UNIVERSIDAD DE JAÉN

FACULTAD DE CIENCIAS DE LA SALUD

DEPARTAMENTO DE CIENCIAS DE LA SALUD

TESIS DOCTORAL

DIFERENCIACIÓN DE CÉLULAS MADRE COMO MODELO EXPERIMENTAL DE TERAPIA EN ENFERMEDADES PREVALENTES: OSTEOARTRITIS Y PATOLOGÍA CARDIOVASCULAR

PRESENTADA POR:

ELENA LÓPEZ RUIZ

DIRIGIDA POR:

DRA. DÑA. MACARENA PERÁN QUESADA

DR. D. JUAN ANTONIO MARCHAL CORRALES

DRA. DÑA. MARÍA ÁNGEL GARCÍA CHAVES

JAÉN, 21 DE FEBRERO DE 2014

ISBN 978-84-8439-840-0

El doctorando **Elena López Ruiz** y los directores de la tesis **Macarena Perán Quesada, Juan Antonio Marchal Corrales y María Ángel García Chaves** garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Jaén, 21 Febrero 2013

Director/es de la Tesis:

Fdo.: Macarena Perán Quesada

Fdo.: Juan Antonio Marchal Corrales

Fdo.: María Ángel García Chaves

Doctorando:

Fdo.: Elena López Ruiz

La doctoranda **Elena López Ruiz** ha disfrutado de una beca de investigación F.P.D.I. *Formación de Personal Docente e Investigador predoctoral en áreas de conocimiento consideradas deficitarias por necesidades docentes* (convocatoria de 10 de febrero de 2009 –BOJA nº 47 de 10 de marzo).
Para optar a la mención de "Doctor Internacional", la doctoranda realizó, durante el periodo de formación, una estancia de tres meses en el laboratorio de la Dr. Cay Kielty en la Facultad de Ciencias de la Universidad de Manchester.

Los resultados de esta Tesis Doctoral han sido aceptados en las siguientes publicaciones que cumplen con los criterios de calidad exigidos (ver anexo):

- Peran M, Garcia MA, Lopez-Ruiz E, Jimenez G, Marchal JA. 2013. How Can Nanotechnology Help to Repair the Body? Advances in Cardiac, Skin, Bone, Cartilage and Nerve Tissue Regeneration. Materials 6:1333-1359

- Lopez-Ruiz E, Peran M, Cobo-Molinos J, Jimenez G, Picon M, Bustamante M, Arrebola F, Hernandez-Lamas MC, Delgado-Martinez AD, Montanez E, Marchal JA. 2013. Chondrocytes extract from patients with osteoarthritis induces chondrogenesis in infrapatellar fat pad-derived stem cells. Osteoarthritis Cartilage 21:246-258.

- Peran M, Lopez-Ruiz E, Gonzalez-Herrera L, Bustamante M, Valenzuela A, Marchal JA. 2013. Cellular extracts from post-mortem human cardiac tissue direct cardiomyogenic differentiation of human adipose tissue-derived stem cells. Cytotherapy 15:1541-1548.

- López-Ruiz E, Perán M, Picón-Ruiz M, García MA, Carrillo E, Jiménez-Navarro M, Hernández MC, Prats I, De Teresa E, Marchal JA. 2014. Cardiomyogenic differentiation potential of human endothelial progenitor cells isolated from myocardial infarction patients. Cytotherapy. Submitted.

AGRADECIMIENTOS

Durante estos años en los que he estado realizando mi tesis son muchas las personas que me han ayudado tanto laboral como personalmente, por eso quiero expresarles mi más sincero agradecimiento ya que sin ellos no hubiera podido realizar este trabajo.

En primer lugar empezaré por mis directores de tesis, gracias por vuestra confianza y por haberme brindado la oportunidad de conocer este mundo de la ciencia. Agradezco a Macarena todos sus consejos, su eficacia y a que con su gusto por las cosas bien hechas ha sido una motivación para esforzarme y sacar lo mejor de mí, sin "la Macarena" no lo hubiera conseguido. Igualmente, agradecer a Juan su esfuerzo, sus inquietudes científicas y su optimismo que siempre son una motivación para seguir trabajando. No solo científicamente, sino también personalmente me considero afortunada por haberos tenido como directores de tesis y haber compartido con vosotros todo este tiempo.

A Marian y Houria por su apoyo científico y personal.

Al profesor David Tosh por haberme dado la oportunidad de trabajar en su laboratorio.

A Steve, mi tutor en la Universidad de Manchester, por confiar en mí y por el tiempo dedicado para mi formación.

A Milán y Gema que comenzaron siendo compañeras de trabajo y se han convertido en dos grandes amigas, a las dos gracias por aguantar todos mis pisotones, por ser como sois y saber cuándo es necesario comer "un poco" de chocolate.

A mis amigos y compañeros de laboratorio que tantos buenos y regulares momentos hemos pasado. A Paola por su sonrisa y naturalidad, a Esther por demostrar que tiene mucha clase, a Alberto y Jesús por vuestra ayuda y por estar siempre dispuestos a tomar un café y hacer todo más llevadero y a Cythia por los buenos ratos en campana.

A Aitor, primero en apodarme "La doñi" (no entraremos en detalles del porqué), Javi, Sabi y Laura, mis pipiolos de máster a los que guardo un gran cariño.

A Blanca, Salva, Gloria, Javi, Ruth, Manuel, Carmen, Macarena, Ana, Mohamed, y Marina que de una forma u otra me han ayudado durante esta etapa.

A Gabi y Yolanda por su amistad ya que sin ellas mi estancia en Bath no hubiera sido lo mismo.

A mis compañeros de carrera Valentín, Pilar, Alberto, Silvia, Javi, Elena y Paty.

A Ángela, por su buen humor, su alegría y estar siempre cuando lo he necesitado, tras todo este tiempo de convivencia, ya eres como una hermana para mí.

A Miryam, Ana, Elena V, Mari Carmen, Mª Lola, Nieves y Carla por ser mis amigas desde siempre y hasta siempre.

A toda mi familia y en especial a mi hermana, a mi abuela y a Santi, gracias por vuestro cariño. También gracias a mi sobrina Laura porque soy su "tita prefe" aunque todavía no se haya enterado.

A Juan Ignacio, por estar siempre ahí, por animarme y ser un gran apoyo.

A mis padres por su ejemplo de esfuerzo y constancia. Gracias por vuestro amor, por la educación que me habéis dado y por ayudarme en todas mis decisiones.

“Todo parece imposible hasta que se hace”

Nelson Mandela

NOTA

A lo largo del texto se han utilizado las abreviaturas de varios términos en inglés. Esto es debido al uso extendido de la lengua inglesa en publicaciones científicas y al uso de abreviaturas en inglés de forma cotidiana en el lenguaje científico. En el glosario, al final del trabajo, se detalla la descripción en inglés y en castellano de cada uno de las abreviaturas. Asimismo, en este glosario se recogen las abreviaturas de los términos usados en castellano.

ÍNDICE

SUMMARY

Cell based therapy is an emerged discipline in the field of regenerative medicine born with the aim to restore damage or lost tissue. Three pillars supports tissue regeneration, i) a suitable cell source; ii) cell differentiation strategies; and iii) bio-compatible scaffolds that maintain the 3D structural architecture of the tissue.

It is broadly known the limited regeneration capacity of cartilage and cardiac tissue. The main goal of the present study was to develop strategies to direct cell differentiation toward cardiac and cartilage linage to repair and/or regenerate the damage tissue.

Adult stem cells and progenitor cells can be easily isolated and expanded in culture. A key feature of these cells is their plasticity, which allows their conversion to different cell types. Here, we have studied the potential differentiation capacity of four different cell sources i) human adipose derived stem cells (hASCs) isolated from lipoaspirates; ii) hASCs obtained from infrapatellar fat pad (IFPSCs) of patients with osteoarthritis (OA), iii) endothelial progenitor cells (EPCs) derived from umbilical cord blood (UCB) and iv) EPCs isolated from peripheral blood (PB) of patients suffering from acute myocardial infarction (AMI).

Our first objective was to induce chondrogenic differentiation of autologous IFPSCs of patients with OA using cellular extracts-based transdifferentiation method. Cells exposed to chondrocyte extracts acquired a characteristic morphological and ultrastructural chondrocyte phenotype that was confirmed by ultrastructural observations and histological, immunological and molecular proves. We demonstrate that extracts obtained from chondrocytes of osteoarthritic knees promote chondrogenic differentiation of autologous IFPSCs. Moreover, combination of transdifferentiated IFPSCs with biodegradable 3D poly (DL-lactic-co-glycolic acid) (PLGA) scaffolds was an efficient system for the maintenance and maturation of cartilage tissue.

Our second objective was to study the potential capacity of postmortem cardiac tissue to direct cardiac differentiation of hASCs *in vitro*. We used heart tissue collected from autopsies to obtain cell extracts and conditioned medium and both approaches were tested for cardiac induction. Gene expression analyses proved that human cardiac postmortem tissue maintain genetic integrity. Furthermore, hASCs exposed to the cell extracts or conditioned medium for two weeks achieved the appearance of myotube-like

structures and were positive for cardiac markers proved by immunofluorescence and by reverse transcription-polymerase (RT-PCR) chain reaction. In conclusion postmortem human cardiac tissue can induce hASCs differentiation into myocardial like cells.

Our third objective was to compare the cardiac differentiation capacity of EPCs isolated from PB of patients suffering from AMI with EPCs obtained from UCB. We characterized EPCs from both origins using endothelial markers and endothelial cell colony-forming units (CFU-EC) assay. Cardiac differentiation capacity of EPCs was assessed by immunofluorescence and RT-PCR after 5-azacytidine (5-aza) induction. We found no significant differences between the number of CFU-EC in subjects with AMI and samples from UCB. Moreover, 5-aza induced the appearance of myotube-like structures and the positive expression of cardiac markers in a similar pattern for both cell sources, indicating a comparable acquisition of a cardiac-like phenotype.

In conclusion, here we have implemented novel strategies to direct cell differentiation. The finding included in this study could represent a potential therapeutic approach in cardiovascular diseases (CVD) and osteochondral injuries.

INTRODUCCIÓN

1. Medicina regenerativa

La capacidad para recuperarse después de un daño difiere ampliamente entre unos organismos y otros. Algunos invertebrados, como la planaria, tiene gran capacidad de regeneración, incluso es capaz de regenerar la cola a partir de la cabeza y viceversa, mientras que ciertos vertebrados, como la salamandra, puede regenerar estructuras complejas, como las extremidades o la médula espinal (Sugimoto et al., 2011). Sin embargo, la mayoría de los mamíferos adultos carecen de este alto grado de plasticidad. De hecho, los seres humanos tienen una capacidad de auto-regeneración muy limitada, pudiendo mantener una homeostasis tisular, que es evidente en tejidos como la piel, la sangre, o el hígado, pero que se da, aunque en menor grado, en todos los tejidos del organismo. Con tales restricciones, la pérdida de una cantidad importante de tejido queda muy lejos de poder ser reparada por mecanismos de reparación endógena.

La sugerente idea de poder activar y controlar la regeneración tisular ha llevado al desarrollo de la medicina regenerativa, disciplina que nace con el objetivo de desarrollar terapias para remplazar tejidos u órganos lesionados como resultado de daños, defectos congénitos o enfermedades crónicas (Warren, 2013). Su finalidad no es solamente reemplazar, sino además recuperar la función biológica perdida, ya sea proporcionando los elementos necesarios *in vivo*, y/o estimulando la capacidad de regeneración intrínseca del propio organismo (Mason and Dunnill, 2008).

Conseguir la regeneración del tejido dañado es un reto al que se enfrentan profesionales de muy diversos ámbitos entre los que se encuentran biólogos celulares/moleculares, químicos, físicos, nano-tecnólogos, ingenieros de tejidos y profesionales clínicos entre otros (Corona et al., 2010).

La clave para la regeneración de tejidos puede plantearse en tres simples postulados, i) se necesita una fuente celular; ii) las células deben mantener la diferenciación que las haga funcionales; iii) se debe lograr mimetizar la estructura tridimensional del tejido, para lo cual se precisa de soportes celulares biocompatibles (Peran et al., 2012).

Es obvio que dependiendo del daño tisular o del tejido afectado no siempre será necesario que se cumplan los tres postulados mencionados. En algunos casos, como en los trasplantes de médula ósea, bastaría con sustituir la población de células dañadas para restaurar la función tisular. Así, en función del alcance del daño de un órgano o tejido, existe un incremento paralelo en cuanto a la complejidad de las tecnologías que se aplican para restaurar su función (Figura 1). Las estrategias actuales van desde métodos que mejoran la capacidad de regeneración endógena del cuerpo para su auto-reparación (se incluyen, células, terapia génica o terapia con medicamentos) a métodos que combinan varias tecnologías, es decir, células, biomateriales, biorreactores, genes y terapia con medicamentos, que pueden llegar hasta la creación de órganos para su remplazo (Greenwood et al., 2006).

Por tanto, podemos concluir que en medicina regenerativa, disciplina en continua evolución, se están desarrollando nuevas estrategias para tratar patologías que las terapias convencionales actuales no son capaces de resolver.

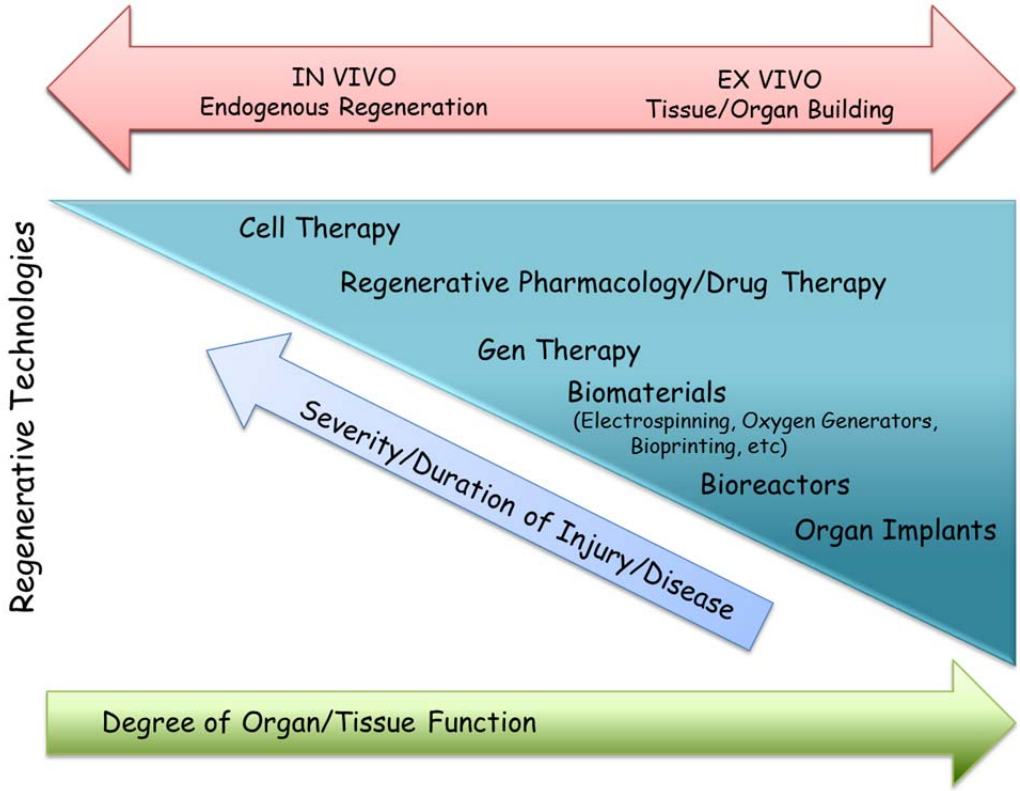


Figura 1. Representación esquemática del espectro de tecnologías aplicadas en medicina regenerativa. Figura adaptada de Corona et al., 2010.

Centrándonos en la fuente celular indicada para la regeneración tisular, podemos diferenciar entre fuentes alogénicas (procedentes de donantes) o autólogas (las células del huésped), prefiriéndose las células autólogas ya que no presentan riesgo de ser rechazadas por el sistema inmune del paciente. En este sentido, las células madre somáticas y las células progenitoras son candidatas a destacar debido a que pueden ser aisladas de distintas fuentes del propio paciente y han demostrado su capacidad para diferenciarse hacia linajes celulares específicos. La gran plasticidad que demuestran estás células han promovido un elevado número de estudios con el fin de diferenciarlas hacia diversos tejidos con capacidad de regeneración limitada. Se parte de la premisa de que las células comprometidas o diferenciadas hacia un linaje específico deben integrarse mejor en el tejido de destino que aquellas células no diferenciadas (Steinert et al., 2012). Enfermedades degenerativas con alta prevalencia en la población, tales como las patologías osteocondrales o las enfermedades cardiovasculares, podrían beneficiarse de las terapias celulares si son llevadas con existo a la práctica clínica. Por consiguiente, el estudio de estrategias de diferenciación celular es clave para la producción del tipo celular deseado, así como también lo es el desarrollo de biomateriales que faciliten su implantación en el organismo.

2. Fuentes celulares

La elección de la fuente celular apropiada para ser trasplantada en el tejido dañado a menudo supone una tarea complicada. Como se ha mencionado anteriormente, las células deben ser preferiblemente de origen autólogo o no inmunogénicas, altamente proliferativas, tener capacidad para ser diferenciadas en el tipo celular requerido, a la vez de ser capaces de mantener su estado de diferenciación, y además no deben presentar riesgo de formación de teratomas cuando son trasplantadas. Son muchos y variados los estudios que se están realizando en este campo, en los cuales se emplean diversos tipos celulares que van desde las células madre de origen embrionario (ESCs); las células madre pluripotentes inducidas (iPS cells), las células madre adultas, entre las que destacan las células madre mesenquimales (MSCs) y las células progenitoras, como las EPCs.

2.1. Células madre

Las células madre (SC) se definen como aquellas células que independientemente de su origen ontogénico y tisular tienen capacidad de dividirse y generar células hijas con sus mismas características (división simétrica) o bien originar células progenitoras (división asimétrica), las cuales pueden dar lugar a células diferenciadas, (Figura 2) (Thomson et al., 1998; Pittenger et al., 1999; Odorico et al., 2001; Ratajczak et al., 2008).

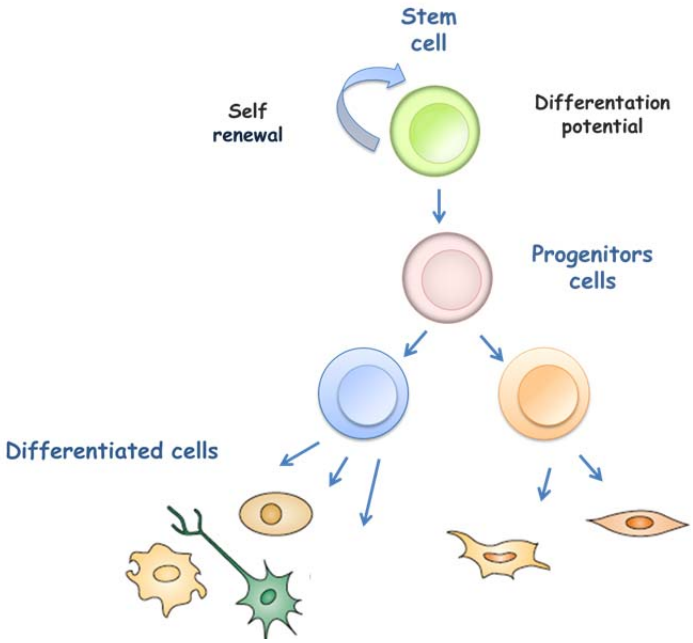


Figura 3. Características fundamentales de las células madre. Adaptada de Collas and Hakelien, 2003.

Desde que en 1961 los científicos James Till y Ernest McCulloch demostraran la existencia de las células madre hematopoyéticas (HSCs) (Becker et al., 1963), el estudio de las SC ha despertado el interés de la comunidad científica. Su capacidad de renovación, su alta plasticidad y la posibilidad de ser expandidas *in vitro* sin duda convierten a estas células en una atractiva herramienta en medicina regenerativa (Ehnert et al., 2009). De hecho, las SC han creado muchas expectativas por su potencial para el tratamiento de un amplio número de enfermedades degenerativas, como el Parkinson o enfermedades isquémicas, como el infarto cerebral o de miocardio. Tanto es así que se prevé que en el futuro estas células puedan convertirse en fuentes celulares disponibles para el remplazo y trasplante de diferentes tejidos como el nervioso, el muscular, el sanguíneo, el hepático, el condral, el pancreático y el cardiovascular (Chung et al.,

2004; Nagaya et al., 2004; Yokoo et al., 2008; Madec et al., 2009; Quevedo et al., 2009; Vija et al., 2009; Wu et al., 2013).

El potencial de diferenciación de las SC puede variar dependiendo de la madurez de los tejidos en los que residen y su origen (Ilic and Polak, 2011). Por ello, se han clasificado en base a estas premisas.

2.1.1. Clasificación basada en su potencial

En función de su potencial de diferenciación las SC se han clasificado en totipotentes, pluripotentes, multipotenes o unipotentes:

- Células madre totipotentes: estas células pueden diferenciarse en tejidos embrionarios y extraembrionarios como el trofoectodermo, es decir son capaces de generar un organismo completo. El zigoto es considerado la única célula totipotente.

- Células madre pluripotentes: son aquellas células con capacidad de renovación y que pueden diferenciarse en cualquiera de las tres capas embrionarias (ectodermo, endodermo y mesodermo). Las ESCs y las iPS cells son las células que forman este grupo. Sin embargo, las iPS cells, son consideradas un grupo aparte por no existir de forma natural y ser obtenidas mediante la reprogramación de células somáticas.

- Células madre multipotentes: son células con capacidad de renovación y que se diferencian únicamente hacia células de su mismo linaje o capa embrionaria. Un conocido ejemplo son las HSCs que pueden diferenciarse hacia linajes mieloide y linfoide.

- Células madre unipotentes: son aquellas células que solo generan un tipo celular en particular. Por ejemplo, las células madre musculares tienen capacidad de renovación y de diferenciarse en un solo tipo celular.

2.1.2. Clasificación basada en su origen

Las SC son clasificadas en función del momento del desarrollo (ontogenia) en cuatro tipos: células madre embrionarias, células madre fetales, células madre neonatales y células madre adultas.

2.1.2.1. Células madre embrionarias

Estas células madre pluripotentes se han estudiado como posible fuente celular en medicina regenerativa por su gran capacidad de diferenciación hacia las tres capas germinales y por su alta proliferación *in vitro*. Las ESCs son capaces de generar todos los tipos de células del cuerpo, excepto el cordón umbilical, el trofoblasto y estructuras asociadas. Sin embargo, se ha visto que estas células pueden desarrollar tumores *in vivo*, lo que limita su futura aplicabilidad clínica.

Los primeros trabajos con estas células aparecen en 1981, cuando por primera vez se consiguieron aislar y cultivar satisfactoriamente de la masa interna de blastocistos murinos (Evans and Kaufman, 1981; Martin, 1981). Trascurrió más de una década hasta que, en 1998, el científico americano James Thomson y su equipo establecieron la primera línea de ESCs humanas proporcionando así la posibilidad de usar estas células como fuente para terapia celular (Thomson et al., 1998). Fue entonces cuando, a raíz de este descubrimiento, surgió la controversia sobre la necesaria destrucción de embriones tempranos para su obtención, lo cual ha desatado un importante debate político y ético que perdura hasta la actualidad. Son muchos los detractores, los problemas legales y burocráticos que la aplicación de ESCs humanas conlleva, asimismo, estas células no constituyen una fuente celular del propio paciente y podrían causar problemas de rechazo (Nikol'skii et al., 2007; Hipp and Atala, 2008).

Como resultado de todas estas dificultades se está enlenteciendo el desarrollo de ensayos clínicos que utilicen ESCs como terapia, no obstante en la actualidad existen ensayos clínicos en fase I y II que se están llevando a cabo, basados en el uso de células epiteliales pigmetarias de la retina derivadas de ESCs en pacientes con distrofia macualar [Clinical trials.com].

Dejando a un lado su posible aplicación en terapia celular, la investigación con estas células también se enfoca hacia el *screening* de fármacos, modelos para el estudio de la especificación tisular y para el estudio de enfermedades que se desarrollan en estadios prenatales.

2.1.2.5. Células madre pluripotentes inducidas

En un estudio sin precedentes dirigido por el premio Nobel Shinya Yamanaka, ha sido demostrado que las células somáticas pueden ser reprogramadas en células pluripotentes mediante el uso de factores transcripcionales asociados a la pluripotencia (Takahashi and Yamanaka, 2006). Estas células pluripotenciales inducidas denominadas iPS cells, presentan características similares a las ESCs a la vez que su uso no conlleva dilemas éticos o políticos.

Los científicos apuntan a su utilidad como herramientas para el estudio de enfermedades humanas y el desarrollo de fármacos, así como para estudios de toxicología (Drews et al., 2012). Con dichas finalidades, se han generado líneas celulares de pacientes con enfermedades tales como la distrofia muscular de Duchenne y Becker, la enfermedad de Huntington, el síndrome del Cromosoma X Frágil, la Adrenoleucodistrofia o la Neurofibromatosis (Volarevic et al., 2011). Sin embargo, la terapia celular con estas células está aún muy lejos de ser llevada a la práctica clínica, ya que la posible formación de teratomas continúa siendo un problema sin resolver.

2.1.2.2. Células madre fetales

Estas células son obtenidas de múltiples tejidos durante el desarrollo fetal (entre el día 14 hasta el momento del nacimiento). No poseen tanto potencial de diferenciación como las ESCs pero por otro lado no han levantado tanta controversia como estas. Son obtenidas de embarazados interrumpidos, aunque también se han aislado de pequeñas muestras rutinarias de líquido amniótico y de las vellosidades coriónicas (Weber et al., 2011). Hoy día muchas de las líneas de células madre neuronales que se han generado poseen un origen fetal e incluso han sido probadas en ensayos clínicos con humanos en ciertos países (Ilic and Polak, 2011). Asimismo, células mesenquimales con una alta capacidad de proliferación y baja inmunogenenicidad han sido obtenidas de diversos

tejidos fetales, incluso diversos estudios han documentado que albergan un gran potencial terapéutico en el tratamiento del cáncer, ya que pueden migrar hacia del sitio del tumor y reducir su tamaño (Bitsika et al., 2013).

2.1.2.3 Células madre neonatales

Estas células pueden obtenerse de la placenta y del cordón umbilical (UC). Al ser aisladas de tejidos que son desechados como residuos biológicos no plantean controversia ética alguna.

El trasplante de SC procedentes de UC es una terapia que actualmente se lleva a cabo con bastante existo para tratar enfermedades congénitas o adquiridas de la médula ósea, tales como las leucemias agudas, gracias a que la sangre de este tejido es una fuente rica en células madre hematopoyéticas. Asimismo, de la sangre de UC se obtienen otras células con gran potencial para su uso en medicina regenerativa tales como las MSCs o las EPCs (Pelosi et al., 2012).

Recientemente se está prestando especial atención a las SC extraídas de la gelatina de Wharton presente en la matriz extracelular del UC. Tales células han demostrado ser multipotentes y presentan marcadores típicos de las MSCs. Diversos estudios apuntan que su capacidad de diferenciación también se extiende hacia distintos linajes, por ello, gracias a su alta capacidad de proliferación *in vitro* y sus funciones inmunomoduladoras, estas células se han convertido en candidatas para su uso en medicina regenerativa (Anzalone et al., 2010). De manera que no es de extrañar que las empresas privadas, además de conservar las células del UC, hayan comenzado a ofrecer la criopreservación de estas células para su posible uso en terapias celulares en un futuro (Batsali et al., 2013).

Igualmente, se han podido obtener MSCs y epiteliales de la placenta y sus membranas (Parolini et al., 2008). Experimentos *in vitro* con estas células han mostrado que se expanden más rápido, poseen menos inmunogenicidad y son menos imnosupresivas que sus homólogas adultas (Barlow et al., 2008; Murphy and Atala, 2013).

2.1.2.4. Células madre adultas

Las células madre adultas están presentes, en diverso grado, en todos los tejidos somáticos y órganos del cuerpo de un adulto. Estas células son las responsables de la renovación y reparación del tejido dañado o envejecido.

Entre las células madre adultas, las HSCs de la médula ósea fueron las primeras en ser descubiertas, seguidas de las células madre mesenquimales y desde entonces hasta la actualidad se ha probado que existe una población de células no diferenciadas en todos los tejidos del organismo. Generalmente, estas células se localizan en un nicho, es decir en un microambiente que determinará su estado de activación, asegurando así un equilibrio entre el mantenimiento del pool de SC y la producción de células progenitoras implicadas en la diferenciación de un tejido. Estas células, solo pueden generar una gama de tipos celulares en función del tejido en particular en el que residen. Motivo por el cual también han sido denominadas como células madre residentes. Así, algunas células madre adultas son multipotentes, como las MSCs y las HSCs, mientras que otras se cree que son precursoras directas de las células del tejido en el que se encuentran, es decir unipotentes (Ooi et al., 2006).

Las células madre adultas son una fuente celular bastante propicia para su aplicación en clínica ya que además de no suscitar debates éticos, constituyen una fuente celular abundante, de fácil disponibilidad y presentan una gran plasticidad. Por ello, están siendo objeto de multitud de estudios para el tratamiento autólogo de enfermedades degenerativas, autoinmunes y congénitas. Muchos de estos estudios consisten en experimentos *in vivo* (Lagasse et al., 2000; Orlic et al., 2001; Di Campli et al., 2004) y ensayos clínicos destinados a la regeneración de órganos dañados como el corazón, hígado, riñón o cerebro (Tendera et al., 2009; Howe et al., 2011; Wojakowski et al., 2012). Estas células están siendo aisladas principalmente de tejidos tales como la médula ósea, la sangre periférica (PB) o el tejido adiposo.

El transplante de HSCs es uno de los más exitosos ejemplos de terapia celular, ya que no se precisa de su expansión *ex vivo* y no hay necesidad de reconstituir órganos complejos. Esta terapia se aplica con bastante frecuencia para prolongar la vida de los pacientes con enfermedades hematopoyéticas tales como la leucemia, o para reconstituir

el sistema inmune en pacientes inmunocomprometidos después de terapias de radiación y/o quimioterapia (Gratwohl et al., 2008).

2.1.2.4.1. Células madre mesenquimales

Las células madre mesenquimales son un tipo destacado de células madre multipotentes capaces de diferenciarse en diversos tejidos de origen mesodérmico. Se ha establecido que las MSCs tienen potencial de diferenciación hacia osteoblastos, condroblastos, adipocitos, fibroblastos y miocitos (Pittenger et al., 1999), (Figura 3). Sin embargo, muchos investigadores han reportado que su alto grado de plasticidad también les confiere capacidad para diferenciarse hacia otros linajes no mesodérmicos como el páncreas, el hígado, o cerebro (Sasaki et al., 2008; Snykers et al., 2009; Bae et al., 2011), afirmaciones que han sido motivo de gran controversia.

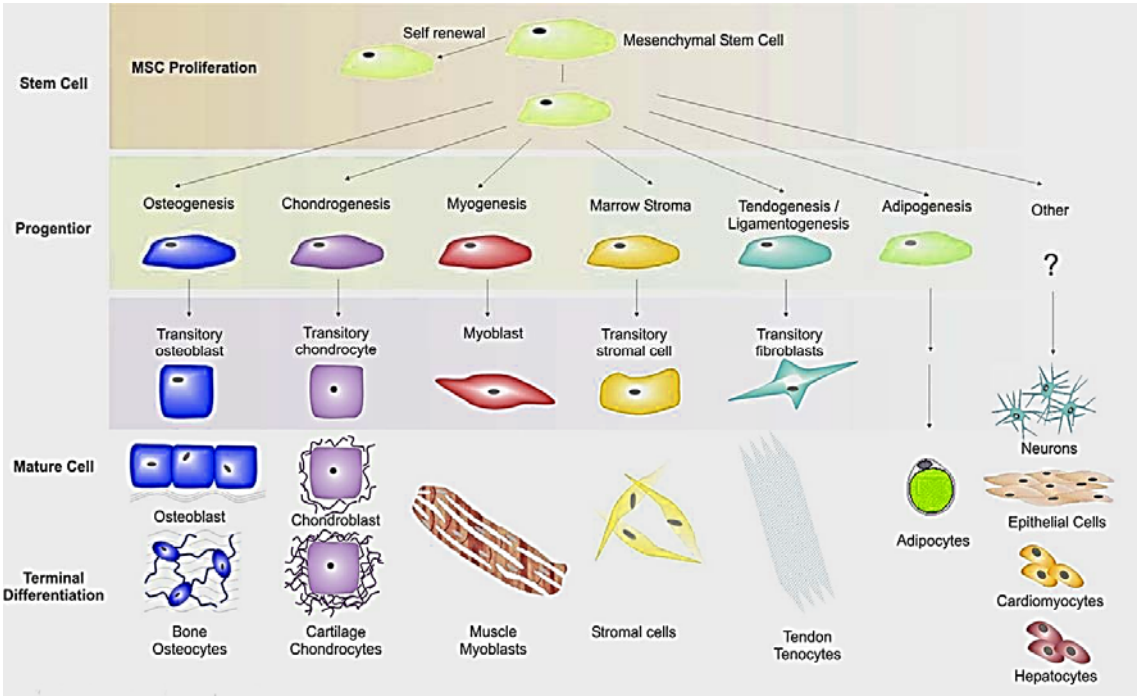


Figura 3. Potencial de diferenciación de las MSCs hacia distintos linajes celulares. Las MSCs son multipotentes y poseen la capacidad de proliferar y comprometerse a diferentes tipos de células en función de las condiciones ambientales. Fuente Kalinina et al., 2011.

Identificadas por primera vez en la médula ósea por Friedenstein y su equipo, fueron descritas como células madre no hematopoyéticas aisladas del estroma capaces de

adherirse y formar colonias a partir de una sola célula. Los clones iniciales se expandían formando colonias compuestas por células de aspecto fibroblastoide, de ahí el término de unidades formadoras de colonias de fibroblastos (CFU-F). Después de unos pocos días, las células empezaban a proliferar y podían diferenciarse en células maduras de los linajes mesenquimales tales como osteoblastos (Friedenstein et al., 1970).

El término de células madre mesenquimales fue propuesto por Caplan en referencia a su capacidad de diferenciación hacia los distintos linajes mesodérmicos (Caplan, 1991), aunque por otro lado los hematólogos las denominaron células del estroma medular debido a que servían de soporte para alimentar el cultivo de las células hematopoyéticas (Eaves et al., 1991). Hoy día, la polémica en torno a la nomenclatura de estas células sigue siendo centro de intenso debate. Recientemente la Sociedad Internacional de Terapia Celular (ISCT) ha revisado dicho término y ha introducido el término "células estromales mesenquimales multipotentes" para referirse a las células cultivadas *in vitro*, y dejar el término stem para designar a los precursores/ células madre *in vivo*. Sin embargo, ambos términos conservan el mismo acrónimo (Horwitz et al., 2005). Por tanto, debido a la gran aceptación que ha alcanzado el término "células madre mesenquimales" y a que sigue siendo el más utilizado como término general, será el que empleemos en el presente trabajo.

El aislamiento y caracterización de las MSCs está ampliamente extendido en los laboratorios de cultivo celular de todo el mundo. Si bien es cierto que no existe un método universalmente aceptado para su aislamiento, expansión e identificación, si existe una lista de criterios establecidos por la ISCT para definir los cultivos primarios *in vitro* de las MSCs que incluyen los siguientes: i) ser adherentes al plástico, ii) expresar los marcadores de superficie CD73, CD90 y CD105 y no expresar los marcadores CD45, CD34, CD14 o CD11b, CD79 o CD19 y HLA-DR, y por último, iii) tener capacidad de diferenciación hacia los tipos celulares de tres linajes mesodérmicos (osteoblastos, adipocitos y condroblastos) (Dominici et al., 2006).

Las MSCs resultan muy interesantes para la terapia celular ya que además de su capacidad de diferenciación, también destacan por sus propiedades inmunomoduladoras (Ortiz et al., 2007; Dayan et al., 2011) y efectos antiinflamatorios (Chen and Tuan, 2008). Se ha demostrado que las MSCs carecen o muestran una baja expresión del

complejo mayor de histocompatibilidad II (Parolini et al., 2008) y es debido a esta baja inmugenicidad por la que se están realizando estudios para la implantación de MSCs alogénicas. Además, se ha comprobado que las MSCs liberan diversos factores, tales como HGF, EGF, VEGF y sFRP-4 (Katsha et al., 2011), por lo que a través del contacto directo célula a célula o de sus efectos paracrinos, las MSCs pueden modular el microambiente y activar las células progenitoras endógenas para contribuir en la restauración de los tejidos locales (Koelling and Miosge, 2009; Li et al., 2009; Hatzistergos et al., 2010). Asimismo, estas células tienen la capacidad de movilizarse desde la médula ósea a la circulación sanguínea para acudir a zonas donde se ha producido un daño y contribuir de este modo a la restauración tisular. Todas estas propiedades hacen que las MSCs sean las candidatas elegidas con mayor frecuencia para estudios clínicos dirigidos hacia el tratamiento de enfermedades autoinmunes y degenerativas [Clinical trials.com]. De hecho, existen numerosos estudios *in vitro* y con modelos animales en los que estas células se utilizan como terapia celular y para la reconstrucción de tejidos (Wong et al., 2008; Hamou et al., 2009; de la Garza-Rodea et al., 2011). Se ha demostrado que son capaces de mejorar lesiones presentes en casi todos los órganos importantes, incluyendo el cerebro, hígado, el riñón, corazón y los pulmones (Si et al., 2011; Dimarino et al., 2013; Zhu et al., 2013)

Las MSCs obtenidas de diversas fuentes comparten las características principales señaladas con anterioridad, sin embargo es de reseñar que pueden presentar variaciones en cuanto al fenotipo, la morfología, el potencial de proliferación y la capacidad de diferenciación. Por ejemplo, se ha demostrado que las MSCs de tejido sinovial pueden ser más eficaces en la reparación de los defectos del cartílago que las MSCs de otros tejidos como la médula ósea (Jones et al., 2008). Asimismo, la tasa de proliferación en MSCs, procedentes de UC es mayor en comparación con el tejido adiposo y la médula ósea, presentando esta última la tasa más baja (Baksh et al., 2007; Jin et al., 2013). Por otro lado, mientras que la frecuencia de las MSCs procedentes de la médula ósea (BM-MSCs) corresponde a 1 en 50.000-100.000 células del total presentes en la médula ósea (Fraser et al., 2006; Kern et al., 2006), la frecuencia de MSCs en tejido adiposo es de 1 por cada 100 células, lo cual supone una diferencia de casi 500 veces más cantidad de células en el tejido adiposo. Esta variabilidad entre unas mesenquimales y otras puede deberse, en parte, a que proceden de distintos microambientes o al empleo de distintos protocolos de expansión.

Aunque las MSCs poseen una gran capacidad de renovación *in vitro*, esta parece no ser indefinida. De hecho, existen estudios que muestran que las MSCs entran en senescencia tras haber duplicado su población aproximadamente unas 20 veces, perdiendo finalmente su capacidad de diferenciación (Digirolamo et al., 1999). Por otro lado, el cultivo prologado de las MSCs también es causa de alteraciones genéticas que pueden modificar sus propiedades. Los riesgos inherentes de una larga expansión en cultivo podrían evitarse con el uso de una fuente celular abundante como por ejemplo el tejido adiposo.

2.1.2.4.1.1. Células madre derivadas de tejido adiposo

El tejido adiposo presenta varias ventajas en relación con las otras fuentes de MSCs. En primer lugar, es un tejido muy abundante que se encuentra en todos los individuos y cuya extracción puede realizarse al propio paciente mediante la administración de anestesia local (Zuk et al., 2002). En segundo lugar, las células mesenquimales se aíslan fácilmente de los lipoaspirados, se cultivan y proliferan *in vitro* sin requerimientos especiales. Y por último, al ser extraídas del propio paciente no presentan problemas de respuesta inmune, por lo que constituyen una alternativa muy válida para terapia celular (Zuk et al., 2001).

Estas células madre mesenquimales aisladas de tejido adiposo han sido nombradas con diversos términos que van desde preadipocitos, células del estroma, células procesadas de lipoaspirado, células madre multipotentes derivadas de tejido adiposo (hMADS).o células madre adultas derivadas de tejido adiposo. Sin embargo, en la conferencia de "International Fat Applied Technology Society", se llegó al conceso para la recomendación del término "células madre derivadas de tejido adiposo" (ASCs) (Locke et al., 2011; Bourin et al., 2013).

Además de su capacidad de diferenciación hacia linajes mesodérmicos se ha descrito que son capaces de diferenciarse en células de orígenes endodérmico y ectodérmico tales como las células neuronales, células pancreáticas endocrinas, hepatocitos, células epiteliales y cardiomiocitos (Peran et al., 2010; de Girolamo et al., 2013).

La obtención de estas células se lleva a cabo mediante la digestión del tejido adiposo con colagenasa y su posterior centrifugación de la cual se obtiene un pellet de células conocido como la fracción del estroma vascular (SVF). La SVF recién aislada contiene una mezcla de células, que no sólo incluyen ASCs, sino que también contiene células endoteliales, células del músculo liso, pericitos, fibroblastos y células circulantes tales como leucocitos, HSCs o EPCs. Para obtener una fracción más purificada el método más común es el cultivo prolongado de la SVF, que se basa en la capacidad de las ASCs por competir con otras poblaciones celulares bajo condiciones de cultivo prolongadas en el tiempo (Tholpady et al., 2006). La expresión de marcadores estromáticos incrementa con la realización de varios pases, lo que resulta en una población menos heterogénea (Mitchell et al., 2006).

Se ha descrito que del tejido adiposo de la articulación de la rodilla, conocido como la grasa infrapatelar de Hoffa, se pueden aislar una población de ASCs denominadas "infrapatelar stem cells" (IFPSCs). Dicha grasa es generalmente extraída de la articulación de pacientes sometidos a artroscopia o a cirugía para la implantación de prótesis de rodilla (English et al., 2007; Buckley et al., 2010b). Resultados preliminares de un ensayo clínico con pacientes con OA demuestran que la inyección en la articulación de IFPSCs autólogas es un procedimiento seguro que mejora el dolor y la funcionabilidad de la rodilla osteoartrítica (Koh and Choi, 2012).

2.2. **Células progenitoras**

Como hemos mencionado anteriormente el potencial de diferenciación de las SC puede variar dependiendo de la madurez de los tejidos y su origen. Conforme las SC pierden potencial se van comprometiendo hacia una línea celular más específica, es decir, hacia las llamadas células progenitoras, las cuales se consideran un tipo intermedio entre las células madre y las células diferenciadas (Steindler, 2007). Por ejemplo, las células progenitoras hematopoyéticas tienen restringida su diferenciación hacia el linaje linfoide o bien hacia el linaje mieloide, mientras que las células madre hematopoyéticas pueden diferenciarse en cualquier tipo celular de ambos linajes siendo capaces de regenerar, a largo plazo, todo el sistema hematopoyético. Las células progenitoras tienen capacidad de proliferación y pueden diferenciarse hacia una o varias células maduras con marcadores de linaje específicos. Las células maduras resultantes son

células funcionalmente activas que también pueden proliferar. Las células progenitoras son las responsables de mantener una fisiología normal y compensada para dar respuesta a las necesidades fisiológicas diarias

2.2.1. **Células progenitoras endoteliales**

Las EPCs son células progenitoras capaces de diferenciarse en células endoteliales maduras (Asahara et al., 2011). Las EPCs fueron identificadas por primera vez en 1997 cuando Asahara y su equipo aislaron de la sangre periférica de adulto una población celular derivada de la médula ósea capaz de diferenciarse en células endoteliales y formar nuevos vasos en zonas de isquemia (Asahara et al., 1997).

El descubrimiento de las EPCs supuso un nuevo paradigma para la reparación endotelial y la vasculogénesis postnatal. De tal forma que hasta hace poco tiempo se pensaba que la revascularización del tejido isquémico ocurría a través de la migración y proliferación de células endoteliales maduras presentes en los tejidos cercanos, mediante el proceso conocido como "angiogénesis", sin embargo, estas células tienen un potencial de proliferación bajo, por lo que su capacidad para reemplazar otras células endoteliales dañadas y crear nuevos vasos es muy limitada. Actualmente, se sabe que después de una lesión isquémica las EPCs migran desde la médula ósea hasta los sitios de neovacularización, donde se diferencian en células endoteliales, imitando así un proceso que ocurre durante la embriogénesis, la "vasculogénesis" (Krenning et al., 2009b). Las EPCs se movilizan desde de la medula ósea a la circulación en respuesta al estrés y/o a factores liberados como consecuencia de un daño. Los factores más relevantes conocidos para estimular la movilización de EPCs incluyen el VEGF, SDF-1, MCP-1 y G-CSF (Cottler-Fox et al., 2003; Hattori et al., 2003). El factor de crecimiento del endotelio vascular (VEGF) destaca por ser un factor clave en la mediación de la proliferación, diferenciación y quimiotaxis de las células endoteliales (Asahara et al., 1999b).

Existe bastante controversia en cuanto al aislamiento y caracterización de las EPCs, como resultado, no existiendo una definición clara para estas células. En la actualidad, se ha aceptado que las EPCs son un grupo heterogéneo de células que exhiben una

variedad de funciones dependiendo del método de aislamiento y cultivo (Vaughan and O'Brien, 2012).

Aunque no hay unos marcadores específicos para las EPCs y muchos de ellos coinciden con marcadores de células madre hematopoyéticas y células endoteliales, comúnmente han sido caracterizadas por: aislarse a partir de las células mononucleares (MNC) de la sangre, adherirse a fibronectina, la captación de lipoproteínas de baja densidad acetiladas (LDL), unirse a lectinas como Ulex europaeus agglutinin I (UEA-I) y expresar marcadores de expresión de proteínas de células endoteliales como CD31, CD144 (vascular endotelial [VE]-cadherina), el factor de von Willebrand (vWF) y la sintasa de óxido nítrico endotelial celular (eNOS) (Krenning et al., 2009b).

En cuanto a su aislamiento, es necesaria la extracción de las MNC sanguíneas mediante un gradiente de densidad con ficol, tras el cual pueden seguirse varios protocolos. Por un lado, se puede seguir un protocolo de selección con un marcador de superficie, generalmente el CD34, o bien combinaciones de ellos (mediante sorter o columnas magnéticas) o por otro lado, cultivar las MNC directamente en placas cubiertas con fibronectina para facilitar su adherencia (Asahara et al., 1997; Peichev et al., 2000). Se ha visto que tanto las células seleccionadas con CD34, como aquellas sin selección previa, incrementan la expresión de marcadores endoteliales con el tiempo en cultivo. Se ha estimado que aproximadamente en el día 6 adquieren un fenotipo hacia un linaje endotelial y se vuelven positivas para VEGF, VE-cadherina, UEA-I y LDL (Urbich and Dimmeler, 2004).

Independientemente del método de aislamiento, dos tipos de células parecen surgir del cultivo de las MNC, por lo que se ha desarrollado una clasificación que define las EPCs como EPCs tempranas y EPCs tardías o maduras. Las EPCs entre los días 4 y 7 se consideran EPCs tempranas y después de 7 días comienzan a perderse, por tanto la población resultante de EPCs tardías se convierte en la población que prevalece (Vaughan and O'Brien, 2012).

Las EPCs tempranas poseen características parecidas a los monocitos (Hirschi et al., 2008). Estas células también son conocidas como unidades formadoras de colonias (CFU), EPC CD14+ (Krenning et al., 2009a) o células circulantes angiogénicas (CACs)

(Akita et al., 2003). Se caracterizan por formar grupos de células con una morfología en forma de huso que aparecen después de tan solo 4-7 días de cultivo. Las EPCs CD14+ poseen un potencial transitorio proliferativo *in vitro* y no se les puede realizar pases (Zhang et al., 2006). Mientras que el cultivo durante períodos de tiempo más largos (7-21 días) da lugar a las EPCs tardías, también llamadas "outgrowth endothelial cells" (OECs) o endothelial outgrowth cells (EOCs) que muestran morfologías de adoquines o empalizadas y poseen capacidad de proliferación y de formar vasos, por lo que son consideredas más parecidas a las células de tipo endotelial (Ingram et al., 2004). Las EPCs tardías también son conocidas como EPC CD34+ (van der Strate et al., 2007), ya que se piensa que se originan a partir de las células madre hematopoyéticas CD34 + (Yoder et al., 2007).

Las EPCs pueden ser aisladas de la médula ósea (BM-EPCs), la placenta, la sangre periférica (PB-EPCs) y del cordón umbilical (UCB-EPCS). Aunque estudios recientes han reportado su aislamiento en otras localizaciones como nichos perivasculares, hígado o intestino o tejido adiposo (Basile and Yoder, 2014). Entre estas fuentes, la sangre circular periférica y la sangre de cordón umbilical destacan por ser fáciles de obtener, a partir de procedimientos no invasivos y por sus propiedades angiogénicas (Mead et al., 2008; Strunk, 2011). Sin embargo, se ha demostrado que existen algunas diferencias entre ellas, por ejemplo las EPCs obtenidas de sangre de cordón umbilical poseen una mayor tasa de replicación en comparación con las EPCs aisladas de PB (Brown et al., 2010). Ambas fuentes expresan similares marcadores de células endoteliales pero se ha visto que las UCB-EPCs presentan mayor expresión de CXCR4 que las BM-EPC, un receptor de quimioquinas implicadas en la capacidad de llamada (*homing*) y además menos marcadores del estroma (CD105, CD73) (Finney et al., 2006). Existe aproximadamente 7 veces más cantidad de EPCs en UCB que en PB, no obstante el número de EPCs en sangre puede incrementar cuando se movilizan de la médula ósea mediante diversos factores de crecimiento, citoquinas, drogas u hormonas (Dzau et al., 2005; Zammaretti and Zisch, 2005).

Factores tales como el ejercicio y la edad puede aumentar y disminuir respectivamente la cantidad de EPCs en sangre (Alobaid et al., 2005; Dzau et al., 2005). Por ejemplo, se ha evidenciado que las EPCs de pacientes diabéticos proliferan un 48% menos que las EPCs procedentes de pacientes sanos (Tepper et al., 2002). Por otro lado, el número de

EPCs circulantes se correlaciona negativamente con diversos factores de riesgo, como la aterosclerosis y con trastornos asociados con la disfunción vascular (Hill et al., 2003; Aicher et al., 2005). Por el contrario, el insulto isquémico induce la movilización de EPCs de la médula ósea elevando el número de EPCs en sangre que acude a los sitios de la neovascularización (Shintani et al., 2001; Massa et al., 2005).

Las EPCs que se movilizan al tejido isquémico pueden permanecer en el espacio intersticial y mejorar la angiogénesis mediante la secreción de proteínas citoprotectoras, citoquinas y factores de crecimiento que promueven la proliferación y la migración de células endoteliales locales. Entre los factores que secretan se incluye el VEGF, el factor de crecimiento de hepatocitos (HGF), angiopoyetina-1 (Ang1), eNOS, la óxido nítrico sintasa inducible (iNOS), SDF-1α, y el factor de crecimiento insulina 1 (IGF-1) (Jujo et al., 2008).

Debido a que las EPCs contribuyen de forma directa o indirecta a la neovascularización en el adulto, poseen gran potencial para su uso en terapias celulares para el tratamiento de diversas enfermedades isquémicas.

3. Diferenciación Celular

La diferenciación celular es un proceso por el cual las células se vuelven más especializadas de forma que adquieren una nueva identidad. Los estados de diferenciación a menudo se consideran consolidados e irreversibles, sin embargo en algunos casos el balance de los factores de transcripción pueden cambiar y las células pueden interconvertirse. De hecho, los estados de diferenciación estables están controlados por mecanismos dinámicos que pueden ser alterados bajo ciertos estímulos obteniendo como resultado un nuevo patrón de genes de expresión, convirtiéndose así un tipo celular en otro.

Desde que se conoce que los estados de diferenciación celular no son inamovibles han surgido nuevos puntos vista sobre la plasticidad de las células animales (Eguchi and Kodama, 1993; Slack and Tosh, 2001). En este sentido, varios procesos se han asociado con la regeneración, estos incluyen procesos en los que células diferenciadas pueden

convertirse en células progenitoras, (desdiferenciación), células diferenciadas pueden convertirse en otro tipo celular (transdiferenciación) o bien células somáticas pueden ser inducidas hacia la pluripotencia (reprogramación) (Eguizabal et al., 2013), (Figura 4).

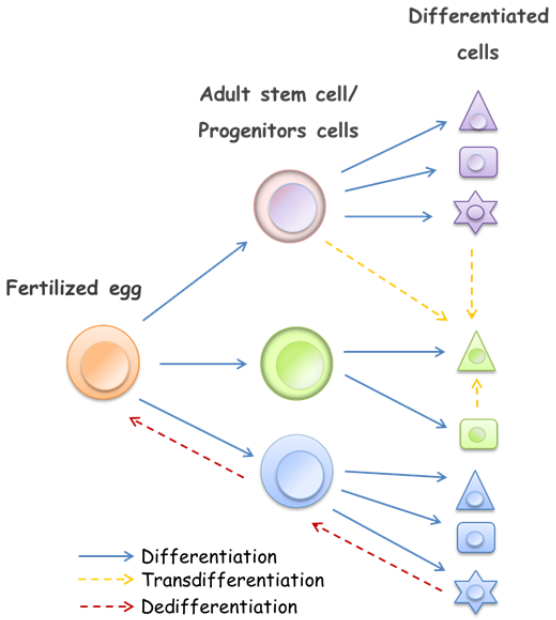


Figura 4. Diagrama esquemático de la diferenciación celular, transdiferenciación y desdiferenciación. Figura adaptada de Sugimoto et al., 2011.

3.1. Desdiferenciación

Es el proceso por el cual células diferenciadas con funciones específicas revierten hacia un fenotipo más temprano que posee mayor plasticidad. La desdiferenciación es un proceso crucial que ocurre principalmente de forma natural en algunos sistemas de regeneración, los cuales suelen pertenecer a formas de vida simples, como gusanos, anfibios e incluso plantas.

El proceso de desdiferenciación conlleva la pérdida de características distintivas del tipo celular, tales como marcadores estructurales típicos o el reingreso en el ciclo celular de células que normalmente no son capaces de dividirse. Si bien, la entrada en el ciclo celular no ocurre en todos los casos (Monje et al., 2010), sí parece estar relacionada con los procesos de regeneración, de forma que la proliferación celular permite obtener células que sustituyen a las que se han perdido.

Un conocido ejemplo de desdiferenciación natural consiste en la capacidad de regeneración miocárdica de varias especies de vertebrados no mamíferos tales como el pez cebra, el cual es capaz de regenerar totalmente su corazón después de la amputación de hasta un 20% del ventrículo (Jopling et al., 2010). Durante la regeneración del corazón, los cardiomiocitos del pez cebra se desdiferencian facilitando así su reingreso en el ciclo celular. Los cardiomiocitos adyacentes al sitio del daño comienzan un proceso de desdiferenciación de forma que desmontan sus estructuras sarcoméricas, dejan de expresar ciertos genes cardiacos y re-expresan genes típicos de inmadurez, tales como *GATA-4* (Kikuchi et al., 2010). A continuación, los cardiomiocitos entran en el ciclo celular para finalmente diferenciarse en cardiomiocitos maduros que sustituyen a las células perdidas (Jopling et al., 2010).

En los mamíferos, después de un proceso de desdiferenciación la capacidad de regeneración es limitada (Eguizabal et al., 2013). A pesar de ello, se ha observado en un modelo de ratón que células de Schwann poseen la capacidad natural para regenerarse tras un daño, previo paso de un proceso de desdiferenciación (Mirsky et al., 2008).

Por otro lado, la desdiferenciación *in vitro* puede llevarse a cabo mediante condiciones de cultivo específicas. De esta forma, se ha demostrado que células terminalmente maduras sin capacidad de proliferación pueden revertir su fenotipo y adquirir características de células madre multipotentes. Por ejemplo, se ha evidenciado que los adipocitos humano son capaces de desdiferenciarse dando lugar a fibroblastos libres de lípidos, llamados DFAT con potencial de diferenciaciación en adipocitos, osteocitos, condrocitos y miocitos (Roobrouck et al., 2011; Shen et al., 2011). Asimismo, la adquisición de plasticidad *in vitro* hacia un progenitor o células multipotentes se ha observado en células foliculares humanas (Suzuki et al., 2011), keratinocitos humanos (Sun et al., 2011), células β (Talchai et al., 2012) y condrocitos (de la Fuente et al., 2004).

Existen estudios *in vitro* que muestran como MSCs diferenciadas hacia un fenotipo son capaces de desdiferenciarse y adquirir un fenotipo más primitivo (Song et al., 2006). Este es el caso de las MSCs de fluido amniótico en las que se ha observado un proceso de desdiferenciación después de haber sido diferenciadas hacia adipocitos, por el cual incrementaban su expresión para marcadores de pluripotencia (Zagoura et al., 2013).

Se necesitan más estudios para comprender este proceso ambiguo que hasta lo que hoy conocemos es una estrategia de algunos sistemas animales para volver a adquirir plasticidad y así dar lugar a nuevos tejidos. Debido a que estudios *in vitro* e *in vivo* han demostrado que la desdiferenciación se produce durante la regeneración de los tejidos, la comprensión de este fenómeno podría ayudar a investigar los genes implicados en el mantenimiento de la función celular, así como a promover la regeneración en tejidos que carecen de esta capacidad.

3.2. Transdiferenciación

Este proceso consiste en un cambio irreversible de una célula diferenciada en otra, lo que resulta en la pérdida de un fenotipo y la ganancia de otro (Tosh and Slack, 2002; Rawlins and Hogan, 2006).

Los criterios establecidos para reconocer un proceso como una transdiferenciación consisten en: i) definir claramente el fenotipo de la célula antes y después de la transdiferenciación mediante cambios morfológicos, a nivel molecular y bioquímicos, y ii) establecer la relación de linaje entre los dos tipos celulares (Eguchi, 1986; Eguchi and Kodama, 1993).

A menudo se piensa que la transdiferención es más fácil si la célula de partida y la célula final comparten una misma historia evolutiva. Tal es el caso del hígado y del páncreas, los cuales mantienen una estrecha relación evolutiva (Burke et al., 2006). Como resultado de esta relación, se ha demostrado la transdiferenciación de hepatocitos adultos a células pancreáticas, así como células exocrinas pancreáticas en células β productoras de insulina (Zhou et al., 2008; Eberhard et al., 2010). Sin embargo, son cada vez más los estudios que demuestran que las células de partida pueden convertirse con éxito en células que proceden de una capa germinal distinta. Esto ocurre por ejemplo en la generación de células progenitoras sanguíneas a partir de fibroblastos dérmicos humanos (Szabo et al., 2010).

Durante la transdiferenciación un conjunto de genes dejan de expresarse mientras que un nuevo conjunto comienza a hacerlo, esto implica que los productos de la célula inicial pueden permanecer al mismo tiempo que se están expresando los de la segunda.

No obstante, en algunos sistemas de regeneración natural se ha observado que ocurre un proceso de desdifenciación como paso previo a la transdiferenciación (Eguchi et al., 1974; Roberson et al., 2004; Jarriault et al., 2008). De hecho, la primera vez que se observó de forma natural el fenómeno de la transdiferenciación fue hace más de 100 años, cuando se descubrió que la lente del tritón se regenera tras ser eliminada. Esto es debido a que las células epiteliales pigmentadas del iris dorsal entran en el ciclo celular, pierden su pigmentación y finalmente se transdiferencian en células del cristalino (Eguchi et al., 1974).

Mientras que la transdiferenciación natural tiene lugar en algunos vertebrados e invertebrados durante los procesos de desarrollo y regeneración en respuesta a determinadas señales locales, la transdiferenciación inducida se considera una tecnología prometedora que se basa en la manipulación *in vitro* de genes o el tratamiento de células con ciertos componentes para promover así la conversión de un tipo celular en otro con fines tales como el estudio del desarrollo, modelos de enfermedades, investigación de fármacos o remplazo celular (Sisakhtnezhad and Matin, 2012).

La transdiferenciación *in vitro* e *in vivo* se ha logrado mediante el uso de uno o varios genes maestros. La primera vez que se demostró la viabilidad de este proceso fue en 1987 cuando se descubrió que la expresión forzada del gen MyoD era suficiente para convertir fibroblastos de ratón en myofibroblastos (Davis et al., 1987). Asimismo, la expresión de este gen puede inducir un fenotipo muscular en otros tipos celulares tales como adipocitos, células de músculo liso o hepatocitos. Los miofibroblastos generados expresan marcadores específicos de este fenotipo celular como la miosina de cadena pesada (*MHC*) y la miosina de cadena corta 2 (*MLC2*) (Taylor and Jones, 1979; Weintraub et al., 1989; Tapscott, 2005).

Estudios recientes muestran que la transdiferenciación de células somáticas es posible sin pasar por un estado de pluripotencia mediante el uso de factores de transcripción específicos que definen una identidad celular (Ieda et al., 2010). Por ejemplo, se ha descrito con éxito distintas combinaciones de factores para la reprogramación de: células pancreáticas exocrinas en células β (*PDX1, NGN3* y *MAFA*) (Zhou et al., 2008), fibroblastos en neuronas (*ASCL1, BRN2*, también conocido como *POU3F2* y *MYT1L*)

(Vierbuchen et al., 2010) y fibroblastos en cardiomiocitos (*GATA-4, Mef2c* y *TBX5*) (Ieda et al., 2010).

Estos experimentos han arrojado esperanza a la idea de que algún día la transdiferenciación terapeútica sea posible. En ese caso, sus aplicaciones clínicas serían numerosas, por ejemplo en órganos como el corazón, donde tras una lesión isquémica se generan fibroblastos que podrían ser transdiferenciados hacia cardiomiocitos. Sin embargo, para lograr la implantación de esta prometedora terapia regenerativa se deben lograr grandes mejoras con respecto a la eficiencia y la seguridad de esta técnica. Ejemplos de estudios de transdiferenciaciones en mamíferos se han incluido en la tabla 1.

Tabla 1. Ejemplos de factores de transcripción implicados en la transdiferenciación:

Starting Cell Type	Final Cell Type	Transcription Factors implicated	References
Adult hepatic oval stem cells	Pancreatic insulin secreting cells	-*LIF and* high glucose	(Yang et al., 2002)
Adult hepatocytes	Pancreatic cells	*PDX1*	(Meivar-Levy et al., 2007)
Pancreatic exocrine cells /Fibroblast	Pancreatic insulin secreting cells	*PDX1, NGN3 and MAFA*	(Zhou et al., 2008)
B cells	Macrophages	*E2A, EBF,* and *PU.1/CEBPα- and CEBPβ*	(Xie et al., 2004)/ (Rodriguez-Ubreva et al., 2012)
Fibroblasts	Blood Progenitors	*OCT4* + citokines	(Szabo et al., 2010)
Fibroblasts	Neurons	*ASCL1, BRN2* and *MYTL1*	(Vierbuchen et al., 2010)
Fibroblast	Cardiomyocytes	*GATA-4, Mef2c* and *TBX5*	(Ieda et al., 2010)
Fibroblasts, pigment, nerve, fat, liver cells	Myofribroblast	*MYO*	(Davis et al., 1987)
Fibroblasts	Hepatocyte	*HNF4A PLUS FOXA1, FOXA2* or *FOXA3*	(Sekiya and Suzuki, 2011)
Myoblasts	Adipocytes	*PPARΓ, C/EBPA*	(Rosen et al., 2002)

A pesar de que la transdiferenciación se considera la conversión irreversible de una célula diferenciada en otra distinta a la original, el término transdiferenciación se emplea igualmente para definir la diferenciación de células madre adultas, siempre que cambien su "destino" y se diferencien en células un linaje distinto del que estaban predestinadas (Song and Tuan, 2004; Song et al., 2006; Barzilay et al., 2009b; Peran et al., 2011).

3.3. Reprogramación hacia la pluripotencia

La reprogramación es el proceso en el que una célula completamente diferenciada es inducida hacia la pluripotencia adquiriendo el potencial para poder ser diferenciada en cualquier tipo celular.

La reprogramación puede lograrse mediante la transferencia de núcleos somáticos, fusión celular con ESCs, exposición a extractos celulares de células madre o mediante la introducción en células somáticas de factores de transcripción implicados en el mantenimiento de la pluripotencia de las ESCs, dando lugar a las iPS cells (Sumer et al., 2011). La reprogramación con factores de transcripción comenzó con el uso de 4 factores (*Oct4, Sox2, cMyc*, and *Klf4*) definidos por el Dr Yamanaka (Takahashi and Yamanaka, 2006) que fueron introducidos en fibroblastos de ratón. Desde entonces esta técnica se ha ido modificando en cuanto al número de factores de transcripción, la estrategia de entrada y el tipo celular empleado (Bayart and Cohen-Haguenauer, 2013). Por ejemplo, con el fin de evitar inserciones en el genoma o mutaciones no deseadas, en lugar de la introducción de ADN con vectores virales, se está estudiando el uso de ARNm sintético, transgenes escindibles, proteínas recombinantes y genes episomales. Por otro lado, se ha sugerido que cuanta más inmadura es la célula menos factores son necesarios para su reprogramación.

Es de reseñar que las MSCs han sido uno de los tipos celulares empleados para ser reprogramadas en iPS cells (Park et al., 2008a; Park et al., 2008b; Buccini et al., 2012). Recientemente, MSCs de ratón han sido inducidas hacía células madre pluripotentes mediante una transducción retroviral de los factores *Oct4, Sox2, Klf4* y *c-Myc.* Además, estas MSCs pluripotentes llamadas MIPS, han sido diferenciadas con existo hacia progenitoras cardiacas mostrando latidos espontáneos y la expresión de factores de

transcripción y proteínas específicas cardiacas incluyendo *GATA-4, Mef2C, Nkx2.5*, la cadena pesada de la miosina, troponin I y la troponina T. Tras 4 semanas de implantación en un modelo de ratón con infarto de miocardio, no se observó la formación de tumores pero sí una mejora general de la función cardiaca (Chen et al., 2013).

4. Principales métodos para inducir la diferenciación

Debido a su gran plasticidad, las SC pueden ser diferenciadas mediante el uso de diferentes metodologías. A continuación se resumen algunas de las técnicas más comunes empleadas para dirigir la diferenciación de las SC. Dado que han sido objeto de estudio de esta tesis, prestaremos especial interés a los métodos dirigidos hacia la diferenciación condrocítica y cardiomiocítica.

4.1. Medio de cultivo definido

Una opción habitualmente utilizada para inducir la diferenciación son los medios de cultivo suplementados con una gran variedad de moléculas biológicas tales como las citoquinas, factores de crecimiento o compuestos químicos sintéticos o naturales.

4.1.1. Factores de crecimiento y citoquinas

Entre los factores de crecimiento con efectos reguladores en las SC más destacados se encuentran los siguientes: los miembros de la familia del factor de crecimiento transformante beta (TGF-β), factores de crecimiento de la insulina (IGF), los factores de crecimiento de los fibroblastos (FGF), el factor de crecimiento epidérmico (EGF), el factor de crecimiento derivado de plaquetas (PDGF), el factor de crecimiento endotelial vascular (VEGF), y la familia de factores de crecimiento conocidos como Wnt (Liu et al., 2009).

Durante el proceso de condrogénesis están implicados numerosas citoquinas y factores de crecimiento, muchos de los cuales han mostrado tener funciones similares e incluso participar en la diferenciación osteogénica. Las moléculas inductivas más potentes para

dirigir la diferenciación condrogénic son: la familia de TGF- β, incluyendo el TGF-β1, TGF-β2 y TGF-β3, así como las proteínas morfogenéticas óseas (BMPs), conocidas por su participación en la formación de cartílago. Dentro de la familia TGF- β se incluyen otras citoquinas como la activina y el GDF-5 (Sun et al., 2012). Los efectos de estas citoquinas se transducen principalmente a través de dos vías de señalización intracelular, una de ellas incluye las moléculas de señalización de la familia SMAD (Song et al., 2009), mientras que en la otra participa la proteína quinasa activada por mitógenos (MAPK) (Bobick and Kulyk, 2008). Otras citoquinas conocidas por tener un papel importante en la condrogénesis son las formadas por las diversas isoformas del factor de crecimiento de fibroblastos (FGF) (Ellman et al., 2013) y el IGF-1. Asimismo se han identificado diversas proteínas capaces de estimular la condrogénesis que incluyen la prolactina, la interleucina-1β, Cyr61, HBGAM, y la hormona de crecimiento (Mahmoudifar and Doran, 2012). La combinación de factores de crecimiento y/o citoquinas parece que aumenta la capacidad de diferenciación condrocítica de las MSCs (Puetzer et al., 2010; Danisovic et al., 2012). De hecho recientemente se ha demostrado que citoquinas quiméricas, formadas por la unión de activina y BMP-2 y de activina y Nodal inducen activamente la diferenciación condrocítica de ASCs (Esquivies et al., 2013; Peran et al., 2013b).

