Universidad de Navarra

Facultad de Ciencias

APLICACIÓN TERAPÉUTICA DE LA BIOINGENIERÍA MEDIANTE LA COMBINACIÓN DE CÉLULAS MADRE Y MATRICES EXTRACELULARES EN UN MODELO DE INFARTO DE MIOCARDIO EN RATA

Miriam Araña Ciordia

Universidad de Navarra

Facultad de Ciencias

APLICACIÓN TERAPÉUTICA DE LA BIOINGENIERÍA MEDIANTE LA COMBINACIÓN DE CÉLULAS MADRE Y MATRICES EXTRACELULARES EN UN MODELO DE INFARTO DE MIOCARDIO EN RATA

Memoria presentada por Dª. Miriam Araña Ciordia para aspirar al grado de Doctor por la Universidad de Navarra

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Terapia Celular y autorizamos su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, 29 de Septiembre de 2011

Dr. Felipe Prósper Cardoso

(Director de la Tesis Doctoral)

Dra. Beatriz Pelacho Sámper

(Co-directora de la Tesis Doctoral)

A mis padres y abuelos

Agradecimientos

Quisiera agradecer con estas palabras a todas aquellas personas que me han apoyado y ayudado en este largo camino:

Mi interés por la biología tiene mucho que ver con todo lo que ha rodeado y rodea mi vida. El entorno que enmarca mi casa en Etxarri-Aranatz desde la cual puedo admirar Urbasa, San Miguel de Aralar o San Donato, mi familia que día a día me ha inculcado el amor por la naturaleza o los profesores que así han sabido trasmitirlo. Gracias a mis abuelos, eskerrik asko amiña-aittuna, que me han emocionado con sus historietas y me han enseñado que es muy poco lo que necesitamos para ser felices. A mis padres, trabajadores incansables, eskerrik asko, por su eterna confianza y por ofrecerme la oportunidad de estudiar lo que yo elegí y de llegar hasta aquí. A mis tres herman@s, Belén, Adrián eta Rubén, por ser ¡la alegría de mi huerta!

A Xabi, que fue la primera persona que me transmitió *in situ* la pasión por la ciencia y el trabajo, y me adentró en este laboratorio.

A mis directores, Felipe y Bea, por la confianza depositada y por las innumerables cosas que me han enseñado durante estos años. Fue en cuarto de carrera la primera vez que tuve una reunión con Felipe (hace 7 años ya) para realizar prácticas en su laboratorio durante un mes de ese verano. Él me dijo que como mínimo tenían que ser dos meses porque en uno sólo no daba tiempo para mucho… finalmente, han sido unos cuantos más, y me alegro, porque ha dado tiempo para mucho más. Me acuerdo también de la primera conversación con Bea, sin ella ni yo saber que se convertiría en la persona que junto con Felipe dirigiría mi tesis, me dijo que para dar ese paso tenía que gustarte la investigación puesto que requiere un gran esfuerzo e intenso trabajo. Una vez más, no se equivocaba, pero indudablemente

gracias a su ayuda y apoyo incondicional ha sido un camino mucho más fácil y muy enriquecedor.

Durante estos años hemos creado una pequeña familia en el grupo de “cardio”, Manu, mi hermano mayor (y que pronto va a ¡¡hacerme tía!!), siempre dispuesto a ayudar, escuchar y responder a mis preguntas, e incluso hacerme rabiar… Olalla, alegre y trabajadora, fui la primera en “entrevistarla” y convencerla para que viniera con nosotros, ¡gran adquisición!; Edurne, la todoterreno, mi mano derecha en muchas ocasiones; Laura, de humor ácido pero dulce personalidad; Sheyla y Anama, excelentes compañeras de las que me queda mucho por aprender; Fabio, Teresa y Esther, que nos enseñan que existe algo más allá de la terapia celular; Natalia, richarachera, que últimamente ayuda a clonarme y sacar adelante varias cosas a la vez.

A todos los que han formado y forman parte del laboratorio porque gracias a ellos cada día me acuesto habiendo aprendido algo más, Maitane, Mariajo, Arantxa, Inés, Lucía, Salomón, Silvia, Pablo, María Gutiérrez, Silvia M, Maialen, Federico, María Jiménez, Miguel, Saray, Ángelo, Juanro, Natalia Z, Quique, Tania, Goretti, Marifé, Pili, María G, Adriana, Idoia, Xabi, Leire, Edurne SJ, Amaia, Cristina, Nico, Andoni, Esti, Montse, Ana, Miriam B, Virginia, María JL, Neira, y especialmente a aquellos que forman parte de este trabajo, Gloria y Juanjo en el modelo animal, Iñaki y Fany en el estudio mecánico, Cristina en las citometrías, y personal de morfología, imagen y animalario.

Sentía gran admiración por el grupo ya antes de entrar y ha sido un orgullo para mí el poder formar parte de este gran equipo.

A la Universidad de Navarra, CIMA, Gobierno de Navarra e Instituto de Salud Carlos III, por darme la oportunidad de desarrollar mi formación.

A mis amigas de Etxarri, Paky, Loreto, Maite, Ainhoa, Vanessa y Sole, a las que conozco desde los tres años, a mis compis de carrera, Ana, Amaia, Elena, Nerea, María y Paola, a mis compañeras de piso Amaia, Cristina y Rosana, y al resto de amig@s de Mundaka y Pamplona (este verano os he echado de menos, pero ¡pronto nos veremos!) por preocuparos por mí y mis animalitos... sois l@s mejores.

Y sobre todo a Asier, por estar aquí, en mi corazón.

ABREVIATURAS

αMEM: Medio mínimo esencial

αSMA: Alfa actina de músculo liso

λ: Deformación

σ: Tensión

A: Área

ACE: Enzima convertidora de angiotensina

ACE I: Enzima convertidora de angiotensina I

ACE II: Enzima convertidora de angiotensina II

ACEi: Inhibidor de la enzima convertidora de angiotensina

AD-CMG: Célula cardiomiogénica derivada de tejido adiposo

ADN: Ácido desoxirribonucleico

ADSC: Célula madre derivada de tejido adiposo

ANGII: Angiotensina II

ANP: Péptido natriurético atrial

APOLLO: Trasplante de células madre derivadas del tejido adiposo 3D en pacientes con infarto agudo de miocardio con elevación ST I

ARB: Bloqueantes del receptor tipo 1 de la angiotensina II

ARN: Ácido ribonucleico

ASTAMI: Trasplante de células madre autólogas en infarto agudo de miocardio con elevación ST I

AT-R: Receptor de angiotensina

ATP: Adenosín tri-fosfato

BMC: Células de médula ósea

BNP: Péptido natriurético cerebral

BOOST I: Transferencia de médula ósea para mejorar la regeneración de infarto con elevación ST I

BSA: Seroalbúmina bovina

CM: Cardiomiocito

CML: Célula muscular lisa

Co: Control

CPrC: Células progenitoras circulantes

CSC: Célula madre cardiaca

DAB: Diaminobencidina

DAPI: 4',6-diamidino-2-fenilindol

DVItd: Diámetro telediastólico

DVIts: Diámetro telesistólico

E_I: Módulo elástico

E_{II}: Módulo tangente

ECM: Matriz extracelular

EDC: 1-etil-3-(3-dimetilaminopropil) carbodiimida

EDTA: Ácido etilendiaminotetraacético

eGFP: Proteína verde fluorescente mejorada

EP: Endopeptidasas

EPC: Célula precursora endotelial

ESC: Célula madre embrionaria

F: Carga

FEVI: Fracción de eyección del ventrículo izquierdo

FGF: Factor de crecimiento de fibroblastos

FITC: Isotiocianato de fluoresceína

HGF: Factor de crecimiento hepático

HIF-1α: Factor inducible por hipoxia 1α

HSC: Célula madre hematopoyética

IC: Intracoronario

IGF-1: Factor de crecimiento similar a insulina **IL1β:** Interleuquina 1β

IL-6: Interleuquina 6

IL-8: Interleuquina 8

IL-10: Interleuquina 10

IM: Infarto de miocardio

iPS: Célula pluripotente inducida

l_0: Longitud inicial

LDL: Lipoproteína de baja densidad

Long: Longitudinal

m: Meses

MAGIC: Injerto de mioblastos autólogos en cardiomiopatía isquémica

MAPC: Célula progenitora adulta multipotente

Mb: Membrana

MCP-1: Proteína quimioatrayente de monocitos

MIAMI: Célula inducible multilinaje adulta aislada en médula

min: Minuto

MMP: Metaloproteinasa de matriz

MNC-MO: Células mononucleadas derivadas de la médula ósea

MO: Médula ósea

MSC: Célula madre mesenquimal

NE: Norepinefrina

PBS: Tampón fosfato salino

PDGF: Factor de crecimiento derivado de plaquetas

PE: Ficoeritrina

PGA: Ácido poliglicólico

PGCL: Ácido poliglicólico-co-prolactona

PLA: Ácido poliláctico

PLGA: Ácido poliláctico-glicólico

PRECISE: Tratamiento con células madre derivadas del tejido adiposo 3D en pacientes con miocardio isquémico no revascularizable

P/S: Penicilina-estreptomicina

RAS: Sistema renina-angiotensina

REPAIR-AMI: Reinfusión de células progenitoras enriquecidas y remodelado de infarto en infarto de miocardio agudo

SCF: Factor de célula madre

SD: Desviación estándar

SD: Sprague-Dawley

SDS: Dodecilsulfato sódico

SDF-1: Factor derivado estromal 1

Sk-34: Célula madre multi-miogénica derivada de músculo esquelético CD34 positiva

SPARC: Proteína secretada ácida rica en cisteína

SPOC: Precursores esqueléticos de cardiomiocitos

SVF: Fracción estromal vascular

TBS: Tampón tris-salino

TCo: Transcoronario

TdT: Deoxinucleotidil transferasa terminal

TEp: Transepicárdico

TGF-β: Factor de crecimiento transformador β

TIMP: Inhibidor tisular de metaloproteinasa

TLR: Receptor de tipo Toll

TNFα: Factor de necrosis tumoral alfa

Trans: Transversal

TSP-2: Trombospondina 2

TSP-1: Trombospondina 1

TUNEL: Marcación de la UTP final mediada por la transferasa desoxinucleotidil terminal

u: Extensión

VEGF: Factor de crecimiento endotelial vascular

VSEL: Célula madre muy pequeña similar a embrionaria

Vtd: Volumen telediastólico

Vts: Volumen telesistólico

ÍNDICE

1. INTRODUCCIÓN

1.1. Las enfermedades cardiovasculares: epidemiología y factores de riesgo

Las enfermedades cardiovasculares constituyen la primera causa de morbilidad y mortalidad en el mundo [1] y representan, según el último informe de la Organización Mundial de la Salud, el 29% de los fallecimientos a escala global, lo que equivale aproximadamente a más de 17 millones de muertes anuales (http://www.who.int/mediacentre/factsheets/fs317/es/index.html). Dentro de estas enfermedades, la isquemia cardiaca es la principal causa de mortalidad, constituyendo un 12,2% del total, seguida por los accidentes cerebro-vasculares, con un 9,7%. Los principales riesgos que predisponen al infarto de miocardio son la arteriosclerosis, la hipercolesterolemia, la homocisteinemia, la diabetes mellitus o la edad. Ciertos hábitos modificables como el tabaquismo, el consumo excesivo de bebidas alcohólicas, la obesidad y los altos niveles de estrés, pueden incrementar la probabilidad de padecer un infarto.

1.2. Fisiopatología del infarto de miocardio

La isquemia cardiaca o infarto de miocardio (IM) se produce como consecuencia de la obstrucción de los vasos coronarios y, por tanto, de la supresión del flujo sanguíneo. La falta de aporte de oxígeno al músculo cardiaco deriva en una rápida muerte celular y consiguiente pérdida de la contractilidad muscular, lo cual puede llegar a provocar el fallo cardiaco post-infarto.

La principal causa de la obstrucción del flujo sanguíneo en la isquemia cardiaca es la aterosclerosis coronaria [2]. Ésta es una enfermedad inflamatoria crónica, que se caracteriza por el desarrollo de placas de ateroma en las arterias, como respuesta a una agresión del endotelio vascular. Dicha agresión está mediada por diversos factores, tales como la hipertensión, la presencia de lipoproteínas o ácidos grasos libres y el tabaquismo, entre otros. Como consecuencia de ello, las células endoteliales de la pared arterial expresan moléculas de adhesión de células leucocitarias, y se

producen cambios en su permeabilidad que favorecen la entrada de partículas lipídicas LDL (*low-density lipoprotein*) a la túnica íntima. Además, los monocitos atraviesan el endotelio y penetran en la túnica íntima, donde diferencian hacia macrófagos tisulares que fagocitan las lipoproteínas, para transformarse en células espumosas. Por otro lado, las células musculares de la túnica media migran hacia la túnica íntima, donde proliferan y producen proteínas de matriz extracelular tales como el colágeno, la elastina y los proteoglicanos. En esta matriz, se acumulan también restos lipídicos provenientes de la apoptosis de las células espumosas de la zona. Este proceso da lugar a la formación de la placa de ateroma, la cual puede causar estenosis arterial o incluso trombosis si la placa se rompe, provocando la interrupción del flujo sanguíneo (Figura 1.1).

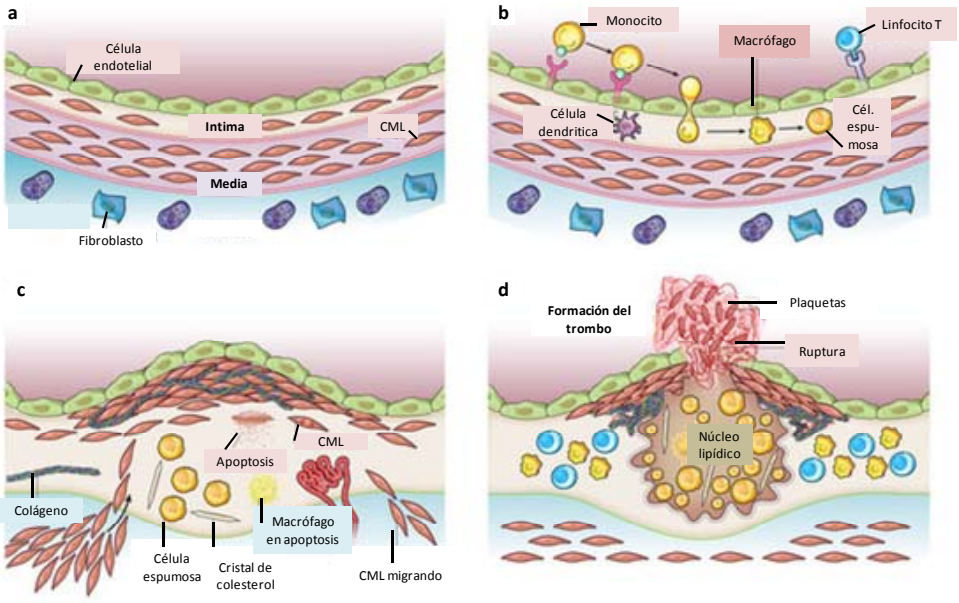


Figura 1.1. Fases en el desarrollo de la aterosclerosis (adaptado de *Libby P et al., 2011, Nature*). a. Arteria sana formada por la túnica íntima, túnica media y adventicia. **b.** Primeras fases de la aterosclerosis: los leucocitos sanguíneos se adhieren a las células endoteliales activadas y migran hacia la túnica íntima. Los monocitos maduran hacia macrófagos que captan lípidos y se convierten en células espumosas. **c.** Migración de las células musculares lisas (CML) de la túnica media hacia la túnica íntima donde aumenta la síntesis de moléculas de matriz extracelular (colágeno, elastina y proteoglicanos). Con la apoptosis de macrófagos y CML, se liberan restos lipídicos y se acumulan en la región central de la placa. **d.** Ruptura de la placa y formación del trombo que obstaculiza el flujo sanguíneo.

La falta de riego sanguíneo en el corazón tiene graves consecuencias. Es indispensable que el aporte de oxígeno al corazón sea constante, ya que presenta estrictos requerimientos metabólicos y en condiciones anaerobias, no posee la capacidad de generar suficiente energía para mantener su funcionalidad [3]. En caso de oclusión, antes del transcurso de 10 segundos comienza la acumulación de productos derivados del metabolismo anaeróbico y la depleción de ATP, y, tras 60 segundos, el cardiomiocito (CM) sufre cambios importantes tales como la hinchazón mitocondrial y celular y la disminución del glucógeno. A partir de los 20-40 minutos, el daño producido es irreversible y, en el transcurso de 4-6 horas tras la oclusión, el músculo cardiaco sufre ya una evidente necrosis.

Como mecanismo de respuesta al daño isquémico, se activan una serie de procesos de remodelado que, si bien no logran regenerar el daño provocado, lo contrarrestan parcialmente y evitan la ruptura tisular. Este proceso se da en tres fases que se solapan entre sí: una primera fase inflamatoria, una segunda fase de proliferación y una fase final de maduración de la cicatriz.

-Fase inflamatoria: Con la muerte celular del CM, el contenido intracelular se libera, iniciándose una respuesta inflamatoria por activación de los mecanismos innatos de la inmunidad. Se activa el sistema de complemento [4], se genera una elevada producción de especies reactivas de oxígeno y se activan las vías mediadas por TLR (*Toll-like receptor*), induciendo la liberación de numerosas citoquinas como el TNFα (*Tumoral Necrosis Factor*), la interleuquina-6 (IL-6)) [5] y las moléculas con actividad quimiotáctica, MCP-1 (*Monocyte Chemoattractant Protein-1*) o SCF (*Stem Cell Factor*) [6]. Dichas señales inducen una serie de cambios en el CM a nivel funcional y ultraestructural [7, 8], que generan una primera adaptación al daño tisular, principalmente a través de la hipertrofia y expresión de proteínas fetales. Por otra parte, en la matriz extracelular se activa la acción de metaloproteinasas (MMPs) [9], las cuales promueven la degradación de ésta, favoreciendo así la infiltración de neutrófilos y células mononucleadas responsables de eliminar las células necróticas de la zona [10]. Además, el aumento de la permeabilidad de la microvasculatura favorece

la extravasación de fibrinógeno y fibronectina plasmática, los cuales formarán una primera matriz provisional.

-Fase proliferativa: Durante esta etapa se activan mecanismos que inducen la revascularización de la zona dañada, y en la que el endotelio cardiaco y las células inflamatorias liberan factores quimiotácticos tales como el SDF-1 (*Stromal Derived Factor-1*) o la interleuquina-8 (IL-8), implicados en el reclutamiento de progenitores vasculares a la zona dañada (revisado en [11]). Entre dichos progenitores, se ha demostrado en ratones que en una segunda fase del proceso inflamatorio, es atraída una subpoblación de macrófagos caracterizada por la baja expresión de Ly-6C y que juega un papel importante en el proceso de revascularización [12]. Además, dicha subpoblación libera altos niveles de TGF-β1 (*Transforming Growth Factor*-β1), citoquina implicada en la atracción de fibroblastos del propio tejido y de la médula ósea (MO) [13], así como en su diferenciación hacia miofibroblastos. Por otro lado, los fibroblastos y macrófagos reclutados en la zona isquémica son los encargados del depósito de fibronectina, formando así una segunda matriz provisional. Éstos también liberan factores tales como el TGF-β1 [14], la interleuquina-10 (IL-10) [15] y los inhibidores tisulares de metaloproteinasas (TIMPs), que inhiben la actividad proinflamatoria, para permitir así el comienzo de la tercera fase.

-Fase de maduración: Durante esta última fase se produce una estabilización de la matriz provisional, depositada durante las fases tempranas. Los miofibroblastos [16] desempeñan un papel crucial en este momento, liberando TIMPs que detienen la degradación de la matriz extracelular y produciendo colágeno que contribuirá a la formación de la cicatriz, lo que impide una mayor dilatación ventricular. Finalmente, los miofibroblastos, gracias a su capacidad de contracción, contribuyen a la retracción del área infartada.

Como se puede comprobar, el proceso de remodelado cardiaco incluye complejos patrones de estimulación celular y molecular, que se integran en un intento de mantener la funcionalidad del corazón (Figura 1.2.). Sin embargo, existe una limitación importante para su recuperación, que es la reducida capacidad de

regeneración del tejido cardiaco [17, 18], lo que hace inevitable la permanencia de la cicatriz en el miocardio.

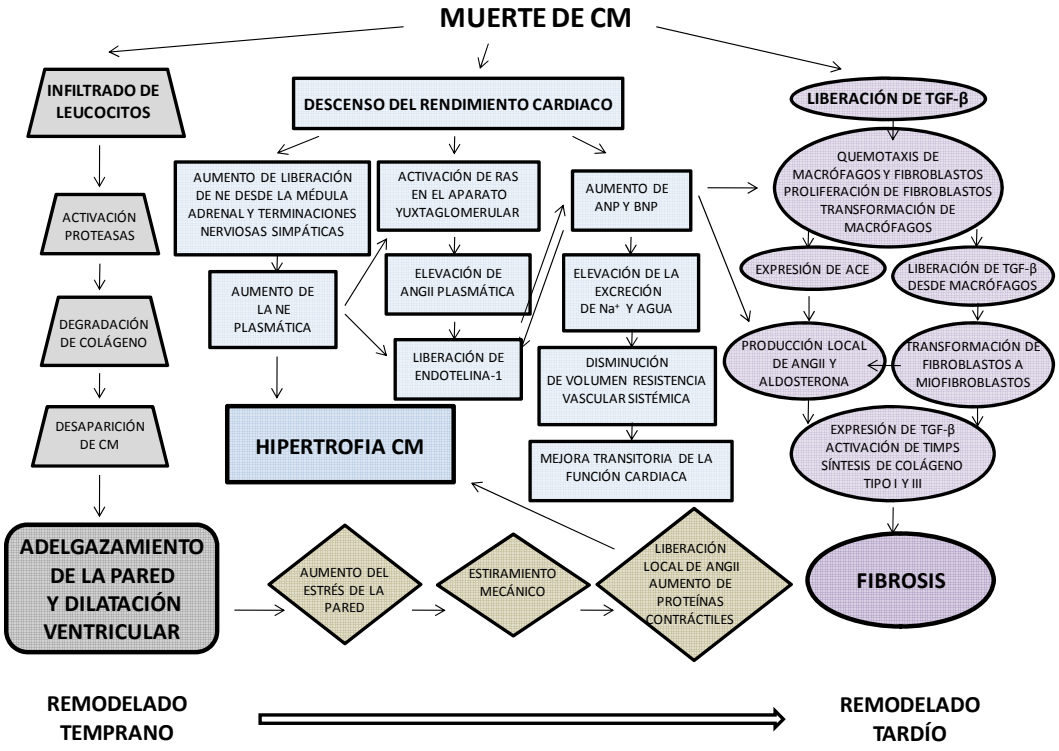


Figura 1.2. Representación de los procesos implicados en el remodelado cardiaco (adaptado de *Sutton MG et al., 2000, Circulation*). NE: norepinefrina; ANP: péptido natriurético atrial; BNP: péptido natriurético cerebral; ANGII: angiotensina II; TGF-β: *Transforming Growth Factor*-β; CM: cardiomiocito; ACE: enzima convertidora de angiotensina; TIMPS: inhibidores tisulares de metaloproteinasas de matriz; MMP: metaloproteinasa de matriz.

A pesar de que se ha comprobado la existencia en el corazón de células madre capaces de dar lugar a CM [19], esta capacidad no es suficiente a nivel fisiológico para rescatar el daño producido en el órgano. Así, el delicado equilibrio existente entre los diversos procesos que interactúan (la protección, la reparación y la hipertrofia), suele degenerar con el paso del tiempo en una situación de cronificación de las señales adaptativas anteriormente mencionadas, lo que deriva en una excesiva fibrosis e hipertrofia del tejido, las cuales, finalmente, pueden desencadenar en un fallo cardiaco irreversible [20].

1.3. Tratamiento del infarto de miocardio

1.3.1. Tratamientos clásicos: farmacología y clínica

En la fase aguda, el tratamiento del IM se basa en la reperfusión de la arteria coronaria obstruida, para evitar así complicaciones tales como la disfunción sistólica del ventrículo izquierdo, incrementando así la supervivencia del paciente tanto a corto como a largo plazo. El tratamiento de reperfusión incluye el tratamiento médico con fármacos trombolíticos, la revascularización coronaria percutánea mediante la angioplastia coronaria, la implantación de "stents" (convencionales y farmacoactivos) y, rara vez, la cirugía urgente de derivación aortocoronaria.

Tras el tratamiento agudo, diversos fármacos han demostrado efectos beneficiosos a corto y largo plazo sobre la supervivencia, el remodelado ventricular y la calidad de vida de los pacientes con IM. Así, se ha demostrado que la inhibición del sistema simpático nervioso o del sistema renina-angiotensina (RAS) previenen el remodelado ventricular izquierdo y reducen la mortalidad post-infarto.

Los inhibidores de la enzima convertidora de angiotensina (ACE) inhiben la síntesis de la angiotensina II (ANGII), un potente péptido de acción vasoconstrictora e hipertrófica que se libera en el miocardio tras el infarto (Figura 1.3.). El beneficio clínico incluye una reducción de la mortalidad [21], de la rehospitalización [22] y de la progresión de la insuficiencia cardiaca [22-25]. Por otro lado, el bloqueo de los receptores de ANGII también impide los efectos nocivos que su acumulación provoca, y ha demostrado ser eficaz en diversos ensayos clínicos realizados [26, 27]. Así, los inhibidores ACE son considerados como terapia de primera línea tras el IM, y los bloqueantes del receptor de ANGII son los fármacos de elección si aparece intolerancia a los inhibidores ACE.

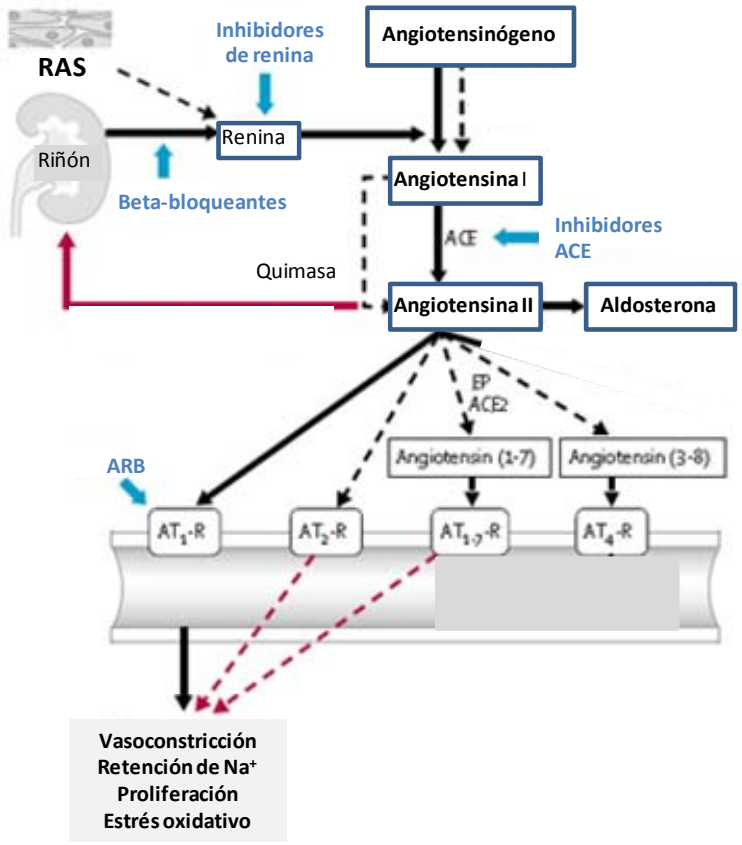


Figura 1.3. Sistema Renina-Angiotensina-Aldosterona (adaptado de *Staessen et al., 2006, Lancet*). Los beta-bloqueantes, inhibidores de renina, inhibidores de la enzima convertidora de angiotensina (ACE) y los bloqueantes del receptor tipo 1 de la angiotensina II (ARB) reducen la actividad del sistema renina-angiotensina (RAS). AT-R: receptor de la angiotensina; EP: endopeptidasas. Las flechas negras señalan inducción y las rojas inhibición. Las líneas punteadas describen vías de señalización alternativas.

Se utilizan también los antagonistas de la aldosterona, ya que su síntesis provoca un aumento del colágeno, la retención de sodio, pérdida de magnesio y potasio e hipertrofia ventricular, entre otros efectos. Tras la aparición del estudio EPHESUS [28], se ha recomendado su uso, en adición a los inhibidores ACE y los agentes beta-bloqueantes, en los casos de infarto agudo de miocardio y disfunción ventricular izquierda con signos de insuficiencia cardiaca o diabetes mellitus, ya que mejoran la supervivencia y disminuyen la muerte súbita cardiaca y los episodios de hospitalización por empeoramiento de la insuficiencia cardiaca [28, 29].

Por otro lado, los agentes beta-bloqueantes están indicados en todo paciente tras un IM, ya que disminuye el consumo metabólico de oxígeno y, a largo plazo, favorecen la contractilidad cardiaca [30]. Debido a que la acción de los beta-bloqueantes puede ser bifásica, con mejoría a largo plazo pero con un posible empeoramiento inicial, el tratamiento debe iniciarse bajo un minucioso control.

Existen además otros tratamientos que se utilizan como terapia preventiva, entre ellos, los fármacos antiagregantes, como la aspirina o el clopidogrel, o las estatinas, las cuales, además de disminuir los niveles séricos de colesterol, ejercen un efecto antiinflamatorio y vasoprotector, previniendo el desarrollo de la aterosclerosis y disminuyendo por tanto el riesgo de posteriores eventos coronarios. En los casos más graves, el trasplante de corazón suele ser la opción elegida [31].

Gracias al desarrollo y a la administración de estos fármacos, se ha conseguido prolongar y mejorar considerablemente la calidad de vida de los pacientes. Sin embargo, estos tratamientos no logran inducir la regeneración del tejido dañado ni evitan totalmente su progresiva degeneración, lo que hace necesaria en algunos casos la intervención quirúrgica, la cual, aunque eficaz, tampoco ejerce una acción regeneradora. Con este último fin se han desarrollado nuevas terapias, tales como la terapia proteica, la terapia génica y la terapia celular, las cuales se describen a continuación.

1.3.2. La terapia génica y la terapia proteica

Tanto la terapia génica como la terapia proteica se basan en la utilización de factores proteicos para el tratamiento de la enfermedad. Las citoquinas o factores de crecimiento pueden ser administrados como proteínas recombinantes (terapia proteica) o transfiriendo los genes que las codifican mediante plásmidos o vectores virales (terapia génica). En el ámbito de la isquemia cardiaca, la aplicación de este tipo de tratamientos se ha centrado principalmente en la inducción de la revascularización del tejido isquémico, logrando con ello, la protección/rescate del tejido dañado.

En el caso de la terapia génica, se han utilizado vectores con genes que codifican para proteínas pro-angiogénicas, tales como el $VEGF_{165}$ (*Vascular Endothelial Growth Factor-165*), el FGF-2 y 4 (*Fibroblast Growth Factor)*, o el HIF-1α (*Hypoxia-inducible Factor)* (revisado en [32]). En el caso de la terapia proteica, también se han inyectado, de forma intravenosa o intracardiaca, proteínas de acción similar, tales como el VEGF y el PDGF (*Plaquetal Derived Growth Factor*). Estos tratamientos han resultado efectivos en varios de los modelos animales estudiados [33, 34]. Sin embargo, los resultados obtenidos en los estudios clínicos no han sido satisfactorios (revisado en [35]). Posibles causas de la ineficacia de estos tratamientos han podido ser la baja eficacia de la administración de plásmidos o vectores virales así como la corta vida media y alta inestabilidad de las proteínas inyectadas, respectivamente. Además, en el caso de la terapia génica, puede producirse una cierta inmunogenicidad hacia las proteínas víricas (revisado en [36]) que, junto con el riesgo de la integración genómica en algunos casos, limitan su aplicación en la clínica. Por otro lado, en estos estudios se analizó el efecto de una única citoquina, siendo esperable que se requiriera la combinación de varias para inducir de forma más potente la revascularización del tejido.

En vista de estas limitaciones, se han desarrollado sistemas de liberación controlada que permiten liberar dichos factores de forma sostenida y estable, ensayándose, entre otros, la utilización de liposomas, micro- y nanopartículas e hidrogeles. Así, por ejemplo, se han utilizado micropartículas cargadas con VEGF que, tras su inyección en el corazón, han ejercido un efecto angiogénico constante, induciendo la revascularización del tejido y un remodelado cardiaco positivo [37, 38]. Se han obtenido efectos similares mediante la inyección de liposomas [39] e hidrogeles [40]. En otros estudios se han utilizado hidrogeles de alginato capaces de liberar de forma secuencial una combinación de citoquinas pro-angiogénicas tales como VEGF, TGF-β y PDGF, con las que se ha comprobado la inducción de la revascularización en un modelo de inyección subcutánea [41]. Se han realizado también estudios con otros factores, tales como el IGF-1 (*Insulin Growth Factor-1)* y el HGF (*Hepatic Growth*

Factor), esta vez inyectados en un modelo de IM agudo en rata, en el que se ha comprobado un efecto positivo a nivel de revascularización y remodelado del corazón, así como un incremento en la supervivencia y proliferación de las células cardiacas [42]. Hasta el momento, la mayoría de estos estudios se han realizado en modelos animales, aunque ya se han comenzado algunos ensayos clínicos, en los que se ha demostrado la seguridad y factibilidad del estudio tras la administración de bFGF en microcápsulas de alginato [43] o como el denominado ALCADIA (ClinicalTrials.gov Identifier: NCT00981006), dirigido al tratamiento de la cardiomiopatía isquémica y en el cual se ha combinado la administración de hidrogeles cargados con bFGF y células madre cardiacas.

1.3.3. La terapia celular

La búsqueda de una terapia regenerativa capaz de restablecer un tejido sano ha potenciado enormemente la investigación en el área de las células madre, las cuales presentan capacidad de auto-renovación y diferenciación hacia otros tipos celulares.

En el caso de la isquemia cardiaca, la célula ideal debería presentar el potencial de diferenciar hacia CM funcionales con capacidad de acoplarse electromecánicamente al tejido cardiaco circundante, así como de formar nuevos vasos que sustenten el tejido regenerado.

1.3.3.1. Tipos celulares y potencial cardiovascular

El potencial de diferenciación de las células madre varía según el tipo celular, en función de lo cual se han clasificado (yendo de mayor a menor capacidad) en: **células madre totipotentes,** que son aquellas células capaces de diferenciar tanto a tipos celulares de tejido embrionario como extra-embrionario; **células madre pluripotentes,** aquellas con capacidad de diferenciar a células tanto de tipo somático como germinal (dicha capacidad es propia de las células de la masa interna del blastocisto); **células madre multipotentes,** aquellas cuya capacidad de diferenciación

se restringe a los tipos celulares derivados de su misma capa embrionaria (es el caso de la mayoría de las células madre obtenidas a partir de los distintos tejidos adultos); y **células madre unipotentes,** aquellos progenitores capaces de derivar a un único tipo celular (Figura 1.4.).

Por otro lado, se ha establecido una división en función de su origen tisular, diferenciando así las células madre embrionarias (obtenidas a partir del blastocisto) de las células madre adultas (obtenidas de un tejido adulto). Es importante destacar el descubrimiento de la reprogramación de células adultas hacia estadios de desarrollo más tempranos y con características similares a las de las células madre embrionarias. Estas células se han denominado iPS (*induced Pluripotent Stem Cells*). Las características y aplicaciones en la enfermedad cardiovascular de todos estos tipos celulares se describen a continuación.

1.3.3.1.a. Células madre embrionarias

Las células madre embrionarias (ESC) son células pluripotentes provenientes de la masa celular interna del blastocisto [44, 45] que se caracterizan por su capacidad de auto-renovación, inmortalidad y pluripotencia. Son numerosos los trabajos que han descrito su diferenciación hacia tipos celulares pertenecientes a las tres capas embrionarias, incluyendo células con fenotipo cardiovascular que han sido obtenidas tanto *in vitro* [46, 47] como *in vivo* [48, 49]. Además, se ha demostrado, en modelos de IM murinos, que los CM derivados de las ESC, implantados en el corazón infartado, son capaces de injertar y madurar [50-52], induciendo la regeneración del corazón y un beneficio funcional del mismo [50, 53]. Sin embargo, a pesar de su gran potencial de diferenciación, las células embrionarias presentan importantes limitaciones para su uso clínico, siendo las más importantes, junto con los aspectos éticos, la tumorogenicidad y la inmunogenicidad.

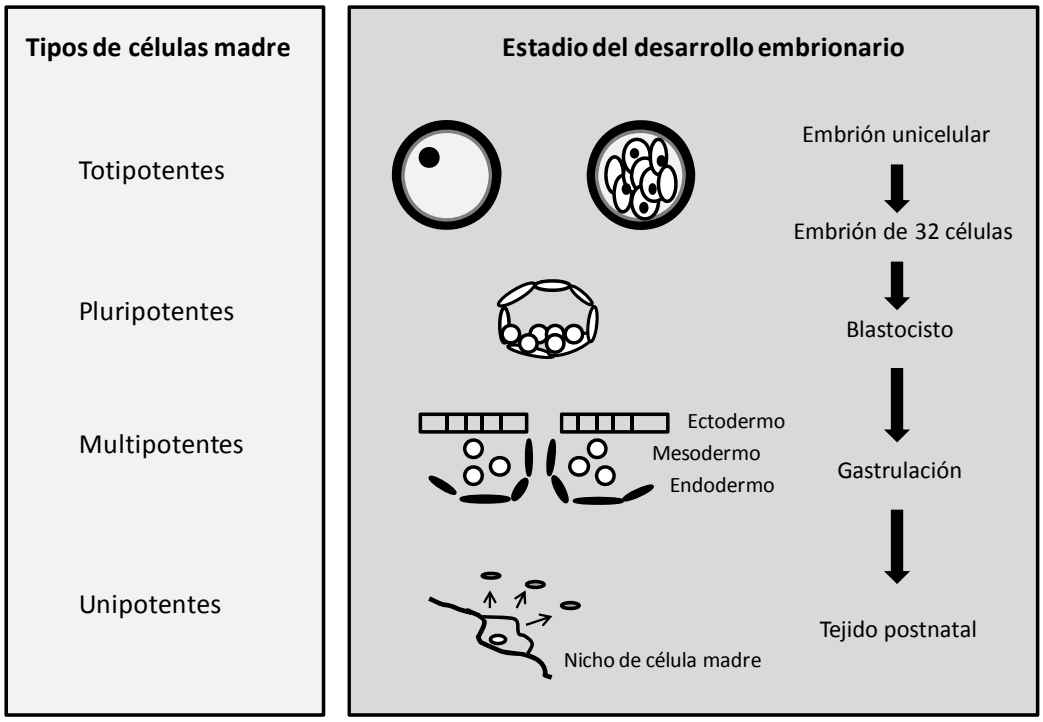


Figura 1.4. Esquema del potencial de diferenciación de las células madre durante las diferentes fases del desarrollo (adaptado de *Zimmermann Wh et al., 2011, Journal of Molecular and Cellular Cardiology*). Las células madre totipotentes se pueden aislar del embrión temprano de hasta 32 células mediante una biopsia del blastómero. Las células madre pluripotentes, comúnmente denominadas células madre embrionarias, derivan de la masa interna del blastocisto. Las células madre multipotentes se obtienen de una de las tres capas germinales y exhiben una diferenciación restringida hacia la propia capa germinal. Las células madre multipotentes también pueden aislarse en el estadio postnatal. Las células madre unipotentes pierden el potencial de transdiferenciación hacia otros linajes.

Se han descrito varias alternativas para evitar el riesgo tumoral, como la diferenciación *in vitro* y la estricta selección de las células diferenciadas [54] y para la inducción de la tolerancia inmunológica, mediante su co-inyección con células mesenquimales [55], el trasplante de precursores hematopoyéticos derivados de las ESC, la reprogramación nuclear para la generación de una fuente de ESC autóloga o la manipulación genética de los genes del complejo mayor de histocompatibilidad para la producción de una línea ESC [56], entre otros (revisado en [57]).

1.3.3.1.b. Células reprogramadas: Células pluripotentes inducidas y transdiferenciación directa

En el año 2006, el laboratorio del Dr. Yamanaka demostró por primera vez que una célula somática puede ser reprogramada al estado embrionario mediante la sobreexpresión de los factores de pluripotencialidad y autorenovación celular, Oct3/4, Sox2, Kfl4 y c-Myc. Estas células recibieron el nombre de células madre pluripotenciales inducidas (iPS), y presentaban características morfológicas y propiedades de pluripotencialidad similares a las de las células madre embrionarias.

Estos experimentos fueron originalmente realizados en fibroblastos de ratón, pero tan sólo un año más tarde se obtuvieron iPS humanas utilizando la misma combinación de factores [58-60] así como otras combinaciones (Oct4, Sox2, Nanog y Lin28) [61]. A partir de la publicación de estos trabajos, se han generado iPS de fibroblastos de diferentes especies, incluyendo el perro, el cerdo, la rata y los primates, y también se han generado a partir de otros muchos tipos celulares tales como queratinocitos, células $CD34^+$ de cordón umbilical, melanocitos, células madre neuronales, células derivadas de líquido amniótico y células mesenquimales derivadas de la grasa o de la pulpa dental (revisado en [62, 63]). Además, se han introducido nuevas variantes para inducir la reprogramación, como la generación de iPS sin la utilización del oncogen c-Myc [64, 65] o mediante la utilización de plásmidos o moléculas de ARN y proteínas, como alternativa a los vectores virales [66].

Los mecanismos por los cuales se desencadena la reprogramación todavía no se conocen en profundidad, aunque sí se ha determinado el papel que las modificaciones epigenéticas tienen en dicho proceso [67, 68].

De forma análoga a las células ESC, se ha demostrado la capacidad de diferenciación de las iPS hacia CM con capacidad funcional [69-71], cuyo potencial se ha comprobado en un modelo de IM agudo [69, 70]. Debido a su similitud, se han estudiado y comparado los fenotipos de CM derivados de ESC e iPS, mostrando un fenotipo muy similar entre ambos [72, 73]. A diferencia de las ESC, las iPS no presentan implicaciones éticas para su uso y pueden utilizarse como terapia autóloga. Su

capacidad tumorogénica, sin embargo, sigue representando un problema de cara a su posible aplicación terapéutica.

Existe una alternativa a esta limitación, que es la posibilidad de la transdiferenciación directa hacia células cardiacas. La transdiferenciación fue descrita inicialmente por el grupo del Dr. Takeuchi y colaboradores a partir de mesodermo de ratón, mediante la sobreexpresión de los factores de transcripción Gata4 y Tbx5 y una subunidad del complejo BAF de remodelado de la cromatina (Baf60c) [74]. Posteriormente se ha conseguido reproducir este método utilizando fibroblastos adultos [75], en este caso mediante la sobreexpresión de los factores de transcripción Mef2C, Gata4 y Tbx5.

1.3.3.1.c. Células madre adultas

Las células derivadas de tejidos adultos presentan generalmente una capacidad de diferenciación más limitada, pero poseen sin duda una serie de ventajas a nivel práctico tales como la inocuidad en su extracción y posterior aplicación, así como la posibilidad de su utilización como terapia autóloga, lo que ha facilitado su introducción en la práctica clínica.

Son muchos los tipos celulares derivados de tejidos adultos que se encuentran en estudio, destacando principalmente los derivados del músculo cardiaco y esquelético, de la MO y del tejido adiposo (Figura 1.5.).

- **Células madre cardiacas**

La presencia en el corazón de progenitores cardiacos fue demostrada por primera vez por el grupo del Dr. Anversa [19]. Las células aisladas se caracterizaban por la expresión del marcador **c-Kit** y la ausencia del marcador Sca-1, y se demostró un incremento de la población en el momento agudo del infarto. Dichos progenitores eran aislados a partir de biopsias de corazón, pudiendo ser cultivados y expandidos *in vitro* para su posterior implante en el tejido dañado. Se comprobó así su diferenciación

hacia CM, músculo liso y endotelio vascular *in vivo,* reemplazando la mayor parte del tejido isquémico y mejorando la función ventricular en un modelo murino de IM agudo [76, 77].

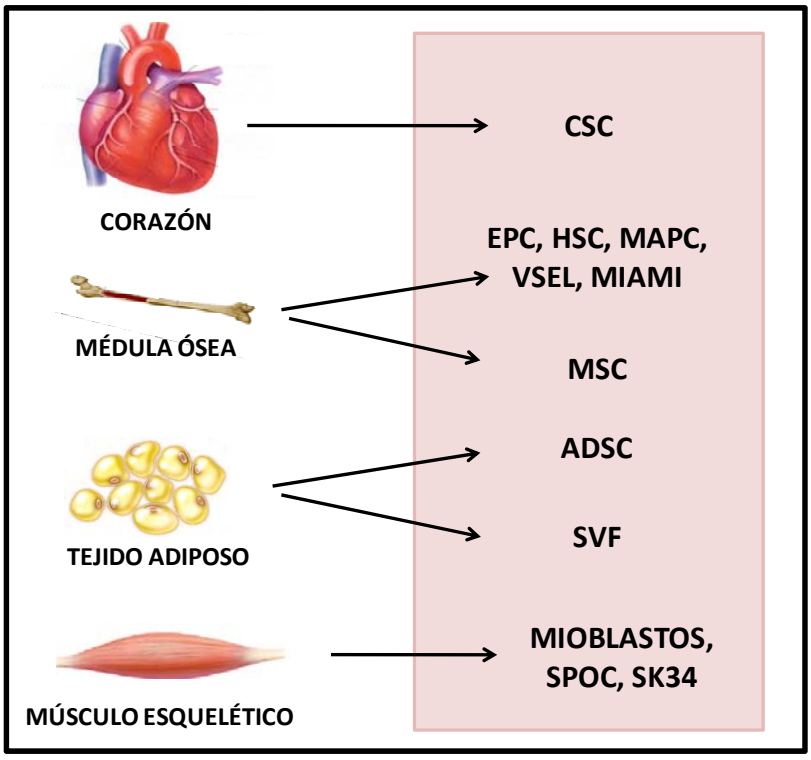


Figura 1.5. Tipos de células madre derivadas de tejidos adultos. CSC: *cardiac stem cells*; EPC: *endothelial progenitor cell*; HSC*: hematopoietic stem cells*; MAPC: *multipotent adult progenitor cell*; VSEL: *very small embryonic-like stem cell*; MIAMI: *marrow-isolated adult multilineage inducible cell*; MSC: *mesenchymal stem cell*; ADSC: *adipose-derived stem cell;* SVF: *stromal vascular fraction*; SPOC: *skeletal-based precursors of CM*; Sk-34: *skeletal muscle-derived multi-myogenic stem cell CD34*$^{+}$.

Por otro lado, el grupo del Dr. Schneider describió la presencia en el corazón del ratón de otra población celular, de fenotipo **Sca-1^{+}/c-kit^{-}** y con capacidad de diferenciación a CM tras su estimulación *in vitro* con 5-azacitidina. Se demostró además su capacidad de injerto y la expresión de marcadores cardiacos en un modelo *in vivo* de IM [78].

También han sido aislados otros tipos celulares, como la población **Sca-1^{+}/cKit^{LOW}/Abgc-2^{+}**, con capacidad de diferenciación a músculo cardiaco tras su co-

cultivo con CM [79, 80] y la población **Islet-1^{+}** [81, 82], también con potencial cardiomiogénico. Por último, se ha descrito la obtención de progenitores cardiacos que pueden ser cultivados en suspensión formando lo que se ha denominado como **cardioesferas**. Éstas son aisladas a partir de biopsias de tejido muscular cardiaco atrial o ventricular y, una vez procesadas, son cultivadas *in vitro*, de modo que forman agregados celulares compuestos por un núcleo de células proliferativas c-Kit^{+} y por células periféricas con potencial cardiovascular [83]. Se ha demostrado además que, cuando éstas son trasplantadas en modelos murinos de IM e incluso en modelos pre-clínicos de cerdo, son capaces de regenerar el tejido cardiaco dañado e inducir un efecto positivo sobre el remodelado tisular y la función cardiaca [84-86].

En vista de la existencia y diversidad fenotípica de todas estas poblaciones de progenitores cardiacos, sería importante caracterizarlas en mayor profundidad para determinar con mayor precisión el papel que desempeñan en el corazón isquémico y poder así estimularlas para regenerar el tejido dañado.

- Células madre derivadas del músculo esquelético

Los mioblastos o células satélite son células progenitoras presentes en el músculo esquelético que están implicadas en su regeneración, mediante un proceso secuencial de activación, diferenciación a célula muscular madura y fusión con las miofibras circundantes [87]. Los miofibroblastos han sido uno de los primeros tipos celulares estudiados como terapia para el IM, ya que se planteó la hipótesis de que pudieran transdiferenciar *in vivo* hacia CM [88] y, por tanto, lograran reparar el corazón isquémico. Estudios posteriores demostraron, sin embargo, su capacidad de diferenciación exclusiva hacia el linaje muscular esquelético [89, 90]. Además, la falta de expresión de las uniones *gap* requeridas para el acoplamiento electromecánico con los CM [91], hace que éstos no sean capaces de sincronizarse en la contracción [92, 93], pudiendo incluso generar eventos de arritmias [94].

A pesar de estas limitaciones, diversos estudios realizados en modelos animales han demostrado que el trasplante de mioblastos induce un efecto beneficioso en la

función ventricular [95, 96], atribuída principalmente a mecanismos de tipo trófico implicados en la revascularización del área infartada y en la reorganización de la matriz extracelular [97].

Además de los mioblastos, se han aislado y estudiado otras poblaciones menos frecuentes en el músculo esquelético, que se han denominado como células **SPOC** (*Skeletal-based precursors of cardiomyocytes*) y **Sk-34** (*Skeletal muscle-derived multi-myogenic stem cells CD34*$^{+}$). En ambos casos se demostró su potencial cardiomiogénico al ser trasplantadas en un modelo de IM murino [98, 99], aunque su presencia en el músculo esquelético humano todavía no se ha demostrado.

- Células madre derivadas de la médula ósea

La médula ósea ha sido una de las fuentes celulares más estudiadas en el ámbito de la regeneración cardiaca, debido a la facilidad de su extracción, a la posibilidad de aplicación autóloga y a la presencia de diversas poblaciones celulares con gran potencial de diferenciación [100-103]. Son diversos los trabajos realizados *in vivo* en modelos de IM, tanto en rata [104-106], ratón [107] o cerdo [108, 109], en los que se ha demostrado su beneficio tanto a nivel funcional como histológico, con una mejora de la perfusión y del remodelado del tejido cardiaco infartado.

Son muchos los estudios realizados en diversos modelos animales en los que se ha analizado el potencial de las células mononucleadas derivadas de la MO (MNC-MO). Así, por ejemplo, se ha descrito que, cuando éstas son trasplantadas en un modelo de infarto crónico en rata, inducen un beneficio funcional cardiaco, así como un efecto positivo sobre el remodelado ventricular [110]. Por otro lado, también se ha observado que la infusión temprana de esta población celular en un modelo de isquemia-reperfusión en rata, ejerce un efecto protector sobre el tejido, disminuyendo el grado de apoptosis y necrosis del mismo [111]. Además, se ha confirmado en modelos pre-clínicos en cerdo que su trasplante favorece la angiogénesis tisular y disminuye el tamaño de infarto [112], ejerciendo un beneficio funcional cardiaco [113].

Además de la población mononucleada total también se ha analizado el potencial de subpoblaciones de la MO de distintos orígenes (hematopoyético, endotelial o mesenquimal, entre otros), que se describen a continuación.

Células madre hematopoyéticas (HSC): la presencia de progenitores cardiacos en la MO fue demostrada por primera vez por el grupo del Dr. Anversa. En este estudio se mostró cómo la subpoblación de fenotipo **lin$^-$/c-kit$^+$**, era capaz de regenerar el tejido miocárdico en corazones previamente infartados, logrando inducir una mejora funcional dos semanas después del implante [103, 107]. Estos resultados sin embargo han sido difícilmente reproducidos por otros grupos [114-116]. Así, el Dr. Balsam y colaboradores no encontraron CM derivados de las HSC tras su inyección en corazones infartados pese a la mejora de la función ventricular [114]. El Dr. Nygren y colaboradores tampoco observaron diferenciación de las HSC hacia CM y determinaron la posibilidad de fusión de las HSC con los CM [115].

Las células progenitoras endoteliales (EPC) fueron originalmente descritas como progenitores que expresan los marcadores de superficie KDR, CD34 y AC133 [117] y que se movilizan desde la MO en respuesta a estímulos tales como la isquemia o el daño vascular. Estas células pueden favorecer la angiogénesis [118, 119] a través de su contribución directa a la formación de la vasculatura, pero también mediante su acción paracrina, al proporcionar señales de supervivencia a las células dañadas [120] y reclutar e inducir la proliferación de nuevos progenitores presentes en el organismo. En modelos de isquemia periférica se observó que su trasplante mejoraba el grado de perfusión [121]. También en modelos de IM, donde inducían un beneficio funcional, acompañado de una reducción de la apoptosis y del contenido de colágeno [122, 123], principalmente por su acción trófica [124].

Por otro lado, el cultivo *in vitro* de la fracción MNC-MO da lugar a las **células madre mesenquimales (MSC)**. Éstas son células multipotentes que se caracterizan por presentar un fenotipo positivo para los marcadores CD44, CD73, CD90 y CD105, y negativo para CD34, CD45 y MHC-II, y que además se caracterizan por su capacidad de diferenciación hacia hueso, cartílago y grasa (revisado en [125]). En algunos casos,

también se ha descrito su contribución vascular [126, 127]. A pesar de esta limitada capacidad de diferenciación hacia el linaje cardiaco, han demostrado ejercer un importante efecto en diversos modelos de isquemia, gracias a su actividad paracrina a nivel de revascularización y remodelado tisular (revisado en [128]). Se ha demostrado la acción pro-angiogénica de las MSC mediante la liberación de un amplio panel de citoquinas tales como FGF, VEGF, MMP, PDGF, TGF-β, IL-1 o angiopoietina (revisado en [129]). Además, se les atribuyen otros efectos tales como la protección y estimulación de la proliferación de los CM, mediadas por la proteína Sfrp2 [130] o la disminución de la proliferación y de la síntesis del colágeno de tipo I y III por parte de los fibroblastos cardiacos [131, 132]. Por otro lado, tras ser implantadas en modelos de IM se ha observado una mejora en la función cardiaca asociada a un aumento de la angiogénesis [105, 110], a la estimulación de precursores cardiacos [108] y a la disminución del tamaño de infarto y fibrosis [110]. Además, cabe destacar su capacidad de modulación de la respuesta inmune [133-135]. Dicha propiedad se ha descrito *in vitro* ya que no provocan reactividad cruzada con linfocitos e inhiben la respuesta proliferativa de éstos a los mitógenos. Además, no expresan la molécula HLA-DR, y todo ello, supone una ventaja para el posible trasplante alogénico facilitando así su implantación en la clínica [136-138]. De ese modo, células mesenquimales bien caracterizadas y obtenidas a partir de donantes jóvenes y sanos, podrían utilizarse para tratar de inmediato a distintos pacientes, incluyendo aquellos con infarto agudo. Hay que tener en cuenta sin embargo, que estudios recientes han sugerido que esta capacidad puede perderse *in vivo* [139-141]. Así por ejemplo, en un modelo inmunocompetente de cerdo, se demostró la activación de la respuesta celular y humoral como consecuencia del implante de MSC [141]. En cualquier caso, puesto que las MSC no contribuyen a la regeneración del corazón mediante su diferenciación, sino que ejercen un efecto temporal de tipo paracrino, esta acción podría ser lo suficientemente prolongada como para ejercer un beneficio cardiaco. Además, existe la posibilidad de la inmunosupresión del paciente o de la selección de las células

donantes mediante estudios de histocompatibilidad. En cualquier caso, este aspecto requiere ser estudiado en mayor profundidad.

Por último, se ha descrito también la presencia en la MO de poblaciones raras con capacidad pluripotente, como son las denominadas MAPC (*Multipotent Adult Progenitor Cells*), las VSEL (*Very small embryonic-like stem cells*) y las MIAMI (*Marrow-isolated adult multilineage inducible cells*). En el caso de las **MAPC**, éstas fueron aisladas por el laboratorio de la Dra. Verfaillie a partir de distintas especies: roedor [142], cerdo [143] y humano [144]. Las MAPC se caracterizan por presentar la capacidad de diferenciar hacia tipos celulares pertenecientes a las tres capas embrionarias y proliferar durante más de 100 duplicaciones sin acortamiento de los telómeros. Su potencial beneficioso se demostró, entre otros, en modelos animales de isquemia periférica y cardiaca [145-149], destacando su contribución a la vasculatura y al músculo, así como su efecto paracrino en la angiogénesis tisular [144, 150]. Otra población celular, las **VSEL**, fue aislada a partir de MO de ratón adulto y se caracteriza por la expresión de marcadores típicos de células embrionarias tales como Oct-4 y SSEA-1, así como por su pluripotencialidad tanto *in vitro* [151] como *in vivo* [152, 153]. En un modelo de IM, las VSEL produjeron un beneficio funcional, y aunque en bajo número, se encontraron células que expresaban marcadores de células vasculares y musculares [152, 153]. Por último, la población denominada **MIAMI** [154] se caracteriza por la expresión de Oct-4, Rex-1 y telomerasa, y aunque su potencialidad cardiomiogénica no ha sido aún demostrada, sí se comprobó su potencial vascular en un modelo murino de isquemia periférica [155].

- Células madre derivadas del tejido adiposo

El tejido adiposo es un tejido complejo que contiene diversas poblaciones celulares maduras, tales como los adipocitos, fibroblastos, células vasculares y hematopoyéticas, así como una fracción celular no adipocítica denominada fracción estromal vascular (*Stromal Vascular Fraction*, **SVF**) [156, 157] que se caracteriza por ser un compartimento rico en células con capacidad multi/pluripotente [158, 159]. En

esta fracción tisular se han encontrado precursores hematopoyéticos [160], adipocíticos [161], vasculares (endoteliales [162] y de músculo liso [163]), e incluso cardiacos [164-166] y de músculo esquelético [167] (Figura 1.6.). Además, la SVF posee una potente actividad paracrina, capaz de modular procesos de hematopoyesis [160, 168], de angiogénesis y de supervivencia celular [169, 170]. En los estudios realizados *in vivo*, se ha comprobado el efecto beneficioso de su trasplante en modelos animales de isquemia periférica [162, 169] y de IM [171, 172], provocando una mejora funcional y un efecto cardioprotector [173]. Estudios realizados a largo plazo en modelos de IM crónico [174] y agudo [175] han mostrado un beneficio tanto de la función como del metabolismo cardiaco. Dicha mejora se asocia a un aumento en el grado de revascularización y a un efecto positivo en el remodelado.

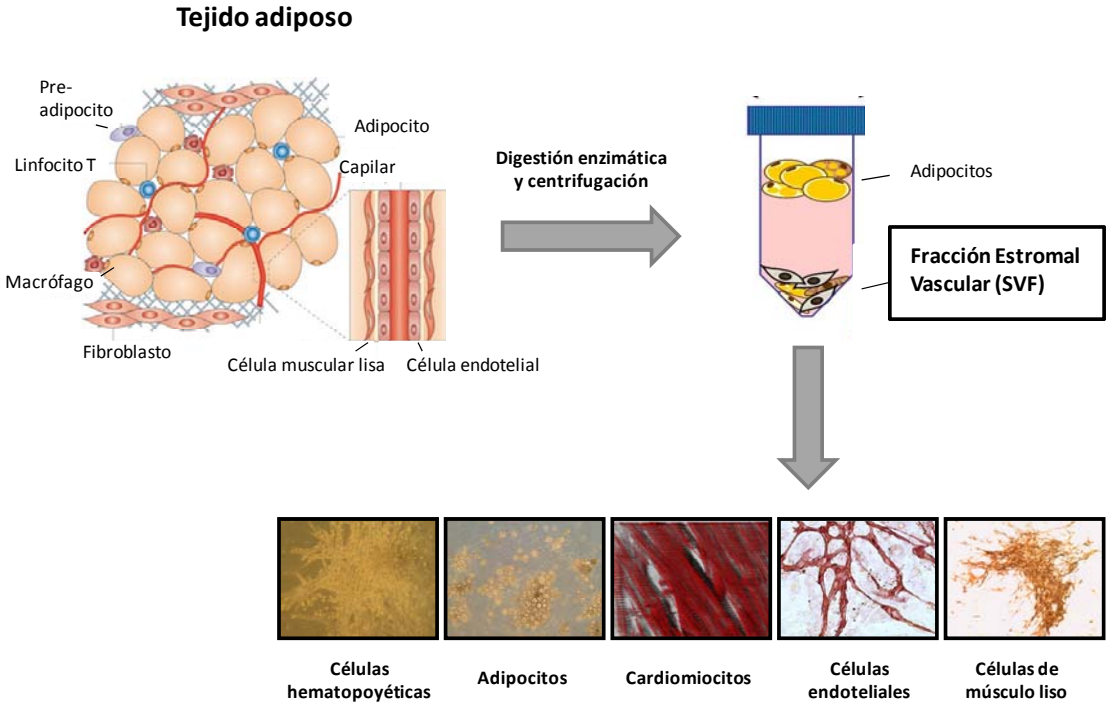


Figura 1.6. Fracción estromal vascular (SVF). Obtenida a partir de tejido adiposo mediante tratamiento mecánico y enzimático, la SVF ha demostrado ser capaz de diferenciar a distintos tipos celulares de interés para la terapia en el infarto de miocardio: músculo cardiaco, endotelio y músculo liso entre otros.

Por otro lado, el cultivo *in vitro* de la SVF da lugar a una población celular más homogénea y con propiedades mesenquimales que se ha denominado **ADSC** (*Adipose Derived Stem Cells)* [158]. Las ADSC presentan un perfil fenotípico característico de poblaciones mesenquimales ($CD73^+$, $CD90^+$, $CD105^+$, $CD45^-$, $CD34^-$, $CD11b^-$, $CD79^-$, $CD19^-$, $HLA\text{-}DR^-$), así como su capacidad de diferenciación hacia tejido óseo, cartílago, tendón, músculo esquelético y grasa [176-178]. A pesar de las similitudes existentes, se han descrito diferencias entre las poblaciones mesenquimales derivadas de la MO y del tejido adiposo tanto a nivel genómico y proteómico, como funcional [179, 180], y que sugieren un mayor compromiso de la población MSC hacia un linaje osteoblástico y/o condrogénico que las ADSC.

El potencial terapéutico de las ADSC ha sido demostrado en modelos animales de isquemia periférica [169, 181] y cardiaca [182-184]. En los modelos de IM en rata [183, 184] se ha determinado una mejora en la función cardiaca y perfusión asociada a un aumento en la vascularización del tejido isquémico y a una disminución de la fibrosis tisular. Además, se han descrito resultados que apoyan el efecto de la población ADSC en la mejora de la función cardiaca y la revascularización del tejido en modelos pre-clínicos en cerdo de IM agudo [182] y crónico (datos no publicados obtenidos en nuestro laboratorio). Por otro lado, en un trabajo comparativo del Dr. Kim y colaboradores, se comprobó un mayor efecto angiogénico de las ADSC que de las MSC [181]. Cabe destacar, por último, el supuesto efecto inmunomodulador [137, 185, 186] de la población ADSC, que supondría una ventaja añadida de cara a su posible aplicación alogénica en la terapia clínica.

1.3.3.2. Primeras aproximaciones al paciente: ensayos clínicos

A pesar de que todavía es necesario investigar y comprender en mayor profundidad el comportamiento y los mecanismos por los que actúan las células madre, el beneficio funcional observado en los distintos modelos animales realizados ha promovido el desarrollo de ensayos clínicos en pacientes, realizados principalmente con mioblastos esqueléticos y diversas poblaciones celulares derivadas de la MO.

Recientemente se han comenzado también algunos ensayos con células madre cardiacas y derivadas del tejido adiposo que se comentarán a continuación.

1.3.3.2.a. Ensayos clínicos realizados con mioblastos esqueléticos

En el año 2000 se comenzó el primer ensayo clínico realizado con mioblastos esqueléticos, para el cual se reclutaron diez pacientes con fallo cardiaco severo (fracción de eyección del ventrículo izquierdo, FEVI ≤ 35%) [94]. El tratamiento mejoró significativamente la función cardiaca, analizada un año después del implante y que se mantuvo hasta seis años después [187]. Sin embargo, cinco de los pacientes sufrieron taquicardias e incluso tres de ellos experimentaron eventos arrítmicos graves, lo que replanteó la seguridad del tratamiento. Afortunadamente, estudios posteriores mostraron la seguridad del mismo, demostrado una mejora de la función y perfusión cardiaca sin eventos graves de arritmias [188-194]. Así, en el estudio publicado por el Dr. Gavira y colaboradores [193], no se describieron episodios de arritmias en el seguimiento a un año, y se detectó además de un incremento en la FEVI, una mejora en la contractilidad de la pared y en la viabilidad y perfusión del tejido. Otros ensayos publicados [191, 195] confirmaron dicha mejora funcional dos años después del implante de mioblastos (Tabla 1.1.).

A pesar de que los datos obtenidos por estos grupos apuntaban hacia un efecto beneficioso de la terapia con mioblastos, el limitado número de pacientes tratados y la ausencia de un grupo placebo no permitieron obtener resultados concluyentes. Por ello, se realizó un ensayo que se denominó como estudio MAGIC (*Myoblast Autologous Grafting in Ischemic Cardiomyopathy*), a doble-ciego, aleatorizado, con grupo placebo y en el que se incluyeron varias dosis de tratamiento [196]. En este estudio se confirmó, tras seis meses de seguimiento, la seguridad del tratamiento, ya que no se evidenciaron diferencias entre los grupos a nivel de los sucesos de arritmias, ni en el ratio de supervivencia de los pacientes. Sin embargo, no se observó una mejora de la función ventricular.

Tabla 1.1. Ensayos clínicos realizados con mioblastos esqueléticos. Abreviaturas: Co: control; m: meses; TEp: transepicárdico; TCo: transcoronario; Vtd: Volumen telediastólico; Vts: Volumen telesistólico; DVIts: Diámetro telesistólico; DVItd: Diámetro telediastólico.

Autor (Año)	Nº pacientes (Tratado/Co)	Vía	Seguimiento (Meses)	% ΔFEVI (Basal vs. Tratado) (*p* en grupo tratado)	Otros resultados funcionales
Menasché *et al* [94] (2003) Hagegé *et al* [187] (2006)	10/0	TEp con o sin CABG	10,9 52	10,9m: 8(*) 52m: 4(ns)	10,9 m: ↑movilidad regional de la pared
Smits *et al* [188] (2003)	5/0	TEp	3 6	3m: 5(**) 6m: 9(ns)	↑movilidad regional de la pared
Ince *et al* [189] (2004)	6/6	TEp	12	8(*) Co: -3	
Siminiak *et al* [190] (2004)	10/0	TEp con o sin CABG	4 12	4m : 7(*) 12m : 7(*)	↑movilidad regional de la pared
Chachques *et al* [195] (2004)	20/0	TEp con o sin CABG	9, 19	24(*)	↑movilidad regional de la pared, y viabilidad tisular
Siminiak *et al* [194] (2005)	9/0	TCo	2,5	3-8 en 6-9 pacientes (sin análisis estadístico)	Mejora sintomática
Gavira *et al* [193] (2006)	12/14	TEp + CABG	3 12	3m : 8(*) 12m : 9(**) Co-12m : 3(ns)	↑movilidad regional de la pared, viabilidad tisular y perfusión
Biagini *et al* [192] (2006)	10/0	TEp	1, 3, 6, 12	12m: 6(**)	↑movilidad regional de la pared; ↓Vts, ≈Vtd
Menasché *et al* [196] (2008) MAGIC	30 dosis alta/33 dosis baja/ 34 Co	TEp + CABG	6	dosis alta: 0.8(ns)	≈movilidad regional de la pared; ↓Vts (Dosis alta)
Dib *et al* [197] (2009) CAUSMIC	12/11	TEp	12	ND	↓ DVIts , ↓DVItd, mejora en las medidas de voltaje
Duckers *et al* [198] (2011) SEISMIC	26/14	TEp	6	6m:(ns)	Mejora sintomática

Por otro lado, los resultados de otros dos ensayos más recientes (CAUSMIC y SEISMIC) indicaban la seguridad del tratamiento en la práctica clínica [197, 198]. Finalmente, de cara a confirmar la posible seguridad y eficacia del tratamiento con mioblastos, se ha iniciado un ensayo multicéntrico en el que se ha incluido un número de pacientes mucho mayor (330 pacientes de Norte América y Europa), que se ha denominado "ensayo MARVEL", diseñado como un estudio controlado por placebo, realizado a doble-ciego y aleatorizado.

1.3.3.2.b. Ensayos clínicos realizados con células madre derivadas de la médula ósea

Los primeros ensayos realizados con células madre derivadas de la MO tuvieron como objetivo demostrar la factibilidad y seguridad de la terapia [199-205]. Se realizaron estudios muy diversos en los que se analizaron distintos tipos celulares (células mononucleadas, fracciones seleccionadas de MO, MSCs, células mobilizadas, etc), inyectados a través de distintas rutas de infusión (percutánea, endocárdica vía catéter, epicárdica por cirugía transtorácica, etc) y con los que se trataron distintos tipos de pacientes. Esto ha dificultado enormemente una comparación directa de los mismos, aunque sí que ha confirmado la inocuidad del tratamiento. Posteriormente, se han realizado estudios aleatorizados, dirigidos a determinar la eficacia del tratamiento.

Así, el primer ensayo realizado con este tipo celular, denominado BOOST I (*Bone Marrow Transfer to Enhance ST-elevation Infarct Regeneration*), concluyó que la aplicación intracoronaria de células mononucleadas totales provocaba una mejora de la función cardiaca tras seis meses de tratamiento. Sin embargo, cabe destacar que este beneficio resultó ser transitorio, ya que dicha mejora desaparecía a los dieciocho meses [206-208]. Otro estudio con MSC también demostró su beneficio sobre la función del corazón, pero, dado que el periodo de seguimiento de los pacientes fue tan sólo de seis meses, no ha sido posible demostrar su efecto a largo plazo [209]. En 2006, el grupo del Dr. Janssen publicó los resultados del primer estudio aleatorizado realizado a doble-ciego y controlado por placebo. En este estudio se trataron pacientes que habían sufrido un IM agudo, con MNC-MO inyectadas tras 24 horas de su reperfusión. Aunque los resultados indicaron una disminución del tamaño de infarto y una mejora de la función sistólica regional, no se pudo demostrar un efecto positivo global a los cuatro meses [210]. Por otro lado, en ese mismo año se publicaron dos ensayos clínicos con resultados opuestos. Por un lado, las conclusiones del estudio REPAIR-AMI (*Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction*) indicaron que, cuatro meses después de la infusión de las células, el grupo tratado presentaba una mejora de la función ventricular, así como

una disminución del tamaño de infarto [211, 212], mientras que en el estudio ASTAMI (*Autologous Stem cell Transplantation in Acute Myocardial Infarction*), a pesar de que se observó una mejora en el tiempo de ejercicio, no se encontraron diferencias significativas entre el grupo tratado con células y el grupo placebo [213]. Estos resultados contradictorios han podido deberse al procesamiento y a la dosis de las células trasplantadas, que fue de dos a cuatro veces menor en el estudio ASTAMI. Finalmente, en el estudio realizado por el grupo del Dr. Meluzin, se concluía que dicho beneficio podía ser dependiente de la dosis celular, ya que, tras trasplantar una dosis de 10 y 100 millones de MNC, se observó tan sólo una mejora en los individuos tratados con la mayor dosis [214, 215].

Por otro lado, ensayos clínicos realizados recientemente también han obtenido resultados opuestos. En el estudio FINCELL [216], se concluyó que el trasplante de MNC-MO inducía un aumento significativo en la función ventricular tras seis meses del tratamiento. En cambio, en los ensayos REGENT y HEBE no se encontraron diferencias significativas entre los grupos a los seis y cuatro meses, respectivamente [217, 218].

Como conclusión de todos los estudios realizados, en 2007 se publicó un interesante meta-análisis, resultante de los diez ensayos clínicos más relevantes llevados a cabo hasta el momento con células MNC-MO, en el que se concluía que su aplicación terapéutica en el IM ejercía efectos positivos a nivel funcional y de remodelado cardiaco, dependiente de la dosis celular empleada [219]. Otros meta-análisis realizados posteriormente han corroborado la factibilidad y seguridad de la terapia, así como la posibilidad de un beneficio cardiaco frente a los tratamientos convencionales [220, 221].

En su conjunto, todos estos resultados (Tabla 1.2.) indican que, aunque las expectativas resultan prometedoras, para demostrar la eficacia de la utilización de células de la MO como tratamiento del IM, es necesario llevar a cabo estudios multicéntricos aleatorizados con un seguimiento de los pacientes a largo plazo.

Tabla 1.2. Ensayos clínicos con células derivadas de MO. Abreviaturas: Co: control; BMC: células de médula ósea; CPrC: células progenitoras circulantes; m: meses; IC: intracoronario Vtd: Volumen telediastólico; Vts: Volumen telesistólico.

Autor (Año)	Nº pacientes (Tratado/Co)	Tipo celular	Vía	Seguimiento (Meses)	% ΔFEVI (Basal vs. Tratado) (p en grupo tratado)	Otros resultados funcionales
Strauer *et al* [200] (2002)	10/10	BMC	IC	3	4 vs 5(ns)	↓Vts ≈ Vtd; ↑movilidad regional de la pared y perfusión; ↓tamaño de infarto
Assmus, Britten, Schachinger *et al* [199, 201, 202] (2002-4) TOPCARE-AMI	19/11	BMC/CPrC	IC	4, 12	2,5 vs 8,5(**)	↓Vts, ≈ Vtd; ↑movilidad regional de la pared; ↓tamaño de infarto
Fernández-Avilés *et al* [203] (2004)	20/13	BMC	IC	6	5,8(**)	↓ Vts, ≈ Vtd; ↑grosor pared infartada
Bartunek *et al* [205] (2005)	19/16	CD133	IC	4	4,3 vs 7,1(*)	≈ Vtd; ↑perfusión
Woolert, Schaefer *et al* [207, 208] (2006) BOOST I	30/30	BMC	IC	6 18	6m: 0,7 vs 6,7(**) 18m: 3,1 vs 5,9(ns)	↓tamaño de infarto
Chen *et al* [209] (2004)	34/35	MSC	IC	6	6 vs 8(*)	↓ Vts ; ↑movilidad regional de la pared y perfusión
Jannsens *et al* [210] (2006)	66	BMC	IC	4	2,2 vs 3,3(ns)	↑perfusión, viabilidad tisular; ↓tamaño infarto
Lunde *et al* [213] (2006-7) ASTAMI	47/50	BMC	IC	6	6,7 vs 8(ns)	≈ Vts, ≈ Vtd; ≈perfusión; ≈tamaño de infarto
Schachinger, Erbs *et al* [211, 212] (2006-7) REPAIR-AMI	101/103	BMC	IC	4, 12	3 vs 5,5(*)	≈Vts, ≈ Vtd; ≈tamaño de infarto
Meluzin *et al* [214, 215] (2006-7)	22 dosis alta/22 dosis baja/22 Co	BMC	IC	3, 6, 12	3m: 3 vs 6(ns) 6m: 0 vs 7 (dosis alta)(*) 12m: 0 vs 7 (dosis alta)(*)	↓ Vts, ≈ Vtd; ↑perfusión
Huikuri *et al* [216] (2008) FINCELL	39/38	BMC	IC	6	6m: 1 vs 7(*)	
Van der Laan *et al* [217] (2008) HEBE	189	BMC/PBMC	IC	4	4m:(ns)	No hay cambios en la función ventricular izquierda
Tendera *et al* [218] (2009) REGENT	46/51/20	BMC/BMC $CXCR4^{+}CD34^{+}$	IC	6	6m:(ns)	

1.3.3.2.c. Ensayos clínicos realizados con otras poblaciones celulares

En vista de los resultados experimentales, se han iniciado también ensayos clínicos con células madre derivadas de tejido adiposo, como el estudio PRECISE (*3D adipose-derived stem cells in the treatment of patients with non-revascularizable ischemic myocardium*), liderado por el grupo del Dr. Fernández-Avilés (ClinicalTrials.gov Identifier: NCT00426868) y el estudio APOLLO (*3D adipose-derived stem-cell transplant in the treatment of patients with an acute ST-elevation MI*), liderado por el Dr. Serruys (ClinicalTrials.gov Identifier NCT00442806). Sin duda, los resultados que se obtengan de estos ensayos arrojarán luz sobre la capacidad de las células madre del tejido adiposo para su aplicación terapéutica en la clínica.

Por otro lado, se han comenzado dos ensayos clínicos con células madre cardiacas: en el primer ensayo se están administrando células c-kit$^+$ como tratamiento coadyuvante en pacientes sometidos a cirugía de *bypass* coronario (ClinicalTrials.gov identifier NCT00474461) y en el otro, se están inyectando cardiosferas de origen autólogo en pacientes con un IM reciente (ClinicalTrials.gov identifier NTC00893360).

1.3.3.3. Ventajas y limitaciones de la terapia celular

Una de las conclusiones fundamentales a la que se ha llegado al analizar el potencial terapéutico de las células madre en los modelos experimentales y en los posteriores ensayos clínicos, es que, pese a que se ha comprobado su capacidad de diferenciación *in vitro*, esta diferenciación celular no siempre se da *in vivo*. La falta de un microambiente adecuado, como la presencia de una matriz extracelular adecuada, unos niveles óptimos de oxígeno y nutrientes para la supervivencia celular y de factores y citoquinas específicos que dirijan la diferenciación, dificultan enormemente el injerto y la diferenciación en el tejido, impidiendo la regeneración de éste. Sin embargo, a pesar de no cumplirse las expectativas referentes al efecto regenerador de las células madre mediante su diferenciación *per se*, sí que se ha demostrado un importante beneficio consecuencia de su acción paracrina [97, 128, 129, 222].

Así, múltiples estudios han confirmado que, mediante la secreción de un amplio panel de factores y citoquinas, las células trasplantadas son capaces de activar procesos de revascularización, supervivencia celular y/o un remodelado positivo del corazón. Se ha demostrado que diversas poblaciones de células madre (MSC y MNC-MO, ADSC o incluso mioblastos esqueléticos) secretan factores de tipo pro-angiogénico tales como VEGF, FGF-1 y PDGF, implicados en procesos de angiogénesis y arteriogénesis [97, 146, 147, 222]. También ejercen diversos efectos sobre la matriz extracelular mediante la secreción de MMPs, encargadas de la degradación de la misma, así como de sus inhibidores específicos, los TIMPs, de modo que modulan la regulación de los procesos de fibrosis y cicatrización del tejido [97, 174]. Por último, otros factores como IGF-1 o el propio VEGF han demostrado ejercer un papel protector sobre células vasculares y CM, rescatándolos de su entrada en apoptosis [169, 223].

Un aspecto interesante que refuerza la hipótesis del efecto paracrino en la terapia celular es el hecho de que distintas poblaciones celulares sean capaces de ejercer una acción relativamente similar sobre el corazón infartado, y que, por tanto, no sea tan relevante el tipo celular implantado como el patrón de factores liberados por sí mismo. Así, en estudios en los que se ha comparado el efecto terapéutico de diversas poblaciones celulares simultáneamente, se ha observado un efecto más potente con algunas de ellas (por ejemplo de las MAPC frente a las células MNC-MO o fibroblastos), como consecuencia del tipo y concentración de las citoquinas que secretan [146].

Otro parámetro importante es la capacidad de injerto celular y supervivencia en el tejido, siendo ésta una de las limitaciones más importantes con las que se ha encontrado la terapia celular. Así, se ha observado que menos del 30% de las células trasplantadas sobreviven más de 48 horas después del implante, y que éstas desaparecen progresivamente por las condiciones de hipoxia, inflamación y fibrosis del tejido que las rodea. Además, debido a la vía de administración utilizada habitualmente en los trasplantes (inyección intramiocárdica de las células en suspensión), se pierde un alto porcentaje de las mismas, tanto en el momento de la

inyección como posteriormente, por la falta de adhesión a la matriz y de un aporte adecuado de oxígeno y nutrientes en el tejido [224].

A pesar de estas limitaciones, hay que destacar que, en general, se ha conseguido ejercer un efecto beneficioso sobre la función cardiaca, por lo que cabe esperar que, al incrementar el injerto y supervivencia celular, se potencie su efecto terapéutico. Con este objetivo se han realizado nuevas aproximaciones, tales como la preparación de soportes compuestos por distintos biomateriales, o incluso la creación de construcciones tisulares más complejas que puedan ser trasplantadas en el corazón, favoreciendo así su regeneración. En esta nueva etapa se ha impulsado la combinación de la terapia celular con la bioingeniería, lo que permite la construcción de modelos *in vitro* más relevantes y mejora la aplicación *in vivo* de las células madre en la terapia regenerativa.

1.3.4. Nuevas aplicaciones de la terapia celular: la bioingeniería

En el ámbito de la ingeniería de tejidos y la terapia celular, se han desarrollado diversos métodos para favorecer el injerto y la supervivencia celular.

Estudios iniciales han demostrado que la inyección de células en combinación con biomateriales, tales como la gelatina, la fibrina o el colágeno, han permitido un incremento del injerto celular, ejerciendo como consecuencia de ello, un mayor beneficio funcional en el corazón [225, 226]. Además, la administración de las células en la zona dañada, mediante, por ejemplo, su previa adhesión a un sustrato, ha demostrado no sólo una mayor supervivencia de las mismas sino también un efecto "terapéutico" más homogéneo en todo el tejido. En vista de estos resultados iniciales, se ha potenciado la utilización de soportes de diverso tipo, entre los cuales destacan el uso de matrices biodegradables, en las cuales se retienen o sobre las que se cultivan las células, la creación de tejidos sin soporte, compuestos únicamente por matriz secretada por las propias células, o incluso la obtención de matrices a partir de tejidos u órganos decelularizados, que posteriormente se han recubierto con las células de interés. Los estudios más relevantes se comentan a continuación.

1.3.4.1. Soportes biocompatibles

Gracias a la combinación de distintos biomateriales y poblaciones celulares, se están obteniendo parches celulares que favorecen el injerto de las células en el tejido. Se ha trabajado principalmente con biomateriales sintéticos (poliésteres como el ácido poliglicólico (PGA), ácido poliláctico (PLA) o el copolímero ácido poliláctico-glicólico (PLGA) y polilactonas como por ejemplo, la prolactona) y con componentes de la matriz extracelular (colágeno, fibrina, gelatina, matrigel o alginato), caracterizados todos ellos por su biocompatibilidad y biodegradabilidad.

Kellar y colaboradores implantaron un parche formado por PLGA y fibroblastos humanos (Dermagraft®, Advaced BioHealing, USA) en un modelo de IM en ratón, observándose una inducción de la revascularización del tejido [227] y una mejora en la función ventricular [228] un mes tras el implante. También se detectaron efectos similares al trasplantar un parche compuesto por ácido poliglicólico-co-prolactona (PGCL) sembrado con MNC-MO en un modelo de IM en rata. En este caso, además, se observó un remodelado positivo del corazón [229]. El grupo de Lesman y colaboradores, ha utilizado una combinación de PLGA y matrigel y tres tipos de células de origen humano (ESC, células endoteliales y fibroblastos embrionarios) para crear un tejido cardiaco vascularizado y con capacidad proliferativa [230]. Este parche cardiaco es capaz de crear un injerto estable en el corazón de rata y se ha observado la contribución de la vasculatura preexistente a la perfusión del tejido [231].

Otros biomateriales ampliamente utilizados son los derivados de componentes biológicos de la matriz extracelular, como el alginato, un polisacárido formado por ácido manurónico y gulurónico. Así por ejemplo, se han formado geles porosos de alginato en combinación con CM fetales de rata [232], que se implantaron en un modelo murino de IM observándose la revascularización del parche. Pese a ello, el injerto no fue estable ya que las células cardiacas implantadas fueron reemplazadas por colágeno y no se observó una integración estructural del parche en el tejido. Aún así, se comprobó una mejora de la función cardiaca consecuencia del remodelado positivo del corazón y de la revascularización del mismo, indicando una vez más el

efecto paracrino más que regenerativo de las células trasplantadas. Ryu y colaboradores inyectaron MNC-MO o MNC-MO embebidas en fibrina, obteniéndose una mayor revascularización y consiguiente regeneración en el grupo implantado con fibrina [226], confirmando la eficacia de este método. Simpson y colaboradores en cambio, utilizaron el colágeno de tipo I en forma líquida como soporte en la inyección de MSC en corazones con IM en rata. Este colágeno es líquido a temperatura ambiente y gelifica a 37ºC. En este estudio, también se observó un incremento del injerto celular y por tanto, un efecto paracrino más potente y duradero. Esto se tradujo además en una mejora en la función cardiaca [233].

