

Terapia celular: de la investigación básica a la transfusión sanguínea

COORDINADORES: L. BLANCO. *Valladolid*
P. ORTIZ. *Barcelona*

Resumen del simposio

Aunque la terapia celular aparece en la literatura como un procedimiento terapéutico novedoso, la utilización de células como tratamiento en ciertas enfermedades es un hecho que se han planteado los científicos desde antiguo. Ya en el en el siglo XV, Paracelso hacía referencia a la utilización de células de la misma estirpe para curar enfermedades (“el corazón cura al corazón, el pulmón cura al pulmón, el bazo cura al bazo...; lo igual cura lo igual”).

La transfusión sanguínea es el primer procedimiento de terapia celular y también data de antiguo. Ya en 1667, J. B. Denis transfundió a un paciente enfermo mental sangre de un carnero, y desde entonces esta forma de terapia celular ha progresado y perfeccionado hasta convertirse hoy día en una práctica habitual.

El pasado siglo se intentó la infusión de células de otros tejidos. En el año 1912, Kuettner mantenía la teoría y ensayaba la posibilidad de cortar los órganos en pequeñas piezas e inyectarlos disueltos en solución salina, aunque fue unos años más tarde, en 1931, cuando Niehans puso en práctica esta teoría inyectando pequeños trocitos de paratiroides de buey disueltos en salino e inyectados a su vez a una paciente con hipoparatiroidismo grave. La paciente recuperó la función paratiroidea y vivió más de 30 años. Podemos decir que 1931 fue el año del nacimiento de la terapia celular tal y como la entendemos en la actualidad. Después del éxito del primer tratamiento, Niehans continuó experimentando con inyecciones de células vivas extraídas de otros órganos de animales sanos.

Fue también a principios del siglo XX cuando Alexander Maximov planteó su teoría sobre la existencia de células pluripotenciales circulantes que no sólo eran capaces de regenerar células iguales, sino que también podían regenerar células de otros órganos y tejidos.

En la segunda mitad del siglo XX, los estudios sobre progenitores hematopoyéticos, sobre todo en relación con los trasplantes, han sido clave para conocer el proceso de diferenciación celular y han sentado las bases de la terapia celular.

La diferenciación hematopoyética se produce durante el desarrollo embrionario y en el adulto bajo circunstancias de estimulación que inducen la aparición de células precursoras en diferentes estadios (*stem cells*, o células madre). Estas células son capaces de responder dividiéndose y madurando a células funcionales pudiendo regenerar medula ósea normal. Durante la división, la diferenciación y el desarrollo celular juegan un papel intercurrente numerosos factores extrínsecos como son las citocinas, los factores de crecimiento y las hormonas.

El hecho de que la transfusión sanguínea de glóbulos rojos está experimentando un incremento progresivo en los últimos años, junto a una estabilización en el número de donaciones de sangre, y, por otro lado, la idea de poder disponer de glóbulos rojos suficientes, sin riesgo de transmitir enfermedades infecciosas y con determinado grupo sanguíneo han dado lugar a la aplicación de los conocimientos sobre el origen de las células madre hematopoyéticas, sobre las señales que utiliza el embrión para generarlas y/o multiplicarlas y sobre cómo los afecta el microambiente medular, para mimetizar la eritropoyesis *ex vivo* y llegar a la producción de glóbulos rojos destinados a transfusión.

En este simposio, de rabiosa actualidad, profundizaremos en estos temas y en el estado actual de esta manufacturación de glóbulos rojos y los problemas que se deberán salvar, de la mano de grandes expertos en estas áreas.

MECANISMOS QUE REGULAN LA GENERACIÓN DE CÉLULAS MADRE HEMATOPOYÉTICAS EN EL EMBRIÓN DE RATÓN: ¿QUÉ SE NECESITA PARA PRODUCIR CÉLULAS MADRE HEMATOPOYÉTICAS?

A. BIGAS SALVANS

Coordinadora del Grupo de Investigación en Células Madre y Cáncer. Programa de Investigación en Cáncer. IMIM-Hospital del Mar. Parc de Recerca Biomèdica. Barcelona

Introducción

Las células madre hematopoyéticas son las responsables de la formación de células sanguíneas especializadas durante toda la vida. Para ello, estas células deben tener la capacidad de autorenovación, así como de diferenciación hacia los distintos tipos celulares. La capacidad de autorenovación indefinida es la que diferencia a estas células de los progenitores multipotentes. Históricamente se han desarrollado diversas técnicas para caracterizar y distinguir las células madre de otros precursores hematológicos indiferenciados. Actualmente, se considera que una célula madre hematopoyética es aquella que es capaz de reconstituir la hematopoyesis al ser transplantada a un organismo inmunodeprimido¹. Estas células residen en la médula ósea de una persona adulta y se perpetúan mediante autoreplicación. Sin embargo, en algún momento debe existir un precursor a partir del cual las células madre del adulto se generan. Actualmente hay evidencias de que esta célula precursora existe, al menos, durante la vida embrionaria, ya que se ha demostrado que las células hematopoyéticas de un ratón adulto provienen de un precursor con características endoteliales (VE-cad+ aunque también Runx+) que se formó durante el desarrollo embrionario y que muy probablemente coincide con el denominado *hemangioblasto*²⁻⁵. Estas investigaciones son muy importantes para demostrar que el proceso de la hematopoyesis embrionaria esconde las claves para saber generar células madre hematopoyéticas con capacidad de formar todos los tipos de células hematológicas a largo plazo (Figura 1).

Hematopoyesis primitiva en el saco vitelino

¿Qué sabemos sobre la hematopoyesis en el embrión humano? Al igual que en el ratón, hay una

primera etapa embrionaria que se caracteriza por la formación de algunos tipos de células sanguíneas, mayoritariamente eritrocitos y macrófagos. Este proceso tiene lugar durante un corto periodo de tiempo y se conoce como hematopoyesis primitiva. Estas células sanguíneas primitivas se originan en el saco vitelino, que es la estructura más externa de origen embrionario, probablemente para facilitar el acceso del oxígeno a los eritrocitos. Estas células serán las encargadas de distribuir oxígeno al embrión cuando éste haya crecido suficiente como para que no pueda conseguirlo por difusión celular. Esto ocurre a partir de los 21 días de desarrollo en el embrión humano (día 8,5 en ratón), que es cuando se establece la circulación entre el embrión y el saco vitelino mediante las arterias y venas vitelinas, y al mismo tiempo se observa la conexión entre el embrión y la placenta (mediante las arterias y venas umbilicales). Cuando estas conexiones se han establecido, las células hematopoyéticas primitivas originadas previamente en el saco embrionario están disponibles para circular hasta el embrión y cubrir las necesidades iniciales de oxígeno y nutrientes. El proceso de la hematopoyesis primitiva, aunque muy importante durante el desarrollo, es un proceso transitorio e independiente del que formará las células hematopoyéticas que mantendrán al