Para el desarrollo de medios de cultivo dirigidos a la diferenciación cardiomiogénica también son de especial interés la familia de TGF-β1, BMP-2 y BMP-4. Recientemente, se ha desarrollado un cóctel de proteínas similares a las producidas por el endodermo para dirigir el fenotipo de las células progenitoras mesenquimales hacia un fenotipo cardíaco. Dicho coctel consiste en una combinación de TGF-β1, BMP-4, activina A, ácido retinoico, IGF-1, FGF-2, alfa-trombina y la interleucina-6. Las MSCs tratadas con dicho cóctel fueron inyectadas en el miocardio de ratones infartados y tras un año de seguimiento se observó una mejora de la funcionabilidad cardiaca en comparación con los controles. Asimismo, las MSCs inducidas expresaban marcadores cardiacos demostrando así su prediferenciación hacia MSCs cardiopoyéticas (Behfar et al., 2010).

4.1.2. Productos químicos/ sintéticos

Al igual que los factores de crecimiento, otros productos químicos tienen un papel crítico en la determinación del linaje de las SC. Por ejemplo, se ha comprobado que

medios de cultivo suplementados con nicotinamida y β-mercaptoetanol inducen la diferenciación de las MSCs de rata en células de islotes β (Chen et al., 2004).

Por otro lado, la incubación de las MSCs con dexametasona , insulina, isobutil metil xantina y la indometacina promueve la diferenciación hacia el linaje adipogénico. De forma que, MSCs tratadas con estos compuestos acumulan en su interior vacuolas ricas en lípidos y expresan el receptor activador del proliferador de peroxisomas (*PPAR*) (*PPARγ2*), la lipoproteína lipasa (*LPL*) y la proteína de unión a ácidos grasos (*aP2*) (Pittenger et al., 1999).

La diferenciación de MSCs en células similares a las neuronas también ha sido descrita mediante el uso de compuestos químicos simples como el dimetil sulfóxido (DMSO) y el hidroxianisol butilado, así como con la adición de ácido retinoico que facilita su diferenciación neuronal (Woodbury et al., 2000; Safford et al., 2002). Sin embargo, estas células no se han considerado como auténticas neuronas por carecer de los canales iónicos dependientes de voltaje necesarios para la generación de potenciales de acción (Hofstetter et al., 2002).

Por otro lado, el método clásico para la diferenciación osteogénica *in vitro* de las MSCs implica la incubación de una monocapa confluente de MSCs con combinaciones de dexametasona, beta-glicerofosfato y ácido ascórbico durante varias semanas (Okamoto et al., 2002). La diferenciación osteogénica se ha demostrado mediante el análisis de los depósitos mineralizados, la medición de la actividad de la fosfatasa alcalina y por el estudio de la expresión de genes específicos osteogénicos mediante RT-PCR (Zakharov Iu and Makarova, 2013).

Entre los productos químicos sintéticos conocidos por promover la diferenciación cardiomiogénica se encuentran la 5-aza, la anfotericina B (Wakitani et al., 1995), el ácido ascórbico, el ácido retinoico (Zandstra et al., 2003), DMSO y dinorfina B (Ventura and Maioli, 2000). La 5-aza es un nuclesido sintético que se utiliza comúnmente como un inhibidor de la metilación del ADN ya que inhibe la ADN metiltransferasa causando la desmetilazion del ADN y la reactivación de genes silenciados (Christman, 2002). Se trata de un potente inductor de la diferenciación cardiomiogénica tanto en células madre embrionarias como en adultas (Rangappa et al.,

2003). Las MSCs tratadas con 5-aza muestran una morfología parecida a los cardiomiocitos y expresan marcadores y genes específicos de diferenciación cardiaca (Wakitani et al., 1995; Kadivar et al., 2006), incluso se ha observado que son capaces de transformase en células contráctiles similares a los cardiomiocitos (Rangappa et al., 2003; Strem et al., 2005; Choi et al., 2010).

Del mismo modo han sido descrito diversos compuestos químicos que promueven la diferenciación condrogénica *in vitro* entre ellos se encuentra la dexametasona y el ácido ascórbico como los más destacados y el uso de otros como la hormona tiroidea, la 1,25-dihidroxi vitamina D3, la prostaglandina E2, la estaurosporina, AMPc dibutryl, concavalina A, vanadato, FK506 y suplementos (insulina, transferrina, ácido selenioso, ácido linoleico y BSA) (Sottile et al., 2002; Heng et al., 2004). La dexametasona es un glucocorticoide sintético reconocido como un potente inductor de la diferenciación condrogénica en MSCs humanas, al igual que el ácido ascórbico (vitamina C), del cual es sabido que estimula la diferenciación condrogénica promoviendo la síntesis de 1,25-dihidroxi-vitamina D3 y la producción de matriz del cartílaginosa (Heng et al., 2004).

4.2. Factores de transcripción

Se ha evidenciado que la expresión de uno o varios genes maestros en las SC pueden activar un fenotipo celular específico. De hecho, la sobreexpresión de solo uno factor de transcripción ha logrado promover la diferenciación de MSCs en células β pancreáticas, células neuronales y células endoteliales e incluso conservarla tras ser extraídas del cultivo e implantadas en animales (Li et al., 2007; Kim et al., 2008; Barzilay et al., 2009a).

Entre los factores de trascripción implicados en la diferenciación condrogénica se encuetran *Sox-9*, *Sox-5*, y *Sox-6*, que son inducidos por la activación de varias vías de señalización intracelulares, como la vía de proteínas quinasas activadas por mitógenos (MAPK), y Smads. La inducción de estos factores resulta en la síntesis de proteínas de la matriz extracelular (ECM), tales como el colágeno de tipo II, el proteoglicano agrecano y la proteína de la matriz oligomérica del cartílago (COMP), requerida para la formación del cartílago. Los factores de transcripción *Sox-5 y Sox-6* se expresan aguas abajo de *Sox-9* y difieren de *Sox-9* en que no poseen un dominio de transactivación, por

tanto, *Sox-5* y *Sox-6* no afectan a la expresión de genes directamente, aunque se cree que pueden alterar la expresión génica mediante el reclutamiento de otros activadores de la transcripción (Frith and Genever, 2008). De hecho, la mutación de *Sox-9* durante la formación de las condesaciones mesenquimales en el desarrollo embrionario detiene la condrogenesis (Akiyama et al., 2002). Se ha demostrado tanto *in vitro* como *in vivo* que la expresión de *Sox-9* en MSCs, mediante la transfección con virus (Cao et al., 2011; Yang et al., 2011b) e incluso con nanopartículas (Jeon et al., 2012), induce la diferenciación hacia condrocitos.

Otro gen maestro, en este caso de la osteogénesis es *Runx2*. No obstante, *Runx2* también posee un papel relevante en la regulación de la condrogénesis ya que mientras *Sox-9* es expresado en la condrogénesis temprana, *Runx2* es expresado durante la etapa hipertrófica de la condrogénesis tardía activando otros genes de la matriz del cartílago, tales como el colágeno tipo I, α1 (*COL1A1*), Indian hedgehog (*Ihh*), osteopontina (Yamashita et al., 2009). Por tanto, la sobreexpresión de *Runx2* en los condrocitos conduce a la osificación endocondral (Enomoto-Iwamoto et al., 2001).

En cuanto a la diferenciación cardiaca, los genes *GATA-4*, *Nkx 2.5* y *Mef2c*, son considerados genes maestros por su papel en la cardiomiogénesis (Zheng et al., 2003). Durante estadios tempranos del desarrollo embrionario *Nkx2.5* comienza a promover la diferenciación del mesodermo en tejido cardíaco. *Nkx 2.5* regula la expresión de myocardin, necesario para la cardiomyogenesis (Ueyama et al., 2003), tal es la relevancia de este factor que se ha demostrado que su mutación detiene el desarrollo embrionario (Lyons et al., 1995). Por otro lado, la mutación de *GATA-4*, resulta en cardiopatías congénitas incluidas deficiencias en las válvulas y septos cardiacos (Rajagopal et al., 2007). *GATA-4* es también considerado uno de los factores anti-apostóticos que regulan la supervivencia de los miocitos cardíacos (Kim et al., 2003). Se ha evidenciado que la sobreexpresión de *GATA-4* en MSCs aumenta la supervivencia y el potencial angiogénico de estas células en el miocardio isquémico (Li et al., 2010). Por otro lado, *Mef2c* participa en la miogénesis temprana siendo una diana transcripcional directa de la famila *MyoD I* (Dodou et al., 2003). Se ha observado que *Mef2c* puede inducir la miogénesis esquelética cuando se sobre-expresa en células multipotentes (Ridgeway et al., 2000). En consecuencia, combinaciones con estos tres

factores han sido empleadas para la adquisición del fenotipo cardiaco en SC (Yamada et al., 2007; Behfar et al., 2008; Arminan et al., 2009; Li et al., 2010; Xu et al., 2012)

4.3. Extracto celular

El método del extracto celular, desarrollado en el laboratorio de Philip Collas, consiste en introducir los componentes intracelulares de un tipo celular en otro tipo celular distinto para dirigir la diferenciación de la célula diana hacia la estirpe de la célula origen. Este método requiere que la célula receptora esté reversiblemente permeabilizada para que los componentes nucleares y citoplasmáticos puedan entrar en la célula donante. Dicha permeabilización reversible se realiza mediante la streptolisina O (SLO), un miembro de las citolisinas dependientes de colesterol (Walev et al., 2001). Tras la exposición al extracto se añade calcio al medio para reparar las lesiones inducidas por el SLO en la membrana plasmática (Hakelien et al., 2002; Landsverk et al., 2002).

Este método se ha empleado tanto para modificar el fenotipo de células adultas diferenciadas, como por ejemplo la conversión de células HEK293T hacia un fenotipo linfoide (Hakelien et al., 2006) o el uso de extracto de células pancreáticas para transdiferenciar células hepáticas en células productoras de insulina (Peran et al., 2011), como para dirigir la diferenciación de SC. En este sentido, se ha demostrado la expresión de proteínas cardiacas en MSCs humanas tras la exposición a un extracto celular obtenido de cardiomiocitos de rata (Gaustad et al., 2004) e incluso de muestras humanas de tejido cardiaco (Peran et al., 2010).

4.4. Cocultivos y medio condicionado

En los mamíferos, la interacción entre las células está mediada por factores solubles liberados por las células al medio extracelular. Las proteínas secretadas pueden incluir proteínas de la matriz extracelular, proteinasas, factores de crecimiento, hormonas proteicas, citoquinas inmunorreguladoras, quimiocinas y otras moléculas bioactivas que tienen un impacto directo sobre el fenotipo de las células diana (Skalnikova et al., 2011).

Los cocultivos son empleados para estudiar como las moléculas solubles liberadas por una célula pueden inducir la diferenciación de otra. Para ello, las células con el fenotipo de interés son cultivadas en unas membranas con poros de entre 0.4–0.3 μm que permiten el intercambio de medio pero sin establecer contacto directo entre ambos tipos celulares.

Por otro lado, también se realizan cocultivos en los que las células crecen juntas sin la presencia de una membrana de separación. Para distinguir las poblaciones, estas son marcadas con distintos fluorocromos, tales como DAPI, DIL o con anticuerpos específicos. Varios estudios avalan este sistema y afirman que el contacto célula a célula es necesario para lograr la diferenciación *in vitro* de las SC (Iijima et al., 2003). No obstante, es muy importante diferenciar bien ambas poblaciones y realizar estudios en profundidad para discernir con claridad si se trata de un proceso de diferenciación o una fusión celular.

Se ha comprobado que el cocultivo de MSCs con cardiomiocitos de rata es capaz de inducir la expresión de marcadores fenotípicos cardiacos en las MSCs, aunque en este estudio además de la presencia de factores solubles se hizo necesario el contacto intracelular de las MSC con los cardiomiocitos (Rangappa et al., 2003; Wang et al., 2006; Plotnikov et al., 2008). De hecho, durante la organogénesis, las uniones intercelulares son factores clave, en este sentido algunos autores sugieren que la diferenciación por contacto resulta en una comunicación nanotubular que permite el transporte y el intercambio de proteínas intracelulares, moléculas e incluso orgánulos entre ambos tipos celulares. Por tanto, a través del contacto físico directo, las células crearían un espacio intracelular común, donde pueden influenciarse mutuamente (Koyanagi et al., 2005).

En cuanto a la diferenciación condrogénica se ha visto que el cocultivo en transwell de condrocitos y MSCs induce la expresión de marcadores de condrocitos en las MSCs como respuesta a los factores solubles secretados al medio por los condrocitos (Chen et al., 2009)

Otra variante del cocultivo es el uso del medio condicionado, este medio es recolectado de las células hacia cuyo fenotipo se quiere dirigir la diferenciación, cultivando las

células que se quiere diferenciar, con dicho medio. El medio condicionado contiene el secretoma de las células, es decir, las proteínas que contienen un péptido señal y que se procesan a través del retículo endoplásmico y aparato de Golgi a través de la vía de secreción clásica. Además, también se incluyen las proteínas desprendidas de la superficie de la célula y las proteínas intracelulares liberadas a través de vía de secreción no clásica o exosomas (Dowling and Clynes, 2011).

El uso de medio condicionado de cardiomiocitos de tejido cardiaco isquémico y condrocitos en cultivo también se ha visto que pueden inducir respectivamente la diferenciación cardiomiocítica y condrocítica (Hwang et al., 2007; Ramesh et al., 2012).

Por otro lado, las proteínas liberadas en el medio condicionado por las células en cultivo son una fuente de material para los experimentos de identificación de proteínas inductivas. De esta forma, el medio condicionado de células embrionarias inducidas a cardiomiocitos se ha utilizado en la busqueda de factores cardioinductivos (Arrell et al., 2008). Asimismo, en los últimos años, el medio condicionado se está empleando como alternativa útil para la identificación de biomarcadores candidatos mediante el uso de análisis proteómico (Skalnikova et al., 2011).

4.5. Soportes 3D

Como se comentó anteriormente, el tercer pilar para la regeneración de tejidos (en la mayoría de los casos) es la creación de estructuras tridimensionales que favorezcan el crecimiento celular y que mantengan una ambiente extracelular que sea lo más parecido posible al nicho en el cual las células se encuentran en vivo.

En el organismo, las células se encuentran insertas en la ECM formando estructuras complejas y típicas de cada determinado tejido. Este ambiente tridimensional permite la interacción dinámica entre las células y la ECM que facilita la comunicación de señales para el crecimiento, diferenciación, supervivencia y organización de las células presentes en el tejido (Hynes, 1999). Las células están en constante interacción con el microambiente que las rodea, reaccionando ante los cambios, y enviando señales que afectan a células vecinas. Todo esto juega un papel fundamental en el desarrollo

prenatal y en el mantenimiento postnatal de las funciones celulares (Discher et al., 2005; Calve et al., 2010).

El objetivo principal de la ingeniería de tejidos es el desarrollo de soportes celulares que reproduzcan estructuras tridimensionales con tamaño, arquitectura y propiedades físicas específicas que imiten el ambiente natural de las células con el fin de promover ciertas funciones como la adhesión celular, la movilidad y la diferenciación celular (Li et al., 2007 562; Engel et al., 2008; de Rezende et al., 2011 609).

Los requisitos clave que debe de tener un soporte son biocompatibilidad, porosidad controlada, permeabilidad, así como adecuadas propiedades mecánicas y una velocidad de degradación similar el tejido diana. Estos soportes tridimensionales se pueden dividir en sintéticos y naturales.

Los soportes-3D naturales proceden de una fuente animal o humana y están basados en componentes de la ECM, como el colágeno, el alginato, el matrigel, la agarosa o el quitosano (Freyman et al., 2001; Nahmias et al., 2005; Wang et al., 2005; Williams, 2009).

Recientemente, los tejidos descelularizados se han convertido en un material prometedor para su uso en medicina regenerativa. La eliminación de los antígenos celulares hace que el tejido descelularizado sea prácticamente inmunogénico, lo que permite su implantación sin problemas de rechazo (Cardinal and Williams, 2009). Uno de los ejemplos más destacados fue realizado en 2008 cuando se implantó una traquea descelularizada, posteriormente recelularizada en un bioreactor, con células del propio paciente, para sustituir el bronquio principal izquierdo que se encontraba dañado (Macchiarini et al., 2008). Hasta el momento el paciente se encuentra en buen estado y no ha necesitado fármacos inmunosupresores. Otros ejemplos de soportes naturales aplicados en clínica son los vasos xenogénicos para la restauración de la función vascular (Kakisis et al., 2005) (Kakisis et al., 2005 161)o el uso de dermis descelularizadas para tratar quemaduras (Badylak et al., 2009)

A pesar que los materiales naturales han demostrado resultados prometedores en la reparación de tejidos, pueden presentar algunas desventajas relacionadas con sus

propiedades mecánicas, su rápida degradación y su posible inmunogenicidad. Por lo tanto se están estudiando alternativas sintéticas con el objetivo de minimizar estos inconvenientes.

Los soportes-3D sintéticos, se construyen con materiales sintéticos o una combinación entre naturales y sintéticos. El ácido polihidróxido, politetrafluoroetileno, el acero de titanio o la cerámica son ejemplos de algunos de los polímeros sintéticos que presenta mayor biocompatibilidad (Hutmacher, 2000; Place et al., 2009). Entre los materiales sintéticos más utilizados en la fabricación de soportes a nanoescala destacan el ácido poliglicólico (PGA), el ácido poliláctico (PLA), el ácido poliláctico-coglicólico (PLGA), el alcohol de polivinilo (PVA), la policaprolactona (PCL), o el alcohol polivinílico (PVA) (Peran et al., 2012).

Por otra parte, en los soportes se pueden incorporar funciones adicionales como nanotopografías, revestimientos de proteínas o la conjugación química de moléculas de señalización específicas (Carletti et al., 2011; Chung and King, 2011; Prabhakaran et al., 2011) Se ha visto que en injertos vasculares de PLGA y stent de titanio se puede mejorar las funciones de las células endoteliales si las superficies presentan rugosidades a escala nanométrica (Miller et al., 2007; Lu et al., 2008).

4.5.1. Aplicación de soportes 3D en enfermedades osteocondrales

Se ha demostrado que materiales naturales tales como alginato, agarosa, chitosán, sulfato de condroitina, ácido hialurónico , colágeno, fibrina o fibroína de seda pueden potenciar la producción de colágeno de tipo II y glicosaminoglicanos sulfatados en las SC (Vinatier et al., 2009). Sin embargo, a pesar de su biocompatibilidad, su potencial para el uso clínico en lesiones condrales está limitado debido a que presentan una pobre resistencia mecánica, pueden causar inmunogenicidad y porque sin los reactivos químicos adecuados, pueden degradarse muy rápido tras ser implantados (Lee and Shin, 2007)

En cambio, los materiales sintéticos presentan característica más adecuadas para la regeneración de cartílago ya que se puede controlar mejor su degradación y son más fáciles de moldear (Capito and Spector, 2003). Los más utilizados son el PLA, el PGA,

el PLGA, el PCL, el poliumarato de propileno y el polietilenglicol (PEG) (Alves da Silva et al., 2010; Dahl et al., 2011)

Para imitar mejor el ambiente de la ECM se está combinando el uso de materiales sintéticos y biológicos como el sulfato de condroitina, el ácido hialurónico o el colágeno, dentro de los soportes celulares. De esta forma, se pretende fomentar la especificidad tisular y facilitar su integración *in vivo* (Coburn et al., 2012). Igualmente, para mantener o inducir el fenotipo condrocítico se han incluido factores implicados en la condrogénesis tales como TGF-β3, TGF-β1 o el *Sox-9* (Kim et al., 2011; Park et al., 2011a; Jeon et al., 2012). Por ejemplo, se han combinado hidrogeles que contenían MSCs con nanopartículas que contenía el factor de transcripción TGF-β3 (Park et al., 2011b). En otro estudio, Jung y su equipo crearon un scaffold de PLGA y fibrina con nanoparticulas de heparina que liberaba de manera controlada TGF-β1 consiguiendo inducir la diferenciación condrogénica de ASCs *in vitro* e *in vivo* (Jung et al., 2009).

Por otro parte, con el objetivo de reparar defectos osteocondrales varios autores han hecho eco de la necesidad de crear soportes bifásicos que reproduzcan la estructura anatómica osteocartilaginosa (Jiang et al., 2007; Liu et al., 2013; Shimomura et al., 2014). De hecho, varios soportes están siendo probados en clínica, algunos combinan colágeno e hidoxiapatita (Kon et al., 2014) y otros emplean PLGA (Chiang et al., 2013). Los soportes bifásicos de PGLA son reabsorbibles y porosos para permitir la infiltración de elementos biológicos como sangre, proteínas y células. Los resultados preliminares en pacientes con pequeñas lesiones ostecondrales demuestran la seguridad de estos soportes y presentan mejoras en cuanto a la sintomatología y la función, ofreciendo una alternativa a técnicas como la microfractura o injertos osteocondrales (Dhollander et al., 2012; Bekkers et al., 2013). Sin embargo, también existen casos en los que no se ha logrado su integración y han tenido que retirar el implante. Los médicos aconsejan evaluar a los pacientes a más largo plazo para dar tiempo a que el cartílago articular regenerado madure antes de considerar la realización de procedimientos de artroplastia irreversibles (Carmont et al., 2009).

4.5.2. Aplicación de soportes 3D en enfermedades cardiovasculares

Aunque la inyección de células madre en el corazón ha demostrado un incremento en la función cardiaca, uno de los aspectos que está por mejorar de esta técnica es la implantación de las células en el tejido. Teniendo en cuenta dicho inconveniente y el hecho de que muchas enfermedades cardiovasculares conducen a lesiones en el corazón, con la consiguiente necesidad de sustituir estructuras funcionales del corazón, tales como válvulas o incluso todo el corazón, se está prestando especial atención a la creación de tejido cardiaco a través de la bioingeniería. Hasta la actualidad, se ha conseguido crear *in vitro* injertos cardiacos contráctiles que se postulan como un sistema para la sustitución de miocardio infartado para mejorar la función cardíaca.

Para regenerar el corazón hace falta encontrar polímeros con propiedades mecánicas elásticas y dúctiles específicas. Por ejemplo, para imitar la anisotropía necesaria del tejido cardiaco la combinación de PCL y gelatina han dado buenos resultados mejorando la adhesión y la alineación de los cardiomiocitos en el soporte (Kai, et al., 2011). Se ha llegado aún más lejos desarrollando soportes biocompatibles que no solo poseen buenas propiedades físico-química y mecánicas, sino que también presentan la capacidad de diferenciar las células en cardiomiocitos. En este sentido, Gupta y su equipo (Gupta et al., 2011) han creado un soporte con la combinación de PEG-PCL-CPCL y un inhibidor de BMP, que promueve la diferenciación de las ESCs hacia cardiomiocitos funcionales. Asimismo, se ha diseñado un soporte que combina la fibrina y PLGA, que es capaz de estimular la diferenciación de MSCs hacia cardiomiocitos (Sreerekha et al., 2013). Además, alternativas como las válvulas del corazón creadas mediante la ingeniería de tejidos o la inyección de nanomateriales para mejorar la función de las válvulas cardíacas defectuosas, son algunas de las nuevas estrategias que están emergiendo para mejorar las terapias actuales en la cirugía cardíaca valvular. De hecho, estas válvulas se han probado en modelos animales mostrando excelente remodelación de los tejidos. Un ejemplo exitoso de válvula cardíaca ha sido demostrado por Kalfa y su equipo, que utilizó polidioxanona (PDO) para construir válvulas bio-absorbibles que apoyaron el crecimiento de MSCs. 8 meses después de la implantación en el corazón de corderos en crecimiento, estos soportes de PDO estaban completamente degradados y sustituidos por tres capas viables de tejido (Kalfa et al., 2010).

5. Patologías condrales

El cartílago es un tejido avascular compuesto de condrocitos atrapados en una matriz extracelular rica en proteoglicanos y colágenos. Los mecanismos innatos de reparación en el cartílago son limitados debido a la escasez / ausencia de células madre residentes y a la falta de un sistema vascular y linfático. Por tanto, los eventos normales que ocurren durante la reparación de los tejidos, como la inflamación o la formación de un coágulo de fibrina, no suceden en las lesiones del cartílago. Sólo los condrocitos y sinoviocitos que residen en el entorno local pueden llenar los defectos mediante una lenta proliferación y deposición de matriz (Wu et al., 2013). Como consecuencia de la limitada capacidad de regeneración del cartílago, las lesiones condrales suponen un importante problema clínico y social, con un alto impacto económico.

Los defectos del cartílago son características comunes en enfermedades de las articulaciones, tales como la OA (Noel et al., 2002). La OA consiste en un proceso degenerativo crónico que se caracteriza por el deterioro progresivo del cartílago, remodelación osea subcondral, pérdida del espacio articular, osteofitosis marginal, y la pérdida de la función articular (Wieland et al., 2005). Se estima que la mayoría de la población mayor de 65 años de edad presenta OA en al menos una articulación.

Las intervenciones actuales para la OA tienen como objetivo principal aliviar los síntomas, reducir el dolor y controlar la inflamación con medicamentos antiinflamatorios no esteroideos, esteroides, o ácido hialurónico , los cuales tienen poco impacto en la progresiva degeneración de los tejidos de la articulación (Qi et al., 2012).

Los tratamientos quirúrgicos más populares para los defectos del cartílago son la micro-perforación, la mosaicoplastia y la implantación de condrocitos autólogos (ACI). La técnica de micro-perforación, también conocida como la microfractura, consiste en inducir pequeñas fracturas en la placa de hueso subcondral mediante la perforación de pequeños agujeros que permiten que la sangre y la médula ósea se filtre en la lesión. Esto crea un coágulo de sangre que contiene células madre mesenquimales. Las MSCs finalmente sanan el defecto con tejido cicatrizal que consiste en una mezcla de tejido fibroso, fibrocartílago y cartílago hialino (Gilbert, 1998). Esta técnica tiene una alta tasa de existo en pacientes jóvenes, sin embargo la calidad del nuevo tejido depende de

muchos factores como la edad, la localización o el postoperatorio. Además, las propiedades mecánicas del tejido de la cicatriz son inferiores en comparación con el cartílago nativo y puede predisponer a la articulación a un inicio de OA a medio y largo plazo.

Por otro lado, la mosaicoplastia es una técnica reconstructiva que consiste en extraer cilíndricos de cartílago hialino junto con el hueso subcondral subyacente, de un área no afectada, para ser implantados en la lesión condral. Sin embargo, diversos estudios sugieren otras técnicas más efectivas como el ACI (Bentley et al., 2012). ACI, fue introducido por primera vez por Brittberg y su grupo en 1994 (Brittberg et al., 1994), consiste en llenar los defectos del cartílago con condrocitos autólogos que se expanden *in vitro*. El procedimiento clásico incluye la extirpación artroscópica de biopsias en áreas de cartílago sano que llevan poco peso, el aislamiento y la expansión de condrocitos en el laboratorio para su posterior implantación en la lesión, que finalmente quedará cubierta por una membrana de periostio suturada a los tejidos sanos circundantes (Wu et al., 2013).

Actualmente, una nueva técnica denominada implante de condrocitos autólogos inducida por matriz (MACI) es cada vez más popular. En esta técnica en lugar de inyectar los condrocitos como una suspensión celular, estos son cultivados en una bicapa de colágeno tipo I / tipo III de origen porcino. A continuación, la membrana MACI se asegura directamente a la lesión, mediante pegamento de fibrina, sin necesidad de una cubierta (Bartlett et al., 2005). Estudios clínicos, con un período de seguimiento de 2 a 10 años, indican que el 90 % de los pacientes tratados con esta técnica desarrolló tejido bien integrado en los sitios de la lesión (Peterson et al., 2003). A pesar del éxito en la práctica clínica, estas técnicas presentan algunos inconvenientes que limitan su aplicación. Por ejemplo, la tasa de existo desciende severamente con la edad, por lo que no se aplica en pacientes mayores de 50 años. Al mismo tiempo conlleva otros inconvenientes como los costosos procedimientos quirúrgicos, la morbilidad del sitio donante y la desdiferenciación de los condrocitos durante la expansión *in vitro* (Wu et al., 2013), (Figura 5).

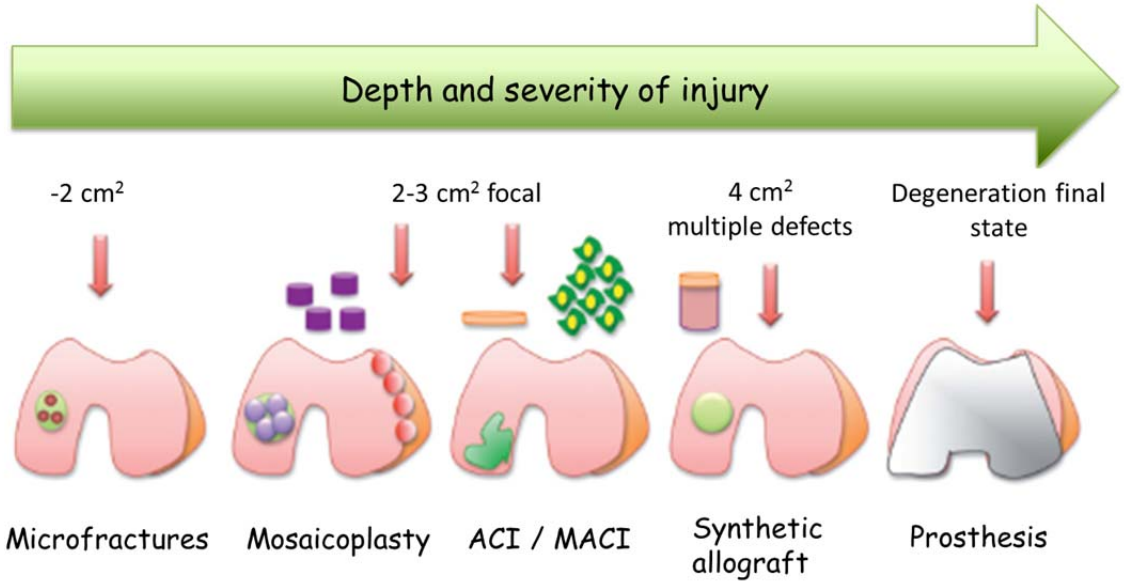


Figura 5. Indicaciones generales para las lesiones condrales. Adaptada de Álvarez E et al; 2010.

En los casos de degeneración severa de la articulación, esta debe ser sustituida por una articulación protésica. Sin embargo, los componentes de las prótesis sufren un alto desgaste y son más rígidas que el cartílago, asimismo carecen de muchas propiedades que posee una articulación natural como la lubricación, deformidad y capacidad de absorción a los golpes Gomoll et al., 2010.

6. Enfermedades cardiovasculares

Las enfermedades cardiovasculares son la principal causa de muerte y discapacidad en todo el mundo. Causa 9.4 millones y medio de muertes al año y además se prevé que aumente sustancialmente su prevalencia y coste en el futuro. Tanto es así, que se ha calculado que en 2030 morirán cerca de 23,3 millones de personas a causa de CVD (WHO, 2004), (Figura 6).

Las ECV se deben a trastornos del corazón y los vasos sanguíneos, entre ellos se incluyen las cardiopatías coronarias (ataques cardiacos), las enfermedades cerebrovasculares (apoplejía), el aumento de la tensión arterial (hipertensión), las

vasculopatías periféricas, las cardiopatías reumáticas, las cardiopatías congénitas y la insuficiencia cardiaca.

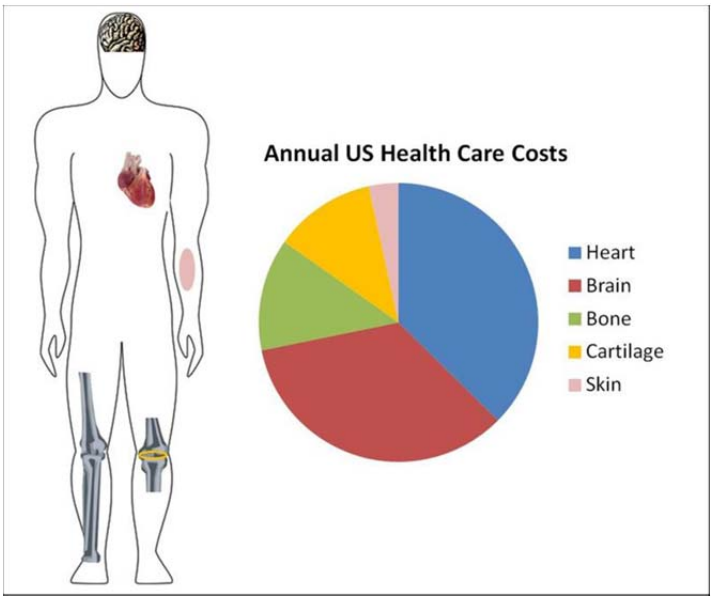


Figura 6. Diagrama representativo que muestra el coste anual en Estados Unidos de las principales enfermedades relacionadas con la degeneración del tejido. El coste se muestra en miles de millones de dólares. Fuente Peran et al., 2013a.

Se estima que de los ocho factores principales de riesgo, seis de ellos pueden prevenirse. Tres de estos factores de riesgo modificables, se asocian a factores biológicos tales como la hipertensión, la diabetes y el colesterol anormal. Otros tres factores modificables, considerados factores de estilo de vida, incluyen el consumo de tabaco, la inactividad física y la obesidad. Los únicos dos factores de riesgo no modificables son la edad y la historia familiar de enfermedad cardíaca temprana o derrame cerebral (Thayer et al., 2010).

Después de una lesión de miocardio/ infarto de miocardio, los fibroblastos cardíacos migran a la zona lesionada y proliferan generando una matriz extracelular que forma una cicatriz. Aunque la cicatriz restaura la estructura del corazón, esta no es funcional. Asimismo, debido a que la cicatriz es avascular, los miocitos residuales del interior o del borde de la cicatriz son isquémicos y pueden convertirse en focos de arritmia. Por consiguiente, este proceso resulta en la pérdida de función del corazón y finalmente en insuficiencia cardíaca.

La limitada capacidad de regeneración del corazón humano explica por qué el infarto de miocardio a menudo culmina en insuficiencia cardíaca y muerte súbita (van den Borne et al., 2010 4820). A su vez, la pérdida de vascularización puede inducir graves consecuencias como un aumento del tamaño de la lesión, pérdida celular por apostosis e incluso un incremento de las posibilidades de un fallo orgánico. Por tanto, la vascularización del tejido durante estos eventos es muy importante. Numerosos medicamentos y dispositivos mecánicos pueden mejorar temporalmente la función cardiaca, no obstante tales enfoques no sustituyen el músculo cardíaco perdido y son inevitablemente transitorios. Actualmente los esfuerzos médicos se centran en mejorar los tratamientos y encontrar opciones para los trasplantes de corazón, los cuales están indicados en pacientes que han sufrido un fallo cardiaco y no responden a terapias convencionales. Sin embargo, aparte de la necesidad de encontrar un donante oportuno, existen otras dificultades tales como los riesgos tanto preoperatorios como postoperatorios, así como el gran coste que supone debido al incierto tiempo de seguimiento que conlleva este procedimiento (de Mora-Martin et al., 2011).

Por lo tanto, se están desarrollando nuevas terapias con el objetivo de permitir la regeneración del corazón. Por ejemplo, se ha creado un dispositivo de asistencia ventricular, el LVAD, que se trata de una pequeña bomba que eleva la función del corazón, y reduce la tensión en el ventrículo izquierdo, lo cual parece ayudar al corazón a recuperarse por sí mismo. Normalmente, el LVAD se usa como "puente" para mantener en funcionamiento un corazón enfermo hasta tanto haya disponible un órgano para trasplante.

Además, se están desarrollando terapias celulares para la regeneración de la masa miocárdica y para para la inducción de neovascularización. Entre estos métodos destacan la cardiomioplastia celular (inyección de las células), cardiomioplastia de tejido (implante de injerto cardíaco diseñado mediante bioingeniería) e ingeniería in situ (implantación de soportes). Algunas técnicas ya han sido evaluadas en el ámbito clínico, como por ejemplo la inyección de miocitos o MSCs de médula ósea, observado una mejoría en la función cardiaca. Para mejorar la implantación celular se están desarrollando láminas de células que son crecidas encima de un polímero sensible a la temperatura. El modelo de sistema sensible a la temperatura más ampliamente estudiado ha sido el poli(N-isopropilacrilamida) (PIPAAm) desarrollado por Okano (Miyagawa et

al., 2005), este polímero pasa de ser hidrófobo a hidrófilo cuando se baja la temperatura. Este cambio permite que las células puedan extraerse sin destruir la ECM y las interacciones de célula-célula. Ensayos clínicos con láminas de mioblastos autólogos se han iniciado en pacientes con patologías cardiacas mostrando una mejora de los síntomas (Sawa et al., 2012). Otras estrategias que se están estudiando es la combinación de células, como mioblastos, en las láminas junto con factores angiogénicos o células que induzcan la vascularización (Sawa and Miyagawa, 2013).

HYPOTHESIS

Despite significant therapeutic advances, degenerative diseases continue to be leading causes of health problems worldwide. The currently available pharmacological and surgical therapies are unable to repair tissues with limited regenerative capacity such as heart or cartilage. Therefore, the effort to development novel therapeutics approaches has increased in the last years.

Adult stem cells and progenitor cells are attractive candidates for cell therapy approaches. These cells can be easily isolated from the patient and have demonstrated their ability to differentiate into specific cell lineages. Therefore, exploring conditions that promote differentiation of SC is essential to achieve specific linage cells that could be used to replace and/or regenerate related damaged tissues.

The present work is based on the following hypothesis:

1. Chondral defects suppose a challenging clinical problem aggravated by the increase in elderly population in developed countries. Infrapatellar fat pad of patients with OA contains multipotent and highly clonogenic hASCs that can be isolated by low invasive methods (English et al., 2007). Moreover, nuclear and cytoplasmic cellular extracts has been showed to be effective in induction of cell differentiation and reprogramming. Here, we hypothesize that hASCs obtained from fat pad, the same joint microenvironment, of patients with OA, can undergo chondrogenesis when stimulated with an extract prepared from autologous chondrocytes obtained from patients' cartilage tissue. Moreover, as tissues are three-dimensional structures, scaffolds of biocompatible materials may provide a three dimensional environment that enhance chondrogenesis by increasing the production of extracellular matrix and maintaining the chondrocytic phenotype of these autologous cells.

2. Related heart diseases are the major cause of death throughout the world. One of the biggest problems is the inability of cardiac muscle to regenerate after massive loss of cardiomyocytes that follows myocardial infarction. Conditioned media and cell extract from rat or even human cardiac tissue have proved to induce differentiation of MSCs into cardiomyocyte-like cells (Liu et al., 2008; Peran et al., 2010; Schittini et al., 2010). However, limitations to obtain large amounts of human cardiac tissue are obvious. Since postmorten cardiac tissue can be obtained more easily than biopsies from patients,

we hypothesize that postmorten human cardiac tissue could be used to obtain cell extracts and conditioned medium in order to direct cardiomyogenic differentiation of human hASCs

3. Pathological events such as isquemic heart disease require both the regeneration of lost myocytes and neovascularization. EPCs are known to play a beneficial role by promoting postnatal vasculogenesis. In addition, it has been shown that ischemic insult induces the mobilization of EPCs from the bone marrow in response to growth factors, cytokines and hormones that are released from the target tissue (Shintani et al., 2001; Asahara et al., 1999b). However, little is known about the potential of EPCs to restore heart damage tissue. We hypothesize that EPCs isolated from PB of patients suffering from AMI could show a similar cardiac differentiation capacity to EPCs obtained from UCB.

OBJECTIVES

1-To induce chondrogenic differentiation of autologous IFPSCs obtained from patients with OA using cellular extracts-based method.

2-To test the ability of biodegradable PLGA cylindrical implants, constructed with a porous cartilage phase, as 3-D scaffolds to support chondrogenic differentiation.

3- To study the potential capacity of postmortem cardiac tissue to direct cardiac differentiation of hASCs by two different approaches: i) cell extract method and ii) conditioned medium.

4- To explore the cardiac differentiation capacity of EPCs isolated from PB of AMI patients and EPCs obtained from UCB.

.

MATERIALS AND METHODS

1. Origen of tissue samples

1.1. Articular cartilage tissue

Articular cartilage was obtained from 18 patients with knee OA during joint replacement surgery, isolated from the femoral side, selecting the non-overload compartment: lateral condyle in varus knees and medial condyle in the valgus cases. None of the patients had a history of inflammatory arthritis or crystal-induced arthritis and only cartilage that macroscopically looked relatively normal was used for this study. Ethical approval for the study was obtained from the Ethics Committee of the Clinical University Hospital of Málaga, Spain. Informed patient consent was gained for all samples used in this study.

1.2. Adipose tissue from Hoffa's fat pad

Hoffa's fat pad was harvested from the interior of the capsule excluding vascular areas and synovial regions. Samples were collected at the moment of joint arthroplasty from OA patient, after signed an informed consent, approved by the Ethics Committee of the Clinical University Hospital of Málaga, Spain. Samples were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) with 100 U/ml penicillin and 100 μg/ml streptomycin until processing.

1.3. Subcutaneous adipose tissue

Subcutaneous adipose tissue was obtained from patients undergoing liposuction procedure after informed consent from all patients and approval from the Ethics Committee of Clinical University Hospital of Málaga, Spain.

1.4. Myocardial tissue

Myocardial tissue samples were obtained from five forensic autopsies, no more than 12 hours postmortem. Approximately 100 mg of tissue were taken in each case from the lateral wall of the left ventricle and were kept at -80ºC until analyses. Each myocardial

tissue sample was used individually. Ethic Committee of Granada University approved the procedure.

1.5. Peripheral blood of patients who have been diagnosed of acute myocardial infarction

We studied 24 patients admitted with a diagnosis of AMI, defined as an acute coronary syndrome with ST elevation (STEMI) with suggestive chest pain and an elevation of at least 3 mm in the ST segment in at least three precordial leads, with no history of ischemic heart disease and within 8 h of symptoms. Preinfarction angina was defined as the presence of at least one chest pain < 30 min, the week before the onset of the infarction. Patients were excluded if they had had chest pain compatible with angina for more than 1 week before the infarction or if they had underlying structural heart disease (cardiomyopathy or important valve disorders). Moreover, patients with history of rheumatoid arthritis, hepatic, hematological, or coagulation disorders, cancer or other acute or chronic inflammatory diseases such diabetes mellitus were not included in the study. Informed consents were obtained from all patients and heparinized blood samples (20 ml) were drawn from all subjects

1.6. Umbilical cord blood samples

Human UCB samples (n=25) were obtained from the Centro Regional de Transfusión Sanguínea y Tejidos de Málaga, Málaga, Spain, according to institutional guidelines. Samples were generally processed within 24-48 h of collection.

2. Isolation and culture of primary cells

2.1. Isolation and culture of human articular chondrocytes

Articular cartilage was minced and digested overnight in an over-night 0.08% collagenase IV (Sigma) digestion at 37°C with gentle agitation. Cells were centrifuged and rinsing to remove the collagenase. The remaining cells were then plated in cultured flasks with chondrocytes media: DMEM (Sigma) supplemented with 20% fetal bovine

serum (FBS, Lonza, Basel, Switzerland), 7 ml human insulin (Astrapid; Novo Nordisk, Bagsvaerd, Denmark), 6 µl DNase I (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2. After 24 hours the medium was replaced with fresh medium supplemented with 10% FBS. Chondrocytes were cultured for a maximum of three weeks before the experiments to avoid dedifferentiation phenomenon (de la Fuente et al., 2004).

2.2. Isolation and culture of adipose derived stem cells

Samples of adipose tissue from lipoaspirates or from Hoffa's fat pad tissue were finely minced and digested using an enzymatic solution of 1 mg/ml collagenase type IA (Sigma) at 37 ºC for 1 h on a shaker. After digestion, collagenase was removed by a single wash in sterile phosphate-buffered saline (PBS), followed by two further washes in DMEM supplemented with 10% FBS. The cell pellet was resuspended in DMEM (Sigma) containing 10% FBS and 1% penicillin/ streptomycin and cultured at 37ºC in 5% CO2. After 48 hours the medium was removed to discard non adherent cells. At 80% of confluency the cells were released with trypsin–EDTA (Sigma) and subcultured.

2.3 Isolation and culture of endothelial progenitor cells

Samples of PB from AMI patient and from UCB were processed by density-gradient centrifugation with Histopaque-10771 (Sigma) for 25 minutes at 1500 r/min and washed three times in phosphate-buffered saline (PBS) with 2% FBS. MNC obtained were plated on fibronectin coated 6-well dishes (BD) at a density of 5×10^6 cells per well in Endothelial Cell Basal Medium, EBM-2 (Clonetics) supplemented with EGM-2 SingleQuot Kit (Clonetics) which contain Hydrocortisone, hFGF-B, VEGF, R3-IGF-1, Ascorbic Acid, Heparin, FBS, hEGF and GA-1000. After 2 days of culture, non-adherent cells were re-plated on fibronectin coated 24-well dished (BD) at a density of 1×10^6/well with 1 ml of fresh complete endothelial growth media, then cells were incubated at 37ºC at 5% CO2 with 95% humidity.

The numbers of colonies were counted under a phase-contrast microscope at days 5 and 10 of culture. Five randomly selected microscopic fields were evaluated, and colonies were calculated in each PB from AMI patients or UCB samples.

3. Cell characterization

3.1. Flow cytometry analisys of chondrocytes IFPSCs and hASCs

The immunophenotype of articular chondrocytes, IFPSCs, hASCs s was analyzed by flow cytometry (FACS). Cells were washed and resuspended in phosphate buffered saline (PBS) with 2% bovine serum albumin BSA (Sigma, St. Louis, MO), and 2 mM ethylenediaminetetraacetic acid (EDTA, Sigma). Cells were incubated in the dark for 30 minutes at 4º C with the appropriate fluorochrome-conjugated monoclonal antibodies. The markers used were: CD133-PE (Miltenyi), CD105-APC, CD90-FITC (eBioscience Inc., San Diego, CA), KDR-APC (R&D System, Minneapolis, MN), CD34-FITC, CD31, CD45-APC-Cy7, CD 73-PE and CXCR4-PE, CXCR4-APC (BD Biosciences, San Jose, CA). For the determination of Collagen II expression cells were, first, fixed and permeabilized with Fix and Perm® reagent (Invitrogen, Carlsbad, CA, USA), then incubated with the primary monoclonal antibody (AbCam, Cambridge, UK) for 20 min, washed and finally incubated with a secondary FITC-conjugate monoclonal antibody (Sigma) for 30 min. All cells were washed in PBS and analyzed in a FACSCanto II cytometer (BD Biosciences).

3.2. Quantification of endothelial markers in mononuclear cells

MNC isolated by density gradient centrifugation with Histopaque-1077 from PB of AMI patient and from UCB, were washed and resuspended in phosphate-buffered saline (PBS) with 2% bovine serum albumin (Sigma), and 2mM ethylene diamine tetra acetic acid (EDTA, Sigma). Cells were incubated in the dark at 4ºC for 45 min with the following fluorochrome-conjugated monoclonal antibodies: CD133-PE (Miltenyi), KDR-APC (R&D System, Minneapolis, MN), CD34-FITC, and CXCR4-PE (BD Biosciences, San Jose, CA). Cells were then washed in PBS and analyzed in a fluorescence activated cell sorting (FACS) Canto II cytometer equipped with the FACS

Diva analysis software (BD Biosciences). Data obtained were expressed as mean ± standard error (SE) from 4 independent experiments performed in triplicate ($P<0.05$).

3.3. Differentiation assays of adipose derived stem cells

hASCs isolated from lipoaspitaes or for Hoffa's fat pad tissue were plated at 2 x 103 cells/cm^2 in DMEM (Sigma) containing 10% FBS with penicillin and streptomycin at 100 µg/ml and allowed to adhere for 24 hours. The culture medium was then replaced with specific inductive media. For adipogenic, osteogenic and chondrogenic differentiation, cells were cultured for two weeks in Adipogenic MSCs Differentiation BulletKit, Osteogenic MSCs Differentiation BulletKit (Lonza, Basel, Switzerland) and NH ChondroDiff Medium (Miltenyi Biotec, Auburn, CA, USA), respectively. Differentiated cell cultures were stained with Oil Red O (Amresco, Solon, OH, USA) for adipogenic differentiation, Alizarin Red (Lonza) for osteogenic differentiation or Toluidine Blue (Sigma) for chondrogenic differentiation.

3.4. Assessment of acetylated low-density lipoprotein uptake and Ulex europaeus-lectin 1 binding

After 7 days of cell culture, EPCs were identified by uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine percholorate-labeled acetylated low-density lipoprotein (Dil-ac-LDL, Molecular Probes, America) and adherence of fluorescein isothiocyanate-conjugated Ulex europaeus lectin 1. The adherent cells were incubated with DiI-ac-LDL at 37°C for 2 h. Thereafter, cells were washed with PBS and fixed with 2% formaldehyde for 10 min and washed again with PBS. Fluorescein isothiocyanate-conjugated Ulex europaeus lectin (FITC-UEA-l, Vector, America) was added and incubated for 1 hour at 25°C. Culture was then washed twice and visualized with a Leica DM 5500B (Solms, Germany) fluorescent microscope, software Meta Systems Isis.

4. Cell differentiation

4.1. Cell differentiation by cell extract-based method

4.1.1. Preparation of cell extract

Cell extract was obtained from cultured chondrocytes and from postmortem cardiac tissue. For chondrocytes extract preparation, cultured chondrocytes were harvested by trypsin digestion solution and pelleted at 500,000 cells. For cardiac tissue extract, approximately 50-70 mg of postmortem cardiac tissue was first disaggregated by physical and enzymatic methods. Chondrocytes and isolated myocardiocytes were washed twice in cold PBS and once in cold cell lysis buffer (50 mM NaCl, 5 mM MgCl 2, 100mM HEPES, pH 8.2, 1mM dithiothreitol and 0.1mM phenylmethylsulfonyl flouoride; Sigma). Cells were centrifuged at 800g, resuspended in 1.5 volumes of cell lysis buffer containing protease inhibitor cocktail (Sigma) and allowed to swell on ice for 45 min. Cells were homogenized by pulse-sonication and the lysate sedimented at 15.000 g for 15 min at 4°C. The supernatant (cell extract) was collected and used fresh.

4.1.2. Cell permeabilization

To evaluate the efficiency of SLO permeabilization $3x10^5$ IFPSCs and hASCs were exposed to several concentration of SLO ranged from 0.230-305 ng/ml and incubated with Texas red- conjugated 70,000 Mr dextran (Invitrogen). The uptake of the dye was observed by phase contrast and epifluorescence microscopy after 2 hours and 24 hours of SLO permeabilization.

IFPSCs and hASCs were harvested by trypsin digestion and washed twice in ice-cold PBS and once in ice-cold Hanks' balanced salt solution (HBSS; Gibco-BRL, Paisley, UK). Cells were permeabilized by Streptolysin O (SLO; Sigma) exposition. Therefore, cells were pelleted at 300 000 cells/reaction in 1.5-mL tubes and suspended in 488 µL ice-cold HBSS, placed in a water bath at 37°C for 2 min and a final concentration of 185 ng/ml and 230 ng/ml of SLO for IFPSCs and hASCs was added respectively. Samples were incubated for 30 min at 37°C.

4.1.3. Target cell incubation with cell extract

Permeabilized IFPSCs and hASCs were centrifuged at 300 g for 5 min at 4°C in a swing out rotor. The supernatant was removed and 500 µL extract containing 20 µL 1mmol/L of each nucleotide triphosphate set (Roche, Indianapolis, USA) added. Cells were incubated with the extracts for 1 h at 37°C. DMEM/10% FBS containing 2 mmol/L $CaCl_2$ was added and the cells were then transferred to 25 cm^2 culture flasks. 4 h later, dead (floating) cells and the Ca^{2+} containing medium were removed and replaced with fresh DMEM containing 10% FBS and antibiotics. Damaged or dead cells, marked with Trypan blue (Sigma), represented 15-20% of the total. Cells were cultured for 2 weeks until use. Control cells were either non-permeabilized and non-extract exposed cells or permeabilized cells not exposed to the extracts. Cell viability was assessed by phase contrast microscopy after 2 weeks of extract exposure by counting cells in four different regions of the dish and calculating the average.

4.2. Cell differentiation by conditioned medium

To prepare cardiac tissue-derived conditioned medium 500-700 mg of postmortem right ventricle tissue was cut into approximately 1 mm^3 pieces, and washed three times with PBS. Tissue fragments were placed into 75 cm^2 culture flasks containing 20 ml of DMEM containing 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and cultured as explants. After two days the medium was collected, and centrifuged at 2000g for 10 min followed by filtering the supernatant through a 0.22 µm filtration unit (Millipore, Bedford, MA). The media was stored at 4°C until use.

To induce cardiac differentiation of hASCs, $8x10^4$ cells per well were plated on a 6-well plate and incubated in cardiac tissue-derived conditioned medium for 2 weeks.

4.3. Cell differentiation by 5-azacytidine induction

EPCs were seeded in per- manox-chamberslides (Nunc, Wiesbaden, Germany) at a concentration of 50,000 cells. Twenty-four hours after seeding, cells were washed with PBS twice and were incubated for 24h in endothelial growth media serum-free containing 10µM 5-aza. Then, cells were washed and medium was replaced with fresh

endothelial growth media containing FBS to prevent cell death due to prolonged exposure to 5-aza and incubated in a CO_2 incubator. Cells were cultured for two weeks and medium was changed every 3 days (Kadivar et al., 2006).

5. *In vitro* primary culture on scaffold

Chondrocytes extract exposed IFPSCs and control cells were seeded in biodegradable cylindrical scaffold constructed of a porous cartilage phase and a porous bone phase (5 mm in diameter x 10 mm deep). The cartilage phase of the scaffold was cut to 3 mm in diameter and 3 mm in height under sterile conditions on a petri dish and was plated in 96-well plates. The synthetic material is a blend of poly DL-lactide-co-glycolide, polyglycolide fibers and surfactant (TruFit CB Plug; Smith & Nephew, London, UK). Cells suspensions containing 6.000 cells in 50 µl of medium were slowly dropped onto the surface of each scaffold incubated in 96-well plates for 4 h at 37ºC and then analyzed under the inverted microscope to check cell adhesion to the polymer surface. After that, 50 µl of fresh medium was added in each well plate. Cell culture medium was changed every two days.

6. Molecular analysis

6.1. RNA extraction

6.1.1. RNA extraction from cultured cells

Total cellular RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's recommendations.

6.1.2. RNA extraction from tissue

30 mg of myocardial tissue were disrupted and homogenized using the TissueLyser LT (Qiagen Hilden, Germany), and subsequently, RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen). RNA quantity and quality were assessed by a NanoDrop 2000-C (Thermo, Waltham, MA, USA). RNA integrity was evaluated using

chip-based capillary electrophoresis with Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara CA, USA).

6.2. Reverse transcription–polymerase chain reaction

Total RNA was reverse transcribed using the Reverse Transcription System kit (Promega, Madison, WI, USA) and the PCR reaction was performed with ReddyMix PCR Master Mix (Thermo, Waltham, MA, USA). After the initial denaturation (2 min at 94°C), 33 cycles were performed (30 s at 94°C, 50 s for annealing temperature and 1 min at 72°C) for all set of primers except for β-actin, which was 25 cycles. Primer sequences and annealing temperatures can be found in Table 2. The PCR products were visualized on 1% agarose gels containing 0.1 mg/ml ethidium bromide using ultraviolet light.

Table 2. Primer sequences and annealing temperatures for RT-PCR:

Target gene	Primers (forward and reverse)	Annealing temperature
Collagen type I	GAG AGC ATG ACC GAT GG GTG ACG CTG TAG GTG AA	56
Collagen type II	GAC AAT CTG GCT CCC AAC ACA GTC TTG CCC CAC TTA C	56
Collagen type X	GCC CAC TAC CCA ACA C TGG TTT CCC TAC AGC TGA	60
Aggrecan I	GTC TCA CTG CCC AAC TAC GGA ACA CGA TGC CTT TCA C	56
Sox-5	ATC CCA ACT ACC ATG GCA GCT GAT ACC TGC ATT GCA GCT	60
Sox-6	GCA GTG ATC AAC ATG TGG CCT TTC ATC ATG CGCTGC CAG TAG	60
Sox-9	GAG CAG ACG CAC ATC TC CCT GGG ATT GCC CCG A	56
Gapdh	GAA GGT GAA GGT CGG AGT C GAA GAT GGT GAT GGG ATT TC	60
β-actin	ATCATGTTTGAGACCTTCAA CATCTCTTGCTCGAAGTCCA	45
α-cardiac-actin	ATCTCTGCTGGCCATGAAAC GATGAGGGAAGGTGGTTTGG	53
Cardiac troponin I	CCCTGCACCAGCCCCAATCAGA CGAAGCCCAGCCCGGTCAACT	64
Mef2c	AGTGGTTTCCGTAGCAACTCCT TAGTGCAAGCTCCCAACTGACT	62
GATA-4	TCCCTCTTCCCTCCTCAAATTCA GCGTGTAAAGGCATCTG	52
Cardiac troponin T	AGA GCG GAA AAG TGG GAA GA CTG GTT ATC GTT GAT CCT GT	53

6.3. Reverse transcription and real-time PCR analysis

QuantiTect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis. Real-time PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) on a 7500 device (Applied Biosystem, Foster City, CA, USA). For all samples, a relative quantitative gene expression analysis of cardiac troponin I (TNNI3), myosin light chain 3 (MYL3) and Glyceraldehide-3phosphate dehydrogenase (GAPDH) were carried out in triplicate. Primers were commercially supplied by Qiagen (QuantiTect Primer Assay reference numbers: *TNNI3* QT00084917; *MYL3* QT00090223; *GAPDH* QT01192646).

7. Immunocytochemistry

For immunofluorescence staining, cells were fixed with 4% PFA in PBS for 20 min at room temperature. When staining intracellular antigens, cells were permeabilized with 0.1% Triton X-100 for 15 min. Following, cells were blocked in 2% blocking buffer solution (Roche) for 1 h at room temperature. Primary antibodies were incubated overnight at 4ºC; when needed, secondary antibodies were incubated at room temperature for 2 hours. Afterwards, they were washed three times in PBS and the slides were mounted using Vectashield containing DAPI. Photographs were taken with a Leica DM 5500B (Solms, Germany) fluorescent microscope, software Meta Systems Isis.

Primary antibodies used for immunocytochemistry were i) endothelial markers: KDR-APC (R&D System, Minneapolis, MN) and CD133-PE (Miltenyi) diluted 1:200; ii) cardiac markers: desmin (rabbit monoclonal; Sigma) diluted 1:20, human cardiac-specific troponin I (mouse monoclonal; Research Diagnostics, Flanders, NJ, USA), sarcomeric α-actinin (mouse monoclonal; Sigma) diluted 1:200, human cardiac-specific troponin T (rabbit polyclonal; Abcam, Cambridge, UK) diluted 1:100, and iii) condrogenic marker: anti-Collagen type II (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100.

Secondary antibodies used were: Fluorescein Isothiocyanate (FITC) or Tetramethyl Rhodamine Isothiocyanate (TRITC) (Santa Cruz, CA, USA) diluted 1:200.

8. Transmission electron microscopy

Cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4; Sigma) for 1 h at room temperature and post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (Sigma) for 1 h at room temperature, and dehydrated in ethanol. Cells were detached from the culture vessel with propylene oxide and embedded in Epon 812. After polymerization, the plastic was removed and ultrathin sections were cut parallel and perpendicular to the surface of the flask. The sections were contrasted with uranyl acetate lead citrate and examined with a LEO 906E transmission electron microscope (TEM).

9. Environmental scanning electron microscope analysis

Samples were fixed in 3% glutaraldehyde during 2 hour at 4ºC and then were rinsed several times with sodium cacodylate. The samples were kept refrigerated intosodium cacodylate buffer. The observations started at an initial water vapour pressure of 5.7 tors. At this pressure, a liquid water phase was present in the sample (100% RH). Then, vapour pressure was decreasing slowly until the surfaces of the samples were visible (4.5 and 5 tors). Accelerating voltages varied between 10 and 15 kV we obtained a good image resolution using small beam current (spot size 3-3.5) and a working distance of 5-6 mm. The Environmental scanning electron microscope (ESEM) used in this work was a Quanta 400 (FEI) located at the Centro de Instrumentación Científica of the University of Granada.

10. Histological analysis

For histological analysis, cells seeded on glass coverslips and cell-seeded PLGA sections were washed with PBS and fixed in 4% paraformaldehyde in PBS for 20 min or overnight at 4ºC, respectively. Then, samples cells seeded on glass coverslips were assayed for cartilaginous matrix production by toluidine blue. While cell-seeded PLGA were dehydrated in graded alcohols, washed with xylene, and embedded in paraffin using standard procedures. Sections of 5 mm were cut from the paraffin blocks and

placed on glass slides. Safranin-O staining and toluidine blue were then performed. For safranin-O staining, sections were deparaffinized with xylene and ethanol, treated with aqueous safranin-O (0.1%) (Panreac) for 30min, and washed with distilled water. For toluidine blue staining, sections were deparaffinized with xylene and ethanol, treated with 0,04% Toluidine O Blue (Sigma) solution for 20 min and washed with distilled water. Sections were dehydrated and cleaned in ascending concentrations of ethanol and xylene before coverslips were mounted (DPX mountant, BDH). Observation under light microscopy and photographs were taken with Nikon Eclipse 50i microscope.

11. Statistics

Data were subjected to 1-way ANOVA followed by Fisher's test for comparison between any 2 means. Differences of $P<0.05$ were considered significant. Values are expressed as mean±SE.

RESULTS

CHAPTER I:

Chondrocytes extract from patients with osteoarthritis induces chondrogenesis in infrapatellar fat pad-derived stem cells

Chondrocytes extract from patients with osteoarthritis induces chondrogenesis in infrapatellar fat pad-derived stem cells

I.1. Cell isolation and characterization of chondrocytes

FACS characterization showed that *ex-vivo* cultured chondrocytes expressed the surface markers CD73 (99.84%), CD90 (97.25%), CD105 (99.74%) and the intracellular protein collagen type II (60%), while lacked expression for both hematopoietic and endothelial cell markers CD45 (<1%), CD34 (1.4%), CD133 (1.82%), CXCR4 (2.27%), KDR (7.63%), CD31 (1.53%) (Figure 1A).

Light microcopy observation showed that chondrocytes at day one were attached to the flask surface as cell-clusters of rounded shapes (Figure 1B). After a week in culture, cells displayed a polygonal shape appearing some of them with a star-like morphology (Figure. 1C). These stellated like-cells occupied the entire surface and reached confluence after two weeks (Figure 1D).

1.2. Cell isolation and characterization of IFPSCs

Isolated IFPSCs presented a spindle shape fibroblastic morphology. FACS characterization demonstrated a positive expression of the surface markers CD73 (>99.80%), CD90 (>99.42%), CD105 (>99.91%) and a negative expression for CD45 (1.16%), CD34 (8.16%), CD133 (1.41%), CXCR4 (1.97%), KDR (1.49%) and CD31 (1,18%) as shown previously (Gimble et al., 2007). In addition, IFPSCs showed a slightly expression of Collagen type II (13%) (Figure 2A). IFPSCs treated with conditioned media displayed characteristics of adipogenic, osteogenic and chondrogenic differentiation after staining (Figure 2B).

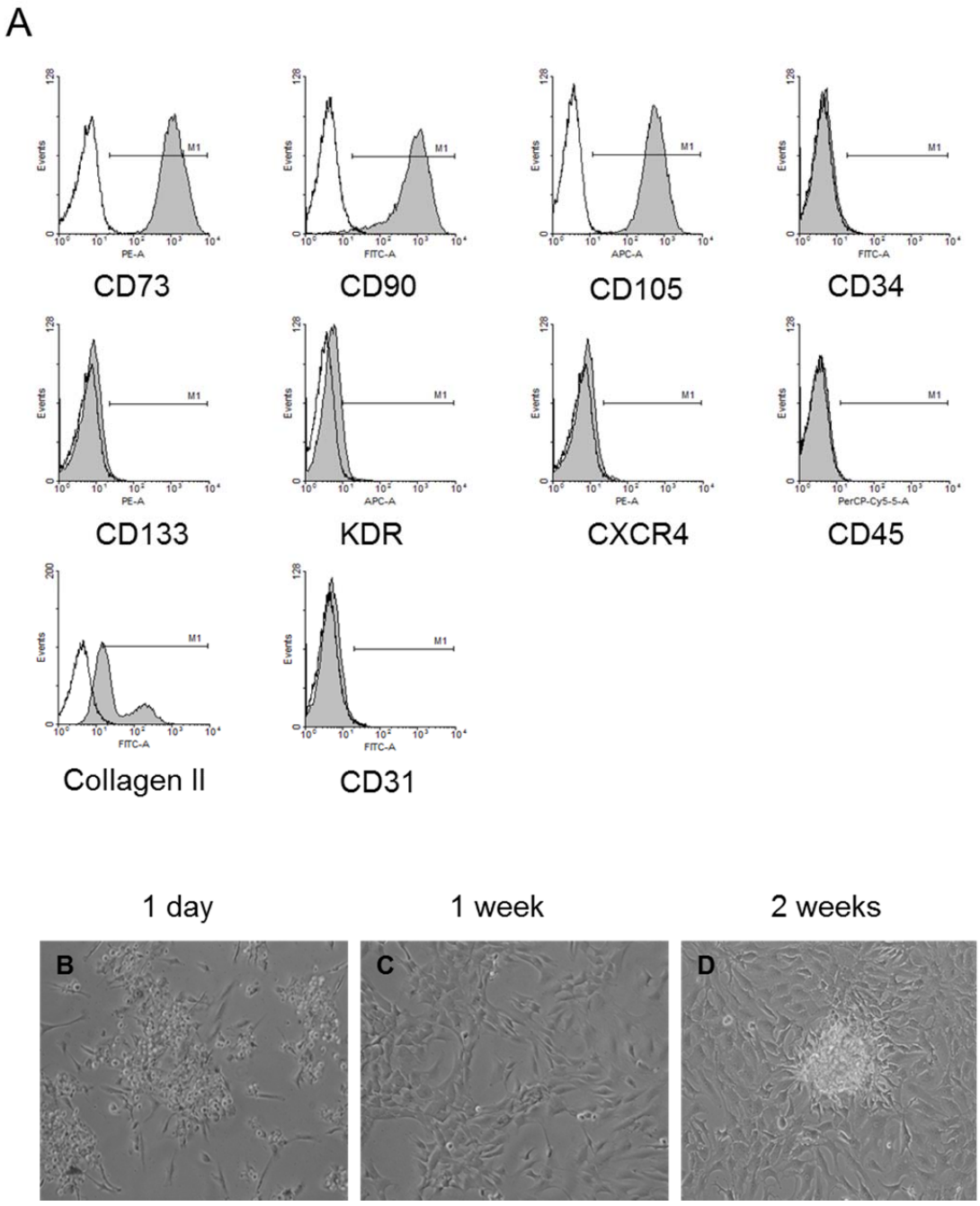


Figure 1. Phenotypic characterization of chondrocytes. (**A**) Chondrocytes were cultured for two weeks and then tested for mesenchymal surface markers (CD105, CD73 and CD90), hematopoietic and endothelial markers (CD133, CD34, KDR, CD45, CD31 and CXCR4) and for collagen II marker by flow cytometry. White histograms identify the isotype controls (negative). Phase-contrast light microscopy of cultured human articular chondrocytes for 1 (**B**), 7 (**C**) and 14 days (**D**). Original magnification: 10x for B and 20x for C and D.