Otros grupos han tratado de favorecer una distribución más homogénea de las células trasplantadas, y se han utilizado matrices sólidas de distinto tipo, entre ellas de colágeno, de modo que las células podían ser sembradas *in vitro* sobre la membrana, para ser implantadas posteriormente en el corazón infartado. Siguiendo esta aproximación, se analizó, en un modelo de IM en ratón, el potencial de las MNC de cordón umbilical previamente cultivadas sobre una membrana de colágeno y aplicadas a modo de parche sobre el corazón [225]. Una vez más, se detectó un incremento en el nivel de injerto y supervivencia de las mismas así como una mejora de la función cardiaca. En vista de estos y otros resultados obtenidos en estudios animales (revisado en [234]), el grupo del Dr. Chachques comenzó en el año 2007 el primer ensayo clínico (fase I) realizado con este tipo de matrices. Para ello, 20 pacientes con IM fueron trasplantados con parches compuestos por MNC-MO y membranas de colágeno [235]. Es importante destacar que no se detectaron efectos adversos como consecuencia del trasplante, demostrando así la factibilidad y seguridad del procedimiento. Además, se obtuvieron indicios positivos de la eficacia del tratamiento, ya que se observó una mejora funcional y un remodelado positivo de los corazones tratados un año después del implante [236]. La realización de estudios aleatorizados a doble-ciego con un número mayor de pacientes confirmará dicho beneficio.

Finalmente, se han realizado recientemente estudios en los que se han utilizado técnicas como el *electrospinning* o *microtemplating*, para crear matrices con

un patrón dirigido y favorecer así la disposición y diferenciación celular del implante en el tejido huésped. Se han creado por ejemplo, hidrogeles de poli(2-hidroxietil-metacrilato-co-ácido metacrílico) organizados en canales que favorecen la disposición paralela de los CM depositados sobre dicha matriz de forma similar a como se organizan en el corazón. Además, se han incluido microporos esféricos e interconectados para favorecer la angiogénesis y reducir la formación de estructuras cicatrizantes tras su implante en el corazón [237].

Por último, se han creado parches de tejido contráctil *in vitro* utilizando varias poblaciones celulares. Por ejemplo, el Dr. Zimmermann y colaboradores han creado estructuras tridimensionales compuestas por CM y otras poblaciones cardiacas embebidas en colágeno de tipo I y matrigel, condicionadas mediante tensión mecánica para la estimulación contráctil de los mismos [238, 239]. Así, tras su trasplante en corazones infartados, éstos contribuían a la capacidad contráctil del corazón sin evidencia de arritmias, previniendo además la dilatación de la pared ventricular [239]. Recientemente, otro grupo ha formado un tejido cardiaco de origen humano combinando CM derivados de ESC e iPS y células endoteliales embebidos en una matriz tridimensional de colágeno. Se ha demostrado su capacidad de injerto y supervivencia celular, al ser trasplantado en corazones de ratas atímicas [240]. Además se observó una mayor formación de vasos de origen humano en los animales a los cuales se implantó, también en el parche, MSC o fibroblastos embrionarios de ratón.

1.3.4.2. Sistemas sin soporte

Otros grupos se han centrado en la aproximación contraria, es decir, en la creación de parches celulares sin matrices que actúen de soporte, evitándose así una posible reacción inmunológica frente al material utilizado. Gracias a la técnica desarrollada en el laboratorio del Dr. Okano, es posible, mediante la utilización de placas termosensibles (compuestas por el polímero poli(*N*-isopropil-acrilamida)), cultivar células hasta una alta confluencia para después, al someter la placa a una temperatura menor, desprender fácilmente la monocapa celular formada [241]. Así, se

ha comprobado, en un modelo de IM agudo en rata, que la aplicación de una monocapa celular de ADSC favorece la revascularización del tejido, preservando la pared ventricular y, en definitiva, promoviendo la mejora de la función cardiaca [242]. Además, existe la posibilidad de combinar varias láminas celulares al adherir unas sobre otras, incrementando así el número de células implantadas sobre la región de tejido dañado [243]. En general, cuando se crean parches de un grosor superior a las 200 µm, la mayor limitación viene dada por el índice de difusión del oxígeno y de los nutrientes esenciales, ya que se ha detectado necrosis celular en las regiones centrales de parches de mayor grosor. Por ello, como ya hemos comentado anteriormente, uno de los aspectos fundamentales que se debe considerar a la hora de crear parches tridimensionales es la revascularización de los mismos. Así, al igual que en los estudios con células ADSC, también se han creado mediante esta técnica parches compuestos por diversas capas de CM neonatales combinados con células endoteliales. Se ha observado que al implantarlos en un modelo de infarto en rata, éstos son capaces de integrarse en el tejido gracias a la formación de redes vasculares funcionales que se conectan a la vasculatura general. Como consecuencia de ello, se produce una mejora funcional un mes tras el implante [244].

Recientemente, también se han creado parches con CM derivados de ESC combinados con células endoteliales derivadas de cordón umbilical y fibroblastos embrionarios y que pueden adquirir capacidad contráctil y ser eléctricamente estimulados a un ritmo determinado. Se ha demostrado que estos parches son capaces de injertar *in vivo*, aunque su posible beneficio al ser aplicados en un modelo de IM aún no ha sido demostrado [245, 246].

Por otro lado, también se han trasplantado cardioesferas, que como ya se ha comentado anteriormente, son agregados celulares con potencial cardiovascular [83]. Tras su implante en el corazón infartado, se ha demostrado su efecto positivo tanto en el remodelado tisular como en la función cardiaca [84, 85].

1.3.4.3. Matrices decelularizadas

Otra estrategia que está actualmente en desarrollo, es la decelularización de tejidos u órganos, de modo que la matriz resultante puede ser recelularizada con las células deseadas y trasplantada a modo de parche sobre el epicardio. La ventaja de utilizar estas membranas es que contienen componentes naturales de la matriz extracelular, tales como el colágeno o los glicosaminoglicanos, lo que favorece la adherencia y supervivencia celular. Además, generalmente provocan un menor rechazo inmunológico que otros materiales sintéticos. Se han decelularizado y empleado matrices derivadas del omentum pericárdico, el pericardio bovino o la submucosa del intestino delgado, entre otros.

En el caso del omentum, éste comenzó ya a utilizarse para el tratamiento del IM en los años 30, con el objetivo precisamente de promover la revascularización del tejido cardiaco. Recientemente, el grupo de Ueyama y colaboradores combinó el omentum con una lámina de hidrogel enriquecido además con el factor proangiogénico bFGF, y comprobaron que, al trasplantarlo en un modelo de IM en conejo, se inducía de forma muy potente la revascularización del tejido y, en consecuencia, la mejora de la función cardiaca [247].

En otros estudios se ha utilizado pericardio bovino decelularizado y microseccionado en varias capas formando un "*sandwich*" intercalado con láminas de MSC. Así, en un modelo de IM en rata, se determinó una mejora funcional tres meses tras el implante, consecuencia, al menos en parte, de la revascularización del tejido cardiaco. Se comprobó además, el efecto paracrino de las MSC, detectándose la secreción de los factores cardioprotectores IGF-1 y HGF [248, 249].

Por último, el grupo de Tan y colaboradores, ha creado un parche decelularizado a partir de la submucosa del intestino delgado y recelularizado con MSC de conejo. Tras una semana de cultivo *in vitro*, la matriz formada e implantada en un modelo de IM en conejo, indujo una mejora de la función contráctil del corazón un mes tras el implante [250]. Al igual que en los demás estudios, los análisis histológicos demostraron un aumento en la vascularización del tejido.

A la vista de todos estos resultados, se puede concluir que la utilización de los distintos parches celulares con sus ventajas y limitaciones (Tabla 1.3.) puede resultar en general beneficiosa, especialmente cuando las áreas dañadas del corazón son relativamente pequeñas.

Tabla 1.3. Parches celulares, ventajas y limitaciones. Abreviaturas: Matriz extracelular (MEC).

Aplicaciones	**Ventajas**	**Desventajas**
Parches: biomateriales porosos	-Soporte estructural -Mayor retención celular en combinación con componentes de MEC -Acondicionamiento mecánico y eléctrico -Aumenta la vascularización -Mejora de la función y remodelado	-Distribución irregular -Supervivencia limitada -Material biodegradable que desencadena respuesta inflamatoria -No se integra en el miocardio -Cirugía para su implante
Parches: hidrogeles/derivados de MEC	-Retención celular y distribución regular -Acondicionamiento mecánico y eléctrico -Aumenta la vascularización -Mejora de la función y remodelado -Injerto cardiaco en el miocardio	-Supervivencia limitada -Inmunogenicidad cuando son productos derivados animales -No se integra en el miocardio -Cirugía para su implante
Sistemas sin soporte	-No se induce respuesta inflamatoria ni inmunogenicidad -Potencial angiogénico *in vitro* -Aumenta la vascularización -Integración vascular con el miocardio -Mejora de la función y remodelado	-Acondicionamiento mecánico limitado -Cirugía para su implante
Matrices decelularizadas	-No se induce respuesta inflamatoria ni inmunogenicidad -Acondicionamiento mecánico y eléctrico -Aumenta la vascularización -Mejora de la función y remodelado	-Distribución irregular -Diferenciación celular e injerto limitado -Cirugía para su implante

Sin embargo, en casos en los que el corazón ha presentado episodios recurrentes de IM y comienza a no ser funcional, se precisa el empleo de técnicas más drásticas como el trasplante de corazón. Desafortunadamente, la disponibilidad de

corazones sanos y compatibles es muy limitada, y pese a los avances que se están realizando en el desarrollo de corazones mecánicos, éstos todavía presentan una serie de problemas asociados, como el tromboembolismo o la limitada calidad de vida del paciente. La idea de crear un nuevo corazón mediante la decelularización de corazones de cadáveres o incluso provenientes de cerdos, para su posterior recelularización con células cardiovasculares derivadas de células madre, resulta muy atractiva. Se ha demostrado que, mediante la perfusión del corazón con el detergente SDS al 0,1%, es posible eliminar todo el componente celular sin dejar trazas de ADN, pero conservándose a su vez intacta la estructura y composición de la matriz. Además, mediante la utilización de un bioreactor, se ha conseguido su revascularización con células endoteliales y la formación de una capa muscular contráctil con capacidad de bombeo [251].

A pesar de lo llamativo de estos resultados, son muchos los aspectos referentes a la estructura del corazón obtenido que todavía necesitan ser controlados, tales como la creación de una pared de mayor grosor (ya que el conseguido hasta ahora es muy inferior al de un corazón normal), con una capacidad contráctil adecuada y estable, para así evitar posibles casos de arritmias. Además, de cara a la recelularización del mismo, todavía es necesario disponer de una fuente celular de CM óptima, que pueda ser fácilmente obtenida y expandida, y que presente características de célula madura y funcional. Actualmente, este aspecto todavía supone gran reto, ya que, por un lado, la obtención de progenitores cardiacos no es eficiente debido a la falta de una completa caracterización y, por otro, su obtención a partir de células pluripotentes tales como las ESC o iPS, presenta riesgos de tumorogenicidad, incluyendo además los de tipo inmunológico y ético en el caso de las ESC. En cualquier caso, la posibilidad de crear órganos con esta tecnología representa un nuevo horizonte en la búsqueda de nuevas terapias para el tratamiento de las enfermedades cardiovasculares entre otras patologías.

2. HIPÓTESIS

Los estudios realizados hasta el momento han demostrado el efecto beneficioso que el trasplante de las células madre, incluidas las derivadas del tejido adiposo (ADSC), ejercen sobre el corazón infartado. En general, a pesar de su capacidad de diferenciación, los mecanismos principales por los que inducen tal efecto son de tipo trófico, liberando factores que favorecen el remodelado positivo y la revascularización del corazón.

La aplicación de las células madre presenta, sin embargo, importantes limitaciones que disminuyen su efectividad, tales como el bajo grado de injerto y de supervivencia celular, que se deben en gran medida, a los métodos de trasplante habitualmente utilizados. Hasta el momento, las células han sido generalmente inyectadas en suspensión, dificultando su retención y su supervivencia inducida por señales dependientes de anclaje a la matriz extracelular.

La hipótesis de este estudio es que el trasplante en el corazón de las células ADSC adheridas a una matriz biocompatible podría incrementar su permanencia en el tejido y posibilitar una distribución más homogénea de las mismas, potenciando así su beneficio terapéutico en el tratamiento del infarto de miocardio.

3. OBJETIVOS

Los objetivos del presente trabajo han sido los siguientes:

1. Aislar y caracterizar la población de células madre derivadas del tejido adiposo (ADSC), a partir de grasa de rata Sprague-Dawley.

2. Caracterizar el comportamiento biológico y mecánico de distintos tipos de membranas de colágeno y determinar su biocompatibilidad *in vivo*.

3. Analizar de forma comparativa, el potencial terapéutico de las ADSC en el corazón, al ser trasplantadas como parche celular (mediante su previa adhesión a una membrana de colágeno) o inyectadas sin soporte, en un modelo de infarto crónico de miocardio en rata.

4. Determinar los mecanismos implicados en la posible acción terapéutica de las membranas celularizadas con ADSC.

4. MATERIAL Y MÉTODOS

4.1. Obtención, cultivo y caracterización de las células ADSC

Las ADSC se obtuvieron mediante el cultivo *in vitro* de la fracción estromal vascular (SVF) aislada a partir de tejido adiposo de rata. La obtención, el procesamiento de las muestras y el cultivo de las células se realizó bajo condiciones de esterilidad.

4.1.1. Obtención y cultivo de las células ADSC

La SVF se aisló a partir de tejido adiposo de ratas macho Sprague-Dawley (SD) de 10-12 semanas. Se utilizaron ratas transgénicas que expresan de forma ubicua la proteína eGFP, la cual está controlada bajo el promotor de la β-actina.

Los animales se sacrificaron mediante inhalación de CO_2. A continuación, se les extrajo el tejido adiposo de la zona inguinal, y éste, se trituró y se digirió con colagenasa tipo I (2 mg/ml) (Gibco; ref. 17100-017) diluida en α-MEM (Gibco; ref. 32561), mediante incubación a 37ºC, durante 30 minutos y en agitación. A continuación, la muestra se filtró con un filtro de 40 µm (Falcon, ref. 2340) y se centrifugó a 600g durante 7 minutos. Posteriormente, la muestra se trató con una solución de lisis eritrocitaria (31 mM NH_4Cl, 2 mM $KHCO_3$ y 0,02 mM EDTA) durante 5 minutos a temperatura ambiente y se centrifugó nuevamente a 600g durante 7 minutos. Finalmente, las células obtenidas se sembraron a una densidad de 7.500 células/cm^2 en medio de cultivo compuesto por α-MEM (Gibco) suplementado con 10% de suero de ternera fetal (Biochrom; ref. S0115) y 1% de penicilina-estreptomicina (P/S) (Lonza; ref. 17602E). Cuando las células alcanzaron una confluencia del 80%, se lavaron con PBS (Lonza; ref. BE17-512F) e incubaron con tripsina/EDTA (0,05% tripsina; 200 mg/L EDTA) (Lonza; ref. 17161E) durante 5 minutos a 37ºC, inactivándola posteriormente con medio de cultivo. Las células se cultivaron a una confluencia de 4.000 células/cm^2 y cada 3-5 días de cultivo, se tripsinizaron y sembraron nuevamente a la densidad celular indicada.

Las ADSC se cultivaron a 37ºC en atmósfera húmeda con un 5,5% de CO_2 durante 3 semanas (correspondiente a 7-8 duplicaciones celulares), para obtener una población homogénea de fenotipo característico de célula mesenquimal. Todos los experimentos se realizaron con ADSC mantenidas en cultivo durante 3 semanas.

4.1.2. Caracterización de las células ADSC

El fenotipo de la población ADSC obtenida tras 3 semanas de cultivo se analizó mediante citometría de flujo. Para ello, se resuspendieron 250.000 células en 100 µl de PBS y se incubaron, durante 15 minutos y en oscuridad, con 10 µl de los anticuerpos marcados con el fluorocromo PE: anti-RT1A (BD; ref. 559993), anti-RT1B (BD; ref. 554929), anti-CD31 (BD; ref. 555027), anti-CD44H (BD; ref. 550974), anti-CD45R (BD; ref. 554881), anti-CD73 (BD; ref. 551124), anti-CD90 (BD; ref. 554898) y el isotipo control (BD; ref. 555584). A continuación, las células se lavaron con 2 ml de PBS y se centrifugaron a 600g durante 7 minutos, tras lo cual se fijaron con 300 µl de formaldehido al 4%.

Además se confirmó la expresión de la proteína eGFP de las ADSC. Como control negativo, se utilizaron células ADSC aisladas de tejido adiposo de ratas SD salvajes que no expresaban la proteína eGFP.

Las muestras se analizaron utilizando el citómetro de flujo FACSCanto (BD) y el programa *Cellquest* (BD).

4.2. Caracterización de las membranas de colágeno

4.2.1. Propiedades de las membranas de colágeno

Se analizaron 4 tipos de membranas de colágeno que fueron preparadas por la empresa VISCOFAN, S.A. Las membranas, compuestas por colágeno de tipo I (20 g/m^2), se prepararon a partir de piel bovina y presentaban un grosor de 20 µm. Los 4 tipos de membranas diferían entre sí en su grado de entrecruzamiento (generado por el agente

entrecruzante 1-Etil-3-(3-dimetilaminopropil)-carbodiimida (EDC; $C_8H_{17}N_3$)), de modo que la membrana #406 carecía de él, mientras que las membranas #407, #408 y #409 presentaban niveles crecientes de entrecruzamiento (Tabla 4.1.).

Tabla 4.1. Grado de entrecruzamiento de las membranas #406, #407, #408 y #409. Ratio molar EDC:Colágeno. EDC: 1-Etil-3-(3-dimetilaminopropil)-carbodiimida.

# Membrana	EDC : Colágeno
406	0:1
407	2:1
408	5:1
409	10:1

4.2.2. Cultivo de ADSC sobre las membranas de colágeno

La celularización de las membranas con ADSC se realizó mediante la utilización de bastidores creados a partir de tubos de plástico de 1,5 ml (Sarstedt; ref. 175508) recortados y autoclavados. Con este sencillo método se consiguió optimizar la adherencia de las células a la membrana, evitando la pérdida parcial de células como consecuencia de su adhesión a los pocillos de cultivo (Figura 4.1.). Las membranas carecen de poros y por lo tanto, las células se adhieren a su superficie formando una monocapa. El área final de la membrana que queda en contacto con las células en el bastidor es de 0,8 cm^2.

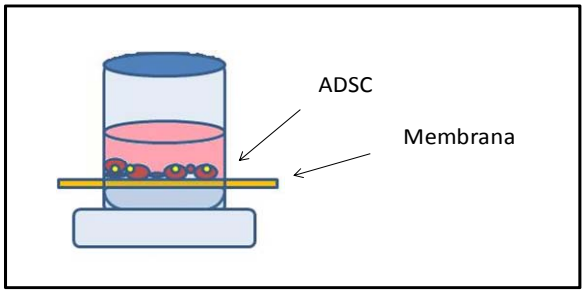


Figura 4.1. Cultivo de ADSC sobre membrana en bastidor.

Previamente al montaje de los bastidores, las membranas se hidrataron en PBS (Lonza) durante 10-12 horas. Las ADSC se cultivaron en el bastidor con 500 µl de medio de cultivo en estufa a 37ºC y 5,5% de CO_2.

4.2.3. Ensayos *in vitro*

4.2.3.1. Ensayo in vitro de proliferación celular

Las ADSC se sembraron sobre los 4 tipos de membrana (#406, #407, #408 y #409) o sobre placas de cultivo de 48 pocillos (Corning; ref. 3548) (utilizados como control del crecimiento celular), a una densidad de 40.000 células/cm^2. Las células se cultivaron en estufa a 37ºC y 5,5% de CO_2 en medio de cultivo de ADSC. Transcurridos 1, 4 y 7 días, se tripsinizaron para realizar el contaje celular. Para ello, las membranas se incubaron en agitación durante 10 minutos en tripsina/EDTA (0,5% tripsina; 2 g/L EDTA) (Gibco; ref. 15400), y tras este tiempo, se inactivó la tripsina con suero de ternera fetal (Biochrom), se recogieron las células, se centrifugaron a 600g durante 7 minutos y se resuspendieron en 100 µl de PBS (Lonza). Finalmente, para cuantificar el número de núcleos presentes en la muestra, se les añadió 100 µl de tampón de lisis (Chemometec; ref. 910-0003) y 100 µl de reactivo estabilizante, (Chemometec; ref. 910-0002) y se realizó el contaje mediante el aparato NucleoCounter (Chemometec).

Se realizaron 3 experimentos independientes incluyendo triplicados para cada condición. Los resultados se expresaron como número de células/cm^2.

4.2.3.2. Ensayo in vitro de adhesión celular

4.2.3.2.a. Cuantificación de células adheridas a la membrana

Las células ADSC se sembraron sobre las membranas o en placas de cultivo de 48 pocillos (Corning) (como control de adhesión celular), a una densidad de 100.000, 250.000 y 500.000 células/membrana o pocillo. Las células se cultivaron en estufa a 37ºC y 5,5% de CO_2 en medio de cultivo de ADSC. Las ADSC adheridas se recogieron

tras 24 horas y se cuantificaron con el aparato Nucleocounter (Chemometec) (ver apartado 4.2.3.1).

Se realizaron 4 experimentos independientes incluyendo triplicados para cada condición. Los resultados se expresaron como número de células adheridas a las 24 horas.

4.2.3.2.b. Inmunofluorescencia de células adheridas a la membrana

Por otro lado, se determinó la disposición de las ADSC en la membrana, para lo cual se realizó una inmunofluorescencia frente a vimentina (proteína del citoesqueleto presente en células mesenquimales). Para ello, se retiró el medio de cultivo de los bastidores, se realizaron 2 lavados con PBS (Lonza) durante 5 minutos, y se fijaron con zinc-formalina (Thermo Scientific; ref. 5701ZF) durante 10 minutos a temperatura ambiente. A continuación, tras 2 lavados con PBS, las células se permeabilizaron con PBS-Tween®20 (Sigma; ref. STBB3609) al 0,1% durante 15 minutos, se bloquearon las uniones inespecíficas con gelatina de pescado (Sigma; ref. G7765) al 0,4% durante 1 hora, y se incubaron con el anticuerpo primario anti-vimentina (Dako; ref. M0725) diluido 1:100 en gelatina de pescado, durante 3 horas a temperatura ambiente. Posteriormente, se realizó un lavado con PBS y 3 lavados de 5 minutos cada uno con PBS-Tween®20 (Sigma) al 0,1%, para retirar el exceso de anticuerpo primario. A continuación, las células se incubaron nuevamente con gelatina de pescado (Sigma) al 0,4% durante 15 minutos y se incubaron, durante 1 hora en cámara húmeda, con el anticuerpo secundario marcado con el fluorocromo Alexa fluor 594 (Invitrogen; ref. A11032) diluido 1:500 en gelatina de pescado. Finalmente, tras un lavado en PBS, se desmontaron los bastidores y se colocó la membrana en un portaobjetos con medio de montaje que incluía, para realizar el marcaje nuclear, DAPI al 25% (Vectashield-Dapi, Vector Laboratories; ref. H1200) diluido en una solución de PBS:glicerol (1:1). Se incluyó como control negativo de inmunofluorescencia, una membrana celularizada a la cual no se le añadió el anticuerpo primario.

La adquisición de imágenes se realizó para cada condición con el objetivo de 20x, con la cámara AxioCam MR3 (Zeiss) acoplada al microscopio Zeiss AxioImager M1 (Zeiss).

Para el análisis del grosor de la monocapa celular formada, las membranas se fijaron con zinc-formalina, se lavaron y permeabilizaron siguiendo el protocolo de inmunofluorescencia y se tiñeron los núcleos con TOPRO-3 (Molecular probes; ref. T3605) 0,02 mM en una solución de PBS:glicerol (1:1).

Se realizaron 8 medidas del grosor de la monocapa celular en las imágenes obtenidas con el objetivo de 20x, para cada confluencia celular. Los resultados se expresaron como grosor de la monocapa celular (µm). La adquisición de imágenes se realizó con el programa AIM 4.2 (Zeiss) en el microscopio confocal LSM 510 META (Zeiss).

4.2.3.3. Ensayo in vitro de apoptosis celular: TUNEL

Las células ADSC se sembraron sobre las membranas colocadas en bastidores a una densidad de 100.000, 250.000 y 500.000 células/membrana. Éstas se cultivaron durante 24 horas en estufa a 37ºC y 5,5% de CO_2 en medio de cultivo de ADSC.

El grado de apoptosis celular fue cuantificado mediante la técnica de Tunel, para lo cual se utilizó un kit específico (*In Situ Cell Death Detection Kit*; Roche; ref. 12156792). Esta técnica está basada en la detección *in situ* de núcleos en apoptosis mediante la unión de nucleótidos marcados con la enzima deoxinucleotidil transferasa terminal (TdT) a los extremos libres 3´-OH de los fragmentos de ADN, generados durante la apoptosis.

Para realizar dicho marcaje, las células se lavaron 3 veces con PBS (Lonza) durante 2 minutos cada una y se fijaron con paraformaldehído (Sigma; ref. P6148) al 4% durante 20 minutos a temperatura ambiente. Tras la fijación, las células se volvieron a lavar 3 veces con PBS durante 2 minutos, se permeabilizaron con PBS-Tritón X100 (Sigma; ref. 8532) al 0,5% durante 4 minutos y se lavaron nuevamente con PBS durante 2 minutos. A continuación, se añadieron 80 µl de la mezcla de reactivos

de Tunel (dilución 1:10 de la enzima TdT en su tampón) y se incubó durante 1 hora en cámara húmeda y en oscuridad a 37ºC. Tras un nuevo lavado en PBS, la membrana se montó sobre un portaobjetos para posteriormente marcar los núcleos con medio de montaje Dapi-Vectashield (Vector laboratories; ref. H1200) al 25% en una solución de glicerol:PBS (1:1). El control positivo de apoptosis se correspondió con ADSC tratadas durante 20 horas con un estímulo apoptótico (medio de cultivo sin suero y peróxido de hidrógeno 50 µM).

Se tomaron 8 imágenes de cada membrana, con el objetivo de 10x. Las imágenes se cuantificaron manualmente y los resultados se expresaron como porcentaje del número de células Tunel positivas respecto al número total de células. Se realizaron 3 experimentos independientes por triplicado. La adquisición de imágenes se realizó con cámara AxioCam MR3 (Zeiss) acoplada al microscopio Zeiss AxioImager M1 (Zeiss).

4.2.3.4. Microscopía electrónica de la membrana celularizada

Las membranas celularizadas con ADSC o sin celularizar, se analizaron mediante microscopía electróncia. En el primer caso, se sembraron 500.000 ADSC sobre la membrana #406 y se cultivaron durante 24 horas en estufa a 37ºC y 5,5% de CO_2 en medio de cultivo de ADSC.

Tanto las membranas sin celularizar como las membranas sembradas con ADSC se fijaron con glutaraldehído (Electron Microscopy Sciences; ref. 16210) al 2,5% durante 2 horas y se lavaron 3 veces con PBS (Lonza) durante 10 minutos. Posteriormente, las muestras se deshidrataron mediante pases sucesivos por etanol de 30º, 50º, 70º, 90º, 96º y absoluto, durante 15 minutos en cada alcohol. El posterior procesamiento de las muestras para las técnicas de microscopia electrónica se realizó según lo descrito en el apartado 4.7.3.

4.2.3.5. Ensayo in vitro del comportamiento mecánico de las membranas

Se analizó el comportamiento mecánico de los 4 tipos de membrana de colágeno (#406, #407, #408 y #409) así como de la membrana #406 celularizada con ADSC. En los experimentos de membrana celularizada, se sembraron 500.000 células sobre la membrana, 24 horas antes del análisis mecánico. Las membranas sin células se hidrataron durante 24 horas con medio de cultivo en estufa, a una temperatura de 37ºC y 5,5% de CO_2.

El análisis del comportamiento mecánico de las membranas se realizó mediante un ensayo de tracción uniaxial en el cual se midieron la elongación de la muestra *u* (mm) y la carga aplicada *F* (N), valores a partir de los cuales se calcularon la tensión ($\sigma=F/A$) y deformación ($\lambda=1+u/l_0$) del material (siendo *A* el área transversal de la muestra y l_0 la longitud inicial de la misma antes del ensayo). Estos valores son representados gráficamente, para obtener así el módulo elástico (módulo tangente al 1%, E_I) y el módulo tangente (módulo tangente en la parte lineal de la curva, E_{II}) (Figura 4.2.).

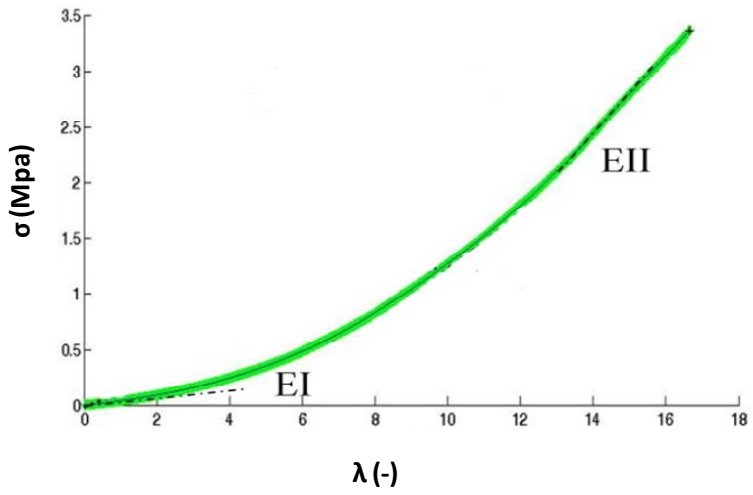


Figura 4.2. Cálculo de los módulos tangentes EI y EII a partir de la curva tensión (σ) deformación (λ).

Los ensayos se realizaron en un equipo INSTRON MicroTester 5548 con un video-extensómetro láser (para medir el desplazamiento). Las muestras se ensayaron en una cámara con un humidificador, para garantizar el 100% de humedad y evitar la deshidratación de las mismas. El ensayo tuvo lugar a una velocidad de desplazamiento de 1 mm/min con un preacondicionamiento consistente en una carga y descarga de tres ciclos, a un nivel de deformación del 10%, las muestras, posteriormente se elongaron hasta su ruptura.

Las muestras fueron cortadas en dos direcciones perpendiculares entre sí, denominadas longitudinal y transversal, mediante un troquel para garantizar la homogeneidad del tamaño y dimensiones de las mismas (20 mm x 2 mm). Para cada tipo de membrana, se realizaron 6 experimentos independientes en cada dirección (longitudinal y transversal). Los resultados se mostraron como la media de los módulos tangentes para cada tipo de membrana y en cada dirección del ensayo.

4.3. Trasplante de membranas celularizadas en un modelo de infarto crónico de miocardio en rata

4.3.1. Modelo de infarto crónico de miocardio en rata

El protocolo utilizado para la generación del modelo *in vivo* de infarto de miocardio y el resto de los procedimientos animales realizados fueron aprobados por el Comité Ético de Experimentación Animal (CEEA) de la Universidad de Navarra. Los animales utilizados se mantuvieron en condiciones estándar de estabulación (humedad, temperatura, alimentación, luz, etc) en el animalario de la Universidad de Navarra.

El modelo de infarto de miocardio crónico se realizó en ratas hembra Sprague-Dawley (Harlan-IBERICA, Barcelona) de 8 semanas (250 g), la intervención se llevó a cabo en condiciones de esterilidad y bajo anestesia inhalatoria. Como agente anestésico se empleó isoflurano (Esteve veterinaria; ref. 5910A) al 4% en una cámara

de inducción con oxígeno. Antes de la intervención, se administraron los analgésicos ketoprofeno (Ketofen 1%, Jesús Guerrero; ref. MR47) 5 mg/Kg (por vía subcutánea) y fentanilo (Fentanest, Kern Pharma; ref.756650) 0,15 mg/Kg (por vía intraperitoneal). Las ratas se intubaron y ventilaron mecánicamente a 90 ciclos/min y el isoflurano se mantuvo al 1,5-2% durante la operación. La intervención se realizó mediante toracotomía izquierda a nivel del cuarto espacio intercostal y consiguiente apertura del pericardio, tras lo cual se procedió a la ligación permanente de la arteria coronaria descendente anterior con hilo prolene 7/0 (Ethicon; ref. W8702). Tras la ligación, se procedió al cierre de la incisión por planos, y el abdomen y la piel se suturaron con hilo de vicryl 4/0 (Ethicon; ref. V304). Se comprobó la reanimación de los animales de la anestesia y se les mantuvo durante 24 horas en la jaula con manta térmica para su restablecimiento tras la operación.

Las ratas operadas recibieron antibioticoterapia general con enrofloxacina (25 mg/kg) (Alsir® Lechones 5 mg, Esteve veterinaria; ref. 0901-ESP) en el agua de bebida durante 7 días consecutivos y analgesia mediante inyección subcutánea de ketoprofeno (Jesús Guerrero) durante 2 días consecutivos.

4.3.2. Análisis de la biocompatibilidad de las membranas con el tejido cardiaco

Para determinar la biocompatibilidad de las distintas membranas (#406, #407, #408 y #409) con el tejido cardiaco, éstas se implantaron en los corazones tras 5 semanas de haber sido provocado el infarto. Se trasplantaron 24 animales (6 animales/grupo) y se sacrificaron 2, 7 y 30 días después del implante.

Las ratas se operaron mediante esternotomía para la realización del implante. Para ello, el animal anestesiado, intubado y conectado al respirador mecánico (siguiendo las mismas pautas de anestesia, analgesia y antibioticoterapia descritas en el modelo de infarto de miocardio), se colocó en posición supina y se le depiló y desinfectó la zona del esternón. Una vez abierta la cavidad torácica, se liberó el pericardio y se pasó una sutura de tracción intramural prolene 7/0 (Ethicon) en la zona

del ventrículo izquierdo sana. Tras localizar la zona infartada, se implantaron las membranas mediante sutura en 5 puntos del miocardio con hilo prolene 7/0 (Ethicon). Las dimensiones de la membrana fueron de 1 x 1 cm. En todos los casos, tras comprobar que no había sangrado, se retiró la sutura de tracción y se procedió al cierre del abdomen y piel por planos, con vicryl 4/0 (Ethicon). Cuando los animales se recuperaron de la anestesia, se retiró el tubo endotraqueal y se devolvieron a su jaula donde se les mantuvo en observación y con manta térmica durante 24 horas para asegurar su restablecimiento tras la operación.

4.3.3. Trasplante de ADSC adheridas a membrana o mediante inyección

Para determinar el potencial terapéutico de las membranas celularizadas con ADSC, se infartaron un total de 116 ratas y se incluyeron en el estudio aquellos animales que habían sobrevivido y presentaban una fracción de eyección ventricular < 45% (ver apartado 4.4.) 1 mes después del infarto (n=68). Los animales se dividieron aleatoriamente en 4 grupos experimentales: grupo control (medio), grupo tratado con ADSC, grupo trasplantado con membrana (#406), y grupo trasplantado con membrana (#406) celularizada con ADSC.

Las ratas se operaron siguiendo el protocolo de cirugía anteriormente explicado (aptdo. 4.3.2.). En el caso de las células, éstas se resuspendieron en 80 µl de α-MEM (Gibco) y se inyectaron subepicárdicamente en 4 puntos de la zona de peri-infarto, con aguja de insulina de 29 G (BD Micro-Fine; ref. 324892). El grupo control, recibió 4 inyecciones de medio α-MEM (Gibco), de 20 µl cada una. En el caso de las membranas (celularizadas o no), se implantaron sobre la zona infartada mediante sutura en 5 puntos del miocardio. Las dimensiones de la membrana fueron de 1 x 1 cm.

Las membranas celularizadas se prepararon sembrando 500.000 ADSC sobre las membranas (#406) y se mantuvieron en cultivo durante 24 horas para su correcta adhesión antes del implante. En el grupo de animales implantados con ADSC, se inyectaron 350.000 células, ya que éste resultaba ser el número final de células

adheridas a la membrana tras 24 horas de su cultivo sobre la membrana (ver apartado de resultados 5.3.1. (experimentos de adhesión a día 1)).

4.4. Estudio de la función cardiaca mediante ecocardiografía

La adquisición de imágenes para el estudio de la función cardiaca se realizó con los animales anestesiados con isoflurano al 2% (Esteve Veterinaria) colocados en decúbito lateral. Se practicaron registros de ecocardiograma bidimensional, modo M y medidas de ecocardiograma-Doppler.

Los parámetros de remodelado ventricular analizados fueron los diámetros y volúmenes telesistólicos (DVIts-Vts) y telediastólicos (DVItd-Vtd), de acuerdo con las recomendaciones de la Sociedad Americana de Ecocardiografía [252]. La fracción de eyección ventricular izquierda (FEVI) se obtuvo a partir de una proyección paraesternal en el eje corto según fórmula de Teichholz, definido como el cambio de volumen del ventrículo izquierdo dividido por el volumen inicial [253].

Excepto la FEVI, el resto de los parámetros analizados se corrigieron con el peso del animal y todos los datos ecocardiográficos se registraron a frecuencias cardiacas similares. Las medidas se promediaron con tres ciclos cardiacos consecutivos. Los estudios ecocardiográficos fueron realizados por el mismo investigador que realizó las medidas en modo ciego y se realizaron previamente a la aplicación del tratamiento y 4 meses tras el implante. Sólo los animales con una FEVI inferior al 45% tras un mes de haber sido provocado el infarto fueron incluidos en el estudio. Los estudios ecocardiográficos se realizaron con un equipo de ultrasonidos Sonos 4500 (Philips) acoplado a un transductor lineal de 12 MHz y con capacidad Doppler.

4.5. Estudio del comportamiento mecánico del corazón

Los animales de los distintos grupos se sacrificaron 4 meses tras el implante. Para el sacrificio, los animales se anestesiaron con una mezcla de un anestésico disociativo, ketamina (Imalgene 1000 Merial Laboratorios), a una concentración de 75 mg/kg, y de un tranquilizante agonista Z-2 adrenérgico, xilacina (Rompun 2% Bayer; ref. Q-0615-035), a una concentración de 10 mg/Kg. Tras obtener los corazones, se retiraron las aurículas, y se cortaron muestras rectangulares desde la parte superior hacia el ápice en la zona del infarto cerca de la ligadura y en zonas paralelas, con el objetivo de comprobar la evolución de las propiedades mecánicas del tejido cardiaco ventricular de la zona infartada y peri-infarto (Figura 4.3.).

En total, se obtuvieron, de cada corazón, 5 muestras paralelas de dimensiones aproximadas de 15 mm de longitud y 5 mm de ancho. Para cada grupo se ensayaron 6 animales, haciendo un total de 30 muestras por grupo.

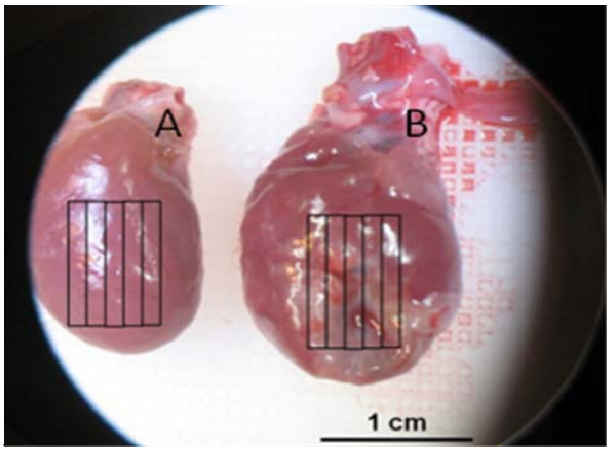


Figura 4.3. Esquema de la recogida de muestras de los corazones. A. corazón sin infartar; **B**. corazón infartado.

Los ensayos se realizaron siguiendo el mismo protocolo utilizado para realizar las medidas de las membranas en el equipo INSTRON MicroTester 5548 con un video-

extensómetro láser en una cámara con un humidificador (ver apartado 4.2.3.5.). Los ensayos se realizaron a una velocidad de desplazamiento de 1 mm/min con un precondicionado que consistió en una carga y descarga de tres ciclos a un nivel de deformación del 10% y después se elongaron hasta rotura. De nuevo se obtuvieron las gráficas tensión ($\sigma=F/A$) frente deformación ($\lambda=1+u/l_0$) para cada zona del ensayo, y se compararon el módulo tangente en la parte lineal de la curva (E_{II}), así como las curvas de comportamiento de cada grupo.

Se analizaron los resultados obtenidos cuando las muestras se sometieron a una deformación del 20-50%. Con deformaciones menores al 20%, las fibras de colágeno no aportan apenas rigidez y los resultados de los módulos elásticos entre grupos suelen ser similares. En cambio, cuando la deformación es mayor (20-50%), el colágeno sí aporta su máxima rigidez y las posibles diferencias entre grupos se hacen más evidentes y comparables. No se considerarán los valores obtenidos con deformaciones mayores al 50%, por no ser fisiológicamente representativos.

4.6. Obtención y procesamiento de tejidos

Los animales de los distintos grupos fueron sacrificados 4 meses después de haber realizado el implante. Para el sacrificio, los animales se anestesiaron previamente con la mezcla de ketamina (75 mg/Kg) y xilacina (10 mg/Kg). A continuación, se inyectó en los corazones 1 ml de cloruro de cadmio ($CdCl_2$ Sigma; 202908) 1 mM (provocando la parada del corazón en diástole), y se perfundieron durante 5 minutos con PBS (Lonza). Finalmente, se fijaron con zinc-formalina (Thermo Scientific) durante 15 minutos (en el caso de aquellos destinados al análisis mediante técnicas histológicas), o con zinc-formalina (Thermo Scientific) y glutaraldehído (Electron Microscopy Sciences) al 0,1% (en aquellos que serían analizados por microscopía electrónica).

Se procesaron 5-6 corazones por grupo animal tras 4 meses del implante y 2-3 corazones por grupo animal 1 y 4 semanas post-implante, para la realización de los

posteriores análisis histológicos. Los corazones se extrajeron, se cortaron transversalmente en 3 secciones de unos 4 mm de grosor cada una y se fijaron de manera adicional durante 24 horas en zinc-formalina (Thermo Scientific). A continuación, tras 3 lavados de 5 minutos con PBS (bioMerieux; ref. 75511) y 1 lavado de 5 minutos con agua destilada, los tejidos se deshidrataron en etanol al 70º durante 24 horas para su posterior inclusión en parafina. De cada sección del corazón, se realizaron 60 cortes de 5 µm de grosor, en 6 series de 10 cortes cada una.

4.7. Técnicas histológicas

Se analizaron 18 cortes seriados para cada una de las técnicas histológicas realizadas. En primer lugar, se procedió al desparafinado de las muestras. Para ello, se mantuvieron en una estufa a 56ºC durante 15 minutos y, para eliminar los restos de parafina, se sumergieron en xilol (Panreac) durante 30 minutos. A continuación, se mantuvieron en etanol absoluto (Merck) durante 2 minutos y se hidrataron mediante pases sucesivos, de 2 minutos cada uno, en diluciones decrecientes de alcohol en agua: 96º, 80º y 70º y finalmente, agua destilada. Una vez hidratadas, se siguieron los protocolos correspondientes de tinción, inmunohistoquímica o inmunofluorescencia.

4.7.1. Tinciones histológicas

4.7.1.1. Tinción de hematoxilina-eosina

Las muestras desparafinadas e hidratadas se tiñeron en una solución de hematoxilina de Harris (Merck; ref. 1082530) durante 7 minutos. Se lavaron con abundante agua y, a continuación, se introdujeron en etanol 70º con unas gotas de ácido clorhídrico (Panreac; ref. 141020) al 37%. Las preparaciones se diferenciaron en carbonato de litio (Panreac; ref. 141391) saturado y se lavaron en agua durante 5 minutos. Finalmente, se tiñeron en una solución de eosina (Merck; ref. 115935) al 0,5% a pH5 durante 5 segundos. El exceso de eosina se retiró con agua y se procedió a la

deshidratación de las muestras mediante pases sucesivos por alcohol de 96º, alcohol absoluto y xilol antes de ser montadas en solución DPX (BDH; ref. 361254D).

4.7.1.2. Tinción de rojo sirio

Las muestras desparafinadas e hidratadas se tiñeron con una solución de rojo sirio (Sigma; ref. 365548) al 0,1% en ácido pícrico (Sigma; ref. 80452) saturado durante 30 minutos a temperatura ambiente. Posteriormente, se deshidrataron en alcohol absoluto (Merck) y xilol (Panreac), y se montaron en solución DPX (BDH).

4.7.2. Inmunohistoquímica e inmunofluorescencia

En el caso de las técnicas inmunohistoquímicas, se procedió a eliminar la actividad peroxidasa endógena para evitar su interferencia en el revelado. Para ello, las muestras desparafinadas (apartado 4.7.), se mantuvieron durante 2 minutos en etanol y 20 minutos en oscuridad en una solución de metanol (Sigma; ref. 34940): peróxido de hidrógeno (Panreac; ref.141077) al 10% (v:v). Tras la hidratación de las muestras mediante pases de 2 minutos cada uno, por diluciones decrecientes de alcohol en agua: 96º, 80º y 70º, se mantuvieron durante 5 minutos en agua destilada y 5 minutos en tampón tris-salino (TBS) (TBS: Tris 50 mM, NaCl al 0,9%, a pH 7,36) con Tween®20 (Sigma; ref. STBB3609) al 0,1%.

En función del anticuerpo empleado y para facilitar la accesibilidad de los antígenos, el tejido se sometió a un pretratamiento térmico con microondas (un ciclo de 10 minutos a máxima potencia (800 W) y un ciclo de 10 minutos a mínima potencia (80 W)) en el tampón requerido (Tabla 4.2.), tras el cual, se dejaron enfriar durante 5 minutos en TBS-Tween®20 al 0,1%. A continuación, las muestras se incubaron con BSA (Sigma; ref. A6003) al 5% en TBS, durante 30 minutos a temperatura ambiente, para bloquear las uniones inespecíficas y seguidamente, se incubaron con el anticuerpo primario correspondiente, diluido a la concentración adecuada (Tabla 4.2.) en TBS y BSA al 1%, durante toda la noche a 4ºC. Finalmente, tras 3 lavados de 5 minutos en

TBS-Tween®20 al 0,1%, éstas se incubaron con el anticuerpo secundario marcado con Peroxidasa o la molécula FITC según el caso.

En el caso de las técnicas de inmunohistoquímica se utilizó el sistema de amplificación de señal Envision-peroxidasa (Dako; ref. K4003). Tras 30 minutos de incubación a temperatura ambiente, se realizaron 3 lavados de 5 minutos en agitación en TBS-Tween®20 al 0,1% y se procedió al revelado con el cromógeno diaminobencidina (DAB) (Dako; ref. K3466). El tiempo de revelado de las muestras se estableció con un control positivo y la reacción se detuvo con agua. Las muestras se contrastaron con hematoxilina de Harris (Sigma) diluida 1:1 en agua destilada. Para el montaje con DPX (BDH), las muestras se deshidrataron mediante pases sucesivos por alcohol de 96º, absoluto y xilol. Como control negativo se utilizó el mismo tejido al cual no se añadió el anticuerpo primario y un tejido que no expresase el antígeno de interés.

Tabla 4.2. Anticuerpos empleados en las técnicas de inmunohistoquímica e inmunofluorescencia.

Anticuerpo	Casa comercial (Ref.)	Concentración	Liberación de antígeno	Amplificación de la señal
eGFP	Invitrogen (#A11122)	1:500	No	Sí
Caveolina-1	Cell signaling (#3238)	1:125	Microondas Citrato pH6	No
α-Actina de músculo liso (αSMA)-Cy3	Sigma (#6198)	1:500	Microondas Citrato pH6	No

En las técnicas de inmunofluorescencia, para el revelado de los anticuerpos primarios no conjugados a fluorocromos, se empleó un anticuerpo secundario dirigido frente a ratón y marcado con FITC (Jackson Immunoresearch; ref. 711-096-152) que se

utilizó a una dilución 1:200 en TBS y BSA al 1% e incubaron durante 1 hora a temperatura ambiente y en cámara húmeda. Finalmente, las preparaciones se montaron con medio Vectashield-Dapi (Vector Laboratories) al 25% en glicerol:PBS (1:1).

4.7.3. Microscopía electrónica

La membrana #406 celularizada con ADSC o sin celularizar, fue procesada para su análisis mediante técnicas de microscopia electrónica.

Para ello, las muestras se post-fijaron en tetróxido de osmio (OsO_4) al 1% y glucosa al 7% durante 30 minutos, se deshidrataron, se incluyeron en durcupan (Sigma; ref. 44610) y se dejaron polimerizar durante 3 días en estufa a 65ºC. A continuación, se realizaron los cortes semifinos (de 1,5 µm de grosor) y se tiñeron con azul de toluidina (Sigma; ref. 89640) para realizar posteriormente los cortes ultrafinos (de 80 nm de grosor), que se contrastaron con citrato de plomo (0,1 M nitrato de plomo (Merck; ref. 107398), 0,3 M citrato de sodio (Merck; ref. 112005) y 1N hidróxido de sodio (Merck; ref. 106498)) en agua destilada.

Se utilizó el microscopio electrónico FEI Tecnai Spirit G2 para examinar las muestras.

4.7.4. Análisis histológicos

La adquisición de imágenes para los análisis histológicos se realizó con la cámara AxioCam ICc3 (Zeiss), en el caso de las preparaciones teñidas mediante técnicas de inmunohistoquímica o tinciones convencionales, o con la cámara AxioCam MR3 (Zeiss), en el caso de las preparaciones teñidas mediante técnicas de inmunofluorescencia. Ambas cámaras se encontraban acopladas al microscopio Zeiss AxioImager M1 (Zeiss).

4.7.4.1. Cuantificación del grado de inflamación tisular

Para la cuantificación de la inflamación, se utilizaron cortes de corazón teñidos con hematoxilina-eosina. Dicha tinción nos permite distinguir las zonas de infiltrado leucocitario correspondientes a las zonas de inflamación (que adquieren un intenso color morado debido a la alta densidad de núcleos en la zona que tienen naturaleza ácida y se tiñen con la hematoxilina que es un componente básico) a diferencia de las zonas de no inflamación correspondientes con el músculo cardiaco (zonas más rosadas con un menor número de núcleos y mayor extensión de componentes citoplasmáticos, que por ser elementos de naturaleza básica se teñirán con el colorante ácido, la eosina). Este método de cuantificación de la inflamación ha sido descrita en otros trabajos y se considera un análisis semi-cuantitativo [146, 254, 255] (Figura 4.4.), alternativo por ejemplo a la detección de células $CD45^{+}$, que en rata no ha sido posible realizar debido a la imposibilidad de encontrar un anticuerpo específico para dicho marcador.

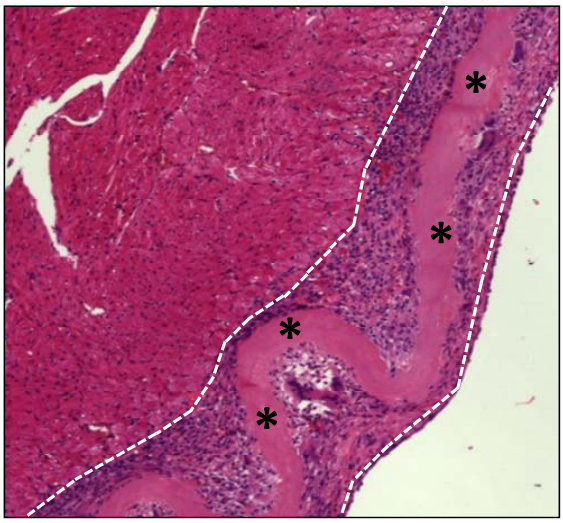


Figura 4.4. Análisis semi-cuantitativo del grado de inflamación producido por las membranas implantadas en un modelo *in vivo* con la tinción hematoxilina-eosina. Imagen representativa de la cuantificación de las zonas de inflamación (delimitadas por el punteado blanco, donde se aprecia una mayor densidad celular correspondiente al infiltrado de las células inflamatorias). Los asteriscos (*) señalan la membrana.

Se tomaron imágenes en mosaico de secciones transversales del corazón teñidas con hematoxilina-eosina, con el objetivo de 2,5x. Se analizaron 18 series para cada animal y las imágenes captadas se cuantificaron con el programa *AnalySIS* (Olympus Biosystem, Soft Imaging System GmbH), que permite seleccionar las áreas de inflamación. Los datos se mostraron como porcentaje de inflamación por área de ventrículo.

4.7.4.2. Cuantificación del tamaño de infarto

Se cuantificó el tamaño de infarto en las preparaciones teñidas con rojo sirio. La tinción de rojo sirio permite distinguir la zona del tejido infartado (teñida en rojo), del músculo cardiaco no dañado (teñida en amarillo).

Se tomaron imágenes en mosaico de secciones transversales del corazón, con el objetivo de 2,5x. Se realizaron 12 series para cada animal y las imágenes captadas se cuantificaron con el programa *AnalySIS*. Los datos obtenidos (área infartada y área no infartada) se mostraron como el porcentaje de infarto en el ventrículo izquierdo.

4.7.4.3. Cuantificación del grado de fibrosis tisular

Para la cuantificación de la fibrosis tisular, se utilizaron cortes de corazón teñidos con la tinción de rojo sirio. La tinción de rojo sirio permite distinguir las fibras de colágeno (teñidas en rojo) de la zona no fibrótica o músculo (teñida en amarillo).

Se fotografiaron las zonas de peri-infarto de las secciones de los corazones de 12 series (2 fotografías/sección), con el objetivo de 10x. Las imágenes captadas se cuantificaron con el programa *AnalySIS*. Los datos se mostraron como porcentaje de área de fibrosis en zona peri-infarto respecto al área total del tejido.

4.7.4.4. Cuantificación del grado de revascularización tisular

Se cuantificó la densidad capilar (angiogénesis) mediante el marcaje de caveolina-1, así como de los vasos arteriales (arteriogénesis), mediante el marcaje de

actina de músculo liso (αSMA); ambos marcajes se realizaron mediante técnicas de inmunofluorescencia.

Se analizaron 12 series en cada una de las cuales se tomaron 2 fotografías de la región de peri-infarto, con el objetivo de 20x. Las imágenes captadas se cuantificaron con un programa desarrollado por la Unidad de Imagen del CIMA con la plataforma *Matlab*. Los datos se mostraron como número de vasos caveolina-$1^{+}/mm^{2}$ (con un diámetro de 5 a 15 µm) y como número de vasos $\alpha SMA^{+}/mm^{2}$.

4.7.4.5. Cuantificación del injerto celular

El injerto celular se determinó mediante la cuantificación en 12 series, de las células eGFP positivas marcadas mediante inmunohistoquímica, en el tejido cardiaco. Los datos se mostraron como porcentaje de células positivas para el marcaje eGFP respecto al número total de células implantadas.

4.8. Análisis estadístico

El análisis estadístico se realizó utilizando el programa SPSS 17.0 para Windows. La normalidad de las muestras se comprobó mediante los tests de Shapiro-Wilk y Kolmogorov-Smirnov. En aquellas muestras que seguían una distribución normal, las comparaciones entre más de dos grupos se realizaron utilizando un ANOVA, y las comparaciones entre dos grupos, utilizando el T test para dos muestras independientes o pareadas. En aquellas muestras en las que fue necesario utilizar estadística no paramétrica, las comparaciones entre grupos se realizaron utilizando el test de Kruskal-Wallis, y para las comparaciones entre dos grupos, utilizando el test U de Mann-Whitney. Para las comparaciones no paramétricas de dos muestras pareadas se utilizó el test de Wilcoxon.

En todos los casos, las diferencias entre los grupos se consideraron significativas para $p<0,05$, y muy significativas para $p<0,01$. Los datos se mostraron como media ± desviación estándar.

5. RESULTADOS

5.1. Cultivo celular y caracterización de las células ADSC

Para la obtención de las ADSC, se aisló la fracción estromal vascular a partir de grasa inguinal de ratas Sprague-Dawley macho, adultas (8 semanas de edad) y transgénicas para la proteína eGFP (1-2 x 10^6 células/gramo de tejido adiposo procesado), la cual se mantuvo en cultivo durante 3 semanas. La población obtenida se caracterizó mediante citometría de flujo para los marcadores de célula mesenquimal CD44, CD73 y CD90, de célula endotelial CD31, de célula hematopoyética CD45 y de los complejos de inmunohistocompatibilidad RT1A y RT1B.

La población ADSC resultó positiva para los marcadores CD44, CD73, CD90 y RT1A, y negativa para CD31, CD45 y RT1B, confirmando su fenotipo mesenquimal (Figura 5.1.).

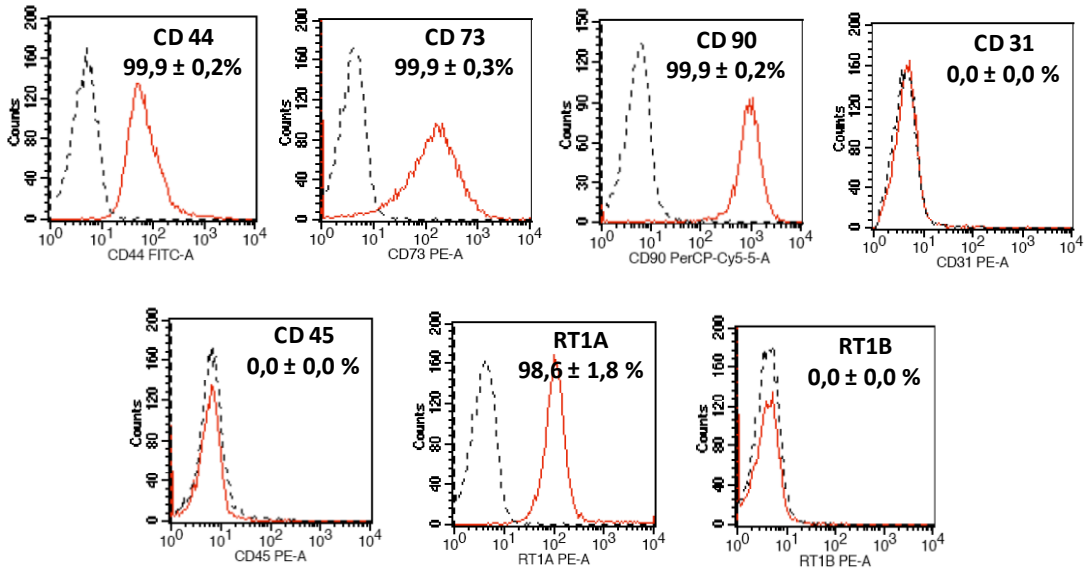


Figura 5.1. Caracterización fenotípica. Las ADSC se marcaron con los anticuerpos frente a CD44, CD73, CD90, CD31, CD45, RT1A y RT1B (línea roja), o con los controles de isotipo (línea negra). Los porcentajes de expresión corresponden a los valores obtenidos (media ± SD) en 5 poblaciones distintas de ADSC.

Además, la expresión de la proteína eGFP, fue monitorizada a lo largo del cultivo y en el momento previo al implante, siendo en todos los casos superior al 83%.

5.2. Caracterización de las membranas de colágeno

En primer lugar, se analizó el comportamiento mecánico de las membranas así como la capacidad de adhesión y proliferación de las ADSC sobre ellas. Además, se determinó la biocompatibilidad de las membranas con el tejido cardiaco en el modelo de IM, para lo cual se analizó el grado de reabsorción de la membrana en el tejido cardiaco y la posible reacción inflamatoria inducida por las mismas tras su trasplante.

5.2.1. Comportamiento mecánico de las membranas

Las 4 membranas analizadas mostraron una respuesta altamente no lineal típica de los tejidos biológicos blandos con una zona inicial de baja pendiente, y por tanto baja rigidez, donde es necesaria muy poca fuerza para producir un alargamiento de la malla (E_I, zona exponencial) y una zona lineal con una pendiente más grande, y por tanto elevada rigidez donde para producir el mismo alargamiento es necesaria mucha mayor fuerza (E_{II}) (Figura 5.2.). Este comportamiento es debido a que las fibras de colágeno de la membrana inicialmente onduladas y aleatoriamente orientadas no aportan mucha rigidez al material, mientras que cuando se incrementa el alargamiento se va produciendo dicha orientación y las fibras de colágeno aportan su máxima resistencia.

La membrana sin entrecruzamiento (#406) presentaba un grado de rigidez significativamente menor que el resto de membranas (#407, #408 y #409) (p<0,05) (Figura 5.2.), así como un comportamiento anisótropo entre la dirección longitudinal y transversal (p<0,05) (Tabla 5.1.).

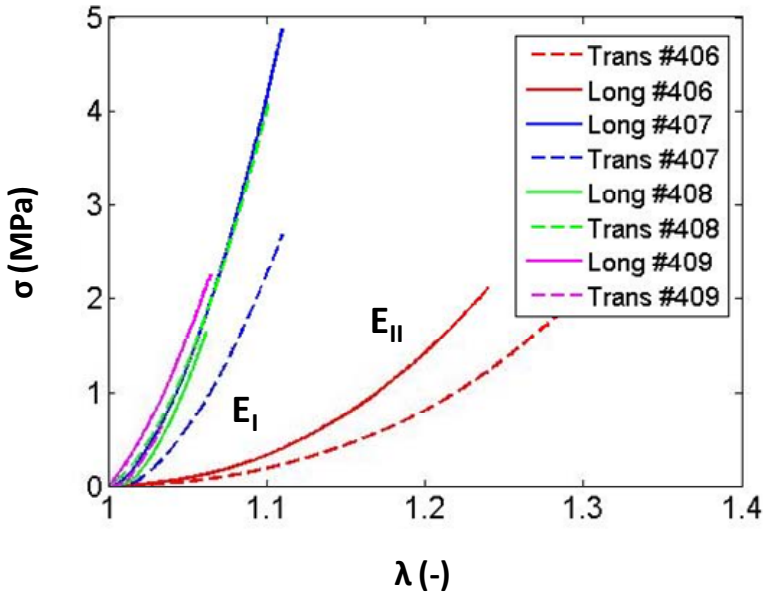


Figura 5.2. Comportamiento mecánico de las membranas #406, #407, #408 y #409. Curvas de tensión-deformación de los 4 tipos de membranas en dirección longitudinal (Long) y transversal (Trans). La membrana #406 presenta un comportamiento menos rígido que el resto de las membranas con entrecruzamiento (#407, #408 y #409) (p<0,05) y una marcada anisotropía (p<0,05). También se determinó un comportamiento anisótropo en la membrana #407 (p<0,05). Se realizaron 6 experimentos independientes en cada dirección para cada tipo de membrana.

En cambio, en las membranas con entrecruzamiento (#407, #408 y #409) no se observaron diferencias significativas en su rigidez y en cuanto a la anisotropía, tan sólo se detectó en la membrana con menor entrecruzamiento (#407) (p<0,05) pero no en las otras dos membranas con mayor entrecruzamiento (#408 y #409) (Tabla 5.1.).

Tabla 5.1. Módulos elásticos tangentes (MPa) de las membranas #406, #407, #408 y #409. Análisis en la dirección longitudinal (Long) y transversal (Trans), (media ± SD). Diferencias estadísticamente significativas entre las dos direcciones (anisotropía) (*p<0,05; **p<0,01).

	E_I Long	E_I Trans	p	E_{II} Long	E_{II} Trans	p
#406	2,79 ± 0,46	0,96 ± 0,76	0,080	26,64 ± 1,98	7,78 ± 4,25	0,005**
#407	15,24 ± 7,71	3,52 ± 3,03	0,006**	86,84 ± 27,26	48,61 ± 20,82	0,010*
#408	8,32 ± 11,93	15,64 ± 10,46	0,689	79,07 ± 22,84	70,97 ± 26,87	0,897
#409	21,59 ± 11,61	14,01 ± 7,44	0,104	77,89 ± 26,32	63,89 ± 15,25	0,181

5.2.2. Biocompatibilidad de las membranas con las células ADSC

Con el fin de determinar el grado de biocompatibilidad de las 4 membranas (#406, #407, #408 y #409) con las células ADSC, se analizó el grado de adhesión y proliferación de las células sobre las mismas. Para ello, las ADSC se cultivaron sobre los 4 tipos de membranas a una densidad de 40 x 10^3 células/cm^2 y se determinó su densidad celular a lo largo del tiempo (días 1, 4 y 7) (Figura 5.3.).

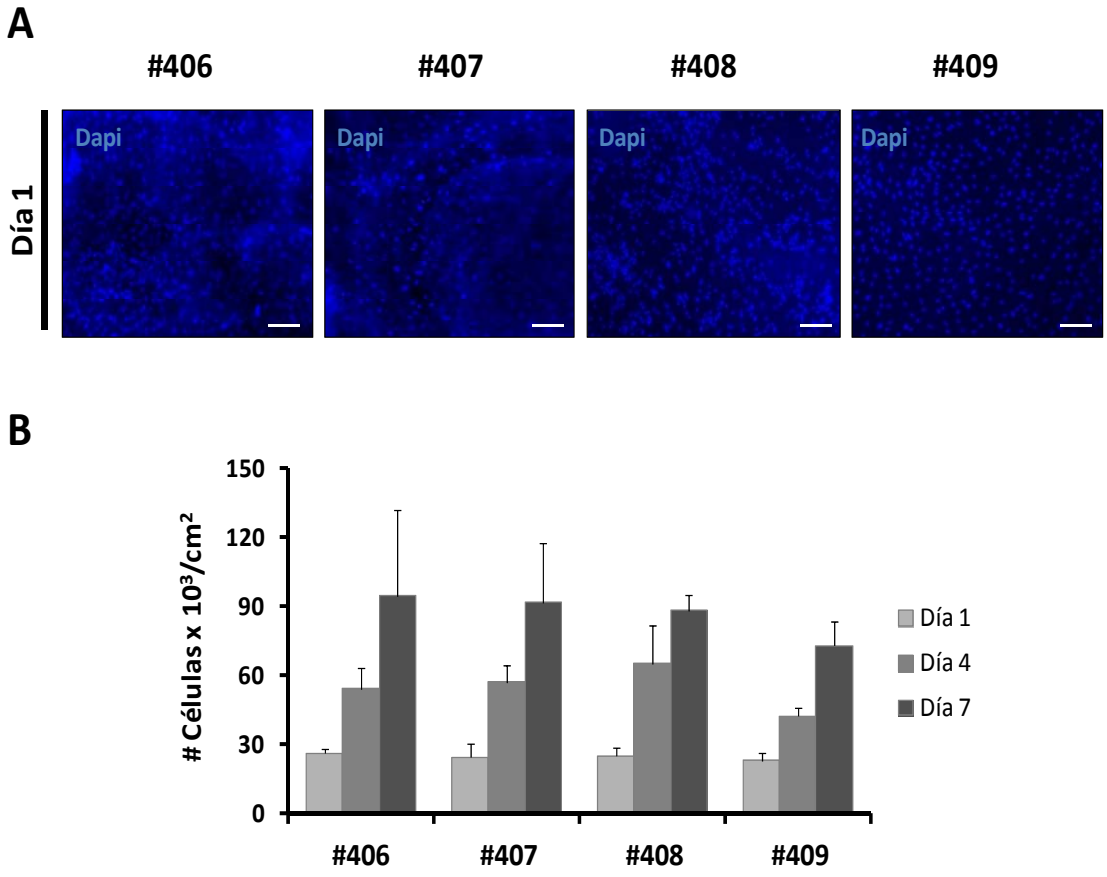


Figura 5.3. Adhesión y proliferación de las ADSC sobre las distintas membranas de colágeno (#406, #407, #408 y #409). A. Imágenes representativas de la adhesión de las ADSC a las membranas 24 horas tras el cultivo. Núcleos marcados con Dapi. **B.** No se detectaron diferencias significativas en el grado de adhesión y proliferación de las ADSC sobre los 4 tipos de membranas a día 1, 4 y 7 (p = n.s.). Se realizaron 3 experimentos independientes incluyendo un triplicado para cada condición. Barras de escala: 100 µm.

Las ADSC mostraron una correcta adhesión en los 4 tipos de membranas, no encontrándose diferencias estadísticamente significativas en el número de células adheridas tras 24 horas de cultivo sobre las mismas (**Día 1:** #406: 26,0 ± 2,1 x 10^3 células/cm^2; #407: 24,3 ± 6,1 x 10^3 células/cm^2; #408: 24,9 ± 3,7 x 10^3 células/cm^2; #409: 23,0 ± 3,1 x 10^3 células/cm^2; *p* = n.s.). Además, el grado de proliferación de las ADSC sobre los 4 tipos de membranas fue similar entre ellas (**Día 4:** #406: 54,2 ± 9,1 x 10^3 células/cm^2; #407: 57,1 ± 7,3 x 10^3 células/cm^2; #408: 65,0 ± 16,4 x 10^3 células/cm^2; #409: 42,1 ± 3,9 x 10^3 células/cm^2; *p* = n.s.; **Día 7:** #406: 94,7 ± 37,1 x 10^3células/cm^2; #407: 91,7 ± 25,6 x 10^3células/cm^2; #408: 88,2 ± 6,8 x 10^3células/cm^2; #409: 72,8 ± 10,7 x 10^3; células/cm^2; *p* = n.s.), lo cual indica una adecuada y similar biocompatibilidad de los 4 tipos de membranas con las células ADSC (Figura 5.3.).

5.2.3. Biocompatibilidad de las membranas *in vivo*

Para el análisis de la biocompatibilidad de las membranas con el tejido cardiaco, se llevó a cabo un estudio piloto donde se implantaron los 4 tipos de membranas (#406, #407, #408 y #409) en un modelo de IM crónico en rata. Para ello, se trasplantaron 6 animales con cada tipo de membrana y se sacrificaron tras 2, 7 y 30 días del implante.

5.2.3.1. Efecto del entrecruzamiento en la adhesión y reabsorción de la membrana

En primer lugar, se evaluó el grado de adhesión y reabsorción de las membranas al corazón a lo largo del tiempo. Dos días después de realizar el implante, se observó a nivel macroscópico la total adhesión de la membrana #406 al corazón (Figura 5.4.), pero no así de las membranas con entrecruzamiento. Incluso 1 mes después del implante, ninguna de las otras 3 membranas se encontró completamente adherida al tejido.

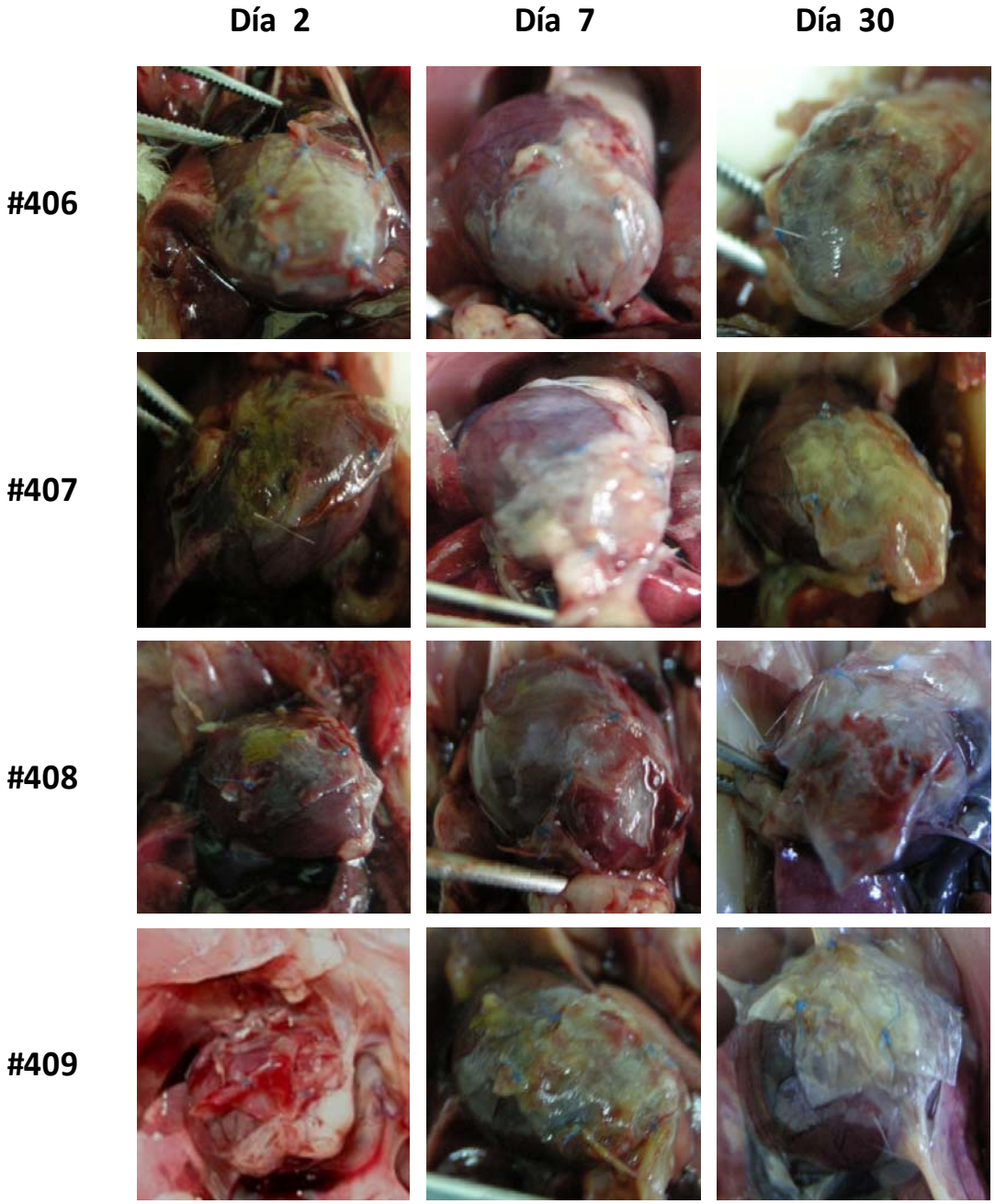


Figura 5.4. Adhesión de las membranas #406, #407, #408 y #409 *in vivo*. Imágenes representativas de los corazones 2, 7 y 30 días después del implante. A nivel macroscópico se observa una correcta adhesión de la membrana #406 al corazón.

Estas observaciones se corroboraron a nivel histológico, donde se confirmó una mayor reabsorción y degradación de la membrana sin entrecruzamiento (#406) (Figura 5.5.).

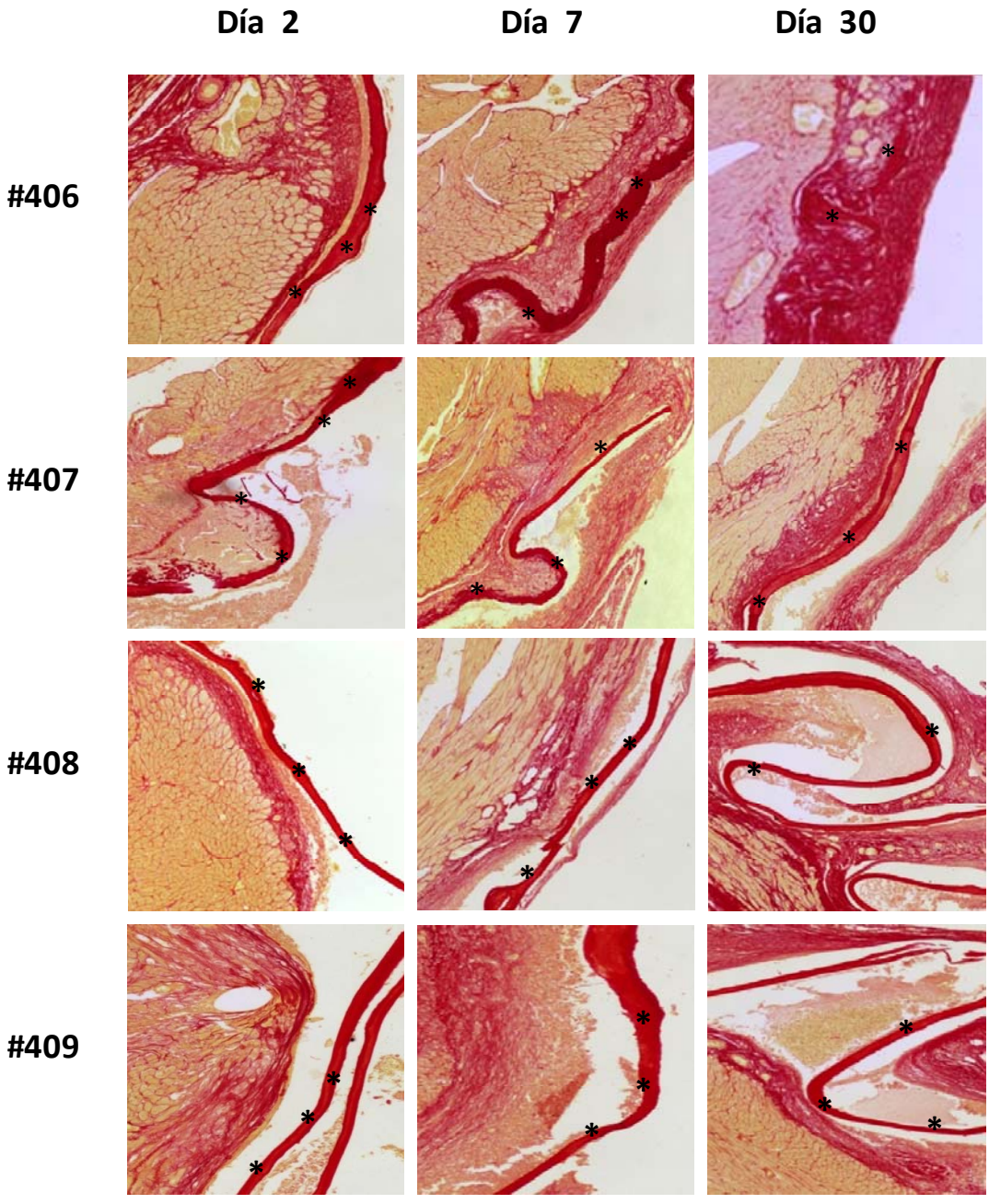


Figura 5.5. Reabsorción de las membranas #406, #407, #408 y #409 *in vivo*. Imágenes representativas de secciones de los corazones teñidos con rojo sirio, 2, 7 y 30 días después del implante. En las secciones de los corazones trasplantados se observó una correcta adhesión y reabsorción a lo largo del tiempo de la membrana #406. Los asteriscos (*) indican la posición de la membrana.

5.2.3.2. Efecto del entrecruzamiento en la inflamación

Las cuatro membranas estudiadas (#406, #407, #408 y #409) están compuestas por colágeno de tipo I obtenido a partir de piel bovina. Sin embargo, a pesar de estar compuestas por colágeno, que es una proteína de la matriz extracelular muy conservada entre especies, como ésta ha sido sometida a diversos métodos físico-químicos de procesamiento y entrecruzamiento, es posible que provoque una cierta respuesta inmunológica frente a cuerpo extraño. Por ello, se analizó el grado de inflamación producido por las diferentes membranas tras 7 y 30 días del implante (Figura 5.6.).

Tal y como se puede observar en la Figura 5.6., las 4 membranas indujeron una reacción inflamatoria moderada en el tejido cardiaco, y se detectaron diferencias estadísticamente significativas entre sí, de modo que los corazones implantados con la membrana #406 (sin entrecruzamiento) presentaban un menor grado de inflamación que los tratados con las membranas entrecruzadas a día 7 (#406: 10,2 ± 2,1%; #407: 16,3 ± 2,9%; #408: 15,9 ± 4,8%; #409: 17,4 ± 4,1%; (Membrana no entrecruzada (#406) *vs*. Membranas entrecruzadas (#407, #408 y #409): $p<0,05$).

Se observó además una disminución en el grado de inflamación de los tejidos tratados con cualquiera de los 4 tipos de membranas a lo largo del tiempo, siendo el grado de inflamación a día 30 significativamente menor (prácticamente nulo) en el grupo implantado con la membrana #406 con respecto al resto de membranas (#406: 1,3 ± 1,3%; #407: 9,4 ± 3,0%; #408: 7,0 ± 2,1%; #409: 9,8 ± 2,5%; (Membrana no entrecruzada (#406) *vs*. Membranas entrecruzadas (#407, #408 y #409): $p<0,01$).

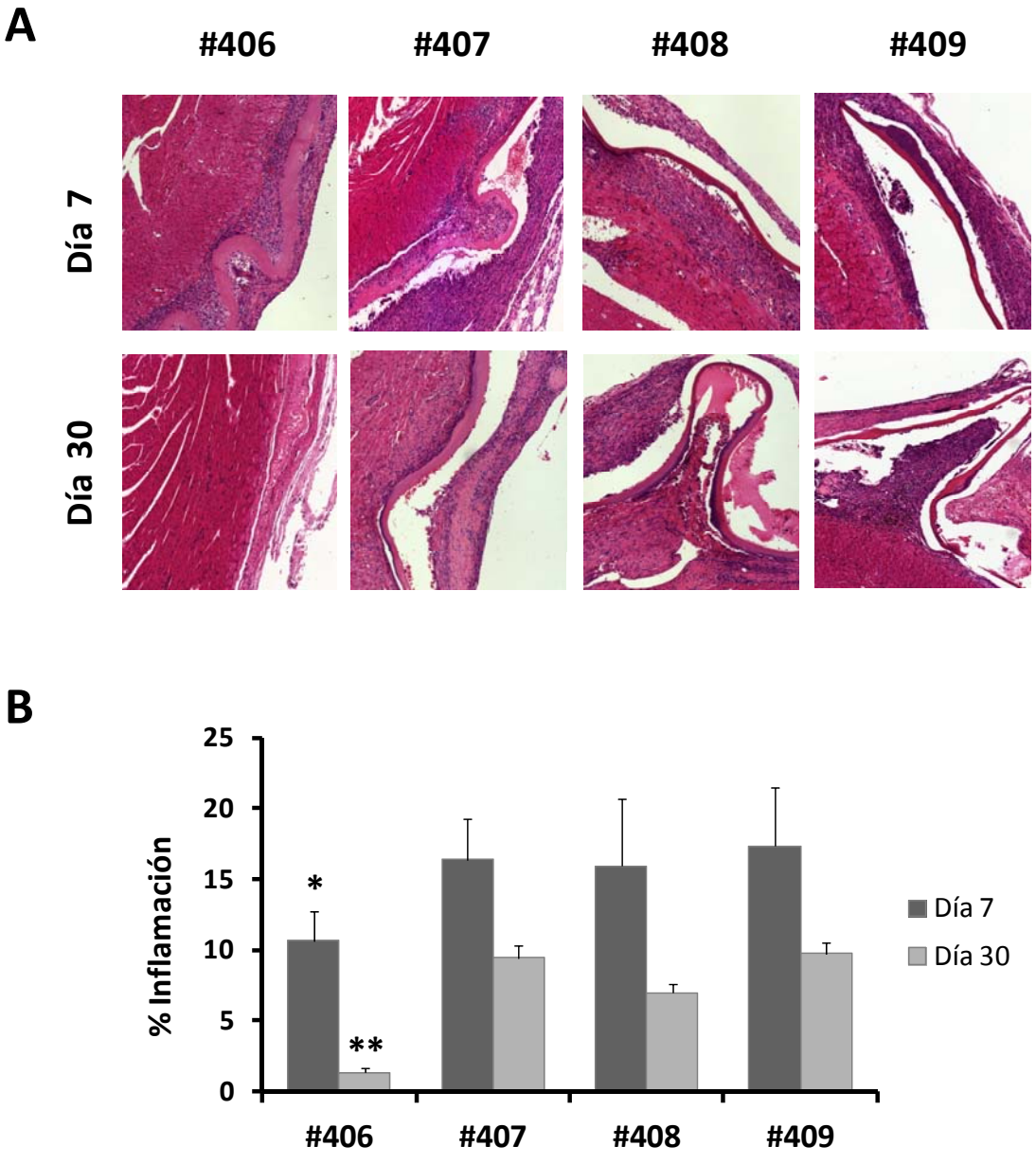


Figura 5.6. Análisis de la inflamación producida por las membranas #406, #407, #408 y #409. **A.** Imágenes representativas de secciones de los corazones con la tinción hematoxilina-eosina, 7 y 30 días después del implante. **B.** Cuantificación del grado de inflamación producido por las membranas #406, #407, #408 y #409. Se detectó un menor grado de inflamación en los corazones trasplantados con la membrana #406 respecto al resto de membranas, tanto a día 7 (*p<0,05) como a día 30 post-implante (**p<0,01).

Como conclusión, la membrana de colágeno sin entrecruzamiento (#406) presentó un comportamiento menos rígido que el resto de las membranas y resultó ser biocompatible tanto con las células ADSC como con el tejido cardiaco, en el que presentaba una rápida adhesión y reabsorción, así como un bajo grado de inflamación, que resultó ser significativamente menor que el provocado por las membranas con entrecruzamiento (#407, #408 o #409). En vista de estos resultados, se escogió la membrana #406 como soporte de las ADSC para el posterior estudio *in vivo.*

5.3. Celularización y caracterización de la membrana de colágeno

5.3.1. Elección de la confluencia celular para la celularización de la membrana

Una vez seleccionada la membrana de colágeno #406, se determinó la densidad celular más adecuada para la celularización de la misma. Para ello, se analizaron tanto el grado de adhesión como de apoptosis celular a 3 confluencias celulares distintas (100, 250 y 500 x 10^3 células/membrana (área de la membrana: 0,8 cm^2)).

Para el análisis de la adhesión, se determinó el número de células adheridas a la membrana tras 24 horas de cultivo, no encontrándose diferencias estadísticamente significativas entre el número de ADSC adheridas al pocillo de plástico (control) o a la membrana #406, en ninguna de las confluencias celulares analizadas (**Control *vs.* Membrana: 100 x 10^3:** 102,4 ± 28,2 x 10^3 células *vs.* 84,1 ± 20,4 x 10^3 células; p = n.s.; **250 x 10^3:** 194,6 ± 30,0 x 10^3 células *vs*. 188,7 ± 60,4 x 10^3 células; p = n.s.; **500 x 10^3:** 363,4 ± 71,1 x 10^3 células *vs.* 378,0 ± 91,4 x 10^3 células; p = n.s.) (Figura 5.7.).

