebra was made and a cord contusion of 200Kdyns was induced using an Infinite Horizon Impactor device (Precision System and Instrumentation, Kentucky, USA). The animals were transplanted acutely, 30 min after the contusion, and divided in 4 groups. Two groups of rats received vehicle (VE, n=8) or MSC (MSC, n=8) plus i.p. saline injection. Other two groups of rats were injected with vehicle (VE-FK506, n=7) or MSC suspension (MSC-FK506, n=7) plus i.p. FK506 injection. For the cell survival study two additional groups were transplanted with GFP+ MSC and one received saline (gMSC, n=10) and the other received FK506 (gMSC-FK506, n=10).

Spinal cord injections were made using a glass needle (100μm internal diameter, Eppendorf,

Hamburg, Germany) coupled to a 10µl Hamilton syringue (Hamilton #701, Hamilton Co, Reno, NV, USA). Three µl of the corresponding cell suspension or vehicle (L15) were intraspinally injected at the epicenter and at 2mm rostrally and caudally, for a total of 450,000 cells per rat. A perfusion speed of 2µl/min was controlled by an automatic injector (KDS 310 Plus, Kd Scientific, Holliston, MA, USA), and the tip was maintained inside the tissue 3min after each injection to avoid liquid reflux. The wound was sutured by planes and the animals allowed to recover in a warm environment. An i.p. bolus of saline or FK506 (2mg/kg) was administered immediately after the operation and additional injections of saline or FK506 (1mg/kg) were given once a day until the end of the follow-up. To prevent infection, amoxicilin (500 mg/l, Normon) was given in the drinking water for one week. Postoperative analgesia was provided with buprenorphine (0.05mg/kg). Bladders were expressed twice a day until reflex voiding of the bladder was re-established.

Functional assessment

Open-field locomotion. Motor behavior was tested before surgery and at 3, 7, 14, 21, 28, 35 and 42 days postoperation (dpo). Animals were placed individually in a circular enclosure and allowed to move freely for 5 minutes. Two observers evaluated locomotion during open-field walking and scored the hindlimb performance, according to the BBB-scale, ranging from 0 (no movement) to 21 (normal movement) (Basso et al., 1995).

Treadmill locomotion. The maximal walking speed, gait pattern and interlimb coordination were assessed at end time point using the Digigait Imaging system (Mouse Specifics Inc., Boston, MA) as previously described (Redondo-Castro et al., 2013). Briefly, images captured by a video camera mounted below a transparent treadmill belt were digitized at 140 frames per second, and a minimum of 10 sequential step cycles analyzed. For the maximal speed analysis, the treadmill speed was progressively increased from 0 cm/s until the animal was not able to maintain running. For gait and interlimb coordination the rats were recorded at 20 cm/s, if the animals were able to run at this speed. The start and ending times of the stance phase of each stride were extracted and a foot gait diagram was depicted (McEwen and Springer, 2006). Quantitative values for the gait parameters (stride duration, stance duration and swing duration) and the stance/swing ratio were calculated. To assess the coordination of locomotion the regularity index (RI) was calculated. A regular step pattern implies fully coordinated locomotion, in which each paw is placed one time every four footprints, and is placed or raised at a given time. The RI was calculated as the percentage of correct step sequences with respect to the total number of step cycles (Cheng et al., 1997; Hamers et al., 2006).

Sensory tests. Were performed before surgery and at 42 dpo in both hindpaws. The response to mechanical stimuli was evaluated with an electronic Von Frey algesimeter (Bioseb, Chaville, France). The spring metallic filament connected to a force sensor was applied to the medial part of the hindpaw and the pressure gradually increased until the rat withdrew its paw. The threshold was determined as the force until withdrawal. The response to hot stimuli was determined by using a Plantar algesimetrer (Ugo Basile, Comerio, Italy). The rats received a radiant hot stimulus in the hindpaw and the latency time until the animal withdrew the limb was used to determine the hot pain threshold. A cut-off time was set at 20s to prevent tissue damage (Casals-Diaz et al., 2009). Five trials separated by 10 min resting periods for each hindpaw were performed in both sensory tests. The value for each test was the mean of both hindpaws.

Electrophysiological studies

For the electrophysiological tests the animals were anesthetized with pentobarbital (30 mg/kg i.p.), placed prone onto a metal plate and skin temperature maintained above 32°C. An electromyograph (Sapphyre 4ME, Vickers) was used.

Motor evoked potentials (MEPs) were elicited by transcranial electrical stimulation with two needle electrodes were placed subcutaneously over the skull, the anode over the sensorimotor cortex and the cathode on the hard palate. Single electrical pulses of supramaximal intensity (25 mA, 100 μs) were applied, and the MEPs were recorded with needle electrodes from tibialis anterior (TA), gastrocnemius medialis (GM) and plantar (PL) muscles (García-Alías et al., 2004; Valero-Cabré et al., 2004). Peripheral motor nerve conduction tests were performed by stimulating the sciatic nerve with single electrical pulses (100 μs at supramaximal intensity) delivered by needles at the sciatic notch, and recording the compound muscle action potentials (CMAPs) of TA, GM and PL muscles by means of needle electrodes. The recording active electrode was inserted on the belly of the muscle and the reference at the fourth toe. The direct M wave and the reflex H wave (electrophysiological analogue of the stretch reflex) were recorded in the same sweep. The latency and the onset-to-peak amplitude of the maximal MEP, M and H waves were measured. The H/M amplitude ratio was calculated (Valero-Cabré et al., 2004).

Histology

The end time point of the animals for evaluation of treatment was 42 days after injury. The rats were deeply anesthetized (pentobarbital 60 mg/kg i.p.) and intracardially perfused with 4% paraformaldehyde in PBS. The spinal cord segment from 1cm rostral to 1cm caudal of the injury epicenter (2cm total length) was harvested and post-fixed in the same fixative solution for 24h and cryopreserved in 30% sucrose. For evaluation of the injury transversal spinal cord sections 30μm thick were cut with a cryotome (Leica CM190, Leica Microsystems, Wetzlar, Germany) and distributed in 15 series of 24 sections (separated by 450μm) each. One series of sections was re-hydrated in water, submerged in hematoxylin Harris solution (Fluka, Sigma) for 5 min, washed in water 2 times followed by 1% HCl in ethanol solution during 30s. The sections were washed with water again and stained with Eosin Y (Merck Millipore, Dramstadt, Germany) during 6 min. The sections were dehydrated and mounted with DPX (Sigma).
Analysis of spared tissue and injury size was made using 19 transversal cord sections (separated by 450μm between pairs) of each animal. Images of transversal sections were taken at 40x (Olympus BX51) through a digital camera (Olympus DP50). The area of spared tissue, of the cavity and the total spinal cord section were delineated and measured using ImageJ software (NIH, Bethesda MA, USA). The volumes of the graft, spared tissue, cavity and total spinal cord injured segment were calculated using the Cavalieri's correction of morphometric volume (Rosen and Harry, 1990).

Cell survival assessment

Subgroups of animals injected with GFP-MSC were sacrificed at 2, 7, 14, 21 and 42 days after injection (n=2 for each time point). Perfusion and processing of the spinal cord was as above. Longitudinal sections of 30μm thickness of the spinal cord segment were cut and distributed in 12 series of 8 sections (separated by 360μm) each. Sections were blocked with TBS 0.3% with Triton 5% in fetal bovine serum and incubated for 24h at 4ºC with primary antibody rabbit anti-GFP (1/200, Life Technologies). After washes, sections were incubated for 2h at room temperature with biotinilated conjugated donkey anti-rabbit antibody (1/200, Vector, Burlingame, CA, USA), and then with AlexaFluor 488 conjugated streptoavidine (1/200, Jackson Immunoresearch, West Grove, PA,

USA). Slides were dehydrated and mounted with Citoseal 60 (Thermo Fisher Scientific, Madrid, Spain).

Analysis of the GFP+ area was performed on 8 spinal cord sections (separated by 360μm between pairs) of each animal. Consecutive images of the spinal cord injured segment were taken at 40x with the same setting and the total section was mounted using Photoshop software (Adobe Systems Inc.). Microphotographs were transformed to a grey scale and analyzed using ImageJ software. The GFP labeled area for each section was measured after defining a threshold for background correction. The longest distance of the GFP labeled area from the epicenter was measured in the three directions, rostral to caudal (X), anterior to posterior (Y) and medial to lateral (Z).

Data analysis

Quantitative data from spared tissue, cavity and total spinal injured segment volume, % volume of spared tissue, stance/swing ratio, RI, sensory perception and electrophysiology tests were analyzed by one way ANOVA. Data of graft volume were analysed using two way ANOVA. Statistical analyses of BBB score were performed by two way ANOVA of repeated measures. Bonferroni's post hoc test for comparative pairs of groups was used where needed. Data from maximal treadmill speed was evaluated as survival curve by log-rank test. A p value lowere than 0.05 was considered as significant.

Results

Immunosuppression with FK506 enhance MSC survival in the injured spinal cord

Figure 1 shows the localization of the grafted cells in the spinal cord injured segment after transplantation. GFP labeled cells were observed inside the spinal cord parenchyma located around the injection sites and distributed in the tissue injured area (Fig. 1A,B). The stereological volume of GFP immunofluorescence signal was calculated at 2, 7, 14, 21 and 42 dpo (Fig. 1C). Both groups of transplanted animals showed a large volume of GFP signal at 2 and 7 days after injury (Fig. 1B, C). However, in group gMSC the GFP volume markedly decreased already at 14 days after transplantation, and GFP signal practically disappeared at 21days. The gMSC-FK506 animals showed a significantly larger volume occupied by the transplanted cells from 14 to 42 dpo. Morevoer, group gMSC showed a reduction with time in the X (Fig. 1G), Y (Fig. 1H) and Z (Fig. 1I) directions of space, further illustrating the progressive disappearance of the GFP+MSC, while the administration of FK506 maintained the maximal extension of the GFP signal in the X and Y direction, with limited reduction in the Z direction of space, confirming the longer presence of the MSC with immunosuppression.

MSC transplant combined with FK506 slightly improves the locomotor outcome after SCI

The BBB open field locomotion score was used to test the overground voluntary locomotion activity after SCI. All contused rats displayed immediate but temporary hindlimb paralysis (BBB 0 score) followed by a fast recovery during the next two weeks. Regardless of intervention, all the animals achieved a plateau without further significant recovery during the last 3 weeks of follow-up with a mean BBB score about 10-11 points (occasional/frequent plantar stepping without fore-hind limb coordination). During the early phase, the vehicle plus FK506 showed lower BBB score than the other groups at 7 days. No significant differences were found between groups during the plateau phase (Fig. 2A). In the BBB subscore, a measure of walking ability (Basso, 2004), the MSC-FK506 group had higher punctuations than the other three groups during the last 3 weeks of follow-up (Fig. 2B).

In the treadmill testing, at 42 days after SCI all the rats were able to support body weight in stance and run at least at 5 cm/s. The proportion of

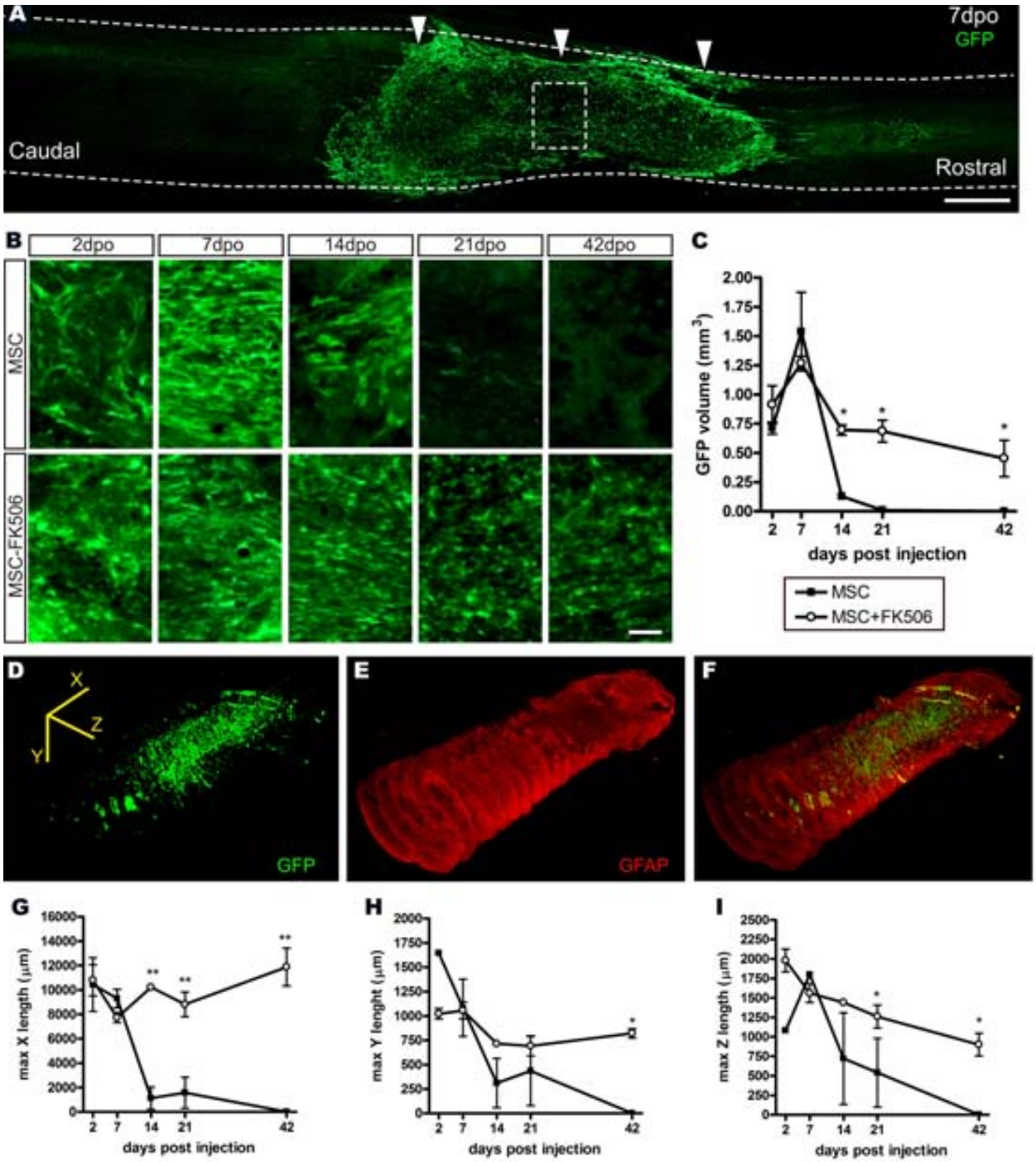

Fig. 1. Grafted MSC survival in the injured spinal cord using GFP labeling. GFP+ MSC grafted cells (green) were localized 7 days after transplantation into the spinal cord (A) surrounding the injection sites (white arrows) and inside the injured area. The dotted line in A delimits the spinal cord longitudinal section. The density of GFP+ cells in the gMSC group (without immunosuppression) markedly decreased from 7 to 21 days after injection (B), while in the gMSC-FK506 group (with immunosuppression) the presence of GFP+ MSC remained at least until 42 days after injection (B). The GFP labeled volume was quantified as a measure of the volume that grafted cells occupied inside the spinal cord. We observed a progressive reduction of the graft volume at 14, 21 and 42 days in the gMSC group in comparison with the gMSC-FK506 group (C). Representative 3D reconstruction of GFP (D), GFAP (E) and merge (F). The maximum distance of the GFP signal from the epicenter was measured in the directions X or rostral to caudal (G), Y or anterior to posterior (H) and Z or medial to lateral (I), showing larger extension of the grafted MSC with FK506 treatment. Scale bar = 1000 µm in A; 100 µm in B.

animals able to walk was reduced with increasing treadmill speed. Both MSC transplanted groups had significantly more rats able to walk at higher speed than the vehicle groups (long-rank test, $p<0.05$, respectively) (Fig. 2C,D). Both vehicle injected groups reached a median speed of 20 cm/s, while the MSC and MSC-FK506 groups achieved a median of 37.5 and 32.5 cm/s respectively. No significant differences were observed between FK506 administered and not administered groups.