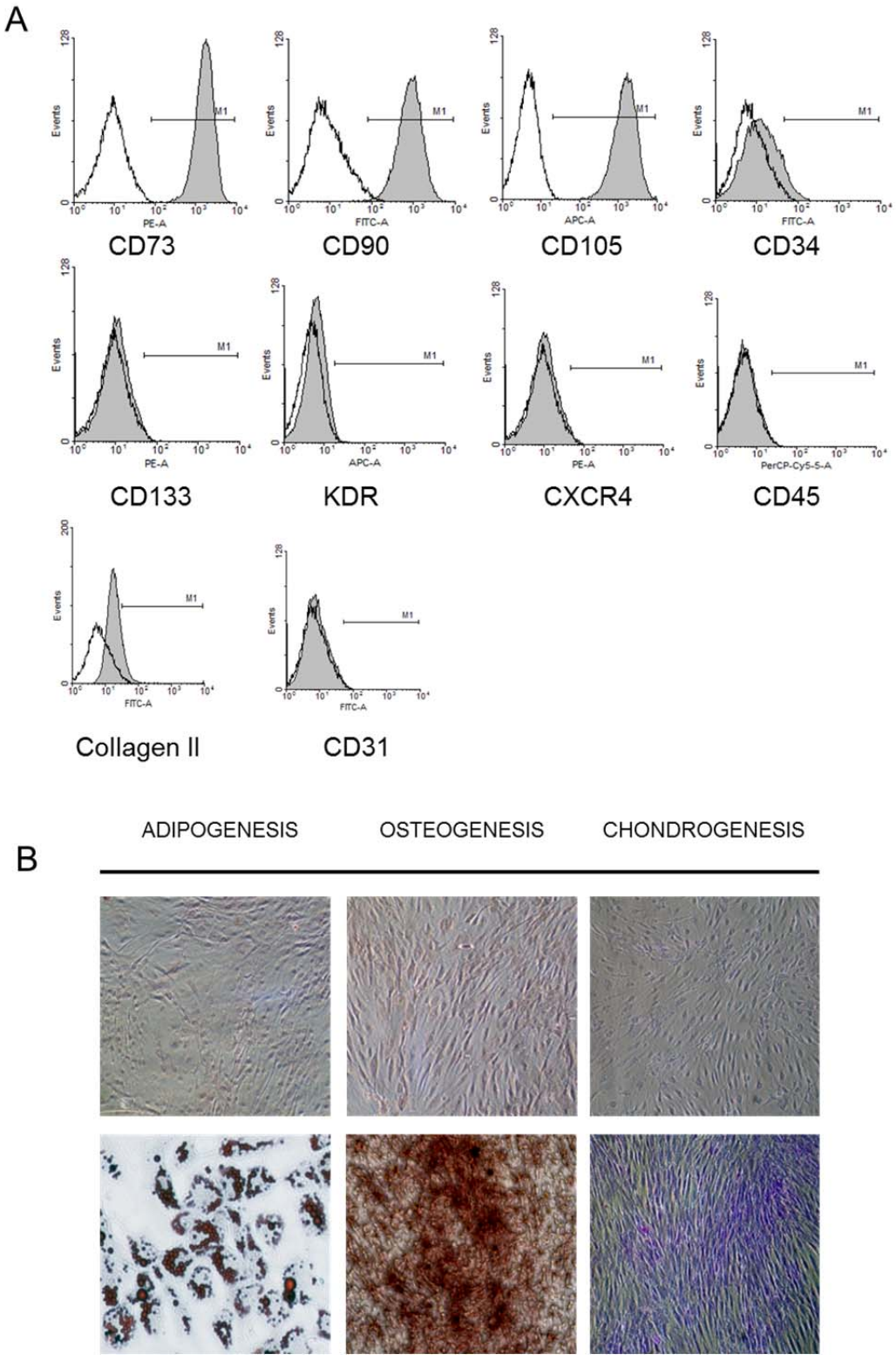


Figure 2. Phenotypic characterization and differentiation potential of IFPSCs. (**A**) IFPSCs were cultured for two week and then tested for mesenchymal surface markers (CD105, CD73 and CD90), hematopoietic and endothelial markers (CD133, CD34, KDR, CD45, CD31 and CXCR4) and for collagen II by flow cytometry. (**B**) The differentiation potential of IFPSCs towards adipogenic, chondrogenic and osteogenic lineage was confirmed by Oil Red O, Toluidine Blue and Alizarin Red S staining, respectively. Upper

pictures show negative controls, cells cultured in normal medium for 2 weeks and then histochemically stained. Original magnification: 10 x.

I.3. Cell permeabilization assay

Previous to perform the extract reprogramming experiment we optimize IFPSCs permeabilization procedure by testing different concentrations of the SLO toxin ranged from 0,230 to 305 ng/ml. Among the tested concentration, 185 ng/ml of SLO was the most efficient, permeabilizing 80-95% of the cells (Figure 3). Results showed that 20-25% of the cells survived to the extract exposure while 80% of control cells (permeabilized but not exposed to extract) were viable.

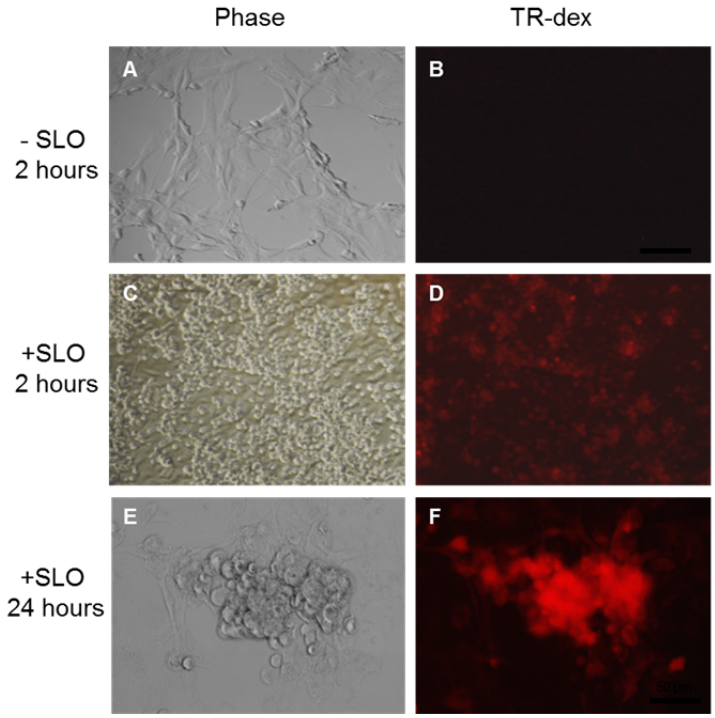


Figure 3. Reversible cell permeabilization, uptake of a Texas red-conjugated dextran by IFPSCs**.** Intact IFPSC (-SLO) or SLO-permeabilized IFPSC (+SLO). Cells were incubated for 30 min in HBSS containing 50 µg/ml of Texas red- conjugated 70,000 *M*r dextran, resealed with 2 mM $CaCl_2$ and cultured for 2 hours and 24 hours before observation by phase contrast and epifluorescence microscopy. Original magnification: 10x for A-D and 20x for E-F.

I.4. Phenotypic changes after exposition to chondrocyte extracts

Phase microscopy observation of cultured chondrocytes showed a typical polygonal morphology with a high rate of star-like cells (Figure 4A). On the other hand, permeabilized but not exposed to extract cells (control IFPSCs) displayed a characteristic mesenchymal shape consisting in flatter cells with a spindle-like morphology (Figure 4B). It can be appreciated that IFPSCs acquired a chondrocyte-like phenotype after exposure to the autologous cell extract, showing an increased number of smaller polygonal and star-shaped cells (Figure 4C)

Furthermore, toluidine blue assay demonstrated a characteristic positive metachromatic staining in both chondrocytes and IFPSCs treated with extracts which evidenced the synthesis of glycosaminoglycans (*GAG*) (Figure 4D and F), respectively when compared with control cells (Figure 4E).

I.5. Ultrastructural analysis

TEM showed that cultured chondrocytes possessed an euchromatic nuclei, a cytoplasm rich in rough endoplasmic reticulum (RER), many transport vesicles, lipid droplets (LI) and proteoglycan granules. Moreover, ECM produced by cultured chondrocytes contained a homogeneous population of fibrillar collagens (FC) with typical striation (Figure 4G and J). Control IFPSCs exhibited morphological features typical of MSCs, including various mitochondrial profiles, small vacuoles and a nucleus with multiple nucleoli (Figure 4H and K). In contrast, extract exposed IFPSCs seemed to have undergone chondrogenic differentiation characterized by nuclei with prominent nucleoli, extended cistern of RER, Golgi apparatus (GA) and vacuoles and fine cytoplasmatic processes (Figure 4I and L). Moreover, a characteristic feature present in chondrocytes and found in extract exposed IFPSCs (Figure 4I and L), but not in control (Figure 4H and K), was the presence of proteoglycan granules in both cytoplasm and ECM. Rounded-shaped lipid vesicles of variable size were also presents in differentiated cells.

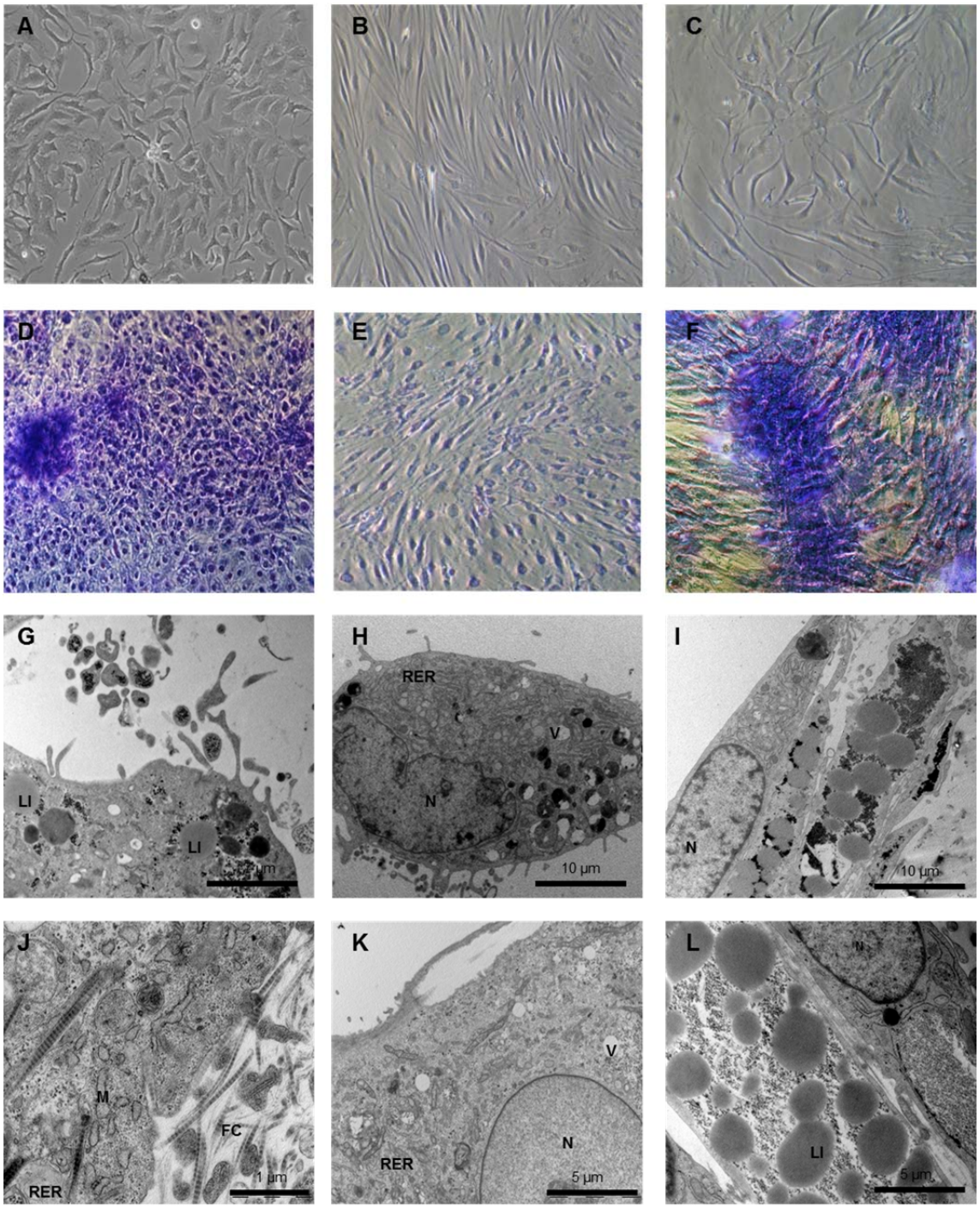


Figure 4. Morphological analysis of control IFPSCs (permeabilized but not exposed to extract cells) and extract exposed cells. Phase-contrast light microscopy of chondrocytes (**A**), control IFPSCs (**B**) and extracts-treated IFPSCs (**C**). GAG synthesized was visualized by Toluidine blue staining in chondrocytes (**D**) and in IFPSCs under chondrogenic differentiation (**F**), but not in control cells (**E**). TEM analysis of chondrocytes (**G** and **J**) showing a cytoplasm containing abundant RER, LI, proteoglycan granules and a homogeneous distribution of FC. Control IFPSCs with their complement of cytoplasmic organelles, such as RER, GA and vacuoles (**H** and **K**). Finally, IFPSCs exposed to the extract showed rounded-shaped

lipid vesicles and proteoglycan granules in both cytoplasm and ECM (**I** and **L**). Original magnification 20 x for A-F.

I.6.Transdifferentiated IFPSCs increased expression of chondrogenic markers

After two weeks of exposure to extracts, we analysed the expression of collagen II by immunofluorescence. Extract exposed IFPSCs (Figure 5C, F and I) showed a strong expression of collagen II protein with a similar pattern of cultured chondrocytes (Figure 5A, D and G). Collagen type II was confined in both cytoplasm and ECM. However, non-treated IFPSCs displayed a weak staining for the chondrogenic marker collagen II (Figure 5B, E and H).

In addition, differential expression of selected chondrogenic key markers was evaluated by RT-PCR (Figure 5J). After 14 days, extract-treated cells showed increased gene expression for *Col2A1, Acan*, *Col10, L-Sox5, Sox6* and *Sox9*, in comparison with control IFPSCs, which showed a low expression for *Acan* and *L-Sox5* and a weak expression for *Col2A1, ColX,* and *Sox6*. Chondrocytes exhibited higher expression levels of these genes than exposed cells. No significant changes were observed in the expression of *Col1* in all samples. Interestingly, extract not exposed cells showed a basal expression for *Col2A1*, *Sox9*, *Acan*, *Sox6* and *ColX*.

I.7. Cell attachment and distribution in the scaffold

Chondrocytes, extract exposed cells and control cells were seeded in the cylindrical scaffold for 5 days and outside and inside layers of each scaffold were examined by ESEM (Figure 6). All cells were able to attach on the polymer surface by filopodia and connected with each other. Chondrocytes acquired a predominantly spherical or sometimes fusiform and were surrounded with a dense matrix that extended between the cells to form a continuous network of cell and matrix (Figure 6A and B). In contrast, control non-exposed IFPSCs showed a flattened morphology with a high number of prolongations and secrete some dense material (Figure 6C). Moreover, the cross-section images clearly showed that these control cells were able to penetrate within scaffold (Figure 6D). Interestingly, extract exposed IFPSCs were able to grow covering the scaffold pores and to secrete a high dense extracellular matrix that formed a

homogeneously compact surface (Figure 6E and J). Furthermore, histological and immunohistochemical analysis of scaffold sections clearly showed cartilage-specific extracellular components produced by the IFPSCs exposed to the extract (Figure 7).

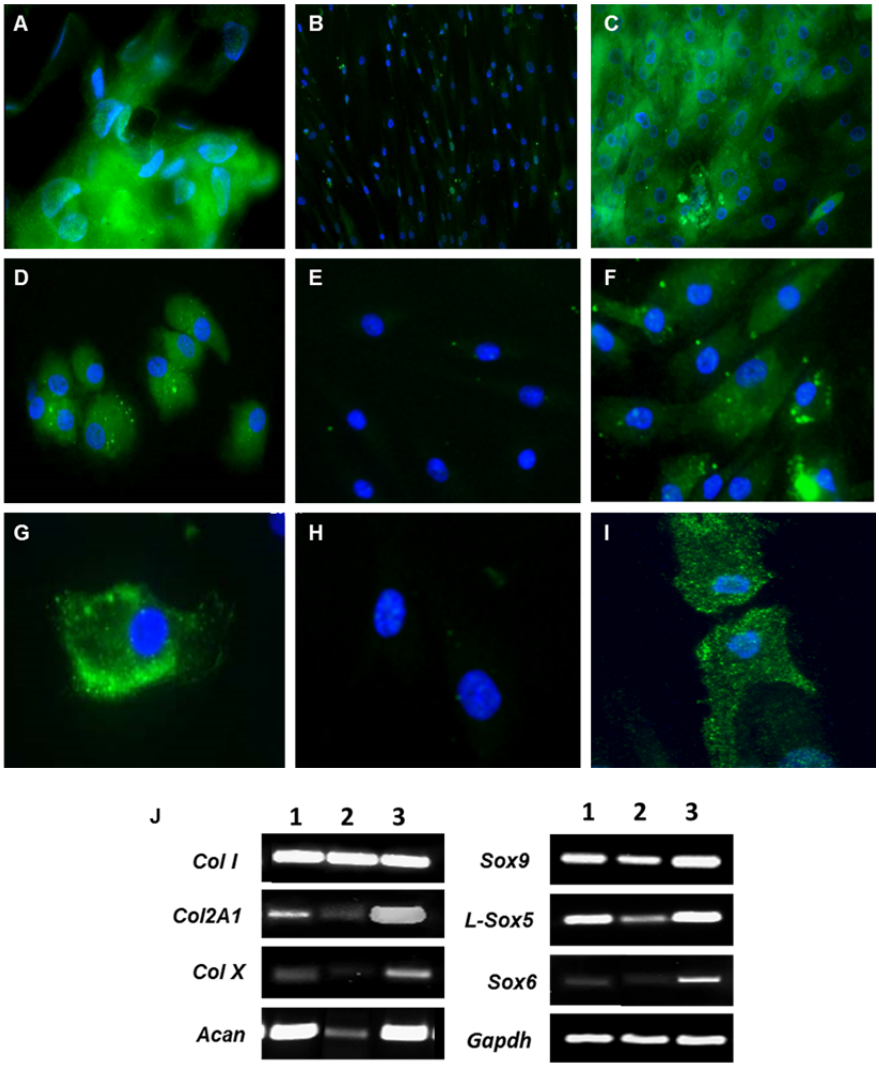


Figure 5. Immunofluorescence of collagen II and gene expression of chondrogenic markers. Type II collagen indirect immunofluorescence of chondrocytes (**A**, **D** and **G**), Control IFPSCs (**B**, **E** and **H**) and IFPSCs exposed to the extract (**C**, **F** and **I**). Expression of cartilage-specific collagen II protein with intense green staining can be appreciated on treated cells, showing the characteristic collagen fibbers framework of cartilage. Original magnification 63 × for A and G-I; 10x for B; 20x for C; 40x for D-F. RT-PCR analysis of chondrogenic markers (**J**). Two weeks in culture chondrocytes used as positive control (lane3) and extracts-exposed IFPSCs (lane1) showed increased gene expression for *Col2A1, Acan*, *Col10, L-Sox5, Sox6* and *Sox9*. Basal expression of these genes was seen in control IFPSCs (lane 2). Expression of *Gapdh* was used as an internal control. Experiments were performed in triplicate, were carried out at least twice and yielded similar results.

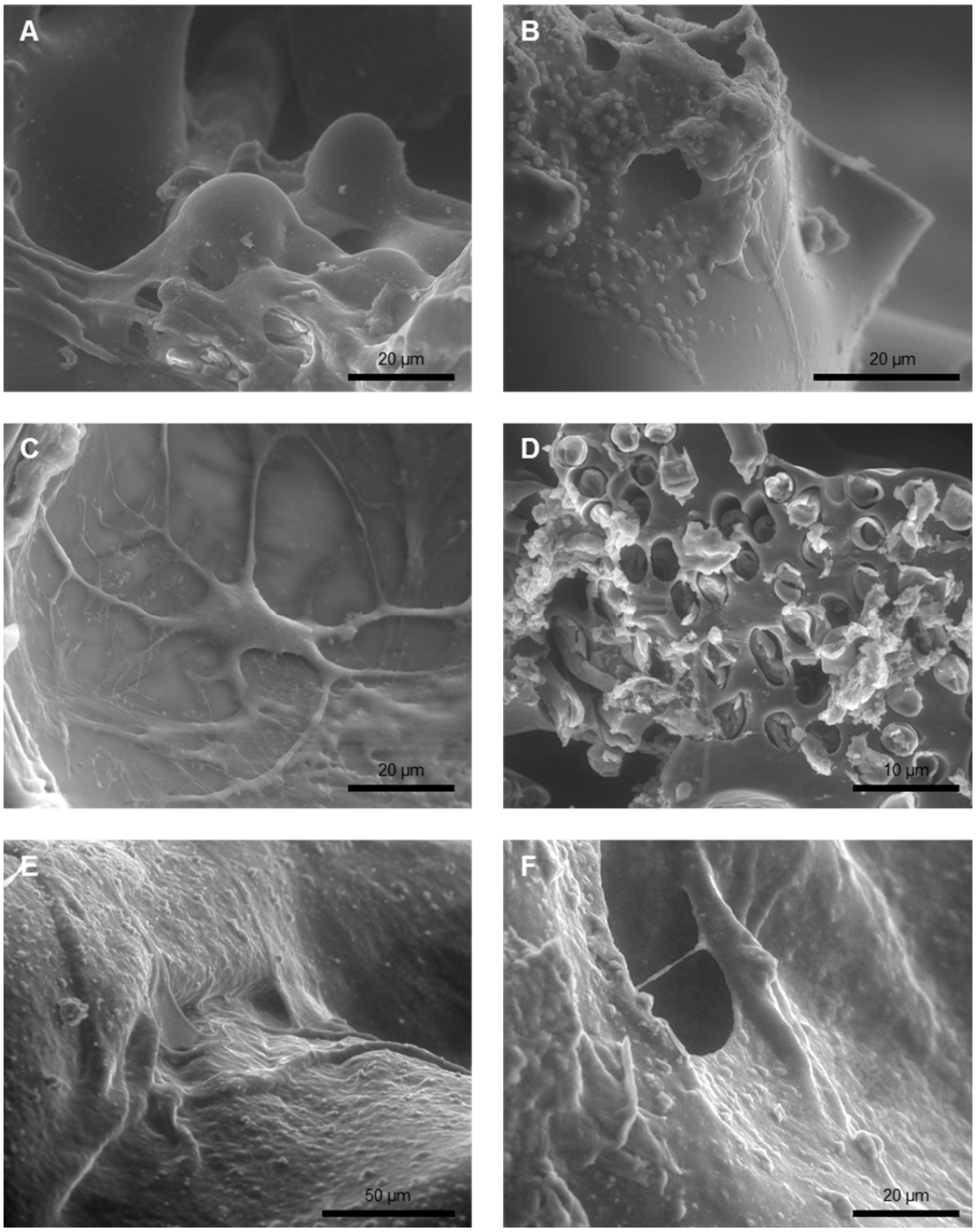


Figure 6. Scanning electron micrograph of human of chondrocytes (**A** and **B**), control IFPSCs (**C** and **D**) and extracts-treated IFPSCs (**E** and **F**) after 5 days growing on the PLGA scaffold. Numerous cells firmly adhere to the scaffold and appeared to be suspended within the lumen or crawled around the walls. Chondrocytes show a round shaped morphology and a rough surface. Chondrocytes and extracts-treated IFPSCs appeared surrounded by matrix.

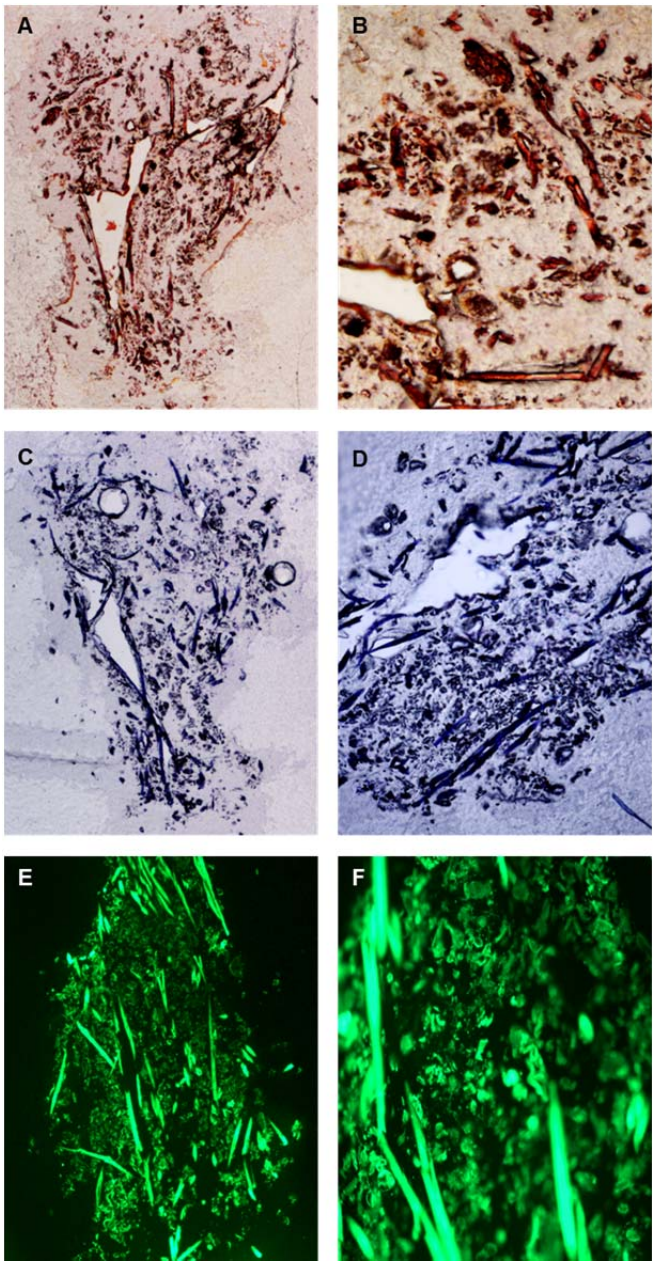


Figure 7. Analysis for cartilage-specific extracellular components of PLGA scaffolds sections seeded with transdifferentiated IFPSCs. Safranin O staining (**A** and **B**) and touidine blue staining (**C** and **D**) revealing glycosaminoglycans production. Indirect immunofluorescence visualized in green for cartilage-specific collagen II protein (**E** and **F**). 5x original magnification for: A, C, E; 10x original magnification for: B and D; 20x F.

CHAPTER II:

Cellular extract from postmortem human cardiac tissue direct cardiomyogenic differentiation of human adipose derived stem cells

Cellular extract from postmortem human cardiac tissue direct cardiomyogenic differentiation of human adipose derived stem cells

II.1. Cell isolation and characterization of hASCs

hASCs obtained following isolation from human lipoaspirates showed a typical spindle shape that acquired a homogeneous appearance after 3 weeks in culture. FACS characterization demonstrated a positive expression of the surface markers CD73 (>99.42%), CD90 (>99.74%), CD105 (>99.89%) and a negative expression for CD34 (1.61%), CD45 (0.75%), CD133 (1.15%), CXCR4 (1.14%) and KDR (1.14%) and CD31 (1.4%) (Figure 1A). To test the potential of these stem cells to differentiate along mesenchymal lineages, hASCs were treated with conditioned media to induce adipogenic, osteogenic and chondrogenic differentiation. hASCs were able to express all three phenotypes when cultured with the appropriate media (Figure 1B).

II.2. RNA integrity and gene expression analysis of human cardiac postmortem tissues

Frist, the quality and integrity of the RNA extracted from 5-12 h postmortem tissues was evaluated using the RNA Integrity Number (RIN). RIN is an algorithm based on a selection of features that provides information about the RNA integrity: i) proportion of large molecules compared to smaller ones; ii) the state of the degradation process and iii) how far the degradation proceeded. The samples are assigned to 10 different categories ranging from 1 (worst) to 10 (best) (Schroeder et al., 2006). Samples showed a high degree of RNA integrity with RIN values ranged from 9.5 to 8.5 and with a 260/280 ratio of 2 (Figure 2A).

Second, gene expression analysis of cardiac markers such as *TNNI3* and *MYL3* was performed by real-time PCR. The threshold cycle (Ct) represents the PCR cycle at which an increase in SYBR Green fluorescence above a base line signal can first be detected. Figure 2B shows Ct values obtained for the genes *TNNI3, MYL3* and *GAPDH* in the 5 different autopsy samples.

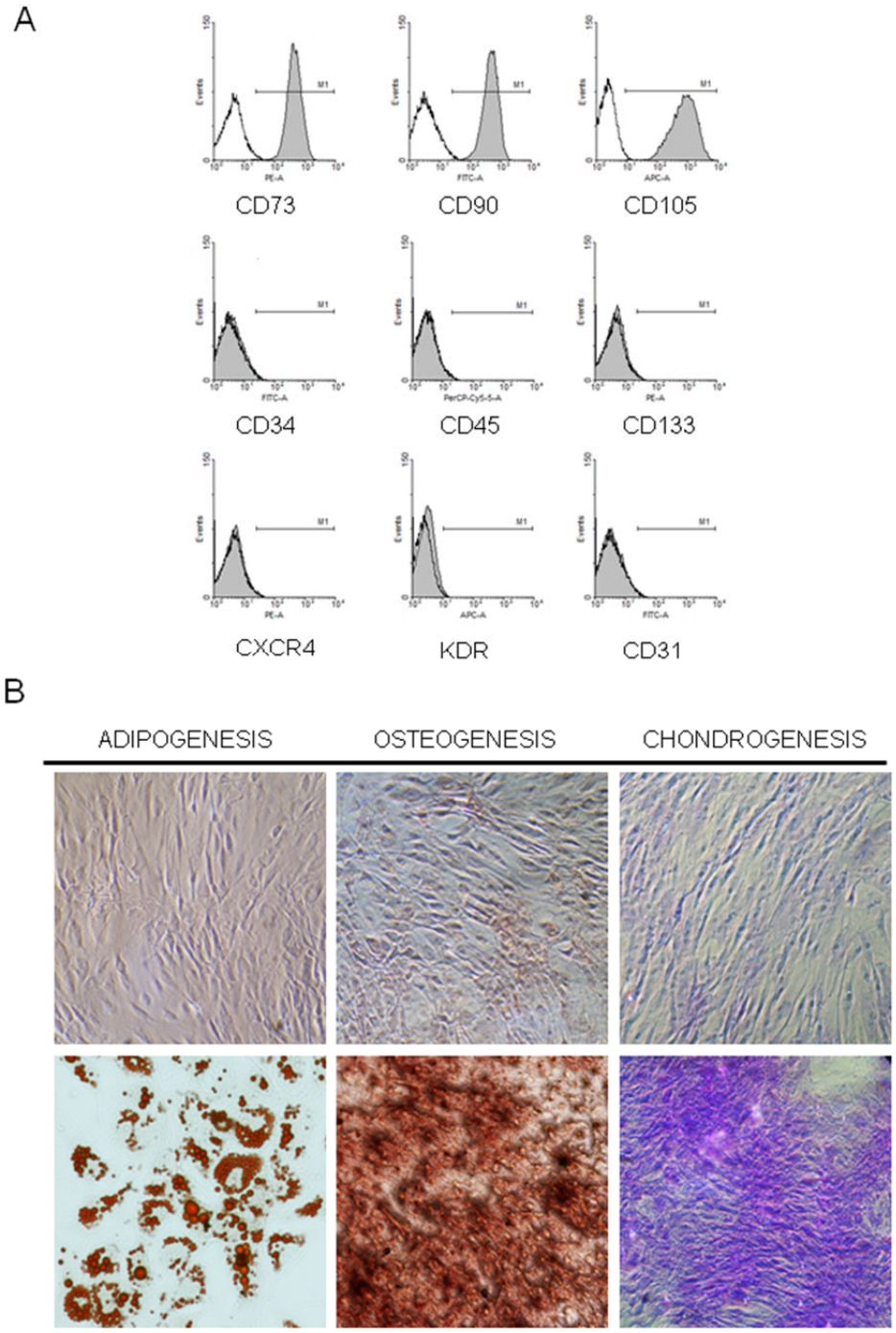


Figure 1. Phenotypic characterization and differentiation potential of hASCs. (**A**) hASCs were cultured for three weeks and then tested for mesenchymal surface markers (CD105, CD73 and CD90), hematopoietic and endothelial markers (CD133, CD34, KDR, CD45, CXCR4 and CD31) by flow cytometry. (**B**) The differentiation potential of hASCs towards adipogenic, chondrogenic and osteogenic lineage was confirmed by Oil Red O, Toluidine Blue and Alizarin Red S staining, respectively. Upper pictures show negative controls, cells cultured in normal medium for 2 weeks and then histochemically stained. Original magnification: 10 x.

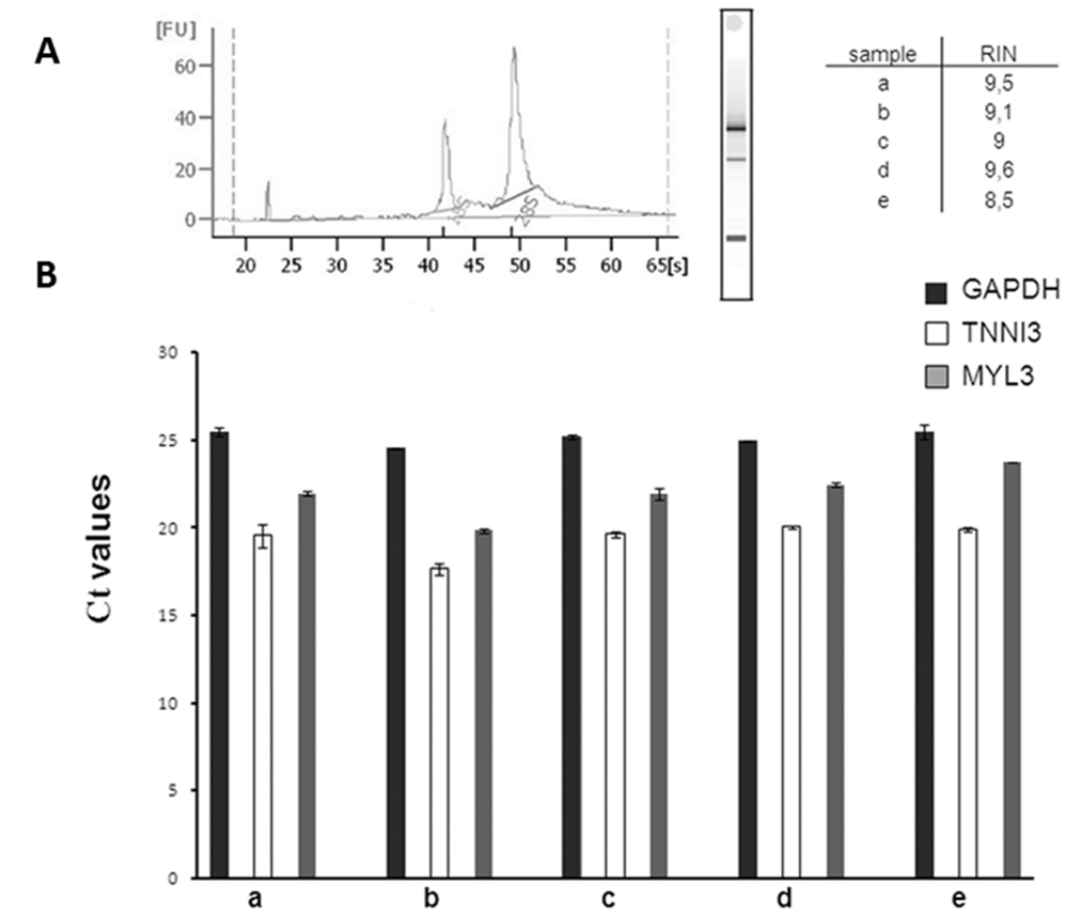


Figure 2. (**A**) Postmortem cardiac tissue RNA integrity, from left to right representative RIN graphic for a RNA sample with a RIN value of 9.5, and RIN values for all the samples (**B**) Real-time PCR gene expression analysis of cardiac troponin I (*TNNI3*), myosin light chain 3 (*MYL3*) and Glyceraldehide-3phosphate dehydrogenase (*GAPDH*) in 5 samples (represented a-e) obtained from postmortem cardiac tissue.

II.3. Expression of cardiac markers in hASCs after differentiation

In this study, we tested the cardiac differentiation potential of two different methodologies, both based on the use of postmortem cardiac tissue. Gene expression analysis of mRNA levels and immunofluorescence studies revealed expression of cardiac-specific markers in hASCs after exposition to the conditioned medium or to the cardiac cellular extract.

Figure 3 shows cardiomyocyte-related genes expression after cardiomyogenesis induction. Expression of early (*GATA-4* or *Mef2c*) and late (*α-cardiac actin* and *cardiac troponin I*) cardiomyocyte development markers, were found in treated cells.

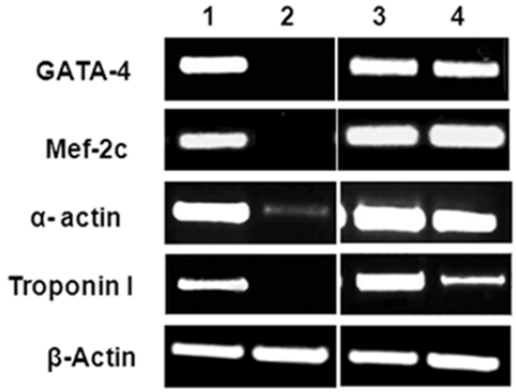


Figure 3. RT-PCR analysis of expression of cardiomyocyte marker genes. Isolated cardiomyocytes used as a positive control (lane 1), control hASCs (lane 2), cardiomyocytes-conditioted medium treated cells (lane 3) and cells treated with the cardiomyocytes extract (lane 4) expressed *GATA-4* (194bp), *Mef2c* (230 pb), *cardiac α-actin* (400 bp) and *cardiac troponin I* (233bp). Results are representative of three independent experiments.

Indirect immunofluorescence analyses of human cardiac-troponins (I and T), sarcomeric α-actinin and desmin protein distribution revealed clear differences between treated and untreated hASCs (Figures 4, 5). After induction by the cellular extract or by the conditioned medium we found a high percentage of cells (50-60%) that were positive for troponin I with elongated nuclei, as shown by DAPI staining (Figures 4C, 4D and 5D). Desmin expression was strongly positive, with a markedly filament organization in treated hASCs (Figures 4E and 5C, 5E) when compared with control cells (Figures 4A-B and 5A-B). Although, there was a weak or not significant staining for sarcomeric α-actinin in non-induced cells (Figures 4G-H and 5G-H), higher expression of this protein was observed in the exoskeleton of cells treated with both differentiation methods (Figures 4J and 5J). Finally, Troponin T was highly expressed in treated cells exposed to the cardiac extract (Figure 4I and K) or cultured in conditioned medium (Figure 5I and K) with almost 65% of positive cells in both methods.

Morphological changes were also noticeable after treatment with cardiac postmortem tissue by both methodologies. Closer inspection of Figures 4 and 5 showed that treated cells extended their cytoplasmic processes with adjacent cells and achieved a myotube-

like morphology, while control cells maintained their spindle-like morphology. In addition, multinucleated cells were detected after the induction (Figure 5J-L).

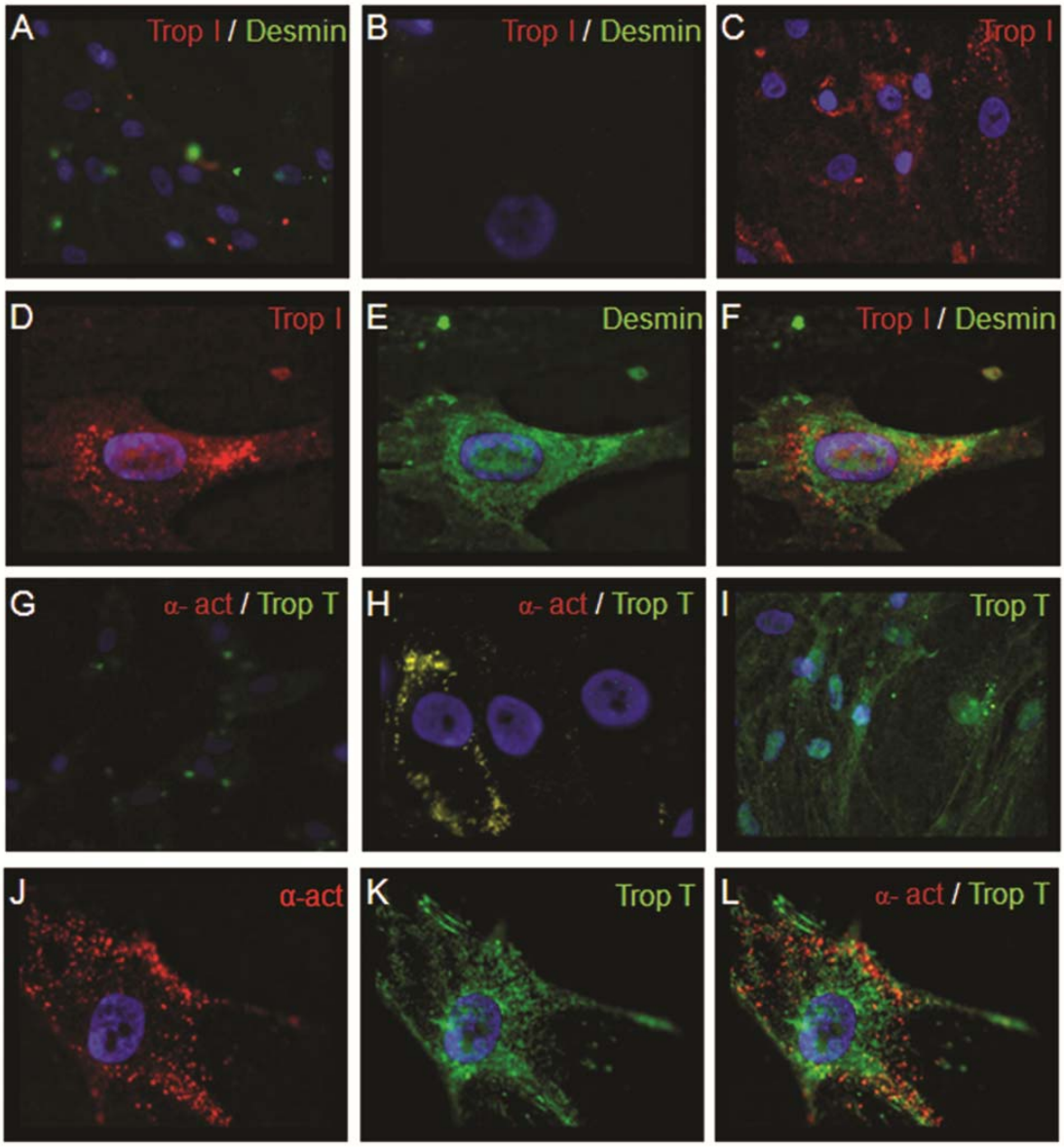


Figure 4. Immunofluorescence analysis of cardiac markers in hASCs after extract induction. (**C-F** and **I-L**) Cardiomyogenic differentiation of hASCs after 2 weeks of being exposed to cellular extract obtained from human autopsies. (**A-B** and **G-H**) Control non-treated cells. Red label for expression of troponin I (Trop I) and α-actinin (α-act) and green label for expression of desmin and toponin T (Trop T). Nuclei were stained with DAPI. Original magnification x40 for A, C, G and I; x63 for B, D, E, F, H, J, K and L.

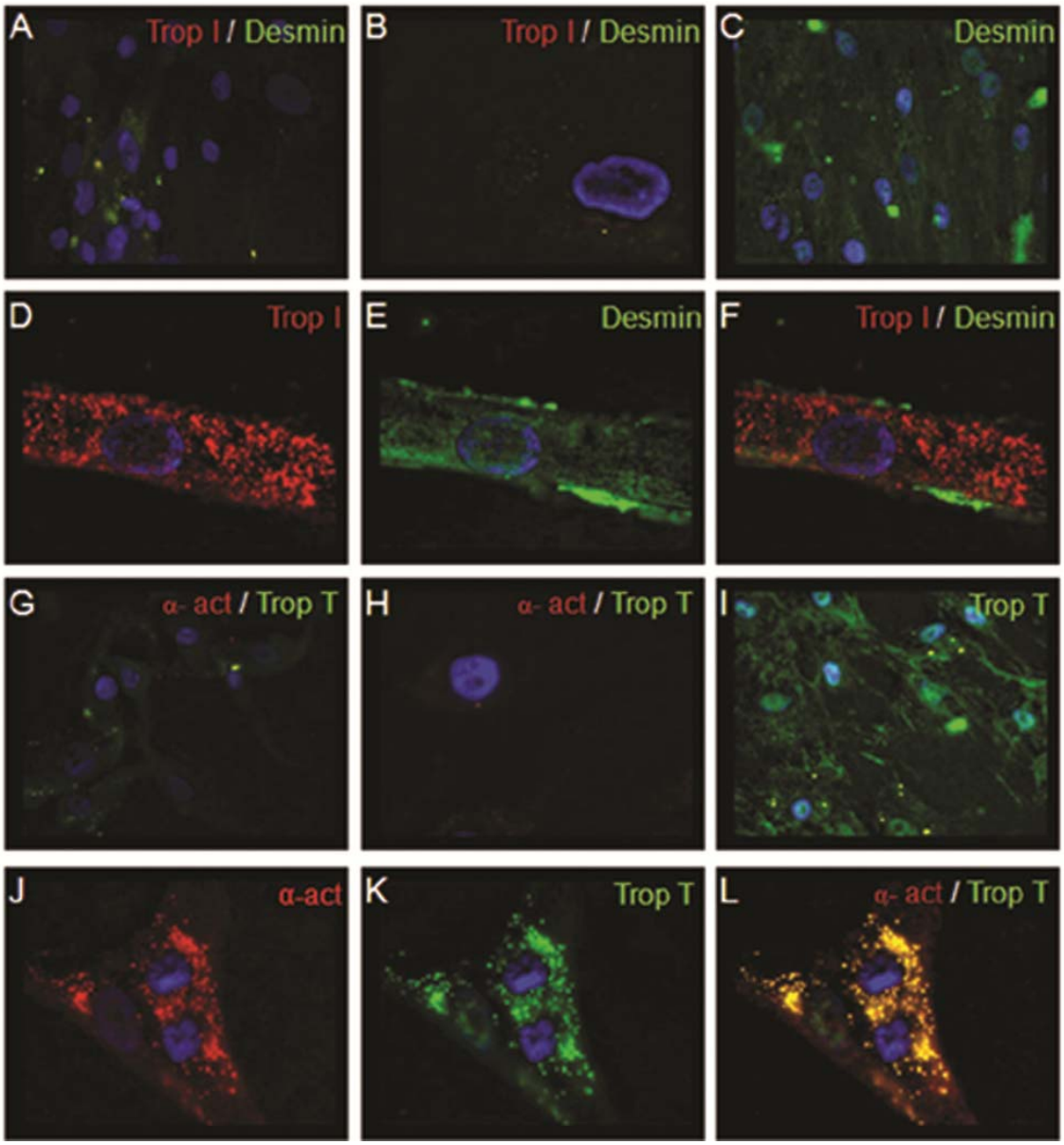


Figure 5. Immunofluorescence detection of cardiac differentiation markers of hASCs cultured in conditioned medium. (**C-F** and **I-L**) hASC after 2 weeks of being exposed to human cardiac explant-conditioned medium. (**A-B** and **G-H**) Control hASCs. Red label for expression of troponin I (Trop I) and α-actinin (α-act) and green label for expression of desmin and toponin T (Trop T). Nuclei were stained with DAPI. Original magnification x40 for A, C, G and I; x63 for B, D, E, F, H, J, K and L

CHAPTER III:

Cardiomyogenic differentiation potential of human endothelial progenitor cells isolated from acute myocardial infarction patients

Cardiomyogenic differentiation potential of human endothelial progenitor cells isolated from acute myocardial infarction patients

III.1. Characterization of total MNC from UCB and PB of AMI patients

Firstly, we analyzed by flow cytometry the percentage of expression of proliferative and endothelial markers in freshly isolated total MNC from both origins. The cell surface adhesion molecule CD34 was expressed by 1.3 ± 0.12% and 1.46 ± 0.21% of total MNC from AMI-PB and UCB, respectively (Figure 1A). In addition, we found a higher expression of CD133 in UCB (6.1 ± 0.79%) than in AMI-PB (2.5 ± 0.18%) ($P< 0.05$) (Figure 1B). Furthermore, the chemokine receptor CXCR4 was notably expressed on freshly isolated MNC from both origins, with an expression of 80.2 ± 5.6% for UCB and 58.6 ± 3.1% for AMI-PB (Figure 1C). Finally, we found significant differences when the vascular endothelial growth factor receptor KDR was studied (Figure 1D), with 6.1 ± 0.89% expression in MNC from PB of AMI patients in comparison with a 2.5 ± 0.02% from the UC.

III.2. Endothelial cells colony-forming units

Isolated MNC were further processed and since mature circulating EC attach within the first 48 hours of culture, we discard this population by collecting only non-adherent cells and replanting then to fibronectin-coated-well plates. Initially, isolated cells had a rounded morphology and after two days of culture formation colonies was detected (Figure 2A), which consisted of rounded ball-like clusters with elongated sprouting cells at the periphery. These clusters were classified as endothelial cell colony-forming units (CFU-EC). At day 5 CFU-EC increased in size and appear with a more defined morphology while on day 7, spindle-like EPCs sprouted out from the edge of the clusters and spread over the entire culture well surface (Figure 2A). CFU-ECs were counted manually in a minimum of four wells at day 5 and 10 and no significant differences ($P = 0.5$) were found between CB- and AMI-EPCs. In addition, CFU-EC number peaked at day 5 and significantly decreased ($P <0.05$) by day 10 in both cell sources (Figure 2B).

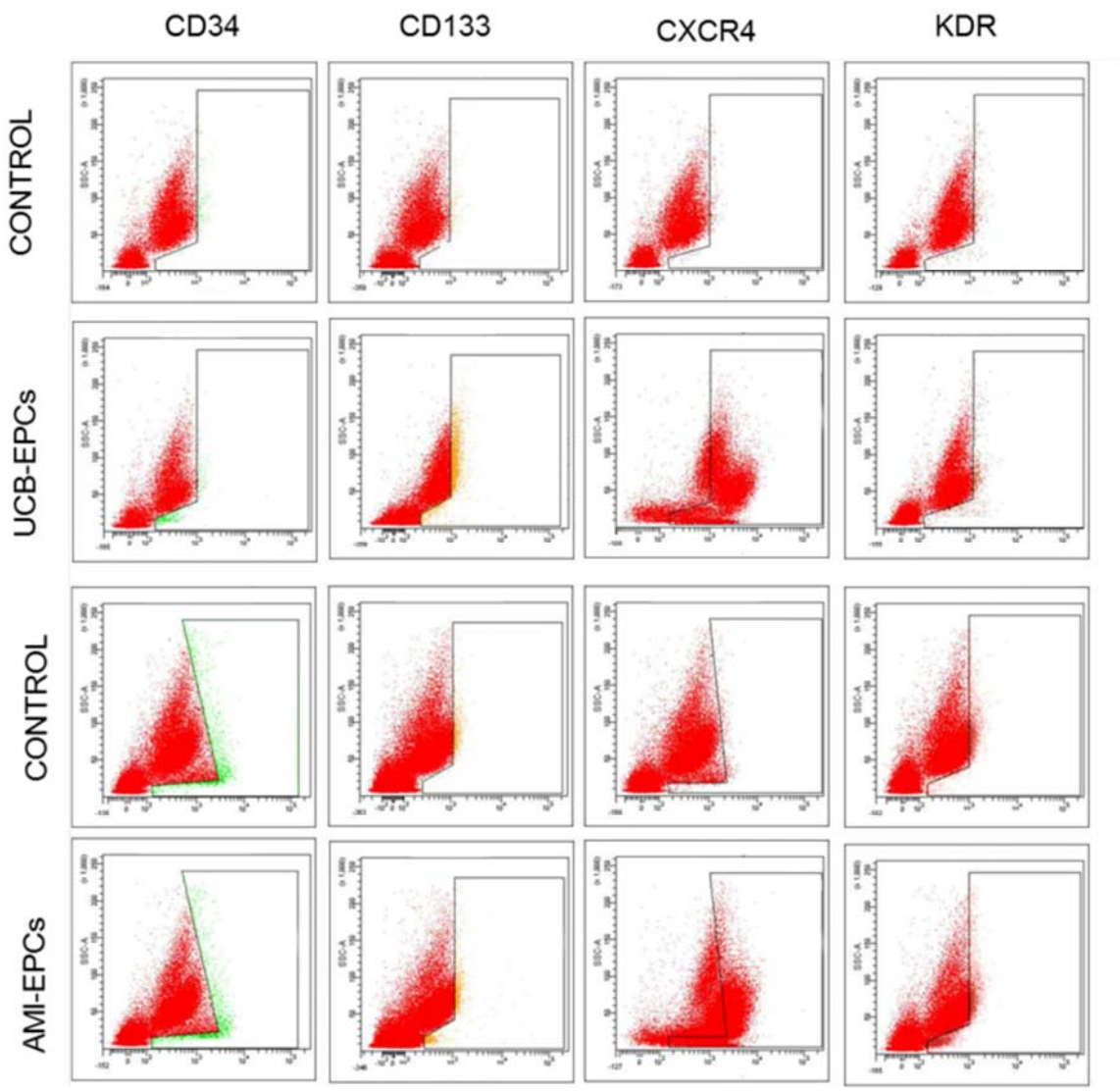


Figure 1. Representative graphics of FACS analysis of isolated MNC from umbilical UCB-EPC and from PB of patients diagnosed of AMI.

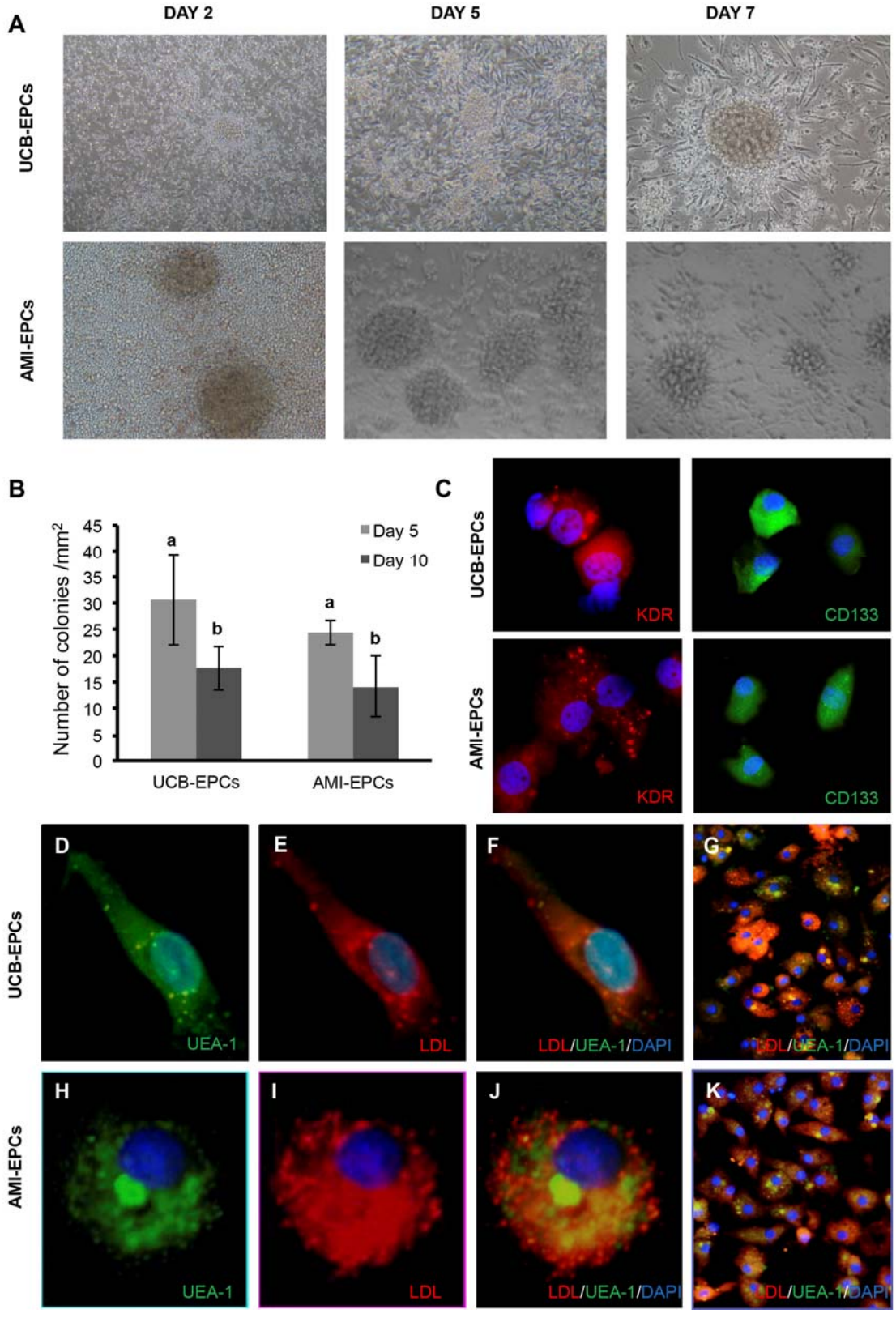


Figure 2. Characterization of EPCs derived from UCB (UCB-EPCs) or PB of AMI patients (AMI-EPCs). (**A**) Morphology of the UCB-EPCs and AMI-EPCs at days 2, 5 and 7 of culture. (**B**) Assessment of CFU-EC at 5 and 10 days later of the isolation. Different letters stand for significant differences (One-way

ANOVA; P<0.05). (**C**) Immunofluorescence of the UCB-EPCs and AMI-EPCs showing positive staining for endothelial markers CD133 and KDR. (**D-K**) Cell surface binding of UEA-1 and DiI-ac-LDL incorporation. Original magnification : 10x for A; 20x for G and K; 40x for C and 100x for D-F and H-J.

III.3. Endothelial markers expression

The attached cells were characterized, as previously shown (Asahara, Murohara et al. 1997), by the expression of the typical endothelial markers KDR and CD133. Immunofluorescence phenotyping revealed that both the CB and AMI-EPCs highly expressed these endothelial markers (Figure 2C). In addition we tested diI-acLDL and UEA-1 binding as specific functional markers of EC. Representative imagines of UEA-1 cell surface binding and dil-acLDL incorporation are shown in Figure 2D-K, demonstrating that AMI-EPCs and UCB-EPCs displayed, in a similar pattern, the functional phenotypes of EPCs.

III.4. Differentiation of EPCs towards cardiomyocites

To investigate the differentiation potential towards cardiomyocytes of adult circulating EPCs from patients who had suffered AMI, we treated cells with DNA demethylation agent 5-aza. After 5-aza exposition, EPCs morphology changed dramatically in both PB-AMI and UCB sources from spindle-like cells towards elliptical and elongated shape cells with a marked size enhancement. Furthermore, cells arranged in parallel and formed myotube-like structures, while control cells maintained their spindle-like morphology (Figure 3A-F).

The acquisition of a cardiomyogenic differentiation phenotype was confirmed by immunocytochemical and RT-PCR analysis. Cardiac-specific markers, troponin I, and the myocyte-specific proteins sarcomeric α-actinin, desmin and cardiac troponin T were detected after epigenetic reprogramming of EPCs by 5-aza (Figure 3G and 3H-O).

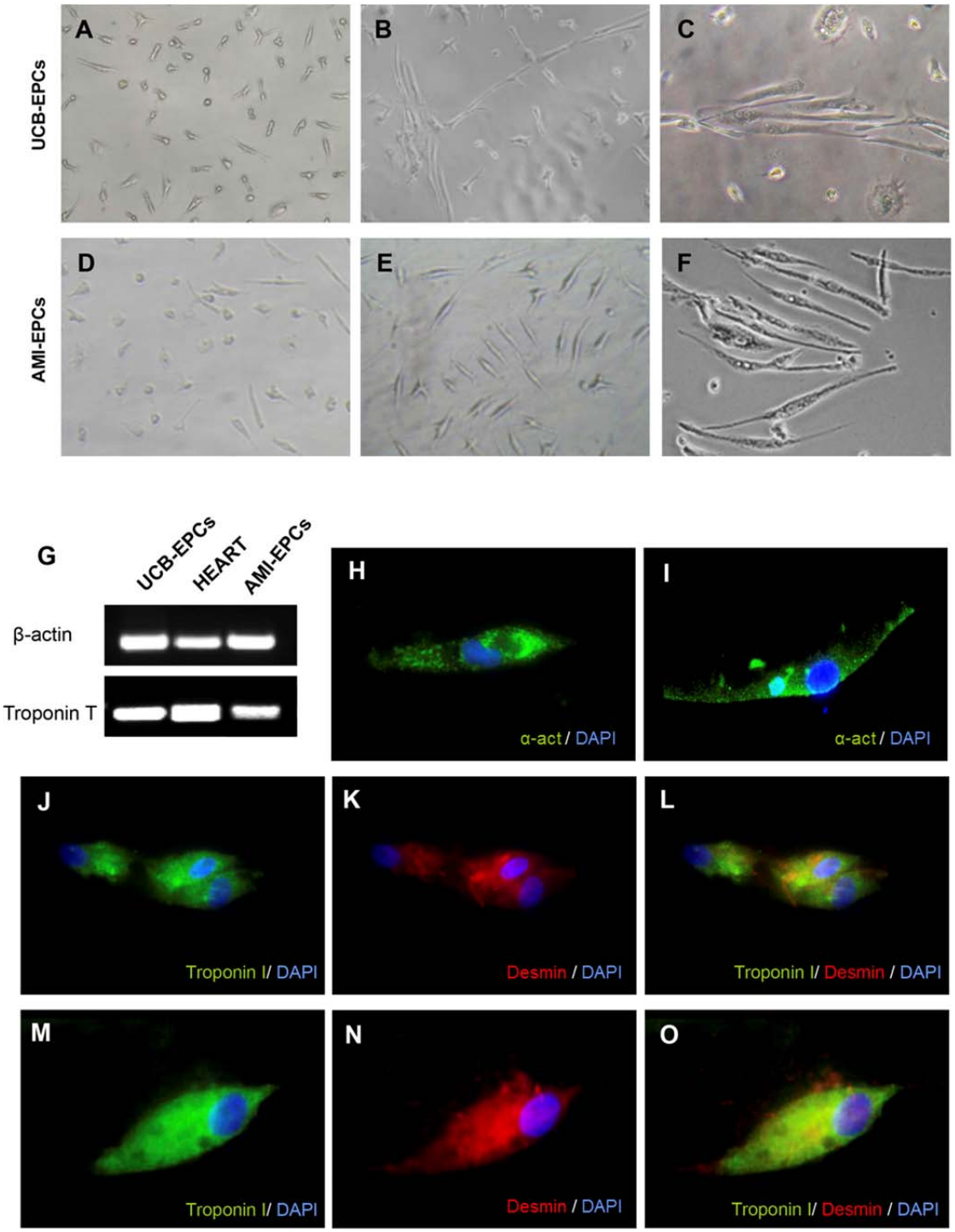


Figure 3. Phenotypic changes and gene expression induced by 5-aza in EPCs (**A, D**) Phase-contrast light microscopy of non-treated EPCs (control EPCs). (**B**, **C**, **E** and **F**) EPCs exposed to 5-aza appeared enlarged, with an elliptical shape and formed myotube-like structures. (**G**) RT-PCR analysis revealed the expression of cardiac Troponin T in cells treated with 5-aza. Human heart tissue was used as a positive control. (**H-O**) Fluorescence immunostaining of differentiated EPCs toward cardiomyocytes after 2 weeks of treatment. Green label for sarcomeric α-actinin and troponin I expression and red label for desmin expression. Nuclei stained with DAPI. Original magnification: 10x for A, B, D and E; 20x C and F; 40x H-L and 63x M-O.

DISCUSSION

Chondrocytes extract from patients with osteoarthritis induces chondrogenesis in infrapatellar fat pad derived stem cells

Mature hyaline cartilage has a very low self-repair potential due to its intrinsic properties. For this reason, researchers have focus in the search of methods to induce complete cartilage repair. In the last years, tissue engineering strategies combining cell therapy and scaffolds has emerged as a promising new approach for the treatment of articular cartilage defects (Albrecht et al., 2011; Peran et al., 2012).

Cellular reprogramming based on cell extracts has shown that differentiated cells can be transdifferentiated in other differentiated cell types or dedifferentiated into pluripotent cells (Collas and Taranger, 2006; Peran et al., 2011). Moreover, we have demonstrated that using extracts from adult human heart tissue MSCs induced differentiation towards cardiomyocytes (Peran et al., 2010).

Recently, it has been shown that IFPSCs undergo chondrocyte differentiation, do not seem to have any age related decline in proliferative potential and are easily accessible with less discomfort to the patients (Buckley et al., 2010a; Khan et al., 2012). In this work we analyse the chondrogenic potential of MSCs obtained from Hoffa's fat pad of patients with OA after the exposition to an extract, containing the intracellular components of chondrocytes.

We observed that human articular chondrocytes obtained from elderly patients maintained a chondrocyte phenotype in agreement with Varghese et al. (Varghese et al, 2007). Chondrocytes and IFPSCs characterization by flow cytometry showed similar expression of MSCs surface makers CD90, CD105 and CD73. CD90 has been suggested as a new target to induce re-differentiation of de-differentiated human chondrocytes in cartilage regeneration procedures and CD105 could be used as a molecular marker predictive of the capacity of cultured chondrocytes to form cartilage *in vivo* (Diaz-Romero et al., 2005). Also, collagen II was highly expressed in chondrocytes, and showed a basal expression in IFPSCs. In fact, mature articular joint develops from embryonic mesodermal precursors that differentiate into chondroprogenitors and ultimately into mature adult chondrocytes (O'Sullivan et al.,

2011). In addition, we found that IFPSCs were able to differentiate into specific lineages suggesting that fat pad cell population, as shown previously even in OA, contains a population of highly proliferative and multipotent MSCs (Wickham et al., 2003).

IFPSCs exposed to chondrocyte extracts showed characteristic morphological changes suggesting the acquisition of a chondrocyte-like cell phenotype that was confirmed by the increased proteoglycan formation (Pabbruwe et al., 2009) in both toluidine staining and TEM observations, in agreement with chondrocytes typical features (Fioravanti et al., 2005). Further studies to demonstrate if the chondrocyte extract induce modifications in the expression of adipogenic and/or osteogenic markers will be of interest.

Collagen type II, a marker for hyaline cartilage together with aggrecan, are the predominant proteins in the extracellular matrix of cartilage (Yamashita et al., 2009). Immunostaining of extracts-treated IFPSCs showed an increased expression of collagen II. Similarly, PCR analysis displayed an up-regulation of *Col2a1* and others chondrocyte-marker genes including *Sox9, L-Sox5* and *Acan*. *Sox9* is the master transcription factor for chondrogenesis, which acts in early stages of chondrocyte differentiation by directly induction of type II collagen (Lefebvre et al., 1997) and is expressed in the mesenchymal condensations (Lefebvre et al., 1998). Moreover, it has been previously demonstrated that *Sox9* in concert with *L-Sox5* and *Sox6*, regulates cartilage formation and maintains the chondrocyte phenotype in the mature cartilage by activating expression of several cartilage-specific genes, including genes *Col2a1*, *Col9a1* and *Col11a1*, *Acan* and *Comp* (Han and Lefebvre, 2008). In treated IFPSCs *Sox9* and its cofactor *L-sox5* expressions were elevated and a slightly induction of *Sox6* was found.

Some OA characteristics are the expression of hypertrophy markers such as collagen X with a concomitant decrease in the synthesis of type II collagen and aggrecan (Steinert et al., 2009). In our study chondrocytes isolated from patients with OA showed increased *Col10* expression, however IFPSCs exposed to the extract displayed a marked reduction. This fact together with the increased expression of *Sox9*, *L-Sox5*, *Sox6* and *Col2a1*, suggest the efficacy of this methodology to promote mature chondrogenesis.

Nevertheless, the use of *Col10* mRNA as a marker of chondrogenic hypertrophy for *in vitro* studies has been questioned (Mwale et al., 2006).