Se observó que al aumentar el número de células sembradas se producía una disminución en el porcentaje de ADSC adheridas a la membrana (**100 x 10^3:** 84 ± 20%; **250 x 10^3:** 75 ± 24%; **500 x 10^3:** 76 ± 18%), sin embargo, estos resultados eran similares a los obtenidos tras sembrar las células en las placas de cultivo, (**100 x 10^3:** 102 ± 28%; **250 x 10^3:** 78 ± 12%; **500 x 10^3:** 73 ± 14%), por lo que el efecto era independiente de la membrana.

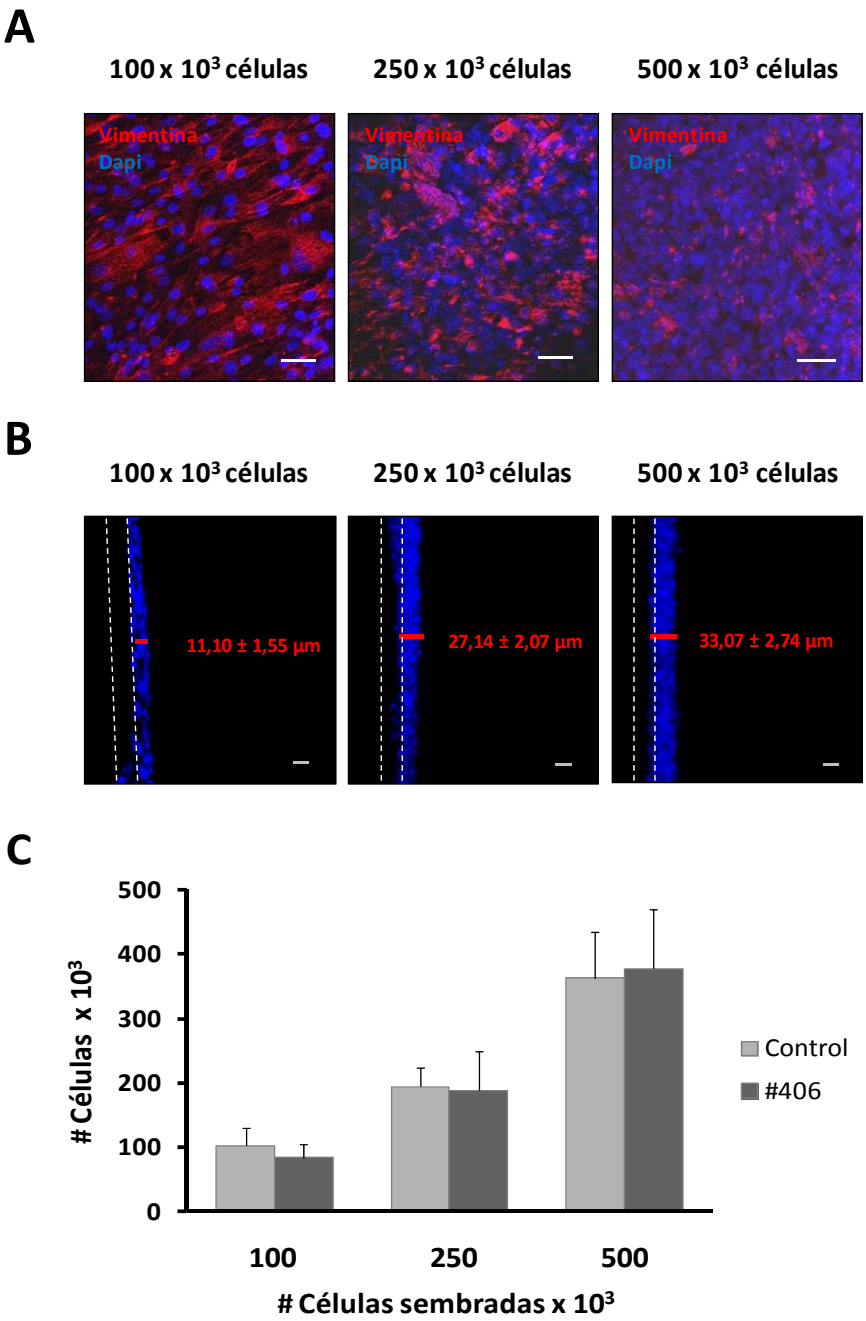


Figura 5.7. Adhesión de las ADSC a la membrana #406. **A.** Imágenes representativas de inmunofluorescencia frente a vimentina, muestran la disposición de las células ADSC sobre la membrana. Núcleos contrastados con DAPI. **B.** Imágenes de microscopía confocal muestran el grosor del parche celular formado (µm). Núcleos contrastados con TOPRO. La membrana, no teñida y de un grosor de 20 µm, se ha representado con un punteado blanco. **C.** Ensayo de adhesión de las ADSC sembradas a distintas densidades celulares (100, 250 y 500 x 10^3 células/membrana #406 o pocillo control). No se detectaron diferencias significativas en el grado de adhesión de las ADSC sembradas a las distintas confluencias celulares, sobre la membrana #406 o sobre la placa de cultivo utilizada como control (p = n.s.), Barras de escala: 50 µm **(A)**, 20 µm **(B)**. Se realizaron 4 experimentos independientes incluyendo un triplicado para cada condición.

Además, se determinó la disposición de las ADSC sobre la matriz de colágeno mediante la inmunotinción para la proteína del citoesqueleto vimentina, observándose una distribución homogénea y confluente de las células. También, se cuantificó mediante microscopía confocal, el grosor del parche celular formado (Figura 5.7.). Así, cuando se cultivaron 100 x 10^3 ADSC/membrana se formó una monocapa de un grosor de 11,10 ± 1,55 µm**,** que se duplicó cuando se cultivaron 250 x 10^3 ADSC/membrana, alcanzando un grosor de 27,14 ± 2,07 µm que se triplicó hasta 33,07 ± 2,74 µm en la densidad celular más alta.

Finalmente, se determinó mediante la técnica de Tunel, el grado de apoptosis de las ADSC sembradas a las distintas densidades (100, 250 y 500 x 10^3 células/membrana). No se encontraron diferencias estadísticamente significativas entre las mismas (**100 x 10^3:** 0,31 ± 0,15%; **250 x 10^3:** 0,28 ± 0,18%; **500 x 10^3:** 0,23 ± 0,09%; *p* = n.s.) (Figura 5.8.), lo que indicaba que el aumento de la densidad celular no inducía un mayor grado de muerte celular.

Debido a que no se observaron diferencias estadísticamente significativas en el grado de adhesión de las ADSC a la membrana en comparación con las placas de cultivo utilizadas como control, y a que además, no se detectó un aumento significativo en el grado de apoptosis entre las tres confluencias celulares testadas, se seleccionó la densidad celular más alta para los implantes en el modelo *in vivo* (500 x 10^3 ADSC/membrana).

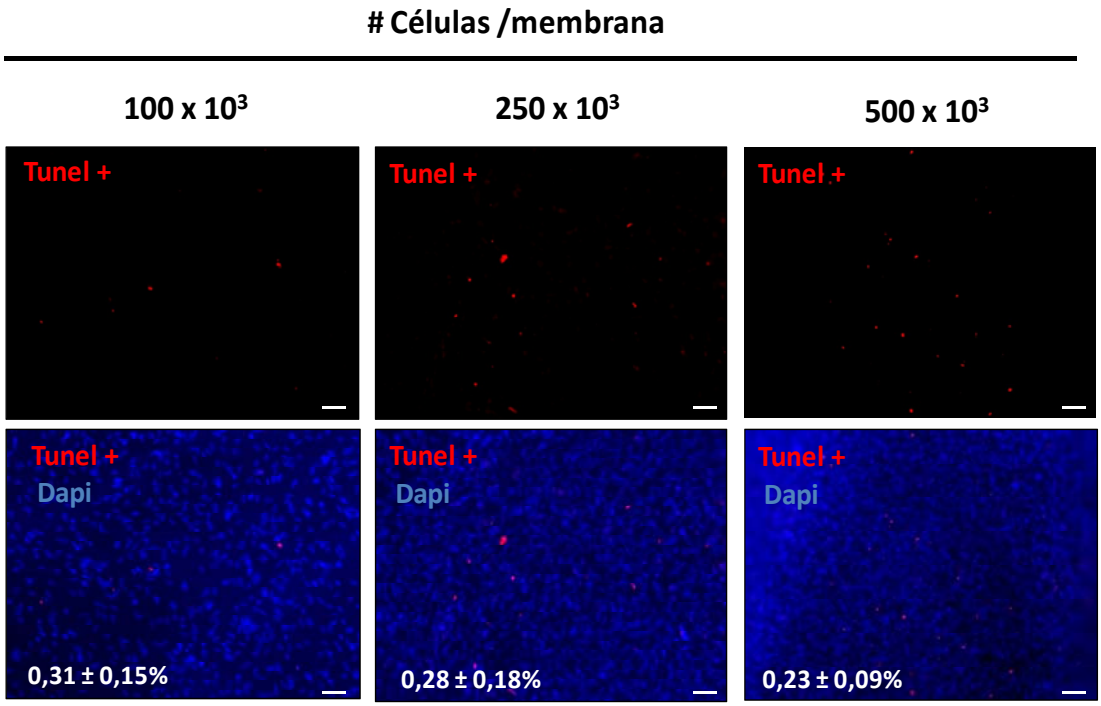


Figura 5.8. Cuantificación de la apoptosis celular mediante Tunel. Imágenes representativas de la técnica de Tunel (Rojo: Fragmentos de ADN; Dapi: Núcleos). No se detectaron diferencias en el porcentaje de células en apoptosis en las distintas confluencias celulares analizadas (100, 250 y 500 x 10^3 células/membrana) sobre la membrana #406 (*p* = n.s.). Se realizaron 3 experimentos independientes incluyendo un triplicado para cada condición. Se muestra el porcentaje de células en apoptosis (media ± SD). Barras de escala: 100 µm.

5.3.2. Comportamiento mecánico de la membrana celularizada

Se analizó el comportamiento mecánico de la membrana #406 cultivada con o sin ADSC durante 24 horas, observándose que las membranas celularizadas mostraban una menor rigidez (E_I= 1,65 ± 0,27 MPa y E_{II}= 13,56 ± 4,44 MPa) que aquellas que no habían sido cultivadas con células (E_I= 2,79 ±0,46 MPa y E_{II}= 26,64 ± 1,98 MPa), (p<0,01 en ambos casos) (Figura 5.9.).

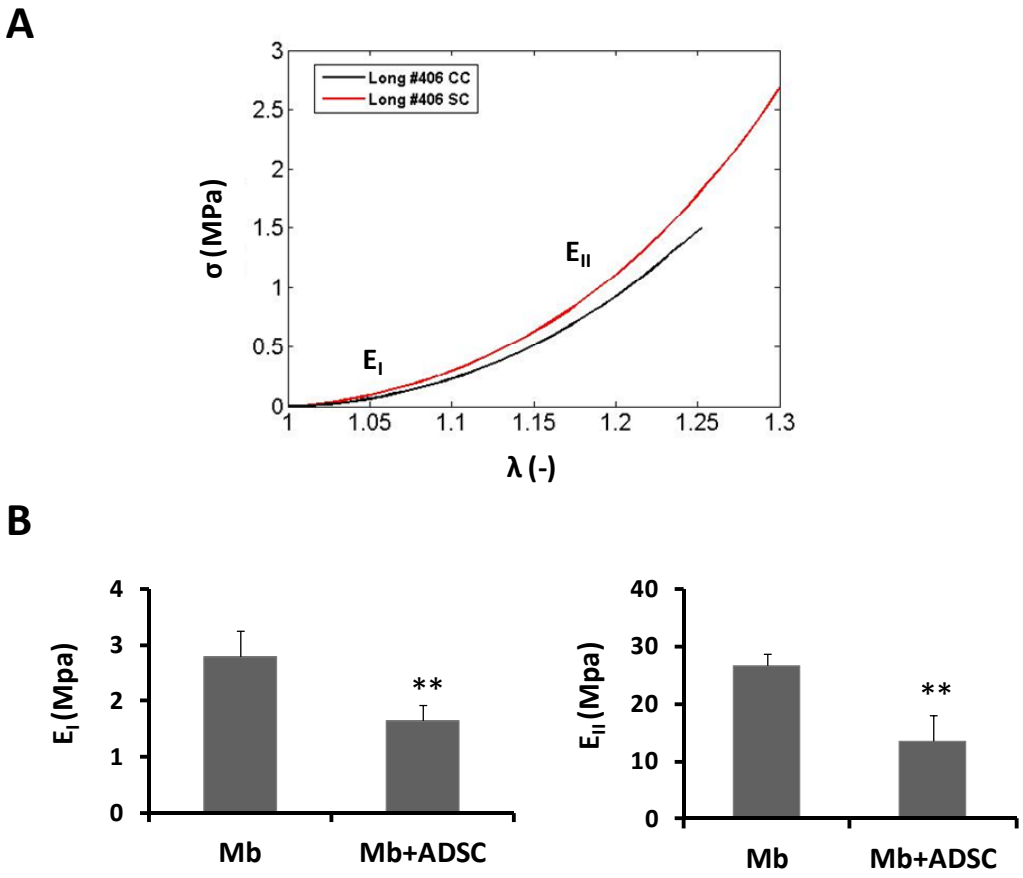


Figura 5.9. Comportamiento mecánico de la membrana celularizada. A. Curvas de tensión-deformación en la dirección longitudinal, de la membrana #406 sembrada con o sin ADSC durante 24 horas. La membrana celularizada (en negro) presenta una menor rigidez que la membrana sin celularizar (en rojo) (p<0,01). **B**. Módulos tangentes E_I y E_{II} para las membranas sin células (Mb) y celularizadas (Mb+ADSC) (unidades en MPa). Se realizaron 5 experimentos para cada tipo de membrana.

5.3.3. Microscopía electrónica de las membranas

Por último, se analizó mediante microscopía electrónica la disposición de las ADSC cultivadas durante 24 horas a una densidad de 500 x 10^3 células/membrana (#406), confirmando lo observado anteriormente mediante las técnicas de inmunofluorescencia para vimentina. Las ADSC forman una capa celular confluente y homogéneamente distribuida sobre la membrana (Figura 5.10.).

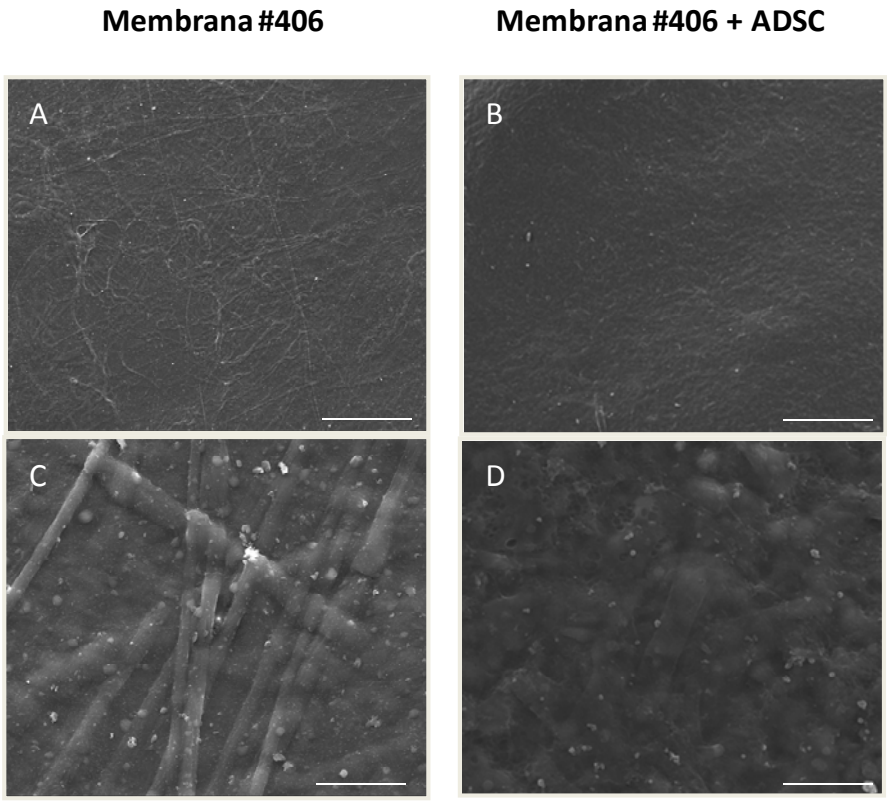


Figura 5.10. Microscopía electrónica de las membranas celularizadas. Imágenes representativas de microscopía electrónica para la membrana #406 **(A y C)** y para la membrana #406 celularizada con ADSC **(B y D)**, tras 24 horas de cultivo. Barras de escala: 250 µm **(A y B)**; 25 µm **(C y D)**.

5.4. Trasplante de la membrana celularizada en un modelo de infarto de miocardio crónico

Una vez caracterizada la membrana (#406) celularizada (500 x 10^3 ADSC/membrana), se realizó un estudio *in vivo* para valorar el posible beneficio de su implante en el corazón infartado. Se analizaron diversos aspectos tales como la función cardiaca, el comportamiento mecánico y el remodelado del corazón.

5.4.1. Trasplante de ADSC con o sin soporte en un modelo de infarto de miocardio crónico

Se quiso determinar, en un modelo en rata de infarto de miocardio crónico, el posible beneficio terapéutico del implante de las ADSC adheridas a un soporte de colágeno o inyectadas de forma directa en el miocardio. Según los estudios previos realizados *in vitro*, el número de ADSC adheridas a la membrana en el momento del implante resultó ser de 350 x 10^3 células (ver apartado 5.3.1.), por lo que se inyectó un número similar en el grupo de ADSC sin membrana. Además, se incluyó un grupo de referencia en el que se implantó la membrana #406 sin celularizar y otro grupo control, al que tan sólo se le inyectó medio de cultivo. Los implantes se realizaron tras 5 semanas de haber sido provocado el infarto, asegurando así la cronificación del daño.

5.4.2. El tratamiento con la membrana celularizada con ADSC induce una mejora funcional a largo plazo

Se analizó la posible mejora funcional y el remodelado cardiaco a largo plazo (4 meses post-trasplante) mediante análisis ecocardiográfico, para lo cual se determinaron los parámetros de fracción de eyección del ventrículo izquierdo (FEVI), y los volúmenes y diámetros telesistólicos y diastólicos, comparándose los valores obtenidos a los 4 meses respecto a los valores preimplante.

La FEVI basal de las ratas sanas (71,6 ± 2,5%) disminuyó hasta un 34,5 ± 2,1% tras 4 semanas de haber sido provocado el infarto. Los animales infartados se distribuyeron aleatoriamente en 4 grupos, entre los cuales no se observaron diferencias estadísticamente significativas en los valores de FEVI pre-implante (Control: 35,1% ± 2,5%; Membrana: 37,0 ± 1,4%; ADSC: 34,6 ± 1,9%; Membrana+ADSC: 31,3 ± 2,4%, p = n.s.), lo que indica la homogeneidad de los grupos.

Cuando se comparó el valor de la FEVI en el momento pre-implante respecto al valor de la FEVI a los 4 meses dentro de cada grupo, sólo en el grupo tratado con la membrana celularizada se observó un aumento estadísticamente significativo (**Pre- *vs.***

Post-implante: Control: 35,1 ± 2,5% *vs.* 38,8 ± 3,2%; *p* = n.s.; **Membrana:** 37,0 ± 1,4% *vs.* 37,4 ± 3,1%, *p* = n.s.; **ADSC:** 34,6 ± 1,9% *vs.* 39,5 ± 3,7%, *p* = n.s.; **Membrana celularizada con ADSC:** 31,3 ± 2,4% *vs.* 47,6 ± 3,9%, *p*<0,01) (Tabla 5.2.).

Tabla 5.2. Valores pre-implante y post-implante (4 meses) de la fracción de eyección del ventrículo izquierdo (FEVI), diámetros telesistólicos (DVIts), diámetros telediastólicos (DVItd), volúmenes telesistólicos (Vts), y volúmenes telediastólicos (Vtd). Diferencias estadísticamente significativas (*p<0,05; **p<0,01). (Media ± SEM). Abreviaturas: Co: control; Mb: membrana.

	Co	Mb	ADSC	Mb+ADSC
FEVI (%)				
Pre-implante	35,13 ± 2,51	36,96 ± 1,44	34,55 ± 1,87	31,26 ± 2,41
Post-implante	38,83 ± 3,15	37,37 ± 3,13	39,50 ± 3,71	47,61 ± 3,86**
DVITs (mm)				
Pre-implante	0,77 ± 0,02	0,72 ± 0,02	0,72 ± 0,02	0,71 ± 0,02
Post-implante	0,76 ± 0,04	0,72 ± 0,03	0,74 ± 0,04	0,67 ± 0,04
DVITd (mm)				
Pre-implante	0,90 ± 0,02	0,85 ± 0,02	0,84 ± 0,02	0,81 ± 0,02
Post-implante	0,89 ± 0,03	0,86 ± 0,03	0,89 ± 0,03	0,85 ± 0,03
Vts (ml)				
Pre-implante	1,02 ± 0,07	0,85 ± 0,06	0,84 ± 0,06	0,82 ± 0,06
Post-implante	1,02 ± 0,14	0,89 ± 0,09	0,96 ± 0,12	0,77 ± 0,13
Vtd (ml)				
Pre-implante	1,56 ± 0,10	1,33 ± 0,08	1,29 ± 0,09	1,19 ± 0,07
Post-implante	1,56 ± 0,16	1,39 ± 0,11	1,54 ± 0,14*	1,38 ± 0,13

Asimismo, en las comparaciones realizadas entre grupos, se observaron diferencias estadísticamente significativas en la variación de la FEVI post *vs.* pre-implante, en el grupo implantado con la membrana celularizada con respecto al resto de los grupos (**%ΔFEVI; Control:** 3,7 ± 3,5%; Membrana: 0,4 ± 4,28%; **ADSC:** 5,0 ± 3,6%; **Membrana celularizada con ADSC:** 16,4 ± 3,3%; *p*<0,05). No se observaron diferencias significativas en el grupo de ADSC ni en el de la membrana sin celularizar con respecto al control (Figura 5.11.).

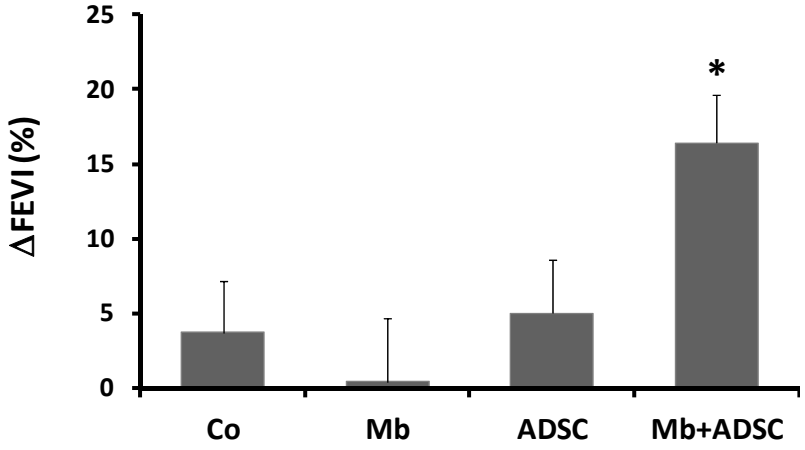


Figura 5.11. La membrana celularizada con ADSC induce una mejora en la función cardiaca. La FEVI fue monitorizada mediante ecocardiografía, previamente al implante y 4 meses tras éste (ΔFEVI: FEVI 4 meses – FEVI basal) en los grupos animales trasplantados con el medio como control (Co), la membrana #406 (Mb), las ADSC o la membrana #406 celularizada (Mb+ADSC). Tan sólo se detectó un aumento significativo en el %ΔFEVI al comparar el grupo tratado con la membrana celularizada con respecto al resto de grupos (*p <0,05).

Por otro lado, no se detectaron cambios significativos en otros parámetros ecocardiográficos, tales como los diámetros y volúmenes telesistólicos (DVIts, Vts) y telediastólicos (DVItd, Vtd) (Tabla 5.2.). En el caso de las ADSC, se observó un mayor grado de dilatación ventricular consecuencia del remodelado tisular negativo que se desencadena tras el infarto.

5.4.3. El tratamiento con la membrana celularizada con ADSC promueve el remodelado cardiaco

5.4.3.1. Comportamiento mecánico del corazón

Se analizó el comportamiento mecánico del tejido cardiaco de las ratas infartadas tratadas con la membrana celularizada, con ADSC, con membrana o medio de cultivo. Además se incluyó un grupo de animales infartados sin tratamiento y otro sano. Todos los animales se sacrificaron a la misma edad. En el análisis del comportamiento mecánico del tejido se consideraron únicamente los datos de las

curvas de la zona infartada central, debido a la variabilidad en la extensión del infarto en las muestras (Figura 4.3.).

Como cabía esperar, los corazones del grupo de ratas infartadas presentaban unos valores de rigidez significativamente mayores que los corazones del grupo de ratas no infartadas (E_{II} Ratas Infartadas: 0,54 ± 0,14 MPa *vs.* E_{II} Ratas Sanas: 0,08 ± 0,05 MPa, p<0,01), lo cual indica un comportamiento mecánico más rígido en los corazones con infarto, debido a la acumulación de colágeno en el área dañada (Figura 5.12.), consecuencia a su vez de los procesos de remodelado desencadenados tras producirse el infarto.

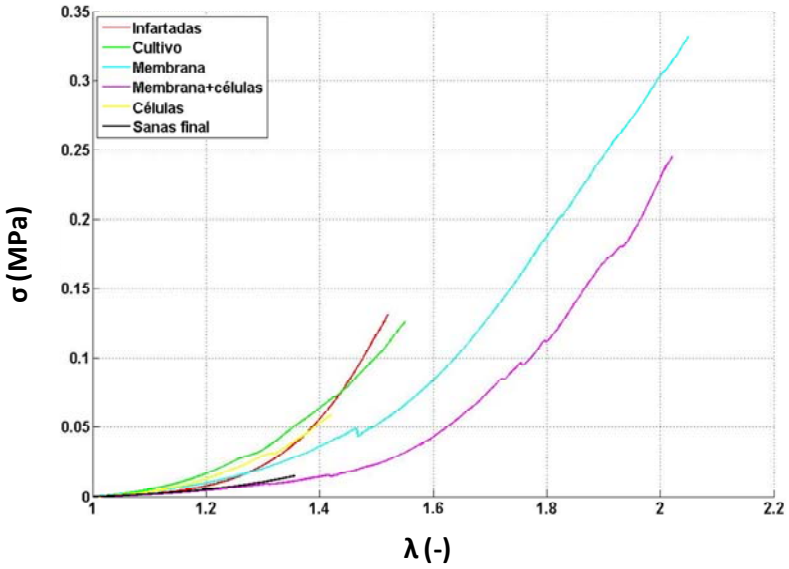


Figura 5.12. El tratamiento con la membrana celularizada con ADSC promueve una mejora en el comportamiento mecánico del corazón. Curvas de tensión-deformación de los corazones de los distintos grupos ensayados (Ratas sanas sin infartar, ratas infartadas sin administración de tratamiento, ratas infartadas e implantadas con la membrana #406, ADSC, medio o membrana celularizada con ADSC). Los corazones implantados con la membrana celularizada presentaron un comportamiento mecánico similar al de los corazones de ratas sanas, no así los grupos implantados con ADSC o con la membrana no celularizada.

Por otro lado, el tratamiento de los corazones con las células ADSC, inyectadas en suspensión subepicárdicamente, no fue capaz de revertir de forma significativa dicha rigidez, observándose, al igual que en los grupos tratados con medio o con la membrana sin celularizar, una significativa rigidez en el tejido cardiaco (E_{II} Ratas sanas: 0,08 ± 0,05 MPa *vs.* E_{II} ADSC: 0,41 ± 0,09 MPa, $p<0,01$; *vs.* E_{II} Medio: 0,36 ± 0,23 MPa, $p<0,01$; *vs.* E_{II} Membrana: 0,20 ± 0,10 MPa, $p<0,01$). Es interesante destacar que en el grupo tratado con la membrana celularizada con ADSC, sí que se observó la efectividad del tratamiento celular, ya que los corazones presentaban niveles de rigidez similares a los determinados en los corazones sanos (E_{II} Membrana celularizada con ADSC *vs.* Ratas sanas: 0,09 ± 0,05 MPa *vs.* 0,08 ± 0,05 MPa; p = n.s.), demostrando el efecto protector de dicho tratamiento.

5.4.3.2. Remodelado tisular

Se analizó el tamaño de infarto y el contenido de colágeno en las zonas infartadas de los corazones en los 4 grupos de estudio.

Respecto al tamaño de infarto, no se detectó una disminución significativa, en los corazones tratados con ADSC trasplantadas de forma directa o sobre el soporte de colágeno (**Control:** 21,3 ± 4,0%; **Membrana:** 18,7 ± 2,3%; **ADSC:** 14,2 ± 3,1%; **Membrana celularizada con ADSC:** 16,5 ± 6,6%; p = n.s.). Por otro lado, sí se observó una disminución estadísticamente significativa en el contenido de colágeno de la zona peri-infarto del grupo tratado con la membrana celularizada con ADSC con respecto al grupo control (medio) no detectada en los otros dos grupos (membrana no celularizada y ADSC) (**Control:** 28,6 ± 2,8%; **Membrana:** 34,7 ± 4,6%; **ADSC:** 31,7 ± 2,1%; **Membrana celularizada con ADSC:** 20,6 ± 2,0%; $p<0,05$) (Figura 5.13).

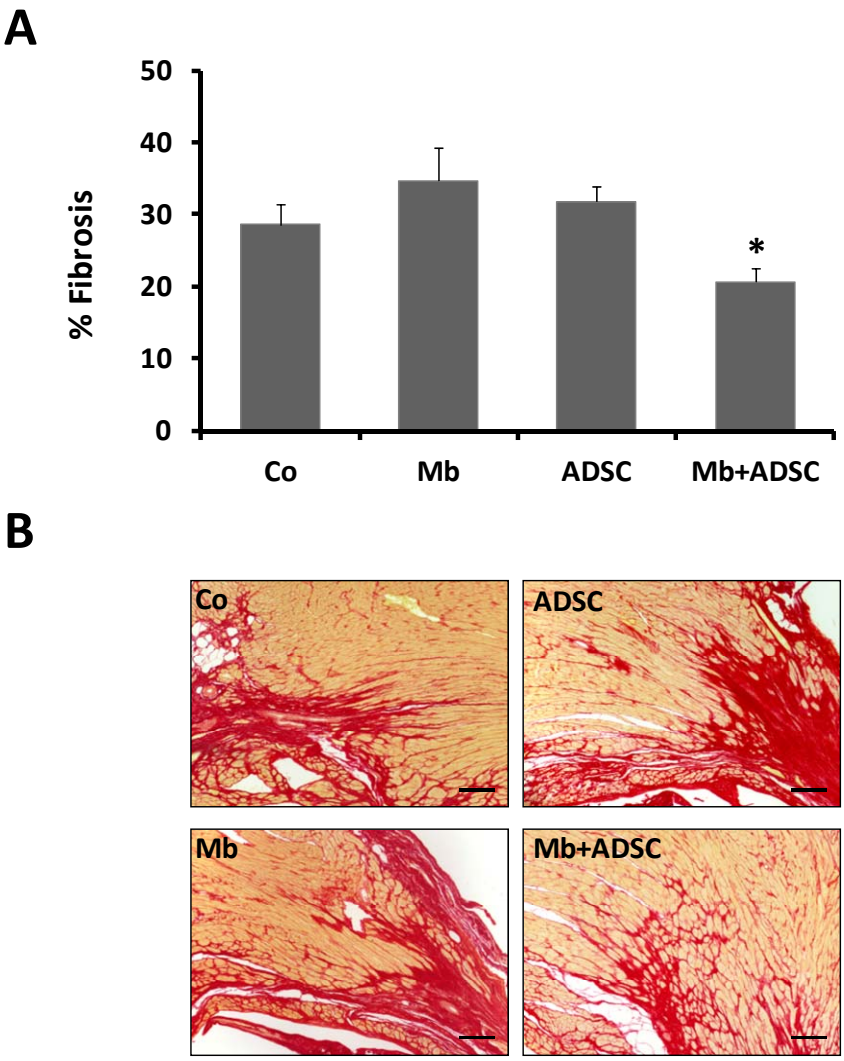


Figura 5.13. El trasplante de la membrana celularizada con ADSC afecta positivamente al remodelado tisular. A. Cuantificación, en secciones del corazón teñidas con rojo sirio, del grado de fibrosis en la zona peri-infarto 4 meses tras el implante de medio (grupo control, Co), membrana (Mb), ADSC o la membrana celularizada con ADSC (Mb+ADSC). **B.** Imágenes representativas de la tinción de rojo sirio. Se observó una disminución significativa en el grado de fibrosis en el grupo implantado con ADSC sobre la membrana #406 en comparación con el grupo control (**p*<0,05). Barras de escala: 150 µm.

5.4.4. Injerto de las ADSC

Por otro lado, para comprobar si el soporte de colágeno favorecía la retención e injerto celular en el corazón, se determinó la presencia de células ADSC-eGFP^{+} en los

grupos inyectados con células ADSC o trasplantados con ADSC previamente cultivadas sobre la membrana #406.

La detección inmunohistoquímica de la proteína eGFP en los corazones de los animales sacrificados 4 meses post-trasplante, no reveló la presencia de ADSC, a tan largo plazo, ni en el grupo de inyección ni en el grupo implantado con las membranas celularizadas. En cambio, sí que se observó injerto celular en el grupo tratado con la membrana celularizada tras 1 semana (25,3 ± 7,0%) y 4 semanas (6,4 ± 4%) del implante. Por el contrario, no se detectó injerto en el grupo inyectado con ADSC tan siquiera tras 1 semana de su inyección (Figura 5.14.).

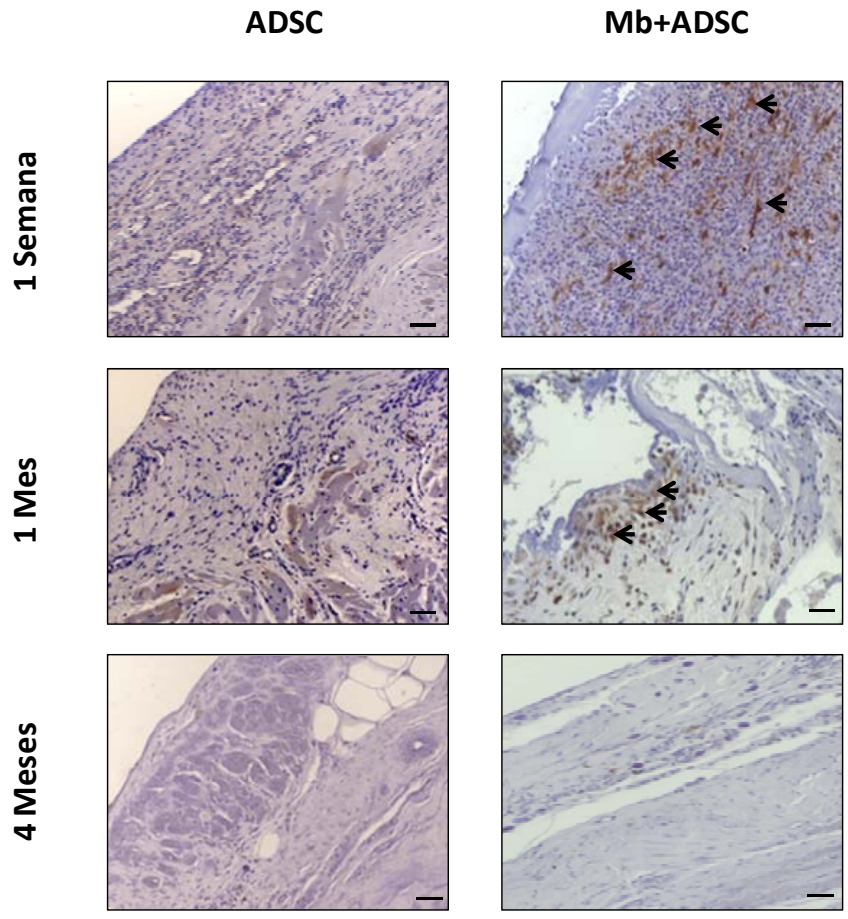


Figura 5.14. Injerto de ADSC-eGFP. Imágenes representativas de secciones de corazón teñidas mediante inmunohistoquímica frente a la proteína $eGFP^+$. Se observó injerto celular en el grupo tratado con la membrana celularizada tras 1 y 4 semanas del implante. No se observó injerto celular en el grupo tratado con ADSC. Las flechas indican células GFP^+ localizadas en el tejido cardiaco. Barra de escala: 50 μm.

Así, a pesar de las condiciones isquémicas del corazón que limitan la capacidad de supervivencia de las células trasplantadas, la utilización de este sistema de matriz como vehículo del implante celular permite una acción paracrina más homogénea y mucho más prolongada en el tiempo (de al menos 1 mes) responsable a su vez del beneficio funcional y del remodelado observado.

5.4.5. El tratamiento con la membrana celularizada con ADSC promueve la revascularización del tejido cardiaco

Finalmente, se evaluó el posible efecto paracrino que las ADSC pueden ejercer mediante la liberación de factores angiogénicos, en la potenciación de la revascularización del tejido cardiaco tras el IM. Mediante técnicas de inmunofluorescencia se marcaron las células endoteliales (caveolina-1^+) y murales (αSMA^+), y se analizó la presencia de capilares (vasos caveolina-1^+ con un diámetro de 5-15 µm) (angiogénesis) y vasos de mayor calibre (arteriolas y arterias) marcados positivamente para αSMA (arteriogénesis).

El análisis de la angiogénesis en la zona peri-infarto mostró un incremento significativo en el número de capilares caveolina-1^+/mm^2 (Figura 5.15.A.) en el grupo tratado con la membrana celularizada, en comparación con el grupo control (**Control:** 413 ± 33 capilares/mm^2; **Membrana:** 333 ± 40 capilares/mm^2; **ADSC:** 333 ± 40 capilares/mm^2; **Membrana celularizada con ADSC:** 487 ± 60 capilares/mm^2; p<0,05). Dicho aumento no se detectó en el grupo tratado con ADSC sin soporte ni con la membrana no celularizada.

Además, el análisis del grado de arteriogénesis (Figura 5.15.B.), mostró un aumento significativo en el número de vasos αSMA^+ en el grupo tratado con la membrana celularizada con ADSC (**Zona peri-infarto; Control:** 93 ± 7 vasos/mm^2; **Membrana:** 107 ± 20 vasos/mm^2; **ADSC:** 113 ± 20 vasos/mm^2; **Membrana celularizada con ADSC:** 160 ± 27 vasos/mm^2; p<0,01) con respecto al grupo control. Dicho aumento tampoco se detectó en el grupo tratado con las ADSC o la membrana sin celularizar.

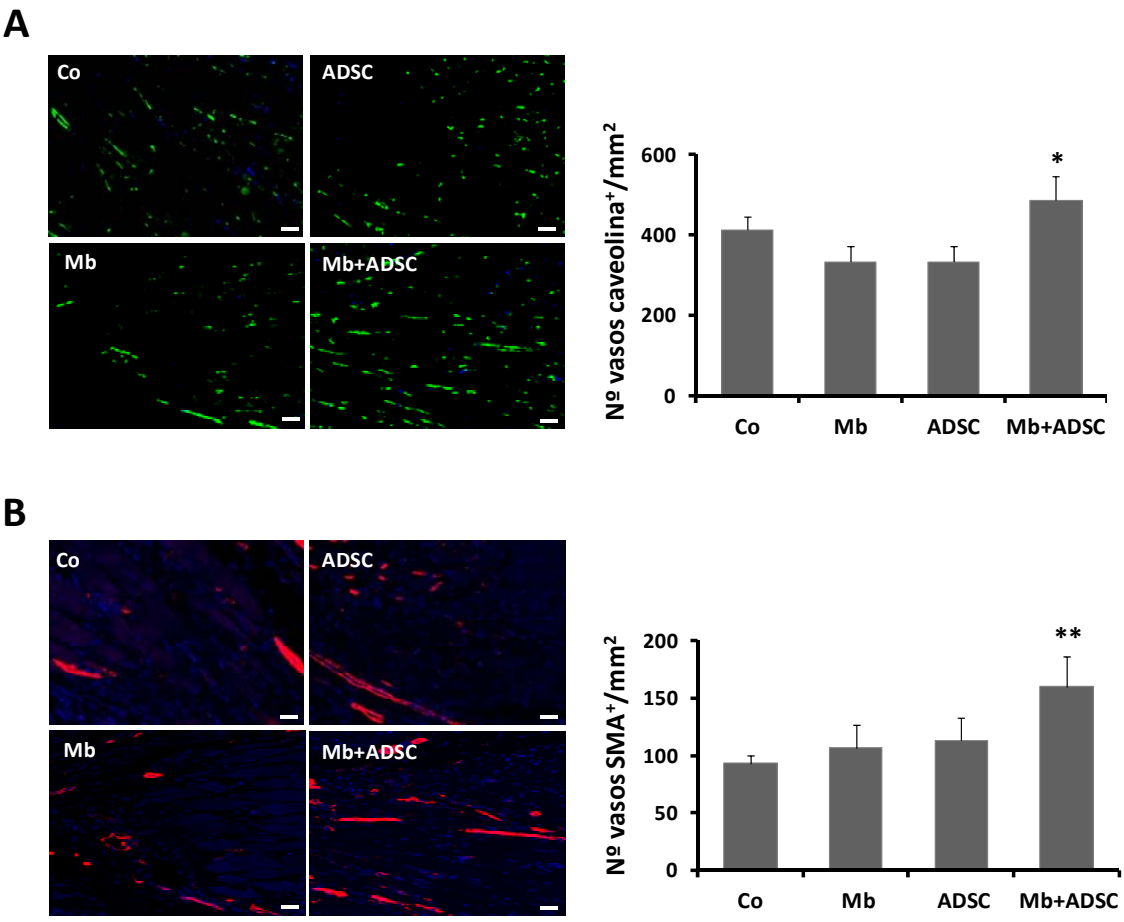


Figura 5.15. Revascularización del tejido cardiaco. La densidad de capilares y arteriolas/arterias se cuantificó como número de vasos caveolina-1^{+}/mm^{2} (diámetro: 5-15 µm) **(A)** y vasos αSMA^{+}/mm^{2} **(B)** respectivamente, en las áreas de peri-infarto, 4 meses tras el implante de medio (grupo control), membrana, ADSC o membrana celularizada con ADSC. Imágenes representativas de las inmunofluorescencias para caveolina-1 (verde) **(A)** y αSMA (rojo) **(B)**, marcaje nuclear con Dapi (azul). Se determinó un aumento significativo en el número de vasos de pequeño (*p<0,05) y gran calibre (**p<0,01) en el grupo implantado con la membrana celularizada en comparación con el grupo control. Abreviaturas: Co: control; Mb: membrana. Barra de escala: 50 µm.

En resumen, el tratamiento del infarto de miocardio con membranas de colágeno celularizadas con ADSC ejerce un efecto positivo sobre el mismo y superior a la inyección subepicárdica de las células, induciendo una mejora de la función cardiaca y un efecto positivo a nivel de remodelado tisular y revascularización del tejido.

6. DISCUSIÓN

Numerosos estudios tanto experimentales (revisado en [256, 257]) como clínicos (revisado en [221, 258, 259]) han mostrado el efecto beneficioso que la terapia celular ejerce como tratamiento de diversas enfermedades, entre ellas, el infarto de miocardio. Sin embargo, dicha terapia presenta todavía una serie de limitaciones importantes, como el bajo grado de injerto celular y supervivencia en el tejido, que disminuyen la efectividad de la misma. Además, se ha observado una correlación positiva entre el número de células implantadas y el beneficio ejercido por éstas [260, 261], lo cual confirma la necesidad de desarrollar nuevas estrategias que favorezcan el injerto celular.

Con este último objetivo, hemos combinado la terapia celular con la bioingeniería, creando así membranas celularizadas que pudieran facilitar la implantación de las células en el tejido cardiaco. Su beneficio terapéutico ha sido analizado en un modelo de infarto de miocardio crónico en rata, ya que, a pesar del interés que dicha terapia también puede ejercer al ser aplicada en el infarto agudo, existe un número creciente de pacientes crónicos que requieren de un tratamiento alternativo al ofrecido hasta el momento.

En este estudio, la población celular de elección ha sido el de las células madre derivadas del tejido adiposo o ADSC. Estudios comparativos realizados en nuestro laboratorio con distintas poblaciones celulares derivadas tanto de la MO (MNC, MAPC, MSC) como del tejido adiposo (SVF, ADSC y AD-CMG (CM derivados de la SVF mediante diferenciación *in vitro*), han mostrado el gran potencial terapéutico de las células mesenquimales con respecto a otras poblaciones celulares. Así por ejemplo, de entre las distintas poblaciones derivadas de la MO estudiadas, no se ha observado una mejora funcional en los animales tratados con MAPC tras un mes de su inyección (aunque sí se observó su efecto protector a nivel del remodelado tisular) [145], y cuando se ha analizado el potencial de las células MNC frente a las MSC, se ha comprobado que a pesar de que ambas inducen un beneficio funcional, la población MSC ejerce un efecto más potente, al inducir además un efecto positivo sobre el remodelado, asociado a un incremento del metabolismo tisular [110]. Por otro lado, al

comparar el potencial regenerador de las células MNC-MO con la población mesenquimal, en este caso derivada del tejido adiposo (ADSC), sólo se ha observado una mejora funcional en el grupo tratado con las ADSC, detectándose un incremento del metabolismo tisular cardiaco así como una disminución en el grado de fibrosis tisular y un incremento de la angiogénesis en el tejido cardiaco [183]. Además, al estudiar comparativamente las poblaciones mesenquimales derivadas de la MO o del tejido adiposo, se ha mostrado un mayor efecto angiogénico en el caso de las ADSC [181]. Por último, no se ha observado un efecto positivo tras el trasplante de la población cardiomiogénica derivada del tejido adiposo (AD-CMG), probablemente debido a su baja capacidad de supervivencia en el tejido isquémico, consecuencia de su alta sensibilidad a la hipoxia [183].

Todos estos resultados previos indican el beneficio terapéutico de las ADSC, lo cual, unido a la fácil obtención y manipulación de las células e incluso a su posible aplicación alogénica, podría facilitar su utilización como agente terapéutico en la clínica. En este estudio, además de confirmar dicho beneficio, hemos conseguido potenciar significativamente su efecto, gracias a la utilización de membranas de colágeno, las cuales hemos demostrado son compatibles tanto con las células ADSC como con el tejido cardiaco. Dicha biocompatibilidad se ve favorecida por la composición de la membrana, colágeno de tipo I, que constituye el componente más abundante de la matriz extracelular del corazón y que se ha demostrado facilita la adhesión celular, aporta estabilidad mecánica y presenta una baja inmunogenicidad y un excelente grado de biodegradación [262]. Debido a estas propiedades, el colágeno ha sido utilizado en diversos estudios relacionados con las enfermedades cardiovasculares [225, 233, 263-266]. Así por ejemplo, se han inyectado distintos tipos celulares (células mesenquimales o cardiomiocitos) en combinación con colágeno líquido, obteniendo un mayor injerto de este modo que al utilizar sólo medio de cultivo como vehículo de inyección. Sin embargo, a pesar de la ventaja de este método, no se logró evitar la distribución de las células hacia otros tejidos [265]. Por ello, en nuestro estudio se ha utilizado un soporte de colágeno sólido en lugar del

biomaterial líquido que sí ha evitado dicha migración (datos no mostrados), favoreciendo también una distribución más homogénea de las células. Además, como ya se mencionaba anteriormente, mediante la utilización de la membrana, se ha conseguido incrementar la supervivencia de las células trasplantadas *in vivo*, al evitar, gracias a su previa adhesión a un sustrato, la inducción de su muerte por anoikis (revisado en [267]).

En este estudio, se ha comprobado la biocompatibilidad de la membrana con las células, ya que éstas presentaban una correcta adhesión y proliferación sobre la misma. Además, la biocompatibilidad fue similar en los cuatro tipos de membrana, confirmando, tal y como se ha descrito previamente [266, 268], que el agente utilizado para entrecruzar las membranas de colágeno (EDC) no ejerce toxicidad sobre las células. Dicho agente entrecruza moléculas de colágeno mediante la formación de isopéptidos, impidiendo la despolimerización y la posible liberación de reactivos potencialmente citotóxicos [269]. Además, el subproducto que se forma en la reacción de entrecruzamiento es la urea que no ejerce citotoxicidad [270, 271] y puede eliminarse fácilmente en la rutina de lavado de las matrices. Sin embargo, debe considerarse que una vez que las membranas son implantadas *in vivo*, el biomaterial puede ejercer una respuesta pro-inflamatoria de cuerpo extraño en el tejido [272, 273]. Se ha demostrado que tras el implante, se genera una matriz provisional alrededor del biomaterial, en la que se acumulan mitógenos, citoquinas y factores de crecimiento que pueden favorecer la actividad de los macrófagos, que a su vez modulan a otras poblaciones celulares que participan en la respuesta inmunológica [274]. En el estudio piloto *in vivo* en el cual se implantaron los cuatro tipos de membranas de colágeno, además de la inflamación generada por la propia intervención y la sutura de las matrices al corazón, se observó un mayor grado de inflamación en los corazones implantados con las membranas de colágeno entrecruzado que con la de colágeno no entrecruzado. Considerando que, la técnica de cuantificación de inflamación empleada presenta ciertas limitaciones, aunque ha sido previamente utilizada en otros estudios [146, 254, 255], y que, por otro lado, no ha

sido posible realizar un marcaje fiable para células inflamatorias $CD45^+$ en rata, sería interesante, en futuros estudios, confirmar dichos resultados mediante la determinación, en muestras de suero, de marcadores de inflamación tales como la proteína C reactiva o la proteína sérica amiloide A [275].

Cabe destacar que, además de la baja respuesta inflamatoria observada con la membrana no entrecruzada, se confirmaron otros aspectos importantes, tales como su rápida adherencia y degradación *in vivo*. En este sentido se observó una menor adherencia y degradación en las membranas con entrecruzamiento, tal y como se ha descrito en otros estudios [268]. El hecho de que las membranas con entrecruzamiento estén presentes en el tejido durante un periodo más prolongado y de que no se integren en el mismo, también podría inducir la respuesta inflamatoria hacia las mismas [276].

Por otro lado, en este estudio, se ha comprobado que el tratamiento con la membrana de colágeno celularizada con ADSC induce una mejora de la función cardiaca, que además está asociada a un remodelado tisular positivo. Debe destacarse también que tras el tratamiento con la matriz celularizada, el comportamiento mecánico de los corazones se asemejaba a un corazón sano, beneficio que no se obtuvo al implantar las ADSC sin soporte o la membrana sin celularizar. Hay que considerar que pese a que el potencial regenerativo cardiovascular de las ADSC ya ha sido demostrado en otros trabajos [181, 182, 265], incluyendo los realizados por nuestro propio grupo [183], en este estudio no se ha detectado beneficio alguno en los animales tratados con las ADSC sin membrana, esto es debido al bajo número de células inyectado (350.000 células) en comparación con los trabajos anteriores. En cambio, cuando el mismo número de células fue implantado sobre una matriz de colágeno, éstas sí fueron capaces de generar dicho beneficio, con un incremento de la fracción de eyección de alrededor del 50%, valor similar al que se determinó cuando se trasplantaron 10 millones de SVF mediante inyección directa [174]. También se detectó una mejora similar cuando los corazones fueron tratados con un número mayor de células ADSC (1 millón) [183], aunque en ese caso dicha mejora fue

detectada un mes después del tratamiento, y su efecto a largo plazo no fue determinado. Puede concluirse por tanto, que el implante de ADSC adheridas a un soporte de colágeno, prolonga su acción beneficiosa, destacando el hecho de que tal efecto se consigue obtener con un número de células significativamente menor, lo cual facilitaría su posible uso en la clínica.

Además, la membrana utilizada en este estudio presenta una serie de propiedades favorables tales como su biocompatibilidad, biodegradación, correcta adhesión e integración en el tejido y moderado rechazo inmunológico, que la hace óptima para su futura utilización clínica. Por otro lado, también es importante destacar que la mejora funcional observada tras el implante de la membrana celularizada con ADSC se determinó cuatro meses después del implante, demostrando el beneficio del tratamiento a largo plazo. Este aspecto es de gran relevancia ya que la mayoría de los estudios experimentales descritos hasta el momento han analizado el potencial regenerativo de la terapia celular en periodos más cortos de tiempo (uno o dos meses post-implante como máximo) y es importante conocer, de cara a considerar su posible aplicación en el paciente, si el tratamiento aplicado ejerce un beneficio sostenido. Cabe destacar también, que aunque no se ha observado injerto celular a tan largo plazo (cuatro meses post-implante), sí se ha conseguido prolongar en gran medida la permanencia de las células en el tejido (al menos un mes tras su implante), ya que cuando las células ADSC se inyectaron subepicárdicamente, no se detectaron siquiera una semana tras su implante. También, estudios previos realizados por nuestro grupo en los que se inyectó de forma directa un mayor número de células ADSC (1 millón) en el mismo modelo animal, mostraron que dichas células no fueron capaces de sobrevivir más allá de dos semanas. Tampoco en otros estudios en los que se inyectaron células mesenquimales (1 millón) embebidas en colágeno líquido, se detectaron células injertadas tras un mes de su implante [233, 265], lo que demuestra la ventaja de utilizar una membrana sólida como soporte.

Por otro lado, hay que considerar que aunque la supervivencia celular se ve dificultada por el ambiente hipóxico y fibrótico del corazón [183], la expresión de

transgenes exógenos como la proteína GFP puedan provocar una respuesta inmunológica, acelerando la desaparición de las células en el tejido [277]. Para evitar este problema, se han utilizado inmunosupresores tales como la ciclosporina. En este estudio, sin embargo, no hemos empleado dicho fármaco ya que se han descrito efectos secundarios sobre la contractilidad cardiaca que podrían afectar a los resultados del estudio [278]. Además, hemos comprobado en estudios anteriores que la ciclosporina no parece ejercer una acción inmunosupresora completa puesto que no evita la acción de las células del linaje monocito/macrófago responsables de la fagocitosis de las células trasplantadas [110]. Finalmente, puesto que en este estudio se ha trabajado con un modelo alogénico, es interesante determinar el potencial de las células mesenquimales sin un tratamiento de inmunosupresión para analizar su posible potencial clínico y dilucidar así su capacidad inmunomoduladora. Sería interesante, ahora que se ha determinado el efecto beneficioso de las ADSC, estudiar en mayor profundidad las respuestas inmunológicas desencadenadas por las mismas y realizar un análisis comparativo de su potencial en un trasplante autólogo.

Por otro lado, profundizando en los mecanismos de acción responsables del remodelado positivo del corazón e inducción de la revascularización tisular observada, cabe sugerir que éstos sean principalmente de tipo paracrino. En este sentido, se ha descrito en numerosos trabajos la implicación de las células madre mesenquimales en procesos de angiogénesis y arteriogénesis, relacionados con la liberación de factores pro-angiogénicos y pro-arteriogénicos como las angiopoietinas, VEGF, bFGF y HGF [129, 279], implicados a su vez en la mejora funcional cardiaca tras la isquemia [110, 280]. En concreto, se ha demostrado que las ADSC secretan factores de actividad pro-angiogénica tales como VEGF, HGF e IGF-1 [169, 281, 282] e incluso se ha observado un mayor efecto pro-angiogénico de la población ADSC en comparación con la población MSC [181, 182]. Además, la liberación de estas citoquinas pro-angiogénicas por parte de las células ADSC puede verse favorecida en el ambiente isquémico y fibrótico, mediante la estimulación generada por la hipoxia [169], TNFα [282] o bFGF entre otros [170]. También se ha demostrado que las ADSC pueden movilizar células

endoteliales y de músculo liso [283] hacia el tejido isquémico, inhibir la apoptosis de las células endoteliales y favorecer su proliferación [169].

Otro factor importante relacionado con el beneficio funcional es la inducción de la contractilidad cardiaca, tal y como se ha descrito en un modelo de conejo**,** en el que la inyección de MSC mejoraba la función hemodinámica al inducir la disminución de la expresión de los β-adrenoreceptores y el consiguiente aumento de la contractilidad de los CM [284]. Finalmente, respecto al remodelado tisular, se ha descrito una disminución de la fibrosis tisular como consecuencia del trasplante de las ADSC, efecto que se ha observado también en estudios similares realizados con MSC [131, 261, 285, 286]. Dicha disminución puede deberse a que las células mesenquimales favorecen la degradación de la matriz extracelular mediante la inducción de la secreción de MMPs e inhibición de la expresión del colágeno por parte de los fibroblastos cardiacos, cuya proliferación también inhiben [131, 261]. Otra acción mediada por la población mesenquimal en relación con la fibrosis, es la atenuación del aumento de la expresión cardiaca del colágeno de tipo I y III, de TIMP1 y TGF-β en el IM [132, 287]. También se ha descrito su efecto en la inhibición de la inflamación local debido a la disminución de la expresión de citoquinas pro-inflamatorias tales como TNFα, IL-1α e IL-6, implicadas todas ellas en el remodelado ventricular [132]. Además, se ha descrito la importancia de los componentes de la matriz extracelular cardiaca en relación con el comportamiento mecánico del corazón y de su remodelado. Así por ejemplo, se ha comprobado que la trombospondina-2 (TSP-2) se expresa durante el proceso de maduración de la cicatriz, actuando a su vez, en la regulación de la actividad MMP2 [288]. Otra proteína de esa familia, la TSP-1, es liberada por células inflamatorias en el momento agudo del IM e induce la expresión de TGF-β1, previniendo la expansión del tejido isquémico [289]. También se ha estudiado el papel de la familia de las SPARC (*Secreted Protein Acidic and Rich Cysteine*) en el remodelado cardiaco, describiéndose la participación de esta proteína en el ensamblaje y morfología de las fibrillas de colágeno [290]. Además, SPARC favorece la actividad de TGF-β1 y protege al corazón de la dilatación y su posterior disfunción ventricular [291]. Sería interesante analizar en

futuros estudios el papel que dichas proteínas pueden desempeñar en el remodelado tisular así como la posible implicación de las ADSC en la actividad de las mismas.

Por otro lado, pese a que el miocardio está compuesto por diversos componentes de la matriz extracelular tales como el colágeno, la elastina y los proteoglicanos, prácticamente todos los estudios mecánicos se han centrado en la implicación del colágeno, por ser éste el más abundante [292]. Además, el colágeno es un componente con una alta fuerza de extensión y pequeños cambios en su concentración y disposición pueden alterar el comportamiento mecánico del corazón. Como ya se ha comentado, en este estudio se ha observado que el tejido cardiaco de los animales tratados con la membrana celularizada con ADSC presenta un comportamiento mecánico similar al del tejido cardiaco sano (menos rígido), hecho que no se ha observado en el grupo tratado con las ADSC inyectadas sin soporte, en el que se ha detectado un comportamiento mecánico similar al del tejido cardiaco infartado no tratado (más rígido). Tampoco se ha observado una mejora en el comportamiento mecánico de los corazones tratados con la membrana sin celularizar. En este sentido, una mayor concentración de colágeno de tipo I, un mayor diámetro o un mayor grado de entrecruzamiento de las fibras de colágeno, podrían ser los causantes de la rigidez del tejido cardiaco (revisado en [292, 293]). Sería interesante realizar un análisis más exhaustivo de las modificaciones que han tenido lugar en el tejido cardiaco a nivel de la matriz extracelular, para determinar las posibles modificaciones de concentración y disposición del colágeno en la misma tras el tratamiento con la membrana de colágeno celularizada con ADSC.

En cualquier caso, en vista de todos los resultados obtenidos, podemos concluir que las células ADSC, implantadas sobre la matriz de colágeno, ejercen en un modelo crónico de IM en rata, un efecto beneficioso a largo plazo, a nivel de función, remodelado y comportamiento mecánico.

Estos estudios presentan un gran interés de cara a su potencial aplicación clínica, aunque obviamente, es necesario confirmar previamente su beneficio en un modelo pre-clínico como es el cerdo. Hasta el momento, se han realizado varios

ensayos pre-clínicos en los que se ha determinado el potencial de las ADSC inyectadas intramiocárdicamente. Valina y colaboradores inyectaron ADSC o MSC en un modelo de IM agudo en cerdo, observando una mejora funcional del corazón tratado con ambas poblaciones un mes post-implante. Pese a no encontrar diferencias significativas entre los dos grupos en relación a la función ventricular, sólo en el grupo implantado con ADSC se detectó un incremento de la densidad capilar y un aumento en el grosor de la pared ventricular [182]. Por otro lado, nuestro grupo ha realizado un estudio en un modelo de isquemia-reperfusión en cerdo con seguimiento a tres meses en el cual se ha observado una mejora funcional relacionada con un remodelado ventricular positivo y revascularización del tejido, como consecuencia del trasplante de las ADSC (datos no publicados, ver Anexo II). En relación con los resultados positivos obtenidos en estos modelos pre-clínicos tras el trasplante de las ADSC, puede esperarse que dichos resultados también se vean potenciados por el uso de un soporte de colágeno tal y como se ha observado en el modelo en rata. Siguiendo en esta línea, en nuestro laboratorio ya se ha comenzado un estudio en cerdo y se considera factible que, de obtenerse también resultados positivos, pudiera trasladarse su uso a la clínica. Cabe destacar que, hasta el momento, no se han realizado ensayos clínicos con ADSC pero sí con células mesenquimales derivadas de la MO, con las que se ha demostrado la seguridad de su trasplante [209, 294, 295]. Los ensayos fase II/III confirmarán su potencial terapéutico aunque los resultados obtenidos hasta el momento en los ensayos previos sugieren dicho beneficio. Por otro lado, tan sólo se ha publicado un ensayo clínico en el que se han combinado células madre con algún tipo de biomaterial, en este caso, una membrana de colágeno celularizada con 100 millones células mononucleadas derivadas de la MO. En este ensayo fase I, denominado como MAGNUM, se reclutaron 20 pacientes con IM crónico que fueron trasplantados con la membrana celularizada o con las células sin dicho soporte. Los resultados han mostrado la factibilidad y seguridad del procedimiento [236]. Además, se observó un remodelado ventricular positivo y un aumento de la función diastólica en los pacientes tratados con la membrana celularizada.

Por otro lado, en vista de todos estos resultados, deben plantearse nuevas estrategias terapéuticas que consigan mejorar en mayor medida la efectividad de la terapia celular. Además, como ya se ha comentado a lo largo del trabajo, existen distintas poblaciones celulares que ejercen su potencial terapéutico a través de diversos mecanismos. Como consecuencia de ello, es necesario establecer cuál es la función específica que ha de ejercer la terapia celular en el tratamiento de la enfermedad y elegir la población celular que mejor se ajuste a dicho objetivo. Este esfuerzo debería centrarse fundamentalmente en dos aspectos que se comentan a continuación.

En primer lugar, se ha comprobado que el potencial terapéutico que ejercen la mayoría de las células madre adultas es de tipo paracrino y que su contribución mediante la diferenciación es muy baja o prácticamente inexistente. Sin embargo, existen otras poblaciones celulares como las ESC, las recientemente descubiertas iPS o los progenitores cardiacos, que han demostrado tener un mayor potencial de diferenciación cardiovascular. Por ello, cuando se plantea la protección/regeneración del tejido isquémico hay que tener en cuenta las distintas propiedades que caracterizan a cada población celular. En el caso de querer ejercer un efecto positivo en el remodelado tisular tras la isquemia cardiaca, podrían aplicarse las células mesenquimales, teniendo en cuenta su potencial paracrino y sus supuestas propiedades inmunomoduladoras. En cambio, para la regeneración del músculo cardiaco, serían necesarias poblaciones celulares capaces de derivar hacia células cardiacas, aunque las poblaciones descritas hasta el momento presentan ciertas limitaciones. En el caso de las células madre cardiacas, es necesaria una caracterización más detallada de las mismas que permita optimizar su purificación a partir del tejido cardiaco humano. En cuanto a las ESC, existen barreras éticas y de inmunogenicidad que limitan su uso, así como de tumorogenicidad, barrera que las iPS también comparten. El desarrollo de nuevos protocolos de purificación de células [54] previamente diferenciadas *in vitro* podría solventar este último problema, aunque serían necesarios controles muy estrictos para su posterior aplicación en la clínica.

También existe la posibilidad de la obtención directa de CM a partir de células somáticas mediante la sobre-expresión de distintos factores [75], aunque dichos protocolos todavía requieren ser optimizados.

El segundo aspecto a tener en cuenta de cara a incrementar el potencial de la terapia celular, es el modo de administración de las células en el implante. En los estudios realizados hasta el momento, se han utilizado sobre todo vías de administración directas como la inyección intravenosa, intramuscular o intracoronaria. En la terapia con MSC, se ha observado que en la inyección intravenosa el número de células que injertan en el corazón es muy bajo, distribuyéndose además, hacia otros órganos como el pulmón, hígado o riñón [265, 296]. La inyección intramuscular de MSC se ha descrito como la más eficaz, aunque también está limitada por el bajo número de células que sobreviven e injertan en el tejido cardiaco. Además, esta vía de administración es adecuada para pacientes con IM crónico pero no para pacientes con IM agudo, en el que la vía intracoronaria es la que se utiliza habitualmente, a pesar de que existe un cierto riesgo de formación de trombos debido al gran tamaño de las células implantadas. Una alternativa a la administración celular clásica y que favorece la supervivencia e injerto celular tras el trasplante, es precisamente la utilización de biomatrices, que como hemos demostrado en este trabajo, permiten la interacción de las señales de supervivencia célula-célula y una distribución más homogénea de las mismas.

Por otro lado, de cara a favorecer la aplicación alogénica de las células, incluso en el caso de las células mesenquimales cuya capacidad inmunomoduladora no parece tan evidente *in vivo* [139-141], se han desarrollado estrategias alternativas para su administración y protección frente al sistema inmune, como son la encapsulación de las células o la utilización de hidrogeles (revisado en [297]). Además, los avances conseguidos en la micro y nanotecnología están permitiendo potenciar el efecto terapéutico conseguido con los parches celularizados. Por ejemplo, se han desarrollado nuevas estrategias como la creación de parches de colágeno embebidos con el anticuerpo Sca-1 que favorecen el reclutamiento de células progenitoras [264],

o con el factor de crecimiento VEGF-165, para generar la vascularización del parche *in vivo* [263]. Estos parches se han combinado además con células endoteliales para incrementar su vascularización [243, 244]. También, se han creado construcciones celulares más complejas combinando distintas poblaciones que sustenten las diversas necesidades de la regeneración cardiaca. El grupo del Dr. Zimmermann ha creado parches tisulares con propiedades cardiacas y con capacidad contráctil *in vivo,* utilizando una matriz compuesta por colágeno y matrigel y células cardiacas de distinto origen [238, 239]. En esta línea, se está investigando la implicación de las propiedades mecánicas de los materiales que se utilizan como soporte en la diferenciación celular (revisado en [298]). Estos nuevos conocimientos permitirían dirigir la diferenciación celular hacia el fenotipo de interés para el tratamiento. Así por ejemplo, se ha descrito recientemente la generación de construcciones tisulares que simulan las propiedades mecánicas y estructurales del miocardio, induciendo la diferenciación de las MSC hacia distintos linajes celulares, la cual se ve favorecida cuando la distribución celular es tridimensional [299].

En vista de todos estos avances y resultados, podemos concluir que la terapia celular presenta un gran potencial como tratamiento de la enfermedad cardiovascular y que, desarrollada en combinación con la bioingeniería, la micro y nanotecnología, puede generar alternativas más eficaces para la protección y regeneración del tejido cardiaco.

7. CONCLUSIONES

Las conclusiones obtenidas en el presente trabajo son las siguientes:

1. Las membranas de colágeno de tipo I (406, #407, #408 y #409) son, independientemente de su grado de entrecruzamiento, biocompatibles con las células madre derivadas del tejido adiposo (ADSC), ya que permiten la adhesión uniforme de las células a su superficie, sobre la que se mantienen viables.

2. Las ADSC presentan, además, una correcta capacidad de proliferación sobre los cuatro tipos de membranas. Dicho aspecto confirma la biocompatibilidad de las matrices con las células.

3. A nivel de comportamiento mecánico, el grado de rigidez de la membrana sin entrecruzamiento (#406) es significativamente menor que el de las membranas con entrecruzamiento (#407, #408 y #409). Además, dicha rigidez disminuye cuando la membrana es celularizada con ADSC. Por otro lado, se ha detectado el marcado carácter anisotrópico de dicha membrana.

4. *In vivo*, el trasplante de las membranas de colágeno sobre el corazón infartado induce una moderada inflamación en el tejido. El trasplante de la membrana #406 provoca una reacción inflamatoria significativamente menor que el resto de las membranas, que es prácticamente inexistente tras el mes de su implante. Además, dicha membrana presenta una rápida adhesión y reabsorción en el corazón, no observada con las membranas entrecruzadas.

5. El trasplante de ADSC en un soporte biocompatible de colágeno (membrana #406) induce, a largo plazo (cuatro meses), una mejora significativa de la función ventricular en un modelo de infarto crónico de miocardio en rata. Dicha mejora no se detecta, sin embargo, al implantar las células sin soporte o al trasplantar la membrana no celularizada.

6. El tratamiento del corazón infartado con la membrana celularizada con ADSC induce, a diferencia de las células sin soporte o de la membrana no celularizada, una mejora significativa del comportamiento mecánico del corazón, así como una disminución significativa del grado de fibrosis tisular.

7. El trasplante de la membrana celularizada con ADSC induce de forma significativa la revascularización, tanto a nivel angiogénico como arteriogénico, de las áreas de peri-infarto del tejido cardiaco. En cambio, dicha efecto no se produce al trasplantar las células sin soporte o la membrana no celularizada.

8. El trasplante de las células ADSC previamente adheridas a un soporte favorece una mayor supervivencia y distribución más homogénea de las mismas en el tejido, ejerciendo así una acción trófica más potente y prolongada en el tiempo y por tanto, un mayor efecto terapéutico de las mismas.

8. BIBLIOGRAFÍA

1. Roger, V.L., et al., *Heart disease and stroke statistics--2011 update: a report from the American Heart Association.* Circulation, 2011. **123**(4): p. e18-e209.
2. Libby, P., P.M. Ridker, and G.K. Hansson, *Progress and challenges in translating the biology of atherosclerosis.* Nature, 2011. **473**(7347): p. 317-25.
3. Jennings, R.B., et al., *Development of cell injury in sustained acute ischemia.* Circulation, 1990. **82**(3 Suppl): p. II2-12.
4. Rossen, R.D., et al., *Mechanism of complement activation after coronary artery occlusion: evidence that myocardial ischemia in dogs causes release of constituents of myocardial subcellular origin that complex with human C1q in vivo.* Circ Res, 1988. **62**(3): p. 572-84.
5. Deten, A., et al., *Cardiac cytokine expression is upregulated in the acute phase after myocardial infarction. Experimental studies in rats.* Cardiovasc Res, 2002. **55**(2): p. 329-40.
6. Frangogiannis, N.G., C.W. Smith, and M.L. Entman, *The inflammatory response in myocardial infarction.* Cardiovasc Res, 2002. **53**(1): p. 31-47.
7. Mann, D.L., *Mechanisms and models in heart failure: A combinatorial approach.* Circulation, 1999. **100**(9): p. 999-1008.
8. Heusch, G., R. Schulz, and S.H. Rahimtoola, *Myocardial hibernation: a delicate balance.* Am J Physiol Heart Circ Physiol, 2005. **288**(3): p. H984-99.
9. Cleutjens, J.P., et al., *Regulation of collagen degradation in the rat myocardium after infarction.* J Mol Cell Cardiol, 1995. **27**(6): p. 1281-92.
10. Ertl, G. and S. Frantz, *Healing after myocardial infarction.* Cardiovasc Res, 2005. **66**(1): p. 22-32.
11. Nian, M., et al., *Inflammatory cytokines and postmyocardial infarction remodeling.* Circ Res, 2004. **94**(12): p. 1543-53.
12. Nahrendorf, M., et al., *The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions.* J Exp Med, 2007. **204**(12): p. 3037-47.
13. van Amerongen, M.J., et al., *Bone marrow-derived myofibroblasts contribute functionally to scar formation after myocardial infarction.* J Pathol, 2008. **214**(3): p. 377-86.
14. Bujak, M. and N.G. Frangogiannis, *The role of TGF-beta signaling in myocardial infarction and cardiac remodeling.* Cardiovasc Res, 2007. **74**(2): p. 184-95.
15. Frangogiannis, N.G., et al., *IL-10 is induced in the reperfused myocardium and may modulate the reaction to injury.* J Immunol, 2000. **165**(5): p. 2798-808.
16. Sun, Y., et al., *Infarct scar as living tissue.* Basic Res Cardiol, 2002. **97**(5): p. 343-7.
17. Beltrami, A.P., et al., *Evidence that human cardiac myocytes divide after myocardial infarction.* N Engl J Med, 2001. **344**(23): p. 1750-7.
18. Pasumarthi, K.B. and L.J. Field, *Cardiomyocyte cell cycle regulation.* Circ Res, 2002. **90**(10): p. 1044-54.
19. Beltrami, A.P., et al., *Adult cardiac stem cells are multipotent and support myocardial regeneration.* Cell, 2003. **114**(6): p. 763-76.
20. Mann, D.L., *Stress-activated cytokines and the heart: from adaptation to maladaptation.* Annu Rev Physiol, 2003. **65**: p. 81-101.
21. *Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). The CONSENSUS Trial Study Group.* N Engl J Med, 1987. **316**(23): p. 1429-35.

22. *Effect of ramipril on mortality and morbidity of survivors of acute myocardial infarction with clinical evidence of heart failure. The Acute Infarction Ramipril Efficacy (AIRE) Study Investigators.* Lancet, 1993. **342**(8875): p. 821-8.
23. Pfeffer, M.A., et al., *Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction. Results of the survival and ventricular enlargement trial. The SAVE Investigators.* N Engl J Med, 1992. **327**(10): p. 669-77.
24. Ambrosioni, E., C. Borghi, and B. Magnani, *The effect of the angiotensin-converting-enzyme inhibitor zofenopril on mortality and morbidity after anterior myocardial infarction. The Survival of Myocardial Infarction Long-Term Evaluation (SMILE) Study Investigators.* N Engl J Med, 1995. **332**(2): p. 80-5.
25. Torp-Pedersen, C. and L. Kober, *Effect of ACE inhibitor trandolapril on life expectancy of patients with reduced left-ventricular function after acute myocardial infarction. TRACE Study Group. Trandolapril Cardiac Evaluation.* Lancet, 1999. **354**(9172): p. 9-12.
26. Dickstein, K. and J. Kjekshus, *Effects of losartan and captopril on mortality and morbidity in high-risk patients after acute myocardial infarction: the OPTIMAAL randomised trial. Optimal Trial in Myocardial Infarction with Angiotensin II Antagonist Losartan.* Lancet, 2002. **360**(9335): p. 752-60.
27. Pfeffer, M.A., et al., *Valsartan, captopril, or both in myocardial infarction complicated by heart failure, left ventricular dysfunction, or both.* N Engl J Med, 2003. **349**(20): p. 1893-906.
28. Pitt, B., et al., *Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction.* N Engl J Med, 2003. **348**(14): p. 1309-21.
29. Hayashi, M., et al., *Immediate administration of mineralocorticoid receptor antagonist spironolactone prevents post-infarct left ventricular remodeling associated with suppression of a marker of myocardial collagen synthesis in patients with first anterior acute myocardial infarction.* Circulation, 2003. **107**(20): p. 2559-65.
30. Bristow, M.R., *beta-adrenergic receptor blockade in chronic heart failure.* Circulation, 2000. **101**(5): p. 558-69.
31. Tiyyagura, S.R. and S.P. Pinney, *Left ventricular remodeling after myocardial infarction: past, present, and future.* Mt Sinai J Med, 2006. **73**(6): p. 840-51.
32. Simons, M. and J.A. Ware, *Therapeutic angiogenesis in cardiovascular disease.* Nat Rev Drug Discov, 2003. **2**(11): p. 863-71.
33. Lopez, J.J., et al., *VEGF administration in chronic myocardial ischemia in pigs.* Cardiovasc Res, 1998. **40**(2): p. 272-81.
34. Hao, X., et al., *Angiogenic and cardiac functional effects of dual gene transfer of VEGF-A165 and PDGF-BB after myocardial infarction.* Biochem Biophys Res Commun, 2004. **322**(1): p. 292-6.
35. Yla-Herttuala, S., J.E. Markkanen, and T.T. Rissanen, *Gene therapy for ischemic cardiovascular diseases: some lessons learned from the first clinical trials.* Trends Cardiovasc Med, 2004. **14**(8): p. 295-300.
36. Zaiss, A.K. and D.A. Muruve, *Immune responses to adeno-associated virus vectors.* Curr Gene Ther, 2005. **5**(3): p. 323-31.
37. Formiga, F.R., et al., *Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia-reperfusion model.* J Control Release, 2010. **147**(1): p. 30-7.

38. Chung, Y.I., et al., *Efficient revascularization by VEGF administration via heparin-functionalized nanoparticle-fibrin complex.* J Control Release, 2010. **143**(3): p. 282-9.
39. Scott, R.C., et al., *Targeting VEGF-encapsulated immunoliposomes to MI heart improves vascularity and cardiac function.* FASEB J, 2009. **23**(10): p. 3361-7.
40. Matsusaki, M., et al., *Controlled release of vascular endothelial growth factor from alginate hydrogels nano-coated with polyelectrolyte multilayer films.* J Biomater Sci Polym Ed, 2007. **18**(6): p. 775-83.
41. Freeman, I. and S. Cohen, *The influence of the sequential delivery of angiogenic factors from affinity-binding alginate scaffolds on vascularization.* Biomaterials, 2009. **30**(11): p. 2122-31.
42. Ruvinov, E., J. Leor, and S. Cohen, *The promotion of myocardial repair by the sequential delivery of IGF-1 and HGF from an injectable alginate biomaterial in a model of acute myocardial infarction.* Biomaterials, 2011. **32**(2): p. 565-78.
43. Laham, R.J., et al., *Local perivascular delivery of basic fibroblast growth factor in patients undergoing coronary bypass surgery: results of a phase I randomized, double-blind, placebo-controlled trial.* Circulation, 1999. **100**(18): p. 1865-71.
44. Thomson, J.A., et al., *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.
45. Doetschman, T.C., et al., *The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium.* J Embryol Exp Morphol, 1985. **87**: p. 27-45.
46. Kehat, I., et al., *Electromechanical integration of cardiomyocytes derived from human embryonic stem cells.* Nat Biotechnol, 2004. **22**(10): p. 1282-9.
47. He, J.Q., et al., *Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization.* Circ Res, 2003. **93**(1): p. 32-9.
48. Hodgson, D.M., et al., *Stable benefit of embryonic stem cell therapy in myocardial infarction.* Am J Physiol Heart Circ Physiol, 2004. **287**(2): p. H471-9.
49. Behfar, A., et al., *Stem cell differentiation requires a paracrine pathway in the heart.* FASEB J, 2002. **16**(12): p. 1558-66.
50. van Laake, L.W., et al., *Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction.* Stem Cell Res, 2007. **1**(1): p. 9-24.
51. Behfar, A., et al., *Cardiopoietic programming of embryonic stem cells for tumor-free heart repair.* J Exp Med, 2007. **204**(2): p. 405-20.
52. Dai, W., et al., *Survival and maturation of human embryonic stem cell-derived cardiomyocytes in rat hearts.* J Mol Cell Cardiol, 2007. **43**(4): p. 504-16.
53. Laflamme, M.A., et al., *Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts.* Nat Biotechnol, 2007. **25**(9): p. 1015-24.
54. Kang, N.Y., et al., *Embryonic and induced pluripotent stem cell staining and sorting with the live-cell fluorescence imaging probe CDy1.* Nat Protoc, 2011. **6**(7): p. 1044-52.
55. Puymirat, E., et al., *Can mesenchymal stem cells induce tolerance to cotransplanted human embryonic stem cells?* Mol Ther, 2009. **17**(1): p. 176-82.
56. Taylor, C.J., et al., *Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching.* Lancet, 2005. **366**(9502): p. 2019-25.
57. Odorico, J.S., D.S. Kaufman, and J.A. Thomson, *Multilineage differentiation from human embryonic stem cell lines.* Stem Cells, 2001. **19**(3): p. 193-204.

58. Park, I.H., et al., *Reprogramming of human somatic cells to pluripotency with defined factors.* Nature, 2008. **451**(7175): p. 141-6.
59. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. **131**(5): p. 861-72.
60. Aasen, T., et al., *Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes.* Nat Biotechnol, 2008. **26**(11): p. 1276-84.
61. Yu, J., et al., *Induced pluripotent stem cell lines derived from human somatic cells.* Science, 2007. **318**(5858): p. 1917-20.
62. Masip, M., et al., *Reprogramming with defined factors: from induced pluripotency to induced transdifferentiation.* Mol Hum Reprod, 2010. **16**(11): p. 856-68.
63. Stadtfeld, M. and K. Hochedlinger, *Induced pluripotency: history, mechanisms, and applications.* Genes Dev, 2010. **24**(20): p. 2239-63.
64. Nakagawa, M., et al., *Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts.* Nat Biotechnol, 2008. **26**(1): p. 101-6.
65. Wernig, M., et al., *c-Myc is dispensable for direct reprogramming of mouse fibroblasts.* Cell Stem Cell, 2008. **2**(1): p. 10-2.
66. Okita, K., et al., *Generation of mouse induced pluripotent stem cells without viral vectors.* Science, 2008. **322**(5903): p. 949-53.
67. Kim, K., et al., *Epigenetic memory in induced pluripotent stem cells.* Nature, 2010. **467**(7313): p. 285-90.
68. Polo, J.M., et al., *Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells.* Nat Biotechnol, 2010. **28**(8): p. 848-55.
69. Martinez-Fernandez, A., et al., *iPS programmed without c-MYC yield proficient cardiogenesis for functional heart chimerism.* Circ Res, 2009. **105**(7): p. 648-56.
70. Martinez-Fernandez, A., et al., *c-MYC independent nuclear reprogramming favors cardiogenic potential of induced pluripotent stem cells.* J Cardiovasc Transl Res. **3**(1): p. 13-23.
71. Mauritz, C., et al., *Generation of functional murine cardiac myocytes from induced pluripotent stem cells.* Circulation, 2008. **118**(5): p. 507-17.
72. Zwi, L., et al., *Cardiomyocyte differentiation of human induced pluripotent stem cells.* Circulation, 2009. **120**(15): p. 1513-23.
73. Zhang, J., et al., *Functional cardiomyocytes derived from human induced pluripotent stem cells.* Circ Res, 2009. **104**(4): p. e30-41.
74. Takeuchi, J.K. and B.G. Bruneau, *Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors.* Nature, 2009. **459**(7247): p. 708-11.
75. Ieda, M., et al., *Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors.* Cell, 2010. **142**(3): p. 375-86.
76. Dawn, B., et al., *Cardiac stem cells delivered intravascularly traverse the vessel barrier, regenerate infarcted myocardium, and improve cardiac function.* Proc Natl Acad Sci U S A, 2005. **102**(10): p. 3766-71.
77. Rota, M., et al., *Local activation or implantation of cardiac progenitor cells rescues scarred infarcted myocardium improving cardiac function.* Circ Res, 2008. **103**(1): p. 107-16.
78. Oh, H., et al., *Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12313-8.

79. Martin, C.M., et al., *Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart.* Dev Biol, 2004. **265**(1): p. 262-75.
80. Pfister, O., et al., *CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation.* Circ Res, 2005. **97**(1): p. 52-61.
81. Laugwitz, K.L., et al., *Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages.* Nature, 2005. **433**(7026): p. 647-53.
82. Bu, L., et al., *Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages.* Nature, 2009. **460**(7251): p. 113-7.
83. Messina, E., et al., *Isolation and expansion of adult cardiac stem cells from human and murine heart.* Circ Res, 2004. **95**(9): p. 911-21.
84. Li, T.S., et al., *Cardiospheres recapitulate a niche-like microenvironment rich in stemness and cell-matrix interactions, rationalizing their enhanced functional potency for myocardial repair.* Stem Cells, 2010. **28**(11): p. 2088-98.
85. Lee, S.T., et al., *Intramyocardial injection of autologous cardiospheres or cardiosphere-derived cells preserves function and minimizes adverse ventricular remodeling in pigs with heart failure post-myocardial infarction.* J Am Coll Cardiol, 2011. **57**(4): p. 455-65.
86. Chimenti, I., et al., *Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice.* Circ Res, 2010. **106**(5): p. 971-80.
87. Collins, C.A., et al., *Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche.* Cell, 2005. **122**(2): p. 289-301.
88. Chiu, R.C., A. Zibaitis, and R.L. Kao, *Cellular cardiomyoplasty: myocardial regeneration with satellite cell implantation.* Ann Thorac Surg, 1995. **60**(1): p. 12-8.
89. Reinecke, H., V. Poppa, and C.E. Murry, *Skeletal muscle stem cells do not transdifferentiate into cardiomyocytes after cardiac grafting.* J Mol Cell Cardiol, 2002. **34**(2): p. 241-9.
90. Reinecke, H., et al., *Evidence for fusion between cardiac and skeletal muscle cells.* Circ Res, 2004. **94**(6): p. e56-60.
91. Reinecke, H., et al., *Electromechanical coupling between skeletal and cardiac muscle. Implications for infarct repair.* J Cell Biol, 2000. **149**(3): p. 731-40.
92. Leobon, B., et al., *Myoblasts transplanted into rat infarcted myocardium are functionally isolated from their host.* Proc Natl Acad Sci U S A, 2003. **100**(13): p. 7808-11.
93. Rubart, M., et al., *Spontaneous and evoked intracellular calcium transients in donor-derived myocytes following intracardiac myoblast transplantation.* J Clin Invest, 2004. **114**(6): p. 775-83.
94. Menasche, P., et al., *Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction.* J Am Coll Cardiol, 2003. **41**(7): p. 1078-83.
95. Jain, M., et al., *Cell therapy attenuates deleterious ventricular remodeling and improves cardiac performance after myocardial infarction.* Circulation, 2001. **103**(14): p. 1920-7.
96. Menasche, P., *Skeletal myoblast transplantation for cardiac repair.* Expert Rev Cardiovasc Ther, 2004. **2**(1): p. 21-8.
97. Perez-Ilzarbe, M., et al., *Characterization of the paracrine effects of human skeletal myoblasts transplanted in infarcted myocardium.* Eur J Heart Fail, 2008. **10**(11): p. 1065-72.