The analysis of gait parameters and interlimb coordination were performed at 20 cm/s treadmill speed using the Digigait system (Redondo-Castro et al., 2013) (Fig. 3). Due to the reduced number of control injured animals that achieved 20cm/s running, and that the non-transplanted groups did not present statistical differences in any parameter,

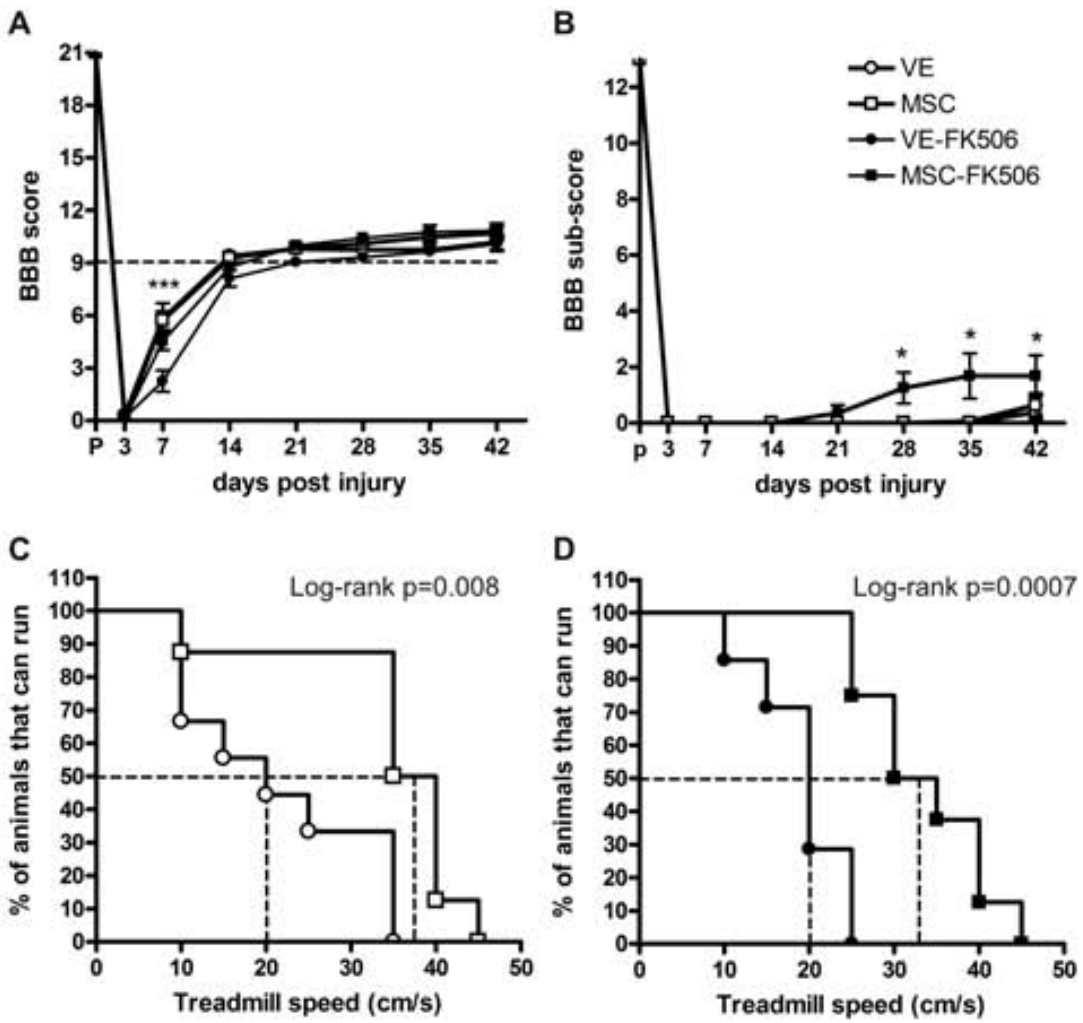

Fig. 2. Gross functional locomotion test results. The open field locomotion was evaluated weekly after the injury using the BBB score (A) and subscore (B). After injury all the rats showed temporal paralysis in the hindlimbs with partial recovery durig the next 2-3 weeks. The dotted line indicates a relevant point in the BBB scale (9 points) regarding the capacity of the rats to support their body weight. In the BBB score, the vehicle plus FK506 group showed lower value at 7 days compared to the other groups (*** $p<0.001$ VE-FK506 group vs. other groups). The MSC-FK506 group showed slight improvement in the BBB subscore during the 3 last weeks compared to the other groups (* $p<0.05$ MSC-FK506 group vs. other groups). The percentage of animals that were able to run at increasing treadmill speeds was analyzed as survival curves (C and D). A significantly higher proportion of rats in groups MSC and MSC plus FK506 was able to maintain running at higher speeds than with vehicle and vehicle plus FK506. The p value for each comparison represent the statistical significance of the log-rank test.

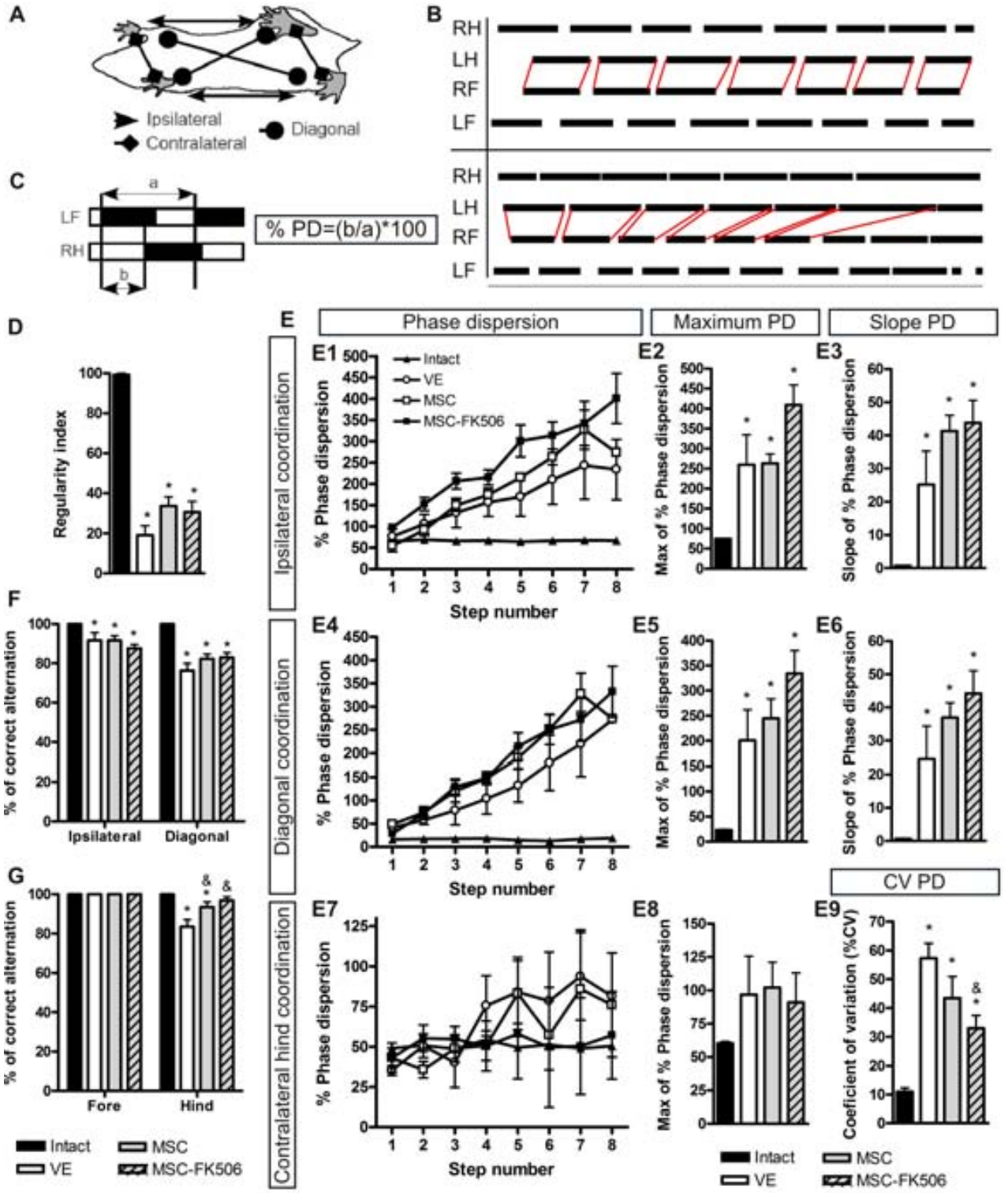

Fig. 3. Coordination of locomotion after SCI and MSC transplants. The coordination of the locomotion on a treadmill was calculated for the ipsilateral, diagonal and contralateral relationships of the limbs (A) at 42 days after SCI. Representative foot-print diagram from a rat before injury (B, top panel) and 42 days after injury (B, bottom panel). The delay between steps (red lines) was constant in intact animals (B, top panel) but progressively increased with stepping after the SCI (B, bottom panel), indicating uncoordination. The regular step pattern (D) and ipsilateral (F), diagonal and contralateral (G) alternations were represented ►

as the percentage of correct stepping sequence. The interlimb coordination (E) was also estimated and represented as the percentage of phase dispersion (E1, E4, E7), the maximum percentage of phase dispersion (E2, E5, E8), the slope of the ipsilateral (E3) and diagonal (E6) coordination and the percentage of coefficient of variation of the contralateral hindlimb coordination (E8). * p<0.05 intact group vs. other groups. In G, & p<0.05 MSC and MSC-FK506 groups vs. VE group. In E9, & p<0.05 MSC-FK506 group vs. VE group. RH: right hindlimb, LH: left hindlimb, RF: right forelimb, LF: left forelimb.

we analyzed together the animals of both vehicle groups. Following SCI, the animals increased the forelimb and reduced the hindlimb step number. As a consequence, the stance and swing time for each step decreased in forelimbs, while the stance time increased in hindlimbs, resulting in an increase of the stance/swing ratio for both fore and hindlimbs in comparison with intact rats. There were no significant differences between treatment groups in the gait parameters (data not shown). On the other hand, the regularity index for alternating step pattern, used as a measure of coordination, was markedly reduced in all the groups after injury (Fig. 3D). The uncoordinated walking was also reflected by partial loss of alternation stepping between ipsilateral and diagonal pairs of limbs (Fig. 3F). Regarding the contralateral relationship of the hindlimbs, a reduction in alternation was observed in the VE and MSC groups but not in the MSC-FK06 group (Fig. 3G), indicating improvement of the correct step sequence of the hindlimbs in this group. These changes were corroborated with the phase dispersion measures, an indicator of the step time delay between two pairs of limbs (Fig. 3B, E). After SCI, both antero-posterior coordinations, ipsilateral and diagonal, were affected as a progressive increase in the delay or phase dispersion (Fig. 3E1, E4). As a result significant increase of the maximal phase dispersion and higher slope in comparison with normal values were observed in all SCI rats (Fig. 3E2, E3, E5, E6). In contrast, the hind contralateral phase dispersion only slightly increased after injury (Fig. 3E7 and E8). The coefficient of variation of the contralateral hindlimbs phase dispersion, a measure of the regularity in the coordination (Redondo-Castro et al., 2013), increased after injury (Fig. 3E9), but the MSC-FK506 showed significantly less increase than the other injured grups. All these results denote a slight, but significant improvement of locomotor performance by the MSC transplantation and enhanced by MSC-FK506 combination.

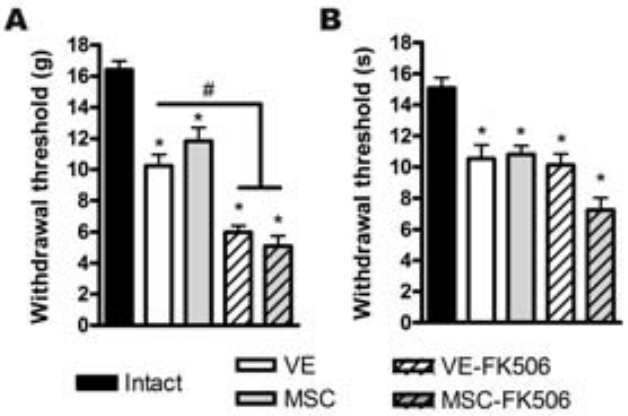

Fig. 4. Sensory test results after SCI and MSC transplants. The response to noxious mechanical stimulus was assessed using an electronic Von Frey device (A) and to heat stimulus using the plantar test (B). After the injury, the threshold of paw withdrawal were reduced in all the groups, indicating hypersensitivity in front to noxious mechanical and thermal stimuli. The rats of groups VE-FK506 and MSC-FK506 showed lower threshold in the Von Frey test compared to VE and MSC goups, indicative of higher mechanical hypersensitivity. * p<0.05 injured group vs. intact group; # p<0.05 MSC-FK506 and VE-FK506 groups vs. MSC and VE groups.

FK506 administration induce higher hypersensitibity to mechanical stimulus

The sensory responses to mechanical (Fig. 4A) and hot noxious stimuli (Fig. 4B) were assessed at the end of the follow-up. Before the injury, the normal withdrawal threshold was around 16g in the Von Frey and 15s in the Plantar test. After the injury, a decrease of withdrawal thresholds in both

Von Frey and Plantar test was observed in all the animals. However, the hypersensitivity to mechanical stimulus induced by the injury was more important in both groups of rats that were administered with FK506 compared to those that did not receive immunosuppression. In the Plantar test, no statistical differences were observed between injured groups.

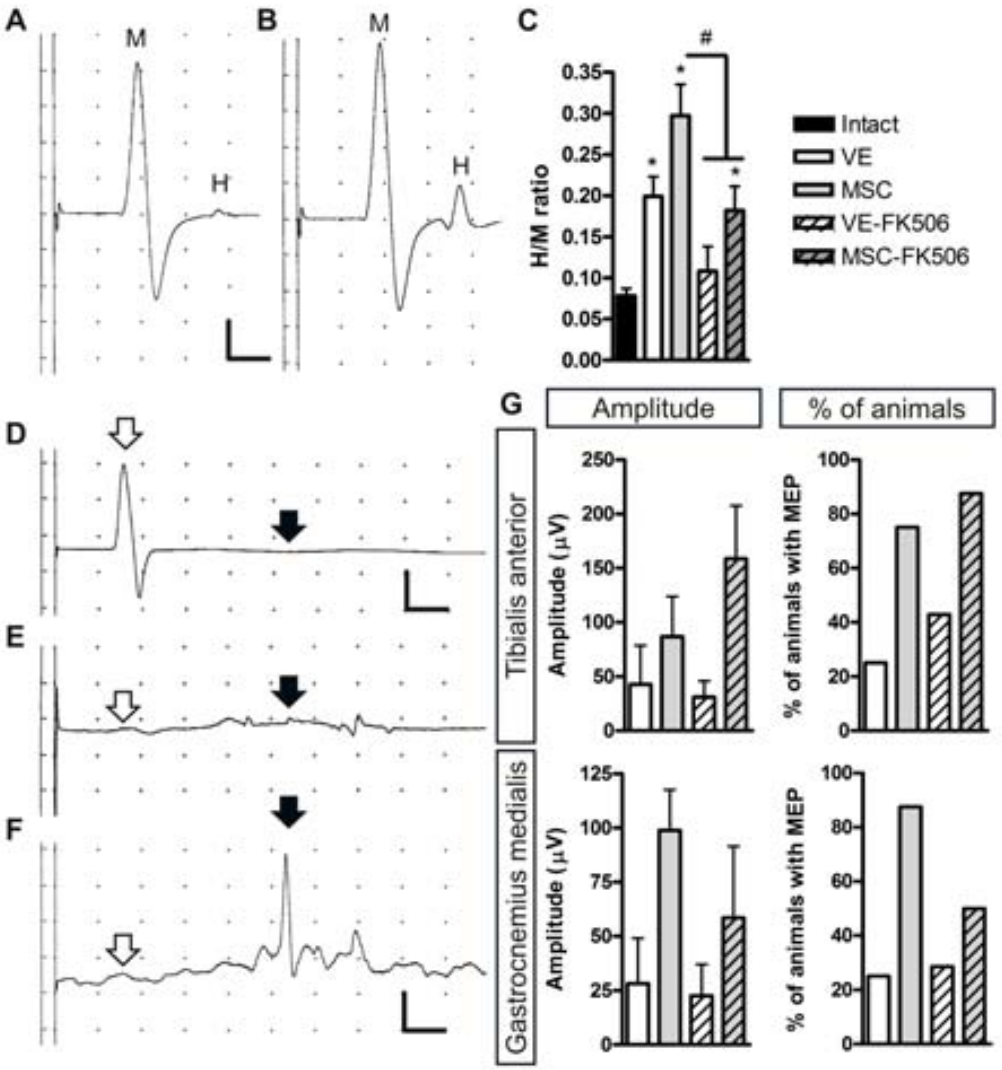

Fig. 5. Electrophysiological results after SCI and MSC transplants. CMAPs recorded in plantar muscle before (A) and after (B) SCI. After SCI no changes were observed in the amplitude of the M wave, but the H wave increased. The increase of the H/M ratio, indicative of hyperreflexia in the lumbar spinal segments was higher in the MSC group than in VE, VE-FK506 and MSC-FK506 groups (C). Note that VE-FK506 treated animals maintained normal levels of the H/M ratio. Representative recordings of MEPs of tibialis anterior muscle (TA) before injury (D), after injury and vehicle injection (E) and after injury and MSC graft (F). In intact rats MEP had a latency about 6 ms (D, white arrow). After injury this early response disappeared, and a second slower response was recorded with a latency about 20 ms (E and F, black arrow). The amplitude of the late MEP component and the percentage of animals that presented this response from TA and GM muscles are represented for all treatments (G). In C, * $p<0.05$ injured groups vs. intact rats; # $p<0.05$ MSC group vs. VE-FK506 and MSC-FK506 groups. In A and B, vertical bar = 2mV, horizontal bar = 2ms. In D, vertical bar = 10mV, horizontal bar = 5ms. In E and F, vertical bar = 50μV, horizontal bar = 5ms.

MSC transplantation induces increase of hyperreflexia and recovery of MEP

Spinal reflexes. Electrical stimulation of the sciatic nerve evoked in all the animals two consecutive muscle responses, the M and the H waves. At the end of the follow-up, 6 weeks after injury, neither the amplitude nor the latency of the M waves were significantly changed in the SCI rats. In contrast, the H wave amplitude was increased (Fig. 5B), so the H/M amplitude ratio markedly increased after injury in all animals, except in the VE-FK506 group with respect to preoperative values (Fig. 5C), indicating hyperreflexia. The MSC treated animals, both with and without FK506 treatment showed higher H/M ratio than the respective vehicle groups although without significant differences. Moreover, the H/M ratio of the MSC group was significantly higher than of groups MSC-FK506 and VE-FK506. This results indicate a enhanced hyperreflexia with the MSC transplant that is reduced by FK506 administration.

MEP. In all the injured rats the normal MEP was abolished during the follow-up, and only a few animals presented a small response (less than 50μV) at the normal latency at 42 dpo (Fig. 5E and F). A late MEP response (latency around ~20ms) appeared after injury in TA and GM muscles (Fig. 5E and F). In the MSC transplanted groups, with and without FK506 treatment, the mean amplitude of this second component was higher than in the corresponding vehicle groups (Fig. 5G). Moreover, the proportion of animals that presented MEPs was higher in the MSC transplant groups compared to VE injected groups (Fig. 5G). These results indicate that cell transplantation may exert protection of descending spinal pathways.