Type I collagen is categorized as a fibril-forming collagen and is usually produced when cells go into fibroblastic or osteoblastic differentiation (Dessau et al., 1980). In our experiment, *Col1a1* transcripts were constitutively expressed in chondrocytes and IFPSCs. After exposure to the extracts this expression was not affected. Type I collagen participates in regulating mesenchymal condensation and the onset of chondrogenic differentiation (Chen et al., 2012). Moreover, *Col1* expression increase has been often closely related to chondrogenic differentiation *in vitro* (Tallheden et al., 2004). The basal expression of characteristic chondrogenic markers in IFPSCs can be explained according to their micro-environmental niche. Recently, it has been demonstrated that non-cartilaginous knee joint tissues such as infrapattelar fat pad possess significant chondrogenic potentials and this may be associated with the proximity to the niche they reside (O'Sullivan et al., 2011; Toghraie et al., 2011; Buckley and Kelly, 2012). In addition, IFPSCs offers a number of practical advantages such as they can be extracted from the same patient, avoiding rejection or side effects of immunosuppressive medication administration. Moreover, they are easily accessible, with less discomfort to the patient, as they can be obtained from osteoarthritic knee during knee arthroscopy. Furthermore, the fat pad has a greater yield of MSCs than bone marrow (Khan et al., 2007; Khan et al., 2008).

It has been shown that in comparison with monolayer 3-D matrices, which are developed to mimic the extracellular environment, could maintain better chondrocyte phenotypes and play a critical role in supporting chondrogenesis (Benya and Shaffer, 1982; Peran et al., 2012). Therefore, we growth transdifferentiated IFPSCs in 3-D PLGA scaffolds, a cylindrical implant constructed with a porous cartilage phase which have been used before as cell-free for cartilage repair in surgery (Melton et al., 2010). Nano-structured PLGA surfaces have been shown to accelerate chondrocyte adhesion and proliferation, as well as extracellular matrix production (Park et al., 2005). Our results showed that 3D PLGA scaffold was able to support growth and cell expansion and facilitate their free diffusion throughout the structure. These preliminary results demonstrated the affinity of IFPSCs for PLGA-based scaffolds and its ability to support chondrogenic differentiation.

Transdifferentiation studies have supported the notion that cell fate is controlled by master switch genes and that one or two factors can be sufficient to direct cells from one lineage to another (Li et al., 2005). Transdifferentation of the IFPSCs into chondrocytes can involve both the suppression and regulation of different genes in the cells, implying that genes from both cell types are co-expressed at some point. Further studies are necessaries to identify the factors and molecules presents in the extract that should enhance our understanding of the mechanisms involved in chondrocyte differentiation and development.

In conclusion, our results confirmed that extracts obtained from chondrocytes of osteoarthritic knees promote chondrogenic differentiation of IFPSCs. This chodrogenesis was not depended of exogenous growth factor induction, neither of the use of viral vectors. To our knowledge there have not been done experiments of IFPSCs programmed differentiation into chondrocytes using this methodology. Moreover, we show here, for the first time, that combination of transdifferentiated IFPSCs with biodegradable 3D PLGA scaffolds can serve as an efficient system for the maintenance and maturation of cartilage tissue. These findings encourage *in vivo* implantation studies to corroborate its usefulness to repair articular surface in OA.

Cellular extract from postmortem human cardiac tissue direct cardiomyogenic differentiation of human adipose derived stem cells

Related heart diseases are the major cause of death throughout the world (Ortegon et al., 2012). Due to the reduced regenerative potential of the heart, survivors of myocardial infarction often present left ventricular (LV) dysfunction that reduces quality of life and prognosis. Nowadays, treatments for AMI mainly consist on pharmacological approaches or cardiac transplantation for those patients with the most severe types of cardiac failure. Regarding to pharmacological treatments, the development of new cardioactive drugs is a complicated process in which preclinical toxicity assays are a critical step. Cell-based screening assays are currently used to study disease mechanisms and to test the effect of novel drugs and/or genomics treatments. The aim of the present study was to develop new strategies to obtain cardiomiocyte-like cells

that could be used for drug screening, based on the use of cardiac tissue obtained from postmortem human samples.

First, we tested the genetic integrity of the autopsy-derived samples and demonstrated the viability of using this tissue source. In agreement with others studies, we found that RNA could be extracted in adequate quality from human postmortem tissue (Heinrich et al., 2007; Vennemann and Koppelkamm, 2010), in fact, examination of gene expression levels at the time point of death has become an innovative tool in autopsies. In addition, we demonstrated the maintenance of cardiac markers expression in the postmortem tissue. Previously, gene expression of beta-glucuronidase (*GUSB*), nitric oxide synthase 3 (*NOS3*), *COL1A1* and collagen 3 (*COL3A1*) was shown using mRNA extracted from postmortem heart tissue (Partemi et al., 2010).

Others studies have showed the possibility of obtaining cardiomyocyte-like cells by the use of rat (Gaustad et al., 2004; Xie et al., 2006) our even human cardiac tissue (Peran et al., 2010) obtained from patients undergoing coronary artery bypass surgery. Due to the obvious limitations to obtain large amounts of tissue from cardiovascular patients the present study offers a suitable alternative. Up to 12 hours autopsy-derived tissue seams to maintain genetic integrity and protein integrity and could represent a suitable tissue source for *in vitro* cardiac differentiation induction.

ESCs have shown great potential to improve drug screening. In fact, the effect that some drugs produce in cardiomyocytes was similar to the one produced in cardiomyocytes derived from ESCs (He et al., 2003; Reppel et al., 2004). Furthermore, ESCs have proved to be a suitable model to test the development of cardiac arrhythmias as a pharmacological side effect (Caspi et al., 2009). To avoid ethical restrictions, iPS cells have become, in no time, the ideal alternative to ESCs. With the generation of disease-specific iPS cells lines from patients with a variety of diseases (Park et al., 2008a) new expectations have emerged, not only in basic research and regenerative medicine but also in a pharmacological context. Nevertheless the use of ESCs or iPS cells is restricted to a few laboratories around the world, not only because of the difficulties due to a very restrictive legislation, which limit their use, but even more important, for the high cost that ESCs and/or iPS cells culture represents.

Here, we have used hASCs that can be easily isolated and rapidly expanded *ex vivo* without provoking ethical controversy, and demonstrated their cardiac differentiation capacity using two different approaches. After two weeks of cardiac induction by cell extract or conditioned medium, clear differences between treated and untreated hASCs were observed. Treated cells expressed cardiomyocyte-related genes such as *GATA-4, Mef2c,* α-cardiac actin and cardiac troponin. *GATA-4*, and *Mef2c*, are cardiac specific transcription factors implicated in cardiac commitment and differentiation lineage (Kodama et al., 2002; He et al., 2002). It has been shown that *GATA-4* enhances MSCs capacity to differentiate into cardiac cell phenotypes (Li et al., 2011; Xu et al., 2012) even in the microenvironment after myocardial infarction (Gao et al., 2011). Moreover, *GATA-4* is one of the three transcription factors necessary to transdifferentiate somatic cells into cardiomiocytes (Sayed et al., 2013).

In our study, immunofluorescence analyses showed troponin I expression and others cardiac related proteins including troponin T, α-sarcomeric actinin and the presence of a desmin filaments organization. The expression of α-sarcomeric actinin and troponin I, two late cardiomyocyte-specific markers, has been proved to be restricted to cardiomyocytes rather than skeletal muscle (Makino et al., 1999). Furthermore, the expression of cardiomyocyte-specific markers was accompanied by noticeable morphological changes consisted in the appearance of multinucleated cells and a myotube-like morphology, suggesting cardiac differentiation.

Cardiac tissue extracts or cell lysates, from normal and even infarcted myocardial tissue of rats have been proved before to contribute to the differentiation of BM-MSCs into cardiomyocyte-like cells (Liu et al., 2008). These findings raise the hypothesis that soluble signaling molecules produced by cardiac cells after death are ideal inducers of hASCs differentiation in myocardial-like cells. In fact, up to 20 proteins including, cytokines, growth factors and myocardial related proteins have been identified to be released into the culture medium by human cardiac explants (Schittini et al., 2010). In our study, we demonstrated that the postmortem cardiac tissue maintains its integrity and provides the paracrine mechanisms necessaries to promote cardiomyogenic differentiation without direct cell-to-cell contact between cardiomyocytes and hASCs.

Previous studies have shown that cells can be isolated from postmortem tissues (Machalinski et al., 2003; Erker et al., 2010). In fact, viable hepatocytes can be isolated from cadaveric human liver after 24 hours of cold ischemia (Erker et al., 2010). In addition, stem cells isolated from postmortem tissues (up to 48h after death) are currently used for experimental and clinical purposes (Blazar et al., 1986; Kapelushnik et al., 1998; Palmer et al., 2001; Xu et al., 2003; Liu et al., 2006). Interestingly, viable and functional skeletal myogenic cells from humans up 17 days postmortem have been recently isolated (Latil et al., 2012).

It has been suggested that cardiomyocytes (Kacimi et al., 1998; Shen et al., 2000), and cardiac fibroblasts (Porter and Turner, 2009) secrete cytokines and chemokines in response to various stimuli such as ischemia or mechanical stress to the heart. Here, postmortem cardiac tissue might respond to the stress caused by the natural cardiac arrest and the hypoxic state that precedes natural death liberating factors. In fact, ischemic myocardium produces several cytokines or transcription factors, such as VEGF and SDF-1 that promote and increase stem cell survival (Leu et al., 2011) and it has been proved *in vivo* and *ex vivo* that induce differentiation of MSCs into cardiomyocyte-like cells (Toma et al., 2002; Elmadbouh et al., 2007; Das et al., 2009). In addition, factors such as TGF-β and BMP2, released by cardiac myocytes or fibroblasts (Roberts et al., 1992; Sun et al., 1993; Izumi et al., 2001) are secreted chronically after myocardial infarction (Hao et al., 1999; Bujak and Frangogiannis, 2007; Frantz et al., 2008). These factors are required in early cardiogenesis and have been seen to induce actively up-regulated expression of cardiac transcription factors in stem cells (Behfar et al., 2002; Kruithof et al., 2006; Zhang et al., 2012; Mohanty et al., 2013). Moreover, guided cardiopoiesis has been implemented with mesenchymal stem cells by using a cardiogenic cocktail compound of TGFβ1, BMP-2/4, FGF-2/4, IL-6, IGF-1/2, VEGF-A, EGF, and activin-A (Behfar et al., 2008).

In conclusion, the results of the present study show that postmortem cardiac tissue can induce hASCs to express cardiac specific contractile proteins *in vitro*. This study confirms that the soluble signaling molecules produced by cardiac cells after death are also ideal inducers of hASCs differentiation in myocardial-like cells. Further studies are needed to identify the signal regulation and factors involved in the hASCs differentiation. The methodology described here would serve as a useful and non

expensive *in vitro* model to obtain cardiomyocyte-like cells that could be used for cardioactive drugs toxicity assays.

Cardiomyogenic differentiation potential of human endothelial progenitor cells isolated from acute myocardial infarction patients

Myocardial infarction is the result of coronary arteries obstruction with the consequently reduction of blood supply to the heart muscle and the massive loss of cardiomyocytes that are replaced by a non-functional scar tissue (Laflamme and Murry, 2005). In humans, after an acute ischemic event BM-EPCs are mobilized in response to growth factors, cytokines and hormones that are released from the target tissue (Asahara et al., 1999a; Asahara et al., 1999b). These progenitor cells have the potential to differentiate into mature endothelial cells and to play a role in promoting postnatal vasculogenesis even in pathological events that require neovascularization (Asahara et al., 1997; Quirici et al., 2001; Young et al., 2007). Although it has been demonstrated that EPCs play a beneficial role in treatment of ischemic disease, the potential of circulating EPCs, from patients that have suffered an infarct, to restore heart damage tissue need further investigation. Here, we have tested the cardiac differentiation potential of circulating EPCs from PB of patients who have been diagnosed of AMI and compared with EPCs isolated from UCB.

First, we characterized total MNC from UCB and PB of AMI patients. Flow cytometric analysis revealed that freshly isolated total MNC from both origins express about 10-fold excess of CD34+ cells when compared with MNC of healthy subjects (Murohara, 2010). Recently, Yang et al showed that CD34+ cells could represent a functional EPC population in bone marrow and have beneficial therapeutic effects in myocardial infarction (Yang et al., 2011a). In fact, others studies demonstrated mobilization of CD34+ MNC in patients with AMI (Shintani et al., 2001). According with the immaturity of the MNC from UCB, we obtain higher expression of CD133 in UCB than in PB, in fact CD133 is a marker for stemness that represents hematopoietic/endothelial progenitor fraction (Salven et al., 2003; Urbich and Dimmeler, 2004). In addition, CXCR4 marker, notably expressed on hematopoietic stem cells, was highly expressed on MNC from both origins. This endothelial marker has previously been shown to play

a key role in their homing and mobilization towards ischemic tissue (Mohle et al., 1998). Moreover, over-expression of CXCR4 in mesenchymal stem cells enhances *in vivo* engraftment into the ischemic heart and subsequently improves functional recovery via increasing myoangiogenesis (Zhang et al., 2008). In contrast, KDR expression was higher in MNC from PB than in UC which is related with the fact that KDR has been proved to be responsible for VPF/VEGF-stimulated endothelial cells proliferation and migration (Zeng et al., 2002). These results indicate that peripheral blood from AMI patients seems to be a valuable source for obtaining EPCs comparable with umbilical cord blood.

After MNC characterization, cells were cultured and evaluated for the formation of endothelial-progenitor-cell colonies. Rounded ball-like clusters with elongated sprouting cells at the periphery were visible at day two and were classified as CFU-EC. CFU-EC size, forming frequency and time of appearance were similar between PB-EPCs and UCB-EPCs. For both cells sources a decrease in CFU-EC number was detected after one week when cells start to grow homogeneously over the entire culture. This results contrast with others studies which have found that EPC colonies derived from UCB emerged earlier and in a major number than colonies obtained from healthy adult peripheral blood (Kim et al., 2013). Since in our study we did not detect these variances our data suggest that the cardiac insult enhances EPC population in AMI patient PB.

Derived EPCs from AMI patient PB and UCB exhibited similar characteristics concordant with an endothelial phenotype. In both cases, typical endothelial cobblestone morphology could be detected in addition to a positive surface-marker expression for endothelial cell markers (CD133, KDR) and ac-LDL uptake. Asahara et al (1997) showed for the first time that peripheral blood contains $CD34^+$ bone marrow (BM)-derived cells, which could be characterized by the co-expression of others markers such as CD133, CD34, KDR or VE-cadherin (Massa et al., 2005; Asahara et al., 2011).

Circulating EPCs are known to play an important role in the pathogenesis and prognosis of cardiovascular diseases (Antonio et al., 2010; Bakogiannis et al., 2012) (Vasa et al., 2001; Briguori et al., 2010). Interestingly, recent studies in animal models of ischemic

cardiomyopathy suggest that transplanted EPCs can improve heart recovery after injury (Ott et al., 2005; Takamiya et al., 2006; Hu et al., 2009). Moreover, improvement in myocardial function after transplantation of autologous BM-derived stem/progenitor cells including EPCs, have been reported in clinical trials (Losordo et al., 2011), nevertheless a long-term follow-up of these patients still needs to be evaluated. On the basis of these facts, we investigated the differentiation cardiac potential of adult circulating EPCs from patients who had suffered AMI by treating them with 5-aza. This agent is known as a chemical inhibitor of DNA methyltransferase which blocks DNA methylation and consequently promotes demethylation, a key step required for epigenetic modification of cells. 5-aza has been shown before to induce cardiomyocyte differentiation of MSCs (Yoon et al., 2005; Ye et al., 2006; Burlacu et al., 2008) and EPCs (Thal et al., 2012). Our results showed that 5-aza treatment resulted in distinctive morphological changes in both PB-AMI and UCB-EPCs, consisting in increased elongated shape cells which dispose in parallel and formed myotube-like structures. Moreover, expression of the cardiac-specific markers troponin I, and the myocyte-specific proteins α-sarcomeric actinin, desmin and cardiac troponin T demonstrated the acquisition of a cardiomyogenic differentiation phenotype. Interestingly, we found that cells isolated from both sources showed a similar pattern of cardiac markers expression.

Previous studies have suggested that EPCs isolated from mouse bone marrow and from human PB could differentiate into cardiomyocytes under certain *in vitro* conditions (Condorelli et al., 2001; Badorff et al., 2003; Thal et al., 2012). For instance, EPCs obtained from PB of healthy adult volunteers and patients with cardiovascular disease have been proved to transdifferentiate into cardiac myocytes when cocultured with rat cardiomyocytes (Badorff et al., 2003). In addition, the potential of human umbilical cord-derived mesenchymal stem cells to differentiate into cells with characteristics of cardiomyocytes have been shown before (Kadner et al., 2002). The differentiation capacity of EPCs into cardiomyogenic cells reported in this study agrees with the work by Thal *et al*. (2012), suggesting that epigenetic reprogramming of EPCs could enhance their therapeutic efficacy by enhancing their plasticity and functions. Since endothelial cells, vascular smooth muscle cells, and cardiomyocytes all differentiate from a common progenitor in the mesoderm, evidences suggest that under appropriate culture conditions, such as paracrine factors (growth factors and cytokines) and epigenomic

agents (5-aza), endothelial cells could recapitulate their cardiomyogenic potential (Thal et al., 2012).

Furthermore, it has been shown that intracoronary infusion of autologous EPCs in patients with acute myocardial infarction enhances myocardial regeneration (Leistner et al., 2011). Therefore, it is reasonable to speculate that EPCs differentiated towards cardiomyocyte-like cells could improve the therapeutic effect of the cell transplantation. In conclusion, our finding suggests that EPCs isolated from patients that have suffered myocardial insult is a valuable EPCs source with potential to differentiate toward cardiac-like cells. Since the ultimate aim of cardiac regenerative medicine is to replace both microvasculature and lost cardiomyocytes, autologous AMI-EPCs, which possess vascular and cardiac tissue regenerative potential, could be an excellent candidate for cell based therapies.

CONCLUSIONS

1.- Adipose derived stem cells isolated from the knee of osteoarthritis patients represent an optimal cell source with possible cell-based therapy implications in cartilage repair that could overcome the limitations of autologous chondrocyte implantation.

2.- Intracellular extracts obtained from chondrocytes isolated from osteoarthritis knee promote chondrogenic differentiation of autologous stem cells derived from the infrapatellar fat pad.

3.- Biodegradable 3D scaffolds support cell growth and extracellular matrix deposition of adipose stem cells differentiated toward chondrocytes. The combination of pre-differentiated cells with biodegradable scaffolds is as an efficient system for the maintenance and maturation of cartilage tissue.

4.- Human cardiac tissue obtained from forensic autopsies maintains genetic integrity up to 12 h after death.

5.- The use of post-mortem cardiac tissue is a viable alternative to cardiac tissue obtained from patient's biopsies. Cell extract method or conditioned media from autopsies samples are useful and non-expensive methodologies to obtain *in vitro* human cardiomyocyte-like cells, which could be employed for cardioactive drugs toxicity assays.

6.- Postmortem human cardiac tissue provide the necessary factors to induce adipose derived stem cells differentiation into cardiomyocyte-like cells, which indicates that the soluble signaling molecules produced by cardiac cells after death are inducers of hASCs cardiac differentiation.

7.- Peripheral blood obtained from AMI patients is a valuable source of functional EPCs comparable with umbilical cord blood, suggesting that the cardiac insult enhances EPC population.

8.- EPCs isolated from patients after cardiac insult have cardiogenic potential similar than EPCs isolated from umbilical cord blood. The vascular and cardiac tissue regenerative potential of EPCs are relevant attributes for cell based therapies.

REFERENCES

Aicher A, Zeiher AM, Dimmeler S. 2005. Mobilizing endothelial progenitor cells. Hypertension 45:321-325.

Akita T, Murohara T, Ikeda H, Sasaki K, Shimada T, Egami K, Imaizumi T. 2003. Hypoxic preconditioning augments efficacy of human endothelial progenitor cells for therapeutic neovascularization. Lab Invest 83:65-73.

Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. 2002. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev 16:2813-2828.

Albrecht C, Tichy B, Nurnberger S, Hosiner S, Zak L, Aldrian S, Marlovits S. 2011. Gene expression and cell differentiation in matrix-associated chondrocyte transplantation grafts: a comparative study. Osteoarthritis Cartilage 19:1219-1227.

Alobaid N, Alnaeb ME, Sales KM, Seifalian AM, Mikhailidis DP, Hamilton G. 2005. Endothelial progenitor cells and their potential clinical applications in peripheral arterial disease. Endothelium 12:243-250.

Álvarez E, Ripoll PL, Restrepo A , Forriol F. 2010. Revisión de la reparación del cartílago. Posibilidades y resultados. Trauma Fund MAPFRE (2010) Vol 21 nº 2:117-134.

Alves da Silva ML, Martins A, Costa-Pinto AR, Costa P, Faria S, Gomes M, Reis RL, Neves NM. 2010. Cartilage tissue engineering using electrospun PCL nanofiber meshes and MSCs. Biomacromolecules 11:3228-3236.

Antonio N, Fernandes R, Rodriguez-Losada N, Jimenez-Navarro MF, Paiva A, de Teresa Galvan E, Goncalves L, Ribeiro CF, Providencia LA. 2010. Stimulation of endothelial progenitor cells: a new putative effect of several cardiovascular drugs. Eur J Clin Pharmacol 66:219-230.

Anzalone R, Lo Iacono M, Corrao S, Magno F, Loria T, Cappello F, Zummo G, Farina F, La Rocca G. 2010. New emerging potentials for human Wharton's jelly mesenchymal stem cells: immunological features and hepatocyte-like differentiative capacity. Stem Cells Dev 19:423-438.

Arminan A, Gandia C, Bartual M, Garcia-Verdugo JM, Lledo E, Mirabet V, Llop M, Barea J, Montero JA, Sepulveda P. 2009. Cardiac differentiation is driven by NKX2.5 and GATA-4 nuclear translocation in tissue-specific mesenchymal stem cells. Stem Cells Dev 18:907-918.

Arrell DK, Niederlander NJ, Faustino RS, Behfar A, Terzic A. 2008. Cardioinductive network guiding stem cell differentiation revealed by proteomic cartography of tumor necrosis factor alpha-primed endodermal secretome. Stem Cells 26:387-400.

Asahara T, Kawamoto A, Masuda H. 2011. Concise review: Circulating endothelial progenitor cells for vascular medicine. Stem Cells 29:1650-1655.

Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM. 1999a. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 85:221-228.

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. 1997. Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964-967.

Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM. 1999b. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 18:3964-3972.

Badorff C, Brandes RP, Popp R, Rupp S, Urbich C, Aicher A, Fleming I, Busse R, Zeiher AM, Dimmeler S. 2003. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. Circulation 107:1024-1032.

Badylak SF, Freytes DO, Gilbert TW. 2009. Extracellular matrix as a biological scaffold material: Structure and function. Acta Biomater 5:1-13.

Bae KS, Park JB, Kim HS, Kim DS, Park DJ, Kang SJ. 2011. Neuron-like differentiation of bone marrow-derived mesenchymal stem cells. Yonsei Med J 52:401-412.

Bakogiannis C, Tousoulis D, Androulakis E, Briasoulis A, Papageorgiou N, Vogiatzi G, Kampoli AM, Charakida M, Siasos G, Latsios G, Antoniades C, Stefanadis C. 2012.

Circulating endothelial progenitor cells as biomarkers for prediction of cardiovascular outcomes. Curr Med Chem 19:2597-2604.

Baksh D, Yao R, Tuan RS. 2007. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. Stem Cells 25:1384-1392.

Barlow S, Brooke G, Chatterjee K, Price G, Pelekanos R, Rossetti T, Doody M, Venter D, Pain S, Gilshenan K, Atkinson K. 2008. Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. Stem Cells Dev 17:1095-1107.

Bartlett W, Skinner JA, Gooding CR, Carrington RW, Flanagan AM, Briggs TW, Bentley G. 2005. Autologous chondrocyte implantation versus matrix-induced autologous chondrocyte implantation for osteochondral defects of the knee: a prospective, randomised study. J Bone Joint Surg Br 87:640-645.

Barzilay R, Ben-Zur T, Bulvik S, Melamed E, Offen D. 2009a. Lentiviral delivery of LMX1a enhances dopaminergic phenotype in differentiated human bone marrow mesenchymal stem cells. Stem Cells Dev 18:591-601.

Barzilay R, Melamed E, Offen D. 2009b. Introducing transcription factors to multipotent mesenchymal stem cells: making transdifferentiation possible. Stem Cells 27:2509-2515.

Basile DP, Yoder MC. 2014. Circulating and tissue resident endothelial progenitor cells. J Cell Physiol 229:10-16.

Batsali AK, Kastrinaki MC, Papadaki HA, Pontikoglou C. 2013. Mesenchymal stem cells derived from Wharton's Jelly of the umbilical cord: biological properties and emerging clinical applications. Curr Stem Cell Res Ther 8:144-155.

Bayart E, Cohen-Haguenauer O. 2013. Technological overview of iPS induction from human adult somatic cells. Curr Gene Ther 13:73-92.

Becker AJ, Mc CE, Till JE. 1963. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature 197:452-454.

Behfar A, Faustino RS, Arrell DK, Dzeja PP, Perez-Terzic C, Terzic A. 2008. Guided stem cell cardiopoiesis: discovery and translation. J Mol Cell Cardiol 45:523-529.

Behfar A, Yamada S, Crespo-Diaz R, Nesbitt JJ, Rowe LA, Perez-Terzic C, Gaussin V, Homsy C, Bartunek J, Terzic A. 2010. Guided cardiopoiesis enhances therapeutic benefit of bone marrow human mesenchymal stem cells in chronic myocardial infarction. J Am Coll Cardiol 56:721-734.

Behfar A, Zingman LV, Hodgson DM, Rauzier JM, Kane GC, Terzic A, Puceat M. 2002. Stem cell differentiation requires a paracrine pathway in the heart. FASEB J 16:1558-1566.

Bekkers JE, Bartels LW, Vincken KL, Dhert WJ, Creemers LB, Saris DB. 2013. Articular cartilage evaluation after TruFit plug implantation analyzed by delayed gadolinium-enhanced MRI of cartilage (dGEMRIC). Am J Sports Med 41:1290-1295.

Bentley G, Biant LC, Vijayan S, Macmull S, Skinner JA, Carrington RW. 2012. Minimum ten-year results of a prospective randomised study of autologous chondrocyte implantation versus mosaicplasty for symptomatic articular cartilage lesions of the knee. J Bone Joint Surg Br 94:504-509.

Benya PD, Shaffer JD. 1982. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215-224.

Bitsika V, Vlahou A, Roubelakis MG. 2013. Fetal mesenchymal stem cells in cancer therapy. Curr Stem Cell Res Ther 8:133-143.

Blazar BR, Lasky LC, Perentesis JP, Watson KV, Steinberg SE, Filipovich AH, Orr HT, Ramsay NK. 1986. Successful donor cell engraftment in a recipient of bone marrow from a cadaveric donor. Blood 67:1655-1660.

Bobick BE, Kulyk WM. 2008. Regulation of cartilage formation and maturation by mitogen-activated protein kinase signaling. Birth Defects Res C Embryo Today 84:131-154.

Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM. 2013. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science

(IFATS) and the International Society for Cellular Therapy (ISCT). Cytotherapy 15:641-648.

Briguori C, Testa U, Riccioni R, Colombo A, Petrucci E, Condorelli G, Mariani G, D'Andrea D, De Micco F, Rivera NV, Puca AA, Peschle C. 2010. Correlations between progression of coronary artery disease and circulating endothelial progenitor cells. FASEB J 24:1981-1988.

Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. 1994. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 331:889-895.

Brown MA, Zhang L, Levering VW, Wu JH, Satterwhite LL, Brian L, Freedman NJ, Truskey GA. 2010. Human umbilical cord blood-derived endothelial cells reendothelialize vein grafts and prevent thrombosis. Arterioscler Thromb Vasc Biol 30:2150-2155.

Buccini S, Haider KH, Ahmed RP, Jiang S, Ashraf M. 2012. Cardiac progenitors derived from reprogrammed mesenchymal stem cells contribute to angiomyogenic repair of the infarcted heart. Basic Res Cardiol 107:301.

Buckley CT, Kelly DJ. 2012. Expansion in the presence of FGF-2 enhances the functional development of cartilaginous tissues engineered using infrapatellar fat pad derived MSCs. J Mech Behav Biomed Mater 11:102-111.

Buckley CT, Vinardell T, Kelly DJ. 2010a. Oxygen tension differentially regulates the functional properties of cartilaginous tissues engineered from infrapatellar fat pad derived MSCs and articular chondrocytes. Osteoarthritis Cartilage 18:1345-1354.

Buckley CT, Vinardell T, Thorpe SD, Haugh MG, Jones E, McGonagle D, Kelly DJ. 2010b. Functional properties of cartilaginous tissues engineered from infrapatellar fat pad-derived mesenchymal stem cells. J Biomech 43:920-926.

Bujak M, Frangogiannis NG. 2007. The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. Cardiovasc Res 74:184-195.

Burke ZD, Shen CN, Ralphs KL, Tosh D. 2006. Characterization of liver function in transdifferentiated hepatocytes. J Cell Physiol 206:147-159.

Burlacu A, Rosca AM, Maniu H, Titorencu I, Dragan E, Jinga V, Simionescu M. 2008. Promoting effect of 5-azacytidine on the myogenic differentiation of bone marrow stromal cells. Eur J Cell Biol 87:173-184.

Calve S, Odelberg SJ, Simon HG. 2010. A transitional extracellular matrix instructs cell behavior during muscle regeneration. Dev Biol 344:259-271.

Cao L, Yang F, Liu G, Yu D, Li H, Fan Q, Gan Y, Tang T, Dai K. 2011. The promotion of cartilage defect repair using adenovirus mediated Sox9 gene transfer of rabbit bone marrow mesenchymal stem cells. Biomaterials 32:3910-3920.

Capito RM, Spector M. 2003. Scaffold-based articular cartilage repair. IEEE Eng Med Biol Mag 22:42-50.

Caplan AI. 1991. Mesenchymal stem cells. J Orthop Res 9:641-650.

Cardinal KO, Williams SK. 2009. Assessment of the intimal response to a protein-modified stent in a tissue-engineered blood vessel mimic. Tissue Eng Part A 15:3869-3876.

Carletti E, Motta A, Migliaresi C. 2011. Scaffolds for tissue engineering and 3D cell culture. Methods Mol Biol 695:17-39.

Carmont MR, Carey-Smith R, Saithna A, Dhillon M, Thompson P, Spalding T. 2009. Delayed incorporation of a TruFit plug: perseverance is recommended. Arthroscopy 25:810-814.

Caspi O, Itzhaki I, Kehat I, Gepstein A, Arbel G, Huber I, Satin J, Gepstein L. 2009. In vitro electrophysiological drug testing using human embryonic stem cell derived cardiomyocytes. Stem Cells Dev 18:161-172.

Coburn JM, Gibson M, Monagle S, Patterson Z, Elisseeff JH. 2012. Bioinspired nanofibers support chondrogenesis for articular cartilage repair. Proc Natl Acad Sci U S A 109:10012-10017.

Collas P, Hakelien AM. 2003. Teaching cells new tricks. Trends Biotechnol 21:354-361.

Collas P, Taranger CK. 2006. Epigenetic reprogramming of nuclei using cell extracts. Stem Cell Rev 2:309-317.

Condorelli G, Borello U, De Angelis L, Latronico M, Sirabella D, Coletta M, Galli R, Balconi G, Follenzi A, Frati G, Cusella De Angelis MG, Gioglio L, Amuchastegui S, Adorini L, Naldini L, Vescovi A, Dejana E, Cossu G. 2001. Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardium regeneration. Proc Natl Acad Sci U S A 98:10733-10738.

Corona BT, Ward CL, Harrison BS, Christ GJ. 2010. Regenerative medicine: basic concepts, current status, and future applications. J Investig Med 58:849-858.

Cottler-Fox MH, Lapidot T, Petit I, Kollet O, DiPersio JF, Link D, Devine S. 2003. Stem cell mobilization. Hematology Am Soc Hematol Educ Program:419-437.

Chen CC, Liao CH, Wang YH, Hsu YM, Huang SH, Chang CH, Fang HW. 2012. Cartilage fragments from osteoarthritic knee promote chondrogenesis of mesenchymal stem cells without exogenous growth factor induction. J Orthop Res 30:393-400.

Chen FH, Tuan RS. 2008. Mesenchymal stem cells in arthritic diseases. Arthritis Res Ther 10:223.

Chen LB, Jiang XB, Yang L. 2004. Differentiation of rat marrow mesenchymal stem cells into pancreatic islet beta-cells. World J Gastroenterol 10:3016-3020.

Chen M, Lu Y, Hamazaki T, Tsai H, Erger K, Conlon T, Elshikha AS, Li H, Srivastava A, Yao C, Brantly M, Chiodo V, Hauswirth WW, Terada N, Song S. 2013. Reprogramming adipose tissue derived mesenchymal stem cells (AT-MSCs) into pluripotent stem cells by a mutant AAV vector. Hum Gene Ther Methods.

Chen WH, Lai MT, Wu AT, Wu CC, Gelovani JG, Lin CT, Hung SC, Chiu WT, Deng WP. 2009. In vitro stage-specific chondrogenesis of mesenchymal stem cells committed to chondrocytes. Arthritis Rheum 60:450-459.

Chiang H, Liao CJ, Hsieh CH, Shen CY, Huang YY, Jiang CC. 2013. Clinical feasibility of a novel biphasic osteochondral composite for matrix-associated autologous chondrocyte implantation. Osteoarthritis Cartilage 21:589-598.

Choi YS, Dusting GJ, Stubbs S, Arunothayaraj S, Han XL, Collas P, Morrison WA, Dilley RJ. 2010. Differentiation of human adipose-derived stem cells into beating cardiomyocytes. J Cell Mol Med 14:878-889.

Christman JK. 2002. 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. Oncogene 21:5483-5495.

Chung NG, Jeong DC, Park SJ, Choi BO, Cho B, Kim HK, Chun CS, Won JH, Han CW. 2004. Cotransplantation of marrow stromal cells may prevent lethal graft-versus-host disease in major histocompatibility complex mismatched murine hematopoietic stem cell transplantation. Int J Hematol 80:370-376.

Chung S, King MW. 2011. Design concepts and strategies for tissue engineering scaffolds. Biotechnol Appl Biochem 58:423-438.

Dahl JP, Caballero M, Pappa AK, Madan G, Shockley WW, van Aalst JA. 2011. Analysis of human auricular cartilage to guide tissue-engineered nanofiber-based chondrogenesis: implications for microtia reconstruction. Otolaryngol Head Neck Surg 145:915-923.

Danisovic L, Varga I, Polak S. 2012. Growth factors and chondrogenic differentiation of mesenchymal stem cells. Tissue Cell 44:69-73.

Das H, George JC, Joseph M, Das M, Abdulhameed N, Blitz A, Khan M, Sakthivel R, Mao HQ, Hoit BD, Kuppusamy P, Pompili VJ. 2009. Stem cell therapy with overexpressed VEGF and PDGF genes improves cardiac function in a rat infarct model. PLoS One 4:e7325.

Davis RL, Weintraub H, Lassar AB. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51:987-1000.

Dayan V, Yannarelli G, Billia F, Filomeno P, Wang XH, Davies JE, Keating A. 2011. Mesenchymal stromal cells mediate a switch to alternatively activated monocytes/macrophages after acute myocardial infarction. Basic Res Cardiol 106:1299-1310.

de Girolamo L, Lucarelli E, Alessandri G, Avanzini MA, Bernardo ME, Biagi E, Brini AT, D'Amico G, Fagioli F, Ferrero I, Locatelli F, Maccario R, Marazzi M, Parolini O,

Pessina A, Torre ML, Italian Mesenchymal Stem Cell G. 2013. Mesenchymal stem/stromal cells: a new "cells as drugs" paradigm. Efficacy and critical aspects in cell therapy. Curr Pharm Des 19:2459-2473.

de la Fuente R, Abad JL, Garcia-Castro J, Fernandez-Miguel G, Petriz J, Rubio D, Vicario-Abejon C, Guillen P, Gonzalez MA, Bernad A. 2004. Dedifferentiated adult articular chondrocytes: a population of human multipotent primitive cells. Exp Cell Res 297:313-328.

de la Garza-Rodea AS, van der Velde I, Boersma H, Goncalves MA, van Bekkum DW, de Vries AA, Knaan-Shanzer S. 2011. Long-term contribution of human bone marrow mesenchymal stromal cells to skeletal muscle regeneration in mice. Cell Transplant 20:217-231.

de Mora-Martin M, Perez-Ruiz JM, Delgado-Prieto JL, Urbano-Carrillo CA. 2011. Comorbidity in patients admitted to a department of cardiology due to heart failure. Rev Esp Cardiol 64:75.

de Rezende AA, e Silva ML, Tavares DC, Cunha WR, Rezende KC, Bastos JK, Lehmann M, de Andrade HH, Guterres ZR, Silva LP, Spano MA. 2011. The effect of the dibenzylbutyrolactolic lignan (-)-cubebin on doxorubicin mutagenicity and recombinogenicity in wing somatic cells of Drosophila melanogaster. Food Chem Toxicol 49:1235-1241.

Dessau W, von der Mark H, von der Mark K, Fischer S. 1980. Changes in the patterns of collagens and fibronectin during limb-bud chondrogenesis. J Embryol Exp Morphol 57:51-60.

Dhollander AA, Liekens K, Almqvist KF, Verdonk R, Lambrecht S, Elewaut D, Verbruggen G, Verdonk PC. 2012. A pilot study of the use of an osteochondral scaffold plug for cartilage repair in the knee and how to deal with early clinical failures. Arthroscopy 28:225-233.

Di Campli C, Piscaglia AC, Pierelli L, Rutella S, Bonanno G, Alison MR, Mariotti A, Vecchio FM, Nestola M, Monego G, Michetti F, Mancuso S, Pola P, Leone G, Gasbarrini G, Gasbarrini A. 2004. A human umbilical cord stem cell rescue therapy in a murine model of toxic liver injury. Dig Liver Dis 36:603-613.

Diaz-Romero J, Gaillard JP, Grogan SP, Nesic D, Trub T, Mainil-Varlet P. 2005. Immunophenotypic analysis of human articular chondrocytes: changes in surface markers associated with cell expansion in monolayer culture. J Cell Physiol 202:731-742.

Digirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ. 1999. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 107:275-281.

Dimarino AM, Caplan AI, Bonfield TL. 2013. Mesenchymal Stem Cells in Tissue Repair. Front Immunol 4:201.

Discher DE, Janmey P, Wang YL. 2005. Tissue cells feel and respond to the stiffness of their substrate. Science 310:1139-1143.

Dodou E, Xu SM, Black BL. 2003. mef2c is activated directly by myogenic basic helix-loop-helix proteins during skeletal muscle development in vivo. Mech Dev 120:1021-1032.

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

Dowling P, Clynes M. 2011. Conditioned media from cell lines: a complementary model to clinical specimens for the discovery of disease-specific biomarkers. Proteomics 11:794-804.

Drews K, Jozefczuk J, Prigione A, Adjaye J. 2012. Human induced pluripotent stem cells--from mechanisms to clinical applications. J Mol Med (Berl) 90:735-745.

Dudics V, Kunstar A, Kovacs J, Lakatos T, Geher P, Gomor B, Monostori E, Uher F. 2009. Chondrogenic potential of mesenchymal stem cells from patients with rheumatoid

arthritis and osteoarthritis: measurements in a microculture system. Cells Tissues Organs 189:307-316.

Dzau VJ, Gnecchi M, Pachori AS, Morello F, Melo LG. 2005. Therapeutic potential of endothelial progenitor cells in cardiovascular diseases. Hypertension 46:7-18.

Eaves CJ, Cashman JD, Sutherland HJ, Otsuka T, Humphries RK, Hogge DE, Lansdorp PL, Eaves AC. 1991. Molecular analysis of primitive hematopoietic cell proliferation control mechanisms. Ann N Y Acad Sci 628:298-306.

Eberhard D, O'Neill K, Burke ZD, Tosh D. 2010. In vitro reprogramming of pancreatic cells to hepatocytes. Methods Mol Biol 636:285-292.

Eguchi G. 1986. Instability in cell commitment of vertebrate pigmented epithelial cells and their transdifferentiation into lens cells. Curr Top Dev Biol 20:21-37.

Eguchi G, Abe SI, Watanabe K. 1974. Differentiation of lens-like structures from newt iris epithelial cells in vitro. Proc Natl Acad Sci U S A 71:5052-5056.

Eguchi G, Kodama R. 1993. Transdifferentiation. Curr Opin Cell Biol 5:1023-1028.

Eguizabal C, Montserrat N, Veiga A, Izpisua Belmonte JC. 2013. Dedifferentiation, transdifferentiation, and reprogramming: future directions in regenerative medicine. Semin Reprod Med 31:82-94.

Ehnert S, Glanemann M, Schmitt A, Vogt S, Shanny N, Nussler NC, Stockle U, Nussler A. 2009. The possible use of stem cells in regenerative medicine: dream or reality? Langenbecks Arch Surg 394:985-997.

Ellman MB, Yan D, Ahmadinia K, Chen D, An HS, Im HJ. 2013. Fibroblast growth factor control of cartilage homeostasis. J Cell Biochem 114:735-742.

Elmadbouh I, Haider H, Jiang S, Idris NM, Lu G, Ashraf M. 2007. Ex vivo delivered stromal cell-derived factor-1alpha promotes stem cell homing and induces angiomyogenesis in the infarcted myocardium. J Mol Cell Cardiol 42:792-803.

Engel E, Michiardi A, Navarro M, Lacroix D, Planell JA. 2008. Nanotechnology in regenerative medicine: the materials side. Trends Biotechnol 26:39-47.

English A, Jones EA, Corscadden D, Henshaw K, Chapman T, Emery P, McGonagle D. 2007. A comparative assessment of cartilage and joint fat pad as a potential source of cells for autologous therapy development in knee osteoarthritis. Rheumatology (Oxford) 46:1676-1683.

Enomoto-Iwamoto M, Enomoto H, Komori T, Iwamoto M. 2001. Participation of Cbfa1 in regulation of chondrocyte maturation. Osteoarthritis Cartilage 9 Suppl A:S76-84.

Erker L, Azuma H, Lee AY, Guo C, Orloff S, Eaton L, Benedetti E, Jensen B, Finegold M, Willenbring H, Grompe M. 2010. Therapeutic liver reconstitution with murine cells isolated long after death. Gastroenterology 139:1019-1029.

Esquivies L, Blackler A, Peran M, Rodriguez-Esteban C, Izpisua Belmonte JC, Booker E, Gray PC, Ahn C, Kwiatkowski W, Choe S. 2013. Designer Nodal/BMP2 chimeras mimic Nodal signaling, promote chondrogenesis, and reveal a BMP2-like structure. J Biol Chem.

Evans MJ, Kaufman MH. 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154-156.

Finney MR, Greco NJ, Haynesworth SE, Martin JM, Hedrick DP, Swan JZ, Winter DG, Kadereit S, Joseph ME, Fu P, Pompili VJ, Laughlin MJ. 2006. Direct comparison of umbilical cord blood versus bone marrow-derived endothelial precursor cells in mediating neovascularization in response to vascular ischemia. Biol Blood Marrow Transplant 12:585-593.

Fioravanti A, Cantarini L, Chellini F, Manca D, Paccagnini E, Marcolongo R, Collodel G. 2005. Effect of hyaluronic acid (MW 500-730 kDa) on proteoglycan and nitric oxide production in human osteoarthritic chondrocyte cultures exposed to hydrostatic pressure. Osteoarthritis Cartilage 13:688-696.

Frantz S, Hu K, Adamek A, Wolf J, Sallam A, Maier SK, Lonning S, Ling H, Ertl G, Bauersachs J. 2008. Transforming growth factor beta inhibition increases mortality and left ventricular dilatation after myocardial infarction. Basic Res Cardiol 103:485-492.

Fraser JK, Wulur I, Alfonso Z, Hedrick MH. 2006. Fat tissue: an underappreciated source of stem cells for biotechnology. Trends Biotechnol 24:150-154.

Freyman TM, Yannas IV, Yokoo R, Gibson LJ. 2001. Fibroblast contraction of a collagen-GAG matrix. Biomaterials 22:2883-2891.

Friedenstein AJ, Chailakhjan RK, Lalykina KS. 1970. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3:393-403.

Frith J, Genever P. 2008. Transcriptional control of mesenchymal stem cell differentiation. Transfus Med Hemother 35:216-227.

Gao XR, Tan YZ, Wang HJ. 2011. Overexpression of Csx/Nkx2.5 and GATA-4 enhances the efficacy of mesenchymal stem cell transplantation after myocardial infarction. Circ J 75:2683-2691.

Gaustad KG, Boquest AC, Anderson BE, Gerdes AM, Collas P. 2004. Differentiation of human adipose tissue stem cells using extracts of rat cardiomyocytes. Biochem Biophys Res Commun 314:420-427.

Gilbert JE. 1998. Current treatment options for the restoration of articular cartilage. Am J Knee Surg 11:42-46.

Gimble JM, Katz AJ, Bunnell BA. 2007. Adipose-derived stem cells for regenerative medicine. Circ Res 100:1249-1260.

Gomoll AH, Madry H, Knutsen G, van Dijk N, Seil R, Brittberg M, Kon E. 2010. The subchondral bone in articular cartilage repair: current problems in the surgical management. Knee Surg Sports Traumatol Arthrosc 18:434-447.

Gratwohl A, Baldomero H, Frauendorfer K, Rocha V, Apperley J, Niederwieser D. 2008. The EBMT activity survey 2006 on hematopoietic stem cell transplantation: focus on the use of cord blood products. Bone Marrow Transplant 41:687-705.

Greenwood HL, Singer PA, Downey GP, Martin DK, Thorsteinsdottir H, Daar AS. 2006. Regenerative medicine and the developing world. PLoS Med 3:e381.

Gupta MK, Walthall JM, Venkataraman R, Crowder SW, Jung DK, Yu SS, Feaster TK, Wang X, Giorgio TD, Hong CC, Baudenbacher FJ, Hatzopoulos AK, Sung HJ. 2011. Combinatorial polymer electrospun matrices promote physiologically-relevant cardiomyogenic stem cell differentiation. PLoS One 6:e28935.

Hakelien AM, Kuntziger T, Gaustad KG, Marstad A, Collas P. 2006. In vitro reprogramming of nuclei and cells. Methods Mol Biol 348:259-268.

Hakelien AM, Landsverk HB, Robl JM, Skalhegg BS, Collas P. 2002. Reprogramming fibroblasts to express T-cell functions using cell extracts. Nat Biotechnol 20:460-466.

Hamou C, Callaghan MJ, Thangarajah H, Chang E, Chang EI, Grogan RH, Paterno J, Vial IN, Jazayeri L, Gurtner GC. 2009. Mesenchymal stem cells can participate in ischemic neovascularization. Plast Reconstr Surg 123:45S-55S.

Han Y, Lefebvre V. 2008. L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by securing binding of Sox9 to a far-upstream enhancer. Mol Cell Biol 28:4999-5013.

Hao J, Ju H, Zhao S, Junaid A, Scammell-La Fleur T, Dixon IM. 1999. Elevation of expression of Smads 2, 3, and 4, decorin and TGF-beta in the chronic phase of myocardial infarct scar healing. J Mol Cell Cardiol 31:667-678.

Hattori K, Heissig B, Rafii S. 2003. The regulation of hematopoietic stem cell and progenitor mobilization by chemokine SDF-1. Leuk Lymphoma 44:575-582.

Hatzistergos KE, Quevedo H, Oskouei BN, Hu Q, Feigenbaum GS, Margitich IS, Mazhari R, Boyle AJ, Zambrano JP, Rodriguez JE, Dulce R, Pattany PM, Valdes D, Revilla C, Heldman AW, McNiece I, Hare JM. 2010. Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. Circ Res 107:913-922.

He JQ, Ma Y, Lee Y, Thomson JA, Kamp TJ. 2003. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. Circ Res 93:32-39.

He Q, Mendez M, LaPointe MC. 2002. Regulation of the human brain natriuretic peptide gene by GATA-4. Am J Physiol Endocrinol Metab 283:E50-57.

Heinrich M, Matt K, Lutz-Bonengel S, Schmidt U. 2007. Successful RNA extraction from various human postmortem tissues. Int J Legal Med 121:136-142.

Heng BC, Cao T, Lee EH. 2004. Directing stem cell differentiation into the chondrogenic lineage in vitro. Stem Cells 22:1152-1167.

Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. 2003. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 348:593-600.

Hipp J, Atala A. 2008. Sources of stem cells for regenerative medicine. Stem Cell Rev 4:3-11.

Hirschi KK, Ingram DA, Yoder MC. 2008. Assessing identity, phenotype, and fate of endothelial progenitor cells. Arterioscler Thromb Vasc Biol 28:1584-1595.

Hofstetter CP, Schwarz EJ, Hess D, Widenfalk J, El Manira A, Prockop DJ, Olson L. 2002. Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. Proc Natl Acad Sci U S A 99:2199-2204.

Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A. 2005. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy 7:393-395.

Howe AJ, Shand JA, Menown IB. 2011. Advances in cardiology: clinical trial update. Future Cardiol 7:299-310.

Hu CH, Li ZM, Du ZM, Zhang AX, Yang DY, Wu GF. 2009. Human umbilical cord-derived endothelial progenitor cells promote growth cytokines-mediated neorevascularization in rat myocardial infarction. Chin Med J (Engl) 122:548-555.

Hutmacher DW. 2000. Scaffolds in tissue engineering bone and cartilage. Biomaterials 21:2529-2543.

Hwang NS, Varghese S, Puleo C, Zhang Z, Elisseeff J. 2007. Morphogenetic signals from chondrocytes promote chondrogenic and osteogenic differentiation of mesenchymal stem cells. J Cell Physiol 212:281-284.

Hynes RO. 1999. Cell adhesion: old and new questions. Trends Cell Biol 9:M33-37.

Ieda M, Fu JD, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG, Srivastava D. 2010. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell 142:375-386.

Iijima Y, Nagai T, Mizukami M, Matsuura K, Ogura T, Wada H, Toko H, Akazawa H, Takano H, Nakaya H, Komuro I. 2003. Beating is necessary for transdifferentiation of skeletal muscle-derived cells into cardiomyocytes. FASEB J 17:1361-1363.

Ilic D, Polak JM. 2011. Stem cells in regenerative medicine: introduction. Br Med Bull 98:117-126.

Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, Pollok K, Ferkowicz MJ, Gilley D, Yoder MC. 2004. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood 104:2752-2760.

Izumi M, Fujio Y, Kunisada K, Negoro S, Tone E, Funamoto M, Osugi T, Oshima Y, Nakaoka Y, Kishimoto T, Yamauchi-Takihara K, Hirota H. 2001. Bone morphogenetic protein-2 inhibits serum deprivation-induced apoptosis of neonatal cardiac myocytes through activation of the Smad1 pathway. J Biol Chem 276:31133-31141.

Jarriault S, Schwab Y, Greenwald I. 2008. A Caenorhabditis elegans model for epithelial-neuronal transdifferentiation. Proc Natl Acad Sci U S A 105:3790-3795.

Jeon SY, Park JS, Yang HN, Woo DG, Park KH. 2012. Co-delivery of SOX9 genes and anti-Cbfa-1 siRNA coated onto PLGA nanoparticles for chondrogenesis of human MSCs. Biomaterials 33:4413-4423.

Jiang CC, Chiang H, Liao CJ, Lin YJ, Kuo TF, Shieh CS, Huang YY, Tuan RS. 2007. Repair of porcine articular cartilage defect with a biphasic osteochondral composite. J Orthop Res 25:1277-1290.

Jin HJ, Bae YK, Kim M, Kwon SJ, Jeon HB, Choi SJ, Kim SW, Yang YS, Oh W, Chang JW. 2013. Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. Int J Mol Sci 14:17986-18001.

Jones EA, Crawford A, English A, Henshaw K, Mundy J, Corscadden D, Chapman T, Emery P, Hatton P, McGonagle D. 2008. Synovial fluid mesenchymal stem cells in health and early osteoarthritis: detection and functional evaluation at the single-cell level. Arthritis Rheum 58:1731-1740.

Jopling C, Sleep E, Raya M, Marti M, Raya A, Izpisua Belmonte JC. 2010. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. Nature 464:606-609.

Jujo K, Ii M, Losordo DW. 2008. Endothelial progenitor cells in neovascularization of infarcted myocardium. J Mol Cell Cardiol 45:530-544.

Jung Y, Chung YI, Kim SH, Tae G, Kim YH, Rhie JW. 2009. In situ chondrogenic differentiation of human adipose tissue-derived stem cells in a TGF-beta1 loaded fibrin-poly(lactide-caprolactone) nanoparticulate complex. Biomaterials 30:4657-4664.

Kacimi R, Karliner JS, Koudssi F, Long CS. 1998. Expression and regulation of adhesion molecules in cardiac cells by cytokines: response to acute hypoxia. Circ Res 82:576-586.

Kadivar M, Khatami S, Mortazavi Y, Shokrgozar MA, Taghikhani M, Soleimani M. 2006. In vitro cardiomyogenic potential of human umbilical vein-derived mesenchymal stem cells. Biochem Biophys Res Commun 340:639-647.

Kadner A, Hoerstrup SP, Tracy J, Breymann C, Maurus CF, Melnitchouk S, Kadner G, Zund G, Turina M. 2002. Human umbilical cord cells: a new cell source for cardiovascular tissue engineering. Ann Thorac Surg 74:S1422-1428.

Kafienah W, Mistry S, Dickinson SC, Sims TJ, Learmonth I, Hollander AP. 2007. Three-dimensional cartilage tissue engineering using adult stem cells from osteoarthritis patients. Arthritis Rheum 56:177-187.

Kakisis JD, Liapis CD, Breuer C, Sumpio BE. 2005. Artificial blood vessel: the Holy Grail of peripheral vascular surgery. J Vasc Surg 41:349-354.

Kalfa D, Bel A, Chen-Tournoux A, Della Martina A, Rochereau P, Coz C, Bellamy V, Bensalah M, Vanneaux V, Lecourt S, Mousseaux E, Bruneval P, Larghero J, Menasche P. 2010. A polydioxanone electrospun valved patch to replace the right ventricular outflow tract in a growing lamb model. Biomaterials 31:4056-4063.

Kalinina NI, Sysoeva VY, Rubina KA, Parfenova YV, Tkachuk VA. 2011. Mesenchymal stem cells in tissue growth and repair. Acta Naturae 3:30-37.

Kapelushnik J, Aker M, Pugatsch T, Samuel S, Slavin S. 1998. Bone marrow transplantation from a cadaveric donor. Bone Marrow Transplant 21:857-858.

Katsha AM, Ohkouchi S, Xin H, Kanehira M, Sun R, Nukiwa T, Saijo Y. 2011. Paracrine factors of multipotent stromal cells ameliorate lung injury in an elastase-induced emphysema model. Mol Ther 19:196-203.

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

Khan WS, Adesida AB, Hardingham TE. 2007. Hypoxic conditions increase hypoxia-inducible transcription factor 2alpha and enhance chondrogenesis in stem cells from the infrapatellar fat pad of osteoarthritis patients. Arthritis Res Ther 9:R55.

Khan WS, Adesida AB, Tew SR, Longo UG, Hardingham TE. 2012. Fat pad-derived mesenchymal stem cells as a potential source for cell-based adipose tissue repair strategies. Cell Prolif 45:111-120.

Khan WS, Tew SR, Adesida AB, Hardingham TE. 2008. Human infrapatellar fat pad-derived stem cells express the pericyte marker 3G5 and show enhanced chondrogenesis after expansion in fibroblast growth factor-2. Arthritis Res Ther 10:R74.

Kikuchi K, Holdway JE, Werdich AA, Anderson RM, Fang Y, Egnaczyk GF, Evans T, Macrae CA, Stainier DY, Poss KD. 2010. Primary contribution to zebrafish heart regeneration by GATA-4(+) cardiomyocytes. Nature 464:601-605.

Kim J, Jeon YJ, Kim HE, Shin JM, Chung HM, Chae JI. 2013. Comparative proteomic analysis of endothelial cells progenitor cells derived from cord blood- and peripheral blood for cell therapy. Biomaterials 34:1669-1685.

Kim JH, Park JS, Yang HN, Woo DG, Jeon SY, Do HJ, Lim HY, Kim JM, Park KH. 2011. The use of biodegradable PLGA nanoparticles to mediate SOX9 gene delivery in human mesenchymal stem cells (hMSCs) and induce chondrogenesis. Biomaterials 32:268-278.

Kim SS, Yoo SW, Park TS, Ahn SC, Jeong HS, Kim JW, Chang DY, Cho KG, Kim SU, Huh Y, Lee JE, Lee SY, Lee YD, Suh-Kim H. 2008. Neural induction with neurogenin1 increases the therapeutic effects of mesenchymal stem cells in the ischemic brain. Stem Cells 26:2217-2228.

Kim Y, Ma AG, Kitta K, Fitch SN, Ikeda T, Ihara Y, Simon AR, Evans T, Suzuki YJ. 2003. Anthracycline-induced suppression of GATA-4 transcription factor: implication in the regulation of cardiac myocyte apoptosis. Mol Pharmacol 63:368-377.

Kodama H, Hirotani T, Suzuki Y, Ogawa S, Yamazaki K. 2002. Cardiomyogenic differentiation in cardiac myxoma expressing lineage-specific transcription factors. Am J Pathol 161:381-389.

Koelling S, Miosge N. 2009. Stem cell therapy for cartilage regeneration in osteoarthritis. Expert Opin Biol Ther 9:1399-1405.

Koh YG, Choi YJ. 2012. Infrapatellar fat pad-derived mesenchymal stem cell therapy for knee osteoarthritis. Knee 19:902-907.

Kon E, Filardo G, Di Martino A, Busacca M, Moio A, Perdisa F, Marcacci M. 2014. Clinical results and MRI evolution of a nano-composite multilayered biomaterial for osteochondral regeneration at 5 years. Am J Sports Med 42:158-165.

Koyanagi M, Brandes RP, Haendeler J, Zeiher AM, Dimmeler S. 2005. Cell-to-cell connection of endothelial progenitor cells with cardiac myocytes by nanotubes: a novel mechanism for cell fate changes? Circ Res 96:1039-1041.

Krenning G, van der Strate BW, Schipper M, van Seijen XJ, Fernandes BC, van Luyn MJ, Harmsen MC. 2009a. CD34+ cells augment endothelial cell differentiation of CD14+ endothelial progenitor cells in vitro. J Cell Mol Med 13:2521-2533.

Krenning G, van Luyn MJ, Harmsen MC. 2009b. Endothelial progenitor cell-based neovascularization: implications for therapy. Trends Mol Med 15:180-189.

Kruithof BP, van Wijk B, Somi S, Kruithof-de Julio M, Perez Pomares JM, Weesie F, Wessels A, Moorman AF, van den Hoff MJ. 2006. BMP and FGF regulate the differentiation of multipotential pericardial mesoderm into the myocardial or epicardial lineage. Dev Biol 295:507-522.

Laflamme MA, Murry CE. 2005. Regenerating the heart. Nat Biotechnol 23:845-856.

Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M. 2000. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 6:1229-1234.

Landsverk HB, Hakelien AM, Kuntziger T, Robl JM, Skalhegg BS, Collas P. 2002. Reprogrammed gene expression in a somatic cell-free extract. EMBO Rep 3:384-389.

Latil M, Rocheteau P, Chatre L, Sanulli S, Memet S, Ricchetti M, Tajbakhsh S, Chretien F. 2012. Skeletal muscle stem cells adopt a dormant cell state post mortem and retain regenerative capacity. Nat Commun 3:903.

Lee SH, Shin H. 2007. Matrices and scaffolds for delivery of bioactive molecules in bone and cartilage tissue engineering. Adv Drug Deliv Rev 59:339-359.

Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. 1997. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. Mol Cell Biol 17:2336-2346.

Lefebvre V, Li P, de Crombrugghe B. 1998. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO J 17:5718-5733.

Leistner DM, Fischer-Rasokat U, Honold J, Seeger FH, Schachinger V, Lehmann R, Martin H, Burck I, Urbich C, Dimmeler S, Zeiher AM, Assmus B. 2011. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI): final 5-year results suggest long-term safety and efficacy. Clin Res Cardiol 100:925-934.

Leu S, Kao YH, Sun CK, Lin YC, Tsai TH, Chang LT, Chua S, Yeh KH, Wu CJ, Fu M, Yip HK. 2011. Myocardium-derived conditioned medium improves left ventricular function in rodent acute myocardial infarction. J Transl Med 9:11.

Li H, Zuo S, He Z, Yang Y, Pasha Z, Wang Y, Xu M. 2010. Paracrine factors released by GATA-4 overexpressed mesenchymal stem cells increase angiogenesis and cell survival. Am J Physiol Heart Circ Physiol 299:H1772-1781.

Li H, Zuo S, Pasha Z, Yu B, He Z, Wang Y, Yang X, Ashraf M, Xu M. 2011. GATA-4 promotes myocardial transdifferentiation of mesenchymal stromal cells via up-regulating IGFBP-4. Cytotherapy 13:1057-1065.

Li WC, Yu WY, Quinlan JM, Burke ZD, Tosh D. 2005. The molecular basis of transdifferentiation. J Cell Mol Med 9:569-582.

Li Y, Zhang R, Qiao H, Zhang H, Wang Y, Yuan H, Liu Q, Liu D, Chen L, Pei X. 2007. Generation of insulin-producing cells from PDX-1 gene-modified human mesenchymal stem cells. J Cell Physiol 211:36-44.

Li Z, Guo J, Chang Q, Zhang A. 2009. Paracrine role for mesenchymal stem cells in acute myocardial infarction. Biol Pharm Bull 32:1343-1346.

Liu M, Yu X, Huang F, Cen S, Zhong G, Xiang Z. 2013. Tissue engineering stratified scaffolds for articular cartilage and subchondral bone defects repair. Orthopedics 36:868-873.

Liu X, Zhu Y, Gao W. 2006. Isolation of neural stem cells from the spinal cords of low temperature preserved abortuses. J Neurosci Methods 157:64-70.

Liu XN, Yin Q, Zhang H, Zhu SJ, Wei YJ, Hu SS. 2008. Tissue extracts from infarcted myocardium of rats in promoting the differentiation of bone marrow stromal cells into cardiomyocyte-like cells. Biomed Environ Sci 21:110-117.

Liu ZJ, Zhuge Y, Velazquez OC. 2009. Trafficking and differentiation of mesenchymal stem cells. J Cell Biochem 106:984-991.

Locke M, Feisst V, Dunbar PR. 2011. Concise review: human adipose-derived stem cells: separating promise from clinical need. Stem Cells 29:404-411.

Losordo DW, Henry TD, Davidson C, Sup Lee J, Costa MA, Bass T, Mendelsohn F, Fortuin FD, Pepine CJ, Traverse JH, Amrani D, Ewenstein BM, Riedel N, Story K, Barker K, Povsic TJ, Harrington RA, Schatz RA. 2011. Intramyocardial, autologous CD34+ cell therapy for refractory angina. Circ Res 109:428-436.

Lu J, Rao MP, MacDonald NC, Khang D, Webster TJ. 2008. Improved endothelial cell adhesion and proliferation on patterned titanium surfaces with rationally designed, micrometer to nanometer features. Acta Biomater 4:192-201.

Lyons I, Parsons LM, Hartley L, Li R, Andrews JE, Robb L, Harvey RP. 1995. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. Genes Dev 9:1654-1666.

Macchiarini P, Jungebluth P, Go T, Asnaghi MA, Rees LE, Cogan TA, Dodson A, Martorell J, Bellini S, Parnigotto PP, Dickinson SC, Hollander AP, Mantero S, Conconi MT, Birchall MA. 2008. Clinical transplantation of a tissue-engineered airway. Lancet 372:2023-2030.

Machalinski B, Paczkowski M, Kawa M, Paczkowska E, Ostrowski M. 2003. An optimization of isolation of early hematopoietic cells from heparinized cadaveric organ donors. Transplant Proc 35:3096-3100.

Madec AM, Mallone R, Afonso G, Abou Mrad E, Mesnier A, Eljaafari A, Thivolet C. 2009. Mesenchymal stem cells protect NOD mice from diabetes by inducing regulatory T cells. Diabetologia 52:1391-1399.

Mahmoudifar N, Doran PM. 2012. Chondrogenesis and cartilage tissue engineering: the longer road to technology development. Trends Biotechnol 30:166-176.

Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. 1999. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103:697-705.

Martin GR. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78:7634-7638.

Mason C, Dunnill P. 2008. A brief definition of regenerative medicine. Regen Med 3:1-5.

Massa M, Rosti V, Ferrario M, Campanelli R, Ramajoli I, Rosso R, De Ferrari GM, Ferlini M, Goffredo L, Bertoletti A, Klersy C, Pecci A, Moratti R, Tavazzi L. 2005. Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction. Blood 105:199-206.

Mead LE, Prater D, Yoder MC, Ingram DA. 2008. Isolation and characterization of endothelial progenitor cells from human blood. Curr Protoc Stem Cell Biol Chapter 2:Unit 2C 1.

Meivar-Levy I, Sapir T, Gefen-Halevi S, Aviv V, Barshack I, Onaca N, Mor E, Ferber S. 2007. Pancreatic and duodenal homeobox gene 1 induces hepatic dedifferentiation by suppressing the expression of CCAAT/enhancer-binding protein beta. Hepatology 46:898-905.

Melton JT, Wilson AJ, Chapman-Sheath P, Cossey AJ. 2010. TruFit CB bone plug: chondral repair, scaffold design, surgical technique and early experiences. Expert Rev Med Devices 7:333-341.

Miller DC, Haberstroh KM, Webster TJ. 2007. PLGA nanometer surface features manipulate fibronectin interactions for improved vascular cell adhesion. J Biomed Mater Res A 81:678-684.

Mirsky R, Woodhoo A, Parkinson DB, Arthur-Farraj P, Bhaskaran A, Jessen KR. 2008. Novel signals controlling embryonic Schwann cell development, myelination and dedifferentiation. J Peripher Nerv Syst 13:122-135.

Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, Di Halvorsen Y, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM. 2006. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells 24:376-385.

Miyagawa S, Sawa Y, Sakakida S, Taketani S, Kondoh H, Memon IA, Imanishi Y, Shimizu T, Okano T, Matsuda H. 2005. Tissue cardiomyoplasty using bioengineered contractile cardiomyocyte sheets to repair damaged myocardium: their integration with recipient myocardium. Transplantation 80:1586-1595.

Mohanty S, Bose S, Jain KG, Bhargava B, Airan B. 2013. TGFbeta1 contributes to cardiomyogenic-like differentiation of human bone marrow mesenchymal stem cells. Int J Cardiol 163:93-99.

Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L. 1998. The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood 91:4523-4530.

Monje PV, Soto J, Bacallao K, Wood PM. 2010. Schwann cell dedifferentiation is independent of mitogenic signaling and uncoupled to proliferation: role of cAMP and JNK in the maintenance of the differentiated state. J Biol Chem 285:31024-31036.

Murohara T. 2010. Cord blood-derived early outgrowth endothelial progenitor cells. Microvasc Res 79:174-177.

Murphy SV, Atala A. 2013. Amniotic fluid and placental membranes: unexpected sources of highly multipotent cells. Semin Reprod Med 31:62-68.

Mwale F, Stachura D, Roughley P, Antoniou J. 2006. Limitations of using aggrecan and type X collagen as markers of chondrogenesis in mesenchymal stem cell differentiation. J Orthop Res 24:1791-1798.

Nagaya N, Fujii T, Iwase T, Ohgushi H, Itoh T, Uematsu M, Yamagishi M, Mori H, Kangawa K, Kitamura S. 2004. Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. Am J Physiol Heart Circ Physiol 287:H2670-2676.

Nahmias Y, Schwartz RE, Verfaillie CM, Odde DJ. 2005. Laser-guided direct writing for three-dimensional tissue engineering. Biotechnol Bioeng 92:129-136.

Nikol'skii NN, Gibai IA, Somova NV. 2007. [Human embryonic stem cells. Problems and perspectives]. Tsitologiia 49:529-537.

Noel D, Djouad F, Jorgense C. 2002. Regenerative medicine through mesenchymal stem cells for bone and cartilage repair. Curr Opin Investig Drugs 3:1000-1004.

O'Sullivan J, D'Arcy S, Barry FP, Murphy JM, Coleman CM. 2011. Mesenchymal chondroprogenitor cell origin and therapeutic potential. Stem Cell Res Ther 2:8.

Odorico JS, Kaufman DS, Thomson JA. 2001. Multilineage differentiation from human embryonic stem cell lines. Stem Cells 19:193-204.

Okamoto T, Aoyama T, Nakayama T, Nakamata T, Hosaka T, Nishijo K, Nakamura T, Kiyono T, Toguchida J. 2002. Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. Biochem Biophys Res Commun 295:354-361.