98. Winitsky, S.O., et al., *Adult murine skeletal muscle contains cells that can differentiate into beating cardiomyocytes in vitro.* PLoS Biol, 2005. **3**(4): p. e87.
99. Tamaki, T., et al., *Cardiomyocyte formation by skeletal muscle-derived multi-myogenic stem cells after transplantation into infarcted myocardium.* PLoS One, 2008. **3**(3): p. e1789.
100. Mezey, E., et al., *Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow.* Science, 2000. **290**(5497): p. 1779-82.
101. Lagasse, E., et al., *Purified hematopoietic stem cells can differentiate into hepatocytes in vivo.* Nat Med, 2000. **6**(11): p. 1229-34.
102. Krause, D.S., et al., *Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell.* Cell, 2001. **105**(3): p. 369-77.
103. Orlic, D., et al., *Bone marrow cells regenerate infarcted myocardium.* Nature, 2001. **410**(6829): p. 701-5.
104. Zhang, S., et al., *Long-term effects of bone marrow mononuclear cell transplantation on left ventricular function and remodeling in rats.* Life Sci, 2004. **74**(23): p. 2853-64.
105. Numasawa, Y., et al., *Treatment of Human Mesenchymal Stem Cells with Angiotensin Receptor Blocker Improved Efficiency of Cardiomyogenic Transdifferentiation and Improved Cardiac Function via Angiogenesis.* Stem Cells.
106. Shinmura, D., et al., *Pretreatment of human mesenchymal stem cells with pioglitazone improved efficiency of cardiomyogenic transdifferentiation and cardiac function.* Stem Cells. **29**(2): p. 357-66.
107. Rota, M., et al., *Bone marrow cells adopt the cardiomyogenic fate in vivo.* Proc Natl Acad Sci U S A, 2007. **104**(45): p. 17783-8.
108. Hatzistergos, K.E., et al., *Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation.* Circ Res. **107**(7): p. 913-22.
109. Li, T.S., et al., *The safety and feasibility of the local implantation of autologous bone marrow cells for ischemic heart disease.* J Card Surg, 2003. **18 Suppl 2**: p. S69-75.
110. Mazo, M., et al., *Transplantation of mesenchymal stem cells exerts a greater long-term effect than bone marrow mononuclear cells in a chronic myocardial infarction model in rat.* Cell Transplant, 2010. **19**(3): p. 313-28.
111. Lovell, M.J., et al., *Bone marrow mononuclear cells reduce myocardial reperfusion injury by activating the PI3K/Akt survival pathway.* Atherosclerosis, 2010. **213**(1): p. 67-76.
112. Waksman, R., et al., *Transepicardial autologous bone marrow-derived mononuclear cell therapy in a porcine model of chronically infarcted myocardium.* Cardiovasc Radiat Med, 2004. **5**(3): p. 125-31.
113. Sheu, J.J., et al., *Six-month angiographic study of immediate autologous bone marrow mononuclear cell implantation on acute anterior wall myocardial infarction using a mini-pig model.* Int Heart J, 2009. **50**(2): p. 221-34.
114. Balsam, L.B., et al., *Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium.* Nature, 2004. **428**(6983): p. 668-73.
115. Nygren, J.M., et al., *Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation.* Nat Med, 2004. **10**(5): p. 494-501.
116. Murry, C.E., et al., *Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts.* Nature, 2004. **428**(6983): p. 664-8.

117. Urbich, C. and S. Dimmeler, *Endothelial progenitor cells functional characterization.* Trends Cardiovasc Med, 2004. **14**(8): p. 318-22.
118. Rubart, M. and L.J. Field, *Cardiac regeneration: repopulating the heart.* Annu Rev Physiol, 2006. **68**: p. 29-49.
119. Young, P.P., D.E. Vaughan, and A.K. Hatzopoulos, *Biologic properties of endothelial progenitor cells and their potential for cell therapy.* Prog Cardiovasc Dis, 2007. **49**(6): p. 421-9.
120. Narmoneva, D.A., et al., *Endothelial cells promote cardiac myocyte survival and spatial reorganization: implications for cardiac regeneration.* Circulation, 2004. **110**(8): p. 962-8.
121. Kalka, C., et al., *Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3422-7.
122. Kocher, A.A., et al., *Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function.* Nat Med, 2001. **7**(4): p. 430-6.
123. Schuh, A., et al., *Transplantation of endothelial progenitor cells improves neovascularization and left ventricular function after myocardial infarction in a rat model.* Basic Res Cardiol, 2008. **103**(1): p. 69-77.
124. Cho, H.J., et al., *Role of host tissues for sustained humoral effects after endothelial progenitor cell transplantation into the ischemic heart.* J Exp Med, 2007. **204**(13): p. 3257-69.
125. Caplan, A.I. and J.E. Dennis, *Mesenchymal stem cells as trophic mediators.* J Cell Biochem, 2006. **98**(5): p. 1076-84.
126. Liu, J.W., et al., *Characterization of endothelial-like cells derived from human mesenchymal stem cells.* J Thromb Haemost, 2007. **5**(4): p. 826-34.
127. Lozito, T.P., et al., *Mesenchymal stem cell modification of endothelial matrix regulates their vascular differentiation.* J Cell Biochem, 2009. **107**(4): p. 706-13.
128. Mirotsou, M., et al., *Paracrine mechanisms of stem cell reparative and regenerative actions in the heart.* J Mol Cell Cardiol, 2011. **50**(2): p. 280-9.
129. Kinnaird, T., et al., *Bone-marrow-derived cells for enhancing collateral development: mechanisms, animal data, and initial clinical experiences.* Circ Res, 2004. **95**(4): p. 354-63.
130. Mirotsou, M., et al., *Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair.* Proc Natl Acad Sci U S A, 2007. **104**(5): p. 1643-8.
131. Ohnishi, S., et al., *Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions.* FEBS Lett, 2007. **581**(21): p. 3961-6.
132. Guo, J., et al., *Anti-inflammation role for mesenchymal stem cells transplantation in myocardial infarction.* Inflammation, 2007. **30**(3-4): p. 97-104.
133. Yagi, H., et al., *Mesenchymal stem cells: Mechanisms of immunomodulation and homing.* Cell Transplant. **19**(6): p. 667-79.
134. Le Blanc, K. and O. Ringden, *Immunomodulation by mesenchymal stem cells and clinical experience.* J Intern Med, 2007. **262**(5): p. 509-25.
135. Ren, G., et al., *Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression.* Stem Cells, 2009. **27**(8): p. 1954-62.

136. Puissant, B., et al., *Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells.* Br J Haematol, 2005. **129**(1): p. 118-29.
137. Yanez, R., et al., *Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versus-host disease.* Stem Cells, 2006. **24**(11): p. 2582-91.
138. Gonzalez, M.A., et al., *Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells.* Arthritis Rheum, 2009. **60**(4): p. 1006-19.
139. Huang, X.P., et al., *Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair.* Circulation, 2011. **122**(23): p. 2419-29.
140. Eliopoulos, N., et al., *Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice.* Blood, 2005. **106**(13): p. 4057-65.
141. Poncelet, A.J., et al., *Although pig allogeneic mesenchymal stem cells are not immunogenic in vitro, intracardiac injection elicits an immune response in vivo.* Transplantation, 2007. **83**(6): p. 783-90.
142. Jiang, Y., et al., *Pluripotency of mesenchymal stem cells derived from adult marrow.* Nature, 2002. **418**(6893): p. 41-9.
143. Zeng, L., et al., *Multipotent adult progenitor cells from swine bone marrow.* Stem Cells, 2006. **24**(11): p. 2355-66.
144. Aranguren, X.L., et al., *In vitro and in vivo arterial differentiation of human multipotent adult progenitor cells.* Blood, 2007. **109**(6): p. 2634-42.
145. Agbulut, O., et al., *Can bone marrow-derived multipotent adult progenitor cells regenerate infarcted myocardium?* Cardiovasc Res, 2006. **72**(1): p. 175-83.
146. Pelacho, B., et al., *Multipotent adult progenitor cell transplantation increases vascularity and improves left ventricular function after myocardial infarction.* J Tissue Eng Regen Med, 2007. **1**(1): p. 51-9.
147. Aranguren, X.L., et al., *Multipotent adult progenitor cells sustain function of ischemic limbs in mice.* J Clin Invest, 2008. **118**(2): p. 505-14.
148. Aranguren, X.L., et al., *MAPC transplantation confers a more durable benefit than AC133+ cell transplantation in severe hind limb ischemia.* Cell Transplant, 2011. **20**(2): p. 259-69.
149. Dimomeletis, I., et al., *Assessment of human MAPCs for stem cell transplantation and cardiac regeneration after myocardial infarction in SCID mice.* Exp Hematol, 2010. **38**(11): p. 1105-14.
150. Luttun, A., et al., *Differentiation of multipotent adult progenitor cells into functional endothelial and smooth muscle cells.* Curr Protoc Immunol, 2006. **Chapter 22**: p. Unit 22F 9.
151. Kucia, M., et al., *A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow.* Leukemia, 2006. **20**(5): p. 857-69.
152. Dawn, B., et al., *Transplantation of bone marrow-derived very small embryonic-like stem cells attenuates left ventricular dysfunction and remodeling after myocardial infarction.* Stem Cells, 2008. **26**(6): p. 1646-55.

153. Zuba-Surma, E.K., et al., *Transplantation of expanded bone marrow-derived very small embryonic-like stem cells (VSEL-SCs) improves left ventricular function and remodelling after myocardial infarction.* J Cell Mol Med, 2011. **15**(6): p. 1319-28.
154. D'Ippolito, G., et al., *Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential.* J Cell Sci, 2004. **117**(Pt 14): p. 2971-81.
155. Rahnemai-Azar, A., et al., *Human marrow-isolated adult multilineage-inducible (MIAMI) cells protect against peripheral vascular ischemia in a mouse model.* Cytotherapy. **13**(2): p. 179-92.
156. Casteilla, L., et al., *Plasticity of adipose tissue: a promising therapeutic avenue in the treatment of cardiovascular and blood diseases?* Arch Mal Coeur Vaiss, 2005. **98**(9): p. 922-6.
157. Prunet-Marcassus, B., et al., *From heterogeneity to plasticity in adipose tissues: site-specific differences.* Exp Cell Res, 2006. **312**(6): p. 727-36.
158. Zuk, P.A., et al., *Human adipose tissue is a source of multipotent stem cells.* Mol Biol Cell, 2002. **13**(12): p. 4279-95.
159. Katz, A.J., et al., *Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS) cells.* Stem Cells, 2005. **23**(3): p. 412-23.
160. Corre, J., et al., *Human subcutaneous adipose cells support complete differentiation but not self-renewal of hematopoietic progenitors.* J Cell Physiol, 2006. **208**(2): p. 282-8.
161. Rodeheffer, M.S., K. Birsoy, and J.M. Friedman, *Identification of white adipocyte progenitor cells in vivo.* Cell, 2008. **135**(2): p. 240-9.
162. Miranville, A., et al., *Improvement of postnatal neovascularization by human adipose tissue-derived stem cells.* Circulation, 2004. **110**(3): p. 349-55.
163. Chang, S.A., et al., *Impact of myocardial infarct proteins and oscillating pressure on the differentiation of mesenchymal stem cells: effect of acute myocardial infarction on stem cell differentiation.* Stem Cells, 2008. **26**(7): p. 1901-12.
164. Planat-Benard, V., et al., *Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells.* Circ Res, 2004. **94**(2): p. 223-9.
165. Sliwa, A., et al., *Differentiation of human adipose tissue SVF cells into cardiomyocytes.* Genes Nutr, 2009. **4**(3): p. 195-8.
166. Bai, X., et al., *Genetically selected stem cells from human adipose tissue express cardiac markers.* Biochem Biophys Res Commun, 2007. **353**(3): p. 665-71.
167. Di Rocco, G., et al., *Myogenic potential of adipose-tissue-derived cells.* J Cell Sci, 2006. **119**(Pt 14): p. 2945-52.
168. Han, J., et al., *Adipose tissue is an extramedullary reservoir for functional hematopoietic stem and progenitor cells.* Blood, 2010. **115**(5): p. 957-64.
169. Rehman, J., et al., *Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells.* Circulation, 2004. **109**(10): p. 1292-8.
170. Kilroy, G.E., et al., *Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors.* J Cell Physiol, 2007. **212**(3): p. 702-9.
171. Yamada, Y., et al., *Cardiac progenitor cells in brown adipose tissue repaired damaged myocardium.* Biochem Biophys Res Commun, 2006. **342**(2): p. 662-70.
172. Sumi, M., et al., *Transplantation of adipose stromal cells, but not mature adipocytes, augments ischemia-induced angiogenesis.* Life Sci, 2007. **80**(6): p. 559-65.

173. Premaratne, G.U., et al., *Stromal vascular fraction transplantation as an alternative therapy for ischemic heart failure: anti-inflammatory role.* J Cardiothorac Surg. **6**: p. 43.
174. Mazo, M., *Transplantation of Adipose derived-Stromal Vascular Fraction in a model of chronic myocardial infarction induces long-term improvement of cardiac function through differentiation and paracrine activity.* Cell Transplantation, 2011.
175. Schenke-Layland, K., et al., *Adipose tissue-derived cells improve cardiac function following myocardial infarction.* J Surg Res, 2009. **153**(2): p. 217-23.
176. Dicker, A., et al., *Functional studies of mesenchymal stem cells derived from adult human adipose tissue.* Exp Cell Res, 2005. **308**(2): p. 283-90.
177. Zuk, P.A., et al., *Multilineage cells from human adipose tissue: implications for cell-based therapies.* Tissue Eng, 2001. **7**(2): p. 211-28.
178. Lee, R.H., et al., *Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue.* Cell Physiol Biochem, 2004. **14**(4-6): p. 311-24.
179. Kern, S., et al., *Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue.* Stem Cells, 2006. **24**(5): p. 1294-301.
180. Noel, D., et al., *Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials.* Exp Cell Res, 2008. **314**(7): p. 1575-84.
181. Kim, Y., et al., *Direct comparison of human mesenchymal stem cells derived from adipose tissues and bone marrow in mediating neovascularization in response to vascular ischemia.* Cell Physiol Biochem, 2007. **20**(6): p. 867-76.
182. Valina, C., et al., *Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular function, perfusion, and remodelling after acute myocardial infarction.* Eur Heart J, 2007. **28**(21): p. 2667-77.
183. Mazo, M., et al., *Transplantation of adipose derived stromal cells is associated with functional improvement in a rat model of chronic myocardial infarction.* Eur J Heart Fail, 2008. **10**(5): p. 454-62.
184. Li, B., et al., *Adipose tissue stromal cells transplantation in rats of acute myocardial infarction.* Coron Artery Dis, 2007. **18**(3): p. 221-7.
185. Constantin, G., et al., *Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis.* Stem Cells, 2009. **27**(10): p. 2624-35.
186. Mitchell, J.B., et al., *Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers.* Stem Cells, 2006. **24**(2): p. 376-85.
187. Hagege, A.A., et al., *Skeletal myoblast transplantation in ischemic heart failure: long-term follow-up of the first phase I cohort of patients.* Circulation, 2006. **114**(1 Suppl): p. I108-13.
188. Smits, P.C., et al., *Catheter-based intramyocardial injection of autologous skeletal myoblasts as a primary treatment of ischemic heart failure: clinical experience with six-month follow-up.* J Am Coll Cardiol, 2003. **42**(12): p. 2063-9.
189. Ince, H., et al., *Transcatheter transplantation of autologous skeletal myoblasts in postinfarction patients with severe left ventricular dysfunction.* J Endovasc Ther, 2004. **11**(6): p. 695-704.
190. Siminiak, T., et al., *Autologous skeletal myoblast transplantation for the treatment of postinfarction myocardial injury: phase I clinical study with 12 months of follow-up.* Am Heart J, 2004. **148**(3): p. 531-7.

191. Dib, N., et al., *Safety and feasibility of autologous myoblast transplantation in patients with ischemic cardiomyopathy: four-year follow-up.* Circulation, 2005. **112**(12): p. 1748-55.
192. Biagini, E., et al., *Stress and tissue Doppler echocardiographic evidence of effectiveness of myoblast transplantation in patients with ischaemic heart failure.* Eur J Heart Fail, 2006. **8**(6): p. 641-8.
193. Gavira, J.J., et al., *Autologous skeletal myoblast transplantation in patients with nonacute myocardial infarction: 1-year follow-up.* J Thorac Cardiovasc Surg, 2006. **131**(4): p. 799-804.
194. Siminiak, T., et al., *Percutaneous trans-coronary-venous transplantation of autologous skeletal myoblasts in the treatment of post-infarction myocardial contractility impairment: the POZNAN trial.* Eur Heart J, 2005. **26**(12): p. 1188-95.
195. Chachques, J.C., et al., *Autologous human serum for cell culture avoids the implantation of cardioverter-defibrillators in cellular cardiomyoplasty.* Int J Cardiol, 2004. **95 Suppl 1**: p. S29-33.
196. Menasche, P., et al., *The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation.* Circulation, 2008. **117**(9): p. 1189-200.
197. Duckers, H.J., et al., *Final results of a phase IIa, randomised, open-label trial to evaluate the percutaneous intramyocardial transplantation of autologous skeletal myoblasts in congestive heart failure patients: the SEISMIC trial.* EuroIntervention. **6**(7): p. 805-12.
198. Dib, N., et al., *One-year follow-up of feasibility and safety of the first U.S., randomized, controlled study using 3-dimensional guided catheter-based delivery of autologous skeletal myoblasts for ischemic cardiomyopathy (CAuSMIC study).* JACC Cardiovasc Interv, 2009. **2**(1): p. 9-16.
199. Assmus, B., et al., *Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI).* Circulation, 2002. **106**(24): p. 3009-17.
200. Strauer, B.E., et al., *Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans.* Circulation, 2002. **106**(15): p. 1913-8.
201. Britten, M.B., et al., *Infarct remodeling after intracoronary progenitor cell treatment in patients with acute myocardial infarction (TOPCARE-AMI): mechanistic insights from serial contrast-enhanced magnetic resonance imaging.* Circulation, 2003. **108**(18): p. 2212-8.
202. Schachinger, V., et al., *Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial.* J Am Coll Cardiol, 2004. **44**(8): p. 1690-9.
203. Fernandez-Aviles, F., et al., *Experimental and clinical regenerative capability of human bone marrow cells after myocardial infarction.* Circ Res, 2004. **95**(7): p. 742-8.
204. Kuethe, F., et al., *Lack of regeneration of myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans with large anterior myocardial infarctions.* Int J Cardiol, 2004. **97**(1): p. 123-7.
205. Bartunek, J., et al., *Intracoronary injection of CD133-positive enriched bone marrow progenitor cells promotes cardiac recovery after recent myocardial infarction: feasibility and safety.* Circulation, 2005. **112**(9 Suppl): p. I178-83.

206. Wollert, K.C., et al., *Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial.* Lancet, 2004. **364**(9429): p. 141-8.
207. Meyer, G.P., et al., *Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial.* Circulation, 2006. **113**(10): p. 1287-94.
208. Schaefer, A., et al., *Impact of intracoronary bone marrow cell transfer on diastolic function in patients after acute myocardial infarction: results from the BOOST trial.* Eur Heart J, 2006. **27**(8): p. 929-35.
209. Chen, S.L., et al., *Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction.* Am J Cardiol, 2004. **94**(1): p. 92-5.
210. Janssens, S., et al., *Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial.* Lancet, 2006. **367**(9505): p. 113-21.
211. Erbs, S., et al., *Restoration of microvascular function in the infarct-related artery by intracoronary transplantation of bone marrow progenitor cells in patients with acute myocardial infarction: the Doppler Substudy of the Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) trial.* Circulation, 2007. **116**(4): p. 366-74.
212. Schachinger, V., et al., *Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction.* N Engl J Med, 2006. **355**(12): p. 1210-21.
213. Lunde, K., et al., *Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction.* N Engl J Med, 2006. **355**(12): p. 1199-209.
214. Meluzin, J., et al., *Autologous transplantation of mononuclear bone marrow cells in patients with acute myocardial infarction: the effect of the dose of transplanted cells on myocardial function.* Am Heart J, 2006. **152**(5): p. 975 e9-15.
215. Meluzin, J., et al., *Three-, 6-, and 12-month results of autologous transplantation of mononuclear bone marrow cells in patients with acute myocardial infarction.* Int J Cardiol, 2008. **128**(2): p. 185-92.
216. Huikuri, H.V., et al., *Effects of intracoronary injection of mononuclear bone marrow cells on left ventricular function, arrhythmia risk profile, and restenosis after thrombolytic therapy of acute myocardial infarction.* Eur Heart J, 2008. **29**(22): p. 2723-32.
217. van der Laan, A., et al., *Bone marrow cell therapy after acute myocardial infarction: the HEBE trial in perspective, first results.* Neth Heart J, 2008. **16**(12): p. 436-9.
218. Tendera, M., et al., *Intracoronary infusion of bone marrow-derived selected CD34+CXCR4+ cells and non-selected mononuclear cells in patients with acute STEMI and reduced left ventricular ejection fraction: results of randomized, multicentre Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction (REGENT) Trial.* Eur Heart J, 2009. **30**(11): p. 1313-21.
219. Lipinski, M.J., et al., *Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials.* J Am Coll Cardiol, 2007. **50**(18): p. 1761-7.
220. Martin-Rendon, E., et al., *Autologous bone marrow stem cells to treat acute myocardial infarction: a systematic review.* Eur Heart J, 2008. **29**(15): p. 1807-18.

221. Zhang, C., et al., *Efficacy and safety of intracoronary autologous bone marrow-derived cell transplantation in patients with acute myocardial infarction: insights from randomized controlled trials with 12 or more months follow-up.* Clin Cardiol, 2010. **33**(6): p. 353-60.
222. Uemura, R., et al., *Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling.* Circ Res, 2006. **98**(11): p. 1414-21.
223. Davis, M.E., et al., *Local myocardial insulin-like growth factor 1 (IGF-1) delivery with biotinylated peptide nanofibers improves cell therapy for myocardial infarction.* Proc Natl Acad Sci U S A, 2006. **103**(21): p. 8155-60.
224. Muller-Ehmsen, J., et al., *Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium.* J Mol Cell Cardiol, 2002. **34**(2): p. 107-16.
225. Cortes-Morichetti, M., et al., *Association between a cell-seeded collagen matrix and cellular cardiomyoplasty for myocardial support and regeneration.* Tissue Eng, 2007. **13**(11): p. 2681-7.
226. Ryu, J.H., et al., *Implantation of bone marrow mononuclear cells using injectable fibrin matrix enhances neovascularization in infarcted myocardium.* Biomaterials, 2005. **26**(3): p. 319-26.
227. Kellar, R.S., et al., *Scaffold-based three-dimensional human fibroblast culture provides a structural matrix that supports angiogenesis in infarcted heart tissue.* Circulation, 2001. **104**(17): p. 2063-8.
228. Kellar, R.S., et al., *Cardiac patch constructed from human fibroblasts attenuates reduction in cardiac function after acute infarct.* Tissue Eng, 2005. **11**(11-12): p. 1678-87.
229. Piao, H., et al., *Effects of cardiac patches engineered with bone marrow-derived mononuclear cells and PGCL scaffolds in a rat myocardial infarction model.* Biomaterials, 2007. **28**(4): p. 641-9.
230. Caspi, O., et al., *Tissue engineering of vascularized cardiac muscle from human embryonic stem cells.* Circ Res, 2007. **100**(2): p. 263-72.
231. Lesman, A., et al., *Transplantation of a tissue-engineered human vascularized cardiac muscle.* Tissue Eng Part A, 2010. **16**(1): p. 115-25.
232. Leor, J., et al., *Bioengineered cardiac grafts: A new approach to repair the infarcted myocardium?* Circulation, 2000. **102**(19 Suppl 3): p. III56-61.
233. Simpson, D., et al., *A tissue engineering approach to progenitor cell delivery results in significant cell engraftment and improved myocardial remodeling.* Stem Cells, 2007. **25**(9): p. 2350-7.
234. Schussler, O., et al., *3-dimensional structures to enhance cell therapy and engineer contractile tissue.* Asian Cardiovasc Thorac Ann, 2011. **18**(2): p. 188-98.
235. Chachques, J.C., et al., *Myocardial assistance by grafting a new bioartificial upgraded myocardium (MAGNUM clinical trial): one year follow-up.* Cell Transplant, 2007. **16**(9): p. 927-34.
236. Chachques, J.C., et al., *Myocardial Assistance by Grafting a New Bioartificial Upgraded Myocardium (MAGNUM trial): clinical feasibility study.* Ann Thorac Surg, 2008. **85**(3): p. 901-8.
237. Madden, L.R., et al., *Proangiogenic scaffolds as functional templates for cardiac tissue engineering.* Proc Natl Acad Sci U S A, 2010. **107**(34): p. 15211-6.
238. Zimmermann, W.H., et al., *Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes.* Biotechnol Bioeng, 2000. **68**(1): p. 106-14.

239. Zimmermann, W.H., et al., *Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts.* Nat Med, 2006. **12**(4): p. 452-8.
240. Tulloch, N.L., et al., *Growth of engineered human myocardium with mechanical loading and vascular coculture.* Circ Res, 2011. **109**(1): p. 47-59.
241. Shimizu, T., et al., *Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces.* Circ Res, 2002. **90**(3): p. e40.
242. Miyahara, Y., et al., *Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction.* Nat Med, 2006. **12**(4): p. 459-65.
243. Asakawa, N., et al., *Pre-vascularization of in vitro three-dimensional tissues created by cell sheet engineering.* Biomaterials, 2010. **31**(14): p. 3903-9.
244. Sekine, H., et al., *Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts.* Circulation, 2008. **118**(14 Suppl): p. S145-52.
245. Stevens, K.R., et al., *Scaffold-free human cardiac tissue patch created from embryonic stem cells.* Tissue Eng Part A, 2009. **15**(6): p. 1211-22.
246. Stevens, K.R., et al., *Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue.* Proc Natl Acad Sci U S A, 2009. **106**(39): p. 16568-73.
247. Ueyama, K., et al., *Development of biologic coronary artery bypass grafting in a rabbit model: revival of a classic concept with modern biotechnology.* J Thorac Cardiovasc Surg, 2004. **127**(6): p. 1608-15.
248. Wei, H.J., et al., *Bioengineered cardiac patch constructed from multilayered mesenchymal stem cells for myocardial repair.* Biomaterials, 2008. **29**(26): p. 3547-56.
249. Chen, C.H., et al., *Porous tissue grafts sandwiched with multilayered mesenchymal stromal cell sheets induce tissue regeneration for cardiac repair.* Cardiovasc Res, 2008. **80**(1): p. 88-95.
250. Tan, M.Y., et al., *Repair of infarcted myocardium using mesenchymal stem cell seeded small intestinal submucosa in rabbits.* Biomaterials, 2009. **30**(19): p. 3234-40.
251. Ott, H.C., et al., *Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart.* Nat Med, 2008. **14**(2): p. 213-21.
252. Lang, R.M., et al., *Recommendations for chamber quantification: a report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology.* J Am Soc Echocardiogr, 2005. **18**(12): p. 1440-63.
253. Meller, J., M.V. Herman, and L.E. Teichholz, *Noninvasive assessment of left ventricular function.* Adv Intern Med, 1979. **24**: p. 331-57.
254. Mizutani, M. and T. Matsuda, *Liquid photocurable biodegradable copolymers: in vivo degradation of photocured poly(epsilon-caprolactone-co-trimethylene carbonate).* J Biomed Mater Res, 2002. **61**(1): p. 53-60.
255. Chin, L., et al., *Characterization of and host response to tyramine substituted-hyaluronan enriched fascia extracellular matrix.* J Mater Sci Mater Med, 2011. **22**(6): p. 1465-77.
256. Segers, V.F. and R.T. Lee, *Stem-cell therapy for cardiac disease.* Nature, 2008. **451**(7181): p. 937-42.

257. Laflamme, M.A. and C.E. Murry, *Heart regeneration.* Nature, 2011. **473**(7347): p. 326-35.
258. Psaltis, P.J., et al., *Concise review: mesenchymal stromal cells: potential for cardiovascular repair.* Stem Cells, 2008. **26**(9): p. 2201-10.
259. Wollert, K.C. and H. Drexler, *Cell therapy for the treatment of coronary heart disease: a critical appraisal.* Nat Rev Cardiol, 2010. **7**(4): p. 204-15.
260. Sekiya, N., et al., *Layered implantation of myoblast sheets attenuates adverse cardiac remodeling of the infarcted heart.* J Thorac Cardiovasc Surg, 2009. **138**(4): p. 985-93.
261. Mias, C., et al., *Mesenchymal stem cells promote matrix metalloproteinase secretion by cardiac fibroblasts and reduce cardiac ventricular fibrosis after myocardial infarction.* Stem Cells, 2009. **27**(11): p. 2734-43.
262. Song, E., et al., *Collagen scaffolds derived from a marine source and their biocompatibility.* Biomaterials, 2006. **27**(15): p. 2951-61.
263. Miyagi, Y., et al., *Biodegradable collagen patch with covalently immobilized VEGF for myocardial repair.* Biomaterials, 2011. **32**(5): p. 1280-90.
264. Shi, C., et al., *Stem-cell-capturing collagen scaffold promotes cardiac tissue regeneration.* Biomaterials, 2011. **32**(10): p. 2508-15.
265. Danoviz, M.E., et al., *Rat adipose tissue-derived stem cells transplantation attenuates cardiac dysfunction post infarction and biopolymers enhance cell retention.* PLoS One, 2010. **5**(8): p. e12077.
266. Kutschka, I., et al., *Collagen matrices enhance survival of transplanted cardiomyoblasts and contribute to functional improvement of ischemic rat hearts.* Circulation, 2006. **114**(1 Suppl): p. I167-73.
267. Robey, T.E., et al., *Systems approaches to preventing transplanted cell death in cardiac repair.* J Mol Cell Cardiol, 2008. **45**(4): p. 567-81.
268. Zhu, Y., et al., *Collagen-chitosan polymer as a scaffold for the proliferation of human adipose tissue-derived stem cells.* J Mater Sci Mater Med, 2009. **20**(3): p. 799-808.
269. Park, S.N., et al., *Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide cross-linking.* Biomaterials, 2002. **23**(4): p. 1205-12.
270. Wissink, M.J., et al., *Endothelial cell seeding on crosslinked collagen: effects of crosslinking on endothelial cell proliferation and functional parameters.* Thromb Haemost, 2000. **84**(2): p. 325-31.
271. Ochoa, I., et al., *Mechanical properties of cross-linked collagen meshes after human adipose derived stromal cells seeding.* J Biomed Mater Res A, 2010. **96**(2): p. 341-8.
272. Badylak, S.F. and T.W. Gilbert, *Immune response to biologic scaffold materials.* Semin Immunol, 2008. **20**(2): p. 109-16.
273. Badylak, S.F., et al., *Macrophage phenotype as a determinant of biologic scaffold remodeling.* Tissue Eng Part A, 2008. **14**(11): p. 1835-42.
274. Anderson, J.M., A. Rodriguez, and D.T. Chang, *Foreign body reaction to biomaterials.* Semin Immunol, 2008. **20**(2): p. 86-100.
275. Malle, E. and F.C. De Beer, *Human serum amyloid A (SAA) protein: a prominent acute-phase reactant for clinical practice.* Eur J Clin Invest, 1996. **26**(6): p. 427-35.
276. Badylak, S.F., D.O. Freytes, and T.W. Gilbert, *Extracellular matrix as a biological scaffold material: Structure and function.* Acta Biomater, 2009. **5**(1): p. 1-13.

277. Lin, Y., M. Vandeputte, and M. Waer, *Natural killer cell- and macrophage-mediated rejection of concordant xenografts in the absence of T and B cell responses.* J Immunol, 1997. **158**(12): p. 5658-67.
278. Laudi, S., et al., *Worsening of long-term myocardial function after successful pharmacological pretreatment with cyclosporine.* J Physiol Pharmacol, 2007. **58**(1): p. 19-32.
279. Dzau, V.J., et al., *Therapeutic potential of endothelial progenitor cells in cardiovascular diseases.* Hypertension, 2005. **46**(1): p. 7-18.
280. Zhou, Y., et al., *Direct injection of autologous mesenchymal stromal cells improves myocardial function.* Biochem Biophys Res Commun, 2009. **390**(3): p. 902-7.
281. Moon, M.H., et al., *Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia.* Cell Physiol Biochem, 2006. **17**(5-6): p. 279-90.
282. Wang, M., et al., *Human progenitor cells from bone marrow or adipose tissue produce VEGF, HGF, and IGF-I in response to TNF by a p38 MAPK-dependent mechanism.* Am J Physiol Regul Integr Comp Physiol, 2006. **291**(4): p. R880-4.
283. Planat-Benard, V., et al., *Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives.* Circulation, 2004. **109**(5): p. 656-63.
284. Dhein, S., et al., *Effects of autologous bone marrow stem cell transplantation on beta-adrenoceptor density and electrical activation pattern in a rabbit model of non-ischemic heart failure.* J Cardiothorac Surg, 2006. **1**: p. 17.
285. Berry, M.F., et al., *Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance.* Am J Physiol Heart Circ Physiol, 2006. **290**(6): p. H2196-203.
286. Nagaya, N., et al., *Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy.* Circulation, 2005. **112**(8): p. 1128-35.
287. Xu, X., et al., *Effects of mesenchymal stem cell transplantation on extracellular matrix after myocardial infarction in rats.* Coron Artery Dis, 2005. **16**(4): p. 245-55.
288. Swinnen, M., et al., *Absence of thrombospondin-2 causes age-related dilated cardiomyopathy.* Circulation, 2009. **120**(16): p. 1585-97.
289. Frangogiannis, N.G., et al., *Critical role of endogenous thrombospondin-1 in preventing expansion of healing myocardial infarcts.* Circulation, 2005. **111**(22): p. 2935-42.
290. Bornstein, P. and E.H. Sage, *Matricellular proteins: extracellular modulators of cell function.* Curr Opin Cell Biol, 2002. **14**(5): p. 608-16.
291. Schellings, M.W., et al., *Absence of SPARC results in increased cardiac rupture and dysfunction after acute myocardial infarction.* J Exp Med, 2009. **206**(1): p. 113-23.
292. Holmes, J.W., T.K. Borg, and J.W. Covell, *Structure and mechanics of healing myocardial infarcts.* Annu Rev Biomed Eng, 2005. **7**: p. 223-53.
293. Lopez, B., et al., *Role of lysyl oxidase in myocardial fibrosis: from basic science to clinical aspects.* Am J Physiol Heart Circ Physiol, 2011. **299**(1): p. H1-9.
294. Katritsis, D.G., et al., *Transcoronary transplantation of autologous mesenchymal stem cells and endothelial progenitors into infarcted human myocardium.* Catheter Cardiovasc Interv, 2005. **65**(3): p. 321-9.
295. Chen, S., et al., *Intracoronary transplantation of autologous bone marrow mesenchymal stem cells for ischemic cardiomyopathy due to isolated chronic occluded left anterior descending artery.* J Invasive Cardiol, 2006. **18**(11): p. 552-6.

296. Freyman, T., et al., *A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction.* Eur Heart J, 2006. **27**(9): p. 1114-22.
297. Hernandez, R.M., et al., *Microcapsules and microcarriers for in situ cell delivery.* Adv Drug Deliv Rev, 2010. **62**(7-8): p. 711-30.
298. Discher, D.E., P. Janmey, and Y.L. Wang, *Tissue cells feel and respond to the stiffness of their substrate.* Science, 2005. **310**(5751): p. 1139-43.
299. Guan, J., et al., *The stimulation of the cardiac differentiation of mesenchymal stem cells in tissue constructs that mimic myocardium structure and biomechanics.* Biomaterials, 2011. **32**(24): p. 5568-80.

9. ANEXOS

ANEXO I

Decision Letter (CT-0309.R2)

From: Amit.Patel@hsc.utah.edu

To: fprosper@unav.es

Subject: Cell Transplantation - Decision on Manuscript CT-0309.R2

Body: @@date to be populated upon sending@@

Dear Dr. Prosper:

It is a pleasure to accept your manuscript entitled "Adipose Stromal Vascular Fraction improves cardiac function in chronic myocardial infarction through differentiation and paracrine activity" in its current form for publication in the journal "Cell Transplantation". The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

For publication purposes, we will require 2 hard copies of your manuscript, along with any figures as high quality glossy prints (since the publishers will scan in the figures) and a CD containing the entire manuscript in a non-PDF format. Please send this to:
Dr. David Eve, Ph.D
Associate Editor,
Cell Transplantation - The Regenerative Medicine Journal
Center of Excellence for Aging and Brain Repair
Department of Neurosurgery & Brain Repair, MDC-78
University of South Florida
12901 Bruce B Downs Blvd
Tampa, FL 33612

Once the hard copies have been received and checked, a copyright transfer agreement will be requested. On receipt your manuscript can be forwarded to the publishers and queued for publication, as well as a black and white pre-copyedited Epub generated. At the proofing stage, the publishers will ask you to confirm color figure authorization charges as well as payment of the open access fees, both of which are required before your manuscript can be published.

Thank you for your fine contribution. On behalf of the Editors of "Cell Transplantation", we look forward to your continued contributions to the journal "Cell Transplantation".

Sincerely,
Prof. Amit Patel
Section Editor, Cell Transplantation
Amit.Patel@hsc.utah.edu, kathy.anglesey@hsc.utah.edu

Date Sent: 02-Apr-2011

CELL TRANSPLANTATION
The Regenerative Medicine Journal

Adipose Stromal Vascular Fraction improves cardiac function in chronic myocardial infarction through differentiation and paracrine activity

Journal:	*Cell Transplantation*
Manuscript ID:	CT-0309.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	08-Mar-2011
Complete List of Authors:	Mazo, Manuel; Clínica Universidad de Navarra Cemnorain, Arantxa; Clínica Universidad de Navarra, University of Navarra, Hematology and Cell Therapy Gavira, Juan José; Clínica Universidad de Navarra, University of Navarra, Department of Cardiology and Cardiovascular Surgery Abizanda, Gloria; Clínica Universidad de Navarra, University of Navarra, Hematology and Cell Therapy Araña, Miriam; Foundation for Applied Medical Research, Division of Cancer, Hematology and Cell Therapy Casado, Mayte; Instituto Cavanilles, University of Valencia, Department of Cell Biology Soriano, Mario; Instituto Cavanilles, University of Valencia, Department of Cell Biology Hernández, Salomón; Foundation for Applied Medical Research, Division of Cancer, Hematology and Cell Therapy Moreno, Cristina; Clínica Universidad de Navarra, University of Navarra, Immunology Service Ecay, Margarita; Clínica Universidad de Navarra, University of Navarra, Department of Nuclear Medicine and MicroPET Research Unit CIMA-CUN Albiasu, Edurne; Foundation for Applied Medical Research, Division of Cancer, Hematology and Cell Therapy Belzunce, Miriam; Foundation for Applied Medical Research, Division of Cardiovascular Diseases Orbe, Josune; Foundation for Applied Medical Research, Division of Cardiovascular Diseases Paramo, Jose; Foundation for Applied Medical Research, Division of Cardiovascular Diseases Merino, Juana; Clínica Universidad de Navarra, University of Navarra, Immunology Service Penuelas, Ivan; CUN; Clínica Universidad de Navarra, University of Navarra, Department of Nuclear Medicine and MicroPET Research Unit CIMA-CUN

	García-Verdugo, José Manuel; Instituto Cavanilles, University of Valencia, Department of Cell Biology Pelacho, Beatriz; CIMA, Stem Cell Laboratory; Foundation for Applied Medical Research, Division of Cancer, Hematology and Cell Therapy Prosper, Felipe; Clinica Universidad de Navarra, Hematology; Clínica Universidad de Navarra, University of Navarra, Hematology and Cell Therapy
Keywords:	adipose cells, paracrine, infarction, remodeling
Abstract:	AIMS: Fresh adipose-derived cells have been shown to be effective in the treatment of acute myocardial infarction (MI), but their role in the chronic setting is unknown. We sought to determine the long-term effect of the adipose derived-stromal vascular fraction (SVF) cell transplantation in a rat model of chronic MI. METHODS AND RESULTS: MI was induced in 82 rats by permanent coronary artery ligation and 5 weeks later, rats were allocated to receive an intra-myocardial injection of 107 GFP-expressing fresh SVF-cells or culture media as control. Heart function and tissue metabolism were determined by echocardiography and 18F-FDG-microPET, respectively, and histological studies were performed for up to 3 months after transplantation. SVF induced a statistically significant long-lasting (3 months) improvement in cardiac function and tissue metabolism that was associated with increased revascularization and positive heart remodeling, with a significantly smaller infarct size, thicker infarct wall, lower scar fibrosis and lower cardiac hypertrophy. Importantly, injected cells engrafted and were detected in the treated hearts for at least 3 months, directly contributing to the vasculature and myofibroblasts and at negligible levels to cardiomyocytes. Furthermore, SVF-release of angiogenic (VEGF and HGF) and pro-inflammatory (MCP-1) cytokines, as well as TIMP1 and TIMP4, was demonstrated in vitro and in vivo, strongly suggesting that they have a trophic effect. CONCLUSIONS: These results show the potential of SVF to contribute to the regeneration of ischemic tissue and to provide a long-term functional benefit in a rat model of chronic MI, by both direct and indirect mechanisms.

Adipose Stromal Vascular Fraction improves cardiac function in chronic myocardial infarction through differentiation and paracrine activity

Manuel Mazo[1], Arantxa Cemborain[1], Juan José Gavira[2], Gloria Abizanda[1], Miriam Araña[1], Mayte Casado[5], Mario Soriano[5], Salomón Hernández[1], Cristina Moreno[3], Margarita Ecay[4], Edurne Albiasu[1], Miriam Belzunce[6], Josune Orbe[6], José Antonio Páramo[6] Juana Merino[3], Iván Peñuelas[4], José Manuel García Verdugo[5], Beatriz Pelacho[1*], Felipe Prosper[1*]

[1]Hematology and Cell Therapy and Foundation for Applied Medical Research, Division of Cancer, [2] Department of Cardiology and Cardiovascular Surgery, [3]Immunology Service and [4]Department of Nuclear Medicine and MicroPET Research Unit CIMA-CUN, Clínica Universitaria, University of Navarra, Spain, [5]Department of Cell Biology, Instituto Cavanilles, University of Valencia, Spain, [6]Foundation for Applied Medical Research, Division of Cardiovascular Diseases.

Running Title: SVF repairs myocardium.

Address for Correspondence:

Felipe Prósper MD
Hematology and Cell Therapy
Clínica Universitaria
Av. Pío XII 36, Pamplona 31008,
Navarra, Spain
Phone 34 948 255400 Fax 34 948 296500
e-mail: fprosper@unav.es

Beatriz Pelacho PhD
Foundation for Applied Medical Research
Av. Pio XII 57, Pamplona 31008,
Navarra, Spain
Phone 34 948 194700
e-mail: bpelacho@unav.es

* BP and FP contributed equally to this work and should be considered equal last authors

Word count: Abstract: 249 words; body text (excluding supplements): 5349 words

This work was supported in part by ISCIII PI050168, PI070474, CP09/00333 and ISCIII-RETIC RD06/0014, MICCIN PLE2009-0116, and PSE SINBAD, Gobierno de Navarra (Departamento de Educación), Comunidad de Trabajo de los Pirineos (CTP), European Union Framework Project VII (INELPY), Caja de Ahorros de Navarra (Programa Tu Eliges: Tu Decides) and the "UTE project CIMA".

AIMS: Fresh adipose-derived cells have been shown to be effective in the treatment of acute myocardial infarction (MI), but their role in the chronic setting is unknown. We sought to determine the long-term effect of the adipose derived-stromal vascular fraction (SVF) cell transplantation in a rat model of chronic MI.

METHODS AND RESULTS: MI was induced in 82 rats by permanent coronary artery ligation and 5 weeks later, rats were allocated to receive an intra-myocardial injection of 10^7 GFP-expressing fresh SVF-cells or culture media as control. Heart function and tissue metabolism were determined by echocardiography and ^{18}F-FDG-microPET, respectively, and histological studies were performed for up to 3 months after transplantation. SVF induced a statistically significant long-lasting (3 months) improvement in cardiac function and tissue metabolism that was associated with increased revascularization and positive heart remodeling, with a significantly smaller infarct size, thicker infarct wall, lower scar fibrosis and lower cardiac hypertrophy. Importantly, injected cells engrafted and were detected in the treated hearts for at least 3 months, directly contributing to the vasculature and myofibroblasts and at negligible levels to cardiomyocytes. Furthermore, SVF-release of angiogenic (VEGF and HGF) and pro-inflammatory (MCP-1) cytokines, as well as TIMP1 and TIMP4, was demonstrated *in vitro* and *in vivo*, strongly suggesting that they have a trophic effect.

CONCLUSIONS: These results show the potential of SVF to contribute to the regeneration of ischemic tissue and to provide a long-term functional benefit in a rat model of chronic MI, by both direct and indirect mechanisms.

INTRODUCTION

Myocardial infarction is a leading cause of mortality in western countries (19). Large cardiomyocyte (CM) loss provoked by ischemia leads to impairment of cardiac contractility, loss of pump force and subsequent risk of heart failure. Although new therapeutic strategies have contributed to improve survival and quality of life (3), regeneration of the myocardium remains an elusive goal which leaves organ transplantation the only alternative for chronic end-stage patients.

Over the last decade, the application of stem cells has opened new perspectives in the form of regenerative therapy whereby healthy cells can substitute the damaged tissue. A wide array of cell types has been tested in various animal models of MI, with bone marrow mononuclear cells and skeletal myoblasts reaching the stage of clinical trials (36). However, the results obtained with these two cell populations have shown only mild effects and, although long-term studies with larger cohorts of patients are required, new cell sources or approaches are sought in order to demonstrate the feasibility of this therapy.

Recent studies have demonstrated the capacity of stem/progenitor cells contained in the stromal fraction of the adipose tissue (SVF), not only in terms of their differentiation capacity (17), but also because they produce a wide array of growth factors and cytokines (8). Given that this cell population has been mainly tested in models of acute MI (43,44), the goal of the present study was to investigate the potential benefit of transplanting freshly isolated white adipose tissue-derived SVF cells in a rat model of chronic MI, focusing particularly on their long-term engraftment and differentiation capacity as well as their potential to restore the functional capacity of the heart.

MATERIAL AND METHODS

Ethics Statement

All experiments were performed in accordance with the guidelines of the National Society for Medical Research and Institute of Laboratory Animal Resources. All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals.

Isolation and characterization of injected cells

SVF cells were isolated from 4 to 8 weeks old Sprague-Dawley eGFP rats as previously described (29), with slight modifications as follows. Briefly, rats were killed by cervical dislocation under CO_2 anesthesia. Inguinal adipose depots were isolated, carefully minced and digested for 30 minutes at 37 ºC in DMEM-F12 (Invitrogen, Leiden, The Netherlands) containing 2% collagenase A (Roche, Madrid, Spain). After centrifugation of the pelleted stromal vascular fraction (SVF), the reaction was stopped by adding DMEM supplemented with 10% fetal calf serum to the sample, which was subsequently filtered through 100- and 40-µm filters. Cells were washed in PBS and incubated in lysis buffer in order to eliminate contaminating erithrocytes obtaining aproximatelly 1.15 x 10^6 cells/gr of white adipose tissue. Viability was shown to be around 98% as determined by trypan blue dye exclusion. Characterization of SVF was performed by FACS analysis, staining cells with antibodies against RT1A, RT1B, CD11b, CD31, CD44, CD45, CD73, CD90 and CD106 (all purchased from BD, Madrid, Spain). Expression of GFP was confirmed by FACS and direct visualization under a microscope equiped with epifluorescence, and ensured to be around 90% in all cases.

Experimental model

A total of 82 female Sprague-Dawley rats (Harlan IBERICA S.L. Barcelona, Spain) underwent coronary artery ligation of the left coronary artery as previously described (9,18). Only those surviving animals (n=57) in which LVEF decreased below 40% at 1 month post-MI were included in the study (n=55). Heterogeneicity of the groups was excluded as no statistically significant differences were found in pre-implantation EF values between SVF treated animals and the control group (Control: 29.87±2.84%; SVF: 26.46±2.26%; P=0.29). A similar lack of significant variability intra-group was detected (Variances: Control: 80.48; SVF: 70.5; Levene´s test for equality of variances: P=0.678).

Five weeks post-myocardial infarction, rats were reoperated by sternotomy and randomized to receive 10^7 cells per heart (total volume: 70 µl) of freshly isolated SVF from male GFP-rats (n=29) or media alone (n=24), in 2 points of the infarct border region. No differences in early mortality after cell injection were detected in the cell treated group versus the control group (only 1 out of 29 animals died during the injection versus none in the control group with 24 animals). All animals were daily immune-suppressed with cyclosporin A (20 mg/kg/d i.p. Sandimmun, Novartis) from two days pre-transplantation until sacrifice at 1,3 days and 1,2,4 weeks (2-4 animals/time point) and 3 months (10 animals).

Tissue processing and immunostaining

After sacrificed, the rat hearts were excised, fixed in 4 % paraformaldehyde for 4 hours at 4ºC, and cut in 3 equal size blocks (apical, mid-ventricular and basal). Hearts were dehydrated in ethanol 70% (4°C o/n) and embedded in paraffin. For histological analysis 5 µm sections were performed. Cell detection was based on the presence of GFP-positive signals by inmunohistochemical methods (anti-GFP, Invitrogen, or chicken anti-GFP (Abcam, Cambridge, UK), diluted 1:500 in TBS) or immunofluoresecence. For immunofluorescence, a tyramide amplifying kit (Invitrogen) or a secondary antibody anti-chicken IgY coupled to 655 nm emitting quantum dots were used following manufacturer's instructions. Immunolabeling was performed with antibodies against α-Smooth Muscle Actin (α-SMA) (diluted 1:1000 in TBS, Sigma, Madrid, Spain), cardiac troponin T (cTT) (diluted 1:100 in TBS; Labvision), ventricular myosin (diluted 1:2 in TBS; BioCytex), Ki67 (diluted 1:100 in PBS; Dako), Caveolin-1 (diluted 1:100 in TBS, BD), Connexin-43 (diluted 1:200 in TBS, Sigma), Laminin (diluted 1:25 in TBS, Sigma), bFGF (diluted 1:100 in TBS, BD), HGF (diluted 1:100 in TBS, Assay Designs, Ann Harbor, MI, USA), active caspase 3 (diluted 1:100 in TBS, Cell Signalling, Beverly, MA, USA), MCP1 (diluted 1:100 in TBS, Abcam), VEGF (diluted 1:50 in TBS, Abcam), SM Myosin (diluted 1:400 in TBS, Abcam), TIMP1 (diluted 1:50, Abcam), TIMP4 (diluted 1:50, Abcam), MMP2 (diluted 1:100, Abcam), MMP9 (diluted 1:100, R&D) and MMP14 (diluted 1:100, Abcam). Secondary antibodies labeled with AlexaFluor-594 or AlexaFluor-488 were purchased from Molecular Probes (Invitrogen) if needed. EnVision™-HRP conjugated system (Dako) was used as secondary reagent for immunohistochemistry. For confocal microscopy, a LSM 510 META (Carl Zeiss, Minneapolis, USA) microscope was used.

For Sirius Red staining, sections were deparaffinized and immersed in 0.1% Fast Red (Sigma) in a saturated solution of picric acid, for 90 minutes, differentiated 2 minutes in HCl (Sigma) 0.01N, dehydrated and mounted in DPX. Hematoxylin-Eosin staining was performed as detailed elsewhere. Briefly, sections where stained in Ehrlich´s hematoxylin (Sigma) for 7 minutes, differentiated through water-HCl and Li_2CO_3 solutions, immersed in Eosin (Sigma) for 10 seconds, dehydrated and mounted in DPX.

Electron microscopy

For electron microscopy studies, hearts were fixed with 4% paraformaldehyde 0.5% glutaraldehyde. Tissues were cryoprotected in 25% sucrose for 48 hours and cut in 100 µm sections. Sections were post-fixed with 2% osmium, rinsed, dehydrated and embedded in araldite (Durcupan, Fluka). Semithin sections (1.5 µm) were cut with a diamond knife and stained lightly with 1% toluidine blue. Later ultra-thin (0.08 µm) sections were cut with a diamond knife, stained with lead citrate (Reynolds solution) and examined under a FEI Tecnai G2 Spirit transmision electron microscopy. Ultra-thin sections were washed in phosphate buffer (PB), blocked in 0.3% bovine serum albumin-C (BSA, Aurion, Netherlands) and incubated in primary chicken anti-GFP antibody (Aves Labs; 1:200 in PB for 3 days at 4°C). Sections were washed in PB, blocked in 0.5% BSA and 0.1% fish gelatin (1 h, room temperature) and incubated in colloidal gold-conjugated anti-chicken secondary antibody (1:50 for 24 h). Sections were washed in PB and 2% sodium acetate. Silver enhancement was performed (as per Aurion instructions) and washed again in 2% sodium acetate. To stabilize, silver particles samples were immersed in 0.05% gold chloride (10 min at 4°C), washed in sodium thiosulfate then washed in PB and postfixed in 2% glutaraldehyde (30 min). Sections were contrasted with 1% osmium and 7% glucose and embedded in araldite. Second, semi-thin 1.5 µm sections were prepared, selected at the light microscope level and re-embedded for ultra-thin sectioning at 70 nm. Photomicrographs were obtained under a FEI microscope (Tecnai-Spirit) using a digital camera (Morada, Soft-imaging System).

Morphometric analysis

Quantification of vascular density was performed in animals sacrificed 3 months post-transplantation. For capillary density (capillaries/mm^2), serial sections 30 µm apart were stained with caveolin-1 and infarct border images were analyzed. Arteriolar density

(arterioles/mm^2) and arteriolar area (μm^2) were quantified in the same way after staining with anti-alpha smooth muscle actin-Cy3 in the following sections. Pictures were taken on a Nikon Eclipse E800 microscope equipped with epifluorescence optics and digital images were analyzed using imaging software (Jay Image), or on a Zeiss LSM 510 META laser confocal microscope. The degree of fibrosis was determined by quantification of collagen deposition stained by Sirius red staining of serial sections as previously described (6). Infarct size was assessed as the mean % of infarcted area vs. total LV area, ventricular infarct wall thickness as the average of repetitive wall thickness measurements per section and fibrosis degree was measured in high power photographs within the infarct as % of collagen area (red) *vs.* total tissue area (yellow). AnalySIS^R software was used for these analyses. Measurement of CM hypertrophy was performed on laminin-stained sections. High-power field images were acquired and CM cross sectional area was quantified using Image J software. A minimum of 100 CM per heart were analyzed.

Cell engraftment was quantified after GFP-immunostaining. Positive cells were only counted if their nucleus were identifiable. Tissue sections were screened and GFP-cells quantified from the first to the last section with engrafted cells. Percentage of surviving cells was referred to the quantity of injected cells (10^7cells). Cell differentiation was measured in confocal laser microscopy images by counting total GFP-positive cells and those double-positive for GFP and lineage specific markers. GFP- and non-GFP expresing adipocytes were counted by means of their characteristical morphology with AxioVision 4.6 (Zeiss, Madrid, Spain). Both differentiation and adipocytes are thus expressed as relative to the number of engrafted cells. Fifteen serial sections were analyzed, taking photographs of the whole left ventricle. Finally, cell proliferation and apoptosis were quantified by counting PCNA- or Activated Caspase-3 positive cells in serial sections double-stained for GFP and PCNA or GFP and activated Caspase-3 respectively. All photographs were taken on a Zeiss LSM 510 META laser confocal microscope.

Echocardiographic studies

Animals were slightly anesthetized prior to study with 2% isoflurane (Forane®. Lab. ABBOTT S.A, Madrid, Spain) in 100% O_2 gas and placed in the supine or lateral position on a warming pad for transthoracic two-dimensional echocardiography, M-

mode recordings, and Doppler ultrasound measurements as described (1). Echocardiography was performed using a Sonos 4500 ultrasound system (Philips) with a 12 MHz linear array transducer and Doppler measurement. Left ventricular remodeling was assessed by measuring end systolic and diastolic volumes and diameters, according to the American Society of Echocardiology. The left ventricular ejection fraction was determined in parasternal short axes (21) and diastolic function was assessed by measuring E and A waves of the mitral filling pattern by pulsed echo-Doppler technique in four-chamber apical views. Echocardiographic studies were performed in 10 animals/group at baseline (before infarct), before cell transplantation and at day 90 postransplant by the same investigator blinded to the group of treatment. Measurements were done in three cycles and the mean value was obtained.

^{18}F-FDG PET imaging protocols, image reconstruction and semiquantitative evaluation

Immediately before injection of cells and 90 days later, animals were subjected to PET analysis using the technique previously described by our group (27). Rats were anesthetized with 2% isoflurane in 100% O_2 gas and after tail-vein injection of ^{18}F-FDG (75 MBq in 100-200 µL) immediately awakened and placed back in the cage. Two hours after tracer injection, animals were anesthetized with isoflurane, placed prone on the PET scanner cradle and kept during the overall study under continuous influx of the anesthetic. A static 60-minute study (sinogram) was acquired in a Mosaic (Philips) small animal dedicated imaging tomograph. No transmission scan was performed. Scanner efficiency normalization, dead time and decay corrections were applied during reconstruction. Images were reoriented for further processing and polar maps obtained using the specific cardiac imaging software package of the PET scanner. After reorientation of transaxial images into short- and long-axis slices polar maps were generated and divided into 17 different ROIs. Individual quantification of the ^{18}F-FDG uptake in each of them was calculated. The total number of counts obtained for each of the ROIs, was divided by its corresponding area to obtain counts per area unit. For each PET study, the maximal value of the 17 ROIs was considered as 100% and the remaining data transformed into percentage values. All further calculations and statistical analysis were performed on these sets of re-scaled numerical data.

Cytokine array

To obtain culture supernatants, freshly isolated rat SVF were cultured in DMEM/10% FBS at a density of $5x10^5$ cells/cm^2 until reach confluence (72h), under normoxic or hypoxic (5% O_2) conditions. Then, media was changed for DMEM/5% FBS and collected and frozen 48h later for Cytokine arrays or proliferation studies. Also, DMEM/5% FBS media was kept in the incubator during 48h as control media. Cytokine levels were measured by a cytokine antibody array (Rat Array-I, Ray Biotech, Norkross, GA, USA) according to the manufacturer's instructions.

***In vitro* proliferation studies**

Rat smooth muscle cells (RAO Cell Applications, CA, USA) and murine endothelial cells (MS-I, ATCC, VA, USA) were plated in 24 well plates at a density of $2.5x10^3$, $5x10^3$ cells/well repectively, and cultured in the presence of control media (NCM) or SVF conditioned media (CM) obtained under normoxic or hypoxic conditions (75% of the media). After 60 hours, the number of viable cells was quantified by a luminiscent cell viability assay (CellTiter-Glo™, Promega, USA) following manufacturer's protocol. Four independent experiments were performed and every cell type grown in CM or NCM was seeded in quadruplicate.

Gelatin zymography

Serum samples prediluted 1:50 in saline were mixed with equal amounts of sodium dodecyl sulfate (SDS) sample buffer (Novex, Carlsbad, CA, U.S.A.) and electrophoresed on 10% SDS-polyacrylamide gels (Novex) containing 1 mg/mL gelatin as the protease substrate. Following electrophoresis, gels were placed in 2.7% TritonX-100 for 1 hour to remove SDS, and then incubated for 20 hours at 37°C in developing buffer (50 mmol/L Tris base, 40 mmol/L HCl, 200 mmol/L NaCl, 5 mmol/L $CaCl_2$, and 0.2% Briji 35; Novex). After incubation, gels were stained with gel code blue stain reagent (Pierce) for 1 hour followed by destaining.

Statistical analysis

Normal distribution was demonstrated by Shapiro-Wilk and Komogorov-Smirnov tests. All data are expressed as mean±SD. Comparisons were performed using the paired or unpaired t-test, or ANOVA plus Tukey´s HSD. Statistical analysis was performed with the SPSS 17.0 software and differences were considered statistically significant when

1 P<0.05

RESULTS

Cell characterization

FACS analysis of SVF cells showed a heterogeneous population comprised of stromal, hematopoietic and endothelial progenitor cells, among others, which proved positive for RT1A, low positive for RT1B, CD31, CD44, CD45, CD73 and CD90 and negative for CD11b and CD106 (Fig.1).

SVF-cells induce a long-lasting improvement in cardiac function and tissue metabolism

The long-term functional efficacy of SVF-cells was assessed by echocardiography. No significant differences at baseline were found between Control and SVF groups for any of the echocardiographic parameters. The results indicate that rats treated with SVF showed an improvement in cardiac function at 3 months, demonstrated not only by the increase in ejection fraction (from 26.46±1.32% at preimplant to 38.25±4.01% at 3 months; $P<0.01$) and fractional shortening (FS) (10.16±1.32% at preimplant to 16.3±2.01% at 3 months; $P=0.006$), but also by prevention of ventricle dilation as indicated by the limitation of the increase in the telesystolic diameter (ESD) and volume (ESV) (Table 1). By contrast, in the control group, no significant improvement in LVEF (from 29.87±2.84% at preimplant to 28.1±1.59% at 3 months; $P=0.63$) and FS (from 12.3±1.27% at preimplant to 11.34±0.77% at 3 months; $P=0.586$) was detected, while ESD and ESV deteriorated severely, indicating deleterious progression of the remodeling process (Table 1).

Consistent with the improvement in cardiac function, microPET studies revealed a significant increase in tissue metabolism within infarcted areas in animals treated with SVF-cells (from 50.6±4.4% at preimplant to 60.6±5.3% at 3 months; $P<0.05$), whereas no significant changes were detected in control animals (from 45.3±2.5% at preimplant to 42.7±1.9% at 3 months) (Fig.2).

Fate of transplanted SVF-cells

Immunohistological detection of GFP throughout the experiment (1-3 days, 1-2 weeks, and 1-3 months) revealed no positive GFP-signal in control hearts as expected (Fig.3A-F). Conversely, the presence of SVF-derived GFP cells was demonstrated at all the time-points analyzed in all cell-treated hearts (29/29) (Fig.3G-L). Cells progressively

changed their morphology from an initial fibroblastic appearance (Fig.3G-J, asterisks) towards a more differentiated one (Fig.3K-L, arrowheads). Apoptosis (Fig.3M-R), although detected early after cell transplantation (1 day: 10.2±2.2%; 3 days: 2.2±0.5%, referred to total engrafted cells) disappeared after one week, whereas relatively high levels of proliferating GFP-cells were detected within the area of injection during the first few days (1 day: 18.7±2.8%; 3 days: 22.4±1.2%, referred to total engrafted cells) and decreased afterwards (Fig.3S-X). Quantification of cell engraftment indicated low retention of injected cells 1 day after transplantation (7.4±1.1% engrafted cells) which decreased further at later time-points (Fig.3Y). Remarkably, cells could still be detected at 3 months (1.3±0.5% of total implanted cells).

SVF-cells positively affect vascularization through differentiation and paracrine mechanisms

Immunostaining demonstrated the potential of SVF-cells to differentiate towards endothelial (caveolin-1$^+$) (Fig.4A-C) and mural cells (αSMA$^+$) (Fig.4D-F) which was further confirmed by electron microscopy (TEM). Importantly, colloidal gold-stained endothelial cells formed functional vessels, as depicted by the presence of erythrocytes within the lumen (Fig.4C). Mural GFP$^+$αSMA$^+$ cells displayed pericytic morphology, surrounding newly formed capillaries (Fig.4F). Interestingly, the percentage of cells that acquired a vascular phenotype increased over time to a total of 5.2±1.8% and 9.4±2.1% GFP-positive endothelial (Fig.4G) and smooth muscle cells (Fig.4H) respectively, at 3 months. Moreover, transplanted cells expressed the pro-angiogenic cytokines VEGF, HGF and the pro-inflammatory cytokine MCP-1 (Fig.4I-K respectively, arrowheads). These factors were also secreted by host-derived cells in the proximity of the transplant (Fig.4I-K, asterisks) and the peri-infarct zone (Fig.4L-N), thus amplifying the paracrine effect induced by SVF cells. No positive cells were detected in the scar area or in the control groups (data not shown). This *in vivo* secretion of VEGF and MCP-1 was confirmed by cytokine array of the SVF-conditioned media (Fig.5A), which also induced a significant proliferation of vascular cells (endothelial and smooth-muscle cells) (Fig.5B). Given the putative vasculogenic capacity of the transplanted SVF-cells, we analyzed this aspect in more depth. As shown in Fig.6A, a higher vessel density was observed in the areas of engraftment, which again suggested the trophic effect of these cells. TEM of transplanted areas also revealed a high density of vessels (Fig.6B,C) which, while functional, had an extremely thin wall, and marked them as newly

formed/developing vessels. A significant increase in tissue vascularization was detected 3 months after transplant, both at the capillaries (small caliber-caveolin-1-positive vessels in control group: 683.9±37.8 capillaries/mm^2; SVF group: 883.9±42.7 capillaries/mm^2; P<0.05) (Fig.6E-F) and larger vessels (αSMA-positive vessels in control group: 46.3±1.9 vessels/mm^2; SVF-group: 76.4±3.4 vessels/mm^2; P<0.01) (Fig.6G-I). Thus, SVF-transplantation not only directly contributes to form new functional blood vessels, but also stimulates a response that increases the vascularization of the peri-infarct through paracrine mechanisms.

SVF transplantation strongly influences positive tissue remodeling

Despite this process of vascular differentiation, the great majority of transplanted cells (30.0±5.4% of engrafted cells at 3 months) retained a fibroblastic phenotype (Fig.7A-E). Some of the transplanted cells acquired characteristics of myofibroblasts (Fig.7F) with expression of αSMA (Fig.7G). They could be distinguished from vascular cells by absence of expression of other smooth muscle markers such as SM Myosin (not shown) and also by TEM analysis (Fig.7H-I), although their levels remained relatively low through the duration of the study (1.1±0.4% at 3 months) (Fig.7J). As the main mediators of remodeling processes, engrafted cells were screened for the expression of MMPs and their natural inhibitors TIMPs, demonstrating that SVF cells *in vitro* and *in vivo* expressed TIMP1 (Fig.7K; Fig.5A) and TIMP4 (Fig.7L) but not other MMPs (2, 9 and 14) or TIMP2, as shown by double immunofluorescence at 3 months. Moreover, immunofluorescence for TIMP4 revealed that SVF-derived cells caused a marked increase in the expression of this inhibitor, not only in the area of injection, but also in the remote myocardium (Fig.7M,N). Also, cell-injection correlated with a reduction in the expression of MMP2 in the peri-infacted area (Fig.7O,P). These results were further confirmed by zimographies performed on peripheral blood sera of SVF- and medium-treated rats, which showed a significant decrease in MMP2 activity in the SVF-treated animals (Pre-implant: 0.81±0.15%; 3 months: 0.62±0.10%; P=0.02) unlike control animals where no changes were found (Pre-implant: 0.79±0.05%; 3 months: 0.79±0.07%; P=0.98).

These changes were consistent with the morphometric analysis: transplantation of SVF-cells induced positive remodeling of the heart in comparison with control animals, suggesting a protective effect exerted by the cells. Thus, a significant smaller infarct

size (Medium: 19.7±1.2%; WAT-SVF: 10.9±3.4; P=0.001), greater infarct wall thickness (Medium: 432.6±60.9µm; WAT-SVF: 739.6±104.1µm; P=0.03) (Fig.7Q-S) with lower scar collagen content (Medium: 60.9±1.7%; WAT-SVF: 46.8±5.6%; P=0.001) (Fig.7T-V) and a lower degree of cardiac hypertrophy (Medium: 293.3±51.0µm^2; WAT-SVF: 205.9±34.3µm^2; P=0.02) (Fig.7W-Y) were all detected 3 months after SVF treatment. On the other hand, no significant direct contribution of SVF to the cardiac tissue was detected. GFP-MLC2v double-positive cells were found but the percentage was indeed very low (0.017±0.001% of engrafted cells at 2 weeks) indicating that the differentiation of SVF cells to cardiomyocytes is at most a rare event (Fig.8).

Finally, no ectopic tissue formation, such as bone, cartilage or tumor, was detected in any of the transplanted animals (not shown) and, although a relatively high percentage of the engrafted cells differentiated towards adipocytes (23.6±7.7% after 3 months) (Fig.9), there was no significant increase in adipose cells in the SVF-injected hearts *versus* the media-injected ones (not shown), suggesting that there was no significant induction of adipogenesis in the transplanted hearts.

DISCUSSION

Although the unexpected vasculogenic and cardiomyogenic potential of adipose tissue-derived stem cells has prompted their use in models of acute MI (43), significantly less information has been gathered regarding their use in chronic models. Thus, unlike recent studies in which subpopulations of adipose derived stem cells (22,44) or previously *in vitro* cultured SVF-cells were used (18,43), our research tested freshly isolated SVF, since it may contain several stem/progenitor cell populations (17) and can also be readily obtained from patients without the need for extensive processing. Albeit stromal-cultured cells may benefit from their mesenchymal nature, they may also loose the potential to give rise to all the lineages needed for the healing of the disease. Moreover, fresh-adipose cells could be implemented as an autologous therapy not only for the chronic but for the acutely infarcted patients as well. The results of our study demonstrate that transplantation of SVF-cells in chronically infarcted tissue promotes a persistent benefit in cardiac function and metabolism, by inducing tissue revascularization and protection against deleterious tissue remodeling. Furthermore, an ongoing clinical trial (PRECISE trial), in which SVF-cells have induced a beneficial effect in chronically-infarcted patients, support the effectiveness and feasibility of this therapy.

Furthermore, in contrast to previous studies in which culture adipose cells were used, our results indicate that SVF-cells responded *in vivo* to tissue signals that induced their differentiation towards cardiovascular cells (15,18). In fact, the differentiation capacity of SVF into endothelial (30) and smooth muscle (11,41) cells was consistent with recent *in vitro* and *in vivo* studies which demonstrated direct incorporation of the SVF cells into mature vessels, both capillaries and arterioles/arteries, in a hind-limb ischemia model (22,41). Similarly, cardiac-like progenitors have also been isolated *in vitro* from fresh SVF (26,29) but not from cultured adipose cells. In this sense, despite the low percentage, our study provides proof of the concept that SVF can also differentiate *in vivo* into cardiomyocytes. An important question that remains to be answered is which population of cells within the SVF is endowed with cardiac potential.

An overwhelming issue in cell therapy remains the low engraftment of transplanted cells, which diminishes the efficiency of cell therapy (reviewed in (34)). Recent studies have demonstrated that a combination of pro-survival factors (13) or the use of a matrix

support (12,23,37) favors their survival. Remarkably, in our study, despite the fact that "bare" cells were directly injected into a chronically infarcted myocardium (a more severe situation than the acute model), they could be detected up to 3 months later in relatively high levels in comparison with reported findings with other (stem) cell types (34). On the other hand, the low degree of engraftment indicates that despite the direct contribution of the transplanted cells, they cannot be responsible for the beneficial effect, making more likely that the paracrine effect is the mechanism which explains the functional results observed (32). SVF transplantation strongly induced revascularization of the heart tissue along with the secretion of the angiogenic factors VEGF and HGF, together with the inflammatory factor MCP-1. MCP-1 is known to be involved in the recruitment of a subpopulation of monocytes that positively affect the initiation of angiogenesis (2,5), which was clearly detected in the transplanted cells, adding support to the trophic hypothesis. The *in vitro* culture studies with SVF-conditioned media further confirm this mechanism.

In this regard, several reports have demonstrated the capacity of the SVF or SVF-derived cells to secrete cytokines with angiogenic properties like VEGF (15,25,33) and HGF (10) and to directly enhance the chemotaxis of progenitors by producing SDF-1 (38), possibly contributing to the increased vasculogenesis of the heart (33). Additionally, HGF could also exert a wide array of protective effects beyond induction of angiogenesis. In cardiomyopathic hamster hearts, treatment with HGF not only decreased cardiac hypertrophy, tissue fibrosis, remodeling and dysfunction, but also induced a significant benefit in cardiac function (24). Along similar lines, we have recently demonstrated in a chronic model of MI in rats that the functional benefit associated with skeletal myoblast transplant is at least partially related to the production of angiogenic cytokines by the transplanted cells (28).

On the other hand, as an evolving tissue, the remodeling heart is a complex system in which MMPs and TIMPs are tightly regulated (40). Even minor changes in their balance can end up in major histopathological and functional consequences, as reported in animal models (4) and patients (16). TIMP1 is downregulated in ischemic cardiomyopathy (16) and is able to inhibit CM apoptosis (39). TIMP4, on the other hand, is preferentially expressed in the heart (14) and has been reported to be downregulated in cardiac diseases (16,35). TIMP4 inhibits the activity of MMP2 (7) so

it is plausible that upregulation of TIMP4 in transplanted SVF-cells and injured tissues may be responsible for the downregulation of MMP2 in the peri-infarct region and the significant decrease in peripheral-serum MMP2 activity. This could intrinsically have therapeutic implications, as MMP2 is upregulated in MI (40) and may also influence cardiac regeneration by inactivating SDF1 (20), thus decreasing progenitor homing to the damaged tissue. The paracrine potential of TIMP4 may be related to its antithrombotic features (31) and its ability to regulate proper cardiac fibroblast behavior (42). Globally, SVF-derived regulation of TIMP/MMP can induce a protective balance upon injured hearts which, together with the increased revascularization of the tissue that could rescue myogenic cells at the border zone of the infarct, would avoid the progressive deleterious remodeling of the heart (as shown by detection of a smaller infarct size, thicker infarct wall and lower degree of scar fibrosis and cardiac hypertrophy in the SVF-treated hearts).

Finally, although no adverse effects were detected in the SVF-transplanted animals and no local induction of adipogenesis of host cells was induced in the hearts, it would be interesting to analyze the effects of SVF cells depleted for the pre-adipocytic population (sorted i.e. for their pref-1 specific marker) in order to elucidate the best treatment and potential clinical application.

In summary, this report demonstrates the long-term benefit of SVF-cell injection in chronic myocardial infarction and provides evidence for the *in vivo* multilineage capacity and paracrine activity of this population. Furthermore, compared with ADSC (cultured adipose-derived stem cells), the significantly faster and inexpensive processing of the cells makes them much more affordable in the clinical setting. If proven safe and effective, SVF would offer an attractive population to consider for future clinical applications for cardiac repair.

REFERENCES

1. Agbulut, O.; Mazo, M.; Bressolle, C.; Gutierrez, M.; Azarnoush, K.; Sabbah, L.; Niederlander, N.; Abizanda, G.; Andreu, E. J.; Pelacho, B.; Gavira, J.J.; Pérez-Ilzarbe, M.; Peyrard, S.; Bruneval, P.; Samuel, J-S.; Soriano-Navarro, M.; García-Verdugo, J. M.; Hagège, A. A.; Prósper, F.; Menasché, P. Can bone marrow-derived multipotent adult progenitor cells regenerate infarcted myocardium? Cardiovasc. Res. 72(1):175-183; 2006.
2. Capoccia, B. J.; Gregory, A. D.; Link, D. C. Recruitment of the inflammatory subset of monocytes to sites of ischemia induces angiogenesis in a monocyte chemoattractant protein-1-dependent fashion. J. Leukoc. Biol. 84(3):760-768; 2008.
3. Cleland, J. G.; Coletta, A. P.; Abdellah, A. T.; Cullington, D.; Clark, A. L.; Rigby, A. S. Clinical trials update from the American Heart Association 2007: CORONA, RethinQ, MASCOT, AF-CHF, HART, MASTER, POISE and stem cell therapy. Eur. J. Heart Fail. 10(1):102-108; 2008.
4. Deten, A.; Volz, H. C.; Briest, W.; Zimmer, H. G. Cardiac cytokine expression is upregulated in the acute phase after myocardial infarction. Experimental studies in rats. Cardiovasc. Res. 55(2):329-340; 2002.
5. Dewald, O.; Zymek, P.; Winkelmann, K.; Koerting, A.; Ren, G.; Abou-Khamis, T.; Michael, L. H.; Rollins, B. J.; Entman, M. L.; Frangogiannis, N. G. CCL2/Monocyte Chemoattractant Protein-1 regulates inflammatory responses critical to healing myocardial infarcts. Circ. Res. 96(8):881-889; 2005.
6. Gavira, J. J.; Perez-Ilzarbe, M.; Abizanda, G.; Garcia-Rodriguez, A.; Orbe, J.; Paramo, J. A.; Belzunce, M.; Rabago, G.; Barba, J.; Herreros, J.; Panizo, A.; García de Jalón, J. A.; Martínez-Caro, D.; Prósper, F. A comparison between percutaneous and surgical transplantation of autologous skeletal myoblasts in a swine model of chronic myocardial infarction. Cardiovasc. Res. 71(4):744-753; 2006.
7. Hernandez-Barrantes, S.; Shimura, Y.; Soloway, P. D.; Sang, Q. A.; Fridman, R. Differential roles of TIMP-4 and TIMP-2 in pro-MMP-2 activation by MT1-MMP. Biochem. Biophys. Res. Commun. 281(1):126-130; 2001.
8. Hong, S. J.; Traktuev, D. O.; March, K. L. Therapeutic potential of adipose-derived stem cells in vascular growth and tissue repair. Curr. Opin. Organ Transplant.15(1):86-91; 2010.
9. Jennings, R. B.; Murry, C. E.; Steenbergen, C., Jr.; Reimer, K. A. Development of cell injury in sustained acute ischemia. Circulation 82(3 Suppl):II2-12; 1990.
10. Kilroy, G. E.; Foster, S. J.; Wu, X.; Ruiz, J.; Sherwood, S.; Heifetz, A.; Ludlow, J. W.; Stricker, D. M.; Potiny, S.; Green, P.; Halvorsen, Y-D. C.; Cheatham, B.; Storms, R. W.; Gimble, J. M. Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors. J. Cell. Physiol. 212(3):702-709; 2007.
11. Kim, Y. M.; Jeon, E. S.; Kim, M. R.; Jho, S. K.; Ryu, S. W.; Kim, J. H. Angiotensin II-induced differentiation of adipose tissue-derived mesenchymal stem cells to smooth muscle-like cells. Int. J. Biochem. Cell Biol. 40(11):2482-2491; 2008.
12. Kitabayashi, K.; Siltanen, A.; Patila, T.; Mahar, M. A.; Tikkanen, I.; Koponen, J.; Ono, M.; Sawa, Y.; Kankuri, E.; Harjula, A. Bcl-2 expression enhances myoblast sheet transplantation therapy for acute myocardial infarction. Cell Transplant. 19(5):573-588; 2010.

13. Laflamme, M. A.; Chen, K. Y.; Naumova, A. V.; Muskheli, V.; Fugate, J. A.; Dupras, S. K.; Reinecke, H.; Xu, C.; Hassanipour, M.; Police, S.; O´Sullivan, C.; Collins, C.; Chen, Y.; Minami, E.; Gill, E. A.; Ueno, S.; Yuan, C.; Gold, J.; Murry, C. E. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat. Biotechnol. 25(9):1015-1024; 2007.
14. Leco, K. J.; Apte, S. S.; Taniguchi, G. T.; Hawkes, S. P.; Khokha, R.; Schultz, G. A.; Edwards, D. R. Murine tissue inhibitor of metalloproteinases-4 (Timp-4): cDNA isolation and expression in adult mouse tissues. FEBS Lett. 401(2-3):213-217; 1997.
15. Li, B.; Zeng, Q.; Wang, H.; Shao, S.; Mao, X.; Zhang, F.; Li, S.; Guo, Z. Adipose tissue stromal cells transplantation in rats of acute myocardial infarction. Coron. Artery Dis. 18(3):221-227; 2007.
16. Li, Y. Y.; Feldman, A. M.; Sun, Y.; McTiernan, C. F. Differential expression of tissue inhibitors of metalloproteinases in the failing human heart. Circulation 98(17):1728-1734; 1998.
17. Madonna, R.; De Caterina, R. Adipose tissue: a new source for cardiovascular repair. J. Cardiovasc. Med. 11(2):71-80; 2010.
18. Mazo, M.; Planat-Benard, V.; Abizanda, G.; Pelacho, B.; Leobon, B.; Gavira, J. J.; Penuelas, I.; Cemborain, A.; Penicaud, L.; Laharrague, P.; Joffre, C.; Boisson, M.; Ecay, M.; Collantes, M.; Barba, J.; Castiella, L.; Prósper, F. Transplantation of adipose derived stromal cells is associated with functional improvement in a rat model of chronic myocardial infarction. Eur. J. Heart Fail. 10(5):454-462; 2008.
19. McMurray, J. J.; Pfeffer, M. A. Heart failure. Lancet 365(9474):1877-1889; 2005.
20. McQuibban, G. A.; Butler, G. S.; Gong, J. H.; Bendall, L.; Power, C.; Clark-Lewis, I.; Overall, C. M. Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. J. Biol. Chem. 276(47):43503-43508; 2001.
21. Meller, J.; Herman, M. V.; Teichholz, L. E. Noninvasive assessment of left ventricular function. Adv. Intern. Med. 24:331-357; 1979.
22. Miranville, A.; Heeschen, C.; Sengenes, C.; Curat, C. A.; Busse, R.; Bouloumie, A. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. Circulation 110(3):349-355; 2004.
23. Miyahara, Y.; Nagaya, N.; Kataoka, M.; Yanagawa, B.; Tanaka, K.; Hao, H.; Ishino, K.; Ishida, H.; Shimizu, T.; Kangawa, K.; Sano, S.; Okano, T.; Kitamura, S.; Mori, H. . Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat. Med. 12(4):459-465; 2006.
24. Nakamura, T.; Matsumoto, K.; Mizuno, S.; Sawa, Y.; Matsuda, H.; Nakamura, T. Hepatocyte growth factor prevents tissue fibrosis, remodeling, and dysfunction in cardiomyopathic hamster hearts. Am. J. Physiol. Heart Circ. Physiol. 288:H2131–H2139; 2005.
25. Nie, C.; Yang, D.; Xu, J.; Si, Z.; Jin, X.; Zhang, J. Locally Administered Adipose-derived Stem Cells Accelerate Wound Healing through Differentiation and Vasculogenesis. Cell Transplant. 2010.
26. Palpant, N. J.; Yasuda, S.; MacDougald, O.; Metzger, J. M. Non-canonical Wnt signaling enhances differentiation of Sca1+/c-kit+ adipose-derived murine stromal vascular cells into spontaneously beating cardiac myocytes. J. Mol. Cell. Cardiol. 43(3):362-370; 2007.

27. Penuelas, I.; Abizanda, G.; Garcia-Velloso, M. J.; Gavira, J. J.; Marti-Climent, J. M.; Ecay, M.; Collantes, M.; Garcia de Jalon, J. A.; Garcia-Rodriguez, A.; Mazo, M.; Barba, J.; Richter, J. A.; Prósper, F. 18F-FDG metabolism in a rat model of chronic infarction: a 17-sector semiquantitative analysis. Nuklearmedizin 46(4):149-154; 2007.

28. Perez-Ilzarbe, M.; Agbulut, O.; Pelacho, B.; Ciorba, C.; San Jose-Eneriz, E.; Desnos, M.; Hagege, A. A.; Aranda, P.; Andreu, E. J.; Menasche, P.; Prósper, F. Characterization of the paracrine effects of human skeletal myoblasts transplanted in infarcted myocardium. Eur. J. Heart Fail. 10(11):1065-1072; 2008.

29. Planat-Benard, V.; Menard, C.; Andre, M.; Puceat, M.; Perez, A.; Garcia-Verdugo, J. M.; Penicaud, L.; Casteilla, L. Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. Circ. Res. 94(2):223-229; 2004.

30. Planat-Benard, V.; Silvestre, J. S.; Cousin, B.; Andre, M.; Nibbelink, M.; Tamarat, R.; Clergue, M.; Manneville, C.; Saillan-Barreau, C.; Duriez, M. and others. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 109(5):656-663; 2004.