MSC transplantation exerts neuroprotection

To determine the capacity of the cells to provide tissue protection, we evaluated the amount of spared (Fig. 6) and injured (Fig. 7) tissue at 42 days after surgery. Regarding the cross-section area of the spinal cord, we observed a progressive reduction from both rostral and caudal segments to the epicenter after injury (Fig. 6E). The amount of spared tissue area was higher in both MSC groups with respect to vehicle injected groups, independently of the administration of FK506, in both rostral and caudal areas to the epicenter (Fig. 6E). At the center of the injury there were no differences between groups in the amount of spared tissue. We found a higher percentage of spared tissue volume with both MSC (83.8±5.2%) and MSC-FK506 (84.6±.38%) transplants in comparison to both vehicle (72.84±6%) and vehicle plus FK506 injection (71.6±9%) (Fig. 6F). When comparing both vehicle groups no differences were observed, suggesting that FK506 did not influence cord tissue preservation. Accordingly, the amount of injured tissue (Fig. 7) was significantly lower in the MSC transplant groups than in vehicle groups, but no differences between MSC groups were observed (Fig. 7I and J). The stereological estimation of the cavity formed in the injured spinal cord revealed a significant reduction of the empty space after MSC transplantation than in control injured groups (Fig. 7I and K). This reduction was highest in the MSC-FK506 group. In addition, in rats receiving FK506 after MSC transplantation the cavity was filled by a dense tissue (Fig. 7D, F and H), a finding that was not observed in any of the other groups (Fig. 7A-C, E and G). These data indicate that the MSC graft allows the rescue of some damaged tissue independently of immunosuppression, while combined MSC and FK506 treatment also induced filling of the spinal cord cavity.

Discussion

MSC transplantation has demonstrated potential therapeutic applications in CNS disorders, including traumatic SCI (Parr et al., 2007; Hernández et al., 2011; Wright KT, El Masri W, Osman A, Chowdhury J, 2011; Mothe and Tator, 2012). However, the immunogenic rejection of

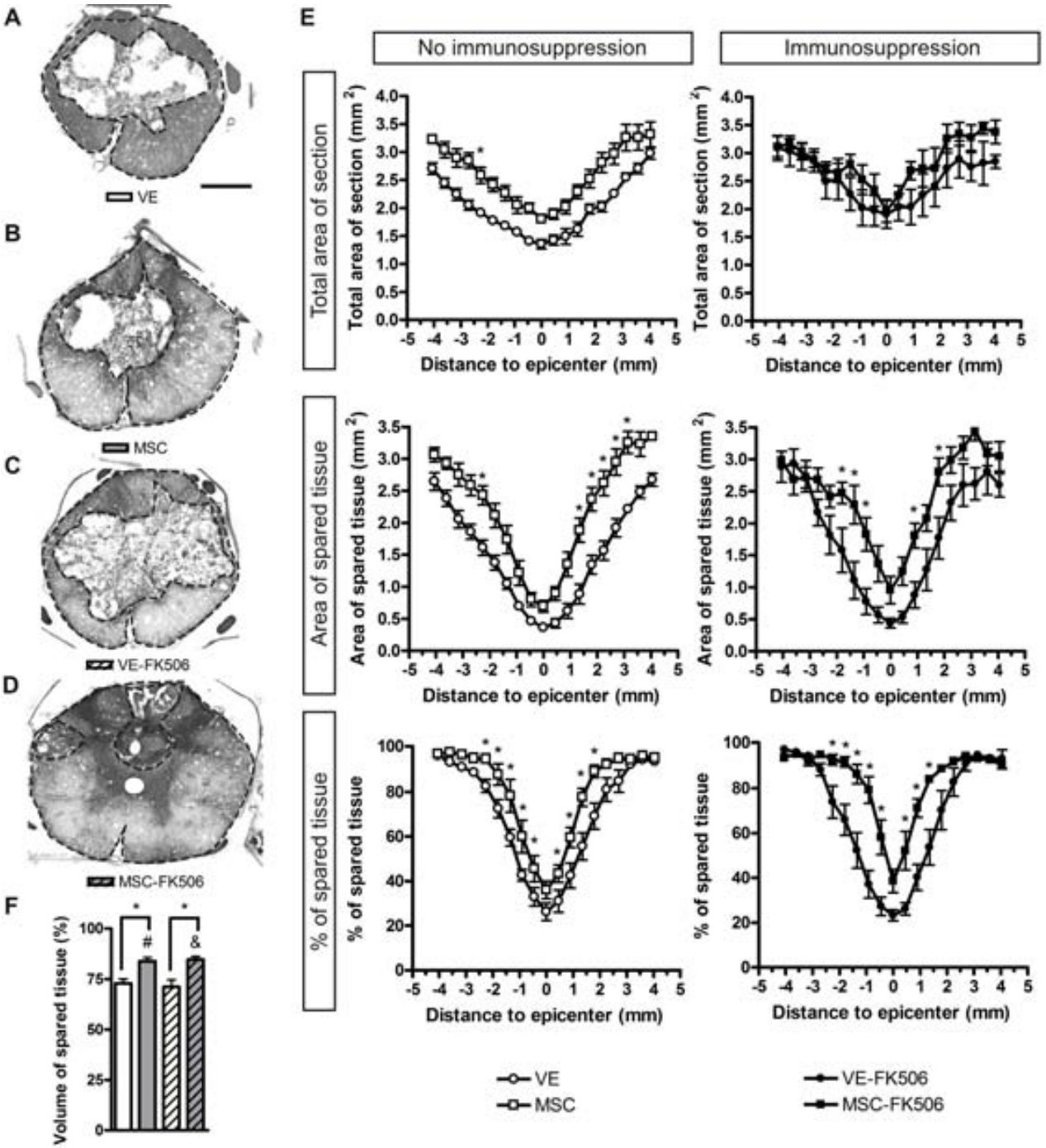

Fig. 6. Spared tissue analysis of injured spinal cord after MSC transplant. Measurements of spared tissue and total cord section areas were made on hematoxylin-eosin stained transversal sections of the injured spinal cord segment (A-D, representative sections taken 2mm rostral to epicenter). The spared tissue area was also represented as the percentage with respect to the total section area (E). The comparative plots were divided according to saline (no immunosuppression) (E, left column) and FK506 (immunosuppression) (E, right column) treatment after transplant. * $p<0.05$ group MSC vs. group VE and $p< 0.05$ group MSC-FK506 vs. group VE-FK506. The volume of spared tissue was also calculated (F); # $p< 0.05$ group MSC vs. group VE-FK506, & $p<0.05$ group MSC-FK506 vs. group VE. Dotted lines in A-D delineate the spared tissue and the total cord section.

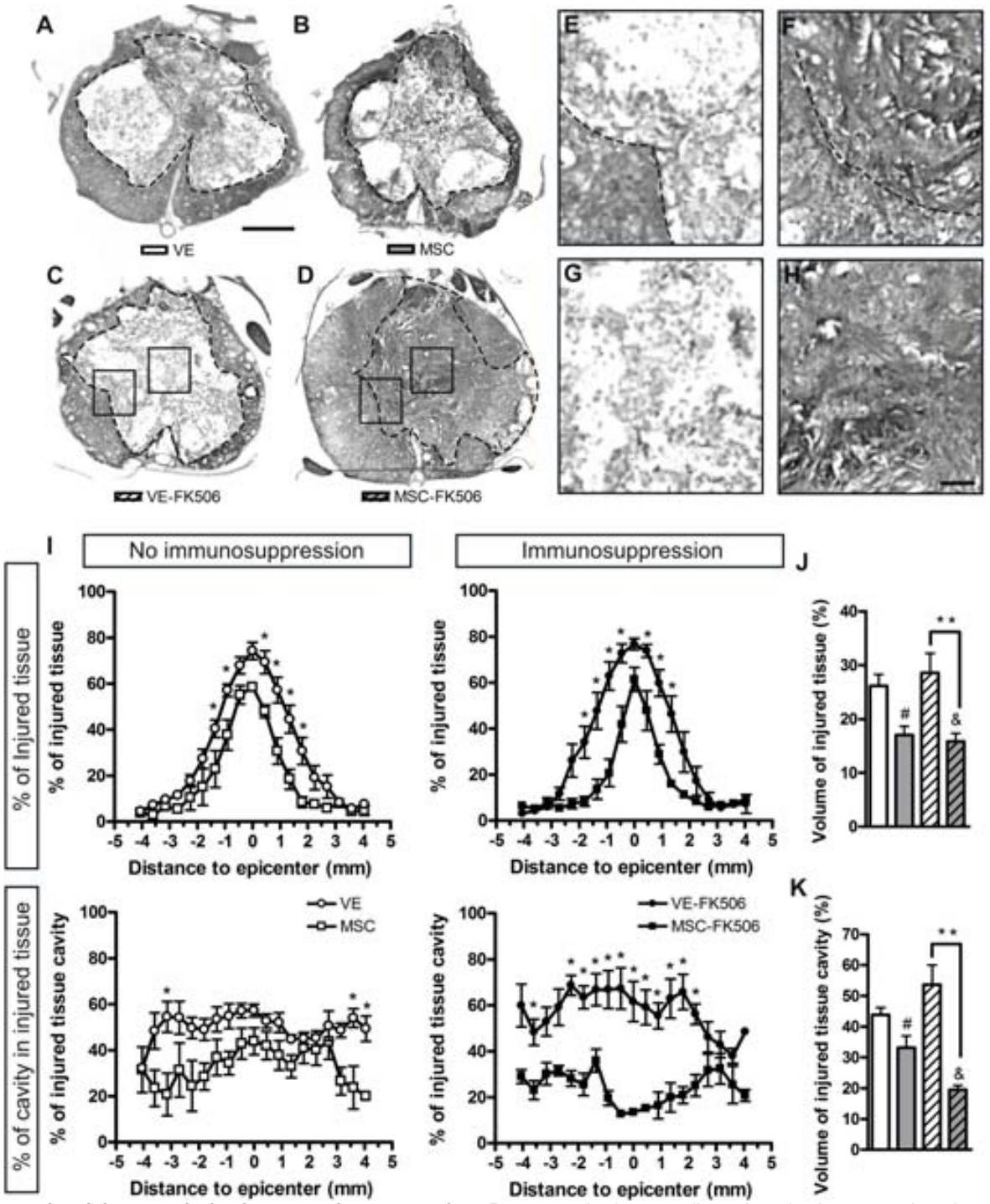

Fig. 7. Injured tissue analysis after SCI and MSC transplant. Representative hematoxylin-eosin stained transversal sections of the injured spinal cord segment in each group studied (A-D). Magnification of the spared-injured tissue interface (E, left box in C; F, left box in D) and of the injured tissue (G, right box in C; H, right box in D). Measurements of injured tissue and cavity areas were made (I). The injured tissue was represented as the percentage with respect to the total section area (see Fig. 6E) and the percentage of cavity were calculated with respect to the injured tissue area. The analysis was divided according to saline (no immunosuppression) (I, left column) and FK506 (immunosuppression) (I, right column) treatment after SCI and transplant. The volumes of the injured tissue (J) and cavity (K) were also calculated and the percentage represented. * $p<0.05$ group MSC vs. group VE and $p< 0.05$ group MSC-FK506 vs. group VE-FK506; In F and K, ** $p< 0.01$ group VE-FK506 vs. MSC-FK506, # $p< 0.05$ group MSC vs. group VE-FK506, & $p<0.05$ group MSC-FK506 vs. group VE. Dotted line in A-F delimits the injured tissue.

engrafted MSC after SCI has received less attention (Nauta and Fibbe, 2009). Our study shows long-term survival of engrafted MSC after SCI by application of immunosuppressive treatment with FK506. As a result of this prolonged survival, we observed a slight improvement of locomotor recovery provided by the graft and the formation of compact tissue inside the cavity resulting from the spinal cord contusion. Furthermore, other effects can be attributed to the immunosuppressive treatment, such as the reduction of both hyperreflexia and hyperalgesia induced by the injury.

Immunosuppression increases MSC graft survival

Survival of MSC grafted in the injured spinal cord of immune competent rats was significantly compromised from early time points, and resulted in total graft erradication by 6 weeks after transplantation. In contrast, immunosuppression with FK506 resulted in longer survival of the engrafted MSC, at least during the 6 weeks of the follow up. Although a number of works noted that MSC are immunosuppressive in vitro (Bartholomew et al., 2002; Tse et al., 2003; Nauta and Fibbe, 2009), it is not yet clear that this capability is sufficient to prevent their rejection by the host after transplantation in a hostile environment such as the injured spinal cord. Experimental results suggest that MSCs may also behave as non-professional antigen-presenting cells (APCs), inducing an immune response (Rasmusson et al., 2003; Spaggiari et al., 2006). In agreement with these observations, subcutaneous allografts of MSC in MHC class I and MHC class II mismached mice induce a specific immune reaction (Nicoletta, 2005). This reaction against to engrafted MSC has been also observed in the intact spinal cord from rat (Swanger et al., 2005) and human donors (Ronsyn et al., 2007), with an important recruitment of macrophages, that lead to a dramatic reduction in the number of cells between 2 and 4 weeks after injection in comparison to a syngeneic transplant (Swanger et al., 2005). Furthermore, the administration of cyclosporine A, an immuno-suppressive agent, prolongs the survival of the MSC grafts (Swanger et al., 2005; Ronsyn et al., 2007), confirming the immune rejection induced in the intact spinal cord. After SCI, a limited survival of MSC graft has been reported in xenogeneic transplants of human MSC (Himes et al., 2006) and allogeneic transplants in contused rats (Abrams et al., 2009; Nandoe Tewarie et al., 2009) and dogs (Jung et al., 2009). The long-term survival of the MSC graft boosted by immunosuppression is indicative of graft rejection induced by the immune system. Interestingly, survival of the grafted MSC during the first week after transplantation did not seem affected by immunosuppression (see Fig. 1), suggesting that rejection of the cells starts after a few days of injection, coinciding with an increase of immune genes expression (unpublished results).

Positive functional effects of MSC transplant combined with immunosuppression after SCI

After SCI, the destruction of the spinal cord architecture severely compromises neurological functions below the level of the injury. Our detailed functional analysis showed initial complete paralysis of the hindlimbs in all the injured rats, followed by partial recovery of motor skills, loss of MEP responses, hyperreflexia and hypersensitivity to mechanical and thermal stimuli. The transplantation of MSC after SCI improved the recovery of locomotion in terms of better treadmill speed walking. This recovery was improved in animals with MSC and FK506 treatment, as reflected by slight improvements in the BBB subscore, increased speed walking and hindlimbs coordination pattern. It has been reported that MSC transplantation in acute and subacute phases of SCI results in improvement of locomotor performance in mice (Boido et al., 2012), rats (Chopp et al., 2000; Hofstetter et al., 2002; Nakajima et al., 2012; Quertainmont et al., 2012)

and dogs (Lim et al., 2007; Jung et al., 2009). However, such recovery is limited by the restricted survival of the grafted MSC. Our findings demonstrate enhanced recovery when the survival of the grafted cells was increased by immunosuppression. The better survival of autologous versus allogeneic MSC grafts results in enhanced locomotor skills in SCI dogs (Jung et al., 2009), confirming the importance of long-term graft survival for recovery. This conclusion was also reported for fibroblasts (Hayashi et al., 2005) and olfactory ensheathing cells (López-Vales et al., 2006) transplantation after SCI, as well as for nerve allografts after sciatic nerve transection (Navarro et al., 2001; Udina et al., 2003a), in which immunosuppression improved the neurological functional recovery induced by the grafted cells.

The enhanced locomotor performance induced by the MSC graft was paralleled by partial recovery of MEPs, demonstrating increased descending information accross the injury to the lumbar cord segments. Nevertheless, the recovered MEPs were considerably smaller in amplitude in comparison to intact rats, and we did not find differences between immunosuppressed and non-immunosuppressed grafted groups. The 200kdyn contusion injury destroys the ventral and lateral foniculi of the white matter (Cao et al., 2005), necessary for normal overground locomotion and MEP responses (Magnuson et al., 1999; Loy et al., 2002; Cao et al., 2005). Our histological results showed tissue protection provided by the grafted MSC, but the transplantation failed to preserve more tisssue at the epicenter of the injury. Consequently, the descending pathways remained largely affected, compromising locomotion and MEP recordings.

Besides the immunosuppressive effects as an immunophilin ligand, FK506 has diverse neuroprotective actions after central and peripheral neural injuries (Gold et al., 2004; Voda et al., 2005; Saganová et al., 2012), by inhibiting apoptosis and necrosis (Herr et al., 1999; Furuichi et al., 2004), reducing inflamation and macrophage/microglia activity (López-Vales et al., 2005; Guzmán-Lenis et al., 2008) and limiting leukocytes accumulation (Tsujikawa et al., 1998). After SCI the beneficial effects of the FK506 appears to be dependent on the injury model. Thus, improvement of locomotor function by FK506 administration was found following photochemical (López-Vales et al., 2005, 2006) and hemisection (Voda et al., 2005) injuries, but not after contusion (Voda et al., 2007) and compression (Saganová et al., 2009) injuries. Furthermore, the neuroprotective effects of FK506 are also dependent on dose and timing of administration (Wakita et al., 1998; Udina et al., 2002, 2003b; Saganová et al., 2012). We selected a dose of 1mg/kg/day according to previous reports, since this dose has cell graft protective action (Udina et al., 2003b; Hayashi et al., 2005) but does not induce functional and histological benefits in a SCI model similar to ours (Saganová et al., 2009). Our findings confirm these previous observations in that the dose used of FK506 improved graft survival but without any significant effects in locomotor recovery and tissue sparing.

Following a SCI, the spinal reflexes in segments caudal to the lesion become more excitable. The hyperreflexia is attributed to the loss of inhibitory descending pathways (Calancie et al., 1993) and changes in motoneurons excitability (Chen et al., 2001). Hyperreflexia was increased in MSC transplanted animals but not in immunosuppressed MSC grafted rats in comparison to control injured rats. Remarkably, in both MSC groups, the H reflex was higher than in the respective vehicle groups. However, the control animals that received FK506 did not show hyperreflexia after SCI, corroborating a direct effect of FK506 on the spinal reflex responses (López-Vales et al., 2005). In contrast, the mechanical but not the thermal hypersensitivity induced by the SCI was greater in the animals that received FK506 administration, independently of the MSC transplant. This observation is contrary to the findings of Voda et al. (2007) that daily injections of FK506 reduced the hypersensitivity to

mechanical stimulation. This discordance may be explained by the different doses used and the fact that these authors started FK506 administration before the injury. Nevertheless, in both studies FK506 treatment did not influence changes in heat stimuli perception. Altogether, these results suggest some effects of FK506 on the spinal circuitry after SCI, but the mechanisms have not yet been addressed.