Ooi OC, Al Habib HF, Almsherqi ZA, El Oakley RM. 2006. Stem cell transplantation: potential impact on heart failure. Cell Tissue Bank 7:307-317.

Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. 2001. Bone marrow cells regenerate infarcted myocardium. Nature 410:701-705.

Ortegon M, Lim S, Chisholm D, Mendis S. 2012. Cost effectiveness of strategies to combat cardiovascular disease, diabetes, and tobacco use in sub-Saharan Africa and South East Asia: mathematical modelling study. BMJ 344:e607.

Ortiz LA, Dutreil M, Fattman C, Pandey AC, Torres G, Go K, Phinney DG. 2007. Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. Proc Natl Acad Sci U S A 104:11002-11007.

Ott I, Keller U, Knoedler M, Gotze KS, Doss K, Fischer P, Urlbauer K, Debus G, von Bubnoff N, Rudelius M, Schomig A, Peschel C, Oostendorp RA. 2005. Endothelial-like cells expanded from CD34+ blood cells improve left ventricular function after experimental myocardial infarction. FASEB J 19:992-994.

Pabbruwe MB, Esfandiari E, Kafienah W, Tarlton JF, Hollander AP. 2009. Induction of cartilage integration by a chondrocyte/collagen-scaffold implant. Biomaterials 30:4277-4286.

Palmer TD, Schwartz PH, Taupin P, Kaspar B, Stein SA, Gage FH. 2001. Cell culture. Progenitor cells from human brain after death. Nature 411:42-43.

Park GE, Pattison MA, Park K, Webster TJ. 2005. Accelerated chondrocyte functions on NaOH-treated PLGA scaffolds. Biomaterials 26:3075-3082.

Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, Lensch MW, Cowan C, Hochedlinger K, Daley GQ. 2008a. Disease-specific induced pluripotent stem cells. Cell 134:877-886.

Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. 2008b. Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451:141-146.

Park JS, Yang HN, Woo DG, Jeon SY, Do HJ, Lim HY, Kim JH, Park KH. 2011a. Chondrogenesis of human mesenchymal stem cells mediated by the combination of SOX trio SOX5, 6, and 9 genes complexed with PEI-modified PLGA nanoparticles. Biomaterials 32:3679-3688.

Park JS, Yang HN, Woo DG, Jeon SY, Park KH. 2011b. Chondrogenesis of human mesenchymal stem cells in fibrin constructs evaluated in vitro and in nude mouse and rabbit defects models. Biomaterials 32:1495-1507.

Parolini O, Alviano F, Bagnara GP, Bilic G, Buhring HJ, Evangelista M, Hennerbichler S, Liu B, Magatti M, Mao N, Miki T, Marongiu F, Nakajima H, Nikaido T, Portmann-Lanz CB, Sankar V, Soncini M, Stadler G, Surbek D, Takahashi TA, Redl H, Sakuragawa N, Wolbank S, Zeisberger S, Zisch A, Strom SC. 2008. Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. Stem Cells 26:300-311.

Partemi S, Berne PM, Batlle M, Berruezo A, Mont L, Riuro H, Ortiz JT, Roig E, Pascali VL, Brugada R, Brugada J, Oliva A. 2010. Analysis of mRNA from human heart tissue and putative applications in forensic molecular pathology. Forensic Sci Int 203:99-105.

Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, Rafii S. 2000. Expression of VEGFR-2 and AC133 by circulating human

CD34(+) cells identifies a population of functional endothelial precursors. Blood 95:952-958.

Pelosi E, Castelli G, Testa U. 2012. Human umbilical cord is a unique and safe source of various types of stem cells suitable for treatment of hematological diseases and for regenerative medicine. Blood Cells Mol Dis 49:20-28.

Peran M, Garcia MA, Lopez-Ruiz E, Bustamante M, Jimenez G, Madeddu R, Marchal JA. 2012. Functionalized nanostructures with application in regenerative medicine. Int J Mol Sci 13:3847-3886.

Peran M, Garcia MA, Lopez-Ruiz E, Jimenez G, Marchal JA. 2013a. How Can Nanotechnology Help to Repair the Body? Advances in Cardiac, Skin, Bone, Cartilage and Nerve Tissue Regeneration. Materials 6:1333-1359.

Peran M, Marchal JA, Lopez E, Jimenez-Navarro M, Boulaiz H, Rodriguez-Serrano F, Carrillo E, Sanchez-Espin G, de Teresa E, Tosh D, Aranega A. 2010. Human cardiac tissue induces transdifferentiation of adult stem cells towards cardiomyocytes. Cytotherapy 12:332-337.

Peran M, Ruiz S, Kwiatkowski W, Marchal JA, Yang SL, Aranega A, Choe S, Izpisua Belmonte JC. 2013b. Activin/BMP2 chimeric ligands direct adipose-derived stem cells to chondrogenic differentiation. Stem Cell Res 10:464-476.

Peran M, Sanchez-Ferrero A, Tosh D, Marchal JA, Lopez E, Alvarez P, Boulaiz H, Rodriguez-Serrano F, Aranega A. 2011. Ultrastructural and molecular analyzes of insulin-producing cells induced from human hepatoma cells. Cytotherapy 13:193-200.

Peterson L, Minas T, Brittberg M, Lindahl A. 2003. Treatment of osteochondritis dissecans of the knee with autologous chondrocyte transplantation: results at two to ten years. J Bone Joint Surg Am 85-A Suppl 2:17-24.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143-147.

Place ES, George JH, Williams CK, Stevens MM. 2009. Synthetic polymer scaffolds for tissue engineering. Chem Soc Rev 38:1139-1151.

Plotnikov EY, Khryapenkova TG, Vasileva AK, Marey MV, Galkina SI, Isaev NK, Sheval EV, Polyakov VY, Sukhikh GT, Zorov DB. 2008. Cell-to-cell cross-talk between mesenchymal stem cells and cardiomyocytes in co-culture. J Cell Mol Med 12:1622-1631.

Porter KE, Turner NA. 2009. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol Ther 123:255-278.

Prabhakaran MP, Ghasemi-Mobarakeh L, Ramakrishna S. 2011. Electrospun composite nanofibers for tissue regeneration. J Nanosci Nanotechnol 11:3039-3057.

Puetzer JL, Petitte JN, Loboa EG. 2010. Comparative review of growth factors for induction of three-dimensional in vitro chondrogenesis in human mesenchymal stem cells isolated from bone marrow and adipose tissue. Tissue Eng Part B Rev 16:435-444.

Qi Y, Feng G, Yan W. 2012. Mesenchymal stem cell-based treatment for cartilage defects in osteoarthritis. Mol Biol Rep 39:5683-5689.

Quevedo HC, Hatzistergos KE, Oskouei BN, Feigenbaum GS, Rodriguez JE, Valdes D, Pattany PM, Zambrano JP, Hu Q, McNiece I, Heldman AW, Hare JM. 2009. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. Proc Natl Acad Sci U S A 106:14022-14027.

Quirici N, Soligo D, Caneva L, Servida F, Bossolasco P, Deliliers GL. 2001. Differentiation and expansion of endothelial cells from human bone marrow CD133(+) cells. Br J Haematol 115:186-194.

Rajagopal SK, Ma Q, Obler D, Shen J, Manichaikul A, Tomita-Mitchell A, Boardman K, Briggs C, Garg V, Srivastava D, Goldmuntz E, Broman KW, Benson DW, Smoot LB, Pu WT. 2007. Spectrum of heart disease associated with murine and human GATA-4 mutation. J Mol Cell Cardiol 43:677-685.

Ramesh B, Bishi DK, Rallapalli S, Arumugam S, Cherian KM, Guhathakurta S. 2012. Ischemic cardiac tissue conditioned media induced differentiation of human mesenchymal stem cells into early stage cardiomyocytes. Cytotechnology 64:563-575.

Rangappa S, Fen C, Lee EH, Bongso A, Sim EK. 2003. Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes. Ann Thorac Surg 75:775-779.

Ratajczak MZ, Zuba-Surma EK, Wysoczynski M, Wan W, Ratajczak J, Wojakowski W, Kucia M. 2008. Hunt for pluripotent stem cell -- regenerative medicine search for almighty cell. J Autoimmun 30:151-162.

Rawlins EL, Hogan BL. 2006. Epithelial stem cells of the lung: privileged few or opportunities for many? Development 133:2455-2465.

Reppel M, Boettinger C, Hescheler J. 2004. Beta-adrenergic and muscarinic modulation of human embryonic stem cell-derived cardiomyocytes. Cell Physiol Biochem 14:187-196.

Ridgeway AG, Wilton S, Skerjanc IS. 2000. Myocyte enhancer factor 2C and myogenin up-regulate each other's expression and induce the development of skeletal muscle in P19 cells. J Biol Chem 275:41-46.

Roberson DW, Alosi JA, Cotanche DA. 2004. Direct transdifferentiation gives rise to the earliest new hair cells in regenerating avian auditory epithelium. J Neurosci Res 78:461-471.

Roberts AB, Roche NS, Winokur TS, Burmester JK, Sporn MB. 1992. Role of transforming growth factor-beta in maintenance of function of cultured neonatal cardiac myocytes. Autocrine action and reversal of damaging effects of interleukin-1. J Clin Invest 90:2056-2062.

Rodriguez-Ubreva J, Ciudad L, Gomez-Cabrero D, Parra M, Bussmann LH, di Tullio A, Kallin EM, Tegner J, Graf T, Ballestar E. 2012. Pre-B cell to macrophage transdifferentiation without significant promoter DNA methylation changes. Nucleic Acids Res 40:1954-1968.

Roobrouck VD, Clavel C, Jacobs SA, Ulloa-Montoya F, Crippa S, Sohni A, Roberts SJ, Luyten FP, Van Gool SW, Sampaolesi M, Delforge M, Luttun A, Verfaillie CM. 2011. Differentiation potential of human postnatal mesenchymal stem cells, mesoangioblasts, and multipotent adult progenitor cells reflected in their transcriptome and partially influenced by the culture conditions. Stem Cells 29:871-882.

Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez FJ, Spiegelman BM. 2002. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev 16:22-26.

Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM, Rice HE. 2002. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun 294:371-379.

Salven P, Mustjoki S, Alitalo R, Alitalo K, Rafii S. 2003. VEGFR-3 and CD133 identify a population of CD34+ lymphatic/vascular endothelial precursor cells. Blood 101:168-172.

Sasaki M, Abe R, Fujita Y, Ando S, Inokuma D, Shimizu H. 2008. Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. J Immunol 180:2581-2587.

Sawa Y, Miyagawa S. 2013. Present and Future Perspectives on Cell Sheet-Based Myocardial Regeneration Therapy. Biomed Res Int 2013:583912.

Sawa Y, Miyagawa S, Sakaguchi T, Fujita T, Matsuyama A, Saito A, Shimizu T, Okano T. 2012. Tissue engineered myoblast sheets improved cardiac function sufficiently to discontinue LVAS in a patient with DCM: report of a case. Surg Today 42:181-184.

Sayed N, Wong WT, Cooke JP. 2013. Therapeutic Transdifferentiation: Can we Generate Cardiac Tissue Rather Than Scar after Myocardial Injury? Methodist Debakey Cardiovasc J 9:210-212.

Schittini AV, Celedon PF, Stimamiglio MA, Krieger M, Hansen P, da Costa FD, Goldenberg S, Dallagiovanna B, Correa A. 2010. Human cardiac explant-conditioned medium: soluble

factors and cardiomyogenic effect on mesenchymal stem cells. Exp Biol Med (Maywood) 235:1015-1024.

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T. 2006. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 7:3.

Sekiya S, Suzuki A. 2011. Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 475:390-393.

Shen JF, Sugawara A, Yamashita J, Ogura H, Sato S. 2011. Dedifferentiated fat cells: an alternative source of adult multipotent cells from the adipose tissues. Int J Oral Sci 3:117-124.

Shen JG, Quo XS, Jiang B, Li M, Xin W, Zhao BL. 2000. Chinonin, a novel drug against cardiomyocyte apoptosis induced by hypoxia and reoxygenation. Biochim Biophys Acta 1500:217-226.

Shimomura K, Moriguchi Y, Murawski CD, Yoshikawa H, Nakamura N. 2014. Osteochondral tissue engineering with biphasic scaffold: Current strategies and techniques. Tissue Eng Part B Rev.

Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K, Shimada T, Oike Y, Imaizumi T. 2001. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation 103:2776-2779.

Si YL, Zhao YL, Hao HJ, Fu XB, Han WD. 2011. MSCs: Biological characteristics, clinical applications and their outstanding concerns. Ageing Res Rev 10:93-103.

Sisakhtnezhad S, Matin MM. 2012. Transdifferentiation: a cell and molecular reprogramming process. Cell Tissue Res 348:379-396.

Skalnikova H, Motlik J, Gadher SJ, Kovarova H. 2011. Mapping of the secretome of primary isolates of mammalian cells, stem cells and derived cell lines. Proteomics 11:691-708.

Slack JM, Tosh D. 2001. Transdifferentiation and metaplasia--switching cell types. Curr Opin Genet Dev 11:581-586.

Snykers S, De Kock J, Rogiers V, Vanhaecke T. 2009. In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. Stem Cells 27:577-605.

Song B, Estrada KD, Lyons KM. 2009. Smad signaling in skeletal development and regeneration. Cytokine Growth Factor Rev 20:379-388.

Song L, Tuan RS. 2004. Transdifferentiation potential of human mesenchymal stem cells derived from bone marrow. FASEB J 18:980-982.

Song L, Webb NE, Song Y, Tuan RS. 2006. Identification and functional analysis of candidate genes regulating mesenchymal stem cell self-renewal and multipotency. Stem Cells 24:1707-1718.

Sottile V, Halleux C, Bassilana F, Keller H, Seuwen K. 2002. Stem cell characteristics of human trabecular bone-derived cells. Bone 30:699-704.

Sreerekha PR, Menon D, Nair SV, Chennazhi KP. 2013. Fabrication of electrospun poly (lactide-co-glycolide)-fibrin multiscale scaffold for myocardial regeneration in vitro. Tissue Eng Part A 19:849-859.

Steindler DA. 2007. Stem cells, regenerative medicine, and animal models of disease. ILAR J 48:323-338.

Steinert AF, Proffen B, Kunz M, Hendrich C, Ghivizzani SC, Noth U, Rethwilm A, Eulert J, Evans CH. 2009. Hypertrophy is induced during the in vitro chondrogenic differentiation of human mesenchymal stem cells by bone morphogenetic protein-2 and bone morphogenetic protein-4 gene transfer. Arthritis Res Ther 11:R148.

Steinert AF, Rackwitz L, Gilbert F, Noth U, Tuan RS. 2012. Concise review: the clinical application of mesenchymal stem cells for musculoskeletal regeneration: current status and perspectives. Stem Cells Transl Med 1:237-247.

Strem BM, Zhu M, Alfonso Z, Daniels EJ, Schreiber R, Beygui R, MacLellan WR, Hedrick MH, Fraser JK. 2005. Expression of cardiomyocytic markers on adipose tissue-derived cells in a murine model of acute myocardial injury. Cytotherapy 7:282-291.

Strunk D. 2011. Endothelial progenitor cells: quod erat demonstrandum? Curr Pharm Des 17:3245-3251.

Sugimoto K, Gordon SP, Meyerowitz EM. 2011. Regeneration in plants and animals: dedifferentiation, transdifferentiation, or just differentiation? Trends Cell Biol 21:212-218.

Sumer H, Liu J, Verma PJ. 2011. Cellular reprogramming of somatic cells. Indian J Exp Biol 49:409-415.

Sun JZ, Li XY, Sporn MB, Schneider MD, Roberts R, Bolli R. 1993. Effect of transforming growth factor-beta 1 on myocardial stunning in the intact dog. J Mol Cell Cardiol 25:379-386.

Sun X, Fu X, Han W, Zhao Y, Liu H, Sheng Z. 2011. Dedifferentiation of human terminally differentiating keratinocytes into their precursor cells induced by basic fibroblast growth factor. Biol Pharm Bull 34:1037-1045.

Sun Z, Zhang Y, Yang S, Jia J, Ye S, Chen D, Mo F. 2012. Growth differentiation factor 5 modulation of chondrogenesis of self-assembled constructs involves gap junction-mediated intercellular communication. Dev Growth Differ 54:809-817.

Suzuki K, Mitsutake N, Saenko V, Suzuki M, Matsuse M, Ohtsuru A, Kumagai A, Uga T, Yano H, Nagayama Y, Yamashita S. 2011. Dedifferentiation of human primary thyrocytes into multilineage progenitor cells without gene introduction. PLoS One 6:e19354.

Szabo E, Rampalli S, Risueno RM, Schnerch A, Mitchell R, Fiebig-Comyn A, Levadoux-Martin M, Bhatia M. 2010. Direct conversion of human fibroblasts to multilineage blood progenitors. Nature 468:521-526.

Takahashi K, Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663-676.

Takamiya M, Okigaki M, Jin D, Takai S, Nozawa Y, Adachi Y, Urao N, Tateishi K, Nomura T, Zen K, Ashihara E, Miyazaki M, Tatsumi T, Takahashi T, Matsubara H. 2006. Granulocyte colony-stimulating factor-mobilized circulating c-Kit+/Flk-1+ progenitor cells regenerate endothelium and inhibit neointimal hyperplasia after vascular injury. Arterioscler Thromb Vasc Biol 26:751-757.

Talchai C, Xuan S, Lin HV, Sussel L, Accili D. 2012. Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. Cell 150:1223-1234.

Tallheden T, Karlsson C, Brunner A, Van Der Lee J, Hagg R, Tommasini R, Lindahl A. 2004. Gene expression during redifferentiation of human articular chondrocytes. Osteoarthritis Cartilage 12:525-535.

Tapscott SJ. 2005. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. Development 132:2685-2695.

Taylor SM, Jones PA. 1979. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 17:771-779.

Tendera M, Wojakowski W, Ruzyllo W, Chojnowska L, Kepka C, Tracz W, Musialek P, Piwowarska W, Nessler J, Buszman P, Grajek S, Breborowicz P, Majka M, Ratajczak MZ. 2009. 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 30:1313-1321.

Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP, Gurtner GC. 2002. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation 106:2781-2786.

Thal MA, Krishnamurthy P, Mackie AR, Hoxha E, Lambers E, Verma S, Ramirez V, Qin G, Losordo DW, Kishore R. 2012. Enhanced angiogenic and cardiomyocyte differentiation capacity of epigenetically reprogrammed mouse and human endothelial progenitor cells augments their efficacy for ischemic myocardial repair. Circ Res 111:180-190.

Thayer JF, Yamamoto SS, Brosschot JF. 2010. The relationship of autonomic imbalance, heart rate variability and cardiovascular disease risk factors. Int J Cardiol 141:122-131.

Tholpady SS, Llull R, Ogle RC, Rubin JP, Futrell JW, Katz AJ. 2006. Adipose tissue: stem cells and beyond. Clin Plast Surg 33:55-62, vi.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. Science 282:1145-1147.

Toghraie FS, Chenari N, Gholipour MA, Faghih Z, Torabinejad S, Dehghani S, Ghaderi A. 2011. Treatment of osteoarthritis with infrapatellar fat pad derived mesenchymal stem cells in Rabbit. Knee 18:71-75.

Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. 2002. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 105:93-98

Tosh D, Slack JM. 2002. How cells change their phenotype. Nat Rev Mol Cell Biol 3:187-194.

Ueyama T, Kasahara H, Ishiwata T, Nie Q, Izumo S. 2003. Myocardin expression is regulated by Nkx2.5, and its function is required for cardiomyogenesis. Mol Cell Biol 23:9222-9232.

Urbich C, Dimmeler S. 2004. Endothelial progenitor cells: characterization and role in vascular biology. Circ Res 95:343-353.

van den Borne SW, Diez J, Blankesteijn WM, Verjans J, Hofstra L, Narula J. 2010. Myocardial remodeling after infarction: the role of myofibroblasts. Nat Rev Cardiol 7:30-37.

van der Strate BW, Popa ER, Schipper M, Brouwer LA, Hendriks M, Harmsen MC, van Luyn MJ. 2007. Circulating human CD34+ progenitor cells modulate neovascularization and inflammation in a nude mouse model. J Mol Cell Cardiol 42:1086-1097.

Varghese S, Theprungsirikul P, Sahani S, Hwang N, Yarema KJ, Elisseeff JH. 2007. Glucosamine modulates chondrocyte proliferation, matrix synthesis, and gene expression. Osteoarthritis Cartilage 15:59-68.

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

Vaughan EE, O'Brien T. 2012. Isolation of circulating angiogenic cells. Methods Mol Biol 916:351-356.

Vennemann M, Koppelkamm A. 2010. mRNA profiling in forensic genetics I: Possibilities and limitations. Forensic Sci Int 203:71-75.

Ventura C, Maioli M. 2000. Opioid peptide gene expression primes cardiogenesis in embryonal pluripotent stem cells. Circ Res 87:189-194.

Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M. 2010. Direct conversion of fibroblasts to functional neurons by defined factors. Nature 463:1035-1041.

Vija L, Farge D, Gautier JF, Vexiau P, Dumitrache C, Bourgarit A, Verrecchia F, Larghero J. 2009. Mesenchymal stem cells: Stem cell therapy perspectives for type 1 diabetes. Diabetes Metab 35:85-93.

Vinatier C, Mrugala D, Jorgensen C, Guicheux J, Noel D. 2009. Cartilage engineering: a crucial combination of cells, biomaterials and biofactors. Trends Biotechnol 27:307-314.

Volarevic V, Ljujic B, Stojkovic P, Lukic A, Arsenijevic N, Stojkovic M. 2011. Human stem cell research and regenerative medicine--present and future. Br Med Bull 99:155-168.

Wakitani S, Saito T, Caplan AI. 1995. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 18:1417-1426.

Walev I, Bhakdi SC, Hofmann F, Djonder N, Valeva A, Aktories K, Bhakdi S. 2001. Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. Proc Natl Acad Sci U S A 98:3185-3190.

Wang T, Xu Z, Jiang W, Ma A. 2006. Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. Int J Cardiol 109:74-81.

Wang Y, Kim UJ, Blasioli DJ, Kim HJ, Kaplan DL. 2005. In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. Biomaterials 26:7082-7094.

Warren V. 2013. Understanding regenerative medicine: a commissioner's viewpoint. Regen Med 8:227-232.

Weber B, Zeisberger SM, Hoerstrup SP. 2011. Prenatally harvested cells for cardiovascular tissue engineering: fabrication of autologous implants prior to birth. Placenta 32 Suppl 4:S316-319.

Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Adam MA, Lassar AB, Miller AD. 1989. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc Natl Acad Sci U S A 86:5434-5438.

Wickham MQ, Erickson GR, Gimble JM, Vail TP, Guilak F. 2003. Multipotent stromal cells derived from the infrapatellar fat pad of the knee. Clin Orthop Relat Res:196-212.

Wieland HA, Michaelis M, Kirschbaum BJ, Rudolphi KA. 2005. Osteoarthritis - an untreatable disease? Nat Rev Drug Discov 4:331-344.

Williams DF. 2009. On the nature of biomaterials. Biomaterials 30:5897-5909.

Wojakowski W, Landmesser U, Bachowski R, Jadczyk T, Tendera M. 2012. Mobilization of stem and progenitor cells in cardiovascular diseases. Leukemia 26:23-33.

Wong CY, Cheong SK, Mok PL, Leong CF. 2008. Differentiation of human mesenchymal stem cells into mesangial cells in post-glomerular injury murine model. Pathology 40:52-57.

Woodbury D, Schwarz EJ, Prockop DJ, Black IB. 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364-370.

Wu L, Cai X, Zhang S, Karperien M, Lin Y. 2013. Regeneration of articular cartilage by adipose tissue derived mesenchymal stem cells: perspectives from stem cell biology and molecular medicine. J Cell Physiol 228:938-944.

Xie H, Ye M, Feng R, Graf T. 2004. Stepwise reprogramming of B cells into macrophages. Cell 117:663-676.

Xie XJ, Wang JA, Cao J, Zhang X. 2006. Differentiation of bone marrow mesenchymal stem cells induced by myocardial medium under hypoxic conditions. Acta Pharmacol Sin 27:1153-1158.

Xu M, Millard RW, Ashraf M. 2012. Role of GATA-4 in differentiation and survival of bone marrow mesenchymal stem cells. Prog Mol Biol Transl Sci 111:217-241.

Xu Y, Kimura K, Matsumoto N, Ide C. 2003. Isolation of neural stem cells from the forebrain of deceased early postnatal and adult rats with protracted post-mortem intervals. J Neurosci Res 74:533-540.

Yamada Y, Sakurada K, Takeda Y, Gojo S, Umezawa A. 2007. Single-cell-derived mesenchymal stem cells overexpressing Csx/Nkx2.5 and GATA-4 undergo the stochastic cardiomyogenic fate and behave like transient amplifying cells. Exp Cell Res 313:698-706.

Yamashita S, Andoh M, Ueno-Kudoh H, Sato T, Miyaki S, Asahara H. 2009. Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes. Exp Cell Res 315:2231-2240.

Yang J, Ii M, Kamei N, Alev C, Kwon SM, Kawamoto A, Akimaru H, Masuda H, Sawa Y, Asahara T. 2011a. CD34+ cells represent highly functional endothelial progenitor cells in murine bone marrow. PLoS One 6:e20219.

Yang L, Li S, Hatch H, Ahrens K, Cornelius JG, Petersen BE, Peck AB. 2002. In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci U S A 99:8078-8083.

Yang Z, Huang CY, Candiotti KA, Zeng X, Yuan T, Li J, Yu H, Abdi S. 2011b. Sox-9 facilitates differentiation of adipose tissue-derived stem cells into a chondrocyte-like phenotype in vitro. J Orthop Res 29:1291-1297.

Ye NS, Chen J, Luo GA, Zhang RL, Zhao YF, Wang YM. 2006. Proteomic profiling of rat bone marrow mesenchymal stem cells induced by 5-azacytidine. Stem Cells Dev 15:665-676.

Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT, Ingram DA. 2007. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 109:1801-1809.

Yokoo T, Kawamura T, Kobayashi E. 2008. Kidney organogenesis and regeneration: a new era in the treatment of chronic renal failure? Clin Exp Nephrol 12:326-331.

Yoon J, Min BG, Kim YH, Shim WJ, Ro YM, Lim DS. 2005. Differentiation, engraftment and functional effects of pre-treated mesenchymal stem cells in a rat myocardial infarct model. Acta Cardiol 60:277-284.

Young PP, Vaughan DE, Hatzopoulos AK. 2007. Biologic properties of endothelial progenitor cells and their potential for cell therapy. Prog Cardiovasc Dis 49:421-429.

Zagoura DS, Trohatou O, Bitsika V, Makridakis M, Pappa KI, Vlahou A, Roubelakis MG, Anagnou NP. 2013. AF-MSCs fate can be regulated by culture conditions. Cell Death Dis 4:e571.

Zakharov Iu M, Makarova EB. 2013. [Regulation of osteogenic differentiation of mesenchimal stem sells of bone marrow]. Ross Fiziol Zh Im I M Sechenova 99:417-432.

Zammaretti P, Zisch AH. 2005. Adult 'endothelial progenitor cells'. Renewing vasculature. Int J Biochem Cell Biol 37:493-503.

Zandstra PW, Bauwens C, Yin T, Liu Q, Schiller H, Zweigerdt R, Pasumarthi KB, Field LJ. 2003. Scalable production of embryonic stem cell-derived cardiomyocytes. Tissue Eng 9:767-778.

Zeng H, Zhao D, Mukhopadhyay D. 2002. KDR stimulates endothelial cell migration through heterotrimeric G protein Gq/11-mediated activation of a small GTPase RhoA. J Biol Chem 277:46791-46798.

Zhang D, Fan GC, Zhou X, Zhao T, Pasha Z, Xu M, Zhu Y, Ashraf M, Wang Y. 2008. Over-expression of CXCR4 on mesenchymal stem cells augments myoangiogenesis in the infarcted myocardium. J Mol Cell Cardiol 44:281-292.

Zhang SJ, Zhang H, Wei YJ, Su WJ, Liao ZK, Hou M, Zhou JY, Hu SS. 2006. Adult endothelial progenitor cells from human peripheral blood maintain monocyte/macrophage function throughout in vitro culture. Cell Res 16:577-584.

Zhang Z, Li H, Ma Z, Feng J, Gao P, Dong H. 2012. Efficient cardiomyogenic differentiation of bone marrow mesenchymal stromal cells by combination of Wnt11 and bone morphogenetic protein 2. Exp Biol Med (Maywood) 237:768-776.

Zheng B, Wen JK, Han M. 2003. Regulatory factors involved in cardiogenesis. Biochemistry (Mosc) 68:650-657.

Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. 2008. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455:627-632.

Zhu XY, Lerman A, Lerman LO. 2013. Concise review: mesenchymal stem cell treatment for ischemic kidney disease. Stem Cells 31:1731-1736.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. 2002. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279-4295.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. 2001. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211-228.

GLOSSARY

5 aza: 5-azacitidina, del inglés "5-azacytidine".

Acan: Aggrecan.

ACI: Implantación de condrocitos autólogos, del inglés "autologous chondrocyte implantation".

AMI: Acute myocardial infarction.

AMPc: Adenosín monofosfato cíclico.

Ang1: Angiopoyetina-1.

aP2: Proteína de unión a ácidos grasos.

ARNm: Ácido ribonucleico mensajero.

ASCL1: Achaete–scute homologue 1.

ASCs: Células madre derivadas de tejido adiposo, del inglés "adipose derived stem cells".

BM-EPCs: Células progenitoras endoteliales procedentes de la médula ósea, del inglés "endotelial progenitor cells from bone marrow".

BM-MSCs: Células madre mesenquimales procedentes de la médula ósea, del inglés" mesenchymal stem cells from bone marrow".

BMPs: Proteínas morfogenéticas óseas, del inglés "bone morphogenic proteins".

BRN2 (*POU3F2*): Brainspecific homeobox.

BSA: Albumina serica bovina.

CACs: Células circulantes angiogénicas.

CFU-EC: Unidades formadoras de colonias de células endoteliales, del inglés "endothelial cell colony-forming units".

CFU-F: Unidades formadoras de colonias de fibroblastos, del inglés "fibroblast colony-forming units ".

Col10: Collagen type X

Col1A1: Colágeno tipo I α1, del inglés "Collagen, type I, alpha 1".

Col2A1: Colágeno 2, del inglés "Collagen, type II, alpha 1".

COL3A1: Collagen, type III, alpha 1.

COMP: Proteína de la matriz oligomérica del cartílago, del inglés "cartilage oligomeric. matrix protein".

Ct: threshold cycle.

CVD: Enfermedades cardiovasculares, del inglés "cardiovascular diseases".

Cyr61: Cysteine-rich, angiogenic inducer, 61.

DAPI: 4',6-diamidino-2-phenylindole.

DFAT: Dedifferentiated fat cells.

DMEM: Dulbecco's modified Eagle's médium.

DMSO: Dimetil sulfóxido.

EC: Endothelial cells.

ECM: Matriz extracelular, del inglés "extracelular matrix".

EGF: Factor de crecimiento epidérmico.

eNOS: Sintasa de óxido nítrico endotelial, del inglés "endothelial nitric oxide synthase".

EOCs: Endothelial outgrowth cells.

EPCs: Células progenitoras endoteliales, del inglés "endothelial progenitor cells".

es"outgrowth endothelial cells" (OECs) o endothelial outgrowth cells (EOCs)

ESCs: Células madre embrionarias, del inglés "embrionic stem cells"

FACS: Flow cytometry

FBS: Fetal bovine serum.

FGF: Factores de crecimiento de los fibroblastos.

FITC: Fluorescein Isothiocyanate.

Foxa: Forkhead box protein A.

GA: Golgi apparatus.

Gapdh: Glyceraldehyde 3-phosphate dehydrogenase.

GATA-4: GATA binding protein 4

G-CSF: Factor estimulante de colonias de granulocitos.

GDF5: Growth differentiation factor 5.

GUSB: Gene expression of beta-glucuronidase.

hASCs: Células madre derivadas de tejido adiposo humano, del inglés " human adipose derived stem cells".

HBSS: Hanks' balanced salt solution

HEK293T: Human embryonic kidney 293 cells.

HGF: Factor de crecimiento de hepatocitos, del inglés " hepatocyte growth factor".

hMADS: Células madre multipotentes derivadas de tejido adiposo humano.

Hnf4a: Hepatocyte nuclear factor 4, alpha.

HSCs: Células madre hematopoyéticas, del inglés "hematopoietic stem cells".

IFPSCs: Células madre de la almohadilla de la grasa infrapatelar, del inglés "infrapatellar fat pad stem cells"

IGF: Factores de crecimiento de la insulina, del inglés "insulin-like growth factor"

Ihh: Indian hedgehog

iNOs: Óxido nítrico sintasa inducible, del inglés "nitric oxide synthase 3" (NOS3).

iPS cells: Células madre pluripotentes inducidas , del inglés "induced pluripotent stem cells".

ISCT: Sociedad Internacional de Terapia Celular Sociedad, del inglés "International society of cell therapy"

Klf4: Kruppel-like factor 4.

LDL: Lipoproteínas de baja densidad acetiladas

LI: Lipid droplets.

LIF: Leukemia inhibitory factor.

LPL: Lipoproteína lipasa.

LV: Ventrículo izquierdo, del inglés "left ventricular".

LVAD: Dispositivo de asistencia ventricular, del inglés "left ventricular assist device".

MACI: Implante autólogo de condrocitos inducido en matriz extracelular , del inglés Matrix-induced chondrocyte implantation".

Mafa: V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A.

MAPK: Mitogen-activated protein kinase.

MCP-1: Monocyte chemoattractant protein-1.

Mef2-c: Myocyte-enhancer factor-2c.

MHC: Miosina de cadena pesada, del inglés "myosin heavy chain".

MI: Infarto agudo de miocardio, del inglés "acute myocardial infarction"

MIPS: Mesenchymal stem cells derived iPS cells.

MLC2: Miosina de cadena corta 2, del inglés "myosin light chain 2".

MNC: Mononucleated cells, del inglés " mononucleated cells".

MSCs: Células madre mesenquimales, del inglés "mesenchymal stem cells".

MYL3: Myosin light chain 3.

MyoD: Proteína 1 de diferenciación miogénica.

MYT1L: Myelin transcription factor 1-like.

Ngn3: Neurogenin 3.

NOS3: Nitric oxide synthase 3.

OA: Osteoarthritis.

Oct4: Octamer-binding transcription factor 4.

OECs: outgrowth endothelial cells.

PB: Sangre periférica, del inglés "peripheral blood".

PB-EPCs: Células progenitoras endoteliales de sangre periférica, del inglés "endothelial progenitors cells from peripheral blood".

PBS: Phosphate-buffered saline.

PCL: Policaprolactona.

PDGF: Factor de crecimiento derivado de plaquetas.

PDO: Polidioxanona, del inglés "polydioxanone".

Pdx1: Pancreatic and duodenal homeobox 1

PEG: Polietilenglicol.

PFA: Paraformaldehyde.

PGA: Ácido poliglicólico.

PIPAAm: Poli(N-isopropilacrilamida).

PLA: Ácido poliláctico.

PLGA: Ácido poliláctico-glicólico, del inglés "polylactic-co-glycolic acid"

POU3F2: POU class 3 homeobox 2.

PPAR: Receptor activador del proliferador de peroxisomas, del inglés "peroxisome proliferator-activated receptor"

PVA: Alcohol polivinílico.

RER: Rough endoplasmic reticulum.

RIN: RNA integrity number.

RT-PCR: Reverse transcription-polymerase.

SDF-1: Stromal cell-derived factor 1.

SEM: environmental scanning electron microscope analysis.

sFRP-4: Secreted frizzled-related protein 4.

SLO: Streptolisina O, del inglés streptolysin O.

Sox 5 (L-Sox5): SRY (sex determining region Y)-box 5.

Sox2: SRY (Sex determining Region Y)-box 2.

Sox6: SRY (sex determining region Y)-box 6.

Sox9: SRY (sex determining region Y)-box 9.

SVF: Fracción del estroma vascular.

Tbx5: T-box transcription factor 5.

TEM: Transmission electron microscopy.

TGF-β: Factor de crecimiento transformante, beta, del inglés "transforming growth factor beta".

TNNI3: Cardiac troponin I type 3.

TRITC: Tetramethyl rhodamine isothiocyanate.

UC: Cordón umbilical, del inglés "umbilical cord".

UCB: Sangre de cordon umbilical, "umbilical cord blood".

UCB-EPCS: Células progenitoras endoteliales de cordón umbilical, del inglés "endotelial progenitors cells from umbilical cord".

UEA-I: Ulex europaeus agglutinin 1.

VE-cadherina: Vascular endotelial.

VEGF: Factor de crecimiento endotelial vascular, del inglés "vascular endothelial growth factor".

VPF: Vascular permeability factor.

vWF: Factor de von willebrand.

WHO: World Health Organization.

OTHERS STUDIES IN WHICH THE PH.D STUDENT HAS PARTICIPATE

Peran M, Garcia MA, Lopez-Ruiz E, Bustamante M, Jimenez G, Madeddu R, Marchal JA. 2012. Functionalized nanostructures with application in regenerative medicine. Int J Mol Sci 13:3847-3886.

García M, Carrilo E, Ramírez A, Jiménez G, López-Ruiz E, Perán M, Picón M, J Campos, Boulaiz H, Marchal JA. 2012. Apoptosis as a Therapeutic Target in Cancer and Cancer Stem Cells: Novel Strategies and Futures Perspectives. INTECH. Apoptosis and Medicine. ISBN 978-953-51-0701-9.

Bustamante M, Marchal JA, Perán M, López E, Álvarez P, Rodriguez- Serrano F, Carrillo E, Aránega A. Therapeutic approaches in regenerative medicine for cardiovascular diseases: from bench to benside. INTECH. Advances in Regenerative Medicine. ISBN 978-953-307-732-1.

Peran M, Sanchez-Ferrero A, Tosh D, Marchal JA, Lopez E, Alvarez P, Boulaiz H, Rodriguez-Serrano F, Aranega A. 2011. Ultrastructural and molecular analyzes of insulin-producing cells induced from human hepatoma cells. Cytotherapy 13:193-200.

Peran M, Marchal JA, Lopez E, Jimenez-Navarro M, Boulaiz H, Rodriguez-Serrano F, Carrillo E, Sanchez-Espin G, de Teresa E, Tosh D, Aranega A. 2010. Human cardiac tissue induces transdifferentiation of adult stem cells towards cardiomyocytes. Cytotherapy 12:332-337.

Peran M, Rodriguez-Serrano F, Boulaiz H, Marchal JA, Lopez E, Picon M, Alvarez P, Rama AR, Ortiz R, Ramirez A, Aranega A. 2009. Fluorescence Recovery after Photobleaching. Cell Movement: New Research Trends: 331-343.

ANNEXE OF ARTICLES INCLUDED IN THIS THESIS

Materials **2013**, *6*, 1333-1359; doi:10.3390/ma6041333

OPEN ACCESS

materials

ISSN 1996-1944

www.mdpi.com/journal/materials

Review

How Can Nanotechnology Help to Repair the Body? Advances in Cardiac, Skin, Bone, Cartilage and Nerve Tissue Regeneration

Macarena Perán [1,*], María Angel García [2], Elena Lopez-Ruiz [1], Gema Jiménez [3] and Juan Antonio Marchal [3,4,*]

[1] Department of Health Sciences, University of Jaén, Campus Las Lagunillas, S/N, Jaén 23071, Spain; E-Mail: elruiz@ujaen.es

[2] Research Unit, University Hospital "Virgen de las Nieves", Avda. de las Fuerzas Armadas, 2, Granada 18014, Spain; E-Mail: mangelgarcia@ugr.es

[3] Biopathology and Regenerative Medicine Institute (IBIMER), University of Granada, Avda. del Conocimiento S/N. CP Armilla, Granada 18100, Spain; E-Mail: gemajg@correo.ugr.es

[4] Department of Human Anatomy and Embryology, University of Granada, Avda. De Madrid, 11, Granada 18012, Spain

***** Authors to whom correspondence should be addressed; E-Mails: mperan@ujaen.es (M.P.); jmarchal@ugr.es (J.A.M.); Tel.: +34-953-21-29-21 (M.P.); +34-958-24-93-21 (J.A.M.); Fax: +34-953-21-29-43 (M.P.); +34-958-24-62-96 (J.A.M.).

Received: 6 January 2013; in revised form: 20 March 2013 / Accepted: 20 March 2013 / Published: 28 March 2013

Abstract: Nanotechnologists have become involved in regenerative medicine *via* creation of biomaterials and nanostructures with potential clinical implications. Their aim is to develop systems that can mimic, reinforce or even create *in vivo* tissue repair strategies. In fact, in the last decade, important advances in the field of tissue engineering, cell therapy and cell delivery have already been achieved. In this review, we will delve into the latest research advances and discuss whether cell and/or tissue repair devices are a possibility. Focusing on the application of nanotechnology in tissue engineering research, this review highlights recent advances in the application of nano-engineered scaffolds designed to replace or restore the followed tissues: (i) skin; (ii) cartilage; (iii) bone; (iv) nerve; and (v) cardiac.

Keywords: nanotechnology; bio-scaffold; tissue engineering; nanostructures

---

1. Introduction

The populations of developed countries are rapidly aging, causing an increment in age-related diseases like osteoarthritis, osteoporosis, or Parkinson's disease. In addition to accidents, as well as poor health habits such as tobacco and stress, tissue or organ dysfunction can be provoked or even lost. To date, donor organ transplantation is the usual procedure to restore or enhance life expectancy. Nevertheless, this option is limited due to short supply and life-long immune suppression issues, as well as other associated side effects.

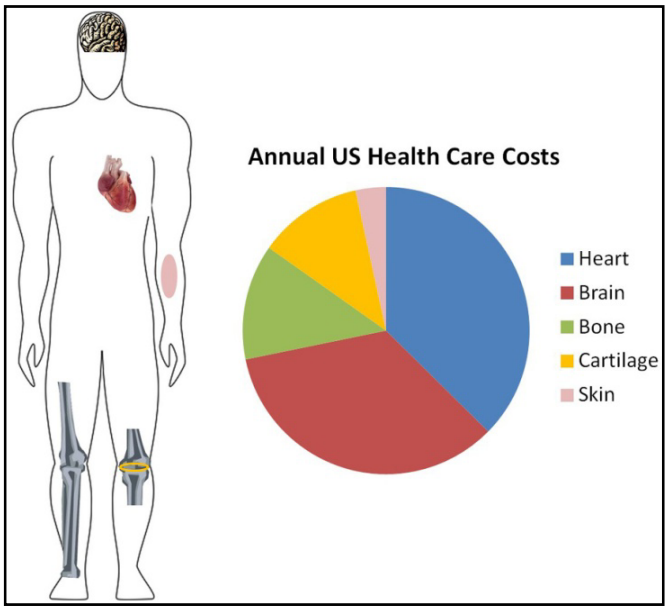
Tissue engineering, which is an alternative option for organ transplantation, is currently demonstrating great promise with first-in-man successful stories of tissue engineered implants [1]. This interdisciplinary science has as ultimate goal to design artifacts that (i) mimic natural tissues characteristics; (ii) fill up a space until the damage tissue is regenerated; (iii) temporarily replace tissue functions and; (iv) serve as a guide for tissue ingrowths. With this aim, nanotechnologists are applying their knowledge and experience using materials in a scale of less than 100 nanometers, to design and manufacture scaffolds that can replace the natural extra cellular matrix until host cells can repopulate and redo a new natural matrix. In addition, the biological substitutes should match the mechanical properties as the tissue is replacing to avoid mismatch between the synthetic graft and the surrounding native tissue. Furthermore, the materials used to construct the scaffold need to be biocompatible. The material must therefore be non-toxic, and its presence in the body should not elicit an immunological response. If the material used is biodegradable, its degradation kinetics should match the rate of tissue regeneration to ensure an optimal healing process. Other important parameters are adequate porosity to facilitate the delivery of nutrients to the regenerating cells, and appropriate nanotopography to promote cell adhesion and proliferation [2].

Natural or synthetic scaffolds have been tested in order to produce a clinically useful tissue scaffold of a target tissue or organ. Examples of natural scaffolds that have been applied clinically include decellularized dermis to treat burn injuries, as well as decellularized small intestine, ureter, or xenogeneic vessels to restore vascular function [3,4]. Although these materials have shown promising results in tissue repair, they have some drawbacks regarding mechanical properties, degradation, immunogenicity and cross-contamination. On the other hand, synthetic scaffolds have been constructed using synthetic materials or a combination between natural and synthetic materials and have demonstrated promising results in tissue repair [2]. The most commonly used natural biopolymers include demineralized bone matrix, agarose, collagen, hyaluronan, basement membrane, and alginate. Synthetic polymers include degradable polyesters, such as polyglycolic acid (PGA), polylactic acid (PLA), and their copolymers, poly (D,L-lactide-coglycolide) (PLGA). These biodegradable polymers have a long history of clinical use and are currently employed in various tissue engineering applications [5].

To construct suitable synthetic bio-scaffolds, the most widely chosen technique is electrospinning. This method allows the production of nanofibrous scaffolds with specific and desired properties and functionality. Importantly, nanofibrous scaffolds possess an extremely high surface-to-volume ratio, tunable porosity, and malleability to conform to a wide variety of sizes and shapes with a desirable 3D pattern [6].

In this work we have focused our attention on five tissues whose degeneration or dysfunction lead to chronic health problems and cause a steep increase in health care costs (Figure 1).

Figure 1. Representative figure showing the annual cost in the United Stated of America for main diseases related to tissue degeneration. The cost is shown in billions of US dollars.



In the United States, chronic wounds affect around 6.5 million patients and an excess of $25 billion is spent annually [7]. In addition, the direct and indirect health care costs associated with all forms of arthritis is approximately 86 billion dollars per year [8]. Furthermore, nearly $95 billion of health care dollars are used annually to treat patients with osteoporosis-related fractures, excluding the expenses caused by fractures in healthy young people from accidents [9]. The costs increase significantly when it comes to related brain diseases (such as Alzheimer's disease; blindness, deafness, brain injury; epilepsy, multiple sclerosis, Parkinson's disease, spinal cord injury, or stroke) with an estimated cost each year of $273 billion [10]. Finally, cardiovascular diseases have an overall cost per year of $273 billion [11].

This review provides an overview of the progress of nanotectnology application in tissue engineering research, highlighting recent advances in the application of nano-engineered scaffolds designed to replace or restore tissues like (i) skin; (ii) cartilage; (iii) bone; (iv) nerve; and (v) cardiac.

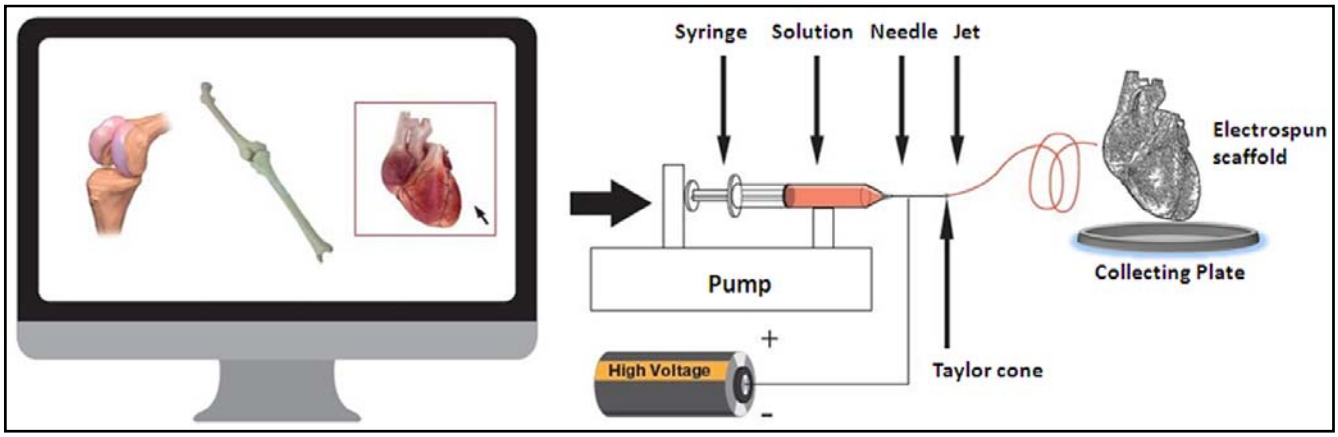
2. Electrospinning

Polymeric nanofibers can be processed by a number of techniques such as drawing, template synthesis, phase separation, self-assembly and electrospinning. Among these methods, the most successful for tissue engineering applications is the electrospinning process. The main advantage of this method is that electrospun scaffolds can be characterized by a complex micro-scale structure responsible for its macroscopic mechanical behavior. In this sense, various parameters can be controlled in the process of nanofiber creation: (i) polymer solution parameters (viscosity, surface tension, conductivity, *etc.*); (ii) electrospinning process parameters (voltage, federate, tip-to-collector

distance, *etc.*); and, (iii) ambient conditions (humidity). This allows the creation of various types of nanofibers with different thickness, pattern and forms which can be used to create various types of scaffolds [12].

In brief, electrospinning consists of a pipette to hold the polymer solution (spinneret), two electrodes, a high voltage power supply and a grounded collecting plate (usually a metal screen, plate, or rotating mandrel) (Figure 2). The production of polymer filaments is done by the electrostatic force, through the electrically charged jet of polymer solution. The polymer drops from the tip of the pipette and is drawn into a fiber due to the high voltage. The jet is electrically charged and the nanofibers are formed by the narrowing of the ejected jet stream as it undergoes increasing surface charge density due to evaporation of the solvent. The fiber is then collected as a mesh of fibers on the surface of a grounded target, the collecting plate, thus forming the electrospun scaffold [6]. A very important feature of this technique is that it provides a large surface area-to-volume ratio, which facilitates cellular uptake and nutrient diffusion. In addition, the fiber diameters can be controlled to mimic the extracellular matrix (ECM) fibrous architecture [13]. Moreover, electrospinning produces nanoarchitecturely patterned fibers in random or aligned form, which greatly influence the cell orientation and function [14,15].

Figure 2. Schematic diagram of the electrospinning process showing a glass syringe containing polymer solution; a nanofiber jet; a copper collecting plate and a power supply.



Since the physical and biological properties of the electrospun nanofibrous scaffolds—including hydrophilicity, mechanical modulus and strength, biodegradability, biocompatibility, and specific cell interactions—can be controlled, the biomedical applications of functional nanofibrous scaffolds have great potential. Those biological parameters are determined by the polymer chemical compositions. Playing with polymer physics, copolymerization and polymer blending to combine different polymers can yield new material properties. In addition, the performance of the electrospun scaffold can be further controlled by adjusting the diameter and morphology of the nanofibers, desirable 3-D patterns (e.g., layered structures) and the porosity through the electrospinning processing technology. Thus, by selecting a combination of proper components and by adjusting the component ratio, properties of electrospun scaffolds can be tailored with desired new functions [6]. Technological advances could lead to the development of a versatile electrospinning system able to create different tissues scaffold (Figure 1).

3. Skin Regeneration by Nanotechnological Approaches

The primary function of the skin is to act as a barrier; consequently, any related problem such as burns, chronic wound, ulcers or accidents can cause serious health complications. The apparently simple structure of the skin, consisting of two layers, the epidermis and the dermis, and its easy target localization, has encouraged the search for therapeutic alternatives. In this regard, nanobiotechnology emerges as a promising hope to improve wound healing and skin restoration.

Skin tissue engineering is based in the creation of scaffolds that must share the followed minimal characteristics: (i) biocompatibility; (ii) support for cell attachment and proliferation; and, (iii) to imitate the ECM as closely as possible [16,17]. One of the main difficulties found in the application of this artificial skin is the problems related to adhesion and integration of the scaffolds to the topography of the wound, while maintaining physical and mechanical properties [18,19].

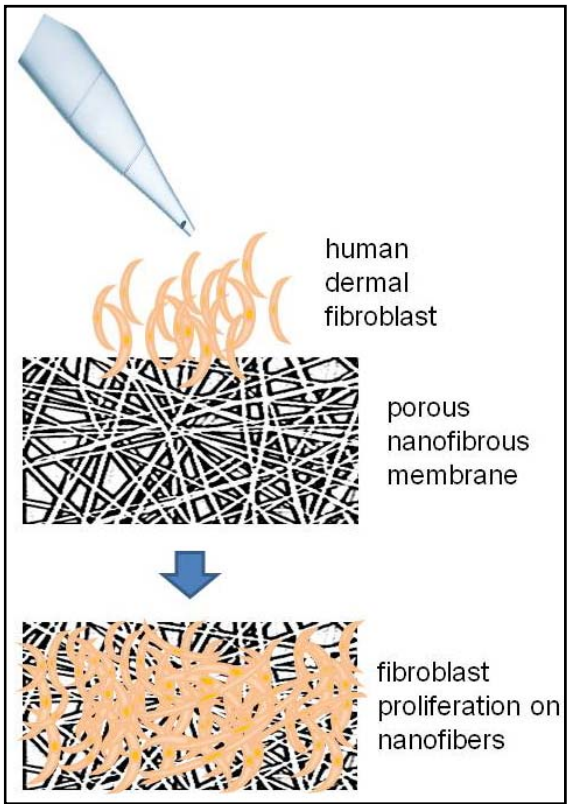
Electrospun nanofibrous scaffolds have been created to mimic the three-dimensional fiber network of the collagen fibrous structure. They are composed of collagen fibers that are formed hierarchically by nanometer-scale multi-fibrils and have been proved to support cell adhesion, proliferation, and differentiation mimicking the fibrous architecture of the ECM [5,20]. In addition, the electrospinning technique allows for control over the desired pore diameter, distribution, total volume, total area, and, consequently, the final porosity of the structure. A pioneering *in vivo* study has shown the benefits of covering wounds with polyurethane membranes produced via electrospinning. These membranes increased the epithelialization rate and formed a well-organized dermis [21]. The electrospun nanofibrous membrane could control water loss by evaporation, was permeable to oxygen, and promoted fluid drainage ability, while inhibited exogenous microorganism invasion. Other examples of good antibacterial activity was shown using collagen/chitosan-immobilized polypropylene wound-dressing membranes, demonstrating an excellent remodeling effect after histological examination with respect to the construction of vein, epidermis, and dermis at 21 days after skin injury [22].

Fibroblasts are the cell type best indicated for wound healing proposes [23]. In fact, seeding fibroblasts into dermal substitutes have been shown to improve wound healing [24]. In this respect, poly(L-lactic acid)-*co*-poly(ε-caprolactone) and gelatin (PLACL-G-P) nanofibrous scaffolds provided enough space for fibroblast ingrowth and induced the formation of a dermal substitute [25]. Figure 3 shows a schematic example of an electrospun-scaffold for the wound-healing proposes. In addition, the incorporation of collagen to the polycaprolactone-nanofibrous membrane improved attachment and proliferation of fibroblast [26]. Other studies have used a scaffold with mesenchymal stem cells (MSCs), and found an increased proliferation rate and differentiation of MSCs when combining poly (l-lactic acid) poly-*co*-(3-caprolactone) with a biomaterial [27]. In another assay, three-dimensional chitosan nanofibers were implanted in mice to cover full-thickness skin wounds and were able to induce a faster regeneration of both the epidermis and dermis compartments when compared to other structures such as sponges [28].

The potential clinical application of exogenous growth factors to chronic wounds, in an effort to accelerate healing, is not new, and in fact dates back more than 10 years [29]. Recently, electrospun fibers with a core-sheath structure and loaded with basic fibroblast growth factor-encoding plasmid were found in diabetic mice. The gradual growth factor released revealed significantly higher wound

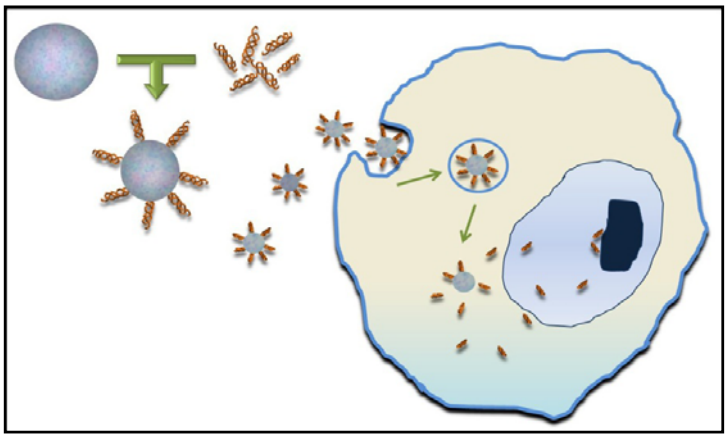
recovery rate with improved vascularization, enhanced collagen deposition and maturation, complete re-epithelialization and formation of skin appendages [30].

Figure 3. Human dermal fibroblast can be cultured on electrospun nanofibrous membrane to create *in vitro* allogeneic dermal substitutes.



The development of nanotechnology has also allowed the creation of nanoparticles (NPs) that act as a vehicle and carrier of biological factors that induce skin regeneration (Figure 4). In fact, several promising results have been obtained in studies using NP bearing: growth factors, thrombin, nitric oxide, opioids or protease inhibitors [31]. For example, thrombin-conjugated iron oxide NPs improved tensile strength of the wounds, thereby indicating a significant acceleration of the healing process [32].

Figure 4. Synthetic nanoparticles are able to conjugate peptides, growth factors, nitric oxide or other molecules onto the particle surface and act as delivery vehicles.



4. Nanotechnological Advances in Cartilage Repair

Cartilage injuries lead to joint pain and loss of function. Mature hyaline cartilage has a very low self-repair potential due to its intrinsic properties. For this reason, researchers have focused in the search of methods to reproduce the tissue characteristics of hyaline cartilage and induce complete cartilage repair. A new approach for the treatment of articular cartilage defects is the use of biocompatible scaffolds [33]. There are plenty of polymers, but only some of them are suitable for cartilage tissue engineering. Natural materials used in the field of cartilage engineering include alginate, agarose, chitosan, chondroitin sulfate, hyaluronan, collagen, fibrin gelatine and silk fibroin. It has been demonstrated that these natural material could potentiate the production of collagen type II and sulfated glycosaminoglycans by both chondrocytes and stem cells [34]. Nevertheless, despite their biocompatibility, their potential for clinical use is limited by poor mechanical strength, immunogenicity and their rapid degradation upon implantation if not are cross-linked with appropriate chemical reagents [35]. Instead, synthetic materials present more easy molding characteristics, relatively easy production and the ability to control dissolution and degradation [36]. The synthetic materials most widely used are poly (a-hydroxy acids), especially poly (lactic acid) (PLA), poly (glycolic acid) (PGA) and their co-polymers (PLGA), poly (ε-caprolactone) (PCL), poly(propylene fumarate) and polyethylene glycol (PEG) [37,38].

ECM of cartilage tissue is comprised of collagen and proteoglycans which are nanometers in scale. Thus scaffolds for cartilage tissue engineering have to be accomplished on the nanoscale to achieve similar mechanical and physical properties to native tissue. To better recapitulate the ECM environment for cartilage tissue engineering, researchers have introduced several biological signals, including chondroitin sulfate (CS), hyaluronic acid and collagen, into tissue-engineered scaffolds. Recently, nanofibrous scaffolds composed of poly (vinyl alcohol) (PVA), a hydrophilic synthetic biodegradable polymer, and chondroitin sulfate, have been shown to enhance tissue formation *in vitro* and also *in vivo*, when these were implanted into rat osteochondral defects [39]. In addition, combination of PVA-PCL electrospun nanofiber scaffolds with MSCs showed improvement on tissue healing compared with those which received cell-free scaffolds, suggesting their potential as a suitable graft for articular cartilage reconstruction [40].

Another strategy for cartilage tissue engineering is to induce chondrogenic differentiation of adult stem cells by the delivery of growth factors included in nanoparticles that are embedded into the scaffolds. For example the continuous and controlled release of TGF-β1 from a heparin-functionalized NP within a fibrin/PLCL scaffold was proved to enhance and maintain chondrogenic differentiation of implanted cells [41]. In addition, biodegradable PLGA NPs have been used as gene delivery vehicles to induce chondrogenesis in hMSCs [42,43]. Recently, Jeon *et al.* pretreated PLGA NPs with PEI to modify the particle surface and conjugate SOX9 and a small interfering RNA of the Cbfa-1 gene expressed during osteogenesis [44]. *In vivo* results of injected MSCs that were encapsulated in fibrin hydrogels and transfected with PEI/SOX9 plus a Cbfa-1 siRNA showed a markedly increased expression of genes associated with chondrogenesis, whereas genes and proteins associated with osteoblasts did not show the same expression increment [44]. Others studies also demonstrate that combination of hMSCs, encapsulated in fibrin hydrogel, and NPs containing a specific growth factor represent a suitable niche for the differentiation of transplanted hMSCs [45,46]. Furthermore, scaffolds

can be chemically modified to contain bioactive molecules such as peptides or heparin for creating a better microenvironment of cell adhesion and growth for use in cell therapy applications [41,47–49].

Surface modifications allow introducing nanofeatures into the scaffolds. For example, surface modifications with nano-hydroxyapatite (NHA) have been demonstrated to facilitate and promote cartilage regeneration. Actually, a PLGA/NHA scaffold seeded with MSCs was evaluated in a rat model. After implantation, articular osteochondral defects were filled up with smooth and hyaline-like cartilage with abundant glycosaminoglycan and collagen type II deposition, but deficient in collagen type I [50].

In addition, for the repair of osteochondral units, several authors have highlighted the need for biphasic scaffolds to reproduce the osteocartilaginous anatomical structure. In fact, a bilayer porous PLGA calcium-sulfate biopolymer (TruFit) is largely commercialized for clinical application. Although preclinical experimentation is promising [32], information on the long-term durability is still not available.

The safety and performance of the newly developed type I collagen hydroxyapatite nanostructured biomimetic osteochondral scaffold have been tested in a pilot clinical study. Clinical evaluation by magnetic resonance imaging showed that this scaffold promoted bone and cartilage tissue restoration [51].

Even though later advances in scaffold design for cartilage repair are relevant, improvement of mechanical strength, cell adherence, viability and metabolism of the neocartilage constructs are still necessary for their translation into clinical use.

5. Applying Nanotechnology to Bone Reconstruction

Trauma, pathological degeneration or congenital deformities make bone one of the most commonly transplanted tissues worldwide [52–54]. Autologous bone grafting and bone allografts are the usual treatment for reconstruction of skeletal defects. However, open surgery involves a considerable risk of morbidity and implant failure in patient population. As a result of these limitations, the engineering of new bone to replace the damaged bone based on synthetic biomaterials such as metals, polymers, porous ceramics, hydroxyapatite, collagen sponges or hydrogels, among others, have been developed in the past few years [55].

Despite substantial progress, the construction of structures able to provide the suitable physical and biological properties of the bone still presents challenges. Bone is comprised of hierarchically arranged collagen fibrils, hydroxyapatite and proteoglycans [56]. To mimic the natural bone nanocomposite architecture, novel biomaterials and nanofabrication techniques are currently being employed and many different nanostructures have already been designed and tested. Electrospinning has been extensively applied to create bone nanofiber scaffolds and biomaterials typically used for this purpose, including synthetic organic polymers such as PCL [57], PLGA, PLLA and natural polymers, such as chitosan [58] and silk fibroin [59]. The combination of synthetic and natural materials has also been studied, in fact, electrospun poly(L-lactic acid)/collagen nanofibrous scaffold have been shown to significantly induce osteogenic differentiation of human MSCs and the formation of bone minerals [60]. Recently, bioactive macromolecules like poly-benzyl-L-glutamate and nanohydroxyapatite have been introduced on the surface of polymeric nanofibers, and were proven to

regulate and improve specific biological functions like adhesion, proliferation and differentiation of adipose-derived stem cells [61].

Among the materials used for bone-reconstruction, PLLA is a biocompatible polymer with the advantage of being highly biodegradable. For this reason, PLLA have received the approval of the Food and Drug Administration (FDA) to be use in bone reconstructive surgery [62]. PLLA nanofibers are often functionalized to improve their biological performance with peptides such as RGD (Arg-Gly-Asp); with osteogenic molecules such as hydroxyapatite; or with proteins such as collagen and the growth factor bone morphogenic protein 2 (BMP-2) [63]. In fact, direct incorporation of BMP-2 into PLLA nanofibers enhances the osteoinductivity of the scaffolds. It has been shown that PLLA nanofibers facilitate colonization of bone defects and in combination with BMP-2 increase bone generation, PLLA/BMP-2 implants being able to close critically sized calvarial defects within eight weeks [62].

Current orthopedic implants fail in an appropriate osteo-integration limiting implant lifespan. Recent studies are focused in altering the surface topography of materials at nanoscale level to secure integration with the surrounding tissue and to avoid extrusion and movement. Indeed, nanotopography has been shown to influence the type, quantity and conformation of adsorbed protein, and control cellular adhesion to the surface [64]. Titanium, as a biocompatible material, has been used to enhance implant incorporation in bone for dental, craniofacial, and orthopedic applications. Studies have demonstrated that nanoporous titanium dioxide (TiO_2) surface modification alters nanoscale topography improving soft tissue attachment on titanium implants surface [65,66]. For example, the uses of nanoporous TiO_2 surface-modified implants, in a human dental clinical study, showed that TiO_2 thin film increased adherence in early healing of the human oral mucosa and reduced marginal bone resorption [67].

Nanostructured implant surfaces are also known to enhance osteoblast activity. Using a hydrothermal technique, a simple one-step wet chemical method, non-periodic nanostructures have been developed to surface modify metallic titanium implants. Among the nanomorphologies tested, the nanoleafy pattern showed the strongest influence on protein adsorption, *in vitro* osteoblast cell proliferation and differentiation. *In vivo*, these nanostructures have also demonstrated a higher percentage of bone contact without producing any inflammatory response. These results point to the importance of specific nanomorphologies in controlling tissue integration [68].

In another study, the effect on osteoblast differentiation of TiO_2 nanofiber meshes, fabricated using an electrospinning method to create different surface micro-roughness and nanofiber diameters, was evaluated. Osteoblast differentiation and local factor production were regulated by both roughness surface and the nanotopography, indicating that scaffold structural characteristics alone can be used to drive cell differentiation and create an osteogenic environment without the use of exogenous factors [69].

Nanotube structures have also been shown to have great potential for bone regeneration; their shape is very similar to that of the nanofibers with the only difference that the nanotubes are hollow. Bioactive helical rosette nanotubes are self-assembled nanomaterials, formed in water from synthetic DNA base analogs that mimic the helical nanostructure of collagen in bone. This technology has been used to create a biomimetic nanocomposite combined with nanocrystalline hydroxyapatite, and biocompatible hydrogels which increased osteoblast adhesion [70]. Rosette nanotubes have also been

incorporated into various natural and synthetic biomaterial scaffolds. For example, combination of hydrogels, specifically poly(2-hydroxyethyl methacrylate) (pHEMA) and hydroxyapatite (HA) NP, have been demonstrated to improve long-term functions of osteoblasts by increasing the collagen synthesis, alkaline phosphatase activity, and calcium deposition [71].

Carbon nanotubes (CNTs) are other suitable scaffold materials that have proved to support osteoblast proliferation. Indeed, CNTs possess exceptional mechanical, thermal, and electrical properties, facilitating their use as reinforcements or, in combination with other biomaterials, to improve and to support bone growth [72,73]. In fact, Li *et al.* have showed that CNTs could induce ectopic bone formation in the dorsal musculature of mice, suggesting that these nanotubes might not only improve cell attachment and proliferation but also differentiate the inducible cells derived from soft tissues to osteogenic cells [65].

Clinical therapies implying the use of nanotechnology in bone regeneration are still in the beginning stages. Considering that hydroxyapatite is one of the major components in the bone matrix, synthetic nanocrystalline HA has been used to construct scaffold for bone substitutes. Recently, the bone healing ability of a nanocomposite (DBSint®), approved for clinical use, constituted by biomimetic nanostructured Mg-hydroxyapatite and human demineralized bone matrix has been investigated. The clinical-radiographic and histomorphometry study in subjects undergoing high tibial osteotomy, demonstrated that these nanocomposites are safe and effective. However, non-reabsorbed graft remnants surrounded by soft tissues were observed, thus the benefits of this approach in a long-term outcome are still unclear and require further investigation [74]. Schwarz *et al.* undertook a four-year study of patients treated of moderate intrabony peri-implantitis defects using either a nanocrystalline hydroxyapatite or a natural bone mineral (BioOsss spongiosa granules) in combination with a collagen membrane (BioGides) and found bone reconstruction [75].