31. Radomski, A.; Jurasz, P.; Sanders, E. J.; Overall, C. M.; Bigg, H. F.; Edwards, D. R.; Radomski, M. W. Identification, regulation and role of tissue inhibitor of metalloproteinases-4 (TIMP-4) in human platelets. Br. J. Pharmacol. 137(8):1330-1338; 2002.

32. Ramos, G. A.; Hare, J. M. Cardiac cell-based therapy: cell types and mechanisms of actions. Cell Transplant. 16(9):951-961; 2007.

33. Rehman, J.; Traktuev, D.; Li, J.; Merfeld-Clauss, S.; Temm-Grove, C. J.; Bovenkerk, J. E.; Pell, C. L.; Johnstone, B. H.; Considine, R. V.; March, K. L. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation 109(10):1292-1298; 2004.

34. Robey, T. E.; Saiget, M. K.; Reinecke, H.; Murry, C. E. Systems approaches to preventing transplanted cell death in cardiac repair. J. Mol. Cell. Cardiol. 45(4):567-81; 2008.

35. Schulze, C. J.; Wang, W.; Suarez-Pinzon, W. L.; Sawicka, J.; Sawicki, G.; Schulz, R. Imbalance between tissue inhibitor of metalloproteinase-4 and matrix metalloproteinases during acute myocardial [correction of myoctardial] ischemia-reperfusion injury. Circulation 107(19):2487-2492; 2003.

36. Segers, V. F.; Lee, R. T. Stem-cell therapy for cardiac disease. Nature 451(7181):937-942; 2008.

37. Sekine, H.; Shimizu, T.; Hobo, K.; Sekiya, S.; Yang, J.; Yamato, M.; Kurosawa, H.; Kobayashi, E.; Okano, T. Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts. Circulation 118(14 Suppl):S145-152; 2008.

38. Sengenes, C.; Miranville, A.; Maumus, M.; de Barros, S.; Busse, R.; Bouloumie, A. Chemotaxis and differentiation of human adipose tissue CD34+/CD31- progenitor cells: role of stromal derived factor-1 released by adipose tissue capillary endothelial cells. Stem Cells 25(9):2269-2276; 2007.

39. Singla, D. K.; Singla, R. D.; McDonald, D. E. Factors released from embryonic stem cells inhibit apoptosis in H9c2 cells through PI3K/Akt but not ERK pathway. Am. J. Physiol. Heart Circ. Physiol. 295(2):H907-913; 2008.

40. Spinale, F. G. Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function. Physiol. Rev. 87(4):1285-1342; 2007.

41. Sumi, M.; Sata, M.; Toya, N.; Yanaga, K.; Ohki, T.; Nagai, R. Transplantation of adipose stromal cells, but not mature adipocytes, augments ischemia-induced angiogenesis. Life Sci. 80(6):559-565; 2007.
42. Tummalapalli, C. M.; Heath, B. J.; Tyagi, S. C. Tissue inhibitor of metalloproteinase-4 instigates apoptosis in transformed cardiac fibroblasts. J Cell. Biochem. 80(4):512-521; 2001.
43. Valina, C.; Pinkernell, K.; Song, Y. H.; Bai, X.; Sadat, S.; Campeau, R. J.; Le Jemtel, T. H.; Alt, E. Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular function, perfusion, and remodelling after acute myocardial infarction. Eur. Heart J. 28(21):2667-2677; 2007.
44. Yamada, Y.; Wang, X. D.; Yokoyama, S.; Fukuda, N.; Takakura, N. Cardiac progenitor cells in brown adipose tissue repaired damaged myocardium. Biochem. Biophys. Res. Commun. 342(2):662-670; 2006.

FIGURE LEGENDS

Figure 1: Phenotypic characterization of SVF cells. SVF cells were stained with antibodies against RT1A, RT1B, CD11b, CD31, CD44, CD45, CD73, CD90 and CD106 (red line) or isotype controls (dashed lines).

Figure 2: MicroPET evaluation of tissue metabolism measured by ^{18}F-FDG-uptake. **(A)** ^{18}F-FDG-uptake was evaluated by microPET before and after 3 months of transplantation. Results are expressed as the percentage (mean±SD) of ^{18}F-FDG-uptake in the infarcted area. *P<0.05 (3months *versus* pre-implant). **(B)** Representative images of tissue-polar maps reconstructions, 3 months after SVF-cells or medium injection.

Figure 3: Engraftment of SVF-cells. SVF-engraftment was examined between 1-90 days post-transplantation. Representative images of animals injected with medium **(A-F)** or SVF **(G-L)** are shown. Cell engraftment was detected at all time-points by GFP-immune staining. Undifferentiated cells were mainly identified at early time-points **(G-J,asterisks)** whereas multiple GFP-derived phenotypes were present at 1 and 3 months **(K,L,arrowheads)**. GFP$^+$C3a$^+$-apoptotic cells were detected up to 3 days **(M,N,asterisks)** but not thereafter **(O-R)**. Engrafted proliferating GFP$^+$Ki67$^+$-cells **(asterisks)** were identified at different time-points **(S-X)**. Quantification (mean±SD) of SVF-engraftment (*versus* total injected cells) -apoptosis and -proliferation (*versus* number of engrafted cells) **(Y)**. Scale bars: 20µm.

Figure 4: SVF-cells differentiate towards vascular phenotypes *in vivo*. Endothelial and mural-cell phenotypes were tested by immunohistochemistry **(A,D)** and double immunofluorescence for GFP and lineage-specific markers **(B,E)** was tested by . Representative pictures are shown **(arrowheads)**. TEM-analysis confirmed the results, finding endothelial- and pericytic-GFP-colloidal gold-positive cells **(C,F arrowheads; e: erythrocyte)**. Quantification of transplanted cell differentiation towards endothelium and smooth muscle was performed by counting the number of cells double-positive for GFP and lineage specific markers (Caveolin-1 and α-SMA respectively). Levels of endothelial cells **(G)** and pericytes **(H)** were expressed as a percentage of total number of engrafted cells. Results represent the mean±SD. Expression of VEGF **(I, arrowheads)**, HGF **(J, arrowheads)** and MCP1 **(K, arrowheads)** in transplanted cells

and in host cells in the proximity of the transplant **(I-K, asterisks)** and in the peri-infarct area (**L-N, asterisks**). Scale bars: 15 µm : **(A,B,D,E)**, 2 µm **(C,F)**, 25 µm **(I-N)**, 8 µm **(I-K, inserts)**, 50 µm.

Figure 5: Cytokine release pattern of the SVF conditioned media and *in vitro* proliferative effect. Angiogenic and inflammatory cytokine levels were analized utilizing arrays specific for rat (RayBio-Rat-I). To obtain culture supernatants, freshly isolated rat SVF were cultured in DMEM/10% FBS at a density of $5x10^5$ cells/cm^2 until reach confluence (72h), under normoxic or hypoxic (5% O_2) conditions. Then, media was changed for DMEM-5% FBS and collected and frozen 48h later. Frames highlight factors that are not present in control arrays (incubated with DMEM/5%FBS) **(A)**. The vasculogenic potential of the SVF secreted factors was confirmed *in vitro* by culturing the endothelial (MS-I) and smooth muscle cell (RAO) lines for 60h in the presence of non-conditioned media (NCM) or SVF-conditioned media obtained under hypoxic or normoxic conditions and viable cell number quantified. Representative pictures show the cell density after treatment. The graphs represent the mean fold increase (*versus* NCM) ±SD, quantified in 4 independent experiments, each performed in quadruplicates (**: P<0.01) **(B)**.

Figure 6. SVF- injection induces increased vasculogenesis *in vivo*. Higher vessel density was detected inside the engrafted area and in the vicinity **(A)** which was confirmed by TEM, showing a multitude of vessels in the peri-infarct area with an extremely thin but active cytoplasm **(B)**, characteristic of neovessels. TEM analysis of treated tissues also allowed reconstruction by serial imaging, showing a higher degree of vascularization within the area of injection **(C)**. Capillary and arterioles/arteries densities (mean±SD) were respectively determined by quantification of caveolin-1 positive capillaries/mm^2 **(D-F)** and αSMA-positive arterial vessels/mm^2 **(G-I)** in the perinfarcted area of animals treated with media **(D,G)** or SVF **(E,H)**, 3 months after transplantation. *P<0.05 and **P<0.01. Scale bars: **(A)**, 20 µm **(B)**, 100 µm **(C)**, 25 µm **(D,E)**, 100 µm **(G,H)**.

Figure 7: SVF-cells positively affect tissue remodeling i*n vivo*. A percentage of engrafted cells retained a fibrocytic phenotype **(A)**, not staining with antibodies specific for smooth or cardiac muscle or endothelium **(B)**. After colloidal-gold staining for GFP,

this subset of cells were also found by TEM **(C,D).** Some fusiform cells **(F)** stained positive for αSMA (and negative for SM Myosin), identifying them as myofibroblast **(G),** which was confirmed by TEM **(H,I)**. Quantification of transplanted cell differentiation towards fibroblasts and myofibroblasts was performed by counting the number of GFP positive cells and double-positive for GFP and α-SMA. Levels of fibroblasts **(E)** and myofibroblasts **(J)** were expressed as a percentage of the total number of engrafted cells. Fibroblastic GFP-cells expressed TIMP1 **(K)** and TIMP4 **(L)**. Cell-injection increased TIMP4 expression in the myocardium **(N)** in comparison with the non-treated ones **(M)** and also decreased MMP2 in the peri-infarcted area of the SVF-hearts **(P)** *versus* the control hearts **(O)**. Infarct size (percentage of infarcted area *vs.* total left ventricle area; %IA *vs.* LVA)**(Q-S)** and scar fibrosis (percentage of collagen volume fraction; %CVF)**(T-V)**, were determined in Sirius Red serial stained sections while hypertrophy was determined by quantification of the cardiomyocyte size (μm^2) in laminin immunostained serial sections **(W-Y)**, 3 months post-transplantation. Results represent the mean±SD. *P<0.05; **P<0.01. Scale bars: 15 µm **(A,B,F,G)**, 2 µm **(C,H)**, 0.25 µm **(D,I)**, 25 µm **(K,L)**, 2 mm **(M,N)**, 50 µm **(O,P,T-X)**, 250 µm **(Q,R)**.

Figure 8: SVF-derived cells differentiate into cardiomyocyte-like cells *in vivo*. Confocal laser images demonstrated presence of GFP^+ cells with the morphology of CM, positive for the cardiac marker MCL2v **(A)**. Quantification of cardiac differentiated cells was assessed by counting the number of double-positive cells (GFP and MLC2v), and expressed as a percentage of total number of engrafted cells **(B).** Results represent the mean±SD. Scale bars: 15 µm.

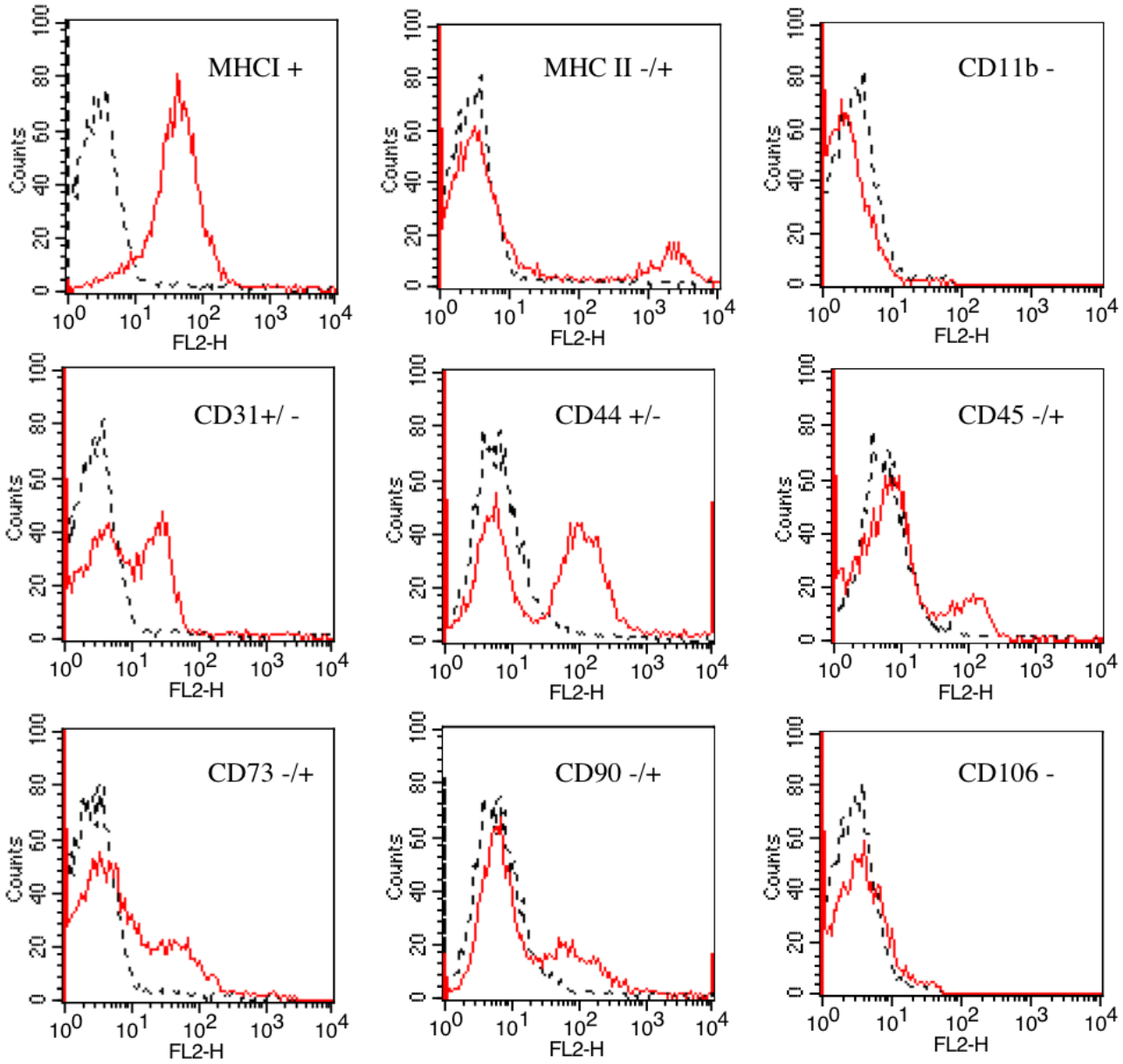
Figure 9: SVF-derived cells give rise to adipocytes. Transplanted cells also gave rise to adipocytes **(A)**, which was further confirmed by TEM showing colloidal gold-stained cells with characteristics of adipose cells **(B)**. Quantification between 1 week and 3 months was assessed by counting the number of GFP^+ cells with morphology of adipocytes and expressed as a percentage of total number of engrafted cells **(C).** Results represent the mean±SD. Scale bars: 15 µm **(A)**, 2 µm **(B)**, 1 µm **(B, insert)**.

TABLES

Table 1: Echocardiography studies

EF: Ejection Fraction; FS: Fractional Shortening; EDD: end-diastolic diameter; EDV: end-diastolic volume; ESD: end-systolic diameter; ESV: end-systolic volume. Values are mean (95% confidence interval). *P<0.05; **P<0.01 (3months *versus* pre-implant).

		PRE-IMPLANT	3 MONTHS	*P*
LVEF (%)	CONTROL	29.87 (23.45,36.29)	28.1 (24.51,31.69)	0.633
	SVF	26.46 (17.48,32.02)	38.25 (28.77,47.73)	0.005**
FS (%)	CONTROL	12.3 (9.42,15.18)	11.34 (9.61,13.07)	0.586
	SVF	10.16 (7.04,13.29)	16.3 (11.54,21.05)	0.006**
EDD (cm)	CONTROL	0.80 (0.73,0.87)	0.90 (0.81,0.98)	0.098
	SVF	0.83 (0.79,0.86)	0.87 (0.79,0.97)	0.259
ESD (cm)	CONTROL	0.69 (0.64,0.73)	0.78 (0.70,0.87)	0.023*
	SVF	0.74 (0.63,0.86)	0.74 (0.62,0.85)	0.818
EDV (ml)	CONTROL	1.07 (0.95,1.34)	1.52 (1.07,1.96)	0.105
	SVF	1.28 (0.87,1.70)	1.48 (1.03,1.96)	0.218
ESV (ml)	CONTROL	0.74 (0.63,0.85)	1.10 (0.76,1.45)	0.040*
	SVF	0.99 (0.61,1.36)	0.96 (0.57,1.34)	0.790



SVF

Fig.1

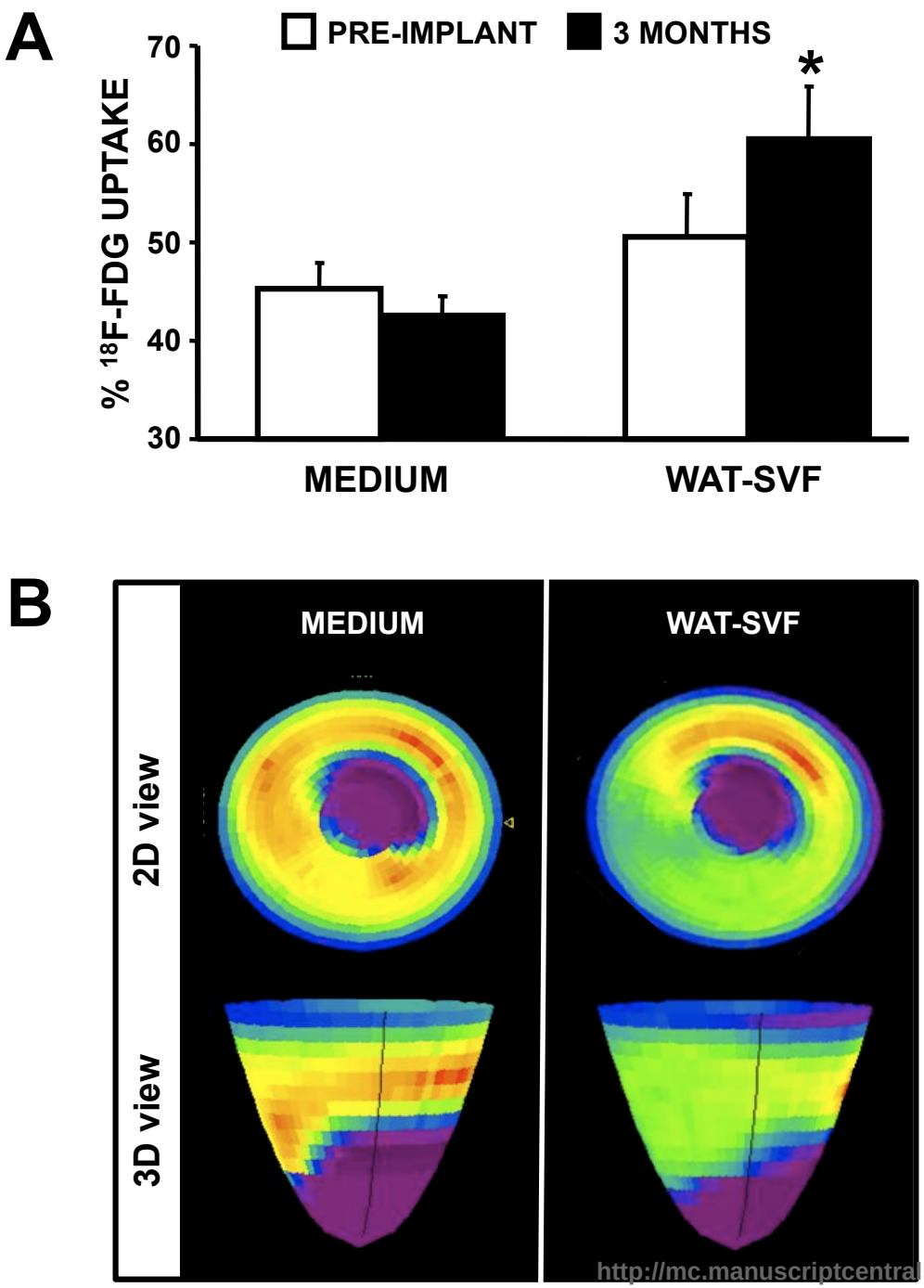


Fig. 2

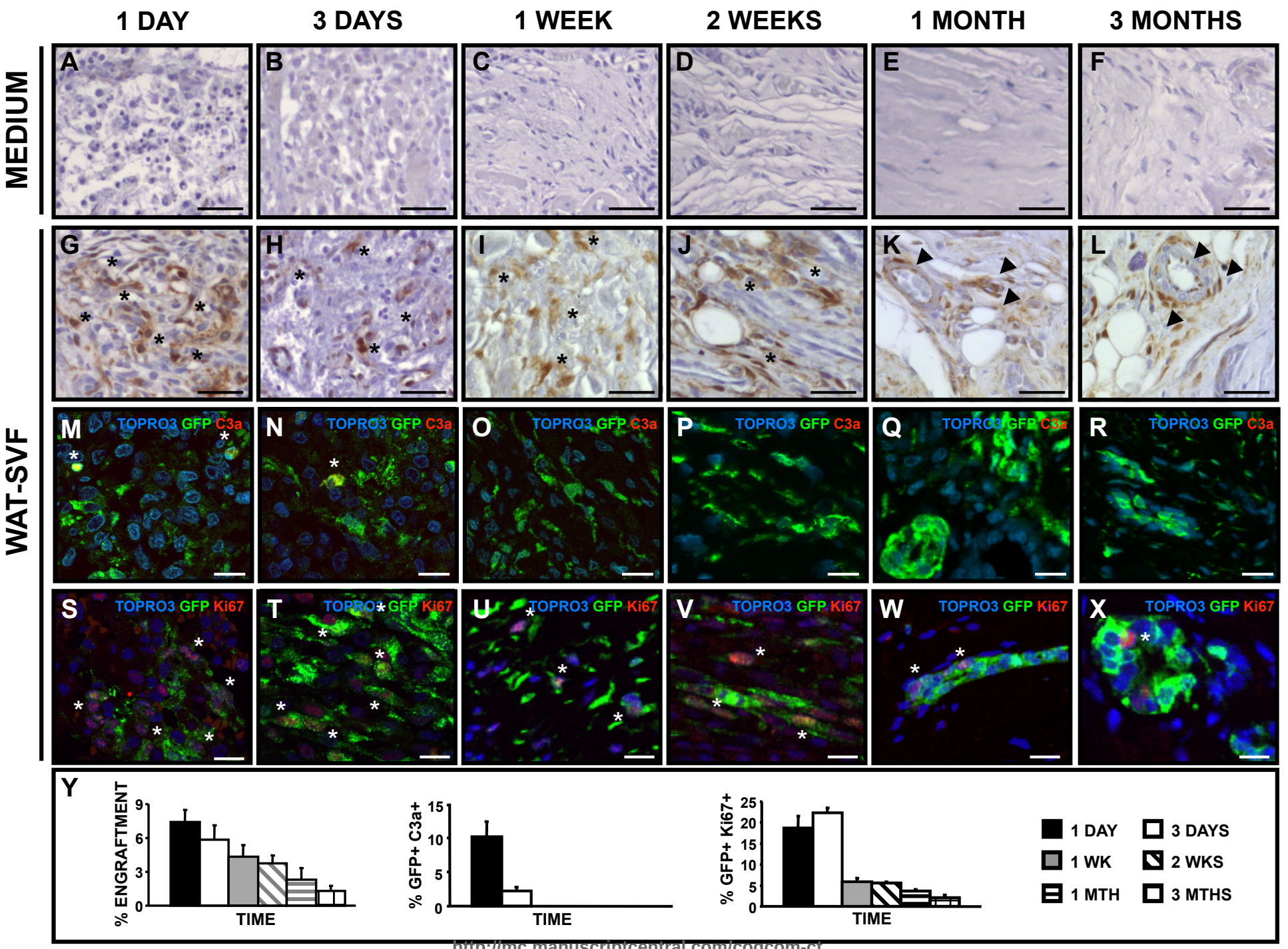

1 DAY
3 DAYS
1 WEEK
2 WEEKS
1 MONTH
3 MONTHS
MEDIUM
WAT-SVF
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
TOPRO3 GFP C3a
TOPRO3 GFP Ki67
Y
% ENGRAFTMENT
% GFP+ C3a+
% GFP+ Ki67+
TIME
1 DAY
3 DAYS
1 WK
2 WKS
1 MTH
3 MTHS

Fig. 3

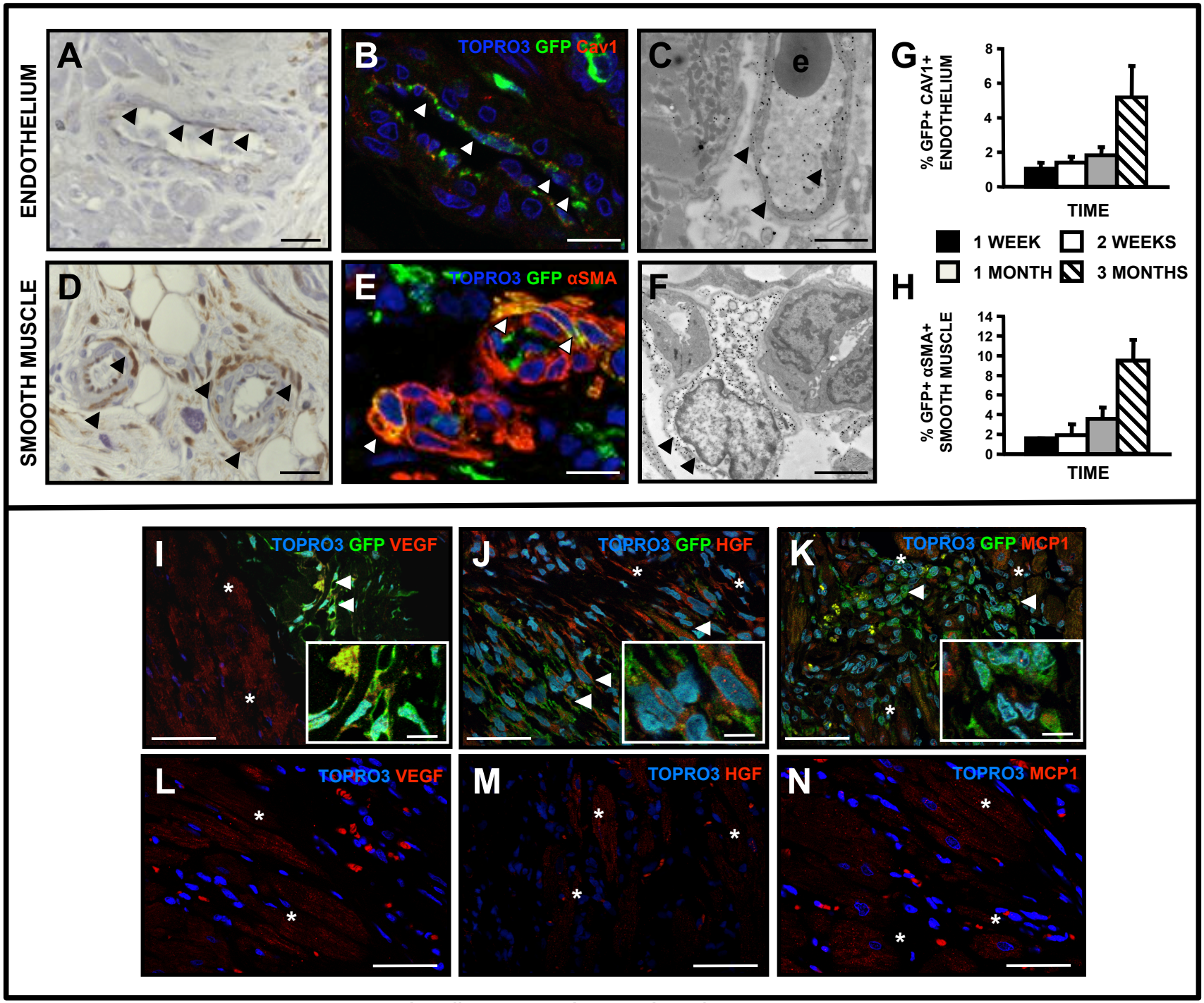

ENDOTHELIUM
SMOOTH MUSCLE
A
B
TOPRO3 GFP Cav1
C
e
D
E
TOPRO3 GFP αSMA
F
G
% GFP+ CAV1+ ENDOTHELIUM
TIME
1 WEEK
2 WEEKS
1 MONTH
3 MONTHS
H
% GFP+ αSMA+ SMOOTH MUSCLE
TIME
I
TOPRO3 GFP VEGF
J
TOPRO3 GFP HGF
K
TOPRO3 GFP MCP1
L
TOPRO3 VEGF
M
TOPRO3 HGF
N
TOPRO3 MCP1

Fig. 4

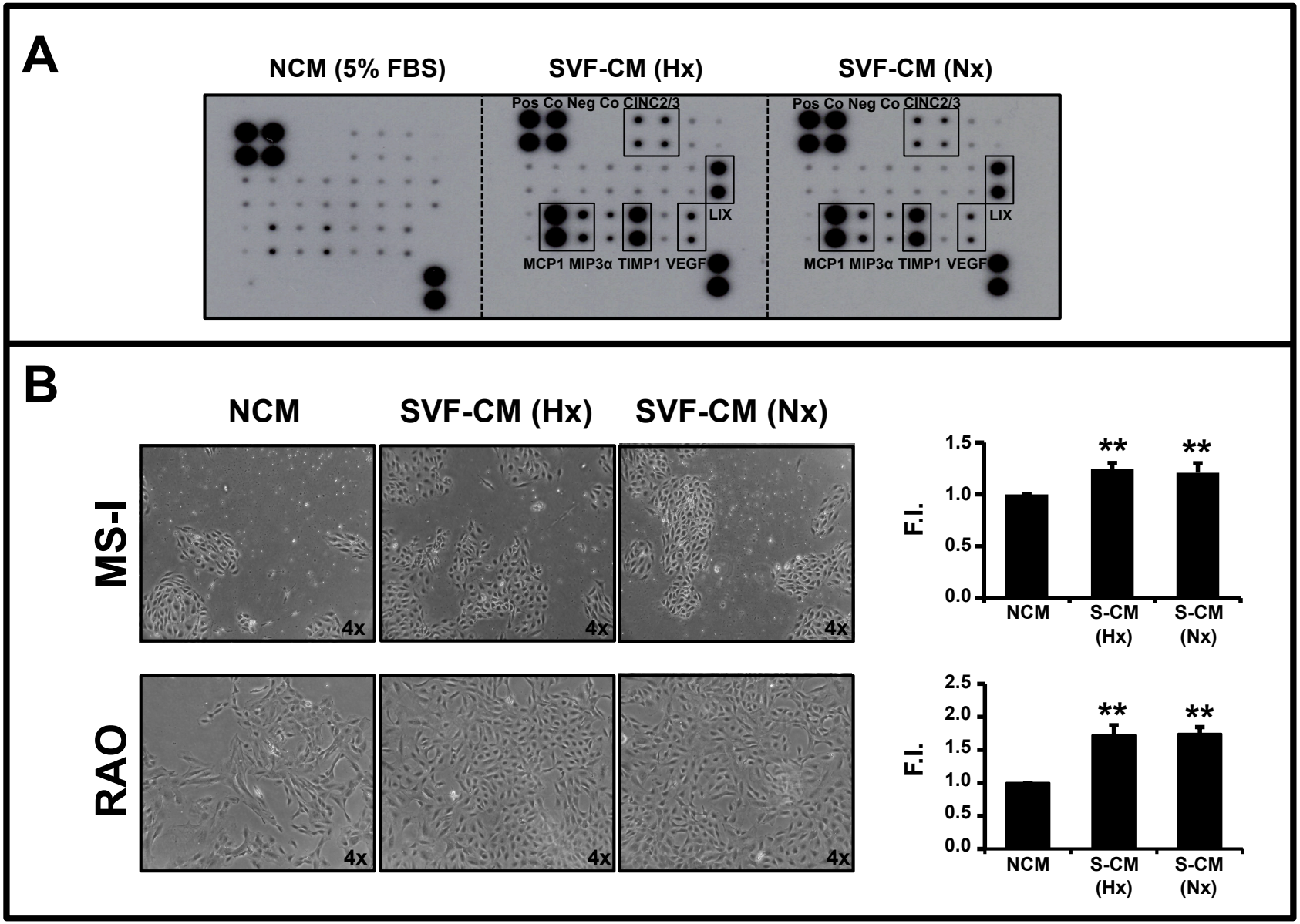

A
NCM (5% FBS)
SVF-CM (Hx)
SVF-CM (Nx)
Pos Co Neg Co CINC2/3
LIX
MCP1 MIP3α TIMP1 VEGF
B
NCM
SVF-CM (Hx)
SVF-CM (Nx)
MS-I
RAO
4x
F.I.
1.5
1.0
0.5
0.0
**
NCM
S-CM (Hx)
S-CM (Nx)
2.5
2.0

Fig. 5

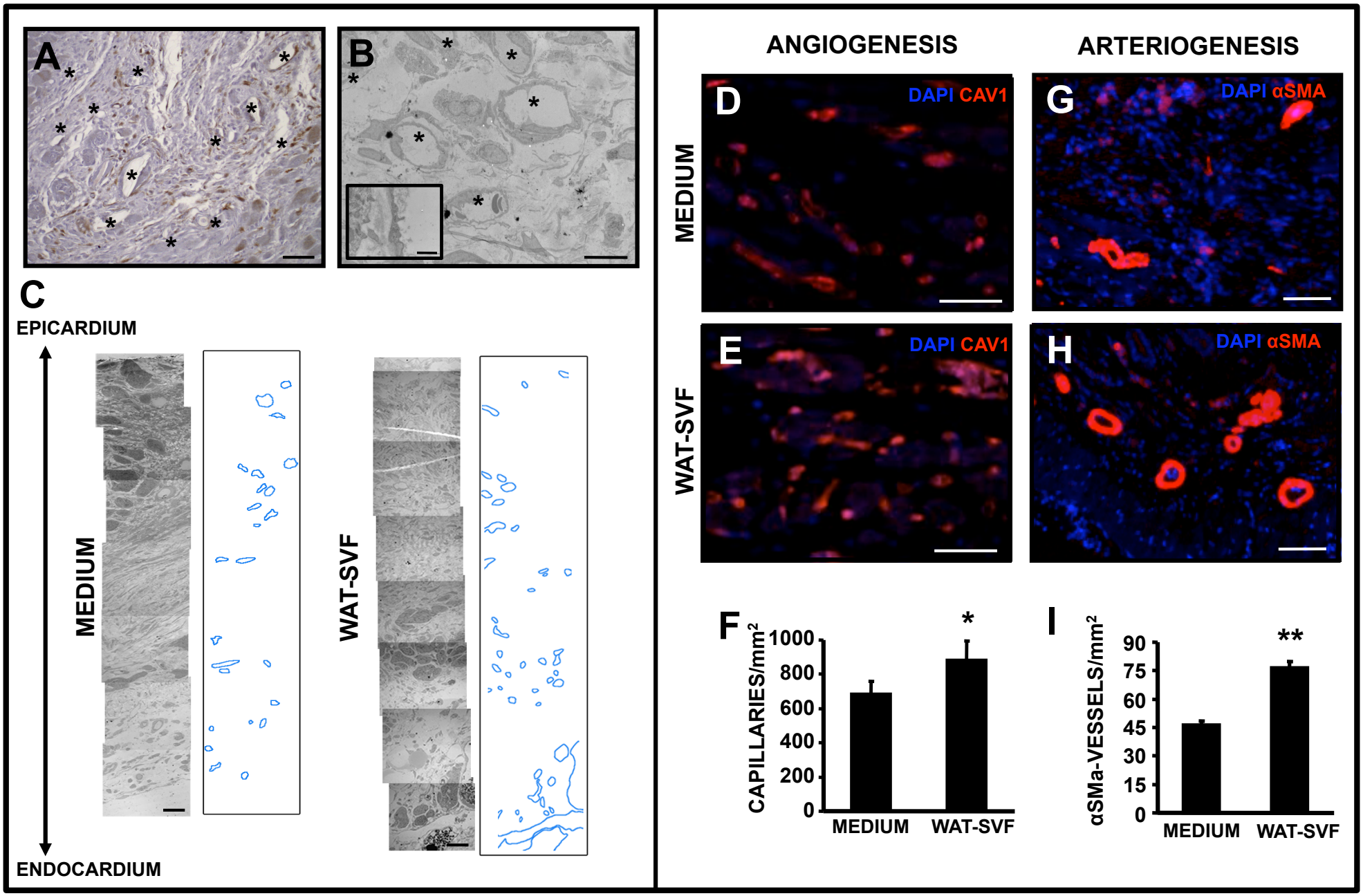

A
B
C
EPICARDIUM
MEDIUM
WAT-SVF
ENDOCARDIUM
ANGIOGENESIS
ARTERIOGENESIS
D
DAPI CAV1
G
DAPI αSMA
MEDIUM
E
DAPI CAV1
H
DAPI αSMA
WAT-SVF
F
CAPILLARIES/mm²
1000
800
600
400
200
0
*
MEDIUM
WAT-SVF
I
αSMa-VESSELS/mm²
90
75
60
45
30
15
0
**
MEDIUM
WAT-SVF

Figure 6

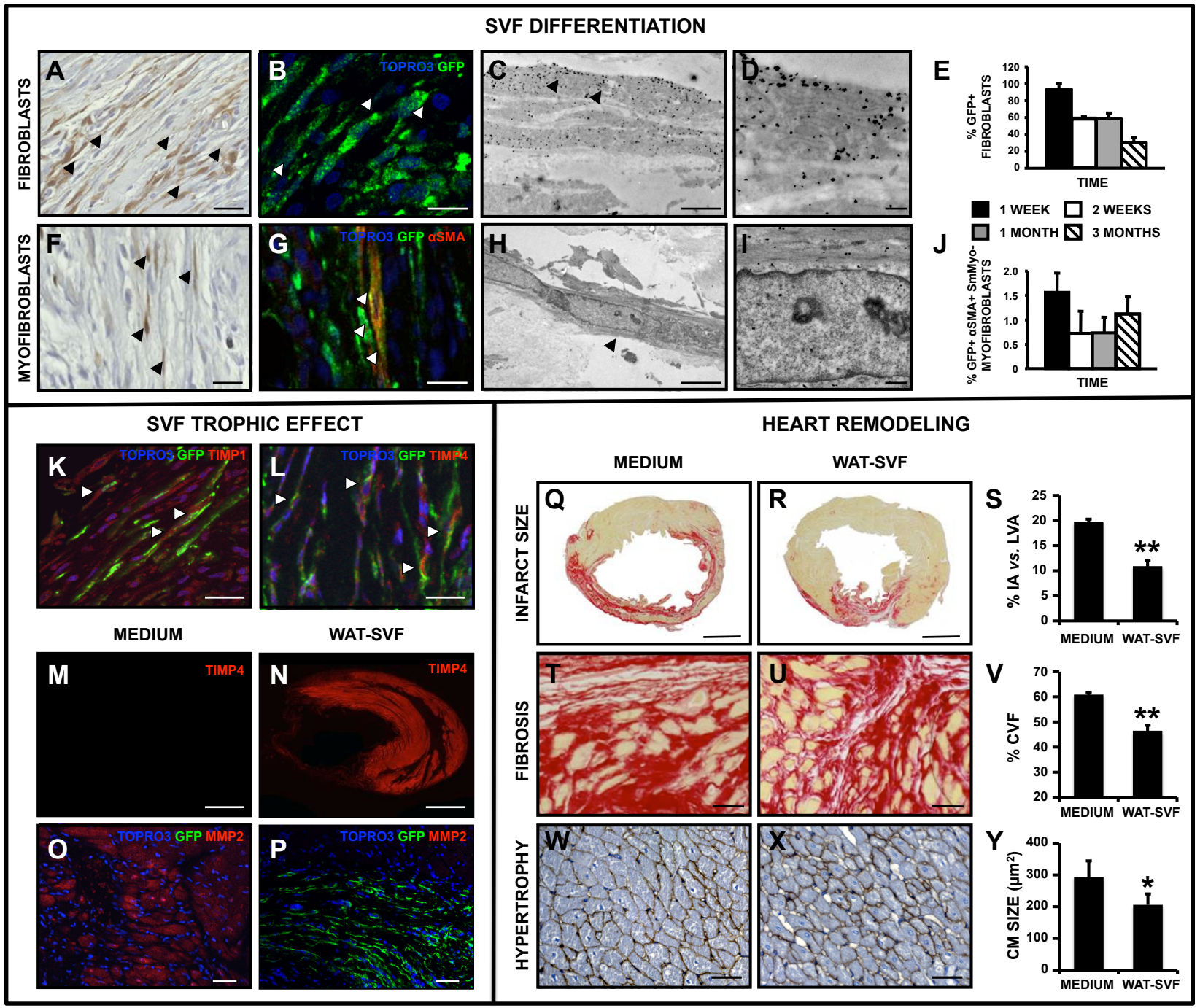

SVF DIFFERENTIATION
FIBROBLASTS
MYOFIBROBLASTS
A
B
TOPRO3 GFP
C
D
E
% GFP+ FIBROBLASTS
TIME
F
G
TOPRO3 GFP αSMA
H
I
J
1 WEEK
2 WEEKS
1 MONTH
3 MONTHS
% GFP+ αSMA+ SmMyo- MYOFIBROBLASTS
TIME
SVF TROPHIC EFFECT
K
TOPRO3 GFP TIMP1
L
TOPRO3 GFP TIMP4
MEDIUM
WAT-SVF
M
TIMP4
N
TIMP4
O
TOPRO3 GFP MMP2
P
TOPRO3 GFP MMP2
HEART REMODELING
MEDIUM
WAT-SVF
INFARCT SIZE
Q
R
S
% IA vs. LVA
MEDIUM
WAT-SVF
FIBROSIS
T
U
V
% CVF
MEDIUM
WAT-SVF
HYPERTROPHY
W
X
Y
CM SIZE (μm²)
MEDIUM
WAT-SVF

Fig. 7

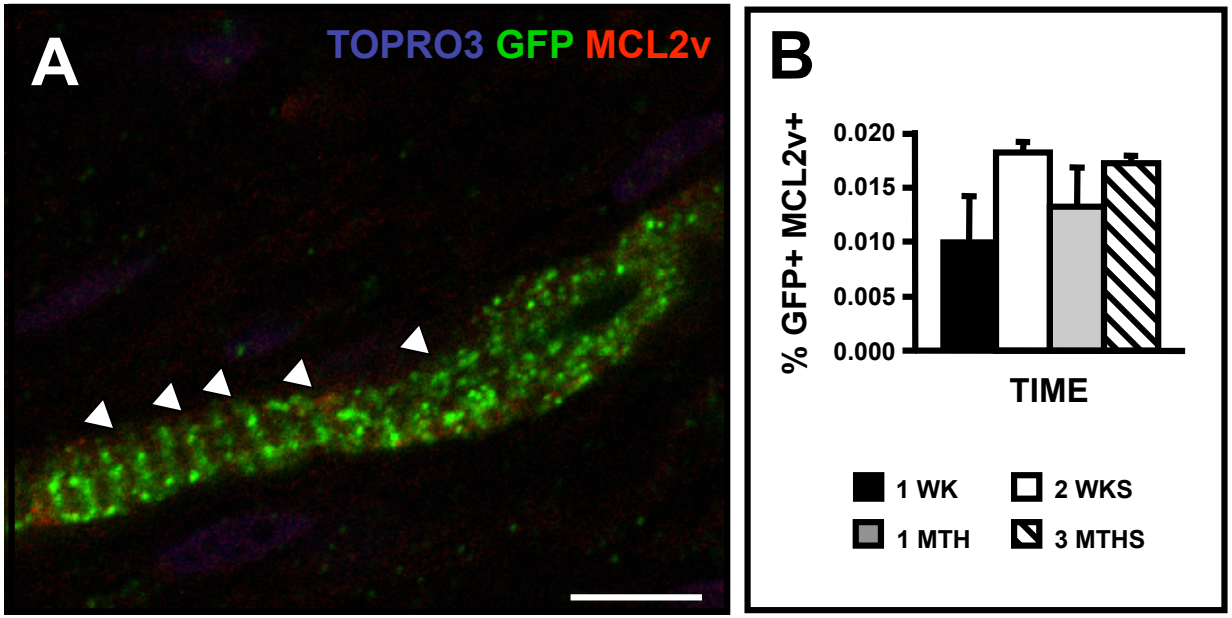

A
TOPRO3 GFP MCL2v
B
% GFP+ MCL2v+
0.020
0.015
0.010
0.005
0.000
TIME
1 WK
2 WKS
1 MTH
3 MTHS

Fig. 8

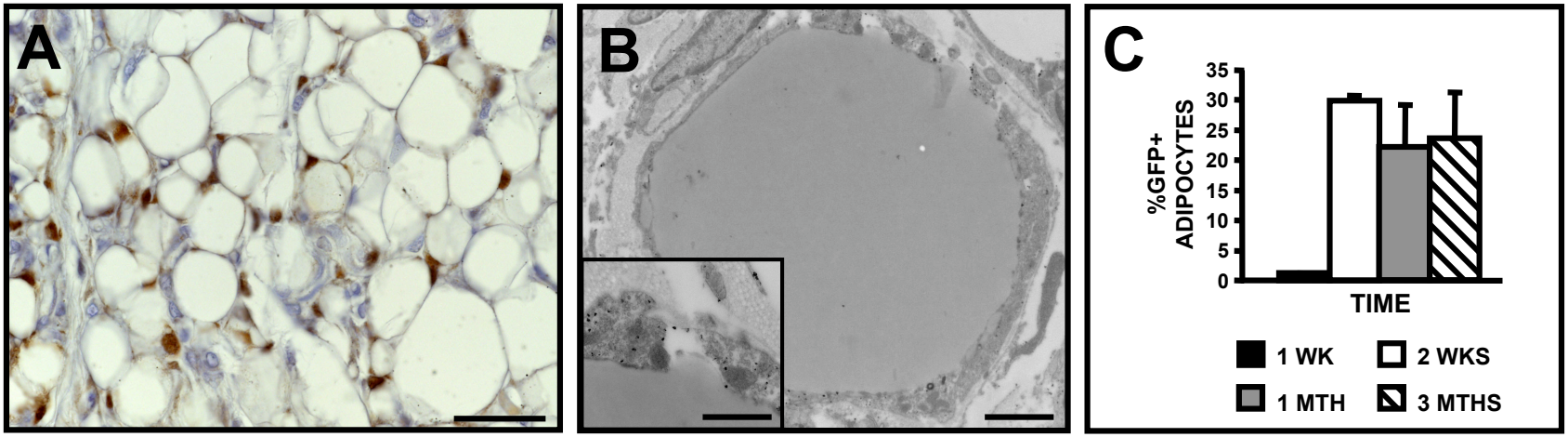
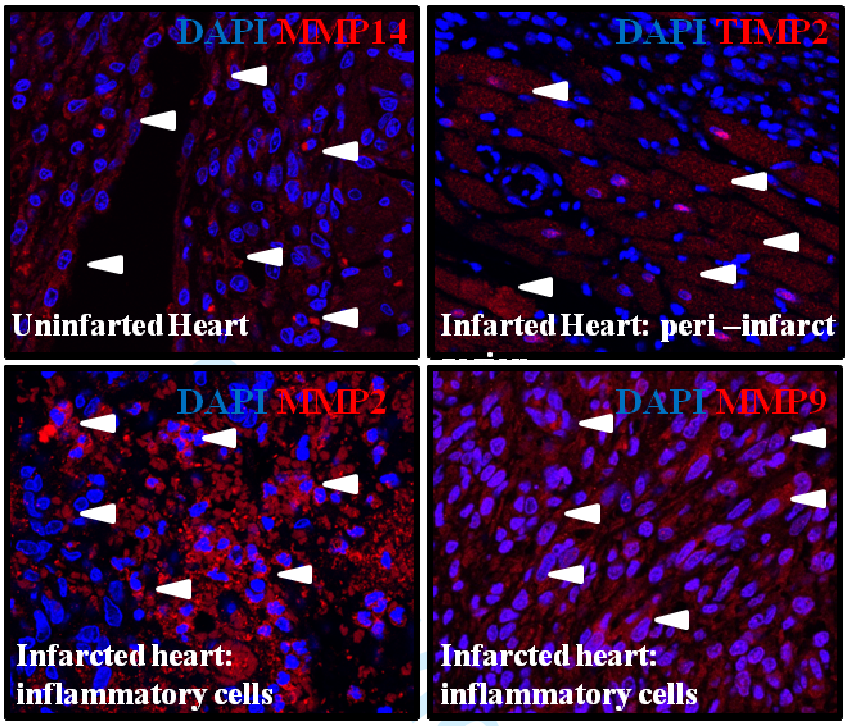

A
B
C
%GFP+ ADIPOCYTES
35
30
25
20
15
10
5
0
TIME
1 WK
2 WKS
1 MTH
3 MTHS

Fig. 9

FIGURE R1 (in answer to Reviewer 2, question 2)



ANEXO II

CELL TRANSPLANTATION
The Regenerative Medicine Journal

Treatment of reperfused ischemia with adipose-derived stem cells in a preclinical swine model of myocardial infarction

Journal:	*Cell Transplantation*
Manuscript ID:	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Mazo, Manuel; Clínica Universidad de Navarra, Hematology and Cell Therapy Hernández, Salomón; Universidad Panamericana Gavira, Juan José; Clínica Universidad de Navarra, University of Navarra, Department of Cardiology and Cardiovascular Surgery Abizanda, Gloria; Clínica Universidad de Navarra, Hematology and Cell Therapy Araña, Miriam; Clínica Universidad de Navarra, Hematology and Cell Therapy Lopez, Tania; Clínica Universidad de Navarra, Hematology and Cell Therapy Moreno, Cristina; Clínica Universidad de Navarra, University of Navarra, Immunology Service Merino, Juana; Clínica Universidad de Navarra, University of Navarra, Immunology Service Martino-Rodríguez, Alba; University of Zaragoza, Animal Pathology Uixeira, Alicia; University of Zaragoza, Animal Pathology García de Jalón, José A.; University of Zaragoza, Animal Pathology Martínez-Caro, Diego; Clínica Universidad de Navarra, Department of Cardiology and Cardiovascular Surgery Prosper, Felipe; Clínica Universidad de Navarra, Hematology and Cell Therapy
Keywords:	Adipose stem cells, Cardiac ischemia/reperfusion, Angiogenesis, Heart remodeling
Abstract:	AIMS: To determine the long-term effect of transplantation of adipose-derived stromal cells (ADSC) in a preclinical model of ischemia/reperfusion (I/R). METHODS AND RESULTS: I/R was induced in 20 Goettingen minipigs by 120 minutes coronary artery occlusion followed by reperfusion. Nine days later, animals were allocated to receive trans-endocardial injection of a mean of 213.6±41.78 million GFP-expressing ADSC

	(n=10) or culture medium as control (n=10). Heart function, cell engraftment and histological analysis were performed 3 months after transplantation. Transplantation of ADSC induced a statistically significant long-lasting (3 months) improvement in cardiac function and geometry in comparison with control animals. Functional improvement was associated with an increase in angiogenesis and vasculogenesis and a positive effect on heart remodeling with a decrease in fibrosis and cardiac hypertrophy in animals treated with ADSC. Despite the lack of cell engraftment after 3 months, ADSC transplantation induced changes in the ratio between MMP/TIMP. CONCLUSION: Our results indicate that transplantation of ADSC, despite the lack of long-term significant cell engraftment, increases vessel density and prevents adverse remodeling in a clinically relevant model of myocardial infarction, strongly suggesting a paracrine mediated effect. ADSC thus constitute an attractive candidate for the treatment of myocardial infarction. Mazo ADSC Abstract_CT.doc

Treatment of reperfused ischemia with adipose-derived stem cells in a preclinical swine model of myocardial infarction

Manuel Mazo[1], Salomón Hernández[2], Juan José Gavira[3], Gloria Abizanda[1], Miriam Araña[1], Tania López-Martínez[2], Cristina Moreno[4], Juana Merino[4], Alba Martino-Rodríguez[5], Alicia Uixeira[5], José A. García de Jalón[5], Diego Martínez-Caro[3], and Felipe Prosper[1,*]

[1]Hematology and Cell Therapy, Clinica Universidad de Navarra, Pamplona, Spain

[2]Univesidad Panamericana, Mexico City

[3]Department of Cardiology and Cardiovascular Surgery, Clinica Universidad de Navarra, Pamplona, Spain

[4]Immunology Service, Clinica Universidad de Navarra, Pamplona, Spain

[5]Department of Animal Pathology, Veterinary Faculty, University of Zaragoza

Running Title: ADSC in preclinical I/R

Address for Correspondence:
Felipe Prósper MD, PhD
Hematology and Cell Therapy
Clínica Universitaria
Av. Pío XII 36, Pamplona 31008,
Navarra, Spain
Phone 34 948 255400 Fax 34 948 296500
e-mail: fprosper@unav.es

Word count: Abstract: 200; body text: 4863

ABSTRACT

AIMS: To determine the long-term effect of transplantation of adipose-derived stromal cells (ADSCs) in a preclinical model of ischemia/reperfusion (I/R).

METHODS AND RESULTS: I/R was induced in 20 Goettingen minipigs by 120 minutes coronary artery occlusion followed by reperfusion. Nine days later, animals were allocated to receive trans-endocardial injection of a mean of 213.6±41.78 million green fluorescent protein (GFP)-expressing ADSCs (n=10) or culture medium as control (n=10). Heart function, cell engraftment and histological analysis were performed 3 months after transplantation.

Transplantation of ADSCs induced a statistically significant long-lasting (3 months) improvement in cardiac function and geometry in comparison with control animals. Functional improvement was associated with an increase in angiogenesis and vasculogenesis and a positive effect on heart remodeling with a decrease in fibrosis and cardiac hypertrophy in animals treated with ADSCs. Despite the lack of cell engraftment after 3 months, ADSC transplantation induced changes in the ratio between MMP/TIMP.

CONCLUSION: Our results indicate that transplantation of ADSCs, despite the lack of long-term significant cell engraftment, increases vessel density and prevents adverse remodeling in a clinically relevant model of myocardial infarction, strongly suggesting a paracrine mediated effect. ADSCs thus constitute an attractive candidate for the treatment of myocardial infarction.

Key-words: Adipose stem cells; Cardiac ischemia/reperfusion; Angiogenesis; Heart remodeling.

INTRODUCTION

Over the last decade, the use of multiple types of stem cells for cardiovascular diseases has extensively been tested in a broad range of experimental settings from *in vitro* assays to clinical trials (20). Although the desired goal of tissue regeneration has not really been achieved, most studies have suggested that transplantation of stem cells is associated with a modest beneficial effect on cardiac function. The limited cell engraftment and differentiation of transplanted cells into cardiomyocytes (4, 14) points towards a paracrine mechanism of action in which transplanted cells are able to secrete or induce host-secretion of certain molecules with therapeutic capacity (10).

Among the various cell types, ADSCs represent an attractive source of stem cells for cardiovascular repair (17). ADSCs are endowed with a remarkable differentiation potential as well as the capacity to release significant amounts of growth factors involved in healing and tissue regeneration (22, 23, 25). Moreover, adipose tissue stands out for being easy to harvest and having high-efficiency stem cell culture. In spite of these facts, only a limited number of studies have been performed using ADSCs in models of cardiac disease (17) and particularly in only two reports have adipose-derived stem cells been applied to large animal models of MI (29, 26).

With few exceptions, studies either in rodents or large animals have employed protocols that cannot be translated directly into the human setting owing to, for instance, the need for the prolonged culture of stem cells required to obtain enough cells for transplantation in an acute myocardial infarct model (29). In the current study, we compared the potential of ADSCs to prevent cardiac derangement and to contribute to improving cardiac function in a swine model of ischemia-reperfusion. ADSCs were

obtained at the time of cardiac ischemia and injected within a clinically meaningful time-frame after a short culture without multiple passages.

METHODS

Animal procedures and study design

Adults male and female Goettingen Minipigs (average weight, 60-80 Kg) procured from our breeding center were maintained in the animal facilities of CIFA (GLP accredited center at the University of Navarra, Spain). All the study was performed in accordance with the US National Institutes of Health guidelines, the Declaration of Helsinki and approved by the Ethics Comitee for Animal Experimentation from the University of Navarra. In each procedure, animals were pre-medicated with a combination of 15 mg/kg ketamine (Imalgene 1000, MERIAL) and 2 mg/Kg azaperon (Stresnil, Esteve Veterinaria). After sedation, animals received 5 mg/kg of etomidate (Hypnomidate, Janssen-CILAG) and 0.03 mg/kg cisatracurium besylate (Nimbex, GlasoSmithKline) i.v. and were endotracheally intubated and mechanically ventilated with supplementary oxygen. During surgery, anesthesia was maintained with a combination of 3% isofluorane (ISOFLO, Abbott) and 0.01 mg/kg/h fentanyl (Fentanest, Kern Pharma) administered by continuous infusion and ECG, heart rate, SpO2, EtCO2 and rectal temperature were controlled with a multiparameter monitor. At the end of the procedure and after extubation, all animals received the non-steroidal analgesic ketoprofen (Ketofen 10%, MERIAL) 3 mg/kg/24h i.m. for 3 days as well as the antibiotic amoxicillin (Clamoxyl L.A, Pfizer) 7 mg/kg/24h for 5 days.

Myocardial infarction was induced as previously described (9). Briefly, a 7-Fr introducer sheath was placed by dissection in the left carotid artery and 1.5mg/kg heparin (Heparina 1%, Rovi) was intravenously infused. A 7-Fr coronary artery-guiding catheter was placed within the ostium under fluoroscopic guidance using a mobile C-arm (Powermobil, Siemens) and myocardial infarction was produced by occluding the

left anterior descending coronary artery with a balloon catheter 2.5 just below the second diagonal branch. Temporary and complete occlusion was performed by balloon dilatation (8 atm) and maintained for 120 minutes, followed by reperfusion, as demonstrated by coronary angiography and ST-segment elevation and reversion in the electrocardiogram. Lidocaine (B.Braun) 2 mg/kg iv and advanced life support were used when needed. Finally, the delivery catheter was removed, the carotid artery ligated, and the cut down site sutured. After induction of myocardial infarction, adipose tissue was collected after cleaning and disinfection of the right abdominal zone. An incision was performed in the skin of approximately 20 cm in length, which allowed us to dissect and obtain between 90-120gr of abdominal subcutaneous fat.

Nine days after I/R, animals were randomized to receive medium or ADSC transplantation. A percutaneous access through dissection of the femoral artery was performed. Treatment was intramyocardially delivered under simultaneous fluoroscopic and echocardiography (HP SONOS 4500) guidance using the Myocath® catheter (Bioheart, FL, USA). Multiple injections (average of 20 per time point of 0.25–0.5 ml per injection) were performed in each animal in and around the infarct area.

Cell culture and characterization

Adipose tissue biopsies and serum were obtained from pigs at the time of I/R. Samples were processed as previously described (22) with minor modifications. Briefly, fat was carefully minced and digested in 2 mg/ml collagenase type I (Invitrogen, Barcelona, Spain) for 45 minutes at 37ºC. After serial filtering through 100 µm and 40 µm mesh (BD, Madrid, Spain), cells were suspended in erythrocyte lysing solution (155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA) for 5 minutes, centrifuged and counted. The

resulting stromal vascular fraction (SVF) was plated in a 10-tray cell culture factory (Thermo, Barcelona, Spain) in α-MEM (Invitrogen) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and 1% antibiotics (Invitrogen). Approximately 2 million cells/gr of adipose tissue were obtained. After 2 days, cells were washed in PBS (Invitrogen) and infected as described below. 48 hours prior to cell injection, medium was replaced with α-MEM supplemented with antibiotics and 10% autologous serum to avoid unwanted immune reactions. Cells were characterized by FACS analysis using antibodies against SLA-DR, CD29, CD31 and CD90 (all from BD) before plating and before injection.

Lentiviral production and ADSC infection

Lentiviruses were produced in 293T cells by cotransfection of 4 plasmids as previously described (6). Briefly, the packaging plasmids MDL.RRE and pRSV.REV, and the VSV-G envelope protein expression plasmids were mixed with the PPT.CMV plasmid (which encodes GFP under the control of the constitutively expressed CMV promoter) and 293T cells were transiently transfected using calcium chloride. Medium was changed every 24 hours. After 72 and 96 hours post-transfection, supernatants were collected, filtered through 0.45 μm and concentrated by ultracentrifugation at 50000g for 120 minutes, and stored at -80ºC. Two days after ADSC trasnplantation, one viral production (from 40 100-mm dishes) was resuspended in 150 ml of basal medium, added to the culture factory and incubated for 1 hour at 37ºC with gentle agitation. Finally, 550 ml of complete medium were added.

Assessment of ventricular function and arrhythmias

Animals under general anesthesia were placed in the left lateral decubitus position and transthoracic two-dimensional echocardiography was performed using a Sonos 4500 ultrasound system (Philips) and a 4 MHz linear array transducer. Left ventricular remodeling was assessed by measuring end-systolic and end-diastolic volumes and diameters, according to the American Society of Echocardiography and adjusted for animal weight at the time of analysis. Assessment of diastolic function was performed by mitral filling pulse Doppler and mitral annulus tissue Doppler. Left ventricular ejection fraction (LVEF) was determined in parasternal short axis (18) due to the unreliability of the 4-camera approach in swine (11, 30). Echocardiogram was performed at baseline (before MI), before the implantation procedure, and at the time of sacrifice (3 months post-transplantation) by two investigators blinded to the type of treatment. Major arrhythmic events (ventricular tachycardia and ventricular fibrillation) during procedures were registered in all groups.

Histological processing and immune-staining

After sacrifice with pentobarbital and a saturated solution of potassium chloride the heart was excised and fixed in formalin and paraffin embedded for histological analysis. Location of MI was visually assessed. Sampling of tissues consisted of scar surrounded by a ring of viable myocardium. After paraffin embedding, 5 μm sections were stained with hematoxylin-eosin and Gallego Tricromic for qualitative assessment and with the appropriate antibodies. These included anti-GFP (Invitrogen), anti-MMP2, TIMP1 and TIMP2 (Abcam, Cambridge, UK). EnVision™-HRP conjugated system (Dako, Barcelona, Spain) was used as secondary reagent and Diaminobenzidine (Dako) as chromogen.

Vasculogenesis (arterioles and arteries) was evaluated in paraffin-embedded sections by measuring the area occupied by smooth muscle-covered vessels in the infarct border using an anti-α-smooth muscle actin antibody coupled to Cy3 (Sigma, Madrid, Spain). Angiogenesis was assessed by counting the density of small vessels (capillaries/mm^2) stained with *Bandeiraea simplicifolia* lectin-I (BSL-I, Sigma) and using Vectastain® (Vector, Barcelona, Spain) as secondary reagent and Diaminobenzidine as chromogen. The degree of fibrosis was determined by quantification of collagen deposition stained by Sirius Red staining. Briefly, sections were deparaffinized and immersed in 0.1% Fast Red (Sigma) in a saturated solution of picric acid, for 90 minutes, differentiated for 2 minutes in HCl (Sigma) 0.01N, dehydrated and mounted in DPX. Measurement of cardiomyocyte (CM) hypertrophy was performed on laminin-stained (anti-Laminin, Sigma) sections. High-power field images were acquired and CM cross sectional area was quantified using Image J software. A minimum of 120 CM per heart were analyzed. A minimum of 40 sections per animal were analyzed for each morphological measurement. All microphotographs were obtained on a Nikon Eclipse E800 microscope and analyzed with a computerized system (Axiovision 4.6, Zeiss, Germany). Sampling and measuring was performed in all cases by an investigator blinded to the treatment of individual animals

PCR analysis

DNA was extracted from paraffin-embedded tissue. 50-80 slides per heart (peri-infarct region) were deparaffinized and hydrated. Tissue was carefully separated from glass and DNA extracted with DNAeasy Mini Kit (Quiagen, Venlo, The Netherlands), following the manufacturer's instructions. GFP expression was analyzed by PCR (GFP-upstream primer: 5′-GCACCATCTTCTTCAAGGAC-3′; GFP-downstream primer: 5′-

ACTTGTACAGCTCGTCCATG-3′). Amplification conditions were as follows: 95 °C for 2 min followed by 40 cycles consisting on 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s, plus an extension phase of 72 °C for 10 min. All samples were also amplified for GAPDH (GAPDH-upstream primer: 5′-ACCTGCCGCCTGGAGAAACC-3′; GAPDH-downstream primer: 5′-GACCATGAGGTCCACCACCCTG-3′) as control for the presence of amplified DNA.

Serum zymography

Protein in serum samples was quantified by the Bradford method (Thermo). 100 µg of total protein were diluted in saline and mixed with equal amounts of sodium dodecyl sulfate (SDS) sample buffer (Novex, Carlsbad, CA, U.S.A.) and electrophoresed on 10% SDS-polyacrylamide gels (Novex) containing 1 mg/mL gelatin as the protease substrate. Following electrophoresis, gels were placed in 2.7% TritonX-100 for 1 hour to remove SDS, and then incubated for 20 hours at 37°C in developing buffer (50 mmol/L Tris base, 40 mmol/L HCl, 200 mmol/L NaCl, 5 mmol/L $CaCl_2$, and 0.2% Brij 35; Novex). After incubation, gels were stained with gel code blue stain reagent (Pierce) for 1 hour followed by de-staining. Quantification was performed using QuantityOne™ software.

Statistical analysis

All data are expressed as mean ± SD. Comparisons were performed using the paired or unpaired t-test. In case of non-normal distribution (demonstrated by Shapiro-Wilk and Komogorov-Smirnov tests), Wilcoxon or Mann Whitney U were used. Statistical analysis was performed with the SPSS 17.0 software and differences were considered statistically significant when $P<0.05$.

RESULTS

Phenotypic characterization and transplantation of ADSCs

Flow cytometry analysis showed fresh SVF cells to be a heterogeneous population with low expression of the endothelial cell marker CD31 and higher CD90 and CD29 expression consistent with stromal cells (Figure 1A). After 9 days in culture, a more homogenous population of cells with no expression of SLA-DR or CD31 was obtained (Figure 1B). Animals received a mean of 213.6±41.78 millions of ADSCs. GFP-expression measured by flow cytometry and immunocytochemistry was consistently above 50% of transplanted cells (Figure 1C).

Transplantation of ADSCs improve cardiac function in a preclinical model of ischemia reperfusion

Cardiac function was assessed by echocardiography at baseline before MI, at day 9 before cell transplant and at 3 months after transplant. Ischemia-reperfusion induced a statistically significant decrease in LVEF in comparison with baseline values, which was similar between ADSC treated animals and controls (baseline LVEF was 76±4% in the control group and 76.66±3.98% in ADSC treated animals; day +9 LVEF was 47.77±8.45% in the control group and 46.69±5.62% in ADSC treated animals; p=0.0001 between baseline and day +9). Reperfusion alone was associated with an improvement in LVEF from 47.77±8.45% to 55.79±6.39%; p=0.026 3 months after transplantation (Figure 2A), consistent with early reperfusion induced preservation of cardiac function (31). However, when ADSCs were injected, transplanted animals showed a greater increase on cardiac contractility (from 46.69±5.62% to 64.73±5.14% at 3 months; p=0.0001) (Figure 2A). The increase in LVEF was significantly greater in animals treated with ADSC in comparison with the control group (Control: 8.02±2.94;

ADSC: 18.04±2.16; p=0.021) (Figure 2B). ADSC transplant was also associated with a statistically significant prevention of ventricle geometry worsening, as indicated by the significant decrease in end-systolic diameter (ESD, from 3.27±0.71 cm before transplant to 2.69±0.45 cm at 3 months; p=0.029) and both end-diastolic and end-systolic volumes (EDV, from 87.06±10.34 ml to 64.67±4.64 ml at 3 months; p=0.038; ESV, from 46.44±8.03 ml to 25.58±4.76 ml at 3 months; p=0.028) (Figure 2D-F), thus suggesting an effect on the remodeling process.

Transplantation of ADSCs promotes angiogenesis and vasculogenesis and inhibits cardiac remodeling in infarcted hearts

To evaluate whether changes in cardiac performance could be related to cell-derived effects upon tissue architecture, we measured infarct collagen content (fibrosis), vessel density (capillaries and arteries/arterioles) and myocyte hypertrophy in the border zone of the infarct. Fibrosis was significantly reduced in ADSC treated animals (control: 57.79±5.18%; ADSC: 47.84±7.13%; p=0.007) (Figure 3A), while tissue vascularization was significantly improved in animals treated with ADSCs as indicated by an increase in the area occupied by smooth muscle-covered vessels (control: 2.54±1.08%; ADSC: 5.44±2.28%; p=0.037) (Figure 3B), and in the capillary-density (control: 153.28±29.3 vessels/high power field, HPF; ADSC: 235.22±53.28 vessels/HPF; p=0.003) (Figure 3C). Finally, CM-hypertrophy was decreased in the ADSC transplanted animals (control: 460.27±80.47 μm^2; ADSC: 206.38±32.66 μm^2; p=0.0001) (Figure 3D).

Due to the multipotentiality of ADSCs, transplantation could be associated with differentiation of ADSCs into unwanted tissues such as adipose cells or other mesoderm derived tissues (1). However, no evidence of ectopic adipose, bone or cartilage tissue

was observed in any of the treated animals (Figure 4A). Three months after medium- or cell-transplantation, only minor foci of inflammatory cells were found in the myocardium, with no differences between ADSC and control groups (Figure 4A). However, no trace of GFP-expressing cells could be found either by immunohistology or by PCR (Figure 4B).

We next sought to examine whether the improvement in cardiac function and remodeling could be related to changes in the composition of matrix metalloproteinases (MMPs) or their inhibitors (TIMPs) induced by transplantation of ADSCs. In this case both the border zone and the non-infarcted area were analyzed. Although we found no significant differences at 3 months between control and ADSC treated animals in the expression of MMP2, the most abundant metalloproteinases in the porcine heart (5), (Figure 5A-D), expression of TIMP1 and 2 were down-regulated in CM in the non-infarcted area in the control group (Figure 5E-F and I-J) in comparison with animals treated with ADSC. Furthermore, an increased number of TIMP1 and 2-positive vessels were found in the infarcted border zone of ADSC-transplanted animals (Figure 5G-H and K-L). A decrease in the activity of MMP2 was found by serum zymography (Figure 5M). As the proteinase activity depends on the balance between MMPs and TIMPs, these results suggest that ADSCs induced an increase in the net activity of MMP-2, thus limiting the adverse remodeling of the heart.

DISCUSSION

Despite the impressive results obtained with stem cell therapy in animal models of myocardial infarction (24), transfer to the bedside has not fulfilled expectations and only modest results comparable to those of conventional treatments have been obtained. Some of the lessons learnt from previous research have recently been highlighted (19) and indicate that an important reason for discrepancies in the results resides in the divergences between animal models and the human disease these models try to mirror. For instance, in the acute MI setting the use of culture cells (such as ADSCs or MSCs) would not be feasible unless allogeneic or previously stored cells were to be used. This is the case with the only 2 studies using adipose cells for the treatment of acute and subacute MI in large animal models (29, 26) in which cells were cultured several weeks before transplant. An additional limitation of both studies was the short term follow-up of 30 and 21 days respectively which prevents conclusions regarding the long-term effect of cells from being drawn, an important issue considering that clinical trials have shown that the effect of stem cell transplantation can be transitory, as was the case with the BOOST trial (28). In our study, pigs were transplanted 9 days after harvesting ADSCs and were followed for 3 months, which takes into account 2 of the aforementioned limitations, providing evidence for the improvement of cardiac function in a model that better resembles the human setting. The value of our model is also supported by the fact that the control group also experienced an improvement in cardiac function consistent with the reperfusion effect. Unlike previous studies (29, 26), we were unable to detect ADSCs at 3 months despite the long term functional effect observed, which could be explained on the basis of the longer follow up in our study.

Transplantation of ADSCs had an effect on two of the main detrimental factors related to the disease: the lack of sufficient blood-supply and the late-remodeling. However, the current study only allows us to speculate about a paracrine mechanism of action. ADSCs have consistently been shown to contribute to cardiac regeneration by the release of the proangiogenic factors vascular endothelial growth factor and hepatocyte growth factor, a potential that is enhanced under hypoxia conditions as is the case in ischemia (25, 12).

Adverse remodeling is one of the major players in the transition from MI to heart failure (7). In our model, transplantation of ADSCs induced a decrease in collagen content. The lack of differences in MMP2 activity suggests that the net effect of collagen decrease was not due to an increase digestion but rather to prevention of collagen deposition. The decrease in TIMP-1 and 2 in animals treated with ADSCs could contribute to decrease collagen deposition. In fact the MMPs/TIMPs system is tightly regulated in the myocardium and the misbalance could result in severe organ damage (13, 3). Thus, ADSCs may be contributing to correction of the imbalance that happens after MI thus preventing ventricular dilatation (27, 15), which in turn could lead to a less stressed ventricle and reduced cardiomyocyte hypertrophy.

The lack of meaningful long term engraftment remains a major caveat in cell therapy which has been observed uniformly for every type of cell that has been used in animal models (2). Several mechanisms have been implicated in the cell attrition observed after transplantation such as cell apoptosis due to the hypoxic milieu or immune rejection associated with the GFP protein (2). Different means to improve engraftment have been tested including genetic manipulations (21). Nevertheless, improvement in engraftment

remains a moving target in the field. Based on the results from a number of studies including ours, an increase in engraftment would be expected to improve the functional results. Simple strategies such as repeated injections of cells are associated with a modest improvement in function and engraftment but seem not be a definitive solution (8).

The results of two randomized placebo-controlled clinical trials with adipose-derived stem cells that have recently been completed, APOLLO (3D adipose-derived stem-cell transplantation in the treatment of patients with an acute ST-elevation myocardial infarction) and PRECISE (3D adipose-derived stem-cells in the treatment of patients with nonrevascularizable ischemic myocardium), should provide important information regarding the usefulness of this therapy and possibly reinforce its applicability. In this sense, the present report sheds light on the behavior of ADSC-therapy in a clinically relevant model of reperfused MI, showing that transplantation of ADSCs induces a significant benefit in cardiac contractility. This effect is related to prevention of cardiac remodeling, an increase in damaged-tissue perfusion and a decrease in myocyte hypertrophy.

FUNDING

This work was supported in part by Ministerio de Ciencia e Innovacion: ISCIII PI070474, CP09/00333 and RETIC RD06/0014, PLE2009-0116, and FEDER funds from Comunidad de Trabajo de los Pirineos (CTP), European Union Framework Project VII (INELPY), Caja de Ahorros de Navarra (Programa Tu Eliges: Tu Decides) and the "UTE project CIMA".

ADKNOWLEDGEMENTS

The authors would like to thank Beatriz Pelacho for her critical reading of this work.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Breitbach, M.; Bostani, T.; Roell, W.; Xia, Y.; Dewald, O.; Nygren, J. M.; Fries, J. W.; Tiemann, K.; Bohlen, H.; Hescheler, J.; Welz, A.; Bloch, W.; Jacobsen, S. E.; Fleischmann, B. K. Potential risks of bone marrow cell transplantation into infarcted hearts. Blood 110:1362-1369; 2007.
2. Bubnic, S. J.; Nagy, A.; Keating, A. Donor hematopoietic cells from transgenic mice that express GFP are immunogenic in immunocompetent recipients. Hematology 10:289-295; 2005.
3. Deten, A.; Holzl, A.; Leicht, M.; Barth, W.; Zimmer, H. G. Changes in extracellular matrix and in transforming growth factor beta isoforms after coronary artery ligation in rats. J.Mol.Cell.Cardiol. 33:1191-1207; 2001.
4. Dimmeler, S.; Burchfield, J.; Zeiher, A. M. Cell-based therapy of myocardial infarction. Arterioscler.Thromb.Vasc.Biol. 28:208-216; 2008.
5. Dubois, C.; Liu, X.; Claus, P.; Marsboom, G.; Pokreisz, P.; Vandenwijngaert, S.; Depelteau, H.; Streb, W.; Chaothawee, L.; Maes, F.; Gheysens, O.; Debyser, Z.; Gillijns, H.; Pellens, M.; Vandendriessche, T.; Chuah, M.; Collen, D.; Verbeken, E.; Belmans, A.; Van de Werf, F.; Bogaert, J.; Janssens, S. Differential effects of progenitor cell populations on left ventricular remodeling and myocardial neovascularization after myocardial infarction. J.Am.Coll.Cardiol. 55:2232-2243; 2010.
6. Dull, T.; Zufferey, R.; Kelly, M.; Mandel, R. J.; Nguyen, M.; Trono, D.; Naldini, L. A third-generation lentivirus vector with a conditional packaging system. J.Virol. 72:8463-8471; 1998.

7. Ferrari, R.; Ceconi, C.; Campo, G.; Cangiano, E.; Cavazza, C.; Secchiero, P.; Tavazzi, L. Mechanisms of remodelling: A question of life (stem cell production) and death (myocyte apoptosis). Circ.J. 73:1973-1982; 2009.

8. Gavira, J. J.; Nasarre, E.; Abizanda, G.; Perez-Ilzarbe, M.; de Martino-Rodriguez, A.; Garcia de Jalon, J. A.; Mazo, M.; Macias, A.; Garcia-Bolao, I.; Pelacho, B.; Martinez-Caro, D.; Prosper, F. Repeated implantation of skeletal myoblast in a swine model of chronic myocardial infarction. Eur.Heart J. 31:1013-1021; 2010.

9. Gavira, J. J.; Perez-Ilzarbe, M.; Abizanda, G.; Garcia-Rodriguez, A.; Orbe, J.; Paramo, J. A.; Belzunce, M.; Rabago, G.; Barba, J.; Herreros, J.; Panizo, A.; de Jalon, J. A.; Martinez-Caro, D.; Prosper, F. A comparison between percutaneous and surgical transplantation of autologous skeletal myoblasts in a swine model of chronic myocardial infarction. Cardiovasc.Res. 71:744-753; 2006.

10. Gnecchi, M.; Zhang, Z.; Ni, A.; Dzau, V. J. Paracrine mechanisms in adult stem cell signaling and therapy. Circ.Res. 103:1204-1219; 2008.

11. Kerut, E. K.; Valina, C. M.; Luka, T.; Pinkernell, K.; Delafontaine, P.; Alt, E. U. Technique and imaging for transthoracic echocardiography of the laboratory pig. Echocardiography 21:439-442; 2004.

12. Li, T. S.; Mikamo, A.; Takahashi, M.; Suzuki, R.; Ueda, K.; Ikeda, Y.; Matsuzaki, M.; Hamano, K. Comparison of cell therapy and cytokine therapy for functional repair in ischemic and nonischemic heart failure. Cell Transplant. 16:365-374; 2007.

13. Li, Y. Y.; Feldman, A. M.; Sun, Y.; McTiernan, C. F. Differential expression of tissue inhibitors of metalloproteinases in the failing human heart. Circulation 98:1728-1734; 1998.

14. Martin-Rendon, E.; Brunskill, S. J.; Hyde, C. J.; Stanworth, S. J.; Mathur, A.; Watt, S. M. Autologous bone marrow stem cells to treat acute myocardial infarction: A systematic review. Eur.Heart J. 29:1807-1818; 2008.

15. Matsumura, S.; Iwanaga, S.; Mochizuki, S.; Okamoto, H.; Ogawa, S.; Okada, Y. Targeted deletion or pharmacological inhibition of MMP-2 prevents cardiac rupture after myocardial infarction in mice. J.Clin.Invest. 115:599-609; 2005.

16. Mazo, M.; Planat-Benard, V.; Abizanda, G.; Pelacho, B.; Leobon, B.; Gavira, J. J.; Penuelas, I.; Cemborain, A.; Penicaud, L.; Laharrague, P.; Joffre, C.; Boisson, M.; Ecay, M.; Collantes, M.; Barba, J.; Casteilla, L.; Prosper, F. Transplantation of adipose derived stromal cells is associated with functional improvement in a rat model of chronic myocardial infarction. Eur.J.Heart Fail. 10:454-462; 2008.

17. Mazo, M.; Gavira, J. J.; Pelacho, B.; Prosper, F. Adipose-derived stem cells for myocardial infarction. J.Cardiovasc.Transl.Res. 4: 145-153; 2011.

18. Meller, J.; Herman, M. V.; Teichholz, L. E. Noninvasive assessment of left ventricular function. Adv.Intern.Med. 24:331-357; 1979.

19. Menasche, P. Cardiac cell therapy: Lessons from clinical trials. J.Mol.Cell.Cardiol. 50:258-265; 2011.

20. Passier, R.; van Laake, L. W.; Mummery, C. L. Stem-cell-based therapy and lessons from the heart. Nature 453:322-329; 2008.

21. Penn, M. S.; Mangi, A. A. Genetic enhancement of stem cell engraftment, survival, and efficacy. Circ.Res. 102:1471-1482; 2008.

22. Planat-Benard, V.; Menard, C.; Andre, M.; Puceat, M.; Perez, A.; Garcia-Verdugo, J. M.; Penicaud, L.; Casteilla, L. Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. Circ.Res. 94:223-229; 2004.

23. Planat-Benard, V.; Silvestre, J. S.; Cousin, B.; Andre, M.; Nibbelink, M.; Tamarat, R.; Clergue, M.; Manneville, C.; Saillan-Barreau, C.; Duriez, M.; Tedgui, A.; Levy, B.; Penicaud, L.; Casteilla, L. Plasticity of human adipose lineage cells toward endothelial cells: Physiological and therapeutic perspectives. Circulation 109:656-663; 2004.

24. Reffelmann, T.; Kloner, R. A. Cellular cardiomyoplasty--cardiomyocytes, skeletal myoblasts, or stem cells for regenerating myocardium and treatment of heart failure? Cardiovasc.Res. 58:358-368; 2003.

25. Rehman, J.; Traktuev, D.; Li, J.; Merfeld-Clauss, S.; Temm-Grove, C. J.; Bovenkerk, J. E.; Pell, C. L.; Johnstone, B. H.; Considine, R. V.; March, K. L. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation 109:1292-1298; 2004.

26. Rigol, M.; Solanes, N.; Farre, J.; Roura, S.; Roque, M.; Berruezo, A.; Bellera, N.; Novensa, L.; Tamborero, D.; Prat-Vidal, C.; Huzman, M. A.; Batlle, M.; Hoefsloot, M.; Sitges, M.; Ramirez, J.; Dantas, A. P.; Merino, A.; Sanz, G.; Brugada, J.; Bayes-Genis, A.; Heras, M. Effects of adipose tissue-derived stem cell therapy after myocardial infarction: Impact of the route of administration. J.Card.Fail. 16:357-366; 2010.

27. Roten, L.; Nemoto, S.; Simsic, J.; Coker, M. L.; Rao, V.; Baicu, S.; Defreyte, G.; Soloway, P. J.; Zile, M. R.; Spinale, F. G. Effects of gene deletion of the tissue inhibitor of the matrix metalloproteinase-type 1 (TIMP-1) on left ventricular geometry and function in mice. J.Mol.Cell.Cardiol. 32:109-120; 2000.

28. Schaefer, A.; Meyer, G. P.; Fuchs, M.; Klein, G.; Kaplan, M.; Wollert, K. C.; Drexler, H. Impact of intracoronary bone marrow cell transfer on diastolic

function in patients after acute myocardial infarction: Results from the BOOST trial. Eur.Heart J. 27:929-935; 2006.

29. Valina, C.; Pinkernell, K.; Song, Y. H.; Bai, X.; Sadat, S.; Campeau, R. J.; Le Jemtel, T. H.; Alt, E. Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular function, perfusion, and remodelling after acute myocardial infarction. Eur.Heart J. 28:2667-2677; 2007.

30. Weidemann, F.; Jamal, F.; Sutherland, G. R.; Claus, P.; Kowalski, M.; Hatle, L.; De Scheerder, I.; Bijnens, B.; Rademakers, F. E. Myocardial function defined by strain rate and strain during alterations in inotropic states and heart rate. Am.J.Physiol.Heart Circ.Physiol. 283:H792-9; 2002.

31. White, H. D.; Chew, D. P. Acute myocardial infarction. Lancet 372:570-584; 2008.

FIGURE LEGENDS

Figure 1: FACS analysis of ADSC

Cells were labeled with antibodies against SLA-DR, CD29, CD31 and CD90 (red line) or isotype controls (dashed lines), at the time of harvesting **(A)** and after 9 days in culture **(B)**. **(C)** GFP-expression measured by immunocytochemistry (ICC) and FACS. A representative experiment is shown**.**

Figure 2: Transplantation of ADSC induces an improvement in cardiac function and geometry

Cardiac function was measured by echocardiography before transplant and 3 months after transplant in the control and ADSC treated animals. **(A)** Left ventricular ejection fraction (LVEF). **(B)** ΔLVEF between control and ADSC treated animals. **(C)** End diastolic diameter (EDD). **(D)** End systolic diameter (ESD). **(E)** End diastolic volume (EDV). **(F)** End systolic volume (ESV). Results are shown as mean±SD. *p<0.05; **p<0.01 between pre-transplant and 3 months after ADSC- or medium-transplantation.

Figure 3: ADSC transplant is associated with an increase in vasculogenesis and a decrease in cardiac fibrosis

5μm sections obtained from the infarct border zone of animals sacrificed at 3 months after transplant were analyzed. Fibrotic index **(A)**, capillary **(B)** and arterioles/arteries **(C)** densities, and CM hypertrophy **(D)** were determined by quantification of Sirius Red, caveolin-1 positive capillaries/HPF, αSMA-positive arterial vessels/mm^2 and laminin-positive CM in animals treated with media or ADSC. Results represent the mean±SD. A representative image is shown for each analysis. *p<0.05;**p<0.01.