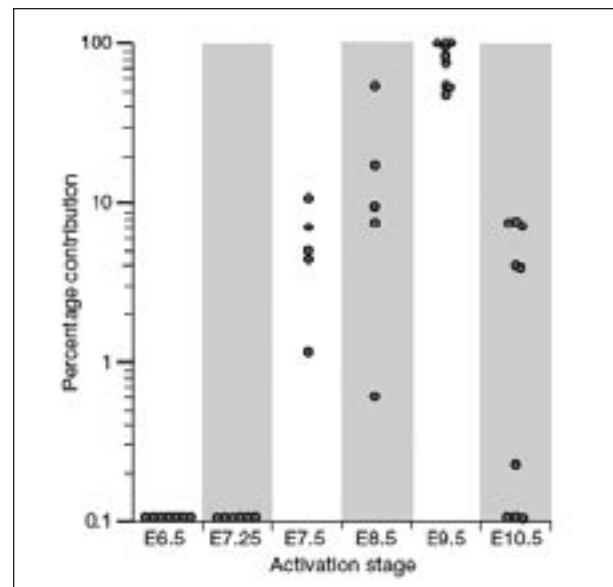


Figura 1. La gráfica muestra el porcentaje de células hematopoyéticas β -galactosidas+ en un ratón adulto que provienen de unos precursores marcados a distintos días de desarrollo embrionario (eje X, E6.5 a E10.5). Estos estudios demuestran que células Runx1+ marcadas genéticamente en un embrión de día 9.5 (aproximadamente) son precursoras del 100% de las células sanguíneas que se encuentran en el ratón adulto). Publicado por Samokhvalov et al., *Nature* 2007.

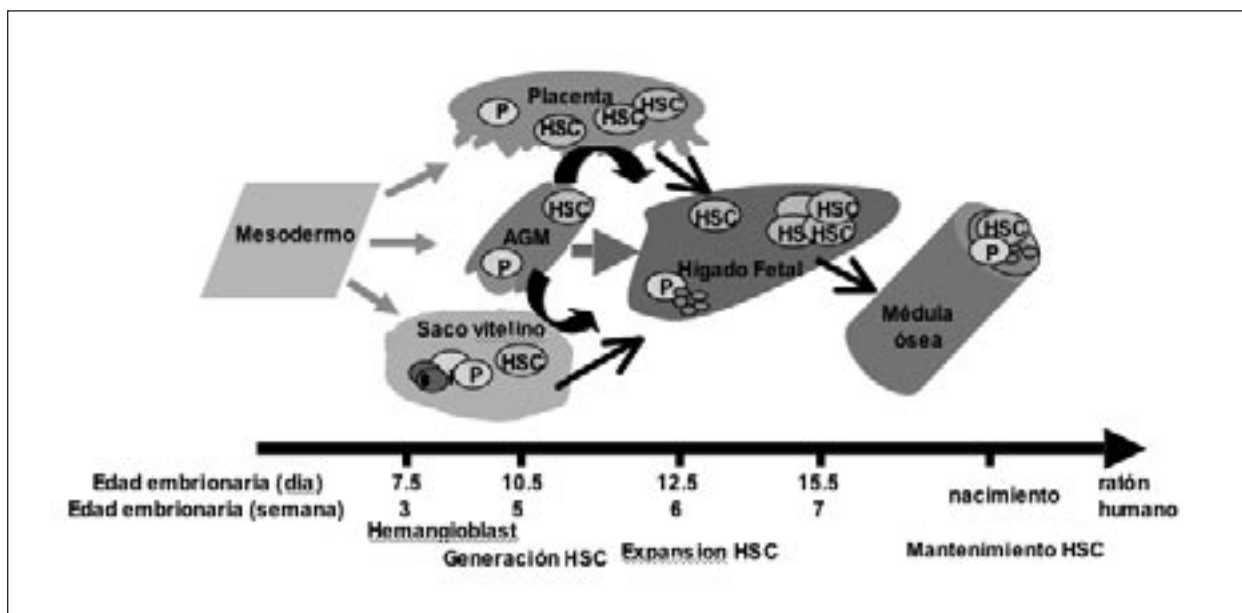


Figura 2. Representación de las distintas fases y nichos hematopoyéticos durante el desarrollo hematopoyético en el embrión de ratón y humano (a partir de Mikkola *et al.*,⁽¹²⁾). El proceso de formación de estas células se ha caracterizado en los últimos años gracias en parte al estudio de ratones mutantes que presentan alteraciones en las primeras etapas de la hematopoyesis. Asimismo, los embriones mutantes para genes como AML1/Runx1 o GATA2 entre otros, carecen de células hematopoyéticas capaces de reconstituir un organismo adulto^(13,14). Se considera que la expresión de estos genes es parcialmente responsable de la ejecución del programa hematopoyético.

organismo adulto. Más adelante, sin embargo, el saco embrionario puede tener capacidad para formar otras células que sean precursoras de las células madre adultas. Esta posibilidad está todavía en discusión^{5,6}.

Hematopoyesis definitiva durante el desarrollo embrionario

A partir de día 19 de desarrollo embrionario humano (día 10 en el ratón), en la región caudal de la aorta dorsal, donde se localiza la gónada y el mesonefros (AGM), se observan células hematopoyéticas indiferenciadas que, a diferencia de las células hematopoyéticas primitivas, tienen la capacidad de regenerar la hematopoyesis de forma permanente al ser trasplantadas a un ratón SCID irradiado⁷. Esta característica es la que identifica a las células madre hematopoyéticas y las primeras células de este tipo que se detectan en el embrión, se generan en esta región de la AGM, asociadas al endotelio de la aorta⁸. Últimamente, se ha descrito que simultáneamente se originan células de las mismas características en la placenta y en las arterias vitelinas y umbilicales, indicando que muy probablemente las condiciones para generar células madre hematopoyéticas se consiguen en diversas estructuras arteriales del embrión^{9,11} (Figura 2).

Vías de señalización molecular que regulan la formación de células madre hematopoyéticas en el embrión

Nuestro laboratorio está investigando el papel que algunas vías de señalización como Notch y Wnt tienen en el proceso de formación de las células madre hematopoyéticas. Como es lógico, los experimentos funcionales deben hacerse en ratón para después buscar las comparaciones en el caso de células humanas.