6. Nanotechnology for Nerve Regeneration

Incomplete recovery from peripheral nerve injuries can produce a diversity of negative outcomes, including numbness, impairment of sensory or motor function, the possibility of developing chronic pain, and devastating permanent disability. The gold standard treatment is surgery, which requires an autologous nerve graft. Nevertheless, many complications are related to this technique including the sacrifice of the donor nerve function, limited availability of donor tissue, and formation of potentially painful neuromas. Consequently, it is needed to develop new strategies to create nerve artificial prosthesis to solve nerve donor-associated complications.

One of the biggest challenges in peripheral nerve tissue engineering is to create an artificial nerve graft that could mimic the ECM and assist in nerve regeneration. Bio-composite nanofibrous scaffolds made from synthetic and natural polymeric blends provide suitable substrate for tissue engineering and it can be used as nerve guides eliminating the need for autologous nerve grafts. Nanotopography or orientation of the fibers within the scaffolds greatly influences the nerve cell morphology and outgrowth, and the alignment of the fibers ensures better contact guidance of the cells. A bioartificial nerve conduit must meet the overall requirements of a suitable bio-scaffold; it must therefore be biodegradable, biocompatible and non-immunogenic. Furthermore, nerve conduits should also be

engineered to achieve specific characteristics such as to possess an adequate tensile strength without compromising flexibility [76].

It is essential to select the appropriate material in order to reproduce the specific characteristic of the native nerve. In this respect, numerous materials, synthetic and natural, have been tested for manufacturing nerve conduits.

Aliphatic polyesters are biocompatible polymers that can be synthesized into fibers via electrospinning [76]. Actually, several PGA and PCL nerve guidance conduits have been approved by the FDA for clinical use in peripheral nerve repair [77]. A successful example is Neurotube®, an absorbable woven PGA mesh tube designed for peripheral nerve repair or reconstruction (Synovis Micro Companies Alliance, Birmingham, AL). The efficacy of this scaffold has been demonstrated by a large multicenter clinical trial, reporting very encouraging results for digital nerve reconstruction [78]. Further clinical reports investigating the Neurotube® conduit in motor reconstruction of the spinal accessory nerve, several facial nerves and forearm median nerves showed promising clinical outcomes. Nevertheless, although Neurotube® is the preferred synthetic nerve conduit among surgeons, there are still some issues that decrease their efficiency, such as the high rate of degradation that reduces its mechanical properties, and the formation of acidic degradation deposits.

To solve these limitations and to improve efficacy, more recently, numerous PGA derivative compounds and combinations have begun to emerge. For example, neural stem cells and Schwann cells have been cultured in combined PLGA conduits and neurotrophin-3 (NT-3) to generate NT-3-loaded PLGA carriers *in vitro* [79]. In addition, the incorporation of growth factor-microspheres or VEGF-microspheres to PLGA has proved to increase nerve formation when grafted to a rat model [80]. Another study showed nerve regeneration after two months of PLGA tube, containing rat dental pulp cells embedded into a collagen gel, transplantation [81]. Finally, introduction of autologous MSCs to PLGA scaffolds improved the repair and rehabilitation of a large gap after peripheral nerve injury in dogs [82] and rats [83].

Another biocompatible polymer that has gained considerable interest in nerve regeneration research field is PCL. The main advantages of PCL are that can be easily manipulated with low processing costs. Its high processability is attributed to the fact that PCL is very soluble in a wide range of organic solvents and, moreover, its crystalline nature enables easy formability at relatively low temperatures. Its degradation products are less acidic than PLA and non-toxic, which causes less damage to the surrounding tissue environment and do not trigger an inflammatory response [84]. Neurolac® (Polyganics Inc., the Netherlands) is a PCL nerve conduit that has also been approved by the FDA. A randomized clinical trial, with 30 patients suffering from hand nerve lesions, has been conducted to test the nerve reconstruction capacity of Neurolac®. Patients were randomized for treatment either with autologous nerve grafts or with Neurolac®. Although some complications were reported for the Neurolac®-treated group, none were directly related to the use of the PLC device. Interestingly, the recovery of sensibility between groups was comparable [85], however Neurolac® showed some limitation, principally, its high rigidity [83].

Another strategy for nerve regeneration is the combination of PCL devices with cells or natural materials. For instance, MSCs grown in the nerve scaffold has been proved to improve nerve regeneration after a nerve transection in mice [86]. On the other hand, collagen/PCL fibrous scaffold

successfully supported nerve regeneration through an 8-mm sciatic nerve gap in adult rats, achieving similar electrophysiological and muscle reinnervation results as autografts [87].

Peptide amphiphiles (PAs) are peptides with the ability to form spontaneous self-assembly nanofibers and a dual functionality of simultaneously being hydrophobic and hydrophilic. This balance of polarity between attractive and repulsive forces within the nanomolecular construct further alludes to their novel properties [76]. In fact, the nanofiber self-assembly framework of PAs has proved to promote the migration and proliferation of neural cells [88]. PAs can also function as efficient drug and gene delivery platforms. Actually, various therapeutic agents can be incorporated into PAs to augment the recovery process and minimize immune response. Some examples of PAs, with potential to be a candidate of nerve conduits, is IKVAV, a pentapeptide, made up of a sequence of amino acids Ile-Lys-Val-Ala-Val, first identified in the A chain of laminin. IKVAV is a neurite-promoting laminin epitope, and it has been demonstrated to upregulate the proliferation of neural cells [76]. Another is RGD (Arg-Gly-Asp) a tripeptide able to mediate peripheral neuron regeneration, in fact integration of RGD into PAs promoted cell proliferation and differentiation [89]. A plethora of peptide sequences can also be incorporated into PAs, making these nanofibers extremely versatile and customizable [76].

Natural materials offer increased levels of biocompatibility, decreased toxicity and enhanced migration of support cells when compared with synthetic materials. Collagen has widespread use as a biological material including peripheral nerve repair, because, when purified, it is weakly antigenic. Diffusion processes through collagen matrices are facilitated by its smooth microgeometry and transmural permeability. In addition, the adhesive property of collagen for different cell types also permits enhanced survival and proliferation [84]. NeuraGen® (Integra Life Sciences Corporation, Plainsboro, NJ, USA) was the first semi-permeable Type I collagen nerve guidance conduit to receive approval from the FDA. The clinical experience using NeuraGen® was reported by Taras *et al.* in a medical study on peripheral nerve reconstruction [90]. Patients tolerated splinting and resisted exercise without negative clinical consequences. In another study, NeuraGen® has been compared with direct suture repair, in patients with complete traumatic nerve injuries. Results showed that patients who received NeuraGen® had lower post-operative pain than those treated with direct suture repair. The overall study conclusion was that entubulation nerve repair using the NeuraGen® is as effective method of joining severed nerves as direct microsurgical suture for short gap graft repair (data presented in the American Society for Peripheral Nerve) (revised in Kehoe *et al.*) [84]. To improve NeuraGen's® limitations, such as the long duration of its biodegradation, other collage Type I derivate structures have been designed;however, no conclusive clinical data have yet been documented [84].

Recently, composite materials based on the coupling of conductive organic polymers and carbon nanotubes have shown to possess properties of the individual components and the benefit of a synergistic effect. For instance, multi-wall carbon nanotube (MWCNT)/polymer composites are hybrid materials that combine numerous mechanical, electrical and chemical properties and can be used for the development of nerve guidance channels to promote nerve regeneration. This biomaterial is a suitable substrate that increases electronic interfacing between neurons and can be employed to create micro-machined electrodes with potential applications in neural regeneration, prosthetic devices and brain implants [91].

The use of micro-electromechanical systems stimulation, through modulation of ions around the nerve, is a novel nanotechnology strategy for modulating nerve impulse activation. These findings

have potentially significant implications for the design of special nano-enhanced materials that could be used to promote nerve regeneration and rehabilitation [92].

Bridging larger nerve gaps between proximal and distal ends requires exogenous tubular constructs with uniaxially aligned topographical cues to promote the axonal regrowth. In this respect, electrospun nanofibrous scaffolds are a good candidate to fill up the gap of the injured nerve. Recently, it has been demonstrated that the alignment of nanofibers has a significant influence on the adhesion and proliferation of Schwann cells. The axially aligned nanofibers were shown to mimic the fibrin cable architecture and, thereby, this approach may represent an ideal scaffold for extending the growth of axonal processes [93].

Additionally, flexible nerve agent sensors, based on hydroxylated poly(3,4-ethylenedioxythiophene) nanotubes with surface substructures such as nanonodules and nanorods have been explored. The surface substructures can be grown on a nanofiber surface by controlling critical synthetic conditions during vapor deposition polymerization on the polymer nanotemplate, leading to the formation of multidimensional conducting polymer nanostructures where hydroxyl groups are found to interact with the nerve agents. Representatively, the sensing response of dimethyl methylphosphonate, as a simulant for sarin, is highly sensitive and reversible from the aligned nanotubes and the sensor has excellent mechanical bendability and durability [94].

7. Nanotecnology for Cardiac Tissue Regeneration

Heart stroke and valvular heart disease are a significant cause of morbidity and mortality worldwide. Myocardial infarction results in reduced cardiac function due to cardiomyocyte death. As the proliferative potential of the terminally differentiated cardiomyocytes is low, the heart is unable to repair itself and, after damage, non-functional scar tissue is formed. On the other hand, damage or defect in one of the four heart valves can ultimately lead to heart failure. Classical replacement surgery involves the implantation of mechanical valves or biological valves (xeno- or homografts).

Engineering the heart represents a real challenge for a new branch of multidisciplinary researchers whose goal is regenerating the damaged cardiac tissue. Certainly, it is not a simple matter of patching the damaged tissue. The elasticity and contractive properties of this perfect pump have to be guaranteed in order to avoid complications, such as arrhythmias or dysfunction, which could prevent the correct impulsion of blood to the entire body [95,96].

Cell injection directly into the heart has proven to revolutionize the treatment of heart disease [97,98]. Some clinical trials have been conducted injecting autologous stem cells derived from bone marrow, and some benefits such as improvement in left ventricular ejection fraction and concomitant increase in myocardial perfusion have been proven [99–101]. Moreover, enhancement in myocardial oxygen consumption [102] and in the contractility properties of the scarred area has been demonstrated [103]. A recent meta-analysis performed to evaluate the effectiveness of adult bone marrow-derived stem cell injection to treat acute myocardial infarction concluded that a moderate and significant improvement in global heart function was achieved after the stem cell therapy [104]. However, although beneficial, the effects of stem/progenitor cell administration on cardiac function in the clinical setting have not quite fulfilled expectations [102].

Considering the drawbacks of cell implantation and the fact that many cardiovascular diseases can lead to heart damage from the necessarily replaced functional structures of the heart—such as valves, or even the whole heart—there is a great need for approaches that create cardiac tissue via bioengineering. Advances in nanotechnology have allowed researchers to fabricate scaffolds with the aim to mimic the natural cell environment with the physical properties that influence the physiological behavior of the tissue. Thus far, contractile cardiac grafts have been created *in vitro* and are postulated as a system for replacing infarcted myocardium and to enhance cardiac function. Furthermore, tissue engineering of heart valves or injection of nanomaterials to improve the function of faulty heart valves, are newly emerging alternatives that improve current modes of therapy in valvular heart surgery.

The use of a degradable, nanofibrous scaffold made by electrostatic fiber spinning have been postulated to be a feasible method to produce cardiac grafts with clinically relevant dimensions [105]. A variety of biomaterials have been tested, alone or in combinations, to fulfill the requirements of myocardial regeneration. The priority is to find polymers with specific elastic and ductile mechanical properties that can, for example, mimic the necessary anisotropy of cardiac tissue with the combination of PCL and gelatin, and be properly oriented, yield excellent results and improve adhesion and alignment of cardiomyocytes in the nanofibrous mesh [106]. A very interesting study shows that nanofibrous scaffolds made of L-lactic acid with trimethylene carbonate (P(L)LA-*co*-TMC) promote cardiomyocyte proliferation and efficiently preserve cell morphology, without hampering expression of sarcomeric alpha actinin marker, thus demonstrating its potential as a synthetic biomaterial for myocardial tissue engineering [107].

Furthermore, a recent study revealed the proper ratio of poly(1,8-octanediol-*co*-citrate) (POC) and poly(L-lactic acid)-*co*-poly-(3-caprolactone) (PLCL) to obtain a nanoscaffold with mechanical properties, such as elasticity and tensile strength, similar to the cardiac tissue [96,108].

Going even further, researchers have developed a biocompatible scaffold that not only has good physical, chemical and mechanical properties, but also present the ability to differentiate cells to cardiomyocytes. In this respect, Gupta *et al.* have created a scaffold combining PEG-PCL-CPCL with an inhibitor of bone morphogenetic protein (BMP) which promotes differentiation of ESCs toward functional cardiomyocytes [109]. In addition, Sreerekha *et al.* showed that a scaffold that combines fibrin and PLGA was able to stimulate cardiomyocyte MSC differentiation [110]. Other studies have used a chitosan nanoscaffold coated with fibronectin and proposed a cardiomyocyte-fibroblast co-culture system that resulted in a cardiac tissue-like structure, where cardiomyocytes maintained their morphology and polarity and contracted synchronously [111]. Recently, cardiac patches of PEG nanoscaffold, embedded in a fibrin hydrogel together with cardiac progenitor cells, were implanted in the ventricle wall of a rat infarction model. The engraftment improves the infarcted area, increasing cell viability and ECM collagen organization [112].

Another nanotechnological variant consists in the use of NPs, which present important advantages for a targeted therapy. For instance NPs can easily cross the endothelium, and can be administrated by a non-invasive procedure, intravenously or by inhalation [113,114]. Numerous *in vivo* studies have illustrated the advantages of the use of NPs as complementary therapy in cardiovascular diseases. For example, phosphatidylserine liposomes administered intravenously to a myocardial infarction mouse model, proved to be capable of promoting angiogenesis, remodeling and to prevent ventricular dilatation [115]. In addition, NPs have been employed to allow factor and/or cytokine administration.

Anti-P-selectin-conjugated liposomes containing VEGF, improved cardiac function and vascular structure in mice after myocardial infarction [116].

Actually, the two main nanotechnological strategies for heart valve disease treatment are: (i) the use of tissue engineering to produce fully functional heart valve; or (ii) the employment of NPs to alter the physical structure and behavior of faulty valves.

Biological valves are used for valve replacement in surgical therapy for end-stage valvular diseases. But biological prostheses lead to complications such as limited lifespan of the implant due to deterioration and calcification of the valve structure, together with problems related to immunological response [117]. Nano-engineered heart valves are a promising approach to overcome the limitations of conventional heart valve prostheses. In fact, these valves have been tested in animal models showing excellent tissue remodeling. For instance, fibrin scaffold in combination with arterial-derived cells were inserted in a sheep model and, after three months, the fibrin scaffold was replaced by new tissue containing mature collagen along with functional blood vessels [118]. Another successful example of a nano-engineered heart valve has been shown by Kalfa *et al.*, who used polydioxanone (PDO) and the electrospun technique to construct bioabsorbable valved patches that supported MSCs growth [119]. After eight months of implantation in the heart of growing lambs, the PDO scaffold was completely degraded and replaced by a viable, three-layered, endothelialized tissue.

One important aspect that has much to do with the efficiency of artificial valves is the procedure employed by culture cells on the scaffolds. In a very recent study, Aleksieva *et al.* have evaluated which culture conditions are optimal for seeding cells onto polyurethane heart valve scaffolds [120]. They compared static cultivation with dynamic cultivation using a conditioning bioreactor. Bioreactors are devices in which biological and/or biochemical processes are manipulated through close control of environmental and process-bound factors such as pH, temperature, pressure, and nutrient and waste flow [121]. After growing endothelial cells and fibroblasts onto the valve scaffolds they evaluated cell confluence, ECM formation and inflammatory response. The study concluded that the use of the bioreactor improved cell attachment to the polyurethane structure and the mechanical properties of the valve scaffold.

In these examples, cell growth is supported *in vitro* and later cell-containing scaffolds are grafted to the animal model. *In situ* tissue engineering represents a new approach in which nude scaffolds are implanted and signaling component presented in the functionalized scaffold guide cell homing, adhesion and growth. The ultimate goal is to achieve complete cellularization of the graft, the production of a new matrix and, finally, tissue formation. Although *in situ* tissue engineering for heart valve reconstruction is an attractive alternative, more studies are needed to elucidate the *in vivo* feasibility of the approach [122].

The second main approach based on nanotechnology directed to improve heart valve diseases conditions is the use of NPs. Atherosclerosis processes that involve heart valve degeneration are the target of functionalized NPs that can be used as a vehicle for drug delivery [123–126]. In addition, NPs can be used to reduce the risk of thromboembolic events after heart valve surgery [127]. For instance, PLA matrix systems incorporated with PLGA NPs containing nitric oxide donors have been developed for prevention of heart valve complications through sustained and controlled release of nitric oxide [128].

8. Clinical Trials

To evaluate the efficacy and safety of nanotechnological devices for application in regenerative therapy it is necessary to perform clinical trials with a large cohort of patients. A clinical trial is conducted through five consecutive phases defined by the FDA [129]. Briefly: (i) Phase 0: Exploratory study involving very limited human exposure to the drug, with no therapeutic or diagnostic goals; (ii) Phase 1: Studies that are usually conducted with healthy volunteers and that emphasize safety; (iii) Phase 2: Studies that gather preliminary data on effectiveness. Safety continues to be evaluated, and short-term adverse events are studied; (iv) Phase 3: Studies that gather more information about safety and effectiveness by studying different populations and different dosages and by using the drug/medical device in combination with other drug/medical device; and, (v) Phase 4: Studies occurring after FDA has approved a drug/medical device for marketing. These studies gather additional information about safety, efficacy, or optimal use.

Here, ClinicalTrials Website [130] was used to look for nanotechnological applications that are currently being evaluated by a clinical trial. The clinical trials we report here have been the result of a search using the terms: nanotechnology, scaffold, devices in conjunction with skin, cartilage, bone, nerve and heart. Table 1 summarized some of the most significant outcomes of our search.

Related to skin tissue, a clinical trial that evaluates the use of *silk sericin scaffold* to wound dressing is being carried out (NCT01539980). Another advanced wound care device that is being clinically tested is *Integra(TM) Flowable Wound Matrix*. Based on collagen technology, this invention provides a scaffold for cellular invasion and capillary growth (NCT01108263). In addition, a multilayer transdermal patch designed by electrospinning, which produces a continuous and stable nitric oxide release, is currently evaluated. The study tries to determine the efficacy of this novel nitric oxide topical donor for the treatment of cutaneous leishmaniasis (NCT00317629). Other clinical trials are evaluating the use of silver NP-coated charcoal dressings with the aim of preventing infection and enhancing recovery in patients with skin burns (NCT01598493, NCT01598480).

In the field of cartilage regeneration, we found a clinical trial that evaluates the regenerative potential of a sponge-like scaffold *BioCart™II* seeded with chondrocytes, to treat cartilage defects of the femoral condyle (NCT00729716). Aa cartilage-repair device called *Kensey Nash Corp* is also being evaluated for clinical use. This device is implanted in the bone below the area of damaged cartilage, the aim of the inventors is that the grafted scaffold will absorb blood and bone marrow from the bone and produce the healing of the damaged cartilage area (NCT01183637).

Interestingly, for bone reconstruction therapy, there is a study that aims to pre-engineer large synthetic bone grafts, to seed then with fat-derived progenitor cells differentiated into osteoblasts and to study the vascularization process *in vivo* (NCT01218945). Another study is a prospective trial to test the safety and feasibility of injectable scaffold (*Ignite®)* in combination with progenitor cells (NCT01435434).

An ambitious clinical study that compares different nerve conduit devices for peripheral nerve gap repairs is actually in theirs beginnings. The hollow tube nerve conduits that are going to be evaluated in this trial are Neurotube, NeuroLac, NeuraGen, NeuroMatrix and NeuroFlex (NCT01573650). In addition, we have found a clinical trial that will evaluate nerve repair using a fibrin conduit device to treat patients with traumatic peripheral nerve injury of the finger (NCT01573650).

Table 1. Clinical trials on the use of nanotechnological devices for tissue regeneration.

ClinicalTrials.gov Identifier	Clinical trial name	Nanotechnology	Tissue	Status/Phase
NCT01539980	Clinical Study on Silk Sericin Wound Dressing for Split-thickness Skin Graft Donor Sites Treatment	Device: Sericin scaffold	Skin	Phase 1 Phase 2
NCT01108263	Use of INTEGRA™ Flowable Wound Matrix to Manage Diabetic Foot Ulcers	INTEGRA™ Flowable Matrix (Collagen)	Skin	Phase 4
NCT00317629	Controlled Nitric Oxide-Releasing Patch *Versus* Meglumine Antimoniate in the Treatment of Cutaneous Leishmaniasis	Electrospinning-controlled nitric oxide releasing patch	Skin	Phase 3
NCT00729716	Comparison of BioCart™II With Microfracture for Treatment of Cartilage Defects of the Femoral Condyle	BioCart™II scaffold	Cartilage	Phase 2
NCT01183637	Evaluation of an Acellular Osteochondral Graft for Cartilage Lesions Pilot Trial (EAGLE Pilot)	bioresorbable scaffold	Bone/ Cartilage	Phase 2
NCT01218945	Development of Bone Grafts Using Adipose-Derived Stem Cells and Different Scaffolds	Bone scaffold	Bone	recruiting participants
NCT01435434	Mononucleotide Autologous Stem Cells and Demineralized Bone Matrix in the Treatment of Non-Union/Delayed Fractures	Ignite®ICS injectable scaffold	Bone	Not yet recruiting
NCT00948025	A Comparative Post-Marketing Study of Commercially Available Peripheral Nerve Gap Repair Options (CHANGE)	Device: Hollow tube nerve conduit, synthetic or biosynthetic	Nerve	Active, not recruiting Phase 4
NCT01573650	Optimization of Peripheral Nerve Reconstruction: A Non-Inferiority Trial	Device: Fibrin Conduit	Nerve	not yet open for participant recruitment
NCT01270139	Plasmonic Photothermal Therapy of Flow-Limiting Atherosclerotic Lesions With Silica–Gold Nanoparticles: a First-in-Man Study	Silica–gold nanoparticles. Iron bearing nanoparticles.	Heart	Has results Phase 1–2
NCT00124943	A Phase I/II Safety Trial of Intracoronary Administration of Systemic Nanoparticle Paclitaxel (ABI-007) for the Prevention of In-Stent Restenosis	Nanoparticle paclitaxel	Heart	Has results Phase 1–2

In the field of cardiac tissue regeneration we have not find any data of clinical trials conducted to test heart scaffold devices or synthetic heart valve substitutes. We reported here two clinical trials that evaluate the use of nanoparticules to prevent or treat heart disease-related complications. The first one studies the possible beneficial implications of *silica-gold NPs* in the prevention of atherosclerosis and treatment of its complications (NCT01270139). The second clinical trial evaluates *NP paclitaxel* preliminary efficacy for the prevention of in-stent restenosis (NCT00124943).

9. Conclusions

The field of nanotechnology is advancing quickly. This interdisciplinary approach is leading to a rapid expansion and development in the fabrication of biomimetic scaffolds for tissue engineering. Many studies have been conducted in the search for appropriate materials to create a scaffold that may play an active role in the regeneration process instead of simply being a cell carrier or tissue template. The advantages of nanomaterials as therapeutic and diagnostic tools are vast, due to design flexibility, small sizes, large surface-to-volume ratio, and ease of surface modification.

The potential of these bio-devices has shown promising results *in vitro*, and some of them have also been successfully tested *in vivo* with animal models. Nevertheless, the gap between laboratory and medical application of these nanotechnological advances is still wide. Although some successful devises have already being tested in clinical trials and the data produced by these studies is highly encouraging, the safety of nanomedicine is not yet fully defined and more clinical studies still need to be conducted to translate nanotechnological devices to the clinic.

Nanotechnologists, cell biologists and medical doctors have begun to walk the path toward a personalized medicine with the hope of improving the treatment of many diseases. The advanced applications of this approach to regenerative medicine will undoubtedly transform the fundamentals of diagnosis, treatment, and prevention of diseases, becoming an inevitable part of our life.

Acknowledgments

This work was supported in part by grants from the Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, excellence project number CTS-6568) and the Instituto de Salud Carlos III (Fondo de Investigación Sanitaria, FEDER funds, grant number PI10/02295).

References

1. de Mel, A.; Seifalian, A.M.; Birchall, M.A. Orchestrating cell/material interactions for tissue engineering of surgical implants. *Macromol. Biosci.* **2012**, *12*, 1010–1021.
2. Peran, M.; Garcia, M.A.; Lopez-Ruiz, E.; Bustamante, M.; Jimenez, G.; Madeddu, R.; Marchal, J.A. Functionalized nanostructures with application in regenerative medicine. *Int. J. Mol. Sci.* **2012**, *13*, 3847–3886.
3. Badylak, S.F.; Freytes, D.O.; Gilbert, T.W. Extracellular matrix as a biological scaffold material: Structure and function. *Acta Biomater.* **2009**, *5*, 1–13.
4. Kakisis, J.D.; Liapis, C.D.; Breuer, C.; Sumpio, B.E. Artificial blood vessel: the Holy Grail of peripheral vascular surgery. *J. Vasc. Surg.* **2005**, *41*, 349–354.
5. Li, W.J.; Laurencin, C.T.; Caterson, E.J.; Tuan, R.S.; Ko, F.K. Electrospun nanofibrous structure: A novel scaffold for tissue engineering. *J. Biomed. Mater. Res.* **2002**, *60*, 613–621.
6. Liang, D.; Hsiao, B.S.; Chu, B. Functional electrospun nanofibrous scaffolds for biomedical applications. *Adv. Drug Deliv. Rev.* **2007**, *59*, 1392–1412.
7. Sen, C.K.; Gordillo, G.M.; Roy, S.; Kirsner, R.; Lambert, L.; Hunt, T.K.; Gottrup, F.; Gurtner, G.C.; Longaker, M.T. Human skin wounds: a major and snowballing threat to public health and the economy. *Wound Repair Regen.* **2009**, *17*, 763–771.

8. Clark, K.L. Nutritional considerations in joint health. *Clin. Sports Med.* **2007**, *26*, 101–118.
9. Viswanathan, H.N.; Curtis, J.R.; Yu, J.; White, J.; Stolshek, B.S.; Merinar, C.; Balasubramanian, A.; Kallich, J.D.; Adams, J.L.; Wade, S.W. Direct healthcare costs of osteoporosis-related fractures in managed care patients receiving pharmacological osteoporosis therapy. *Appl. Health Econ. Health Policy* **2012**, *10*, 163–173.
10. Jamison, D.T.; Breman, J.G.; Measham, A.R.; Alleyne, G.; Claeson, M.; Evans, D.B.; Jha, P.; Mills, A.; Musgrove, P. *Priorities in Health*; World Bank: Washington, DC, USA, 2006.
11. Heidenreich, P.A.; Trogdon, J.G.; Khavjou, O.A.; Butler, J.; Dracup, K.; Ezekowitz, M.D.; Finkelstein, E.A.; Hong, Y.; Johnston, S.C.; Khera, A.; *et al.* Forecasting the future of cardiovascular disease in the United States: A policy statement from the American Heart Association. *Circulation* **2011**, *123*, 933–944.
12. Ramakrishna, S.; Fujihara, K.; Teo, W.E.; Lim,T.C.; Ma, Z. *An Introduction to Electrospinning and Nanofibers*; World Scientific Publishing Co.: Hackensack, NJ, USA, 2005.
13. Pham, Q.P.; Sharma, U.; Mikos, A.G. Electrospinning of polymeric nanofibers for tissue engineering applications: a review. *Tissue Eng.* **2006**, *12*, 1197–1211.
14. Venugopal, J.; Ramakrishna, S. Applications of polymer nanofibers in biomedicine and biotechnology. *Appl. Biochem. Biotechnol.* **2005**, *125*, 147–158.
15. La Francesca, S. Nanotechnology and stem cell therapy for cardiovascular diseases: Potential applications. *Methodist Debakey Cardiovasc. J.* **2012**, *8*, 28–35.
16. Mohamed, A.; Xing, M.M. Nanomaterials and nanotechnology for skin tissue engineering. *Int. J. Burns Trauma* **2012**, *2*, 29–41.
17. Huang, S.; Fu, X. Naturally derived materials-based cell and drug delivery systems in skin regeneration. *J. Control. Release* **2010**, *142*, 149–159.
18. Stynes, G.; Kiroff, G.K.; Morrison, W.A.; Kirkland, M.A. Tissue compatibility of biomaterials: Benefits and problems of skin biointegration. *ANZ J. Surg.* **2008**, *78*, 654–659.
19. Liu, W.; Cao, Y.L. Application of scaffold materials in tissue reconstruction in immunocompetent mammals: Our experience and future requirements. *Biomaterials* **2007**, *28*, 5078–5086.
20. Tran, P.A.; Zhang, L.; Webster, T.J. Carbon nanofibers and carbon nanotubes in regenerative medicine. *Adv. Drug Deliv. Rev.* **2009**, *61*, 1097–1114.
21. Khil, M.S.; Cha, D.I.; Kim, H.Y.; Kim, I.S.; Bhattarai, N. Electrospun nanofibrous polyurethane membrane as wound dressing. *J. Biomed. Mater. Res. B Appl. Biomater.* **2003**, *67*, 675–679.
22. Wang, C.C.; Su, C.H.; Chen, C.C. Water absorbing and antibacterial properties of N-isopropyl acrylamide grafted and collagen/chitosan immobilized polypropylene nonwoven fabric and its application on wound healing enhancement. *J. Biomed. Mater. Res. A* **2008**, *84*, 1006–1017.
23. van den Bogaerdt, A.J.; van Zuijlen, P.P.; van Galen, M.; Lamme, E.N.; Middelkoop, E. The suitability of cells from different tissues for use in tissue-engineered skin substitutes. *Arch. Dermatol. Res.* **2002**, *294*, 135–142.
24. Ojeh, N.O.; Frame, J.D.; Navsaria, H.A. *In vitro* characterization of an artificial dermal scaffold. *Tissue Eng.* **2001**, *7*, 457–472.

25. Chandrasekaran, A.R.; Venugopal, J.; Sundarrajan, S.; Ramakrishna, S. Fabrication of a nanofibrous scaffold with improved bioactivity for culture of human dermal fibroblasts for skin regeneration. *Biomed. Mater.* **2011**, *6*, 015001:1–015001:10.
26. Venugopal, J.R.; Zhang, Y.; Ramakrishna, S. *In vitro* culture of human dermal fibroblasts on electrospun polycaprolactone collagen nanofibrous membrane. *Artif. Organs* **2006**, *30*, 440–446.
27. Jin, G.; Prabhakaran, M.P.; Ramakrishna, S. Stem cell differentiation to epidermal lineages on electrospun nanofibrous substrates for skin tissue engineering. *Acta Biomater.* **2011**, *7*, 3113–3122.
28. Tchemtchoua, V.T.; Atanasova, G.; Aqil, A.; Filee, P.; Garbacki, N.; Vanhooteghem, O.; Deroanne, C.; Noel, A.; Jerome, C.; Nusgens, B.; *et al.* Development of a chitosan nanofibrillar scaffold for skin repair and regeneration. *Biomacromolecules* **2011**, *12*, 3194–3204.
29. Lenz, G.; Mansson, P. Growth factors as pharmaceuticals. *Pharm. Technol.* **1991**, *15*, 34–38.
30. Yang, Y.; Xia, T.; Chen, F.; Wei, W.; Liu, C.; He, S.; Li, X. Electrospun fibers with plasmid bFGF polyplex loadings promote skin wound healing in diabetic rats. *Mol. Pharm.* **2012**, *9*, 48–58.
31. Tocco, I.; Zavan, B.; Bassetto, F.; Vindigni, V. Nanotechnology-based therapies for skin wound regeneration. *J. Nanomater.* **2012**, doi:10.1155/2012/714134.
32. Ziv-Polat, O.; Topaz, M.; Brosh, T.; Margel, S. Enhancement of incisional wound healing by thrombin conjugated iron oxide nanoparticles. *Biomaterials* **2010**, *31*, 741–747.
33. Lopez-Ruiz, E.; Peran, M.; Cobo-Molinos, J.; Jimenez, G.; Picon, M.; Bustamante, M.; Arrebola, F.; Hernandez-Lamas, M.C.; Delgado-Martinez, A.D.; Montanez, E.; Marchal, J.A. Chondrocytes extract from patients with osteoarthritis induces chondrogenesis in infrapatellar fat pad-derived stem cells. *Osteoarthr. Cartil.* **2013**, *21*, 246–258.
34. Vinatier, C.; Bouffi, C.; Merceron, C.; Gordeladze, J.; Brondello, J.M.; Jorgensen, C.; Weiss, P.; Guicheux, J.; Noel, D. Cartilage tissue engineering: Towards a biomaterial-assisted mesenchymal stem cell therapy. *Curr. Stem Cell Res. Ther.* **2009**, *4*, 318–329.
35. Lee, S.H.; Shin, H. Matrices and scaffolds for delivery of bioactive molecules in bone and cartilage tissue engineering. *Adv. Drug Deliv. Rev.* **2007**, *59*, 339–359.
36. Capito, R.M.; Spector, M. Scaffold-based articular cartilage repair. *IEEE Eng. Med. Biol. Mag.* **2003**, *22*, 42–50.
37. Alves da Silva, M.L.; Martins, A.; Costa-Pinto, A.R.; Costa, P.; Faria, S.; Gomes, M.; Reis, R.L.; Neves, N.M. Cartilage tissue engineering using electrospun PCL nanofiber meshes and MSCs. *Biomacromolecules* **2010**, *11*, 3228–3236.
38. Dahl, J.P.; Caballero, M.; Pappa, A.K.; Madan, G.; Shockley, W.W.; van Aalst, J.A. Analysis of human auricular cartilage to guide tissue-engineered nanofiber-based chondrogenesis: Implications for microtia reconstruction. *Otolaryngol. Head Neck Surg.* **2011**, *145*, 915–923.
39. Coburn, J.M.; Gibson, M.; Monagle, S.; Patterson, Z.; Elisseeff, J.H. Bioinspired nanofibers support chondrogenesis for articular cartilage repair. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 10012–10017.
40. Shafiee, A.; Soleimani, M.; Chamheidari, G.A.; Seyedjafari, E.; Dodel, M.; Atashi, A.; Gheisari, Y. Electrospun nanofiber-based regeneration of cartilage enhanced by mesenchymal stem cells. *J. Biomed. Mater. Res. A* **2011**, *99*, 467–478.

41. Jung, Y.; Chung, Y.I.; Kim, S.H.; Tae, G.; Kim, Y.H.; Rhie, J.W. *In situ* chondrogenic differentiation of human adipose tissue-derived stem cells in a TGF-beta1 loaded fibrin-poly(lactide-caprolactone) nanoparticulate complex. *Biomaterials* **2009**, *30*, 4657–4664.
42. Park, J.S.; Yang, H.N.; Woo, D.G.; Jeon, S.Y.; Do, H.J.; Lim, H.Y.; Kim, J.H.; Park, K.H. Chondrogenesis of human mesenchymal stem cells mediated by the combination of SOX trio SOX5, 6, and 9 genes complexed with PEI-modified PLGA nanoparticles. *Biomaterials* **2011**, *32*, 3679–3688.
43. Kim, J.H.; Park, J.S.; Yang, H.N.; Woo, D.G.; Jeon, S.Y.; Do, H.J.; Lim, H.Y.; Kim, J.M.; Park, K.H. The use of biodegradable PLGA nanoparticles to mediate SOX9 gene delivery in human mesenchymal stem cells (hMSCs) and induce chondrogenesis. *Biomaterials* **2011**, *32*, 268–278.
44. Jeon, S.Y.; Park, J.S.; Yang, H.N.; Woo, D.G.; Park, K.H. Co-delivery of SOX9 genes and anti-Cbfa-1 siRNA coated onto PLGA nanoparticles for chondrogenesis of human MSCs. *Biomaterials* **2012**, *33*, 4413–4423.
45. Park, J.S.; Yang, H.N.; Woo, D.G.; Chung, H.M.; Park, K.H. *In vitro* and *in vivo* chondrogenesis of rabbit bone marrow-derived stromal cells in fibrin matrix mixed with growth factor loaded in nanoparticles. *Tissue Eng. A* **2009**, *15*, 2163–2175.
46. Park, J.S.; Yang, H.N.; Woo, D.G.; Jeon, S.Y.; Park, K.H. Chondrogenesis of human mesenchymal stem cells in fibrin constructs evaluated *in vitro* and in nude mouse and rabbit defects models. *Biomaterials* **2011**, *32*, 1495–1507.
47. Na, K.; Kim, S.; Park, K.; Kim, K.; Woo, D.G.; Kwon, I.C.; Chung, H.M.; Park, K.H. Heparin/poly(L-lysine) nanoparticle-coated polymeric microspheres for stem-cell therapy. *J. Am. Chem Soc.* **2007**, *129*, 5788–5789.
48. Hsu, S.H.; Huang, T.B.; Cheng, S.J.; Weng, S.Y.; Tsai, C.L.; Tseng, C.S.; Chen, D.C.; Liu, T.Y.; Fu, K.Y.; Yen, B.L. Chondrogenesis from human placenta-derived mesenchymal stem cells in three-dimensional scaffolds for cartilage tissue engineering. *Tissue Eng. A* **2011**, *17*, 1549–1560.
49. Liu, L.; Wang, Y.; Guo, S.; Wang, Z.; Wang, W. Porous polycaprolactone/nanohydroxyapatite tissue engineering scaffolds fabricated by combining NaCl and PEG as co-porogens: structure, property, and chondrocyte-scaffold interaction *in vitro*. *J. Biomed. Mater. Res. B Appl. Biomater.* **2012**, *100*, 956–966.
50. Xue, D.; Zheng, Q.; Zong, C.; Li, Q.; Li, H.; Qian, S.; Zhang, B.; Yu, L.; Pan, Z. Osteochondral repair using porous poly(lactide-*co*-glycolide)/nano-hydroxyapatite hybrid scaffolds with undifferentiated mesenchymal stem cells in a rat model. *J. Biomed. Mater. Res. A* **2010**, *94*, 259–270.
51. Kon, E.; Delcogliano, M.; Filardo, G.; Busacca, M.; Di Martino, A.; Marcacci, M. Novel nano-composite multilayered biomaterial for osteochondral regeneration: A pilot clinical trial. *Am. J. Sports Med.* **2011**, *39*, 1180–1190.
52. Arrington, E.D.; Smith, W.J.; Chambers, H.G.; Bucknell, A.L.; Davino, N.A. Complications of iliac crest bone graft harvesting. *Clin. Orthop. Relat. Res.* **1996**, 300–309.
53. Giannoudis, P.V.; Dinopoulos, H.; Tsiridis, E. Bone substitutes: An update. *Injury* **2005**, *36*, S20–S27.

54. Miyazaki, M.; Tsumura, H.; Wang, J.C.; Alanay, A. An update on bone substitutes for spinal fusion. *Eur. Spine J.* **2009**, *18*, 783–799.
55. Kneser, U.; Schaefer, D.J.; Polykandriotis, E.; Horch, R.E. Tissue engineering of bone: The reconstructive surgeon's point of view. *J. Cell. Mol. Med.* **2006**, *10*, 7–19.
56. Rho, J.Y.; Kuhn-Spearing, L.; Zioupos, P. Mechanical properties and the hierarchical structure of bone. *Med. Eng. Phys.* **1998**, *20*, 92–102.
57. Yoshimoto, H.; Shin, Y.M.; Terai, H.; Vacanti, J.P. A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering. *Biomaterials* **2003**, *24*, 2077–2082.
58. Frohbergh, M.E.; Katsman, A.; Botta, G.P.; Lazarovici, P.; Schauer, C.L.; Wegst, U.G.; Lelkes, P.I. Electrospun hydroxyapatite-containing chitosan nanofibers crosslinked with genipin for bone tissue engineering. *Biomaterials* **2012**, *33*, 9167–9178.
59. Kim, K.H.; Jeong, L.; Park, H.N.; Shin, S.Y.; Park, W.H.; Lee, S.C.; Kim, T.I.; Park, Y.J.; Seol, Y.J.; Lee, Y.M.; *et al.* Biological efficacy of silk fibroin nanofiber membranes for guided bone regeneration. *J. Biotechnol.* **2005**, *120*, 327–339.
60. Nguyen, L.T.; Liao, S.; Chan, C.K.; Ramakrishna, S. Enhanced osteogenic differentiation with 3D electrospun nanofibrous scaffolds. *Nanomedicine* **2012**, *7*, 1561–1575.
61. Ravichandran, R.; Venugopal, J.R.; Sundarrajan, S.; Mukherjee, S.; Ramakrishna, S. Precipitation of nanohydroxyapatite on PLLA/PBLG/Collagen nanofibrous structures for the differentiation of adipose derived stem cells to osteogenic lineage. *Biomaterials* **2012**, *33*, 846–855.
62. Schofer, M.D.; Roessler, P.P.; Schaefer, J.; Theisen, C.; Schlimme, S.; Heverhagen, J.T.; Voelker, M.; Dersch, R.; Agarwal, S.; Fuchs-Winkelmann, S.; Paletta, J.R. Electrospun PLLA nanofiber scaffolds and their use in combination with BMP-2 for reconstruction of bone defects. *PLoS One* **2011**, *6*, e25462.
63. Schofer, M.D.; Tunnermann, L.; Kaiser, H.; Roessler, P.P.; Theisen, C.; Heverhagen, J.T.; Hering, J.; Voelker, M.; Agarwal, S.; Efe, T.; *et al.* Functionalisation of PLLA nanofiber scaffolds using a possible cooperative effect between collagen type I and BMP-2: Impact on colonization and bone formation *in vivo*. *J. Mater. Sci. Mater. Med.* **2012**, *23*, 2227–2233.
64. Dalby, M.J.; Gadegaard, N.; Curtis, A.S.; Oreffo, R.O. Nanotopographical control of human osteoprogenitor differentiation. *Curr. Stem Cell Res. Ther.* **2007**, *2*, 129–138.
65. Felice, P.; Pistilli, R.; Piattelli, M.; Soardi, E.; Corvino, V.; Esposito, M. Posterior atrophic jaws rehabilitated with prostheses supported by 5 × 5 mm implants with a novel nanostructured calcium-incorporated titanium surface or by longer implants in augmented bone. Preliminary results from a randomised controlled trial. *Eur. J. Oral Implantol.* **2012**, *5*, 149–161.
66. Peltola, T.; Jokinen, M.; Rahiala, H.; Patsi, M.; Heikkila, J.; Kangasniemi, I.; Yli-Urpo, A. Effect of aging time of sol on structure and *in vitro* calcium phosphate formation of sol-gel-derived titania films. *J. Biomed. Mater. Res.* **2000**, *51*, 200–208.
67. Wennerberg, A.; Frojd, V.; Olsson, M.; Nannmark, U.; Emanuelsson, L.; Johansson, P.; Josefsson, Y.; Kangasniemi, I.; Peltola, T.; Tirri, T.; *et al.* Nanoporous TiO(2) thin film on titanium oral implants for enhanced human soft tissue adhesion: a light and electron microscopy study. *Clin. Implant. Dent. Relat. Res.* **2011**, *13*, 184–196.

68. Rani, V.V.; Vinoth-Kumar, L.; Anitha, V.C.; Manzoor, K.; Deepthy, M.; Shantikumar, V.N. Osteointegration of titanium implant is sensitive to specific nanostructure morphology. *Acta Biomater.* **2012**, *8*, 1976–1989.
69. Wang, X.; Gittens, R.A.; Song, R.; Tannenbaum, R.; Olivares-Navarrete, R.; Schwartz, Z.; Chen, H.; Boyan, B.D. Effects of structural properties of electrospun TiO_2 nanofiber meshes on their osteogenic potential. *Acta Biomater.* **2012**, *8*, 878–885.
70. Zhang, L.; Chen, Y.; Rodriguez, J.; Fenniri, H.; Webster, T.J. Biomimetic helical rosette nanotubes and nanocrystalline hydroxyapatite coatings on titanium for improving orthopedic implants. *Int. J. Nanomed.* **2008**, *3*, 323–333.
71. Sun, L.; Zhang, L.; Hemraz, U.D.; Fenniri, H.; Webster, T.J. Bioactive rosette nanotube-hydroxyapatite nanocomposites improve osteoblast functions. *Tissue Eng. A* **2012**, *18*, 1741–1750.
72. Sahithi, K.; Swetha, M.; Ramasamy, K.; Srinivasan, N.; Selvamurugan, N. Polymeric composites containing carbon nanotubes for bone tissue engineering. *Int. J. Biol. Macromol.* **2010**, *46*, 281–283.
73. Li, X.; Liu, H.; Niu, X.; Yu, B.; Fan, Y.; Feng, Q.; Cui, F.Z.; Watari, F. The use of carbon nanotubes to induce osteogenic differentiation of human adipose-derived MSCs *in vitro* and ectopic bone formation *in vivo*. *Biomaterials* **2012**, *33*, 4818–4827.
74. Dallari, D.; Savarino, L.; Albisinni, U.; Fornasari, P.; Ferruzzi, A.; Baldini, N.; Giannini, S. A prospective, randomised, controlled trial using a Mg-hydroxyapatite-demineralized bone matrix nanocomposite in tibial osteotomy. *Biomaterials* **2012**, *33*, 72–79.
75. Schwarz, F.; Sahm, N.; Bieling, K.; Becker, J. Surgical regenerative treatment of peri-implantitis lesions using a nanocrystalline hydroxyapatite or a natural bone mineral in combination with a collagen membrane: a four-year clinical follow-up report. *J. Clin. Periodontol.* **2009**, *36*, 807–814.
76. Tan, A.; Rajadas, J.; Seifalian, A.M. Biochemical engineering nerve conduits using peptide amphiphiles. *J. Control. Release* **2012**, *163*, 342–352.
77. Kehoe, S.; Zhang, X.F.; Boyd, D. Composition-property relationships for an experimental composite nerve guidance conduit: Evaluating cytotoxicity and initial tensile strength. *J. Mater. Sci. Mater. Med.* **2011**, *22*, 945–959.
78. Weber, R.A.; Breidenbach, W.C.; Brown, R.E.; Jabaley, M.E.; Mass, D.P. A randomized prospective study of polyglycolic acid conduits for digital nerve reconstruction in humans. *Plast Reconstr. Surg.* **2000**, *106*, 1036–1045; discussion 1046–1048.
79. Xiong, Y.; Zhu, J.X.; Fang, Z.Y.; Zeng, C.G.; Zhang, C.; Qi, G.L.; Li, M.H.; Zhang, W.; Quan, D.P.; Wan, J. Coseeded Schwann cells myelinate neurites from differentiated neural stem cells in neurotrophin-3-loaded PLGA carriers. *Int. J. Nanomed.* **2012**, *7*, 1977–1989.
80. de Boer, R.; Borntraeger, A.; Knight, A.M.; Hebert-Blouin, M.N.; Spinner, R.J.; Malessy, M.J.; Yaszemski, M.J.; Windebank, A.J. Short- and long-term peripheral nerve regeneration using a poly-lactic-co-glycolic-acid scaffold containing nerve growth factor and glial cell line-derived neurotrophic factor releasing microspheres. *J. Biomed. Mater. Res. A* **2012**, *100*, 2139–2146.

81. Sasaki, R.; Aoki, S.; Yamato, M.; Uchiyama, H.; Wada, K.; Ogiuchi, H.; Okano, T.; Ando, T. PLGA artificial nerve conduits with dental pulp cells promote facial nerve regeneration. *J. Tissue Eng. Regen. Med.* **2011**, *5*, 823–830.
82. Xue, C.; Hu, N.; Gu, Y.; Yang, Y.; Liu, Y.; Liu, J.; Ding, F.; Gu, X. Joint use of a chitosan/PLGA scaffold and MSCs to bridge an extra large gap in dog sciatic nerve. *Neurorehabil. Neural Repair* **2012**, *26*, 96–106.
83. Kang, K.N.; Kim da, Y.; Yoon, S.M.; Lee, J.Y.; Lee, B.N.; Kwon, J.S.; Seo, H.W.; Lee, I.W.; Shin, H.C.; Kim, Y.M.; *et al.* Tissue engineered regeneration of completely transected spinal cord using human mesenchymal stem cells. *Biomaterials* **2012**, *33*, 4828–4835.
84. Kehoe, S.; Zhang, X.F.; Boyd, D. FDA approved guidance conduits and wraps for peripheral nerve injury: A review of materials and efficacy. *Injury* **2012**, *43*, 553–572.
85. Meek, M.F.; Coert, J.H. Synthetic nerve guide implants in humans: A comprehensive survey. *Neurosurgery* **2007**, *61*, E1340; doi:10.1227/01.neu.0000306132.21108.25.
86. Frattini, F.; Lopes, F.R.; Almeida, F.M.; Rodrigues, R.F.; Boldrini, L.C.; Tomaz, M.A.; Baptista, A.F.; Melo, P.A.; Martinez, A.M. Mesenchymal stem cells in a polycaprolactone conduit promote sciatic nerve regeneration and sensory neuron survival after nerve injury. *Tissue Eng. A* **2012**, *18*, 2030–2039.
87. Yu, W.; Zhao, W.; Zhu, C.; Zhang, X.; Ye, D.; Zhang, W.; Zhou, Y.; Jiang, X.; Zhang, Z. Sciatic nerve regeneration in rats by a promising electrospun collagen/poly(epsilon-caprolactone) nerve conduit with tailored degradation rate. *BMC Neurosci.* **2011**, doi:10.1186/1471-2202-12-68.
88. Beniash, E.; Hartgerink, J.D.; Storrie, H.; Stendahl, J.C.; Stupp, S.I. Self-assembling peptide amphiphile nanofiber matrices for cell entrapment. *Acta Biomater.* **2005**, *1*, 387–397.
89. Liu, W.Q.; Martinez, J.A.; Durand, J.; Wildering, W.; Zochodne, D.W. RGD-mediated adhesive interactions are important for peripheral axon outgrowth *in vivo*. *Neurobiol. Dis.* **2009**, *34*, 11–22.
90. Taras, J.S.; Nanavati, V.; Steelman, P. Nerve conduits. *J. Hand Ther.* **2005**, *18*, 191–197.
91. Antoniadou, E.V.; Ahmad, R.K.; Jackman, R.B.; Seifalian, A.M. Next generation brain implant coatings and nerve regeneration via novel conductive nanocomposite development. *Conf. Proc. IEEE Eng. Med. Biol. Soc.* **2011**, doi:10.1109/IEMBS.2011.6090884.
92. Ibrahim, A.; Ohshima, H.; Allison, S.A.; Cottet, H. Determination of effective charge of small ions, polyelectrolytes and nanoparticles by capillary electrophoresis. *J. Chromatogr. A* **2012**, *1247*, 154–164.
93. Subramaniam, R.; Astot, C.; Juhlin, L.; Nilsson, C.; Ostin, A. Determination of S-2-(*N*,*N*-diisopropylaminoethyl)- and S-2-(*N*,*N*-diethylaminoethyl) methylphosphonothiolate, nerve agent markers, in water samples using strong anion-exchange disk extraction, in vial trimethylsilylation, and gas chromatography-mass spectrometry analysis. *J. Chromatogr. A* **2012**, *1229*, 86–94.
94. Kwon, O.S.; Park, S.J.; Lee, J.S.; Park, E.; Kim, T.; Park, H.W.; You, S.A.; Yoon, H.; Jang, J. Multidimensional conducting polymer nanotubes for ultrasensitive chemical nerve agent sensing. *Nano Lett.* **2012**, *12*, 2797–2802.

95. Tandon, V.; Zhang, B.; Radisic, M.; Murthy, S.K. Generation of tissue constructs for cardiovascular regenerative medicine: From cell procurement to scaffold design. *Biotechnol. Adv.* **2012**, In Press.
96. Prabhakaran, M.P.; Kai, D.; Ghasemi-Mobarakeh, L.; Ramakrishna, S. Electrospun biocomposite nanofibrous patch for cardiac tissue engineering. *Biomed. Mater.* **2011**, *6*, 055001:1–055001:12.
97. Kocher, A.A.; Schuster, M.D.; Szabolcs, M.J.; Takuma, S.; Burkhoff, D.; Wang, J.; Homma, S.; Edwards, N.M.; Itescu, S. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat. Med.* **2001**, *7*, 430–436.
98. Penn, M.S.; Francis, G.S.; Ellis, S.G.; Young, J.B.; McCarthy, P.M.; Topol, E.J. Autologous cell transplantation for the treatment of damaged myocardium. *Prog. Cardiovasc. Dis.* **2002**, *45*, 21–32.
99. Fuchs, S.; Kornowski, R.; Weisz, G.; Satler, L.F.; Smits, P.C.; Okubagzi, P.; Baffour, R.; Aggarwal, A.; Weissman, N.J.; Cerqueira, M.; *et al.* Safety and feasibility of transendocardial autologous bone marrow cell transplantation in patients with advanced heart disease. *Am. J. Cardiol.* **2006**, *97*, 823–829.
100. van Ramshorst, J.; Bax, J.J.; Beeres, S.L.; Dibbets-Schneider, P.; Roes, S.D.; Stokkel, M.P.; de Roos, A.; Fibbe, W.E.; Zwaginga, J.J.; Boersma, E.; *et al.* Intramyocardial bone marrow cell injection for chronic myocardial ischemia: a randomized controlled trial. *JAMA* **2009**, *301*, 1997–2004.
101. Tse, H.F.; Thambar, S.; Kwong, Y.L.; Rowlings, P.; Bellamy, G.; McCrohon, J.; Thomas, P.; Bastian, B.; Chan, J.K.; Lo, G.; *et al.* Prospective randomized trial of direct endomyocardial implantation of bone marrow cells for treatment of severe coronary artery diseases (PROTECT-CAD trial). *Eur. Heart J.* **2007**, *28*, 2998–3005.
102. Perin, E.C.; Silva, G.V.; Henry, T.D.; Cabreira-Hansen, M.G.; Moore, W.H.; Coulter, S.A.; Herlihy, J.P.; Fernandes, M.R.; Cheong, B.Y.; Flamm, S.D.; *et al.* A randomized study of transendocardial injection of autologous bone marrow mononuclear cells and cell function analysis in ischemic heart failure (FOCUS-HF). *Am. Heart J.* **2011**, *161*, 1078–1087.
103. Williams, A.R.; Trachtenberg, B.; Velazquez, D.L.; McNiece, I.; Altman, P.; Rouy, D.; Mendizabal, A.M.; Pattany, P.M.; Lopera, G.A.; Fishman, J.; *et al.* Intramyocardial stem cell injection in patients with ischemic cardiomyopathy: Functional recovery and reverse remodeling. *Circ. Res.* **2011**, *108*, 792–796.
104. Clifford, D.M.; Fisher, S.A.; Brunskill, S.J.; Doree, C.; Mathur, A.; Watt, S.; Martin-Rendon, E. Stem cell treatment for acute myocardial infarction. *Cochrane Database Syst. Rev.* **2012**, doi:10.1002/14651858.CD006536.pub3.
105. Shin, M.; Ishii, O.; Sueda, T.; Vacanti, J.P. Contractile cardiac grafts using a novel nanofibrous mesh. *Biomaterials* **2004**, *25*, 3717–3723.
106. Kai, D.; Prabhakaran, M.P.; Jin, G.; Ramakrishna, S. Guided orientation of cardiomyocytes on electrospun aligned nanofibers for cardiac tissue engineering. *J. Biomed. Mater. Res. B Appl. Biomater.* **2011**, *98B*, 379–386.

107. Mukherjee, S.; Gualandi, C.; Focarete, M.L.; Ravichandran, R.; Venugopal, J.R.; Raghunath, M.; Ramakrishna, S. Elastomeric electrospun scaffolds of poly(L-lactide-co-trimethylene carbonate) for myocardial tissue engineering. *J. Mater. Sci. Mater. Med.* **2011**, *22*, 1689–1699.
108. Prabhakaran, M.P.; Nair, A.S.; Kai, D.; Ramakrishna, S. Electrospun composite scaffolds containing poly(octanediol-co-citrate) for cardiac tissue engineering. *Biopolymers* **2012**, *97*, 529–538.
109. Gupta, M.K.; Walthall, J.M.; Venkataraman, R.; Crowder, S.W.; Jung, D.K.; Yu, S.S.; Feaster, T.K.; Wang, X.; Giorgio, T.D.; Hong, C.C.; Baudenbacher, F.J.; Hatzopoulos, A.K.; Sung, H.J. Combinatorial polymer electrospun matrices promote physiologically-relevant cardiomyogenic stem cell differentiation. *PLoS One* **2011**, *6*, e28935:1–e28935:12.
110. Sreerekha, P.R.; Menon, D.; Nair, S.V.; Chennazhi, K.P. Fabrication of Electrospun Poly (Lactide-co-Glycolide)-Fibrin Multiscale Scaffold for Myocardial Regeneration *In Vitro*. *Tissue Eng. A* **2013**, *19*, 849–859.
111. Hussain, A.; Collins, G.; Yip, D.; Cho, C.H. Functional 3-D cardiac co-culture model using bioactive chitosan nanofiber scaffolds. *Biotechnol. Bioeng.* **2013**, *110*, 637–647.
112. Kim, D.H.; Kshitiz; Smith, R.R.; Kim, P.; Ahn, E.H.; Kim, H.N.; Marban, E.; Suh, K.Y.; Levchenko, A. Nanopatterned cardiac cell patches promote stem cell niche formation and myocardial regeneration. *Integr. Biol.* **2012**, *4*, 1019–1033.
113. Dvir, T.; Bauer, M.; Schroeder, A.; Tsui, J.H.; Anderson, D.G.; Langer, R.; Liao, R.; Kohane, D.S. Nanoparticles targeting the infarcted heart. *Nano Lett.* **2011**, *11*, 4411–4414.
114. Yang, X. Nano- and microparticle-based imaging of cardiovascular interventions: Overview. *Radiology* **2007**, *243*, 340–347.
115. Harel-Adar, T.; Ben Mordechai, T.; Amsalem, Y.; Feinberg, M.S.; Leor, J.; Cohen, S. Modulation of cardiac macrophages by phosphatidylserine-presenting liposomes improves infarct repair. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 1827–1832.
116. Scott, R.C.; Rosano, J.M.; Ivanov, Z.; Wang, B.; Chong, P.L.; Issekutz, A.C.; Crabbe, D.L.; Kiani, M.F. Targeting VEGF-encapsulated immunoliposomes to MI heart improves vascularity and cardiac function. *FASEB J.* **2009**, *23*, 3361–3367.
117. Huang, G.; Rahimtoola, S.H. Prosthetic heart valve. *Circulation* **2011**, *123*, 2602–2605.
118. Flanagan, T.C.; Sachweh, J.S.; Frese, J.; Schnoring, H.; Gronloh, N.; Koch, S.; Tolba, R.H.; Schmitz-Rode, T.; Jockenhoevel, S. *In vivo* remodeling and structural characterization of fibrin-based tissue-engineered heart valves in the adult sheep model. *Tissue Eng. A* **2009**, *15*, 2965–2976.
119. Kalfa, D.; Bel, A.; Chen-Tournoux, A.; Della Martina, A.; Rochereau, P.; Coz, C.; Bellamy, V.; Bensalah, M.; Vanneaux, V.; Lecourt, S.; *et al.* A polydioxanone electrospun valved patch to replace the right ventricular outflow tract in a growing lamb model. *Biomaterials* **2010**, *31*, 4056–4063.
120. Aleksieva, G.; Hollweck, T.; Thierfelder, N.; Haas, U.; Koenig, F.; Fano, C.; Dauner, M.; Wintermantel, E.; Reichart, B.; Schmitz, C.; Akra, B. Use of a special bioreactor for the cultivation of a new flexible polyurethane scaffold for aortic valve tissue engineering. *Biomed. Eng. Online* **2012**, *11*, 92.

121. Martin, I.; Wendt, D.; Heberer, M. The role of bioreactors in tissue engineering. *Trends Biotechnol.* **2004**, *22*, 80–86.
122. Mol, A.; Smits, A.I.; Bouten, C.V.; Baaijens, F.P. Tissue engineering of heart valves: advances and current challenges. *Expert Rev. Med. Devices* **2009**, *6*, 259–275.
123. Winter, P.M.; Neubauer, A.M.; Caruthers, S.D.; Harris, T.D.; Robertson, J.D.; Williams, T.A.; Schmieder, A.H.; Hu, G.; Allen, J.S.; Lacy, E.K.; *et al.* Endothelial alpha(v)beta3 integrin-targeted fumagillin nanoparticles inhibit angiogenesis in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **2006**, *26*, 2103–2109.
124. Winter, P.M.; Caruthers, S.D.; Zhang, H.; Williams, T.A.; Wickline, S.A.; Lanza, G.M. Antiangiogenic synergism of integrin-targeted fumagillin nanoparticles and atorvastatin in atherosclerosis. *JACC Cardiovasc. Imaging* **2008**, *1*, 624–634.
125. Peters, D.; Kastantin, M.; Kotamraju, V.R.; Karmali, P.P.; Gujraty, K.; Tirrell, M.; Ruoslahti, E. Targeting atherosclerosis by using modular, multifunctional micelles. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 9815–9819.
126. Sharma, G.; She, Z.G.; Valenta, D.T.; Stallcup, W.B.; Smith, J.W. Targeting of Macrophage Foam Cells in Atherosclerotic Plaque Using Oligonucleotide-Functionalized Nanoparticles. *Nano Life* **2010**, *1*, 207–214.
127. Ji, J.; Ji, S.Y.; Yang, J.A.; He, X.; Yang, X.H.; Ling, W.P.; Chen, X.L. Ultrasound-targeted transfection of tissue-type plasminogen activator gene carried by albumin nanoparticles to dog myocardium to prevent thrombosis after heart mechanical valve replacement. *Int. J. Nanomed.* **2012**, *7*, 2911–2919.
128. Acharya, G.; Hopkins, R.A.; Lee, C.H. Advanced polymeric matrix for valvular complications. *J. Biomed. Mater. Res. A* **2012**, *100*, 1151–1159.
129. U.S. Food and Drug Administration. The FDA's Drug Review Process: Ensuring Drugs Are Safe and Effective. Avaliable online: http://www.fda.gov/drugs/resourcesforyou/consumers/ucm143534.htm (aceessed on 25 March 2013).
130. ClinicalTrials Home Page. Avaliable online: http://www.clinicaltrials.gov/ (aceessed on 25 March 2013).

Osteoarthritis and Cartilage 21 (2013) 246–258

Osteoarthritis and Cartilage

Chondrocytes extract from patients with osteoarthritis induces chondrogenesis in infrapatellar fat pad-derived stem cells

E. López-Ruiz †[a], M. Perán †[a], J. Cobo-Molinos †, G. Jiménez ‡§, M. Picón ‡, M. Bustamante ||, F. Arrebola ¶, M.C. Hernández-Lamas #, A.D. Delgado-Martínez †, E. Montañez ††, J.A. Marchal ‡§*

† *Department of Health Sciences, University of Jaén, Jaén E-23071, Spain*
‡ *Biopathology and Regenerative Medicine Institute (IBIMER), Centre for Biomedical Research, University of Granada, Granada E-18100, Spain*
§ *Department of Human Anatomy and Embryology, Faculty of Medicine, University of Granada, Granada E-18012, Spain*
|| *Biosciences Institute, University College Cork, Cork, Ireland*
¶ *Department of Histology, Faculty of Medicine, University of Granada, Granada E-18071, Spain*
# *Málaga Tissue Bank, Centro Regional de Transfusión, Málaga, Spain*
†† *Department of Orthopedic Surgery and Traumatology, Virgen de la Victoria University Hospital, Málaga, Spain*

ARTICLE INFO

Article history:
Received 27 July 2012
Accepted 11 October 2012

Keywords:
Infrapatellar fat pad-derived stem cells
Cell extracts
Osteoarthritis (OA)
Chondrogenic differentiation
Poly (DL-lactic-co-glycolic acid) (PLGA) scaffolds

SUMMARY

Objective: Infrapatellar fat pad of patients with osteoarthritis (OA) contains multipotent and highly clonogenic adipose-derived stem cells that can be isolated by low invasive methods. Moreover, nuclear and cytoplasmic cellular extracts have been showed to be effective in induction of cell differentiation and reprogramming. The aim of this study was to induce chondrogenic differentiation of autologous mesenchymal stem cells (MSCs) obtained from infrapatellar fat pad (IFPSCs) of patients with OA using cellular extracts-based transdifferentiation method.

Design: IFPSCs and chondrocytes were isolated and characterized by flow cytometry. IFPSCs were permeabilized with Streptolysin O and then exposed to a cell extract obtained from chondrocytes. Then, IFPSCs were cultured for 2 weeks and chondrogenesis was evaluated by morphologic and ultrastructural observations, immunologic detection, gene expression analysis and growth on 3-D poly (DL-lactic-co-glycolic acid) (PLGA) scaffolds.

Results: After isolation, both chondrocytes and IFPSCs displayed similar expression of MSCs surface makers. Collagen II was highly expressed in chondrocytes and showed a basal expression in IFPSCs. Cells exposed to chondrocyte extracts acquired a characteristic morphological and ultrastructural chondrocyte phenotype that was confirmed by the increased proteoglycan formation and enhanced collagen II immunostaining. Moreover, chondrocyte extracts induced an increase in mRNA expression of chondrogenic genes such as *Sox9*, *L-Sox5*, *Sox6* and *Col2a1*. Interestingly, chondrocytes, IFPSCs and transdifferentiated IFPSCs were able to grow, expand and produce extracellular matrix (ECM) on 3D PLGA scaffolds.

Conclusions: We demonstrate for the first time that extracts obtained from chondrocytes of osteoarthritic knees promote chondrogenic differentiation of autologous IFPSCs. Moreover, combination of transdifferentiated IFPSCs with biodegradable PLGA 3D scaffolds can serve as an efficient system for the maintenance and maturation of cartilage tissue. These findings suggest its usefulness to repair articular surface in OA.

Introduction

Chondral defects suppose a challenging clinical problem aggravated by the increase in elderly population in developed countries. Consequently, many strategies including arthroplasty, the stimulation of reparative tissues through arthroscopic, abrasion, drilling or microfracture[1] have been developed to treat the injured cartilage. However, these procedures cannot reproduce successfully the tissue characteristics of hyaline cartilage[2,3]. New biological based strategies such as the replacement of damaged

* Address correspondence and reprint requests to: J.A. Marchal, Department of Human Anatomy and Embryology, Faculty of Medicine, University of Granada, Avda de Madrid s/n, Granada E-18012, Spain. Tel: 34-958-249-321; Fax: 34-958-246-296.
E-mail addresses: elruiz@ujaen.es (E. López-Ruiz), mperan@ujaen.es (M. Perán), jcobos@ujaen.es (J. Cobo-Molinos), gemajg@correo.ugr.es (G. Jiménez), mpicon@ugr.es (M. Picón), 111224080@umail.ucc.ie (M. Bustamante), fav@ugr.es (F. Arrebola), carmen.hernandez.lamas.sspa@juntadeandalucia.es (M.C. Hernández-Lamas), adelgado@ujaen.es (A.D. Delgado-Martínez), elvira.montanez.sspa@juntadeandalucia.es (E. Montañez), jmarchal@ugr.es (J.A. Marchal).
[a] These authors contributed equally to this work.

1063-4584/$ – see front matter © 2012 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.joca.2012.10.007

surface by healthy one (osteochondral grafts)[4] or the implantation of cultured expanded autologous chondrocytes have been used for cartilage repair[5]. Nevertheless, the difficulties to obtain sufficient amounts of autologous chondrocytes[6] or the dedifferentiation process that chondrocytes undergo after serial passages in monolayer culture[7] are the main disadvantages that limit their use.

MSCs represent an attractive cell source for cartilage regeneration cell therapy approach. These multipotent cells can be easily isolated from different sources such as bone marrow, fat tissue, umbilical cord or peripheral blood among others[8] and present a high proliferation capacity. The chondrogenic potential of MSCs derived from bone marrow have been demonstrated; [9] however, the extraction procedure is invasive and increases morbidity in patients with osteochondral disease. Therefore, it is necessary to search new sources of MSCs that could be isolated by less invasive methods. In this respect, MSCs obtained from adipose tissue represent a good alternative and recently, it has been shown that Hoffa's fat pad in patients with osteoarthritis (OA), contains multipotent and highly clonogenic adipose-derived stem cells[10,11]. These cells can be obtained from the articulation of the patient in a single surgical procedure[12,13].

Last years, many protocols for inducing adult stem cells to differentiate *in vitro* across germinal boundaries, a process referred to as transdifferentiation, has been described[14–16]. There are different strategies for achieving adult cell reprogramming or transdifferentiation: somatic nuclear transfer, cell fusion, ectopic expression of master switch genes and cell extract based methods[17]. The use of nuclear and cytoplasmic extracts has been showed to be effective in induction of cell differentiation and cell reprogramming[18,19]. Among potential benefits of the extract approach are the use of autologous material to direct differentiation and to avoid exogenous genes, recombinant proteins like exogenous growth factors or viral delivery methods.