Figure 4: Transplantation of ADSC is not associated with ectopic tissue formation and long term engraftment

(A) No differences in amounts of adipocytes, microcalcifications or inflammatory infiltrates were found between control and ADSC treated animals.. (B) ADSC cell engraftment was not found 3 months after transplant either by immune-staining or PCR analysis for GFP protein.

Figure 5: Transplantation of ADSC is associated with changes in MMP/TIMP balance

Immune-histochemistry using antibodies against MMP-2 **(A-D)**, TIMP1 **(E-H)** and TIMP2 **(I-L)** was performed in 5µm sections obtained from the non-infarcted and the infarct border zone of animals sacrificed at 3 months after transplantation of ADSC or media control. No differences in the expression of MMP-2 were found between ADSC or control animals pigs in the non-infarcted myocardium **(A,B)** or in the border zone **(C,D)**. Expression of TIMP1 and 2 was down-regulated in CM **(arrowheads in E and I)** and an increase in TIMP1- or 2-expressing vessels was found in the border zone of ADSC treated animals **(asterisks in G,H,K and L)**. Serum MMP2-activity by gelatin zymography was also diminished in ADSC-treated pigs **(M)**. Results are shown as mean±SD. *$p<0.05$.

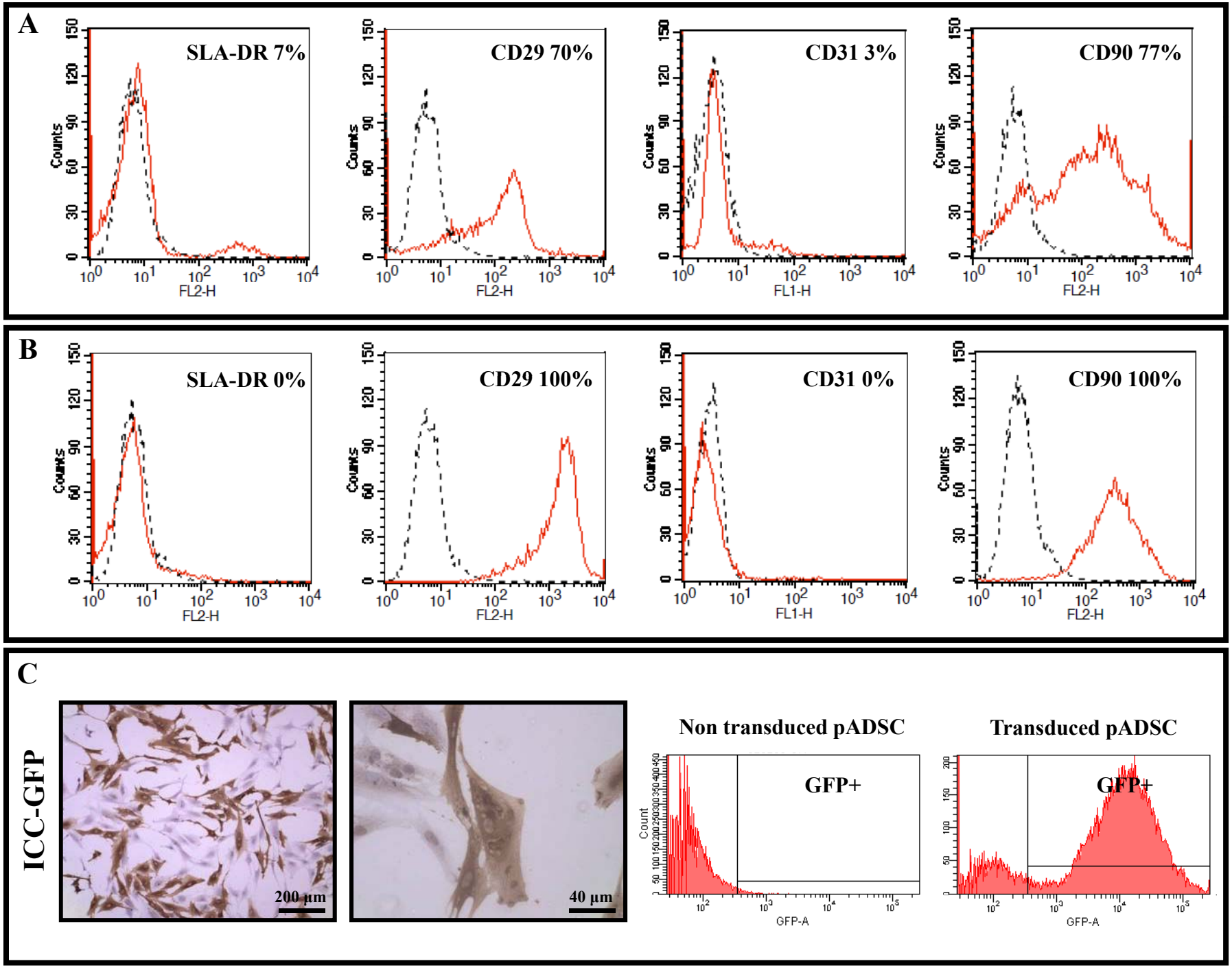

A
SLA-DR 7%
CD29 70%
CD31 3%
CD90 77%
Counts
FL2-H
FL1-H
B
SLA-DR 0%
CD29 100%
CD31 0%
CD90 100%
C
ICC-GFP
200 µm
40 µm
Non transduced pADSC
Transduced pADSC
GFP+
GFP+
Count
GFP-A

Figure 1

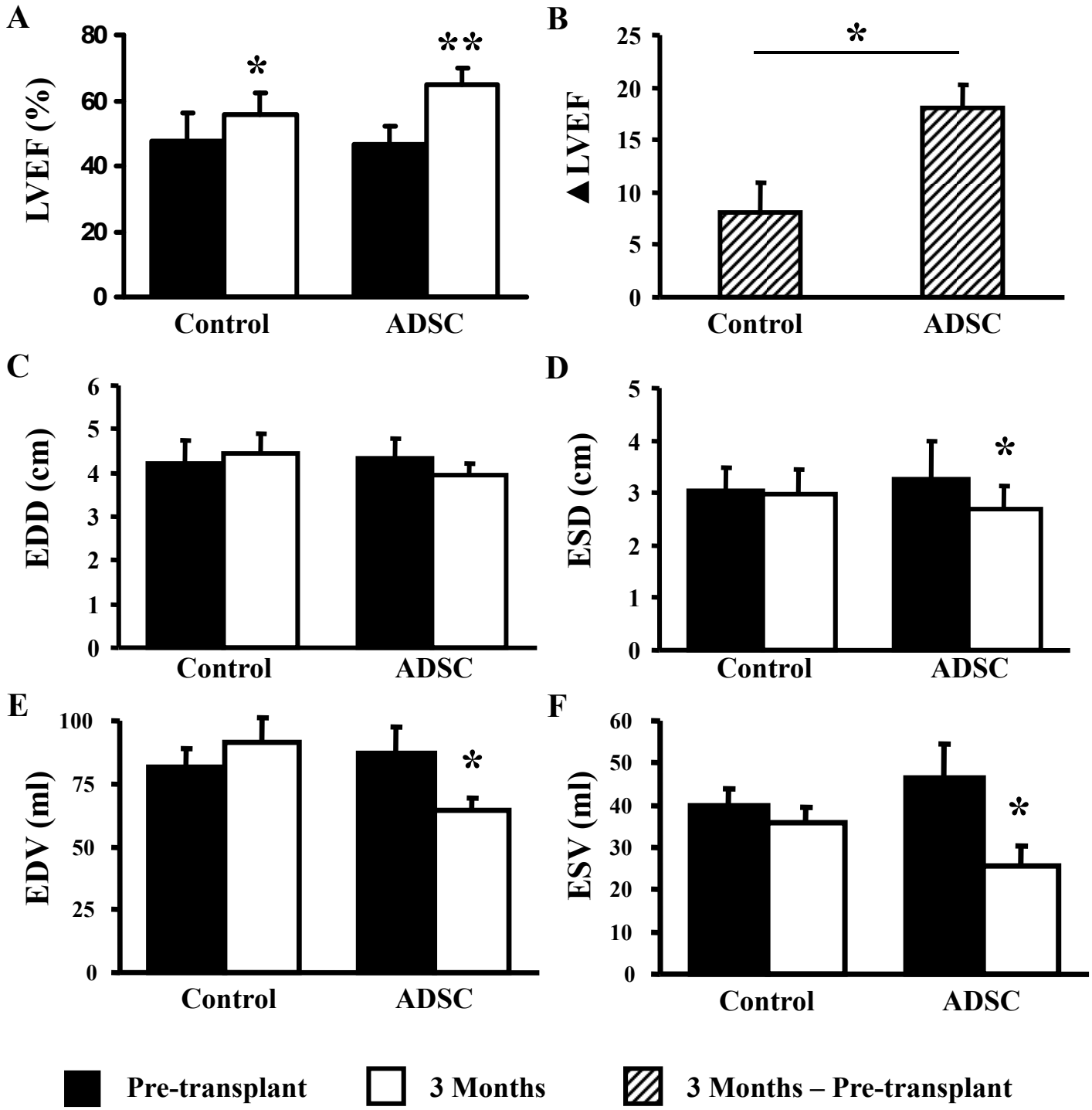

A
LVEF (%)
80
60
40
20
0
*
**
Control
ADSC
B
▲LVEF
25
20
15
10
5
0
*
Control
ADSC
C
EDD (cm)
6
5
4
3
2
1
0
Control
ADSC
D
ESD (cm)
5
4
3
2
1
0
*
Control
ADSC
E
EDV (ml)
100
75
50
25
0
*
Control
ADSC
F
ESV (ml)
60
50
40
30
20
10
0
*
Control
ADSC
Pre-transplant
3 Months
3 Months – Pre-transplant

Figure 2

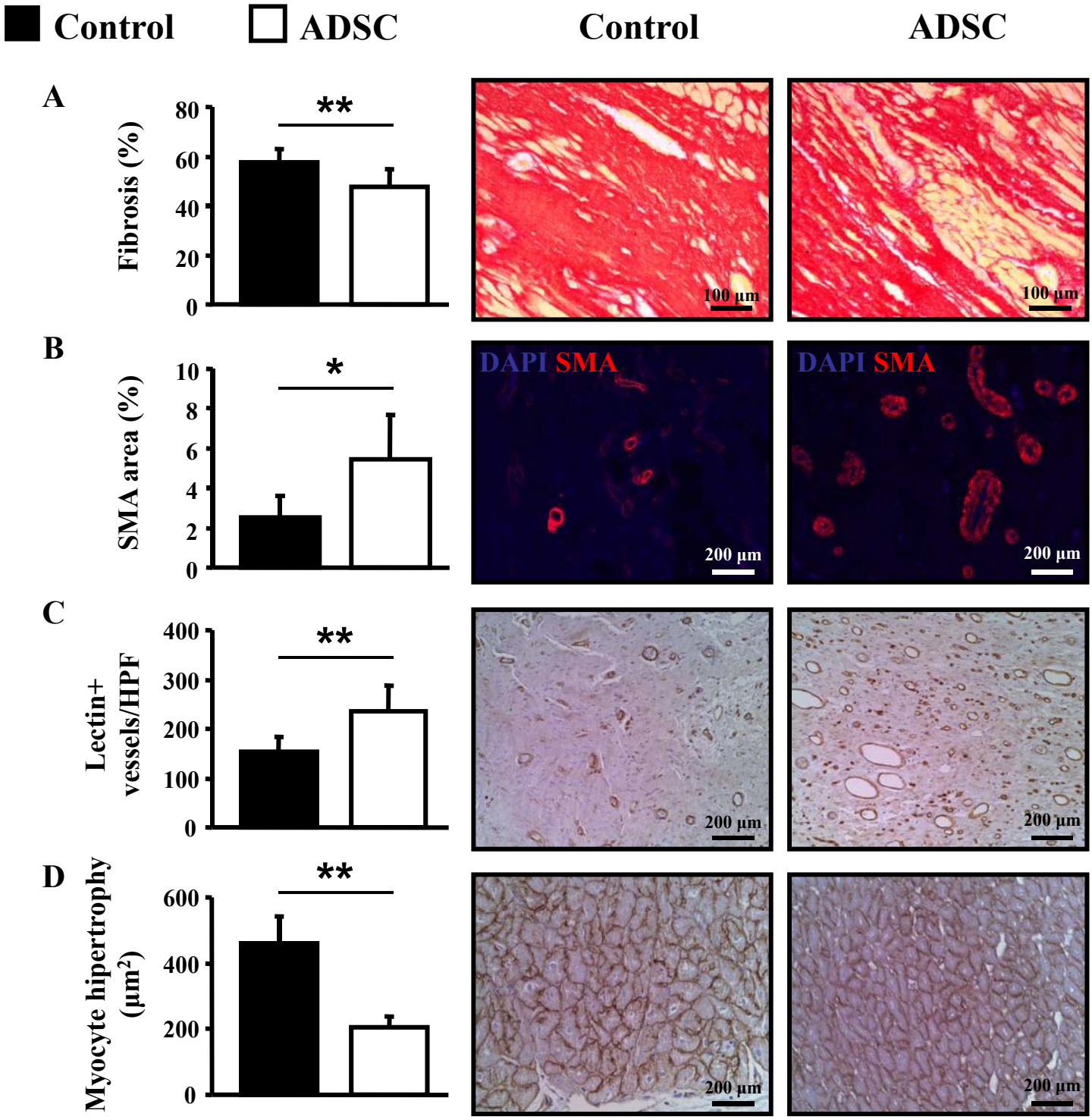

Control
ADSC
Control
ADSC
A
Fibrosis (%)
80
60
40
20
0
**
100 µm
100 µm
B
SMA area (%)
10
8
6
4
2
0
*
DAPI SMA
200 µm
DAPI SMA
200 µm
C
Lectin+ vessels/HPF
400
300
200
100
0
**
200 µm
200 µm
D
Myocyte hipertrophy (µm²)
600
400
200
0
**
200 µm
200 µm

Figure 3

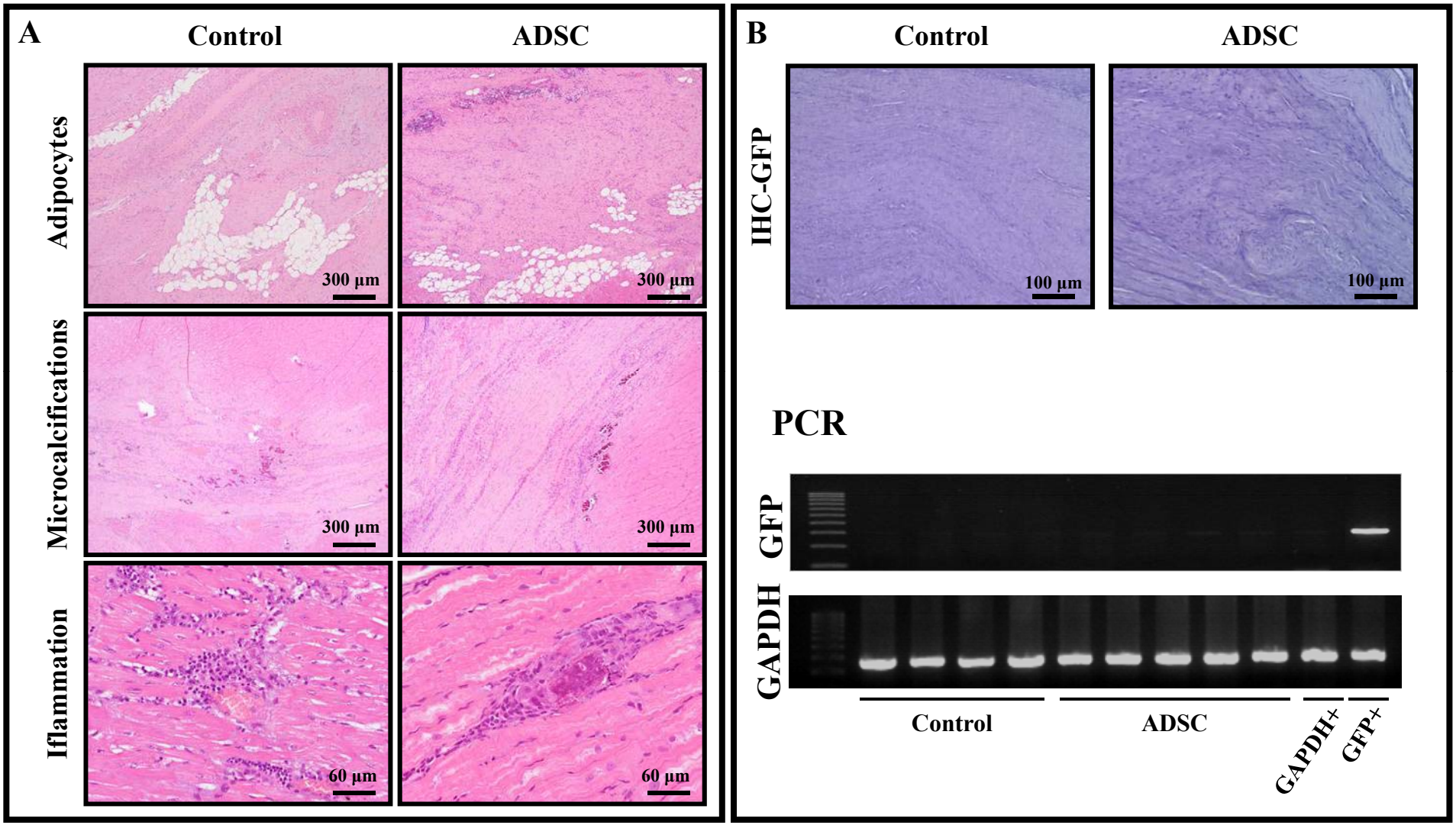

A
Control
ADSC
Adipocytes
Microcalcifications
Inflammation
300 μm
300 μm
300 μm
300 μm
60 μm
60 μm
B
Control
ADSC
IHC-GFP
100 μm
100 μm
PCR
GFP
GAPDH
Control
ADSC
GAPDH+
GFP+

Figure 4

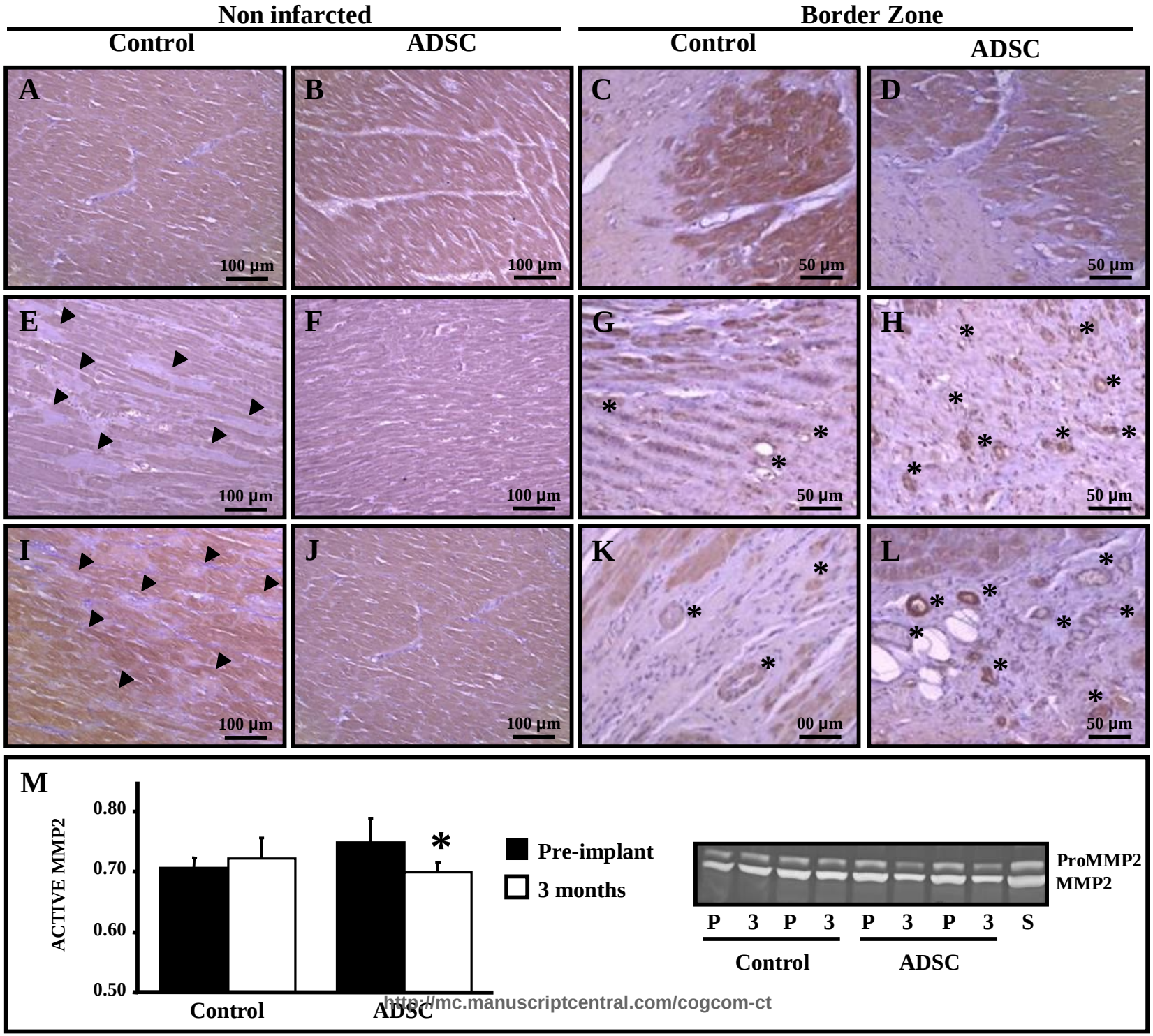


Figure 5

ANEXO III

Cell Transplantation, Vol. 20, pp. 259–269, 2011

0963-6897/11 $90.00 + .00
DOI: 10.3727/096368910X516592
E-ISSN 1555-3892
www.cognizantcommunication.com

MAPC Transplantation Confers a More Durable Benefit Than AC133$^+$ Cell Transplantation in Severe Hind Limb Ischemia

Xabier L. Aranguren,*‡[1] Beatriz Pelacho,*[1] Ivan Peñuelas,† Gloria Abizanda,* Maialen Uriz,* Margarita Ecay,† María Collantaes,† Miriam Araña,* Manu Beerens,‡ Giulia Coppiello,‡ Inés Prieto,* Maitane Perez-Ilzarbe,* Enrique J. Andreu,* Aernout Luttun,‡ and Felipe Prósper*

*Hematology Service and Cell Therapy, Foundation for Applied Medical Research, Division of Cancer, University of Navarra, Pamplona, Spain
†Department of Nuclear Medicine, Clínica Universitaria, University of Navarra, Pamplona, Spain
‡Center for Molecular and Vascular Biology, Catholic University of Leuven, Leuven, Belgium

There is a need for comparative studies to determine which cell types are better candidates to remedy ischemia. Here, we compared human AC133$^+$ cells and multipotent adult progenitor cells (hMAPC) in a mouse model reminiscent of critical limb ischemia. hMAPC or hAC133$^+$ cell transplantation induced a significant improvement in tissue perfusion (measured by microPET) 15 days posttransplantation compared to controls. This improvement persisted for 30 days in hMAPC-treated but not in hAC133$^+$-injected animals. While transplantation of hAC133$^+$ cells promoted capillary growth, hMAPC transplantation also induced collateral expansion, decreased muscle necrosis/fibrosis, and improved muscle regeneration. Incorporation of differentiated hAC133$^+$ or hMAPC progeny into new vessels was limited; however, a paracrine angio/arteriogenic effect was demonstrated in animals treated with hMAPC. Accordingly, hMAPC-conditioned, but not hAC133$^+$-conditioned, media stimulated vascular cell proliferation and prevented myoblast, endothelial, and smooth muscle cell apoptosis in vitro. Our study suggests that although hAC133$^+$ cell and hMAPC transplantation both contribute to vascular regeneration in ischemic limbs, hMAPC exert a more robust effect through trophic mechanisms, which translated into collateral and muscle fiber regeneration. This, in turn, conferred tissue protection and regeneration with longer term functional improvement.

Key words: Angiogenesis; Stem cells; Critical limb ischemia; Multipotent adult progenitor cells (MAPC); AC133$^+$ cells

INTRODUCTION

Peripheral vascular disease (PVD) caused by inadequate blood supply to the limbs is a severe health problem in the Western world, affecting 3–10% of the population above the age of 60 (5). Therapeutic revascularization techniques, like laser revascularization, endovascular treatment, or bypass surgery, can in some cases restore flow and prevent gangrene formation/amputation. Nevertheless, for many patients, these techniques are not applicable due to the diffuse nature or the distal location of the obstructions or coexistence of comorbidities. For these no-option patients, alternatives have been proposed, mainly focused on the induction of tissue revascularization by angiogenic gene/protein delivery or by cell therapy. Unfortunately, clinical trials based on growth factor delivery have not yielded the expected results, thereby exposing the need for alternatives (5,13). Evidence indicating that adult vasculogenesis—vessel formation from endothelial progenitor cells (EPC)—is a process that contributes to therapeutic neovascularization (6) has fueled the interest in the use of this alternative paradigm for therapeutic revascularization.

Different (stem) cell populations have been tested in animals for treatment of hind limb ischemia, like embryonic stem cells (ESC) (10), unselected bone marrow mononuclear cells (MNC) (17), peritoneal macrophages (16), adipose tissue-derived stem cells (ADSC) (26), mesenchymal stem cells (MSC) (18), mature endothelial cells (EC) (19,35), or EPC (34,35). Overall, results have

Received November 13, 2009; final acceptance June 9, 2010. Online prepub date: August 17, 2010.
[1]These authors provided equal contribution to this work.
Address correspondence to Felipe Prósper, Hematology and Cell Therapy Area, Clínica Universitaria, University of Navarra, Av Pio XII 36, Pamplona 31009, Spain. Tel: +34-948-255400; Fax: +34-948-296500; E-mail: fprosper@unav.es *or* Aernout Luttun, Center for Molecular and Vascular Biology, Katholieke Universiteit Leuven, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium. Tel: +32-16-34-57-72; Fax: +32-16-34-59-90; E-mail: aernout.luttun@med.kuleuven.be

shown that whereas mature EC transplantation does not induce functional improvement, a variable degree of recovery has been demonstrated when MNC or EPC were transplanted. In contrast to earlier reports, more recent mechanistic studies have revealed that the degree of direct effects of stem/progenitor cells on revascularization may be not as robust, and in some cases even negligible (19). Nevertheless, as a result of the promising preclinical results, at least 40 clinical trials with (stem) cells have already been started in PVD patients (5). Most of them have evaluated the potential of cord blood cells, peripheral blood-derived MNC, or total bone marrow (2). A few clinical trials have been performed using enriched stem cell populations like $CD34^+$ or $AC133^+$ cells demonstrating a clinical benefit [reviewed in (5,13)]. The mean follow-up time in the majority of these studies was rather short, precluding conclusions on longer term benefits or possible side effects, the latter as reported in one recent study (22).

Taking together the existing preclinical and clinical data, further studies should primarily focus on defining the type of stem cell that can exert a greater benefit in patients with critical limb ischemia as well as identifying the mechanism responsible for this effect. In order to do so, direct comparative studies involving different cell types are required. Along this line, we recently demonstrated the superior effect of murine multipotent adult progenitor cells (mMAPC) compared to unfractionated murine bone marrow cells in a mild hind limb ischemia model, reminiscent of intermittent claudication (4). In the current study, we tested the potential of human MAPC (hMAPC) in a severe model of limb ischemia (resembling critical limb ischemia) and compared it with human $AC133^+$ cells, which have already been used in clinical trials. We also compared the main mechanisms of action of both cell types.

MATERIALS AND METHODS

Cell and Tissue Processing

Human bone marrow, muscle biopsies, and umbilical cord blood (UCB) cells were obtained after informed consent from donor or mother according to the guidelines from the Committee on the Use of Human Subjects in Research from the Clínica Universitaria, Pamplona. UCB mononuclear cells were separated by Ficoll Hypaque centrifugation (specific gravity, 1077; Sigma, St. Louis, MO, USA) and $hAC133^+$ cells were selected using micromagnetic beads (Miltenyi Biotec, Germany) with autoMACS columns (Miltenyi Biotec) as described (3). More than 90% cell purity was obtained after column selection. hMAPC were obtained according to published protocols (3,4). Primary human umbilical vein endothelial cells (HUVEC), umbilical arterial smooth muscle cells (SMC), and skeletal myoblasts (SkM) were prepared from human umbilical cords and muscle biopsies as previously described (3,15). Briefly, for SkM isolation, human muscle biopsies were minced and digested with trypsin-EDTA (0.5 mg/ml trypsin/0.53 mM EDTA) and collagenase (1.5 mg/ml) and isolated cells were grown in Ham-F12 media (GIBCO-BRL) supplemented with 20% fetal calf serum (FCS) and 1% penicillin/streptomycin (GIBCO-BRL). HUVEC cells were grown in F12K medium (ATCC) supplemented with serum (10%), heparin (0.1 mg/ml), and ECGS (0.03 mg/ml) and SMC were grown in DMEM high glucose (GIBCO-BRL) supplemented with 10% serum. Cells were grown under normoxic conditions.

In Vivo Studies

Surgery. Severe hind limb ischemia was induced under anesthesia (75 mg/kg ketamine/10 mg/kg xylazine) in 6–10-week-old male nude Balbc mice by excision of the left iliac artery as previously described (25). The mice were given postoperative analgesia (5 mg/kg ketoprofen, every day for 3 days). One million cells resuspended in 30 μl of PBS, or PBS alone were injected in four equal fractions in the adductor and quadriceps region of the left limb, 24 h after surgery. As undifferentiated hMAPC express very low levels of MHC-I and are thus sensitive to natural killer (NK) cell-mediated clearance (32), all mice were intraperitoneally injected with 20 μl of a specific anti-NK antibody (28) (anti-asialo GM1 antibody; Wako Chemicals) 1–2 h before transplantation and every 10 days thereafter. Mice were housed in specific pathogen-free conditions and all procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Navarra.

Live Imaging and Assessment of Limb Perfusion. Limb perfusion assessment was performed by microPET as described by our group (25). Briefly, microPET imaging was performed 10 min after ^{13}N-ammonia injection 1, 7, 15, and 30 days after surgery. For quantitative analysis and comparisons among subjects, evaluation of perfusion in both hind limbs was carried out as follows: regions of interest (ROIs) were drawn on coronal 1-mm-thick microPET images over the hind limbs, and activity concentration per area unit calculated as a measurement of perfusion. The ratio between left (ischemic) and right (nonischemic) hind limbs was used in all cases for comparisons and obtained data were exported to the PMOD software package for quantification.

Histological Analysis

At sacrifice (day 15 and day 30), mice were anesthetized with 60 mg/kg Nembutal and perfused with Tris-buffered saline (TBS)-adenosine and followed by zinc-

paraformaldehyde. Following dissection, muscles were postfixed 24 h and divided in two equal pieces to be processed for paraffin embedding. H&E and Sirius red stainings were performed as described (3). Those areas with presence of fat cells replacing muscle fibers, inflammatory infiltrates, or "ghost" cells (muscle cells without nucleus) were considered necrotic areas. For immunofluorescence and immunohistochemistry, antibodies against α-smooth muscle actin (α-SMC, unconjugated, DAKO; or Cy3 or FITC conjugated, Sigma), human CD31 (DAKO or Pharmingen), UEA lectin (biotin, TRITC, or FITC, conjugated; Sigma), mouse BS-I lectin (Sigma), human vimentin (DAKO), and desmin (DAKO) were used as primary antibodies. Secondary antibodies coupled to FITC or peroxidase enzyme were purchased from Molecular Probes. For quantification, analyses were done on cross sections with regular intervals spanning 2 mm. When technically feasible, entire cross sections were evaluated on lower power field images, or when higher magnification was required several (usually 3–4 per section) randomly chosen higher power fields covering a large part of each cross section were analyzed. Pictures for morphometric analysis were taken using a Zeiss Axio Imager connected to an Axiocam MRc5 camera (Zeiss, Zaventem, Belgium) and analysis was performed using Image J, KS300 (Leica, Brussels, Belgium).

In Vitro Proliferation and Apoptosis Assays

Proliferation. hMAPC were cultured in MAPC medium (3) with 2% FCS but without cytokines at a density of 17,500 cells/cm^2, and hAC133$^+$ cells were cultured in DMEM high glucose supplemented with 2% FCS at a density of 10^5/cm^2. Conditioned media (CM) were collected after 48 h. Corresponding media incubated without cells for 48 h served as controls [nonconditioned media (NCM)]. Early passages of HUVEC, SMC, or SkM were seeded in 24-well plates at a cell density of 1,250 cells/cm^2 (HUVEC and SkM) or 625 cells/cm^2 (SMC), respectively, and cultured in the presence of hMAPC or hAC133$^+$ CM or NCM. In order to obtain the same cell concentration as hMAPC, hAC133$^+$ CM was previously diluted and all media were supplemented with extra serum to reach a 6% FCS concentration. After 72 h, the number of viable cells was quantified by a luminiscent cell viability assay (Cell Titer-Glo™, Promega, USA) following the manufacturer's protocol. Four independent experiments were performed and every cell type grown in CM or NCM was seeded in quadruplicate.

Apoptosis. CM from hMAPC and hAC133$^+$ were collected as described before, after 12-h culture in non-serum-containing media. Endothelial and skeletal cells were seeded in 96-well plates at a density of 10^3 cells/cm^2 (HUVEC) or 5,000 cells/cm^2 (SMC and SkM) and cultured under hypoxic conditions (1% O_2) in the presence of CM, NCM, or in normal cell culture conditions (CCM) (20% O_2 and cell culture media) during 24 h for HUVEC and 72 h for SMC and SkM cells. For this assay, no serum was added to the CM. The degree of cell apoptosis was measured by an ELISA kit that quantifies the presence of oligonucleosomal fragments (Roche Applied Science, Barcelona, Spain). The apoptosis control corresponds to cells cultured under hypoxia and NCM. Under this condition apoptosis was clearly detected and considered 100%. The rest of the samples were referred to that value, in order to determine the degree of "protection" conferred by the conditioned media. Four independent experiments were performed on cells seeded in quadruplicate.

RNA Isolation, qRT-PCR, and ELISA

Total RNA from hAC133$^+$ and hMAPC was extracted using the RNAeasy minikit (Qiagen). mRNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) and cDNA underwent 40 cycles of amplification (ABI PRISM 7700, Perking Elmer/Applied Biosystems). SYBR Green Universal Mix PCR reaction buffer (Applied Biosystems) was used for quantification, normalizing data using *GAPDH* as housekeeping gene. Cytokine levels present in hMAPC or hAC133$^+$ conditioned and nonconditioned medias were quantified by ELISA. For human VEGF, bFGF, and Ang-2, ELISA kits were purchased from R&D Systems and ELISA procedures were followed according to the manufacturer's instructions.

Statistical Analysis

All data are expressed as mean ± SEM or median (Q1; Q3). Comparisons for repeated measurements were performed by ANOVA or Kruskal-Wallis tests. Shapiro-Wilk test was used to verify that the data had a Gaussian distribution, which justifies the use of a parametric test. Tukey, Tukey's b, and Tamhane's T2 post hoc tests were performed after ANOVA and *U*-Mann-Whitney 1:1 with signification correction by Bonferroni's method for the nonparametric tests. Statistical analysis was performed with Instat 3.0 software and differences were considered statistically significant when $p < 0.05$.

RESULTS

hMAPC Have a More Durable Effect on Limb Reperfusion Than hAC133$^+$ Cells

The revascularization potential of hAC133$^+$ cells and hMAPC was compared in a severe ischemia model induced by iliac artery excision. After 24 h, such injury caused a dramatic decrease in blood perfusion (~70%)

in the ischemic limb (Fig. 1). By day 15, PBS-injected animals presented a spontaneous recovery in blood flow (54 ± 3% at 15 days vs. 23 ± 3% at 24 h). In comparison with this group, a significantly greater recovery was detected in the animals transplanted either with hAC133$^+$ cells or hMAPC (PBS: 54 ± 3%; hAC133$^+$: 74 ± 7%, $p < 0.05$; hMAPC: 64 ± 4%, $p < 0.05$). Importantly, 30 days posttransplantation, while animals treated with hMAPC presented a significantly higher blood flow, perfusion recovery was no longer significantly different from controls in the animals treated with hAC133$^+$ (PBS: 66 ± 3%; hAC133$^+$ cells: 73 ± 5%, $p = NS$; hMAPC: 77 ± 3%, $p < 0.05$) (Fig. 1), suggesting that hMAPC exerted a more robust and prolonged effect on reperfusion than hAC133$^+$ cells.

hMAPC and hAC133$^+$ Cells Have a Differential Effect on Blood Vessel Growth

We first examined whether the different effects on reperfusion could be explained by histological assessment of blood vessel growth. In the ischemic adductor region, vascular expansion occurs by a combination of three mechanisms: vasculogenesis (vascular precursor incorporation into growing vessels), angiogenesis (capillary growth), and adaptive arteriogenesis (collateral expansion). First, direct contribution of hMAPC or hAC133$^+$ vascular progeny was studied at 30 days after surgery. As we have previously reported, direct contribution of hMAPC to both EC (human specific CD31 and UEA positive) and SMC layers (human vimentin and α-actin double positive) of the newly formed vessels was confirmed in the adductor region. However, this contribution was limited (Fig. 2A–C) (4). On the other hand, hAC133$^+$ cells also contributed to the EC lineage, although they did not differentiate into SMC (Fig. 2D–F). The remaining hAC133$^+$ cell fraction at 30 days was too low to allow for reliable quantification. Thus, for both cell types, vascular incorporation was low, which makes it unlikely that vasculogenesis was the main revascularization mechanism.

Next, we quantified the effect of cell transplantation on capillary growth and collateral expansion. Both cell types had a significant effect on both parameters; however, they had a differential effect on these processes. While hAC133$^+$ cells mainly affected capillary growth (small CD31$^+$ vessels/mm^2: 290 ± 36 in PBS; 759 ± 96 in hAC133$^+$, $p < 0.01$ and 493 ± 45 in hMAPC, $p < 0.01$; hMAPC vs. hAC133$^+$, $p < 0.05$) (Fig. 2G–J), the effect of hMAPC on collateral expansion was much more apparent (α-actin-coated vessel area %: 0.32 ± 0.10 in PBS; 0.59 ± 0.10 in hAC133$^+$, $p < 0.05$ and 0.72 ± 0.14 in hMAPC, $p < 0.05$; hMAPC vs. hAC133$^+$, $p < 0.05$) (Fig. 2K–N).

Unlike hAC133$^+$ Cells, hMAPC Significantly Improve Muscle Viability and Regeneration

In a next series of analyses, we determined whether there was a correlation between revascularization and the degree of tissue damage or regeneration, by analyzing muscle necrosis, fibrosis, and regeneration 30 days posttransplantation. Necrosis and fibrosis were significantly lower in the hMAPC-treated but not in hAC133$^+$-treated animals, compared to PBS [% necrosis: 31 ± 5 in PBS; 27 ± 5 in hAC133$^+$, $P = NS$ and 12 ± 5 in hMAPC, $p < 0.05$; hMAPC vs. hAC133$^+$, $p < 0.05$ (Fig. 3A–D); % fibrosis: 15 ± 2 in PBS; 12 ± 3 in hAC133$^+$, $P = NS$ and 8 ± 2 in hMAPC, $p < 0.05$; hMAPC vs. hAC133$^+$, NS (Fig. 3E–H)]. Moreover, also by day 15, necrosis and fibrosis were significantly lower in the hMAPC-treated animals than in the hAC133$^+$-treated ones (data not shown). Finally, we evaluated the degree of muscle regeneration (myogenesis), defined by the presence of fibers with strong positivity for desmin (14). Unlike hAC133$^+$ cell-injected muscles, at day 30, hMAPC-treated muscles showed a statistically significant higher expression of this marker compared with nontreated limbs [% desmin area: 1.15 ± 0.15 in PBS; 1.51 ± 0.13 in hAC133$^+$, $p = NS$; 1.71 ± 0.17 in hMAPC, $p < 0.05$; hMAPC vs. hAC133$^+$, NS) (Fig. 3I–L), a difference that was already detected at day 15 (data not shown). These results suggest a unique capacity of hMAPC to contribute to muscle regeneration compared to hAC133$^+$ cells.

hMAPC Secrete Factors That Protect Against Apoptosis and Stimulate Proliferation

Because direct contribution to vessels was limited, we hypothesized that the beneficial effects on revascularization and muscle regeneration were due to trophic effects on endogenous cells. Therefore, we measured growth factor secretion and performed experiments with CM on vascular and muscular cells. Quantification by qRT-PCR showed a higher expression of proangiogenic and arteriogenic factors ($VEGF_{165}$, bFGF, and PlGF) in hMAPC versus hAC133$^+$ cells (hMAPC vs. hAC133$^+$ cells: % expression vs. universal RNA: $VEGF_{165}$: 236 ± 144 vs. 8.4 ± 0.4; PlGF: 1828 ± 2407 vs. 10 ± 5; bFGF: 452 ± 106 vs. 12 ± 17). Protein secretion, determined by ELISA, confirmed the higher secretion of $VEGF_{165}$, bFGF, and PlGF by hMAPC (hMAPC vs. hAC133$^+$ cells: pg/10^5 cells: $VEGF_{165}$: 8066 ± 636 vs. undetectable; bFGF: 20 ± 3 vs. undetectable; PlGF: 11 ± 5 vs. undetectable).

To determine whether the release of growth factors by the transplanted cells could be responsible for the observed beneficial effect on vascular/muscular regeneration in vivo, we analyzed the potential of hAC133$^+$ cell-

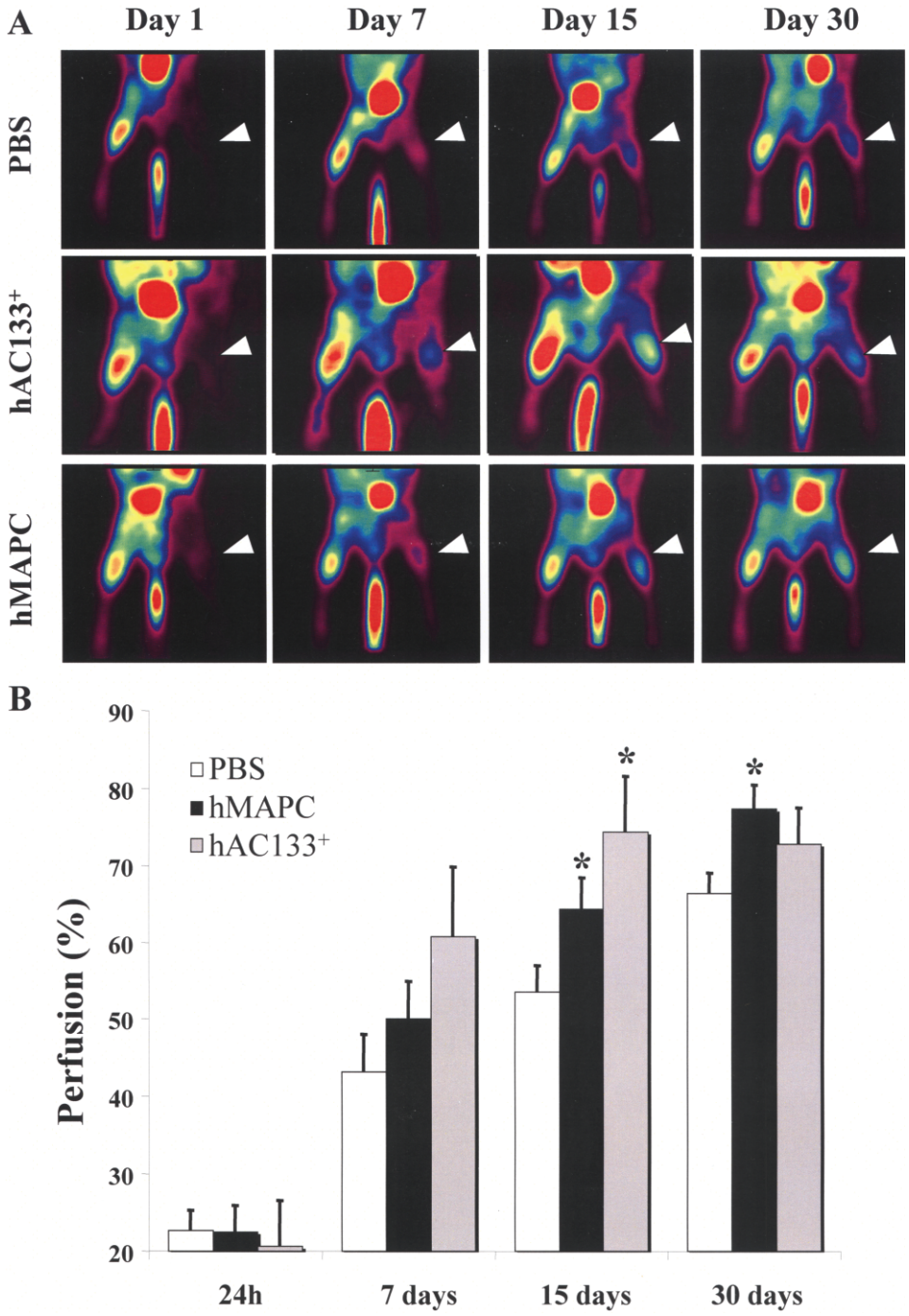


Figure 1. hMAPC have a more durable effect on limb reperfusion than hAC133+ cells. (A) Representative ^{13}N-ammonia PET images taken 1, 7, 15, and 30 days after ischemia and transplantation of PBS, hAC133+ cells, or hMAPC are shown. Arrowheads indicate the ischemic limb. (B) ^{13}N-Ammonia uptake in animals treated with PBS (white bars), hAC133+ cells (gray bars), and hMAPC (black bars) are shown at the different time points as the mean% ± SEM blood perfusion relative to the nonischemic limbs ($N = 7–10$ mice/group). *$p < 0.05$.

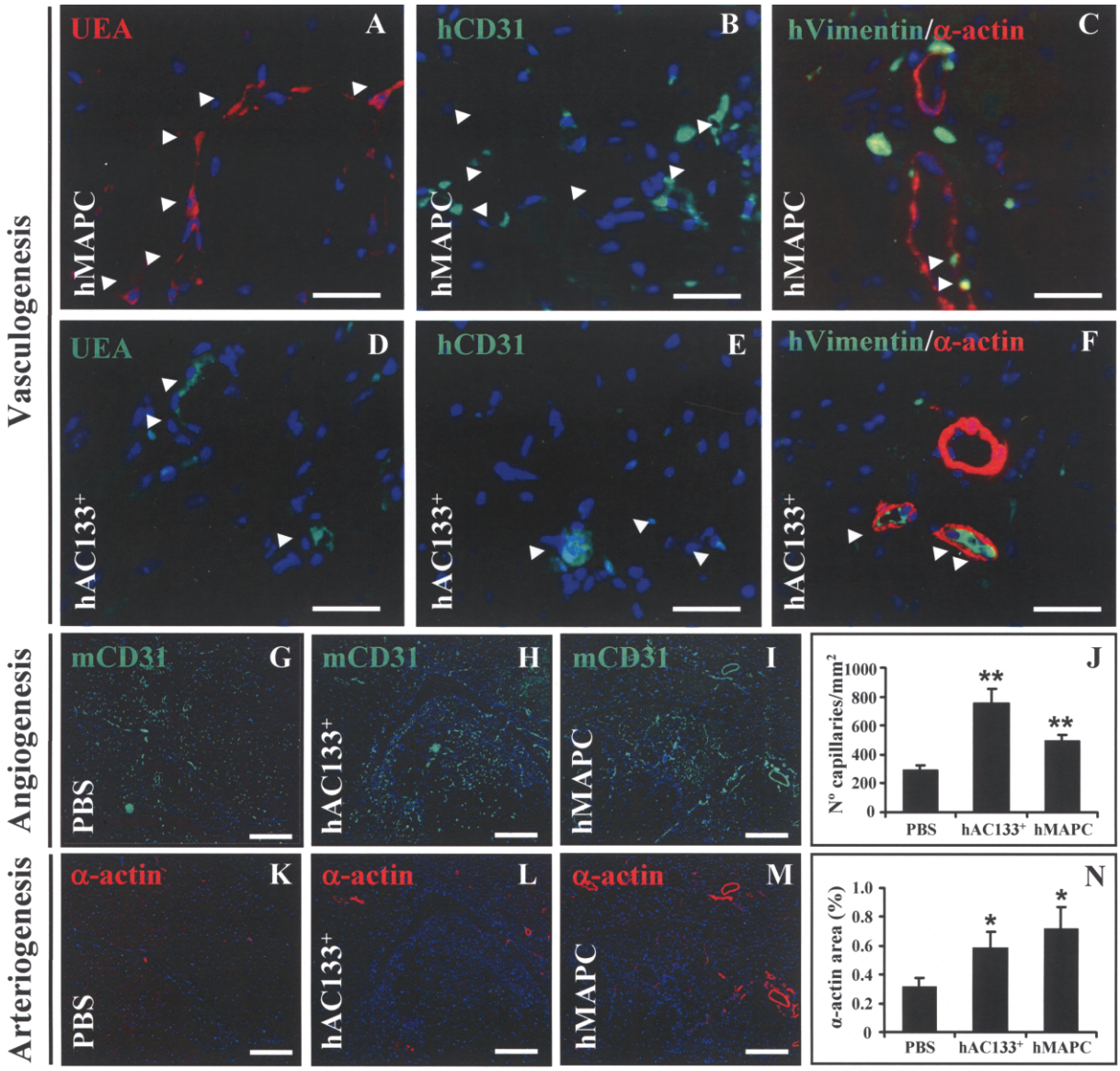


Figure 2. hMAPC and hAC133⁺ cells have a differential effect on blood vessel growth. (A–F) Analysis of direct contribution (vasculogenesis) by immunofluorescence-stained cross sections of hMAPC-transplanted (A–C) or hAC133⁺-transplanted (D–F) adductors for UEA lectin, only recognizing human EC (red in A, green in D), human-specific CD31 (green in B, E) indicating the endothelial identity of the transplanted cells (arrowheads), and double staining for human vimentin (recognizing human mesodermal cells like EC and smooth muscle cells) (green in C, F) and smooth muscle α-actin (recognize both human and mouse smooth muscle cells) (red in C, F) showing colocalization in hMAPC but not in hAC133⁺ cells. (G–J) Analysis of endogenous angiogenesis status. Representative pictures of capillary density (revealed by mouse-specific CD31 in green) in PBS (G), hAC133⁺ (H), or hMAPC-treated (I) quadriceps muscles and corresponding quantification (J; data expressed as number of capillaries/muscle area in mm^2; mean ± SEM). (K–N) Representative pictures of collaterals (arteriogenesis) [revealed by (mouse and human) smooth muscle α-actin in red] in PBS (K), hAC133⁺ (L), or hMAPC-treated (M) quadriceps muscles and corresponding quantification (N; data expressed as fractional area in % ± SEM; $N = 4–6$ mice per group). DAPI was used for nuclear staining in (A–I, K–M). *$p < 0.05$ and **$p < 0.01$ versus PBS. Scale bars: 50 μm (A–F) and 400 μm (G–I, K–M).

or hMAPC-derived CM to induce proliferation and/or to inhibit apoptosis of vascular/muscular cells. Culture of EC and SMC in the presence of hMAPC but not hAC133+ CM induced a significant increase in their proliferation rate in comparison with the NCM (EC proliferation relative to NCM: 1.0 ± 0.2-fold for hAC133+ CM, $p = \text{NS}$ and 6.5 ± 0.8-fold for hMAPC-CM, $p < 0.05$; SMC proliferation relative to NCM: 0.9 ± 0.1-fold for hAC133+ CM, $p = \text{NS}$ and 1.4 ± 0.2-fold for hMAPC-CM, $p < 0.05$) (Fig. 4A, B. In addition, while hMAPC-CM almost completely abrogated the apoptosis induced by hypoxia (1% O_2) and serum deprivation in the EC and SMC, no significant protection was observed when cells were cultured in the presence of hAC133+ CM [% EC apoptosis vs. NCM (100%): 7 ± 3 for CCM (normoxia and 10% FCS), $p < 0.01$, 84 ± 8 for hAC133+ CM, $p = \text{NS}$ and 35 ± 12 for hMAPC-CM, $p < 0.05$; % SMC apoptosis vs. NCM (100%): 18 ± 1 for CCM, $p < 0.01$, 87 ± 5 for hAC133+ CM, $p = \text{NS}$ and 21 ± 10 for hMAPC CM, $p < 0.05$] (Fig. 4C). Finally, the effect of CM on SkM proliferation was moderate in the case of hMAPC CM with a trend towards statistical significance (data not shown); however, unlike hAC133+ CM, hMAPC CM exerted a potent antiapoptotic effect on SkM when these were exposed to hypoxia or serum deprivation [% SkM apoptosis vs. NCM (100%): 3 ± 3 for CCM, $p < 0.01$, 97 ± 16 for hAC133+ CM, $p = \text{NS}$, and 20 ± 8 for hMAPC CM, $p < 0.01$] (Fig. 4C). Thus, hMAPC, but not hAC133+ cells, had a beneficial effect on vascular/muscular cell proliferation and/or survival through paracrine effects.

DISCUSSION

The already high incidence of PVD will further increase due to a growing number of patients that carry risk factors (e.g., diabetes, hypertension) predisposing them for developing PVD. At the same time, for many of these patients these risk factors may limit the therapeutic options for revascularization. The demonstration of postnatal vasculogenesis (6) fueled a boost in preclinical studies as well as a rapid clinical translation of this concept. However, many issues need to be resolved be-

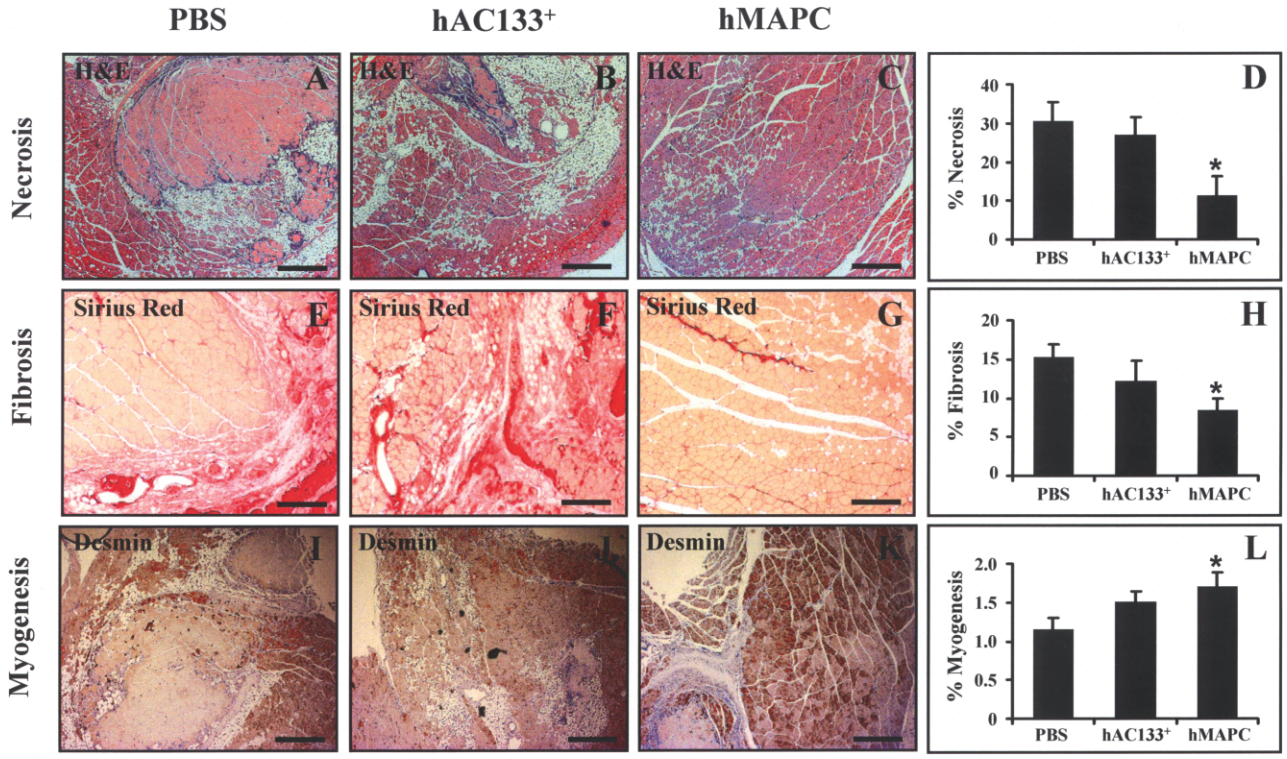


Figure 3. Unlike hAC133+ cells, hMAPC significantly improve muscle viability and regeneration. (A–D) Representative pictures of necrosis (defined by dead muscle cells, fat, and inflammatory clusters on H&E) in PBS (A), hAC133+ (B), or hMAPC-treated (C) quadriceps muscles and corresponding quantification (D); data expressed as fractional area in % ± SEM ($N = 4$–6 mice per group). (E–H) Representative pictures of fibrosis (revealed by Sirius red staining) in PBS (E), hAC133+ (F), or hMAPC-treated (G) quadriceps muscles and corresponding quantification (H); data expressed as fractional area in % ± SEM ($N = 4$–6 mice per group). (I–L) Representative pictures of myogenesis (revealed by desmin staining) in PBS (I), hAC133+ (J), or hMAPC-treated (K) quadriceps muscles and corresponding quantification (L; data expressed as fractional area in % ± SEM; $N = 4$–6 mice per group). *$p < 0.05$ versus PBS. Scale bars: 200 μm (E–G) and 400 μm (A–C, I–K).

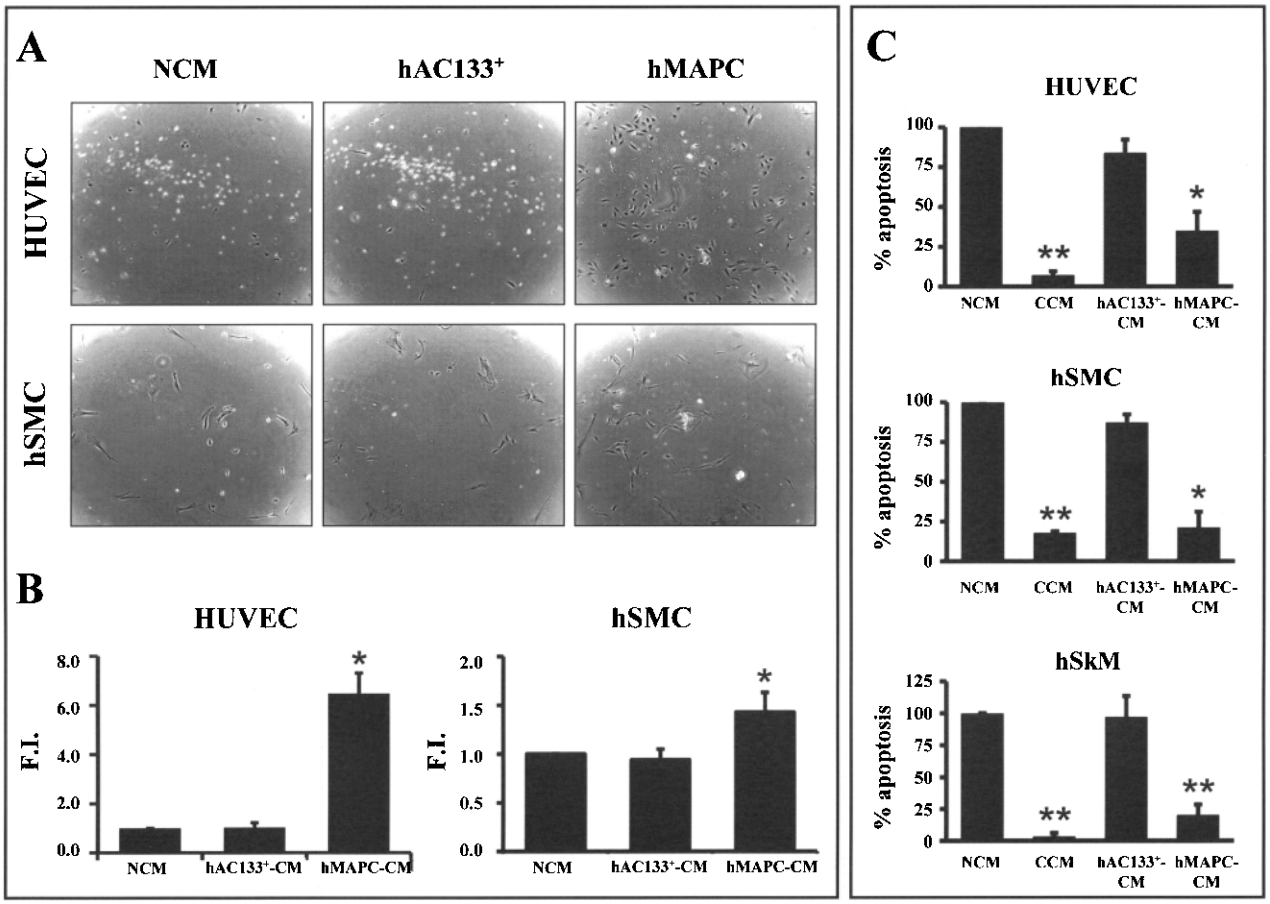


Figure 4. hMAPC secrete factors that protect against apoptosis and stimulate proliferation. (A, B) HUVEC and SMC were cultured in NCM, hAC133$^+$ CM, or hMAPC CM for 72 h. Cell proliferation is shown as fold increase in comparison with proliferation in NCM. The mean ± SEM of four different experiments in quadruplicate is shown as well as representative pictures showing the cell density differences after 72 h. *$p < 0.05$. (C) Apoptosis of HUVEC, SMC, and skeletal myoblasts (SkM) induced by hypoxia and serum deprivation was measured by ELISA after 24 h (HUVEC) or 72 h (SMC and SkM) in culture in the presence of NCM, CCM (cell culture media and normoxia), hAC133$^+$ CM, or hMAPC-CM. Values are expressed as percentage of apoptosis relative to the control group (cells in hypoxic conditions cultured with NCM) where apoptosis was considered 100%. The mean ± SEM of four different experiments in quadruplicate is shown. *$p < 0.05$; **$p < 0.01$.

fore cell transplantation becomes routine practice for PVD treatment. In this context, we performed a recent study in which we compared murine unfractionated bone marrow cells with mMAPC in a model reminiscent of intermittent claudication, a milder PVD form (4). The current study was intended to further extend these studies in order to increase our understanding of the role of stem cells in ischemic disease: by comparing two populations of human-derived cells; by following up the effects of transplantation for longer term; by testing the cells in a severe limb ischemia model, reminiscent of critical limb ischemia (which is the main target for cell therapy); and by performing mechanistic studies to get insight into the way these cells may have beneficial effects. We found that hMAPC had a more durable effect on revascularization than hAC133$^+$ cells. In addition, unlike hAC133$^+$ cells, hMAPC stimulated muscle regeneration to a statistically significant extent in this severe ischemic setting.

We chose to compare hMAPC with hAC133$^+$ cells because the latter are already used in clinical PVD trials (8,9,20). AC133 (or prominin-1) is a surface antigen expressed on precursors with hematopoietic, neuronal, endothelial, or myogenic potential (30). Our study is not the first to evaluate these cells in a preclinical PVD model; however, in most of the studies, their potential was not compared to that of other stem cell sources and the study settings were also different (7,27,31). Unlike in the majority of these studies, we used a severe model (by more proximal ligation/excision combined with an immune-deficient and low collateral reserve BalbC genetic background) and followed the effects on blood

flow recovery beyond 3 weeks of transplantation. While hMAPC had a sustained and progressive effect on perfusion recovery, the initial benefit of hAC133$^+$ cell transplantation was lost at 1 month, with a regression of the capillary vessels from day 15 to day 30. Interestingly, the most recent clinical study with the latter cells also showed that the initially observed functional improvement was no longer significant after a 1-year follow-up (8).

Several reasons may explain the temporary nature of the hAC133$^+$ cell treatment effect. One may be the limited engraftment of the cells in the inflammatory ischemic environment. This may be overcome by delivering the cells on a matrix scaffold, as recently demonstrated in ischemic rat limbs (31). As for the clinical studies, another problem relates to the functional deficit of EPC derived from patients with risk factors (12,23). Recent studies have revealed that these functionally deficient cells can be "reeducated" by pretreatment with (angiogenic) growth factors (33). In the current study we propose another reason for the temporal benefit of hAC133$^+$ cells compared to the sustained effect of hMAPC (i.e., a differential mechanism of revascularization). While hAC133$^+$ mainly affected capillary growth, hMAPC had a significant effect on collateral growth. The latter is responsible for delivering the bulk flow and hence may represent a more crucial requirement for long-term revascularization of the ischemic tissue.

The importance of muscle regeneration is often not considered when evaluating cell therapy studies for limb ischemia. However, from our earlier comparative studies, it was apparent that, even in case of a milder form of ischemia, cells with a combined vascular and muscular regeneration effect, such as undifferentiated mMAPC, had a more durable effect on limb functional recovery compared to cells with a restricted vascular regenerative effect, such as vascular predifferentiated mMAPC (4). Most likely, muscle regenerative effects are even more desirable in more severe forms of ischemia, such as in the model used in the current study. While this has not been shown in a limb ischemia model, hAC133$^+$ cells have been recently reported to contribute to skeletal muscle regeneration in a model of muscle injury (29). Here, there was a trend towards stimulation of muscle regeneration (estimated by the appearance of desmin-positive regenerating fibers) following hAC133$^+$ cell treatment; however, this did not reach statistical significance. hMAPC, on the other hand, did stimulate myogenesis to a significant extent, confirming our previous observations (4). Therefore, despite higher perfusion capacity and greater capillary density induced by day 15 in the AC133$^+$-treated tissues, no significant effect on muscle necrosis was observed, suggesting that the indirect effect exerted by the hMAPC in muscle regeneration was critical for limb recovery. Muscle regeneration was observed even macroscopically in hMAPC-treated limbs, suggesting a more durable effect of this cell population in comparison with AC133$^+$ cells. Additional studies in large animal models will contribute valuable information for future application of hMAPC in patients.

The direct contribution of hAC133$^+$ or hMAPC to muscle fibers has not been analyzed in the current study. However, we have previously demonstrated a limited contribution of mMAPC to skeletal muscle in a similar hind limb model, even though mostly due to fusion processes (4). Although we cannot rule out a direct muscle (trans)differentiation of hMAPC or even hAC133$^+$ cells, the limited engraftment suggests that muscle regeneration was mainly derived from stimulation of endogenous cells induced by factors secreted from hMAPC.

The existence of stem/progenitor cells with broad differentiation potential, such as ESC, and more recently induced pluripotency cells has raised many hopes for regenerative approaches, because one cell type would be sufficient to supply cells for many different damaged tissue types. However, in the last 5 years this paradigm has been shifting, because many studies have now shown that the main benefit of stem cell transplantation may rather result from their trophic activity. Also in the context of ischemia, the potential of stem cells to confer functional and perfusional improvement by the production of cytokines is by no means a new concept (18,19). In our previous study (4), we pointed out that also mMAPC have a beneficial effect on vessel and muscle regeneration mainly by secreting growth factors such as VEGF and IGF-1. Here, we show that also hMAPC secrete significant amounts of $VEGF_{165}$, PlGF, and bFGF, while hAC133$^+$ cells did not produce these factors at levels that were detectable by our ELISA assays. Accordingly, CM from hMAPC but not hAC133$^+$ cells stimulated EC, SMC, and SkM proliferation and/or survival. Our failure to detect VEGF or other cytokines in hAC133$^+$ CM may appear in conflict with a recent study; however, they used cells from a different source (fetal aorta versus cord blood in our study) (7).

Patients with myocardial infarction (MI) can also benefit from treatment with endothelial progenitor cells. In fact, functional improvement has been shown after MAPC or AC133 treatment (1,11,21,24), although unfortunately, a direct comparison between the different cell types has not been performed. In accordance with our results in the limb ischemia model, low/absent engraftment and differentiation potential of both cell types have been demonstrated, suggesting that paracrine mechanisms are responsible of the benefit observed. In fact, we have shown that secretion of angio/arteriogenic factors by MAPC are associated with revascularization of the ischemic tissue (24).

In summary, our study demonstrates that locally in-

jected hMAPC but not hAC133+ cells have a long-term effect on limb revascularization and, in addition, contribute to muscle regeneration. We believe that these results set the stage for future clinical trials with hMAPC in patients with critical limb ischemia.

ACKNOWLEDGMENTS: This work was supported by grants from the MICCIN PI050168, PI070474, PSE SINBAD, and RD06/0014; Comunidad de Trabajo de los Pirineos (CTP); European Union Framework Project VI (StrokeMAP) and VII (INELPY); Government of Navarra (Department of Education) the "UTE project CIMA'; the American Heart Association (A.L. and B.P.); "Excellentie financiering KULeuven (EF/05/013)" (A.L.).

REFERENCES

1. Agbulut, O.; Vandervelde, S.; Al Attar, N.; Larghero, J.; Ghostine, S.; Leobon, B.; Robidel, E.; Borsani, P.; Le Lorc'h, M.; Bissery, A.; Chomienne, C.; Bruneval, P.; Marolleau, J. P.; Vilquin, J. T.; Hagege, A.; Samuel, J. L.; Menasche, P. Comparison of human skeletal myoblasts and bone marrow-derived CD133+ progenitors for the repair of infarcted myocardium. J. Am. Coll. Cardiol. 44: 458–463; 2004.
2. Amann, B.; Luedemann, C.; Ratei, R.; Schmidt-Lucke, J. A. Autologous bone marrow cell transplantation increases leg perfusion and reduces amputations in patients with advanced critical limb ischemia due to peripheral artery disease. Cell Transplant. 18:371–380; 2009.
3. Aranguren, X. L.; Luttun, A.; Clavel, C.; Moreno, C.; Abizanda, G.; Barajas, M. A.; Pelacho, B.; Uriz, M.; Arana, M.; Echavarri, A.; Soriano, M.; Andreu, E. J.; Merino, J.; Garcia-Verdugo, J. M.; Verfaillie, C. M.; Prosper, F. In vitro and in vivo arterial differentiation of human multipotent adult progenitor cells. Blood 109: 2634–2642; 2007.
4. Aranguren, X. L.; McCue, J. D.; Hendrickx, B.; Zhu, X. H.; Du, F.; Chen, E.; Pelacho, B.; Penuelas, I.; Abizanda, G.; Uriz, M.; Frommer, S. A.; Ross, J. J.; Schroeder, B. A.; Seaborn, M. S.; Adney, J. R.; Hagenbrock, J.; Harris, N. H.; Zhang, Y.; Zhang, X.; Nelson-Holte, M. H.; Jiang, Y.; Billiau, A. D.; Chen, W.; Prosper, F.; Verfaillie, C. M.; Luttun, A. Multipotent adult progenitor cells sustain function of ischemic limbs in mice. J. Clin. Invest. 118:505–514; 2008.
5. Aranguren, X. L.; Verfaillie, C. M.; Luttun, A. Emerging hurdles in stem cell therapy for peripheral vascular disease. J. Mol. Med. 87:3–16; 2009.
6. Asahara, T.; Murohara, T.; Sullivan, A.; Silver, M.; van der Zee, R.; Li, T.; Witzenbichler, B.; Schatteman, G.; Isner, J. M. Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967; 1997.
7. Barcelos, L. S.; Duplaa, C.; Krankel, N.; Graiani, G.; Invernici, G.; Katare, R.; Sir Katare,agusa, M.; Meloni, M.; Campesi, I.; Monica, M.; Simm, A.; Campagnolo, P.; Mangialardi, G.; Stevanato, L.; Alessandri, G.; Emanueli, C.; Madeddu, P. Human CD133+ progenitor cells promote the healing of diabetic ischemic ulcers by paracrine stimulation of angiogenesis and activation of Wnt signaling. Circ. Res. 104:1095–1102; 2009.
8. Burt, R. K.; Testori, A.; Oyama, Y.; Rodriguez, H. E.; Yaung, K.; Villa, M.; Bucha, J. M.; Milanetti, F.; Sheehan, J.; Rajamannan, N.; Pearce, W. H. Autologous peripheral blood CD133+ cell implantation for limb salvage in patients with critical limb ischemia. Bone Marrow Transplant. 45:111–116; 2010.
9. Canizo, M. C.; Lozano, F.; Gonzalez-Porras, J. R.; Barros, M.; Lopez-Holgado, N.; Briz, E.; Sanchez-Guijo, F. M. Peripheral endothelial progenitor cells (CD133 +) for therapeutic vasculogenesis in a patient with critical limb ischemia. One year follow-up. Cytotherapy 9:99–102; 2007.
10. Cho, S. W.; Moon, S. H.; Lee, S. H.; Kang, S. W.; Kim, J.; Lim, J. M.; Kim, H. S.; Kim, B. S.; Chung, H. M. Improvement of postnatal neovascularization by human embryonic stem cell derived endothelial-like cell transplantation in a mouse model of hindlimb ischemia. Circulation 116:2409–2419; 2007.
11. Das, H.; George, J. C.; Joseph, M.; Das, M.; Abdulhameed, N.; Blitz, A.; Khan, M.; Sakthivel, R.; Mao, H. Q.; Hoit, B. D.; Kuppusamy, P.; Pompili, V. J. Stem cell therapy with overexpressed VEGF and PDGF genes improves cardiac function in a rat infarct model. PLoS One 4:e7325; 2009.
12. Dimmeler, S.; Vasa-Nicotera, M. Aging of progenitor cells: Limitation for regenerative capacity? J. Am. Coll. Cardiol. 42:2081–2082; 2003.
13. Emmerich, J. Current state and perspective on medical treatment of critical leg ischemia: Gene and cell therapy. Int. J. Low. Extrem. Wounds 4:234–241; 2005.
14. Gallanti, A.; Prelle, A.; Moggio, M.; Ciscato, P.; Checcarelli, N.; Sciacco, M.; Comini, A.; Scarlato, G. Desmin and vimentin as markers of regeneration in muscle diseases. Acta Neuropathol. 85:~–92; 1992.
15. Herreros, J.; Prosper, F.; Perez, A.; Gavira, J. J.; Garcia-Velloso, M. J.; Barba, J.; Sanchez, P. L.; Canizo, C.; Rabago, G.; Marti-Climent, J. M.; Hernandez, M.; Lopez-Holgado, N.; Gonzalez-Santos, J. M.; Martin-Luengo, C.; Alegria, E. Autologous intramyocardial injection of cultured skeletal muscle-derived stem cells in patients with non-acute myocardial infarction. Eur. Heart J. 24:2012–2020; 2003.
16. Hirose, N.; Maeda, H.; Yamamoto, M.; Hayashi, Y.; Lee, G. H.; Chen, L.; Radhakrishnan, G.; Rao, P.; Sasaguri, S. The local injection of peritoneal macrophages induces neovascularization in rat ischemic hind limb muscles. Cell Transplant. 17:211–222; 2008.
17. Ikenaga, S.; Hamano, K.; Nishida, M.; Kobayashi, T.; Li, T. S.; Kobayashi, S.; Matsuzaki, M.; Zempo, N.; Esato, K. Autologous bone marrow implantation induced angiogenesis and improved deteriorated exercise capacity in a rat ischemic hindlimb model. J. Surg. Res. 96:277–283; 2001.
18. Iwase, T.; Nagaya, N.; Fujii, T.; Itoh, T.; Murakami, S.; Matsumoto, T.; Kangawa, K.; Kitamura, S. Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. Cardiovasc. Res. 66:543–551; 2005.
19. Kinnaird, T.; Stabile, E.; Burnett, M. S.; Shou, M.; Lee, C. W.; Barr, S.; Fuchs, S.; Epstein, S. E. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. Circulation 109: 1543–1549; 2004.
20. Kolvenbach, R.; Kreissig, C.; Ludwig, E.; Cagiannos, C. Stem cell use in critical limb ischemia. J. Cardiovasc. Surg. (Torino) 48:39–44; 2007.
21. Leor, J.; Guetta, E.; Feinberg, M. S.; Galski, H.; Bar, I.; Holbova, R.; Miller, L.; Zarin, P.; Castel, D.; Barbash, I. M.; Nagler, A. Human umbilical cord blood-derived CD133+ cells enhance function and repair of the infarcted myocardium. Stem Cells 24:772–780; 2006.

22. Miyamoto, K.; Nishigami, K.; Nagaya, N.; Akutsu, K.; Chiku, M.; Kamei, M.; Soma, T.; Miyata, S.; Higashi, M.; Tanaka, R.; Nakatani, T.; Nonogi, H.; Takeshita, S. Unblinded pilot study of autologous transplantation of bone marrow mononuclear cells in patients with thromboangiitis obliterans. Circulation 114:2679–2684; 2006.
23. Oliveras, A.; Soler, M. J.; Martinez-Estrada, O. M.; Vazquez, S.; Marco-Feliu, D.; Vila, J. S.; Vilaro, S.; Lloveras, J. Endothelial progenitor cells are reduced in refractory hypertension. J. Hum. Hypertens. 22:183–190; 2008.
24. Pelacho, B.; Nakamura, Y.; Zhang, J.; Ross, J.; Heremans, Y.; Nelson-Holte, M.; Lemke, B.; Hagenbrock, J.; Jiang, Y.; Prosper, F.; Luttun, A.; Verfaillie, C. Multipotent adult progenitor cell transplantation increases vascularity and improves left ventricular function after myocardial infarction. J. Tissue Eng. Regen. Med. 1:51–59; 2007.
25. Penuelas, I.; Aranguren, X. L.; Abizanda, G.; Marti-Climent, J. M.; Uriz, M.; Ecay, M.; Collantes, M.; Quincoces, G.; Richter, J. A.; Prosper, F. (13)N-ammonia PET as a measurement of hindlimb perfusion in a mouse model of peripheral artery occlusive disease. J. Nucl. Med. 48: 1216–1223; 2007.
26. Planat-Benard, V.; Silvestre, J. S.; Cousin, B.; Andre, M.; Nibbelink, M.; Tamarat, R.; Clergue, M.; Manneville, C.; Saillan-Barreau, C.; Duriez, M.; Tedgui, A.; Levy, B.; Penicaud, L.; Casteilla, L. Plasticity of human adipose lineage cells toward endothelial cells: Physiological and therapeutic perspectives. Circulation 109:656–663; 2004.
27. Sanchez-Guijo, F. M.; Oterino, E.; Barbado, M. V.; Carrancio, S.; Lopez-Holgado, N.; Muntion, S.; Hernandez-Campo, P.; Sanchez-Abarca, L. I.; Perez-Simon, J. A.; Miguel, J. F.; Brinon, J. G.; Canizo, M. C. Both CD133$^+$ cells and monocytes provide significant improvement for hindlimb ischemia, although they do not transdifferentiate into endothelial cells. Cell Transplant. 19:103–112; 2010.
28. Seaman, W. E.; Sleisenger, M.; Eriksson, E.; Koo, G. C. Depletion of natural killer cells in mice by monoclonal antibody to NK-1.1. Reduction in host defense against malignancy without loss of cellular or humoral immunity. J. Immunol. 138:4539–4544; 1987.
29. Shi, M.; Ishikawa, M.; Kamei, N.; Nakasa, T.; Adachi, N.; Deie, M.; Asahara, T.; Ochi, M. Acceleration of skeletal muscle regeneration in a rat skeletal muscle injury model by local injection of human peripheral blood-derived CD133-positive cells. Stem Cells 27:949–960; 2009.
30. Shmelkov, S. V.; St Clair, R.; Lyden, D.; Rafii, S. AC133/CD133/Prominin-1. Int. J. Biochem. Cell Biol. 37:715–719.; 2005.
31. Suuronen, E. J.; Veinot, J. P.; Wong, S.; Kapila, V.; Price, J.; Griffith, M.; Mesana, T. G.; Ruel, M. Tissue-engineered injectable collagen-based matrices for improved cell delivery and vascularization of ischemic tissue using CD133+ progenitors expanded from the peripheral blood. Circulation 114:I138–144; 2006.
32. Tolar, J.; O'Shaughnessy, M. J.; Panoskaltsis-Mortari, A.; McElmurry, R. T.; Bell, S.; Riddle, M.; McIvor, R. S.; Yant, S. R.; Kay, M. A.; Krause, D.; Verfaillie, C. M.; Blazar, B. R. Host factors that impact the biodistribution and persistence of multipotent adult progenitor cells. Blood 107:4182–4188; 2006.
33. Walter, D. H.; Rochwalsky, U.; Reinhold, J.; Seeger, F.; Aicher, A.; Urbich, C.; Spyridopoulos, I.; Chun, J.; Brinkmann, V.; Keul, P.; Levkau, B.; Zeiher, A. M.; Dimmeler, S.; Haendeler, J. Sphingosine-1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway via the S1P3 receptor. Arterioscler. Thromb. Vasc. Biol. 27:275–282; 2007.
34. Yang, C.; Zhang, Z. H.; Li, Z. J.; Yang, R. C.; Qian, G. Q.; Han, Z. C. Enhancement of neovascularization with cord blood CD133+ cell-derived endothelial progenitor cell transplantation. Thromb. Haemost. 91:1202–1212; 2004.
35. Yoon, C. H.; Hur, J.; Park, K. W.; Kim, J. H.; Lee, C. S.; Oh, I. Y.; Kim, T. Y.; Cho, H. J.; Kang, H. J.; Chae, I. H.; Yang, H. K.; Oh, B. H.; Park, Y. B.; Kim, H. S. Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: The role of angiogenic cytokines and matrix metalloproteinases. Circulation 112:1618–1627; 2005.

ANEXO IV

blood

2007 109: 2634-2642
Prepublished online Nov 7, 2006;
doi:10.1182/blood-2006-06-030411

In vitro and in vivo arterial differentiation of human multipotent adult progenitor cells

Xabier L. Aranguren, Aernout Luttun, Carlos Clavel, Cristina Moreno, Gloria Abizanda, Miguel A. Barajas, Beatriz Pelacho, Maialen Uriz, Miriam Araña, Ana Echavarri, Mario Soriano, Enrique J. Andreu, Juana Merino, Jose Manuel Garcia-Verdugo, Catherine M. Verfaillie and Felipe Prósper

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published semimonthly by the American Society of Hematology, 1900 M St, NW, Suite 200, Washington DC 20036.

In vitro and in vivo arterial differentiation of human multipotent adult progenitor cells

Xabier L. Aranguren,[1] Aernout Luttun,[2,3] Carlos Clavel,[1] Cristina Moreno,[1,4] Gloria Abizanda,[1] Miguel A. Barajas,[1,2] Beatriz Pelacho,[2] Maialen Uriz,[1] Miriam Araña,[1] Ana Echavarri,[1] Mario Soriano,[5] Enrique J. Andreu,[1] Juana Merino,[4] Jose Manuel Garcia-Verdugo,[5] Catherine M. Verfaillie,[2] and Felipe Prósper[1]

[1]Hematology Service and Cell Therapy, Clínica Universitaria, Foundation for Applied Medical Research, Division of Cancer, University of Navarra, Pamplona, Spain; [2]Stem Cell Institute, University of Minnesota Medical School, Minneapolis; [3]Center for Molecular and Vascular Biology, Catholic University of Leuven, Belgium; [4]Immunology Service, Clínica Universitaria, University of Navarra, Pamplona, Spain; [5]Department of Cell Biology, Instituto Cavanilles, University of Valencia, Spain

Many stem cell types have been shown to differentiate into endothelial cells (ECs); however, their specification to arterial or venous endothelium remains unexplored. We tested whether a specific arterial or venous EC fate could be induced in human multipotent adult progenitor cells (hMAPCs) and AC133$^+$ cells (hAC133$^+$). In vitro, in the presence of VEGF$_{165}$, hAC133$^+$ cells only adopted a venous and microvascular EC phenotype, while hMAPCs differentiated into both arterial and venous ECs, possibly because hMAPCs expressed significantly more sonic hedgehog *(Shh)* and its receptors as well as Notch 1 and 3 receptors and some of their ligands. Accordingly, blocking either of those pathways attenuated in vitro arterial EC differentiation from hMAPCs. Complementarily, stimulating these pathways by addition of Delta-like 4 (Dll-4), a Notch ligand, and Shh to VEGF$_{165}$ further boosted arterial differentiation in hMAPCs both in vitro and in an in vivo Matrigel model. These results represent the first demonstration of adult stem cells with the potential to be differentiated into different types of ECs in vitro and in vivo and provide a useful human model to study arteriovenous specification. (Blood. 2007;109:2634-2642)

Introduction

The vascular system is a bipolar complex network of arteries that transport oxygen-rich blood to all tissues and veins that bring oxygen-deprived blood back to the heart.[1] Because of this bipolar set-up, arteries and veins feature anatomic and physiological differences. Unlike venous endothelium, arterial endothelium is surrounded by several layers of smooth muscle cells (SMCs), separated by elastic laminae, and embedded in a thick layer of fibrillar collagen.[2] Moreover, both vessel types have a differential susceptibility to atherosclerotic disease, possibly due to exposure to different levels of shear stress. Arterial and venous endothelial cells (ECs) also have a distinct molecular signature, and such molecular specification occurs before the onset of blood flow.[3] Indeed, arteriovenous (AV) specification of ECs is accomplished early in development and is associated with the expression of a specific complement of factors: venous endothelium is characterized by the expression of EphB4,[4] Lefty-1,[5] Lefty-2,[5] COUP-TFII,[6] and MYO1-β,[5] and arterial ECs express high levels of Notch 1 and 4,[7] Dll-4,[8] EphrinB1 and EphrinB2,[4] Jagged-1 and -2,[7] connexin-40, and Hey-2 (gridlock zebrafish ortholog).[9,10] Studies in *Xenopus,* zebrafish, and mice have revealed that, besides blood flow,[11] vessel-intrinsic cues and—later in development—signals from outside the vasculature[12,13] are implicated in defining arterial or venous fate such as members of the TGF-β pathway,[14,15] VEGF isoforms,[13,16-18] neuropilins,[17] angiopoietins,[19] the Notch pathway,[7,9,20-22] the patched pathway,[20] and COUP-TFII, a member of the orphan nuclear receptor superfamily.[6]

Although it has been shown that some of these pathways are well conserved from zebrafish to mouse, less information is available on whether they have a similar role in humans. Because these molecular differences between arterial and venous ECs exist independently of blood flow and some of these factors work in an EC-intrinsic way,[2] it should be possible to manipulate some or all of these to endow ECs with an arterial or venous fate. Consistent with this notion, recent studies have suggested that arterial markers can be induced in primary mature ECs.[5,13,21,23,24]

Many different stem cell populations, including bone marrow (BM) mononuclear cells, AC133$^+$ endothelial progenitor cells, and embryonic stem cells have the potential to differentiate in vitro and in vivo into mature and functional ECs.[4,25-28] We have recently described another stem cell population, multipotent adult progenitor cells (MAPCs), that differentiates into most somatic cell types, including functional ECs, in vitro and in vivo.[29-33] The question of whether and how these stem cells can be coaxed into arterial or venous ECs has thus far not been addressed. In this study, we analyzed the in vitro and in vivo arterial and venous endothelial differentiation of human MAPCs (hMAPCs) and hAC133$^+$ cells.