En estudios publicados recientemente hemos demostrado que la vía de Notch debe activarse para que las células hematopoyéticas de la aorta puedan formarse. Notch es una proteína que se encuentra en la membrana de muchas células y que interactúa con otras proteínas que se encuentran en células adyacentes (ligandos). Estas interacciones célula-célula tienen como resultado el procesamiento de Notch y su internacilización al núcleo de la célula donde funciona como factor de la transcripción. En el caso de la hematopoyesis embrionaria en la aorta, sabemos que Notch tiene que interactuar con Jagged1 para que se pueda formar hematopoyesis¹⁷.

Otra línea de investigación importante en el laboratorio consiste en identificar la función de la vía de señalización de Wnt que es importante en este proceso (Figura 3).

Aplicaciones de la investigación embrionaria en la generación de tejido sanguíneo

La disponibilidad de células madre hematopoyéticas es actualmente el punto limitante para el trasplante hematológico de muchos enfermos sin donantes compatibles. En los últimos años, y gracias al desarrollo de los bancos de células de cordón umbilical, se ha aumentado ligeramente el número de donantes para ciertos tipos de trasplantes. Sin embargo, para mejorar esta situación de forma permanente, necesitamos investigar sobre nuevas fuentes de células madre. En este sentido, las células derivadas de células madre embrionarias (células ES, del inglés *embryonic stem*) representan una nueva expectativa a la solución de este problema. Hoy en día podemos generar muchos tipos celulares a partir de células madre embrionarias. Se han generado todos los tipos celulares hematopoyéticos, e incluso células que son capaces de reconstituir ratones inmunodeprimidos (SCID), indicando que el proceso de generar células madre hematopoyéticas a partir de estas células es posible¹⁸. Sin embargo, la reproducibilidad y eficiencia de este proceso hace que sea imposible pensar en su utilización terapéutica. En resumen, no se ha conseguido generar células madre hematopoyéticas de forma reproducible a partir de células madre embrionarias. Gran parte de este fracaso es debido a que no conocemos cómo se generan estas células, y para ello debemos estudiar cómo se hace en el embrión e intentar seguir los mismos pasos en el laboratorio (Figura 4).

Problemas asociados a la hematopoyesis embrionaria: ¿dónde se generan las leucemias? ¿Existen células preleucémicas en el embrión?

Investigaciones recientes demuestran que existen algunas aberraciones cromosómicas asociadas a leucemias que se originan durante la fase fetal o embrionaria. Se ha observado que si un gemelo monoplacental desarrolla leucemia a una edad temprana (antes de los 4 años), el otro tiene más posibilidades de padecer ese mismo tipo de leucemia comparado con el resto de la población¹⁹. Además en pacientes que han desarrollado leucemias, se han encontrado en muestras de sangre obtenidas al nacer alteraciones genéticas asociadas a leucemias, indicando que estas alteraciones se han originado durante el periodo embrionario o

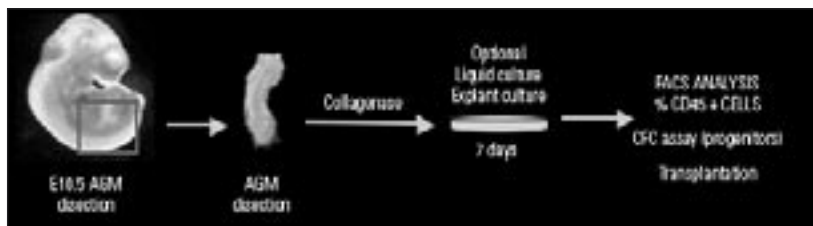


Figura 3. Protocolo experimental para analizar las células hematopoyéticas que se originan en la aorta de un embrión de ratón. Estas células, una vez obtenidas, se disgregan mediante incubación en colagenasa y se pueden trasplantar a ratones irradiados para estudiar las HSC.

fetal. Estos descubrimientos ponen de manifiesto la necesidad de controlar la seguridad de muestras de sangre de cordón umbilical al ser utilizadas para trasplante, muy especialmente en posibles casos de trasplantes autólogos para pacientes que han desarrollado leucemias.

Conclusiones

- Las células madre hematopoyéticas se generan a partir de precursores endoteliales durante el periodo de desarrollo embrionario.
- Durante la etapa embrionaria distintos tejidos vasculares/arteriales tienen la capacidad de generar células madre hematopoyéticas incluida la arteria umbilical, vitelina, placenta y sobre todo la aorta dorsal. Investigar y conocer los mecanismos moleculares que utiliza el embrión para formar las células hematopoyéticas es un paso imprescindible para generar estas células en el laboratorio a partir de precursores o células embrionarias.

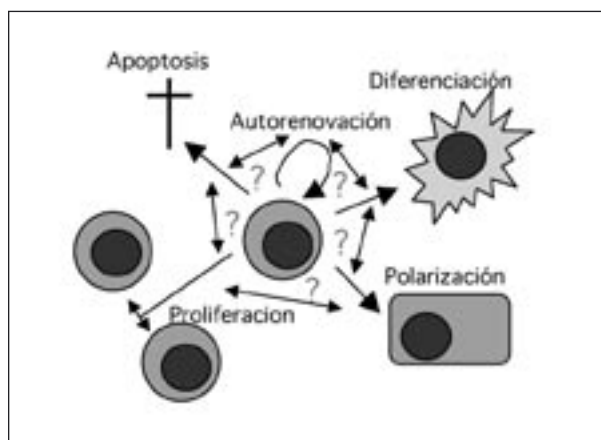


Figura 4. Diferentes procesos biológicos a los que pueden optar las células madre: autorreproducción, diferenciación, muerte celular o apoptosis, proliferación o migración.

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BACK AND FORTH WITH THE STEM CELL NICHE: HOW RHO GTPASES CONTROL STEM CELL TRAFFICKING

J.A. CANCELAS

*Division of Experimental Hematology,
Cincinnati Children's Research Foundation,
University of Cincinnati Medical Center,
Cincinnati (OH, USA)*

Introduction

In adults, hematopoietic stem cells and progenitors (HSC/P) reside in the BM and are largely absent from PB¹. HSC/P transplants are used to replace the endogenous hematopoiesis of patients in the treatment of cancer and some genetic disorders²⁻⁸. The success of stem cell transplants depends on the number and quality of HSC infused, a receptive host BM and, in the case of allogeneic transplants, on the immunotolerance of the recipient for the progeny engrafted stem cells.