Neuroprotective effects of MSC transplant combined with immunosuppression after SCI

Our histological results indicate that a MSC transplantation induced spinal cord tissue protection and reduction of the damage expansion, in agreement with previous reports (Ankeny et al., 2004; Nandoe Tewarie et al., 2009; Quertainmont et al., 2012). However, no differences were observed between both MSC grafted groups despite the addition of FK506. This may indicate that the main actions of the MSC for the protection of the injured tissue were done early after transplantation, when the grafted cells survival was the same with and without immunosuppression. Recently it has been demonstrated that autologous MSC survive better than allograft cells when transplanted after SCI in dogs, but no more protected tissue was found (Jung et al., 2009). On the other hand, immunosuppression allowed the formation of a dense and compact tissue filling the cavity formed after the cord contusion, which might be constituted by survived MSC and MSC-extracellular matrix, similar to what was seen in the dogs with a MSC autograft (Jung et al., 2009). Thus, the MSC can provide a scaffold for axonal growth (Hofstetter et al., 2002; Wright et al., 2007), that may improve the chances for late recovery suggesting that this tissue could support regenerating axons. Nevertheless, this tissue may also become a tumor (Ronsyn et al., 2007), an undesirable effect for clinical applications.

In conclusion, the findings of this study support the notion that allogeneic MSC grafts trigger an immune response in the hosts and that immunosuppression rescue engrafted MSC from immune rejection. Nevertheless, the longer graft survival only resulted in a limited enhancement of locomotor recovery, and did not increase tissue protection. In addition, the treatment with FK506 as an immunosuppressant agent may produce changes in the spinal circuitry caudal to the injury, that deserve further investigation. On the other hand, the tissue formed instead of the cavity with MSC under immunosuppression could act as a bridge for axonal regeneration, an interesting feature for combined therapies in long-term treatments. Considering these results, allogeneic MSC transplant plus immunosuppressive treatment may be a feasible strategy to improve the outcomes in regenerative therapies for SCI.

Acknowledgments

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RESULTS: CHAPTER 5

Neuroprotection and axonal regeneration after lumbar ventral root avulsion by reimplantation and mesenchymal stem cells transplant combined therapy

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ORIGINAL ARTICLE

Neuroprotection and Axonal Regeneration After Lumbar Ventral Root Avulsion by Re-implantation and Mesenchymal Stem Cells Transplant Combined Therapy

Abel Torres-Espín · Dora Luz Corona-Quintanilla · Joaquim Forés · Ilary Allodi · Francisco González · Esther Udina · Xavier Navarro

Abstract Ventral spinal root avulsion causes complete denervation of muscles in the limb and also progressive death of segmental motoneurons (MN) leading to permanent paralysis. The chances for functional recovery after ventral root avulsion are very poor owing to the loss of avulsed neurons and the long distance that surviving neurons have to re-grow axons from the spinal cord to the corresponding targets. Following unilateral avulsion of L4, L5 and L6 spinal roots in adult rats, we performed an intraspinal transplant of mesenchymal stem cells (MSC) and surgical re-implantation of the avulsed roots. Four weeks after avulsion the survival of MN in the MSC-treated animals was significantly higher than in vehicle-injected rats (45 % *vs* 28 %). Re-implantation of the avulsed roots in the injured spinal cord allowed the regeneration of motor axons. By combining root re-implantation and MSC transplant the number of surviving MN at 28 days post-injury was higher (60 %) than in re-implantation alone animals (46 %). Electromyographic tests showed evidence of functional re-innervation of anterior tibialis and gastrocnemius muscles by the regenerated motor axons only in rats with the combined treatment. These results indicate that MSC are helpful in enhancing neuronal survival and increased the regenerative growth of injured axons. Surgical re-implantation and MSC grafting combined had a synergic neuroprotective effect on MN and on axonal regeneration and muscle re-innervation after spinal root avulsion.

Keywords Ventral root · Motoneuron · Neuroprotection · Axonal regeneration · Stem cells · Surgical repair

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A. Torres-Espín · D. L. Corona-Quintanilla · I. Allodi · F. González · E. Udina · X. Navarro
Group of Neuroplasticity and Regeneration, Institute of Neurosciences, Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Bellaterra, Spain

D. L. Corona-Quintanilla
Centro Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala, Tlaxcala, Mexico

J. Forés
Hand and Peripheral Nerve Unit, Hospital Clínic i Provincial, Universitat de Barcelona, Barcelona, Spain

X. Navarro (✉)
Unitat de Fisiologia Mèdica, Edif. M, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain
e-mail: xavier.navarro@uab.cat

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Introduction

Avulsion of the spinal roots causes disconnection between the peripheral and the central nervous systems. The avulsion of dorsal roots leads to anesthesia, sensory dysfunction, and neuropathic pain in the affected limb, while ventral root avulsion (VRA) induces muscular denervation, atrophy, and paralysis. Moreover, the avulsion of the ventral roots results in a marked and progressive death of the axotomized motoneurons (MN) [1–3]. Nevertheless, surviving MN maintain their capacity for axonal re-growth [4–8], so that if the avulsed ventral root is surgically re-implanted into the spinal cord segment, MN can regenerate their axons and also have an increased probability of survival, particularly when re-implantation is performed soon after injury [9, 10]. In patients with brachial plexus avulsion, direct re-implantation of injured roots has allowed some functional recovery [11]. Similarly, in animal models of VRA at cervical and sacral levels, re-implantation of avulsed roots increased MN survival and allowed muscle re-

innervation although with limited functional recovery [10, 12–14]. However, the chances for functional recovery after lumbar VRA and surgery repair are very poor [14, 15]. The lower capacity of lumbar MN to re-innervate distal target muscles may be due to the long distance that surviving neurons have to re-grow [16, 17]. Besides the necessity of axons to re-innervate their target muscles, survival of the avulsed MN is fundamental. In order to prevent MN death most studies have focused on the neuroprotective role of different neurotrophic factors. Thus, exogenous application of brain-derived neurotrophic factor (BDNF) [15, 18] or glial cell line-derived neurotrophic factor (GDNF) [15, 19, 20] has been shown to partially promote survival of neurons and axonal regeneration after spinal root injuries. However, the efficacy of these factors depends on their continuous supply and periodic injections might be necessary. Another possibility is the transplantation of cells that secrete neurotrophic factors. Thus, after VRA local injection of stem cells that express BDNF and GDNF exerted protection against MN death and reduced astrocyte reaction [21].

Mesenchymal stem cells (MSC) are pluripotent stem cells that can differentiate into mesodermal tissue, such as bone, muscle, cartilage, and fat [22]. Transplantation of MSC has been demonstrated to produce beneficial effects in animal models of central and peripheral neural insults. Thus, after spinal cord injury [23–26] and brain stroke [27, 28] focal injection of MSC improves functional recovery and reduces tissue damage and neuronal death. Furthermore, in experimental peripheral nerve injuries, MSC grafts have the capacity to enhance axonal regeneration [29, 30].

As surgical re-implantation of avulsed roots is mandatory for allowing axons to regenerate along the peripheral nerve and reach muscles and then obtain functional recovery, and the results are generally poor or absent after lumbosacral root avulsion, the aim of this work was to complement the surgical repair of avulsed lumbar spinal roots with a MSC transplant into the injured segments of the spinal cord. The re-implantation of avulsed roots will establish reconnection of MN with the spinal nerves, whereas the transplanted MSC will facilitate neuronal survival and axonal regeneration. We demonstrate that either root re-implantation or MSC transplants have beneficial effects regarding MN survival, whereas combination of both strategies showed a synergistic effect and enhanced axonal regeneration.

Materials and Methods

Cell Cultures

MSC Cultures

Primary MSC cultures were obtained from P22 Sprague–Dawley rats. Rats were euthanized with carbon dioxide (CO_2). Tibias and femurs were placed on cool phosphate buffered saline (PBS), and epiphyses were removed. The diaphyses of bones were flushed with PBS and the marrow was homogenized mechanically. The cell mixture was filtered through a 70-μm nylon mesh and recovered by centrifugation for 10 min at 1500 rpm (231 g). The pellet was re-suspended in growth medium: alpha modification minimum essential medium (Life Technologies, Grand Island, NY, USA) supplemented with 20 % heat-inactivated fetal bovine serum (Lonza, Verviers, Belgium), 2 mM L-glutamine (Life Technologies) and 100 units/ml penicillin/streptomycin (Life Technologies, 100×); and plated in 100-mm culture dishes (Iwaki, Asahi Technoglass, Chiba, Japan) at 5×10^6 cells/cm^2. After 24 h, the supernatant containing non-adherent cells was removed and fresh medium was added. When the culture was near confluence, every 4–5 days, the cells were detached using PBS with 0.05 % trypsin (Life Technologies) and 0.04 % ethylenediaminetetraacetic acid (Sigma, St Louis, MO, USA) and re-plated at 5000 cells/cm^2. Cells were passaged 3–4 times, and expanded to 80–90 % confluence.

Fibroblast Cultures

Fibroblasts were obtained from P22 rat sciatic nerves. The epineurium was taken with the help of microscissors and fine forceps, and enzymatically dissociated in 1 ml Hank's salt solution (Ca^{2+} and Mg^{2+}-free) with the addition of 0.25 % trypsin, 1 mg/ml collagenase A and 1 mg/ml of DNAse-I at 37 °C for 1 h. Then, the tissue was dissociated mechanically with a Pasteur pipette, and 13 ml DF10S medium was added to stop the enzymatic reaction. After centrifugation at 900 rpm (83 g) for 10 min, the pellet was re-suspended in 1 ml DF10S medium and cells were counted in a Neubauer chamber. Cells were plated at a concentration of 350–500 cells/mm^2, and maintained at 37 °C and 5 % CO_2, with changes of the medium every 3 days.

Cell Labeling

For pre-labeling the cells we used a lentiviral vector encoding green fluorescence protein (GFP) under THE EF1α promoter. The cells in passage 2 were plated at 2000 cells/cm^2 and incubated with the lentivirus at a multiplicity of infection of 10 for 48 h. Then, the medium was changed and the cells cultured as described above. The cells for transplantation were detached and re-suspended in L15 medium (Life Technologies) at 50,000 cells/μl—the maximal cell concentration that we found in pilot assays that does not compromise cell survival during injection—and maintained in ice during the surgery.

Spinal Cord Slices Culture

Sprague–Dawley rats at day 7 post-natally were decapitated and the spinal cord lumbar segments were dissected, placed in cold Gey's balanced salt solution (Sigma) enriched with 6 mg/ml glucose, and cleaned from blood and meningeal debris. Spinal cords were then cut with a McIlwain Tissue Chopper (Mickle Laboratory Engineering, Surrey, UK) into 350-μm thick slices. A volume of 450 μl of type I collagen solution (BD Biosciences, Erembodegem, Belgium) at a concentration of 3.4 mg/ml supplemented with 10 % fibronectin (BD Biosciences) was mixed with 50 μl of 10× basal Eagle's medium (Life Technologies) and 2 μl of 7.5 % sodium bicarbonate solution (Life Technologies) [31]. Single 30-μl drops were deposited on poly-d-lysine- (1μg/ml; Sigma) coated coverslips, which were placed in Petri dishes or 24-well multidishes (Iwaki) and kept in the incubator at 37 °C and 5 % CO_2 for 2 hours to induce collagen gel formation. Spinal cord slices were then placed on gelled collagen droplets and covered by a second 30-μl droplet of the same collagen solution. The embedded samples were incubated with Neurobasal medium (Life Technologies), supplemented with B27 (Life Technologies), glutamine and penicillin/streptomycin (Sigma). After 1 day in culture, the medium of spinal cord cultures was changed by a penicillin/streptomycin-free medium. Spinal cord slices were cultured for 4 days and fixed with 4 % paraformaldehyde at 4 °C for 20 min, and immunostained as described previously [31] to assess neurite growth. For MSC or fibroblast co-culture, an adequate amount of GFP-labeled cell suspension in culture medium was gently mixed into the collagen matrix to get a final density of 5×10^4 in each volume of matrix used to embed the spinal cord slice.

Analysis of Neurite Outgrowth

Spinal cord cultures were stained with anti-neurofilament antibody RT97 (1:200, Developmental Studies Hybridoma Bank) in order to label neurons and growing neurites. Microphotographs for quantitative analysis were taken at 20× with a digital camera (Olympus DP50; Olympus, Hamburg Germany) attached to the microscope (Olympus BX51), acquired in Adobe Photoshop CS4 to photomerge them automatically, and analyzed with the aid of ImageJ software (National Institutes of Health; available at http://rsb.info.nih.gov/ij/). The conventional Sholl [32, 33] method was adapted for spinal cord slice cultures to count the number of neurites crossing concentric circles at specific lengths from the ventral root exit. The length of the longest neurite in the cultures was also measured for at least 15 samples per condition. For the arborization area, the microphotographs were transformed to a gray 8-bit image, and the labeled area was assessed after defining a threshold for background correction using ImageJ software.

Surgical Procedure and Cell Transplantation

A total of 75 adult female Sprague–Dawley rats (9 weeks old; 250–300 g) were used in this study and housed with free access to food and water at a room temperature of 22±2 °C under a 12:12 hour light–dark cycle. The experimental protocols were approved by the animal ethics committee of the Universitat Autonoma de Barcelona in accordance with the European Directive 86/609/EEC. In the root avulsion alone study, the animals were distributed randomly in avulsion (AV, n=15), avulsion and vehicle injection (n=12) or avulsion and MSC transplantation groups (AV-MSC, n=12). In the avulsion and surgical repair study, the animals were assigned to avulsion with direct re-implantation and vehicle injection (AV-RE, n=15) or avulsion with direct re-implantation and MSC transplantation (AV-RE-MSC, n=15). Moreover, to follow the MSC transplanted into the spinal cord, 6 animals were injected with GFP^+-MSC after VRA.

Under anesthesia with ketamine (90 mg/kg) and xylacine (10 mg/kg), a unilateral intravertebral avulsion of L4, L5, and L6 roots was done as described previously [1, 3]. Briefly, a midline skin incision was made and the paravertebral muscles were retracted to expose the spinal column. A unilateral laminectomy was made at the right side and L4, L5, and L6 ventral and dorsal roots were identified. A moderate traction was applied, detaching both the ventral and dorsal roots from the spinal cord. In the avulsion groups, the roots were withdrawn approximately 5 mm to avoid axonal re-growth. In the avulsion-re-implanted groups, the three ventral and dorsal root stumps were inserted, just after avulsion, in the spinal cord lateral surface of the corresponding segments and secured with a 10–0 suture. In the injected groups, a total of 3 μl of vehicle (L15) or MSC suspension (50,000 cells/μl) was injected into the lateral funiculus of the avulsed cord. Using a glass needle (100 μm internal diameter; Eppendorf, Hamburg, Germany) coupled to a 10 μl Hamilton syringe, three 1-μl injections were performed, each one into each avulsed spinal cord segment. A perfusion speed of 2 μl/min was controlled by an automatic injector (KDS 310 Plus; Kd Scientific, Holliston, MA, USA), and the needle tip was maintained inside the tissue 3 min after each injection to avoid leaking. The wound was sutured by planes and the animals allowed to recover in a warm environment. At 2 weeks after surgery rats were evaluated by behavioral locomotion and electrophysiological techniques in order to confirm the deficits caused by L4–L6 root avulsion in the hindlimb. If the behavioral-electrophysiological tests

suggested an incomplete injury of L4, L5, and L6 roots, the animal was not included in the study. Thus, a total of 10 animals was excluded from the experiments.

Behavioral Tests

The locomotor behavior of the operated animals was monitored weekly. A subscale of the Basso, Beattie and Bresnahan (BBB) locomotor score [34] relevant for the lumbar root avulsion model was used for evaluating the hindlimb function, as described previously [3, 20]. The voluntary movements of the ankle, knee, and hip joints of the avulsed limb were evaluated, with the scores for each being (0) = no spontaneous movement of the joint, (1) = slight movement of the joint and (2) = normal movement of the joint. The final score for the avulsed limb was the accumulative scores for the 3 joints. The normal score for an intact limb was 6 points. The toe-spreading response was also assessed while lifting the rat by the tail.

Electrophysiological Tests

All the animals were tested at 2 weeks in order to characterize the extent of muscle denervation of the hindlimb muscles after VRA; in addition, rats with root reimplantation repair were also evaluated at 4 and 8 weeks after the injury to assess re-innervation of target muscles. For the electrophysiological tests, animals were anesthetized with pentobarbital (30 mg/kg i.p.), placed prone onto a metal plate and skin temperature maintained above 32 °C. Motor-evoked potentials (MEPs) were elicited by transcranial electrical stimulation of the brain. Two needle electrodes were placed subcutaneously over the skull—the anode over the sensorimotor cortex and the cathode on the hard palate. Single electrical pulses of supramaximal intensity (25 mA, 100 μs) were applied, and the MEPs were recorded with monopolar needle electrodes from tibialis anterior (TA) , gastrocnemius medialis (GM), and interossei plantar (PL) muscles [35, 36]. Peripheral motor nerve conduction tests were performed by stimulating the sciatic nerve with single electrical pulses (100 μs at supramaximal intensity) delivered by monopolar needles placed at the sciatic notch and recording the compound muscle action potentials (CMAP) of TA, GM, and PL muscles by means of needle electrodes. The active electrode was inserted on the belly of the muscle and the reference at the fourth toe [3, 33]. Signals were amplified, filtered (bandpass 1–5000 Hz), displayed on an oscilloscope (Sapphire 4ME; Medelec-Vickers, Woking, Surrey, UK) and measured.

Retrograde Axonal Tracing

One week before sacrificing the animals of the re-implantation study, the retrotracer FluroroRuby (Life Technologies) was applied in the sciatic nerve. Under anesthesia with ketamine/xylacine (90/10 mg/kg i.p.) both left and right sciatic nerves were exposed at the mid-thigh. The sciatic nerve was cut and the proximal stump submerged in 5 % FluoroRuby solution for 1 h. Then, the area was flushed with saline and the wound sutured by planes.