Materials and methods

Additional and extended descriptions of methods are included in Document S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Submitted June 21, 2006; accepted October 29, 2006. Prepublished online as *Blood* First Edition Paper, November 7, 2006; DOI 10.1182/blood-2006-06-030411.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Cell populations

Samples were obtained after informed consent from donor or mother according to the guidelines from the Committee on the Use of Human Subjects in Research at the Clínica Universitaria, Pamplona, Spain. For hAC133$^+$ cells, BM and umbilical cord blood mononuclear cells were separated by Ficoll Hypaque centrifugation (specific gravity, 1077; Sigma, St Louis, MO), and hAC133$^+$ cells were selected using the autoMACS with the AC133 Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).[34] The purity of hAC133$^+$ cells was more than 90% in all samples by flow cytometry. New hMAPC clones were established, based on protocols described previously,[29-33] at the University of Navarra using BM from donors aged 18 to 54 years. BM mononuclear cells were depleted of CD45$^+$ and glycophorin A–positive cells using micromagnetic beads (Miltenyi Biotec) or directly after Ficoll-Paque. Characterization of hMAPCs was done by fluorescence-activated cell sorter (FACS) phenotyping, pluripotency marker expression analysis, and 3-lineage differentiation (Document S1, Figure S1, Table S1). Primary human umbilical vein ECs (HUVECs) and human umbilical artery ECs (HUAECs) were prepared in-house from human umbilical cords.

Endothelial differentiation/specification of hAC133$^+$ cells and hMAPCs

Human AC133$^+$ cells. For hAC133$^+$ cells, a total of 10^5/cm^2 were plated on fibronectin (50 μg/mL)–coated culture vessels in IMDM (Gibco BRL, Carlsbad, CA) supplemented with 20% fetal calf serum (FCS) (Gibco BRL), 50 ng/mL $VEGF_{165}$ (R&D Systems, Minneapolis, MN), 10 ng/mL bFGF (Sigma), and 1% penicillin/streptomycin. Cultures were maintained by media exchange every 4 to 5 days.

Human MAPCs. The hMAPCs were plated at 30×10^3 to 40×10^3/cm^2 in growth media: 58% low-glucose DMEM (Gibco BRL), 40% MCDB-201 (Sigma), 2% FCS (Biochrom, Cambridge, United Kingdom), ITS+1 (Sigma), 10^{-8} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 1% penicillin/streptomycin (Gibco BRL), and 10 ng/mL each of PDGF-BB and EGF during 24 hours, and then media were exchanged with the same media without FCS, EGF, and PDGF-BB but now with 100 ng/mL $VEGF_{165}$. Media were changed every 4 to 5 days. Arterial-specific differentiation was boosted by addition of different combinations of the following growth factors: $VEGF_{165}$ in different combinations with Dll-4, Jagged-1, or Shh (all from R&D Systems at 100 ng/mL). Blocking of patched and Notch pathways was performed using cyclopamine[35] (Biomol, Plymouth Meeting, PA) at 5 μM (added every 4 to 5 days with medium change) and 1 μM γ-secretase inhibitor L-685458 (Bachem, King of Prussia, PA),[36] respectively.

FACS analysis

For fluorescence-activated cell sorter (FACS) analysis, cells were detached with 0.05% trypsin-EDTA and washed with phosphate-buffered saline (PBS). The following antibodies were used: CD31-PE, CD34-APC, $\alpha_V\beta_3$-PE, CD73-PE, CD45-PerCP, CD90-APC, HLA-DR–PE, HLA-DP–PE, HLA-DQ–PE, HLA-A–PE, HLA-B–PE, HLA-C–PE, CD44-PE, CD13-PE, CD36-FITC, CD16+CD56-PE, CD3-FITC, CD19-APC, CD11B-PE, CD11C-APC, CD14-APC, CD14 FITC (all from BD PharMingen, San Diego, CA), CD105-PE (Ancell, Bayport, MN), and CD133/1-PE and CD133/1APC (Miltenyi Biotec), and their corresponding isotype controls (all from BD PharMingen). From 50 000 to 200 000 cells were incubated with primary antibody for 15 minutes in the dark at room temperature. Cells were fixed with 4% paraformaldehyde at 4°C. Syto was used to determine cell viability when necessary. Samples were analyzed using a FACS Calibur (BD PharMingen) cell sorter and Cellquest (BD PharMingen) software.

Immunofluorescent and histochemistry staining and analysis

A list of primary antibodies is provided in Table S2. Secondary antibodies coupled to FITC or PE were from Molecular Probes (Eugene, OR). For immunofluorescent staining, samples were fixed with 4% paraformaldehyde at 20°C and, in case of intracellular molecules, permeabilized with 0.1% Triton X-100. Blocking solution consisted of PBS, 1% bovine serum albumin, and 10% donkey serum. Primary antibodies were diluted in blocking solution and applied overnight at 4°C. After incubation, nonspecific binding was washed with a solution of PBS and 0.1% Tween 20. Secondary antibody at a dilution 1:1000 in PBS was applied for 1 hour at 4°C. Nonadherent antibody was washed with PBS and 0.1% Tween 20, after which samples were mounted using DAPI (Vector Laboratories, Burlingame, CA) or TO-PRO-3 iodide (Molecular Probes, Leiden, The Netherlands) as nuclear marker. For controls, cells were labeled with unspecific immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with the secondary antibody. For immunohistochemistry staining, the Envision (DAKO, Glostrup, Denmark) and ABC (Vector Laboratories) systems were used. Sirius red[37] and orcein[38] staining were performed as described. To quantify the percentage of cultured cells expressing arterial or venous markers, the number of positive cells in 20 randomly selected fields were scored and divided by the total number of cells.

Images were generated using a Zeiss microscope (Zeiss, Jena, Germany) connected to a monochrome fluorescence camera (Coolsnap SF; Photometrics, ____, Germany) and a CCD camera (SPOT; Diagnostic Instruments, Sterling Heights, MI), equipped with 20×/0.75 NA dry, 40×/0.75 NA dry, and 63×/1.40 NA oil objectives (Zeiss). Morphometric analyses were performed using MetaMorph software (version 6.3r6; Molecular Devices, Downington, PA). Immersion oil was purchased from Zeiss.

RNA isolation and real-time and quantitative RT-PCR

Total RNA was obtained using the RNeasy Mini extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A more detailed description of methods and the primers used for reverse transcriptase–polymerase chain reaction (RT-PCR) and real-time PCR are shown in Document S1 and Table S3, respectively.

In vitro EC functional tests

To analyze acetylated-LDL uptake, cells were washed and 10 μg/mL acetylated-LDL-DiI (Biomedical Technologies, Stoughton, MA) was added in IMDM or MAPC differentiation medium (described under "Human MAPCs," above). Cells were incubated for 2 hours at 37°C, washed, fixed, and viewed under a fluorescent microscope using DAPI as nuclear marker. For the in vitro Matrigel assay, 1 mm cold (4°C) Matrigel (BD Pharmingen) was incubated for 30 minutes at 37°C. After gelification, 30×10^3 to 50×10^3 cells differentiated for 14 days were plated in differentiation media on Matrigel. After 24 to 48 hours, tube formation was analyzed.

Electron microscopy

For ultrastructural studies, samples were washed in PBS and fixed with 2% glutaraldehyde. Samples were postfixed with 1% osmium, rinsed, dehydrated, and embedded in Araldite (Fluka, Buchs, Switzerland). Semithin sections (1.5 μm) were cut with a diamond knife and stained lightly with 1% toluidine blue. Then, semithin sections were reembedded in an araldite block and detached from the glass slide by repeated freezing (liquid nitrogen) and thawing. The block with semithin sections was cut into ultrathin (50 to 70 nm) sections, stained with lead citrate, and examined under a Jeol (Tokyo, Japan) JEM-1010 electron microscope.

ELISA

To assess cytokine production of undifferentiated cells, hMAPCs were plated in triplicate at 30×10^3 to 40×10^3/cm^2 at day 0 in cytokine-less expansion media, and supernatant was collected 60 hours later and frozen. To assess cytokine production in differentiated cells, cells were plated in triplicate for endothelial differentiation as described above and media were collected after 7 and 14 days and frozen. Enzyme-linked immunosorbent assay (ELISA) kits were from R&D Systems, and the procedure was performed according to the manufacturer's recommendations.

In vivo Matrigel model

For the in vivo Matrigel plug assay, 10-week-old nude mice were injected subcutaneously in the back with 0.5 mL cold growth factor–reduced Matrigel containing 300 ng/mL $VEGF_{165}$ alone, or 300 ng/mL $VEGF_{165}$ + 100 ng/mL Shh + 100 ng/mL Dll-4, combined or not with 0.5×10^6 undifferentiated hMAPCs or $hAC133^+$ cells (unlabeled or labeled with carboxyfluorescein succinimidyl ester [CFSE; Molecular Probes] or Resovist [Schering, Berlin, Germany] as described[39]). In some animals, 0.1×10^6 or 2.5×10^6 cells were injected. Ten days after injection, animals were perfusion fixed and Matrigel plugs were removed and processed for paraffin or OCT embedding. Tissue sections were examined and photographed under a fluorescence microscope (Zeiss) or a confocal microscope. Ultrastructural analysis was performed as described above. In vivo live imaging was performed under anesthesia using a Leica (Weztlar, Germany) dissection microscope.

Results

Isolation and qualification of hMAPCs

For the current studies, 8 new clones of hMAPCs very similar in their characteristics were used. They were established at the University of Navarra using methods as previously described[32,33] and maintained for more than 50 to 80 population doublings (Table S1). The phenotype of most cells within these clones was $CD90^+$, $CD13^+$, $CD44^{low}$, $MHC\text{-}I^-$, $\alpha_v\beta_3{}^-$, $CD73^-$, $AC133^-$, $CD105^-$, $MHC\text{-}II^-$, $CD36^-$, $CD45^-$, and $CD34^-$ (Figure S1A), largely consistent with their initial characterization.[33] RT-PCR demonstrated presence of the transcription factors Oct3/4, Rex-1, and nanog as well as hTERT (Figure S1B), and cells stained positive for SSEA-4, Oct3/4, and nanog but not SSEA-1 proteins (Figure S1C-F). We further qualified the cells by showing differentiation into mesodermal (ECs, Figures S2J-R and S3; SMCs, Figure S1G), endodermal (hepatocytes, Figure S1H), and ectodermal (neurons, Figure S1I) cell types.

$VEGF_{165}$ induces $hAC133^+$ cells and hMAPCs to differentiate into functional ECs

First, we compared the ability of hMAPCs and cord blood– or BM-derived $hAC133^+$ cells, a cell population previously shown to be enriched for endothelial, neuronal, and hematopoietic progenitors,[25,26] to differentiate into functional mature endothelium. Culture of $hAC133^+$ cells in the presence of $VEGF_{165}$ induced down-regulation of AC133 and hematopoietic markers CD45 and CD34 and up-regulation of mature EC markers, CD105 and $\alpha_v\beta_3$ (Figure S2A-B), as described.[26] Most of the cells (about 80%; Table S4) in 21-day cultures (which we further designate "hAC133-ECs") expressed von Willebrand factor (VWF), angiopoietin receptors Tie-1 and -2, and VEGF receptors 1 and 2 (Flt-1 and KDR, respectively) (Figure S2C-G) and were functional as demonstrated by acetylated-LDL uptake (Figure S2H; Table S4) and vascular tube formation on Matrigel (Figure S2I). A fraction (about 20%) of $hAC133^+$ cells differentiated to hematopoietic cells, indicated by coexpression of the $CD45^+$ antigen and other myeloid-monocytic antigens (Figure S4). Unlike human EC controls, which mostly showed a stellate shape in culture (Figure S5), hAC133-ECs were mainly rounded (FigS2C-H). Most (more than 80%) hMAPCs cultured in the presence of $VEGF_{165}$ acquired EC markers, including KDR, Flt-1, Tie-1, Tie-2, CD105, VE-cadherin, CD31, VWF, and $\alpha_v\beta_3$, while expression of hematopoietic markers CD34 and CD45 was very low or absent (Figures S2J-P and S3; Table S4). The resultant hMAPC-ECs were functional as shown by acetylated-LDL uptake (Figure S2Q; Table S4) and vascular tube formation on Matrigel (Figure S2R). While no SMC α-actin–positive cells were observed in $hAC133^+$ cell–derived cultures, hMAPCs gave rise to SMC α-actin–positive cells representing 5% of the differentiated cells (not shown). For hMAPCs, the increase in mRNA expression varied between different endothelial genes from 5-fold *(CD31)* to more than 600-fold *(Flt-1)* (Figure S3).

$VEGF_{165}$ supports arterial differentiation of hMAPCs but not $hAC133^+$ cells

Studies in zebrafish and mice have suggested that $VEGF_{165}$ would be part of a cascade involving Shh, Notch, and EphrinB2 that induces arterial differentiation in vivo and that $VEGF_{165}$ alone may be sufficient to induce an arterial phenotype in vivo.[20,40] To study whether $VEGF_{165}$ was able to support arterial EC differentiation from stem cells in vitro, we determined using quantitative (Q)–RT-PCR and/or immunofluorescence if the arterial markers Hey-2, Dll-4, EphrinB2, and EphrinB1 and the venous marker EphB4 were expressed in hMAPC-ECs and hAC133-ECs generated in the presence of $VEGF_{165}$. Low transcripts levels for arterial- and venous-specific genes were detected in either cell population before differentiation. In $hAC133^+$ cells, $VEGF_{165}$ treatment decreased arterial markers while venous markers remained stable. In contrast, $VEGF_{165}$ treatment induced significant levels of the arterial markers *Hey-2, Dll-4,* EphrinB1, and EphrinB2 as well as the venous marker *EphB4* in hMAPCs (Figures 1A and S6). Ten- to 100-fold differences were observed in arterial gene expression between hAC133-ECs and hMAPC-ECs. At the protein level, determined by immunofluorescence, expression of arterial Hey-2 (48.3% ± 3.5% of the cells were $Hey2^+$), EphrinB1 (65.8% ± 4%), and venous EphB4 (31.2% ± 2.8%) was found in hMAPC-ECs at day 14 (Figure 1B-D), while no protein expression was detected at baseline (not shown). Interestingly, while most hAC133-ECs expressed CD36, suggesting a microvascular phenotype, hMAPC-ECs were mostly $CD36^-$, suggesting a macrovascular phenotype[41-43] (Figure 1E). Together, this suggests the ability of hMAPCs but not $hAC133^+$ cells to differentiate into arterial ECs in addition to venous ECs.

Notch/patched pathway members are differentially expressed in hMAPCs and $hAC133^+$ cells

Because AV specification during zebrafish and mouse embryogenesis is in large part mediated by Notch and Shh in a cell-intrinsic manner,[2] we compared the expression of Notch and its ligands, Jagged and Dll, and Shh and its receptor patched in undifferentiated hMAPCs and $hAC133^+$ cells by Q-RT-PCR. Expression of *Shh* was restricted to hMAPCs (Figure 2A), and the expression of its receptors patched 1 and patched 2 was significantly higher in hMAPCs compared with $hAC133^+$ cells (Figure 2A). Likewise, Notch-1 was uniquely expressed in hMAPCs (Figure 2B), and Dll-3, Jagged-1, and Notch-3 were more highly expressed in hMAPCs than $hAC133^+$ cells (Figure 2B), while Dll-1 and Notch-4 were expressed preferentially in $hAC133^+$ cells (Figure 2B). Expression of Jagged-2, Notch-2, and Dll-4 was not significantly different between the 2 undifferentiated cell populations (Figure 2B). To determine whether the different response to $VEGF_{165}$ in hMAPCs and $hAC133^+$ cells could be due to differences in VEGF receptor or endogenous $VEGF_{165}$ expression, we compared their expression levels by Q-RT-PCR. Baseline endogenous $VEGF_{165}$ expression was similar in both cell populations as well as expression of Flt-1 and KDR (not shown). Thus, the

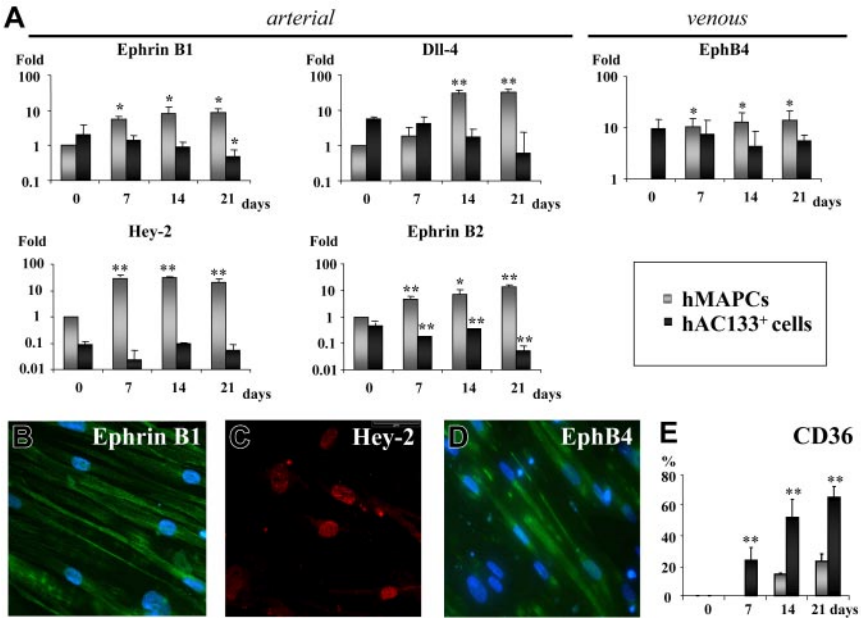


Figure 1. $VEGF_{165}$ induces arterial specification of hMAPCs but not hAC133$^+$ cells. (A) Q-RT-PCR for arterial (EphrinB1, Dll-4, Hey-2, EphrinB2) and venous markers *(EphB4)* on hAC133$^+$ cell–derived ECs (■) or hMAPC-derived ECs (▨) at different time points (0, 7, 14, and 21 days) after the start of the differentiation process. While hMAPCs up-regulated arterial and venous markers during the differentiation process, hAC133$^+$ cell–derived ECs showed reduced arterial marker expression. Expression levels are presented as fold increase (in logarithmic scale) in comparison with baseline levels and were normalized by using GAPDH as housekeeping gene. The mRNA levels in undifferentiated hMAPCs were considered as 1. Expression between baseline levels (day 0) and day 7, 14, and 21 for each cell population was compared (*$P < .05$; **$P < .01$). (B-D) Immunofluorescent staining of hMAPC-derived ECs. After 14 days, hMAPCs were positive for arterial markers EphrinB1 (B), Hey-2 (C), and venous marker EphB4 (D) (see text for percentage of positive cells). A representative example from 3 different clones is shown. (E) Comparative expression, plotted as percentage of total number of cells, based on FACS analysis, of the microvascular-specific marker CD36 in hMAPC (▨) and hAC133$^+$ cell–derived ECs (■) (**$P < .01$ versus hMAPC-ECs). The mean (± SEM) of 3 (A) or 5 (E) different experiments in triplicate is shown. Magnification ×40.

expression of Shh and patched, several of the Notch ligands, and receptors in hMAPCs may at least in part account for the ability of hMAPCs but not hAC133$^+$ cells to differentiate along the arterial EC lineage.

Shh or Notch pathway blocking attenuates arterial EC differentiation in hMAPCs

To investigate the causal involvement of Notch and/or Shh/patched pathways in arterial EC differentiation from hMAPCs, we manipulated each of them separately. Specific blocking of Shh signaling by cyclopamine-mediated inhibition of the patched receptor complex[35] significantly decreased expression of arterial EC markers Hey-2, EphrinB1, and EphrinB2 and simultaneously slightly (but not statistically significant) increased expression of the venous marker EphB4 (Figure 3A). A more pronounced attenuation of arterial EC marker expression was observed by blocking the Notch pathway using an inhibitor for γ-secretase, essential for Notch receptor activation[36] (Figure 3A). Compared with either inhibitor alone, combining cyclopamine and γ-secretase inhibitor further significantly decreased expression of Hey-2 ($P < .05$) but not of EphrinB1 or EphrinB2 ($P > .05$) (Figure 3A). These results suggest that arterial specification in hMAPCs in the presence of $VEGF_{165}$ is at least in part mediated by the patched and Notch pathways.

Simultaneous Notch and patched activation boosts arterial EC fate in hMAPCs

To further evaluate the role of Notch and patched in arterial specification of hMAPCs, we evaluated the effect of $VEGF_{165}$ (alone or combined with either Dll-4 or Jagged-1) and Shh (alone or in combination) (Figure S6). Of all combinations tested, addition of Dll-4 and Shh most efficiently increased expression of Hey-2 along with down-regulation of venous marker EphB4, indicating a preferential differentiation toward arterial endothelium (Figures 3A and S6). We analyzed the latter condition ($VEGF_{165}$ + Shh + Dll-4) in more detail by examining the expression of additional arterial specific genes, showing that in addition to Hey-2, Jagged-2 and ALDH1A1 were also up-regulated, in agreement with previous work with HUVECs.[5] Increased expression of arterial markers was associated with decreased levels of additional venous-specific Lefty-1 and Lefty-2 transcripts (Figure 3B). While Notch and

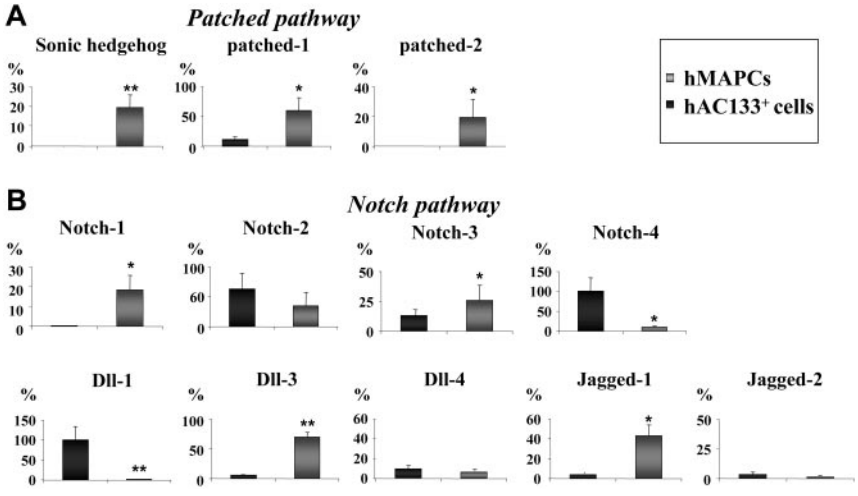


Figure 2. Notch and patched pathway members are differentially expressed in hMAPCs and hAC133$^+$ cells. Q-RT-PCR analysis of members of the patched pathway (Shh, patched-1, and patched-2) (A) and the Notch pathway (Notch-1, -2, -3, and -4 and ligands Dll-1, -3, -4, and Jagged-1 and -2) (B) known to be involved in AV specification. Note differences in expression (see "Results") between hMAPCs compared with hAC133$^+$ cells. The mRNA levels in all panels are expressed in percentage versus a positive control (total RNA) and were normalized by using GAPDH as housekeeping gene. The mean (± SEM) of 3 different experiments in triplicate is shown. *$P < .05$; **$P < .01$ versus hAC133$^+$ cells.

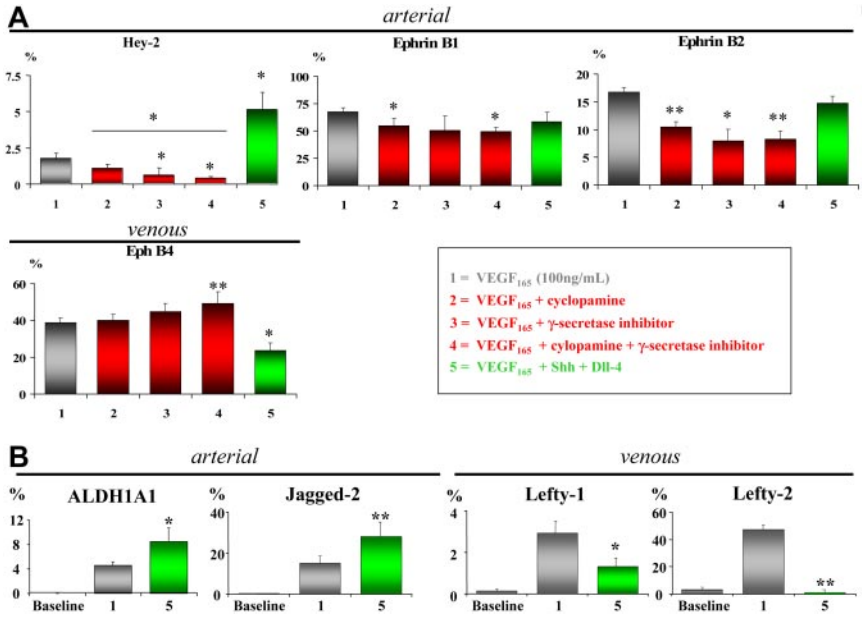


Figure 3. Blockage of Notch/patched pathway attenuates while stimulation increases arterial EC differentiation of hMAPCs. (A) Q-RT-PCR analysis for arterial (EphrinB1, Hey-2, and EphrinB2) and venous markers (EphB4) on hMAPC-derived ECs (gray bars) after 14 days of differentiation using blocking (red bars) or activating (green bars) treatments (as indicated in the box). Shh blocking, Notch blocking, or a combination of both significantly reduced expression of arterial EC markers paralleled by an increase in venous marker expression compared with $VEGF_{165}$ alone (gray bars). Conversely, addition of Shh and Dll-4 to $VEGF_{165}$ further significantly increased expression of arterial marker Hey-2 while significantly decreasing venous marker EphB4. $^{*}P < .05$; $^{**}P < .01$ versus $VEGF_{165}$ alone; $^{*}P < .05$ condition 4 versus condition 2. (B) Q-RT-PCR analysis of additional arterial (*ALDH1A1*, Jagged-2) and venous markers (Lefty-1, Lefty-2) in hMAPC-derived ECs cultured in $VEGF_{165}$ alone (gray bars) or combined with Shh and Dll-4 (green bars). Note the significant up-regulation of arterial markers and simultaneous down-regulation of venous markers in the combination cocktail as compared with $VEGF_{165}$ alone. The mRNA levels in all panels are expressed as mean percentage of HUAECs (arterial markers) and HUVECs (venous markers) and were normalized using GAPDH as housekeeping gene. Baseline corresponds to undifferentiated hMAPCs. The mean (± SEM) of 3 different experiments is shown. $^{*}P < .05$; $^{**}P < .01$ versus $VEGF_{165}$ alone.

patched activation enhanced arterial markers in hMAPCs, a similar effect was not observed in $hAC133^{+}$ cells (Figure S6).

Shh and Dll-4 boost arterial EC differentiation of hMAPCs and arterial-like vessel growth in vivo

To determine whether the same factors could also induce hMAPC differentiation into arterial endothelium in vivo, we injected 0.5×10^6 undifferentiated hMAPCs in growth factor–reduced Matrigel containing either $VEGF_{165}$ ("standard media") or $VEGF_{165}$ + Shh + Dll-4 ("arterial media") under the skin of nude mice (n = 6 per group). To account for effects of the admixed cytokines on host cells, we also included the corresponding "cytokine-alone" groups. To track the cells following implantation, hMAPCs were labeled with CFSE or iron particles (Resovist)[39,44] before injection. Irrespective of the cytokine cocktail used, localized areas of CFSE-labeled cells (Figure 4A) and single Resovist-labeled hMAPC-derived cells (Figure 4B) persisted for at least 10 days in the Matrigel plug as determined by in vivo live imaging and electron microscopy, respectively. Most implanted cells expressed (human) CD31 and (human) VE-cadherin (Figure 4C-D) and Fli-1 (not shown), indicating their EC identity. The hMAPC-ECs contributed to 2.3% ± 0.7% of the vessels in the Matrigel plugs with standard media and 3.1% ± 1.3% in arterial media, indicating that most cells of blood vessels in the Matrigel plugs were host derived. Dose-response studies revealed that engraftment strongly correlated with cell dose and cell dose with vascularity (Figure S7; Table S5).

Following transplantation with all cell doses (0.5×10^6 to 2.5×10^6 cells), several hMAPC-ECs generated in the presence of the arterial cytokine combination—while arterial marker expression was only detected with the highest cell dose with $VEGF_{165}$ alone (Figure S8)—expressed both human-specific lectin UEA and human-specific arterial markers Hey-2 and EphrinB1, as shown by immunohistochemistry (Figure 4E-F) and double immunofluorescence confocal microscopy (Figure 4G-H), demonstrating differentiation of hMAPCs in vivo to arterial endothelium. Importantly, these arterial hMAPC-ECs contributed to vessels functionally connected to the host vasculature as demonstrated by the presence of erythrocytes in their lumen (Figure 4F). Also, in some animals, we injected TRITC-labeled UEA lectin (that specifically binds to human ECs) in the tail vein 30 minutes before killing. Colabeling of TRITC-UEA with CFSE (with which the hMAPCs were labeled) confirmed that the hMAPC-EC–containing vessels were connected to the host vasculature (Figure 4I). Although transplanted $hAC133^{+}$ cells gave rise to $CD31^{+}$ UEA lectin-positive ECs, the latter did not express arterial markers, nor were they associated with larger vessels (Figure S9). Thus, as in vitro, unlike $hAC133^{+}$ cells, hMAPCs—and more so with the arterial cytokine mix—could be specified to functional arterial ECs in vivo.

In addition to its arterial differentiation effect on hMAPC-ECs, the arterial cytokine mix also induced the formation of arterial-like vessels in which both implanted and host cells participated. Indeed, coating with host α-actin–positive SMCs of human EC-containing vessels was frequently observed in the Matrigel plugs containing the arterial cytokine combination, as shown by double confocal immunofluorescence (Figure 4J). Moreover, in addition to a significant increase in total vessel number (number of lectin-positive vessels: 124 ± 16/mm^2 in arterial media versus 74 ± 10/mm^2 in standard media; $P < .05$), the arterial mix also significantly increased the fraction of vessels coated with SMCs (32% ± 5% in arterial media versus 15% ± 4% in standard media; $P < .05$; Figure 5A-C) as well as the diameter of these vessels (20.1 ± 4.2 μm versus 14.7 ± 3.7 μm; $P = .01$). Consistent with an effect of the cytokine mix on host cells, without addition of hMAPCs more α-actin coated vessels were also seen in Matrigels containing arterial versus standard media. However, when hMAPCs were coimplanted, they had an additive effect on SMC coating (Figure 5C), in part by in situ differentiation to SMCs (not shown). Significantly more deposition of Sirius red–positive fibrillar collagen (Figure 5D-F) and orcein-positive elastin (not shown), both characteristics of arteries, could be detected surrounding the newly formed EC channels when hMAPCs and the arterial cytokine combination was used. Again, even in the absence of hMAPCs, there was more collagen deposition with the arterial mix than with standard media, although it was significantly higher when hMAPCs were coimplanted (Figure 5F). In addition to a direct contribution

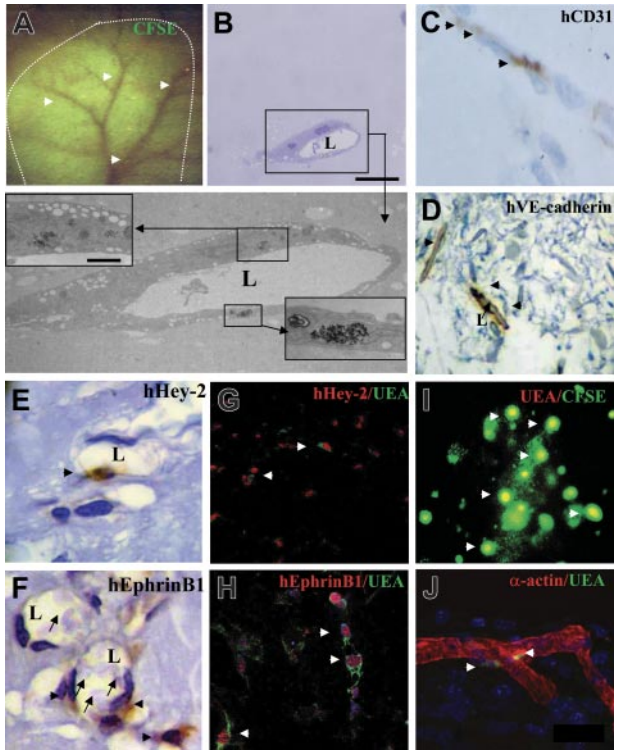


Figure 4. Shh and Dll-4 boost functional arterial hMAPC-EC differentiation in vivo. (A) Live in vivo imaging of a Matrigel plug containing $VEGF_{165}$ and hMAPCs labeled with CFSE 10 days after subcutaneous implantation. Note the localized CFSE-labeled area (outlined by a dashed white line) located in the Matrigel in the vicinity of a large vascular tree (arrowheads) from the overlying host skin. (B-J) Histologic analysis on cross-sections through Matrigel plugs containing hMAPCs and $VEFG_{165}$ (B-D) or hMAPCs and $VEGF_{165}$ + Shh+ Dll-4 ("arterial cytokine mix" [E-J]). (B) Electron microscopy showing a capillary composed of a Resovist-labeled hMAPC-derived EC in Matrigel plugs. A semithin section (B, top panel), an ultrathin section (B, bottom panel), and a detail of iron particles (insets in lower panel of B) are shown. (C-F) Immunohistochemical staining of 3 μm paraffin cross-sections through Matrigel plugs for human-specific CD31 (C) and human-specific VE-cadherin (D) (both indicating their EC identity) and human-specific Hey-2 (E) and human-specific EphrinB1 (F) (both indicating their arterial EC identity). Arrows indicate red blood cells in vessel lumen. (G-H) Double confocal immunofluorescence staining of 40 μm cryopreserved cross-sections through Matrigel plugs with human endothelial-specific lectin UEA (green) and Hey-2 (red) (G) or UEA (green) and Ephrin B1 (red) (H). Topro (blue) was used for nuclear staining. (I) High-resolution live in vivo imaging of a Matrigel plug containing $VEGF_{165}$ and hMAPCs labeled with CFSE 10 days after subcutaneous implantation and 30 minutes after intravenous injection of UEA lectin. Note colocalization (yellow; indicated by arrowheads) of CFSE-labeled cells (green) and UEA lectin (red) area, indicating that the vessels containing CFSE-labeled cells were connected to the host vascular system. (J) Double confocal immunofluorescence staining of 40 μm cryopreserved cross-sections through Matrigel plugs with human endothelial-specific lectin UEA (green) and α-actin (red), showing hMAPC-ECs (arrowheads) coated by α-actin–positive SMCs. Topro (blue) was used for nuclear staining. "L" in panels B and D-F indicates the vessel lumen. Magnifications ×63 (E-F), ×40 (C-D,J), and ×20 (G-I). Scale bars in panel B: 10 μm (semithin); 2.5 μm (ultrathin); 1 μm (upper inset); 0.5 μm (lower inset).

to arterial growth, we hypothesized that the additive effect of hMAPCs on SMCs and extracellular matrix (ECM) coating was due to trophic effects by secretion of arteriogenic cytokines (similar to what has been described for other stem cells[45]). In support of such a hypothesis, an increase in the production of PDGF-BB and TGF-β1, factors known to stimulate SMC proliferation and ECM production, was observed after in vitro culture of hMAPCs in the presence of the standard media and more so in the presence of the arterial cytokine combination (Figure 5G-H). Electron microscopic analysis further confirmed the differences in complexity and caliber between vessels formed in Matrigel plugs with standard versus arterial media (Figure 6). Together, these data indicate that the arterial cytokine combination induced the formation of vessels with arterial characteristics and that the presence of hMAPCs had an additive effect on this process.

Discussion

In the last decade, our knowledge about the molecular differences between arterial and venous ECs and the pathways underlying their specification during development has rapidly increased (reviewed by Lawson et al,[20] Harvey and Oliver,[46] Shawber and Kitajewski,[47] and Iso et al[48]). That such specification is not only important during development but also in adult life is perhaps most clearly demonstrated by the occurrence of diseases restricted to arteries (eg, atherosclerosis) or veins (eg, varicose veins). Therefore, knowledge about the mechanisms that underlie EC specification may allow us to design more optimal therapeutic regimens for those diseases. Unfortunately, most of our understanding about AV specification is derived from animal studies. Therefore, the experiments presented here with human stem cells provide a valuable model for the elucidation of mechanisms involved in EC differentiation and AV specification in humans.

Developmental studies in zebrafish[9,20] and *Xenopus*[18] have demonstrated that AV specification occurs very early, at the level of the angioblast, the embryonic EC precursor. Currently, it is not known what initially determines arterial and venous angioblasts. Until recently, it was suggested that VEGF—the most widely explored angiogenic growth factor—equally affects proliferation and migration of all EC (precursor) types. However, evidence has now been presented that VEGF preferentially stimulates the growth of arterial ECs (and their precursors) during development (reviewed by Torres-Vazquez et al[2]) and adulthood.[40] Several hypotheses have been put forward to explain VEGF's preference, but none of them has been unequivocally proven.[2,20] Arterial angioblasts may respond differently because they are, as shown in *Xenopus*[18] and zebrafish,[9] the first ones to migrate and thus encounter the VEGF signal earlier. Alternatively, specific responses may be elicited by the need for a coreceptor for $VEGF_{165}$ to engage the downstream Notch pathway. Recent evidence has shown that NP-1, which only binds the $VEGF_{165}$ isoform, might be a candidate coreceptor.[6,17] Although our in vitro system did not allow addressing the spatially different responses of arterial and venous angioblasts to VEGF, our findings support the notion that, like in zebrafish and mouse development, there are separate arterial and venous endothelial precursors in the adult human. Under conditions in which hMAPCs gave robust arterial EC differentiation, $hAC133^+$ cells appeared to have lost that ability both in vitro and in vivo and therefore could be considered venous EPCs. In contrast, hMAPCs are less mature than EPCs[32] and may constitute a cell population that precedes AV specification and can therefore differentiate to both arterial and venous ECs in the presence of $VEGF_{165}$. Noteworthy in this context is the fact that $hAC133^+$ cells gave rise to venous and $CD36^+$ microvascular ECs, while hMAPCs differentiated mainly into $CD36^-$ macrovascular ECs.

During zebrafish development, VEGF is part of an arterial EC inductive sequence involving Shh (acting upstream from VEGF) and Notch (acting downstream),[20] a cascade that is at least in part conserved in mouse development (reviewed by Harvey and Oliver[46]). Using hMAPCs, we demonstrate both by positive and negative manipulation that the Shh and Notch elements of this cascade are also active during arterial EC specification from adult human stem cells. Although we show that $VEGF_{165}$, Shh, and Dll-4

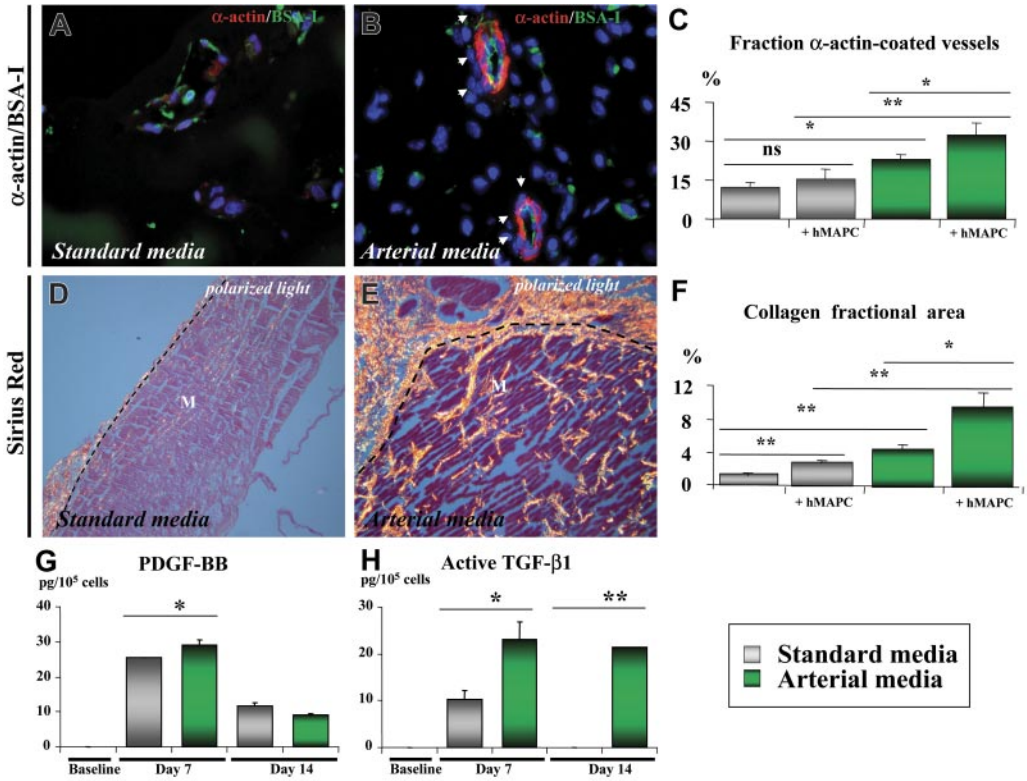


Figure 5. Shh and Dll-4 together with hMAPCs additively increase formation of arterial-like vascular structures in vivo. (A-F) Histologic analysis on cross-sections through Matrigel plugs containing hMAPCs and $VEFG_{165}$ (A,D) or hMAPCs and $VEGF_{165}$ + Shh + Dll-4 (B,E). (A-B) Double immunofluorescent staining of 3 μm paraffin cross-sections through Matrigel plugs stained with SMC α-actin (red) and BS-I lectin (staining ECs in green) showing more SMC-coated (indicated by arrowheads) vessels when the arterial media were used (B) in comparison with standard media (A). (C) Diagram comparing the fraction of SMC-coated vessels (expressed as percentage ± SEM versus the total number of vessels) for the conditions outlined in the box; $*P < .05$; $**P < .01$. Note the additive effect of hMAPCs. (D-E) Sirius red staining (visualized by polarized light microscopy) indicating abundant and thick (orange-red birefringent) fibrillar collagen around vessels in Matrigels containing hMAPCs combined with the arterial mix (E) as compared with the less abundant and thinner collagen in Matrigels containing hMAPCs combined with $VEGF_{165}$ alone (D); dashed lines indicate the edge of the Matrigel (M). (F) Diagram comparing the collagen fractional area (expressed as percentage ± SEM versus the total area) for the conditions outlined in the legend; $*P < .05$; $**P < .01$. Note the additive effect of hMAPCs. (G,H) ELISA for PDGF-BB (G) and active TGF-β1 (H) on cell supernatants of undifferentiated hMAPCs ("baseline") or differentiated for 7 or 14 days to ECs either in standard media (gray bars) or arterial media (green bars). While PDGF-BB production was only slightly and temporarily higher, active TGF-β1 production was significantly higher in arterial media versus standard media. $*P < .05$; $**P < .01$. Data are expressed as picogram per 10^5 cells and represent the mean (± SEM) of 3 experiments performed in triplicate.

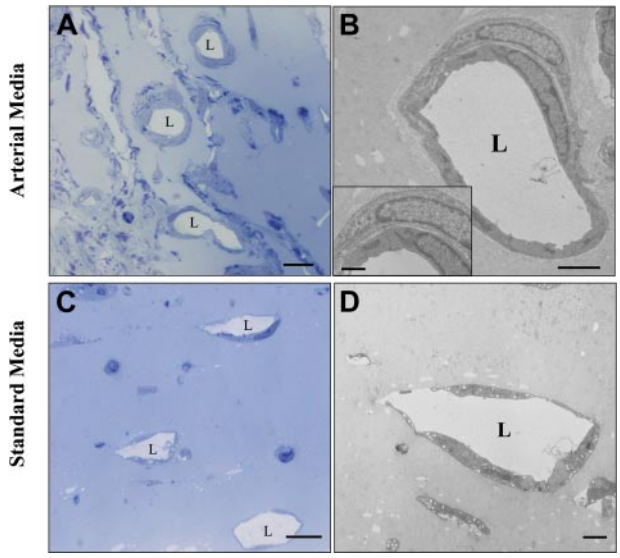


Figure 6. Ultrastructural comparison between the vessel make-up in Matrigel plugs injected with hMAPCs in arterial or standard media. Ultrastructural analysis of Matrigel plugs injected subcutaneously with hMAPCs combined with arterial (A-B) or standard media (C-D). Semithin sections of a Matrigel plug (A,C) and an ultrathin section of an artery-like tube (B) with a detail showing an SMC around an EC (inset) and a veinlike tube (D). "L" indicates the vessel lumen. Scale bars: 10 μm (A,C); 2.5 μm (B); 2 μm (D); 1 μm (B, inset).

may have an additive effect on arterial EC differentiation, additional mechanistic studies will help to determine whether they work in a similar cascade. The involvement of those pathways was further substantiated by the differential expression of many of the pathway members in hMAPCs and $hAC133^+$ cells. Increased expression of the patched receptors in hMAPCs may be the result of an autocrine positive feedback loop[49,50] driven by hMAPC-derived Shh. Intriguingly, while Notch-1 was exclusively expressed in hMAPCs, Notch-4 was significantly underexpressed in hMAPCs in comparison with $hAC133^+$ cells. Recent studies have, however, demonstrated the redundancy between Notch-1 and -4, supporting the notion that Notch-1 may be sufficient to induce arterial EC differentiation in hMAPCs.[51] The fact that not all arterial markers were elevated in hMAPCs under the influence of $VEGF_{165}$ (combined or not with Notch and patched ligands) and that arterial differentiation was not completely abrogated by Notch and/or Shh blocking may reflect the existence of additional VEGF/Notch/patched-independent pathways in arterial differentiation. We did, for instance, not fully explore the potential involvement of TGF-β, COUP-TFII, adrenomedullin, or angiopoietins, known to play a role in AV fate decisions.[7,14,22,40]

During development and in primary ECs,[52] Notch activation has been associated with endothelial-to-mesenchymal transformation. Despite the ability of hMAPCs to robustly differentiate into SMCs

under other conditions (Figure S1; Document S1), the conditions used for EC differentiation induced SMC differentiation of hMAPCs in vitro and in vivo to a low degree (not shown). This lack of significant SMC (trans)differentiation, however, allowed us to conclude that changes in expression of certain Notch components (like Jagged-1 and Ephrin B2, which have been shown to be expressed also in arterial SMCs[48,53]) were EC specific in our system. However, we cannot exclude that activation of Notch in fully EC-differentiated MAPCs may induce such transformation. Dll-4–induced Notch activation in ECs was recently shown to down-regulate EC proliferation, maybe due to an attenuated responsiveness to VEGF.[54] However, despite this inhibitory effect on EC proliferation, Notch activation was still compatible with up-regulation of arterial EC markers, such as Hey-2, similar to what we and others documented in in vitro studies.[55,56] Shh has also been shown to support neuronal differentiation,[57] yet we did not observe the formation of neurons in our system despite the intrinsic ability of hMAPCs for neuronal differentiation, the latter of which requires a different cytokine cocktail (Figure S1; Document S1).

In agreement with the in vitro data, hMAPCs but not hAC133$^+$ cells were capable of arterial EC differentiation in vivo. Indeed, the combination of $VEGF_{165}$, Shh, and Dll-4 boosted arterial EC differentiation from coimplanted hMAPCs. In addition, this arterial "cocktail" induced a different vascular pattern than $VEGF_{165}$ alone (ie, vessels were larger in diameter, more frequently coated with host SMCs, and contained elastin and more fibrillar collagen—altogether features consistent with arterial vessels). Other studies have shown that in the setting of ischemia, vessels formed in the presence of Shh had similar characteristics as the ones described here (ie, larger-diameter vessels and more often coated with SMCs).[50,58] In our study, injection of the arterial cocktail without hMAPCs also induced effects on the endogenous mouse vascular cells but, importantly, the effects were more pronounced in the presence of hMAPCs. We hypothesize that, in addition to their direct contribution to arterial endothelium, hMAPCs (similar to other stem cells[45]), and more so when cultured in arterial media, had trophic effects on endogenous cells through secretion of PDGF-BB and TGF-β1. Interestingly, as previously shown in tumors, Shh in the arterial mix or produced by hMAPCs could potentially enhance PDGF receptor expression on the host vasculature and thereby enhance its response to the hMAPC-derived PDGF-BB signal.[59]

Together, our data show that coaxing human stem cells in vitro and in vivo (along with endogenous vascular cells) specifically into an arterial phenotype is feasible, thereby providing a model to study AV specification in humans. Moreover, our findings may also be of importance for improvement and optimization of strategies for vascular regeneration in ischemic patients and for the design of EC-coated artificial arterial grafts.

Acknowledgments

This work as supported in part by grants from the Health Department of the Government of Navarra (4/2004), Spanish Ministerio de Ciencia y Tecnología (SAF 2002-04574-C02), Ministerio de Sanidad PI050168, FEDER (INTERREG IIIA) the UTE project CIMA, the American Heart Association (A.L., B.P.), Excellentie financiering KULeuven (EF/05/013) (A.L.), and the Belgian American Educational Foundation (BAEF) (A.L.).

Authorship

Contribution: X.L.A. and C.C. performed and designed research, collected data, and analyzed data; A.L., C.M.V., and F.P. designed research, analyzed data, and wrote the manuscript; C.M., G.A., M.A.B., B.P., M.U., M.A., A.E., M.S., E.J.A., and J.M. performed research and collected data; and J.M.G.-V. contributed analytical tools and collected and interpreted data.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

X.L.A., A.L., and C.C. contributed equally to this study.

Correspondence: Felipe Prósper, Hematology and Cell Therapy Area, Clínica Universitaria, University of Navarra, Av Pio XII 36, Pamplona 31009, Spain; e-mail: fprosper@unav.es.

References

1. Carmeliet P. Manipulating angiogenesis in medicine. J Intern Med. 2004;255:538-561.
2. Torres-Vazquez J, Kamei M, Weinstein BM. Molecular distinction between arteries and veins. Cell Tissue Res. 2003;314:43-59.
3. Jain RK. Molecular regulation of vessel maturation. Nat Med. 2003;9:685-693.
4. Bagley RG, Walter-Yohrling J, Cao X, et al. Endothelial precursor cells as a model of tumor endothelium: characterization and comparison with mature endothelial cells. Cancer Res. 2003;63:5866-5873.
5. Chi JT, Chang HY, Haraldsen G, et al. Endothelial cell diversity revealed by global expression profiling. Proc Natl Acad Sci U S A. 2003;100:10623-10628.
6. You LR, Lin FJ, Lee CT, DeMayo FJ, Tsai MJ, Tsai SY. Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. Nature. 2005;435:98-104.
7. Villa N, Walker L, Lindsell CE, Gasson J, Iruela-Arispe ML, Weinmaster G. Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. Mech Dev. 2001;108:161-164.
8. Shutter JR, Scully S, Fan W, et al. Dll4, a novel Notch ligand expressed in arterial endothelium. Genes Dev. 2000;14:1313-1318.
9. Zhong TP, Childs S, Leu JP, Fishman MC. Gridlock signalling pathway fashions the first embryonic artery. Nature. 2001;414:216-220.
10. Zhong TP, Rosenberg M, Mohideen MA, Weinstein B, Fishman MC. gridlock, an HLH gene required for assembly of the aorta in zebrafish. Science. 2000;287:1820-1824.
11. le Noble F, Moyon D, Pardanaud L, et al. Flow regulates arterial-venous differentiation in the chick embryo yolk sac. Development. 2004;131:361-375.
12. Othman-Hassan K, Patel K, Papoutsi M, Rodriguez-Niedenfuhr M, Christ B, Wilting J. Arterial identity of endothelial cells is controlled by local cues. Dev Biol. 2001;237:398-409.
13. Mukouyama YS, Shin D, Britsch S, Taniguchi M, Anderson DJ. Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. Cell. 2002;109:693-705.
14. Waite KA, Eng C. From developmental disorder to heritable cancer: it's all in the BMP/TGF-beta family. Nat Rev Genet. 2003;4:763-773.
15. Sorensen LK, Brooke BS, Li DY, Urness LD. Loss of distinct arterial and venous boundaries in mice lacking endoglin, a vascular-specific TGFbeta coreceptor. Dev Biol. 2003;261:235-250.
16. Stalmans I, Ng YS, Rohan R, et al. Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. J Clin Invest. 2002;109:327-336.
17. Mukouyama YS, Gerber HP, Ferrara N, Gu C, Anderson DJ. Peripheral nerve-derived VEGF promotes arterial differentiation via neuropilin 1-mediated positive feedback. Development. 2005;132:941-952.
18. Cleaver O, Krieg PA. VEGF mediates angioblast migration during development of the dorsal aorta in Xenopus. Development. 1998;125:3905-3914.
19. Moyon D, Pardanaud L, Yuan L, Breant C, Eichmann A. Selective expression of angiopoietin 1 and 2 in mesenchymal cells surrounding veins and arteries of the avian embryo. Mech Dev. 2001;106:133-136.
20. Lawson ND, Vogel AM, Weinstein BM. sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. Dev Cell. 2002;3:127-136.
21. Liu ZJ, Shirakawa T, Li Y, et al. Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. Mol Cell Biol. 2003;23:14-25.
22. Yurugi-Kobayashi T, Itoh H, Schroeder T, et al. Adrenomedullin/cyclic AMP pathway induces

Notch activation and differentiation of arterial endothelial cells from vascular progenitors. Arterioscler Thromb Vasc Biol. 2006;26:1977-1984.

23. Masood R, Xia G, Smith DL, et al. Ephrin B2 expression in Kaposi sarcoma is induced by human herpesvirus type 8: phenotype switch from venous to arterial endothelium. Blood. 2005;105:1310-1318.
24. Shawber CJ, Das I, Francisco E, Kitajewski J. Notch signaling in primary endothelial cells. Ann N Y Acad Sci. 2003;995:162-170.
25. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science. 1997;275:964-967.
26. Gehling UM, Ergun S, Schumacher U, et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood. 2000;95:3106-3112.
27. Salven P, Mustjoki S, Alitalo R, Alitalo K, Rafii S. VEGFR-3 and CD133 identify a population of CD34+ lymphatic/vascular endothelial precursor cells. Blood. 2003;101:168-172.
28. Pelosi E, Valtieri M, Coppola S, et al. Identification of the hemangioblast in postnatal life. Blood. 2002;100:3203-3208.
29. Jiang Y, Henderson D, Blackstad M, Chen A, Miller RF, Verfaillie CM. Neuroectodermal differentiation from mouse multipotent adult progenitor cells. Proc Natl Acad Sci U S A. 2003;100(suppl 1):11854-11860.
30. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002;418:41-49.
31. Schwartz RE, Reyes M, Koodie L, et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest. 2002;109:1291-1302.
32. Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest. 2002;109:337-346.
33. Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood. 2001;98:2615-2625.
34. de Wynter EA, Buck D, Hart C, et al. CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors. Stem Cells. 1998;16:387-396.
35. Watkins DN, Berman DM, Burkholder SG, Wang B, Beachy PA, Baylin SB. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. Nature. 2003;422:313-317.
36. Dahlqvist C, Blokzijl A, Chapman G, et al. Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. Development. 2003;130:6089-6099.
37. Luttun A, Lupu F, Storkebaum E, et al. Lack of plasminogen activator inhibitor-1 promotes growth and abnormal matrix remodeling of advanced atherosclerotic plaques in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2002;22:499-505.
38. Salvato G. Quantitative and morphological analysis of the vascular bed in bronchial biopsy specimens from asthmatic and non-asthmatic subjects. Thorax. 2001;56:902-906.
39. Arbab AS, Bashaw LA, Miller BR, Jordan EK, Bulte JW, Frank JA. Intracytoplasmic tagging of cells with ferumoxides and transfection agent for cellular magnetic resonance imaging after cell transplantation: methods and techniques. Transplantation. 2003;76:1123-1130.
40. Visconti RP, Richardson CD, Sato TN. Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). Proc Natl Acad Sci U S A. 2002;99:8219-8224.
41. Petzelbauer P, Bender JR, Wilson J, Pober JS. Heterogeneity of dermal microvascular endothelial cell antigen expression and cytokine responsiveness in situ and in cell culture. J Immunol. 1993;151:5062-5072.
42. Ades EW, Candal FJ, Swerlick RA, et al. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. J Invest Dermatol. 1992;99:683-690.
43. Swerlick RA, Lee KH, Wick TM, Lawley TJ. Human dermal microvascular endothelial but not human umbilical vein endothelial cells express CD36 in vivo and in vitro. J Immunol. 1992;148:78-83.
44. Arbab AS, Bashaw LA, Miller BR, et al. Characterization of biophysical and metabolic properties of cells labeled with superparamagnetic iron oxide nanoparticles and transfection agent for cellular MR imaging. Radiology. 2003;229:838-846.
45. Kinnaird T, Stabile E, Burnett MS, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res. 2004;94:678-685.
46. Harvey NL, Oliver G. Choose your fate: artery, vein or lymphatic vessel? Curr Opin Genet Dev. 2004;14:499-505.
47. Shawber CJ, Kitajewski J. Notch function in the vasculature: insights from zebrafish, mouse and man. Bioessays. 2004;26:225-234.
48. Iso T, Hamamori Y, Kedes L. Notch signaling in vascular development. Arterioscler Thromb Vasc Biol. 2003;23:543-553.
49. Marigo V, Johnson RL, Vortkamp A, Tabin CJ. Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. Dev Biol. 1996;180:273-283.
50. Pola R, Ling LE, Silver M, et al. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. Nat Med. 2001;7:706-711.
51. Carlson TR, Yan Y, Wu X, et al. Endothelial expression of constitutively active Notch4 elicits reversible arteriovenous malformations in adult mice. Proc Natl Acad Sci U S A. 2005;102:9884-9889.
52. Noseda M, McLean G, Niessen K, et al. Notch activation results in phenotypic and functional changes consistent with endothelial-to-mesenchymal transformation. Circ Res. 2004;94:910-917.
53. Gale NW, Baluk P, Pan L, et al. Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. Dev Biol. 2001;230:151-160.
54. Williams CK, Li JL, Murga M, Harris AL, Tosato G. Upregulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. Blood. 2006;107:931-939
55. Lawson ND, Scheer N, Pham VN, et al. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. Development. 2001;128:3675-3683.
56. Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. Genes Dev. 2004;18:901-911.
57. Benedito R, Duarte A. Expression of Dll4 during mouse embryogenesis suggests multiple developmental roles. Gene Expr Patterns. 2005;5:750-755
58. Kusano KF, Allendoerfer KL, Munger W, et al. Sonic hedgehog induces arteriogenesis in diabetic vasa nervorum and restores function in diabetic neuropathy. Arterioscler Thromb Vasc Biol. 2004;24:2102-2107.
59. Ruiz i Altaba A, Sanchez P, Dahmane N. Gli and hedgehog in cancer: tumours, embryos and stem cells. Nat Rev Cancer. 2002;2:361-372.

ANEXO V

Title	Mesenchymal Stem Cells and cardiovascular disease: a bench to bedside roadmap
Journal	Stem Cells International
Issue	Clinical Perspectives of Mesenchymal Stem Cells (CPM)
Additional Files	Reply to review reports
Manuscript Number	175979.v2 (Review Article)
Resubmitted On	2011-10-13
Authors	**Manuel Mazo**, Miriam Araña, Beatriz Pelacho, F. Prosper
Editor	Wolfgang Wagner (Assigned on 2011-10-13)
Editorial Recommendation	Publish Unaltered (Made On 2011-10-13)
Editorial Decision	Accepted (Made On 2011-10-13)

Mesenchymal Stem Cells and cardiovascular disease: a bench to bedside roadmap

Manuel Mazo, Miriam Araña, Beatriz Pelacho, Felipe Prosper

Hematology and Cell Therapy, Clinica Universidad de Navarra, Pamplona, Spain

Address for Correspondence:

Felipe Prósper MD, PhD

Hematology and Cell Therapy

Clínica Universitaria

Av. Pío XII 36, Pamplona 31008,

Navarra, Spain

Phone 34 948 255400 Fax 34 948 296500

e-mail: fprosper@unav.es

ABSTRACT

In recent years, the incredible boost in stem cell research has kindled the expectations of both patients and physicians. Mesenchymal progenitors, owing to their availability, ease of manipulation and therapeutic potential, have become one of the most attractive options for the treatment of a wide range of diseases, from cartilage defects to cardiac disorders. Moreover, their immunomodulatory capacity has opened up their allogenic use, consequently broadening the possibilities for their application. In this review, we will focus on their use in the therapy of myocardial infarction, looking at their characteristics, *in vitro* and *in vivo* mechanisms of action, as well as clinical trials.

INTRODUCTION

Although traditionally regarded as a health concern related particularly to the industrialized world, cardiovascular diseases are now the first cause of death worldwide [1], with myocardial infarction (MI) resulting in 12.8% of deaths. Aside from changes in ways of life associated with economic and social development, one of the main reasons is the fact that MI is an evolving disease. After the ischemic event, anaerobic conditions rapidly induce massive cell death, not only involving cardiomyocytes (CM), but also vascular cells. Although the organism tries to exert a compensatory activity (reviewed in [2]) during the first stages of the disease and may even manage to partially restore functionality, the resulting scar is never repopulated, relentlessly leading the patient towards the setting of heart failure. Thus, though not conventionally regarded as such, cardiac disease is a degenerative affection in which lack of sufficient contractile and vascular cells leads to a decompensated neurohormonal microenvironment [3] which further impairs both organ function and cell survival.

Although the existence of stem cells has been a well-known fact for nearly half a century [4], it is in the last 15 years that the field has experienced a major boost. Their capacity for differentiation has made stem cells outstanding candidates for the treatment of degenerative diseases, substituting for cells lost during the course of the disorder. Consequently, cardiac diseases and MI have been the object of intense research [5]. Among the cell-types studied, mesenchymal stem cells (MSCs) are strong candidates for success in the MI-setting. In the following pages, we will discuss their capacities as well as pre- and clinical investigations in which these cells have been employed.

ORIGIN, TYPES AND CHARACTERISTICS

The studies by Friedenstein and colleagues are regarded as one of the first reports on MSCs [4]. In these, the clonogenic potential of a population of bone marrow (BM)-derived stromal cells, described as colony-forming unit-fibroblasts, was examined. BM is indeed one of the best-known sources of progenitor cells, MSC being among them [6]. Although this is not entirely understood, BM-MSCs are thought to act as supporters and nurturers of other cells within the marrow [7-9], possibly in a location close to blood vessels [10]. However, there is a relatively small population (0.01%-0.0001% of

nucleated cells in human BM [11]), so MSCs can be easily purified by plastic-adherence and expanded after BM-extraction. Similarly, but adding simple mechanical and enzymatic processing, a mixed cell population (called stromal vascular fraction, SVF) can be isolated from adipose depots, which, after *in vitro* culture and homogenization gives rise to the mesenchymal progenitors from this tissue, also termed adipose-derived stem cells (ADSC) [12]. Adipose tissue is regarded as a much richer source of progenitors, harboring 100 to 500 times the numbers seen in BM [13]. However, despite similarities in phenotype, differentiation or growth kinetics, there are certain differences at a functional, genomic and proteomic level [9,14], suggesting a degree of higher commitment of BM-MSC to chondrogenic and osteogenic lineages than ADSC [15].

Adipose tissue and BM are the most widely researched sources of mesenchymal progenitors because they are easy to harvest, and owing to the relative abundance of progenitors and the lack of ethical concerns. Nevertheless, MSCs have been ubiquitously found in a variety of locations, as cord blood [16], dental pulp [17], menstrual blood [18] or heart [19] among others (reviewed in [20]). This wide variety of origins, methodologies and acronyms prompted standardization in 2005 by the International Society for Cellular Therapy, which set the minimum requirements for MSC definition, namely plastic-adherence, surface antigen profile (presence of CD73, CD90 and CD105, and lack of expression of HLA-DR, CD11b, CD14, CD19, CD34, CD45 and CD79alpha) and multipotent differentiation capacity (towards osteoblasts, chondrocytes and adipocytes) [21]. Still, caution must be taken as some reports fail to meet these criteria, and MSC is often employed for “Marrow Stromal Cell”, “Mesenchymal Stromal Cell” or “Marrow Stem Cell”. Accordingly, a clarification was published in which MSC was defined as “Multipotent Mesenchymal Stromal Cells” [22], adding the supportive property to the required characteristics [23].

WHAT DO MSCs HAVE TO OFFER TO CARDIAC REGENERATION

When considering the goal of cardiac tissue regeneration, the desired objective must encompass 3 objectives: the production of a replacement myocardial mass, the formation of a functional vascular network to sustain it and the returning of the impaired ventricle to its proper geometry. Cell therapy may theoretically affect those

processes in two ways: either by direct differentiation of transplanted cells towards the desired lineages or by their production of molecules with therapeutic potential.

BM-MSC have shown their *in vitro* capacity to give rise to endothelial cells (EC) [24,25] and smooth muscle (SMC) [24]. Cardiomyocyte differentiation has proved more problematic, as either demethylating agents have been employed [26], or it has been inefficient and incomplete [27,28]. On the other hand, the cardiac potential of ADSC is better documented *in vitro*, showing their capacity to give rise to CM, either by the use of DMSO [29] or CM extracts [30]. In addition, ADSC seems to harbor a progenitor subset characterized by the expression of Nkx2.5 and Mcl2v [31] and whose differentiation relies on the autocrine/paracrine activity of vascular endothelial growth factor (VEGF) [32]. SMC [33] and EC [34] have been obtained from adipose cells, yet a cautionary note must be struck, as some of these studies either rely on subpopulations of freshly-isolated cells or culture them in differentiation-promoting medium before purifying the mesenchymal population [35,36]. Finally, other mesenchymal progenitors have also been differentiated to CM or CM-like cells, such as menstrual blood-derived MSC [18] or umbilical cord-MSC [37].

However, although it is extremely interesting, this differentiation potential must cope with two opposing factors. First, patients receiving stem cell therapy are severely diseased and usually elderly, two factors that have an outstanding impact on stem cell function. For instance, a decrease in the numbers and functionality of circulating endothelial progenitors is directly related to cardiovascular risks and smoking [38,39] and age has also been shown to impair the angiogenic capacity of both ADSC [40] and BM-MSC [41]. Second, the small percentage of engrafted cells (see [42] for a review) coupled to the huge catastrophe caused by an MI (the loss in some cases of over 1 billion CM [43]) and the low rate of differentiation achieved even under *in vitro* controlled conditions makes the adding of such small number of cells a therapeutically-inefficient approach.

On the other hand, secretion of beneficial molecules has been demonstrated to be able to exert a positive effect, even when a few engrafted cells are left [44]. These molecules can induce a benefit either by increasing tissue-perfusion, decreasing collagen deposition and fibrosis, enhancing host-cell survival or attracting/regulating endogenous progenitors. Thus, Chen and coworkers compared the expression profile of BM-MSC

and dermal fibroblasts [45], showing that mesenchymal progenitors secreted a higher amount several molecules, including the potent proangiogenic cytokine VEGF or the chemotactic stromal derived factor 1 (SDF1). Conditioned medium from BM-MSC induced the recruitment of EC and macrophages, as well as improved wound healing. Moreover, it has recently been shown that serum-deprived BM-MSC acquire EC-features as well as increasing the release of VEGF or hepatocyte growth factor (HGF), another potent angiogenic molecule [46], both of which have been reported to be secreted by ADSC [32,47,48]. Moreover, Dr March's group demonstrated that ADSC have a pericytic nature and are able to form and stabilize functional vascular networks when mixed with endothelial progenitors [49]. Also, BM-MSCs show a potent antifibrotic action, as their conditioned medium decreases cardiac fibroblast proliferation and expression of collagen types I and III [50,51] and increases secretion of antifibrotic molecules such as matrix metalloproteinases (MMP) 2, 9 and 14 [52]. These cells express 5 types of MMPs (2, 13 and membrane type-MMPs 1, 2 and 3) and are able to cross through type I collagen membranes [53], which theoretically would allow their trafficking across the infarction-derived scar. Likewise, ADSC produce transforming growth factor (TGF) β1 [54], a potent regulator of fibrosis. Taken as a whole, these examples demonstrate that mesenchymal progenitors are potent paracrine mediators with a considerable capacity to impact infarct evolution.

One last noteworthy competence is the ability of BM-MSC and ADSC to modulate the immune response. Marrow-derived mesenchymal progenitors inhibit the proliferation of activated T cells and the formation of cytotoxic T cells [55], inducing an anti-inflammatory phenotype, which would allow their allogenic use and significantly broaden the scope of their applicability. However, Huang *et al* reported that differentiation reduced their capacity of immunological escape [56], related to an increase of immunostimulatory molecules MHC-Ia and II and a decrease in the immunosuppressive MHCIb. Along similar lines, McIntosh and coworkers reported that ADCS beyond passage 1 (and thus devoid of contaminating differentiated cells [57]) failed to elicit a response from allogenic T cells [58], but this attribute may be diminished under inflammatory stimuli, as shown *in vitro* [59].

Finally, since the onset of induced pluripotent cells (iPS) [60], mesenchymal cells have been investigated [61,62] due to their relatively easy harvest and higher potency than other cell types (e.g.: dermal fibroblasts), which shows an increased efficiency, even in

the absence of the oncogene c-Myc. Their supportive capacities have also made them good candidates to replace mouse cells as feeders [63,64].

MSC IN ANIMAL MODELS OF MI

However, in spite of all the positive characteristics of mesenchymal progenitors already depicted, their *in vivo* testing in animal models of the disease is compulsory. In this regard, 3 different settings can be found. First, the acute setting, in which cells are transplanted within hours of the MI. Here, the inflammatory microenvironment and the necrotic/apoptotic signals released from resident cells [65,66] are the main opposing forces to the therapeutic activity of cells. Nevertheless, homing signals [67] and an anti-fibrotic milieu [68] may have a positive influence. Also, from a practical point of view, dealing with acute models offers the advantage of subjecting animals to only one surgery, as at the time of the MI (or minutes after it), the cells are applied, thus decreasing mortality and invasiveness. As a consequence, the majority of published reports use acute models [37,69-84]. Most studies (with the exception of the two by van der Bogt and colleagues [74,77]) have consistently demonstrated that the treatment induces a significant benefit for cardiac function, mainly through paracrine mechanisms that induce an increase in tissue perfusion and a decrease in the size of the scar and collagen content.

Similar results have been obtained in a second setting, the chronic one. Here, the repair processes that take place after ischemia have been completed, the scar has matured, and although a new network of blood vessels has been created, this is disorganized and inadequate [85,86]. These facts impose a great burden upon cell-survival. However, it must be taken into account that the generation of homogeneous populations as BM-MSC or ADSC needs weeks of *in vitro* culture, thus, unless used in the allogeneic setting, there is no possibility of the bedside translation of use of mesenchymal progenitors in the acute setting. In spite of this difficulty, fewer reports deal with this issue [87-90]. Compared to results in the acute setting, mesenchymal cell therapy of chronically infarcted hearts has a positive effect upon organ contractility and histology.

As a third and intermediate position, the so-called sub-acute model represents a situation where angiogenic processes are still on course, either through endothelial

progenitors [91] or macrophages [92], and the receding of inflammation plus the increase in fibrotic processes are also on course. As with chronic models, there are few reports in this setting [17,18,93,94], but again the benefit and mechanisms appear to be consistent.

Nevertheless, analyzing in more depth the studies mentioned above, it is possible to find a fair amount of information on how mesenchymal progenitors behave when injected into the diseased heart has been gathered. Lee *et al* showed that transplantation of BM-MSC into chronically infarcted rabbit hearts induced an increase in the concentration of SDF1 that elicited the chemotaxis of host-derived BM progenitors (CD34+, CD117+, STRO1+) and was related to a functional benefit, a decrease in infarct size and improvement in tissue vascularization [89]. Li and coworkers demonstrated that the functional enhancement was accompanied by the augmented expression of the pro-survival gene Akt [95] whereas Mias and colleagues showed that the benefit upon contractility and remodeling *in vivo* was accompanied *in vitro* by a plethora of anti-fibrotic actions [52]. In a sheep model of MI, the group of Dr Spinale monitored the evolution of MMPs and their inhibitors, demonstrating a relationship with the number of transplanted cells [75]. Resembling their *in vitro* behavior, several publications have demonstrated the association between pro-angiogenic activity *in vitro* and secretion (either direct or host-derived) of angiogenic cytokines as VEGF, HGF or insulin-like growth factor 1 (IGF1) among others [17,84,93,96,97]. Whether these capacities are related to the claimed pericytic-nature of these cells [10,48,49] remains to be resolved.

Immune modulation (reviewed in [98]) in theory provides the means for the allogenic use of MSCs and as an off-the-shelf product (expanded prior to the onset of the ischemia and applicable on demand). Two reports have compared the effects of allogenic versus syngenic injection of BM-MSC in rat model of MI, with conflicting results. Imanishi *et al* [78] demonstrated that both autologous and allogeneic cells improved cardiac function 4 weeks after transplantation, remained in the damaged tissues and did not stimulate rejection. Huang and coworkers, on the other hand [56], followed animals for up to 6 months. Syngenic cells stimulated cardiac recovery, but the effect of the allogenic treatment was transitory (significant 3 months post-injection but not at 6) and BM-MSC disappeared earlier than their syngenic counterpart. However, this difference can be attributed to methodological discrepancies regarding time of transplantation (acute versus chronic respectively) or follow-up (1 versus 6 months).

Equivalent and importantly, results from clinically-relevant large animal models of MI in which allogenic cells have been employed have revealed either positive [99,100] or no functional outcome [79]. On the other hand, when autologous ADSCs or BM-MSCs are used [72,83,101,102], reports have shown a robust and consistent functional recovery after cell transplantation. Thus, strict considerations about building up animal models must be taken into account.

PROBLEMS, SOLUTIONS

Despite all the optimism, stem cell therapy shows certain caveats that are amenable to improvement, namely lack of substantial engraftment and cell-persistence, high levels of death and low *in vivo* differentiation capacity. Some approaches to try to remedy these problems have included the use of genetic manipulation, *in vitro* pre-treatment of cells or biomaterials. In this sense, the CXCR4/SDF1 axis has been greatly exploited. Ma *et al* investigated the peak of cardiac SDF1 expression [103] in rat MI, finding that injected cells at that time point (1 day post-infarction) increased cell engraftment and tissue angiogenesis. Cheng and coworkers transplanted BM-MSC engineered to overexpress the receptor CXCR4, strengthening cell-homing to the injured tissue after tail vein-injection [104]. The same group combined BM-MSC peripheral injection with administration of granulocyte colony-stimulating factor, which *in vitro* increased CXCR4 expression. However, although engraftment was increased, no effect of cardiac function was found [105]. Huang and associates demonstrated that overexpression of the chemokine receptor CCR1 but not CXCR2 was associated with improved survival and grafting in a mouse model of MI, which also restored functionality [106].

Cell survival in the infarcted myocardium is jeopardized by hypoxia, inflammation or oxidative stress. Liu *et al* engineered BM-MSC to overexpress angiogenin [107], which improved hypoxic resistance in culture and was translated into an increase in cell engraftment and functional- and histological-recovery induction. Cell-overexpression of hemeoxygenase-1 through adenoviral transfection showed superior therapeutic capacity, mainly through protection from inflammation and apoptosis [108], whereas targeted Akt-overproduction in MSC restored cardiac function 2 weeks post-MI through paracrine actions, including protection from hypoxia-induced apoptosis, release of cytokines and preservation of tissue metabolism [109-111]. Others have explored anti-

oxidants, like Song *et al* who published that reactive oxygen species (ROS) diminished BM-MSC adherence to the substrate, but when treated with a ROS scavenger (N-acetyl-L-cysteine), engraftment was improved and the increase in fibrosis and infarct size prevented [112]. Hsp20 overexpression also protected MSC from oxidative stress and improved their beneficial activities [97].

However, viral or genetic modification of cells implies certain risks that currently make it difficult for a devised therapy to reach the bedside. Bioengineering uses biocompatible materials to improve or direct cell therapy and either synthetic or naturally-derived systems have been employed. Jin and coworkers seeded BM-MSC on poly(lactide-co-1-caprolactone) patches which when applied on a rat cryoinjury model were able to improve cardiac function and decrease infarct size [113]. Porcine small intestine submucose, a decellularized substrate, has been employed to treat a rabbit model of chronic MI, showing a significant benefit upon contractility and histology, as well as cell migration towards the injured tissue [114]. The cell sheet technology allows increasing thickness through stacking of constructs, as shown by Chen *et al* [115], where its transplantation in rat syngenic model of cardiac ischemia improved cardiac function as well as paracrine secretion of therapeutic molecules by grafted cells. Dr Mori´s group compared the transplantation of a cell sheet seeded with ADSC versus fibroblasts, showing the superior effect of the mesenchymal progenitors [116]. Recently, autologous ADSC were transplanted along with allogenic ESC-derived CD15+ cardiac progenitors in a monkey model of infarction, demonstrating the safety of the procedure, although the functional outcome was not analyzed [117].

MESENCHYMAL PROGENITORS AND CLINICAL APPLICATION

Several clinical trials have been performed with autologous BM-MSC, proving their safety when transplanted in patients with either acute or chronic myocardial infarction [118-120]. Moreover, the first clinical trial designed as a randomized study showed an improvement in the cardiac function 3 months after BM-MSC intracoronary infusion in patients with acute MI [120]. In view of the encouraging results of the previous clinical trials, new phase-I/II studies have been initiated, including the Transendocardial Autologous Cells (hMSC or hBMC) in Ischemic Heart Failure Trial (TAC-HFT; www.clinicaltrials.org/NCT00768066), the Prospective Randomised study Of MSC

THErapy in patients Undergoing cardiac Surgery (PROMETHEUS) trial (www.clinicaltrials.org/NCT00587990) and the Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis (POSEIDON) pilot study (www.clinicaltrials.org/NCT01087996) [121], among others.

BM-MSCs from allogeneic origin have been tested as an off-the-shelf cell product. The first phase-I, randomized, double-blind, placebo-controlled, dose-escalation study was performed in 53 patients with acute MI, who intravenously received one of three doses of BM-MSCs (0.5, 1.6 or 5.0 x 10^6 BM-MSC/Kg body weight) derived from a single cell donor (Prochymal; Osiris therapeutics, Inc.) or placebo [122]. Safety of the procedure was proven, showing fewer episodes of ventricular tachycardia and even a better lung function in the cell treated group. Also, renal, hepatic and hematologic laboratory indexes were similar in the two groups and no patient developed tumors. Importantly, a significant increase was detected in the ejection fraction (EF) of the treated patients. In a magnetic resonance imaging substudy, cell treatment, but not placebo, increased left ventricular ejection fraction and led to a reversal of adverse remodeling after 6 months of treatment. Now, a phase-II multicentre trial of ProchymalTM has been started (www.clinicaltrials.org/NCT00877903).

Furthermore, BM-MSC safety has been tested in patients with moderate to severe chronic heart failure in a phase-II, randomized, single-blind, placebo-controlled, dose-escalation, multicenter study. In this clinical trial, the patients received an endoventricular injection of an allogeneic BM-MSC product (Revascor, Mesoblast Ltd.) along the infarct border zone and no procedure-related complications were reported. Analysis of the data obtained after 6 months of follow-up (www.mesoblast.com/newsroom/asx-announcements/archives) showed a significant decrease in the number of patients who developed any severe or major adverse cardiac event, such as composite of cardiac death, heart attack or need for coronary revascularization procedures. Moreover, the first cohort in the study (n=20 patients) (which received the low dose of the cell treatment) showed a significantly greater increase in the EF when compared with the control group [123].

On the other hand, regarding other sources of MSC such as adipose tissue, no clinical trials have been initiated yet, despite the fact that the beneficial potential of ADSC has been pre-clinically demonstrated [83]. Until now, only the non-cultured adipose stromal

vascular fraction is being tested at the clinical level. The first study, a double-blind, placebo-controlled trial named APOLLO (www.clinicaltrials.org/NCT00442806; [124]) where AMI patients received autologous adipose derived stem cells by intracoronary infusion, was proved safe. Now a phase II/III ADVANCE trial has been initiated to evaluate their efficacy (www.clinicaltrials.org /NCT01216995).

In general, the results obtained from the many clinical trials performed, either with MSCs or other stem cell populations (mainly BM-derived cells and skeletal myoblasts), have taught us several important lessons that will help to design and interpretate the following clinical trials: (i): cell treatment is not equally efficacious in all the patients. In general, it seems that the worse the heart damage (meaning severely decreased post-revascularization LVEF or high degree of infarct transmurality), the better the benefit induced by the transplanted cells seems to be [125-127]; (ii): Cell dose and timing for treatment are critical. Thus, a meta-analysis of the results obtained in the most relevant clinical trials performed in acute MI patients treated with BM cells has shown a significantly greater effect in those patients that received high cell doses (10^8 cells). Also, the same study showed a greater beneficial effect when cells are infused during the first week after the infarct [128]; (iii): Autologous treatment is not necessarily the best. Until now, most of the clinical studies have been designed for autologous cell application in order to avoid the immunorejection of the transplanted cells. However, it has to be borne in mind that stem cells derived from aged-patients with risk of atherosclerosis or other diseases might be defective, and thereby, treatment with them might not be as efficacious as with cells derived from young healthy donors [129-131]. In that sense, the use of MSCs which present immunomodulatory properties [132] could be of great relevance. Thus, advantages of allogeneic MSC treatment would be that, together with the putative greater paracrine effect that allogeneic cells derived from a healthy donor could exert, a fully tested clinical grade ready to use allogeneic cell product could be available for any patient. Importantly, patients with acute MI could also be eligible for such treatment. Furthermore, the logistical complexity and manufacturing costs that autologous cell preparation implies would be significantly reduced by the allogeneic application. However, caution should be taken when taking into consideration the issues related to their immune privilege explained above.

Thus, although it is mandatory to better understand the mechanisms involved in the MSC phenotype switch and to elucidate how this could affect the cells' potential

benefit, it has to be considered that, in any case, because MSC would not differentiate towards cardiovascular cells and would act as a paracrine factor source [111], their permanent presence in the heart might not be necessary for therapeutic purposes. In that case, a temporarily action should be sufficient for exerting their benefit. Phase-II clinical trials are currently assessing the efficacy of the allogeneic MSC treatment, together with the long-term safety. If allogeneicity of the cells diminishes their effectiveness, several options could be considered, like temporal patient immunosupression and/or donor-recipient HLA-II mismatch minimizing. As a consequence, the increase in the rate of engraftment of transplanted cells is so far one of the main challenges. As already indicated, the use of scaffolds could improve this factor. Interestingly, a clinical trial has been performed in 15 patients with chronic MI who were treated with a collagen scaffold previously seeded with bone marrow mononuclear cells [133]. The celularized patch was implanted onto the pericardium and no adverse events were reported, showing the feasibility and safety of the treatment. Furthermore, a limiting effect in ventricular wall remodeling and an improved diastolic function was detected. These positive results will probably promote new larger randomized controlled trials, where mesenchymal and other stem cell populations might be tested in combination with scaffolds, thus leading to a further step in the therapeutic use of stem cells.

CONCLUSION

Mesenchymal cells have raised substantial interest in recent years due to their potential and versatility. Although we are only now starting to understand the mechanisms by which they repair or induce the repair of damaged organs, their pleiotropic activity and the technical ease of manipulation makes them good candidates for the treatment of the MI. Though waiting for randomized, double-blinded, placebo-controlled clinical trials in which large cohorts of patients could participate, the available data demonstrates the safety of the therapy and points towards a positive effect, further encouraging new investigations. The addition of the latest improvements in the field, including *in vitro* conditioning and bioengineering, will surely suppose a further step towards finding an optimized treatment. However, certain issues, mainly immunomodulatory-capacity and allogenic use, need to be better understood.