While BM harvests were previously utilized to collect transplantable hematopoietic stem cells, current clinical practice mostly applies the biological phenomenon of HSC/P mobilization from the marrow into the peripheral blood (PB) to allow leukapheresis harvest⁹, as it does not require general anesthesia and is typically associated with shorter periods of post-pancytopenia^{8,10}. Administration of cytokines, such as Granulocyte-Colony Stimulating Factor (G-CSF)³ or Stem Cell Factor (SCF)¹¹, chemokine-receptor inhibitors (e.g. AMD3100)¹² and cytotoxic drugs (e.g. cyclophosphamide)³⁻⁷ have been used clinically to increase the number of circulating HSC/P. A goal of PBSCT for hemato-oncological diseases is to optimize the number of HSC to ensure low levels of the morbidity and mortality associated with rescue in the myeloablative setting^{6,10}. In addition, PBSC are ideal targets to be utilized in cell and gene therapies¹³. The administration of G-CSF is currently the major method for mobilization of HSC/P for clinical usage. However, over 40% of patients who have undergone intensive chemotherapy, and between 10% and 20% of all patients and normal individuals, fail to mobilize sufficient numbers of HSC/P for successful PBSCT⁹.

HSC homing and engraftment are crucial to successful transplantation, and clinical engraftment is severely compromised when donor-cell numbers are limited. For instance, the low number of HSC/P appears to limit the use of umbilical cord blood for transplantation of adult patients, where limited HSC dose appears to be associated with delayed engraftment and unacceptably high rates of graft failure¹⁴⁻¹⁸. Another

example is found in the current design of cell/gene therapy protocols, which require large amounts of HSC/P for *ex vivo* manipulation and subsequent reinfusion. The fact that chemotherapy-based protocols may be inadequate or unacceptable to mobilize stem cells in many immunodeficiencies and other non-malignant hematological diseases, makes the search for other methods of mobilization highly desirable. The current alternative is the use of cytokine-induced mobilization, but this is hampered by a high variability in the efficiency of mobilization⁹. Several investigators¹⁹⁻²¹ have suggested that mobilized PB stem cells may also contribute to the generation of non-lymphohematopoietic tissues. While controversial²²⁻²⁴, these data, suggest potential additional therapeutic application of mobilized PBSC. Thus, increasing the HSC/P availability, by improving stem cell mobilization, and functional modifications of the HSC/P to facilitate their homing and engraftment abilities, might provide an answer to cases of absent or poor engraftment after HSC/P transplantation.

Mechanisms of HSC/P Homing and Retention in bone marrow

Bone marrow engraftment in the context of HSC/P transplantation is based on the ability of intravenously administered cells to lodge in the medullary cavity and be retained in the appropriate marrow space, a process referred to as homing. It is hypothesized that homing is a multistep process, encompassing a sequence of highly regulated events that mimic the migration of leukocytes to inflammatory sites. This process includes a first phase of tethering and rolling of cells to the endothelium via E- and P-selectins, firm adhesion to the vessel wall via integrins, transendothelial migration, and chemotaxis through the extracellular matrix (ECM) to the endosteal space²⁵⁻²⁷. A second phase involves the interaction of specific HSC/P surface receptors, such as $\alpha_4\beta_1$ -integrin receptor with stromal vascular-cell-cell adhesion molecule-1 (VCAM-1) and fibronectin in the ECM²⁸. After intravenous infusion, HSC/P can be temporarily detected in other organs, such as liver, lung and kidneys, but disappear from these sites within 48 h after transplantation. In contrast, the retention of HSC/P in BM is sustained and appears specific. The factors that influence this specific retention have been studied recently and probably involve the interplay between chemokines, growth factors, proteolytic enzymes, and adhesion molecules²⁹. Among the chemokines, CXCL12, also called Stromal Derived Factor-1 α (SDF-1 α), and its receptor, CXCR4, play key roles in human HSC trafficking and repopulation³⁰. CXCL12 α is expressed by both human

and murine BM endothelium and stroma^{31,32} and is a powerful chemoattractant of HSC/P^{33,34}. CXCL12 may also regulate survival of HSC/P^{35,36}. CXCL12 induces the integrin-mediated firm arrest of human HPCs, facilitates their transendothelial migration^{27,31}, regulates homing³⁷ and BM engraftment³⁸. Furthermore, CXCL12 is also required for the retention of murine HSC/P within the BM^{39,40}. A second factor critical for HSC/P survival and engraftment is SCF, the ligand for the receptor tyrosine kinase, c-kit, which is expressed on BM stromal cells. A transmembrane isoform of SCF, membrane-bound SCF (transmembrane, tm-SCF), has recently been shown to be critical in the lodgment and retention of HSC within the hematopoietic microenvironment, although it does not appear to play a role in the homing of transplanted cells to BM⁴¹. Integrin adhesion molecules also play important roles. Among them, $\alpha_4\beta_1$ -integrin is probably the best characterized⁴²⁻⁴⁵ and is discussed below.

Mechanisms of HSC/P Mobilization and Trafficking

HSC/P mobilization is also a dynamic and complex process⁴⁶⁻⁴⁸. HSC/P must exit the stem cell niche in the BM, migrate through the marrow sinusoidal endothelium, and circulate in the blood. Circulating HSC/P and BM-adherent HSC/P appear to be interchangeable. It has been shown in parabiotic mice that HSC/P can leave their niche without induction, traffic through the bloodstream, and finally migrate into BM of the conjoined animal⁴⁹⁻⁵². This suggests that HSC/P trafficking is a physiological phenomenon. If so, circulating HSC/P would be predicted to move into the BM microenvironment through transendothelial migration directed by chemoattractants, and finally anchor within the extravascular BM space where proliferation and differentiation occur. In this process, adhesion molecules, chemokine receptors and integrin signaling require signal integration that drives cytoskeleton rearrangements, gene transcription activation, cell survival and cell cycle activation.

At the molecular level, the interaction between CXCL12 and the G-coupled chemokine receptor CXCR4 has been recognized as pivotal in stem cell mobilization. As HSC/P are known to migrate towards a positive gradient of CXCL12⁵³, it has been suggested that treatment with G-CSF, cyclophosphamide or interleukin (IL)-8 leads to a reduction of CXCL12 in BM, resulting in a positive gradient towards PB and induction of HSC/P migration towards PB. Raising the plasma levels of CXCL12, by intravenous injection of CXCL12-expressing adenovirus⁵⁴ or sulfated polysaccharides⁵⁵, or by inhibition of the

CXCR4 receptor^{12,56,57}, leads to mobilization of HSC/P. G-protein inhibition by pertussis toxin⁵⁸ induces a similar mobilization effect, probably by interfering with the CXCR4 signaling pathway.