In this study, we used cellular extracts-based transdifferentiation method to induce chondrogenic differentiation of autologous MSCs obtained from infrapatellar fat pad (IFPSCs) of patients with OA. Here, we demonstrate for the first time, that IFPSCs can undergo chondrogenesis when stimulated with an extract prepared from autologous chondrocytes obtained from patients' cartilage tissue.

Materials and methods

Patients

Both human IFPSCs and articular cartilage were obtained from 18 patients with knee OA during joint replacement surgery. Ethical approval for the study was obtained from the Ethics Committee of the Clinical University Hospital of Málaga, Spain. Informed patient consent was obtained for all samples used in this study. None of the patients had a history of inflammatory arthritis or crystal-induced arthritis. Human articular cartilage was obtained from the femoral side, selecting the non-overload compartment: lateral condyle in varus knees and medial condyle in the valgus cases. Only cartilage that macroscopically looked relatively normal was used for this study. Hoffa's fat pad was harvested from the interior of the capsule excluding vascular areas and synovial regions. Samples collected at joint arthroplasty were transported to the laboratory in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) with 100 U/ml penicillin and 100 μg/ml streptomycin.

Isolation of human articular chondrocytes

Articular cartilage was minced and digested overnight in an overnight 0.08% collagenase IV (Sigma) digestion at 37°C with gentle agitation. Cells were centrifuged and rinsing to remove the collagenase. The remaining cells were then plated in cultured flasks with chondrocytes media:DMEM (Sigma) supplemented with 20% FBS (Lonza, Basel, Switzerland), 7 ml human insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark), 6 μl DNase I (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. After 24 h the medium was replaced with fresh medium supplemented with 10% FBS. Chondrocytes were cultured for a maximum of 3 weeks before the experiments to avoid dedifferentiation phenomenon[7].

Isolation of MSCs from IFPSCs

Fat tissue finely minced was digested using enzymatic solution of 1 mg/ml collagenase type IA (Sigma) and incubated on a shaker at 37°C for 1 h. After digestion, collagenase was removed by a single wash in sterile phosphate-buffered saline (PBS), followed by two further washes in DMEM supplemented with 10% FBS. The cell pellet was resuspended in DMEM (Sigma) containing 10% FBS and 1% penicillin/streptomycin, added to tissue culture flasks, and cultured at 37°C in 5% CO_2. After 48 h the medium was removed to discard non-adherent cells. At 80% of confluency the cells were released with trypsin–ethylenediaminetetraacetic acid (EDTA) (Sigma) and subcultured.

Characterization of articular chondrocytes and IFPSCs

The immunophenotype of articular chondrocytes and cultured IFPSCs was analyzed by flow cytometry (FACS). MSC marker phenotyping was performed as previously described[20]. For the determination of collagen II expression cells were, first, fixed and permeabilized with Fix and Perm® reagent (Invitrogen, Carlsbad, CA, USA), then incubated with the primary monoclonal antibody (AbCam, Cambridge, UK) for 20 min, washed and finally incubated with a secondary FITC-conjugate monoclonal antibody (Sigma) for 30 min. All cells were washed in PBS and analyzed in a FACSCanto II cytometer (BD Biosciences).

Differentiation assays of IFPSCs

Human IFPSCs were plated at 2×10^3 cells/cm^2 in DMEM (Sigma) containing 10% FBS with penicillin and streptomycin at 100 μg/ml and allowed to adhere for 24 h. The culture medium was then replaced with specific inductive media to demonstrate adipogenic, osteogenic and chondrogenic differentiation potential as previously reported[20]. In brief, for adipogenic, osteogenic and chondrogenic differentiation, cells were cultured for 2 weeks in Adipogenic MSCs Differentiation BulletKit, Osteogenic MSCs Differentiation BulletKit (Lonza, Basel, Switzerland) and NH ChondroDiff Medium (Miltenyi Biotec, Auburn, CA, USA), respectively.

Cell permeabilization assay and exposure to cell extract of IFPSCs

Prior IFPSCs incubation with the nuclear and cytoplasmic extracts, the efficiency of Streptolysin O (SLO; Sigma) treatment was evaluated following a modification of the protocol described in[21]. Briefly, 3×10^5 IFPSCs were permeabilized with several concentration of SLO and exposed to Texas red-conjugated 70,000 *M*r dextran (Invitrogen). The uptake of the dye was observed by phase contrast and epifluorescence microscopy after 2 h and 24 h of SLO permeabilization. Chondrocytes extract preparation and exposure to IFPSCs were performed as described elsewhere[22] with slight modifications (See Supplementary data). 1,666 chondrocytes per 1,000 IFPSCs were used per reaction. After *in vitro* reprogramming in cellular extracts, cells were cultured for 2 weeks until use. We

used permeabilized cells not exposed to the extracts as control. Cell viability was assessed by phase contrast microscopy after 2 weeks of extract exposure by counting cells in four different regions of the dish and calculating the average.

Transmission electron microscopy (TEM)

Cells were assayed for by TEM as previously reported[17] and sections were examined with a LEO 906E transmission electron microscope.

Histological and immunohystochemical analysis

Cells seeded on glass coverslips and cell-seeded poly (DL-lactic-co-glycolic acid) (PLGA) sections were assayed for cartilaginous matrix production by toluidine blue and safranin O staining. Type II collagen expression was determined by immunofluorescence as previously described[17]. A primary monoclonal antibody anti-collagen type II (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used and photographs were taken with a Leica DM 5500B (Solms, Germany) fluorescent microscope, software Meta Systems Isis.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from control SLO-permeabilized cells, SLO-permeabilized cells exposed to the extract and from cultured chondrocytes (positive control) was extracted using TriReagent (Sigma). RNA was reverse transcribed using the Reverse Transcription System kit (Promega, Madison, WI, USA) and the PCR reaction was performed with ReddyMix PCR Master Mix (Thermo, Waltham, MA, USA). After the initial denaturation (2 min at 94°C), 33 cycles were performed (30 s at 94°C, 50 s for annealing temperature and 1 min at 72°C) for all set of primers except for β-actin, which was 25 cycles. Primer sequences and annealing temperatures can be found in Table I. The PCR products were visualized on 1% agarose gels containing 0.1 mg/ml ethidium bromide using ultraviolet light.

In vitro primary culture on scaffold

We used a biodegradable cylindrical implant constructed of a porous cartilage phase and a porous bone phase (5 mm in diameter × 10 mm deep). The cartilage phase of the scaffold was cut to 3 mm in diameter and 3 mm in height under sterile conditions on a Petri dish and was plated in 96-well plates. The synthetic material is a blend of poly DL-lactide-co-glycolide, polyglycolide fibers and surfactant (TruFit CB Plug; Smith & Nephew, London, UK). Cells suspensions containing 6,000 cells in 50 μl of medium were slowly dropped onto the surface of each scaffold incubated in 96-well plates for 4 h at 37°C and then analyzed under the inverted microscope to check cell adhesion to the polymer surface. After that, 50 μl of fresh medium was added in each well plate. Cell culture medium was changed every 2 days.

Table I
Sequences of the primers used in the RT-PCR reactions

Target gene	Primers (forward and reverse)
Collagen type I	GAG AGC ATG ACC GAT GG
	GTG ACG CTG TAG GTG AA
Collagen type II	GAC AAT CTG GCT CCC AAC
	ACA GTC TTG CCC CAC TTA C
Collagen type X	GCC CAC TAC CCA ACA C
	TGG TTT CCC TAC AGC TGA
Aggrecan I	GTC TCA CTG CCC AAC TAC
	GGA ACA CGA TGC CTT TCA C
Sox-5	ATC CCA ACT ACC ATG GCA GCT
	GAT ACC TGC ATT GCA GCT
Sox-6	GCA GTG ATC AAC ATG TGG CCT
	TTC ATC ATG CGCTGC CAG TAG
Sox-9	GAG CAG ACG CAC ATC TC
	CCT GGG ATT GCC CCG A
Gapdh	GAA GGT GAA GGT CGG AGT C
	GAA GAT GGT GAT GGG ATT TC

Environmental scanning electron microscope (ESEM) analysis

After 3 days of culture, samples were fixed in 3% glutaraldehyde during 2 h at 4°C and then were rinsed several times with sodium cacodylate. The samples were kept refrigerated into PBS. The observations started at an initial water vapor pressure of 5.7 tors. At this pressure, a liquid water phase was present in the sample (100% RH). Then, vapor pressure was decreasing slowly until the surfaces of the samples were visible (4.5 and 5 tors). Accelerating voltages varied between 10 and 15 kV we obtained a good image resolution using small beam current (spot size 3–3.5) and a working distance of 5–6 mm. The ESEM used in this work was a Quanta 400 (FEI) located at the Centro de Instrumentación Científica of the University of Granada.

Results

Cell isolation and characterization of chondrocytes

FACS characterization showed that *ex-vivo* cultured chondrocytes expressed the surface markers CD73 (99.84%), CD90 (97.25%) CD105 (99.74%) and the intracellular protein collagen type II (60%), while lacked expression for both hematopoietic and endothelial cell markers CD45 (<1%), CD34 (1.4%), CD133 (1.82%), CXCR4 (2.27%), KDR (7.63%) [Fig. 1(A)].

Light microcopy observation showed that chondrocytes at day one were attached to the flask surface as cell-clusters of rounded shapes [Fig. 1(B)]. After a week in culture, cells displayed a polygonal shape appearing some of them with a star-like morphology [Fig. 1(C)]. These stellated-like cells occupied the entire surface and reached confluence after 2 weeks [Fig. 1(D)].

Cell isolation and characterization of IFPSCs

Isolated IFPSCs presented a spindle shape fibroblastic morphology. FACS characterization demonstrated a positive expression of the surface markers CD73 (>99.80%), CD90 (>99.42%), CD105 (>99.91%) and a negative expression for CD45 (1.16%), CD34 (8.16%), CD133 (1.41%), CXCR4 (1.97%) and KDR (1.49%) as shown previously[23]. In addition, IFPSCs showed a slightly expression of collagen type II (13%) [Fig. 2(A)]. IFPSCs treated with conditioned media displayed characteristics of adipogenic, osteogenic and chondrogenic differentiation after staining [Fig. 2(B)].

Cell permeabilization assay

Previous to perform the extract reprogramming experiment we optimize IFPSCs permeabilization procedure by testing different concentrations of the SLO toxin ranged from 0.230–305 ng/ml. Among the tested concentration, 185 ng/ml of SLO was the most efficient, permeabilizing 80–95% of the cells [Fig. 3]. Results showed that 20–25% of the cells survived to the extract exposure while 80% of control cells (permeabilized but not exposed to extract) were viable.

Phenotypic changes after exposition to chondrocyte extracts

Phase microscopy observation of cultured chondrocytes showed a typical polygonal morphology with a high rate of star-like cells [Fig. 4(A)]. On the other hand, permeabilized but not exposed to

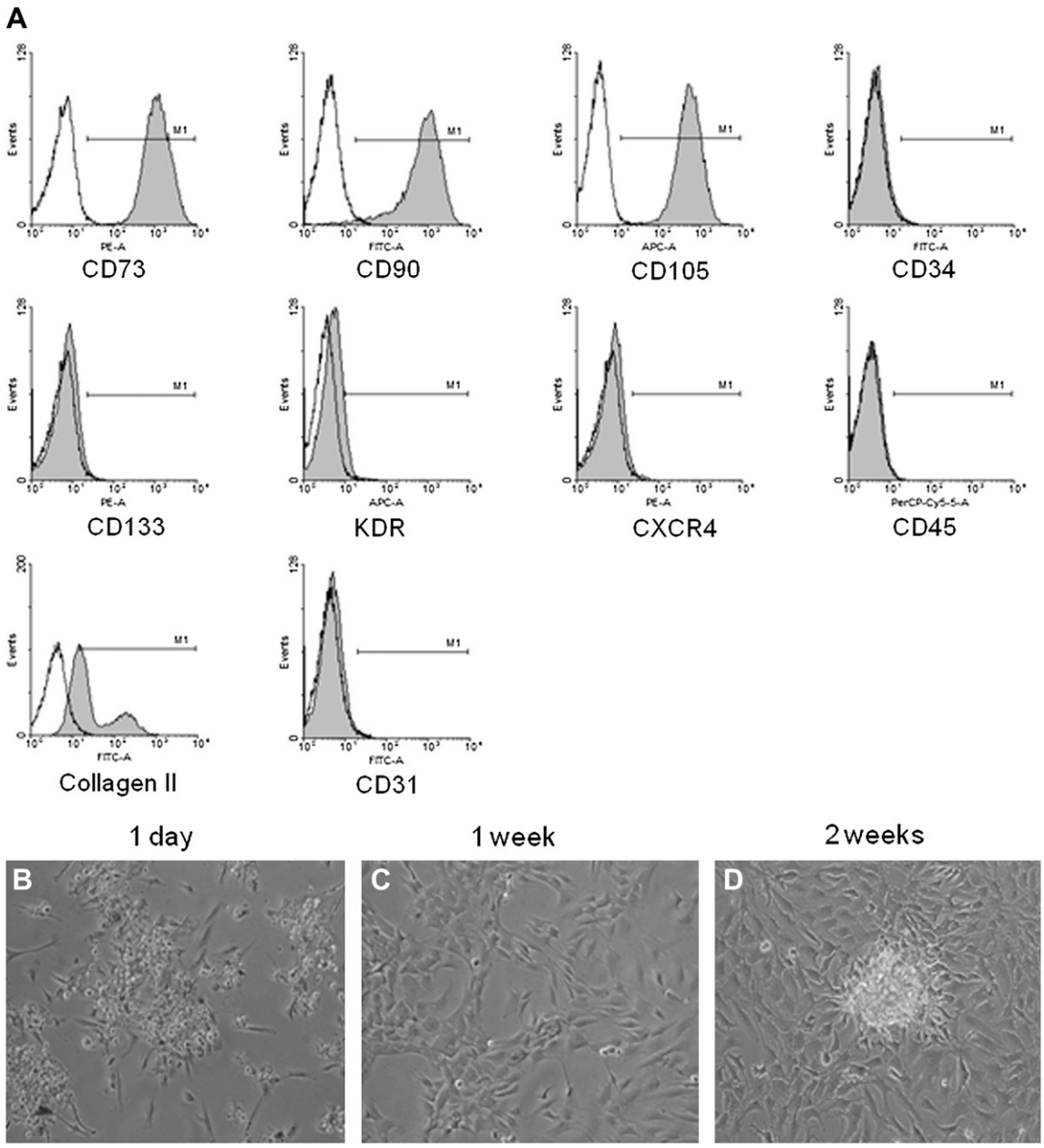


Fig. 1. Phenotypic characterization of chondrocytes. (A) Chondrocytes were cultured for 2 weeks and then tested for mesenchymal surface markers (CD105, CD73 and CD90), hematopoietic and endothelial markers (CD133, CD34, KDR, CD45 and CXCR4) and for collagen II by flow cytometry. White histograms identify the isotype controls (negative). (B–D) Phase-contrast light microscopy of cultured human articular chondrocytes for 1 (B), 7 (C) and 14 days (D). Original magnification: 10× for B and 20× for C and D.

extract cells (control IFPSCs) displayed a characteristic mesenchymal shape consisting in flatter cells with a spindle-like morphology [Fig. 4(B)]. It can be appreciated that IFPSCs acquired a chondrocyte-like phenotype after exposure to the autologous cell extract, showing an increased number of smaller polygonal and star-shaped cells [Fig. 4(C)].

Furthermore, toluidine blue assay demonstrated a characteristic positive metachromatic staining in both chondrocytes and IFPSCs treated with extracts which evidenced the synthesis of glycosaminoglycans (*GAGs*) [Fig. 4(D and F), respectively] when compared with control cells [Fig. 4(E)].

Ultrastructural analysis

TEM showed that cultured chondrocytes possessed an euchromatic nuclei, a cytoplasm rich in rough endoplasmic reticulum (RER), many transport vesicles, lipid droplets (LI) and proteoglycan granules. Moreover, ECM produced by cultured chondrocytes contained a homogeneous population of fibrillar collagens (FC) with typical striation [Fig. 4(G and J)]. Control IFPSCs exhibited morphological features typical of MSCs, including various mitochondrial profiles, small vacuoles and a nucleus with multiple nucleoli [Fig. 4(H and K)]. In contrast, extract exposed IFPSCs seemed to have undergone chondrogenic differentiation characterized by nuclei with prominent nucleoli, extended cistern of RER, Golgi apparatus (GA) and vacuoles and fine cytoplasmatic processes [Fig. 4(I and L)]. Moreover, a characteristic feature present in chondrocytes and found in extract exposed IFPSCs [Fig. 4(I and L)], but not in control [Fig. 4(H and K)], was the presence of proteoglycan granules in both cytoplasm and ECM. Round-shaped lipid vesicles of variable size were also present in differentiated cells.

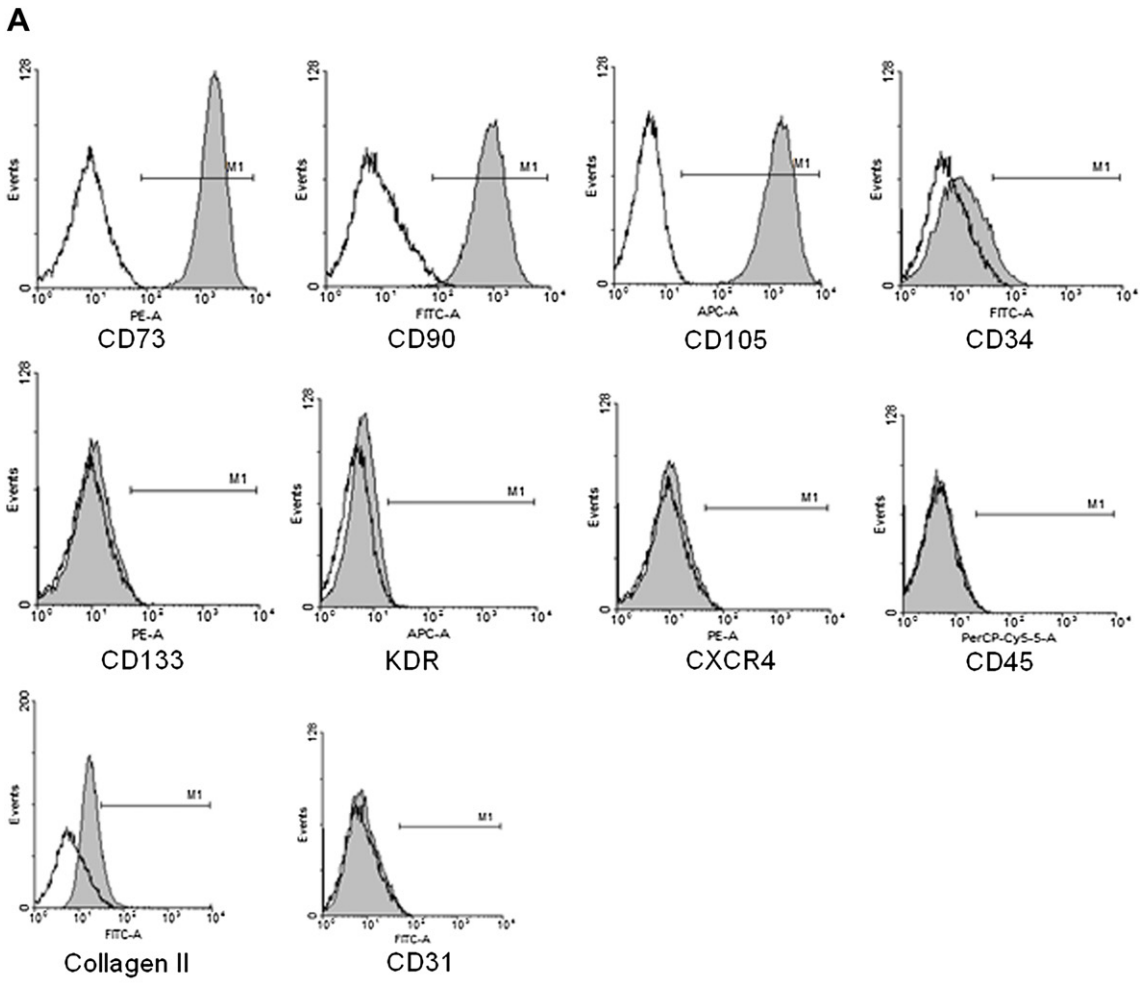


Fig. 2. Phenotypic characterization and differentiation potential of IFPSCs. (A) IFPSCs were cultured for 2 weeks and then tested for mesenchymal surface markers (CD105, CD73 and CD90), hematopoietic and endothelial markers (CD133, CD34, KDR, CD45 and CXCR4) and for collagen II by flow cytometry. (B) The differentiation potential of IFPSCs towards adipogenic, chondrogenic and osteogenic lineage was confirmed by Oil Red O, toluidine blue and Alizarin Red S staining, respectively. Upper pictures show negative controls, cells cultured in normal medium for 2 weeks and then histochemically stained. Original magnification: 10×.

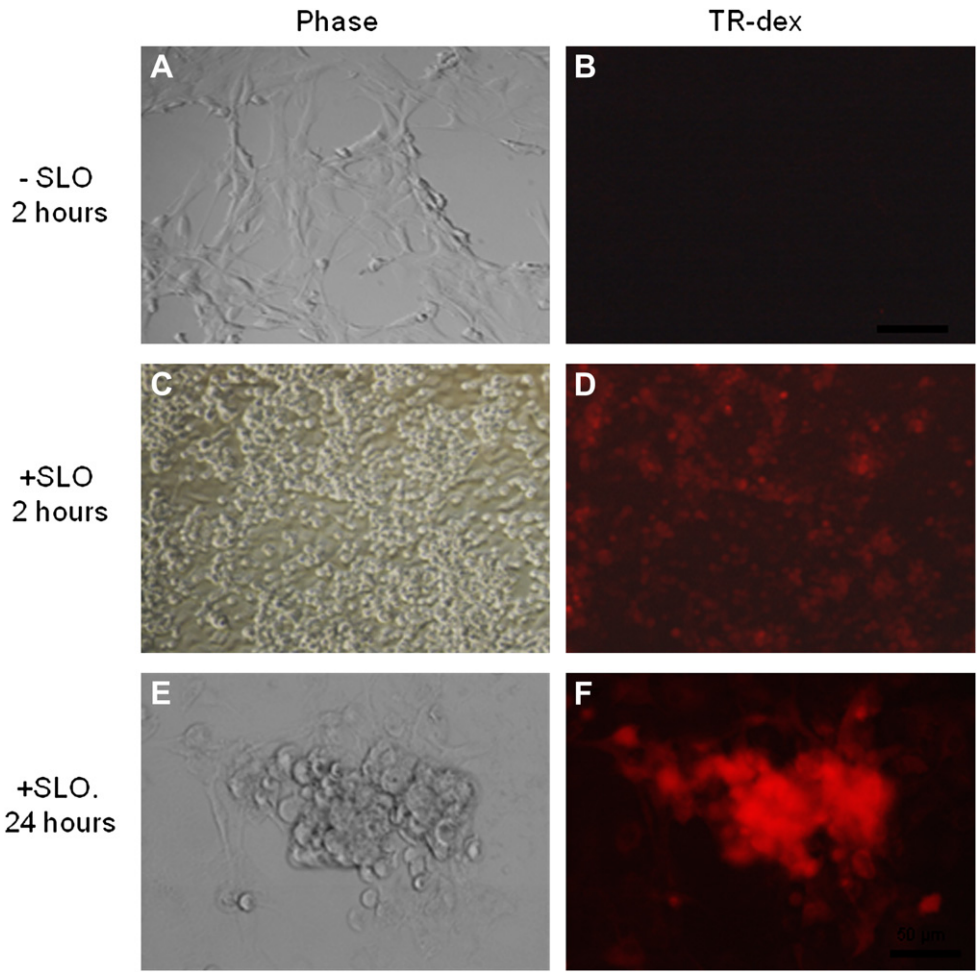


Fig. 3. Reversible cell permeabilization, uptake of a Texas red-conjugated dextran by IFPSCs. Intact IFPSC (−SLO) or SLO-permeabilized IFPSC (+SLO). Cells were incubated for 30 min in HBSS containing 50 µg/ml of Texas red-conjugated 70,000 *Mr* dextran, resealed with 2 mM $CaCl_2$ and cultured for 2 h and 24 h before observation by phase contrast and epifluorescence microscopy. Original magnification: 10× for A–D and 20× for E–F.

Transdifferentiated MSCs increased expression of chondrogenic markers

After 2 weeks of exposure to extracts, we analyzed the expression of collagen II by immunofluorescence. Extract exposed IFPSCs [Fig. 5(C, F and I)] showed a strong expression of collagen II protein with a similar pattern of cultured chondrocytes [Fig. 5(A, D and G)]. Collagen type II was confined in both cytoplasm and ECM. However, non-treated IFPSCs displayed a weak staining for the chondrogenic marker collagen II [Fig. 5(B, E and H)].

In addition, differential expression of selected chondrogenic key markers was evaluated by RT-PCR [Fig. 5(F)]. After 14 days, extract-treated cells showed increased gene expression for *Col2A1*, *Acan*, *Col10*, *L-Sox5*, *Sox6* and *Sox9*, in comparison with control IFPSCs, which showed a low expression for *Acan* and *L-Sox5* and a weak expression for *Col2A1*, *ColX*, and *Sox6*. Chondrocytes exhibited higher expression levels of these genes than exposed cells. No significant changes were observed in the expression of *Col1* in all samples. Interestingly, extract not exposed cells showed a basal expression for *Col2A1*, *Sox9*, *Acan*, *Sox6* and *ColX*.

Cell attachment and distribution in the scaffold

Chondrocytes, extract exposed cells and control cells were seeded in the cylindrical scaffold for 5 days and outside and inside layers of each scaffold were examined by ESEM [Fig. (6)]. All cells were able to attach on the polymer surface by filopodia and connected with each other. Chondrocytes acquired a predominantly spherical or sometimes fusiform and were surrounded with a dense matrix that extended between the cells to form a continuous network of cell and matrix [Fig. 6(A and B)]. In contrast, control non-exposed IFPSCs showed a flattened morphology with a high number of prolongations and secrete some dense material [Fig. 6(C)]. Moreover, the cross-section images clearly showed that these control cells were able to penetrate within scaffold [Fig. 6(D)]. Interestingly, extract exposed IFPSCs were able to grow covering the scaffold pores and to secrete a high dense extracellular matrix (ECM) that formed a homogeneously compact surface [Fig. 6(E and F)]. Furthermore, histological and immunohistochemical analyses of scaffold sections clearly showed cartilage-specific extracellular components produced by the IFPSCs exposed to the extract (Fig. 7).

Discussion

Mature hyaline cartilage has a very low self-repair potential due to its intrinsic properties. For this reason, researchers have focus in the search of methods to induce complete cartilage repair. In the last years, tissue engineering strategies combining cell therapy and scaffolds have emerged as a promising new approach for the treatment of articular cartilage defects[24,25].

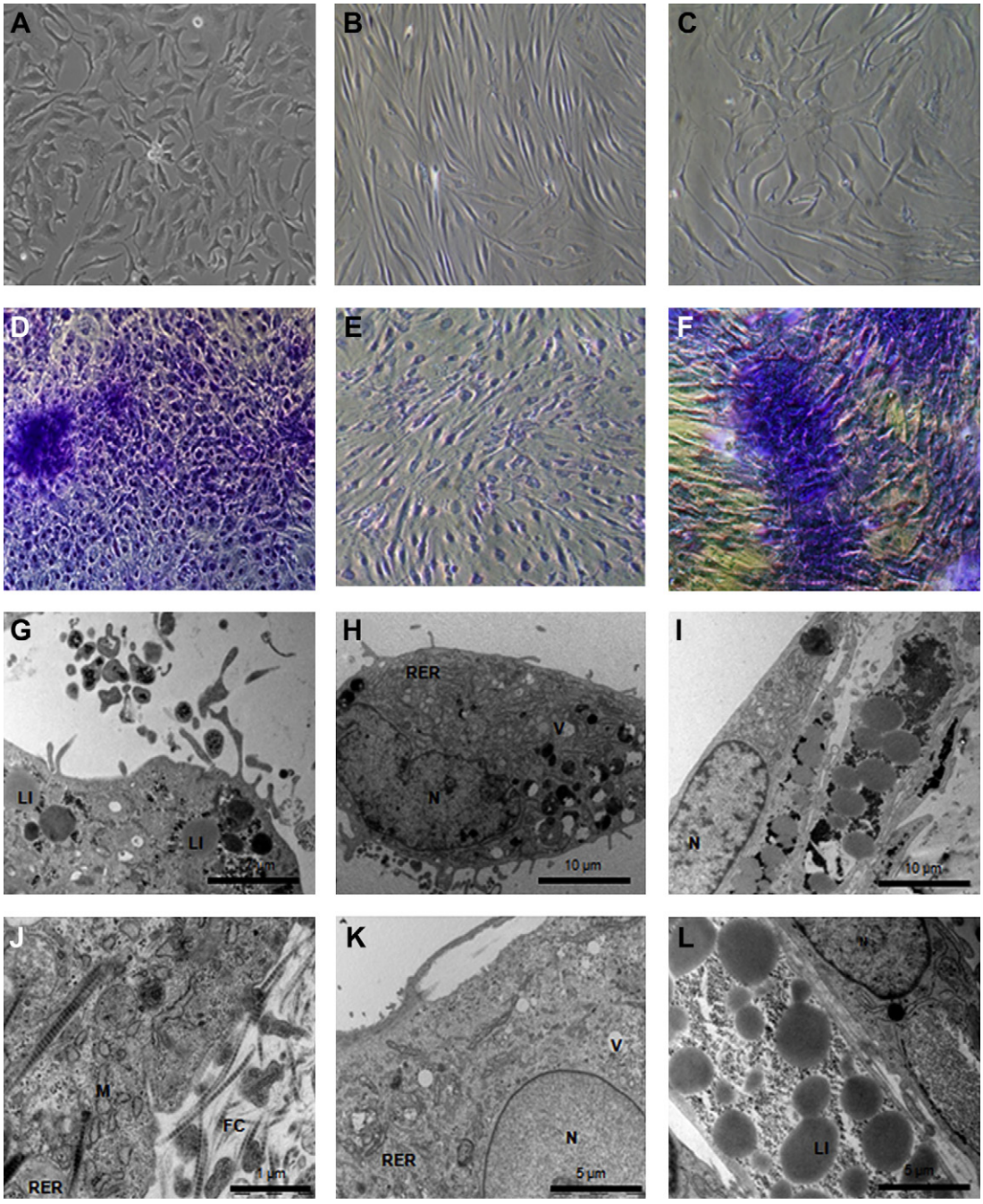
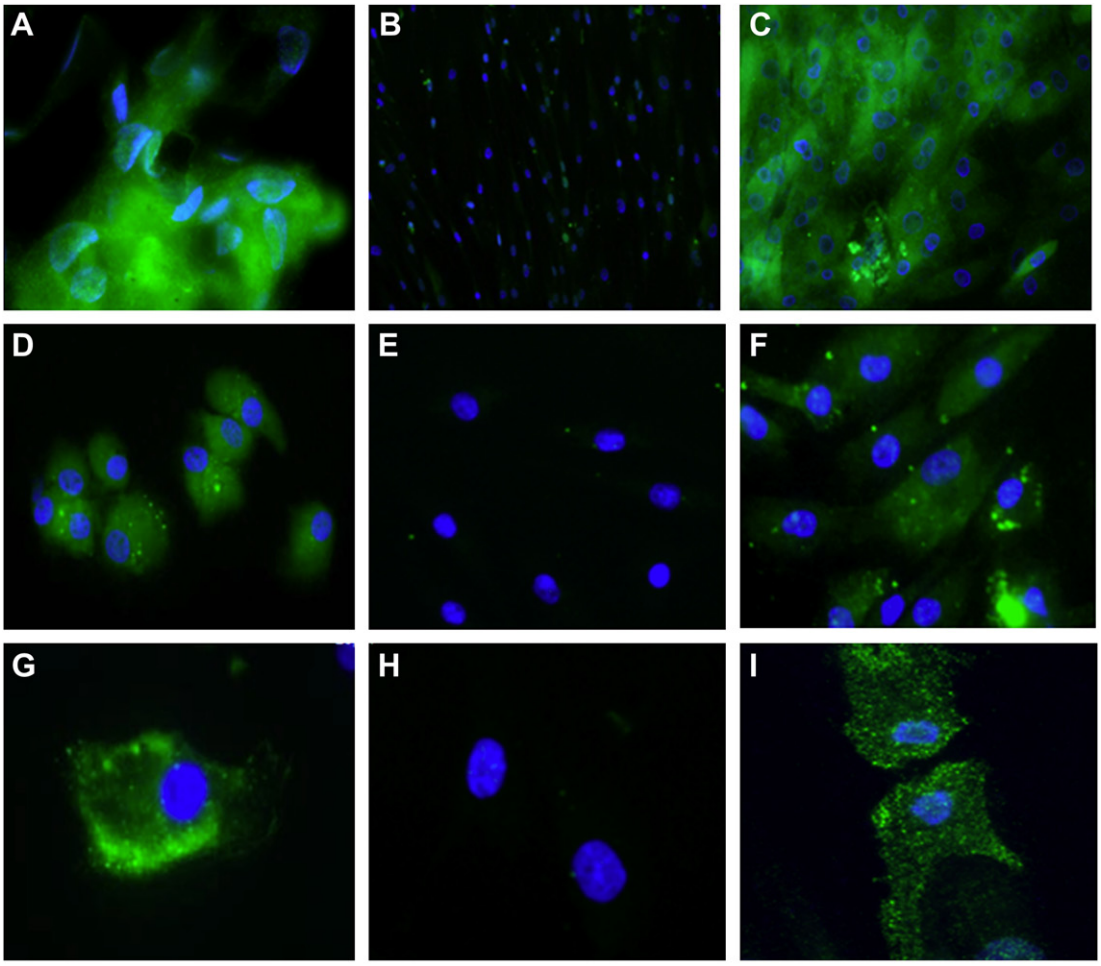


Fig. 4. Morphological analysis of control IFPSCs (permeabilized but not exposed to extract cells) and extract exposed cells. Phase-contrast light microscopy of chondrocytes (A), control IFPSCs (B) and extracts-treated IFPSCs (C). GAG synthesized was visualized by toluidine blue staining in chondrocytes (D) and in IFPSCs under chondrogenic differentiation (F), but not in control cells (E). TEM analysis of chondrocytes (G and J) showing a cytoplasm containing abundant RER, LI, proteoglycan granules and a homogeneous distribution of FC. Control IFPSCs with their complement of cytoplasmic organelles, such as RER, GA and vacuoles (H and K). Finally, IFPSCs exposed to the extract showed rounded-shaped lipid vesicles and proteoglycan granules in both cytoplasm and ECM (I and L). Original magnification 20× for A–F.

Cellular reprogramming based on cell extracts has shown that differentiated cells can be transdifferentiated in other differentiated cell types or dedifferentiated into pluripotent cells[17,19]. Moreover, we have demonstrated that using extracts from adult human heart tissue MSCs induced differentiation toward cardiomyocytes[26].

Recently, it has been shown that IFPSCs undergo chondrocyte differentiation, do not seem to have any age related decline in proliferative potential and are easily accessible with less discomfort to the patients[27,28]. In this work we analyze the chondrogenic potential of MSCs obtained from Hoffa's fat pad of patients with OA after the exposition to an extract, containing the intracellular components of chondrocytes.

We observed that human articular chondrocytes obtained from elderly patients maintained a chondrocyte phenotype in agreement with[29]. Chondrocytes and IFPSCs characterization by flow cytometry showed similar expression of MSCs surface makers CD90, CD105 and CD73. CD90 has been suggested as a new target to induce re-differentiation of dedifferentiated human chondrocytes in cartilage regeneration procedures and CD105 could be used as a molecular marker predictive of the capacity of cultured chondrocytes to form



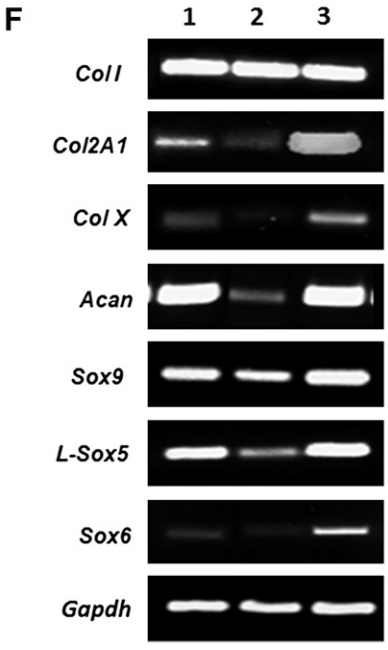


Fig. 5. Immunofluorescence of collagen II and gene expression of chondrogenic markers. Type II collagen indirect immunofluorescence of chondrocytes (A, D and G), control IFPSCs (B, E and H) and IFPSCs exposed to the extract (C, F and I). Expression of cartilage-specific collagen II protein with intense green staining can be appreciated on treated cells, showing the characteristic collagen fibers framework of cartilage. Original magnification 63× for A and G–I; 10× for B; 20× for C; 40× for D–F. RT-PCR analysis of chondrogenic markers (F). Two weeks in culture chondrocytes used as positive control (lane 3) and extracts-exposed IFPSCs (lane 1) showed increased gene expression for *Col2A1*, *Acan*, *Col10*, *L-Sox5*, *Sox6* and *Sox9*. Basal expression of these genes was seen in control IFPSCs (lane 2). Expression of *Gapdh* was used as an internal control. Experiments were performed in triplicate, were carried out at least twice and yielded similar results.

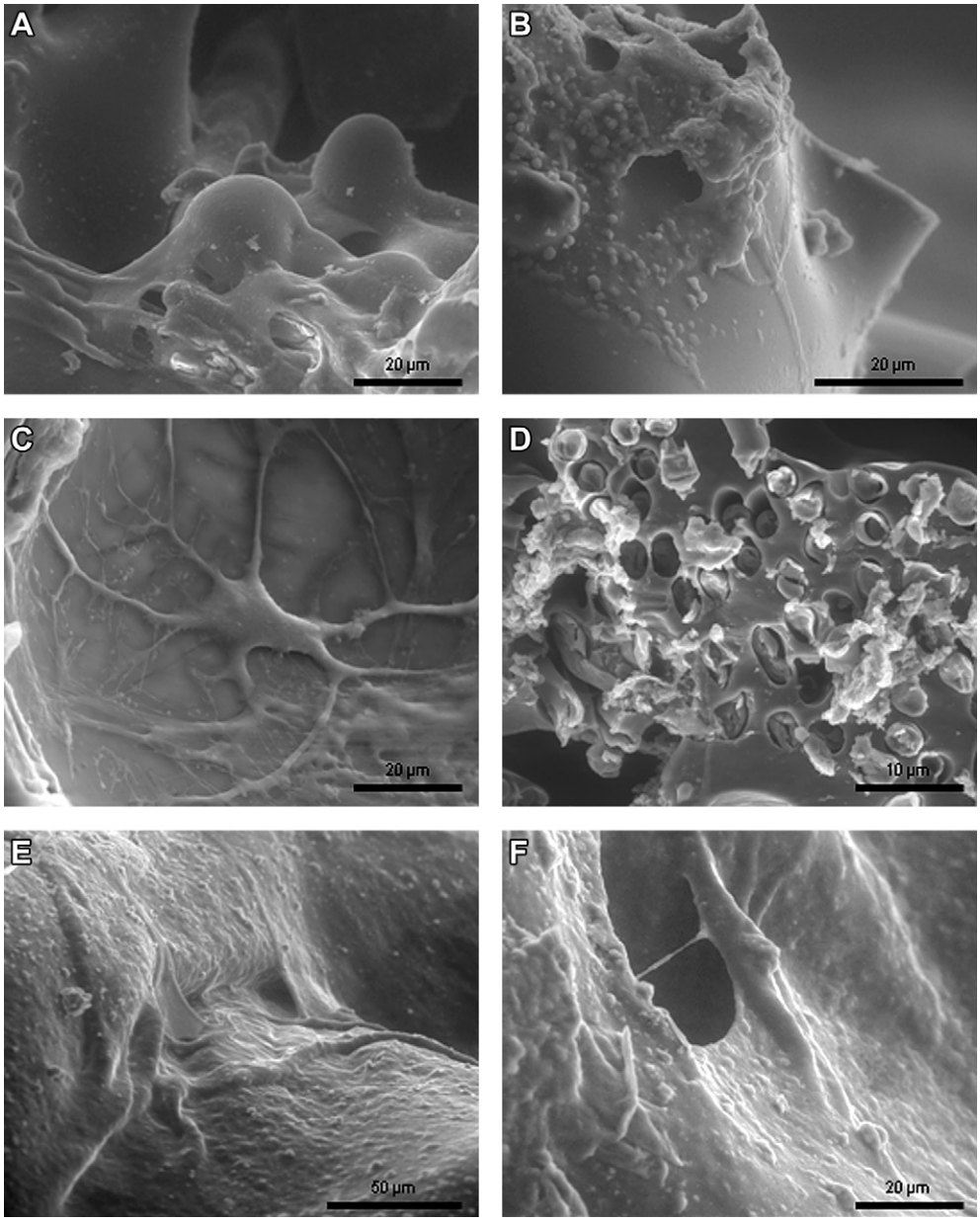


Fig. 6. Scanning electron micrograph of human of chondrocytes (A and B), control IFPSCs (C and D) and extracts-treated IFPSCs (E and F) after 5 days growing on the PLGA scaffold. Numerous cells firmly adhere to the scaffold and appeared to be suspended within the lumen or crawled around the walls. Chondrocytes show a round shaped morphology and a rough surface. Chondrocytes and extracts-treated IFPSCs appeared surrounded by matrix.

cartilage *in vivo*[30]. Also, collagen II was highly expressed in chondrocytes, and showed a basal expression in IFPSCs. In fact, mature articular joint develops from embryonic mesodermal precursors that differentiate into chondroprogenitors and ultimately into mature adult chondrocytes[31]. In addition, we found that IFPSCs were able to differentiate into specific lineages suggesting that fat pad cell population, as shown previously even in OA, contains a population of highly proliferative and multipotent MSCs[32].

IFPSCs exposed to chondrocyte extracts showed characteristic morphological changes suggesting the acquisition of a chondrocyte-like cell phenotype that was confirmed by the increased proteoglycan formation[33] in both toluidine staining and TEM observations, in agreement with chondrocytes typical features[34]. Further studies to demonstrate if the chondrocyte extract induce modifications in the expression of adipogenic and/or osteogenic markers will be of interest.

Collagen type II, a marker for hyaline cartilage together with aggrecan, are the predominant proteins in the ECM of cartilage[35]. Immunostaining of extracts-treated IFPSCs showed an increased expression of collagen II. Similarly, PCR analysis displayed an up-regulation of *Col2a1* and others chondrocyte-marker genes including *Sox9*, *L-Sox5* and *Acan*. *Sox9* is the master transcription factor for chondrogenesis, which acts in early stages of chondrocyte differentiation by directly induction of type II collagen[36] and is expressed in the mesenchymal condensations[37]. Moreover, it has been previously demonstrated that *Sox9* in concert with *L-Sox5* and *Sox6*, regulates cartilage formation and maintains the chondrocyte phenotype in the mature cartilage by activating expression of several cartilage-specific genes, including genes *Col2a1*, *Col9a1* and *Col11a1*, *Acan* and *Comp*[38]. In treated IFPSCs *Sox9* and its cofactor *L-sox5* expressions were elevated and a slightly induction of *Sox6* was found.

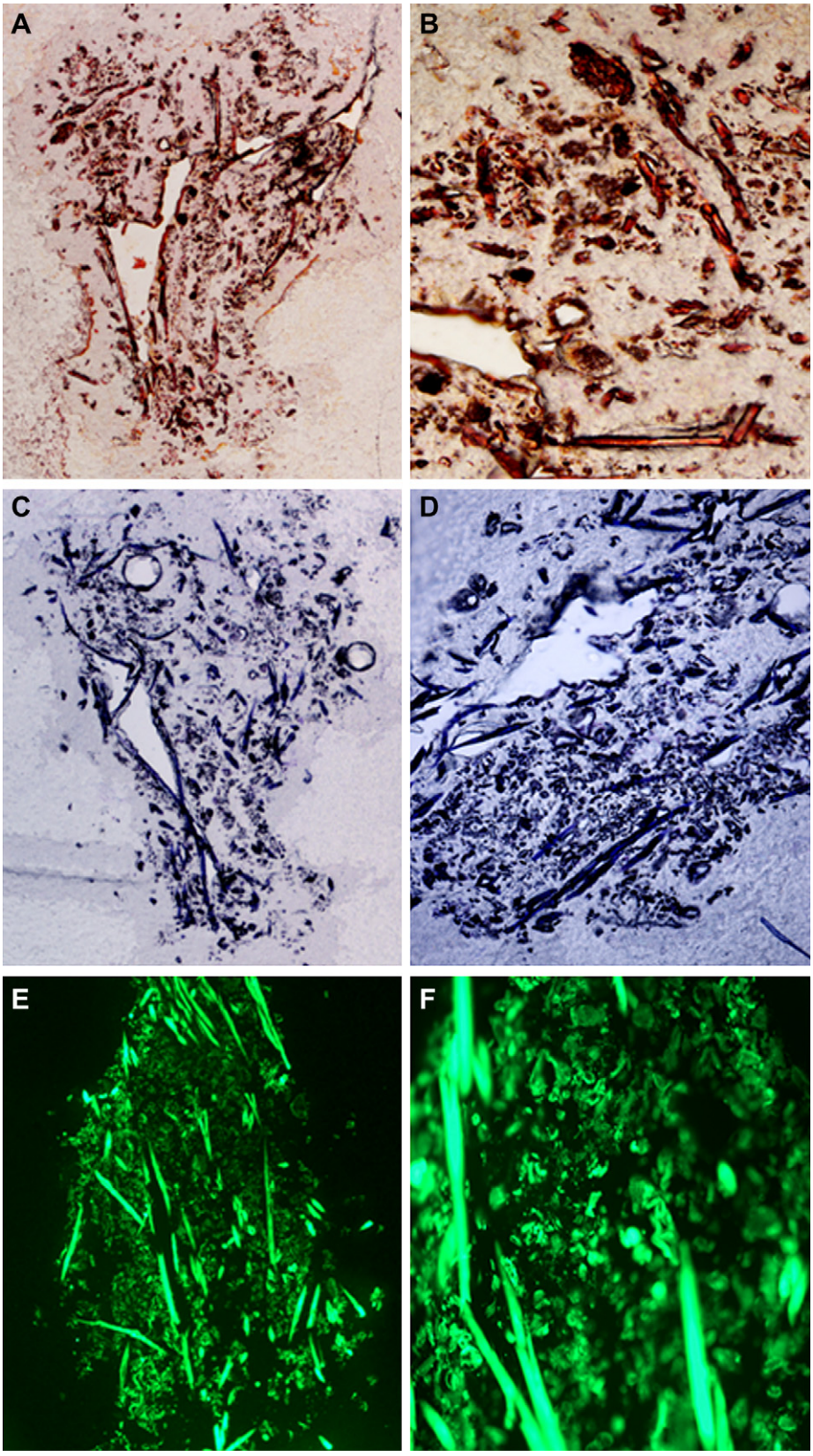


Fig. 7. Analysis for cartilage-specific extracellular components of PLGA scaffolds sections seeded with transdifferentiated IFPSCs. Safranin O staining (A and B) and toluidine blue staining (C and D) revealing *GAGs* production. Indirect immunofluorescence visualized in green for cartilage-specific collagen II protein (E and F). 5× original magnification for: A, C, E; 10× original magnification for: B and D; 20× F.

Some OA characteristics are the expression of hypertrophy markers such as collagen X with a concomitant decrease in the synthesis of type II collagen and aggrecan[39]. In our study chondrocytes isolated from patients with OA showed increased *Col10* expression, however IFPSCs exposed to the extract displayed a marked reduction. This fact together with the increased expression of *Sox9*, *L-Sox5*, *Sox6* and *Col2a1*, suggest the efficacy of this methodology to promote mature chondrogenesis. Nevertheless, the use of *Col10* mRNA as a marker of chondrogenic hypertrophy for *in vitro* studies has been questioned[40].

Type I collagen is categorized as a fibril-forming collagen and is usually produced when cells go into fibroblastic or osteoblastic differentiation[41]. In our experiment, *Col1a1* transcripts were constitutively expressed in chondrocytes and IFPSCs. After exposure to the extracts this expression was not affected. Type I collagen participates in regulating mesenchymal condensation and the onset of chondrogenic differentiation[42]. Moreover, *Col1* expression increase has been often closely related to chondrogenic differentiation *in vitro*[43]. The basal expression of characteristic chondrogenic markers in IFPSCs can be explained according to their micro-environmental niche. Recently, it has been demonstrated that non-cartilaginous knee joint tissues such as IFP possess significant chondrogenic potentials and this may be associated with the proximity to the niche they reside[31,44,45]. In addition, IFPSCs offer number of practical advantages such as they can be extracted from the same patient, avoiding rejection or side effects of immunosuppressive medication administration. Moreover, they are easily accessible, with less discomfort to the patient, as they can be obtained from osteoarthritic knee during knee arthroscopy. Furthermore, the fat pad has a greater yield of MSCs than bone marrow[46,47].

It has been shown that in comparison with monolayer 3-D matrices, which are developed to mimic the extracellular environment, could maintain better chondrocyte phenotypes and play a critical role in supporting chondrogenesis[24,48]. Therefore, we growth transdifferentiated IFPSCs in 3-D PLGA scaffolds, a cylindrical implant constructed with a porous cartilage phase which have been used before as cell-free for cartilage repair in surgery[49]. Nano-structured PLGA surfaces have been shown to accelerate chondrocyte adhesion and proliferation, as well as ECM production[50]. Our results showed that 3D PLGA scaffold was able to support growth and cell expansion and facilitate their free diffusion throughout the structure. These preliminary results demonstrated the affinity of IFPSCs for PLGA-based scaffolds and its ability to support chondrogenic differentiation.

Transdifferentiation studies have supported the notion that cell fate is controlled by master switch genes and that one or two factors can be sufficient to direct cells from one lineage to another[51]. Transdifferentation of the IFPSCs into chondrocytes can involve both the suppression and regulation of different genes in the cells, implying that genes from both cell types are co-expressed at some point. Further studies are necessaries to identify the factors and molecules presents in the extract that should enhance our understanding of the mechanisms involved in chondrocyte differentiation and development.

In conclusion, our results confirmed that extracts obtained from chondrocytes of osteoarthritic knees promote chondrogenic differentiation of IFPSCs. This chondrogenesis was not depended of exogenous growth factor induction, neither of the use of viral vectors. To our knowledge there have not been done experiments of IFPSCs programmed differentiation into chondrocytes using this methodology. Moreover, we show here, for the first time, that combination of transdifferentiated IFPSCs with biodegradable 3D PLGA scaffolds can serve as an efficient system for the maintenance and maturation of cartilage tissue. These findings encourage *in vivo* implantation studies to corroborate its usefulness to repair articular surface in OA.

Author contributions

ELR: design study, data acquisition, data analysis and interpretation, drafting the article, final approval of submitted manuscript. MP: design study, data acquisition, data analysis and interpretation, drafting the article, final approval of submitted manuscript. JCM: design study, data acquisition, drafting the article, final approval of submitted manuscript. GJ: data acquisition, data analysis and interpretation, drafting the article, final approval of submitted manuscript. MP: data acquisition, data analysis and interpretation, drafting the article, final approval of submitted manuscript. MB: data acquisition, data analysis, drafting the article, final approval of submitted manuscript. FA: data acquisition, data analysis, drafting the article, final approval of submitted manuscript. MCHL: data acquisition, data analysis, provision of study materials, drafting the article, final approval of submitted manuscript. ADDM: data analysis and interpretation, provision of patients, drafting the article, final approval of submitted manuscript. EM: design study, data acquisition, data interpretation, provision of patients, drafting the article, final approval of submitted manuscript. JAM: conception and design of the study, data acquisition, data analysis and interpretation, obtaining of funding, drafting the article, final approval of submitted manuscript.

Role of the funding source

This work was supported in part by grants from the Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, excellence project number CTS-6568) and the Instituto de Salud Carlos III (Fondo de Investigación Sanitaria, FEDER funds, grant number PI10/02295). We acknowledge the Junta de Andalucía through excellence project number CTS-6568 for providing a fellowship granted to GJ.

Conflict of interest

None of the authors have a conflict of interest to declare.

Acknowledgment

We gratefully acknowledge Isabel Sánchez-Almazo from the Centro de Instrumentación Científica for excellent technical assistance with ESEM studies.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2012.10.007.

References

1. Ronn K, Reischl N, Gautier E, Jacobi M. Current surgical treatment of knee osteoarthritis. Arthritis 2011;2011:454873.
2. Curl WW, Krome J, Gordon ES, Rushing J, Smith BP, Poehling GG. Cartilage injuries: a review of 31,516 knee arthroscopies. Arthroscopy 1997;13:456–60.
3. Groen JW, Stevens M, Kersten RF, Reininga IH, van den Akker-Scheek I. After total knee arthroplasty, many people are not active enough to maintain their health and fitness: an observational study. J Physiother 2012;58:113–6.
4. Hardingham TE, Oldershaw RA, Tew SR. Cartilage, SOX9 and Notch signals in chondrogenesis. J Anat 2006;209:469–80.
5. Ochi M, Uchio Y, Kawasaki K, Wakitani S, Iwasa J. Transplantation of cartilage-like tissue made by tissue engineering

in the treatment of cartilage defects of the knee. J Bone Joint Surg Br 2002;84:571–8.
6. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331:889–95.
7. de la Fuente R, Abad JL, Garcia-Castro J, Fernandez-Miguel G, Petriz J, Rubio D, *et al*. Dedifferentiated adult articular chondrocytes: a population of human multipotent primitive cells. Exp Cell Res 2004;297:313–28.
8. Ding DC, Shyu WC, Lin SZ. Mesenchymal stem cells. Cell Transplant 2011;20:5–14.
9. Im GI, Kim DY, Shin JH, Hyun CW, Cho WH. Repair of cartilage defect in the rabbit with cultured mesenchymal stem cells from bone marrow. J Bone Joint Surg Br 2001;83:289–94.
10. English A, Jones EA, Corscadden D, Henshaw K, Chapman T, Emery P, *et al*. A comparative assessment of cartilage and joint fat pad as a potential source of cells for autologous therapy development in knee osteoarthritis. Rheumatology (Oxford) 2007;46:1676–83.
11. Buckley CT, Vinardell T, Thorpe SD, Haugh MG, Jones E, McGonagle D, *et al*. Functional properties of cartilaginous tissues engineered from infrapatellar fat pad-derived mesenchymal stem cells. J Biomech 2010;43:920–6.
12. Jurgens WJ, van Dijk A, Doulabi BZ, Niessen FB, Ritt MJ, van Milligen FJ, *et al*. Freshly isolated stromal cells from the infrapatellar fat pad are suitable for a one-step surgical procedure to regenerate cartilage tissue. Cytotherapy 2009;11:1052–64.
13. Dragoo JL, Samimi B, Zhu M, Hame SL, Thomas BJ, Lieberman JR, *et al*. Tissue-engineered cartilage and bone using stem cells from human infrapatellar fat pads. J Bone Joint Surg Br 2003;85:740–7.
14. Peran M, Marchal JA, Rodriguez-Serrano F, Alvarez P, Aranega A. Transdifferentiation: why and how? Cell Biol Int 2011;35:373–9.
15. Phinney DG, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair – current views. Stem Cells 2007;25:2896–902.
16. Eberhard D, Tosh D. Transdifferentiation and metaplasia as a paradigm for understanding development and disease. Cell Mol Life Sci 2008;65:33–40.
17. Peran M, Sanchez-Ferrero A, Tosh D, Marchal JA, Lopez E, Alvarez P, *et al*. Ultrastructural and molecular analyzes of insulin-producing cells induced from human hepatoma cells. Cytotherapy 2011;13:193–200.
18. Hakelien AM, Gaustad KG, Collas P. Transient alteration of cell fate using a nuclear and cytoplasmic extract of an insulinoma cell line. Biochem Biophys Res Commun 2004;316:834–41.
19. Collas P, Taranger CK. Epigenetic reprogramming of nuclei using cell extracts. Stem Cell Rev 2006;2:309–17.
20. Marchal JA, Picon M, Peran M, Bueno C, Jimenez-Navarro M, Carrillo E, *et al*. Purification and long-term expansion of multipotent endothelial-like cells with potential cardiovascular regeneration. Stem Cells Dev 2012;21:562–74.
21. Hakelien AM, Gaustad KG, Collas P. Modulation of cell fate using nuclear and cytoplasmic extracts. Methods Mol Biol 2006;325:99–114.
22. 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.
23. Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. Circ Res 2007;100:1249–60.
24. Peran M, Garcia MA, Lopez-Ruiz E, Bustamante M, Jimenez G, Madeddu R, *et al*. Functionalized nanostructures with application in regenerative medicine. Int J Mol Sci 2012;13:3847–86.
25. Albrecht C, Tichy B, Nurnberger S, Hosiner S, Zak L, Aldrian S, *et al*. Gene expression and cell differentiation in matrix-associated chondrocyte transplantation grafts: a comparative study. Osteoarthritis Cartilage 2011;19:1219–27.
26. Peran M, Marchal JA, Lopez E, Jimenez-Navarro M, Boulaiz H, Rodriguez-Serrano F, *et al*. Human cardiac tissue induces transdifferentiation of adult stem cells towards cardiomyocytes. Cytotherapy 2010;12:332–7.
27. Khan WS, Adesida AB, Tew SR, Longo UG, Hardingham TE. Fat pad-derived mesenchymal stem cells as a potential source for cell-based adipose tissue repair strategies. Cell Prolif 2012;45:111–20.
28. Buckley CT, Vinardell T, Kelly DJ. Oxygen tension differentially regulates the functional properties of cartilaginous tissues engineered from infrapatellar fat pad derived MSCs and articular chondrocytes. Osteoarthritis Cartilage 2010;18:1345–54.
29. Varghese S, Theprungsirikul P, Sahani S, Hwang N, Yarema KJ, Elisseeff JH. Glucosamine modulates chondrocyte proliferation, matrix synthesis, and gene expression. Osteoarthritis Cartilage 2007;15:59–68.
30. Diaz-Romero J, Gaillard JP, Grogan SP, Nesic D, Trub T, Mainil-Varlet P. Immunophenotypic analysis of human articular chondrocytes: changes in surface markers associated with cell expansion in monolayer culture. J Cell Physiol 2005;202:731–42.
31. O'Sullivan J, D'Arcy S, Barry FP, Murphy JM, Coleman CM. Mesenchymal chondroprogenitor cell origin and therapeutic potential. Stem Cell Res Ther 2011;2:8.
32. Wickham MQ, Erickson GR, Gimble JM, Vail TP, Guilak F. Multipotent stromal cells derived from the infrapatellar fat pad of the knee. Clin Orthop Relat Res 2003;412:196–212.
33. Pabbruwe MB, Esfandiari E, Kafienah W, Tarlton JF, Hollander AP. Induction of cartilage integration by a chondrocyte/collagen-scaffold implant. Biomaterials 2009;30:4277–86.
34. Fioravanti A, Cantarini L, Chellini F, Manca D, Paccagnini E, Marcolongo R, *et al*. Effect of hyaluronic acid (MW 500–730 kDa) on proteoglycan and nitric oxide production in human osteoarthritic chondrocyte cultures exposed to hydrostatic pressure. Osteoarthritis Cartilage 2005;13:688–96.
35. Yamashita S, Andoh M, Ueno-Kudoh H, Sato T, Miyaki S, Asahara H. Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes. Exp Cell Res 2009;315:2231–40.
36. Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. Mol Cell Biol 1997;17:2336–46.
37. Lefebvre V, Li P, de Crombrugghe B. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO J 1998;17:5718–33.
38. Han Y, Lefebvre V. L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by securing binding of Sox9 to a far-upstream enhancer. Mol Cell Biol 2008;28:4999–5013.
39. Steinert AF, Proffen B, Kunz M, Hendrich C, Ghivizzani SC, Noth U, *et al*. Hypertrophy is induced during the in vitro chondrogenic differentiation of human mesenchymal stem cells by bone morphogenetic protein-2 and bone morphogenetic protein-4 gene transfer. Arthritis Res Ther 2009;11:R148.

40. Mwale F, Stachura D, Roughley P, Antoniou J. Limitations of using aggrecan and type X collagen as markers of chondrogenesis in mesenchymal stem cell differentiation. J Orthop Res 2006;24:1791–8.
41. Dessau W, von der Mark H, von der Mark K, Fischer S. Changes in the patterns of collagens and fibronectin during limb-bud chondrogenesis. J Embryol Exp Morphol 1980;57:51–60.
42. Chen CC, Liao CH, Wang YH, Hsu YM, Huang SH, Chang CH, *et al*. Cartilage fragments from osteoarthritic knee promote chondrogenesis of mesenchymal stem cells without exogenous growth factor induction. J Orthop Res 2012;30:393–400.
43. Tallheden T, Karlsson C, Brunner A, Van Der Lee J, Hagg R, Tommasini R, *et al*. Gene expression during redifferentiation of human articular chondrocytes. Osteoarthritis Cartilage 2004;12:525–35.
44. Toghraie FS, Chenari N, Gholipour MA, Faghih Z, Torabinejad S, Dehghani S, *et al*. Treatment of osteoarthritis with infrapatellar fat pad derived mesenchymal stem cells in rabbit. Knee 2011;18:71–5.
45. Buckley CT, Kelly DJ. Expansion in the presence of FGF-2 enhances the functional development of cartilaginous tissues engineered using infrapatellar fat pad derived MSCs. J Mech Behav Biomed Mater 2012;11:102–11.
46. Khan WS, Adesida AB, Hardingham TE. Hypoxic conditions increase hypoxia-inducible transcription factor 2alpha and enhance chondrogenesis in stem cells from the infrapatellar fat pad of osteoarthritis patients. Arthritis Res Ther 2007;9:R55.
47. Khan WS, Tew SR, Adesida AB, Hardingham TE. Human infrapatellar fat pad-derived stem cells express the pericyte marker 3G5 and show enhanced chondrogenesis after expansion in fibroblast growth factor-2. Arthritis Res Ther 2008;10:R74.
48. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215–24.
49. Melton JT, Wilson AJ, Chapman-Sheath P, Cossey AJ. TruFit CB bone plug: chondral repair, scaffold design, surgical technique and early experiences. Expert Rev Med Devices 2010;7:333–41.
50. Park GE, Pattison MA, Park K, Webster TJ. Accelerated chondrocyte functions on NaOH-treated PLGA scaffolds. Biomaterials 2005;26:3075–82.
51. Li WC, Yu WY, Quinlan JM, Burke ZD, Tosh D. The molecular basis of transdifferentiation. J Cell Mol Med 2005;9:569–82.

Cytotherapy, 2013; 15: 1541–1548

International Society for Cellular Therapy
ISCT®

Cellular extracts from post-mortem human cardiac tissue direct cardiomyogenic differentiation of human adipose tissue-derived stem cells

MACARENA PERÁN[1], ELENA LÓPEZ-RUIZ[1], LUCAS GONZÁLEZ-HERRERA[2], MILÁN BUSTAMANTE[3], AURORA VALENZUELA[2] & JUAN ANTONIO MARCHAL[4,5]

[1]Department of Health Sciences, University of Jaén, Spain, [2]Department of Forensic Medicine, Faculty of Medicine, University of Granada, Spain, [3]Biosciences Institute, University College Cork, Cork, Ireland, [4]Department of Human Anatomy and Embryology, Faculty of Medicine, University of Granada, Spain, and [5]Biopathology and Medicine Regenerative Institute (IBIMER), Granada, Spain

Abstract

Background aims. Human adipose tissue-derived stem cells (hASCs) can be easily (and inexpensively) expanded in culture, and their high plasticity allows their conversion to different cell types. We study the potential capacity of postmortem cardiac tissue to direct cardiac differentiation of hASCs *in vitro*. *Methods.* Cardiac tissue collected from autopsies was used to obtain cell extracts and conditioned medium, and both approaches were tested for cardiac induction. *Results.* Gene expression analyses proved that post-mortem human cardiac tissue maintains genetic integrity. hASCs exposed to the cell extracts or conditioned medium for 2 weeks achieved the appearance of myotube-like structures and were positive for cardiac markers such as sarcomeric α-actinin, cardiac troponin I and T and desmin as proved by immunofluorescence. In addition, differentiated cells showed increased expression of cardiomyocyte-related genes analyzed by reverse transcriptase polymerase chain reaction (*GATA-4*, myocyte-enhancer factor-2c, α-cardiac actin and cardiac troponin I). *Conclusions.* For the first time, post-mortem human cardiac tissue was used to induce hASC differentiation into myocardial-like cells. The methodology described here would serve as a useful model to obtain cardiomyocyte-like cells *in vitro*.

Key Words: *cardiomyogenic differentiation, cell extract, conditioned medium, human adipose stem cells, postmortem tissue*

Introduction

Related heart diseases are the major cause of death throughout the world (1). The development of new cardioactive drugs is a complicated process in which pre-clinical toxicity assays are a critical step. At the present time, cell-based screening assays are used to study disease mechanisms and to test the effect of novel drugs or gene treatments. Different cell sources have been used to obtain human cardiomyocyte-like cells. Studies using human embryonic stem cells (ESCs) have shown great potential for improving drug screening. The effect that some drugs produce in cardiomyocytes was similar to the effect produced in cardiomyocytes derived from ESCs (2,3). In addition, ESCs have proved to be a suitable model to test the development of cardiac arrhythmias as a pharmacologic side effect (4). To avoid ethical restrictions, induced pluripotent stem cells (iPSCs) have become the ideal alternative to ESCs. With the generation of disease-specific iPSC lines from patients with various diseases (5), new expectations have emerged, not only in basic research and regenerative medicine but also in a pharmacologic context. However, the use of ESCs or iPSCs is restricted to a few laboratories around the world, not only because of the difficulties secondary to a very restrictive legislation, which limit their use, but also, and even more important, because of the high cost of ESC and iPSC culture.

Previous studies showed the possibility of obtaining cardiomyocyte-like cells by the use of cell extracts from rat cardiomyocytes (6). More recently, our group demonstrated an improvement of this methodology using human cardiac tissue to direct differentiation of human adipose tissue-derived stem cells (hASCs) toward cardiomyocytes (7). In this study, the cardiac tissue was obtained from patients undergoing coronary artery bypass surgery, with limitations to obtaining large amounts of tissue. Post-mortem human cardiac tissue could be a suitable alternative; it has been demonstrated that robust gene

Correspondence: Dr **Macarena Perán**, Department of Health Sciences, University of Jaén, Jaén 23071, Spain. E-mail: mperan@ujaen.es

(Received 21 March 2013; accepted 22 June 2013)

http://dx.doi.org/10.1016/j.jcyt.2013.06.016

expression can be obtained using RNA from autopsy-derived tissue 24 hours after autolysis (8).

Mesenchymal stromal cells transplanted into infarcted myocardium have been shown to acquire the phenotypic characteristics of cardiomyocytes (9,10), suggesting paracrine (i.e., cytokine and chemokine) effects of the cardiac tissue in the implanted cells. Conditioned media, derived by *in vitro* culturing cells dissociated from the heart, have been proven to induce differentiation of bone marrow-derived mesenchymal stromal cells (11).

In this study, we used for the first time post-mortem cardiac tissue to induce hASC differentiation into myocardial-like cells. First, genetic integrity of human cardiac post-mortem tissue was evaluated by real-time polymerase chain reaction (PCR) analyses. Second, two different approaches were implemented: (i) cell extract method and (ii) conditioned medium. To confirm the cardiac phenotype achieved by the hASCs, immunofluorescence analyses were performed. In addition, gene expression was evaluated by reverse transcriptase PCR to determine the expression of several cardiac-specific makers.

Methods

Isolation and culture of hASCs

The hASCs used in this study were isolated from human adipose tissue and characterized as previously shown (12). Phenotypic characterization and differentiation potential of isolated hASCs are shown in supplementary Figure 1. Cells were cultured with high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Merelbeke, Belgium) at 37°C in a humidified atmosphere containing 5% CO_2.

Samples

Myocardial tissue samples were obtained from five forensic autopsies, ≤12 h post-mortem. Approximately 100 mg of tissue was taken in each case from the lateral wall of the left ventricle and was kept at −80°C until analysis. Each myocardial tissue sample was used individually.