Tissue Processing for Histology

The end time point of the avulsion and vehicle injection and AV-MSC groups was at 28 days post-operation (dpo) (n=10 for each group), while the animals of groups AV, AV-RE, and AV-RE-MSC were divided and the tissues processed at 28 (n=8 for each group) and 52 dpo (n=5 for each group). For animals injected with GFP^+-MSC the end time points were 7, 14 and 28 dpo (n=2 for each). The rats were anesthetized deeply (pentobarbital 60 mg/kg i.p.) and intracardially perfused with 4 % paraformaldehyde in PBS. The spinal cord L4, L5, L6, and S1 segments (1 cm total length) and the sciatic nerve from lumbar plexus to sciatic notch were harvested and post-fixed in the same fixative solution for 24 h and cryopreserved in 30 % sucrose. Coronal spinal cord sections 30 μm thick were cut with a cryostat and distributed in 10 series of 8 sections (separated by 440 μm) each. The sciatic nerves were cut at 15 μm and distributed in 5 series of 8 sections (separated by 75 μm). For morphological evaluation of sciatic nerve cross-sections, a segment of the nerve at the mid-thigh was harvested and post-fixed in glutaraldehyde/paraformaldehyde (3 %/3 %) in cacodylate buffer solution (0.1 M, pH 7.4) overnight at 4 °C, post-fixed in 2 % osmium tetroxide, dehydrated through ethanol series, and embedded in epon (Electron Microscopy Sciences, Hatfield, PA, USA) resin. Transverse semithin sections (0.5 μm thick) were cut with an ultramicrotome, stained with toluidine blue, and examined by light microscopy.

Motoneuron Number Counts

One series of 8 sections of the spinal cord was incubated for 20 min with fluorescence Nissl labeling solution (Life Technologies) following the manufacturer's protocol. The number of alpha MN present was estimated by the stereological optical dissector method as described elsewhere [2, 3]. MN were identified by their localization in the lateral ventral horn of lumbar spinal cord sections and only MN with diameters higher than 30 μm, a prominent nucleolus, and polygonal shape were counted. Sequential microphotographs covering the lateral ventral horn were taken at 400× and a 30-μm square grid was superimposed onto each one [37]. Size exclusion was used to selectively count the population of alpha MN avoiding inclusion of gamma MN, interneurons, and glial cells in the counts. In the case of re-

implanted animals, the total number of FluoroRuby retrolabeled neurons in each section was also counted. The mean number of MN per section was calculated. For comparisons, the estimated number of MN present in the ventral horn of the avulsed side was expressed as a percentage of the contralateral side.

Immunohistochemistry and Image Analysis

Sections from animals of the different groups taken at different time points were processed in parallel for immunohistochemistry. Tissue sections were blocked with tris-buffered saline-0.3 % Triton-5 % fetal bovine serum and incubated for 2 days at 4 °C with primary antibodies goat anti-choline acetyltransferase (ChAT) (1/50; Millipore, Billerica, MA, USA) to label motor fibers and mouse anti-glial fibrillary acidic protein (GFAP) (1/1000; Dako, Glostrup, Denmark) to label astrocytes. After washes, sections were incubated for 2 h at room temperature with biotinylated conjugated donkey anti-goat or anti-mouse antibodies (1/200; Vector, Burlingame, CA, USA) and followed by 2 h with AlexaFluor 488 or AlexaFluor 565-conjugated streptabidine (1/200; Jackson Immunoresearch, West Grove, PA, USA). Slides were dehydrated and mounted with Citoseal 60 (Thermo Fisher Scientific, Madrid, Spain). The sections of GFP^+-MSC-injected rats were mounted directly without processing after cutting. Images were captured with a digital camera (Olympus DP50) attached to a microscope (Olympus BX51). Analysis of glial reactivity was performed using 10 spinal cord sections (440 μm between pairs) of each animal. Images of the ventral area of the spinal cords were taken at 400× with the same setting. The microphotographs were transformed to a gray scale and analyzed using ImageJ software. Immunoreactivity was assessed by calculating the integrated density of a region of interest measured after defining a threshold for background correction. The regions of interest were selected on the gray matter of the ventral horn and had an area of 0.34 mm^2. The integrated density is the area above the threshold for the mean density minus the background. To count the number of ChAT + fibers in the nerve sections, images at 400× were taken of 1 series of 8 sections. The mean number of fibers per section was calculated in proximal, medial, and distal parts of the studied nerve segment.

Data Analysis

Data are expressed as mean values and the standard error. For statistical analysis one way analysis of variance was used for parameters measured at only one time point, and two way analysis of variance for repeated measures for time-depending parameters. Post hoc Bonferroni test for comparative pairs of groups was used. In all the comparisons, an α of 5 % was considered as significant.

Results

Spinal Cord Organotypic and Cell Co-cultures

As *in vitro* approximation of MN axotomy, we cultured spinal cord slices in a three-dimensional matrix in the presence of MSC, fibroblasts or control vehicle. Figure 1 shows representative micrographs of the MSC and control cultures where neurites from MN growing outside the spinal cord are observed. After 4 days in culture, the presence of MSC significantly increased the amount of axons that grew a longer distance from the spinal cord exit ($p<0.05$ between 300 and 550 μm) (Fig. 1g). The maximal length of the growing neurites was also higher in the MSC co-cultures than in controls (956 μm±87 μm *vs* 648 μm±70 μm, 149 % ±15 with respect to control, $p=0.01$). There was also an increase, although not significant, in the arborization of neurites—a relative measure of the amount and branching of neurites. While the presence of MSC enhanced neurite outgrowth, no significant differences were observed between fibroblast co-culture and control cultures regarding neurites number ($p>0.05$ for all the distances), maximal length (91 %±15 % of control, $p=0.76$) and arborization (182 %±29 % with respect to control, $p=0.11$). These observations indicate that MSC have the intrinsic capability to enhance regeneration of motor axons, while fibroblasts do not affect motor axons growth. Interestingly, neurites that grew out of the spinal cord slice into the three-dimensional matrix plus MSC regenerated without close association with MSC (Fig. 1c, d), indicating that direct contact between cells and axons is not needed.

MSC Transplantation After VRA

GFP^+-MSC were found in the injured side of L4, L5, and L6 spinal segments at 7 days after injection (Fig. 2b). Most cells migrated from the injection site and were localized in the ventral root exit zone. At longer times the GFP labeling was reduced inside the spinal cord and no migration out of the injury area was observed. Thus, at 14 days after injury (Fig. 2c) the amount of GFP was less than at 7 days, whereas at 28 days only a few GFP-positive cells were found (Fig. 2d, e).

Using fluorescence Nissl staining we counted the number of MN in coronal sections of L4, L5, and L6 segments of the spinal cord in both the avulsed and the contralateral side. In the intact animals, the number of MN was similar in both sides of the spinal cord. One month after VRA, only about 30 % of the MN were present in the injured spinal cord segments of non-treated rats, while animals transplanted with MSC had a significantly higher number of surviving MN (28.9 %±4.4 % and 46.8 %±4.3 % for AV and AV-MSC groups respectively) (Fig. 2f–i).

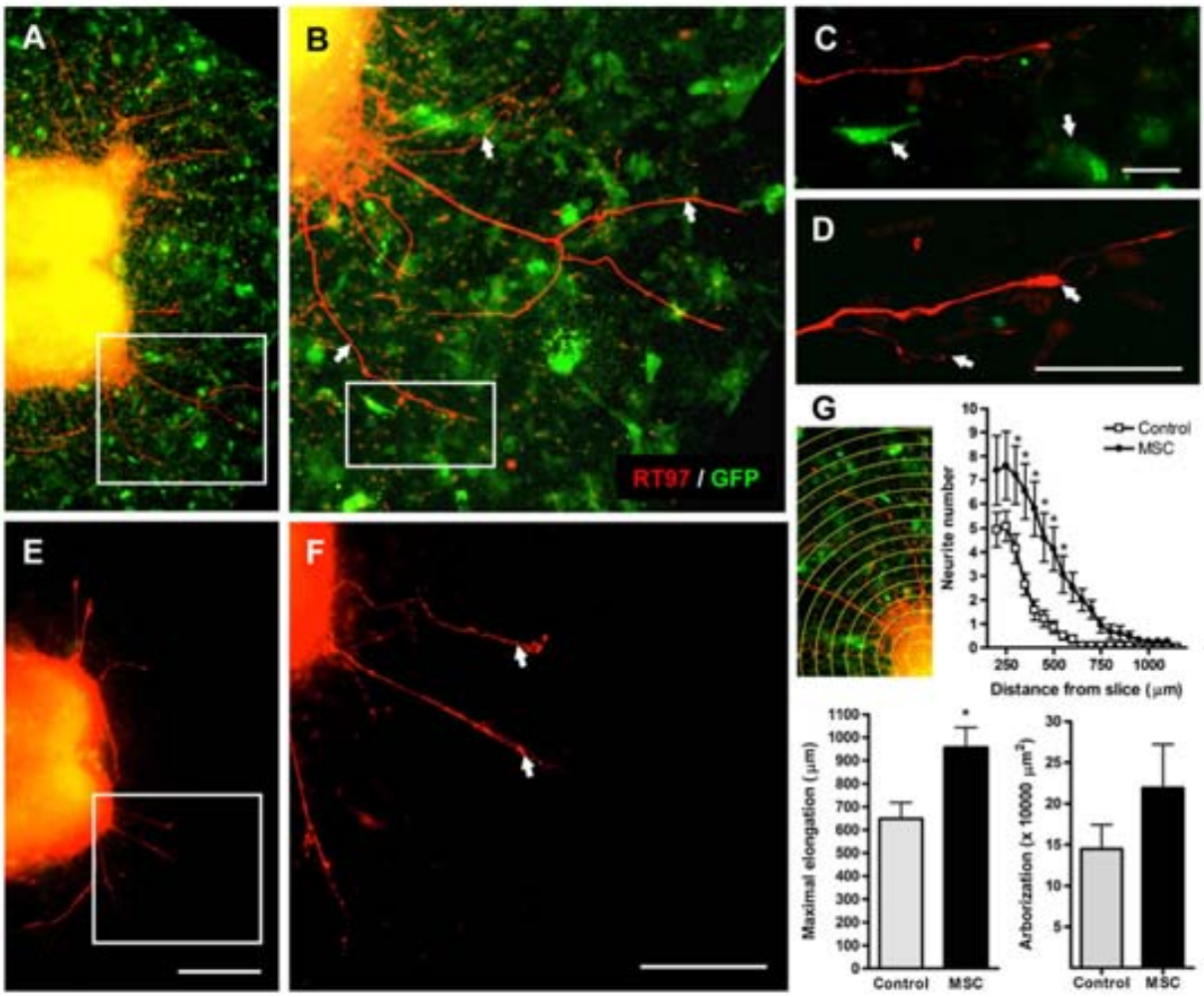


Fig. 1 Organotypic culture of spinal cord slices and co-culture with mesenchymal stem cells (MSC). After 4 days *in vitro* neurites grow longer out of the spinal cord in the three-dimensional collagen matrix (arrows in B and F, immunolabeling of RT97 in red) in green fluorescence protein (GFP) + MSC co-culture (**a**, **b** is higher magnification image of the box in **a**) than in control condition (**e**, higher magnification in **f**). (**c**), (**d**) (higher magnifications of box in **b**) show the tip of a regenerating neurite (arrow in **d**) that grows without contacting the MSC (arrow in **c**). Quantification of the number of neurites that grew to specific distances from the spinal cord slice (**g**, top graph), counted using the Sholl method, the maximal elongation (**g**, bottom left graph), and arborization (**g**, bottom right graph) of the neurites shows an increased neurite growth in co-culture of MSC and spinal cord slices with respect to culture alone. Scale bar = 500 μm in (**a**) and (**e**), 250 μm in (**b**) and (**f**), and 50 μm in (**c**) and (**d**)

The immunoreactivity of GFAP was measured in the same lumbar segments to quantify the astrocyte reaction. Because no changes were found between injured segments in each animal, we represent the mean values of the 3 segments. At 28 days after VRA we found an increased GFAP immunoreactivity in the ipsilateral side compared with the contralateral intact side, indicative of hypertrophy and reactivity of astrocytes. The animals treated with MSC showed significantly reduced GFAP immunoreactivity than non-treated rats (Fig. 2j–m), with levels similar to intact animals (200.8 %±37.9 and 96.4 %±6.2, for AV and AV-MSC groups respectively).

MN Survival After Root Re-implantation and MSC Transplant

We used fluorolabeling against Nissl bodies to identify the MN in both re-implantation plus vehicle and re-implantation plus MSC groups at 28 and 56 days after the injury (Fig. 3). Re-implantation of the avulsed roots increased MN survival (46.3 %±4.2 %) compared with animals with avulsion alone at 28 days. The number of MN at the end of the follow-up was reduced significantly compared with the counts at 4 weeks, suggesting a retardation in MN death (Fig. 3f). At this time point, the number of surviving MN with re-

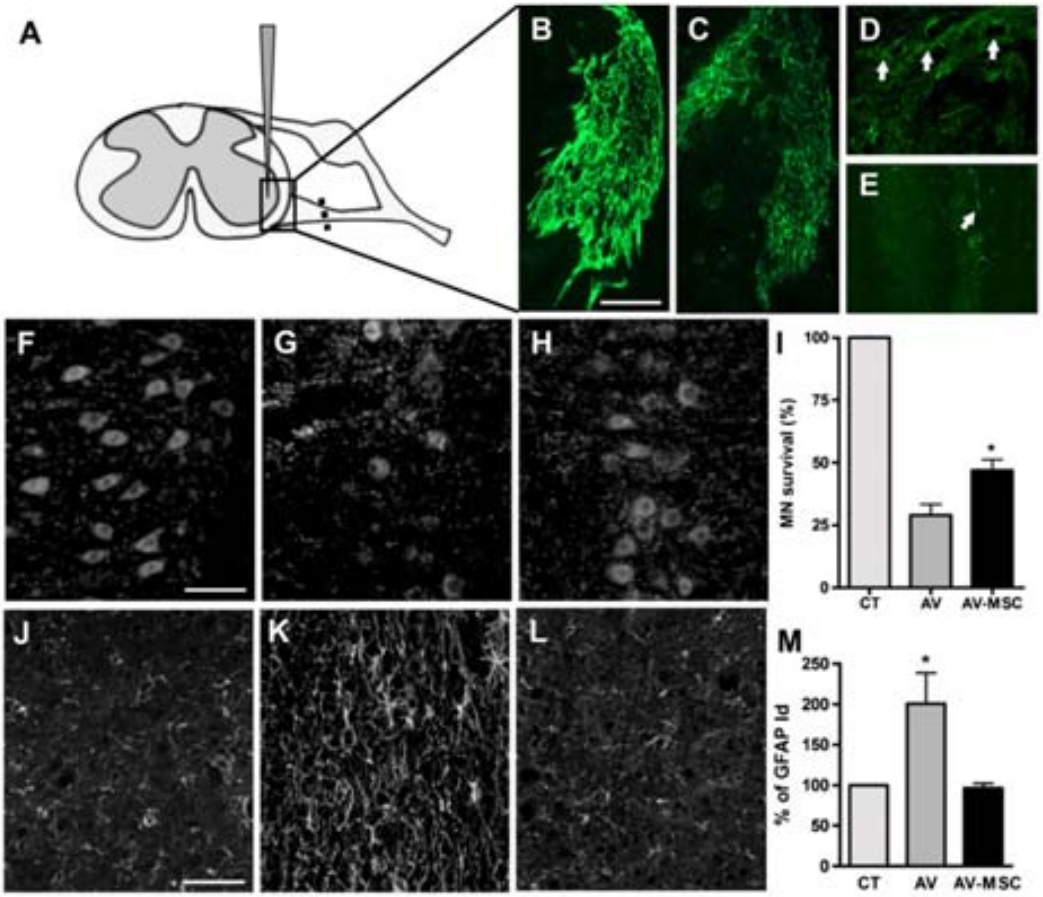


Fig. 2 Mesenchymal stem cell (MSC) transplantation, motoneuron (MN) survival, and astrogliosis after ventral root avulsion. Diagram of the site of intraspinal injection after injury (**a**). Green fluorescence protein $(GFP)^+$ MSC were found 1 week after treatment (**b**) in the injection site (box in **a**). A reduction of GFP labeling was observed at 2 weeks (**c**), and at 4 weeks only a few GFP^+ cells were found (**d** and **e**, arrows). Representative micrographs showing MN labeled with fluorescence Nissl stain of intact (**f**), root avulsed (**g**, group AV), and avulsed plus MSC graft (**h**, group AV-MSC) rats. Number of surviving MN (**i**), represented as percentage of MN in the avulsed side with respect to the non-injured side, show significant preservation by the MSC transplant. Immunohistochemistry against glial fibrillary acidic protein (GFAP) was used to assess the degree of astrogliosis. GFAP immunoreactivity, calculated as percentage of integrated density in the avulsed side (**k**: group AV; **l**: group AV-MSC) with respect to the non-injured side (**j**), show a significant increase after root avulsion that was prevented by the MSC transplant. *$p<0.05$ group AV versus group AV-MSC. Scale bar = 200 μm

implantation (10.4 %±0.3 %) was similar to that of non-repaired rats (9.8 %±1.9 %). Thus, early re-implantation of the avulsed roots enhances survival of MN during the first month after surgery, but is not effective to prevent the delayed MN death. The combination of root re-implantation with MSC injection provided a significant increase in MN survival at 4 weeks (60.9 %±7.4 %) and also at 8 weeks (26.2 %±7.1 %) in comparison with re-implantation repair alone and non-treated VRA (Fig. 3f).