Functional blocking of $\alpha_4\beta_1$ -integrin (receptor for VCAM-1 and fibronectin) alone or together with $\alpha_1\beta_2$ -integrins or the functional blocking of the β_2 integrin LFA-1 (leukocyte function-associated antigen-1) by antibodies, results in mobilization of HSC/P^{59,60}. HSC/P accumulate in the PB soon after gene deletion in inducible $\alpha_4\beta_1$ -integrin deficient mice. Although their numbers gradually stabilize at a lower level, progenitor cell influx into the circulation continues at above-normal levels for more than 50 wks with a concomitant progressive accumulation of spleen HSC/P⁴⁴.

Playing an important and independent role in HSC/P mobilization is the interaction between SCF and its receptor, c-kit. SCF/c-kit interaction plays a critical role in G-CSF-mediated mobilization^{61,62}, and SCF in combination with G-CSF has been shown to enhance HSC/P mobilization⁶³. As mentioned above, tm-SCF has been shown to be critical in retaining HSC/P in BM⁴¹.

A key observation is that Rac proteins, members of the RHO GTPase family of small, Ras-like molecules are downstream signaling components of all of these surface proteins (β_1 -integrins, CXCR4 and c-kit) and thus represent a convergence of biochemical pathways critical for engraftment and mobilization⁶⁴⁻⁶⁶. Indeed, combined deficiency of Rac1 and Rac2 induces a striking mobilization of HSC/P into circulation while Rac1-deficiency is associated with engraftment failure in murine transplant models⁶⁷.

RHO GTPases as Signal Integrators

The signals involved in engraftment and mobilization drive cytoskeleton rearrangements, gene transcription activation, cell survival and cell cycle activation. In 1992, Ridley and Hall^{68,69} reported that cytoskeleton rearrangements are controlled by members of the RHO GTPase family. The Rho family of small monomeric GTPases, members of the Ras superfamily, includes 22 genes coding for at least 25 proteins. Based on sequence identity, domain structure and function, the Rho proteins can be divided into 6 families, including: Rac, RhoA related proteins, Cdc42 group, TC10 and TCL, Rnd, the RhoBTB subset and the Miro subfamily (ref.). RHO proteins are molecular switches that cycle between inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound states. In the GTP-bound form, RHO proteins interact with their specific effector or target molecules to trigger diverse cellular responses. Activation of RHO GTPases is induced by a divers-

ty of Dbl-family guanine nucleotide exchange factors (GEFs)⁷⁰ that are activated by receptor-dependent kinases. Negative regulation of GTPases is achieved by acceleration of the intrinsic GTPase function of the Rho proteins by GTPase activating proteins (GAPs) or sequestering and stabilizing the inactive, GDP-bound state by GDP dissociation inhibitors (GDIs). In contrast to these regulatory mechanisms controlling most Rho GTPases, members of the RhoE subfamily, including the recently described hematopoietic-specific RhoH, have been demonstrated incapable of GTP hydrolysis and thus remain constitutively GTP-bound. These constitutively active GTPases are therefore, thought to be regulated on the transcriptional or post-translational level.

Members of the Rho GTPase family show high conservation (40 - 95%) at the amino acid level. The best studied members of this family are Rac1, RhoA and Cdc42. In fibroblasts and leucocytes, Rho activation induces the assembly of filamentous (F)-actin and myosin filaments (stress fibers), while Rac and Cdc42 promote the formation of surface protrusions and ruffling (lamellipodia) and finger-like membrane extensions (filopodia) respectively, both actin containing structures. Rho GTPase-induced F-actin assembly is accompanied by clustering of integrin receptors in membrane structures overlying the F-actin. In several cell systems, RHO GTPases thus integrate stimuli from the activation of tyrosine kinase receptors^{71,72}, chemokine receptors⁷³ and integrin receptors⁷⁴, which are key regulators of both homing and mobilization.

Rac RHO GTPases in HSC/P homing, retention and migration

The Rac GTPase subfamily includes three highly homologous proteins: Rac1, Rac2 and Rac3. Rac1 is ubiquitously expressed while Rac2 is expressed only in hematopoietic cells⁷⁵. Rac3 appears expressed in a variety of cell lines and appears to play an important role in myelopoiesis (ref.) and in the development of p190-BCR/ABL-dependent leukemogenesis⁷⁶, but little is known about its expression in other primary tissues and its function in stem cells. Rac proteins play a major role in the development of lamellipodia and membrane ruffling and also activate a number of kinase pathways.

Many of the stimuli that have been shown crucial for HSC homing and retention, and specifically the interaction CXCL12 α /CXCR4, signal through Rac RHO GTPases. The first observations were based on the use of very unspecific tools that affected different Rac RHO GTPases. The high level of homology among RHO GTPases and the resultant lack of discriminating tools, such as specific antibodies, did not

allow a conventional analysis of the differential role of Rac proteins.

Nishita *et al.* showed that CXCL12 activates Rac1 and Cdc42 in Jurkat T cells in a pertussis toxin (PTX)-sensitive fashion, whereas RhoA activation was PTX-insensitive (88), which suggested specific signaling via Gi proteins toward Rac and Cdc42, but not to RhoA. Specifically in HSC/P, Whetton *et al.*, based on the use of Clostridium difficile toxin B (an inactivator of Rho, Rac and Cdc42) and the use of a downstream inhibitor of RhoA, showed that the migratory potential of primitive hematopoietic cells is lower than compared to more mature hematopoietic cells⁷⁷ and that this migration can be boosted by stimulating the cells with lysophospholipids (lysophosphatidic acid, sphingosine-1-phosphate) in combination with CXCL12 and that Rho GTPases were implicated in their signaling. We have observed that either Rac1 or Rac2-deficiencies substantiate a migration impairment of hematopoietic stem cells through CXCL12 gradients^{67,78}. Interestingly, the deficiency of Vav-1 (a hematopoietic-specific Rac GEF, but able to activate both Rac1 and Rac2) induces a severe migration impairment through a CXCL12 α gradient and in T cells appears to have a pivotal role in the transmission of signals from the CXCR4 receptor⁷⁹.