SOURCE OF FUNDING

Work in the laboratory of FP is supported by grants from the EU (FP7 INELPY), Instituto de Dalud Carlos III (ISCIII-RETIC RD06/0014 PI10/01621 and CP09/00333 (FIS). Ministero de Ciencia e Innovacion Programa de Internacionalización CARDIOBIO

FIGURE LEGENDS

Figure 1. Standarized requirements for MSC definition. Prerequisite for MSC denomination includes surface antigen profile, capacity or differentiation into mesenchymal lineages, stromal supportiveness and *in vitro* growth on plastic adherence.

Figure 2. Main MSC actions on injuried myocardium. Mesenchymal progenitors transplanted onto the ischemic myocardium are able to secrete a plethora of therapeutic molecules (paracrine activity), differentiate towards cardiac lineages or modulate immune response, promoting both a anti-inflammatory microenviroment and an increased tolerance of transplanted cells, encouraging the healing of the damaged tissue and avoiding its transition to a scarred muscle. Abbreviations: IGF1: insulin-like growth factor 1; SDF1: stromal derived factor 1; VEGF: vascular endothelial growth factor; HGF: hepatocyte growth factor; EC: endothelial cell; MMP: matrix metalloproteinase; CM: cardiomyocyte; SMC: smooth muscle cell; DC: dendritic cell; Pro-inf.: pro-inflammatory; Anti-infl.: anti-inflammatory; MI: myocardial infarction.

REFERENCES

[1] World Health Organization. World Health Statistics 2008. , 2008.

[2] Mazo M, Pelacho B, Prosper F. Stem cell therapy for chronic myocardial infarction. J Cardiovasc Transl Res 2010;3:79-88.

[3] Frangogiannis NG. Chemokines in the ischemic myocardium: from inflammation to fibrosis. Inflamm Res 2004;53:585-95.

[4] Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation 1968;6:230-47.

[5] Pelacho B, Prosper F. Stem Cells and Cardiac Disease: Where are We Going? Curr Stem Cell Res Ther 2008;3:265-76.

[6] Clavel C, Verfaillie CM. Bone-marrow-derived cells and heart repair. Curr Opin Organ Transplant 2008;13:36-43.

[7] Walenda T, Bork S, Horn P, Wein F, Saffrich R, Diehlmann A et al. Co-culture with mesenchymal stromal cells increases proliferation and maintenance of haematopoietic progenitor cells. J Cell Mol Med 2010;14:337-50.

[8] Jones DL, Wagers AJ. No place like home: anatomy and function of the stem cell niche. Nat Rev Mol Cell Biol 2008;9:11-21.

[9] Wagner W, Roderburg C, Wein F, Diehlmann A, Frankhauser M, Schubert R et al. Molecular and secretory profiles of human mesenchymal stromal cells and their abilities to maintain primitive hematopoietic progenitors. Stem Cells 2007;25:2638-47.

[10] Cai X, Lin Y, Friedrich CC, Neville C, Pomerantseva I, Sundback CA et al. Bone marrow Derived Pluripotent Cells are Pericytes which Contribute to Vascularization. Stem Cell Rev Rep 2009.

[11] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-7.

[12] Mazo M, Gavira JJ, Pelacho B, Prosper F. Adipose-derived stem cells for myocardial infarction. J Cardiovasc Transl Res 2011;4:145-53.

[13] Casteilla L, Planat-Benard V, Laharrague P, Cousin B. Adipose-derived stromal cells: Their identity and uses in clinical trials, an update. World J Stem Cells 2011;3:25-33.

[14] Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006;24:1294-301.

[15] Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. Circ Res 2007;100:1249-60.

[16] Yang SE, Ha CW, Jung M, Jin HJ, Lee M, Song H et al. Mesenchymal stem/progenitor cells developed in cultures from UC blood. Cytotherapy 2004;6:476-86.

[17] Gandia C, Arminan A, Garcia-Verdugo JM, Lledo E, Ruiz A, Minana MD et al. Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction. Stem Cells 2008;26:638-45.

[18] Hida N, Nishiyama N, Miyoshi S, Kira S, Segawa K, Uyama T et al. Novel Cardiac Precursor-Like Cells from Human Menstrual Blood-Derived Mesenchymal Cells. Stem Cells 2008.

[19] Carlson S, Trial J, Soeller C, Entman ML. Cardiac mesenchymal stem cells contribute to scar formation after myocardial infarction. Cardiovasc Res 2011;91:99-107.

[20] Ding DC, Shyu WC, Lin SZ. Mesenchymal stem cells. Cell Transplant 2011;20:5-14.

[21] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-7.

[22] Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC et al. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy 2005;7:393-5.

[23] Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 2007;131:324-36.

[24] Lozito TP, Taboas JM, Kuo CK, Tuan RS. Mesenchymal stem cell modification of endothelial matrix regulates their vascular differentiation. J Cell Biochem 2009;107:706-13.

[25] Liu JW, Dunoyer-Geindre S, Serre-Beinier V, Mai G, Lambert JF, Fish RJ et al. Characterization of endothelial-like cells derived from human mesenchymal stem cells. J Thromb Haemost 2007;5:826-34.

[26] Xu W, Zhang X, Qian H, Zhu W, Sun X, Hu J et al. Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro. Exp Biol Med (Maywood) 2004;229:623-31.

[27] Yan X, Lv A, Xing Y, Liu B, Hou J, Huang W et al. Inhibition of p53-p21 pathway promotes the differentiation of rat bone marrow mesenchymal stem cells into cardiomyocytes. Mol Cell Biochem 2011;354:21-8.

[28] Arminan A, Gandia C, Garcia-Verdugo JM, Lledo E, Mullor JL, Montero JA et al. Cardiac transcription factors driven lineage-specification of adult stem cells. J Cardiovasc Transl Res;3:61-5.

[29] van Dijk A, Niessen HW, Zandieh Doulabi B, Visser FC, van Milligen FJ. Differentiation of human adipose-derived stem cells towards cardiomyocytes is facilitated by laminin. Cell Tissue Res 2008.

[30] Gaustad KG, Boquest AC, Anderson BE, Gerdes AM, Collas P. Differentiation of human adipose tissue stem cells using extracts of rat cardiomyocytes. Biochem Biophys Res Commun 2004;314:420-7.

[31] Bai X, Pinkernell K, Song YH, Nabzdyk C, Reiser J, Alt E. Genetically selected stem cells from human adipose tissue express cardiac markers. Biochem Biophys Res Commun 2007;353:665-71.

[32] Song YH, Gehmert S, Sadat S, Pinkernell K, Bai X, Matthias N et al. VEGF is critical for spontaneous differentiation of stem cells into cardiomyocytes. Biochem Biophys Res Commun 2007;354:999-1003.

[33] Kim YM, Jeon ES, Kim MR, Jho SK, Ryu SW, Kim JH. Angiotensin II-induced differentiation of adipose tissue-derived mesenchymal stem cells to smooth muscle-like cells. Int J Biochem Cell Biol 2008;40:2482-91.

[34] Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R et al. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 2004;109:656-63.

[35] Fischer LJ, McIlhenny S, Tulenko T, Golesorkhi N, Zhang P, Larson R et al. Endothelial differentiation of adipose-derived stem cells: effects of endothelial cell growth supplement and shear force. J Surg Res 2009;152:157-66.

[36] Sengenes C, Miranville A, Maumus M, de Barros S, Busse R, Bouloumie A. Chemotaxis and differentiation of human adipose tissue CD34+/CD31- progenitor cells: role of stromal derived factor-1 released by adipose tissue capillary endothelial cells. Stem Cells 2007;25:2269-76.

[37] Chang SA, Lee EJ, Kang HJ, Zhang SY, Kim JH, Li L et al. Impact of Myocardial Infarct Proteins and Oscillating Pressure on the Differentiation of Mesenchymal Stem Cells: Effect of Acute Myocardial Infarction on Stem Cell Differentiation. Stem Cells 2008;26:1901-12.

[38] Kondo T, Hayashi M, Takeshita K, Numaguchi Y, Kobayashi K, Iino S et al. Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. Arterioscler Thromb Vasc Biol 2004;24:1442-7.

[39] Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 2001;89:E1-7.

[40] Madonna R, Renna FV, Cellini C, Cotellese R, Picardi N, Francomano F et al. Age-dependent impairment of number and angiogenic potential of adipose tissue-derived progenitor cells. Eur J Clin Invest.

[41] Liang H, Hou H, Yi W, Yang G, Gu C, Lau WB et al. Increased expression of pigment epithelium-derived factor in aged mesenchymal stem cells impairs their therapeutic efficacy for attenuating myocardial infarction injury. Eur Heart J 2011.

[42] Haider HK, Ashraf M. Strategies to promote donor cell survival: Combining preconditioning approach with stem cell transplantation. J Mol Cell Cardiol 2008.

[43] Robey TE, Saiget MK, Reinecke H, Murry CE. Systems approaches to preventing transplanted cell death in cardiac repair. J Mol Cell Cardiol 2008.

[44] Fedak PW. Paracrine effects of cell transplantation: modifying ventricular remodeling in the failing heart. Semin Thorac Cardiovasc Surg 2008;20:87-93.

[45] Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS ONE 2008;3:e1886.

[46] Oskowitz A, McFerrin H, Gutschow M, Carter ML, Pochampally R. Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic. Stem Cell Res 2011;6:215-25.

[47] Kilroy GE, Foster SJ, Wu X, Ruiz J, Sherwood S, Heifetz A et al. Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors. J Cell Physiol 2007;212:702-9.

[48] Traktuev DO, Merfeld-Clauss S, Li J, Kolonin M, Arap W, Pasqualini R et al. A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. Circ Res 2008;102:77-85.

[49] Traktuev DO, Prater DN, Merfeld-Clauss S, Sanjeevaiah AR, Saadatzadeh MR, Murphy M et al. Robust functional vascular network formation in vivo by cooperation of adipose progenitor and endothelial cells. Circ Res 2009;104:1410-20.

[50] Li L, Zhang S, Zhang Y, Yu B, Xu Y, Guan Z. Paracrine action mediate the antifibrotic effect of transplanted mesenchymal stem cells in a rat model of global heart failure. Mol Biol Rep 2009;36:725-31.

[51] Ohnishi S, Sumiyoshi H, Kitamura S, Nagaya N. Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions. FEBS Lett 2007;581:3961-6.

[52] Mias C, Lairez O, Trouche E, Roncalli J, Calise D, Seguelas MH et al. Mesenchymal Stem Cells Promote Matrix Metalloproteinase Secretion By Cardiac Fibroblasts And Reduce Cardiac Ventricular Fibrosis After Myocardial Infarction. Stem Cells 2009.

[53] Rogers TB, Pati S, Gaa S, Riley D, Khakoo AY, Patel S et al. Mesenchymal stem cells stimulate protective genetic reprogramming of injured cardiac ventricular myocytes. J Mol Cell Cardiol.

[54] Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation 2004;109:1292-8.

[55] Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815-22.

[56] Huang XP, Sun Z, Miyagi Y, McDonald Kinkaid H, Zhang L, Weisel RD et al. Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. Circulation 2010;122:2419-29.

[57] Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A et al. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells 2006;24:376-85.

[58] McIntosh K, Zvonic S, Garrett S, Mitchell JB, Floyd ZE, Hammill L et al. The immunogenicity of human adipose-derived cells: temporal changes in vitro. Stem Cells 2006;24:1246-53.

[59] Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Pescatori M, Stubbs AP et al. Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. Clin Exp Immunol.

[60] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663-76.

[61] Tat PA, Sumer H, Jones KL, Upton K, Verma PJ. The efficient generation of induced pluripotent stem (iPS) cells from adult mouse adipose tissue-derived and neural stem cells. Cell Transplant 2010;19:525-36.

[62] Sun N, Panetta NJ, Gupta DM, Wilson KD, Lee A, Jia F et al. Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. Proc Natl Acad Sci U S A 2009;106:15720-5.

[63] Mamidi MK, Pal R, Mori NA, Arumugam G, Thrichelvam ST, Noor PJ et al. Co-culture of mesenchymal-like stromal cells derived from human foreskin permits long term propagation and differentiation of human embryonic stem cells. J Cell Biochem 2011;112:1353-63.

[64] Hwang ST, Kang SW, Lee SJ, Lee TH, Suh W, Shim SH et al. The expansion of human ES and iPS cells on porous membranes and proliferating human adipose-derived feeder cells. Biomaterials 2010;31:8012-21.

[65] Nian M, Lee P, Khaper N, Liu P. Inflammatory cytokines and postmyocardial infarction remodeling. Circ Res 2004;94:1543-53.

[66] Mann DL. Mechanisms and models in heart failure: A combinatorial approach. Circulation 1999;100:999-1008.

[67] Penn MS. Importance of the SDF-1:CXCR4 axis in myocardial repair. Circ Res 2009;104:1133-5.

[68] Cleutjens JP, Kandala JC, Guarda E, Guntaka RV, Weber KT. Regulation of collagen degradation in the rat myocardium after infarction. J Mol Cell Cardiol 1995;27:1281-92.

[69] Ii M, Horii M, Yokoyama A, Shoji T, Mifune Y, Kawamoto A et al. Synergistic effect of adipose-derived stem cell therapy and bone marrow progenitor recruitment in ischemic heart. Lab Invest 2011;91:539-52.

[70] Gaebel R, Furlani D, Sorg H, Polchow B, Frank J, Bieback K et al. Cell origin of human mesenchymal stem cells determines a different healing performance in cardiac regeneration. PLoS One 2011;6:e15652.

[71] Bai X, Yan Y, Coleman M, Wu G, Rabinovich B, Seidensticker M et al. Tracking long-term survival of intramyocardially delivered human adipose tissue-derived stem cells using bioluminescence imaging. Mol Imaging Biol 2011;13:633-45.

[72] Dubois C, Liu X, Claus P, Marsboom G, Pokreisz P, Vandenwijngaert S et al. Differential effects of progenitor cell populations on left ventricular remodeling and myocardial neovascularization after myocardial infarction. J Am Coll Cardiol 2010;55:2232-43.

[73] Yang YJ, Qian HY, Huang J, Li JJ, Gao RL, Dou KF et al. Combined Therapy With Simvastatin and Bone Marrow-Derived Mesenchymal Stem Cells Increases Benefits in Infarcted Swine Hearts. Arterioscler Thromb Vasc Biol 2009.

[74] van der Bogt KE, Schrepfer S, Yu J, Sheikh AY, Hoyt G, Govaert JA et al. Comparison of transplantation of adipose tissue- and bone marrow-derived mesenchymal stem cells in the infarcted heart. Transplantation 2009;87:642-52.

[75] Dixon JA, Gorman RC, Stroud RE, Bouges S, Hirotsugu H, Gorman JH,3rd et al. Mesenchymal cell transplantation and myocardial remodeling after myocardial infarction. Circulation 2009;120:S220-9.

[76] Bai X, Yan Y, Song YH, Seidensticker M, Rabinovich B, Metzele R et al. Both cultured and freshly isolated adipose tissue-derived stem cells enhance cardiac function after acute myocardial infarction. Eur Heart J 2009.

[77] van der Bogt KE, Sheikh AY, Schrepfer S, Hoyt G, Cao F, Ransohoff KJ et al. Comparison of different adult stem cell types for treatment of myocardial ischemia. Circulation 2008;118:S121-9.

[78] Imanishi Y, Saito A, Komoda H, Kitagawa-Sakakida S, Miyagawa S, Kondoh H et al. Allogenic mesenchymal stem cell transplantation has a therapeutic effect in acute myocardial infarction in rats. J Mol Cell Cardiol 2008;44:662-71.

[79] Hashemi SM, Ghods S, Kolodgie FD, Parcham-Azad K, Keane M, Hamamdzic D et al. A placebo controlled, dose-ranging, safety study of allogenic mesenchymal stem cells injected by endomyocardial delivery after an acute myocardial infarction. Eur Heart J 2008;29:251-9.

[80] Hale SL, Dai W, Dow JS, Kloner RA. Mesenchymal stem cell administration at coronary artery reperfusion in the rat by two delivery routes: A quantitative assessment. Life Sci 2008.

[81] Carr CA, Stuckey DJ, Tatton L, Tyler DJ, Hale SJ, Sweeney D et al. Bone marrow-derived stromal cells home to and remain in the infarcted rat heart but fail to improve function: an in vivo cine-MRI study. Am J Physiol Heart Circ Physiol 2008;295:H533-42.

[82] Cai L, Johnstone BH, Cook TG, Tan J, Fishbein MC, Chen PS et al. IFATS Series: Human Adipose Tissue-Derived Stem Cells Induce Angiogenesis and Nerve Sprouting Following Myocardial Infarction, in Conjunction with Potent Preservation of Cardiac Function. Stem Cells 2008.

[83] Valina C, Pinkernell K, Song YH, Bai X, Sadat S, Campeau RJ et al. Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular function, perfusion, and remodelling after acute myocardial infarction. Eur Heart J 2007;28:2667-77.

[84] Li B, Zeng Q, Wang H, Shao S, Mao X, Zhang F et al. Adipose tissue stromal cells transplantation in rats of acute myocardial infarction. Coron Artery Dis 2007;18:221-7.

[85] Virag JI, Murry CE. Myofibroblast and endothelial cell proliferation during murine myocardial infarct repair. Am J Pathol 2003;163:2433-40.

[86] Sun Y, Kiani MF, Postlethwaite AE, Weber KT. Infarct scar as living tissue. Basic Res Cardiol 2002;97:343-7.

[87] Song H, Cha MJ, Song BW, Kim IK, Chang W, Lim S et al. Reactive Oxygen Species Inhibit Adhesion of Mesenchymal Stem Cells Implanted into Ischemic Myocardium via Interference of Focal Adhesion Complex. Stem Cells.

[88] Mazo M. Transplantation of Mesenchymal Stem Cells exerts a greater long-term effect than Bone Marrow Mononuclear Cells in a chronic myocardial infarction model in rat. - 2009;Accepted.

[89] Lee BC, Hsu HC, Tseng WY, Chen CY, Lin HJ, Ho YL et al. Cell therapy generates a favourable chemokine gradient for stem cell recruitment into the infarcted heart in rabbits. Eur J Heart Fail 2009;11:238-45.

[90] Mazo M, Planat-Benard V, Abizanda G, Pelacho B, Leobon B, Gavira JJ et al. Transplantation of adipose derived stromal cells is associated with functional improvement in a rat model of chronic myocardial infarction. Eur J Heart Fail 2008;10:454-62.

[91] Jujo K, Ii M, Losordo DW. Endothelial progenitor cells in neovascularization of infarcted myocardium. J Mol Cell Cardiol 2008;45:530-44.

[92] Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med 2007;204:3037-47.

[93] Wang L, Deng J, Tian W, Xiang B, Yang T, Li G et al. Adipose-derived stem cells are an effective cell candidate for treatment of heart failure: an MR imaging study of rat hearts. Am J Physiol Heart Circ Physiol 2009;297:H1020-31.

[94] Amado LC, Saliaris AP, Schuleri KH, St John M, Xie JS, Cattaneo S et al. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc Natl Acad Sci U S A 2005;102:11474-9.

[95] Li H, Malhotra D, Yeh CC, Tu R, Zhu BQ, Birger N et al. Myocardial survival signaling in response to stem cell transplantation. J Am Coll Surg 2009;208:607-13.

[96] Cho J, Zhai P, Maejima Y, Sadoshima J. Myocardial injection with GSK-3beta-overexpressing bone marrow-derived mesenchymal stem cells attenuates cardiac dysfunction after myocardial infarction. Circ Res 2011;108:478-89.

[97] Wang X, Zhao T, Huang W, Wang T, Qian J, Xu M et al. Hsp20-Engineered Mesenchymal Stem Cells are Resistant to Oxidative Stress Via Enhanced Activation of Akt and Increased Secretion of Growth Factors. Stem Cells 2009.

[98] Le Blanc K. Mesenchymal stromal cells: Tissue repair and immune modulation. Cytotherapy 2006;8:559-61.

[99] Schuleri KH, Amado LC, Boyle AJ, Centola M, Saliaris AP, Gutman MR et al. Early improvement in cardiac tissue perfusion due to mesenchymal stem cells. Am J Physiol Heart Circ Physiol 2008;294:H2002-11.

[100] Amado LC, Schuleri KH, Saliaris AP, Boyle AJ, Helm R, Oskouei B et al. Multimodality noninvasive imaging demonstrates in vivo cardiac regeneration after mesenchymal stem cell therapy. J Am Coll Cardiol 2006;48:2116-24.

[101] Rigol M, Solanes N, Farre J, Roura S, Roque M, Berruezo A et al. Effects of adipose tissue-derived stem cell therapy after myocardial infarction: impact of the route of administration. J Card Fail 2010;16:357-66.

[102] Zhou Y, Wang S, Yu Z, F. HR,Jr, Sachdev V, Vincent P et al. Direct injection of autologous mesenchymal stromal cells improves myocardial function. Biochem Biophys Res Commun 2009;390:902-7.

[103] Ma J, Ge J, Zhang S, Sun A, Shen J, Chen L et al. Time course of myocardial stromal cell-derived factor 1 expression and beneficial effects of intravenously administered bone marrow stem cells in rats with experimental myocardial infarction. Basic Res Cardiol 2005;100:217-23.

[104] Cheng Z, Ou L, Zhou X, Li F, Jia X, Zhang Y et al. Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance. Mol Ther 2008;16:571-9.

[105] Cheng Z, Liu X, Ou L, Zhou X, Liu Y, Jia X et al. Mobilization of mesenchymal stem cells by granulocyte colony-stimulating factor in rats with acute myocardial infarction. Cardiovasc Drugs Ther 2008;22:363-71.

[106] Huang J, Zhang Z, Guo J, Ni A, Deb A, Zhang L et al. Genetic modification of mesenchymal stem cells overexpressing CCR1 increases cell viability, migration,

engraftment, and capillary density in the injured myocardium. Circ Res 2010;106:1753-62.

[107] Liu XH, Bai CG, Xu ZY, Huang SD, Yuan Y, Gong DJ et al. Therapeutic potential of angiogenin modified mesenchymal stem cells: Angiogenin improves mesenchymal stem cells survival under hypoxia and enhances vasculogenesis in myocardial infarction. Microvasc Res 2008.

[108] Zeng B, Chen H, Zhu C, Ren X, Lin G, Cao F. Effects of combined mesenchymal stem cells and heme oxygenase-1 therapy on cardiac performance. Eur J Cardiothorac Surg 2008.

[109] Gnecchi M, He H, Melo LG, Noiseaux N, Morello F, de Boer RA et al. Early beneficial effects of bone marrow-derived mesenchymal stem cells overexpressing Akt on cardiac metabolism after myocardial infarction. Stem Cells 2009;27:971-9.

[110] Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F et al. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. Faseb J 2006;20:661-9.

[111] Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 2005;11:367-8.

[112] Song H, Cha MJ, Song BW, Kim IK, Chang W, Lim S et al. Reactive oxygen species inhibit adhesion of mesenchymal stem cells implanted into ischemic myocardium via interference of focal adhesion complex. Stem Cells 2010;28:555-63.

[113] Jin J, Jeong SI, Shin YM, Lim KS, Shin H, Lee YM et al. Transplantation of mesenchymal stem cells within a poly(lactide-co-epsilon-caprolactone) scaffold improves cardiac function in a rat myocardial infarction model. Eur J Heart Fail 2009;11:147-53.

[114] Tan MY, Zhi W, Wei RQ, Huang YC, Zhou KP, Tan B et al. Repair of infarcted myocardium using mesenchymal stem cell seeded small intestinal submucosa in rabbits. Biomaterials 2009;30:3234-40.

[115] Chen CH, Wei HJ, Lin WW, Chiu I, Hwang SM, Wang CC et al. Porous tissue grafts sandwiched with multilayered mesenchymal stromal cell sheets induce tissue regeneration for cardiac repair. Cardiovasc Res 2008.

[116] Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H et al. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med 2006;12:459-65.

[117] Bel A, Planat-Bernard V, Saito A, Bonnevie L, Bellamy V, Sabbah L et al. Composite cell sheets: a further step toward safe and effective myocardial regeneration by cardiac progenitors derived from embryonic stem cells. Circulation 2010;122:S118-23.

[118] Chen S, Liu Z, Tian N, Zhang J, Yei F, Duan B et al. Intracoronary transplantation of autologous bone marrow mesenchymal stem cells for ischemic cardiomyopathy due to isolated chronic occluded left anterior descending artery. J Invasive Cardiol 2006;18:552-6.

[119] Katritsis DG, Sotiropoulou PA, Karvouni E, Karabinos I, Korovesis S, Perez SA et al. Transcoronary transplantation of autologous mesenchymal stem cells and endothelial progenitors into infarcted human myocardium. Catheter Cardiovasc Interv 2005;65:321-9.

[120] Chen SL, Fang WW, Ye F, Liu YH, Qian J, Shan SJ et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol 2004;94:92-5.

[121] Hare JM. Translational development of mesenchymal stem cell therapy for cardiovascular diseases. Tex Heart Inst J 2009;36:145-7.

[122] Hare JM, Traverse JH, Henry TD, Dib N, Strumpf RK, Schulman SP et al. A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J Am Coll Cardiol 2009;54:2277-86.

[123] Dib N, Henry T, DeMaria A, Itescu S, McCarthy MM, Jaggar SC. The first US study to assess the feasibility and safety of endocardial

delivery of allogenic mesenchymal precursor cells in patient with heart failure: Three-month interim analysis. Circ 2009;120:S810.

[124] Duckers HJ, Houtgraaf J, van Geuns RJ, van Dalen BD, Regar E, van der Giessen W. First-in-man experience with intracoronary infusion of adipose-derived regenerative cells in the treatment of patients with ST-elevation myocardial infarction: The Apollo trial. Circ 2010;120:A12225.

[125] Schaefer A, Zwadlo C, Fuchs M, Meyer GP, Lippolt P, Wollert KC et al. Long-term effects of intracoronary bone marrow cell transfer on diastolic function in patients after acute myocardial infarction: 5-year results from the randomized-controlled BOOST trial--an echocardiographic study. Eur J Echocardiogr 2010;11:165-71.

[126] Miettinen JA, Ylitalo K, Hedberg P, Kervinen K, Niemela M, Saily M et al. Determinants of Functional Recovery after Myocardial Infarction of Patients Treated with Bone Marrow Derived Stem Cells after Thrombolytic Therapy. Heart 2009.

[127] Stamm C, Kleine HD, Choi YH, Dunkelmann S, Lauffs JA, Lorenzen B et al. Intramyocardial delivery of CD133+ bone marrow cells and coronary artery bypass grafting for chronic ischemic heart disease: safety and efficacy studies. J Thorac Cardiovasc Surg 2007;133:717-25.

[128] Martin-Rendon E, Brunskill SJ, Hyde CJ, Stanworth SJ, Mathur A, Watt SM. Autologous bone marrow stem cells to treat acute myocardial infarction: a systematic review. Eur Heart J 2008;29:1807-18.

[129] Li TS, Kubo M, Ueda K, Murakami M, Mikamo A, Hamano K. Impaired angiogenic potency of bone marrow cells from patients with advanced age, anemia, and renal failure. J Thorac Cardiovasc Surg 2010;139:459-65.

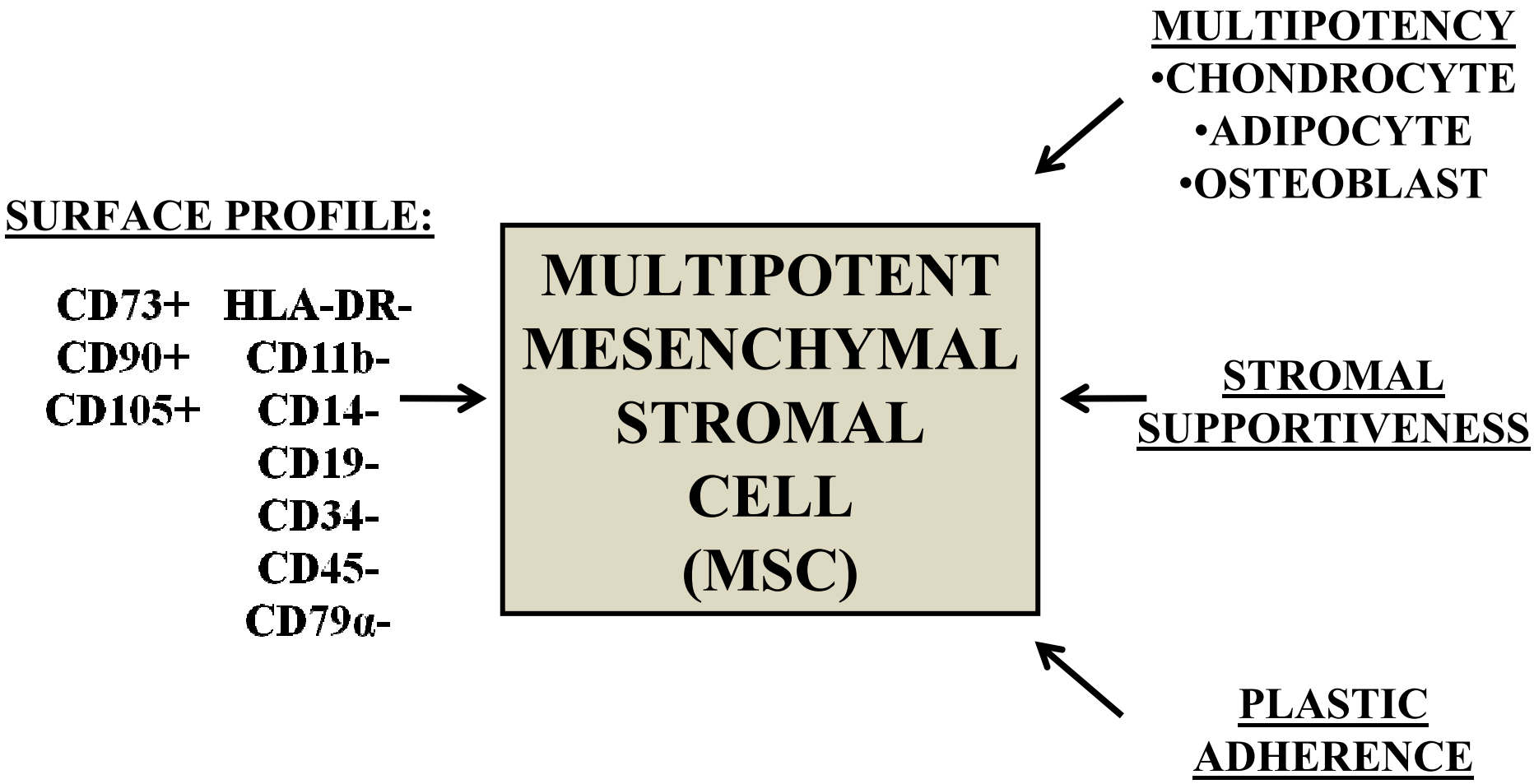
[130] Kissel CK, Lehmann R, Assmus B, Aicher A, Honold J, Fischer-Rasokat U et al. Selective functional exhaustion of hematopoietic progenitor cells in the bone marrow of patients with postinfarction heart failure. J Am Coll Cardiol 2007;49:2341-9.

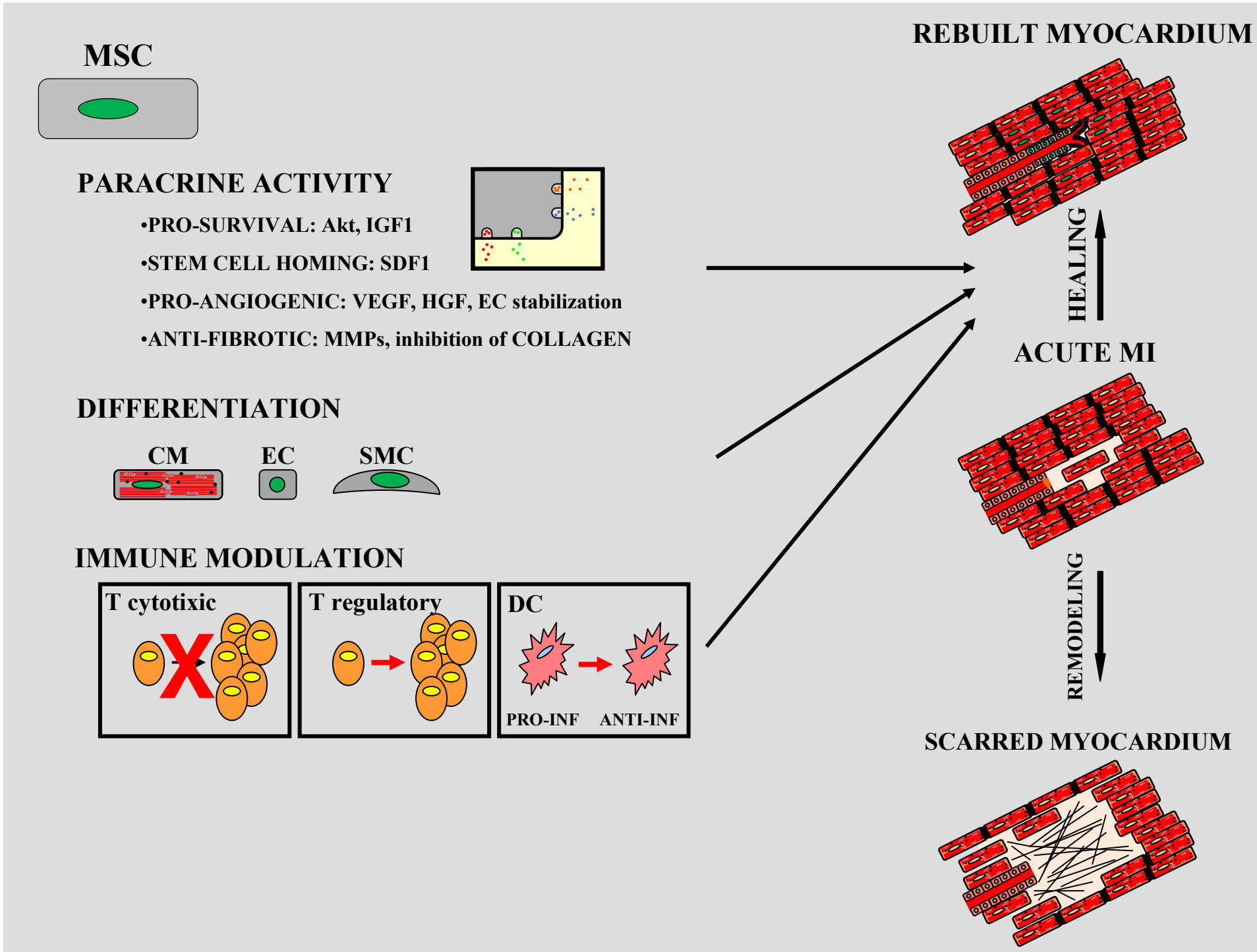
[131] Sorrentino SA, Bahlmann FH, Besler C, Muller M, Schulz S, Kirchhoff N et al. Oxidant stress impairs in vivo reendothelialization capacity of endothelial progenitor cells from patients with type 2 diabetes mellitus: restoration by the peroxisome

proliferator-activated receptor-gamma agonist rosiglitazone. Circulation 2007;116:163-73.

[132] Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. Blood 2007;110:3499-506.

[133] Chachques JC, Trainini JC, Lago N, Masoli OH, Barisani JL, Cortes-Morichetti M et al. Myocardial assistance by grafting a new bioartificial upgraded myocardium (MAGNUM clinical trial): one year follow-up. Cell Transplant 2007;16:927-34.


MULTIPOTENCY
•CHONDROCYTE
•ADIPOCYTE
•OSTEOBLAST
SURFACE PROFILE:
CD73+ HLA-DR-
CD90+ CD11b-
CD105+ CD14-
CD19-
CD34-
CD45-
CD79α-
MULTIPOTENT MESENCHYMAL STROMAL CELL (MSC)
STROMAL SUPPORTIVENESS
PLASTIC ADHERENCE


MSC
PARACRINE ACTIVITY
•PRO-SURVIVAL: Akt, IGF1
•STEM CELL HOMING: SDF1
•PRO-ANGIOGENIC: VEGF, HGF, EC stabilization
•ANTI-FIBROTIC: MMPs, inhibition of COLLAGEN
DIFFERENTIATION
CM
EC
SMC
IMMUNE MODULATION
T cytotixic
T regulatory
DC
PRO-INF
ANTI-INF
REBUILT MYOCARDIUM
HEALING
ACUTE MI
REMODELING
SCARRED MYOCARDIUM

ANEXO VI

Mesenchymal Stem Cells for cardiac repair. Preclinical models of disease.

Manuel Mazo, Miriam Araña, Beatriz Pelacho, Felipe Prosper

Hematology and Cell Therapy, Clínica Universidad de Navarra, University of Navarra and Foundation for Applied Medical Research, Pamplona, Spain

Address for Correspondence:

Felipe Prósper MD, PhD

Hematology and Cell Therapy

Clínica Universitaria

Av. Pío XII 36, Pamplona 31008,

Navarra, Spain

Phone 34 948 255400 Fax 34 948 296500

e-mail: fprosper@unav.es

ABSTRACT

In recent years, the incredible boost in stem cell research has kindled the expectations of both patients and physicians. Mesenchymal progenitors, owing to their availability, ease of manipulation and therapeutic potential, have become one of the most attractive options for the treatment of a wide range of diseases, from cartilage defects to cardiac disorders. Moreover, their immunomodulatory capacity has opened up their allogenic use, consequently broadening the possibilities for their application. In this review, we will focus on their use in the therapy of animal preclinical models of myocardial infarction, with special focus on their characteristics and their *in vitro* and *in vivo* mechanisms of action.

INTRODUCTION

In spite of the increasing knowledge of its causes, development and treatment, myocardial infarction (MI) remains the greatest health concern worldwide [1]. Following the ischemic event, the damage inflicted upon cardiac cells rapidly becomes irreversible, leading to massive death and loss not only of muscular but also vascular cells [2]. Although clinical practice has raised the expectancy of surviving an MI and is able to mitigate its progression [3], the mammalian (and therefore the human) heart lacks a significant capacity to self-heal, leaving a chronically scarred organ with a limited and waning functionality, which will finally lead to heart failure and the dichotomy of transplantation versus death.

During the last 15 years, the boost in stem cell research has ignited the expectations of patients and scientists alike. Although the initial hypothesis was that these plasticity-endowed cells could mend what disease had broken through direct replacement of lost cell populations [4], an increasing body of evidence points towards their capacity to secrete therapeutic molecules as a potent regenerative tool [5]. Among the different cell types assayed, mesenchymal stem cells (MSC) are one of the most interesting and intensively investigated. In the following pages we will revise what characteristics make mesechymal progenitors so appealing for cardiac regeneration as well as discuss the available data from studies in which MSC have been employed to treat animal models of MI.

ORIGIN, CHARACTERISTICS AND ISOLATION OF MSC

In 1974, Friedenstein and colleagues published one of the first reports on MSC [6,7], demonstrating the clonogenic potential of multipotent cells in the marrow, which they termed colony forming unit-fibroblasts. Since then, our knowledge of these cells has greatly increased, and MSCs are now viewed as a mesodermal-derived population, ubiquitously found in a variety of tissues, most abundantly bone marrow (BM) and adipose tissue (AT), but also in dental pulp, menstrual and cord blood, among other sites [8-12]. Nevertheless, their physiological function is still not well established, mainly due to the lack of an appropriate marker for their *in vivo* identification and the difficulty of performing adequate lineage-tracing experiments. Although the possibility

of their residence within the proximity of vessels has been hinted [13], this has not been extensively proved. It is widely assumed, however, that MSCs give support and nurture to other cells, with several studies identifying their role in endogenous tissue repair processes (reviewed in [14]). This plethora of origins and capacities has impelled standardization, as the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed in 2006, concluding that the minimum requirements to define MSC [15] are: first, cells must be plastic-adherent under currently established culture conditions; second, MSC should express CD73, CD90 and CD105, and lack expression of HLA-DR, CD11b, CD14, CD19, CD34, CD45 or CD79-alpha; finally, they must be able to differentiate to osteoblasts, chondrocytes and adipocytes *in vitro*. Still, caution must be taken as some reports fail to meet these criteria, and MSC is often employed for "Marrow Stromal Cell", "Mesenchymal Stromal Cell" or "Marrow Stem Cell". Accordingly, a clarification was published in which MSC was defined as "Multipotent Mesenchymal Stromal Cells" [16], adding the supportive property to the required characteristics [17]. Nevertheless, tissue of origin gives isolated progenitors a distinct therapeutic potential, making comparisons between different populations difficult. For example, the relative abundance of mesenchymal progenitors in BM versus AT (0.01%-0.0001% of cells in the marrow [18] and 100 to 500 times that number in adipose depots [8]) may importantly impact cell growth kinetics and other characteristics, as has been proved at a functional, genomic and proteomic level [19,20], suggesting a higher degree of commitment of BM-MSC to chondrogenic and osteogenic lineages than adipose-derived MSC (termed adipose-derived stem cells, ADSC) [21]. However, whether these differences are due to true divergences in the nature of MSC and ADSC or to the *in vitro* processing also remains to be resolved.

The isolation of mesenchymal progenitors is nowadays a straightforward technique which commonly relies on obtaining an intermediate mixture of progenitors. When the starting sample is BM or blood, a Ficoll gradient centrifugation or similar process is employed to remove most contaminating erythrocytes [11,12,22], rendering the mononuclear fraction. AT processing requires careful mincing plus an enzymatic (usually collagenase) digestion of the specimen to segregate adipocytes from the stromal vascular fraction [23]. After *in vitro* culture under similar conditions, the mononuclear and the stromal vascular faction give rise to BM-MSC and ADSC

respectively. Thus, unless allogenically employed, the use of mesenchymal progenitors implies several weeks of processing prior to their application to the patient. This issue will be further discussed bellow, as it has fundamental implications for their use in the cardiovascular field.

THE TRIPLE GOAL OF CARDIAC REGENERATION AND MSCs

The reconstruction of the diseased left ventricle (LV) is a final goal with three main aims: (1) the regeneration of the cardiac muscle, (2) the formation of a mature, functional vascular network to irrigate it and (3) the return of the damaged tissue to its former geometry. Failure in accomplishing any of the three would impair the outcome of the therapy. Stem cells, and mesenchymal progenitors among them, can contribute to these mainly through two mechanisms: certain differentiation to the desired phenotypes and, of more significance, releasing of molecules with therapeutic potential. Further increasing their capacities, mesenchymal cells have also the ability to regulate the immune response, which further widens their possible applications.

Transdifferentiation of MSC to the desired phenotypes has been documented. BM-MSC have shown their capacity to give rise either to endothelial cells (EC) [24,25] or smooth muscle cells (SMC) [24], but the obtaining of CM has been contested due to the immaturity of the resulting cells [26,27] or the use of demethylating agents [28]. Similarly, although ADSC are able to differentiate into vascular cells [29,30], the obtaining of CM has been accomplished under treatment with DMSO [31]. Other types of MSC have also been differentiated to CM or CM-like cells, such as menstrual blood-derived MSC [11] or umbilical cord-MSC [12]. In recent years AT has been proposed as a much richer source of stem cells with cardiac and endothelial potential than the BM, yet a cautionary note must be risen as these studies either rely on freshly isolated cell subpopulations or they culture cells under differentiating promoting conditions without true prior mesenchymal enrichment [32-34], so direct comparison between those and BM-MSC is not possible. To date, though angiogenic potential of MSC is better established and is probably related to the pericytic nature of these cells [35,36], their differentiation into cardiac myocytes is still heavily challenged.

However, the suitable exploitation of the differentiation capacity of MSC in MI still presents certain caveats. First, patients receiving stem cell therapy are severely diseased and usually elderly, two factors that have an outstanding impact on stem cell function. For instance, a decrease in the numbers and functionality of circulating endothelial progenitors is directly related to cardiovascular risk and smoking [37,38] and age has also been shown to impair the angiogenic capacity of both BM-MSC and ADSC [39,40]. Second, cell engraftment is poor, with few cells capable of long-term persistence (reviewed in [41]) in the scarred myocardium. Third, the loss of a significant mass of contractile cells and their associated vessels imposes a burden that is almost impossible to overcome. The myocardium hosts on average 20 million cardiomyocytes (CM) per gram of tissue which, considering a human left ventricle (LV) of about 400 g, means that in cases when an MI damages 25% of it, about 1 billion contractile cells are lost, as well as other components of the cardiac architecture (fibroblasts, endothelial and smooth muscle cells, etc.) [42]. Thus all of these factors, coupled to the low rates of cell differentiation achieved even under *in vitro* controlled conditions, make the rebuilding of the LV through the addition of such a small number of cells a rather naïve strategy.

On the other hand, MSC have been shown to be first-class paracrine secretors, being able to induce a significant effect upon the damaged heart, even when low numbers of cells manage to engraft [43]. Angiogenic molecules like vascular endothelial growth factor (VEGF) or hepatic growth factor (HGF) have been shown to be secreted by either BM-MSC or ADSC [44-48]. Additionally, ADSC have been reported to form and stabilize functional vascular networks when mixed with endothelial progenitors [49] and Chen *et al* showed that BM-MSC were a more potent source of pro-angiogenic cytokines than dermal fibroblasts, exerting a stronger effect upon the recruitment of EC and macrophages and improving wound healing [46]. The role of mesenchymal progenitors such as fibrosis-regulators is similarly extensively documented. Conditioned medium from BM-MSC decreases cardiac fibroblast proliferation and modifies the secretory equilibrium towards an anti-fibrotic milieu, with diminished expression of collagen types I and III [50,51] and increased secretion of matrix metalloproteinases (MMP) 2, 9 and 14 [52]. Likewise, ADSC produce transforming growth factor (TGF) β1, one of the most potent regulators of fibroblast behavior and fibrosis [53]. Beside perfusion promotion and collagen architecture regulation, MSC are able to migrate to the sites of injury due to their expression of the chemokine receptor CXCR4 [54] and

traffic through type I collagen membranes as demonstrated by the expression of five types of MMPs (2, 3 and membrane type-MMPs 1, 2 and 3) [55]. These two features would theoretically allow MSC to home to and migrate through the scarred myocardium. Taken as a whole, all of these exemplify the plethora of paracrine-mediated actions that mesenchymal progenitors can set into motion.

As already noted, immune regulation is one of the most interesting competences of MSC (reviewed in [56]). Marrow-derived MSC inhibit the proliferation of activated T cells and the formation of cytotoxic T cells [57], inducing an anti-inflammatory phenotype. This unique capacity significantly broadens the scope of MSC use, as it opens the way to allogenic applications or even to their use as "inducers of tolerance" to other cell types [58]. However, some concerns have been reported. Huang *et al* reported that differentiation reduced the capacity of immunological escape of BM-MSC [59], related to an increase in immunostimulatory molecules MHC-Ia and II and a decrease in the immunosuppressive MHC-Ib. Along similar lines, McIntosh and coworkers reported that ADCS beyond passage 1 (and thus devoid of contaminating differentiated cells [60]) failed to elicit a response from allogenic T cells [61], but this attribute may be diminished under inflammatory stimuli, as shown *in vitro* [62]. As a consequence, the true ability of MSC to be tolerated by the host´s immune system is still debated, and has proved not so significant as initially thought [63].

Finally, since the groundbreaking report in 2006 by Yamanaka and coworkers [64] where induction of pluripotency in adult (stem) cells by overexpression of the pluripotency transcription factors Oct3/4, Sox2, Klf4 and c-Myc was shown, mesenchymal cells have been investigated intensively [65,66]. Advantages like the ease of harvest and culture together with a higher potency than other cell types (e.g.: dermal fibroblasts), has made them good candidates for iPS derivation

MSC IN PRECLINICAL MODELS OF MI

The assessment of stem cells in animal models of MI is a compulsory test before any further step is taken. Results from clinical trials that have already finished have demonstrated the critical impact that not only the treatment, but most importantly, the type of disease where cells are applied on, has on the outcome of the study (reviewed in

[67]). Although cardiovascular diseases have been widely modeled, stem cell therapy has been mostly centered on permanent/transient models of myocardial ischemia, with few reports dealing with other settings [68]. However, a distinction between three different models can be made: acute, sub-acute and chronic models of MI.

In the acute form, cells are transplanted within hours of the induction of ischemia. Here, cell therapy must cope with a pro-inflammatory microenvironment and the necrotic/apoptotic molecules released from dying cells [69,70]. Nevertheless, both the presence of homing signals and an anti-fibrotic milieu can be a positive counterbalance [71,72]. The great majority of published reports has relied on this model [12,73-88] due to the fact that transplanting cells at the time of coronary occlusion (or minutes/hours after it) subjects animals to only one operation and consequently decreases the mortality associated with the process. With the exception of studies by van der Bogt and colleagues [78,81], all specialists have consistently demonstrated that therapy with MSC induces a significant benefit upon cardiac function, which has mainly been attributed to the paracrine activity of the cells that induce an increase in tissue perfusion and a decrease in the size of the scar and collagen content.

In spite of these results, implementation of a therapy based on transplanting mesenchymal progenitors (a population that needs several weeks of *in vitro* purification) on the acute phase of the disease is not possible unless allogenically employed. Strikingly, far fewer studies have dealt with the opposite situation, the chronic phase [22,23,89,90], where the repair processes take place after the ischemia has been completed: inflammation has receded, the scar has matured and although a new vascular network has been developed, it is disorganized and insufficient [91,92]. Notwithstanding the great burdens that these conditions impose upon transplanted cells, the abovementioned studies have demonstrated that, as shown for the acute phase, MSCs exert a positive action on cardiac contractility and histology, again mostly due to the secretion of therapeutic molecules and the action on resident cells.

Finally, a third, intermediate setting can also be found: the sub-acute. In this, angiogenic processes are still on course, either through endothelial progenitors [93] or macrophages [94], and the receding of inflammation is coupled to an increase in fibrotic processes. Again, reports in models of sub-acute MI are scarce [10,11,95,96], but the benefit and mechanisms, as in acute and chronic models, appear to be consistent.

Thus, a fair amount of information is available, supporting the capacity of mesenchymal progenitors to exert a benefit on cardiac function. The mechanistic basis of this is not yet fully explained and it seems to be far more complex than what was expected. However, it is widely accepted that MSC exert their influence upon cardiac contraction not by directly resupplying the tissue with new CM, but through more subtle actions on organ architecture and histology. Some of the abovementioned studies provide crucial hints. Lee and coworkers showed that transplantation of BM-MSC in a rabbit model of chronic MI induced an increase in the concentration of stromal derived factor (SDF) 1, which elicited the chemotaxis of host-derived BM progenitors (CD34+, CD117+, STRO1+) and was related to a functional benefit, a decrease in infarct size and improvement in tissue vascularization [90], as similarly shown by Suzuki *et al* in a swine model of hibernating myocardium [97]. Li and collaborators demonstrated that the enhanced contractility was accompanied by an increase in the pro-survival gene Akt [98], whereas Mias *et al* showed that this functional improvement and remodeling reversal *in vivo* was coupled to a myriad of *in vitro* anti-fibrotic actions [52]. The Spinale group monitored the evolution of MMPs and their inhibitors after infusion of BM-MSC in a model of sheep MI, establishing a relationship between the number of transplanted cells and enzymatic levels [79]. Resembling what was found *in vitro*, several reports have associated the pro-angiogenic activity of the cells with the secretion (either directly or host-derived) of potent angiogenic cytokines such as VEGF, HGF or insulin-like growth factor (IGF) 1 among others [10,88,95,99,100].

THE PROBLEMS AND THEIR SOLUTIONS

Despite the initial optimism, the fire that fueled the field in its beginnings has cooled down. Although positive, results from clinical trials have not been as good as it had been anticipated (reviewed in [67]) and the desired goal of replacing the scar with a functional myocardium has not yet been achieved. Cell therapy presents certain caveats that need to be improved before the goal of regeneration is achieved. There are two main difficulties: lack of efficient differentiation and low survival/engraftment capacity. As one of the spearheads of cell therapy, MSC are currently undergoing intense investigation to solve both of these issues (Figure 1).

The question of improving cell survival and permanence at the site of injury has been dealt with by two chief approaches: cell modification and tissue engineering. Albeit with certain constraints, genetic manipulation of MSC has undergone profound investigation. Among the many possibilities, the CXCR4/SDF1 axis has been shown to have a capital role in cell trafficking and homing. As a consequence, many reports have exploited it, trying to improve the therapy. Ma *et al* investigated the peak of cardiac SDF1 expression after MI in rat, showing that injecting cells at that time point (1 day post-infarction) significantly enhanced cell engraftment and angiogenesis [101]. Cheng and collaborators injected BM-MSC engineered to overexpress the receptor CXCR4, strengthening cell-homing to the injured tissue [54]. The same group combined BM-MSC peripheral injection with administration of granulocyte colony-stimulating factor (G-CSF). Although G-CSF was able *in vitro* to increase CXCR4 expression and *in vivo* to augment cell engraftment, no effect on cardiac function was found [102]. Recently, the group led by Dzau showed that overexpression of the chemokine receptor CCR1, but not CCR2, was associated with improved survival and grafting in a mouse model of MI, as well as functional restoration [103].

Similarly, the issue of cell survival has been dealt with through genetic approaches. Liu *et al* engineered BM-MSC to overexpress angiogenin, which conferred an improved resistance to hypoxia and was translated into an increase in cell engraftment and functional/histological recovery [104]. Adenoviral-transfection of hemeoxygenase-1 resulted in a superior therapeutic effect, mainly through protection from inflammation and apoptosis [105]. Akt-overproduction in MSC was able to restore cardiac function 2 weeks post-MI via paracrine actions which included protection from hypoxia-induced apoptosis, release of cytokines and preservation of tissue metabolism [106-108]. The use of antioxidants, aimed at diminishing injury by reactive oxygen species (ROS), has proved useful, as demonstrated by Song and collaborators who showed that ROS inhibited BM-MSC adherence to the substrate, whereas pretreatment with the ROS scavenger N-acetyl-L-cysteine improved engraftment and precluded the increase of fibrosis and infarct size [89]. Hsp20-mediated protection from ROS also enhanced the therapeutic activity of MSC due to increased expression of Akt and secretion of growth factors [100].

However, genetic modification of stem cells, though possible, implies risks that make it difficult for a treatment to reach the bedside. Bioengineering, on the other hand, makes

use of biocompatible materials to improve or direct cell therapy through providing anchorage to transplanted cells as well as mechanically helping the diseased ventricle. Naturally-derived materials have been employed intensively. Porcine small intestine submucose, a decellularized substrate, seeded with BM-MSC, has been used to treat chronic MI in rabbit, resulting in a significant benefit for cardiac function and histology, as well as improved cell retention and migration towards the injured tissue [109]. Jin and coworkers applied patches of poly(lactide-co-ε-coprolactone) overlaid with BM-MSC on a rat cryoinjury model, where treatment improved functionality and decreased infarct size [110]. The cell sheet technology allows increasing the thickness of the constructs through stacking. Thus, Chen and coworkers transplanted 5-fold layers of marrow-derived MSC on a rat model of cardiac ischemia, showing that the treatment improved cardiac function along with paracrine secretion of therapeutic molecules by grafted cells [111]. Mori´s group compared the transplantation of a cell sheet composed of ADSC versus fibroblasts, demonstrating the superior effect of the mesenchymal progenitors [112]. Recently, autologous ADSC were co-transplanted with allogenic human embryonic stem cell-derived CD15+ cardiac progenitors in a monkey model of MI, providing proof of the safety of the procedure, although functionality was not analyzed due to the small number of treated animals [58].

Stem cell differentiation, though shown not to be necessary for a therapy to induce significant benefits [22,23], is one of the most desired goals in the field. If transplanted cells are not able to replace ischemia-damaged cells, the treatment may limit infarct expansion, but the damaged tissue will not be substituted, leaving the organ chronically scarred. As a consequence, some reports have aimed at increasing MSC differentiation *in vitro* prior to its transplantation. Hahn and coworkers incubated cells in a mixture of fibroblast growth factor 2, IGF1 and bone morphogenetic protein 2 (all of them well known promoters of cardiac differentiation) [113]. In culture, pre-treated cells, though not differentiated, were able to protect neighboring CM from hypoxia via enhanced gap junctional communication, which was *in vivo* translated to a significant benefit in cardiac function even in comparison with untreated cells. Cho *et al.* lentivirally transduced BM-MSC to overexpress GSK3β under the rationale of increasing cell differentiation to CM. When transplanted in a mouse model of MI, the treatment showed an enhanced efficacy as well as an improved cell differentiation capacity [99]. Tissue architecture and mechanical properties have also of late been revealed as

principal mediators of cell differentiation [114,115]. Although few, some published reports are starting to investigate the effect of substrate geometry and rigidity on MSC differentiation, with striking results [115,116].

Finally, the immune modulation capacity of mesenchymal progenitors (reviewed in [56]), which in theory provides the means for the allogenic use of MSCs and as an off-the-shelf product (expanded prior to the onset of the ischemia and applicable on demand), needs to be carefully revised. Two reports have compared the effects of allogenic versus syngenic injection of BM-MSC in rat model of MI, with conflicting results. Imanishi *et al* [82] demonstrated that both autologous and allogenic cells improved cardiac function 4 weeks after transplantation, remained in the damaged tissues and did not stimulate rejection. Huang and coworkers, on the other hand [59], followed animals for up to 6 months. Syngenic cells stimulated cardiac recovery, whereas the effect of the allogenic treatment was transitory (significant 3 months post-injection but not at 6) and BM-MSC disappeared earlier than their syngenic counterpart. However, this difference can be attributed to methodological discrepancies regarding the time of transplantation (acute versus chronic respectively) or the follow-up (1 versus 6 months). Importantly, results from clinically-relevant large animal models of MI in which allogenic cells have been employed have revealed either positive [117,118] or no functional outcome [83]. On the other hand, when autologous ADSCs or BM-MSCs were used [76,86,119,120], reports have shown a robust and consistent functional recovery after cell transplantation. Thus, although consistently demonstrated *in vitro*, *in vivo* immune modulation by MSC must be accurately assayed.

CONCLUSION

Mesenchymal cells have received substantial interest in recent years as a potential therapy for MI. Endowed with certain differentiation ability, potent paracrine activity and capacity for immune modulation, MSC have been one of the major candidates for reaching the bedside. Nevertheless, the information already gathered from pre- and clinical studies has significantly shifted this picture to one where immune modulation in the setting of MI must be carefully revised and *in vitro* transdifferentiation has not been translated into any significant contribution to vascular or cardiac lineages. In spite of these shortcomings, the paracrine action of transplanted cells is sufficiently potent to be

capable of inducing an important benefit in terms not only of cardiac contractility but also histology. The next challenge faced by both scientists and clinicians is to better understand the pleiotropic capacities of MSC before further improvements in therapy can be achieved.

SOURCE OF FUNDING

This work was supported by grants to FP (FP7 INELPY, Instituto de Salud Carlos III (ISCIII-RETIC RD06/0014 (FIS) and Ministerio de Ciencia e Innovación Programa de Internacionalización CARDIOBIO) and BP (Instituto de Salud Carlos III (PI10/01621 and CP09/00333 (FIS)).

FIGURE LEGEND

Figure 1. Different strategies for an improved cell therapy of MI. After MI, albeit significant preventing acute death and transiently increasing contractile capacity, conventional medical treatment does not restore organ architecture. MSC transplantation is able to induce a partial recovery of cardiac function and limit infarct expansion. However, low engraftment and differentiation impair the therapy. Four systems aim at improving this situation and manage total tissue regeneration: tissue engineering, genetic modification of cells, pretreatment with protective molecules and *in vitro* predifferentiation towards the desired lineages.

REFERENCES

[1] World Health Organization. World Health Statistics 2008. , 2008.

[2] Mazo M, Pelacho B, Prosper F. Stem cell therapy for chronic myocardial infarction. J Cardiovasc Transl Res 2010;3:79-88.

[3] White HD, Chew DP. Acute myocardial infarction. Lancet 2008;372:570-84.

[4] Reinecke H, Minami E, Zhu WZ, Laflamme MA. Cardiogenic differentiation and transdifferentiation of progenitor cells. Circ Res 2008;103:1058-71.

[5] Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 2008;103:1204-19.

[6] Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 1974;2:83-92.

[7] Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 1974;17:331-40.

[8] Mazo M, Gavira JJ, Pelacho B, Prosper F. Adipose-derived stem cells for myocardial infarction. J Cardiovasc Transl Res 2011;4:145-53.

[9] Bianco P, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell 2008;2:313-9.

[10] Gandia C, Arminan A, Garcia-Verdugo JM, Lledo E, Ruiz A, Minana MD et al. Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction. Stem Cells 2008;26:638-45.

[11] Hida N, Nishiyama N, Miyoshi S, Kira S, Segawa K, Uyama T et al. Novel Cardiac Precursor-Like Cells from Human Menstrual Blood-Derived Mesenchymal Cells. Stem Cells 2008.

[12] Chang SA, Lee EJ, Kang HJ, Zhang SY, Kim JH, Li L et al. Impact of Myocardial Infarct Proteins and Oscillating Pressure on the Differentiation of Mesenchymal Stem

Cells: Effect of Acute Myocardial Infarction on Stem Cell Differentiation. Stem Cells 2008;26:1901-12.

[13] Cai X, Lin Y, Friedrich CC, Neville C, Pomerantseva I, Sundback CA et al. Bone marrow derived pluripotent cells are pericytes which contribute to vascularization. Stem Cell Rev 2009;5:437-45.

[14] Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem 2006;98:1076-84.

[15] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-7.

[16] Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC et al. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy 2005;7:393-5.

[17] Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 2007;131:324-36.

[18] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-7.

[19] Wagner W, Roderburg C, Wein F, Diehlmann A, Frankhauser M, Schubert R et al. Molecular and secretory profiles of human mesenchymal stromal cells and their abilities to maintain primitive hematopoietic progenitors. Stem Cells 2007;25:2638-47.

[20] Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006;24:1294-301.

[21] Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. Circ Res 2007;100:1249-60.

[22] Mazo M. Transplantation of Mesenchymal Stem Cells exerts a greater long-term effect than Bone Marrow Mononuclear Cells in a chronic myocardial infarction model in rat. - 2009;Accepted.

[23] Mazo M, Planat-Benard V, Abizanda G, Pelacho B, Leobon B, Gavira JJ et al. Transplantation of adipose derived stromal cells is associated with functional improvement in a rat model of chronic myocardial infarction. Eur J Heart Fail 2008;10:454-62.

[24] Lozito TP, Taboas JM, Kuo CK, Tuan RS. Mesenchymal stem cell modification of endothelial matrix regulates their vascular differentiation. J Cell Biochem 2009;107:706-13.

[25] Liu JW, Dunoyer-Geindre S, Serre-Beinier V, Mai G, Lambert JF, Fish RJ et al. Characterization of endothelial-like cells derived from human mesenchymal stem cells. J Thromb Haemost 2007;5:826-34.

[26] Yan X, Lv A, Xing Y, Liu B, Hou J, Huang W et al. Inhibition of p53-p21 pathway promotes the differentiation of rat bone marrow mesenchymal stem cells into cardiomyocytes. Mol Cell Biochem 2011;354:21-8.

[27] Arminan A, Gandia C, Garcia-Verdugo JM, Lledo E, Mullor JL, Montero JA et al. Cardiac transcription factors driven lineage-specification of adult stem cells. J Cardiovasc Transl Res;3:61-5.

[28] Xu W, Zhang X, Qian H, Zhu W, Sun X, Hu J et al. Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro. Exp Biol Med (Maywood) 2004;229:623-31.

[29] Kim YM, Jeon ES, Kim MR, Jho SK, Ryu SW, Kim JH. Angiotensin II-induced differentiation of adipose tissue-derived mesenchymal stem cells to smooth muscle-like cells. Int J Biochem Cell Biol 2008;40:2482-91.

[30] Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R et al. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 2004;109:656-63.

[31] van Dijk A, Niessen HW, Zandieh Doulabi B, Visser FC, van Milligen FJ. Differentiation of human adipose-derived stem cells towards cardiomyocytes is facilitated by laminin. Cell Tissue Res 2008.

[32] Leobon B, Roncalli J, Joffre C, Mazo M, Boisson M, Barreau C et al. Adipose-derived cardiomyogenic cells: in vitro expansion and functional improvement in a mouse model of myocardial infarction. Cardiovasc Res 2009;83:757-67.

[33] Fischer LJ, McIlhenny S, Tulenko T, Golesorkhi N, Zhang P, Larson R et al. Endothelial differentiation of adipose-derived stem cells: effects of endothelial cell growth supplement and shear force. J Surg Res 2009;152:157-66.

[34] Sengenes C, Miranville A, Maumus M, de Barros S, Busse R, Bouloumie A. Chemotaxis and differentiation of human adipose tissue CD34+/CD31- progenitor cells: role of stromal derived factor-1 released by adipose tissue capillary endothelial cells. Stem Cells 2007;25:2269-76.

[35] Cai X, Lin Y, Friedrich CC, Neville C, Pomerantseva I, Sundback CA et al. Bone marrow Derived Pluripotent Cells are Pericytes which Contribute to Vascularization. Stem Cell Rev Rep 2009.

[36] Jain R, Ellika SK, Scarpace L, Schultz LR, Rock JP, Gutierrez J et al. Quantitative estimation of permeability surface-area product in astroglial brain tumors using perfusion CT and correlation with histopathologic grade. AJNR Am J Neuroradiol 2008;29:694-700.

[37] Kondo T, Hayashi M, Takeshita K, Numaguchi Y, Kobayashi K, Iino S et al. Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. Arterioscler Thromb Vasc Biol 2004;24:1442-7.

[38] Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 2001;89:E1-7.

[39] Liang H, Hou H, Yi W, Yang G, Gu C, Lau WB et al. Increased expression of pigment epithelium-derived factor in aged mesenchymal stem cells impairs their therapeutic efficacy for attenuating myocardial infarction injury. Eur Heart J 2011.

[40] Madonna R, Renna FV, Cellini C, Cotellese R, Picardi N, Francomano F et al. Age-dependent impairment of number and angiogenic potential of adipose tissue-derived progenitor cells. Eur J Clin Invest.

[41] Haider HK, Ashraf M. Strategies to promote donor cell survival: Combining preconditioning approach with stem cell transplantation. J Mol Cell Cardiol 2008.

[42] Robey TE, Saiget MK, Reinecke H, Murry CE. Systems approaches to preventing transplanted cell death in cardiac repair. J Mol Cell Cardiol 2008.

[43] Fedak PW. Paracrine effects of cell transplantation: modifying ventricular remodeling in the failing heart. Semin Thorac Cardiovasc Surg 2008;20:87-93.

[44] Oskowitz A, McFerrin H, Gutschow M, Carter ML, Pochampally R. Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic. Stem Cell Res 2011;6:215-25.

[45] Traktuev DO, Merfeld-Clauss S, Li J, Kolonin M, Arap W, Pasqualini R et al. A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. Circ Res 2008;102:77-85.

[46] Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS ONE 2008;3:e1886.

[47] Kilroy GE, Foster SJ, Wu X, Ruiz J, Sherwood S, Heifetz A et al. Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors. J Cell Physiol 2007;212:702-9.

[48] Song YH, Gehmert S, Sadat S, Pinkernell K, Bai X, Matthias N et al. VEGF is critical for spontaneous differentiation of stem cells into cardiomyocytes. Biochem Biophys Res Commun 2007;354:999-1003.

[49] Traktuev DO, Prater DN, Merfeld-Clauss S, Sanjeevaiah AR, Saadatzadeh MR, Murphy M et al. Robust functional vascular network formation in vivo by cooperation of adipose progenitor and endothelial cells. Circ Res 2009;104:1410-20.

[50] Li L, Zhang S, Zhang Y, Yu B, Xu Y, Guan Z. Paracrine action mediate the antifibrotic effect of transplanted mesenchymal stem cells in a rat model of global heart failure. Mol Biol Rep 2009;36:725-31.

[51] Ohnishi S, Sumiyoshi H, Kitamura S, Nagaya N. Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions. FEBS Lett 2007;581:3961-6.

[52] Mias C, Lairez O, Trouche E, Roncalli J, Calise D, Seguelas MH et al. Mesenchymal Stem Cells Promote Matrix Metalloproteinase Secretion By Cardiac Fibroblasts And Reduce Cardiac Ventricular Fibrosis After Myocardial Infarction. Stem Cells 2009.

[53] Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation 2004;109:1292-8.

[54] Cheng Z, Ou L, Zhou X, Li F, Jia X, Zhang Y et al. Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance. Mol Ther 2008;16:571-9.

[55] Rogers TB, Pati S, Gaa S, Riley D, Khakoo AY, Patel S et al. Mesenchymal stem cells stimulate protective genetic reprogramming of injured cardiac ventricular myocytes. J Mol Cell Cardiol.

[56] Le Blanc K. Mesenchymal stromal cells: Tissue repair and immune modulation. Cytotherapy 2006;8:559-61.

[57] Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815-22.

[58] Bel A, Planat-Bernard V, Saito A, Bonnevie L, Bellamy V, Sabbah L et al. Composite cell sheets: a further step toward safe and effective myocardial regeneration by cardiac progenitors derived from embryonic stem cells. Circulation 2010;122:S118-23.

[59] Huang XP, Sun Z, Miyagi Y, McDonald Kinkaid H, Zhang L, Weisel RD et al. Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. Circulation 2010;122:2419-29.

[60] Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A et al. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells 2006;24:376-85.

[61] McIntosh K, Zvonic S, Garrett S, Mitchell JB, Floyd ZE, Hammill L et al. The immunogenicity of human adipose-derived cells: temporal changes in vitro. Stem Cells 2006;24:1246-53.

[62] Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Pescatori M, Stubbs AP et al. Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. Clin Exp Immunol.

[63] Puymirat E, Geha R, Tomescot A, Bellamy V, Larghero J, Trinquart L et al. Can mesenchymal stem cells induce tolerance to cotransplanted human embryonic stem cells? Mol Ther 2009;17:176-82.

[64] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663-76.

[65] Tat PA, Sumer H, Jones KL, Upton K, Verma PJ. The efficient generation of induced pluripotent stem (iPS) cells from adult mouse adipose tissue-derived and neural stem cells. Cell Transplant 2010;19:525-36.

[66] Sun N, Panetta NJ, Gupta DM, Wilson KD, Lee A, Jia F et al. Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. Proc Natl Acad Sci U S A 2009;106:15720-5.

[67] Menasche P. Cardiac cell therapy: lessons from clinical trials. J Mol Cell Cardiol 2011;50:258-65.

[68] de Macedo Braga LM, Lacchini S, Schaan BD, Rodrigues B, Rosa K, De Angelis K et al. In situ delivery of bone marrow cells and mesenchymal stem cells improves cardiovascular function in hypertensive rats submitted to myocardial infarction. J Biomed Sci 2008;15:365-74.

[69] Nian M, Lee P, Khaper N, Liu P. Inflammatory cytokines and postmyocardial infarction remodeling. Circ Res 2004;94:1543-53.

[70] Mann DL. Mechanisms and models in heart failure: A combinatorial approach. Circulation 1999;100:999-1008.

[71] Penn MS. Importance of the SDF-1:CXCR4 axis in myocardial repair. Circ Res 2009;104:1133-5.

[72] Cleutjens JP, Kandala JC, Guarda E, Guntaka RV, Weber KT. Regulation of collagen degradation in the rat myocardium after infarction. J Mol Cell Cardiol 1995;27:1281-92.

[73] Ii M, Horii M, Yokoyama A, Shoji T, Mifune Y, Kawamoto A et al. Synergistic effect of adipose-derived stem cell therapy and bone marrow progenitor recruitment in ischemic heart. Lab Invest 2011;91:539-52.

[74] Gaebel R, Furlani D, Sorg H, Polchow B, Frank J, Bieback K et al. Cell origin of human mesenchymal stem cells determines a different healing performance in cardiac regeneration. PLoS One 2011;6:e15652.

[75] Bai X, Yan Y, Coleman M, Wu G, Rabinovich B, Seidensticker M et al. Tracking long-term survival of intramyocardially delivered human adipose tissue-derived stem cells using bioluminescence imaging. Mol Imaging Biol 2011;13:633-45.

[76] Dubois C, Liu X, Claus P, Marsboom G, Pokreisz P, Vandenwijngaert S et al. Differential effects of progenitor cell populations on left ventricular remodeling and myocardial neovascularization after myocardial infarction. J Am Coll Cardiol 2010;55:2232-43.

[77] Yang YJ, Qian HY, Huang J, Li JJ, Gao RL, Dou KF et al. Combined Therapy With Simvastatin and Bone Marrow-Derived Mesenchymal Stem Cells Increases Benefits in Infarcted Swine Hearts. Arterioscler Thromb Vasc Biol 2009.

[78] van der Bogt KE, Schrepfer S, Yu J, Sheikh AY, Hoyt G, Govaert JA et al. Comparison of transplantation of adipose tissue- and bone marrow-derived mesenchymal stem cells in the infarcted heart. Transplantation 2009;87:642-52.

[79] Dixon JA, Gorman RC, Stroud RE, Bouges S, Hirotsugu H, Gorman JH,3rd et al. Mesenchymal cell transplantation and myocardial remodeling after myocardial infarction. Circulation 2009;120:S220-9.

[80] Bai X, Yan Y, Song YH, Seidensticker M, Rabinovich B, Metzele R et al. Both cultured and freshly isolated adipose tissue-derived stem cells enhance cardiac function after acute myocardial infarction. Eur Heart J 2009.

[81] van der Bogt KE, Sheikh AY, Schrepfer S, Hoyt G, Cao F, Ransohoff KJ et al. Comparison of different adult stem cell types for treatment of myocardial ischemia. Circulation 2008;118:S121-9.

[82] Imanishi Y, Saito A, Komoda H, Kitagawa-Sakakida S, Miyagawa S, Kondoh H et al. Allogenic mesenchymal stem cell transplantation has a therapeutic effect in acute myocardial infarction in rats. J Mol Cell Cardiol 2008;44:662-71.

[83] Hashemi SM, Ghods S, Kolodgie FD, Parcham-Azad K, Keane M, Hamamdzic D et al. A placebo controlled, dose-ranging, safety study of allogenic mesenchymal stem cells injected by endomyocardial delivery after an acute myocardial infarction. Eur Heart J 2008;29:251-9.

[84] Hale SL, Dai W, Dow JS, Kloner RA. Mesenchymal stem cell administration at coronary artery reperfusion in the rat by two delivery routes: A quantitative assessment. Life Sci 2008.

[85] Carr CA, Stuckey DJ, Tatton L, Tyler DJ, Hale SJ, Sweeney D et al. Bone marrow-derived stromal cells home to and remain in the infarcted rat heart but fail to improve function: an in vivo cine-MRI study. Am J Physiol Heart Circ Physiol 2008;295:H533-42.

[86] Cai L, Johnstone BH, Cook TG, Tan J, Fishbein MC, Chen PS et al. IFATS Series: Human Adipose Tissue-Derived Stem Cells Induce Angiogenesis and Nerve Sprouting Following Myocardial Infarction, in Conjunction with Potent Preservation of Cardiac Function. Stem Cells 2008.

[87] Valina C, Pinkernell K, Song YH, Bai X, Sadat S, Campeau RJ et al. Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular

function, perfusion, and remodelling after acute myocardial infarction. Eur Heart J 2007;28:2667-77.

[88] Li B, Zeng Q, Wang H, Shao S, Mao X, Zhang F et al. Adipose tissue stromal cells transplantation in rats of acute myocardial infarction. Coron Artery Dis 2007;18:221-7.

[89] Song H, Cha MJ, Song BW, Kim IK, Chang W, Lim S et al. Reactive oxygen species inhibit adhesion of mesenchymal stem cells implanted into ischemic myocardium via interference of focal adhesion complex. Stem Cells 2010;28:555-63.

[90] Lee BC, Hsu HC, Tseng WY, Chen CY, Lin HJ, Ho YL et al. Cell therapy generates a favourable chemokine gradient for stem cell recruitment into the infarcted heart in rabbits. Eur J Heart Fail 2009;11:238-45.

[91] Virag JI, Murry CE. Myofibroblast and endothelial cell proliferation during murine myocardial infarct repair. Am J Pathol 2003;163:2433-40.

[92] Sun Y, Kiani MF, Postlethwaite AE, Weber KT. Infarct scar as living tissue. Basic Res Cardiol 2002;97:343-7.

[93] Jujo K, Ii M, Losordo DW. Endothelial progenitor cells in neovascularization of infarcted myocardium. J Mol Cell Cardiol 2008;45:530-44.

[94] Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med 2007;204:3037-47.

[95] Wang L, Deng J, Tian W, Xiang B, Yang T, Li G et al. Adipose-derived stem cells are an effective cell candidate for treatment of heart failure: an MR imaging study of rat hearts. Am J Physiol Heart Circ Physiol 2009;297:H1020-31.

[96] Amado LC, Saliaris AP, Schuleri KH, St John M, Xie JS, Cattaneo S et al. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc Natl Acad Sci U S A 2005;102:11474-9.

[97] Suzuki G, Iyer V, Lee TC, Canty JM,Jr. Autologous Mesenchymal Stem Cells Mobilize cKit+ and CD133+ Bone Marrow Progenitor Cells and Improve Regional Function in Hibernating Myocardium. Circ Res 2011.

[98] Li H, Malhotra D, Yeh CC, Tu R, Zhu BQ, Birger N et al. Myocardial survival signaling in response to stem cell transplantation. J Am Coll Surg 2009;208:607-13.

[99] Cho J, Zhai P, Maejima Y, Sadoshima J. Myocardial injection with GSK-3beta-overexpressing bone marrow-derived mesenchymal stem cells attenuates cardiac dysfunction after myocardial infarction. Circ Res 2011;108:478-89.

[100] Wang X, Zhao T, Huang W, Wang T, Qian J, Xu M et al. Hsp20-Engineered Mesenchymal Stem Cells are Resistant to Oxidative Stress Via Enhanced Activation of Akt and Increased Secretion of Growth Factors. Stem Cells 2009.

[101] Ma J, Ge J, Zhang S, Sun A, Shen J, Chen L et al. Time course of myocardial stromal cell-derived factor 1 expression and beneficial effects of intravenously administered bone marrow stem cells in rats with experimental myocardial infarction. Basic Res Cardiol 2005;100:217-23.

[102] Cheng Z, Liu X, Ou L, Zhou X, Liu Y, Jia X et al. Mobilization of mesenchymal stem cells by granulocyte colony-stimulating factor in rats with acute myocardial infarction. Cardiovasc Drugs Ther 2008;22:363-71.

[103] Huang J, Zhang Z, Guo J, Ni A, Deb A, Zhang L et al. Genetic modification of mesenchymal stem cells overexpressing CCR1 increases cell viability, migration, engraftment, and capillary density in the injured myocardium. Circ Res 2010;106:1753-62.

[104] Liu XH, Bai CG, Xu ZY, Huang SD, Yuan Y, Gong DJ et al. Therapeutic potential of angiogenin modified mesenchymal stem cells: Angiogenin improves mesenchymal stem cells survival under hypoxia and enhances vasculogenesis in myocardial infarction. Microvasc Res 2008.

[105] Zeng B, Chen H, Zhu C, Ren X, Lin G, Cao F. Effects of combined mesenchymal stem cells and heme oxygenase-1 therapy on cardiac performance. Eur J Cardiothorac Surg 2008.

[106] Gnecchi M, He H, Melo LG, Noiseaux N, Morello F, de Boer RA et al. Early beneficial effects of bone marrow-derived mesenchymal stem cells overexpressing Akt on cardiac metabolism after myocardial infarction. Stem Cells 2009;27:971-9.

[107] Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F et al. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. Faseb J 2006;20:661-9.

[108] Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 2005;11:367-8.

[109] Tan MY, Zhi W, Wei RQ, Huang YC, Zhou KP, Tan B et al. Repair of infarcted myocardium using mesenchymal stem cell seeded small intestinal submucosa in rabbits. Biomaterials 2009;30:3234-40.

[110] Jin J, Jeong SI, Shin YM, Lim KS, Shin H, Lee YM et al. Transplantation of mesenchymal stem cells within a poly(lactide-co-epsilon-caprolactone) scaffold improves cardiac function in a rat myocardial infarction model. Eur J Heart Fail 2009;11:147-53.

[111] Chen CH, Wei HJ, Lin WW, Chiu I, Hwang SM, Wang CC et al. Porous tissue grafts sandwiched with multilayered mesenchymal stromal cell sheets induce tissue regeneration for cardiac repair. Cardiovasc Res 2008.

[112] Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H et al. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med 2006;12:459-65.

[113] Hahn JY, Cho HJ, Kang HJ, Kim TS, Kim MH, Chung JH et al. Pre-treatment of mesenchymal stem cells with a combination of growth factors enhances gap junction formation, cytoprotective effect on cardiomyocytes, and therapeutic efficacy for myocardial infarction. J Am Coll Cardiol 2008;51:933-43.

[114] Engler AJ, Carag-Krieger C, Johnson CP, Raab M, Tang HY, Speicher DW et al. Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. J Cell Sci 2008;121:3794-802.

[115] Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell 2006;126:677-89.

[116] Forte G, Carotenuto F, Pagliari F, Pagliari S, Cossa P, Fiaccavento R et al. Criticality of the Biological and Physical Stimuli Array Inducing Resident Cardiac Stem Cell Determination. Stem Cells 2008.

[117] Schuleri KH, Amado LC, Boyle AJ, Centola M, Saliaris AP, Gutman MR et al. Early improvement in cardiac tissue perfusion due to mesenchymal stem cells. Am J Physiol Heart Circ Physiol 2008;294:H2002-11.

[118] Amado LC, Schuleri KH, Saliaris AP, Boyle AJ, Helm R, Oskouei B et al. Multimodality noninvasive imaging demonstrates in vivo cardiac regeneration after mesenchymal stem cell therapy. J Am Coll Cardiol 2006;48:2116-24.

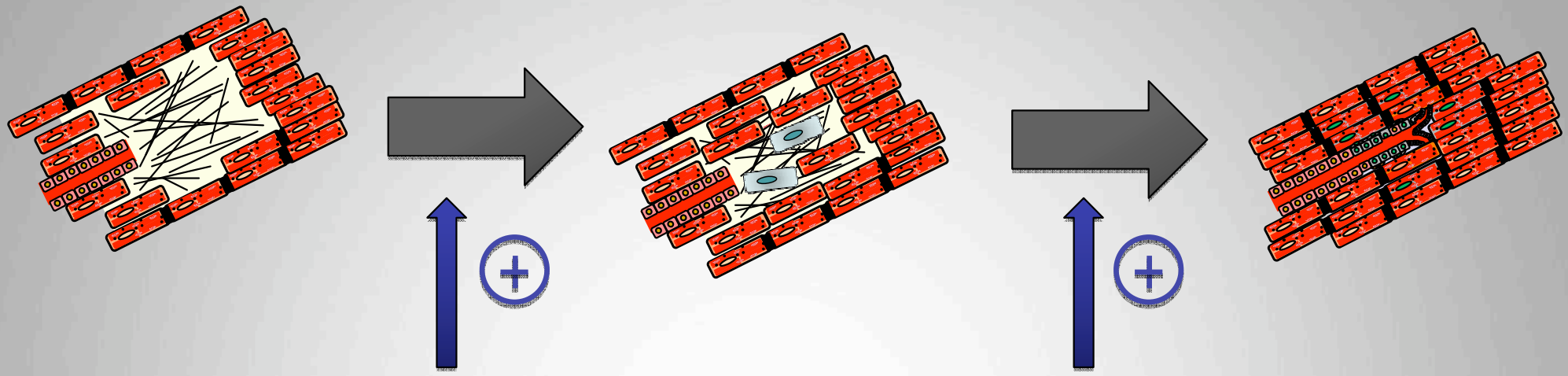
[119] Rigol M, Solanes N, Farre J, Roura S, Roque M, Berruezo A et al. Effects of adipose tissue-derived stem cell therapy after myocardial infarction: impact of the route of administration. J Card Fail 2010;16:357-66.

[120] Zhou Y, Wang S, Yu Z, F. HR,Jr, Sachdev V, Vincent P et al. Direct injection of autologous mesenchymal stromal cells improves myocardial function. Biochem Biophys Res Commun 2009;390:902-7.

SCARRED MYOCARDIUM

PARTIALLY SCARRED MYOCARDIUM

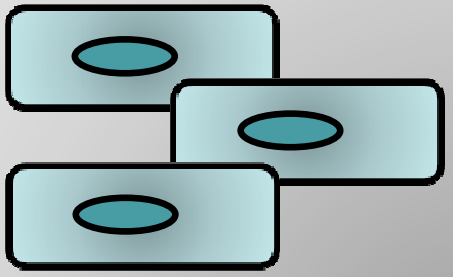
REBUILT MYOCARDIUM



CELL THERAPY:

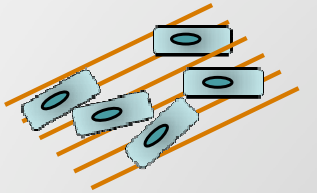
CONVENTIONAL CELL TRANSPLANTATION

MSC

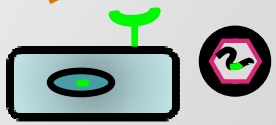


CELL THERAPY:

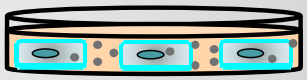
IMPROVED METHODOLOGY



TISSUE ENGINEERING



GENETIC MODIFICATION



IN VITRO PRETREATMENT

EC

CM

SMC

PREDIFFERENTIATION