Motor Axon Regeneration After Root Re-implantation and MSC Transplant

To assess axonal growth in the avulsed and re-implanted roots, immunohistochemistry for ChAT in samples of the sciatic nerve was performed. We observed ChAT + fibers in the sciatic nerve segment from the lumbar plexus to the sciatic notch level, confirming regeneration of motor fibers. In this segment (length = 2 cm), the numbers of ChAT + fibers per section were counted at 3 points—in the proximal, medial, and distal parts. Contralateral intact sciatic nerves showed a homogeneous distribution and a large number of fibers (Fig. 4a). In the injured nerve, 4 weeks after root re-implantation, the number of ChAT+fibers was lower in both groups, with and without MSC transplant, (Fig. 4b, c) than in contralateral intact nerves. At the same time, in the sciatic nerve of the animals with VRA without re-implantation no ChAT+fibers were found, confirming the inability of axonal regeneration without repair of the avulsed roots. A decrease in the number of ChAT+fibers was observed from proximal

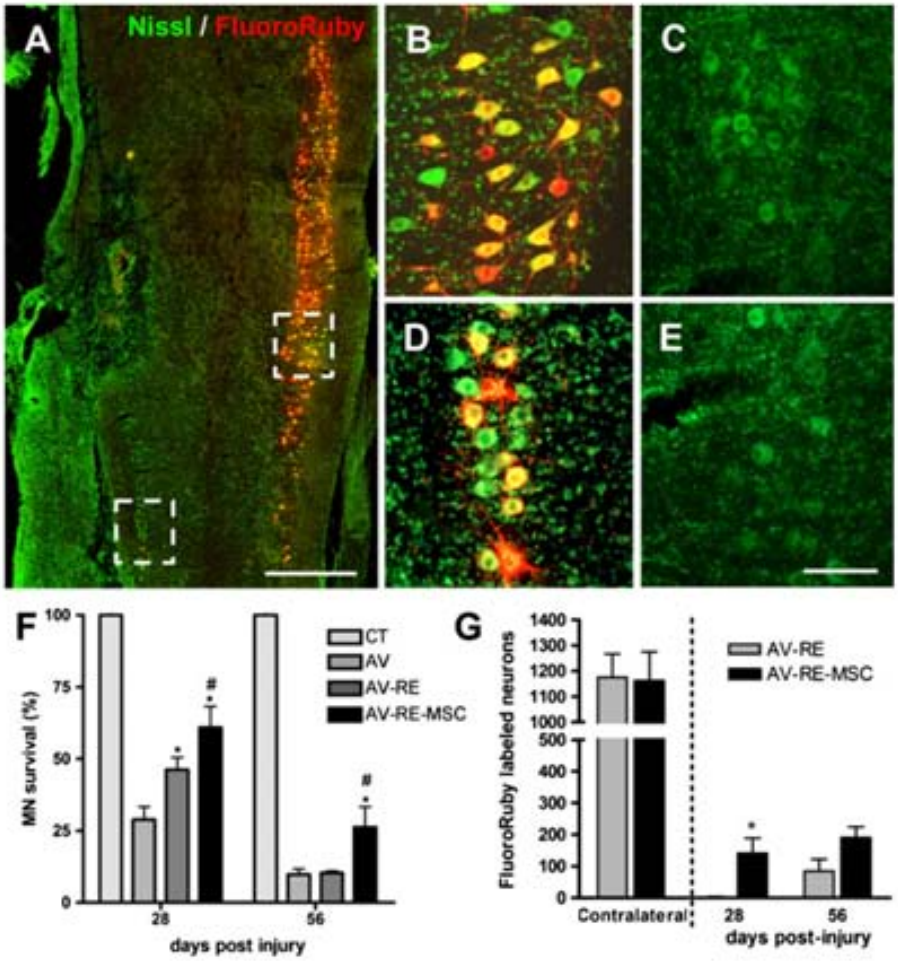


Fig. 3 Motoneuron (MN) survival and axonal projection after root avulsion and re-implantation. Spinal MN were labeled with fluorescence Nissl stain (in green), and MN that were able to project their axons to the sciatic nerve (8 cm distance) were also retrolabeled with FluoroRuby (in red). Co-labeling (in yellow) shows the surviving MN that regenerated their axons in the sciatic nerve. **a** Low magnification micrography of an injured animal showing the non-injured side (right) and the injured and re-implanted side (left). **b** Magnification of the right-hand box in (**a**) showing the intact MN of the contralateral side. Magnification of the left-hand box in (**a**), injured side, **c** of a rat of group avulsion with direct re-implantation (AV-RE), **d** of a rat of group AV-RE-mesenchymal stem cells (MSC), and (**e**) of a rat of group avulsion (AV). Scale bar = 1 mm in (**a**) and 200 μm in (**b**–**e**). The percentage of MN survival was increased significantly by root re-implantation and further increased when combined with a MSC transplant (**f**). *$p<0.05$ AV versus other groups, #$p<0.05$ AV-RE-MSC versus AV-RE. The number of MN labeled with FluoroRuby was increased in animals of group AV-RE-MSC compared with group AV-RE (**g**). *$p<0.05$ AV-RE-MSC versus AV-RE

to distal along the studied nerve segment in both re-implanted groups. Although more axons were found in group AV-RE-MSC than in group AV-RE at 4 weeks at mid and distal levels, the differences were not significant. At 8 weeks the number of ChAT + fibers in both re-implanted groups was similar than at 4 weeks in the 3 distances measured, and no differences between groups were found (Fig. 4f).

To quantify the number of MN that regenerated their axons in the re-implanted root to the sciatic nerve we applied FluoroRuby retrotracer at the sciatic nerve 8 cm from the spinal cord. In the control re-implanted group, we observed back-labeled MN in the injury side only at 8 weeks, indicating that some motor axons were able to extend 8 cm distal to the spinal cord, whereas the animals of the re-implanted plus MSC group already had some labeled MN at 4 weeks (Fig. 3d), and slightly increased the number at 8 weeks (Fig. 3g). These data indicate that axonal growth was faster in the presence of MSC in the spinal cord, at least during the first 4 weeks. When comparing the number of regenerating MN with that of surviving MN measured by Nissl staining, 20 % of the MN were able to project their

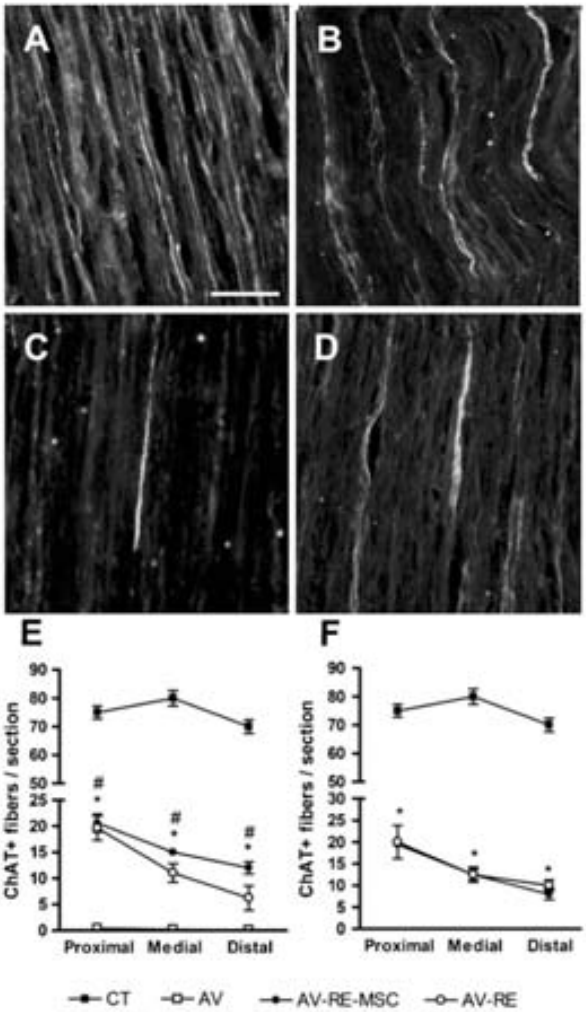


Fig. 4 Choline acetyltransferase-positive (ChAT+) motor fibers regenerated in the segment from lumbar plexus to sciatic notch. In the contralateral non-injured nerve (**a**) there was a large number of ChAT + motor fibers with a longitudinal and parallel arrangement. After ventral root avulsion and re-implantation some motor fibers were found into the proximal (**b**) and the distal (**c**, **d**) part of the studied nerve segment at 28 days. Quantification of the number of motor axons per section at 28 days (**e**) and 56 days (**f**) showed that the number of ChAT + fibers in the injured side decreased with the distance from the spinal cord. In the ventral root avulsion without re-implantation group no motor fibers were observed at 28 days after injury. $^{\#}p<0.05$ avulsion (AV) group versus avlusion with direct re-implantation (AV-RE) and AV-RE-mesenchymal stem cell (AV-RE-MSC) groups. $^{*}p<0.05$ contralateral (CT) versus avulsed groups. Scale bar=200 μm

axons at 4 weeks and 43 % at 8 weeks in the MSC transplanted animals, whereas in the re-implantation alone group 60 % of surviving MN (about 2.5 times less in number) were labeled with FluoroRuby at 8 weeks. The higher proportion of regenerating MN in the re-implantation alone group with respect to the MSC-treated animals is a consequence of the lower MN survival in the former group. These results suggest that the grafted MSC accelerated axonal growth, but at 8 weeks after treatment the effect of the cells was more important on MN survival than on axonal regeneration. Nevertheless, more MN regenerated their axons in the MSC-injected group than in the re-implantation alone group.

In the distal part of the sciatic nerve, taken at 8–9 cm from the spinal cord, a histological study of cross-sections was performed (Fig. 5). After VRA there were areas in the tibial (Fig. 5a, c, e) and peroneal (not shown in detail) branches with a high density of non-injured axons, with morphological appearance and myelin sheaths similar to axons of intact nerves (Fig. 5b, d). In other distinct areas, which corresponded well for the tibial nerve to the location of motor fascicles [38], there were a few axons preserved, many figures of axonal degeneration, and some phagocytizing macrophages (Fig. 5c, f). Similar evidences of denervation were observed in areas of the peroneal nerve, but not in the sural nerve (Fig. 5a, see 'S'), indicative of the preservation of sensory axons. At 4 and 8 weeks after root re-implantation and MSC injection, myelinated fibers of small and medium size, clustered in small fascicles, and frequent small endoneurial vessels were present in the degenerated area corresponding to regenerative units [39, 40] (Fig. 5g), while in the re-implantation alone rats such regenerative units were found only at 8 weeks.

Electrophysiological Outcome After Root Re-implantation and MSC Transplant

We stimulated at the sciatic nerve and at the brain levels to evoke CMAPs and MEPs, respectively, of GM, TA, and PL muscles. After L4–L6 root avulsion, we still recorded a CMAP component with normal latency and small amplitude in the TA and GM muscles, an artefact consequence of cross-linking with non-denervated proximal muscles (Fig. 6, white arrowhead). Four weeks after root avulsion, some animals of the AV-RE-MSC group presented a second CMAP that was polyphasic, with a long latency and very low amplitude in TA and GM muscle recordings (Fig. 6, black arrowhead), indicative of muscle re-innervation [11, 40, 41]. At 8 weeks all the transplanted animals showed responses of re-innervation in the TA and GM muscles, whereas the PL muscle remained completely denervated. In the group AV-RE with re-implantation alone, a few animals showed evidence of re-innervation only at 8 weeks after injury. The amplitudes of polyphasic CMAPs in MSC-treated rats averaged 260 μV±96 μV in TA and 236 μV± 118 μV in GM muscle at 4 weeks, and 263 μV±95 μV for TA, and 276 μV±30 μV for GM muscle at 8 weeks (Fig. 6), being, at the two time points, higher in the MSC-treated group than in the control re-implanted group.

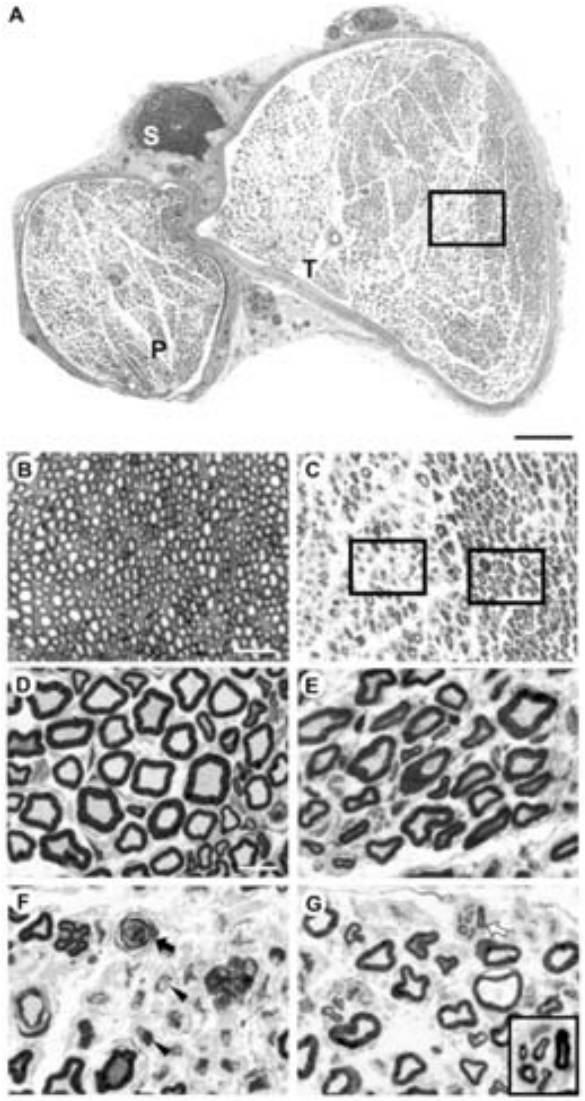


Fig. 5 Histology of the distal sciatic nerve. **a** Section of the distal sciatic nerve showing the division in tibial (T), peroneal (P), and sural (S) branches. The distribution of myelinated axons in intact tibial nerves was homogeneous (**b** and higher magnification in **d**), while in avulsed nerves (**a**, box magnified in **c**) there were distinct areas regarding the morphological appearance of the myelinated fibers. Some areas were occupied by non-injured axons (right-hand box in **c** and magnification in **e**), similar to an intact nerve (**b** and magnification in **d**). In other areas there were overt signs of axonal degeneration (left-hand box in **c** and magnification in **f**), showing empty endoneural tubules (black arrowhead in **f**) and degenerating axons (black arrow in **f**). In these areas there were also clusters of small myelinated axons (white arrow in **g**, further magnified in the inset) with typical characteristics of regenerative units. Scale bar = 100 μm in (**a**) 50 μm in (**b** and **c**), and 10 μm in (**d**–**g**)

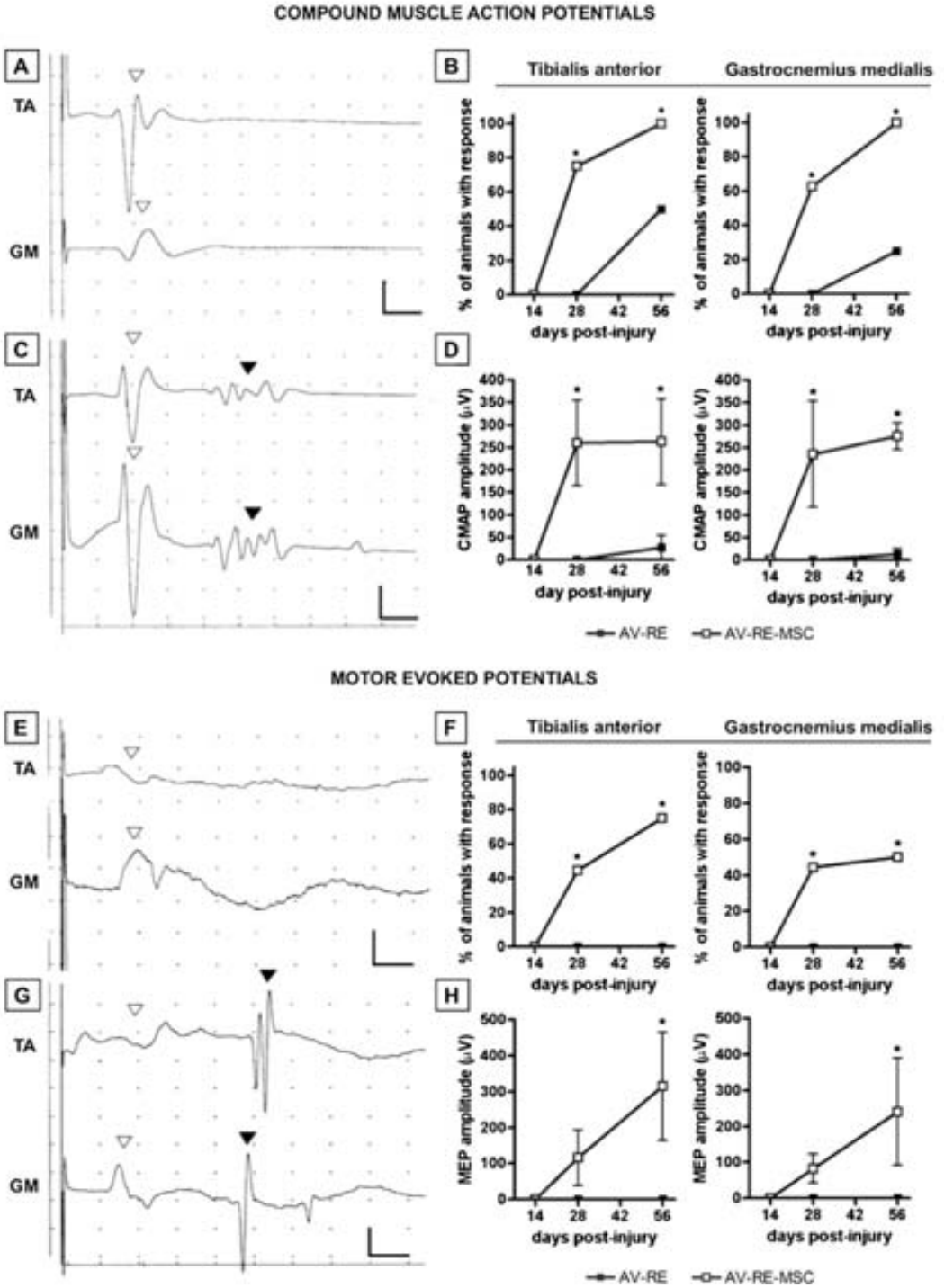
Regarding the MEPs we found a polyphasic response of delayed latency at 4 weeks after injury only in MSC transplanted animals (Fig. 6). At 8 weeks, only 1 animal of the AV-RE group presented a polyphasic re-innervation potential in both TA and GM muscles. However, MEPs were recorded in most MSC-treated rats. The polyphasic MEPs increased in amplitude from 4 weeks to 8 weeks in the AV-RE-MSC group and were significantly higher than in control re-implanted rats. These results indicate that after root re-implantation and MSC transplantation some motor axons were able to reach their targets and re-innervate the muscles earlier, and in a considerably higher proportion of animals than in non-transplanted rats.