Specifically, Rac Rho GTPases in HSC/P constitute an example of homologous proteins, which share some functions but are completely specific for other cell-specific relevant functions. Our group has devoted a special emphasis over the past years to specifically analyze the differential role in genetically targeted animals of the very homologous Rac1 and Rac2 GTPases in migration and homing of hematopoietic cells. By using genetically targeted mice and hematopoietic reconstitution models, we have been able to delineate some of the specific functions of Rac1 and Rac2 in HSC/P, as well as in more mature hematopoietic cells, including B cells^{80,81}, T cells^{81,82}, mast cells⁸³⁻⁸⁵, eosinophils⁸⁶ and platelets⁸⁷.

An example of well-studied Rac-dependent functions is Rac2 in neutrophils. We were able to show that Rac2-deficient mice displayed an impaired host defense with diminished L-selectin mediated tethering, reduced chemotaxis through formyl-methionyl-leucyl-phenylalanine (fmlp) gradients and reduced actin polymerization and oxidative burst that correlated with low activation levels of mitogen-activated protein kinases (MAPK)⁸⁸. Mutational analysis of a patient with phagocytic immunodeficiency allowed us and others to identify a mutation in the Rac2 gene (D57N) resulting in expression of a dominant-negative version of the Rac2 protein^{89,90}. This patient suffered this disease despite normal activation of Rac1. Rac2-deficient mice display neutrophilia but normal myeloid progenitor proliferation *in vitro*.

From our studies, it has become clear that effects of Rac1 or Rac2 deficiencies are in many cases due to specific defects, rather than to an overall reduction of Rac levels. These differences may be due to either differential GEF-dependent activation or specific intracellular localization. In myeloid cells, specificity of function of Rac1 versus Rac2 maps to the carboxy-terminus and the regulation of intracellular GTPase localization⁹¹ which may be dependent on interaction with other proteins.

In HSC/P, non-specific Rac dominant-negative mutant expression induces a HSC engraftment defect⁹². A detailed study of the stem cell compartment in Rac2^{-/-} mice allowed us to observe that Rac2-deficient HSC show normal short-term engraftment but display an abnormal interaction of HSC with the hematopoietic microenvironment, which leads to defective hematopoiesis in quantitative cobblestone-area forming cell (CAFC) assays and long-term engraftment assays⁹³. At the same time, mimicking the phenotype of $\alpha 4$ deletion or anti- $\alpha 4$ antibody administration^{44,94}, Rac2-deficient mice displayed an increased pool of circulating HSC/P (around 2-3 fold), which suggested that Rac2 is required to retain cells in the bone marrow microenvironment and this effect may be specifically dependent on $\alpha_4\beta_1$ -integrin-mediated adhesion to fibronectin but not on $\alpha_5\beta_1$ -integrin. Hypermotility of Rac2-deficient HSC/P appeared to be due to an unbalanced compensatory activation of Rac1 and Cdc42 and increased F-actin polymerization following CXCL12 α activation⁷⁸.

We have recently approached to the analysis of the specific role of Rac1 in stem cell homing and migration. Rac1^{-/-} HSC are incapable of short term engraftment and demonstrate reduced *in vitro* growth associated with impaired growth factor-stimulated p27^{kip1} down-regulation and Cyclin D1 induction⁶⁷, while Rac2^{-/-} HSC, as mentioned earlier, show normal short-term engraftment but demonstrate defective growth factor rescue of apoptosis *in vitro*. The basis of the severe engraftment defect of Rac1^{-/-} HSC appears to be related to a homing/microlocalization defect in the bone marrow cavity for primitive CAFC and selected HSC/P *in vivo*⁹⁵, which correlates with the finding that signaling pathways downstream of the receptor tyrosine c-kit, the chemokine receptor CXCR4 and $\beta 1$ integrins, previously implicated in lodging and engraftment^{31,41,42} are defective in Rac-deficient HSC/P *in vitro*⁶⁴⁻⁶⁶. Importantly, the combination of defects in these two pathways contributes to the profound defect in growth *in vitro* and *in vivo* seen in Rac1^{-/-}; Rac2^{-/-} HSCs and their massive egress from bone marrow to peripheral blood^{67,95}. This is likely due to strongly reduced mediated adhesion in the Rac1^{-/-}/Rac2^{-/-} cells. Single deficiency of Rac1 or Rac2 leads to impaired *in vitro* migration to-

ward CXCL12 α , a response that is further reduced when both GTPases are absent, indicating that for migration, these GTPases are partially redundant. Reintroduction of Rac1 expression in circulating double Rac1/Rac2-deficient HSC/P allows their engraftment in lethally irradiated recipients⁹⁵. In addition, we have shown that the reversible inhibition of Rac activation by a novel, rationally designed small molecule (NSC23766) which had been designed to interfere the binding between Rac proteins and several GEFs⁹⁶ induces HSC/P mobilization in a “poor-mobilizer” murine model that correlates in time and fashion with p21-activated kinase inhibition⁹⁵.

An example of applicability of Rac targeting is Fanconi anemia (FA). FA is an inherited disorder characterized by early-onset progressive bone marrow (BM) failure, congenital abnormalities and predisposition towards cancer⁹⁷. FA group A is the predominant complementation group (>60% of all diagnosed FA patients). The only curative therapy currently available is allogeneic stem cell transplantation from a non-affected donor. Unfortunately, the availability of unaffected sibling donors is low for the majority of patients and the disease-free survival rate for transplant using a matched unrelated donor is not optimal, ranging from 15 to 67%^{98,99}. Since many children with FA are diagnosed prior to the onset of severe pancytopenia¹⁰⁰ a possible novel experimental therapy could use autologous hematopoietic stem cells (HSC) prior to BM failure, for corrective molecular intervention. Studies which examine the feasibility of collecting HSC/P have shown that stem cell mobilization using granulocyte colony-stimulating factor (G-CSF) is not robust in mice¹⁰¹, or in FA patients which may require prolonged periods of daily apheresis procedures to obtain clinically relevant numbers of HSC^{102,103}. Xenogeneic homing of FA patient BM progenitors appears decreased¹⁰⁴. FA-A cells also showed defects in both cell-cell and cell-matrix adhesion. Complementation of FA-A deficiency by reexpression of *FANCA* readily restored adhesion of FA-A cells. A significant decrease in the activity of the Rho GTPase Cdc42 was found associated with these defective functions in patient-derived cells, and expression of a constitutively active Cdc42 mutant was able to rescue the adhesion defect of FA-A cells. Interestingly, inhibition of Rac GTPases by *in vivo* administration of the small molecule NSC23766 is able to rescue part of the progenitor mobilization failure of FA-A cells in a murine model of HSC/P mobilization induced by G-CSF¹⁰⁵.