RNA extraction and determination of quantity, quality and integrity

For molecular analysis, 30 mg of each myocardial tissue sample was taken. The frozen tissue samples were disrupted and homogenized using the TissueLyser LT (Qiagen, Hilden, Germany), and subsequently RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen). RNA quantity and quality were assessed by a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was evaluated using chip-based capillary electrophoresis with Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara CA, USA).

Reverse transcriptase and real-time PCR analysis

QuantiTect Reverse Transcription Kit (Qiagen) was used for complementary DNA synthesis. Real-time PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) on a 7500 device (Applied Biosystems, Foster City, CA, USA). For all samples, a relative quantitative gene expression analysis of cardiac troponin I (*TNNI3*), myosin light chain 3 (*MYL3*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was carried out in triplicate. Primers were commercially supplied by Qiagen (QuantiTect Primer Assay reference numbers: *TNNI3*, QT00084917; *MYL3*, QT00090223; *GAPDH*, QT01192646).

Differentiation of hASCs into cardiomyocyte-like cells by cell extract method

Cell extract method was performed as described before (7). Briefly, post-mortem cardiac tissue was disaggregated by physical and enzymatic methods. Isolated cells were washed twice in cold phosphate-buffered saline (PBS) and then in cold cell lysis buffer (50 mmol/L sodium chloride, 5 mmol/L magnesium chloride, 100 mmol/L *N*-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid, pH 8.2, 1 mmol/L dithiothreitol and 0.1 mmol/L phenylmethylsulfonyl fluoride; all Sigma-Aldrich). Cells were centrifuged at 800*g*, resuspended in 1.5 volumes of cell lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich) and allowed to swell on ice for 45 min. Cells were homogenized by pulse sonication, and the lysate was sedimented at 15,000*g* for 15 min at 4°C. The supernatant was collected and used fresh. For exposure to cell extract, hASCs were harvested by trypsin digestion and washed twice in ice-cold PBS and once in ice-cold Hank's Balanced Salt Solution (Gibco-BRL, Paisley, UK). Cells were pelleted at 300,000 cells/reaction in 1.5-mL tubes and suspended in 488-µL ice-cold Hank's Balanced Salt Solution, placed in a water bath at 37°C for 2 min and a final concentration of 230 ng/mL streptolysin O (Sigma-Aldrich) was added. Samples were incubated for 30 min at 37°C. Cells were centrifuged at 300*g* for 5 min at 4°C in a swing out rotor. The supernatant was removed, and 500 µL extract containing 20 µL 1 mmol/L of each nucleotide triphosphate set (Roche,

Indianapolis, Indiana, USA) was added. Cells were incubated with the extracts for 1 h at 37°C. DMEM/10% FBS containing 2 mmol/L calcium chloride was added, and the cells were transferred to 25 cm^2 culture flasks. Dead (floating) cells and the Ca^{2+}-containing medium were removed 4 h later and replaced with fresh DMEM containing 10% FBS and antibiotics. Damaged or dead cells, marked with trypan blue (Sigma-Aldrich), represented 15–20% of the total. Cells were cultured for 2 weeks until use. Control cells were either non-permeabilized and non-extract exposed cells or permeabilized cells not exposed to the extracts. Cell viability was assessed by phase contrast microscopy after 2 weeks of extract exposure by counting cells in four different regions of the dish and calculating the average.

Differentiation of hASCs into cardiomyocyte-like cells by conditioned medium

To prepare cardiac tissue-derived conditioned medium, 500–700 mg of post-mortem right ventricle tissue was cut into approximately 1-mm^3 pieces, and washed three times with PBS. Tissue fragments were placed into 75 cm^2 culture flasks containing 20 mL of DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin and cultured as explants. After 2 days, the medium was collected and centrifuged at 2000*g* for 10 min followed by filtering the supernatant through a 0.22-μm filtration unit (Millipore, Bedford, MA, USA). The medium was stored at 4°C until use. To induce cardiac differentiation of hASCs, 8×10^4 cells per well were plated on a six-well plate and incubated in cardiac tissue-derived conditioned medium for 2 weeks.

Immunofluorescence

Cells seeded on glass coverslips were washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were then permeabilized with 0.1% Triton X-100 (Sigma) for 15 min, washed three times with PBS and blocked in 2% blocking buffer solution (Roche) for 1 h at room temperature. Cells were incubated overnight in primary antibody diluted in blocking buffer solution at 4°C, washed three times in PBS and incubated for 2 h with secondary (fluorescein isothiocyanate-conjugated or tetramethylrhodamine isothiocyanate-conjugated; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies diluted 1:200 in blocking buffer solution. Afterward, they were washed three times in PBS, and the coverslips were mounted on slides with 4,6-diamino-2-phenylindole (DAPI)-containing mounting solution (Ultra Cruz mounting medium; Santa Cruz Biotechnology). Controls were performed with non-treated cells. Antibodies used were desmin (1:20, mouse monoclonal; Sigma), human cardiac-specific troponin I (1:100, mouse monoclonal; Research Diagnostics, Flanders, NJ, USA), human cardiac-specific troponin T (1:100, rabbit polyclonal; Abcam, Cambridge, UK) and sarcomeric α-actinin (1:200, mouse monoclonal; Sigma). Photographs were taken with a Leica DM 5500B (Leica Camera AG, Solms, Germany) fluorescent microscope, using Isis software (MetaSystems, Oberkochen, Germany). Figures were processed with Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

Reverse transcriptase PCR

After induction of differentiation, total RNA from treated and non-treated hASCs was extracted using an RNeasy Mini Kit (Qiagen). Complementary DNA synthesis was carried out from 1 μg of RNA sample using the Reverse Transcription System (Promega, Madison, WI, USA), and the PCR reaction was performed with ReddyMix PCR Master Mix (Thermo Fisher Scientific). After the initial denaturation (2 min at 94°C), 33 cycles were performed (30 s at 94°C, 50 s for annealing temperature and 1 min at 72°C) for all sets of primers except for β-actin, which was 25 cycles. Primer sequences and annealing temperatures were 5′ATCATGTTTGAGACCTTCAA3′ and 5′CATCTCTTGCTCGAAGTCCA3′ (45°C) for β-actin, 5′ATCTCTGCTGGCCATGAAAC3′ and 5′GATGAGGGAAGGTGGTTTGG3′ (53°C) for cardiac α-actin, 5′CCCTGCACCAGCCCCAATCAGA3′ and 5′CGAAGCCCAGCCCGGTCAACT3′ (64°C) for cardiac troponin I, 5′AGTGGTTTCCGTAGCAACTCCT3′ and 5′TAGTGCAAGCTCCCAACTGACT3′ (62°C) for *Mef2c* and 5′TCCCTCTTCCCTCCTCAAATTCA3′ and 5′GCGTGTAAAGGCATCTG3′ (52°C) for *GATA-4*. The PCR products were visualized on 1% agarose gels containing 0.1 mg/mL ethidium bromide using ultraviolet light.

Results and discussion

RNA integrity and gene expression analysis of human cardiac post-mortem tissues

First, we evaluated the quality and integrity of the RNA extracted from tissues 5–12 h post-mortem using the RNA Integrity Number (RIN). RIN is an algorithm based on a selection of features that provides information about the RNA integrity: (i) proportion of large molecules compared with smaller ones, (ii) the state of the degradation process and (iii) how far the degradation proceeded. The samples are assigned to 10 different categories ranging from

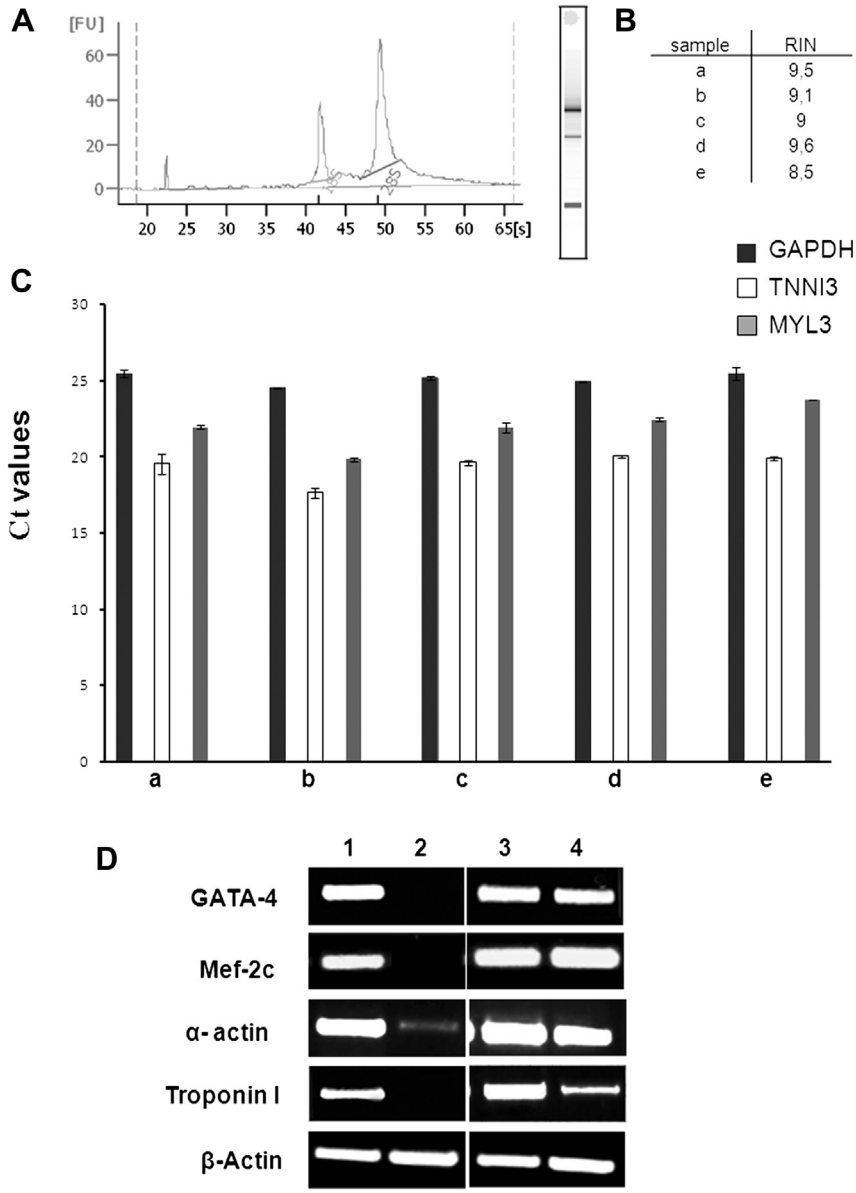


Figure 1. (A) Post-mortem cardiac tissue RNA integrity. Representative RIN graphic for RNA sample with RIN value of 9.5. (B) RIN values for all the samples. (C) Real-time PCR gene expression analysis of *TNNI3*, *MYL3* and *GAPDH* in five samples (a–e) obtained from post-mortem cardiac tissue. (D) Reverse transcriptase PCR analysis of expression of cardiomyocyte marker genes. Isolated cardiomyocytes used as a positive control (lane 1), control hASCs (lane 2), cardiomyocyte-conditioned medium treated cells (lane 3) and cells treated with the cardiomyocyte extracts (lane 4) expressing *GATA-4* (194 bp), *Mef-2c* (230 bp), cardiac α-actin (400 bp) and cardiac troponin I (233 bp). Results are representative of three independent experiments.

1 (worst) to 10 (best) (13). Our samples showed a high degree of RNA integrity with RIN values ranging from 9.5 to 8.5 and with a 260/280 ratio of 2 (Figure 1A). These findings demonstrated the viability of using post-mortem tissue as a reliable source of genetic material. Examination of gene expression levels at the time point of death has become an innovative tool in autopsies because RNA of adequate quality can be extracted from human post-mortem tissue (14,15).

Second, we performed gene expression analysis of cardiac markers such as *TNNI3* and *MYL3* by real-time PCR. The threshold cycle represents the PCR cycle at which an increase in SYBR Green fluorescence above a baseline signal can first be detected. Figure 1B shows threshold cycle values obtained for the genes *TNNI3*, *MYL3* and *GAPDH* in the five different autopsy samples. This result is indicative and demonstrates the maintenance expression of cardiac markers in the post-mortem tissue. Gene expression of beta-glucuronidase (*GUSB*), nitric oxide synthase 3 (*NOS3*), collagen 1 (*COL1A1*) and collagen 3 (*COL3A1*) has been previously shown using messenger RNA extracted from post-mortem

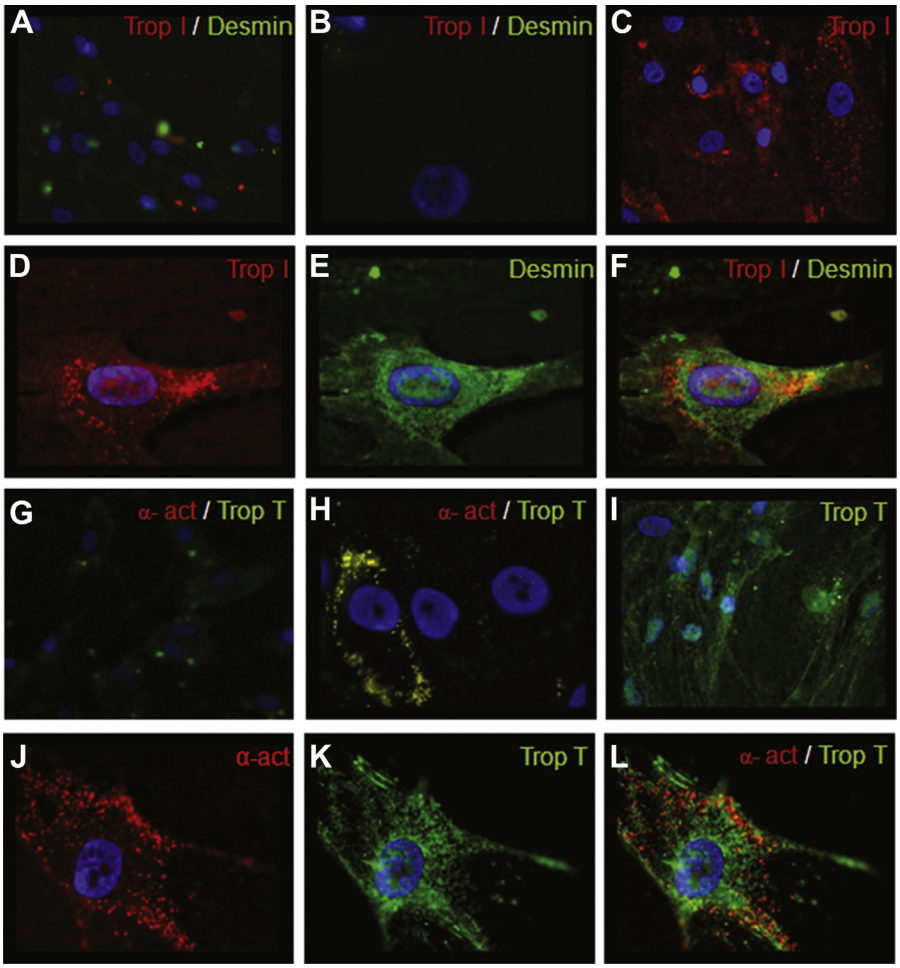


Figure 2. (A–L) Immunofluorescence analysis of cardiac markers in hASCs after extract induction. (C–F and I–L) Cardiomyogenic differentiation of hASCs after 2 weeks of being exposed to cellular extracts obtained from human autopsies. (A, B, G, H) Control non-treated cells. Red label for expression of troponin I (Trop I) and α-actinin (α-act) and green label for expression of desmin and troponin T (Trop T). Nuclei were stained with DAPI. (Original magnification ×40 for A, C, G and I and ×63 for B, D, E, F, H, J, K and L.)

cardiac tissue (16). Autopsy-derived tissue 24 h old seems to maintain genetic integrity and protein integrity and could represent a suitable tissue source for *in vitro* hASCs differentiation toward cardiomyocytes.

Expression of cardiac markers in hASCs after differentiation

In this study, we tested the cardiac differentiation potential of two different methodologies, both based on the use of post-mortem cardiac tissue. Gene expression analysis of messenger RNA levels and immunofluorescence studies revealed expression of cardiac-specific markers in hASCs after exposition to the conditioned medium or to the cardiac cellular extract.

Figure 1C shows cardiomyocyte-related gene expression after cardiomyogenesis induction. Expression of early (*GATA-4* or *Mef2-c*) and late (cardiac α-actin and cardiac troponin I) cardiomyocyte development markers was found in treated cells. *GATA4* and *Mef2c* are cardiac-specific transcription factors, which have been reported to be implicated in cardiac commitment and differentiation (17). In addition, the expression of sarcomeric α-actinin and troponin I, two cardiomyocyte-specific markers, has been proved to be restricted to cardiomyocytes rather than skeletal muscle (18).

Indirect immunofluorescence analyses of human cardiac troponins (I and T), sarcomeric α-actinin and desmin protein distribution revealed clear differences between treated and untreated hASCs (Figures 2, 3). After induction by the cellular extract or by the conditioned medium, we found a high percentage of cells (50–60%) that were positive for troponin I with elongated nuclei, as shown by DAPI staining (Figures 2C,D, 3D). Desmin expression was strongly positive, with a marked filament organization in treated hASCs (Figures 2E, 3C,E) compared with control cells (Figures 2A,B, 3A,B). Although there

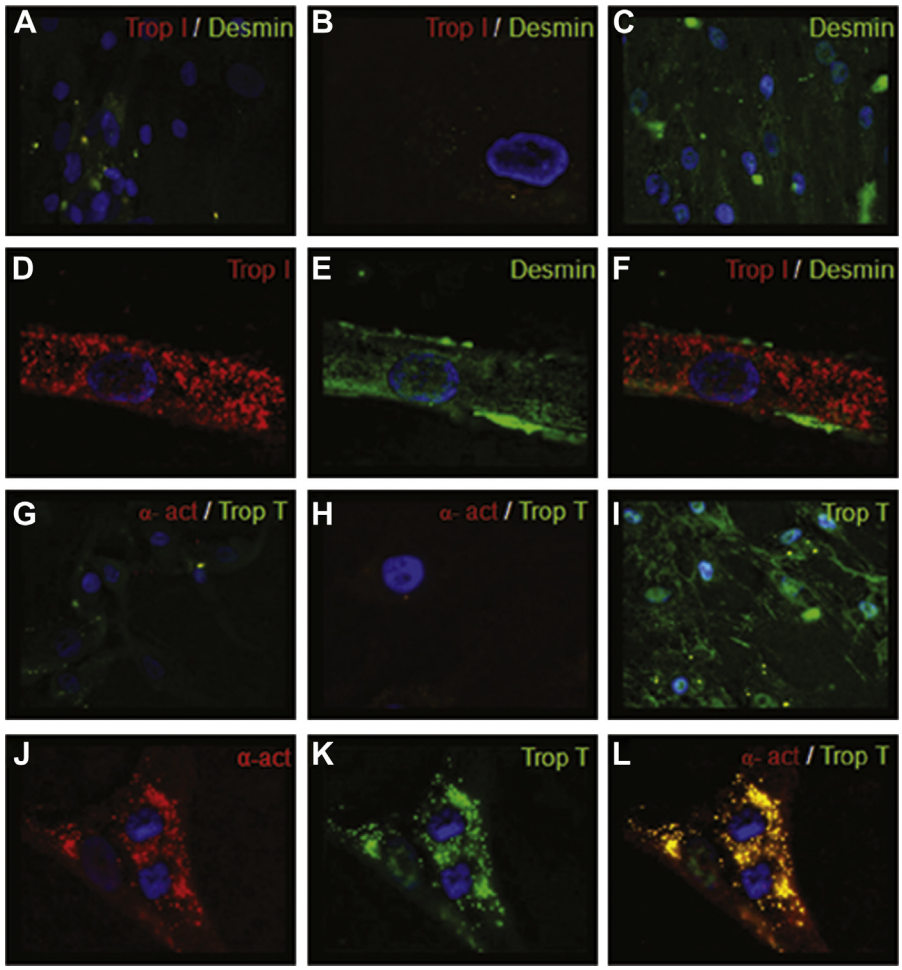


Figure 3. (A–L) Immunofluorescence detection of cardiac differentiation markers of hASCs cultured in conditioned medium. (C–F and I–L) hASCs after 2 weeks of being exposed to human cardiac explant-conditioned medium. (A, B, G, H) Control hASCs. Red label for expression of troponin I (Trop I) and α-actinin (α-act) and green label for expression of desmin and troponin T (Trop T). Nuclei were stained with DAPI. (Original magnification ×40 for A, C, G and I and ×63 for B, D, E, F, H, J, K and L.)

was a weak or non-significant staining for sarcomeric α-actinin in non-induced cells (Figures 2G,H, 3G,H), higher expression of this protein was observed in the exoskeleton of cells treated with both differentiation methods (Figures 2J, 3J). Finally, troponin T was highly expressed in treated cells exposed to the cardiac extract (Figure 2I,K) or cultured in conditioned medium (Figure 3I,K) with almost 65% of positive cells in both methods.

Morphologic changes were also noticeable after treatment with cardiac post-mortem tissue by both methodologies. Closer inspection of Figures 2 and 3 showed that treated cells extended their cytoplasmic processes with adjacent cells and achieved a myotube-like morphology, whereas control cells maintained their spindle-like morphology. In addition, multinucleated cells were detected after the induction (Figure 3J–L).

Cardiac tissue extracts or cell lysates from normal and infarcted myocardial tissue of rats have been proven to contribute to the differentiation of bone marrow-derived stromal cells into cardiomyocyte-like cells (19). These findings raise the hypothesis that soluble signaling molecules produced by cardiac cells after death are ideal inducers of hASC differentiation in myocardial-like cells. There have been 20 proteins including cytokines, growth factors and myocardial related proteins identified to be released into the culture medium by human cardiac explants (20). In our study, we demonstrated that the post-mortem cardiac tissue maintains its integrity and provides the paracrine mechanisms necessary to promote cardiomyogenic differentiation without direct cell-to-cell contact between cardiomyocytes and hASCs.

Previous studies have shown that cells can be isolated from post-mortem tissues (21,22). Viable hepatocytes can be isolated from cadaveric human liver after 24 h of cold ischemia (21). In addition, stem cells isolated from post-mortem tissues (48 h after death) are currently used for experimental and clinical purposes (23–27). Viable and functional

skeletal myogenic cells from humans 17 days post-mortem have been isolated (28).

It has been suggested that cardiomyocytes (29,30) and cardiac fibroblasts (31) secrete cytokines and chemokines in response to various stimuli, such as ischemia or mechanical stress to the heart. Post-mortem cardiac tissue might respond to the stress caused by the natural cardiac arrest and the hypoxic state that precedes natural death liberating factors. Ischemic myocardium produces several cytokines or transcription factors such as vascular endothelial growth factor and stromal cell derived factor that promote and increase stem cell survival (32) and, as has been proven *in vivo* and *ex vivo*, that induce differentiation of mesenchymal stromal cells into cardiomyocyte-like cells (33–35).

In conclusion, the results of the present study show that post-mortem cardiac tissue can induce hASCs to express cardiac-specific contractile proteins *in vitro*. This study confirms that the soluble signaling molecules produced by cardiac cells after death are also ideal inducers of hASC differentiation into myocardial-like cells. The methodology described here would serve as a useful and inexpensive *in vitro* model to obtain cardiomyocyte-like cells that could be used for cardioactive drug toxicity assays.

Acknowledgments

This work was supported in part by grants from the Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, excellence project number CTS-6568).

Disclosure of interest: The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

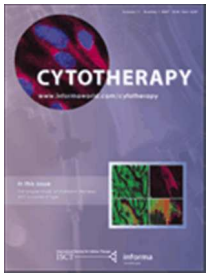
References

1. Mendis S, Puska P, Norrving B, editors. Global atlas on cardiovascular disease prevention and control. Geneva: World Health Organization; 2011.
2. He JQ, Ma Y, Lee Y, Thomson JA, Kamp TJ. Human embryonic stem cells develop into multiple types of cardiac myocytes. Circ Res. 2003;93:32–9.
3. Reppel M, Boettinger C, Hescheler J. Beta-adrenergic and muscarinic modulation of human embryonic stem cell-derived cardiomyocytes. Cell Physiol Biochem. 2004;14:187–96.
4. Caspi O, Itzhaki I, Kehat I, Gepstein A, Arbel G, Huber I, et al. In vitro electrophysiological drug testing using human embryonic stem cell derived cardiomyocytes. Stem Cells Dev. 2009;18:161–72.
5. Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, et al. Disease-specific induced pluripotent stem cells. Cell. 2008;134:877–86.
6. 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.
7. Perán M, Marchal JA, López E, Jiménez-Navarro M, Boulaiz H, Rodríguez-Serrano F, et al. Human cardiac tissue induces transdifferentiation of adult stem cells towards cardiomyocytes. Cytotherapy. 2010;12:332–7.
8. Gupta S, Halushka MK, Hilton GM, Arking DE. Postmortem cardiac tissue maintains gene expression profile even after late harvesting. BMC Genomics. 2012;13:26.
9. Wang JS, Shum-Tim D, Galipeau J, Chedrawy E, Eliopoulos N, Chiu RC. Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential clinical advantages. J Thorac Cardiovasc Surg. 2000;120:999–1005.
10. Sun CK, Chang LT, Sheu JJ, Chiang CH, Lee FY, Wu CJ, et al. Bone marrow-derived mononuclear cell therapy alleviates left ventricular remodeling and improves heart function in rat-dilated cardiomyopathy. Crit Care Med. 2009;37:1197–205.
11. Xie XJ, Wang JA, Cao J, Zhang X. Differentiation of bone marrow mesenchymal stem cells induced by myocardial medium under hypoxic conditions. Acta Pharmacol Sin. 2006;27:1153–8.
12. López-Ruiz E, Perán M, Cobo-Molinos J, Jiménez G, Picón M, Bustamante M, et al. Chondrocytes extract from patients with osteoarthritis induces chondrogenesis in infrapatellar fat pad-derived stem cells. Osteoarthritis Cartilage. 2013;21:246–58.
13. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol. 2006;31:7–3.
14. Heinrich M, Matt K, Lutz-Bonengel S, Schmidt U. Successful RNA extraction from various human postmortem tissues. Int J Legal Med. 2007;121:136–42.
15. Vennemann M, Koppelkamm A. mRNA profiling in forensic genetics I: possibilities and limitations. Forensic Sci Int. 2010; 203:71–5.
16. Partemi S, Berne PM, Batlle M, Berruezo A, Mont L, Riuró H, et al. Analysis of mRNA from human heart tissue and putative applications in forensic molecular pathology. Forensic Sci Int. 2010;203:99–105.
17. Kodama H, Hirotani T, Suzuki Y, Ogawa S, Yamazaki K. Cardiomyogenic differentiation in cardiac myxoma expressing lineage-specific transcription factors. Am J Pathol. 2002;161: 381–9.
18. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest. 1999;103:697–705.
19. Liu XN, Yin Q, Zhang H, Zhang H, Zhu SJ, Wei YJ, et al. Tissue extracts from infarcted myocardium of rats in promoting the differentiation of bone marrow stromal cells into cardiomyocyte-like cells. Biomed Environ Sci. 2008;21: 110–7.
20. Schittini AV, Celedon PF, Stimamiglio MA, Krieger M, Hansen P, da Costa FD, et al. Human cardiac explant-conditioned medium: soluble factors and cardiomyogenic effect on mesenchymal stem cells. Exp Biol Med (Maywood). 2010;235:1015–24.
21. Erker L, Azuma H, Lee AY, Guo C, Orloff S, Eaton L, et al. Therapeutic liver reconstitution with murine cells isolated long after death. Gastroenterology. 2010;139:1019–29.
22. Machalinski B, Paczkowski M, Kawa M, Paczkowska E, Ostrowski M. An optimization of isolation of early hematopoietic cells from heparinized cadaveric organ donors. Transplant Proc. 2003;35:3096–100.
23. Blazar BR, Lasky LC, Perentesis JP, Watson KV, Steinberg SE, Filipovich AH, et al. Successful donor cell engraftment in a recipient of bone marrow from a cadaveric donor. Blood. 1986;67:1655–60.
24. Kapelushnik J, Aker M, Pugatsch T, Samuel S, Slavin S. Bone marrow transplantation from a cadaveric donor. Bone Marrow Transplant. 1998;21:857–8.

25. Liu X, Zhu Y, Gao W. Isolation of neural stem cells from the spinal cords of low temperature preserved abortuses. J Neurosci Methods. 2006;157:64–70.
26. Palmer TD, Schwartz PH, Taupin P, Kaspar B, Stein SA, Gage FH. Cell culture: progenitor cells from human brain after death. Nature. 2001;411:42–3.
27. Xu Y, Kimura K, Matsumoto N, Ide C. Isolation of neural stem cells from the forebrain of deceased early postnatal and adult rats with protracted post mortem intervals. J Neurosci Res. 2003;74:533–40.
28. Latil M, Rocheteau P, Châtre L, Sanulli S, Mémet S, Ricchetti M, et al. Skeletal muscle stem cells adopt a dormant cell state post mortem and retain regenerative capacity. Nat Commun. 2012;3:903.
29. Kacimi R, Karliner JS, Koudssi F, Long CS. Expression and regulation of adhesion molecules in cardiac cells by cytokines: response to acute hypoxia. Circ Res. 1998;82:576–86.
30. Shen JG, Quo XS, Jiang B, Li M, Xin W, Zhao BL. Chinonin, a novel drug against cardiomyocyte apoptosis induced by hypoxia and reoxygenation. Biochim Biophys Acta. 2000; 1500:217–26.
31. Porter KE, Turner NA. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol Ther. 2009;123: 255–78.
32. Leu S, Kao YH, Sun CK, Lin YC, Tsai TH, Chang LT, et al. Myocardium-derived conditioned medium improves left ventricular function in rodent acute myocardial infarction. J Transl Med. 2011;9:11–29.
33. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation. 2002;105:93–8.
34. Elmadbouh I, Haider H, Jiang S, Idris NM, Lu G, Ashraf M. Ex vivo delivered stromal cell-derived factor-1alpha promotes stem cell homing and induces angiomyogenesis in the infarcted myocardium. J Mol Cell Cardiol. 2007;42:792–803.
35. Das H, George JC, Joseph M, Das M, Abdulhameed N, Blitz A, et al. Stem cell therapy with overexpressed VEGF and PDGF genes improves cardiac function in a rat infarct model. PLoS One. 2009;4:e7325.

Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jcyt.2013.06.016



Cardiomyogenic differentiation potential of human endothelial progenitor cells isolated from myocardial infarction patients

Journal:	*Cytotherapy*
Manuscript ID:	Draft
Article Type:	Short Communication
Date Submitted by the Author:	n/a
Complete List of Authors:	López Ruiz, Elena; University of Jaén, Department of Health Sciences Peran, Macarena; Jaén University, Health Science Picón, Manuel; University of Miami, Braman Family Breast Cancer Institute, Sylvester Comprehensive Cancer Center García, María-Angel; Virgen de las Nieves University Hospital, Oncology Carrillo, Esmeralda; University of Granada, Human Anatomy and Embryology Jiménez Navarro, Manuel; Heart Unit, University Hospital ''Virgen de la Victoria'', Hernandez, M Carmen; Banco de Cordón Umbilical de Andalucia, Prats, Isidro; Cord Blood Bank, Centro Regional de Transfusión Sanguínea, de Teresa, Eduardo; Heart Unit, University Hospital ''Virgen de la Victoria'', Marchal, Juan-Antonio; University of Granada, Human Anatomy and Embriology
Keywords:	AMI-EPCs, UCB-EPCs, cardiomyogenic differentiation, 5-azacytidine

Cardiomyogenic differentiation potential of human endothelial progenitor cells isolated from myocardial infarction patients

Running title: Cardiomyogenic potential of AMI-EPCs

LÓPEZ-RUIZ E[1,*], PERÁN M[1,2,*], PICÓN-RUIZ M[2,3], GARCÍA MA[4,5], CARRILLO E[2,6], JIMÉNEZ-NAVARRO M[7], HERNÁNDEZ MC[8], PRATS I[8], DE TERESA E[7], MARCHAL JA[2,5,6, †]

[1]Department of Health Sciences, University of Jaén, Jaén, Spain; [2]Biopathology and Medicine Regenerative Institute (IBIMER), University of Granada, Granada, Spain; [3]Braman Family Breast Cancer Institute, Sylvester Comprehensive Cancer Center, University of Miami, Miller School of Medicine, Miami, FL, USA; [4]Department of Oncology, Virgen de las Nieves, University Hospital, Granada, Spain; [5]Biosanitary Institute of Granada (ibs.GRANADA), Granada, Spain; [6]Department of Human Anatomy and Embryology, University of Granada, Granada, Spain; [7]Heart Unit, University Hospital ''Virgen de la Victoria'' Malaga, Spain; [8]Cord Blood Bank, Centro Regional de Transfusión Sanguínea, Málaga, Spain.

***** *This authors equally contributed to this work*

[†]**Address correspondence**: Prof. Juan Antonio Marchal, MD, PhD, E-mail: jmarchal@ugr.es. Phone number: +34958249321.

.

Abstract

Background aims. Endothelial progenitors cells (EPCs) are known to play a beneficial role by promoting postnatal vasculogenesis in pathological events, such as isquemic heart disease and peripheral artery disease. However, little is known about the potential of EPCs to restore heart damage tissue. Here we compared the cardiac differentiation capacity of EPCs isolated from peripheral blood (PB) of patients suffering from acute myocardial infarction (AMI) with EPCs obtained from umbilical cord blood (UCB).

Methods. EPCs from both origins were isolated by density gradient centrifugation and them, characterized using endothelial markers (UEA-1 lectin, CD133, and KDR) and endothelial cell colony-forming units (CFU-EC) assay. Cardiac differentiation capacity of EPCs was assessed by immunofluorescence and RT-PCR after 5-azacytidine (5-aza) induction.

Results. No significant differences were observed between the number of CFU-EC in PB of AMI patients and samples from UCB. Moreover, 5-aza induced the appearance of myotube-like structures and the positive expression of sarcomeric α-actinin, cardiac troponin I and T and desmin in a similar pattern for both cell sources, indicating a comparable acquisition of a cardiac phenotype.

Conclusion. For the first time we have compared the cardiomyogenic *in vitro* potential of EPCs derived from AMI patients and from UCB. Our data indicate that EPCs obtained from both origins have similar plasticity and functions and suggest their therapeutic efficacy in cardiac cell therapy.

Key Words: AMI-EPCs, UCB-EPCs, cardiomyogenic differentiation, 5-azacytidine.

Introduction

Myocardial infarction is the result of coronary arteries obstruction with the consequently reduction of blood supply to the heart muscle and the massive loss of cardiomyocytes that are replaced by a non-functional scar tissue [1]. Asahara et al (1997) showed for the first time that peripheral blood (PB) contains $CD34^+$ bone marrow (BM)-derived cells, called as endothelial progenitor cells (EPCs) which can be characterized by the expression of several markers such as CD133, CD34, KDR or VE-cadherin [2, 3]. These progenitor cells have the potential to differentiate into mature endothelial cells (EC) and to play a role in promoting postnatal vasculogenesis even in pathological events that require neovascularization [4-6]. The significantly increased of progenitor cells following myocardial infarction [3, 7, 8], indicate that circulating EPCs play an important role in the pathogenesis and prognosis of cardiovascular diseases [9-12].

The *in vitro* and *in vivo* angiogenic properties of EPCs isolated from circulating PB and umbilical cord blood (UCB) have been shown before [13, 14]. Recent studies in animal models of ischemic cardiomyopathy suggest that transplanted EPCs can improve heart recovery after injury [15-17]. Moreover, improvement in myocardial function after transplantation of autologous BM-derived stem/progenitor cells including EPCs, have been reported in clinical trials [18], nevertheless a long-term follow-up of these patients still needs to be evaluated. Others studies suggest that EPCs isolated from mouse BM and from human PB could differentiate into cardiomyocytes under certain *in vitro* conditions [19-21]. In summary, although it has been demonstrated that EPCs play a beneficial role in treatment of ischemic disease, the potential of circulating EPCs from patients that have suffered an infarct to restore heart damage tissue need further investigation. Here, we have tested the cardiac differentiation potential of circulating

EPCs from PB of patients who have been diagnosed of acute myocardial infarction (AMI) and compared with EPCs from UCB.

Materials and Methods

Patients

We studied 24 patients admitted with a diagnosis of AMI, defined as an acute coronary syndrome with ST elevation (STEMI) with suggestive chest pain and an elevation of at least 3 mm in the ST segment in at least three precordial leads, with no history of ischemic heart disease and within 8 h of symptoms. Preinfarction angina was defined as the presence of at least one chest pain < 30 min, the week before the onset of the infarction. Patients were excluded if they had had chest pain compatible with angina for more than 1 week before the infarction or if they had underlying structural heart disease (cardiomyopathy or important valve disorders). Moreover, patients with history of rheumatoid arthritis, hepatic, hematological, or coagulation disorders, cancer or other acute or chronic inflammatory diseases such diabetes mellitus were not included in the study. Informed consents were obtained from all patients and heparinized blood samples (20 ml) were drawn from all subjects. Human UCB samples (n=25) were obtained from the Centro Regional de Transfusión Sanguínea y Tejidos de Málaga, Málaga, Spain, according to institutional guidelines. Samples were generally processed within 24-48 h of collection. Myocardial tissue samples were obtained from forensic cadaver autopsies no more than 12 hours postmortem as described previously [22].

Quantification of endothelial markers in mononuclear cells

Mononuclear cells (MNC) isolated by density gradient centrifugation with Histopaque-1077 from PB of AMI patient and from UCB, were washed and resuspended in phosphate-buffered saline (PBS) with 2% bovine serum albumin (Sigma), and 2mM ethylene diamine tetra acetic acid (EDTA, Sigma). Cells were incubated in the dark at 4ºC for 45 min with the following fluorochrome-conjugated monoclonal antibodies: CD133-PE (Miltenyi), KDR-APC (R&D System, Minneapolis, MN), CD34-FITC, and CXCR4-APC (BD Biosciences, San Jose, CA). Cells were then washed in PBS and analyzed in a fluorescence activated cell sorting (FACS) Canto II cytometer equipped with the FACS Diva analysis software (BD Biosciences). Data obtained were expressed as mean ± standard error (SE) from 4 independent experiments performed in triplicate ($P<0.05$).

Isolation and culture of EPCs

Samples of PB from AMI patient and from UCB were processed by density-gradient centrifugation with Histopaque-10771 (Sigma) for 25 minutes at 1500 r/min and washed three times in phosphate-buffered saline (PBS) with 2% FBS. Cells were further processed as described before [23]. Briefly, 5×10^6 isolated MNC were plated per well of a fibronectin coated 6-well dishes (BD) in Endothelial Cell Basal Medium, EBM-2 (Clonetics) supplemented with EGM-2 Single Quot Kit (Clonetics) which contain Hydrocortisone, hFGF-B, VEGF, R3-IGF-1, Ascorbic Acid, Heparin, FBS, hEGF and GA-1000. After 2 days of culture non-adherent cells were re-plated on fibronectin coated 24-well dished (BD) at a density of 1×10^6/well with 1 ml of fresh complete endothelial growth media, then cells were incubated at 37ºC at 5% CO2 with 95% humidity.

The numbers of colonies were counted under a phase-contrast microscope at days 5 and 10 of culture. Five randomly selected microscopic fields were evaluated, and colonies were calculated in each PB from AMI patients or UCB sample.

Assessment of acetylated low-density lipoprotein uptake and Ulex europaeus-lectin 1 binding (UEA-l)

After 7 days of cell culture, EPCs were identified by uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine percholorate-labeled acetylated-low density lipoprotein (DiI-ac-LDL, Molecular Probes, America) and adherence of fluorescein isothiocyanate-conjugated Ulex europaeus lectin (FITC-UEA-l, Vector, America). The adherent cells were incubated with Dil-ac-LDL at 37°C for 2 h. Thereafter, cells were washed with PBS and fixed with 2% formaldehyde for 10 min and washed again with PBS. FITC-UEA-l was added and incubated for 1 hour at 25°C. Culture was then washed twice and visualized with a Leica DM 5500B (Solms, Germany) fluorescent microscope, software Meta Systems Isis.

Cardiomyogenic differentiation of EPCs

EPCs were seeded in per-manox-chamber slides (Nunc, Wiesbaden, Germany) at a concentration of 50,000 cells. Twenty-four hours after seeding, cells were washed with PBS twice and were incubated for 24h in endothelial growth media serum-free containing 10μM 5-azacytidine (5-aza). Then, cells were washed and medium was replaced with fresh endothelial growth media containing FBS to prevent cell death due to prolonged exposure to 5-aza and incubated in a CO_2 incubator. Cells were cultured for 2 weeks and medium was changed every 3 days [24].

Immunocytochemistry

For immunofluorescence staining, cells were fixed with 4% PFA in PBS for 20 min at room temperature. When staining intracellular antigens, cells were permeabilized with 0.1% Triton X-100 for 15 min. Following, cells were blocked in 2% blocking buffer solution (Roche) for 1 h at room temperature. Primary antibodies were incubated overnight at 4ºC; when needed, secondary antibodies were incubated at room temperature for 2 hours. Slides were mounted using Vecta shield containing DAPI. Photographs were taken with a Leica DM 5500B (Solms, Germany) fluorescent microscope, software Meta Systems Isis.

Antibodies used for immunocytochemistry included fluorochrome-conjugated monoclonal antibodies: KDR-APC (R&D System, Minneapolis, MN) and CD133-PE (Miltenyi) (dilution 1:200); primary monoclonal antibodies used for cardiac differentiation: desmin (rabbit monoclonal; Sigma), human cardiac-specific troponin I (mouse monoclonal; Research Diagnostics, Flanders, NJ, USA) and sarcomeric α-actinin (mouse monoclonal; Sigma) (dilution 1:100) secondary antibodies: Fluorescein Isothiocyanate (FITC) or Tetramethyl Rhodamine Isothiocyanate (TRITC) (Santa Cruz, CA, USA) (dilution 1:200).

Reverse transcriptase–polymerase chain reaction (RT-PCR)

For RT-PCR analysis, total RNA from 5-aza treated UCB-EPCs and from 5-aza treated AMI-EPCs was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA extracted from human heart tissue was used as a positive control. RNA was checked on 1% agarose gel. The cDNA reaction was performed using 0.5–2 μg total RNA with primers from SuperScriptII-kit (Invitrogen, Paisley, UK) according to the manufacturer'sinstructions. Forward and reverse

sequence for *cardiac troponin T* were T 5'-AGA GCG GAA AAG TGG GAA GA-3' and 5'-CTG GTT ATC GTT GAT CCT GT-3' (235bp) and for β-actin 5'-ATC ATG TTT GAG ACC TTC AA-3' and 5'-CAT CTC TTG CTC GAA GTC CA-3' (316 bp). The PCR reaction was performed with Reddy Mix PCR Master Mix (Thermo, Waltham, MA, USA). After the initial denaturation (2 min at 94°C), 35 cycles were performed (20 s at 94°C, 20 s at 53°C, for *cardiac troponin T* and 20 s at 45°C and 1 min at 72°C for β-actin). The PCR products were run on 1% agarose gel.

Statistics

Data were subjected to 1-wayANOVA followed by Fisher's test for comparison between any 2 means. Differences of $P<0.05$ were considered significant. Values are expressed as mean ± SE.

Results and Discussion

The aim of the study was to isolate circulating EPCs from PB of patients who have been diagnosed of AMI and to compare their differentiation and plasticity potential towards cardiomyocyte lineage with EPCs isolated from UCB.

In humans, bone marrow EPCs are mobilized after an acute ischemic event in response to growth factors, cytokines and hormones that are released from the target tissue [25, 26]. Firstly, we analyzed by flow cytometry the percentage of expression of proliferative and endothelial markers in freshly isolated total MNC from both origins. The cell surface adhesion molecule CD34 was expressed by 1.3 ± 0.12% and 1.46 ± 0.21% of total MNC from AMI-PB and UCB, respectively (Figure 1A) about 10-fold excess of CD34+ cells when compared with MNC of healthy subjects [27]. Recently,

Yang et al showed that CD34+ cells could represent a functional EPC population in bone marrow and have beneficial therapeutic effects in myocardial infarction [28]. In fact, others studies demonstrated mobilization of CD34+MNC in patients with AMI [8]. In addition, we found a higher expression of CD133 in UCB (6.1 ± 0.79%) than in AMI-PB (2.5 ± 0.18%) ($P<0.05$) (Figure 1B) that correlates with the immaturity of UCB-MNC as CD133 is a marker for stemness that represents a hematopoietic/endothelial progenitor fraction [29-31]. Furthermore, the chemokine receptor CXCR4 was notably expressed on freshly isolated MNC from both origins, with an expression of 80.2 ± 5.6% for UCB and 58.6 ± 3.1% for AMI-PB (Figure 1C). CXCR4 is highly expressed on hematopoietic stem cells and has previously been shown to play a key role in their homing and mobilization towards ischemic tissue [32]. Moreover, over-expression of CXCR4 in mesenchymal stem cells (MSCs) enhances *in vivo* engraftment into the ischemic heart and subsequently improves functional recovery via increasing myoangiogenesis [33]. Finally, we found significant differences when the vascular endothelial growth factor receptor KDR was studied (Figure 1D), with 6.1 ± 0.89% expression in MNC from PB of AMI patients in comparison with a 2.5 ± 0.02% from the UC. In fact KDR has been proved to be responsible for VPF/VEGF-stimulated EC proliferation and migration [34]. These results indicate that blood from AMI patients seems to be a valuable source for obtaining EPCs comparable with UCB.

Isolated MNC were further processed and since mature circulating EC attach within the first 48 hours of culture, we discard this population by collecting only non-adherent cells and replanting then to fibronectin-coated-well plates. Initially, isolated cells had a rounded morphology and after two days of culture formation of colonies was detected (Figure 2A), which consisted of rounded ball-like clusters with elongated sprouting cells at the periphery. These clusters were classified as endothelial cell colony-forming

units (CFU-EC). At day 5 CFU-EC increased in size and appear with a more defined morphology while on day 7, spindle-like EPCs sprouted out from the edge of the clusters and spread over the entire culture well surface (Figure 2A). CFU-ECs were counted manually in a minimum of four wells at day 5 and 10 and no significant differences (P = 0.5) were found between CB- and AMI-EPCs. In addition, CFU-EC number peaked at day 5 and significantly decreased ($P < 0.05$) by day 10 in both cell sources (Figure 2B).

Others studies have found that EPC colonies derived from UCB emerged earlier and in a major number than colonies obtained from healthy adult PB [35]. Since in our study we did not detect these variances our data suggest that the cardiac insult enhances EPC population in AMI patient PB.

The attached cells were characterized, as previously shown [4], by the expression of the typical endothelial markers KDR and CD133. Immunofluorescence phenotyping revealed that both the CB-and AMI-EPCs highly expressed these endothelial markers (Figure 2C). In addition we tested dil-ac-LDL and UEA 1 binding as specific functional markers of EC. Representative imagines of UEA-1 cell surface binding and dil-ac-LDL incorporation are shown in Figure 2D-K, demonstrating that AMI-EPCs and UCB-EPCs displayed, in a similar pattern, the functional phenotypes of EPCs.

To investigate the differentiation potential towards cardiomyocytes of adult circulating EPCs from patients who had suffered AMI, we treated cells with DNA demethylation agent 5-aza. This agent has been shown to induce cardiomyocyte differentiation of MSCs [36-40] and in EPCs [21]. After 5-aza exposition, EPCs morphology changed dramatically in both PB-AMI and UCB sources from spindle-like cells towards elliptical and elongated shape cells with a marked size enhancement. Furthermore, cells

arranged in parallel and formed myotube-like structures, while control cells maintained their spindle-like morphology (Figure 3A-F).

The acquisition of a cardiomyogenic differentiation phenotype was confirmed by immunocytochemical and RT-PCR analysis. Cardiac-specific markers, troponin I, and the myocyte-specific proteins sarcomeric α-actinin, desmin and cardiac troponin T were detected after epigenetic reprogramming of EPCs by 5-aza (Figure 3G and 3H-O). The potential of human UCB-MSCs to differentiate into cells with characteristics of cardiomyocyte have been shown before [24, 41]. Moreover, EPCs obtained from PB of healthy adult volunteers and patients with coronary artery disease have also been proved to transdifferentiate into cardiac myocytes after coculture with neonatal rat cardiomyocytes [19]. Since EC, vascular smooth muscle cells, and cardiomyocytes all differentiate from a common progenitor in the mesoderm, evidences suggest that reprogramming EC back to an earlier state in mesodermal development could recapitulate their cardiomyogenic potential [21]. This fact also could explain why EPCs when are cultured under appropriate culture conditions such as paracrine factors (growth factors and cytokines) and epigenomic agents (5-aza), or exposed to the proper microenvironment *in vivo,* differentiate into cardiomyocyte-like cells. The process of tissue auto-regeneration is highly complex, where implicated cells dedifferentiate, transdifferentiate or differentiate from stem cells sitting within the adult tissue [42].

Here we prove for the first time that EPC isolated from AMI patients have similar cardiogenic potential than EPCs isolated from UCB. In fact, Figure 3 clearly shows that cells isolated from both sources showed a similar pattern of cardiac markers expression. In agrement, intracoronary infusion of autologous EPCs in patients with AMI seams to enhance myocardial regeneration [43].

In conclusion, our finding suggests that EPCs isolated from patients that have suffered myocardial insult is a valuable EPCs source with the potential to differentiate toward cardiac-like cells. Since the ultimate aim of cardiac regenerative medicine is to replace both microvasculature and lost cardiomyocytes, autologous AMI-EPCs, which posses vascular and cardiac tissue regenerative potential, could be an excellent candidate for cell based therapies.

Figure legends

Figure 1. Representative graphics of FACS analysis of isolated MNC from umbilical UCB-EPC and from PB of patients diagnosed of AMI.

Figure 2. Characterization of EPCs derived from UCB or PB of AMI patients (AMI-EPCs). (**A**) Morphology of the UCB-EPCs and AMI-EPCs at days 2, 5 and 7 of culture. (**B**) Assessment of CFU-EC at 5 and 10 days later of the isolation. Different letters stand for significant differences (One-way ANOVA; $P<0.05$). (**C**) Immunofluorescence of the UCB-EPCs and AMI-EPCs showing positive staining for endothelial markers CD133 and KDR. (**D-K**) Cell surface binding of UEA-1 and Dil-ac-LDL incorporation. Original magnification : 10x for A; 20x for G and K; 40x for C and 100x for D-F and H-J.

Figure 3. Phenotypic changes and gene expression induced by 5-aza in EPCs. (**A, D**) Phase-contrast light microscopy of non-treated EPCs (control EPCs). (**B**, **C**, **E** and **F**) EPCs exposed to 5-aza appeared enlarged, with an elliptical shape and formed myotube-like structures. (**G**) RT-PCR analysis revealed the expression of cardiac troponin T in cells treated with 5-aza. Human heart tissue was used as a positive control. (**H-O**) Fluorescence immunostaining of differentiated EPCs toward cardiomyocytes after 2

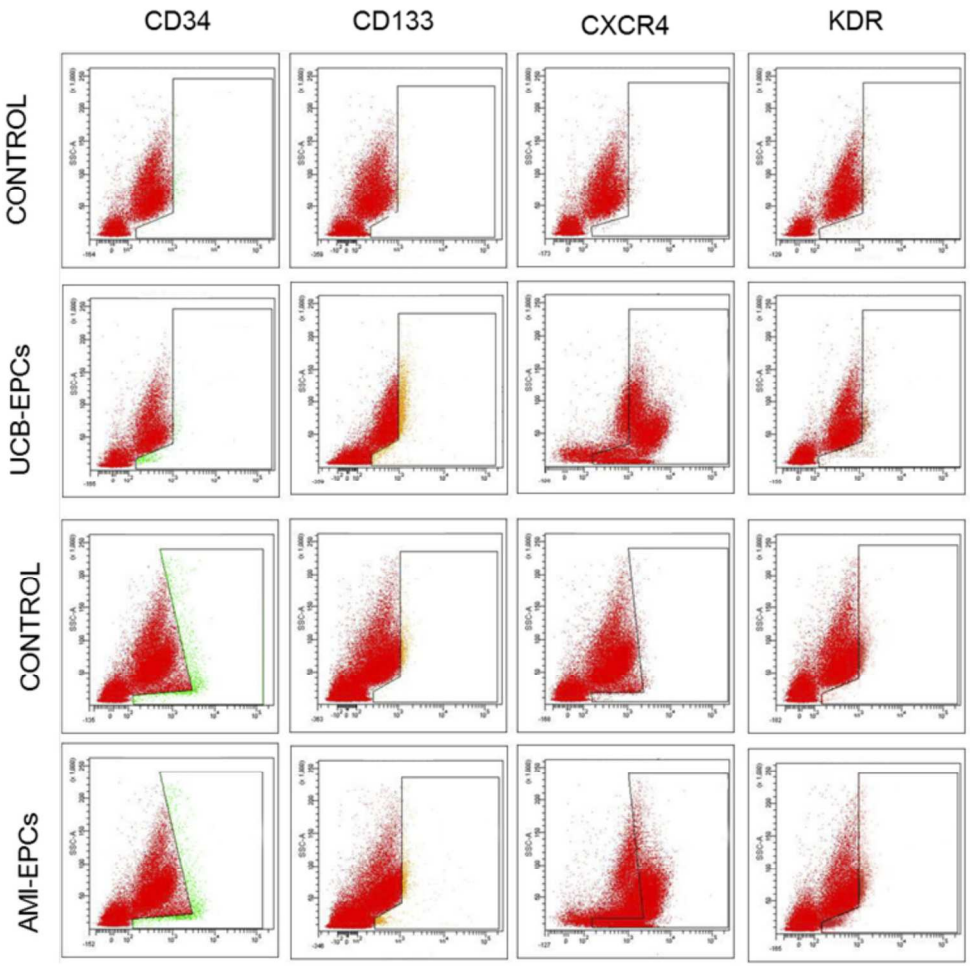
weeks of treatment. Green labels for sarcomeric α-actinin and troponin I expression and red label for desmin expression. Nuclei stained with DAPI. Original magnification: 10x for A, B, D and E; 20x C and F; 40x H-L and 63x M-O

References

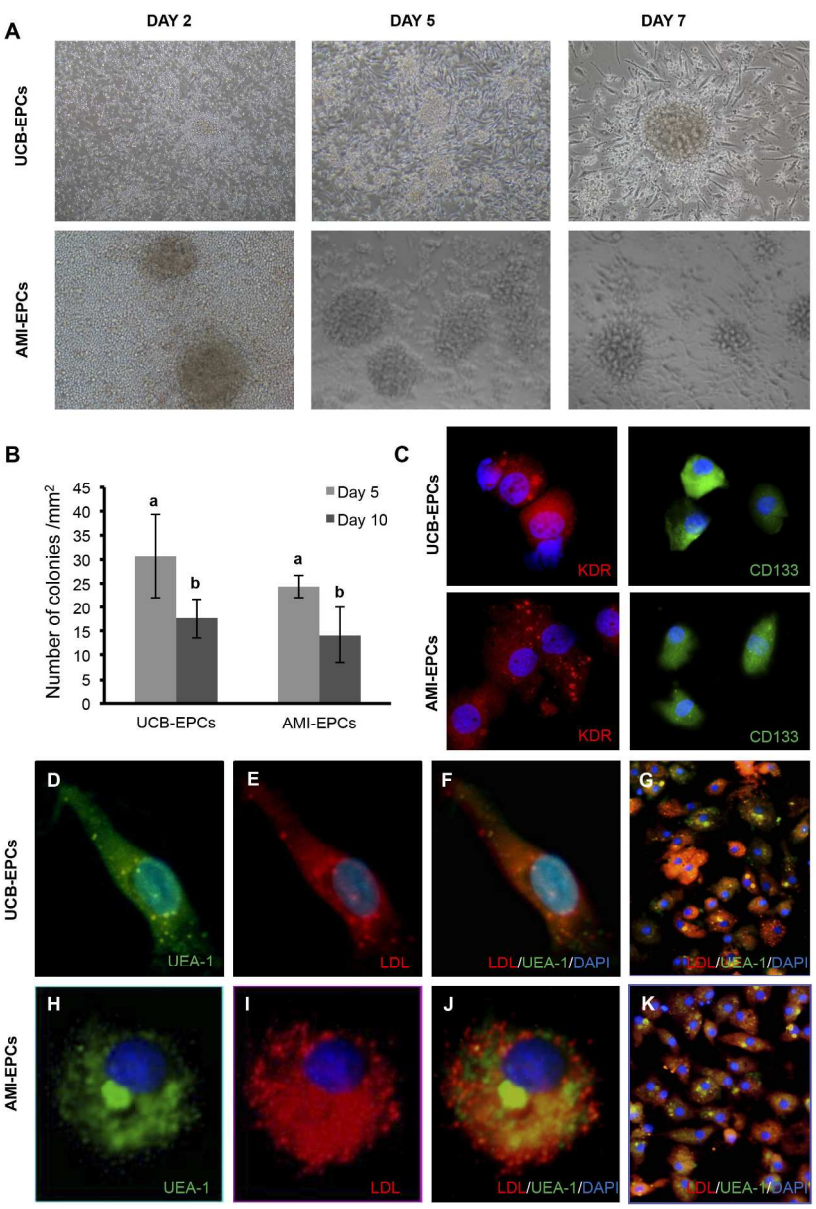
1. Laflamme, M.A. and C.E. Murry, *Regenerating the heart.* Nat Biotechnol, 2005. **23**(7): p. 845-56.
2. Asahara, T., A. Kawamoto, and H. Masuda, *Concise review: Circulating endothelial progenitor cells for vascular medicine.* Stem Cells, 2011. **29**(11): p. 1650-5.
3. Massa, M., et al., *Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction.* Blood, 2005. **105**(1): p. 199-206.
4. Asahara, T., et al., *Isolation of putative progenitor endothelial cells for angiogenesis.* Science, 1997. **275**(5302): p. 964-7.
5. Quirici, N., et al., *Differentiation and expansion of endothelial cells from human bone marrow CD133(+) cells.* Br J Haematol, 2001. **115**(1): p. 186-94.
6. 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.
7. Navarro-Sobrino, M., et al., *Mobilization, endothelial differentiation and functional capacity of endothelial progenitor cells after ischemic stroke.* Microvasc Res, 2010. **80**(3): p. 317-23.
8. Shintani, S., et al., *Mobilization of endothelial progenitor cells in patients with acute myocardial infarction.* Circulation, 2001. **103**(23): p. 2776-9.
9. Antonio, N., et al., *Stimulation of endothelial progenitor cells: a new putative effect of several cardiovascular drugs.* Eur J Clin Pharmacol, 2010. **66**(3): p. 219-30.
10. Bakogiannis, C., et al., *Circulating endothelial progenitor cells as biomarkers for prediction of cardiovascular outcomes.* Curr Med Chem, 2012. **19**(16): p. 2597-604.
11. Briguori, C., et al., *Correlations between progression of coronary artery disease and circulating endothelial progenitor cells.* FASEB J, 2010. **24**(6): p. 1981-8.
12. Vasa, M., et al., *Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease.* Circ Res, 2001. **89**(1): p. E1-7.
13. Mead, L.E., et al., *Isolation and characterization of endothelial progenitor cells from human blood.* Curr Protoc Stem Cell Biol, 2008. **Chapter 2**: p. Unit 2C 1.
14. Huang, G.P., et al., *Ex vivo expansion and transplantation of hematopoietic stem/progenitor cells supported by mesenchymal stem cells from human umbilical cord blood.* Cell Transplant, 2007. **16**(6): p. 579-85.
15. Ott, I., et al., *Endothelial-like cells expanded from CD34+ blood cells improve left ventricular function after experimental myocardial infarction.* FASEB J, 2005. **19**(8): p. 992-4.

16. Hu, C.H., et al., *Human umbilical cord-derived endothelial progenitor cells promote growth cytokines-mediated neorevascularization in rat myocardial infarction.* Chin Med J (Engl), 2009. **122**(5): p. 548-55.
17. Takamiya, M., et al., *Granulocyte colony-stimulating factor-mobilized circulating c-Kit+/Flk-1+ progenitor cells regenerate endothelium and inhibit neointimal hyperplasia after vascular injury.* Arterioscler Thromb Vasc Biol, 2006. **26**(4): p. 751-7.
18. Losordo, D.W., et al., *Intramyocardial, autologous CD34+ cell therapy for refractory angina.* Circ Res, 2011. **109**(4): p. 428-36.
19. Badorff, C., et al., *Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes.* Circulation, 2003. **107**(7): p. 1024-32.
20. Condorelli, G., et al., *Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardium regeneration.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10733-8.
21. Thal, M.A., et al., *Enhanced angiogenic and cardiomyocyte differentiation capacity of epigenetically reprogrammed mouse and human endothelial progenitor cells augments their efficacy for ischemic myocardial repair.* Circ Res, 2012. **111**(2): p. 180-90.
22. Peran, M., et al., *Cellular extracts from post-mortem human cardiac tissue direct cardiomyogenic differentiation of human adipose tissue-derived stem cells.* Cytotherapy, 2013. **15**(12): p. 1541-8.
23. Hill, J.M., et al., *Circulating endothelial progenitor cells, vascular function, and cardiovascular risk.* N Engl J Med, 2003. **348**(7): p. 593-600.
24. Kadivar, M., et al., *In vitro cardiomyogenic potential of human umbilical vein-derived mesenchymal stem cells.* Biochem Biophys Res Commun, 2006. **340**(2): p. 639-47.
25. Asahara, T., et al., *Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization.* Circ Res, 1999. **85**(3): p. 221-8.
26. Asahara, T., et al., *VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells.* EMBO J, 1999. **18**(14): p. 3964-72.
27. Murohara, T., *Cord blood-derived early outgrowth endothelial progenitor cells.* Microvasc Res, 2010. **79**(3): p. 174-7.
28. Yang, J., et al., *CD34+ cells represent highly functional endothelial progenitor cells in murine bone marrow.* PLoS One, 2011. **6**(5): p. e20219.
29. Urbich, C. and S. Dimmeler, *Endothelial progenitor cells: characterization and role in vascular biology.* Circ Res, 2004. **95**(4): p. 343-53.
30. Salven, P., et al., *VEGFR-3 and CD133 identify a population of CD34+ lymphatic/vascular endothelial precursor cells.* Blood, 2003. **101**(1): p. 168-72.
31. Dimmeler, S. and A.M. Zeiher, *Vascular repair by circulating endothelial progenitor cells: the missing link in atherosclerosis?* J Mol Med (Berl), 2004. **82**(10): p. 671-7.
32. Mohle, R., et al., *The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1.* Blood, 1998. **91**(12): p. 4523-30.
33. Zhang, D., et al., *Over-expression of CXCR4 on mesenchymal stem cells augments myoangiogenesis in the infarcted myocardium.* J Mol Cell Cardiol, 2008. **44**(2): p. 281-92.
34. Zeng, H., D. Zhao, and D. Mukhopadhyay, *KDR stimulates endothelial cell migration through heterotrimeric G protein Gq/11-mediated activation of a small GTPase RhoA.* J Biol Chem, 2002. **277**(48): p. 46791-8.
35. Kim, J., et al., *Comparative proteomic analysis of endothelial cells progenitor cells derived from cord blood- and peripheral blood for cell therapy.* Biomaterials, 2013. **34**(6): p. 1669-85.

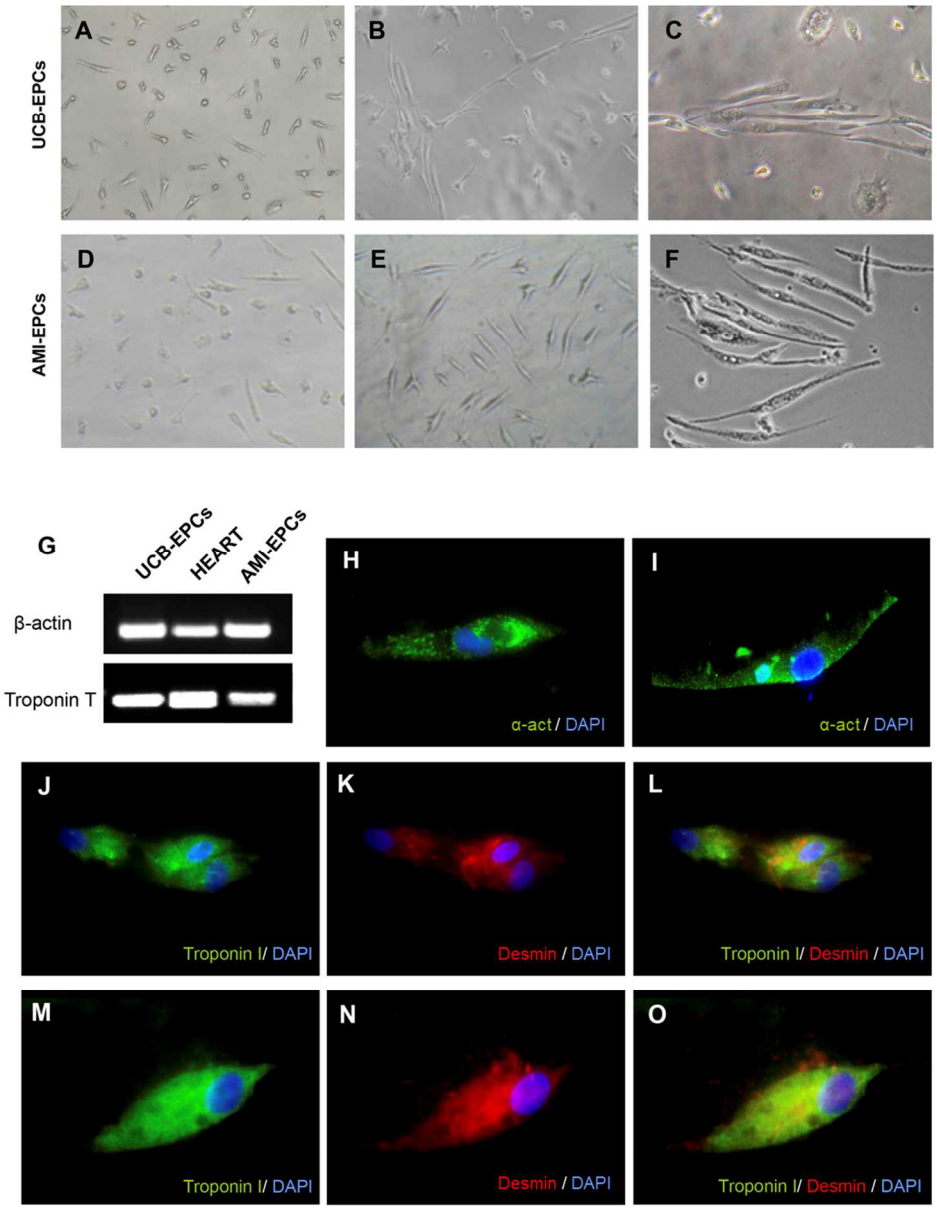
36. Burlacu, A., et al., *Promoting effect of 5-azacytidine on the myogenic differentiation of bone marrow stromal cells.* Eur J Cell Biol, 2008. **87**(3): p. 173-84.
37. Makino, S., et al., *Cardiomyocytes can be generated from marrow stromal cells in vitro.* J Clin Invest, 1999. **103**(5): p. 697-705.
38. Ye, N.S., et al., *Proteomic profiling of rat bone marrow mesenchymal stem cells induced by 5-azacytidine.* Stem Cells Dev, 2006. **15**(5): p. 665-76.
39. Yoon, J., et al., *Differentiation, engraftment and functional effects of pre-treated mesenchymal stem cells in a rat myocardial infarct model.* Acta Cardiol, 2005. **60**(3): p. 277-84.
40. Marchal, J.A., et al., *Purification and long-term expansion of multipotent endothelial-like cells with potential cardiovascular regeneration.* Stem Cells Dev, 2012. **21**(4): p. 562-74.
41. Kadner, A., et al., *Human umbilical cord cells: a new cell source for cardiovascular tissue engineering.* Ann Thorac Surg, 2002. **74**(4): p. S1422-8.
42. Sugimoto, K., S.P. Gordon, and E.M. Meyerowitz, *Regeneration in plants and animals: dedifferentiation, transdifferentiation, or just differentiation?* Trends Cell Biol, 2011. **21**(4): p. 212-8.
43. 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.



156x151mm (300 x 300 DPI)



186x266mm (300 x 300 DPI)



190x241mm (150 x 150 DPI)