To assess functional recovery after treatments, a score of hindlimb movement was used (Fig. 7). After injury, the animals lost the ability to place the paw of the avulsed limb and toe-spreading responses. Moreover, the mobility of ankle and knee joints were lost and reduced at the hip joint. Despite the electrophysiological outcome found after cell transplant, no significant recovery of the hind paw motion was observed in any group during the follow-up, except for a mild recovery in the capacity of placing the paw on the ground.

Discussion

The avulsion of ventral roots from the spinal cord surface causes massive loss of MN, with 60–80 % death after 3–4 weeks [1, 3]. Strategies aimed to enhance survival of MN are mandatory as a first step in attempts to permit functional recovery after these injuries, which usually have devastating effects in the motor control of the affected territories. In this study we show that a focal transplantation of MSC, without other intervention, increases MN survival by 46 % at least 4 weeks after VRA. Furthermore, when the MSC transplant was combined with direct re-implantation of the avulsed roots, neuronal survival increased and was accompanied by enhancement of axonal regeneration through the re-implanted roots.

Fig. 6 Electrophysiological results after root avulsion and re-implantation. Results of compound muscle action potentials (CAMP) (**a**–**d**) and motor-evoked potentials (MEP) (**e**–**h**) recorded in tibialis anterior (TA) and gastronemius medialis (GM) muscles at 14, 28 and 56 days after injury and re-implantation. In all the recordings a remnant, cross-talk response from proximal muscles was observed (white arrowhead). A second polyphasic component characteristic of re-innervation (black arrowhead) was found in most animals treated with mesenchymal stem cells (MSC) at 28 days after injury (**c**, **g**). At the same time, re-innervation responses were not found in animals without MSC (**a**, **e**). For both CAMP and MEP, the percentage of animals with re-innervation responses (**b**, **f**) and the amplitudes of each response (**d**, **h**) were increased by a MSC transplant. *$p<0.05$ avulsion with direct re-implantation (AV-RE) versus AV-RE-MSC. In (**a**) and (**e**), vertical bar = 500 μV, horizontal bar = 20 ms. In (**e**) and (**g**), vertical bar = 100 μV, horizontal bar = 50 ms


COMPOUND MUSCLE ACTION POTENTIALS
A
TA
GM
B
Tibialis anterior
Gastrocnemius medialis
% of animals with response
days post-injury
C
D
CMAP amplitude (μV)
day post-injury
AV-RE
AV-RE-MSC
MOTOR EVOKED POTENTIALS
E
F
G
H
MEP amplitude (μV)

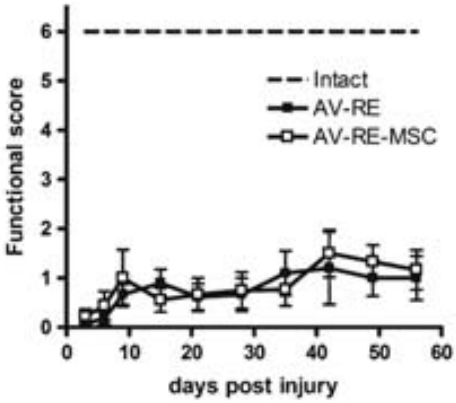


Fig. 7 Functional score of hindlimb movements. Intact animals had 6 points in the score scale (dotted line). After root avulsion and re-implantation there was complete paralysis of the hindlimb. Slight functional improvement was observed 10 days after injury; however, the animals performed around 1 point during the follow-up, without differences between avulsion with direct re-implantation (AV-RE) and AV-RE-mesenchymal stem cell (MSC) groups

MSC Rescue Motoneurons and Reduce Reactive Astrocytosis After VRA

Our results indicate that an MSC graft increases preservation of approximately half of the avulsed MN during the first month after injury. This finding is in concordance with a previous report about 67 % MN survival improvement by BDNF-producing MSC transplants after lumbar VRA [21]. Although the mechanisms through which MSC provide such beneficial effect are still not known, some hypotheses are under investigation. The neuroprotective effect induced by these cells might be mediated by the direct secretion or the induction in the transplanted tissue of some neurotrophic factors, such as BDNF and GDNF [21]. An exogenous application of BDNF protects MN from avulsion injury and sustains regeneration of motor axons, especially when BDNF is administered chronically [15, 18, 42–44]. However, the application of high doses of BDNF after sciatic nerve injury has detrimental effects on axonal regeneration [45]. The expression of the high affinity BDNF receptor, TrkB, is reduced in MN after VRA [46], while the expression of p75, the low affinity receptor for the neurotrophin family including BDNF, is increased [47]. Blockade of p75 with specific antibodies reverts the detrimental outcome induced by high doses of BDNF, and thus demonstrates a role of p75 in this effect [45]. Similarly to BDNF, GDNF can also support MN survival and regeneration [15, 20, 44, 48]. In this case, mRNA levels of both GDNF receptors—high affinity receptor GFRa1 and low affinity receptor c-ret—increase in MN during some weeks after axotomy and avulsion lesions [44].

Besides the neuroprotective role of the MSC transplant, we also observed a reduction in astrocyte reactivity secondary to the injury. This is in agreement with previous works, that reported reduced astrocyte activation, characterized by GFAP immunoreactivity, 2 weeks after VRA and MSC transplantation [21]. This effect has been described also after spinal cord injury [49] and brain ischemia [27, 50]. MSC are able to modulate the inflammation process [51], which, in turn, can contribute to prevent the astrocyte reaction after injury. Moreover, this effect could also be consequence of the increased neuronal survival and tissue protection induced by the transplanted MSC.

MSC Accelerate Axonal Regeneration Through the Re-implanted Root

MN survival is an important goal after avulsion of ventral roots, but surgical reconnection of the nerve stump with the spinal cord surface is essential to allow axonal regeneration of injured motor axons to target muscles. We observed that acute re-implantation of avulsed roots near the root exit zone increases MN survival and also allows motor axon regrowth. After 4 weeks of re-implantation some motor axons were observed in the lumbar plexus, 4 cm distal to the spinal cord. However, the amount of motor fibers at 8 weeks at the same distance was similar to that at 4 weeks, as described previously [16]. The combination of re-implantation with MSC transplantation further increased MN survival and axonal regeneration compared to the group with re-implantation alone. Although the numbers of motor fibers in the lumbar plexus were similar in both re-implanted group, more axons reached long-distance growth in the MSC-transplanted animals, as observed in the retrotracing study. Thus, we found that some motor axons were able to reach the distal sciatic nerve, approximately 8 cm from the spinal cord at 4 weeks only in MSC transplanted animals. With these results, we can estimate an axonal growth rate of approximately 1.4 mm/day for control re-implanted rats and 2.8 mm/day for the combined therapy group. Therefore, a focal transplant of MSC into the spinal cord enhances the growth rate of motor axons during the first weeks after re-implantation. Moreover, the presence of regenerative axons at the distal sciatic nerve only in re-implanted animals with MSC injection corroborate the longer axonal regeneration induced by these cells. This ability of MSC to potentiate axonal growth has been also described when the cells are transplanted distal to the MN soma. Thus, an increased rate of regeneration by MSC grafts was reported after sciatic nerve transection and repair with synthetic tubes [30] or after facial nerve transection [52]. In our *in vitro* model, we also observed that MSC enhance axonal regeneration, thus confirming the *in vivo* findings.

The electrophysiological results also evidenced faster re-innervation of target muscles in the denervated hindlimb in the group with a MSC transplant after root re-implantation.

Muscle re-innervation by regenerating axons is characterized at the initial stages by polyphasic electromyographic responses, with a long latency and low amplitude, that tend to progress to a monophasic CMAP of increasing amplitude and near normal latency with advancing re-innervation [41]. In the present study, after re-implantation of L4, L5, and L6 roots, some polyphasic responses were recorded in the TA and GM muscles, but not in the more distal PL muscles, in a few animals 8 weeks after injury, indicating functional re-innervation by the regenerated motor axons. Interestingly, the combination of MSC transplantation with re-implantation accelerated the electromyographic responses from 8 to 4 weeks, and increased the amplitude of responses in TA and GM muscles. Nevertheless, the CMAPs did not show much progression during this time, indicating that only a limited number of motor axons were able to re-grow and reach the muscles in the hindlimb.

The capacity of MSC to enhance axonal regeneration may be owing to their ability to increase the expression of neurotrophic factors, such as GDNF and BDNF [21, 53], which are known to promote axonal growth in both *in vitro* [31] and *in vivo* [44] regeneration models. Similarly, application of exogenous GDNF [15, 19, 20] and BDNF [15, 18] in the spinal cord after axotomy improves axonal regeneration. However, excessive levels of BDNF or GDNF, induced by gene vectors, may result in abnormal growth of axons, forming a neuroma at the site of the factor secretion [20]. It is plausible that stem cells provide a better regulation of neurotrophin levels by paracrine and autocrine mechanisms. Nevertheless, our results show that the positive effect was of limited scope, as the number of motor fibers reaching the distal sciatic nerve and the muscles did not increase significantly from 4 to 8 weeks after repair and transplant. This suggests that the early potentiation of axonal regeneration by the MSC decreased from 4 to 8 weeks, likely owing to the low number of transplanted cells found at 4 weeks in the avulsed segments grafted. In fact, the reduction of MN survival from 4 to 8 weeks may be another consequence of the loss of MSC and their trophic action with time.

The inability of transplanted MSC to sustain neuronal survival and regeneration for longer periods of time may be a consequence of their poor survival within the spinal cord. As described previously, the survival of cells transplanted in the spinal cord after injuries is limited independently of cell type, which indicates that the spinal cord environment after damage is hostile to cell transplants in general [54–56]. Despite the immunomodulatory properties of MSC [51], survival of MSC grafted in the injured spinal cord has been reported to decrease with time [49, 54, 57]. Our observations after spinal root avulsion are in agreement with those previous reports. The reduction of the transplanted cells in time, together with the limited proportion of MN that were able to regenerate the axons distally, explain the lack of functional recovery of hindlimb movements during the 8 weeks of follow-up. In fact, the achieved re-innervation of the hindlimb muscles was still very low in comparison with an intact limb (less than 1 % considering the CMAPs amplitude), and likely insufficient to allow enough muscle contraction for limb movements. In addition, the lack of selective re-innervation of appropriate muscles by regenerating motor axons after severe nerve lesions, such as VRA, often impedes the coordinated muscle contractions needed for an adequate joint movement, despite good muscle re-innervation [58]. Slight functional recovery has been described with re-implantation after cervical spinal roots avulsion [11–13], but is very limited after lumbar root avulsion [15, 48]. Two main factors are determinant of successful muscle re-innervation and functional recovery: the distance from the spinal cord to target muscles and the time-depending capacity of nerves to support axonal regeneration [17]. Following axotomy, acute denervated Schwann cells of the distal nerve stump switch to an immature proliferative state, secrete pro-regenerative neurotrophic factors and sustain axonal growth. In fact, the beneficial effects of root re-implantation would be to provide neurotrophic action from the Schwann cells [17]. However, after prolonged periods of denervation, Schwann cells decrease their pro-regenerative capability, and there is a temporal decrease of neurotrophic factors, especially GDNF and BDNF [16], and an increase of inhibitory molecules in the denervated distal nerve stump [59]. Therefore, the rate of axonal growth declines markedly [16]. Thus, although an MSC transplant into the spinal cord enhances the regeneration of MN close to the transplant site, it can not compensate the reduced potential of long-time denervated nerves to sustain regeneration over longer time and distance. Therefore, a further approach may be needed by manipulating the trophic support in the distal peripheral nerve in addition to extending the time of the focal action provided by the intraspinal cell graft.

Therapeutic Perspectives

Nowadays the repair of spinal root injuries to allow for some degree of functional recovery is challenging. Surgical repair for the reconnection of the detached roots into the correct point of the spinal cord, by direct re-implantation of the root stumps or by nerve grafting [11], seems mandatory. However, owing to the long distance that avulsed axons need to re-grow and the chronic atrophy of the limb muscles when regenerating axons may re-innervate the targets, surgical repair is insufficient. The use of cell therapy combined with surgical repair could be a good approach for these injuries. In this work we demonstrate that focal grafting of MSC extends motoneuron survival and enhances axonal regeneration compared with root re-implantation alone. Although other cell types, such as Schwann cells [60] and olfactory

ensheathing cells [61], have been shown to support axonal regeneration and could also be used, the easier procurement of MSC make them more clinically feasible [62]. Nevertheless, the translation of this cell therapy needs more investigation to define the optimal conditions. More information about the time-window for grafting, the number of injections, the quantity of cells, and the phenotype of the transplanted cells needs to be gathered from pre-clinical studies. The evidences for increased neuronal survival and faster axonal regeneration and muscle re-innervation constitute a proof-of-concept that a MSC transplantation combined with surgical root re-implantation, and later rehabilitation interventions, provides beneficial effects for the treatment of spinal root lesions.

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Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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Discusión

En la presente memoria se recogen los estudios que hemos realizados en cuanto a la terapia celular para el tratamiento agudo de lesiones traumáticas que afectan a la médula espinal. Con el trabajo expuesto se ha demostrado que el trasplante celular reduce en parte los efectos negativos producidos por una lesión de médula espinal, sea ésta por acción directa o por un daño indirecto como la avulsión radicular, y se ha indagado en los mecanismos moleculares que subyacen los efectos del trasplante celular.

Tratamiento de las lesiones directas de médula espinal por contusión mediante el trasplante de MSC o de OEC

La terapia celular ha sido en los últimos años una de los principales áreas en la búsqueda de tratamientos efectivos para lesiones directas de la médula espinal. A pesar de los numerosos estudios realizados, a día de hoy no se ha determinado la estrategia celular más adecuada para el tratamiento de estas dolencias. Dentro del abanico de posibilidades, probablemente las células más estudiadas, tanto en la investigación preclínica como en la clínica, son las células estromales/madre mesenquimales (MSC) y las células de la glia envolvente del bulbo olfativo (OEC) (Syková et al., 2006; Sahni and Kessler, 2010; Hernández et al., 2011; Mothe and Tator, 2012). Pero ¿cuál de estos dos tipos celulares ejerce mayor beneficio como terapia para lesiones traumáticas de la médula espinal? Ambos tipos celulares han demostrado su eficiencia disminuyendo los procesos perjudiciales que se dan después de la lesión medular y proporcionando una mejora parcial de las funciones afectadas (Li et al., 1997; Chopp et al., 2000; Ramón-Cueto et al., 2000; Hofstetter et al., 2002; Lu et al., 2002; Verdú et al., 2003; García-Alías et al., 2004; Neuhuber et al., 2005; Himes et al., 2006; López-Vales et al., 2006; Amemori et al., 2010; Quertainmont et al., 2012). No obstante, algunos estudios no consiguieron mostrar una recuperación funcional clara con el uso de estas estrategias (Takami et al., 2002; Resnick et al., 2003; Barakat et al., 2005; Collazos-Castro et al., 2005; Pearse et al., 2007; Nandoe Tewarie et al., 2009). En nuestro estudio, tan solo los animales tratados con MSC en un tiempo agudo después de la lesión presentaron una ligera mejoría, mientras que las OEC trasplantadas no parecen producir un beneficio funcional. Uno de los aspectos que más pueden influir en la discrepancia de resultados entre diversos autores es el modelo de lesión. Nuestros estudios se realizaron en una lesión inducida por contusión, siendo probablemente el modelo que más se asemeja a la patología de la mayoría de personas aquejadas por una lesión medular (Bunge et al., 1997). En este aspecto, mientras que los resultados experimentales obtenidos con el trasplante de MSC en lesiones medulares por contusión parecen ser consistentes (Chopp et al., 2000; Hofstetter et al., 2002; Neuhuber et al., 2005; Himes et al., 2006; Quertainmont et al., 2012), en lo que se refiere a las OEC, los resultados son algo dispares. Así, la mayoría de trabajos que han mostrado una mejora funcional fueron realizados en modelos de sección completa (Li et al., 1998; Ramón-Cueto et al., 2000; López-Vales et al., 2006, 2007; Franssen et al., 2007), hemisección (Deumens et al., 2006) o por lesiones neurotóxicas como la fotoquímica (Verdú et al., 2003; Gracía-Alías et al., 2004; López et al., 2006). En contra, la mayoría de los trabajos de trasplante de OEC en modelos de contusión no observaron ningún efecto positivo en la recuperación de las capacidades motoras afectadas (Takami et al., 2002; Resnick et al., 2003; Barakat et al., 2005; Collazos-Castro et al., 2005; Pearse et al., 2007). Así pues, mientras que las MSC pueden resultar útiles en varios modelos de lesión, incluyendo la contusión, las OEC pueden tener una limitación de uso en función del tipo de lesión. Por lo tanto, determinar qué tipo celular otorga mejores beneficios en función de la severidad y naturaleza de la lesión permite acotar

procedimientos clínicos concretos según estas variables.