Although outside the scope of this review, the role of Rac activation in distinct stem cell-initiated and how Rac targeting may impair the pathogenesis in distinct stem cell-initiated diseases, like chronic myelogenous leukemia and MLL-rearranged acute myelogenous leukemia¹⁰⁶⁻¹⁰⁹ has been demonstrated.

Other RHO GTPases and their crosstalk with Rac in HSC/P homing and migration

Much less is known about the role of other RHO GTPases in HSC/P homing and migration. Cross-talk among different small RhoGTPases and Ras members would explain the role of these proteins in apparently contradictory functions¹¹⁰. In fibroblasts, introduction of constitutive active or dominant negative mutants of Cdc42, Rac and RhoA were shown to effect activation or inhibition of each other. Growth factor receptor induced activation of Cdc42 has been shown to activate Rac, which in turn stimulates Rho activity resulting in cytoskeletal remodeling.

Overexpression of Cdc42-activating or dominant-negative mutants in fibroblasts have shown that Cdc42 plays an essential role in cell-growth control by regulating G₁-S cell-cycle transition^{111,112}. In embryonic stem cells, however, gene targeting of Cdc42 did not affect cell growth or replication¹¹³. Recently, Wang *et al.*¹¹⁴ have shown in a gain-of-function model for Cdc42 (Cdc42GAP^{-/-}), that hyperactivation of Cdc42 induces anemia with a significant reduction in fetal liver and bone marrow cellularity, decreased erythroid/myeloid progenitor content and decreased HSC/P content. The decrease in HSC/P number was associated with increased apoptosis of the Cdc42GAP^{-/-} HSC/Ps and activation of JNK-mediated apoptotic machinery, showing impaired cortical F-actin assembly, deficiency in adhesion and migration, and defective short- and long-term competitive repopulation ability. The mechanism of how gain-of-function of Cdc42 induces a hematopoietic impairment is not clear yet. HSC from Cdc42^{-/-} mice also show defective migration and adhesion, which is associated with abnormal F-actin assembly, homing, and engraftment/retention in the BM. Cdc42^{-/-} mice show increased numbers of circulating HSC and developmental abnormalities in myelopoiesis and erythropoiesis^{115,116}. Interestingly, HSC aging has also been related to Cdc42 activity. Adhesion of HSC to the hematopoietic microenvironment appears to be reduced while G-CSF-induced mobilization is increased in aged HSC compared with young HSCs and this difference may be, at least partly, a consequence of the increased activity of the small Rho GTPase Cdc42 in aged HSCs¹¹⁷.

The RhoA subfamily members when overexpressed as activated proteins contribute to contractility and formation of stress fibers and focal adhesions, which are mainly responsible for strong attachment to the underlying extracellular matrix. Different Rho proteins have been associated with different proliferation effects. While RhoA and RhoC are usually growth-promoting, RhoB inhibits cell growth^{118,119} and has been shown to be downregulated in cancer cells¹²⁰. In some cells, including hematopoietic cells,

Rac and Rho GTPases provide inhibitory crosstalk in these functions. In fact, Ras-induced membrane ruffling due to activation of Rac leads subsequently to formation of stress fibers in a Rho-dependent fashion¹²¹. These observations delineated a signaling pathway where activated Rac induced Rho activation.

RhoA leads to stress fiber formation and cell shape changes, although most of these studies have been performed on fibroblasts. In fibroblasts, activation of RhoA has been reported to decrease the expression of Cdk inhibitors and to shorten G1¹²². Using the same cell types, inactivation of RhoA has been shown to induce the expression of cyclin D–Cdk4 complexes in early G1 phase and promote a rapid G1/S phase transition^{123,124}.

Transforming growth factor- β -induced activation of RhoA has been shown in mammary cells to stimulate the nuclear translocation of p160 ROCK, a known target of RhoA, which results in cell cycle arrest by decreasing the activity of Cdc25A phosphatase and decreasing Rb phosphorylation¹²⁵. Therefore, the effect of RhoA GTPase activity on cell cycle and proliferation appears both cell type and agonist specific. In contrast with the published role of RhoA in fate determination and differentiation in mesenchymal stem cells¹²⁶, inhibition of RhoA activity through expression of the dominant negative mutant RhoAN19 via retrovirus-mediated gene transfer was associated with a significant enhancement of HSC engraftment and reconstitution *in vivo*¹²⁷. Increased engraftment of HSC expressing RhoAN19 was associated with increased cyclin D1 expression and enhanced proliferation and cell cycle progression of hematopoietic progenitor cells *in vitro*, despite this enhanced engraftment *in vivo*. Consistent with studies reported in fibroblast cells¹²⁸, RhoA was essential for normal adhesion and migration of hematopoietic progenitor cells *in vitro*. Decreased activity of RhoA GTPase resulted in defective $\alpha4\beta1$ and $\alpha5\beta1$ integrin mediated adhesion and impaired CXCL12-directed migration of hematopoietic progenitor cells *in vitro*. These results are surprising given the role of adhesion and migration in HSC engraftment. Taken together, these data suggest that RhoA GTPase plays a crucial role in HSC engraftment, although the mechanism of enhanced engraftment seen with expression of the DN RhoA protein is unclear. In the context of previous reports describing Rac GTPase function in HSC^{67,95}, these studies suggest that inhibition in Rac activity may enhance mobilization, whereas inhibition of RhoA may augment HSC engraftment.

A distinct type of Rho proteins is constituted by RhoH. RhoH is a hematopoietic-specific, GTPase-deficient member of the Rho GTPase family. RhoH has been shown to modulate Rac signaling¹²⁹⁻¹³¹. The biochemical clue on how RhoH antagonizes Rac came

from studies in primary HSC/P, where RhoH deletion leads to increased chemotaxis and chemokinesis towards the chemoattractant CXCL12. This hypermigratory response is due to increased Rac1 activity and translocation of Rac1 protein to the cell membrane, where it colocalizes with cortical F-actin and lipid rafts. Expression of a Rac dominant-negative mutant inhibits the cortical F-actin assembly and chemotaxis of wild-type and RhoH-deficient HSC/P to the same extent. Conversely, overexpression of RhoH in HSC/P blocks the membrane translocation of Rac1 and impairs cortical F-actin assembly and chemotaxis in response to CXCL12 stimulation. At a molecular level, subcellular localization and inhibitory function of RhoH in HSC/P appear to be regulated by C-terminal motifs, including a CKIF prenylation site, required for specific membrane localization where targets Rac¹²⁹.

In summary, Rho GTPases play a crucial role in the integration and modulation of signals from cortical receptors, specifically chemokine receptor CXCR4, c-kit and $\beta1$ -integrins, and the analysis of their specific functions may help us to delineate specific pathways and molecular targets for pharmacologic intervention in stem cell and gene therapy.

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¿ES POSIBLE PRODUCIR COMPONENTES SANGUÍNEOS *IN VITRO* A PARTIR DE CÉLULAS MADRE?

A. PLA, J.J. CAIRÓ, J. GARCÍA

XCELIA. División de Terapias Avanzadas. Banc de Sang i Teixits de Catalunya. Barcelona

La transfusión sanguínea ha sido, desde siempre, objeto de intensa actividad científica, hecho que ha permitido la aparición de sucesivas generaciones de productos, a medida que la técnica ha ido avanzando, cada vez más especializados y efectivos. Los primeros productos disponibles, consistentes en concentrados de eritrocitos, plaquetas y derivados plasmáticos, dieron paso a las tecnologías de manipulación de progenitores hematopoyéticos, y éstas, a la utilización de diversas fuentes de progenitores hematopoyéticos mínimamente manipulados como producto terapéutico (médula ósea, sangre periférica movilizada y cordón umbilical). En el momento actual, y gracias a los avances producidos en el campo de la biología de las células madre hematopoyéticas, se está evaluando una nueva generación de terapias personalizadas, encaminadas a combatir diversas patologías de carác-

ter inmunológico u oncológico. Siguiendo este camino de continua evolución, y teniendo en cuenta el complicado contexto en que se encuentra hoy en día la donación de sangre, el desarrollo de nuevas tecnologías capaces de responder a las necesidades de la transfusión de emergencia son de gran interés estratégico. La estabilización del nombre de donaciones, el aumento continuo en la demanda de unidades para transfundir y el cada vez más elevado coste de producción por bolsa asociado a los constantes incrementos de las medidas de seguridad transfusional, justifican los esfuerzos y hacen muy atractivas las aplicaciones tecnológicas que puedan generar sustitutos a la donación convencional. La posibilidad de disponer de una fuente de células rojas liberada de la donación, con capacidad de responder a las necesidades de los diversos grupos sanguíneos y que carezca de los riesgos asociados a transmisión de enfermedades infecciosas que comporta la transfusión tradicional, representaría un gran avance en el campo de la medicina transfusional.

Las principales aproximaciones tecnológicas dirigidas a generar alternativas a la donación desarrolladas hasta el momento se han focalizado en la generación de sustitutos sintéticos. Estos productos basados en soluciones capaces de transportar oxígeno mediante la utilización de perfluorocarbonos o soluciones de hemoglobina estabilizadas, a pesar del gran esfuerzo y tiempo invertidos en su desarrollo, han producido por el momento resultados poco esperanzadores. Por ejemplo, los ensayos clínicos realizados con sustitutos de los hematíes en base a hemoglobina han mostrado que pueden inducir elevaciones de la presión arterial, o disminución de la perfusión de los tejidos, entre otros efectos adversos. También se ha asociado la administración de estos productos a un incremento en el riesgo de mortalidad de hasta un 30% y un aumento de la posibilidad de padecer infarto de miocardio del 3%¹.

Como aproximación alternativa a los sustitutos sintéticos, la obtención de hematíes a partir de células madre representa una posibilidad esperanzadora. El mejor conocimiento de la biología de las células madre hematopoyéticas, su interacción con el nicho hematopoyético y los factores que gobiernan las rutas de diferenciación hacia los diversos linajes han permitido dilucidar algunos de los requerimientos necesarios para mimetizar la eritropoyesis *ex vivo*³. En este tipo de aproximaciones, uno de los principales problemas que se ha debido afrontar ha sido la enucleación. Este fenómeno, cuyos mecanismos no son plenamente conocidos, ha podido finalmente ser reproducido *ex vivo* gracias al reconocimiento de la importancia del nicho hematopoyético y el entorno de señales que lo configuran. Con esta pieza del rompecabezas se ha abierto el camino a la producción de hematíes a partir de di-

versas fuentes de células madre. Diversos trabajos han mostrado la factibilidad técnica de obtener células rojas *ex vivo* a partir fuentes de células madre hematopoyéticas adultas y embrionarias. Como dato relevante, cabe destacar que las células obtenidas a partir de progenitores hematopoyéticos de sangre de cordón, muestran características funcionales muy similares a los hematíes normales⁴. Tienen el mismo contenido en hemoglobina, la misma morfología, tienen capacidad de captación y liberación de oxígeno y, en el aspecto de propiedades elásticas y de comportamiento *in vivo*, han demostrado ser capaces de sobrevivir en huéspedes murinos inmunosuprimidos. Independientemente de la fuente celular empleada, las tecnologías disponibles para la obtención de hematíes sintéticos se basan en los mismos principios; la maduración secuencial mediante factores solubles y la inducción de la enucleación mediante un cocultivo en capa estromal o mediante la señales aportadas por factores humorales. Estas metodologías se caracterizan por generar las células rojas a costa del agotamiento de las células madre hematopoyéticas de partida o en el caso de las embrionarias de las células madre hematopoyéticas generadas en cultivo, hecho que requiere de un continuo flujo de entrada de nuevas células en el proceso. Como alternativa a esta limitación se están intentado establecer líneas eritroides derivadas de células madre embrionarias con el objetivo de obtener una célula que se capaz de diferenciarse hacia eritrocitos funcionales y a la vez retenga la capacidad de ser expandida de forma masiva⁶.

Los procesos actuales permiten obtener el equivalente de dos a cuatro concentrados de hematíes por unidad de cordón umbilical. En contraste, la generación fisiológica de las células sanguíneas en la médula ósea se produce de forma extremadamente efec-

tiva y exquisitamente regulada de forma que en el humano adulto se generan alrededor de diez mil millones de células rojas cada hora. Esta diferencia abismal entre las eficiencias celulares subyacentes a los procesos de generación *in vivo* o *ex vivo* de hematíes pone de manifiesto las importantes limitaciones de las tecnologías de producción existentes. Por lo tanto, a pesar del importante avance realizado, es necesario destacar que la aplicación a gran escala de estas tecnologías requiere de la solución de problemas complejos. Entre éstos, cabe destacar el coste prohibitivo de los medios y factores de crecimiento empleados, la compleja ingeniería requerida para estandarizar los procesos y la baja tasa de conversión entre el número de células de entrada al proceso (progenitores hematopoyéticos) respecto del número de hematíes obtenidos.

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