El objetivo máximo de los tratamientos para lesiones de médula espinal es una recuperación de las funciones perdidas. Es por ello que la investigación preclínica se ha focalizada mayoritariamente en los aspectos funcionales y en la mejora de las habilidades motoras afectadas. No obstante, uno de los efectos más consensuado de estas células en la bibliografía es su capacidad de proteger el tejido medular dañado. Este efecto se ha podido observar tanto con el trasplante de MSC (Ankeny et al., 2004; Isele et al., 2007; Abrams et al., 2009; Quertainmont et al., 2012) como con el de OEC (Ramón-Cueto et al., 2000; Ruitenberg et al., 2003; Verdú et al., 2003; García-Alías et al., 2004; López-Vales et al., 2006, 2007; Franssen et al., 2007)), independientemente de la vía de administración y el modelo de lesión. Nuestros experimentos corroboraron una vez más los resultados publicados en que el trasplante de ambas células permitía una mayor preservación de tejido, limitando así el daño secundario de la lesión medular. A pesar de la ello, los resultados funcionales encontrados fueron, como hemos comentado anteriormente, limitados. La explicación más obvia es la limitada protección del parénquima medular a nivel del epicentro de la lesión. Mientras que el trasplante de ambos tipos de células inducía una reducción del daño en los segmentos circundantes al centro de la lesión, tanto a nivel caudal como rostral, en la zona de impacto no se encontraron mejoras significativas. Tan solo los animales que habían recibido las MSC en un tiempo agudo presentaron una cierta recuperación de los potenciales motores evocados. Esto indicaría o bien una mayor preservación de axones descendentes o bien una recuperación de vías dañadas por remielinización o por regeneración axonal. Sea cual sea el motivo, la pequeña cantidad de tejido preservado en el epicentro de la lesión no parece permitir una recuperación funcional satisfactoria. Además de las controversias ya expuestas sobre el modelo de lesión, otro de los factores influyentes en muchos tratamientos es la dosis, y la terapia celular no está exenta de ello. El número de células inyectadas intramedularmente varía entre los numerosos trabajos, abarcando desde pocas decenas de miles (Ruitenberg et al., 2003; Koda et al., 2005) hasta varios millones (Ohta et al., 2004; Zurita et al., 2006; Pearse et al., 2007). Además, los trasplantes se han realizado mediante una única inyección en el epicentro (Lu et al., 2002; Ankeny et al., 2004) o varias a lo largo de la zona de lesión (Hofstetter et al., 2002; Ramon-Cueto et al., 2002), lo que concentra o diluye la cantidad de células en el tejido medular. En nuestros experimentos el total de células que recibió cada animal fue de 450.000 repartidas por igual en tres puntos de inyección. Esto supone tan solo 150.000 células en el epicentro, donde el daño es mayor. En comparación con algunos de los trabajos que encontraron una mejora funcional clara con el trasplante de MSC o OEC después de una lesión medular por contusión, la cantidad de células que nosotros hemos usado es menor. Así pues, el número de células trasplantadas en el centro de la lesión puede ser determinante para el éxito funcional del tratamiento.

La información sobre los mecanismos por los cuales estas células ejercen su acción protectora es aún escasa. Como reveló el estudio génico, ambos tipos celulares son capaces de modificar el ambiente perjudicial generado después de la lesión, iniciando procesos ligados a la regeneración tisular.

El trasplante temprano de MSC promueve procesos de remodelación de la matriz extracelular, especialmente aquellos que implican una mayor deposición de colágeno I, importante durante los procesos de reparación tisular, y los relacionados con la formación de tejidos fibrosos como la metaloproteinasa 13 y la fibronectina. Además, el trasplante agudo de MSC también

induce cambios en la expresión génica que sugieren un mayor reclutamiento de células inflamatorias necesarias para la eliminación del tejido destruido, así como procesos de respuesta a hipoxia y la producción de nuevos vasos sanguíneos que reducirían el ambiente isquémico generado después de la lesión. Estos datos concuerdan con algunos trabajos previos donde el trasplante de MSC resultó en cambios de la matriz extracelular y angiogenesis (Chopp et al., 2000; Hofstetter et al., 2002; Neuhuber et al., 2005; Himes et al., 2006; Amemori et al., 2010; Quertainmont et al., 2012). Curiosamente, con el trasplante de las MSC en un periodo más tardío después de la lesión (a los 7 días) nuestros resultados mostraron una dinámica contraria a la observada con el trasplante inmediato. De este modo, después del trasplante retardado pudimos observar la reducción de la expresión de genes asociados a procesos de reparación tisular como los de morfogénesis, remodelación de la matriz extracelular, angiogénesis y de respuesta a hipoxia. Además, algunos genes relacionados con la respuesta inflamatoria implicada en la reparación de tejidos resultaron expresados al alza y otros a la baja. Esto reflejaría una modulación de los procesos inflamatorios por parte de las MSC después de su trasplante, reafirmando resultados publicados previamente (Nakajima et al., 2012). Todos estos datos sugieren una capacidad de las MSC para modular los procesos de reparación, aumentándolos en los momentos iniciales y reduciéndolos posteriormente. Esta capacidad plástica de las MSC podría ser una consecuencia de su papel fisiológico. Algunos autores han sugerido que las células mesenquimales presentes en los tejidos podrían ser uno de los componentes celulares más importantes durante los procesos de reparación en tejidos dañados (Caplan, 2007, 2011; Laird et al., 2008).

En cuanto a las OEC, al igual que sucede con las MSC, su presencia en los tiempos tempranos después de la lesión induce una expresión al alza de genes relacionados con procesos asociados a la reparación tisular, incluyendo inflamación, morfogénesis y desarrollo de órganos y tejidos, crecimiento y proliferación celular, respuesta a substancias orgánicas y al nivel de nutrientes, producción de nuevos vasos sanguíneos, respuesta a hipoxia y una reorganización de la matriz extracelular. Por contra, el trasplante de OEC en momentos más tardíos de la lesión causa una reducción de algunos de estas vías de reparación. De acuerdo con nuestros resultados, estudios previos demostraron que las OEC son capaces de expresar genes relacionados con la reparación tisular, la reestructuración de la matriz extracelular, moléculas de adhesión (Kafitz and Greer, 1998; Vincent et al., 2005; Franssen et al., 2008) y la respuesta inflamatoria (Vincent et al., 2005). A pesar de estos cambios, el trasplante de OEC promueve una activación temprana de la respuesta inmunitaria adaptativa que sugiere una rápida respuesta de rechazo. Esta actuación por parte del tejido huésped explicaría la pobre integración que tienen las OEC en la médula espinal, la escasa migración después de su trasplante y su aislamiento con la formación de acúmulos (Ruitenberg et al., 2002; Lu et al 2006). Además de todos estos cambios, es interesante mencionar como algunos de los genes, cuya expresión aumenta en los trasplantes retardados de OEC, están implicados en procesos de diferenciación y maduración de neuronas. Esta acción sobre precursores neurales se observó recientemente in vitro, sugiriendo una acción paracrina de las OEC relacionada con la diferenciación neuronal (Duan et al., 2011). En resumen, la presencia temprana de las MSC o de las OEC en la médula espinal después de una lesión por contusión puede potenciar o acelerar algunos de los procesos necesarios para la reparación del tejido dañado. Esto permitiría una recuperación más rápida de la homeostasis y un incremento de la remodelación de la matriz extracelular, limitando la expansión de la lesión.

Por otro lado, una disminución de la reparación en las fases más tardías evitaría, en parte, la cronificación de algunos de estos procesos que no permiten una correcta regeneración del tejido. Dada esta capacidad dinámica de respuesta, la hipótesis de una acción paracrina por parte de las MSC y de las OEC va tomando cada vez más forma.

A pesar de que se ha demostrado el potencial protector del trasplante de MSC o de OEC en fases tempranas después de la lesión, a día de hoy las mejoras funcionales publicadas en modelos de lesión medular por contusión son escasas. Se ha sugerido que uno de los factores que podría estar limitando el beneficio del trasplante de éstas células es la reducida supervivencia que presentan después del trasplante. Nuestros estudios de seguimiento de las células injertadas demostraron que tanto las MSC como las OEC desaparecen a las pocas semanas después de su trasplante. Esta elevada muerte es independiente de las células trasplantadas, sugiriendo un ambiente hostil para el injerto generado en las lesiones por contusión (Barakat et al., 2005; Pearse et al., 2007; Ronsyn et al., 2007). Los resultados obtenidos en el estudio de la expresión génica después del trasplante mostraron una rápida activación de vías metabólicas asociadas a los procesos inmunitarios de respuesta a agentes externos. Esto señalaría un rechazo del tejido huésped, siendo posiblemente el motivo de la limitada supervivencia de las células trasplantadas. Además, el aumento de la supervivencia de las MSC trasplantadas con la combinación de un inmunosupresor, como es el FK506, confirmaría estos resultados. Cabe destacar que el aumento de la expresión de genes relacionados con el rechazo es más rápido en los animales trasplantados con OEC que en los trasplantados con MSC. Esto podría explicar el reducido numero de genes que varían su expresión una semana después del trasplante de OEC en comparación con el trasplante de MSC. A pesar de ello, los resultados de la supervivencia de las células no mostraron diferencias entre ambas. El hecho de que las respuestas inmunitarias contra las MSC sean más tardías que frente a las OEC puede ser consecuencia de la capacidad inmunomoduladora e inmunoprivilegiada (Nauta and Fibber, 2009) que las MSC poseen. No obstante, a diferencia de lo que sucede en otros órganos, esta habilidad de las MSC para sortear las respuestas inmunitarias no evita su desaparición después de ser trasplantadas en la médula espinal lesionada. Como hemos comentado anteriormente, la corta supervivencia del injerto podría reducir el éxito de la terapia. Sin embargo, nuestro resultados del trasplante de MSC con un tratamiento de inmunosupresión, que ha permitido una buena supervivencia de las células, no han sido todo lo satisfactorio que se esperaría, con mínimas diferencias frente al trasplante sin inmunosupresión. Por tanto, la prolongación de la presencia de las células no conlleva a una mejora substancial, señalando que probablemente el efecto de las MSC sea más importante durante las etapas tempranas de la lesión, al menos en cuanto a protección de tejido. Un dato a destacar con la inmunosupresión es la formación de un tejido compacto que rellena la zona de lesión en lugar de la cavidad habitual presente en los animales no tratados o trasplantados pero no inmunosuprimidos. Por ahora desconocemos la naturaleza de este tejido y si puede o no ejercer de andamio para el crecimiento y la regeneración de axones. En un futuro esperamos poder responder a esa pregunta y de ser así, buscar terapias combinadas con un trasplante inicial de MSC, inmunosupresión y estrategias que incrementen la regeneración axonal.

Tratamiento de las lesiones de avulsión mediante la reparación quirúrgica y el trasplante de MSC

La avulsión de las raíces ventrales desde la superficie de la médula espinal causa una perdida progresiva de motoneuronas, con la consecuente afectación permanente de las funciones motoras y una atrofia de los órganos denervados (Koliatsos et al., 1994; Martin et al., 1999; Natsume et al., 2002; Hoang et al., 2003; Penas et al., 2009). Las estrategias que tienen como objetivo aumentar la supervivencia de las MN son obligadas como primer paso para conseguir una recuperación funcional. Como hemos discutido anteriormente, el trasplante de MSC en fase aguda después de una lesión de médula espinal por contusión puede ser beneficioso en algunos aspectos, mayoritariamente los relacionados con una protección tisular. Es por ello que nos planteamos el trasplante de MSC en modelos de avulsión como terapia neuroprotectora. En nuestro estudio hemos demostrado como el trasplante de MSC incrementa la supervivencia de las MN, al menos durante las 4 primeras semanas después de la lesión. Además, la combinación del trasplante de estas células con la reimplantación directa de las raíces avulsionadas aumenta aún más la supervivencia de las MN, y se acompaña de una mayor regeneración axonal a través de las raíces reparadas. A pesar de los resultados obtenidos, con una mayor supervivencia de MN, una aceleración en la regeneración axonal y una leve reinervación muscular, los animales no presentaron una mejora en la capacidad motora de la extremidad inferior afectada por la avulsión. Así pues, el incremento de la supervivencia de las MSC y la combinación con tratamientos de rehabilitación, que reduzcan la atrofia muscular permitiendo una reinervación más eficiente, podría resultar en una mejora funcional. Además, averiguar los mecanismos de acción de las MSC permitirá la optimización y el diseño de tratamientos farmaco-celulares.

A modo de conclusión, la terapia celular ha demostrado ser una estrategia potencialmente útil en el tratamiento de dolencias que afectan a la médula espinal, especialmente las lesiones a consecuencia de traumatismos. No obstante, aventurarse hoy a defender un tipo celular es, como mínimo, arriesgado. Mientras que el beneficio de un trasplante de MSC en lesiones por contusión parece ser mayor que el tratamiento con OEC, éstas últimas han demostrado su utilidad en lesiones menos destructivas como podrían ser las laceraciones de la médula espinal. Además, hay que tener en cuenta el carácter multifactorial y dinámico de los procesos fisiopatológicos que subyacen de la lesión medular. Por lo tanto, una línea a seguir es desarrollar el trasplante celular como complemento a otros tratamiento y no como una terapia única, buscando estrategias que de manera conjunta abarquen un mayor número de objetivos.

Conclusiones

•El cultivo de MSC procedente de animales jóvenes presenta características similares a las descritas para MSC obtenidas de individuos adultos.

•La adición de MSC, previamente aisladas en cultivo, a cultivos organotípicos de secciones de médula espinal induce el crecimiento de un mayor número de neuritas que alcanzan distancias más largas en el mismo tiempo.

•La eficiencia de un trasplante celular en lesiones de médula espinal depende de varios factores, entre ellos las condiciones de cultivo y preparación de las células a trasplantar, así como variables intrínsecas al donante como la edad del mismo.

•Tanto las MSC como las OEC trasplantadas después de una lesión de médula espinal por contusión presentan una supervivencia limitada en el tiempo, desapareciendo prácticamente por completo a las tres semanas después de ser trasplantadas.

•El trasplante agudo o subagudo de MSC o de OEC después de una lesión por contusión reduce el daño tisular sufrido por la médula espinal, principalmente en las zonas rostrales y caudales al epicentro de la lesión.

•A pesar del beneficio tisular, los trasplantes realizados, a excepción del trasplante agudo de MSC, no consiguieron una mejora funcional substancial en cuanto a locomoción, respuestas electrofisiológicas y sensibilidad a diferentes estímulos.

•El trasplante agudo de MSC resultó en una mejora funcional aparente, incrementando la velocidad máxima a la que pueden correr los animales tratados, permitiendo la reaparición de potenciales motores evocados y aumentando la hiperreflexia espinal.

•Las lesiones de médula espinal por contusión inducen grandes cambios en el transcriptoma medular, incrementado y reduciendo la expresión de un gran número de genes desde tiempos tempranos después del daño.

•El trasplante de MSC y de OEC produce modificaciones en los cambios génicos inducidos por la lesión, potenciando mecanismos de reparación tisular con el trasplante agudo y reduciendo estos mecanismos después de un trasplante subagudo. A pesar de que muchos de estos cambios son similares, la vía principal de acción por la cual las células ejercen estos cambios parecen ser específicas al tipo celular.

•La presencia de las MSC o de las OEC en el parénquima medular después de una lesión induce la sobreexpresión de genes relacionados con una activación del sistema inmune, principalmente las vías de respuesta a organismos externos. Esto podría indicar una respuesta de rechazo contra las células trasplantadas y una explicación a la rápida desaparición de las mismas.

•La administración de un tratamiento inmunosupresor tras el trasplante agudo de MSC en animales con lesión de médula espinal permitió prolongar la supervivencia del injerto, al menos durante las 6 semanas de seguimiento. Esto confirma que la desaparición de las células trasplantadas se debe, en parte, a una respuesta inmunitaria de rechazo.

•La combinación del trasplante agudo de MSC con el tratamiento inmunosupresor potencia la recuperación de las funciones locomotoras. No obstante, no hay cambios en la recuperación de los potenciales evocados motores respecto a los

animales con un trasplante agudo de MSC sin inmunosupresión.

•La administración de FK506 como agente inmunosupresor reduce la hiperreflexia medular e incrementa la sensibilidad a estímulos mecánicos después de la lesión medular, tanto en los animales inyectados con vehículo como en los que recibieron un trasplante agudo de MSC.

•A pesar de las diferencias funcionales encontradas con el trasplante de MSC con o sin tratamiento inmunosupresor, ambos tratamientos parecen potenciar una reducción del daño tisular en el mismo grado.

•El trasplante agudo de MSC combinado con inmunosupresión permite la formación de un tejido denso en el centro de la lesión, en lugar de la cavidad característica de las lesiones medulares por contusión en rata. Esta neoformación tisular podría ser ventajosa a más largo plazo para posibilitar la regeneración axonal.

•El trasplante agudo de MSC después de una avulsión radicular de los segmentos medulares L4, L5 y L6 permite una mayor supervivencia de las motoneuronas lesionadas, acompañada de una reducción de la reactividad astroglial inducida por la lesión.

•La combinación del reimplante quirúrgico con el trasplante agudo de MSC después de una avulsión de raíces espinales incrementa la supervivencia de las motoneuronas, induce una mayor velocidad de regeneración axonal y la reinervación parcial de los músculos diana, en comparación con el tratamiento quirúrgico simple.

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Abreviaturas

AMPA receptor
α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ASIA
American Spinal Injury Association

BDNF
Brain-Derived Neurotrophic Factor

CNTF
Ciliary Neurotrophic Factor

CSPG
Chondroitin Sulfate Proteoglycan

ESC
Embrionic Stem Cells

GAP-43
Growth Associeted Protein 43

GDNF
Glial cell line-Derived Neurotrophic Factor

GFAP
Glial Fibrillary Acidic Protein

GM-CSF
Granulocyte Macrophage Colony-Stimulating Factor

HSC
Hematopoietic Stem Cells

Hsp27
Heat Shock 27kDa Protein

IFN-γ
Interferon gamma

IGF-1
Insulin Growth Factor 1

IL-1β
Interleukin 1 beta

IL-6
Interleukin 6

iPSC
induced Pluripoten Stem Cells

MAG
Myelin-Associated Glycoprotein

MN
MotoNeurona

MSC
Mesenchymal Stromal/Stem Cells

NGF
Nerve Growth Factor

NMDA receptor
N-Methyl-D-Aspartate receptor

NSC
Neural Stem Cells

NT-3
NeuroTrophin 3

OEC
Olfactory Ensheathing Cells

OMGP
Oligodendrocyte-Myelin GlycoProtein

ROS
Reactive Oxygen Species

SC
Schwann Cells

SNC
Sistema Nervioso Central

SNP
Sistema Nervioso Periférico

TGF-β
Transforming Growth Factor beta

TNFα
Tumor Necrosis Factor alpha

UCB
Umbilical Cord Blood

VEGF
Vascular Endothelial Growth Factor